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Essential Oil of *Achillea ligustica* (Asteraceae) as an Antifungal Agent against Phytopathogenic Fungi

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The essential oil from the inflorescences of Ligurian yarrow (*Achillea ligustica* All., Asteraceae) naturalized in Central Italy was investigated in order to evaluate the antifungal activity against five phytopathogenic fungi. The composition of the essential oil characterized by GC-FID and GC-MS, revealed linalool (24.8%), viridiflorol (9.6%), β-pinene (6.4%), 1,8-cineole (5.8%) and terpinen-4-ol (5.3%) as the major constituents. The antifungal activity was evaluated by the *in vitro* disc agar diffusion test and Agar vapor assay on 5 microbial strains. The essential oil showed a good antifungal activity against all fungal strains, being a valid candidate in the fight against plant pathogens that harm many traditional crops. The possible incorporation of this essential oil into antifungal preparations with a low environmental impact is hypothesized.

Keywords: *Achillea ligustica*, Antifungal activity, Phytopathogenic fungi, Essential oil composition, Linalool.

In recent years, scientists have focused on increasing the food production needed for the rapid expansion of the world population. Unfortunately, there are significant yield losses due to insects and plant diseases caused by fungi, bacteria and viruses. Fungi and bacteria also have unfavorable effects on the quality, safety and preservation of food [1,2]. Plants are constantly exposed and threatened by a variety of pathogenic microorganisms present in their environments and phytopathogenic fungi pose serious problems to the crops of economically important plants [3-5]. In an effort to combat diseases, the plants have devised several mechanisms, in particular, producing compounds to repel microbial invaders. However, despite the existence of defense mechanisms, the plants are continually exposed to the attack of plant pathogenic fungi. For this reason, chemical fungicides in current agriculture are widely used and the excessive use of them has caused major problems such as the deterioration of human health and the environment, and a number of problems including a developed resistance to fungicides. The increase of fungal resistance to classical drugs, the treatment costs and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies [6,7].

In the past decade, due to concerns regarding safety of the synthetic antimicrobial agents, the scientific research has been focused on the potential application of plant essential oils as alternative chemical control measures. Indeed, essential oils have a broad spectrum of antifungal properties and they are environmentally friendly (biodegradable, do not leave toxic residues or by-products in the environment) [8-11].

The genus *Achillea* (Asteraceae) includes more than 80 species widespread all around the northern hemisphere. Among them, *Achillea ligustica* All. is a perennial pubescent herbaceous plant growing spontaneously throughout the western Mediterranean region. In Italy it grows especially in the Tyrrenian area from Liguria to Sicily. The essential oil of this plant has been used in herbal, food and cosmetic applications. In previous investigations the chemical composition of *A. ligustica* essential oil showed a strong variability, depending on geographical factors [12,13]. Notably, the essential oil composition from *A. ligustica* naturalized in Marche region (central Italy) after cultivation was dominated by β-pinene (11.7%), viridiflorol (9.8%), terpinen-4-ol (8.0%), linalool (7.7%), germacrene D (7.0%), and 1,8-cineole and these data are reported in Table S1 in the Supplementary data Section. The qualitative and quantitative differences were observed with respect to naturally occurring populations [13-17]. The essential oil of Ligurian yarrow showed potential as an anticariogenic agent to be incorporated in oral hygiene products [18] and as a botanical insecticide against *Culex quinquefasciatus* and *Musca domestica* [19].

This study was undertaken in order to evaluate the *in vitro* antifungal activity of the same sample of essential oil. For the purpose, five phytopathogenic fungi, namely *Fusarium avenaceum*, *Fusarium graminearum*, *Fusarium semitectum Alternaria solani* and *Phytopthora cryptogea,* damaging the traditional crops of the central Italy territory and its agro-food structure, were selected.

Table 1: Effect of *Achillea ligustica* essential oil on *in vitro* growth of selected phytopathogenic fungi (% inhibition).

Fungi	Positive control 1 (100 ppm)	Positive control 2 (100 ppm)	Essential oil 250 ppm	500 ppm	1000 ppm	1500 ppm
F. graminearum	100*	100*	61.9± 3.2$_a$	80.1±4.1$_a$	100°	100*
F. semitectum	100*	100*	65.2±3.1$_{ab}$	68.1±3.1$_b$	100°	100*
F. avenaceum	100*	100*	69.7±3.1$_b$	80.2±3.9$_a$	100°	100*
P. cryptogea	100*	100*	78.3±4.1$_c$	100*	100*	100*
A. solani	100*	100*	88.2±3.5$_d$	100*	100*	100*

The values are the average of three determinations ± standard error of the mean (SEM)
Different letter within a column indicate statistically significant differences between the mean ($p<0.05$)
°fugistatic activity; *fungicidal activity. Positive control 1 is represented by Nystatin and positive control 2 is represented by Benomyl

Kordali *et al.* [20,21] evaluated the antifungal activities of some oxygenated monoterpenes, including camphor, carvone, 1,8-cineole, fenchone, geraniol, linalool and menthol against 31 phytopathogenic fungi. They stated that some of monoterpenes had potent inhibitory effects against most of the tested fungal species. In our

oil, the main component was linalool (24.79%) and the fraction of the oxygenated monoterpenes represented the main fraction (49.15%) [13]. The efficacy of *A. ligustica* essential oil on the phytopathogenic fungi tested may be due to oxygenated monoterpenes and in particular, linalool, terpinen-4-ol and 1,8-cineole that act in synergies.

Table 2: Screening of MIC and MFC of *Achillea ligustica* essential oil

Fungi	MIC (µg/mL)	MFC(µg/mL)
F. graminearum	750_a	1200_c
F. semitectum	750_a	1200_c
F. avenaceum	750_a	1200_c
P. cryptogea	270_b	300_d
A. solani	270_b	300_d

Different letter within a column indicate statistically significant differences between the mean ($p<0.05$)

In fact, the presence of a synergistic effect seems to be supported by the results obtained in the vapor phase assay carried out with the *A. ligustica* essential oil and its main component linalool. Data reported in Table 3 indicate that the *A. ligustica* oil manifested its antifungal activity at a minimum inhibitory dose of 30.0 µL/400 mL air. The fungi failed to restore growth even after the lid of Petri dishes were opened for 4 min, after the six days incubation period, indicating a fungicidal activity of the oil.

Table 3: Linear growth of the tested fungi exposed to *Achillea ligustica* oil vapors.

Fungi	Essential oil (µL/400 mL air space)				
	10.0	15.0	20.0	30.0	40.0
F. graminearum	$8.0\pm0.4_a$	$8.0\pm0.4_a$	$8.0\pm0.4_a$	0.0	0.0
F. semitectum	$8.0\pm0.4_a$	$8.0\pm0.4_a$	$8.0\pm0.4_a$	0.0	0.0
F.avenaceum	$8.0\pm0.4_a$	$8.0\pm0.4_a$	$8.0\pm0.4_a$	0.0	0.0
P. cryptogea	$7.0\pm0.3_a$	$7.0\pm0.3_a$	$5.0\pm0.2_b$	0.0	0.0
A. solani	$7.0\pm0.3_a$	$7.0\pm0.3_a$	$5.0\pm0.2_b$	0.0	0.0

The values indicate the diameter of linear growth (mm). The values are the means of three determinations ± SD. Different letter within a column indicate statistically significant differences between the mean ($p<0.05$)

Table 4: Linear growth of the tested fungi exposed to linalool.

Fungi	Linalool (µL/400 mL air space)		
	5.00*	7.50°	10.00˙
F. graminearum	$9.7\pm0.4_a$	$9.2\pm0.5_a$	$5.6\pm0.3_a$
F. semitectum	$9.7\pm0.4_a$	$9.2\pm0.5_a$	$5.6\pm0.2_a$
F. avenaceum	$9.7\pm0.5_a$	$9.2\pm0.4_a$	$5.6\pm0.3_a$
P. cryptogea	$9.7\pm0.5_a$	$7.6\pm0.3_a$	$3.5\pm0.2_b$
A. solani	$9.7\pm0.4_a$	$7.6\pm0.3_a$	$3.5\pm0.2_a$

The values indicate the diameter of linear growth (mm). The values are the means of three determinations ± SD. Different letter within a column indicate statistically significant differences between the mean ($p<0.05$); *Amount of linalool present in 20µl of essential oil; ° Amount of linalool present in 30µl of essential oil;˙ Amount of linalool present in 40µl of essential oil.

Afterwards, we also examined the antifungal activity against all tested fungi, of two mixtures: terpinen-4-ol + 1,8 cineole (mixture 1) and linalool+terpinen-4-ol + 1,8 cineole (mixture 2) at the same concentrations present in the essential oil.

In fact, the fungistatic or fungicidal activity was not detected at 10 µL/400 mL air of linalool (corresponding to the linalool contained in 40.0 µL/400 mL air of essential oil).

Afterwards, we also examined the antifungal activity against all tested fungi, of two mixtures: terpinen-4-ol + 1,8 cineole (mixture 1) and linalool+terpinen-4-ol + 1,8 cineole (mixture 2) at the same concentrations present in the essential oil.

The first combination shown an inhibitory effect against all fungi similar to that exerted by linalool alone (Table 5) while the second combination showed an excelling antifungal efficacy comparable with than exerted by the essential oil (Table 6).

Table 5: Linear growth of the tested fungi exposed to mixture 1 (terpinen-4-ol + 1,8-cineole)at the same concentration present in the oil.

Fungi	Mixture 1 (terpinen-4-ol + 1,8-cineole) (µL/400 mL air space)		
	2.20*	3.30°	4.40˙
F. graminearum	$9.5\pm0.5_a$	$9.1\pm0.4_a$	$5.4\pm0.4_a$
F. semitectum	$9.6\pm0.4_a$	$9.0\pm0.5_a$	$5.5\pm0.5_a$
F. avenaceum	$9.5\pm0.6_a$	$9.0\pm0.5_a$	$5.4\pm0.4_a$
P. cryptogea	$9.3\pm0.6_a$	$7.3\pm0.3_a$	$3.1\pm0.2_b$
A. solani	$9.3\pm0.4_a$	$7.3\pm0.3_a$	$3.1\pm0.3_b$

The values indicate the diameter of linear growth (mm). The values are the means of three determinations ± SD. Different letter within a column indicate statistically significant differences between the mean ($p<0.05$); *Amount of mixture 1 present in 20µl of essential oil; °Amount of mixture 1 present in 30µl of essential oil; ˙Amount of mixture 1 present in 40µl of essential oil

Table 6: Linear growth of the tested fungi exposed to mixture 2 (linalool +terpinen-4-ol + 1,8-cineole)at the same concentration present in the oil.

Fungi	Mixture 2 (linalool+terpinen-4-ol + 1,8-cineole) (µL/400 mL air space)		
	7.20*	10.80°	14.40˙
F. graminearum	$8.5\pm0.4_a$	$5.2\pm0.2_a$	$1.5\pm0.1_a$
F. semitectum	$8.5\pm0.4_a$	$5.2\pm0.3_a$	$1.7\pm0.1_a$
F. avenaceum	$8.5\pm0.5_a$	$5.2\pm0.3_a$	$1.6\pm0.1_a$
P. cryptogea	$5.5\pm0.3_b$	$2.4\pm0.1_b$	0.0
A. solani	$5.5\pm0.3_b$	$2.3\pm0.1_b$	0.0

The values indicate the diameter of linear growth (mm); The values are the means of three determinations ± SD; Different letter within a column indicate statistically significant differences between the mean ($p<0.05$);*Amount of mixture 2 present in 20 µL of essential oil; °Amount of mixture 2 present in 30 µL of essential oil; ˙Amount of mixture2 present in 40 µL of essential oil

In conclusion, the essential oil was found to have a significant antifungal activity against all tested fungi. These results are not in agreement with the data reported by Tuberoso *et al*. [14] and this difference could be explained by the different origin of sample. affecting the oil chemical profile. This research has highlighted the potential use of this oil in limiting stem infections caused by phytopathogenic fungi that most commonly infect crops in our territory, providing a possible introduction into preparations of plant protection products. In this regard, the essential oil from Ligurian yarrow appears to be scalable on an industrial level because of its fair yield and availability of the raw material (both from spontaneous and cultivated plants). Currently, in all sectors of Italian agriculture, for quality agriculture (also in accordance with European regulations), the use of metabolites and/or plant biocidal molecules is identified as an important parameter in reducing the impact on agro-ecosystems [22]. In this way, the use of fungicides obtained from vegetable matrices is considered as a parameter capable of characterizing and further qualifying the final product.

However, further studies are required to determine whether it could have value in the management of weed control and fungal plant infectious diseases, and also phytotoxicity against the cultivated plants.

Experimental

Plant material: A population naturalized after cultivation at the Botanical Garden of the University of Camerino (central Italy, 600 m above sea level, GPS coordinates: N43°08'02" E 13°04'06") was studied in this work. Flowering aerial parts were collected during the blooming period in June 2009. The plant was botanically confirmed by F. Maggi using available literature. Plant material was air dried at room temperature protected from light for one week [13,23].

Isolation of the volatile oil: The essential oil was isolated by hydrodistillation of the flowering aerial parts in a Clevenger-type

apparatus for 4 h as described by Maggi *et al*. [13]. The oil yield was calculated on a dry weight basis.

Chemical analysis: GC analyses were performed by GC-FID and GC-MS using a HP-5 capillary and a HP-5MS columns, respectively. The utilized temperature programs were described by Maggi *et al* in 2009 [13]. The identification of volatile components was based on computer matching with the WILEY275, NIST05 and ADAMS libraries, as well as by comparison of the mass spectra and retention indices (RI), with those reported in the literature and as described by Maggi *et al* [13,24].

Antimicrobial screening: *In vitro* antifungal activity of the *A. ligustica* essential oil against the phytopathogenic fungi was carried out according to the agar dilution method and agar vapor assay [25,26]. The phytopathogenic fungi were cultured in appropriate culture media (for *Fusarium* species and *A. solani* the culture medium was PDA potato dextrose agar; for *Phytopthora cryptogea* the culture medium was V8- Juice Agar).

Agar dilution method: Briefly, potato dextrose agar (PDA) and V8-Juice Agar plates were prepared using 9 cm diameter glass Petri dishes. The essential oil was dissolved in absolute ethyl alcohol and 5% Tween 20 (Fluka) was added in order to obtain an emulsion and aliquots of this emulsion were added to the culture medium at a temperature of 40-45°C, then poured into Petri dishes (Ø9 cm) [27,25]. Concentrations of 250, 500, 1000 e 1500 ppm were tested. A disc (5mm diameter) of the fungal species was cut from 1-week-old cultures PDA plates (for *Fusarium* species and *A. solani*) and on V8-Juices agar medium (for *Phytopthora cryptogea*) and then the mycelia surface of the disc was placed upside down on the center of a dish with fungal species in contact with growth medium on the dish. Then, the plates were incubated in the dark at 22±2°C. Controls consisted of 250, 500, 1000 and 1500 ppm of the emulsion above described, where the essential oil was replaced with sterile distilled water. In addition, Nystatin and Benomyl were used as reference fungicides. The treatments were incubated under controlled temperature conditions of 22±1°C in the dark. Colony growth diameter was measured after the fungal growth in the control treatments had completely covered the Petri dishes.

The percentage of growth inhibition by treatment was calculated from the following equation: Mycelial growth inhibition (%) = [(DC-DT/DC]x 100 [25], where DC and DT are average diameter of fungal colony of control and treatment, respectively.

The fungicidal activity of the oil was determined using the technique of Carta and Thompson [28,29]. The mycelia disks were transferred from Petri dishes in which no growth was observed (total inhibition =100) onto fresh plates of culture medium, in order to verify after 7 days the fungistatic or fungicidal activity of such inhibition. All experiments were carried out in triplicate.

Determination of minimum inhibitory concentration (MICs): The MIC values (µg/mL) were determined by the dilution method. Dilutions of the oil emulsions were made in the culture medium over the concentration range of 250 ppm (250 µL/mL) and 1000 ppm (1000 µL/mL) for all tested fungi. MICs were determined as the lowest concentration with no visible growth. All experiments were performed in triplicate.

Determination of minimum fungicidal concentration: MFCs were obtained using a membrane filtration method [30] and were calculated from 1000 ppm to 1500 ppm for all species of *Fusarium* and from 500 ppm to 1500 ppm for *A. solani* and *P. cryptogea*. All experiments were performed in triplicate.

Vapor-phase antifungal activity: the essential oil and linalool were dissolved in 100 µL ethyl acetate, applied to a filter paper (110 mm diameter) and air-dried for 1 min. The filter paper was mounted on the inverted lid in a Petri dish (140 mm x 23 mm, which offers 400 mL air space after addition of culture medium). Disc of the tested fungi was placed at the center of each dish. The sealed dishes were incubated for 6 days at 25°C after that the linear growth of the mycelium was measured. The reported data were the mean of three replicates. Control samples containing water instead of the oil and/or linalool were used. The inhibition dose of the oil was expressed as µL/ 400 mL air space.

Statistical analysis: Analysis of variance was performed by one-way ANOVA and by Duncan's post hoc test. Statistical differences at $p < 0.05$ were considered to be significant.

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