SCHOOL OF ADVANCED STUDIES

UNIVERSITY OF CAMERINO

PhD Course in Chemical and Pharmaceutical Sciences and Biotechnology

Curriculum: Pharmaceutical, Nutraceutical and Food Sciences

XXXV Cycle

Medicinal and food plants as sources of biopesticides and biologically active compounds: a focus on *Carlina acaulis* and *Acmella oleracea*

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Academic years 2020–2022

To you,

who never really left me,

as promised.

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List of Publications

Publications included in the Thesis and adapted for each Chapter

Benelli, G., Pavoni, L., Zeni, V., Ricciardi, R., Cosci, F., Cacopardo, G., ... & Lucchi, A. (2020). Developing a highly stable *Carlina acaulis* essential oil nanoemulsion for managing *Lobesia botrana*. *Nanomaterials*, *10*(9), 1867.

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disease vector mosquito, and their non-target impact. *Industrial Crops and Products*, *176*, 114413.

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Abstract

Botanical products have been employed since the early human history as foods, medicinal and insecticidal agents and this is ascribed to their richness in biologically active compounds. Regarding the use as insecticidal agents, they are nowadays gaining increasing scientific interest since the misuse and overuse of conventional pesticides led to the onset of resistance and negative effects for human health and the environment. Botanical pesticides, also known as biopesticides, are considered as alternatives to traditional pesticides due to the lack of persistence into the environment, selectivity towards non-target pests, and low toxicity on humans. From this scenario derives the increasing motivation of researching new and effective botanical pesticides obtainable by extraction from the plant matrix or also by synthesis of more active derivatives. This was one main purpose of the work carried out by our research group and herein reported. The main studies conducted on the Asteraceae plants *Carlina acaulis* L. and *Acmella oleracea* (L.) R. K. Jansen are described in the first two Chapters of this Thesis. In detail, Chapter I reports all the scientific investigations carried out on *C. acaulis*, which is a traditional medicinal plant growing in the European mountainous regions. The most interesting product of this plant is its roots essential oil (EO) (0.97% w/w yield), characterized by the predominance of the polyacetylene carlina oxide (> 95%). In this regard, an optimization of the EO extraction procedure through Microwave-Assisted Extraction (MAE) was performed using a one-step statistical approach (Design of Experiments, DoE) and compared to the traditional hydrodistillation (HD). This study pointed out that MAE is more efficient than conventional HD in terms in terms of EO yield (0.65 and 0.49% for MAE and HD, respectively) and extraction time (210 min for MAE).

C. acaulis EO, carlina oxide, and an EO-nanoemulsion (EO-NE) were also tested on several bacterial and fungal strains. Indeed, they were extremely active on the Gram-positive bacteria strains tested, with a complete inefficacy against Gram-negative bacteria for the EO and carlina oxide.

Nevertheless, the main studies carried out on *C. acaulis* were focused on its promising pesticidal activity. Indeed, its derived products were assayed on vectors, agricultural, and stored-product pests. Regarding vectors, *C. acaulis*-derived products (EO-microemulsion (EO-ME), EO-NE, and EO) were assayed on *Culex quinquefasciatus* Say larvae, the main vector of lymphatic filariaris and Zika virus. This study proved the high efficacy of the EO-ME on the vector, also with sublethal doses. The EO was also tested on human keratinocytes and fibroblasts and Winstar rats showing mild toxicity (LD_{50} on rats of 1098 mg kg^{-1}). The LD_{50} on rats increased above 5 g/kg when the EO was encapsulated into ME. Concerning the agricultural pests, different *C. acaulis*-derived products were assayed on *Lobesia botrana* (Denis & Schiffermüller, 1775), *Bactrocera oleae* (Rossi), *Ceratitis capitata* (Wiedemann), *Meloidogyne incognita*, *Xylosandrus compactus* (Eichhoff), and

Tetranychus urticae Koch, which are pests affecting different crops worldwide and leading to severe economic losses. From these studies, a marked activity on all the agricultural pests assayed was detected.

In addition, diverse *C. acaulis*-derived products were tested on stored-product pests as *Acarus siro* L., *Alphitobius diaperinus* (Panzer), *Oryzaephilus surinamensis* L., *Prostephanus truncatus* (Horn), *Rhyzopertha dominica* (F.), *Sitophilus oryzae* L., *Tribolium confusum* Jacquelin du Val, *Tribolium castaneum* (Herbst), *Tenebrio molitor* L., and *Trogoderma granarium* Everts. These pests usually affect stored products leading to economic damages but also to negative effects on the consumers' health. The bioactivity detected was extremely promising against all pests taken into consideration. During these studies, also the non-target toxicity on *Neoseiulus californicus* (McGregor) and *Daphnia magna* Straus was assessed resulting moderate. The development of a synthetic approach to produce carlina oxide analogues and the assessment of their insecticidal activity and toxicity are also reported in Chapter I. Between the analogues synthetized, the *m*-chloro substituted resulted more active on *Cx. quinquefasciatus* and less toxic on human keratinocytes (HaCaT cell line) than its precursor carlina oxide.

On the other hand, Chapter II reports all the research carried out on *A. oleracea*, which is a traditional medicinal plant native to Brazil but spread worldwide as a crop. The *A. oleracea*-derived products' (EO, EO-NE, and extracts) biological activities have been mainly linked to the presence of *N*-alkylamides, with a focus on spilanthol that resulted the most abundant. Firstly, MAE and HD were used for the EO extraction, and a comparison of the obtained products was performed. This study pointed out that MAE is more efficient than HD for the obtaining of the EO in terms of yield (0.47 and 0.22% w/w, respectively) and spilanthol content (11.7 and 2.3%, respectively). The EO, EO-NE, and a *n*-hexane-extract were efficient against *Cx. quinquefasciatus* larvae, with spilanthol and the *n*-hexane extract as the most active products. In addition, the safety for mammal cells of the EO and spilanthol was also proved together with the protection from lipopolysaccharide (LPS)-induced inflammation. Based on these findings, an optimization of the formulative parameters of the *n*-hexane extract and spilanthol into MEs and NEs was performed and from this study the optimal formulative parameters were found only for NEs. Moreover, seen the promising activity displayed by the *n*-hexane extract, an optimization study for the extraction of spilanthol from the raw material was assessed. In this regard, Soxhlet extraction with methanol resulted the best extractive procedure, even if the *n*-hexane extract displayed the highest concentration of spilanthol. These methanol and *n*-hexane extracts were assayed on several stored-product pests as *O. surinamensis*, *T. granarium*, *T. castaneum*, *T. confusum*, *T. molitor*, *A. diaperinus*, *A. siro*, and *Cryptolestes ferrugineus* (Stephens). This study pointed out the higher effectiveness of the *n*-hexane extract than the methanol extract and this result confirmed that the concentration of spilanthol is fundamental for the biological activity.

Lastly, Chapter III reports the results of the research activity carried out abroad at the Laboratory of Organic Chemistry of Prof. Nuno Maulide (University of Vienna) for a period of four months. In detail, the goal was to develop a new synthetic approach to produce α-aminated amides through electrophilic amide activation with triflic anhydride. Initially, the reaction was investigated for protected α-aminated amides and, from the preliminary screening of protecting groups (PGs), benzoyl (Bz) resulted the best in terms of yield. The reaction displayed good functional group tolerance, except for bulky amides. Moreover, a protocol was developed and applied for the synthesis of 'free' α-aminated amides, and the reaction was screened on different substrates with acceptable yields.

CHAPTER I: *Carlina acaulis*

1. Introduction

1.1. Taxonomy

Carlina acaulis L. is a member of the Asteraceae family and belongs to the Cardueae tribe and Carlinae subtribe (Table 1) (Badry et al., 2020).

For this species, two subspecies have been reported, namely *C. acaulis* L. subsp. *acaulis* that is steamless and *C. acaulis* L. subsp. *simplex* (Waldst. and Kit.) Nyman that is endowed with a short stem (Tutin et al., 1972).

Table 1. *Carlina acaulis* taxonomy.

Regnum	Plantae
Divisio	Tracheophyta
Sub-divisio	Spermatophyta
Class	Magnoliopsida
Superordo	Asteranae
Ordo	Asterales
Familia	Compositae Giseke
Tribus	Cardueae Cass.
Genus	*Carlina* L.
Species	*C. acaulis*

Even though previous studies reported this plant with different names, i.e., *C. acaulos magno flore albo*, *C. acaulos gummifera*, *Chamaeleon exiguus*, or *C. acaulis septentrionaleum*, botanical descriptions indicate that they all belong to the same species (Miller 1754; Strzemski et al., 2019). The first complete monograph of the plant was presented by Meusel and Kästner (1990) and contained the description of several features, from the taxonomy to its morphology and secondary metabolites content. The term 'acaulis' means 'without stem', denoting that the flower heads are directly connected to the basal leaf rosette (Figure 1). The origin of the name 'Carlina' is unclear. It seems to derive from a legend linked to the Charlemagne army or from a correlation with the *Carduus* genus (Rejewski, 1996; Rieder er al., 1979). The currently accepted names for *C. acaulis* are *C. acaulis* subsp. *acaulis*, *C. acaulis* var. *acaulis*, *C. alpina* Jacq., *C. subacaulis* DC., *C. cirsioides* Klokov, *C. officinalis* Bubani, and *Chromatolepis acaulis* Dula (http://www.worldfloraonline.org/taxon/wfo-0000090349).

Figure 1. Flower head of *Carlina acaulis*.

1.2. Habitat and distribution

C. acaulis finds its geographic origin in northern Europe, and its natural habitat consists of rocky soils, pastures, and dry meadows from 0 to 2800 m a.s.l.. It grows preferentially on calcareous soils in the mountains, rarely occurring in lowland areas (Meusel and Kästner, 1990; Pavela et al., 2020; Tutin et al., 1972) and it is mainly widespread in southern and north-eastern Poland (Zając and Zając, 2001). However, *C. acaulis* is also distributed in southern European countries such as Austria, France, Italy, and Romania (Figure 2) (Tutin et al., 1972).

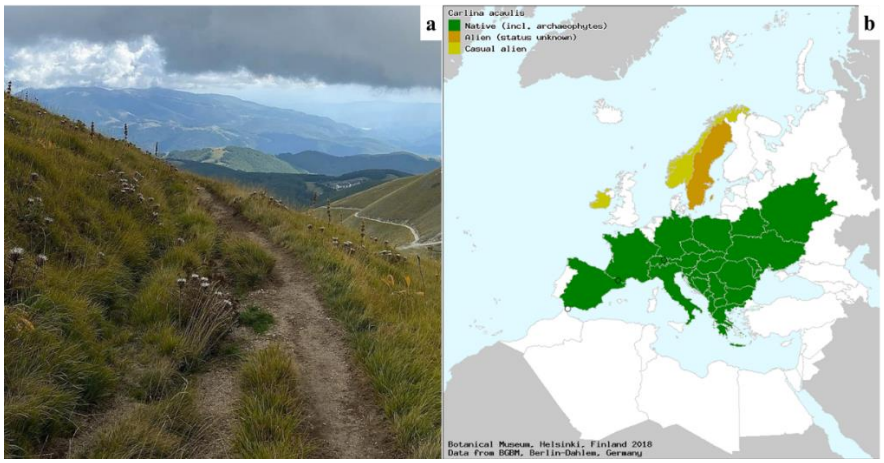

Figure 2. *Carlina acaulis* habitat (**a**) and distribution (**b**) (https://europlusmed.org/cdm_dataportal/taxon/8ebb6829-8ad7-4a67-9498-66a0ed6b7bd1#distribution).

1.3. Morphological and anatomical features

C. acaulis consists of a basal rosette presenting a 20 cm diameter, with spiky and pinnatilobate leaves. It presents a blossom, which ends up into a huge flowerhead showing silver white ray flowers, arranged around a yellow-brown inner disc. The capitula present a diameter of 25-50 mm (Erzsebet et al., 2009; Trejgell et al., 2009a). The stem is usually short, with a maximum height of

15 cm, even if the plant is usually acaulescent. Usually and in some conditions, the head closes for the protection of the pollen. This aspect is traditionally used for the prediction of rain. The massive plant rhizome is placed vertically inside the ground and the roots assume a colour from grey-brown to dark-brown, presenting a longitudinal fold. The fruits or cypselae, which are incorrectly called seeds, are oblong, with lengths and widths of 4-6 and 1 mm, respectively. Moreover, they are brown and coated with silver hairs and they assume an elliptical shape in the cross section (Strzemski et al., 2020) (Figure 3).

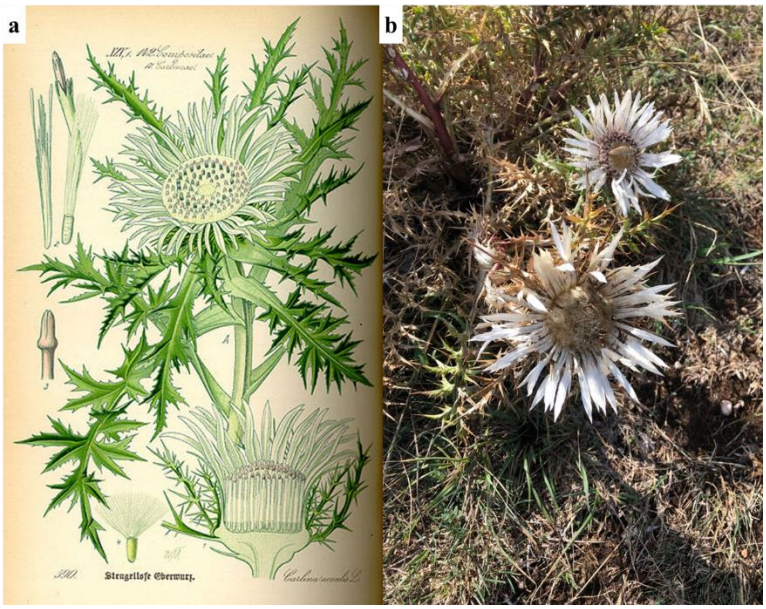

Figure 3. Illustration of *Carlina acaulis* (**a**) (Thomé, 1885), *Carlina acaulis* plant (**b**).

From a microscopical and anatomical point of view, the roots are characterized by a thin-walled cork and a durable cortex. The secondary phloem is dominant and presents bundles of fibres and simple and reticulate vessels. *C. acaulis* roots are also characterized by the presence of secretory ducts of SD1 and SD2a type located in the phloem and by medullary rays, where the essential oil (EO) is stored (Figure 4) (Fritz and Saukel, 2011).

The leaves are amphistomatic and present anomocytic stomata, with a smooth cuticle on the surface and sporadic non-glandular hairs (Đorđević et al., 2004).

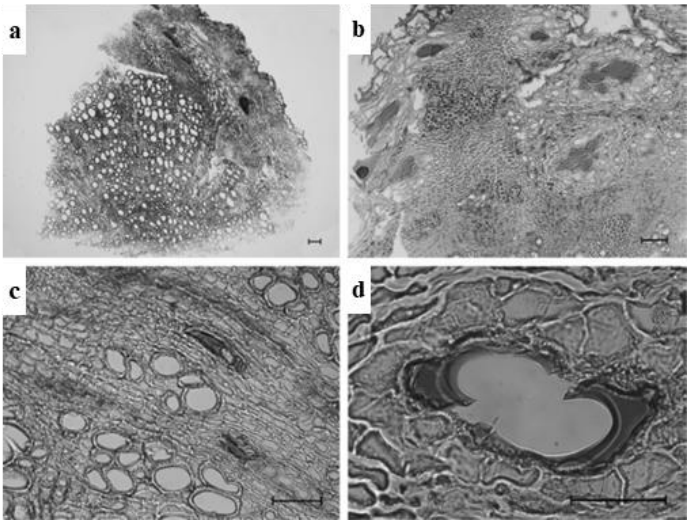

Figure 4. Microscopical analysis of *Carlina acaulis* roots. Overview of the roots (**a**); secondary phloem (**b**); SD1 secretory ducts (**c, d**) (Fritz and Saukel, 2011).

1.4. Ethnobotanical uses

C. acaulis usage as remedy for different diseases has been described since antiquity in ancient texts. Theophrastus identified two different plant types, which were useful against skin exudates and as anthelminthic agents. According to the author, the chopped root was also employed to treat leishmaniosis and leprosy. Moreover, it has been reported that the root boiled in combination with sulphur and a resin was effective for scabies. Also, Pliny suggested that this plant was useful for the healing of ulcers and that the root aroma could be a deterrent for scorpions, while Discorides described the diuretic use of a decoction of the entire plant (Strzemski et al., 2019).

C. acaulis has great importance in the European traditional medicine, particularly in Poland, where it is extracted with wine and consumed as a stimulant for the brain and digestive system. Moreover, it is also traditionally reported as a laxative, emetic, anthelminthic, and diuretic agent, but also as effective for the cure of male impotence (Strzemski et al., 2019). The use of this plant in ethnomedicine is also widely spread in Italy, where it is employed as cholagogue, antibacterial, and diuretic agent (Guarrera, 2003; Menale et al., 2006). In Montenegro, Bosnia and Herzegovina the aerial parts are employed for the treatment of gastritis, while in Serbian culture the roots are considered a remedy for skin disorders, such as ulcers, eczema, and acne (Jarić et al., 2007; Redžić, 2007; Rexhepi et al., 2013; Šarić-Kundalić et al., 2010). The roots and inflorescences of this plant are edible, and they have been consumed as a food since ancient times (Pavela et al., 2020). For instance, the smell of the root favoured its use for the preparation of desserts in southern Italy (Guarino et al., 2008), while the receptacle is utilized in substitution of artichoke (Abbet et al., 2014) besides also for the preparation of snacks and alcoholic beverages (Armand, 1993; Pieroni and Giusti, 2009). It is worthy of being mentioned that *C. acaulis* root and the derived EO are inserted in the Italian list of botanicals to be used in food supplements and in the BELFRIT list,

which are harmonized lists of plants whose use in food supplements is allowed (Cousyn et al., 2013; http://www.gazzettaufficiale.it/eli/gu/2018/09/26/224/sg/pdf).

1.5. Phytochemistry

C. acaulis possesses a rich phytocomplex. The main polysaccharide found in the roots is inulin (~ 20%) (Raynaud and Rasolojaona, 1979). The oil obtained from the plant seeds is constituted of fatty acids, mainly represented by linoleic acid (50-52%), while stearic, palmitic, and oleic acids are present in lower amounts (< 10%).

Moreover, this plant bears a large variety of secondary metabolites belonging to different chemical classes, namely polyphenols, volatile compounds, triterpenes, and polyacetylenes. Figure 5 resumes the main metabolites found in *C. acaulis*.

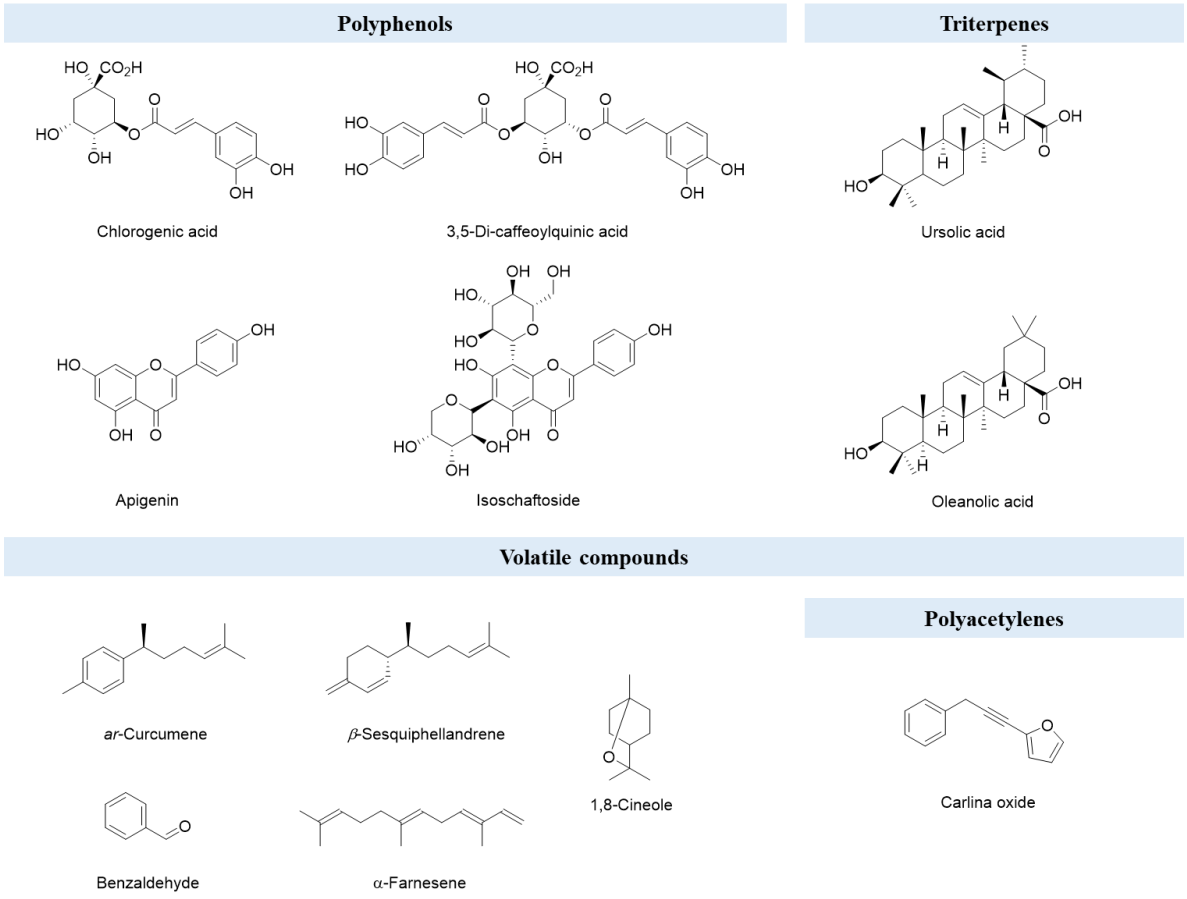

Figure 5. Main secondary metabolites found in *Carlina acaulis*.

1.5.1 Polyphenols

Polyphenols are polyhydroxylated phytochemicals largely produced by plants that can be divided into three subclasses, namely phenolic acids, flavonoids, and stilbenoids. These compounds are

responsible for different biological activities displayed by *C. acaulis*, such as the radical scavenging, antioxidant, antimicrobial, and anti-inflammatory properties. Between the phenolic acids, the main compounds are chlorogenic acid, mainly found in the leaves, and 3,5-di-caffeoylquinic acid, mainly concentrated in the roots (Strzemski et al., 2019). In addition, it has been found that chlorogenic acid is highly present in *C. acaulis* cypsela, with a concentration of 22 g/kg, greater than that of plants belonging to the same family (Strzemski et al., 2020). Among the chlorogenic acids found in this plant, also 5-*O*-caffeoylquinic, 3-*O*-caffeoylquinic, *cis*-5-*O*-caffeoylquinic, 4-*O*-feruloyl-5-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, caffeoyl-epi-quinic, 4-*O*-dicaffeoylquinic, 5-*O*-feruloylquinic, *cis*-3-*O*-feruloylquinic, A *cis*-3,5-*O*-dicaffeoylquinic, *cis*-4-*O*-feruloylquinic, *cis*-5-*O*-feruloylquinic, 5-*O*-*p*-coumaroylquinic, 3-*O*-feruloylquinic, *cis*-5-*O*-*p*-coumaroylquinic, A *cis*-3, 4,5-*O*-dicaffeoylquinic, and 3-*O*-caffeoyl-4-*O*-feruloylquinic acids were identified in the leaves (Jaiswal et al., 2011). *C. acaulis* herb was also found to contain some flavonoids, such as isoschaftoside, apigenin 7-*O*-glucoside, homoorientin, vitexin, orientin, and apigenin (Đorđević et al., 2012; Raynaud and Rasolojaona, 1979).

1.5.2 Triterpenes

Triterpenes represent a large class of natural products, and they are thirty-carbon compounds derived from five-carbon isoprene units. These molecules are also precursors of different compounds, as plant phytosterols or hormones, and their scaffolds are usually tetracyclic or pentacyclic (Ghosh, 2016). Triterpenes are an abundant group of secondary metabolites produced by *C. acaulis* (Mioc et al., 2022; Strzemski et al., 2020), and they are mainly present in the leaves, flowerheads, stems, and roots in lower amounts. These molecules are highly concentrated in the plant waxes as defensive agents against insects, and they are involved in allelopathy phenomena (Strzemski et al., 2016). The presence of these compounds is responsible for some of the biological activities showed by the plant, such as the anthelmintic, diuretic, and anti-inflammatory properties (Herrmann et al., 2011; Menale et al., 2006; Miller, 1754; Strzemski et al., 2019). Pentacyclic triterpenes are the main representatives of this class of compounds found in *C. acaulis* and the most abundant are ursolic and oleanolic acids (Strzemski et al., 2016, 2019, 2021). The different cultivation of *C. acaulis* influences the content of these molecules, which seem to be highly produced in hydroponic and in field cultivations (Strzemski et al., 2020), while their production is reduced in the *in vitro* micropropagation cultivation technique. In addition to these compounds, also lupeol, lupeole acetate, α-amyrin, β-amyrin, β-amyrin acetate, and betulinic acid have been detected (Strzemski et al., 2016).

1.5.3 Volatile compounds

Volatile compounds are low-molecular weight lipophilic substances usually synthetized by plants to adapt to the environment and more than 1700 of these compounds have been characterized (Knudsen and Gershenzon, 2006). These molecules are usually terpenoids, benzenoids, phenylpropanoids, amino acid derivatives, and fatty acids (Dudareva et al., 2004). The main volatile compounds of *C. acaulis* are found in its roots EO (1-2%) (Đorđević et al., 2012), whose chemical composition is characterized by the predominance of a polyacetylene, known as carlina oxide. This compound accounts for the 80% (Herrmann et al., 2011) to the 99% (Benelli et al., 2021; Kavallieratos et al., 2022a; Stojanović-Radić et al., 2012) of the total composition. Minor constituents are *ar*-curcumene, benzaldehyde, *β*-sesquiphellandrene, (*E,Z*)-*α*-farnesene, and 1,8-cineole (Chalchat et al., 1996). Regarding carlina oxide, it represents a chemotaxonomic marker of the genus. In fact, this polyacetylene has been found in other *Carlina* species in high amount, such as *Carlina acanthifolia* All. (Đorđević et al., 2007), *Carlina vulgaris* L. (Belabbes et al., 2019), *Carlina diae* (Rech. f.) Meusel and Kästner (Bohlmann et al., 1981), and *Carlina hispanica* Lam. (Achiri et al., 2021) (Table 2). This compound has also been found in other plants of the same family, such as *Carthamus caeruleus* L. (Mami et al., 2019) and *Atractylis gummifera* L. (Mejdoub et al., 2019).

Table 2. Chemical constitution of the EOs obtained from *Carlina* genus plants (Spinozzi et al., 2023).

Species	Geographic origin	Part	Main components	Reference
Carlina acaulis	Albania (wild)	Root	Carlina oxide (94.6-98.8%), benzaldehyde (0.9-3.1), *ar*-curcumene (0.29-0.72%), *β*-sesquiphellandrene (0.05-0.2%), acetophenone, benzyl methyl ketone, carvone.	Pavela and Benelli, 2016; Benelli et al., 2019a, 2020, 2021, 2022; Pavela et al., 2020; Rizzo et al., 2021; Rosato et al., 2021; Kavallieratos et al., 2020, 2022a, 2022b
Carlina acaulis	Serbia (wild)	Root	Carlina oxide (97.2%), benzaldehyde, *ar*-curcumene, *β*-sesquiphellandrene, heptane (0.05%), 1,8 cineole, 1-phenyl-2-propane, (*Z,E*)-*α*-farnesene.	Chalcat et al., 1996
Carlina acaulis	Poland (cultivated)	Root	Carlina oxide (96.2%), *ar*-curcumene (0.56), *β*-sesquiphellandrene (0.16%).	Wnorowski et al., 2020

Carlina acanthifolia	Serbia (wild)	Root	Carlina oxide (91.5%), *ar*-curcumene (1.6), *β*-sesquiphellandrene (2.8%), trans-*β*-farnesene (0.4%), *γ*-curcumene (1.1%), *α*-zingiberene (2.4%), *β*-bisabolene (0.1%), *cis-trans*-farnesal (0.1%).	Đorđević et al., 2005, 2007
Carlina acanthifolia	Poland (cultivated)	Root	Carlina oxide (98.96%), *β*-sesquiphellandrene (0.2%), *ar*-curcumene (0.59%), *α*-zingiberene (0.24%), benzaldehyde (0.57%).	Strzemski et al., 2017
Carlina vulgaris	Algeria (wild)	Root	Carlina oxide (33.7%) 13-methoxy carlina oxide (11.5%).	Belabbes et al., 2019
Carlina hispanica	Algeria (wild)	Root	Carlina oxide (81.1%).	Achiri et al., 2021

1.5.4 Polyacetylenes

Polyacetylenes are natural compounds with diverse biological activities found in plants, fungi, sponges, lichens and moss, and marine algae. They are widely distributed in plants belonging to the family of Apiaceae, Araliaceae, Asteraceae, Campanulaceae, Santalaceae, and Olacaceae, and they are usually characterized by the presence of two or more carbon-carbon triple bonds (Bohlmann, 1988). In detail, over half of the acetylenes extracted and characterized derives from plants of the Asteraceae family (Christensen and Lam, 1990; 1991a; 1991b; 1992). The building blocks for the biosynthesis of polyacetylenes are saturated fatty acids, which are synthetized by head-to-tail addition of malonyl units to a progressively formed acyl chain (Minto and Blacklock, 2008; Negri, 2015). Different enzymes are then responsible for the functionalization of these fatty acids, but the key step of the biosynthesis of polyacetylenes is the oxidation of already formed double bonds into triple bonds. For this process, different biosynthetic routes have been proposed and they are resumed in Figure 6a (Bu'Lock and Smith, 1967; Fleming and Harley-Mason, 1963). The products of this pathway are stearolic, tariric, and crepenynic acids, which then undergo to diverse chemical transformations leading to a large variety of polyacetylenes (Figure 6b,c) (Minto and Blacklock, 2008). These molecules are characterized by a broad chemical variety and they are usually classified in accordance with their structure in acyclic C_8-C_{13} acetylenes, acyclic C_{14}-C_{18} acetylenes, acetylenes with the allene structure, and aromatic and heterocyclic acetylenes (Christensen and Brandt, 2006). These groups are further divided into subgroups according to the presence of specific functional groups, such as sulfoxides and sulfones, pyrans and furans, thiophenes and thioesters (Bohlmann et al., 1988; Christensen, 1998; Lindstedt and Steen, 1975).

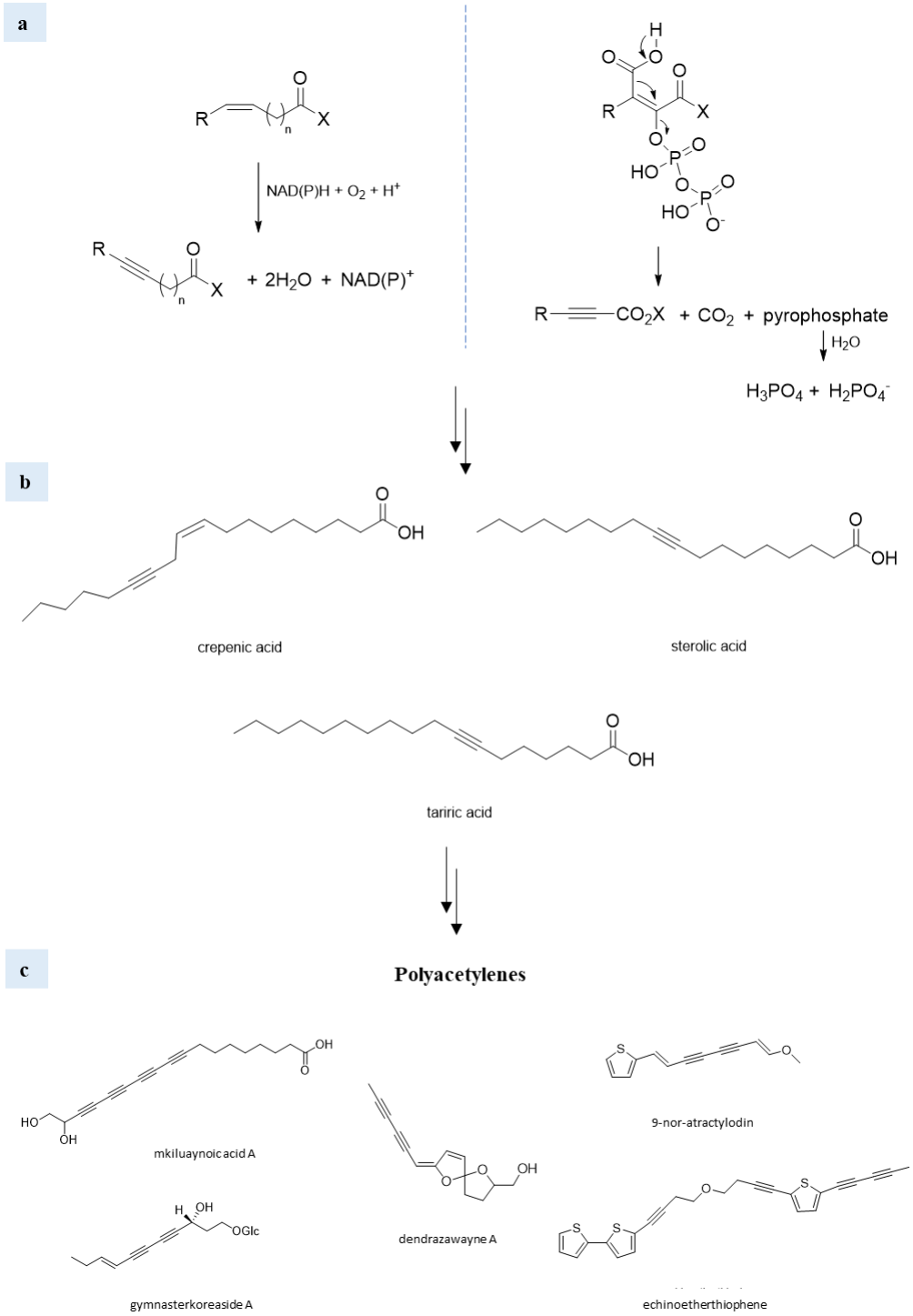

Figure 6. Proposed mechanisms for the formation of the acetylenic bonds (**a**) (Minto and Blacklock, 2008); mono-acetylenic intermediates biosynthesis (**b**) (Negri, 2015); natural polyacetylenes (**c**) (Spinozzi et al., 2023).

Aromatic polyacetylenes are largely distributed in the Asteraceae family, and they seem to be responsible for the linking of aromatic polyacetylenes with heterocyclic compounds with higher toxicity (Bohlmann et al., 1988). As previously mentioned in Section 1.5.3, the main polyacetylene

from *C. acaulis* is 2-(3-phenylprop-1-yn-1-yl)furan, known as carlina oxide (Figure 7), which was obtained for the first time in 1889 and which became one of the most known polyacetylenes (Semmler, 1889). This compound has a boiling point of 112-115°C, a refractive index (RI) of 1.5875 and a density of 1.063 g/mL at 25°C (Rosato et al., 2021).

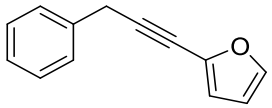

Figure 7. Carlina oxide chemical structure.

Carlina oxide can be extracted from the plant roots using different extraction techniques. The most common extraction procedure is hydrodistillation (HD), which is employed for the obtaining of carlina oxide-rich EOs and hydrosols (Benelli et al., 2019a; Mami et al., 2020; Pavela et al., 2020). Moreover, organic solvents, as ethanol or methanol, can be used for the extraction of this polyacetylene from the plant through different extractive systems, such as Soxhlet or ultrasounds (Đorđević et al., 2012; Link, 2016; Strzemski et al., 2019). The characterization of this compound can be achieved using diverse analytical techniques, such as NMR, IR, GC-MS, GC-FID but also HPLC-PDA (Đorđević et al., 2005; Pavela et al., 2021; Wnorowski et al., 2020).

The many biological activities shown by *C. acaulis* and described in Section 1.7 are surely due to the presence of carlina oxide. The biological properties displayed by polyacetylenes can be ascribed to their marked chemical reactivity and instability, mainly caused by the presence of C-C triple bonds. In detail, these compounds are subjected to fast oxidation, especially after UV light exposition, but are also deeply susceptible to the medium pH (Minto and Blacklock, 2008). Several studies also reports that this class of compounds is activated by sunlight wavelength less than 400 nm, leading to an improvement of their toxicity (Arnason et al., 1981). In fact, polyacetylenes are also considered photosensitizers that lead to the photodynamic degradation of membranes (Waksmundzka-Hajnos et al., 2008). Even if the carlina oxide mode of action has not been proved, its numerous bioactivities, especially the insecticidal property, could be ascribed to the presence of the C-C triple bond that is activated by UV light exposure and leads to the generation of radicals (Wink, 2012). Moreover, Herrmann et al. (2011) hypothesized a possible interaction of this polyacetylene with the active center of the trypanothione reductase, where the triple bond can link irreversibly to the SH groups, blocking the normal function of the enzyme. However, these hypotheses should be experimentally confirmed with further studies.

1.6. Cultivation and micropropagation

Even if *C. acaulis* is usually collected in its natural habitat, its presence is often limited. Moreover, the plant is a protected species in several countries (Vangendt et al., 2014). For this reason, different studies have been conducted on its cultivation and micropropagation, namely in field cultivation, hydroponic cultivation, and *in vitro* cultures, to find alternative methods for the employment of this plant in a larger scale (Figure 8).

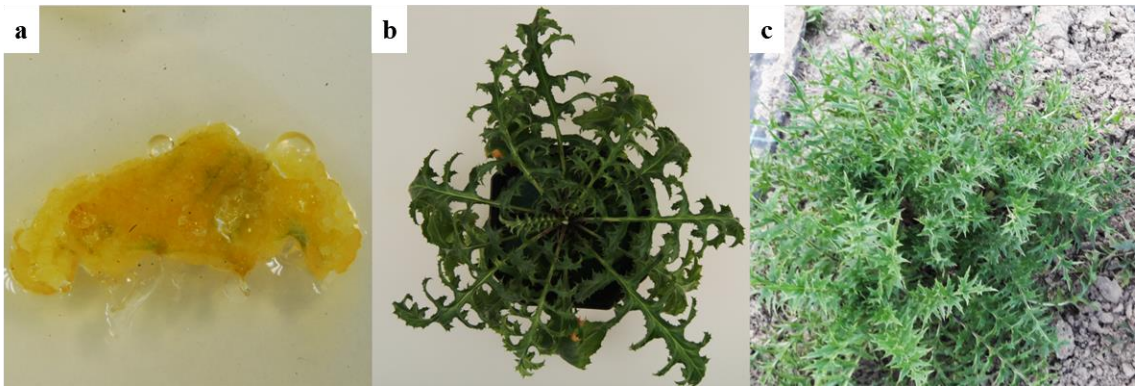

Figure 8. *In vitro* culture (**a**), hydroponic cultivation (**b**), and in field cultivation (**c**) (Strzemski et al., 2021).

1.6.1 In field cultivation

The production of this plant can be easily obtained by generative propagation since its seeds have a fast germination capacity and seedlings can be obtained in short periods. A first study on the plant cultivation was provided in 1805 by Kluk, who described the plant as effortlessly growing even in poor soils and as a promising source of food for its edible receptacles. The only reported study on *C. acaulis* cultivation was furnished by Strzemski et al. (2021), who evaluated the influence of nitrogen fertilization on the content of the main bioactive compounds. The study showed that the plant yield was negatively affected by nitrogen, while changes in metabolites content were not observed. Their content was only dependent on the biomass yield. This work demonstrated for the first time the possibility of developing a low-cost cultivation of this plant.

1.6.2 Hydroponic cultures

This kind of cultivation permits a complete control of the growth conditions for the plant, such as temperature, humidity, nutrients, and day length. Thanks to their reproducibility, these cultures are also employed for the correct identification of the nutritional necessities of the plant and also for the evaluation of stress factors on the plant growth. Strzemski et al. (2019) compared the content of

bioactive metabolites in plants deriving from hydroponic cultures, from in field cultivations, and from callus lines. The root contents of chlorogenic and 3,5-di-caffeoylquinic acids were comparable for plants deriving from hydroponics and field cultivations, as also the leaves content. However, carlina oxide content was significantly higher in plants grown in field, demonstrating the necessity of environmental conditions for the production of carlina oxide.

1.6.3 *In vitro* cultures

As for the hydroponic, *in vitro* cultures furnish stable conditions for the growth of the plant since they allow to control the growing conditions and the biosynthesis of some metabolites. This kind of cultures was used to obtain from *C. acaulis* leaves and roots callus lines to induce the production of carlina oxide, pentacyclic triterpenes, and chlorogenic acids (Strzemski et al., 2019). However, the study revealed that the content of these compounds was higher in plants from hydroponic and in field cultivations. In addition, carlina oxide was not detected in any of the callus lines produced (Strzemski et al., 2019). Nevertheless, these *in vitro* cultures were effective for the micropropagation of the plants (Trejgell et al. 2009b), which were then transferred to the field, flowered, and produced seeds. In conclusion, this technique could be employed for the micropropagation of *C. acaulis* plants.

1.7. Biological activities

The large employment of *C. acaulis* in traditional medicine is surely linked to its biological activities, which are reported below.

Antifungal and antibacterial activity

C. acaulis-derived products showed promising antifungal and antibacterial properties. In fact, Herrmann et al. (2011) evaluated the activity of the *n*-hexane extract and carlina oxide on the Gram-negative *Pseudomonas aeruginosa* ATCC 27853 (MIC of 4 mg mL^{-1} and 60 μg mL^{-1}, respectively), *Klebsiella pneumoniae* ATCC 700603 (MIC of 4 mg mL^{-1} and 60 μg mL^{-1}, respectively), and *Escherichia coli* ATCC 25922 (MIC of 8 mg mL^{-1} and 60 μg mL^{-1}, respectively. The authors also tested the two products on the Gram-positive *Streptococcus pyogenes* ATCC 12344, with MIC values of 0.125 mg mL^{-1} and 15 μg mL^{-1} for the *n*-hexane extract and carlina oxide, respectively. In addition, also the antifungal properties were assessed on *Candida glabrata* ATCC MYA 2950, with MIC of 0.5 mg mL^{-1} and 15 μg mL^{-1} (for the *n*-hexane extract and carlina oxide, respectively) and *Candida albicans* ATCC 90028, with MIC of 0.25 mg mL^{-1} and 15 μg mL^{-1} (for the *n*-hexane extract and carlina oxide, respectively). Similar studies were also performed on *C. acaulis* EO, that was tested on the fungal strain *C. albicans* ATCC 1023, showing a MIC of 0.19

μL mL^{-1}. Moreover, the EO was also evaluated on bacterial strains, as *Staphylococcus aureus* ATCC 6538 (MIC of 0.02 μL mL^{-1}), *Proteus vulgaris* ATCC 8427 (MIC of 0.09 μL mL^{-1}), *K. pneumoniae* ATCC 10031 (MIC of 0.78 μL mL^{-1}), *P. aeruginosa* ATCC 9027 (MIC of 0.09 μL mL^{-1}), and *E. coli* ATCC 25922 (MIC of 0.39 μL mL^{-1}) (Stojanović-Radić et al., 2012).

Antitrypanosomal activity

Herrmann et al. (2011) also reported a marked antitrypanosomal activity for *C. acaulis*. In detail, the *n*-hexane extract was the most active between all the plant extracts tested, with an IC_{50} of 3.7 μg mL^{-1}. In addition, carlina oxide resulted also extremely active, displaying an IC_{50} of 1.0 μg mL^{-1}.

Antiviral activity

Recently, a strong inhibitory action on SARS-CoV-2 was reported for the polyacetylene carlina oxide, which was proved to inhibit the interaction between the virus and the correlated human receptor through a bioluminescent immunoassay (Wnorowska et al., 2022). In detail, the study reported a dose-effect inhibition of the interaction between the human angiotensin-converting enzyme 2 (ACE2), responsible for the virus entry into the cells, and the receptor binding domain (RBD) of the viral spike protein. The IC_{50} for the RBD:ACE2 binding was of 234.2 μg mL^{-1} (Wnorowska et al., 2022).

Cytotoxicity

Carlina oxide was also reported for its cytotoxicity on some melanoma cell lines, namely C32, UACC-647, and UACC-903. However, this antitumour activity was accompanied by a toxicity on BJ normal fibroblast cells, for which an induction of necrosis and apoptosis was noticed (Wnorowski et al., 2020). The cytotoxicity of carlina oxide was also tested by Benelli et al. (2019a) on human colon cancer cell lines (HCT116), human breast cancer cell line (MDA-MB 231), and human dermis fibroblasts (HuDe), demonstrating a lower cytotoxicity compared to that of cisplatin but also a lack of selectivity for normal and cancer cell lines.

Antioxidant activity

Carlina oxide and the dichloromethane extract from *C. acaulis* showed an antioxidant activity on *Caenorhabditis elegans* model, for which a reduction of the oxidative stress of 64 and 47% was noticed, respectively. In the same study, the activation of the transcription factor (DAF-16) responsible for anti-stress gene expression was also reported (Link et al., 2016). Moreover, the extract obtained from the herb of the plant displayed a radical scavenging activity with an IC_{50} of 8.00 μg mL^{-1} and a concentration-dependent antioxidant property in 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay, with a DPPH neutralisation of 50% at 72.00 μg mL^{-1}. The roots

and herb extracts also had a gastroprotective action in gastric ulcers caused by ethanol-induced stress but also reduced carrageenan-induced oedema (Đorđević et al., 2012).

Insecticidal activity

Despite the numerous biological activities displayed by *C. acaulis*, worthy of mention is undoubtedly the marked insecticidal property of the EO and of its main constituent. This was investigated for the first time by Benelli et al. (2019), who demonstrated a significant larvicidal activity against *Culex quinquefasciatus* Say (Diptera: Culicidae) for the EO and carlina oxide. Moreover, also Pavela et al. (2020) proved the efficacy of the EO on *Musca domestica* L. (Diptera: Muscidae) adults, showing promising efficacy also on the following generations of individuals.
The insecticidal activity displayed by *C. acaulis* EO is undoubtedly linked to the presence of carlina oxide, whose possible mode of action has been reported in Section 1.5.4.

2. Microwave-assisted extraction (MAE) of *Carlina acaulis* essential oil (EO): a Fractional Factorial Design (FFD) optimization study

2.1. Introduction

EOs are natural products that have always aroused great interest for their application in diverse areas, due to their numerous biological properties. The definition reported by the International Organization for Standardization (ISO) describes the EO as a "product obtained from a natural raw material of plant origin by steam distillation, by mechanical processes from the epicarp of citrus fruits or by dry distillation, after separation of the aqueous phase – if any – by physical processes" (https://www.iso.org/obp/ui#iso:std:iso:9235:ed-3:v1:en). For the obtaining of these products, conventional and innovative methods can be exploited and the choice of the extraction technique is crucial, since it can influence the quantity and quality of the EO (Bilia et al., 2014; El Asbahani et al., 2015). Classical methods are based on the contact of the plant with aqueous steam in the case of steam distillation (SD) or on the direct contact of the plant with water in the case of hydrodistillation (HD). In both extraction techniques, aqueous steam crosses the plant matrix and allows the transport of volatile molecules into a condenser (Figure 9) (El Asbahani et al., 2015).

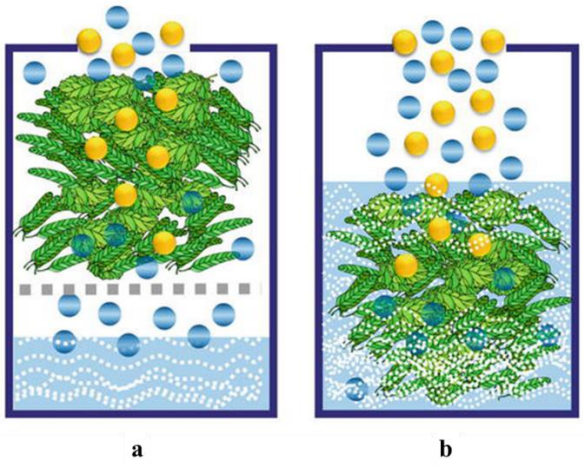

Figure 9. Steam distillation (SD) (**a**) and hydrodistillation (HD) (**b**) working principles for essential oils (EOs) extraction (Stashenko and Martínez, 2019).

SD and HD are the most used traditional techniques for the extraction of EOs, since they are easy to execute, reproducible, and cheap (Golmakani et al., 2008; Guan et al., 2007; Rasul, 2018). However, these techniques are linked to different limitations, such as long extraction times, large water and energy consumption, and the risk of degradation for thermo-sensitive compounds (El Asbahani et al., 2015). Moreover, these processes can be adjusted and modified in terms on EO

yield and chemical composition only by few adjustable parameters (Golmakani et al., 2017; Yang et al., 2014). A modern technique for EOs extraction is Microwave-Assisted Extraction (MAE), which relies on the direct action of microwaves on the plant material that converts part of the absorbed electromagnetic energy into heat energy (Letellier and Budzinski, 1999). Differently from traditional heating that is based on convection and conductive processes, during MAE the heating is achieved in a selective and focused manner, with low energy and heat loss during the extraction. Plant cells involved in the extraction contain water, which is the target of microwave heating. The latter causes an increase in the dipole rotation and in the kinetic energy of the molecules. This leads to an increased temperature inside the cells and to an increase in the pressure on the cell wall caused by the evaporation of the water molecules. Therefore, the rupture of the plant cells occurs, leading to the evaporation of volatile compounds (Figure 10) (Wang and Weller, 2006).

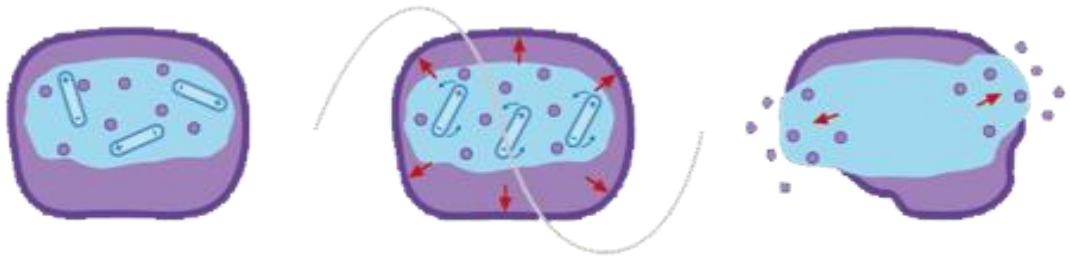

Figure 10. Microwave-assisted extraction (MAE) effects on plant cells.

MAE is affected by several factors and the right selection of these factors can positively influence the yield of extraction, but also the concentration of analytes in the EO.

- Extraction time

  Time, like every other aspect influencing extraction techniques, is a factor influencing MAE. In general, the extension of the extraction period promotes the complete exhaustion of the plant matrix, even if it sometimes increases the risk of deterioration phenomena. Typically for MAE, an extraction time of 15-20 min is sufficient, but even 40 sec have been shown to provide great recovery (Li et al., 2004; Wang et al., 2007). On the other hand, in the extraction of *Artemisia annua* L., a 92% EO yield was achieved in 12 min and after that time the extraction yield decreased due to the heat degradation of many compounds (Hao et al., 2002). Indeed, the extraction time is also related to other factors and its change does not always considerably enhance recovery. For instance, Barbero et al. (2006) performed several extractions of pepper samples to assess the time required to achieve full recovery of the

volatile compounds. The results revealed that the increase of the extraction time did not result in a significant increase in capsaicinoids recovery.

- Microwave power

Microwave power and irradiation time are two parameters that largely influence the extraction process. The combination of low or moderate microwave power with longer exposure times and vice versa may be a useful approach for several plant matrices. For instance, it has been shown that for the extraction of ginsenosides, the increased microwave power improved the recovery during short extraction times (Shu et al., 2003).

Sometimes thermal deterioration can be a problem linked to the use of high power over an extended time. However, some reports suggest that increasing the power from 400 to 1200 W has no significant impact on the stability of some compounds, as for instance, on the yield of flavonoids extraction from *Saussurea medusa* Maxim (Gao et al., 2006). To minimize excessive heating and matrix deterioration, the microwave power must be properly selected (Mandal et al., 2007).

- Temperature

Temperature is also a key factor, particularly when the extraction is performed with closed vessel systems, since it can rise much over the boiling point of the solvent (Letellier and Budzinski, 1999).

Extraction efficiencies usually increase with the temperature since the release of volatile compounds from the matrix is enhanced. In closed systems, an increase of the temperature is also associated with an increase of pressure, and this can also cause safety problems (Mandal et al., 2007).

- Effect of the sample characteristics

Sample quality and sample treatment are other considerable factors that impact on MAE. Usually, for greater contact surface area greater extraction efficiencies are achieved since microwaves manage to penetrate deeper smaller particles. For this reason, preventive shredding and homogenization of the plant matrix is strongly advised. Sometimes, also the soaking of the dried plant material in water can enhance the extraction yields (Mandal et al., 2007).

MAE has been deeply confronted with traditional extraction processes, and several differences have been pointed out, as reported below.

- Extraction time

In HD the heating is achieved by a gradual heat transfer from the heating media to the inner part of the sample, while in MAE heating is volumetrically distributed within the radiation.

Moreover, microwave heating depends on ionic conduction and dipole rotation, and it is directly associated with the dielectric constant of the medium (Chemat et al., 2009). Since the main solvent used is water, which has a high dielectric constant, the MAE process is characterized by a quick heating. This leads to a dramatic reduction in the time of EO extraction. In this regard, Drinic et al. (2020) demonstrated that, even at the lowest microwave power (180 W), the overall extraction time resulted much shorter than that of HD. This was also reported by other studies (Ferhat et al., 2007; Golmakani and Rezaei, 2008; Rezvanpanah et al., 2008).

- EO yield

The study of Drinic et al. (2020) on *Origanum vulgare* L. also demonstrated that the EO yield from MAE is influenced by the microwave power chosen for the extraction. In fact, from microwave powers of 180, 360, and 600 W corresponded EO yields of 2.55, 5.67, and 7.10%, respectively. The yield obtained with HD was, in that case, 5.81%. In addition, Chemat et al. (2009) attributed the increase in the yield related to a higher microwave power as a synergistic impact of temperature and mass gradients.

- EO chemical composition

MAE and HD usually lead not only to different extraction times or EO yields, but also to different EO chemical compositions. In fact, Drinic et al. (2020) also proved that MAE EO of *O. vulgare* was richer in oxygenated monoterpenes and poorer in monoterpene hydrocarbons with respect to that obtained from HD. Moreover, a MAE optimization study on *Trachyspermum ammi* L. demonstrated that the optimized MAE conditions were able to produce an EO with in a higher yield and concentration of thymol compared with HD (Mazzara et al., 2021).

- Environmental impact

In some cases, MAE resulted more 'environmental-friendly' than HD. In fact, Drinić et al. (2020) demonstrated that MAE leads to lower CO_2 emissions and electrical consumption in comparison to HD. In detail, the MAE process allowed to save energy and CO_2 emissions by 75%. Extraction time affects CO_2 emissions and electrical usage, and, since MAE is a faster process for the isolation of the EOs, it can be considered a more 'environmental-friendly' technique.

C. acaulis EO is of great importance mostly for its insecticidal properties and is conventionally obtained by traditional HD (Pavela et al., 2020). Recently, since advanced extraction methods in terms of higher EO yield, reduced costs, and time of extraction have been developed, the study herein presented aimed to analyse the most influential parameters of MAE process to understand

their effects on the latter and ultimately optimize the EO yield and its carlina oxide content. In this regard, the Design of Experiment (DoE) represents a rather convenient set of methodologies since it allows to reduce the number of experimental procedures and understand of the process with numerous data obtained. In this work, by means of experimentation, an optimization of the extraction protocol of *C. acaulis* EO with MAE was performed for the first time and compared to traditional HD.

2.2. Materials and methods

2.2.1 Plant material and sample preparation

C. acaulis roots were obtained from A. Minardi & Figli (Bagnacavallo, Ravenna, Italy, 48012). EO extractions were carried out on samples as received or pre-treated, via milling and/or moistening, according to the screening design experimental conditions (Section 2.2.3). The milling process was performed once for the whole material, corresponding to 2000 g and using a shredder (Molino Trita Piante, Albrigi Luigi Srl, Verona, Italy, code E0585). The effect of milling on sample particle size is reported in Figure 11. The moistening treatment was performed for 16 h before the extraction in distilled water, and the same quantity required for the extraction runs during the screening experiments was used (Section 2.2.3).

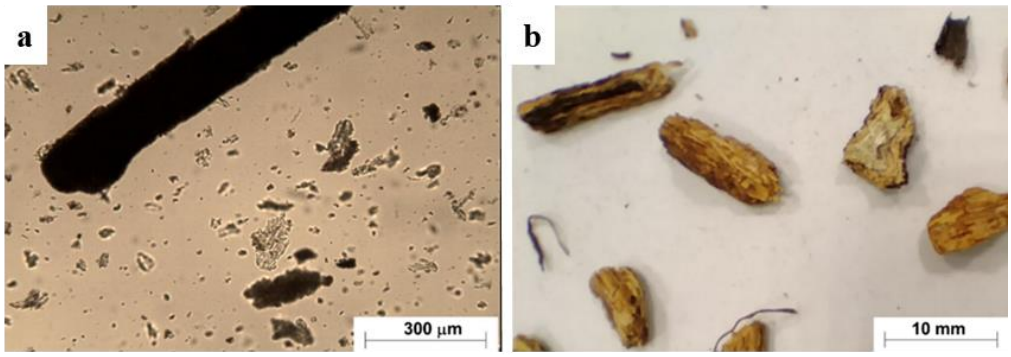

Figure 11. Images of milled (**a**) and as received (**b**) roots. Image **a** was acquired with a microscope (MT9000, Meiji Techno Co. Ltd, JP) equipped with a 3-megapixel CMOS camera (Invenio 3S, DeltaPix, DK) and objective lens of 10X. Image **b** of as received sample was acquired with a mobile phone camera equipped with CMOS 13 megapixel (4208x3120) sensor. Images were processed and calibrated with image pro-plus software.

2.2.2 Microwave-assisted extraction (MAE)

The EO was extracted using a Milestone ETHOS X (Milestone, Italy) microwave extractor, composed of a 2.45 GHz microwave reactor. The latter was furnished with two magnetrons reaching together a maximum power of 1800 W and of an infrared sensor monitoring the temperature. The experiments were carried out in a 5 L glass reactor closed with a glass lid at

atmospheric pressure. The system included a stainless-steel Clevenger-type device above the oven ('Fragrances set-up') linked to a Chiller (Smart H150-2100S, Labtech srl, Sorisole, Bergamo, Italy), which kept the water temperature at 8°C (Figure 12).

Figure 12. Milestone ETHOS X (Milestone, Italy) microwave extractor.

All the 16 screening runs (Section 2.2.3) were carried out on 1 kg of loaded sample, consisting of water and roots in different percentages (W) and varying extraction time (ET), microwave irradiation power (MP), and pre-treatments (milling (Mi) and moistening (Mo)). Experimental runs were performed by applying a continuous microwave irradiation or splitting the whole process in two irradiation steps reaching the time requested to lower the temperature in the reactor to 50°C (Cycle). Between the first and second step the weight of the loaded sample was checked and restored by the addition of water, if necessary. The experimental values of the variables (also defined factors) W, MP, ET, Mi, Mo, and Cycle are reported in the next Section.

At the end of each experiment, the EO was separated from the aqueous layer and collected in glass vials that were then protected from light exposure and kept at -20°C until further chemical analysis.

2.2.3 Design of experiment (DoE) - Screening design

A two-level quarter Fractional Factorial Design (FFD) was employed for the identification of the parameters potentially affecting the MAE process. FFD was defined by:

$$N = l^{f-p/2}$$

where *N* is the number of experimental runs, *l* is the number of levels (2 in this case), *f* the number of factors (6 in this case), and *p* is the partitioning of the design. Moreover, it was decided to fraction the original factorial design to a quarter, so the value of *p* was 4. The FFD selected required 16 experimental runs which have been determined using the generators G1 = 234 and G2 = 1234 and were characterized by a resolution of IV (Table 3). The resolution describes how much the effects are aliased with other effects in an FFD, and it is the ability of the design to lead to an independent estimation of the main effects and their interactions. A resolution of IV represents a good compromise for a screening design since it allows an independent estimation of the main effects, while two-factor interactions are aliased between them (Lewis et al., 1998).

Table 3. Experimental conditions both in uncoded and coded variables of the sixteen runs carried out according to the screening design.

Run	Uncoded variables						Coded variables					
	MP (W/g)	ET (min)	W (%)	Mo	Mi	Cycles	MP	ET	W	Mo	Mi	Cycles
1	1.00	90	65	N	N	N	-	-	-	-	-	-
2	1.45	90	65	N	Y	N	+	-	-	-	+	-
3	1.00	210	65	N	Y	Y	-	+	-	-	+	+
4	1.45	210	65	N	N	Y	+	+	-	-	-	+
5	1.00	90	85	N	Y	Y	-	-	+	-	+	+
6	1.45	90	85	N	N	Y	+	-	+	-	-	+
7	1.00	210	85	N	N	N	-	+	+	-	-	-
8	1.45	210	85	N	Y	N	+	+	+	-	+	-
9	1.00	90	65	Y	N	Y	-	-	-	+	-	+
10	1.45	90	65	Y	Y	Y	+	-	-	+	+	+
11	1.00	210	65	Y	Y	N	-	+	-	+	+	-
12	1.45	210	65	Y	N	N	+	+	-	+	-	-
13	1.00	90	85	Y	Y	N	-	-	+	+	+	-
14	1.45	90	85	Y	N	N	+	-	+	+	-	-
15	1.00	210	85	Y	N	Y	-	+	+	+	-	+
16	1.45	210	85	Y	Y	Y	+	+	+	+	+	+

Each of the 16 runs was carried out for each of the selected variables and employed two different values (levels): experimental values (uncoded variables) and coded notations (coded variables).

The factor W represents the % of water in 1 kg of extracted sample, while for the categorical factors (Mo, Mi, and Cycle) the unloaded values of Y or N identified if the condition was applied or not. Each extraction run was evaluated in terms of the following responses:

- EO yield (%): $\frac{g\ of\ EO}{g\ of\ dry\ biomass}\ x\ 100$;
- EO content of carlina oxide (Section 2.2.5);
- EO density (g/cm^3) (Section 2.2.5);
- EO RI (Section 2.2.5).

All the results of the 16 runs were analysed by multilinear regression using a liner model:

$$y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i$$

where y is the response, β_0 is the model constant, and β_i is the coefficient corresponding to the variables x_i (linear terms). The fitting procedure of the experimental results was then assessed through the analysis of variance (ANOVA) coefficient and residual analysis. Minitab 18 statistical software was used for the performance of the screening design and for data analysis.

2.2.4 Hydrodistillation (HD)

For a comparison with the MAE validation run, an HD was performed, applying the extraction parameters used for MAE. The extraction time was 210 min, and 150 g of *C. acaulis* dried roots were placed in a 2 L round flask with distilled water and heated using a mantle system Falc MA (Falc Instruments, Treviglio, Italy) combined with a glass Clevenger-type apparatus (Figure 13). At the end of the HD process, the EO was collected and stored as described in Section 2.2.2.

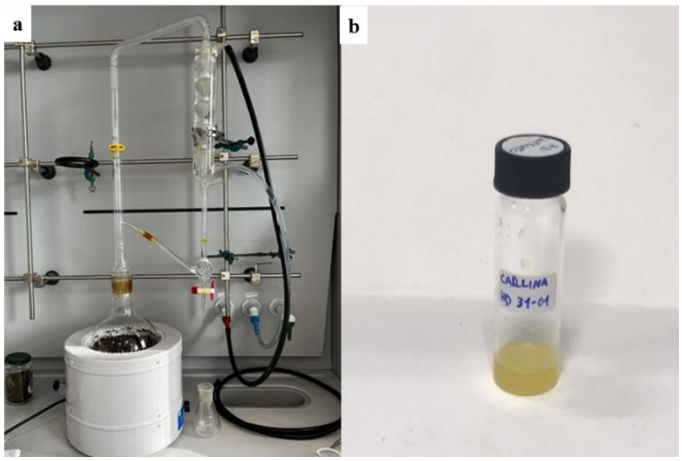

Figure 13.: Hydrodistillation (HD) apparatus (**a**); *Carlina acaulis* essential oil (EO) extracted through HD (**b**).

2.2.5 Analysis of EOs

- Density determination

The density of the 16 MAE EOs and of the HD EO was determined using a digital densimeter with an oscillating U-tube (DA-100M, Mettler Toledo; Figure 14) at 25°C.

Figure 14. U-tube DA-100M from Mettler Toledo.

- Refractive index (RI)

RI was calculated by Abbe refractometer (NAR-1T LIQUID, ATAVO CO ltd; Figure 15) at 20°C.

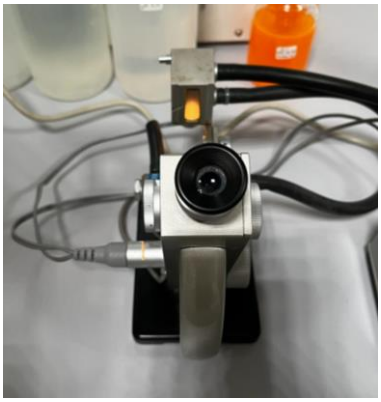

Figure 15. Abbe refractometer (NAR-1T LIQUID, ATAVO CO ltd).

- GC-MS analysis

The EOs were analysed using an Agilent 8890 gas chromatograph (GC) equipped with a single quadrupole 5977B mass spectrometer (Santa Clara, California, USA) and an autosampler PAL RTC120 (CTC Analytics AG, Zwingen, Switzerland) (Figure 16). The ionization was obtained by using an electron impact (EI) source. The injector temperature was set at 280°C, and the gas carrier

was helium at a flow rate of 1 mL min^{-1}. The separation of the molecules was achieved using an HP-5 MS capillary column (30 m, 250 µm i.d., 0.25 µm film thickness).

For EOs quantitative analysis, the column was thermostated at 60°C, then raised up to 120°C at 7°C/min, then to 280°C at 20°C/min and held for 10 min, and finally to 300°C at 20°C/min and held for 1 min. The run time was of 28.571 min. The transfer line was set at 280°C and the temperature of the ionization source and the mass analyzer were set at 230 and 150°C, respectively. The acquisition has been carried out in SCAN mode (29-400 *m/z*). The EOs were diluted (1:2000) in HPLC-grade diethylether containing 200 ppm of undecane as internal standard and 1 µL of this solution was injected in split mode (1:50). On the other hand, for EOs qualitative analysis the temperature of the HP-5 MS column was initially set at 60°C for 5 min, then to 220°C at 4°C/min, to 280°C at 11°C/min and held for 15 min, and lastly to 300°C at 15°C/min and held for 0.5 min. The run time was 67 min. The other GC-MS parameters were the same reported above for the quantitative analysis of the EOs. The transfer line was set at 280°C, and the temperature of the ionization source and the mass analyzer were set at 230 and 150°C, respectively. The acquisition has been carried out in SCAN mode (29-400 *m/z*) and the EOs were diluted (1:100) in *n*-hexane (Merk, Italy), and 1 µL injected in split mode (1:50). The chromatogram analysis followed that previously reported (Benelli et al., 2019a).

Figure 16. GC-MS system used for the essential oils (EOs) analysis.

- GC-MS carlina oxide quantification

Preparation of standard solutions

A stock solution containing 1000 ppm of carlina oxide was prepared in HPLC-grade diethylether. Other standard solutions of carlina oxide were prepared diluting the stock solution to 100, 200, 300, 400, 600, and 800 ppm. Stock solution containing 10149 ppm of undecane was prepared in HPLC-

grade diethylether. This solution was used for the dilution of 200 ppm. The solutions were stored at -20°C in glass vials until chemical analysis.

Linearity of the quantification method

The linearity was evaluated by injecting standard solutions at different concentrations of carlina oxide (100, 200, 300, 400, 600, 800, and 1000 ppm), containing 200 ppm of undecane as internal standard. The calibration curve was constructed by plotting the carlina oxide peak areas against the response factor (RF), calculated as the ratio between carlina oxide and undecane areas. The linear regression equation obtained was $y = 0.0054x - 0.6766$, while the calibration curve showed a coefficient of determination (R^2) of 0.9939 (Figure 17).

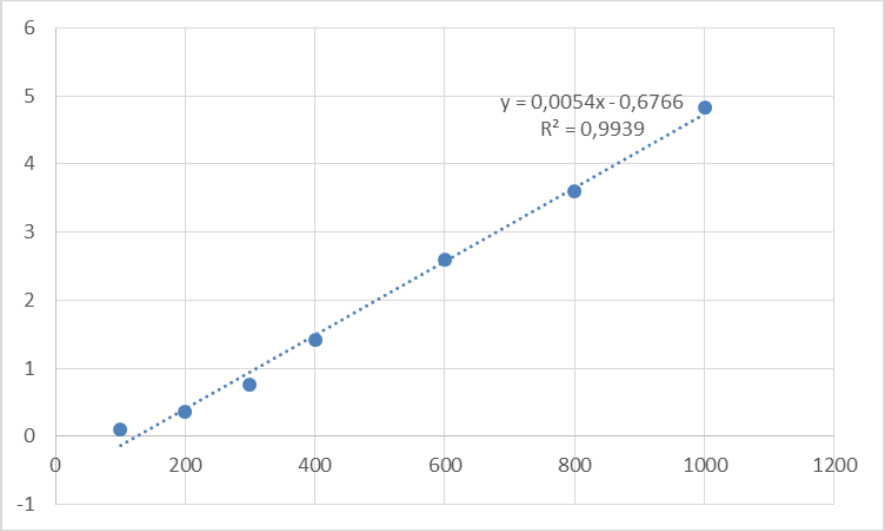

Figure 17. Calibration curve developed for GC-MS quantification of carlina oxide.

2.3. Results and discussion

2.3.1 Preliminary screening

The DoE approach is an effective and easy instrument for the identification of the connection between causes (factors or variables) and responses. It usually allows the improvement of a process by defining the optimal experimental conditions. Therefore, this strategy was applied for the identification of the significant parameters influencing the quantity and quality of *C. acaulis* root EO obtained through MAE. In the screening step, the factors ET, MP, W, Mi, Mo, and Cycles were studied. The data distributions of all the responses (EO yield, carlina oxide concentration, density, and RI) determined for the EOs (16 experimental runs of the screening design) were examined before the FFD analysis using box plots (Figure 18).

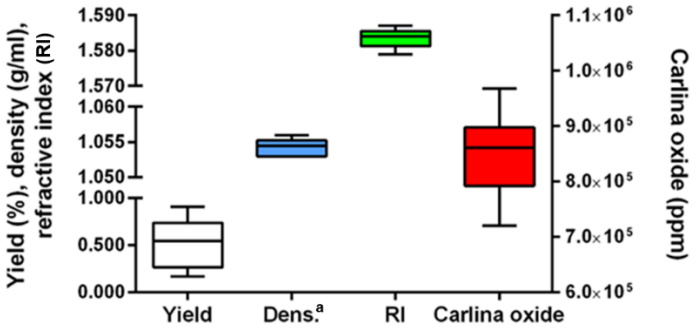

Figure 18. Box plots showing the distribution of the values of the four responses measured. The horizontal line in within the box represents the median value, while the maximum and minimum values recorded are represented by the whiskers.

The measured RI (1.585 and 1.584 for MAE and HD EO, respectively) and density values (1.056 and 1.054 g cm^{-3} for MAE and HD EO, respectively) were all very similar for the 16 EOs, with differences at the third decimal digit, the lowest sensitivity limit of the equipment utilized. Hence, they could not be regarded as reliable for a discrimination between the EOs, and they have not been further investigated in the study. This result was not surprising as it has been previously obtained studying MAE of *T. ammi* EO (Mazzara et al., 2021). Since *T. ammi* and *C. acaulis* EOs are both characterized by the presence of a single component (thymol and carlina oxide, respectively), it is likely that RI and density did not significantly vary (at least at the level of the second decimal digit) as a function of the extraction condition. Conversely, the Box Plot showed a significative variation of the EO yield and carlina oxide content among the 16 experiments, ranging from 0.17 to 0.91% and from $7.2 \cdot 10^5$ to $9.7 \cdot 10^5$ ppm (72-97%), respectively. Consequently, they were the only two parameters considered for FFD analysis.

However, the regression analysis (detailed results are reported in Section S1.2 of the Supplementary Material) pointed out that carlina oxide concentration could not be totally described by the model used (not statistically significant regression detected). This result suggested that the data variability observed (Figure 18) was due to the intrinsic variability of the samples or depended on other factors that were not investigated in the study and that are currently unknown. Such result has been previously reported in the literature from an evaluation of the effect of MAE operating conditions on the EOs composition of *Cannabis sativa* L. and *T. ammi* (Fiorini et al., 2020; Mazzara et al., 2021). However, the above-mentioned study was carried out using a different DoE design. Conversely, the model fitting for the EO yield results was statistically significant. The adjusted determination coefficient (R^2_{adj}) was 0.69, and the residuals analysis did not show issues or anomalies (Section S1.1). In these conditions, the regression model was reliable, at least for a screening. Concerning the model coefficient analysis, it pointed out that the only statistically significant parameter was the ET, showing a positive correlation with the EO yield. None of the

other factors studied had a significant effect on the EO yield, as reported in the Pareto plots (Figure 19).

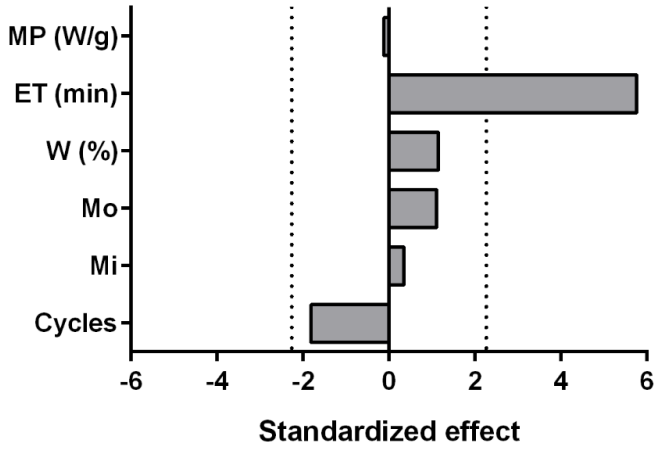

Figure 19. Pareto plots showing the factors influence on the essential oil (EO) yield as determined in the Fractional Factorial Design (FFD). The dot lines represent the statistically significative limits (t-value$_{\alpha/2}$ of a t-distribution with degrees of freedom equal to the degrees of freedom for the error term) when the variables are reported in term of standardized effect (t-value of the coefficient). The abbreviations are the following: MP, microwave power; ET, extraction time; W, percentage of water added to the plant roots; Mo, moistening process; Mi, milling process.

The results obtained for W, Mo, Mi, and cycles confirmed previous literature data. In fact, it was displayed that these factors were irrelevant for EO yield of *T. ammi* schizocarps (Mazzara et al., 2021). This could be linked to the nature of the samples that in both studies were hard and fibrous. On the contrary, the MP result was rather surprising considering the literature findings (Chemat et al., 2005; Fiorini et al., 2020), but the *p*-value (0.911) obtained did not allow a different interpretation. Finally, sample conditions at the end of the extraction should be considered and discussed. Specifically, samples 3, 4, 11, and 12 (Table 3) were burned or partially burned. By a simple observation of the extraction parameters reported in the above-mentioned Table, all these samples shared two experimental conditions: long ET (210 min) and low W (65%). None of the un-burned samples were processed applying the same values of ET and W at the same time. The sample burning was not an independent variable and could not directly be considered during regression analysis. For these reasons, the eventual effect of burning on EO yield and carlina oxide concentration was evaluated using parametric hypothesis tests such as T-Test or ANOVA. The results of hypothesis tests (Figure 20a-21a) clearly indicated that EO yield and carlina oxide concentration were not affected by the sample burning. In addition, a comparison was carried out dividing the un-burned samples in two different groups according to the ET (Figure 20b-21b). The results suggested that a different ET determined a different EO yield but not a different carlina oxide concentration, independently by sample burning and confirming both the null relevance of

the burning process and the FFD results. However, it should be highlighted that the exact moment of burning was not identified (surely between 90 and 210 min), so the finding that burning did not affect the quality and quantity of EOs was valid only for a maximum extraction time of 210 min.

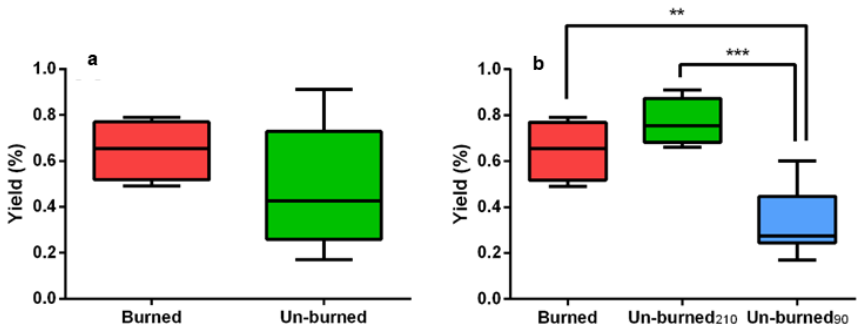

Figure 20. Box plots showing the essential oil (EO) yield values distributions for burned and un-burned samples (**a**) and for burned vs un-burned samples subjected to microwave irradiation for 90 or 210 min (**b**). The two groups in panel **a** were compared with unpaired t-test, while the three groups of panel **b** with ANOVA followed by post-hoc Tukey's test. The significance was reported in term of *p*-value as follows: no asterisks for $p > 0.05$; * for $0.05 < p < 0.01$; for ** $0.01 < p < 0.001$; *** for $p < 0.001$. The horizontal line in within the box is the median value, while the whiskers represent the maximum and minimum values recorded. In the box plots the top of the rectangle indicates the third quartile, the horizontal line within the rectangle is the median, the bottom of the rectangle indicates the first quartile, while the whiskers represent the maximum and minimum values recorded.

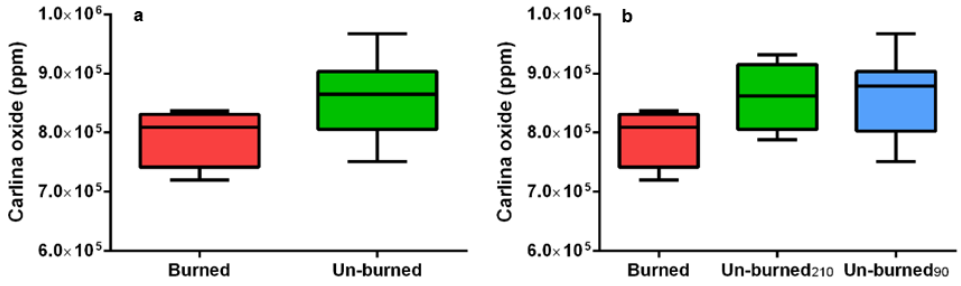

Figure 21. Box plots showing carlina oxide concentration values distributions for burned and un-burned samples (**a**) and for burned vs un-burned samples subjected to microwave irradiation for 90 or 210 min (**b**). The two groups in panel **a** were compared with unpaired t-test, while the three groups of panel **b** with ANOVA. The significance was reported in term of *p*-value as follows: no asterisks for $p > 0.05$; * for $0.05 < p < 0.01$; for ** $0.01 < p < 0.001$; *** for $p < 0.001$. The horizontal line in within the box is the median value, while the whiskers represent the maximum and minimum values recorded. In the box plots the top of the rectangle indicates the third quartile, the horizontal line within the rectangle is the median, the bottom of the rectangle indicates the first quartile, while the whiskers represent the maximum and minimum values recorded.

2.3.2 Effect of the extraction time

The screening design purpose is useful for the identification of the factors that affect the studied process rather than providing a full description of how it works. Usually, after the identification of few relevant factors, the process is deeply studied using a more suitable DoE, as Central Composite

Design (CCD) (Mazzara et al., 2021). This strategy aims to optimize the cost/benefit ratio of the full project. However, further DoE study was not required in this work since the preliminary screening demonstrated in a reliable manner that the ET is the only significant parameter. Consequently, to deeply define the effect of ET on the EO yield, further experimental runs were carried out setting constant the un-relevant parameters (MP, W, Mo, Mi, and Cycles) and varying the ET from 90 to 330 min. Mo, Mi, and Cycles were not performed (levels NA in Table S1, Section S1.3 of Supplementary Material), while MP and W were set to values that avoided samples burning (1 W g^{-1} and 85% for MP and W, respectively; Table S1, Section S1.3 of Supplementary Material). The effects on EO yield and carlina oxide concentration of the new conditions applied are reported in Figure 22.

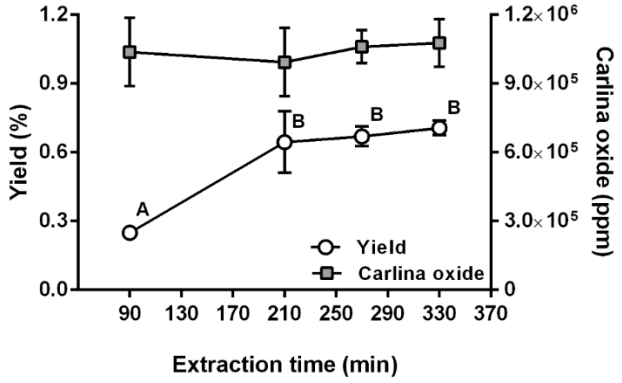

Figure 22. Effect of the extraction time on the essential oil (EO) yield and carlina oxide concentration during the extraction runs carried out subsequently to Fractional Factorial Design (FFD). The results of different time points for both responses were compared with ANOVA and, if the differences were statistically significant, further analyses using Tukey's test were performed. In the graph, groups having the same letters are not statistically different, while different letters indicate statistically differences (results of Tukey's test). The absence of letters indicates no statistical differences when tested with ANOVA.

Carlina oxide concentration remained constant independently on the extraction length (no statistically significant differences were identified using ANOVA), confirming the results of the screening also for longer ET. Concerning the EO yield, it grew up to 0.65% around 210 min (the differences between 90 and 210 were statistically significant according to post-hoc Tukey's test), remaining almost stable for longer times (the yields at 210, 270, and 330 min were not statistically different, while all the 3 EO yield values statistically differed with the values at 90 min, according to Tukey's test). None of the samples reported in Figure 22 burned during the extraction. Finally, to be certain that no more EOs could be extracted, the last run at 330 min was re-started after its completion and no additional EO was collected. According to these results, 210 min was identified as the ideal length for the extraction process of EO's from *C. acaulis* root using MAE.

Unfortunately, no other studies on EO extraction from *C. acaulis* using MAE are available, making the reported study the first to be undertaken. Consequently, a comparison of the results obtained

with other works is not possible. However, studies on MAE of many plants have been reported in literature using a similar DoE approach, to which it is possible to compare the parameters evaluated and their impact on EO yield and chemical composition. Mazzara et al. (2021) used a two-step DoE strategy to minimize the number of tests and increase the cost/benefit ratio in a comparable study using *T. ammi* schizocarps. Individual value plots were used to assess the responses determined for each of the 16 experimental runs. The EO yield exhibited significant variance throughout all experimental runs, differently from the density and RI values. Moreover, the ET resulted the most significant variable, followed only by Cycles. However, in this study it was concluded that the increased EO yield obtained for multi-cycle extraction procedures was most likely attributable to an increase in ET rather than an influence of the Cycles themselves. Furthermore, correlations between the three major compounds, namely thymol, *p*-cymene, and *γ*-terpinene were detected and evaluated. Their levels resulted modified as ET and MP values changed. For example, the most active ingredient, thymol, rose in concentration as MP increased and within a short period of time, exhibiting an inverse trend to *p*-cymene and *γ*-terpinene. Fiorini et al. (2020) reported another study using a two-step DoE on dry inflorescences of *C. sativa*, and the results showed that the MAE treatment, using high MP and relatively long ET, significantly increased the content of cannabidiol (CBD) in the EO, maintaining high EO yield values when compared to conventional HD. DoE data obtained in the study seemed to indicate that an increase in EO yield might be related to an increase in CBD recovery.

2.3.3 Comparison of MAE and HD

To have an equal measure of comparison of the EO yield obtained through MAE, a conventional HD run was performed under the same conditions of the optimized MAE extraction protocol. Such conditions included:

- W/R ratio: 85-15%;
- ET: 210 min.

The EO yield from HD resulted of 0.49%, differently from MAE which gave 0.65% of yield at the above-reported conditions. This comparative run validated the MAE conditions described above, demonstrating that an optimization of the protocol for *C. acaulis* EO extraction was achieved. Moreover, the quantitative GC-MS analysis of the HD EO revealed no significant differences in carlina oxide concentration if compared with the MAE EO run chosen for the comparison. In addition, HD and MAE EOs were also analysed qualitatively through GC-MS analysis and no significant differences in the chemical compositions were found. In fact, HD and MAE EOs resulted characterized by carlina oxide (accounting for 97.9% and 98.6% of the total composition, respectively), benzaldehyde (1.4% and 0.6%, respectively), and *ar*-curcumene (0.1% and 0.3%,

respectively). The total of identified compounds was 99.3 and 99.5% for HD and MAE EOs, respectively (Figure 23). The chemical compositions found in this study were comparable with that reported in the literature (Benelli et al., 2019a; Pavela et al., 2020).

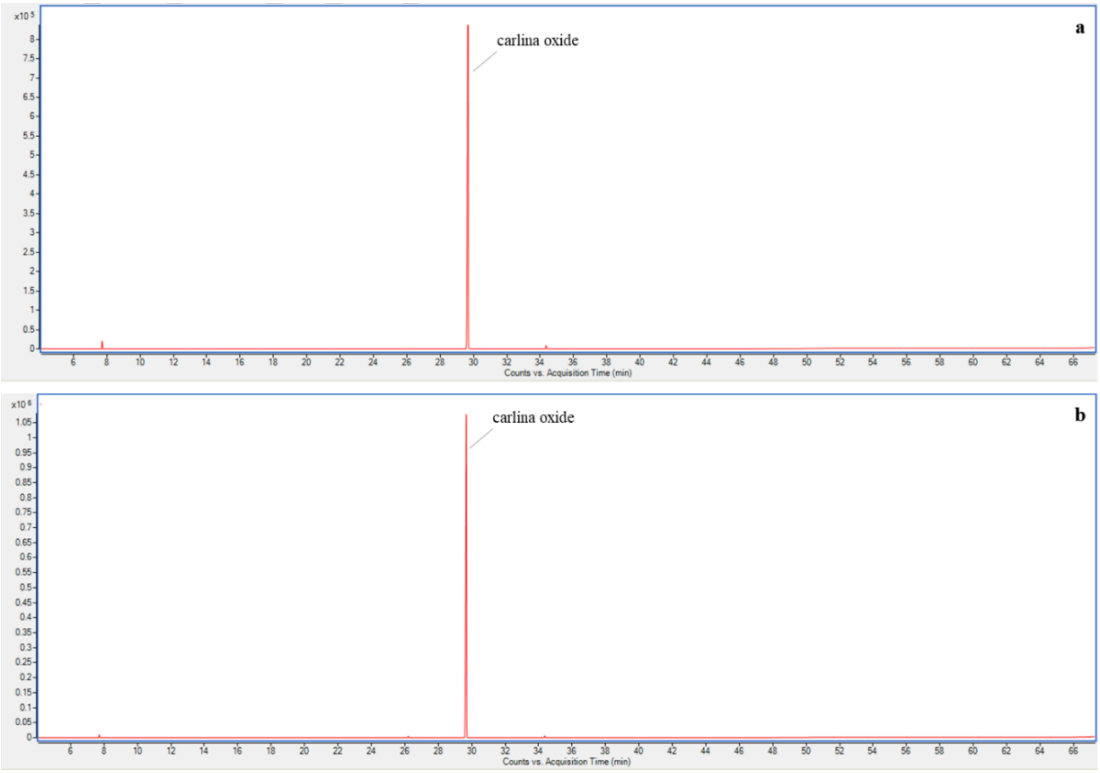

Figure 23. Chromatograms of the essential oils (EOs) from hydrodistillation (HD) (**a**) and microwave-assisted extraction (MAE) (**b**) deriving by qualitative GC-MS analysis.

Nonetheless, further trials for extraction of this EO with MAE should be performed, for example, by changing some external parameters (ex. cooling water temperature), to investigate the possibility of a further refining of the protocol and, consequently, of the EO yield.

2.4. Conclusions

Given that EOs are valuable products to be exploited in pharmaceutical, nutraceutical, cosmeceutical, and agrochemical industries, it may be worthwhile to explore and implement new and eco-friendly extraction methods. In the presented study, the optimization of MAE was carried out utilizing a one-step statistical technique (DoE), which allowed to relate the characteristics of the produced EOs with the applied experimental conditions using mathematical models. The goal of this study was to optimize the extraction procedure of *C. acaulis* EO, with an EO yield equivalent to or greater than the one obtained by conventional HD. Even though DoE approach is

based on statistical analysis and prediction, the preliminary screening results allowed to optimize the protocol only by the measure of ET, and thus skipped complex data analysis such as Surface Response Methodology and CCD, which were inevitably required for other previously mentioned works (Fiorini et al., 2020; Mazzara et al., 2021). In conclusion, MAE is a novel and effective extraction approach that provides a more stringent control of the factors involved in the process if compared to traditional extraction methods. By the optimization of *C. acaulis* root EO extractive process, it can be stated that MAE resulted in more efficient than conventional HD in terms of significant savings of energy and water consumption, obtaining a comparable yield. This approach might be effective for the implementation of the production of *C. acaulis* EO, which is gaining popularity in the bio-agricultural domains due to its promising insecticidal properties.

3. General procedures for biological assays

Unless otherwise stated, the following general experimental procedures were employed in the biological studies reported in the following Sections. The results herein presented are comparable for all the studies performed.

3.1. *Carlina acaulis* essential oil (EO) isolation

3.1.1 Materials and methods

The dry roots of *C. acaulis* were always acquired from A. Minardi & Figli (Bagnacavallo, Ravenna, Italy). The EO was obtained through HD from the roots preventively reduced to a 1.5 mm size following the procedure described in Section 2.2.1. For the extraction, 1 kg of roots was soaked for 16 h with 7 L of distilled water and into a 10 L round bottom flask. The HD process was conducted using the distillation system described in Section 2.2.4 and the storage of the EO followed the procedure described in the same Section.

3.1.2 Results

The EO presented a yellowish color and a density of 1.063 g mL^{-1}. The HD extraction yield was of 0.97% (w/w).

3.2. *Carlina acaulis* essential oil (EO) chemical characterization

3.2.1 Materials and methods

The chemical composition of *Carlina acaulis* EO was investigated through GC-MS accordingly to the qualitative analysis procedure described in Section 2.2.5.

3.2.2 Results

C. acaulis EO chemical composition was mainly characterized by carlina oxide (97.8%) (Figure 24), while minor constituents were benzaldehyde (0.9%), *ar*-curcumene (0.7%), *β*-sesquiphellandrene (0.2%), and *α*-zingiberene (0.1%). The total of identified compounds was 99.8% and this composition was in accordance with literature (Benelli et al., 2019a).

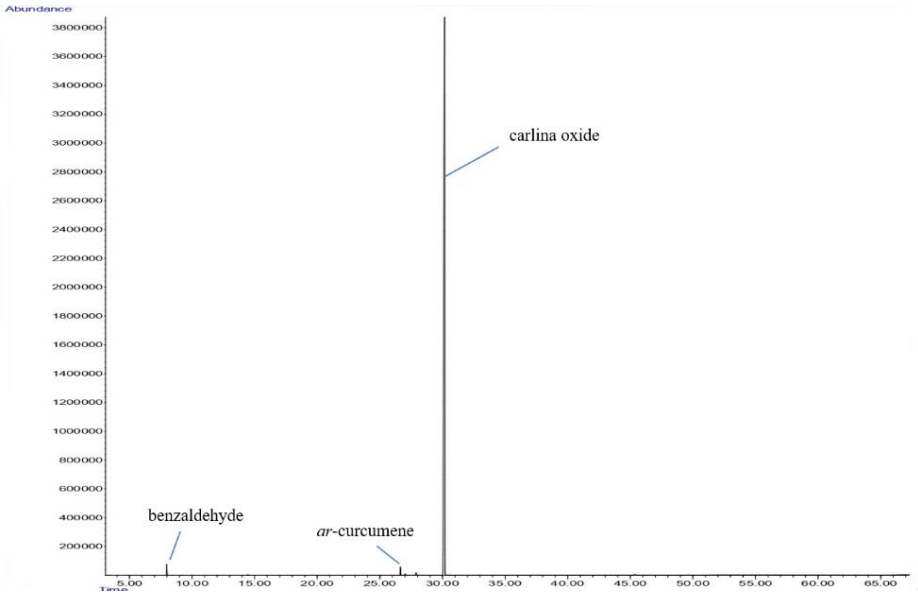

Figure 24. Chemical composition of *Carlina acaulis* essential oil (EO).

3.3. Carlina oxide isolation

Part of the obtained EO (1.403 g) was subjected to silica gel (70 g) column chromatography (70−230 mesh, 60 Å, Merck) using *n*-hexane (Merk, Italy) as eluent. Carlina oxide (1.306 g) was isolated in different fractions, which were collected and evaporated to dryness through a Rotary evaporator (Buchi Rotavapor R-200, Büchi Labortechnik AG, Flawil, Switzerland). Residues of the eluent were removed under high vacuum. Carlina oxide was then characterized through NMR analysis and mass spectrometry (MS). The structural data obtained were linear with those already reported (Benelli et al., 2019a).

4. Antibacterial and antifungal activities of *Carlina acaulis* essential oil (EO) and its nanoemulsion (NE)

4.1. Introduction

Antibiotic resistance consists of the bacteria's resistance to the effect of antibiotic agents and allows the persistence of the infections, leading to an increase of the risk of negative clinical outcomes and, in some cases, of death. Moreover, new mechanisms of resistance are continuously emerging, enhancing the possibility of propagation of new resistant strains (Barbieri et al., 2017). Thus, the increasing antibiotic resistance represents a source of great concern for the contemporary world. The ability of antibiotics to treat infectious diseases is gradually diminishing and this is the reason why research is intensely focusing on the discovery of novel and effective antibiotic compounds. In this context, plants can be considered as promising sources of antibiotic molecules. This is linked to the fact that they have developed an intricate defence system to adapt and survive to biotic and abiotic stresses (Ballhorn et al., 2009). In response to these stresses, several responses are produced, as, for instance, those against fungal and bacterial infections. Indeed, plants usually activate a series of processes, as the synthesis of specific secondary metabolites with protective and defensive functions (Zaynab et al., 2018). Numerous of these molecules have been widely employed for the development of novel antibiotic agents. In fact, between the nine classes of antibiotics, six are represented by natural-derived compounds, while only three have been totally developed through chemical synthesis (Rossiter et al., 2017). Moreover, the use of nanosystems is of crucial importance to improve the stability and physio-chemical properties of antibacterial and antifungal agents, but also to increase their ability to be delivered to the infection site and to reduce their side effects (Patra et al., 2018). In detail, surface charge, particle size, and solubility of nanosystems are key characteristics involved in processes as the biodistribution, intracellular uptake, or clearance. Furthermore, the nanometric size of the particles is responsible for efficient drug loading, while the zeta (ζ)-potential and surface charge guide the interactions with the tissues or cells of the action site (Dacoba et al., 2017; Lombardo et al., 2019). Moreover, nanosystems have a high hydrophobicity that allows them to easily target the drug delivery within the bacterial membrane (Patra et al., 2018).

As described in Section 1.4, *C. acaulis* has been widely used in traditional medicine to treat infections and several studies have been reported confirming the antifungal and antibacterial properties of the EO, extracts, and carlina oxide (Section 1.7). These activities certainly depend on the presence of carlina oxide, since polyacetylenes are well-known antibacterial and antifungal compounds. In view of its potential as an antifungal and antibacterial agent, this work aimed to evaluate the antimicrobial and antifungal properties of *C. acaulis* EO and carlina oxide against different bacteria and yeasts. In addition, the EO was encapsulated into a nanoemulsion (NE) and

tested on the same fungal and bacterial strains, to evaluate the improvement of the physio-chemical properties and biological activity.

4.2. Materials and methods

4.2.1 Nanoemulsions (NEs) development and characterization

C. acaulis EO NE was produced using a high-pressure homogenizer and the protocol reported by Rosi Cappellani et al. (2018), with some modifications: 6% w/w of EO, 2% w/w of ethyl oleate and 3% w/w of surfactant (Polysorbate 80, Merk) aqueous solution were mixed under high-speed stirring (Ultraturrax T25 basic, IKA® Werke GmbH & Co.KG, Staufen, Germany) for 5 min at 9500 rpm. The emulsion was then homogenized using a French Pressure Cell Press (American Instrument Company, AMINCO, Maryland) for 4 cycles at the pressure of 130 MPa. The visual analysis and characterization of the NE was achieved with a polarizing optical microscope (MT9000, Meiji Techno Co Ltd, JP) furnished of a 3-megapixel CMOS camera (Invenio 3S, DeltaPix, DK).

The measures of particle size were performed with dynamic light scattering (DLS) analyses with a Zetasizer nanoS (Malvern Instrument, UK) equipped with a backscattered light detector operating at 173°. The sample (1 mL) was placed into a cuvette and analysed at 25°C, following a temperature equilibration time (180 s).

4.2.2 Nanoemulsions (NEs) stability studies

Long-term stability

The NEs were stored at room temperature and 12:12 h (L:D) for up to six months. The physio-chemical stability of the NEs was evaluated performing DLS analysis at various time points: 0 day (t0), 1 month (t30), 3 months (t90), 6 months (t180), and 9 months (t270).

Accelerated stability test

The NEs thermodynamic stability was assessed through a test consisting of three steps (centrifugation, heating/cooling cycles, and freeze/thaw cycles) and following the procedure developed by Alkilani et al. (2018) applying certain modifications.

- Centrifugation: the sample was subjected to centrifuge at 9000 G for 30 min. If no phase separation was observed, the next step was performed.

- Heating-cooling cycle: the NEs were exposed to three cycles of changing temperature, from 4 to 40 °C, with a period of storage of 48 h at each temperature. If the stability of the resulting NEs was assessed, the next step was performed.

- Freeze-thaw cycle: 3 freeze-thaw cycles between -21 °C and +25 °C were accomplished, with a period of storage of 48 h at each temperature.

After the three steps test, the NEs were analysed by visual inspection and DLS.

4.2.3 Antimicrobial test

The antifungal and antibacterial properties of *C. acaulis* products were evaluated on Gram-positive and Gram-negative bacteria, and yeast. The microorganism isolation and identification (by API systems) was performed at the Department of Biomedical Science and Human Oncology (Hygiene Section) of the University of Bari (Italy). The isolates were provided by patients in the intensive care unit of the same Department. The ATCC strains and bacteria derived from clinical isolation used in this study are reported in Table 4.

Table 4. ATCC strains and bacteria derived from clinical isolation.

Strains from ATCC	
Acinetobacter baumannii	ATCC 19606
Bacillus cereus	ATCC 10876
Enterococcus faecalis	ATCC 29212
Escherichia coli	ATCC 25922
	ATCC 35218
	ATCC 25922
Klebsiella pneumoniae	ATCC 13883
Pseudomonas aeruginosa	ATCC 27853
Staphylococcus aureus	ATCC 29213, ATCC 6538, ATCC 6538P, ATCC 25923, ATCC 43300 methicillin resistant (MRSA)
Strains from clinical isolation	
Corynebacterium striatum	RM
Cytrobacter freundii	IG
Enterococcus faecalis	BS, 2011, 1011
Staphylococcus lugdunensis	-
Staphylococcus sciuri	-
Staphylococcus warneri	-
Proteus mirabilis	IG
Serratia marcescens	IG
Staphylococcus aureus	IG24, IG23, IAC, TER

Moreover, also some clinically isolated strains with a severe threat of antibiotics multi-resistance were also used in this work, and they are reported in Table 5.

Table 5. Clinically isolated strains with severe threat of antibiotics multi resistance.

Strains from clinical isolation	
Acinetobacter baumannii	BS
Escherichia coli	ESBL
Klebsiella pneumoniae	BS

On the other hand, yeast strains used in this study are reported in Table 6.

Table 6. Yeast strains from ATCC and from clinical isolation.

Strains from ATCC	
Candida albicans	ATCC 10231, ATCC 90028
Candida glabrata	ATCC 15126
Candida kefyr	ATCC 204093
Candida krusei	ATCC 6258
Candida tropicalis	ATCC 750
Strains from clinical isolation	
Candida albicans	A18, 10A12,810
Candida krusei	31A29
Candida parapsilosis	11A13, 1A1, 911, 910
Candida tropicalis	810

The antifungal and antibacterial activities were evaluated through modified Minimum inhibitory concentration (MIC) determinations and agar diffusion, according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2008, 2015). MIC was defined as the lowest concentration that did not result in any visible growth of the bacterial strains compared to their growth in the control well. MIC values are reported in mg mL^{-1}, and Rifaximin (μg mL^{-1}) and Amphotericin (μg mL^{-1}) were used as controls.

Two-fold serial dilutions in ethanol (Merk, Italy) of the EO and carlina oxide were prepared and plated for bacteria in the suitable test medium in concentrations between 0.17-10.9, 0.17-10.7 mg mL^{-1}, respectively. For the tests on yeast, other dilutions were made: 0.17-5.4 mg mL^{-1} for the EO and 0.02-0.16 mg mL^{-1} for carlina oxide, while the NE doses ranged between 0.5-500 mg for bacteria and yeast, respectively and they were used without solvent. The solvent was assayed to assess the presence of adverse effects on bacteria and yeast at its maximum concentration along with the medium.

The culture media used for bacterial strains was on Mueller Hinton Agar (MHA, Oxoid) and the bacterial suspensions were constituted of 2-3 colonies of each strain taken from an MHA plate and dissolved in 2 mL of Mueller Hinton Broth (MHB, Oxoid). The MICs for the EO and carlina oxide on bacteria were determined by broth microdilution method, using 96-well plates and according to CLSI regulations (CLSI, 2008, 2015) with some modifications.

Bacterial cell suspension turbidity was evaluated using a spectrophotometric method (Thermo Spectro Nic, Genesis 20) at 625 nm. Then, the standardized suspension was diluted 1:100 with MHB to obtain 1-2 x 10^6 CFU mL^{-1}. All wells were seeded with 100 µL of inoculum, and some wells were prepared with only inoculated broth and used as control growth. The plates were incubated at 37 ˚C for 24 h, and MIC was determined by using an antibacterial assay repeated in duplicate. The MHB medium well control, 0.1% (v/v) Tween 80, and ethanol (Merk, Italy) 3% (without the EO) was used as positive growth control.

Concerning the fungal strains, these were subcultured twice on Sabouraud dextrose agar before the tests. The fungal suspension was removed from its frozen stock at -70°C, and the yeast cells were washed for 4 times in sterile saline. The strains were inoculated in tubes containing 5 mL of Sabouraud dextrose broth, and then left in incubation at 35 °C for 48 h under stirring. MIC values were determined by broth microdilution method, in accordance with CLSI protocols for yeasts (CLSI, 2008, 2015). The EO was prepared for the antifungal tests as described for the antibacterial assays. A small amount of inoculum from incubated tubes was dissolved in RPMI 2% glucose and then spectrophotometrically adjusted to 0.5 x 103 to 2.5x 103 CFU mL^{-1} (McFarland, turbidity standard). The initial inoculum was confirmed by plating serial dilutions and determining the colony counts. A total of 0.1 mL of each yeast suspension was dispensed into serially diluted wells containing the drugs or the EO, achieving the final drug concentration. After the addition of 0.1 mL of inoculum, the plates were incubated at 36 °C for 48 h. MIC was defined as the lowest concentration of the mixtures at which no visible growth of the fungal strains could be detected compared to their growth in the negative control well. MIC values are reported in mg mL^{-1} for the EO, NE, and carlina oxide and in µg mL^{-1} for Rifaximin and Amphotericin. The results of Agar diffusion method are reported as mm of the inhibition zone. The MIC and Agar diffusion determinations were realized in triplicate in 3 independent tests, and the results reported are an average of the 3 evaluations.

4.3. Results and discussions

4.3.1 Preparation and characterization of the essential oil (EO) nanoemulsion (NE)

The NE was able to encapsulate the 6% w/w of EO, 2% w/w of ethyl oleate and 3% w/w of surfactant (Polysorbate 80, Merk). The NE sample showed the maintenance of the average size of

the internal droplets below 200 nm (Z-average from DLS in the range 114-136 nm) and the absence of oil droplets above 1 μm (observed from optical microscope). The value of 200 nm represents the upper-established limit for this kind of formulations (Huang et al., 2010). The physical stability of the NE was also confirmed up to 9 months, without showing phase separation and creaming. No increase in the mean particle size (Z-average) and in the polidispersity index (PDI) occurred as resulted from DLS measurements (Table 7).

Table 7. Variation of Z-Average (nm) and polydispersity index (PDI) for the *Carlina acaulis* essential oil (EO)-nanoemulsion (NE) over time (t0-t270 days).

Time (days)	Z-Average (nm)	PDI[a]
t0	134.62 ± 2.22	0.268 ± 0.009
t30	129.63 ± 1.11	0.265 ± 0.005
t90	121.5 ± 0.78	0.260 ± 0.008
t180	117.63 ± 2.23	0.256 ± 0.009
t270	114.7 ± 0.69	0.262 ± 0.002

[a]PDI, polydispersity index.

Given the findings obtained in the study, the *C. acaulis* EO-based NE could be considered a physio-chemical stable nanosystem.

4.3.2 Antimicrobial assays

C. acaulis EO and carlina oxide resulted active against the screened Gram-positive bacterial strains, with MIC values between 2.7-10.9 mg mL^{-1} and 0.33-5.35 mg mL^{-1}, respectively. Noteworthy is also the marked action on clinical bacterial isolates with significative virulence, resulting carlina oxide more active than the EO. However, these results were also accompanied by a total inefficacy of both products on the Gram-negative bacteria assayed. About the EO-NE, it was active on Gram-positive bacterial strains and the MIC values varied between 7.5 and 60 mg mL^{-1}. The action against the Gram-negative strains resulted lower, with MIC values between 125-500 mg mL^{-1} (Table 8).

Table 8. MIC and agar diffusion ADIFF results of *Carlina acaulis* essential oil (EO), nanoemulsion (NE), carlina oxide, and Rifaximin against different bacterial species.

	MIC[a] EO (mg mL^{-1})	**ADIFF[b] EO (mm)**	**MIC[c] NE (mg mL^{-1})**	**MIC[d] Carlina oxide (mg mL^{-1})**	**MIC[e] Rifaximin (µg mL^{-1})**
Bacillus cereus 10876	5.4	0.9	15	na[f]	0.1
Corynebacterium striatum RM	5.4	1.3	15	1.33	0.4
Enterococcus faecalis 1011	10.9	1.0	60	5.35	0.4
Enterococcus faecalis 29212	5.4	0.8	60	10.7	0.1
Staphylococcus aureus 23	10.9	0.8	60	5.35	0.1
Staphylococcus aureus 24	5.4	1.0	15	0.33	0.4
Staphylococcus aureus 25923	2.7	0.7	15	0.33	0.1
Staphylococcus aureus 29213	5.4	1.1	15	0.33	0.1
Staphylococcus aureus 43300	2.7	0.9	7.5	1.33	3.1
Staphylococcus aureus 6538	2.7	1.0	7.5	1.33	0.1
Staphylococcus aureus 6538P	5.4	1.0	15	0.67	0.1
Staphylococcus aureus IAC	5.4	0.9	7.5	0.67	0.1
Staphylococcus aureus TER	10.9	0.7	15	1.32	0.1
Staphylococcus lugdunensis	10.9	1.0	15	1.33	0.1
Staphylococcus sciuri	10.9	1.0	15	2.77	0.1
Staphylococcus warneri	5.4	1.0	15	1.33	0.1
Acinetobacter baumannii BS	na	na	15	na	25.0
Citrobacter freundii IG	na	na	500	na	12.5
Escherichia coli 25922	na	na	125	na	6.3
Escherichia coli 35218	na	na	125	na	6.3
Escherichia coli ESBL	na	na	250	na	6,3
Klebsiella pneumoniae 13883	na	na	500	na	12.5
Klebsiella pneumoniae BS	na	na	125	na	25.0
Proteus mirabilis	na	na	500	na	6.3
Pseudomonas aeruginosa 27853	na	na	250	na	6.3
Serratia marcescens IG	na	na	500	na	6.3

[a]Minimum inhibitory concentration (MIC) of the essential oil (EO); [b]Agar diffusion (ADIFF) test of the EO; [c]MIC of the nanoemulsion (NE); [d]MIC of carlina oxide; [e]MIC of Rifaximin; [f]na, not active.

Conversely, a strong activity of the EO and carlina oxide was achieved on fungal strains, being both able to inhibit all *Candida* spp. isolates' growth. The EO showed a moderate action only on some fungal strains, while the fungicidal activity of carlina oxide was higher (Table 9).

Table 9. MIC and ADIFF findings of *Carlina acaulis* essential oil (EO), nanoemulsion (NE), carlina oxide, and amphotericin against the fungal strains.

	MIC[a] EO (mg mL^{-1})	**ADIFF[b] EO (mm)**	**MIC[c] NE (mg mL^{-1})**	**MIC[d] Carlina oxide (mg mL^{-1})**	**MIC[e] Amphotericin B (µg mL^{-1})**
Candida albicans ATCC 10231	0.68	1.30	1.9	0.04	1.0
Candida albicans ATCC 90028	1.35	1.00	1.9	0.04	1.0
Candida glabrata ATCC 15126	2.70	1.22	0.9	0.04	0.5
Candida kefyr ATCC 200,0493	2.70	0.91	0.9	0.02	0.5
Candida krusei ATCC 6258	2.70	1.10	0.9	0.02	1.0
Candida albicans 10A12	2.70	1.20	0.9	0.04	1.0
Candida krusei 31A29	0.67	1.52	1.9	0.04	1.0
Candida parapsilosis 11A13	0.67	1.41	1.9	0.08	0.5
Candida parapislosis 1A1	0.34	1.41	1.9	0.04	1.0
Candida parapsilosis 910	1.35	1.22	1.9	0.04	0.5
Candida parapsilosis 911	1.35	1.21	1.9	0.04	0.5
Candida tropicalis 810	0.36	0.90	1.9	0.08	1.0

[a]Minimum inhibitory concentration (MIC) of the essential oil (EO); [b]Agar diffusion (ADIFF) test of the EO; [c]MIC of the nanoemulsion (NE); [d]MIC of carlina oxide; [e]MIC of Amphotericin B.

Carlina oxide demonstrated higher efficacy against yeasts clinically isolated, as *Candida* species. Regarding the EO-NE, it was active with MIC between 0.9 and 1.9 mg mL^{-1}.

Despite some antifungal and antibacterial activities of *C. acaulis* have been already reported, this work presented a deep investigation of these properties for different *C. acaulis*-derived products. In addition, *C. acaulis* EO-NE has been studied against several bacterial and fungal strains for the first time. The results obtained underline the activity of the EO and carlina oxide against Gram-positive bacteria, with a more intense action for carlina oxide. The strongest activity of carlina oxide was also confirmed on fungal strains, even if also the EO displayed an antifungal action. This result was not surprising, since polyacetylenes are promising antibacterial and antifungal agents. For instance, polyacetylenic carboxylic acids produced by *Mitrephora celebica* Scheff. showed an activity against the methicillin-resistant *S. aureus* and *Mycobacterium smegmatis* (Zgoda et al., 2001). In a similar way, pentayne diol, which is a polyacetylene extracted from *Bidens pilosa* L., resulted to be active against Gram-positive and Gram-negative bacteria, such as the methicillin-resistant *S.*

aureus and the vancomycin-resistant *Enterococcus faecalis*, but also on fungal species such as *C. albicans* (Tobinaga et al. 2009).

This work also confirmed that the encapsulation of the EO into a NE causes an enhancement of its antifungal and antibacterial activities. For Gram-positive bacteria, EO-NE MIC values were higher than those obtained for the EO (7.5 and 60 and 0.68–2.9 mg mL^{-1}, respectively). However, it should be taken into consideration that only 6% (w/w) of EO was loaded into the formulation, which, consequently, resulted more effective than the EO itself. Moreover, the NE displayed an antibacterial activity against Gram-negative strains, while the EO alone resulted not active. This greater activity of NE compared to that of EO was also detected on fungal strains. The results underline the crucial significance of nanoformulations for the enhancement of the physio-chemical properties and stability of natural products, as also of their biological activities. In the case of *C. acaulis* EO, the improved activity of its NE could be linked to its best solubility in the aqueous phase, but also to enhanced interactions with bacterial and fungal cells.

4.4. Conclusions

Antibiotics overuse both in humans and animals, caused the manifestation of multidrug resistance in numerous microorganisms (van den Bogaard and Stobberingh, 2000). Therefore, there is the necessity for alternative agents to substitute common antibiotics and research interest is focusing on botanical products, with a special focus on EOs. These mixtures of volatile compounds have displayed antimicrobial properties, resulting active also on multidrug-resistant bacteria (Kon and Rai, 2012).

C. acaulis EO, carlina oxide, and the EO-NE resulted extremely active on the Gram-positive bacteria strains tested (MIC values between 2.7-10.9 mg mL^{-1}, 0.33-5.35 mg mL^{-1}, and 7.5-60 mg mL^{-1}, respectively), with a complete inefficacy against the Gram-negative bacteria for the EO and carlina oxide. In conclusion, promising antifungal and antibacterial activities were revealed for *C. acaulis* EO and carlina oxide. In addition, the NE, whose EO concentration was 6%, resulted more effective than the pure EO, proving that formulating systems can also enhance the biological properties of the encapsulated products. This study confirmed once again the importance of natural products as resources of new antimicrobial agents.

5. *Carlina acaulis*-derived products insecticidal activities and safety profile

5.1. Introduction

During the last decades, the cheapness and ease of use of pesticides led to their uncontrolled and excessive use. However, the abuse of synthetic pesticides caused a selection of more pesticide-tolerant insects, leading to the development of pesticide-resistant species (Ahmed et al., 2021). Moreover, this misuse can be responsible for the onset of several human diseases, especially caused by the development of resistant insect vectors and by the occurrence of pesticide toxicity. In addition, this is often accompanied by negative environmental consequences (Damalas and Koutroubas, 2016; Mahmood et al., 2016; Sande et al., 2011). Furthermore, many synthetic insecticides are not biodegradable, leading to the contamination of soils and groundwater (Shabana et al., 2017; Wimalawansa and Wimalawansa; 2014). Despite the increasing evidence of the damage to the environment and human health, insecticides' global use has continuously increased over the last 50 years, especially in Brazil and China (https://ourworldindata.org/grapher/pesticide-use-tonnes?time=1990..2014). To partially solve this problem, new insecticidal products with lower health and environmental impact have been developed, but a stricter regulatory examination for the approval of pesticides have been globally established, resulting in less products available to fight insects and pests (Carvalho, 2017). For instance, Demeton® and Alphadime® (alpha-cypermethrin + dimethoate) are two insecticides not allowed to be used anymore (Business Daily, 2013, 2014). From this scenario comes the motivation of developing novel, effective, safe, and eco-friendly pesticides. Some of the pest management sectors that need less hazardous pesticides encompass the control of vectors of human and animal diseases, the management of stored-product pests, agricultural pests, structural (wood-destroying) pests, decorative plants pests, and ectoparasites of domestic animals (Isman, 2020a). In this regard, plants have been shown to contain several bioactive compounds effective for the treatment of arthropod pests and vectors (Raja, 2014; Thiruppathi et al., 2010). Plants as *Tanacetum cinerariifolium* (Trevir.) Sch.Bip. (pyrethrum) or *Azadirachta indica* A.Juss. (from which neem is obtained) represent just two of the many natural products employed as insecticides. The main botanical insecticides derive from Apiaceae, Asteraceae, Caesalpinaceae, Cupressaceae, Myristicaceae, Piperaceae, Rutaceae, Solanaceae, and Zingiberaceae families (Chougule and Andoji, 2016; Gakuubi et al., 2016; Vidyasagar and Tabassum, 2016). Among plant-derived products, EOs need particular attention and, even if their use as insecticidal agents is known from antiquity, research on their pesticidal activity just started in the 1900s (Theis and Lerdau, 2003). They gained more interest in the last years for their encouraging insecticidal properties, moderate toxicity, and safety, and surely also for the less rigorous legislations for their approval due to their recognised traditional uses (Isman, 2000). EOs usually have good biodegradability and low persistence, and are easily extractable and cheap (Abad

et al., 2007; Mossa, 2016). However, these products are also characterized by issues linked to their physio-chemical properties, such as their instability, low water solubility, and volatility. To overcome these problems, diverse encapsulating systems have been developed in recent years (Maes et al., 2019). Even if there is great enthusiasm around EOs as biopesticides, just few of them have seen a real commercial application, as reported in Table 10.

Table 10. Commercial insecticides/miticides constituted by essential oils (EO) or by their bioactive compounds (Isman, 2020a,b).

Product	Producer	Active ingredient(s)	Field rate (L/ha)	Crop(s)	Target pests
EcoTrol™	KeyPlex (USA)	10% rosemary EO[a], 5% geraniol 2% peppermint EO	1.43–5.7	berries, citrus, grapes, tree fruits, vegetables	aphids, spider mites, thrips, leafhoppers, whiteflies, mealybugs, scales, lygus bugs, early stage lepidopteran larvae
TetraCURB™	Kemin (USA)	50% rosemary EO, 3% clove EO, 1.95% peppermint EO	0.25–2.0% in water	food and nonfood cultivations, indoor and outdoor	spider mites
Requiem®	Bayer (USA)	59.7% α-terpinene, 22.4% *p*-cymene, 17.9% D-limonene	1.17–9.35	grapes, oil seeds, tree fruits, vegetables	spider mites, thrips, whiteflies, leafhoppers, psyllids
Prev-Am®	Oro Agri (RSA)	5–6% orange EO	3.65–14.6	berries, cereals, grapes tree fruits, vegetables	aphids, mites, whiteflies, psyllids, mealy bugs, thrips, scales, leafhoppers, armyworms and loopers
Eco-oil®	Organic Crop Protectants (AUS)	2% blend of tea tree (*Melaleuca alternifolia* (Maiden & Betche)) and eucalyptus EOs	0.5–1.0% in water	berries, citrus, vegetables	spider mites, aphids, greenhouse whiteflies, mealybugs, citrus leafminer
Akabrown®	Green Corp Biorganiks (MEX)	1.25% cinnamon EO, 1.0% peppermint EO, 0.5% clove EO, 0.25% oregano EO	1–1.5	berries, vegetables	spider mites

[a]EO, essential oil.

For this reason, there is a strong necessity to implement the discovery and production of botanical pesticides, in terms of availability of raw materials, costs and regulatory issues. With respect to *C. acaulis*, no data on the insecticidal potential of its EO and carlina oxide have been available until 2019, when the group of Benelli et al. (2019a) investigated the insecticidal activity of these two

products on *Cx. quinquefasciatus*. The study demonstrated a high toxicity on the 3rd instar larvae of the mosquito, both for the EO and carlina oxide, with LC_{50} values of 1.31 and 1.39 μg mL^{-1}, respectively. Moreover, the larvae were exposed for 24 h to the two products and a decrease in larval survival over time was detected at all the concentrations assayed. Moreover, even if the treated larvae were then moved to a tank filled with clean water 24 h post-exposure, further mortality was observed in the following 5 days (Benelli et al., 2019a). Following the previous work, Pavela et al. (2020) evaluated the efficacy of *C. acaulis* EO on *M. domestica*, which is a vector of pathogens responsible for the outcome of several human and animal pathologies (WHO, 1991), besides causing microbial contamination and food deterioration (Fotedar, 2001; Palacios et al., 2009). The EO resulted extremely effective in the topical assays on both sexes of the insect. The LD_{50} were 2.74 and 5.96 μg fly^{-1} for males and females, respectively. Moreover, the exposure to LD_{30} values caused a reduction of the adult longevity in female individuals, a reduction of eggs laid, and a decreased vitality of the following generation of individuals, with an enhanced mortality of the pupae. These studies also revealed a low non-target toxicity. In fact, the study conducted by Benelli et al. (2019a) demonstrated low non-target toxicity on *Daphnia magna* Straus (Cladocera: Daphniidae) adults, on which the EO and carlina oxide were less toxic than the control cypermethrin. These products were also tested on different HuDe cells, which are human dermis fibroblasts cell lines, and carlina oxide showed a toxicity lower than that of cisplatin. These promising results encouraged our research group to perform a deep investigation of the insecticidal properties of *C. acaulis*-derived products on vectors (Section 5.2), agricultural pests (Section 5.3), and stored-product pests (Section 5.4). In some of the following studies reported, the effects on non-target species were also taken into consideration. Indeed, besides insecticidal assays, also toxicity studies on human cells and mammals were performed to assess the potential safety of some *C. acaulis* derived products. All the results obtained are exposed in the following Sections.

Vectors

5.2. *Culex quinquefasciatus*

Cx. qunquefasciatus (Figure 25) is identified as the main vector of lymphatic filariasis (Vadivalagan et al., 2017) and Zika virus (Benelli and Romano, 2017; Guo et al., 2016; van den Hurk et al., 2017). Moreover, it is also reported as arbovirus vector, being responsible for the transmission of West Nile, Western equine, and St. Louis encephalitis (Samy et al., 2016).

Figure 25. *Culex quinquefasciatus*.

The strategies for the management of this species are based on the use of synthetic insecticides that are accompanied by the quick onset of pesticide resistance and serious consequences for the human health and the environment (Mariappan and Tyagi, 2018). Given the importance of formulating systems for the enhancement of the physio-chemical and biological properties of natural products, in this study nanocarrier-based formulations were developed, namely micro- (MEs) and nano-emulsions (NEs), encapsulating *C. acaulis* EO or carlina oxide as active ingredients. Their insecticidal activities were evaluated against *Cx. quinquefasciatus*. Moreover, *C. acaulis*-derived products were tested on different human cell lines and Wistar rats.

5.2.1 Materials and methods

Preparation and characterization of micro- (MEs) and nano-emulsions (NEs)

MEs were prepared accordingly to the procedures developed by Cespi et al. (2017) for the encapsulation of the EO of *Smyrnium olusatrum* L., by a spontaneous emulsification process originating from the blending of an oily phase and an aqueous phase. Distilled water was added slowly to the oily phase under stirring, in the quantity necessary for obtaining of the final concentration. The oily phase was constituted as follows: EO or a mixture of EO-ethyl oleate (Crodamol™), Polysorbate 80 (TEGO® SMO 80; Evonik Industries, Essen, DE), glycerol (ACEF, Fiorenzuola d'Arda, IT), and ethanol (6:1 ratio). NEs were obtained following the procedure

described in Section 4.2.1. Table 11 reports the compositions of MEs, NEs and their relative controls.

Table 11. Composition of microemulsions (MEs) (M1-M2), nanoemulsions (NEs) (N1-N2), and ME/NE controls (C_M - C_N).

Sample	Polysorbate 80 (%)	Alcoholic phase[a] (%)	Active ingredient (%)		H_2O (%)
			C. acaulis EO	Carlina oxide	
M1	13	35	0.5	-	51.5
M2	13	35	-	0.5	51.5
C_M	13	35	-	-	52
N1	0.33	-	0.5	-	99.17
N2	0.33	-	-	0.5	99.17
C_N	0.33	-	-	-	99.67

[a]Alcoholic phase: 30% of glycerol and 5% of ethanol 96%.

The characterization and analysis of MEs and NEs followed the procedure reported in Section 4.2.1 and 4.2.2, at different time points: 0 day (t0), 1 month (t1), 3 months (t3) and 6 months (t6).

Mosquito rearing

Cx. quinquefasciatus mosquitoes derived from a laboratory colony of the Crop Research Institute (Prague, Czech Republic). The mosquito individuals were raised under monitored conditions for more than 20 generations, and never subjected to insecticides prior to the tests. Larvae were nourished with dog biscuits and yeast powder (3:1 ratio), and maintained at 25 ± 2 °C, 70 ± 5% RH, and a photoperiod of 16:8 (L:D) h.

Larvicidal activity

The acute toxicity on *Cx. quinquefasciatus* 3rd instar larvae was evaluated 24 h post-exposure. The larvicidal assays were performed following WHO Standard Procedures (1996) (Pavela et al., 2015), with some changes (Benelli et al., 2018). The EO was dissolved in dimethyl sulfoxide (DMSO) for the preparation of a serial dilution of test concentrations. For the assay, to 224 mL of distilled water was added to 1 mL of serial dilutions and slightly mixed to homogenize the test solution at the following concentrations: 0.3; 0.5; 0.8; 1.0; 1.5, and 1.8 µL L^{-1}. MEs and NEs (M1, M2, N1 and N2, respectively) were tested following the same procedure described above, with the exception that NEs were blended into the water directly and without the dilution in DMSO to achieve a serial dilution of 200, 400, 600, 800 a 1 000 µL L^{-1}. C_M and C_N represent the negative controls for MEs and NEs, respectively. For EO and carlina oxide experiments, water plus DMSO at the maximum quantity employed to formulate the active ingredients was assayed as a negative control. In all the experiments, the mosquito larvae were moved through distilled water into a bowl containing the prepared test solution (final surface area of 125 cm^2; 25 larvae $beaker^{-1}$, 4 replications). The assays

were then positioned in a growth chamber (16:8 (L:D), 25 ± 1 °C). No food was furnished to the larvae, and mortality was measured 24 h post-exposure.

Sublethal toxicity

Diverse lethal concentrations (LC) at different exposure times were assayed to evaluate larval and pupal mortality and the percentage of new emerged *Cx. quinquefasciatus* adults. 3rd Instar larvae of *Cx. quinquefasciatus* (200 larvae replicate^{-1}) were subjected to the LC_{16}, LC_{30}, LC_{50}, and LC_{90} of the most effective larvicidal emulsion (M1) and *C. acaulis* EO for 1, 2, 3, 4, 5, 6, and 7 h. The application methods followed those reported in the previous paragraph. Later, the larvae were moved to clean water and nourished with standard food. The mortality was measured daily for 18 days. Larval mortality in the development period, total larval and pupae mortality, and percentage of emerged adults of *Cx. quinquefasciatus* were evaluated. Each concentration was tested in 4 replicates. Mosquito mortality or adults emerged were reported as mean values (%) ± SE. Then, the treated insects were moved to a growth chamber (25 ± 1 °C; 16:8 (L:D) h).

Dermal toxicity of *Carlina acaulis* essential oil (EO) and its microemulsion (ME)

Cell lines

Immortalized human keratinocytes (HaCaT) and primary human fibroblast (NHF A12) cell lines, deriving from IFOM (Rome, Italy), were cultured in Dulbecco's Modified Eagle Medium (DMEM) added of 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU mL^{-1} penicillin/streptomycin and maintained at 37 °C with 5% CO_2 and 95% of humidity.

MTT assay

For the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, three thousand cells per well were seeded in 96-wells plate (final volume of 100 µL well^{-1}) and left in incubation for one day. After that time, the EO (1.69-863.74 µg mL^{-1}), the ME and its relative control (M1 and C_M, 205.08-105000 µg mL^{-1}) were added, and each treatment consisted of six replicates. After 24 h, 0.8 mg mL^{-1} of MTT (Merk) was added to the media for the evaluation of cell viability. The supernatant was removed 3 h later, and the pellet of salt crystals was dissolved in 100 µL well^{-1} of DMSO. An ELISA reader microliter plate (BioTek Instruments, Winooski, VT) was used for the measure of the absorbance of the sample against a background control was measured at 570 nm.

Acute oral toxicity in rats

Acute toxicity studies were performed on female Wistar rats of the weight of 250-300 g. After the period of habituation, rats were single housed and kept in a 12 h light/dark cycle at the temperature of 20–22 °C and humidity of 45–55%, with food and water ad libitum. Animals' treatment was performed following the guidelines of the European Community Council Directive for Care and Use of Laboratory Animals and acute toxicity was measured according to the methods described in OECD 425 main test guidelines accordingly to an Up-and-Down Procedure (UDP) (OECD, 2008). Before the treatment, seven female Wistar rats were fasted overnight and treated with fixed doses of the EO (175, 550, and 2000 mg kg^{-1}), which were made by diluting it in 2% Tween 80 vehicle. The administration was performed by gavage. The first animal was treated with 175 mg kg^{-1} dose of the EO, and if it survived after 48 h, the next dose of 550 mg kg^{-1} was given to the second animal. If the second animal survived after 48 h, the next animal was given a dose of 2000 mg kg^{-1}. When the third animal died within 48 h, the 550 mg kg^{-1} dose was repeated and if the fourth animal survived within 48 h, the 2000 mg kg^{-1} dose was repeated. If the fifth animal died within 48 h, the 550 mg kg^{-1} dose was repeated; when the sixth animal survived within 48 h, the 2000 mg kg^{-1} dose was repeated. At the end, a 2000 mg kg^{-1} dose was administered to the seventh rat that died within 48 h. Following OECD 425 main test guidelines, the test was terminated when 5 reversals (response followed by non-response or vice versa) were observed. An individual observation of the animals was done after dosing for signs of toxicity during the first 30 min and then periodically during the first 48 h. The surviving animals were observed daily thereafter for 14 days. The activities of the central and autonomic nervous system (tremors, convulsions, straub, sedation, anaesthesia and ataxia, coma, lacrimation, cyanosis, ptosis, salivation, and piloerection) were observed during the study. The LD_{50} of M1 was measured by limit test at 5000 mg kg^{-1} according to OECD 425 guidelines. A first animal received a dose of 5000 mg kg^{-1} by gavage. If the first animal, which received a dose of 5000 mg kg^{-1} by gavage, survived after 48 h, two additional animals were dosed. Both animals survived after 48 h and the test was stopped. Moreover, one rat was also treated with the ME control (C_M) and no evident signs of toxicity were observed.

Statistical analysis

In mosquito larvicidal tests, if control mortality reached 20%, Abbott's formula was used (Abbott, 1925). The LC_{16}, LC_{30}, $LC_{50,}$ and LC_{90} values were calculated using probit analysis of dose-mortality data and associated 95% confidence limits for each treatment (Finney, 1971). Concerning sub-lethal toxicity tests of M1 and the EO, data (%) were converted by arcsine$\sqrt{}$ and analysed by ANOVA followed by Tukey's HSD test ($p \leq 0.05$). Data concerning rat mortality were analysed by "Acute Oral Toxicity (Guidelines 425) Statistical Program" (AOT425StatPgm).

5.2.2 Results and discussion

Micro- (MEs) and nano-emulsions (NEs) characterization

The quali-quantitative composition of MEs was established thanks to an initial screening, in which the amount (%) of *C. acaulis* EO, the total oily phase, and Tween 80 were examined for a total of 11 formulations. The samples were kept under monitoring for a period of 1 month and, after that time, the thermodynamically stable ME resulted M1, containing 0.5% w/w of EO, 13% w/w of Polysorbate 80, 35% of alcoholic phase, and 51.5% of water (Table 11). This M1 was characterized by optical microscopy and then through DLS analysis. Figure 26 shows the presence of an isotropic system that confirmed the formation of a microemulsified sample (Mcclements, 2012).

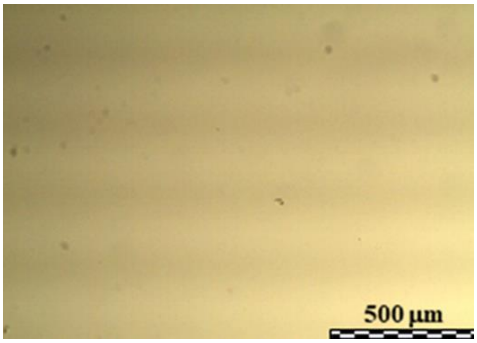

Figure 26. Optical microscope image of microemulsion M1.

The nanometric size range of the M1 was confirmed by DLS analysis, with a bimodal size distribution centred at 37.84 nm (80%) and 408 nm (20%). Based on these results, a ME containing carlina oxide was formulated at the same conditions of M1 (Sample M2), replacing the EO with the pure carlina oxide. Again, the formation of a microemulsified system was confirmed by optical microscope image and DLS analysis. M2 showed a bimodal distribution where the two populations had a medium diameter of 41.92 nm (85%) and 666 nm (15%). The physio-chemical stability of the two MEs (M1 and M2, respectively) was observed for up to 6 months of storage at room temperature. They were analysed by DLS at different time points: 0 day (t0), 1 month (t1), 3 months (t3), and 6 months (t6) (Figure 27), demonstrating a thermodynamical stability for the considered period, even if a slight increase in the size of the dispersed phase of M1 was detected.

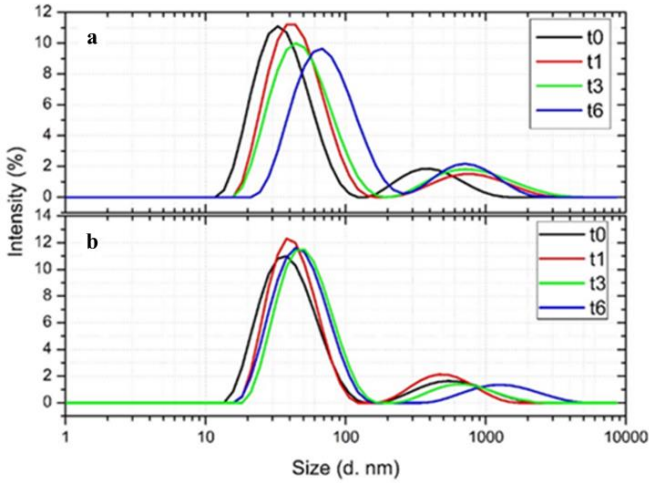

Figure 27. Dynamic light scattering (DLS) analysis of microemulsions M1 (**a**) and M2 (**b**) at different time points: 0 day (t0), 1 month (t1), 3 months (t3), 6 months (t6).

NEs containing the EO (N1) and carlina oxide (N2) were also formulated, for an evaluation of the influence of the encapsulation method on the insecticidal activity of *C. acaulis* derived products. The great advantage of NEs in comparison with MEs is that they encapsulate the oil phase (that in the case of EOs is the active ingredient) with a lower amount of surfactant (Pavoni et al., 2019a). For this reason, NEs could be considered a greener substitute of MEs, because surfactants are reported to be potentially dangerous to the environment (Wilhelm et al., 1993). NEs were prepared accordingly to the procedure previously described for MEs. Remarkably, NEs were obtained only using the 0.33% (w/w) of Tween 80 (Table 11). Both NEs (N1 and N2) displayed a monomodal size distribution centred between 140 and 145 nm, which was validated by the PDI values of 0.2 and 0.18, respectively. These values resulted lower compared with those of MEs, proving that the ME system showed a bimodal size distribution of the dispersed phase. Moreover, also both NEs displayed good stability for a storage time of 6 months at room temperature (Figure 28).

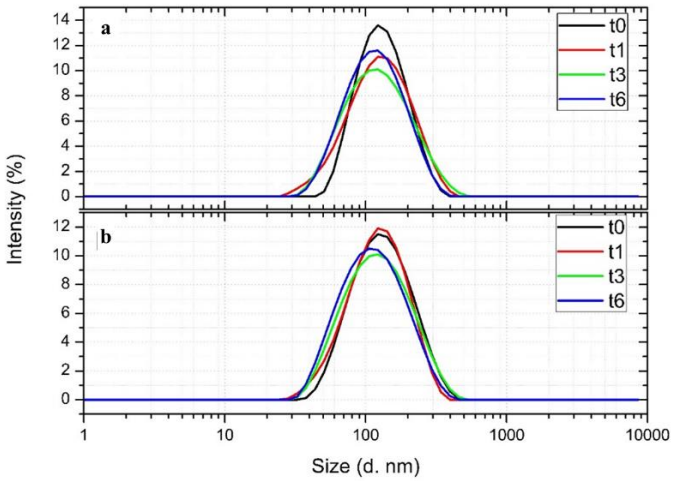

Figure 28. Dynamic light scattering (DLS) analysis of nanoemulsions N1 (**a**) and N2 (**b**) at different time points: 0 day (t0), 1 month (t1), 3 months (t3), 6 months (t6).

From a technological point of view, the two formulations (MEs and NEs, respectively) revealed similarities and differences. Firstly, both formulations showed a size of dispersed phase below 200 nm, which is the established limit for the definition of a nanosystem (Huang et al., 2010). MEs showed minor droplet size in comparison with NEs, and this result is in accordance with the literature (McClements et al., 2012). Moreover, MEs and NEs differ for the methods of preparation and their composition. First, MEs form spontaneously while NEs usually need an external energy input to reach the final colloidal system. Moreover, MEs showed a limited amount of encapsulated oil phase with respect to NEs.

Acute toxicity on *Culex quinquefasciatus*

The lethal concentrations calculated for MEs, NEs, EO, and carlina oxide are reported in Table 12. The efficacy of the EO and of carlina oxide depended on the kind of formulation used for their encapsulation. M1 resulted the most efficient with an $LC_{50}(_{90})$ value of 579.1 (791.3) µL L^{-1}, corresponding to the value of 2.3 (3.9) µL L^{-1} of EO. M2 was slightly less effective, with an $LC_{50}(_{90})$ value of 628.3 (939.8) µL L^{-1}, corresponding to the 3.1 (4.7) µL L^{-1} of carlina oxide. However, the activities of M1 and M2 were not significantly different. In this work, LC_{50} values for the EO and carlina oxide were also estimated as 1.3 and 1.4 µL L^{-1}, respectively (Table 12). The N2 provided the lowest efficacy ($LC_{50}(_{90})$ of 897.1 (1,135.1) µL L^{-1}, corresponding approximately to 4.5 (5.7) µL L^{-1} of the EO. The higher larvicidal activity of MEs compared to NEs could be linked to their different particle sizes. In detail, MEs showed a droplet mean diameter of around 40 nm, while NEs of around 140 nm. Anjali et al. (2012) reported the droplets size-mortality relationship for the larvicidal activity of neem oil nanosystems on *Cx. quinquefasciatus*, observing that the neem oil-based nanosystem, with a medium diameter of 31 nm, led to higher larval mortality compared to those with a mean diameter of 93 and 251 nm. So, $LC_{50}(_{90})$ values decrease as the droplets size decreases demonstrating that the reduced EO droplets of MEs could allow a more effective penetration of the active ingredient.

Table 12. Lethal concentrations calculated for the microemulsions (MEs) (M1, M2), nanoemulsions (NEs) (N1, N2), the essential oil (EO), and carlina oxide against *Culex quinquefasciatus*.

Product	**LC_{16}[a] (µL L^{-1})**	**CI_{95}[b]**	**LC_{30}[a] (µL L^{-1})**	**CI_{95}[b]**	**LC_{50}[a] (µL L^{-1})**	**CI_{95}[b]**	**LC_{90}[a] (µL L^{-1})**	**CI_{95}[b]**	**χ^{2}[c] (*d.f.*=3)[d]**	***P*-value[e]**
M1	384.5 ± 25.7	278.4-395.7	509.7 ± 16.2	468.8-528.7	579.1 ± 30.9	482.3-623.9	791.3 ± 39.5	659.8-912.3	6.161	0.195 *ns*
M2	452.1 ± 20.8	398.7-502.3	525.9 ± 29.5	511.1-598.7	628.3 ± 15.2	518.1-698.5	939.8 ± 28.3	897.5-1026.4	5.221	0.235 *ns*
N1	617.8 ± 28.6	587.5-657.4	684.4 ± 31.5	658.5-701.9	767.1 ± 24.2	619.8-788.7	1015.1 ± 28.6	923.7-1258.7	7.251	0.318 *ns*
N2	747.3 ± 22.3	698.8-785.8	815.7 ± 19.9	793.5-850.1	897.1 ± 19.6	859.3-936.3	1135.1 ± 38.6	1073.3-1226.4	1.765	0.628 *ns*
EO	0.5 ± 0.0	0.4-0.5	0.9 ± 0.1	0.8-1.0	1.3 ± 0.1	1.2-1.4	1.8 ± 0.1	1.7-1.9	2.351	0.925 *ns*
carlina oxide	0.4 ± 0.0	0.3-0.5	0.8 ± 0.1	0.6-0.9	1.4 ± 0.2	1.3-1.5	2.1±0.2	1.3-2.3	1.521	0.935 *ns*

[a]LC, lethal concentration killing 16%, 30%, 50% or 90% or the exposed larval population; [b]CI_{95}, 95% confidence interval; [c]χ^{2}, chi-square; [d]d.f., degrees of freedom; [e]ns, not significant ($P > 0.05$).

Sublethal effects of microemulsion (M1) and essential oil (EO) on *Culex quinquefasciatus*

The work also focused on the effects of lethal and sublethal concentrations, together with various exposure times, on *Cx. quinquefasciatus* larvae for the most efficient ME, namely M1, and for *C. acaulis* EO. The most significant effect in terms of larval mortality resulted that of M1 compared to the EO. The LC_{16} of M1 (384.5 µL L^{-1}, corresponding approximately to the equivalent of 1.9 µL L^{-1} of EO encapsulated) produced more than 78% mortality of the larvae after 1 h of exposure, and fatal consequences were detected when larvae were subjected to this ME for 5 h. The exposure of larvae to the LC_{30} led to 100% mortality after 2 h of exposure, and exposure to the LC_{50} of this ME showed fatal larval effects just after 1 h of exposure. However, even if the EO was the active constituent of the ME, this activity was not detected after its application. In fact, the application of 0.5 µL L^{-1} EO (corresponding to the LC_{16}) resulted in low *Cx. quinquefasciatus* mortality, ranging between 6.7 and 36.7%, while the application of the LC_{90} (1.8 µL L^{-1}) led to the highest mortality of 91.7% only 7 h post-exposure. For a deep understanding of the difference of activity of the EO and ME, Figures 29, 30, 31, and 32 illustrate the development of mean cumulative larval mortality over time for individual concentrations and various exposure times. Based on the comparison of cumulative mortality over time between M1 and the EO, while the EO showed an increase in mortality approximately for the first 7 days from termination of exposure, with only exceptional mortality afterwards, M1 displayed an increase in mortality throughout the observation period, i.e. until the larvae become pupae (Figures 29, 30, 31, and 32).

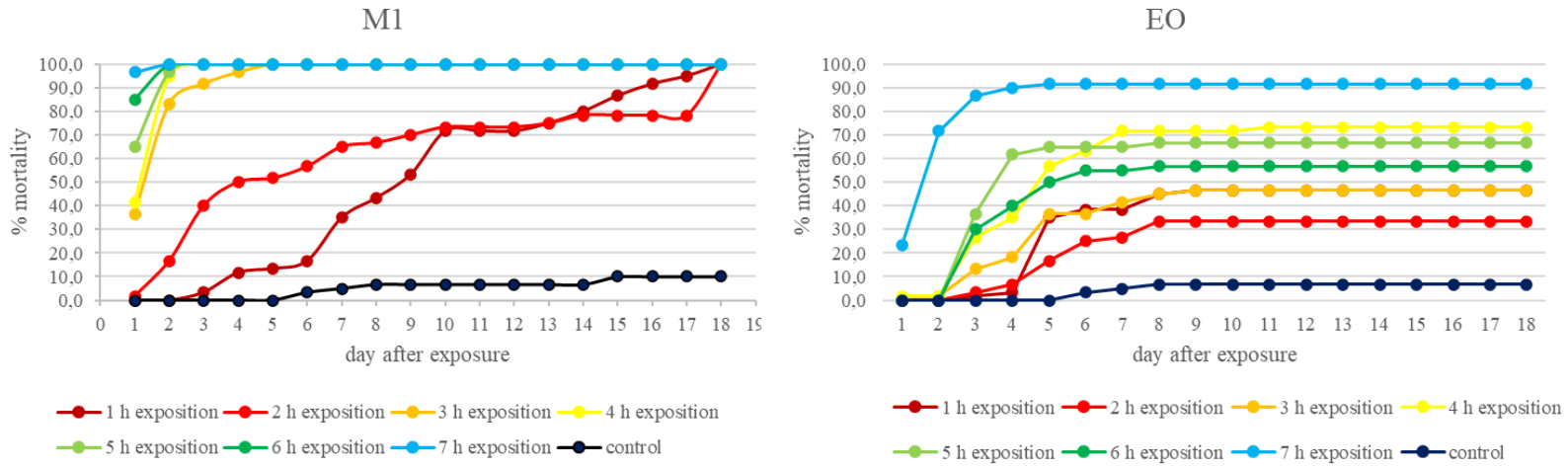

Figure 29. Mortality over time of *Culex quinquefasciatus* larvae after treatment with microemulsion M1 and essential oil (EO) concentrations corresponding to LC_{90}.

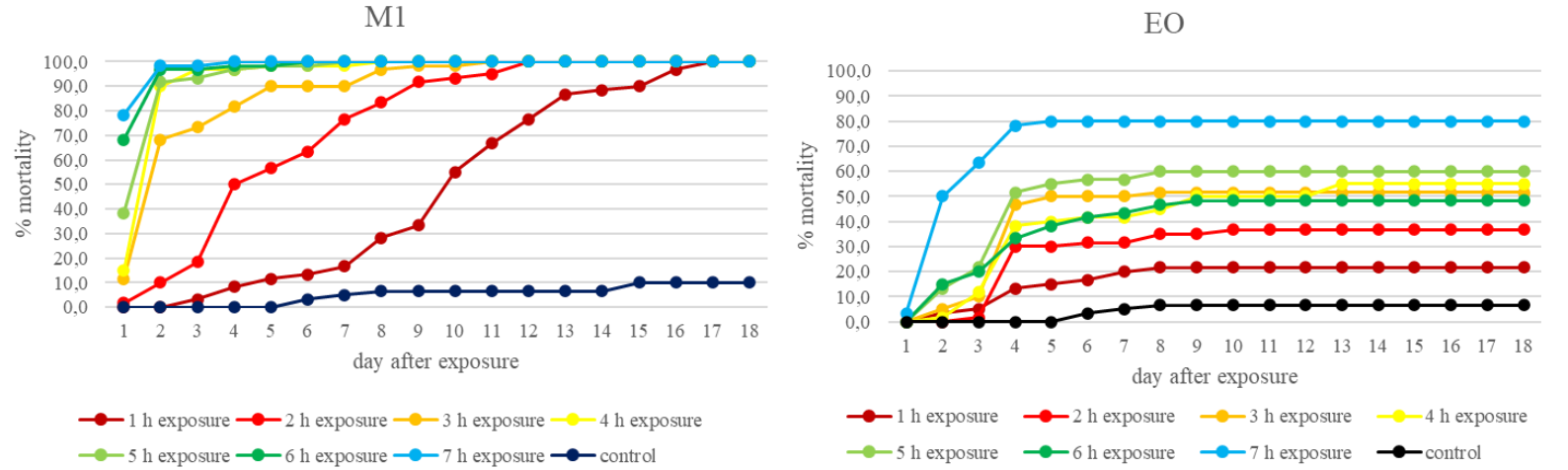

Figure 30. Mortality over time of *Culex quinquefasciatus* larvae after treatment with microemulsion M1 and essential oil (EO) concentrations corresponding to LC_{50}.

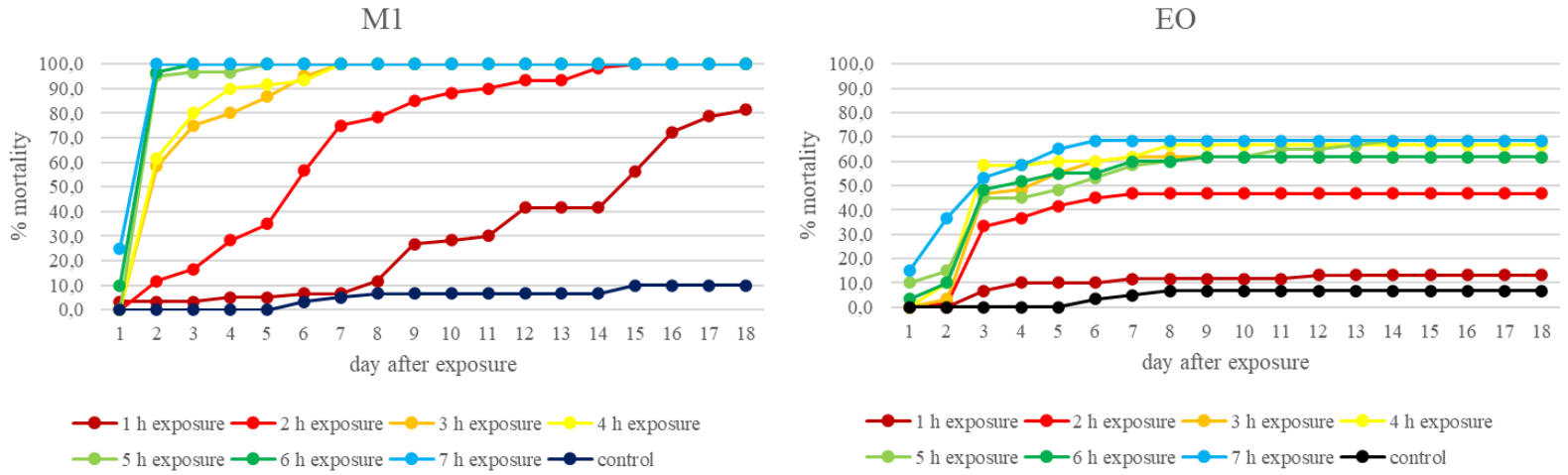

Figure 31. Mortality over time of *Culex quinquefasciatus* larvae after treatment with microemulsion M1 and essential oil (EO) concentrations corresponding to LC_{30}

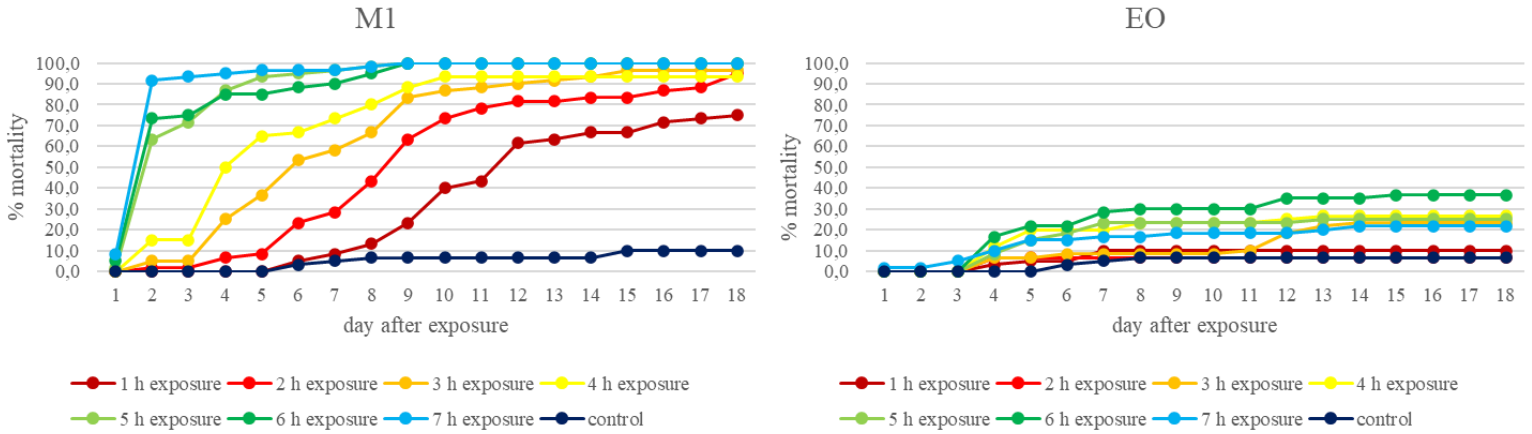

Figure 32. Mortality over time of *Culex quinquefasciatus* larvae after treatment with microemulsion M1 and essential oil (EO) concentrations corresponding to LC_{16}.

The results obtained in this work suggested that treatment with M1, even with low concentrations (LC_{16}), had fatal consequences for *Cx. quinquefasciatus* larvae that were probably linked to an improved dispersion in water and diffusion of the active ingredient through the cuticle or spiracles of the larvae (Du et al., 2016). Nevertheless, this theory should be verified with further studies. In conclusion, M1 caused a significantly higher efficacy compared to the EO and can be judged as a prototype formulation more effective than its active ingredient alone. Since the effects of EOs tested in sublethal doses or concentrations on fertility, fecundity, and natality have also been reported for other EOs (Benelli and Pavela, 2018), it is crucial to further analyse this effect also to non-target organisms.

Dermal toxicity of *Carlina acaulis* essential oil (EO) and its microemulsion (M1)

The EO and M1 were also evaluated on two normal human cell lines, namely HaCaT (immortalized human keratinocytes cell line) and NHF A12 (primary human fibroblast cell line). The cells were subjected to diverse concentrations of EO (from 1.69 to 863.74 µg mL^{-1}) and ME (from 205.8 to 105,000 µg mL^{-1}) for a period of 24 h. The results indicated that the EO has a dose-dependent cytotoxicity on both cell lines, with an IC_{50} of 88.31 ± 1.3 µg mL^{-1} and 115.92 ± 6.1 µg mL^{-1} on HaCaT and NHF A12, respectively (Figure 33a). On the other hand, M1 treatment gave an IC_{50} of 1457.4 ± 63 µg mL^{-1} and of 5392.8 ± 315 µg mL^{-1} on HaCaT and NHF A12 cells, respectively (Figure 33b).

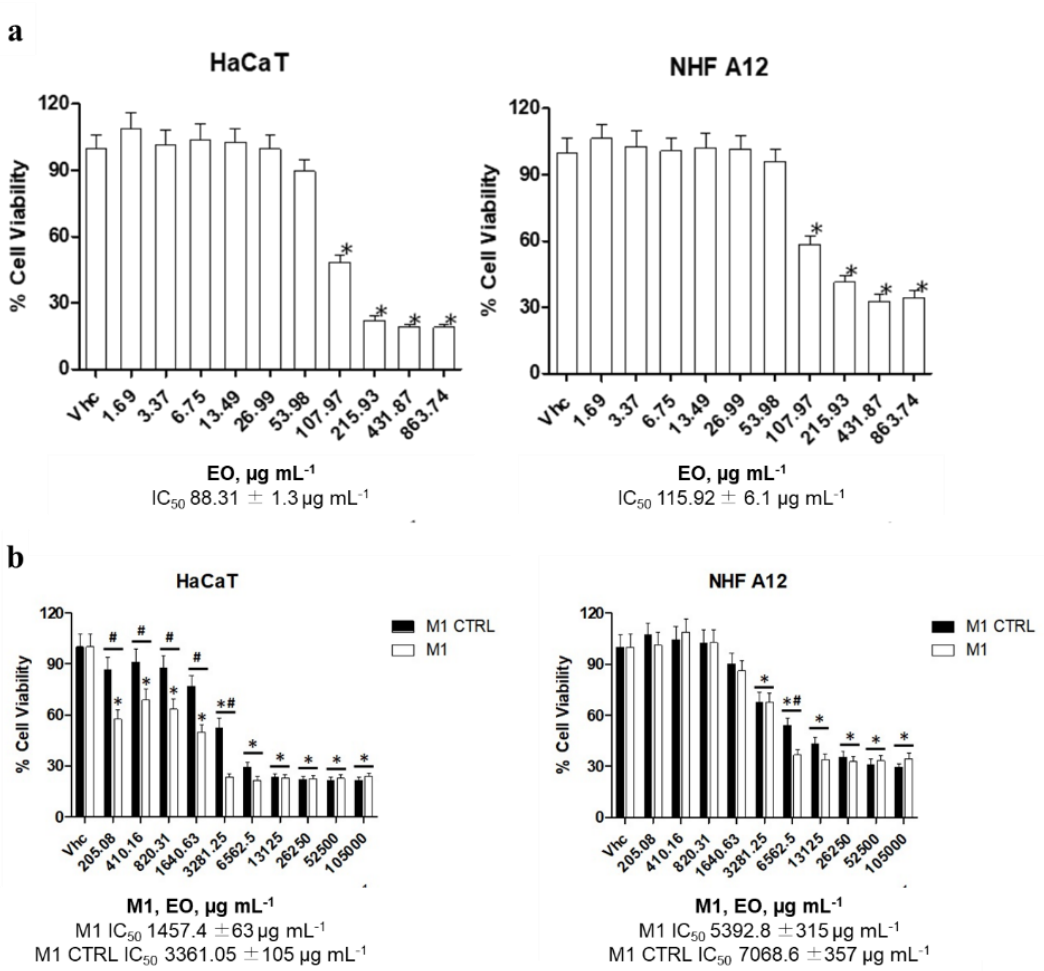

Figure 33. Essential oil **(**EO) cytotoxic effect. Data shown are expressed as mean ± standard error (SE) of three separate experiments. *$p < 0.05$ vs Vhc (**a**); Cytotoxic effect of microemulsion M1 and M1 control (M1 CTRL). Data shown are expressed as mean ± SE of three separate experiments. *$p < 0.05$ vs Vhc, #$p < 0.05$ vs M1 CTRL (**b**).

These results demonstrated a mild toxicity of the EO on the two cell lines tested and that this toxicity became negligible when the EO was formulated into a ME. In detail, cell viability at 100 µg mL^{-1} of EO was reduced to 34.51% and 22.75% for HaCaT and NHF A12 cells, respectively. Consequently, this product cannot be defined as cytotoxic according to the ISO guidelines (ISO 20093-5 2009). The results reported in this study are not linear with those reported by Wnorowski et al. (2020). In fact, carlina oxide was tested in a concentration range of 3.125–50 µg mL^{-1} on BJ normal foreskin fibroblasts, and a concentration-dependent increase in necrotic effects was found. However, in that case the IC_{50} concentration on this cell line was not determined and its rate of toxicity was not estimated according to the ISO guidelines (ISO 20093-5 2009).

Acute oral toxicity in rats

From the acute oral toxicity determination of *C. acaulis* EO a LD_{50} of 1098 mg kg^{-1} was determined, with an approximate 95% confidence interval of 550–2000 mg kg^{-1}. Moreover, the dose of 550 mg kg^{-1} did not lead to any symptoms of toxicity in rats, while for the dose of 2000 mg kg^{-1} the following toxicity signs were progressively observed starting approximately 24 h after the administration: sedation, tremors, ptosis, ataxia, and coma. These data provide new information regarding the potential toxicity of *C. acaulis* EO (Hermann et al. 2011; Stojanović-Radić et al. 2012) and permit its classification as moderately toxic, especially in comparison with the EOs derived from other aromatic plants such as *Thymus vulgaris* L. (LD_{50} = 2840 mg kg^{-1}), *M. alternifolia* (Maiden & Betche) Cheel (LD_{50} = 1900 mg kg^{-1}), *Zingiber officinale* Roscoe (LD_{50} = 3400 mg kg^{-1}), and *Foeniculum vulgare* Mill. (LD_{50} = 3120 mg kg^{-1}). *C. acaulis* EO is a 'monocomponent' EO, so the overall toxicity may be ascribed to carlina oxide. Regarding this polyacetylene, the LD_{50} measured after oral administration overlapped with those reported for some EOs constituents such as dillapiol (LD_{50}= 1000 mg kg^{-1}), cinnamaldehyde (LD_{50} = 1160 mg kg^{-1}), and thymol (LD_{50}= 980 mg kg^{-1}) (Dev and Koul 1997; Isman and Machial 2006). On the other hand, the LD_{50} was considerably higher than that of pulegone (LD_{50} = 150 mg kg^{-1}), α-thujone (LD_{50} = 45 mg kg^{-1}), and thymoquinone (LD_{50} = 794 mg kg^{-1}) (Al-Ali et al. 2008; Maggi and Benelli 2018). These results confirmed that *C. acaulis* EO is reasonably safe when administered orally to rats. Moreover, when the EO is encapsulated in MEs and NEs at 0.5% concentration, it completely fulfils regulatory agencies' requirements for insecticidal formulations (Isman and Machial, 2006). The LD_{50} of ME M1 was higher than 5000 mg kg^{-1} and no adverse effects were detected after one week, demonstrating to be safe up to this concentration.

5.2.3 Conclusions

In this work, a formulation encapsulating 0.5% of *C. acaulis* EO was produced and demonstrated high efficacy on *Cx. quinquefasciatus* larvae. Moreover, sublethal toxicity experiments demonstrated the promising efficacy of M1 application, while EO did not show a long-lasting larvicidal effect. More research still needs to be performed to comprehend the more intense toxicity of ME over NE. From a toxicological point of view, *C. acaulis* EO and its M1 showed slight toxicity to human keratinocytes and fibroblasts. Moreover, the EO seemed to have a mild toxicity on rats, with LD_{50} values linear to those of other EOs constituents, such as thymol and cinnamaldehyde (Pavela and Benelli 2016). Based on the requirements of regulatory agencies, the LD_{50} of the encapsulated *C. acaulis* EO (ME) was also measured, indicating the safety of this formulation (LD_{50} > 5000 mg kg^{-1}) for human health.

Agricultural pests

5.3 *Lobesia botrana*

Lobesia botrana (Denis & Schiffermüller, 1775) (Lepidoptera: Tortricidae) (Figure 34), also known as the European grapevine moth (EGVM), is a pest affecting the grapevine worldwide, with a widespread economic importance.

Figure 34. *Lobesia botrana*.

Its larvae eat grape bunches (*Vitis vinifera* L.), decreasing the yield and enhancing the predisposition to bacterial and fungal infections (Ioriatti et al., 2011). The tools that have been employed for decades for the management of this pest enclose mating disruption agents and biopesticides (mainly *Bacillus thuringiensis*) (Benelli et al., 2019b; Ifoulis et al., 2004; Lucchi and Benelli, 2018). However, since its management often needs the employment of chemicals (Civolani et al., 2014; Pavan et al., 2014; Thiéry et al., 2018; Vassiliou, 2011), the search for valid and sustainable alternatives is a key challenge. In the work herein presented, a *C. acaulis* EO-based NE was developed. The EO and the EO-NE were assayed for their toxicity against *L. botrana* 1st instar larvae.

5.3.1 Materials and methods

Preparation and characterization of *Carlina acaulis* essential oil-nanoemulsion (EO-NE)

NEs were prepared and characterized accordingly to the procedure described in Sections 4.2.1 and 4.2.2. Their stability studies were performed accordingly to those previously described (Section 4.2.2).

Lobesia botrana mass-rearing

L. botrana young instars tested in this study derived from a laboratory mass-rearing kept at the Laboratory of Entomology, University of Pisa. Adults were raised into a plastic bottle and fed with a liquid diet and eggs were collected every 2 days and transferred into a plastic tray, previously drilled to allow airflow. Each tray was provided with a piece of artificial food medium. Semi-synthetic larval diet was prepared accordingly to Gabel et al. (1980) recipe. The emerged adults were moved into a new PVC bottle. The rearing was maintained at a constant temperature (25 ± 1 °C), R.H. 70 ± 10% and 16:8 (L:D) photoperiod.

Insecticidal activity on *Lobesia botrana*

C. acaulis EO and its NE were tested on *L. botrana* adapting the procedure of Bosch et al. (2007). A 32 µL-drop of NE or EO formulation was placed on a piece of semi-synthetic diet (4 x 4 x 1 cm), properly distributed, and allowed to dry for 2 h. Sixteen 1st instar larvae (L1) of *L. botrana* were put on each portion of the diet and individualized inside a gelatine capsule (00, Fagron, Quarto Inferiore, Bologna, Italy). Each portion of the diet with the larvae was arranged in a closed plastic box to prevent desiccation. Larval mortality was monitored after 96 h. At this point, gelatine capsules were removed, and a binocular microscope was used for the inspection of larvae inside the diet. A larva was judged dead if it did not react to a gentle touch with a small brush. Different concentrations of the EO (1, 2.5, 6, 7.5, 8, 10, 30 µL mL^{-1}) and NE (5, 7.5, 8, 10, 30, 60 µL mL^{-1}) were tested. The above-described method was evaluated testing positive and negative controls. The positive control was Spinosad (Laser®, Dow) a commercial insecticide tested at the tab dose (15 mL/hL); the negative control was 0.17% Tween 80 + 99.83% of H_2O for NE, and H_2O + DMSO - same concentration of the EO. Three replications for each concentration of EO, NE, and positive and negative control were performed. All tests were done at the constant temperature of 22 ± 1 °C, R.H. 45 ± 5 %, and photoperiod 16:8 (L:D).

Statistical analysis

L. botrana mortality (%) was arcsine√ converted before the performance of an analysis of variance (ANOVA, two factors as fixed effects), followed by Tukey's honestly significant difference (HSD) test ($p < 0.05$). The mortality determined experimentally was adjusted with Abbott's formula. LC_{30}, LC_{50}, and LC_{90} with associated 95% confidence interval (CI) and chi-squares, were calculated using probit analysis (Finney et al., 1971). For all analyses, JMP 9 (SAS) software was employed and $p = 0.05$ was chosen as the threshold to determine significant differences.

5.3.2 Results

Preparation and characterization of the essential oil-nanoemulsion (EO-NE)

The NE was able to encapsulate 6% (w/w) of *C. acaulis* EO and 4% (w/w) of surfactant (Tween 80, Sigma-Aldrich). The sample showed a monomodal size distribution in the nanometric range. The droplets' population had a mean diameter centred around 140 nm and DLS analysis registered a Z-Average and PDI (Polydispersity Index) values of 98.85 and 0.33, respectively. Moreover, the EO-NE showed good stability at room temperature, evaluated for a storage period of 6 months. The thermodynamic stability of the system was also proved by the accelerated stability test.

Insecticidal activity on *Lobesia botrana*

Mortality of *L. botrana* larvae was directly proportional to the concentration of the EO and EO-NE used for the tests. A substantial larvicidal effect was detected from the concentration of 2.5 and 8.0 µL mL^{-1} for the EO and EO-NE, respectively. Similar EO concentrations displayed better larvicidal activity over EO-NE. As reported in Table 13, LC_{50} was reached, testing the concentrations of 7.29 ± 0.25 and 9.04 ± 0.39 µL mL^{-1} for the EO and EO-NE, respectively. Complete mortality was only reached testing the positive control that consisted of a semi-synthetic diet added with spinosad (Laser®) at the label dose.

Table 13. Larvicidal activity of *Carlina acaulis* essential oil (EO) and essential oil nanoemulsion (EO-NE) on 1st instar larvae of *Lobesia botrana*.

Tested product	LC_{10} ± SE (CI_{95})[a] (µL mL^{-1})	LC_{30} ± SE (CI_{95}) (µL mL^{-1})	LC_{50} ± SE (CI_{95}) (µL mL^{-1})	LC_{90} ± SE (CI_{95}) (µL mL^{-1})	χ^2	*p*-value
EO	4.87 ± 0.49 (3.9-5.4)	6.19 ± 0.31 (5.6-6.5)	7.29 ± 0.25 (6.9-7.6)	10.92 ± 1.40 (9.7-13.6)	1.158	0.563 n.s.[b]
EO-NE	6.24 ± 0.58 (5.1-6.8)	7.77 ± 0.33 (7.2-8.1)	9.04 ± 0.39 (8.6-9.7)	17.70 ± 4.48 (15.4-27.5)	1.257	0.262 n.s.

[a]CI_{95}, 95% confidence interval; [b]n.s., not significant ($p > 0.05$).

Avgin et al. (2008) evaluated different EOs obtained from some aromatic plants such as *F. vulgare*, *Mentha* x *piperita* L., *T. vulgaris, Carum carvi* L., and *Rosmarinus officinalis* L. on field-collected grapes. From this work it was observed that *C. carvi* EO was the most effective, reaching > 96% mortality on *L. botrana* larvae at a concentration of 25 µL on 20 g of grapes. Most of the works on EOs efficacy on *L. botrana* has been performed to investigate the influence of these products on adults' behaviour (Cattaneo et al., 2014). The study herein reported is the first that evaluated EO efficacy on the mortality of freshly hatched *L. botrana* larvae. Moreover, a lower toxicity of the EO-NE in respect to that of EO was proved. Indeed, the EO concentration to reach 90% of larval

mortality (LC_{90}) was 10 µL mL^{-1}, while the same mortality was not reached with the highest concentration (60 µL mL^{-1}) of NE. Also, the LC_{50} of EO-NE resulted lower than that of EO. Despite this unexpected result, *C. acaulis*-based NE still represents an encouraging option for the development of promising eco-friendly tools for pest management, because of its low LC_{50} value (always < 10 µL mL^{-1}) as well as for its higher in field stability if compared to the unformulated EO (Pavoni et al., 2019a).

This study opens the way for a novel standpoint of *L. botrana* management employing botanical pesticides and further studies need to be also performed on the adulticidal activity of *C. acaulis* EO against this pest.

5.3.3 Conclusions

The present work underlines the encouraging potential of *C. acaulis* root EO as an effective ingredient for botanical insecticide development against *L. botrana* displaying high insecticidal efficacy against 1st instar larvae. In addition, the results promoted the real-world use of the *C. acaulis* EO through its encapsulation into a nanoformulation. An EO-based NE ensures the maintenance of the insecticidal activity guaranteeing dispersibility in the environment and its stability over time. Even if the results presented promote the employment of *C. acaulis* EO in the agricultural field, further studies should be performed for the evaluation of its eco-toxicological profile. Equally, more studies are needed to investigate lethal and sub-lethal concentrations effects on fertility, longevity, and behaviour of *L. botrana*.

5.4. *Bactrocera oleae*

Bactrocera oleae (Rossi) (Diptera: Tephritidae), also known as the olive fruit fly (Figure 35), is one of the principal pests affecting olive groves worldwide (Daane and Johnson, 2010).

Figure 35. *Bactrocera oleae*.

The olive fruit fly induces damages thanks to the susceptibility of the olive cultivar (Malheiro et al., 2015a; Rizzo et al., 2012) and leads to yield reduction, table olives devaluation, and oil quality decrease (Caleca et al. 2017; Gucci et al. 2012; Tzanakakis, 2006). Despite its high economic impact, there are limited data on the employment of natural environmental-friendly active ingredients, as EOs, in the attract and kill control protocols against the olive fruit fly (Canale et al., 2013a,b; Rizzo et al., 2020). In this work, *C. acaulis* EO and carlina oxide were tested in terms of toxicity, electroantennographic (EAG), and behavioral responses to adult olive fruit flies.

5.4.1 Materials and methods

Olive fruit fly rearing

Olive fruit fly adults tested in the ingestion toxicity and electrophysiology trials derived from pupae of *B. oleae* nurtured from drupes of two Sicilian organic olive groves (Caccamo and S. Giuseppe Jato, Palermo Province, Italy). Pupae were kept in plastic containers (30 × 30 x 15 cm) in a dark climatic room at the temperature of 8 °C, and groups of about 2000 pupae each were transferred weekly inside plastic boxes (30 × 30 x 30 cm) and placed in a climatic room at 21 ± 1 °C with a 16:8 (L:D) photoperiod. Before starting the tests, flies were nourished with 10% organic honey aqueous solution. Olive fruit flies tested in the behavioral trials were obtained from pupae collected in a Tuscan olive-mill (November 2020). Pupae maintenance was performed at 22 °C ± 1, 50–60% RH, and natural photoperiod till adult emergence. Olive fruit fly adults were nourished with a dry diet (yeast extract and sucrose mixture, 1:10 w/w ratio), and water was provided through a cotton wick (Canale and Benelli, 2012; Canale et al., 2013a,b).

Ingestion toxicity assays

The ingestion toxicity assays were performed following the work of Rizzo et al. (2020). The tests were performed on groups of 10 adults (both sexes, 10–15 days old) casually selected from the main rearing boxed and put into transparent plastic cages (450 mL). The olive fruit flies were nurtured with different concentrations of *C. acaulis* EO or carlina oxide, which were mixed with 2 mL of an aqueous emulsion of 2% of carboxy-methylcellulose sodium salt (Sigma-Aldrich®, medium viscosity), 12.5% of sucrose, and 1% of protein bait Nu-Bait® (Biogard). The mucilage was furnished through a bakelite cup (Ø = 30 mm), and closed with a cotton disk (Ø = 30 mm). A negative control, consisting of the viscous carrier without EO or the carlina oxide, was employed for every assay. For the EO, the subsequent concentrations (w/v) were tested: 0.007813, 0.015625, 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1%; while for carlina oxide: 0.015625, 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1% (w/v). Dead insects were counted daily, and the last control was done at 4

days. In every bioassay, *B. oleae* mortality was checked after 96 h, performing 5 replicates for each EO or carlina oxide concentration, over different days to account any daily variability. The tests were performed under controlled laboratory conditions [21 ± 1 °C, 45 ± 10 % R.H., 16:8 (L:D)].

Electrophysiological experiments

For electrophysiological experiments, antennal sensitivity to increasing concentrations of the EO and carlina oxide was evaluated on 15 to 20-day-old adults (males and females) of *B. oleae* according to the methods previously reported (Canale et al., 2013a,b; Germinara et al., 2009; Rotundo et al., 2001). To generate dose–response curves, solutions from 0.001 to 100 µg μL^{-1} of EO and carlina oxide in *n*-hexane were prepared and stored at -20 °C before the tests. A specimen was inserted in a plastic pipette tip (0.1 mL) with a cut end to allow the protrusion of the head. The electrodes were two glass capillaries filled with 0.1 M KCl saline solution. The indifferent electrode was placed into the insect head, while the recording electrode was placed in contact with an antenna tip. Moreover, electrical continuity between the antennal preparation and an AC/DC UN-6 amplifier in DC mode associated to a PC equipped with the EAG 2.0 program (Syntech Laboratories, Hilversum, The Netherlands) was maintained through AgCl-coated silver wires.

Stimuli consisted of 10 µL of a *n*-hexane solution of *C. acaulis* EO or carlina oxide adsorbed onto a filter paper (Whatman no. 1, Brentford, UK) strip (1 cm^2) inserted into a Pasteur pipette (15 cm long). The stimuli were insufflated into a stream of charcoal-filtered humidified air (500 mL min^{-1}) passing in a stainless-steel delivery tube (Ø = 1 cm) with the outlet positioned at 1 cm from the antenna. During 1 s, 2.5 cm^3 of vapor from an odor cartridge were added.

Control consisted of 10 µL of *n*-hexane and 10 µL of a 10 µg μL^{-1} (*Z*)-3-hexen-3-ol standard solution stimuli, which was applied at the beginning of the experiment and after each group of three test stimuli. Stimuli were given in intervals of 1 min. Ascending doses of test stimuli were applied on five males and females' antennae.

Behavioral assays

The assay of the purified carlina oxide was performed to exclude the possible interference minor components of the EO, such as benzaldehyde and *ar*-curcumene in the behavioral assays.

The potential attractive or repellence activity of carlina oxide formulated at the LC_{90} obtained in the ingestion toxicity assay was assessed towards both sexes of *B. oleae* in Y-tube bioassays, accordingly to Canale et al. (2015). The solution was prepared through emulsification of carlina oxide (at the LC_{90}) with DMSO (1:1) and by subsequent solubilization in deionized water. Together with the formulation, a negative control consisting of the same solution free of carlina oxide was also tested. Illumination conditions and Y-tube device employed in the experiment were illustrated

in the work of Canale et al. (2015), with a purified air flux of 1 mL min^{-1}. Temperature and R.H. were set at 23 °C ± 1 and 45% ± 5, respectively. At the beginning of each assay, the fly was placed into the central arm of the Y-tube. The potential attractiveness of carlina oxide was estimated on mated males and females and the formulation was assayed with 5 mL dose. The sample was positioned on a filter paper dish (Ø = 1 cm, Whatman no. 1) and, after evaporation of the solvent (20 s), the cue was placed into a Drechsel bottle (500 mL). A similar filter paper dish was prepared with the negative control and placed into the second Drechsel bottle (500 mL), representing the clean air control. Only first choices where the fly walked in each arm and stayed there for at least 30 s were observed. For each *B. oleae*, the first choice and the time passed in each arm were noted. Flies that were not moving within 3 min from their release were discarded (Carpita et al., 2012; Canale et al., 2013a,b). For each replicate, the olfactometer arms were flipped of 180°, Y-tube was cleaned as previously described (Carpita et al., 2012) and the chemicals were replaced. 40 replicates with responsive flies were carried out and, for each of them, each fly was replaced with a new fly of the same age. Both sexes were tested daily randomly (Ngumbi et al., 2012).

Statistical analysis

The Abbott's formula (1925) was employed for the correction of the experimental mortality and if the control mortality was higher than 20% data were discharged. Values of LC_{10}, LC_{30}, LC_{50}, and LC_{90} with associated 95% confidence interval (CI) and χ^2 were calculated through probit analysis (Finney, 1978). The amplitude (mV) of the EAG response was calculated following the procedure of Raguso and Light (1998), and the resulting EAG response was adjusted on the base of the reduction of the EAG response to the standard stimulus (Otter et al., 1991). The Student's t-test (p = 0.05) for independent samples was used for the comparison of male and female EAG responses to each test stimulus. In behavioural assays, a likelihood χ^2 test with Yates' correction (p = 0.05) was employed for comparing the proportion of flies choosing carlina oxide or the negative control. For both male and female flies, the time spent in the chosen arm was evaluated with the Wilcoxon test (p = 0.05). The statistical analyses were performed using JMP® 13 (SAS), SPSS (version 10.0.7 for Windows, SPSS Inc., Chicago, IL, USA), and Minitab Inc., State College, PA, USA.

5.4.2 Results and discussion

Ingestion toxicity bioassays

From the ingestion toxicity bioassay, the LC_{50} values of *C. acaulis* EO and carlina oxide were 706.155 and 1052.376 ppm, respectively (Table 14). Carlina oxide resulted less toxic than the EO. The study herein reported showed that *C. acaulis* EO and its main bioactive compound had a significant concentration-dependent toxicity to both sexes of *B. oleae*. Even if the EO is composed

mainly of carlina oxide, the polyacetylene resulted less toxic to the tephritid than the EO (Table 14). The different efficacies obtained could be linked to the presence of EO minoritarian compounds that could have the role of enhancing the activity of the main constituent. The insecticidal potential of EOs incorporated in protein baits against the olive fruit fly has been investigated before by Canale et al. (2013a,b), but also by Rizzo et al. (2020), who focused on the toxicity of the EOs from *R. officinalis*, *Hyptis suaveolens* (L.) Poiteau, *Lavandula angustifolia* Miller., *Pimpinella anisum* L., *Thymbra spicata* L. *Ocimum gratissimum* L., and *T. ammi*. In this regard, *C. acaulis* EO showed an LC_{50} (706.15 ppm) lower than those found for EOs from *R. officinalis* (5107 ppm), *H. suaveolens* (4922 ppm), *L. angustifolia* (6272 ppm) and *T. spicata* (2509 ppm). In addition, the LC_{50} of carlina oxide was comparable to that of the EOs from *O. gratissimum* (925 ppm), while the LC_{50} of *C. acaulis* EO was comparable to those of the EOs of *T. ammi* (633 ppm) and *P. anisum* (771 ppm) (Canale et al., 2013a,b; Rizzo et al., 2020).

Table 14. Lethal concentrations of *Carlina acaulis* essential oil (EO) and carlina oxide formulated in protein baits against adults of *Bactrocera oleae*.

Tested product	**LC_{10}[a] (95%CI[b]) (ppm)**	**LC_{30} (95%CI) (ppm)**	**LC_{50} (95%CI) (ppm)**	**LC_{90} (95%CI) (ppm)**	**Intercept ± SE**	**Slope ± SE**	**Goodness of fit χ^2 (d.f.)**
EO	190.823 (96.319-288.602)	413.400 (268.900-547.482)	706.155 (530.213-880.887)	2613.202 (1991.810-3939.835)	2.595± 0.318	2.255± 0.309	12.838 (7) p=0.076 n.s[c]
carlina oxide	352.606 (240.896-463.244)	672.744 (521.039-821.618)	1052.376 (865.805-1254.674)	3140.863 (2530.428-4178.891)	2.638± 0.259	2.698± 0.271	7.446 (6) p=0.282 n.s.

[a]LC, lethal concentration killing 10% (LC^{10}), 30% (LC^{50}), 50% (LC^{50}), or 90% (LC^{90}) of the exposed population; [b]95% CI, 95% confidence interval; [c]n.s., not significant ($p > 0.05$).

Electroantennographic (EAG) experiments

Figure 36 shows the EAG responses of *B. oleae* adults (males and females) to increasing concentrations of the EO and carlina oxide.

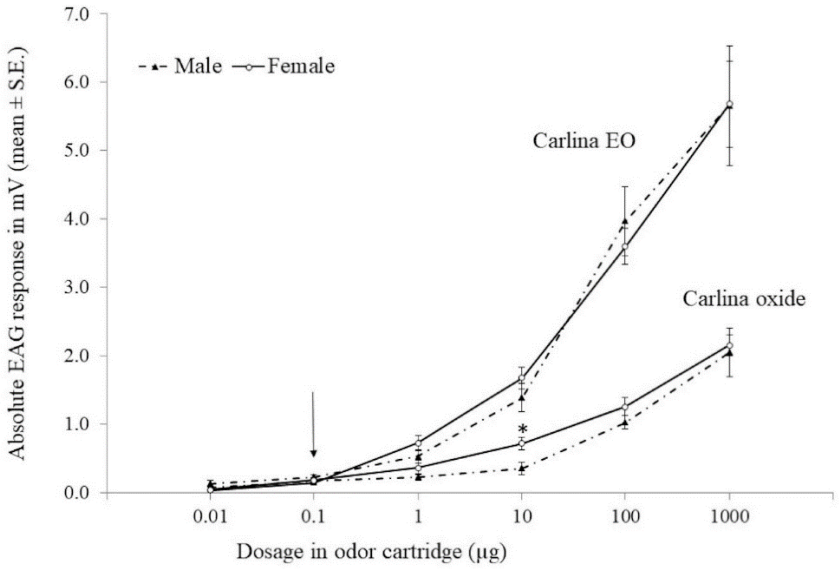

Figure 36. Electroantennographic (EAG) dose-response curves of *Bactrocera oleae* males and females (n=5) to *Carlina acaulis* essential oil (EO) and carlina oxide. The arrows indicate the activation thresholds. The asterisk indicates significant differences between male and female EAG responses to carlina oxide at $p = 0.05$.

Both stimuli caused EAG dose-dependent responses in the antennae of males and females, for which the activation threshold was 0.1 µg. The mean EAG responses of males and females to the treatment with a dose of EO and carlina oxide of 1000 µg was higher than those with the 100 µg dosage, indicating the absence of receptors saturation at the lowest dose.

The EAG tests demonstrated that *C. acaulis* EO and carlina oxide stimulated in a dose-dependent manner the peripheral olfactory system of olive fruit fly adults. In the dose range of 0.01 to 1000 µg, the EO and carlina oxide elicited dose-dependent EAG responses (0.03–5.67 and 0.07–2.11 mV, respectively), showing a strong antennal sensitivity to both stimuli. Moreover, no significant differences were detected between the EAG responses of males and females to most of the doses tested. However, in both sexes, a strongest EAG response caused by the EO than that caused by carlina oxide was detected. These differences could be linked to the presence in the EO of minoritarian compounds, as benzaldehyde, *ar*-curcumene, and *β*-sesquiphellandrene, deriving the electroantennogram from the summation of receptor potentials caused by olfactory stimuli from various sensilla on the antennae (Schneider, 1962).

Benzaldehyde was also detected in some olive cultivars extracts that resulted to cause electrophysiological responses in male and female antennae of *B. oleae* (Malheiro et al., 2015b)

Moreover, the electrophysiological and behavioural influence of terpenes derived from plants on tephritid flies, including *B. oleae*, has also been proved (Anfora et al., 2012).

Behavioural assays

Regarding the behavioural assays, the Y-tube tests showed that both males and females of *B. oleae* were not attracted by carlina oxide (Figure 37).

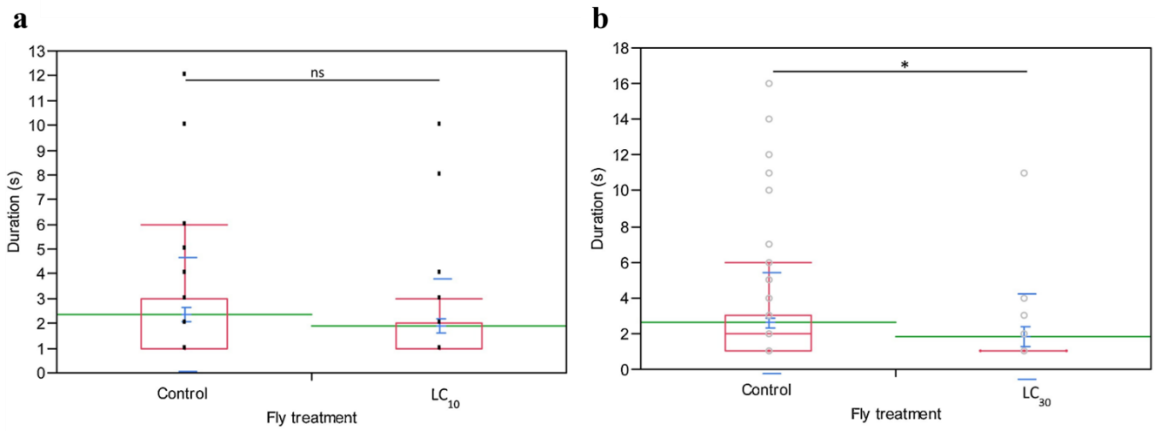

Figure 37. Time spent in the chosen arm by (**a**) males and (**b**) females of *Bactrocera oleae* during Y-tube tests. Red boxplots indicate the median (solid line) within each box and the range of dispersion (lower and upper quartiles and outliers). Means and standard errors are represented by green lines and blue T-bars, respectively. Above the boxplots, ns indicates no significant difference over the control (Wilcoxon test, p >0.05).

Even if the EO and carlina oxide showed a marked electrophysiological activity in the dose range tested, they displayed a neutral effect on the fly behaviour. Behaviourally inactive compounds as active ingredients for insecticidal products can be considered advantageous for lure-and-kill approaches, because they do not deter insects from feeding on the protein bait.

5.4.3 Conclusions

This study evaluated the potential of *C. acaulis*-derived products as effective constituents for the development of lure-and-kill instruments for the management of *B. oleae*. The results presented underlined that *C. acaulis* EO and carlina oxide have an adulticidal action at rather low concentrations. Moreover, it was highlighted that the LC_{90} of carlina oxide does not cause any behavioural attraction or repellence in this olive pest, despite the strong EAG responses noted for males and females. Further studies on the safety and potential sublethal effects of *C. acaulis* EO

and carlina oxide should be performed as well as insights on the insecticidal activity of nanoformulations against this pest.

5.5. *Ceratitis capitata*

Ceratitis capitata (Wiedemann) (Diptera: Tephritidae) (Figure 38) is a cosmopolitan polyphagous species also known as Mediterranean fruit fly or medfly, which affects more than 300 host plants (Guillem-Amat et al., 2020; Liquido et al., 2017).

Figure 38. *Ceratitis capitata*.

Control programs of *C. capitata* are still based on the employment of traditional insecticides. However, their overuse caused the onset of medfly resistance to deltamethrin, pyrethroid, spinosyn, and organophosphate insecticides (Demant et al., 2019; Magaña et al., 2007, 2008; Vontas et al., 2011). Integrated Pest Management (IPM) protocols for the management of *C. capitata* often rely on the combination of chemicals with protein-baits or female attractants, with an "attract and kill" approach (Asadi et al., 2020; Siciliano et al., 2014). This strategy also reduces the impact of pesticides on non-target organisms (Gregg et al., 2018). In this context, the use of EOs is continuously increasing. In the studies reported below, the ingestion toxicity of *C. acaulis* EO and carlina oxide in "attract and kill" formulations was assayed on medfly adults, as also their sublethal effects on the medfly aggressive behaviour. Moreover, to further investigate the mammal safety of the EO, its acute toxicity was evaluated on the liver, stomach, and kidney of rats after oral administration.

5.5.1 Materials and methods

Medfly rearing

The medflies are derived from a stock culture kept at the Entomology Section of the University of Pisa. Cylindrical PVC cages, each containing about 2000 flies (sex ratio 1:1), constituted the rearing production unit. The rearing of medfly followed the procedure developed by Canale and Benelli (2012). Mass-rearing conditions consisted of 25 ± 1 °C and 45% R.H., with a photoperiod of 16:8 (L:D). Adult medflies used in bioassays were 10–12 days old.

Ingestion toxicity assay

Following the method by Benelli et al. (2012), an ingestion toxicity assay was performed using 30 *C. capitata* adults (of both sexes). These were put into a plastic container (600 mL) with a thin mesh on the top for air exchange. The food of medflies, nourished for 96 h, consisted of 2 mL of mucilage containing 0.0039, 0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1% of the EO and carlina oxide. The mucilage was prepared by emulsifying the EO or carlina oxide with DMSO (1:1), 2% of carboxymethylcellulose sodium salt, 12.5% of sucrose and 1% of protein bait (NuBait, Biogard, Italy). The mucilage was furnished inside a bakelite cup (Ø =30 mm) and, to avoid insects drowning, a cotton disk (Ø =30 mm) was used as coverage. For each test, a negative control was performed assaying just the viscous carrier without *C. acaulis* products. In all the tests, medfly mortality was examined after 96 h, and 4 replicates were performed for each EO or carlina oxide concentration, over diverse days. Experiments were performed under controlled laboratory conditions [21 ± 1 °C, 45 ± 10% R.H., 16:8 (L:D)]

Impact on aggressive behaviour

In this test, the effect of feeding medflies with *C. acaulis* EO and carlina oxide at their respective LC_{10} and LC_{30} was evaluated in terms of aggressive behavior. The observations were done on adult medflies separated by sex. Each test consisted of an arena represented by a plastic container with a volume of 600 mL covered by a piece of glass. Inside the arena, a lemon twig (~15 cm, 1 leaf/twig) was placed and 4 adult males or females were introduced. Two were fed on the EO or carlina oxide mucilage for 96 h before the test and the other two with the control viscous carrier for the same time. In each replicate, two tests lasted 30 min and comprised an initial phase of 10 min allowing the insect to adapt. An aggressive behavior occurred when a medfly (treated or control) approached the other and displayed an aggressive interaction as described by Benelli et al. (2015). The aggression escalations were expressed in terms of “aggression score” as detailed by Benelli et al. (2015). The intensity of the aggressive events was evaluated, and each display ordered in an

escalation from 0 to 12 (i.e. minimum to maximum aggressiveness) (Benelli et al., 2015). At the end of each test, the lemon twig was removed, and the arena was washed with hot water and mild soap (Benelli et al., 2015; Carpita et al., 2012). Experiments were conducted under controlled laboratory conditions (21 ± 1 °C, 45 ± 10% R.H., 16:8 (L:D).

Rats

For the toxicity studies on rats, the EO was tested, to have enough material for the performance of the *in vivo* tests, and since the EO was mainly constituted by carlina oxide, whose purification requires additional costs. The study employed female Wistar rats weighting 250–300 g. Each animal was kept in a single cage and in cycles of 12 h of dark followed by 12 h of light at a temperature and R.H. of 20-22 °C and 44-45%, respectively. Food and water were furnished ad libitum. Housing and experiments were carried out accordingly to the guidelines of the European Community Council Directive Care and Use of Laboratory Animals (Ministry of Health Authorization n° 1D580.22)

Acute toxicity procedures

For the assessment of acute toxicity, the EO was dissolved in 2% of Tween 80, used as a vehicle, and the administration was performed by gavage. Rats were divided in four groups, composed of four individuals. To the first group was administered with the vehicle; the second one received 250 mg of EO per kg by oral administration; the third one 500 mg per kg; and the fourth group 1000 mg per kg. After the administration, each animal was observed for the first 30 min to detect signs of toxicity and, then periodically, for the remaining 48 h before tissues were harvested. The death of animals was registered if it occurred. The noted signs of toxicity regarded the autonomous and central nervous system activities and consisted of ataxia, ptosis, tremors, convulsions, cyanosis, piloerection, coma, lacrimation, and salivation. Each rat was weighted after 48 h from the administration of the doses and then sacrificed. Afterwards, the organs (stomach, liver, and kidney) were excised through surgery and their characteristics and weight were recorded. Organs were then stored in fixative Bouin's solution for 6 h, then dehydrated in ethanol gradually from 70% to absolute, and finally cleared in xylene for the paraffin embedding. Consecutive sections of 5 μm were treated with haematoxylin and eosin dye (H&E), observed through a light microscope Leica DMR (Germany) connected by a DS-R12 Nikon camera to the computer and analyzed using a NIS Elements Nikon image analyzer software. At the stomach level, the presence of ulcer, inflammatory aggregate, and elements of necrosis were observed. For the liver, the following parameters were observed: degeneration of hepatocytes, presence of apoptotic cells or hepatic

necrosis, inflammatory elements, vacuolization, and dilated sinusoids. In the kidney, signs of glomerular and tubular alterations, and infiltration were analyzed.

Data analysis

Experimental mortality was corrected with Abbott's formula (Abbott, 1925) if it ranged from 1 to 20%, while if control mortality was > 20% the experiments were repeated. Ingestion LC_{50} and LC_{90} with associated 95% confidence interval (CI) and chi-squares were calculated using probit analysis (Finney, 1978). Chi-square tests with Yates' correction (Rohlf and Sokal, 1981) were used to analyze the differences in the total number of medflies performing aggressively. The influence of the EO or carlina oxide treatment on the duration of the aggressive behavior was analyzed using the weighted generalized linear model (GLZ) described by Benelli et al. (2015). Differences among the aggression scores were evaluated using the Kruskal-Wallis test ($p = 0.05$). Statistical analyses were performed using JMP® 9 (SAS) and Minitab Inc., State College, PA. For rat acute toxicity, one-way ANOVA was employed to analyze rat body weight data as the main effects. When appropriate, Tukey's multiple test was used as post-hoc test ($\alpha = 0.05$). GraphPad Prism 8 software (San Diego, CA) was used for analyzing these data.

5.5.2 Results and discussion

Ingestion toxicity

Probit analysis demonstrated the efficacy of *C. acaulis* EO and carlina oxide as promising products to be employed in "attract and kill" formulations (Table 15). For the EO the LC_{50} was achieved with 1094 ppm, while for carlina oxide the LC_{50} was reached with 1273 ppm.

Table 15. Ingestion toxicity on *Ceratitis capitata* adults of *Carlina acaulis* essential oil (EO) and carlina oxide in protein baits.

Tested product	**LC_{10}[a] (95%CI[b]) (ppm)**	**LC_{30} (95%CI) (ppm)**	**LC_{50} (95%CI) (ppm)**	**LC_{90} (95%CI) (ppm)**	**χ^{2c} (d.f.)d *p*-value**
EO	388 (274-496)	716 (570-860)	1094 (917-1293)	3082 (2480-4140)	1.502 (7) $p = 0982$ ns[e]
carlina oxide	555 (394-698)	906 (725-971)	1273 (1078-1480)	2922 (2413-3839)	5.860 (7) $p = 0.556$ ns

[a]LC, lethal concentration killing 10 %, 30 %, 50 %, 90 % of exposed flies; [b]95 % CI, lower and upper limits of the 95 % confidence interval; [c]χ^2, chi-square; [d]d.f., degrees of freedom; [e]ns, not significant ($p > 0.05$).

To the best of our knowledge, this is the first study on the adulticidal activity of *C. acaulis* and carlina oxide on *C. capitata* flies. However, other EOs have been tested on this target. The LC_{50} found in this work for the EO was lower than those obtained with the EOs from *H. suaveolens*, *L. angustifolia* L., *Thuja occidentalis* L., and *M. alternifolia* for which the LC_{50} values were higher than 5000 ppm (Benelli et al., 2012; 2013). Since in this study the purified carlina oxide displayed a promising LC_{50} against adults of *C. capitata*, it can be deduced that the overall toxicity of *C. acaulis* EO is mainly related to the presence and bioactivity of this polyacetylene.

Aggressive behaviour

Number of aggressive events

In this study, the *C. capitata* aggressive behaviour was observed at a population level in terms of intensity and duration of the event and in terms of differences among male and female individuals. The EO formulations showed a dose-dependent effect in the reduction of aggressiveness in medflies, which resulted less aggressive than those treated with the control (Figure 39).

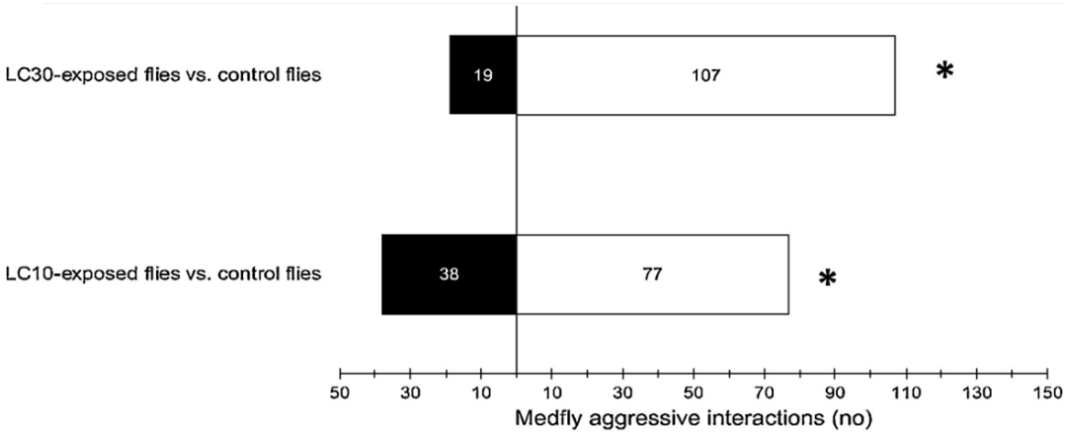

Figure 39. Total number of aggressive behaviours of medflies exposed to LC_{10} and LC_{30} of the essential oil (EO) vs. control flies. The asterisk shows a significant difference over the control (χ^2 test with Yates' correction, $p < 0.05$).

On the other hand, both sexes of medflies fed with carlina oxide-formulations showed an aggressive behaviour comparable to the control medflies, apart from females treated with the LC_{10}. In this case, there was a significative difference in the number of aggressive interactions compared to those of the control individuals (Figure 40).

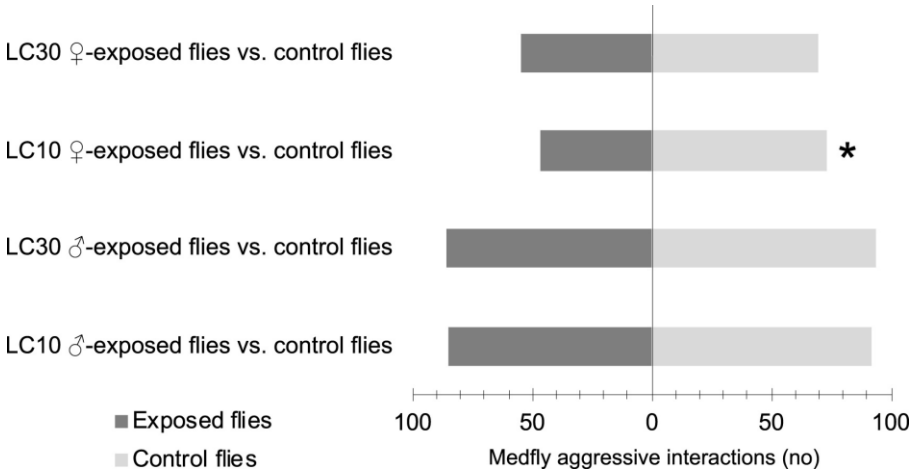

Figure 40. Overall abundance of aggressive behaviours performed by medfly adults fed on LC_{10} and LC_{30} of carlina oxide vs. control flies. The asterisk shows a significant difference over the control (χ^2 test with Yates' correction, $p < 0.05$).

Asymmetries in aggressive interactions

In this study, it was also possible to evaluate deeply the directionality of the aggressive behaviour and to distinguish *C. capitata* individuals carrying out the aggression and those suffering it. No significant differences were observed testing different concentrations of the EO (Figure 41).

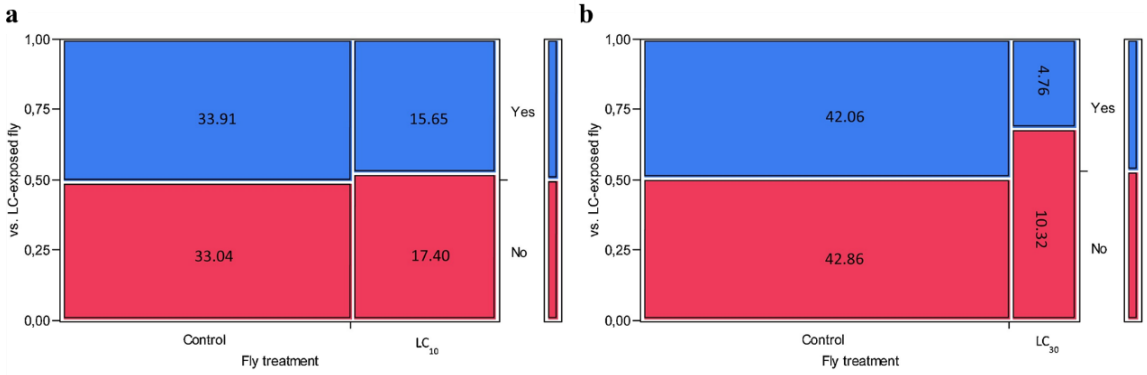

Figure 41. Mosaic diagram of the aggressive interactions in medflies after feeding on the essential oil (EO) formulated in protein baits. The bar on the right (yes/no) indicates the directionality of the action, i.e. whether it has been directed or not towards the target subject. Medflies exposed to LC_{10} of the EO (**a**); medflies exposed to LC_{30} of the EO (**b**).

Regarding carlina oxide, the tested LC significantly influences the directionality of the aggressive behaviours. Both sexes feed on LC_{30} were more attacked by control flies and, in a similar way, also individuals fed on carlina oxide LC_{10}. Overall, the exposure to both concentrations of carlina oxide affected the willingness of receiving attacks from control flies by the treated ones (Figure 42).

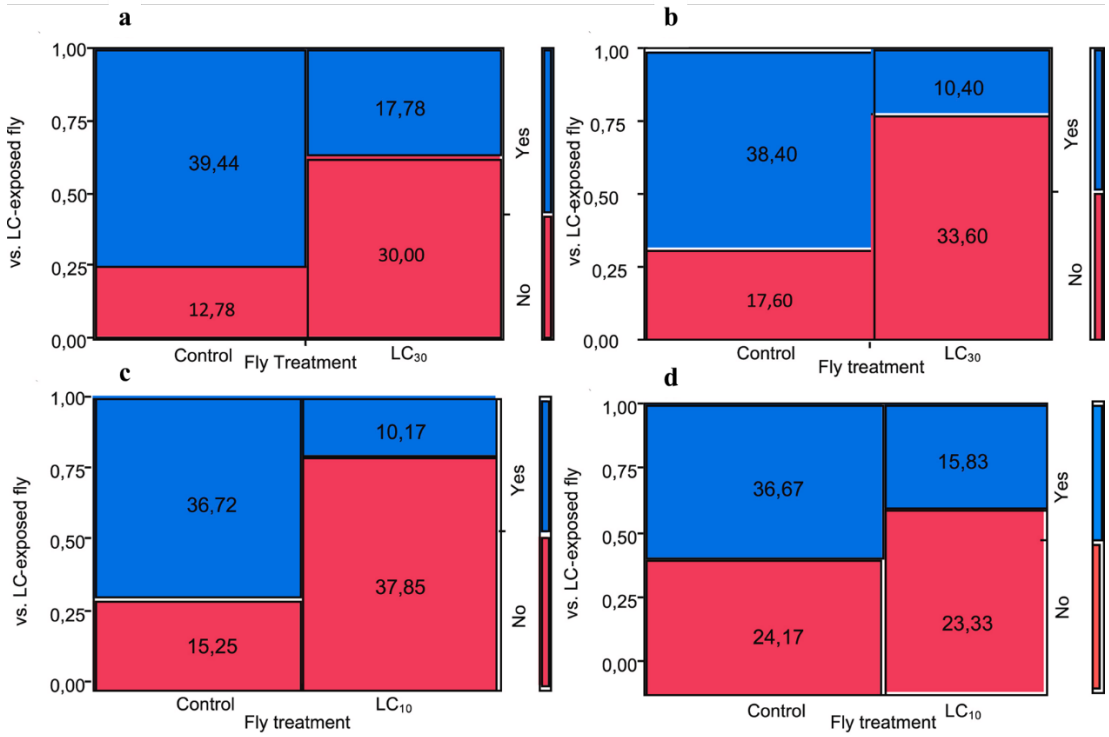

Figure 42. Mosaic diagram of the aggressions in medfly adults after being fed on carlina oxide. The bar on the right (yes/no) shows the directionality of the action, i.e., whether it has been directed or not towards the target subject. Males fed on carlina oxide LC_{30} (**a**); females fed on carlina oxide LC_{30} (**b**); males fed on carlina oxide LC_{10} (**c**), females fed on carlina oxide LC_{10} (**d**). The value within each mosaic specifies the percentage of aggressions. The size of each various mosaic varies according to the number of individuals who have shown aggressive behaviour.

Sex of the fighting flies

Aggressive interactions can also vary in relation to the sex of the involved fly. For the treatment with the EO, no significant differences were found between the two sexes fed on LC_{10} and LC_{30} (Figure 43).

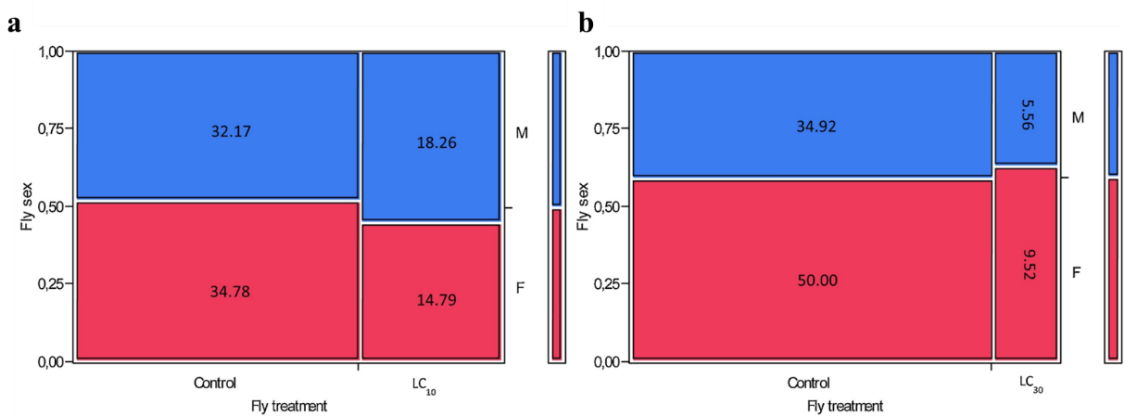

Figure 43. Mosaic diagram of the aggressive interactions in male and female medflies feeding on the essential oil (EO) formulated in protein baits. Medflies exposed to LC_{10} (**a**); medflies exposed to LC_{30} (**b**). The bars on the right represent the percentage of males and females out of the total number of individuals tested. The numbers inside each box display the percentage of aggressive interactions based on gender (red = female; blue = male). The size of the various mosaic tiles changes accordingly to the number of individuals who have shown aggressive interactions.

Regarding carlina oxide, no significant differences between the sexes were detected for both LC_{10} and LC_{30} (Figure 44).

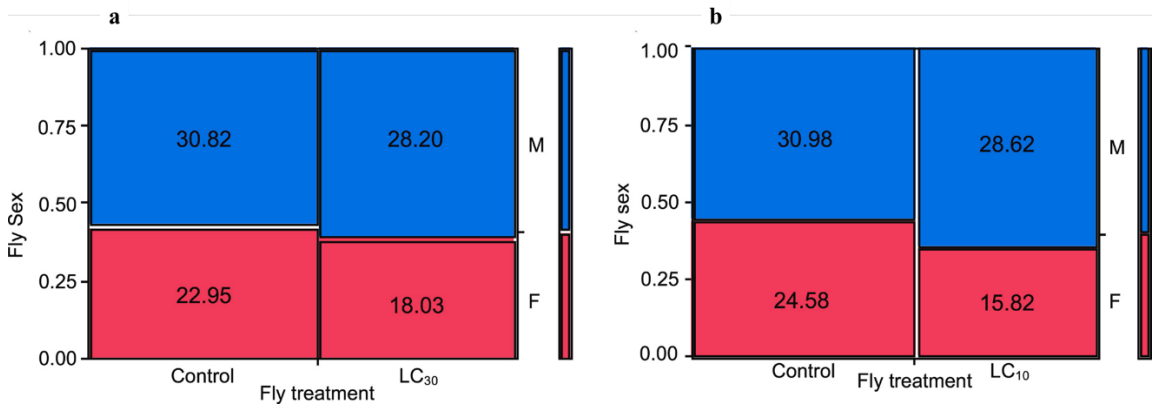

Figure 44. Mosaic diagram of the aggressions in adult males and females medflies feeding on carlina oxide. Adults fed on LC_{30} (**a**); adults fed on LC_{10} (**b**). The bars on the right denote the percentage of males and females out of the total number of individuals tested. The numbers inside each box show the percentage of aggressions based on gender (red = female; blue = male). The size of each mosaic tile changes accordingly to the number of adults that have shown aggressive behaviour.

Aggression score

Regarding the treatment with the EO and carlina oxide, no significant differences were found between *C. capitata* fed on the different EO or carlina oxide concentrations and control.

Aggression duration

Significative differences were found between *C. capitata* individuals fed on LC_{30} of the EO with respect to the control individuals. The duration of the aggressive interactions was shorter for individuals fed on low concentrations of the EO (Figure 45).

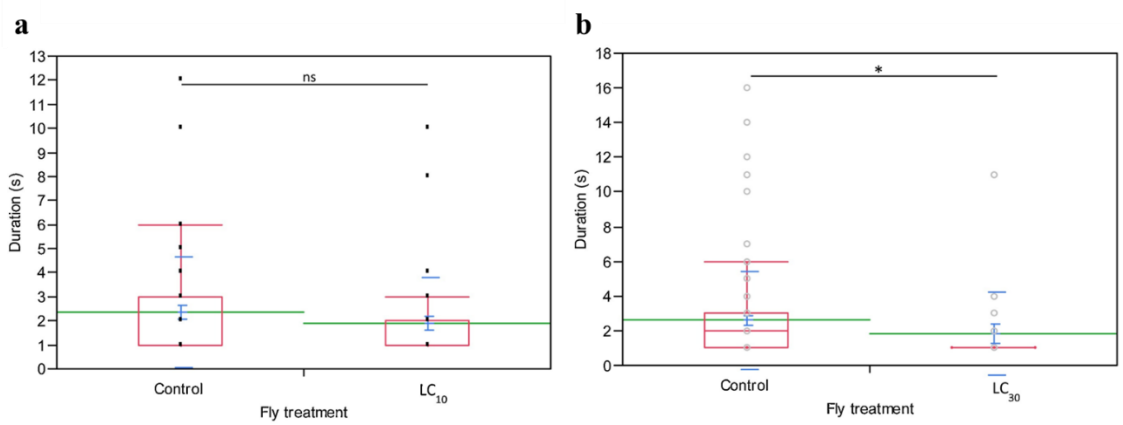

Figure 45. Aggression duration in individuals exposed or not to LC_{10} and LC_{30} of the essential oil (EO). Individuals exposed to LC_{10} (**a**); individuals exposed to LC_{30} (**b**). Each box plot shows the median (red line) and its dispersion range (lower, upper quartile and extreme values, outliers). The average is specified with a green line, the standard error is a blue T-bar. *, a significant difference was detected (Kruskal-Wallis test, $p < 0.05$); ns, not significant ($p > 0.05$).

Regarding carlina oxide, no significative differences in terms of duration of aggressive events were detected on medfly adults fed on carlina oxide in confront with the control ones (Figure 46).

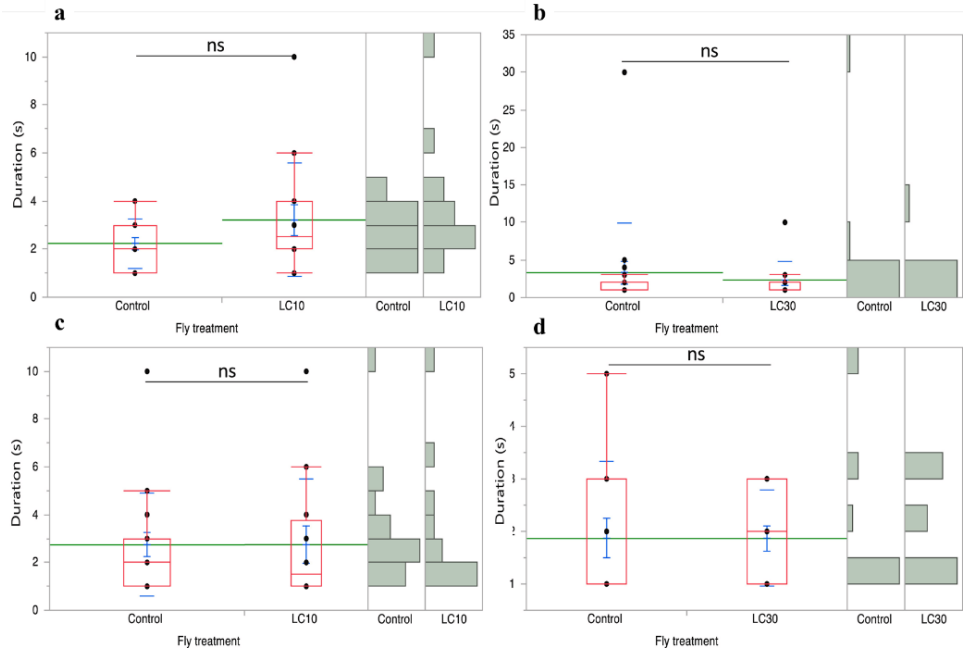

Figure 46. Aggression duration in male and female medflies fed or not to LC_{10} and LC_{30} of carlina oxide. Males fed on LC_{10} (**a**); males fed on LC_{30} (**b**); females fed on LC_{10} (**c**); females fed on LC_{30} (**d**). Each box plot indicates the median (red line) and its dispersion range (lower, upper quartile and extreme values, outliers). The mean is indicated with a green line, the standard error is a blue T-bar; ns, not significant (Kruskal-Wallis test, $p > 0.05$).

The research on the impact of sublethal doses of EOs on the behaviour of insects is poor (Lee et al., 2020a; Toledo et al., 2020). Recently, it was demonstrated that exposure to a mixture of EOs (such as *Cedrus atlantica* (Endl.) Manetti ex Carriere, *Corymbia citriodora* (Hook.) K.D. Hill & L.A.S.Johnson, and *Cymbopogon citratus* (DC.) Stapf) is not able to lower the parasitization efficacy of *Psyttalia concolo*r (Szépligeti), which is an endoparasitoid that attacks medfly larvae. Moreover, Silva et al. (2018) showed that treatment with sublethal concentrations of *Aristolochia trilobata* L. EO were able to change the swimming actions of *Aedes aegypti* L. (Diptera: Culicidae) larvae. Regarding the effect of carlina oxide, in this study it was surprising to note that a significative decrease in medfly aggressive behaviour was reached only for female individuals fed on LC_{10} vs. control flies. However, the test of the EO led to a reduction of medfly aggressive interactions along with shorter aggressive times in medflies treated with both sublethal concentrations of the EO. The differences between carlina oxide and the EO could be ascribed to the presence of minor constituents in the EO, such as benzaldehyde and *ar*-curcumene. These two compounds have an insecticidal action and can also enhance the penetration of carlina oxide into the insect cuticle (Benelli et al., 2017a, b; Yuan et al., 2019).

Since there are no evidences on the impact of the exposure to EOs on intraspecific aggressions of insects, this study embodies the first contributions in this field of research.

Acute toxicity on rats

In the rats treated with the dose of 1000 mg kg^{-1} of *C. acaulis* EO, corresponding to LD_{50}, different signs of toxicity were detected, as sedation, tremors, ataxia, and ptosis. No evident signs of toxicity were found for the administration of lower doses, and no significative changes in the body and organs weight were observed after acute administration of the diverse doses of the EO. The analysis of the anatomy of the inner wall of the stomach revealed hyperaemia, with a sign of necrosis that was evident in rats treated with the highest dose of the EO (Figure 47).

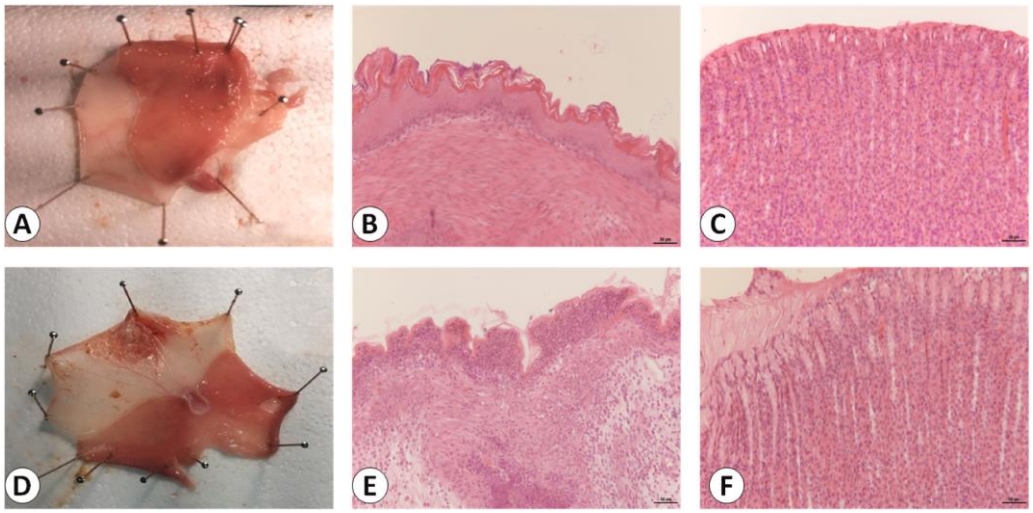

Figure 47. Inner wall of the stomach (**a**, **d**) and sections staining with haematoxylin and eosin at the levels of the fundus (**b**, **e**) and body (**c**, **f**) of animal administered with vehicle (**a**–**c**) and *Carlina acaulis* essential oil (EO) (**d**, **e**, **f**) at the dose of 1000 mg kg^{-1}.

Analysis of 5 μm sections of the organs stained with H&E permitted to compare their structures and to evaluate the presence of injuries linked to the treatment with the EO. The results indicated that stomachs did not have significant injuries for both the groups 'vehicle' and groups treated with 250 and 500 mg kg^{-1} of EO. Only in the stomachs of two of the four rats administered with 1000 mg kg^{-1} of EO signs of necrosis were found. In addition, a morphological analysis of gastric mucosae of the body showed the absence of morphological alterations for rats administered with vehicle of 250 mg kg^{-1} and 500 mg kg^{-1} of EO. Only the highest dose of EO was able to induce the formation of ulcers. From the gross anatomical analysis of liver, it emerged that the hepatic parenchyma was not affected by different dosages of EO. On the other hand, rats treated with the

dose of 1000 mg kg^{-1} showed in the liver vacuolations in a perivascular central zone, dilated sinusoid, degenerated hepatic cord, and apoptotic cells (Figure 48).

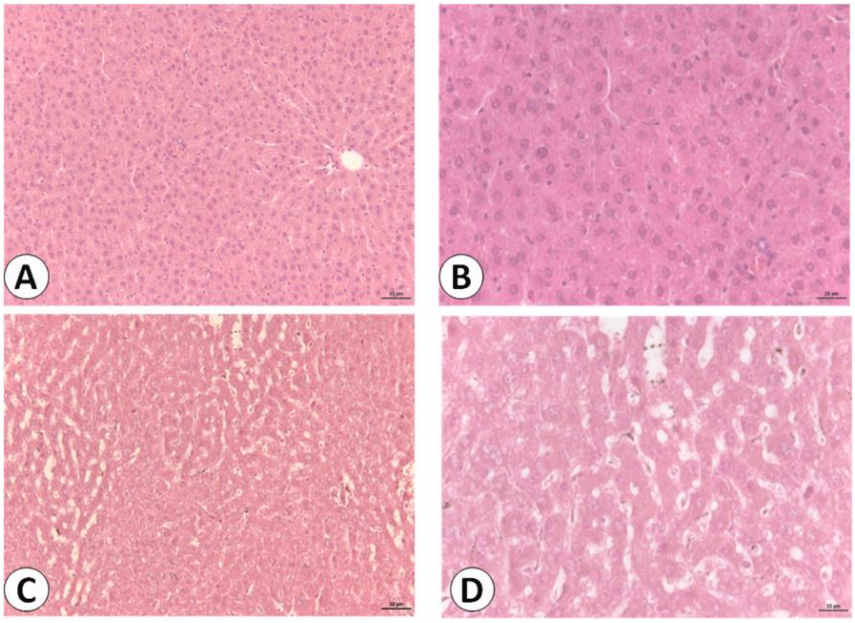

Figure 48. Sections of the liver stained with haematoxylin and eosin of animal treated with vehicle (**a**, **b**) and 1000 mg kg^{-1} of *Carlina acaulis* essential oil (EO) (**c**, **d**).

Moreover, also the kidney was histologically studied in micrometers sections and that of the renal cortex displayed convoluted tubules (Figure 49a, b) and normal renal corpuscles. Animals treated with the highest dose of the EO (1000 mg kg^{-1}) presented oedematous alterations in the renal interstitium, even if there was the absence of mononucleate cellular infiltrates. Even if glomeruli appeared normal, in capillary and Bowman's spaces an increased dilatation was detected (Figure 49d). Doses of 500 and 1000 mg kg^{-1} of the EO led to an increased luminal diameter of renal tubules (arrow in Figure 49c).

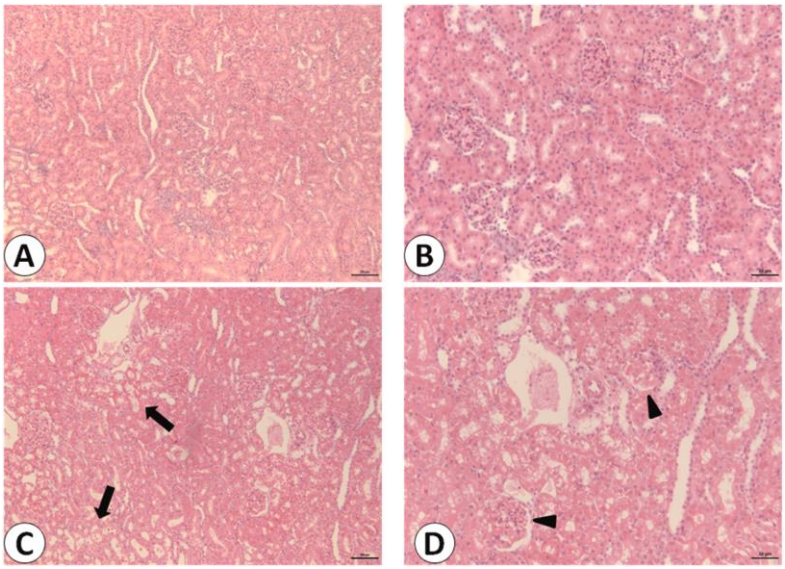

Figure 49. Sections of the cortical layer of kidney stained with haematoxylin and eosin of rats administered with vehicle (**a**, **b**) and 1000 mg kg^{-1} of *Carlina acaulis* essential oil (EO) (**c**, **d**).

Regarding the toxicity to mammals evaluated in this study, variations in the weight of body and organs are usually considered indicators of toxicity (Michael et al., 2007; OECD, 2008). In this work, no changes were observed for both body and organs weight.

Neurological toxicity signs were detected only after the administration of the highest dose (1000 mg kg^{-1}) of EO and increasing symptoms of ptosis, tremors, sedation, and ataxia were noted over 48 h of observation. Neurotoxicity is a common consequence of the treatment with EOs, since they can cross the brain-blood barrier thanks to their lipophilicity. Some EO compounds have been reported to induce neurotoxicity, as, for instance, thujone which is reported to cause excitations and convulsions (PMID: 23201408). In a similar way, camphor and 1,8-cineole, which are frequent constituents of rosemary and eucalyptus EOs are responsible for the induction of seizures (PMID: 19893077). However, since no symptoms of neurotoxicity were noted at the lowest dose tested, *C. acaulis* EO can be considered to have a relatively safe profile, since its concentration in insecticidal formulations would be lower than those assayed in this study. The histopathological results obtained on the organs studied indicated a moderate toxicity of the EO at the highest dose of 1000 mg kg^{-1}. This toxicity was not detected for the dosage of 500 mg kg^{-1}. Based on the evidence obtained in this study, *C. acaulis* EO is slightly toxic to rats, with LD_{50} that overlapped with those of some EOs constituents, as cinnamaldehyde and thymol (Pavela and Benelli, 2016). In conclusion, the *in vivo* toxicological study herein reported showed a low oral toxicity for *C. acaulis* EO. The LD_{50} of *C. acaulis* EO resulted higher compared to that of plant extracts bearing polyacetylenes. For instance, a study on the toxicity of *Bupleurum longiradiatum* Turcz. (Apiaceae) displayed that the extract has high toxicity on rats, with LD_{50} values of 37.5-77.7 mg kg^{-1} (You et al., 2002).

5.5.3 Conclusions

In this study, the possibility of employing *C. acaulis*-derived products for the development of "attract and kill" formulations has been outlined. The results presented underlined that carlina oxide can influence the directionality of aggressive behaviour, with the attacks of control medflies directed mostly to flies previously treated on carlina oxide concentrations. Further studies are needed both to understand the possible interactions between the EO minor constituents and to assess the toxicity on invertebrates that represent biocontrol agents of *C. capitata*. In addition, since the *in vivo* toxicological assays demonstrated that the EO could provoke modest neurological signs and moderate effects on the stomach, liver, and kidney only at the highest dose (1000 mg kg^{-1}), this study demonstrated the safety of this natural product that could be used in "attract and kill" approaches for the management of major agricultural pests.

5.6. *Meloidogyne incognita*

Meloidogyne (Figure 50) species spp are obligate sedentary endoparasites of numerous botanical species and well-known plant pests, leading to severe economic losses around the world (Abad et al., 2003; Jones et al., 2013).

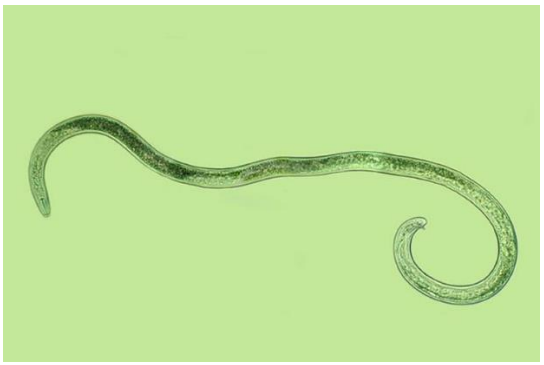

Figure 50. *Meloidogyne incognita*.

The control of these nematodes is traditionally achieved using synthetic formulates. However, the use of most of these products is nowadays banned given their hazards to human health and the environment (Council directive 91/414/EEC,1991; Directive 2009/128/EC, 2009). Even if chemical products remain the first option, the developed nematicide degradation mechanisms by soil biota (Gallego et al., 2019) are raising serious concerns regarding the efficacy of these products. In this context, EOs are reported as good nematicidal agents (Andrés et al., 2012; Gahukar, 2012; Suteu et al., 2020). The present study investigated the nematicidal activity of *C. acaulis* EO on the nematode *Meloidogyne incognita* in terms of second stage juveniles (J2) paralysis *(in vitro* assays) and biological cycle arrest in tomato host roots (*in vivo* assays). Moreover, a *C. acaulis* EO-NE was

developed to improve the physio-chemical properties of the EO, but also its nematicidal activity also in in field conditions.

5.6.1 Materials and methods

Preparation and characterization of *Carlina acaulis* essential oil-nanoemulsion (EO-NE)

C. acaulis EO-NE (6% of EO) was prepared and characterized accordingly to the procedures described in Section 4.2.1 and 4.2.2.

Nematode rearing

The *M. incognita* population used in the assays derived from two single egg masses of Greek origin and it was preserved on plants of tomatoes, *Solanum lycopersicum* L. cv. Belladonna. When the biological cycle was completed on the tomato root, second stage parasite juveniles (J2) nematodes were obtained accordingly to the procedure of Hussey (1973) and used for the tests.

Paralysis effect of the essential oil (EO) on *Meloidogyne incognita* second stage juveniles (J2)

C. acaulis EO efficacy in terms of *M. incognita* J2 paralysis was assessed using *in vitro* assays, calculating also the EC_{50} values. The range level of 15 to 250 μL L^{-1} was used for the performance of the dose-response bioassays. The test solutions were prepared dissolving the EO in ethanol and then reaching the final volume with Tween 90 aqueous solution. The content of ethanol and Tween 90 was 1 and 0.3% (v/v) in the immersion wells, respectively. The control was represented by distilled water. For the *in vitro* assay, 15 J2 individuals were placed in each well of a Cellstar 96-well plates (Greiner bio-one). Plates were covered using lids and kept at 28 °C in constant darkness. The test solutions and nematode suspension were placed in a test well at a 1:1 (v/v) ratio. Any vapor drift in the plates was evidenced using borders adjacent to the immersion wells. Moreover, the closure of the 96 well plates was performed to avoid evaporation phenomena. The observation of nematodes was conducted at 40x using an inverted microscope (Euromex, The Netherlands) at two time points (40x after 24 h and 48 h). After the observation, the individuals were divided into two different groups, namely motile or paralyzed. The J2 were then transferred into water to verify the motility regain after further 24 h, but this regain was not evident and J2 individuals were considered dead. The paralysis values are presented as before rinsing. J2 individuals that never regained activity were considered dead. The experiments were replicated 6 times and each test was performed twice.

Soil drip irrigation with the essential oil (EO) for the control of *Meloidogyne incognita*

A soil of pH 7.8 and 1.3% of organic matter was sampled from a wild field at the Benaki Phytopathological Institute and was passed through a 3-mm sieve, being then air dried overnight. A mix of that soil with sand (2:1 w/w) was prepared and referred as 'soil'. The latter was then distributed into 6 plastic bags of the weight of 1 kg each representing the experimental treatments. Afterwards, the soil was inoculated with 2500 *M. incognita* J2 kg^{-1}. After an appropriate mixing and an incubation at room temperature overnight, each plastic bag was employed for transplanting 5 tomato plants Belladonna 7-week-old into 5 separate plastic pots that represented treatment replicates. The EO test solutions were applied through drip irrigation. In detail, 20 mL of test solution was applied on the pot surface around the plant stem, achieving final test concentrations of 30, 40, 50, 60, and 70 μL kg^{-1} soil. Experiments were performed at 27°C, 60% RH at 16 h photoperiod for 40 days. The pots containing the tomato plants were watered (20 mL) every 3 days. After 40 days of experiments, the tomato roots were washed. Afterwards, shoots were removed from the roots, and root staining was performed (Bybd et al., 1983) in order to count the number of *M. incognita* female individuals per g of root at 10× magnification control. The potential phytotoxicity on tomato plants was evaluated by registering the weight of fresh roots and stems. Each experiment was performed twice, and the treatments were conducted accordingly to a completely randomized design (CRD) with 5 replicates each.

Soil drip irrigation with the essential oil-nanoemulsion (EO-NE) to control *Meloidogyne incognita*

A pot experiment was performed using the above-mentioned soil and respecting the procedure described in the previous paragraph. The treatments were done by applying the EO-NE at the $EC_{50,}$ and EC_{50} x2 values found for the EO, calculating the EO percentage in the EO-NE. The control was represented by water and by the carrier used for the development of the formulations. The experiment was performed at 27°C, 60% R.H. at 16:8 h (L:D) photoperiod for 40 days. Each pot was watered as described in the previous paragraph. The evaluation of the results was performed in terms of *M. incognita* females per gram of root, fresh stems, and roots weight. Each experiment was conducted as described above.

Data analysis

Regarding the *in vitro* bioassays, the *M. incognita* natural death or paralysis was rectified and submitted to nonlinear regression analysis (Seefeldt et al., 1995). The EC_{50} value was calculated by the mean value of the 6 replicates for each concentration and also considering the immersion time. For the *in vivo* assay and the calculation of the EC_{50}, the results were presented as a reduction % of

females per g of root, corrected to the control using the Abbott's formula. Moreover, the EO-NE efficacy was expressed as a reduction (%) of females per g of root corrected to the control by the Abbott's formula. No differences were noticed between the two controls used for the experiment. The treatments of means were compared with Tukey's HSD test at $P \leq 0.05$.

5.6.2 Results and discussion

Preparation and characterization of *C. acaulis* EO-NE

NEs were prepared and characterized accordingly to the procedure described in Section 4.3.1.

Paralysis activity of the essential oil (EO) against *Meloidogyne incognita*

Regarding the assessment of the paralysis activity of *C. acaulis* EO, the J2 individuals were submerged in the EO test solutions, and the dose and time had a considerable effect, testing the concentrations between 15 and 250 µL L^{-1}. The EC_{50} was of 72.85 µL L^{-1} after 24 h, and it increased to 67.45 µL L^{-1} the day after (Table 16).

Table 16. EC_{50} (µL L^{-1}) values on *Meloidogyne incognita* calculated after immersion in *Carlina acaulis* essential oil (EO) test solutions.

EO	Exposure time	EC_{50} (µL L^{-1})	SE[a]	CI_{95}%
activity in the treatment well	1 day	72.85	9.59	53.012 - 92.689
	2 days	67.45	6.10	54.822 – 80.084

[a]SE, standard error.

Regarding the EO fumigant activity on *M. incognita* J2, substantial effects of the assayed dose and time were detected. In fact, a 51% nematicidal efficacy was calculated 48 h after J2 immersion in 250 µL L^{-1} of the EO (Table 17).

Table 17. Fumigant effect of J2s in test solutions of *Carlina acaulis* essential oil (EO) for 1 and 2 days, and J2 paralysis caused by fumigant activity at 125 and 250 µL L^{-1} after 1 and 2 days.

EO	Exposure time	J2 paralysis (125 µL L^{-1}) ± SD[a]	J2 paralysis (250 µL L^{-1}) ± SD
fumigant activity	1 day	18 ± 3.2	29 ± 6.3
	2 days	57 ± 5.0	51 ± 11

[a]SE, standard error.

The results obtained underline the significant nematicidal activity of *C. acaulis* EO against *M. incognita*, with the paralysis of J2 individuals and the arrest of their biological cycle into the roots

of the host plant. This efficacy is linked to the presence of carlina oxide, whose hypothetic mechanism of action is discussed in Section 1.5.4, but also to a coadjuvant action of the minor constituents of the EO. For instance, benzaldehyde has already been reported to be a nematicidal agent against this pest (Barros et al., 2019). This is the first study investigating the nematicidal activity of *C. acaulis* EO on *M. incognita*, on which the paralysis efficacy (EC_{50} of 67.45 µL L^{-1} after 24 h) resulted considerable. Other EOs have been tested on this pest. For instance, *Origanum dictamnus* L., *O. vulgare*, *Melissa officinalis* L., and *Mentha pulegium* L. displayed EC_{50} values of 1.72, 1.55, 6.15, and 3.15 µL mL^{-1} after 96 h, respectively (Ntalli et al., 2010). In other immersion tests, the EC_{50} values after 96 h were 231, 269, 807 and 1116 µg mL^{-1} for *F. vulgare*, *P. anisum*, *Eucalyptus melliodora* A.Cunn. ex Schauer, and *Pistacia terebinthus* L. EOs, respectively (Ntalli et al., 2011).

Efficacy of the essential oil (EO) against *Meloidogyne incognita*: dose-response pot bioassay

In the *in vivo* bioassay, an EC_{50} of 40 µL kg^{-1} (Table 18) and a dose-dependent effect were found. Regarding roots and fresh stems weights, no differences were detected (data not shown).

Table 18. Efficacy of drip irrigated *Carlina acaulis* essential oil (EO) on tomatoes infested by *Meloidogyne incognita*, calculated from female counts per g of root EC_{50} (µL kg^{-1}) values.

EC_{50} (µL kg^{-1}) (Abbott: females g^{-1} root)	**SE[a]**	**CI_{95}%**
40.31	1.52	37.17-43.46

[a]SE, standard error

The efficacy of *C. acaulis* EO in arresting the biological cycle of the pest inside the host (EC_{50} of 40 µL kg^{-1}) was similar to that of to the *Eucalyptus globulus* Labill. and *Pelargonium asperum* Willd., which significantly reduced the multiplication of the nematode and gall formation on tomato roots at 50, 100, and 200 µL kg^{-1} soil. Lower bioactivity was detected for *M. piperita*, *Eucalyptus citriodora* Hook., and *Ruta graveolens* L. EOs, which were suppressive at concentrations higher than 50 µL kg^{-1} soil (Laquale et al., 2015).

Efficacy of essential oil-nanoemulsion (EO-NE): *in vivo* pot bioassay

The EO-NE was assayed in *in vivo* bioassays at the test concentration representing the EC_{50} of the EO (40 µL kg^{-1}, EO-NE40) and the doubled EC_{50} value (80 µL kg^{-1}, EO-NE80). The test showed that there was a minor number of counted females per g of root with respect to the untreated control (Figure 51).

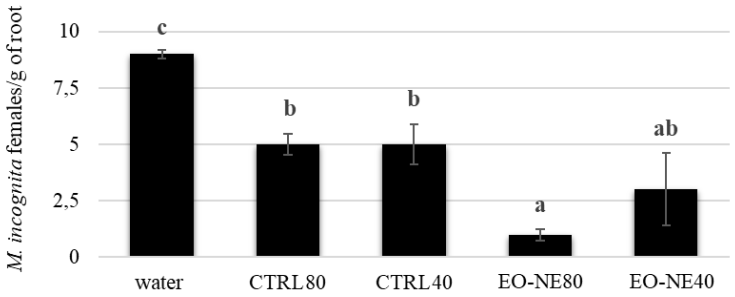

Figure 51. *Meloidogyne incognita* female counts per g of root, with standard errors, after treatment with the two concentrations of *Carlina acaulis* essential oil (EO)-nanoemulsion (EO-NE) (40 and 80 µL kg^{-1} of EO). The respective carrier concentrations and water control are also displayed.

After the treatment with the EO-NE40, 3 females per g of root were found (efficacy of 66%), while for the water control 9 females per g of root. Conversely, the EO at the concentration of 40 µL kg^{-1} showed an efficacy of 50%. Consequently, the EO formulated into the NE (6% of EO) presented an enhanced bioactivity. Also, for the carrier control a significant efficacy was noticed, if compared to that of water control, but this efficacy was lower than that of the EO-NE. No differences were found between the roots and fresh stems weights (data not shown).

Since the volatility, solubility, and instability of EOs play a crucial role on their effectiveness, *C. acaulis* EO-NE was developed and tested. The results obtained in this study suggest that the NE was able to increase the EO bioactivity against *M. incognita*. This enhanced efficacy could be linked to the small droplets size (Z-average values ≤ 140 nm) and narrow distribution (PDI ≤ 0.27) of the EO-NE, which could enhance the contact of the EO and its penetration inside the pest. Similarly, formulations of *C. acaulis* EO enhanced its larvicidal activity on *Cx. quinquefasciatus* and reduced the acute toxicity on rats (as displayed in Section 5.2.2). Other formulations containing natural products displayed similar nematicidal activities. For instance, silver nanoparticles of *Senna siamea* (Lam.) H.S.Irwin & Barneby leaf extract were active at 50 ppm against this pest being also able to enhance the host plant growth (Danish et al., 2021).

5.6.3 Conclusions

The results presented in this study demonstrate the efficacy and the potential of *C. acaulis* EO against *M. ingognita*. Moreover, the importance of the EO formulation was also displayed, being the NEs able to increase the EO bioactivity. However, further research is still needed for the assessment of these nanosystems' efficacy in the field conditions and also of the effects on soil micro-communities.

5.7. *Xylosandrus compactus*

Xylosandrus compactus (Eichhoff) (Figure 52) is a species native to Southeastern Asia but distributed in numerous continents (Urvois et al., 2022).

Figure 52. *Xylosandrus compactus*.

This pest affects more a large variety of plant species belonging to 62 different families (Greco and Wright, 2015). *X. compactus* raised serious concerns especially for its threat to coffee production in East and Central Africa (FAO, 2013).

For the management of this pest, there is a general lack of knowledge on effective and environmentally management tools. Among them, there is the 'push–pull' strategy, which combines a repellent agent to 'push' individuals away from the host plant and an attractant agent to 'pull' them into an artificial trap (Cook et al., 2007). In this study, the potential of *C. acaulis* EO-NEs as repellents was studied on *X. compactus* dispersing females in terms of the choice behaviour, host-colonization, and progeny production. Finally, the EO-NE was tested in a semi-field experiment with potted plants.

5.7.1 Materials and methods

Preparation and characterization of *Carlina acaulis* essential oil nanoemulsion (EO-NE)

The EO required for the final concentrations of 3, 1 or 0.5% (w/w) in the formulations was combined with ethyl oleate as cosolvent (ethyl oleate-EO weight ratio of 0.33). This mixture was added dropwise to a Tween 80 (Sigma-Aldrich) aqueous solution under high-speed stirring (Ultraturrax T25 basic, IKAfi Werke GmbH& Co.KG, Staufen, Germany) for 5 min at 9500 rpm. The weight ratio between Tween 80 and EO was maintained at 0.66 in all formulations. The characterization of the NEs was performed accordingly to the procedure described in Section 4.2.2.

In vitro choice bioassays

The evaluation of the effects of the treatment of laurel stems with the EO-NE on the choice behavior of *X. compactus* adult females was performed by means of dual choice bioassays. The individuals' choice between the stems treated with the EO-NE and with the control was evaluated inside experimental arenas consisting of plastic boxes (175 × 120 × 70 mm). The arenas were furnished with a window (135 × 80 mm) closed with a fine mesh net (0.25 × 0.25 mm) to promote ventilation but also to avoid escaping phenomena. Laurel stems (8-12 mm Ø) deriving from potted healthy plants (pot Ø = 15 cm) were sliced in sections 80 mm long, sealed with Parafilm® strips at the ends, left soaking in 10% ethanol for 2 h, and then dried for 30 min. These stems were then soaked into the EO-NEs for 10 s and then left drying for 30 min. These stems were then moved into the arenas. Control stems were soaked in the control NE, constituted only by ethyl oleate and Tween 80 in the same ratios of the EO-NE. For the assessment of *X. compactus* response, the control and EO-NE treated stems were placed in the opposite sides of the arena, at the center of which 5 adult females were placed. The choice between the two stems was considered if females dug the entry hole and was examined 24, 48, and 72 h after the beetle release. Three different concentrations of the EO into the NE were tested, namely 0.5, 1, and 3% (w/w), and 10 replicates were performed (EO 0.5% treated stems *vs* control; EO 1% treated stems *vs* control; EO 3% treated stems *vs* control). The arenas were maintained in darkness at 25 ± 1 °C and 60 ± 10% R.H. No beetle choice was recorded if the females were not found boring entry holes in one of the two stems after 72 h, and they were not considered for further data analysis. The EO was tested initially at the highest concentration, i.e., 3%.

In vitro no-choice bioassays

The EO-NE impact on *X. compactus* survival and host-colonization was estimated by means of no-choice bioassays with the same experimental protocols of the previous paragraph. This test differs from the previous for the use of two stems both treated with the same EO-NE concentration or control. In addition, the check of beetles' survival was also performed 48 h after their release. As previously described, the EO was tested at 3 different concentrations into the NE (0.5, 1, and 3% w/w). In detail, 10 replicates (arenas) were conducted and 50 beetle females for each tested EO concentration of the different EO-NEs and 50 beetle females for each of the relative control NEs were used.

Essential oil-nanoemulsion (EO-NE) impact on beetle progeny production

The potential impact of EO-NE on *X. compactus* reproduction was analyzed allowing adult females to infest stems treated with the EO-NE under similar conditions to the laboratory rearing. In detail,

EO-NE treated stems were managed as above and were individually disposed into glass tubes (open at both ends) and a single female individual per replicate was released. The glass tube ends were closed with a fine mesh net (0.25 × 0.25 mm) to promote the ventilation and prevent escaping phenomena. Tubes were maintained in darkness at 25 ± 2 °C and 60 ± 10% R.H. for 4 weeks. After 28 days, stems were dissected and examined through a stereomicroscope. For each infested stem section, the brood size, as the total number of offspring produced per female, was recorded. These tests were performed using the highest EO concentration (i.e., 3%). In detail, 43 replicates were performed for the EO-NE and 43 replicates for the control NE. Beetle females that had not created entry holes or found dead were not considered for data analysis.

Choice bioassays *in semi-field* conditions

A bioassay *in semi-field* conditions was performed for the confirmation of the potential repellency of *C. acaulis* EO-NE at the concentration of 3%. For the bioassay, pesticide-free potted laurel plants (pot Ø = 15 cm) two years old and 1.20-1.40 m tall were used. Two days after the imposed flooding, plants were separated in groups and sprayed with EO-NEs solutions from a distance of 0.3 m through a hand sprayer (50 mL). Then they were left drying under semi-field conditions for 2 h before being used in bioassay. Control plants were sprayed with control NE. The EO-NE treated plant and the control treated plant were placed oppositely in the net cages (75 × 55 × 150 cm) and 20 females individuals were released into the center of each cage. Net cages were placed 2 m from each other and *X. compactus* choice between one of the two laurel plants (EO-NE treated vs control) was evaluated after 48 h. In detail, stems of both laurel plants were dissected, and the entry holes were counted in each stem. For each of the tested combinations (*C. acaulis* EO 3% treated plants *vs* control), 8 replicates (cages) were performed. Beetle females that had not created entry holes or found dead were not considered for data analysis.

Data analysis

Chi-squared goodness-of fit test was used for the analysis of the number of *X. compactus* choices in bioassays on the beetle choice behavior for the assessment of the beetle response to different sources. EO effects on the survival of the individuals, host colonization, and progeny production were analyzed using one-way ANOVA followed by Tukey's HSD post hoc tests ($p \leq 0.05$) for multiple mean comparisons among treatments. Regarding the choice bioassays in semi-field, the non-parametric Kruskal-Wallis test ($p < 0.05$) was employed to compare the treatments since the obtained data did not fulfil the assumptions for analysis of variance. SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for the performance of statistical analyses.

5.7.2 Results and discussion

Preparation and characterization of *C. acaulis* EO-NE

The results obtained from NEs preparation and characterization were in accordance to those described in Section 4.3.1.

In vitro choice bioassays

X. compactus females showed a marked preference for control laurel stem sections in confront with those treated with EO-NE (Figure 53).

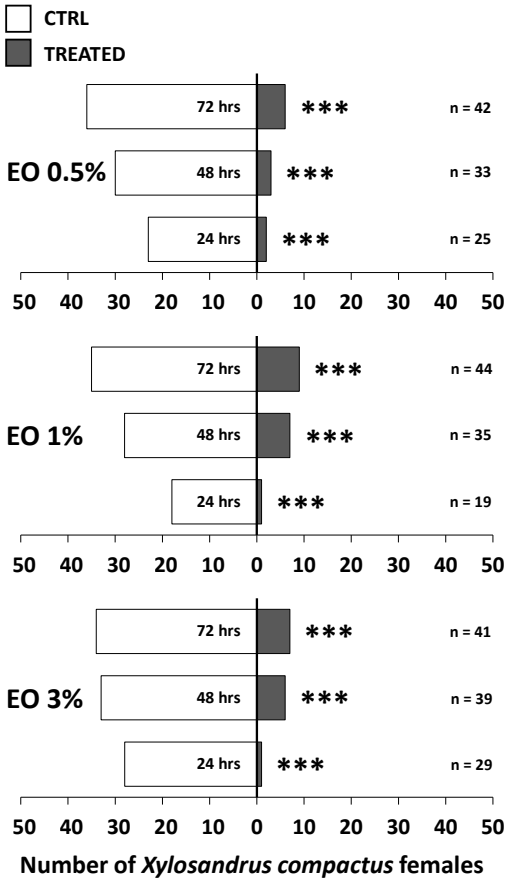

Figure 53. Number of *Xylosandrus compactus* females choosing laurel stem sections untreated (ctrl) or treated with increasing concentrations of *Carlina acaulis* essential oil (EO), 24, 48, and 72 h after being released in the experimental arena. Asterisks specify significant differences (likelihood chi-squared test): *** = $p < 0.001$; ns, not significant. N, number of beetles choosing a source.

A significant repellent activity was observed in all choice bioassays performed with *C. acaulis* EO-NE treated stems at all the concentrations assayed: 0.5% (24 hrs: $\chi^2 = 17.64$, $p < 0.001$; 48 hrs: $\chi^2 = 22.09$, $p < 0.001$; 72 hrs: $\chi^2 = 21.43$, $p < 0.001$), 1% (24 hrs: $\chi^2 = 15.21$, $p < 0.001$; 48 hrs: $\chi^2 =$

12.60, $p < 0.001$; 72 hrs: $\chi^2 = 15.36$, $p < 0.001$), and 3% (24 hrs: $\chi^2 = 25.14$, $p < 0.001$; 48 hrs: $\chi^2 = 18.69$, $p < 0.001$; 72 hrs: $\chi^2 = 17.78$, $p < 0.001$).

Choice bioassays conducted with laurel stems treated with *C. acaulis* EO-NE compared with untreated ones displayed a strong repellence for *X. compactus* females. To the best of our knowledge, this is the first study that evaluates *C. acaulis* EO-NE impact against an ambrosia beetle. Even if the main goal of this study was the evaluation of the EO-NE influence on choice behavior, the achieved results also pointed out a possible contact toxicity effects of this EO on *X. compactus*. This repellent effect was also detected for *R. officinalis* cultivar verbenone, which showed similar repellent effects in the same study.

In vitro no-choice bioassays

C. acaulis EO-NE treatment led to a significant reduction of *X. compactus* survival, with a mortality rate (average ± S.E.) of 38.00 ± 5.27% (48 h after treatment), when tested at a concentration of 3% ($F_{3,36} = 12.67$; $P < 0.001$), compared with control stems (6.00 ± 3.06%). On the contrary, no significant differences in terms of obtained cumulative mortality (48 h after treatment) were found between control stems and the EO-NE.

The treatment also showed a significant effect in terms of the percentage of beetle females boring galleries (Figure 54).

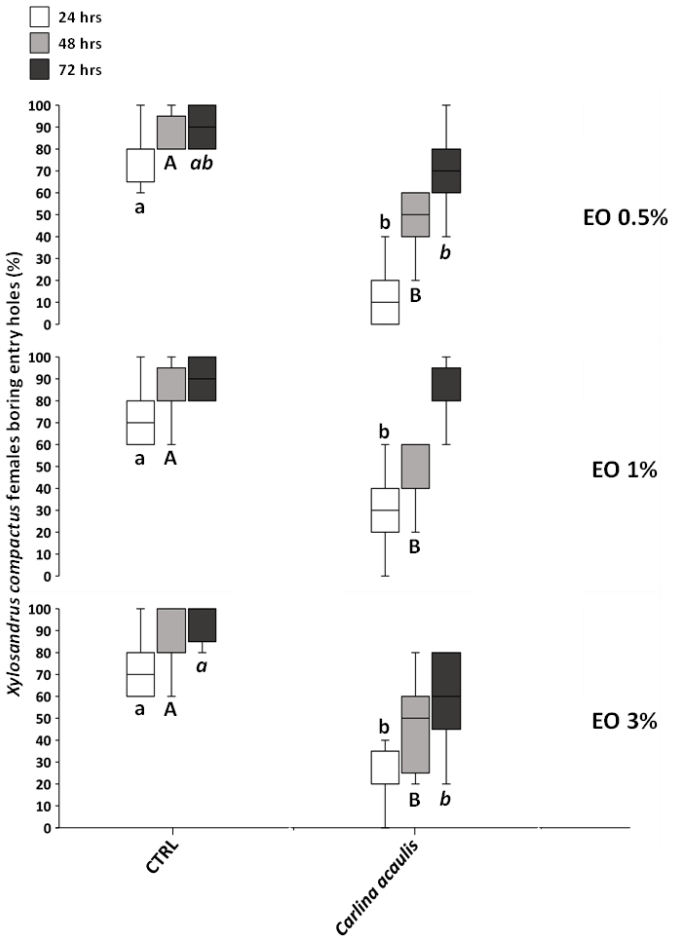

Figure 54. Box-plot representations of the impact of the essential oil-nanoemulsion (EO-NE) at different EO concentrations (i.e., 0.5%, 1% and 3%) on *Xylosandrus compactus* host colonization (percentages of beetles boring entry holes) after 24, 48, and 72 h of exposure to treated laurel stems. Within the same exposure time and concentration, means (±SE, standard error) with different letters are significantly different according to Tukey's HSD post hoc test at $p \leq 0.05$.

In detail, a lower number of female individuals dug entry holes in laurel stems treated with the EO-NE compared with stems treated with control both 24 h (EO-NE 0.5%: $F_{3,36} = 25.66$; $p < 0.001$; EO-NE 1%: $F_{3,36} = 14.73$; $p < 0.001$; EO-NE 3%: $F_{3,36} = 11.74$; $p < 0.001$) and 48 h (EO-NE 0.5%: $F_{3,36} = 10.59$; $p < 0.001$; EO-NE 1%: $F_{3,36} = 10.02$; $p < 0.001$; EO-NE 3%: $F_{3,36} = 9.82$; $p < 0.001$) after treatment. This trend was also confirmed at the 72 h observation time interval only at the EO-NE with concentrations of EO 0.5% and 3%.

Since only the EO-NE containing the 3% of the EO displayed high levels of mortality of female individuals (40%), and no sublethal effects were noticed, further studies investigating more EO concentrations in the NE should be performed.

Essential oil (EO) impact on beetle progeny production

A total of 72.1 and 53.49% of the tested *X. compactus* females bored entry holes in control stems or *C. acaulis* EO-NE, respectively. No significant differences were found between the brood size

produced by female individuals that survived to EO-NE treatment and those of the control. The number of offspring produced per *X. compactus* females was 37.3 ± 2.5 for control stems and 36.5 ± 2.3 for EO-NE treated stems, respectively.

Choice bioassays *in semi-field* conditions

In the *semi-field* assays, *X. compactus* females showed a marked preference for untreated (control) laurel plants rather than EO-NE treated plants (Figure 49). In detail, the number of females infesting plants was 6.13 ± 2.23 and 2.13 ± 1.25 for untreated and treated plants, respectively (Figure 55).

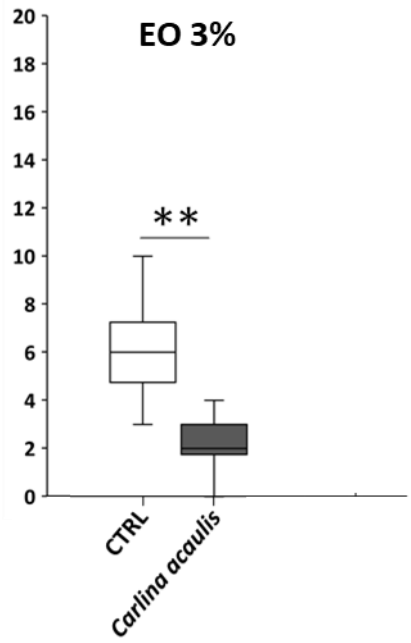

Figure 55. Box plot showing the impact of the essential oil-nanoemulsion (EO-NE) on *Xylosandrus compactus* choice behavior. The number of beetle individuals boring entry holes (preference) in laurel plants treated with EO-NE (*Carlina acaulis*) or untreated control

5.7.3 Conclusions

The results obtained in this study provided new insights for the employment of EOs as repellent agents of the invasive ambrosia beetle *X. compactus*. In detail, this study on *C. acaulis* EO-NE on *X. compactus* was accompanied by the evaluation of the bioactivity of other EOs from *R. officinalis* cv verbenone, *Laurus nobilis* L., *Cupressus sempervirens* L., and *Betula alba* L. (data not shown). Between all the EO-NEs tested, only that from *R. officinalis* showed a comparable efficacy to that of *C. acaulis* EO-NE. This underlines the promising potential of *C. acaulis* for the management of agricultural pests. In addition, the findings reported give rise to new perspectives on the use of

these natural products in the management strategies that aim at altering the beetle orientation behavior. Though, more studies should be performed to confirm the efficacy of *C. acaulis* EO-NE in in field conditions. Moreover, future research should also focus on the employment of this EO in 'push-pull' strategies, since it was demonstrated the reduction of the number of individuals infesting susceptible laurel stems without a complete prevention of attacks to the host plants.

5.8. *Tetranychus urticae*

The two-spotted spider mite (TSSM) *Tetranychus urticae* Koch (Acariformes, Tetranychidae) (Figure 56) is a pest affecting several crops worldwide in open fields and greenhouses (Attia et al., 2013; Knapp et al., 2006; Pascual-Ruiz et al., 2013; Perring et al., 1987).

Figure 56. *Tetranychus urticae*.

Even if *Phytoseiulus persimilis* Athias-Henriot and *Neoseiulus californicus* (McGregor) have been successfully employed for management programs of TSSM infestations (Abad-Moyano et al., 2010; Easterbrook et al., 2001; Greco et al., 1999), biocontrol is often not successful on some crops (Cédola et al., 2001; Kennedy, 2003; Nihoul, 1992; van Lenteren and Woets, 1988), and farmers are often obliged to the use of synthetic acaricides of the second and third generation. However, the onset of resistance and non-target negative consequences led to the search of alternative products also of natural origin (Ibrahim and Yee, 2000). The present work focused on the evaluation of *C. acaulis* EO effects on *T. urticae* eggs, nymphs, and adults, with the adoption also of a demographic approach (Tsolakis and Ragusa, 2008; Venzon et al., 2005; Walthal and Stark, 1997). Moreover, since the evaluation of non-target effects is essential for the employment of botanical products, the toxicity of *C. acaulis* EO was assayed on *N. californicus* females.

5.8.1 Materials and methods

Mite species rearing

T. urticae derived from weeds and *Solanum melongena* L., near Palermo, Italy (38° 1'41.49"N 13° 1'55.61"E, June 2020) and were maintained in laboratory cultures on potted bean plants (*Phaseolus vulgaris* L.). *N. californicus* derived from strawberries infested by *T. urticae* at Partinico (Palermo) (38° 4'22.21'' N 13° 6' 12.27''E, December 2018) and was raised in plexiglass arenas. The food for *N. californicus* consisted of various stages of *T. urticae* and a mixture of pollens (*Carpobrotus edulis* (L.) N.E.Br., *Oxalis pes-caprae* L., *Typha latifolia* L.). The two rearings were kept in a conditioned room at a temperature of 25 ± 1 °C, RH of 70 ± 5%, and 16:8 L:D. Both rearings were renewed or supplemented with field-collected specimens.

Toxicity of *Carlina acaulis* essential oil (EO) on *Tetranychus urticae*

Assays were performed on eggs, nymphs, and adults of *T. urticae* and *C. acaulis* EO was employed at the following concentrations in acetone: 312.5, 625, 1250, 2500, and 5000 ppm. The experimental units (EUs) were constituted by a bean leaf disk (Ø 3 cm) with the abaxial surface up, put on wet cotton saturated every day with distilled water, in a Petri dish (Ø 100mm, h 10 mm). The solution (8 mL) was sprayed using the Potter Precision Spray Tower (Potter, 1952), at 1 PSI (6.89 kPa) of pressure on each EU. Control was constituted by only acetone.

Toxicity assays on *T. urticae* were conducted accordingly to the procedure of Tsolakis and Ragusa (2008). To obtain a cohort of TSSM eggs, 3 females were put on each EU, allowed to lay eggs for 24 h and then removed. Ten eggs/EU were kept for treatments. Replication of 30 times was performed for each test and ended after 7 days (when more than of 95% of eggs hatched in the control). This procedure was used for the obtaining of coetaneous nymphs. Each toxicity test was performed in 5 replicates on 5 nymphs/EU and the test lasted 4 days.

For the obtaining of a cohort of females of the same age, 50 females were transferred with a fine brush (4/0) on the abaxial surface of 5 bean leaves (10 females/leaf), allowed to lay eggs for 24 h and then removed. The mites obtained from the eggs were reared until reaching adulthood. Then 60 young females (max 24 h old) were transferred on the EUs (one female/EU + one male) to guarantee mating. After 48 h males were removed, and fertilized females were employed for the assays. Each test was repeated 60 times and ended after 4 days. Nymphs and adult females' mortality (M) was registered every day. In addition, the number of eggs/female/day was registered over the test period. A measure of the EO's effects on reproduction (R) was obtained with the quotient of the total eggs/female for treated and untreated. Thus, the total toxic effect (E) of *C. acaulis* EO was calculated using the formula E = 100% - (100% - M) x R (Overmeer and van Zon 1982). Four categories of toxicity were applied in this study: 1 = no toxic (< 25% of mortality), 2 =

slightly toxic (25 – 50%), 3 = moderately toxic (51 – 75%), 4 = very toxic (> 75%). For *N. californicus* the following categories of toxicity were applied: 1 = harmless (< 30% of mortality), 2 = slightly harmful (30-79%), 3 = moderately harmful (80-99%), 4 = harmful (>99%).

Effect of *Carlina acaulis* essential oil (EO) on *Tetranychus urticae* population growth rate

For the determination of *C. acaulis* EO effect on the populations growth of *T. urticae* the instantaneous rate of increase (ri) was calculated. This index measures the population increase or decrease and is calculated by adopting the following equation:

$$r_i = \frac{\ln\left(\frac{Nf}{No}\right)}{\Delta t},$$

where *Nf* indicates the final number of mites, *No* is the initial number of mites and *Δt* denotes the time the experiment lasted. Moreover, r_i is a rate of population increase, like that obtained with the intrinsic rate of increase (rm) (Walthall and Stark, 1997). Positive values of r_i show a growing population, $r_i = 0$ indicates a stable population, while negative values of r_i indicate a declining population directed toward extinction (Walthall and Stark, 1997). The instantaneous rate of increase was calculated after 4 days.

Side effects of *Carlina acaulis* essential oil (EO) on *Neoseiulus californicus*

Young females of *N. californicus* were obtained as follows: 100 eggs were moved to a new arena, provided with abundant mixture of pollens until the reaching of adulthood. Newly emerged males and females were moved with a fine brush (4/0) into a new arena for 48 h to provide mating (sex ratio females:males 3:1). Then, one female was transferred on each EU for subsequent assays.

Four different tests were performed on *N. californicus* females: a) sprayings, made directly on the EUs containing each 1 female (30 replications); b) spraying on leaf disc, where the female was placed 4 min after spraying when the leaf disk was dry (15 replications); c) spraying on a female which was then moved on untreated leaf disc (30 replications); d) spraying on leaf disc, where the female was placed 48 h after (15 replications). For the above-mentioned assays, 5000 ppm concentration of EO was employed, since it was the only one that caused a mortality of 95% on *T. urticae* females. Once the sprayed surface of the leaf disk was dry, food constituted by pollen grains was furnished. The assays lasted 3 days and were performed at a temperature 25 ± 1°C, R.H. of 70 ± 5%, and photoperiod of 16:8 (L:D).

N. californicus fresh eggs employed for toxicity tests were obtained as follows. Firstly, 50 females from breedings were put on a new arena and allowed to lay eggs for 24 h. Then, 1 egg was

transferred with a fine brush (4/0) on each EU and sprayed with *C. acaulis* EO at 1250 ppm, which was the concentration leading to 95% mortality on *T. urticae* eggs. Each test was repeated 30 times and ended after 3 days.

The mortality of the eggs has been considered when more than 95% of eggs hatched in the control (after 3 days). The mortality of motile stages was registered every day during the test period. Natural and induced mortalities were considered, while if mites were found drowned in the wet cotton wool surrounding the leaf disc, this was ascribed to the repellent effect of the EO.

Y-tube olfactometer bioassays on *Neoseiulus californicus* adults

Y-tube apparatus

Attraction or repellency caused by *C. acaulis* EO on adult females of *N. californicus* was evaluated through two-choice tests conducted in a Y-tube olfactometer, following the method by Fonseca et al. (2020) with some changes. In detail, two behavioral assays were performed: (a) preliminary test on filter paper (50 replicates/dose); (b) spraying on a bean leaf disc and performing the test 15 min and 48 h after spraying (30 replicates/dose). The two highest doses tested on *T. urticae* females (2500 and 5000 ppm) were assayed. All the bioassays were carried out at a temperature of 24 ± 1 °C and R.H. of 70 ± 10%.

Briefly, the system was constituted by a Plexiglas unit (200 × 190 × 15 mm) with a central tube (90 mm × 15 mm) and two lateral arms (75 mm × 15 mm), which were connected to a Drechsel bottle (250 mL) enclosing the odor source. A Teflon connection provided humidified (70 ± 10%) and purified air at 2 mL min^{-1} was provided for both behavioral assays. The unit was closed with a glass panel and the olfactometer was placed horizontally, at about 80 cm from the ground. A vertically hanging red led light (12 W, 1050 lm) above the olfactometer unit (height 60 cm) provided illumination. In both behavioral assays, the filter paper or the bean leaf were wet with 10 µL of the EO and the respective control. To allow the detection of any possible symmetry, every 5 females the arena was flipped to 180°, cleaned with *n*-hexane, rinsed with warm water at 35-40 °C, then immersed in a water bath with mild soap for about 5 min, washed with hot water, and finally rinsed with distilled water at room temperature (Carpita et al., 2012). Afterwards, the substances were renewed.

Preliminary bioassay

For the preliminary bioassay, two concentrations (2500 and 5000 ppm) of *C. acaulis* EO were formulated with Tween 80 (3%) (1:1) and distilled water, and then applied on a filter paper (20 x 20 mm, Whatman 1). Also, negative controls, as the solution without the EO, were prepared. The females of *N. californicus* were introduced separately in the main arm of the olfactometer, and to

every female it was permitted to choose reaching the end of one of the two arms. After the choice, the female was removed for the introduction of a new female. Each observation lasted 5 minutes and if no movement was detected within 3 min the females were removed and scored as no choice occurred. A total of 50 adult females of *N. californicus* were tested for both EO concentrations.

Carlina acaulis essential oil (EO) bioactivity throughout the time

The repellent or attractive effect of *C. acaulis* EO was also evaluated throughout the time. The Y-tube bioassay was performed also in this case, according to the procedure described in the previous paragraph. Fresh bean leaf discs (Ø = 20 mm) were employed for the evaluation of the attractive or repellent activity of *C. acaulis* EO towards *N. californicus* females throughout time. In detail, 10 µL of EO solutions and the respective negative control were sprayed on leaf discs through an airbrush (Badger Air Brush 200-9-GFX, Chicago, USA), and they were left drying to the air for 1 h. The bioactivity of the two doses was evaluated at two timepoints: 0 h (t0) and 48 h (t1) from the spraying treatment. At the end of the first bioassay, leaf discs were maintained in the fridge for 48 h. A total of 30 adult females of *N. californicus* were assayed for the EO doses.

Statistical analyses

All data on mortality for *T. urticae* and *N. californicus*, mean oviposition rate and mean hatching time of *T. urticae* eggs and repellence towards *N. californicus* females were converted through an arcsine-square-root equation prior to General Linear Model (GLM) analysis. Mortality data were previously corrected using Abbott's formula (Abbott, 1925). If significant differences were detected, Tukey's studentized range honest significant differences (HSD) test ($p = 0.05$) was used for the separation of the means. The Jackknife method was used to create pseudo values (Meyer et al., 1986) for the instantaneous rate of increase data. On these values, goodness-of-fit tests to ascertain the normality of distribution, and Analyses of Variance followed by t-Student tests, were carried out. Differences were judged as significant when 95% of fiducial limits were not overlapping. Lethal concentrations necessary for a 10% (LC_{10}), 30% (LC_{30}), 50% (LC_{50}), and 90% (LC_{90}) of mortality were estimated using the probit model implemented in the Minitab program, adopting a 95% confidence level. Minitab 17.0 software (Minitab Inc., State College, PA, USA) was used for the computation of all analyses.

Concerning behavioral assays, a contingency analysis was performed for the analysis of the biological activity of *C. acaulis* EO towards *N. californicus* adults. If Pearson χ^2 test was significant, a residual analysis was also performed, for the determination of which category majorly contributed to rejecting the null hypothesis. All statistical analyses on behavioral tests were performed using RStudio software; $p = 0.05$ was set as the threshold.

5.8.2 Results and discussion

Toxicity of *Carlina acaulis* essential oil (EO) on *Tetranychus urticae*

From the GLM analysis conducted on survival rates of TSSM, it emerged that survival rates of all motile stages at the end of the experiments were different depending on the various concentrations tested ($F_{5, 528} = 58.44$; $p < 0.001$) and the two ontogenetic stages ($F_{1,528} = 13.74$; $p < 0.001$). Moreover, no interactions between dose and stage were recorded ($F_{5,528} = 0.63$; $p = 0.677$), suggesting that each dose exerted the same action on females and nymphs (Table 19).

The highest dose tested (5000 ppm) led to complete mortality of all female individuals during the first 2 days, while the dose of 2500 ppm led to similar mortality values after 4 days. The other concentrations tested caused less toxic effects, with increasing mortality over time. Tukey pairwise tests indicated three main groups, considering the two stages together: a) control, b) 625 and 1250 ppm and c) 2500 and 5000 ppm. The dose of 312.5 ppm resulted similar to both the first and second group ($p < 0.05$). All the dead motile stages were detected on the leaf disc, and no mites were noticed on the wet cotton wool surrounding the disc leaf. This underlined that there was no repellent effect by the EO on *T. urticae*.

Table 19. Susceptibility of females and nymphs of *Tetranychus urticae* to different concentrations of *Carlina acaulis* essential oil (EO).

Ontogenetic stage (n. tested)	Concentrations tested (ppm)	Survival/day (in % mean ± SE)				Mean Survival Time (days)	Mortality at the end of tests	Class
		1	2	3	4	Mean±SE	(%)	
Females	Control	100.0 ± 0.0	98.3 ± 1.67	96.7 ± 2.34	85.0 ± 4.65	3.80 ± 0.070^{a*}	15.0 ± 4.65^{a}	1
(n = 60)	312.5	90.0 ± 3.91	80.0 ± 5.21	75.0 ± 5.64	68.3 ± 6.06	3.13 ± 0.185^{abc}	31.7 ± 6.06^{a}	2
	625	86.7 ± 4.43	81.7 ± 5.04	68.3 ± 6.06	48.3 ± 6.51	2.85 ± 0.184^{bc}	51.7 ± 6.51^{ab}	2
	1250	83.3 ± 4.85	75.0 ± 5.64	56.7 ± 6.45	48.3 ± 6.51	2.63 ± 0.200^{c}	51.7 ± 6.51^{ab}	2
	2500	61.7 ± 6.33	26.7 ± 5.76	11.7 ± 4.18	10.0 ± 3.90	1.10 ± 0.159^{d}	90.0 ± 3.91^{cd}	3
	5000	38.3 ± 6.33	0.0	0.0	0.0	0.38 ± 0.063^{d}	100.0 ± 0.0^{d}	4
Nymphs	Control	100.0 ± 0.0	96.7 ± 3.33	90.0 ± 4.47	90.0 ± 4.47	3.78 ± 0.133^{ab}	10.0 ± 4.47^{a}	1
(n = 30)	312.5	100.0 ± 0.0	100.0 ± 0.0	93.3 ± 4.22	86.7 ± 6.67	3.80 ± 0.101^{ab}	13.3 ± 6.67^{ab}	1
	625	93.3 ± 4.22	90.0 ± 4.47	76.7 ± 8.03	73.3 ± 6.67	3.33 ± 0.216^{abc}	26.7 ± 6.67^{bc}	1
	1250	80.0 ± 5.16	73.3 ± 9.89	66.7 ± 13.3	66.7 ± 13.3	2.90 ± 0.301^{abc}	33.3 ± 13.3^{bc}	2
	2500	46.7 ± 11.2	36.7 ± 8.03	26.7 ± 6.67	16.7 ± 3.33	1.27 ± 0.291^{d}	83.3 ± 3.33^{d}	3
	5000	16.7 ± 6.15	16.7 ± 6.15	10.0 ± 4.47	10.0 ± 4.47	0.53 ± 0.234^{d}	90.0 ± 4.47^{d}	3

*Within a column, different letters show significant differences (ANOVA followed by Tukey pairwise comparisons $p < 0.05$).

The GLM analysis conducted on mean survival time displayed significant differences for the trend of survival depending on the EO doses ($F_{15,1439}$= 3.5; p <0.001), which is the TSSM death that occurred at different timepoints regardless of the effect registered for each dose at the end of the experiment.

Concerning the mean survival time of motile stages, the GLM analysis demonstrated that the survival time differed significantly between the two stages ($F_{1,539}$ = 6.62; p < 0.001) and among the EO doses ($F_{5,539}$ = 97.76; p < 0.001), but no interaction doses x stage was detected ($F_{5,539}$ = 0.88; p = 0.497). According to Tukey pairwise comparisons, no significant effects of the lowest EO dose (312.5 ppm) on females and of the lower three EO doses (312.5, 625, and 1250 ppm) on nymphs were detected if compared with control. Nevertheless, the two higher EO doses (2500 and 5000 ppm) significantly lowered the mean survival time of both motile stages (Table 19). The higher EO dose (5000 ppm) displayed very toxic effects (class 4) after 4 days on females but moderately toxic effects (class 3) on juvenils of *T. urticae*. Conversely, the two lowest doses (625 and 312.5 ppm) showed no toxicity for juveniles but slight toxicity for *T. urticae* females.

Moreover, for the different doses of the EO it was also noticed a secondary effect on the oviposition performance of treated TSSM females (Figure 57).

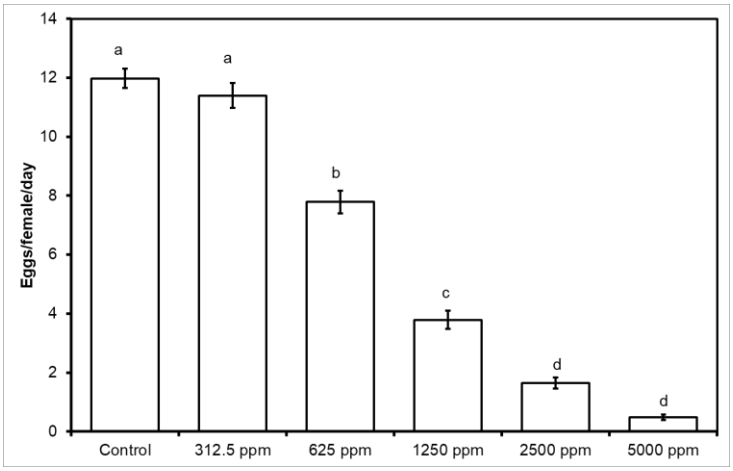

Figure 57. Rate (mean ± SE) of oviposition of *Tetranychus urticae* exposed to different concentrations of *Carlina acaulis* essential oil (EO). Above each column different letters suggest significant differences, GLM followed by HSD Tukey test, p < 0.05.

In fact, all the doses assayed, with the exception of the lowest one (312.5 ppm), significantly lowered the daily oviposition rate of the mite ($F_{5,872}$ = 105.63; p < 0.001). This effect obviously influenced the total toxic effect (E) of the various concentrations (Figure 58).

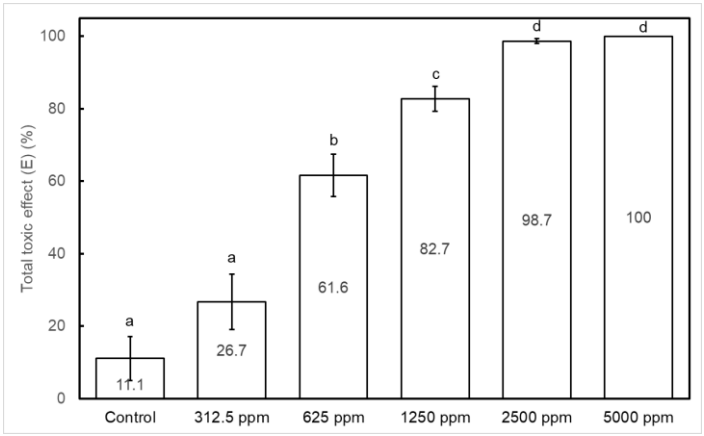

Figure 58. Total toxic effect (E) (mortality and reduction of fertility) of *Carlina acaulis* essential oil (EO) on *Tetranychus urticae* female individuals. Different letters above each column suggest significant differences, GLM followed by HSD Tukey test, $p < 0.05$.

Regarding the effects on eggs, all the EO doses tested led to marked negative effects towards TSSM eggs ($F_{5,179} = 455.0$; $p < 0.001$) (Table 20), while slight toxicity on eggs was detected with the dose of 312.5 ppm (22% of mortality). The other EO doses led to a mortality ranging from 83.7 to 100% (Table 20). In addition, the mean hatching time was significantly increased by the increasing EO doses tested ($F_{4,609} = 57.83$; $p < 0.001$) (Table 20).

Regarding the lethal concentration (LC) of the EO causing a 50% of mortality (LC_{50}), it has been computed by Probit analysis for all the ontogenetic stages of *T. urticae* assayed in this work. Egg mortality does not fit a linear model, while mortalities of females and nymphs fit with both the normal and Weibull distributions. The latter was adopted for the definition of the percentiles for the two motile stages, while the normal distribution for eggs. LC_{50} obtained for *T. urticae* nymphs and females was 1825.0 and 1145.1 ppm, respectively (Table 21), demonstrating the higher susceptibility of females in confront with nymphs ($z = -2.53$, $p = 0.012$). Concerning the eggs, the lethal concentrations reported in Table 21 must be considered indicative, due to the lack of fit to the linear model of the Probit analysis ($\chi^2 = 84.0$, $p < 0.001$).

Table 20. Susceptibility of *Tetranychus urticae* eggs to different concentrations of *Carlina acaulis* essential oil (EO).

EO concentration (ppm)	Cumulative percentage of hatched eggs (mean ± SE) Elapsed days after egg laying				Hatching time (days) (mean ± SE)	Mortality (%)
	4	5	6	7		
Control	93.7 ± 1.55	97.7 ± 0.92	98.3 ± 0.84	100.0 ± 0.0	4.10 ± 0.026^{a*}	0.0^{a}
312.5	55.7 ± 4.69	71.3 ± 3.77	76.7 ± 3.44	78.0 ± 2.89	4.39 ± 0.044^{b}	22.0 ± 2.89^{b}
625	2.7 ± 1.43	13.3 ± 2.81	16.3 ± 3.13	16.3 ± 3.13	5.02 ± 0.085^{c}	83.7 ± 3.13^{c}
1250	0.0	2.0 ± 0.88	4.3 ± 1.14	4.3 ± 1.14	5.60 ± 0.131^{d}	95.7 ± 1.14^{d}
2500	0.0	1.0 ± 0.56	2.3 ± 0.92	2.3 ± 0.92	5.57 ± 0.202^{cd}	97.7 ± 0.92^{de}
5000	0.0	0.0	0.0	0.0	0.0^{e}	100.0 ± 0.0^{e}

*Different letters within a column show significant differences (one-way ANOVA followed by Tukey pairwise comparisons $p < 0.05$); 300 eggs were tested for each essential oil (EO) concentration.

Table 21. Lethal concentrations (LC) of *Carlina acaulis* essential oil (EO) against different ontogenetic stages of *Tetranychus urticae*.

Ontogenetic stages	LC_{10} ppm (95% CI)	LC_{30} ppm (95% CI)	LC_{50} ppm (95% CI)	LC_{90} ppm (95% CI)	LC_{95} ppm (95% CI)	Intercept ± SE	Slope ± SE	χ^2 (d.f.)
Females	181.5	560.7	1145.1	5137.4	7434.5	6.21436±0.601	3.2447±0.045	7.85 (7) $p = 0.346$
	(116.4-254.0)	(415.5-708.4)	(918.1-1395.1)	(3880.2-7516.7)	(5330.9-11893.5)			
Nymphs	256.1	852.3	1825.0	9042.3	13409.4		3.4594±0.075	
	(162.2-366.8)	(604.5-1152.1)	(1333.6-2520.9)	(5848.0-16332.0)	(8195.0-26771.3)			
Eggs	196.2	313.8	434.3	961.3	1204.2	-9.79632±0.560	3.7138±0.203	84.03(3)
	(170.8-220.1)	(286.1-339.6)	(405.5-462.6)	(887.2-1055.6)	(1093.5-1351.0)			$p < 0.001$

C. acaulis EO displayed a higher toxicity against females rather than nymphs of *T. urticae* (LC_{50} values of 1145.1 and 1825 ppm for females and nymphs, respectively). However, the influence on the mean survival time resulted the same on both the ontogenetic stages mentioned above. Regarding the toxic effects on *T. urticae* females, other Compositae plant species have been studied. For instance, Chiasson et al. (2001) indicated lethal effects on females with EOs extracted from *T. vulgare* and *A. absinthium* (95.6 and 92.8% of mortality, respectively). Though, it is important to underline that the concentrations used for the two EOs were 16-fold higher (80,000 ppm) than the highest one (5,000 ppm) used in our study. Moreover, *C. acaulis* EO displayed 26-fold higher toxicity if compared to *Lippia gracilis* Schauer EO, 5-fold more toxic than the synthetic acaricide fenpyroxymate and 34-fold more toxic than Azadirachtin (Born et al., 2018). In addition to the toxic effects, a negative effect on the daily oviposition rate was also noticed by all the concentrations tested, with the exception of the lowest one. A marked reduction of the oviposition rate for the tetranychid females has also been reported for aqueous and acetonic extracts obtained from *A. absinthium* (Tsolakis and Ragusa, 2004), and for EOs of *Piper aduncum* L., *Melaleuca leucadendra* L., and *Schinus terebinthifolius* Raddi (Araújo et al., 2020).

Effect of *Carlina acaulis* essential oil (EO) on the growth rate *Tetranychus urticae* population

The various EO doses significantly affected the instantaneous rate of population growth ($F_{5,359}$ = 752.69; $p < 0.001$). In detail, the growth rate determined for the lowest EO dose (312 ppm) was not different from that of the control (r_i = 0.847 and 0.955, respectively). A growth index was achieved also with the 625 ppm concentration (r_i =0.343), even if it resulted statistically different from the lowest one. Conversely, negative values of growth rate were obtained with the other concentrations (r_i =-0.202, -0.775, and -0.991 for the 1250, 2500, and 5000 ppm, respectively), displaying a decreasing trend of the population (Figure 59).

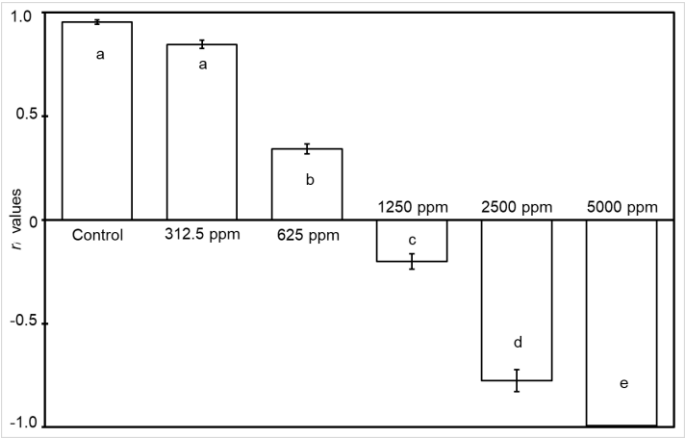

Figure 59. Instantaneous rate of increase values calculated for different doses of *Carlina acaulis* essential oil (EO). Different letters inside each column indicate significant differences among tests (GLM followed by Tukey for $p < 0.05$).

Over time, the negative effect of *C. acaulis* EO on females of *T. urticae* also influenced the population growth rate. The negative values recorded for the three highest concentrations, showed a combined effect of toxicity and decreased fecundity of females, as shown by the total effect of *C. acaulis* EO on females. However, no repellent effects on *T. urticae* have been detected. This indicates that no disturbing smells are contained in *C. acaulis* EO for all mobile stages. Different plant EOs have been reported for their repellent action on *T. urticae* (Araújo et al., 2012; Farahani et al., 2020; Motazedian et al., 2012; Mozaffari et al., 2013; Tsolakis and Ragusa, 2008), but *C. acaulis* EO failed for this activity against *T. urticae*.

Side effects of *Carlina acaulis* essential oil (EO) on *Neoseiulus californicus*

The detected side effects of *C. acaulis* EO on females of *N. californicus* are reported in Table 22. The GLM analysis demonstrated that all included factors significantly influenced the overall effect (mortality + repellence) on females of *N. californicus* (Test: $F_{4,715} = 19.29$; Time: $F_{2,715} = 84.41$; mortality or repellence effect: $F_{1,715} = 85.64$; $p < 0.001$ for all factors).

The mortality of female individuals occurred completely in the first day, and the four tests were not significantly different from those of the control displaying a harmless effect (class 1). Though, a marked repellent effect was noticed when females were directly sprayed and remained on the sprayed leaf disc, but also when unsprayed females were positioned on freshly sprayed leaf discs (Tests A and B, Table 22). In addition, a substantial repellent effect was also detected when treated females were placed on untreated leaf discs (Test C, Table 22). Importantly, the repellent effect of *C. acaulis* EO lasted for two days. In fact, when unsprayed females were put on treated leaf discs after two days (Test D, Table 22), only a slight repellent effect was detected within the first day ($p < 0.05$), and the oviposition rate was not different for that of the control.

Table 22. Side effects of *Carlina acaulis* essential oil (EO) on females of *Neoseiulus californicus* in the four different tests and in the control.

		Daily percentage of mortality (mean ± SE)			**Class**	**Daily percentage of repellence (mean ± SE)**			**Eggs/ female/ day**	**Total negative effect E (%)**
Test	**(n.)**	**1**	**2**	**3**		**1**	**2**	**3**		
A. Spraying on female placed on leaf disc	30	10.0 ± 5.57[a*]	-	-	1	90.0 ± 5.57[a]	-	-	0.00[a]	100.0[a]
B. Spraying on leaf disc; the female has been placed on leaf disc 4 minutes after	15	6.7 ± 6.67[a]	0.0	0.0	1	80.0 ± 10.7[a]	13.3 ± 9.09[a]	-	0.02[a]	99.5[a]
C. Spraying on female which placed afterwards on an untreated leaf disc	30	6.7 ± 4.63[a]	0.0	0.0	1	23.3 ± 7.85[b]	30.0 ± 8.51[a]	0.0	0.09[a]	94.3[b]
D. Spraying on leaf disc; the female has been placed on leaf disc 48 h after	15	0.0[a]	0.0	0.0	1	13.3 ± 9.09[b]	0.0[b]	0.0	0.62[b]	14.7[c]
Control	30	6.7 ± 4.63[a]	0.0	0.0	1	0.0[c]	0.0[b]	0.0	0.63[b]	6.7[c]

*Different letters within a column show significant differences. GLM followed by Tukey Pairwise Comparisons for $p < 0.05$ was performed on data.

The EO did not cause side effects on egg hatching. In fact, in treated eggs a hatching of 100% was registered (F1,166 = 0.57; p = 0.453) (Table 23). Nevertheless, even if the highest hatching percentages were recorded in the control (93.4%) during the 2nd day, in the 3rd day a 70.0% of hatching was detected in treated eggs (Table 23), demonstrating that *C. acaulis* EO led to a slow rate of the egg-hatching (F2,166 = 57.4; $p < 0.001$).

Table 23. Side effects of *Carlina acaulis* essential oil (EO) on eggs of *Neoseiulus californicus.*

Treatment	**Daily percentage of hatched eggs (mean ± SE)**			**Hatching (%) (mean ± SE)**
	1	2	3	
1250 ppm	13.3±6.31^{a*}	16.67±6.92^{a}	70.0±4.66^{a}	100.0
Control	3.3±3.33^{b}	93.4±4.63^{b}	3.3±3.33^{b}	100.0

Thirty eggs were tested for the control and 1250 ppm concentration. GLM followed by Tukey Pairwise Comparisons was performed on data. *Different letters denote significant differences for $p < 0.05$.

Y-tube olfactometer bioassays on *Neoseiulus californicus* adults

Preliminary bioassays

From the contingency analysis significative difference among mite choices were detected at the different concentrations tested (χ^2 = 46.192, d.f. = 2, $p < 0.001$) (Figure 60).

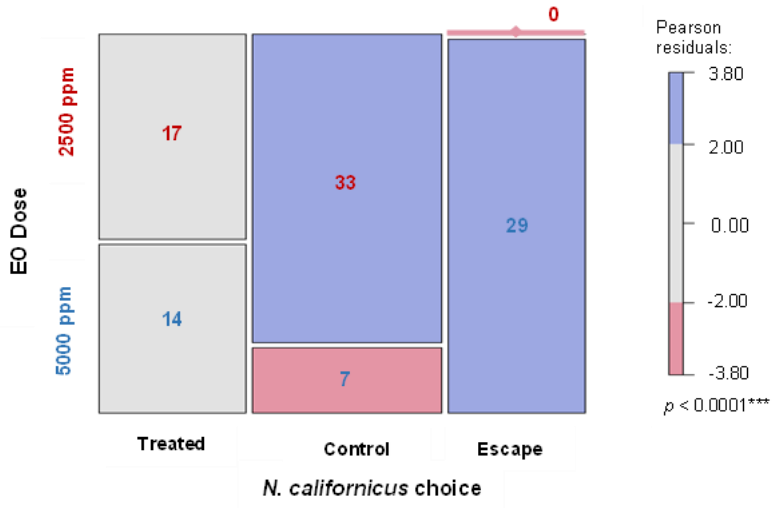

Figure 60. Mosaic plot visualizing the contingency analysis relating to dose and choice of preliminary bioassays. Graphical display of the association between the choice of the predatory mite and the dose of *Carlina acaulis* essential oil (EO) (based on a Pearson chi-squared test of independence, p-value reported in the plot). The area of each tile is proportional to the count of choices made by the predatory mite. The shading of the cells refers to the sign and magnitude of the respective Pearson residuals. Additionally, the number of observations per cell is presented; each value is color-coded according to the dose ($p < 0.05$).

The residual analysis showed a correlation between the escape of females of *N. californicus* and the highest dose, which indicates a strong repellent activity of the EO at 5000 ppm. In fact, 58% of escape of *N. californicus* females was detected just after the release. Conversely, a positive correlation between the choice of the control arm and the lowest dose (2500 ppm) was noticed.

Carlina acaulis EO bioactivity throughout the time

As in our preliminary bioassays, significant differences were detected between the highest concentration tested and the choice made by *N. californicus* immediately after the spray of the EO on the leaf (t0) (χ^2 = 24.649, d.f. = 2, p < 0.0001). The residual analysis underlined an association between mite response and dose (5000 ppm), while at 2500 ppm the repellent effect of the EO may be mitigated by the volatile compounds released by the leaf (Figure 61).

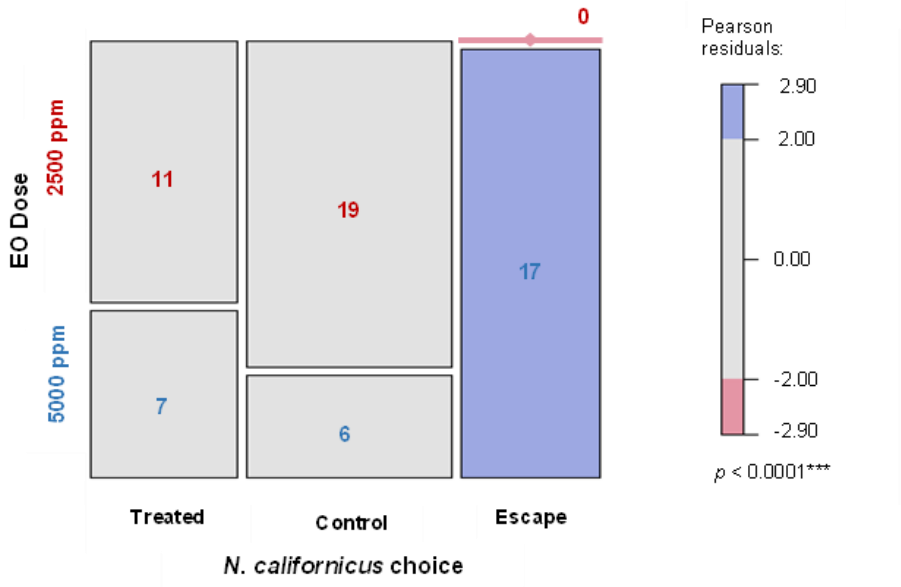

Figure 61. Mosaic plot visualizing the contingency analysis relating to dose and choice of *Carlina acaulis* essential oil (EO) bioactivity on the fresh bean leaf after fresh leaf treatment (t0). Graphical display of the association between the choice of the predatory mite and the dose of *Carlina acaulis* EO (based on a Pearson chi-squared test of independence, *p*-value reported in the plot). The area of each tile is proportional to the count of choices made by the predatory mite. The shading of the cells refers to the sign and magnitude of the respective Pearson residuals. Additionally, the number of observations per cell is presented; each value is color-coded according to the dose (p < 0.05).

On the other hand, not significant differences were found between the dose and the choice made by the mite (χ^2 = 0.7213, d.f. = 2, p = 0.6972) analysing the data after 48 h (t1), meaning that the EO repellent effect ended (Figure 62).

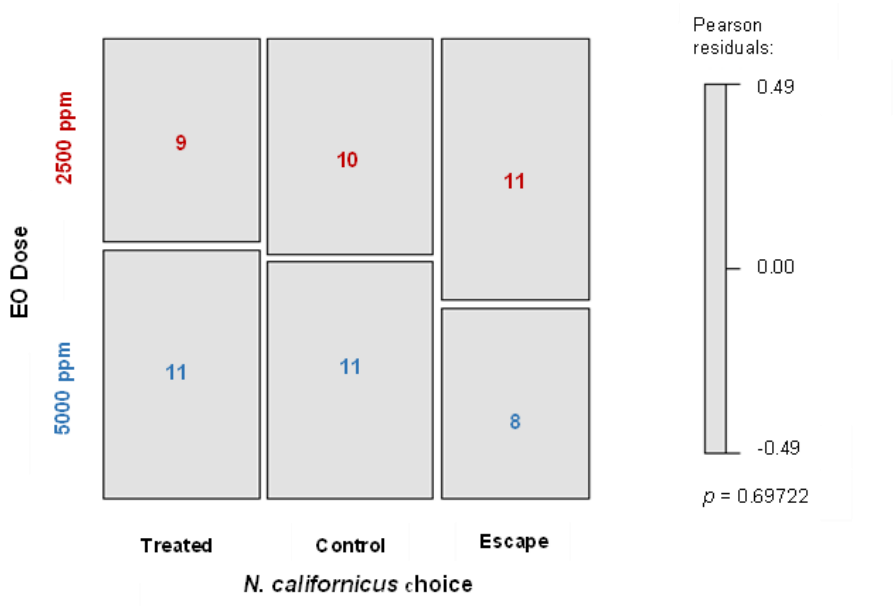

Figure 62. Mosaic plot visualizing the contingency analysis relating to dose and choice of *Carlina acaulis* essential oil (EO) bioactivity on the fresh bean leaf after 48h (t1). Graphical display of the association between the choice of the predatory mite and the dose of *C. acaulis* EO (based on a Pearson chi-squared test of independence, *p*-value reported in the plot). The area of each tile is proportional to the count of choices made by the predatory mite. Additionally, the number of observations per cell is presented; each value is color-coded according to the dose ($p > 0.05$).

The results obtained with Y-tube assay showed a marked repellent activity of the highest concentration of *C. acaulis* EO at the time of application (t0), which then was lowered or even disappeared after 48 h.

C. acaulis showed a repellent effect on *N. californicus*, but it did not display a toxic effect against phytoseiid females. The different behavioral responses of the two mites assayed with the same EO concentration could be ascribed to the kind of their chemoreceptors. Lately, Su et al. (2021) reported that predatory mites rely prevalently on gustatory receptors (GRs) and ionotropic receptors (IRs) for chemosensation. The repellent effect displayed by *C. acaulis* EO terminated after 48 h. This EO also proved to be harmless also towards *N. californicus* eggs, but the doses tested led to a significative delay in the eggs' hatching, and this effect has also been detected on the tetranychid eggs. The harmless effects on *N. californicus*' eggs compared to the high toxicity against the *T. urticae* ones could be ascribed to the different exposure times as also detected for *P. persimilis* eggs treated with caraway EO (Tsolakis and Ragusa, 2008). In fact, *N. californicus* eggs hatched within 2 days, while the tetranychid ones after 4-5 days, and this time probably led the active ingredient to penetrate better the chorion and to block the development of the embryo.

5.8.3 Conclusions

C. acaulis EO demonstrated to be a candidate active ingredient for the development of products to be used in the management of this agricultural pest. Our results showed that *C. acaulis* EO can

lower the longevity and fecundity of *T. urticae* females. However, more studies on resistance onset to this product, along with semi-field and field studies, should be performed for a complete evaluation of the EO against this agricultural pest.

5.9. Stored-products pests

The management of arthropod pests affecting stored products represents a great challenge in terms of reduction of pesticide resistance and exposure to hazardous agents (Athanassiou et al., 2018; Nauen et al., 2022; Sakka et al., 2020). The attack of these pests to stored products represents a great threat to their long-term conservation (Pimentel, 1991), but also to human health. In fact, it has been observed that some of them are also the cause of some allergic reactions to stored foods (Van-Lynden-van Nes et al., 1996). The stored-products pests employed for the bioassays with *C. acaulis*-derived products are reported below.

- *Acarus siro* L. (Astigmatina: Acaridae) (Figure 63), known as flour mite, is a pest affecting different types of grains, cheese, and flour (OConnor, 2009).

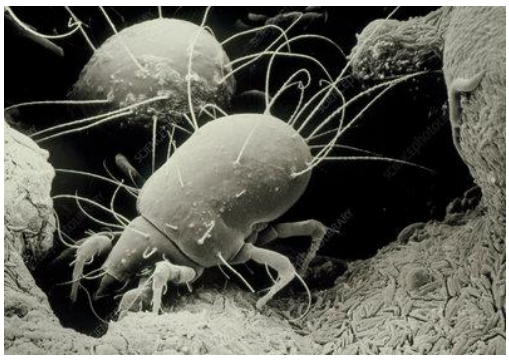

Figure 63. *Acarus siro*.

It causes a reduction of food weight (Žďárková, 1967), affects the germination (Parkinson, 1990), alters the organoleptic features (Brückner and Heethoff, 2016; Tuma et al., 1990), and the nutritional values (Yassin, 2015). This pest also causes skin inflammations and allergies (Halliday, 2003).

- *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) (Figure 64), known as a lesser mealworm or litter beetle, is a pest attacking production facilities (Axtell, 1994) that reproduces inside farms, nourishing itself on cracked eggs, poultry manure, and other organic material (Axtell and Arends, 1990; Pfeiffer and Axtell, 1980; Rueda and Axtell, 1997).

Figure 64. *Alphitobius diaperinus*.

Its presence also affects the economy of the production of stored products, causing changes in the organoleptic properties and reduced weight of the products (Despins and Axtell, 1994). It also represents a reservoir for diverse avian pathogens (Axtell and Arends, 1990; Axtell, 1994).

- *Oryzaephilus surinamensis* L. (Coleoptera: Silvanidae) (Figure 65), also called the saw-toothed grain beetle, is a pest affecting amylaceous products and cereals commodities, but also dried fruits, tobacco, sugar, nuts, and seeds (Hill, 2002; Kumar, 2017; Mahroof and Hagstrum, 2012).

Figure 65. *Oryzaephilus surinamensis*.

- *Prostephanus truncatus* (Horn) (Coleoptera: Bostrychidae) (Figure 66) is a pest of great importance for dried roots of cassava and stored maize (Gueye et al., 2008; Hill, 2002; Hodges et al., 1985) and is subjected to quarantine measures in some countries (Myers and Hagstrum, 2012; Tyler and Hodges, 2002).

Figure 66. *Prostephanus truncatus*.

It is also able to survive on different non-maize flour and grains, being these commodities a contribution to its reproduction and expansion.

- *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) (Figure 67), also called lesser grain borer, is a primary pest affecting cereals, rice, and wheat worldwide. It is able to adapt to dry environments, and larvae and adults cause quantitative and qualitative damages to stored-products (Evans, 1983).

Figure 67. *Rhyzopertha dominica*.

- *Sitophilus oryzae* L. (Coleoptera: Curculionidae) (Figure 68), is a widely distributed pest that affects stored grain. Adults nourish themselves mainly on the endosperm, causing a reduction of the carbohydrate levels, while larvae feed on the germ leading to reduced values of vitamins and proteins. These insects affecting germs cause higher germination loss than other kinds of pests (Dal Bello et al., 2000).

Figure 68. *Sitophilus oryzae*.

- *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) (Figure 69), also known as the confused flour beetle, is a secondary pest affecting flours, dried fruits, nuts, cereals, and spices worldwide and can be found in pet stores, mills, and warehouses (Hagstrum and Subramanyam, 2009; Robinson, 2005).

Figure 69. *Tribolium confusum*.

Its presence is reported to cause allergic reactions and itching after the ingestion of infected flour (Krinsky, 2019; Robinson, 2005).

- *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Figure 70), also known as the redflour beetle, affects a wide variety of goods worldwide and is the most pervasive pest in storage amenities (Kumar et al., 2017).

Figure 70. *Tribolium castaneum*.

As in the case of the pest mentioned above, it also produces food degradation and itching, due to the production of quinones (Hagstrum and Subramanyam, 2009; Mahroof et al., 2012; Robinson, 2005).

- *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) (Figure 71), also known as the yellow meal worm beetle, is a secondary cosmopolitan species, with a larger diffusion than the aforementioned pests (Robinson, 2005).

Figure 71. *Tenebrio molitor*.

It infects meals, dried fruit, and flour, and is largely found in food shops and storage units (Hagstrum and Subramanyam, 2009). It is the cause of the onset of asthma and other diseases as allergies (Krinsky, 2019).

- *Trogoderma granarium* Everts (Coleoptera: Dermestidae) (Figure 72) is a stored-product pest subjected to stringent phytosanitary concerns (Hill, 2002; Myers and Hagstrum, 2012) and has been classified as one of the most hazardous alien species worldwide.

Figure 72. *Trogoderma granarium***.**

It nourishes itself on numerous foods, as amylaceous containing products and cereals, but also non-grain goods (Athanassiou et al., 2016; Bhattacharya and Pant, 1968; Degri and Zainab, 2013; Lindgren and Vincent, 1959; Viljoen, 1990).

The studies reported below investigate the insecticidal action of *C. acaulis* EO, its NE (for the studies on *T. confusum, T. castaneum,* and *T. molitor*), and of its spray solution against stored-products pests mentioned above.

5.9.1 Materials and methods

Preparation and characterization of *C. acaulis* EO-NE

C. acaulis EO-NE (6% of EO) was prepared and characterized accordingly to the procedure described in Section 4.2.1 and 4.2.2.

Insects and mite species

Insects and mite species derived from the Laboratory of Agricultural Zoology and Entomology, Agricultural University of Athens. Regarding the insect's species, the colonies were maintained at 30 °C, with 55% or 65% R.H. and constant darkness (Boukouvala et al., 2019a, 2019b; Kar et al., 2021; Kavallieratos et al., 2013, 2017a, 2017b, 2019; Nika et al., 2021; Renault et al., 2004; Rice and Lambkin, 2009; Skourti et al., 2019; Wakil et al., 2022), while colonies of the mite species were kept at 25 °C, 80% RH and constant darkness (Kavallieratos et al., 2018a). *T. castaneum*, and *T. confusum* were raised on wheat flour with an additional 5% of brewer's yeast (Boukouvala et al., 2019a, 2019b; Skourti et al., 2019). *T. molitor* was reared on oat bran and cuts of potato (De Vosjoli, 2007; Kavallieratos et al., 2019), while *P. truncatus* and *T. granarium* on whole maize and wheat, respectively. The media used for the rearing of *O. surinamensis* were oat flakes, brewer's yeast cracked, and wheat (5:1:5 ratio) (Nika et al., 2021), while those for *A. diaperinus* were wheat bran and yeast (3:1 ratio) with diced apples (Kavallieratos et al., 2022b; Rice and Lambkin, 2009).

R. dominica and *S. oryzae* were grown on wheat kernels (Kavallieratos et al., 2017b; Wakil et al., 2022), while *A. siro* on oat flakes, brewer's yeast, and wheat germ (1:10:10 ratio) (Kavallieratos et al., 2018a). Regarding *T. confusum* and *T. castaneum*, the adults used for the study were less than 2 weeks old, while larvae 3rd or 4th instars old. *T. molitor* larvae were 10–14 mm long (Kavallieratos et al., 2013; 2019). *P. truncatus* adults were less than 1 week old (Kavallieratos et al., 2018a), while *T. granarium* adults were less than 24 h old, and the larvae were 2–4 mm long (Athanassiou et al., 2015; Kavallieratos et al., 2016, 2017a). Adults of *O. surinamensis*, *R. dominica*, and *S. oryzae* were less than 2 weeks old (Kavallieratos et al., 2019; Nika et al., 2021; Skourti et al., 2019; Wakil et al., 2022), while *A. diaperinus* adults were less than 1 week old (Kavallieratos et al., 2022b; Rees, 2004).

Concerning the larvae of the above-mentioned insect species, they were 3rd instar for *O. surinamensis* (Kavallieratos et al., 2013; Nika et al., 2020; Skourti et al., 2019), smaller than 7 mm of length for *A. diaperinus* (Kavallieratos et al., 2022b; Rees, 2004), or 10 to 14 mm long for *T. molitor* (Kavallieratos et al., 2019).

The unsexed individuals of *A. siro* were from 1 to 21 days old, while nymphs and adults were recognized by external morphology (Hughes, 1976; Kavallieratos et al., 2018a; Nesvorna and Hubert, 2014).

Commodity

Mortality bioassays were conducted by using hard wheat, *Triticum durum* Desf. (var.Claudio) (Poaceae), which was clean and free of pesticides and contaminations. For the mortality bioassays on *P. truncatus*, maize, *Zea mays* L. (var. Dias), without contaminations or pesticides, was used. Before the experiments, the moisture content of grains (13.1%) was determined using a moisture meter (mini GAC plus,Dickey-John Europe). Wheat or maize were dried in an oven at 50 °C or by incorporation of distilled water on the base of its original moisture content (Athanassiou et al., 2016; Kavallieratos et al., 2012).

Toxicity assays

Regarding EO-spray solutions, these were prepared at two different concentrations: 500 or 1000 ppm (500 or 1000 μL kg^{-1} wheat), respectively. The total volume of sprayed solutions was 750 μL. For the preparation of 500 ppm solution, 125 μL of the EO were solubilized in pure ethanol (125 μL), while for the preparation of 1000 ppm solution 250 μL of EO were diluted in pure ethanol (250 μL). Then 500 or 250 μL of Tween 80 (0.3% v/v in water) were added to 500 and 1000 ppm solutions to reach the volume of 750 μL (Kavallieratos et al., 2021a). The control was represented by wheat of the weight of 0.25 kg sprayed with: a) pure ethanol, (b) water, (c) positive control

Actellic EC at the label dose of 5 ppm (= 5 μL kg^{-1} wheat) containing the active ingredient pirimiphos-methyl (50%), and (c) carrier control (Tween 80 plus ethanol plus water). For *P. truncatus* and *T. granarium*, the control was represented by: (a) pure ethanol, (b) water, and (c) carrier control (Tween 80 plus ethanol plus water). Regarding the testing of *C. acaulis* EO-NE (6% w/w) on *T. confusum*, *T. castaneum*, and *T. molitor*, the following concentrations were used: 500 μL kg^{-1} wheat (500 ppm) and 1000 μL kg^{-1} wheat (1000 ppm) (Kavallieratos et al., 2021a). These concentrations were selected through preliminary tests. Test solutions were prepared in water at the final volume of 750 μL and were sprayed on wheat samples weighting 0.25 kg positioned on plates. The controls were samples of wheat (weighting 0.25 kg) sprayed with: (a) water, (b) 4% surfactant dispersed in water (carrier control), and (c) Actellic EC, containing 50% pirimiphos-methyl active ingredient (Syngenta) (positive control) at the dose of 5 μl kg^{-1} wheat (5 ppm) (Kavallieratos et al., 2019). The controls and the tested concentrations of *C. acaulis* EO-NE or EO were sprayed on the wheats using diverse airbrushes. Then, for the improvement of the EO-NE or EO spray solutions over the wheat, the samples were placed inside a 1-L glass vessels and submitted to shaking for 10 min. This procedure was performed also for the controls. Three samples, weighting each 10 g (for insects) or 1 g (for mites), were picked up from the vessel containing the treatment or the control using different scoops. Sample weights were measured with a Precisa XB3200D compact balance (AlphaAnalytical Instruments). The samples were then moved to glass vials of 12.5 cm height x 7.5 cm diameter (for the insect species) and of 6.0 cm height x 2.7 cm diameter (for the mite species), furnished of 1.5-cm diameter circular opening covered with a gauze to assure a sufficient aeration inside the vials. Moreover, to avoid beetle escape polytetrafluoroethylene (60 wt % dispersion in water; Sigma-Aldrich) was placed on the upper internal sides of the vials. Then, 10 nymphs, larvae, or adults of each arthropod were placed separately into the vials. Then, the vials were put into incubators at the temperature of 30 °C and RH of 65% (for the insect species) and of 25°C and R.H. of 80% (for the mite species) for the whole experimental period (Kavallieratos et al., 2018a). For *A. diaperinus*, each sub replication consisted of 10 vials containing one individual each to avoid cannibalism (Kavallieratos et al., 2022b; Szczepanik et al., 2016). Mortality was determined at x57 total magnification with Olympus stereomicroscope (SZX9) after 4, 8, and 16 h and after 1, 2, 3, 4, 5, 6, and 7 days of exposure. The detection of movements of each insect was performed through a fine brush (Cotman 111 No 000, Winsor and Newton). The above-descripted procedure was repeated 3 times, using new vials, insects or mites, and wheat samples.

Data analysis

Mortality resulted less than 5% in the controls sprayed with water for all the insect and mite species and no correction was required.

Mortality in the carrier controls constituted by 4% surfactant dispersed in water was higher than 5% for the tested tenebrionid species and mortality rates were adjusted using Abbott's formula (Abbott, 1925). To normalize the variance, mortality data were log(x+1) converted before analysis (Scheff and Arthur, 2018; Zar, 2010). Statistical analyses were performed by following the repeated-measures model independently for each tested species and life stage (Sall, 2005). The repeated factor was the exposure interval, while mortality was the response variable. Concentration and insect species/developmental stage were the main effects. The associated interactions of the main effects were embodied in the analysis. All analyses were conducted with JMP v.14 software. Means were separated using the Tukey–Kramer honestly significant difference (HSD) test at a 0.05 significance level (Sokal and Rohlf, 1995).

5.9.2 Results and discussion

Preparation and characterization of *Carlina acaulis* essential oil-nanoemulsion (EO-NE)

NEs were prepared and characterized obtaining results linear with those of Section 4.3.1.

Toxicity assays

For all tested insect and mite species, main effects and associated interactions were significant between and within exposure intervals, all main effects and associated interactions were significant ($p < 0.05$) except concentration x developmental stage for *O. surinamensis*.

Oryzaephilus surinamensis

C. acaulis EO showed a high effectiveness on *O. surinamensis* adults (Table 24) since the concentration of 1000 ppm led to 93.3% mortality 6 days post-exposure and to 96.7% mortality 7 days post-exposure. The concentration of 500 ppm did not lead to significant mortality also after 7 days from the exposure (22.2% of mortality). *C. acaulis* EO at 1000 ppm and 7 days post-exposure was also more effective than the positive control pirimiphos-methyl (mortality of 7.8%). The EO also displayed high larvicidal effects on *O. surinamensis*. After 2 days from the treatment, the EO at 1000 ppm caused the complete mortality of all individuals. Levels of mortality were also elevated at 500 ppm, in fact 83.3% of mortality was reached 7 days post-exposure. Also in this case, the EO was more effective than the positive control pirimiphos-methyl (mortality of 63.3% 7 days post-exposure). The efficacy reached by *C. acaulis* EO has not been achieved by other botanical insecticides. For instance, the NE containing the EO of *Mentha longifolia* L. led to a mortality of 63.3 and 86.7% on adults and larvae of O. *surinamensis* after 7 days of exposure, respectively (Kavallieratos et al., 2022c). On the other hand, the EO from *T. vulgare* caused a

mortality of 13.3 and 93.3% on adults and larvae, respectively (Kavallieratos et al., 2021b). The knowledge on the use of synthetic insecticides for the protection of grain from this species is limited. Spinosad and methoprene applied on wheat at different concentrations were able to kill just 10.0–32.2% of *O. surinamensis* adults 14 days post-exposure (Athanassiou et al., 2011). Moreover, it was also demonstrated that 1 mg kg^{-1} of spinosad applied on four different classes of wheat caused 2.7 (hard red winter), 7.8 (hard red winter), 5.6 (hard red spring) and 74.9% (durum) mortality on *O. surinamensis* adults 7 days post-exposure (Fang et al., 2002).

Alphitobius diaperinus

For *A. diaperinus*, the EO was not effective on adults, leading to low mortality (Table 25). However, this product led to high toxicity on larvae, for which complete mortality (100%) was reached after 4 days (at 500 ppm) and 2 days (at 1000 ppm) from the exposure. The EO was even more effective on larvae than the positive control, which led to 37.8% mortality 7 days post-exposure. From the experiments on this species, a higher tolerance of adults with respect to larvae was noted. Recently, Kavallieratos et al. (2022a) reported that chlorfenapyr had a lower activity on adults rather than on larvae. Moreover, etofenprox and piperonyl butoxide plus acetamiprid plus d-tetramethrin resulted to 57.8 and 58.9% of mortalities, respectively (Kavallieratos et al., 2022a). In our experiments, the mortality 3 days post-exposure was of 100%. This could be partially explained by the fact that adults and larvae have differences in terms of structure and physiology of the cuticula (Armold et al., 1969; Hackman, 1964; Mewis and Ulrichs, 2001; Neville and Farner, 1975).

Table 24. Mean (%) mortality ± standard errors (SE) of *Oryzaephilus surinamensis* adults and larvae after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil (EO) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	**4 h**	**8 h**	**16 h**	**1 day**	**2 days**	**3 days**	**4 days**	**5 days**	**6 days**	**7 days**	***F***	***P***
Adults												
500 ppm	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	7.8 ± 3.6^{ABb}	13.3 ± 5.0^{Ab}	15.6 ± 5.3^{Ab}	17.8 ± 5.5^{Ab}	20.0 ± 6.9^{Ab}	22.2 ± 7.4^{Ab}	13.2	< 0.01
1000 ppm	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	2.2 ± 1.5^{C}	4.4 ± 2.4^{C}	31.1 ± 4.2^{Ba}	63.3 ± 8.2^{ABa}	78.9 ± 6.6^{Aa}	85.6 ± 5.6^{Aa}	93.3 ± 4.7^{Aa}	96.7 ± 2.4^{Aa}	109.5	< 0.01
p.c.	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	1.1 ± 1.1^{B}	1.1 ± 1.1^{B}	1.1 ± 1.1^{Bb}	1.1 ± 1.1^{Bc}	5.6 ± 2.4^{ABb}	$6.7 \pm 2.9A^{Bc}$	7.8 ± 2.8^{Ab}	7.8 ± 2.8^{Ab}	3.2	< 0.01
F	-	-	1.1	2.6	21.6	39.3	18.8	20.8	83.2	18.9		
P	-	-	0.35	0.13	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		
Larvae												
500 ppm	1.1 ± 1.1^{Eb}	7.8 ± 3.2^{Db}	18.9 ± 5.6^{Cb}	31.1 ± 5.6^{BCb}	57.8 ± 7.0^{ABa}	$66.7 \pm 7.6A^{Ba}$	70.0 ± 7.1^{ABa}	73.3 ± 5.8^{ABa}	76.7 ± 6.9^{ABa}	83.3 ± 5.8^{Ab}	39.8	< 0.01
1000 ppm	26.7 ± 6.7^{Ba}	54.4 ± 9.7^{Aa}	75.6 ± 10.0^{Aa}	86.7 ± 6.7^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	10.2	< 0.01
p.c.	1.1 ± 1.1^{Eb}	$1.1 \pm 1.1^{E}b$	1.1 ± 1.1^{Ec}	$4.4 \pm 1.8^{DE}c$	11.1 ± 2.6^{CDb}	17.8 ± 3.2^{BCb}	30.0 ± 4.7^{ABCb}	34.4 ± 5.0^{ABb}	45.6 ± 6.3^{ABb}	63.3 ± 3.3^{Ac}	32.0	< 0.01
F	13.8	28.2	51.1	41.8	29.9	22.9	33.4	29.9	20.2	20.5		
P	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		

For each developmental stage, within each row, means followed by the same uppercase letter are not significantly different, in all cases *df* = 9, 89; Tukey HSD test at *P* = 0.05. For each developmental stage, within each column, means followed by the same lowercase letter are not significantly different, in all cases *df* = 2, 26; Tukey's HSD test at *P* = 0.05. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Table 25. Mean (%) mortality ± standard errors (SE) of *Alphitobius diaperinus* adults and larvae after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil (EO) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{Bb}	0.0 ± 0.0^{Bb}	1.1 ± 1.1^{Bb}	7.8 ± 2.2^{Ab}	13.3 ± 3.3^{Ab}	15.2	< 0.01
1000 ppm	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	1.1 ± 1.1^{Bab}	2.2 ± 2.2^{Bb}	4.4 ± 2.9^{Bb}	15.6 ± 2.9^{Aab}	25.6 ± 3.4^{Aa}	26.5	< 0.01
p.c.	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	4.4 ± 1.8^{Ba}	8.9 ± 2.0^{Ba}	18.9 ± 3.5^{Aa}	22.2 ± 3.6^{Aa}	23.3 ± 3.7^{Aab}	49.5	< 0.01
F	-	-	-	-	-	3.7	12.7	19.7	4.6	4.0		
P	-	-	-	-	-	0.04	< 0.01	< 0.01	0.02	0.03		
Larvae												
500 ppm	4.4 ± 1.8^{Dab}	15.6 ± 2.9^{Ca}	32.2 ± 4.0^{Ba}	47.8 ± 4.9^{ABa}	87.8 ± 4.7^{Aa}	98.9 ± 1.1^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	42.9	< 0.01
1000 ppm	8.9 ± 2.0^{Da}	22.2 ± 3.2^{Ca}	42.2 ± 4.7^{BCa}	65.6 ± 8.2^{ABa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	27.0	< 0.01
p.c.	2.2 ± 1.5^{Cb}	5.6 ± 2.4^{Cb}	7.8 ± 2.8^{Cb}	8.9 ± 2.6^{BCb}	18.9 ± 3.5^{ABb}	24.4 ± 2.4^{Ab}	26.7 ± 2.9^{Ab}	28.9 ± 2.6^{Ab}	34.4 ± 3.8^{Ab}	37.8 ± 2.8^{Ab}	15.8	< 0.01
F	3.4	5.6	15.8	21.3	90.6	268.9	183.2	197.0	97.2	193.0		
P	0.05	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		

For each developmental stage, within each row, means followed by the same uppercase letter are not significantly different, in all cases df = 9, 89; Tukey HSD test at P = 0.05. For each developmental stage, within each column, means followed by the same lowercase letter are not significantly different, in all cases df = 2, 26; Tukey's HSD test at P = 0.05. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Rhyzopertha dominica

R. dominica adults (Table 26) were susceptible to the treatment, which caused 96.7% mortality (at 1000 ppm) after 2 days from the exposure. The mortality was lower at 500 ppm after the same time from the treatment (56.7%). The complete mortality (100%) was achieved at 1000 ppm after 4 days from the initial exposure. Pirimiphos-methyl was less effective (82.2% mortality) than the EO at both the concentrations tested. The efficacy showed by *C. acaulis* EO was quite elevated. In fact, for instance, diatomaceous earths Protect-It (150 ppm), imidacloprid (1.25, 2.5 or 5 ppm), and their combinations led to 35.3–82.3% mortality after 4 days of exposure (Wakil et al., 2021). Moreover, Boukouvala and Kavallieratos (2022) demonstrated that etofenprox sprayed on wheat at 10 ppm led to low mortality rates (21.7–56.7%) to adults of this species after 7 days of exposure. Additionally, *Beauveria bassiana*, fipronil and their mixes killed 24.5–94.3% of *R. dominica* individuals at the same time interval (Wakil et al., 2022).

Sitophilus oryzae

On *S. oryzae* adults the EO at 1000 ppm led to high efficacy, with 94.4 and 100.0% mortality, 4 and 6 days post-exposure, respectively (Table 27). By the end of the test (7 days post-exposure), the concentration of 500 ppm led to 86.7% mortality, while the positive control led to 90.0% mortality, respectively. The control of this pest has been reported as particularly challenging. For instance, a recent study reported that linalool or nerolidol applied at 25 μL mL^{-1} on rice led to 79.7 and 58.2% mortalities of *S. oryzae* adults 7 days post-exposure, respectively (Akinbuluma et al., 2022). Moreover, it has been reported that the combination of spinosad, lufenuron, chlorfluazuron, and hexaflumuron led to mortality values of *S. oryzae* between 2.7 and 56.3%, 7 days post-exposure (Gad et al., 2022). On the other hand, mortality values of this pest using etofenprox at 10 ppm were between 12.8-31.7%, 7 days after the exposure (Boukouvala and Kavallieratos, 2022).

Table 26. Mean (%) mortality ± standard errors (SE) of *Rhyzopertha dominica* adults after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil (EO) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	1.1 ± 1.1^{Eb}	11.1 ± 2.0^{Db}	25.6 ± 2.9^{Ca}	40.0 ± 2.4^{BCa}	56.7 ± 2.4^{ABb}	74.4 ± 5.6^{ABb}	86.7 ± 4.4^{a}	96.7 ± 2.4^{Aa}	98.9 ± 1.1^{Aa}	98.9 ± 1.1^{Aa}	101.2	< 0.01
1000 ppm	10.0 ± 2.4^{Da}	27.8 ± 3.2^{Ca}	50.0 ± 4.7^{BCa}	72.2 ± 3.6^{ABa}	96.7 ± 3.3^{Aa}	97.8 ± 2.2^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	39.5	< 0.01
p.c.	0.0 ± 0.0^{Db}	2.2 ± 1.5^{CDc}	4.4 ± 1.8^{Cb}	5.6 ± 1.8^{Cb}	20.0 ± 2.9^{Bc}	32.2 ± 3.2^{ABc}	46.7 ± 3.7^{ABb}	57.8 ± 5.5^{Ab}	71.1 ± 3.9^{Ab}	82.2 ± 3.6^{Ab}	53.4	< 0.01
F	16.0	25.1	32.0	39.8	78.6	65.6	57.1	30.7	41.8	18.7		
P	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		

Means followed by the same uppercase letter within each row are not significantly different in all cases *df* = 9, 89; Tukey HSD test at *P* = 0.05. Means followed by the same lowercase letter within each column are not significantly different, in all cases *df* = 2, 26; Tukey's HSD test at *P* = 0.05. Where no letters exist, no significant differences were recorded.

Table 27. Mean (%) mortality ± standard errors (SE) of *Sitophilus oryzae* adults after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil (EO) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{Db}	30.0 ± 5.3^{Cb}	60.0 ± 8.2^{Bab}	68.9 ± 6.3^{ABb}	77.8 ± 5.2^{ABb}	86.7 ± 5.0^{Ab}	86.7 ± 5.0^{Ab}	511.5	< 0.01
1000 ppm	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{Db}	47.8 ± 3.2^{Ca}	81.1 ± 5.1^{Ba}	94.4 ± 4.4^{ABa}	95.6 ± 3.4^{ABa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	3599.4	< 0.01
p.c.	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	10.0 ± 2.4^{Da}	24.4 ± 2.4^{Cb}	41.1 ± 3.5^{BCb}	48.9 ± 2.6^{ABc}	66.7 ± 3.3^{ABb}	76.7 ± 3.3^{Ab}	90.0 ± 3.3^{Aab}	199.3	< 0.01
F	-	-	-	26.5	6.8	8.3	19.3	10.7	9.8	3.7		
P	-	-	-	< 0.01	0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.04		

Means followed by the same uppercase letter within each row are not significantly different in all cases *df* = 9, 89; Tukey HSD test at *P* = 0.05. Means followed by the same lowercase letter within each column are not significantly different, in all cases *df* = 2, 26; Tukey's HSD test at *P* = 0.05. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Prostephanus truncatus

Concerning *P. truncatus* adults, the EO was highly effective, leading to 92.2 and 100% mortality after two days at 500 and 1000 ppm, respectively. For the 500 ppm concentration, the mortality reached the 98.9% after 5 days of exposure (Table 28). Concerning the management of this pest through EOs, different studies are available. For instance, the EO of *Acorus calamus* (L.) led to a decrease of the maize damage caused by the feeding activity of this pest at the concentration of 0.01% w/w (Schmidt and Streloke, 1994). In addition, the EO from *Plectranthus glandulosus* Hook f. caused 100% mortality against *P. truncatus* 7 days post-exposure at 80 μL 40 g^{-1} maize (Nukenine et al., 2010). From an applicative point of view, *C. acaulis* EO proved to be a reliable tool for the management of the above-mentioned pest comparably to synthetic insecticides, even if they are effective at lower concentrations. For example, 98.3% mortality of *P. truncatus* adults was achieved with maize treated with 1 ppm of chlorfenapyr after 14 days from the exposure (Kavallieratos et al., 2011). Another study reported that liquid spinetoram or liquid spinosad applied on maize were able to lead to almost complete mortality of the pest 7 days post-exposure (Athanassiou and Kavallieratos, 2014).

Trogoderma granarium

For *T. granarium* (Table 29), adult mortality was of 42.2% at 1000 ppm, but it increased after 1 day of exposure to 77.8%. At the end of the experiments, 98.9% of mortality was reached at 1000 ppm. On larvae, the EO showed moderate rates of mortalities at both the concentrations tested 7 days post-exposure (56.7 and 61.1% for 500 and 1000 ppm, respectively). Even if no data are available on the employment of EOs against *T. granarium*, several studies have been reported on the contact toxicity or repellent activity of EOs against this pest. For instance, it has been reported that 1 mL of *Schinus molle* L. EO applied on filter paper led to 90% mortality against the adults of this species (Abdel Sattar et al., 2010). Moreover, it was also reported that the EOs from *E. globulus* and *Origanum syriacum* L. led to complete mortality of *T. granarium* adults at 50 and 30 μL cm^{-3} air after 2 days of exposure (Tayoub et al., 2012). On the other hand, also Nenaah (2014) reported that the EO of *Achillea biebersteinii* Afan was able to kill the 83.2% of *T. granarium* larvae after 7 days of topical application. Moreover, the EO was able to perform a marked repellent and fumigant activity on both larvae and adults. Kavallieratos et al. (2017a) evaluated six different insecticides on both larvae and adults of this pest and demonstrated that only pirimiphos-methyl and SilicoSec were highly effective on adults, while no activity was detected on larvae for the insecticides tested, except for pirimiphos-methyl.

Table 28. Mean (%) mortality ± SE (standard errors) of *Prostephanus truncatus* adults over selected time intervals in maize treated with *Carlina acaulis* essential oil (EO).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	$16.7 \pm 3.3^{C*}$	$44.4 \pm 6.3^{B*}$	$92.2 \pm 4.3^{A*}$	$97.8 \pm 2.2^{A*}$	$97.8 \pm 2.2^{A*}$	$98.9 \pm 1.1^{A*}$	$98.9 \pm 1.1^{A*}$	$98.9 \pm 1.1^{A*}$	235.6	< 0.01
F	-	-	19.6	46.4	324.1	850.0	626.3	567.4	292.1	130.9		
P	-	-	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		
1000 ppm	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	$34.4 \pm 4.8^{C*}$	$84.4 \pm 4.1^{B*}$	$100.0 \pm 0.0^{A*}$	$100.0 \pm 0.0^{A*}$	$100.0 \pm 0.0^{A*}$	$100.0 \pm 0.0^{A*}$	$100.0 \pm 0.0^{A*}$	$100.0 \pm 0.0^{A*}$	467.9	< 0.01
F	-	-	46.8	381.4	2958.4	3136.0	2704.0	384.0	153.0	9.1		
P	-	-	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		

Within each column, asterisks indicate significant differences, *df* = 1, 17; Tukey's HSD test at *P* = 0.05. Within each row, means followed by the same uppercase letter are not significantly different, *df* = 9, 89; Tukey's HSD test at *P* = 0.05. Where dashes exist, no analysis was conducted.

Table 29. Mean (%) mortality ± SE (standard errors) of *Trogoderma granarium* adults and larvae over selected time intervals in wheat treated with the *Carlina acaulis* essential oil (EO).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	2.2 ± 1.5^{Dd}	4.4 ± 1.8^{Dd}	30.0 ± 3.7^{Cd}	41.1 ± 3.1^{BCc}	52.2 ± 2.8^{ABc}	56.7 ± 2.4^{Ac}	58.9 ± 2.6^{Ac}	58.9 ± 2.6^{Ac}	60.0 ± 2.4^{Ac}	61.1 ± 2.6^{Ab}	77.1	< 0.01
F	298.4	429.6	376.5	166.2	204.6	233.3	208.9	194.0	222.2	210.1		
P	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		
1000 ppm	22.2 ± 6.4^{Ec}	42.2 ± 6.6^{Dc}	67.8 ± 4.9^{Cbc}	77.8 ± 4.0^{BCb}	91.1 ± 2.6^{ABab}	96.7 ± 2.9^{ABa}	97.8 ± 2.8^{Aa}	97.8 ± 2.8^{Aa}	98.9 ± 3.1^{Aa}	98.9 ± 3.1^{Aa}	42.3	< 0.01
F	54.2	64.2	91.3	99.2	94.8	94.1	39.0	23.7	21.7	11.4		
P	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		
Larvae												
500 ppm	1.1 ± 1.1^{Dc}	1.1 ± 1.1^{Dd}	12.2 ± 3.2^{CDcd}	17.8 ± 4.3^{BCDbc}	31.1 ± 6.1^{ABCc}	38.9 ± 6.1^{ABCc}	43.3 ± 7.6^{ABc}	43.3 ± 7.6^{ABc}	48.9 ± 7.9^{Ac}	56.7 ± 7.6^{Ab}	11.8	< 0.01
F	20.5	35.7	47.4	55.1	79.1	85.7	66.6	65.9	61.8	66.9		
P	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		
1000 ppm	2.2 ± 1.5^{Cc}	4.4 ± 2.4^{Cc}	30.0 ± 5.0^{BCc}	41.1 ± 6.3^{ABc}	52.2 ± 7.2^{ABc}	56.7 ± 7.5^{ABb}	58.9 ± 7.2^{Ab}	58.9 ± 7.2^{Ab}	60.0 ± 6.9^{Ab}	61.1 ± 6.3^{Ab}	14.1	< 0.01
F	45.6	74.0	81.5	50.8	50.8	53.2	50.7	50.5	50.9	46.0		
P	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		

Within each column, means followed by the same lowercase letter are not significantly different, *df* = 7, 71; Tukey's HSD test at *P* = 0.05. Within each row, means followed by the same uppercase letter are not significantly different, *df* = 9, 89; Tukey's HSD test at *P* = 0.05. Where no letters exist, no significant differences were recorded.

Tribolium castaneum

Regarding *T. castaneum* adults, the EO (at 1000 ppm) led to complete mortality (100%) after 5 days from the initial exposure (Table 30). The concentration of 500 ppm showed an increase of mortality from 93.3 up to 97.8% from 4-7 days after initial exposure. On the other hand, the EO-NE showed lower mortality values on adults at both the concentrations tested if compared to the EO (Table 31). Positive control did not lead to any mortality on *T. castaneum* adults. For *T. castaneum* larvae, the EO showed complete mortality (100%) 16 h (at 1000 ppm) and 2 days (at 500 ppm) after the exposure (Table 30). For the EO-NE high levels of mortality (75.3 and 93.9% for 500 and 1000 ppm, respectively) were reached at 7 days post-exposure (Table 31). Pirimiphos-methyl gave significantly less mortality levels if compared to both the EO and the EO-NE (Table 30 and 31).

Since *T. castaneum* adults are more tolerant than larvae (Arthur, 2010; Athanassiou and Kavallieratos, 2014; Boukouvala and Kavallieratos, 2022; Deb and Kumar, 2020; Lee et al., 2020a), the effectiveness of *C. acaulis* EO (100% mortality reached even at 500 ppm) makes it a useful tool for managing these species without targeting a certain developmental stage. Regarding *C. acaulis* EO-NE, its efficacy on *T. castaneum* adults (23.3% at 1000 ppm and 7 days post-exposure) was comparable to that of spinosad applied at 1 ppm (mortality between 1.8 and 17.8% on different strains of *T. castaneum* adults) (Sehgalet al., 2013) and to that of diverse diatomaceous earths (A2, A3, MN51, and Chemutsi) (mortality between 0% and 21.8%) (Machekano et al., 2017). *C. acaulis* EO-NE tested in our study was more effective on *T. castaneum* larvae than Nimbecidine ECoil at 10.0% oil diluted in distilled water with an emulsifier. In fact, the NE led to 75.3 and 93.9% mortalities at 500 and 1000 ppm, respectively, 7 days post-exposure. On the other hand, Nimbecidine ECoil led to 30% mortality 6 days post-exposure (Younus et al., 2020).

Table 30. Mean (%) mortality ± standard errors (SE) of *Tribolium castaneum* adults and larvae after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil (EO) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	4.4 ± 1.8^{Dab}	17.8 ± 2.8^{Ca}	30.0 ± 2.4^{BCb}	50.0 ± 3.3^{ABb}	77.8 ± 6.6^{Ab}	85.6 ± 4.8^{Ab}	93.3 ± 3.7^{Aa}	94.4 ± 2.9^{Aa}	94.4 ± 2.9^{Aa}	97.8 ± 2.2^{Aa}	56.5	< 0.01
1000 ppm	10.0 ± 3.3^{Ca}	31.1 ± 5.9^{Ba}	52.2 ± 6.2^{ABa}	82.2 ± 6.0^{Aa}	97.8 ± 2.2^{Aa}	97.8 ± 2.2^{Aa}	98.9 ± 1.1^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	30.3	< 0.01
p.c.	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	-	-
F	6.2	144.8	649.9	1518.2	2371.9	5097.0	10564.6	20132.3	20132.3	35241.8		
P	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		
Larvae												
500 ppm	11.1 ± 2.6^{Cb}	31.1 ± 2.6^{Ba}	60.0 ± 2.4^{Aa}	98.9 ± 1.1^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	10.1	< 0.01
1000 ppm	28.9 ± 2.6^{Ca}	68.9 ± 4.6^{Ba}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	137.0	< 0.01
p.c.	0.0 ± 0.0^{Cc}	4.4 ± 1.8^{Bb}	7.8 ± 2.8^{Bb}	22.2 ± 3.6^{Ab}	35.6 ± 3.4^{Ab}	37.8 ± 4.3^{Ab}	38.9 ± 3.9^{Ab}	45.6 ± 5.0^{Ab}	52.2 ± 4.7^{Ab}	53.3 ± 5.0^{Ab}	40.4	< 0.01
F	50.2	43.2	37.6	82.6	100.1	69.2	88.2	45.8	56.8	48.0		
P	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		

For each developmental stage, within each row, means followed by the same uppercase letter are not significantly different, in all cases $df = 9, 89$; Tukey HSD test at $P = 0.05$. For each developmental stage, within each column, means followed by the same lowercase letter are not significantly different, in all cases $df = 2, 26$; Tukey's HSD test at $P = 0.05$. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Table 31. Mean (%) mortality ± standard errors (SE) of *Tribolium castaneum* adults and larvae after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil-nanoemulsion (EO-NE) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{Bb}	1.1 ± 1.1^{Bb}	2.2 ± 1.5^{Bab}	2.2 ± 1.5^{Bb}	2.2 ± 1.5^{Bb}	10.0 ± 2.9^{Ab}	4.8	<0.01
1000 ppm	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	1.1 ± 1.1^{DE}	4.4 ± 2.4^{CDEa}	6.7 ± 2.4^{BCDEa}	10.0 ± 3.7^{BCDa}	13.3 ± 3.3^{ABCa}	15.6 ± 2.9^{ABa}	23.3 ± 3.3^{Aa}	12.5	<0.01
p.c.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0c	-	-
F	-	-	-	1	3.9	6.2	5.1	12.7	22.3	30.7		
P	-	-	-	0.38	0.03	<0.01	0.02	<0.01	<0.01	<0.01		
Larvae												
500 ppm	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	3.3 ± 1.7^{CD}	7.8 ± 3.2^{C}	24.8 ± 5.4^{B}	48.8 ± 9.5^{AB}	61.5 ± 10.4^{ABab}	68.3 ± 10.9^{AB}	73.7 ± 9.8^{Aa}	75.3 ± 9.4^{Aa}	43.7	<0.01
1000 ppm	0.0 ± 0.0^{C}	2.2 ± 1.5^{BC}	6.7 ± 2.9^{BC}	11.6 ± 5.0^{B}	31.0 ± 3.9^{A}	56.9 ± 9.1^{A}	71.7 ± 10.9^{Aa}	76.8 ± 11.0^{A}	82.6 ± 7.8^{Aa}	93.9 ± 3.2^{Aa}	36.4	<0.01
p.c.	0.0 ± 0.0^{D}	2.2 ± 1.5^{D}	7.8 ± 2.2^{C}	17.8 ± 3.6^{BC}	23.3 ± 4.1^{AB}	28.9 ± 5.1^{AB}	34.4 ± 4.4^{AB}b	40.0 ± 4.4^{AB}	42.2 ± 3.6^{Ab}	47.8 ± 4.0^{Ab}	34.4	<0.01
F	-	1.1	1.0	2.2	0.90	2.3	3.5	3.2	8.2	12.1		
P	-	0.34	0.39	0.13	0.42	0.13	0.05	0.06	<0.01	<0.01		

For each developmental stage, within each row, means followed by the same uppercase letter are not significantly different, in all cases $df = 9, 89$; Tukey HSD test at $P = 0.05$. For each developmental stage, within each column, means followed by the same lowercase letter are not significantly different, in all cases $df = 2, 26$; Tukey's HSD test at $P = 0.05$. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Tribolium confusum

T. confusum adults were susceptible to the EO (Table 32). In fact, complete mortality was reached 4 (at 1000 ppm) and 7 days (at 500 ppm) post-exposure. For the EO-NE (Table 33), no mortality was observed 4 days after the exposure at both the concentrations tested. By the end of the experiment, the EO-NE led to 12.2 and 21.4% mortality at 500 and 1000 ppm, respectively. The positive control was also quite ineffective 7 days post-exposure. *T. confusum* larvae were highly susceptible to the EO. In fact, 100% mortality was reached after 16 h (1000 ppm) and 1 day (500 ppm) of exposure (Table 32). For the EO-NE, the mortality of the larvae was low 1-day post-exposure, but it increased during the days of experiments. After 7 days of exposure 78.0 and 98.9% mortality were reached at 500 and 1000 ppm, respectively (Table 33). Pirimiphos-methyl led to lower mortality after 7 days of exposure, if compared to both the EO and EO-NE (Table 32 and 33).

As previously described for *T. castaneum* adults, also *T. confusum* adults are more tolerant than larvae (Arthur, 2010; Athanassiou and Kavallieratos, 2014; Boukouvala and Kavallieratos, 2022; Deb and Kumar, 2020; Lee et al., 2020a) and in this case *C. acaulis* EO (100% mortality reached even at 500 ppm) can be considered an effective tool for managing these species. Regarding *C. acaulis* EO-NE, it showed higher mortality of *T. confusum* adults (21.4%) than some pyrrole derivatives, for which the toxicity was lower than 20% (Boukouvala et al., 2016, 2019a).

Table 32. Mean (%) mortality ± standard errors (SE) of *Tribolium confusum* adults and larvae after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil (EO) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	3.3 ± 1.7^{B}	62.2 ± 3.6^{Ab}	80.0 ± 3.7^{Ab}	91.1 ± 3.9^{Ab}	94.4 ± 3.4^{Aa}	98.9 ± 1.1^{Aa}	100.0 ± 0.0^{Aa}	291.5	< 0.01
1000 ppm	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	1.1 ± 1.1^{C}	5.6 ± 2.4^{B}	77.8 ± 4.3^{Aa}	96.7 ± 1.7^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	172.4	< 0.01
p.c.	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{Bc}	0.0 ± 0.0^{Bc}	0.0 ± 0.0^{Bc}	0.0 ± 0.0^{Bb}	0.0 ± 0.0^{Bb}	3.3 ± 1.7^{Ab}	4.0	< 0.01
F	-	-	1.0	2.8	2870.6	7612.2	10507.1	13632.9	158313.7	91.2		
P	-	-	0.38	0.08	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		
Larvae												
500 ppm	24.4 ± 4.4^{C}	52.2 ± 4.7^{Ba}	81.1 ± 4.6^{ABa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	18.5	< 0.01
1000 ppm	48.9 ± 2.6^{C}	88.9 ± 4.6^{Ba}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	92.3	< 0.01
p.c.	3.3 ± 1.7^{D}	6.7 ± 2.4^{Db}	15.6 ± 2.4^{Cb}	16.7 ± 2.4^{BCb}	21.1 ± 2.0^{ABCb}	26.7 ± 2.9^{ABCb}	33.3 ± 2.4^{ABCb}	40.0 ± 2.4^{ABb}	44.4 ± 3.8^{Ab}	51.1 ± 2.0^{Ab}	24.5	< 0.01
F	24.0	38.3	139.8	1250.0	242.2	110.3	215.0	241.3	95.2	293.0		
P	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		

For each developmental stage, within each row, means followed by the same uppercase letter are not significantly different, in all cases $df = 9, 89$; Tukey HSD test at $P = 0.05$. For each developmental stage, within each column, means followed by the same lowercase letter are not significantly different, in all cases $df = 2, 26$; Tukey's HSD test at $P = 0.05$. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Table 33. Mean (%) mortality ± standard errors (SE) of *Tribolium confusum* adults and larvae after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil-nanoemulsion (EO-NE) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	4.4 ± 1.8^{Bab}	8.9 ± 2.0^{Aa}	12.2 ± 1.5^{Ab}	28.2	<0.01
1000 ppm	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	8.9 ± 2.0^{Ba}	14.4 ± 1.8^{Aa}	21.4 ± 1.1^{Aa}	105.6	<0.01
p.c.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{c}	-	-
F	-	-	-	-	-	-	-	8.9	37.9	750.4		
P	-	-	-	-	-	-	-	<0.01	<0.01	<0.01		
Larvae												
500 ppm	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	2.2 ± 1.5^{DE}	8.9 ± 2.6^{CD}	18.9 ± 4.2^{BC}	42.2 ± 8.9^{AB}	49.6 ± 11.3^{AB}	60.0 ± 12.6^{AB}	69.8 ± 10.8^{A}	78.0 ± 8.3^{Ab}	33.9	<0.01
1000 ppm	0.0 ± 0.0^{D}	1.1 ± 1.1^{D}	4.4 ± 2.4^{D}	14.4 ± 2.9^{C}	27.8 ± 3.2^{BC}	45.3 ± 5.0^{AB}	52.8 ± 4.6^{AB}	66.5 ± 5.9^{AB}	83.0 ± 4.3^{A}	98.9 ± 1.1^{Aa}	72.7	<0.01
p.c.	0.0 ± 0.0^{F}	2.2 ± 1.5^{EF}	5.6 ± 1.8^{DE}	12.2 ± 3.2^{CD}	20.0 ± 4.1^{BC}	32.2 ± 3.2^{AB}	40.0 ± 3.3^{AB}	51.1 ± 3.5^{AB}	55.6 ± 2.9^{A}	62.2 ± 2.8^{Ab}	43.5	<0.01
F	-	1.1	1.0	0.8	1.8	0.8	0.9	0.9	2.9	10.3		
P	-	0.35	0.39	0.46	0.19	0.47	0.43	0.41	0.08	<0.01		

For each developmental stage, within each row, means followed by the same uppercase letter are not significantly different, in all cases $df = 9, 89$; Tukey HSD test at $P = 0.05$. For each developmental stage, within each column, means followed by the same lowercase letter are not significantly different, in all cases $df = 2, 26$; Tukey's HSD test at $P = 0.05$. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Tenebrio molitor

Regarding the EO toxicity on *T. molitor* adults, no mortality was detected 1 day after initial exposure (Table 34). However, 81.1% of adult mortality was reached at 1000 ppm at the end of the experiments, followed by pirimiphos-methyl (55.6%), and by the EO at 500 ppm concentration (27.8%). For EO-NE (Table 35), mortality rates resulted low after 2 days of exposure at both the concentrations tested. By the end of the trials, EO-NE showed a mortality of 41.9 and 85.2% at 500 and 1000 ppm, respectively. Pirimiphos-methyl showed higher mortality values than the EO-NE. Larvae of *T. molitor* were susceptible to the EO, and complete mortality was achieved after 4 and 7 days of exposure at 1000 and 500 ppm, respectively. The mortality caused by the positive control was lower after 7 days of initial exposure (Table 34). Regarding EO-NE, very low mortality rates were registered even at the end of the trials (Table 35). In fact, the positive control showed the highest larvicidal action.

C. acaulis EO was highly effective against *T. molitor l*arvae. Different studies reported the low effectiveness of different insecticides. For instance, Kavallieratos et al. (2019) demonstrated the low action of spinosad, deltamethrin, and silicoSec applied on wheat (mortalities of 8.9, 6.7, and 5.6%, respectively). Low efficacy was also detected for some plant-derived products. For example, Ntalli et al. (2021) tested different compounds and only 2-undecanone managed to kill 87.8% of individuals 7 days post-exposure. The other compounds, such as *(E)*-anethole, furfural, (*E*,*E*)-2,4-decadienal, (*E*)-2-decenal, and acetic acid caused a mortality between 16.7 and 80.0%. Regarding EOs tested on *T. molitor*, *T. vulgare* EO caused 8.9% mortality 7 days post-exposure (Kavallieratos et al., 2021b). Even if *C. acaulis* EO did not lead to complete mortality, its insecticidal activity was even higher than that of other formulations applied on wheat at 1000 ppm.

For instance, *Pogostemon cablin* (Blanco) Benth. EO showed a mortality of 21.1%, *Elettaria cardamomum* (L.) Maton EO of 23.3%, *Syzygium aromaticum* (L.) Merr. & L.M.Perry EO of 43.3%, *Coriandrum sativum* L. EO of 30.0%, *Melaleuca cajuputi* Powell EO of 50.0%, *Boswellia carteri* Birdw. EO of 33.3%, *C. citriodora* EO of 40.0%, *T. vulgaris* EO of 40.0%, *Copaifera officinalis* L. EO of 46.7%, and *Santalum album* L. EO of 60.0% 7 days post-exposure (Kavallieratos et al., 2020, 2021b, 2022d; Ntalli et al., 2021). Concerning *C. acaulis* EO-NE, the mortality of adults displayed in our study (85.2%) resulted higher than that caused by deltamethrin (24.4%), spinosad (35.6%), silicoSec (44.4%), and pirimiphos-methyl (66.7%) 7 days post-exposure and applied at the label dose (Kavallieratos et al., 2019). The mortality caused by the above-mentioned insecticidal products on larvae was lower than 29%. The comparison between the efficacy of this EO-NE and that of other EO-based NEs is difficult, since this research field is still unexplored.

Table 34. Mean (%) mortality ± standard errors (SE) of *Tenebrio molitor* adults and larvae after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil (EO) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	2.2 ± 1.5^{C}	5.6 ± 2.4^{BC}	14.4 ± 4.1^{ABb}	20.0 ± 5.5^{Ab}	24.4 ± 6.3^{Ab}	27.8 ± 6.4^{Ab}	16.6	< 0.01
1000 ppm	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	7.8 ± 2.2^{C}	12.2 ± 3.2^{C}	30.0 ± 3.7^{Ba}	43.3 ± 5.8^{ABa}	60.0 ± 7.8^{ABa}	81.1 ± 6.3^{Aa}	79.6	< 0.01
p.c.	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	7.8 ± 2.2^{C}	15.6 ± 2.9^{B}	31.1 ± 3.5^{Aa}	51.1 ± 3.9^{Aa}	52.2 ± 3.6^{Aa}	55.6 ± 4.8^{Aa}	102.6	< 0.01
F	-	-	-	-	2.7	3.1	5.9	8.3	7.1	8.7		
P	-	-	-	-	0.09	0.06	0.01	< 0.01	< 0.01	< 0.01		
Larvae												
500 ppm	0.0 ± 0.0^{D}	1.1 ± 1.1^{CD}	5.6 ± 2.4^{Cab}	12.2 ± 2.8^{Ba}	43.3 ± 3.7^{Ab}	81.1 ± 8.1^{Aa}	91.1 ± 4.8^{Aa}	94.4 ± 3.8^{Aa}	98.9 ± 1.1^{Aa}	100.0 ± 0.0^{Aa}	75.7	< 0.01
1000 ppm	1.1 ± 1.1^{D}	2.2 ± 1.5^{CD}	6.7 ± 2.4^{Ca}	16.7 ± 3.7^{Ba}	87.8 ± 6.4^{Aa}	98.9 ± 1.1^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	65.0	< 0.01
p.c.	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{Bb}	0.0 ± 0.0^{Bb}	0.0 ± 0.0^{Bc}	3.3 ± 1.7^{Bb}	8.9 ± 2.0^{Ab}	8.9 ± 2.0^{Ab}	8.9 ± 2.0^{Ab}	15.6 ± 2.4^{Ab}	19.8	< 0.01
F	1.0	1.1	4.1	19.0	1141.9	76.9	48.5	49.7	51.4	190.7		
P	0.38	0.35	0.03	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		

For each developmental stage, within each row, means followed by the same uppercase letter are not significantly different, in all cases $df = 9, 89$; Tukey HSD test at $P = 0.05$. For each developmental stage, within each column, means followed by the same lowercase letter are not significantly different, in all cases $df = 2, 26$; Tukey's HSD test at $P = 0.05$. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Table 35. Mean (%) mortality ± standard errors (SE) of *Tenebrio molitor* adults and larvae after 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil-nanoemulsion (EO-NE) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	4 h	8 h	16 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	*F*	*P*
Adults												
500 ppm	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	1.1 ± 1.1^{E}	3.3 ± 1.7^{DE}	4.4 ± 1.8^{CDE}	8.9 ± 2.6^{CDb}	12.2 ± 2.8^{BCb}	21.1 ± 2.0^{ABb}	36.1 ± 2.0^{Ab}	41.9 ± 4.1^{Ac}	26.0	<0.01
1000 ppm	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	2.2 ± 1.5^{E}	5.6 ± 2.4^{DE}	12.2 ± 2.2^{CD}	17.8 ± 3.2^{Cab}	22.2 ± 4.0^{BCab}	32.5 ± 2.1^{ABCa}	53.1 ± 3.6^{ABa}	85.2 ± 2.5^{Aa}	38.0	<0.01
p.c.	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	3.3 ± 1.7^{C}	10.0 ± 2.9^{B}	24.4 ± 2.9^{Aa}	34.4 ± 2.9^{Aa}	43.3 ± 3.7^{Aa}	50.0 ± 3.3^{Aa}	60.0 ± 2.9^{Ab}	75.6	<0.01
F	-	-	1.1	0.2	2.5	4.7	4.4	15.4	8.9	35.8		
P	-	-	0.35	0.81	0.10	0.02	0.02	<0.01	<0.01	<0.01		
Larvae												
500 ppm	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	1.1 ± 1.1^{B}	1.1 ± 1.1^{B}	1.1 ± 1.1^{B}	3.3 ± 1.7^{AB}	7.8 ± 2.2^{Ab}	10.0 ± 2.4^{Ab}	11.1 ± 3.1^{Ab}	8.3	<0.01
1000 ppm	0.0 ± 0.0^{D}	1.1 ± 1.1^{D}	1.1 ± 1.1^{D}	2.2 ± 1.5^{CD}	2.2 ± 1.5^{CD}	2.2 ± 1.5^{CD}	6.7 ± 1.7^{BC}	14.4 ± 1.8^{ABa}	17.8 ± 2.8^{ABa}	18.9 ± 3.5^{Aab}	18.7	<0.01
p.c.	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	1.1 ± 1.1^{CD}	4.4 ± 1.8^{BC}	6.7 ± 2.4^{B}	12.2 ± 1.5^{Aab}	16.7 ± 2.4^{Aa}	25.6 ± 2.9^{Aa}	34.0	<0.01
F	-	1.0	1.0	1.1	0.3	1.3	1.0	4.5	3.4	5.1		
P	-	0.38	0.38	0.35	0.77	0.28	0.38	0.02	0.05	0.01		

For each developmental stage, within each row, means followed by the same uppercase letter are not significantly different, in all cases $df = 9, 89$; Tukey HSD test at $P = 0.05$. For each developmental stage, within each column, means followed by the same lowercase letter are not significantly different, in all cases $df = 2, 26$; Tukey's HSD test at $P = 0.05$. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Acarus siro

C. acaulis EO showed high mortality on *A. siro* adults and nymphs (Table 36). At the end of the experiments, 91.1% mortality was reached at 1000 ppm, while the positive control led just to 63.3% of adult mortality. Regarding nymphs, high mortality (95.6%) was reached at 1000 ppm at the end of the trials. Knowledge of the use of botanical pesticides on this species is limited.

It has been reported that NEs containing *T. ammi* and *P. anisum* EOs and sprayed on wheat led to 29.8–38.1 and 21.1–86.6% mortality on adults and nymphs, respectively, 7 days after the exposure (Kavallieratos et al., 2022d). In addition, also *M. longifolia* EO and its NE have been reported for their toxicity on this pest, leading to 87.8 and 82.2% (adults) or 67.8 and 65.6% (nymphs), respectively (Kavallieratos et al., 2022c). Some natural pesticides have been considered for the management of this pest (i.e., zeolites), but they were not able to lead to mortality higher of 61.5 and 66.7% on adults and nymphs, respectively (Kavallieratos et al., 2018b).

Table 36. Mean (%) mortality ± standard errors (SE) of *Acarus siro* adults and nymphs 4–16 h, and 1–7 days in wheat treated with *Carlina acaulis* essential oil (EO) distributed at two concentrations and with pirimiphos-methyl (positive control, which correspond to the abbreviation p.c.).

Exposure	**4 h**	**8 h**	**16 h**	**1 day**	**2 days**	**3 days**	**4 days**	**5 days**	**6 days**	**7 days**	***F***	***P***
Adults												
500 ppm	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	2.2 ± 1.5^{DEb}	2.2 ± 1.5^{DEb}	7.8 ± 2.8^{CDb}	10.0 ± 2.4^{BCb}	15.6 ± 2.4^{ABb}	18.9 ± 3.1^{ABb}	31.1 ± 3.1^{Ac}	26.2	< 0.01
1000 ppm	0.0 ± 0.0^{F}	0.0 ± 0.0^{F}	0.0 ± 0.0^{F}	5.6 ± 1.8^{Eab}	16.7 ± 2.9^{Da}	26.7 ± 2.4^{CDa}	35.6 ± 1.8^{BCa}	50.0 ± 2.4^{ABCa}	64.4 ± 2.4^{ABa}	91.1 ± 3.1^{Aa}	158.2	< 0.01
p.c.	0.0 ± 0.0^{F}	0.0 ± 0.0^{F}	0.0 ± 0.0^{F}	8.9 ± 2.0^{Ea}	13.3 ± 2.4^{DEa}	24.4 ± 3.4^{CDa}	31.1 ± 2.6^{BCa}	40.0 ± 3.7^{ABCa}	48.9 ± 3.5^{ABa}	63.3 ± 3.7^{Ab}	144.0	< 0.01
F	-	-	-	3.4	28.1	12.3	14.1	38.7	46.8	69.4		
P	-	-	-	0.05	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		
Nymphs												
500 ppm	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{Db}	4.4 ± 1.8^{Cb}	20.0 ± 2.4^{Ba}	30.0 ± 1.7^{ABa}	34.4 ± 2.4^{ABb}	37.8 ± 3.6^{ABb}	40.0 ± 4.1^{Ab}	43.3 ± 3.7^{Ab}	131.7	< 0.01
1000 ppm	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	12.2 ± 2.2^{Da}	22.2 ± 3.2^{Ca}	37.8 ± 4.9^{BCa}	56.7 ± 4.7^{ABa}	71.1 ± 3.9^{Aa}	82.2 ± 3.6^{Aa}	87.8 ± 3.6^{Aa}	95.6 ± 1.8^{Aa}	194.4	< 0.01
p.c.	0.0 ± 0.0^{E}	0.0 ± 0.0^{E}	2.2 ± 1.5^{DEb}	5.6 ± 2.4^{DEb}	8.9 ± 2.6^{CDb}	16.7 ± 2.9^{BCb}	21.1 ± 3.1^{ABc}	35.6 ± 4.1^{ABb}	41.1 ± 4.6^{ABb}	46.7 ± 4.7^{Ab}	33.1	< 0.01
F	-	-	20.5	9.5	10.8	12.0	36.9	30.5	31.1	39.8		
P	-	-	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01		

For each developmental stage, within each row, means followed by the same uppercase letter are not significantly different, in all cases $df = 9, 89$; Tukey HSD test at $P = 0.05$. For each developmental stage, within each column, means followed by the same lowercase letter are not significantly different, in all cases $df = 3, 35$; Tukey HSD test at $P = 0.05$. Where no letters exist, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

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5.9.3 Conclusions

To conclude, our studies offered detailed information on the efficacy of *C. acaulis* EO for the management of different developmental stages of several stored-product pests, displaying its high activity regardless of the developmental stage in the majority of the assayed pests. Moreover, on *T. confusum*, *T. granarium*, and *T. molitor* has also been investigated the efficacy of the EO-NE, which resulted effective on adults of *T. molitor* and larvae of *T. confusum* and *T. granarium*. The encapsulation of EOs has been described as a promising tool for the management of different arthropods affecting stored grains, as demonstrated for *Hazomalania voyronii* (Jum.) Capuron and *M. longifolia* EO-NEs (Kavallieratos et al., 2021a, 2022c). The *C. acaulis* EO should be tested more for its potential against stored-products pests, but also NEs development and commercialization should also be boosted at a regulatory level.

6. Total synthesis of carlina oxide analogues and evaluation of their insecticidal potential and safety profile

6.1. Introduction

Agriculture and public health are enormously influenced by the use of pesticides, which play a crucial role for the increase of food and fiber production but also for the improvement of human health with the reduction of the onset of vector-borne diseases (Blindauer et al., 1999). However, the misuse and overuse of pesticides have caused several negative consequences, such as their accumulation in food, water, and soil but also effects on human health, on the ecosystem, and the increase of pesticide resistance (Mossa et al., 2018; Souto et al., 2021). In this context, botanical pesticides are increasingly gaining more attention, due to their promising insecticidal properties, often linked to low environmental and health impacts (Isman, 2020a,b). The promising insecticidal activity of *C. acaulis* reported in the previous Sections surely revolves around carlina oxide. This compound also displayed moderate toxicity on human cells and on rats. Considering that the encouraging outcomes obtained with *C. acaulis*-derived products arise from the presence of the above-mentioned polyacetylene, the aim of this work was the development of a synthetic protocol to produce carlina oxide analogues and the investigation of their insecticidal activity. The latter was evaluated and compared with that of carlina oxide and *C. acaulis* EO in terms of topical toxicity on the housefly *M. domestica* and in terms of larvicidal activity, sublethal toxicity, and influence on adult emergence on *Cx. quinquefasciatus* 3rd instar larvae. Moreover, the cytotoxicity of these compounds was also evaluated on HaCat cells and compared to that of carlina oxide.

6.2. Materials and methods

6.2.1 Synthesis of carlina oxide analogues

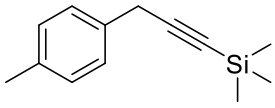
Experimental general

All reagents and solvents were purchased from Merk KGaA (Darmstadt, Germany) and used without additional purification, except tetrahydrofuran THF (freshly distilled over metallic sodium) and toluene (dried over 3 Å molecular sieves). ^{1}H (400 MHz) and ^{13}C (100 MHz) spectra were acquired on a Varian Mercury 400 (Varian, Inc., Palo Alto, CA, USA). IR spectra (cm^{-1}) were recorded with a Perkin-Elmer FT-IR spectrometer Spectrum Two UATR (Perkin Elmer, Inc., Waltham, MA, USA). GC-MS analyses were performed using a Hewlett-Packard GC/MS 6890N working with the EI technique (70 eV). Compounds purity assessment was accomplished through GC-MS and NMR analyses.

Step I: Synthesis of compounds 3a-f

Compounds **3a-f** were synthetized according to the procedure of Hameury et al. (2009) with some modifications. i-PrMgCl (2 M in THF, 20.0 mmol, 4.0 equiv) was slowly added at 0°C to a solution of ethynyltrimethylsilane (20.0 mmol, 4.0 equiv) in THF (10 mL). The reaction mixture was stirred for 30 min at 0°C and for 30 min at r.t. Then, CuBr-dimethylsulfide complex (3 mmol, 0.6 equiv) was added all at once, and the mixture was stirred at r.t. for 30 min before the addition of substrates **2a-f** (5.0 mmol). The reaction was refluxed for 16 h. After that time, the solution was cooled to r.t. and poured into a saturated aqueous solution of NH_4Cl (200 mL). The aqueous phase was extracted with Et_2O (2 × 250 mL), and the organic layers were washed with H_2O (200 mL), dried over $MgSO_4$, filtered, and concentrated. The crude products were purified by silica gel chromatography (100% hexane) to afford the products in different yields.

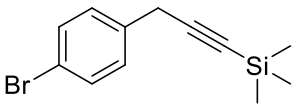
Trimethyl(3-(p-tolyl)prop-1-yn-1-yl)silane (**3a**)



3a

Colourless oil (174 mg, 86% yield). NMR spectra were in accordance with data reported in the literature (Louvel et al., 2013). IR (neat) 2959, 2176, 1514, 1418, 1249, 1030, 1020, 838, 793, 758, 638, 476 cm^{-1}. MS (EI): m\z = 202 (M^+), 187 (100%), 172, 157, 128, 73.

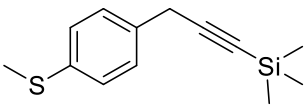
(3-(4-Bromophenyl)prop-1-yn-1-yl)trimethylsilane (**3b**)



3b

Colourless oil (216 mg, 81% yield). ^1H NMR (500 MHz, $CDCl_3$) δ 7.49 – 7.46 (m, 1H), 7.46 – 7.44 (m, 1H), 7.24 (d, J = 8.4 Hz, 2H), 3.62 (s, 2H), 0.21 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.88, 131.55, 129.63, 129.00, 128.30, 120.43, 103.50, 87.46, 25.71, 0.05. IR (neat) 2959, 2178, 1515, 1405, 1248, 1071, 1028, 1012, 841, 791, 759, 635, 474 cm^{-1}. MS (EI): m\z = 267 (M^+), 252 (100%), 222, 194, 172, 128, 73.

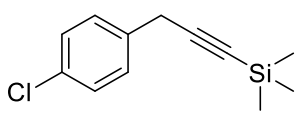
Trimethyl(3-(4-(methylthio)phenyl)prop-1-yn-1-yl)silane (**3c**)



3c

Colourless oil (169 mg, 72% yield). ^1H NMR (500 MHz, $CDCl_3$) δ 7.31 – 7.27 (m, 2H), 7.27 – 7.23 (m, 2H), 3.63 (s, 2H), 2.50 (s, 3H), 0.21 (s, 9H),. ^{13}C NMR (101 MHz, $CDCl_3$) δ 136.63, 133.60, 128.60, 127.23, 104.34, 87.19, 25.90, 16.39, 0.31. IR (neat) 2958, 2175, 1492, 1404, 1248, 1091, 967, 908, 839, 794, 732, 642, 484 cm^{-1}. MS (EI): m\z = 234 (M^+, 100%), 187, 159, 109, 73.

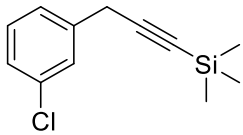
(3-(4-Chlorophenyl)prop-1-yn-1-yl)trimethylsilane (**3d**)



3d

Colourless oil (174 mg, 78% yield). NMR spectra were in accordance with data reported in literature, as well as IR spectrum (Larsen et al., 2009). MS (EI): m\z = 222 (M^+), 207 (100%), 179, 128, 73.

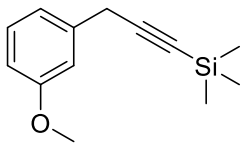
(3-(3-Chlorophenyl)prop-1-yn-1-yl)trimethylsilane (**3e**)



3e

Colourless oil (138 mg, 62% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.34 (m, 1H), 7.26 – 7.22 (m, 3H), 3.63 (s, 2H), 0.20 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.58, 134.52, 129.91, 128.33, 127.04, 126.28, 103.42, 87.90, 26.14, 26.09, 26.03, 0.28, 0.26. IR (neat) 2959, 2178, 1431, 1250, 1075, 1029, 1010, 840, 759, 649, 474 cm^{-1}. MS (EI): m\z = 222 (M^+), 207 (100%), 179, 128, 73.

(3-(3-Methoxyphenyl)prop-1-yn-1-yl)trimethylsilane (**3f**)



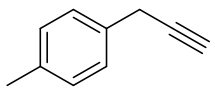
3f

Yellow oil (181 mg, 83% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 (s, 1H), 6.95 – 6.92 (m, 2H), 6.91 (dt, J = 1.6, 0.8 Hz, 1H), 3.81 (s, 3H), 3.64 (s, 2H), 0.19 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.95, 138.13, 129.64, 120.46, 113.65, 112.39, 104.35, 87.25, 55.38, 26.40, 0.32. IR (neat) 2958, 2176, 1435, 1249, 1026, 1015, 840, 759, 650, 441 cm^{-1}. MS (EI): m\z = 218 (M^+), 189 (100%), 173, 73.

Step II: Synthesis of compounds 4a-f

The synthesis of compounds **4a-f** was performed following the work of Louvel et al. (2013). Compound **5** was synthetized according to the procedure previously reported by Gilman and Wright (1933), and its NMR spectra and MS spectra were linear with those reported.

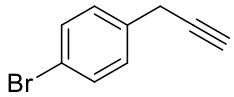
1-Methyl-4-(prop-2-yn-1-yl)benzene (**4a**)



4a

Colourless oil (130 mg, quantitative yield). NMR spectra were in accordance with data reported in the literature (Yang and Ge, 2022). IR (neat) 2256, 1489, 1157, 1125, 990, 915, 732, 660, 597, 493 cm^{-1}. MS (EI): m\z = 130 (M^+), 105 (100%), 91.

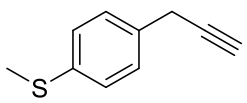
1-Bromo-4-(prop-2-yn-1-yl)benzene (**4b**)



4b

Yellow oil (168 mg, 86% yield). NMR spectra were in accordance with data reported in the literature (Kinena et al., 2018). IR (neat) 2256, 1488, 1220, 1157, 989, 910, 729, 652, 595, 490 cm^{-1}. MS (EI): m\z = 195 (M^+), 115 (100%), 89, 63.

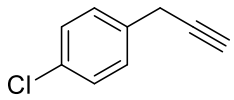
Methyl(4-(prop-2-yn-1-yl)phenyl)sulfane (**4c**)



4c

Yellow solid (153 mg, 94% yield). NMR spectra were in accordance with data reported in the literature (Yang and Ge, 2022). IR (neat) 2253, 1492, 1210, 1153, 985, 904, 727, 649, 592, 488 cm^{-1}. MS (EI): m\z = 162 (M^+), 147, 115 (100%), 89, 63.

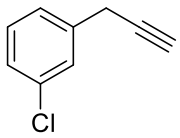
1-Chloro-4-(prop-2-yn-1-yl)benzene (**4d**)



4d

Yellow oil (122 mg, 81% yield). NMR spectra were in accordance with data reported in the literature, as well as IR spectrum (Henrion et al., 2013). MS (EI): m\z = 150 (M^+), 115 (100%), 89, 63.

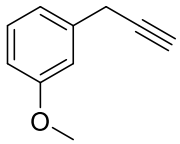
1-Chloro-3-(prop-2-yn-1-yl)benzene (**4e**)



4e

Yellow oil (151 mg, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.36 (m, 1H), 7.25 – 7.22 (m, 3H), 3.59 (d, J = 2.7 Hz, 2H), 2.22 (t, J = 2.7 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.39, 138.24, 134.62, 129.98, 127.20, 126.28, 81.22, 71.36, 24.72. IR (neat) 2154, 1491, 1212, 1160, 990, 906, 730, 652, 593, 489 cm^{-1}. MS (EI): m\z = 150 (M^+), 115 (100%), 89, 63.

1-Methoxy-3-(prop-2-yn-1-yl)benzene (**4f**)



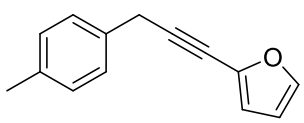
4f

Yellow oil (135 mg, 92% yield). NMR spectra were in accordance with data reported in the literature (Konno et al., 2015). IR (neat) 3292, 2958, 2835, 1600, 1585, 1488, 1454, 1212, 1159, 996, 737, 638, 489 cm^{-1}. MS (EI): m\z = 146 (M^+, 100%), 131, 115, 103, 89, 63.

Step III: Synthesis of compounds 6a-f

Compounds **6a-f** were synthetized accordingly to the methodology developed by Tomas-Mendivil et al. (2015), except for the products' purification, which was performed through silica gel column chromatography (100% hexane).

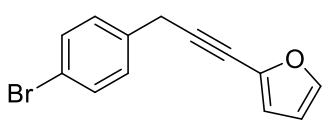
2-(3-(p-Tolyl)prop-1-yn-1-yl)furan (**6a**)



6a

Yellow oil (66 mg, 33.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (m, 1H), 7.27 (d, J = 7.9 Hz, 2H), 7.15 (d, J = 7.7 Hz, 2H), 6.52 (d, J = 2.6 Hz, 1H), 6.39 – 6.35 (m, 1H), 3.81 (s, 2H), 2.34 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 142.72, 137.21, 136.19, 132.66, 129.09, 127.66, 113.96, 110.49, 92.07, 72.52, 25.22, 21.10, 20.84. IR (neat) 3032, 2921, 2216, 1513, 1486, 983, 900, 793, 738, 592, 475 cm^{-1}. MS (EI): m\z = 196 (M^+, 100%), 181, 167, 152, 128, 115, 91, 51.

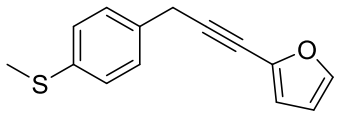
2-(3-(4-Bromophenyl)prop-1-yn-1-yl)furan (**6b**)



6b

Yellow oil (107 mg, 42% yield, purity > 90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (t, J = 2.0 Hz, 1H), 7.48 (d, J = 2.0 Hz, 1H), 7.39-7.38 (m, 1H), 7.29-7.27 (m, 2H), 6.56 (d, J = 3.2 Hz, 1H), 6.40 (dd, J = 3.3, 1.9 Hz, 1H), 3.83 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 143.13, 138.17, 137.13, 134.95, 130.46, 129.72, 129.66, 123.21, 114.46, 110.74, 91.18, 73.35, 25.35. IR (neat) 3033, 2925, 2218, 1515, 1488, 986, 906, 796, 740, 596, 477 cm^{-1}. MS (EI): m\z = 261.5 (M^+), 181 (100%), 168, 152, 115 91, 51.

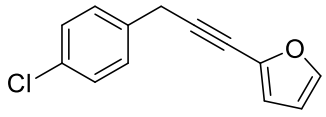
2-(3-(4-(Methylthio)phenyl)prop-1-yn-1-yl)furan (**6c**)



6c

Yellow solid (96 mg, 42% yield, purity > 80%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (dd, J = 1.8, 0.7 Hz, 1H), 7.35 – 7.31 (m, 2H), 7.28 – 7.27 (m, 1H), 7.27 – 7.25 (m, 1H), 6.55 (d, J = 3.4 Hz, 1H), 6.39 (dd, J = 3.4, 1.9 Hz, 1H), 3.83 (s, 2H), 2.50 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 143.01, 137.31, 136.81, 132.94, 128.50, 128.40, 127.21, 127.12, 114.29, 110.71, 91.80, 73.01, 25.34, 16.19. IR (neat) 3035, 2253, 1492, 1093, 985, 904, 727, 649, 592 cm^{-1}. MS (EI): m\z = 228 (M^+), 181 (100%), 152, 126, 91, 51.

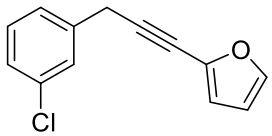
2-(3-(4-Chlorophenyl)prop-1-yn-1-yl)furan (**6d**)



6d

Yellow oil (82 mg, 38% yield) ^{1}H NMR (400 MHz, CDCl3) δ 7.39-7.37 (m, 1H), 7.37 (dd, J = 1.9, 0.7 Hz, 1H), 7.28 (s, 1H), 7.27-7.25 (m, 2H), 6.55 (d, J = 3.4 Hz, 1H), 6.38 (dd, J = 3.4, 1.9 Hz, 1H), 3.82 (s, 2H). ^{13}C NMR (101 MHz, CDCl3) δ 143.35, 138.12, 137.30, 134.68, 130.11, 128.44, 127.32, 126.42, 114.86, 110.94, 91.19, 73.68, 25.76. IR (neat) 3034, 2925, 2218, 1596, 1574, 1431, 1077, 986, 904, 775, 741, 592, 432 cm^{-1}. MS (EI): m\z = 216 (M^+), 181 (100%), 152, 127, 92, 51.

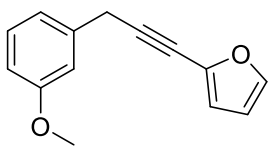
2-(3-(3-Chlorophenyl)prop-1-yn-1-yl)furan (**6e**)



6e

Yellow oil (48 mg, 22% yield, purity > 80%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.38 (m, 1H), 7.37 (dd, J = 1.9, 0.7 Hz, 1H), 7.27 (t, J = 1.4 Hz, 1H), 7.27 – 7.25 (m, 2H), 6.55 (d, J = 3.4 Hz, 1H), 6.38 (dd, J = 3.4, 1.9 Hz, 1H), 3.83 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.43, 138.10, 137.29, 134.67, 130.10, 128.42, 127.31, 126.41, 114.85, 111.05, 91.17, 73.66, 25.75. IR (neat) 3004, 1598, 1577, 1474, 1432, 1264, 1078, 895, 855, 777, 703, 643, 436 cm^{-1}. MS (EI): m\z = 216 (M^+, 100%), 152, 127, 92, 51.

2-(3-(3-Methoxyphenyl)prop-1-yn-1-yl)furan (**6f**)



6f

Yellow oil (134 mg, 63% yield) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (dd, J = 1.9, 0.7 Hz, 1H), 7.25 (s, 1H), 6.99 – 6.97 (m, 1H), 6.97 – 6.94 (m, 1H), 6.80 (dd, J = 8.0, 2.3 Hz, 1H), 6.53 (d, J = 3.4 Hz, 1H), 6.37 (dd, J = 3.4, 1.9 Hz, 1H), 3.83 (s, 2H), 3.82 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.05, 143.21, 137.67, 137.52, 129.82, 120.57, 114.50, 113.96, 112.45, 110.93, 92.02, 73.20, 55.46, 26.06. IR (neat) 2938, 2835, 1600, 1585, 1487, 1464, 1453, 1257, 1152, 1048, 984, 900, 775, 737, 592, 432 cm^{-1}. MS (EI): m\z = 212 (M^+, 100%), 181, 169, 152, 127, 115, 51.

6.2.2 Houseflies

The used houseflies, *M. domestica*, (females, 3–5 days old), were obtained from an established laboratory colony (Crop Research Institute, Czech Republic, >20 generations). The houseflies were reared as detailed by Pavela (2013). The flies were treated and maintained at 25 ± 1 °C, 50–70% RH, and using the 16:8 (L:D) photoperiod. Larvae were reared in a mixture of sterilized bran, milk powder, and water; adults were provided with ad libitum access to water and to milk powder.

6.2.3 Mosquitoes

The mosquito larvae of *Cx. quinquefasciatus* (3rd instar) were obtained from an established laboratory colony (Crop Research Institute, Czech Republic, > 20 generations) as well. The larvae were fed on dog biscuits and yeast powder in a 3:1 ratio. Adults received a 10% sucrose solution and a 1-week-old chick for blood feeding. The mosquitoes were held at 25 ± 2 °C and 70 ± 5% R..H., using the photo regime of 16:8 (L:D) h.

6.2.4 Topical bioassays on *Musca domestica*

Acute topical toxicity of *C. acaulis* EO, carlina oxide, and its analogues on *M. domestica* adult females was evaluated according to the method by Pavela (2013). The females were anaesthetized using carbon dioxide. The compounds were diluted in acetone (p.a. purity, Sigma Aldrich, Czech Republic), using a concentration series prepared to obtain the following doses upon application of 1 μL onto the housefly pronotum: 1, 3, 5, 8, 12, 15, 18, 20, 25, 30, 35 and 40 μg per fly. To calculate the lethal doses alone, 5-6 doses were selected, which caused mortality in the range of 20% to 90%. The doses were applied using a micro-electric applicator; the control flies were treated only with

acetone. A number of 20 adults was used for every dose. After evaporation of acetone (approximately after 3-5 min), the flies were transferred to air-permeable plastic boxes (10 x 15 x 8 cm) containing food in the form of a 20% sugar solution (w:v). The experiment was located in an air-conditioned room at 25 ± 1 °C, 70 ± 3% R.H., and 16:8 h (L:D). The entire experiment was repeated 4 times. Adult mortality was assessed after 24 h from treatment.

6.2.5 Acute toxicity on *Culex quinquefasciatus* larvae

Acute toxicity of *C. acaulis* EO, carlina oxide, and its analogues diluted in DMSO (Sigma-Aldrich, Czech Republic) for *Cx. quinquefasciatus* larvae was assessed following the WHO (1996) procedure with minor modifications by Benelli et al. (2019a).

Experimental treatment was prepared as follows: 1 mL of serial dilution was dissolved using DMSO in 224 mL of distilled water using a 500 mL glass bowl and shaken to produce a homogeneous test solution. The tested concentrations were: 0.5, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, and 2.6 µg mL^{-1}, while each concentration was replicated 4 times. Distilled water containing the same amount of DMSO as that used to dissolve the compounds was used as the negative control. The *Cx. quinquefasciatus* larvae were transferred into water in the bowl containing the prepared test solution (25 larvae/beaker). For every sample concentration, 4 duplicate trials (100 larvae per single replication) were carried out, while each trial included a negative control composed of distilled water with the same amount of DMSO as the test sample. The assays were placed in a growth chamber [16:9 (L:D), 25 ± 1 °C]; mortality was recorded after 24 h.

6.2.6 Sublethal effect against *Culex quinquefasciatus* larvae

To assess the effect of sublethal doses (LC_{30}) of *C. acaulis* EO, carlina oxide, and its analogues on *Cx. quinquefasciatus*, 3rd instar larvae were exposed to each product for 24 h (the used concentrations are presented in Table 39). Subsequently, the surviving larvae were transferred to clean water and provided with a standard diet – dog biscuits and yeast powder. The application methods are described in the paragraph above concerning the larvicidal tests. Larval and pupal mortality and hatched adults were evaluated and 4 replicates were completed for each product tested. Again, the treated insects were placed in a growth chamber (25 ± 1 °C; 16:9 (L:D)).

6.2.7 Larval mortality dynamics in time, upon application of LC_{90}

The rate of mortality increase in time upon application of LC_{90} concentrations was evaluated as follows. The EO, carlina oxide, or respective analogues were mixed in water using an identical method as described above for acute toxicity tests. The initial number of larvae was also identical

(25/replication). The used concentrations are presented in Table 40. Mortality was assessed in time intervals of 4, 8, 12, 16, 20, and 24 h from introducing the larvae in water contaminated with respective substances. Larvae not responding to mechanical stimuli were considered dead. At the time of every evaluation, the dead larvae were removed using a brush and 4 replicates were performed for each products tested. The treated insects were placed in a growth chamber (25 ± 1 °C; 16:9 (L:D)).

6.2.8 Cytotoxicity assays

Cell lines

Immortalized human keratinocytes cell line (HaCaT), provided by IFO (Istituti Fisioterapici Ospitalieri, Rome, Italy), were cultured in DMEM enriched with 10% fetal bovine serum (FBS), 100 IU mL^{-1} penicillin/streptomycin, 2 mM L-glutamine and kept at 37 °C with 5% CO_2, and 95% humidity.

Cell viability assay

Cells were seeded at density of $2x10^3$/well in a 96-wells plate with a final volume of 100 µL. After overnight incubation, cells were treated with different concentration of carlina oxide analogues, *C. acaulis* EO, and carlina oxide (up to 100 µg mL^{-1}) for 3 days and then cytotoxicity was evaluated by adding MTT. After 3 h, the salt crystals were dissolved in 100 µL/well of DMSO. An ELISA reader microliter plate (BioTek Instruments, Winooski, VT) was used to measure the absorbance of samples at 570 nm against a control.

6.2.9 Statistical analysis

The mortality was corrected using the Abbott's formula (Abbott, 1925). Subsequently, $LD_{50(90)}$ and $LC_{50(90)}$ were estimated by probit analysis (Finney, 1971).

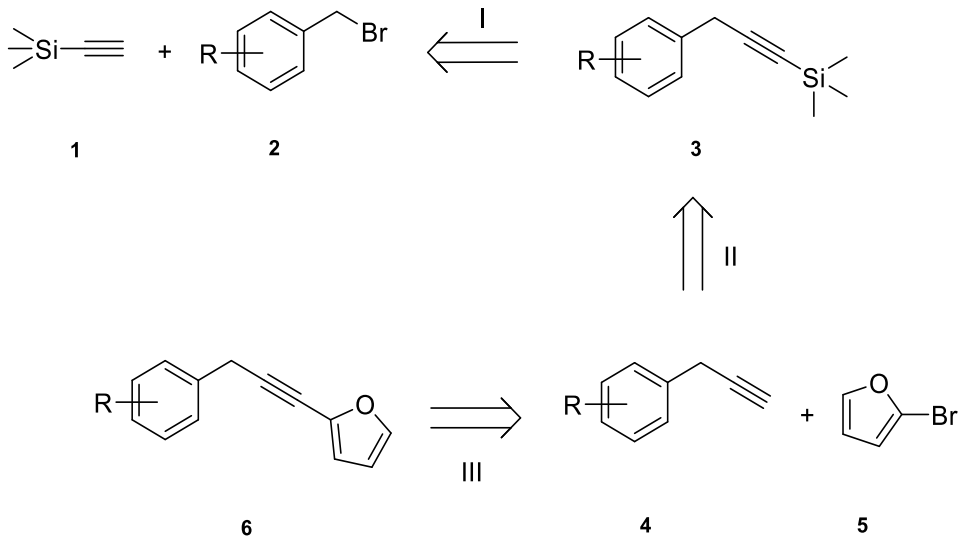
Furthermore, the data in % were transformed using the arcsine square root transformation before being analyzed using ANOVA and Tukey's HSD test ($P \leq 0.05$).

The data of cell cytotoxicity represent the mean with standard deviation (SD) of at least 3 independent experiments. The statistical significance was determined by one-way ANOVA, using Tukey's HSD multiple comparisons test ($p < 0.05$). IC_{50} was calculated using GraphPad Prism software.

6.3. Results and discussion

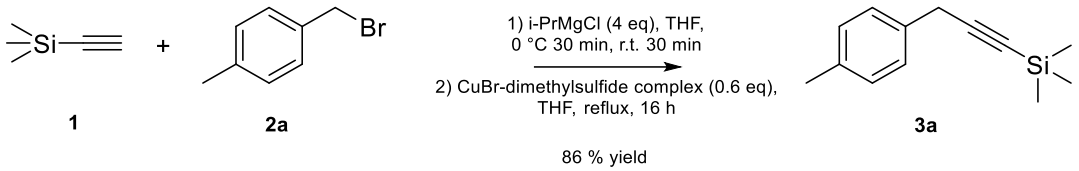
6.3.1 Synthesis of carlina oxide analogues

On the base of the promising insecticidal activities of carlina oxide, a new synthetic protocol for the synthesis of some analogues has been developed, starting from differently substituted benzyl bromide substrates (Scheme 1).



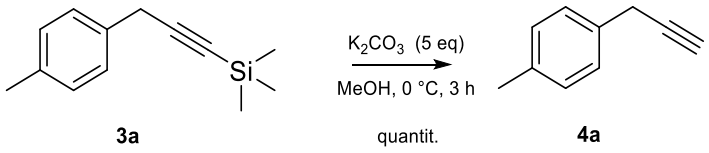
Scheme 1. Retrosynthetic analysis of the synthetic approach.

The developed process involved three steps: (I) the substitution reaction of ethynyltrimethylsilane on the benzyl bromide substrate, (II) the deprotection of the terminal C–H of the alkyne, and (III) the Sonogashira coupling between the differently substituted terminal alkynes and 2-bromofuran. The model substrate used for the assessment of this synthetic approach was 4-methylbenzyl bromide (compound **2a**). Inspired by the work of Hameury et al. (2009), the reaction of **1** with **2a** (I Step) was investigated using initially ethylmagnesium bromide (EtMgBr) as Grignard reagent and copper bromide (CuBr) as catalyst, but no reactivity was observed. After different attempts, characterised by the change of the Grignard reagent or of the catalyst, the use of isopropylmagnesium chloride (i-PrMgCl) and of copper bromide-dimethylsulfide complex in tetrahydrofuran (THF) for 16 h led to **3a** formation in 86% yield (Scheme 2).



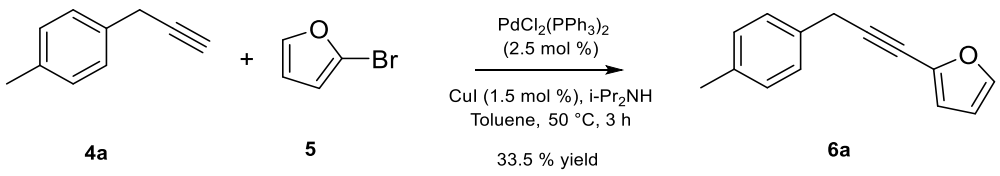
Scheme 2. Substitution reaction of ethynyltrimethylsilane on 4-methylbenzylbromide.

At this point, on the basis of the study of Konno et al. (2015) tetrabutylammonium fluoride (TBAF) was initially chosen as the C-H bond deprotecting agent for the synthesis of **4a** (II Step), and several optimization efforts were executed for the achievement of the optimal reaction conditions, but no product formation was observed. Thus, following the work of Louvel et al. (2013), this step was performed using potassium carbonate (K_2CO_3) in methanol (MeOH) for 3 h at 0 °C with quantitative yield (Scheme 3).



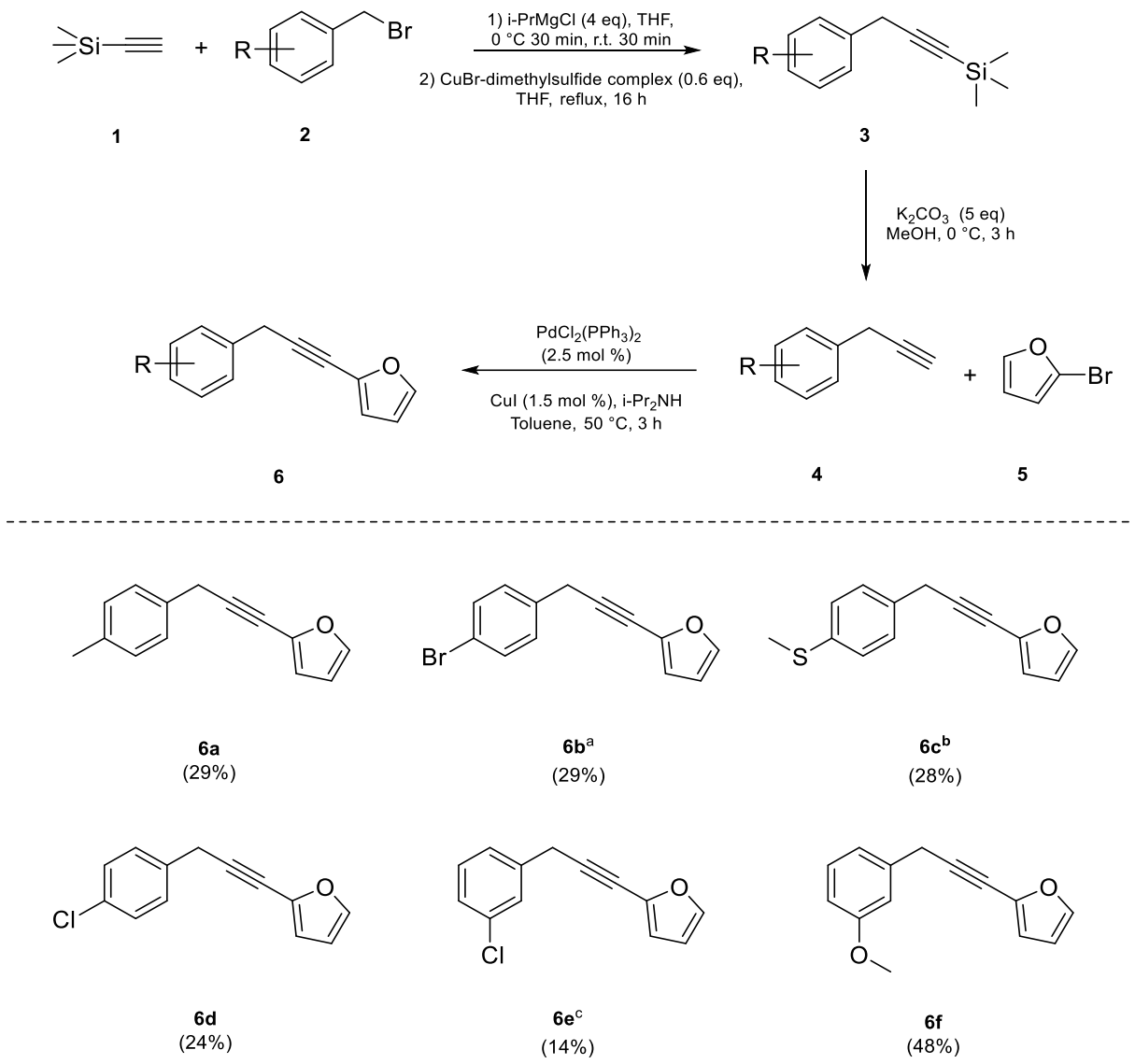
Scheme 3. Synthesis of the alkyne **4a**.

Finally, for the synthesis of compound **6a** (III Step) a Sonogashira coupling between **4a** and 2-bromofuran was performed according to the procedure previously reported by Tomas-Mendivil et al. (2015), using palladium(II)bis(triphenylphosphine) dichloride ($PdCl_2(PPh_3)_2$), copper iodide (CuI), and diisopropylamine (i-Pr_2NH) in toluene at 50°C for 3h. This step led to product formation in 33.5 % yield (Scheme 4).



Scheme 4. Synthesis of compound **6a**.

This synthetic protocol developed for the synthesis of compound **6a**, was applied to other substituted benzyl bromides to obtain compounds **6b-6e** in different overall yields (Scheme 5).



Scheme 5. Substrate scope demonstration. [a]Product > 90% pure, [b,c] product > 80% pure.

With this work, a new synthetic approach to produce carlina oxide analogues was developed, and six target compounds were obtained over three steps, using commercially available starting materials. Chemical modifications of carlina oxide were previously performed by Mami et al. (2021). The authors developed an hemisynthesis to produce carlina oxide analogues by functionalization of the polyacetylene structure on the furan ring, with yields ranging from 17 to 30%. Also, for this study, the yields reported were quite low. Even if an overall yield optimization is still needed for the study presented, it could be the starting point for the synthesis of other derivatives functionalized at the benzyl moiety, opening the way also to structure-relationship studies.

6.3.2 Topical bioassays on *Musca domestica*

Carlina oxide analogues displayed high efficacy in terms of *M. domestica* female mortality. The estimated lethal doses are presented in Table 37. As indicated by the results, all the tested analogues showed an efficacy comparable to that of purified carlina oxide for which LC_{50} was estimated as 5.5 µg female $^{-1}$ and LD_{90} as 11.5 µg female^{-1}. Only **6b** and **6d** provided significantly poorer efficacy; for $LD_{50(90)}$ of other products, the Cl_{95} values overlapped in at least one LD parameter, and thus they cannot be considered as significantly more effective, although, for example, LD_{50} for **6a** was estimated as 2.9 µg female $^{-1}$.

Table 37. Mortality of *Musca domestica* (female adults) after topical application and 24 h exposition.

	LD_{50} (µg female^{-1})	**CI_{95}**	**LD_{90} (µg female^{-1})**	**CI_{95}**	**Chi***	**Df**	***p*-value**
Carlina acaulis EO[a]	5.3	3.2-7.1	11.7	8.9.21.8	6.451	3	0.521
Carlina oxide	5.5	3.3-7-5	11.5	8.6-18.7	3.562	3	0.128
6a	2.9	1.3-5-7	16.3	12.7-25.8	3.502	3	0.321
6b	10.1	8.1-15.6	46.5	35.8-56.2	1.835	3	0.607
6c	9.7	7.2-12.9	55.1	38.7-65.9	4.218	4	0.377
6d	11.6	10.3-13.1	24.5	20.4-32.1	1.861	3	0.601
6e	5.1	4.1-6.2	18.4	16.2-18.5	3.814	3	0.282
6f	4.7	3.8-5-8	15.8	12.3-23.7	1.262	3	0.531

[a]EO, essential oil. LD_{30}, LD_{50} and LD_{90} values in µg female^{-1} and CI_{95} – 95% confidence intervals, essential oils activity is considered significantly different when the 95% CI fail to overlap. * Chi-square value, not significant (ns) at $p > 0.05$ level.

This is the first study reporting the carlina oxide analogues activity on *M. domestica* and a comparison with literature in not easy. However, Metcalf and Fukuto (1962) reported the excellent activity achieved on topical bioassays on *M. domestica* by phenyl diethyl phosphates *meta*-substituted with the sulfurpentafluoro group, which is an electron-withdrawing moiety. Since this effect was not detected for the **6e** analogue, bearing a chlorine atom in the *meta* position, other mechanisms could be responsible for the bioactivity of carlina oxide, and its analogues on this target, and more studies should be performed.

6.3.3 Acute toxicity on *Culex quinquefasciatus* larvae

The lethal concentrations estimated for *Cx. quinquefasciatus* larvae are shown in Table 38. All of the tested analogues showed very promising mortality. Nevertheless, based on $LC_{50(90)}$ comparison, **6e** was found to provide a significantly higher efficacy, exhibiting a significantly lower lethal

concentration ($LC_{50(90)}$ = 0.71(0.85) µg mL^{-1}) compared to the EO or carlina oxide, which showed $LC_{50(90)}$ 1.21(2.28) µg mL^{-1} and 1.31(2.31) µg mL^{-1}, respectively.

Table 38. Mortality of *Culex quinquefasciatus* larvae (3rd instar) – 24 h exposition.

	LC_{50}	**CI_{95}**	**LC_{90}**	**CI_{95}**	**Chi***	***p*-level**	**Df**
Carlina acaulis EO[a]	1.21	1.12-1.31	2.28	2.27-2.59	1.721	0.786	4
Carlina oxide	1.31	1.12-1.47	2.31	1.96-3.05	7.869	0.163	5
6a	2.25	1.47-2.69	3.61	2.74-6.41	4.446	0.188	3
6b	1.44	1.23-1.86	2.41	2.11-3.28	3.852	0.421	3
6c	1.21	1.11-1.31	2.36	2.13-2.67	3.251	0.128	5
6d	1.11	0.93-1.23	2.12	1.95-2.23	2.529	0.896	4
6e	0.71	0.55-0.78	0.85	0.81-1.25	2.189	0.334	3
6f	0.73	0.62-1.11	1.43	1.22-2.02	3.181	0.364	3

[a]EO, essential oil. LC_{50} and LC_{90} values in µg ml^{-1} and CI_{95} – 95% confidence intervals, compounds activity is considered significantly different when the 95% CI fail to overlap. * Chi-square value, not significant (ns) at $p > 0.05$ level.

The introduction of a substituent on the benzyl ring differently modulated the biological activity of the analogues. In detail, the *meta*-substitution led to the best LC_{50} values. Probably, this kind of substitution is crucial for the exploitation of the larvicidal activity on *Cx. quinquefasciatus* and the mechanism of action should be further investigated. The best acute toxicity was observed by the *meta*-chloro analogue. Indeed, the chlorine substituent has been shown to be essential for several insecticidal compounds as DDT, but also for natural products used as antibiotics and anti-tumour agents, such as clindamycin and vancomycin (Birkenmeyer and Kagan, 1970; Harris et al., 1985) or cryptophycin and clavulon (Golakoti et al., 1995; Nagaoka et al., 1985). The presence of chlorine on an aromatic moiety generally causes an increase of lipophilicity, non-bonding interactions with the binding sites, prevention of metabolic hydroxylation at that position, and increase of the electrophilicity of proximate parts of the molecule due to its electron-withdrawing effects (Naumann, 2000). Regarding this last feature, other studies reported the importance of the presence of electron-withdrawing groups in the *meta*-position of benzyl or phenyl moieties. For instance, Metcalf and Fukuto (1962) reported the excellent activity achieved on *M. domestica* by *meta*-substituted phenyl diethyl phosphates and demonstrated that this enhancement of efficacy was due to the electron-withdrawing effect of the *m*-sulfurpentafluoro moiety. Nishiwaki et al. (2000) synthesized differently substituted benzyl derivatives of chloronicotinyl insecticides and demonstrated that the most active was *m*-cyano derivative. Also in this case, this moiety presented an electron-withdrawing nature. However, in the case of the carlina oxide analogues activity herein reported, also the *m*-methoxy analogue showed a good toxicity on *Cx. quinquefasciatus* larvae, almost comparable to that of the chlorine-substituted one. The methoxy group is an electron-

donating moiety, and this suggests that maybe the real key feature affecting the activity is the presence in the *meta*-position of groups enhancing the lipophilicity of the molecule (Pavić et al., 2014). However, this hypothesis should be further investigated.

6.3.4 Sublethal effect against *Culex quinquefasciatus* larvae

Application of the sublethal concentration (LC_{30}) obtained in the acute toxicity assays caused subsequent significant mortality of *Cx. quinquefasciatus* larvae (except for compound **6f**) despite a relatively short exposure period (24 h), and compared to untreated larvae, a significantly reduced percentage of adults then hatched (Table 39). Nevertheless, differences could be observed between the individual tested products. **6b** provided the highest efficacy, causing mortality of 71% larvae and hatching of only 28.7% adults upon application in the concentration of 1 µg mL^{-1}; this result was identical to that observed for the EO and even significantly better compared to the natural carlina oxide.

Table 39. The effect of sublethal concentrations (LC_{30}) of *Carlina acaulis* essential oil (EO), carlina oxide, and its synthetized analogues (exposure time 24 h) on larval and pupal mortality and on the percentage of successfully emerged *Culex quinquefasciatus* adults.

Compounds	Concentration used (µl L^{-1})	Larval mortality (% ± SE)	Pupal mortality (% ± SE)	Hatched adults (% ± SE)
Carlina acaulis EO	0.9	79.0 ± 5.7^{e}	0.7 ± 0.9	20.3 ± 5.2^{a}
Carlina oxide	0.8	58.3 ± 7.4^{d}	0.03 ± 0.5	41.3 ± 6.9^{b}
6a	1.5	53.0 ± 2.2^{d}	0.0 ± 0.0	47.0 ± 2.2^{b}
6b	1.0	71.0 ± 5.0^{e}	0.3 ± 0.5	28.7 ± 4.7^{a}
6c	0.8	32.7 ± 3.4^{c}	0.7 ± 0.5	66.7 ± 3.9^{c}
6d	0.8	33.8 ± 3.1^{c}	0.5 ± 0.2	69.5 ± 3.2^{c}
6e	0.5	39.0 ± 8.6^{c}	1.3 ± 0.4	59.7 ± 8.2^{c}
6f	0.5	8.7 ± 2.4^{b}	0.3 ± 0.5	91.0 ± 2.8^{d}
Control		3.0 ± 2.2^{a}	0.0 ± 0.0	97.0 ± 2.2^{d}
ANOVA $F_{8, 32}$, P [a]		98.3; 0.000	ns	184.3;0.000

[a]ANOVA parameters. In the same column, means followed by different letters are significantly different (ANOVA, Tukey's HSD test, $p < 0.05$). EO = essential oil; ns = not significant ($p > 0.05$).

The results reported above pointed out a better efficacy of compound **6b**, bearing a bromine atom in the *para*-position with respect to the *meta*-chloro substituted analogue (**6e**) that was the most active in the acute toxicity assays. This difference could suggest a major effectiveness of bromine-substituted analogues over time with respect to chlorine-substituted ones.

6.3.5 Larval mortality dynamics in time, upon application of LC_{90}

The dynamics of *Cx. quinquefasciatus* larval mortality increase upon application of a concentration corresponding to LC_{90} values are presented in Table 40.

No or almost no mortality was observed for the first 8 hours from application. A dynamic increase in larval mortality was observed only 12 h from exposure, and fatal mortality occurred within 24 hours from application in all variants except **6e,** which caused only 86.7% mortality, and **6b,** which caused only 55.1% larval mortality. **6a** showed the highest dynamics, exhibiting the same course of the mortality increase rate as the EO, although it should be noted that a much higher applied concentration was used, i.e., 3.6 μg mL^{-1}, while the EO was applied in the concentration of 2.3 μg mL^{-1}.

Table 40. Dynamics of *Culex quinquefasciatus* larval mortality after LC_{90} application.

Compounds concentration (μl L^{-1})	**Time**					
	4 h	**8 h**	**12 h**	**16 h**	**20 h**	**24 h**
Carlina acaulis EO (2.3)	0.0 ± 0.0	0.0 ± 0.0[a]	78.6 ± 4.1[f]	100 ± 0.0[e]	100 ± 0.0[d]	100 ± 0.0[d]
Carlina oxide (2.3)	0.0 ± 0.0	0.0 ± 0.0[a]	62.9 ± 2.8[g]	96.7 ± 2.4[e]	100 ± 0.0[d]	100 ± 0.0[d]
6a (3.6)	0.0 ± 0.0	0.0 ± 0.0[a]	71.9 ± 4.1[f]	100 ± 0.0[e]	100 ± 0.0[d]	100 ± 0.0[d]
6b (2.4)	0.0 ± 0.0	5.2 ± 1.2[b]	28.9 ± 2.5[d]	45.0 ± 4.1[b]	46.7 ± 4.1[b]	55.1 ± 4.1[b]
6c (2.4)	0.0 ± 0.0	0.0 ± 0.0[a]	12.8 ± 1.2[b]	93.3 ± 6.2[e]	98.3 ± 2.4[d]	100 ± 0.0[d]
6d (2.1)	0.0 ± 0.0	0.0 ± 0.0[a]	43.8 ± 3.5[e]	78.7 ± 4.7[c]	95.2 ± 1.5[d]	100 ± 0.0[d]
6e (1.0)	0.0 ± 0.0	0.0 ± 0.0[a]	18.9 ± 2.1[c]	75.0 ± 8.2[c]	81.7 ± 2.4[c]	86.7 ± 6.1[c]
6f (1.5)	0.0 ± 0.0	0.0 ± 0.0[a]	45.7 ± 3.2[e]	86.7 ± 4.7[c]	98.3 ± 1.2[d]	100 ± 0.0[d]
control	0.0 ± 0.0	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0± 0.0[a]
ANOVA $F_{8, 32}$, P [a]	ns	7.0; 0.005	395.6; 0.000	874.2; 0.000	1,528.2; 0.000	1,335.3; 0.000

[a]ANOVA parameters. In the same column, means followed by different letters are significantly different (ANOVA, Tukey's HSD test, $p < 0.05$). EO = essential oil; ns=not significant ($p > 0.05$).

In this case and conversely, to the acute toxicity results, the less active analogues after 24 h resulted to be **6e** (86.7% mortality) and **6b** (55.1% mortality). These analogues bear a chlorine and a bromine atom in the *meta*- and *para*-positions, respectively. These results suggest that probably the presence of an electron-withdrawing group on the benzyl moiety does not have a positive effect on larval mortality over time when treated with concentrations corresponding to LC_{90} values.

6.3.6 Cell viability assay

Results from the cytotoxic assay showed that **6a** and **6d** induce a significant reduction in cell viability (HaCat cell line) compared to carlina oxide and *C. acaulis* EO with an IC_{50} of 20.26 ± 1.2 and 9.18 ± 0.3 μg mL^{-1}, respectively, compared to 34.85 ± 2.4 μg mL^{-1} for carlina oxide and 54.05

± 5.0 μg mL^{-1} for the EO. Moreover, **6f** showed a similar effect to carlina oxide with an IC_{50} of 37.39 ± 2.8 μg mL^{-1}. On the other hand, **6b**, **6c,** and **6e**, with an IC_{50} of 60.38 ± 3.5, 52.68 ± 3.7, and 58.40 ± 3.1 μg mL^{-1} respectively, showed a better safety profile compared to carlina oxide but not to EO which exerted a similar effect (Table 41, Figure 56).

Table 41. *Carlina acaulis* essential oil (EO), carlina oxide, and carlina oxide analogues cytotoxic effects.

HaCat	**IC_{50} ± SD (μg mL^{-1})**
Carlina acaulis EO[a]	54.05 ± 5.0[a]
Carlina oxide	34.85 ± 2.4[b]
6a	20.26 ± 1.2[d]
6b	60.38 ± 3.5[c]
6c	52.68 ± 3.7[a]
6d	9.18 ± 0.3[e]
6e	58.40 ± 3.1[c]
6f	37.39 ± 2.8[b]

[a]EO, essential oil. Data shown are expressed as mean ± SD of three separate experiments. *$p < 0.05$ vs Vhc. IC_{50} ± SD within a column followed by the same letter do not differ significantly (ANOVA, Tukey's HSD test, $p \leq 0.05$).

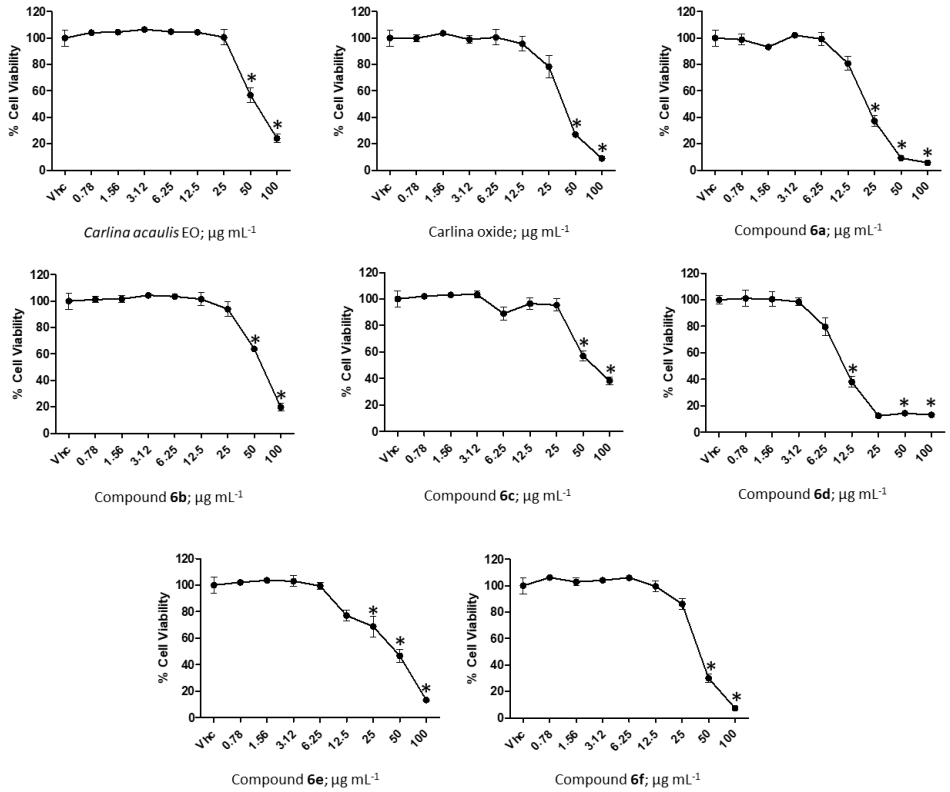


Figure 73. MTT Viability assay after treatment with different concentrations of *Carlina acaulis* essential oil (EO), carlina oxide, and carlina oxide analogues. Cell viability was evaluated after 24 h post-treatment. * $p < 0.05$.

C. acaulis EO was already tested on two cell lines, namely HaCaT and NHFA12, showing a moderate toxicity. In that study, also its ME was evaluated, showing negligible toxicity and demonstrating that the formulation of this EO could reduce its cytotoxicity (Pavela et al., 2021). Thus, once encapsulated into nanocarriers (at 5-10% level) such as nanoemulsions, nanoparticles or liposomes, also the carlina oxide analogues are expected to be not cytotoxic according to the Interantional Standard Organization (ISO) guidelines (ISO 20093-5, 2009), where the reduction of cell viability should not exceed 70% at 100 ppm (Pavela et al., 2021).

6.4. Conclusions

The need for new, effective, and safe insecticides is enormously growing, due to the increasing pesticide resistance caused by their misuse and the negative consequences that derive from it. Natural products could represent one of the keys for the resolution of these problems. *C. acaulis* EO has already shown great insecticidal potential against several insect pests and vectors. Since its activity is linked to the predominant presence of carlina oxide, the aim of this work was the synthesis of carlina oxide analogues for the investigation of their activity compared to their precursor. This study demonstrated the efficacy of carlina oxide, but also highlighted the possibility of generating some analogues with better insecticidal activity, as some of them resulted in a higher efficacy against *M. domestica* and *Cx. quinquefasciatus*. Moreover, since the safety of insecticidal products is of crucial importance for their employment at an industrial level, the cytotoxicity of these products was evaluated on HaCaT cell line, and some of the analogues tested showed a better safety profile than carlina oxide itself. The results obtained in this work open the way for the development of carlina oxide analogues, which could be the starting point to produce compounds with higher insecticidal activity than the precursor, but also for structure-relationship studies on carlina oxide and for the investigation of its mechanism of action. A future perspective linked to this work will also be the evaluation of an industrial scale-up of the synthesis of carlina oxide most promising derivatives. This is due to the fact that nowadays the employment of *C. acaulis* at industrial level is limited by the scarce availability of the raw material, since the plant is a protected species in several European countries.

CHAPTER II: *Acmella oleracea*

1. Introduction

1.1. Taxonomy

Acmella oleracea (L.) R. K Jansen belongs to the Asteraceae (Composite) family and is the most known cultured species of the *Acmella* genus. Concerning its name, there is a general confusion in literature since the genus *Acmella* is often taxonomically confused with the *Spilanthes* genus. This mistaken identity led to several taxonomic reclassifications and name revisions (Paulraj et al., 2013). Indeed, this species is frequently described as *Spilanthes oleracea* L. (Martins et al., 2012), *Spilanthes acmella* L. (Lee et al., 2015), and *A. oleracea* (L.) R. K Jansen (Simas et al., 2013). Nevertheless, anatomical, morphological, phytochemical, and chromosomal research revealed that the two genera, namely *Acmella* and *Spilanthes* are different. This is due to the presence of eight distinct morphological characteristics and specific chromosomes (Paulraj et al., 2013). The accepted name of this plant is *A. oleracea* (L.) R. K Jansen, of the genus *Acmella*, included in the Asteraceae family (Mabberley, 2017) (Table 1).

Table 1. *Acmella oleracea* taxonomy.

Regnum	Plantae
Divisio	Tracheophyta
Sub-divisio	Spermatophyta
Class	Magnoliopsida
Superordo	Asteranae
Ordo	Asterales
Familia	Compositae Giseke
Tribus	Heliantheae Cass.
Genus	*Acmella* Pers.
Species	*Acmella oleracea* (L.) R. K Jansen

The common names used to indicate this plant are: jambú, akmaella, agrião do pará, brede mafane, spot flower, and paracress (Uthpala and Navaratne, 2021) (Figure 1).

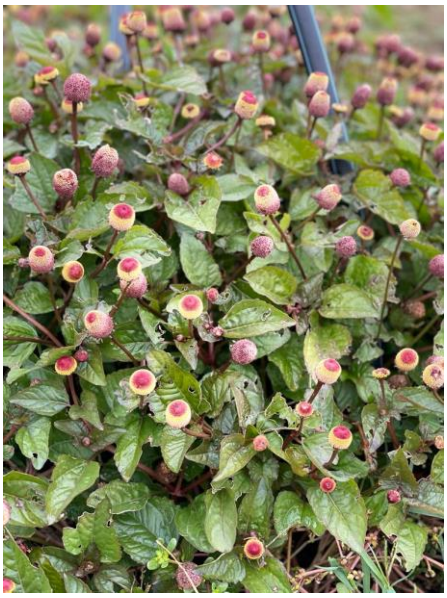


Figure 1. *Acmella oleracea* plant.

1.2. Habitat and distribution

It is believed that the origin of jambú is associated with the cultivation of *Acmella alba* (L'Hér.) R. K. Jansen in the central areas of Perù (Jansen, 1985). However, sometimes its native origin has been described to be Brazil, where it is cultivated as decorative and medicinal plant and where it is known as jambú (Lim, 2016; Prachayasittikul et al., 2013). Despite its unclear and unknown origin, *A. oleracea* is distributed and cultivated worldwide (Figure 2). This plant is distributed in Mexico, Perù, Brazil, Bolivia, USA, northern Australia, Southeast Asia, and Africa (Dubey et al., 2013; Hind and Biggs, 2003; Hossan et al., 2010; Jansen, 1985).

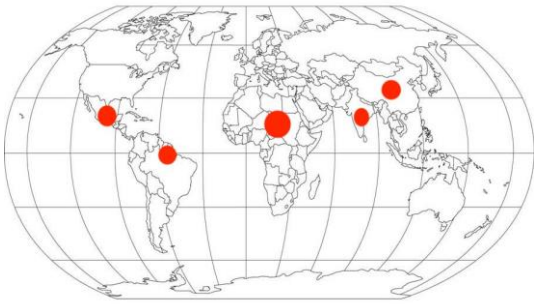


Figure 2. Global distribution of *Acmella oleracea*.

A. oleracea is a perennial herb in the Mediterranean area, where it is spread thanks to its ease of cultivation and growth into wild areas (Lalthanpuii et al., 2016). Due to this great adaptation capability, this plant has been introduced in the tropics and subtropics

of East Africa, since it also grows in humid areas and secondary woodland margins in Uganda, Kenya, and Tanzania (Lim, 2016). This kind of distribution is surely connected to the tendency of the plant to develop and grow in sun exposed areas, while it suffers warmer climates (Sahu et al., 2011). This plant grows in drained soils rich in organic elements up to 1200 m a.s.l. (Lim, 2016). It can be found in pastures, rice paddies, villages, old glades, and rocky riverbanks (Sahu et al., 2011).

1.3. Cultivation and micropropagation

This plant is usually planted in the field by direct sowing. It grows preferentially in soils rich of organic substances, and nitrogen (N) plays a crucial role in the correct development of the plantation (Nascimento et al., 2020).

A. oleracea crops must be constantly watered to avoid a quick drying and should be planted in areas exposed to sun, since they are very sensible to a total shade. Even if the temperature for the germination of seeds is usually 21 °C, this plant can also germinate in winter if protected by glass. It is usually sowed in April, and flowering starts in July and finishes in October. Jambú presents a phenological cycle of 6-8 months and propagates by cutting or seeds. It does not generate roots at the nodes and does not spread much. For this reason, dense cultivations are usually suggested (Hind and Biggs, 2003; Uthpala and Navaratne, 2021) (Figure 3).

Figure 3. *Acmella oleracea* in field crop.

Since this plant has several biological properties, it has aroused great interest also from industries of different fields (Silveira et al., 2018). For this reason, studies on its cultivation have been

performed, in order to increase crop productivity and to quali- and quantitatively standardize its secondary metabolites. For instance, a recent work (Borges et al., 2013) valued the effect of urea and bovine manure on the plant yield and pointed out that the use of mineral fertilizers leads to higher yields in confront to the manure one. Another study (Sousa et al., 2018) evaluated the treatment of the *A. oleracea* crop with leaves of *Syzygium malaccense* (L.) Merr. & L.M.Perry, *Mangifera indica* L., *Inga edulis* Mart., and *Zoysia japonica* Steud. together with a vermicompost, and the optimal dose of this fertilizing mixture was of 10 kg m^{-2}. Concerning the effect on the production of secondary metabolites, it has been demonstrated that the treatment with an organic fertilizer leads to higher contents of flavonoids, *N*-alkylamides, carotenoids, phenols, spermine, and spermidine if compared to a traditional fertilizing agent (Borges, 2012; da Silva Borges et al., 2016). Regarding *N*-alkylamides, also the use of biostimulants has been evaluated for the enhancement of their production. However, the use of a triacontanol-based blend together with a plant tissue extract did not lead to an increase in these metabolites, but only to an increase in the crop yield (Sut et al., 2020).

In addition, methods different from the conventional cultivation systems have been studied. For instance, some authors proposed the use of hydroponic cultivations in order to deal with crop losses due to insects and the onset of plant pathologies. For the hydroponic cultures, different parameters have been taken into consideration in response to growing concentrations of N into the fertilizing solution (Borges et al., 2013). The study pointed out that different N doses lead to diverse growth, plant yields, and post-harvest quality and demonstrated that the optimum dose for high performances is 21 mmol L^{-1} (Sampaio et al., 2021). Nascimento et al. (2020) estimated the effect of the kind of cultivation, conventional or hydroponic, on the leaves, flowers, and stems chemical composition. Conventional crops were able to lead to aerial parts richer in polyphenols and flavonoids than the hydroponic ones, with the exception of flowers. In order to develop an alternative approach to the above-mentioned cultivations of jambú, research also focused on the finding of the best conditions for the plant micropropagation. In fact, a first attempt was performed trying the micropropagation using axillary buds as explants, using a Murashige and Skoog (MS) medium additioned of 2.0 mg L^{-1} of N^6-benzyladenine (Haw and Keng, 2003). Another efficient *in vitro* propagation protocol was developed through the use of a transverse thin-cell layer (tTCL) culture system. In this case, explant source was represented by nodal segments and a MS medium with 5.0 mg dm^{-3} of 6-benzylaminopurine (BAP) was used to promote shoot regeneration (Singh et al., 2009). The study of Singh and Chaturvedi also evaluated the use of a micropropagation protocol starting from the cultures from nodal segments. For the shoot multiplication, MS medium supplemented with 5 μM BAP was employed (Singh and Chaturvedi, 2008). In addition, Leng et al. (2011) carried out a study for the detection of *N*-alkylamides and other secondary metabolites from the mother plant, *in vitro* seedlings, callus cultures, and cultures in the cell suspension. This procedure led for the first time to the detection of spilanthol and *N*-isobutyl-(*2E,4Z,8Z,10E*)-

dodecatetraenamide as main *N*-alkylamides in micropropagated plantlets (Leng et al., 2011). Some years later, Franca and co-workers (2016) assessed spilanthol content in jambú plants grown using three different cultivation systems: *in vitro*, acclimatized, and in field. The data obtained proved that the acclimatized plants had a higher content of spilanthol, followed by the *in vitro* culture and, finally, by the in field crop (Franca et al., 2016). Another research work determined the best dose of growth regulators for the *in vitro* cultivation of the plant and developed an efficient procedure for its micropropagation. The best treatment resulted to be a MS medium with the use of 0.125 mg L^{-1} of the BAP (Almeida et al., 2020). Table 2 resumes the studies carried out on *A. oleracea* cultivation techniques.

Table 2. Research carried out on *Acmella oleracea* cultivation techniques.

Cultivation	Treatment/Medium	Shoot Formation	Plant yield	Minerals/ Secondary Metabolites	References
In field	mineral fertilizer	nr [a]	4.40 kg m^{-2}	high N and K content	Borges et al., 2013
In field	organic fertilizer	nr	2,78 kg m^{-2}	high N and P content	Borges et al., 2013
In field	organic fertilizer and vermicompost	nr	2.90–3.87 kg m^{-2}	nr	Souto et al., 2018
In field	organic fertilization	nr	nr	high levels of total phenolics and carotenoids	da Silva Borges et al., 2016
In field	conventional fertilizer	nr	nr	high levels of nitrates, vitamin C, and organic N	da Silva Borges et al., 2016
In field	biostimulant	nr	enhanced of 22–25%	maintained levels of alkylamides and polyphenols	Sut et al., 2020
In field	organic fertilization	nr	nr	spilanthol concentration: 4.65%	Franca et al., 2016
Acclimatized	in soil	nr	nr	spilanthol concentration: 9.54%	Franca et al., 2016
Hydroponic culture	nitrogen-rich nutrient solution	nr	enhanced (at 21 mmol L^{-1} of N)	nr	Sampaio et al., 2021
In vitro (axillary buds)	MS[b] medium supplemented with 2.0 mg L^{-1} of N^6-benzyladenine	multiple-shoot formation from each axillary bud	nr	nr	Haw et al., 2003
In vitro (nodal segments)	MS medium supplemented with 0.1 mg L^{-1} kinetin	93.33% rooted plants	nr	nr	Malosso et al., 2008
In vitro (nodal segments)	MS medium with 5.0 mg dm^{-3} of BAP[c]	97% of shoot regeneration	nr	nr	Singh et al., 2009
		multiple-shoot regeneration	nr	nr	Singh et al., 2008
In vitro (nodal segments)	MS medium without growth regulators	nr	nr	spilanthol content: 0.98%	Franca et al., 2016
In vitro (nodal segments)	MS medium with the use of 0.125 mg L^{-1} of the BAP	average of 2.2 sprouts/explant	nr	nr	Almeida et al., 2020

[a] nr, not reported; [b] Murashige and Skoog; [c] 6-benzylaminopurine.

1.4. Morphological and anatomical features

Jambú is an annual plant reaching a maximum height of 40-60 cm (Srinath and Laksmi, 2014). It usually presents decumbent to ascending stems (diameter from 2.5 to 7.5 mm), without the presence of roots at nodes, and with a glabrous texture colored from green to red. The petioles are long, from 2 to 6.5 cm and are glabrous, winged, or slightly pilose. The plant presents ovate to deltate leaf blades, while the capitula are large and discoid with a maximum height and diameter of 23.5 and 17 mm, respectively. Peduncles are 3.5–12.5 cm long and with a maximum diameter of 17 mm, being usually glabrous. The heads are discoid, and the receptacle, which sometimes

encompasses two heads, is 8.3–21.5 mm high and with a maximum diameter of 8.5 mm (Figure 4). The yellow corollas of *A. oleracea* are from 2.7–3.3 mm long, with the tube usually 0.5–0.7 mm long and with a diameter of 0.2–0.4 mm. The plant encompasses stamens 1.4–1.7 mm long with black anthers. The ciliate achenes are 2–2.5 mm long, with a width of 0.9–1.1 mm. Finally, the pappus is characterized by two bristles of 0.5–1.5 mm and 0.3–1.3 mm, respectively (Jansen, 1985).

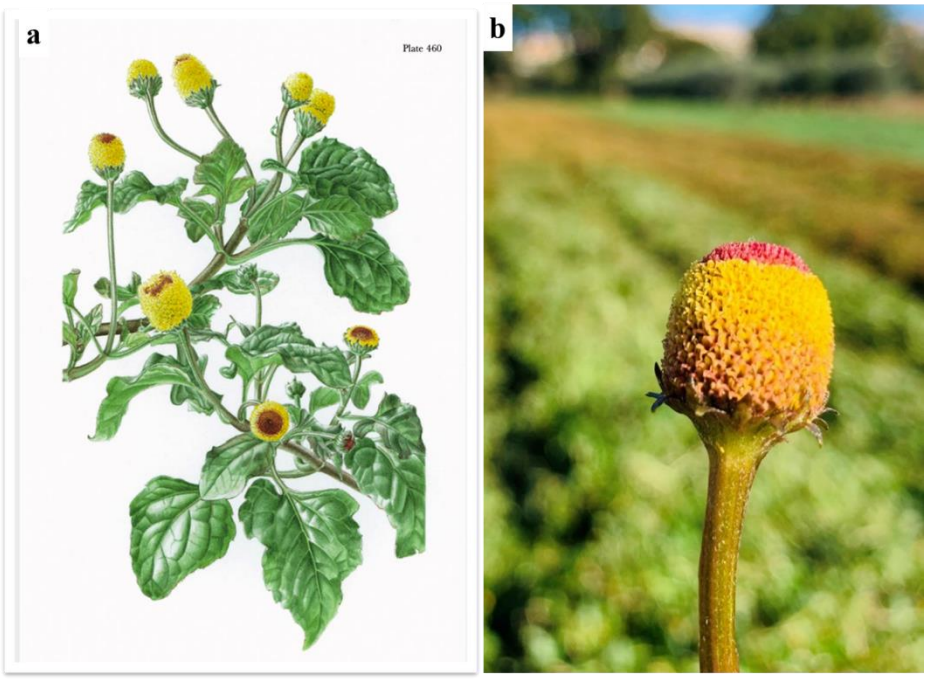


Figure 4. Illustration of *Acmella oleracea* (**a**) (Hind and Biggs, 2003). Head (capitulum) of *Acmella oleracea* plant (**b**).

1.5. Ethnobotanical uses

A. oleracea has been cultivated since ancient times as decorative plant. In fact, it was already cultured in 1700 in a botanical garden in St. Vincent, Kingstown (Saint Vincent and Grenadine) (Hind and Biggs, 2003). Its use as an ornamental plant is due to the fact that it presents cylindrical and attractive flower heads composed of red or purplish paleae, contrasting with the yellow colors of flowers (Jansen, 1985).

This plant found large employment also in cuisine from ancient tribal communities to contemporary generations since its parts, namely the tips of young shoots and leaves, are eaten raw or cooked (Dubey et al., 2013; Lim, 2014). Lamarck in his Encyclopedie Methodique (Lamarck, 1785) reported for the first time the flavor of this plant, which was described as spicy and unpleasant. However, jambú is added in first and second dishes in Brazil, Japan, Indonesia, and India, and is additioned to salads thanks to its spicy aroma (Bailey, 1949; Burdock, 2016; Chung, 2008; Ochse and Bakhuizen, 1980). Moreover, in Taiwan, this plant accompanies cooked duck,

rice dishes (Pará rice), and pizza (Bailey, 1949). It is also employed as a spice (Ramsewak et al., 1999), and its extracts are used to flavour foods worldwide (Nascimento et al., 2020).

Despite the applications described above, *A. oleracea* is also traditionally used as a medicinal herb, especially for the treatment of toothache (Hind and Biggs, 2003). For this reason, it is also well-known as the 'toothache plant' (Lalthanpuii and Lalchhandama, 2019, 2020; Purushothaman et al., 2018). In fact, it is usually applied directly to the site of pain for its anaesthetic properties (Dubey et al., 2013). It is also traditionally formulated in tinctures or in oral gel. Typically, a recommended medicinal use consists in chewing jambú roots, leaves, and flower heads, even if it has been reported that fresh capitula have a more intense effect (Nascimento et al., 2020). Moreover, leaves and flowers decoctions represent an effective treatment for toothache, constipation, and stomatitis (Uthpala and Navaratne, 2021). *A. oleracea* is also employed as an additive to antiseptic agents in mouthwash and toothpaste (Gilbert and Favoreto, 2010). The use of jambú involves also its property as an appetite stimulant in Japan and Brazil (Singh and Chaturvedi, 2010; Yamane et al., 2016), and in Cameroon it is a remedy for snakebite. In addition, in India flower heads are an effective remedy for children's stuttering, while the leaves and flowers are used to treat leukorrhea in Bangladesh (Dubey et al., 2013). In India, *A. oleracea* is employed as aphrodisiac agent for the enhancement of desire and improvement of male sexual functions (Lalthanpuii et al., 2016). The applications of this plant in traditional medicine are wide and include also uses as: antiprotozoal, tonic, anti-inflammatory, antimalarial, anticonvulsant, diuretic, antipyretic, antifungal, antidiarrheal, antiulcer, and diuretic agent. Jambú has also been reported as a traditionally employed insecticidal agent (Araújo et al., 2021; Lalthanpuii et al., 2016; Prachayasittikul et al, 2013).

1.6. Commercial applications

A. oleracea finds several and diverse commercial applications, and this is due to its classification as 'safe' by the 'Flavor and Extract Manufacturers Association' (FEMA) in 2000 (FEMA, 2000) and by the 'European Food Safety Authority' (EFSA) (2015). In 2012 it was included by the Italian Ministry of Health in the list of plant substances and preparations (botanicals) used in food supplements (http://www.gazzettaufficiale.it/eli/gu/2018/09/26/224/sg/pdf), while in 2014 it was encompassed in the BELFRIT list (https://www.trovanorme.salute.gov.it/norme/renderNormsanPdf?anno=0&codLeg=48636&parte=3&series).

This plant is largely employed in the cosmetic field. In fact, many oral hygiene products have been produced and commercialized, as mouthwashes, oral gel, and toothpastes, as Buccaldol® from Alphamega (France) and Indolphal® from ID Phar (Belgium), but also antiseptic dyes such as Vogel® (Silveira et al., 2018). Recently, *A. oleracea* extract has also been employed to produce

anti-wrinkle creams such as Nealim® (Demarne and Passaro, 2009) and anti-age creams such as Gatuline®, SYN-COLL® e ChroNOline® (Rani et al., 2019; Veryser et al., 2014). In addition, the extract is employed for the production of Mitidol® (Indena S.p.A, Milan, Italy), which is a nutraceutical product that is used to treat aches, pain, and inflammation (Rondanelli et al., 2020).

1.7. Secondary metabolites

A. oleracea encompasses a wide range of secondary metabolites, and its phytochemistry has gained large research interest. All the parts of the plant were proved to contain flavonoids, saponins, alkaloids, tannins, and steroid glycosides, while leaves and flowers, rather than stems, were reported as the main source of phenolics (Abeysiri et al., 2013). In detail, a large number of triterpenoids have been detected, as *β*-sitostenone, 3-acetyl aleuritolic acid, and stigmasterol. In addition, steroid glycosides (stigmasteryl-3-*O*-*β*-D-glucopyranoside and *β*-sitosteryl-3-*O*-*β*-D-glucopyranoside) were found in the plant. Among phenolic compounds, *trans*-ferulic, *trans*-isoferulic, and vanillic acids were detected together with a coumarin (scopoletin) (Prachayasittikul et al., 2009). In addition, fatty acids such as *n*-tetradecanoic and *n*-hexadecanoic acids have been identified (Singh et al., 2009).

Jambú is also characterized by a volatile fraction, represented by the EO, obtainable from the inflorescences and mainly constituted by germacrene D, *β*-pinene, myrcene, (*E*)-caryophyllene, caryophyllene oxide, *β*-phellandrene, spilanthol, and acmellonate (Baruah and Leclercq, 1993; Spinozzi et al., 2021). Figure 5 resumes the main secondary metabolites found in *A. oleracea* (Spinozzi et al., 2022).

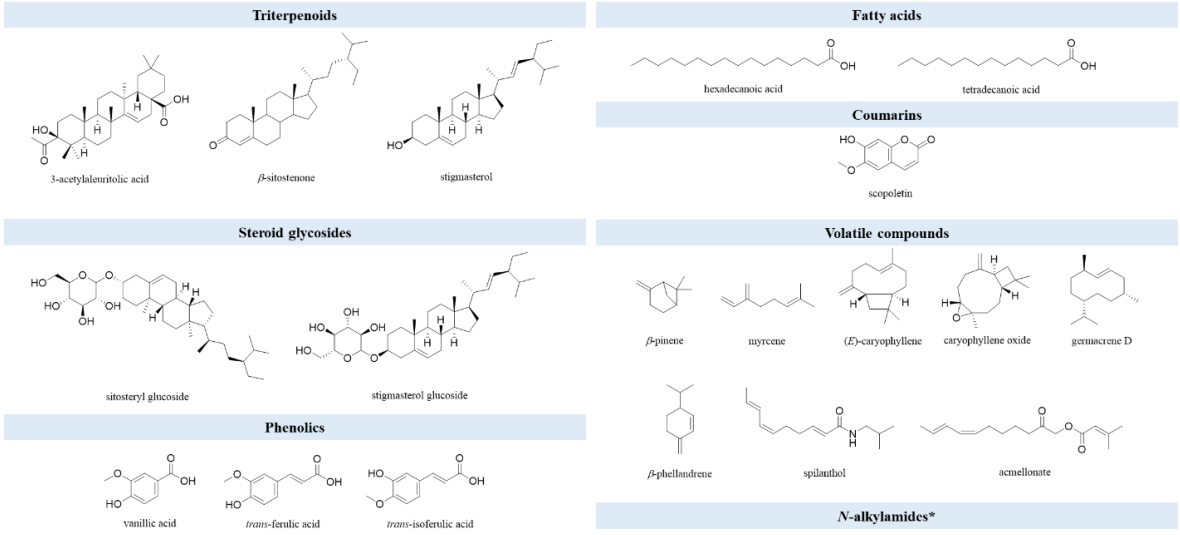


Figure 5. Main secondary metabolites found in *Acmella oleracea*.

1.7.1. *N*-alkylamides

The most interesting metabolites in terms of biological activities are *N*-alkylamides. These compounds are defined as pseudo-alkaloids since they present a structural similarity to this chemical class. *N*-alkylamides are fatty acid amides, and their general chemical structure is displayed in Figure 6.

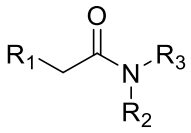


Figure 6. General chemical structure of *N*-alkylamides.

The structure includes one amidic nitrogen atom bonded to two moieties: R_2 represents the remaining of an amino acid, while R_3 is generally an H atom. On the other hand, R_1 is usually a fatty acid chain.

From a biosynthetic point of view, *N*-alkylamides derive from a condensation reaction between a decarboxylated amino acid and a fatty acid chain, which usually derives from unsaturated fatty acids as linolenic, linoleic, and oleic acids. In addition, 23 chemically distinct amine fractions have been identified. They match to different fatty acids and conduct to various *N*-alkylamides. Most of these fractions are derived from the decarboxylation of amino acids (Sharma and Arumugam, 2021). The biosynthetic route of *N*-alkylamides is reported in Figure 7 (Boonen et al., 2012; Cortez-Espinosa et al., 2011; Greger, 2015; Rizhsky et al., 2016; Shephard, 2013).

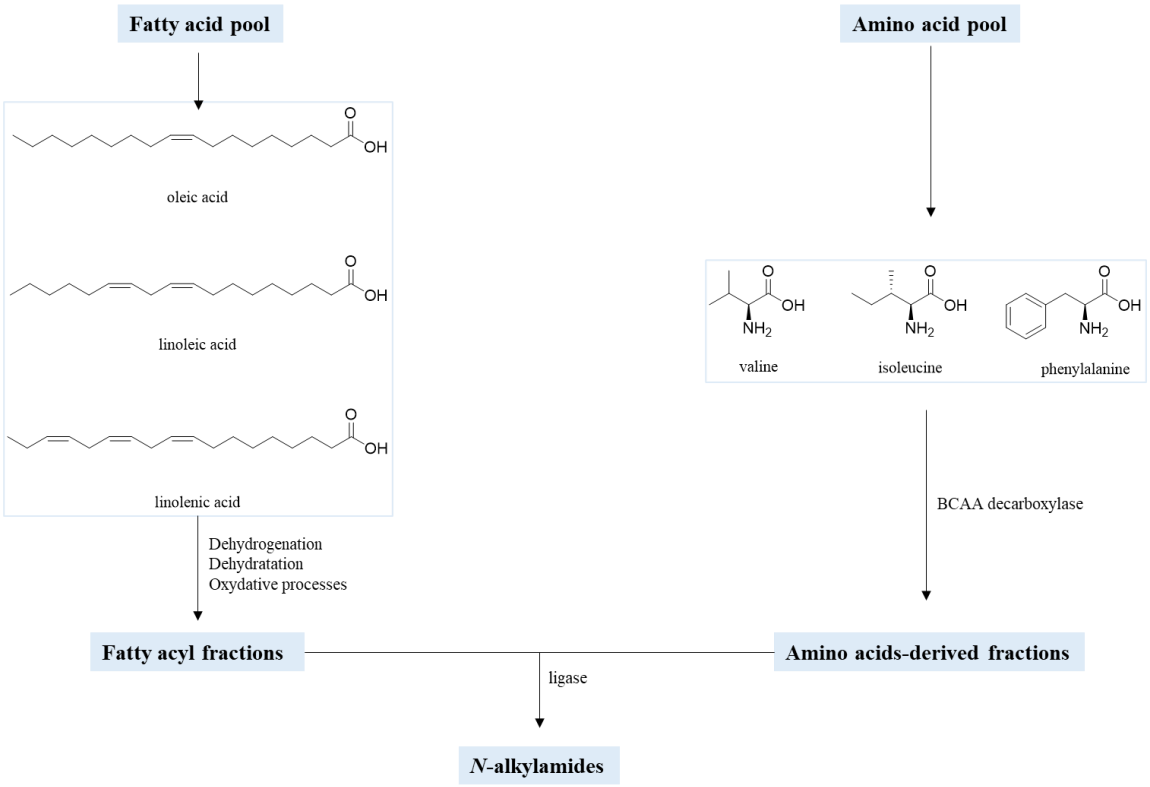


Figure 7. Deduced biosynthetic pathway for *N*-alkylamides.

The *N*-alkylamides characterized in *A. oleracea* are reported in Figure 8, and are classified as isobutylamides, methylbutylamides, and phenylbutylamides depending on the side chain linked to the nitrogen atom of the amidic moiety (Cheng et al., 2015; Savic et al., 2021a).

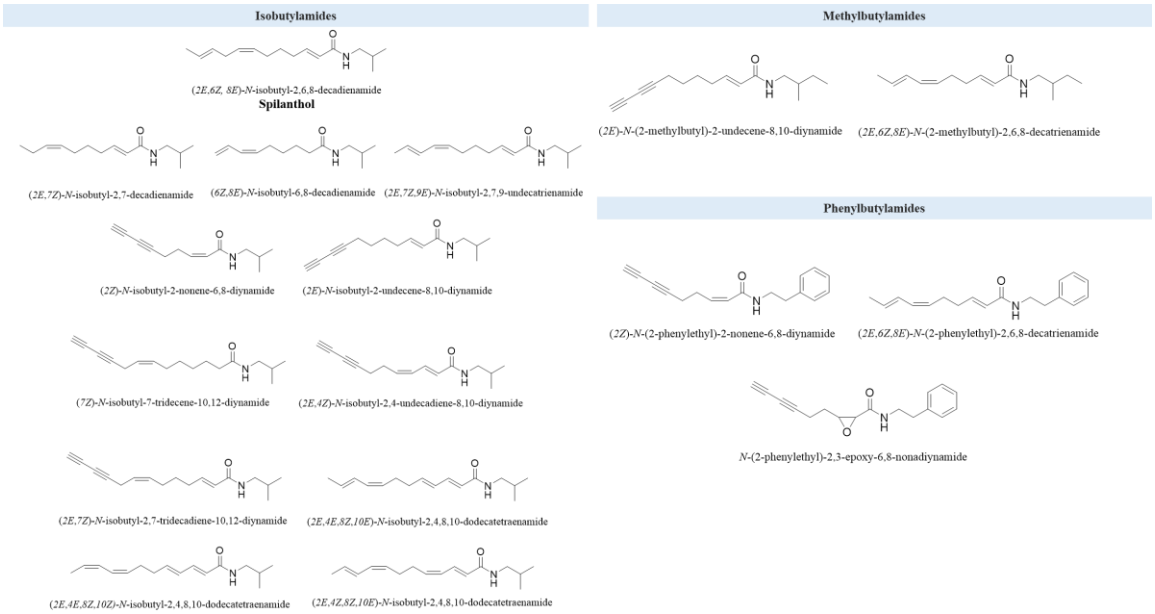


Figure 8. Main *N*-alkylamides found in *Acmella oleracea*.

Among the various *N*-alkylamides found in *A. oleracea,* spilanthol is surely the most abundant one (Figure 9).

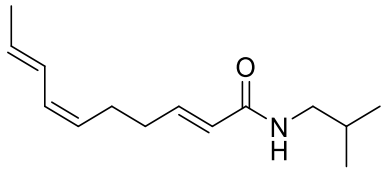


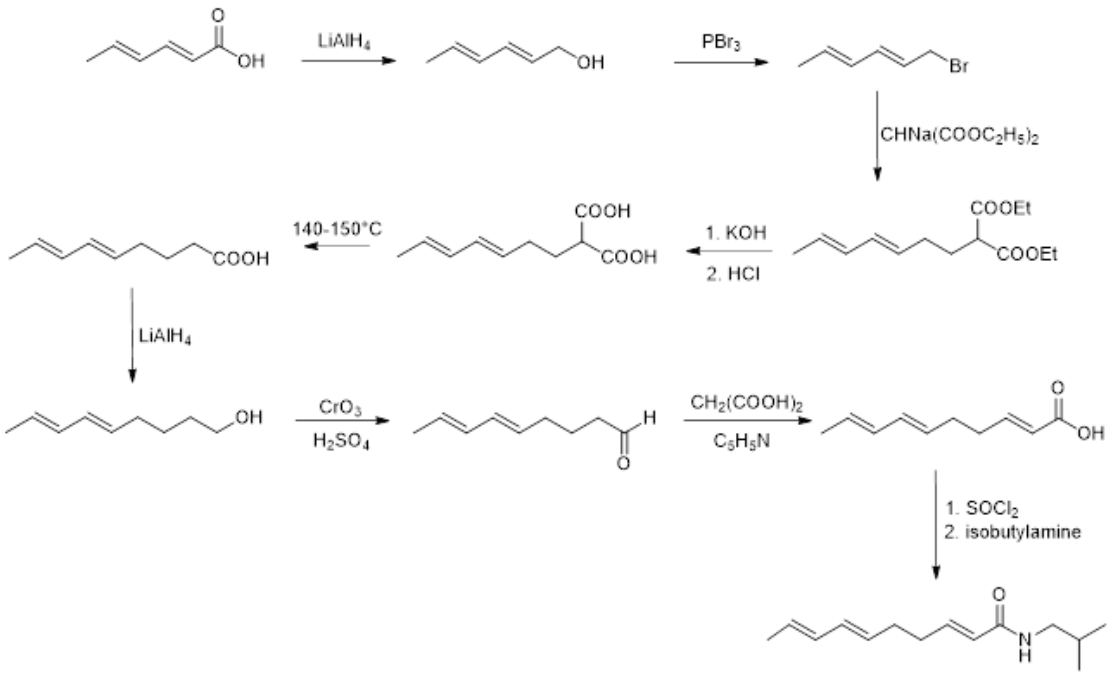
Figure 9. Chemical structure of spilanthol.

Spilanthol is an olefinic *N*-alkylamide endowed with an isobutyl side chain and is the main one responsible for the biological activities and sensory effects characteristic of jambú. This compound is also known by the IUPAC name of (*2E,6Z,8E*)-*N*-isobutyl-2,6,8-decatrienamide (Molinatorres et al., 1996), while its common name is 'affinin' (Prachayasittukal et al., 2013). This molecule has been described as a pale-yellow oily compound with the following physio-chemical features:

- chemical formula of $C_{14}H_{23}NO$,
- boiling point of 165 °C,
- melting point of 23 °C,
- RI at 298 °C of 1.5135,
- maximum UV absorption at 220 nm.

Spilanthol is an amphiphilic molecule encompassing a relatively polar amidic moiety and an apolar acyclic chain with three unsaturations. This chemical nature ensures its extraction with solvents with different polarity, as methanol or ethanol, rather than *n*-hexane or supercritical CO_2 (Abeysinghe et al., 2020). The first isolation of spilanthol dates back to 1903 (Gerber, 1903). Generally, this compound can be purified from *A. oleracea* extracts by TLC, HPLC, or silica gel column chromatography (Moreno et al., 2012).

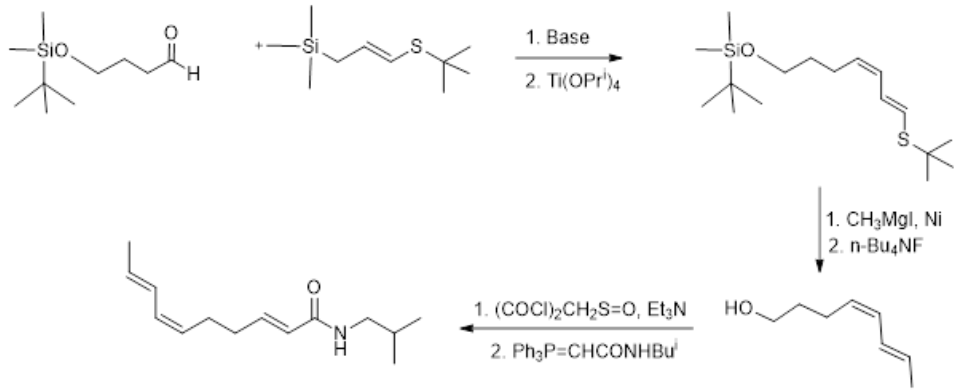
However, given the biological importance displayed by this *N*-alkylamide, some synthetic approaches have also been developed. In 1955, it was provided a nine-steps synthesis for spilanthol, even if, at that time, its stereochemistry was still unknown, and the process led to the formation of all-*E* isoform of spilanthol (Jacobson, 1955) (Scheme 1).



Scheme 1. Synthesis of all-*E* spilanthol isomer (Jacobson, 1955).

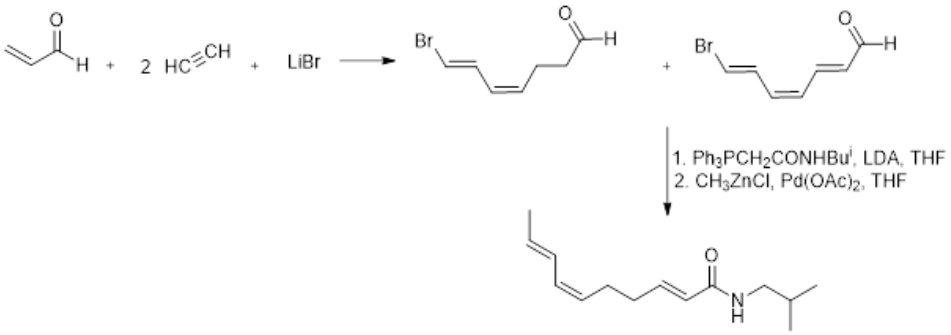
The exact stereochemistry of spilanthol was described only in 1963. The *E* conformation of two double bonds was proved through infrared evidence, and with the analogy with neoherculin, whose stereochemistry was already known. Then, the (2*E*,6*Z*,8*E*)-trienamide stereochemistry was confirmed by comparison of melting point and infrared spectra with the compound isolated from the plant (Crombie et al., 1963).

Afterwards, Ikeda et al. (1984) developed the synthesis using a convergent approach by the addition of an allyltitanium species over an aldehyde as a key step (Scheme 2) (Ikeda et al., 1984). The product was obtained in 88% purity.



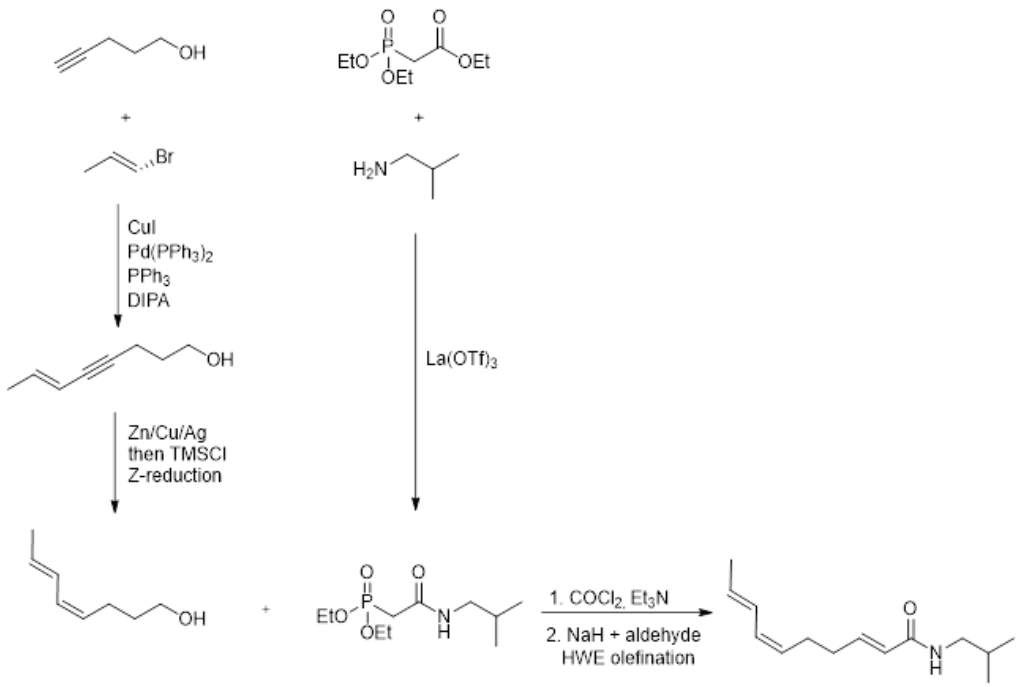
Scheme 2. Ikeda et al. (1984) procedure using allyltitanium species as a catalyst (Ikeda et al., 1984).

Wang and co-workers (1998) developed a shorter approach for the synthesis of this compound with the cotrimerization of acrolein with two equivalents of acetylene as a crucial transformation for the assembly of the conjugated diene fragment with the required geometry. The yield was 43% (Scheme 3).



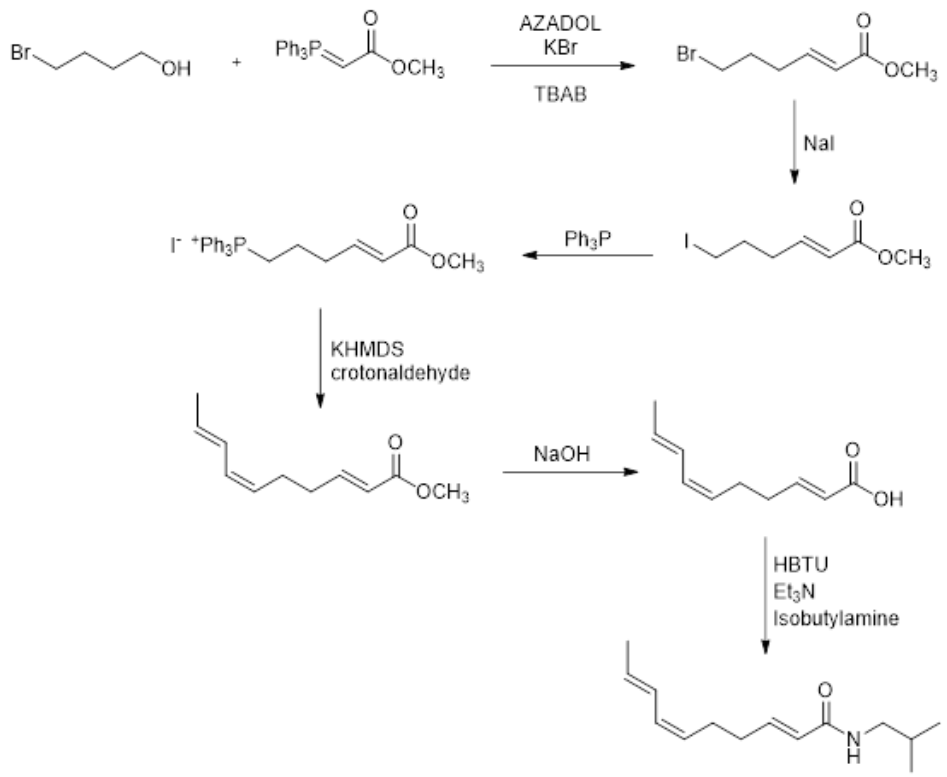
Scheme 3. Spilanthol synthesis reported by Wang et al. (1998).

Some years later, Takasago International Corporation (WO2009/091040A1; US2010/0184863A1; US2011/0105773A1; WO2011/007807A1; US2012/0116116A1) developed an alternative synthesis for the *N*-alkylamide characterized by intermediate easy purification but by poor control over the stereochemistry of the double bond formation. Alonso et al. (2018) reported a 5-step synthesis using a Horner-Wadsworth-Emmons (HWE) olefination reaction for the formation of the 2*E* double bond: this reaction allowed an easier purification of the product compared to Ikeda and Wang protocols (Scheme 4) (Alonso et al., 2018).



Scheme 4. Synthesis of spilanthol through HWE olefination (Alonso et al., 2018).

Recently, Nakamura et al. (2020) reported the synthesis of spilanthol through a highly selective Wittig reaction that led to the desired *Z*-form tetraene. This was achieved through the use of a freshly synthesized phosphonium salt with low deliquescence and long-term stability. The yield achieved was 47% (Scheme 5).



Scheme 5. Synthesis of spilanthol reported by Nakamura et al. (2020).

Regarding the mechanisms of action through which spilanthol exerts its biological activities, they have been widely investigated. Firstly, the lipophilicity surely has a fundamental role for the properties displayed by this compound. For instance, an *in vitro* permeability test demonstrated that the *N*-alkylamide is able to cross the CaCo-2 cell monolayer cultures through a mechanism of passive diffusion and proved spilanthol permeation through the oral mucosa and human skin (Boonen et al., 2010a,b). Moreover, it was also demonstrated that this compound is able to reach blood circulation and to cross of the blood-brain barrier in assays on rats and mice (Veryser et al., 2016). Among spilanthol biological activities, the anti-nociceptive is surely one of the most studied. Déciga-Campos et al. (2010) proved that this property is due to serotoninergic, opioidergic, and GABAergic system activation and that it also implies in the NO/cGMP/potassium channel pathway. On the other hand, the spilanthol anti-inflammatory effect has been reported to be linked to NO-suppressive effects, but also to the prevention of NO-dependent cell death. Moreover, it has also been demonstrated the compound's ability to lower the iNOS mRNA expression and protein, leading to an anti-inflammatory action (Bakondi et al., 2019). The anti-inflammatory action is also linked to the reduction of pro-inflammatory cytokines (PGE2, MCP-1, TNF-α, COX-2, and ICAM-1) expression, inhibiting the NF-κB and MAPK signalling pathways and repressing the monocyte adhesion to IL-1β-stimulated A549 cells (Huang et al., 2018a,b). Spilanthol also displays anti-aflatoxigenic and antifungal properties. A study on *Aspergillus parasiticus* proved

spilanthol ability to downregulate the aflD and aflR genes that participate in aflatoxins biosynthetic pathway (Buitimea-Cantúa et al., 2020). The diuretic action of this *N*-alkylamide seems to be linked to the lowering of the basal phosphorylation level of NKCC2 in mouse kidney slices and in NKCC2-expressing HEK293 cells. On the other hand, affinin antimutagenic activity was proved to be linked to the reduction of 2AA- and NOR-induced mutations in *Salmonella typhimurium*, but also to the reduction of the DNA damage linked to norfloxacin and to the mutations produced by 2-aminoanthracene (2AA) (Arriaga-Alba et al., 2013). Surely, spilanthol is also known for its insecticidal action on different pests and vectors (Table 3).

Table 3. Literature data on the toxicity of spilanthol against arthropod pests and vectors.

Species	Instar	Efficacy	Reference
Aedes aegypti L.	Larvae	LD_{50} = 6.25 µg mL^{-1}	Ramsewak et al., 1999
		LD_{100} = 12.5 µg mL^{-1}	
		LC_{50} = 7.38 mg L^{-1}	Hernández-Morales et al., 2015
Anopheles albimanus Wiedemann	Larvae	LC_{50} = 4.24 mg L^{-1}	Hernández-Morales et al., 2015
Dermacentor nitens Neumann	Larvae	100 % mortality at 12.5 mg mL^{-1}	Cruz et al., 2016
Lipaphis erysimi Kalt	-	mortality of 83 %	Gouvêa et al. 2019
Periplaneta americana L.	Adults	LD_{50} = 2.46 µg g^{-1}	Kadir et al., 1989
Plutella xylostella L.	Larvae	LC_{50} = 1.49 µg L^{-1}	Sharma et al., 2012
		LC_{90} = 1.99 µg L^{-1}	
Rhipicephalus microplus	Larvae	100 % mortality at 1.6 mg mL^{-1}	Cruz et al., 2016
	Engorged females	92.9% mortality 20.0 mg mL^{-1}	
Solenopsis saevissima (Fr. Smith)	Adults	LD_{50} = 0.18 µg mg^{-1}	Moreno et al., 2012
		LD_{80} = 1.12 µg mg^{-1}	
Tetragonisca angustula Latreille	Adults	LD_{50} = 0.35 µg mg^{-1}	Moreno et al., 2012
		LD_{80} = 0.81 µg mg^{-1}	
Tuta absoluta (Meyrick)	Adults	LD_{50} = 0.13 µg mg^{-1}	Moreno et al., 2012

Even if the insecticidal mode of action is not completely clear, it is believed that this *N*-alkylamide affects the central nervous system. This hypothesis is supported by the fact that *A. oleracea* extracts induced abnormal movements in a cockroach (Barbosa et al., 2016a). Moreover, it is also believed that this compound is able to affect the histogenesis processes and histolysis, since it caused rapid mortality on some larvae tested (Spinozzi et al., 2021). In addition, this compound contains an aliphatic chain with three conjugated double bonds at positions 2*E*, 6*Z*, and 8*E*. Greger (1984) suggested that these unsaturations play a crucial role for its insecticidal mechanism of action, even if it not clear yet. This hypothesis has also been confirmed by the study of Hernández-Morales et

al. (2015) that showed that the unsaturations are necessary for the insecticidal activities against *An. albimanus* and *Ae. aegypti*.

Structurally similar amides isolated from Piperaceae plants also displayed a promising insecticidal activity. For instance, piperamides isolated from *Piper nigrum* L. proved their insecticidal effects on various mosquito larvae with diverse mechanisms of action. These involve insects detoxifying system neutralization by affecting the activity of monooxygenase, oxidoreductase, and glutathione S-transferase, but also neurotoxicity due to the nicotinic acetylcholine receptors activation, antifeedant activity, and embryonic development interruption (Pavela et al., 2019a). Since spilanthol has a structural similarity with piperamides found in the black pepper, it could function as an insecticidal agent involving similar pathways. However, deeper research should be carried out to confirm this hypothesis.

1.8. Biological activities of *Acmella oleracea*

A. oleracea encompasses a wide range of biological activities.

- *Anesthetic activity*

This property represents the reason why *A. oleracea* has been used since ancient times for the treatment of toothache (Vishwanathan et al., 2021). The anesthetic mechanism of action has been investigated and depends on spilanthol (de Freitas-Blanco et al., 2019), which leads to an enhanced release of gamma-aminobutyric acid (GABA) (Rios et al., 2007), interacts with transient receptor potential vanilloid type 1 (TRPV1) and TRPA1 receptors (Nomura et al., 2013), promotes the production of prostaglandins, activates the serotoninergic (Acosta-Madrid et al., 2009) and the opioidergic (Ong et al., 2011) systems, and negatively affects the activity of the voltage-gated Na^+ channels (Rondanelli et al., 2020).

- *Cytotoxic activity*

A. oleracea methanol extract demonstrated a high cytotoxic activity on cancer cell line V79, with an IC_{50} value of 54.341 µg mL^{-1} (Lalthanpuii et al., 2018).

- *Anti-inflammatory activity*

The anti-inflammatory properties of *A. oleracea* have been resumed by Rondanelli et al. (2020). In fact, it has been reported that this plant and its major metabolites can exert an anti-inflammatory activity in various experimental models and with numerous and diverse modes of action. In addition, Stein et al. (2021) confirmed the anti-inflammatory activity of jambú derived-products through *in vitro* and *in vivo* experiments demonstrating a decrease of the expression and production of nitric oxide (NO), catalase (CAT), and superoxide anion dismutase (SOD). Moreover, these

products significantly reduced the formation of oedema, the release of NO, and cell tissue infiltration, without leading to hepatic or renal toxicity.

- *Antioxidant activity*

Abdul Rahim et al. (2021) resumed all the experiments performed in cell and cell-free systems up to 2021. The majority of these studies demonstrated a marked antioxidant property for all the parts of *A. oleracea*. The antioxidant activity seems to be linked to phenolic compounds, flavonoids, and triterpenoids, which are some of the secondary metabolites found in the plant. For instance, phenolic compounds showed marked protective effects in SH-SY5Y cells that were previously treated with H_2O_2 by the up-regulation of SIRT1 and FoxO3a expressions (Abdul Rahim et al., 2021).

- *Antimicrobial activity*

This property appears to be highly species specific since *A. oleracea* demonstrated positive results only on some of the numerous bacteria and fungi assayed. For instance, it resulted active on *Salmonella typhi* (MIC of 31.25 µg mL^{-1}), *S. aureus* (MIC of 1.250 µg mL^{-1}), *C. albicans* (MIC of 2.50 µg mL^{-1}), *Bacillus subtilis* (MIC of 1.250 µg mL^{-1}), *E. coli* (MIC of 1.250 µg mL^{-1}), *Streptococcus mutans* and *Lactobacillus* species (zones of inhibition of 21-29 mm at 20 mg mL^{-1}), and *P. aeruginosa* (MIC of 1.250 µg mL^{-1}) (de Alcantara et al., 2015; Onoriode et al., 2018; Uthpala et al., 2021). *A. oleracea* also displayed an antibiofilm action against *S. mutans* biofilms (MIC of 125 µg mL^{-1}) (Peretti et al., 2021).

- *Healing activity*

Recently, also a healing activity has been reported for *A. oleracea*. In detail, its effect on collagen content and organization has been tested in rats. Yamane et al. (2016) demonstrated that jambú facilitates collagen deposition and shortens the length of the healing process. Furhtermore, Moro et al. (2021) proved that *A. oleracea* can increase collagen molecular organization and content.

- *Aphrodisiac activity*

The use of jambú as an aphrodisiac in folk medicine led research to investigate this property (Regadas, 2008; Sharma et al., 2011, 2013). Lira Batista et al. (2021) reviewed research works published up to 2021, assuming that jambú acts by oral and topical administration, with different modes of action. Topically, the aphrodisiac action is probably linked to the tingling effect on the glans, which is produced by potassium channels inhibition and by the NO and prostacyclin release. Conversely, the oral effect could rely on increased testosterone levels, but the exact mode of action is still unknown.

- *Antiwrinkle activity*

A serum containing an *A. oleracea* extract displayed a positive effect on skin hydration levels of human volunteers and ameliorated facial expression lines and wrinkles on the lip and eye contours (Savic et al., 2021a).

- *Anthelmintic activity*

Jambú extracts were active against both the cestode *Taenia tetragona* Molin and the nematode *Ascaridia perspicillum* (Rud.) (Burdock, 2010). The ethanol extract of *A. oleracea* also showed a spasmolytic effect in an isolated rat ileum. The mode of action was linked to its capability to cause ileal smooth muscle relaxation by the blockage of voltage-dependent calcium channels (Duangjai et al., 2021).

- *Tyrosinase inhibitory activity*

A. oleracea was also demonstrated to interact with the enzyme tyrosinase. In detail, it has been found that the enzyme inhibition and activation strongly relied on spilanthol concentration in the extract assayed. In this regard, the hexane fraction, which contained 84% spilanthol, displayed an activation capacity toward the enzyme, while the dichloromethane fraction an inhibitory activity (Barbosa et al., 2016b).

- *Antimalarial activity*

The *A. oleracea* extract displayed an antimalarial property. In detail, the aqueous extract of Jambú inflorescences exhibited a moderate activity against *Plasmodium falciparum* Welch, which represents the main cause of malaria development (Chaniad et al., 2022).

- *Diuretic activity*

A. oleracea has been known in traditional medicine as a diuretic agent. Both *A. oleracea* extracts and spilanthol inhibit the $Na^+/K^+/Cl^-$ co-transporter in the ascending loop of nephron, increasing natriuresis and kaleuresis. The mode of action has been deeply investigated recently (Gerbino et al., 2016). It has been found that exposition to spilanthol leads to a reduction of the basal phosphorylation level of NKCC2, whose action is linked to the urine concentrating mechanism. In addition, this *N*-alkylamide has been found to inhibit vasopressin induced AQP2 translocation in mouse CD cells. Finally, spilanthol showed to reduce or reverse basal and agonist-increased cAMP levels involving increases in intracellular Ca^{2+} (Gerbino et al., 2016).

- *Antispasmodic activity*

In addition to the above-mentioned properties, treatments based on jambú were shown to reduce gastric lesions with cellular proliferation induction (Maria-Ferreira et al., 2014) and displayed an antispasmodic property in gastrointestinal disorders (Duangjai et al., 2021). These activities could be due to a cellular proliferation increase, reduction of inflammation processes, maintenance of gastric mucus, and modulation of the antioxidant mechanisms (Maria-Ferreira et al., 2014).

- Insecticidal activity

Insect vectors

A promising insecticidal activity has been reported for *A. oleracea* against numerous insect species. Between them, mosquitoes are the main vectors of human pathogens around the world (Benelli et al., 2016). The extract obtained from *A. oleracea* has been reported to be active against the malaria vectors *Anopheles stephensi* (Liston) (Diptera: Culicidae) (Pandey et al., 2007) and *Anopheles culicifacies* (species C) (Giles) (Diptera: Culicidae) (Pandey et al., 2007), *Cx. quinquefasciatus* (De Araújo et al., 2018; Pandey et al., 2007), and *A. aegypti* (Araújo et al., 2020; De Araújo et al., 2018; Maria-Ferreira et al., 2014). The use of *A. oleracea* appears more efficient than other plant extracts, as demonstrated by Pandey et al. (2007). Jambú *n*-hexane extract demonstrated its effectiveness resulting the most active for the induction of complete lethality at minimum concentrations (Pandey et al., 2007). Regarding *An. culicifacies* larvae, the LC_{50} was 0.87 μg mL^{-1}; for *An. Stephensi* 4.57 μg mL^{-1}, and for *Cx. quinquefasciatus* 3.11 μg mL^{-1} (Benelli et al., 2019c; Pandey et al., 2007). Similar results have also been presented by De Araújo et al. (2018) using an hydroethanolic extract of the plant (LC_{50} = 32.40 μg mL^{-1}).

Extracts deriving from different solvents lead to different results (De Araújo et al., 2020). For instance, the *n*-hexane extract resulted more toxic against *Ae. aegypti* larvae (LC_{50} = 2.23 μg mL^{-1}) than the methanolic and ethanolic extracts.

Insect pests

The extract rich of spilanthol derived from *A. oleracea* flowers was reported as active against several insect pests. For instance, Kadir et al. (1989) demonstrated that spilanthol has an insecticidal activity on *Periplaneta americana* (L.) (Blattodea: Blattidae), through the penetration inside the insect integumental system and interference with the central nervous system.

Topical application of *A. oleracea* extract caused mortality on the *S. littoralis* (LD_{50} = 68.1 μg $larva^{-1}$) (Benelli et al., 2019c), *Tuta absoluta* (Meyrick) (LD_{50} = 0.13 μg mg^{-1}) (Moreno et al., 2012), and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), where it was observed an increasing mortality over time (LC_{50} = 5.14 and 5.04 g L^{-1} for crude methanol and n-hexane seed extracts, respectively) (Sharma et al., 2012). The EO also displayed marked toxicity on adult females of *M. domestica* (LD_{50} 44.3 μg $adult^{-1}$) (Benelli et al., 2019c).

The feeding substrate also gave promising results against *Myzus persicae* (Sulzer) (Aphididae: Hemiptera) and *Lipaphis erysimi* (Kaltenbach) (Aphididae: Hemiptera), for which lower insect fecundity was noticed (Gouvêa et al., 2019). Ogban et al. (2015) also reported the toxicity of *A. oleracea* extract on *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae) adults, with a significant adult mortality. Table 4 resumes the insecticidal potential of *A. oleracea*-derived products on insect pests and vectors. All these studies demonstrate the possibility of employing

jambú as an insecticidal agent and represent the basis for its insecticidal activity investigation from our research group.

Table 4. Insecticidal activity of *Acmella oleracea*-derived products on insect pests and vectors (Spinozzi et al., 2022).

Target			
Species and instar	**Treatment**	**Efficacy**	**References**
Periplaneta americana adults	*A. oleracea* Spilanthol crude extract	LC_{50} =2.46 μg/g	Kadir et al., 1989
Anopheles culifacies late third/early fourth instar larvae	*A. oleracea* hexane extract	LC_{90} = 1.92 μg/mL LC_{50} = 0.87 μg/mL	Pandey et al., 2007
Anopheles stephensi late 3rd /early 4th instar larvae	*A. oleracea* hexane extract	LC_{90} = 7 μg/mL LC_{50} = 4.57 μg/mL	Pandey et al., 2007
Culex quinquefasciatus late 3rd /early 4th instar larvae	*A. oleracea* hexane extract	LC_{90} = 8.89 μg/mL LC_{50} = 3.11 μg/mL	Pandey et al., 2007
Tuta absoluta 2nd instar larvae	*A. oleracea* hexane extract	LC_{80} = 2.94 μg/mL LC_{50} = 1.83 μg/mL	Moreno et al., 2012
Pluella xylostella 2nd instar larvae	*A. oleracea* crude extract, methanol and hexane extract	LC_{50} = 1.49 x 10^{-3} μg/mL LC_{50} = 5.14 x 10^{-3} μg/mL	Sharma et al., 2012
Sitophilus zeamais adults	*A. oleracea* plant powder	100% mortality after 96 h of exposure to 1% (w/w)	Ogban et al., 2015
Culex quinquefasciatus 3rd instar larvae	*A. oleracea* hydroethanolic extract	LC_{90} = 1.92 μg/mL LC_{50} = 32.40 μg/mL	De Araújo et al., 2018
Aedes aegypti 3rd instar larvae	*A. oleracea* hexane extract, topical application	LT_{90} = 57.05 h LC_{50} = 44.3 μg/adult	De Araújo et al., 2018
Culex quinquefasciatus 3rd instar larvae	*A. oleracea* EO[a]	LC_{90} = 68.24 μg/mL LC_{50} = 42.2 x 10^{-4} μg/mL	Benelli et al., 2019c
Musca domestica adult females	*A. oleracea* EO topical application	LC_{90} = 73.6 x 10^{-4} mg/mL LC_{50} = 44.3 μg/adult	Benelli et al., 2019c
Spodoptera littoralis 3rd instar larvae	*A. oleracea* EO topical application	LC_{90} = 87.5 μg/adult LC_{50}=68.1 μg/larva	Benelli et al., 2019c
Myzus persicae nymphs	*A. oleracea* ethanol extract on kale leaves: 0.01 g/mL	LC_{90} =132.1 μg/larva LT_{50} = 3.29 h	Gouvêa et al., 2019
Liphapis erysimi nymphs	*A. oleracea* ethanol extract on kale leaves: 0.01 g/mL	LT_{90} = 24.85 h LT_{50} = 8.85 h	Gouvêa et al., 2019
Aedes aegypti 3rd instar larvae	*A. oleracea* hydroethanolic extract (1), hexane extract (2), methanolic extract (3)	LC_{90} = 87.5 μg/adult LC_{50}= 1) 28.42 μg/Ml 2) 23.2 μg/Ml 3) 39.67 μg/mL LC_{90} = 1) 63.45 μg/mL 2) 39.98 μg/mL 3) 63.34 μg/mL	De Araújo et al., 2020

[a]EO, essential oil.

- Acaricidal activity

Lethal effects against ticks

A. oleracea n-hexane extract was reported as highly effective on *Rhipicephalus microplus* (Canestrini) (Ixodida: Ixodidae) larvae at concentrations higher than 1.6 mg mL^{-1} (LC_{50} = 0.8 mg mL^{-1}) and against engorged females (LC_{50} of 79.7 mg mL^{-1}). This acaricidal property was also displayed by the methanolic extract of this plant on both *R. microplus* and *Dermacentor nitens* (Acari: Ixodidae), with mortality values higher than 90% from concentrations of 1.6 and 6.2 mg mL^{-1}, respectively. In this regard, Castro et al. (2014) confirmed the previously reported data also testing the *n*-hexane extract finding a mortality of 93% for *R. microplus* larvae with a dose of 1.6 mg mL^{-1}. What determines the lethal effect is surely the spilanthol concentration in the extract. As proved by Marchesini et al. (2018), the dichloromethane fraction containing 99% of spilanthol showed the highest activity among all the fraction tested, resulting in a mortality of *R. microplus* larvae higher than 80% at a concentration of 0.4 mg mL^{-1}. The methanolic extract of jambú was also effective on the immature stages of *Amblyomma sculptum* (Berlese) (Ixodida: Ixodidae) (Marchesini et al., 2020), with 100% mortality of engorged larvae and nymphs from concentrations of 12.5 and 200.0 mg mL^{-1}, respectively (Marchesini et al., 2018).

Sublethal effects against ticks

The acaricidal potential of *A. oleracea*-based products (Table 5) is expressed in terms of lethal and sublethal effects, and it depends mainly on spilanthol concentration. For *A. oleracea*-based products malformations and sublethal effects have been investigated. For instance, *A. oleracea n*-hexane extract assayed through an immersion test on *Rhipicephalus sanguineus* engorged females caused alterations on generative and digestive cells at concentrations of 18, 36, and 45 mg mL^{-1} (de Oliveira et al., 2021). Regarding sublethal effects, several works on *A. oleracea n*-hexane extract demonstrated a marked cytotoxicity on germ cells of *R. sanguineus* (Latreille) (Ixodida: Ixodidae) and to cause alterations in the generative and digestive cells of semi-engorged females (de Oliveira et al., 2021). Moreover, *A. oleracea* extract damaged the midgut epithelial cells together with the female fertility (de Oliveira et al., 2019). In males of *A. cajennense* exposed to *A. oleracea* ethanolic extract, the compromission of the reproductive system was noticed, with a reduction of the production of the elements constituting the sperm fluid (Anholeto et al., 2017). *A. oleracea* ethanolic extract has been reported as significantly cytotoxic, causing alterations on germinative cells, morphological changes in the oocytes, disorganization, and cytoplasmic vacuolation, together with a reduction of the number of yolk granules and germ vesicle fragmentation (Anholeto et al., 2018).

Table 5. Sublethal effects of *A. oleracea* extracts on tick species.

Target			
Species	**Treatment**	**Sublethal effects**	**References**
Amblyomma cajennense males	immersion test ethanolic extract	damage on reproductive system	Anholeto et al., 2017
Amblyomma cajennense semi-engorged females	immersion test ethanolic extract	alterations on germinative cells	Anholeto et al., 2018
Rhipicephalus sanguineus semi-engorged females	immersion test ethanolic extract	alterations on generative and digestive cells	Benelli et al., 2019c
Rhipicephalus sanguineus semi-engorged females	immersion test *n*-hexane extract	alterations on generative and digestive cells	de Oliveira et al., 2021
Rhipicephalus sanguineus engorged females	immersion test *n*-hexane extract	alterations on generative and digestive cells	de Oliveira et al., 2021

Data reported in literature underline that the insecticidal activity of *A. oleracea* is strongly connected to the presence of spilanthol, which showed a marked activity on different targets, arthropod and insects previously reported.

- Side effects on non-target species

To the best of our knowledge, the effects of *A. oleracea* and its main constituents on non-target species have been scarcely investigated. The zebrafish embryo test has been employed for assaying the toxic effect of spilanthol on animals. According to the study of Ponpornpisit et al. (2011) the extract of this plant was not toxic to zebrafish, but de Souza et al. (2019) observed a significant increase of egg deposition that caused less resources and more mortality in eggs.

2. General procedures

Unless otherwise stated, the following general experimental procedures were employed in the biological studies reported in the following Sections.

2.1. Plant material

A. oleracea plants derived from a plantation of Dr. Ettore Drenaggi in the flat ground of the Middle-end section of the riverbed of the Musone river, in Castelfidardo (AN) at 38 m a.s.l. The harvest was manual and was conducted at 70% of the flowering phase between July and October 2020 (for the studies reported in Sections 3 and 4) and between July and October 2021 (for the study reported in Section 5). For all the studies reported herein, flowering aerial parts of *A. oleracea* were employed. The plant material was dried in an open environment protected from the sun and in an open environment with no solar protection (greenhouse with plastic coating), for about 5-6 days for the study reported in Section 3. The drying of the plant material employed for the studies reported in Sections 4 and 5 was performed at 38 °C for 72 h. Unless otherwise specified, the plant material was then shredded with a plant grinder (Albrigi, mod. E0585, Stallavena, Verona Italy) with a 1.5 mm pore sieve.

3. Spilanthol-rich essential oil (EO) from *Acmella oleracea*: insecticidal, cytotoxic and anti-inflammatory activities evaluation

3.1. Introduction

As described in Chapter 1, Section 2.1, EOs are natural products that recently gained great research interest since they encompass a wide range of biological properties. The choice of the correct extraction parameters and the right extraction process significantly influence the yield of EO obtained and its chemical composition (Bilia et al., 2014; El Asbahani et al., 2015). EOs are usually obtained with traditional techniques as SD or HD, which are cheap, reproducible, and easy to be performed (Golmakani et al., 2008; Guan et al., 2007; Rasul, 2018). Nevertheless, they present several limitations: long extraction times, huge energy and water utilization, and risk of thermal degradation of sensitive compounds (El Asbahani et al., 2015). Furthermore, for these extraction processes only few parameters can be adjusted and changed to modify the EO yield and composition (Golmakani et al., 2017; Yang et al., 2014). MAE is an innovative extraction technique that allows EOs extraction through the action of microwaves that are absorbed by the plant material and converted into heat energy (Letellier and Budzinski, 1999). MAE is characterized by the presence of different factors affecting the yield of extraction but also the chemical constitution of the EOs obtained. These factors have been deeply described in Chapter 1, Section 2.1, and they are: extraction time, microwave power, temperature, and the effect of the sample characteristics.

Usually, MAE presents several differences if compared to traditional distillation processes, such as extraction time, EO yield, EO chemical composition, and environmental impact.

Regarding *A. oleracea*, from the stems, leaves, and inflorescences of this plant it is possible to obtain an EO, which is usually extracted through HD. This EO is gaining increasing research interest thanks to the numerous biological properties that it displayed, especially the insecticidal activity. This property is increasingly attracting considerable interest, especially for the management of mosquitoes, for which the onset of extremely invasive species (Wilke et al., 2020), together with insecticide resistance, have been reported (Hemingway et al., 2016).

The main objective of the study herein reported was to adopt for the first time MAE for the extraction of *A. oleracea* EO and to confront it with the traditional HD in terms of EO yield and chemical composition. The obtained EO and its NE, together with the main *N*-alkylamide (spilanthol) detected and a *n*-hexane extract, were tested for their larval toxicity on *Cx. quinquefasciatus*, for which Integrated Vector Management (IVM) strategies must be urgently improved (Wilke et al., 2020). Moreover, the sublethal effects of these jambú-derived products were studied in terms of larval mortality, emergence, fertility, fecundity, and natality of the newly generated adults. Finally, to support the safe use of these botanicals for a real-world application,

their cytotoxicity on mammal cells, as well as anti-inflammatory effects in microglial cells and the anti-acetylcholinesterase activity were evaluated as well.

3.2. Materials and methods

3.2.1 Chemicals and reagents

Standards used for the chemical characterization of the EOs and extract were all of an analytical grade and were purchased from Merck (Milan, Italy). Solvents were also purchased from Merk (Milan, Italy), as also high glucose Dulbecco's modified Eagle medium (DMEM), streptomycin, penicillin, glutamine, Lipopolysaccharide (LPS) from *E. coli* serotype O127:B8, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), MTT, L-glutamine, H_2O_2, DMSO. Low-Endotoxin FBS was acquired from Euroclone (Milan, Italy).

3.2.2 Microwave-assisted extraction (MAE)

For MAE, the plant material was used frozen instead of dried. The extraction was conducted with a Milestone ETHOS X (Milestone, Italy) advanced microwave extraction system. In detail, 1.750 kg of plant material were left thawing in the 12 L glass reactor (Pyrex) for 30 min prior to the extraction, which was then performed at 1800 W for 160 min with the 'Fragrances set-up'. Then, the obtained EO was separated from the aqueous layer, dehydrated with Na_2SO_4, collected in a PTFE-silicon septum vial, and stored at 4 °C. The yield of extraction was 0.47% (w/w dw). The calculation of the moisture content in the biomass was calculated by drying the plant in an oven at 110 °C for 24 h.

3.2.3 Hydrodistillation (HD)

For the HD, fresh plant material was employed (2 kg) and extracted with 12 L of distilled water for 4 h until no more EO was condensed. The HD system consisted of a Clevenger-type apparatus and was heated in a mantle system Falc MA (Falc Instruments, Treviglio, Italy). Then, the EO was collected, and stored at 4 °C in PTFE/silicon cap vial. The yield of extraction was 0.22% (w/w dw). The moisture content was calculated as reported in the previous paragraph.

3.2.4 Preparation of *Acmella oleracea n*-hexane extract

Aerial parts and inflorescences of *A. oleracea* were shredded using a shredder (Albrigi, mod. E0585, Stallavena, Verona, Italy) and then extracted (450 g of plant material) using *n*-hexane (1:10 w/v), in an ultrasound bath (Analogic ultrasonic bath Mod. AU-220, ARGOLAB, Carpi, Italy) at

40 °C for 3 h. Then, the extract was filtered, and the solution evaporated until dryness using a rotary evaporator (Section 3.3) at 40 °C. Then the removal of chlorophyll was performed as follows. The extract (12.9 g) was dissolved into *n*-hexane (50 mL), added with activated charcoal (6.45 g, 50% w/w of the dry extract (DE)), and then placed into a warm bath at 30 °C with constant stirring for one hour. Then, the mixture was filtered on Celite® and evaporated to dryness (10 g of extract obtained). The obtained extract was stored at 4 °C until use.

3.2.5 Isolation and characterization of spilanthol

Spilanthol (158 mg) was purified from *A. oleracea* hexane extract (1 g) through a flash silica gel column chromatography (230-400 mesh, Merck), employing a gradient solvent system of *n*-hexane/ethyl acetate 90:10 to 80:20. The *N*-alkylamide chemical structure was confirmed by NMR and MS analyses, which were consistent with those reported by Alonso et al. (2018). For the assessment of the purity of spilanthol, HPLC-DAD analysis was also carried out. For the analysis, spilanthol (3.6 mg) was solubilized in methanol (2 mL), and then the analysis was performed using a Phenomenex Luna C_{18} column (4.6 x 150 mm). The chosen injection volume was 0.5 µL, and the flow rate was set at 1 mL min^{-1}. The mobile phase consisted of acetonitrile:water (70:30), and the purity was evaluated at different wavelengths (210, 220, 230, 240, 250, 270 nm). Spilanthol displayed a purity of 100% and 96.60% at 270 and 210 nm, respectively.

3.2.6 Gas chromatography–mass spectrometry (GC–MS) analysis of *Acmella oleracea* essential oil (EO)

The chemical analysis of the two EOs obtained from *A. oleracea* by MAE and HD was performed with a gas chromatograph coupled to a mass spectrometer detector (GC-MS, Agilent 6890N equipped with a 5973N single quadrupole detector). The analysis conditions used and the identification of the EO chemical constituents were performed as described in Chapter I, Section 2.2.5.

3.2.7 Quantification of the *Acmella oleracea* essential oil (EO) marker compounds by GC-FID analysis

The HD and MAE EOs and the *n*-hexane extract were analysed through GC coupled to flame ionization detector (FID) for the quantification of *α*-pinene, *β*-pinene, myrcene, 1,8-cineole, (*E*)-*β*-ocimene, terpinolene, (*E*)-caryophyllene, humulene, germacrene D, caryophyllene oxide, and spilanthol. An Agilent 6850 GC series GC-FID was used for the analysis and the EO (50 µL) was dissolved in 5 mL of analytical grade *n*-hexane, and the solution (0.5 µL) injected into the GC.

Conversely, for the analysis of the *n*-hexane extract a preliminary transmethylation was performed. In detail, 15 mg of extract were solubilized in 1 mL of *n*-hexane. Afterwards, 2 N KOH solution in methanol (0.1 mL) was additioned and the solution was mixed with a vortex device for 2 min. Finally, the reaction was quenched by the addition of 1.5 mL of a saturated NaCl solution, the mixture was shaken for 2 min, and then centrifuged for 5 min (5000 rpm). The organic phase was recovered, and each sample was analysed in duplicate (injection volume of 0.5 µL). The GC method was the same for all the samples. The carrier gas (hydrogen) was generated using a generator PGH2-250 (DBS Analytical Instruments, Vigonza, Italy) and flowed at 3.7 mL min^{-1}. The injector temperature was 300 °C, and the split mode (1:30) was employed. The analytical conditions used for separation and quantification of *A. oleracea* EO main constituents were the same as those previously reported (Fiorini et al., 2020), with the additional analysis of spilanthol. If the analytical standards were not available, the calibration curves of representative compounds for each chemical class have been employed for quantitative purposes.

3.2.8 Chiral chromatography

The EOs were also analyzed by chiral chromatography. In detail, the enantiomeric distribution of *α*-pinene, *β*-pinene, limonene, *β*-phellandrene, (*E*)-caryophyllene, and caryophyllene oxide was taken into consideration and analysed through GC-MS furnished of a chiral column (HP 20B, 30 m l. x 0.25 mm i.d., 0.25 µm f.t.) from Agilent Technologies (Santa Clara, California, USA). The GC-MS analytical conditions were the same as those reported in the previous paragraph. The identification of the enantiomers of the above compounds was performed by comparing the Retention Time (RT) and Retention Index (RI) (calculated with respect to a mixture of C_7-C_{30} *n*-alkanes, Merck, Milan, Italy) with those of analytical standards (Merck, Milan, Italy) and with data reported in the literature.

3.2.9 NMR and HPLC-MS analyses of *Acmella oleracea n*-hexane extract

The main *N*-alkylamides occurring in *A. oleracea n*-hexane extract were analysed by NMR using a protocol previously developed (Sut et al., 2020). In detail, *A. oleracea n*-hexane extract (10-15 mg) was solubilized in deuterated chloroform (1 mL), adding caffein (1.0-1.5 mg) as internal standard. ^{1}H NMR was acquired at room temperature with calibrated P1 and optimized spectral width, using 6 s of D1 for a complete relaxation of the different proton signals. Data were employed for the calculation of the amount of total *N*-alkylamides (mainly represented by spilanthol) using the integral signal of H-2 of alkylamides (δ 5.81, CH, d) and one of the signals of methyl group of caffein (δ 3.61, CH_3, s). Integrals normalisation was performed on the base of signal multiplicity (3H for methyl group, ^{1}H for the *N*-alkylamide CH) and molecular weight of the compounds. For

the quantification of *N*-alkylamides, spilanthol (MW of 221 g mol^{-1}) resulted the predominant compound and was used as a reference to calculate the total amount of *N*-alkylamides. Regarding *N*-alkylamides analysis by LC-MS, the extract was solubilised in methanol (10 mg/10 mL) by 10 min sonication, then centrifuged at 13000 g for 10 min, and the supernatant used for the analysis. A Phenomenex RP-MAX 150 x 3.0 mm (4 μm particle size) column was employed as the stationary phase, while the mobile phase was constituted by acetonitrile (A) and water 0.1% formic acid (B), with the following gradient: min 0 (15% A, 85% B); min 33 (85% A, 15% B); min 33.5 (15% A, 85% B) with 4 min of riequilibration time. The flow was set at 0.4 mL min^{-1} and the injection volume was of 10 μL. *N*-alkylamides were identified in positive mode through the MS 500 ion Trap in a range of 100-700 Da in TDDS mode (Turbo Detection Data Scanning). Peak assignation was achieved comparing fragments of ions with those reported by Boonen et al. (2010). Each compound amount was calculated on the base of the relative peak area, while total *N*-alkylamides were calculated by NMR.

3.2.10 *Acmella oleracea* essential oil (EO) formulation

Encapsulation of the Acmella oleracea essential oil (EO) in nanoemulsions (NEs)

A. oleracea EO-NEs were prepared using a high-energy method. EO and ethyl oleate (ratio of 0.5) were slowly added to a Polysorbate 80 (P80, Sigma-Aldrich) water solution under stirring at high-velocity (Ultraturrax T25 basic, IKAfi Werke GmbH & Co.KG, Staufen, Germany) for 5 min at 9500 rpm. The emulsion homogenization was achieved using the French Pressure Cell Press (American Instrument Company, AMINCO, MY, USA), operating at a pressure of 130 MPa for four cycles. NEs were prepared by varying the percentage of the oil phase from 6 to 9% (w/w) and the percentage of P80 from 0.2 to 0.5% (w/w), as reported in Table 6.

Table 6. Composition of nanoemulsions (NEs) containing *Acmella oleracea* essential oil (EO).

NE[a]	*A. oleracea* EO[b]	Ethyl oleate	OP[c]	P80[d]	EO/Ethyl oleate ratio	P80/OP ratio
NE_1	0.4	0.2	0.6	0.2	0.5	0.33
NE_2	0.4	0.2	0.6	0.5	0.5	0.83
NE_3	0.6	0.3	0.9	0.2	0.5	0.22
NE_4	0.6	0.3	0.9	0.5	0.5	0.55
NE_5	0.5	0.25	0.75	0.35	0.5	0.46

[a]NE, nanoemulsion; [b]EO, essential oil, [c]OP, total oil phase; [d]P80, Polysorbate 80.

The visual inspection of NEs was performed using a MT9000 polarizing optical microscope (Meiji Techno Co Ltd., Japan) endowed with a CMOS camera (Invenio 3S, DeltaPix, Denmark) at 10X

magnification. The evaluation of internal phase droplet size was performed by a Zetasizer nanoS (Malvern Instruments, Worcestershire, UK) coupled with a backscattered light detector working at 173°. Analytical conditions were the same as those reported in Chapter I, Section 4.2.2. Observation by optical microscope and DLS analyses were performed at different timepoints (i.e. 0, 15, 30, 90 and 360 days, T0, T15, T30, T90, T360, respectively).

Head Space-Gas-Chromatography/Mass spectrometry (HS-GC/MS) analysis of nanoemulsions (NEs)

The stability of the *A. oleracea* EO NEs was assessed over time in terms of the vapor phase of pure and encapsulated *A. oleracea* EO, using an autosampler (Perkin-Elmer Headspace Turbomatrix 40) coupled to a GC-MS Clarus 500 (Waltham, MA, USA) (Garzoli et al., 2020). For the determination of Volatile Organic Compounds (VOCs), the sample (2 mL) was placed into 20 mL vials furnished of headspace PTFE-coated silicone septa. The headspace parameters were: 80 °C thermostating temperature for 20 min; 3.0 min pressurization time, and 0.3 min of injection time. The separation of analytes was achieved using a Varian Factor Four VF-1 fused-silica capillary column (length 60 m x 0.32 mm ID x 1.0 μm film thickness) and helium (1.0 mL min^{-1}) was the carrier gas. The oven temperature was set at 60 °C, to reach then 220 °C at a rate of 5 °C min^{-1} and held 30 min. The injector temperature was set at 280 °C, the ionization energy was 70 eV, ion source temperature was 200 °C, and the mass range of 40.0–500.0 AMU. The components were identified by the comparison of their mass spectra with those reported in MS libraries (ADAMS, WILEY 275 and NIST 17). In addition, RI were calculated as described in the previous paragraph. VOCs percentage was calculated by peak area normalization and data were analysed by One-way ANOVA followed by post-hoc Tukey HSD test ($p < 0.05$ as significant).

3.2.11 Insecticidal assays

Acute toxicity on Culex quinquefasciatus larvae

Mosquitoes were reared using the same protocol reported Chapter I, Section 5.2.1. *A. oleracea*-derived products (EO, EO-NE, *n*-hexane extract, and spilanthol) were diluted in DMSO, then in water (i.e. the EO and spilanthol), or directly in water (i.e., the EO-NE and the extract) and serial dilutions of test concentrations were prepared: a series of 5 concentrations ranging from 10 to 30 μL L^{-1}, from 300 to 900 μL L^{-1}, from 5 to 20 μL L^{-1} and from 1 to 10 μL L^{-1} for the EO, EO-NE, extract and spilanthol, respectively). Larvicidal assays were performed as previously described (Chapter I, Section 5.2.1). Decis Forte containing 100 g L^{-1} (10.5%) of deltamethrin (Bayer S.A.S., France) was employed as the positive control (Rahman and Howlader, 2018).

Effect of lethal concentrations on larval development and fertility of new adults

Early 3rd instar larvae of *Cx. quinquefasciatus* were moved to a plastic bowl (20 × 20 × 20 cm) containing water (3 L). After 1 h, the LC_{30} obtained for the EO, EO-NE, extract, and spilanthol (i.e., 12.4, 343.7, 7.3, and 2.3 μL L^{-1}, respectively) were added to the water. EO, extract, and spilanthol were diluted in DMSO. The negative control was represented by water with the same amount of DMSO used for the dilution of EO, extract, and spilanthol. Each trial (consisting of 100 larvae) was conducted thrice. After 24 h from the treatment, larvae were moved into clean water furnished of dog biscuits and yeast powder (3:1, w:w) as larval food, for the observation of adult emergence. Mortality was measured 24 and 48 h post-treatment. In addition, final mortality, emerged adults (%), and their sex were observed (Benelli et al., 2017a). The procedure developed by Benelli et al. (2017a) was employed for the evaluation of the LC_{30} exposure effects on the mosquito fecundity, fertility, and natality. In detail, adults of both sexes were placed in breeding cages (25 × 25 × 30 cm) and fed, according to Benelli et al. (2017a). The oviposition was evaluated in a bowl (10 cm of diameter) containing water placed in the cage, and the number of laid eggs was measured daily using a Leica light microscope. Each experiment was performed 4 times in a growth chamber (16:8 (L:D), 25 ± 1 °C) (Benelli et al., 2017a).

Statistical analysis

Statistical analysis was performed as described in Chapter I, Section 5.2.1. Differences among means were considered significative if $p < 0.05$.

3.2.12 Acetylcholinesterase (AChE) Inhibitory Activity

Acetylcholinesterase (AChE) inhibitory activity of *A. oleracea* EO, spilanthol, and *A. oleracea* EO-NE samples was evaluated through the Ellman assay (Ellman et al., 1961) with some changes (Benelli et al., 2019a). All samples were diluted in methanol.

3.2.13 Cytotoxicity and anti-inflammatory effects of *Acmella oleracea*-derived products on human cells

Cell viability assay

Human malignant melanoma cell line (A375), human breast adenocarcinoma cell line (MDA-MB 231), and normal human fibroblast cell line (NHF-A12) were cultured in DMEM, adding 1 mM sodium pyruvate. Murine microglial cells (BV-2) were provided by Prof. Elisabetta Blasi, University of Modena and Reggio Emilia, Italy, and cultured in DMEM. All the media were added

with 10% heat-inactivated fetal bovine serum (HI-FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. Cells incubation was performed at a temperature of 37°C with 5% CO_2. A375 cells, MDA-MB 231 cells, and NHF-A12 were submitted to different doses of *A. oleracea* EO (1.95-500 µg mL^{-1}), spilanthol (3.125–800 µg mL^{-1}), and *A. oleracea* extract (µg mL^{-1}), solubilized in DMSO for 72 h and then diluted in the medium. *A. oleracea* EO-NE (7.8-2000 µg mL^{-1}) was diluted directly in the medium. The BV-2 cells were treated with diverse doses of *A. oleracea* EO (0.5-500 µg mL^{-1}), *A. oleracea* EO-NE (0.001-10 µg mL^{-1}), and spilanthol (10-200 µM) for 24 h and then activated with 100 ng µg mL^{-1} LPS for 24 h. At the end of each assay, cell viability was estimated by the reduction of MTT to formazan by mitochondrial succinate dehydrogenase following the protocol previously reported (Nkuimi Wandjou et al., 2020). The measure of absorbance performed at 540 nm with a microplate spectrophotometer FLUOstar Omega (BMG Labtech). The cytotoxicity calculation was performed with GraphPad Prism 5 software and it was reported as the concentration of the sample reducing cell growth by 50% (IC_{50}). Cell viability of BV-2 cells was expressed as % of control cells.

DCFH-DA assay

The determination of intracellular Reactive Oxygen Species (ROS) formation in microglial BV-2 cells was analyzed using the fluorescent DCFH-DA probe (Marrazzo et al., 2019). After the different experiments, 10 µM DCFH-DA in DMEM 1% FBS without phenol red were added in each well and cells. 30 min later, DCFH-DA solution was replaced with PBS. Cell fluorescence was quantified ($\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 535 nm) on a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, PerkinElmer).

Real-Time polymerase chain reaction (PCR)

The total RNA from BV-2 cells was extracted using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). RNA quality was evaluated by measuring A_{260}/A_{280} and A_{260}/A_{230} with NanoVue Spectrophotometer (GE Healthcare, Milano, Italy). iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA) was employed to reverse-transcribed 1 µg of total RNA to cDNA. To carry out PCR in a total volume of 10 µL were added 12.5 ng of cDNA, 5 µL SsoAdvanced Universal SYBR Green Supermix (BIO-RAD), and 500 nM of each primer. In different primers used (SIGMA-ALDRICH, Milan, Italy) are reported in Table S2 of Supplementary Material. GAPDH was considered as the reference gene. SsoAdvanced Universal SYBR Green Supermix (BioRad) was used for real-time PCR performance. The polymerase was activated at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Expression levels were normalized in respect to the reference gene and compared with control cells using the $2^{-\Delta\Delta CT}$ method.

3.3. Results and Discussion

3.3.1 Chemical analyses of *Acmella oleracea* essential oils (EO) obtained by hydrodistillation (HD) and Microwave-Assisted Extraction (MAE)

MAE led to a higher extraction yield than the conventional HD, 0.47 and 0.22% (w/w), respectively. The results of the chemical analysis of the two EOs are reported in Table 7.

Table 7. Chemical composition of the essential oils of *Acmella oleracea* from hydrodistillation (HD) and microwave-assisted extraction (MAE).

No.	Components[a]	RI[b]	RI Lit.[c]	Relative peak area (%)		ID[d]
				HD	MAE	
1	(2*E*)-hexenal	847	846	-	0.1	RI,MS
2	*n*-hexanol	864	863	-	tr[e]	RI,MS
3	*α*-thujene	921	924	0.1	tr	RI,MS
4	*α* –pinene	926	932	2.1	1.7	Std,RI,MS
5	Camphene	939	946	Tr	tr	Std,RI,MS
6	sabinene	965	969	0.7	1.5	Std,RI,MS
7	*β*-pinene	967	974	14.7	10.8	Std,RI,MS
8	myrcene	989	988	17.4	12.3	Std,RI,MS
9	*p*-mentha-1(7),8-diene	1001	1004	0.3	0.2	RI,MS
10	*α*-terpinene	1013	1014	0.2	tr	Std,RI,MS
11	*p*-cymene	1021	1020	0.2	tr	Std,RI,MS
12	*β*-phellandrene	1024	1025	6.5	4.9	RI,MS
13	(*Z*)-*β*-ocimene	1036	1032	6.0	3.8	Std,RI,MS
14	(*E*)-*β*-ocimene	1046	1044	0.1	tr	Std,RI,MS
15	*γ*-terpinene	1054	1054	0.3	tr	Std,RI,MS
16	terpinolene	1084	1086	tr	0.1	Std,RI,MS
17	allo-ocimene	1128	1128	0.3	0.1	RI,MS
18	terpinen-4-ol	1171	1174	0.5	tr	Std, RI,MS
19	*δ*-elemene	1330	1335	0.3	0.8	RI,MS
20	*α*-copaene	1366	1374	0.1	0.2	RI,MS
21	*β*-elemene	1384	1389	0.3	0.2	RI,MS
22	cyperene	1386	1398	tr	0.2	RI,MS
23	(*Z*)-caryophyllene	1396	1408	0.4	-	RI,MS
24	(*E*)-caryophyllene	1408	1417	19.4	19.4	Std,RI,MS
25	*β*-copaene	1418	1430	0.1	0.1	RI,MS
26	*γ*-elemene	1426	1434	0.3	0.1	RI,MS
27	*α*-humulene	1441	1452	1.3	1.1	Std, RI,MS
28	germacrene D	1470	1484	11.8	15.2	RI,MS
29	bicyclogermacrene	1486	1500	-	0.3	RI,MS
30	1-pentadecene	1492	1493	-	5.5	RI,MS
31	(*Z*,*E*)-*α*-farnesene	1493	1491	2.1	2.7	RI,MS
32	*δ*-amorphene	1498	1511	0.1	-	RI,MS
33	*γ*-cadinene	1503	1513	0.3	tr	RI,MS
34	(*E*,*E*)-*α*-farnesene	1505	1505	0.6	0.4	RI,MS
35	*δ*-cadinene	1515	1522	1.1	0.2	RI,MS
36	kessane	1520	1529	1.4	0.5	RI,MS
37	germacrene B	1543	1559	0.4	0.2	RI,MS
38	(*E*)-nerolidol	1560	1561	0.7	0.2	Std,RI,MS
39	caryophyllene oxide	1569	1583	0.9	0.3	Std,RI,MS
40	(*Z*,*Z*)-1,8,11-heptadecatriene	1660	1664	-	0.2	RIMS
41	spilanthol	1887	1888[f]	2.3	11.7	MS[g]
42	acmellonate	1997	-	2.1	2.5	MS[h]
43	*n*-tricosane	2297	2300	tr	0.2	RI,MS

44	*n*-pentacosane	2497	2500	tr	0.1	RI,MS
	Essential oil yield (%, w/w)			0.22	0.47	
	Total identified (%)			95.3	97.8	
	Grouped compounds (%)					
	Monoterpene hydrocarbons			48.8	35.5	
	Oxygenated monoterpenes			0.5	tr	
	Sesquiterpene hydrocarbons			38.6	41.2	
	Oxygenated sesquiterpenes			2.9	0.9	
	Alkylamides			2.3	11.7	
	Others			2.2	8.6	

[a]The order of elution derives from an HP-5MS column (30 m x 0.25 mm, 0.1 μm). [b]The linear retention index is in accordance with Van den Dool and Kratz (1963). [c]RI has been taken from ADAMS and/or NIST17 and FFNSC3 libraries. dIdentification performed using commercial standards or comparing the mass spectrum (MS) or the retention index (RI) with those reported in literature. [e]tr, traces.

As reported in Table 7, the major constituents of the HD EO were monoterpene hydrocarbons (48.8%), sesquiterpene hydrocarbons (38.6%), oxygenated sesquiterpenes (2.9%), alkylamides (2.3%), and oxygenated monoterpenes (0.5%). The total compounds identified represented the 95.3% of the total EO composition. On the contrary, MAE EO was mainly constituted by sesquiterpene hydrocarbons (41.2%), monoterpene hydrocarbons (35.5%), alkylamides (11.7%), oxygenated sesquiterpenes (0.9%), and oxygenated monoterpenes in traces. The total of identified compounds accounted for the 97.8%. The major compounds found in both EOs were (*E*)-caryophyllene, *β*-phellandrene, myrcene, germacrene D, spilanthol, *β*-pinene, and (*Z*)-*β*-ocimene. However, from a comparison of the two chemical constitutions, it was clear that spilanthol percentage was higher in the MAE EO with respect to HD EO (11.7 vs 2.3%, respectively), as shown in Figure S2 (Supplementary Material). In addition, other differences were found between HD and MAE EOs represented by the content of *β*-pinene (14.7 and 10.8%, respectively), sabinene (0.7 and 1.5%, respectively), myrcene (17.4 and 12.3%, respectively), and germacrene D (11.8 and 15.2%, respectively). Moreover, a ketol ester, (*7Z*,*9E*)-2-oxo-undeca-7,9-dienyl 3-methylbut-2-enoate, also known as acmellonate and with a structure similar to that of spilanthol, was identified confronting the MS fragmentation pattern reported by Ley et al. (2006). This study demonstrated the above-mentioned properties of MAE for the enhancement of the EO extraction yield and the modulation of its chemical composition. In fact, MAE led to a higher EO extraction yield, which resulted double to that of HD. Furthermore, the EO yield obtained through MAE resulted higher than that obtained from another study on the HD EO obtained from the dry plant (Benelli et al., 2019c). This lead to conclude that MAE is more efficient in terms of EO yields than HD for the *A. oleracea* EO extraction. Additionally, MAE resulted an efficient technique to obtain a jambú EO richer in spilanthol, *β*-pinene, myrcene, sabinene, and germacrene D. The results obtained in our study were linear with those from the work of Benelli et al. (2019c), in which the *A. oleracea* HD EO contained similar amounts of spilanthol, (*Z*)-*β*-ocimene, *δ*-elemene, germacrene D and (*Z*,*E*)-*α*-farnesene if compared with the HD EO obtained in our study.

Another aim of this study was the quantification of *A. oleracea* marker compounds through GC-FID analysis in both the EOs. Figure 10 shows the main differences between HD and MAE EOs in terms of marker compounds content. The major difference resulted in the spilanthol percentages (13.31 and 2.24% in MAE and HD EOs, respectively). Regarding the other compounds, (*E*)-caryophyllene, *α*-humulene, and germacrene D content was higher in the MAE EO (16.34, 10.42, and 7.60%, respectively). On the contrary, myrcene and *β*-pinene content resulted higher in the HD EO than in the MAE EO (13.30 and 16.50%, respectively).

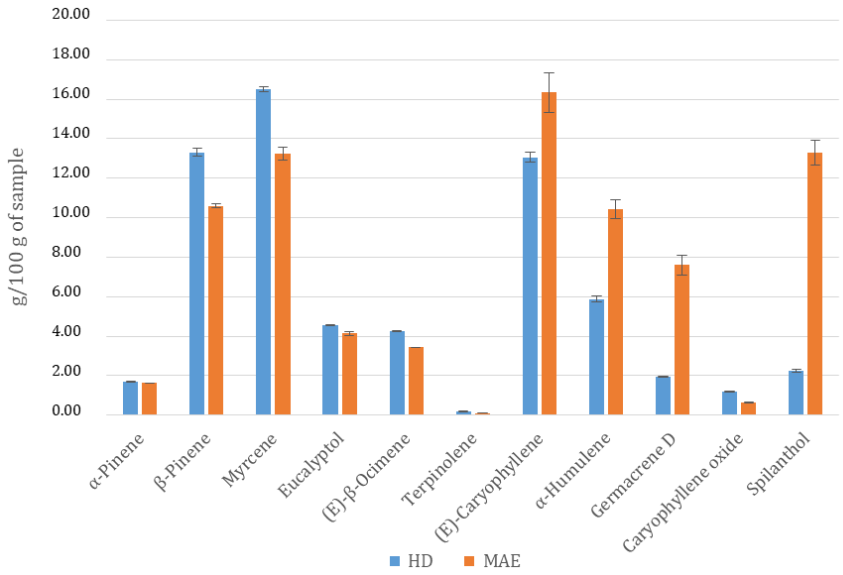


Figure 10. GC-FID quantified marker compounds in *Acmella oleracea* essential oils (EOs) obtained by hydrodistillation (HD, blue) and microwave-assisted extraction (MAE, orange). Data were analysed by one-way ANOVA, highlighting various degrees of significance (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) between HD and MAE

The results herein reported indicate that MAE, in which the extraction is based on a deeper penetration of microwaves through the plant matrix, is able to enhance the extraction of spilanthol. Probably, HD lets to lower contents of spilanthol since it is a process leading to a strong oxidation of the fatty matter from which spilanthol is usually produced (Barbosa et al., 2017).

MAE EO was analysed in terms of enantiomeric distribution and enantiomeric excess (EE) of their chiral components. Table 8 reports the compounds that were successfully separated and identified.

Table 8. Enantiomeric distribution of the chiral components of *Acmella oleracea* essential oil obtained from microwave-assisted extraction (MAE).

Enantiomer compound	RT[a]	RI[b]	% in EO[c]	Enant. %[d]	EE %[e]
(-)-*α*-pinene	15.604	1022	0.99	95.2	90.5
(+)-*α*-pinene	15.949	1027	0.05	4.8	-
(+)-*β*-pinene	19.238	1081	0.02	0.2	-
(-)-*β*-pinene	19.395	1083	12.05	99.8	99.6
(+)-limonene	20.220	1097	0.57	84.6	69.2
(-)-limonene	20.517	1101	0.10	15.4	-
(+)-*β*-phellandrene	21.297	1114	0.34	12.7	-
(-)-*β*-phellandrene	21.541	1117	2.30	87.3	74.5
(+)-(*E*)-caryophyllene	-	-	-	-	-
(-)-(*E*)-caryophyllene	44.688	1498	14.71	100	100
(-)-caryophyllene oxide	57.472	1741	0.05	100	100
(+)-caryophyllene oxide	-	-	-	-	-

[a]Retention time of the different enantiomers from the chiral column (HP chiral 20B). [b]Linear retention index calculated using a *n*-alkanes mixture (C_7-C_{30}). [c]Absolute content of enantiomer in the essential oil determined by GC-MS analysis. [d] Relative content of enantiomeric pairs. [e] Enantiomeric excess.

In detail, the assignation of the enantiomers of both *α*-pinene and *β*-pinene was in accordance with the work of Dahmane et al. (2015), in which the same stationary phase was employed for the separation. Regarding the order of elution of the enantiomers of (*E*)-caryophyllene, limonene, and caryophyllene oxide, this was in accordance with data previously reported (Fiorini et al., 2020). The order of elution of *β*-phellandrene enantiomers was established accordingly to that reported by Yassaa and Williams (2005). The reported data point out that both enantiomers of the monoterpenes *α*-pinene, *β*-pinene, limonene, and *β*-phellandrene are contained in the EO. Concerning *α*-pinene, the (-)-enantiomeric form was predominant (EE of 90.5%), as also for *β*-pinene whose EE of the (-)-form was of 99.6%. For limonene, the (+)-enantiomeric form was present in an EE of 69.2%, while for *β*-phellandrene the EE of the (-)-form was of 74.5%. On the contrary, the sesquiterpene (*E*)-caryophyllene and the oxygenated sesquiterpene caryophyllene oxide were detected only in the (-)-form.

3.3.2 GC-FID, NMR, and HPLC-MS analyses of *Acmella oleracea n*-hexane extract

The quantification of spilanthol in the *n*-hexane extract by GC-FID analysis led to a concentration of 22.39 g/100 g of DE. The *n*-hexane extract was then analysed by ^{1}H-NMR, which was characterized by signals ascribable to *N*-alkylamides. In detail, spilanthol resulted the most abundant member of this class of compounds. The ^{1}H-NMR was performed by choosing the signal attributed to the H-2, since it was the only doublet common to almost all the *N*-alkylamides in the plant. The total amount of *N*-alkylamides expressed as spilanthol content in the extract was of 50.9% (Figure 11).

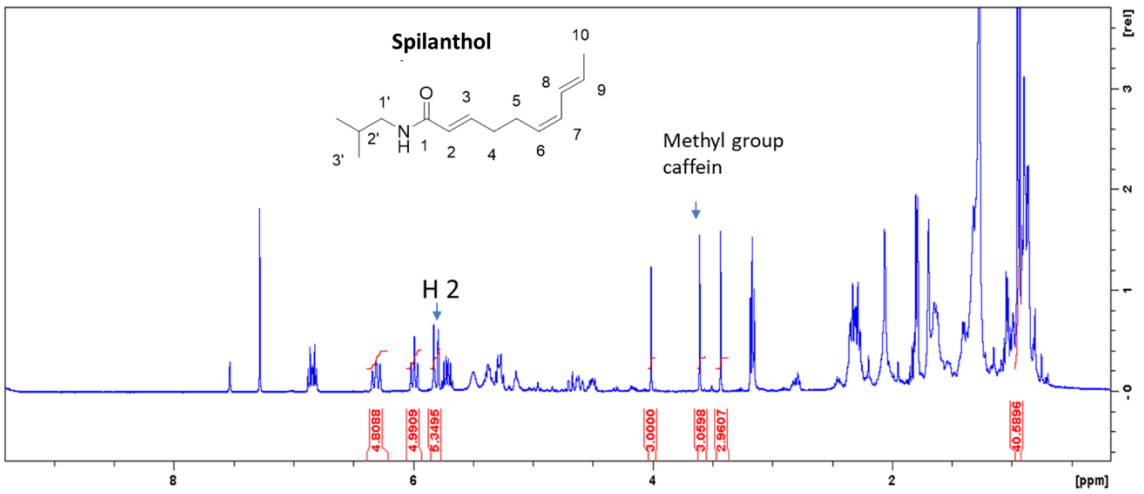


Figure 11. ^{1}H NMR of the of *Acmella oleracea n*-hexane extract and caffeine; integral of signal H-2 of spilanthol and methyl group of caffeine are reported.

An LC-MS method was employed for the assignment of *N*-alkylamides in the hexane extract, and fragmentation pathway of each ion was confronted with that reported by Boonen et al. (2010a), tentatively assigning each peak as reported in Table 9.

Table 9. Identification and quantification of *N*-alkylamide derivatives in *Acmella oleracea n*-hexane extract. Retention time (RT) and $[M+H]^+$ for each compound were reported.

$[M+H]^+$	RT	Identification	%
204	21	(2*Z*)-*N*-isobutyl-2-nonene-6,8-diynamide	0.18
232	23.9	(2*E*)-*N*-isobutyl-2-undecene-8,10-diynamide	0.43
222	25	(2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol)	42.67
246	25.8	(2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide	0.11
258	26.1	(2*E*,7*Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide	0.43
224	27.1	(2*E*,7*Z*)-*N*-isobutyl-2,7-decadienamide	0.89
236	27.1	(2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide	6.10
248	28	(2*E*,4*E*,8*Z*,10*Z*)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide	0.09

The most abundant *N*-alkylamide resulted spilanthol (42.67%), followed by (*2E,6Z,8E*)-*N*-(2-methylbutyl)-*2,6,8*-decatrienamide (6.1%) and (*2E,7Z*)-*N*-isobutyl-2,7-decadienamide (0.89%). The results were linear with those reported by Boonen et al. (2010a), who analyzed the ethanolic extract of *A. oleracea*, revealing that the main *N*-alkylamide was spilanthol, followed by (2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide. In addition, our data are confirmed also by the work of Barbosa et al. (2016b), which focused on the analysis of *A. oleracea* methanolic extract and reported spilanthol and (2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide as the main *N*-alkylamides.

3.3.3 Development and characterization of *A. oleracea* EO nanoemulsions

For the encapsulation *A. oleracea* EO, different formulations were prepared varying the total oil phase amount (0.6, 0.75 and 0.9% w/w; EO to ethyl oleate ratio of 0.5) and the percentage of the surfactant (P80 at 2, 3.5 and 5% w/w) in order to optimize the number of constituents and to produce stable *A. oleracea* EO-based NEs. All the NEs (NE_1–NE_5) appeared as isotropic samples, with no oil droplets above 1000 nm right after preparation and also up to 1-year of storage (Figure 12a). This result pointed out the effectiveness of high-pressure homogenization for the droplet size reduction and the formation of nano-emulsified systems.

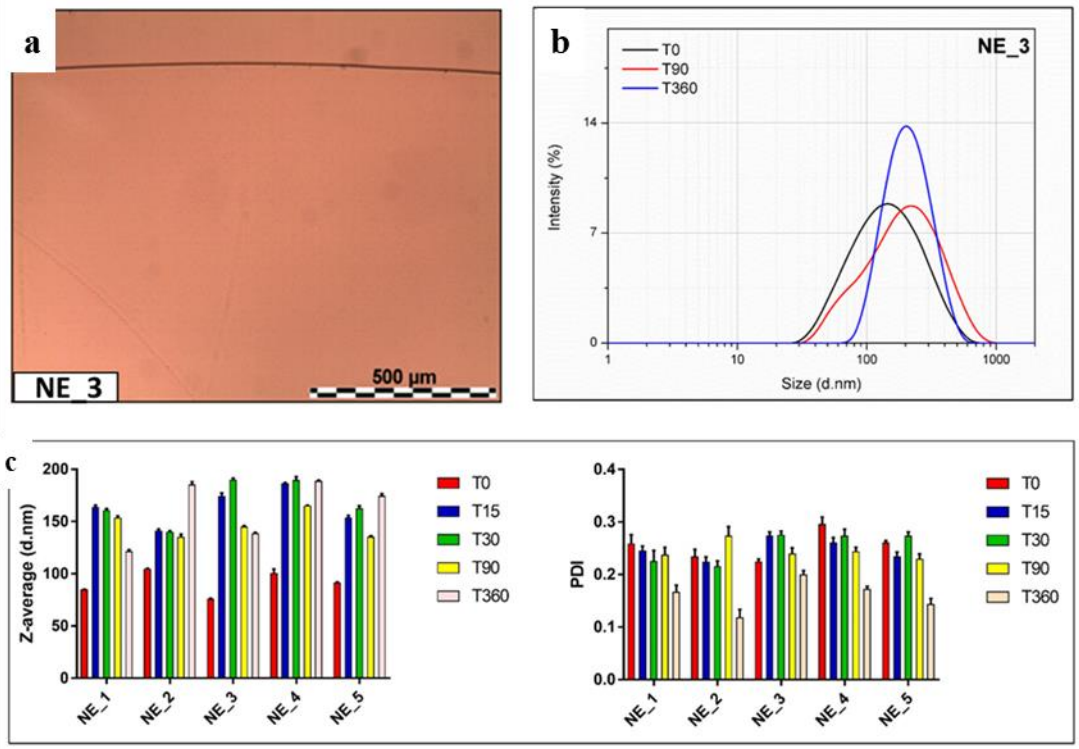


Figure 12. (**a**) Polarised optical microscope image (10x of magnification) of the *Acmella oleracea* essential oil-based nanoemulsion NE_3 stored for 1 year at room temperature. (**b**) Droplet size distribution (intensity %) from DLS for the nanoemulsion NE_3 recorded at different stability time points (0, 90 and 360 days, i.e. T0, T90 and T360 days, respectively). (**c**) Z-average (d.nm) and polydispersity index (PDI) from DLS for all prepared NEs (NE_1–NE_5) at different stability time points (0, 15, 30, 90, and 360 days, i.e., T0, T15, T30, T90 and T360, respectively).

In addition, a monomodal droplet size distribution < 1000 nm was registered for all NEs, as revealed by DLS analysis at different timepoints up to 1 year (Figure 12b). The NEs were also compared in terms of Z-average (d.nm) and polydispersity index (PDI) (Figure 12c). In detail, the droplet size was lower than 100 nm for all formulations right after the formulation (T0), increasing up to 120-200 nm after 15 days (T15) and stayed in this range for all the observation times up to 1 year (T360). PDI values were between 0.2-0.3 for all NEs up to 3 months (T0-T90), showing a

reduction (0.1-0.2) for a longer time up to 1-year (T360). The results obtained represented the confirmation of the physical stability of all the NEs at least up to 1-year. The data obtained led to the choice of NE_3 for further investigations. This NE_3 was constituted by the highest amount of oil phase (9% w/w) and the lowest amount of surfactant (2% w/w of P80). NE_3 chemical stability over time was analysed through headspace (HS)-GC/MS analysis, which revealed only the most volatile compounds (Figure 13).

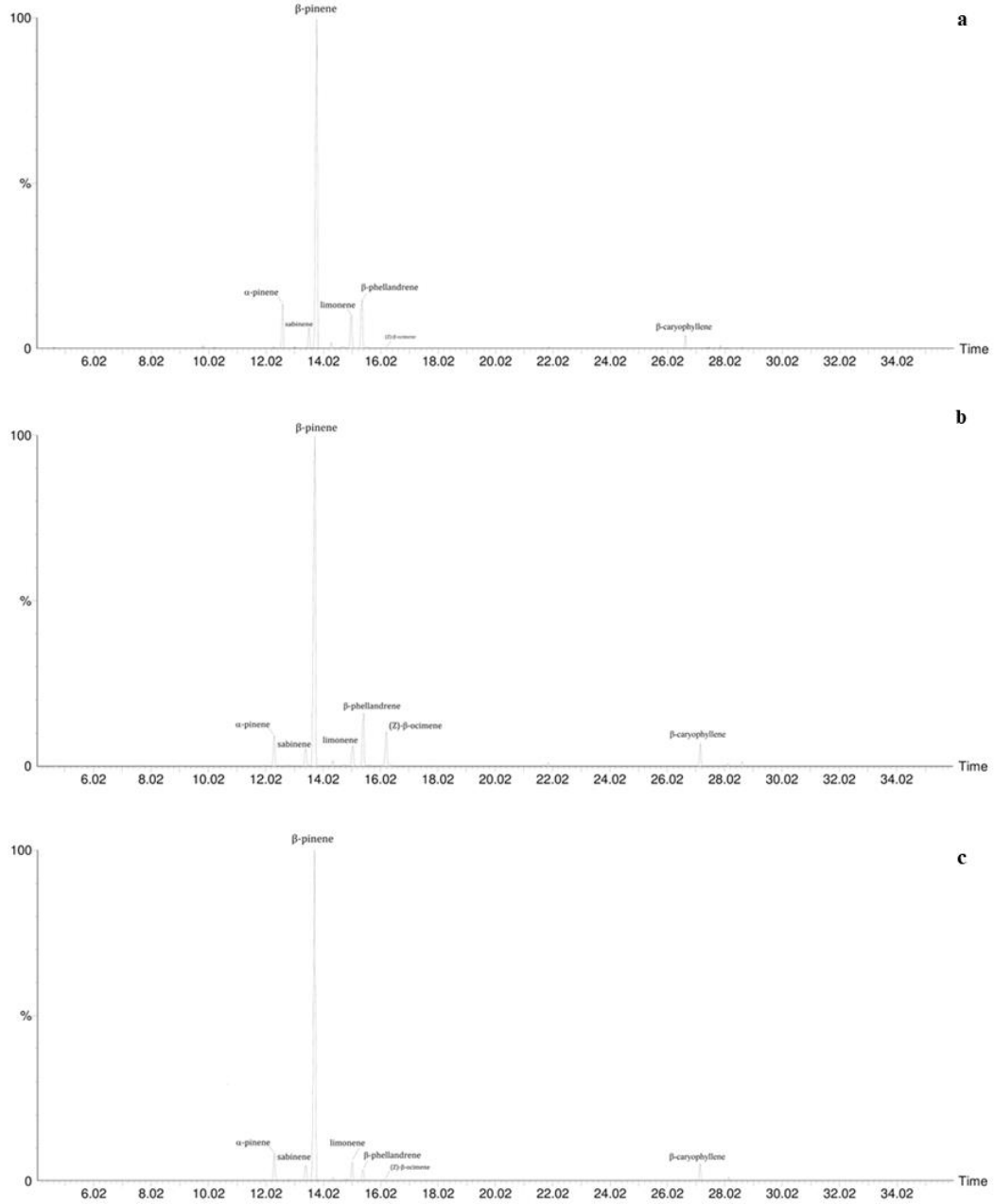


Figure 13. HS-GC-MS chemical fingerprint of the vapour phase of *Acmella oleracea* essential oil (**a**), its nanoemulsion at time 0 (**b**), and after 4 months of storage (**c**).

However, sesquiterpenes and spilanthol could not be found in the NE headspace due to their higher boiling points. The GC-MS chemical fingerprints of the vapor phase of *A. oleracea* EO and its nanoencapsulated form were overlapping, pointing out that the NE retained the whole EO. The main compounds found in the vapor phase resulted *β*-pinene (66.5 and 65.6%, respectively, semi-quantitative data), *β*-phellandrene (9.3 and 10.0%, respectively), *α*-pinene (8.0 and 5.3%, respectively), limonene (6.0 and 4.8%, respectively), sabinene (4.2 and 3.4%, respectively), (*E*)-caryophyllene (3.1 and 3.0%, respectively), and other minor components. In the pure EO and its encapsulated form a prevalence of monoterpene hydrocarbons (96.0 and 96.3%, respectively) rather than sesquiterpene hydrocarbons (4.0 and 3.7%, respectively) was noticed. The EO formulated into the NE was also analysed four months after its formulation for the evaluation of its stability over time. Statistical analysis showed the absence of significant differences between the means ± SD of all compounds. This suggested that storage did not modify the chemical composition of the EO formulated into the NE.

3.3.4 Insecticidal activity

A. oleracea-derived products were tested on *Cx. quinquefasciatus* 3rd instar larvae, and they all showed relevant activity. Spilanthol resulted the most effective product (LC_{50} value of 3.1 µL L^{-1}), followed by the *n*-hexane extract (LC_{50} = 9.5 µL L^{-1}), and the EO (LC_{50} = 16.1 µL L^{-1}). A significant difference among the tested products was detected, since 95% confidence intervals failed to overlap (Table 10).

Table 10. Insecticidal activity of *Acmella oleracea*-derived botanicals against *Culex quinquefasciatus* 3rd instar larvae.

Tested product[a]	**LC_{30}**[b] **(µL L^{-1})**	**CI_{95}**[c]	**LC_{50} (µL L^{-1})**	**CI_{95}**	**LC_{90} (µL L^{-1})**	**CI_{95}**	**χ^2**	***df***[d]	***p*-value**
EO	12.4	6.7-15.7	16.1	15.9-24.1	29.8	25.7-36.5	3.038	3	0.385 ns[e]
EO-NE	343.7	271.5-358.7	407.5	392.8-466.9	617.7	582.6-712.3	3.957	4	0.412 ns
Hexane extract	7.3	3.8-8.1	9.5	8.9-12.7	17.7	15.5-28.9	4.074	3	0.253 ns
Spilanthol	2.3	2.1-2.5	3.1	2.8-3.4	6.3	5.6-7.3	4.721	4	0.317 ns
Positive control Deltamethrin	3.8	3.2-5.6	15.3	10.1-17.5	56.5	48.9-67.2	2.859	3	0.523 ns

[a]EO, essential oil; NE, nanoemulsion; [b]LC, lethal concentration killing 50% (LC_{50}) or 90% (LC_{90}) of the exposed mosquito larvae; [c]CI_{95}, 95% confidence interval; [d]*df* , degrees of freedom; [e]ns, not significant ($p > 0.05$).

Worthy of notice is that spilanthol and the *n*-hexane extract resulted slightly more effective than the positive control deltamethrin (LC_{50} of 15.3 µL L^{-1}), while overlapping LC_{50} values with the EO were registered. Moreover, when the EO is formulated into a NE an LC_{50} of 407.5 µL L^{-1} was reached (Table 10). However, the lethal concentrations of the EO-NE should be recalculated,

considering that only the 6% of the EO was encapsulated. So, it can be concluded that the EO and the EO-NE presented lethal concentrations ($LC_{50(90)}$) not significantly different. Literature data on *A. oleracea* demonstrated the broad insecticidal activity of its EO against mosquitoes, such as *Cx. quinquefasciatus* (LC_{50} = 42.2 mg L^{-1}), as well as on *M. domestica* females (LD_{50} = 44.3 µg $adult^{-1}$) and *S. littoralis* 3rd instar larvae (LD_{50} = 68.1 µg $larva^{-1}$) (Benelli et al., 2019c). The main *N*-alkylamide of *A. oleracea* was also tested alone for the investigation of its insecticidal activity. In detail, spilanthol demonstrated high efficacy against larvae of *P. xylostella* (LC_{50} = 1.5 g L^{-1}) (Sharma et al., 2012) and *T. absoluta* (LD_{50} = 0.13 µg mg^{-1}) (Moreno et al., 2012). In addition, this compound resulted also active on *A. aegypti* larvae (12.5 µg mL^{-1}) (Ramsewak et al., 1999) and its 7.5 ppm concentration led to 100% mortality of larvae of *Culex*, *Aedes*, and *Anopheles* mosquitoes; while its lower doses were effective also on eggs and pupae (Saraf and Dixit, 2002). On the other hand, spilanthol and other *N*-alkylamides from *Heliopsis longipes* (A.Gray) S.F.Blake roots were active both on *An. albimanus* and *Ae. aegypti* 3rd instar larvae with LC_{50} values of 4.2 and 7.3 mg L^{-1}, respectively (Hernández-Morales et al., 2015). In this context, the herein reported study supports the hypothesis of spilanthol major contribution for the mosquitocidal activity of *A. oleracea*-derived products. Overall, further efforts to shed light on the toxicity of spilanthol are needed, since the insecticidal mechanism of action of this *N*-alkylamide is still not clarified at all.

Cx. quinquefasciatus larvae biology significantly changed when the individuals were treated with the LC_{30} of the EO, EO-NE, hexane extract, and spilanthol (12.4, 343.7, 7.3, and 2.3 µL L^{-1}, respectively), with the onset of further mortality and a significant decrease in adult emergence rates (Table 11).

Table 11. Sublethal effect of *Acmella oleracea*-derived products formulated at their LC_{30} on *Culex quinquefasciatus* larval mortality as well as on emergence, fecundity, fertility, and natality of the new generation adults.

Treatment [a]	**Tested concentration (µL L^{-1})**	**Larval mortality (%)** [b]			**Emergence of adults (%)** [b]			**Fecundity and fertility indicators** [b]		**Natality** [b]	
		24 h	**48 h**	**Total**	**Female**	**Male**	**Total**	**Fecundity (no. eggs/female)**	**Fertility (egg hatchability %)**	**F_1 generation larvae (no) out of 100 treated larvae**	**Natality inhibition over the control (%)**
EO	12.4	5.6 ± 1.2^{a}	8.0±1.4^{b}	18.1±3.6^{b}	45.9±1.8^{b}	34.2±1.7^{b}	80.2±4.5^{cd}	81.9±12.7	95.5±1.3^{bc}	3.230.7±239.7^{b}	24.1±8.5
EO-NE	343.7	20.6±2.7^{b}	23.5±1.5^{c}	33.3±2.9^{c}	31.5±1.2^{a}	32.2±1.5^{b}	63.8±1.5^{b}	103.7±17.2	71.2±5.4^{a}	1.483.8±258.8^{a}	65.1±7.9
Hexane extract	7.3	31.7±3.5^{c}	33.1±3.8^{d}	41.5±3.3^{d}	35.4±1.7^{a}	20.1±2.1^{a}	55.5±3.2^{a}	78.9±12.1	92.0±0.3^{b}	1.444.2±138.5^{a}	66.1±9.4
Spilanthol	2.3	3.7±0.5^{a}	6.2±0.2^{b}	19.5±2.2^{b}	45.8±1.1^{b}	31.8±2.5^{b}	77.7±1.6^{c}	101.2±15.9	90.0±1.1^{b}	3.241.2±202.5^{b}	23.9±3.9
Control	-	0.0±0.0^{a}	0.0±0.0^{a}	8.8±5.5^{a}	45.1±2.1^{b}	43.9±2.3^{c}	89.0±5.5^{d}	109.0±12.2	97.4±1.8^{c}	4.243.4±189.5^{c}	-
ANOVA $F_{4.15}$ and *P*-value	-	122.2, <0.0001	148.5, <0.0001	95.0, <0.0001	147.5, <0.0001	185.7, <0.0001	227.8, <0.0001	ns	128.7, <0.0001	162.5, <0.0001	-

[a]*Cx. quinquefasciatus* 3rd instar larvae were exposed to concentrations corresponding to the estimated LC_{30} for each *Acmella oleracea*-derived products (i.e. 12.4, 343.7, 7.3, and 2.3 µL L^{-1} for EO, EO-NE, hexane extract and spilanthol, respectively); EO, essential oil; NE, nanoemulsion. [b]Mean% (±SE) within a column followed by the same letter do not differ significantly according to Tukey's HSD test at $p < 0.05$ (% = arcsine transformed data); ns, not significant ($p > 0.05$).

The treatment with the EO, EO-NE, *n*-hexane extract, and spilanthol with the calculated LC_{30} also led to a reduction of adult fertility, in terms of egg hatchability (%) and overall abundance of F_1 larvae if compared with the individuals treated with the control (Table 11). The EO-NE resulted the *A. oleracea*-derived product able to cause the highest reduction of egg hatchability (%) (71.2 vs. 97.4% of the control). Regarding the natality, both the EO-NE and the *n*-hexane extract caused an inhibition of natality higher than 65% over mosquitoes treated with the control (Table 11). As indicated above, the tested NE-EO displayed a higher effect at the LC_{30} compared with the EO itself. This observation has been confirmed also by other studies that demonstrated that EO-loaded NEs usually give a higher efficacy rather than that of the EO (Bai and McClements 2016; Oliveira et al. 2016). The use of EO-NEs usually leads to some advantages with respect to the use of pure EOs, as the enhancement of the hydrophilicity and stability of EOs, but also to improved biological activities (Pavela et al., 2019b). The improved effect of the NE could also be ascribed to the nanoscale particle size (Oliveira et al. 2016) and to a long-term delivery of EOs.

Moreover, the EO-NE was the most effective at LC_{30} than the pure spilanthol. In this regard, probably other compounds contained in the EO, such as terpenoids, could increase the efficacy. Data reported in the literature suggest that terpenoids could act on insects through neurotoxicity or by interacting with receptors of the insect nervous system (Isman, 2020b). Some of these molecules are reported to inhibit the AChE enzyme, which has a crucial role in neuro-neuronal and neuromuscular junctions in both insects and mammals (Isman and Tak, 2017; Jankowska et al., 2018). For this reason, *A. oleracea* EO, *n*-hexane extract, and spilanthol were tested against AChE using galantamine as the positive control (Table 12).

Table 12. Acetylcholinesterase inhibitory properties of *Acmella oleracea* essential oil (EO) and spilanthol.

Treatment[a]	**IC_{50} (mg ml^{-1})**	**mg GEIC/g[b]**
EO	1.20 ± 0.08	0.5±0.01
Spilanthol	15.54±1.8	0.038±0.01
EO-NE	NA[c]	NA[c]
Hexane extract	NA[c]	NA[c]
Positive control		
Galantamine	0.6 (± 0.02) 10^{-3}	

[a]EO, essential oil; NE, nanoemulsion. [b]GEIC, galantamine-equivalent inhibition capacity. [c]na, not active.

A. oleracea EO showed an IC_{50} of 1.2 mg mL^{-1}, resulting 13-fold more active than the pure spilanthol (IC_{50}=15.54 mg mL^{-1}). In both cases, the inhibitory concentrations were far distant from that of galantamine (IC_{50}= 0.6 10^{-3} mg ml^{-1}). This confirms the previous hypothesis on the role of terpenoids in the enhancement of the insecticidal activity. In fact, the analysis of the EO chemical

composition showed the presence of monoterpene hydrocarbons (*α*- and *β*-pinene, and *β*-phellandrene) and sesquiterpene hydrocarbons (germacrene D and (*E*)-caryophyllene) that may contribute to the AChE inhibitory activity (Bonesi et al., 2010; Politeo et al., 2011). For example, (*E*)-caryophyllene displayed an AChE inhibitory activity with an IC_{50} value of 20 μg mL^{-1} (Borrero et al., 2019). If compared with the insecticidal EO from *C. acaulis* (Benelli et al., 2019a), the *A. oleracea* EO displayed a two-fold higher IC_{50} value. Thus, it is possible to assume that *A. oleracea* EO could act with a different mode of action compared to *C. acaulis* EO and that it could be the result of complex interactions of terpenoids with spilanthol.

The results obtained from the insecticidal assays can be considered as extremely promising for the development of new larvicidal agents based on *A. oleracea*.

3.3.5 Cytotoxic and anti-inflammatory activity of *Acmella oleracea* derivatives on human cells

Since botanical insecticides must also be evaluated in terms of safety to humans, in this work the cytotoxic effect of *A. oleracea*-derived products was also evaluated on normal human fibroblast cell line (NHF-A12) and two human tumour cell lines (A375 and MDA-MB 231) by MTT assay. The results displayed a moderate cytotoxic activity of *A. oleracea* EO against all cell lines with an accentuated activity on tumour MDA-MB 231 cells (IC_{50} value of 87.80 μg mL^{-1}) (Table 13).

Table 13. *In vitro* growth inhibition of cells by *Acmella oleracea* essential oil (EO), nanoemulsion (NE), spilanthol, and *n*-hexane extract.

Treatment[a]	**Cell line (IC_{50} μg mL^{-1})**[b]		
	NHF-A12[c]	**MDA-MB 231**[d]	**A375**[e]
EO	266.3	87.80	130.9
95% CI [f]	256.2–276.7	72.5–106.3	126.1–136.0
EO-NE	246.1	291.9	245.3
95% CI	229.2–264.3	274.9–309.8	226–266.3
Spilanthol	166.0	526.2	287.2
95% CI	124.3-182.7	475.6-582.4	243.7-332.3
Hexane extract	120.6	> 200	> 200
95% CI	98.14-154.05	-	-
Positive control			
Cisplatin	1.21	2.23	0.46
95% CI	1.05-1.53	1.99–2.37	0.33–0.54

[a]EO, essential oil; NE, nanoemulsion. [b]IC_{50}, the concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation). [c]Normal human fibroblast. [d]Human breast adenocarcinoma. [e]Human malignant melanoma. [f]CI Confidence interval.

A. oleracea EO displayed a lower toxicity on normal human fibroblasts than on treated cancerous cell lines, with an IC_{50} value of 266.3 μg mL^{-1}. According to the guidelines of the International

Organization for Standardization (ISO, 20093–5, 2009), *A. oleracea* EO cannot be considered toxic to normal cells. In addition, spilanthol did not display considerable toxicity on the experimental model of this study, since the IC_{50} values ranged from 166.0 to 526.2 µg mL^{-1} for NHF-A12 and MDA-MB 231, respectively. These data are linear with the work of Gerbino et al. (2016), that reported an IC_{50} value for spilanthol of 260 µg mL^{-1} in HEK293 cells. Regarding the other *A. oleracea*-derived products, a slightly higher toxicity was detected for the hexane extract (IC_{50} of 120.6 µg/mL). This could probably be linked to the presence of other metabolites that could enhance spilanthol cytotoxicity. The toxicity of the EO-NE was assayed on the same cell pattern. As reported in Table 13, a moderate activity on all cell lines was detected, with IC_{50} values ranging from 245.3 to 291.9 µg mL^{-1}. Conversely, *A. oleracea* EO resulted more effective on breast adenocarcinoma than malignant melanoma cells. The major compounds found in the MAE EO resulted to be (*E*)-caryophyllene, germacrene D, *β*-pinene, myrcene, and spilanthol and they have been reported as cytotoxic on different tumour cell lines (Setzer, et al. 2006; Venditti et al., 2013; Willig, et al., 2019; Woguem et al., 2014). Regarding the safety of *A. oleracea*-derived products, these displayed low cytotoxicity, but more investigations must be performed.

The anti-inflammatory property of *A. oleracea* EO, EO-NE, and spilanthol was assessed using murine microglial BV-2 cells, which are the counterpart of macrophages in the brain and in physiological conditions are involved in the protection of the central nervous system against exogenous damages (Wake et al., 2019). Though, the BV-2 cells' excessive activation can lead to neurotoxicity for the disproportionate production of pro-inflammatory agents like IL-6, IL-1β, nitric oxide, TNF-α, and ROS (Block and Hong, 2005). Moreover, during the last years, it was proven a key role of microglia in disorders such as Alzheimer's or Parkinson's (Bartels et al., 2020; Dansokho and Heneka, 2018; Kaminska et al., 2016).

Firstly, the activity of *A. olearacea* EO and spilanthol was assessed, and BV-2 cells were treated with different doses of EO and spilanthol for 24 h, measuring cell viability by MTT assay. Figure 14 displayed that *A. oleracea* EO was not cytotoxic up to 50 µg mL^{-1} (Figure 14a), while spilanthol was not cytotoxic at the tested concentrations (Figure 14b).

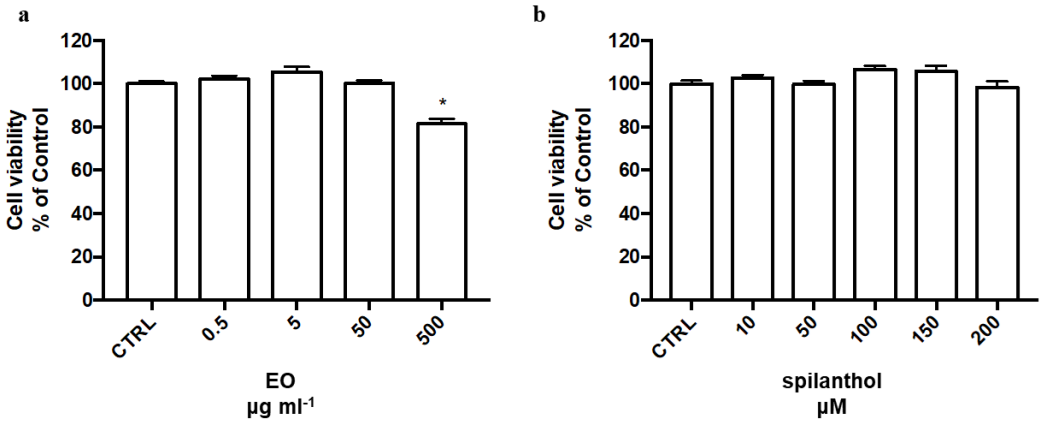


Figure 14. Cytotoxicity of *Acmella oleracea* essential oil (EO) and spilanthol in BV-2 cells. Cells were treated with (**a**) 0.5–500 µg mL^{-1} EO and (**b**) 10-200 µM spilanthol for 24 h, and MTT test was used to evaluate cell viability. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. *$p < 0.05$ compared to control (CTRL).

In addition, EO and spilanthol ability to reduce inflammation was assayed by the treatment of BV-2 cells with different doses of the two products for 24 h before the induction of inflammation through the activation cells with LPS (Figure 15). *In vitro*, LPS is commonly employed as an inflammatory agent since it activates the pro-inflammatory pathway in microglial cells (Ding et al., 2018; Lee et al., 2020b).

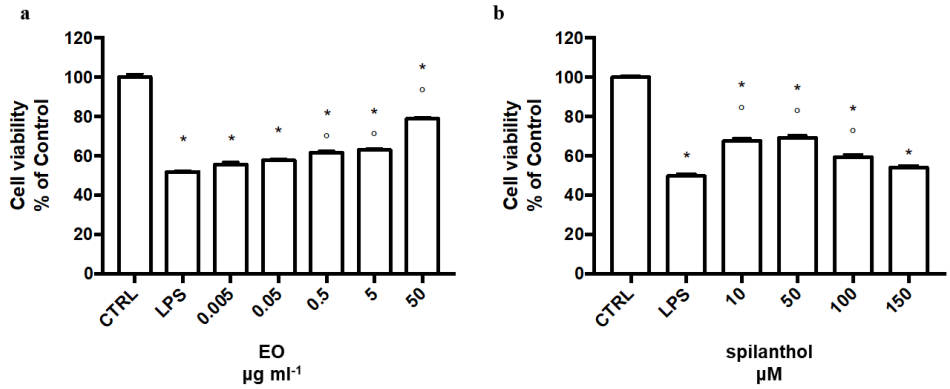


Figure 15. Anti-inflammatory activity of *Acmella oleracea* essential oil (EO) and spilanthol against LPS in BV-2 cells. Cells were treated with (**a**) 0.005–50 µg mL^{-1} EO and (**b**) 10-150 µM spilanthol for 24 h, exposed to 100 ng mL^{-1} LPS for a further 24 h, and MTT test was used to evaluate cell viability. Each bar represents means ± SEM of at least 4 independent experiments. Data were analyszed by one-way ANOVA followed by Bonferroni's test. *$p < 0.05$ compared to control (CTRL); °$p < 0.05$ compared to LPS.

In the control cells, LPS caused a significant reduction of cell viability. On the other hand, the treatment with the EO (0.5-50 µg mL^{-1}) led to an improvement of cell viability if compared with control cells, and this indicated the ability of the EO to counteract the damage caused by LPS (Figure 15a). Regarding spilanthol, at 10, 50, and 100 µM it caused a cell viability increase with

respect to the LPS activated cells (Figure 15b). Based on these data, the concentrations of EO and spilanthol chosen for the next experiments were 50 µg mL^{-1} and 10 µM, respectively.

Since it has been reported that LPS also cause oxidative stress increasing the production of ROS (He et al., 2019; Yang et al., 2018), the EO and spilanthol antioxidant potential was investigated on BV-2 cells with LPS-triggered oxidative stress. The cells were treated with the EO (50 µg mL^{-1}) and spilanthol (10 µM), and then activated with 100 ng mL^{-1} LPS for 24 h. ROS levels were measured by DCFH-DA assay (Figure 16).

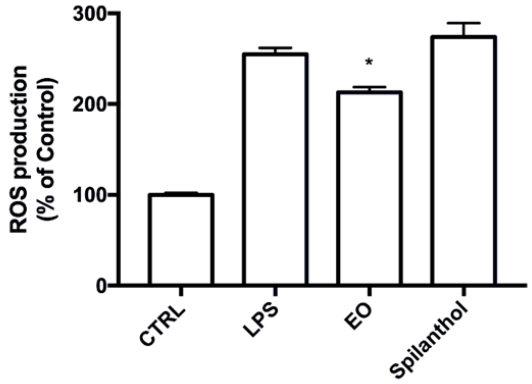


Figure 16. Effect of *Acmella oleracea* essential oil (EO) and spilanthol on intracellular reactive oxygen species (ROS) production in LPS-activated BV-2 cells. Cells were pre-treated with 50 µg mL^{-1} of EO or 10 µM spilanthol for 24 h before activation with 100 ng mL^{-1} LPS. After 24 h intracellular ROS were measured using the peroxide-sensitive fluorescent probe DCHF-DA. Each column represents the mean ± SD of three independent experiments. Data were analyszed by one-way ANOVA followed by Bonferroni's test. *$p < 0.05$ compared to control (CTRL); °$p < 0.05$ compared to LPS.

Remarkably, the EO was the only product that significantly decreased ROS levels when compared with LPS exposed cells, while spilanthol did not influence this parameter.

Moreover, for a deeper understanding of the EO and spilanthol anti-inflammatory mode of action, the expression of pro-inflammatory genes (TNF-α, iNOS, IL-1β, and COX-2) was evaluated by RT-PCR analysis (Figure 17).

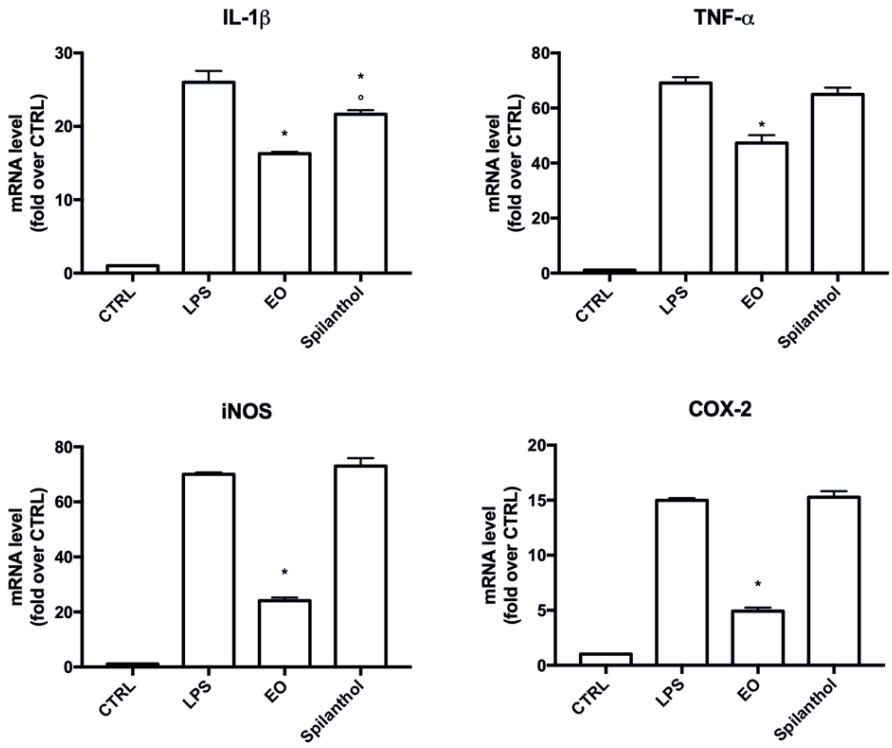


Figure 17. Expression of IL-1, TNF-, iNOS, and COX-2 in BV-2 cells treated with *Acmella oleracea* essential oil (EO) and spilanthol. Cells were treated with EO 50 µg mL^{-1} of EO or spilanthol 10 µM for 24 h, exposed to 100 ng mL^{-1} LPS for 24 h, and real time-PCR was performed. Data are expressed as relative abundance compared to untreated cells. Each bar represents the mean ± SEM of three independent experiments. Data were analysed by a one-way ANOVA followed by Bonferroni's test. ° $p < 0.05$ vs. control (CTRL), * $p < 0.05$ vs. LPS.

The expression of IL-1β, TNF-α, iNOS, and COX-2 were significantly up-regulated in LPS-activated BV-2 cells. As shown in Figure 17, the treatment with the EO significantly lowered the expression of all the tested genes, with a marked effect on two anti-inflammatory enzymes, namely iNOS and COX-2. Conversely, spilanthol did not show any modulation of the mRNA levels of COX-2, TNF-α, and iNOS, but significantly down-regulated the expression of IL-1β. Studies reported in the literature attribute the anti-inflammatory activity of *A. oleracea* to spilanthol (Abdul Rahim et al., 2021; Bakondi et al., 2019; Huang et al., 2018a,b; Stein et al., 2021; Wu et al., 2008). However, the results indicated that the EO counteracted neuroinflammation with a more marked effect with respect to spilanthol, suggesting that other compounds could be involved in the anti-inflammatory activity. In addition, this is the first time that *A. oleracea* anti-inflammatory action is assessed on microglia cells, with the EO leading to a marked down-regulation of the expression of IL-1β, TNF-α, COX-2, and iNOS in LPS activated cells, while spilanthol exerted this action only on TNF-α. However, other studies reported the anti-inflammatory activity of this compound. For instance, Bakondi et al. (2019) demonstrated that that spilanthol inhibits the expression of iNOS and the production of NO in RAW macrophages. Wu et al. (2008) demonstrated that this *N*-alkylamide inhibits the activation of NF-κB downregulating the IL-1 β, IL-6, COX-2, iNOS, and TNF-α. Spilanthol (50-200 µM) was also assayed on vascular smooth muscle cells (VSMC) on

which inflammation was induced demonstrating the ability to reduce NO production, chymase expression, and antioxidant enzyme catalase up-regulation (Stein et al., 2021). The anti-inflammatory activity of spilanthol was also demonstrated on HaCaT keratinocytes by enhancement of heme oxygenase 1 expression levels, inhibition of pro-inflammatory ICAM-1, MCP-1, IL-6, IL-8, and COX-2, and inhibition of the pJNK-MAPK signaling pathway (Huang et al., 2018a,b). Even if the above-mentioned studies are in contrast with the data herein reported, the differences could be ascribed to the experimental protocol followed, since this represented the first study assessing the anti-inflammatory activity of this compound on BV-2 cells. In addition, the concentrations tested in this study were quite lower compared to those of the mentioned works. However, the strongest activity detected for *A. oleracea* EO with respect to the pure *N*-alkylamide suggests that other compounds could participate to this property. For instance, (*E*)-caryophyllene, *β*-pinene, and myrcene from various *Pinus* species demonstrated an anti-inflammatory action on LPS-stimulated macrophages, with a reduction of IL-6 secretion (Basholli-Salihu et al., 2017). Moreover, the EO of *Schinus polygama* (Cav.) Cabrera, mainly constituted by *β*-pinene, proved its ability to counteract inflammation in guinea pigs (Erazo et al., 2006). The EO from *Eremanthus erythropappus* (DC.) MacLeish, mainly characterized by myrcene, *β*-pinene, (*E*)-caryophyllene, and germacrene D, counteracted inflammation in rats inhibiting carrageenan-induced paw oedema (Sousa et al., 2008). Also, the EO from *Liquidambar formosana* Hance, which was rich in *β*-pinene, displayed this anti-inflammatory property in mouse macrophages stimulated by LPS (Hua et al., 2014). Moreover, (*E*)-caryophyllene displayed its anti-inflammatory action by inhibiting the cytokines with the involvement of the arachidonic acid and histamine pathways (de Morais Oliveira-Tintino et al., 2018).

For a further investigation of the use of *A. oleracea* EO to counteract inflammation, the action of EO-NE treatment in LPS activated microglial cells was evaluated. Firstly, the cytotoxic effect of the formulated EO was assessed by treating cells with 0.001-10 mg mL^{-1} of EO-NE for 24 h (Figure 18a).

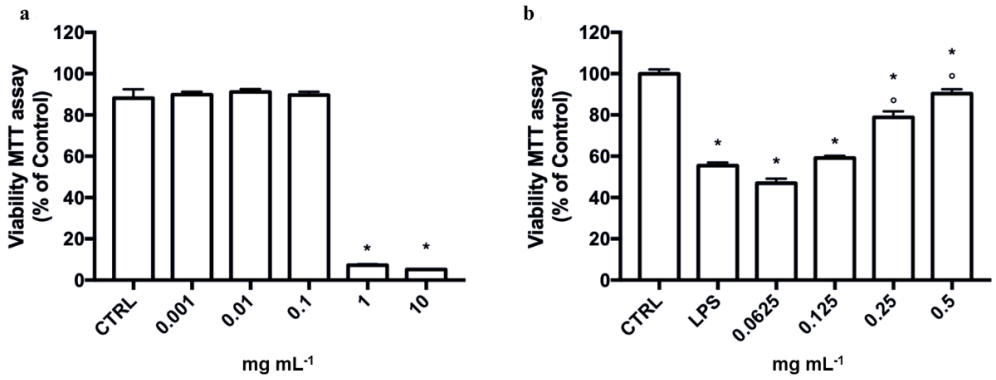


Figure 18. Effect of *Acmella oleracea* essential oil nanoemulsion (EO-NE) on BV-2 cells. Cells were treated with (**a**) 0.001–10 mg mL^{-1} EO-NE for 24 h and cell viability was evaluated by MTT assay, (**b**) 0.0625-0.5 mg mL^{-1} EO-NE, activated with 100 ng mL^{-1} LPS for 24 h and cell viability was evaluated by MTT assay. Each bar represents means ± SEM of at least four independent experiments. Data were analysed by one-way ANOVA followed by (**a**) Dunnett's test. (**b**) Bonferroni's test *$p < 0.05$ compared to CTRL, °$p < 0.05$ compared to LPS.

The EO-NE was able to maintain cell viability to values comparable to control cells up to 0.1 mg mL^{-1}, while the cell viability was strongly reduced at 1 and 10 mg mL^{-1} with respect to controls. The EO-NE anti-inflammatory activity was investigated with a pre-treatment of the cells with different doses of EO-NE before their activation with LPS (Figure 18b). Remarkably, EO-NE highly increased cell viability if compared to LPS exposed cells by almost 30% at 0.25 mg mL^{-1} and 40% at 0.5 mg mL^{-1}. Worthy of notice is that these EO-NE concentrations contain 15 and 30 µg mL^{-1} of EO, respectively, and this suggests that the formulation of the EO can enhance the bioactivity of this product, increasing its availability at a cellular level. This hypothesis is supported by the fact that 1 mg mL^{-1} of EO-NE encapsulated 60 µg mL^{-1} of EO, and this dose was not cytotoxic in the previous experiment.

3.4. Conclusions

This study demonstrated the higher effectiveness of MAE for the obtaining of *A. oleracea* EO with respect to traditional HD. This EO was also richer in spilanthol, the main *N*-alkylamide found in *A. oleracea*. The EO, its NE, spilanthol, and an *A. oleracea n*-hexane extract were tested on *Cx. quinquesciatus*. Regarding acute toxicity, spilanthol and the *n*-hexane extract were the two *A. oleracea*-derived products that displayed the lowest LC_{50} values (3.1 and 9.5 µL L^{-1}, respectively) and, consequently, promising larvicidal efficacy. On the other hand, this study also pointed out the promising efficacy of the EO-NE on larval development, fecundity, fertility, and natality of the assayed insect when applied at the LC_{30} concentrations and for short exposure times. Moreover, the safety for mammal cells of the EO and spilanthol was also demonstrated together with the

protection from LPS-induced inflammation. These results lead to the basis for the implementation of *A. oleracea*-derived products as tools for the management of mosquitoes.

4. Development of nanoformulations based on *Acmella oleracea n*-hexane extract and spilanthol

4.1. Introduction

Natural products have always had a crucial role in the pharmaceutical, nutraceutical, cosmetic but also agricultural fields (Dayan et al., 2009; Dini and Laneri, 2021; Talib et al., 2020). This is linked to the large variety of bioactive compounds encompassed in their phytocomplex. However, the use of these products is often limited due to their physio-chemical properties, and one of the major challenges for their real-world application is their efficient delivery. In fact, they are usually lipophilic and not stable since they are affected by degradation processes (Gunasekaran et al., 2014). So, their poor water solubility and inappropriate molecular size result in low absorption and reduced physiologic availability (Jia and Zhao, 2009). Consequently, several formulation systems have been developed (Ahmed et al., 2021) aiming to improve water solubility, lower degradation processes, decrease any possible toxic effects, improve the biological availability and, if possible, also the bioactivity of the formulated products (Kesarwani and Gupta, 2013). One of the most common formulative system is nanoencapsulation. Nanotechnology is usually referring to the use of nanomaterials between 1 and 100 nm size. Thanks to their dimensions in the nanometric range, nanocarriers enhance the stability, the physio-chemical properties, and the bioactivity of botanical products. Examples of nanocarriers of natural products are polymeric nanoparticles, lipidic nanocarriers, MEs and NEs, and cyclodextrins (Bilia et al., 2018).

Among the various nanosystems, MEs and NEs are the easiest to formulate and handle. Moreover, they can be produced at low costs (Pavoni et al. 2019a,b). These formulations are self-emulsifying colloidal systems constituted by two liquid phases that are immiscible. Despite the prefixes ‘micro-’ and ‘nano-’, these systems are characterized by an internal phase which is a dispersion of nanometric droplets (Anton and Vandamme, 2011), and the main differences consist mainly in the quantitative composition and free energy of the system. The latter is a crucial parameter for their preparation and stability (Pavoni et al., 2020). In detail, NEs have a lower surfactant-to-oil ratio (SOR), generally between 1 and 2, than MEs (SOR > 2) (Rao and McClements, 2011), and this is an advantage in terms of sustainability and safety. Conversely, the formation of MEs is favoured because they form spontaneously, while NEs formation need an external input to overcome the high energy barriers (McClements, 2012).

Several formulative studies have been performed on the encapsulation of natural products into MEs or NEs. For instance, Piazzini et al. (2017a,b) developed MEs and NEs that improved the solubility and the absorption of *Vitex agnus-castus* L. (VAC) and *Silybum marianum* L. extracts. Furthermore, Deepa et al. (2012) demonstrated that the NE containing *Phyllanthus amarus* Schumach. & Thonn. extract resulted more active than the extract alone.

On this base and since spilanthol and the *n*-hexane extract resulted the most promising *A. oleracea*-derived products in terms of acute larval toxicity on *Cx. quinquefasciatus* (Section 3.3.4), the aim of this work was the development of MEs and NEs containing the above-mentioned *N*-alkylamide and the *n*-hexane extract.

4.2. Materials and methods

4.2.1 Preparation of *Acmella oleracea n*-hexane extract

Leaves and flowers of *A. oleracea* were extracted using *n*-hexane (1:10 w/v), following the procedure and using the same plant amount described in Section 3.2.4. The amount of extract obtained was of 11.5 g. The removal of chlorophyll was performed following the procedure described in Section 3.2.4. From the extract without chlorophyll, also waxes were removed throughout winterization. In detail, 9.1 g of *n*-hexane extract was dissolved in *n*-hexane (1:10 w/v) and stored at 4 °C for 24 h, allowing the precipitation of waxes, which were then removed through filtration. This operation was repeated twice to remove all the waxes from the extract. After the filtration process, it was evaporated until dryness (8.5 g) and stored at 4°C until further use.

4.2.2 Isolation and characterization of spilanthol

The purification of spilanthol (170 mg) from *A. oleracea n*-hexane extract (1.3 g) and its characterization followed the procedure described in Section 3.2.5.

4.2.3 Nanoemulsions (NEs) development

The NEs were prepared according to the procedure described in Chapter I, Section 4.2.1. All the samples were prepared by changing the relative formulative parameters, especially the ethyl oleate/extract ratio, and the surfactant/oil phase ratio in order to optimize the formulative process (Table 14).

Table 14. Composition (% w/w) of the nanoemulsions (NEs).

Sample	*Acmella oleracea n*-hexane extract	Spilanthol	Ethyl oleate	Tween 80	H_2O
NE_1	1	-	-	0.33	98.67
NE_2	1	-	1	0.50	97.50
NE_3	1	-	0.5	0.50	98.00
NE_4	1	-	1	0.25	97.75
NE_5	1	-	1	0.50	97.50
NE_6	1	-	1	0.75	97.25

NE_7	-	1	1	0.50	97.50
NE_8	1	-	2	0.75	96.25
NE_9	1	-	1	0.50	97.50[a]
NE_10	1[b]	-	1	0.50	97.50
NE_11	2[b]	-	2	1.00	95.00

[a]water added of methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate. [b]*Acmella oleracea n*-hexane extract obtained through a winterization process.

4.2.4 Microemulsions (MEs) development

MEs were prepared according to the procedure described in Section 5.2.1. The oily phase was constituted by *A. oleracea n*-hexane extract or spilanthol, Tween 80 (TEGO® SMO 80; Evonik Industries, Essen, DE), and a mixture of glycerol (ACEF, Fiorenzuola d'Arda, IT) and ethanol, at 6:1 ratio. All the samples were prepared by changing the relative formulative parameters in order to optimize the formulative process (Table 15).

Table 15. Composition (% w/w) of the microemulsions (MEs).

Sample	*Acmella oleracea n*-hexane extract	Spilanthol	Ethyl oleate	Tween 80	Alcoholic mixture	H_2O
ME_1	1	-	1	13	35	50
ME_2	1	-	2	13	35	49
ME_3	0.5	-	0.5	13	35	51
ME_4	1	-	1	10	35	53
ME_5	1	-	1	15	35	47
ME_6	0.5	-	0.5	13	-	86
ME_7	0.5[a]	-	0.5	13	35	51
ME_8	-	1	1	13	35	50

[a]*Acmella oleracea n*-hexane extract obtained through a winterization process.

4.2.5 Characterization of micro- (MEs) and nanoemulsions (NEs)

MEs and NEs were characterized accordingly to the procedure described in Section 4.2.2. The DLS analysis was repeated at different timepoints for NEs (0 days (t0), 30 days (t30), and 90 days (t90)), while for MEs only at t0.

4.3. Results and discussion

4.3.1 Formulative study and stability of nanoemulsions (NEs)

The first part of the study focused on the assessment of the best formulative parameters for the preparation of NEs. For the initial formulation, the first sample (NE_1) was prepared adopting an

extract/surfactant ratio of 3:1 and using as oily phase only the *n*-hexane extract. These conditions were considered not sufficient for the obtaining of the NE, since the sample presented solid particles formation, which was confirmed by microscopic analysis (Figure 19).

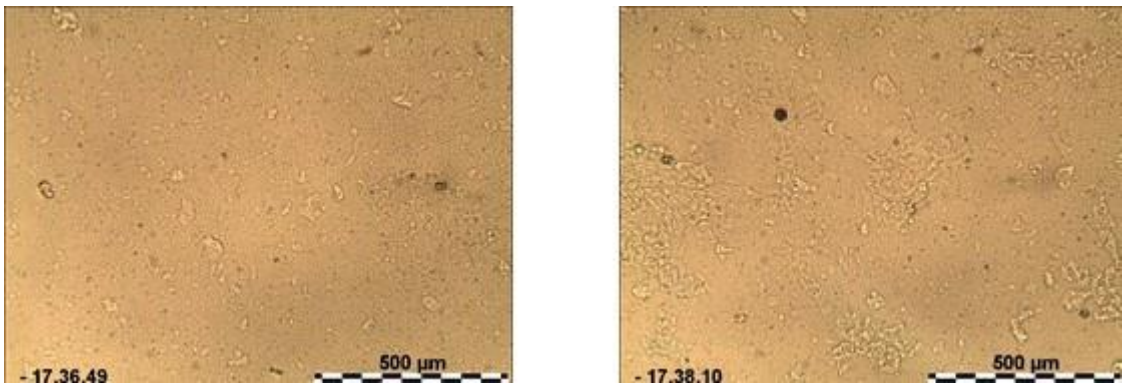


Figure 19. Image of NE_1 derived from the analysis at the polarized light microscope.

For this reason, the sample NE_2 was prepared using in the oily phase also the ethyl oleate at 1% (w/w). These conditions did not lead to solid particles formation. This preliminary result led to the conclusion that the NEs containing the *A. oleracea n*-hexane extract needed the presence of ethyl oleate as solubilizing agent. The amount of ethyl oleate was also reduced (NE_3) at 0.5%, but this quantity was not sufficient for a complete solubilization of the extract (Figure 20).

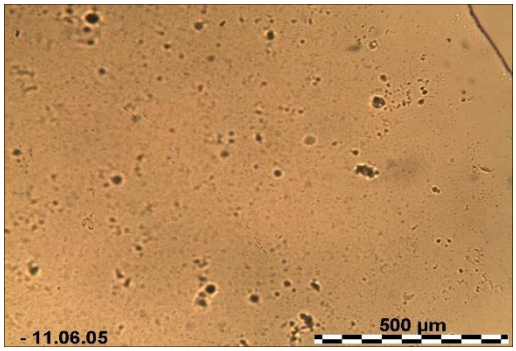


Figure 20. Image of NE_3 derived from the analysis at the polarized light microscope after homogenization.

This confirmed that the minimum amount of ethyl oleate to be used in this kind of formulations was 1%. Then, the number of French Pressure Cell Press cycles was investigated to obtain a droplet nanometric size distribution for the NEs. The optimized number of cycles leading to the formation of a nanodispersed system was 5, since no droplet of dimensions higher than 1 µm were observed (Figure 21). This result confirms that the French Pressure Cell Press is a performing instrument for the development of NEs.

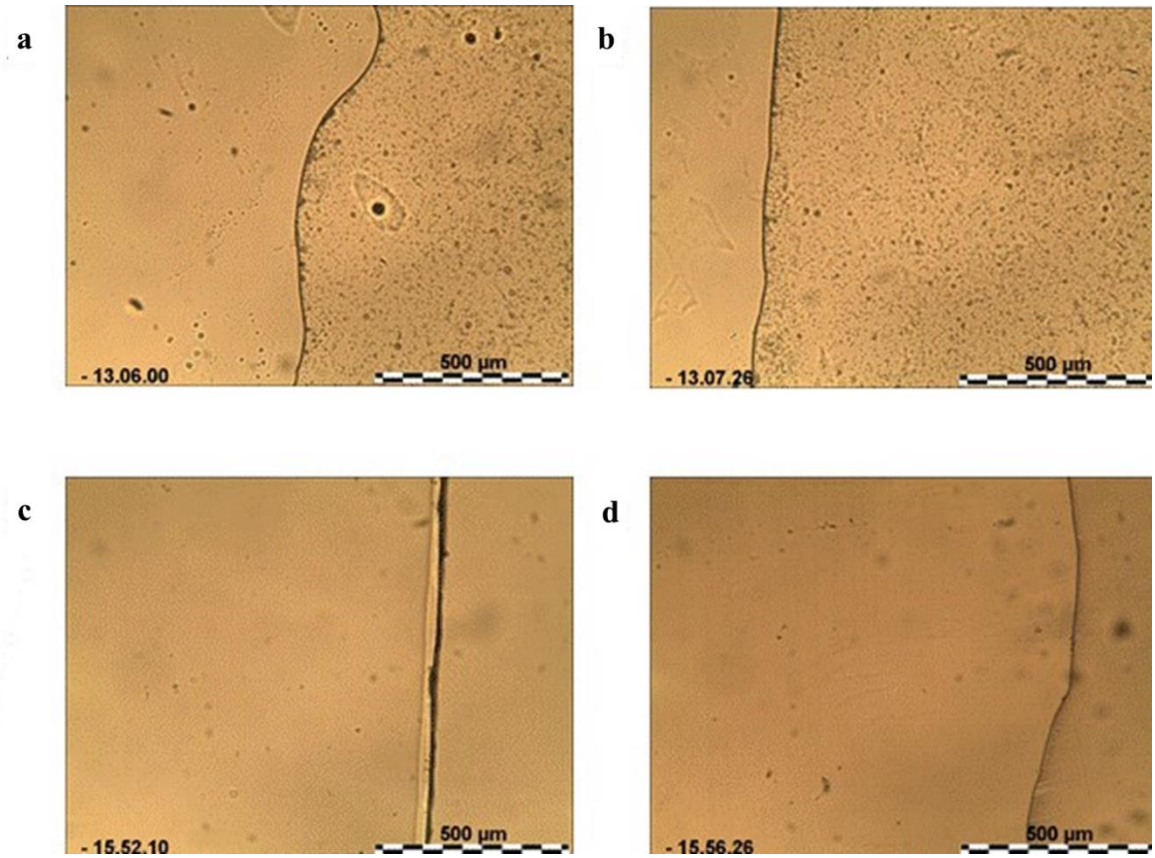


Figure 21. Images derived from the analysis at the polarized light microscope of nanoemulsions (NEs) produced by 3 (**a**,**b**) and 5 cycles of French Pressure Cell Press (**c**,**d**), respectively.

Then, a different amount of surfactant together with the oil phase/surfactant ratio were evaluated. The NEs prepared contained 0.25, 0.50, and 0.75% (NE_4, NE_5, and NE_6, respectively) of surfactant. The DLS analyses of the samples at t0 led Z-average and PDI values that are reported in Table 16.

Table 16. Z-average and Polydispersity Index (PDI) values obtained after Dinamic Light Scattering (DLS) analysis of nanoemulsions (NEs) NE_4, NE_5, and NE_6 at t0.

	Z-Average (nm)					**PDI**				
	Test1	**Test2**	**Test3**	**Average[a]**	**SD[b]**	**Test1**	**Test2**	**Test3**	**Average**	**SD**
NE_4	213.7	217.7	215.4	215.6	2.007786	0.277	0.273	0.276	0.27	0.002082
NE_5	159.8	159.5	160	159.7	0.2516611	0.151	0.154	0.160	0.155	0.004583
NE_6	178.2	177.6	182.7	179.5	2.787472	0.264	0.331	0.367	0.33	0.002082

[a]average obtained from three different tests; [b]SD, standard deviation.

The three NEs showed a monomodal size distribution of droplets of the dispersed phase (Figure 22). Between all the samples at t0, the NE_5, which contained an amount of surfactant of 0.5%, resulted to be the best formulation. In fact, it showed a PDI less than 0.155, which represents a stricter droplets dimensions range with respect to the other formulations analyzed. The PDI is an indicator of the system quality, and its value is usually between 0 and 1. The smaller is its value the more the system is monodispersed (Sadeghi et al., 2015). Moreover, NE_5 was characterized by a Z-average of 159.7 nm.

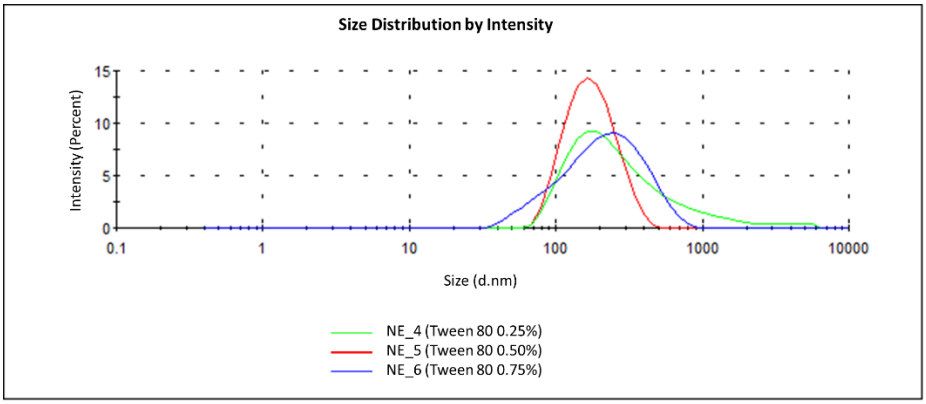


Figure 22. Dinamic Light Scattering (DLS) of nanoemulsions (NEs) NE_4, NE_5, and NE_6.

The physio-chemical stability of the three samples was assessed throughout time, after their storage at room temperature for 3 months. In detail, the samples were analysed by DLS at t0, t30, and t90. In Figure 23 (a,b) the changes of the Z-average and PDI values throughout time are reported.

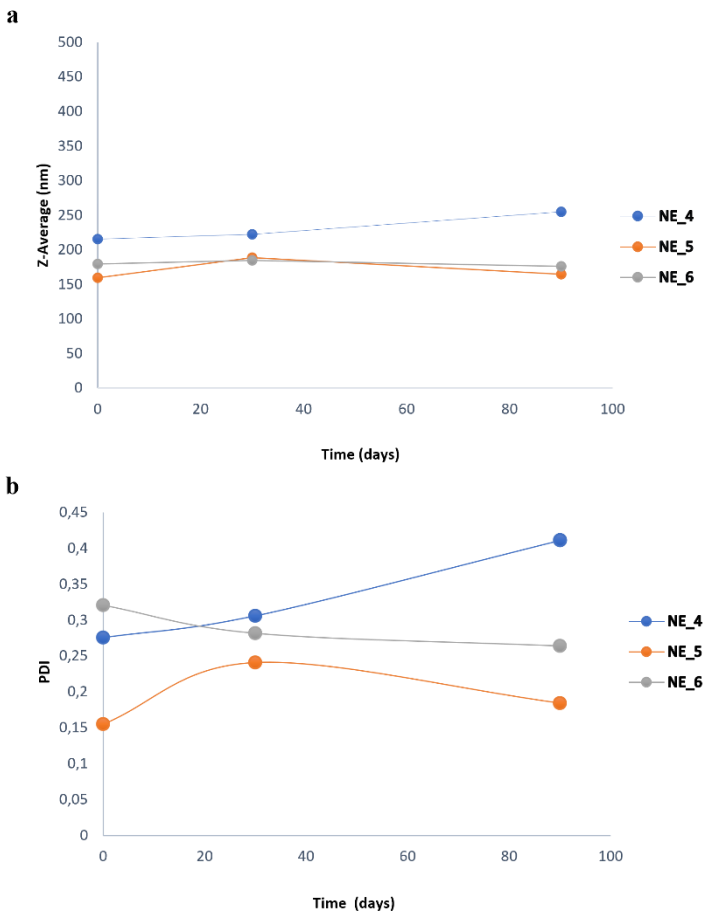


Figure 23. Average trend of Z-average (**a**) and Polydispersity Index (PDI) (**b**) throughout time (t0, t30, t90) of nanoemulsions (NEs) NE_4, NE_5, and NE_6.

Between the formulations, NE_5 resulted to be the more stable. On the other hand, NE_4 displayed a marked increase of PDI and Z-average at increasing times, and, for this reason, resulted the less stable formulation. Moreover, it displayed a particle size distribution higher than 1000 nm. Regarding NE_6, even if it did not display significative variations in the PDI and Z-average values, these resulted higher than those of NE_5.

During the observation time and after 14 days from the preparation, the formation of particles not related to creaming or sedimentation phenomena was detected in all the three NEs (Figure 24).

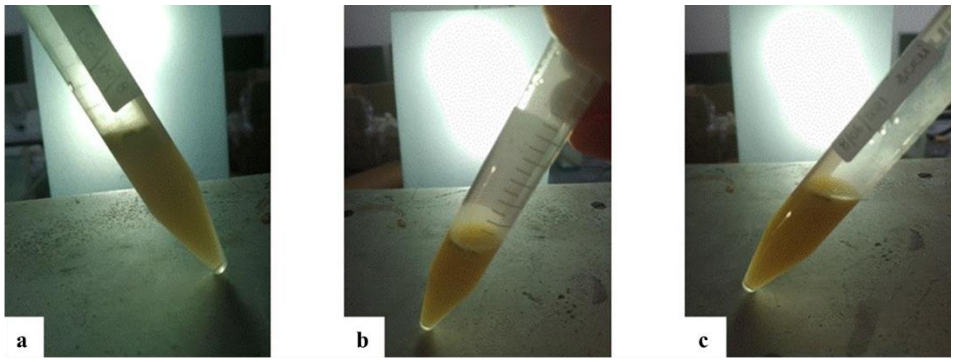


Figure 24. Solid particles detected in nanoemulsions (NEs) NE_4 (**a**), NE_5 (**b**), and NE_6 (**c**).

To overcome this phenomenon, the formulative parameters were adapted with three different strategies. Firstly, it was chosen to employ the co-solvent action of ethyl oleate, increasing its content in the NE to 2%, but maintaining constant the oil phase/surfactant ratio (NE_8). Secondly, no quantitative variation of the NE composition was performed, but water added of methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate (NE_9) was employed for the formulation. Lastly, for the NE preparation, a *n*-hexane extract without waxes was employed. This extract was produced with the winterization procedure described in Section 4.2.1 (NE_10). The NEs (NE_8, NE_9, and NE_10, respectively) were observed for 14 days until 3 months and did not show any particles formation (Figure 25).

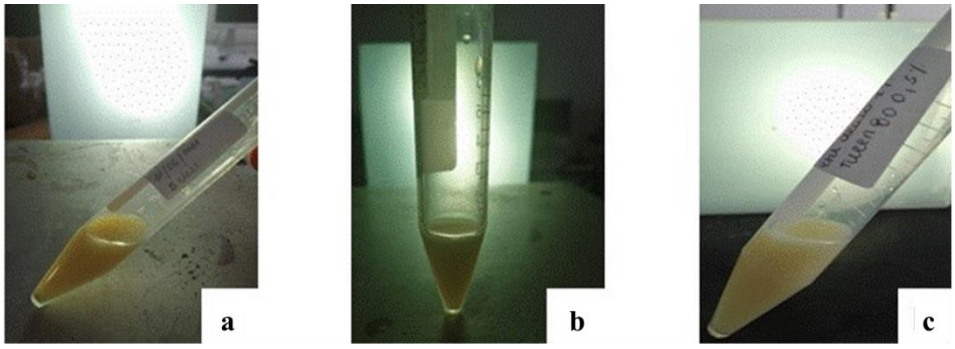


Figure 25. Nanoemulsions (NEs) NE_8 (**a**), NE_9 (**b**), and NE_10 (**c**) without solid particles formation up to 3 months.

These formulations were also analysed at the DLS at t0 and t30, showing good stability and a particle size distribution in the nanometric range (Figure 26a,b,c).

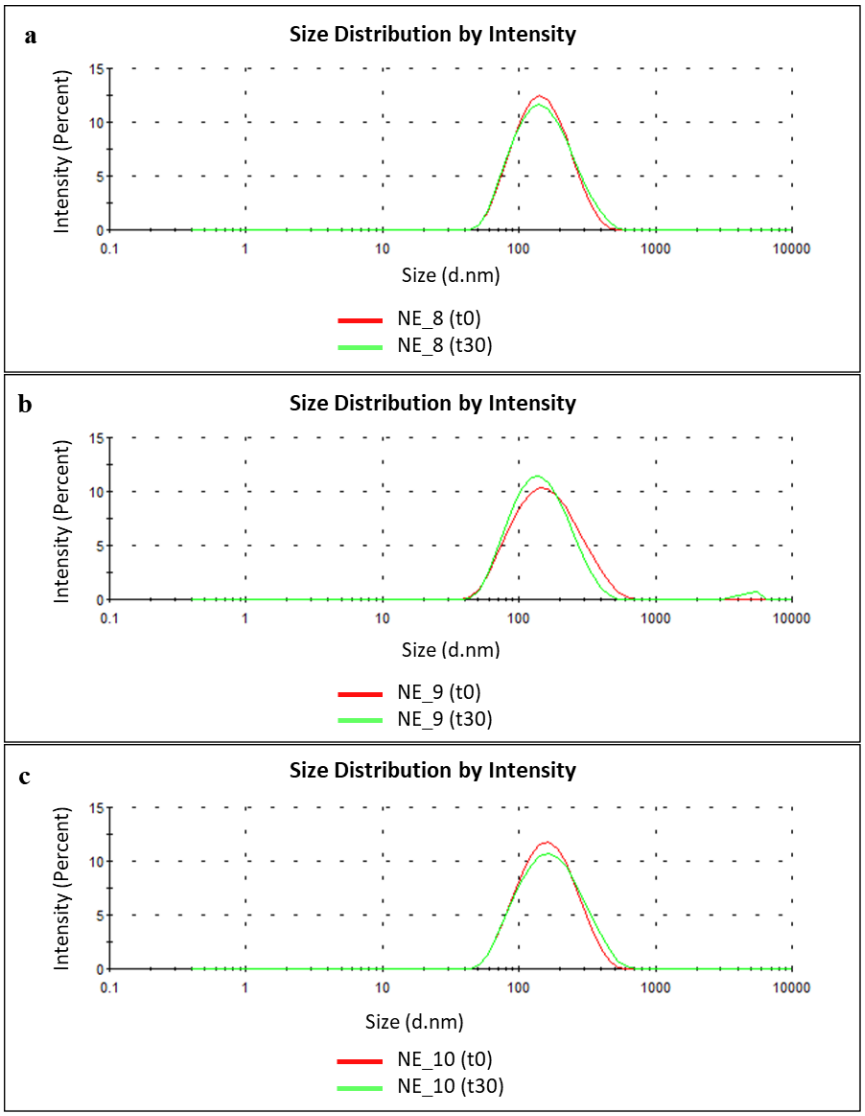


Figure 26. Dinamic Light Scattering (DLS) analyses of nanoemulsions (NEs) NE_8 (**a**), NE_9 (**b**), and NE_10 (**c**) at t0 and t30.

Once the best formulation in terms of composition was determined, namely NE_5, this was chosen as a model for the development of spilanthol NE (NE_7). The latter was characterized by spilanthol (1% w/w), surfactant (0.5% w/w), and ethyl oleate (1%). This formulation was examined at the DLS at t0, t30, and t90 showing good stability over time and an adequate particle distribution between 60 and 300 nm (Figure 27). Moreover, differently from the NEs containing the *n*-hexane extract, no particles formation was noticed up to 3 months. This could be linked to the fact that spilanthol is an isolated and pure compound, while the *n*-hexane extract is a complex mixture of molecules characterized by diverse physio-chemical properties.

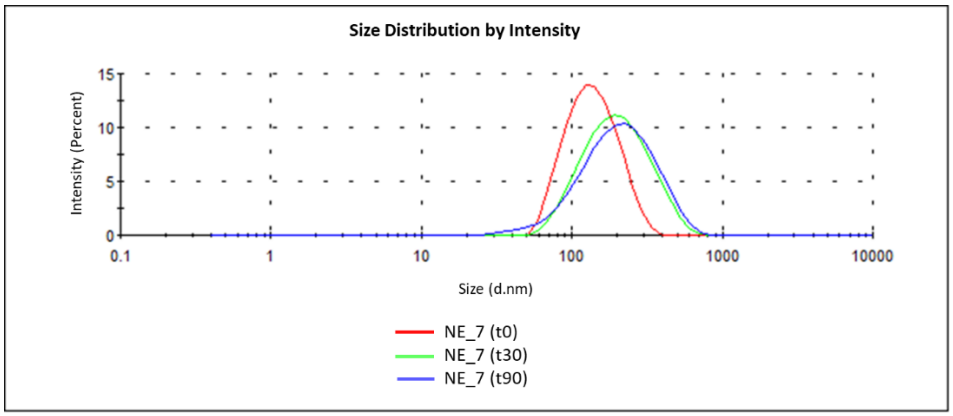


Figure 27. Dinamic Light Scattering (DLS) analysis of nanoemulsion (NE) NE_7 at t0, t30, and t90.

The study on NEs was concluded by the preparation of NE_11, in which the *n*-hexane extract free of waxes was encapsulated and in which the amount of extract, surfactant, and ethyl oleate was doubled, keeping constant the oil phase/surfactant and extract/surfactant ratios with respect to NE_5. This formulation was prepared to confirm that the formulative parameters optimization was effective. This NE was analysed at the DLS at t0 and t30, showing a particle size in the nanometric range and a good stability (Figure 28).

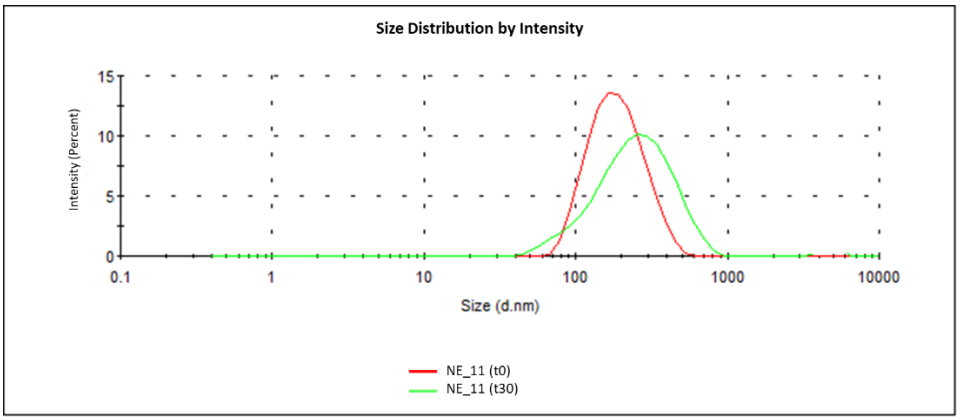


Figure 28. Dinamic Light Scattering (DLS) analysis of nanoemulsion (NE) NE_11 at t0 and t30.

4.3.2 Formulative study and stability of microemulsions (MEs)

The optimization of the formulative parameters to produce the MEs started with the ME_1. For this ME, it was established an extract/surfactant ratio of 1:13. However, this formulation did not show a particle size distribution in the micrometric range, and it was not possible to define it a ME (Figure 29).

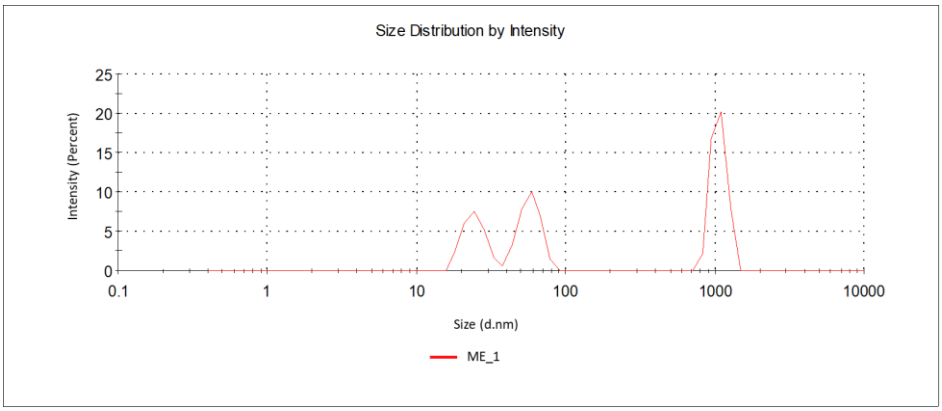


Figure 29. Dinamic Light Scattering (DLS) analysis of microemulsion (ME) ME_1 at t0.

For this reason, the other MEs (ME_2, ME_3, ME_4, ME_5, ME_6, and ME_7) were prepared changing diverse formulative parameters as the *n*-hexane extract content, the content of surfactant (10, 13, and 15%), the content of ethyl oleate (0.5, 1.0, and 2.0%), or the eventual presence of the alcoholic mixture. However, the several attempts performed did not lead to encouraging results. In fact, in all the samples the particle size distribution resulted out of the micrometric range, as showed by the DLS analysis (Figure 30).

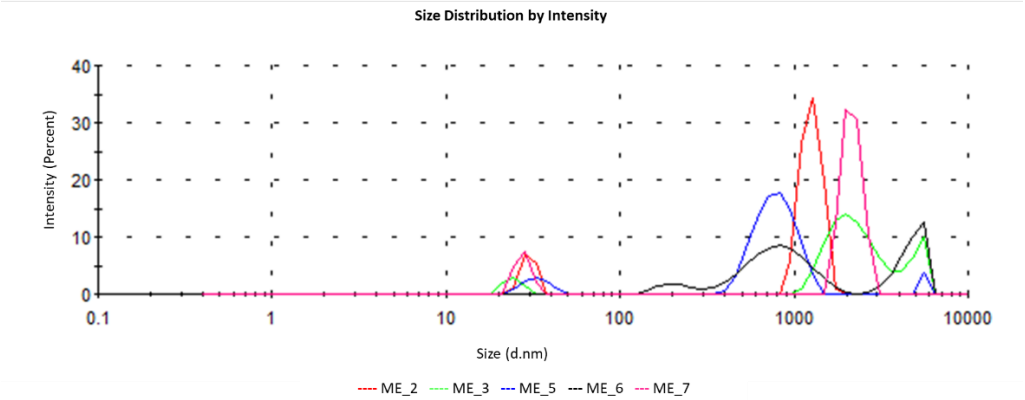


Figure 30. Dinamic Light Scattering (DLS) analysis at t0 of microemulsion (ME) ME_2, ME_3, ME_5, ME_6, and ME_7.

Moreover, these systems displayed PDI values higher than 0.55 and Z-averages higher than 1000 nm (Table 17). For this reason, they could not be defined MEs.

Table 17. Polydispersity Index (PDI) and Z-average values at t0 of microemulsions (MEs) ME_1, ME_2, ME_3, ME_5, ME_6, and ME_7.

	Z-Average (nm)					PDI[a]				
	Test1	**Test2**	**Test3**	**Media**	**SD[b]**	**Test1**	**Test2**	**Test3**	**Media**	**SD**
ME_1	6103	4069	4662	4945	1046	0.416	0.646	0.647	0.569	0.133
ME_2	2121	2285	2717	2374	307.9	1.000	0.971	0.936	0.969	0.032
ME_3	2703	2251	2010	2321	351.8	0.854	0.445	0.561	0.62	0.211
ME_4	-	-	-	-	-	-	-	-	-	-
ME_5	2548	1400	1542	1830	625.8	0.945	0.888	1.000	0.944	0.056
ME_6	954	967	855	927	61.1	0.606	1.000	1.000	0.868	0.227
ME_7	4218	4133	6044	4798	1080	0.467	0.546	1.000	0.671	0.288

[a]PDI, polydispersity Index; [b]SD, standard deviation.

Following the formulative parameters for ME_1, a ME encapsulating spilanthol (ME_8) was formulated, to evaluate if the chemical nature of the extract and spilanthol could influence the realization of MEs. The sample analysed at the DLS at t0 showed a particle size distribution lower than 1000 nm, conversely to the ME_1. However, at t7 and t14 the sample showed an increase in the particle size distribution (>1 μm) (Figure 31).

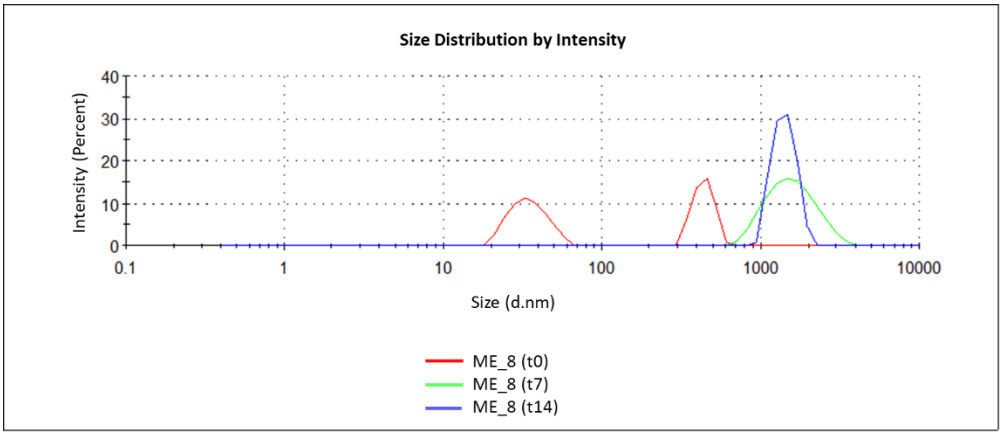


Figure 31. Dinamic Light Scattering (DLS) at t0, t7, and t14 of microemulsion (ME) ME_8.

Moreover, ME_8 showed a Z-average increase and a reduction of PDI during time, probably linked to the presence of droplets of sample of bigger dimensions with respect to those measurable by DLS (Table 18).

Table 18. Z-average and Polydispersity Index (PDI) values of microemulsion (ME) ME_8 at t0, t7, and t14.

	Z-Average (nm)					PDI[a]				
	Test1	**Test2**	**Test3**	**Media**	**SD[b]**	**Test1**	**Test2**	**Test3**	**Media**	**SD**
t0	584.1	575.2	478.2	545.8	58.74	0.749	0.747	0.791	0.7623	0.0248
t7	1378	1348	1375	1361	16.52	0.184	0.207	0.190	0.1936	0.0119
t14	1291	1433	1446	1390	85.98	0.276	0.191	0.203	0.2233	0.0460

[a]PDI, polydispersity Index; [b]SD, standard deviation.

All the formulated MEs showed a turbidity on their upper side, besides the loss of transparency after their preparation. So, also for spilanthol, it was not possible to find formulative parameters to produce MEs.

This study is innovative since no other studies on MEs and NEs containing *A. oleracea n*-hexane extract have been performed and an optimization of the formulative parameters have never been carried out. However, other kinds of *A. oleracea* formulations are reported in literature. For instance, Vlachojannis et al. (2018) developed a tincture of jambú and tested it to assess its antimicrobial activity. Moreover, some marketed products contain nanostructured lipid carriers of *A. oleracea* extract, as Intensive Serum Nanorepair Q10® developed by Dr. Rimpler (Wedemark, Germany) and employed for cosmetic applications (Montenegro et al., 2017). An optimization approach for the development of *A. oleracea* formulation was reported by Savic et al. (2021b). In this case, a systematic DoE was employed, allowing the identification of the best formulative parameters and the preparation of an anti-wrinkle serum. Regarding spilanthol formulation, some studies reported the encapsulation of this compound into different formulative systems. For instance, Yang et al. (2019) developed a spilanthol emulsion, investigating its effect at a molecular level and screening its formula using an *in vitro* skin permeation and retention study.

4.4. Conclusions

This work focused on the optimization of the formulative parameters to produce MEs and NEs. In detail, the NEs optimization study led to the identification of the best NE in terms of stability and particle size distribution. In fact, NE_5 characterized by 1% of *n*-hexane extract or spilanthol, 1% of ethyl oleate, 0.5% of surfactant, and 97.5% of water, led to the best results. Regarding MEs formulations, they could not be obtained, with a particle size distribution higher than the micrometric order. More studies on the formation of MEs should be performed. Moreover, studies on the biological activities of these *A. oleracea* formulations should be carried out, and the potential insecticidal activities of the above-mentioned NEs are under investigation.

5. *Acmella oleracea* extracts' insecticidal activity against noxious arthropods attacking stored products

5.1. Introduction

As reported in Chapter I, Section 5.9, *O. surinamensis, T. granarium, T. castaneum, T. confusum, T. molitor, A. diaperinus,* and *A. siro* are worldwide pests affecting stored products such as nuts, cereals, spices, coffee, chocolate, but also processed foods and flours (Hagstrum and Subramanyam, 2009; Hagstrum et al., 2013; Hill, 2003; Kumar, 2017; Rees, 2004; Robinson, 2005). Moreover, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae) is a global secondary pest, affecting dried root crops, oilseeds, grains and their derivatives, and nuts (Hagstrum et al., 2013; Hill, 2002; Rees, 2004). *A. oleracea* extracts have been widely investigated for their insecticidal properties on diverse insects of economic importance, such as mosquitoes, houseflies, and moths (Dubey et al., 2013; Kadir et al., 1989; Moreno et al., 2012; Sharma et al., 2012). However, no data are available against stored-product pests. Seen the results obtained from the study of jambú-derived products against *Cx. quinquefasciatus* (Section 3.3.4), the objective of the present study was to determine the pesticidal activity of *A. oleracea n*-hexane and methanol extracts, deriving from a screening of extraction solvents and extraction techniques and targeting the above-mentioned arthropods.

5.2. Materials and methods

5.2.1 Plant extraction

Screening of extraction solvents

Concerning the extraction solvents screening, the plant matrix (20 g) was extracted in an ultrasound bath (Section 3.2.4) at room temperature for 1 h using different solvents: *n*-hexane, methanol, ethanol, dichloromethane, petroleum ether, and ethyl acetate (Sigma-Aldrich, Milan, Italy) always in a plant/solvent ratio of 1:10 (w/v). Then, the extracts obtained were subjected to filtration on a cotton filter and evaporated to dryness with a Rotavapor (Buchi Rotavapor R-200, Büchi Labortechnik AG, Flawil, Switzerland) at 40°C. All the extractions were carried out in duplicate. The yields obtained, expressed in % w/w of DE, are reported in Table 19.

Optimization of the extraction procedure

The screening of extraction techniques was performed employing the instruments available in the laboratory. In detail, the plant material (20 g) was extracted with the solvent (plant/solvent ratio of

1:10 (w/v)) which led to the highest extraction yield (Table 19) and spilanthol recovery from the plant biomass (Table 21). The procedures employed for each extraction technique are described below.

Procedure A – Ultrasound bath: plant material was extracted with the selected solvent in a flask inserted in the ultrasound bath described in Section 3.2.4. The extraction was carried out at room temperature for 1 and 3 h, respectively.

Procedure B – Ultrasound extractor: for this extraction, plant matrix was extracted with the selected solvent in an ultrasound extractor (US2020, Albrigi Luigi) employing the program H + M (high power and mixing) for 1 h at room temperature.

Procedure C – Magnetic stirring: the extraction of the plant material with the selected solvent was performed with magnetic stirring at 500 rpm at room temperature, for 1 and 3 h, respectively.

Procedure D - Soxhlet extraction: for this extraction, a higher amount of plant material was used, due to operating requirements of the sonication apparatus. The plant matrix was extracted with the selected solvent through a Soxhlet apparatus for 8 h.

Each extraction procedure was performed in duplicate, and the extracts were evaporated as described in Section 3.2.4. The yields obtained (% w/w of DE) are reported in Table 19.

Table 19. Yield obtained for the screening of solvents and of extraction techniques.

Solvent[a]	**Yield (%, w/w)[b] ± SD[c]**
n-hexane	2.7 ± 0.28
ethanol	3.9 ± 0.11
methanol	11 ± 0.07
dichloromethane	5.5 ± 0.12
petroleum ether	1.9 ± 0.20
ethyl acetate	4.2 ± 0.21
extraction technique	
ultrasound bath (1 h)	10 ± 0.26
ultrasound bath (3 h)	11 ± 1.4
ultrasound extractor	7.6 ± 0.35
magnetic stirring (1 h)	12 ± 0.33
magnetic stirring (3 h)	12 ± 0.57
soxhlet	26 ± 0.66

[a]Solvent used for the extraction of the plant material in an ultrasound bath for 1 h at room temperature (plant/solvent ratio of 1:10 (w/v)). [b]Relative yield values (%) are mean of two independent analyses.[c]SD, standard deviation.

5.2.2 Development of HPLC-DAD-MS method

Chemical and reagents

Spilanthol used for the analysis was purified accordingly to the procedure described in Section 3.2.5. Acetonitrile (HPLC-grade) employed for the analysis was purchased from Sigma-Aldrich (Milan, Italy).

Preparation of samples and standard solutions

The stock solutions were prepared solubilizing spilanthol in HPLC-grade methanol and reaching the concentrations of 1700 and 850 mg L^{-1}. These stock solutions were stored at –20 °C in glass vials until chemical analysis. The other standard solutions were prepared by dilution of the stock solutions to 170, 17, 1.7 and 85, 8.5 mg L^{-1}, respectively. The DEs were solubilized in acetonitrile to reach the concentration of 1000 mg L^{-1}. Then, they were vortexed for 1 min and further placed in an ultrasound bath (Section 3.2.4) for 5 min. Finally, they were filtered using a 0.2 μm syringeless filter.

HPLC-DAD-MS conditions

The HPLC instrument was an Agilent 1100 series (Agilent Technologies, Santa Clara, CA, USA), consisting of a photodiode array detector (DAD), an autosampler, a binary solvent pump, and an ion-trap mass spectrometer (with electrospray ion source) LC/MSD Trap SL Agilent Technologies, controlled by LCMSD (Agilent, v.6.2) and ChemStation (Agilent, v.01.03) software. The chromatographic separation was performed on a Luna C18 column (4.6 x 150 mm, i.d., particle size 5 μm), purchased from Phenomenex (Chesire, UK), which operated at 35 °C. Analysis was conducted with a mobile phase consisting of acetonitrile and water. A linear gradient starting with 20% acetonitrile was set to reach 80% at 20 min and then hold for 20 min. Afterwards, the column was reconditioned in 5 min and held at the reconditioning gradient for 15 min. The flow rate was 1 mL min^{-1} and the volume of injection was 2 μL. The detection systems were DAD and ITMS. DAD was utilized for the quantification of spilanthol in the extracts deriving from the screenings and other *N*-alkylamides in the extracts selected for the biological assays. Chromatograms were recorded at 220 nm. All the compounds were confirmed by HPLC-MS analysis. The mass spectrometry system included an electrospray ionization (ESI) source functioning in positive ionization mode. Nitrogen was used as drying gas at 325 °C, at a flow rate of 12 L min^{-1}, and was also chosen as nebulizer gas at 70 psi. The capillary voltage was of 3500 V. The mass scan was set in the range 50-900 *m/z* with a target mass of 222 *m/z*. The DEs were prepared at 1000 mg L^{-1} in acetonitrile.

Method validation

Each solution was injected twice (n = 2). The relative standard deviation percentage (RSD %) was calculated for each sample to evaluate the precision of the obtained data. The HPLC-DAD-MS method was validated in terms of linearity, limits of detection (LODs), and limits of quantification (LOQs). The linearity was estimated by injecting standard solutions at various concentrations of spilanthol (1.7, 8.5, 17, 85, 170, 850, 1700 mg L^{-1}). Spilanthol calibration curve was developed by plotting the analyte peak areas against the analyte concentrations and was used for the quantification of spilanthol and other *N*-alkylamides. The repeatability of the method was evaluated injecting each standard solution 3 times in HPLC in the same day (intraday), while the 850 mg L^{-1} solution was injected 3 times in 3 consecutive days (inter-day). The repeatability was evaluated in terms of relative standard deviation (RSD %) (Table 20).

Table 20. Method repeatability evaluation.

Concentration (mg L^{-1})	Area	Mean area[a]	SD intraday[b]	SD interday[c]	RSD % intraday[d]	RSD % interday[e]
1700	11153.7	11121.9	45.0		0.40	
	11090.1					
850	5612.9	5622.5	2.2	9.833	0.04	0.32
	5616.0					0.07
	5638.3					
	5621.5					
	5623.8					
170	1140.7	1140.6	0.1		0.01	
	1140.5					
85	596.9	596.9	0.0		0	
	596.9					
17	119.8	119.9	0.1		0.12	
	120.0					
8.5	54.6	55.0	0.6		5.15	
	55.4					
1.7	10.9	11.1	0.3		2.86	
	11.3					

[a] Mean area is the mean of two independent analyses. [b] SD intraday, SD calculated injecting each standard solution 3 times in HPLC in the same day (intraday). [c] SD interday, standard deviation calculated injecting 850 mg L^{-1} solution 3 times in 3 consecutive days (inter-day). [d] RSD % intraday, relative SD calculated for each standard solution injected in the same day (intraday). [e] RSD % intraday, relative SD calculated injecting 850 mg L^{-1} solution 3 times in 3 consecutive days (inter-day).

A signal-to-noise ratio (S/N) 3:1 was accepted to evaluate the LOD, while a signal-to-noise ratio 10:1 was considered for the determination of LOQ.

5.2.3 Insect and mite species

C. ferrugineus, O. surinamensis, T. granarium, T. castaneum, T. confusum, T. molitor, A. diaperinus, and *A. siro* used in this study were collected from mass-rearing of the Laboratory of Agricultural Zoology and Entomology, Agricultural University of Athens. *C. ferrugineus* and the other pests were cultured as described in Chapter I, Section 5.9.1. Also, the rearing conditions followed those reported in Chapter I, Section 5.9.1. The selection of adult participants was random, and the age of the individuals was the same reported in Chapter I, Section 5.9.1, with the exception of *C. ferrugineus* (less than 2 weeks old). Larval study subjects were between the 3rd and 4th larval instar (*C. ferrugineus, O. surinamensis, T. castaneum, T. confusum*), between 10 and 14 mm long (*T. molitor*), shorter than 3 or 7 mm (*T. granarium* and *A. diaperinus*, respectively). The selection of *A. siro* was also random, and the age of individuals was the same reported in Chapter I, Section 5.9.1. Nymphs and adults were recognized by external morphology (Hughes, 1976; Kavallieratos et al., 2018a; Nesvorna and Hubert, 2014).

Grain

The commodity used for the study was *T. durum* as reported in Chapter I, Section 5.9.1.

Bioassays

Preliminary trials were conducted on all arthropod pests to select the two extract concentrations: 0.5 g extract kg^{-1} wheat (500 ppm) and 1 g extract kg^{-1} wheat (1000 ppm). The solutions were prepared as follows: for 500 ppm, 0.125 g of extract and 0.625 mL of Tween 80 were mixed and vortexed until the complete solubilization; while for 1000 ppm, 0.25 g of extract and 1.25 mL of Tween 80 were combined. Afterwards, distilled water was added (5.375 and 4.75 mL for 500 and 1000 ppm, respectively). The 6 mL solutions were separately sprayed on 0.25 kg wheat, laid on trays, with a unique A BD-134K airbrush (Fengda, UK). Regarding carrier controls, 0.25 mL of Tween 80 were mixed with 4.75 mL of distilled water, while for controls, 6 mL of distilled water were sprayed employing different airbrushes on different trays to avoid contamination. After the spraying, lots of wheat were submitted to a 10 min handshake in order to assure the homogeneous distribution of the extracts/controls to the whole commodity. Three samples of 10 g and 1 g were obtained from the lots, for the insect and mite pests, respectively, weighted on a unique paper on an electronic compact Precisa XB3200D balance (Alpha Analytical Instruments, Greece). Afterwards, the 10 g and 1 g samples were moved into glass containers (12.5 cm height and 7.5 cm diameter, and 6.0 cm height and 2.7 cm diameter, for the insect and mite species, respectively). The first type of containers was characterized by lids with a circular whole of 1.5 cm diameter, covered with cloth; while the second type was characterized by drilled lids, to ensure the aeration of their inside

space. To avoid escaping phenomena, polytetrafluoroethylene (Sigma-Aldrich Chemie GmbH, Germany) was used to polish the top inside part of them. Ten arthropod individuals of each pest/developmental stage were transferred into the containers, which were then placed into incubators set at a temperature of 30 °C and 65% of RH (insects) or of 25 °C and 80% of RH (mite). The data of mortality were registered after 4, 8, and 16 h and 1 to 7 days, under a stereomicroscope (Olympus SZX9, Bacacos S.A., Greece). Individuals were considered dead if the absence of movements was detected and a brush was employed to slightly poke the pest individuals. The same preparation was repeated three more times for both concentrations/extracts/controls with fresh grains, individuals, and containers. In total, 8,640 pests were used for these trials (3 replicates x 3 sub-replicates x 10 individuals x 6 concentrations/extracts (500 ppm, 1000 ppm, carrier control, control) x 16 species/developmental stages).

Data analysis

Data analysis followed that already reported in Chapter I, Section 5.9.1.

5.3. Results and discussion

5.3.1 HPLC-DAD-MS quantification method

The linear regression equation obtained for the spilanthol calibration curve was y = 6.5441x + 19.094 and was characterized by a coefficient of determination (R^2) of 1 (Figure 32). The values obtained for LOQ and LOD were 0.153 mg L^{-1} and 0.046 mg L^{-1}, respectively.

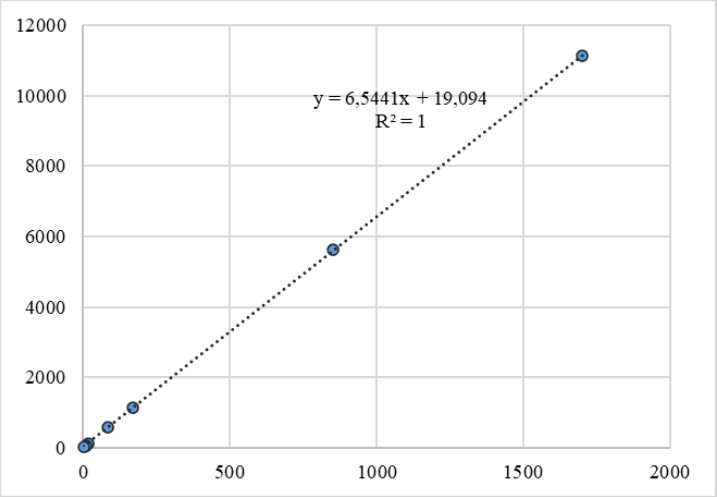


Figure 32. Calibration curve of spilanthol obtained through HPLC-DAD analysis.

5.3.2 Screening of *Acmella oleracea* extractive solvents and extraction techniques

Firstly, the objective of this screening was the investigation of the spilanthol extraction capacity of solvents of diverse polarity, as *n*-hexane, ethanol, methanol, dichloromethane, petroleum ether, and ethyl acetate. This screening pointed out significant differences in extraction yields (Table 19). In fact, the best extraction solvent was methanol with 11 ± 0.07% yield, followed by dichloromethane (5.5 ± 0.12%), ethyl acetate (4.2 ± 0.21%), ethanol (3.9 ± 0.11%), *n*-hexane (2.7 ± 0.28%), and petroleum ether (1.9 ± 0.20%). The HPLC-DAD results derived from the analysis of the DEs are reported in Table 21.

Table 21. Results of the first screening of different extractive solvents.

Solvent	Concentration (g/100 g DE[a])[b] ± SD[c]	RSD %[d]	Absolute amount of spilanthol extracted (g/100 g DB)[e]
n-hexane	20.9 ± 2.4	11.7	0.6
ethanol	11.4 ± 0.6	5.5	0.4
methanol	15.9 ± 2.6	16.4	1.3
dichloromethane	17.7 ± 2.6	14.5	0.9
petroleum ether	19.7 ± 0.3	1.3	0.4
ethyl acetate	16.5 ± 0.4	2.4	0.7

[a]DE, dry extract. [b]Mean concentration (g/100 g DE) of spilanthol found in each dry extract and is the mean of two independent analyses. [c]SD, standard deviation. [d]RSD %, relative SD. [e]Amount of spilanthol (g/100 g) extracted from dry biomass.

Concerning spilanthol concentration (g/100g DE), the best extract resulted to be the *n*-hexane extract (20.9 ± 2.4 g/100 g DE), followed by petroleum ether (19.7 ± 0.3 g/100g DE) and dichloromethane (17.7 ± 2.6 g/100g DE) extracts. Ethyl acetate, methanol and ethanol extracts displayed lower concentrations (16.5 ± 0.4, 15.9 ± 2.6, 11.4 ± 0.6 g/100 g DE, respectively). However, considering the absolute amount of spilanthol extracted from the plant matrix the best extractive solvent for spilanthol (Table 21) resulted to be methanol, with the absolute amount of the *N*-alkylamide extracted from the plant of 1.3%, followed by dichloromethane (0.9%), ethyl acetate (0.7%), and *n*-hexane (0.6%). The worst extractive solvents were ethanol and petroleum ether with an amount of spilanthol extracted both of 0.4%. The results achieved with the first screening led to the use of methanol as an extractive solvent for the subsequent screening of extraction techniques, since it showed the highest spilanthol extraction capacity from the plant matrix.

So, the aim of this second screening was the identification of the best extraction technique leading to the highest recovery of spilanthol from the plant material. Diverse extraction techniques were assessed employing methanol as an extractive solvent: ultrasound bath, ultrasound extractor, magnetic stirring, and Soxhlet. This study led to different extraction yields that are reported in Table 19. The highest yield was obtained using Soxhlet (26 ± 0.66%), followed by that obtained

from magnetic stirring for 3 and 1 h (12 ± 0.33-0.57%), and by that from the ultrasound bath extraction of 3 and 1 h (11 ± 1.4 and 10 ± 0.26%, respectively). Finally, the yield obtained from the ultrasound extractor was of 7.6 ± 0.35%. The results obtained from spilanthol quantitative analysis of the extracts are reported in Table 22.

Table 22. Results of the second screening of extraction techniques.

Extraction technique	Concentration (g/100 g DE[a])[b] ± SD[c]	RSD %[d]	Absolute amount of spilanthol extracted (g/100 g DB)[e]
Ultrasound bath 1 h	11.8 ± 0.3	2.7	1.2
Ultrasound bath 3 h	11.3 ± 0.8	7.4	1.3
Ultrasound extractor	12.3 ± 0.3	2.5	0.9
Magnetic stirring 1 h	10.9 ± 0.5	4.9	1.3
Magnetic stirring 3 h	11.3 ± 0.7	6.3	1.4
Soxhlet	7.6 ± 0.3	3.4	1.9

[a]DE, dry extract. [b]Mean concentration (g/100 g DE) represents the mean concentration of spilanthol found in each dry extract and is the mean of two independent analyses. [c]SD, standard deviation. [d]RSD %, relative SD. [e]Amount of spilanthol (g/100 g) extracted from dry biomass.

From the Table 22, the extract with the highest concentration of spilanthol was the one resulting from the extraction with the ultrasound extractor (12.3 ± 0.3 g/100 g DE), followed by those resulting from the extractions with ultrasound bath for 1 and 3 h (11.8 ± 0.3 and 11.3 ± 0.8 g/100 g DE, respectively) and with magnetic stirring for 3 h (11.3 ± 0.7 g/100 g DE). Extracts resulting from the magnetic stirring for 1 h and Soxhlet showed the lowest concentration of the *N*-alkylamide (10.9 ± 0.5 and 7.6 ± 0.3 g/100 g DE, respectively). On the other hand, the absolute amount of spilanthol extracted from the plant matrix had a completely different trend. Indeed, Soxhlet resulted the technique with the highest recovery of spilanthol from the plant (absolute amount extracted of 1.9%), and magnetic stirring of 3 and 1 h gave a similar recovery of the *N*-alkylamide (1.4 and 1.3%, respectively). The ultrasonic bath extractions of 3 and 1 h gave a recovery of spilanthol of 1.3 and 1.2%, respectively. The ultrasound extractor led to the lowest recovery of the *N*-alkylamide from the plant (0.9%). To conclude, from this second screening performed, the best technique for the highest recovery of spilanthol from the plant matrix resulted to be Soxhlet extraction.

For the evaluation of *A. oleracea* extracts activity on the above-mentioned arthropods, two extracts were selected, deriving in both cases from Soxhlet extraction. Firstly, it was chosen the methanol extract since methanol resulted the most efficient solvent in terms of spilanthol recovery from the plant. In addition, also a *n*-hexane extract was produced, because this was the solvent leading to the extract with the highest concentration of this *N*-alkylamide. Moreover, *n*-hexane is the most used solvent in previous entomological investigations of *A. oleracea* (Castro et al., 2014; Marchesini et al., 2020).

5.3.3 *N*-Alkylamides quantification *Acmella oleracea* extracts

For the *n*-hexane and methanol extracts selected for the efficacy evaluation against pests, a quali-quantitative determination of the main *N*-alkylamides was performed through the HPLC-DAD-MS method. Different *N*-alkylamides (Table 23, Figure 33) were identified through the evaluation of the MS spectra.

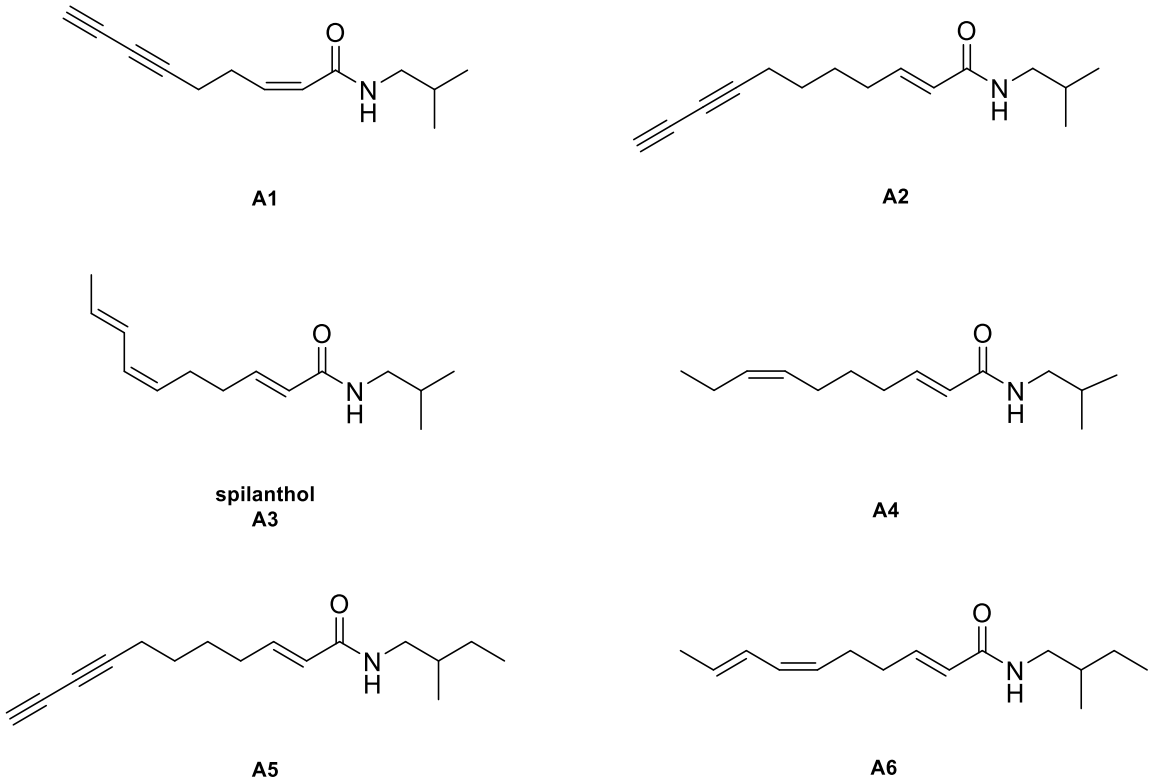


Figure 33. Main *N*-alkylamides found in *Acmella oleracea n*-hexane and methanol extracts.

The (*2Z*)-*N*-isobutyl-2-nonene-6,8-diynamide (A1) presence was confirmed from $[M + H]^+$ and $[M + NH_4]^+$ ions, which were 204.7 and 222.7 *m/z*, respectively (Figure S3.1, Supplementary Material). The ions 232.6 and 254.6 *m/z* ($[M + H]^+$ and $[M + Na]^+$, respectively) confirmed the presence of (*2E*)-*N*-isobutyl-2-undecene-8,10-diynamide (A2) (Figure S3.2, Supplementary Material); while the ions 222.5 and 244.5 *m/z* ($[M + H]^+$ and $[M + Na]^+$, respectively) were indicative of the presence of (*2E,6Z,8E*)-*N*-isobutyl-2,6,8-decatrienamide (A3) or spilanthol (Figure S3.3, Supplementary Material).

The *N*-alkylamides (*2E,7Z*)-*N*-isobutyl-2,7-decadienamide (A4) and (*2E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (A5) coeluted from the column. For A4, 224.6 and 246.6 *m/z* (corresponding to $[M + H]^+$ and $[M + Na]^+$ ions) were detected; in addition 246.6 *m/z* corresponded also to the $[M + H]^+$ ion of A5, for which also 268.6 *m/z* ($[M + Na]^+$ ion) was detected. Finally,

236.6 and 258.6 *m/z* ($[M + H]^+$ and $[M + Na]^+$ ions) (Figure S3.4, Supplementary Material) were detected for the alkylamide (*2E,6Z,8E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide (A6).

Table 23. Results of the *N*-alkylamides HPLC-DAD-MS quantification in *Acmella oleracea n*-hexane and methanol extracts.

	***N*-Alkylamide**	***n*-Hexane extract**			**Methanol extract**		
		Concentration (g/100 g DE[a])[b] ± SD[c]	**RSD %[d]**	**Absolute amount of *N*-alkylamides extracted (g/100 g DB)[e]**	**Mean concentration (g/100 g DE) ± SD**	**RSD %**	**Absolute amount of *N*-alkylamides extracted (g/100 g DB)[e]**
A1	(*2Z*)-*N*-isobutyl-2-nonene-6,8-diynamide	0.4 ± 0.1	16.7	< 0.1	0.1 ± 0.0	0.0	< 0.1
A2	(*2E*)-*N*-isobutyl-2-undecene-8,10-diynamide	0.3 ± 0.0	0.0	< 0.1	0.1 ± 0.0	0.0	< 0.1
A3	(*2E,6Z,8E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol)	24.3 ± 1.3	4.0	1.3	7.6 ± 0.3	3.4	1.9
A4	(*2E,7Z*)-*N*-isobutyl-2,7-decadienamide	0.2 ± 0.0	0.0	< 0.1	0.1 ± 0.0	0.0	< 0.1
A5	(*2E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide						
A6	(*2E,6Z,8E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide	1.7 ± 0.3	13.0	0.1	0.5 ± 0.0	0.0	< 0.1

[a]DE, dry extract. [b]Average concentration (g/100 g DE) represents the average concentration of *N*-alkylamides found in each dry extract and is the mean of two independent analyses. [c]SD, standard deviation. [d]RSD %, relative SD. [e]Amount of *N*-alkylamides (g/100 g) extracted from dry biomass.

Regarding the quantification of *N*-alkylamides in the *A. oleracea* extracts, the *n*-hexane extract resulted to be more concentrated. Spilanthol was the main compound in both *n*-hexane and methanol extracts, resulting in 24.3 ± 1.3 and 7.6 ± 0.3 g/100 g DE, respectively, followed by A6 (1.7 ± 0.3 and 0.5 ± 0.0 g/100 g DE, respectively). A1, A2, and A3 were detected in minor amounts in both extracts, as reported in Table 23. On the contrary, methanol extraction was more effective than *n*-hexane in terms of *N*-alkylamides recovery from the plant material, especially spilanthol (1.9 and 1.3%, respectively), as also demonstrated by the solvents screening reported in Section 5.3.2. The total amount of the other *N*-alkylamides recovered was < than 0.1%.

Concerning the screening of *A. oleracea* extraction, different solvents have been screened for the extraction of spilanthol, and *n*-hexane (Ramsewak et al., 1999), ethanol (Simas et al., 2013), and methanol are the most frequently employed (Mbeunkui et al., 2011). Several studies also described the application of combination of solvents to extract the *N*-alkylamide, such as ethanol:water (7: 3, v/v) (Martins et al., 2012) or ethanol:*n*-hexane (3:7, v/v) (Costa et al., 2013). Balieiro et al. (2020) compared the influence of acetonitrile, methanol, dichloromethane, and ethanol on spilanthol extraction and, using a response surface methodology and HPLC-DAD analysis, pure ethanol resulted to be the best extractive solvent.

Concerning the extraction techniques screening, the use of microwave-assisted extraction (Franca et al., 2016), supercritical CO_2 extraction (Dias et al., 2012), and Soxhlet (Bakondi et al., 2019) have been reported. For instance, Franca et al. (2016) demonstrated that microwave-assisted extraction led to the highest recovery of spilanthol if compared with normal maceration. Bellumori et al. (2022) recently screened three different *A. oleracea* extraction procedures using ethanol 80% v/v as extractive solvent: sonication at 60 °C for 10 min, magnetic stirring for 50 min followed by sonication for 10 min, and sonication for 10 min at room temperature. In this study, it was proved that sonication at 60 °C for 10 min was the best extractive technique and that a fractionation step with *n*-hexane to the obtained ethanolic extract led to an enriched *N*-alkylamides fraction. The results reported by Bellumori et al. (2022) suggested that *n*-hexane gives an extract enriched in spilanthol and this is linear with the results presented in our study, even if a different extractive approach was used. From the study of Bellumori et al. (2022) it is also clear that magnetic stirring is not the best spilanthol extractive technique, as also evident from our study (Table 21). These results were also linear with those published by Grymel et al. (2022). Indeed, between Soxhlet extraction, magnetic stirring at high temperature and room temperature, and maceration at room temperature, Soxhlet resulted in the best extraction technique for the highest recovery of the *N*-alkylamide from the plant.

Regarding the identification of the *N*-alkylamides reported in our study, the results presented are quite linear with those reported by Bae et al. (2010), even if they identified 3 more *N*-alkylamides from a 75% ethanol extract: (*2E,4Z*)-*N*-isobutly-2,4-undecadiene-8,10-diynamide, (*2E,7Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide, (*2E,4E,8Z,10E*)-*N*-isobutyl-dodeca-2,4,8,10-

tetraenamide. In addition, Cheng et al. (2015) isolated and characterized from an *A. oleracea* ethanolic extract another *N*-alkylamide, namely (*2E,5Z*)-*N*-isobutylundeca-2,5-diene-8,10-diynamide that was not found in the herein reported study. Moreover, (*2E,4Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide, (*2E,7Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide, (*2E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, *N*-phenethyl-2,3-epoxy-6,8-nonadiynamide,and (*2E,4E,8Z,10Z*)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide were identified by Boonen et al. (2010a) in an *A. oleracea* ethanolic extract, differently from the results presented in our study.

5.3.4 Efficacy of *Acmella oleracea* extracts against arthropod pests

Cryptolestes ferrugineus

Regarding *C. ferrugineus* adults, the main effects were significant between exposure intervals (Table S3, Supplementary Material). The *n*-hexane extract at 1000 ppm was highly effective against *C. ferrugineus* adults killing 91.1% after 1 day of exposure (Table 24). One day after, the *n*-hexane extract at 500 and 1000 ppm caused 96.7 and 100.0% mortality of the individuals, respectively. On the contrary methanol extract did not reach a mortality higher than 70.0% (1000 ppm) after the same time. Indeed, it led to 100.0% mortality only on the 4th and 6th day of the experimentation for the 500 and 1000 ppm concentration, respectively.

Concerning *C. ferrugineus* larvae, between and within exposure intervals all main effects and their interactions were significant, as shown in Table S3, Supplementary Material. The 2nd day both concentrations of the *n*-hexane extract showed moderate efficacy, with 52.2% (500 ppm) and 75.6% (1000 ppm) mortality (Table 24). At the 7th day, *n*-hexane extract killed 96.7 and 98.9% of larvae at 500 and 1000 ppm, respectively. On the other side, methanol extract at 1000 ppm led to 100% larval mortality at the same exposure period. The concentration of 500 ppm of methanol extract caused 86.7% mortality at the end of the experiments.

Table 24. Mean (%) mortality ± standard error (SE) of *Cryptolestes ferrugineus* adults and larvae after 4 h, 8 h, 16 h, and 1–7 days on wheat treated with *Acmella oleracea n*-hexane and methanol extracts at two different concentrations.

	***n*-Hexane extract**		**Methanol extract**		***F***	***P***
	500 ppm	**1000 ppm**	**500 ppm**	**1000 ppm**		
Adults						
4 h	0.0 ± 0.0^{Db}	3.3 ± 1.7^{Ca}	0.0 ± 0.0^{Cb}	0.0 ± 0.0^{Eb}	4.0	0.02
8 h	12.2 ± 2.2^{Ca}	23.3 ± 4.1^{Ba}	2.2 ± 1.5^{Cb}	3.3 ± 1.7^{Db}	10.1	< 0.01
16 h	40.0 ± 3.7^{Ba}	55.6 ± 6.5^{Aa}	5.6 ± 2.9^{Cc}	12.2 ± 1.5^{Cb}	26.2	< 0.01
1 day	73.3 ± 4.7^{Aa}	91.1 ± 3.5^{Aa}	17.8 ± 4.3^{Bb}	24.4 ± 2.4^{Bb}	22.5	< 0.01
2 days	96.7 ± 1.7^{Aa}	100.0 ± 0.0^{Aa}	60.0 ± 7.3^{Ab}	70.0 ± 5.3^{Ab}	13.9	< 0.01
3 days	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	83.3 ± 6.0^{Ab}	92.2 ± 4.3^{Aab}	4.2	0.01
4 days	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	93.3 ± 3.7^{Ab}	100.0 ± 0.0^{Aa}	3.1	0.04
5 days	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	97.8 ± 1.5^{A}	100.0 ± 0.0^{A}	2.3	0.10
6 days	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	-	-
7 days	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	100.0 ± 0.0^{A}	-	-
F	211.1	45.0	71.1	162.8		
P	< 0.01	< 0.01	< 0.01	< 0.01		
Larvae						
4 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
8 h	0.0 ± 0.0^{C}	1.1 ± 1.1^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	1.0	0.41
16 h	1.1 ± 1.1^{Cb}	20.0 ± 3.3^{Ca}	0.0 ± 0.0^{Db}	0.0 ± 0.0^{Db}	86.2	< 0.01
1 day	16.7 ± 4.1^{Bb}	42.2 ± 4.7^{Ba}	0.0 ± 0.0^{Dc}	1.1 ± 1.1^{Dc}	61.0	< 0.01
2 days	52.2 ± 5.2^{Aa}	75.6 ± 6.5^{Aa}	8.9 ± 3.1^{Cb}	12.2 ± 2.8^{Cb}	16.3	< 0.01
3 days	73.3 ± 3.7^{Aa}	87.8 ± 5.7^{Aa}	22.2 ± 3.2^{Bb}	23.3 ± 2.9^{Bb}	48.0	< 0.01
4 days	87.8 ± 2.8^{Aa}	91.1 ± 6.1^{Aa}	45.6 ± 2.9^{Abb}	54.4 ± 3.8^{Ab}	28.3	< 0.01
5 days	93.3 ± 2.4^{Aa}	95.6 ± 3.4^{Aa}	65.6 ± 4.4^{Ab}	67.8 ± 2.8^{Ab}	18.5	< 0.01
6 days	96.7 ± 2.4^{Aa}	98.9 ± 1.1^{Aa}	81.1 ± 4.8^{Ab}	92.2 ± 2.8^{Aab}	6.4	< 0.01
7 days	96.7 ± 2.4^{Aab}	98.9 ± 1.1^{Aa}	86.7 ± 4.7^{Ab}	100.0 ± 0.0^{Aa}	4.8	0.01
F	195.3	244.5	143.9	155.6		
P	< 0.01	< 0.01	< 0.01	< 0.01		

Within each row, means followed by the same lowercase letter are not significantly different (df = 3, 35; Tukey's HSD test at $P = 0.05$). Within each column, means followed by the same uppercase letter are not significantly different (df = 9, 89; Tukey's HSD test at $P = 0.05$). Where no letters, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Tenebrio molitor

Concerning *T. molitor* adults, the type of extract, concentration, exposure, and exposure x extract were significant between and within exposure intervals (Table S3, Supplementary Material). For 8 and 16 h, no mortality was noticed for *n*-hexane and methanol extracts at the concentrations tested (Table 25). The *n*-hexane extract mortality rates were low for the whole experimental interval at 500 and 1000 ppm, not exceeding 13.3 and 24.4%, respectively. For methanol extract, complete mortality was reached after 5 days at 1000 ppm, while after a 7-day exposure, 91.1% of the individuals were dead at 500 ppm.

Regarding *T. molitor* larvae, all main effects and interactions were significant between and within exposure intervals, except extract x concentration (Table S3, Supplementary Material). During the first 2 days of the test, methanol extract led to 55.6% of larval mortality *T. molitor* at 1000 ppm, while mortalities of the 500 ppm concentration ranged between 0.0 and 16.7% (Table 25). After 5 days of tests, both *n*-hexane and methanol extracts at 1000 ppm provided high mortalities (95.6 and 93.3%, respectively). At the end of the experiments, 500 and 1000 ppm of *n*-hexane extract and 500 and 1000 ppm of methanol extract caused 86.7, 100.0, 80.0, and 97.8% larval mortality, respectively.

Table 25. Mean (%) mortality ± standard error (SE) of *Tenebrio molitor* adults and larvae after 4 h, 8 h, 16 h, and 1–7 days on wheat treated with *Acmella oleracea n*-hexane and methanol extracts at two different concentrations.

	***n*-Hexane extract**		**Methanol extract**		***F***	***P***
	500 ppm	**1000 ppm**	**500 ppm**	**1000 ppm**		
Adults						
4 h	0.0 ± 0.0^{B}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{E}	-	-
8 h	0.0 ± 0.0^{B}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{E}	-	-
16 h	0.0 ± 0.0^{Bb}	0.0 ± 0.0^{Db}	3.3 ± 1.7^{Dab}	4.4 ± 1.8^{Da}	3.6	0.02
1 day	1.1 ± 1.1^{Bc}	3.3 ± 1.6^{CDbc}	8.9 ± 2.0^{Cab}	10.0 ± 1.7^{Ca}	7.5	< 0.01
2 days	2.2 ± 1.5^{ABb}	5.6 ± 1.8^{BCDb}	36.7 ± 6.0^{Ba}	43.3 ± 4.7^{Ba}	28.9	< 0.01
3 days	3.3 ± 1.7^{ABb}	6.7 ± 2.4^{ABCDb}	63.3 ± 6.5^{ABa}	68.9 ± 4.2^{ABa}	34.0	< 0.01
4 days	4.4 ± 1.8^{ABb}	8.9 ± 2.0^{ABCb}	72.2 ± 6.2^{ABa}	93.3 ± 2.9^{Aa}	36.2	< 0.01
5 days	5.6 ± 2.4^{ABb}	12.2 ± 2.8^{ABCb}	80.0 ± 5.8^{ABa}	100.0 ± 0.0^{Aa}	29.8	< 0.01
6 days	8.9 ± 3.5^{ABb}	17.8 ± 4.0^{ABb}	86.7 ± 4.4^{Aa}	100.0 ± 0.0^{Aa}	20.0	< 0.01
7 days	13.3 ± 4.4^{Ab}	24.4 ± 3.8^{Ab}	91.1 ± 2.6^{Aa}	100.0 ± 0.0^{Aa}	16.5	< 0.01
F	3.8	9.6	101.2	138.5		
P	< 0.01	< 0.01	< 0.01	< 0.01		
Larvae						
4 h	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
8 h	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
16 h	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
1 day	0.0 ± 0.0^{D}	1.1 ± 1.1^{D}	0.0 ± 0.0^{D}	2.2 ± 1.5^{C}	1.3	0.28
2 days	2.2 ± 1.5^{CDc}	16.7 ± 3.7^{Cb}	0.0 ± 0.0^{Dc}	55.6 ± 2.4^{Ba}	54.6	< 0.01
3 days	4.4 ± 1.8^{Cc}	44.4 ± 4.4^{Bab}	18.9 ± 2.0^{Cb}	76.7 ± 3.7^{ABa}	40.6	< 0.01
4 days	17.8 ± 3.2^{Bb}	76.7 ± 5.0^{ABa}	44.4 ± 3.8^{Ba}	88.9 ± 3.9^{ABa}	20.0	< 0.01
5 days	44.4 ± 3.4^{Ac}	95.6 ± 2.4^{Aa}	66.7 ± 2.9^{Ab}	93.3 ± 2.9^{ABa}	54.6	< 0.01
6 days	68.9 ± 4.2^{Ab}	97.8 ± 2.2^{Aa}	75.6 ± 3.8^{Ab}	94.4 ± 2.4^{ABa}	16.1	< 0.01
7 days	86.7 ± 3.3^{Abc}	100.0 ± 0.0^{Aa}	80.0 ± 4.4^{Ac}	97.8 ± 1.5^{Aab}	10.2	< 0.01
F	82.9	216.7	1659.0	375.9		
P	< 0.01	< 0.01	< 0.01	< 0.01		

Within each row, means followed by the same lowercase letter are not significantly different (df = 3, 35; Tukey's HSD test at $P = 0.05$). Within each column, means followed by the same uppercase letter are not significantly different (df = 9, 89; Tukey's HSD test at $P = 0.05$). Where no letters, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Oryzaephilus surinamensis

Between and within exposure intervals, all main effects and interactions were significant for *O. surinamensis* adults (Table S3, Supplementary Material). Both extracts tested at 1000 ppm led to around 50.0% mortality the 2nd day of the experiments, while to around 90% mortality at the 5th day (Table 26). After 7 days of experiments, *n*-hexane and methanol extracts led to 90.0 and 97.8% adult mortality, respectively. High mortality levels were also reached for *n*-hexane and methanol extracts at 500 ppm (87.8 and 82.2%, respectively).

Concerning *O. surinamensis* larvae, the main effects were significant between and within exposure intervals (Table S3, Supplementary Material). The *n*-hexane extract caused 53.3% mortality after 2-day exposure at 1000 ppm, while all the other treatments led to low mortality values (10.0-27.8%) (Table 26). The same concentration of the *n*-hexane extract caused 90.0% larval mortality after 5-days of exposure. At the end of the experiments, mortalities of 94.4 and 95.6% were reached at 500 and 1000 ppm of *n*-hexane extract. Conversely, methanol extract did not lead to mortality higher than 75.6 (at 500 ppm) and 86.7% (1000 ppm) for the same interval.

Table 26. Mean (%) mortality ± standard error (SE) of *Oryzaephilus surinamensis* adults and larvae after 4 h, 8 h, 16 h, and 1–7 days on wheat treated with *Acmella oleracea n*-hexane and methanol extracts at two different concentrations.

	***n*-Hexane extract**		**Methanol extract**		***F***	***P***
	500 ppm	**1000 ppm**	**500 ppm**	**1000 ppm**		
Adults						
4 h	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	-	-
8 h	0.0 ± 0.0^{Db}	3.3 ± 1.7^{Ca}	0.0 ± 0.0^{Cb}	0.0 ± 0.0^{Cb}	4.0	0.02
16 h	0.0 ± 0.0^{Db}	17.8 ± 2.8^{Ba}	0.0 ± 0.0^{Cb}	2.2 ± 1.5^{BCb}	50.2	< 0.01
1 day	7.8 ± 2.8^{Cbc}	30.0 ± 5.0^{Ba}	0.0 ± 0.0^{Cc}	8.9 ± 3.9^{Bb}	13.7	< 0.01
2 days	21.1 ± 4.2^{Bb}	58.9 ± 5.4^{Aa}	24.4 ± 6.5^{Bb}	47.8 ± 3.6^{Aa}	7.6	< 0.01
3 days	42.2 ± 4.0^{ABb}	75.6 ± 7.3^{Aa}	63.3 ± 5.0^{Aa}	67.8 ± 5.2^{Aa}	7.9	< 0.01
4 days	61.1 ± 4.8^{Ab}	86.7 ± 5.3^{Aa}	77.8 ± 4.9^{Aa}	87.8 ± 3.6^{Aa}	7.3	< 0.01
5 days	83.3 ± 5.8^{A}	90.0 ± 5.0^{A}	78.9 ± 4.6^{A}	92.2 ± 2.8^{A}	1.7	0.19
6 days	87.8 ± 6.4^{A}	90.0 ± 5.0^{A}	80.0 ± 4.7^{A}	94.4 ± 1.8^{A}	1.5	0.23
7 days	87.8 ± 6.4^{A}	90.0 ± 5.0^{A}	82.2 ± 4.3^{A}	97.8 ± 1.5^{A}	1.7	0.18
F	137.1	109.0	266.2	100.5		
P	< 0.01	< 0.01	< 0.01	< 0.01		
Larvae						
4 h	0.0 ± 0.0^{E}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{E}	-	-
8 h	0.0 ± 0.0^{E}	1.1 ± 1.1^{D}	0.0 ± 0.0^{D}	1.1 ± 1.1^{E}	0.7	0.58
16 h	3.3 ± 2.4^{Eb}	13.3 ± 2.4^{Ca}	0.0 ± 0.0^{Db}	3.3 ± 1.7^{DEb}	10.0	< 0.01
1 day	17.8 ± 3.6^{Dab}	28.9 ± 3.5^{Ba}	2.2 ± 1.5^{Dc}	6.7 ± 1.7^{Dbc}	14.0	< 0.01
2 days	27.8 ± 4.0^{CDab}	53.3 ± 4.1^{ABa}	10.0 ± 1.7^{Cc}	17.8 ± 2.8^{Cbc}	16.5	< 0.01
3 days	37.8 ± 3.6^{BCb}	74.4 ± 4.8^{Aa}	13.3 ± 1.7^{Cd}	23.3 ± 3.3^{BCc}	40.4	< 0.01
4 days	55.6 ± 4.1^{ABCb}	86.7 ± 4.4^{Aa}	28.9 ± 2.0^{Bc}	53.3 ± 3.7^{ABb}	40.6	< 0.01
5 days	70.0 ± 4.7^{ABa}	90.0 ± 3.7^{Aa}	46.7 ± 5.0^{ABb}	67.8 ± 5.2^{Aa}	11.8	< 0.01
6 days	84.4 ± 5.0^{ABa}	92.2 ± 3.6^{Aa}	64.4 ± 7.5^{ABb}	83.3 ± 3.7^{Aa}	5.2	< 0.01
7 days	94.4 ± 3.8^{Aa}	95.6 ± 2.9^{Aa}	75.6 ± 6.0^{Ab}	86.7 ± 2.9^{Aab}	5.0	0.01
F	100.0	146.4	125.9	69.5		

Within each row, means followed by the same lowercase letter are not significantly different (df = 3, 35; Tukey's HSD test at $P = 0.05$). Within each column, means followed by the same uppercase letter are not significantly different (df = 9, 89; Tukey's HSD test at $P = 0.05$). Where no letters, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Trogoderma granarium

Regarding *T. granarium* adults, between and within exposure intervals, main effects and interactions were significant (Table S3, Supplementary Material). At the 3rd day of the tests, mortality values were between 33.3% (for 500 ppm of *n*-hexane extract) and 64.4% (for 1000 ppm of methanol extract) (Table 27). High mortality levels were achieved testing both extracts at 1000 ppm (94.4% for the *n*-hexane and 93.3% for the methanol extract) after 6 days of tests, while 100.0% and 97.8% mortality were reached for the *n*-hexane and methanol extract after 7 days, respectively.

Between and within exposure intervals, concentration, exposure, and exposure x extract were significant in the case of *T. granarium* larvae (Table S3, Supplementary Material). After a day of exposure, *n*-hexane extract did not lead to a mortality higher than 8.9% (at 1000 ppm), while both methanol concentrations did not cause any effect (Table 27). Mortalities of *T. granarium* larvae were moderate (52.2 and 60.0% for the *n*-hexane and methanol extracts at 1000 ppm) at the end of the experiments. The larval mortality at 500 ppm for both extracts was of 27.8% for the same interval.

Table 27. Mean (%) mortality ± standard error (SE) *of Trogoderma granarium* adults and larvae after 4 h, 8 h, 16 h, and 1–7 days on wheat treated with *Acmella oleracea n*-hexane and methanol extracts at two different concentrations.

	***n*-Hexane extract**		**Methanol extract**		***F***	***P***
	500 ppm	**1000 ppm**	**500 ppm**	**1000 ppm**		
Adults						
4 h	0.0 ± 0.0^{E}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{C}	-	-
8 h	0.0 ± 0.0^{Eb}	0.0 ± 0.0^{Db}	0.0 ± 0.0^{Db}	3.3 ± 1.7^{Ca}	4.0	0.02
16 h	1.1 ± 1.1^{Eb}	2.2 ± 1.5^{CDb}	0.0 ± 0.0^{Db}	14.4 ± 2.9^{Ba}	16.3	< 0.01
1 day	5.6 ± 1.8^{Db}	6.7 ± 2.9^{Cb}	2.2 ± 1.5^{Db}	21.1 ± 3.9^{Ba}	7.4	< 0.01
2 days	16.7 ± 2.4^{Cc}	24.4 ± 3.8^{Bbc}	32.2 ± 3.2^{Cab}	46.7 ± 4.4^{Aa}	12.2	< 0.01
3 days	33.3 ± 3.7^{BCb}	44.4 ± 5.6^{ABab}	46.7 ± 5.0^{BC}ab	64.4 ± 6.0^{Aa}	6.7	< 0.01
4 days	45.6 ± 3.8^{ABb}	77.8 ± 4.6^{Aa}	53.3 ± 5.5^{ABCb}	77.8 ± 6.0^{Aa}	11.8	< 0.01
5 days	54.4 ± 4.1^{ABc}	87.8 ± 3.6^{Aa}	66.7 ± 4.7^{ABbc}	83.3 ± 5.3^{Aab}	11.7	< 0.01
6 days	56.7 ± 4.4^{ABb}	94.4 ± 2.9^{Aa}	80.0 ± 4.7^{ABa}	93.3 ± 3.3^{Aa}	20.1	< 0.01
7 days	85.6 ± 3.8^{Abc}	100.0 ± 0.0^{Aa}	83.3 ± 4.7^{Ac}	97.8 ± 2.2^{Aab}	6.3	< 0.01
F	112.1	98.1	253.9	80.4		
P	< 0.01	< 0.01	< 0.01	< 0.01		
Larvae						
4 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
8 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
16 h	0.0 ± 0.0^{Cb}	4.4 ± 1.8^{Ca}	0.0 ± 0.0^{Db}	0.0 ± 0.0^{Db}	6.4	< 0.01
1 day	3.3 ± 1.7^{BCb}	8.9 ± 2.0^{Ba}	0.0 ± 0.0^{Db}	0.0 ± 0.0^{Db}	11.2	< 0.01
2 days	10.0 ± 3.3^{ABCb}	33.3 ± 3.3^{Aa}	3.3 ± 1.7^{CDb}	7.8 ± 2.8^{Cb}	8.1	< 0.01
3 days	14.4 ± 3.4^{ABb}	37.8 ± 3.6^{Aa}	7.8 ± 2.2^{BCb}	20.0 ± 4.4^{Bab}	6.6	< 0.01
4 days	16.7 ± 4.1^{ABb}	46.7 ± 3.3^{Aa}	16.7 ± 2.9^{ABb}	28.9 ± 4.8^{ABab}	4.8	0.01
5 days	26.7 ± 5.8^{Aab}	50.0 ± 4.1^{Aa}	17.8 ± 2.8^{ABb}	35.6 ± 5.6^{ABab}	3.7	0.02
6 days	27.8 ± 6.0^{Ab}	52.2 ± 3.2^{Aa}	24.4 ± 3.4^{ABb}	50.0 ± 5.5^{Aa}	3.7	0.02
7 days	27.8 ± 6.0^{Ab}	52.2 ± 3.2^{Aa}	27.8 ± 4.7^{Ab}	60.0 ± 5.3^{Aa}	4.1	0.02
F	12.6	78.0	21.1	89.5		
P	< 0.01	< 0.01	< 0.01	< 0.01		

Within each row, means followed by the same lowercase letter are not significantly different (df = 3, 35; Tukey's HSD test at $P = 0.05$). Within each column, means followed by the same uppercase letter are not significantly different (df = 9, 89; Tukey's HSD test at $P = 0.05$). Where no letters, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Tribolium castaneum

Extract, exposure, and exposure x extract were significant for *T. castaneum* adults between and within exposure intervals (Table S3, Supplementary Material). Both concentrations of the *n*-hexane extract did not lead to any mortality 16 h after the exposure, as also the concentrations of methanol extract after one day (Table 28). Generally, the mortality values were quite low on adults. In fact, they were not higher than 15.6 and 18.9% at the end of the experiments for 1000 ppm of *n*-hexane and methanol extracts, respectively.

For *T. castaneum* larvae, the main effects and interactions were significant between and within exposure intervals, except for extract x concentration (Table S3, Supplementary Material). The *n*-hexane extract led to 54.4% mortality at 1000 ppm and after 2-day exposure (Table 28). At the 5th day, 1000 ppm of *n*-hexane extract caused 95.6% larval mortality, while the other treatments led to 21.1-70.0% mortality. At the end of the experiments, 1000 ppm of the *n*-hexane extract led to 100% larval mortality, followed by 500 ppm (87.8%). Conversely, 1000 and 500 ppm of methanol extract caused 76.7 and 38.9% mortality, respectively.

Table 28. Mean (%) mortality ± standard error (SE) of *Tribolium castaneum* adults and larvae after 4 h, 8 h, 16 h, and 1–7 days on wheat treated with *Acmella oleracea n*-hexane and methanol extracts at two different concentrations.

	***n*-Hexane extract**		**Methanol extract**		***F***	***P***
	500 ppm	**1000 ppm**	**500 ppm**	**1000 ppm**		
Adults						
4 h	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	-	-
8 h	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	-	-
16 h	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	-	-
1 day	1.1 ± 1.1^{ABab}	4.4 ± 1.8^{ABa}	0.0 ± 0.0^{B}	0.0 ± 0.0^{C}	4.1	0.01
2 days	1.1 ± 1.1^{AB}	7.8 ± 3.2^{AB}	4.4 ± 2.4^{AB}	5.6 ± 1.8^{B}	1.7	0.18
3 days	5.6 ± 3.4^{ABb}	$8.9 \pm 3.5A^{Bab}$	$5.6 \pm 2.4^{ABa}b$	12.2 ± 1.5^{Aa}	3.1	0.04
4 days	6.7 ± 3.3^{AB}	10.0 ± 3.3^{A}	7.8 ± 3.2^{AB}	13.3 ± 1.7^{A}	2.3	0.10
5 days	7.8 ± 3.2^{AB}	12.2 ± 3.2^{A}	8.9 ± 3.1^{A}	14.4 ± 1.8^{A}	1.9	0.15
6 days	12.2 ± 4.9^{AB}	15.6 ± 4.4^{A}	8.9 ± 3.1^{A}	16.7 ± 1.7^{A}	1.7	0.19
7 days	13.3 ± 4.7^{A}	15.6 ± 4.4^{A}	8.9 ± 3.1^{A}	18.9 ± 1.1^{A}	1.8	0.16
F	4.1	6.1	5.1	81.4		
P	< 0.01	< 0.01	< 0.01	< 0.01		
Larvae						
4 h	0.0 ± 0.0^{E}	0.0 ± 0.0^{D}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	-	-
8 h	0.0 ± 0.0^{E}	2.2 ± 1.5^{D}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	2.3	0.10
16 h	0.0 ± 0.0^{Eb}	15.6 ± 2.9^{Ca}	0.0 ± 0.0^{Cb}	3.3 ± 1.7^{Db}	20.7	< 0.01
1 day	12.2 ± 2.8^{Db}	31.1 ± 4.6^{Ba}	0.0 ± 0.0^{Cc}	12.2 ± 2.8^{Cb}	20.9	< 0.01
2 days	17.8 ± 2.8^{CDb}	54.4 ± 3.8^{ABa}	2.2 ± 1.5^{Cc}	16.7 ± 3.3^{BCb}	22.0	< 0.01
3 days	33.3 ± 5.0^{BCa}	70.0 ± 5.8^{Aa}	5.6 ± 2.4^{BCb}	30.0 ± 4.1^{ABa}	26.3	< 0.01
4 days	58.9 ± 4.2^{ABa}	82.2 ± 4.3^{Aa}	16.7 ± 4.4^{ABb}	37.8 ± 4.7^{ABa}	13.7	< 0.01
5 days	70.0 ± 5.0^{ABa}	95.6 ± 2.4^{Aa}	21.1 ± 4.8^{Ab}	57.8 ± 4.7^{Aa}	16.1	< 0.01
6 days	83.3 ± 4.7^{Aa}	98.9 ± 1.1^{Aa}	35.6 ± 6.9^{Ab}	68.9 ± 3.5^{Aa}	19.7	< 0.01
7 days	87.8 ± 4.3^{Aa}	100.0 ± 0.0^{Aa}	38.9 ± 8.1^{Ab}	76.7 ± 4.4^{Aa}	16.7	< 0.01
F	105.9	105.4	27.9	57.1		
P	< 0.01	< 0.01	< 0.01	< 0.01		

Within each row, means followed by the same lowercase letter are not significantly different (df = 3, 35; Tukey's HSD test at $P = 0.05$). Within each column, means followed by the same uppercase letter are not significantly different (df = 9, 89; Tukey's HSD test at $P = 0.05$). Where no letters, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Tribolium. confusum

Extract, concentration (between exposure intervals), and exposure (within exposure intervals) were significant for *T. confusum* adults (Table S3, Supplementary Material). Regarding adults, no mortality was detected for 16 h, 8 h, and 2 days after the exposure to both concentrations of *n*-hexane extract and methanol extracts (Table 29). At the end of the experiments, mortality values ranged between 1.1% (500 ppm of methanol extract) and 18.9% (1000 ppm of *n*-hexane extract).

Regarding *T. confusum* larvae, concentration, exposure, exposure x extract and exposure x extract x concentration were significant between and within exposure intervals (Table S3, Supplementary Material). For larval mortality, 1000 ppm of *n*-hexane and methanol extracts led to 58.9% and 33.3% mortality, respectively after 3 days of experiments (Table 29). At the end of the tests, 1000 ppm of *n*-hexane extract led to 88.9% mortality, while 500 ppm to 85.6%. At the same exposure interval, 57.8 and 80.0% of larval mortality was detected after their exposure to 500 and 1000 ppm of methanol extract, respectively.

Table 29. Mean (%) mortality ± standard error (SE) of *Tribolium confusum* adults and larvae after 4 h, 8 h, 16 h, and 1–7 days on wheat treated with *Acmella oleracea n*-hexane and methanol extracts at two different concentrations.

	***n*-Hexane extract**		**Methanol extract**		***F***	***P***
	500 ppm	**1000 ppm**	**500 ppm**	**1000 ppm**		
Adults						
4 h	0.0 ± 0.0	0.0 ± 0.0^{B}	0.0 ± 0.0	0.0 ± 0.0^{B}	-	-
8 h	0.0 ± 0.0	0.0 ± 0.0^{B}	0.0 ± 0.0	0.0 ± 0.0^{B}	-	-
16 h	0.0 ± 0.0	2.2 ± 1.5^{AB}	0.0 ± 0.0	0.0 ± 0.0^{B}	2.3	0.10
1 day	1.1 ± 1.1^{ab}	4.4 ± 1.8^{ABa}	0.0 ± 0.0^{b}	0.0 ± 0.0^{Bb}	4.1	0.01
2 days	2.2 ± 1.5^{ab}	7.8 ± 3.6^{ABa}	0.0 ± 0.0^{b}	0.0 ± 0.0^{Bb}	3.7	0.02
3 days	2.2 ± 1.5	8.9 ± 4.6^{AB}	1.1 ± 1.1	2.2 ± 1.5^{AB}	1.3	0.29
4 days	4.4 ± 2.4	11.1 ± 4.6^{AB}	1.1 ± 1.1	4.4 ± 1.8^{AB}	1.7	0.18
5 days	4.4 ± 2.4	14.4 ± 6.7^{AB}	1.1 ± 1.1	5.6 ± 2.4^{AB}	1.8	0.17
6 days	5.6 ± 3.4^{ab}	18.9 ± 6.1^{Aa}	1.1 ± 1.1^{b}	1.1 ± 1.1^{b}	4.3	0.01
7 days	6.7 ± 3.3^{ab}	18.9 ± 6.1^{Aa}	1.1 ± 1.1^{b}	7.8 ± 2.8^{Aab}	4.0	0.02
F	1.7	3.9	0.6	4.5		
P	0.12	< 0.01	0.83	< 0.01		
Larvae						
4 h	0.0 ± 0.0^{E}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
8 h	0.0 ± 0.0^{E}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	2.2 ± 1.5^{D}	2.3	0.10
16 h	0.0 ± 0.0^{Eb}	3.3 ± 1.7^{Dab}	10.0 ± 6.7^{CDab}	11.1 ± 3.5^{Ca}	3.4	0.03
1 day	7.8 ± 1.5^{D}	11.1 ± 3.1^{C}	16.7 ± 7.1^{BC}	17.8 ± 3.2^{BC}	0.7	0.55
2 days	27.8 ± 3.2^{C}	33.3 ± 5.0^{B}	23.3 ± 6.2^{AB}	31.1 ± 3.5^{AB}	1.6	0.21
3 days	46.7 ± 3.7B^{Cab}	58.9 ± 6.1^{ABa}	32.2 ± 6.4^{ABc}	33.3 ± 3.3^{ABbc}	7.1	< 0.01
4 days	64.4 ± 4.1^{ABa}	70.0 ± 6.5^{ABa}	36.7 ± 7.6^{Ab}	40.0 ± 3.7^{ABb}	9.5	< 0.01
5 days	76.7 ± 5.3^{ABa}	77.8 ± 4.9^{Aa}	40.0 ± 7.6^{Ab}	45.6 ± 4.7^{Ab}	12.1	< 0.01
6 days	83.3 ± 4.4^{ABa}	84.4 ± 2.9^{Aa}	48.9 ± 7.5^{Ab}	66.7 ± 4.7^{Aa}	11.7	< 0.01
7 days	85.6 ± 3.8^{Aa}	88.9 ± 2.6^{Aa}	57.8 ± 8.3^{Ab}	80.0 ± 4.1^{Aa}	8.7	< 0.01
F	239.2	93.1	36.0	44.4		
P	< 0.01	< 0.01	< 0.01	< 0.01		

Within each row, means followed by the same lowercase letter are not significantly different (df = 3, 35; Tukey's HSD test at $P = 0.05$). Within each column, means followed by the same uppercase letter are not significantly different (df = 9, 89; Tukey's HSD test at $P = 0.05$). Where no letters, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Acarus siro

Concerning *A. siro* adults, the main effects were significant between exposure intervals, while only exposure and exposure x concentration were significant within exposure intervals (Table S3, Supplementary Material). After 1 day, no mortality was detected at all tested extracts and relative concentrations at 1000 ppm (Table 30). However, after 5 days both extracts led to a mortality higher than 50.0%. At the end of the experiments, 100.0% (for 1000 ppm of *n*-hexane extract) and 94.4% (for 1000 ppm of methanol extract) adult mortality was detected, while 500 ppm of the extracts provided moderate mortalities: 45.6 and 57.8% for the *n*-hexane and methanol extracts, respectively.

For *A. siro* nymphs, only the concentration resulted significant between exposure intervals (Table S3, Supplementary Material). Within exposure intervals, all main effects were important. No mortality was detected for 2 days for all tested extracts and relative concentrations (Table 30). Moderate mortality was detected at the end of the studies, not exceeding 50.0 and 68.9% for 1000 ppm of *n*-hexane and methanol extracts, respectively.

Table 30. Mean (%) mortality ± standard error (SE) of *Acarus siro* adults and nymphs after 4 h, 8 h, 16 h, and 1–7 days on wheat treated with *Acmella oleracea n*-hexane and methanol extracts at two different concentrations.

	***n*-Hexane extract**		**Methanol extract**		***F***	***P***
	500 ppm	**1000 ppm**	**500 ppm**	**1000 ppm**		
Adults						
4 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
8 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
16 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
1 day	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
2 days	2.2 ± 1.5^{BC}	3.3 ± 1.7^{D}	3.3 ± 1.7^{CD}	6.7 ± 1.7^{C}	1.4	0.26
3 days	4.4 ± 2.4^{BC}	13.3 ± 3.3^{C}	8.9 ± 2.6^{C}	12.2 ± 2.8^{BC}	2.5	0.07
4 days	7.8 ± 2.7^{Bb}	26.7 ± 2.9^{Ba}	16.7 ± 2.9^{Ba}	25.6 ± 6.5^{Ba}	8.4	< 0.01
5 days	27.8 ± 3.6^{Ab}	55.6 ± 5.0^{ABa}	45.6 ± 5.3^{Ab}	57.8 ± 7.4^{Aa}	5.9	< 0.01
6 days	35.6 ± 3.8^{Ac}	83.3 ± 2.9^{Aa}	52.2 ± 5.2^{Ab}	80.0 ± 4.4^{Aa}	21.2	< 0.01
7 days	45.6 ± 5.0^{Ab}	100.0 ± 0.0^{Aa}	57.8 ± 6.2^{Ab}	94.4 ± 2.9^{Aa}	19.6	< 0.01
F	42.7	106.4	69.7	114.7		
P	< 0.01	< 0.01	< 0.01	< 0.01		
Nymphs						
4 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
8 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
16 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
1 day	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
2 days	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
3 days	4.4 ± 1.8^{Bb}	13.3 ± 2.4^{Ba}	5.6 ± 2.4^{Cb}	13.3 ± 3.3^{Ca}	2.9	0.05
4 days	7.8 ± 1.5^{B}	20.0 ± 3.3^{B}	16.7 ± 3.7^{B}	30.0 ± 5.8^{BC}	2.6	0.07
5 days	15.6 ± 1.8^{Ac}	36.7 ± 4.1^{Aab}	24.4 ± 4.1^{ABbc}	48.9 ± 4.8^{ABa}	14.4	< 0.01
6 days	24.4 ± 2.9^{Ab}	47.8 ± 4.0^{Aa}	26.7 ± 4.4^{ABb}	57.8 ± 4.3^{Aa}	13.4	< 0.01
7 days	26.7 ± 2.4^{Ac}	50.0 ± 3.7^{Ab}	38.9 ± 2.6^{Ab}	68.9 ± 6.1^{Aa}	24.0	< 0.01
F	60.4	209.1	63.7	94.9		
P	< 0.01	< 0.01	< 0.01	< 0.01		

Within each row, means followed by the same lowercase letter are not significantly different (df = 3, 35; Tukey's HSD test at $P = 0.05$). Within each column, means followed by the same uppercase letter are not significantly different (df = 9, 89; Tukey's HSD test at $P = 0.05$). Where no letters, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

Albopictus diaperinus

Regarding *A. diaperinus* adults, between and within exposure intervals, none of the main effects or interactions were significant (Table S3, Supplementary Material). For the entire experimental period, no mortality was detected for the *n*-hexane extract at 500 and 1000 ppm and for the methanol extract at 500 ppm (Table 31). Methanol extract at 1000 ppm led only to 3.3% mortality 7 days post-exposure.

For *A. diaperinus* larvae, concentration was significant between exposure intervals, while within exposure intervals, all main effects and interactions were significant (Table S3, Supplementary Material). Moderate mortality was detected after 3 days of experiments, ranging from 46.7 to 71.1% for 500 ppm of methanol extract and 1000 ppm of *n*-hexane extract (Table 31). A mortality of 100% was detected for a concentration of 1000 ppm of *n*-hexane extract, 1000 ppm of methanol extract, and 500 ppm of *n*-hexane extract after 5, 6, and 7 days of exposure. The methanol extract tested at 500 ppm did not exceed 83.3% mortality at the end of the experiments.

Table 31. Mean (%) mortality ± standard error (SE) of *Alphitobius diaperinus* adults and larvae after 4 h, 8 h, 16 h, and 1–7 days on wheat treated with *Acmella oleracea n*-hexane and methanol extracts at two different concentrations.

	***n*-Hexane extract**		**Methanol extract**		***F***	***P***
	500 ppm	**1000 ppm**	**500 ppm**	**1000 ppm**		
Adults						
4 h	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-	-
8 h	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-	-
16 h	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-	-
1 day	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 1.1	1.0	0.41
2 days	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 1.1	1.0	0.41
3 days	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 1.1	1.0	0.41
4 days	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 1.1	1.0	0.41
5 days	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 1.1	1.0	0.41
6 days	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.2 ± 1.5	2.3	0.10
7 days	0.0 ± 0.0	1.1 ± 1.1	1.1 ± 1.1	3.3 ± 1.7	1.5	0.24
F	-	1.0	1.0	1.0		
P	-	0.45	0.45	0.46		
Larvae						
4 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{E}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
8 h	0.0 ± 0.0^{C}	0.0 ± 0.0^{E}	0.0 ± 0.0^{D}	0.0 ± 0.0^{D}	-	-
16 h	0.0 ± 0.0^{Cb}	5.6 ± 1.8^{Da}	0.0 ± 0.0^{Db}	2.2 ± 1.5^{CDab}	5.3	0.01
1 day	1.1 ± 1.1^{Cb}	16.7 ± 2.9^{Ca}	6.7 ± 1.7^{Cab}	6.7 ± 3.3^{Cab}	6.5	< 0.01
2 days	21.1 ± 3.9^{Bb}	44.4 ± 4.8^{Ba}	24.4 ± 2.4^{Bab}	36.7 ± 4.4^{Bab}	4.1	0.01
3 days	56.7 ± 4.7^{Aab}	71.1 ± 5.4^{ABa}	46.7 ± 2.9^{Ab}	65.6 ± 4.4^{ABa}	5.3	0.01
4 days	73.3 ± 5.0^{Ab}	92.2 ± 2.8^{ABa}	60.0 ± 3.3^{Ab}	91.1 ± 4.8^{Aa}	12.5	< 0.01
5 days	85.6 ± 4.1^{Aa}	100.0 ± 0.0^{Aa}	64.4 ± 4.1^{Ab}	98.9 ± 1.1^{Aa}	23.8	< 0.01
6 days	94.4 ± 2.4^{Aa}	100.0 ± 0.0^{Aa}	75.6 ± 3.4^{Ab}	100.0 ± 0.0^{Aa}	26.0	< 0.01
7 days	100.0 ± 0.0^{Aa}	100.0 ± 0.0^{Aa}	83.3 ± 5.0^{Ab}	100.0 ± 0.0^{Aa}	10.2	< 0.01
F	206.2	116.3	197.5	114.4		
P	< 0.01	< 0.01	< 0.01	< 0.01		

Within each row, means followed by the same lowercase letter are not significantly different (df = 3, 35; Tukey's HSD test at $P = 0.05$). Within each column, means followed by the same uppercase letter are not significantly different (df = 9, 89; Tukey's HSD test at $P = 0.05$). Where no letters, no significant differences were recorded. Where dashes exist, no statistical analysis was performed.

In the continuous research for sustainable control tools against arthropods infesting agricultural products, *A. oleracea* has been considered as a promising source of pesticides (Spinozzi et al., 2022). Concerning *A. oleracea* toxicity on arthropod pests, the results presented in our study point out the high efficacy of the *n*-hexane and methanol extracts against many of the tested pests at their different developmental stages. In almost all tested pests, the *n*-hexane extract was more toxic than the methanol extract. Recently, De Araújo et al. (2020) also outlined this trend testing different *A. oleracea* extracts (i.e., *n*-hexane, hydroethanolic, and methanolic) against *A. aegypti* larvae, proving the highest effectiveness of the *n*-hexane extract if compared to the others. Furthermore, the *n*-hexane extract killed more *T. absoluta* than the ethanol one (Moreno et al., 2012). The highest activity of the *n*-hexane extract could be ascribed to the highest spilanthol content (24.3 ± 1.3 g/100 g DE), if compared with the methanolic one (7.6 ± 0.3 g/100 g DE) (Table 23). This was also suggested by Marchesini et al. (2020) that found that the more spilanthol is contained in the extract, the higher the efficacy against *R. microplus* larvae is observed. The mechanism of action of this *N*-alkylamide has been summarized in Section 1.7.1, and its action on the above-mentioned pests could be ascribed to feeding toxicity and contact toxicity.

Regarding the insecticidal activity of *A. oleracea*-derived products against different targets, this has already been reported in Section 1.8, and this study offers novel results on the potential employment of *A. oleracea* extracts for the management of arthropod pests. Indeed, a different susceptibility among the tested pests at their different developmental stages was detected. For instance, 500 ppm of the *n*-hexane extract led to 100.0% mortality of *A. diaperinus* larvae while 0.0% adults after 7 days of experiments. This phenomenon has been previously detected in several insects and is often due to the structure of the cuticula, setae, and behaviour of each stage (Andersen, 2010).

T. granarium larvae (that have many large setae) were more tolerant than *T. confusum*, *T. castaneum*, *A. diaperinus*, *O. surinamensis*, and *T. molitor* larvae (that have few small setae). This could be ascribed to setae, that could act as protective barrier between the body and the treated surface (Athanassiou et al., 2006; Carlson and Ball, 1962; Hadaway, 1959; Peterson, 1948, 1951; Rees, 2004). One of the most important findings of the present study were the high mortality level of both *A. oleracea* extracts against *T. molitor* larvae, which reached 100.0 and 97.8% mortality after 7-day exposure to the *n*-hexane and methanol extracts, respectively. However, only the methanol extract was highly effective on the adults of the same species, and it can be assumed that this stage is tolerant to other constituents of *A. oleracea n*-hexane extract since adults represent a very susceptible stage to numerous synthetic and natural insecticides (Kavallieratos et al., 2019, 2021a, 2022c). Adults of *A. diaperinus* have shown various susceptibility/tolerance levels. For instance, they did not exceed 23.3, 25.6, and 31.1% mortality when they were exposed to pirimiphos-methyl, *C. acaulis* EO, and deltamethrin, respectively (Kavallieratos et al., 2022b,d).

These data are linear with the results obtained in the presented work since the tested extracts barely led to mortality to this stage.

Regarding *C. ferrugineus*, it has been reported that adults were more susceptible than larvae to *Citrus hystrix* DC., *Euodia suaveolens* (Hochr.) Bakh. f., *Cinnamomum verum* J.Presl, *Syzygium aromaticum* (L.) Merr. & L.M.Perry, and *Cymbopogon nardus* (L.) Rendle EOs in fumigant bioassays (Ikawati et al., 2020). However, in the presented work, both stages of *C. ferrugineus* were highly susceptible, even if larvae needed more time of exposure for complete mortality. *O. surinamensis* and *T. granarium* stages evaluated in this study followed the general trends already reported. In fact, both stages of *O. surinamensis* were susceptible to several green insecticides like the *C. acaulis* and *M. longifolia* EOs (Kavallieratos et al., 2022c, 2022d), while larvae of *T. granarium* resulted more tolerant than the adults (Ali et al., 2022; Kavallieratos et al., 2017c, Kousar et al., 2021; Saad et al., 2022). *A. siro* life stages susceptibility did not follow a trend since nymphs showed higher tolerance than adults when treated with the *A. oleracea* extracts. However, previous work reported high susceptibility of both stages when exposed to *C. acaulis* EO (Kavallieratos et al., 2022c), or when treated with three Apiaceae EO-based NEs (Kavallieratos et al., 2022d).

Regarding the evaluation of *A. oleracea*-derived products as pesticides, it is crucial to analyze their toxicity to non-target. In this context, it has been reported that the extracts are non-toxic towards non-target organisms, as *Chlorella vulgaris* Beijerinck (Chlorellales: Chlorellaceae) (De Araújo et al., 2020), or have low risk towards *Tetragonisca angustula* (Latr.) (Hymenoptera: Apidae) and *Solenopsis saevissima* (Smith) (Hymenoptera: Formicidae) (Moreno et al., 2012).

5.4. Conclusions

This work focused on optimizing the extraction procedure for spilanthol and other *N*-alkylamides from *A. oleracea* and Soxhlet resulted to be the most efficient technique. Methanol was the best solvent for the highest recovery of spilanthol from the plant, while *n*-hexane was the solvent leading to the extract most concentrated of this compound. So, the two selected extracts were tested on several stored-product pests resulting effective against *C. ferrugineus* adults and larvae, A. *diaperinus* larvae, *T. granarium* adults, *O. surinamesis* adults and larvae, *A. siro* adults, *T. confusum*, *T. molitor*, and *T. castaneum* larvae. In general, the *n*-hexane extract resulted more active than the methanol one, and this was probably due to the higher content of spilanthol. In contrast, the extracts were not efficient against *A. diaperinus*, *T. confusum*, and *T. castaneum* adults. Further research on these extracts, their fractions, and spilanthol, as well as on their modes of action and the effectiveness of formulations, are still needed to evaluate their pesticidal potential when applied to additional durable food commodities. In conclusion, the main findings of this work open new perspectives for the employment of *A. oleracea* in the agrochemical field.

Conclusions and future perspectives on *Carlina acaulis* and *Acmella oleracea*

The studies presented in the previous Chapters enclose all the scientific investigations carried out on *C. acaulis* and *A. oleracea* at the University of Camerino, c/o Chemistry Interdisciplinary Project (ChIP) research Center, in collaboration with several research groups from the University of Pisa, Agricultural University of Athens, Crop Research Institute of Prague, and Research Centre for Plant Protection and Certification of Bagheria, Italy. These two plants have in common not only the family (Asteraceae), but also the large employment as traditional remedies and foods. The research performed demonstrated their high value in terms of biological activities, with a special focus on the pesticidal action. Regarding *C. acaulis*-derived products, a strong activity on the vector *Cx. quinquefasciatus* was detected but also on agricultural pests as *L. botrana*, *B. oleae*, *C. capitata*, *M. incognita*, *X. compactus*, and *T. urticae* and on stored-product pests as *A. siro*, *A. diaperinus*, *O. surinamensis*, *P. truncatus*, *R. dominica*, *S. oryzae*, *T. confusum*, *T. castaneum*, *T. molitor*, and *T. granarium*. The bioactivity of the *C. acaulis* products tested is associated to the presence of the polyacetylene carlina oxide, which was isolated and tested as well, showing a marked pesticidal action. These studies also pointed out a moderate non-target toxicity on *N. californicus*, but also a mild toxicity on human cells and rats. In addition, several carlina oxide analogues were synthetized to overcome the plant availability issues and to produce compounds more active than carlina oxide itself. Among the analogues synthetized, the *m*-chloro substituted resulted more active on *Cx. quinquefasciatus* and less toxic to human cells than its precursor.

The studies on *A. oleracea*-derived products (EO, EO-NE, and extracts) revealed a great potential against *Cx. quinquefasciatus* and several stored-product pests as *O. surinamensis*, *T. granarium*, *T. castaneum*, *T. confusum*, *T. molitor*, *A. diaperinus*, *A. siro*, and *C. ferrugineus*. In this case, spilanthol, the main *N*-alkylamide found in the plant, is responsible for this activity. In addition, an optimization study for spilanthol extraction from the plant was assessed and Soxhlet extraction with methanol resulted the best extractive procedure. Moreover, it was also demonstrated the possibility to encapsulate these plants' products into NEs or MEs, often enhancing their bioactivity and lowering their toxicity. Furthermore, MAE technique was tested on both plants, resulting more efficient in terms of time of extraction for *C. acaulis* EO, and EO yield and spilanthol content for *A. oleracea* EO. Future studies on *C. acaulis* will focus on the evaluation of a possible scale-up of the synthesis of carlina oxide analogues and set up of a plant cultivation system. Regarding *A. oleracea*, novel studies will aim to test the insecticidal activities of *n*-hexane extract and spilanthol NEs on different targets. Moreover, further non-target toxicity studies will be performed to assess the safety of *A. oleracea*-derived products as botanical pesticides. Overall, from the reported studies, it can be concluded that *C. acaulis* and *A. oleracea* are promising candidates for the development of novel, safe, and effective botanical pesticides.

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Supplementary material

S1. Microwave-assisted extraction of *Carlina acaulis* essential oil (EO): a Fractional Factorial Design (FFD) optimization study

S1.1. Preliminary screening: EO yield

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	0,663500	0,110583	6,53	0,007
Linear	6	0,663500	0,110583	6,53	0,007
MP (w/g)	1	0,000225	0,000225	0,01	0,911
ET (min)	1	0,562500	0,562500	33,20	0,000
W (%)	1	0,022500	0,022500	1,33	0,279
Moistening	1	0,021025	0,021025	1,24	0,294
Milling	1	0,002025	0,002025	0,12	0,737
Cycles	1	0,055225	0,055225	3,26	0,104
Error	9	0,152475	0,016942		
Total	15	0,815975			

Model Summary

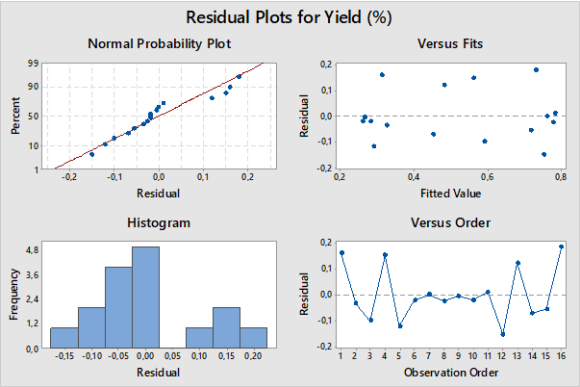
S	R-sq	R-sq(adj)	R-sq(pred)
0,130160	81,31%	68,86%	40,94%

Coded Coefficients

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		0,5212	0,0325	16,02	0,000	
MP (w/g)	-0,0075	-0,0037	0,0325	-0,12	0,911	1,00
ET (min)	0,3750	0,1875	0,0325	5,76	0,000	1,00
W (%)	0,0750	0,0375	0,0325	1,15	0,279	1,00
Moistening	0,0725	0,0362	0,0325	1,11	0,294	1,00
Milling	0,0225	0,0112	0,0325	0,35	0,737	1,00
Cycles	-0,1175	-0,0587	0,0325	-1,81	0,104	1,00

Regression Equation in Uncoded Units

Yield (%) = -0,208 - 0,017 MP (w/g) + 0,003125 ET (min) + 0,00375 W (%) + 0,0362 Moistening + 0,0112 Milling - 0,0587 Cycles



S1.2. Preliminary screening: Concentration of carlina oxide

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	31191321689	5198553615	1,31	0,344
Linear	6	31191321689	5198553615	1,31	0,344
MP (w/g)	1	11376445728	11376445728	2,86	0,125
ET (min)	1	5915077728	5915077728	1,49	0,254
W (%)	1	1582289881	1582289881	0,40	0,544
Moistening	1	3685859216	3685859216	0,93	0,361
Milling	1	5725177470	5725177470	1,44	0,261
Cycles	1	2906471666	2906471666	0,73	0,415
Error	9	35786679737	3976297749		
Total	15	66978001426			

Model Summary

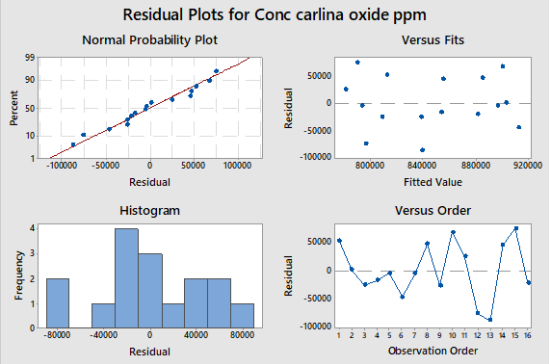
S	R-sq	R-sq(adj)	R-sq(pred)
63057,9	46,57%	10,95%	0,00%

Coded Coefficients

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		846621	15764	53,70	0,000	
MP (w/g)	53330	26665	15764	1,69	0,125	1,00
ET (min)	-38455	-19227	15764	-1,22	0,254	1,00
W (%)	19889	9945	15764	0,63	0,544	1,00
Moistening	-30356	-15178	15764	-0,96	0,361	1,00
Milling	37832	18916	15764	1,20	0,261	1,00
Cycles	26956	13478	15764	0,85	0,415	1,00

Regression Equation in Uncoded Units

Conc carlina oxide ppm = 674929 + 118512 MP (w/g) - 320 ET (min) + 994 W (%) - 15178 Moistening + 18916 Milling + 13478 Cycles



S1.3. Effect of the extraction time

Table S1. Parameters and results of extraction time study. Abbreviation for coded variables: MP (microwave power); ET (extraction time).

MP (W)	ET (min)	Water (g)	Root (g)	Mi	Mo	Cycles	EO Yield (%)	carlina oxide (ppm)
1000	90	850	150	NA	NA	NA	0.26	1.18E+06
1000	90	850	150	NA	NA	NA	0.24	9.32E+05
1000	210	850	150	NA	NA	NA	0.55	1.10E+06
1000	210	850	150	NA	NA	NA	0.74	8.88E+05
1000	270	850	150	NA	NA	NA	0.64	1.11E+06
1000	270	850	150	NA	NA	NA	0.73	1.01E+06
1000	330	850	150	NA	NA	NA	0.72	1.15E+06
1000	330	850	150	NA	NA	NA	0.73	1.00E+06

S1.4. Evaluation of the intrinsic variability of carlina oxide concentration

To test if the data distributions of the carlina oxide in the FFD runs were due to due to the intrinsic variability of the samples or depended on other factors, runs reported in Table S2 were compared with those obtained during FFD runs. The runs in Table S1 were all repetition of the same experiment (with the exception of the ET that it is not statistically significant), and they were all carried out in a small temporal window (4 days), so they represent a reliable measurement of the intrinsic variabilities.

S2. Spilanthol-rich essential oil (EO) from *Acmella oleracea*: insecticidal, cytotoxic and anti-inflammatory activities evaluation

Table S2. List of primers used for real-time PCR.

Gene	Primer
GAPDH forward	5'ACCACAGTCCATGCCATCAC3'
GAPDH reverse	5'TCCACCACCCTGTTGCTGTA3'
IL-1β forward	5'GTTCCCATTAGACAACTGCACTACAG3'
IL-1β reverse	5'GTCGTTGCTTGGTTCTCCTTGTA3'
TNFα forward	5'CCCCAAAGGGATGAGAAGTTC3'
TNFα reverse	5'CCTCCACTTGGTGGTTTGCT3'
iNOS forward	5'CCTCCTCCACCCTACCAAGT3'
iNOS reverse	5'CACCCAAAGTGCTTCAGTCA3'
COX2 forward	5'TGGGGTGATGAGCAACTATT3'
COX2 reverse	5'AAGGAGCTCTGGGTCAAACT3'

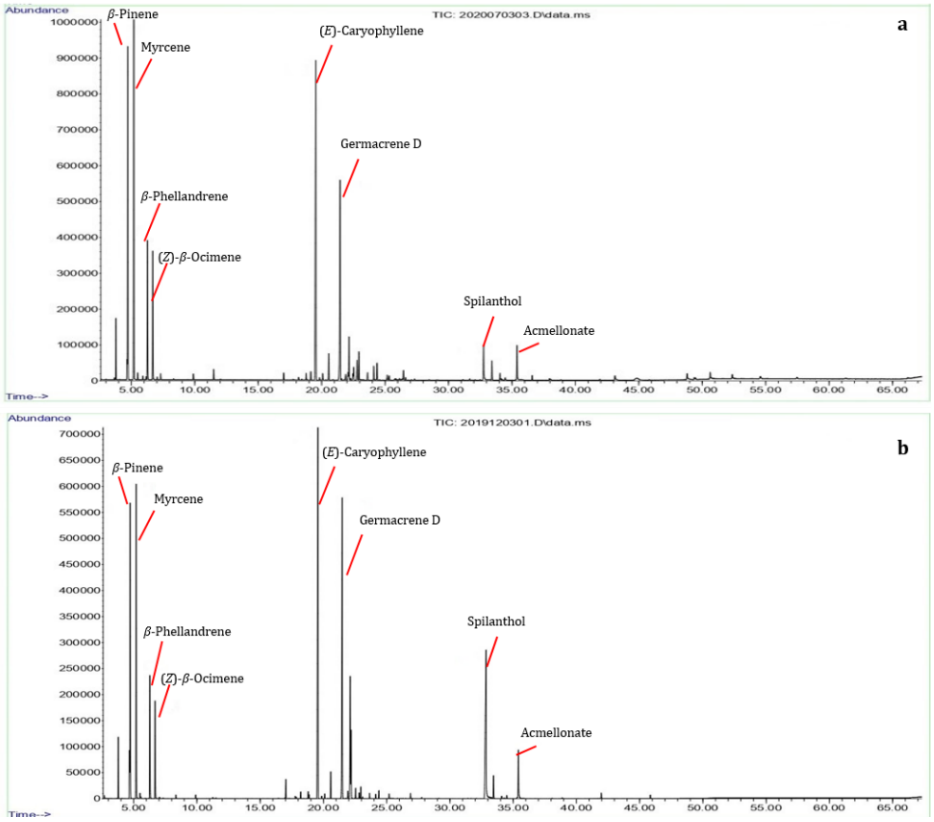


Figure S2. GC-MS chromatograms of *Acmella oleracea* essential oil obtained by hydrodistillation (HD, **a**) and microwave-assisted extraction (MAE, b).

S3. *Acmella oleracea* extracts' insecticidal activity against noxious arthropods attacking stored products

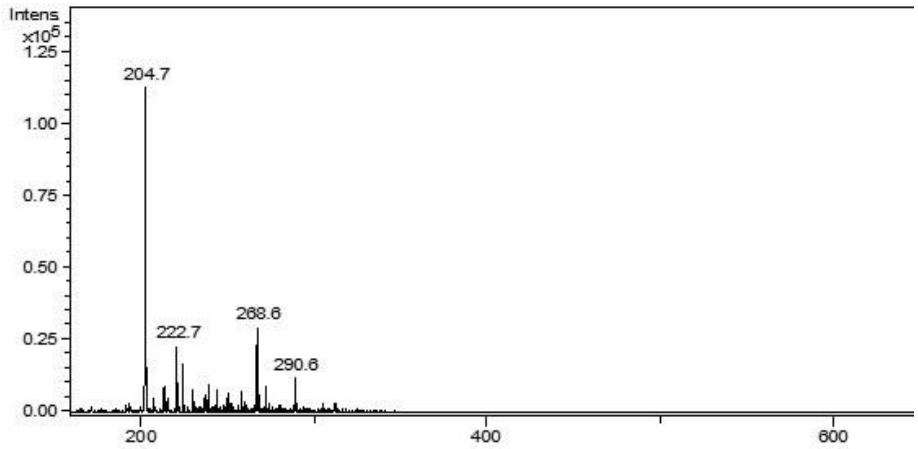


Figure S3.1. (*2Z*)-*N*-isobutyl-2-nonene-6,8-diynamide (A1) MS spectrum.

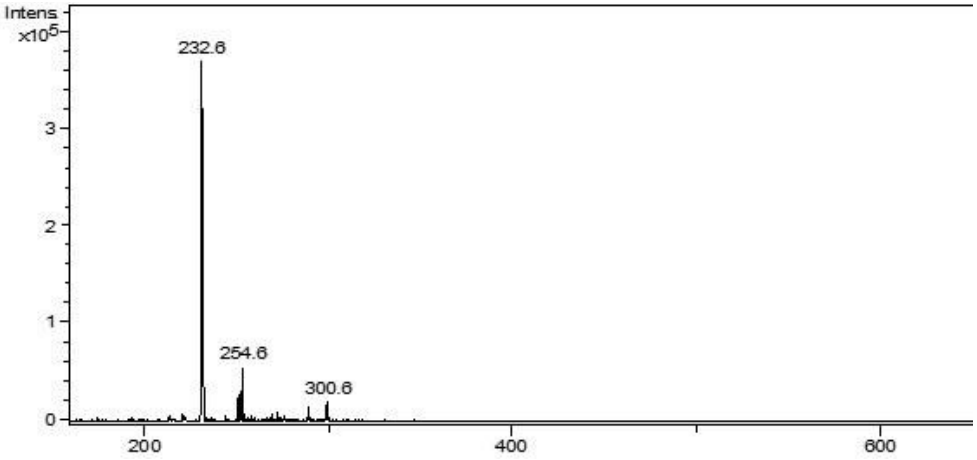


Figure S3.2. (*2E*)-*N*-isobutyl-2-undecene-8,10-diynamide (A2) MS spectrum.

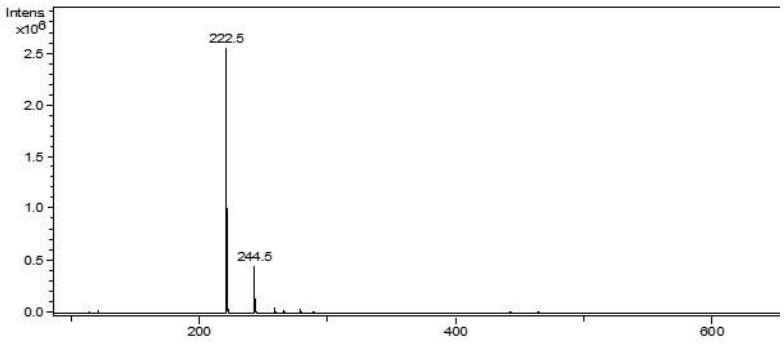


Figure S3.3. (*2E,6Z,8E*)-*N*-isobutyl-2,6,8-decatrienamide or spilanthol (A3) MS spectrum.

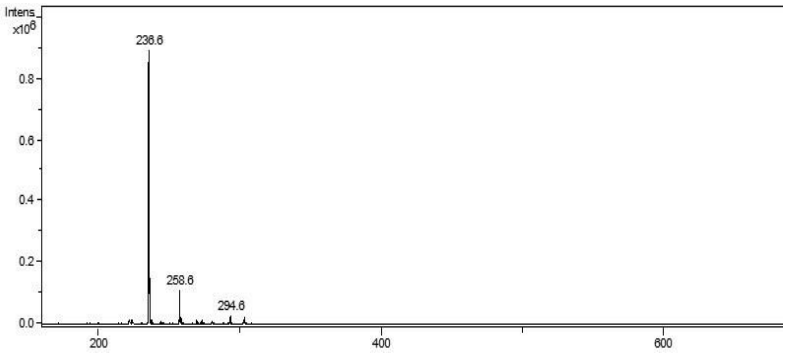


Figure S3.4. (*2E,6Z,8E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide (A6) MS spectrum.

Table S3. MANOVA parameters for the main effects and associated interactions leading to the observed mortality rates of adults and larvae of *Cryptolestes ferrugineus*, *Tenebrio molitor*, *Oryzaephilus surinamensis*, *Trogoderma granarium*, *Tribolium castaneum*, *Tribolium confusum*, and *Alphitobius diaperinus*, and *Acarus siro* adults and nymphs between and within exposure intervals (error df = 32).

		Between exposures				**Within exposures**			
		Intercept	**Extract**	**Concentration**	**Extract x concentration**	**Exposure**	**Exposure x extract**	**Exposure x concentration**	**Exposure x extract x concentration**
	df	1	1	1	1	9	9	9	9
Cryptolestes ferrugineus adults	*F*	7822.8	66.6	8.6	0.4	191.1	19.4	1.5	2.3
	P	< 0.01	< 0.01	0.01	0.52	< 0.01	< 0.01	0.22	0.05
Cryptolestes ferrugineus larvae	*F*	5742.5	175.5	19.1	5.4	11971.0	26.0	9.0	8.4
	P	< 0.01	< 0.01	< 0.01	0.03	< 0.01	< 0.01	< 0.01	< 0.01
Tenebrio molitor adults	*F*	472.8	108.6	4.2	1.3	51.7	12.2	0.7	0.5
	P	< 0.01	< 0.01	0.05	0.26	< 0.01	< 0.01	0.69	0.88
Tenebrio molitor larvae	*F*	5399.2	19.5	164.6	0.2	7192.1	14.1	26.6	18.7
	P	< 0.01	< 0.01	< 0.01	0.67	< 0.01	< 0.01	< 0.01	< 0.01
Oryzaephilus surinamensis adults	*F*	4691.9	15.0	47.5	6.2	2364.6	5.5	9.2	6.1
	P	< 0.01	< 0.01	< 0.01	0.02	< 0.01	< 0.01	< 0.01	< 0.01
Oryzaephilus surinamensis larvae	*F*	4752.2	71.5	39.5	< 0.1	4458.2	10.0	6.7	1.1
	P	< 0.01	< 0.01	< 0.01	0.87	< 0.01	< 0.01	< 0.01	0.37
Trogoderma granarium adults	*F*	3597.6	11.3	29.6	7.3	5082.8	11.0	8.6	6.9
	P	< 0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	< 0.01
Trogoderma granarium larvae	*F*	444.1	6.4	18.2	1.6	51.1	4.6	2.2	1.8
	P	< 0.01	0.02	< 0.01	0.21	< 0.01	< 0.01	0.06	0.13
Tribolium castaneum adults	*F*	89.3	0.6	5.9	0.1	10.7	2.6	0.6	1.3
	P	< 0.01	0.47	0.02	0.74	< 0.01	0.03	0.82	0.30
Tribolium castaneum larvae	*F*	1481.3	60.0	55.6	2.5	1278.2	5.7	6.5	3.2
	P	< 0.01	< 0.01	< 0.01	0.12	< 0.01	< 0.01	0.01	0.01
Tribolium confusum adults	*F*	25.2	4.6	5.5	0.3	3.3	1.4	1.6	0.6
	P	< 0.01	0.05	0.03	0.58	0.01	0.26	0.17	0.81
Tribolium confusum larvae	*F*	2020.9	0.5	4.2	0.7	1340.1	11.4	2.0	2.2
	P	< 0.01	0.51	0.05	0.42	< 0.01	< 0.01	0.09	0.06
Acarus siro adults	*F*	1156.8	4.1	18.2	1.9	884.4	0.6	7.9	1.1
	P	< 0.01	0.05	< 0.01	0.17	< 0.01	0.76	< 0.01	0.37
Acarus siro nymphs	*F*	1283.1	1.8	24.2	0.26	1013.1	3.7	7.8	0.4
	P	< 0.01	0.19	< 0.01	0.61	< 0.01	0.01	< 0.01	0.94
Alphitobius diaperinus adult	*F*	2.9	1.9	1.9	1.3	0.5	0.2	0.2	0.2
	P	0.10	0.17	0.17	0.27	0.86	0.99	0.99	0.99
Alphitobius diaperinus larvae	*F*	4555.2	1.4	21.7	3.9	11241.0	2.7	7.9	5.4
	P	< 0.01	0.25	< 0.01	0.06	< 0.01	0.03	< 0.01	< 0.01

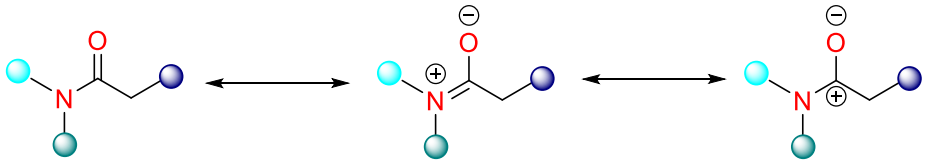
CHAPTER III: Research abroad

The research abroad was carried out at the Laboratory of Organic Chemistry of Prof. Nuno Maulide at the University of Vienna for a period of four months.

1. Introduction

1.1. Amide activation

Between carbonyl compounds, amides are usually considered to be unreactive, especially if compared to acyl halides, anhydrides, ketones, and esters. This is linked to the amidic resonance, namely the delocalization of the lone pair of electrons at nitrogen on the π* system (Bennet et al., 1990; Greenberg et al., 2003; Slebocka-Tilk and Brown, 1987), which is responsible for the enhanced stabilization of the carbonyl carbon (Scheme 1) (Pauling, 1960).

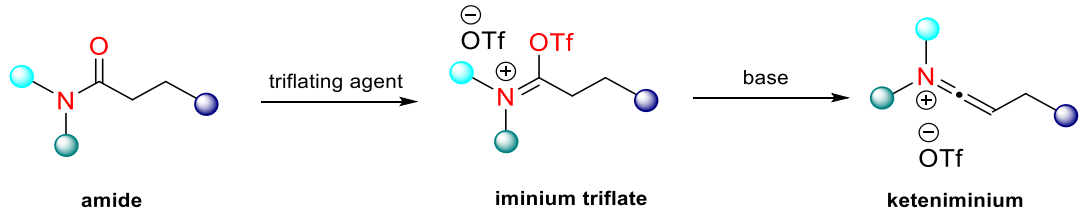


Scheme 1. Amidic resonance structures.

Even if there is the general consideration of the amide moiety as a not exceptionally useful functional group, its stability has been demonstrated as a valuable feature to be employed in several synthetic routes (Ruider and Maulide, 2015; Pace et al., 2014). Moreover, amides are highly occurring moieties in biologically active compounds, such as pharmaceutical molecules and natural products. For this reason, the activation and functionalization of amides have been deeply studied and improved in the last 140 years. Therefore, several methods of amide activation are reported in the literature, such as the Vilsmeier−Haack and Bischler−Napieralski reactions (Bischler and Napieralski, 1893; Vilsmeier and Haack, 1927), the Hofmann rearrangement (Hofmann, 1881), activation with strong electrophiles (Speziale, 1963), or transition-metal-catalyzed insertion to the C-N bond (Hie et al., 2015).

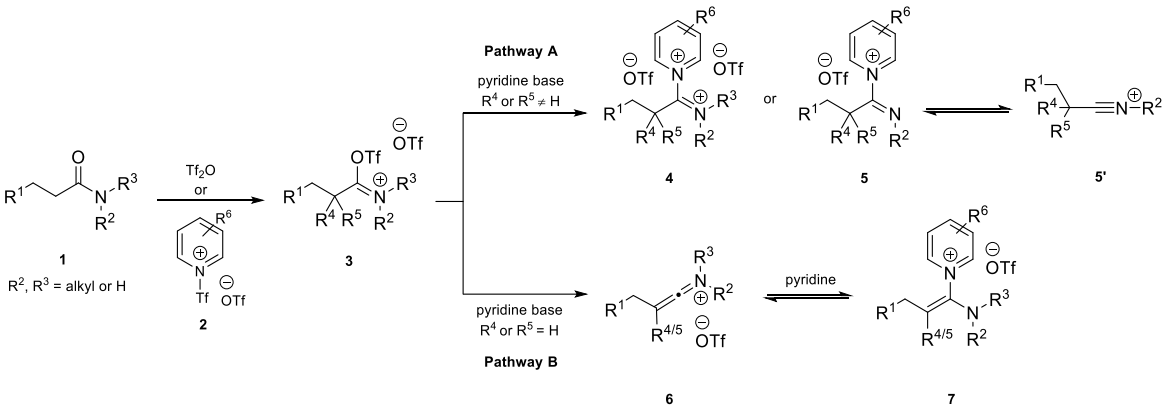
However, between these numerous procedures, amide activation through trifluoromethanesulfonic anhydride (triflic anhydride) treatment led to the development of several new synthetic routes. The reaction of amides with triflic anhydride leads to their *in situ* conversion into *O*-trifluoromethanesulfonyloxyiminium trifluoromethanesulfonates (iminium triflates), which

represent more electrophilic amides and that can be used for the formation of versatile keteniminium ions (Kaiser and Maulide, 2016) (Scheme 2). This progressively enhanced electrophilicity is the starting point of several versatile reactions.



Scheme 2. Enhancement of reactivity in amide activation.

Numerous spectroscopic studies have been conducted for the complete investigation of the reaction mechanism (Charette and Grenon, 2001; White et al., 2015) (Scheme 3). Firstly, the formation of the iminium triflate **3** occurs often through the presence of a triflating agent, the *N*-trifluoromethylsulfonyl)pyridinium triflate **2**, and then two different reaction pathways take place, according to the nature of the α-substituents and to the degree of substitution of the amide nitrogen (pathways A and B, Scheme 3).



Scheme 3. Amide activation with triflic anhydride and a pyridine base (Kaiser and Maulide, 2016).

The lack of protons in the α-position causes the nucleophilic substitution of the triflate by the pyridine base, leading to the formation of the pyridinium intermediates **4** or **5**, which is in equilibrium with the nitrilium ion **5′** (Scheme 3, Pathway A). On the other hand, the presence of protons in the α-position leads to deprotonation of the α-carbon, with the formation of a keteniminium triflate **6**, which can combine with pyridine base to give the keteneaminal intermediate **7** (Scheme 3, Pathway B) (Kaiser and Maulide, 2016). Differently from amide

activation with triflic anhydride that proceeds with or without the pyridine derivative, the equilibrium between the keteniminium and pyridinium strongly depends on the properties of the base. Usually, the addition of unsubstituted pyridine is an irreversible process with strong nucleophiles, while the addition of sterically hindered pyridines or pyridines bearing an electron-withdrawing substituent in position 2 activates the equilibrium and leads to the permanent presence of the keteniminium triflate (Movassaghi and Hill, 2006; Peng et al., 2014).

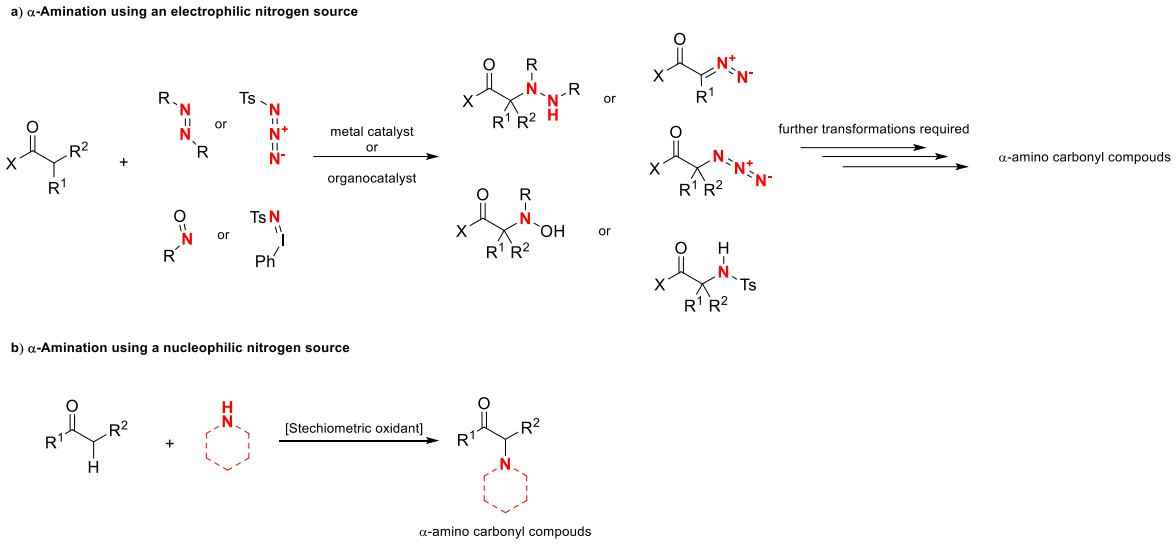
The strong adaptability and selectivity of triflic anhydride for the activation of carboxamides represents one of the guiding themes of the Maulide research group, which led throughout the years to the use of amides as precursors to produce useful intermediates, demonstrating the wide range of molecules that can be pursued by this kind chemistry.

1.2. α-Amination of carbonyl compounds: a focus on amides

Since α-amino acids are frequent motives for many natural products of medical, agricultural, and food interest, the synthesis of α-amino carbonyl compounds has gained great interest in synthetic chemistry research (Albericio and Kruger, 2012; Newman and Cragg, 2016; Stevenazzi et al., 2014).

Direct α-amination represents the most convenient strategy for the synthesis of α-amino carbonyl compounds in terms of flexibility and versatility. However, some classical methods do not lead directly to α-aminated carbonyls, but to α-hydrazinyl or α-oxy-amino compounds that must be submitted to more chemical transformations for the achievement of the α-aminated products (de la Torre et al., 2017; Tona et al., 2016). Between these methods, α-amination with electrophilic nitrogen sources is surely the most frequent (Scheme 4a) (Fu et al., 2013; Shevchenko et al., 2015; Tona et al., 2016; Vilaivan and Bhanthumnavin, 2010; Yang and Toste, 2015; Zhou et al., 2014).

A different approach involves the coupling of an amine with a carbonyl compound using oxidative conditions (Scheme 4b). In this case, α-amino carbonyl compounds can be directly achieved, but this method is applicable only to secondary amines (Jia et al., 2014; Jiang et al., 2014; Tian et al., 2012).



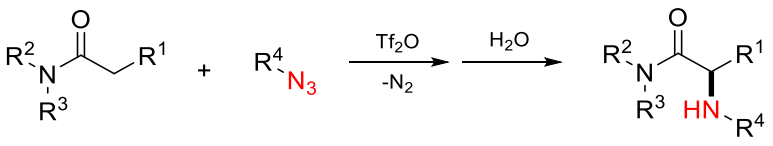
Scheme 4. Classical methods for α-amination of carbonyl compounds (de la Torre et al., 2017; Tona et al., 2016).

However, the application of direct α-amination to amides still represents a great challenge in organic synthesis.

For instance, Harschneck et al. (2012) developed an oxidative approach for the azidation of β-keto amides using NaN_3 as an azide source (Harschneck et al., 2012). However, this process does not lead directly to α-amino compounds, which can be obtained only with further transformations.

Recently, Bell-Tyrer et al. developed a novel procedure for the synthesis of α-amino amides through (3+2) vinyl azide-enolate cycloaddition leading to the formation of imine derivatives that give the corresponding amines after reduction or hydrolysis (Bell-Tyrer et al., 2022).

Maulide group also developed a metal-free method for the direct α-amination of amides using azides through electrophilic amide activation. This chemo- and stereoselective process takes place under mild conditions and leads to the release of nitrogen gas (Scheme 5) (de la Torre et al., 2017).



Scheme 5. Direct α-amination of amides developed by Maulide group.

This procedure showed large functional group tolerance and an important chemoselectivity for the amidic moiety in the presence of an alkyl nitrile, a ketone, an ester, or halides. Moreover, the reaction was also possible on cyclic and acyclic amides, demonstrating its high versatility and applicability to different substrates (de la Torre et al., 2017).

2. Materials and methods

2.1. Preparation of amides

General procedure A

To a solution of the amine (1.00 equiv.) and triethylamine (2.00 equiv.) in DCM (0.1 M) at 0 °C, the corresponding acyl chloride (1.20 equiv.) was added dropwise, and the reaction mixture was then left stirring 16 h at 25 °C. Then, $NaHCO_3$ aq. was added, and after extraction of the aqueous phase with DCM, the organic phases were combined and dried over Na_2SO_4. The crude product was purified by flash column chromatography on silica gel (ethyl acetate/heptane) to afford the desired compound.

General procedure B

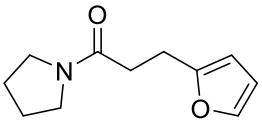
To a solution of the carboxylic acid (1.00 equiv.), triethylamine (1.00 equiv.), 4-dimethylaminopyridine (DMAP, 10 mol%) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl, 1.50 equiv.) in DCM (0.1 M), the corresponding amine (1.20 equiv.) was added, and the solution was stirred at 25 °C dor 16 h. Then, the organic solution was extracted sequentially with HCl aq. 0.5 M, $NaHCO_3$ aq. and NaCl aq. The solution was then dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting crude material was purified by flash column chromatography on silica gel (ethyl acetate/heptane) to afford the desired compound.

General procedure C

To a solution of the amide (1.00 equiv.) and $Pd(OAc)_2$ (0.100 equiv.) in DMSO/water (0.330 M, 10/1, v/v) under oxygen atmosphere was added TFA (1.00 equiv.). The mixture was heated to 70 °C and stirred for 16 h. After letting it cool to 25 °C, it was diluted with water and then extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified through flash column chromatography on silica gel (ethyl acetate/heptane) to afford the desired compound.

Characterization of the prepared amides

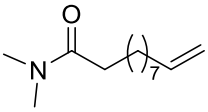
ELSP134, 3-(furan-2-yl)-1-(pyrrolidin-1-yl)propan-1-one



Prepared according to the General Procedure B in 56% yield (391 mg, 2.02 mmol) as a white solid.

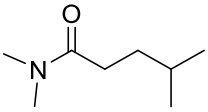
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.28 (m, 1H), 6.29 – 6.25 (m, 1H), 6.04 – 6.00 (m, 1H), 3.46 (t, J = 6.8 Hz, 2H), 3.35 (t, J = 6.7 Hz, 2H), 3.04 – 2.95 (m, 2H), 2.59 (t, J = 8.8, 6.8 Hz, 2H), 1.88 (dp, J = 35.4, 6.6 Hz, 4H). All analytical data were in linear with data reported in the literature (WO2008011478, 2018).

ELSP145, *N*,*N*-dimethylundec-10-enamide



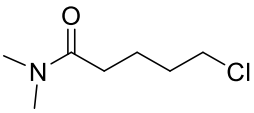
Prepared according to the General Procedure A in 79% yield (748 mg, 3.54 mmol) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (ddt, J = 16.9, 10.2, 6.7 Hz, 1H), 5.03 – 4.88 (m, 2H), 3.00 (s, 3H), 2.94 (s, 3H), 2.33 – 2.26 (m, 2H), 2.03 (q, J = 6.9 Hz, 2H), 1.67 – 1.57 (m, 2H), 1.41 – 1.24 (m, 10H). All analytical data were in linear with data reported in the literature (Porte et al., 2020).

ELSP146, *N*,*N*,4-trimethylpentanamide



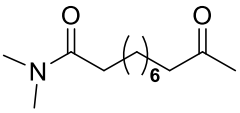
Prepared according to the General Procedure A in 81% yield (524 mg, 3.66 mmol) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.00 (s, 3H), 2.93 (s, 3H), 2.33 – 2.26 (m, 2H), 1.63 – 1.48 (m, 3H), 0.90 (dd, J = 6.4, 0.7 Hz, 6H). All analytical data were in linear with data reported in the literature (Li et al., 2020).

ELSP150, 5-chloro-*N*,*N*-dimethylpentanamide



Prepared according to the General Procedure A in 72% yield (588 mg, 3.59 mmol) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.56 (t, J = 6.3 Hz, 2H), 3.00 (s, 3H), 2.94 (s, 3H), 2.34 (t, J = 7.0 Hz, 2H), 1.89 – 1.74 (m, 4H). All analytical data were in linear with data reported in the literature (Buswell et al., 2004).

ELSP152, *N*,*N*-dimethyl-10-oxoundecanamide

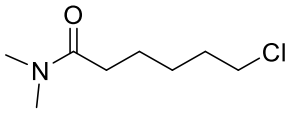


Prepared according to the General Procedure C in 56% yield (543 mg, 2.39 mmol) as a colourless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.99 (s, 3H), 2.93 (s, 3H), 2.40 (t, *J* = 7.4 Hz, 2H), 2.31 – 2.25 (m, 2H), 2.12 (s, 3H), 1.65 – 1.50 (m, 4H), 1.35 – 1.23 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.5, 173.3, 43.9, 37.4, 35.45, 33.5, 30.0, 29.56, 29.34, 29.34, 29.2, 25.23, 23.9.

IR (neat) νmax (cm^{-1}): 2928, 2853, 1714, 1648, 1501, 1494, 1465, 1412, 1396, 1356, 1161. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{13}H_{25}NNaO_2^+$) requires 250.1778 *m/z*, found 250.1778 *m/z* **.**

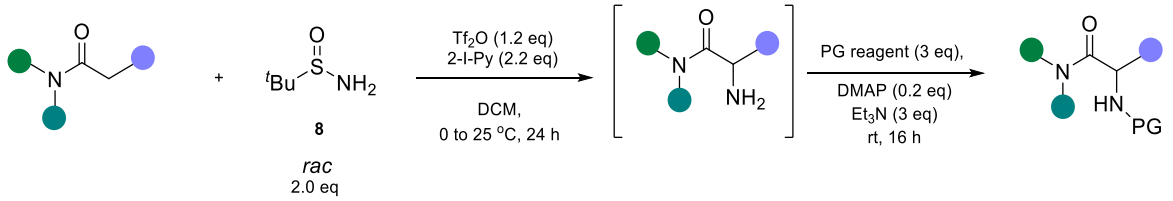
ELSP164, 6-chloro-*N*,*N*-dimethylhexanamide



Prepared according to the General Procedure B in 91% yield (647 mg, 3.64 mmol) as a colourless oil. ^{1}H NMR δ 3.53 (t, J = 6.7 Hz, 2H), 2.99 (s, 3H), 2.93 (s, 3H), 2.31 (t, J = 7.5 Hz, 2H), 1.86 – 1.73 (m, 2H), 1.71 – 1.60 (m, 2H), 1.54 – 1.42 (m, 2H). All analytical data were in linear with data reported in the literature (Porte et al., 2020).

2.2. Protected α-aminated amides synthesis

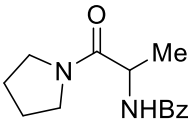
General procedure



To a mixture of amide (0.200 mmol, 1.00 equiv.), 2-iodopyridine (46.8 µL, 0.440 mmol, 2.20 equiv.) in DCM (1.00 mL) in a flame-dry Schlenk tube was added trifluoromethanesulfonic anhydride (40.7 µL, 0.240 mmol, 1.20 equiv.) dropwise under Ar at 0 °C. After stirring for 15 min at 0 °C, a solution of *tert*-butylsulfinamide (48.5 mg, 0.400 mmol, 2.00 equiv.) in DCM (1.0 mL) was added and the reaction stirred at 25 °C. After that time, triethylamine (84.0 µL, 0.600 mmol, 3.00 equiv.), DMAP (4.85 mg, 0.040 mmol, 0.200 equiv.) and the corresponding protecting group reagent (PG reagent: the corresponding acyl chloride (for benzoyl, tosyl, and nosyl protection) or anhydride (for *tert*-butyloxycarbonyl protection) (0.600 mmol, 3.00 equiv.) were subsequently added and after stirring at 25 °C for 16 hours, $NaHCO_3$ aq. (ca. 5 mL) was added to the mixture. The biphasic mixture was separated, and the aqueous phase was extracted with DCM (3 x 5 mL). The combined organic layers were dried over anhydrous $MgSO_4$, and the crude product was purified by column chromatography.

Characterization of α-aminated amides

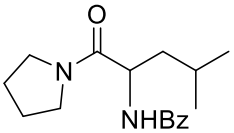
ELSP130, *N*-(1-oxo-1-(pyrrolidin-1-yl)propan-2-yl)benzamide



Prepared according to the General Procedure (using BzCl as a protective agent) in 45% yield (28.0 mg, 0.090 mmol) as a white solid. Purified with two subsequent purifications (EtOAc/Hept, 7:3; then ACN).

Rf = 0.16 (EtOAc/Hept, 7:3) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 – 7.77 (m, 2H), 7.51 – 7.46 (m, 1H), 7.44 – 7.39 (m, 2H), 7.31 (brd, J = 6.5 Hz, 1H), 4.91 (p, J = 6.9 Hz, 1H), 3.71 – 3.63 (m, 1H), 3.59 – 3.42 (m, 3H), 2.04 – 1.96 (m, 2H), 1.93 – 1.85 (m, 2H), 1.44 (d, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.1, 166.5, 134.3, 131.6, 128.6, 127.2, 47.4, 46.5, 46.2, 26.2, 24.2, 18.6. IR (neat) νmax (cm^{-1}): 3309, 3293, 2975, 2952, 2929, 2874, 1628, 1579, 1535, 1488, 1438, 1341, 716, 694. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{14}H_{18}N_2NaO_2^+$) requires 269.1260 *m/z*, found 269.1267 *m/z*.

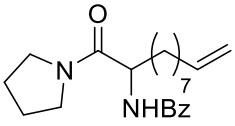
ELSP132, *N*-(4-methyl-1-oxo-1-(pyrrolidin-1-yl)pentan-2-yl)benzamide



Prepared according to the General Procedure (using BzCl as a protective agent) in 44% yield (16.0 mg, 0.090 mmol) as a white solid.

Rf = 0.38 (EtOAc/Hept, 7:3) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 – 7.79 (m, 2H), 7.53 – 7.46 (m, 1H), 7.45 – 7.39 (m, 2H), 7.04 (brd, *J* = 8.4 Hz, 1H), 5.04 (td, *J* = 9.4, 4.0 Hz, 1H), 3.77 (dt, *J* = 10.0, 6.7 Hz, 1H), 3.57 – 3.39 (m, 3H), 2.05 – 1.95 (m, 2H), 1.94 – 1.84 (m, 2H), 1.83 – 1.70 (m, 2H), 1.58 – 1.48 (m, 1H), 1.04 (d, *J* = 6.4 Hz, 3H), 0.95 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.5, 167.4, 134.6, 132.0, 128.9, 127.6, 50.0, 46.9, 46.5, 43.0, 26.5, 25.3, 24.6, 23.9, 22.5. IR (neat) νmax (cm^{-1}): 3312, 3286, 2953, 2926, 2870, 1622, 1578, 1533, 1488, 1433, 1341, 1292, 716, 693. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{17}H_{24}N_2NaO_2^+$) requires 311.1730 m/z, found 311.1734 m/z.

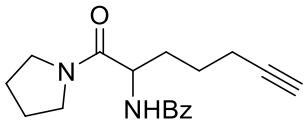
ELSP135, *N*-(1-oxo-1-(pyrrolidin-1-yl)undec-10-en-2-yl)benzamide



Prepared according to the General Procedure (using BzCl as a protective agent) in 40% yield (22.2 mg, 0.080 mmol) as a white solid. Purified with two subsequent purifications (EtOAc/Hept, 7:3; then DCM/ACN, 2:1). Rf = 0.44 (EtOAc/Hept, 7:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 – 7.77 (m, 2H), 7.52 – 7.45 (m, 1H), 7.45 – 7.38 (m, 2H), 7.11 (brd, J = 8.1 Hz, 1H), 5.78 (ddt, J = 16.9, 10.2, 6.7 Hz, 1H), 5.00 – 4.87 (m, 3H), 3.73 (dt, J = 10.1, 6.6 Hz, 1H), 3.58 – 3.41 (m, 3H), 2.05 – 1.94 (m, 4H), 1.93 – 1.78 (m, 4H), 1.42 – 1.26 (m, 10H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7, 166.9, 139.3, 134.3, 131.7, 128.6, 127.2, 114.3, 51.2, 46.7, 46.1, 33.9, 33.1, 29.5, 29.4, 29.1, 29.0, 26.2, 25.4, 24.3. IR (neat) νmax (cm^{-1}): 3324, 2974, 2925, 2876, 2854, 1656, 1624, 1579, 1535, 1451, 1341, 910, 739, 693. HRMS

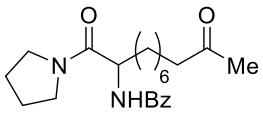
(ESI$^+$): exact mass calculated for [M+Na]$^+$ ($C_{22}H_{32}N_2NaO_2^+$) requires 379.2356 m/z, found 379.2359 m/z.

ELSP131, *N*-(1-oxo-1-(pyrrolidin-1-yl)hept-6-yn-2-yl)benzamide



Prepared according to the General Procedure (using BzCl as a protective agent) in 50% yield (29.0 mg, 0.100 mmol) as a white solid. Purified with two subsequent purifications (EtOAc/Hept, 7:3; then DCM/ACN, 2:1). Rf = 0.30 (EtOAc/Hept, 7:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 – 7.79 (m, 2H), 7.52 – 7.47 (m, 1H), 7.45 – 7.40 (m, 2H), 7.13 (brd, *J* = 7.9 Hz, 1H), 4.98 (td, *J* = 8.2, 4.5 Hz, 1H), 3.71 (dt, *J* = 10.1, 6.6 Hz, 1H), 3.59 – 3.42 (m, 3H), 2.29 (ddd, *J* = 9.1, 7.1, 2.6 Hz, 2H), 2.04 – 1.96 (m, 3H), 1.95 (t, *J* = 2.6 Hz, 1H), 1.92 – 1.86 (m, 2H), 1.86 – 1.77 (m, 1H), 1.68 – 1.61 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 167.0, 134.2, 131.8, 128.7, 127.2, 83.9, 69.1, 50.6, 46.7, 46.2, 31.8, 26.2, 24.3, 24.1, 18.1. IR (neat) νmax (cm^{-1}): 3296, 2972, 2950, 2874, 1624, 1578, 1535, 1488, 1452, 1342, 1307, 738, 693. HRMS (ESI$^+$): exact mass calculated for [M+Na]$^+$ ($C_{18}H_{22}N_2NaO_2^+$) requires 321.1573 *m/z*, found 321.1579 *m/z*.

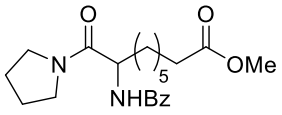
ELSP137, *N*-(1,10-dioxo-1-(pyrrolidin-1-yl)undecan-2-yl)benzamide



Prepared according to the General Procedure (using BzCl as a protective agent) in 41% yield (23.0 mg, 0.080 mmol) as a white solid.

Rf = 0.23 (EtOAc/Hept, 7:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 – 7.79 (m, 2H), 7.51 – 7.45 (m, J = 7.3 Hz, 1H), 7.44 – 7.38 (m, J = 7.4 Hz, 2H), 7.15 (brd, J = 8.1 Hz, 1H), 4.95 (td, J = 7.8, 5.4 Hz, 1H), 3.73 (dt, J = 10.1, 6.6 Hz, 1H), 3.57 – 3.41 (m, 3H), 2.38 (t, J = 7.4 Hz, 1H), 2.10 (s, 1H), 1.99 (dt, J = 13.2, 6.6 Hz, 1H), 1.89 (dd, J = 13.9, 7.0 Hz, 1H), 1.57 – 1.48 (m, J = 14.4, 7.3 Hz, 1H), 1.42 – 1.32 (m, 2H), 1.30 – 1.22 (m, J = 8.4 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.4, 170.7, 166.9, 134.3, 131.7, 128.6, 127.2, 51.2, 46.7, 46.1, 43.8, 33.0, 30.0, 29.4, 29.3, 29.1, 26.2, 25.3, 24.3, 23.9. IR (neat) νmax (cm^{-1}): 3293, 2926, 2854, 1714, 1625, 1579, 1535, 1488, 1458, 1436, 1341, 1312, 715, 694. HRMS (ESI$^+$): exact mass calculated for [M+Na]$^+$ ($C_{22}H_{32}N_2NaO_3^+$) requires 395.2305 *m/z*, found 395.2311 *m/z*.

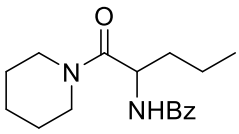
ELSP136, methyl 8-benzamido-9-oxo-9-(pyrrolidin-1-yl)nonanoate



Prepared according to the General Procedure in 55% yield (32.0 mg, 0.110 mmol) as a white solid. Rf = 0.30 (EtOAc/Hept, 7:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 – 7.78 (m, 2H), 7.51 – 7.45 (m, 1H), 7.44 – 7.39 (m, 2H), 7.09 (brd, J = 8.1 Hz, 1H), 4.95 (td, J = 7.8, 5.2 Hz, 1H), 3.72 (dt, J = 10.1, 6.6 Hz, 1H), 3.64 (s, 3H), 3.56 – 3.40 (m, 3H), 2.27 (t, J = 7.5 Hz, 2H), 1.98 (dd, J = 13.1, 6.5 Hz, 2H), 1.89 (dd, J = 14.0, 7.0 Hz, 2H), 1.86 – 1.79 (m, 2H), 1.64 – 1.54 (m, 2H), 1.43 – 1.35 (m, 2H), 1.34 – 1.26 (m, J = 11.2, 6.9 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.3, 170.6, 166.9, 134.3, 131.7, 128.6, 127.2, 51.6, 51.2, 46.7, 46.1, 34.1, 33.0, 29.2, 29.0, 26.2, 25.2, 24.9, 24.3. IR (neat) νmax

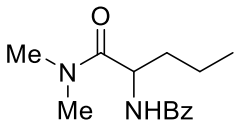
(cm^{-1}): 3290, 2931, 2856, 1735, 1622, 1579, 1534, 1488, 1451, 1435, 1253, 1170, 715, 694. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{21}H_{30}N_2NaO_4^+$) requires 397.2098 *m/z*, found 397.2100 *m/z*.

ELSP141, *N*-(1-oxo-1-(piperidin-1-yl)pentan-2-yl)benzamide



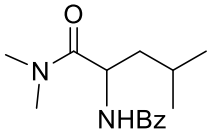
Prepared according to the General Procedure (using BzCl as a protective agent) in 49% yield (18.0 mg, 0.100 mmol) as a white solid. Purified with two subsequent purifications (EtOAc/Hept, 7:3; then DCM/ACN, 1.5:1). Rf = 0.48 (EtOAc/Hept, 7:3). ^1H NMR (400 MHz, $CDCl_3$) δ 7.85 – 7.80 (m, 2H), 7.51 – 7.45 (m, 1H), 7.45 – 7.38 (m, 2H), 7.29 (brd, J = 7.6 Hz, 1H), 5.15 (td, J = 7.6, 4.6 Hz, 1H), 3.66 – 3.55 (m, 2H), 3.51 (dd, J = 15.3, 9.2 Hz, 2H), 1.87 – 1.75 (m, 1H), 1.70 – 1.54 (m, 7H), 1.45 – 1.38 (m, 2H), 0.93 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.2, 166.8, 134.4, 131.6, 128.6, 127.2, 49.2, 46.7, 43.4, 35.7, 26.6, 25.7, 24.6, 18.5, 14.1. IR (neat) νmax (cm^{-1}): 3293, 2934, 2854, 1619, 1578, 1534, 1488, 1441, 1322, 1246, 1134, 1014, 712, 639. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{17}H_{24}N_2NaO_2^+$) requires 311.1730 *m/z*, found 311.1737 *m/z*.

ELSP142, *N*-(1-(dimethylamino)-1-oxopentan-2-yl)benzamide



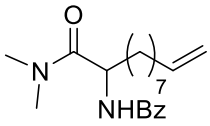
Prepared according to the General Procedure (using BzCl as a protective agent) in 70% yield (20.0 mg, 0.140 mmol) as a white solid. Purified with two subsequent purifications (EtOAc/Hept, 7:3). Rf = 0.32 (EtOAc/Hept, 7:3). ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 – 7.79 (m, 2H), 7.55 – 7.47 (m, 1H), 7.46 – 7.40 (m, 2H), 7.12 (brd, J = 6.9 Hz, 1H), 5.17 (td, J = 7.8, 4.9 Hz, 1H), 3.15 (s, 3H), 3.00 (s, 3H), 1.87 – 1.76 (m, J = 1H), 1.73 – 1.56 (m, 1H), 1.50 – 1.37 (m, 2H), 0.95 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.2, 166.9, 134.4, 131.7, 128.7, 127.2, 49.3, 37.3, 35.9, 35.5, 18.7, 14.1. IR (neat) νmax (cm^{-1}): 3292, 2959, 2931, 1629, 1578, 1535, 1488, 1400, 1119, 713, 693. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{14}H_{20}N_2NaO_2^+$) requires 271.1417 *m/z*, found 271.1424 *m/z*.

ELSP148, *N*-(1-(dimethylamino)-4-methyl-1-oxopentan-2-yl)benzamide



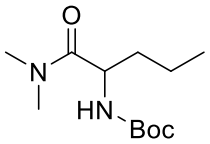
Prepared according to the General Procedure (using BzCl as a protective agent) in 64% yield (20.0 mg, 0.130 mmol) as a white solid. Purified with two subsequent purifications (EtOAc/Hept, 7:3; then DCM/ACN, 2:1). Rf = 0.37 (EtOAc/Hept, 7:3). ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 – 7.78 (m, J = 5.3, 3.3 Hz, 2H), 7.51 – 7.45 (m, 1H), 7.45 – 7.37 (m, 2H), 7.06 (brd, J = 8.2 Hz, 1H), 5.22 (ddd, J = 9.8, 8.7, 3.9 Hz, 1H), 3.14 (s, 3H), 2.98 (s, 3H), 1.80 – 1.69 (m, 1H), 1.68 – 1.58 (m, 1H), 1.55 – 1.45 (m, 1H), 1.05 (d, J = 6.5 Hz, 3H), 0.93 (d, J = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.7, 167.0, 134.3, 131.7, 128.6, 127.2, 47.8, 42.9, 37.2, 35.9, 24.9, 23.6, 22.1. IR (neat) νmax (cm^{-1}): 3325, 3309, 2956, 2932, 1630, 1602, 1579, 1536, 1489, 1448, 1400, 1309, 741, 693. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{15}H_{22}N_2NaO_2^+$) requires 285.1573 *m/z*, found 285.1582 *m/z*.

ELSP147, *N*-(1-(dimethylamino)-1-oxopent-4-en-2-yl)benzamide



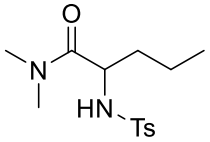
Prepared according to the General Procedure (using BzCl as a protective agent) in 35% yield (15.0 mg, 0.07 mmol) as a white solid. Purified with two subsequent purifications (EtOAc/Hept, 7:3; then DCM/ACN, 2:1). Rf = 0.48 (EtOAc/Hept, 7:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 – 7.78 (m, 2H), 7.52 – 7.46 (m, 1H), 7.46 – 7.39 (m, 2H), 7.13 (brd, J = 7.9 Hz, 1H), 5.79 (ddt, J = 16.9, 10.2, 6.7 Hz, 1H), 5.15 (td, J = 7.8, 4.9 Hz, 1H), 5.02 – 4.87 (m, 2H), 3.14 (s, 3H), 3.00 (s, 3H), 2.01 (dd, J = 14.3, 6.9 Hz, 2H), 1.42 – 1.31 (m, 6H), 1.29 – 1.20 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.2, 166.9, 139.3, 134.3, 131.7, 128.6, 127.2, 114.3, 49.4, 37.3, 35.9, 33.9, 33.3, 29.6, 29.4, 29.1, 29.0, 25.3. IR (neat) νmax (cm^{-1}): 3325, 2925, 2854, 1630, 1602, 1579, 1535, 1488, 1399, 1344, 1261, 908, 741, 713, 693. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{20}H_{30}N_2NaO_2^+$) requires 353.2199 *m/z*, found 353.2206 *m/z*.

ELSP153, *tert*-butyl (1-(dimethylamino)-1-oxopentan-2-yl)carbamate



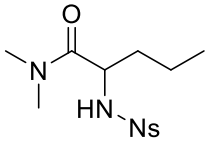
Prepared according to the General Procedure (using Boc anhydride as a protective agent) in 66% yield (19.0 mg, 0.130 mmol) as a white solid. Rf = 0.42 (EtOAc/Hept, 6:4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.35 (brd, J = 8.3 Hz, 1H), 4.60 (td, J = 8.4, 4.9 Hz, 1H), 3.06 (s, 3H), 2.95 (s, 3H), 1.66 – 1.46 (m, 2H), 1.42 (s, 9H), 1.39 – 1.32 (m, 2H), 0.92 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.6, 155.7, 79.5, 50.0, 37.2, 35.8, 35.7, 28.5, 18.7, 14.0. IR (neat) νmax (cm^{-1}): 3322, 2978, 2960, 2933, 1710, 1703, 1640, 1491, 1454, 1416, 1392, 1274, 1168, 1109, 1020, 740. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{12}H_{24}N_2NaO_3^+$) requires 267.1679 *m/z*, found 267.1688 *m/z*.

ELSP160, *N*,*N*-dimethyl-2-((4-methylphenyl)sulfonamido)pentanamide



Prepared according to the General Procedure (using TsCl as a protective agent) in 55% yield (33.0 mg, 0.110 mmol) as a white solid. Rf = 0.32 (EtOAc/Hept, 6:4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 – 7.65 (m, J = 8.3 Hz, 2H), 7.29 – 7.26 (m, 1H), 7.25 – 7.24 (m, 1H), 5.61 (brd, J = 9.3 Hz, 1H), 4.05 (dt, J = 9.3, 6.2 Hz, 1H), 2.79 (s, 3H), 2.62 (s, 3H), 2.39 (s, 3H), 1.53 – 1.34 (m, 4H), 0.89 (t, J = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.1, 143.5, 136.9, 129.4, 127.4, 52.7, 36.7, 35.6, 35.5, 21.6, 18.5, 13.6. IR (neat) νmax (cm^{-1}): 3325, 2960, 2873, 1641, 1598, 1495, 1457, 1397, 1337, 1163, 1120, 1093, 935, 816, 666. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{14}H_{22}N_2NaO_3S^+$) requires 321.1243 *m/z*, found 321.1252 *m/z*.

ELSP162, *N*,*N*-dimethyl-2-((4-nitrophenyl)sulfonamido)pentanamide

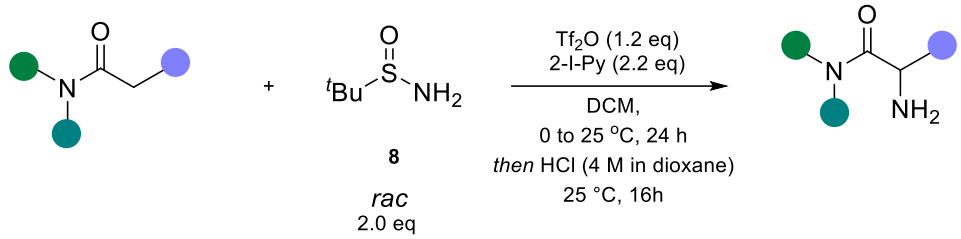


Prepared according to the General Procedure (using NsCl as a protective agent) in 53% yield (35.0 mg, 0.110 mmol) as a white solid. Rf = 0.42 (EtOAc/Hept, 6:4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 – 8.29 (m, J = 8.8 Hz, 2H), 8.02 –

7.97 (m, J = 8.8 Hz, 2H), 5.91 (brd, J = 9.2 Hz, 1H), 4.22 – 4.15 (m, 1H), 2.89 (s, 3H), 2.69 (s, 3H), 1.57 – 1.33 (m, 4H), 0.91 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 150.1, 146.0, 128.6, 124.2, 53.2, 36.8, 35.8, 35.4, 18.5, 13.6. IR (neat) νmax (cm^{-1}): 3257, 3237, 2960, 2932, 2873, 1636, 1605, 1526, 1397, 1348, 1170, 1090, 991, 857, 738, 682, 619. HRMS (ESI^+): exact mass calculated for $[M+Na]^+$ ($C_{13}H_{19}N_3NaO_5S^+$) requires 352.0938 *m/z*, found 352.0946 *m/z*.

2.3. 'Free' α-aminated amides synthesis

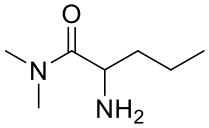
General procedure



To a mixture of amide (0.200 mmol, 1.00 equiv.), 2-iodopyridine (46.8 µL, 0.440 mmol, 2.20 equiv.) in DCM (1.00 mL) in a flame-dry Schlenk tube was added trifluoromethanesulfonic anhydride (40.7 µL, 0.240 mmol, 1.20 equiv.) dropwise under Ar at 0 °C. After stirring for 15 min at 0 °C, a solution of *tert*-butylsulfinamide (48.5 mg, 0.400 mmol, 2.00 equiv.) in DCM (1.00 mL) was added, and the reaction stirred at 25 °C. After 24 hours, a 4M HCl solution in dioxane (0.500 mL, 2.00 mmol, 10.0 equiv.) was added, and the biphasic mixture was vigorously stirred for 16 h at 25 °C. The reaction was diluted with DCM (ca. 3 mL) and carefully quenched with saturated K_2CO_3 aq. (ca. 3 mL) and extracted 3 x 5 mL with DCM. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and the solvent removed under reduced pressure. Purification by column chromatography gave the pure product.

Characterization of 'free' α-aminated amides

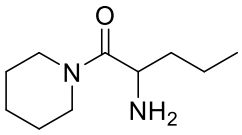
ELSP155, 2-amino-*N*,*N*-dimethylpentanamide



Prepared according to the General Procedure in 58% yield (17.0 mg, 0.120 mmol) as a light-yellow oil. Rf = 0.32 (EtOAc/MeOH/NH_4OH, 8:2:0.1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.19 (brs, 2H), 4.40 (t, J = 6.1 Hz, 1H), 3.08 (s, 3H), 2.98 (s, 3H), 1.87 – 1.77 (m, 2H), 1.55 – 1.42 (m, 2H), 0.98 – 0.94 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7, 50.7, 37.4, 36.2, 33.3, 18.2, 13.9. IR (neat) νmax (cm^{-1}): 3258, 2962, 2874,

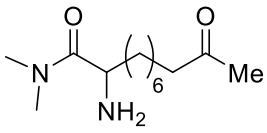
1655, 1506, 1405, 1260, 1218, 1155, 1065, 1026, 748, 692. HRMS (ESI^+): exact mass calculated for $[M+H]^+$ ($C_7H_{17}N_2O$) requires 145.1335 *m/z*, found 145.1338 *m/z*.

ELSP163, 2-amino-1-(piperidin-1-yl)pentan-1-one



Prepared according to the General Procedure in 44% yield (16.0 mg, 0.090 mmol) as a light-yellow oil. Rf = 0.44 (EtOAc/MeOH/NH_4OH, 8:2:0.1). ^1H NMR (400 MHz, $CDCl_3$) δ 3.84 – 3.72 (m, 1H), 3.61 – 3.49 (m, 2H), 3.45 – 3.36 (m, 2H), 2.74 (brs, 2H), 1.67 – 1.43 (m, 10H), 0.93 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.6, 50.7, 46.4, 43.5, 36.9, 26.6, 25.7, 24.7, 18.9, 14.1. IR (neat) νmax (cm^{-1}): 3243, 2959, 2934, 2855, 1647, 1634, 1442, 1249, 1219, 1121, 1012, 853, 770. HRMS (ESI^+): exact mass calculated for $[M+H]^+$ ($C_{10}H_{21}N_2O^+$) requires 185.1648 *m/z*, found 185.1654 *m/z*.

ELSP157, 2-amino-*N*,*N*-dimethyl-10-oxoundecanamide

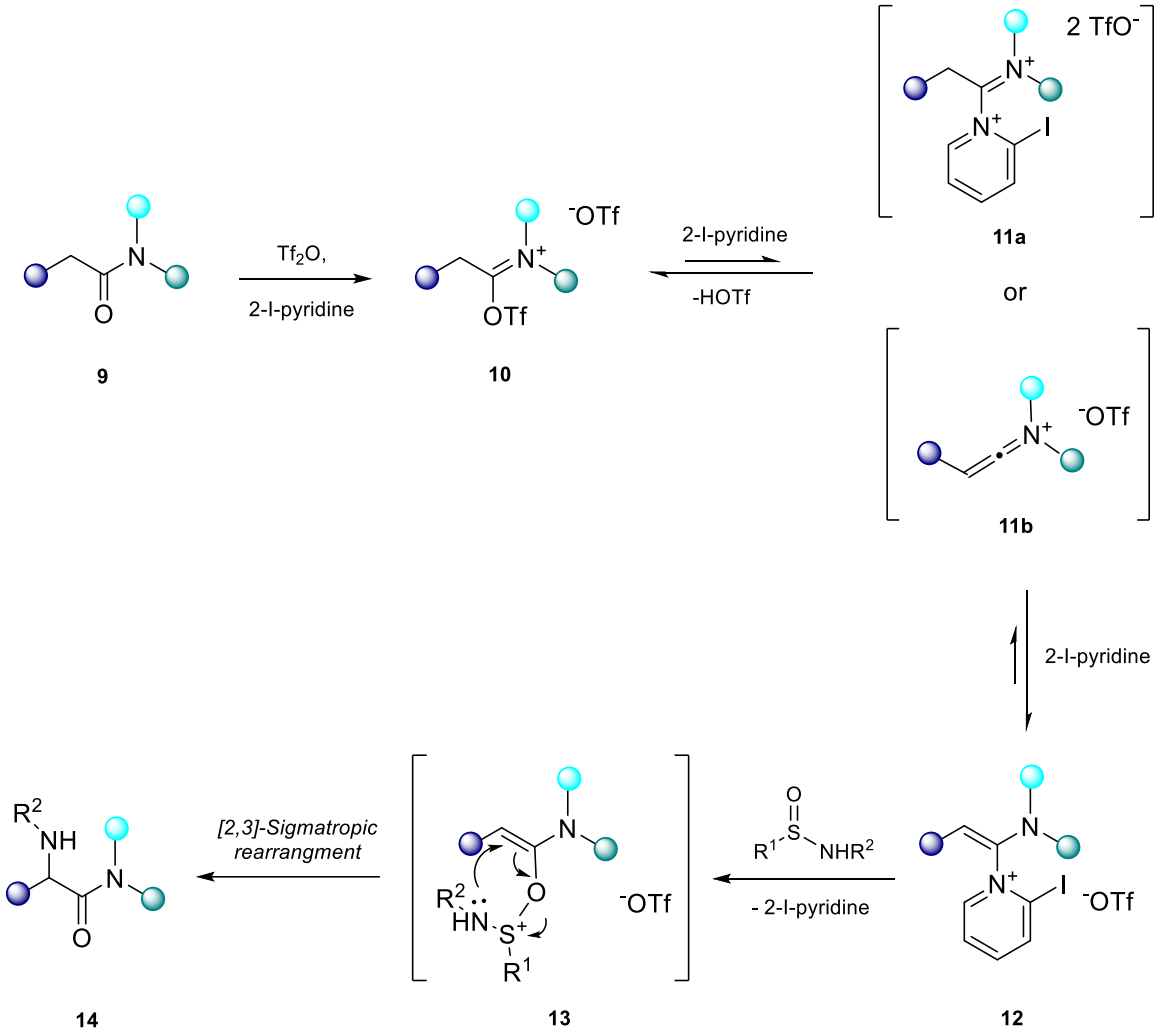


Prepared according to the General Procedure in 38% yield (18.0 mg, 0.080 mmol) as a light-yellow oil. Rf = 0.32 (EtOAc/MeOH/NH_4OH, 8:2:0.1). ^1H NMR (400 MHz, $CDCl_3$) δ 3.72 – 3.63 (m, 1H), 3.02 (s, 3H), 2.95 (s, 3H), 2.39 (t, J = 7.4 Hz, 2H), 2.11 (s, 3H), 2.03 (brs, 2H), 1.59 – 1.50 (m, 2H), 1.47 – 1.37 (m, 2H), 1.31 – 1.26 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.4, 175.5, 51.2, 43.8, 36.9, 35.9, 35.3, 30.0, 29.4, 29.4, 29.2, 25.8, 23.9. IR (neat) νmax (cm^{-1}): 3246, 2928, 2853, 1713, 1646, 1641, 1462, 1399, 1368. HRMS (ESI^+): exact mass calculated for $[M+H]^+$ ($C_{13}H_{27}N_2O_2^+$) requires 243.2067 *m/z*, found 243.2073 *m/z*.

3. Results and discussion

On the basis of recent results gained by the Maulide group on this kind of chemistry, the work was based on the investigation of amide α-amination through electrophilic amide activation and exploiting the weakness of the S-O bond of *tert*-butylsulfinamide for the formation of α-amino amides in an electrophilic rearrangement strategy.

Going into details on the mechanistic insights of these reactions, initial amide activation with triflic anhydride leads to the formation of intermediate **10**, which is then converted into the iminium intermediate **11a** or keteniminium triflate **11b**. The second equivalent of 2-iodopyridine causes the formation of compound **12**. The choice of this kind of base is crucial since it is also a good leaving group that can be displaced by a *tert*-butylsulfinamide to produce the intermediate **13**. This intermediate undergoes to [2,3]-sigmatropic rearrangement, and compound **14** is formed (Scheme 6). This mechanism was proposed following the work previously published by the group (Peng et al., 2014), where α-arylation of amides using diphenyl sulfoxide was investigated.

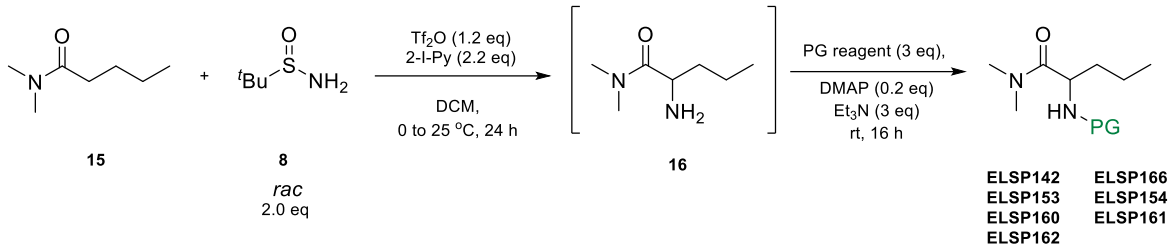


Scheme 6. Proposed mechanism for α-amination of amides using *tert*-butylsulfinamide.

The α-amination work was mainly divided into two main projects. The first and most articulate one relied on the application of the α-amination reaction for the synthesis of protected α-amino amides, since 'free' α-amino products are usually quite difficult to handle and to demonstrate the reaction applicability also to complex synthetic processes, for which the use of protecting groups (PGs) is often needed. The second project was focused on the development of an α-amination procedure for the production and isolation of 'free' α-amino amides.

3.1. Protected α-aminated amides synthesis

The initial scope was based on the application of the α-amination reaction following the procedure already developed by the α-amination team (Scheme 7).



Scheme 7. General procedure developed for the synthesis of protected α-amino compounds.

A screening of protecting groups was performed (tosyl (Ts), nosyl (Ns) and *tert*-butyloxycarbonyl (Boc)), as shown in Figure 1.

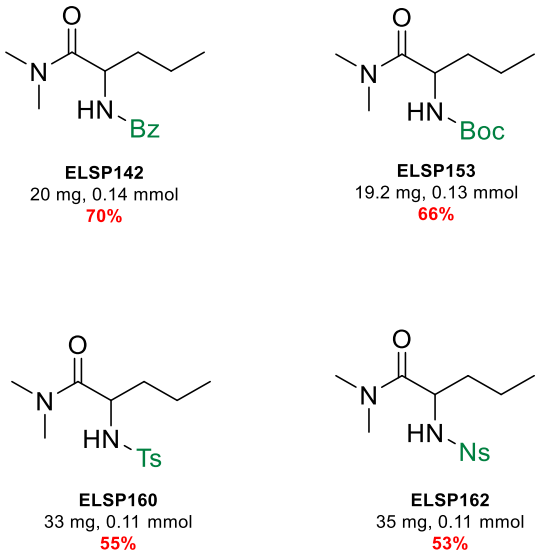


Figure 1. Protecting groups screened for α-amination.

However, some other protecting groups gave low conversion (carboxybenzyl (Cbz), acetyl (Ac)) (< 50% NMR yield), as shown in Figure 2.

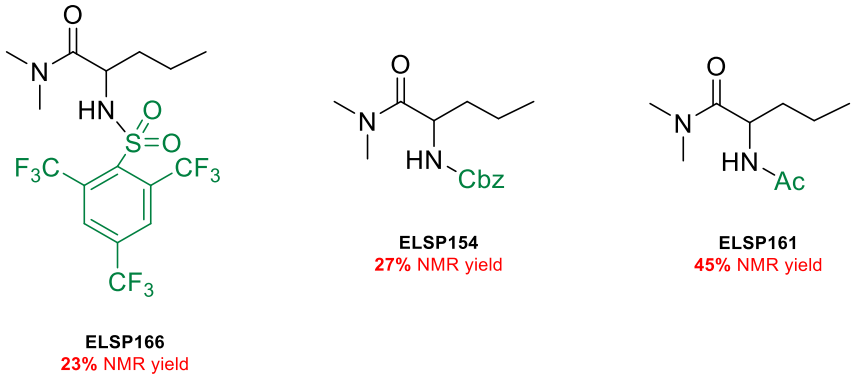


Figure 2. Protecting groups with low conversion.

Since Bz gave the highest yield, it was chosen as PG for the reaction scope, which started from some differently functionalized amides (ELSP130-137), obtaining acceptable crude NMR yields (Figure 3).

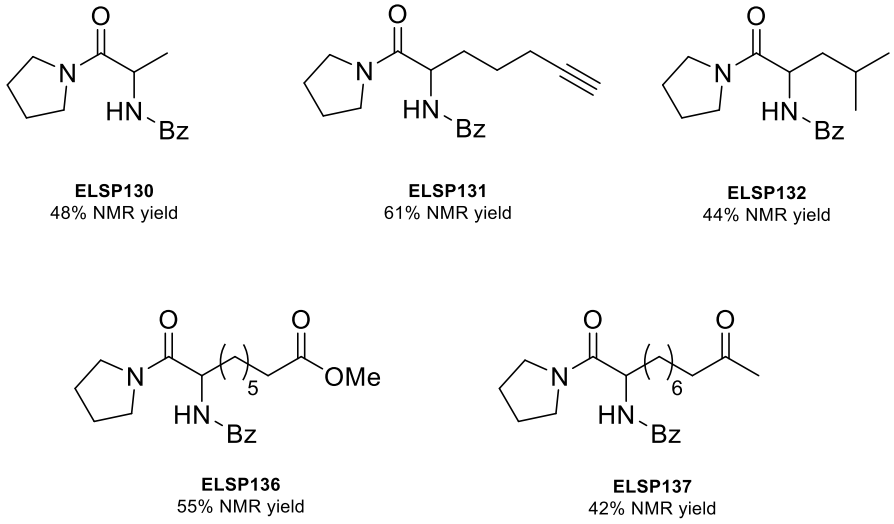


Figure 3. α-Amination on differently functionalized amides.

However, some purification issues were found for all the synthetized products since their retention factor (Rf) is similar to that of the starting material. This required an optimization of the purification conditions, consisting of at least two subsequent chromatographic columns, using the more appropriate eluent for each substrate. The purification details for each product are reported in Section 2.

Consequently, different Bz protected α-aminated amides were synthetized, with good functional group tolerance, and an overview is reported in Figure 4.

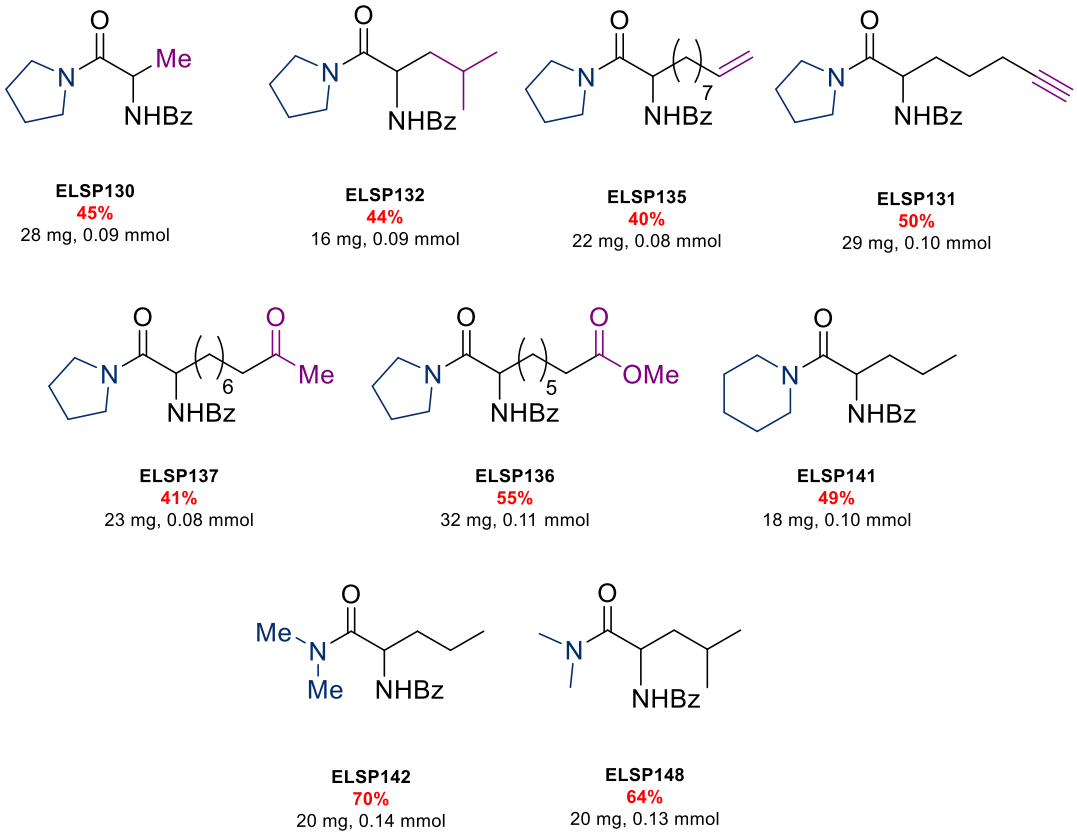


Figure 4. Benzoyl protection scope for α-amination.

Moreover, for some amides (Figure 5) low conversion or low isolated yields were obtained.

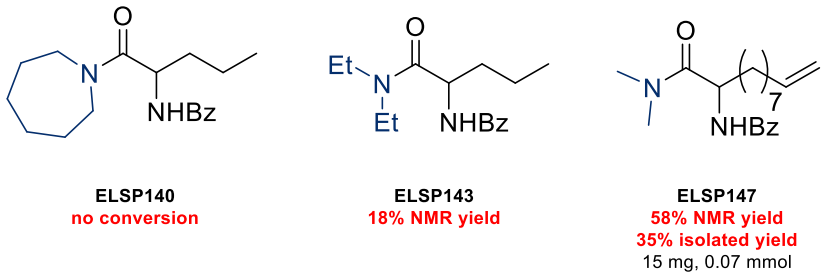
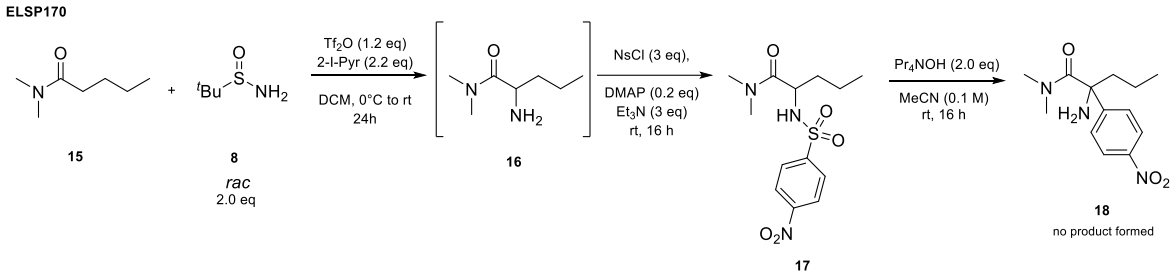


Figure 5. Benzoyl protection for α-amination with low conversion or low isolated yield.

This low conversion was not surprising for ELSP140, for which the steric hindrance of the azepane ring surely played a crucial role. The result obtained for ELSP143 was quite surprising, but, even if the reaction was repeated, the same low conversion was gained. For ELSP147, the low isolated

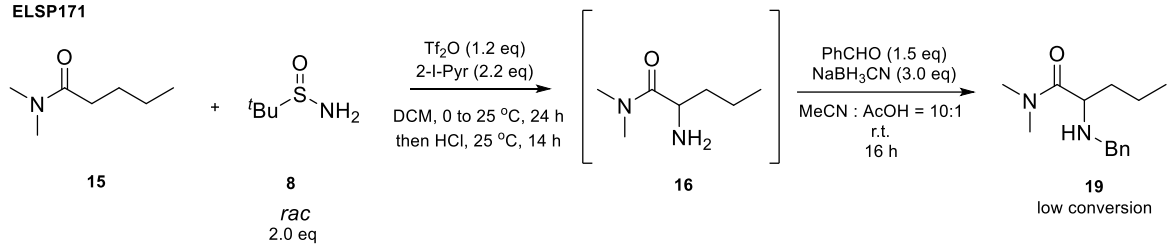
yield could be linked to the four columns performed for its purification, which resulted trickier than that of the other substrates.

On some protected α-aminated amides, some reactions were performed. For instance, on Ns-protected α-aminated amide, the Smiles-rearrangement was tried, as shown in Scheme 8.



Scheme 8. Smiles-rearrangement tried on nosyl-protected α-aminated amide.

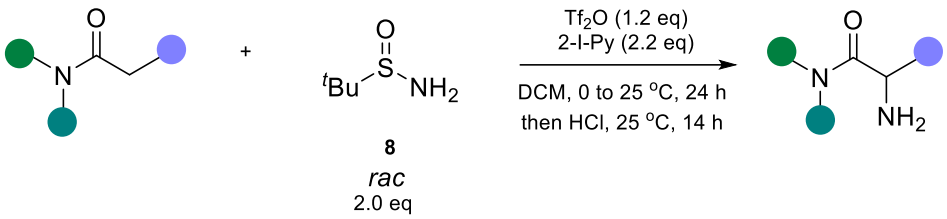
The reaction was initially performed at 25 °C for 16 h, but no product formation was observed. From LC-MS analysis only unreacted starting material was detected. So, the reaction was repeated this time heating at 60°C for 16 h. Again, only starting material was detected after LC-MS analysis. Moreover, benzyl (Bn) protection through reductive amination was also performed (Scheme 9), but LC-MS analysis of the crude revealed low product formation.



Scheme 9. Benzyl-protection through reductive amination.

3.2. 'Free' α-amination

The second project of the work focused on the application of the 'free' α-amination reaction (Scheme 10), which was developed by the α-amination team



Scheme 10. 'Free' α-amination reaction conditions.

Some α-aminated amides were synthetized, and an overview is reported in Figure 6.

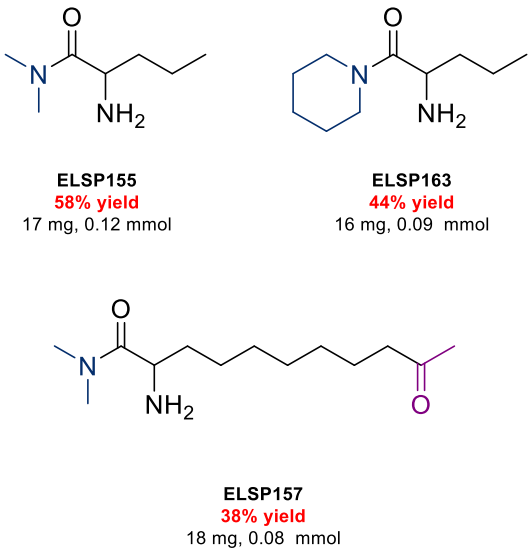


Figure 6. α-Aminated amides synthetized.

For ELSP157 the conversion resulted quite low, and this was confirmed by LC-MS analysis, which revealed the presence of a large amount of unreacted starting material.

Moreover, for some substrates, the 'free' α-amination resulted in quite messy (Figure 7).

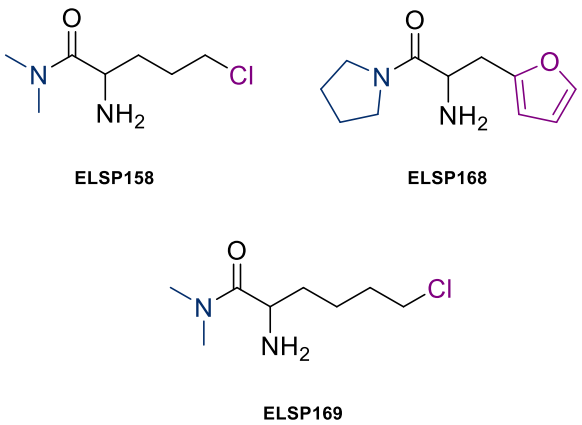


Figure 7. α-Amination reactions with low conversion.

For ELSP158, the LC-MS analysis revealed the formation of the corresponding 'free' α-aminated amide and of the cyclized product (Figure 8), but the crude of the reaction looked quite messy:

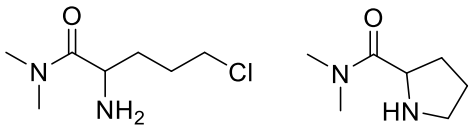


Figure 8. Products formed from ELSP158 'free' α-amination.

ELSP169 gave a low isolated yield (< 20%), but the LC-MS analysis of the crude was quite promising, as also the TLC. The reaction will be repeated by the α-amination team.

4. Conclusions

The research carried out at the Laboratory of Organic Chemistry of Prof. Nuno Maulide (University of Vienna) had as its main objective the application of amide activation reaction through triflic anhydride to differently substituted amides. The goal was to develop a new synthetic approach for producing α-aminated amides in one pot. The initial screening of PG led to the identification of Bz as the best PG in terms of yield, and the reaction displayed good functional group tolerance, except for bulky amides. Moreover, a protocol was developed and applied to produce 'free' α-aminated amides, and the reaction was screened on some substrates with acceptable yields. This new synthetic approach could lead to several synthetic applications and the production of α-aminated precursors employable for the synthesis of biologically active compounds. Moreover, a future perspective will also be the application of this procedure to the *N*-alkylamide spilanthol in order to synthetize several analogues and test their insecticidal potential.

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