SUPPLEMENTARY MATERIAL

Hierarchical cluster analysis and chemical characterization of *Myrtus communis* L. essential oil from Yemen region and its antimicrobial, antioxidant and anti-colorectal adenocarcinoma properties

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Abstract

The hydrodistilled essential oil obtained from the dried leaves of *Myrtus communis*, collected in Yemen, was analyzed by GC–MS. Forty-one compounds were identified, representing 96.3% of the total oil. The major constituents of essential oil were oxygenated monoterpenoids (87.1%), linalool (29.1%), 1,8-cineole (18.4%), α-terpineol (10.8%), geraniol (7.3%) and linalyl acetate (7. 4%). The essential oil was assessed for its antimicrobial activity using a disc diffusion assay and resulted in moderate to potent antibacterial and antifungal activities targeting mainly *Bacillus subtilis, Staphylococcus aureus* and *Candida albicans.* The oil moderately reduced the diphenylpicrylhydrazyl radical (IC_{50}= 4.2µl/ml or 4.1 mg/ml). *In vitro* cytotoxicity evaluation against HT29 (human colonic adenocarcinoma cells) showed that the essential oil exhibited a moderate antitumor effect with IC_{50} of 110 ± 4 µg/ml. Hierarchical cluster analysis of *M. communis* has been carried out based on the chemical compositions of 99 samples reported in the literature, including Yemeni sample.

Keywords: *Myrtus communis*, linalool, cytotoxic, Yemen, Hierarchical cluster analysis

Experimental

Plant material: The leaves of *M. communis* with blue-black berries morph were collected in the early morning from the Alselw district, Taiz province, Yemen, on 13th August 2011. The plant was identified by Dr. Hassan M. Ibrahim of the Botany Department, Faculty of Sciences, and Sana'a University. A voucher specimen (YMP-comp-14) has been deposited at the Pharmacognosy Department, Sana'a University, Yemen.

Volatile oil extraction: Dried leaves from *M. communis* were hydrodistilled for 3 h in a Clevenger type apparatus according to the European Pharmacopoeia (European Pharmacopoeia 1997). The obtained oil was subsequently dried over anhydrous Na_2SO_4 and kept at 4°C until analysis.

GC-MS analysis: The essential oil of *M. communis* was analyzed by GC-MS using an Agilent 6890 GC with Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV), scan range = 45-400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC column was a HP-5ms fused silica capillary with a (5% phenyl)-polymethylsiloxane stationary phase, film thickness of 0.25 μm, a length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 48.7 kPa and a flow rate of 1.0 mL/min. Inlet temperature was 200°C and interface temperature 280°C. The GC oven temperature program was used as follows: 40°C initial temperature, hold for 10 min; increased at 3°C/min to 200°C; increased 2°/min to 220°C. A 1 % w/v solution of the sample in CH_2Cl_2 was prepared and 1 μL was injected using a splitless injection technique. Identification of the oil components was based on their retention indices determined by reference to a homologous series of *n*-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature (Adams 2007), as stored in the MS library [NIST database (G1036A, revision D.01.00) / ChemStation data system (G1701CA, version C.00.01.080]. The percentages of each component are reported as raw percentages based on total ion current (TIC) without standardization. The essential oil composition of *M. communis* is summarized in Table S1.

Antimicrobial activity: The antimicrobial activity of the essential oil was evaluated by the agar disc diffusion method, and the minimal inhibitory concentration (MIC) was determined using a broth dilution method, as previously described (Ali et al. 2012).The microorganisms used were *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 25619, *Staphylococcus aureus*

ATCC 29737, *Bacillus subtilis* ATCC 6633, and *Candida albicans* ATCC 2091. Muller Hinton Agar (MHA) (Merck, Darmstadt, Germany) was used for bacterial culture at 37ºC. Sabouraud dextrose agar (Merck, Darmstadt, Germany) was used to culture *Candida albicans*.

Determination of antioxidant activity: For the preliminary test, analytical TLC on silica gel plates was developed under appropriate conditions after application of 5 μL of oil solution, dried and sprayed with DPPH solution (0.2%, MeOH). Five minutes later, active compounds appeared as yellow spots against a purple background. Estimation of a radical scavenging effect was carried out by using a DPPH free radical scavenger assay in 96-well microtiter plates (MTP) according to the modified method (Mohamad et al.2004).A solution of DPPH (Sigma-Aldrich, Germany) was prepared by dissolving 5 mg DPPH in 2 mL of methanol, and the solution was kept in the dark at 4°C until use. Stock solutions of the samples were prepared at 2 mg/mL and diluted to different concentrations. Methanolic DPPH solution (5 μL) was added to each well. The plate was shaken for 2 min to ensure thorough mixing before being wrapped in aluminum foil and stored in the dark. A methanolic solution of DPPH served as control. After 30 min the optical density (OD) of the solution was measured at a wavelength of 517 nm using a microtiter plate ELISA reader (Thermo, Finland) and the percentage decolorization calculated as an indication of the antioxidant activity of the sample. Each experiment was made at least in triplicate and IC_{50} values were calculated. Ascorbic acid (Sigma-Aldrich, Germany) was used as a positive control. DPPH scavenging activity is usually presented by an IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution.

Cytotoxicity test: HT29 cells were grown in a 5% CO_2 environmentat 37°C in RPMI 1640 medium without L-glutamine, supplementedwith 10% fetal bovine serum, 1% (200 mM) L-alanyl-L-glutamineand 1.6% HEPES (1 M). Cells were plated into 96-well cell culture plates at 1.5×10^3 cells (HT29) per well. The volume in each well was 100 μL. After 24 h, supernatant fluid was removed by suction and replaced with 100 μL growth medium containing 0.1 μL of a DMSO solution of the essential oil, giving final concentrations of 25, 50 and 100 μg/mL for each well. Solutions were added to wells in 3 replicates. Medium controls without cells and DMSO controls (0.5 μL DMSO/mL) were used. Digitonin (125 μM) was used as a positive control (Rashan et al. 2011). After the addition of oils, plates were incubated for 72 h at 37°C in 5% CO_2; medium was then removed by suction, and 100 μL of fresh medium was added to each well. In order to establish percent growth inhibition rates, the XTT assay for cell viability was

carried out according to Cell Proliferation Kit II protocol, (Roche) (Scudiere et al. 1988). After colorimetric readings were recorded (Molecular Devices Spectra MAX M5 microplate reader, 490 nm), average absorbance, growth inhibition and standard deviations were determined. The IC_{50} value was determined using the Reed-Muench method (Reed and Muench 1938).

Hierarchical Cluster Analysis: A total of 98 M. communis essential oil compositions from the published literature (Akin et al. 2010, Asllani 2000, Bazzali et al. 2012, Berka-Zougali et al. 2012, Boelens & Jimenez 1992, Bouzabata et al. 2015, Bouzouita et al. 2003, Brada et al. 2012, Bradesi et al. 1997, Cherrat et al. 2014, Chryssavgi et al. 2008, Conti et al. 2010, Curini et al. 2003, Djenane et al. 2011, Farah et al. 2006, Flamini et al. 2004, Ghannadi & Dezfuly 2011, Ghnaya et al. 2013, Hennia et al. 2015, Hsouna et al. 2014, Jamoussi et al. 2005, Khan et al. 2014, Koukos et al. 2001, Mahboubi & Bidgoli 2010, Messaoud et al. 2005, Mimica-Dukić et al. 2010, Moghrani & Maachi 2008, Mulas & Melis 2011, Özek et al. 2000, Pereira et al. 2009, Rahimmalek et al. 2013, Rasooli et al. 2002, Senatore et al. 2006, Tuberoso et al. 2006, Wannes et al. 2007, 2010, Weherstahl et al. 1994, Yadegarinia et al. 2006, Zomorodian et al. 2013), as well as the EOMC composition from this study were treated as operational taxonomic units (OTUs). The percentage composition of 30 major essential oil components (α-pinene, 1,8-cineole, linalool, limonene, α-terpineol, myrtenyl acetate, linalyl acetate, geranyl acetate, methyl eugenol, α-terpinyl acetate, terpinen-4-ol, β-ocimene, β-pinene, p-cymene, geraniol, γ-terpinene, terpinolene, β-caryophyllene, δ-3-carene, α-thujene, myrtenol, myrcene, isobutyl isobutyrate, neryl acetate, borneol, caryophyllene oxide, cis-verbenol, camphene, estragole, and bornyl acetate) was used to determine the chemical relationship between the various M. communis essential oil samples by agglomerative hierarchical cluster (AHC) analysis using the XLSTAT software, version 2015.4.01. Pearson correlation was selected as a measure of similarity, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition. The cluster analysis dendrogram is shown in Fig. S4.

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Table S 1: Essential oil composition of *Myrtus communis*

	RI[a]	RI[b]	Compound	Area (%)
1	912	911	Isobutyl isobutyrate	0.1
2	**942**	**939**	**α-Pinene**	**2.3**
3	993	990	Myrcene	Tr[c]
4	1025	1024	*p*-Cymene	0.1
5	1028	1029	Limonene	0.4
6	1032	**1031**	**1,8-Cineole**	**18.4**
7	1039	1037	(*Z*)-β-Ocimene	Tr
8	1049	1050	(*E*)-β-Ocimene	Tr
9	1073	1072	*cis*-Linalool oxide (furanoid)	0.1
10	1089	1086	*trans*-Linalool oxide (furanoid)	0.1
11	1104	**1096**	**Linalool**	**29.1**
12	1113	1116	*endo*-Fenchol	Tr
13	1138	1139	*trans*-Pinocarveol	0.3
14	1165	1169	Borneol	0.1
15	1167	1166	δ-Terpineol	0.2
16	1177	1177	Terpinen-4-ol	0.6
17	1186	1182	*p*-Cymen-8-ol	Tr
18	1192	**1188**	**α-Terpineol**	**10.8**
19	1198	1196	Estragole (= Methyl chavicol)	0.2
20	1217	1216	*trans*-Carveol	0.1
21	1227	1229	Nerol	0.4
22	1253	**1252**	**Geraniol**	**7.3**
23	1258	**1257**	**Linalyl acetate**	**7.4**
24	1271	1267	Geranial	0.1
25	1299	1298	*trans*-Pinocarvyl acetate	Tr
26	1302	1299	Carvacrol	Tr
27	1323	1324	Methyl geranate	0.4
28	**1350**	**1349**	**α-Terpinyl acetate**	**6.2**
29	1366	1361	Neryl acetate	1.7
30	**1386**	**1381**	**Geranyl acetate**	**3.8**
31	**1407**	**1403**	**Methyl eugenol**	**4.0**
32	1419	1419	(*E*)-Caryophyllene	Tr
33	1454	1455	Geranylacetone	Tr
34	1487	1488	(*E*)-β-Ionone	0.1
35	1496	1492	δ-Selinene	Tr
36	1516	1515	Geranyl isobutanoate	Tr
37	1522	---	Unidentified	1.6
38	1546	1547	Flavesone	0.5
39	1584	1583	Caryophyllene oxide	0.9
40	1609	1608	Humulene epoxide II	0.2
41	1636	1640	Caryophylla-4(12),8(13)-dien-5-ol	Tr
42	1645	---	Unidentified	0.9
43	1655	1659	Selin-11-en-4α-ol	0.4

44	1718	---	Unidentified	0.7
			Monoterpene hydrocarbons	2.8
			Oxygenated monoterpenoids	87.1
			Sesquiterpene hydrocarbons	Tr
			Oxygenated sesquiterpenoids	1.5
			Phenylpropanoids	4.2
			Others	0.7
			Total Identified (41)	96.3

[a] Retention indices relative to a series of normal alkanes on an HP-5ms column.
[b] Retention indices from Adams (2007).
[c] Tr = "trace" (< 0.05%).

Table S2: Inhibition zone (mm) of Myrtle Oil:

Plant	Conc.	Inhibition zones in (mm)					
		bacteria					Fungus
		Gram (+ve)		Gram (-ve)		Resistant bacteria	
		S.a	*B.s*	*E.c*	*P.a*	*MRSA*	*C.a*
M.communis	10 µl/disc $8.6*10^3$mcg/disc	14	**48**	10	-ve	11	**19**
Ampicillin Disc	10 mcg/disc	39	32	22	17	----	-----
Gentamicin Disc	10 mcg/disc	23- 1	35	27	23	-----	-----
Enoxacine	10mcg/disc	----	----	----	-----	22	----
Nystatin Disc	100 unit/disc	-----	------	----	-----	- ----	27
Sterile Disc	----	-ve	-ve	-ve	-ve	-ve	-ve

Mean zone of inhibition in mm, S.a = *S.aureus* ; B.s =*Bacillus subtilis*; E.c = *Ecoli*; P.a = *Pseudomonas aerogenosa*; C.a= *Candida albicans*; *MRSA *:Methicillin Resistant Staphylococcus.*

Table S3: Minimum inhibitory concentration of Myrtle oil:

Sample	MIC (μg/ml)					
	Bacteria					Fungi
	Gram (+ve)		Gram (-ve)		Resistant bacteria	
	S. a	**B. s**	**E. c**	**P. a**	**MRSA**	**C. a**
M.communis	10 ($8.6*10^3$)	5 ($4.3*10^3$)	-------	-------	-----	10 ($8.6*10^3$)
Growth control	+ve	+ve	+ve	+ve	+ve	+ve
Sterility control	-ve	-ve	-ve	-ve	-ve	-ve

MIC = minimal inhibitory concentration (μg/mL); S.a = *S.aureus* ; B.s =*Bacillus subtilis*; E.c = *Ecoli*; P.a = *Pseudomonas aerogenosa*; C.a= *Candida albicans*; *MRSA*: Methicillin Resistant Staphylococcus*.

Figure S4: Dendrogram obtained from the agglomerative hierarchical cluster analysis of 99 *Myrtus communis* essential oil compositions: