Estimation of phytosterols using gas chromatography and synthesis of silver nanoparticles in *Centella asiatica*

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Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled "**Estimation of phytosterols using gas chromatography and synthesis of silver nanoparticles in *Centella asiatica*". (DTU/14/M.TECH./085)** in the partial fulfilment of the requirements for the reward of the degree of Master of Technology, 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 honouring of any other degree.

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DECLARATION

This is to certify that the thesis of Major Project II entitled "**Estimation of phytosterols using gas chromatography and synthesis of silver nanoparticles in *Centella asiatica***" (DTU/14/M.Tech./085) in the partial fulfilment of the requirements for the reward of the degree of Mater of Technology, Delhi Technological University (Formerly Delhi college of Engineering, University of Delhi), is an authentic record of the my own work carried out under the guidance of my project supervisor ***Dr. Navneeta Bharadvaja***, Assistant Professor, Department of Biotechnology, DTU. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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ABSTRACT

In the *Ayurvedic* system of medicine, *Centella asiatica (*gotu kola) is one of the important rejuvenating herbs for nerve and brain cells and is believed to be capable of increasing intelligence, longevity, and memory. It contains several active constituents and the most important bioactive compounds are triterpenoid saponins, including asiaticoside, centelloside, madecassoside, and asiatic acid. It possesses anti-leprotic, anti-viral, anti-bacterial, anti-tumor activities. Due to its medicinal importance, this plant is being overexploited and to conserve this plant it is necessary to micropropagate this plant in laboratory with high biomass yeild. ***In the present investigation***, estimation of phytosterols was done in five accessions of *Centella asiatica* and a comparative study for shoot multiplication was performed. For shoot culture, explants of different accessions of *C. asiatica* were inoculated in different media i.e. MS, Gamborg's B5 and Nitsch which were supplemented with 1 mg/l BAP. The cultures were incubated at 25 ± 2 °C with photoperiod of 16 hours. After eight week of incubation period it was found that MS media showed highest growth in all the accessions. Estimation of stigmasterol was done and it was found the accession no.342109 showed highest amount of stigmasterol content i.e. 4.55±0.70µg/200mg of leaves. It was also found that all the accession contains β-sitosterol and tocopherol. In qualitative analysis of phytochemicals ethanolic, methanolic and aqueous extract of *Centella asiatica* (whole plant) were used. In case of ethanolic and methanolic extract it showed positive results for terpenoid, steroid, flavonoid and coumarins whereas in case of aqueous extract it showed positive results for terpenoid, steroid, flavonoid, coumarins and tannin. Further synthesis of silver nanoparticles was done and characterization of synthesized nanoparticles was done by using UV-VIS spectroscopy, FT-IR and SEM.

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LIST OF ABBREVIATIONS

NBPGR -National Bureau of Plant Genetic Resources

BAP- 6-Benzylaminopurine

°C-degree Celcius

µg – microgram

µL- microlitre

mg- milligram

ml-mililiter

mM- millimolar

ANOVA- Analysis of Variance

INTRODUCTION

Medicinal plants are the traditional source of many pharmaceutically important compounds. In recent times, they are utilized by the pharmaceutical companies for the preparation of several formulations. In the present time there has been an increase in the use of herbal products around the world. In the last 20 years about 28% of new chemical compounds that are launched into the market are accounted by the natural products (Ahmad, 1993). World Health Organisation (WHO) also stated that more than 80% of the world's population relies on the herbal medicines (Singh, 2012).

Importance of medicinal plants is due to the presence of specific chemical compounds that produce a physiological effect on the human body. These bioactive chemical constituents of plants include saponin, flavanoids, alkaloids, sterols, tannins, phenols (Singh, 2012).

Medicinal plant based drugs have an advantage over the other drugs because they are simple, effect and offer broad spectrum of activity. Furthermore they have very less adverse side effects as compare to the chemotherapeutic drugs (Ahmad, 1993).

India is rich in medicinal plant diversity and since the ancient times use of drugs of herbal origin is prevalent in traditional system of medicines such as *Ayurveda* and *Unani.* There are about 426 biomes which comprises of different habitat diversity that give rise to the richest centres for plant genetic resources in the world (Ahmad, 1993). Out of 18,665 flowering species, only about 3000 plants has been used for the various formulations in classic system of medicines such as *Ayurveda, Siddha* and *Unani* (Singh, 2012)..

1.1 *Centella asiatica*

Centella asiatica is one of the important traditional medicinal plant belonging to family *Apiaceae* and commonly known as 'Gotu kola', 'Indian Pennywort' or 'Mandookaparni' in India. It is an important perennial medicinal herb found in the tropical and subtropical countries like India, Sri Lanka and Bangladesh. *C. asiatica* contains several triterpene, saponins like asiaticoside, asiatic acid, sapogenins, madecassic acid, vellarin, adecassoside, glycosides and centelloside (Glasby, 1991).

Leaves contains high amount of triterpenoids (Zainol et. al., 2008). It possesses several important properties like antileprotic, antistress, antifeedent, antituberculosis activities, wound-healing properties (Chakraborty et al., 1996, Srivastava et al., 1997), antibacterial, and fungicidal activity (Oyedeji et al., 2015). It is used in the treatment of leprosy, wound, cancer, fever, allergies (Imandar et al., 1996), abscesses, asthma, catarrh, convulsions, dysentery, eczema, gonorrhea, hypertension, bronchitis, headache, jaundice, pleuritis, rheumatism, ulcers, spasms, tuberculosis, urethritis, etc (Hausen et al., 1993). Leaves of this

plant are rich in vitamin B and C. and minerals such as magnesium, potassium, calcium, phosphorus and aluminium (Herbert et al., 1994). It is also used as brain tonic and blood purifier (Jorge et al., 2005). *C. asiatica* contains various flavonoids which include quercetin and kaempferol, rutin and naringin (Zainol et al., 2003).

The annual requirement of *Centella asiatica* was around 12,700 tonnes of dry biomass valued at rupees 1.5 billion (Ahmad, 1993). National Medicinal Plants Board, Govt of India, has projected a combined demand of Centella and Brahmi of 6621.8 MT with an annual growth rate of 20.1% till year 2004-05. This requirement is rising sharply in view of the popularity of the mandukaparni-based drugs.

1.2 Micropropagation

Micropropagation is a technique to produce a large number of progeny plants under laboratory conditions. It plays an important role in the conservation and enhancement of valuable medicinal plants (Govarthanan et al., 2015). In this study, the purpose of using different accession was to choose the best accession for the phytocompounds production. An accession refers to the collection of plant material from a single species which is collected at one time from a specific geographical location. Each accession is an attempt to capture the diversity present in a given plant population. Accession number is given a unique identifier, and it is used to maintain associated information in the database. It exhibits significant variations in morphological parameters like growth of leaf, flowering, stomatal frequency, etc. Effect of different media on the shoot multiplication of this plant provides an opportunity to explore the role of media on the enhancement of *in vitro* culture of different accessions of *C. asiatica.*

1.3 Phytochemicals Screening

Phytochemicals are natural bioactive compounds found in plants. These work with nutrients and fiber to form an integral part of defence system against various diseases. The most important bioactive constituents of this plant are saponin (asiaticoside, medecassic acid, asiatic acid and madecassoside), terpenoid, steroid, flavanoids, and tannins (Jiang et al., 2005, Kuroda et al., 2001). These compounds are responsible for the wide therapeutic activity. However there is a significant difference in the active constituent content among samples from different locations. Qualitative analysis reveals the presence of different phytocompounds in this plant.

1.4 Plant sterols (Phytosterols)

Phytosterols are naturally occurring cholesterol equivalent in plants. They plays an important role in reducing the blood cholesterol levels and prevent circulatory and heart

diseases in human, this was first this was first demonstrated in humans in 1953 (Pollak, 1953). Most abundant phytosterols in plant are stigmasterols, campesterol, β-sitosterol, tocopherol and avenasterol. Stigmasterols has potential to cure Alzheimer's disease, similarly tocopherol used in foods as antioxidant, they can prevent oxidative stress. Triterpenic steroids reported to occur in this plant are stigmasterol and sitosterol.

1.5 Synthesis of Nanoparticles

In recent times nanotechnology is one of the fast growing fields. It mainly concern to the synthesis and designing of nanomaterials within the range of 1-100nm. Nanoparticles exhibit new properties based on specific characteristics such as, size, morphology and distribution, if compared to with the large particles of bulk material. Nanoparticles present a high surface to volume ration with decreasing size. Generally nanomaterials synthesized by using physical and chemical methods but the by-products from these methods are toxic in nature and the process is also costly. To overcome this problem there is a need of environment friendly methods of synthesis. At present there are two ways to synthesis nanomaterials i.e. either using microorganisms or using medicinal plant extract (Singhal et al., 2011). Several nanoparticles have been synthesized by this plant like silver, gold, copper oxide, etc. Using plant for nanoparticles synthesis can be an advantageous over other biological processes by eliminating the elaborate process of maintaining cell culture. Among several metal nanoparticles, silver nanoparticles have attained a special focus due to its stability, good conductivity and antimicrobial activity.

1.6 Objectives of the study

Due to the medicinal importance of *Centella asiatica* several research and industrial communities exploited this plant available in wild. This exploitation declined its population due to which International Union for Conservation of Nature and Natural resources (IUCN) listed it as threatened plant and endangered species (Singh et. al., 2010). This situation demands a quick action over its exploitation as well as its conservation by conventional and biotechnological techniques. Tissue culture techniques can play an important role in the clonal multiplication of elite clones of this plant as well as conservation of its germplasm.

Based on these objectives, this study focused on following major points:

1. Micropropagation of potential accessions of this plant.
2. Estimation of several phytocompounds such as terpenoid, steroid, saponin, flavonoid, tannin, glycosides, coumarins and alkaloids.
3. Estimation of stigmasterol using Gas Chromatography.
4. Evaluation of potential of different accessions for the synthesis of silver nanoparticles and characterization of synthesized silver nanoparticles using UV-VIS spectrophotometer, FT-IR, SEM.

REVIEW OF LITERATURE

2.1 Chemical Constituent

Centella asiatica contains various chemical constituents which includes triterpenoid saponins (asiaticoside, masecassoside, asiatic acid and medecassic acid) (Jiang et al., 2005, Kuroda et al., 2001), polyacetylenes (Schulte et al., 1973), flavones (Prum et al., 1983), phytosterols and lipid (Kapoor et al., 2003).

Bosse et al., 1979 reported the amount of four active compounds which are asiatic acid (29-30%), madecassic acid (29-30%), madecassoside (1%) and asiaticoside (40%). In addition they also contains total phenolics about 23000mg/100gm (Brinkhaus et al., 2000)

Saponins

Various saponins have been isolated from this plant which includes Asiaticoside, Madecassoside, Brahmoside, Centelloside, Thakuniside, etc (Srivastava et al., 1997)

- **Asiaticoside**

  Asiaticoside was first isolated from leaves of Centella asiatica more than fifty six years ago by Polonoski (1951). It is one of the principle terpenoids of this plant; it acts as antibacterial and fungicidal agents against pathogens and fungi (Hausen, 1993). It helps in collagen I synthesis in human (Bonte F, Dumas M, Chaudagne C and Maybeck A (1994), Influence of Asiatic acid, madecassic acid and asiaticoside on human collagen I synthesis, Planta Med, 60 (2), 133-135) [4]. It is clinically used as a wound healing agent in combination with madecassic and asiatic ascids (Hausen, 1993).

- **Asiatic acid**

  It is a pentacycluc triterpene coumpounds and the aglycone of asiaticoside. It exhibits bioactive efficacy (Park et al., 2007), and also known to control cell division in human melanoma, heptoma cells and cytotoxic activity on fibroblast cells (Coldern et al., 2003). It shows protective activity against UV induced photoaging, would healing, induces cell cycle arrest and anti-proliferative effects on human breast, gastric and urine cancer cells (Park et al., 2006).

- **Madecassoside**

  It is a glycoside that act as a strong anti-inflammatory agent (Si-Qi and Huei-Fang, 1981)

- **Madecassic acid**
  Its wound healing property has been attributed to its ability to stimulate collagen synthesis. (Si-Qi and Huei-Fang, 1981)

Triterpenic acids

Several pentacyclic triterpenic acids have been isolated and characterized from this plant. They occur either in free state or as aglycones of the naturally occurring saponins.e.g. asiatic acid, madasiatic acid, brahmic acid, isobrahmic acid, thankunic acid, betulic acid , centoic acid, centellic acid, 6b- Hydroxiasiatic acid & terminolic acid. (Srivastava et al., 1997)

Phytosterols constituents

The plant is reported to possess Stigmasterol, Campesterol, Beta-sitosterol and stigma sterol-b-D-glucopyranoside (Srivastava et al., 1997).

Nitrogen containing constituents

An alkaloid hydrocotylin, C22H33O8N, melting point 110-12ºC, has been isolated from this plant with 0.0016% yield. The plant also yields glycine, aspartic acid, glutamic acid, alanine and phenylalanine. (Srivastava et al., 1997)

Flavanoids

It was found that leaves contain 3- glucosylquercetin, 3-glucosylkaepferol and 7-glucosylkaempferol. (Srivastava et al., 1997)

2.2 Pharmacological Activities

Wound healing- Titrated extract of Centella asiatica which consist of mixture of three triterpenes (asiaticoside, asiatic acid and madecassic acid) stimulates glycosaminoglycan and collagen synthesis in rats (Maquart et al., 1999). Asiaticoside and asiatic acid were more active than madacassic acid in wound healing thus it appers to be an effective treatment of wound healing disturbances (Brinkhaus et al., 2000).

Central Nervous System- Mook-Jung et al., 1999 reported that asiaticoside derivatives reduce or inhibits H_2O_2 induced cell death and lower the intracellular free radical concentration and protect against the effects of beta amyloid neurotoxicity. *Centella asiatica* extract was found to increase brain GABA levels (Chatterjee et al., 1992).

Memory enhancing- Aqueous extract of *Centella asiatica* showed significant effect in memory enhancement. This positive effect is due to the presence of brahminoside,

brahmic acid and brahmoside in plant (Bradwejn et al., 2000 and Cesarone et al., 2001). In a study different doses of fresh leaf juice of *Centella asiatica* were given to seven day old neonatal rats for different time periods. These rats were then subjected to the spatial learning and passive avoidance tests along with the age matched normal and saline control rats. Results showed that there was an improvement in spatial learning performance and enhanced memory retention in neonatal rats treated with higher doses. These results indicates that fresh leaf juice of *Centella asiatica* enhance memory retention (Rao et al., 2005)

Antibacterial- Wei et al., 2008 reported that methanolic extract of *Centella asiatica* showed inhibition zone against *V. alginolyticus, V. vulnificus* and *Streptococcus sp.* Sankar et al., 2010 reported that methaqnolic extract of *Centella asiatica* showed antibacterial activity against three Vibrio species that are *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* but acetone, chloroform and hexane extract was not showed antibacterial activity against these species.

Antioxidant- In a study it was reported that asiaticoside significantly increased the levels of catalase, superoxide dismutase, glutathione peroxidise, ascorbic acid and vitamin E in excision type cutaneous wounds in rats. The level of antioxidant activity was highest during the initial stages of treatment (Shukla et al., 1999)

Hepatitis- It was found that titrated extract of Centella asiatica helps in the improvement in chronic hepatic disorders (Darnis et al., 1979).

Cardiovascular- Monteccchio et al., (1991) reported that three week treatment of triterpene fraction of *Centella asiatica* in clients with the post-phlebitic syndrome reduced the number of circulating endothelial cells as compared to the normal one. Cesarone et al., (1992) reported that in a clinical trial Centella asiatica extract found to be efficacious in the treatment of reducing ankle, venous insufficiency, foot swelling , edema, improving capillary filtration rate and microcirculatory parameters.

Neuroprotective effects- Ramanathan et al., (2007) reported that *Centella asiatica* extract protects monosodium glutamate induced neurodegeneration. Water extract of *Centella asiatica* showed neuroprotective efficacy against 3-nitropropionic acid induced oxidative stress in brain of prepubertal mice enhanced glutathione levels, antioxidant defences in brain regions(Shinomol and Muralidhara 2008; Shinomol et al., 2010).

Anti-diabetic- Chauhan et al., (2010) reported that triterpenic fraction of *Centella asiatica* is useful in diabetic microangiopathy by improving the micro-circulation and decreasing the capillary permeability. Also triterpenic fraction of *Centella asiatica* protect against the deterioration. Methanolic and ethanolic extracts had shown

significant protection and lowered blood glucose levels to normal glucose levels in tolerances test.

2.3 Side Effects and Toxicity

Alcoholic extracts of *Centella asiatica* have shown no toxicity at doses of 350 mg/kg when given to rats (Bhavan, 1992). Reported adverse effects include GI upset and nausea. Topical use of the extract has led to reports of rash (Eun et al., 1985). Three cases of jaundice with elevated liver enzymes were reported in Argentina following dosing of Centella. Patients had taken Centella (standardization and dose unknown) for 20-60 days, and recovered on discontinuation of the herb (Jorge et al., 2005).

2.4 Importance of tissue culture

In recent years, there has been an increased interest in *in-vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants (Sahoo and Chand, 1998 and Prakash et al., 1999). Therefore, it is important to develop an efficient micropropagation technique for *C. asiatica* to rapidly disseminate superior clones once they are identified. Tissue culture techniques can play an important role in the clonal propagation of elite clones and germplasm conservation of *C. asiatica*. Shoot regeneration from leaf derived callus (Patra et al., 1998; Banerjee et al., 1999) and stem (Patra et al., 1998) segments of *C. Asiatica* were reported.

All parts of the plant have been used as the explant source. Nodal segments of mature plants have been however used in most cases. Srivastava et al., 1999, reported use of nodal segments in *Ammi majus* L. , nodal explants of *B. monnieri* were propagated *in vitro* using liquid shake cultures (Tiwari et al., 2000); nodal explants were also used for *Eclipta alba* (Gawde and Paratkar, 2004); shoot tip, nodal and internodal segments were reported in *Phyllanthus amarus* (Ghanti et al., 2004).
Stem and leaf explants of green house grown plants were used for the regeneration from callus cultures of *Centella asiatica* (Patra et al., 1998). Banerjee et al., 1999, used 5-6 month old glass house grown plants of Centella for in vitro multiplication from leaf explants. Tiwari et al., 2000 has reported micropropagation of Centella using nodal segments. Explants were collected from natural stands where it grows luxuriously around the marshy fringes of a pond.

Full strength Murashige & Skoog medium (1962) has been used for most of the herbaceous species. Multiple shoots were obtained from shoot tips (1-2 cm) derived from field-grown plants of *Bacopa monnieri* in Murashige and Skoog medium supplemented with 0.5 mg /l BAP within 6 days of culture, whereas in the case of *Paederia foetida* and *Centella asiatica* multiple shoots were obtained from field-

grown plants in MS medium supplemented with 1.0 mg/l BAP within 7 days of culture (Singh et al., 1999). Supplementation of plant growth regulators such as 0.3 mg / l BAP and 0.2 mg / l kinetin have been found to show a good response of shoot proliferation in *Withania somnifera* with a regeneration of 85% (Kulkarni et al., 2000).

Banerjee et al., 1999 reported that in *Centella* initial sprouting required the presence of BAP (2 mg/l) and IBA (0.1 mg/l), however for multiple shoot induction a higher concentration of BAP (3.0 mg/l) and a lower concentration of NAA (0.05 mg/l) is required. Tiwari et al., 2000 reported that the bud break was dependent on BAP, the synergistic combination of 22.2mM BAP and 2.68mM NAA gave optimum result for the shoot number i.e. 4 to 5 shoots per node as well as for optimum frequency i.e. 91%.

Das et al., 2008 reported that MS media fortified with 4.0 mg/l BAP + 0.1 mg/l NAA showed average 10.2±0.38 shoots per explants.

2.5 Role of phytosterols

Phytosterols are naturally occurring bioactive compounds they have the ability to reduce intestinal resorption of cholesterol and are able to cross the blood brain barrier. They have similar structure to cholesterol due to this they may interfere with cholesterol dependent cellular process in brain. Phytosterols were subsequently marketed as a pharmaceutical under the name Cytellin as to treat the elevated cholesterol (Jones, 2007)

Chowdhary et al, 2014 reported the estimation of stigmasterol and it is useful substance in medication of Alzheimer's disease. The stigmasterol obtained from best source is 0.0582 %.

Woyengo et al., 2009 reported that phytosterols has potential to inhibit stomach, lunch, breast and ovarian cancers.

Phytosterols also has the potential to reduce the elevated triglyceride levels which is a risk factor for cardio vascular diseases (Malloy et al., 2001). It was found that the level of triglycerides reduced by 14% by supplementing 1.6 g/day of plant sterols in a fermented milk beverage for six weeks (Plana et al., 2008). Proposed mechanism behind the triglyceride lowering effect of phytosterols is the reduction in triglyceride rich very low density lipoprotein particles produced by liver (Plat et al., 2009).

2.6 Synthesis of silver nanoparticles

Biosynthesis of nanoparticles is an interesting area for the development of new methods of nanomedicine. These particles can be prepared easily by different methods but biological approach is one of the most effective, less time consuming

and ecofriendly. Several nanoparticles have been synthesized by this plant like silver, gold, copper oxide, etc. Among several metal nanoparticles, silver nanoparticles have attained a special focus. Silver is a noble metal, it has potential applications in medicine due to its unique properties like chemically stable, good conductivity, catalytic stability and antimicrobial activity, it increases the oral bioavailability and overcome the poorly water soluble herbal medicines (Gurunathan et al. 2009 and Chen et al. 2005).

Logeswari et al., 2013 reported that aqueous extract was utilized for the synthesis of silver nanoparticles and the size of the silver nanoparticles synthesized by *Centella asiatica* was 33nm and irregular in shape.

Rout et al., 2013 also reported that aqueous leaf extract was utilized for the synthesis of silver nanoparticles and the size was 30-50nm and spherical and cubic in shape.

Palaniselvam et al., 2015 reported that leave extract of *Centella asiatica* used for the silver nanoparticles synthesis and peak obtained at 430nm and the size of synthesized nanoparticles were 50-60nm.

MATERIALS AND MEHODS

3.1 Plant material

Five accessions i.e. 281374, 383913, 342109, 347492, 331514 of *Centella asiatica* belonging to different regions of India were collected from NBPGR, New Delhi and were maintained at the plant tissue culture laboratory of Department of Biotechnology, Delhi Technological University.

3.2 Media Preparation and Inoculation

Table 1:- Composition of MS, B5 and Nitsch media

MS Medium		**B5 Medium**		**Nitsch Medium**	
Components	**mg/L**	**Components**	**mg/L**	**Components**	**mg/L**
Macronutrients		**Macronutrients**		**Macronutrients**	
Ammonium nitrate	1,650.00	Potassium nitrate	2500	Potassium nitrate	950
Potassium nitrate	1,900.00	Ammonium Sulphate	134	Ammonium nitrate	720
Calcium chloride (anhydrous)	332.2	Calcium chloride.$2H_2O$	150	Magnesium sulphate anhydrous	90.34
Magnesium sulfate (anhydrous)	180.7	Magnesium sulphate	122.1	Potassium phosphate monobasic	68
Potassium phosphate monobasic	170	Sodium phosphate monobasic	130.4	**Micronutrients**	
Micronutrients		**Micronutrients**		Manganese sulphate.H_2O	18.94
Manganese sulfate monohydrate	16.9	Manganese sulphate. H_2O	10	Boric acid	10
Ferrous sulfate heptahydrate	27.8	Boric acid	3	Molybdic acid (sodium salt).$2H_2O$	0.25
Zinc sulfate heptahydrate	8.6	Potassium iodide	0.75	Zinc sulphate.$7H_2O$	10
Boric acid	6.2	Molybdic acid (sodium salt).$2H_2O$	0.25	Copper sulphate.$5H_2O$	0.025
Potassium iodide	0.83	Zinc sulphate.$7H_2O$	2	Ferrous sulphate.$7H_2O$	27.85
Sodium molybdate dehydrate	0.25	Copper sulphate.$5H_2O$	0.025	EDTA disodium salt.$2H_2O$	37.25
Cobalt chloride hexahydrate	0.025	Cobalt chloride.$6H_2O$	0.025	**Vitamins**	
Cupric sulfate pentahydrate	0.025	Ferrous sulphate.$7H_2O$	27.8	myo – Inositol	100
Disodium EDTA dehydrate	37.26	EDTA disodium salt.$2H_2O$	37.3	Thiamine hydrochloride	0.5
Vitamins		**Vitamins**		Pyridoxine hydrochloride	0.5

myo-Inositol	100	myo – Inositol	100	Nicotinic acid	5
Nicotinic acid	0.5	Thiamine hydrochloride	10	Folic acid	0.5
Pyridoxine hydrochloride	0.5	Pyridoxine hydrochloride	1	Biotin	0.05
Thiamine hydrochloride	0.1	Nicotinic acid	1	Glycine	2
Sugar		**Sugar**		**Sugar**	
Sucrose	30000	Sucrose	20000	Sucrose	20000

Murashige and Skoog (MS) medium preparation

MS medium was prepared by mixing all the components in 600ml of distilled water in a clean 1000ml beaker. Then pH of the medium was adjusted to 5.8 using 1N HCl/NaOH. Then finally volume was made up to 1000ml with distilled water. Then solidifying agent 0.8% agar was used added. Medium sterilization was done by autoclaving at 15 lbs or 121ºC for 15 minutes. After autoclaving medium was cooled to 45ºC before adding the filter sterilized heat labile supplements (hormones). Then pouring of the culture tube and flasks were done.

Gamborg B5 medium with $CaCl_2$, Vitamins and Sucrose and without Agar preparation

Weighed 23.17 grams of B5 media then suspended in 600ml of distilled water and gently stirring the solution till the powder dissolved completely. Then pH of the medium was adjusted to 5.8 using 1N HCl/NaOH. Then finally volume was made up to 1000ml with distilled water. Medium sterilization was done by autoclaving at 15 lbs or 121ºC for 15 minutes. After autoclaving medium was cooled to 45ºC before adding the filter sterilized heat labile supplements (hormones). Then pouring of the culture tube and flasks were done.

Nitsch Medium with Vitamins and sucrose and without $CaCl_2$ and Agar preparation

Weighed 22.02 grams of Nitsch media then suspended in 600ml of distilled water and gently stirring the solution till the powder dissolved completely. Then pH of the medium was adjusted to 5.8 using 1N HCl/NaOH. Then finally volume was made up to 1000ml with distilled water. Medium sterilization was done by autoclaving at 15 lbs or 121ºC for 15 minutes. After autoclaving medium was cooled to 45ºC before adding the filter sterilized heat labile supplements (hormones). Then pouring of the culture tube and flasks were done.

Inoculation and incubation of explants:

The laminar air flow chamber was properly surface sterilized with alcohol and UV lights for 30 minutes. The explants were trimmed to a suitable size of about 2 cm by

keeping it in sterile Petri-dishes. Then a cut was given on both basal and top portion to remove the undesirable or dead portion. The forceps were rinsed in 70 % ethanol and were flamed and then kept for some time to get cool. Then the lid from the culture tube was removed and mouth of it was flamed to avoid further chances of contamination. Each explant was then aseptically inoculated on the MS, B5 and Nitsch medium containing 1 mg/l BAP in an erect position with long forceps without touching the rim of the culture tube. Then the lid was finally replaced carefully and sealed with the parafilm. The inoculated culture tubes were incubated in the culture room at 25±2°C, with a light intensity of 2500 lux and a photoperiod of 16 hour light, 8 hour dark and 65% humidity.

Statistical analysis:

Visual observations were recorded in terms of number of shoots per explant and the length of each shoot. Experiments were done in triplicates and means of each experiment was carried out, further ANOVA was done to detect the significant differences ($p<0.05$).

3.3 Phytochemicals Analysis

Ethanolic extract preparation:-

Fresh in-vitro grown plant material was ground with mechanical grinder. One gram of plant material was then macerated in 10ml of absolute ethanol for 48 hours and covered properly with aluminium foil and labelled. After 48 hours of extraction, each extract was filtered through the Whatman's filter paper. The filtrate was stored at 4 °C temperature.

Methanolic extract preparation:-

Fresh in-vitro grown plant material was ground with mechanical grinder. One gram of plant material was then macerated in 10ml of methanol for 48 hours and covered properly with aluminium foil and labelled. After 48 hours of extraction, each extract was filtered through the Whatman's filter paper. The filtrate was stored at 4 °C temperature.

Aqueous extract preparation:-

About 100mg of powdered plant materials was mixed with 10 ml of miliQ water and boiled for 20 minutes for the formation of plant extract. Obtained plant extract was filtered through Whatman No.-1 filter paper. Then transferred into autoclaved vials and stored at 4°C for further analysis.

- ***Terpenoid Test (*Salkowski Test*):*** - 100 µl of plant extract was mixed with 2ml of chloroform and 3ml of concentrated sulphuric acid to form a layer. A reddish brown color at interface is formed to show positive result of the presence of terpenoid and triterpenoids.

- ***Steroid test (*Salkowski Test*):*** - 100 µl of plant extract was dissolved in 2ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown color at the interface is an indicative of the presence of steroidal ring.

- ***Saponin test (*Foam test*): -*** Add 100mg of powdered plant material to 10 ml of distilled water. Heat the mixture and observe for persistent froth. Formation of froth indicates the presence of saponins.

- ***Flavonoid test (*NaOH test*)*:** -100 µl of plant extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavanoids.

- ***Tannin test (*$FeCl_3$ test*):*** - 100 µl of plant extract was treated with few drops of freshly prepared 6% $FeCl_3$. Formation of green color indicates the presence of tannin.

- ***Glycosides test (*Fehling's Test*):*** - Equal volume of Fehling A and Fehling B reagents were mixed together and 2ml of it was added to the 100 µl of plant extract and genteelly boiled. A brick red precipitate appeared at the bottom of the test tube indicates the presence of reducing sugars.

- ***Coumarins:*** - 200 µl of plant extract was mixed with few drops of 10% NaOH. Presence of yellow coloration indicates the presence of coumarins.

- ***Alkaloids (*Mayer's Reagent*):*** - One milliliter ethanolic filtrate was taken in test tube and 2 ml of 2N HCL was added to it then solution was shaken vigorously to mix and kept for 5 minutes. Few drops of Mayer's reagent ($HgCl_2$+ KI in water) was added to it. Formation of creamy color precipitation indicates the presence of alkaloids.

3.4 Phytosterols sample preparation

Solutions

I. *2% KOH in ethanol* (Fresh solution of KOH in every extraction).
II. *Internal standard solution*: - prepared fresh internal standard solution in every extraction 3:1 hexane and ethanol solution kept in 4 degree temperature.
III. *Silylating agent*:-prepared fresh solution of silylating agent for every extraction

Sample Preparation

Plant material grinded in pestle and mortal uniformly and then weighted 200mg crushed material. 200µl of internal standard solution then added vertex shortly and 2ml of 2% KOH was added. Closed the cap and then the sample vertexes for 1 min, incubated the tubes for 15min at 80 °C temperature. After incubation again vertex to allow the sample to cool at room temperature for 15 min. 1ml hexane and 1.5 ml water were added and the cap of tubes were closed and then vertex for 30 sec. After this tubes were centrifuged for 4 min at 4000rpm. Upper hexane layer was transfer to polypropylene tube and left to evaporate on a hot plate at 50 °C overnight. 100µl hexane was added to solubilise the dried pellet and dissolved pellet in hexane was transfer to GC vial and then 50 µl silylating agent and incubated for 20-30 minutes at 70-75 °C.

Standard preparation:-

Weighed 5mg of standard stigmasterol (Sigma) and dissolved it in 1ml of internal standard solution. 100µl was taken and 100µl of internal standard added to make concentration of 0.025µg/µl solution. Then 100 µl of silylating agent added and incubated for 20-30 minutes at 70-75 °C.

Gas Chromatography (GC)

GC is the most common method for the phytosterol content and composition analysis (Heupel, 1989). Sample containing at least 50-150µg of mixture of phytosterols can easily analyzed by GC (Winkler et al., 2007). Addition of Internal standard is important for phytosterol analysis to obtain accurate qualitative and quantitative results by the GC. Since retention times often shift from run to run, the main function of internal standard is to aid in identification of phytosterols in unknown samples, based on the relative retention time of phytosterol compared to the internal standard. In addition when using split injection, which is typically in case of phytosterol analysis the amount of sample entering the column, will vary slightly from injection to injection so internal standard is used to compensate for this variation. Internal standard is added to the sample prior to alkaline hydrolysis so that it undergoes the same extraction condition as the sample phytosterols.

GC conditions

An Agilent GC (7890B GC system) equipped with flame ionization detector (FID, H2 flow=40 ml/min, air flow=450 ml/min) was used in this study. The analytical column was DB-5 wax capillary column. Temperatures of injection port and detector were 260 °C and 320 °C. Sample injection volume was 1µl and direct injection mode was used.

3.5 Synthesis of silver nanoparticles

Preparation of aqueous plant extract

In-vitro grown plant materials were dried for seven days. Powdered plant materials were used for the extract preparation. About 100mg of powder was mixed with 10 ml of miliQ water and boiled for 20 minutes for the formation of plant extract. Obtained plant extract was filtered through Whatman No.-1 filter paper. Then transferred into autoclaved vials and stored at 4°C for further analysis.

Preparation of silver nitrate solution

Silver nitrate ($AgNO_3$) was collected from Fisher Scientific. Molecular weight of $AgNO_3$ is 169.87 g/mol. For the preparation of 1mM $AgNO_3$ solution, 16.987 mg of $AgNO_3$ was added to 100ml of mili-Q water and mixed thoroughly. Solution was stored in flask covered with aluminium foil.

Synthesis of silver nanoparticles

For the synthesis of silver nanoparticles, 90 ml of 1mM $AgNO_3$ solution was taken in a autoclaved flask and 10 ml of aqueous plant extract was added to it. Solution was mixed well and kept in incubator shaker at 37°C, 150 rpm for 48 hours. As a result, formation of dark yellowish brown colour indicates the formation of silver nanoparticles.

Characterization of silver nanoparticles

- **UV-Vis spectroscopy**: - Silver nanoparticles exhibits dark yellowish brown color in aqueous solution due to the Surface Plasmon Resonance phenomenon. Thus silver nanoparticles formed were separated from the residual and characterized by UV-VIS absorption spectroscopy. Bio-reduction of pure silver ion was monitored. Deionised water was used as a blank. The wavelength range for the silver nanoparticles detection was 300 to 700nm and the presence of reduced silver ions was highlighted by a peak of absorption in the range of 350 to 500nm.

- **Scanning Electron Microscope (SEM) Analysis: -** Scanning Electron Microscope analysis was done using Hitachi 3700N SEM machine. Thin films of samples were prepared on a carbon coated copper grid by dropping very small amount of sample on the grid, extra solution was removed using a blotting paper and then film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 minutes.

- **Fourier Transmission Infrared Spectroscopy(FT-IR) Analysis: -** FT-IR was used to identify the possible functional groups responsible for the reduction of Ag ions and capping of the bio reduced silver nanoparticles synthesized. In order to determine the functional groups and their possible involvement in the synthesis of silver

nanoparticles, FT-IR analysis was carried out. Liquid samples were used for the analysis. Samples were analyzed using Thermo Scientific Nicolet 380 FT-IR spectrophotometer. Spectrum was recorded in FT-IR in the range of 4000-500 cm^{-1} at a resolution of 4 cm^{-1}. Peaks obtained were plotted as % Transmittance in Y axis and wave number (cm^{-1}) in X axis.

RESULTS AND DISCUSSION

4.1 Effect of different media on shoot multiplication

This study was an attempt to correlate the effect of different media on the shoot multiplication of *Centella asiatica* accessions. To initiate the study, nodal explant were taken from *in-vitro* grown plants. Shoot multiplication of *Centella asiatica* nodal explants cultured on MS, Gamborg's B5 and Nitsch media supplemented with 1.0 mg/L. After two weeks of incubation explants showed the growth response in different media.

After eight weeks of incubation period it was found that MS media showed highest shoot multiplication as compared to Gamborg's B5 and Nitsch media in all the five accessions. In case of MS media, the highest shoot multiplication was observed as follow, 19.6±0.57 in accession no.-342109, 16.6±0.50 in accession no.-347492, 18.3±0.57in accession no.-331514, 16.3±0.57 in accession no.-383913 and 17.3±0.57in accession no.-281374. In case of B5 media highest shoot multiplication was observed as follow 16.3±0.57 in accession no.-342109, 12.3±0.44 in accession no.-347492, 15.6±0.50 in accession no.-331514 and 13.6±0.50 in accession no.-383913 and 13.6±0.44 in accession no.-281374. Whereas Nitsch media showed lowest shoot multiplication in all the five accessions. Details of this study mentioned in Table-2.

Similar results were reported where BAP alone showed the good shoot induction. In general, BAP is the most efficient growth hormone for the shoot proliferation (George et al., 2004). It mimics as an inhibitor agent and function against apical dominance of shoot induction and shoot bud formation (Wang et al., 1991). Tiwari et al., 2000 reported that MS media supplemented with 22.2µM BA+2.68µM NAA showed highest growth where as Karthikeyan et al., 2009 reported that maximum shoot multiplication was observed at 2mg/l BAP.

For statistical significance all the data was analyzed using one way ANOVA ($P<0.05$) and represented as average standard errors. Data showed that $p < 0.05$ for all the treatments and so we reject the null hypothesis, and conclude that there are significant differences.

Table 2:- Effect of different media on number and length of shoot of five different accessions of *Centella asiatica* after four, six and eight weeks of incubation.. Values are expressed as mean ± Standard Error (M ± SE). MS: Murashige and Skoog medium; B5: Gamborg's B5 medium; BAP: 6-Benzyl amino purine.

Accession No.	Media with standard hormones					
	MS + 1 mg/l BAP		B5 + 1 mg/l BAP		Nitsch + 1 mg/l BAP	
	Number of Shoot (M±SE)	Length of Shoot (M±SE)	Number of Shoot (M±SE)	Length of Shoot (M±SE)	Number of Shoot (M±SE)	Length of Shoot (M±SE)
After four weeks of incubation						
342109	10±0.57	2.6±0.53	10±0.57	2.3±0.32	5±0.44	2±0.53
347492	10±0.50	2.3±0.32	7±0.44	1.4±0.32	6±0.57	1.4±0.55
331514	15±0.57	3.4±0.55	10±0.50	1.4±0.55	6±0.57	1.8±0.55
383913	8±0.57	1.9±0.32	6±0.50	1.2±0.53	6±0.44	1.3±0.53
281374	13±0.44	1.5±0.55	10±0.44	2.2±0.32	5±0.57	1.1±0.53
After six weeks of incubation						
342109	14±0.57	3.5±0.53	12±0.57	2.1±0.32	6±0.44	1.9±0.53
347492	13±0.50	2.4±0.32	13±0.44	2.6±0.32	8±0.57	1.2±0.55
331514	18±0.57	4.4±0.55	15±0.50	2.3±0.55	7±0.57	2.2±0.55
383913	14±0.57	3.4±0.32	8±0.57	1.4±0.53	8±0.44	1.4±0.53
281374	16±0.44	5.0±0.55	12±0.44	2.5±0.32	7±0.57	2.6±0.53
After eight weeks of incubation						
342109	19.6±0.57	4.9±0.53	16.3±0.57	2.5±0.32	8.6±0.44	2.0±0.53
347492	16.6±0.50	2.8±0.32	12.3±0.44	2.7±0.32	7.6±0.57	1.5±0.55
331514	18.3±0.57	5.1±0.55	15.6±0.50	2.8±0.55	8.3±0.57	2.3±0.55
383913	16.3±0.57	3.5±0.32	13.6±0.50	2.2±0.53	7.6±0.44	2.0±0.53
281374	17.3±0.57	5.6±0.55	13.6±0.44	2.7±0.32	8.6±0.57	2.9±0.32

ANNOVA Analysis

MS Media

Anova: Single Factor (MS)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	3	55	19.67	0.33
Row 2	3	50	16.67	0.33
Row 3	3	59	18.33	0.33
Row 4	3	49	16.33	0.33
Row 5	3	52	17.33	0.33

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*
Between Groups	22	4	5.5	16.5	0.000211
Within Groups	3.33	10	0.33		
Total	25.33	14			

B5 Media

Anova: Single Factor(B5)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	3	49	16.33	0.33
Row 2	3	41	12.33	1.33
Row 3	3	47	15.67	0.33
Row 4	3	41	13.67	0.33
Row 5	3	37	13.67	0.33

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	32	4	8	15	0.000314
Within Groups	5.33	10	0.53		
Total	37.33333	14			

Nitsch Media

Anova: Single Factor (Nitsch)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	3	22	8.67	0.33
Row 2	3	26	7.33	0.33
Row 3	3	23	7.67	0.33
Row 4	3	22	7.33	0.33
Row 5	3	26	8.67	0.33

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*
Between Groups	5.6	4	1.4	4.2	0.029904
Within Groups	3.33	10	0.33		
Total	8.93	14			

Where,

SS = sum of squares

Df = Degree of freedom

MS = mean square = sum of squares / degree of freedom

F = Mean square (between group) / Mean square (within group)

P- Value is the probability of obtaining that *F* ratio by chance alone.

F tables also usually include the mean squares, which indicates the amount of variance (sums of squares) for that "effect" divided by the degrees of freedom for that "effect."

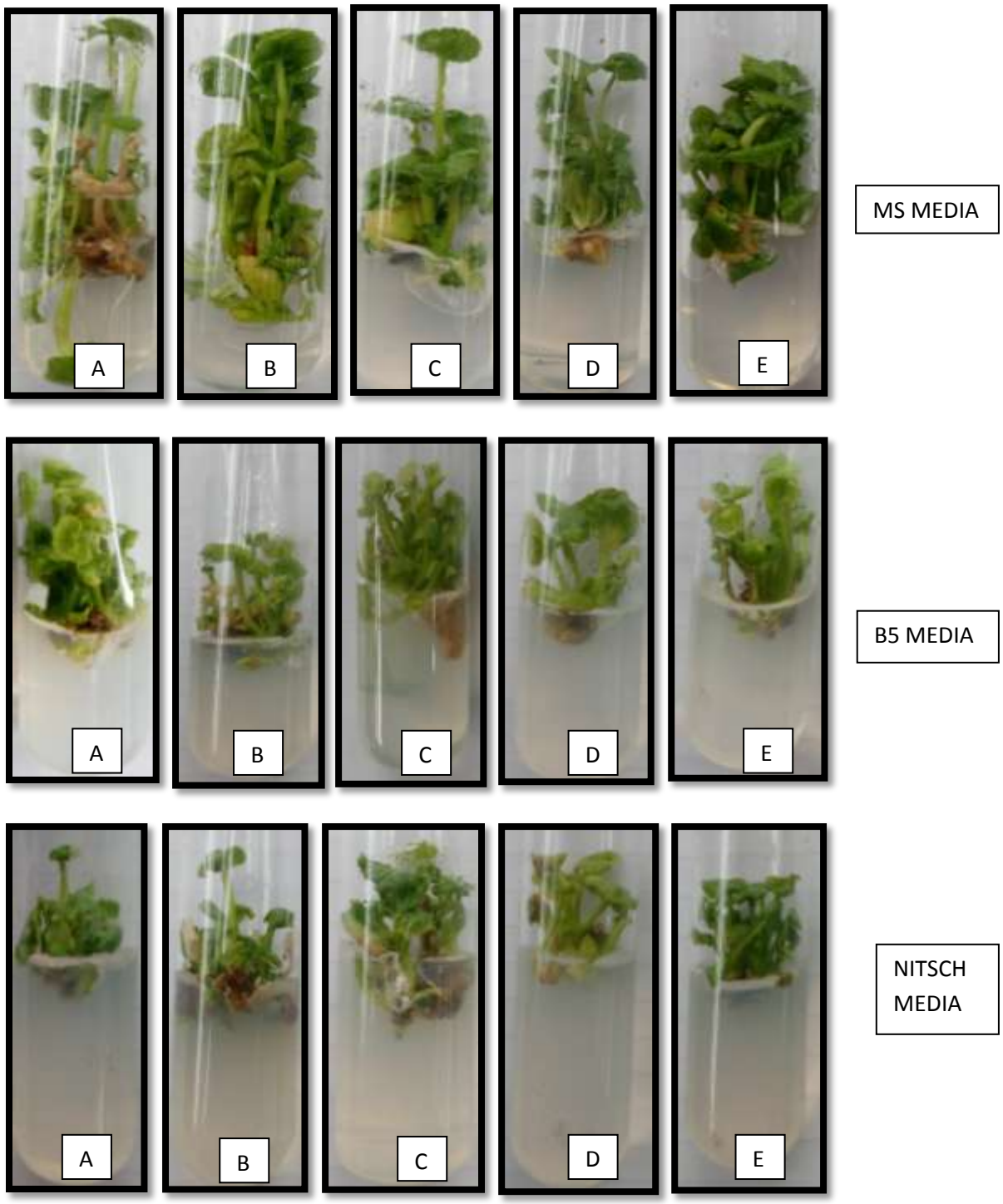


Fig 1:-Effect of different media with 1mg/l BAP on the growth in A) Accession no. - 342109, B) Accession no.- 281374, C) Accession no.- 331514, D) Accession no.- 383913, E) Accession no.- 347492

4.2 Qualitative analysis of phytochemicals

Three different extracts i.e. ethanolic, methanolic and aqueous extract of *Centella asiatica* (whole plant) were used in qualitative analysis of phytochemicals. This study revealed the presence of terpenoid, saponin, steroid, flavonoid, tannin and coumarins. In case of ethanolic and methanolic extract it showed positive results for terpenoid, steroid, flavonoid and coumarins whereas in case of aqueous extract it showed positive results for terpenoid, steroid, flavonoid, coumarins and tannin. These photochemical compounds are major compounds which impart medicinal value of this plant. However all the three extracts showed negative result for glycoside and alkaloids test. Sanjay et al., 2013 used the ethanolic extract for the phytochemicals test where as Singh et al., 2012 used methanolic extract for the phytochemicals test.

Table 3:- Phytochemicals analysis of different accessions of *Centella asiatica* using ethanolic, methanolic and aqueous extract

Extract	Accession	Phytoconstituents							
		Terpenoid	Steroid	Saponin	Flavonoid	Tannin	Glycosides	Coumarins	Alkaloids
ETHANOLIC	342109	+	+	+	+	-	-	+	-
	347492	+	+	+	+	-	-	+	-
	331514	+	+	+	+	-	-	+	-
	383913	+	+	+	+	-	-	+	-
	281374	+	+	+	+	-	-	+	-
METHANOLIC	342109	+	+	+	+	-	-	+	-
	347492	+	+	+	+	-	-	+	-
	331514	+	+	+	+	-	-	+	-
	383913	+	+	+	+	-	-	+	-
	281374	+	+	+	+	-	-	+	-
AQUEOUS	342109	+	+	+	+	+	-	+	-
	347492	+	+	+	+	+	-	+	-
	331514	+	+	+	+	+	-	+	-
	383913	+	+	+	+	+	-	+	-
	281374	+	+	+	+	+	-	+	-

+= Positive -= Negative

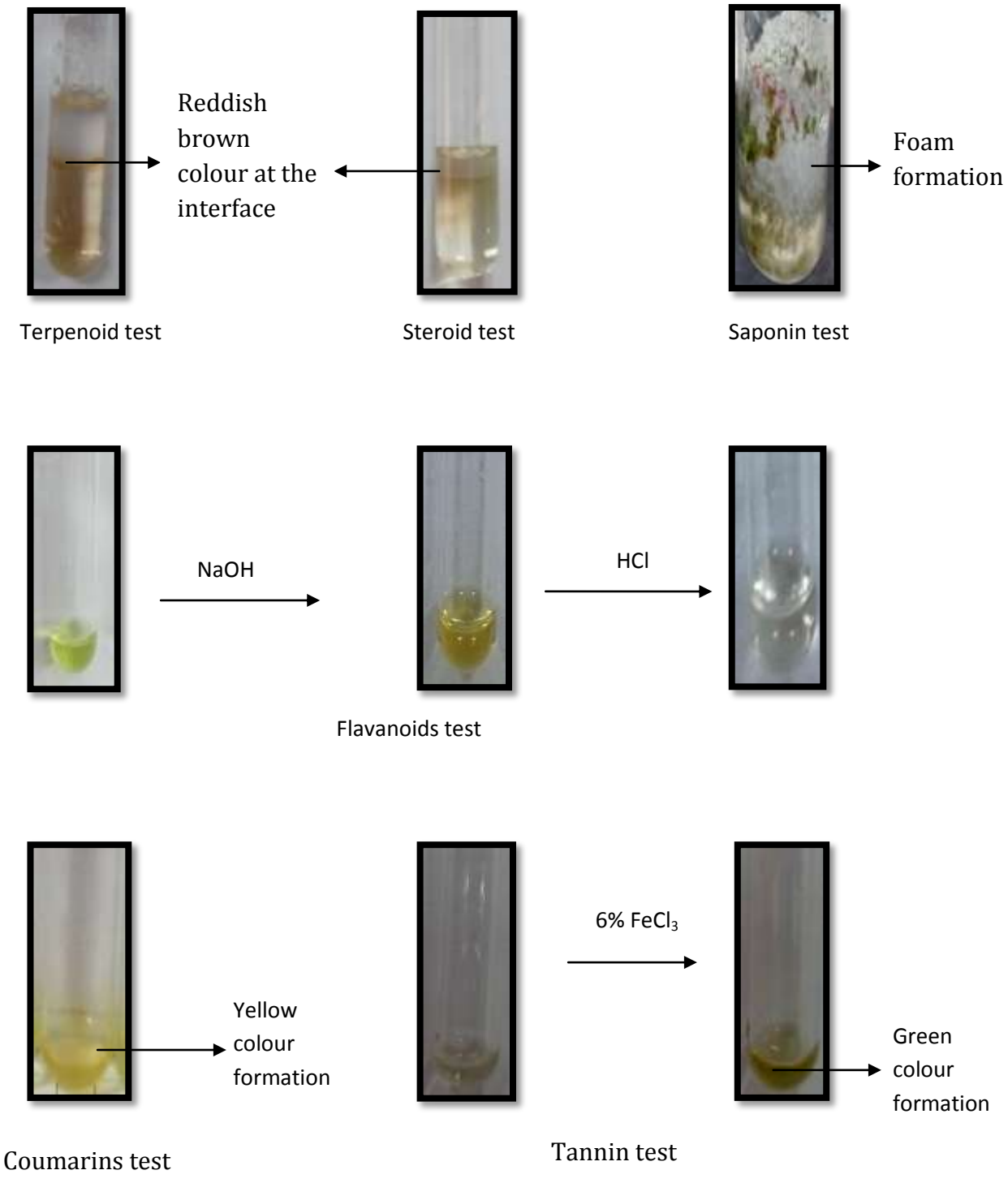


Fig-2:- Phytochemicals test result of terpenoid, steroid, saponin, flavonoid, coumarins and tannin.

4.3 Estimation of phytosterols

Estimation of phytosterol was carried out with the help of Gas Chromatography. Phytosterol analysis revel that the plant contains tocoherols, β-sitosterols and stigmasterols. Variation in the stigmasterol content was determined by using standard stigmasterol (Sigma). Amount of stigmasterol variation is due to the number of factors i.e genetic variations, location etc.

Experimental results showed that out of the five different accessions, accession number 342109 contains highest amount of stigmasterol i.e. 4.55±0.70 µg/200mg of leaves followed by accession number 281374 i.e 1.97±0.72 µg/200mg of leaves followed by accession number 331514 i.e 1.53±0.51 µg/200mg of leaves followed by accession number 383913 i.e 1.31±0.61 µg/200mg of leaves followed by accession number 347492 i.e 0.35±0.75µg/200mg of leaves (Table 4). Time at 8.14-8.171 represents the presence of tocopherol; time at 9.886-9.907 represents the presence of stigmasterol and time at 10.556-10.581 represents the presence of β-sitosterol.

Table 4:- Stigmasterol content of *in- vitro* grown *Centella asiatica* accessions leaves ± Standard deviation of three replicates

Accession no.	Stigmasterol content (µg/200mg of leaves)
342109	4.55±0.70
281374	1.97±0.72
331514	1.53±0.51
383913	1.31±0.61
347492	0.35±0.75

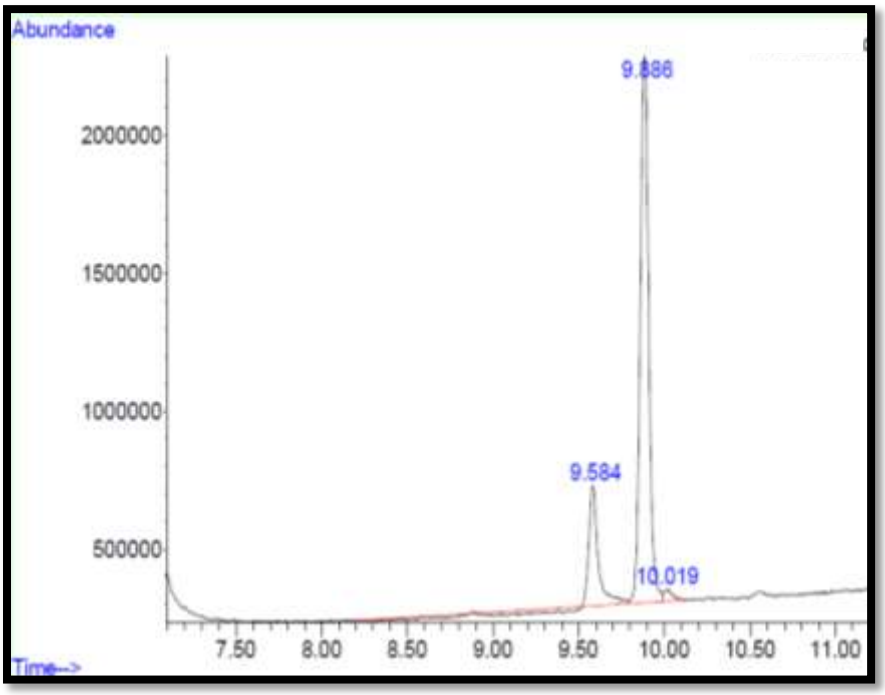


Fig 3:- Chromatogram of Stigmasterol standard

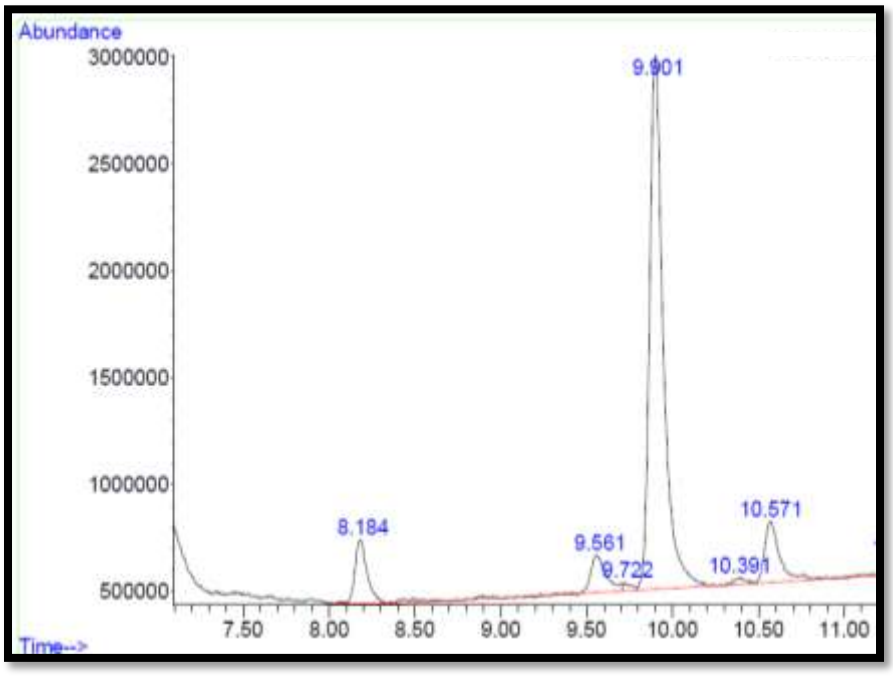


Fig 4:- Chromatogram of accession no.-342109

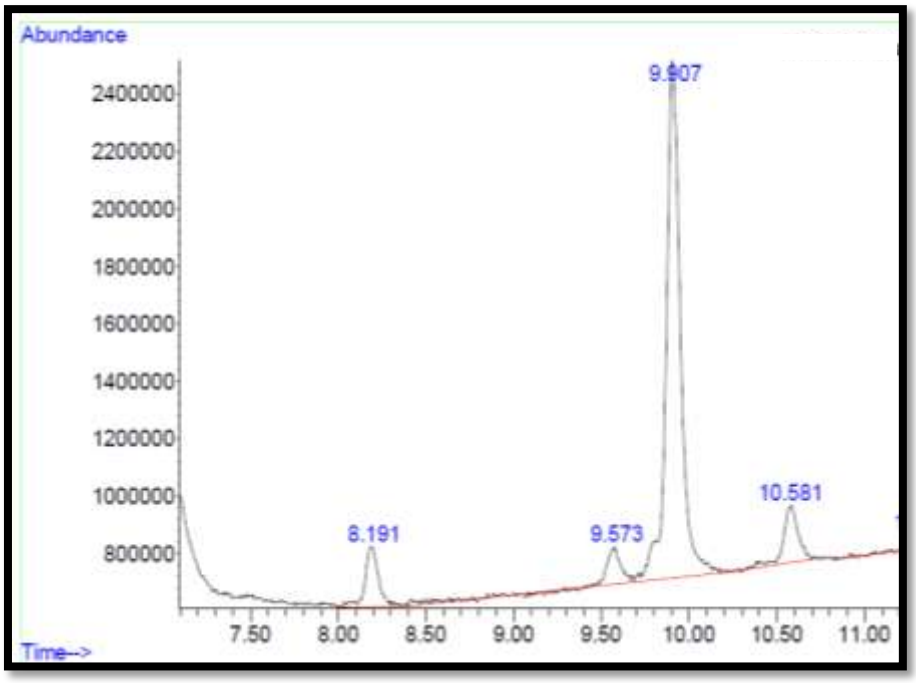


Fig 5:- Chromatogram of accession no.- 281374

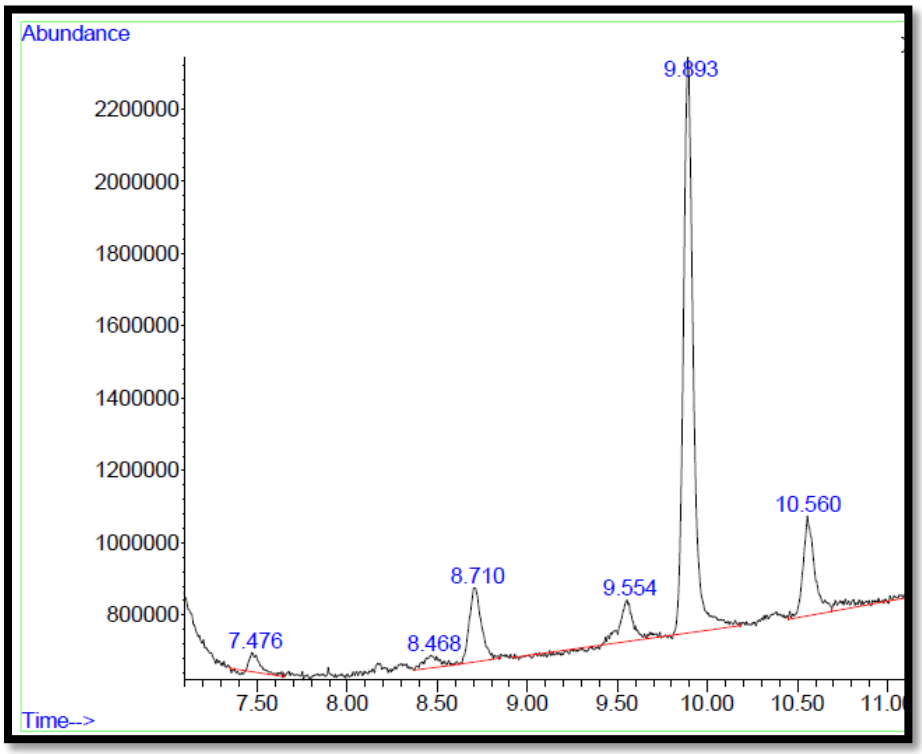


Fig 6:- Chromatogram of accession no.- 331514

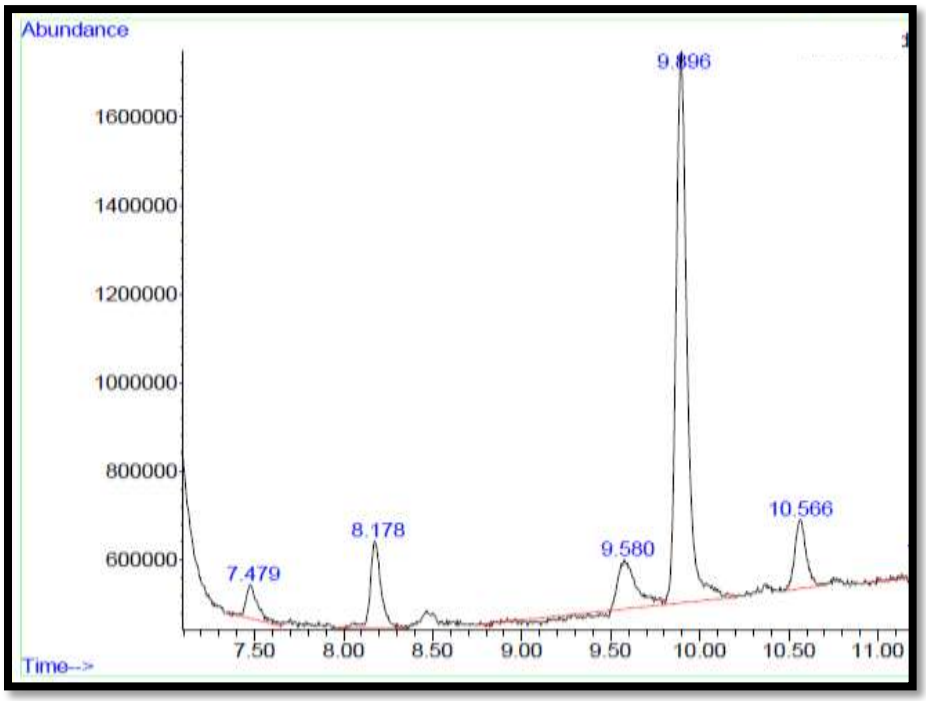


Fig 7:- Chromatogram of accession no.- 383913

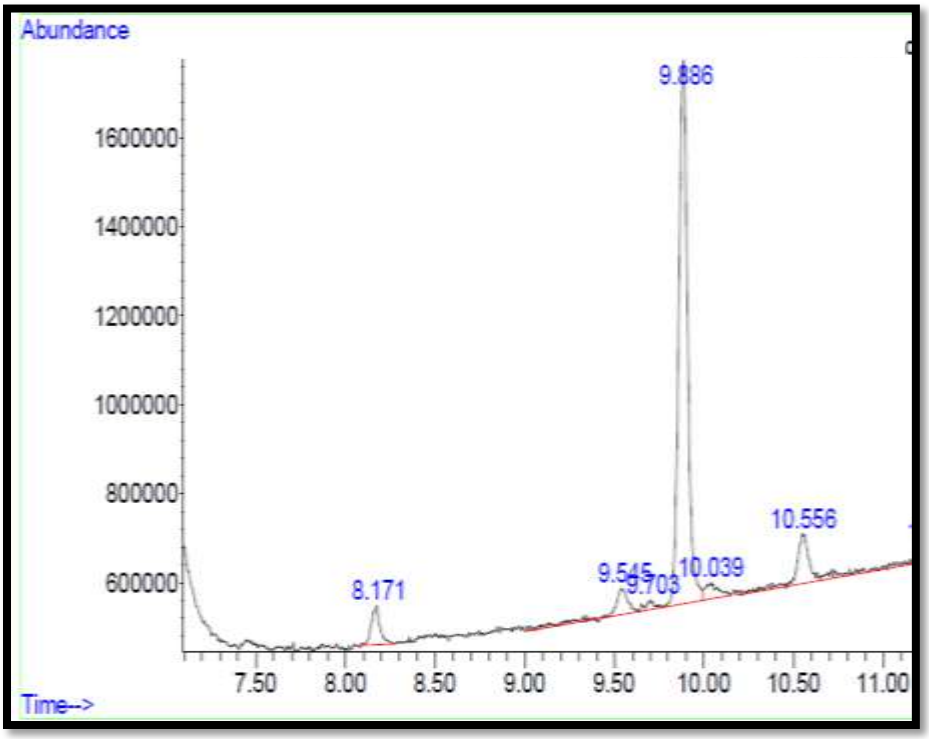


Fig 8:- Chromatogram of accession no.- 347492

4.4 Silver nanoparticles synthesis

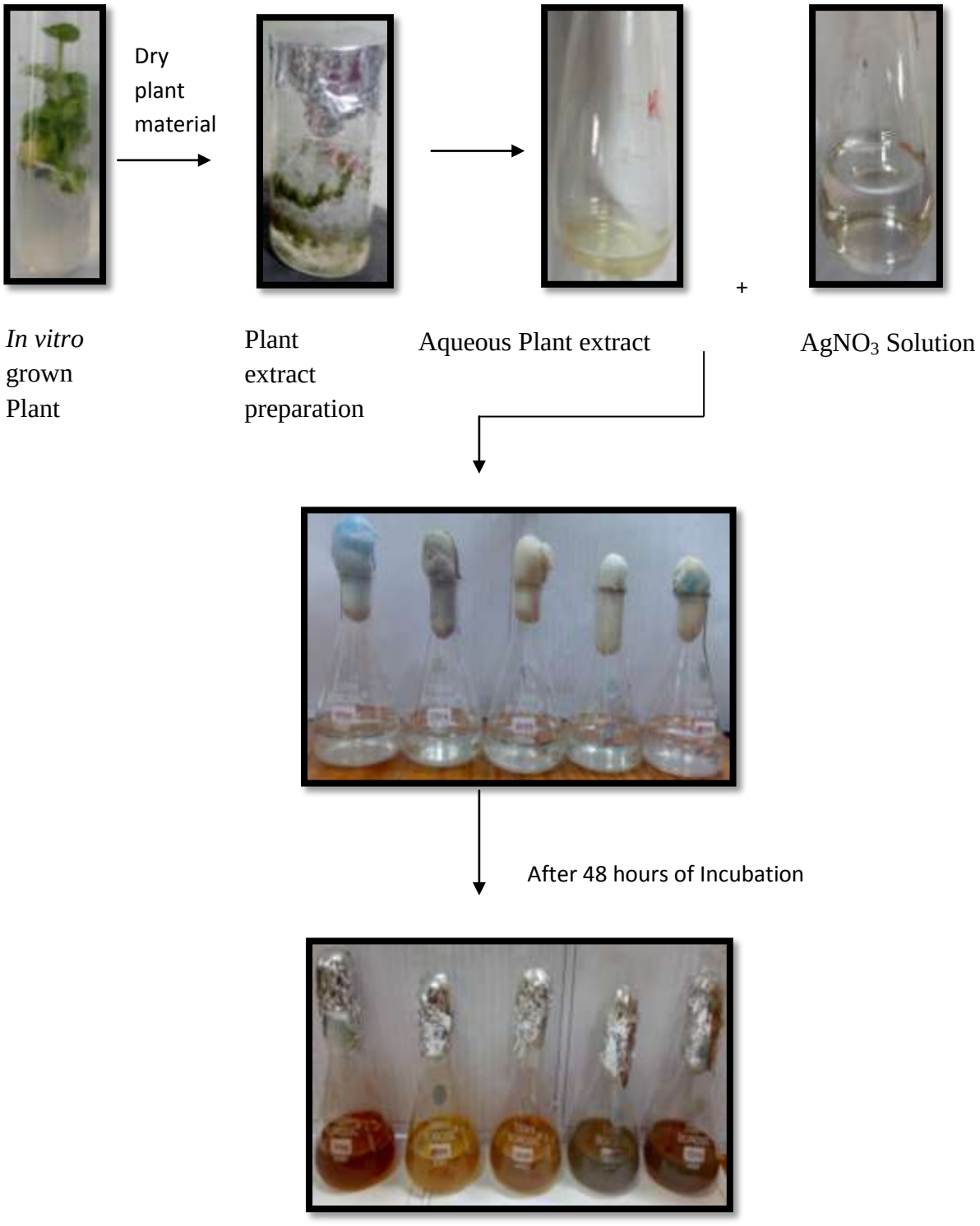


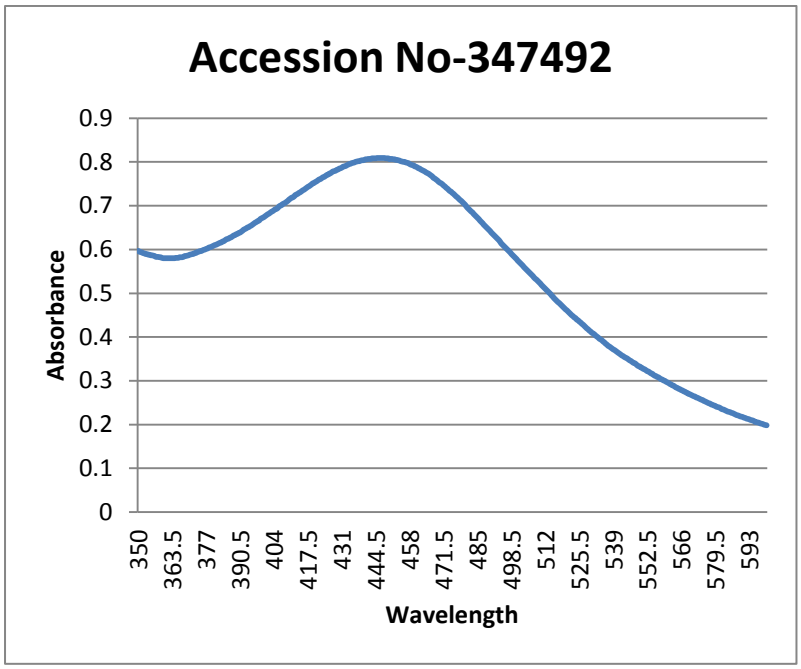
Fig 9:- Synthesis of silver nanoparticles using plant extract

4.4.1 UV-Vis spectroscopy

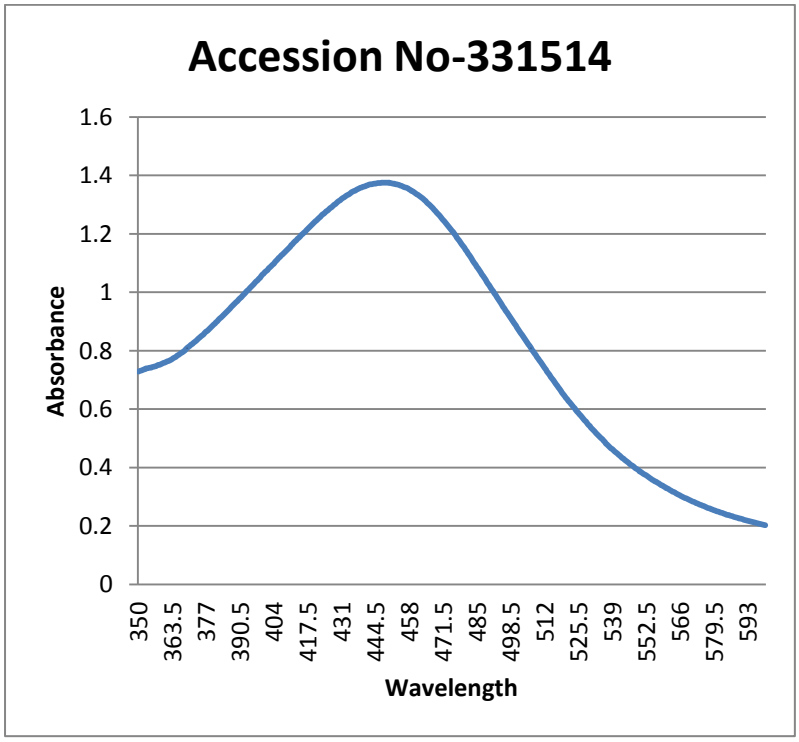
Reduction of silver ions into silver nanoparticles during exposure to plant extracts was observed as a result of change in colour. Change in colour is due to the Surface Plasmon Resonance phenomenon. Metal nanoparticles have free electrons, which give the SPR absorption band due to the combine vibration of electrons of metal nanoparticles in resonance with light wave. Peak of silver nanoparticles were observed at 446.5nm, 447.0nm, 444.0nm, 443.0nm and 437nm in case of accession no- 347492, 331514, 342109, 281374 and 383913. From literature review it was found that silver nanoparticles show peak in a range of 350nm to 500nm. Broadening of peak indicates that the particles are poly dispersed. From this study we found the SPR peak is nearly 437-447 nm for different accessions. The metal particles were observed to be stable in solution even four weeks after synthesis that means the optical properties of nanoparticles in solution with time. Maximum absorbance was observed in the accession no- 383913 which is 1.477.

Table 5:- UV–VIS spectra observation of silver nanoparticles synthesized

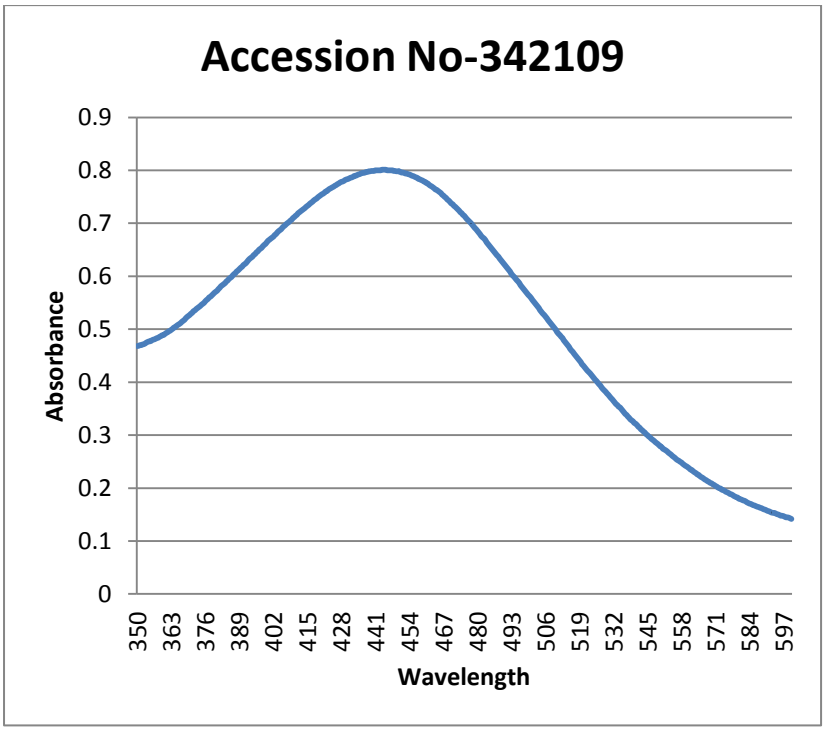
Accession No.	Peak	Absorbance
347492	446.5	0.809
331514	447.0	1.375
342109	444.0	0.801
281374	443.0	1.300
383913	437.5	1.477


Accession No-347492
Absorbance
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
350
363.5
377
390.5
404
417.5
431
444.5
458
471.5
485
498.5
512
525.5
539
552.5
566
579.5
593
Wavelength

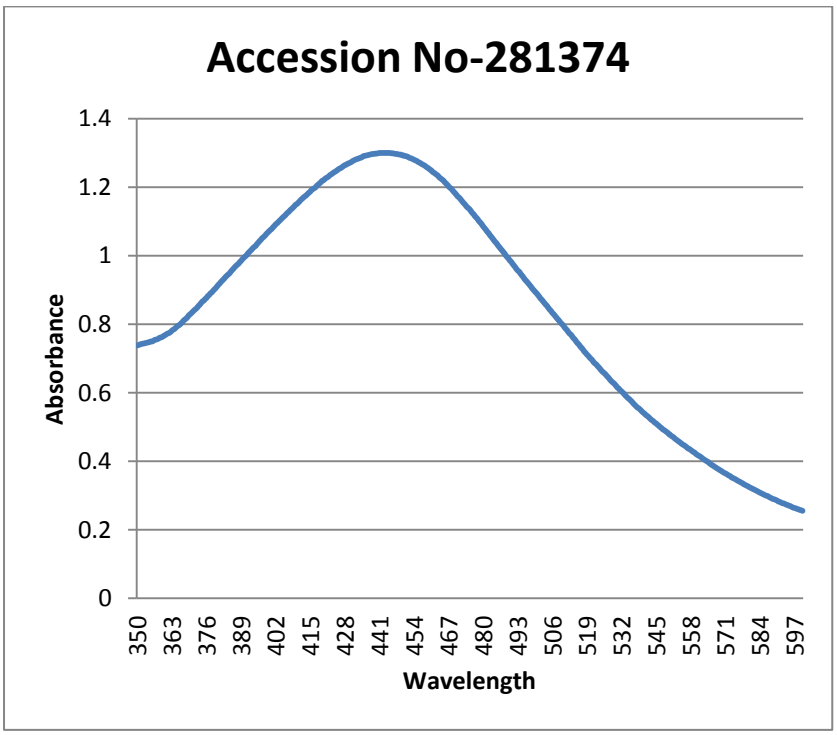
(a)


Accession No-331514
Absorbance
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
350
363.5
377
390.5
404
417.5
431
444.5
458
471.5
485
498.5
512
525.5
539
552.5
566
579.5
593
Wavelength

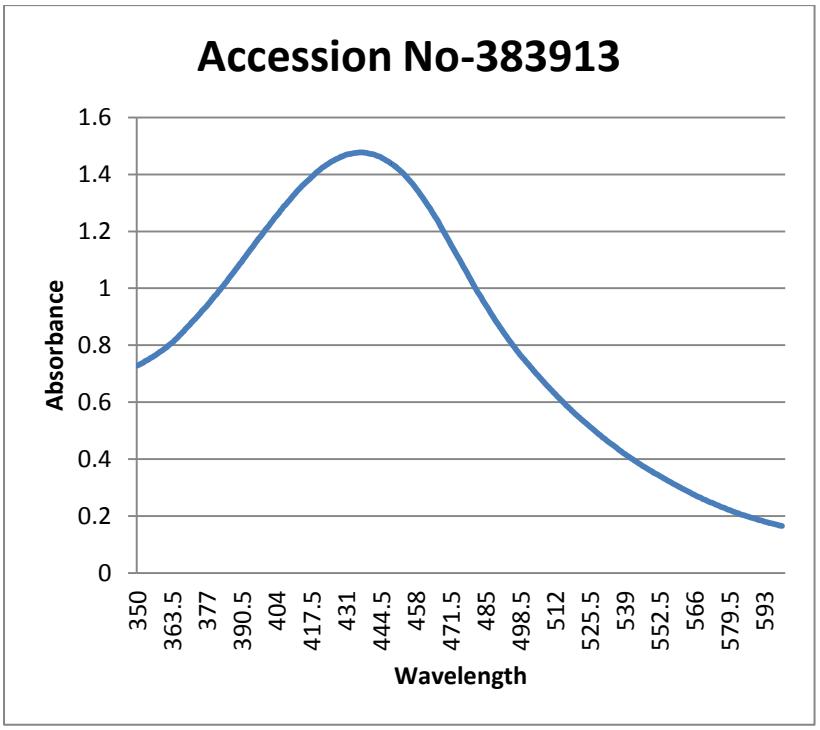
(b)


Accession No-342109
Absorbance
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
350 363 376 389 402 415 428 441 454 467 480 493 506 519 532 545 558 571 584 597
Wavelength

(c)


Accession No-281374
Absorbance
1.4
1.2
1
0.8
0.6
0.4
0.2
0
350 363 376 389 402 415 428 441 454 467 480 493 506 519 532 545 558 571 584 597
Wavelength

(d)



(e)

Fig 10:- UV-Vis spectra of silver nanoparticles synthesized from different accession using plant extracts (a, b, c, d, and e)

4.4.2 Scanning Electron Microscope (SEM) Analysis

SEM provides the details about the morphology of the silver nanoparticles. SEM images were taken at different resolutions i.e. 500nm, 2 µm, 5 µm, 2 µm and 10 µm in case of accession no- 347492, 331514, 342109, 281374 and 383913. Size of synthesized nanoparticles was more than the size of nanoparticles which should be between 1-100nm. Size was more than the desired size due to the protein which were bound in the surface of nanoparticles. Result shows that the particles were of relatively spherical shape in all the accessions i.e. no- 347492, 331514, 342109, 281374 and 383913 (Figure 11).

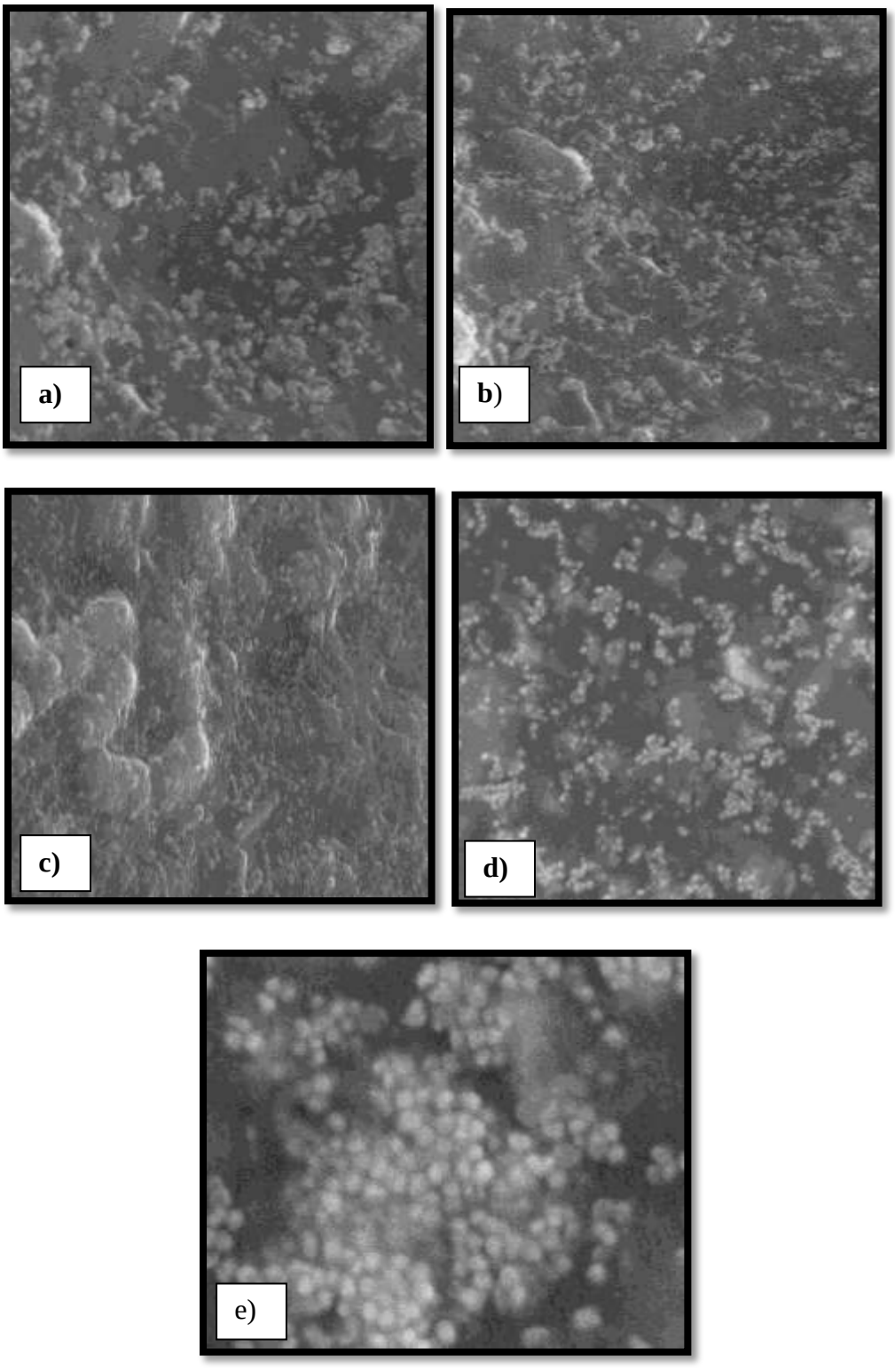
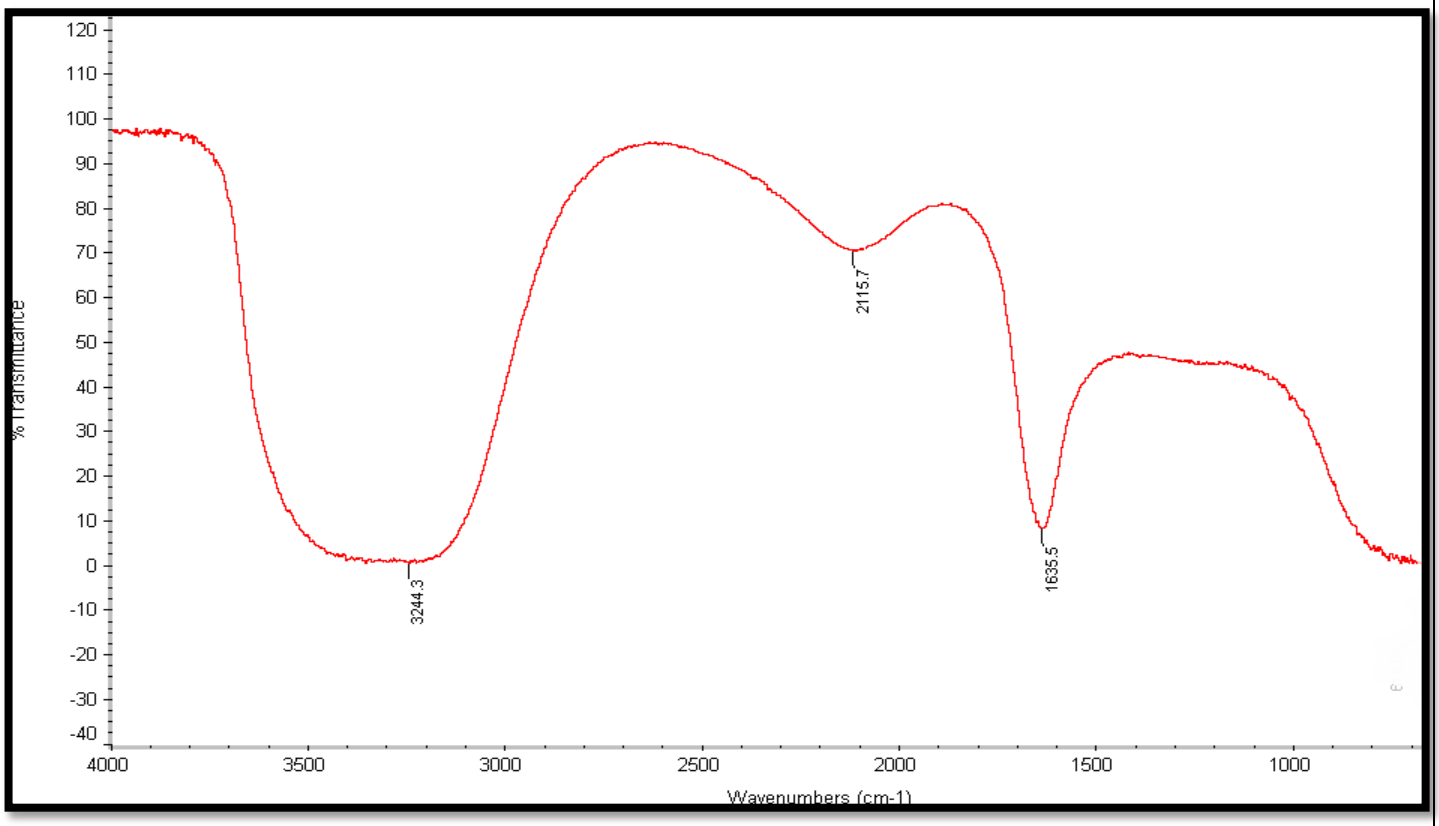


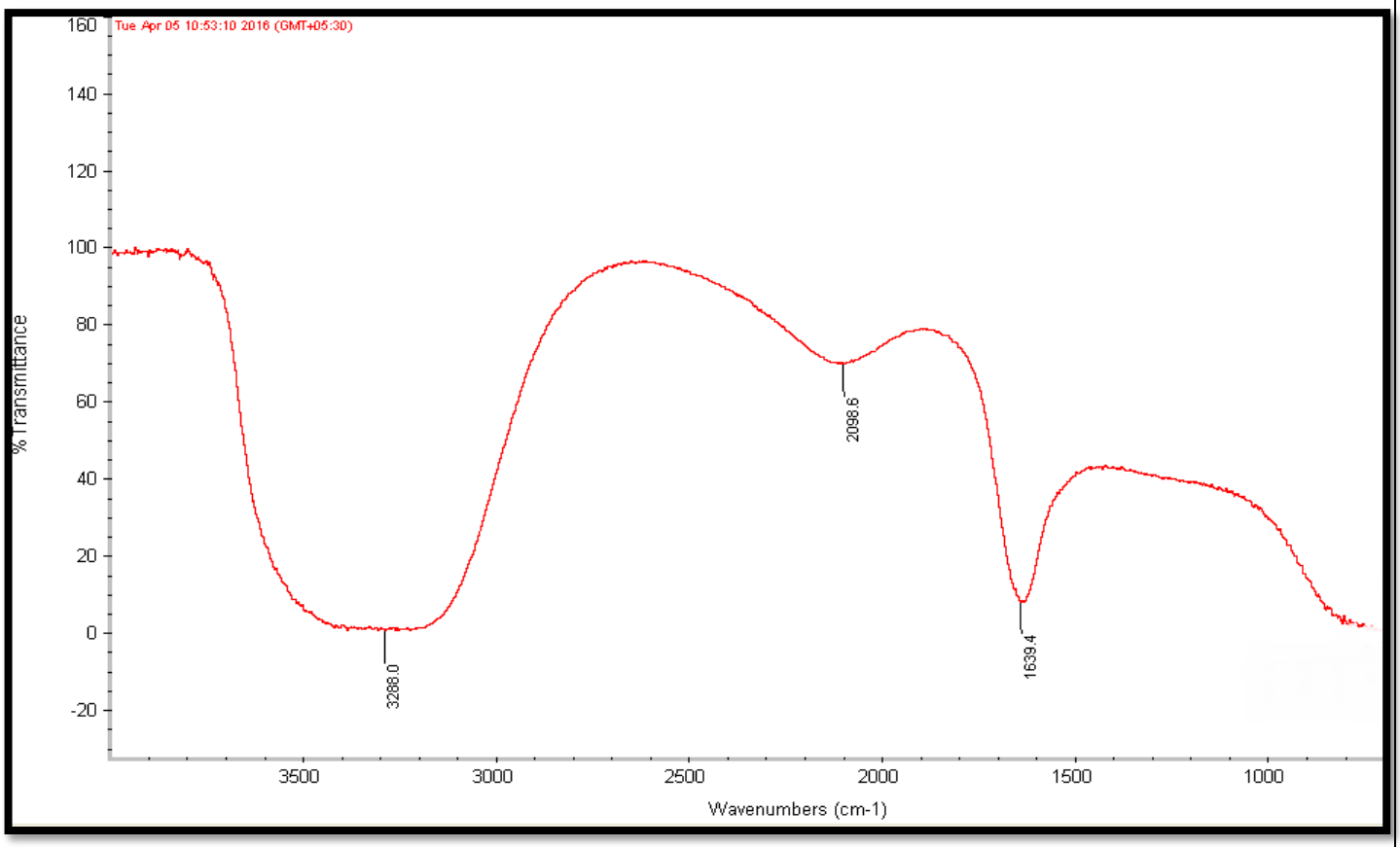
Fig 11:- SEM image of silver nanoparticles. Accession number (a) 281374, (b) 331514, (c) 342109, (d) 347492 and (e) 383913

4.4.3 Fourier Transmission Infrared Spectroscopy (FT-IR) Analysis:

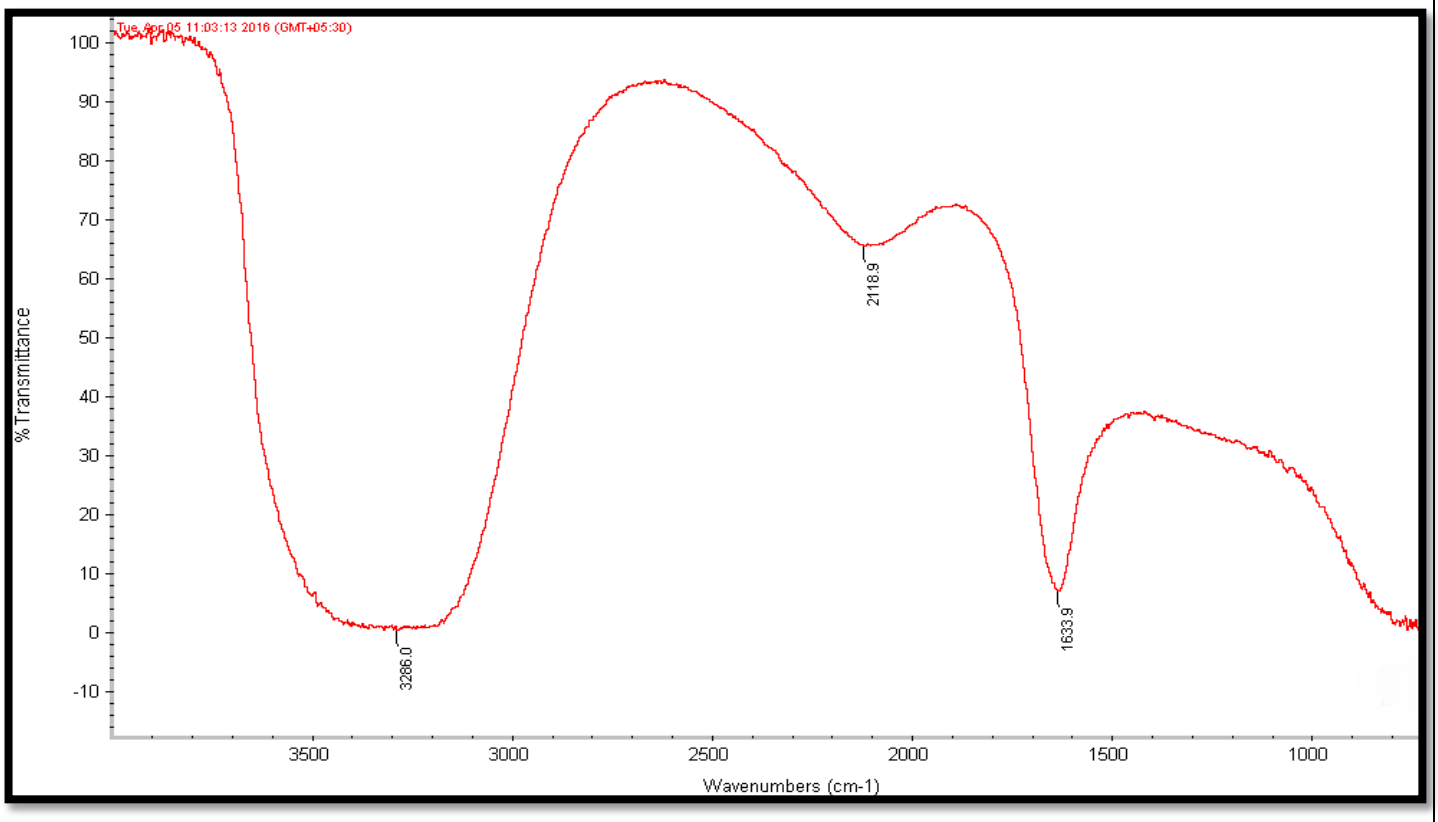
FT-IR measurements were carried out to identify the biomolecules for capping and efficient stabilization of metal nanoparticles synthesised. FT-IR spectrum of silver nanoparticles shows the band between 3207-3288 cm^{-1} corresponds to O-H stretch H-bonded alcohols and phenol. Peaks found around 1633-1639 cm^{-1} corresponds to carbonyl stretch vibrations from carboxylic acid and phenols and N-H identified as amide and arise due to a carbonyl stretch in the amide linkages of the proteins and peaks around 2095-2118 corresponds to C≡C stretch. Therefore synthesised nanoparticles were surrounded by proteins and metabolites such as flavanoids and terpenoids (Palaniselvam et al., 2012). Based on the physical state of the extracts and the characteristic features of the infrared vibrational peaks in the spectra, terpenoids, long chain fatty acids and secondary amide derivatives were the possible compounds in the obtained nanoparticles. Form FT-IR analysis we confirmed that the carbonyl groups from amino acid residues and proteins has stronger ability to bind metal which indicating that the proteins involve in capping of silver nanoparticles and prevents from agglomeration and stabilize the medium.



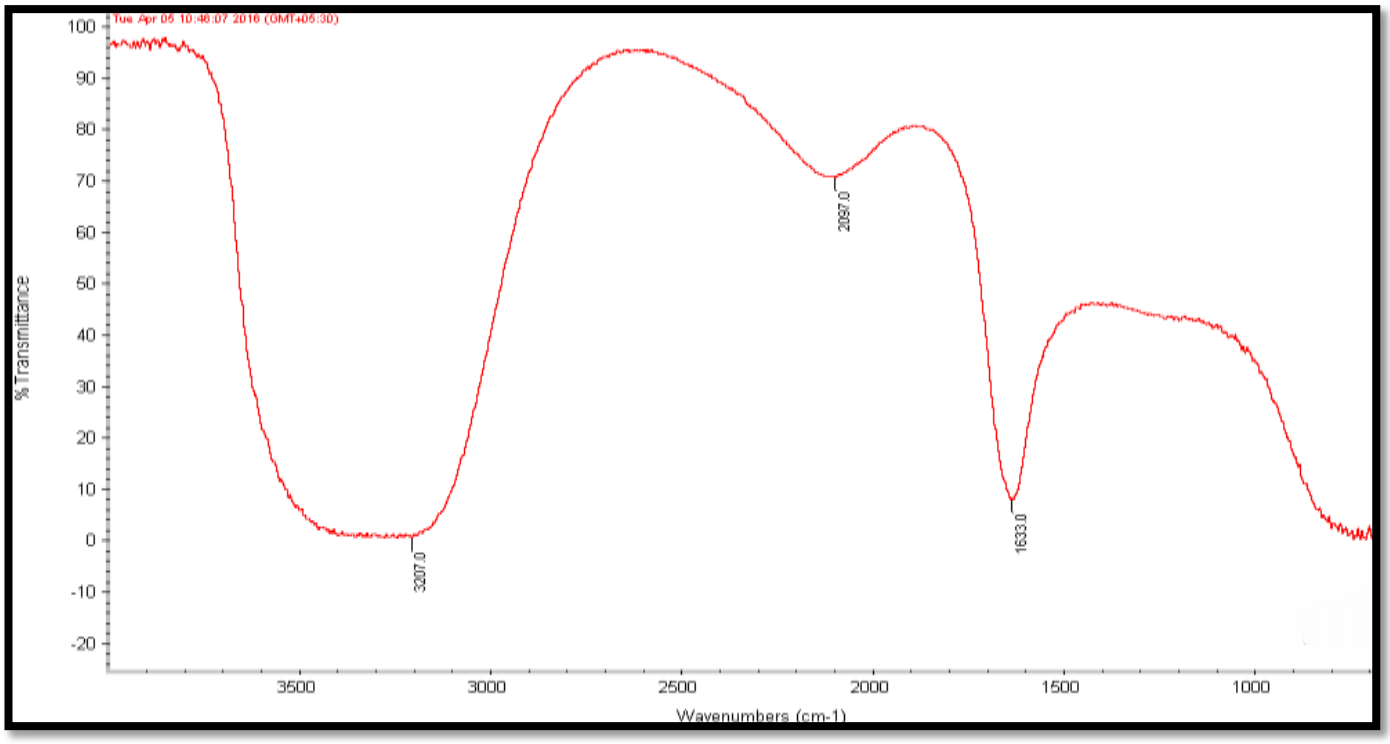
(a)


Tue Apr 05 10:53:10 2016 (GMT+05:30)
% Transmittance
Wavenumbers (cm-1)
3288.0
2098.6
1639.4

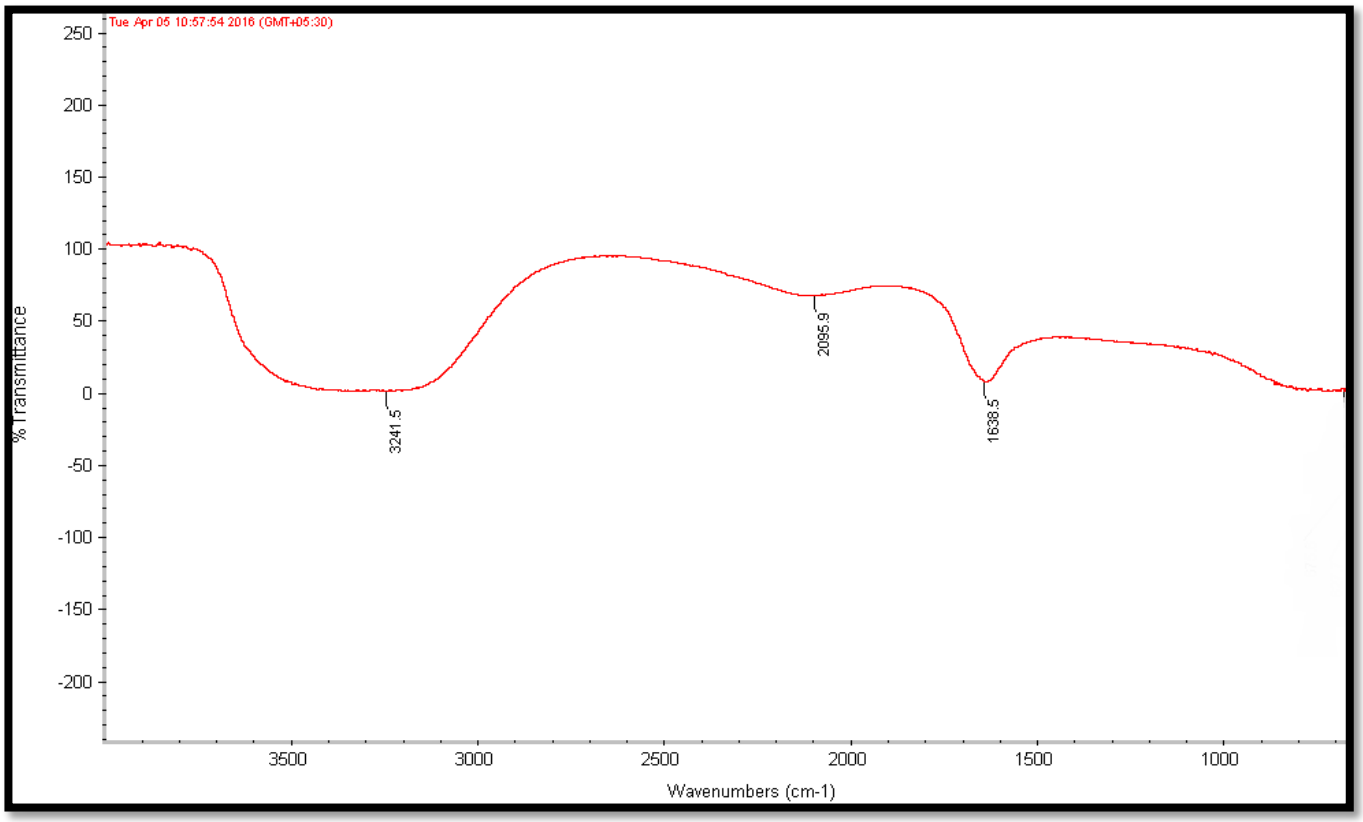
(b)


Tue Apr 05 11:03:13 2016 (GMT+05:30)
% Transmittance
Wavenumbers (cm-1)
3286.0
2118.9
1633.9

(c)



(d)



(e)

Fig 12:- FT-IR Analysis of silver nanoparticles of five different accessions (a) 281374, (b) 331514, (c) 342109, (d) 347492 and (e) 383913

Table 5:- FT-IR analysis of silver nanoparticles

Accession No.	Vibrational peak (cm^{-1})	Vibrational frequencies	References
347492 331514 342109 281374 383913	1635.5 1639.4 1633.9 1633.0 1638.5	carbonyl stretch vibrations from carboxylic acid and phenols N-H	Rout et al.,2013, Logeswari et al., 2013
347492 331514 342109 281374 383913	2115.7 2098.6 2118.9 2097.0 2095.9	could be C≡C	Devaraj et al., 2013
347492 331514 342109 281374 383913	3244.3 3288.0 3286.0 3207.0 3241.5	Free O-H group present in Phenols	Rout et al.,2013

CONCLUSIONS AND FUTURE PERSPECTIVES

Tissue culture techniques play an important role in the clonal multiplication of elite clones of this plant as well as conservation of its germplasm. Due to medicinal importance of *Centella asiatica,* it is overexploited; therefore conservation of this plant is required. In this investigation, it was concluded that the MS medium with concentration of BAP 1 mg/l supports maximum shoot multiplication and length of shoots for all the five accessions of *Centella asiatica*. It was also observed that the maximum number of shoots and length of shoots obtained for accession no.—342109 i.e. 19.6±0.57 (shoot number) and 4.9±0.53 (shoot length) in comparison to the other four accessions. These findings would be useful in conservation and micropropagation of this plant. In future this can be utilized for the mass propagation of the plant.

Qualitative analysis of phytochemicals revealed the presence of terpenoids, steroids, saponin flavonoids, tannins and coumarins in *Centella asiatica.* All the five accession i.e. accession no.—342109, 281374, 331514, 383913 and 347492 showed the presence of these phytocompounds. Presence of these compounds suggests the medicinal properties of this plant. There is a need of further studies to know their biological effects which could be beneficial in the treatment of various diseases.

Gas chromatography analysis showed the presence of phytosterols i.e. stigmasterol, tocopherol and β-sitosterol. Out of the five different accessions, accession no. 342109 showed the high amount of stigmasterol content i.e. 4.55±0.70 µg/200mg of leaves. This suggests that production of phytosterols depends on the growth of the plant. In future the potential accession can be used in the preparation of medication for the treatment of various diseases.

Rapid biological synthesis of silver nanoparticles using *Centella asiatica* aqueous extract provides a simple, efficient and environment friendly method for the synthesis of nanoparticles. All the five accession i.e. accession no.—342109, 281374, 331514, 383913 and 347492 has potential for the silver nanoparticles synthesis. Synthesized nanoparticles were relatively spherical in shape. Nanoparticles were surrounded by a thin layer of proteins and metabolites such as terpenoids having functional groups of alcohols, amines, aldehydes etc., which were found from the characterization using UV-VIS spectroscopy, SEM and FT-IR techniques. It was observed that accession no.-383913 has more potential to synthesized silver nanoparticles as compared to the other accessions. From technological point of view these silver nanoparticles have potential application in biomedical field and this simple procedure has several advantages such as cost effective, compatibility for medical and pharmaceutical applications as we as large scale production.

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