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Chemical composition, Antimicrobial and anti-acetylcholinesterase activities of essential oils from the Tunisian *Asteriscus maritimus* (L.) Less

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Abstract: The chemical composition, the acetylcholinesterase inhibitory effect and the antimicrobial activity of the Tunisian *Asteriscus maritimus* (L.) Less essential oils obtained from flowers, leaves and stems were investigated. According to the GC and GC-MS analysis results, 45 (99.32%), 34 (94%) and 29 compounds (94.95%) were identified, respectively. In the essential oils extracted from the flowers and leaves of *A. maritimus*, oxygenated sesquiterpenes were abundantly found (57.82 and 46.39%, respectively). Hexdecanoic acid was detected in an important amount in leaves and stems (30.35 and 49.18%, respectively), while *β*-eudesmol (24.25%), menthyl acetate (17.03%) and pentadecanal (15.38%) were the most abundant constituents in flowers. To evaluate the *in vitro* antimicrobial activity, all volatiles were tested against some bacterial and fungal agents. It was found that only the volatile oil from flowers exhibited interesting antibacterial and antifungal activities. When screened for their anti-acetylcholinesterase activity, essential oil from flowers exhibited the highest activity (IC_{50} =95 µg/mL). To our knowledge, this is the first report of acetylcholinesterase inhibition and antimicrobial activity of the Tunisian *A. maritimus* essential oils.

Keywords: *Asteriscus maritimus* (L.); essential oil; chemical composition; antimicrobial; anti-acetylcholinesterase.

Introduction:

Asteriscus maritimus (L.) Less, which belongs to the family of the *Asteraceae*, is one of four *Asteriscus* species growing in Tunisia[1]. Flowering from April to September, it is papillose, fragrant with petiolate leaves ranging from elongate to spatulate. The perennial plant produces gold-yellow flowers which are tubular in shape with 5 lobes; achenes 1-5 mm, papus 1-1.5 mm[1]. This species is common in rocky areas of the western portion of the Mediterranean basin, in southern Portugal as well as in western and southern Greece[2].

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In Tunisia, it can be found in Carthage, Zembretta, El Haouaria, Zaghouan, Hammam Sousse and Hergla[1]. A considerable attention has been given to the genus *Asteriscus* from which mainly sesquiterpenes have been reported. Asteriscunolides A-D were previously isolated from the aerial parts of some *Asteriscus* species such as *A. aquaticus*[3] and *A. vogelii*[4]. Other sesquiterpene lactones such as asteriscanolide and aquatolide were obtained from the hexane extract of *A. aquaticus*[3,5]. Flavonoids[6,7], bisabolone hydroperoxides[8] as well as farnesol and thymol derivatives[9] were also described as constituents of extracts from *Asteriscus* plants and reported to possess antimicrobial and hypoglycemic activities[6,7], whereas Asteriscunolides C and D have shown phytotoxic activities and the last one also exhibited cytotoxic effects[4]. While floricultural research on *A. maritimus* has been performed as have mycological studies[10-12] which revealed that it is parasitized by *Entyloma asterisci-maritimi* Vanky, sp. nov. and the fungus *Rhizoctonia solani*[13]. An activity of the lactone fraction in controlling the development of insects in stored food products was also reported[14]. Furthermore, Fraternale *et al.*[2] have focused their attention on the composition of the essential oil isolated from the aerial part of the Italian *A. maritimus*. The presence of myrtenyl acetate (44.2%) as the major component as well as terpinen-4-ol (4.5%) and terphenyl (17.5%) were reported to be associated with the insecticidal activity of the oil against flesh fly larvae and insects.

At present, approximately 3000 essential oils are known, 300 of which are commercially important, especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. Also essential oils have been widely used throughout history for their pharmacological activities such as antibacterial, antifungal, antiviral, antiparasitic, insecticidal and antispasmodic properties[15-18]. Furthermore, plants have been used traditionally to enhance cognitive function and to alleviate other symptoms associated nowadays with Alzheimer's disease[19]. Alzheimer is a progressive degenerative neurologic disorder resulting in impaired memory and behavior. Epidemiological data indicate a potentially considerable increase in the prevalence of the disease over the next two decades[20]. Most treatment strategies have been based on the cholinergic hypothesis which postulated that memory impairments in patients suffering from this disease result from a deficit of cholinergic function in brain. One of the most promising approaches for treating this disease is to enhance the acetylcholine level in the brain by means of acetylcholinesterase (AChE) inhibitors[21]. Several AChE inhibitors are being investigated for the treatment of Alzheimer. Most of the drugs used in this disease therapy are formed by an enzyme inhibitor, e.g. galantamine, isolated from the extract of snowdrop[22] and few reports exist for the inhibitor activity of acetylcholinesterase by essential oils[23,24].

As an extension of the chemical investigation of the genus *Asteriscus*, we report here on the chemical composition of the essential oils from the flowers, leaves and stems of the Tunisian *A. maritimus* by using the most known conventional techniques (GC/FID and GC-MS). In particular, these essential oils were also screened *in vitro* for the first time in Tunisia and, to the best of our knowledge, in the world for their antibacterial, antifungal and anti-acetylcholinesterase activities.

Results and Discussion

Chemical composition. Each part of *A. maritimus* (flowers, leaves and stems) was subjected to hydrodistillation using Clevenger apparatus and yielded yellow oils with characteristic odor. The volatile fraction yield (w/w) differed according to the tested part. It was 0.58% from flowers, 0.32% from leaves and 0.25% from stems. The chemical analysis of the volatile oils was performed by GC-FID and GC-MS. Table 1 shows the chemical composition of the plant volatile fractions in terms of components and classes of compounds.

Table 1. Chemical composition of *Asteriscus maritimus* flowers, leaves and stems volatile compounds

N°	Compound	RI[a] (HP-5)	Proportion (%)[b]		
			Flowers	Leaves	Stems
1	1-Hexanol	873	0.13	-[c]	-
2	Camphene	953	0.22	-	-
3	Myrcene	994	0.29	-	-
4	*α*-Phellandrene	1008	0.57	-	-
5	**Meta-cymene**	1030	**9.34**	-	-
6	Limonene	1034	1.73	-	-
7	2,6-Dimethyl phenol	1103	0.27	-	-
8	4-Ethyl benzaldehyde	1171	0.17	-	-
9	Myrtenol	1194	0.23	-	-
10	Nogigiku alcohol acetate	1237	1.21	-	-
11	**Menthyl acetate**	1294	**17.03**	-	-
12	*(E)*-Methylcinnamate	1379	0.24	-	-
13	*β*-Patchoulene	1389	0.33	-	-
14	Isobornyl isobutyrate	1429	0.17	-	-
15	*β*-Santalene	1462	0.21	-	-
16	*γ*-Gurjunene	1478	0.19	-	-
17	*α*-Curcumene	1487	0.43	-	-
18	Methy isoeugenol	1490	0.43	-	-
19	Bicyclo germacrene	1495	0.60	-	-
20	*α*-Amorphene	1506	1.44	-	-
21	*δ*-Cadinene	1522	0.38	-	-
22	Isoamyl salicylate	1531	2.96	-	-
23	*α*-Agarofuran	1545	0.19	-	-
24	Davanone	1587	3.52	-	-
25	Cedrol	1595	0.13	-	-
26	Hexadecane	1600	0.14	0.87	-
27	Tetradecanal	1616	0.18	-	-
28	1-Epi-cubenol	1623	0.76	-	-
29	Henesol	1635	0.19	0.30	
30	*T*-cadinol	1640	0.6	-	-
31	***β*-Eudesmol**	1650	**24.25**	**3.62**	0.51
32	*α*-Eudesmol	1653	2.23	-	-
33	Dihydro eudesmol	1656	0.56	-	-
34	Citronelly tiglate	1668	7.51	-	-
35	Geranyl tiglate	1697	0.63	-	-
36	**Pentadecanal**	1709	**15.38**	-	-

37	***(E,Z)*-Farnesol**	1743	0.14	**11.99**	0.58
38	*(E,E)*-Farnesol	1748	-	-	1.71
39	Cedryl acetate	1764	0.53	-	-
40	*(Z,E)*-Farnesyl acetate	1822	1.10	-	-
41	*(E,E)*-Farnesyl acetate	1842	0.17	-	-
42	Sclarene	1963	1.62	-	-
43	Methyl octadecanoate	2127	0.17	-	-
44	Hydroxy isopimarene	2132	0.26	-	1.61
45	1-Tricosene	2290	0.33	-	-
46	Pentacosane	2490	0.40	-	1.26
47	3-Carene	1014	-	0.87	-
48	Thymol methyl ether	1275	-	0.64	0.54
49	Isobornyl butyrate	1471	-	2.43	-
50	*α*-Bulnesene	1503	-	0.44	-
51	*γ*-Cadinene	1511	-	0.56	-
52	Elemol	1544	-	0.63	-
53	*Cis*-Nerolidol	1566	-	1.17	0.53
54	Caryophyllene alcohol	1568	-	0.98	-
55	Germacrene-D	1575	-	0.78	0.20
56	Phenyl ethyl tiglate	1583	-	0.81	-
57	***γ*-Eudesmol**	1624	-	**14.99**	**4.45**
58	*α*-Acorenol	1630	-	1.08	-
59	Cubenol	1641	-	2.16	0.52
60	Hexyl salicylate	1675	-	1.58	0.51
61	***α*-Bisabolol**	1681	-	**4.31**	-
62	Ethyl tetradecanoate	1797	-	1.59	0.67
63	Nootkatone	1821	-	1.80	0.59
64	*α*-Vetivone	1835	-	0.68	1.08
65	Nonadecane	1898	-	0.81	1.57
66	Methyl palmitate	1917	-	0.68	1.21
67	Kaurene	2036	-	0.22	0.43
68	**Hexadecanoic acid**	1948	-	**30.35**	**49.18**
69	Hexadecyl acetate	2009	-	0.25	3.73
70	Hydroxy-isopimarene	2133	-	2.16	-
71	**Phytol**	2113	-	**3.22**	**15.57**
72	Methyl linoleate	2087	-	1.02	-
73	**1-Heneicosene**	2095	-	0.33	**6.53**
74	1-Docosene	2185	-	0.24	-
75	*Trans*-Ferrughol	2323	-	0.48	0.21
76	Tetracosane	2394	-	0.83	0.60
77	*(E)*-Nuciferol	1754	-	-	0.32
78	Acorone	1806	-	-	0.46
79	Cedrane diol	1888	-	-	0.28
80	Incensole acetate	2189	-	-	0.23
81	*Trans*-Ferruginol	2323	-	-	0.63
82	Dihydro abietyl alcohol	2364	-	-	0.24
Hydrocarbons			0.87	3.08	9.96
Fatty acid and derivatives			0.17	33.64	50.06
Terpenoids					

Monoterpene hydrocarbons	12.15	0.87	-
Oxygenated monoterpenes and derivatives	22.1	3.03	1.05
Sesquiterpene hydrocarbons	3.58	1.78	0.20
Oxygenated sesquiterpenes and derivatives	57.82	46.39	14.76
Diterpene hydrocarbons	1.62	0.22	0.43
Oxygenated diterpenes and derivatives	0.26	5.86	18.49
Others	0.75	-	-
Total identified	**99.32**	**94**	**94.95**
Yield (w/w)	0.58	0.32	0.25

[a]: Retention index of compounds identified in the Tunisian *A. maritimus* essential oils; [b]Percentages of individual components are given on HP-5 capillary column. [C]:Not identified. Bold type indicates major component;

Forty five constituents were identified in the flowers essential oil representing 99.32% of the total volatiles, 97.53% from which are terpenoids. The oil consisted of 0.87% hydrocarbons, 12.15% monoterpene hydrocarbons, 22.1% oxygenated monoterpenes, 3.58% sesquiterpene hydrocarbons, 57.82% oxygenated sesquiterpenes, 1.62% diterpene hydrocarbons, 0.26% oxygenated diterpenes. *β*-eudesmol (24.25%) has been identified as the chemotype of the oil.

On the other hand, thirty four compounds were identified in the leaves essential oil. This fraction was characterized by a high content of oxygenated terpenes (55.28%) and fatty acid and derivatives (33.3%). Hexadecanoic acid was the major compound (30.35%), followed by *γ*-eudesmol (14.99%), (*E,Z*)-farnesol (11.99%) and *α*-bisabolol (4.31%).

Table 1 also shows that stems essential oil was rich in oxygenated terpenoids (34.3%) whereas hydrocarbon terpenoids were not representative (0.63%). Furthermore, hexadecanoic acid was found to be the major component in stems essential oil (49.18%) afterward phytol (15.57%), 1-heneicosene (6.53%) and *γ*-eudesmol (4.45%).

Oxygenated sesquiterpenes and derivatives represented the most abundant constituents of the essential oils from *A. maritimus* flowers and leaves, while those isolated from leaves and stems were characterized by the presence of a high percentage of fatty acid and derivatives (33.64 and 50.06%, respectively) than in the oil from flowers (0.17%).

The above results show significant differences in the composition of the extracted essential oils as compared to those from the Italian *A. maritimus*[2]. However, myrtenyl acetate (44.2%), terphenyl (17.5%), *(Z)-β*-farnesene (12.9%), myrtenol (5%) and terpinen-4-ol (4.5%) representing the major components in *A. maritimus* aerial part oil were all absent in our samples.

When comparing the chemical composition of the essential oils of leaves, stems, and flowers of the Tunisian *A. maritimus* plants with those of *A. graveolens* from neighbouring countries (Algeria or Morocco), these two species were found to have differences in the major compounds detected. Indeed, leaves and stems oils of *A. graveolens* were characterized by high content of oxygenated sesquiterpenes with 6-oxo- and 6-hydroxycyclonerolidol as major components whereas the flower essential oil was dominated by the new monoterpenic compound cis-8-acetoxychrysanthenyl acetate[25]. However, in our samples, all these compounds were absent.

Antibacterial activity The antibacterial activity of the essential oils from flowers, leaves and stems of *A. maritimus* was tested *in vitro* by using the disc diffusion method. According to the

results given in Table 2, the volatile oil of *A. maritimus* flowers exhibited an interesting antibacterial activity against all bacterial agents tested but not as important as that obtained with the standard reference (ampicillin). The data obtained from MIC estimation shows that the growth of the bacterial strains tested was inhibited by the flower essential oil applied at 4.5 mg/disc (i.e. 4 µL/disc).

Table 2. Growth inhibition zones (the average of three measurements) of flowers, leaves and stems essential oils and minimal inhibitory concentration – MIC (mg/disc) of flowers essential oil from *Asteriscus maritimus*.

	Diameters of the inhibition zone (in mm)					
	Reference products)		Leaves	stems	flowers	MIC of flowers (mg/disc)
Bacterial agents tested	*Ampicillin* (5 mg/mL)	*Carbendazim* (0.5 mg/ mL)				
Pseudomonas sp.	31.3	-[a]	na[b]	na	11[a]	4.5
Burkholderia sp.	32.3	-	na	na	9[a]	4.5
Bacillus sp.	30.3	-	na	na	11.25[a]	4.5
Tested fungi						
Aspergillus flavus	-	12.7	na	na	11[a]	9
A. niger	-	21.3	na	na	10.25[a]	9
Penicillium sp.	-	18.7	na	na	10.25[a]	9
Botrytis cinerea	-	0	na	na	9.75[a]	9
Fusarium oxysporum f. sp. *lycopersici*	-	18	na	na	10.5[a]	9

Values affected with the same letter are not significantly different based on the Student-Newman-Keuls (SNK) test at $P \leq 0.05$; [a]: Not tested; [b]: No activity detected; 1 µL of essential oil from *A. maritimus* flowers corresponds to 1.125 mg

The inherent activity of leaves and stems essential oils can be expected to relate to the chemical configuration of the components, the proportions in which they are present and to the interactions between them[26-28]. An additive effect is observed when the combined effect is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects[29]. Some studies have concluded that whole essential oil have a greater

antibacterial activity than the major components mixed[30,31], which suggests that the minor components are critical to the activity and may have a synergistic effect or potentiating influence. As discussed above, synergism between carvacrol and its biological precursor *p*-cymene has been noted when acting on *Bacillus cereus* vegetative cells. It appears that *p*-cymene, a very weak antibacterial, swells bacterial cell membranes to a greater extent than carvacrol does. By this mechanism *p*-cymene probably enables carvacrol to be more easily transported into the cell so that a synergistic effect is achieved when the two are used together[32.] The antibacterial properties of the volatile fraction of *A. maritimus* flowers may be also associated to the high content of the oxygenated sesquiterpenes (57.82%)[33]. In addition, the antibacterial activity may be related to the presence of *β*-eudesmol (Figure 1) in *A. maritimus* flowers, constituting 24.25%; this compound is already known to exhibit several biological activities especially antimicrobial properties[34].

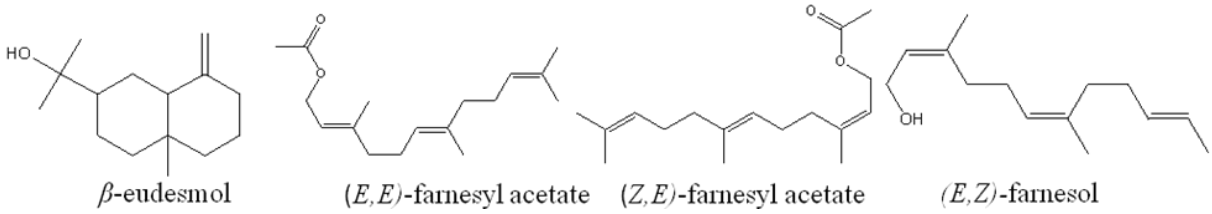

Figure 1. Structures of principal compounds of the essential oil *of Asteriscus maritimus* flowers especially considered to be responsible for the antimicrobial activity

Antifungal activity. According to the results given in Table 2, the volatile oil of *A. maritimus* flowers exhibited an interesting antifungal activity against *Aspergillus flavus*, *A. niger*, *Penicillium* sp., *F. oxysporum* f. sp. *lycopersici* and *Botrytis cinerea* but not as higher as that noted with the standard reference (carbendazim) excepting *B. cinerea* which has developed resistance to this fungicide[35] and was inhibited with *A. maritimus* essential oil. Appreciable antifungal potential of flowers oil could be associated with the presence of oxygenated terpenes, strongly present in *A. maritimus* flowers oil at a rate of 80.18%[36]. As well, according to Lis-Balchin et al.[28] and Pauli[37], oxygenated sesquiterpenes, such as (*Z*,*E*)-farnesyl acetate (1.10%), (*E*,*E*)-farnesyl acetate (0.17%) and (*E*,*Z*)-farnesol (0.14%) (Figure 1) known with their important antifungal activity could contribute to this effect. Table 2 shows that the MIC of the essential oil of *A. maritimus* flowers is 9 mg/disc (i.e. 8 µL/disc).

However, In spite of the presence of oxygenated terpenes in the oils compositions of leaves and stems, known to possess an antifungal activity[36], no antifungal activity were detected. The outcome of a test can be affected by factors such as the method used to extract the essential oil from plant material, the inoculum quantity, the growth phase, the culture medium used, pH, and the incubation time and temperature[38].

Anti-acetylcholinesterase activity. The AChE inhibitory activity of the essential oils of flowers, leaves and stems of *A. maritimus* has never been reported before. Essentials oils of this plant were tested to determine their ability as acetycholinesterase inhibitors and the results are depicted in Table 3.

The greatest inhibitory activity was exhibited by the essential oil of flowers (IC_{50}= 95 ± 0.25 µg/mL). Also, the obtained results revealed that the volatile fraction of leaves and stems of *A. maritimus* could be considered an inhibitor of acetylcholinesterase (IC_{50} = 11 ± 0.68 µg/mL and 13 ± 0.75 µg/mL, respectively). The IC_{50} value of our samples compared to that of the essential oil of *Ferula lutea* (IC_{50} = 70.25 ± 5.41 µg/mL)[39] reinforces this suggestion.

Table 3. Acetylcholinesterase inhibition capacity represented by IC_{50} (µg/mL) of the essential oils of *Asteriscus maritimus* flowers, leaves and stems.

	Acetylcholinesterase inhibition IC_{50} (µg/mL)[a]
Flowers	9.50 ± 0.25
Leaves	11 ± 0.68
Stems	13 ± 0.75
Eserine[b]	0.29 10^{-2}± 0.01

[a]: Values are expressed as means ± standard deviation of three replicates; [b]: Eserine which was used as positive control.

Conclusion

In the current study, the chemical composition of the essential oils of *A. maritimus* has been investigated for the first time in Tunisia. It has been found that essential oils extracted from the flowers and leaves were significantly rich in oxygenated sesquiterpenes (57.82 and 46.39%, respectively). Hexdecanoic acid was detected in an important amount in leaves and stems (30.35 and 49.18%, respectively). The isolated oils were tested for their inhibitory effects against some bacterial and fungal agents and it was found that only the volatile oil from flowers exhibited interesting antibacterial and antifungal activities. Moreover, the volatile fraction of flowers, leaves and stems of *A. maritimus* could be considered an inhibitor of acetylcholinesterase (IC_{50} =95 µg/mL, IC_{50} = 11 ± 0.68 µg/mL and 13 ± 0.75 µg/mL, respectively). To our knowledge, this is the first report of acetylcholinesterase inhibition and antimicrobial activity of the Tunisian *A. maritimus* essential oils.

Experimental Section

Plant material. *A. maritimus* were collected from the region of Hergla (Tunisia), in April 2010 and identified by Professor Féthia HARZALLAH-SKHIRI, in the Laboratory of Plant biology and Botanic, Higher Institute of Biotechnology of Monastir, Tunisia. A voucher specimen (R.A.M-10) was deposited in the same laboratory. The fresh plant was separated in three parts: flowers, leaves and stems. Each part was divided in to little pieces and weighed before the extraction of volatile compounds.

Extraction of essential oils. The fresh flowers (900 g), leaves (800 g) and stems (800 g) were separately submitted to hydrodistillation on a Clevenger-type apparatus for 4 h. The essential

oils were separately collected by decantation, dried over sodium sulphate, weighted and stored in sealed glass vials at 4-5°C prior to analysis.

Chromatographic analysis. The composition of the oils was investigated by GC-FID and GC-MS. The analytical GC was carried out by using a HP5890-series II gas chromatograph (Agilent Technologies, California and USA) equipped with: Flame ionisation detector (FID), HP-5 (30 m × 0.32 mm), 0.25 µm film thickness fused silica capillary column. The carrier gas was nitrogen (1.2 mL/min). The oven temperature programming was 1 min isothermal at 50°C, then 50 to 280°C at a rate of 5°C/min and held isothermal for 1 min. The injection port temperature was 250°C, detector 280°C. The volume injected is 0.1 µL of a 1% solution (diluted in hexane). Percentages of the constituents were calculated by electronic integration of FID peak areas without the use of response factor correction. GC-MS was performed in a Hewlett-Packard 5972 MSD system. An HP-5 MS capillary column (30 m × 0.25 mm, ID, film thickness of 0.25 µm) was directly coupled to the MS. Oven temperature was programmed (50°C for 1 min, then 50 to 280°C at rate of 5°C/min) and subsequently, held isothermal for 20 min. Injector port: 250°C, detector: 280°C, split ratio 1:50. Volume injected: 0.1 µL of 1% solution (diluted in hexane); mass spectrometer: HP5972 recording at 70 eV; scan time: 1.5 s; mass range: 40 to 300 amu. Software adopted to handle mass spectra and chromatograms was a Chemstation.

Identification of the compounds. The identification of the components was based on their GC retention index on an apolar column relative to C_9-C_{30} alkanes; computer matching of spectral MS data with those from Wiley 275 Library and the comparison of the fragmentation patterns with those reported in literature[40,41].

Antibacterial activity.

Bacterial strains. Three bacterial agents, isolated from Tunisian soils, were selected as test microorganisms of the antibacterial activity of *A. maritimus* essential oils: *Pseudomonas* sp. (Pa 499), *Burkholderia* sp. (Bg 35) and *Bacillus* sp. (Bp 420). They were cultured at 25°C on Nutrient Agar (NA) medium for 48 h before use.

Antibacterial assay. The effect of *A. maritimus* oils on bacterial growth inhibition was determined using the agar disc diffusion method[42]. NA medium cooled at 45°C was supplemented with a bacterial suspension (10^6 CFU/mL) and poured into Petri plates. After solidification, sterile Whatman paper discs (diameter 6 mm) were placed at the surface of the culture medium and 8 µL of the essential oil was dropped onto each disc. The negative control plates had no oil added to the filter paper whereas in the positive control plates, discs were impregnated with the same volume (8 µL) of an ampicillin solution (5 mg/mL). The treated Petri dishes were incubated at 25°C for 48 h. The antibacterial activity was evaluated by measuring the diameter of the inhibitory zones formed around the discs. The experiment was duplicated or repeated for precision. The minimal inhibitory concentration (MIC), defined as the lowest concentration of the oil that inhibited visible growth after incubation was carried out by using the disc diffusion method[43,44]. Various increasing volumes of essential oil were tested i.e. 4, 6, 8, 10 and 12 µL per disc. It should be mentioned that 1 µL of *A. maritimus* essential oil corresponds to 1.125 mg.

Antifungal activity.

Test organisms. Five phytopathogenic fungal species were used for the antifungal testing namely: *Aspergillus flavus*, *A. niger*, *Penicillium* sp., *Botrytis cinerea* and *Fusarium*

oxysporum f. sp. *lycopersici*. These fungi were obtained from the Laboratory of Phytopathology of the Regional Center of Research in Horticulture and Organic Agriculture (CRRHAB) of Chott-Mariem, Tunisia. They were cultured at 25°C on potato dextrose agar (PDA) medium one week before use.

Antifungal assay. The disc diffusion method was used for the screening of the antifungal activity of *A. maritimus* essential oils[45]. A conidial suspension of the tested fungi was prepared (10^4-10^5 CFU/mL) and added to PDA medium cooled at 45°C and poured uniformly into Petri plates (diameter 90 mm). Sterilized paper discs (6 mm, Whatman No. 1 filter paper) were impregnated with 8 µL of the volatile oil and placed on the culture plates whereas the negative control plates had no oil added to the filter paper. In the positive control plates, discs were imbibed with the same volume (8 µL) of a carbendazim suspension (0.5 mg/mL, commercial source Bavistin). The diameter of the inhibition zone (mm) around the disc was measured after incubation at 25°C for 4 days and compared with control. The test was performed in triplicate. The MIC was determined as described above in the antibacterial activity section.

Anti-acetylcholinesterase activity. Acetylcholinesterase enzymatic activity was measured as described by Falé et al.[46]. Briefly, 90 µL of 50 mM Tris-HCl buffer, pH=8, 30 µL of sample (75.10^{-2}, $1.5.10^{-1}$, 3.10^{-1}, 6.10^{-1}, 0.125, 0.25, 0.5 and 1 mg/mL) and 7.5 µL of acetylcholinesterase solution containing 0.26 U/mL were mixed in a microwell plate and left to incubate for 15 min. Subsequently, 22.5 µL of a solution of AChI (0.023 mg/mL) and 142 µL of 3 mM DTNB were added. The temperature during the measurement was proximally 25°C. The absorbance was read after 60 mn at 405 nm when the reaction reached equilibrium. A control reaction was carried out using water instead of essential oil and it was considered 100% activity. The percentage Inhibition ((%) IP) is given as follow:

$$(\%)\ IP = 100 - (A_{sample}/A_{control}) \times 100$$

Where A_{sample} is the absorbance of the essential oil containing reaction mixture and $A_{control}$ the absorbance of the reaction without the volatile. Tests were carried out in triplicate and a blank with Tris-HCl buffer instead of enzyme solution was used.

The measurements were repeated three times and values are expressed as IC_{50} ± standard deviation[47]. The IC_{50} value of the positive control, eserine[48,49], was 0.0029 µg/mL.

Statistical analysis. Data were subjected to one-way analysis of variance (ANOVA) by using SPSS 10.0 for Windows according to a completely randomized design where the microorganisms tested were the only fixed factor. In order to compare the microorganism's susceptibility to the essential oil tested, mean diameters were separated using the Student-Newman-Keuls (SNK) test (at $P \leq 0.05$).

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