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Antibiofilm formation, antioxidant and anticholinesterase activities of essential oil and methanol extract of *Marrubium deserti* de Noé

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Abstract
The essential oil obtained from the aerial parts of *Marrubium deserti* de Noé. (Lamiaceae), growing in the North fringe of the Algerian Sahara, was analyzed by GC-MS. Thirty-eight compounds were identified, representing 99.70% of the total oils. The GC-MS analysis revealed the presence of tetracosane, germacrene D, *Δ*-cadinene, *α*-cadinol and *t*-cadinol as the main constituents, representing 31.11%, 7.91%, 6.52%, 6.26% and 5.81%, respectively. Minimum inhibitory concentrations (MICs) of essential oil and methanol extract were calculated by microtitre broth dilution method, and antibiofilm effects by microplate biofilm assay. The highest antibiofilm activity was found to be 69.31% against *Micrococcus luteus* NRRL B-4375 at 25 mg/mL for methanol extract and 36.62% against *Candida albicans* ATCC 10239 at 25 µL/mL concentration for essential oil. The antioxidant activity was determined using three complementary tests namely: β-carotene-linoleic acid, DPPH free radical scavenging, and CUPRAC assays. In β-carotene-linoleic acid assay, both the oil and the extract exhibited good lipid peroxidation inhibition activity, demonstrating 76.81 ± 0.59 and 86.33 ± 0.27% at 200 µg/mL concentration, respectively. In DPPH and CUPRAC assays, however, the methanol extract exhibited high antioxidant activity; however, the essential oil showed weak activity. The *in vitro* anticholinesterase activity, was carried out against acetylcholinesterase and butyrylcholinesterase enzymes spectrophotometrically using Elman method. Methanol extract showed weak acetylcholinesterase and butyrylcholinesterase inhibitory activities, while the essential oil was inactive against both enzymes.

Keywords: Marrubium deserti, antibiofilm, anticholinesterase, antioxidant, essential oil.

1. Introduction
The genus *Marrubium* includes six species and one hybrid in Algeria: *Marrubium vulgare* L., *M. spinum* L., *M. peregrinum* L., *M. alysson* L., *M. alyssoides* Pomel, *M. willkommu* Magn. (*M. supinum* X *vulgare*) and *M. deserti* de Noé. [1], the latter is the subject of this study.
M. deserti is a small perennial shrub (20-30 cm high), very branched, with woolly leaves and stems. The flowers are pale violet or pink. The calyx is bright green and evergreen around the fruit, which is a tetra-achene typical of the Labiatae. The leaves are velvety and opposite, and are generally terminated by three large teeth of variable form [1, 2]. The species is endemic of central and north Algerian Sahara. It grows on desert pastures and flowers in spring (March-April) [1-3]. The plant grows in an arid and semi-arid climate, with an annual rainfall of 100 mm It is usually found in non-saline wadis on gravelly-sandy soils [3, 4]. The plant is used by the

local people in traditional medicine in Algeria. The leaves, stems and flowers are used against intestinal disorders, respiratory diseases, fever, cough, dysmenorrhea, scorpion stings and Allergies [5, 6].
Recently, the flavonoids, phenyl ethanoid and diterpenoids have been isolated from *Marrubium* species which some of them are considered as therapeutic (anti-tumor) and pharmacological [7-11]. In a recent study 6-dehydroxy-19-acetyl-marrubenol, 19-acetyl-marrubenol, 6-acetyl-marrubenol and 16-epoxy-9-hydroxy-labda-13(16), 14-diene, *β*-sitosterol, stigmasterol, *β*-sitosterol 3-*O*-glucoside, and phytol were isolated from *M. deserti* [12]. In other study, marrulibacetal A, desertine, 15-epi-cyllenin A, marrubiin, marrulactone, marrulibacetal, β-stigmasterol, apigenin, apigenin-7-*O*-*β*-neohesperidoside, apigenin-7-*O*-glucoside, terniflorin and apigenin-7-*O*-glucuronide, acteoside and forsythoside B were isolated and elucidated [13]. The chemical composition of essential oil, however, gave six major compounds: germacrene D (45.7%), *β*-bourbonene (4.0%), α-terpinolene (3.9%), *Δ*-cadinene (3.8%), 1-octen-3-ol (3.7%) and α-copaene (3.5%) [14]. The antioxidant effect, antiviral, antibacterial and antigenotoxic activities of aqueous extracts of *M. deserti* were also reported [13, 15-18]. However, to the best of our knowledge, there has been no scientific report on antibiofilm formation and anticholinesterase activities of the extracts of *M. deserti* yet. Therefore, this paper presents the first study on the antibiofilm formation and anticholinesterase activity of essential oil and methanol extract of *M. deserti* together with the chemical composition of hydrodistillated oil of the aerial parts of *M. deserti* growing in El-Oued - Algeria.

2. Experimental methods

2.1. Plant material

The aerial parts (leaves, flowers and steams) of *M. deserti* were collected during the flowering period in April 2012 from Meguibra, El-Oued, Algeria (34°14'0"N, 6°1'0"E) at -11 m altitude and taxonomic identification of plant was confirmed by Dr. Youcef Halis. A voucher sample was deposited in the Laboratory of Biomolecules and Plant Breeding, University of Larbi Ben Mhidi Oum El Bouaghi, Algeria (*M. deserti* voucher number ZA 143).

2.2. Extraction of the essential oil

The essential oil of dried aerial parts (300g) of *M. deserti* was obtained via hydrodistillation by using a Clevenger type apparatus for 4 h. The oil was dried over anhydrous sodium sulphate and stored under nitrogen until required.

2.3. Gas chromatography analysis

GC analysis of the oil were performed using a Shimadzu GC-17 AAF, V3, 230V LV Series (Kyoto, Japan) gas chromatography, equipped with a FID and a DB-1 fused silica column [30m x 0.25 mm (i.d.), film thickness 0.25 μm]; the oven temperature was held at 60°C for 5 min, then programmed to 240°C at 4°C/min and held isothermal for 10 min; injector and detector temperatures were 250°C and 270°C respectively; carrier gas was He at a flow rate of 1.3 mL/min; Sample size, 1.0 μL; split ratio, 50:1. The percentage composition of the essential oil was determined with a Class-GC 10 computer program.

2.4. Gas chromatography–mass spectrometry (GC–MS)

The analysis of the essential oil was performed using a Varian Saturn 2100 (Old York Rd., Ringoes, NJ, USA), ion trap machine, equipped with a DB-1 MS fused silica non-polar capillary column [30 m x 0.25 mm (i.d.), film thickness 0.25 μm]. Carrier gas was helium at a flow rate of 1.4 mL/min. The oven temperature was held at 60°C for 5 min, then increased up to 240°C with 4°C/min increments and held at this temperature for 10 min. Injector and transfer line temperatures were set at 250 and 180°C, respectively. Ion trap temperature was 200°C. The injection volume was 0.2 μL and split ratio was 1:30. EI-MS measurements were taken at 70 eVionisation energy. Mass range was from *m/z* 28 to 650 amu. Scan time was 0.5 s with 0.1 s inter scan delays. Identification of components of the essential oils was based on GC retention indices and computer matching with the Wiley, NIST-2005 and TRLIB Library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature [19] and, whenever possible, by co-injection with authentic compounds.GC and GC-MS spectra were performed at the Department of Chemistry, Faculty of Sciences, Muğla Sıtkı Koçman University.

2.5. Determination of minimum inhibitory concentrations and antibiofilm activity

2.5.1 Microorganisms and condition for cultivation

In the present study, the microorganisms used in the experiments were : Gram positive bacteria (*Staphylococcus aureus* (ATCC 25923, ATCC 6538-P), *Staphylococcus epidermidis* MU 30, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* RSKK 863, *Streptococcus mutans* CNCTC 8/77 and *Micrococcus luteus* NRRL B-4375) and yeast (*Candida albicans* ATCC 10239) were used as test microorganisms. The above-mentioned bacteria except *C. albicans* were grown in nutrient broth (NB, Difco); *C. albicans* was grown in sabouraud dextrose broth (SDB, Difco). *C.albicans* ATCC 10239 was incubated at 30 ± 0.1 °C for 24-48 h. Other bacterial strains were incubated at 37 ± 0.1 °C for 24 h.

2.5.2 Minimal inhibitory concentration (MIC) assay

MICs were determined by a microtitre broth dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [20]. The MIC was defined as the lowest essential oil/extract concentration that yielded no visible growth. The test medium was MHB (Mueller Hinton Broth) and the density of bacteria was 5×10^5 colony-forming units (CFU)/mL. Cell suspensions (100 µL) were inoculated in to the wells of 96-well microplatesin the presence of essential oil with different final concentrations (6.25, 12.5, 25, 50, 80, 160 µL/mL) and in the presence of methanol extract with different final concentrations (1.56, 3.12, 6.25, 12.5, 25, 50 mg/mL). The inoculated microplates were incubated at 37ºC for 24 h before being read.

2.5.3 Effect of essential oil and methanol extract on bacterial biofilm formation

The effect of *M. deserti* essential oil and extract at concentrations including 1, 1/2, 1/4, 1/8 and 1/16 MIC on biofilm-forming ability of test microorganisms were tested with a microplate biofilm assay [21]. Briefly, 1% of overnight cultures of isolates were added into 200 µL of fresh Tryptose-Soy Broth (TSB) supplemented with 0.25% glucose and cultivated in the presence and absence of *M. deserti* essential oil/extract without agitation for 48 h at 37 ºC. The wells containing TSB+cells served as control. After incubation, the wells were washed with water to remove planktonic bacteria. The remaining bacteria were subsequently stained with 0.1% crystal violet solution for 10 min at room temperature. Wells were washed once again to remove the crystal violet solution. 200 µL of 33% glacial acetic acid poured in wells. After shaking and pipetting of wells, 125 µL of the solution from each well transferred to a sterile tube and volume reached to 1 mL with distilled water. Finally optical density (OD) of each well measured at wavelength of 550 nm (Thermo Scientific Multiskan FC, Vantaa, Finland). Percentage of inhibition of the tested extracts was calculated using the formula:

$$Biofilm\ inhibition\ (\%) = \frac{OD_{550Control} - OD_{550Sample}}{OD_{550Control}} \times 100$$

2.6. Anticholinesterase activity

The inhibition activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured by spectrophotometric method developed by Elman et al., in 1961, with slight modification[22, 23].

2.7. Antioxidant activity

2.7.1. Free radical-scavenging activity (DPPH assay)

The free radical scavenging activity was performed using slightly modified DPPH assay [23, 24].

2.7.2. β-Carotene-linoleic acid assay

The slightly modified β-Carotene-linoleic acid test system was used to assay lipid-peroxidation inhibitory activity [23, 25].

2.7.3. Cupric reducing antioxidant capacity (CUPRAC)

The slightly modified Apak's method was used to determine the cupric reducing antioxidant capacity [23, 26].

2.8. Determination of total phenolic compounds

The content of phenolic compounds was determined using Folin–Ciocalteu reagent, and expressed as microgramme of pyrocatechol equivalents [27]. The absorbance was read at 760 nm. The concentration of

phenolic compounds was calculated according to the following equation that was obtained from the standard pyrocatechol graph:

$$Absorbance = 0.006\,\mu g\ pyrocatechol + 0.035 \quad (r^2 = 0.978)$$

2.9. Determination of total flavonoid concentration

Total flavonoid content was determined according to the aluminum method. The results were expressed as quercetin equivalents [28]. The concentration of flavonoid compounds was calculated according to following equation that was obtained from the standard quercetin graph.

$$Absorbance = 0.051\,\mu g\ quercetin + 0.001 \quad (r^2 = 0.999)$$

2.10. Statistical analysis

The antioxidant and the anticholinesterase activity assays were in triplicate analyses. The data were recorded as means ± standard error meaning. Student's *t*-test were used to determine the significant differences between means; $p < 0.05$ were regarded as significant.

3. Results and discussion

3.1. Chemical composition

The essential oil having yellow color was obtained from aerial parts using hydrodistillation (0.15%, v/w) method. Totally 38 components were identified representing 99.70% of the oil (Table 1). *β*-caryophyllene is omnipresent in the essential oils of most *Marrubium* species, followed by germacrene D and bicyclogermacrene [14]. Whereas, the major compounds of essential oil of *M. deserti* were tetracosane (31.11%,), germacrene D (7.91%), *Δ*-cadinene (6.51%), *α*-cadinol (6.26%), and *t*-cadinol (5.81%). bicyclogermacrene (2.84%) and *β*-caryophyllene (1.32%) were also determined as a constituent of the essential oil (**Table 1**). The monoterpenoids represented 9.26% while sesquiterpenoids 16.45% of the total oil. The monoterpenes and sesquiterpenes were in the ratio of 4.32% and 33.85%, respectively.

3.2. Minimum inhibitory concentrations and antibiofilm activity

The antimicrobial activity of the essential oil and methanol extract against 6 bacteria and *Candida albicans* ATCC 10239 was given in **Table 2**. The essential oil inhibited the growth of all microorganisms between 25 and 80 µL/mL concentrations. The MIC values of methanol extract were in the range of 3.25-25 mg/mL. According to the results, *B. subtilis* ATCC 6633 was found as the most susceptible strain against methanol extract. The extract has low activity on the growth of *M. luteus* NRRL B-4375, *S. mutans* CNCTC 8/77 and *S. epidermidis* MU 30 that were only inhibited at high concentration (25 mg/mL). Essential oil at the MIC's inhibited biofilm formations of all microorganisms tested in various percentages. The essential oil exhibited the highest antibiofilm activity against *C. albicans* ATCC 10239 at 25 µg/mL (MIC) concentration with 36.62%.

In the presence of 25 mg/mL extract (MIC), the mean biofilm formation values were equal to 69.31% for *M. luteus* NRRL B-4375, 29.27% for *S. mutans* CNCTC 8/77 and 56.28% for *S. epidermidis* MU 30. The antibiofilm activity of essential oil on tested strains was lower than that of methanol extract.

3.3. Anticholinesterase activity

The anticholinesterase activity of the essential oil and methanol extract against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes was given in **Table 3**. Galantamine was used to compare the activity as a standard drug. The methanol extract exhibited weak inhibitory activity against AChE and BChE enzymes. The IC_{50} values were calculated as 277.4 ± 13.6, and 93.3 ± 0.7µg/mL, respectively. On the other hand, the essential oil was inactive against both AChE and BChE.

3.4. Total phenolic and total flavonoid contents and Antioxidant activity

Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity. The phenolic compounds are also known as powerful chain breaking antioxidants [29]. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily is ingested from a diet rich in stems and vegetables [30]. The concentration of phenolics and flavonoids in the extract were expressed as micrograms of pyrocatechol and micrograms of quercetin equivalents per milligrams of the extract, respectively. The methanol extract had 58.17 ± 0.03 µg pyrocatechol equivalents and 5.2 ± 0.03 µg quercetin equivalents.

Table 1: Chemical composition (%) of the essential oil of *M. deserti*

N°	RI[a]	Compounds	Composition (%)[b]
1	890	cyclofenchene	1.52 ± 0.001
2	932	*α*-thujene	0.52 ± 0.0001
3	936	*α* -pinene	2.15 ± 0.0012
4	978	*β*-pinene	0.82 ± 0.0001
5	1025	*D*-limonene	1.05 ± 0.0011
6	1123	camphor	1.94 ± 0.0013
7	1131	citronellal	0.88 ± 0.0001
8	1149	*n*-nonanol	1.06 ± 0.0014
9	1164	terpinen-4-ol	0.62 ± 0.0001
10	1172	myrtenal	0.72 ± 0.0001
11	1176	*α*-terpineol	0.66 ± 0.0001
12	1178	myrtenol	0.28 ± 0.00001
13	1241	*p*-cumic aldehyde (Cuminal)	0.27 ± 0.00001
14	1243	carvone	1.54 ± 0.0014
15	1271	perillaldehyde	1.70 ± 0.002
16	1290	thymol	1.15 ± 0.0013
17	1351	*α*-cubebene	0.74 ± 0.0001
18	1388	*β*-bourbonene	0.56 ± 0.0001
19	1390	*β*-elemene	3.84 ± 0.002
20	1408	*β*-caryophyllene	1.32 ± 0.0009
21	1460	alloaromadendrene	2.54 ± 0.0014
22	1477	*γ*-gurjunene	0.84 ± 0.0003
23	1481	germacrene D	**7.91 ± 0.0023**
24	1486	eremophilene	1.44 ± 0.0003
25	1494	bicyclogermacrene	2.84 ± 0.003
26	1500	*α*-muurolene	0.69 ± 0.0001
27	1513	*γ*-cadinene	1.95 ± 0.0003
28	1523	*Δ*-cadinene	**6.51 ± 0.003**
29	1545	*α*-calacorene	0.49 ± 0.0001
30	1553	*E*-nerolidol	0.86 ± 0.0001
31	1572	spathulenol	0.96 ± 0.0001
32	1578	caryophyllene oxide	0.90 ± 0.0001
33	1633	*t*-cadinol	**5.81 ± 0.003**
34	1635	*α*-muurolol	1.07 ± 0.0001
35	1643	*α*-cadinol	**6.26 ± 0.003**
36	1648	*allo*-himachalol	2.43 ± 0.0003
37	1653	7-*epi*-*α*-Eudesmol	1.57 ± 0.0001
38	2400	tetracosane	**31.11 ± 0.001**
39	2406	unidentified	0.11 ± 0.0001
40	2418	unidentified	0.13 ± 0.0001
41	2422	unidentified	0.05 ± 0.0000
		Total identified:	99.70
		Monoterpenes:	4,32
		Monoterpenoids:	9,26
		Sesquiterpenes:	33.85
		Sesquiterpenoids:	16,45
		Others:	35.84

[a]Kovats index on DB-1 fused silica column.
[b]The percentage concentrations of the compounds are the means of three parallel measurements ± S.E.M. by GC (p<0.05).

Table 2: MIC and antibiofilm activity results of the essential oil and methanol extract of *M. deserti*

	Essential oil						**Methanol extract**					
	Planktonic	% inhibition on biofilm formation					Planktonic	% inhibition on biofilm formation				
Microorganisms	MIC (µL/mL)	MIC/1	MIC/2	MIC/4	MIC/8	MIC/16	MIC (mg/mL)	MIC/1	MIC/2	MIC/4	MIC/8	MIC/16
Staphylococcus aureus ATCC 25923	50	19.21	-	-	-	-	6.25	28.90	11.65	-	-	-
Staphylococcus aureus ATCC 6538P	80	4.96	-	-	-	-	12.5	27.25	7.37	-	-	-
Staphylococcus epidermidis MU 30	25	29.81	20.33	-	-	-	25	56.28	28.51	10.78	5.31	-
*Bacillus subtilis*ATCC 6633	50	27.98	11.25	-	-	-	3.25	7.57	-	-	-	-
Bacillus cereus RSKK 863	25	26.66	18.25	4.62	-	-	12.5	49.66	25.68	10.85	-	-
Micrococcusluteus NRRL B-4375	25	23.75	10.70	-	-	-	25	69.31	45.46	27.45	3.32	-
Streptococcus mutans CNCTC 8/77	25	20.36	-	-	-	-	25	29.27	10.88	-	-	-
Candida albicans ATCC 10239	25	36.62	17.69	-	-	-	12.5	34.32	18.47	5.51	-	-

- : no inhibition

Table 3: Acetylcholinesterase and butyrylcholinesterase inhibitory activities of the essential oil and methanol extract of *M. deserti*[a]

Samples	**AChE assay**		**BChE assay**	
	Inhibition (%) (200 µg/mL)	IC_{50} (µg/mL)	Inhibition (%) (200 µg/mL)	IC_{50} (µg/mL)
Essential oil	*NA*	*NA*	*NA*	*NA*
MeOH Extract	35.4 ± 1.9	277.4 ± 13.6	53.2 ± 0.1	93.3 ± 0.7
Galantamine[b]	81.4 ± 1.0	5.011 ± 0.09	75.5 ± 1.1	53.9 ± 0. 6

NA: not active
[a] IC_{50} values represent the means ± S.E.M. of three parallel measurements ($p< 0.05$).
[b] Standard drug.

In lipid peroxidation inhibition assay, the essential oil exhibited high inhibition (IC_{50} = 35.9 ± 9.3 µg/mL) against lipid peroxidation, while in DPPH assay, the essential oil demonstrated weak DPPH free readical scavenging activity. As seen it the essential oil composition, there are no phenolic compounds (only thymol) to scavenge the DPPH radicals (**Table 4**). However, the compounds particularly the bicyclic compounds as well as the conjugated mono and sesquiterpenoids are responsible for the lipid peroxidation activity. These compounds can scavenge the singlet oxygen. Therefore, they protect the β-carotene color against bleaching, indirectly. The methanol extract showed highest antioxidant activity both in DPPH assay and in Lipid peroxidation inhibition assay. These results are supported the previously studies, where the polar extracts showed higher antioxidant activity than unpolar extracts [17].

Table 4: Antioxidant activity (%) of the essential oil and methanol extract of *M. deserti* by the DPPH and β-carotene/linoleic acid assays[a]

	DPPH Assay				β-carotene/linoleic acid assay			
	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Essential oil	-	1.34±0.54	3.05±0.58	5.47±0.52	62.29±4.00	72.96±1.37	75.09±0.08	76.81±0.59
MeOH Extract	72.02±0.15	76.77±0.17	77.74±0.26	78.35±0.10	77.62±0.90	81.90±0.45	85.97±0.11	86.33±0.27
BHT[b]	40.43±0.05	53.18±0.51	73.91±0.11	95.1±8,44	93.65±0.06	95.08±0.02	97.83±0,08	99.36±0,09
α-tocopherol	91.16±0.17	92.03±0.55	93.77±0,07	97.25±0,06	92.89±0.27	93.32±0.33	94.22±0,28	96.02±0,30

[a] Values expressed are means ± SEM of three parallel measurements ($p < 0.05$).
[b] BHT: Butylatedhydroxytoluene.

The CUPRAC assay utilized copper(II)-neocuproine (CU(II)-Nc) reagent as the chromogenic oxidizing agent. It is based on the measurement of absorbance at 450 nm by the formation of stable complex between neocuproine and copper (I). As shown in (**Figure 1**). The methanol extract and α-tocopherol had similar activities. Only at 800 µg/mL concentration, the methanol extract (3.81 ± 0.00) exhibited higher reducing power activity than *α*-tocopherol (2.9 ± 0.00) and close activity to that of BHA (3.8 ± 0.00)) at 800 µg/mL. However, the activity of essential oil was lesser than the positive controls.

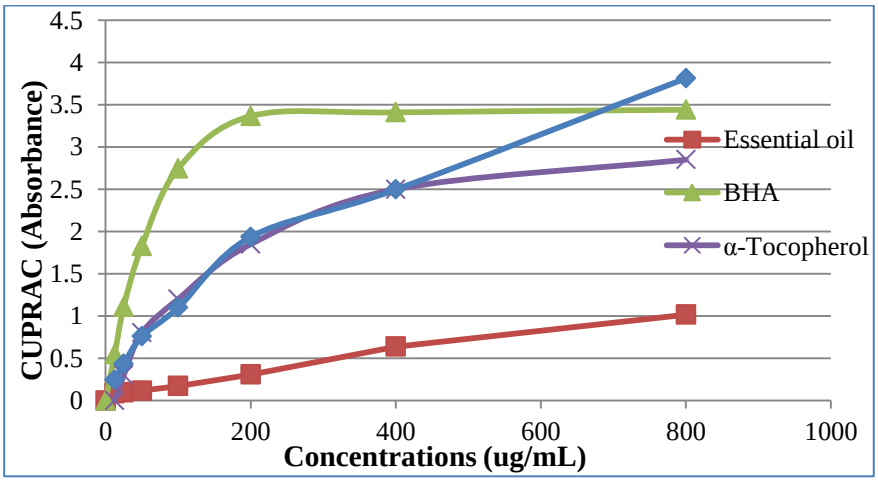

Figure 1: Cupric reducing antioxidant capacity of the methanol extract and essential oil of *M. deserti* Values are means ± S.E.M., $n = 3$, $p < 0.05$, significantly different by Student's *t*-test.

Conclusion

This study deals with the chemical composition of essential oil of *M. deserti* and its biological activities with its methanol extract. Some link could be observed between antioxidant, antibiofilm formation and anticholinesterase activities of methanol extract and essential oil, maybe due the total phenolic and flavonoids contents. The methanolic extract and the oil were found to be effective antioxidants and antimicrobials in different *in vitro* assays and can be suggested as a natural additive in food and pharmaceutical industries. Results obtained from methanol extract were found to be stronger than those obtained from essential oil. However, further studies are necessary to evaluate the origin of the activity.

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