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Essential oil composition of two plants belonging to family Myrtaceae grown in Egypt prepared by different methods and their antibacterial activity

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Abstract

Essential oil of *Callistemon citrinus* and *Melaleuca subulata* leaves were prepared by hydrodistillation (HD) and hydrodistillation assisted by microwave (HDAM) methods. Identification of chemical components of the oils was done using GC/ MS. The yield of *C. citrinus* leaves using HD and HDAM was 0.70% and 0.76% (w/w) respectively, while that of *M . subulata* was 0.73% and 0.81% (w/w) respectively. 1,8-cineole represents the major constituent of *C. citrinus* oil being 37.4% and 56.43% in case of HD and HDAM respectively while α-pinene and 1,8-cineole represent the major constituents for *M. subulata* oil being 47.06% and 16.13% in case of HD and 38.97%, 19.89% in HDAM respectively. The yielded oils showed variable antibacterial activity against the tested microorganisms using agar disc diffusion method.

Key words: 1,8-cineole; α-pinene; *C. Citrinus; Myrtaceae; M. subulata;* antibacterial.

INTRODUCTION

Essential oils obtained from aromatic plants have been known from long time for its use in perfume, pharmaceuticals and food industry as well as alternative medicine [1]. Among the aromatic plants, genus *Callistemon (Myrtaceae)* is commonly known as the bottlebrush. It comprises about 34 species, it is indigenous to Australia, and broadly spread in tropical and subtropical regions as a woody aromatic ornamental tree or shrub [2]. *Callistemon citrinus* (Curtis) Skeels (syn. *C. lanceolatus* D.C. and *Metrosideros Citrine)* is considered to be the most commonly cultivated plant species of the genus *Callistemon.* It is commonly known as Crimson or Lemon Bottlebrush, also it is indigenous to Australia and spread in many regions in the world as a woody aromatic ornamental plant [3, 4]. The plant's leaf is rich in essential oil and is commonly used as a tea [5]. The aerial parts were traditionally used for the treatment of different illnesses. Egyptians are using the essential oils as antimicrobial and an insecticidal agent as well as it is used for the treatment of cough, bronchitis and other purposes [6, 7]. The analysis of the essential oil prepared by hydro distillation from the leaves and flowers collected from different regions of the world revealed that 1,8-cineole is considered to be the major component (47.9 - 82.0%) followed by α- and β-pinenes, α-terpineol, α-phellandrene, limonene, α-terpinene, linalool (3,8-11). In addition, *C. citrinus* oil showed antimicrobial, antinociceptive and anti-inflammatory activities [8-12,13]. *Melaleuca subulata* (Cheel) Craven formally known as *C. subulatus* Cheel [14] is widespread in south eastern Australia. There is only one report about the chemistry of the *C. subulatus* which reveals that the main constituent of the essential oil is 1, 8-cineol (61.3%) [15]. The common method for essential oil preparation by HD, but this technique has many disadvantages due to possible transformation and degradation of oil components by heat [16]. Thus, an alternative technique as HDAM which is characterized by shortened extraction times, high yield of essential oil and the reduction of the organic solvent consumption, to replace HD technique in many different processes in the food and chemical industry was developed.

The analysis of *C. citrinus* leaf oil was reported before using HD technique but in the current study we prepared it by HD to compare the result with HDAM method for the first time, moreover we study the oil prepared by the two method for *M. subulta* leaves grown in Egypt for the first time. Consequently, the two methods were compared in terms of isolation time, yields and composition. In addition, the antibacterial activity of the oils prepared by both methods was evaluated.

MATERIAL AND METHODS

Plant material

C. citrinus (Curtis) Skeels and *M. Subulata* Cheel Craven fresh leaves (500 g) were collected from El Orman Botanical Garden, Giza, Egypt (October, 2017). The leaves were stored in the refrigerator till usage. The taxonomical identification was established by Dr.Trease Labib, former specialist of plant Taxonomy, El Orman Botanical Garden. Voucher specimen [No. 02C ci/2017] and [No.03M su/2017] for *C. Citrinus* and *M. subulata* respectively was deposited at the herbarium of Pharmacognosy department. Faculty of Pharmacy, Helwan University

Chemicals

Alkane mixture of C_8-C_{22} and the external standard C_{12} (40 mg/mL in hexane) were obtained from Sigma-Aldrich (UK).Anhydrous sodium sulphate was purchased from El Nasr company for intermediate chemicals (Giza- Egypt).

Preparation of the oil by hydrodistillation (HD)

C. citrinus and *M. subulata* fresh leaves (150 g each) were suspended in 500 ml distilled water and subjected to hydrodistillation using Clevenger-type apparatus for 4h [17, 18]. The essential oil layer was removed from the aqueous layer. The traces of water

vapour were removed by drying over anhydrous sodium sulphate then filtered. The pure oil then was stored at 4°C until analysis using GC/ Ms. The method was repeated triplicate.

Preparation of the oil by hydrodistillation assisted by microwave (HDAM)

HDAM was carried out in a microwave laboratory oven under atmospheric pressure. 150 g of fresh leaves of each plant were put in 2L two neck-flask then combined with 500 mL distilled water. The flask was placed within the microwave oven cavity and the sample holder was connected to the Clevenger refrigerator that is provided with a glass stop cock and circulating water condenser in order to collect the extracted essential oil. This mixture was heated at a fixed power off 800 W at 100 °C for 20 min which is a sufficient period to extract all the essential oil from the sample. The essential oil was collected and dried over anhydrous sodium sulphate and the pure oil was stored at 4°C [19].

GC/ MS analysis

GC/MS (70eV) data were measured using gas chromatography coupled with a Thermo mass spectrometer detector (ISQ Single Quadru pole Mass Spectrometer). The TG-WAX MS capillary column (30 m x 0.25 mm i.d., 0.25 μm film thicknesses) was coupled directly to the MS. Helium was used as carrier gas (flow rate is 1.0 mL/min. and a split ratio of 1:10). The oven temperature was programmed 60°C for 8 min, then 60- 240°C at 3°C/min and consequently, held isothermal for 10 min. Injector port is 240°C and detector temperature is 240°C. Volume of the sample injected is 0.2 µL of 1% solution in hexane. Scan time 1.5 s, split less time: 0.75. Mass spectra were recorded over 40 - 450 range with EI mode of ionization at 70 eV ionization. The Software used for handling the mass spectra and chromatograms is Chem Station.

Identification of the compounds

Identification of the compounds was carried out through comparing their relative retention indices (R.I.) of GC peaks with those of saturated C_8–C_{22} *n*-alkanes and those of the available authentic standards. Moreover, the identification was confirmed by comparing the mass spectral fragmentation patterns of the compounds with those represented in the data bank (Wiley/NBS library and National Institute of Standards and Technology, NIST) and reported in the literature [20]. Quantification was based on the relative area percentage calculated from the GC detector response without using correction factors. Standards were used for the identification of major constituents.

Antibacterial activity

Gram-positive bacteria; *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus*UW81 and the Gram-negative strains *Escherichia coli*ATCC 12435 and *Pseudomonas aeruginosa* PAO1 were supplied from faculty of pharmacy- Mansoura University. Müller-Hinton Agar (Sigma-Aldrich Company, USA); ampicillin (October pharma, Egypt); ceftriaxone (MUP for Schering-plough, Egypt) and Triphenyltetrazolium chloride (Sigma–Aldrich, Chemical Company USA) were used for antimicrobial evaluation.

Susceptibility test

Determination of the antibacterial activity of the oils was done using agar disc diffusion method [21-22]. The Muller Hinton agar (20 mL) at 37°C was inoculated with 20 µL inoculums of each tested isolate diluted at 0.5McFarlthen mixed well, poured into sterile Petri dish and left until complete solidification. Sterile and dried 4 mm paper discs were saturated with 10 µL sterilized oils then placed on freshly seeded microbial lawns (4 discs in each plate) with a control. All experiments were performed in triplicates. The plates were incubated at 37°C. After 48 h, zones of inhibition developed against the tested bacteria were measured in mm. The results of the antibacterial activity were expressed as resistant, intermediate and sensitive. Ampicillin and ceftriaxone (10 µL, 5 mg/mL) were used as anti-bacterial controls.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of the active samples was measured using microtiter plate micro-dilution method. Two-fold serial dilutions of the active samples were carried out in 100 µLMuller Hinton to reach concentrations from 64 to 1.6 µL then the plates were incubated overnight at 37°C. Triphenyltetrazolium chloride (0.5 w/v) was added to each well; it is reduced to violet colour in microbial growth to assists the detection of the microbial growth. MIC was determined as the lowest concentration of samples with no visible growth.

RESULTS

The oil yield of *C. citrinus* by HD and HDAM methods was 0.70 % and 0.76% (w/w) respectively, while that for *M. subulata* was 0.73% and 0.81% (w/w) respectively (Table 1). The oils were characterized by their yellowish color and strong aromatic odor. A total of 14 and 16 compounds were identified and quantified in the HD and HDAM oils of *C. citrinus* respectively representing 97.32 % and 99.78% of the total oil. 1,8-cineole represent the major compound identified and its percent being 37.41% in HD and 56.43% in HDAM followed by α-terpineol which percent is 13.57% and 10.93% respectively (Table 1). Moreover, α-pinene is considered as the major hydrocarbon compound identified and its percentage become 6.48% and 5.42% in HD and HDAM respectively. In case of *M. subulata*, a total of 13 constituents were identified in both HD and HDAM oil representing 95.18 % and 92.97 % of the total oil respectively. In contrast to *C. citrinus* oil, in which the oxygenated compounds represents the major percentage, the hydrocarbons α-pinene represents major compound in *M.subulata* oil and

its percentage being 47.06% and 38.97% in case of HD and HDAM respectively. In addition, 1, 8-cineole represents the major oxygenated compound with 16.13% and 19.89% for HD and HDAM respectively.

Results of the antibacterial activity were presented in Table 2. It was reported before that the samples showing inhibition zones more than 10 are considered to have strong antibacterial activity while that having 8-10 mm are moderately active [23]. Based on this fact it was found that the essential oil of *C. citrinus* prepared by HDAM showed moderate activity against all the tested bacteria while that for HD showed moderate activity against *B. cereus* and *E. coli* with no activity towards the *P. aeruginosa*. In case of *M. subulata*, the essential oil prepared by HD and HDAM showed strong activity against *S. aureus*. In addition, essential oil of HD and HDAM showed moderate activity against *B. cereus*.

The MICs of essential oil samples were evaluated (Table 3), *S. aureus* showed MIC of 1.28 µL with HD and HDAM oils of *M. subulata* which gave an indication that *S. aureus* was moderately inhibited by it. Furthermore, *E. coli* illustrate a MIC of 1.28 µL HDAM oil of *C. citrinus* which confirm the moderate inhibition of *E. coli* by it.

Table1. Chemical composition *of C. citrinus* and *M. subulata* leaves essential oils prepared by hydrodistillation and microwave assisted extraction.

Compound	Rt	KI ^{cal}	KI ^{Ref}	Percentage composition			
				C. citrinus		*M. subulata*	
				HD	HDAM	HD	HDAM
α-Pinene	4.62	935	939	6.48	5.42	47.06	38.97
Camphene	5.16	949	953	1.88	1.85	--	--
β-Pinene	5.82	981	980	3.00	2.62	1.87	1.38
Myrecene	6.17	985	986	3.47	2.48	0.33	0.50
α-Phellandrene	6.68	999	1005	--	--	3.10	2.98
δ-3-Carene	6.80	1008	1011	0.61	0.49	--	--
α-Terpinene	7.13	1015	1018	1.71	0.73	0.46	0.53
p-Cymene	7.31	1025	1026	--	0.35	2.86	2.50
d-Limonene	7.40	1030	1031	0.65	--	--	--
1,8-Cineole	7.73	1034	1033	37.41	56.43	16.13	19.89
γ-Terpinene	8.50	1061	1062	4.07	2.13	2.47	2.77
α-Terpinolene	9.47	1082	1088	7.79	4.79	3.22	3.75
l-Linalool	10.03	1096	1098	9.15	5.84	0.38	0.93
Borneol	13.06	1164	1165	0.47	0.49	0.52	0.66
4-Terpineol	13.34	1173	1177	7.06	4.74	1.51	2.25
α-Terpineol	14.06	1186	1189	13.57	10.93	15.27	15.86
Ledol	25.91	1561	1565	--	0.26	--	--
Globulol	29.76	1584	1583	--	0.23	--	--
Total % of identified compounds				97.32	99.78	95.18	92.97
Extraction time(min)				240	60	240	60
Oil yield (W/W%)				0.76	0.70	0.81	0.73
Hydrocarbons				29.66	20.86	61.37	53.38
Oxygenated				67.66	78.92	33.81	39.59

Rt = retention time; KI ^{cal} = Calculated Kovats Indices; KI ^{ref} = Experimental Kovats Indices

Table 2. Antibacterial activity of *C. citrinus* and *M. subulata* leaves essential oil.

Isolates	Diameter of the inhibition zone in mm					
	C. citrinus		*M. subulata*		Control	
	HD	HDAM	HD	HDAM	Amp	CTX
S. aureus	-	8	12	12	18	22
B. cereus	8	9	9	10	16	17
E. coli	8	10	-	-	-	16
P. aeruginosa	-	9	-	-	-	14

Results were expressed as mean IZ ± S.D.

Table 3. Minimum inhibitory concentration (MIC) of *C. citrinus* and *M. subulata* leaves essential oil

Isolates	MIC of the tested volatile oil and control					
	C. citrinus		*C. subulatus*		Control	
	HD	HDAM	HD	HDAM	Amp mg/ml	CTX mg/ml
S. aureus	-	2.56	1.28	1.28	1	0.128
B. cereus	5.12	2.56	2.56	2.56	>4	0.128
E. coli	5.12	1.28	-	-	>4	0.5
P. aeruginosa	-	>32	-	-	>4	2

MIC values expressed as µL

DISCUSSION

Regarding the difference in the amount of essential oil extracted by the two methods, it was found that the highest amount was for HDAM followed by HD (Table 1), this could be due to the accelerated rupture of essential oil glands in the presence of microwaves [24], as well as a high dielectric constant of the water allows it to absorb the radiation from the microwaves resulting in a rise in the temperature faster than that in HD, since the high temperature causes the degradation of the plant cells and therefore shortens the extraction time [23]. It is interesting to note that the short distillation time in case of HDAM (20 min) gave high oil yield compared to that obtained after 4 h in case of HD. These results showed a considerable time and energy saving in case of HDAM.

In addition, the percentage of oxygenated compounds was found to be higher in case HDAM than the other method which may be attributed to the rapid heating of polar compounds by microwave, and consequently a shorter extraction time, less amounts of water used. This reduces the level of degradation of oxygenated compounds to minor ones which with less value [25-26]. Plants belonging to family *Myrtaceae* are characterized by the variation in the percentages of the oxygenated compounds and hydrocarbons [15]. Moreover, the difference between the essential oil compositions of the two species being 1, 8-cineole in *C. citrinus* and α-pinene in *M. subulata* (Table1) could be attributed to the environmental factors such as geography, temperature, day length and nutrients [16].

Regarding the difference in the percentage of oxygenated compounds mainly 1,8-cineole and the monoterpene hydrocarbons especially α-pinene between *C. citrinus* and *M. subulata*, it was found that although the essential oils obtained from *C. citrinus* have high content of 1,8-cineole (37.41, 56.43% in case of HD and HDAM respectively) which is well known by its pronounced antimicrobial potentials [27], it showed low anti-bacterial activity. Likewise, the essential oils of *M.subulata*, mainly composed of α-pinene (47.06 and 38.97% in case of HD and HDAM respectively) showed a strong antibacterial activity since that, the α-pinene appears to be able to disintegrate the cellular integrity, and cause the inhibition of respiration and ion transportation process [28], moreover, it was reported that the *Myrcia myrtifolia* essential oil (F. Myrtaceae) contains a high percentage of α-pinene (87.3%) and it exhibits a strong antibacterial activity against *S. aureus* [29].

CONCLUSION

Conventional hydrodistillation (HD) technique has been compared with microwave-assisted hydrodistillation (HDAM) for the preparation of essential oil from *C. citrinus* and *M. subulata* leaves. HDAM method offers important advantages over the traditional HD, shorter extraction times; better yields; environmentally friendly; lower cost and the possibility for a good reproduction of natural aroma.

Conflict of interest

The authors declare no conflict of interest.

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