SUPPLEMENTARY MATERIAL

Chemical composition and antimicrobial activity of *Boswellia serrata* oleo-gum-resin essential oil extracted by superheated steam

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ABSTRACT

Oleo-gum-resin is a complex mixture of essential oils, polysaccharides, and resin acids. The objectives of the present study were to evaluate the variation in chemical components and antimicrobial activity of essential oils extracted by superheated steam at various temperatures. The optimum essential oil yield was obtained at the highest superheated steam temperature (210 ºC). In total, twenty-one compounds were quantified by GC-MS with α-pinene as the major compound, followed by α-thujene, *trans*-verbenol, β-thujone, *p*-cymene, *m*-cymene, and sabinene. Antimicrobial activity was performed by disc diffusion, resazurin microtitre-plate and micro-dilution broth susceptibility assays in which essential oil extracted at 150 ºC and 180 ºC revealed the highest antibacterial and antifungal activity, respectively. It is concluded that superheated steam is an effective method for the isolation of essential oil from oleo-gum-resin that improves the recovery of essential oil as well as antimicrobial activity.

Keywords: Superheated steam, GC-MS, *Boswellia serrata*, Oleo-gum-resin, EO, Antimicrobial Activity.

Experimental

Oleo-gum-resin collection and extraction of essential oil

Two kilogram of *Boswellia serrata* oleo-gum-resin was collected by tapping method from fully grown plants of Zhob district of Balochistan, Pakistan in July-August 2019 (voucher number MPZ 003) verified by Dr. Fahim Arshad, Department of Botany, University of Okara, Pakistan. The oleo-gum-resin sample was washed thrice with double distilled water, air dried under shed for three weeks, ground, and stored in polyethylene bags until further processed. Superheated steam is a type of steam that has temperature higher than normal steam. It is generated by providing extra heat to the normal steam. Extraction was performed in a custom size distillation apparatus made up of a heating mental, round bottom flask, steam flask, steam transfer line, digital thermometer, heating burner, dean stark and condenser. Steam was generated in round bottom flask placed in heating mental. This steam is transfer to steam flask by coiled transfer line. Normal steam is converted into superheated steam by providing extra heat through high temperature controlled burner placed under coiled transfer line. Temperature of superheated steam was checked continuous by digital thermometer attached at the entrance of steam flask. The finely ground (60 mesh) oleo-gum-resin (100 g) was packed in fine cotton cloth, placed in extraction chamber and subjected to superheated steam at 120, 150, 180 and 210 ºC to attained the flow rates of 1.67, 2.92, 5.58 or 9.25 mL/min, respectively for 1 h. Moisture from EOs was removed by adding anhydrous sodium sulfate followed by filtration through Whatman filter paper no. 1 and stored in amber glass vials until further analysis. The process of extraction of oil for each temperature was repeated five times to ensure the reproducibility.

Antimicrobial activity

Microbial strains

EOs were individually tested against *Escherichia coli*, *Aspergillus flavus* , *Staphylococcus aureus* , *Alternaria alternate*, *Bacillus subtilis*, *Fusarium solani* , *Pasteurella multocida* and *Aspergillus niger* collected from Institute of Microbiology, University of Agriculture Faisalabad, Pakistan.

Agar well diffusion method

The antibacterial and antifungal activities of EOs was performed by agar well diffusion method as previously described in literature (Rashid et al. 2013). An overnight culture of the microbial strains, containing 10^8 colony forming units per milliliter was transferred to 25 mL of growth medium solution. Then contents of the flasks were transferred to medium size petri plates. After its solidification at room temperature, sterilize cork borer was used for well formation. These wells were filled with 10 mL of pure EOs extracted by superheated steam at different temperatures and standard drug (1 mg per mL of Ampicillin) for antibacterial activity and (1 mg per mL of Fluconazole). The petri plates were incubated at 37 ºC for 24 h for bacteria and 30 ºC for 40 h for fungal strains. After the incubation period, width of inhibition zones (mm) was measured through digital Vernier caliper.

Resazurin microtitre-plate assay

Minimum inhibitory concentration (MIC) of EOs against different bacterial strains was measured by a modified resazurin microtitre-plate assay as reported in literature (Sarker et al. 2007). Briefly, sample solution of EO was prepared by dissolving 10 mL of EO in 1 mL of 10% DMSO. Similarly, resazurin indicator solution was prepared by dissolving 27 mg of resazurin in 4 mL of sterilized distilled water. About 100 μL of sample solution and standard antibiotic Ampicillin (1 mg/mL in 10% DMSO) was pipetted into the first row of the ninety six well plates. Then 50 mL nutrient broth was added in all wells except first row and two fold serial dilutions were

performed in such a way that each well had 50 μL of the sample mixture. Then 30 μL of 3.3x strength isosensitised broth, 10 μL of resazurin solution and 10 μL (5×10^5 colony forming units per mL) were added in all wells. Plates were incubated at 37 ºC for 24 h. After incubation time, MIC values measured visually. The color change from purple to colorless or pink indicated the bacterial growth, while the lowest concentration at which color change occurred was taken as the MIC value.

Micro-dilution broth susceptibility assay

MIC of EOs extracted by superheated steam at different temperatures against different fungal strains was measured through micro-dilution broth susceptibility assay, as previously described in literature (Dabur et al. 2004). Briefly, sample solution of EOs extracted by superheated steam at different temperatures and standard antibiotic Fluconazole was prepared by dissolving 10 mg of EO and 1 mL of standard antibiotic in 1 mL of 10% DMSO solution, respectively. About100 μL of samples and standard solutions were pipetted into the first row of the 96 well plates. Then 50 μL of Sabouraud dextrose broth was added in all wells except first row, two fold serial dilutions were performed in such a way that each well had 50 μL of the sample mixture. After that 130 μL Sabouraud dextrose broth and 20 μLof microorganism suspension (5×10^5 colony forming units per milliliter) were added in all wells and well plate was incubated at 30 ºC for 48 h. The MIC value was assessed visually. It is the lowest concentration at which complete inhibition of the fungal growth.

Gas chromatography/mass spectrometry (GC-MS) analysis

The volatile components of EOs extracted by superheated steam at different temperatures were determined through Gas Chromatography-Mass Spectrometry (GC–MS) (Shimadzu gas chromatograph GC-2010 system) equipped with QP-2010 plus mass detector and DB-5 capillary

column (50 m × 0.25 mm, film thickness of 0.25 µm). An injection volume of 1 µL EO (diluted with n-hexane 1:10) was injected in to injection port with the help of injection syringe. Column was heated at 60 ºC for 3 min and then gradually increased its temperature up to 240 ºC at the heating rate of 24ºC/min and kept constant for next 10 min. Nitrogen gas was used as the carrier gas that passed through the system at the flow rate of 1.5 mL/min. The MS transfer line temperature was set at 240 ºC. An electron ionization mode (70 eV) was used for MS detection(Ayub et al. 2018). Retention indices of detected compounds were determined by running n-alkanes (C_9-C_{24}) standards under same conditions. Furthermore, these retention indices and mass spectrum data were compared with already published data, NIST and pherobase mass spectral database library (Ayub, Hanif, Sarfraz and Shahid 2018, Hussain et al. 2013). Confirmation of some of the compounds was made by co-injection of authentic standards. The quantification of the constituents of the EOs was performed as described by (Das et al. 2019). Response factors (RFs) were calculated relative to undecane (internal standard) using the equation:

$$RF_c = (A_c / A_{is}) / (C_c / C_{is})$$

Where RF_c is the response factor for the EO component; A_c and A_{is} are the peak areas of the EO component and internal standard, respectively; and C_c and C_{is} are the corresponding concentrations. The RF for small unidentified peaks was assumed to be 1.0. Finally, the RFs were used to calculate the percentage (%) of each of the EOs constituents as follows:

Corrected area = peak area for the component/ response factor for the same component

Percentage (%) = (corrected area for the component / total of corrected areas) x 100

Statistical analysis

All the experiments were performed in triplicate and statistical analysis of the data was performed by analysis of variance (ANOVA) with Post-Hoc Tukey HSD test using STATISTICA 5.5 (Stat Soft Inc., Tulsa, OK, USA) software. A probability value at $p \leq 0.05$ was considered statistically significant. Data are presented as mean values ± standard deviation calculated from triplicate determinations.

Tables

Table S1: GC-MS analysis of *Boswellia serrata* oleo-gum-resin essential oils extracted by superheated steam at different steam temperatures.

Sr. No	Components[A]	RT	RI_{Cal}	RI_{Lit}	Percentage composition of essential oil isolated by superheated steam at different temperatures			
					120 ºC	150 ºC	180 ºC	210 ºC
1	α-Thujene	5.09	923	923	3.72 ± 0.02^{d}	4.49 ± 0.04^{b}	4.64 ± 0.03^{a}	3.90 ± 0.02^{c}
2	α-Pinene	5.32	933	933	89.07 ± 0.12^{a}	78.83 ± 0.06^{c}	77.95 ± 0.11^{d}	76.53 ± 0.11^{b}
3	Camphene	5.82	951	952	0.84 ± 0.00^{c}	1.36 ± 0.03^{a}	1.28 ± 0.01^{b}	1.27 ± 0.00^{b}
4	Verbenene	6.33	971	972	0.25 ± 0.00^{c}	0.44 ± 0.01^{a}	0.45 ± 0.00^{a}	0.37 ± 0.00^{b}
5	Sabinene	6.49	976	976	0.93 ± 0.02^{c}	1.66 ± 0.04^{a}	1.47 ± 0.03^{b}	1.48 ± 0.02^{b}
6	β-Pinene	6.79	988	988	0.48 ± 0.00^{d}	1.11 ± 0.04^{a}	0.63 ± 0.01^{c}	0.77 ± 0.01^{b}
7	m-Cymene	7.96	1023	1023	0.89 ± 0.01^{d}	1.89 ± 0.04^{a}	1.77 ± 0.01^{b}	1.29 ± 0.03^{c}
8	p-Cymene	8.09	1027	1027	0.93 ± 0.02^{d}	1.93 ± 0.05^{b}	2.11 ± 0.04^{a}	1.73 ± 0.01^{c}
9	1,8-Cineole	8.23	1032	1033	0.10 ± 0.00^{c}	0.22 ± 0.01^{a}	0.14 ± 0.00^{b}	0.16 ± 0.00^{b}
10	Linalool	10.70	1099	1098	0.27 ± 0.00^{c}	0.62 ± 0.00^{b}	0.61 ± 0.02^{b}	0.64 ± 0.01^{a}
11	α-Thujone	11.45	1116	1116	0.22 ± 0.00^{d}	0.53 ± 0.02^{b}	0.59 ± 0.02^{a}	0.42 ± 0.01^{c}
12	α-Campholenal	11.81	1125	1125	0.15 ± 0.00^{c}	0.42 ± 0.03^{a}	0.36 ± 0.01^{b}	0.36 ± 0.00^{b}
13	β-Thujone	12.40	1139	1139	0.86 ± 0.02^{d}	1.91 ± 0.01^{c}	2.21 ± 0.06^{a}	1.96 ± 0.02^{b}
14	trans-Verbenol	12.61	1144	1144	0.68 ± 0.01^{d}	1.97 ± 0.03^{c}	2.23 ± 0.01^{b}	3.48 ± 0.05^{a}

15	Isopinocamphone	13.72	1172	1173	---	0.31 ± 0.00^{c}	0.34 ± 0.00^{b}	0.39 ± 0.01^{a}
16	Terpineol-4	14.09	1179	1179	---	0.25 ± 0.00^{a}	0.20 ± 0.00^{b}	0.14 ± 0.00^{c}
17	P-Cymen-8-ol	14.38	1185	1183	---	0.23 ± 0.01^{a}	0.21 ± 0.00^{b}	0.19 ± 0.00^{c}
18	Myrtenol	14.73	1194	1194	0.11 ± 0.00^{c}	0.57 ± 0.02^{b}	0.63 ± 0.01^{a}	1.64 ± 0.00^{a}
19	d-Verbenone	15.24	1205	1205	0.31 ± 0.00^{d}	0.87 ± 0.01^{b}	0.98 ± 0.04^{a}	1.80 ± 0.02^{c}
20	Bornyl acetate	18.64	1284	1285	0.17 ± 0.00^{d}	0.35 ± 0.01^{a}	0.26 ± 0.00^{c}	0.30 ± 0.00^{b}
	Monoterpene hydrocarbon (1-8)				97.11	91.71	90.30	89.34
	Oxygenated Monoterpene (9-20)				2.87	8.25	8.76	11.42
	Total				99.98	99.96	99.06	99.76

Values are mean ± Standard Deviations of three separate determinations.

Different letter in superscripts represent significant difference among *Boswellia serrata* oleo-gum-resin EOs extracted by superheated steam at different steam temperatures.

[A] Compound listed in order of elution from a DB-5 capillary column.

RT= Retention Time

RI_{Lit}= Literature reported retention indices

RI_{Cal}= Retention indices calculated against n-alkanes

Table S2: Antimicrobial activity of *Boswellia serrata* oleo-gum-resin essential oils extracted by superheated steam at different steam temperatures.

Microorganism	Essential oil isolated by superheated steam at different temperatures				Positive control
	120 ºC	150 ºC	180 ºC	210 ºC	
	Inhibition zone (mm)				
Escherichia coli	16.80 ± 0.03^{c}	18.70 ± 0.09^{b}	12.27 ± 0.04^{d}	11.47 ± 0.07^{e}	21.11 ± 0.02^{a}
Staphylococcus aureus	14.15 ± 0.07^{c}	15.35 ± 0.09^{b}	13.24 ± 0.05^{d}	12.45 ± 0.08^{e}	24.38 ± 0.03^{a}
Pastrulla multocida	21.07 ± 0.09^{c}	21.54 ± 0.07^{b}	19.87 ± 0.04^{e}	20.27 ± 0.02^{d}	28.53 ± 0.05^{a}
Bacillus subtilis	13.82 ± 0.03^{c}	14.47 ± 0.05^{b}	11.25 ± 0.02^{e}	12.16 ± 0.07^{d}	22.32 ± 0.04^{a}
Fusarium solani	23.00 ± 0.04^{d}	25.81 ± 0.05^{b}	28.80 ± 0.06^{a}	20.00 ± 0.05^{e}	24.36 ± 0.02^{c}
Aspergillus niger	12.08 ± 0.03^{d}	12.58 ± 0.04^{c}	17.80 ± 0.05^{b}	12.60 ± 0.03^{c}	22.61 ± 0.07^{a}
Alternaria alternata	16.73 ± 0.08^{d}	17.57 ± 0.04^{c}	22.34 ± 0.08^{b}	16.15 ± 0.03^{e}	24.82 ± 0.02^{a}
Aspergillus flavus	12.71 ± 0.03^{d}	15.09 ± 0.04^{c}	15.04 ± 0.04^{c}	17.95 ± 0.05^{b}	23.32 ± 0.07^{a}
	Minimum inhibition concentration (MIC)				
Escherichia coli	98.51 ± 1.98^{c}	84.44 ± 1.68^{d}	197.03 ± 3.94^{b}	281.46 ± 5.62^{a}	5.83 ± 1.16^{e}
Staphylococcus aureus	112.58 ± 2.25^{c}	105.14 ± 1.98^{d}	217.82 ± 2.48^{b}	225.17 ± 2.76^{a}	2.92 ± 0.84^{e}
Pastrulla multocida	70.36 ± 1.40^{c}	63.33 ± 1.26^{d}	84.44 ± 1.68^{a}	77.23 ± 1.54^{b}	8.33 ± 0.95^{e}
Bacillus subtilis	168.88 ± 2.36^{c}	112.58 ± 1.98^{d}	281.47 ± 2.76^{a}	225.17 ± 2.50^{b}	13.33 ± 2.17^{e}
Fusarium solani	56.29 ± 2.40^{b}	35.18 ± 2.20^{c}	28.14 ± 1.72^{d}	84.44 ± 2.48^{a}	5.88 ± 0.16^{e}
Aspergillus niger	281.46 ± 7.02^{a}	225.17 ± 5.63^{b}	112.58 ± 3.82^{c}	225.17 ± 4.92^{b}	8.42 ± 0.21^{d}
Alternaria alternata	140.73 ± 2.81^{b}	112.58 ± 2.05^{c}	70.36 ± 1.40^{d}	168.88 ± 3.34^{a}	5.00 ± 0.10^{e}
Aspergillus flavus	225.17 ± 3.50^{a}	168.88 ± 2.38^{b}	168.88 ± 2.38^{b}	112.58 ± 1.76^{c}	6.66 ± 0.13^{d}

Values are mean ± Standard Deviations of three separate determinations.

Different letter in superscripts represent significant difference among *Boswellia serrata* oleo-gum-resin essential oils extracted by superheated steam at different steam temperatures.

[a] Positive control for bacteria and fungi was Ampicillin and Fluconazole (25 µg/disc), respectively.

Figures

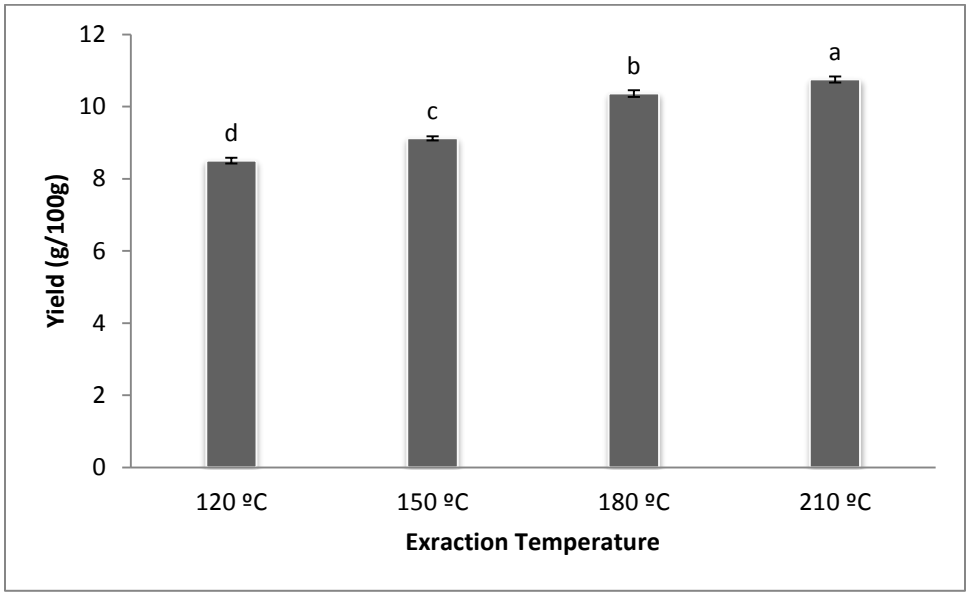

Figure S1: Yield (g/100g) of *Boswellia serrata* oleo-gum-resin essential oils extracted by superheated steam at different temperatures.

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