SUPPLEMENTARY MATERIAL

Chemical constituents of essential oil of endemic *Rhanterium suaveolens* Desf. growing in Algerian Sahara with antibiofilm, antioxidant and anticholinesterase activities

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Abstract

Twenty compounds were detected in the essential oil of *Rhanterium suaveolens* representing 98.01% of the total oil content. Perillaldehyde (45.79%), caryophyllene oxide (24.82%) and β -cadinol (5.61%) were identified as the main constituents. In β -carotene-linoleic acid assay, both the oil and the methanol extract exhibited good lipid peroxidation inhibition activity, with IC₅₀ values of 17.97 ± 5.40 and 11.55 ± 3.39 $\mu\text{g/mL}$, respectively. In DPPH and CUPRAC assays, however, the methanol extract exhibited a good antioxidant activity. The highest antibiofilm activity has been found 50.30% against *Staphylococcus epidermidis* (MU 30) at 20 $\mu\text{g/mL}$ for EO and 58.34% against *Micrococcus luteus* (NRRL B-4375) at 25 mg/mL concentration for methanol extract. The *in vitro* anticholinesterase activity of methanol extract showed a moderate acetylcholinesterase inhibitory (IC₅₀ = 168.76 ± 0.62 $\mu\text{g/mL}$) and good butyrylcholinesterase inhibitory (IC₅₀ = 54.79 ± 1.89 $\mu\text{g/mL}$) activities. The essential oil was inactive against both enzymes.

Keywords: *Rhanterium suaveolens*, Essential oil, Antibiofilm, Anticholinesterase, Antioxidant activity.

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Experimental

Plant material

The aerial parts of *R. suaveolens* (*R. suaveolens* voucher number CAK 16) were collected in the North fringe of the Algerian Sahara during the flowering period in April 2012 near Stile, El-Oued, Algeria (34°18'0"N, 5°54'0"E) at 33 m altitude and taxonomic identification of the plant was confirmed by Dr. Youcef Halice in the Scientific and Technical Research Centre for Arid Areas. The collected plant material was air-dried in darkness at room temperature for three weeks. A voucher specimen was deposited in the herbarium of the Faculty of Life and Natural Sciences of El Oued University (*R. suaveolens* voucher number CAK 4).

Preparation of the extracts

Extraction of the essential oil

The essential oil of dried aerial parts of *R. suaveolens* (400 g) was obtained via hydrodistillation by using a Clevenger type apparatus for 4 hours. The oil was dried over anhydrous sodium sulphate and stored under nitrogen until required.

Preparation of the methanol extract

Total methanol extract of *R. suaveolens* 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 (72 hours × 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.

Gas chromatography

GC analyses 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 [30 m x 0.25 mm (i.d.), film thickness 0.25 µm]; the oven temperature was held at 60°C for 5 minutes., then programmed to 240°C at 4°C/minute and held isothermal for 10 minutes; injector and detector temperatures were 250°C and 270°C respectively; carrier gas was helium at a flow rate of 1.3 mL/minute; 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.

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]. Helium was used as a carrier gas at a flow rate of 1.4 mL/minute. The oven temperature was held at 60°C for 5 minutes, then increased up to 240°C with 4°C/minute increments and held at this temperature for 10 minutes. 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 ionisation 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 (Adams 2007), and whenever possible, by co-injection with authentic compounds.

Anticholinesterase activity

The inhibition activity of Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured by the method developed by Elman et al., in 1961, with slight modification (Ellman et al. 1961; Öztürk et al. 2011), 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 samples (MeOH and EO) were mixed with 150 µL sodium phosphate buffer 100 mM (pH = 8) and 20 µL of enzymes of enzymes solution [AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U)]. After 15 minutes 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 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: $(E - S)/E \times 100$

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 a solvents to dissolve MeOH extract and essential oil and controls.

Determination of total phenolic compounds

The concentration of phenolic content in Methanol extract was expressed as microgrammes of pyrocatechol equivalents, determined with Folin–Ciocalteu reagent (FCR), according to the method of Slinkard and Singleton (1977). 1 mL of the solution containing 1 mg of the tested extract in methanol was added to 46 mL of distilled water and 1 mL of FCR, and mixed thoroughly. After 3

minutes, 3 mL of sodium carbonate (2%) were added to the mixture and shaken intermittently for 2 hours at room temperature. 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:

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

Determination of total flavonoid compounds

Measurement of flavonoid concentration of the extract was based on the method described by Park et al. in 1997 with a slight modification (Park et al. 1997), and result was expressed as quercetin equivalents. An aliquot of 1 mL of the solution (contains 1 mg of extract in methanol) was added to test tubes containing 0.1 mL of 10% aluminium nitrate, 0.1 mL of 1 M potassium acetate and 3.8 mL of ethanol. After 40 minutes at room temperature, the absorbance was determined at 415 nm. Quercetin was used as a standard. The concentration of flavonoid compounds was calculated according to following equation that was obtained from the standard quercetin graph.

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

Antioxidant activity

Free radical-scavenging activity (DPPH assay)

The free radical scavenging activity of the essential oil and methanol extract were determined by the DPPH assay described by Blois with slight modification (Blois 1958; Öztürk et al. 2011). 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:

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

The sample concentration providing 50% free radical scavenging activity (IC₅₀) was calculated from the graph of DPPH Scavenging effect percentage against sample concentration. BHA and α -tocopherol were used as antioxidant standards for comparison of the activity.

β -Carotene-linoleic acid assay

The antioxidant activity of the essential oil and methanol extract of *R. suaveolens* were evaluated using the β -Carotene-linoleic acid test system (Miller 1971; Öztürk et al. 2011). with slight modifications. β -Carotene (0.5 mg) in 1 mL of chloroform was added to 25 μ L 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, was added by vigorous shaking. 4 mL of this mixture was transferred into different test tubes containing different concentrations of the essential oil and MeOH extract. 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 hours 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 minutes). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using following equation:

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

Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity was determined according to the method of Apak et al., with slight modifications (Apak et al. 2004; Öztürk et al. 2011). To each well, in a 96 well plate, 50 μ L 10 mM Cu (II), 50 μ L 7.5 mM neocuproine, and 60 μ L NH₄Ac buffer (1 M, pH 7.0) solutions were added. 40 μ L essential oil/MeOH extract at different concentrations were added to the initial mixture so as to make the final volume 200 μ L. After 1 hour, the absorbance at 450 nm was recorded against a reagent blank by using a 96-well microplate reader. BHT and α -tocopherol were used as antioxidant standards for comparison of the activity.

Determination of minimum inhibitory concentrations and antibiofilm activity

Strains and growth conditions

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 the yeast (*Candida albicans* ATCC 10239). The above-

mentioned microorganisms except *C. albicans* were grown in nutrient broth (NB, Difco); *C. albicans* was grown in sabouraud dextrose broth (SDB, Difco).

Minimal inhibitory concentration (MIC) assay

MICs were determined by a microtitre broth dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (2006). The MIC was defined as the lowest essential oil/extract concentration that yielded no visible growth. The test medium was Mueller-Hinton broth and the density of bacteria was 5×10^5 colony-forming units (CFU)/mL. Cell suspensions (100 μ L) were inoculated in to the wells of 96-well microtitre plates in the presence of essential oil with different final concentrations (5, 10, 20, 40, 60, 80, 100 μ g/mL) and in the presence of methanol extract with different final concentrations (1.56, 3.125, 6.25, 12.5, 25, 50 mg/mL). The inoculated microplates were incubated at 37°C for 24 hours before being read.

Effect of essential oil and methanol extract on bacterial biofilm formation

The effect of *R. suaveolens* essential oil and MeOH extract at concentrations including 1, ½, ¼, 1/8 and 1/16 MIC on biofilm-forming ability of test microorganisms were tested with a microplate biofilm assay (Merritt et al. 2005). Briefly, 1% of overnight cultures of isolates were added into 200 μ L of fresh Tryptose-Soy Broth (TSB) supplemented with 0.25% glucose and cultivated in the presence and absence of *R. suaveolens* essential oil/extract without agitation for 48 hours at 37 °C. 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 minutes at room temperature. Wells were washed once again to remove the crystal violet solution. A volume of 200 μ L of 33% glacial acetic acid were poured in wells. After shaking and pipetting of wells, 125 μ L of the solution from each well were transferred to a sterile tube and volume was adjusted to 1 mL with distilled water. Finally optical density (OD) of each well was measured at 550 nm (Thermo Scientific Multiskan FC, Vantaa, Finland). Percentage of inhibition of the tested extracts was calculated using the formula:

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

Statistical analysis

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

Table S1. The Chemical composition of essential oil of *R. suaveolens*.

Compounds	RI ^a	Composition (%)	Identification method
1 β -Pinene	980	3.21	1,2,3
2 3-Carene	1015	0.33	1,2,3
3 α -Terpinene	1026	0.85	1,2,3
4 Perillaldehyde	1270	45.79	1,2,3
5 γ -Elemene	1298	<i>t</i>	1,2,3
6 Dihydroedulan	1305	0.47	1,2
7 <i>cis</i> -Isoeugenol	1375	0.32	1,2
8 β -citrylideneethanol	1398	1.67	1,2
9 β-Caryophyllene	1415	5.17	1,2,3
10 α -Caryophyllene	1448	<i>t</i>	1,2,3
11 Valencene	1455	0.33	1,2
12 β -Vatirenene	1464	1.12	1,2
13 α -Muurolene	1471	<i>t</i>	1,2
14 α -Selinene	1479	<i>t</i>	1,2
15 α -Irone	1485	1.62	1,2
16 <i>cis</i> - α -Bisabolene	1498	0.78	1,2
17 Caryophyllene oxide	1572	24.82	1,2,3
18 Isoaromadendrene epoxide	1579	0.94	1,2
19 β-Cadinol	1655	5.61	1,2
20 8-Cedren-13-ol	1680	4.98	1,2
Total identified:		98.01	
Monoterpenes:		4.39	
Monoterpenoids:		48.25	
Sesquiterpenes:		7.40	
Sesquiterpenoids:		37.97	

^aKovats index on DB-1 fused silica column. *t*: trace, 1: Mass Spectra comparison
2: retention index literature comparison, 3: Co-injection with authentic compounds.

Table S2. Acetylcholinesterase and butyrylcholinesterase inhibitory activities (%) of the essential oil and methanol extract of *R. suaveolens*.^a

Samples	AChE assay		BChE assay	
	Inhibition (%)	IC ₅₀ (µg/mL)	Inhibition (%)	IC ₅₀ (µg/mL)
	(200 µg/mL)		(100 µg/mL)	
Essential oil	NA	NA	NA	NA
Methanol extract	57.15 ± 0.85	168.76 ± 0.62	69.66 ± 0.06	54.79 ± 1.89
Galantamine ^b	81.4 ± 1.03	5.01 ± 0.09	75.5 ± 1.05	53.9 ± 0.56

NA: not active

^a IC₅₀ values represent the means ± standard deviation of three parallel measurements ($p < 0.05$).

^b Standard drug.

Table S3. Antioxidant activity (%) of the essential oil and methanol extract of *R. suaveolens* by the DPPH and β -carotene/linoleic acid assays^a

Samples	DPPH Assay	β -carotene/linoleic acid assay
	IC ₅₀ (μ g/mL)	IC ₅₀ (μ g/mL)
Essential oil	> 200	17.97 $\pm$ 5.40
Methanol Extract	36.59 $\pm$ 0.16	11.55 $\pm$ 3.39
BHT ^b	45.4 $\pm$ 0.47	1.34 $\pm$ 0.04
α -TOC ^b	7.31 $\pm$ 0.17	2.10 $\pm$ 0.08

^a Values expressed are means $\pm$ SEM of three parallel measurements ($p < 0.05$).

^b Reference compounds, BHT: Butylated hydroxytoluene; α -TOC: α -tocopherol.

Figure S1. Cupric reducing antioxidant capacity of the essential oil and methanol extract of *R. suaveolens* (—♦—Methanol extract—■—Essential oil—▲—BHA—×—α-tocopherol): Values are means ± S.D., $n=3$, $p<0.05$, significantly different by Student's t -test.

Table S4. MIC and antibiofilm activities of the essential oil and methanol extract of *R.suaveolens*.

	Essential oil						Methanol extract					
	Planktonic	%inhibition on biofilms					Planktonic	%inhibition on biofilms				
Microorganism	MIC (µg/mL)	MIC/1	MIC/2	MIC/4	MIC/8	MIC/16	MIC (mg/mL)	MIC/1	MIC/2	MIC/4	MIC/8	MIC/16
<i>Staphylococcus aureus</i> ATCC 25923	20	9.48	-	-	-	-	12.5	20.75	-	-	-	-
<i>Staphylococcus aureus</i> ATCC 6538P	80	4.96	-	-	-	-	12.5	27.25	7.37	-	-	-
<i>Staphylococcus epidermidis</i> MU 30	20	50.30	32.96	-	-	-	25	49.06	19.72	11.92	-	-
<i>Bacillus subtilis</i> ATCC 6633	40	45.55	13.20	-	-	-	1.56	-	-	-	-	-
<i>Bacillus cereus</i> RSKK 863	10	30.35	7.06	-	-	-	12.5	50.49	13.01	8.43	-	-
<i>Micrococcus luteus</i> NRRL B-4375	40	47.43	39.34	11.65	-	-	25	58.34	42.95	38.59	18.36	12.33
<i>Streptococcus mutans</i> CNCTC 8/77	40	24.04	-	-	-	-	6.25	8.86	-	-	-	-
<i>Candida albicans</i> ATCC 10239	80	18.53	-	-	-	-	3.125	21.29	11.42	-	-	-

- : no inhibition

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