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Research Article

COMPARATIVE STUDY OF METABOLITES AND ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OILS EXTRACTED FROM THREE *AMOMUM SUBULATUM* CULTIVARS

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ABSTRACT

Objective: In Sikkim (India), Seremna is a highly growing cultivar of *Amomum subulatum* at lower altitudes. Other popular cultivated varieties are Varlangy and Sawney in the same state but at different altitude. In this study, we evaluate the variation in essential oils, metabolites and antimicrobial activities among *A. subulatum* selected cultivars.

Methods: The composition of essential oil of Varlangy, Seremna, and Sawney was analyzed using gas chromatography–mass spectrometry and comparative antimicrobial activity of oils was explored using agar well diffusion and agar dilution method.

Results: The Seremna cultivar oil was shown the high content of major constituents (1,8-cineole) in comparison to others. Comparative minimum inhibitory concentrations and minimal bactericidal concentration (MBC) or minimal fungicidal concentration against two Gram-positive (Staphylococcus aureus and *Bacillus subtilis*), two Gram-negative (*Klebsiella pneumoniae* and *Escherichia coli*) bacteria, and two (*Candida albicans* and *Aspergillus niger*) fungi were determined. The oil of Seremna showed distinct antibacterial and antifungal activity against all the microorganisms except *B. subtilis* which showed resistance.

Conclusion: The present findings concluded that the high content of the principal compound accelerates the antimicrobial activity of essential oils. The essential oil of Seremna could be a good antimicrobial agent and recommended in the case of infections.

Keywords: *Amomum subulatum*, Cultivars, Essential oil, Gas chromatography–mass spectrometry analysis, Antimicrobial activity.

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INTRODUCTION

Amomum subulatum (commonly known as large cardamom) is a perennial plant widely cultivated in the sub-Himalayan region of North-Eastern part of India (Sikkim), Northern Uttar Pradesh, Arunachal Pradesh, and Mizoram State belongs to the family Zingiberaceae. The worldwide annual production of large cardamom fruits is ~ 12×10^3 mt which is 30% of the total production coming from Sikkim [1]. The capsules/fruits are the main edible source of this plant and worldwide called as Badi elaichi/Kali elaichi (Hindi), Heel kalan (Urdu), Greater/Nepal cardamom (English), Cardamom (French), Ts‟ao-k‟ou (China), and Qakilahe kalan (Persian) [2]. The fruits of *A. subulatum* are mainly used for anti-gonorrhea, kidney stones, respiratory, digestive diseases, and alternative systems of medicine [3]. There are six major cultivars of large cardamom growing in the Sikkim region, i.e., Ramsey, Sawney, Golsey, Varlangy, Bebo, and Seremna [4]. Cultivars such as Seremna, Sawney, and Varlangy are very popular among local growers due to its high productivity and yield [5]. Recent study of bioactive compounds of *A. subulatum* fruit/seeds has shown the different pharmacological activities such as antioxidants, antiulcer, hypolipidemic agents, antimicrobial activity antidiabetics, and hepatoprotective activity by the different investigators [6-9]. A broad citation on the antimicrobial properties of essential oils has been carried out by several investigators. Essential obtained from various medicinal plants such as *Cinnamomum zeylanicum*, *Eugenia caryophyllata*, *Rosmarinus officinalis*, and *Ocimum kilimandscharicum* has been reported to possess significant antimicrobial activities and can serve as a powerful tool to reduce the bacterial resistance [10,11]. Due to lack of technological errors, the essential oil of the plant or bioactive compound of the particular cultivars (Seremna, Sawney, and Varlangy) has not been tested earlier. Several authors considered, the environmental conditions, e.g., type and composition of the soil (edaphic), geographic and genetic variations are the determinant factors influence the secondary metabolites of the essential oils [12-14]. Keeping the view in mind for cardamom pharmacological properties, we have studied the Seremna, Sawney, and Varlangy essential oils by gas chromatography–mass spectrometry (GC–MS) and also observed the effect of their essential oils for antifungal and antibacterial activities collected from Sikkim.

MATERIALS AND METHODS

Sample collection and extraction of essential oils

The fruits of three different cultivars of *A. subulatum* such as Sawney, Seremna, and Varlangy were obtained from Spice Board, ICRI (Indian Cardamom Research Institute), Tadong (Sikkim, India), in the month of November 2016. The hydrodistillation method was used for the extraction of essential oil from three cultivars of Sawney, Seremna, and Varlangy [15]. For each cultivar, 50 g fine powdered were taken for extraction of oil from each cultivar separately for the time of 4 h. The extracted samples were dried by mixing the sodium sulfate (anhydrous) and stored at 4°C for further study. Data were recorded and percentage yields of each cultivar oil were calculated as triplicate.

Chemical characterization: Metabolite identifications through GC–MS

For the comparative qualitative and quantitative study, the essential oils of each cultivar were characterized by GC and GC–MS. GC analysis was carried out using H. P-5890 II apparatus, prepared to splitless injector method using the HP-5M column (0.52 µm film thickness and 25 m × 0.32 mm) and FID (flame ionization detector) with 1 ml/min flow rate of carrier gas (N_2) and injector temperature (250 and 300°C, respectively) in the form of split ratio 1:30. The column temperature

was adjusted and scanned from 40°C to 240°C (4°C/min). 1 µl of the diluted samples (v/v) (1/100 in heptane) were injected manually with a microsyringe.

GC–MS of three cultivars of cardamom, i.e., Sawney, Seremna, and Varlangy was carried out using Hewlett Packard G 1800C Series II GCD, GC–MS system assembled with HP-5MS column (0.25 µm film thickness and 30 m × 0.25 mm) ramping the temperature from 40°C to 260°C was operated between 40 and 450 m/z and mass detector (Ionization energy: 70 eV). The components of essential oil extracted from the Sawney, Seremna, and Varlangy were identified by comparison of their retention indices and mass spectra with standard libraries (16; Wiley6; 1989 and NIST02 2008).

Microorganisms, media, culture, and growth conditions

Bacterial strains, *Staphylococcus aureus* MTCC-902, *Bacillus subtilis MTCC-736, Klebsiella pneumoniae* (MTCC-432), and *Escherichia coli* MTCC-443 and fungus strains, *Candida albicans* MTCC-183 and *Aspergillus niger* MTCC-1344 strains were used for this study to check the oil properties against them. Nutrient broth, Mueller-Hinton agar (MHA), and Sabouraud's dextrose agar (SDA) (HiMedia Laboratories) have been used for the bacterial and fungal growth followed by the standard protocols [17].

The bacteria were cultured at 37ºC on MHA medium and incubated overnight to check its growth in pure culture. Similarly, the fungi were also cultured in SDA medium at 37ºC to further check the fungal hypha growth. To prepare the bacterial and fungal inoculums, the overnight culture of bacteria and fungi was diluted, i.e., 10^8 CFU/ml (0.5 of McFarland) for bacterial and 10^7 CFU/ml for the fungal spores to validate the oil efficiency against these microorganisms. 100 µl inoculum was obtained from the secondary culture of microbes (bacterial and fungal) where spread under sterile condition in laminar hood on MHA and SDA basal media over the entire surface of the Petri plates.

Antimicrobial screening of essential oil

All three essential oils extracted from *A. subulatum* fruit samples were tested for antimicrobial activity using the well diffusion method [18]. The extracted oil (20 mg/ml) of each cultivar of *A. subulatum* (Sawney, Seremna, and Varlangy) was prepared using 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich) solution and tested against the bacterium *S. aureus* and *B. subtilis, K. pneumoniae,* and *E. coli* and fungus *C. albicans* and *A. niger* strains. The antimicrobial activities of the samples were initially evaluated by modifying agar well diffusion assay. Wells (6 mm) of each plate were filled with 15 µl of samples and incubate the plates for 24 h at 37°C; bacteria, 48 h at 37°C; yeast, and 7 days at 28°C for fungi were observed. Gentamicin (GEN-10 µg/ml) and 5-fluorocytosine (5FC, 10 µg/ml) were used as a positive control; similarly, 10% DMSO was used as a negative control. The experiments were repeated thrice, i.e., triplicate manner. The minimum inhibitory concentration (MIC) and minimum bacteriostatic concentration (MBC) were performed using the agar dilution method [19]. Six different dilutions (20, 10, 5, 2.5. 1.25, and 0.625 mg/ml) were prepared and diluted solution (1 ml) of each cultivar oils was separately mixed to 19 ml of MHA hot solution and poured on the sterile Petri plates. After solidification of MHA media, 1 ml inoculum of each microbe was spread on sterile plates with oil and inoculated at 37°C for further observations in terms of antimicrobial activity. For the MIC, the visible growth was observed after 24 h of incubation and for the MBC, visible growth after 5 days of incubation. Similarly, MIC for fungistatic and MIC fungicide (MICF) was observed after incubation for 3 weeks at room temperature.

RESULTS AND DISCUSSION

Percent oil recovery *A. subulatum* cultivars

Collected cultivars from Sikkim have shown the diverse percentage content of the essential oils Table 1. The results of the present study were shown that Seremna has a higher percentage (2.7 ± 0.10) of essential oil than other cultivars; Varlangy (2.5 ± 0.15) and Sawney (1.7 ± 0.20).

Identification of active metabolites in *A. subulatum cultivars*

The active chemical compositions of each cultivar oil are presented in Table 2. 25 active compounds were identified in the Seremna oil (96.49% of total oil), while 25 and 27 compounds were identified in Varlangy and Sawney, respectively. The monoterpene, 1, 8-cineole is recorded highest metabolites in all the cultivars (Seremna, 72.5%; Sawney, 61.83%; and Varlangy, 56.89%). A total of six major components were identified, including 1, 8-cineole and these compounds were limonene, D-nerolidol, α-terpineol, β-pinene, and α-pinene also identified in the present finding (Fig. 1).

In this study, we have observed that the several similar components reported in the essential oil of Nepal *A. subulatum* (1, 8-cineole, alpha-pinene, beta-pinene, alpha-terpineol, etc.) where, 1,8-cineole (60.8%) has also been reported as the main components [20]. Joshi *et al.* studied on the six different cultivars collected from different altitudes of other regions than Sikkim, i.e., Himachal Pradesh, showed that 1,8-cineole (50.55% ± 1.87%–60.46% ± 3.50%) was the main active constituents of *A. subulatum* essential oils. The percentage of the composition of essential oil is varying due to geographical region and its altitude. In the present study, three cultivars were cultivated in different geographical conditions, Seremna is highly cultivated at low altitude (<900 m), Sawney is cultivated at middle altitude (700–1500 m), while Varlangy is cultivated at high altitude (>1500 m) [21]. In our findings, we have also analyzed that 1,8-cineole (eucalyptol) is main compounds of oil and the percentage varies cultivar to cultivar as well as altitude to altitude. This study is also supported by other groups [22,23]. The concentration and

Table 1: Percentage yield of oils of the selected cultivars

Cultivars	Percentage yield
Varlangy	2.5±0.15
Seremna	2.7±0.10
Sawney	1.7±0.20

n=3, mean±SD. SD: Standard deviation

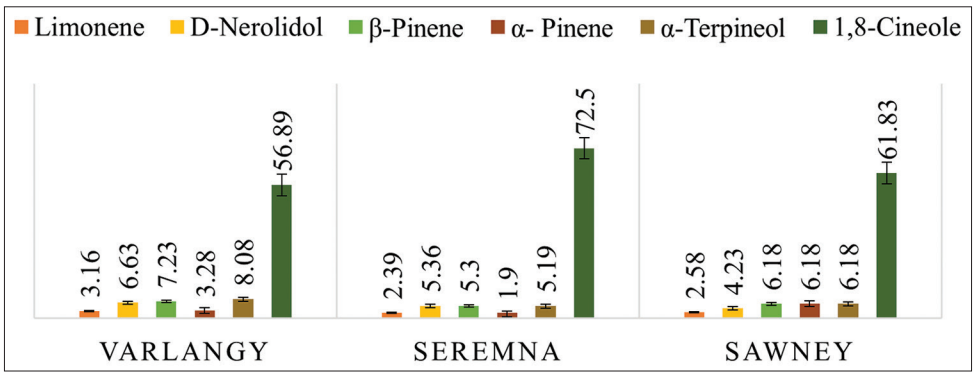

Fig. 1: Major compounds found in the essential oils of *Amomum subulatum* cultivars

the percentage of the other components (α-pinene, β-pinene, terpineol, nerolidol, and limonene) were different from the previous reports [24]. This study is the 1st time reported the chemical composition of Seremna and it was found higher than the other selected cultivars.

Antimicrobial activity

The inhibition zone of different essential oils against selected pathogens is shown in Table 3. Oil of Seremna was shown the good inhibition zones (7.5 ± 1.23–24.28 ± 0.95 mm) against all the microorganisms except *B. subtilis*. The range of inhibition zones of the Varlangy oil was 9.41 ± 0.94–14.25 ± 1.26 mm and Sawney oil was between 10.72 ± 0.96 and 18.49 ± 1.43 mm, low against Gram-negative bacteria (*K. pneumoniae*) but high against Gram-positive bacteria (*S. aureus*). The MIC and MBC values of all essential oils were reported against bacteria in Table 4. The MIC and MICF values of all essential oils were reported against fungal strains in Table 5. The MIC range of Varlangy oil was 2.5–5 mg/ml, Seremna oil was 1.25–2.5 mg/ml, and Sawney oil was 1.25–5 mg/ml for selected fungal strains.

Table 2: Chemical composition of the essential oils obtained from fruits of different cultivars of *Amomum subulatum*

Compounda	Percentage occurrence (%) [b]			RI[c]	Methods[d]
	Varlangy	Seremna	Sawney		
α-Thujene	0.61	0.32	t	928	RI/MS
α-Pinene	3.28	1.7	4.74	936	RI/MS
Camphene	-	0.12	0.14	949	RI/MS
β-Pinene	7.23	5.3	6.18	978	RI/MS
β-Myrcene	0.98	0.36	0.78	986	RI/MS
1-Phellandrene	-	0.13	0.27	1002	RI/MS
α-Terpinene	0.94	-	0.12	1019	RI/MS
p-Cyamin	-	0.12	-	1022	RI/MS
Limonene	3.16	2.39	2.58	1030	RI/MS
1,8-Cineole	56.89	72.5	61.83	1036	RI/MS
γ-Terpinene	0.76	-	0.72	1059	RI/MS
α-Terpinolene	t	0.29	-	1088	RI/MS
trans-Sabinene hydrate	0.82	0.15	0.32	1097	RI/MS
Linalool	0.41	0.17	0.19	1099	RI/MS
Limonene oxide	-	0.11	0.54	1134	RI/MS
Pinocarvone	0.76	0.24	0.25	1166	RI/MS
4- Terpineol	0.98	0.28	0.58	1180	RI/MS
α-Terpineol	8.08	5.19	7.16	1192	RI/MS
trans-Carveol	0.15	-	-	1217	RI/MS
Cis-carveol	0.44	-	t	1229	RI/MS
3-Decyn-2-ol	0	0.21	0.26	1233	RI/MS
2-Decenal	0.15	t	0.32	1263	RI/MS
Geranial	-	-	0.17	1273	RI/MS
1-Undecanol	0.31	-	0.43	1308	RI/MS
Limonene glycol	0.84	0.25	0.73	1320	RI/MS
α-Terpinyl acetate	0.46	0.15	0.55	1350	RI/MS
trans-β-Caryophyllene	0.15	0	0.36	1418	RI/MS
β-Selinene	0.22	0.37	0.32	1467	RI/MS
α-Selinene	-	t	0.14	1484	RI/MS
D-Nerolidol	6.63	4.36	4.53	1562	RI/MS
(+) Spathulenol	0.29	0.86	0.35	1578	RI/MS
Myristic acid	0.96	0.58	0.91	1768	RI/MS
Total identified	95.62	96.49	95.51		
Monoterpene hydrocarbons	16.2	10.44	14.81		
Oxygenated monoterpene	71.05	79.57	74.05		
Sesquiterpene hydrocarbons	0.41	0.4	0.72		
Oxygenated sesquiterpenes	6.92	5.02	4.88		
Fatty acid	0.96	0.58	0.91		

[a]Compounds were recorded to the elution time from a column (HP-5 MS), [b]Percentage peak area (%) was linked to total identified compounds by GC/MS, [c]RI on HP-5 MS column using the homologous series of n-alkane (C8–C28), [d]RI and mass spectra used, t: Less than 0.1%, -: Not identified. GC/MS: Gas chromatography/mass spectrometry, RI: Retention indices

Table 3: Antimicrobial activity (zone of inhibition, mm) of essential oils of different cultivars of *Amomum subulatum*

Microorganisms	Varlangy	Seremna	Sawney	Positive control
Escherichia coli	NI	7.5±1.23	NI	32.06±1.26 (GEM)
Bacillus subtilis	NI	NI	NI	21.16±1.25 (GEM)
Klebsiella pneumoniae	9.41±0.94	12.54±1.25	10.72±0.96	31.17±1.25 (GEM)
Staphylococcus aureus	11.26±0.95	17.15±0.97	15.3±1.64	27.36±1.26 (GEM)
Candida albicans	14.25±1.26	24.28±0.95	18.49±1.43	32.09±1.25 (5-FC)
Aspergillus niger	10.05±1.25	14.72±0.96	12.04±0.95	48.32±1.26 (5-FC)

Values are expressed as mean±SD. ZI: Zone of inhibition, millimeter, NI: No inhibition, GEM: Gentamycin, positive control for antibacterial, 5FC: 5-fluorocytosine, positive control for antifungal, SD: Standard deviation

Table 4: Determination of MIC and MBC (mg/ml) values of the essential oils of different cultivars of *Amomum subulatum*

Bacterial strain	Varlangy oil		Seremna oil		Sawney oil	
	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli	ND	ND	10	ND	ND	ND
Klebsiella pneumoniae	5	<10	2.5	5	5	5
Staphylococcus aureus	5	<10	2.5	5	2.5	10

ND: Did not study, MIC: Minimum inhibitory concentrations, MBC: Minimal bactericidal concentration

Table 5: Determination of minimum inhibitory concentrations and minimum inhibitory concentration fungicide (mg/ml) values of the essential oils of different cultivars of *Amomum subulatum*

Fungi	Varlangy oil		Seremna oil		Sawney oil	
	MIC	MICF	MIC	MICF	MIC	MICF
Candida albicans	2.5	5	1.25	2.5	1.25	5
Aspergillus niger	5	<10	2.5	5	5	10

MICF: Minimum inhibitory concentration fungicide, MIC: Minimum inhibitory concentration

Values are expressed as mean ± standard deviation, Zone of inhibition, millimeter, no inhibition, gentamycin (GEM, Positive control) for antibacterial, and 5-fluorocytosine (5FC, positive control) for antifungal.

The antimicrobial study of oil of Seremna has shown high efficacy against Gram-positive bacteria in comparison to Sawney and Varlangy. The oils of all cultivars were shown a good inhibitory effect against fungal strains which is supported by previously reported studies that the plant parts which have major 1,8-cineole content in the essential oil showed good antibacterial and antifungal activities [25,26]. Oxygenated monoterpene, 1,8-cineole is a non-toxicants be used for the antimicrobial activity [27]. In addition to 1,8-cineole, the other compounds such as α-terpineol, β-pinene, α-pinene, D-nerolidol, and limonene are also contributing in the antimicrobial activity. Seremna essential oil showed the potential bactericidal action against the used bacteria such as *K. pneumonia* and *S. aureus* with 5 mg/ml MBC, while, MBC of Sawney oil reached to 10 mg/ml and Varlangy reached to more than 10 mg/ml and these oils were less sensitive against both Gram-negative (*K. pneumonia*) and Gram-positive strains (*S. aureus*) strains. The present study showed that essential oil of *A. subulatum* was highly active against Gram-positive bacteria and among all selected and among all selected cultivars, Seremna oil was shown comparatively better antibacterial effects than Sawney and Varlangy may be due to the high percentage of the principal compound (1,8-cineole). Seremna oil showed potentially highest fungicide activity against selected fungus (*C. albicans* and *A. niger*) with MICF of 2.5 mg/ml and 5 mg/ml for *C. albicans* and *A. niger,* respectively, while MICF of Sawney oil was 5 mg/ml and 10 mg/ml for *C. albicans* and *A. niger,* respectively. The essential oil of Varlangy was found to be less sensitive against *A. niger* and its MICF value reached to 10 mg/ml. In the previous studies, investigators have reported that Gram-positive bacterial are more sensitive to volatile oils when compared to the Gram negative [25,28]. The plants contain 1,8-cineole as a major content showed good antimicrobial activities against gram-positive bacterial and fungal strains [29]. The antibacterial activity of *A. subulatum* essential oil might be due to the components such as 1,8-cineole, limonene, D-nerolidol, β-pinene, α-pinene, and α-terpineol [30,31].

CONCLUSION

GC–MS study of essential oil of fruits of three different *A. subulatum* cultivars reported 1,8-cineole as the major constituents in all the cultivars. Of the six tested microorganisms, the essential oil of Seremna was found to be active against *K. pneumoniae*, *E. coli*, *C. albicans*, and *A. niger* at concentrations ranging from 1. 25 to 2.5 mg/ml. The highest antimicrobial activity of Seremna oil may be due to the presence of high content of 1,8-cineole.

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AUTHORS' CONTRIBUTIONS

Alam A designed the study project, performed experiments, wrote whole manuscript, and involved in throughout manuscript editing and finalization. Majumdar RS guide and approved the designed project helped in throughout project work. Alam P contributed in manuscript editing and finalization according to journal.

CONFLICTS OF INTEREST

Authors declared no conflicts of interest.

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