ORIGINAL ARTICLE

Biological investigations of antioxidant, antimicrobial properties and chemical composition of essential oil from *Warionia saharae*

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Abstract

Objective: Several aromatic plants and their essential oils are known to possess antimicrobial and antioxidant properties. *Warionia saharae* Benth & Coss, an endemic species of North Africa, is traditionally used in the treatment of inflammatory diseases such as rheumatoid arthritis and for gastrointestinal disorders. The aims of this study were to examine the chemical composition of the essential oil isolated from *W.saharae*, and to test the efficacy of the essential oil as a potential antimicrobial and antioxidant.

Methods: The essential oil was investigated by gas chromatography-mass spectrometry (GC-MS). Thirty-six compounds, accounting 96.8% of total oil with 1.1% oil yield were identified. The major compents of *W.saharae* essential oils were β-eudesmol (24.6%), trans-nerolidol (18.2%), linalool (16.8%), 1,8 cineole (6.2%), camphor (4.6%), p-cymene (3.7%) and terpinen-4-ol (3.6%). In this study, we analyzed biological activities of *Warionia* essential oil from Errachidia region, Morocco. Indeed, we investigated mainly, the antimicrobial activity against four referenced and representative human diseases health bacteria. Also this essential oil was tested against phytopathogenic fungi.

Results: The results showed that *W.saharae* oil exhibited significant antibacterial and antifungal activities; with minimum inhibitory concentrations (MIC) ranging between 0.039 and 0.156 mg/ml for all bacteria and remarkable antifungal effect that exceeds 50% inhibition of mycelial growth for all fungal strains. We also checked whether this oil exhibited an antioxidant property via radical scavenging ability and antioxidant activity, determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and β-carotene bleaching test.

Conclusion: Our results show an important antioxidant property for *W.saharae* essential oil.

INTRODUCTION

The *Warionia saharae*, which belongs to the important Asteraceae's family, is an endemic species of North Africa, characterized by a discerning odor [1]. *W.saharae* was reported for the first time in the Oranais Sahara (Beni Ounif in Algeria) by Dr. Warrion as a shrub of 1 to 3 m of height. The thick trunk, is covered of a gray peel, structural of very wavy terminal leaf bouquets, and of capitulate of yellow flowers. The picking of stems leafed of this bush, clear a very heady and spicy odor, the latex that flows out of injuries of the peel, glue to hands in a very tenacious way [2].

In Morocco, *W.saharae* is growing wild in various regions [3]. The habitat is between schistose rocks [4]. *W.saharae* is known in Morocco by the Berber vernacular names of 'afessas' and 'tazart n-ifiss'. In the Moroccan traditional medicine, the leaves of the plant are used to treat inflammatory diseases such as rheumatoid arthritis, and for gastrointestinal disorders [5].

The chemical composition of *W.saharae* leaves has been investigated and thus twelve new guaianolide type sesquiterpene lactones were identified. Cytotoxic and anti-inflammatory sesquiterpene lactones effects were showed [6, 7]. The chemical composition of the hexanoic extract from *W.saharae* leaves prepared using soxhlet apparatus was reported by Essaqui *et al* [8]. In addition, several studies reported the chemical composition of *W.saharae* essential oil from the leaves [9, 10].

This study deals with the valorization of medicinal and aromatic plants of the oasis Moroccan flora, in order to find new bioactive natural products. Information concerning *in vitro* antioxidant, antimicrobial activities of the essential oil from the *W.saharae* has not been reported earlier.

The aims of this work were to examine the chemical composition of the the essential oil obtained from aerial part of *W.saharae* originated from Southern Moroccan Sahara and investigate their antimicrobial and antioxidant activities.

MATERIAL AND METHODS

Plant material

Flowering parts of *Warionia saharae* were collected in May from natural populations in Errachidia (East of Morocco). The botanical identification was achieved by the National Scientific Institute (Rabat) where voucher specimens were deposited in the Herbarium. The dried plant material is stored in the laboratory at room temperature (25°C) and in the shade before the extraction.

Isolation of the essential oil

The extraction of essential oil of the aerial part of *W.saharae* was conducted by hydrodistillation. The essential oil obtained was dried under anhydrous sodium sulfate and stored at 4°C in the dark before analysis.

Gas chromatography-mass spectrometry

The GC-MS analysis was done on a Trio 1000 mass spectrometer coupled with a model 8000 gas chromatograph (Thermo Scientific, Fisons Instruments) equipped with a HP-5ms capillary spectrometer (30 m long x 0.25 mm diameter, 0.25 µm film tickness). The column temperature program was 60°C for 6 min, with 5°C increases per min to 150°C; which was maintained for 10 min. The carrier gas was helium at a flow rate of 2 ml/min (splitless mode). The detector and injector temperature were maintained at 250 and 225°C respectively. The quadrupole mass spectrometer was scanned over the range 28-400 atomic mass unit at 1 scan per second, with an ionizing voltage of 70 eV, an ionization current of 150 µA. Kovats retention indices were calculated using co-chromatographed standards hydrocarbons.

The individual compounds were identified by MS and their identity was confirmed by comparing their retention indices relatives to C8-C32 *n*-alkanes and by comparing their mass spectra and retention times with those of authentic samples or with data already available in the National Institute of Standards and Technology (NIST) library and literature [11].

Antibacterial activity

Microorganisms; the microorganisms used in this study consisted of two Gram(+) bacteria, *i.e. Staphylococcus aureus* (ATCC 29213) and *Bacillus cereus* (ATCC 29213); and two Gram(-) bacteria, *i.e. Escherichia coli* (ATCC 35218) and *Pseudomonas aeruginosa* (ATCC 27853).

Diffusion method; the qualitative antimicrobial assay of the volatile fraction of *W.saharae* was carried out by the disc diffusion method [12]. It was performed using culture growth at 37°C for 18 h and adjusted to approximately 10^8 colony forming unit per milliliter (CFU/ml). The culture medium used for the bacteria was Mueller Hinton Agar (MHA). Five hundred microliters of the inoculums were spread over plates containing MHA and a Whatman paper disc (6 mm) impregnated with 5, 10, 15 µl of the volatile fraction was placed on the surface of the media. The plates were left 30min at room temperature to allow the diffusion of the oil. They were incubated 24 h at 37°C for the bacteria. After incubation period, the inhibition zone obtained around the disc was measured. Two controls were also included in the test, the first was involving the presence of microorganisms without test material and the second was standard antibiotic: ampicillin used to control the sensitivity of the tested bacteria. The experiments were run in triplicate, and the developing inhibition zones were compared with those of reference discs.

Dilution method; the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of tested volatile fractions were determined using the Mueller Hinton broth (MHB) dilution method [12]. All tests were performed in MHB supplemented with Tween 80 (1%) [10]. Bacterial strains were cultured overnight in MHB at 37°C. Tubes of MHB containing various concentrations of volatile fractions were inoculated with 10 µl of 108 CFU/ml of standardized microorganism's suspensions. Control tubes without tested samples were assayed simultaneously. All samples were tested in triplicate. The MIC was defined as the lowest concentration preventing visible growth [13, 14].

Antifungal activity

Fungal strains; four agricultural pathogenic fungi were obtained from the culture collection at Faculty of Sciences and Techniques, Errachidia. The fungal strains used in the experiments are *Alternaria* sp, *Pencillium expansum*, *Rhizopus stolonifer* and *Botrytis cinerea*.

Antifungal activity tests; the antifungal activity is evaluated as described by Chang *et al* [15]. The quantities of essential oil (100 and 200 µl) are added to 20 ml of sterile potato dextrose agar (PDA). The mixtures were cast on Petri dish. Afterwards, the discs of mycelium each mold 5 mm in diameter cut the device from a culture of 7 days are inoculated in the center of the boxes and then incubated at 25 ± 2°C for 3 days for *Rhizopus* and 7 days for others. Measuring diameters of hyphal growth relative to the control results in applying the following formula:

$$\textbf{Antifungal index (I)} = [1 - (D_a / D_b)] \times 100$$

-D_a; diameter growth dish treated (mm)

-D_b; diameter growth control (mm).

Antioxidant activity

The antioxidant activity was assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and β-carotene bleaching method systems. Data collected for each assay was an average of three experiments.

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Free radical-scavenging assay; the method is based on the reduction of alcoholic DPPH solutions in the presence of a hydrogen donating antioxidant. DPPH solutions show a strong absorption band at 517 nm with a deep violet color. The absorption vanishes and the resulting discoloration is stochiometric with respect to degree of reduction. The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant [16]. 50 µl of the extracted oil dilutions in ethanol was added to 1 ml of 100 µM solution of DPPH. After 30 min of incubation at room temperature, the absorbance was read against a blank at 517 nm (Jenway, UV/Vis 6000). Inhibition (I) of DPPH free radical in percent was calculated as follows:

$$I\ (\%) = (1 - A_{sample}/A_{blank}) \times 100$$

-A_{blank}; absorbance of the control reaction (containing all reagents except the test compound)

-A_{sample}; absorbance of the test compound. Extract concentration providing 50% inhibition

IC_{50} was calculated from the graph plotting inhibition percentage against extract concentration. All tests were carried out in triplicate. The synthetic antioxidant butylated hydroxytoluene (BHT) was used as positive control.

Beta-carotene bleaching assay; the β-carotene method was carried out according Shahidi *et al* [17]. Two milliliters of β-carotene solution (0.2 mg/ml in chloroform) were pipetted into a round-bottomed flask containing 20 µl linoleic acid and 200 µl Tween 20. The mixture was then evaporated at 40°C for 10 min to remove the solvent. Then, the addition of distilled water (100 ml) followed immediately. After agitating the mixture, 1.5 ml aliquot of the resulting emulsion was transferred into test tubes containing 150 µl of extract and the absorbance was measured at 470 nm against a blank consisting of an emulsion without β-carotene. The tubes were placed in a water bath at 50°C and the oxidation of the emulsion was monitored by measuring absorbance at 470 nm after 2 h using spectrophotometry. The same procedure was repeated with BHT as positive control. The antioxidant capacity (AA%) of the solutions tested was calculated via the following formula:

$$AA\% = \frac{\text{β-carotene content after 2 h assay}}{\text{Initial β-carotene content}} \times 100$$

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) and the significance of the differences between means was determined at $P < 0.05$ using Duncan's multiple range tests. Results were expressed as means ± standard deviation of three independent tests. All tests were carried out in an identical condition.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The essential oil of *W.saharae* was extracted by hydrodistillation appearing as blue-green color viscous liquid with a percentage yield of 1.1% (w/w). The volatile components identified by GC-MS, their relative area percentages and their retention times are summarized in Table 1.

Table 1. Constituents of *Warionia saharae* essential oil

Compounds	KI[a] ???	%
α-thujene	934	0.4
α-pinene	938	0.5
Camphene	953	0.8
Sabinene	**973**	**2.4**
β-pinene	980	0.1
β-myrcene	993	0.1
***para*-cymene**	**1025**	**3.7**
Limonene	1030	0.2
1,8-cineole	**1034**	**6.2**
β-ocimene	1042	0.1
Linalool	**1098**	**16.8**
α-thujone	1105	0.3
β-thujone	1115	0.2
Camphor	**1145**	**4.6**
Borneol	1167	0.2
Terpinen-4-ol	**1176**	**3.6**
Terpineol	1189	0.5
trans carveol	1217	0.2
Gernaiol	1235	0.6
Pulegone	1237	0.8
Neral	1240	0.7
Linalyl acetate	1259	1.4
Bornyl acetate	1284	0.9
Carvacrol	1299	1.6
α-terpinyl acetate	1333	0.8
Eugenol	1353	0.6
β-elemene	1387	0.3
β-caryophyllene	1415	0.1
β-farnesene	1452	2.2
δ-cadinene	1526	1.2
***Trans*-nerolidol**	**1566**	**18.2**
Caryophyllene oxide	1580	0.7
α-cadinol	1640	0.8
β-eudesmol	**1650**	**24.6**
α-eudesmol	1652	0.2
14-hydroxy-α-humulene	1780	0.2
Monoterpene hydrocarbons		8.3
Oxygenated monoterpenes		40
Sesquiterpenes hydrocarbons		3.8
Oxygenated sesquiterpenes		44.3
Total identified		96.4

[a]Compounds are listed in order of their elution from an HP-5ms capillary column using the homologous series of n-alkanes.

In this study, 36 components representing 96.8% of the *W.saharae* leaves oil were identified. The chemical composition of the essential oil was dominated highly by the oxygenated sesquiterpenes (44.3%) followed by oxygenated monoterpenes (40.0%). The most abundant compounds were β-eudesmol (24.6%), trans-nerolidol (18.2%) and linalool (16.8%). These three compounds represent 59.6% of the total oils (Fig.1).

These results were in accordance with those previously reported in literature [8, 9, 10]. Indeed, Znini *et al* identified only 3 compounds such as eudesmol, linalool and nerolidol [9]. Thirty compounds amounting 91% of the oil, were identified by Essaqui *et al* [8]; the major components were β-eudesmol, trans-nerolidol and linalool [10]. Among the other chemical components were linalool, 1,8-cineole, camphor, p-cymene, terpinen-4-ol and sabinene. The differences recorded for the chemical composition of the essential oil of *W.saharae* can be according to the genetic characteristics and climatic, seasonal, geographical and geological differences where the plant is collected.

Among identified compound, nerolidol showed anti-leishmanial activity [18] and exhibits antineoplastic activity [19]. This compound is a sesquiterpene present in essential oils of several plants, approved by the US Food and Drug Administration (FDA) as a food flavoring agent. Beta-eudesmol has multiple pharmacological effects; the anti-inflammatory effect of β-eudesmol was shown recently [20].

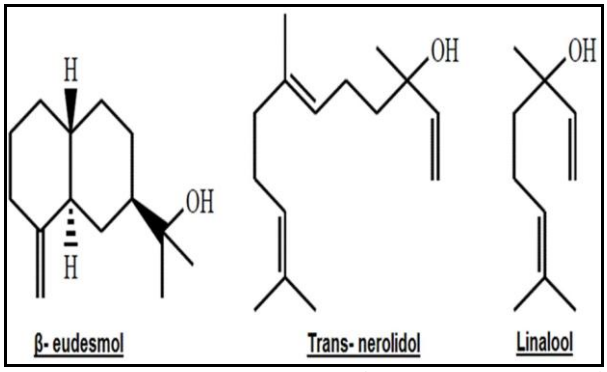

Figure 1. Chemical molecular structure of three major constituents of *W.saharae* essential oil.

Antibacterial activity

The antibacterial activity of *W.saharae* essential oil were evaluated by a paper disc diffusion method against bacterial strains including Gram positive and Gram negative bacteria, as to see in Table 2. *W.saharae* essential oil showed an important antibacterial activity against *S.aureus, B.cereus and P.aeruginosa*, while the growth of *E.coli* was less inhibited. The MICs confirmed this observation with values ranging between 0.039 and 0.156 mg/ml for all strains used; MBC values varied between 0.312 and 1.25 mg/ml.

S.aureus and B.cereus were strongly inhibited by essential oil of *W.saharae*, since MBCs values were lower (0.312 and 0.625 mg/ml, respectively). The antibacterial effect of the oil could be explained by instead of through the disruption of bacteria membrane integrity. Indeed, previous findings revealed that tea tree oil damages the cell membrane structure of *E.coli*, *S.aureus* and *Candida albicans* [21]. It is also possible that the minor components might be involved in some type of synergism with the other active compounds [22].

Antifungal activity

The results of antifungal activity assays showed that the essential oils of *W.saharae* had inhibitory effects on the growth of fungi (Table 3). *Botrytis cinerea* was most suppressed as its growth was mostly reduced, followed by *Penicillium expansum*, *Rhizopus stolonifer* and *Alternaria* sp at 50 ppm; the essential oils of *W.saharae* appeared to be effective against growth of these phytopathogens above 60%. The volatile oils consist of complex mixtures of numerous components. The major or trace compound(s) might give rise to the antifungal activity. Possible synergistic and antagonistic effects of compounds also play an important role in fungi inhibition.

Previous papers on the antifungal activities of essential oils of some species of various genera have shown that they have varying degrees of growth inhibition effects against some agricultural pathogenic fungal species [23].

Table 2. Antibacterial activity of *Warionia saharae* essential oil

Microorganism	Inhibition zone diameter[a]				EO		Amp	
	EO (µg/disc)			Amp (µg/disc)	MIC	MBC	MIC	MBC
	5	10	15					
Gram(-) bacteria								
E.coli ATCC 25922	16.4 ± 0.6	18.7 ± 0.4	20.3 ± 1.2	27.7 ± 1.5	0.156	1.25	1.95	> 3.9
P.aeruginosa ATCC 27853	12.5 ± 0.6	13.1 ± 1.5	17.3 ± 0.4	21 ± 1.2	0.078	> 0.625	3.9	7.81
Gram(+) bacteria								
S.aureus ATCC 25923	20 ± 1.5	24.7 ± 0.4	28.4 ± 0	32.7 ± 0.6	0.039	0.312	6.25	15.62
B.cerus ATCC 29213	18.9 ± 0.4	20.5 ± 0	22.7 ± 1.5	24 ± 1	0.078	0.625	3.9	7.81

[a]Diameter of the zone of inhibition (mm) including disk diameter of 6 mm; Amp, ampicillin; EO, essential oil; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; values given as mg/ml for the essential oils and as µg/ml for antibiotics.

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Table 3. Antifungal activity of *Warionia saharae* essential oil

Fungal species	Oil growth (mm)	Index antifungal (%)	Control growth (mm)
***Alternaria* sp**	21 ± 1	44.44 ± 0.7^{d}	37.8 ± 3.7
Penicillium expansum	23.5 ± 2.3	63.22 ± 0.7^{b}	63.9 ± 7.3
Rhizopus stolonifer	25 ± 0.2	60.75 ± 1^{c}	63.7 ± 7.2
Botrytis cinerea	18.6 ± 1.5	71.02 ± 0.4^{a}	64.2 ± 2.5

Values are means ± standard deviation of three separate experiments; means with different letters are significantly different at $P < 0.05$.

The higher observed activity of essential oil could be attributed to the presence of sesquiterpenes, monoterpene hydrocarbons and oxygenated monoterpenes in the leaves essential oil. The essential oils containing terpenes are reported to possess antimicrobial activity [24], which is in part consistent with our present study.

Antioxidant activity

The principle of antioxidant activity is based on the availability of electrons to neutralize free radicals. In this study, the antioxidant activity of *W.saharae* oil was evaluated by two complementary tests: scavenging of $DPPH^{+}$ free radicals and the β-carotene bleaching test. The results are shown in Table 4.

DPPH is a free radical compound which has been widely used to test the free-radical scavenging ability of various samples. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples [16]. The antioxidant effect of essential oil on DPPH radical scavenging may be due to their hydrogen donating ability and it reduce the stable violet DPPH radical to the yellow DPPH-H. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [25]. DPPH scavenging activity is usually presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Comparison of the DPPH scavenging activity of the *W.saharae* essential oil (26.23 ± 1.25 µg/ml) and those expressed by BHT (7.73 ± 0.11) showed that the essential oil exhibited weaker antioxidant effects than BHT; the antioxidant effect of the oil was about 3 times lower than that of the synthetic antioxidant BHT. The DPPH scavenging ability of this oil can be attributed to the presence of phenolic constituents and to the free radical scavenging activity of some volatile oils [26].

The β-carotene bleaching method is based on the loss of the yellow color of β-carotene due to its reaction with radicals that are formed by linoleic acid oxidation in an emulsion. In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxide arising from linoleic acid oxidation. Beta-carotene undergoes rapid discoloration in the absence of an antioxidant; however, the presence of antioxidant will be minimizing its oxidation. This test measures the potential of the plant to inhibit conjugated diene hydroperoxide formation from linoleic acid oxidation. As can be seen from Table 4, the potential of *W.saharae* to inhibit lipid peroxidation was evaluated using the β-carotene/linoleic acid bleaching test by measuring the antioxidant capacity (AA%), with a value of 60.4 ± 1.02% and 86.5 ± 0.5%, obtained for the oil and the positive control BHT, respectively at the same concentration of 100 µg/ml. This activity was moderately lower than that of BHT and was attributed to the presence of appreciable amount of antioxidant compounds such as 1,8-cineole, α-pinene and carvacrol and to the presence of phenolic compounds [25].

Table 4. Antioxidant capacity of *Warionia saharae*

	W.saharae essential oil	Butylated hydroxytoluene
DPPH (IC_{50} µg/ml DPPH solution)	26.23 ± 1.25	6.2 ± 0.21
Inhibition in linoleic acid system (%)	60.4 ± 1.02	86.5 ± 0.5

Values are means ± standard deviation of three separate experiments.

These research findings lead us to conclude that *W.saharae* essential oil could be considered as potential alternatives for synthetic bactericides and natural antioxidants for use in the food industry along with their possible applications in the pharmaceutical industry for the prevention or treatment of pathogenesis caused by microorganisms and free radicals.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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