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Quantification of β -asarone content in *Acorus calamus* L., an aromatic medicinal plant from the Western Ghats

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Abstract

Acorus calamus L., is an aromatic medicinal plant, widely used in the traditional and modern system of medicine for the treatment of neurological and metabolic disorders. High occurrence of β -asarone in the rhizome of *A. calamus* limits its usage in medicinal preparations due to its toxic and carcinogenic properties. The objective of the study was to collect *A. calamus* accessions growing in the different agroclimatic conditions from the Western Ghats and quantify the β -asarone content. A total of 20 different accessions of *A. calamus* were collected from the Western Ghats and subjected to RP-HPLC analysis. Variations in the β -asarone content (1.35 ± 0.00 mg/g to 71.54 ± 0.00 mg/g) was observed in the collected accessions. The lowest β -asarone content (1.35 ± 0.00 mg/g) was observed in the accession collected from Vattavada region (Idukki dist.) followed by the accession collected from Nelliampathy region, Palakkad (5.54 ± 0.00 mg/g). The highest β -asarone content (71.54 ± 0.01 mg/g) was observed in the accession collected from Kalpetta. The present study identified an elite accession of *A. calamus* with less β -asarone content (1.35 ± 0.00 mg/g) from Vattavada. It could be a most promising raw material that can impart standard quality for medicinal purposes and preparation of polyherbal formulations.

Keywords: *A. calamus*; β -asarone; RP-HPLC, western ghats

Introduction

Acorus calamus L., (Family- Acoraceae) is a high valued aromatic medicinal plant commonly known as 'Sweet flag', distributed in the temperate to sub-temperate regions^[1, 2]. In India, the plants are found predominantly in the swamps, streams and marshy areas of Himalayan and sub-Himalayan regions and some other regions of the Western Ghats and North-East India^[2-5]. Rhizome is the medicinally useful part of the plant and is used for the treatment of neurological and metabolic disorders in the traditional systems of medicine such as Indian and Chinese systems of medicine as well as in modern medicine^[4, 6, 7]. The plant is used as a memory booster, sedative, behaviour-modifying agent, anticonvulsant, anti-rheumatic, anti-arthritic, anti-spasmodic, antibiotic, acetylcholinesterase inhibitory, anti-diabetic, cytoprotective, hypolipidemic, bronchodilatory, anti-inflammatory, antidiarrheal, anti-ulcer and analgesic properties^[8-11]. More than 145 chemical constituents were identified and isolated from different parts (rhizome and leaf) of the medicinal herb. They include phenylpropanoids (α -asarone, β -asarone, eugenol, isoeugenol etc.), sterols, triterpene glycosides, triterpenoid saponins, sesquiterpenoids, monoterpenes, and alkaloids. *A. calamus* species exist in four cytotypes, viz. diploid ($2n=24$), triploid ($3n=36$), tetraploid ($4n=48$) and hexaploid ($6n=72$)^[2, 4, 12, 13]. The chemical constituents and its concentrations in the plant/plant parts depend on the ploidy of the taxa^[2, 13]. Among the chemical constituents, β -asarone (phenylpropanoid) is the principal compound present in the rhizome and leaf of *A. calamus* and it is the most discussed constituent in the plant because of its toxic nature^[13] that can incur chromosomal aberrations, mutation and cancer.

The calamus rhizome (fresh/dried) or essential oil combined with other medicinal herbs, are used for the production of polyherbal formulations (tablets, capsules and powders), which are extensively used for the treatment of neurological and metabolic disorders^[4, 9, 13]. It is also used as a flavour in alcoholic beverages^[13] such as beer, bitters, liqueurs, and vermouths and is used in minute quantities in foods^[14] such as frozen desserts, yoghurts, cakes and confectionery. The Food and Drug Administration (FDA) interdicted the utilization of *A. calamus* owing to the potential carcinogenic effects of β -asarone^[15].

Based on the genotoxicity, the European Medicines Agency (EMA) prefer mostly β -asarone free varieties of *A. calamus* and is set to generally reduce concentrations of β -asarone in herbal medicinal products [16]. According to CFRT (2019) [17], the addition of *A. calamus* to foods in any form is not allowed in the USA. Under these circumstances, it is essential to identify the *A. calamus* plants with very less content of β -asarone which is a prerequisite for medicinal preparations and commercial applications.

Analytical methods such as HPLC, HPTLC, LC-MS and GC-MS have been reported in the literature describing the qualitative and quantitative determination of bioactive compounds in medicinal and aromatic plants, and the precise identification of their structure [18-22]. HPLC is a widely used and valuable analytical technique for quality screening, quantitative analysis, and method development to determine marker compounds in medicinal and aromatic plants. HPLC provide many benefits, including simplicity, robustness, accuracy, sensitivity and affordability [23].

The present study focuses on the quantitative determination of β -asarone content in the rhizome of *A. calamus* accessions collected from different geographical areas in the Western Ghats using RP-HPLC method and for the identification of *A. calamus* accessions that harbour minute quantities of β -asarone.

Materials and Methods

Collection of *A. calamus* accessions from the Western Ghats

Fresh rhizomes of *A. calamus* accessions were collected from different geographical areas of Kerala, Tamil Nadu and Karnataka regions of the Western Ghats, India. The geographic location data such as latitude, longitude and altitude of the collected accessions were recorded using a Global Positioning System (GARMIN-OREGON 550, USA) and a corresponding distribution map was created using Q-Geographic Information System(Q-GIS).

Chemicals and reagents

The β -asarone standard used in the study, methanol and water with HPLC grade were purchased from Merck (Germany). All the chemicals and reagents used for the study were of analytical grade.

Preparation of methanolic extract of the rhizome samples

Freshly collected rhizomes of *A. calamus* accessions from different geographical locations of the Western Ghats were cleaned under running tap water to remove external unwanted materials and then nicked into small pieces and dried under shade for one week. The air-dried rhizomes were ground to powder and the powdered individual samples were subjected to cold extraction using methanol. Powdered rhizome (5 g) samples were taken in a 500 ml conical flask (Borosil, India) to which 200 ml of methanol was added (1:40 w/v). The flask was vortexed for 60 min using a shaker and kept for 24h at room temperature. The extract was filtered using Whatman No.1 filter paper and the crude sample was dried using a rotary evaporator (Heidolph, Germany). A light yellowish-brown coloured concentrate was obtained. The extracts were stored in 15 ml air-tight screw cap bottles (Borosil, India) at -20°C for HPLC analysis.

Preparation of standard and sample solutions

A standard solution of β -asarone was prepared in methanol (mg/ml). Using the standard solution, different concentrations

of standard β -asarone (5, 10, 25, 50 and 100 ppm) was prepared by dissolving it in methanol and filtered through a Whatman Nylon 0.45 μm syringe filter for the construction of a calibration curve. The sample solutions were prepared by dissolving 10mg of crude methanolic extract in 10 ml of methanol and filtered through the Whatman Nylon 0.45 μm syringe filter.

Quantification of β -asarone content using RP-HPLC analysis

A Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method [2, 9] was used for the quantification of β -asarone content in the rhizome of *A. calamus* accessions collected from the Western Ghats. The chromatographic determination was carried out in a Shimadzu-LC-20-AD-HPLC system (Shimadzu, Kyoto, Japan) composed of two pumps, a rhenode injector, C18 column equipped with a PDA detector (SPS-20A). In HPLC analysis, the mobile phase composed of water and methanol in a ratio of 5:95, v/v with a flow rate of 1ml/min. The calibration graph of β -asarone was plotted by injecting different concentrations (5, 10, 25, 50 and 100 ppm) of standard β -asarone at a wavelength of 254nm, with a running time of 8min and the injection volume of 20 μl using a 25 μl capacity syringe (Hamilton, USA). The HPLC system was made stable for 1h before the injection. After constructing the calibration curve, the filtered methanolic extracts were injected to the HPLC system. The system was run at a wavelength of 254 nm, with a running time of 8min and an injection volume of 20 μl . The identification and quantification of β -asarone content was done by comparing the retention times and peak height between the standard and the sample on the HPLC chromatogram.

Linearity and accuracy

The linearity of the method was examined using different concentrations (5, 10, 25, 50 and 100 ppm) of standard β -asarone prepared using HPLC grade methanol and was injected (20 μl) to the HPLC system. The HPLC system was run at 254nm for 8mins and the experiments were replicated thrice. A calibration curve was made by plotting the average peak area (y) against β -asarone concentrations (x). The accuracy of the method was evaluated by consecutive analysis (n=3) of different concentrations (5, 10, 25, 50 and 100ppm) of standard β -asarone.

Statistical analysis

The quantitative determination of β -asarone was performed in triplicates and the results are reported as average \pm standard deviation (SD). All data were statistically analysed by Analysis of Variance (ANOVA) and the means were compared by Duncan's multiple range test ($p<0.05$) using IBM SPSS Statistics 23 (SPSS Inc. Chicago, USA).

Results and Discussion

Twenty different accessions of *A. calamus* were collected from different geographical areas of Kerala (9acc.), Tamil Nadu (9acc.) and Karnataka (2 acc.) regions of the Western Ghats. Natural populations of *A. calamus* were found in the swamps, streams and marshy areas of high-altitude regions (900m above) along with cultivated areas (26m above) in the Western Ghats. Using the recorded geographical locations, a distribution map of *A. calamus* was created with Q-GIS software (Fig 1).

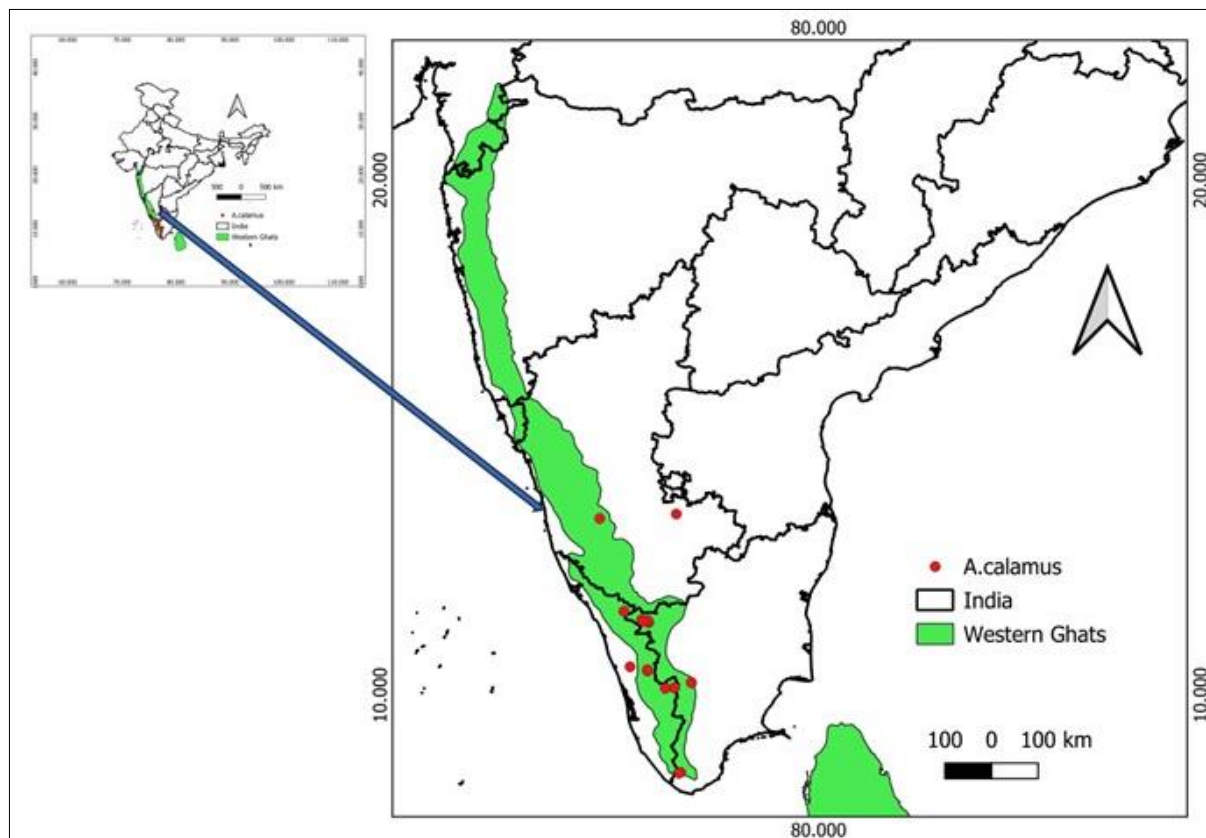


Fig 1: Distribution map of *A. calamus* collected from the Western Ghats

The quantitative determination of β -asarone content in *A. calamus* accessions collected from the different geographical areas of the Western Ghats was carried out through the RP-HPLC method. The HPLC chromatogram of standard β -asarone got separated and showed a sharp peak at a retention time of 4.1 min at a maximum wavelength of 254nm (Fig.2-5) using methanol (95%) and water (5%) as the mobile phase. A linear relation with a good correlation coefficient was obtained when the peak area (y) of β -asarone was plotted against different concentrations (5, 10, 25, 50 and 100 ppm). A correlation coefficient (r^2) of 0.999 was obtained for the calibration curve equation (Fig.2). The β -asarone content of collected accessions from Western Ghats ranged from 1.35 ± 0.00 mg/g- 71.54 ± 0.01 mg/g (Table.1). The lowest β -asarone content was obtained in the *A. calamus* accession collected from Vattavada (ACKLA05) of Munnar region, Idukki district with a concentration of 1.35 ± 0.00 mg/g followed by the accession collected from Muthalamada (ACKLA07) of Nelliampathy region (5.54 ± 0.00 mg/g), of Palakkad district and two accessions collected from Prakashapuram (ACTND01 & ACTND02) of Kodaikanal region (6.37 ± 0.00 mg/g & 6.55 ± 0.00 mg/g), Dindigul district. The highest content (71.54 ± 0.01 mg/g) was obtained from the accession collected from Kalpetta region (ACKLA08), Wayanad district and the plants collected from Patticaud region (ACKLA09), Thrissur district with a concentration of 58.81 ± 0.00 mg/g. High level of β -asarone content (1.35 ± 0.00 mg/g- 71.54 ± 0.01 mg/g) variation was observed in the *A. calamus* accessions collected from Kerala regions (9acc.) of the Western Ghats. The β -asarone content (5acc.) of *A. calamus* accessions collected from Munnar regions, Idukki district ranged from

1.35 ± 0.00 , mg/g- 29.08 ± 0.00 mg/g, (ACKLA01- 14.09 ± 0.00 mg/g, ACKLA02- 9.10 ± 0.00 mg/g, ACKLA03- 29.08 ± 0.00 mg/g, ACKLA04- 16.64 ± 0.00 mg/g, ACKLA04- 1.35 ± 0.00 mg/g), 10.62 ± 0.00 mg/g (ACKLA06) and 5.54 ± 0.00 mg/g (ACKLA07) was observed in the accessions collected from Nelliampathy regions, Palakkad. The β -asarone content of *A. calamus* accession collected from Kalpetta region (ACKLA08), Wayanad district and the accession collected from Patticaud region (ACKLA09), Thrissur district showed 71.54 ± 0.01 mg/g and 58.81 ± 0.00 mg/g respectively and these two accessions showed highest β -asarone content among the accessions collected from Western Ghats. β -asarone content of *A. calamus* accessions collected from Tamil Nadu regions (9acc.) of the Western Ghats ranged from 6.37 ± 0.00 mg/g- 17.23 ± 0.00 mg/g. The β -asarone content of *A. calamus* accessions collected from Ooty regions of Nilgiris district (Tamil Nadu) are Kathadimattam (ACTND03)- 11.68 ± 0.00 mg/g, TR Nagar (ACTND04)- 12.59 ± 0.01 mg/g, Indhunagar (ACTND05)- 6.98 ± 0.00 mg/g; Thalaikundwa (ACTND06)- 7.09 ± 0.00 mg/g & Pykara (ACTND07)- 9.23 ± 0.05 mg/g. The β -asarone content of *A. calamus* accessions collected from Pechippara regions (ACTND08 & ACTND09), Kanyakumari district are 17.23 ± 0.00 mg/g & 17.23 ± 0.00 mg/g respectively. A marginal variation in the β -asarone content was observed in the *A. calamus* accessions (2acc.) collected from Prakashapuram regions (ACTND01- 6.37 ± 0.00 mg/g & ACTND02- 6.55 ± 0.00 mg/g), Kodaikanal, Dindigul district. The rhizome samples collected from Karnataka showed 30.37 ± 0.01 mg/g (Koratagere-ACKND01) and 31.34 ± 0.00 mg/g (Attigundi-ACKND02) of β -asarone content.

Table 1: β -asarone content in *A. calamus* accessions collected from the Western Ghats

Serial Number	Accession Number	Location	Altitude (m)	Locality	District	State	β -asarone (mg/g)
1.	ACKLA01	Kadalar-I	1451	Munnar	Idukki	Kerala	14.09 \pm 0.00 ^h
2.	ACKLA02	Kadalar-II	1758				9.10 \pm 0.00 ^a
3.	ACKLA03	Kundala Dam-I	1758				29.08 \pm 0.01 ^e
4.	ACKLA04	Kundala Dam-II	1754				16.64 \pm 0.00 ^g
5.	ACKLA05	Vattavada	1425	Nelliampathy	Palakkad	Kerala	1.35 \pm 0.00 ^f
6.	ACKLA-06	Muthalamada-I	943				10.62 \pm 0.00 ⁱ
7.	ACKLA-07	Muthalamada-II	936	Kalpetta	Wayanad	Kerala	5.54 \pm 0.00 ^s
8.	ACKLA-08	Kalpetta	921				71.54 \pm 0.01 ^a
9.	ACKLA-09	Patticaud	26				58.81 \pm 0.00 ^b
10.	ACTND-01	Prakashapuram-I	1802	Kodaikanal	Dindigul	Tamil Nadu	6.37 \pm 0.00 ^f
11.	ACTND-02	Prakashapuram-II	1798				6.55 \pm 0.00 ^q
12.	ACTND-03	Kathadimattam	2210	Ooty	Nilgiri		11.68 \pm 0.00 ^k
13.	ACTND-04	TR Nagar	1985				12.59 \pm 0.01 ^j
14.	ACTND-05	Indunagar	2109			6.98 \pm 0.00 ^p	
15.	ACTND-06	Thalaikundwa	2095	Pechippara	Kanyakumari	Tamil Nadu	7.09 \pm 0.00 ^o
16.	ACTND-07	Pykara	2080				9.23 \pm 0.05 ^m
17.	ACTND-08	Mangamala	129				17.23 \pm 0.00 ^f
18.	ACTND-09	Kolinjimala	139	13.90 \pm 0.01 ⁱ			
19.	ACKND-01	Koratagere,	751	Koratagere	Tumkur	Karnataka	30.37 \pm 0.01 ^d
20.	ACKND-02	Attigundi	780	Attigundi	Chikkamagalur		31.34 \pm 0.00 ^c

Values are Mean \pm SD (N=3). Superscript letters with different letters in the same column indicate significant difference ($p < 0.05$) analysed by Duncan's multiple range test.

A. calamus is one of the most important aromatic medicinal herb with high therapeutic values harbouring β -asarone (phenylpropanoid) as its major chemical constituent with potential carcinogenic effect (Avadhani *et al.*, 2016). Because of the presence of β -asarone, the US FDA and European Commission (EC) forbid the utilization of its rhizome/essential oil in food, beverages and herbal formulations and recommended *A. calamus* products with no or negligible

amount of β -asarone [2]. The β -asarone content in *A. calamus* (rhizome/leaf/root) was reported earlier using chromatographic techniques such as GC-MS, HPLC and HPTLC methods. Variations in the β -asarone content was observed in the rhizome samples collected from different geographical locations of North and South-East India through HPLC or HPTLC methods [2, 9, 24, 25].

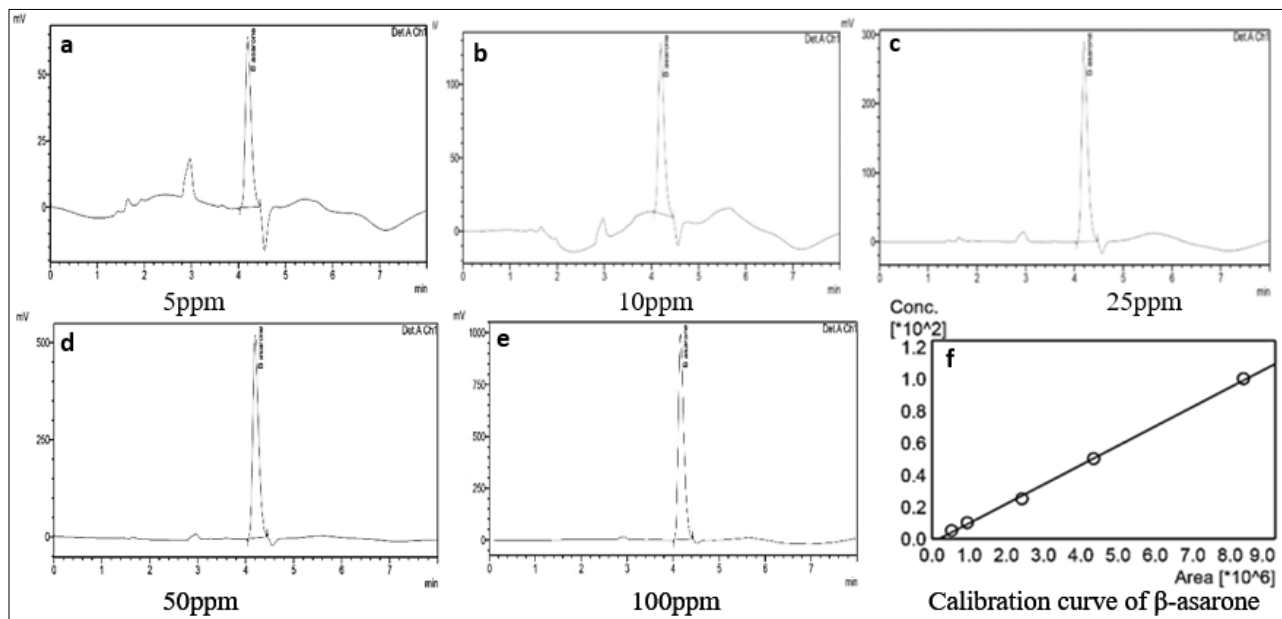


Fig 2: HPLC chromatogram of standard β -asarone; (a) 5ppm, (b) 10ppm, (c) 25ppm, (d) 50ppm, (e) 100ppm and (f) calibration curve of standard β -asarone

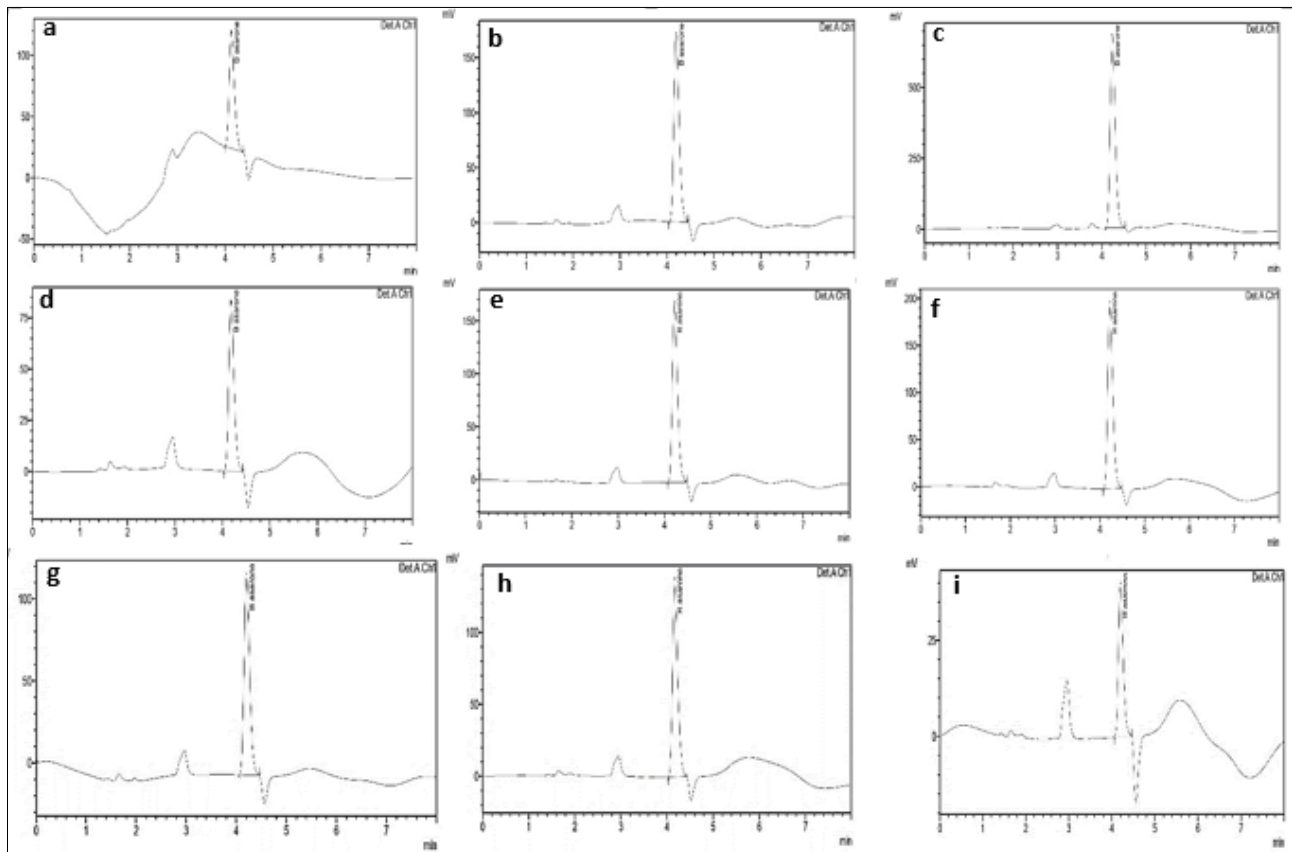


Fig 3: HPLC chromatogram of *A. calamus* collected from Kerala regions of the Western Ghats; (a) Kadalar -I, Munnar (ACKLA01), (b) Kadalar -II, Munnar (ACKLA02), (c) Kundala Dam-I, Munnar (ACKLA03), (d) Kundala Dam-II, Munnar (ACKLA04), (e) Vattavada, Munnar (ACKLA05), (f) Muthalamda, Nelliampathy-I (ACKLA06), (g) Muthalamda, Nelliampathy-II (ACKLA07), (h) Kalpetta, Wayanad (ACKLA08) and (i) Patticaud, Thrissur, (ACKLA09).

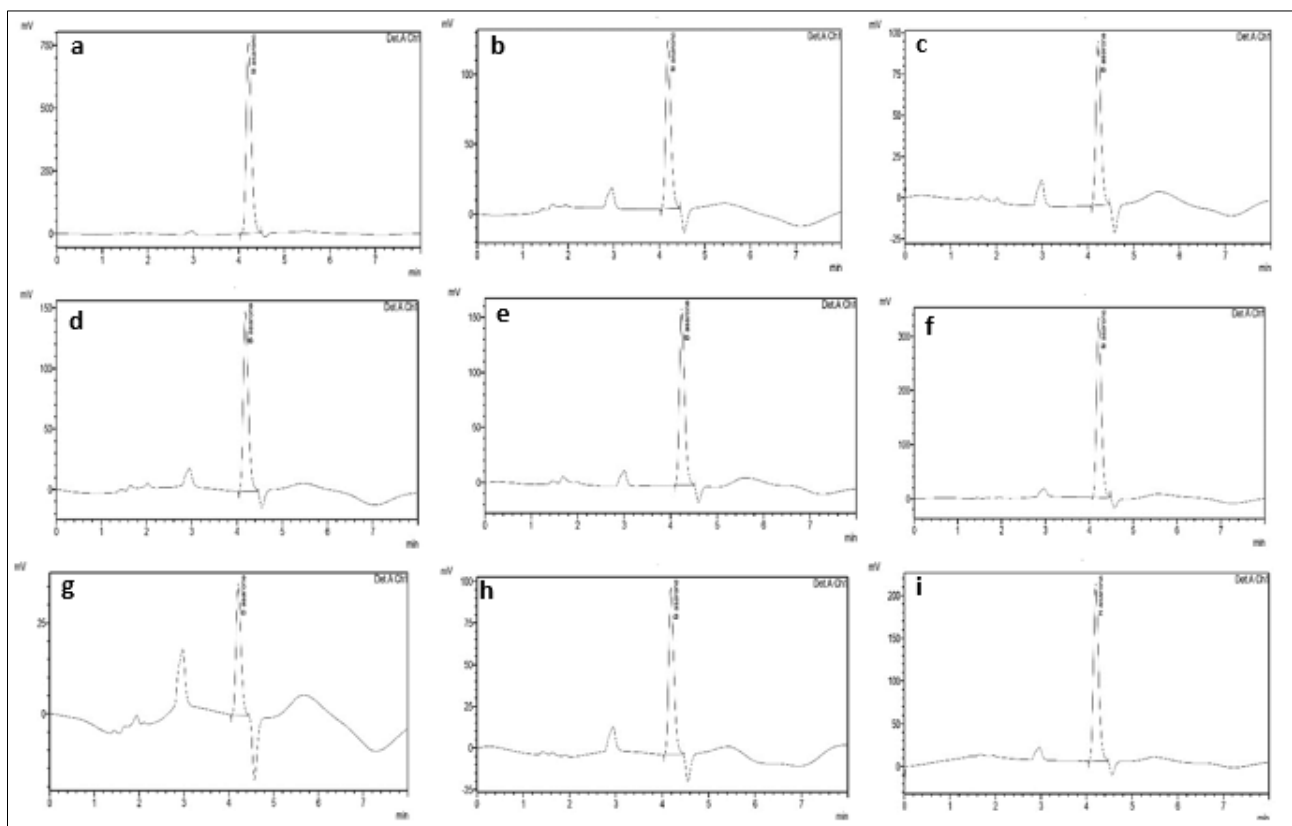


Fig 4: HPLC chromatogram of *A. calamus* collected from Tamil Nadu regions of the Western Ghats; (a) Prakashapuram-I, Kodaikanal (ACTND01), (b) Prakashapuram-II, Kodaikanal (ACTND02), (c) Kathadimattam, Ooty (ACTND03), (d) TR Nagar, Ooty (ACTND04), (e) Indunagar, Ooty (ACTND05), (f) Thalaikundwa, Ooty (ACTND06), (g) Pykara, Ooty (ACTND07), (h) Mangamala, Pechippara (ACTND08) and (i) Kolinjimala, Pechippara (ACTND09).

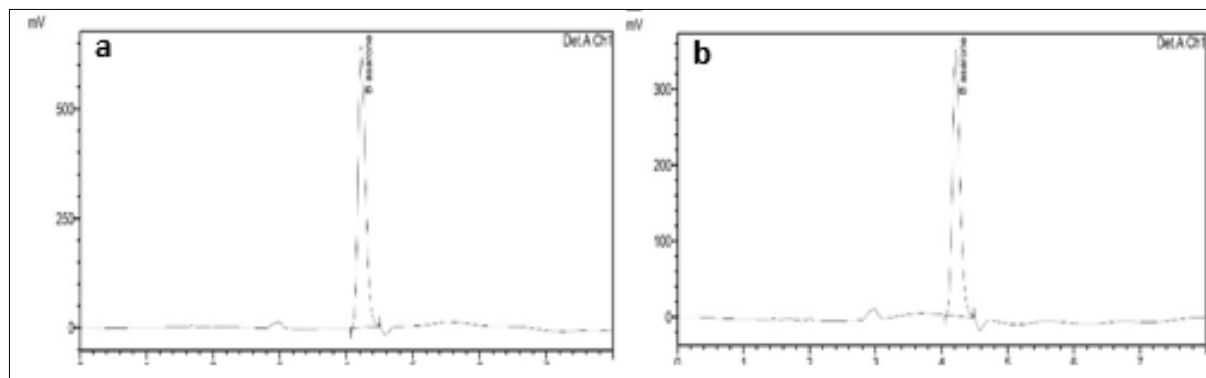


Fig 5: HPLC chromatogram of *A. calamus* collected from Karnataka regions of the Western Ghats; (a) Koratagere, Karnataka (ACKND01) and (b) Attigundi, Karnataka (ACKND02)

The current study is the first to document an *A. calamus* accession with a very low or negligible amount of β -asarone content (1.35 ± 0.00 mg/g) in Vattavada of Munnar region, Kerala and the other accessions listed in Table:1 collected from different geographical locations of the Western Ghats which showed low level of β -asarone content i.e., Nelliampathy region (ACKLA07- 5.54 ± 0.00 mg/g), Kerala, Prakashapuram regions (ACTND01- 6.37 ± 0.00 mg/g & ACTND02- 6.55 ± 0.00 mg/g) and Ooty regions (ACTND05- 6.98 ± 0.00 mg/g & ACTND06- 7.09 ± 0.00 mg/g) of Tamil Nadu. Two accessions collected from Kerala (Wayanad- 71.54 ± 0.00 mg/g and Thrissur- 58.81 ± 0.00 mg/g) regions showed high β -asarone content. Remarkable variations in β -asarone content in the collected accessions from different geographical locations of the Western Ghats was demonstrated in the present study. Avadhani *et al.*, 2016 reported the β -asarone content (2.44 mg/g - 8.30 mg/g) in *A. calamus* accessions from South and North-East India using HPLC analysis. From the earlier reports, the highest β -asarone content was obtained in the sample collected from Belgaum region, Karnataka and Nunmathi region, Assam with a concentration of 8.305 mg/g and 8.24 mg/g and the lowest content (2.44 mg/g) was reported in the sample collected from Dhansiri region, Assam^[2]. The β -asarone content (0.29 mg/g) in the *A. calamus* accession collected from Kerala was reported by Shailajan *et al.*, 2015. Previous reports suggests that variations in the β -asarone content of *A. calamus* depend upon its genetic factor, ploidy, geographical conditions and climatic factors^[2, 4].

Conclusion

The rhizome (dried/fresh) of *A. calamus* is one of the main ingredients in several polyherbal formulations which are used for the treatment of neurological and metabolic disorders and is other alcoholic beverages and food supplements. The FDA, EMA, and CFRT prohibited the use of *A. calamus* in pharmaceutical preparations, alcoholic drinks, and food products due to the high occurrence of β -asarone content in its rhizome which poses potential genotoxicity and carcinogenic effects to the consumer. The present investigation identified an elite clone of *A. calamus* accession with very less quantity of β -asarone from Vattavada, Munnar region of Kerala through RP-HPLC analysis. This elite accession can be used as a quality raw material for the medicinal purpose and production of polyherbal formulations.

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