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Production and quantification of Asiatic acid from *in vitro* raised shoots and callus cultures of *Centella asiatica* (L.) Urban

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Abstract

Centella asiatica (L.) Urban, is one of the important medicinal plants, belonging to the family Apiaceae, with a range of medicinal values. The secondary metabolites produced by *Centella asiatica* are of ursane type and are called as "Centellosides". Asiatic acid is one of the important bioactive compounds produced by the plant, having several medicinal properties. The present study is an attempt towards the production of asiatic acid in the *in vitro* grown shoots as well as callus. *In vitro* grown shoots in semisolid and liquid media produced 1.02± 0.03 mg/gFW and 0.47± 0.08 mg/gFW asiatic acid, respectively, whereas leaf callus produced a maximum amount of 1.46± 0.06 mg/gFW asiatic acid.

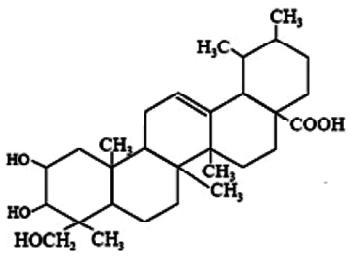
Key words: *Centella asiatica* (L.), Centellosides, Asiatic acid

Introduction

Centella asiatica (L.) Urban also known as "Gotu Kola", is a slender creeping perennial herb, found in shady moist areas of India, Sri Lanka, China, Japan, Madagascar and Australia. It produces a group of compounds collectively called "Centellosides or Centelloids" which are pentacyclic triterpenoid saponins. These active principles have a wide range of pharmaceutical applications in wound healing (Tenni *et al*., 1988; Maquart *et al*., 1999) as antihistamin, antiulcers, antilepsory, treating vein diseases (Brinkhaus *et al*., 2000), memory improvement (Gupta *et al*., 2003; Rao *et al*., 2005), as an antidepressant (Chen *et al*., 2003), antibacterial, antifungal (Ullah *et al*., 2009), for psoriasis (Sampson *et al*., 2001), as anticancer (Babu *et al*., 1995) and as antioxidant (Hussain *et al*., 2007; Kumar and Gupta, 2002) agents.

Centellosides include asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, centellose, asiatic acid *etc*. *C. asiatica* has been reported to contain more than 70 constituents, such as caffeic acid derivatives, flavonoids, quercetin, kaempferol, catechin, rutin, sterols and lipids, some of which have been proven as potent antioxidants (Hussain *et al*., 2007).

The anticancer effect of asiatic acid has been reported in cancer cell lines *via* inhibition of cell cycle progression (Ya *et al*., 2005) and induction of apoptosis (Piyawan *et al*., 2005). The hepatoprotective and antidiabetic activity of asiatic acid has been proved (Byung *et al*., 2007; Wenjie *et al*., 2012; Li *et al*., 2012; Ramachandran and Saravanan, 2012). Owing to its high medicinal value, *Centella asiatica* is being largely depleted from its natural habitats because of the unrestricted exploitation. Thus, the plant has been listed under highly threatened species by the International Union for Conservation for Nature (Pandey *et al*., 1993). Herbal drug technology includes all the steps that are involved in converting botanical materials into medicines, standardization and quality control with integration of modern scientific techniques and traditional knowledge is of great importance (Rasheed *et al*., 2012). The present study is an attempt towards production of asiatic acid under controlled conditions by using tissue culture approach.



Asiatic acid

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Materials and Methods

Plant material

Plants of *Centella asiatica* were collected from Rajdhani Agro Farms, Hyderabad.

Chemicals

All the MS media components, mercuric chloride and HPLC grade solvents were procured from Himedia, India. Plant growth regulators from Duchefa, Netherlands, 0.22µm filters from Millipore and asiatic acid standard from Sigma.

Establishment of in vitro cultures

Nodal parts, leaf, petiole and root sections were used as explants, washed with mild detergent and surface sterilized with the 0.1 % mercuric chloride for 2 mins. Followed by 7 to 8 times washing with sterile distilled water. The cultures were maintained under standard culture conditions (25±2ºC temperature; 16/8 h Light/Dark regime with 40-50 mol m^{-2} S^{-1} Light). *In vitro* cultures were inoculated in liquid as well as semisolid MS media, supplemented with various concentrations of cytokinins [benzyl amino purine (BAP) and kinetin (Kn)].

Callus initiation

In vitro raised plantlets of *C. asiatica* plants were dissected into different parts, *viz.*, leaf, petiole and root and inoculated onto MS basal media, augmented with different concentrations of auxin (2,4-D) and cytokinin (BAP). The calli, obtained from various explants were maintained separately and subcultured every fortnight.

Growth curve of in vitro grown shoots

The growth pattern of *in vitro* grown plants was determined both in semisolid and liquid MS basal media. The nodal explants from the *in vitro* grown plants were incubated under standard culture conditions. The cultures were harvested at regular intervals (4 days) till it reaches decline stage, to study the growth and production kinetics with regard to asiatic acid content.

Growth curve of cell suspensions

Growth pattern of cell suspension cultures of *C. asiaica* was studied by inoculating 10% (w/v) of callus as inoculum in liquid MS medium, augmented with 2, 4- D (1mg/l) and BAP (0.5mg/l). These cultures were incubated in an orbital shaker at 120rmp and 22±2ºC temperature under 16/8 light/dark cycle. The cells were harvested at regular intervals (4 days) to analyse the asiatic acid content.

Quantification of asiatic acid

The *in vitro* shoots (semisolid and liquid) and harvested cells (1g) were ground into a fine paste in a mortar and pestle, using HPLC grade methanol (1 ml). The filtrate was passed through a syringe driven filter of 0.22µm diameter before subjecting to HPLC analysis.

The amount of asiatic acid present in the samples was analysed by modified HPLC method, suggested by Rafamantanana *et al*. (2009). This is reported in Table 1. The analysis was carried out with a Shimadzu-LC-20AD prominence liquid chromatography gradient system equipped with a C18 column. The sample was detected using a SPD-M20A-prominence diode array detector at 206nm. The mobile phase consisted of acetonitrile and water.

Table 1: Gradient conditions for HPLC

Time (min)	Pump A [water (%)]	Pump B [Acetonitrile (%)]
0	80	20
15	65	35
30	35	65
35	20	80
40	20	80
45	80	20
55	80	20

Statistical analysis

All the experiments were performed in triplicate and the data were expressed as mean ± standard deviation. One-way ANOVA analysis followed by the Duncan's test was used to determine significant ($p \leq 0.05$) differences.

Result and Discussion

Explant choice

Various explants of *C. asiatica viz.*, leaf, petiole, node and root were inoculated on to MS basal media, fortified with different concentrations of cytokinins (BAP and Kn). Among different explants used, only nodal explants gave positive response (Figure 1) both in semisolid and liquid MS media as reported earlier (Kim *et al*., 2004 a). These tender shoots gave rise to multiple shoots on repeated subcultures (Figure 2).

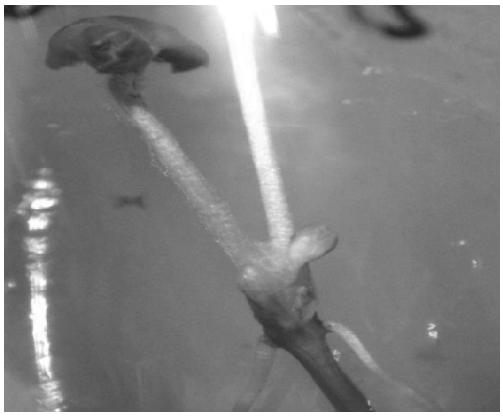


Figure 1: Shoot tips of *C. asiatica* on MS basal media

Figure 2: Liquid culture of *C. asiatica* in MS media

MS basal liquid media proved to be the best for the biomass increase in comparision to the MS basal semisolid media with or without phytohormones. Kim *et al*. (2002) have reported that whole plants derived from nodes are rich in centellosides in comparison to other plant material.

Establishment of *in vitro* cultures

The nodal explants gave new leaflets after 21 days of inoculation. The explants that gave positive response were subcultured every three weeks. Among the various combinations of phytohormones, BAP (1 and 1.5 mg/l) and Kn (1 and 1.5 mg/l) gave the best results (Table 2). The role of BAP and Kn in regulation of internal phytohormone levels for production of multiple shoots has been established in various plants (Das *et al*., 1996; Komalavalli and Rao, 1997; Nguyen and Huynh, 2011). However, there was no significant difference ($p>0.05$) in the morphogenenetic response, obtained on MS basal liquid media and BAP and Kn containing media. Hence, MS basal media was used for further micropropagation.

Table 2: *In vitro* establishment of *C. asiatica* cultures using different cytokinin concentrations with nodal explants

S. No.	BAP (mg/l)	Kn (mg/l)	Growth response
1	0	0	+++
2	0.5	-	+
3	1	-	++
4	1.5	-	++
5	-	0.5	+
6	-	1	++
7	-	1.5	++

* Response + Poor ++ Moderate +++ Excellent

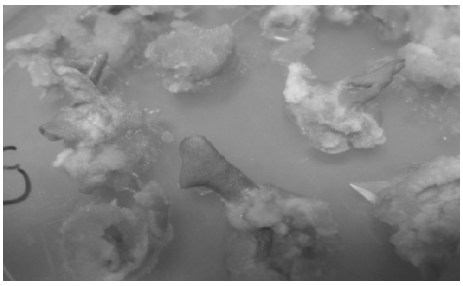
Callus induction

Different plant parts like leaves, petioles and roots of *in vitro* grown plants, were used to initiate the callus. Leaf explants proved to be the best when compared to other parts (Figure 3a, b and c), which was in accordance with the report given by Nath and Burgohain (2005). Among the different phytohormone concentrations, 2,4-D (1 mg/l) in combination with BAP (0.5 mg/l) was found to be most suitable for callus initiation (Table 3). The efficiency of 2,4-D in callus induction has been reported in many plant species (Bhuvaneswari *et al.*, 2012; Suryakala *et al*., 2012; Kiranmayee *et al.*, 2011). Majority of centellosides were reported to be present in the leaves (Kim *et al.*, 2004a; Mercy *et al*., 2012), which are tissue specific, but the advantage of asiatic acid detection in the callus cultures is that, the phytochemical can be produced on a continuous basis, irrespective of its organ specificity. The callus obtained from the leaf explants was used for quantification of asiatic acid by HPLC.

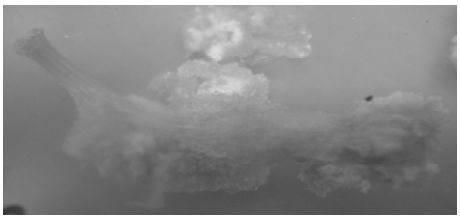
Table 3: Response of different phytohormone concentration and combinatons for callus induction from leaf explant of *C.asiatica*

S. No	2, 4-D (mg/l)	BAP (mg/l)	Response
1	0	0	-
2	0.5	-	-
3	1	-	+
4	1.5	-	++
5	0.5	0.5	+
6	1	0.5	+++
7	1.5	0.5	+++

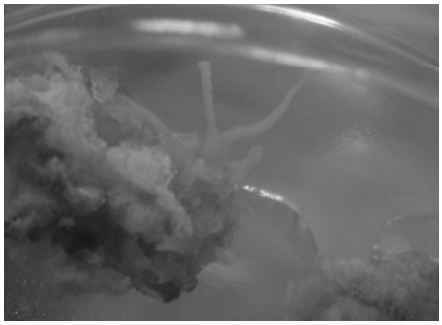
* Response - No Response + Poor ++ Moderate +++ Excellent



(a) Leaf sections



(b) Petiole sections



(c) Root sections

* Observations were taken after 30 days of inoculation

Figure 3: Response of different explants for callus induction in *C. asatica* on MS media supplemented with 2, 4-D (1mg/l) alng with BAP (0.5 mg/l)

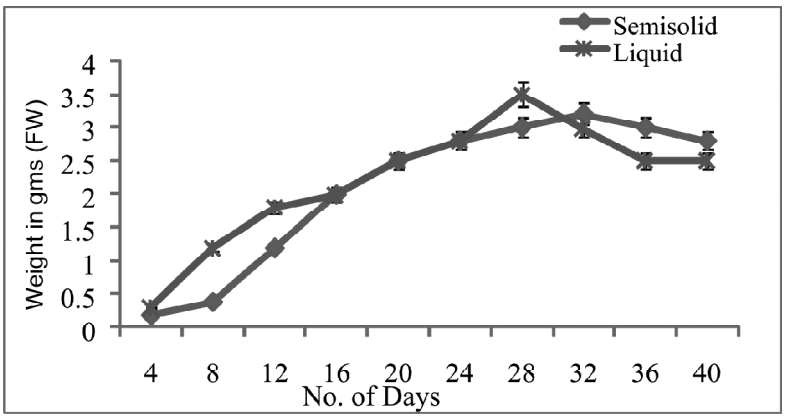


Figure 4: Growth pattern of *C. asiatica in vitro* raised shoots in semisolid and liquid media

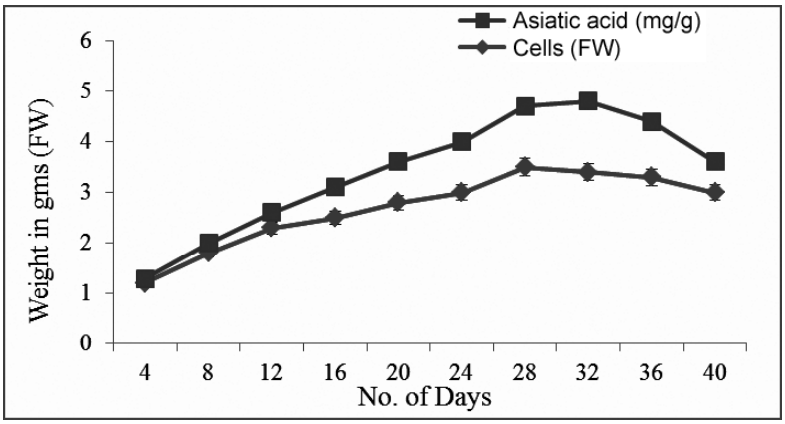


Figure 5: Growth pattern of *C. asiatica* cell suspension cultures

Growth studies

The cell cultures in semisolid and liquid media, followed the sigmoid growth pattern with lag, log, stationary and decline phases. The maximum biomass production in semisolid media was at 32 days, whereas liquid cultures accumulated maximum biomass within 28 days (Figure 4). This result was analogous to the one, obtained by Kim *et al.* in 2004a, where the biomass reached its peak on the 26th day after culture. The cell suspensions of *C. asiatica,* followed the same growth pattern as the shoots with the maximum growth at 28th day of inoculation (Figure 5).

Asiatic acid quantification

The asiatic acid content was quantified by modified HPLC method (Rafamantanana *et al.*, 2009) by comparing the

retention time of samples with that of the asiatic acid standard (Figure 6). Though higher biomass was obtained in liquid cultures, higher quantity of asiatic acid was detected in the cultures propagated on semisolid media. The amount of asiatic acid obtained in semisolid and liquid shoot cultures was found to be 1.02± 0.03 mg/gFW and 0.47± 0.08 mg/gFW respectively (Figures 7 and 8). The production of asiatic acid reached its peak in both the shoot cultures (semisolid and liquid) at the mid stationary phase of growth curve, their accumulation after the growth ceases.

The callus was obtained from leaf explants after inoculation on MS semisolid media, supplemented with 2, 4-D (1 mg/l) and BAP (0.5 mg/l). The callus was further maintained and used for initiation of cell suspension cultures. The presence of asiatic acid in the callus was quantified after 32 days of inoculation. The amount of asiatic acid in the leaf callus was found to be 1.46± 0.06 mg/gFW, calculated from the peak area, obtained after the HPLC analysis (Figure 9). Though many reports were published on the presence of centellosides in *in vitro* shoots as well as whole plants (Kim *et al*., 2004a; Kim *et al*., 2004b; Mercy *et al*., 2012). "There are scanty reports from undifferentiated calli and suspensions (Bonfill *et al*., 2011). In the present study we are reporting high levels of asiatic acid from undifferentiated cultures in comparison to *in vitro* shoot cultures of *Centella asiatica*".

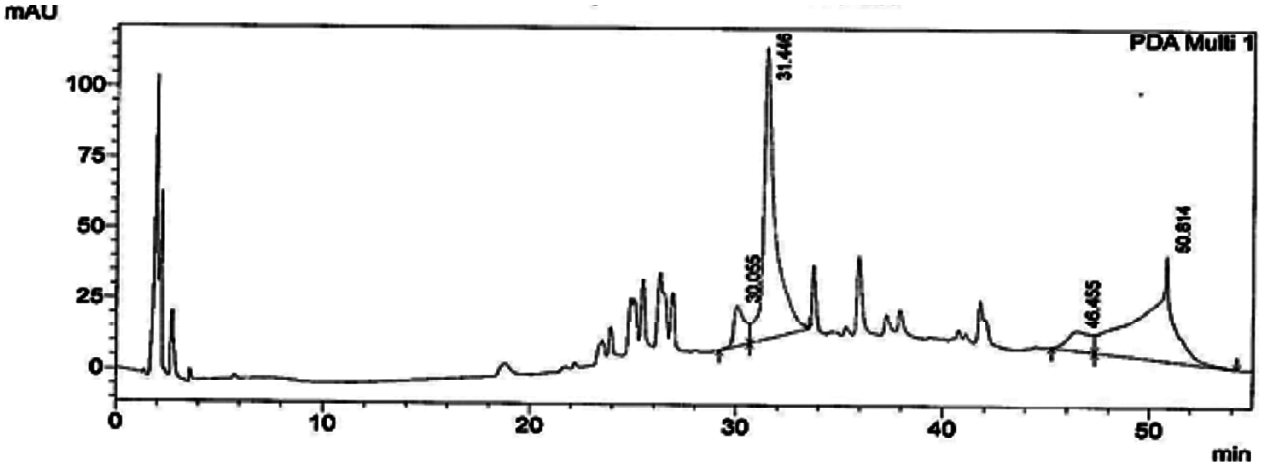


Figure 6: HPLC chromatogram of authentic asiatic acid

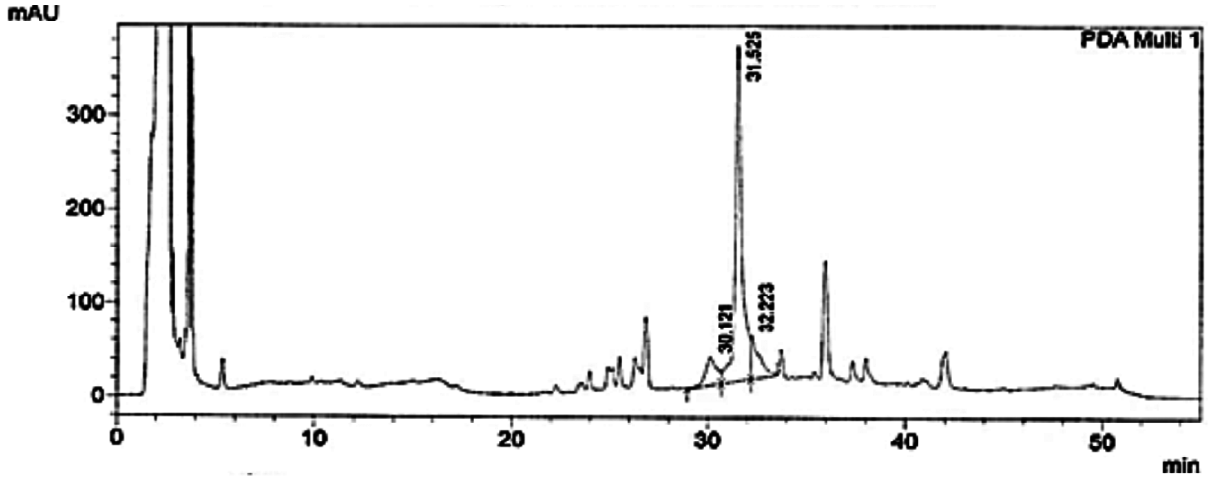


Figure 7: HPLC chromatogram of asiatic acid for plants grown on semisolid media

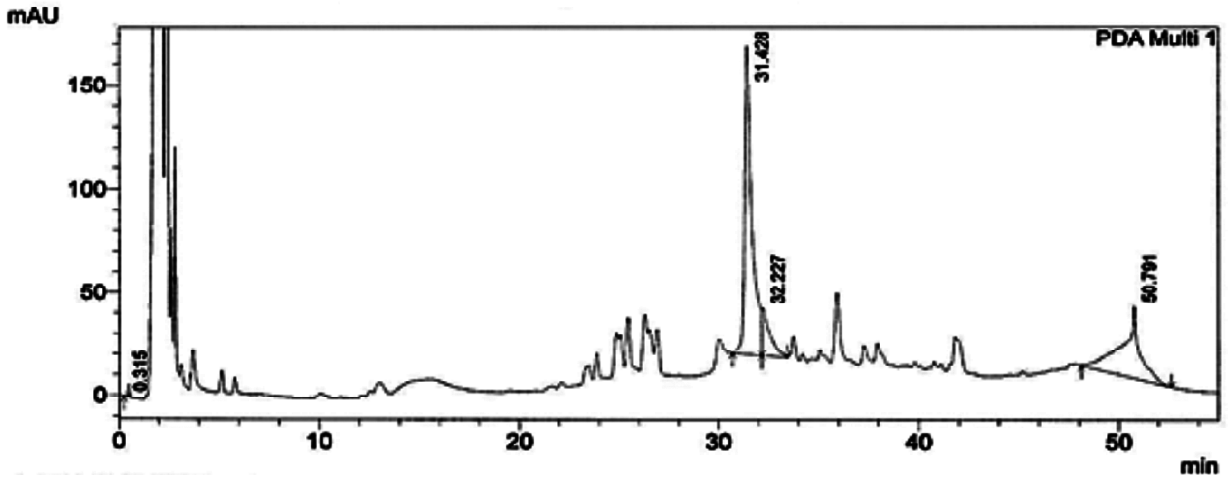


Figure 8: HPLC chromatogram of asiatic acid for plant in liquid media

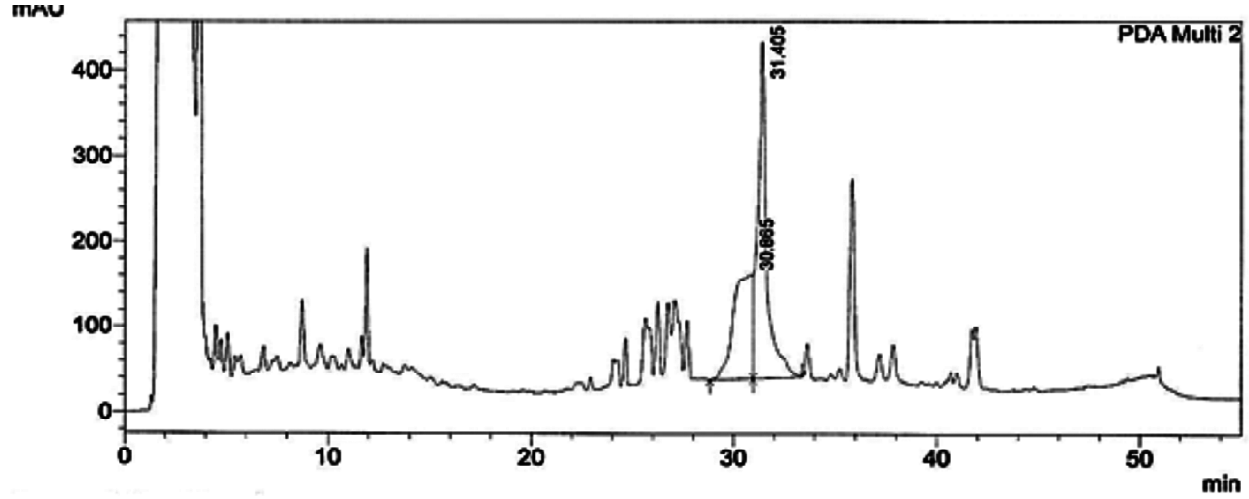


Figure 9: HPLC chromatogram of asiatic acid for cell suspension culture

Conclusion

Among the centellosides, asiatic acid alone has many therapeutic properties like anticancer, antihepatitis, *etc*. Hence, there is a need to produce asiatic acid in bulk amount, which is at present being extracted from the wild plants, obtained from natural habitats. The detection of asiatic acid in both *in vitro* grown shoots and callus cultures, presents a reliable source for continuous production on a large scale to meet the national and international demand. Further, the amount of asiatic acid can be enhanced by elicitation and others approaches for its commercial production.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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