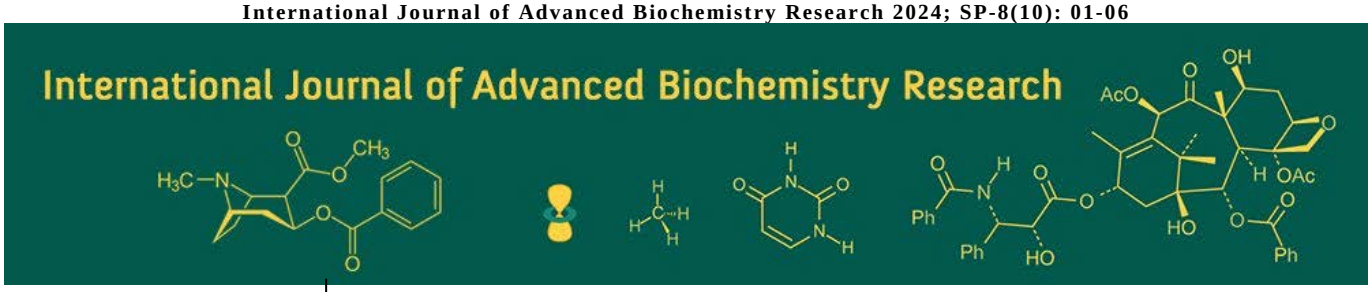

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Variation of essential oil and β-Asarone content among sweet flag (*Acorus calamus* L.) Accessions

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Abstract
In the present in, thirty eight different accessions of *Acorus calamus* L. were estimated by using hydro distillation method. The content of oil in the fresh and dry sweet flag rhizomes was 2.45% and 3.13%, while oil content in fresh and dry sweet flag leaves was 0.41% and 0.60%. The phytochemical analysis of β-asarone content was determined through HPLC method. The β-asarone content ranged from 6.45 mg g^{-1} to 9.96 mg g^{-1} in fresh rhizomes and 10.19 mg g^{-1} to 26.41 mg g^{-1} in dry rhizomes whereas 0.59 mg g^{-1} to 0.10 mg g^{-1} in fresh leaves and 9.01 mg g^{-1} to 1.48 mg g^{-1} in dry leaves. APAc-5 accession recorded highest essential oil content in dry rhizomes (3.13%) and fresh rhizomes (2.45%) whereas APAc-9 accession recorded highest essential oil content from dry leaves (0.60%) and fresh leaves (0.41%). Regarding β-asarone content APAc-8 accession recorded highest in dry rhizomes (26.41 mg g^{-1}), fresh rhizomes (9.96 mg g^{-1}), dry leaves (9.01 mg g^{-1}) and fresh leaves (0.59 mg g^{-1}) whereas the lowest β-asarone content was recorded in dry rhizomes (10.19 mg g^{-1}), fresh rhizomes (6.45 mg g^{-1}), dry leaves (1.48 mg g^{-1}) and fresh leaves (0.10 mg g^{-1}) of TNAc-9.

Keywords: *Acorus calamus* L., rhizomes, leaves, essential oil, β-Asarone, HPLC

Introduction
Acorus calamus L. (Family: Acoraceae) commonly known as sweet flag in English and vasa in Telugu, is one of the important medicinal plant which has been traditionally used in ayurvedic and unani medicines. This perennial herb is common on the banks of streams and in damp marshy places. *Acorus calamus* L. is an aromatic medicinal plant, rich in alkaloids, phenolics and flavonoids. The phyto-chemical ingredients of the plant are utilized worldwide for various purposes. Rhizomes, leaves and roots of the plant are used as traditional medicines in India and China (Wu *et al.*, 1994) [23]. It is widely employed in modern medicine due to its sedative, laxative, diuretic and carminative properties (Tamura *et al.*, 2004) [19].

The essential oil of *Acorus calamus* L. contains various chemical constituents and the proportion of each chemical constituent of the oil particularly β-asarone varied in different genotypes. The essential oil and their components extracted from *Acorus calamus* L. are found helpful in the treatment and removal of cancer (Bayala *et al.*, 2014) [2]. Essential oil plays a crucial role like sex attractants (Howlett, 1915) [7], antioxidant (Dapkevicius *et al.*, 1998) [4], antimicrobial agent (Veldhuizen *et al.*, 2006; Rojas *et al.*, 2007) [21, 17], insecticidal, antifeedant and repellent (Mahmoud and Croteau, 2002; Dudareva *et al.*, 2004) [20, 5] apart from usage in perfume and food industry (Tan and Nishinda, 2012) [20].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 (Venskutonis *et al.*, 2005) [22] 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 (Raina *et al.*, 2003) [16].

Many investigations into sweet flag rhizome essential oil constituents were made in the late 1970s and 1980s, when commercial interest in this species was high. The detection of the mutagenic and carcinogenic effects of β-asarone and other phenylpropene derivatives made the use of the plant less desirable (Goeggelmann and Schimmer, 1983) [6] and limits the use

of *A. calamus* for human purposes. β-asarone is considered a most important constituent in the rhizome essential oil of *A. calamus*. Raw material containing high percentages of β-asarone has been used as an insecticide and insect repellent for stored-product pest control (Jiyavorranant *et al.,* 2003) [8]. Its antifungal and bactericidal properties suggested further uses of the plant as a natural fungicide (Mungkornasawakul *et al.,* 2002; Park *et al.,* 2003) [13, 15]. Moreover, recent investigations demonstrated anti-proliferative, immuno-suppressive and anti-carcinogenic activity of *A. calamus* on human carcinoma cells (Mehotra *et al.,* 2003) [12]. The major compound β-asarone possess antifungal activity (Lee *et al.,* 2004) [9] and it is reported that the tetraploids have higher β-asarone content (70–96%) than the triploids (5–19%) and almost negligible in diploid genotypes. Most of the plants found in Indian subcontinent are predominantly triploids with high β-asarone contents (Ogra *et al.,* 2009) [14].

Material and Methods

Plant Material

The experiment was conducted at Horticultural Research Station, Venkataramannagudem that is situated at 16°83'N latitude and 81°5' E longitude with an altitude of 34 m (112 feet) above the mean sea level. This zone experiences hot, humid summer, mild winter and with an average annual rainfall of 900 mm. The soil is red loamy with good drainage and moderate water holding capacity. The physical composition of the soil was 70 percent sand, 20 percent silt and 15 percent clay. The soil p^H was 5.96 and the EC was 0.3 dSm^{-1}. The available nitrogen, phosphorus and potassium contents were 512 kg, 17.52 kg and 217.5 kg ha^{-1} respectively. The available organic carbon content of the soil was 0.34 percent.

Isolation of essential oil from leaves and rhizomes of *Acorus calamus* L.

Essential oils from rhizomes and leaves of *Acorus calamus* L. were isolated by hydro distillation in a clevenger-type apparatus (Cleavenger, 1928). The plant material (500 g) of fresh rhizomes and leaves was crushed and separately subjected to hydro-distillation in Clevenger apparatus for 3 hours by using the apparatus described in the European Pharmacopoeia by Maisonneuve and Ruffine, 1975 [11]. Later, the collected oil and water solution were separated using separating funnel.

Fresh rhizomes and fresh leaves were shade dried for 10 days to obtain dry rhizomes and dry leaves for oil extraction. Dry rhizomes and dry leaves were crushed into small pieces before oil extraction. The plant material (500 g) was separately subjected to hydro-distillation in Clevenger apparatus for 3 hours by using the apparatus described in the European Pharmacopoeia. Later, the collected oil and water solution were separated using separating funnel.

The essential oils obtained were stored at a low temperature (4°C in refrigerator) for further use. The oil yield (w/w) in rhizomes and leaves from all the thirty eight accessions was recorded and expressed in percentage.

Isolation of β-Asarone from leaves and rhizomes of *Acorus calamus* L.

Sample preparation

Fresh leaves and rhizome sample preparation:

Fresh leaf and rhizomes samples were collected and cleaned, then powdered in willey mill.

Dry leaves and rhizome sample preparation

Leaf and rhizomes samples were collected and cleaned; later shade dried for 10 days. The dried samples were separately grounded into a fine powder by using willey mill and preserved in an air tight container at room temperature.

Preparation of sample for estimation of β-asarone

Powdered samples of 0.1g each (fresh/dry, leaf/rhizome) were taken in a screw cap test tubes and extracted with 10 ml HPLC grade methanol by sonication for 10 minutes. The extract thus obtained was centrifuged at 12,000 rpm for 20 minutes. These extracted samples were then filtered through a millipore filter paper of 0.45 µm size which was finally injected into the HPLC sample injector for estimation of β-asarone (Avadhani *et al.* 2013) [1].

Preparation of Standard solution

Standard β-asarone of 10 mg was accurately weighed and transferred into 10 mL standard volumetric flask which was initially dissolved in minimum quantity of methanol, followed by sonication and then diluted up to the mark with methanol. A standard stock solution of 1000 µg/mL concentration was thus prepared. Working standard solution was prepared by serial dilution of the standard stock solution. Quality control samples were prepared at three concentrations of the linearity range (Shailajan *et al.,* 2015) [18].

Estimation of β-asarone from the samples and formulations of *A. calamus* L.

Relative retention time and relative peak area of each characteristic peak from the samples of *A. calamus* L. and its formulations related to the peak from β-asarone were calculated for quantitative expression of the chemical properties in the chromatographic pattern of *A. calamus* L. using regression equation (Figure 1).

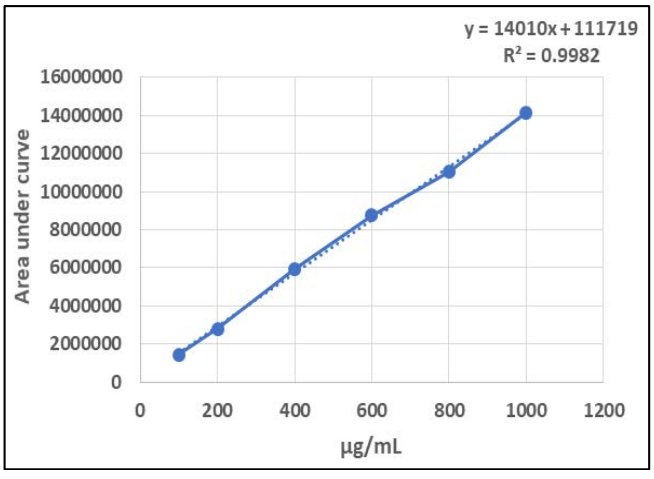

Fig 1: Linearity plot for β-asarone.

HPLC analytical conditions

HPLC consists of LC - 10ATVP pump, a rheodyne injector and SPD M10AVP photodiode array detector. The CLASS-VP 6.12 SP5 integration software was used for analysis. The stationary phase was Phenomenex Luna C 18 (2) (250 x 4.6mm) column having 5µ particle size with a C18 guard column (Phenomenex, 4 mm X 2.0 mm ID). The mobile phase consists of mixture of methanol (HPLC grade, Merck)

and water (5 mM) in the proportion of 65:35 v/v. The column was equilibrated with mobile phase for one hour and then pumped at the rate of 1.0 ml min^{-1}. The standard solutions were injected and the detector response was measured. Plant samples were assayed and the detection was done at 304 nm. By comparing the sample values with the standard values, β-asarone content was estimated and expressed as mg/g on fresh and dry weight basis.

Statistical evaluation

SPSS 23.0 software was used to determine mean, standard deviation and mean difference during the analysis.

Results and Discussion

Essential oil yields of leaves and rhizomes

The essential oils were obtained by conventional hydro-distillation of 38 *A. calamus* rhizomes and leaves on dry and fresh weight basis which were summarized in Table 1 and 2. Oils colour varied from light yellow to light brown with characteristic pleasant, slightly sweet odour. The yield of the essential oil from fresh and dry leaves was found to vary from 0.04% to 0.41% and 0.09% to 0.60% in different accessions with highest yield obtained for APAc-9, followed by APAc-5 (0.37% and 0.48%) and APAc-1 (0.35% and 0.45%). The lowest dry and fresh oil yield found to be 0.04% and 0.09% for the accession Sunitur-4. Whereas the yield of the essential oil for fresh and dry rhizomes varied from 0.38% to 2.45% and 1.78% to 3.13% in 38 accessions with highest oil yield recorded in APAc-5, followed by APAc-19 (2.35% and 2.85%) and APAc-20 (2.32% and 2.84%). The lowest oil yield for both fresh and dry rhizomes found to be recorded in Jorhat AAU-2 accessions (0.38% and 1.78%).

β-asarone content in leaves and rhizomes

β-asarone standard was injected into the HPLC and a standard chromatogram was obtained with an average retention time of 4.14 min for three replications (Figure 2). All the thirty eight accessions of fresh and dry leaves and rhizomes of *Acorus calamus* were also subjected to HPLC analysis which showed a retention time of around 4 minutes. The representative HPLC chromatograms for leaves and rhizomes were shown in Figure 3, 4, 5 and 6.

The concentration of β-asarone from fresh and dry rhizomes varied from around 6.45 to 9.96 mg g^{-1} and 10.19 to 26.41 mg g^{-1} whereas concentration of β-asarone content for fresh and dry leaves varied from 0.10 to 0.59 mg g^{-1} and 1.48 to 9.01 mg g^{-1}. The lowest value was observed for the accession TNAc-9 with a concentration of 6.45 mg g^{-1}, 10.19 mg g^{-1}, 0.10 mg g^{-1} and 1.48 mg g^{-1} of fresh and dry rhizomes and leaves. The details of the observed slides indication the β-asarone content of samples were mentioned in Table 3 and 4.

Table 1: Essential oil content (%) in leaves and rhizomes of sweet flag accessions.

Accessions	Essential oil content in leaves (%)		Essential oil content in rhizomes (%)	
	Dry leaves	Fresh leaves	Dry rhizomes	Fresh rhizomes
APAc-1	0.45	0.35	2.18	1.60
APAc-2	0.25	0.15	2.17	1.57
APAc-3	0.37	0.27	2.71	2.13
APAc-4	0.28	0.17	2.29	1.71
APAc-5	0.48	0.37	3.13	2.45
APAc-6	0.20	0.13	2.05	1.36
APAc-7	0.27	0.16	2.20	1.63
APAc-8	0.17	0.08	1.89	1.16
APAc-9	0.60	0.41	2.17	1.57
APAc-10	0.20	0.14	2.04	1.33
APAc-11	0.27	0.16	2.23	1.63
APAc-12	0.29	0.20	2.30	1.89
APAc-13	0.32	0.22	2.52	2.04
APAc-14	0.37	0.27	2.63	2.12
APAc-15	0.22	0.15	2.06	1.38
APAc-16	0.21	0.15	2.07	1.53
APAc-17	0.16	0.05	1.85	1.00
APAc-18	0.31	0.21	2.40	2.03
APAc-19	0.42	0.30	2.85	2.35
APAc-20	0.38	0.34	2.84	2.32
APAc-21	0.30	0.20	2.36	1.94
APAc-22	0.29	0.20	2.83	1.89
APAc-23	0.19	0.10	2.01	1.22
TNAc-8	0.17	0.08	1.89	1.16
TNAc-9	0.34	0.24	2.59	2.05
TNAc-12	0.15	0.05	1.83	0.73
TNAc-13	0.20	0.10	2.01	1.22
Gubbi	0.18	0.09	1.93	1.20
Symbolia	0.37	0.27	2.74	2.16
Raipur	0.37	0.29	2.79	2.19
Dipholoo-8	0.28	0.19	2.22	1.64
Sonitpur Timisuria-7	0.15	0.05	1.84	0.80
Dibrujoor-1	0.18	0.08	1.94	1.21
Jal Sibsagar-5	0.25	0.15	2.17	1.57
Bokakaatnamte-3	0.35	0.25	2.62	2.06
Golaghat-6	0.20	0.14	2.04	1.33
Sunitur-4	0.09	0.04	2.13	1.55
Jorhat AAU-2	0.16	0.06	1.78	0.38
Mean	0.27	0.18	2.27	1.60
S.E.	0.003	0.002	0.01	0.01
C.D. 5%	0.009	0.008	0.03	0.04

Table 3: β-asarone content in leaves and rhoizomes of sweet flag accessions (mg g^{-1}).

Accessions	β-asarone content in leaves (mg g^{-1})		β-asarone content in rhizomes (mg g^{-1})	
	Dry leaves	Fresh leaves	Dry rhizomes	Fresh rhizomes
APAc-1	6.70	0.56	24.00	9.60
APAc-2	4.78	0.48	20.71	9.26
APAc-3	8.63	0.59	25.29	9.77
APAc-4	5.25	0.50	21.70	9.47
APAc-5	4.50	0.43	17.03	8.77
APAc-6	6.17	0.55	22.89	9.59
APAc-7	6.16	0.54	22.65	9.59
APAc-8	9.01	0.59	26.41	9.96
APAc-9	4.62	0.46	19.75	9.07
APAc-10	6.66	0.56	23.74	9.70
APAc-11	5.66	0.51	22.15	9.49
APAc-12	5.41	0.50	21.92	9.44
APAc-13	6.01	0.52	22.63	9.49
APAc-14	4.44	0.37	15.89	8.79
APAc-15	5.09	0.50	21.33	9.36
APAc-16	5.06	0.49	20.89	9.33
APAc-17	5.79	0.51	22.53	9.52
APAc-18	8.39	0.57	24.57	9.74
APAc-19	4.68	0.48	20.10	9.31
APAc-20	4.60	0.46	18.34	8.86
APAc-21	4.94	0.48	20.77	9.25
APAc-22	4.68	0.48	20.46	9.17
APAc-23	2.75	0.23	11.50	7.78
TNAc-8	3.76	0.25	12.71	7.90
TNAc-9	1.48	0.10	10.19	6.45
TNAc-12	2.99	0.24	12.53	7.86
TNAc-13	4.07	0.29	12.88	8.27
Gubbi	4.53	0.45	17.67	8.29
Symbolia	4.18	0.29	13.84	8.58
Raipur	4.48	0.42	17.01	8.09
Dipholoo-8	2.17	0.14	10.65	7.01
Sonitpur Timisuria-7	2.52	0.21	11.46	7.52
Dibrujoor-1	2.91	0.23	12.01	7.79
Jal Sibsagar-5	2.49	0.21	10.69	7.44
Bokakaatnamte-3	4.24	0.30	14.51	8.53
Golaghat-6	1.66	0.12	10.62	6.65
Sunitur-4	4.44	0.37	15.11	8.58
Jorhat AAU-2	4.04	0.27	12.72	8.09
Mean	4.70	0.40	17.94	8.73
S.E.	0.05	0.004	0.14	0.02
C.D. 5%	0.15	0.012	0.42	0.08

Table 4: β-asarone content in rhizomes of sweet flag accessions (mg g^{-1}).

Accessions	β-asarone content in rhizomes (mg g^{-1})	
	Dry rhizomes	Fresh rhizomes
APAc-1	24.00	9.60
APAc-2	20.71	9.26
APAc-3	25.29	9.77
APAc-4	21.70	9.47
APAc-5	17.03	8.77
APAc-6	22.89	9.59
APAc-7	22.65	9.59
APAc-8	26.41	9.96
APAc-9	19.75	9.07
APAc-10	23.74	9.70
APAc-11	22.15	9.49
APAc-12	21.92	9.44
APAc-13	22.63	9.49
APAc-14	15.89	8.79
APAc-15	21.33	9.36
APAc-16	20.89	9.33
APAc-17	22.53	9.52
APAc-18	24.57	9.74
APAc-19	20.10	9.31
APAc-20	18.34	8.86
APAc-21	20.77	9.25
APAc-22	20.46	9.17
APAc-23	11.50	7.78
TNAc-8	12.71	7.90
TNAc-9	10.19	6.45
TNAc-12	12.53	7.86
TNAc-13	12.88	8.27
Gubbi	17.67	8.29
Symbolia	13.84	8.58
Raipur	17.01	8.09
Dipholoo-8	10.65	7.01
Sonitpur Timisuria-7	11.46	7.52
Dibrujoor-1	12.01	7.79
Jal Sibsagar-5	10.69	7.44
Bokakaatnamte-3	14.51	8.53
Golaghat-6	10.62	6.65
Sunitur-4	15.11	8.58
Jorhat AAU-2	12.72	8.09
Mean	17.94	8.73
S.E.	0.14	0.02
C.D. 5%	0.42	0.08

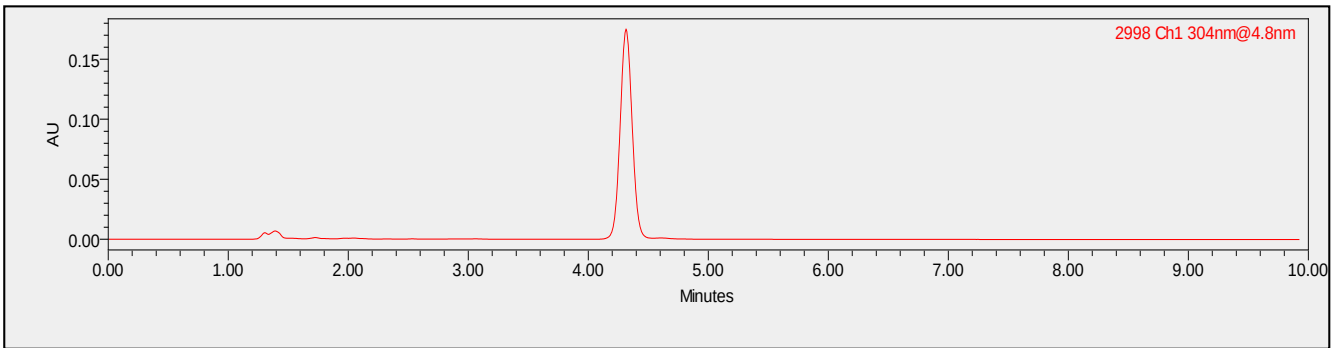

Fig 2: HPLC chromatogram of β-asarone standard.

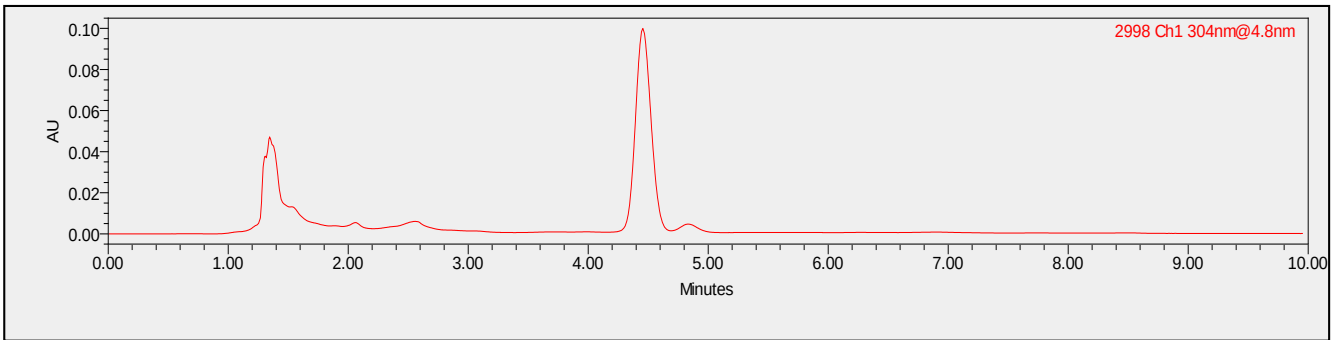

Fig 3: HPLC chromatogram of fresh leaves for β-asarone recorded lowest for TNAc-9 accession.

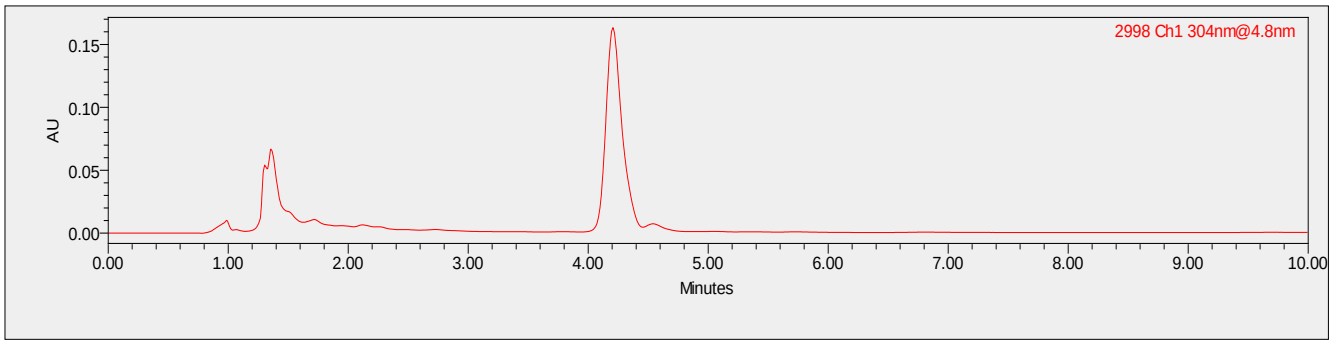

Fig 4: HPLC chromatogram of dry leaves for β-asarone recorded lowest for TNAc-9 accession.

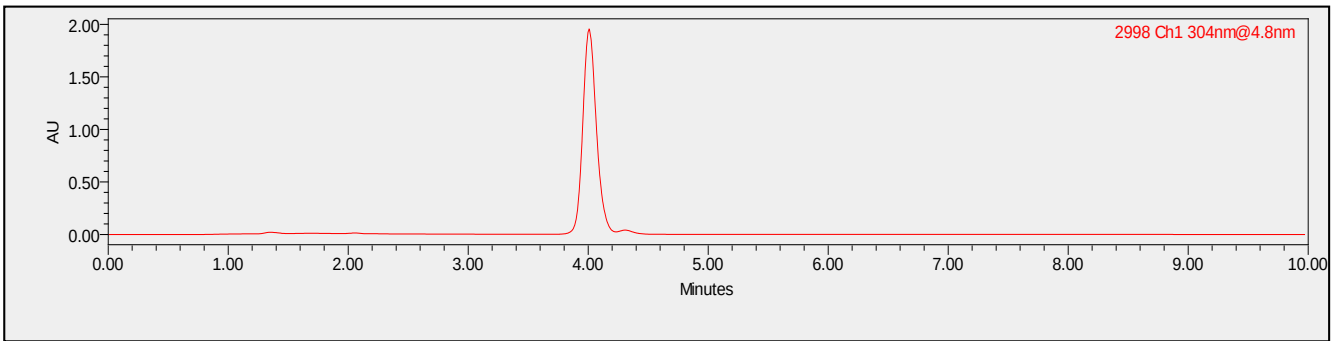

Fig 5: HPLC chromatogram of fresh rhizome for β-asarone recorded lowest for TNAc-9 accession.

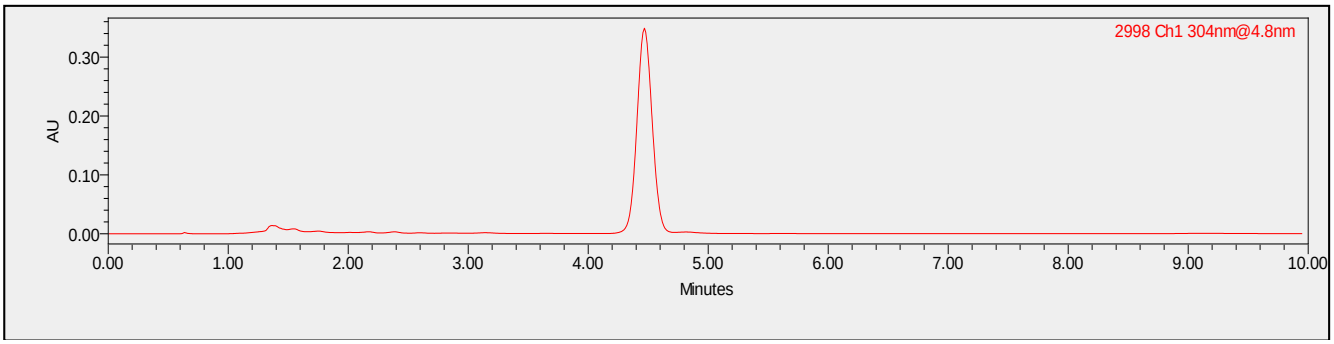

Fig 6: HPLC chromatogram of dry rhizome for β-asarone recorded lowest for TNAc-9 accession

Conclusion

Our study gives a brief insight into the determination of essential oil content and β-asarone content in various accessions of *Acorus calamus* L. through hydro-distillation and HPLC analysis. Among thirty eight sweet flag accessions, APAc-5 recorded maximum essential oil content in dry and fresh rhizomes, while APAc-9 recorded maximum essential oil content in dry and fresh leaves. Whereas β-asarone content was highest in APAc-8 and lowest in TNAc-9 for the data recorded from dry rhizomes, fresh rhizomes, dry leaves and fresh leaves.

Future Scope

1. Studies on seasonal influence on essential oil content and β-asarone content among 38 accessions can be carried out.
2. Cytogenetic studies among 38 sweet flag accessions to determine the correlation between ploidy level and β-asarone content can be done.

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