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ORIGINAL ARTICLE

Chemical composition, antibacterial and antifungal activities of flowerhead and root essential oils of *Santolina chamaecyparissus* L., growing wild in Tunisia

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Abstract The antimicrobial properties of essential oil from various *Santolina* species have not been investigated enough in the previous studies dealing with the biological activities of medicinal plants. In Tunisia, *Santolina chamaecyparissus* L. (Asteraceae) is the only *Santolina* species recorded and is used as vermifuge and emmenagogue. The chemical composition, antibacterial and antifungal properties of essential oils from the flowerheads and roots of spontaneous *S. chamaecyparissus* growing in Tunisia and the chemical composition which leads to the Tunisian chemotype are investigated here for the first time. Essential oils isolated by hydro distillation from flowerheads and roots of *S. chamaecyparissus* were analyzed by GC and GC/MS. Two methods served for antimicrobial assays of the essential oils: diffusion in a solid medium and micro-well dilution assay. Antifungal tests were carried out by the agar incorporation method. Sixty-seven constituents were identified

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from the essential oil of the flowerhead. The major constituents were: 1,8-cineole and β-eudesmol. Two non identified compounds were present at the highest concentration in root oil. Flowerhead oil was characterized by high contents in monoterpenes and sesquiterpenes oxygenated compounds. The flowerhead essential oil demonstrated potent of antibacterial properties against *Pseudomonas aeruginosa* ATCC and *Enterococcus faecalis* ATCC, with MIC of 0.625 μg/ml. These findings demonstrate that the flowerhead essential oils of *S. chamaecyparissus* have excellent antibacterial properties and for this reason they could contribute to decrease the problem of microbial resistance to antibiotics.

1. Introduction

A growing body of evidences suggested that more than three antibiotics derived from microorganisms are initiated every year (Clark, 1996; de Lima Procópio et al., 2012). Novel resources, especially plant, are also being examined. Essential oils, secondary metabolites and medicinally important compounds with or without bioactivity, have been isolated from plants belonging to the Asteraceae family: *Achillea* L., *Anthemis* L., *Artemisia* L., *Balsamita* Desf., *Chrysanthemum* L., *Matricaria* L., *Tanacetum* L., and *Santolina* Tourn (Khallouki et al., 2000). Besides the different strains, several genuses from the family Asteraceae, have been particularly shown to be antimicrobial (Paulo, 2006; Salie et al., 1996; Wetungu et al., 2014).

In the Mediterranean area, it has been found that the genus *Santolina* (Asteraceae, tribe Anthemideae) is characterized with more than 10 species that are distributed widely (Derbesy et al., 1989). Usually plants of the genus *Santolina* cultivate in South Europe and North Africa. As a result, numerous classes of this taxon have been investigated biologically and chemically and give rise to number of mono and sesquiterpenoids along with some other secondary metabolites (Barrero et al., 1998, 1999, 2000; Marco et al., 1993).

In all these *Santolina* species, *Santolina viridis* Wild (South of France and North of Spain), *Santolina pectinata* Lag. (*Iberian peninsula*) and *Santolina chamaecyparissus* L. (Asteraceae) are the most widely spread species around the world. *S. chamaecyparissus* L. is synonym of *Ormenis africana* (Jord. et Fourr.). Out of four species of the genus *Ormenis* only *O. africana* is vivacious (Poli et al., 1997) and others are used in important medicine (Pons Giner and Rios Canavate, 2000). Widely known shrub (*S. Chamaecyparissus*) with yellow inflorescences is utilized in Mediterranean folk medicine because of its analgesic, bactericidal, fungicidal, vermifuge and vulnerary properties (Cuéllar et al., 1998; Grieve, 1984; Bean, 1989). Also it has been suggested that this plant is utilized in phytotherapy for numerous kinds of dermatitis (Giner et al., 1988) and also as a stimulant and a stomachic (Yoganarasimhan, 2000). Although preliminary evidence available in the literature suggested that *S. chamaecyparissus* has anti-inflammatory activity and anti-phospholipase A_2 due to the presence of the isolated active principle nepetin present in the dichloromethane extract (Sala et al., 2000). In Tunisia, *S. chamaecyparissus* is the only *Santolina* species recorded and is used as vermifuge and emmenagogue (Le Floc'h, 1983).

The components of essential oils from diverse species of *Santolina* have been investigated: *Santolina oblongifolia* (De Pascual et al., 1983), *Santolian ligustica* (Flamini et al., 1999), *Santolina rosmarinifolia* (Pala-Paul et al., 2001), *Santolina canescens* (Casado et al., 2001) and *S. chamaecyparissus* (Garg et al., 2001; Lawrence, 1997), and all of these species produced monoterpene oils and demonstrated diverse constituents.

Several volatile oils are recognized to acquire antifungal and antibacterial properties and are potentially applicable as antimycotic agents. Many scientific investigations have been conducted to evaluate the biological activities; of about 60% of the essential oils possess antifungal and 35% of them exhibited antibacterial properties (Chaurasia and Vyas, 1977). A few series of studies have demonstrated the potential antimicrobial effect of essential oils from various *Santolina* species, i.e. *Santolina corsica* oil exhibited an appreciable antibacterial activity against *Staphylococcus aureus* (Rossi et al., 2007) sesquiterpenes from *S. rosmarinifolia* showed significant antimicrobial activity against yeasts while none of the compounds tested showed significant activity besides gram positive and gram negative bacteria (Barrero et al., 1999).

To specifically address the constituents of an essential oil and antimicrobial properties of Tunisian *S. Chamaecyparissus,* we directly examined to investigate the chemical composition, antibacterial and antifungal activities of essential oils isolated by hydrodistillation from the flowerheads and roots of *S. chamaecyparissus* L., growing wildly in Tunisia.

2. Methodology

2.1. Plant material

Plants of *S. chamaecyparissus* were collected at the time of flowering stage from Siliana on the mountain 'Djebel Kesra' located in the West Center of Tunisia at 35.81 (latitude in decimal degrees), 9.36444 (longitude in decimal degrees). The identification of plant material has been demonstrated according to the flora of Tunisia (Pottier-Alapetite, 1981) by the botanist Dr. Fethia Harzallah-Skhiri (Higher Institute of biotechnology, Monastir University, Tunisia).

2.2. Plant extraction and essential oil analysis

2.2.1. Plant extraction

Essential oils from the flower heads and roots of *S. chamaecyparissus* were found by hydrodistillation for 4 h using a Clevenger type apparatus (Benyelles et al., 2014; Suwei and Dongke, 2014; Okoh et al., 2010). Anhydrous sodium sulfate

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served to dry essential oils and stored at 4 °C until use. The yield was calculated according to the plant fresh weight.

2.2.2. Essential oil analysis

The identification of essential oil components was performed by GC and GC/MS. GC diagnostic were carried out on an HP5890-series II gas chromatograph (Agilent Technology, California, USA) with Flame Ionization Detectors (FID) characterized by: a fused silica capillary column, non polar HP-5 and polar HP Innowax (30 m × 0.25 mm ID, film thickness 0.25 μm). The temperature of the oven was 50 °C for 1 min, then it is programmed to achieve heating rate up to 5–240 °C/min and held isothermal for 4 min. Injector temperature: 250 °C, detector temperature: 280 °C; nitrogen used as a carrier gas (1.2 ml/min); and injected 0.1 ml diluted in hexane1%. The percentage of the constituents was calculated by electronic integration of FID peak areas without the use of response factor correction. Hewlett-Packard 5972 MSD system served for GC/MS analyses: HP-5 MS capillary column (30 m × 0.25 mm ID, film thickness of 0.25 mm) was directly coupled to the mass spectrometry. The comparison of the component mass spectra with those in the Wiley 275 GC/MS library and of their retention indices with literature data (Adams, 2001) served to their identification. Retention indices (RI) were calculated by the retention times of a series of n-alkanes.

2.3. Antibacterial activity

2.3.1. Bacterial strains

The antibacterial assays were carried against four gram negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* and *Citrobacter freundii* and two gram positive bacteria: *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection, Rockville, MD).

Inoculums were kept in nutrient agar (Sigma) at 37 °C. Bacterial suspension were prepared in Mueller Hinton Broth (Sigma) and adjusted to a 10^8 cfu/ml for overnight incubation.

2.3.2. Agar diffusion assay

Antibacterial studies have been evaluated by the method of disc-diffusion (Sahin et al., 2003). One millilitre from a bacterial suspension of 10^8 cfu/ml was spread on the surface of both control and test plates.

Under aseptic conditions, 10 μl of essential oil were applied to sterilized Whatman filter paper discs N° 3 (6 mm diameter) and placed on the agar surface. Before incubating at 37 °C, plates were left for 2–3 h at 4 °C to help the diffusion of the oil. Diameters of inhibition were measured in millimetre. Gentamicin (10 μg/disque; Gibco) was used as standard antibiotic for comparison.

2.3.3. Micro-well dilution assay

The flowerhead and root oils from *S. chamaecyparissus* were diluted in ethanol 99° (v/v) to the highest tested concentration of 10 μg/ml. Serial two-fold dilutions (0.078–10 μg/ml) of oils were prepared using nutrient broth. Bacterial strains were inoculated for 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The Minimal Inhibition Concentration (MIC) is defined as the lowest concentration of the essential oil to inhibit the growth of microorganisms and was determined on the basis of micro-well dilution method (Gulluce et al., 2004a,b).

2.4. Antifungal testing

2.4.1. Fungal strains

Seven strains of fungi were used for the antifungal tests, comprising: three dermatophytes (*Trichophyton rubrum*, *Microsporum canis* and *Epidermophyton floccosum*); one opportunist pathogenic yeasts (*Candida albicans*); and three hyphamycetes (*Scytalidium dimidiatum*, *Scopulariopsis brevicaulis* and *Aspergillus fumigatus*). The micro-organisms were collected from Pasteur Institut, Paris (France) or the Microbiology Laboratory, Faculty of Medecine, Besançon (France).

2.4.2. Agar incorporation method

The antifungal assays of *Santolina* essential oils were carried out by agar incorporation method (Benjilali et al., 1986; Yang et al., 1996; Griffin et al., 2000). The essential oils were dissolved in 99% EtOH this solvent was used as a negative control. Then a precise volume is mixed aseptically with 100 ml of Sabouraud glucose agar (SGA) to give final concentrations of 1000, 750, 500, and 250 $\mu L ml^{-1}$. Dermatophytes were inoculated by disposing 5 mm mycelium in the centre of plates while *C. albicans* by adding 1 ml from a blastospore suspension. The Petri dishes were then incubated for 24 h at 37 °C for *Candida* and *Aspergillus* and at 24 °C for 7 days for dermatophytes and *Scopulariopsis*. These tests were carried out in triplicates. Two methods served in the evaluation of the antifungal activity of the essential oil: (1) the minimal inhibitory concentration (MIC), the lowest concentration which inhibits the visible growth of fungi during the incubation period.

(2) the percentage inhibition 1% according to the method of Singh et al. (1993).

3. Results

3.1. Chemical composition of the essential oil

With the help of hydrodistillation of flowerheads, picked from *S. chamaecyparissus* that had yellow colour and an agreeable smell, the yield oil was 0.062% (v/w), volume/fresh weight, as a result of which essential oil is obtained. Whereas the root oil had orange–brown colour and a pungent odour with 0.148% yield oil. The results obtained by qualitative and quantitative analyses by GC and GC/MS are shown in Table 1.

All the compounds acquired are listed in the order of their elution. Sixty-seven constituents were identified representing 99.14% and 99.44% of the total *S. Chamaecyparissus* flowerhead and root oil respectively. The prevalent constituents were 1,8-cineole (12.94%), β-eudesmol (10.49%), terpinene-4-ol (6.97%), γ-cadinene (6.55%), spathulenol (5.80%), camphor (5.27%), germacrene-D (5.03%) and myrtenol (4.26%). Oxygenated monoterpenes represented 36.94% of the total oil, 1.8 cineole (12.9%) and terpinen-4-ol (6.9%) the most abundant compound. Significant amounts of oxygenated sesquiterpenes (29.8%) were mainly represented by *β*-Eudesmol

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Table 1 Composition of *S. chamaecyparissus* flower head and root essential oils.

No	Compounds	RI[a]	Percentage	
			Flowerhead	Root
1	2-Butanone	896	0.04	–
2	α-Pinene	1020	0.09	0.02
3	Camphene	1071	0.09	0.02
4	β-Pinene	1113	0.88	0.04
5	Sabinene	1124	0.56	–
6	Myrcene	1161	2.96	0.10
7	α-Phellandrene	1168	0.22	–
8	α-Terpinene	1186	0.22	–
9	Limonene	1196	0.15	–
10	1,8-Cineole	1214	12.94	0.03
11	*(z)*-*β*-Ocimene	1235	0.00	–
12	δ-Terpinene	1245	1.04	0.03
13	p-Cymene	1269	0.79	–
14	Terpinolene	1286	0.16	–
15	Isoamyl isovalerate	1287	0.29	–
16	Perillene	1306	0.18	–
17	E-2-hexenyl acetate	1327	–	0.03
18	Yomogi alcohol	1382	0.03	–
19	Fenchone	1404	0.03	–
20	Artemisia cetone	1410	0.13	0.06
21	Camphene hydrate	1442	0.06	–
22	Ylangene	1452	0.17	–
23	α-Cubebene	1459	0.05	–
24	α-Ylangene	1467	0.07	–
25	Camphanolene aldehyde	1470	0.09	–
26	α-Copaene	1489	0.01	0.83
27	Artemisia alcohol	1495	0.08	0.03
28	α-Gurjunene	1510	0.08	0.06
29	Camphor	1517	5.27	0.10
30	Linalool	1548	0.33	–
31	Linalyl acetate	1554	0.47	–
32	Elemene	1555	0.13	–
33	Isobornyl acetate	1582	0.50	0.04
34	Bornyl acetate	1586	0.44	0.06
35	Terpinen-4-ol	1600	6.97	0.72
36	Myrtenal	1623	0.69	–
37	Allo-aromadendrene	1638	1.06	0.03
38	Trans pinocarveol	1654	0.58	0.50
39	Isoborneol	1668	0.59	–
40	β-Gurjunene	1677	0.66	0.04
41	α-Terpineol	1693	0.53	0.03
42	Borneol	1702	3.67	0.97
43	Germacrene-D	1703	5.03	0.08
44	δ-Cadinene	1755	0.60	0.23
45	γ-Cadinene	1756	6.55	0.67
46	Cuminaldehyde	1773	1.04	3.72
47	Ar-curcumene	1776	1.18	2.49
48	Myrtenol	1791	4.26	–
49	Carveol	1804	1.14	0.05
50	β-bisabolol	2014	0.55	0.09
51	Ledol	2030	2.45	1.03
52	(E)-Nerolidol	2036	0.38	0.02
53	Elemol	2070	0.61	0.05
54	Viridiflorol	2090	0.67	0.09
55	Cadinol	2145	1.14	0.07
56	Spathulenol	2128	5.80	0.11
57	Patchoulene	2154	0.53	0.07
58	τ-Cadinol	2164	3.21	0.70
59	τ-Muurolol	2187	0.19	0.06
60	α-Cadinol	2200	1.98	0.15
61	Carvacrol	2204	2.51	0.11

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Table 1 (*continued*)

No	Compounds	RI[a]	Percentage	
			Flowerhead	Root
62	α-Bisabolol	2212	2.36	0.06
63	β-Eudesmol	2233	10.49	0.06
64	Phytol	2603	1.18	0.19
65	NI_1 (molecular weight = 200)	2655	0.20	48.13
66	NI_2 (molecular weight = 200)	2699	0.17	37.31
67	Hexadecanoic acid	2845	0.90	0.88
	Total		99.14	99.44
	Monoterpenes oxygenated		36.94	2.52
	Sesquiterpenes oxygenated		29.83	2.50
	Monoterpenes hydrocarbons		7.24	0.22
	Sesquiterpenes hydrocarbons		16.83	3.77
	Aldehydes		1.82	3.73
	Divers		6.10	1.27
	NI		0.38	85.44

[a] RI: retention indices on polar column; (–): compound absent order of elution and percentages (%) of individual components are given on HP Innowax polar column; NI: non identified compounds.

(10.9%) and spathulenol (5.8%). Sesquiterpene hydrocarbons (16.8%) were also observed. The monoterpene hydrocarbons represented only 7.2% of the total oil.

3.2. Antifungal and antibacterial activities

Antifungal activity of the flowerhead and the root essential oils has been reported in Table 2. In the presence of 500 μg/ml of essential oil, the inhibition rate varied from 0 to 89.25%. Out of three dermatophytes tested, the flowerhead oil showed a very strong inhibition rate (73–89.25%); whereas root essential oil showed an average to strong inhibition rate (29.03–68.00%). *E. floccosum* which was the most resistant to the root essential oil (29.03%) was the most sensible to the flowerhead oil (89.25%). We have noticed that there is no inhibition on the pathogenic yeast *C. albicans* at 500 μg/ml, though it was sensible at 1000 μg/ml. The MIC varied from 500 to 1000 μg/ml and from 750 to 1000 μg/ml respectively for the flowerhead and the root essential oils of S. *chamaecyparissus* (Table 2).

Table 2 Antifungal activity of *S. chamaecyparissus* flowerhead and root essential oils using percentage inhibition of micro-organisms and minimum inhibitory concentration in μg/ml.

Microorganisms	Flowerhead essential oil		Root essential oil	
	I%[a]	MIC[b]	I%	MIC
Trichophyton rubrum (B)	85.36	500	68	750
Microsporum canis (IP)	73.00	750	30.95	1000
Epidermophyton floccosum (B)	89.25	500	29.03	1000
Aspergillus fumigatus (B)	82.00	500	50.00	1000
Scopulariopsis brevicaulis (B)	85.70	500	42.85	1000
Scytalidium dimidiatum (IP).	83.82	500	44.11	1000
Candida albicans (B)	0.00	1000	0.00	1000

[a] I%: percentage inhibition of micro-organisms in the presence of 500 μg/ml of essential oil (0–25%, no or little inhibition; 26–50%, average inhibition; 51–75%, strong inhibition).

[b] MIC: minimum inhibitory concentration (μg/ml); strains from IP (Institute Pasteur de Paris, France) and from B (Microbiological laboratory; Faculty of Medicine Besancon, France).

According to the results of the agar diffusion method, *E. faecalis* (ATCC 29212) was the most susceptible microorganism which was strongly inhibited by the flowerhead oil (inhibition zone was 26 mm) (Table 3). As it has been shown in the antifungal tests, bacteria seem to be more sensible to flowerhead than root essential oil.

The concentration rate of MIC has been reported in Table 3. Both EOs of *S. chamaecyparissus* delineate antibacterial activity against all bacterial strains, with MIC values ranging from 0.625 to 10 μg/ml. None of the essential oil tested inhibited *P. aeruginosa* ATCC 27853 by the disc diffusion method, though this germ was sensible to both flowerhead and root Eos by microwell dilution method with MIC of 0.626 μg/ml and 5 μg/ml respectively.

4. Discussion

Compared with previous reports, the composition of the oil that we analyzed greatly differed from the oils of *S. chamaecyparissus* L. collected in Egypt (Aboutabl et al., 1987), in France (Vernin, 1991), in Italy (Tognolini et al., 2006) and in Algeria (Nouasri et al., 2015) (44.5%, 45%, 28.24% and 40.33% respectively). Valencia and Djeddi et al., 2012 studied that *S. chamaecyparissus* L. of Algeria showed a different distribution of components with camphor as the main component at 25% and 31.1% respectively.

In previous reports the oils of *S. canescens*, *S. rosmarinifolia* and *S. oblongifolia* were characterized by santolindiacetylene, acetylenic, and dihydrofuran sesquiterpenes respectively with some interesting similarities existing between the oils of Tunisian *S. chamaecyparissus* and *S. ligustica*, which oil was characterized by myrcene, 1,8-cineole, and terpinen-4-ol (Tirillini et al., 2007). Moreover, key compounds analyzed in the oil from flowerheads of *S. chamaecyparissus* differ from those detected in Tunisian essential oil (Terpinene-4-ol (34%), Borneol (17%), Germacrene D (5%) and γ-Terpinene (7%)) (Fridlender et al., 2002).

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Table 3 *In vitro* antibacterial activity of *Santolina chamaecyparissus* flowerhead and root essential oils.

Bacteria	Growth inhibition zone diameter[a] (mm)			MIC[c]		
	Flowerhead oil[b]	Root oil	Gentamicin	Flowerhead oil	Root	Gentamicin
Escherichia coli (*ATCC* 25922)	15	10	19	1.25	1.25	0.312
Pseudomonas aeruginosa (*ATCC* 27853)	7	NA	14	0.625	5	0.312
Enterococcus faecalis (*ATCC* 29212)	26	7	23	0.625	2.5	0.312
Staphylococcus aureus (*ATCC* 25923)	12	12	19.5	2.5	1.25	0.312
Citobacter freundeii	13	NA	18	10	5	0.625
Proteus mirabilis	12	NA	14	10	>10	1.25

[a] Inhibition zones including the diameter of the paper disc (6 mm).
[b] 10 µl of essential oil/disc.
[c] MIC: Minimal Inhibition Concentration (µg/ml); NA: no activity.

In our study, *S. chamaecyparissus* essential oil demonstrated variability in the quantitative and qualitative contents with the same species growing in other geographic areas. The composition of plants analyzed in the present study was close to that of *S. chamaecyparissus* L. sp. *squarrosa* from Spain (*p*-cymene, 5%), camphor, 25.19%), (bornyl acetate, 9.9%), allo-aromadendrene (19.04%), α-muurolene (7.28%), artemisia cetone (0.64%)) (Villar et al., 1986).

Further investigations of the essential oil composition for a larger number of populations of this species, along with more data on the different species of the genus *Santolina*, can be helpful in chemiotaxonomy.

EO from *Santolina* seems to be more active when using MIC method than disc diffusion method. Agar diffusion technique is considered less suitable to estimate the antimicrobial activity of EOs since the active volatile components are likely to be evaporated together with the dispersing solvent, and their non polar nature prevents them from diffusion through the agar media (Goñi et al., 2009).

As per our knowledge, the antibacterial and antifungal actions of *S. chamaecyparissus* flowerhead and root essential oils, have been reported for the first time. However, few studies on antimicrobial activities of *S. chamaecyparissus* were previously described. An essential oil obtained from the herb of *S. chamaecyparissus* L. is effective in controlling candidiasis both *in vivo* (Suresh et al., 1995), and *in vitro* (Djeddi et al., 2012; Nouasri et al., 2015); while the dichloromethane extracts of *S. chamaecyparissus* L. subsp. *squarrosa* exhibited interesting inhibition against *Rhizopus stolonifer* (Lopez et al., 2008).

Several components detected in the Tunisian *S. chamaecyparissus* flowerhead essential oil have been reported as efficient antibacterial or antifungal agents, such as 1,8-cineole (Cimanga et al., 2002; Tzakou et al., 2001; Pattnaik et al., 1997; Tirillini et al., 1996), α-terpineol, terpinen-4-ol, α-pinene, *β*-pinene, α-phellandrene, and *p*-cymene (Dorman and Deans, 2000). It has been demonstrated in the literature that the inhibitory activity of an essential oil results from a complex interaction between its different constituents, which may produce additive, synergistic or antagonistic effects, even for those present at low concentrations Xianfei et al., 2007; Zakarya et al., 1993), i.e. 1,8-Cineole in combination with camphor has shown higher antimicrobial effects (Viljoen et al., 2003). On the other hand, the compounds present in the greatest proportions are not necessarily responsible for the greatest share of the total activity, and then, the activity could be attributed to the presence of minor components such borneol (3.66%), carvacrol (2.51%) and myrtenal (0.66%) known already to exhibit an antibacterial activity (Zakarya et al., 1993; Viljoen et al., 2003; Onawunmi, 1984).

The type of action of antimicrobial agents depends on the kind of microorganism and evidence designated that in the case of essential oils, it is largely associated with cell membrane damage. Their chemical components are characteristically hydrophobic and will accumulate in the lipid-rich environments of cell membrane structures and cause structural and functional damage. On the other hand, hydrophobicity and ability to damage cell membrane structures are not the only factors involved and it is obvious that toxicity is linked to an optimum range of hydrophobicity (Cox et al., 2000; Lambert et al., 2001).

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