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Elicitation effect on the production of asiaticoside and asiatic acid in shoot, callus, and cell suspension culture of *Centella asiatica*

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ABSTRACT

Centella asiatica is an important medicinal plant which contains various phytocompounds. Asiatic acid and asiaticoside are two major compounds which are responsible for its various pharmaceutical activities. The present study analyzes the effect of elicitor, i.e., methyl jasmonate on the synthesis of asiaticoside and asiatic acid (ATA) in shoot, callus, and cell suspension cultures of *C. asiatica*. A high-performance liquid chromatography analysis showed that the elicitation with 100 µM concentration of methyl jasmonate enhanced asiaticoside content by 69-fold in callus culture, 39-fold in shoot cultures, and ATA by 1.9-fold in cell suspension culture. Thus, elicitation with methyl jasmonate is an effective method of increasing the rate of biosynthesis of asiaticoside and ATA in plant cell cultures of *C. asiatica.*

INTRODUCTION

Centella asiatica is a perennial medicinal plant that belongs to Umbellifere (Apiaceae) family. It is found in tropical and subtropical regions, including India, Sri Lanka, Iran, New Guinea, Australia, Indonesia, and southern and central Africa (Hoang and Nguyen, 2010). This plant usually grows in shady, marshy, damp, and wet places, such as river banks, paddy fields, and higher elevations (Roy and Bharadvaja, 2017a). The flowers are white or light purple to pink in color, sessile arranged in simple umbels, and also bears small oval fruit (Gohil *et al*., 2010). The form and shape of the plant can differ greatly by the environmental conditions. This plant is listed as threatened species by the International Union for Conservation of Nature and Natural Resources (Pandey *et al*., 1993). Because of this, the wild population of *C. asiatica* was banned for commercial applications. Its scarcity is as a result of lack of proper cultivation practices and ruthless collection of herbs for medicinal purposes (Karthikeyan *et al*., 2009). Tissue culture techniques play an important role in clonal multiplication of elite clones as well as conservation of *Centella* germplasm (Roy *et al*., 2016). Plant tissue culture techniques provide an alternative approach to meet the current market demands of secondary metabolites from *Centella* (Prasad *et al*. 2012).

Centella asiatica contains several valuable secondary compounds, including asiaticoside, madecassoside, centelloside, centellose, brahminoside, thankunizide, sceffoleoside, brahmoside, asiatic, centellic, brahmic, and madecassic acid. Among these, the important bioactive compounds are asiatic acid (ATA), asiaticoside, madecassoside, and madecassic acid (Roy and Bharadvaja, 2017b, Kundu *et al*., 2016). Saponin ranges from 1% to 8% of total constituents, based on the plant's origin (Brinkhaus *et al*., 2000). Asiaticoside is the most abundant triterpenoid saponin present in the leaves. It is used in the treatment of psoriasis, leprosy, and eczema due to its potent anti-inflammatory effects and cell proliferative activity (Bonfill *et al*., 2011). ATA helps in controlling the cell division in breast cancer, colon cancer, human hepatoma, and cytotoxic activity on fibroblast cells (Kim *et al*., 2009 , Roy and Bharadvaja, 2017c, Roy *et al*., 2018). Moreover, ATA is known to revitalize the brain and nervous system and delays aging (Brinkhaus *et al*., 2000).

The benefit of plant cell culture is the possibility of manipulating synthesis of desirable compounds within the

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cells (James *et al*., 2008). Elicitors have been found to increase triterpene saponin accumulation in plants and play an important role in signal transduction which regulates all the defense genes in plants (Mangas *et al*., 2006). Metabolic synthesis in cultured cells can be enhanced by elicitor like methyl jasmonate (MeJA) (Suzuki *et al*., 2005). Jasmonates are signaling molecules which leads enhanced production of many secondary metabolites, including triterpenoid saponins of the different plants (Memelink *et al*., 2001). They induce transcriptional activation of genes involved in the formation of secondary metabolites (Yukimune *et al*., 1996). The present study focuses on effects of MeJA on synthesis of asiaticoside and ATA in shoot culture, callus culture, and cell suspension cultures of *C. asiatica.*

MATERIALS AND METHODS

Establishment of shoot culture

In vitro grown culture of *C. asiatica*, accession number 347492, was collected from National Bureau of Plant Genetic Resources, New Delhi, India. The shoot cultures were grown in Murashige and Skoog (MS) media supplemented with 1 mg/l 6-benzylaminopurine (BAP) (Roy *et al*., 2016). Nodes were inoculated vertically into culture tubes and incubated at 26°C ± 2°C under 16-hour photoperiod and light intensity of 3,000 lux with 55%–60% relative humidity. Readings and visual data were recorded.

Establishment of callus culture

Leaf explants of *C. asiatica* were excised from basal shoot culture and edges of the leaves and inoculated onto solid MS medium supplemented with 1 mg/l BAP and 1.5 mg/l naphthalene acetic acid (NAA). The culture was incubated at 26°C ± 2°C under 16-hour photoperiod and light intensity of 3,000 lux with 55%–60% relative humidity. Callus growth and visual data were recorded.

Establishment of cell suspension culture and growth curve analysis

To establish cell suspension culture, approximately 2 g of fresh callus was weighed and finely chopped into small pieces and transferred to 250 ml Erlenmeyer flask containing 50 ml of liquid MS medium supplemented with 3% (w/v) of sucrose, 1 mg/l BAP, and 1.5 mg/l NAA. It was kept in a rotary shaker at 100 rpm in dark conditions at 25°C for 45 days. For growth curve analysis, 5-ml sample was retrieved using sterilized pipette at 3-day interval until the 45th day. To measure wet cell weight, cells were filtered through nylon membrane, washed with distilled water, and weighed immediately. Dry cell weight was determined by drying the collected cells in nylon membrane at 50°C until a constant weight was attained.

Elicitation studies

To study the effect of MeJA on shoot multiplication, MS media was supplemented with 1 mg/l BAP along with different concentrations of MeJA, i.e., 50, 100, 150, 200, and 250 µM. Observations were recorded after 6 weeks of culture period. In callus culture, MS media was supplemented with 1 mg/l BAP and 1.5 mg/l NAA along with 100 µM MeJA. The cell suspension culture was supplemented with the same amount of BAP, NAA, and MeJA.

Quantitative estimation of asiaticoside and asiatic acid

Approximately, 0.5 g of air dried shoots grown in MS media with 1 mg/l BAP (control) and air dried callus grown in MS media with 1 mg/l BAP and 1.5 NAA (control) was taken. Samples were crushed to fine powder and dissolved in 5 ml of methanol:water (9:1) solution. Then, samples were sonicated for 10 minutes and after cooling, samples were filtered through polytetrafluoroethylene (PTFE) syringe filter (0.22 µM) and transferred to glass vials. Similar procedure was used for MeJA-elicited shoot and callus extracts.

For preparing cell suspension culture samples, control cultures were transferred into sterile tubes and centrifuged at 1,500 g for 15 minutes. Approximately, 0.5 g of cells was added in 5 ml of methanol:water (9:1 ratio). After sonication, samples were centrifuged at 1,500 g for 10 minutes, and supernatant was filtered through PTFE syringe filter (0.22 µM). Same procedures were followed for MeJA-treated suspension culture. Samples were stored at −20°C for further analysis. About 1 mg each of asiaticoside standard (Sigma Aldrich) and ATA standard (Sigma Aldrich) was dissolved in 1 ml of methanol:water (9:1) solution. For calibration, standards of asiaticoside and ATA were prepared at different concentrations, i.e., 1, 0.5, and 0.1 mg/ml. For HPLC analysis, C18 column was used, mobile phase was a mixture of methanol and water (70:30) for asiaticoside and (80:20) for ATA. Twenty microliters solution was injected into the column, with a flow rate of 0.5 ml/minute at 26°C. The absorbance was read at 214 nm wavelength.

Statistical analysis

In vitro experiments were performed in triplicates and HPLC analysis of samples was performed in duplicates. The data analysis was performed using one-way analysis of variance using Microsoft Excel 2007. Results were shown in mean ± standard error (M ± SE) with $p \leq 0.05$ and $F_{stat} > F_{critical\ value}$ considered as statistically significant.

RESULTS AND DISCUSSION

Shoot, callus, and cell suspension culture

Shoot cultures grown in different concentration of MeJA (50, 100, 150, 200, and 250 µM) resulted in decreased shoot proliferation compared to control (untreated) one (MS + 1 mg/l BAP) after 6 weeks of incubation (Table 1). The highest shoot multiplication was observed in control one with a shoot number of 31 ± 1 (Fig. 1a). Among the elicitor-treated cultures, shoot cultures supplemented with 1 mg/l BAP + 100 µM MeJA had the highest shoot number of 24 ± 1 (Fig. 1b).

Callus induction from leaf explants of *C. asiatica* in MS media supplemented with 1 mg/l BAP and 1.5 mg/l NAA was observed (Fig. 1c). After 6 weeks of incubation, callus was green colored and compact. However, after 10 weeks of incubation, it turned brown colored. Callus culture treated with 100 µM MeJA resulted in poor callus induction and turned yellow.

For establishment of cell suspension culture, MS liquid media supplemented with 1 mg/l BAP and 1.5 mg/l NAA was

Table 1. Effect of MeJA concentrations on growth of *Centella asiatica* (accession number 347492) supplemented with 1 mg/l 6-benzylaminopurine (BAP), and different concentrations of MeJA (50, 100, 150, 200, and 250 µM). Shoot number and shoot length were recorded after 6 weeks of culture period. Analysis was done in triplicate.

S. no.	Media composition	Shoot number (M ± SE)	Shoot length (cm) (M ± SE)
1	1 mg/l BAP + 50 µM MeJA	13.33 ± 1.52	2 ± 0.2
2	1 mg/l BAP + 100 µM MeJA	24 ± 1	3.4 ± 0.1
3	1 mg/l BAP + 150 µM MeJA	18.66 ± 0.57	2.46 ± 0.35
4	1 mg/l BAP + 200 µM MeJA	16.6 ± 1.52	2.33 ± 0.3
5	1 mg/l BAP + 250 µM MeJA	11 ± 1	1.6 ± 0.2
6	1 mg/l BAP (control)	31 ± 1	3.8 ± 0.25

Figure 1. Shoot culture of Centella asiatica grown in MS media supplemented with (a) 1 mg/l BAP (control) (b) 1 mg/l BAP with 100 µM MeJA (elicited) after 6 weeks of culture period (c) Callus culture treated with 1 mg/l BAP + 1.5 mg/l NAA + 100 µM MeJA after 6 weeks of culture period and (d) Cell suspension culture treated with 1 mg/l BAP + 1.5 mg/l NAA+100 µM MeJA after 6 weeks of culture period.

used. The results after 6 weeks of incubation is shown in Figure 1d. Growth curve was determined using dry weight (Fig. 2a) and wet weight analysis (Fig. 2b) for both control and MeJA-elicited cell suspension culture for 45 days.

In the present study, MeJA has an overall negative effect on shoot proliferation of *C. asiatica* this might be because MeJA disrupts the cortical microtubules of the plants which lead to growth inhibition, induction of leaf senescence, and promotion of ethylene production (Saniewski *et al*., 1987). Previous studies reported that increase concentration of MeJA inhibits growth of the whole plant and cell culture (Kim *et al*., 2004; Mangas *et al*., 2006). Bonfill *et al*. (2006) reported that *Centella* plantlet treated with MeJA showed reduction in growth with over 50% in fresh weight (aerial parts) and also showed symptoms of leaves necrosis at the end of culture period. Although treatment with MeJA resulted to growth inhibition, it was able to increase the

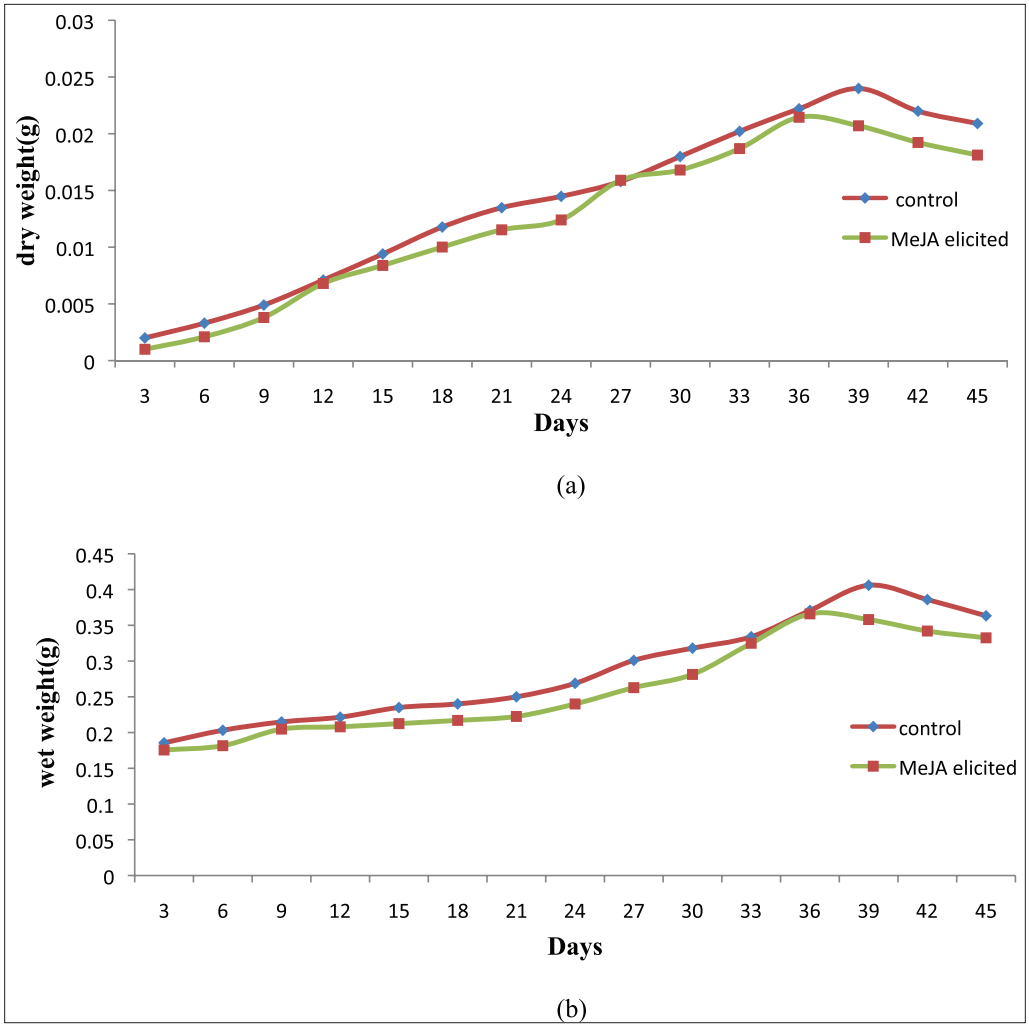

Figure 2. Growth curve of (a) dry weight and (b) wet weight for both control and MeJA-elicited cell suspension cultures treated with 1 mg/l BAP + 1.5 mg/l NAA + 100 µM MeJA up to 45 days. Suspension cultures (treated and untreated) were separated from cells using centrifugation, and wet weight and dry weight were determined and plotted against number of days.

secondary metabolites in *in vitro* cultures (Kim *et al*., 2004) as same happened in our study.

In case of callus culture, change in color of callus may be due to the secretion of phenolic compounds in explant or explant tissues (Dan *et al*., 2000). Previous studies reported that MS medium supplemented with 1 mg/l BAP and 1 mg/l NAA resulted in strong callus induction from petiole explants of *Centella* (Hoang and Nguyen, 2010). MeJA completely ceased the callus growth of *Taxus media var. Hatfieldii* (Furmanowa *et al*., 1997) and callus cultures of *R. cordifolia* in a dose-dependent manner (Bulgakov *et al*., 2002).

For cell suspension culture, a usual type of lag and stationary phase were not observed in the growth curve of both control and MeJA elicited cells. Exponential phase of the cells in the control culture lasted for 39 days and reached death phase by day 42, whereas exponential phase for MeJA elicited cells lasted for 36 days and reached death phase by day 40. Growth curve for MeJA-elicited and control cells were almost similar to each other but exponential phase of elicited cells was shorter than control cells. The growth curve of control and MeJA-elicited cell suspension culture lacks a typical lag phase, and the stationary phase may be due to their shorter duration (Hoang and Nguyen, 2010). The control cells reached death phase by 42 days may be due to the nutrient depletion in the culture media (Bonfill *et al*., 2011). Previous studies reported that viability of cells reduced by more than half of initial population of *C. asiatica* suspension cells at the end of the culture period when elicited with 100 µM MeJA (Bonfill *et al*., 2011).

Quantitative estimation of asiaticoside and asiatic acid by HPLC

The approximate retention time for standard asiaticoside (ASD) was 8.9 minutes (Fig. 3a). It was observed that after treatment with 100 µM MeJA, there was a significant increase in asiaticoside content by 39-fold in shoot culture and about 69-fold in callus extracts (Table 2).

However, the asiaticoside content in cell suspension culture was reduced with MeJA addition. The asiaticoside content

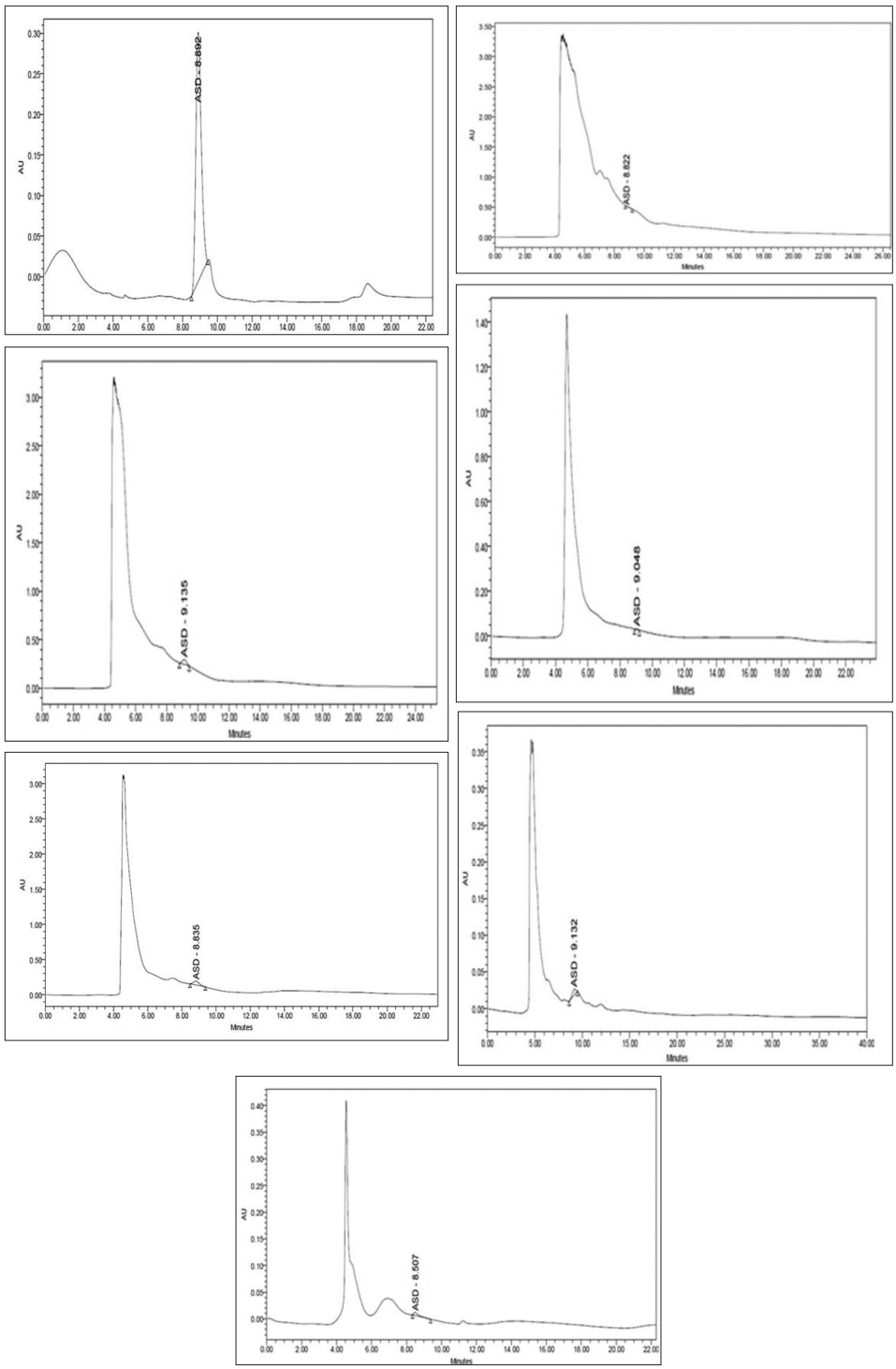

Figure 3. Chromatogram of (a) 1 mg/ml ASD standard (b) control shoot culture (MS +1 mg/l BAP) (c) elicitor treated shoot cultures (1 mg/l BAP + 100µM MeJA) (d) control callus cultures (1 mg/l BAP + 1.5 mg/l NAA) (e) elicitor elicited callus cultures (1 mg/l BAP + 1.5 mg/l NAA + 100 µM MeJA) (f) control cell suspension culture (1 mg/l BAP + 1.5 mg/l NAA) (g) elicited treated cell suspension culture (1 mg/l BAP + 1.5 mg/l NAA+100 µM MeJA).

in cell suspension culture was reduced after MeJA elicitation. Chromatogram of asiaticoside content before and after elicitation in shoot culture, callus culture and cell suspension culture are shown in Figure 3b–g. In *C. asiatica,* βAS (β-amyrin synthase), an important enzyme in the biosynthesis of triterpene saponins gets activated when elicited with MeJA, thereby boosting up the production of asiaticoside in shoot and callus culture (Kim *et al*., 2005). Previous studies indicated that the highest expression of cycloartenol synthase) mRNA transcript in leaves of *C. asiatica* regulates phytosterol synthesis and gets decreased upon addition of MeJA to the medium (Kim *et al*., 2005). This explains that MeJA inhibits the formation of phytosterol and promotes the asiaticoside

Table 2. Asiaticoside estimation in different cultures of *Centella asiatica* treated with 1 mg/l BAP + 100 µM MeJA in case of shoot culture and 1 mg/l BAP + 1.5 mg/l NAA + 100 µM MeJA in case of callus and cell suspension culture. In control cultures MeJA was not present.

Plant cultures	Asiaticoside concentration in control (untreated) culture (mg/ml) (M ± SE)	Asiaticoside concentration in elicitated culture (treated) (mg/ml) (M ± SE)
Shoot culture	0.0036 ± 0.0001	0.1434 ± 0.004
Callus culture	0.0028 ± 0.0001	0.2004 ± 0.0023
Cell suspension culture	0.0323 ± 0.001	0.016 ± 0.0001

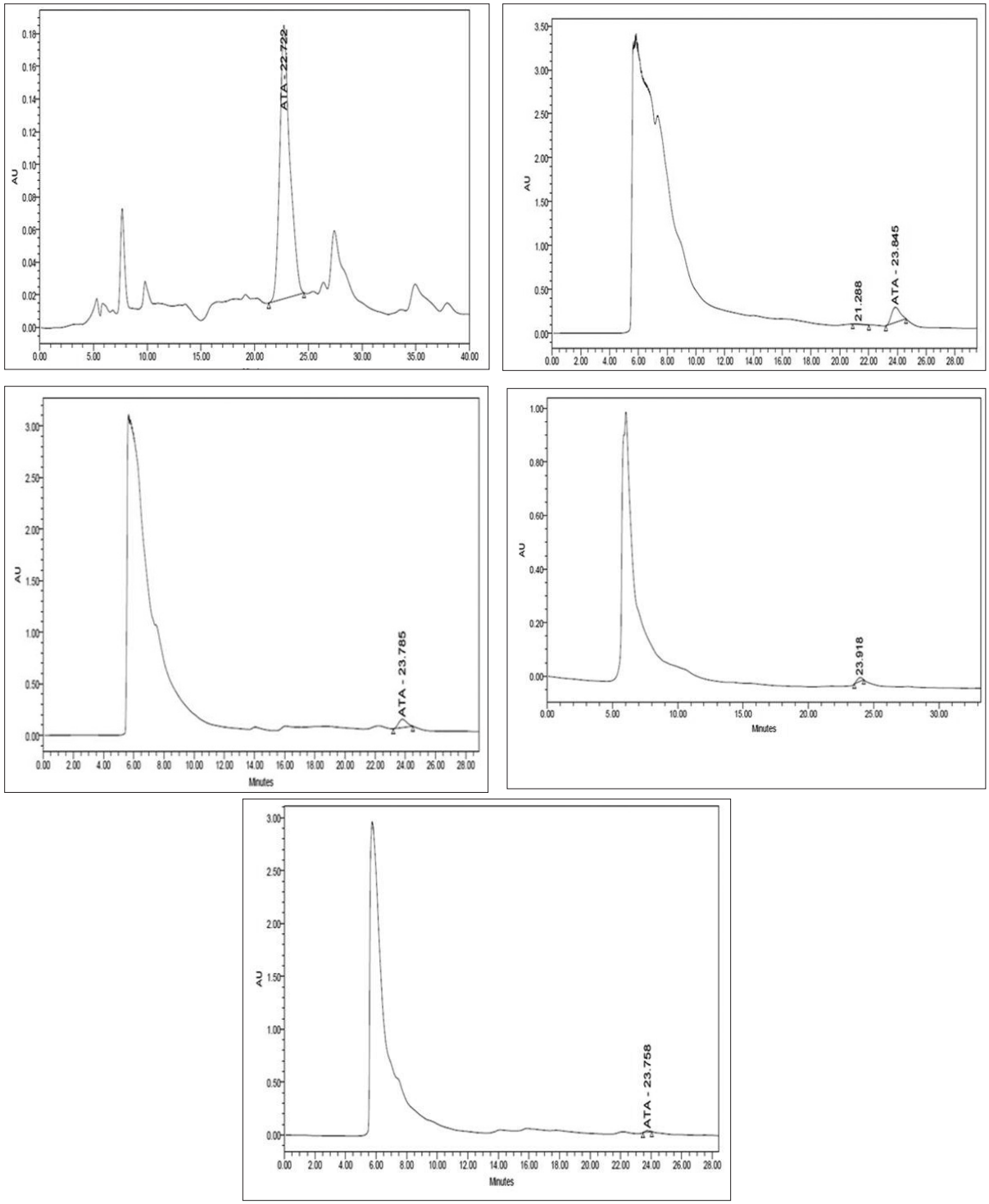

Figure 4. Chromatogram of (a) 1 mg/ml ATA standard (b) control shoot culture (MS + 1 mg/l BAP) (c) elicitor treated shoot cultures (1 mg/l BAP + 100 µM MeJA) (d) control callus cultures (1 mg/l BAP + 1.5 mg/l NAA) (e) elicitor elicited callus cultures (1 mg/l BAP + 1.5 mg/l NAA + 100 µM MeJA) (f) control cell suspension culture (1 mg/l BAP + 1.5 mg/l NAA) (g) elicited treated cell suspension culture (1 mg/l BAP + 1.5 mg/l NAA + 100 µM MeJA).

Table 3. ATA estimation in different cultures of *Centella asiatica* treated with 1 mg/l BAP + 100 µM MeJA in case of shoot culture, and 1 mg/l BAP + 1.5 mg/l NAA + 100 µM MeJA in case of callus and cell suspension culture. In control cultures MeJA was not present. There was no evidence of presence of ATA in both the treated and untreated cell suspension culture.

Plant cultures	ATA concentration in control (untreated) culture (mg/ml) (M ± SE)	ATA concentration in elicitated culture (treated) (mg/ml) (M ± SE)
Shoot culture	0.634 ± 0.026*	0.2681 ±0.010*
Callus culture	0.0339 ± 0.001*	0.0656 ± 0.0026*
Cell suspension culture	0	0

* Statistically significant at $P \leq 0.05$ and $F_{stat} >$ Fcritical value

pathway in shoot and callus cultures. Previous studies had shown that 0.1 mM MeJA is sufficient to upregulate the levels of squalene synthase mRNA and βAS mRNA, and thus enhance the triterpene saponin content in *C. asiatica* (Mangas *et al*., 2006). Asiaticoside was reduced in cell suspension culture after MeJA treatment, maybe because the effect of MeJA is dependent on factors like elicitor's specificity, concentration, duration of treatment, and growth phase of the culture. Previous studies reported that asiaticoside synthesis in *Centella* is highly tissue-specific or organ specific, occurring mainly in the leaves cultured with 100 µM MeJA elicitor (Kim *et al*., 2004). In certain cases, the metabolic flux of specific metabolic pathways gets manipulated in response to elicitors and signal molecules of plant defense responses (James *et al*., 2008). Previous studies reported that callus and cell suspension of *Centella* from Indian origin synthesized asiaticoside (Nath and Buragohain, 2005). So far, biotechnological attempts to over produce asiaticoside through cell or tissue culture have encountered limited success (Kim *et al*., 2002).

The retention time for standard ATA was found to be approximately at 22.6 minutes (Fig. 4a). Treatment with 100 µM MeJA resulted to 1.9-fold increase in ATA in callus extract, whereas in shoot extract ATA was reduced after elicitation (Table 3). ATA was found absent in both control and elicited culture of cell suspension culture. Chromatogram of ATA content before and after elicitation in shoot and callus culture are shown in Figure 4b–e. ATA was not detected in cell suspension culture even after the treatment with MeJA, because plant secondary metabolites are synthesized by specialized cells at particular stage of its development and certain compounds are not synthesized if cells remain undifferentiated as in cell suspension culture (Kim *et al*., 2002). Another important factor for regulation of secondary metabolic processes is the distribution between mRNA transcripts, enzymes, and biosynthetic products within and between cells (James *et al*., 2008). The callus is undifferentiated tissue which has the ability to develop into any plant organ such as root, shoot, or leaf under correct hormone concentrations and this might be the reason for presence of all the triterpenoids in callus and not in cell suspension culture (James *et al*., 2008). Previous studies showed that four triterpenoid, asiaticoside, ATA, madecassic acid, and madecassoside were found in the undifferentiated cultures cells, with higher concentrations in calli as compared to the cell suspensions of *C. asiatica* belonging to two different phenotypes from South Africa (James *et al*., 2008). Another problem is the instability of cell cultures for continued production of secondary products as some cell lines loss their ability to synthesize desired compound after prolonged culture (James *et al*., 2008). Comparative estimation of asiaticoside and ATA are shown in Tables 2 and 3 for shoot, callus, and cell suspension culture, respectively. The highest asiaticoside content was found in MeJA-elicited callus culture, whereas the highest ATA content was found in the control shoot culture.

CONCLUSION

The present study showed that elicitation of MS media with methyl jasmonate caused growth inhibition in shoot, callus, and cell suspension culture of *C. asiatica*. However, biosynthesis of asiaticoside in shoot and callus cultures was enhanced and ATA in case of callus culture. Therefore, this method offers great potential for the enhancement of valuable secondary metabolites, and at the same time, it ensures a sustainable conservation of endangered medicinal plant.

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CONFLICT OF INTEREST

The authors have declared that they have no conflict of interest.

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