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RESEARCH ARTICLE

Chemical and Antibacterial Activity Evaluation of *Alpinia calcarata* and *Alpinia zerumbet* Grown in Foothills Agroclimatic Conditions of Northern India

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Abstract:

Background:

The member of the genus *Alpinia* (family: Zingiberaceae) is used in traditional medicine for various formulations for the food, spices, medicines, and perfume.

Objective:

The present study was carried out to analyse the compositional variability in leaf and rhizome essential oils of *Alpinia calcarata* Roscoe and *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. and to evaluate their antimicrobial activity against eight pathogenic bacteria strains.

Methods:

The essential oils were extracted by hydrodistillation and analysed by GC and GC-MS and the antibacterial activity was evaluated by filter paper disc diffusion and micro dilution broth assay.

Results:

A total of 31 compounds identified, forming 89.26-94.32% composition of *A. calcarata* and *A. zerumbet*. The leaf and rhizome oil of *A. calcarata* and *A. zerumbet* were mainly characterized by 1,8-cineole (15.61-43.63%), β-pinene (5.02-23.52%), terpinen-4-ol (1.00-20.87%), camphor (1.94-11.60%), and (*Z*)-β-ocimene (0.16-11.86%). endo-Fenchyl acetate (13.12-24.39%) was identified as marker constituents of rhizome essential oil of both *A. calcarata* and *A. zerumbet.* The antibacterial assay showed that leaf oil of *A. calcarata* has good activity against *S. mutans,* whereas its rhizome oil possess good activity against *K. pneumoniae, E. coli*, *S. aureus*, and *S. epidermidis*. However, the rhizome oil of *A. zerumbet* showed activity against *S. mutans*, *B. subtilisS. aureus*-2940 and *S. epidermidis.*

Conclusion:

The essential oils of *A. calcarata* and *A. zerumbet* with aroma chemicals *viz*. 1,8-cineole, ocimenes, terpinen-4-ol, α-pinene, β-pinenes and fenchyl acetate and significant antibacterial activities could be used for perfumery and fragrance related formulation.

Keywords: *Alpinia calcarata*, *Alpinia zerumbet*, Essential oil, Antibacterial activity, Endo-fenchyl acetate, Secondary metabolites.

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1. INTRODUCTION

Plants synthesize numerous secondary metabolites *viz.* terpenoids, alkaloids, glycosides, anthocyanins, flavonoids, lignans, tannins, phenolics, caretenoids, steroids and saponins, for diverse applications. Different plants parts (leaves, stem, root, rhizome, flowers, and seeds) of medicinal and aromatic plants accumulate these secondary metabolites of medicinal value and the composition was controlled by genetic factors, geographic and climatic conditions, developmental stage of the plant, extraction methods, *etc* [1 - 5]. Zingiberaceae, the largest monocotyledonous family in India, includes 52 genera and 1400 species distributed in the Indo-Malaysian region of Asia. Among them, 22 genera and 178 species were reported in the north eastern and peninsular regions of India. Various members

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of this family are of economic importance for food-flavour, spice, medicine and source of essential oils and oleoresins of medicinal and export value [6 - 9]. The genus *Alpinia,* consist ca. 230 species worldwide with major distribution in China, India, East Indies, and Polynesia and are used as spices and food additives agent and in the traditional medicine for the treatment of dyspepsia, gastralgia, and sea-sickness and for abdominal colic pains and as digestive, spleen and liver tonic. These are accredited with various pharmacological activities such as antispasmodic, myorelaxant, anti-oxidant, anti-inflammatory, anti-emetic, antiulcer, anti-inflammatory, anti-parasitic, anti-allergic and spasmolytic properties for their use in various indigenous medicinal formulations [10 - 14]. Numerous studies on leaf, flower and rhizome essential oil of *A. calcarata* and *A. zerumbet* have been carried out on its uses and applications in many common and rare health problems. *Alpinia* spp. are characterized by a wide range of volatile compounds in numerous phytochemical studies and mainly found to be dominated by monoterpenoids such as 1,8-cineole, camphor, (*E*)-methyl cinnamate, terpinene-4-ol, and pinenes, ocimenes, and fenchyl acetate as the major constituent distributed in their essential oils [11 - 22]. The chemical composition of the rhizome essential oil of *A. calcarataand A. zerumbet* revealed the presence of oxygenated monoterpene endo-fenchyl acetate as the distinctive marker constituents along with other commonly distributed monoterpenoids [12]. The present experiment focuses on the chemical evaluation & biological activity of two *Alpinia* species *viz*. *A. calcarata* and *A. zerumbet* grown in tarai regions at CIMAP resource center Pantnagar, Uttarakhand, India.

2. MATERIALS AND METHODS

2.1. Plant Material and Extraction of the Essential Oil

Fresh leaves and rhizomes were collected from the crop raised at the experimental field of CSIR-Central Institute of Medicinal and Aromatic Plants, Pantnagar, Uttarakhand. The experimental site is located between coordinates latitude 29.02°N, longitude 79.31°E and an altitude of 237 m above mean sea level The maximum temperature ranges between 35 and 45°C, and minimum between 2 and 5°C. The soil of the experimental site was sandy-loam in texture, with neutral pH and the climate of the region is sub-tropical and humid. The samples were hydrodistilled in a Clevenger-type apparatus for 3 hours. The amount of essential oil was directly measured from the extraction burette and content (%) was calculated as volume (mL) of essential oil per 100 g of fresh plant material. The hydro distilled oil was collected in vials and dehydrated over anhydrous Na_2SO_4 and stored in a cool dark place for further analysis.

2.2. Analysis of the Essential Oil

The essential oils collected were analysed by GC and GC-MS. For the quantitative analysis of the essential oil of leaves and rhizomes of *A. calcarata* and *A. zerumbet*, Gas Chromatography-Flame Ionization Detection (GC-FID) was performed on a Nucon GC-5765, equipped with DB-5 capillary column (30m × 0.25mm i.d. and 0.25 µm film thickness). The oven column temperature ranged from 60°C-230°C, programmed at 3°C/minute, using H_2 as carrier gas, split ratio 1:40 and injection volume 0.02 µL. The detector and injector temperatures were 230°C and 220°C, respectively. The relative amounts of the individual components were calculated based on the relative % peak areas (FID response) in the chromatogram without using a correction factor. GC-MS was performed for the identification of the essential oil constituents.GC-MS analysis of the essential oil samples was carried out on a Clarus 680 GC interfaced with a Clarus SQ 8C mass spectrometer (PerkinElmer) fitted with an Elite-5 MS fused silica capillary column (30m×0.25mm i.d.and 0.25µm film thickness). The oven temperature program ranged was from 60°C to 240°C, at 3°C/minute, and to 270°C at 5°C/minute; 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/minute; ionization energy 70 ev; mass scan ranges 40-450 amu. The essential oil constituents were identified on the basis of retention index, (RI, determined with reference to homologues series of n-alkanes, C_8-C_{30}), co-injection with standard compounds, MS library search (NIST and WILEY), and by comparing with the MS literature data [23].

2.3. Antibacterial Activity Assay

Antibacterial activity of the essential oil was determined by filter paper disc diffusion assay [24]. Inoculums of the test bacteria [Gram-positive: *Staphylococcus aureus* (MTCC 96), *Staphylococcus aureus* (MTCC 2940), *Streptococcus mutans* (MTCC 890), *Staphylococcus epidermidis* (MTCC 435), *Bacillus subtilis* (MTCC 121), and Gram negative: *Klebsiella pneumoniae* (MTCC 109), *Escherichia coli* (MTCC 723), *Pseudomonas aeruginosa* (MTCC 741), *Salmonella typhimurium* (MTCC 98) was prepared equivalent to McFarland Standard 0.5. Uniform bacterial lawns were made using 100 µL inoculums on a nutrient agar plate. Filter paper (Whatman) discs (5.0 mm) soaked with test essential oils were placed overseeded plates. The plates were incubated at 37°C for 24 h. The activity was measured in terms of zone of inhibition (ZI,mm). The net zone of inhibition was determined by subtracting the disc diameter (*i.e*., 5.0 mm) from the total zone of inhibition shown by the test disc in terms of the clear zone around the disc. Norfloxacin was employed as a positive control, while DMSO served as a negative control. The tests were performed in triplicate. The bacterial strains were obtained from the Microbial Type Culture Collection Centre (MTCC), Institute of Microbial Technology (IMT) Chandigarh, India. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by a Micro dilution broth assay using 96 'U' bottom micro-titer plates as per CLSI guidelines [25]. Samples were serially diluted two folds (in the range of 1000-1.95 µg/mL) in Mueller Hinton Broth (MHB). The broth was inoculated with 10.0 µL of diluted 24 h grown culture of test organisms with a titre equivalent to 0.5 McFarland standards. The inoculated plates were then incubated at 37ºC for 16-24 h and the growth was recorded spectrophotometrically at 600 nm using Spectramax 190-microplate reader (Molecular Devices, CA, and USA). The MIC value was determined from the turbidimetric data as the lowest concentration showing growth inhibition equal to or greater than 80% as compared to control. Bactericidal

endpoints were obtained by spread plating known volume (100 µL) from each well on solid media and the endpoint for complete inhibition was defined as the Minimum Bactericidal Concentration (MBC) of test samples in the original tube, which failed to yield discernible growth when sub-cultured. Experimental observations were performed in triplicate to rule out any error during the procedure.

3. RESULTS AND DISCUSSION

The essential oil composition of leaves and rhizomes of *A. calcarata* and *A. zerumbet*, cultivated in Tarai regions of Pantnagar, Uttarakhand, India, were analyzed and compared using capillary GC and GC-MS. The identified constituents with their relative percentage composition are given in Table **1**. The essential oil analysis results in the identification of 31 compounds forming 89.26-94.32% of total identified from the leaves and rhizome of the *A. calcarata* and *A. zerumbet*. The leaf and rhizome oil of *A. calcarata* were mainly characterized by oxygenated monoterpenes (51.49 and 70.94% resp.) represented by1, 8-cineole (36.07 and 43.63% resp.),camphor (11.60 and 4.31% resp.), terpinen-4-ol (1.64 and 1.00% resp.), and monoterpene hydrocarbons (39.60 and 15.4% resp.) with β-pinene (23.52 and 5.02% resp.), α-pinene (7.73 and 3.42% resp.) and camphene (6.49 and 4.97% resp.) as major constituents. In addition to these, other compounds found in significant content in leaf and rhizome oil of *A. calcarata* were viridiflorol (1.92 and 3.36% resp.) and (*E*)-methyl cinnamate (1.03 and 1.54% resp.). Moreover, theleaf and rhizome essential oil of *A. zerumbet* oil were also characterized mainly by oxygenated monoterpenes (55.25 and 58.46% resp.) with 1,8-cineole (26.51 and 15.61% resp.), terpinen-4-ol (20.87 and 6.42% resp.), camphor (1.94 and 3.21% resp.) as major constituents along with monoterpene hydrocarbons *viz*., β-pinene (14.81 and 8.83% resp.), (Z)-β-ocimene (11.86 and 2.84% resp.), *α*-pinene (5.96 and 3.22% resp.). Other compounds found in significant amount were linalool (2.26 and 1.65% resp.), (*E*)-β-ocimene (2.19 and 1.17% resp.), and α-terpineol (2.08 and 1.06% resp.), myrcene (0.49 and 3.49% resp.), and bornyl acetate (0.32 and 5.61% resp.). The results showed that presence of endo-fenchyl acetate (13.12 and 24.39% resp.) and exo-fenchyl acetate (0.10 and 0.16% resp.) as marker constituents of rhizome essential oil of both *A. calcarata* and *A. zerumbet,* whereas these compounds were absent in the leaf oil of *A. calcarata* and *A. zerumbet.* The present results on composition of the leaf and rhizome essential oils of *A. calcarata* and *A. zerumbet* were very close to the earlier reports, showing presence of oxygenated monoterpenoids (1,8-cineole, terpinen-4-ol, endo-fenchyl acetate) and monoterpenoid hydrocarbons (α-pinene, β-pinenes, ocimenes) as characteristics constituents [11, 12]. In one study, the samples collected from southern India of *A. calcarata* showed two different compositions, in one, endo-fenchyl acetate was the major constituent of rhizome essential oil, while in the second geraniol was reported as a major constituent with no endo-fenchyl acetate [13, 14, 18].The presence of monoterpenoids 1,8-cineole, terpinen-4-ol, α-pinene, β-pinene, ocimenes and fenchyl acetate are responsible for their sweet smell and may be utilized for perfumery and fragrance purposes.

Table 1. Comparative leaf and rhizome essential oil composition of two *Alpinia* spp.

S.No.	Compounds	RI[a]	RI[b]	*Alpinia calcarata*		*Alpinia zerumbet*	
				LO	RO	LO	RO
1	α-Thujene	932	931	0.15	0.1	t	0.77
2	α-Pinene	941	939	7.73	3.42	5.96	3.22
3	Camphene	954	953	6.49	4.97	t	5.38
4	β-Pinene	982	980	23.52	5.02	14.81	8.83
5	Myrcene	994	991	0.92	0.95	0.49	3.49
6	α-Phellandrene	1009	1005	t	0.17	t	1.17
7	α-Terpinene	1019	1018	t	-	t	t
8	p-Cymene	1029	1026	t	0.1	t	1.48
9	Limonene	1034	1031	0.24	0.14	t	0.48
10	1,8-Cineole	1038	1033	36.07	43.63	26.51	15.61
11	(*Z*)-β-Ocimene	1042	1040	0.28	0.16	11.86	2.84
12	(*E*)-β-Ocimene	1054	1050	0.27	0.37	2.19	1.17
13	γ-Terpinene	1065	1062	t	t	t	t
14	*cis*-Sabinene hydrate	1069	1068	t	t	1.27	t
15	Linalool	1101	1098	t	0.33	2.26	1.65
16	Camphor	1145	1143	11.6	4.31	1.94	3.21
17	Borneol	1167	1165	0.45	1.78	-	0.13
18	Terpinen-4-ol	1180	1177	1.64	1	20.87	6.42
19	α-Terpineol	1192	1189	t	4.61	2.08	1.06
20	endo-fenchyl acetate	1220	1220	-	13.12	-	24.39
21	*exo*-Fenchyl acetate	1232	1234	-	0.1	t	0.16
22	Bornyl acetate	1285	1285	0.7	0.52	0.32	5.61
23	(*E*)-Methyl cinnamate	1381	1379	1.03	1.54	t	0.22

(Table 1) contd.....

S.No.	Compounds	RI[a]	RI[b]	*Alpinia calcarata*		*Alpinia zerumbet*	
24	β-Caryophyllene	1418	1418	0.23	0.1	1.51	0.1
25	(*Z*)-β-Farnesene	1445	1443	0.1	0.27	t	0.17
26	α-Humulene	1457	1454	0.2	0.13	t	t
27	(*E*)-β-Farnesene	1459	1458	0.11	0.17	t	t
28	Germacrene D	1481	1480	0.16	0.15	0.1	0.18
29	Caryophyllene oxide	1584	1581	0.13	t	1.35	0.19
30	Viridiflorol	1592	1590	1.92	3.36	t	0.28
31	Bulnesol	1672	1666	0.38	1.58	0.31	1.05
32	Monoterpene hydrocarbon	-	-	39.6	15.4	35.31	28.83
33	Oxygenated monoterpene	-	-	51.49	70.94	55.25	58.46
34	Sesquiterpene hydrocarbon	-	-	0.8	0.82	1.61	0.45
35	Oxygenated Sesquiterpene	-	-	2.43	4.94	1.66	1.52
36	**Total identified**	-	-	**94.32**	**92.1**	**93.83**	**89.26**
37	**Essential oil yield (v/w fresh weight basis %)**	-	-	**0.3**	**0.5**	**0.2**	**0.4**

RIa: Retention Index determined with reference to homologous series of n-alkane (C_8–C_{24}) on TG-5 capillary column; RIb: Retention index literature [23]; t = trace (<0.1%); (-): Absent.

Table 2. Antibacterial potential of the essential oils of leaves and rhizome of *Alpinia* spp.

Strains	*Alpinia calcarata* Leaf Oil		*Alpinia calcarata* Rhizome Oil		*Alpinia zerumbet* leaf oil		*Alpinia zerumbet* Rhizome Oil	
	ZOI (mm)	MICs (µg/mL)	ZOI (mm)	MICs (µg/mL)	ZOI (mm)	MICs (µg/mL)	ZOI (mm)	MICs (µg/mL)
Staphylococcus aureus (MTCC-96)	7	500	10	250	6	500	9	500
Staphylococcus aureus (MTCC-2940)	5	1000	8	250	5	1000	12	250
Bacillus subtilis (MTCC-121)	6	1000	7	250	7	1000	14	250
Staphylococcus epidermidis (MTCC- 435)	5	1000	11	125	10	500	11	500
Streptococcus mutans(MTCC-890)	14	500	11	250	5	1000	14	250
Klebsiellapneumoniae(MTCC-109)	6	1000	14	125	na	500	8	500
Escherichia coli (MTCC-723)	6	1000	12	250	na	1000	6	1000
Salmonella typhimurium (MTCC 98)	7	500	na	500	7	500	5	1000

The antibacterial potential of the leaf and rhizome essential oil *of A. calcarata* and *A. zerumbet* was tested for antibacterial potential activity against eight bacterial strains *viz. S. aureus*-96, *S. aureus*-2940, *B. subtilis*, *S. epidermidis*, *S. mutans*, *K. pneumoniae*, *E. coli*and *S. typhimurium*. The leaf and rhizome essential oils of *A. calcarata* and *A. zerumbet* showed varying degree of activities against tested pathogens (Table **2**). The leaf oil of *A. calcarata* shows maximum antibacterial potential activity against *S. mutans* (ZOI: 14 mm, MIC: 500 µg/mL) followed by on *S. aureus*-96 (ZOI: 7 mm, MIC: 500 µg/mL), *S. typhimurium* (ZOI: 7 mm, MIC: 500 µg/mL), *B. subtilis* (ZOI: 6 mm, MIC: 1000 µg/mL), and *S. aureus*-2940 (ZOI: 5 mm, MIC: 1000 µg/mL). However, the rhizome oil of *A. calcarata* shows maximum antibacterial activity against the bacteria *K. pneumoniae* (ZOI: 14 mm, MIC: 125 µg/mL) and significant antibacterial activity against *E. coli* (ZOI: 12 mm, MIC: 250 µg/mL), *S. aureus*-96 (ZOI: 10 mm, MIC: 250 µg/mL), *S. epidermidis* (ZOI: 11mm, MIC: 125 µg/mL), and *S. aureus*-2940 (ZOI: 8 mm, MIC: 250 µg/mL). Moreover, the leaf oil of *A. zerumbet* shows maximumantibacterial potential activity against *S. epidermidis* (ZOI: 10 mm, MIC: 500 µg/mL) followed by*B. subtilis* (ZOI: 7 mm, MIC: 1000 µg/mL), *S. typhimurium* (ZOI: 7 mm, MIC: 500 µg/mL), *S. aureus*-96 (ZOI: 6 mm, MIC: 500 µg/mL), *S. aureus*-2940 (ZOI: 5 mm, MIC: 1000 µg/mL), and*S. mutans* (ZOI: 5 mm, MIC: 1000 µg/mL). However, the rhizome oil of *A. zerumbet* showed maximum antibacterial activity against *S. mutans* (ZOI: 14 mm, MIC: 250 µg/mL) and *B. subtilis* (ZOI: 14 mm, MIC: 250 µg/mL), followed by *S. aureus*-2940 (ZOI: 12 mm MIC: 250 µg/mL),*S. epidermidis* (ZOI: 11 mm, MIC: 500 µg/mL), *S. aureus*-96 (ZOI: 9 mm, MIC: 500 µg/mL), *K. pneumoniae* (ZOI: 8 mm, MIC: 500 µg/Ml), *E. coli* (ZOI: 6 mm, MIC: 1000 µg/mL) and *S. typhimurium* (ZOI:5, MIC: 1000 µg/mL). Results showed that the leaf oil of *A. calcarata* shows very good antibacterial potential activity against*S. mutans* whereas its rhizome oil of significant antibacterial activity against the bacteria *Klebsiella pneumoniae, E. coli*, *S. aureus*-96, and *S. epidermidis*. The rhizome essential oil of A. zerumbet showed potent antibacterial activity against *S. mutans*, *B. subtilis S. aureus*-2940 and *S. epidermidis*; however its leaf oil showed maximumactivity against *S. epidermidis*.

CONCLUSION

The genus *Alpinia* is presently considered as an augmented source of medication in traditional medicine for various health problems. Numerous studies have been carried out on the utility of different parts of the *Alpinia* species *viz*. leaves, roots, rhizomes and flowers. Of all the species, *A. calcarata* and *A. zerumbet* have been widely studied. The study revealed that the

essential oils from leaves and rhizomes of *A. calcarata* and *A. zerumbet* possess aroma chemicals *viz*. 1,8-cineole, ocimenes, terpinen-4-ol, α-pinene, β-pinene and fenchyl acetate for utilization in perfumery and fragrance related formulation. In the southern part of India, these species are in cultivation and used as raw material for phytochemicals and traditional medicine. The antibacterial activity assay also showed that their essential oils possess significant activities against some of the tested pathogenic bacterial strains. The results showed that the future prospect needs to be carried out in these plants for utilizing different plant parts as a prominent source of essential oils and aroma chemicals for product formulation.

ETHICAL STATEMENT

The followed protocols on Plants for this study were carried out in accordance with the CLSI guidelines.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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