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Original Article

Asarone and Shyobunone Content in Indian *Acorus calamus* Fresh Plants and Dried Rhizome Samples

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ABSTRACT

Background: *Acorus calamus,* traditionally used as a neuroprotective, anti-inflammatory, immunomodulatory and analgesic agent, has α-asarone, β-asarone, and shyobunone as its major phytoconstituents. β-asarone is associated with dose-dependent cytotoxicity, mutagenicity and carcinogenicity. Ascertaining content of these phytoconstituents becomes essential for developing safe and efficacious medicines from this plant. **Objectives:** The study aimed to estimate the content of α-asarone, β-asarone and shyobunone in *Acorus calamus* fresh plants and dried rhizome samples collected from different regions of India. **Materials and Methods:** Dried rhizomes and fresh rhizomes and leaves of *Acorus calamus* were collected from various locations in India. Novel, simple, rapid and accurate reverse phase high performance liquid chromatography methods for estimation of α-asarone, β-asarone and shyobunone were developed and validated. 0.05% Ortho Phosphoric Acid (OPA) in water: acetonitrile (55:45) for simultaneous estimation of α-asarone and β-asarone and 0.05% Ortho Phosphoric Acid (OPA) in water, acetonitrile and methanol (15:40:45) was the mobile phase used for analysis of shyobunone. **Results:** The content of α-asarone, β-asarone, and shyobunone in various calamus samples ranged from 0.004-0.10% w/w, 2.30-4.40% w/w and 0.018-0.10%w/w respectively. All rhizome samples were found to contain higher amounts of these phytoconstituents, followed by leaves. Within the dried rhizomes, these phytoconstituents were found to be localized in greater amounts within the wood, as compared to the bark**. Conclusion:** The content of β-asarone in the calamus samples is found to be lower than what has been reported earlier in the Indian varieties, possibly due to spontaneous mutations with alteration in 'ploidy' status.

keywords: *Acorus calamus*, Shyobunone, α-asarone, β-asarone.

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INTRODUCTION

Acorus calamus Linn., commonly referred to as "vacha" and often known as "sweet flag" or "calamus root," is an important and versatile herb with various documented uses in traditional Indian medicinal systems and modern medicine as well.[1-4] It exhibits potent pharmacological effects including anti-inflammatory, antioxidant, antibacterial, anticonvulsant, immunomodulatory, neuroprotective, anti-diabetic, antidiarrheal, antiulcer, and analgesic activities. Vacha is described in the ancient classical Ayurvedic text, Bhavprakash Nighantu, for its use as an appetizer, digestive aid, and nootropic and in the treatment of hysteria, respiratory disorders, throat problems, and for pain relief, including headache, migraine and other neurological conditions including Parkinson's disease.[5]

In India, these botanical specimens are mostly found in the Himalayan and sub-Himalayan zones, as well as in the Western Ghats and North-Eastern India. *Acorus calamus* leaves and rhizomes have been utilized in traditional as well as modern medical treatments.[6] Approximately 145 phytoconstituents including alkaloids, triterpene(camphor), phenylpropanoids (methyleugenol, β-asarone and α-asarone, estragole), triterpenoid saponins, isoflavones(genistin), sterols, monoterpenes (camphene, α-linalool, eucalyptol), and sesquiterpenoids (shyobunone, isoshyobunone) have been extracted from *A. calamus* leaves and rhizomes. The principal compounds amongst these are α-asarone, β-asarone and shyobunone. Figure 1 represents the chemical structure of α-asarone, β-asarone and shyobunone.[6,7]

A. calamus is one of the few herbs known to exhibit differing chromosome numbers, referred to as "ploidy status." The chemical

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composition of calamus varies based on its ploidy status, as illustrated in Table 1.[8,9]

Song B. *et al.* (2015) have reported ploidy-dependent content of β-asarone in the *A. calamus* samples collected from the Indian Himalayan regions, with the diploid and hexaploid cytotypes exhibiting lower content of β-asarone, as compared to the triploid and tetraploid ones. There was also a significant correlation between β-asarone content and its distribution along geographic limits, with environmental factors also exerting a positive influence.[9] R. Kumar *et al.* (2016) corroborated these findings and have reported lower content of β-asarone in diploid *A. calamus* herb samples accessed from western Indian Himalayan regions, as compared to the triploid samples.[10]

β-asarone is reported to exhibit dose-dependent cytotoxicity, genotoxicity and carcinogenetic toxicity, raising potential safety concerns. However, no human studies have been reported till date that confirm these findings.[11-14] Due to the potential carcinogenic effects of β-asarone, the United States Food and Drug Administration has prohibited the use of *A. calamus.*[15] Considering its genotoxicity, the European Medicines Agency prefers *A. calamus* species free from β-asarone and aims to decrease β-asarone content in herbal medicinal products.[16]

In India however, *Acorus calamus* has been extensively utilized in numerous traditional Ayurvedic formulations and has been considered safe for consumption when taken under medical advice.

Table 2 below provides a non-exhaustive list of the Ayurvedic preparations along with details about the content of *A. calamus* rhizome.[17-21]

In recent times, there has been considerable interest in exploring development of *A. calamus*, particularly in the management and treatment of neurological diseases, where the herb shows considerable promise. So long as the content of β-asarone remains low, this herb can be developed as safe and efficacious medicines.

In view of this, the present study aims to evaluate samples of both fresh collection of *A. calamus* rhizome plants as well as dried rhizome samples collected from different locations of India for estimating the content of the major phytoconstituents, α-asarone, β-asarone and shyobunone. Most studies have focused on the study of dried rhizome only and so fresh plants were also taken up for this study. Novel, simple, rapid, accurate and robust reverse phase high performance liquid chromatography methods were developed and validated for these analyses.

MATERIALS AND METHODS

Collection of *A. calamus*

Dried and fresh rhizomes of *A. calamus* were collected from various geographical locations in India. As shown in Table 3, codes were assigned to the samples, indicating the geographical region from where they were collected.

Chemicals and reagents

Pure marker compounds of β-asarone (Lot No. T23E004, vials of 25 mg, and 600 mg) from M/S Natural Remedies, Bangalore, α-asarone (Lot no CFN93217, 20 mg) and Shyobunone (Lot no. CFN98079-20 mg), from M/S Chemfaces, China along with their analytical data were obtained and used. These markers had a peak purity of above 90 %.

HPLC grade solvents, such as acetonitrile and methanol were procured from Thermo Fisher Scientific Pvt. Ltd., Mumbai, India and orthophosphoric acid was procured from S.D. Fine-Chem Ltd., Mumbai, India.

Preparation of ethanolic and aqueous extract of fresh and dried samples of *A. calamus*

The dried rhizome samples of *A. calamus* were weighed and powdered using a mechanical blender. Dried rhizome samples were also cut and separated into bark and wood portions, which were then milled to achieve a fine powder. Powdered samples of whole rhizome, bark and wood were subjected to ethanolic and aqueous extraction using the Soxhlet extraction technique at 50-60°C for 8 hr and hot maceration extraction process for 4 hr at 60-70°C, respectively. The ethanolic extracts were evaporated to dryness using a Rotary evaporator (Rotavapor 210; Buchi, Switzerland), to get a semi-solid paste. Similarly, aqueous extracts were completely dried using an electric water bath (BTI-57, BioTechnics India).

Freshly collected leaves and rhizomes were thoroughly cleaned under running tap water to eliminate adherent material including soil and other matter. They were then finely ground to form a thick paste and extracted in ethanol by the Soxhlet extraction process at temperatures ranging from 50-60°C for 8 hr, followed by evaporation using a Rotary evaporator (Rotavapor 210; Buchi, Switzerland) to obtain semi solid consistency of the extracts. All extracts were stored in airtight vials at 2°C until they were subjected to HPLC analysis.

Instruments

The Liquid chromatographic separations were performed on Shimadzu HPLC system (model-SIL-20AC HT) equipped with a quaternary pump (model-LC 20AD), autosampler (SIL20ACHT), column oven (model-CTD-10AS VP), Diode array detector (model-SPD-M20A) and data acquisition was done using Lab Solution software version 6.12 SPI. Milli Q Water ultra purifier, Water bath (model-BTI 57, Bio technics India), Rotary evaporator (Rotavapor 210; Buchi, Switzerland) and Electronic Weighing Balance (WENSAR) were used for analysis.

Table 1: **Relationship between *A. calamus* cytotypes and the content of α-asarone, β-asarone, and Shyobunone.**

Cytotype	Location	%Yield of essential oil (% v/w)	Content of β-asarone in the oil (% w/v)	Content of α-asarone in the oil (% w/v)	Content of Shyobunone in the oil (% w/v)
Type I-Diploid (*2n*=24) *	Primarily in North America and to some extent in Siberia.	4.7-6.0	Free	Low levels or nil	13-45
Type II-Triploid (*2n*=36) *	Mainly in Europe and to some extent in Himalayan Region of India and Asia.	1.7-4.9	3-19	Not known	3.6-32
Type III-Tetraploidy (*2n*=48) *	Sub-tropical /tropical South and Southeast Asia, and in Temperate Far East Asia. Including the former USSR and Korea.	2.8-9.3	48-84 (and in some locations only 2-3) [USSR and Korea]	2.7-3.7	1.4-6.2
Type IV-Tetraploidy (*2n*=48) *	Some parts of India, Pakistan, and Philippines	2.8-9.3	4-8	2.7-3.7	Not reported.
Type V-Tetraploidy (*2n*=48) *	Japan	2.8-9.3	20-76	Not reported	Low levels. Not reported.
Type VI-Hexaploidy (*2n*=72) *	Kashmir	0.6-3.3	16-20	Not known	Not known

Note: Detailed chemical composition of these *A. calamus* cytotypes is not being given for brevity.

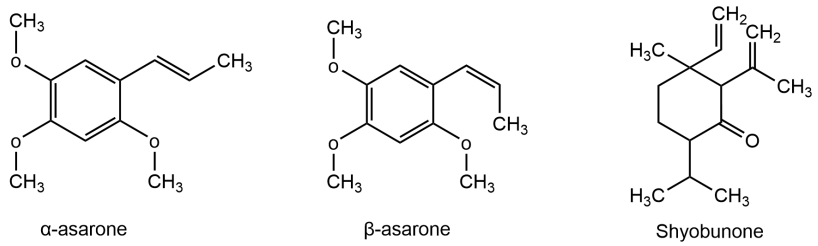

Figure 1: Chemical structures of α-asarone, β-asarone and shyobunone.

Preparation of standard

Standard stock solutions of α-asarone, β-asarone, and shyobunone were prepared separately in methanol (1000 µg/mL). The stock solutions were diluted with methanol to obtain solutions of concentration ranges 0.1-5 µg/mL, 5.75-138 µg/mL and 0.6-10µg/mL for α-asarone, β-asarone, and shyobunone respectively. Before injection, these solutions were filtered through 0.45 µm Whatman Nylon syringe filter and each standard solution was individually injected in six replicates (*n*=6).

Preparation of sample extract solution

Preparation of sample extract stock solution

200 mg of both ethanolic and aqueous extracts were separately diluted with upto 10 mL of methanol to obtain extract stock solutions of concentration 20,000 µg/mL.

Sample preparation for quantification of β-asarone in ethanolic extract

0.25 mL of the sample ethanolic extract stock solution (20,000 µg/mL) was diluted with upto 10 mL of methanol, to give solution of 500 µg/mL concentration. This diluted solution was used for the quantification of β-asarone in the ethanolic extract.

Sample preparation for quantification of α-asarone and shyobunone in ethanolic extract

0.50 mL of the sample ethanolic extract stock solution (20,000 µg/mL) was diluted with upto 10 mL of methanol to give solution of 1000 µg/mL concentration. This diluted solution was used for the quantification of α-asarone and shyobunone in the ethanolic extract.

Sample preparation for quantification of α-asarone, β-asarone, and shyobunone in aqueous extract

0.50 mL of sample aqueous extract stock solution (20,000 µg/mL) was diluted with upto 10 mL of methanol to give solution of 1000 µg/mL concentration. This diluted solution was used for

Table 2: Traditional Ayurvedic preparations containing *A. calamus*.

Name of the Preparation	References	Dosage of the preparation	Quantity of Vacha as per text reference in the preparation	Quantity of Vacha/ dose
Aravindasava	Bhaishjya ratnavali, Balarogadhikara 185	3 to 12 mL	48 g in 35 kg	4 mg to 16 mg
Brahma Rasayan	Astanga Hrudaya Uttar Asthana, Adhyaya 39; 15-19½	12 g	192 g in 110 kg	21 mg
Rasnadi Kvatha Curna (Maha)	Sarangdhara samhita, Madhyama khanda, Adhyaya 2; 89-91½	48 g	1 part of 75	63.84 mg
Maha Yogaraja Guggulu	Sarangdhara samhita, Madhyama Khanda, Adhyaya 7; 56-60	½ to 1 g	3 g in 696 g	2.15 mg to 4.3 mg
Dadhika Ghrta	Ashtangahrudaya, Chikitsa Sthana, Adhyaya 14, 13-19½	12 g	2.4 g in 16 kg	1.8 mg
Dhanvantara Ghrta	Ashtangahrudaya, Chikitsa Sthana, Adhyaya 12, 19-12	48 g	11.63 g in 43.23 kg	13 mg
Nimbadi Curna	Bhaisajyaratnavali, Vataraktadhikara; 31-33	1 to 3 g	12 g of 432 g	27.7 mg to 83.3 mg
Sudarsana Curna	Bhaishjyaratnavali, Jvaradhikara 308-312½	1 to 2 g	1 part of 44	45.4 mg to 90.8 mg
Sanjivani Vati	Sarangdhara samhita, Madhyama Khanda, Adhyaya 7; 18-19	125 mg	1 part of 10	12.5 mg
Pradarantaka Lauha	Bhaishjyaratnavali, Striogadhikara 75-76½	500 mg	1 part of 30	16.65 mg
Kasturyadi (Vayu) Gutika	Sahasrayoga, Gutikaprakarana; 8	125 mg	1 part of 41	3.03 mg
Kankayana Gutika	Bhaishjyaratnavali, Gulmadhikara 56-58½	2 g	48 g in 816 g	118 mg
Khadiradi Gutika (Mukharoga)	Carakasamhita, Cikitsasthana, Adhyaya 26; 206-201½	2 g	12 g in 27.45 kg	0.87 mg

Table 3: Codes assigned to samples as per the geographical region of their collection. The names in parentheses indicate the location of markets from where the drug was procured

Sl. No.	Type of samples	Place/Location	Sample codes
1	Dried samples	Bangalore	BLR-1
2	Dried samples	Bangalore	BLR-2
3	Dried samples	Sagara (Bangalore)	BLR-SG
4	Dried samples	Tumkur (Bangalore)	BLR-TK
5	Dried samples	Tumkur (Delhi)	DEL-TK
6	Dried samples	Tumkur (Virudhunagar)	VDN-TK
7	Dried samples	Dombivli	DOM
8	Dried samples	Himachal Pradesh	HIM
9	Dried samples	New Delhi	DEL
10	Fresh samples	Tumkur, Karnataka	KAR-TK
11	Fresh samples	Tumkur	TK
12	Fresh samples	Munnar, Kerala	KER-MUN
13	Fresh samples	Vellanikara,Trichur, Kerala	KER-VEL-TRI
14	Fresh samples	Kollengodu, Palaghat, Kerala	KER-KOLL-PAL
15	Fresh samples	Neliampatty, Palaghat, Kerala	KER-NEL-PAL
16	Fresh samples	Palampur, Himachal	HIM-PAL

Table 4: Chromatographic conditions of developed RP-HPLC methods for shyobunone and simultaneous estimation of α-asarone and β-asarone in *A. calamus* extract samples.

Parameters	α-asarone and β-asarone	Shyobunone
LC system	Shimadzu HPLC system with quaternary pump (model-LC 20 AD), auto sampler (model-SIL-20AC HT), diode array detector (model-SPD-M20A), column oven (CTD-10AS VP)	
Software	Lab solution software version 6.12 SPI	
Column	Kromasil C18 Column (Dim: 4.6 × 150 mm)	
Mobile Phase	0.05% ortho phosphoric acid (OPA) in water: Acetonitrile (55:45)	0.05% ortho phosphoric acid (OPA) in water, acetonitrile and methanol (15:40:45)
Elution mode	Isocratic	
Column temperature	25°C	
Flow rate	1 mL/min	
Injection volume	10 µL	
Run time	20 min	10 min
Wavelength	254 nm	210 nm
Retention time	β-asarone-12 min 5 sec. α-asarone-14 min 4 sec.	6 min 5 sec.

the quantification of α-asarone, β-asarone and shyobunone in the aqueous extract.

Chromatographic conditions

Novel, simple, rapid, accurate and robust RP-HPLC method was developed for simultaneous estimation of α-asarone and β-asarone. Similarly, a different novel, simple, rapid, accurate and robust RP-HPLC method was developed for analysis of shyobunone. Details of the developed RP-HPLC methods for shyobunone and simultaneous estimation of α-asarone and β-asarone in *A. calamus* extract samples are shown in Table 4.

Method validation

The developed RP-HPLC methods were validated as per International Conference on Harmonization [ICH guidelines Q2 (R1)] guidelines.[22] For parameters including linearity, specificity, precision (inter-day, intra-day, and repeatability), limit of detection, limit of quantification, accuracy (recovery), and robustness.

Quantification of α-asarone, β-asarone, and shyobunone in *A. calamus* extract samples

The novel validated analytical methods were utilized for the simultaneous estimation of α-asarone and β-asarone and shyobunone in ethanolic and aqueous extracts of various samples of *Acorus calamus* collected from different geographical locations in India.

RESULTS

Ethanolic and aqueous extraction of fresh and dried samples of *A. calamus*

The % yield of ethanolic extract of the rhizome, wood and bark part of various plant samples were found to be in the range of 14.8-25.48% w/w, 11.33-27.8% w/w and 10.00-24.48% w/w, respectively. The % yield of aqueous extract of the rhizome, wood and bark part of various plant samples were found to be in the range of 21.9-28.6% w/w, 15.4-24.6% w/w and 10.4-26.6% w/w, respectively. The percent yield of the ethanolic extract from the fresh rhizome and leaves samples ranged from 5.4-36.06% w/w and 2.9-24.66% w/w, respectively.

RP-HPLC Method development and validation for simultaneous estimation of α-asarone and β-asarone and shyobunone

Comprehensive details regarding the calibration curve, linear range, Limit of Detection (LOD), and Limit of Quantification (LOQ) are provided in Table 5. Figures 2 and 3 illustrate the peak purity of the peaks corresponding to the extract chromatograms.

Quantification of α-asarone, β-asarone, and shyobunone in *A.calamus* extract samples

Tables 6-8 and Figures 4-12 illustrate the content of α-asarone, β-asarone and shyobunone found in the ethanolic and aqueous extracts of the various A. calamus samples. Of the alcoholic extracts, the content of α-asarone, β-asarone, and shyobunone in various dried rhizome samples ranged from 0.004-0.10% w/w, 2.30-4.40% w/w, and 0.018-0.10% w/w, respectively. The quantitative analysis of aqueous extracts from various dried rhizome samples identified β-asarone concentrations ranging from 0.06-0.39% w/w, α-asarone from 0.0021-0.0076% w/w, and shyobunone from 0.0006-0.0064% w/w.

As far as the fresh vacha rhizome samples are concerned, the content of α-asarone, β-asarone, and shyobunone was found to range from 0.0024-0.027% w/w, 0.59-4.00% w/w and 0.0074-0.081% w/w respectively. In fresh leaf samples, β-asarone ranged from 0.064-1.31% w/w, α-asarone from 0.0032-0.023% w/w, and shyobunone from 0.0024-0.013% w/w.

DISCUSSION

The RP-HPLC methods for simultaneous estimation of α-asarone and β-asarone and shyobunone were developed, with chromatographic conditions, as shown in Table 4. These methods were validated in accordance with ICH guidelines Q2 (R1). All validation parameters including linearity, specificity, precision (inter-day, intra-day, and repeatability), limit of detection, limit of quantification, accuracy (recovery), and robustness were found to be within acceptable limits. Specificity was evaluated by comparing the peak purity of α-asarone, β-asarone, and shyobunone from the *Acorus calamus* extract sample with standard solutions.

Calibration curves for α-asarone, β-asarone and shyobunone were constructed using standard solutions in the concentration ranges 0.1-5 µg/mL, 5.75-138 µg/mL and 0.6-10 µg/mL, respectively as shown in Table 5. The respective linear regression equations were applied for quantification of these phytoconstituents in the extract samples. Each extract sample was analyzed in triplicates (n=3) to determine the mean content of these phytoconstituents in the plant part powder.

Table 5: Regression data, LOD and LOQ for α-asarone, β-asarone and Shyobunone analyzed by RP-HPLC.

Compounds	Linearity range (µg/mL)	Regression Equation	R^2	LOD (µg/mL)	LOQ (µg/mL)
α-asarone	0.1-5	y=6000000x-1266.4	0.9999	0.05	0.1
β-asarone	5.75-138	y=4000000x+6220.4	0.9998	1.8	5.4
Shyobunone	0.6-10	y=915998x-33.989	0.9997	0.2	0.6

Abbreviations: y, peak area; x, concentration (µg/mL); R^2, coefficient of determination; LOD, limit of detection; LOQ, limit of quantification.

Table 6: Content of α-asarone, β-asarone and shyobunone in ethanolic extract of dried rhizome, wood and bark samples of *A. calamus*.

Sample code	Content of β-asarone in powdered sample (% w/w)			Content of α-asarone in powdered sample (% w/w)			Content of Shyobunone in powdered sample (% w/w)		
	R	W	B	R	W	B	R	W	B
BLR-SG	3.41	2.01	1.30	0.06	0.04	0.014	0.063	0.038	0.013
BLR-2	2.30	1.50	0.99	0.10	0.043	0.03	0.019	0.008	0.0044
DEL-TK	3.20	2.10	0.86	0.02	0.007	0.0043	0.10	0.046	0.030
BLR-1	4.40	3.24	1.10	0.01	0.01	0.003	0.042	0.029	0.0065
HIM	2.83	1.41	0.91	0.01	0.004	0.003	0.022	0.007	0.0047
BLR-TK	4.10	2.50	1.34	0.04	0.02	0.01	0.060	0.024	0.018
VDN-TK	3.10	1.83	1.10	0.012	0.007	0.006	0.10	0.055	0.047
DOM	3.50	1.71	1.70	0.04	0.02	0.01	0.036	0.014	0.012
DEL	3.72	2.40	1.20	0.004	0.003	0.002	0.018	0.008	0.0050

Abbreviations: R, Rhizome; W, Wood; B, Bark.

Table 7: Content of α-asarone, β-asarone and shyobunone in aqueous extract of dried rhizome, wood and bark samples of *A. calamus*.

Sample code	Content of β-asarone in powdered sample (% w/w)			Content of α-asarone in powdered sample (% w/w)			Content of Shyobunone in powdered sample (% w/w)		
	R	W	B	R	W	B	R	W	B
BLR-SG	0.15	0.063	0.044	0.0023	0.0014	0.0013	0.0014	0.00038	0.0003
BLR-2	0.06	0.039	0.011	0.0023	0.0015	0.0012	0.00064	0.00045	0.00013
DEL-TK	0.15	0.079	0.045	0.0025	0.0018	0.0014	0.0064	0.0026	0.0019
BLR-1	0.39	0.20	0.14	0.0076	0.0045	0.0036	0.0020	0.0011	0.00080
HIM	0.06	0.040	0.016	0.0022	0.0018	0.0010	0.00073	0.00042	0.00022
BLR-TK	0.28	0.11	0.052	0.0031	0.0021	0.0010	0.0021	0.00083	0.00034
VDN-TK	0.13	0.070	0.038	0.0021	0.0017	0.0012	0.0049	0.0024	0.0014
DOM	0.17	0.070	0.043	0.0023	0.0014	0.0011	0.0010	0.00029	0.00029
DEL	0.20	0.080	0.052	0.0026	0.0016	0.0012	0.0006	0.0003	0.00016

Abbreviations: R, Rhizome; W, Wood; B, Bark.

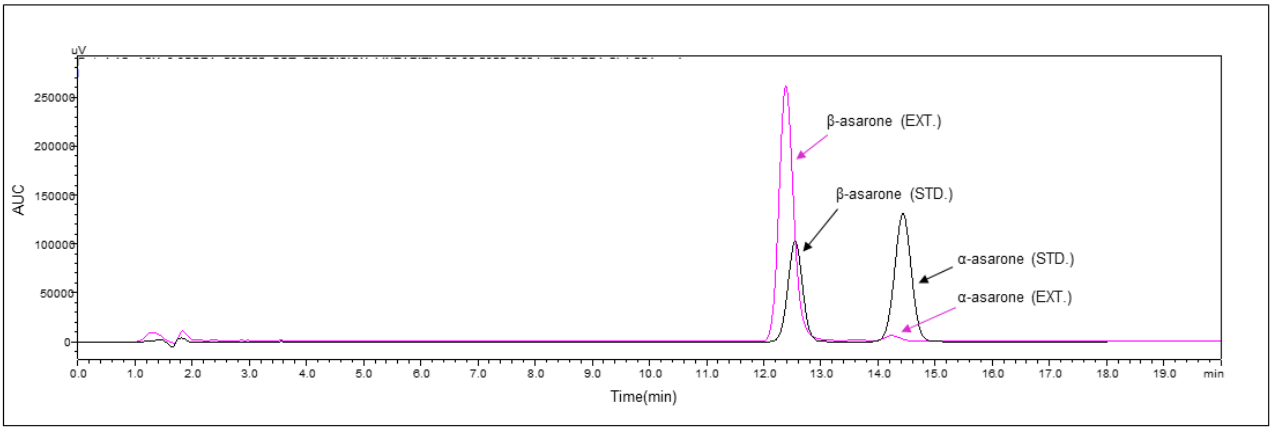

Figure 2: Overlaid chromatograms of 50 µg/mL standard solution of α-asarone and β-asarone and 1000 µg/mL solution of *Acorus calamus* ethanolic extract sample at 254 nm by using mobile phase 0.05% Ortho Phosphoric Acid (OPA) in water: acetonitrile (55:45). Abbreviations: STD: Standard marker compound; EXT: Extract sample.

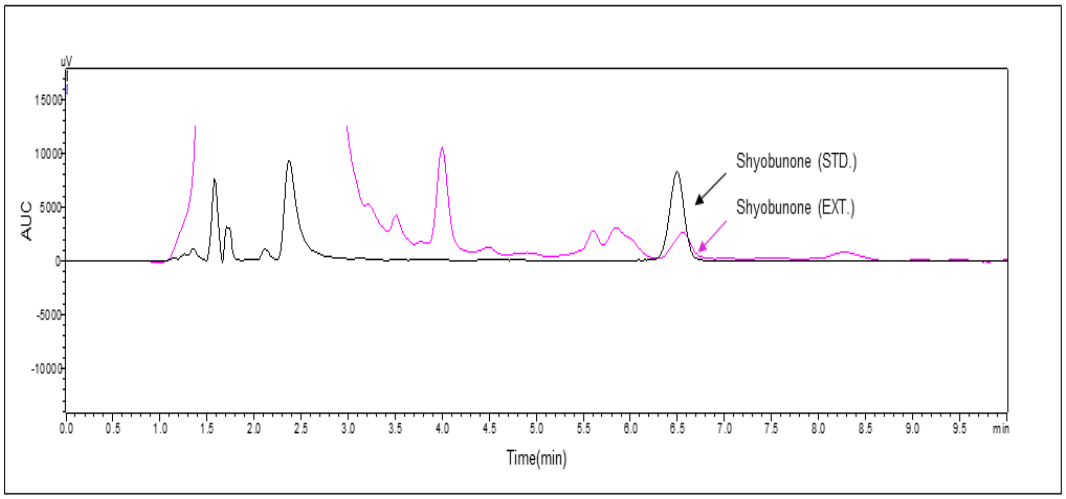

Figure 3: Overlaid chromatograms of 50 µg/mL standard solution of shyobunone and 1000µg/mL solution of *Acorus calamus* ethanolic extract sample at 210 nm by using mobile phase 0.05% Ortho Phosphoric Acid (OPA) in water, acetonitrile and methanol (15:40:45). Abbreviations: STD: Standard marker compound; EXT: Extract sample.

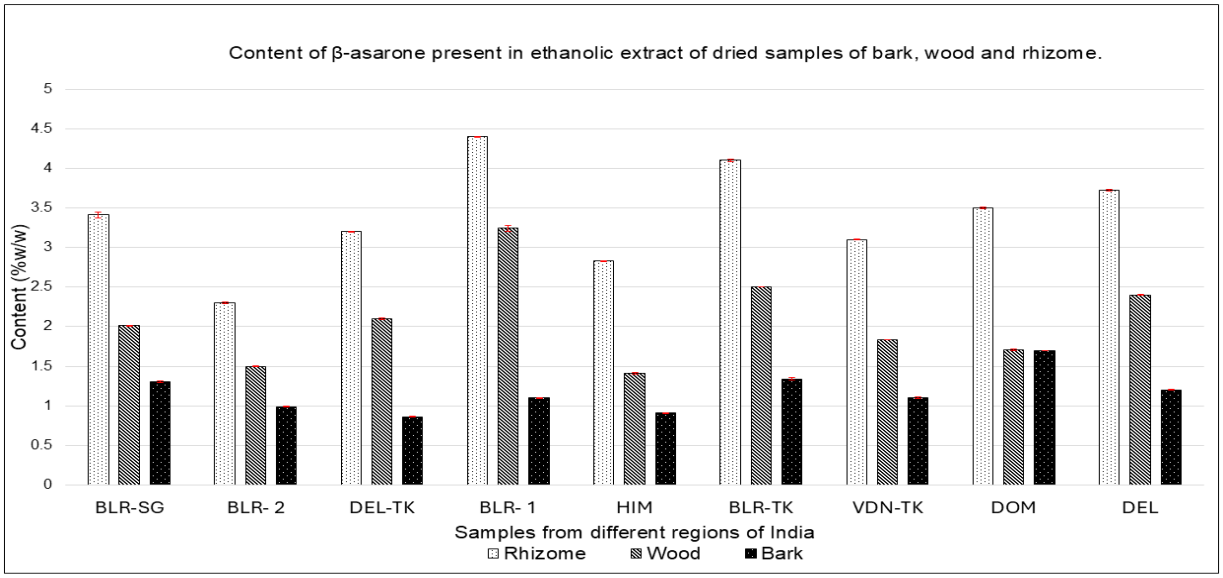

Figure 4: β-asarone content (% w/w) present in ethanolic extract of dried samples of bark, wood and rhizome.

Table 8: Content of α-asarone, β-asarone and shyobunone in ethanolic extract of fresh rhizome and leaf samples of *A. calamus*.

Sample code	Content of β-asarone in paste sample (% w/w)		Content of α-asarone in paste sample (% w/w)		Content of Shyobunone in paste sample (% w/w)	
	Rhizome	Leaves	Rhizome	Leaves	Rhizome	Leaves
KAR-TK	1.90	-	0.0033	-	0.081	-
HIM-PAL	4.00	-	0.027	-	0.067	-
TK	-	0.51	-	0.015	-	0.0099
KER-MUN	0.85	1.31	0.0067	0.023	0.0074	0.013
KER-VEL-TRI	0.59	0.29	0.0024	0.0052	0.013	0.0039
KER-KOLL-PAL	0.73	0.40	0.0071	0.0085	0.011	0.0074
KER-NEL-PAL	1.91	0.064	0.0049	0.0032	0.034	0.0024

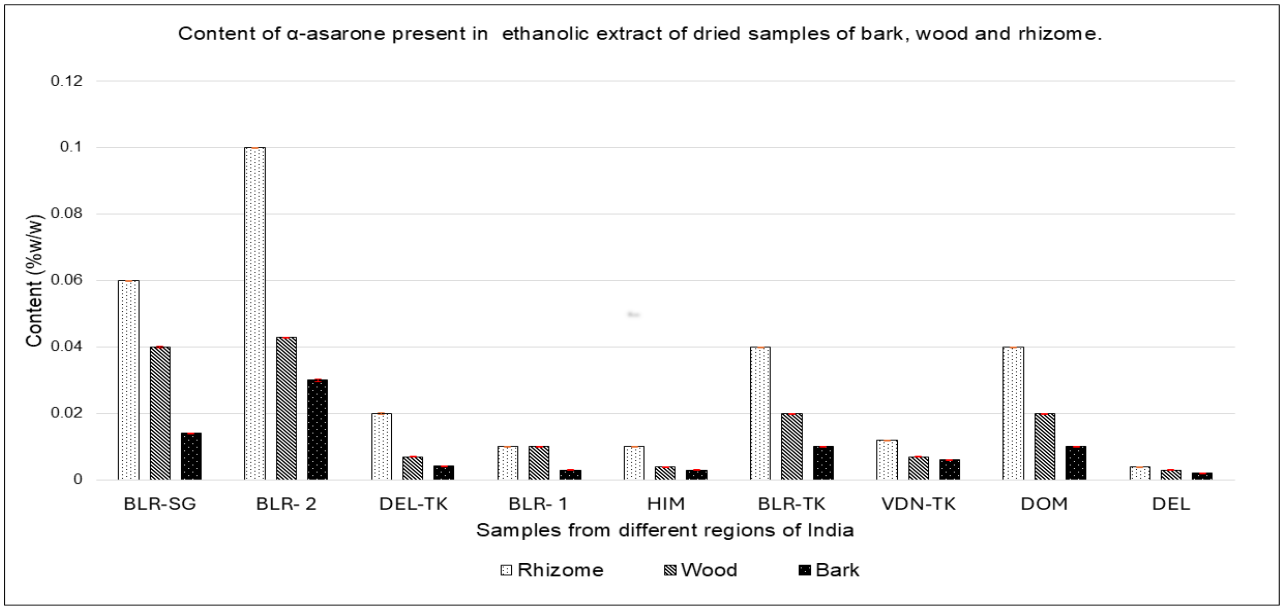

Figure 5: α-asarone content (% w/w) present in ethanolic extract of dried samples of bark, wood and rhizome.

For fresh parts of the plant that contain significant amounts of moisture, the total moisture content was estimated using 'Loss on Drying'.[23] The moisture content in the fresh leaves and rhizomes was accounted for, during estimation of the phytoconstituents' content in their respective plant samples.

Ethanolic extracts of all fresh and dried samples of *A. calamus* showed significantly higher content of α-asarone, β-asarone, and shyobunone compared to the corresponding aqueous extracts of the herb samples, obviously, due to the higher solubility of these phytoconstituents in ethanol.[24,25]

The plant rhizomes from all locations were found to contain higher amounts of all three phytoconstituents, followed by leaves; an exception to this was found to be the sample from Munnar Region of Kerala (KER-MUN), where the leaf part showed greater phytoconstituents' content as compared to the rhizome. It is possible that the asarone(s) may be formed in the leaves and then stored in rhizomes when the plant is fully grown. This is a matter for further study for ascertaining biosynthesis of these chemical compounds in calamus plant parts. Within the rhizome, these phytoconstituents were found to be localized in greater amounts within the wood, as compared to the bark.

The content of α-asarone, β-asarone, and shyobunone in the alcoholic extracts of various dried rhizome samples ranged from 0.004-0.10% w/w, 2.30-4.40% w/w, and 0.018-0.10% w/w, respectively. The dried rhizome samples from the Bangalore Region (BLR-1) showed higher β-asarone content at 4.40% w/w, whereas lower content of β-asarone was observed in dried rhizome samples from Bangalore (BLR-2) at 2.30% w/w. Higher α-asarone content at 0.10% w/w was seen in dried rhizome samples from Bangalore (BLR-2). Dried rhizome samples from Tumkur (Delhi) (DEL-TK) and Tumkur (Virudhunagar) (VDN-TK) reported higher shyobunone content at 0.10% w/w, whereas those from Delhi (DEL) exhibited lower content of α-asarone (0.004% w/w) and shyobunone (0.018% w/w). In fresh rhizome samples, the content of α-asarone, β-asarone, and shyobunone was found to range from 0.0024-0.027% w/w, 0.59-4.00% w/w,

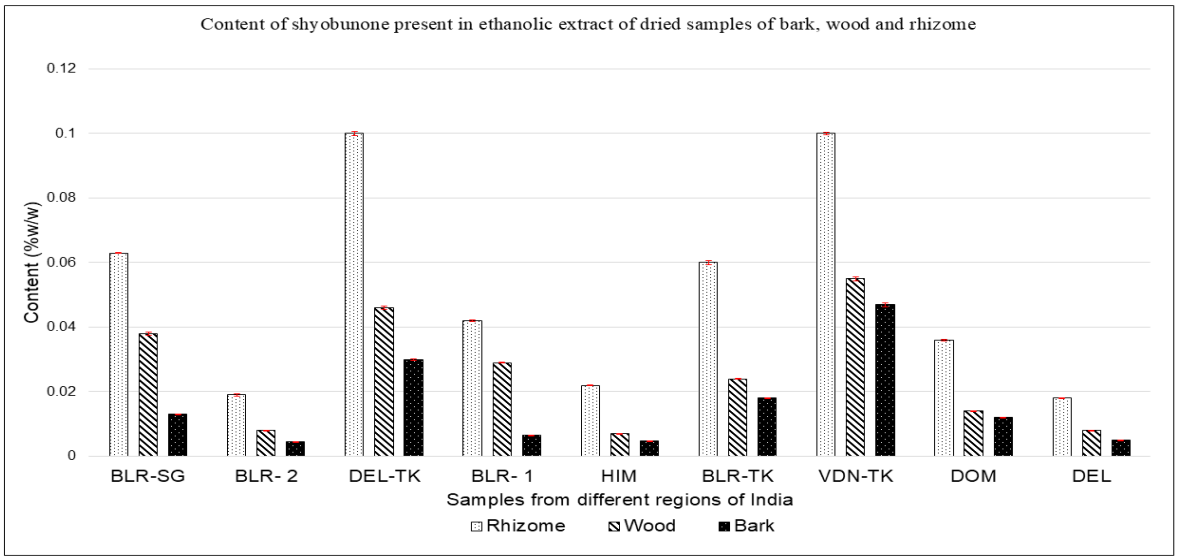

Figure 6: Shyobunone content (% w/w) present in ethanolic extract of dried samples of bark, wood and rhizome.

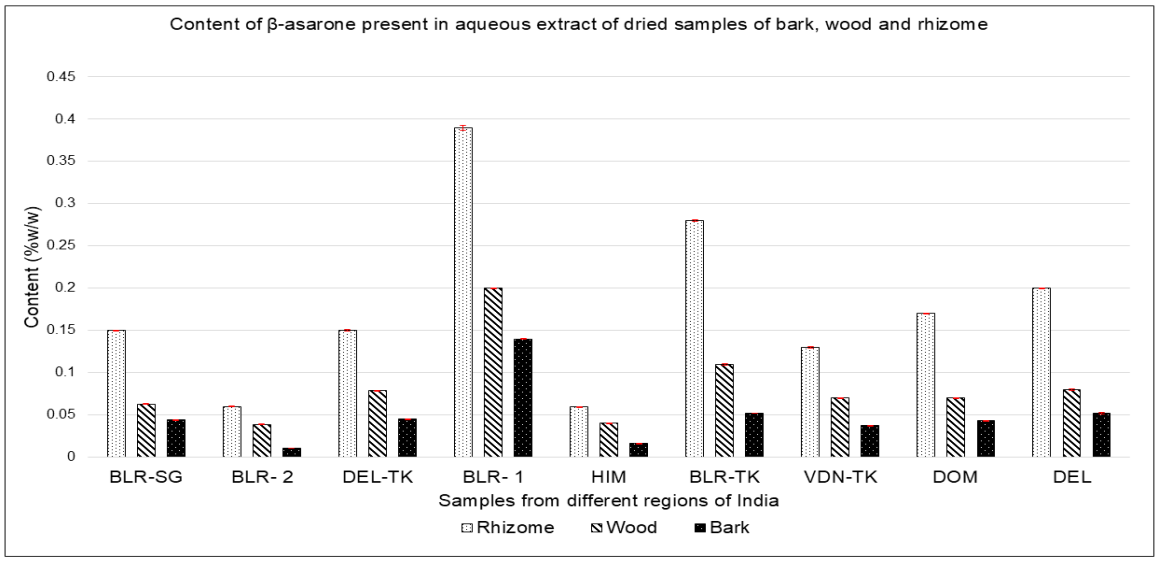

Figure 7: β-asarone content (% w/w) present in aqueous extract of dried samples of bark, wood and rhizome.

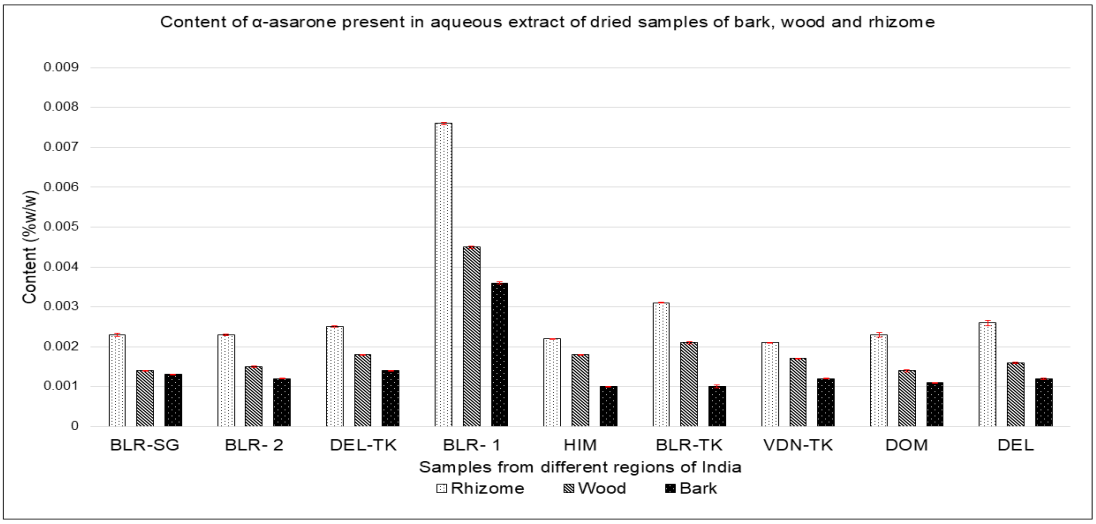

Figure 8: α-asarone content (% w/w) present in aqueous extract of dried samples of bark, wood and rhizome.

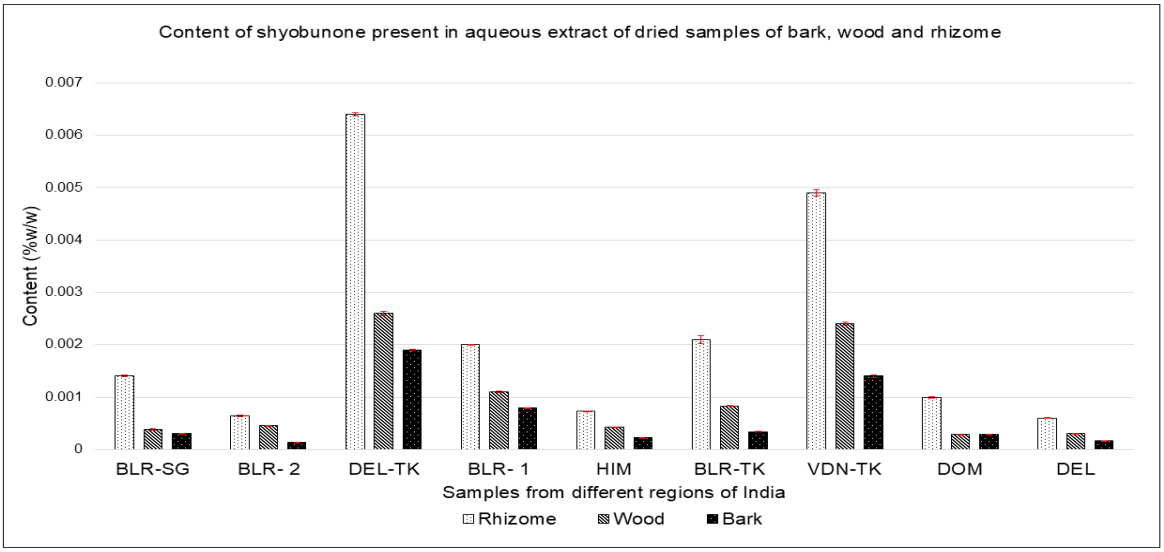

Figure 9: Shyobunone content (% w/w) present in aqueous extract of dried samples of bark, wood and rhizome.

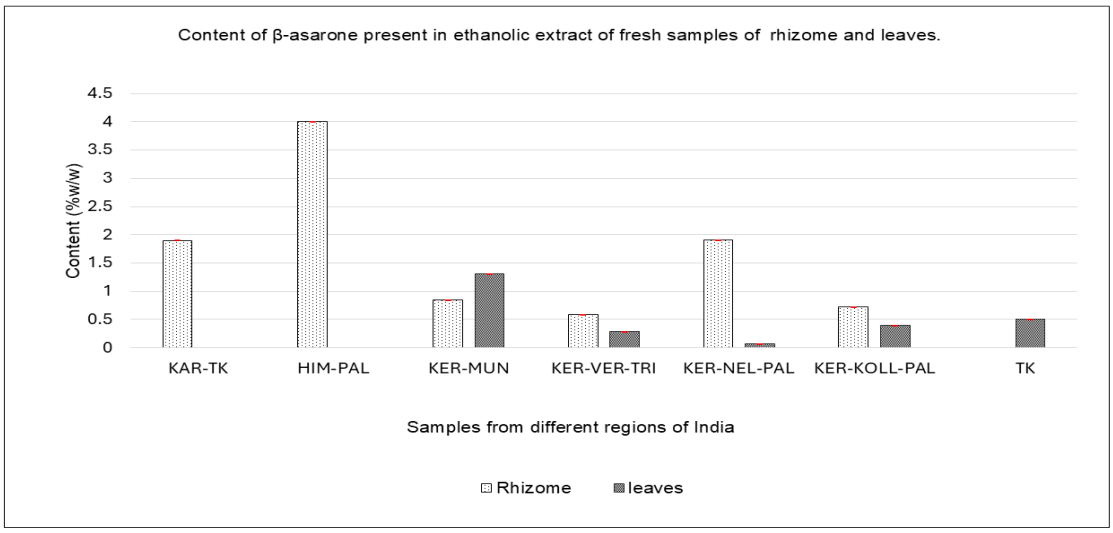

Figure 10: β-asarone content (% w/w) present in ethanolic extract of fresh samples of rhizome and leaves.

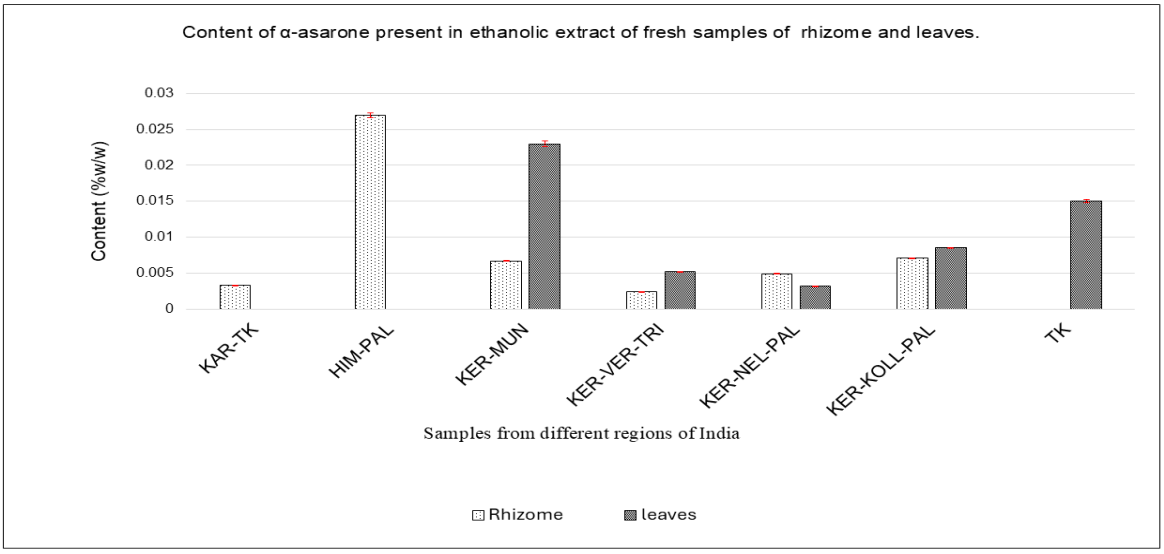

Figure 11: α-asarone content (% w/w) present in ethanolic extract of fresh samples of rhizome and leaves.

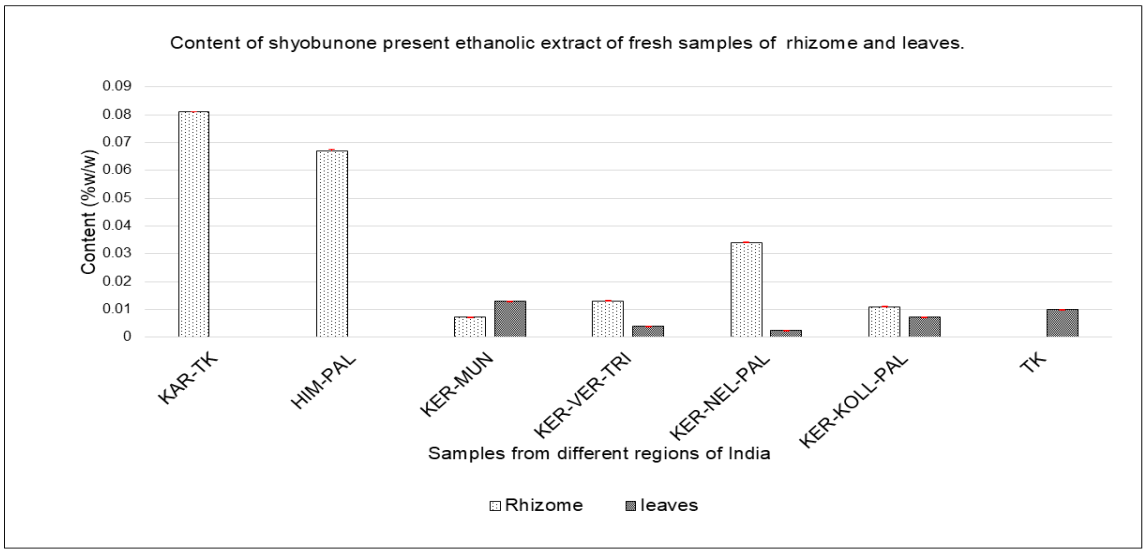

Figure 12: Shyobunone content (% w/w) present in ethanolic extract of fresh samples of rhizome and leaves.

and 0.0074-0.081% w/w, respectively. The fresh rhizome samples from Palampur of Himachal (HIM-PAL) showed higher content of both β-asarone and α-asarone at 4.00% w/w and 0.027% w/w respectively, whereas samples from Vellanikkara, Trichur of Kerala (KER-VEL-TRI) exhibited lower content of both β-asarone (0.59% w/w) and α-asarone (0.0024% w/w). At 0.081% w/w, fresh rhizome samples from Tumkur of Karnataka (KAR-TK) were at the top for content of shyobunone, whereas those from Munnar of Kerala (KER-MUN) made up the lower end at 0.00074% w/w.

In fresh leaf samples, β-asarone ranged from 0.064-1.31% w/w, α-asarone from 0.0032-0.023% w/w, and shyobunone from 0.0024-0.013% w/w. Higher concentrations of β-asarone (1.31% w/w), α-asarone (0.023% w/w), and shyobunone (0.013% w/w) were found in samples collected from Munnar, Kerala. On the other hand, low content of β-asarone (0.064% w/w), α-asarone (0.0032% w/w) and shyobunone (0.0024% w/w) was observed in leaf samples collected from Neliampatty, Palaghat of Kerala (KER-NEL-PAL).

CONCLUSION

Acorus calamus is a versatile herb with reported therapeutic uses in traditional Indian Ayurvedic systems as well as modern system of medicine. In particular, its diverse range of bioactivities, right from anti-inflammatory to immunomodulatory to neuroprotective, make it one of the most valuable herbs that can be deployed in treatment of many modern-day ailments like autoimmune diseases, neurodegenerative diseases and other noncommunicable diseases. The dose-dependent cytotoxicity, mutagenicity and carcinogenicity associated with β-asarone, one of the major constituents in *A. calamus*, has raised concerns with the industry and the drug regulatory bodies world-wide. This has become one of the major factors impeding a wider acceptance and therapeutic usage of calamus. In this paper, an attempt was made to estimate the phytoconstituents' content in *A. calamus* samples sourced from across different geographies of India. Novel, robust, simple and accurate RP-HPLC methods were developed and validated, for analysis of shyobunone and simultaneous estimation of α-asarone and β-asarone. These methods when applied onto the alcoholic extracts of these samples, showed that the content of α-asarone, β-asarone, and shyobunone in all the dried calamus samples ranged from 0.004-0.10% w/w, 2.30-4.40% w/w, and 0.018-0.10% w/w, respectively. Of particular interest is the fact that the content of β-asarone is found to be markedly lower than what has been reported earlier in the Indian varieties.

The dried rhizome of calamus is commonly used in children and infants as a cultural Indian practice. This involves rubbing a clean, dried rhizome on a grinding stone with a few drops of clean water to obtain a paste. The fact that the aqueous extract of the calamus samples showed even lower levels of these phytoconstituents, provides the evidence-based validation for absence of any side-effects whatsoever, when the herb has been so extensively used as a common Indian household remedy and in Ayurvedic medicines.

Another paper has been communicated for publication, by two of the authors where Acorus dried rhizome powder, its extracts in acidic medium and alkaline medium and pure β-asarone have all shown negative results for Ame's test as per OECD guidelines conducted by a GLP-compliant National Laboratory. These testing and results are not reported in this paper for more focus and brevity. This study reports data on all three marker compounds for Indian varieties of *Acorus calamus* only. Due to legal documentation needs and supply chain issues, samples of dried rhizomes from other nations could not be obtained and analysed for comparison.

The set of fresh *A. calamus* samples evaluated in this study, are being studied for their 'ploidy' status, to derive any possible correlation between the reduced content of β-asarone in these

herb samples and possible alteration of their 'ploidy' status. The findings when available will be reported separately.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

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ABBREVIATIONS

RP-HPLC: Reverse phase high performance liquid chromatography; **ICH:** International conference on harmonization; **OECD:** Organisation for economic co-operation and development; **GLP:** Good laboratory practice.

SUMMARY

This study evaluated the content of α-asarone, β-asarone, and shyobunone in fresh and dried *Acorus calamus* samples from various regions in India, using novel and validated Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) methods. Fresh rhizomes, leaves, and dried rhizomes were subjected to both ethanolic and aqueous extraction. The RP-HPLC methods were developed in accordance with ICH guidelines for the simultaneous estimation of α-asarone and β-asarone and a separate method used for shyobunone quantification.

Ethanolic extracts of *Acorus calamus* exhibited significantly higher content of α-asarone, β-asarone, and shyobunone compared to aqueous extracts, with rhizomes consistently containing higher concentrations of these phytoconstituents than leaves. In alcoholic extracts from dried rhizome samples, β-asarone content ranged from 2.30-4.40% w/w, α-asarone from 0.004-0.10% w/w, and shyobunone from 0.018-0.10% w/w. In dried wood samples, β-asarone, α-asarone and shyobunone content ranged from 1.41-3.24% w/w, 0.003-0.043% w/w, and 0.007-0.055% w/w, respectively. In dried bark samples, β-asarone content ranged from 0.86-1.70% w/w, α-asarone from 0.002-0.03% w/w, and shyobunone from 0.0044-0.047% w/w. Of the aqueous extracts, the content of α-asarone, β-asarone, and shyobunone in various dried rhizome samples ranged from 0.0021-0.0076% w/w, 0.06-0.39% w/w, and 0.0006-0.0064% w/w, respectively. In dried wood samples, β-asarone ranged from 0.039-0.20% w/w, α-asarone from 0.0014-0.0045% w/w, and shyobunone from 0.00029-0.0026% w/w. In dried bark samples, α-asarone, β-asarone, and shyobunone ranged from 0.0010-0.0036% w/w, 0.011-0.14% w/w, and 0.00013-0.0019% w/w, respectively. Fresh rhizomes showed lower levels of all three phytoconstituents, with α-asarone ranging from 0.0024-0.027% w/w, β-asarone from 0.59-4.00% w/w, and shyobunone from 0.0074-0.081% w/w. In fresh leaf samples, β-asarone ranged from 0.064-1.31% w/w, α-asarone from 0.0032-0.023% w/w, and shyobunone from 0.0024-0.013% w/w.

Overall, β-asarone levels were notably lower than previously reported in Indian varieties, suggesting genetic or environmental factors may influence phytoconstituent biosynthesis. The observed lower levels of β-asarone in *Acorus calamus* samples from different regions of India suggest reduced carcinogenic risk, making the plant a safer candidate for therapeutic use. This study provides a foundation for future investigations into the role of ploidy status in β-asarone biosynthesis.

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