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Evaluation of the Chemical Variability and Phytochemical Analysis among Indian Germplasm of *Acorus calamus* Linn Using GC- MS and FTIR

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Authors' contributions

This work carried out in collaboration between both authors. Author RK designed the study. Author AK did the experiment planning, design, analysis and drafted manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

The present study was carried out to characterize the bioactive constituents present in essential oil using Fourier transform infrared spectroscopy (FTIR) spectrophotometer and Gas Chromatography –Mass Spectroscopy (GCMS) and evaluation of chemical variability in twenty accessions of *Acorus calamus*, collected from India, and data collected were subjected to principle component analysis. The essential oils were scanned and FTIR analysis indicated the presence of groups such as - C – H stretching, Aldehydic – C – H stretching, Benzene C = C stretching, C – O stretching, Tetra substituted benzene. The GC-MS analysis results revealed the presence of four different compounds namely β-asarone, α-asarone, shyobunone and epicedrol. The main compound observed in all the accessions was β-asarone. Principal component analysis revealed that the first three principal components accounted for 47.80% of the total variation among the accessions. The first component had high positive loadings from epicedrol (0.925), shyobunone (0.659) and α-asarone (0.591) and high negative loadings from β-asarone (-0.953). Most of the variation was

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accounted for by the first principal component (20.97%). The dendogram generated by the UPGMA cluster analysis grouped the twenty accessions into two distinct clusters. The study indicated that there is moderate phytochemical variation within the population evaluated. Chemical compositions were moderately influenced by factors such as plant origins, soil and geographical region, medicinal parts, and storage environment. Hence, this study proposes a base of using *A. calamus* as herbal alternative for the synthesis of various therapeutic agents.

Keywords: Acorus calamus; β-asarone; GC-MS; essential oil composition; principal component analysis (PCA); chemical variability.

1. INTRODUCTION

Acorus calamus (Sweet flag) is a perennial herb, indigenous to Himalayan region through to central Asia and India. It is one of the endangered medicinal plants that grows wild without much consideration [1]. A large number of medicinal plants and their purified constituents have shown valuable therapeutic potentials [2]. Plant natural products have been widely studied and their medicinal properties, such as antibacterial, antifungal, insecticidal and herbicides have been well recognized. Different parts of *A. calamus* such as the rhizome, roots and leaves have been used traditionally from ancient times for the treatment of various ailments and treatment of various disease such as of cough, ,fever, bronchitis, inflammation, depression, tumors, hemorrhoids, skin diseases, numbness, general debility [3]. Some phytochemicals produced by plants are known to possess numerous medicinal activities and used for the development of new drugs [4]. *A. calamus* (Family*: Araceae*) is also known by many other names such as Calamus root, sweet cane, sweet flag, sweet sedge, rat root, flag root, and sweet calomel. Among Indians, the plant is called Bach and Sanskrit it is known as Vacha.

A. calamus prefers swampy or marshy habitats. It is usually grown in abundance in the marshy tracts of Himachal Pradesh, Kashmir, Naga hills, and Manipur and is regularly cultivated in Karnataka and Maharashtra [5]. *A. calamus* exhibits polyploidy in three karyotypes; the diploid karyotype (2n=2x=24) which grows in Asia (Siberia) and in parts of North America; the triploid karyotype (2n=3x=36) is common in Kashmir, India and Central Europe and the tetraploid karyotype (2n=4x=48) grows mainly in East Asia, India, and Japan [6]. In India, the plant is found growing wild as well as cultivated at about altitude of 2200 m in the Himalayas [7]. The diploid plants are known to lack β-asarone, the triploid plants contain a small portion of β-asarone (5%) in their oil and the tetraploid plant oil is high in β- asarone (90-96%) [8]. Studies of phytochemical composition of *Acorus* spp. have revealed that α and β-asarones are the major bioactive components. Because of varying β-asarone content, precise identification of *A. calamus* chemotypes is a prerequisite for commercial application. Various bioactivities like anthelmintic, antibacterial, and antifungal properties are attributed to the presence of β-asarone [9]. Significant works on essential oil of *A. calamus* has been carried out by different workers [10] and it was establish that the chemical compositions are affected by environmental conditions such as geographical origin [11,12]. In addition, β-asarone in the essential oil from *A. calamus* may vary with the locality. This study was carried out to investigate biochemical compositions and β- asarone content in essential oils from accessions of *A. calamus* from the different location in India.

2. MATERIALS AND METHODS

2.1 Plant Materials

Different accessions of *A. calamus* rhizomes collected were at the same stage from the month of May 2013 from different states of India and were maintained in Greenhouse at Maliba campus, Uka Tarsadia University (UTU), Bardoli, Gujarat, India located at 21º.04 N73°.03 E. Bardoli has an average elevation of 22 meters (72 feet) and at an altitude of 34.18 meters above mean sea level (MSL). The voucher specimens were deposited at the herbarium of the same department. The list of accessions used for the study and the location of collections are shown in Table 1.

Accessions were domesticated at the UTU research farm. Rhizomes were transplanted in a field of the UTU farm, Bardoli, in the first week of June 2013. - The plant-to-plant spacing was 30 cm, while row-to-row spacing was 30 cm, replicated thrice in a randomized block design. The field had sandy loam (7.10%) soil containing

N, P, and K at 80, 50 and 150 kg/ ha, respectively, organic carbon at 25% and pH at 7.6. Phosphorus and potash, at rates of 40 kg/ha were applied to the field plot at the time of sowing. Nitrogen at 100 kg/ ha was applied. Leaves emerged next days after transplantation; the plants were irrigated thrice a week in non rainy season and weeded when required. Hydrodistillation was carried out 280 days after the plantation of accessions. Plant morphological characteristics such as leaf length, leaf width, total number of leaves per plant and total numbers of tillers per plant, and rhizomes length, rhizomes width, inter-nodal length, fresh and dry weight of rhizome were also studied.

2.2 Preparation of Extract

The fresh rhizomes were collected and cleaned, which includes scales attached to rhizomes and adventitious roots. The cleansed rhizomes were dried under a shade and ground to coarse particles using a pestle and mortar. The extraction of essential oils was carried out by accurately weighing 100 g of powdered *A. calamus* into 100 ml distilled water and the essential oil was obtained by hydrodistillation for 3 hours in a Clevenger-type apparatus, [13]. The essential oil obtained ranging from 3.5% - 5.5% was separated from the aqueous solution (hydrolate), and oil was stored at 4℃ for further analysis [14].

2.3 FTIR Analysis

To record the FTIR analysis, the essential oil of *A. calamus* plant was dissolved in Petroleum ether [15] to obtain a concentration of 5×10^{-4} µg/ml oils. FTIR analysis was performed using the Perkin Elmer Spectrophotometer (USA). The peak values of the FTIR were recorded. The analysis was performed in triplicate for every accession to the spectrum confirmation.

2.4 GC-MS Analysis

For GC-MS analysis dried rhizome sample were pulverized and 1.0 g was placed into 20 ml of petroleum ether in a 20 ml volumetric flask [16]. The sample mixture was allowed to stand for 24 hrs at room temperature in a volumetric flask. The mixture was extracted for 10-12 min in an ultrasonic bath at room temperature after extraction. 3 g of sodium sulfate was added to the extract to remove trace water, and it was filtered through a 0.2 µm membrane filter. The filtrate (solution) was injected into the GC/ MS system. Analyses by GC were performed using HP 6890 gas chromatograph equipped with a FID detector and a HP-5 fused silica column (30 m X 0.25 mm X 0.25 mm film thickness). Nitrogen was used as a carrier gas. The injector and detector temperature were maintained at 210℃ and 230℃ respectively. The column oven temperature was programmed from 60℃ to 220℃ with an increase rate of 5℃/min. The oven temperature was programmed to increase from 85ºC (for 5 min) at a rate of 10℃/min, this was increased from 90℃ to 140℃ at a rate 10℃/min, and then increased from 140℃ to 280℃ at a rate 20℃/min. Analysis was carried out on a Perkin Elmer mass spectrometer (Model: Claurus 500),attached to a Perkin Elmer Claurus 500 gas chromatograph having 60 m x 0.32 mm x 0.25 mm film thickness column of resting make (Rt-5). Helium was used as the carrier gas (flow rate 1 ml/min).The mass range was scanned from 20-620 Daltons. The oven temperature program range was 60℃ to 220℃ with an increase rate of 3℃/min. Other conditions were the same as described under GC. The identity of the constituents of the extract were retention indices, by comparing their 70eV mass spectra with those reported in literature, and by computer matching with NIST & WILEY libraries, as well as, co-injection with standard compounds also injected.

2.5 Reagents and Apparatus

All reagents used in this study were of analytical grade. Petroleum ether was purchased from Sigma-Aldrich, USA. Standard components, such as α-asarone, β-asarone, epicedrol and shyobunone were purchased from Aldrich (Sigma-Aldrich, USA).

2.6 Data Analysis

All the analyses were carried out in triplicate and phytochemical data were subjected to Factor analysis and principal component analysis using SPSS Software Version 16. The significant levels of the statistical analysis were described at the probability of 0.05 and 0.01.

3. RESULTS

Twenty accessions of various *A. calamus* were collected from different locations (Table 1) in North Eastern, Southern, Western and Eastern parts of India, representing four bio geographical zones (Western Himalaya, Central India, North East India and Eastern Ghats) were analysed. The differences in plant dimensions of *A. calamus* were statistically significant at

(P < 0.01) among the different accessions. The plant heights varied from 41.67 cm in ACPIT to 68.3 cm in ACRRL. Total number of leaves ranged from 53 to 73, with maximum and minimum values recorded in ACRRL, ACRAI, respectively among the samples. The results revealed that essential oil yield varies among the sample based on the location of collection of the accessions. Their morphological characters are presented in Fig. 1. The essential oils isolated in this experiment were light yellow tending to dark yellow at a yield of 3.5% - 5.5% (v/w) among samples based on dry weight. The essential oils of samples were found to vary among the different accessions (Prob. > 0.01). The highest essential oils yield belonged to the samples from ACDAN (5.5%) and the lowest was obtained from ACAK samples (3.5%).

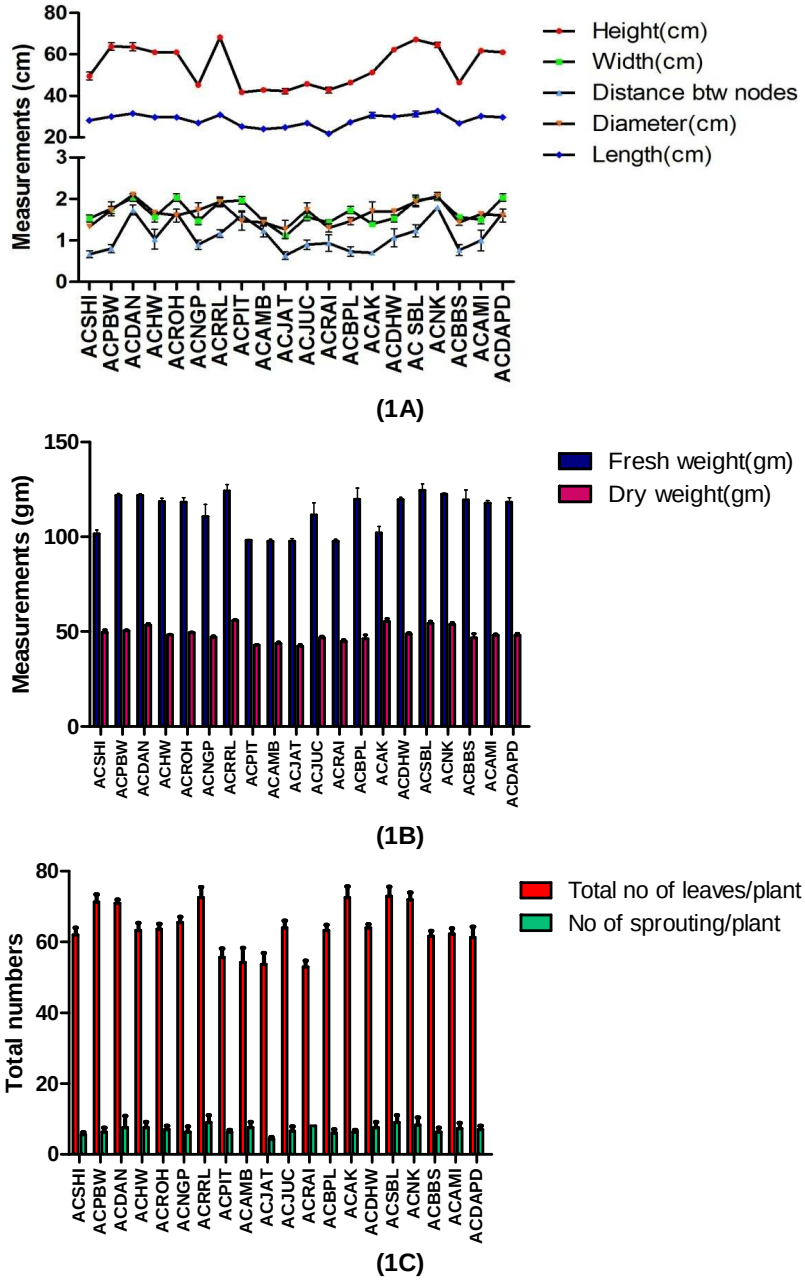


Fig. 1. Morphological characters (1A, 1B and 1C) of *Acorus calamus* accessions collected from different geographical locations in India

Table 1. *Acorus calamus* accessions collected from different geographical locations in India

Code	Voucher number	Place of collection		Locality geographical coordinates		Elevation
		City	State	Latitude (°′N)	Longitude (°′E)	Meters
ACSHI	CGBIBT/ACAK031	Shillong	Meghalaya	25°34' 43. 5828'' N	91°53' 35.7144'' E	1496
ACPBW	CGBIBT/ACAK032	Pantanagar	Uttarakhand	29°1' 15.7368'' N	79°29' 23.0568'' E	243.8
ACDAN	CGBIBT/ACAK033	Dehradun	Uttarakhand	30°18' 5 9.3820'' N	78°1' 55.8912'' E	315
ACHW	CGBIBT/ACAK034	Hardwar	Uttarakhand	29°56' 44.4876'' N	78°9' 51.2928'' E	314
ACROH	CGBIBT/ACAK035	Rohru (Shimla)	Himachal Pradesh	31°12' 7.2000'' N	77°45' 6.8400'' E	1525
ACNGP	CGBIBT/ACAK036	Nagpur	Maharashtra	21°8' 44.8 800'' N	79°5' 17.3580'' E	310.5
ACRRL	CGBIBT/ACAK037	RRL Jammu	Jammu	32°43' 35.767 2'' N	74°51' 25.2936'' E	300
ACPIT	CGBIBT/ACAK038	Pithoragarh	Uttarakhand	29°34 ' 58.2960'' N	80°13' 5.4768'' E	4626
ACAMB	CGBIBT/ACAK039	Ambach	Gujarat	20°23' 20.6196 '' N	72°58' 20.1864'' E	14
ACJAT	CGBIBT/ACAK040	Jatolivillvagham	Uttarakhand	29°17' 40.4196'' N	80°4' 43.0896'' E	1725
ACJUC	CGBIBT/ACAK041	Jalandhar	Punjab	31°19' 33.65 40'' N	75°34' 34.2588'' E	228
ACRAI	CGBIBT/ACAK042	Raipur	Chhattisgarh	21°15' 4. 9824'' N	81°37' 46.7076'' E	298
ACBPL	CGBIBT/ACAK043	Bhopal	Madhya Pradesh	23°15' 35.7588'' N	77°24' 45.4140'' E	427
ACAK	CGBIBT/ACAK044	Akola	Maharashtra	20°41' 60.00 00'' N	77°0' 0.0000'' E	283
ACDHW	CGBIBT/ACAK045	Dharwad	Karnataka	15°27' 32.1 264'' N	75°0' 28.1088'' E	670.75
AC SBL	CGBIBT/ACAK046	Bangalore	Karnataka	12°58' 1 7.7564'' N	77°35' 40.4268'' E	914.4
ACNK	CGBIBT/ACAK047	Nasik	Maharashtra	19°59' 50.83 08'' N	73°47' 23.2872'' E	560
ACBBS	CGBIBT/ACAK048	Bhubaneswar	Odessa	20°17' 45. 8124'' N	85°49' 28.3440'' E	45
ACAMI	CGBIBT/ACAK049	Amravati	Maharashtra	20°56' 1 4.7264'' N	77°46' 46.3836'' E	500
ACDAPD	CGBIBT/ACAK050	Dapoli	Maharashtra	17°45' 32 .0004'' N	73°11' 32.0004'' N	10

The FTIR spectrum of *A. calamus* samples were used to identify the functional group of the active components based on the peak value in the region of infrared radiation. The results of FTIR peak values and functional groups are represented in Table 2. The fingerprint region of the accessions were carried out using essential oil for spectral analysis which revealed essential oil approximately identical with the fingerprint region in a spectrum profile of standard β-asarone. FTIR Spectra of all the accessions showed peaks in the range from 587.72- 2996.41 cm^{-1} and analysis indicated the presence of groups such as - C – H stretching, Aldehydic – C – H stretching, Benzene C = C stretching, C – O stretching and Tetra substituted benzene. However, it is noteworthy to mention here that results obtained from FTIR alone are not significant to prove the existence of compound-classes, especially when it comes to mixture of many compounds.

3.1 GC-MS Analysis

The GCMS fingerprint profile (Fig. 2.) of *A. calamus* showed the relative concentrations (ppm) of various compounds getting eluted as a function of retention time. The heights of the peak indicate total ion chromatograms and the relative concentration of individual component were calculated based on GC peak areas without using correction factors. The results revealed the presence of four different compounds namely β-asarone with retention indices (RI) - 14.878, α-asarone (RI-15.953), shyobunone (RI-13.377), and epicedrol (RI-13.662). The differences in concentrations (ppm) of *A. calamus* were statistically significant at ($P < 0.01$) among the different accessions. All analyzed rhizome samples possessed β-asarone as major constituents, the concentration four essential oil components in different samples *A. calamus* in Fig. 2. It was observed that two accessions ACRRL and ACDHW contained higher amount (10619ppm) of β-asarone while ACPIT and ACAK had the lowest concentration of β-asarone (3956 and 2185ppm respectively). In accession ACROH where all four components were present shown in GC-MS chromatogram (Fig. 5). All the accessions from Uttarakhand showed β-asarone ranges from 5412 – 8494ppm and other minor components like shyobunone, epicedrol were also present in traces in other samples. The mass spectrum of the β-asarone compound with retention time 1488 produced 18 major peaks where molecular ion peak has been at found (m/z) at $[208]^+$. The quantity of β-asarone is higher as compared to previous reports of β-asarone variability in the composition of *A. calamus by* [16].

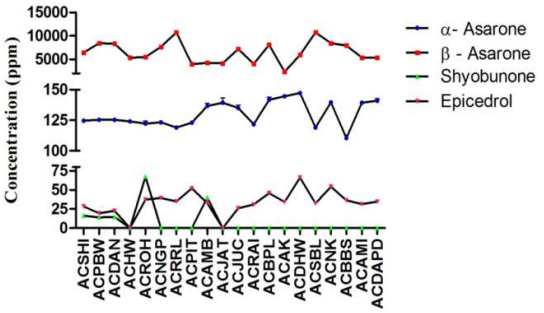


Fig. 2. Concentrations (ppm) of phytochemicals in *Acorus calamus* accessions detected by GCMS

3.2 Chemical Variability

The principal component analysis (PCA) is shown in Table 3. The first three principal components accounted for 47.80% of the total variation among the accessions. Most of the variation was explained by the first principal component (20.97%), followed by the second (14.26%) and the third (12.56%). The first component had high positive loadings from epicedrol (0.925), shyobunone (0.659) and α-asarone (0.591) and high negative loadings from β-asarone (-0.953). The second component had high positive loadings from Distance between nodes (0.495), Rhizome's fresh weight (0.609), Rhizome's dry weight (0.587), Leaves width (0.579). The third component had high positive loadings from Number of tillers per plant (0.602) and high negative loadings from Leaves height (-0.418) and Rhizome's length (-0.662). None of the variables were redundant.

From the dendogram constructed using minimum similarity distance and the UPGMA clustering method (Fig. 4), the first main cluster (A) consisted of five accessions separated into subgroups cluster 1 and 2. Cluster 1 was further subdivided into sub-subgroups ‘a’ and ‘b’, with sub-subgroup I a containing three accessions (ACPIT, ACAMB and ACBPL). ACBPL, ACAMB and ACPIT were 85% similar and closely related to ACAK, with a genetic distance of 2.0. Sub-subgroup I b contained ACAK accessions that were 99% similar and closely related. These accessions had the higher percentage of β-asarone. Cluster 2 contained only one accession ACRAI with (31ppm) of epicedrol.

Table 2. FTIR peak values and functional groups of different accessions of *Acorus calamus*

Peak values	Functional groups
2996.41- 2936.24	- C – H stretching
2835.27	Aldehydic – C – H stretching
1606.48 - 1457.45	Benzene C = C stretching
1264.99 - 1028.90	C – O stretching
930.10 - 761.29	Tetra substituted benzene

The second major cluster (B) was subdivided into two subgroups clusters III and IV. Cluster III contains three accessions ACJAT, ACDHW and ACSBL. Cluster IV sub divided into two subgroups (sub cluster 1 and sub cluster II). Sub cluster 1 divided in two subgroups 'c' which contains ACJAT, ACDHW and subgroups 'd' contains ACSBL respectively, and subcluster II contains two groups Subgroup 'g' contains two subgroup g1 and g 2 having accessions ACBBS, ACAMI, ACNK, ACRRL, ACJUC, ACHW containing higher β- asarone contained and ACDAPD. The subgroup 'h' consists of only one accession ACDAN, which was distantly separated from all others clusters at a genetic distance of 6.

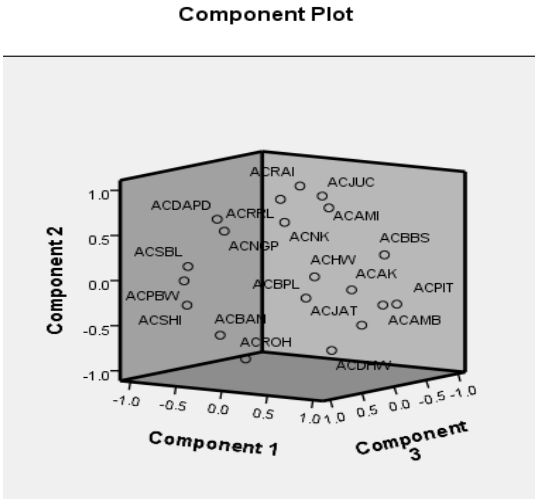


Fig. 3. The ordination of twenty accessions of *A. calamus* on principal component (PC) axes 1, 2 and 3 from cluster analysis using all phytochemical and morphological parameters

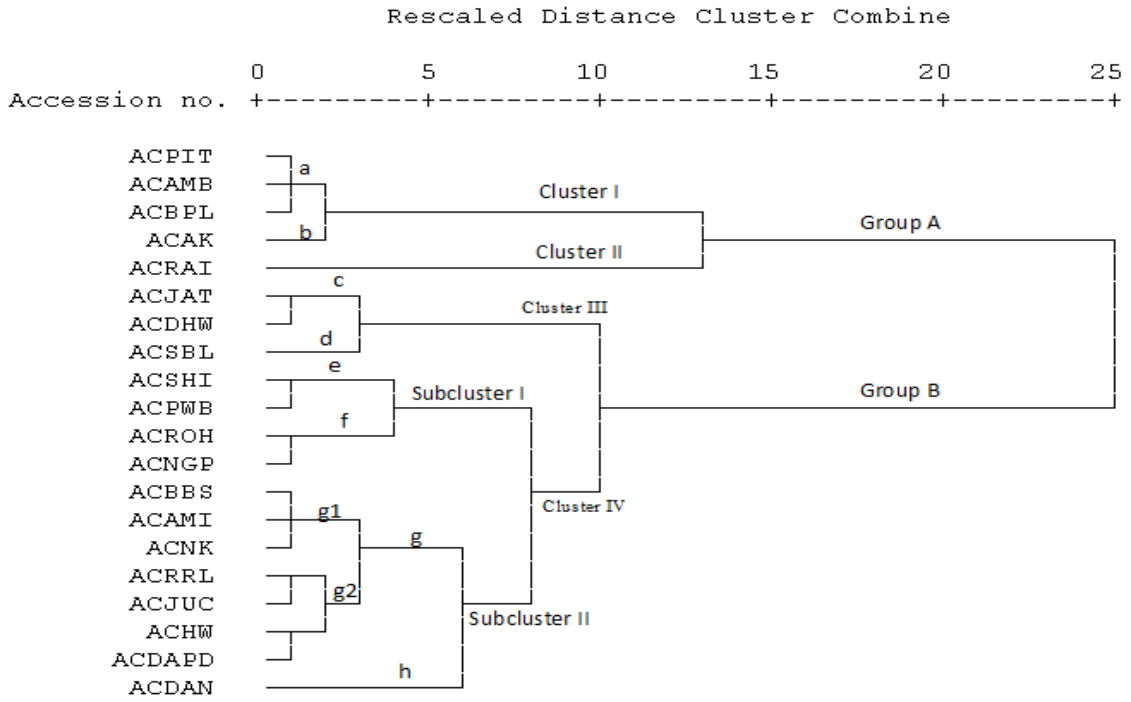
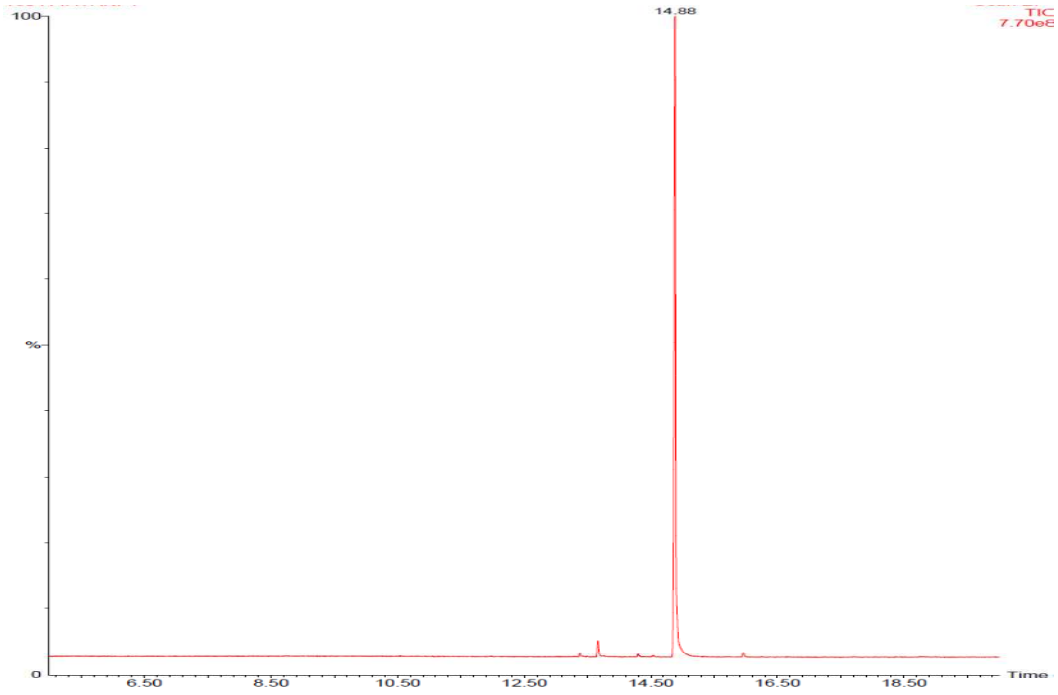


Fig. 4. Dendogram generated based on the UPGMA clustering method, depicting the chemical relationships between twenty accessions of *A. calamus* based on all phytochemical parameters

Table 3. Eigen vectors and percentage variation by the first five principal components between twenty accessions of *A. calamus* using phytochemical data and morphological data studied

Characters	Component				
	PC 1	PC 2	PC 3	PC 4	PC 5
Leaves height	-.120	.454	**-.418**	-.191	.336
Leaves width	.193	**.579**	.092	.296	-.558
Total number of leaves per plant	-.256	-.244	.379	.339	-.021
Number of tillers per plant	-.450	.222	**.602**	-.229	.172
Distance between nodes	.122	**.495**	.116	.635	-.296
Rhizome's diameter	.023	.156	-.425	.353	.294
Rhizome's length	.370	.168	**-.662**	-.348	-.110
Rhizome's fresh weight	.133	**.609**	.375	-.500	.105
Rhizome's dry weight	-.187	**.587**	.482	-.292	.335
Alpha-Asarone	**.591**	-.542	.115	-.107	.307
Beta-Asarone	**-.953**	-.002	-.059	-.071	-.048
Shyobunone	**.659**	.530	-.171	.019	-.138
Epicedrol	**.925**	.133	.084	-.041	-.005
Eigenvalue	4.61	3.13	2.76	2.25	2.12
Individual percentage	20.97	14.26	12.56	10.25	9.66
Cumulative percentage	20.97	35.23	47.80	58.06	67.73

PC: Principal Component



#	Name	RT	Area	Height	BL	Conc	Units	Area/Conc	m/z	Area %
1		13.382	113,603.6	2,938,066	MM	0.00		0.00	TIC	.51
2		13.667	485,794.6	12,268,457	bb	0.00		0.00	TIC	2.19
3		14.878	21,448,192.0	500,388,608	MM	0.00		0.00	TIC	96.52
4		15.963	173,875.0	4,163,829	MM	0.00		0.00	TIC	.78

Fig. 5. GC-MS chromatogram of accession ACROH of *A. calamus*

4. DISCUSSION

Herbal drug development consists of various steps, correct identification of raw materials, chemical quality, pharmacognostics, standardization and safety. Generally, it is important to develop the quality assurance for each botanical drug, therefore; it is the combination of the two methods which has enabled us to understand both the general variability and the specific differences between chemical components.

The essential oils were obtained by conventional hydrodistillation of the rhizomes of *A. calamus*. Our results showed that plant accession of the *A. calamus* represent an accessible source of β-asarone and α – asarone. No systematic trend in essential oil yield and morphological characteristics were observed based on the geographical distribution of the collection accessions. The findings are also in cognisance with the study of [17]. Several studies have reported presence of anti inflammatory effect [18,19]. Also antimicrobial effect of β-asarone have been shown previously [7]. Analysis of the rhizome essential oil of *A. calamus* accessions under FTIR technique showed the presence of phenolic compound. This has also been isolated and characterized [20].

The results of GC-MS analysis identified number of compounds from the GC fractions of the petroleum ether extract of *A. calamus*. The column and temperature program used in our experiment successfully identified a number of compounds. These observations may be due to the nature of biologically active components and the stronger extraction capacity of petroleum ether which could have stimulated the production of a number of active constituents responsible for various medicinal activities [18]. The observation showed the presence of four different compounds namely β-asarone, α-asarone, shyobunone, and epicedrol. All analyzed rhizome accessions contains β-asarone as major constituents. Similar results were also observed while studying with different accessions [16,21].

The component analysis which included morphological and phytochemical studies indicated the presence of 5 major groups among the collected 20 accessions. Phytochemical component in the present study with those from literature showed that geographical distance is one of main reasons for distances in the cluster analysis. Moderate variations observed in *A. calamus* content can be attributed to many factors, such as genetic factors and evolution, environmental conditions, geographic variations, physiological factors and conditions [17]

In addition, dendogram the accessions collected from different states of North India like Uttarakhand (ACDAN), Himachal Pradesh (ACROH), and Karnataka (ACDHW) were grouped in the same cluster (Cluster five). It was observed that these accessions from different states grouped within the same cluster. On the other hand, ACSBL and ACDHW were not grouped into a single cluster, but both accessions were collected from the same place (natural uncultivated lands) of Karnataka similar result were found in different plant [22] Since our-studied natural habitats environmentally were diverse, therefore the selected environmental factors were used to found of probable reasons for *A. calamus* heterogeneity. Results in this part showed that the major components displayed significant correlations with environmental factors. In other words, obtained results illustrated that the environmental factors represent the dominant involved factors similar with other studies [16].

5. CONCLUSION

The chemical profiles of 20 accessions of *A. calamus* collected from Indian germplasm demonstrated similar chromatographic patterns between different geographic origins. The study signified that there is moderate phytochemical variation within the accessions evaluated. Chemical compositions were independent by various factors including origins, soil and cultivation region, medicinal parts, and storage environment. The Principle component analysis (PCA) of the twenty accessions used in the present study and the dendogram of relationships further suggest that there is a moderate level of chemical variability. Phytochemical analysis has valuable potential for domestication and breeding programs as an preliminary source for commercial propagation and cultivation. Therefore this could serve as base for traditional medicines in the cure of disease and further investigation needs to elute novel active compounds.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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