Research Article

HPLC Profiling of B-Asarone Content and Cytogenetic Studies of Medicinally Important Indian *Acorus calamus* l., Accessions

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ABSTRACT

Acorus calamus L. is a well- known herb for its traditional medicinal and pharmaceutical potentials. The rhizome of the plant is mainly used for medicinal purposes because it yields an essential oil known as "Calamus Oil". The main component of the essential oil is β-asarone. In our present study, 20 different species of *Acorus calamus* were collected from different parts of South India and North East India. An attempt has been made to characterize all the accessions on the basis of their ploidy level by cytogenetic studies, and determining β- asarone content by HPLC analysis. All the accessions were screened for their ploidy level by staining the root chromosomes at metaphase stage. The plants observed were either diploid or triploid ruling out any tetraploids. HPLC analysis of powdered rhizome extracts for the β- asarone, indicated that it ranged from 2.2% to 7.2%. Our results also have revealed that there is no correlation between the ploidy status and the content of β-asarone. Indian accessions were found to have low to moderate levels of β- asarone content.

Keywords: *Acorus calamus*, Cytogenetic studies, HPLC analysis, β- asarone.

INTRODUCTION

Sweet flag, *Acorus calamus* L., is a semi-aquatic, tall perennial herbaceous plant. It grows in temperate to sub temperate regions. It is one of the highly valued medicinal and aromatic plants in India commonly known as Bach in Hindi. It is distributed in countries like North America, Canada and Europe. The plant generally grows in high altitude regions. It has a lengthy branched underground rhizome from which long, erect, narrow aromatic leaves have ascended. The plant rarely sets flowers and seeds.

The rich ethno botanical aspects of *A. calamus* have been reviewed.[1-3] On the basis of its ploidy status and geographical distribution, *A. calamus* has been classified as (i) diploid variety (2n = 2x = 24), (ii) triploid variety (2n = 3x = 36), (iii) the tetraploid variety (2n = 4x = 48) and (iv) hexaploid variety (2n = 6x = 72). Reports have stated that the essential oil content and also particularly the β-asarone content depend on the ploidy level of the taxons. It is stated that tetraploids contain highest content of β-asarone with around 70-96%, triploids around 5-19% and diploids 0-2%.[4-6] In the Indian *Acorus calamus* oil, it was reported to have higher percentage of β- asarone and assumed to be of teraploid origin.[7]

The essential oil present in the rhizomes is used for pharmacological purposes. Various reports have indicated that, the rhizome part of the *Acorus calamus* plant has numerous medicinal properties. It has been traditionally used as an anti-rheumatic, anti-arthritic, anti-spasmsodic, anti-diabetic, antibiotic and as a memory booster. They are sold in 'pure' form (fresh or dried plants) or as mixtures or extracts with other herbs, as tablets, capsules, powders, teas, alcohol extracts, etc. The oil of the plant has a characteristic sweet smell by which the name sweet flag was derived. This is due to the major component in the oil a sesquiterpenoid i.e 'β-asarone' [(Z)-1,2,4- trimethoxy-5-prop-1-enyl-benzene].[8,9] The other major compounds detected in the plant include glycosides, flavonoids, saponins, tannins, polyphenolic compounds. The essential oil from the rhizomes is also used in production of beer and alcoholic beverages such as bitters, cordials, vermouths and at lower level in foods such as frozen desserts, yoghurts, cakes and confectionery.[6]

Pharmacological Properties of the Plant

Studies on the properties of the plant have revealed that ethanolic extract of *A. calamus* rhizome display anticellular and immunomodulatory properties.[10] Research on anti-adipogenic properties of *Acorus* spp. for past few years have found that *A. calamus* demonstrate hypolipidemic activity in rats.[11] The saponins found in ethanolic extract of *A. calamus* are found to have hypolipidemic properties. A recent study investigated that *A. calamus* improves postprandial hyperglycemia and cardiovascular complications. For a long time, the radix of *A. calamus* is being used in the therapy of diabetes in traditional folk medicine of America and Indonesia. A recent study investigated that *A. calamus* improves postprandial hyperglycemia and cardiovascular complications.[12]

Another research carried out in vitro has showed that extract of *A. calamus* safeguarded DNA and membrane

damages in murine cells and human peripheral blood leukocytes caused due to γ –radiation.[13] Application of A. calamus rhizome extract has resulted in a significant improvement in neuro-behavioural performances such as, rota-rod performance and grid walking in the experimental rats.[14]

The presence of certain phyto-chemical compounds can be detetcted by means of various analytical and chromatographic techniques, such as the Gas chromatographic–Mass spectrometric (GC–MS), High-Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), Capillary Gas Chromatography, Liquid Chromatography etc. In the present study, we estimated the β- asarone content by HPLC technique and tried to find out whether there is any relationship between the ploidy levels and β-asarone content among 20 different accessions of *Acorus calamus* collected from different geographical areas in India.

MATERIALS AND METHODS

Plant Materials

In our present study 20 different accessions of *Acorus calamus* were collected from different geographical areas of South (Tamil Nadu, Karnataka, Kerala and Andhra Pradesh) and North Eastern parts of India (Assam). The geographic location data (latitude, longitude and altitude) for all twenty *A. calamus* samples were identified using DIVA GIS[15], a computer program for mapping and geographic data analysis. A geographical distribution map for *A. calamus* in various regions of South and North East India was created (Figure 1). The accessions were planted and maintained at the field gene bank of Indian Institute of Horticultural research, Bangalore (India). The detailed passport data along with the morphological features of the accessions were mentioned in Table 1.

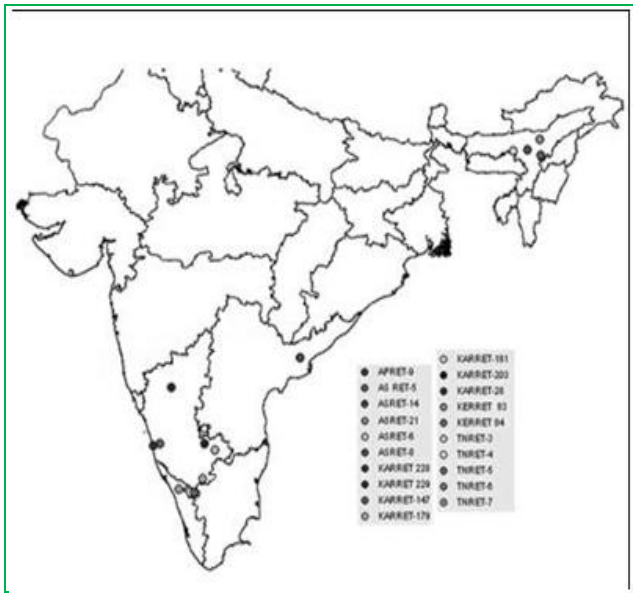

Figure 1: A distribution map showing the *Acorus calamus* accessions collected from 20 different locations [Southern and Eastern India] in the present study.

Sample Preparation for Cytogenetic Analysis

For the study of mitotic divisions in chromosomes, actively growing young root tips of the *Acorus calamus* plant were excised and pretreated with 2 mM solution of 8-hydroxy quinoline for 4 hours at 10°C and subsequently fixed in acetic acid : alcohol (1 : 3) mixture (Carnoy's fixative). These were hydrolyzed in 1 N HCl for 10–15 seconds on a flame prior to staining with aceto-orcein stain. The staining was performed by using 2% acetocarmine. Squash preparations were done by dissecting a well stained portion of the root tip and a drop of 1% aceto-orcein was added. It was covered with a cover slip and gently tapped so that it is adequately pressed. Then temporary slides were prepared by sealing the edges of the coverslip with sealing wax.[11] These slides were observed and photographs were taken by an Olympus IXV0I phase contrast microscope (DSS Imagetech Pvt. Ltd, India).

HPLC Analysis

Preparation of a Standard Solution

β-asarone standard obtained from M/s Natural Remedies, Bangalore, India was used for drawing the calibration curves. Six mg of standard compound was dissolved in 25 ml of methanol (stock solution). Four concentrations of 23 to 92 µg /ml were prepared from stock solution and injected into HPLC to draw the calibration curves. A linear fit was chosen with regression coefficient r^2 of 0.993.

Sample preparation and analysis

All the tuber samples were dried at 40°C for 48 hrs in hot air oven [Serwell Instruments Inc, India]. Dried samples were ground in a Mixer mill MM 400 (RETSCH, Germany) to get fine powder. Approximately 20 mg of the sample powder was taken in a test tube and 15 ml of methanol was added. The test tubes containing sample solutions were sonicated (Vibra cell VC 505, Sonics and Materials, USA) for 5 minutes. The sample solution was filtered using 0.2 µm nylon membrane (Advanced Micro devices India Pvt Ltd., India) and injected in to HPLC. The HPLC analyses were carried out on a Shimadzu Series LC-10A system (Shimadzu, Kyoto, Japan) consisting of a liquid chromatograph connected to a UV-VIS detector (10 A), binary pump and controlled by Shimadzu class VP workstation software. The column used was C18 Gemini, 250 x 4.6 mm, 5 µm (Phenomenex, USA) with security guard column. Samples were injected using a 20 µl loop (Rheodyne, Rohnert Park, CA, USA). The column and guard column were thermostatically controlled at 350°C. The flow rate was 1 ml / min and mobile phase consisted of water with 0.1% trifluoro-acetic acid [TFA] (solvent A) and methanol (solvent B). The instrument was run in an isocratic mode (A: 35; B: 65). The detection was monitored at 210 nm. All solvents used were of HPLC grade.

RESULTS

Cytogenetic Analysis

In all the slide preparations the root metaphase chromosomes were stained and observed, their mitotic divisions were found to be normal. The different stages of mitosis [prophase, anaphase, metaphase and telophase] were regular. No aneuploidy was observed. The size of the chromosomes was considerably very small. Clumping tendency was commonly observed. All the 20 preparations that we observed were either diploid (figure 2a) or triploid (figure 2b). No tetraploid or hexaploids were observed. In all of the 20 accessions observed, four varieties from North-East India i.e from Assam were diploids and the rest from South India were a mix of both diploids and triploids.

Table 1: Place of collection, morphological data, geographical co-ordinates (latitude, longitude) of all 20 *Acorus* accessions

Accession no	Place of collection	Plant height(cm)	leaf length (cm)	leaf width (cm)	No. of leaves	Latitude	Longitude
TNRET-3	Doddabetta, Tamil Nadu	40.0±0.8	36.5±0.5	0.9± 0.0	5.6±0.6	11° 26' 42.1"N	76° 41'14.9"
KARRET-147	Agumbe, Karnataka	37.5±0.4	34.0±1.0	1.0±0.1	5.3±0.6	13°30'31.32" N	75° 5' 45.24" E
KARRET-28	Belgaum, Karnataka	50.2±0.1	45.0±0.5	1.3±0.0	6.6±0.6	15°51'.0"N	74° 30' 0" E
TNRET-5	Doddabetta, Tamil Nadu	47.6±0.3	43.3±1.5	0.7±0.1	4.6±0.6	11° 24' 8.7" N	76° 44' 12.2" E
APRET-9	Rajahmundry,Andhra Pradesh	50.3±0.2	46.6±0.6	0.9±0.1	7.0±1.0	16° 58' 48" N	81° 46' 48" E
TNRET-4	Parsons Valley, Tamil Nadu	45.3±0.1	42.5±0.6	1.2±0.1	6.0±0.0	11° 24' 0" N	76° 42' 0" E
TNRET-6	Ooty, Tamil Nadu	49.5±0.5	46.3±0.3	1.2±0.1	7.3±0.6	11°24'42.2"N	76° 41' 44"E
TNRET-7	Thambettu, Tamil Nadu	48.5±0.2	45.0±1.0	1.0±0.1	6.3±0.6	11° 26' 42.1"N	76° 41' 14.9"E
KARRET-179	Devanahalli ,Karnataka	49.8±0.2	45.7±0.2	1.0±0.1	7.0±0.0	13° 13' 48" N	77° 42' 0" E
KARRET-181	BR,Hills, Karnataka	48.0±0.9	42.9±2.6	1.0±0.2	5.3±0.6	11° 59' 38" N	77° 8' 26" E
KARRET-229	Koratagere, Karnataka	48.5±0.1	44.3±0.6	1.2±0.1	6.3±0.6	13° 31' 12" N	77° 13' 48" E
KERRET-83	Kalpetta, Kerala	48.6±1.5	44.6±0.1	0.9±0.1	7.0±0.0	11°37'21.18"N	76° 4' 52.5" E
KERRET-84	Thrissur, Kerala	50.2±0.1	45.0±0.1	1.4±0.1	6.6±0.6	10° 31' 12" N,	76° 12' 36" E
KARRET-203	Bakala, Karnataka	47.9±0.7	43.6±0.2	1.3±0.0	7.3±0.6	13° 55 '44.7"N	75 °34' 5.2"E
KARRET-228	Attigundi, Karnataka	51.4±0.1	46.2±1.3	1.1±0.1	5.6±0.6	13°25' 44.8'N	75° 44'32.1"E
AS RET-5	Nunmathi, Assam	37.5±0.5	33.0±1.0	0.7±0.0	3.0±0.0	26°5'26"N	91°32'14"E
AS RET-6	Dharbaum, Assam	49.2±0.2	45.4±0.1	1.2±0.0	5.6±0.6	26°4'49"N	91°33'34.6"E
AS RET-8	Sorupthar, Assam	47.0±0	43.6±0.1	1.4±0.1	6.3±1.2	26°19'54.5"N	93°86'24.2"E
AS RET-14	Dhansiri, Assam	40.0±0.5	36.0±0.3	0.9±0.0	6.0±1.0	25°48' 6.2"N	93°36'23.7"E
AS RET-21	Karbi, Assam	37.5±0.2	36.0±3.3	0.9±0.0	4.6±0.6	25°45'55.3"N	93°9'30.6"E

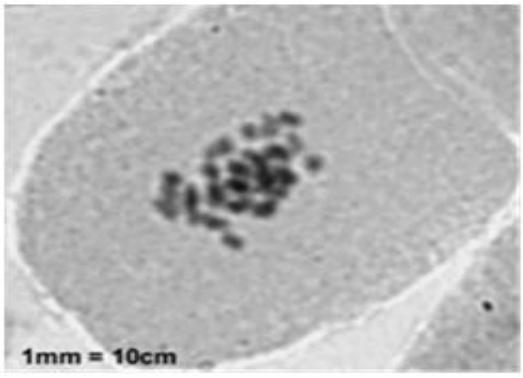

Figure 2a: Microscopical image depicting diploid cytotype of *A.calamus* accession ASRET-14

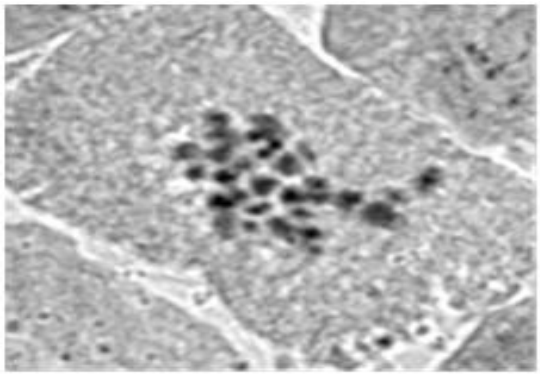

Figure 2b: Microscopical image depicting triploid cytotype of *A.calamus* accession KARRET-229.

Results of HPLC Analysis

β-asarone standard was injected into the HPLC and a standard chromatogram was obtained with an average retention time of 7.14 min for three replications (fig 3). All the 20 accessions of *Acorus calamus* were also subjected to HPLC analysis which showed a retention time of around 7 minutes. The representative HPLC chromatograms for diploid and triploid accessions were shown in Figure 4 and 5). The concentration of β - asarone varied from around 2.80 mg/100mg to 7.3 mg/100mg of the dry weight of the powdered rhizomes. The lowest value was observed for the sample ASRET-14 with concentration of 2.80 mg/100mg and highest was observed for the sample ASRET-6 with a concentration of 7.3 mg/100mg with an average of 5.22mg/100mg for diploids and 4.61mg/100mg for triploids. The details of the observed slides indicating the ploidy level and the β-asarone content of samples were mentioned in Table 2.

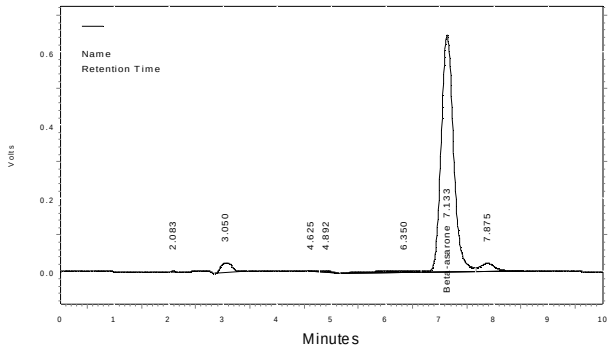

Figure 3: HPLC Chromatogram of the β-asarone standard

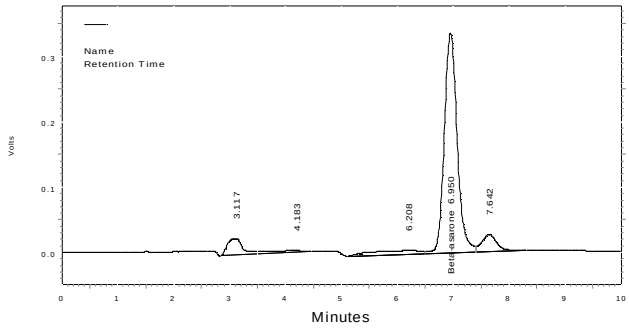

Figure 4: HPLC Chromatogram of the sample KERRET-13 (triploid) showing β-asarone peak

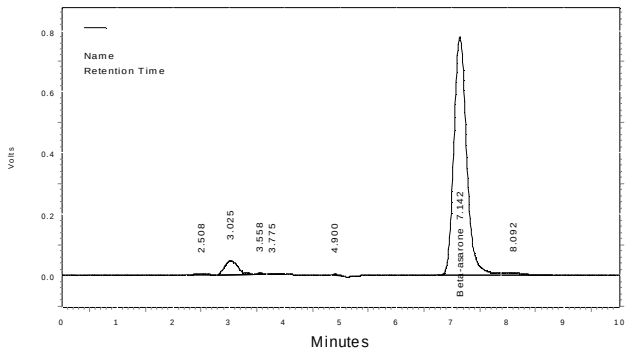

Figure 5: HPLC Chromatogram of the KARRET-181 (Diploid) showing β-asarone peak

DISCUSSION

Acorus calamus or sweet flag a well-known traditional medicinal plant whose essential oil is also used for various purposes. The US FDA and also the European commission (EC) interdicted the utilization of sweet flag owing in therapeutic and food and beverages formulations due to the potential carcinogenic effects of its essential oil, with particular reference to β-asarone which was found to be toxic in rats.[17,18] Since studies have stated that tetraploids contain highest content of β-asarone with around 70-96%, triploids around 5-19% and diploids 0-2% [4-6], the *Acorus calamus* varieties with very low percentage of β-asarone were permitted to be used. There are also reports which state that in reality β-asarone is not actually a carcinogen, but it is a pro-carcinogen that is neither hepatotoxic nor directly hepato-carcinogenic.[19] Studies have reported many pharmaceutical values of the plant extracts.

In our present study, we have compared all the 20 accessions of the *A. calamus* by cytogenetic analysis and also estimated the β-asarone content through HPLC analysis. The ploidy level for each accession was determined. In all the 20 accessions, all the stages of cell division i.e prophase, metaphase, anaphase and telophase were normal. Out of all the slides that were observed 11 were found to be diploids and the rest triploids, ruling out any tetraploids. The accessions from North-East i.e from Assam were found out to diploids and whereas the South Indian varieties were a mixture of both diploids and triploids. The β-asarone content was analysed by HPLC. These results were contradictory to previous reports which have stated that Indian *Acorus* is a tetraploid.[20,3] Our analyses revealed that both the triploid and diploid varieties showed quiet lower concentration of asarone and that there is no significant correlation between the ploidy level and the content of β-asarone in the rhizome samples. These results were on lines to a study by who also have stated that Indian *Acorus Calamus* is not a tetraploid.[16] Our study also indicate that there is a very low range of chemical diversity in the level of β-asarone among the various accessions even though they are collected from different geographical locations and they form two distinct varieties i.e one from North east India and the other from South India. This low level of difference in the varieties may be attributed to the clonal propogation of the plant. Even though the plant reproduces both sexually (by seed) and asexually (by rhizome), the Indian population is derived mostly by clonal propagation as the plant very rarely sets flowers and seeds.

The Indian *Acorus calamus* plants are thought to be derived from a common ancestor plant and then propagated through rhizome stock planted by humans as and when they migrated to different places and cultivated the plant.

Table 2: Ploidy status and β –asarone content of all the 20 accessions

Accession no	Ploidy level	β asarone content in µg/ml (from HPLC analysis)	β-asarone content in % w/w
ASRET-14	Diploid	24.45	2.18
ASRET-21	Diploid	47.5	4.19
TNRET-5	Diploid	68.49	5.92
TNRET-6	Diploid	69.47	5.97
ASRET-6	Diploid	51.99	4.62
TNRET-4	Diploid	50.56	4.29
KARRET-28	Diploid	83.05	7.22
ASRET-5	Diploid	82.43	7.24
APRET-9	Diploid	58.31	4.92
KARRET-181	Diploid	61.81	5.22
KARRET-179	Triploid	56.98	5.07
KERRET-84	Triploid	53.81	4.93
KARRET-229	Triploid	59.17	5.49
TNRET-7	Triploid	52.39	4.69
TNRET-3	Triploid	58.84	4.99
KERRET-83	Triploid	51.11	4.28
KARRET-228	Triploid	62.00	5.27
KARRET-147	Triploid	62.62	5.43
KARRET-203	Triploid	54.64	4.63
ASRET-8	Diploid	52.77	4.52

CONCLUSION

Our study gives a brief insight into the determination of β- asarone content in various accessions of *Acorus calamus* through HPLC analysis. The essential oil present in the *Acorus* rhizomes is used for various pharmacological purposes. Reports also indicated that, the rhizome part of the *Acorus calamus* plant has numerous medicinal properties. Studies have stated that tetraploids contain highest content of β-asarone with around 70-96%, triploids around 5-19% and diploids 0-2%, the *Acorus calamus* varieties with very low percentage of β-asarone were permitted to be used. Our study shows that all the accessions irrespective of their ploidy level and place of collection had considerably low to moderate levels of β-asarone. We can conclude that as long as the content of β–asarone is low, the plants can be cultivated, conserved and also utilized for various pharmaceutical and industrial purposes.

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