

Estimation of Asiaticoside by Using RP-HPLC and FAME Analysis of Medicinally Important Plant *Centella asiatica*

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Abstract

Due to the presence of several bioactive compounds, *Centella asiatica* (L.) is used in traditional medicine for the treatment of various ailments. This usage of plant and its products created a huge demand which required an alternative method of its commercial production so that the loss of natural plant and its genetic pool can be prevented. A different investigation using biotechnological methods provided a wealth of information to enhance the biomass as well as different bioactive compounds. In the present study, we focused and reported the effect of different nitrogen sources on shoot proliferation of *Centella asiatica*. Effect of malt extract as plant elicitor on asiaticoside production as well as fatty acid methyl ester (FAME) profile was also worked out. Four different nitrogen sources i.e., NH_4NO_3 (1.65 g/l), KNO_3 (0.8 g/l), NaNO_3 (1.65 g/l), $\text{Ca}(\text{NO}_3)_2$ (0.825 g/l) on five different accessions of *Centella asiatica* were tested and accession number 347492 (14.66 ± 2.4) provided maximum shoot proliferation with ammonium nitrate. RP-HPLC analysis of accession number 347492 revealed that malt extract as plant extract enhanced 6 times asiaticoside in comparison to standard conditions. GC-MS analysis of five different accessions of *Centella asiatica* concluded that this plant is rich in the content of Pentadecanoic acid, 9, 12 Octadecadienoic acid (Linoleic acid) and 9, 12, 15 Octadecatrienoic acid (Linolenic acid). Most potential fatty acid methyl esters containing plant accession number is 281374.

Keywords *Centella asiatica*; Nitrogen source; Malt extract; Asiaticoside; Fatty acid methyl ester analysis

Introduction

Plants contains various active phytochemicals which includes vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites which are well to do in antioxidant activity [1,2]. *Centella asiatica* (L.) is a polymorphous, perennial, medicinal creeping plant, rooting at nodes and belongs to the family Apiaceae (Umbeliferae). As the plant inhabits a various region in India, it is popular with its regional names such as Thankuni in Bengali, Gotukola in Sinhali, Manimunni in Assam, Valleri in Decan, Mandookaparni in Hindi, Indian pennywort in English [3], etc. This plant is found in abundance on moist, swampy, sandy or clay soils, often in large clumps, forming a dense green carpet. It is also used as a cover crop in plantations. The medicinal value of this plant was revealed in *Charaka Chikitsa* [4] and main active essential elements of *C. asiatica* (L.) are asiatic acid, madecasic acid, asiaticoside, madecassoside, brahmoside, brahmide acid, brahminoside, thankinioside, isothankuniside, centelloside, madasiatic acid, alkaloids, flavanoids, etc. [5-10] which are known to take care of skin problems, to heal wounds, for stimulating the nerves and brain cells, antileprotic, antifilarial, antibacterial, adaptogenic, antifeedant, anti-stress, anti-ulcer, anti-oxidative stress, anti-radiation properties, anti-heavy metal poisoning, antiviral properties, anticancer etc. [11-20].

It was also reported that asiaticoside, one of the important compound of *C. asiatica* shows anti-tumor activity by apoptosis of tumor cells and also used in the healing of leprosy or skin disorder by collagen I synthesis in human [21]. Due to its valuable medicinally

important properties, this plant, as well as its extract, is in huge demand and such demand cannot be fulfilled by natural means. Thus, in the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have more prospective as enhancement to traditional production of bioactive plant metabolites [22,23].

Several groups have worked out that carbon and nitrogen source are essential for the growth of the plant and they also have an important role in metabolic pathways [24] to enhance the formation of auxiliary buds and branching of adventitious roots [25]. Plant cells *in-vitro*, shows physiological and morphological responses to microbial, physical or chemical factors which are known as 'elicitors'. Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plants to ensure their survival. Treatment with elicitors was reported to enhance secondary metabolites in *C. asiatica* [26,27]. Therefore, in the present investigation, the focus has been done to record the effect of different nitrogen sources on shoot multiplication and effect of elicitor such as malt extract on the production of asiaticoside in different accessions of *Centella asiatica*. Another attempt has been made to investigate the fatty acid methyl ester (FAME) profile of this plant under normal environmental conditions. The purpose of this investigation was to find out potential culture conditions as well as potential accession which can provide high yield of plant material as well as active compounds. For this purpose, MS media supplemented with 6 Benzyl amino purine containing four different nitrogen sources $\{\text{NH}_4\text{NO}_3, \text{KNO}_3, \text{NaNO}_3 \text{ and } \text{Ca}(\text{NO}_3)_2\}$ were tested individually. Quantitative analysis of asiaticoside was carried out using the standard protocol of reverse phase high performance liquid chromatography with the plant's accession which

provided best results with malt extract. FAME analysis was done with GC-MS for five different accessions to access the potential fatty acid containing accession of *Centella asiatica*.

Materials and Methods

Plant material

Cultures of five different accessions i.e., 281374, 383913, 342109, 347492, 331514 of *Centella asiatica* were collected from National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India.

In-vitro propagation in different culture conditions

In previous study effect of different carbon source and elicitors on the shoot multiplication of all the five accessions were done [28]. Roy et al. [29] also reported that MS media contains highest number of shoots. In the present study the effect of nitrogen source on shoot multiplication, Murashige and Skoog (MS) [30] (Hi-media) media was prepared and pH was adjusted to 5.8 using 1 N HCl or 1 N NaOH solution. 0.8% plant agar was used to solidify the media. Sterilization of media was done by autoclaving for 20 min at 121°C and 15 lb pressure. MS media was supplemented with 1.5 mg/l 6-Benzylaminopurine (Hi-media) along with different nitrogen sources {NH₄NO₃ (1.65 g/l), KNO₃ (0.8 g/l), NaNO₃ (1.65 g/l), Ca(NO₃)₂ (0.825 g/l)}. After the solidification of media, sterile shoot nodes from each accession were inoculated in culture tubes (25 × 150 mm) separately. The cultures were incubated at 26 ± 2°C under 16 h photoperiod and light intensity of 3000 lux for four weeks. Each and every experiment was done in triplicates. Visual data was recorded after the 4th week of inoculation in terms of number of shoots and length of shoots for *in-vitro* growth measurement.

Estimation of asiaticoside by HPLC

For asiaticoside analysis, plant material of accession number 347492 was used which was found best in a previous study of effect of different plant elicitors on biomass of *C. asiatica*. Best results were found in case of malt extract as plant elicitor. 75 mg of air dried powdered leaf sample of plant was mixed with 20 ml of methanol and sonicated for 10 minutes with occasional shaking. After cooling sample was filtered with 0.45 µm filter paper and final volume made up to 30 ml by mixing additional methanol. Filtered a portion of this solution through a 0.2 µm polytetrafluoroethylene (PTFE) syringe filter and kept for HPLC analysis. 1 mg of standard of asiaticoside (Sigma) was dissolved in 10 ml methanol to prepare standard solution. For the analysis of asiaticoside, reverse phase high performance liquid chromatography (RP-HPLC) was used. HPLC system included a pump, an injection port, column compartment, and UV-VIS detector and fused core C18 HPLC column with chromatographic conditions i.e., mobile phase {Water: Methanol (3:7)}, flow rate (1 ml/min), injected volume (15 µl), column temperature (260°C), run time (10 minutes) and UV detection (220 nm). 15 µl standard solution was injected into HPLC machine and then injected the standard sample extract which was not treated with any plant elicitor. After that samples of accession number 347492 were injected in triplets.

Fatty Acid Methyl Ester (FAME) analysis using GC-MS

For FAME analysis, plants of all five accessions were cultured in MS media supplemented with 1.5 mg/L 6-Benzylaminopurine without using any extra source of carbon, nitrogen and plant elicitors etc.

Sterile shoot nodes from each accession were inoculated in culture tubes (25 × 150 mm) separately at 26 ± 2°C under 16 h photoperiod and light intensity of 3000 lux for four weeks. Each and every experiment was done in triplicates. After four weeks, 100 mg chopped leaves of *Centella asiatica* were transferred to a screw-cap (teflon coated) glass tube and mixed with 1 ml of 2% methanolic HCl. The sample was then incubated at 90°C for an hour. After one hour, 1 ml of 0.9% NaCl was added followed by 2 ml of hexane and centrifuged at 2000 rpm for 2 minutes. The upper (hexane) layer of the sample was transferred into a fresh glass tube and dried under nitrogen flow. Dried sample was then diluted with 100 µl of hexane. 1 µl of samples was then injected into the GC-MS for analysis. Analysis was carried out by GC-MS electron impact ionization method. GC conditions included carrier gas (He), column temperature (50°C to 250°C) and run time (35 minutes). FAME of each accessions were analysed separately in triplets.

Data analysis

Observations were recorded and are presented as means ± standard deviation of 3 biological replicates to estimate the variability between the accessions.

Results

In-vitro propagation in different culture conditions

In previous study it was found that sucrose and malt extract showed highest amount of shoot multiplication [28]. Effect of nitrogen sources on shoot regeneration was observed in five accessions of *Centella asiatica*. Observation was recorded as mean value of triplicate samples after four weeks of inoculation (Table 1). A comparative study of four different nitrogen sources i.e., ammonium nitrate, potassium nitrate, sodium nitrate and calcium nitrate was carried out to test their potential on shoot regeneration. Each accession responded in its own way in MS media containing different nitrogen sources. Among five different accessions, 347492 showed the best result in presence of MS media supplemented with ammonium nitrate as nitrogen source. Maximum number of shoots was 14.66 ± 2.4 while maximum length of shoots 1.61 ± 0.59 cm has been reported in this accession. Although maximum length of shoot was reported in the same accession with sodium nitrate as nitrogen source but number of shoots were less than the number of shoots reported in case of ammonium nitrate. Thus, as combined results of both number and length of shoots, accession number 347492 was a potential accession among five accessions tested. Ammonium nitrate was found as best source of nitrogen among the four different sources tested.

Estimation of asiaticoside by HPLC

Among the five-different accession 347492 shows the best growth in MS media containing malt extract (1 mg/L) and supplemented with BAP (1.5 mg/L) in a previous study. So, the estimation of asiaticoside with and without malt extract was done by reverse phase high performance liquid chromatography (RPHPLC) analysis. Figure 1 show the chromatogram of standard asiaticoside and the retention time of asiaticoside was in 5.2 minutes. If we compare the two chromatograms (Figure 1) with chromatogram of standard asiaticoside, we can see that methanolic extract of malt extract treated sample (Figure 1) contains 8.03% of asiaticoside whereas without treated sample (Figure 1) contains 1.4% of asiaticoside. The

percentages of asiaticoside production in both the treated and untreated samples clearly indicate the 6 times increase in production of asiaticoside. Kim et al. [26] also reported the enhanced production of asiaticoside by the use of elicitors such as methyl jasmonate. They reported 116.8 mg/l production of asiaticoside with the use of 0.1 mM MJ in B5 liquid media. They further increased the yield of asiaticoside by combining 0.1 mM methyl jasmonate with 0.025 mg/l TDZ [thidiazuron, 1-phenyl-3-(1,2,3-thiazol-5-yl) urea] and achieved a production of 342.72 mg/l. Kim et al. [31] also reported the use of methyl jasmonate in the increment of asiaticoside production in hairy root culture of *Centella asiatica*. Satheesan et al. [32] also reported two-fold increase in the asiaticoside production by root colonization of a fungus named *Piriformospora indica*. Fungal mediated elicitation of asiaticoside was also reported by Prasad et al. [27]. Nutrient mediated increment in the asiaticoside production along with high yield of biomass was reported by Prasad et al. [33] wherein they found this increment was due to reduction in the total nitrogen concentration and copper starvation.

Fatty Acid Methyl Ester (FAME) analysis by GC-MS

Table 2 lists the name of fatty acid as well as their relative percentage composition obtained from the gas chromatography mass spectrometry (GC-MS) analysis of the n-hexane extracts of five different accession of *Centella asiatica*. The analysis of fatty acid from five different accession of *Centella asiatica* by GC-MS showed that they contain both saturated and un-saturated fatty acids. In case of saturated fatty acids, three fatty acids i.e., Pentadecanoic, Hexadecanoic and Octadecanoic acid while in case of un-saturated fatty acids Octadecadienoic and Octadecatrienoic acid were reported.

The percentage of Pentadecanoic and Octadecadienoic acids were very high while Octadecatrienoic acid was present in moderate amounts in all five accessions. Percentage of Hexadecanoic and Octadecanoic were very small in comparison to other fatty acids. These fatty acids have good therapeutic value such as Hexadecanoic (Palmitic acid) reduces the risk of cardiovascular disease, Octadecanoic (Stearic acid) used in baked food items, etc. Unsaturated fatty acids, Octadecadienoic (Linoleic acid) and Octadecatrienoic (Linolenic acid) are the most important essential fatty acids as because our body cannot synthesis these fatty acids. Linoleic acid is essential for maintenance of growth and found to be a potent cyclooxygenase-2 (COX-2) catalyzed prostaglandin biosynthesis inhibitor.

Accession no. 281374 contain highest percent of Linoleic acid (approx. 24%) followed by 342109 (20.96%), 383913 (20.72%), 331514 (19.34%) and 347492 (18.62%). When we consider the presence of Linolenic acids, it is recorded that accession no. 347492 contains the highest percent (approx. 10%) followed by 281374 (9.6%), 383913 (7.11%), 383913 (6.41%) and 331514 (4.63%). These potential accessions can be utilized for the high yield of fatty acid production. Very less work has been reported related to the FAME profile of *Centella asiatica* which provided an opportunity to carry out this investigation. Jahan et al. [34] conducted a study related to elemental as well as fatty acid content of four medicinally important plant i.e., *Kaempferia rotunda*, *Cuscuta reflexa*, *Centella asiatica* and *Asparagus racemosus*. Within *Centella asiatica*, they found good amount of Hexadecanoic acid (9.96%), Heptadecanoic acid (3.28%) and Octadecanoic acid (8.34%) but they also reported no presence of 9,12-Octadecadienoic acid. Although in our study, good amount of 9,12-Octadecadienoic acid has been investigated.

Nitrogen Source	Accession Numbers									
	383913		281374		331514		347492		342109	
	A	B	A	B	A	B	A	B	A	B
NH ₄ NO ₃	4.67 ± 0.66	1.4 ± 0.33	6.33 ± 0.88	1.23 ± 0.26	10 ± 1.15	1.49 ± 0.27	14.66 ± 2.4	1.61 ± 0.59	5.33 ± 0.88	1.37 ± 0.62
KNO ₃	7.66 ± 2.18	1.46 ± 0.41	3 ± 0.57	1.35 ± 0.23	7 ± 0.57	0.9 ± 0.22	5.66 ± 0.88	0.93 ± 0.29	7.66 ± 1.2	0.81 ± 0.3
NaNO ₃	9.33 ± 2.4	1.17 ± 0.38	8 ± 0.15	0.76 ± 0.17	4 ± 0.57	0.75 ± 0.14	10 ± 1.15	1.94 ± 0.53	4.66 ± 0.66	0.64 ± 0.19
Ca(NO ₃) ₂	3 ± 0.57	0.25 ± 0.06	4.66 ± 0.66	1.01 ± 0.23	5.6 ± 1.2	0.97 ± 0.36	5.66 ± 1.2	1.04 ± 0.33	3.33 ± 0.88	0.84 ± 0.21

Table 1: Effect of different nitrogen sources on number and length of regenerated shoots in five different accession of *Centella asiatica* after four weeks of inoculation. Values are expressed as mean ± Standard Error (M ± SE). A represents number of shoots (M ± SE); B represents average length of shoot (M ± SE).

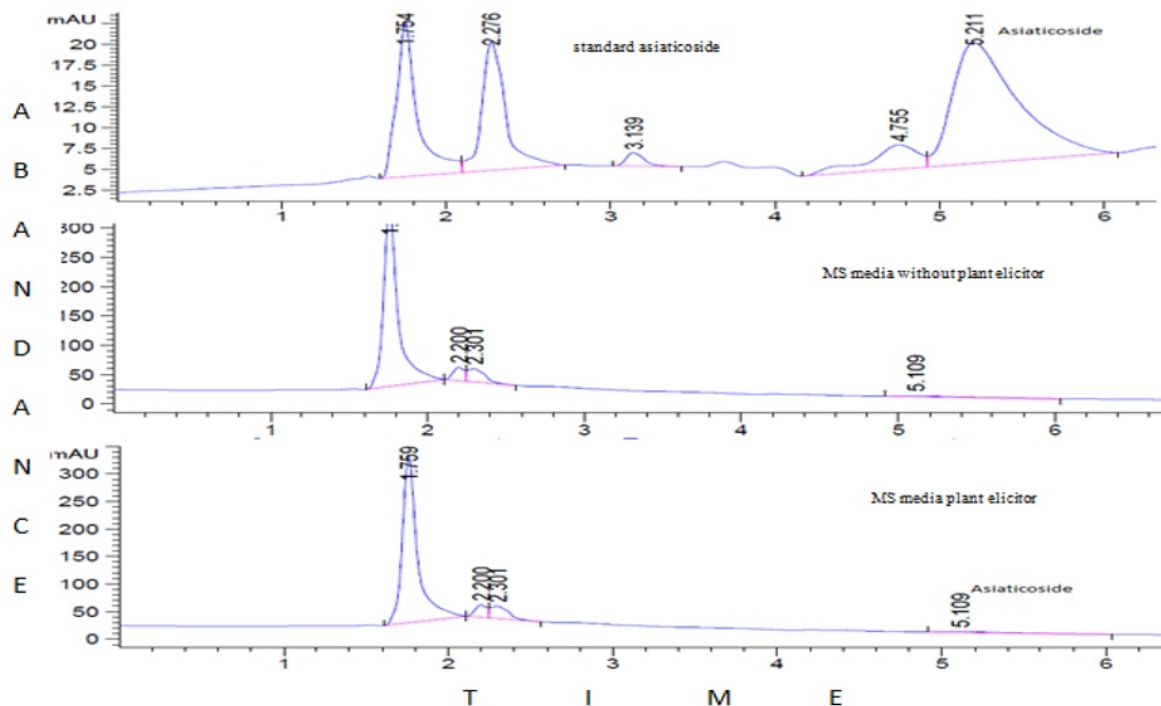


Figure 1: Chromatogram of standard asiaticoside, extract analysed from plant grown in MS media without plant elicitor and extract analysed from plant grown in MS media supplemented with malt extract as plant elicitor. Peaks of asiaticoside were found at 5.211, 5.109, 5.109 minutes in standard, without treated plant and treated plant respectively.

Fatty Acid	Relative %age of different Fatty acid in different accessions of <i>Centella asiatica</i>				
	281374	383913	342109	347492	331514
Saturated Fatty acids					
C15	17.31%	12.73%	15.08%	13.65%	12.54%
C16	1.91%	1.62%	1.90%	1.96%	1.58%
C18	1.70%	1.67%	1.65%	1.68%	1.15%
Un-saturated Fatty acids					
C18:2	23.95%	20.72%	20.96%	18.62%	19.34%
C18:3	9.59%	7.11%	6.41%	9.98%	4.63%

Table 2: Percentage of different saturated and un-saturated methylated fatty acids of five different accession of *Centella asiatica*. C15 - Pentadecanoic acid (Pentadecylic acid) (C₁₅H₃₀O₂), C16 - Hexadecanoic acid (Palmitic acid) (C₁₆H₃₂O₂), C18 - Octadecanoic acid (Stearic acid) (C₁₈H₃₆O₂), C18:2-9,12 Octadecadienoic acid (Linoleic acid) (C₁₈H₃₂O₂) and C18:3-9,12,15 Octadecatrienoic acid (Linolenic acid) (C₁₈H₃₀O₂).

Conclusion

This study concluded that ammonium nitrate is a potential source of nitrogen for the proliferation of shoots in *Centella asiatica*. Accession number 347492 was found best among five different accessions for the maximum production of shoots. Accession number 347492 has also shown the capacity to produce 6 times more asiaticoside production in comparison to the standard environmental conditions when supplemented with malt extract as plant elicitors. GC-MS analysis of

fatty acid methyl ester (FAME) of *Centella asiatica* revealed that accession number 281374 is the potential accession which can provide high yield commercially important fatty acids. These culture conditions and potential accessions can be used for high yield of biomass as well as asiaticoside and fatty acid production under lab conditions which may be beneficial for the fulfilment of their demand.

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References

- Zheng W, Wang SY (2001) Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry* 49: 5165-5170.
- Cai YZ, Sun M, Corke H (2003) Antioxidant activity of betalains from plants of the Amaranthaceae. *Journal of Agricultural and Food Chemistry* 51: 2288-2294.
- Jamil SS, Nizami Q, Salam M (2006) *Centella asiatica* (Linn.) Urban-A Review. *Natural Product Radiance* 6: 158-170.
- Bhavna D, Jyoti K (2011) *Centella asiatica*: The elixer of life. *International Journal of Research in Ayurveda & Pharmacy* 2: 431-438.
- Bonfill M, Mangas S, Cusidó RM, Osuna L, Piñol MT, et al. (2006) Identification of triterpenoid compounds of *Centella asiatica* by thin-layer chromatography and mass spectrometry. *Biomedical Chromatography* 20: 151-153.
- Zhang FL, Wei YJ, Zhu J, Gong ZN (2008) Simultaneous quantitation of three major triterpenoid glycosides in *Centella asiatica* extracts by high performance liquid chromatography with evaporative light scattering detection. *Biomedical Chromatography* 22: 119-124.
- Jiang ZY, Zhang XM, Zhou J, Chen JJ (2005) New triterpenoid glycosides from *Centella asiatica*. *Helvetica Chimica Acta* 88: 297-303.
- Weng XX, Zhang J, Gao W, Cheng L, Shao Y, et al. (2012) Two New Pentacyclic Triterpenoids from *Centella asiatica*. *Helvetica Chimica Acta* 95: 255-260.
- Krishnamurthy RG, Senut MC, Zemke D, Min J, Frenkel MB, et al. (2009) Asiatic Acid, a Pentacyclic Triterpene from *Centella asiatica*, Is Neuroprotective in a Mouse Model of Focal Cerebral Ischemia. *Journal of Neuroscience Research* 87: 2541-2550.
- Subban R, Veerakumar A, Manimaran R, Hashim KM, Balachandran I (2008) Two new flavonoids from *Centella asiatica* (Linn.). *Journal of Natural Medicines* 62: 369-373.
- Singh S, Gautam A, Sharma A, Batra A (2010) *Centella asiatica* (L.): A plant with immense medicinal potential but threatened. *International Journal of Pharmaceutical Sciences Review and Research* 4: 9-17.
- Rao KGM, Rao SM, Rao SG (2006) *Centella asiatica*(L) leaf extract treatment during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborisation in rats. *Evidence Based Complementary and Alternative Medicine* 3: 349-57.
- Gupta R, Flora SJS (2006) Effect of *Centella asiatica* on arsenic induced oxidative stress and metal distribution in rats. *Journal of Applied Toxicology* 26: 213-222.
- Flora SJS, Gupta R (2007) Beneficial Effects of *Centella asiatica* Aqueous Extract against Arsenic-induced Oxidative Stress and Essential Metal Status in Rats. *Phytotherapy Research* 21: 980-988.
- Shinomol GK, Ravikumar H, Muralidhara M (2010) Prophylaxis with *Centella asiatica* Confers Protection to Prepubertal Mice Against 3-nitropropionic-Acid-Induced Oxidative Stress in Brain. *Phytotherapy Research* 24: 885-892.
- Heong S, Ariffin F, Kaur B, Karim AA, Huda N (2011) Antioxidant capacity and phenolic composition of fermented *Centella asiatica* herbal teas. *Journal of the Science of Food and Agriculture* 91: 2731-2739.
- Joy J, Nair CKK (2009) Protection of DNA and membranes from gamma-radiation induced damages by *Centella asiatica*. *Journal of Pharmacology and Pharmacotherapeutics* 61: 941-947.
- Saxena G, Flora SJS (2006) Changes in brain biogenic amines and haem biosynthesis and their response to combined administration of succimers and *Centella asiatica* in lead poisoned rats. *Journal of Pharmacology and Pharmacotherapeutics* 58: 547-559.
- Roy A, Kundu K, Saxena G, Kumar L, Bharadvaja N (2016) Effect of different media and growth hormones on shoot multiplication of in-vitro grown *Centella asiatica* accessions. *Advance Techniques in Biology Med* 4: 172.
- Roy A, Attre T, Bharadvaja N (2017) Anticancer agent from medicinal plants: A Review. New aspects in medicinal plants and pharmacognosy. JB Books Publisher, Poland.
- Bonte F, Dumas M, Chaudagne C, Maybeck A (1994) Influence of asiatic acid, madecassic acid and asiaticoside on human collagen I synthesis. *Planta Medica* 60: 133-135.
- Vanisree M, Lee CY, Lo SF, Nalawade SM, Lin CY, et al. (2004) Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. *Botanical Bulletin Academia Sinica* 45: 1-22.
- Sharma M, Sharma A, Kumar A, Basu SK (2011) Enhancement of secondary metabolites in cultured plant cells through stress stimulus. *American Journal of Plant Physiology* 6: 50-71.
- Panathula CS, Mahadev MD, Naidu CV (2014) Effect of different carbohydrate on in vitro plant regeneration of *Centella asiatica* (L.), an important antijaundice medicinal plant. *International Journal of Medicinal and Aromatic Plants* 4: 41-47.
- Saad AIM, Elshahed AM (2012) Plant tissue culture media. Recent advances in plant in vitro culture. Chapter 2. In Tech Publisher.
- Kim OT, Kim MY, Hong MH, Ahn JC, Hwang B (2004) Stimulation of asiaticoside accumulation in the whole plant cultures of *Centella asiatica* (L.) Urban by elicitors. *Plant Cell Reports* 23: 339-344.
- Prasad A, Mathur A, Kalra A, Gupta MM, Lal RK, et al. (2013) Fungal elicitor-mediated enhancement in growth and asiaticoside content of *Centella asiatica* L. shoot cultures. *Plant Growth Regulation* 69: 265-273.
- Kundu K, Roy A, Saxena G, Kumar L, Bharadvaja N (2016) Effect of Different Carbon Sources and Elicitors on Shoot Multiplication in Accessions of *Centella asiatica*. *Med Aromat Plants* 5: 251.
- Roy A, Bharadvaja N (2017) Effect of different culture medias on shoot multiplication and stigmasterol content in accessions of *Centella asiatica*. *International Journal of Ayurvedic and Herbal Medicine* 7: 2643-2650.
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.
- Kim OT, Bang KH, Shin YS, Lee MJ, Jung SJ, et al. (2007) Enhanced production of asiaticoside from hairy root cultures of *Centella asiatica* (L.) Urban elicited by methyl jasmonate. *Plant Cell Reports* 26: 1941-1949.
- Satheesan J, Narayanan AK, Sakunthala M (2012) Induction of root colonization by *Piriformospora indica* leads to enhanced asiaticoside production in *Centella asiatica*. *Mycorrhiza* 22: 195-202.
- Prasad A, Mathur A, Singh M, Gupta MM, Uniyal GC, et al. (2012) Growth and asiaticoside production in multiple shoot cultures of a medicinal herb, *Centella asiatica* (L.) Urban, under the influence of nutrient manipulations. *Journal of Natural Medicines* 66: 383-387.
- Jahan IA, Akbar PN, Enayetullah M, Ahmmad N, Nuruddin M, et al. (2015) Elemental and Fatty Acid Content of Four Medicinal Plants: *Kaiempferia rotunda*, *Cuscuta reflexa*, *Centella asiatica* and *Asparagus racemosus*. *European Journal of Medicinal Plants* 10: 1-10.