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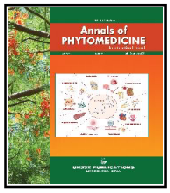

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GC-MS estimation of β-asarone in diploid *Acorus calamus* L. and its callus-mediated regeneration

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Abstract

Acorus calamus L. (Sweet flag) is an important aromatic and medicinal plant used worldwide. Diploid, triploid, tetraploid and hexaploid cytotypes occur in nature and with an increase in the ploidy level, the content of β-asarone increases. A high concentration of β-asarone is reported as toxic and causes mutation, chromosomal anomaly and cancers. Our objective was to assess β-asarone level in diploid cytotype and its callus-mediated regeneration. In this study, we investigated the β-asarone concentration by gas chromatography-mass spectrometry (GC-MS). We further attempted to develop a highly effective as well as consistent callus-mediated large scale production protocol. It was found to contain low concentration of β-asarone (0.67%). Optimum frequency induction of callus was carried out from shoot axillary buds which was cultured on Murashige and Skoog (MS) medium supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid. MS medium fortified with 0.5 mg/l a-naphthaleneacetic acid combined with 2 mg/l 6-benzylaminopurine gave the highest regeneration (90%) results with maximum number of micro-shoots which could grow into independent plantlets. It can be inferred that such a protocol would ensure the availability of elite planting materials of diploid *A. calamus* for large-scale drug manufacturing operations.

1. Introduction

The genus *Acorus* (Family: Acoraceae) has been used conventionally for medicinal purposes globally. The members of the Acoraceae family are rhizomatous or tuberous in nature. According to various reports, till now four species have been identified from this genus (Motley, 1994) in which *A. calamus* is widely studied. The people of Nepal, used to cure respiratory diseases by chewing the rhizome of *A. calamus* (Acharya *et al*., 2005). Other Asian countries like Japan, Korea, and India, also utilize the plant extracts to cure many diseases and decorate the rhizome cubes in their hair and necks to ensure good health (Xiao, 2005). *A. calamus* (Figure 1a) is a perennial coastal plant with branched rhizomes having various medicinal and aromatic properties. The plant is cultivated in India ascending to an altitude of 2200 m. Four cytotypes: diploids (2x=24), triploids (3x=36), tetraploids (4x=48), and hexaploids (6x=72) are recorded. Each cytotype shows significant morphological variation with large differences in the biochemical composition for rhizome and leaves volatile oils. The bioactive compounds present in rhizomes have been reported to show antimicrobial properties (Rajput *et al.,* 2014) and degenerative ailments like cancer and cardiovascular diseases (Mahmoudi *et al.*, 2016). Besides, it is also used in varied skincare and perfumery products. Homeopathy, Siddha and Ayurveda illustrate traditional medicines that have these properties (Ali, 2020). The ethanol extract has been recorded to possess antioxidant properties (Acuna *et al*., 2002). Whereas, aqueous extract was reported to show hypolipidemic activities at a dose of 200 mg/kg. *A. calamus* has the potential to therapeutically manage oxidative stress-related conditions (Balyan and Ali, 2022).

Major bioactive compounds reported from *A. calamus* include α- and β-asarone, acorone, asaryldehyde, methyl isoeugenol, caryophylene and safrol (Bertea *et al*., 2005). Phenolic and flavonoid compounds having pharmaceutical importance were also reported (Mhaveer *et al*., 2014). The percentage of β-asarone content present in the volatile oil differs based on the ploidal level of *A. calamus* (Ahlawat *et al*., 2010). Diploid cytotypes are distinguished by absence or having a meager amount of β-asarone (Ogra *et al*., 2009). Triploid varieties of Europe and North America contain 3-19% β-asarone while tetraploid cytotypes of India, Indonesia and Taiwan have about 96% β-asarone (Ogra *et al*., 2009). Certain accessions collected from Manipur were reported as diploid and triploid cytotypes (Devi *et al*., 2018). A previous investigation by Ahlawat *et al*. (2010) observed *A. calamus* from Manipur as triploids containing approximately 7-8% β-asarone as against 74.0-88.01% in tetraploids from other Indian states. It has been reported that β-asarone could cause cytotoxic effect (Abel, 1987) and moreover it can also cause chromosomal disorders and some types of cancers (Goggelman and Chimmer, 1983). These have indicated that diploid and triploid cytotype of *A. calamus* may be the safest starting resource to be utilized as either pharmaceutical drug or to give flavor to beverages.

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Moreover, medicinal and aromatic plants are integral part of healthcare and spiritual practices in rural India. It is driven by safety, low cost, accessibility and concerns over the adverse effects of conventional medications (Sharma, 2021). Devi *et al*. (2018) assessed 19 populations of *A. calamus* and identified the diploid and triploid accessions based on cytology with RAPD and ISSR markers.

The isolation of several components from the essential oil has been enormously expanded by the development of gas chromatography (Alexander, 1967). There have been many spectroscopic and chromatographic techniques such as TLC, HPTLC, NMR, GC-MS, HPLC and LC-MS for identification, separation and quantification of the rhizomes (Mythili *et al*., 2013; Devi *et al*., 2013). The present investigation aims to develope a fast, well-grounded, low-cost and authenticated analytical procedure for the identification and quantification of β-asarone by GC-MS method.

Since the diploid *A. calamus* with low β-asarone is scantly found in the wild, it is imperative to micropropagate for conservation and sustainable utilization. Until now, few reports are available on micropropagation of triploid cytotypes (Rani *et al*., 2000; Lee and Han, 2011; Sandhyarani *et al*., 2011) using micro shoot induction of the axillary buds obtained from rhizomes. The present investigation is the first report on plant regeneration through callus-mediated somatic embryos of diploid *A. calamus*.

2. Materials and Methods

2.1 Plant samples and cytotype verification

A. calamus accessions were collected from different natural habitats (Figure 2a) of Manipur, India (23°50'-25° 42' N; 92°58'-94°45' E; 772-1169 m altitude) and maintained in the Botanical Garden of Manipur University for future use. The plant was authenticated by Professor G. J. Sharma, Department of Life Sciences (Botany), Manipur University, India (Accession Number: MU/AC-4 for diploid and MU/AC-5 for triploid). Diploid and triploid cytotypes are compared for β-asarone content (Table 1). The natural habitats were mostly associated with marshy swamps, river banks, lakes, streams and ponds. Ploidy verification was carried out according to the protocol of Devi *et al*. (2018).

For the biochemical analysis, the rhizomes (Figure 2c) were washed and cut into smaller pieces and then dried at 45°C separately. For some selected samples, the dried rhizomes (25 g) were extracted in a Soxhlet extractor using ethyl acetate (1000 ml) as solvent and extracts were concentrated using a vacuum evaporator. Extraction procedure was repeated three times for each sample. Then, the oil yield percentage was calculated.

2.2 GC-MS estimation

GC-MS procedures were conducted on Clarus-680 Gas Chromatography and Clarus-600C Mass Spectrophotometry system (Perkin Elmer, USA), column length with 60.0 m, and thickness coating of 250 µm. GC conditions: starting temperature at 60°C for 3 min, incline condition 4°C/min to 200°C, hold for 5 min, ramp 8°C/min to 260°C, split 0:1, sample capacity 2 µl, solvent delay time=10.00 min, transfer temperature = 200°C. Helium with a continuous flow at 1.0 ml/min was employed as carrier gas. MS was run with full scan at 70 eV. Biochemical compounds confirmation was carried out after comparing the relative retention time with standard compounds run under similar conditions. The quantity of β-asarone was calculated from peak areas of GC by normalization method as follows: Percentage content = Area percentage of individual peak/sum of all peak areas × 100.

2.3 Callus-mediated plant regeneration

2.3.1 Culture medium and condition

MS medium (Murashige and Skoog, 1962) with 3% sucrose was used for the experiment. The pH was adjusted to 5.7-5.8 using 1N NaOH or 1N HCl and varying concentrations of 6-benzylaminopurine (BAP) and 1-naphthalene acetic acid (NAA) was added to the medium. The media with 8% agar was autoclaved for 21 min at 121°C. The growth chamber was maintained at a temperature of 25 ± 1°C and 16h photoperiod with 30-35 µmol m^2/s light intensity.

2.3.2 Callus induction and regeneration

Only the leaf base, axillary bud and root tip were collected and surface sterilized for 1 min in a solution containing 1.0% carbendazim 50% and two drops of Tween-20. Further, it was sterilized with 70% alcohol for at least 1 min, followed by 7 min exposure to an aqueous solution of sodium hypochloride (1%) and finally rinsed with sterile distilled water for three times. After sterilization, explants were inoculated on MS basal medium (Raghavan, 2004) fortified with 2,4-dichlorophenoxyacetic acid (0.5-2.5 mg/l) for initial callus induction. Each treatment consisted of 10 explants and the experiments were conducted two times with 3 replicates.

2.3.3 Shoot regeneration and acclimatization

Superior embryogenic and fresh calluses were chosen and inoculated on medium fortified with a varying concentration and combination of BAP (0.5-2.5 mg/l) and NAA (0.5-2.5 mg/l). Root production was facilitated by transferring the regenerated shoots to half-strength basal MS medium fortified with varied combinations of indole-3-butyric acid (IBA) (0.5-2.0 mg/l) and NAA (0.5-2.0 mg/l) for four weeks. Plantlets were transferred and acclimatized in sterilized soil.

2.3.4 Statistical analysis

Each experiment was set up in a completely randomized block design and repeated twice. Data were subjected to analysis of variance (ANOVA) and means were evaluated by Tukey's test of SPSS (Version 14, SPSS Inc., Chicago, USA).

3. Results

3.1 Cytotype verification

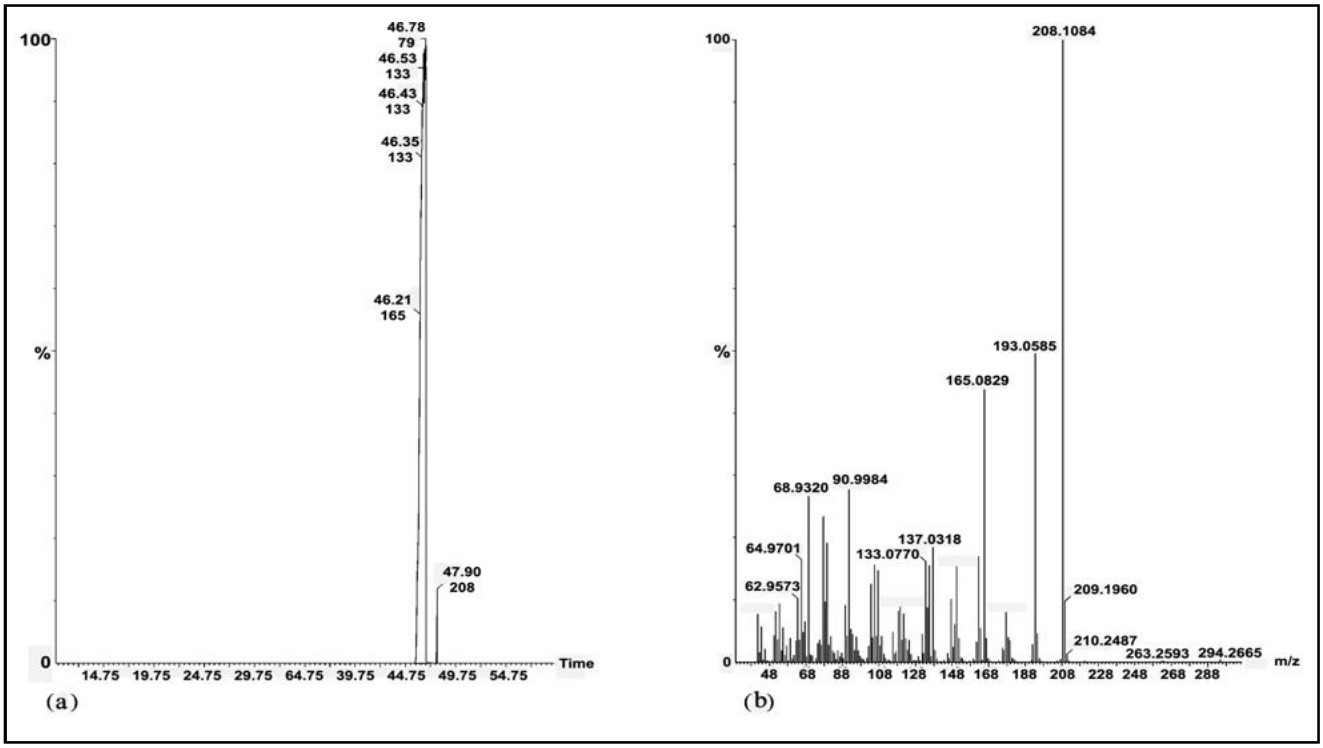
Root tip chromosome count was found as diploids (2n = 24) (Figure 2b).

3.2 GC-MS analysis for β-asarone concentration

The yield of the volatile oil from the hydrodistillation of the rhizome of *A. calamus* (diploid cytotype) was 1.90% (Table 1). When the ethyl acetate extract was analyzed by the GC-MS method β-asarone was identified at retention time (RT) of 47.90 min and its concentration was found to be 0.67% relative to the standard compound. The GC chromatogram along with the mass spectrum of β-asarone is represented in Figures 1a and b.

Table 1: Oil yield and percentage of β-asarone contents in diploid *A. calamus*

Accession code	Ploidy	Oil yield (%)	β-asarone (%)
MU/AC-4	2n = 2x = 24	1.90	0.67
MU/AC-5	2n = 3x = 36	1.07	8.0



Figures 1 (a, b): GC-MS analysis of ethylacetate extract of rhizome of diploid *A. calamus*. (a) GC chromatogram of β-asarone at 47.90 min RT, (b) MS spectra of β-asarone.

3.3 Callus induction and regeneration

Explants of axillary bud exhibited higher callusing formation when compared with leaf base and root tip. When, the explants were grown on MS medium containing 2.0 mg/l 2,4-D axillary bud explants developed callus with a maximum response of 93% (Figure 1d), while leaf base explants exhibited a maximum of 73%. Root tip explants failed to respond to the treatment. Use of 2,4-D at low concentration produced hard callus, however, the callus texture became friable with greenish colour at higher concentration. High concentration (2.5 mg/l), resulted in the production of lower percentage of callus (Table 2). Only the greenish calli were able to regenerate into adventitious shoots on medium with varying combination and concentrations of NAA and BAP (Table 3). Media containing varying concentrations of BAP could induce shoot regeneration with maximum response at 2.0 mg/l (5.8 plants per callus mass). However, the overall best response (90%) with a maximum shoot number (7.8) was found in the medium supplemented with 0.5 mg/l NAA and 2.0 mg/l BAP (Figure 1e). Responsive plantlets were able to give roots on half-strength MS medium containing varying IBA and NAA concentrations (Table 4). Indolebutyric acid at 2.0 mg/l gave the most favourable results with 4.5 roots per shoot. Plantlets with roots were carefully transferred to small PVC pots containing sterilized soil (Figure 1f) and allowed to grow in laboratory condition for 30 days, and then transferred to the field.

Table 2: Influence of various concentrations of 2,4-D on callus induction in diploid *A. calamus* for different explants types

MS + 2,4-D(mg/l)	Types of explant (% callus induction)		
	Leaf base	Axillary bud	Root-tip
0.5	27 ± 0.16^{d}	45 ± 0.18^{d}	–
1.0	54 ± 0.11^{c}	62 ± 0.23^{c}	–
1.5	69 ± 0.09^{b}	74 ± 0.25^{b}	–
2.0	73 ± 0.04^{a}	93 ± 0.27^{a}	–
2.5	50 ± 0.03^{c}	70 ± 0.11^{b}	–

Values are mean ± standard error. Means followed by different letters indicate significant differences among treatments by Tukey's b test (p=0.05). Data recorded after six weeks of inoculation.

Table 3: Influence of BAP and NAA on regeneration percentage and number of callus-derived micro-shoots of *A. calamus*

Plant growth regulators (mg/l)		Regeneration (%)	Number of micro-shoots
BAP	NAA		
-	-	-	-
0.5	-	12 ± 0.17^{g}	2.2 ± 0.12^{g}
1.0	-	33 ± 0.09^{f}	3.5 ± 0.14^{f}
1.5	-	47 ± 0.5^{d}	4.0 ± 0.06^{e}
2.0	-	61 ± 0.06^{c}	5.8 ± 0.06^{c}
2.5	-	43 ± 0.11^{e}	4.6 ± 0.18^{d}
-	0.5	-	-
-	1.0	-	-
-	1.5	-	-
-	2.0	-	-
0.5	0.5	32 ± 0.4^{f}	4.3 ± 0.17^{e}
1.0	0.5	49 ± 0.3^{d}	5.2 ± 0.09^{c}
1.5	0.5	76 ± 0.8^{b}	6.1 ± 0.10^{b}
2.0	0.5	90 ± 0.5^{a}	7.8 ± 0.02^{a}
2.5	0.5	64 ± 0.2^{c}	6.2 ± 0.09^{b}

Values are mean ± standard error. Means followed by different letters indicate significant differences among treatments by Tukey's b test (p=0.05). Data recorded after six weeks of inoculation.

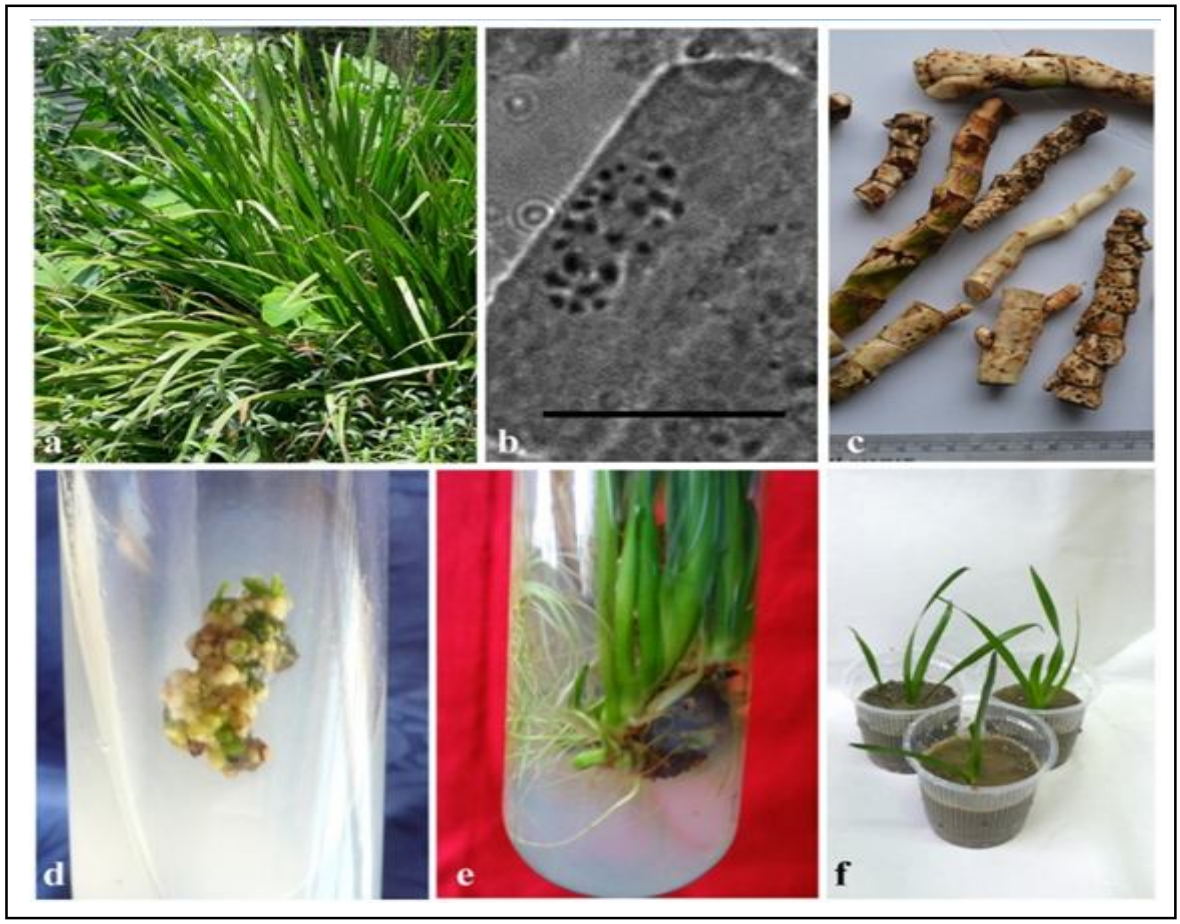


Figure 2 (a-f): Callus-mediated regeneration of diploid *A. calamus*. a: Plants at habitat, b: Photomicrograph of *A. calamus* root-tip chromosomes (2n=2x=24), bar=10 µm, c: Rhizomes, d: Callus showing regeneration of micro-shoots, e: Well-developed shoots with roots on MS medium, f: Hardened plantlets.

Table 4: ***In vitro*** **rooting of** ***A. calamus*** **on half-strength MS medium with various concentrations of IBA and NAA**

Plant growth regulators (mg/l)		Number of roots per shoot
IBA	NAA	
0.5	-	-
1.0	-	1.8 ± 0.7^{c}
1.5	-	2.4 ± 0.8^{ab}
2.0	-	4.5 ± 0.4^{a}
-	0.5	-
-	1.0	-
-	1.5	-
-	2.0	-

Values are mean ± standard error. Means followed by different letters indicate significant differences among treatments by Tukey's b test (p=0.05). Data recorded after four weeks of inoculation.

4. Discussion

Earlier Mcgraw *et al*. (2002) and Bertea *et al*. (2005) reported that Indian *A. calamus* cytotypes possessed excessive β-asarone and tetraploid in nature. Later, the occurrence of triploid and diploid cytotypes was reported and observed that triploid cytotypes contained 82.0 to 89.4% β-asarone in their oil, whereas two diploid cytotypes contained 11.67% and 7.39%, respectively (Ogra *et al*., 2009). Ahlawat *et al*. (2010) found *A. calamus* from Manipur as triploids containing 7.0-7.85% β-asarone. However, in this present study, Devi *et al*. (2018) reported diploid cytotype from Manipur to contain lower β-asarone (0.67%). Since a very low quantity of asarone has potent pharmaceutical applications which is solely found in diploid plant varieties, it will be useful to apply it as a chemical marker (Rasheed *et al*., 2012) and to control its quality (Rasheed *et al*., 2013). Our earlier investigation on genetic diversity assessment using 18 RAPD and 7 ISSR markers was able to cluster separately the diploid and triploid cytotype accessions found in Manipur (Devi *et al*., 2018). Several investigators have been incorporating 2,4-D in micropropagation of various crops including *Arabidopsis* (Raghavan, 2004). Our experimental results indicated that the use of 2.0 mg/l 2,4-D was highly effective in inducing callus formation. However, other investigators obtained good amount of callus induction with optimum concentrations at 1.0 mg/l for *Arabidopsis* (Raghavan, 2004). In conformity with our result, Tao *et al.* (2001) obtained leaf-derived shoot regenerated callus of *Citrus grandis* treated with 1.5 mg/l BAP while Yan *et al*. (2009) described plant shoot regeneration from callus of *Allium chinense* on Gamborg B5 medium fortified with 0.1 mg/l α-naphthaleneacetic acid and 1.0 mg/l 6-benzylaminopurine. On the contrary, Ahmad *et al*. (2010) recorded lower BAP and higher NAA concentration to be the best percentage for microshoot formation from callus of *Ruta graveolens*. Moreover, reports were found that full strength MS medium containing a high amount of nitrogen was found to inhibit root growth (Dubrovsky *et al*., 2009).

5. Conclusion

These studies have notably bestowed the present knowledge of β-asarone content in different cytotypes of *A. calamus*. The diploid cytotypes collected from Manipur, India were found to possess very low β-asarone (0.67%). Different cytotypes show variation in morphology as well as rhizome essential oil concentration. Triploids seem to have limitations in the development of pharmaceutical drugs due to high β-asarone levels and diploids with very low contents are considered to show greater potential as raw materials. The present investigation also successfully established callus mediated regeneration protocol for the elite cytotype (diploid) of *A. calamus*. As the wild populations are highly threatened in fragile wetland ecosystems, strategies are needed for the germplasm conservation of this species. The mass-scale propagation of the diploid cytotypes through plant tissue culture would significantly improve the availability of *A. calamus* for pharmaceutical drug manufacture and therapy.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

References

Abel, G. (1987). Chromosome damage in human lymphocyte induced by β-asarone. Planta Med., **53**:251-253.

Acharya, K.P. and Rokaya, M.B. (2005). Ethnobotanical survey of medicinal plants traded in the streets of Kathmandu Valley. The Sci. World J., **3**(3):44-48.

Acuna, U.M.; Atha, D.E.; Ma, J.; Michael, H.N. and Kennelly, E.J. (2002). Antioxidant capacity of ten edible North-American plants. Phytother. Res., **16**(1):63-65.

Ahlawat, A.; Meenu, K.; Gandhi, R. and Ashok, A. (2010). Genetic diversity in *Acorus calamus* L. as revealed by RAPD markers and its relationship with β-asarone content and ploidy level. Scientia Hort., **124**(2):294-297.

Ahmad, N.; Faisal, M.; Anis, M. and Aref, M. (2010). *In vitro* callus induction and plant regeneration from leaf explants of *Ruta graveolens* L. S. African J. Bot., **76**:597-600.

Alexander, T.S. (1967). The separation and identification of the components of the aromatic ether fraction of essential oils by gas-liquid chromatography. J. Chromatogr., **30**:54-61.

Ali, A. (2020). Herbs that heal: The philanthropic behavior of nature. Ann. Phytomed., **9**(1):7-17.

Balyan, P. and Ali, A. (2022). Comparative analysis of the biological activities of different extracts of *Nigella sativa* L. seeds. Ann. Phytomed., **11**:577-587.

Bertea, C.M.; Azzolin, C.M.; Bossi, S.; Doglia, G. and Maffei, M.E. (2005). Identification of an EcoRI restriction site for a rapid and precise determination of β-asarone free *Acorus calamus* cytotypes. Phytochm., **66**(5):507-514.

Devi, S.A.; Subhasini, S. and Babu, S. (2013). Purification of beta-asarone from *Acorus calamus* L. Res. J. Pharm. Biol. Chem. Sci., **4**:1279-1284.

Devi, N.S.; Kishor, R. and Sharma, G.J. (2018). RAPD and ISSR molecular marker variations in *Acorus calamus* Linn. Eur. J. Biomed. Pharm. Sci., **5**(1):651-658.

Dubrovsky, J.G.; Soukup, A.; Napsucialy-Mendivil, S.; Jeknic, Z. and Ivanchenko M.G. (2009). The lateral root initiation index: An integrative measure of premordium formation. Ann. Bot., **103**:807-817.

Goggelman, W. and Chimmer, O. (1983). Mutagenicity testing of β-asarone and commercial *calamus* drugs with *Salmonella typhimurium*. Mutation Res., **121**(3-4):191-194.

Lander, V. and Schreier, P. (1990). Acorenone and γ-asarone-indicators of the origin of *calamus* oils (*Acorus calamus* Linn.). Flav. Frag J., **6**:75-79.

Lee, J.H. and Han, T.H. (2011). Micropropagation of the plantlets derived from seeds in the genus *Acorus* (*A. calamus* and *A. gramineus*). Hort. Env. Biotechnol. J., **52**(1):89-94.

Hutchings, A.; Scott, A.H.; Lewis, G. and Cunningham, A.B. (1997). Zulu medicinal plants: an inventory. University of Natal Press, Piettermaritzburg, South Africa., **60**:955.

Mahmoudi, S.; Khali, M.; Benkhaled, A.; Benamirouche, K. and Baiti, I. (2016). Phenolic and flavonoid contents, antioxidant and antimicrobial activities of leaf extracts from ten Algerian *Ficus carica* L. varieties. Asia Pacific J. Trop. Biomed., **6**(3):239-45.

Mhaveer, S.; Shahid, H.A. and Sayeed, A. (2014). Production and estimation of asarone in *in vitro* cultures of *Acorus calamus* Linn. Ann. Phytomed., **3**(2):82-86.

Motley, T.J. (1994). The ethnobotany of sweet flag, *Acorus calamus* (Araceae). Econ. Bot., **48**:397-412.

Munshi, M.K.; Roy, P.K.; Kabir, M.H. and Ahmed, G. (2007). *In vitro* regeneration of cabbage (*Brassica oleracea* L. var. *capitata*) through hypocotyls and cotyledon culture. Plant Tiss. Cult. Biotechnol., **17**(2):131-136.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant., **15**(3):473-497.

Mythili, M.N.; Immanuel, S.C.; Rajasekharan, P.E.; Rajasekaran, C.; Tharachand, C. and Rao, V.K. (2013). HPLC profiling of β-asarone content and cytogenetic studies of medicinally important Indian *Acorus calamus* L accessions. Intern. J. Pharm. Sci. Rev. Res., **22**(2):73-78.

Ogra, R.K.; Prashant, M.; Upendra, K.; Sharma, M.; Sinha, A.K. and Ahuja, P.S. (2009). Indian *Acorus calamus* is not a tetraploid. Curr. Sci., **97**:10-11.

Raghavan, V. (2004). Role of 2,4-dichlorophenoxyacetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. American J. Bot., **91**(11):1743-1756.

Rajput, S.B.; Karuppayil, S.M. and Tonge, M.B. (2014). An overview on traditional uses and pharmacological profile of (sweet flag) and other *Acorus species'* phytomedicine. Int. J. Phytothr. Phytopham., **21**(3):268-76.

Rani, A.S.; Subhadra, V.V. and Reddy, V.D. (2000). *In vitro* propagation of *Acorus calamus* Linn.: A medicinal plant. Indian J. Expt. Biol., **38**(7):730-732.

Rasheed, N.M.A.; Nagaiah, K. and Waheed, M.A. (2013). Recent analytical techniques in quality control of indigenous system of medicine. Ann. Phytomed., **2**(1):44-58.

Rasheed, N.M.A.; Nagaiah, K.; Goud, P.R. and Sharma, V.U.M. (2012). Chemical marker compounds and their essential role in quality control of herbal medicines. Ann. Phytomed., **1**(1):1-8.

Sandhyarani, N.; Kishor, R. and Sharma, G.J. (2011). Clonal propagation of triploid *Acorus calamus* Linn using dual phase culture system. J. Crop Sci. Biotechnol., **14**(3):85-95.

Sharma, V. (2021). Ayurveda and remedial plants in medicine. Ann. Phytomed., **10**(1):1-5.

Xiao, F. (2005). The origin and customs of the Dragon Boat Festival. Encyclopedic Knowledge., **12**:57-59.

Yan, C.J. and Zhao Q.H. (1982). Callus induction and plantlet regeneration from leaf n blade of *Oryza sativa* L. Subsp. *indica*. Plant Sci. Lett., **25**:187-192.

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