Asiaticoside and Asiatic acid production in Shoot,Callus and Cell Suspension cultures of *Centella asiatica*

to be submitted as Major Project-II in partial fulfilment of the requirement for the degree of

M. Tech.

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CERTIFICATE

This is to certify **M. Laxmi Krishnan (2K15/IBT/07)** from **M.TECH ,Industrial biotechnology, fourth semester** that the dissertation entitled **Asiaticoside and Asiatic acid production in Shoot, Callus and Cell suspension cultures of *Centella asiatica*** in the fulfillment of the requirements for the reward of the degree of Master of Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honoring of any other degree.

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DECLARATION

I, **M. Laxmi Krishnan**, M.Tech Industrial Biotechnology,Fourth Semester Reg No. **2K15/IBT/07** ,Department of Biotechnology, Delhi Technological University , Delhi declare that my project work titled "**Asiaticoside and Asiatic acid production in Shoot, Callus and Cell suspension cultures of *Centella asiatica***" is an original record and no part of this work report has been submitted for any other degree or diploma . All the given information and works are true to my sense and knowledge.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL FORMS
SC	Shoot culture
CC	Callus Culture
CSC	Cell suspension Culture
BAP	6-benzylaminopurine
ME	Malt extract
ANOVA	analysis of varience
DPPH	2,2-diphenyl-1-picryl-hydrazylhydrate
RP-HPLC	Reversed phase-High performance liquid chromatography
NAA	Naphthaleneacetic acid
IAA	Indole-3-acetic acid
2,4 D	2,4-Dichlorophenoxyacetic acid
M ± SE	Mean ± Standard error
MS media	Murashige and Skoog media
OSC	Oxidosqualene cyclase
ASD	Asiaticoside
ATA	Asiatic acid
GAE	Gallic acid equivalent
QE	Quercetin Equivalent
F-C reagent	Folins Ciocalteau reagent
AA	Antioxidant activity
PTFE	Polytetrafluoroethylene
RPM	Rotation per minute

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Asiaticoside and Asiatic acid production in Shoot, Callus and Cell suspension cultures of *Centella asiatica*

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ABSTRACT

Centella asiatica commonly known as gotu kola, Mandukaparni or Indian pennywort is a small herbaceous perennial medicinal plant belonging to *Apiaceae* family. Its active metabolites are triterpenoids which includes asiaticoside, asiatic acid, madecassoside, madecassic acid which has wide range of pharmaceutical activities like wound healing, memory enhancer, treatment of skin diseases etc. The present work is aimed at comparative estimation of Asiaticoside and Asiatic acid before and after the Methyl jasmonate (MEJA) elicitation in various cell cultures like shoot culture (SC), callus culture (CC) and cell suspension culture (CSC) of *Centella asiatica* . The *in vitro* shoot cultures were grown to study the effects of variations in the nitrogen, carbon, phosphorous, potassium, micronutrient (Manganese) and macronutrient (magnesium) concentrations in MS media and also elicitors like MEJA and malt extract on the shoot multiplication . The callus culture was established using leaf explants in the MS media supplemented with combination of plant hormones like BAP,NAA,2,4 D,IAA . CSC was established using callus as the explants and its growth curve was plotted . By HPLC analysis, maximum asiaticoside content in CSC (0.033mg/ml) followed by shoot and callus culture. When elicited with 100µM MEJA there was 39 fold increase in asiaticoside content(0.146 mg/ml) in shoot and about 68 fold(0.2019 mg/ml) increase in CC. Asiatic acid content was highest in SC (0.653 mg/ml) followed by callus (0.0349 mg/ml) which was enhanced by 2 fold (0.0675 mg/ml) after MEJA elicitation. The antioxidant activity by DPPH assay showed maximum IC 50 value in CSC extract than shoot and callus extracts. The quantitative estimation of total phenolic, flavanoids and tannins indicates highest phytochemicals in SC when compared to CC and CSC.

Keywords: *Centella asiatica,* cell suspension culture, callus culture, Asiaticoside, Asiatic acid

1.0 INTRODUCTION

Centella asiatica , a small edible ,annual herbaceous medicinal plant belonging to Apiaceae family native to India, Sri Lanka,Iran, New Guinea, Australia, Indonesia southern and central Africa(Hoang N. *et al*.,2010) . It is known by various vernacular names at different parts of India and also abroad like Gotu kola , Brahma-Manduki in Hindi, Kodagam in Malayalam, Mandukaparnika in Sanskrit, Indian pennywort in USA , Ghod tapre in Nepal, Fo-ti-tieng in China(Singh S. *et al*.,2010). This plant is listed as an important drug in the Indian Herbal Pharmacopoeia, European Pharmacopoeia, Pharmacopoeia of the People's Republic of China and German Homoeopathic Pharmacopoeia (Schaneberg B.T *et al.*,2002) .In 2006,it was ranked third position in a priority list of most essential Indian medicinal plants based on their pharmaceutical and economic importance and also the demands for its raw material is constantly rising throughout the world (Sivakumar G.*et al*.,2006). The wild population was banned for the commercial applications since the plant has reached a critical level due to lack of proper cultivation practices and ruthless collection of herbs for medicinal purposes (Karthikeyan K. *et al*., 2009). The wild populations growing near filthy ditches are sometimes contaminated with heavy metal and chemical pollutants which affects their medicinal efficacy during its formulations in medicinal products, Thus Plant Tissue culture techniques through some biotechnological intervensions for increasing the biomass and as well as for its secondary metabolites is an alternative approach to meet the current market demands (Prasad A. *et al*.,2012).

Centella contains several valuable secondary compounds or terpenoids, known as centelloids,which includes asiaticoside, madecassoside, centelloside,centellose , brahminoside, thankunizide, sceffoleoside, brahmoside, and asiatic, centellic, brahmic, and madecassic acids. The most biologically active compounds include triterpene saponin like asiatic acid, asiaticoside, madecassoside and madecassic acid, which are responsible for a wide range of therapeutic activity(Govarthanan M *et al.,*2015).Terpene biosynthesis takes place in various steps initially with the formation of the isoprene unit which is formed by two pathways either on mevalonic acid or on 1-deoxyxylulose ,then these units associate to form the isoprenoid backbone which undergoes cyclization to form the carbon skeletons and finally, there are the interrelationship, hydroxylation and oxidation reactions leading to respective terpenoids (James J.T *et al*.,2009).Triterpene consists of 6 isoprene units and have molecular formula C_3OH_{48}.Many pharmacologically important compounds with plant

origin usually have a complex structure making them very difficult to synthesis chemically(James J.,2008)

The most abundant triterpenoid saponin, asiaticoside mainly found in leaves is commercially exploited as wound healing agent in ointments and beauty products like skin nourishing creams, anti ageing serums and memory enhancers for mentally retarded children ,because to its potent anti inflammatory effects and cell proliferative activity , antibacterial, fungicidal effects which have been shown to aid the healing of wounds , ulcers, various skin diseases, mental disorders (Bonfill *et al*.,2011 James J.*et a*l.,2011).Moreover, it is also used for the treatment of various skin diseases including psoriasis, leprosy, eczema, varicose ulcers, diarrhoea, amenorrhea, fever, vein insufficiency, tuberculosis, and diseases related of the female genitourinary tract (Brinkhaus B *et al*., 2000). *Centella asiatica* is well known to revitalize the brain and nervous system, thereby increasing the concentration ,attention span and delays aging (Brinkhaus B. *et al*,2000). It helps in maintenance of connective tissue by tissues by strengthening the weakened vein (Darn is F , 1979, Allegra C.,1981)

The plant cell cultures offers the possibility of acquiring the preferable medicinal compounds at the same time ensuring a sustainable conservation of biodiversity(Phillison *et al* .,1990). Plant cell cultures also provide us with freedom to manipulate the production of desired compound using elicitors, plant growth hormones. Callus culture is an unorganized proliferative mass of cell, tissues or organs grown in asceptical conditions which facilitate the amplification of limiting plant material. It is possible to regenerate the plant from unorganized callus tissues grown from different explants like leaf, petiole by dedifferentiation caused due to by plant growth regulators. Plant regeneration from calli occurs due to *de novo* organogenesis or somatic embryogenesis. Whereas in Cell suspension culture, single cells or small aggregates of cell multiply in an agitated liquid nutrient media to form a suspension . The suspension is usually initiated with inoculums like explants or pieces of undifferentiated and friable callus transferred into liquid media which is agitated at controlled speed and temperature . The advantage of suspension culture is faster growth than callus, better utilization of media nutrients due to agitation and it can provide us with continuous source of natural products (Bonfill M. *et al*, 2011). CSC can be used for large scale culturing of plant cells along with the production of desired secondary metabolites (Bonfill M. *et al*, 2011).

The nutrient media commonly used for plant tissue culture is Murashige and Skoog media,1962 consisting of macro,micro nutrients, minerals, vitamins,plant growth regulators and carbon source. The macroelements are nitrogen ,potassium, phosphorous magnesium,calcium ans sulphur. Nitrogen is vital for overall growth of the plant at it gets initially converted to amino acids and then to proteins and thus in the MS media,it is added as inorganic salts, the nitrate ion and/or the ammonium ion ,balance of which is important. Potassium is the positively charged ion which balances the negative ions in plants. Phosphorus is the fundamental part of nucleic acids and other structural compounds. Magnesium is a cation which balances the negative ions , essential for the functioning of enzymes, and also is a basic component of the chlorophyll molecule. Micronutrient includes the iron,manganese,zinc,copper etc. Manganese is used for enzymatic reactions like photosynthetic and respiratory processes. .The carbon source is usually sugars like sucrose,fructose which maintaines the osmotic potential of the plants. The proper balance of all these nutrient in the MS media contributes to proper growth and metabolism of plant cultures.

Antioxidant is any substance which prevents or retards deterioration richly available in fruits and green leafy vegetables which is known to reduce the risk of cancer, cardiovascular diseases. Some antioxidant components are Ascorbic acid, carotenoids, vitamin E, polyphenols and other phytochemicals. A free redical is substance with unpaired electrons causes damage to lipids,protein,enzyme thus antioxidants prevents these damages by neutralizing them. Phytochemical imparts health benefits beyond basic nutrition(Oomah *et al*.,2000).Several plant extracts and different class of phytochemicals are known to have antioxidant acitivity (Vani,Tripathi.,1997,1996). Flavanoids are potent antioxidant and its capacity depend on their molecular structure and Quercetin is one the most abundant dietary flavonol usually used as standards

1.1 OBJECTIVES :

1. ***In-vitro* propagation:** Effect of varying the concentration of nutrients in MS media like nitrogen ,carbon (sucrose) ,phosphorous, potassium, macronutrient (magnesium)and micronutrient (manganese) and effect of elicitors like Methyl Jasmonate and Malt extract on shoot growth and development was studied.
2. **Reversed-phase High performance Liquid Chromatography** –Quantitative estimation of Asiaticoside and Asiatic acid in Shoot culture, Callus culture and Cell suspension cultures of *Centella asiatica* before and after the elicitation with Methyl Jasmonate using RP-HPLC technique.
3. **Antioxidant activity and Phytochemical analysis:** Quantitative estimation of total phenolic content, total flavanoids and tannin content and antioxidant activity by DPPH assay in shoot, callus, cell suspension extracts.

2.0 REVIEW OF LITERARTURE

2.1 Morphology and origin

Centella asiatica is a tropical medicinal plant that belongs to Umbellifere(Apiaceae) family found in moist places upto an altitude of 1800 (Gohil K.,2010) including parts of India, Malaysia , China,South Africa Indonesia, Madagascar and Sri Lanka, with a long history of therapeutic uses(Jamil *et al.,*2007) . The leaves are small fan or orbicular or reniform shaped with yellowish-green colour, thin, long petioles with seven veins (R. N. Chopra et al.,1956). It has white , light purple-to-pink flowers which are sessile arranged in simple umbels and also bears small oval fruit (Gohil K.,2010).The plant usually grows horizontally combining each other and roots in underground, having long nodes and internodes, through its green to red stolones. The form and shape of the plant can differ greatly determined by the environmental conditions .

2.2 Chemical constituents

2.2.1. Triterpenoids

The terpenoids present in *Centella asiatica* include asiaticoside, madecassoside, brahmoside, thankunizide, centelloside , brahminoside, centellose, and asiatic, brahmic, centellic , and madecassic acids. On hydrolysis Asiaticoside , Madecassoside,centelloside yields the triterpene acids like Asiatic acid , madecassic acid, centellic acid respectively (Singh S.,2010).All of them are present in free form in the plant except for centellic acid (Singh S.,2010) . These saponins present can range for between 1 to 8% of the constituents based on the origin of the Centella plant (Brinkhaus *et al*.,2000). Some factors like climatic conditions, soil texture, and agronomic practices highly influences the bioactive constituent contents of *Centella asiatica*.. The variation in OSC , genetic diversity and other genes contributes to the composition and types of triterpenoid synthesized , presence and enzymatic reactions involved in biosynthesis(James J *et al*.,2009). Its most active metabolites are asiaticoside, asiatic acid, madecassic acid and madecassoside, exhibit many therapeutic properties associated with the treatment of nervous system, skin and blood diseases.

The main compounds found in aerial parts of the plant are asiaticoside followed by madecassoside ,Asiatic acid and madecassic acid(Mangas *et al.,*2006)

a) Asiaticoside

The most abundant triterpenoid saponin, asiaticoside is present mainly in leaves has antibacterial, fungicidal, strong diuretic and due to its potent anti-inflammatory effects and cell proliferative activity which contributes to the wound healing , curing skin diseases, ulcers, eczemas, leprosy, ,vein insufficiency, tuberculosis, and mental disorders, (James J. *et a*l.,2011,Subathra M. *et al*., 2005, Kim W.J *et al*.,2009).

b) Asiatic acid

It was found out that Asiatic acid helps in controlling the cell division in breast cancer, melanoma cells, colon cancer , human hepatoma , cytotoxic activity on fibroblast cells (Kim W.J e*t al*.,2009).Asiatic acid (the aglycon of asiaticoside) inhibites lipopolysaccharide induced Nitric oxide and prostaglandin E2 production than asiaticoside due to its greater anti-inflammatory properties(Yun K *et al*.,2007).

c) Madecassoside :

The Asiaticoside and madecassoside content was found to be maximum in the leaves and less in roots of Centella plant.(Aziz Z.A. *et al,* 2007)**.**

d) Madecassic acid

The effect of madecassoside are due to the metabolic product such as the sapogenin madecassic acid rather than their direct effect (Heon J.,2009). Anti- inflammatory effects of Madecassic acid (the aglycon of madecassoside) was observed in lipopolysaccharide induced macrophage model showed greater inhibitory effects on lipopolysaccharide induced nitric oxide and prostaglandin E2 production than madecassoside (Heon J.,2009) .

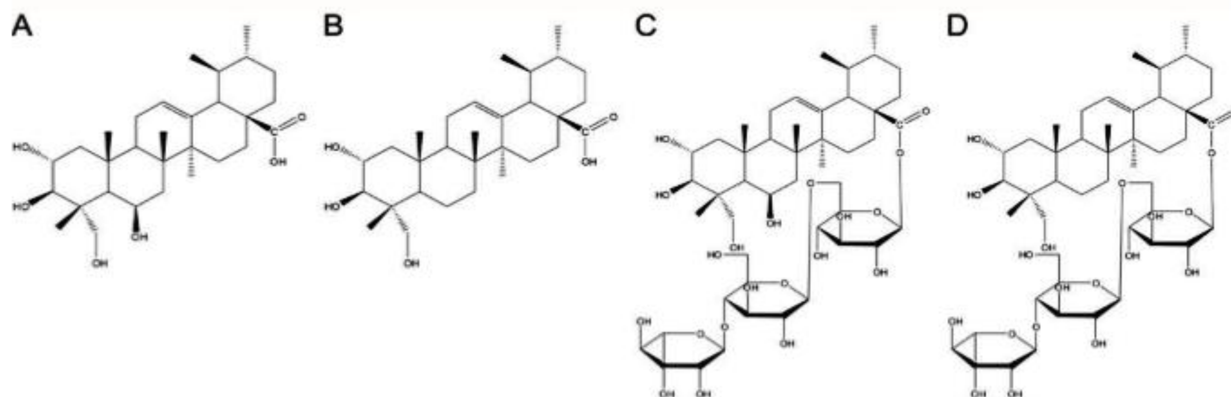


Figure 1: Chemical structures of the most important compounds of *Centella asiatica*: (a)Madecassic acid (b) asiatic acid, (c) madecassoside and (d) asiaticoside (James J et al.,2009).

2.2.3 Flavanoids

It was reported that Flavanoids, 3-glucosylkaemferol , 7-glucosylkaemferol and 3-glucosylquercetin was isolated from the leaves of Centella plant (Rastogi RP.,1969)

2.2.4 Fatty acids

The reported fatty oils in centella extracts includes glycerides of lignoceric, palmitic, linoleic , stearic, oleic and linolenic acids.(Chopra RN,1956)

2.2.5 Phytochemical compounds

It has been reported that *C. asiatica* have abundant phenolic compounds such as rutin, quercetin, luteolin, myricetin, epicatechin, , naringin ,kaempferol, naringenin and catechin (Hussin M.,1993,Bajpai M.,2005.,Mustafa.,2010) which is responsible for its pharmacological functions (Ariffin *et al*.,2011).It was also reported that the plant contains tannins, sugars, inorganic Acids (Kapoor L.,2005) and resin(Chopra RN.,1956), amino acids like phenylalanine ,aspartic acid, α alanine , glutamic acid and glycine (Malhotra *et al.,*1961)

2.2.6 Antioxidant properties

The antioxidant activity of Centella plays a key role in acting against the reactive oxygen species in our body (Hamid ,2002,Mustafa.,2010). It has been reported to work against the oxidative damages caused due to aging in rat brain (Subathra *et al.,*2005). Due to health benefits like antioxidant activity, the usage of Centella in food and beverages has been increased over these years(Hamid *et al*., 2002). The antioxidant activity of Centella extracts (84%) was compared with Vitamin C (88%) and grape seed extract (83%) in a study (Hashim P *et al*.,2011).

2.3 Pharmaceutical properties

Centella asiatica also known as the "Brain food" in India is among the most important herbs for curing skin problems, healing wounds, revitalizing the brain cells and nerves (Singh S. *et al.*,2010).

a) **Wound healing and skin disease** An extract called Madecassol of this plant contains Madecassic acid, Asiaticoside and Asiatic acid helps speeding up the cicatrisation and grafting of wounds . Asiaticoside aids in the extracellular matrix synthesis and fibroblasts proliferation in wound healing and is reported to cause changes in gene expression and induce type 1 collagen synthesis in human fibroblasts (Srivastava R,1997, Rafamantanana M.H *et al*.,2009).
b) **Memory Enhancing**: Centella contains brahminoside,brahmoside, brahmic acid, isobrahmic acid, which has sedative , psychotropic and anticonvulsant properties. It is also useful in the treatment of mental disorders ,dementia and anxiety(Singh S.*et al.*, 2010)**.** Mentat ,a polyherbal formulation causes improvement in attention span, concentration and memory in mentally retarded children (Upadhyay S.K.,2002).
c) **Antidepessent properties**: The Centella extracts have antidepressant activities and reported to have reduced the corticosterone level in the serum(Chen Y *et al.*,2003,2005)
d) **Venous insufficiency:** It is known to strengthen the weakened veins of connective tissues (Allegra C.,1981)and helps in the treatment of scleroderma by stabilizing the connective tissue growth .
e) **Cognitive and antioxidant properties:** a potent antioxidant having neuroprotective effects and is found to have efficiently protected the rat brain against oxidative damage related to

aging. It revitalizes the brain and nervous system, also increases attention span , concentration and slows down aging (Brinkhaus B *et al*.,2000)

f) **Gastric ulcer:** Animal studies have shown that the Centella extracts has inhibited the gastric ulceration in rats caused by cold and restraint stress presumably reduced the damaging effects of free radicals and strengthened the gastric mucosal barrier (Gohil *et al*.,2010)

g) **Anti tumour**: Asiatic acid is reported to have anticancer effect especially against skin cancer(Park BC *et al*.,2005) and is known to control cell division in colon cancer, human hepatoma, and breast cancer (Kim W.J e*t al*.,2009).

h) **Cardioprotective** The whole plant extracts showed cardioprotective activity by limiting the ischemia-reperfusion induced myocardial infraction in rats (Pragada R.R *et al*.,2004)

i) **Radioprotective activity :** It protects against the induced behavioral changes by radiation caused during the clinical radiotherapy (Shobi V *et al.,*2001)

2.4 Plant cell cultures

Plant cell cultures through biotechnological production of metabolites is an attractive alternative, but till date there has been only limited success due to lack of understanding about the metabolite synthesis(Karuppusamy S,2009).Plant cell cultures have been utilised in various studies to enhance secondary metabolites production for medicinal uses(Gaines,2004). There should be better understanding about the biochemical conversions and metabolic pathways of these triterpenoids that will allow the exploitation and manipulation of secondary metabolite synthesis (James J.T *et al*.,2009). The benefit of using of using plant cell culture is the possibility of manipulating the synthesis of desirable compounds within the cells using elicitors and plant hormones(James J.,2008).

2.4.1 In vitro shoot cultures

Tissue culture techniques play an important role in germplasm conservation and clonal propagation of elite clones of Centella(Karthikeyan K. *et al*., 2009). Propagation of medicinal by proliferation is rapid and reliable technique for mass production of the required clones as well as secondary metabolites..Mass propagation of plants through in vitro micro propagation is the most promising and economical technique in tissue culture (Kundu K *et al*.,2016)

2.4.2 Callus cultures

An undifferentiated tissue which has the capability to develop into any of the plant organs like shoot, root or leaf under the optimum plant hormones and growth conditions is known as callus .The four most active triterpenes were detected but in lesser concentrations in the callus samples (James *et al*.,2008).

2.4.3 Cell suspension culture

Cell suspension cultures contains homogeneous cell population, allowing uniform and quick access to nutrition, precursors, growth hormones and signalling compounds for the cells and is more convenient for the large scale production of fine chemicals in bioreactors(Mustafa**.** *et al*.,2010). Cell suspensions and callus developed from Indian origin Centella has been reported to synthesise asiaticoside (Nath , Buragohain .,2005)

2.5 Elicitors

Elicitors are the molecule that enhances the secondary metabolite production under stress during which the plant defense mechanism protects the plant through secondary metabolite production. The metabolic synthesis of cultured cells can be enhanced by elicitor like methyl jasmonate (Suzuki ., 2005,Yoon *et al*. 2000). Jasmonates are molecules known to beneficent elicitors for enhancing many secondary metabolites including triterpenoid saponins of different plant origins (Memelink *et al.,* 2001, Gundlach *et al.,* 1992) .It has been has reported that in vitro plants and hairy root cultures of *C. asiatica* showed increased centelloids when elicited with 100 µM MEJA(Kim O.T *et al.* 2004, 2007, Mangas *et al*. 2006;). Elicitor like Malt extract at 100 mg/L enhances the shoot number, prevents yellowing of leaves and reduces the callus development at the cut end of the nodal explants therefore it is used for rapid proliferation of axillary buds and also to improve the quantity and quality of shoots developed(Sridhar T.M.,2014). Among the elicitors used, MS media supplemented with malt extract (1 mg/ltr) showed maximum number and maximum length of shoots (Kundu K. *et al*.,2016).

2.6 COMMERCIAL APPLICATIONS

The current price of centella extracts in Indian market is Rupees 850 per kilogram. The Himalaya drug company at Banglore, India had introduced many products containing extracts of *Centella asiatica* into the market like Mandukarparni and Mentat which improves the overall mental functions and exhibit significant anti-parkinsonian activity , Abana ,a heart care product helps lowering cholesterol trigylcerides, Gertiforte is an antistress drug.Himalaya has launched various beauty products like Nourishing skin cream for moisturization and protects the skin from harsh climate and pollution (Singh S *et al*.,2010).SD Biotechnologies.co Ltd(Korea) introduced SNP control cream improves drying and delays skin ageing. Paris of Horses biotechnology Co. Ltd. In China has launched Weight loss Tea containing centella extract along with wolfberry fruit ,Chrysanthemum ,Salvia,Pinella extracts for slimming(Singh S *et al*.,2010).

2.7 Clinical studies, dosage and side effects

In a study,hundred pregnant women were compared with the application of skin cream containing Centella extracts, collagen elastin and vitamin E (alpha tocopherol), showed decrease in developing stretch marks(striae gravidarum)(Gohil *et al* .,2005).A study to test antioxidant activity , elderly patients were given centella extracts at dosage of 500 and 750 mg per day for about 90 days showed improved strength and vigor(Gohil.,2010).Typical dosage of single dose capsules is 300 mg to 680 mg, thrice daily or 10mg concentrated extract available in capsules are recommended (Gohil *et al*.,2010). The dried leaves of Centella as tea made by adding one to two teaspoons to about two or three cups of boiling water allowed it to steep for ten to fifteen minutes and three cups per day is suggested (Brinkhaus *et al*.,2000). Till date there has been no known toxicity reported in centella in prescribed doses. The side effects are very rare but high doses may cause and burning sensations , nausea,headache, dizziness and skin allergy (Gohil *et al*.,2010).

3.0 MATERIALS AND METHOD

3.1 Plant Material Collection

The *in vitro* grown cultures of *Centella asiatica* of the accession number 347492 was collected from NBPGR(National bureau of Plant Genetic Resources), New Delhi, India.

3.2 Culture Medium

The constituents of Murashige and Skoog (MS) media as shown in the appendix was prepared according to the respective nutrients manipulated as per the following experiment conditions:

3.2.1 Shoot cultures by varying the ratio of Nitrogen source

The following are the total Nitrogen concentration developed by varying the ratios of ammonium nitrate: potassium nitrate ((NH_4NO_3:KNO_3) in the MS media supplemented with 1mg/ml 6-benzylaminopurine (BAP):

(a)20mM (0:20),(b) 40 mM (20:20), (c) 50mM (20:30) (d) 60mM (20:40) (control) (e) 100mM (40:60)

3.2.2 Shoot cultures by varying the percentage of carbon content

The following are the total sucrose concentration varied in the MS media supplemented with 1mg/l 6-benzylaminopurine (BAP):

(a) 0%,(b)1%,(c)2%,(d) 3%,(e)4%,(f) 5%,(g) 6% ,(e)7%

3.2.3 Shoot cultures by varying potassium content

The following are the total potassium concentration varied in the MS media supplemented with 1mg/l 6-benzylaminopurine (BAP)

a) 0% ,b)30%, c)60% ,d)100% ,e) 150

3.2.4 Shoot cultures by varying phosphorous content

The following are the total phosphorous concentration varied in the MS media supplemented with 1mg/l 6-benzylaminopurine (BAP)

a) 0% ,b)30%, c)60% ,d)100% ,e) 150

3.2.5 Shoot culture by varying macronutrients and micronutrients

a) Macronutrient : $Mg^{(2+)}$

The following are magnesium concentration varied in MS media supplemented with 1mg/l 6-benzylaminopurine (BAP):

(a) 0 mM **(b)** 1 mM **(c)** 2 mM (d). 3 mM

b) Micronutrient :$Mn^{(2+)}$

The following are manganese concentration varied in MS media supplemented with 1mg/l 6-benzylaminopurine (BAP):

(a) 0 µM (b) 100 µM (c)200 µM (d) 300 µM

3.2.6 Multiple Shoot cultures by varying concentration of elicitor and plant hormome

a) The following concentration of Methyl jasmonate was added to MS media supplemented with 1mg/ml 6-benzylaminopurine (BAP):

(a) 50µM ,(b)100µM, (c) 150 µM(d) 200 µM (e)5.250 µM

b) The following concentrations of malt extract and BAP was added to MS media:

1. MS media +1, 1.5, 2 mg/l BAP
2. MS media+ 1mg/l BAP + 0.5, 1,1.5 mg/l ME
3. MS media + 1.5 mg/l BAP + 0.5,1 ,1.5 mg/l ME
4. MS media + 2 mg/l BAP + 0.5, 1 ,1.5 mg/l ME

The pH of the media was adjusted to 5.8-6.0 using 1N hydrochloric acid or IN sodium hydroxide before solidification of agar (0.8 %w/v, plant agar, Hi-media, India). The test tubes and Media prepared were capped with cotton plugs properly and kept for autoclaving at 121° C and 15 lb pressure for 20 minutes for complete sterilization. Then 10 mL of molten media was poured into 50 mL boiling test tubes (Borosil, India) and kept undisturbed for some time for solidification of agar.

3.3 Shoot Multiplication

After solidification of media, sterile shoot nodes of *Centella asiatica* of the the accession number 347492 were inoculated vertically into all the culture tubes (25×150 mm). Each experiment condition was performed in triplicates. Subsequent subculturing were carried out in the same media composition after an interval of every 4 weeks.

3.4 Culture conditions

The culture was incubated at 26±2 ^{o}C under 16 h photoperiod and light intensity of 3000 lux with 55-60% relative humidity for 8 weeks. Readings and visual data was recorded after the every 4,6 and 8th week of inoculation in terms of number of number of shoot and length of shoots for *in vitro* growth measurement.

3.5 Callus Culture medium and Conditions

For callus induction,the Murashige and Skoog (MS) media was used which was supplemented with sucrose(3% w/v) . The pH was adjusted to 5.8-6.0 using 1N HCl or IN NaOH before solidification of agar (0.8 %w/v, plant agar, Hi-media, India). The test tubes and Media prepared were capped with cotton plugs properly and kept for autoclaving at 121° C and 15 lb pressure for 20 minutes for complete sterilization. Then 20-30 mL of molten media were poured into conical flasks (Borosil, India) and kept undisturbed for some time for solidification of agar.

After sterilization, the following combination of plant growth regulator were used for callus induction:

1. MS media + 1 mg/l BAP + 0.5,1,1.5,2 mg/l NAA
2. MS media + 1 mg/l BAP+ 0.5,1 mg/l 2,4 D
3. MS media + 1 mg/l BAP+ 0.5,1 mg/l IAA

For elicitation, along with hormones ,100 µM MEJA was added to the liquid media before solidification. Leaf explants were excised from the growing centella plants and placed in Murashige and skoog(MS) solid medium by carefully removing the edges of the leaves. Each experiment was performed in triplicates. Readings and visual data was recorded after the 6 weeks of inoculation in terms of morphology and callus growth. The culture was incubated at 26±2 ^{o}C under 16 h photoperiod and light intensity of 3000lux with 55-60% relative humidity for 8 weeks.

3.6 Establishing a Cell suspension culture

Approximate 2 g of fresh callus weight is transferred to in 250 ml Erlenmeyer flask containing 50 ml MS liquid medium supplemented with 3%(w/v) of sucrose and plant growth regulator (1 mg/l BAP and 1.5 mg/l NAA) without addition of agar to get a cell suspension culture . The cell suspension culture was established in MS liquid medium supplemented with 1 mg/l BAP and 1.5 mg/l NAA . For elicitation, after approximate 10 days, 100 µM MEJA was added to the liquid media carefully and mixed properly .The callus developed from the solid medium with same hormone concentrations were weighed accurately and then finely chopped into small pieces and inoculated into the liquid MS media. It was kept in a rotary shaker at 100 rpm in the dark conditions at 25°C for approximate 45-50 days.

3.7 Growth curve of cell suspension culture by dry weight and wet weight analysis

From the cell suspension , 5 ml sample were retrieved using sterilized pipette at every 3 days interval until 45 days. The sample was filtered through 0.45 µm nylon membrane using vacuum filtration unit. To measure wet cell weight , the cells in the suspension culture were filtered through the nylon membrane , washed with distilled water and weighed immediately. The dry cell weight was determined by drying the collected cells in nylon membrane at 50°C until a constant weight was attained.

3.8 Preparation of samples for DPPH assay and Phytochemical assays:

3.8.1 Shoot culture

Air dried plant material (0.5g) was weighed and crushed to fine powder and then add 5 ml of 80% methanol was added and mixed properly. Sonicate for 15 minutes and left at room temperature for 24 hrs.The extract was centrifuged for 10 minutes at 1500 g and the supernatant was collected and stored at 4°C until further use.

3.8.2 Callus culture

For callus extracts, approximate 0.5 g of air dried callus was soaked in 5 ml of of 80% methanol and mixed properly, then sonicate for 15 minutes and centrifuge for 10 minutes at 1500 g and the supernatant was collected and stored at 4°C until further use.

3.8.3 Cell suspension culture

For Cell suspension cells extract, the cell suspension was transferred into sterile falcon tubes and centrifuged at 1500g for 15 minutes and then the supernatant was removed so that cells remains at the bottom as pellet. Approximate 0.5 g of cells was weighed and add 5 ml of 80% methanol mix it properly. Then sonicate the samples for 10 minutes . Again , centrifuged the samples at 1500g for 10 minutes and the supernatant was collected and stored at $4^{o}C$ until further use

3.9 Antioxidant activity by DPPH free radical assay

Sample stock solutions of 1mg/ml concentration prepared which were diluted with methanol to final concentrations of 5, 10,25, 50, 125, 250, µg/mL, to make a total volume of 2.5 ml . 1 mL of a 0.3 mM DPPH prepared methanol solution was added to different concentrations of sample solutions and allowed to react at room temperature. Blank was methanol (1.0 mL) and plant extract solution (2.5 mL). The negative control was DPPH solution (1.0 mL) and methanol (2.5 mL) .The positive controls is the standard solutions which is ascorbic acid for which all the above procedure was repeated . After 30 min the absorbance values of standard and samples were measured at 518 nm and converted into the antioxidant activity in percentage using the following formula:

Antioxidant activity (%) = 100- {[(Absorbance of sample - Absorbance of blank})* 100] / Absorbance of control}

The IC50 values was calculated from the plots where the X axis represents the concentration of sample/standard and the Y axis is the percentage of antioxidant activity . Experiment was performed in triplicates.

3.10 Preliminary screening of phyochemicals:

a) Test for Terpenoids (Salkowski test): About 5 ml of sample was mixed with 2 ml of chloroform in a test tube to which 3 ml of concentrated sulphuric acid was carefully added through the sides to form a layer. If reddish brown colour appears at the interface, indicates presence of terpenoids .

b)Test for Saponin . : In a test tube ,few amount of sample was taken and mixed properly with 5 ml of distilled water and was vigorously shaken, if stable foam appears ,then it indicates presence of saponin.

c)Test for Tannins: In a test tube ,about 1 ml of the sample was taken and then add 1 ml of Potassium ferricyanide (0.008 M) then 1 ml of Ferric chloride(0.02 M) containing 0.1 N HCl was added . Blue-black coloration indicates the presence of tannin.

d)Test for Alkaloids: In a test tube few quantity of sample is mixed with 2 ml of Wagner's reagent (2g of iodine and 6g of potassium iodide in 100 ml distilled water) .If Reddish brown coloured precipitate is formed indicates the presence of alkaloids.

e)Test for Flavonoids: In a test tube**,** about 5 ml of dilute ammonia solution was added to small amount of the sample followed by addition of concentrated sulphuric acid. A yellow colour indicates the presence of flavonoids which disappears after some time.

f)Test for Steroids: In a test tube, 1 ml of the sample was taken and dissolved in 5 ml chloroform , then equal volume (5 ml) of concentrated sulphuric acid was carefully added through the sides of test tube. If upper layer turns into red colour and the sulphuric acid layer turns yellow colour with slight green fluorescence which indicates the presence of steroid.

3.11 Flavanoid estimation

The Aluminium chloride colorimetric assay was done to estimate flavanoids .Take 1ml of sample and mix it with 4 ml of distilled water in a test tube . Then, 0.3 ml of prepared sodium nitrite (5 %) was added . 5 minutes later, 0.3 ml of prepared aluminium chloride(10 %) was mixed properly .After 5 minutes, 2 ml of prepared sodium hydroxide (1M) was added and made upto to 10 ml using distilled

water. A standard solution of quercetin of concentration 20, 40, 60, 80 and 100 µg/ml was prepared using the above procedure . The absorbance of the sample and qurecetin standards were determined using an UV or Visible spectrophotometer at 510 nm . The total flavonoid content obtained was expressed in terms of mg of Qurecetin Extract per gram of extract.

3.12 Phenol estimation

The Total phenol content was estimated using Folin-Ciocalteu (F-C) reagent method. Firstly F-C reagent(10%) was prepared by 10 ml of F-C reagent added to 90ml in water. Then sodium carbonate(5%) was prepared by dissolving 3g of sodium carbonate in 50 ml of distilled water . Then in a test tube take about 200µL sample to which 1.5 ml of prepared F-C reagent was added and kept at the dark condition for 5 minutes. After which,1.5 ml of prepared sodium carbonate was added and then mix it properly. All the test tubes were again kept in the dark at dark conditions for about 2 hours. The standard used was Gallic acid whose calibration curve was plotted. About 3 mg of Gallic acid was taken and dissolve it in 10 ml of methanol to get 300 mg/L concentration. Similarly 200, 100, 50 and 25 mg/L concentrations was prepared. Then the above mentioned procedure was followed for all the standard concentrations.The absorbance of all the samples and standards was determined using UV spectrophotometer at a wavelength of 750 nm.

3.13 Tannin estimation

The tannin content was estimated by Folin – Ciocalteu method. About 0.1 ml of the sample extract was treated with 7.5 ml of distilled water , with 0.5 ml of prepared F-C reagent (10%) reagent and add 1 ml of the prepared sodium carbonate solution(35 %) and then dilute it to 10 ml using distilled water. The entire reaction mixture was mixed well and kept at room temperature for 30 min.The gallic acid was used as the standard and prepared to various concentrations like 20, 40, 60, 80 and 100 µg/ml with methanol. Finally the absorbance of all the samples and gallic acid standards were determined using an UV or Visible spectrophotometer at 725 nm . The tannin content obtained was expressed as mg of gallic acid equivalent(GAE) per gram of extract

3.14 Statistical Analysis

All the experiments were performed in triplicates..The results are shown in terms of mean ±standard error (M±SE) and all the means obtained were compared with the One way Analysis of Varience (ANOVA). A probability of $P \leq 0.05$ and $F_{stat} > F_{critical\ value}$ was considered significant.

3.15 Preparation of samples for estimation of Asiaticoside and Asiatic acid using HPLC

3.15.1 Standard preparation:

Asiaticoside :

1) 1)Take 1 mg of asiaticoside standard (Sigma Aldrich) and dissolve in 1 ml of 9:1 ratio HPLC grade methanol : sterile double distilled water to get 1 mg/ml concentration .
2) 2)From 1 mg/ml solution , take 40 µl into another vial and make up with 160 µl methanol: water solution to obtain 0.5 mg/ml concentration
3) Finally from 0.5 mg/ml, take 100 µl standard and add 100 µl methanol: water solution (9:1) to obtain 0.1 mg/ml concentration.

The above procedure was repeated to prepare 0.1 mg/ml,0.5 mg/ml and 1 mg/ml concentrations of Asiatic acid standards.

3.15.2 Sample preparation for shoot and callus cultures:

1) Approximate 0.5 g of air dried shoots (grown in media with 1 mg/l BAP) and callus(grown in media with 1mg/l BAP and 1.5 NAA) was weighed.
2) Both of them were crushed to fine powder and transferred to glass tubes and add 5 ml of HPLC grade methanol: sterile distilled water(9:1) solution, mix it properly.
3) Then sonicate the samples for 10 minutes .
4) After cooling it, the samples were filtered through PTFE (Polytetrafluoroethylene) syringe filter of size 0.22 µM into glass vials .
5) Samples were stored in -4 cold storage until it is used for analysis.

3.15.3 Sample preparation for cell suspension culture:

1) The cell suspension grown in media with 1 mg/l BAP and 1.5 mg/l NAA was transferred into sterile falcon tubes and centrifuged at 1500g for 15 minutes and then the supernatant was removed so that cells remains at the bottom as pellet.
2) Approximate 0.5 g of cells was weighed and add 5 ml of HPLC grade methanol: sterile distilled water(9:1) solution, mix it properly.
3) Then sonicate the samples for 10 minutes .

4) Again , centrifuged the samples at 1500g for 10 minutes and the supernatant obtained was carefully filtered through PTFE (Polytetrafluoroethylene) syringe filter of size 0.22 μM into glass vials .
5) Samples were stored in -4 cold storage until it is used for analysis.

3.15.4 Specification of RP-HPLC system

HPLC system includes:

- C18 column,
- an injection port
- Waters 2998 photodiode array detector
- Waters 515 pump

3.15.5 Chromatographic Conditions:

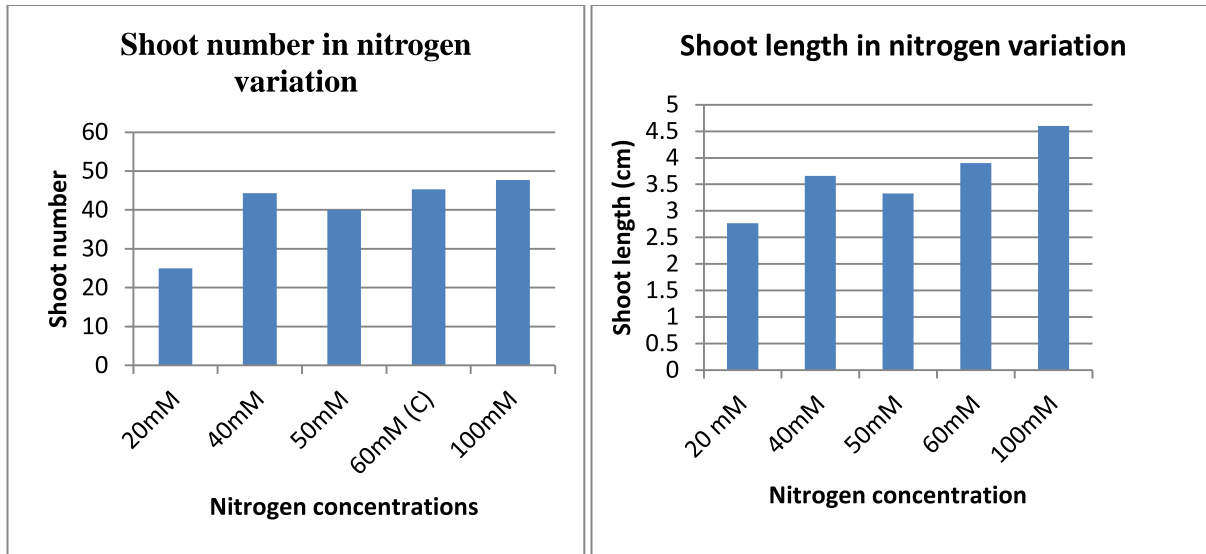
- For asiaticoside ,Mobile phase - HPLC grade Methanol: sterile double distilled water (70:30)
- For Asiatic acid, Mobile phase - HPLC grade Methanol: sterile double distilled water (70:30)
- Detection at wavelength of 214 nm.
- Injection volume: 20 μl
- Flow rate : 0.5 ml/min
- Total Run time: 40 minutes
- Temperature: 26 ^{o}C

4.0 RESULTS AND DISCUSSION

4.1 Effect of variation in nitrogen concentration(NH_4:NO_3) on shoot growth of *Centella asiatica* :

Table 1: Effect of varying the ratios of NH4NO3: KNO3 on shoot number and shoot length after various intervals of time :

Sno	Total nitrogen concentration (NH4NO3: KNO3)	4 weeks		6 weeks		8 weeks	
		Shoot number (M±SE)	Shoot length (cm) (M±SE)	Shoot number (M±SE)	Shoot length (cm) (M±SE)	Shoot number (M±SE)	Shoot length(cm) (M±SE)
1	20 mM (0:20)	11.66± 1.52	1.16±0.152	19±0.5	2.63±0.32	25 ± 2.645	2.76±0.25
2.	40 mM (20:20)	23.33±2.08	3.16±0.288	38.6±0.8	3.43±0.3	44.33 ± 1.52	3.5±0.057
3	50 mM (20: 30)	23.66±2.51	4.16±0.35	33.66±0.1	3.16±0.15	40±2.64	3.33 ±0.15
4.	60 mM (20:40) (Control)	32.66±2.08	3.7±0.72	42±0.4	3.26±0.8	45 ± 1.52	3.9±0.36
5.	100 mM (40:60)	34.33333	3±0.64	43±0.2	4±0.5	47.6 ± 1.52	4.6 ± 0.529



Graph 1: Effect of variation in nitrogen concentration on Shoot number and length after 8 weeks

(a) 20 mM

(b)40mM

(c) 50mM

(d) 60 mM

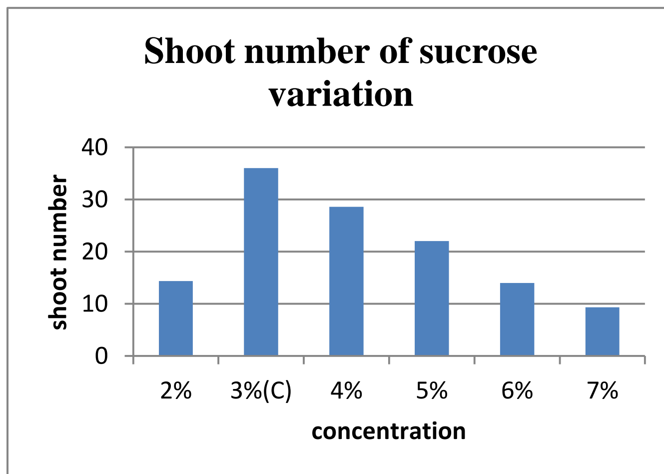
(e)100mM

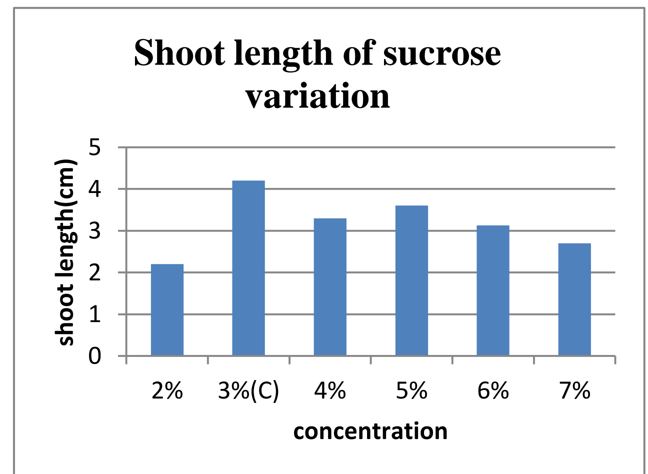
Figure 2: Showing the effect of varying Nitrogen concentration on shoot cultures of *Centella asiatica* after 8 weeks

4.2 Effect of carbon (sucrose) variation on shoot growth of *Centella asiatica*

Table 2: The effect of variation in sucrose concentration in shoot multiplication at various intervals of time :

Sno.	Sucrose concentration	4 weeks		6 weeks		8 weeks	
		Shoot Number M±SE	Shoot Length (cm) M±SE	Shoot Number M±SE	Shoot Length (cm) M±SE	Shoot Number M±SE	Shoot length(cm) M±SE
1	0%	-	-	-	-	-	-
2	1%	-	-	-	-	-	-
3	2%	7.6±1.52	1.5±0.05	9.33±1.52	2.16±0.28	14.3 ± 2.08	2.2 ± 1.1
4	3% (Control)	17.6±2	3.7±0.25	33.66±0.3	4.66±0.11	36±1	4.2±0.3
5	4%	10.3±0.5	1.46±0.3	12±2.64	1.6±0.2	28.66±2.08	3.3± 0.435
6	5%	12.6±2	1.6±0.4	13.66±0.3	2.93±0.3	22±2.64	3.6±0.4
7	6%	8±1	2.4±0.28	11±1	2.5±0.5	14±1	3.13 ± 0.41
5	7%	7±1	1.9±0.45	9.33±0.1	2.7±0.26	9.33± 1.52	2.7±0.26





Graph 2: Effect of variation in sucrose concentration on Shoot number and length after 8 weeks

(a) 2% sucrose

(b)3% sucrose

(c) 4% sucrose

d) 5% sucrose

d) 6% sucrose

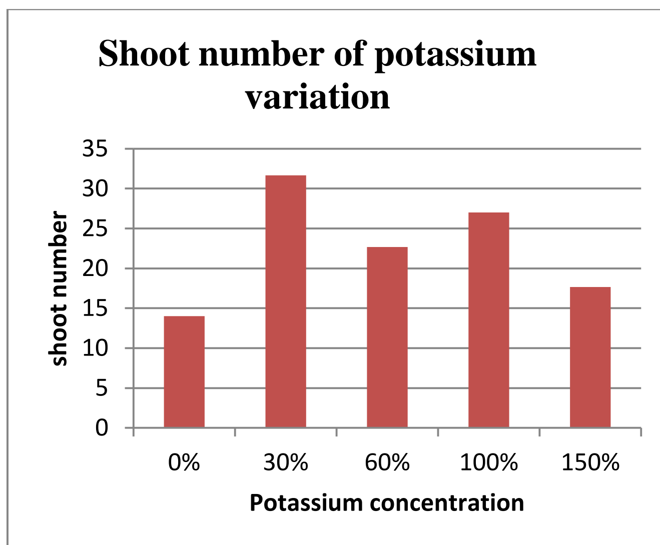
d) 7% sucrose

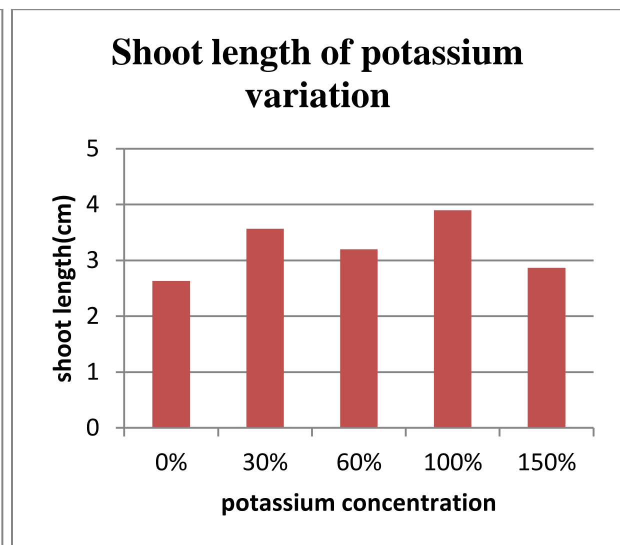
Figure 3: Showing the effect of varying Sucrose concentration on shoot cultures of *Centella asiatica* after 8 weeks.

4.3 Effect of variation in potassium concentrations on shoot growth of *Centella asiatica*

Table 3: The effect of variation in potassium concentration in shoot multiplication at various intervals of time :

SN o.	Total Potassium concentration	4 weeks		6 weeks		8 weeks	
		Shoot number M±SE	Shoot length M±SE	Shoot number M±SE	Shoot length M±SE	Shoot number M±SE	Shoot length M±SE
1	0%	11±0.6	1.3±0.2	12.66± 1.52	2.5±0.251	14±1	2.63±0.15
2	30%	14.66±0.7	2.2±0.6	26.33±0.57	3.4±0.1	31.66±1.52	3.56±0.15
3.	60%	18±1	1.8±0.2	20±1	3.23±0.92	22.6±1.52	3.2±0.88
4	100%	20±0.9	2.7±0.3	25±1	3.8±0.15	27±1.73	3.9±0.1
5	150%	12±0.3	3±0.8	14.66± 1.52	2.6±0.264	17.6± 0.577	2.86±0.23





Graph 3: Effect of variation in potassium concentration on shoot number and length after 8 weeks

(a) 0%

(b)30%

(c)60%

(d) 100%

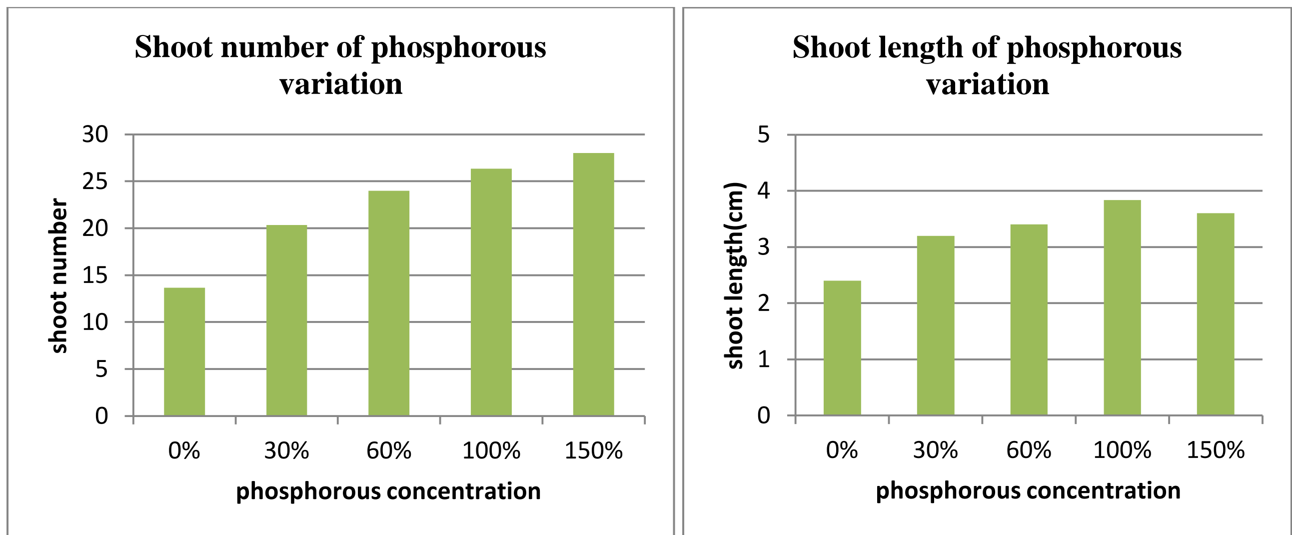
(e) 150%

Figure 4: Showing the effect of varying Potassium concentration on shoot cultures Of *Centella asiatica* after 8 week

4.4 Effect of Phosphorous variation on shoot growth of *Centella asiatica*

Table 4: The effect of phosphorous variation on shoot multiplication at various intervals of time

SNo.	Total Phosphorous concentration	4 weeks		6 weeks		8 weeks	
		Shoot number M±SE	Shoot length M±SE	Shoot number M±SE	Shoot length M±SE	Shoot number M±SE	Shoot length M±SE
1	0%	10±0.2	1.5±0.4	11.5±1	2±0.2	13.66±1.52	2.4±0.17
2	30%	12.6±0.5	2.6±0.8	16±1	2.8±0.51	20.3±0.57	3.2±0.17
3.	60%	10.33±1	2±0.4	20.33±2.08	2.5±0.4	24±1	3.4±0.1
4	100%	16±0.9	2.5±0.34	24.33±1.15	3.2±0.6	26.3±2	3.83±0.15
5	150%	18±0.2	2.2±0.7	25.66±2.08	2.8±0.37	28±1	3.6±0.1



Graph 4: Effect of variation in phosphorous concentration on shoot number and length after 8 weeks

(a)0%

(b)30%

(c)60%

(d) 100%

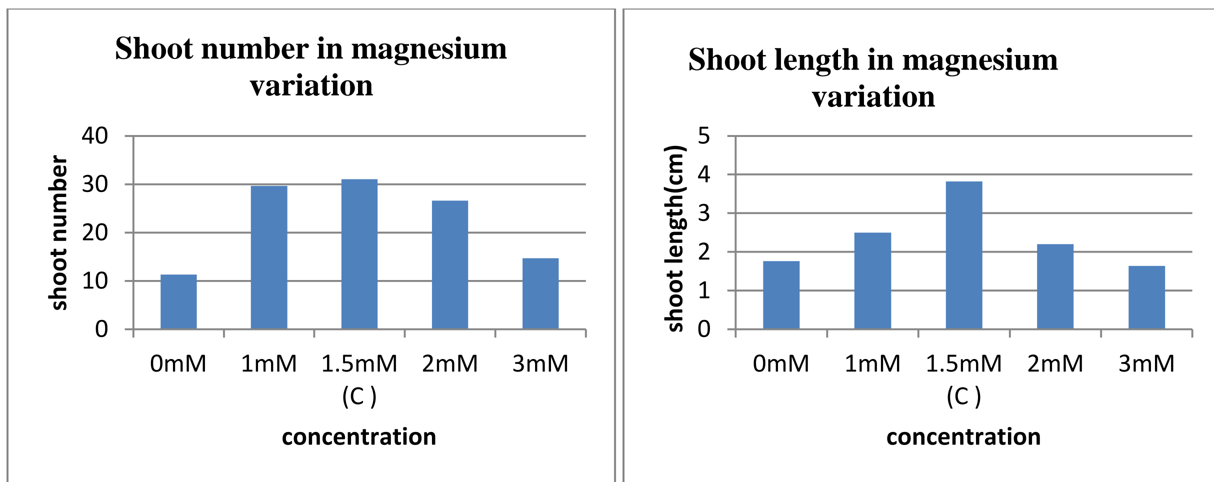
(e)150%

Figure 5: Showing the effect of Phosphorous variation on shoot cultures of *Centella asiatica* after 8 weeks.

4.5 Effect of macronutrient (magnesium) on shoot growth of *Centella asiatica*

Table 5: The following is the effect of varying the magnesium content in shoot multiplication at various intervals of time

SNo.	Total Magnesium concentration	4 weeks		6 weeks		8 weeks	
		Shoot number M±SE	Shoot length M±SE	Shoot number M±SE	Shoot length M±SE	Shoot number M±SE	Shoot length M±SE
1	0mM	4.66±1.52	1.3±0.1	11.3±0.5	1.7±0.17	11.33±1.5	1.76±0.25
2	1mM	13.3±1.52	1.86±0.4	27±0.6	2.43±0.2	29.6±1.52	2.5±0.3
3.	1.5mM (C)	19.6±1	2.2±0.264	28±0.4	2.72±0.8	31±1	3.8±0.25
4	2mM	12.3±2.08	1.7±0.17	18.33±0.7	1.96±0.56	26.6±1.52	2.2±0.4
5	3mM	10.6±2.08	1.23±0.25	11±2.67	1.633±0.15	14.6±1.52	1.633±0.15



Graph 5: Effect of variation in magnesium concentration on shoot number and length after 8 week

(a) 0mM

(b) 1mM

(c) 1.5 mM

2 mM

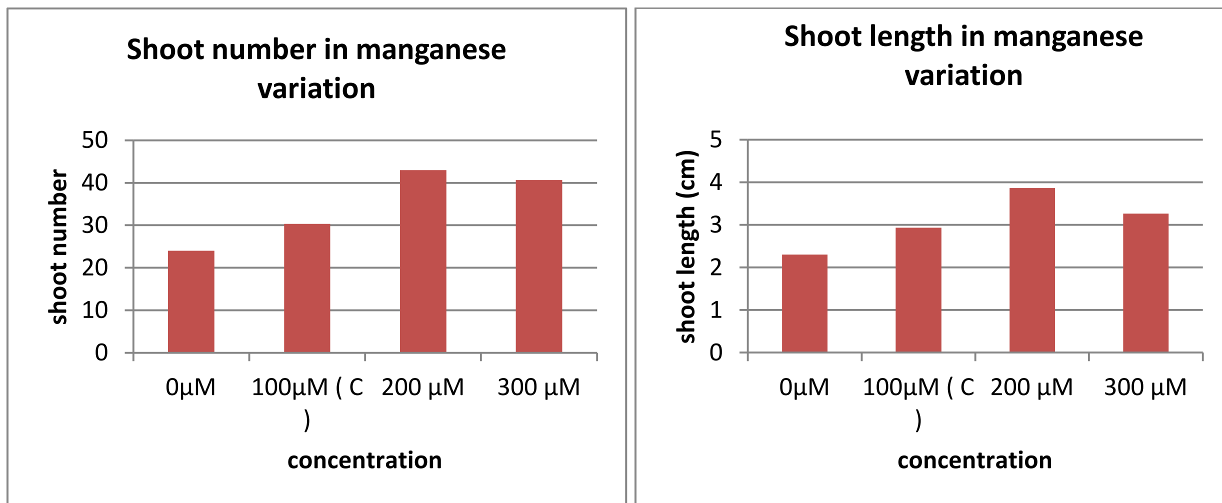
(b) 3mM

Figure 6: Showing the effect of variation of Magnesium concentration on shoot cultures of *Centella asiatica* after 8 weeks.

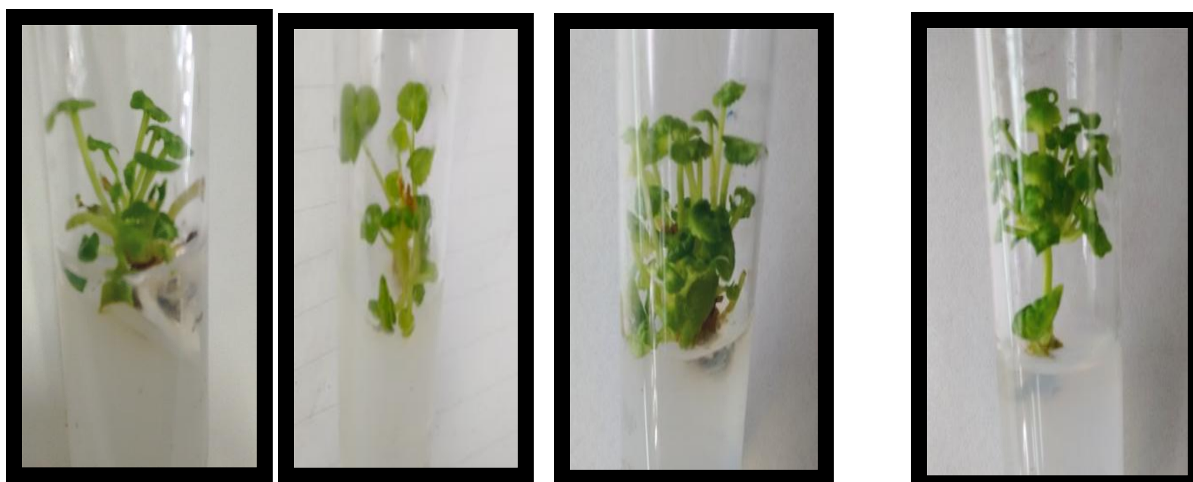
4.6 EFFECT OF MICRONUTRIENT (MANGANESE) ON SHOOT GROWTH OF *Centella asiatica*

Table 6: The following is the effects of variation in manganese content in the shoot multiplication at various intervals of time

SNo.	Total Manganese Concentration in MS media	4 weeks		6 weeks		8 weeks	
		Shoot number	Shoot length(cm)	Shoot number	Shoot length(cm)	Shoot number	Shoot length(cm)
1	0µM	10.66±2	1.3±0.1	14±0.5	1.6±0.17	24±1.73	2.3±0.43
2	100µM (Control)	9.66±115	1.86±0.40	15.33±0.2	2.53±0.25	30.33±1.52	2.93±0.3
3	200 µM	18.3±1.5	2.2±0.26	34±0.8	2.93±0.4	43±2	3.866±0.15
4	300 µM	12.6±2.5	1.23±0.25	22.66±0.1	2.66±0.15	40.66±1.52	3.266±0.25



Graph 6: Effect of variation in manganese concentration on shoot number and length after 8 week



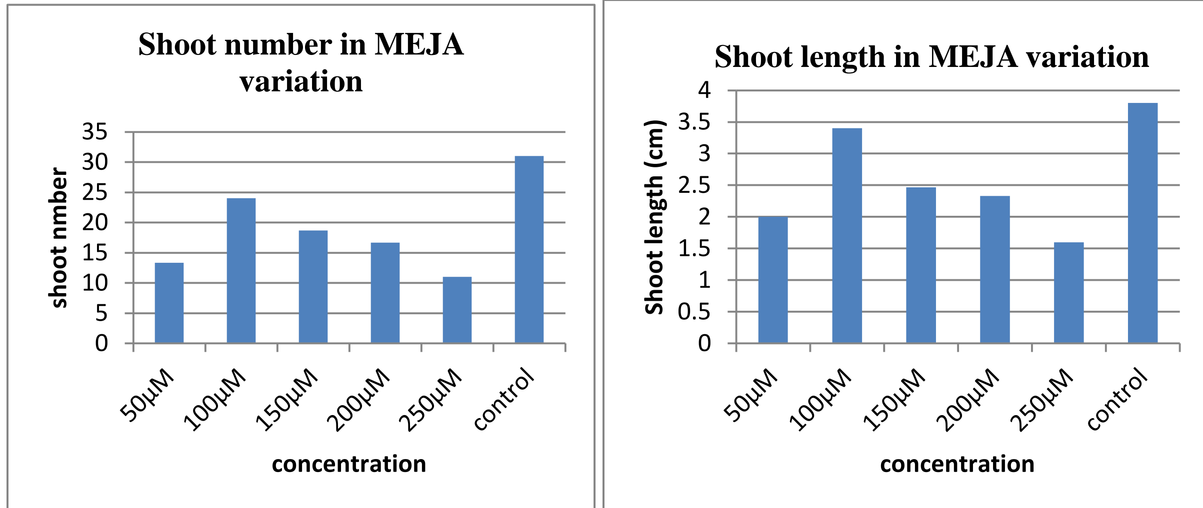
(a) 0 μM (b)100μM (c) 200 μM (d) 300 μM

Figure 7: Showing the effect of variation in Manganese concentration on shoot cultures of *Centella asiatica* after 8 weeks.

4.7 EFFECT OF ELICITOR (METHYL JASMONATE) ON SHOOT GROWTH OF *Centella asiatica*

Table 7: Effect of methyl jasmonate on shoot multiplication at various interval of time

SNo.	Methyl Jasmonate concentration	4 weeks		6 weeks		8 weeks	
		shoot number	Shoot length(cm)	shoot number	Shoot length(cm)	shoot number	Shoot length(cm)
1	50μM	5.66±0.5	1.3±1.86	10.33±0.2	1.766 ±0.25	13.33±1.52	2 ±0.2
2	100μM	12±2	1.8±0.4	18.66±0.57	2.933±0.3	24±1	3.4±0.1
3	150μM	7.3±1.15	2.2±0.26	13.66±0.35	1.633±0.15	18.66±0.57	2.46±0.35
4	200μM	9±0.6	1.23±0.25	11±0.3	2.06±0.3	16.6±1.52	2.33±0.3
5	250μM	6±0.24	1.7±0.17	9.66±0.2	2.16±0.3	11±1	1.6±0.2
6	control	23±0.6	2.8±0.4	26±0.2	3.4±0.1	31±1	3.8±0.25



Graph 5: Effect of methyl jasmonate on shoot multiplication after 8 weeks

(a) 50µM

(b) 100 µM

(c) 150 µM

(d) 200 µM

(e) 250 µM

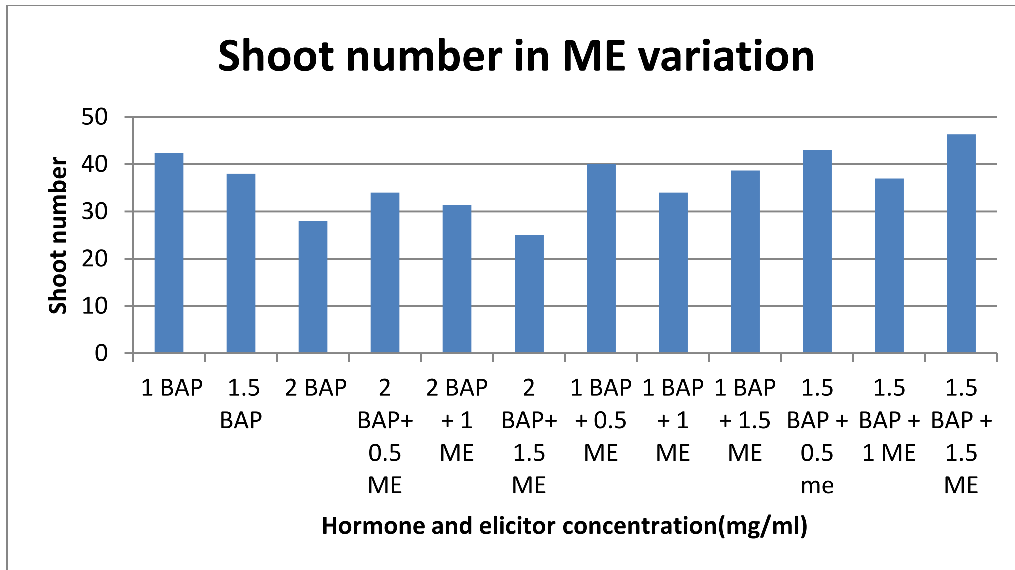
(f) control

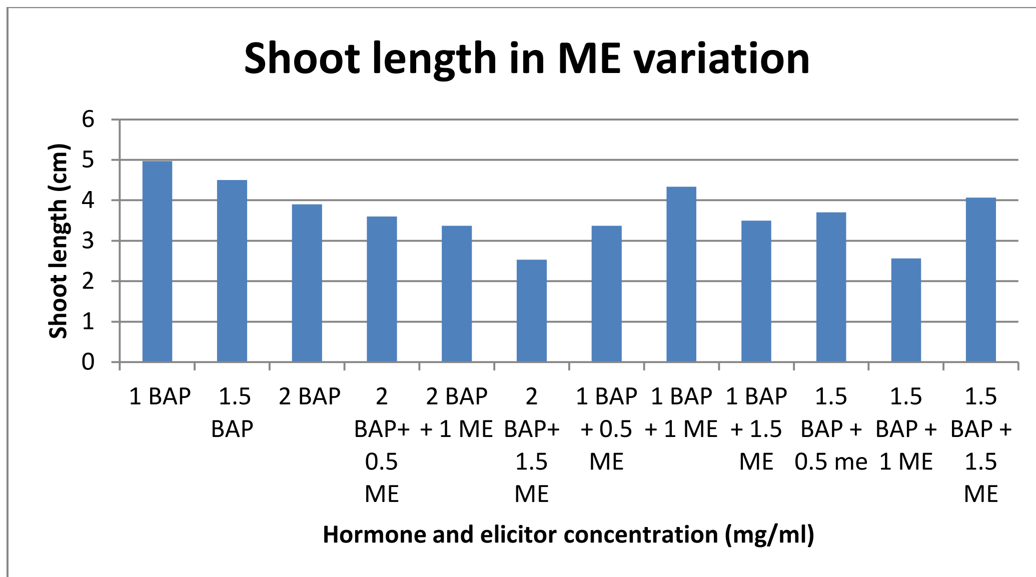
Figure 8: Effect of elicitor MEJA on shoot cultures of *Centella asiatica* after 8 weeks.

4.8 EFFECT OF ELICITOR (MALT EXTRACT) ON SHOOT GROWTH OF *Centella asiatica*

Table 8: The following are results of shoot growth in different concentration of plant growth hormone BAP and elicitor Malt extract in MS media:

SNO	Hormone concentration in MS Media		4 weeks		6 weeks		8 weeks	
	BAP (mg/l)	ME (mg/l)	No. Of shoots (M±SE)	Length of shoots (cm) (M ±SE)	No. Of shoots (M±SE)	Length of shoots (cm) (M ±SE)	No. Of shoots (M±SE)	Length of shoots (cm) (M ±SE)
1	1	0.5	19±2.6	2.5±0.8	32.66±0.5	2.86±0.11	40±1	3.36±0.32
2		1	15±0.8	2.4±1	27±0.13	3.5±0.76	34±2	4.33±0.76
3		1.5	25±1.5	2.6±0.47	28±0.26	3.16±0.76	38.66±0.5	3.5±0.5
4	1.5	0.5	30±0.87	3±0.35	33±0.5	3.36±0.5	43±1	3.7±0.17
5		1	18.6±0.76	2.2±0.6	24±0.1	2.4±0.6	37±1	2.56±0.20
6		1.5	31±0.9	3.9±0.4	34.33±0.12	3.88±0.288	46.33±1.52	4.06±0.11
7	2	0.5	12.33±0.7	2.5±1.8	26.5 ±0.5	3.25±0.2	34±1	3.6±0.85
8		1	13±0.96	2.5±0.8	23.5 ±0.13	3.15±0.4	31±1	3.36±0.51
9		1.5	8±2	2.43±0.9	10±0.45	2.96±0.1	25±1	2.53±0.50
10	1.0	-	27±0.11	3.8±1	33.66±0.4	4.5±0.51	42.33±1.15	4.96 ±0.47
11	1.5	-	19±0.7	3.5±0.3	31.5 ±0.2	4±0.07	38±1	4.5±0.1
12	2.0	-	14±2	3.2±1.5	23.66 ±2.51	3.23±0.1	28±1.73	3.9±0.75

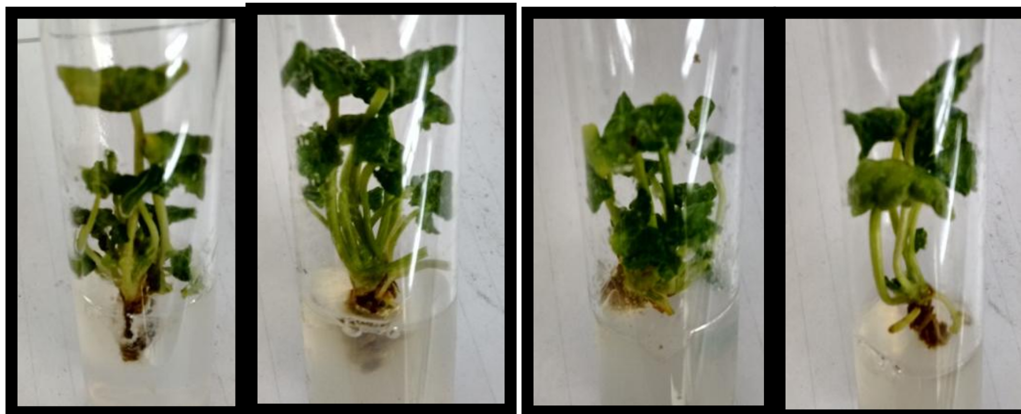




Graph 8: Effect of methyl jasmonate on shoot multiplication after 8 week

a)1mg/l BAP b) 1BAP+0.5 ME c) 1 BAP+1 ME d) 1 BAP+1.5 ME

a)1.5 mg/l BAP b) 1.5 BAP+0.5 ME c) 1.5 BAP+1 ME d) 1.5 BAP+1.5 ME



a)2 mg/l BAP b) 2 BAP+0.5 ME c) 2 BAP+1 ME d) 2 BAP+1.5 ME

Figure 8: Effect of BAP and ME variation on shoot cultures of *Centella asiatica* after 8 week

The experiments was conducted in order to study the effects of varying the nutrients constituents of MS media on two major parameter, shoot number and shoot length of *Centella asiatica* (accession no. 347492) .The highest shoot multiplication was observed in MS media when compared to Gamborg's B5 media and Nitsch media provided with 4 mg/l BAP and 0.4 mg/L NAA for *Centella asiatica* of accession number 347492 (Roy.A et al.,2016) . All the different treatments were supplemented with 1 mg/l BAP .

NITROGEN : The plants were grown in 5 different concentration of nitrogen which was obtained by varying the ratio of ammonium nitrate and potassium nitrate (NH_4NO_3:KNO_3) in MS media. It was observed there was a constant increase in shoot growth throughout the 4 th , 6 th weeks and after 8th week the maximum shoot number(47.6 ± 1.52) and length (4.6 ± 0.529 cm) was seen in media with100mM(40:60) nitrogen concentration followed by 60 mM (20:40 mM)(control) and 40 mM (20:20).Therefore there was better shoot development observed with increase in total nitrogen levels ,more than that in control media(60mM) .There is a need for a proper balance between the two nitrogen sources and neither of the two sources can replace each other in the media (Prasad et al.,2012,Omar.,2005) .It was reported that biomass production did not have much difference in media having 60,40and 20mM total nitrogen content (Prasad et al.,2012).

CARBON : The major carbon source in MS media is sucrose which was varied from 0 to 7% in MS media. The 0% and 1 % failed to support any shoot growth since the nodal explants dried up after the 4 week. The maximum shoots (36±1) and shoot length (4.2±0.3cm) observed in 3 % sucrose (control) was found to be the most optimum carbon composition for efficient plant growth. With increase in sucrose level there was a decrease in shoot number and length that is from 4 to 6 % , steady decrease in shoot growth observed. In 7 % treatment , there was limited growth and the leaves were turning from green to yellow colour by the 6th week due to high sucrose levels that induced stress condition to the plant. Reports indicate that maximum growth rate with 3 times more dry biomass observed in media with 5% sucrose than the control (3 %) followed by 7% which continued to have better growth (Prasad et al.,2012)which was assumed to be associated with better carbon availability and higher osmotic stress(Liu.,2008).Among the carbon sources sucrose(3%) showed highest shoot generation compared to fructose in *Centella asiatica* of accession no. 347492 compared to other accessions (Kundu K. *et al*.,2016).

POTASSIUM: The highest shoot number(31.66±1.52) in 30% potassium treatment followed by 100% and 60% and highest shoot length (3.9±0.1cm) in media supplemented with 100% potassium. Thus better shoot proliferation observed in 30% treatment than the control(100%) media. It was reported that potassium supply induced no significant difference among treatment in the leaf yield until 2 weeks but after 8 weeks highest yield obtained in 30% followed by 60% and 100%,and lowest yield in 0% treatment(Muller V et al.,2013)

PHOSPHOROUS: The herb yield and leaf yield was augmented with increase in Phosphorous supply (Muller V et al.,2013). After 8 weeks,the highest shoot number (28±1) in 150% treatment followed by 100% and 60% and shoot length (3.83±0.15 cm) in 100% phosphorous treatment. Thus increasing phosphorous to 150% lead to better shoot growth than the control media(100%).From previous reports, highest herb and leaf yield were achieved in 150% phosphorous treatment after 8 weeks and lowest yield in 0% treatment (Muller V et al.,2013) .

MACRONUTRIENT: The macronutrient varied in MS media was magnesium which is critical for enzymes functioning. After 8 weeks, maximum shoot number (31±1) and shoot length (3.8±0.25cm) in 1.5 mM(control) magnesium concentration followed by the treatment with 1mM (29,2.53 cm) and 2 mM (26,2.2 cm) magnesium .From previous reports, lowering Magnesium was associated with lowering asiaticoside content but did not have significant effect on growth (Prasad et al.,2012).

MICRONUTRIENT: The micronutrient varied is manganese which is required for respiratory and photosynthetic processes. After 8 weeks, the maximum shoot number (43±2) and shoot length(3.866±0.15 cm) in 200 µM followed by 300 µM and 100 µM managanese concentration respectively in MS media. In 200 µM treatment the shoot development was better than the control media(100 µM). Reports indicate that Manganese did not show any significant effect on growth (Prasad et al.,2012).

ELICITOR- METHYL JASMONATE (MEJA):. Methyl Jasmonate did not have a beneficial effect on shoot development of *Centella asiatica* . In MS media supplemented with 100µM methyl jasmonate ,after 8 weeks the shoot number (24±1) and length (3.4±0.1 cm) was observed whereas other combination showed lesser shoot proliferartion when compared to control media with 1 mg/l BAP without MEJA resulted shoot number (31±1) and shoot length (3.8±0.25cm).From previous reports, the Centella plantlet treated with MEJA showed reduction in growth with over 50% reduction in fresh

weight in the aerial parts and more than 70% decline in root growth and also showed symptoms of necrosis in roots and leaves by the end of the culture period. (Bonfill M et al.,2006)

ELICITOR- MALT EXTRACT (ME): After 8 weeks , the maximum number of shoots (46.33±1.52)observed in the media supplemented with 1.5 mg/l BAP and 1.5 mg/l Malt Extract followed by shoot number (43±1) in media provided with 1.5 mg/l BAP and 0.5 mg/l ME. The maximum length of shoots observed in MS media with 1.0 mg/l BAP (4.96 ±0.47cm) followed by 1.5 mg/l BAP. The maximum shoot multiplication (7 ± 0.22) observed in MS media with the hormone concentration of 1 mg/l BAP after 6 weeks in accession no. 347492 of Centella (Roy.A *et al*.,2016).From the result we can say that media supplemented with elicitor ME showed enhanced the shoot proliferation ,optimum at 1.5 mg/l concentration but increasing upto 2mg/l reduced the shoot proliferation. Moreover, the media provided with only BAP hormone showed improved shoot length. Among the different elicitors like salicylic acid, jasmonic acid used, the MS media which was treated with malt extract (1 mg/l) showed maximum number and maximum length of shoots in *Centella asiatica* (accession number 347492) (Kundu K. *et al*.,2016).

4.9 CALLUS CULTURE:

Table 9: The following is the effect of different plant growth hormones on callus growth:

Growth parameters (mg/l)				**Callus growth**	**Morphology**
BAP	NAA	2,4 D	IAA		
1	0.5	-	-	++++	Medium , Light green and compact
1	1	-	-	+++++	Big , green and compact
1	1.5	-	-	++++++	Big, green color, compact
1	2	-	-	+++	Small, Light green, compact
1	-	0.5	-	++++	Big, light green ,soft
1	-	1	-	+++	Big, Light green ,soft
1	-	-	0.5	++	Small, green, compact
1	-	-	1	+++	Small, light green, compact
1+ 100µM MEJA	-	-	-	++	Small, light green, soft

4.10.1 Effect of various plant regulator hormones on Callus culture

The effects of different combination of plant growth hormones on callus induction from the leaf explants of *Centella asiatica* (accession no. 347492) is shown in Figure 10 and 11. After 14 weeks, the MS media provided with 1mg/l BAP and 1.5 mg/l NAA resulted in maximum induction of callus growth which were morphologically big sized, green coloured whereas the callus developed in the media with 1 mg/l BAP and 0.5mg/l 2,4 D were comparably soft and light green coloured. The media provided with 1 mg/l BAP and 1 mg/l NAA also resulted in the development of big, green and compact callus and the least growth observed in media with 1mg/l BAP and 0.5 mg/l IAA. The other combinations of plant growth regulator resulted in poor growth induction which were small, compact

and light green callus. After 12 weeks ,the callus were turning brown in colour thus were subcultured in fresh medium .The callus which was treated with the elicitor methyl jasmonate were small, light green coloured and soft, thus we can persume that MEJA does not help in induction of better calluses . In Reports , MS medium provided with 1 mg/l BAP and 1 mg/l NAA lead to strong callus induction from petiole explants of Centella after 21 days of culturing, which were yellow coloured, friable and compact(Hoang N et al.,2010).Callus was induced from leaf and stem explants of Centella plant on MS media supplemented with 4 mg/l NAA and 2 mg/l kinetin (Patra *et al*.,1998).

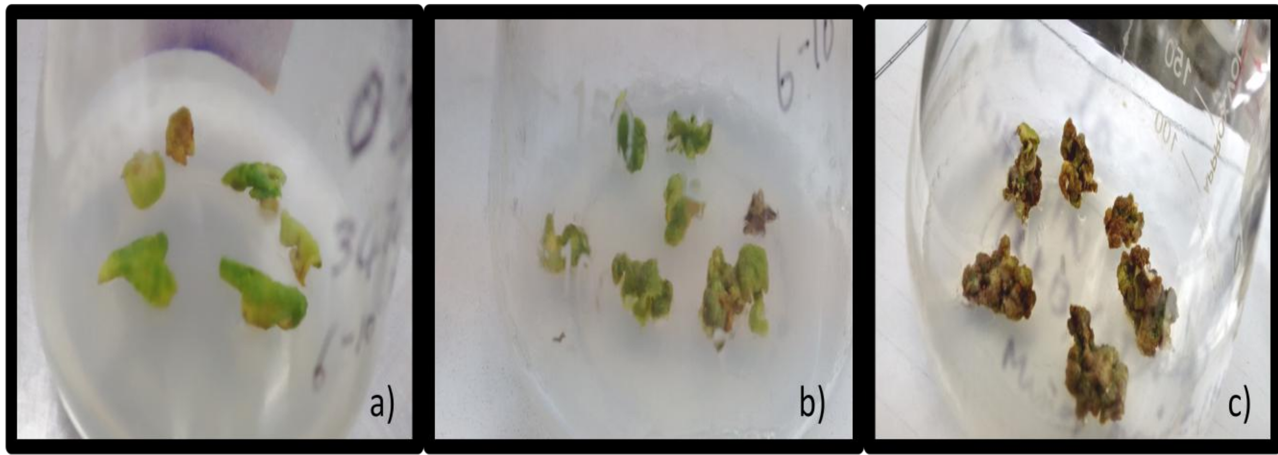


Figure 10:Callus cultures in MS media provided with 1 BAP and 1.5 NAA a) after 3 weeks b) after 8 weeks c) after 14 weeks big ,brown ,compact callus are formed.

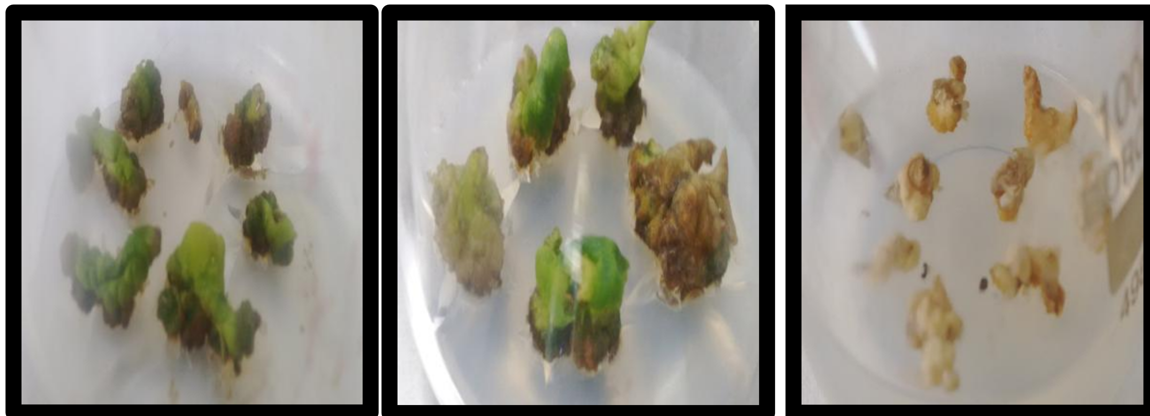


Figure 11:Callus cultures in MS media provided with a) 1 BAP and 0.5 of 2,4 D b) 1BAP and 1 NAA c) 1 BAP , 1.5 NAA with 100µM MEJA after 14 weeks

4.10 CELL SUSPENSION CULTURE

The cell suspension culture was established with the callus which was provided with 1 mg/l BAP and 1.5 mg/l NAA which showed maximum callus induction. The various stages of cells growing in cell suspension is shown in figure 12 and 13.The clear liquid MS media was inoculated with chopped pieces of callus on day 0,cells started growing from day 10,by day 30 a turbid cell suspension was developed and by day 40,white coloured suspended solution with maximum turbidity was observed. Cells from cell suspension culture viewed was under 10X magnification of a light microscope on day 25, cells was observed as heterogenous sized cell population arranged in clumps shown in figure 14.

Cell growth was determined by dry weight and wet weight measurement recorded at every 3 days interval for about 45 days. The elicitor MEJA was added on day 10 as stationary phase is assumed to be starting by the 10th day after inoculation.A growth curve was plotted for control and media elicited with MEJA as shown in graph 9.In dry weight analysis ,for the control media, the exponential phase of growth lasted for approximate 39 days with maximum value of 0.02399g/5ml dry weight and reached death phase by day 45, whereas in media elicited with 100µM MEJA , the exponential phase lasted for 36 day with maximum peak at 0.03048g/5ml dry weight and reached death phase by 42 .In wet weight analysis, the control media reached maximum value of 0.426g/5ml wet weight on day 39 and MeJA elicited reached maximum value of 0.4312 g/5ml wet weight in day 36. The growth curve of the MEJA elicitation and control graph were almost similar to each other but the exponential phase of the elicitated cells was shorter than control .A usual type of lag and stationary phase were not observed may be due to their lesser duration. The cells lost its viability after day 39 may be due to the nutrient depletion. The complete growth curve took approximate 45 days to complete in both the treatments and There was 24 fold increase in dry cell weight from day 3 to day 39 in control and in MEJA elicited there was 30 fold increase in dry cell weight from day 3 to day 36.Therefore,the methyl Jasmonate elicitation has resulted an overall increase of 1.3 folds in dry cell weight.

a)Day 0 b) Day 10 c) Day 25 d) Day 40

Figure 12: Showing cell suspension culture at various intervals of growth period

Figure 13: Showing cell suspension culture elicited with MEJA at various intervals of growth period

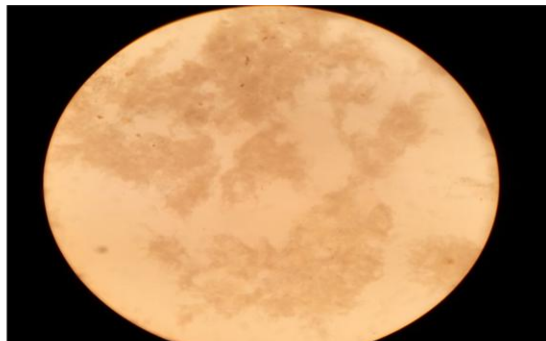
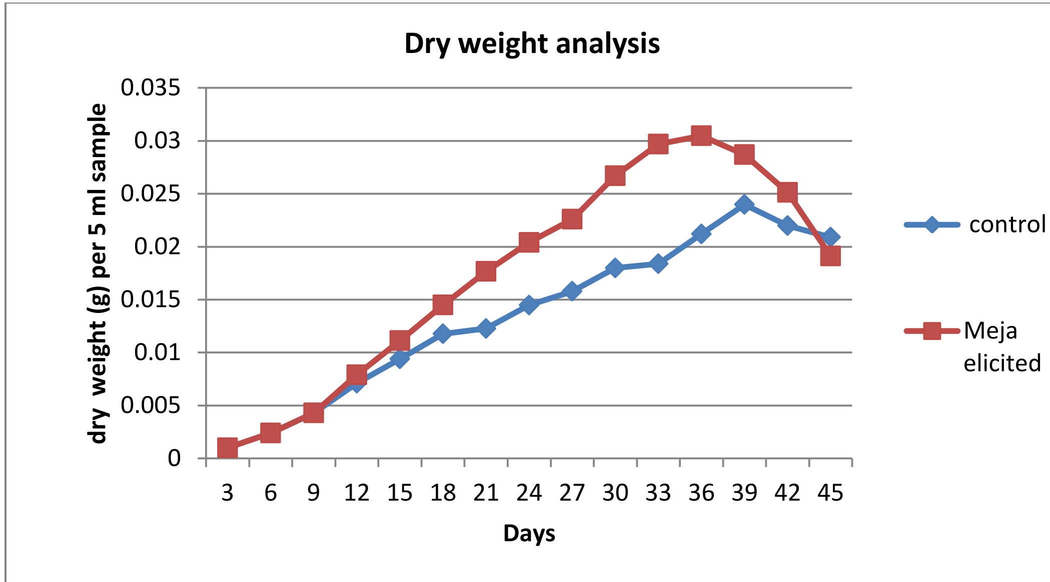
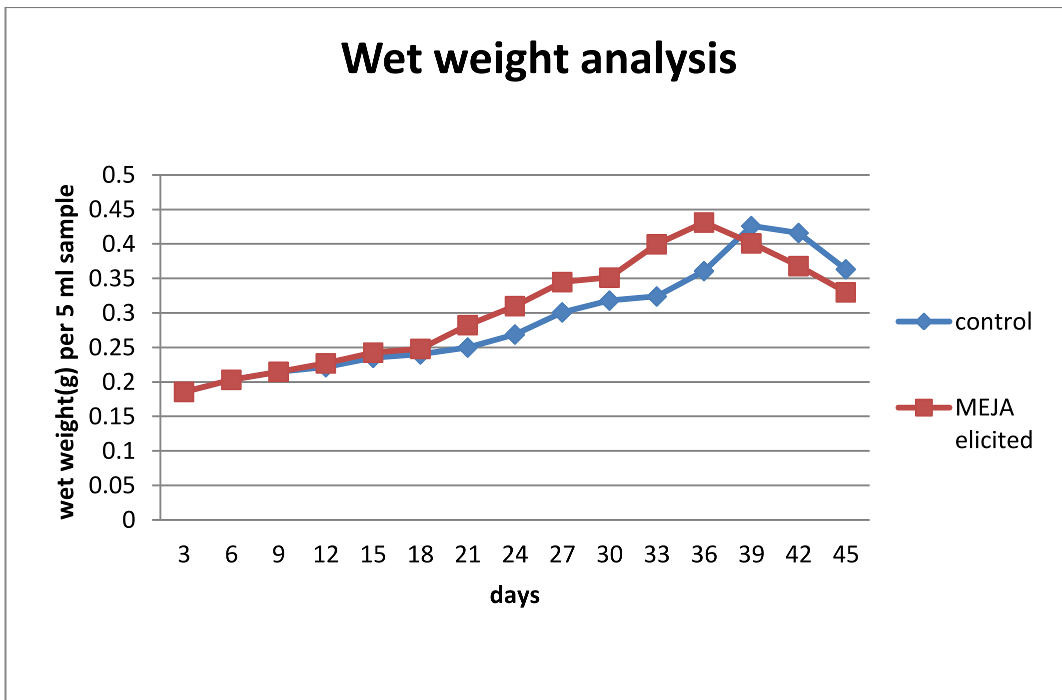


Figure 14 : Cells from the cell suspension culture viewed under 10X magnification in a light microscope on day 30

4.10.1 GROWTH CURVE ANALYSIS





Graph 9: The graphs showing dry weight and wet weight analysis of cell suspension culture

4.11 RESULTS OF HPLC ANALYSIS

4.11.1 Asiaticoside standard chromatogram

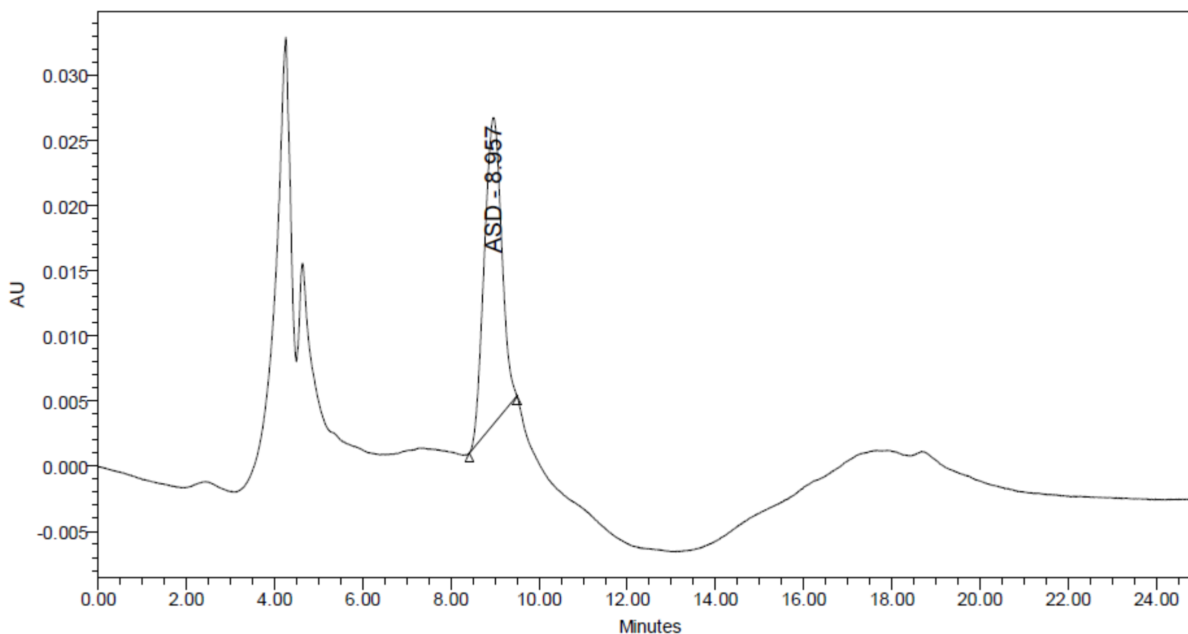


Figure 15 :Chromatogram of 0.1mg/ml asiaticoside standard

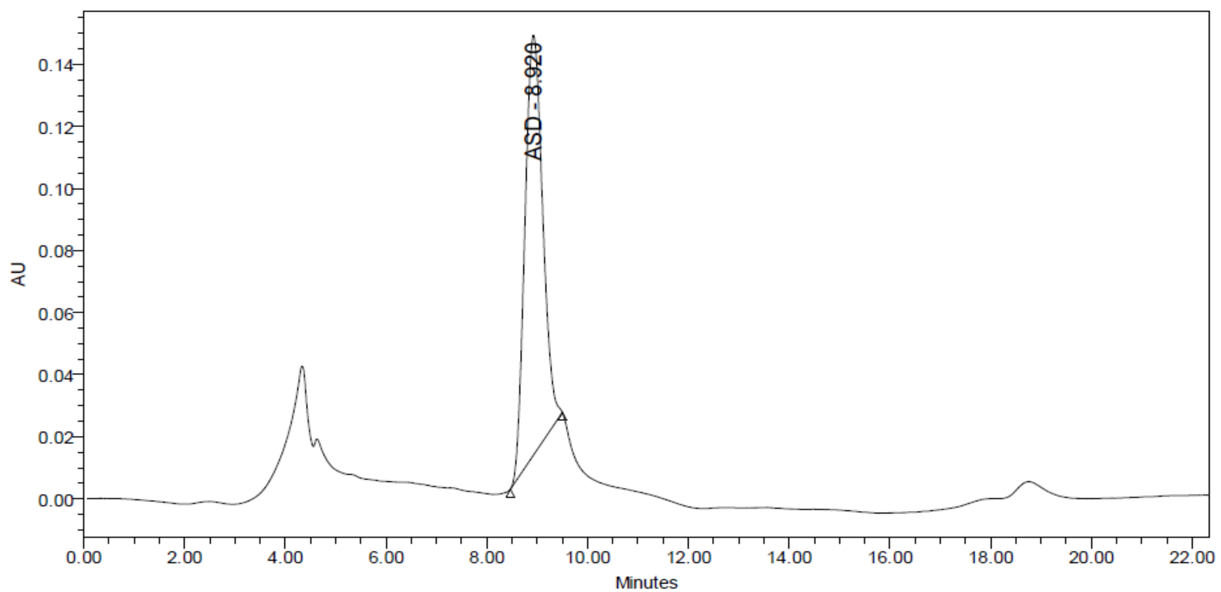


Figure 16 :Chromatogram of 0.5 mg/ml asiaticoside standard

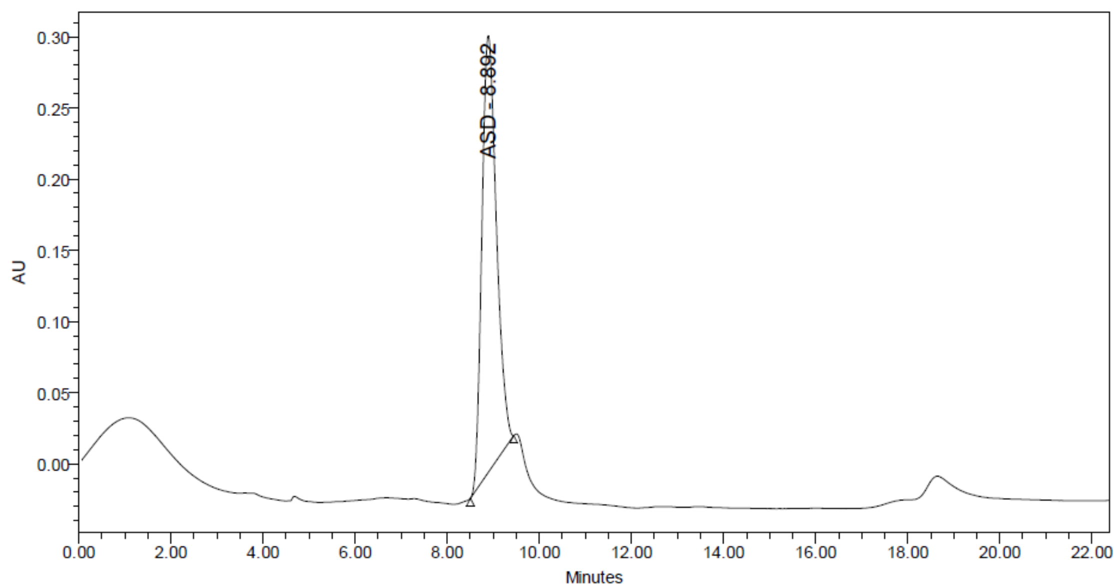


Figure 17: Chromatogram of 1 mg/ml asiaticoside standard

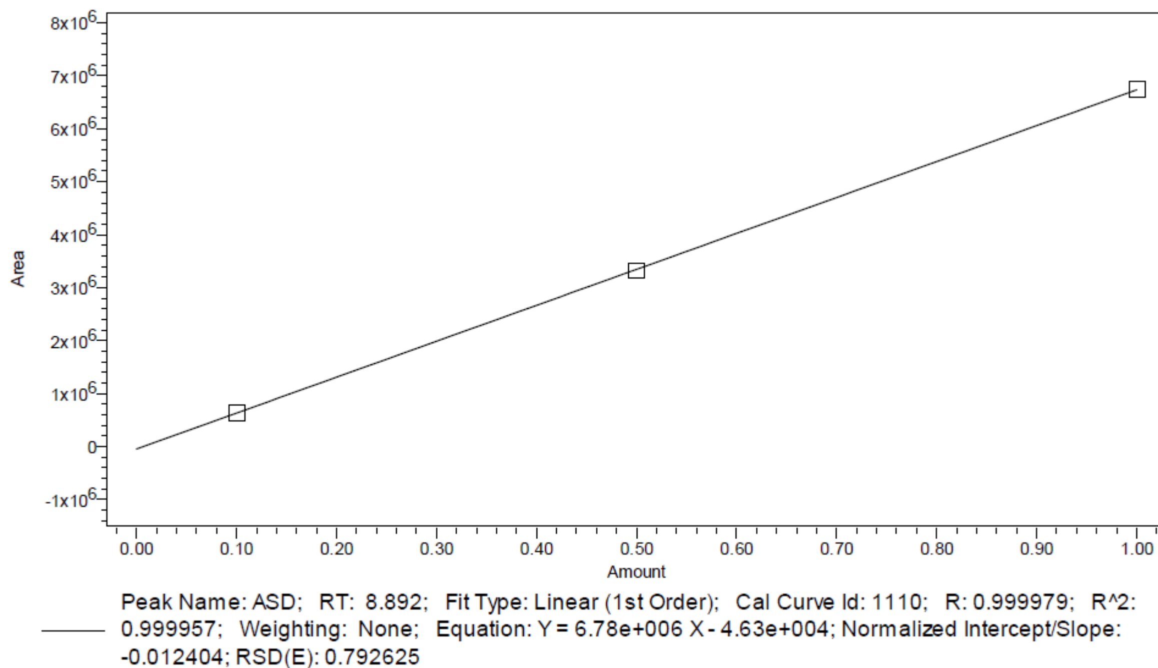


Figure 18 : Calibration curve of the 0.1,0.5 and 1 mg/ml asiaticoside standards

14.11.2 Chromatogram of Samples

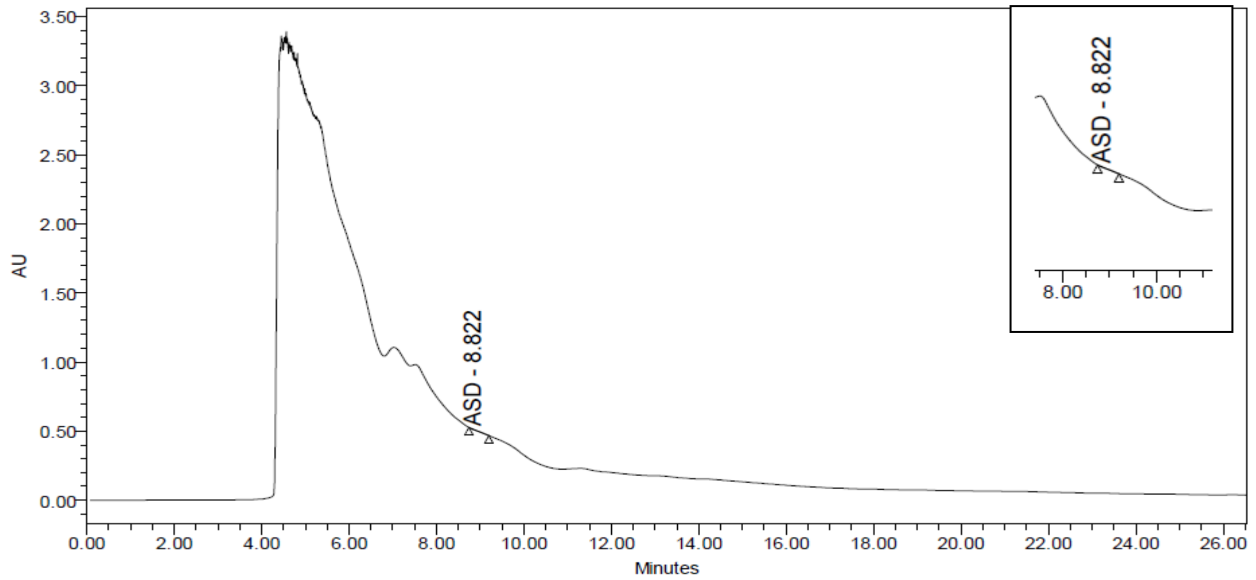


Figure 19: Chromatogram showing Asiaticoside content (0.00377 mg/ml) in plant extract

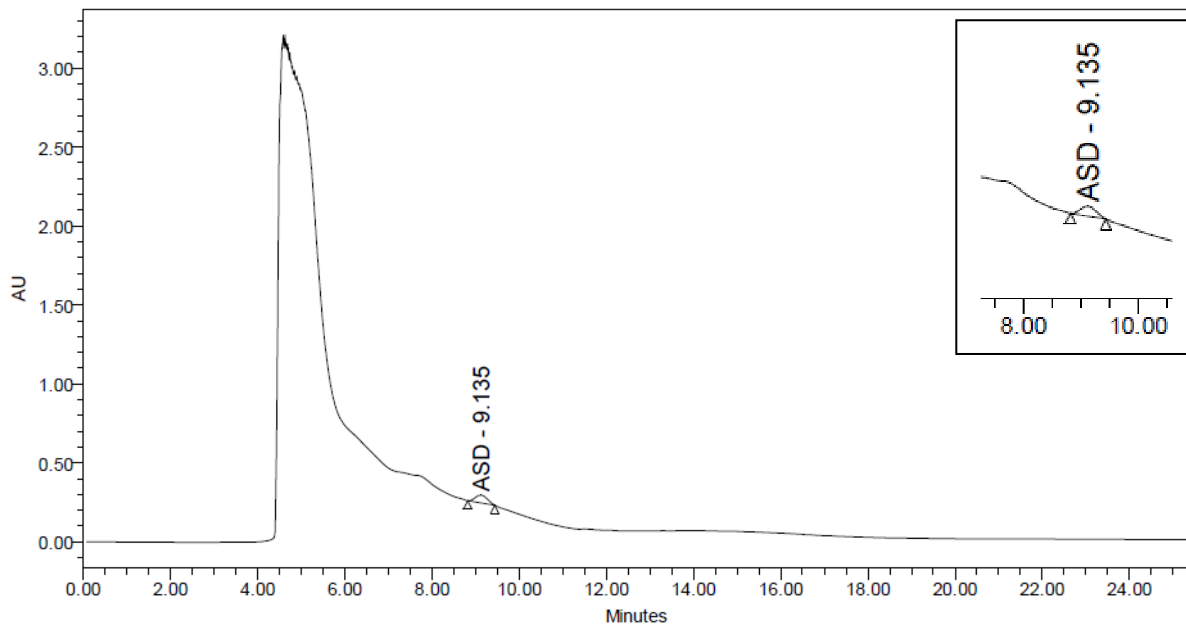


Figure 20: Chromatogram showing Asiaticoside content(0.1467081 mg/ml) in methyl jasmonate elicited plant extract

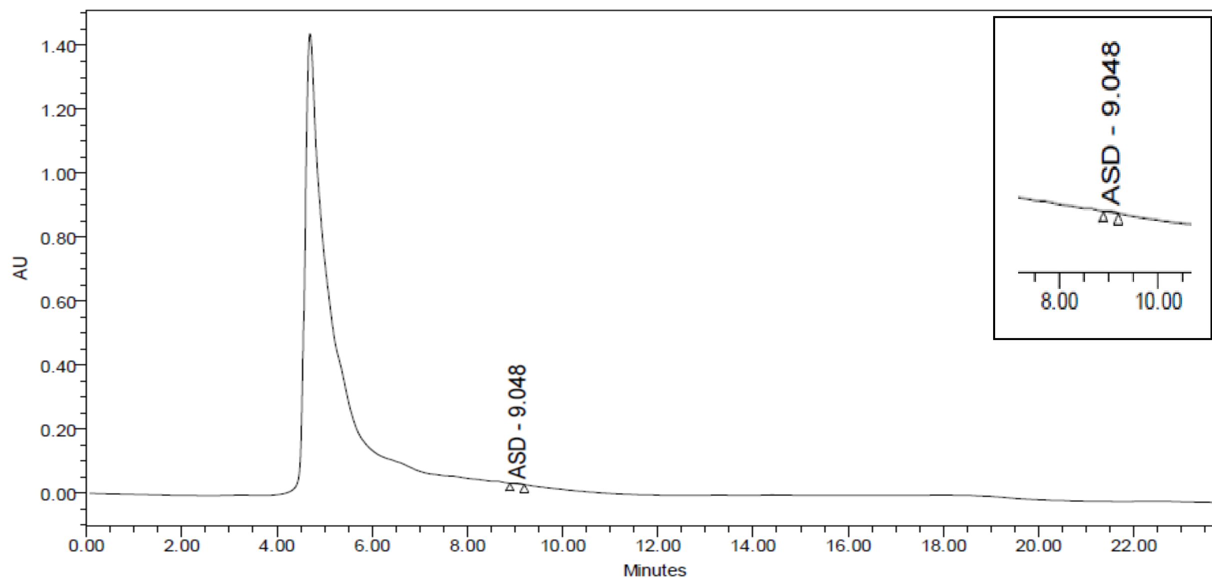


Figure 21 : Chromatogram showing Asiaticoside content(0.0029699 mg/ml) in Callus extract

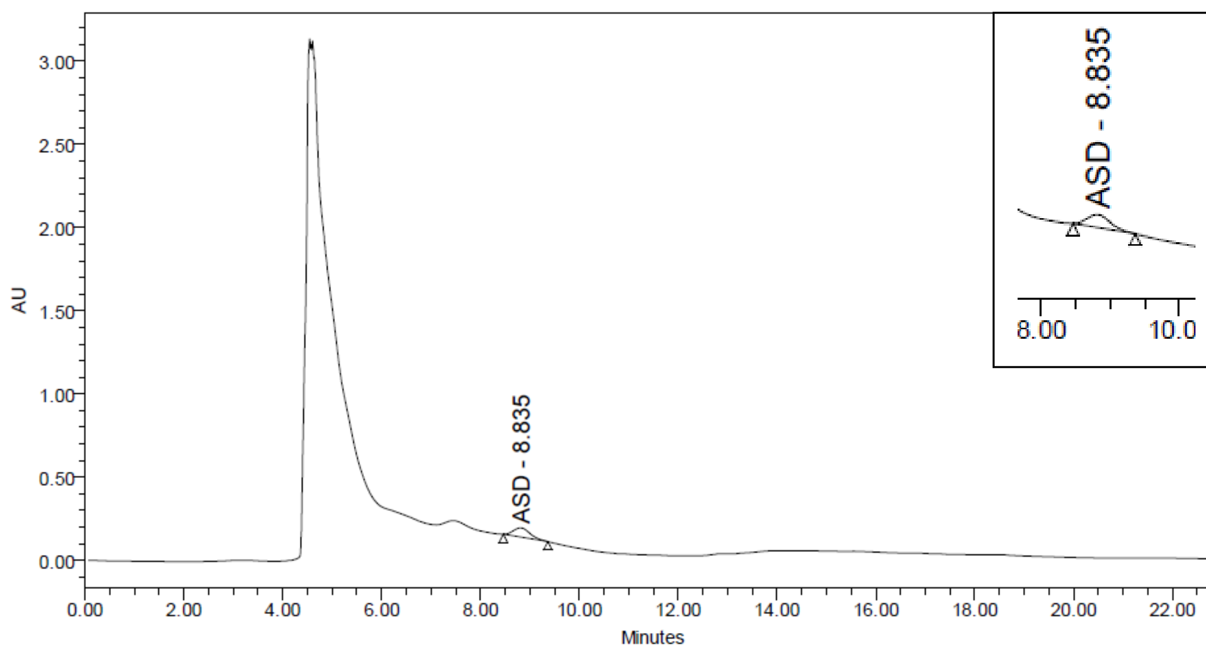


Figure 22: Chromatogram showing Asiaticoside content (0.20195 mg/ml) in methyl jasmonate elicited Callus extract

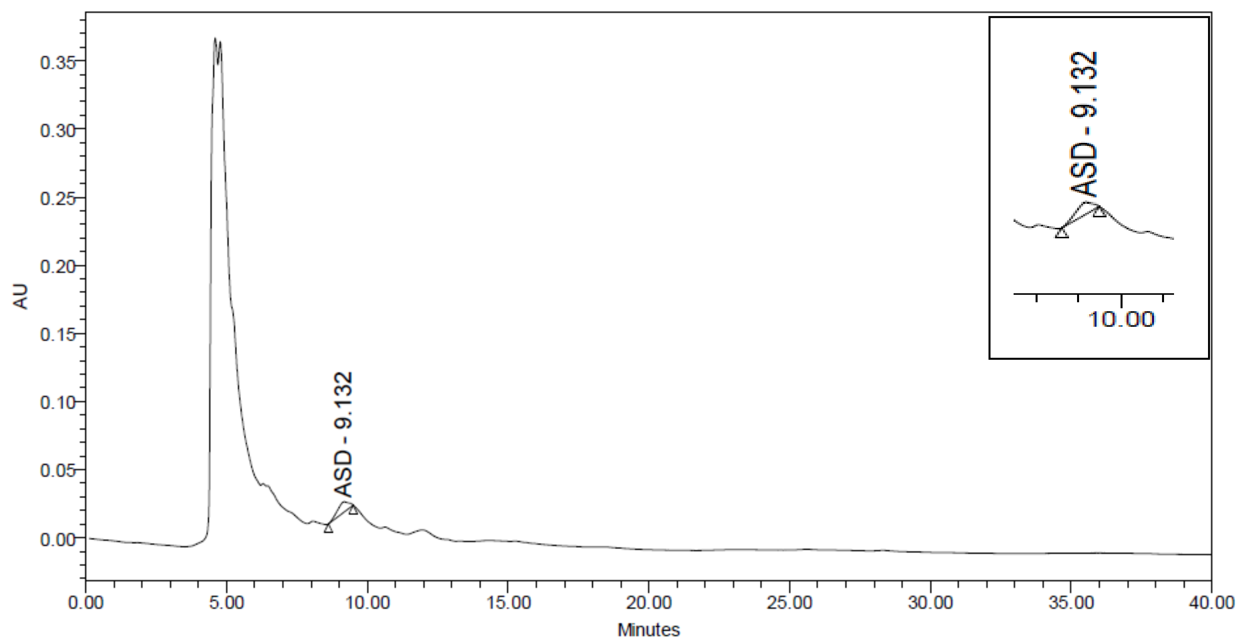


Figure 23: Chromatogram showing Asiaticoside content (0.0333 mg/ml) in Cell suspension cells extract

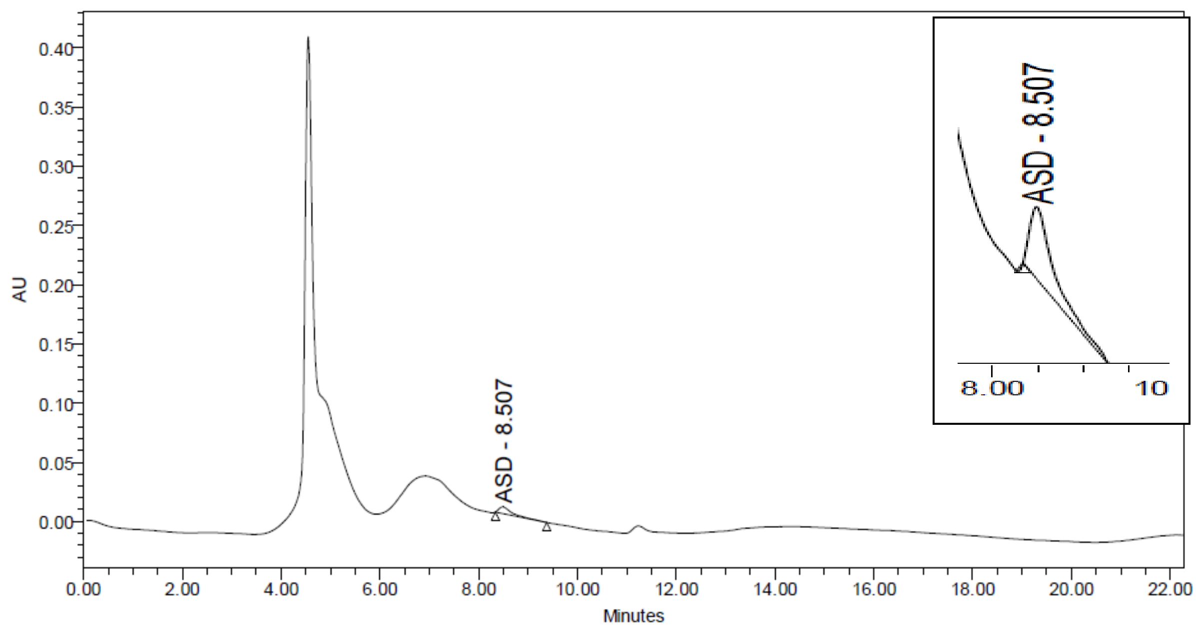


Figure 24: Chromatogram showing Asiaticoside content (0.0161073 mg/ml) in methyl jasmonate elicited Cell suspension cells extract

14.11.3 Quantitative estimation of asiaticoside by RP- HPLC

Using this formula,the concentration of compound in unknown sample was calculated :

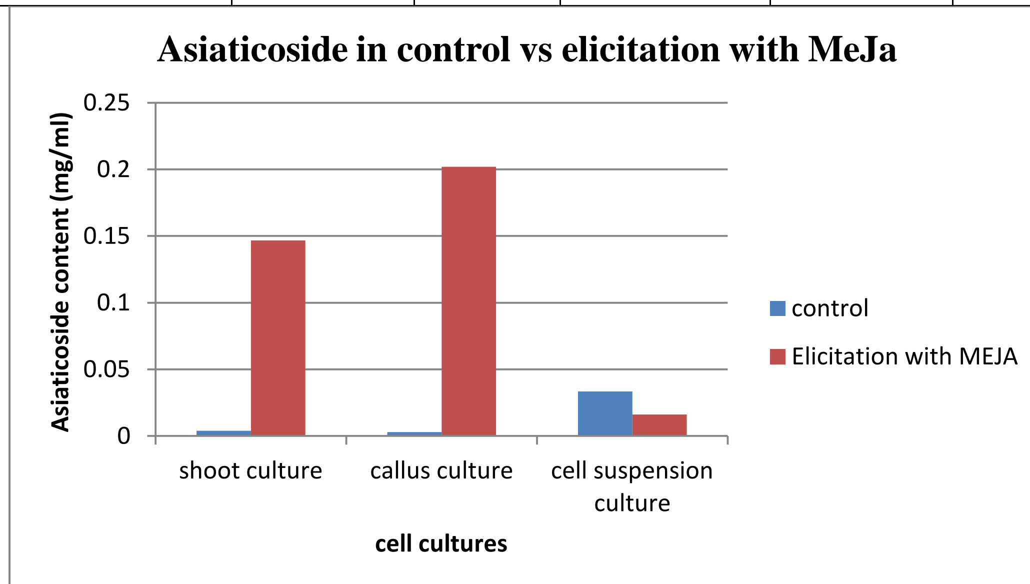
$$\text{Concentration of compound in the sample} = \frac{\text{Concentraion of standard}}{\text{Peak area of standard}} \text{ x Peak area of the sample}$$

The calibration curve for Asiaticoside standard (Sigma Aldrich) at 0.1, 0.5 and 1 mg/ml concentrations with was found to be a straight line and the approximate retention time for the standard asiaticoside was 8.9 minutes. Among the 3 cultures , the cell suspension culture had the maximum asiaticoside content(0.0333 mg/ml) followed by shoot (0.0037mg/ml) and then lastly callus culture (0.0029mg/ml) .It was observed that when elicited with 100µM MEJA , there was a remarkable increase by about 39 fold in asiaticoside content in shoot culture (0.1467 mg/ml) compared to the control(0.0029mg/ml)). Whereas in callus extracts, there was about 68 fold increase in asiaticoside content(0.201 mg/ml) after MEJA elicitation than the control. In cell suspension cells extract ,the methyl jasmonate did not enhance the asiaticoside content, the control had 0.033 mg/ml which was much higher when compared to 0.0161mg/ml after MEJA elicitation.

Reports states that asiaticoside synthesis in Centella is highly tissue-specific occurring mainly in the leaves of about 9.56 mg/g dry weight and only 0.17 mg/g DW in roots cultured with 100 µM MEJA elicitor (Kim *et al*., 2004).The asiaticoside detected in leaves was only 10.55mg/g of dry weight that was grown in MS media provided with 1 mg/l BAP and 1 mg/l NAA, which was significantly less than that of 45.35mg/g dry weight in cell suspension culture(Hoang N. *et al*.,2010).HPLC quantification indicates that leaves of the commercial types of Centella species had higher amounts of triterpenoids when compared to undifferentiated culture cells (James *et al*.,2008). It was reported that the callus and cell suspensions of Centella from Indian origin synthesised asiaticoside.(Nath , Buragohain *et al* ,2005).

TABLE 9:The Asiaticoside content in standards and samples from HPLC shown below:

	ASIATICOSIDE					
	Control			Elicitation with MEJA		
Samples	Retention time (min)	Peak area	Asiaticoside Concentration (mg/ml)	Retention time (min)	Peak area	Asiaticoside Concentration (mg/ml)
Standard	8.957	64476	0.1mg/ml	-	-	-
Standard	8.920	3321784	0.5mg/ml	-	-	-
Standard	8.892	6746212	1mg/ml	-	-	-
Shoot culture	8.822	24338	0.00377	9.135	945924	0.1467081
Callus culture	9.048	19149	0.0029699	8.835	1302116	0.20195
Cell suspension culture	9.132	214771	0.0333 mg/ml	8.507	103855	0.0161073 mg/ml



Graph 10: Comparative study between SC, CC,CSC showing Asiaticoside before and after elicitation with MEJA

4.12.4 Asiatic acid standard chromatogram

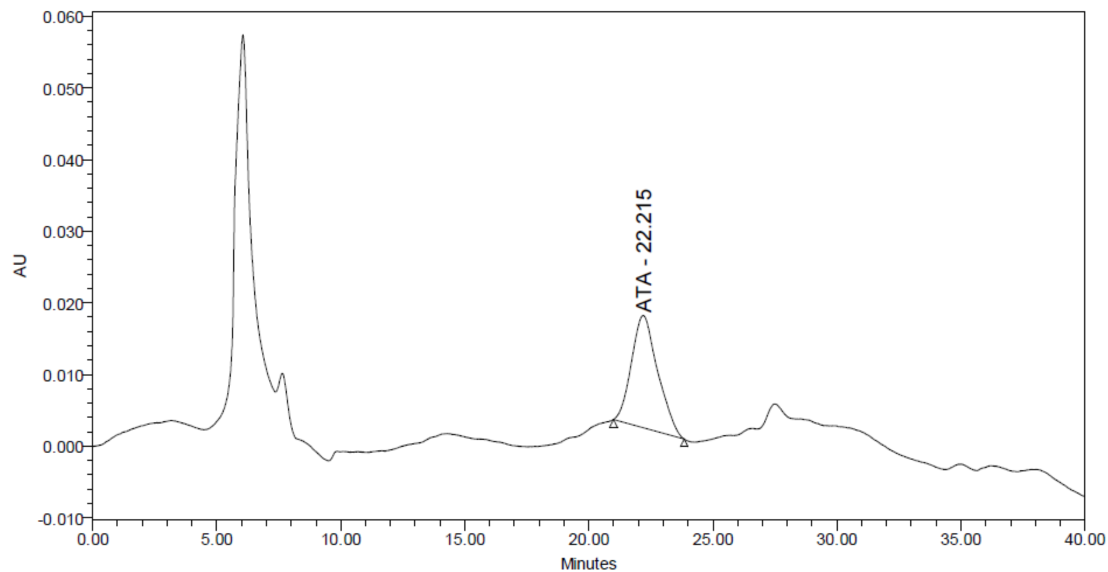


Figure 25: Chromatogram of 0.1 mg/ml Asiatic acid standard

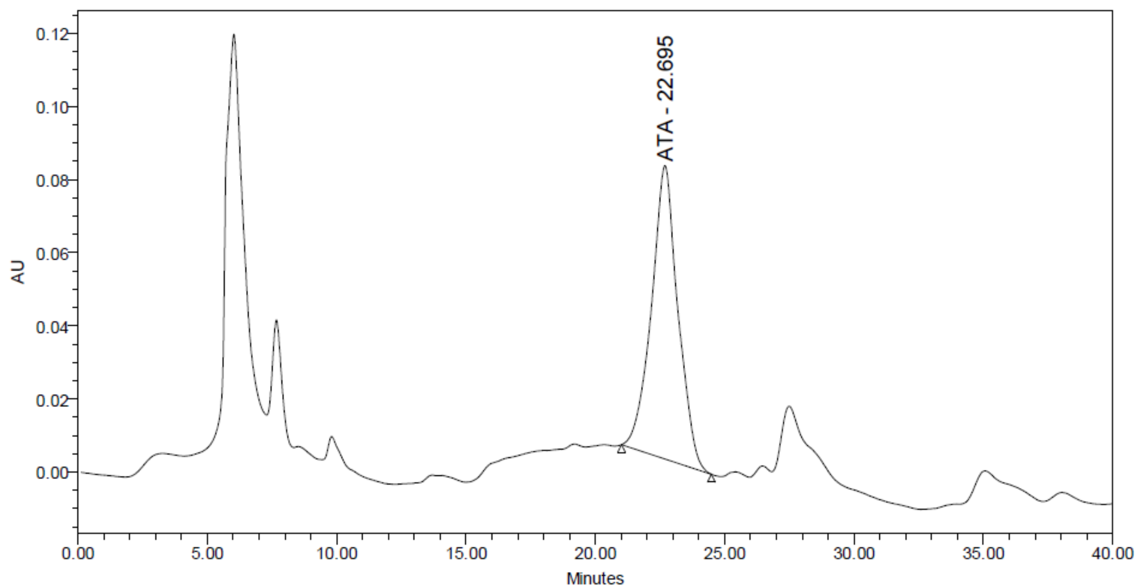


Figure 26: Chromatogram of 0.5 mg/ml Asiatic acid standard

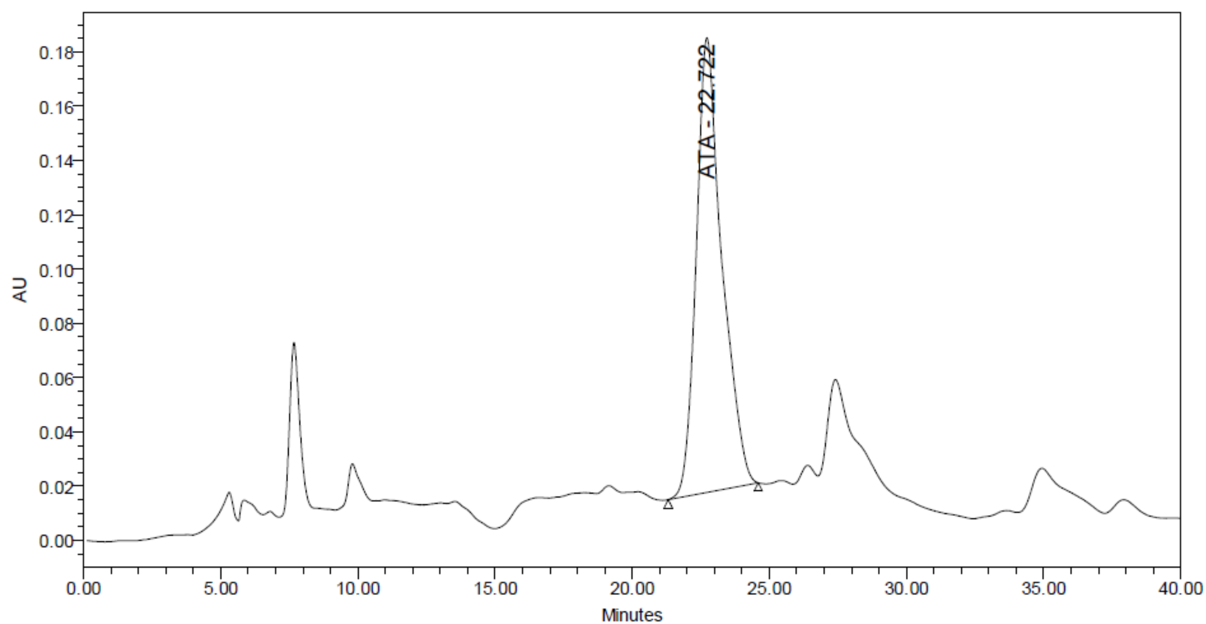


Figure 27: Chromatogram showing 1 mg/ml Asiatic acid standard

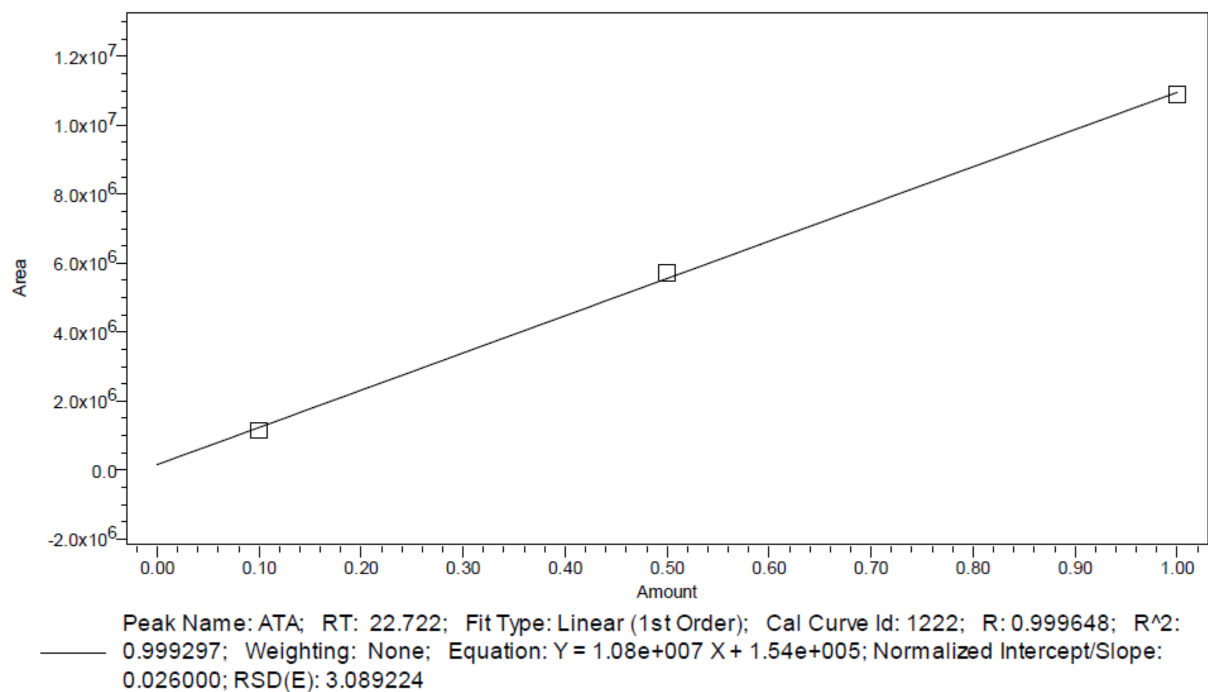


Figure 28: Calibration graph of the 0.1, 0.5 and 1mg/ml standards of Asiatic acid

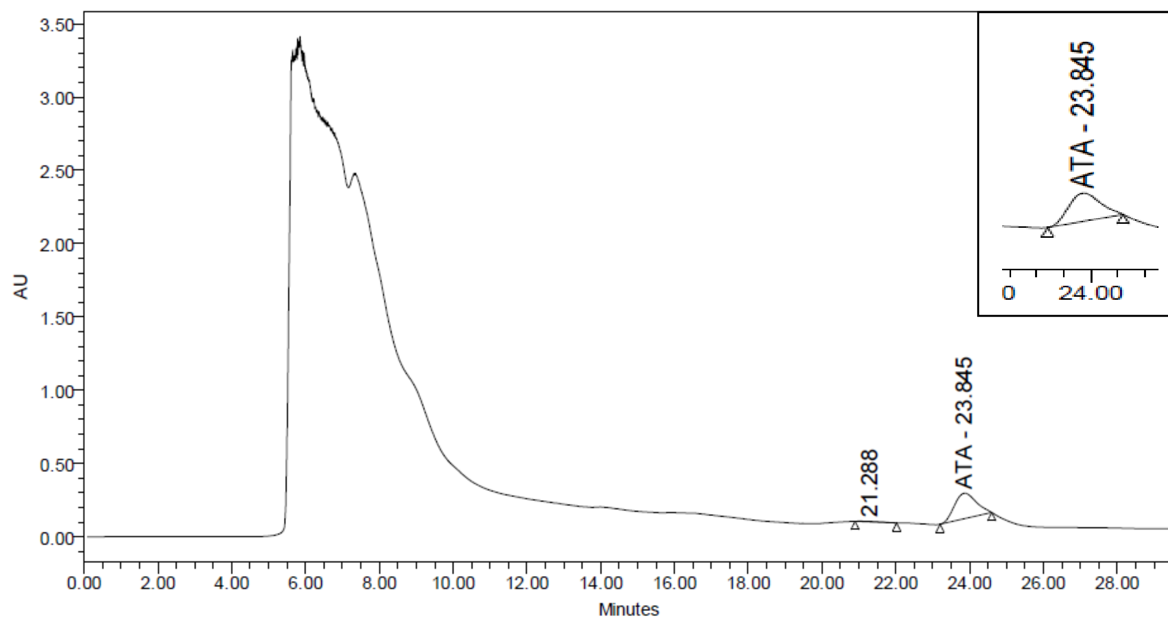


Figure 29: Chromatogram showing Asiatic acid content(0.653087 mg/ml) in plant extract

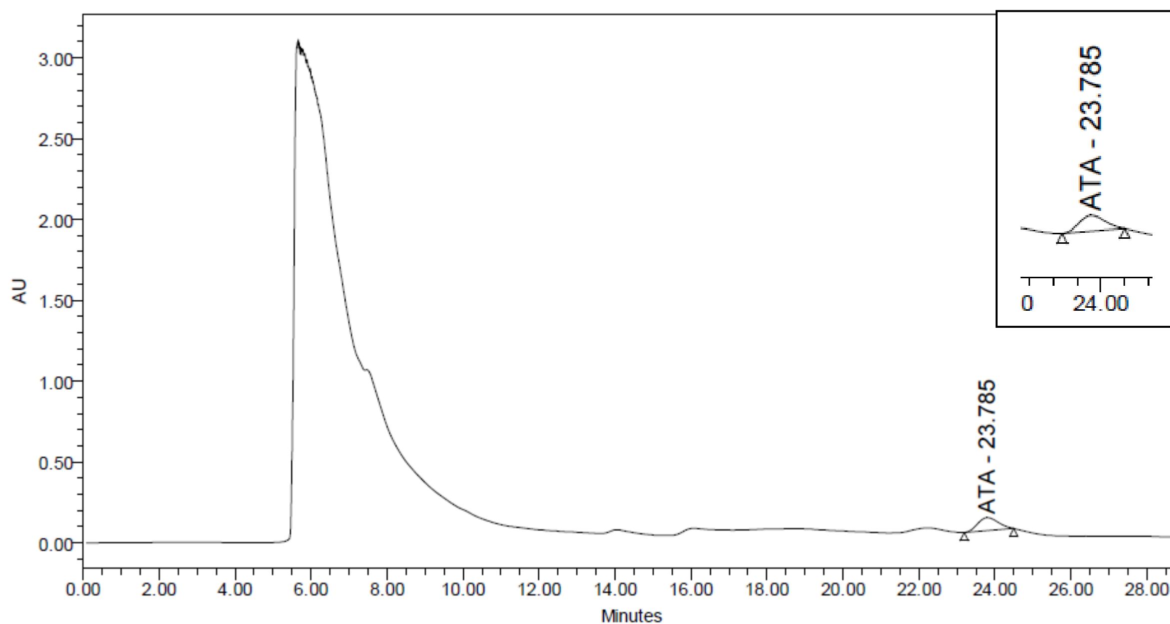


Figure 30: Chromatogram showing Asiatic acid content(0.2757072mg/ml) in methyl jasmonate elicited plant extract

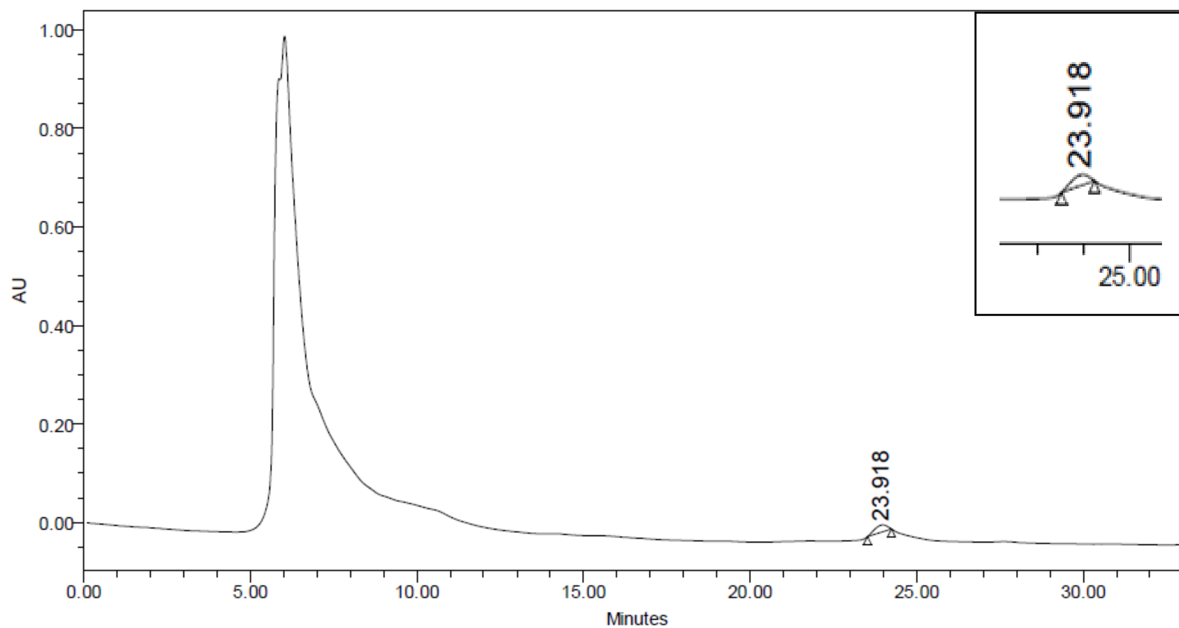


Figure 31: Chromatogram showing Asiatic acid content(0.0349275 mg/ml) in callus extract

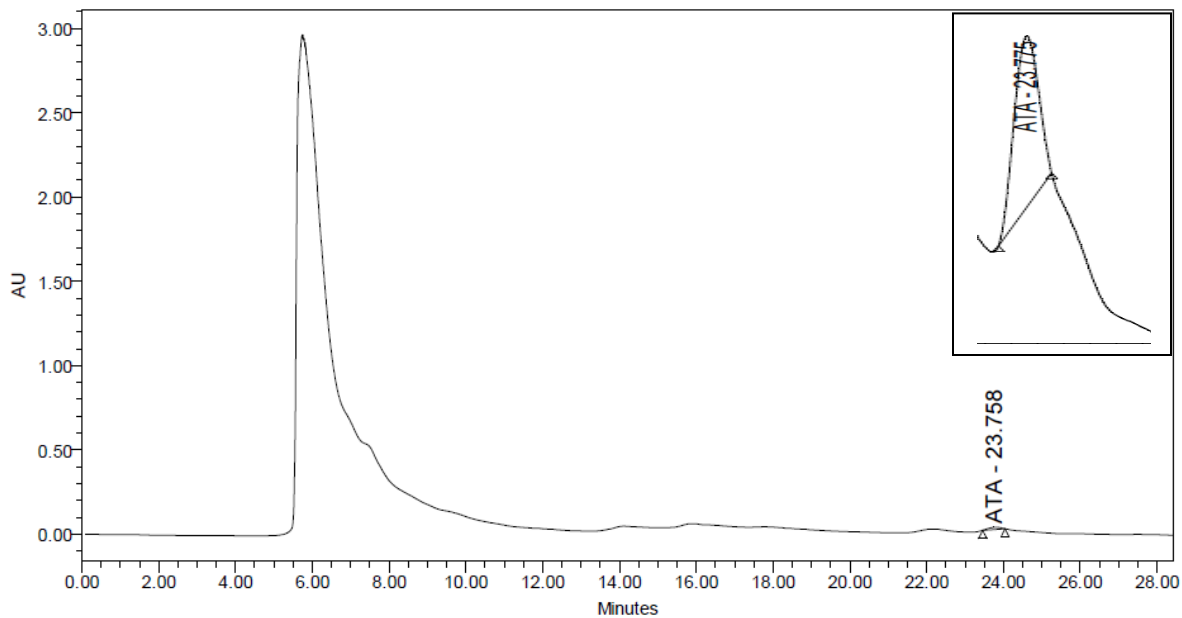


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Figure 32: Chromatogram showing Asiatic acid content(0.067585 mg/ml) in methyl jasmonate elicited callus extract

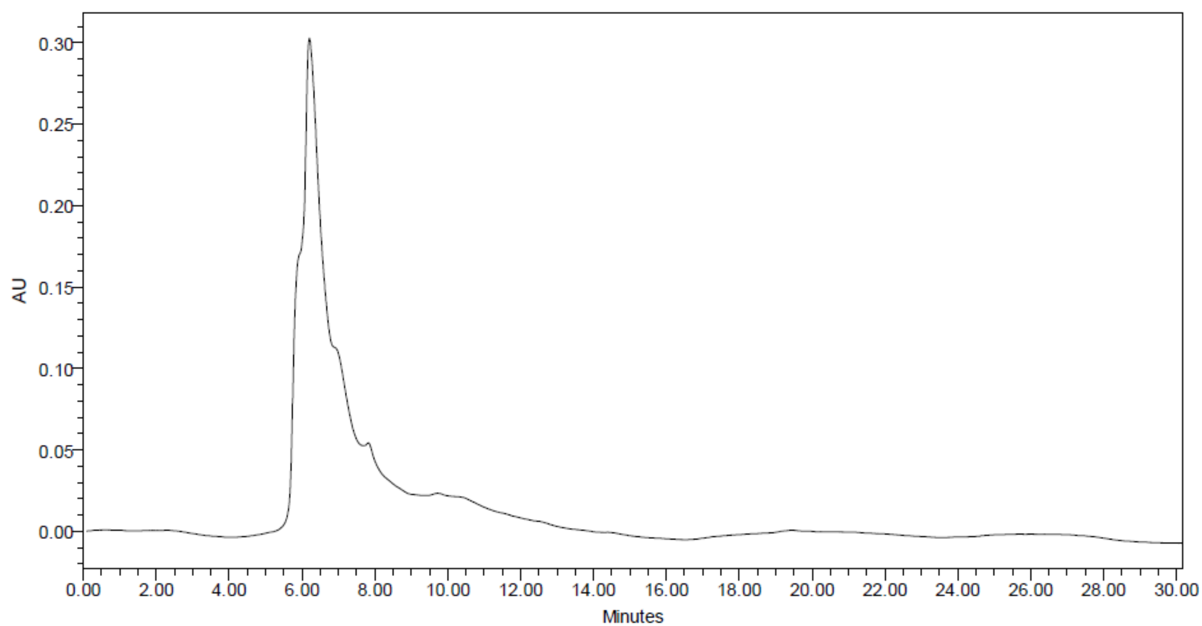


Figure 33: Chromatogram showing no peaks for the Asiatic acid content in cell suspension cells extract

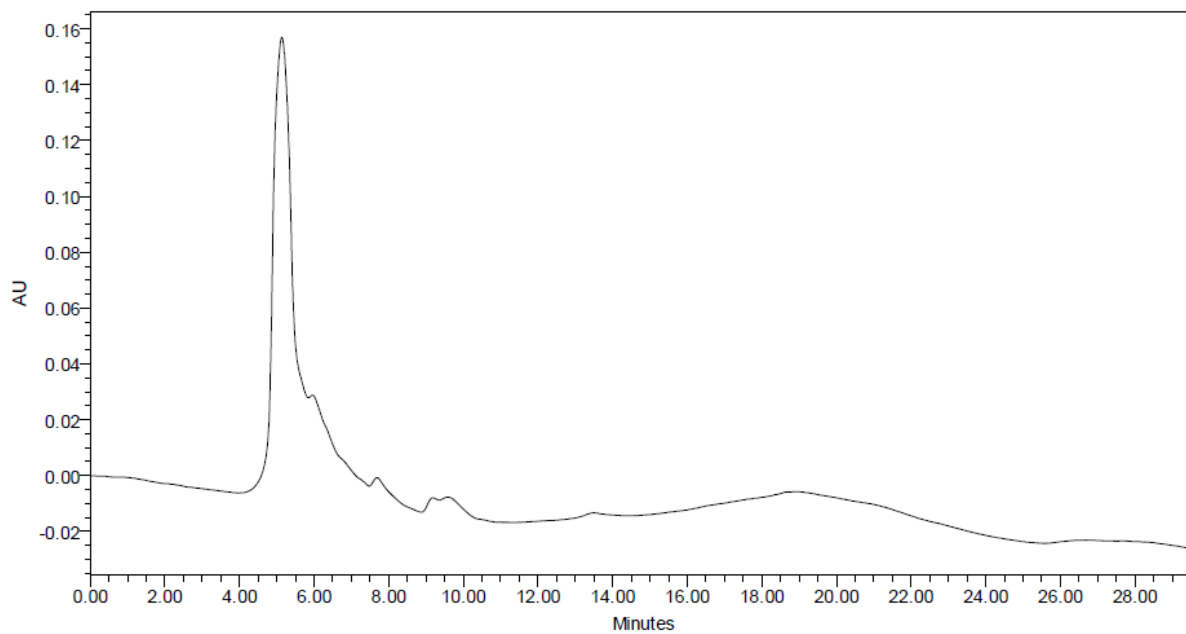


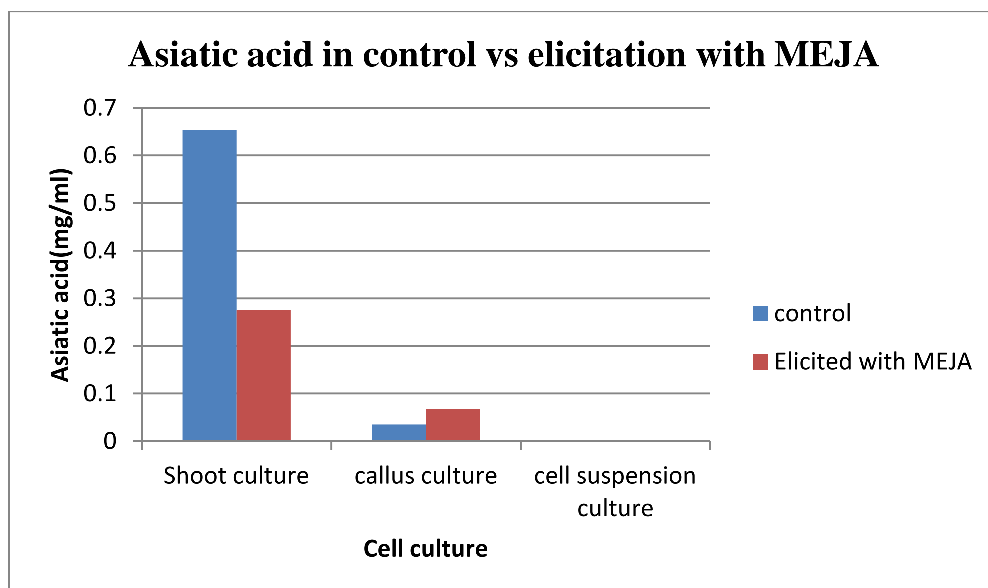
Figure 34: Chromatogram showing no peaks for the Asiatic acid content in methyl jasmonate elicited cell suspension cells extract

4.11.5 Quantitative estimation of Asiatic acid by RP- HPLC

The calibration curve for Asiatic acid standard (Sigma Aldrich) at 0.1, 0.5 and 1 mg/ml concentration was found to be a straight line and the approximate retention time for the standard Asiatic acid was found to be approximate 22.6 minutes.Among the 3 different cultures, the maximum Asiatic acid content was present in shoot extracts (0.653 mg/ml) followed by callus extracts(0.0349 mg/ml). Asiatic acid was not detected in cell suspension cells extracts even after the elicitation with methyl jasmonate may due to low quantities synthesized, or due to the desired compound getting mixed up with unwanted constituents thus the required peaks get merged up with others. The Asiatic acid in shoot extracts in control was tremendously high value of 0.653 mg/ml when compared to 0.2757 mg/ml in MEJA elicted shoot extracts.In the control callus extracts ,0.0349 mg/ml of Asiatic acid content was detected which was enhanced by almost 2 fold to 0.067585 mg/ml with the elicitation of 100µM MEJA.The slight variation in retention times in standards and samples depends on various factors like column condition,time of injection ,temperature, pressure and mobile phase.From reports ,Centella was found to have asiaticoside of 1.97 ± 2.65 mg/mL and madecassoside of 3.10 ± 4.58 mg/mL but low quantities of 0.55 ± 2.29 , Asiatic acid and 0.55 ± 0.89 mg/mL madecassic acid (Hashim P.,2011) . The four active secondary metabolites were present in undifferentiated cultured cells, of which calli contained higher concentrations than that in cell suspensions(James et al.,2008). The order in which metabolites are present in the aerial parts of Centella are asiaticoside following Madecassoside ,Asiatic acid and Madecassic acid(Mangas *et al*.,2006).

TABLE 10 :The Asiatic acid content in standards and samples from HPLC shown below:

ASIATIC ACID						
	Control			Elicitation with MEJA		
	Peak area	Retention Time (min)	Asiatic acid concentration (mg/ml)	Peak area	Retention Time (min)	Asiatic acid concentration (mg/ml)
Standard	1151688	22.215	0.1	-	-	-
Standard	5703111	22.695	0.5	-	-	-
Standard	1088788	22.722	1	-	-	-
Shoot culture	7110744	23.845	0.653087	3001868	23.785	0.2757072
Callus culture	380287	23.918	0.0349275	735861	23.758	0.067585
Cell suspension culture			-	-	-	-



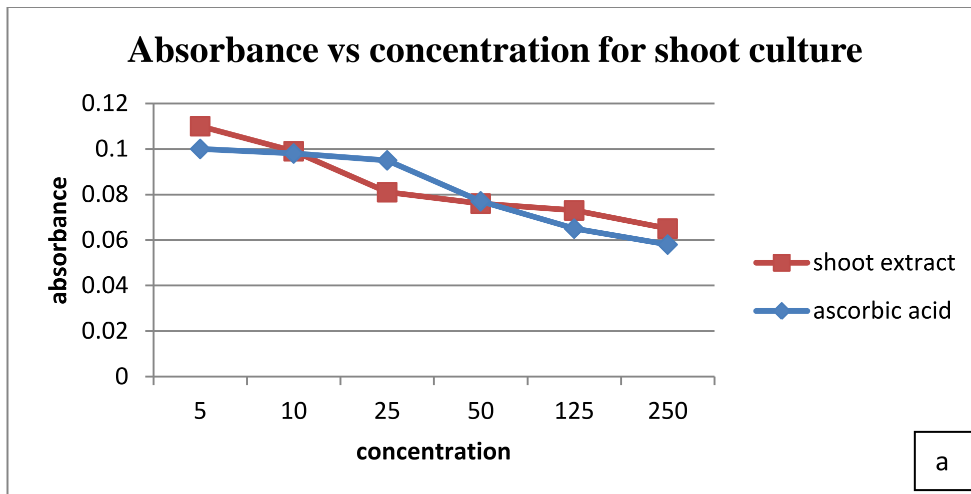
Graph 11: Comparative study between SC, CC,CSC showing Asiatic acid before and after elicitation with MEJA

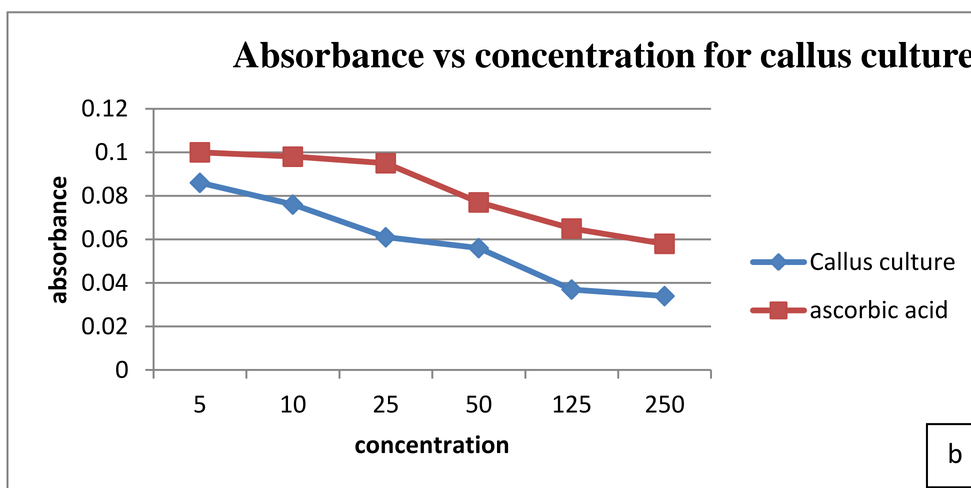
4.12 ANTIOXIDANT ACTIVITY BY DPPH FREE RADIAL ASSAY

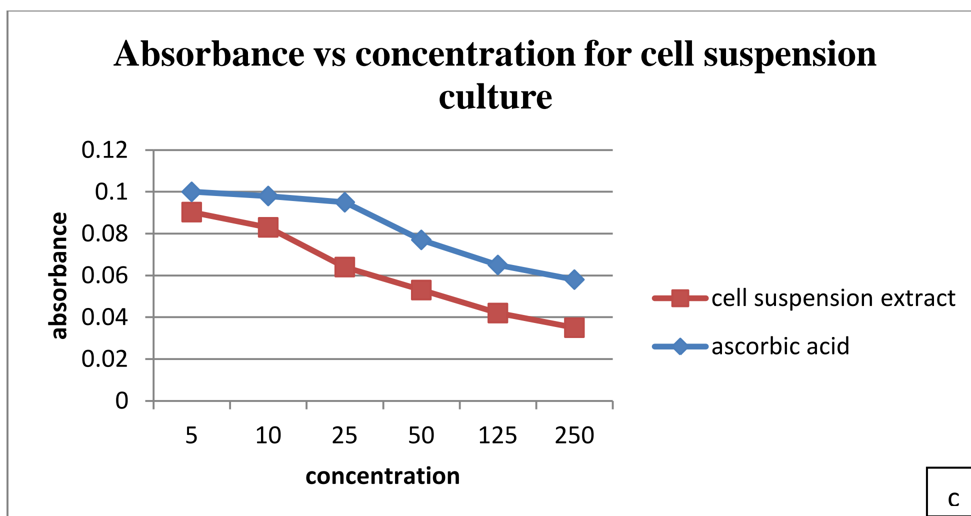
Table 11: The IC 50 values calculated from the graphs are as shown below:

Samples	**IC 50 value(µg/ml)**
Plant extract	19±1
Callus extract	10±2.64
Cell suspension sample	21.25±1.92
Ascorbic acid(standard)	26.25±1.5

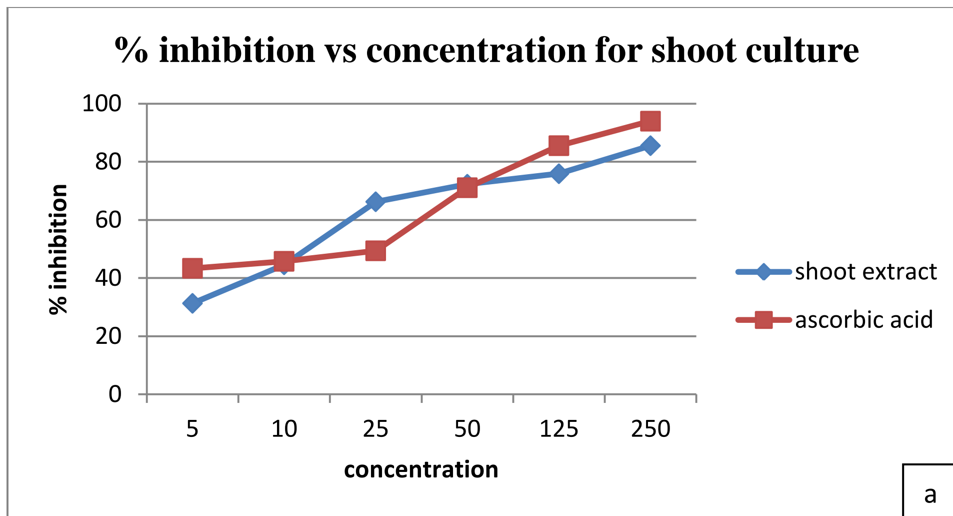
The IC 50 value was calculated from the %inhibition vs concentration graph which indicates the concentration of sample that is required for scavenging 50% of DPPH free radical. The IC50 value was highest of 21.25 µg/ml for cell suspension culture followed by plant extracts 19 µg/ml and then callus extract with 10 µg/ml. The ascorbic acid was taken as the standard with the IC 50 value of 26.25 µg/ml. It was reported that ultrasonic assisted extraction showed highest recovery and highest antioxidant activity with 79% scavenging activity(Nithyanandam *et al*,2014).The IC50 values reported in *C. asiatica* with 100% ethanol extracts is 35.6±1.3 µg/ml , 50% ethanol extract is 7.1±1.5 µg/ml of and water extract is 10.3±1.2 µg/ml (Rahman M. *et al,*2013).

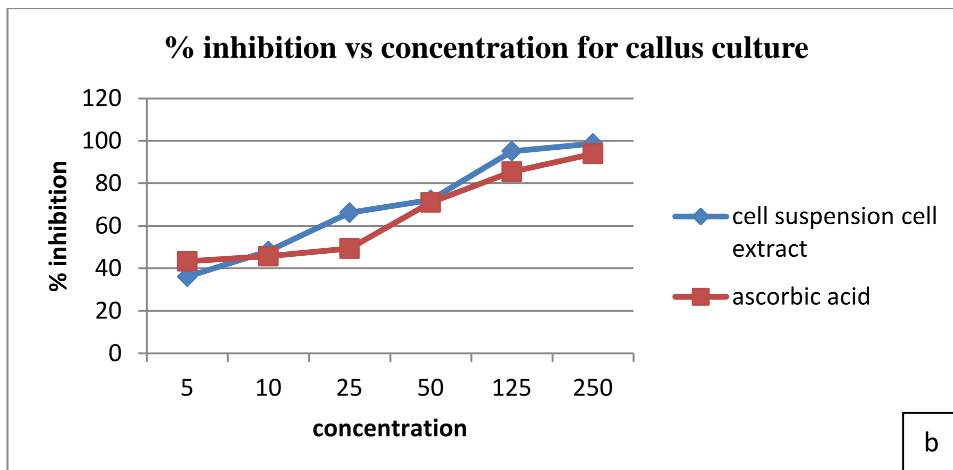


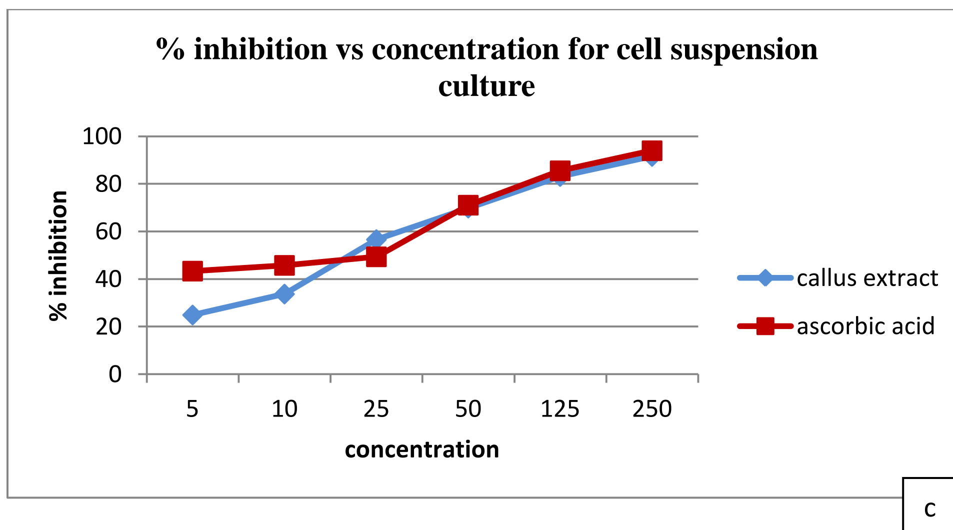




Graph 12: Absorbance vs concentration graph of ascorbic acid and a) shoot extract,b)Callus extract, c) Cell suspension extract







Graph 13: % inhibition vs concentration graph of ascorbic acid and a) shoot extract,b)Callus extract, c) Cell suspension extract

4.13 PHYTOCHEMICAL ESTIMATIONS

4.13.1 Preliminary Phytochemical screening

When preliminary Phytochemical screening was done in shoot,callus and cell suspension extracts, the positive results were obtained in Tannins,flavanoids ,terpenoid,saponin and steroids in all the three cultures and absence of alkaloids.

Table 11 Preliminary Phytochemical screening of methanolic extracts of *Centella asiatica*

Phytochemicals	Plant extract	Callus extract	Cell suspension cells extract
Alkaloids	-	-	-
Tannin	+	+	+
Flavanoids	+	+	+
Terpenoids	+	+	+
Saponins	+	+	+
Steroids	+	+	+

*+ for positive result and – for negative result

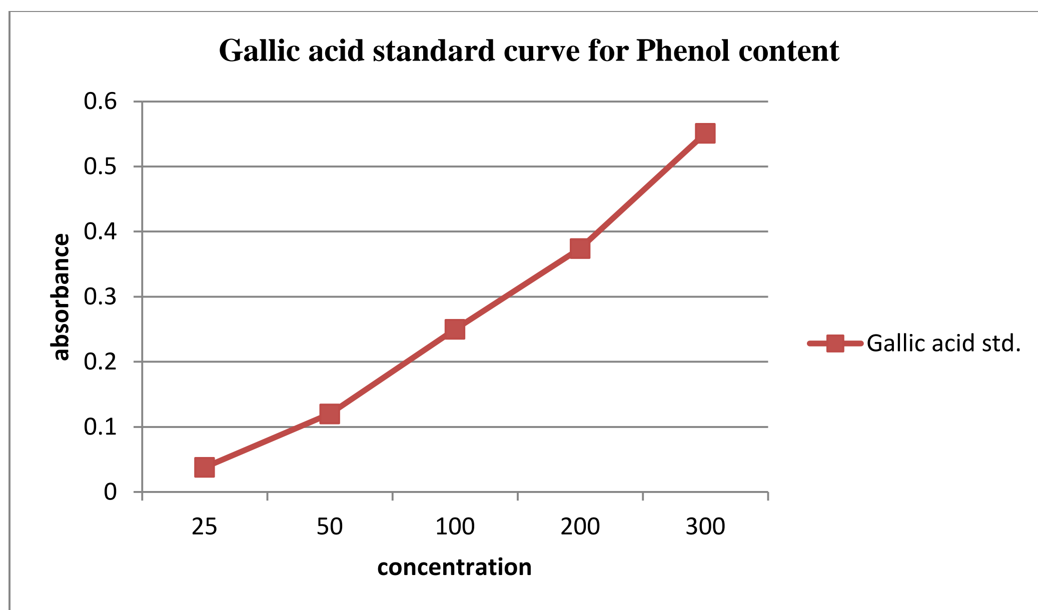
4.14 TOTAL PHENOLIC ESTIMATION

.The polyphenol content expressed in terms of mg of gallic acid equivalent per gram of extract was found to be highest in plant extract of 372.5 mg of GAE/g of extract followed by callus extracts having 182.5 mg of GAE/g of extract and least in cell suspension extract,70 mg of GAE /g of extract.

Table 12: Total phenolic content in SC,CC and CSC

S No.	Samples	Total phenolic content (mg/g)*
1.	Shoot extracts	372.5±2
2.	Callus extracts	182.5±0.78
3.	Cell suspension cells extracts	70±2.28

*Expressed in terms of mg of gallic acid equivalent per gram of extract



Graph 14: Gallic acid standard curve for phenol estimation

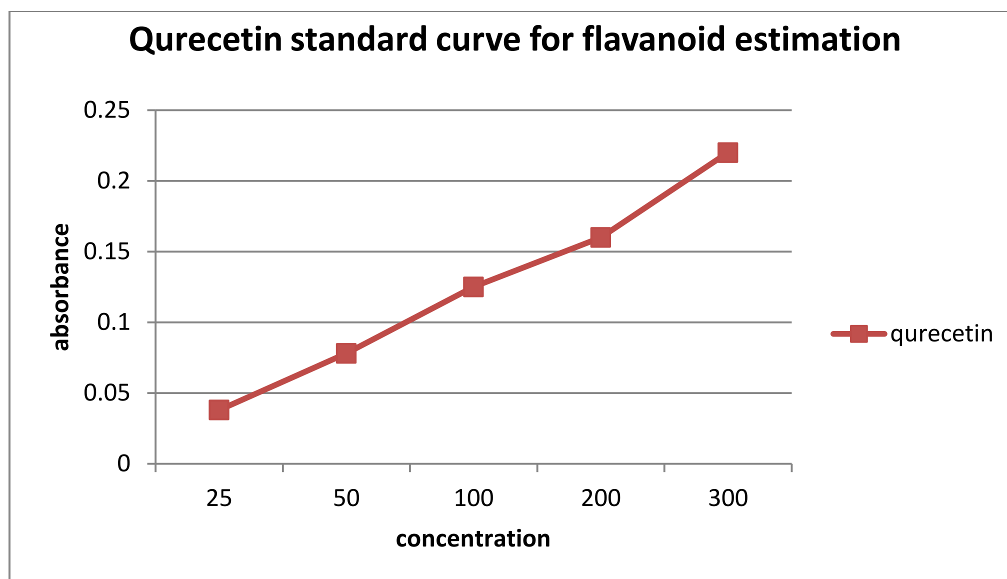
4.15 TOTAL FLAVANOID ESTIMATION

The total flavanoid estimation indicated in terms of qurecetin equivalent per gram of extract with maximum value of 275 ± 0.8 of mg QE/g of extract , following callus 145±0.4 mg QE/g of extract and 100±0.7 QE/g of extract in cell suspension cells.

Table 12:Total flavanoid content in SC,CC and CSC

Sno.	sample	Total flavanoid estimation (mg/g)
1	Plant extract	275 ± 1
2	Callus extract	145±1.28
3	Cell suspension cells extract	100±1

*Expressed as mg of gallic acid equivalent(GAE) per gram of extract



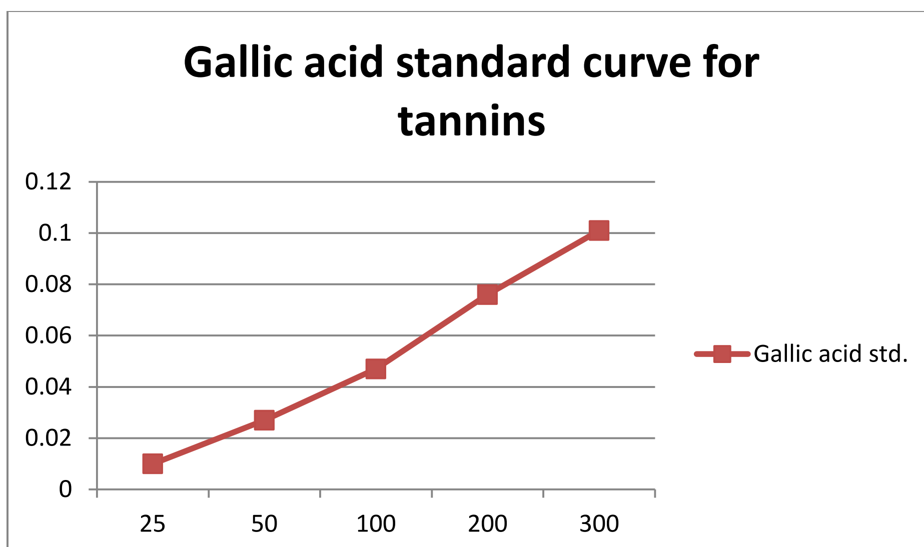
Graph 15: Qurecetin standard curve for phenol estimation

4.16 TOTAL TANNIN ESTIMATION

The Total tannin content estimate using gallic acid as standard found to maximum in shoot extract, 260±0.55 mg of GAE / g of extract, followed by callus, 205±0.7155 mg of GAE /g of extract and 55±0.8 mg of GAE /g of extract in cell suspension extracts.

	Sample	Total tannin content(mg/g)*
1	Plant extract	260±1
2	Callus extract	205±4.15
3	Cell suspension cells extract	55±4.35

*Expressed as mg of gallic acid equivalent per gram of extract



Graph 16: Gallic acid standard curve for tannin estimation

According to reports, Centella extracts by ultrasonic assisted extraction showed Total Phenolic Content of 1350 mg GAE/100 g Dry Weight and Total Flavonoid Content ,599 mg CE/100 g Dry weight ((Nithyanandam et al,2014).The polyphenols in 100% ethanol extact was 21.1±0.1 Pyrogallol Equivalent and flavanoid is 9.3±0.3Quercetin Equivalent(Rahman *et al* .,2013) The polyphenols in Centella was found to be 150 mg tannic acid/100 g for *C. asiatica*(Gupta S *et al*.,2013). The total antioxidant capacity was found to be very less in *C. asiatica* (623.78 μmol of ascorbic acid/g of sample).The IC 50 value was estimated to be 19.89 mg/ml for *Centella asiatica*(Shyamala *et al*.,2005).

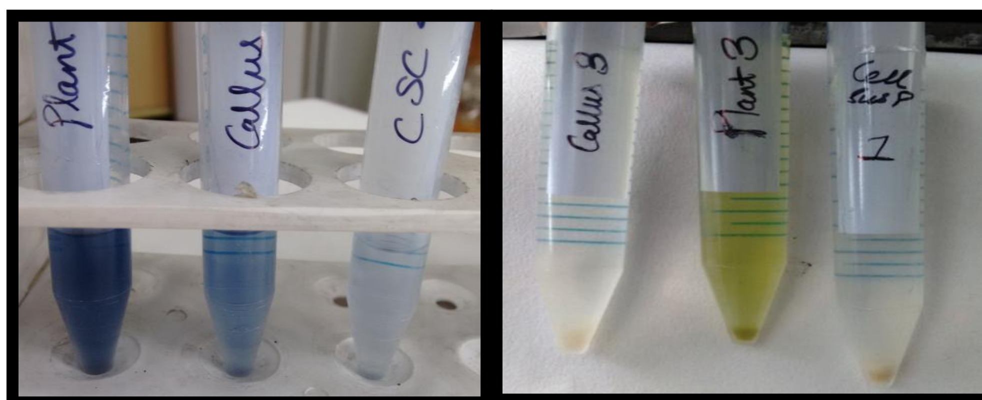
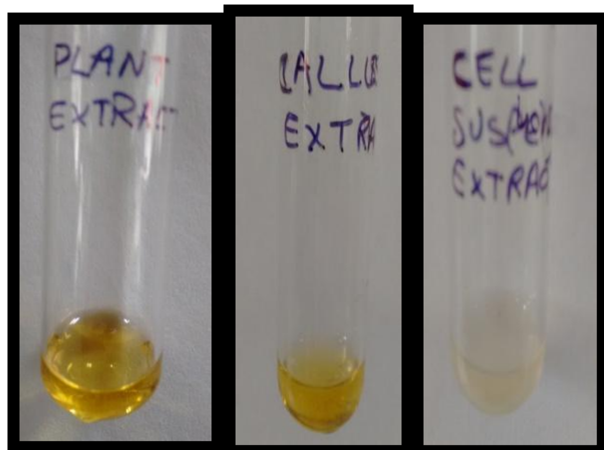


Figure 12: Showing a)Total phenol estimation using F-C technique ,b) Total flavanoid estimation using aluminium chloride technique

Figures of Preliminary Phytochemical screening in shoot, callus and cell suspension cultures are shown below:



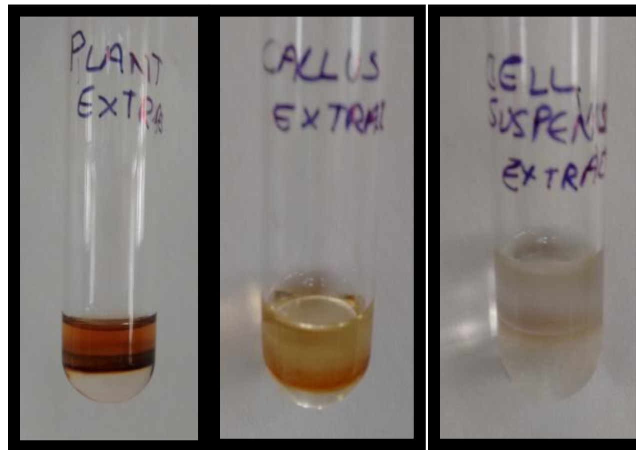
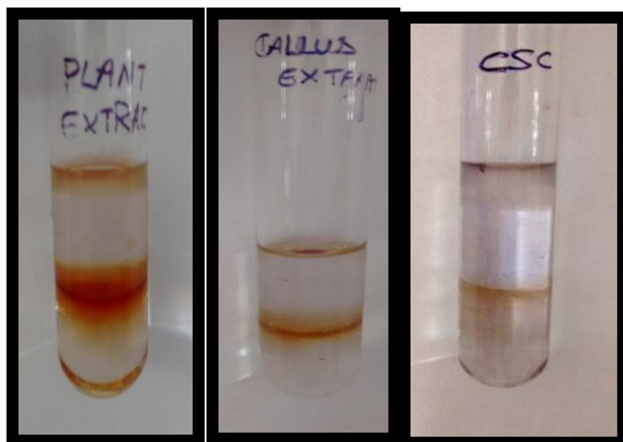


Figure 13:Flavanoid test showed positive results **Figure 14**:Terpenoid test showed positive results



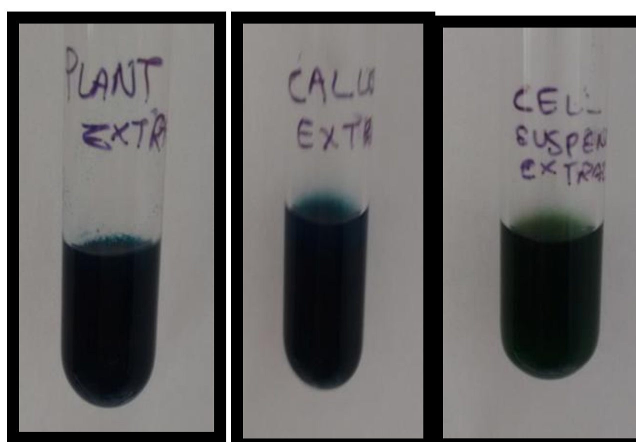


Figure 15:Steroid test showed positive results **Figure 16**:Tannin test showed positive results

4.17 STATISTICAL ANALYSIS

The readings of shoot length and shoot number of the below experimental conditions were compared with the One way Analysis of Varience (ANOVA). A probability of $P \leq 0.05$ and $F_{stat} > F_{critical\ value}$ were obtained in all the cases, thus we can consider all the experimental results as statistically significant.

1. **Nitrogen variation- Shoot number**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
20mM	3	75	25	7
40mM	3	133	44.33333	2.333333
50mM	3	120	40	7
60mM (C)	3	136	45.33333	2.333333
100mM	3	143	47.66667	2.333333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	989.7333	4	247.4333	58.9127	6.48E-07	3.47805
Within Groups	42	10	4.2			
Total	1031.733	14				

2. **Nitrogen variation- Shoot length**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
20 mM	3	8.3	2.766667	0.063333
40mM	3	11	3.666667	0.003333
50mM	3	10	3.333333	0.023333
60mM	3	11.7	3.9	0.13
100mM	3	13.8	4.6	0.28

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.537333	4	1.384333	13.84333	0.000438	3.47805
Within Groups	1	10	0.1			
Total	6.537333	14				

3. Sucrose variation- Shoot number

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
S2	3	43	14.33333	4.333333
S3	3	108	36	1
S4	3	86	28.66667	4.333333
S5	3	66	22	7
S6	3	42	14	1
S7	3	28	9.333333	2.333333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1541.611	5	308.3222	92.49667	3.71E-09	3.105875
Within Groups	40	12	3.333333			
Total	1581.611	17				

4. **Sucrose variation- Shoot length**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
S2	3	6.6	2.2	1.27
S3	3	12.6	4.2	0.09
S4	3	9.9	3.3	0.19
S5	3	10.8	3.6	0.16
S6	3	9.4	3.133333	0.173333
S7	3	8.1	2.7	0.07

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.271111	5	1.454222	4.466894	0.015689	3.105875
Within Groups	3.906667	12	0.325556			
Total	11.17778	17				

5. **Potassium variation - shoot number**

Summary

Groups	*Count*	*Sum*	*Average*	*Variance*
0	3	42	14	1
0.3	3	95	31.66667	2.333333
0.6	3	68	22.66667	2.333333
1	3	81	27	3
1.5	3	53	17.66667	0.333333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	599.6	4	149.9	83.27778	1.23E-07	3.47805
Within Groups	18	10	1.8			
Total	617.6	14				

6. Potassium variation – shoot length

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0	3	7.9	2.633333	0.023333
0.3	3	10.7	3.566667	0.023333
0.6	3	9.6	3.2	0.79
1	3	11.7	3.9	0.01
1.5	3	8.6	2.866667	0.053333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.153333	4	0.788333	4.37963	0.026507	3.47805
Within Groups	1.8	10	0.18			
Total	4.953333	14				

7. Phosphorous variation- Shoot number

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0	3	41	13.66667	2.333333
0.3	3	55	18.33333	2.333333
0.6	3	72	24	1
1	3	79	26.33333	4.333333
1.5	3	84	28	1

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	424.9333	4	106.2333	48.28788	1.66E-06	3.47805
Within Groups	22	10	2.2			
Total	446.9333	14				

8. Phosphorous variation- Shoot length

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0	3	7.2	2.4	0.03
0.3	3	9.1	3.033333	0.063333
0.6	3	9.7	3.233333	0.123333
1	3	11.5	3.833333	0.023333
1.5	3	10.8	3.6	0.01

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.684	4	0.921	18.42	0.000132	3.47805
Within Groups	0.5	10	0.05			
Total	4.184	14				

9. Magnesium variation-shoot number

SUMMARY

Groups	Count	Sum	Average	Variance
0mM	3	34	11.33333	2.333333
1mM	3	89	29.66667	2.333333
1.5mM	3	93	31	1
2mM	3	80	26.66667	2.333333
3mM	3	44	14.66667	2.333333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	980.6667	4	245.1667	118.629	2.21E-08	3.47805
Within Groups	20.66667	10	2.066667			
Total	1001.333	14				

10. Magnesium variation-shoot length

SUMMARY

Groups	Count	Sum	Average	Variance
0mM	3	5.3	1.766667	0.063333
1mM	3	7.5	2.5	0.09
1.5mM	3	11.6	3.866667	0.063333
2mM	3	6.6	2.2	0.16
3mM	3	4.9	1.633333	0.023333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.569333	4	2.392333	29.90417	1.53E-05	3.47805
Within Groups	0.8	10	0.08			
Total	10.36933	14				

11. Manganese variation- shoot number

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0µM	3	72	24	3
100µM (C)	3	91	30.33333	2.333333
200 µM	3	129	43	4
300 µM	3	123	41	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	726.25	3	242.0833	103.75	9.61E-07	4.066181
Within Groups	18.66667	8	2.333333			
Total	744.9167	11				

12. **Manganese variation- shoot length**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0µM	3	6.9	2.3	0.19
100µM (C)	3	8.8	2.933333	0.093333
200 µM	3	11.6	3.866667	0.023333
300 µM	3	9.8	3.266667	0.063333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.849167	3	1.283056	13.87087	0.001553	4.066181
Within Groups	0.74	8	0.0925			
Total	4.589167	11				

13. Elicitor, MEJA-shoot number

Groups	*Count*	*Sum*	*Average*	*Variance*
50µM	3	40	13.33333	2.333333
100µM	3	72	24	1
150µM	3	56	18.66667	0.333333
200µM	3	50	16.66667	2.333333
250µM	3	33	11	1
control	3	93	31	1

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	811.7778	5	162.3556	121.7667	7.46E-10	3.105875
Within Groups	16	12	1.333333			
Total	827.7778	17				

14. Elicitor, Methyl jasmonate- shoot length

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
50µM	3	6	2	0.04
100µM	3	10.2	3.4	0.01
150µM	3	7.4	2.466667	0.123333
200µM	3	7	2.333333	0.093333
250µM	3	4.8	1.6	0.04
control	3	11.6	3.866667	0.063333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11.07778	5	2.215556	35.92793	8.14E-07	3.105875
Within Groups	0.74	12	0.061667			
Total	11.81778	17				

15. Elicitor,Malt extract variation –shoot number

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1 basal	3	14.9	4.966667	0.223333
basal 1.5	3	13.5	4.5	0.01
basal 2	3	11.7	3.9	0.57
2,0.5 me	3	10.8	3.6	0.73
2,1 me	3	10.1	3.366667	0.263333
2,1.5 me	3	7.6	2.533333	0.253333
1,0.5 me	3	10.1	3.366667	0.103333
1,1 me	3	13	4.333333	0.583333
1,1.5 me	3	10.5	3.5	0.25
1.5,0.5 me	3	11.1	3.7	0.03
1.5,1 me	3	7.7	2.566667	0.043333
1.5,1.5 me	3	12.2	4.066667	0.013333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.21333	11	1.564848	6.110037	0.000108	2.216309
Within Groups	6.146667	24	0.256111			
Total	23.36	35				

16. DPPH assay

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
shoot	3	57	19	1
callus	3	30	10	7
csc	3	63.75	21.25	3.7225
ascorbic acid	3	79.7	26.56667	2.263333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	429.4506	3	143.1502	40.94149	3.36E-05	4.066181
Within Groups	27.97167	8	3.496458			
Total	457.4223	11				

17. Phenol estimation

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
shoot	3	1117.5	372.5	4
callus	3	547.5	182.5	0.61
csc	3	210.1	70.03333	5.243333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	140234.8	2	70117.42	21348.33	2.77E-12	5.143253
Within Groups	19.70667	6	3.284444			
Total	140254.5	8				

18. Flavanoid estimation

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
shoot	3	825	275	1
callus	3	435.2	145.0667	1.653333
csc	3	300	100	1

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	49538.68	2	24769.34	20339.78	3.21E-12	5.143253
Within Groups	7.306667	6	1.217778			
Total	49545.98	8				

19. Tannin estimation

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
shoot	5	1041	208.2	13416.7
callus	5	824.1581	164.8316	8076.137
csc	5	224.3589	44.87178	522.4042

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	71578.81	2	35789.41	4.876995	0.028174	3.885294
Within Groups	88060.96	12	7338.414			
Total	159639.8	14				

5.0 CONCLUTION AND FUTURE PERSPECTIVE

The entire study was performed on an important medicinal plant, *Centella asiatica* having multiple therapeutical properties used especially for wound healing ,skin diseases, inflammation, ,memory enhancement . From multiple shoot cultures grown under various nutrient manipulation in MS media, the optimum growth conditions were observed in MS media with 100mM nitrogen,3% sucrose,30% potassium ,150% phosphorous,1.5 mM magnesium and 200µM manganese respectively. The elicitor MEJA did not have positive effect on shoot development in contrast to ME which enhanced shoot proliferation. After successfully establishing the shoot, callus and cell suspension culture ,their HPLC analysis indicates maximum asiaticoside content in cell suspension culture (0.033mg/ml). followed by shoot and then lastly callus. When elicited with 100µM MEJA ,there was about 39 fold increase in asiaticoside content in shoot culture (0.146 mg/ml) and about 68 fold increase in callus culture (0.2019 mg/ml).Asiatic acid content was highest in shoot extracts (0.653 mg/ml) followed by callus extracts(0.0349 mg/ml),which was enhanced by almost 2 fold to 0.067585 mg/ml after MEJA elicitation .Asiatic acid was absent in Cell suspension culture even after elicitation. Antioxidant activity by DPPH assay was performed to observe maximum antioxidants of 21.25 µg/ml in cell suspension. Phytochemical test showed highest flavanoid,tannins and phenols in shoot extracts compared to callus and suspension extracts.

Rapid urbanization, ruthless collection of herbs, pollution and other man-made activities reduces the diversity of medicinal plants in the ecosystem therefore plant tissue culture technologies promises the conservation and enhancement of valuable medicinal plants (Shaik S *et al*.,2011). The recent biotechnological techniques needs to be encouraged to conserve endangered medicinal plants and also enhance their secondary metabolites by conducting genetic variations to withstand stresses and strategies to modify their mechanisms and metabolic pathways involved in the plant metabolites production. Thus we can conclude that plant cell cultures like shoot, callus and cell suspension offers great potential for obtaining required medicinal compounds at the same time ensures a sustainable conservation of endangered medicinal plants like *Centella asiatica* and rational utilisation of biodiversity.

6.0 REFERENCES

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6.0 APPENDIX

Ingrediants of Murashige and Skoog (MS) medium

COMPONENTS	AMOUNTS
Major salts (macronutrients)	
Ammonium nitrate (NH_4NO_3)	1,650 mg/l
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	440 mg/l
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	370 mg/l
Potassium phosphate (KH_2PO_4)	170 mg/l
Potassium nitrate (KNO_3)	1,900 mg/l
Minor salts (micronutrients)	
Boric acid (H_3BO_3)	6.2 mg/l
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.025 mg/l
Cupric sulphate ($CuS_{O4} \cdot 5_{H2}O$	0.025 mg/l
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	27.8 mg/l
Manganese sulphate ($MnSO_4 \cdot 4H_2O$)	22.3 mg/l
Potassium iodide (KI)	0.83 mg/L
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.25 mg/l
Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	8.6 mg/l
Ethylenediaminetetraacetic acid ferric sodium (NaFe-EDTA)	5 mL/L of a stock solution containing 5.57 g FeSO4.7H2O and 7.45 g Na2-EDTA per litre of water.
Vitamins and organics	
Myo-Inositol	100 mg/l
Nicotinic Acid	0.5 mg/l
Pyridoxine	0.5 mg/l
Thiamine · HCl	0.1 mg/l
Glycine	2 mg/l
Lactalbumin Hydrolysate (Edamin) (optional)	1 g/l
Indole Acetic Acid	1-30 mg/l
Kinetin	0.04-10 mg/l