Annals of Phytomedicine 3(2): 82-86, 2014

Journal homepage: www.ukaazpublications.com

ANNALS OF PHYTOMEDICINE
An International Journal

ISSN 2393-9885

Production and estimation of asarone in *in vitro* cultures of *Acorus calamus* Linn.

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Received August 2, 2014: Revised October 14, 2014: Accepted November 30, 2014: Published online December 30, 2014

Abstract

Acorus calamus Linn., a traditionally known drug, used in Ayurvedic and Unani systems of medicine, find its diversified uses in treatment of different neurodisorders. Recently, beta asarone main ingredient of calamus oil is reported to have antiobese action whereas plant extract and alpha asarone have already been reported to contain such activity. The whole plant contains oil glands, containing terpenes that in turn, create difficulty to initiate calli from the explants. However, efforts have been made by employing MS and B5 Gamborg media, supplemented with different growth regulators. The excellent shoot regeneration was observed in MS basal media, supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm). The distinguishing effects were noticed on the growth of the shoots by altering the nutritional content of MS medium. Simultaneously, estimation of its major constituents, alpha and beta asarones was carried out by HPTLC method in different cultured shoots along with assay of total phenolic and flavonoidal contents. The present research work can be used for the production of high content of therapeutically active constituents of calamus as well as for quality control of the drug, using HPTLC method.

Key words: *Acorus calamus* Linn., tissue culture, HPTLC, alpha asarone, beta asarone

1. Introduction

Acorus calamus Linn. belonging to family - Araceae, is an aromatic, semiaquatic or marshy, perennial herb found in both temperate and sub temperate zones. Dried rhizomes of the plant are mainly utilized, containing not less than 1.5% of volatile oil. The plant has been traditionally employed in treatment of various disorders in Ayurvedic and Unani systems of medicine. The rhizomes find their use in Ayurveda in various disorders like epilepsy, schizophrenia, constipation, cough, asthma, weakness of memory (Gupta and Tandon, 2004) *etc*. Calamus found useful in the treatment of bronchial asthma (Rajasekharan and Srivastava, 1977) and possesses various neuropharmacological actions (Martis *et al*., 1991) as anticonvulsants, memory enhancing (Oh *et al*., 2004) as well as antibacterial activities (Ahmad and Aqil, 2007) and found effective as analgesic (Vohra *et al*., 1989). The volatile oil obtained by alcoholic extraction of the rhizome is mainly employed in the pharmaceutical industries (Bertea *et al*., 2005) at commercial scale. The recent report suggested the use of beta asarone as antiobese drug (Lee *et al*., 2011). The calamus extract with saponins also produced hypolipidemic action in rats (Parab and Mengi, 2002), whereas alpha asarone already reported to have hypolipidemic actions (Garduno *et al*., 1997). The diploid variety (American calamus) of the plant possesses almost negligible amount of beta asarone as compare to triploid and tetraploid varieties (Indian calamus) (Stahl and Keller, 1981; Keller and Stahl, 1983). In the present study, *in vitro* cultures were developed and their qualitative and quantitative estimation of alpha and beta asarone was carried out by a HPTLC method reported by our laboratory (Singh *et al*., 2011). For the separation of alpha and beta asarone isomers, caffeine impregnated TLC plates were utilized and quantification was achieved in the both cultured shoots and in the natural leaves. Due to valuable therapeutic role of phenolic and flavonoid contents, it was envisaged to carried out their estimation by using UV spectrophotometer in natural leaves and in 60 days old developed cultured shoots in different hormone combinations. Changes in shoot growth and variation in the chemical composition of cultured shoots were also reported by changing the nutritional contents of the medium.

2. Material and Methods

Fresh leaf explants and rhizomes of calamus were collected from Herbal garden, Jamia Hamdard, New Delhi and authenticated by Dr. H. B. Singh (Head, Raw Material and Herbarium Division, NISCAIR, New Delhi, 110012).

All the chemicals, *viz*., growth hormones and culture media, Murashige and Skoog (MS) medium and Gamborg B5 medium, used for culture media preparation and solvents toluene, ethyl acetate were of analytical grade and obtained from Merck India Company. Standard asarones, rutin and gallic acid were purchased from Sigma Aldrich Pvt. Ltd with purity > 98%.

2.1 Tissue culture study

2.1.1 Preparation of stock solution

The stock solutions of major, minor and vitamins were prepared separately. The stock solutions (01 mg mL^{-1})of auxins were prepared

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by dissolving indole acetic acid (IAA), naphthaline acetic acid (NAA), indole 3 butyric acid (IBA) and 2, 4-dichlorophenoxy acetic acid (2, 4-D) in alcohol whereas cytokinins like kinetin, benzyl adenine were prepared by dissolving them in doubled distilled water, containing few drops of 0.1 N hydrochloric acid. All the stock solutions were stored in refrigerator at 5-6°C, however, they were kept back to the ambient temperature before using them for the media preparation.

2.1.2 Preparation of culture media

MS basal media and Gamborg (B5) media were used for the present study with different hormone combinations with varying concentrations. The different growth regulators were added to the respective tubes after dividing desired volumes from stock media and the pH was adjusted to 5.6, agar 1% was added with gentle heating to the medium and divided equally into all of the culture tubes, and then the tubes were plugged with cotton buns and kept for sterilization in an autoclave.

2.1.3 Preparation of explants for inoculation

Fresh leaves and rhizome explants were washed thoroughly in running tap water for 30 min, soaked in 10 % soap solution for 15 min, followed by washing under running tap water and finally with double distilled water. The explants were transferred in to laminar flow. Surface sterilization of explants was carried out by using 0.1 % w/v solution of mercuric chloride by keeping contact period of 8 min for rhizome and 5 min for leafy explants, respectively. It was then washed with sterile double distilled water to remove sterilizing agent. Appropriate sized explants were then inoculated on a medium under strict aseptic conditions. The inoculated cultures were incubated in the culture room maintained at 25 ± 2°C under fluorescent light with an intensity of 60 UE / M^2/ sec. Photoperiod of 16 h was maintained by keeping relative humidity to 65-75% with the help of an air cooling system. Incubation conditions were maintained at constant rate throughout the experiment.

2.1.4 Development of culture

The initiated cultured shoots from rhizome explants in the selected hormonal combinations were subcultured to fresh media, supplemented with the same hormone combinations for their further development and maintenance. These were subcultured at regular intervals and growth was monitored at the interval of 20 days up to growth period of 60 days.

2.2 Analytical study

2.2.1 Phytochemical screening

The phytochemical screening was carried out in methanolic extracts of cultured shoots and natural leaves to ascertain the presence of phytoconstituents, using standard chemical tests (Ahmad, 2007) for alkaloids, carbohydrates, glycosides, phenolic compounds and tannins, flavonoids, proteins and amino acids, saponins, mucilage, steroids and terpenoids.

2.2.2 Preparation of samples and standards

Extracts were prepared by refluxing 1.0 gm of 60 days old dried cultured shoots with methanol. It was filtered, followed by evaporation on a water bath to get rid of excess of solvent. Residue collected, was further reconstituted in methanol to get 10 mg mL^{-1} concentration. The same procedure was repeated for the preparation of extract of natural leaves. Stock solutions of rutin and gallic acid were prepared to the concentration of 1.0 mg mL^{-1} for carrying out total flavonoidal and phenolic contents, respectively. Methanolic extracts of cultured shoots were taken for the quantitative estimation of alpha and beta asarone. Stock solution of standard alpha and beta asarone (1.0 mg mL^{-1}) were prepared in methanol.

2.2.3 Estimation of total phenolic and total flavonoids

Spectral and absorbance measurements were carried out on a SHIMADZU UV-2401 spectrophotometer, by using 1.0 cm quartz cells for the estimation of total phenolic and flavonoid contents.

Total phenolic contents: Different dilutions of standard gallic acid were prepared in methanol in the concentration range of 25 to 300 µg mL^{-1}. To carry out the estimation, each standard dilution of 0.5 mL was added to 5.0 mL of F.C. reagent (10%) and 4 mL of Na_2CO_3 (1M) solution similarly 0.5 mL of each of sample extracts were taken and followed by addition of same reagents and kept for 15 min for colour development. Absorbance was taken at 765 nm against blank solution containing 0.5 mL of methanol and 5 mL F.C. reagent along with 4 mL of Na_2CO_3 solution.

Total flavonoid content: Different dilutions of standard rutin in methanol were prepared from 10 to 100 µg mL^{-1} of standard rutin in methanol and 0.5 mL of each of dilution was added to 1.5 mL of methanol along with 0.1mL of $AlCl_3$ (0.1gm mL^{-1}) and 0.1 mL of CH_3COONa reagent (1M), followed by addition of 2.8 mL D.D water, similarly 0.5 mL of each of methanolic extracts were taken, followed by the addition of same reagents and kept for 30 min for colour development. Absorbance was taken at 415 nm against blank solution that contained 2.0 mL of methanol, followed by addition of 0.1mL of $AlCl_3$ and 0.1mL of CH_3COONa reagents and 2.8 mL D.D water. Absorbance was taken for standard dilutions and calibration curve was plotted to get linear regression equation. The phenolic and flavonoid contents were calculated in each of the samples by using regression equation obtained from standard calibration curve.

2.3 Quantitative estimation of alpha and beta asarone by HPTLC

2.3.1 HPTLC instrumentation and general conditions

The samples were applied as bands of 4.0 mm width with a Camag microlitre syringe on caffeine impregnated precoated silica gel aluminium plate $60F_{254}$ (10 x 10 cm with 0.2 mm thickness, E. Merck, Germany), using a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 120 nL sec^{-1} was employed and space between two bands was kept at 6.6 mm. Linear ascending development was adopted in twin trough glass chamber, saturated with the mobile phase toluene: ethyl acetate (93:7, v/v). The optimum saturation time for mobile phase was kept 15 min at room temperature. The length of chromatogram was run up to 80 cm subsequent to the development of plate. TLC plates were dried in the current of air with the help of an air dryer. Densitometric

scanning was performed on camag TLC scanner III at the absorbance mode at 313 nm. The source of radiation utilized was deuterium and tungsten lamp. The slit dimension was kept 4.0 × 0.45 mm with scanning speed of 20 mm sec^{-1}.

2.3.2 Quantification of alpha and beta asarone in samples

The 4.0 µL of all the samples for testing were applied in triplicate on caffeine impregnated TLC plate (10 × 10 cm). Quantification was achieved for alpha and beta asarone with Win Cats software by employing regression equation obtained from the calibration curve. Mean of the samples were calculated with respect to peak height and area and the content of the alpha and beta asarone was calculated.

3. Results and Discussion

3.1 Tissue culture study

Literature survey revealed a very little of tissue culture work done on the plant with few reports of micropropagation studies (Rani *et al*., 2000). In the present study, *in vitro* cultured shoots were successfully developed in the new hormone combination, employing MS and B5 Gamborg basal media. It was observed that MS basal media supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm) and supplemented with 1.0 % extra sucrose was found to be best for regeneration of the cultured shoots from rhizome explants. Initiation of shoot regeneration was observed in MS basal medium supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm) and Kinetin (1.0 ppm) + 2, 4-D (2.0 ppm) (Figures 1A and 1B) whereas, MS basal medium supplemented with 6-BA (4.0 ppm) and 6-BA (1.0 ppm) + 2, 4-D (2.0 ppm), showed poor shoot regeneration (Figures 1C and 1D). Developed shoots were subcultured at regular intervals and their growth was monitored. The MS basal medium supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm) was found to be best for the development of shoots. Green healthy shoots measuring 50-60 mm in length (Figure 1E) were obtained at the end of 120 days with hormone combination of Kinetin (1.0 ppm) + 2, 4-D (2.0 ppm). However, MS + 6-BA (4.0 ppm) and MS + 6-BA (1.0 ppm) + 2, 4-D (2.0 ppm), showed development of shoots at very low pace with appearance of yellowish leaves. Explants cultured on MS medium supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm) were found to grow well and upon transference into a big conical flask, green and healthy shoots were obtained, measuring about 185-230 mm in length at the end of 60 days (Figure 1F), whereas MS medium supplemented with Kinetin (1.0 ppm) + 2, 4-D (2.0 ppm) showed the presence of yellowish leaves with slow growth of shoots (Figure 1G) and shoots developed on MS + 6-BA (4.0 ppm) and 6-BA (1.0 ppm) + 2, 4-D (2.0 ppm) were got dried within 45 days. B5 Gamborg basal media supplemented with same hormone was also used for the development of shoots and resulted in stunted growth of shoots and the explants got dried after 40 days (Figure 1H). Changes in the growth of regenerated plant were monitored after increasing the concentration of nutritional contents of MS medium, using the best hormone combination. Effect on shoot growth was observed by increasing the concentration of sucrose (1.0 %), potassium nitrate (600_mg L^{-1}), and potassium dihydrogen phosphate (10_mg L^{-1}) for 60 days. Increased number of regenerated shoots was obtained, resulting in greenish healthy shoots upon increasing sucrose concentration (Figure 1I), whereas with the nitrogen and phosphate additions to the medium, resulted in tiny leaves having same growth pattern (Figures 1J and 1K).

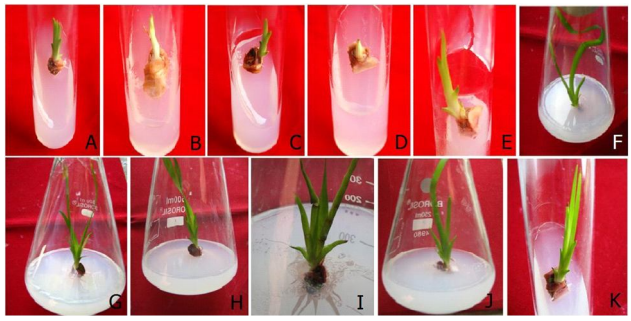

Figure 1: Cultured shoot of different basal medium supplemented with different hormone combinations at different stages

Legend : [A-B-Initiation of shoot in MS basal medium supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm) and Kinetin (1.0 ppm) + 2, 4-D (2.0 ppm), C-D- 6-BA (4.0 ppm) and 6-BA (1.0 ppm) + 2, 4-D (2.0 ppm), E-Green healthy developed shoots at the end of 120 days with Kinetin (1.0 ppm) + 2, 4-D (2.0 ppm), F- developed green and healthy shoots at the end of 60 days MS basal media supplement with 6-BA (4.0 ppm) + IAA (0.5 ppm), G- Yellowish leaves with slow growth observed with Kinetin (1.0 ppm) + 2, 4-D (2.0 ppm), H-B5 Gamborg basal media supplemented with same hormone, showed stunted growth of shoots and dried after 40 days and I-K-Greenish healthy shoots upon increasing sucrose concentration (Figure I), whereas less growth with the nitrogen and phosphate additions (Figures J-K)]

3.2 Analytical studies

3.2.1 Phytochemical screening

The preliminary phytochemical screening was carried out for the presence or absence of secondary metabolites in natural leaves and *in vitro* cultures by performing qualitative chemical tests. All the samples of cultured shoots and natural leaves were found to contain terpenoids, tannins and phenolics, saponins, flavonoids, mucilage and carbohydrates. However alkaloids and steroids were found absent altogether in all of the samples.

3.2.2 Phenolic and flavonoid contents

Phenolics may affect the efficacy of the drug, if they are separated out from the oil the toxicity and sedative action might increase (Dandiya *et al*., 1959). Total phenolic and flavonoid content in natural leaves and *in vitro* cultures were determined by using UV spectrophotometer (Hajaji *et al*., 2010; Singh *et al*., 2012). The standard calibration plot was found to be linear with r^2 = 0.9914 and 0.9994 with regression equation Y= 0.0049x X 0.0021 and Y= 0.0059x for gallic acid and rutin for total phenolic and flavonoid contents, respectively. It was observed that the maximum content of phenolics were present in cultured shoots developed on MS media with 1.0 % of extra sucrose supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm) and minimum in natural leaf extract. The content of total phenolics varied in range of 3.64-5.25 % w/w in natural leaves and *in vitro* cultures (Table 1). Similarly maximum of flavonoid contents were also found in cultured shoots, developed

in MS media with 1% extra sucrose, supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm) and minimum in B5 media, supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm). The content of total flavonoids varied between 4.51 - 6.60 % w/w in natural leaves and *in vitro* cultures (Table 1).

Samples	Content in %w/w ± SD	
	Phenolics	Flavonoids
NL	3.64 ± 0.14	2.51 ± 0.11
ICa	4.32 ± 0.11	2.84 ± 0.07
ICb	3.99 ± 0.08	1.38 ± 0.07
ICc	5.25 ± 0.09	3.60 ± 0.05
ICd	4.80 ± 0.13	2.58 ± 0.06
ICe	4.95 ± 0.13	2.62 ± 0.06

Table 1 : Results of total phenolic and flavonoid analysis in different samples

Legend : *In vitro cultures used (ICa, ICb, ICc, ICd and ICe) are as:*

NL. Natural leaves

ICa. 60 days old regenerated shoot developed in MS basal medium supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm)

ICb.60 days old regenerated shoot developed in B5 basal medium supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm)

ICc. 60 days old regenerated shoot developed in MS basal medium + 1% extra sucrose supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm)

ICd. 60 days old regenerated shoot developed in MS basal medium + 600 mg/L extra KNO_3 supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm)

ICe.60 days old regenerated shoot developed in MS basal medium + 10 mg/L extra KH_2PO_4supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm).

3.2.3 Quantitative estimation of alpha and beta asarone by HPTLC

Very limited reports are available on quantitative estimation of alpha and beta asarone in calamus oil (Chopra *et al.*, 1965; Widmer *et al.*, 2005). In the present investigation, alpha and beta asarones were quantified in cultured shoots and natural leaves, by using a simple, selective, accurate and cost effective HPTLC method developed and reported by the laboratory (Singh *et al.*, 2011). TLC was carried out for the resolution of the sample extracts, employing reported method and the same resulted in form of fine spots of asarones in all the samples. The samples applied on caffeine impregnated precoated TLC plates, which showed well resolved peaks of alpha and beta asarone. The R_f values 0.69 ± 0.02 and 0.74 ± 0.02 were observed in mix standard and samples chromatogram for alpha and beta asarone, respectively (Figure 2). The results were calculated, using regression equation with respect to peak area. The maximum of beta asarone (7.213 % w/w) was found in cultured shoots developed in MS basal media with 1% extra sucrose, supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm), whereas minimum of beta asarone content (3.345 % w/w) was found in cultured shoots, developed in B5 basal medium, supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm), whereas the natural leaves were found to contain beta asarone (3.413 % w/w) (Table 2). Similarly maximum of alpha asarone (0.1103 % w/w) was found in cultured shoots, developed in MS media with 1% extra sucrose supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm) (0.1103 % w/w) and minimum alpha asarone content (0.0381 % w/w) was found in cultured shoots, developed in B5 basal medium supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm). The natural leaves were found to contain 0.0642 % w/w of alpha asarone (Table 2). These analytical studies will help to standardize the potential of developed *in vitro* cultures for the production of pharmacologically active secondary metabolites.

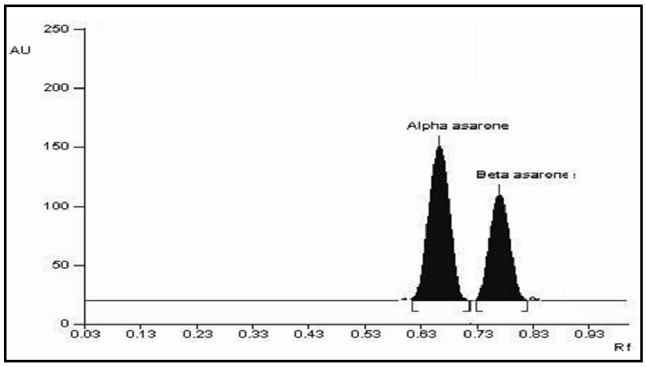

Figure 2 : Typical HPTLC chromatogram of mixed standard of alpha and beta asarone

Sample	Content in %w/w ± SD	
	Beta asarone	Alpha asarone
NL	3.41 ± 0.10	0.064 ± 0.004
ICa	4.16 ± 0.06	0.079 ± 0.003
ICb	3.34 ± 0.10	0.038 ± 0.002
ICc	7.21 ± 0.11	0.110 ± 0.010
ICd	4.76 ± 0.12	0.071 ± 0.006
ICe	5.52 ± 0.15	0.087 ± 0.002

Table 2 : Results of beta and alpha asarone analysis in different samples

In present study, efforts have been made for development of the callus culture from a Rasayana drug calamus (Pushpangadan *et al.*, 2014) for production of secondary metabolites (alpha and beta asarone) by employing MS and B5 Gamborg media, supplemented with different growth regulators, similar to the method reported for asciaticoside by Gandi and Giri (2013). Excellent shoot regeneration was achieved in MS basal media on supplementing 6-BA (4.0 ppm) + IAA (0.5 ppm) like in case of *Bacopa monnieri* (Rao *et al.*, 2012). Addition of 1 % extra sucrose in basal media has shown very promising results on shoot growth as well as on secondary metabolite production. The estimation of its major metabolic constituents such as phenolics and flavonoids by UV method as well as alpha and beta asarones, using simultaneous HPTLC method (Singh *et al.*, 2011) in *in vitro* cultures showed its potential for industrial exploration. Since, asarone is of pharmaceutical importance and is exclusively present in calamus samples; it can be used as a chemical marker (Rasheed *et al.*, 2012) for its quality control (Rasheed *et al.*, 2013; Subramoniam, 2014).

4. Conclusion

Acorus calamus Linn. is a valuable medicinal plant in Ayurvedic and Unani system which is frequently uses in treatment of neuro-

disorders, and its oil have been reported to contain anti-obese property. In present study efforts have been made for development of the callus culture for production of secondary metabolites (alpha and beta asarone) by employing MS and B5 Gamborg media supplemented with different growth regulators. However, it is too difficult to initiate a callus from the terpenoid or volatile oil containing tissues but excellent shoot regeneration was achieved in MS basal media supplemented with 6-BA (4.0 ppm) + IAA (0.5 ppm). The effects of changing different media component on shoot growth as well as on secondary metabolites were studied. The estimation of its major constituents alpha and beta asarones was carried out by using simultaneous HPTLC method in cultured shoots. Additionally, total phenolic and flavonoidal contents of pharmacological importance were also estimated. The work can be utilized to get high content of therapeutically active constituents of calamus as well as for its quality control.

Acknowledgment

Authors are thankful to UGC, Government of India for providing financial assistance for conducting the study.

Conflict of interest

We declare that we have no conflict of interest.

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