

SUPPLEMENTARY MATERIAL

Chemical composition, bactericidal kinetics, mechanism of action, and antiinflammatory activity of *Isodon melissoides* (Benth.) H. Hara essential oil

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Abstract

Present study was aimed to investigate the antibacterial activity, bactericidal mechanism of action, killing kinetics and antiinflammatory activity of *Isodon melissoides* (Benth.) H. Hara essential oil. The gas chromatography-flame ionisation detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS) analysis revealed the presence of carvacrol (45.4 %), *p*-cymene (11.6 %) and thymol (11.3 %) as major constituents of the oil. The oil displayed broad spectrum significant antibacterial activity (MIC: 0.13–8.33 ppm; MBC: 0.13– >33.34 ppm) against test strains. The oil exhibited a time and dose-dependent bactericidal effect. The oil disrupted the cell membrane by changing the cell membrane permeability. The essential oil significantly decreased the overproduction of proinflammatory cytokines in LPS-induced inflammation in HaCaT cells without any cytotoxic effect. *I. melissoides* essential oil can be a promising alternative antimicrobial agent for the control of methicillin resistant *staphylococci* and other pathogenic bacteria tested, and also useful for the topical anti-inflammatory properties.

Keywords: *Isodon melissoides*, carvacrol, methicillin resistant *staphylococci*, bactericidal mechanism, antiinflammatory activity

Experimental

Plant material and isolation of essential oil

The fresh plant material of *I. melissoides* was obtained from the experimental farm of CSIR-CIMAP, Research Centre Pantnagar Uttarakhand. The plant material was hydrodistilled in a Clevenger apparatus for 3h to isolate the essential oil. The essential oil obtained was dried over anhydrous sodium sulphate kept in the cold until further analysis.

GC and GC-MS analysis

The essential oil analysis was carried out using GC-FID and GC-MS techniques. Quantification of the essential oil constituents was done on Centurion Scientific Gas Chromatograph (model CS-5800), equipped with flame ionisation detector (FID) and BP-5 fused silica capillary column (5% phenyl)-polymethylsiloxane stationary phase; 30 m × 0.25 mm internal diameter; film thickness 0.25 µm). Oven temperature was programmed from 60°C to 240°C with increase of 3°C min⁻¹ and final hold time of 10 min. The injector and detector temperatures were 240°C and 250°C, respectively. Nitrogen was used as carrier gas at 1.0 mL min⁻¹. The injection volume was 0.3 µL (diluted in hexane: 1:1) with a split ratio of 1:60. GC-MS analysis was done using a Clarus 680 GC interfaced with a Clarus SQ 8C mass spectrometer of PerkinElmer fitted with Elite-5 MS fused-silica capillary column (5% phenyl)-polymethylsiloxane stationary phase; 30 m × 0.25 mm internal diameter, film thickness 0.25 µm). The oven temperature program was from 60 to 240°C, at 3°C min⁻¹, and programmed to 270°C at 5°C min⁻¹. Injector temperature was 250 °C; transfer line and source temperatures were 220 °C; injection size 0.03 µL neat; split ratio 1:50; carrier gas He at 1.0 mL min⁻¹; ionization energy 70 eV; mass scan range 40-450 amu. Identification of the essential oil constituents was achieved on the basis of retention index (RI), MS Library search (NIST and WILEY), and by comparing RI and mass spectral data with the literature (Adams, 2007; Chaturvedi et al., 2018).

Antibacterial activity

The antibacterial activity of *I. melissoides* essential oil was determined against twelve pathogenic bacterial strains, namely MRSA {methicillin resistant *Staphylococcus aureus* (33591)}, MRSE {methicillin resistant *Staphylococcus epidermidis* (51625)}, MRSA {multi drug resistant *Staphylococcus aureus* (BAA-44)}, *Mycobacterium smegmatis* (UDSC-MC² 155); *Staphylococcus epidermidis* (MTCC-435); *Enterococcus faecalis* (MTCC-439), *Streptococcus mutans* (MTCC-890), *Staphylococcus aureus* (MTCC-96); *Escherichia coli* (MTCC-723); *Escherichia coli* (DH5α), *Streptococcus salivarius* (13419); *Pseudomonas aeruginosa* (27853) using disc diffusion and micro dilution broth assays as per the CLSI guidelines (CLSI, 2006; CLSI, 2012; Chaturvedi et al., 2018). 100 µL of inoculum was carefully taken and spread on the Mueller Hinton agar plates to get uniform lawn. A total of 8 µL of the sample was impregnated on the sterile paper disc (Himedia labs, 6 mm diameter) and placed over the seeded plates. Plates were stored in incubator at 37 °C for 24 h. Net zone of inhibition was determined by subtracting the diameter of disc. The minimum inhibitory concentration (MIC) of the oil samples were determined by broth dilution assay in 96 well micro titre plates. Oil samples were diluted in the range of 100–0.06 µL mL⁻¹. Two fold serial dilutions was carried out for determining the MIC, last two columns of well was served as control, i.e. 11th as positive control for bacterial growth and 12th for plate sterility. 10 µL of freshly prepared working inoculums from overnight culture was inoculated in each well. Plates were stored at 37 °C for 24 hrs. Resazurin dye 0.01% was used as an indicator for the MIC determination. Bactericidal end points

were determined by spreading a known volume (10 µL) from each well on MHA plate. Tetracycline and Vancomycin were used as positive control, while DMSO was considered as negative control. The experimental observations were taken in triplicate. The bacterial strains were obtained from the Microbial Type Culture Collection Centre (MTCC), Institute of Microbial Technology (IMT) Chandigarh, India.

Bactericidal kinetics

The kinetics of bacterial killing of *Staphylococcus aureus* (SA), *Staphylococcus epidermidis* (SE), and *Enterococcus faecalis* (EF) by the essential oil were determined and compared at 2MIC and 4MIC with those of Log-phase culture (1.0×10^7 to 2.0×10^7 CFU/mL) (Pearson et al., 1980). Aliquots of sample were removed after 0.5, 1, 2, 3, and 6 h and diluted in sterile normal saline solution before plating on Mueller-Hinton agar plates, CFU were counted after 24 h of incubation at 37°C. The experiment was repeated three times and the results presented here are from one of the three independent experiments.

The antibacterial mechanism of action

Cell membrane integrity

Leakage of proteins through the membrane

The cell integrity of bacteria was examined by quantifying the release of proteins into the supernatant. The concentrations of proteins in supernatant were determined by using Bradford method (Bradford, 1976). Logarithmic growth phase was estimated for bacterial cells treated with the essential oil at MIC/2, MIC, and 2×MIC except the control, during an incubation period of 24h at 37°C. Micro-organisms were separated by centrifugation (10,000 g) for 5 minutes at 4°C, and the concentrations of cytoplasmic proteins was estimated by measuring the optical density at 595 nm. Results was calculated by the linear equation using Bovine Serum Albumin as standard ($y = 0.003x + 0.308$; $r^2 = 0.998$).

Leakage of DNA and RNA (Cytoplasmic constituent) through the membrane

The integrity of cell membrane was monitored by the release of cytoplasmic constituents of the cell absorbing at 260 nm (Chen and Stuart, 2002). The bacteria were incubated in MHB at 37°C for 12h. Logarithmic growth phase cells of bacteria were treated with the essential oil at MIC/2, MIC, and 2×MIC, except the control. Then the samples were incubated at 37°C for 24h. The samples were then immediately centrifuged (10,000 g) for 5 min at 4°C. To determine the amounts of the DNA and RNA released from the cytoplasm, the supernatant was used to measure the optical density at 260 nm. The results are expressed as the absorbance of the sample (treated)–the absorbance of the control (untreated). Data are expressed as mean \pm SEM (n = 3).

Anti-inflammatory activity assessment of essential oil against LPS-induced inflammation in HaCaT cells

HaCaT, a human keratinocyte cell line was obtained from the National Centre for Cell Sciences Pune, India. Cells were cultured in DMEM supplemented with 10 % fetal bovine serum with antibiotic antimycotic solution in a CO₂ incubator at 37°C with 5 % CO₂ and 90 % relative humidity. HaCaT cells (0.5×10^6 live cells/mL) were treated with *I. melissoides* essential oil at the concentration of 0.3 % and 1 %, respectively, followed by stimulation with LPS for 6 h, separately. Proinflammatory mediators like tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin- β (IL- β) in cell culture supernatant were determined by using human-specific enzyme immunoassay (EIA) kits (BD Biosciences, USA) as per the method described (Singh et al. 2014). The values of TNF- α , and IL-6 were expressed as picograms per millilitre.

Measurement of the cell viability

Effect of essential oil on cell viability was carried out in HaCaT cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Sharma et al., 2012). Peritoneal macrophage cells (0.5×10^6 cells/well) isolated from mice were suspended in RPMI 1640 medium (Sigma, USA) containing 10 % heat-inactivated fetal bovine serum (Gibco, USA) and incubated in a culture 96 well plate at 37°C in 5 % CO₂ in an incubator and left overnight to attach. Cells treated with 1 % DMSO served as a vehicle control for cell cytotoxicity study. The essential oil was dissolved in DMSO. Cells were treated (0.3 % and 1 % essential oil) and incubated for 24 h at 37°C in 5 % CO₂. After incubation, cells with treatment, 20 μ L aliquots of MTT solution (5 mg/mL in PBS) were added to each well and left for 4 h. Then, the MTT containing medium was carefully removed and the cells were solubilized in DMSO (100 μ L) for 10 min. The culture plate was placed on a micro-plate reader (Spectramax; Molecular Devices, USA) and the absorbance was measured at 550 nm. The amount of colour produced is directly proportional to the number of viable cells. Cell cytotoxicity was calculated as the percentage of MTT absorption as follows:

Percentage (%) of survival = (mean experimental absorbance/mean control absorbance) \times 100).

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Table S1: Chemical composition of the essential oil of *Isodon melissoides*

S. no.	Compound ^a	RI ^b	RI ^c	Content (%)	Identification
1.	α -Thujene	930	924	0.2	GC-MS, RI
2.	α -Pinene	938	932	0.3	GC-MS, RI
3.	Camphene	953	946	0.1	GC-MS, RI
4.	Sabinene	978	969	t	GC-MS, RI
5.	1-Octen-3-ol	982	974	0.1	GC-MS, RI
6.	Myrcene	994	988	0.8	GC-MS, RI
7.	3-Octanol	997	988	t	GC-MS, RI
8.	δ -2-Carene	1009	1001	t	GC-MS, RI
9.	α -Phellandrene	1010	1002	0.1	GC-MS, RI
10.	α -Terpinene	1021	1014	1.1	GC-MS, RI
11.	<i>p</i> -Cymene	1026	1020	11.6	GC-MS, RI
12.	Limonene	1032	1024	0.1	GC-MS, RI
13.	β -Phellandrene	1033	1025	t	GC-MS, RI
14.	1,8-Cineole	1034	1026	0.2	GC-MS, RI
15.	(<i>Z</i>)- β -Ocimene	1036	1032	0.1	GC-MS, RI
16.	(<i>E</i>)- β -Ocimene	1050	1044	t	GC-MS, RI
17.	γ -Terpinene	1061	1054	8.1	GC-MS, RI
18.	Acetophenone	1067	1059	0.1	GC-MS, RI
19.	<i>cis</i> -Sabinene hydrate	1065	1072	0.3	GC-MS, RI
20.	Terpinolene	1094	1086	0.1	GC-MS, RI
21.	Linalool	1103	1095	0.8	GC-MS, RI
22.	<i>trans</i> -Sabinene hydrate	1106	1098	t	GC-MS, RI
23.	Camphor	1150	1141	0.2	GC-MS, RI
24.	Menthone	1158	1148	0.1	GC-MS, RI
25.	Borneol	1171	1165	0.5	GC-MS, RI
26.	Menthol	1177	1167	0.3	GC-MS, RI
27.	Terpinen-4-ol	1184	1174	0.3	GC-MS, RI
28.	<i>p</i> -Cymen-8-ol	1189	1179	t	GC-MS, RI
29.	α -Terpineol	1196	1186	t	GC-MS, RI
30.	Thymol methyl ether	1234	1232	t	GC-MS, RI
31.	Carvacrol methyl ether	1245	1241	t	GC-MS, RI
32.	Bornyl acetate	1290	1284	t	GC-MS, RI
33.	Thymol	1293	1289	11.3	GC-MS, RI

34.	Carvacrol	1304	1298	45.4	GC-MS, RI
35.	Thymol acetate	1358	1349	1.6	GC-MS, RI
36.	Eugenol	1364	1356	0.2	GC-MS, RI
37.	Carvacrol acetate	1377	1370	5.2	GC-MS, RI
38.	(<i>E</i>)-Caryophyllene	1426	1417	0.8	GC-MS, RI
39.	α - <i>trans</i> -Bergamotene	1440	1432	0.1	GC-MS, RI
40.	α -Humulene	1461	1452	0.9	GC-MS, RI
41.	Germacrene D	1487	1480	0.1	GC-MS, RI
42.	α -Zingiberene	1499	1493	0.5	GC-MS, RI
43.	β -Bisabolene	1515	1505	3.3	GC-MS, RI
44.	δ -Cadinene	1530	1522	0.2	GC-MS, RI
45.	Caryophyllene oxide	1590	1582	0.6	GC-MS, RI
46.	Humulene epoxide II	1615	1608	0.5	GC-MS, RI
Total identified (%)				96.2	

Note-^aIdentification based on the retention index (RI) and mass spectral data (see experimental section); ^bretention index, determined on BP-5 gas chromatography column; ^cretention index from literature.

Table S2: Antibacterial activity of *Isodon melissoides* essential oil against drug resistant and non-resistant strains

Strain	Essential oil			Standard [†]		
	NZOI (mm)	MIC (ppm)	MBC (ppm)	NZOI (mm)	MIC (ppm)	MBC (ppm)
MRSA (33591)	26	0.52	1.04	16	0.00416	0.01667
SA (BAA-44)	17	0.52	1.04	17	0.00208	0.01667
MRSE (51625)	20	0.26	0.26	16	0.00416	0.01667
MS (UDSC)	18	1.04	>33.34	19	0.00052	0.00416
SE (MTCC435)	19	0.52	0.52	20	0.00052	0.00208
EF (MTCC439)	16	0.13	0.13	21	0.00052	0.00104
SM (MTCC890)	16	0.26	0.26	16	0.00104	0.00208
SA (MTCC96)	19	0.26	0.52	19	0.00104	0.00208
EC (MTCC723)	16	0.26	0.26	23	0.00104	0.00208
EC (DH5 α)	18	0.52	1.04	22	0.00104	0.00208
SS (13419)	22	0.52	1.04	18	0.00208	0.00816
PA (27853)	02	8.33	16.66	14	0.00416	0.00816

Notes-NZOI= Net zone of inhibition; MIC= Minimum inhibitory concentration; MBC= Minimum bactericidal concentration; MRSA: methicillin resistant *Staphylococcus aureus*; BAA-44: multi drug resistant *Staphylococcus aureus*; MRSE: methicillin resistant *Staphylococcus epidermidis*; MS= *Mycobacterium smegmatis*; SE= *Staphylococcus epidermidis*; EF= *Enterococcus faecalis*; SM= *Streptococcus mutans*; SA= *Staphylococcus aureus*; EC= *Escherichia coli*; SS= *Streptococcus salivarius*; PA= *Pseudomonas aeruginosa*; ; [†]vancomycin for MRSA, MRSE & BAA-44 and tetracycline for rest of the strains.

Table S3: Antibacterial activity of *Isodon melissoides* essential oil at 2MIC and 4 MIC

Time (h)	Log CFU/mL								
	Control			2 MIC	4 MIC	2 MIC	4 MIC	2 MIC	4 MIC
	EF	SA	SE	EF	EF	SA	SA	SE	SE
0	7.11	7.11	7.11	7.11	7.11	7.11	7.11	7.11	7.11
0.5	7.27	7.34	7.32	5.49	5.46	5.95	5.53	6.07	5.60
1	7.36	7.63	7.96	5.07	3.62	5.55	4.07	5.59	3.27
2	7.95	7.92	7.83	4.39	3.07	4.46	2.95	4.84	3.17
3	8.57	8.07	8.43	4.07	2.90	3.27	2.84	4.25	2.69
6	9.23	10.44	9.53	3.84	2.47	2.77	2.47	3.11	2.30

Notes- EF= *Enterococcus faecalis*; SA= *Staphylococcus aureus*; SE= *Staphylococcus epidermidis*.

Table S4: Leakage of proteins from bacterial strains treated with *Isodon melissoides* essential oil at MIC/2, MIC and 2MIC

Strains	Leakage of Protein (µg/mL)		
	MIC/2	MIC	2 MIC
<i>Enterococcus faecalis</i>	47.00±10.00	54.16±6.16	67.33±5.66
<i>Staphylococcus epidermidis</i>	45.50±01.83	62.50±4.16	69.83±1.83
<i>Staphylococcus aureus</i>	79.66±3.33	82.33±7.66	83.50±7.50

Notes- The results expressed as mean ±SEM (n=3).

Table S5: Changes in optical density at 260 nm of supernatant release of bacterial strains treated with *Isodon melissoides* essential oil at MIC/2, MIC and 2MIC

Strains	Optical Density (260 nm)		
	MIC/2	MIC	2 MIC
<i>Enterococcus faecalis</i>	0.0255±0.002	0.063±0.002	0.079±0.003
<i>Staphylococcus epidermidis</i>	0.018±0.003	0.0285±0.003	0.052±0.004
<i>Staphylococcus aureus</i>	0.022±0.003	0.025±0.004	0.040±0.004

Notes- The results expressed as mean ±SEM (n=3)

Table S6: Effect of *Isodon melissoides* essential oil on production of pro-inflammatory cytokines in LPS-induced inflammation in HaCaT cells

Treatment	Dose	TNF- α		IL-6		IL- β	
		Pg/ml	% inhibition	Pg/ml	% inhibition	Pg/ml	% inhibition
Normal	–	60.67 \pm 11.05	–	46.33 \pm 7.23	–	15.88 \pm 4.89	–
Vehicle	–	189.33 \pm 4.81 [#]	00	212.94 \pm 9.02 [#]	00	127.46 \pm 8.21 [#]	00
	0.3 %	96.67 \pm 5.36 [*]	48.95 \pm 2.38	136.33 \pm 7.26 [*]	35.49 \pm 5.98	77.96 \pm 3.28 [*]	38.60 \pm 2.12
Essential oil	1 %	83.00 \pm 6.51 [*]	56.15 \pm 3.28	111.30 \pm 7.18 [*]	47.74 \pm 2.36	64.54 \pm 4.00 [*]	49.32 \pm 1.24
Dexamethasone	1 μ g/mL	75.33 \pm 5.33 [*]	60.20 \pm 2.66	52.00 \pm 6.79	75.45 \pm 3.37	53.25 \pm 4.80 [*]	58.23 \pm 2.54

Results are Mean \pm SEM; n= 3; [#](Normal vs Vehicle), ^{*}P<0.05 (Vehicle vs Treatment).

Table S7: Effect of *Isodon melissoides* essential oil on cell viability using MTT assay

Treatment	Dose	% of cell viability
	($\mu\text{g/mL}$)	(Mean \pm SE)
Essential oil	0	100
	0.3%	91.53 \pm 3.55
	1%	86.93 \pm 0.43