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Norsesquiterpene hydrocarbon, chemical composition and antimicrobial activity of *Rhaponticum carthamoides* root essential oil

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ABSTRACT

A detailed analysis of *Rhaponticum carthamoides* (Willd.) Iljin root essential oil was carried out by GC, GC–MS and GC–FTIR techniques. In total, 30 components were identified, accounting for 98.0% of total volatiles. A norsesquiterpene 13-norcypera-1(5),11(12)-diene (22.6%), followed by aplotaxene (21.2%) and cyperene (17.9%), were isolated and their structures confirmed by 1D and 2D-NMR spectra (COSY, ROESY, HSQC, HMBC and INADEQUATE). Selinene type sesquiterpenes and aliphatic hydrocarbons were among minor constituents of the essential oil. The oil exhibited antimicrobial activity against 5 of 9 strains of bacteria and yeast, when tested using broth micro-dilution method. Minimum inhibitory concentrations ranged between 32 and 256 μg/ml.

1. Introduction

The genus *Rhaponticum* Vaill. (= *Stemmacantha* Cass.) belongs to the family Asteraceae and comprises about 25 species of perennial herbs distributed mostly in the Old World from Morocco and Portugal to Eastern Asia and eastern Australia. *Rhaponticum carthamoides* (Willd.) Iljin is a perennial herbaceous plant originating in mountains of Siberia, Middle Asia and Mongolia, currently cultivated throughout the Eastern Europe. As the phylogenetic relationships of the genus are yet unresolved (Hidalgo et al., 2006), this species often occurs in scientific literature equally under synonyms *Leuzea carthamoides* (Willd.) DC., preferred primarily in Eastern Europe and post-Soviet countries, or less common *Stemmacantha carthamoides* (Willd.) Dittrich (Zeleny, 2004).

The roots and rhizomes of the plant have been traditionally used in folk Siberian medicine mainly as tonic, roborant and stimulant (Gammerman and Grom, 1976). Nowadays, it is widely marketed as an adaptogenic or anabolic dietary supplement to athletes (Lafont and Dinan, 2003) and is valued as a rich natural source of ecdysteroids that are present in all parts of the plant. Extracts have been shown to possess wide spectra of biological activities, e.g. radical scavenging and antimicrobial, attributed to the presence of flavonoids, sesquiterpene lactones and polyacetylenes (Opletal et al., 1997).

Essential oils isolated from aerial parts or underground organs of *R. carthamoides* were preliminarily reported (Geszprych and Weglarz, 2002; Belov et al., 1994). Chemistry of volatile oils from aerial or underground organs of other species of the genus has been investigated, *R. uniflorum* (L.) DC. (Liu et al., 1993; Zhu et al., 1991), and recently *R. acaule* DC. (Boussaada et al., 2008). In addition, chemical composition of volatile oils isolated from a vast number of species of closely related genus *Centaurea* have been reported in the past (Senatore et al., 2005, 2006), denoting the importance of volatile constituents research within this taxon.

Present work aims to provide a detailed report on chemical composition of essential oil isolated from the roots of *R. carthamoides*, a commercially and phytotherapeutically most important species of the genus.

2. Results and discussion

2.1. Chemical analysis, isolation and structure elucidation of unknown or tentatively identified constituents

The distillation yielded 0.043% w/w of essential oil of dark yellow color, resembling in its olfactometric properties the smell

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of fresh-cut roots. The oil was identified by means of GC, GC–MS and GC–FTIR and its composition was expressed in percent of the relative peak area from the GC analysis (Table 1). As three of the main constituents, representing 22.6%, 17.9% and 21.2% remained unidentified or tentatively identified, the oil was subjected to column chromatography on silica gel.

Peak **30** was obtained in adequate purity (96%) and was later identified by ^{1}H and ^{13}C NMR as aplotaxene (Fig. 1). Its NMR spectra were in good agreement with the published data (Boland and Jaenicke, 1981).

Compound **11** was identified as cyperene and its structure (Fig. 1) was confirmed by structural analysis on GC–MS, GC–FTIR and comparison with described ^{13}C NMR data (Joseph-Nathan et al., 1984). The use of homo- and heteronuclear 2D-NMR spectra allowed us to make also complete structural assignment of proton signals in $CDCl_3$ (Table 2).

The unknown compound **7** of elemental composition $C_{14}H_{20}$ was further purified by rechromatography on C-18 column and subjected to detailed NMR analysis. The ^{13}C NMR spectrum showed a presence of one tetrasubstituted double bond (δ 140.84 and 127.97) and exomethylene group (δ 163.74 and 96.58), which was confirmed in ^{1}H NMR spectrum (multiplets at δ 4.60 and 4.41) together with one secondary methyl group (doublet at δ 0.87, J = 6.6 Hz) and one methyl group on double bond (signal with fine splitting δ 1.61). Therefore the structure has to contain three cycles and two double bonds that lead to very complex multiplets due to many long-range couplings. The combination of 2D-H,H-COSY, 2D-H,C-HSQC, 2D-H,C-HMBC and 2D-C,C-INADEQUATE spectra allowed us to suggest a structure of 13-norcypera-1(5),11(12)-diene. The NOE contacts observed in 2D-H,H-ROESY spectrum (see Fig. 2) proved relative configurations at C4, C7 and C10 (identical with those in cyperene) and stereochemical assignment of hydrogen atoms of the methylene groups. The complete structural assignment of proton and carbon signals is given in Table 2. Additional independent evidence for suggested structure provided quantum chemical calculations. The geometry optimization and following calculation of ^{1}H and ^{13}C chemical shifts using DFT B3LYP method with basis set 6-311G+(2d,p) (GAUSSIAN 03 program package – Frisch et al., 2003) provided an excellent agreement with experimentally observed NMR data (rms = 0.998 for ^{1}H and 0.999 for ^{13}C chemical shifts – see Fig. 3).

The compound gave following MS and IR spectra: GC–MS 70 eV, *m/z* (rel. int.): 188 $[M]^+$ (56), 173 $[M—CH_3]^+$ (53), 159 $[M—C_2H_5]^+$ (29), 145 (46), 133 (38), 132 (59), 131 (100), 117 (63), 115 (52), 105 (32), 91 (65), 77 (29), 65 (15), 55 (15), 41 (31), GC–FTIR ν_{max}: 3074, 882 (=CH_2), 2935, 2862 (CH_3,CH_2), 1666 (C=C), 1458, 1379, 1325, 1199, 1142, 1069, 981 and 761 cm^{-1}.

In total, 30 components were identified in the essential oil, out of which for one component the correct isomer could not be determined and one was identified tentatively.

Besides the three predominating constituents, representing 61.7% of the total peak relative area, mainly sesquiterpene hydrocarbons were present, selina-3,7-diene **19** (5.2%), rotundene **20** (3.9%), β-selinene **22** (3.8%), α-bulnesene **25** (2.3%), 7-*epi*-α-selinene **26** (2.3%) and β-caryophyllene **14** (1.8%) among the five more abundant. Moreover, we noted the presence of unsaturated aliphatic hydrocarbon pentadec-1-ene **23** (5.2%), unknown isomer of hexadecatriene **27** (0.2%) and heptadeca-1,8-diene **29** (0.7%). Sesquiterpene hydrocarbons were the major group of constituents. The essential oil composition determined by us differed significantly to previous brief reports in the literature (Belov et al., 1994; Geszprych and Weglarz, 2002). Similar tricyclic sesquiterpene skeletons were previously reported in roots of *R. uniflorum* (Liu et al., 1993).

Occurrence of fatty acid-derived long-chain polyacetylenes and unsaturated hydrocarbons in both underground and aboveground organs is typical for family Asteraceae, tribe Cynareae (Christensen and Lam, 1990) and presence of aplotaxene in related *R. centauroides* has been reported (Christensen and Lam, 1989).

Rhaponticum carthamoides is known to contain guaianolide type of sesquiterpene lactones in all parts of the plant and sesquiterpene hydrocarbons present in the essential oil may be intermediates of their biosynthetic pathway. Presence of β-selinene **22**, 7-*epi*-α-selinene, selina-3,7-diene **19** and elemenes **6, 10** (0.4% and 0.9%) may refer to the presence of biosynthetic pathway of germacrene-type of sesquiterpene lactones and possibly to guaianolides, after enzymatic cyclization (de Kraker et al., 2003). Guaianolides are recognized as a typical chemotaxonomical marker of genus *Rhaponticum* (Nowak, 1992).

2.2. Antimicrobial activity of the essential oil

Broth micro-dilution method was used to evaluate antimicrobial activity of the essential oil against 9 standard strains of bacteria (Table 3). The oil exhibited some degree of antimicrobial activity against 4 of 8 potentially pathogenic bacteria tested at minimum inhibitory concentrations (MICs) ranging between 32 and 256 μg/ml and against yeast *Candida albicans*, at concentration of 128 μg/ml. Inhibitory activity of various extracts, fractions (Kokoska et al., 2002, 2005) and isolated compounds (Chobot

Table 1
Composition of essential oil from roots and rhizomes of *R. carthamoides*.

	Component	RI[a]	%[b]	Identification[c]
1	α-Pinene	939	tr	MS, GC
2	β-Pinene	976	tr	MS, GC
3	1-*p*-Menthene	1023	tr	MS, GC
4	Limonene	1031	tr	MS, GC
5	*p*-Cymen-8-ol	1185	0.8	MS, GC
6	δ-Elemene	1337	0.4	MS, GC
7	13-Norcypera-1(5),11(12)-diene	1352	22.6	MS, IR, NMR
8	Cyclosativene	1370	0.2	MS, GC
9	α-Copaene	1376	0.3	MS, GC
10	β-Elemene	1391	0.9	MS, GC
11	Cyperene	1399	17.9	MS, GC, IR, NMR
12	α-Gurjunene	1409	1.4	MS, GC
13	Dehydro isolongifolene[e]	1410	1.5	MS
14	β-Caryophyllene	1418	1.8	MS, GC
15	α-Guaiene	1443	0.7	MS, GC
16	*trans*-β-Farnesene	1456	0.4	MS, GC
17	β-Santalene	1461	1.6	MS, GC
18	Rotundene	1463	1.8	MS, IR
19	Selina-3,7-Diene	1469	5.2	MS, GC, IR
20	Aromadendrene	1473	3.9	MS, GC
21	γ-Himachalene	1476	0.3	MS, GC
22	β-Selinene	1485	3.8	MS, GC
23	Pentadec-1-ene	1494	5.2	MS, GC, IR
24	α-Muurolene	1499	0.3	MS, GC
25	α-Bulnesene	1502	2.3	MS, GC
26	7-*epi*-α-Selinene	1517	2.3	MS, GC
27	Hexadecatriene [d]	1569	0.2	MS, IR
28	Caryophyllene oxide	1581	0.3	MS, GC, IR
29	Heptadeca-1,8-diene	1664	0.7	MS, GC, IR
30	Aplotaxene	1667	21.2	MS, GC, IR, NMR
	Total identified		98.0	
	Monoterpene hydrocarbons		tr	
	Oxygenated monoterpenes		0.8	
	Sesquiterpene hydrocarbons		69.6	
	Oxygenated sesquiterpenes		0.3	
	Others		27.3	

[a] Retention indices relative to C_8–C_{22} *n*-alkanes on DB-5 capillary column.
[b] Quantifications are based upon GC peak integration data.
[c] MS, mass spectrometry, GC, retention indices, IR, infrared spectroscopy, NMR, ^{13}C and ^{1}H nuclear magnetic resonance.
[d] Correct isomer not known.
[e] Tentatively identified; tr = trace (<0.05%).

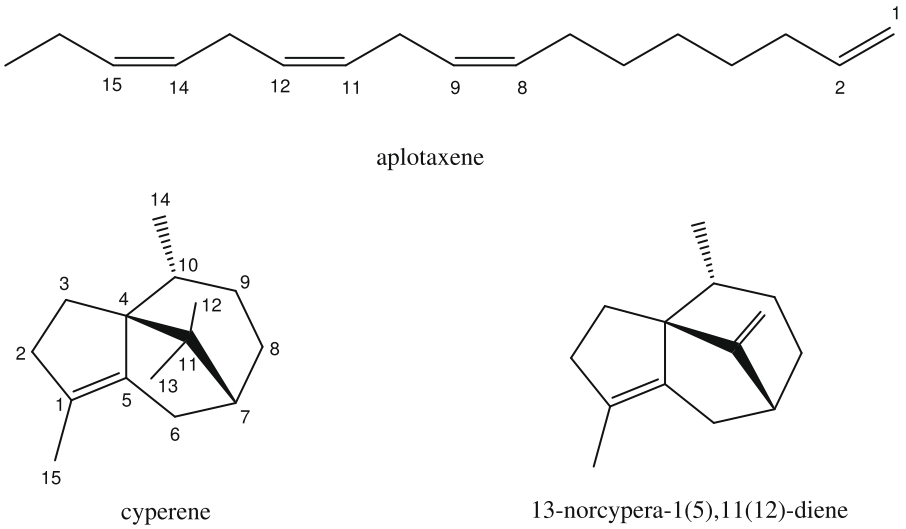

Fig. 1. Structures of aplotaxene, cyperene and newly identified 13-norcypera-1(5),11(12)-diene.

et al., 2003) of *R. carthamoides* against Gram-positive bacteria and fungi has been previously demonstrated. Volatile constituents may play additive role in the activity of the extract.

3. Experimental

3.1. Plant material and essential oil isolation

Roots and rhizomes of *R. carthamoides* were obtained on commercial basis from the breeding company ADAVO Velky Osek, Czech Republic. The plants were authenticated by taxonomist Dr. Zeleny and their voucher specimen has been deposited at the Institute of Tropics and Subtropics of the Czech University of Life Sciences Prague. Approx. 30 kg of roots and rhizomes were finely grinded and subjected to 3 h hydro distillation, using a 62 l stainless steel extractor with a glass trap, a modification of Clevenger apparatus (Albrigi Luigi s.r.l.). The yielded essential oil was dried over anhydrous sodium sulfate and stored at −20 °C prior to analyzes.

3.2. Analytical GC

GC analysis was used to determine the relative proportions of the essential oil constituents and was performed on an Agilent HP 6890 gas chromatograph, equipped with flame ionization detector. Apparatus was equipped with a DB-5 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature program was running from 60 to 250 °C at 3 °C/min and held isothermal for 10 min; injector temperature: 220 °C; detector temperature: 220 °C; carrier gas: N_2, 1 ml/min; splitless injection; injection volume 1 μl.

3.3. GC–MS analysis

GC–MS analysis was carried out using a Finnigan Focus GC (Thermo Scientific) coupled to Fisons MD 800 detector. Apparatus was equipped with a DB-5 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The MS operating conditions were as follows: injector, ion source and transfer line temperatures

Table 2
NMR data of cyperene and 13-norcypera-1(5),11(12)-diene in $CDCl_3$.

Position	Cyperene			13-Norcypera-1(2),11(12)-diene		
	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC
1	–	127.64	H-2β, H-3α, H-15	–	127.97	H-2β, H-3α, H-15
2	α: 2.60 *m* β: 2.18 *m*	42.26	H-3α, H-3β	α: 2.69 *m* β: 2.21 *m*	41.83	H-3α, H-3β, H-15
3	α: 1.40 *m* β: 1.62 *m*	26.28	H-2β	α: 1.84 *ddd* β: 1.76 *dt*	30.53	H-2β
4	–	65.83	H-3α, H-3β, H-6β, H-10, H-12, H-13, H-14	–	65.20	H-3α, H-3β, H-6β, H-9β, H-12a, H-12b, H-14
5	–	142.15	H-2β, H-3α, H-10, H-15	–	140.84	H-2α, H-3α, H-7, H-10, H-15
6	α: 1.765 *dm* β: 2.18 *m*	27.51	H-7, H-8β, H-15	α: 1.96 *m* β: 2.21 *m*	28.31	H-8α, H-8β
7	1.85 *m*	48.70	H-8α, H-8β, H-9β, H-12, H-13	2.865 *m*	46.85	H-8α, H-8β, H-9β, H-12a, H-12b
8	α: 1.28 *m* β: 1.84 *m*	27.93	H-9α, H-9β	α: 1.68 *m* β: 1.715 *m*	35.77	H-9α, H-9β, H-12a
9	α: 1.08 *m* β: 1.42 *m*	28.27	H-7, H-8α, H-10, H-14	α: 1.255 *m* β: 1.485 *m*	28.89	H-7, H-10, H-14
10	1.94 *h*	35.24	H-3α, H-3β, H-8α, H-9α, H-9β, H-14	1.77 *m*	44.71	H-3α, H-3β, H-9α, H-9β, H-12a, H-12b, H-14
11	–	41.36	H-3β, H-8α, H-12, H-13	–	163.74	H−7, H-8α, H-8β, H-10, H-12a, H-12b
12	0.94 *s*	19.35	H-13	4.60 *m* 4.41 *m*	96.58	–
13	0.76 *s*	26.10	H-12	–	–	–
14	0.795 *d*	17.96	H-9α, H-10	0.87 *d*	17.35	H-9α, H-10
15	1.61 *m*	14.17	–	1.61 *m*	14.40	–

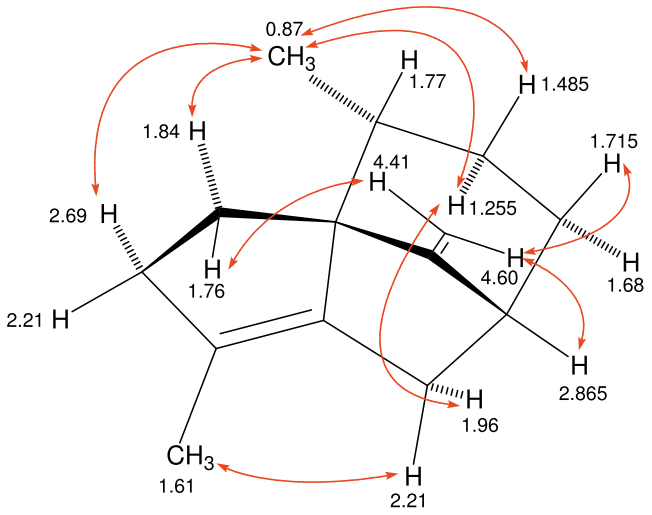

Fig. 2. The observed non-trivial NOE contacts in 2D-H,H-ROESY spectrum of 13-norcypera-1(5),12(13)-diene in $CDCl_3$.

220, 220 and 240 °C, respectively; ionization energy 70 eV; scan time 1 s; mass range 30–350 amu. GC operating conditions were as described above. The sample was injected splitless, diluted in *n*-hexane 1:1000.

Retention indices (RI) were determined relative to the retention times of a series of C_8—C_{22} *n*-alkanes. Retention index, spectra interpretation and computer matching with commercially available databases (NIST 02, Wiley 7) and literature (Adams, 1995; Joulain and Konig, 1998) were used for identification of the majority of constituents.

3.4. GC–FTIR analysis

The IR spectra of most abundant compounds were obtained using a GC–FTIR system consisting of Bruker Equinox 55 spectroscope coupled to Agilent HP 6850 gas chromatograph, equipped with the same column type as mentioned above (i.d. 0.32 mm), under following conditions: injector temperature 220 °C; carrier gas: He, 1 ml/min; oven temperature program 60 to 220 °C at 5 °C/min and held isothermal for 5 min; FT-IR resolution 8 cm^{-1}; light pipe temperature 200 °C. Prior to injection, samples were diluted in *n*-hexane and injected in splitless mode, the injection volume being 1 μl.

Table 3
Antimicrobial activity of the *R. carthamoides* root essential oil against pathogenic microorganisms.

Microorganism	MIC (μg/ml)	
	EO	Ref.
Enterococcus faecalis	256	1
Listeria monocytogenes	128	1
Staphylococcus aureus	32	0.5
Staphylococcus epidermidis	–	1
Streptococcus pyogenes	256	1
Escherichia coli	–	0.015
Pseudomonas aeruginosa	–	0.25
Salmonella enteritidis	–	0.03
Candida albicans	128	4

MIC > 512 μg/ml; EO, essential oil; Ref., reference antibiotics: nystatin for *C. albicans*, ciprofloxacine for other strains.

3.5. Compound isolation

Unknown compound and tentatively identified components were subjected to column chromatography on silica gel (5 × 80 cm; 6 g sample), eluted with pure *n*-hexane (7.5 l). In total, 15 fractions, each 500 ml were collected (F1–F15). Main constituents of the oil were present in fractions F3 (**11**), F4 (**7**) and F10–F14 (**30**). If necessary, fractions were combined and subsequently concentrated in rotary evaporator at 30 °C. Fraction F3 was subsequently purified on low volume silica gel column under same chromatographic conditions, yielding compound **11** (95%, 150 mg), later confirmed by NMR spectra as cyperene. Combined fractions F10–F14 afforded compound **30** in adequate purity (96%, 350 mg). Its structure was later confirmed by NMR spectra as aplotaxene. Fraction 4 subjected to rechromatography on C-18 reverse phase column (0.8 × 20 cm; 180 mg sample), using MeOH as eluent (50 ml). Fractions were collected by 1 ml, F4/23–F4/28 gave compound **7** (~96%, 55 mg).

3.6. NMR measurements

The NMR spectra of cyperene, 13-norcypera-1(5),11(12)-diene and aplotaxene were measured on Bruker AVANCE 500 instrument (^1H at 500.13 MHz and ^{13}C at 125.77 MHz) and/or Bruker AVANCE 600 (^1H at 600.13 MHz and ^{13}C at 150.92 MHz) in $CDCl_3$ at 32 °C. For structural assignment of proton and carbon signals the homonuclear 2D-NMR spectra (H,H-COSY, H,H-ROESY) and heteronuclear 2D-NMR spectra (H,C-HSQC and H,C-HMBC) were used. For

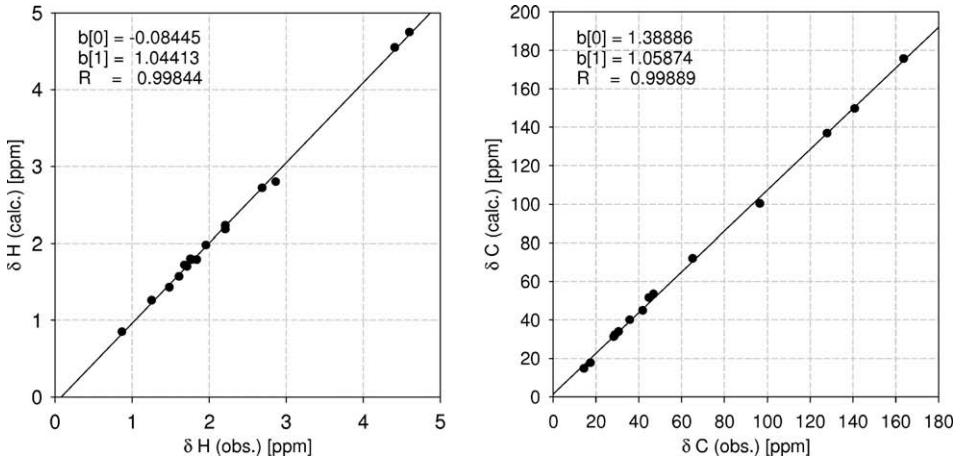

Fig. 3. Comparison of the observed and the calculated ^1H and ^{13}C chemical shifts of 13-norcypera-1(5),12(13)-diene (calculated with DFT B3LYP 6-311G+(2d,p)).

13-norcypera-1(5),11(12)-diene the 2D-C,C-INADEQUATE spectrum was also measured.

3.7. Assay on antimicrobial activity

In vitro antimicrobial activity was determined by the broth micro-dilution method (Jorgensen et al., 1999) using 96-well microtitre plates.

The following strains of bacteria were used: *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* ATCC 12228, *Streptococcuss pyogenes* ATCC 19615. The yeast strain used in this study was *Candida albicans* ATCC 10231. Mueller-Hinton medium was used for all microbial strains, except of for *Streptococcuss pyogenes*, which was grown in Brain-heart infusion broth. The essential oil was resolved in tris-buffered (pH 7.6) corresponding liquid medium, containing 0.2% agar and 0.2% tween 80 for a better dispersion of essential oil in the medium. Antibiotics ciprofloxacine and nystatin were used as a positive control for bacteria and yeast, respectively.

Two-fold dilutions (eleven) of each sample tested were subsequently carried out starting from a concentration 512 μg/ml for extracts and 8 μg/ml for antibiotics. Each well was inoculated with 5 μl of bacterial suspension at a density of 10^7 CFU/ml. The microtitre plates were incubated at 37 °C for 24 h (48 h for the yeast) and then observed for MICs. The growth of microorganisms was observed as turbidity determined by the Multiskan Ascent (Thermo Scientific) microplate reader at 630 nm. MICs were calculated based on the density of the growth control and were the lowest oil concentrations that resulted in 80% reduction in growth compared with that of the extract-free growth control. Tween 80 (0.2%) enriched medium was assayed as negative control and did not inhibit any of tested bacterial strains. All samples were tested in triplicate.

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