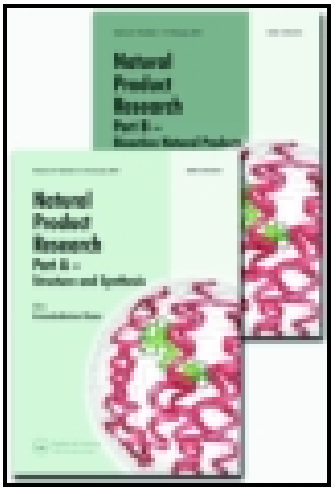

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Antioxidant activity and cytotoxicity on tumour cells of the essential oil from *Cedronella canariensis* var. *canariensis* (L.) Webb & Berthel. (Lamiaceae)

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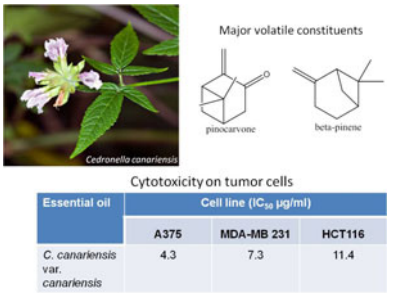

Cedronella canariensis is a lemon-scented species of the family Lamiaceae endemic to the Canary Islands where it is used in the traditional medicine to prepare infusions or inhalations for anti-catarrhal, tonic, diuretic, hypoglycaemiant, hypotensive, anti-inflammatory and decongestant of the respiratory tract. In this work we investigated for the first time the antioxidant activity of the essential oil and its inhibitory effects on tumour cells (A375, MDA-MB-231, HCT 116) proliferation by DPPH, ABTS, FRAP and MTT assays, respectively. The oil, analysed by GC-ionisation flame detector and GC–MS, was characterised by pinocarvone (58.0%) and β-pinene (10.8%) as the major constituents, being typical of the chemotype '*canariensis*'. Noteworthy was the cytotoxic activity of the oil against the tumour cells examined, with IC_{50} values of 4.3, 7.3 and 11.4 μg/mL on A375, MDA-MB-231 and HCT 116 tumour cells, respectively, as well as the scavenging activity against the ABTS radical (IC_{50} of 10.5 μg/mL).

Keywords: *Cedronella canariensis* var. *canariensis*; Lamiaceae; essential oil; antioxidant; cytotoxicity

1. Introduction

Cedronella canariensis (L.) Webb & Berthel. (syn. *Dracocephalum canariense* L.) is a monotypic species of the family Lamiaceae endemic to the Macaronesian region. In the Canary Islands it is present in forests of Tenerife, La Gomera, Gran Canaria, El Hierro and La Palma,

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from 500 to 1550 m of altitude and on sunny rocky slopes (Olesen et al. 1998; Bramwell & Bramwell 2001). It is a perennial herb, sometimes shrubby, growing up to 1 m tall, endowed with serrate, pubescent, aromatic and three palmate leaves, the latter most unusual for a Labiatae, and with whorls of small scented white, pink or purple, typically two lipped, flowers, very attractive to pollinating insects. The special feature of the species is that of emitting a pleasant camphoraceous fragrance. However, some populations of the species are characterised by glabrous leaves emanating an anis-like scent. These observations led to the differentiation of two varieties: *C. canariensis* var. *canariensis* the former, *C. canariensis* var. *anisata* the latter.

C. canariensis, known popularly in the Canary Islands as 'algaritofe', 'garitopa' or 'boca de dragón', is used in traditional medicine as anti-catarrhal, tonic, antimicrobial, analgesic, carminative, diuretic, hypoglycaemiant, hypotensive, anti-inflammatory and decongestant of the respiratory tract. For these purposes, the top leaves are collected before the plant comes into the flowering stage and used to make an invigorating tea called 'the de canaries' and for pot-pourri and inhalations (Facciola 1990; Bean 1996; Pérez de Paz & Hernandez Padron 1999).

From the aerial parts of *C. canariensis*, the monoterpene pinocarvone, ursolic acid and a dimer of pinocarvone known as cedronellone (Carreiras et al. 1987), as well as chavicol and benzyl alcohol glucosides (Coen et al. 1995), were previously isolated. As regards the essential oil, two chemotypes were observed, with the var. *canariensis* being rich in pinocarvone and β-pinene and the var. *anisata* endowed with high levels of methylchavicol (Engel et al. 1991, 1995; López-García et al. 1992). In a previous investigation, the essential oils from the two varieties were proven to exhibit antimicrobial activity with noteworthy inhibition against *Bordetella bronchiseptica* and *Cryptococcus albidus*, thus justifying the popular use of the plants in the treatment of respiratory diseases (López-García et al. 1992). The plant was also proven to exhibit analgesic and anti-inflammatory activities with low toxicity on mice (López-García et al. 1991) as well as hypoglycaemic, diuretic and hypotensor effects in rats (López-García et al. 1996).

The aim of this work was to investigate for the first time the antioxidant activity of the essential oil from *C. canariensis* var. *canariensis* and its cytotoxicity on some tumour cell lines.

2. Results and discussion

2.1. *Essential oil analysis*

The composition of the essential oil from the aerial parts of *C. canariensis* is reported in Table 1. A total of 61 volatile components were identified, corresponding to 92.3% of the total oil composition. The oil composition was dominated by oxygenated monoterpenes, accounting for 69.8%, followed by monoterpenes hydrocarbons (13.9%) and small amounts of sesquiterpene hydrocarbons and oxygenated sesquiterpenes. The major constituents were pinocarvone (58.0%) and β-pinene (10.8%), while all the other components occur in poor percentages, in all cases below 2.8%. Among these, (*E*)-caryophyllene (2.8%), myrtenyl methyl ether (2.7%) and myrtenol (2.6%) were the most abundant. Cedronellone (compound no. 61 in Table 1) is a dimer of *d*-pinocarvone and it is assumed that it can be formed from this monoterpene by a hetero-Diels–Alder reaction (Carreiras et al. 1987). Thermal degradation of cedronellone takes place both during hydrodistillation and in the injector and inside the column during the gas chromatographic run. This was attested by the distension in the base line of cedronellone, giving pinocarvone in the total ion chromatogram (TIC) (Figure 1). Pinocarvone, the main component of the oil, is a common volatile component within the family Lamiaceae, being reported as the main constituent of several essential oils among which hyssop (*Hyssopus officinalis*) is one of the many representatives (Pandey et al. 2014). The abundance of this compound in *C. canariensis* might support its traditional use in the treatment of respiratory diseases (Pérez de Paz & Hernandez Padron 1999) since the pinocarvone-containing plants are known to be used as stimulant and expectorant against cold (Kerrola et al. 1994).

Table 1. Essential oil composition of *C. canariensis* var. *canariensis*.

No.	Component[a]	LRI[b]	LRI lit.[c]		% [d]	ID[e]
			ADAMS	FFNSC2		
1	α-Thujene	917	924	916	0.2	RI, MS
2	α-Pinene	922	932	924	0.7	Std
3	α-Fenchene	933	945	933	tr[f]	RI, MS
4	Camphene	935	946	937	0.1	Std
5	Thuja-2,4(10)-diene	941	953	940	0.0	RI, MS
6	Sabinene	960	969	960	tr	RI, MS
7	β-Pinene	963	974	963	10.8	Std
8	Myrcene	984	988	984	0.3	Std
9	α-Phellandrene	999	1002	998	0.0	Std
10	α-Terpinene	1010	1014	1010	0.2	Std
11	*p*-Cymene	1018	1020	1018	0.1	Std
12	Sylvestrene	1021	1025	1019	0.8	RI, MS
13	1,8-Cineole	1024	1026	1024	0.0	Std
14	γ-Terpinene	1051	1054	1051	0.4	Std
15	Terpinolene	1081	1086	1081	0.3	Std
16	Linalool	1097	1095	1096	0.1	Std
17	*endo*-Fenchol	1105	1114	1123	0.2	RI, MS
18	*trans*-Pinocarveol	1129	1135	1128	0.4	Std
19	Camphene hydrate	1138	1145	1136	0.1	RI, MS
20	Myrtenyl methyl ether	1150		1159[g]	2.7	RI, MS
21	Pinocarvone	1154	1160	1154	58.0	RI, MS
22	Isoborneol	1156	1155	1156	0.4	Std
23	δ-Terpineol	1160	1162		tr	RI, MS
24	*cis*-Pinocamphone	1163	1172	1163	1.6	RI, MS
25	Terpinel-4-ol	1168	1174	1168	1.2	Std
26	*cis*-Pinocarveol	1176	1182		0.2	RI, MS
27	α-Terpineol	1182	1186	1182	1.7	Std
28	Myrtenal	1186	1195	1185	0.5	Std
29	Myrtenol	1187	1194	1187	2.6	RI, MS
30	γ-Terpineol	1198	1199		0.0	RI, MS
31	Methyl myrtenate	1289	1293		0.2	RI, MS
32	Carvacrol	1300	1298	1299	0.0	Std
33	α-Cubebene	1339	1345		0.0	RI, MS
34	α-Ylangene	1359	1373	1358	0.0	RI, MS
35	α-Copaene	1363	1374	1363	0.1	RI, MS
36	β-Bourbonene	1371	1387	1370	0.0	RI, MS
37	(*E*)-β-Damascenone	1376	1383	1376	0.0	RI, MS
38	β-Elemene	1381	1389	1381	0.1	RI, MS
39	(*E*)-Caryophyllene	1403	1417	1404	2.8	Std
40	β-Copaene	1414	1430		0.0	RI, MS
41	Aromadendrene	1423	1439	1427	0.0	RI, MS
42	α-Humulene	1438	1452	1440	0.1	Std
43	γ-Muurolene	1464	1478	1464	0.1	RI, MS
44	Germacrene D	1466	1465	1471	0.2	RI, MS
45	β-Selinene	1470	1489	1488	0.0	RI, MS
46	(*E*)-β-Ionone	1476	1487	1477	0.0	Std
47	α-Muurolene	1488	1500	1488	0.0	RI, MS
48	α-Zingiberene	1490	1493	1490	tr	Std
49	γ-Cadinene	1500	1513	1500	0.0	RI, MS
50	δ-Cadinene	1511	1523	1511	0.2	RI, MS
51	α-Calacorene	1528	1544		0.0	RI, MS
52	Caryophillene oxide	1565	1583	1566	1.1	Std

(*Continued*)

Table 1. (*Continued*)

No.	Component[a]	LRI[b]	LRI lit.[c] ADAMS	LRI lit.[c] FFNSC2	%[d]	ID[e]
53	Salvial-4(14)-en-1-one	1577	1594		0.0	RI, MS
54	Caryophylla-4(12),8(13)-dien-5-ol[h]	1619	1639		0.4	RI, MS
55	α-Cadinol	1640	1654	1640	0.1	RI, MS
56	Selina-3,11-dien-6-α-ol	1642	1642		0.5	RI, MS
57	Eudesma-4(15),7-dien-1β-ol	1670	1688		0.3	RI, MS
58	2-Pentadecanone, 6,10,14-trimethyl-	1838		1838	0.1	RI, MS
59	Hexadecanoic acid	1959	1959	1957	0.9	Std
60	Phytol	2095		2100	0.0	Std
61	Cedronellone	2102			1.2	MS[i]
	Oil yield (%)				1.5	
	Total identified (%)				92.3	
	Grouped compounds (%)					
	Monoterpene hydrocarbons				13.9	
	Oxygenated monoterpenes				69.8	
	Sesquiterpenes hydrocarbons				3.8	
	Oxygenated sesquiterpenes				2.5	
	Others				2.2	

[a] Compounds are listed in order of their elution from a HP-5MS column.
[b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8–C_{30} alkanes.
[c] Relative retention index taken from Adams (2007) and FFNSC2 (2012) for apolar capillary column.
[d] Percentage values are means of three determinations, with an RSD% for the main components below 5% in all cases.
[e] Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries Wiley, Adams, FFNSC2 and NIST 08; RI, by comparison of RI with those reported in the literature (Adams 2007; NIST 08 2008; FFNSC2 2012); Std, by comparison of the retention time and mass spectrum of the available authentic standard.
[f] Tr, traces (mean value below 0.1%).
[g] Retention index value taken from Pandey et al. (2014).
[h] Correct isomer not identified.
[i] MS fragmentation according to Carreiras et al. (1987).

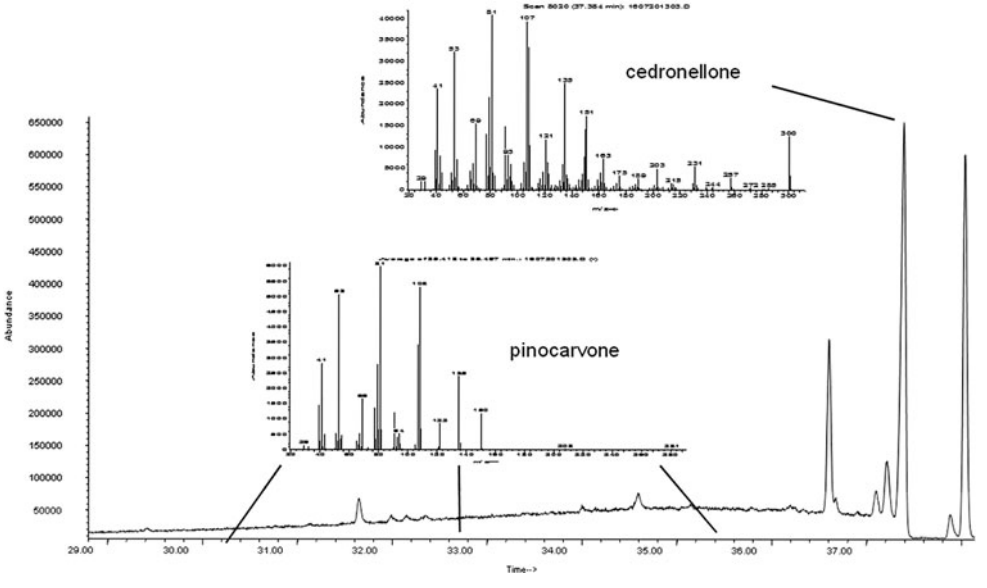

Figure 1. Distension in the baseline due to the thermal degradation of cedronellone ($M^+ = 300$) giving pinocarvone ($M^+ = 150$) in the TIC of the essential oil from *C. canariensis*.

The composition found was consistent with those reported in previous studies (Engel et al. 1991, 1995; López-García et al. 1992), confirming that the var. *canariensis* is characterised by the oxygenated monoterpene pinocarvone, with respect to the var. *anisata* being rich of phenylpropanoids such as methyl chavicol.

2.2. *Antioxidant activity*

The antioxidant scavenger properties and reducing antioxidant power of *C. canariensis* essential oil were proved on DPPH, ABTS and FRAP assays and results are reported in Table 2. The results indicated that the essential oil showed worthy activity against the ABTS radical, with an IC_{50} value of 10.5 μg/mL, about 20 times lower than that of Trolox. In the DPPH assay the antioxidant activity observed was much lower, with an IC_{50} value of 615.5 μg/mL, about 500 times lower than that of Trolox. The DPPH, ABTS and FRAP assays are widely used to evaluate the antioxidant potential of essential oils or plant extracts, and although the mode of action on DPPH and ABTS assays was similar, significant differences in reactions may occur with different antioxidant compounds. Different factors can affect the capacity to react with the two radicals, e.g. the stereoselectivity of radicals, the solubility of tested samples in different experimental conditions and functional groups occurring in bioactive compounds (Wojdylo et al. 2007; Adedapo et al. 2008; Fu et al. 2011). The ferric reducing/antioxidant power (FRAP assay) of *C. canariensis* essential oil (Table 2) was very low (3.8 μmol TE/g). No correlation has been found between the antioxidant activity on the ABTS radical and the essential oil composition. In fact, pinocarvone, the main essential oil component (58.0%), is widely distributed in plants (Kerrola et al. 1994; Jankovsky & Landa 2002; Ozer et al. 2005; Bernotienė & Butkienė 2010), but no antioxidant activity can be related to its high levels observed. However, it is possible that the high levels of oxygenated monoterpenes (69.8%) occurring in *C. canariensis* essential oil may explain, at least in part, the antioxidant activity observed (Ruberto & Baratta 2000).

2.3. *Cytotoxicity on tumour cells*

To investigate the cytotoxic activity of *C. canariensis* essential oil, we evaluated its effect on three human cell lines of different origin, i.e. A375 human malignant melanoma, MDA-MB 231 human breast adenocarcinoma and HCT116 human colon carcinoma, by MTT assay. All cell lines were subjected to growing concentrations of *C. canariensis* essential oil for 72 h. To compare the cytotoxic effects obtained, we used the anticancer drug cisplatin, which is one of the most potent antitumour agents known, displaying clinical activity against a wide variety of solid tumours. The results, collected in Table 3, show that the essential oil exhibited a significant cytotoxicity against the human cancer cells examined. Essential oil induced a concentration-dependent inhibitory effect on all cell lines tested in the dilutions ranging from 0.78 to 200 μg/mL. The essential oil was more active against A375 human melanoma cell line with an IC_{50} of

Table 2. *In vitro* radical scavenging activities of *C. canariensis* essential oil.

		DPPH		ABTS		FRAP
	Polyphenols (μg GA eq/mg)	TEAC[a] (μmol TE/g)	IC_{50}[b] (μg/mL)	TEAC[a] (μmol TE/g)	IC_{50}[b] (μg/mL)	TEAC (μmol TE/g)
C. canariensis oil	23.5 (±9.0)	13.6	615.5 (±76.5)	172.6 (±1.0)	10.5 (±0.06)	3.8 (±1.4)
Trolox			1.3 (±0.01)		0.5 (±0.01)	

[a] TEAC, trolox equivalent (TE) antioxidant concentration.
[b] IC_{50}, the concentration of essential oil/compound that affords a 50% reduction in the assay.

Table 3. *In vitro* cytotoxic activity of *C. canariensis* essential oil.

	Cell line (IC_{50} μg/mL)[a]		
Essential oil	A375[b]	MDA-MB 231[c]	HCT116[d]
C. canariensis	4.3	7.3	11.4
95% CI[e]	3.7–5.1	5.7–9.3	10.5–12.5
Positive control			
Cisplatin	0.4	2.9	2.4
95% CI[e]	0.3–0.5	2.2–3.1	2.0–2.9

[a] IC_{50}, the concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation).
[b] Human malignant melanoma cell line.
[c] Human breast adenocarcinoma cell line.
[d] Human colon carcinoma cell line.
[e] Confidence interval.

4.33 μg/mL. Lower activity was shown on HT116 human colon carcinoma cell line with an IC_{50} of 11.43 μg/mL.

Regarding the active compounds, a low cytotoxic activity on the cell lines tested in this study has been reported recently for β-pinene, which is one of the major essential oil constituents (10.8%) (Woguem et al. 2014). Thus, it is possible that at least part of the effects observed in this work may be due to the presence of this compound. On the other hand, since *d*-pinocarvone is the main component of *C. canariensis* essential oil (58%), we suggested that this monoterpene may also play a major role in inducing the cytotoxic effects on the tumour cells, although we could not find any report in the literature related to the cytotoxic activity of this pure compound. Moreover, it is possible that, like in the case of other plants, a synergistic effect among the different single components present in the essential oil contributes to the observed cytotoxic activity in our study (Wright et al. 2007).

3. Materials and methods

3.1. *Plant material*

Aerial parts of *C. canariensis* were collected in El Monte de las Mercedes (Tenerife, 800 m, Canary Islands, Spain) during blooming in 2013. A voucher specimen of the plant has been deposited in the *Herbarium Universitatis Camerinensis* (included in the online edition of *Index Herbariorum* c/o School of Biosciences and Veterinary Medicine, University of Camerino, Italy), and archived in the anArchive system for botanical data (anArchive system, http://www.anarchive.it). Just after its collection, the plant material was dried in an airy oven at 36°C; its storage was at room temperature in darkness.

3.2. *Extraction of essential oils*

Plant material (200 g) was reduced into a powder, then hydrodistilled in a Clevenger-type apparatus using 6 L of distilled water for 3 h until no more volatile oil was obtained. The essential oil was stored in a sealed vial protected from light at −20°C before chemical analysis and biological assays. The oil yield (1.5%) was estimated on a dry weight basis.

3.3. *GC-FID and GC/MS analyses*

For GC separations, an Agilent 4890D instrument (Palo Alto, CA, USA) coupled to an ionisation flame detector (FID) was used. Volatile components were separated on a HP-5 capillary column (5% phenylmethylpolysiloxane, 30 m, 0.32 mm i.d.; 0.25 mm film thickness; J and W Scientific,

Folsom, CA, USA), with the following temperature programme: 5 min at 60°C, subsequently 4°C/min up to 220°C, then 11°C/min up to 280°C, held for 15 min, for a total run of 65 min. Injector and transfer line temperatures were 280°C; He was used as the carrier gas, at a flow rate of 1.8 mL/min; split ratio, 1:34. A mixture of aliphatic hydrocarbons (C8–C30; Sigma, Milan, Italy) in *n*-hexane was directly injected into the GC under the above temperature programme, in order to calculate the temperature-programmed RIs of peaks in the chromatograms. Oil samples were diluted to 1:100 in hexane and injected at a volume of 1 μL. For each sample, the analysis was repeated three times and the mean value was reported. Data were collected by using HP3398A GC Chemstation software (Hewlett Packard, Rev. A.01.01). Quantification of essential oil components was achieved by FID peak-area internal normalisation without using correction factors. GC–MS analysis was performed on an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer (Palo Alto, CA, USA) using a HP-5 MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 mm film thickness; J and W Scientific) capillary column. The temperature programme was the same as described above. Injector and transfer line temperatures were 280°C; He was used as the carrier gas, at a flow rate of 1 mL/min; split ratio, 1:50; acquisition mass range, 29–400 *m/z*. Mass spectra were acquired in electron-impact mode with an ionisation voltage of 70 eV. The oil sample was diluted to 1:100 in *n*-hexane, and the volume injected was 2 μL. Data were analysed by using MSD ChemStation software (Agilent, Version G1701DA D.01.00). Some major oil constituents were identified by co-injection with authentic standards. Otherwise, the peak assignment was carried out according to the recommendations of the International Organization of the Flavor Industry (http://www.iofi.org/), i.e. by the interactive combination of chromatographic linear retention indices that were consistent with those reported in the literature (Adams 2007; NIST 08 2008; FFNSC2 2012) for apolar stationary phases, and MS data consisting in computer matching with the WILEY275, NIST 08, ADAMS, FFNSC 2 and home-made (based on the analyses of reference oils and commercially available standards) libraries.

3.4. *Determination of total phenolic content and antioxidant activity*

Spectrophotometric methods were employed to evaluate the amounts of total phenols in the extracts and essential oils using Folin–Ciocalteu reagent in $NaHCO_3$ solution by measuring the absorbance at 765 nm (Esparza Rivera et al. 2006). This method gives a general measurement of phenolic content, as it is not completely specific for phenolic compounds and not all phenolic compounds exhibit the same level of activity in the assay. The total phenolic content was expressed as micro gram gallic acid equivalents per mg of extract or essential oil. All tests were conducted in triplicate.

Free radical scavenging activity (DPPH assay) of the extracts and essential oils was assessed on a microplate analytical assay according to the procedures described by Srinivasan et al. (2007), while the total radical scavenging capacity of the same products was measured by the ABTS assay modified as by Re et al. (1999), for application to a 96-well microplate assay. Determination of antioxidant activity by FRAP assay was carried out according to the procedure described by Müller et al. (2011), by monitoring the reduction of Fe^{3+}-tripyridyl triazine (TPTZ) to blue-coloured Fe^{2+}-TPTZ. The ability of the samples tested to scavenge the different radicals in all assays was compared to Trolox used as standard and expressed as tocopherol-equivalent antioxidant capacity μmol TE/g of product. Each experiment was repeated at least three times.

3.5. *MTT assay*

A375 (human malignant melanoma cells) and MDA-MB 231 cells (human breast adenocarcinoma cells) were cultured in Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and supplemented with 10%

heat-inactivated foetal bovine serum (HI-FBS). HCT116 cells (human colon carcinoma cells) were cultured in RPMI1640 medium with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and supplemented with 10% HI-FBS. Cells were cultured in a humidified atmosphere at 37°C in the presence of 5% CO_2. The MTT assay was used as a relative measure of cell viability. In brief, cells were seeded at the density of 2×10^4 cells/mL. After 24 h, samples were exposed to different concentrations of essential oil (0.78–200 μg/mL). Cells were incubated for 72 h in a humidified atmosphere of 5% CO_2 at 37°C. At the end of incubation, the cytotoxic activity was evaluated by the MTT assay (Quassinti et al. 2013). Cisplatin (Sigma) was used as the positive control. Experiments were conducted in triplicate. Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}). The IC_{50} values were determined with GraphPad Prism 4 computer program (GraphPad Software, San Diego, CA, USA).

4. Conclusions

The most important finding of our investigation was that *C. canariensis* essential oil belonging to the chemotype '*canariensis*' shows a good inhibitory activity against three human tumour cell lines. In the plant-screening programme of the National Cancer Institute of the USA, a crude oil is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value, following incubation between 48 and 72 h, is less than 20 μg/mL (Boik 2001). The significant growth-inhibition values detected make the essential oil worthy of further investigation as a promising anticancer agent. Finally, the moderate radical scavenging property exhibited is promising for applications as a food preservative alternative to synthetic substances without important side effects.

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