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ANTIMICROBIAL ACTIVITY OF SUPERCRITICAL CARBON DIOXIDE EXTRACTS OF TUBEROSE (*POLIANTHES TUBEROSA* LINN.*)* FLOWERS AGAINST COMMON PATHOGENS

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ABSTRACT: Tuberose flower extracts were obtained by green technology of Supercritical carbon dioxide (SC-CO_2) extraction and solvent extractions (using *n*-hexane and petroleum ether of b.p. 60-80 °C). Antimicrobial activities of these extracts were tested *in vitro* against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Helicobacter pylori, Vibrio cholerae* and *Candida albicans.* No activity was found against *H. pylori* and *V. cholerae*. Antimicrobial activities of the extracts were established by minimum inhibitory concentration (MIC) assays against the remaining test microorganisms. The SC-CO_2 and solvent extracts showed potency against all test microorganisms. SC-CO_2 extract obtained at 40 °C, 100 bar pressure, 135 min extraction time, and *n*-hexane extract showed most promising results, among all the extracts. However, considering the global need for green extracts for therapeutic applications, SC-CO_2 extraction is the recommended technique for extracting antimicrobials from tuberose flowers. The chemical constituents conferring antimicrobial potencies to the extracts were also identified by GC/MS analyses.

INTRODUCTION: Tuberose (*Polianthes tuberosa* Linn.) is a herb and a member of the Agavaceae family. It is commonly known as *'Rajnigandha'* in India. Bioactive constituents are extracted from herbs conventionally in aqueous and organic solvents which leave artifacts and solvent residues in the extracts, posing health and environmental concerns. These problems have been averted by use of supercritical fluid (SCF) extraction technology. This is a green technology that uses fluids of GRAS status in the supercritical state as extracting solvents. CO_2 is the most commonly used SCF because of its non-toxicity, non-flammability and low critical temperature. Supercritical carbon dioxide (SC-CO_2) extraction has gained paramount importance in extraction of bioactive constituents from several natural matrices, including flowers, such as chrysanthemum [1] and jambú [2].

The present study envisages SC-CO_2 extraction of bioactive constituents from tuberose flowers for possible usage as antimicrobial agents against common pathogenic microorganisms. Reports on antimicrobial activity of tuberose flower extracts against bacteria (by zone of inhibition) [3] and fungi

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(reduction in % mycelia growth) [4] are scanty; moreover, none have reported on MIC (minimum inhibitory concentration) values of the extracts. MIC is regarded as a 'gold standard' to determine susceptibility of microorganisms to antimicrobials [5]. In the present investigation, the extracts obtained by SC-CO_2 have been assessed for antimicrobial efficacies against pathogenic bacteria and fungi, by determining their MIC values, and the compounds attesting to their antimicrobial potencies have been identified by GC/MS. Conventional solvent extraction has also been conducted in this study as an experimental control.

To the best of our knowledge, this paper reports for the first time on MIC values of tuberose extracts. Moreover, there is no literature on SC-CO_2 extraction of antimicrobials from tuberose flowers.

MATERIALS AND METHODS:

Materials: Tuberose flowers (*Polianthes tuberosa* Linn.) of *Calcutta single* variety were procured from cultivators of Barasat, 24 Parganas North, West Bengal (22° 71' N and 88° 51' E, at about 13 m elevation above mean sea level, located in the eastern Gangetic plain of India) in February 2013 and were authenticated by West Bengal Food Processing and Horticulture Development Corporation Limited, Kolkata, India. The flowers were cultivated under tropical-temperate climate (24-32°C, 75-85% RH) in sandy loamy soil of pH 7-8. The flowers were air dried in shade at room temperature (23 ± 2°C) to constant weight. The moisture content of fresh flowers was estimated to be 67.66 % on a dry weight basis, by Bidwell-Sterling method (AOAC 1990; method 925.04) [6].

Petroleum ether (b.p. 60-80°C), *n*-hexane and ethanol (96 %) were procured from M/s Merck Ltd., India. Muller-Hinton agar and broth (MHA and MHB, respectively), Brain Heart Infusion (BHI) agar (M/s Difco Laboratories, MI, USA), horse serum (M/s Invitrogen, NY, USA), IsoVitaleX (M/s Becton-Dickson, MD, USA), Vancomycin, Polymixin B and Trimethoprim (M/s Sigma, MO, USA) were used for microbial culture. CO_2 (food grade) was procured from M/s BOC India Ltd., Kolkata, India. All reagents were of AR grade.

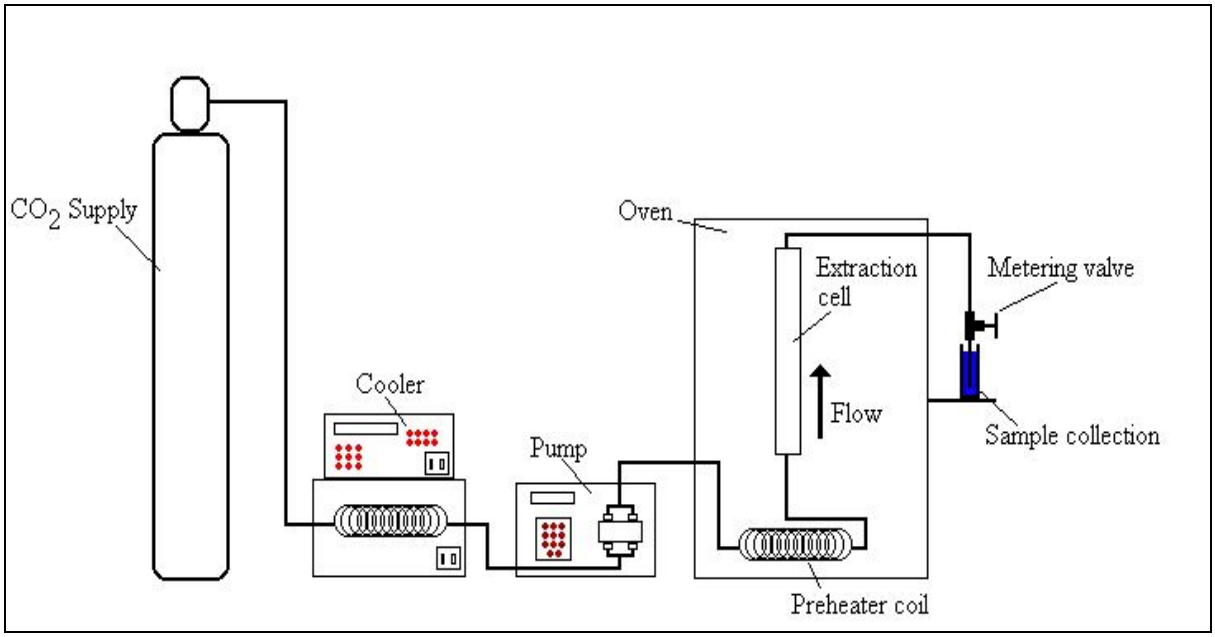

FIGURE 1: SCHEMATIC DIAGRAM OF SC-CO_2 EXTRACTION SYSTEM USED IN THIS INVESTIGATION

Extraction of tuberose extracts using SC-CO_2 and solvent extraction:

SC-CO_2 extraction of tuberose flowers: For SC-CO_2 extraction, a SPE-ED SFE 2 model of M/s Applied Separations, PA, USA was used. The schematic diagram of a SC-CO_2 extraction unit of SPE-ED SFE 2 model employed in this study, is shown in **Fig. 1**. Three different unit operations are involved in this system such as extraction, expansion and separation and their corresponding critical components are high-pressure extractor, a pressure reduction valve and a low pressure extractor, respectively. This system can be performed in either static or in dynamic mode.

The operational limit of the system is 240°C and 690 bar. The sample is charged into a 50 ml extraction vessel (SS 316). The sample bed is firmly packed to ensure that the supercritical fluid diffuses uniformly through the sample matrix. The voidage is filled with *Spe-ed* Matrix (silica matrix). Polypropylene frits are placed on both ends of the sample bed with tight end-fittings.

The extraction vessel is kept in the oven module and the Z-type thermocouple is connected to the vessel body which detects the vessel temperature. The desired temperature for SC-CO_2 extraction is achieved through regulatory switches of oven module. The food grade CO_2 from the cylinder is fed to the extractor through a high pressure modifier pump (Speed MAX P/N 7025) fitted with refrigerated cooling bath to chill the 'pump module' head at -2°C.

CO_2 is passed through the extraction vessel for a definite period of time during which the outlet valve is kept closed. The first mode of extraction is known as static conditioning to allow sufficient contact between sample matrix and extractant. During the dynamic phase of extraction, the outlet valve is kept open, the soluble analytes are portioned from the bulk of the sample matrix into the SC-CO_2, and the extract laden CO_2 is swept through a flow restrictor into a separator or collection vial, kept at ambient conditions. A bubble flow meter attached at the collection end of the extraction module indicates the flow rate of gaseous CO_2. The desired flow rate is achieved through a micrometering valve present in the collection module. At reduced temperature and pressure conditions, the extract precipitates with reduction of fluid density in the separator (collection vial) and CO_2 in gaseous form vents out into the atmosphere.

The batch size, extraction time and flow rate of CO_2 were fixed by preliminary experimental trials. SC-CO_2 extraction was conducted with 20 g dry flowers at extraction conditions of 40°C, 100 bar; 40°C, 200 bar; 60°C, 100 bar; 60°C, 200 bar; 80°C, 200 bar and 80°C, 300 bar; keeping extraction time of 135 min (120 min static time + 15 min dynamic time) constant, at a flow rate of 1 l/min of gaseous CO_2. Three independent extraction runs were conducted for a given set of extraction conditions, with three independent batches of dried tuberose flowers.

All extracts were collected into 96 % ethanol in screw capped glass vials, kept in an ice bath. Post collection, the extracts were purged with nitrogen to evaporate ethanol and the yields were determined gravimetrically. The extracts were then redissolved in 96 % ethanol and stored in N_2 purged screw capped amber colored vials at -18 °C in dark, until further analyses.

Solvent extraction of tuberose flowers: Solvent extractions were carried out in Soxhlet extraction assembly using *n*-hexane and petroleum ether (b.p. 60-80°C), individually, for 8 h. Post extraction, the solvents were evaporated in Rotavac (M/s Eyela Corp., Japan) at 40-45°C, 50 mbar, and the yields were assayed gravimetrically. The extracts were subsequently dissolved in ethanol (96 %) and stored as above.

Antimicrobial activity assay of tuberose extracts: The test microorganisms used in this study included bacteria such as *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Helicobacter pylori* (strain 26695) and *Vibrio cholerae* (strain O1) and a fungi *Candida albicans* (ATCC 10231). These microorganisms were selected since they are the common pathogens known to cause diseases of skin, GI tract and urinary tract infections, enteric ulcer, cholera and candidiasis.

Disc diffusion method was employed for screening antimicrobial potencies of the extracts using MHA for all the microorganisms, except for *H. pylori*. *H. pylori* was cultured on Brain Heart Infusion (BHI) agar supplemented with 5 % Horse serum, 0.4 % IsoVitaleX, Trimethoprim (5 µg/ml), Vancomycin (8 µg/ml) and Polymixin B (10 µg/ml). Autoclaved Whatman No. 1 filter paper discs (5 mm diameter) were each spiked with 10 µl of the extracts, and placed on the microorganism swabbed agar plates and incubated in a BOD incubator at 37°C for 24 h (except for *H. pylori*). The plate with *H. pylori* was incubated at 37°C in microaerophilic atmosphere (5 % O_2, 10 % CO_2, 85 % N_2) (double gas incubator; M/s Heraeus, Langenselbold, Germany) for 3 days. Post incubation, extracts showing inhibition zones were subjected to assay of MIC. *H. pylori* and *V. cholerae* showed no inhibition zone for all the extracts and therefore, were not subjected to MIC assays.

For determination of MIC values, the inoculums of the microorganisms were prepared in MHB and adjusted to 0.5 McFarland turbidity standard (10^6-10^7 cfu/ml of microorganisms). MIC assays were conducted by the 'broth microdilution method' and the microtiter plates were incubated in a BOD incubator at 37°C for 24 h. A microplate reader (M/s Micronaut System, Germany) was used to measure the optical density of the microplate at 0 h and 24 h at 620 nm. To ascertain the static and cidal activities, the extracts showing MIC were transferred from the 96-well microplate on to MHA plates. Microbial growth after 24 h of incubation at 37°C was considered to indicate static and not cidal activity of the extracts against test microorganisms. For MIC assay, positive control used were Gentamycin for bacteria and Amphotericin B for fungi; while ethanol (96 %) used as a diluent for the extracts, was considered as the negative control.

GC/MS analyses of tuberose extracts: To identify the compounds responsible for antimicrobial activity in the tuberose extracts, GC/MS analysis was carried out in Perkin Elmer GC Clarus® 680 chromatograph (M/s Perkin Elmer, MA, USA) with Elite-5 MS (Perkin Elmer, MA, USA) fused silica capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) coupled to a Perkin Elmer Clarus® SQ 8T Mass Detector (M/s Perkin Elmer, MA, USA). The GC injector and MS transfer line temperatures were set to 230°C and 270°C, respectively. The oven temperature was programmed as follows: 50°C for 5 min, then an increase from 50 to 250°C at 5°C / min and finally a fixed isothermal hold at 250°C for 10 min. The carrier gas was He at a flow rate of 1 ml/ min. 1µl of extract was injected at a split ratio of 1:50. The ionization of the sample was performed in the EI ion source (70 eV) and the acquisition mass range was set at 35-500 amu. Identification of components was based on comparison of their mass spectra (using molecular ion [M^+] peak, base peak and the m/z values) with those provided in mass spectra library NIST (2007) and in literature [7, 8]. The compounds whose mass spectra matched with references [9, 10] were only confirmed. The relative peak area percentages (without correction factor) were used to report the abundance of a compound in the extracts.

Statistical analyses: Statistical analyses were carried out by one-way ANOVA at 95 % confidence interval using STATISTICA 8.0 software (Statsoft, OK, USA) to analyze the effects on MIC values of the test microorganisms with the solvents.

RESULTS:

Preliminary screening of extracts by disc diffusion method: The disc diffusion method showed that SC-CO_2 extracts obtained at 40°C, 100 bar; 60°C, 200 bar and 80°C, 300 bar and with *n*-hexane and petroleum ether (b.p. 60-80°C) were potent against the test microorganisms. Therefore, gravimetric yield, color and appearance and GC/MS analyses of only these extracts have been reported.

TABLE 1: YIELD, COLOR AND APPEARANCE OF THE EXTRACTS OBTAINED BY SOLVENT AND SC-CO_2 EXTRACTION TECHNIQUES USED FOR MIC ASSAY

Solvent	Yield (mg g/ dry flowers)[b]	Color and appearance of extract
n-hexane	3.50 ± 0.19[c]	Yellowish orange and slightly waxy[a]
petroleum ether (b.p. 60-80°C)	113.67 ± 9.94[d]	Light orange and waxy[a]
SC-CO_2 (40°C, 100 bar)	9.32 ± 0.85[e]	Light yellow and clear
SC-CO_2 (60°C, 200 bar)	14.34 ± 1.33[f]	Light yellow and clear
SC-CO_2 (80°C, 300 bar)	126.15 ± 10.77[g]	Light green and turbid[a]

[a] Centrifugation was done at 1000 g, 20 min and supernatant was used for GC/MS analyses. [b] Yield of extracts are mean ± SD from three independent extractions. [c, d, e, f, g] Different letters in a column indicate significant difference at $p < 0.05$.

Yield, color and appearance of extracts considered for MIC assay: Among the SC-CO_2 extracts, the highest yield of extract was obtained at 80 °C, 300 bar; and lowest at 40 °C, 100 bar (**Table 1**). We also observe that yield of extract obtained by *n*-hexane was lower than that obtained by petroleum ether.

All SC-CO_2 extracts were clear in appearance and light yellow in color, except that obtained at 80°C, 300 bar which was light green and turbid. The extract obtained using *n*-hexane was yellowish orange and slightly waxy, while a light orange colored waxy extract was obtained with petroleum ether.

Antimicrobial activity of Tuberose extracts by MIC assay:

Antimicrobial activity of extracts obtained by SC-CO_2: ANOVA study concluded that the SC-CO_2 extract obtained at 40°C, 100 bar showed significant difference (p = 0.00) in MIC values obtained for the different test microorganisms, with best activity against *C. albicans* (**Table 2**). The extract obtained at 60°C, 200 bar showed significant difference (p = 0.00) in MIC values and the best potency against *P. aeruginosa.* The extract obtained at 80°C, 300 bar also showed significant difference (p = 0.00) in MIC values with high antimicrobial efficacies against *P. aeruginosa* and *E. coli* (**Table 2**).

Antimicrobial activity of extracts obtained by solvent extraction: From ANOVA study (Table 2), we observed that the extracts obtained by *n*-hexane showed significant differences (p = 0.00) in the MIC values for the different test microorganisms. This extract showed best potency against *P. aeruginosa.* The extracts obtained by using petroleum ether also showed significant difference (p = 0.00) in the MIC values with respect to these microorganisms. This extract showed best antimicrobial activity against *C. albicans*; however, no activity was found against *S. aureus*.

TABLE 2: *IN VITRO* MINIMUM INHIBITORY CONCENTRATION (MIC) VALUES OF TUBEROSE EXTRACTS[a] AGAINST TEST MICROORGANISMS[b]

Test microorganism	MIC[m]						Positive control (µg/ml)
	n-hexane (mg/ml)	petroleum ether (b.p. 60-80°C) (mg/ml)	SC-CO_2 (40°C, 100 bar) (mg/ml)	SC-CO_2 (60°C, 200 bar) (mg/ml)	SC-CO_2 (80°C, 300 bar) (mg/ml)	Negative control (ethanol, 96%) (mg/ml)	
S. aureus	0.49 ± 0.02^{d}	$15.40 \pm 1.48^{d,c}$	1.81 ± 0.06^{d}	3.62 ± 0.10^{d}	3.62 ± 0.12^{d}	11.80 ± 0.12^{d}	$0.50 \pm 0.02^{n,d}$
P. aeruginosa	0.12 ± 0.01^{e}	7.69 ± 0.16^{e}	0.91 ± 0.03^{e}	0.91 ± 0.04^{e}	0.91 ± 0.04^{e}	23.60 ± 2.26^{e}	$0.98 \pm 0.04^{n,e}$
E. coli	0.49 ± 0.03^{d}	7.69 ± 0.18^{e}	1.81 ± 0.08^{f}	1.81 ± 0.06^{f}	0.91 ± 0.04^{e}	47.30 ± 3.82^{f}	$0.42 \pm 0.02^{n,f}$
C. albicans	0.24 ± 0.01^{f}	3.84 ± 0.12^{f}	0.45 ± 0.03^{g}	3.62 ± 0.12^{d}	1.81 ± 0.08^{f}	23.60 ± 2.24^{g}	$3.0 \pm 0.09^{p,g}$

[a] Extracts were dissolved in ethanol for antimicrobial assays.
[b] For *H. pylori* and *V. cholerae* MIC assay was not conducted since they did not show zone of inhibition by disc diffusion method.
[c] MIC of extract higher than that of negative control and therefore not considered to have antimicrobial potency.
[d,e,f,g] Different letters in a column indicate significant difference at $p < 0.05$.
[m] MIC values are mean ± SD of three independent experiments from three independent extracts.
[n] MIC value of Gentamycin (antibacterial positive control).
[p] MIC value of Amphotericin B (antifungal positive control).

GC/MS analyses of tuberose extracts:

GC/MS analyses of SC-CO_2 extracts: Fig. 2a and **Table 3** represent the chromatogram and compound profiles of the SC-CO_2 extract at 40 °C, 100 bar, obtained by GC/MS analysis. Methyl eugenol was found to be the most abundant compound in this extract. The chromatogram shown in **Fig. 2b** and the compound list in **Table 4** describes the SC-CO_2 extract at 60°C, 200 bar. Styrene was found to be the most abundant compound among the compounds identified. From **Fig. 2c** and **Table 5**, for SC-CO_2 extract at 80°C, 300 bar, eugenol was found to be the most abundant compound identified. Overall, SC-CO_2 extract showed lesser proportion of hydrocarbons (hentriacontane) vis-à-vis the solvent extracts, demonstrating increased solubility and selectivity of SC-CO_2 for benzene compounds.

GC/MS analyses of solvent extracts: Fig. 3a represents the chromatogram of the extract obtained by *n*-hexane and **Table 6** lists the peaks of identified compounds in the same. Among the benzene compounds, *trans*-carveol has been tentatively identified and found to be the most abundant. The chromatogram of compounds in petroleum ether (b.p. 60-80°C) extract has been shown in **Fig. 3b** and the identified compounds have been presented in **Table 7**. We found *cis*-3-methyl cyclopentanol to be the most abundant compound in this extract.

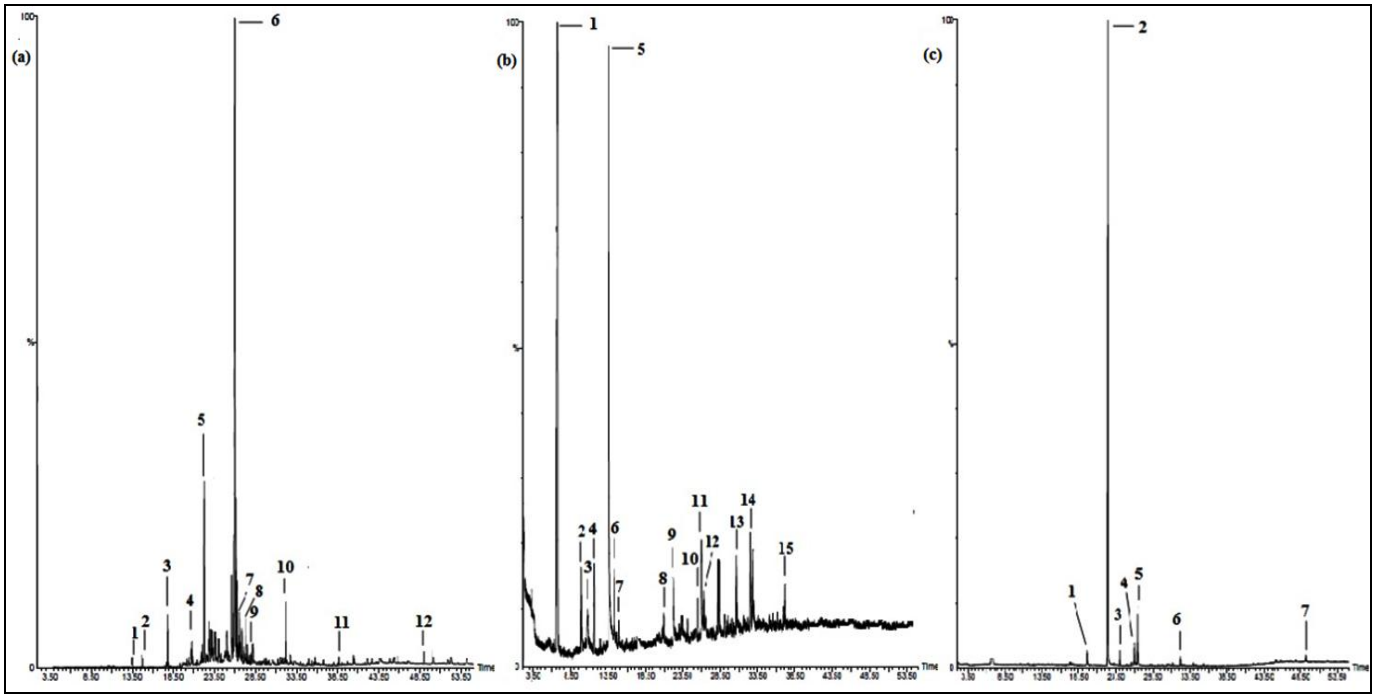

FIGURE 2: GC/MS CHROMATOGRAMS OF TUBEROSE EXTRACTS OBTAINED BY USING SC-CO_2 AT (a) 40°C AND 100 BAR, (b) 60°C, 200 BAR AND (c) 80°C, 300 BAR. LABELS ON THE CHROMATOGRAMS HAVE BEEN EXPLAINED IN TABLES 3, 4 AND 5, RESPECTIVELY.

TABLE 3: LIST OF COMPOUNDS IDENTIFIED BY GC/MS USING SC-CO_2 (40°C, 100 BAR)

Peak No.	R.T. (min)	$[M]^+$ (m/z)	Base Peak (m/z)	% Relative peak area[g]	Identified compounds[a]
1	13.62	120	105	0.17	acetophenone[b]
2	14.59	136	105	0.02	benzoic acid methyl ester
3	17.84	n.a.	n.a.	3.41	unknown
4	22.01	n.a.	n.a.	1.30	unknown
5	22.31	164	164	11.45	eugenol[c]
6	26.02	178	178	62.92	methyl eugenol[d]
7	26.15	204	41	8.87	alpha farnesene[e]
8	26.25	204	69	5.03	beta bisabolene[f]
9	27.53	n.a.	n.a.	1.61	unknown
10	31.19	212	105	4.49	benzyl benzoate
11	38.94	n.a.	n.a.	0.15	unknown
12	49.19	437	57	0.57	hentriacontane

R.T.: Retention time.
n.a.: not applicable.
[a] Identifications were carried out using NIST library (2007), Adams (2007), NIST mass spec data centre (2013) and literature review.
[b,c,d,e,f] Compounds having antimicrobial activity already reported in literature.
[g] Area percentages were reported without correction factor.
m/z values of the identified compounds:
acetophenone: 39, 43, 51, 68, 74, 77, 78, 91, 105, 106, 120.
benzoic acid methyl ester: 38, 51, 77, 105, 106, 136.
eugenol: 43, 51, 55, 65, 77, 81, 91, 103, 122, 131, 137, 149, 163, 164.
alpha farnesene: 39, 41, 43, 55, 67, 69, 79, 93, 107, 119, 135, 147, 161, 189, 204.
beta bisabolene: 39, 41, 43, 55, 67, 69, 79, 91, 93, 109, 119, 135, 147, 161, 189, 204.
benzyl benzoate: 39, 51, 61, 65, 77, 91, 105, 106, 121, 152, 167, 194, 212.

TABLE 4: LIST OF COMPOUNDS IDENTIFIED BY GC/MS USING SC-CO_2 (60°C, 200 BAR)

Peak No.	R.T.(min)	$[M]^+$ (m/z)	Base Peak (m/z)	% Relative peak area[d]	Identified compounds[a]
1	6.74	104	104	58.18	styrene[b]
2	9.79	106	106	2.40	benzaldehyde
3	11.65	146	146	2.65	1,3 dichloro benzene
4	12.45	n.a.	n.a.	0.71	unknown
5	13.62	162	105	19.27	1-phenyl-1-pentanone
6	14.34	n.a.	n.a.	1.94	unknown
7	14.92	n.a.	n.a.	0.72	unknown
8	22.29	164	164	2.20	eugenol[c]
9	23.26	n.a.	n.a.	3.71	unknown
10	25.61	n.a.	n.a.	1.02	unknown
11	25.99	162	57	2.71	1-cholro octadecane
12	26.27	206	191	1.15	2,4 bis (1,1 dimethyl) phenol
13	32.74	238	55	0.97	8-heptadecene
14	32.88	352	57	1.38	pentacosane
15	37.13	437	57	0.93	hentriacontane

R.T.: Retention time.
n.a.: not applicable.
[a] Identifications were carried out using NIST library (2007), Adams (2007), NIST mass spec data centre (2013) and literature review.
[b,c] Compounds having antimicrobial activity already reported in literature.
[d] Area percentages were reported without correction factor.
m/z values of the identified compounds:
styrene: 39, 51, 63, 78, 103, 104.
benzaldehyde: 39, 51, 63, 77, 78, 105, 106.
1,3 dicloro benzene: 51, 55, 74, 75, 111, 113, 146.
1-phenyl pentanone: 41, 51, 53, 77, 78, 105, 106, 120, 162.
1-chloro octadecane: 43, 55, 57, 71, 85, 91, 105, 111, 133, 147, 162.
8-heptadecene: 41, 43, 55, 57, 67, 69, 83, 97, 111, 125, 130, 156, 168, 207, 219, 238.

TABLE 5: LIST OF COMPOUNDS IDENTIFIED BY GC/MS USING SC-CO_2 (80°C, 300 BAR)

Peak No.	R.T. (min)	$[M]^+$ (m/z)	Base Peak (m/z)	% Relative peak area[d]	Identification compounds[a]
1	19.61	134	134	2.63	4-(2-propenyl) phenol
2	22.32	164	164	83.17	eugenol[b]
3	24.05	n.a.	n.a.	1.86	unknown
4	25.96	178	178	3.26	methyl eugenol[c]
5	26.44	n.a.	n.a.	6.13	unknown
6	32.18	212	105	1.08	benzyl benzoate
7	49.20	437	57	1.47	hentriacontane

R.T.: Retention time.
n.a.: not applicable.
[a] Identifications were carried out using NIST library (2007), Adams (2007), NIST mass spec data centre (2013) and literature review.
[b,c] Compounds having antimicrobial activity already reported in literature.
[d] Area percentages were reported without correction factor.
m/z values of the identified compounds:
4-(2-propenyl) phenol: 39, 51, 55, 65, 77, 79, 91, 105, 107, 117, 131, 133, 134.

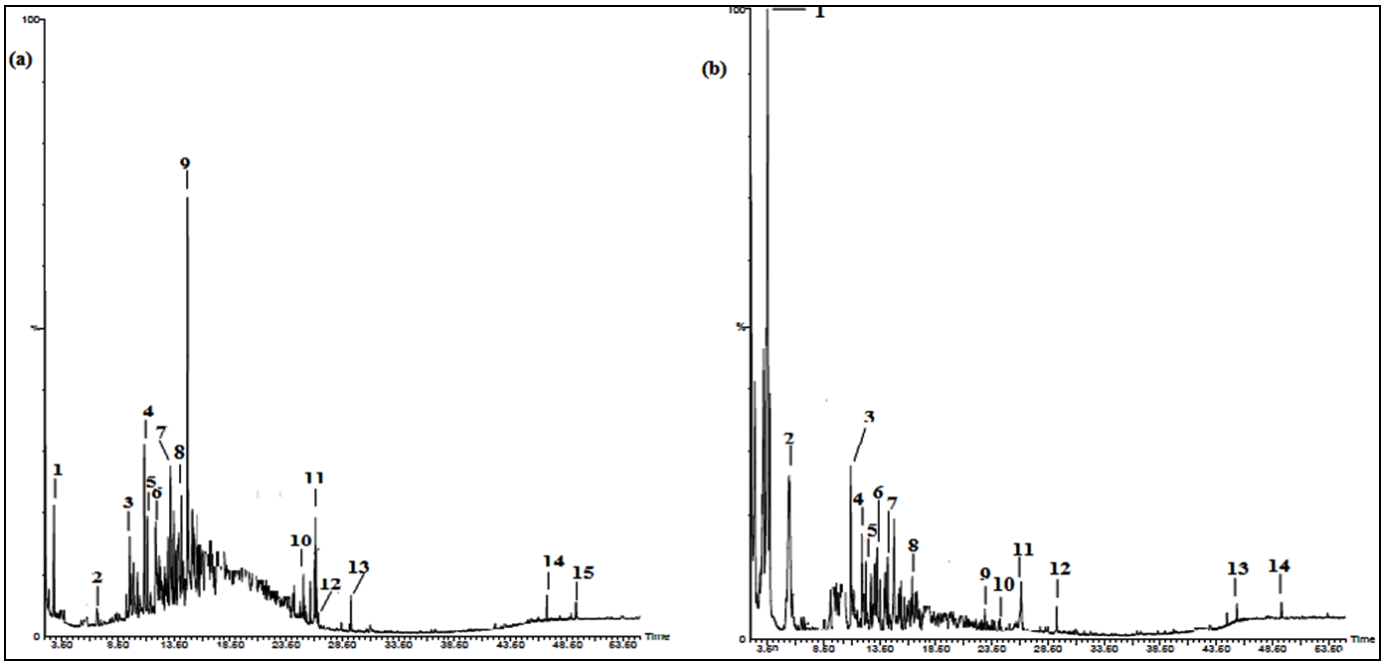

FIGURE 3: GC/MS CHROMATOGRAMS OF TUBEROSE EXTRACTS OBTAINED BY USING SOLVENT EXTRACTION USING (a) *n*-HEXANE (b) PETROLEUM ETHER (B.P. 60-80°C). LABELS ON THE CHROMATOGRAMS HAVE BEEN EXPLAINED IN TABLES 6 AND 7, RESPECTIVELY.

TABLE 6: LIST OF COMPOUNDS IDENTIFIED BY GC/MS USING *n*-HEXANE AS SOLVENT

Peak No.	R.T. (min)	$[M]^+$ (m/z)	Base Peak (m/z)	% Relative peak area[e]	Identified compounds[a]
1	2.80	n.a.	n.a.	13.46	unknown
2	5.76	106	91	5.91	*p*-xylene[b]
3	9.56	120	105	7.11	1-ethyl 3-methyl benzene
4	11.87	120	105	18.04	mesitylene
5	12.18	136	68	0.42	limonene[c]
6	12.56	n.a.	n.a.	2.71	unknown
7	12.68	n.a.	n.a.	6.51	unknown
8	12.85	134	119	3.30	1,4 diethyl benzene
9	13.18	n.a.	152	23.26	*trans*-carveol[f]
10	24.08	n.a.	n.a.	3.93	unknown
11	26.09	178	178	6.40	methyl eugenol[d]
12	26.36	206	191	0.08	2,4 bis (1,1 dimethyl ethyl) phenol
13	29.28	n.a.	n.a.	2.52	unknown
14	45.34	352	57	2.79	pentacosane
15	49.32	436	57	2.87	hentriacontane

R.T.: Retention time.
n.a.: not applicable.
[a] Identifications were carried out using NIST (2007), Adams (2007) NIST mass spec data centre (2013) and literature review.
[b,c,d] Compounds having antimicrobial activity already reported in literature.
[e] Area percentages were reported without correction factor.
[f] Tentative identification was possible since the mass spectra of the compound could not be matched with available standard mass spectra libraries.
m/z values of identified compounds:
p-xylene: 39, 51, 65, 77, 91, 92, 105, 106.
1-ethyl 3-methyl benzene: 39, 51, 65, 77, 91, 105, 120.
mesitylene: 39, 51, 65, 77, 91, 105, 106, 119, 120.
limonene: 41, 53, 65, 67, 68, 79, 80, 91, 92, 93, 94, 107, 121, 136.
1,4 diethyl benzene: 41, 51, 65, 77, 91, 105, 119, 134.
methyl eugenol: 41, 51, 65, 77, 91, 107, 115, 135, 147, 163, 178.
2,4 bis (1,1 dimethyl ethyl) phenol: 41, 57, 74, 91, 107, 115, 147, 163, 175, 191, 206.
pentacosane: 43, 57, 71, 85, 99, 113, 127, 155, 169, 211, 225, 253, 267, 352.
hentriacontane: 45, 55, 57, 69, 71, 85, 99, 113, 127, 155, 169, 197, 211, 239, 253, 281, 436.

TABLE 7: LIST OF COMPOUNDS IDENTIFIED BY GC/MS USING PETROLEUM ETHER, B.P. 60- 80°C AS SOLVENT

Peak No.	R.T. (min)	$[M]^+$ (m/z)	Base Peak (m/z)	% Relative peak area[f]	Identified compounds[a]
1	5.43	100	57	48.02	*cis*-3-methyl cyclopentanol
2	5.77	106	91	0.45	*p*-xylene[b]
3	10.92	120	105	14.27	1,2,4 trimethyl benzene
4	11.93	120	105	5.40	mesitylene
5	12.26	136	68	3.94	limonene[c]
6	12.71	n.a.	n.a.	5.69	unknown
7	13.27	134	119	7.73	1,4 diethyl benzene
8	15.18	n.a.	n.a.	1.26	unknown
9	23.06	151	121	0.52	salicyl hydrazide[d]
10	24.10	157	157	0.89	1,6 dimethyl napthalene
11	26.09	178	178	6.94	methyl eugenol[e]
12	29.27	182	105	2.30	benzophenone
13	45.32	352	57	1.13	pentacosane
14	49.30	437	57	1.32	hentriacontane

R.T.: Retention time.
n.a.: not applicable.
[a] Identifications were carried out using NIST library (2007), Adams (2007), NIST mass spec data centre (2013) and literature review.
[b,c,d,e] Compounds having antimicrobial activity already reported in literature.
[f] Area percentages were reported without correction factor.
m/z values of the identified compounds:
cis-3-methyl cyclopentanol: 41, 56, 57, 68, 71, 82, 100.
1,2,4 trimethyl benzene: 39, 51, 65, 77, 79, 91, 105, 119, 120.
salicyl hydrazide: 39, 53, 65, 76, 93, 121, 122, 151.
1,6 dimethyl napthalene: 49, 69, 85, 98, 115, 141, 156, 157.
benzophenone: 39, 51, 76, 77, 78, 105, 106, 152, 181, 182.

DISCUSSION: For SC-CO_2 extracts, highest gravimetric yield (significant at p = 0.00) was obtained at extraction conditions of 80°C, 300 bar, followed by those at 60°C, 200 bar and 40°C, 100 bar. The highest yield at 80°C, 300 bar may be attributed to extraction of oleoresins which are known to be extracted from plant materials at a pressure regime of 250-350 bar [11]. Moreover, these oleoresins are viscous in nature, which could have rendered the extract waxy and turbid; unlike the extracts obtained at lower pressure regimes. ANOVA study on the yield of the solvent extracts indicated that the gravimetric yield of the petroleum ether extract was significantly higher (p = 0.00) than that of the *n*-hexane extract.

From the overall antimicrobial activities of SC-CO_2 extracts, we found best potency of the extract obtained at 40°C, 100 bar against *C. albicans*. All the SC-CO_2 extracts were equipotent against *P. aeruginosa*. The extract obtained at 80°C, 300 bar was most potent against *E. coli* (**Table 2**). The significant differences in MIC values of the extracts can be attributed to extraction of selective compounds by SC-CO_2 at different temperature-pressure regimes which could be owing to differences in the solubilities of these compounds in the extracting solvent under different extracting conditions. The SC-CO_2 extract at 40°C, 100 bar was found to have maximum potency against all the test microorganisms (**Table 2**). We found that tuberose extracts obtained by SC-CO_2 and solvents, have bacteriostatic and fungistatic activities. This is in agreement to the fact that MIC represents the bacteriostatic and not bactericidal concentration of an antimicrobial agent [12]. Further, GC/MS analyses of the extracts identified compounds attesting to antimicrobial potencies.

In the SC-CO_2 extract at 40°C, 100 bar (**Table 3**), compounds with antimicrobial potency such as acetophenone [13], alpha-farnesene and beta-bisabolene [14], eugenol [15] and methyl eugenol [16] have been identified. For the extract obtained at 60°C, 200 bar, in addition to eugenol, styrene was also detected which is known to be an antimicrobial agent [17], shown in **Table 4**. In the extract obtained at 80°C, 300 bar, the presence of eugenol and methyl eugenol attest to antimicrobial activities (**Table 5**).

Furthermore, in the SC-CO_2 extract at 40 °C, 100 bar, compounds such as beta bisabolene and alpha farnesene have been identified. We hypothesize that these compounds synergistically contribute to the overall antimicrobial potency of the extract which could be selectively extracted under these extracting conditions. These compounds have not been identified in the extracts obtained at 60°C, 200 bar and at 80°C, 300 bar; which establishes selective extraction by SC-CO_2 at 40°C, 100 bar. It can thus be concluded that the extract obtained at 40°C, 100 bar has the best antimicrobial activity (**Table 2**).

GC/MS analyses of *n*-hexane and petroleum ether extracts (**Tables 6 and 7**) confirmed the presence of compounds with antimicrobial potencies such as *p*-xylene [18], limonene [15] and methyl eugenol. Additionally, salicyl hydrazide, a potent antimicrobial [19] was also identified in the petroleum ether extract (**Table 7**).

CONCLUSIONS: MIC assays of SC-CO_2 and solvent extracts of tuberose flowers attested tuberose to be a potential natural source of antimicrobials against *S. aureus*, *P. aeruginosa*, *E. coli* and *C. albicans*; but not against *H. pylori* and *V. cholerae.* The SC-CO_2 extract obtained at 40 °C, 100 bar pressure, 135 min extraction time, and that by *n*-hexane extract showed most promising results as antimicrobials against all test organisms. The chemical compositions of the extracts have been ascertained by GC/MS analysis confirming chemical identities of constituents possessing antimicrobial potencies. Owing to international regulatory concerns, the green SC-CO_2 extract alone is advocated for therapeutic applications. We recommend topical application studies using this extract, since some of its constituents are known to be toxic for internal usage.

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REFERENCES:

1. Wu XL, Li CW, Chen HM, Su ZQ, Zhao XN, Chen JN, Lai XP, Zhang XJ, and Su ZR: Anti-inflammatory effect of supercritical-carbon dioxide fluid extract from flowers and buds of *Chrysanthemum indicum* Linnén. Evidence-Based Complementary and Alternative Medicine 2013; http://dx.doi.org/10.1155/2013/413237.
2. Dias AMA, Santos P, Seabra IJ, Júnior RNC, Braga MEM, and de Sousa HC: Spilanthol from *Spilanthes acmella* flowers, leaves and stems obtained by selective supercritical carbon dioxide extraction. Journal of Supercritical Fluids 2012; 61: 62-70.
3. Kiruthika KA, Jaisheba JA, and Sornaraj R: Evaluation of antibacterial activity of some selected Angiospermic flower extract. International Journal of Chemical Tech Research 2011; 3 (4): 1945-1951.
4. Nidiry ESJ and Babu CSB: Antifungal activity of tuberose absolute and some of its constituents. Phytotheraphy Research 2005; 19: 447-449.
5. Andrews JM: Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy 2001; 48 (S1): 5-16.
6. AOAC, 1990. Official Methods of Analysis. AOAC International, Washington DC, USA, 15th edition.
7. Rakthaworn P, Dilokkunanant U, Sukkatta U, Vajrodaya S, Haruethaitanasan V, and Pitpiangchan P: Extraction methods for tuberose oil and their chemical components. Kasetsart Journal of Natural Science 2009; 43: 204–221.
8. Reverchon E and Della Porta G: Tuberose concrete fractionation by supercritical carbon dioxide. Journal of Agriculture and Food Chemistry 1997; 45: 1356-1360.
9. NIST Mass Spec Data Center. S.E. Stein, director, Mass Spectra. Mallard Gaithersburg *NIST Chemistry WebBook.* In: Linstrom P., Mallard W.G. (Eds.), NIST Standard Reference Database Number 69, National Institute of Standards and Technology, Gaithersburg, MD, USA: [http://webbbok.nist.gov] (accessed August 20, 2013).
10. Adams RP: Identification of essential oil components by gas chromatography/mass spectroscopy, 3rd edition, IL, USA: Allured Publishing Corporation, 2007.
11. Mukhopadhyay M: Natural extracts using supercritical carbon dioxide, 1st edition, FL: Boca Raton, 2000, pp. 177-200.
12. Engelberg C: Strategies to combat bacterial infections. In: Engelberg NC, DiRita V, Dermody TS (Eds.), Schaechter's Mechanism of Microbial Diseases, PA, USA: Lippincott Williams and Wilkins, 5th edition, 1990, pp. 316.
13. Sivakumar PM, Sheshayan G, and Doble M: Experimental and QSAR of acetophenones as antibacterial agents. Chemical Biology and Drug Design 2008; 72 (4): 303-313.
14. Zellagui A, Gherraf N, and Rhouati S: Chemical composition and antibacterial activity of the essential oils of *Ferula vesceriensis* coss et Durr. leaves endemic in Algeria. Organic and Medicinal Chemistry Letters 2012; 2: 3.
15. Bevilacqua A, Corbo MR, and Sinigaglia M: *In vitro* evaluation of the antimicrobial activity of eugenol, limonene and citrus extract against bacteria and yeasts, representative of the spoiling microflora of fruit juices. Journal of Food Protection 2010; 73 (5): 888-894.

16. Anand AK, Mohan M, Haider SZ, and Sharma A: Essential oil composition and antimicrobial activity of *Ocimum* species from Uttarakhand (India). International Journal of Pharmaceutical Science 2011; 3 (3): 223-225.
17. Ersan S, Nacak S, Acar N, and Ozden T: Synthesis and antimicrobial activity of beta-[(2-benzimidazolyl) thio]-beta-benzoyl styrene derivatives. Arzneimittel-Forschung-Drug Research 1997; 47 (6): 73-775.
18. Akpuaka A, Ekwenchi MM, Dashak DA, and Dildar A: Biological activities of characterized isolates of n-hexane extract of *Azadirachta Indica* A. Juss (Neem) leaves. New York Science Journal 2013; 6 (6): 119-124.
19. Patel BP, Patel HS, and Shah PJ: Synthesis, characterization and antibacterial activity of new salicylhydrazide containing azopyrazoles. Orbital- The Electronic Journal of Chemistry 2010; 2: 303-310.

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