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Chemical composition, antioxidant, anticholinesterase, antimicrobial and antibiofilm activities of essential oil and methanolic extract of *Anthemis stiparum* subsp. *sabulicola* (Pomel) Oberpr

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ABSTRACT

Anthemis species are traditionally used to treat infectious and inflammatory processes, among others clinical disturbances. In the current study, the chemical composition, the total phenolic and flavonoid contents, the antioxidant, anticholinesterase, antimicrobial, and antibiofilm activities of *Anthemis stiparum* subsp. *sabulicola* aerial parts methanolic extract (As-ME) and essential oil (As-EO) were investigated. The chemical composition of As-EO was established by GC-MS and GC-FID. Total phenolic and flavonoid contents of As-ME were spectrophotometrically determined. Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging, cupric reducing antioxidant capacity (CUPRAC) and β-carotene bleaching assays were applied to evaluate the antioxidant potential. The anticholinesterase activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes were carried out spectrophotometrically. The antimicrobial activity was assessed by Minimal Inhibitory Concentration (MIC) using broth microdilution method against 7 ATCC® bacterial and one ATCC® yeast reference strains. The antibiofilm effect was determined quantifying the percentage of adhesion inhibition. GC-MS and GC-FID identified 72 compounds (99.02%), being As-EO predominantly constituted by germacrene D (11.13%), t-cadinol (11.01%), camphor (6.73%), spathulenol (6.50%) and isoamyl salicylate (6.45%). The total phenolic and flavonoid contents of As-ME were 13.6 ± 0.03 and 5.9 ± 0.04 pyrocatechol equivalents and quercetin equivalents, respectively. In β-carotene-linoleic acid assay, As-ME showed the best lipid peroxidation inhibition activity with an IC_{50} = 9.96 μg/mL followed by As-EO with an IC_{50} = 619.98 μg/mL. In contrast, in DPPH assay, As-ME and As-EO showed moderate to low activity with an IC_{50} = 92.69 μg/mL for As-ME and 917.69 μg/mL for As-EO. While in CUPRAC assay, As-EO and As-ME indicated a less to moderate reducing activity. As-ME inhibited AChE (IC_{50} = 490.46 μg/mL) and BChE (IC_{50} = 142.07 μg/mL), while As-EO was inactive against AChE and revealed a discreet inhibitory action against BChE (IC_{50} = 212.14 μg/mL). As-ME displayed better antimicrobial activity than As-EO, being active against *Staphylococcus aureus* (ATCC® 25923) and *Bacillus subtilis* (ATCC® 6633), with MIC of 1.56 mg/mL. An expressive fungal adhesion inhibition (80.02%) on *Candida albicans* (ATCC® 10239) was detected with As-ME at 6.25 mg/mL. These results showed that *A. stiparum* subsp. *sabulicola* is a natural source of active compounds with antibiotic and antibiofilm effects against *S. aureus* and *B. subtilis*, and *C. albicans*, respectively, and also presents antioxidant and anticholinesterase properties.

1. Introduction

Reactive oxygen species (ROS) are chemical species that comprise of molecular oxygen [1]. We can find ROS in every aerobic cell and they balance fairly with biochemical antioxidants. In the event that ROS become excessive, or antioxidants become reduced, or both occur, that oxidative stress occurs because the balance gets altered. And oxidative stress is considered as a precursor to Alzheimer's disease (AD), which might be responsible for the dysfunction or death of neuronal cells that contributes to disease pathogenesis [2]. The brain is considered to be

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Table 1
Chemical composition (%) of the essential oil from aerial parts of *A. stiparum* subsp. *sabulicola* using Gas Chromatography with Flame Ionization Detector (GC-FID) and Gas Chromatography Mass Spectrometry (GC-MS).

N°	Compound	Composition (%)	RI[a]	RI[lit.] on DB5	Identification method
1	Cumene	0.49	906	924	1, 2
2	α-Pinene	0.49	936	932	1, 2, 3
3	Camphene	0.24	950	946	1, 2
4	6-Methyl-5-heptene-2-one	4.34	976	981	1, 2
5	*β*-Pinene	0.05	978	974	1, 2, 3
6	o-Cymene	0.05	1009	1022	1, 2
7	Eucalyptol (1,8 Cineol)	0.26	1024	1026	1, 2, 3
8	Limonene	0.06	1025	1024	1, 2, 3
9	γ-Terpinen	0.15	1051	1054	1, 2
10	**Camphor**	**6.73**	1123	1141	1, 2, 3
11	Borneol	0.28	1150	1165	1, 2
12	Isogeranial	0.99	1156	1174	1, 2
13	α-Terpineol	0.10	1176	1186	1, 2, 3
14	2-Decanone or 3-Decanone	0.13	1178	1190	1, 2
15	n-Dodecane	0.10	1200	1200	1, 2
16	δ-Elemene	0.49	1340	1335	1, 2
17	Neryl acetate	0.17	1342	1359	1, 2
18	*β*-Damascenone	0.18	1348	1383	1, 2
19	Decanoic acid	0.29	1349	1364	1, 2
20	α-Copaene	0.62	1379	1374	1, 2
21	Methyleugenol	0.12	1385	1403	1, 2
22	*β*-Bourbonene	0.12	1388	1387	1, 2
23	*β*-Elemene	0.28	1390	1389	1, 2, 3
24	*β*-Caryophyllene	1.97	1408	1417	1, 2, 3
25	*β*-Gurjunene	0.15	1431	1431	1, 2, 3
26	Seychellene	0.64	1447	1444	1, 2
27	α-Humulene	0.33	1455	1452	1, 2
28	Alloaromadendren	0.57	1460	1458	1, 2
29	Aromadendrane < dehydro- >	0.41	1462	1460	1, 2
30	Sesquicineole < dehydro- >	4.46	1469	1469	1, 2
31	Sesquicineole < 7-epi-1,2-dehydro- >	0.16	1471	1471	1, 2
32	**Germacrene D**	**11.13**	1481	1484	1, 2, 3
33	δ-Selinene	0.34	1492	1492	1, 2
34	*β*-Himachalene	5.19	1500	1500	1, 2
35	(E,Z)-α-Farnesene	0.50	1501	1505	1, 2
36	1,5-Cycloundecadiene, 8,8-dimethyl-9-methylene-	0.84	1504	1485	1, 2
37	*β*-Bisabolene	0.18	1505	1505	1, 2
38	γ-Cadinene	1.60	1510	1513	1, 2
39	δ-Cadinene	1.15	1520	1522	1, 2
40	**Isoamyl salicylate**	**6.45**	1523	1535	1, 2
41	*cis*-3-Hexenyl Benzoate	0.74	1545	1565	1, 2
42	γ-Elemene	0.05	1570	1434	1, 2
43	**Spathulenol**	**6.50**	1572	1577	1, 2, 3
44	Caryophyllene oxide	2.56	1578	1582	1, 2, 3
45	Globulol	0.44	1589	1590	1, 2
46	Isoaromadendrene epoxide	1.22	1592	1639	1, 2
47	Ledene oxide-(II)	0.65	1604	1646	1, 2
48	Cubenol	0.56	1630	1645	1, 2
49	Cedren-3-one < 2-epi-α >	2.22	1631	1626	1, 2
50	Hexenyl Phenyl acetate < (3Z)- >	1.50	1635	1632	1, 2
51	**t-Cadinol**	**11.01**	1640	1638	1, 2
52	*β*-Eudesmol	0.53	1641	1649	1, 2
53	α-Cadinol	2.56	1643	1652	1, 2
54	Edusmol < 7-epi-α >	1.41	1653	1662	1, 2
55	Khusinol	0.88	1658	1679	1, 2
56	α-Bisabolol	1.39	1673	1685	1, 2
57	*cis*-Z-α-Bisabolene epoxide	3.80	1704	–	1, 2
58	Nuciferol < (Z)- >	0.32	1713	1724	1, 2
59	Farnesol < 2Z,6E)- >	0.42	1720	1722	1, 2
60	γ-Costol	0.26	1746	1745	1, 2
61	Cedryl acetate	0.26	1769	1767	1, 2
62	Farnesyl acetate < (2Z,6E)- >	0.28	1812	1821	1, 2
63	E−10-Pentadecenol	0.13	1908	–	1, 2
64	Pimaradiene	0.08	1962	1948	1, 2
65	Hexadecanoic acid	0.43	1981	1959	1, 2
66	Thunbergol	0.35	2002	2032	1, 2
67	Hexadecanoic acid, trimethylsilyl ester	0.30	2040	2047	1, 2
68	Heneicosane	0.67	2100	2100	1, 2
69	1,18-Nonadecadien-7,10-dione	0.10	2168	2062	1, 2
70	Epimanool	0.55	2265	2057	1, 2
71	Tricosane	3.29	2306	2300	1, 2
72	Pentacosane	0.78	2500	2500	1, 2
	Total identified:	99.02			

(*continued on next page*)

Table 1 (*continued*)

N°	Compound	Composition (%)	RI[a]	RI[lit.] on DB5	Identification method
	Monoterpenes hydrocarbons:	5.88			
	Oxygenated monoterpenes:	8.36			
	Sesquiterpenes hydrocarbons:	26.54			
	Oxygenated Sesquiterpens:	28.61			
	Others:	29.64			

[a] RI = Retention index; experimentally determined Kováts retention indices; 1: Mass Spectra comparison; 2: retention index literature comparison, 3: Co-injection with authentic compounds.

Table 2
Antioxidant activity (Inhibition%) of *A. stiparum* subsp. *sabulicola* aerial parts methanol extract (As-ME) and essential oil (As-EO) by the DPPH and *β*-carotene/linoleic acid assays.[a]

	Concentration µg/mL	Inhibition%			
		As-ME	As-EO	BHA	α-Tocopherol
DPPH	12.5	13.14 ± 2.60	NA	31.15 ± 0.65	90.7 ± 0.23
	25	17.53 ± 1.91	NA	38.56 ± 0.81	91.16 ± 0.17
	50	31.35 ± 2.34	2.52 ± 0.77	43.78 ± 0.21	92.03 ± 0.55
	100	53.09 ± 2.58	3.96 ± 0.34	59.9 ± 0.35	93.77 ± 0,07
	200	58.56 ± 2.03	5.30 ± 1.12	79.83 ± 0.51	95.9 ± 0.05
	400	67.42 ± 1.45	8.45 ± 0.94	90.58 ± 0.24	96.1 ± 0.9
	800	69.62 ± 1.23	12.54 ± 1.40	94.16 ± 0.15	96.7 ± 0.21
	IC_{50}	**92.69 ± 4.48**	**917.69 ± 68.14**	**45.4 ± 0.47**	**7.31 ± 0.17**
β-carotene/linoleic acid	12.5	58.11 ± 0.28	NA	90.1 ± 0.2	89.15 ± 0.1
	25	75.17 ± 2.78	8.46 ± 2.45	91.56 ± 0.22	90.6 ± 0.3
	50	81.85 ± 2.68	18.39 ± 0.39	92.68 ± 0.3	91.89 ± 0.27
	100	88.56 ± 0.39	33.1 ± 6.07	93.6 ± 0.16	92.1 ± 0.51
	200	90.04 ± 2.64	43.29 ± 4.63	94.8 ± 0.21	93.32 ± 0.33
	400	93.23 ± 0.54	44.37 ± 1.45	95.8 ± 0.15	94.22 ± 0,28
	800	94.03 ± 0.92	52.65 ± 0.75	97.7 ± 0.16	96.02 ± 0,30
	IC_{50}	**9.96 ± 3.36**	**619.98 ± 26.35**	**1.34 ± 0.04**	**2.10 ± 0.08**

Values expressed as absorbance at 450 nm are means ± standard deviation of three parallel measurements. ($p < 0.05$).
[a] Values expressed are means ± SD of three parallel measurements ($p < 0.05$); NA: not active.

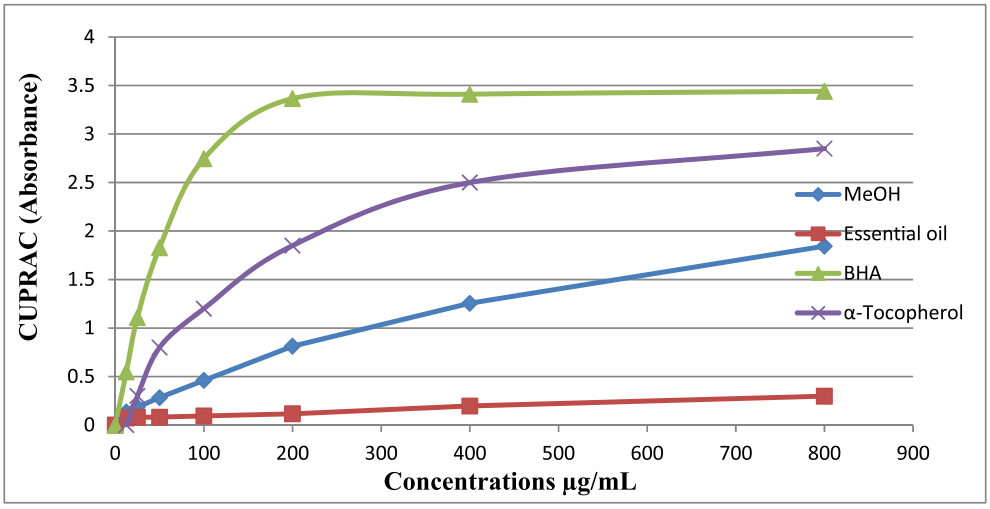

Fig. 1. Cupric reducing antioxidant capacity (CUPRAC) results of *A. stiparum* subsp. *sabulicola* aerial parts methanol extract and essential oil.

vulnerable to the harmful effects of ROS, because of its high rate of metabolism and limited ability of cells to regenerate [3–5]. The absence of Acetylcholine in the brain is often the reason for AD in older patients, particularly for neurodegenerative diseases such as Alzheimer's disease and Parkinson's syndrome. Cell aging in older patients is actually enhanced by Acetylcholinesterase (AChE) activity. In fact, AChE inhibitors are the main target for treating AD [5,6].

Synthetic antioxidants, like butylatedhydroxytoluene (BHT), happen to be the popular choice; and it is other substitutable antioxidants that are more natural are being considered [7,8]. There are such "free radical scavenging enzymes" like superoxide dismutase and peroxidase, and they are related to the behavior of antioxidants in plants; these antioxidants basically contain phenolic compounds, carotenoids, tocopherol, as well as ascorbic acid [9]. Lots of research has indicated that aromatic plants as well as their oils are organisms with really active antioxidants. Phenolic compounds and other chemicals found in various spices and herbs have been shown to demonstrate antioxidant activities [10,11].

It is also true that food borne diseases are an issue for the food industry, consumers, and agencies that cater for food quality. Many

Table 3
Results of acetylcholinesterase and butyrylcholinesterase inhibitory activities of *A. stiparum* subsp. *sabulicola* aerial parts methanol extract (As-ME) and essential oil (As-EO).[a]

		Inhibition%		
	Concentration µg/mL	As-ME	As-EO	Galantamine
AChE	3.125	NA	NA	41.75 ± 0.65
	6.25	NA	NA	52.32 ± 1.20
	12.5	NA	NA	62.21 ± 0.32
	25	NA	NA	68.36 ± 1.10
	50	6.64 ± 1.88	NA	74.38 ± 0.65
	100	16.84 ± 2.52	NA	78.59 ± 0.47
	200	22.18 ± 3.88	NA	80.4 ± 0.9
	IC_{50}	**490.46 ± 76.53**	NA	**5.01 ± 0.09**
BChE	3.125	NA	NA	17.44 ± 1.08
	6.25	NA	NA	21.35 ± 0.66
	12.5	NA	NA	29.62 ± 1.30
	25	14.29 ± 3.08	15.42 ± 0.98	40.59 ± 2.88
	50	25.35 ± 0.71	27.1 ± 2.78	48.73 ± 0.90
	100	36.97 ± 2.77	37.11 ± 0.98	65.02 ± 0.44
	200	-[b]	47.69 ± 0.81	82.2 ± 1.6
	IC_{50}	**142.07 ± 5.41**	**212.14 ± 2.31**	**53.9 ± 0.56**

[a] IC_{50} values represent the means ± SD. of three parallel measurements ($p < 0.05$).
[b] Could not be determined due to turbidity in the well; NA: not active.

researches and efforts have gone into the search for chemicals that would combat these microorganisms that would drop the rate of development of fungus and bacteria in foods and correspondingly improve food quality. It has as well become a thing of concern for consumers about how safe synthetic food preservatives really are. And the consequence of this trend is an elevated demand for natural preservatives [12]. The race also includes the quest for natural antimicrobial substances. It is noteworthy to point out that nowadays, among all substances approved as new antibacterial chemical entities, for example, a significant percentage of them or is natural products or they are derived from prototypes obtained from phytochemicals [13]. The antimicrobials from natural source can come from animals, plants, fungi, bacteria or algae. Some studies that had gone into antimicrobials from plant sources have exhibited some outstanding potency when applied to food and drug industries, and these researches clarified the reason for the observed efficiency [12–14].

On the other hand, microorganisms can produce biofilms, which are a group of sessile bacteria attached to a substrate and to each other, with each cell surrounded by an extracellular [15,16]. This complex of microbes form into a sort of sticky gel made of complex sugar, proteins as well as some other organic substances that stay on wet surfaces, that makes its way into diverse environments ranging from clinical to manufacturing firms, and environments where food and water are respectively prepared and dispensed. Unlike plankton cells in suspension, the bacteria contained within these biofilms have keener capacity to resist antibiotics [17,18]. The fact that most chemical agents are less potent against attached microbes, they are not effective even when introduced into the biofilms. Hydrolyzing the biofilm matrix is mandatory if it must be accessed are degraded. It is necessary to inhibit the growth and improvement of food borne and health-based pathogens like *Staphylococcus aureus* and *Escherichia coli*, but completely eliminating these species fail often and that is because they possess the ability to develop biofilms on surfaces of diverse natures [19,20].

Due to its variety of geographical locations, Algeria has a rich flora of medicinal plants. There are about 3500 plant species reported in Algeria among which 500 are regarded of medicinal values [21]. The genus *Anthemis* L. (tribe Anthemideae Cass.), is the second largest in the *Asteraceae* family consists of more than 210 species [22]. In Algeria, this genus includes about 13 species [21], *Anthemis stiparum* subsp. *sabulicola* (Pomel) Oberpr. is an aromatic and endemic to the North fringe of the Algerian Sahara. Not found neither in the southern regions of Erq (dune sea) and saline areas, vernacular names: Arbian, Itima [23,24]. According to our knowledge, at present time, there is no report in the scientific literature concerning phytochemical or biological activities focusing on this plant. *Anthemis* species are sources of diverse classes of natural products such as sesquiterpene lactones, EOs and flavonoids [25–27], with biological activities including antioxidant, antimicrobial and anti-inflammatory. In Europe, extracts, tinctures, tisanes (teas), and salves of these species are widely used as antibacterial, anti-inflammatory, antispasmodic, and sedative agents. Extracts are used to allay pain and irritation, clean wounds and ulcers, and aid prevention as well as therapy of irradiated skin injuries, treatment of cystitis and dental afflictions [25].

In this context, the current study aimed to characterize the chemical composition, to determine the total phenolic and flavonoid contents and to evaluate the *in vitro* antioxidant, acetylcholinesterase, antimicrobial and antibiofilm potentials of *Anthemis stiparum* subsp. *sabulicola* aerial parts methanol extract (As-ME) and essential oil (As-EO), speculating new approaches to treat infections and avoid food contaminations.

2. Materials and methods

2.1. Plant material

The aerial parts of *A. stiparum* subsp. *sabulicola* were collected in the North fringe of the Algerian Sahara during the flowering period in April 2012 Meguibra, El-Oued, Algeria (34°18′N, 5°54′E) at 33 m altitude. The taxonomic identification of plant was confirmed by Dr. Youcef Halice in the Scientific and Technical Research Centre for Arid Areas and a voucher sample (CAK 2) was deposited in the Laboratory of biology, University of El Oued, Algeria. The collected plant material

Table 4
Minimal Inhibitory Concentration (MIC) and antibiofilm activity results *A. stiparum* subsp. *sabulicola* aerial parts methanol extract (As-ME) and essential oil (As-EO).

Microorganism	As-EO						As-ME					
	Planktonic	% inhibition on biofilms					Planktonic	% inhibition on biofilms				
	MIC µL/mL	MIC	MIC/2	MIC/4	MIC/8	MIC/16	MIC mg/mL	MIC	MIC/2	MIC/4	MIC/8	MIC/16
Staphylococcus aureus subsp. *aureus* (ATCC® 25923™)	25	8.25	NI	NI	NI	NI	1.56	59.06	NI	NI	NI	NI
Staphylococcus aureus subsp. *aureus* (ATCC® 6538™)	100	22.47	9.80	NI	NI	NI	12.5	27.84	11.43	NI	NI	NI
Staphylococcus epidermidis MU 30	25	29.17	NI	NI	NI	NI	25	30.43	16.07	2.27	NI	NI
Bacillus subtilis (ATCC® 6633™)	50	44.44	27.98	7.40	NI	NI	1.56	5.09	NI	NI	NI	NI
Bacillus cereus RSKK 863	100	8.80	NI	NI	NI	NI	12.5	34.33	30.29	20.91	NI	NI
Micrococcus luteus NRRL B-4375	100	45.41	20.21	8.25	NI	NI	12.5	57.96	19.49	3.36	NI	NI
Streptococcus mutans CNCTC 8/77	50	20.21	8.25	NI	NI	NI	12.5	25.80	9.67	NI	NI	NI
Candida albicans (ATCC® 10239™)	50	21.93	4.83	NI	NI	NI	6.25	80.02	37.42	30.26	NI	NI

NI: no inhibition.

was air-dried in darkness at room temperature for three weeks. After drying, the plant material was weighted for the preparation of As-ME and EO.

2.2. Preparation of plant extract and essential oil

2.2.1. Anthemis stiparum subsp. sabulicola aerial parts essential oil

EO of dried aerial parts (300 g) of *A. stiparum* subsp. *sabulicola* (As-EO) was obtained via hydrodistillation using a Clevenger type apparatus for 4 h. The As-EO was a dried over anhydrous sodium sulphate and stored under nitrogen until required.

2.2.2. Anthemis stiparum subsp. sabulicola aerial parts methanol extract

Total methanol extract of *A. stiparum* subsp. *sabulicola* (As-ME) was prepared by maceration technique, the dried and powdered aerial parts of the plant (150 g) were macerated with 300 mL of methanol at room temperature (25 °C) 3 times (24 h × 3). After filtration, The extract was concentrated using a rotary evaporator (Buchi Rotavapor R-200, Flawil, Switzerland) at a maximum temperature of 45 °C, The residuals obtained were stored in a freezer at −20 °C until further study.

2.3. Chromatography with Flame Ionization Detector (GC-FID)

GC analyses of As-EO 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 [30 m × 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 helium at a flow rate of 1.3 mL/min; Sample size, 1.0 μL; split ratio, 50:1. The percentage composition of As-EO was determined with a Class-GC 10 computer program.

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

The analysis of As-EO 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 × 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 eV ionization energy. Mass range was from *m/z* 28–650 amu. Scan time was 0.5 s with 0.1 s inter scan delays.

Identification of components of As-EO 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 [28] and, whenever possible, by co-injection with authentic compounds.

2.5. Determination of total phenolic content

The content of total phenolic of As-ME was determined using Folin–Ciocalteu reagent, and expressed as microgramme of pyrocatechol equivalents [29]. 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:

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

2.6. Determination of total flavonoid content

Total flavonoid content of As-ME was determined according to the aluminum chloride colorimetric method. The results were expressed as quercetin equivalents [30]. The concentration of flavonoid was calculated according to the following equation that was obtained from the standard quercetin graph.

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

2.7. Antioxidant activity

2.7.1. Free radical-scavenging activity (DPPH assay)

The free radical scavenging activity of As-EO and As-ME was determined by the DPPH assay described by Blois, with slight modifications [31,32]. In its radical form DPPH absorbs at 517 nm, but on reduction by an antioxidant or a radical species its absorption decreases. Briefly, a 0.1 mmol L^{-1} solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL of samples solution in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

The capability to scavenge the DPPH radical of an antioxidant was calculated using the following equation:

$$DPPH\ scavenging\ effect\ (\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

The sample concentration providing 50% free radical scavenging activity (IC_{50}) was calculated from the graph of DPPH Scavenging effect percentage against sample concentration. BHA (Butylated hydroxyanisole) and α-tocopherol were used as antioxidant standards for comparison of the activity.

2.7.2. Cupric reducing antioxidant capacity (CUPRAC)

CUPRAC assay was carried out according to Apak et al. method, with slight modifications [31,33]. To each well, in a 96 well plate, 50 μL 10 mM Cu (II), 50 μL 7.5 mM neocuproine, and 60 μL NH_4Ac buffer (1 M, pH 7.0) solutions were added. 40 μL of As-EO or As-ME at different concentrations were added to the initial mixture so as to make the final volume 200 μL. After 1 h, the absorbance at 450 nm was recorded against a reagent blank by using a 96-well microplate reader. BHT (Butylated hydroxytoluene) and α-tocopherol were used as antioxidant standards for comparison of the activity.

2.7.3. β-carotene/linoleic acid co-oxidation assay

The antioxidant activity of As-EO and As-ME was evaluated using the β-carotene-linoleic acid test system [31,34]. with slight modifications. β-Carotene (0.5 mg) in 1 mL of chloroform was added to 25 μL of linoleic acid, and 200 mg of Tween-40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, were added by vigorous shaking. 4 mL of this mixture was transferred into different test tubes containing different concentrations of As-EO and As-ME. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a 96-well microplate reader (SpectraMax 340PC, Molecular Devices, USA). The emulsion system was incubated for 2 h at 50 °C. A blank, devoid of β-carotene, was prepared for back ground subtraction. BHA and α-tocopherol were used as standards.

The bleaching rate (R) of β-carotene was calculated according to the following equation:

$$R = \frac{ln\frac{a}{b}}{t}$$

where: ln = natural logarithme, a = absorbance at time zero, b = absorbance at time t (120 min).

The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using following equation:

$$AA\ (inhibition\%) = \frac{R_{Control} - R_{Sample}}{R_{Control}} \times 100$$

2.8. Anticholinesterase activity assay

The inhibition activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was measured by method developed by Elman et al., in 1961, with slight modification [31,35], using 96-well microplate reader (SpectraMax PC340, Molecular Devices, USA). The substrates of the reaction of both enzymes were acetylthiocholine iodide (0.71 mM) and butyrylthiocholine chloride (0.2 mM). In a 96 well plate, 10 µL of As-EO or As-ME were mixed with 150 µL sodium phosphate buffer 100 mM (pH = 8) and 20 µL of enzymes solution [AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U)]. After 15 min incubation at 25 °C, 10 µL of Ellman's Reagent (DTNB 0.5 mM) and 10 µL of substrates were added, so as to make the final volume of 200 µL. The absorbance was measured at 412 nm. Percentage of inhibition of AChE or BChE was determined by comparison of reaction rates of samples relative to control using the formula: (E − S)/E × 100.where:

E: activity of enzyme with control.
S: activity of enzyme with sample.
The experiments were carried out in triplicate. Galantamine was used as standard.
MeOH and EtOH were used as solvents to dissolve As-EO, As-ME and controls.

2.9. Antimicrobial and antibiofilm activities

2.9.1. Bacterial and fungal strains

Staphylococcus aureus subsp. *aureus* (ATCC® 25923™), *Staphylococcus aureus* subsp. *aureus* (ATCC® 6538™), *Staphylococcus epidermidis* MU 30, *Streptococcus mutans* CNCTC 8/77, *Micrococcus luteus* NRRL B-4375, *Bacillus subtilis* (ATCC® 6633™), *Bacillus cereus* RSKK 863 and *Candida albicans* (ATCC® 10239™) were selected for the *in vitro* antimicrobial activity assessment. The above-mentioned bacteria except *C. albicans* were grown in nutrient broth (NB, Difco); *C. albicans* was grown in sabouraud dextrose broth (SDB, Difco). The cultures of microorganisms were maintained in their appropriate agar slants at 4 °C throughout the study and used as stock cultures.

2.9.2. Determination of Minimal Inhibitory Concentration (MIC)

MICs were determined by a microtitre broth dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [36]. The MIC was defined as the lowest EO/extract concentration that yielded no visible growth. The test medium was MHB 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 microtitre plates in the presence of As-EO with different final concentrations (6.25, 12.5, 25, 50, 80, 160 µL/mL) and in the presence of As-ME with different final concentrations (1.56, 3.12, 6.25, 12.5, 25, 50 mg/mL). The inoculated microplates were incubated at 37 °C for 24 h. The absorbance was measured at 630 nm.

2.9.3. Evaluation of antibiofilm formation activity

The effect of As-EO and As-ME at concentrations of 1, 1/2, 1/4 and 1/8 MIC on biofilm-forming ability of the bacterial and fungal strains selected was tested using a microplate biofilm assay [37]. Briefly, 1% of overnight cultures of the selected strains was added into 200 µL of fresh Tryptose-Soy Broth (TSB) supplemented with 0.25% glucose and cultivated in the presence and absence of As-EO or As-ME without agitation for 48 h at 37 °C. The wells containing TSB + cells were used as control. After incubation, the wells were washed with water to remove planktonic bacteria or yeast cells. The remaining bacteria or yeast 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 was measured at wave length of 550 nm (Thermo Scientific Multiskan FC, Vantaa, Finland). Percentage of inhibition of As-ME and As-EO was calculated using the formula:

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

2.10. Statistical analysis

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

3. Results and discussion

3.1. Chemical composition of Anthemis stiparum subsp. sabulicola aerial parts essential oil (As-EO)

As-EO presented yellow colour and was obtained from aerial parts of *A. stiparum* subsp. *sabulicola* using hydrodistillation method (0.30%, v/w). GC-FID and GC-MS were able to identify 72 components representing 99.02% of As-EO (Table 1). As shown in this Table, the major compounds of As-EO were germacrene D (11.13%), t-cadinol (11.01%), camphor (6.73%), spathulenol (6.50%), and isoamyl salicylate (6.45%).

The oxygenated monoterpenes represented 8.36% while oxygenated Sesquiterpens 28.61% of the total oil. The monoterpenes and sesquiterpenes hydrocarbons were in the ratio of 5.88% and 26.54%, respectively. These results are nearly similar to other studies about EOs of *Anthemis* species, such as, EO of *Anthemis odontostephana* Boiss. var. *odontostephana* growin in Iran [38] and Italian *A. maritima* grown in Pianosa [39] and *A. cotula* from Serbia and Montenegro [40].

Under the phytochemical viewpoint, germacrene D has been identified as the major fraction (8.9%) of the EO from aerial parts of *A. cotula* L. [41], being also observed in many *Anthemis* EOs in different percentages, such as: *A. segetalis* Ten. (12.6%) [42]; *A. auriculata* Boiss. (0.8–9.5%) and *A. chia* L. (0.6–4.6%) from Greece [41]; *A. hyaline* (5.1%) [43] and *A. altissima* L. (6.9%) [44] grown in Iran. In the same way, some reports focusing on EO of *Anthemis* species have been detected t-cadinol with an expressive percentage, for example; in *A. tinctoria* L. var. *parnassica* (11.5%) [41] and *A. segetalis* Ten (4.2%) growing in Montenegro [42].

Other studies concerning the chemical composition of *Anthemis* EOs, such as Hanbali, Mellouki [45], found that camphor (17.5%) was among the major compounds in *A. tenuisecta* growing in Morocco and in *A. cretica* L. subsp. *leucanthemoides* from Turkey with 19.4% [46]. Camphor was determined as a constituent of As-EO 6.73% (Table 1). Despite the fact that isoamyl salicylate was found to be one of the major compounds in As-EO with 6.45%, this compound was not reported from any *Anthemis* EOs. This compound is used in perfumes and fragrances [47]. Finally, borneol and 1,8 cineole were identified as dominant compounds in EOs of *Anthemis* species [42]. Whereas, they were present in lower amounts in our sample with 0.28% and 0.26%, respectively. Changes in the composition of As-EO might have arisen from several different factors such as climatical, seasonal and geographical [48].

3.2. Total phenolic and flavonoid contents and antioxidant activity

Results of antioxidant activity of As-ME and As-EO by the three aforesaid assays are summarized in Table 2 and Fig. 1. As observed in

Table 2, the antioxidant activity increased with the increase of As-ME and As-EO concentration using DPPH and β-carotene/linoleic acid co-oxidation assays. In DPPH assay, As-ME scavenged 69.62 ± 1.23% of DPPH● radical at 800 μg/mL, the higher concentration tested, less than BHT and α-tocopherol, wherase, As-EO showed weak scavenging (12.54 ± 1.40%) at same concentration. In β-carotene/linoleic acid assay, As-ME exhibited similar values when compared with BHT and α-Tocopherol (94.03 ± 0.92%, at 800 μg/mL concentration), while, As-EO inhibited 52.65 ± 0.75%. As displayed by Fig. 1, the CUPRAC assay showed moderate and weak activities for As-ME and As-EO, in this order, when compared with standards.

The concentration of phenolics and flavonoids in the As-ME was expressed as micrograms of pyrocatechol and micrograms of quercetin equivalents per milligrams of the extract, respectively. As-ME had 13.6 ± 0.03 μg pyrocatechol equivalents as its phenolic content, and demonstrated 5.9 ± 0.04 μg quercetin equivalents as its flavonoid content.

From literature, it has been well noted that medicinal plants with high amounts of phenols and flavonoids have potent antioxidant actions [49]. This may explain the antioxidant activity of *A. stiparum* subsp. *sabilucola*. In term of antioxidant potential As-ME, our results are in accordance with those of previous works, indicating close relationship between antioxidant effect and total phenolic and flavonoid contents of *Anthemis* species such as *A. cretica* [50], *A. tinctoria* [51], *A. deserti* [52] and *A. palestina* [53].

3.3. Anticholinesterase activity

The anticholinesterase activity of As-EO and As-ME against AChE and BChE was given in Table 3. Galantamine was the standard drug for comparison. As-ME exhibited a weak inhibitory activity against AChE and BChE, with IC_{50} values of 490.46 ± 76.53, and 142.07 ± 5.41 μg/mL, respectively. Whereas, As-EO was inactive against AChE and exhibited a weak inhibitory activity against BChE with IC_{50} 212.14 ± 2.31 μg/mL.

3.4. Antimicrobial and antibiofilm activities

The antimicrobial As-ME and As-EO against 8 microorganisms were examined and the results are given in Table 4. As-EO inhibited the growth of all microorganisms tested between 25 and 100 μL/mL. MIC of As-EO was 25 μL/mL for *S. aureus* (ATCC® 25923™) and *S. epidermidis* MU 30. These results are consistent with previous studies [54,55], demonstrating that *Anthemis* species EO possess antibacterial activity on *Staphylococcus* spp. As-EO exhibited moderate antifungal activity against *C. albicans* (ATCC®10239™) with MIC value of 50 μL/mL. As-EO at the MIC's inhibited biofilm formations of all microorganisms tested in various percentages. The oil exhibited the highest antibiofilm activity against *M. luteus* NRRL B-4375 at 100 μL/mL (MIC) concentration with 45.41%. In the presence of 50 μL/mL As-EO (MIC), the mean biofilm formation value was equal to 44.44% for *B. subtilis* ATCC 6633. At a concentration of 25 μL/mL As-EO, the biofilm formations of *S.epidermidis* MU 30 and *S.aureus* (ATCC® 25923™) were inhibited to 29.17 and 8.25%, respectively. Oxygenated monoterpenes such as camphor and Oxygenated sesquiterpenes such as t-cadinol and spathulenol, which are representative components in the investigated oil were reported to exhibit antimicrobial activity [56–58]. However, it is difficult to attribute the activity of a complex mixture to a single or particular constituent. Major or trace compounds might give rise to the antimicrobial activity exhibited [59].

According to the results, *S.aureus* (ATCC® 25923™) and *B. subtilis* (ATCC® 6633™) were found as the most susceptible strains against As-ME.

Using As-ME, a MIC of 6.25 mg/mL was observed for *C. albicans* (ATCC® 10239™). On the other hand, As-ME has low activity on the growth of the other strains, which were only inhibited at high concentrations (12.5 and 25 mg/mL). In the presence of 1.56 mg/mL As-ME (MIC), the mean biofilm formation values were equal to 59.06% for *S. aureus* (ATCC® 25923™), and 5.09% for *B. subtilis* (ATCC® 6633™). As-ME showed the strongest antibiofilm activity against the *C. albicans* (ATCC® 10239™) and it was induced 80.02% inhibition of the biofilm when used at MIC. To the best of our knowledge, this is the first report of antimicrobial and antibiofilm activities of As-EO and AS-ME against *Staphylococcus aureus* subsp. *aureus* (ATCC® 25923™), *Staphylococcus aureus* subsp. *aureus* (ATCC® 6538™), *Staphylococcus epidermidis* MU 30, *Streptococcus mutans* CNCTC 8/77, *Micrococcus luteus* NRRL B-4375, *Bacillus subtilis* (ATCC® 6633™), *Bacillus cereus* RSKK 863 and *Candida albicans* (ATCC® 10239™) microbial strains.

4. Conclusion

The aim of this work was to conduct for the first time the chemical composition of the essential oil of *A. stiparum* subsp. *sabulicola* from Algerian Sahara and investigate the antioxidant, anticholinesterase, antimicrobial and antibiofilm activities of its essential oil (As-EO) and methanol extract (As-ME). Our findings indicate that As-ME and As-EO have a potential to be exploit for the development of a new anti-biofilm, as well as antimicrobial agents, and demonstrate the importance of this medicinal plant in pharmaceutical production also in the food industries.

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