Phyto-sterol estimation and Green synthesis of silver nano-particle using leaf extract of *Plumbago zeylanica*

To be submitted as Major project *in partial fulfilment of the requirement for the degree of*

Master of Technology

In

Industrial Biotechnology

Submitted by

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DECLARATION

This is to certify that the major project-2 entitled "**Phyto-sterol estimation and Green synthesis of silver nano-particle using leaf extract of *Plumbago zeylanica***" 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***, Asssistant Professor, Plant Biotechnology, 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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CERTIFICATE

This is to certify that the major report entitled "**Phyto-sterol estimation and Green synthesis of silver nano-particle using leaf extract of *Plumbago zeylanica*" (DTU/14/M.Tech./083)** in the partial fulfilment of the requirements for the reward of the degree of Masters 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 him 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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2K14/IBT/01

CONTENT

LIST OF TABLES

LIST OF FIGURES

Figure-16: Chromatogram of β-Sitosterol from leaf extract of P. zeylanica Accession no.-421418 grown on yeast extract used as an elicitor.

Figure-16: Chromatogram of β-Sitosterol from leaf extract of P. zeylanica Accession no.-421418 grown on yeast extract used as an elicitor.

Figure-17 Chromatogram of β-sitosterol from leaf extract of P. zeylanica Accession no.-421418 grown on yeast extract used as an elicitor.

Figure-18 Chromatogram of β-sitosterol from leaf extract of P. zeylanica Accession no.-421418 grown on yeast extract used as an elicitor.

Figure 19- Chromatogram of β-sitosterol from leaf extract of P. zeylanica accession number 524441 grown on NH_4NO_3 as nitrogen source.

Figure 20- Chromatogram of β-sitosterol from leaf extract of P. zeylanica accession number 524441 grown on NH_4NO_3 as nitrogen source.

Figure- 21 Chromatogram of β-sitosterol from leaf extract of P. zeylanica accession number 524441 grown on NH_4NO_3 as nitrogen source.

Phyto-sterol estimation and Green synthesis of silver nano-particle using leaf extract of *Plumbago zeylanica*

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ABSTRACT

Plants consists of various chemical compounds which have therapeutic value and help to cure various disease including cancer. Since plant products give an advantages of being immune friendly and biocompatible. *Plumbago zeylanica* L. (family: Plumbaginaceae), commonly called "White leadwort" in English and "Chitrak" in Sanskrit (the most ancient Indian language), is a perennial shrub found in peninsular India and West Bengal and is cultivated in medicinal gardens of India. It has antibacterial, hepato- protective and various other properties but the plant is mostly used for its anticancer property. Till date the plants are taken from the wild for research purpose which poses a threat to its survival in the wild. To prevent the extinction, tissue culture can be used as an important tool for its mass propagation and for production of important phyto-compounds having therapeutic significance which can help in further research on the plant without disturbing it in the wild. The biosynthesis of nanoparticles using the plant extract is also helpful in determining the antibacterial and anti-microbial property of the plant. In this study, effect of different nitrogen source and elicitors were recorded on different accessions of *P. zeylanica.* Accession number 524441 was found to be potential accession grown on ammonium nitrate among five different accessions and 421418 grown on plant elicitor yeast extract. Also the estimation of Sterols present in the plant which has been cultured on various conditions has to be done to check the variations in the amount of sterols produced estimation using Gas Chromatography. Along with these investigation, all five accessions were also used for their potential of silver nano-particle synthesis.

INTRODUCTION

Plants have been known as a source of highly useful material to the mankind, may it be food, shelter, medicine etc. Today plants are exploited mainly due to their medicinal property. The compounds isolated from plants are natural and have advantage over synthetic chemical compounds as they are readily available in the nature, since they are natural products so the problem of acquiring resistance against these compounds is minimized to a very great extent. Various plants are used as a source of medicine for diseases such as *R. nasutuskurz* for cervical cancer, *G. glabra* for colon cancer, *P. zeylanica* for Ehrlich ascites carcinoma and many more.

P. zeylanica commonly called "White leadwort" in English and "Chitrak" in Sanskrit, belongs to the family Plumbaginaceae is a perennial shrub which is found in West Bengal and peninsular region of India and also various medicinal gardens cultivate this plant (Sharma A and Singh N, 2015). The plants consists of various bioactive compounds like sterols, saponins, triterpinoids, coumarins naphthoquinones etc. which have been reported to show anti-bacterial, anti-plasmodial, anti-tumour, hepatoprotective, central nervous system stimulatory activity (Kumar et al., 2009 , Wang et al., 2005). *P. zeylanica* contains two highly important compounds namely Plumbagin and β-Sitosterol which is present mainly in root and shoot respectively. β- Sitosterol possesses anti-cancer activity (Von et al 1998, Awad et al., 2000, Zak et al., 2005) also it has anti-diabetic activity (Jagadheshan et al., 2002) .

Tissue Culture in *Plumbago zeylanica*

Due to the high medicinal property of *P. zeylanica* this plant is being over exploited from the wild leading to its extinction. As this plant is of high importance, tissue culture serves as a viable tool for the propagation and conservation. The *in-vitro* propagation offers a powerful tool for germplasm conservation, generation of disease free plants and mass multiplication. Since the seedling derived plants are of heterozygous nature so they are not elite clones. Clonal fidelity of progenies has a great relevance to their active ingredients, which is a key factor initiating the technique of *in-vitro* propagation to save and increase the number of the plants for future research purpose (Kour et al., 2014).

Phytochemicals present in *Plumbago zeylanica*

These are the natural compound present in plants having therapeutic importance synthesized in plants during normal process of metabolism. These compounds are also termed as 'Secondary metabolites' which includes alkaloids, flavonoids, sterols, phenols, coumarins, gums, tannins, terpenes and phenols. The phytochemicals originate in plant food material which when consumed works by nutrients and dietary fibres defending the body against diseases. Various research provides knowledge of phytochemicals as essential dietary content working together to help slowing the aging process and diminishing the hazardous disease like cancer, stroke, cataract, osteoporosis, heart disease etc. (Tyagi and Menghani, 2014). β-Sitosterol is an important compound present in *P. zeylanica* which is having therapeutic advantages for diseases such as hypercholesterolemia, and is also known to have anti-diabetic property.

Silver Nano-particle synthesis in *Plumbago zeylanica*

Silver being a noble metal has been recognized to possess inhibitory property against various medical and industrial process microbes and many bacterial strains. Medical industry utilizes many applications regarding silver and silver nano-particle (Jiang H et al, 2004). Nano-particle is defined as a particle which has size up to 100nm. (Simi CK, Abraham TE, 2007). These particles being very small present a higher surface to volume ratio as compared to that of normal silver particle. The catalytic activity of the particle is dependent of the specific surface area, as the specific surface area increases, the biological effectiveness of the nano-particle increases (William and Wildenberg, 2005). The medical uses of silver nano-particles include ointments and topical creams for prevention of infections during burns and open wounds. Silver nano-particles can be synthesized by different method. The most popular method utilized for silver nano-particle synthesis is chemical method involving harsh and toxic chemicals which poses a great threat to person as well as to environment. So there is a need to synthesize the nano-particle using environment friendly process. Nano-particle synthesis using plant or plant extract, microorganism, and enzyme have been reported to be environment friendly (Konishi Y et al 2007).

Objective of this study

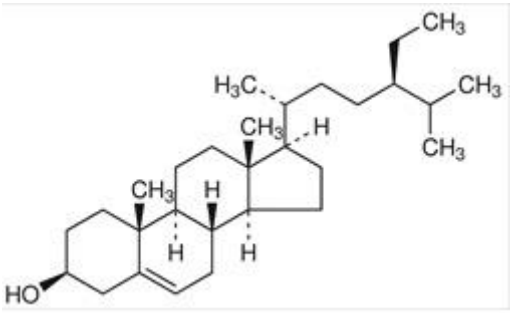
- **Tissue Culture**
  - A- Effect of different elicitors on shoot proliferation of five different accession of *P. zeylanica.*
  - B- Effect of different nitrogen source on growth pattern of five different accessions of *P. zeylanica*.
- **Phytochemical Screening**
  - C- To check the presence of different secondary metabolite in five different accession of *P. zeylanica.*
- **Silver Nano-particle Synthesis**
  - D- Silver nano-particle synthesis using five different accessions of *P.zeylanica.*
- **Estimation of β-sitosterol**
  - E- Qualitative estimation of β-sitosterol in potential accession 421418 and 524441 of *P. zeylanica* using Gas Chromatography.

REVIEW OF LITERATURE

In India *Ayurveda* meaning the science of life, provides medicine to a large population of the country. The World Health Organization nowadays is actively encouraging the use of herbal medicine for ailments which were used by people of various cultures for centuries (Julsing *et al*., 2013).. *P. zeylanica* commonly known as 'chitrak' in Sanskrit and 'lead-wort' in English, it is also known as 'Ceylon lead-wort' as a trade name. This plant is diploid (2n) having a chromosome number of 24, belongs to the family Plumbaginaceae and it has been reported to be used as a key ingredient in various folk medicines of Asia and Africa (Ravikumar and Sudha, 2011). Plumbaginaceae family consists of 280 species and 10 genera, while the genus Plumbago consists of 3 species which are *Plumbago Indica L. (P. Rosea L.) P. Capensis L.* and *P. zeylanica*, and are distributed throughout India. Among these species *P. zeylanica* is found to be mostly present in the central region of Western Ghats.

P. zeylanica consists of a variety of chemical compounds which are having bioactive compounds also known as secondary metabolites used for treatment of many diseases. Presence of alkaloids, carbohydrates, triterpenoids, flavonoids, gums, mucilage, protein, fatty acids and saponin has been reported in petroleum ether, ethanol and aqueous extract of *P. zeylanica* (Kumar *et al*., 2011). All parts of the plant is utilized but the main compounds β-sitosterol and Plumbagin are present in leaf and root respectively (Chaudhari *et al*., 2015)..

β-sitosterol



This compound has been reported to be present in fruits, stem and roots of variou plants and consists ial effect against a diversity of ailments reported in humans. It has been

reported to reduce colon cancer cell, lympholytic leukimia and prostate by improving hepatic activity of the body[Von et.al.1998, Awad et.al.2000, Zak et.al.2005,] As this compound closely resembles the structure of cholesterol which allows it to block the absorption of cholesterol from plasma and intestine[Awad et.al.2008, Frank et.al.2005,] by integrating itself on to membrane of mammalian cell thus it reduces cholesterol and is used for treatment of hypercholestrolemia. β-sitosterol helps in normalizing blood insulin level and blood sugar level in case of Type-II diabetes. It does this by down regulating the level of glucose-6-phosphatase releasing insulin and helps delaying the onset of Type-II diabetes ans aslo age related complications of glucose tolerance[Jagadheshan et.al.2002,]

Pharmaceutical and therapeutic property

Anti-microbial properties

Investigation done by Ahmed *et al.,* 2007using crude alcoholic extract of *P. zeylanica* for its anti-bacterial property against the growth of strains of *E. coli* and *Shigella* showed high activity with MIC of value 0.64-10.24mg/ml when compared with other plant extract. Delayed growth of *E. coli* and *Staphylococcus aureus* when inoculated in antibiotic, but however there was no growth observed in the media containing antibiotic and Plumbagin. The effect of different extracts of *P. zeylanica* leaf and stem showed inhibitory effects against bacteria and fungi using paper disc method, ethanol extract showed activity against micrococcus luteus as investigated by Ravikumar and Sudha, 2011.

Anti-ulcer activity

Study done by Falang *et al*., (2012 reveals the anti-ulcer activity when the aqueous root extract of *P. zeylanica* was administered on aspirin and indo methacine induced acute gastric ulceration in albino lab rats. By determining and comparing the ulcer index, score and percentage protection of the extract with that of the control and negative experiment groups.

Anti- inflammatory

A clinical study conducted on 30 patients who were taken from the OPD and IPD of national institute of Ayurveda, Jaipur by Napalchyal *et al*., (2013) found significant

improvement in pain, swelling, tenderness and dizziness cause due to inflammation of the body parts. Also investigation done by Sheeja *et al*., (2010) and Dang *et al*., (2011) showed that *P. zeylanica*r reduces the oedema thus comforting the body part, it is also investigated to supress the NF-kappa B activation in the tumour cells and also prevention of graft versus host disease (Checker *et al*., 2009).

Hypo-cholesterol emic activity

A clinical study carried by Sharma *et al*., (1991) which utilized the root extract of *P.zeylanica* containing Plumbagin, when administered to the hyper-lipidemic rabbits reduced the serum cholesterol and LDL by a percentage of 53 to 86 and 61 to 91 respectively. The compound Plumbagin restricts the cholesterol and triglyceride accumulation in the liver and aorta.also the investigation done by Ram, 1996 reveals a significant decrease in the serum cholesterol, LDL, cholesterol and triglyceride when 500mg/kg ethanolic extract of *P. zeylanica* was administered to hyper-lipidemic rabbits.

Wound healing activity

Jyothi *et al*., (2013) and Kodati *et al*., (2011) investigated the wound healing activity of extract of *P. zeylanica* and found that the activity is due to the presence of phytochemicals such as terpenoids, alkaloids, flavonoids, saponins etc. and these compounds are responsible for the wound healing activity of the plant. Bryan *et al*., 2012; Schremi *et al*., 2010; Kumar *et al*.2015 reported the evidence of oxidative stress in pathogenesis of non-healing ulcers. As the wound healing mainly depends on low level of oxidant so the antioxidant nature of the plant extract obtained from *P. zeylanica* helps in controlling the wound oxidative stress thus accelerating wound healing.

Cytotoxicity

Plumbagin being a bioactive compound which is present in *P. zeylanica* has been reported to possess the property of modulating cell proliferation, radio-resistance and carcinogenesis. These are regulated by activation of NF-Kappa B which is a transcription factor. Extract of *P. zeylanica* acts by inhibiting the activation which is induced or caused by Carcinogens, TNFs, and other stimulus. The study shows that Plumbagin also suppresses the activation of constitutive NF-Kappa B activation (Sandur *et al.*, 2006).

Anti-Cancer Activity

Study done by Hiradeve *et al.,* (2010) reports the anti-cancer property of *Plumbago zeylanica*. When the ethanolic extract of leaves were administered at a concentration of 200mg/kg which caused the reduction of the tumour volume, packed bed volume, and viable cell count in a dosage dependent manner.

Anti-diabetic activity

Zarmouh et al (2010) studied the effect of *Plumbago zeylanica* extract on diabetic rats. The extract reported to decrease the activity of glucose-6-phosphate and meanwhile increasing the activity of hexokinase when the ethanolic extract at a concentration of 100mg. 200mg/kg along with tolbutamide was administered orally to the streptozotocin treated diabetic rats.

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Materials and Methods

Explant source and Surface sterilization method

The explants were obtained from National Bureau of Plant Genetic Resource [NBPGR] in culture tubes. A total of five accessions of *Plumbago zeylanica* were acquired from NBPGR for our research project. The explants were surface sterilized by rinsing it thoroughly under running tap water for 30min followed by immersing it in detergent solution 5% (v/v) for 5min subsequently placing it in Bevistine a fungicide for 10min on a shaker then the explants were transferred in streptomycin an antibacterial for 15min afterwards again rinsed under running water for 1h to wash the residual sterelent off the explant. In the meantime other equipment were sterilized by autoclaved at a temp. of 121°C and a pressure of 15psi maintained for 20min.

Media Preparation

MS media was prepared by addition of all the components and was sterilized by autoclaving at 121°C, 15psi for 20min. After the media was sterilized it was allowed to cool till the vessel temperature was hand bearable and to it Benzyl Amino Purine [BAP] was added at a conc. of 1.0mg/l. Then the media was carefully poured into the culture tube to avoid spillage and the tubes were left to cool down for agar to get solidified.

***In-vitro* Culture**

500ml of MS media was prepared each for different elicitors Jasmonic acid, Salicylic acid, Yeast Extract and Chitosan. Also 500ml of MS media was prepared each having various nitrogen sources, ammonium nitrate, sodium nitrate and potassium nitrate. The prepared media were autoclaved at 121°C, 15psi for 20min. BAP was added to the autoclaved media inside the laminar air flow chamber after it cooled to a hand bearable temperature. The media was stirred gently to facilitate the proper mixing of BAP in the media. Then media was carefully poured in the culture tubes in front of flame inside the laminar air flow chamber. Afterwards the culture tubes were left overnight to facilitate solidification of agar. Healthy nodal segments of explant was cut using sterilized scalpel and blade into 1.0 to 2.0cm size inoculants. Then these were inoculated in the previously prepared culture tubes containing MS media with 1.0mg/l of BAP. After inoculation the

nodes were incubated at a temp. 25°C for a 16h photoperiod on a culture rack. These nodes were then sub-cultured subsequently to increase the explant population.

Explant inoculation in media containing

A- Elicitors (Jasmonic acid, Salicylic acid, Yeast extract and Chitosan)

B- Nitrogen Source(Ammonium nitrate, sodium nitrate and potassium nitrate)

Sub-cultured nodes of five different accessions 398891, 524441, 421418, 439212, 539866 of *P. zeylanica* were taken as explants for inoculation in media containing different elicitors, and also in media containing different nitrogen source. The inoculation of the explants in each media was done in triplicates. Growth was observed to record the change in phenotype of the inoculated explants on weekly basis.

C-Phytochemical analysis of the different accessions of the explant *P. zeylanica*

All the test were performed using standard procedures using the extract from five different accessions 398891, 524441, 421418, 439212, 53986 of *P. zeylanica.*

1- Test for Reducing Sugar(Fehling's test)

- 500mg of leaf form each accession was taken.
- The leaf was crushed gently and was boiled in water.
- This was then cooled and filtered.
- 5ml of the filtrate was taken as aqueous extract
- The extract was added to the boiling Fehling's solution.

Color change was observed for the presence of reducing sugar.

2- Test for flavonoids

- 4ml of the extracted solution was taken
- This was treated with1.5ml of 50% methanol solution
- The treated solution was then warmed adding metal magnesium to it
- To this solution 4-5 drops of conc. HCl was added

Colour change was observed.

3- Test for alkaloids

- The plant extract was boiled using water bath with 2% hydrochloric acid.

- The solution was cooled to room temperature
- Then few drops of Mayer's reagent was added to the cooled solution

Change in the solution texture was observed.

4- Test for tannins

- The filtered aqueous extract was taken.
- To this solution 0.1% ferric chloride solution was added.

Colour change was observed

5- Test for Terpenoids(Salkowski Test)

- To the aqueous extract 2ml of chloroform was added.
- Then to this solution 3ml of conc. sulphuric acid (H_2SO_4) was added.
- H_2SO_4 was very carefully added to form a layer on top.

Color formation was observed.

D-Bio-synthesis of Silver nanoparticles using leaf extract of five different accessions of *P. zeylanica*

- Leaves of different accessions *of P. zeylanica* were taken.
- These leaves were dried under room temperature for 5days
- Then the dried leaves were crushed using pestle and mortar.
- The crushed material was then boiled in 10ml of distilled water
- Then the extract was left to cool at room temperature
- The cooled extract was filtered into fresh vials
- 8.5mg of silver nitrate was dissolved in 50ml of distilled water
- The extract was then added in the ratio of 1:10.
- This mixture is then incubated at room temperature for overnight.
- Then the sample was analyzed by UV-vis spectrometry and SEM.

E- Estimation of Sterols using Gas Chromatography

- 200mg of leaf sample was taken from each culture condition of each accessions.
- The sample was crushed uniformly using pestle and mortar.
- To this crushed sample 200µl of internal solution was added and shortly vortex.
- 2ml of 2%KOH was added.

- Then the sample was vortex for 1min.
- Then the tubes were incubated at 80°C for 15min.
- The sample was then left to cool at room temperature.
- Now 1ml hexane was added to this cooled solution followed by 1.5ml of water.
- This mixture was then subjected to vortex for 30sec.
- The tube was then centrifuged at 4000rpm for 4min.
- Then the upper hexane layer was transferred to a fresh tube or vial.
- The transferred organic layer was then left overnight to evaporate.
- 100µl of hexane was added to the dried pellet.
- Then the dissolved pellet was transferred to GC vial.
- 50µl of silylating agent was added to the GC vial.
- Incubated at 70°C for 30min.
- Then 1µl of the sample was transferred to GC inlet using micro-syringe.

Result and Discussion

A. Effect of Elicitor on the growth of five different accessions of *P. zeylanica:* Elicitors have been reported by several previous studies to enhance the biomass as well as bioactive compounds in the plant tissue culture. In this study, effect of different elicitors i.e. jasmonic acid, salycilic acid, yeast extract and chitosan on growth of different accession of *P. zeylanica* have been tested. Among these elicitors, maximum number of shoots have been recorded in case of yeast extract in accession number 421418. 5±1 number of shoots along with 23±2 nodes have been found in this accession. Elicitation by yeast extract have also been reported by different investigators. Patulan et al., 2010 reported that, 0.5 mg yeast extract/l and 100 mg chitosan/l were found suitable for plumbagin production in *Drosera burmanii* whole plant cultures after 6 days of elicitation. Yeast extract (0.5 mg/l) provided best results of plumbagin production in roots of *D. burmanii* to 8.8 ± 0.5 mg/g dry wt that was 3.5-fold higher than control plants.

Table-1: Effect of different elicitors on number and length of regenerated shoots, number of nodes in five different accession of *Plumbago zeylanica* after eight weeks of inoculation. Values are expressed as mean ± Standard Error (M ± SE). MS: Murashige and Skoog medium

Experiment detail (elicitor)	Accession Number	Number of Nodes (M±SE)	Number of Shoots (M±SE)	Length of Shoots(cm) (M±SE)
JASMONIC ACID	524441	10±2	2±0	2.367±0.288
	398891	7±2	2±1	3.0167±0.340
	439212	8±2	2±1	1.655±0.394
	539866	6±1	2±1	2.5±0.556
	421418	7±2	2±1	3.378±1.00
SALYCILIC ACID	524441	6±1	2±1	2.689±0.40
	398891	11±2	2±0	2.516±0.368
	439212	6±1	2±0	2.933±0.50
	539866	9±2	2±0	2.667±0.321
	421418	8±1	3±0	3.033±0.107
YEAST EXTRACT	524441	13±2	4±1	2.455±0.236
	398891	19±3	4±2	2.891±1.199
	439212	16±2	3±1	2.652±0.814
	539866	12±2	3±1	2.686±0.480
	421418	23±2	5±1	3.025±0.505
CHITOSAN	524441	13±1	2±1	2.994±0.481
	398891	11±1	2±1	1.894±0.187

	439212	11±0	2±0	2.667±0.175
	539866	8±2	2±0	2.416±0.125
	421418	11±1	2±0	2.916±0.225

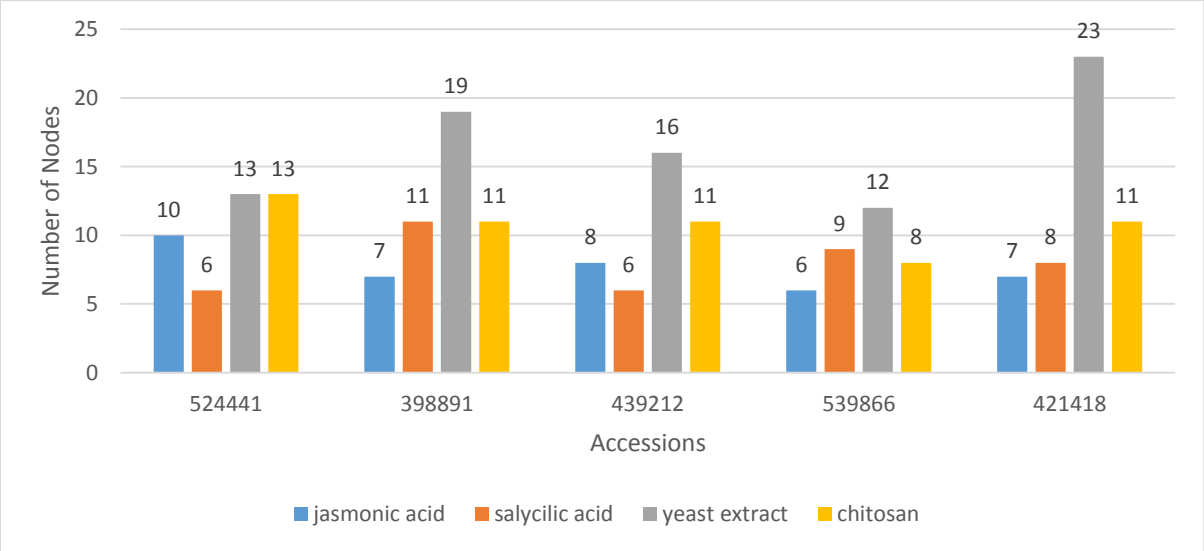


Figure-1: Effect of different elicitors on Number of nodes in five different accessions of *Plumbago zeylanica.*

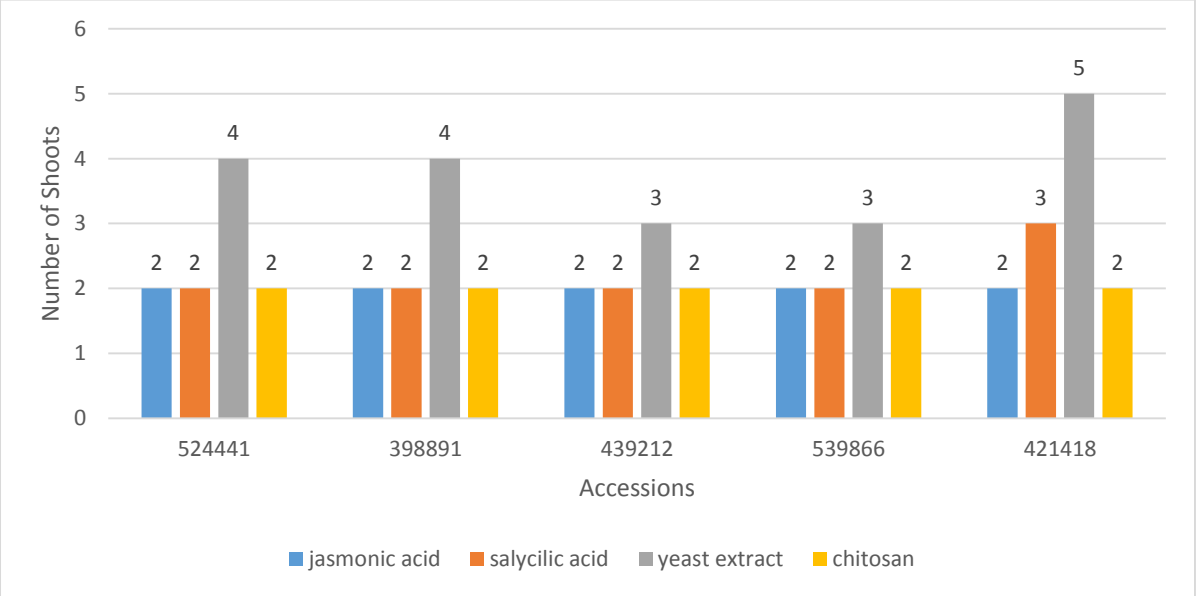


Figure-2: Effect of different elicitors on Number of shoots in five different accessions of *Plumbago zeylanica.*

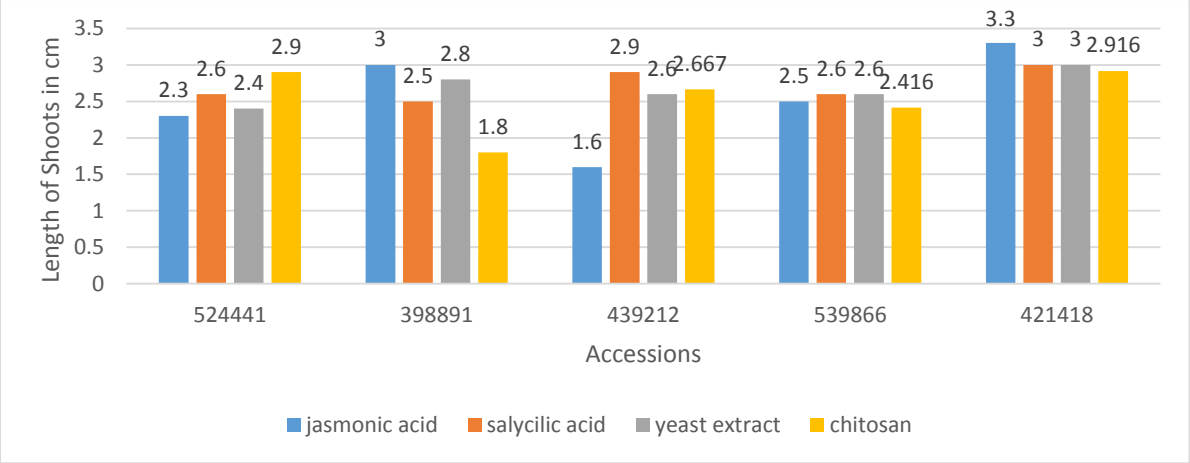
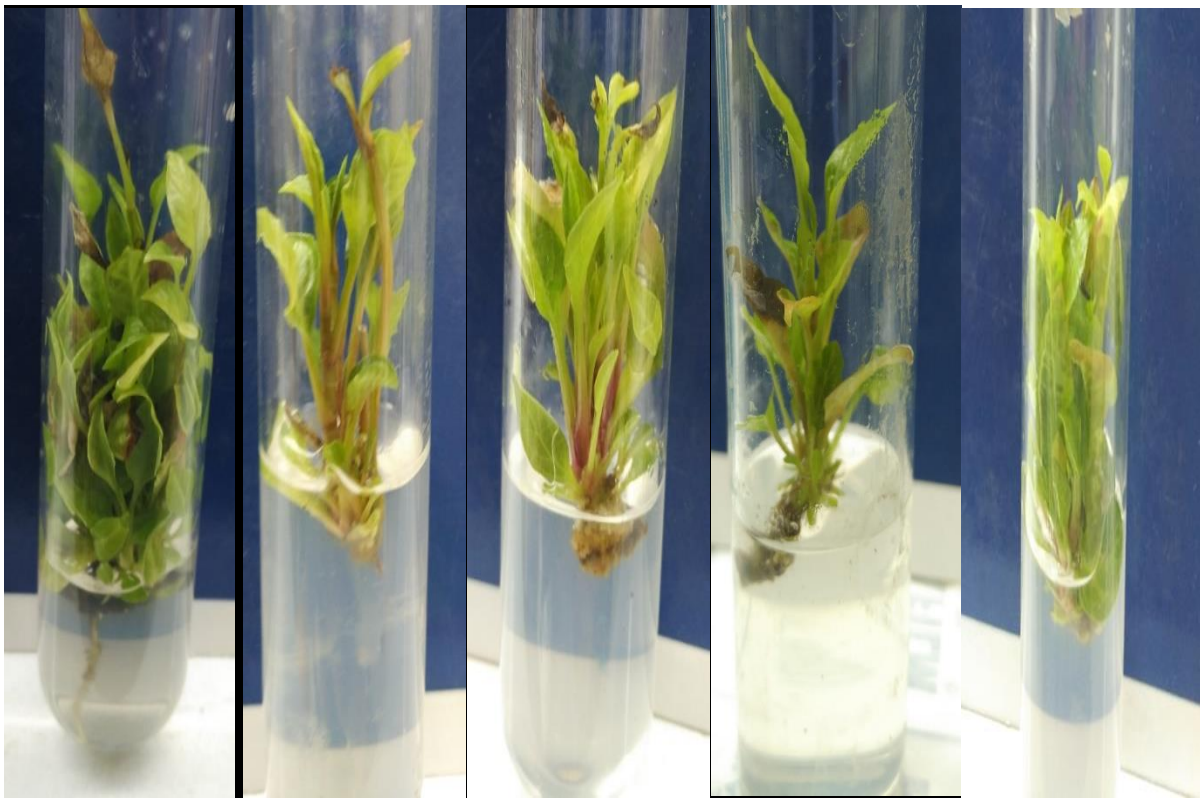


Figure-3: Effect of different elicitors on length of shoots in five different accessions of *Plumbago zeylanica*



Acc. 398891 Acc. 524441 Acc. 421418 Acc. 439212 Acc. 539866

Figure-4: *In vitro* culture of five different accession of *Plumbago zeylanica* in MS media containing Yeast extract as elicitor (After eight weeks of inoculation)

B. Effect of nitrogen source on the growth of five different accessions of *P. zeylanica:* Nitrogen is very essential element for growth. To identify the best source of nitrate, different source of nitrogen i.e. ammonium nitrate, sodium nitrate and potassium nitrate have been tested. The growth observed in this experiment for five different accessions of *P.* zeylanica shows that ammonium nitrate in the range of 16-19 for number

of nodes and 3-5 for number of shoots, sodium nitrate having a range 9-16 for number of nodes and 2-3 for number of shoots while for potassium nitrate, the range was 11-15 for number of nodes and 2-3 for number of shoots. Based on this observation ammonium nitrate is the potential nitrogen source and accession number 524441 provided maximum shoot proliferation in ammonium nitrate. *Chandravanshi et al., 2014* also recorded that maximum production in *P. zeylanica* treated with MS media containing ammonium nitrate and 13.3µM N6-benzyl amino purine and 135.74µM Adenine Sulphate at multiplication stage. Highest rooting average (16.10±1.10) was recorded on 1/2MS medium with 135.74µM AS.

Table-2: Effect of different nitrogen source on number and length of regenerated shoots, number of nodes in five different accession of *Plumbago zeylanica* after eight weeks of inoculation. Values are expressed as mean ± Standard Error (M ± SE). MS: Murashige and Skoog medium.

Experiment detail (Nitrogen Source)	Accession Number	Number of Nodes (M±SE)	Number of Shoots (M±SE)	Length of Shoots(cm) (M±SE)
NH_4NO_3	524441	17±3	4±1	3.179±0.213
	398891	18±2	5±2	3.759±0.418
	439212	16±3	3±0	4.188±0.050
	539866	16±2	3±1	4.111±0.395
	421418	19±3	3±1	3.719±0.807
$NANO_3$	524441	16±2	3±0	3.033±0.360
	398891	12±2	2±0	2.883±0.236
	439212	9±3	2±1	3.194±0.135
	539866	15±2	2±0	4.083±0.665
	421418	12±2	2±1	3.8±0.781
KNO_3	524441	15±3	2±1	2.744±0.350
	398891	11±1	3±1	2.963±0.257
	439212	13±2	2±0	3.35±0.180

	539866	13±3	2±0	3.766±0.284
	421418	12±1	2±0	2.633±0.246

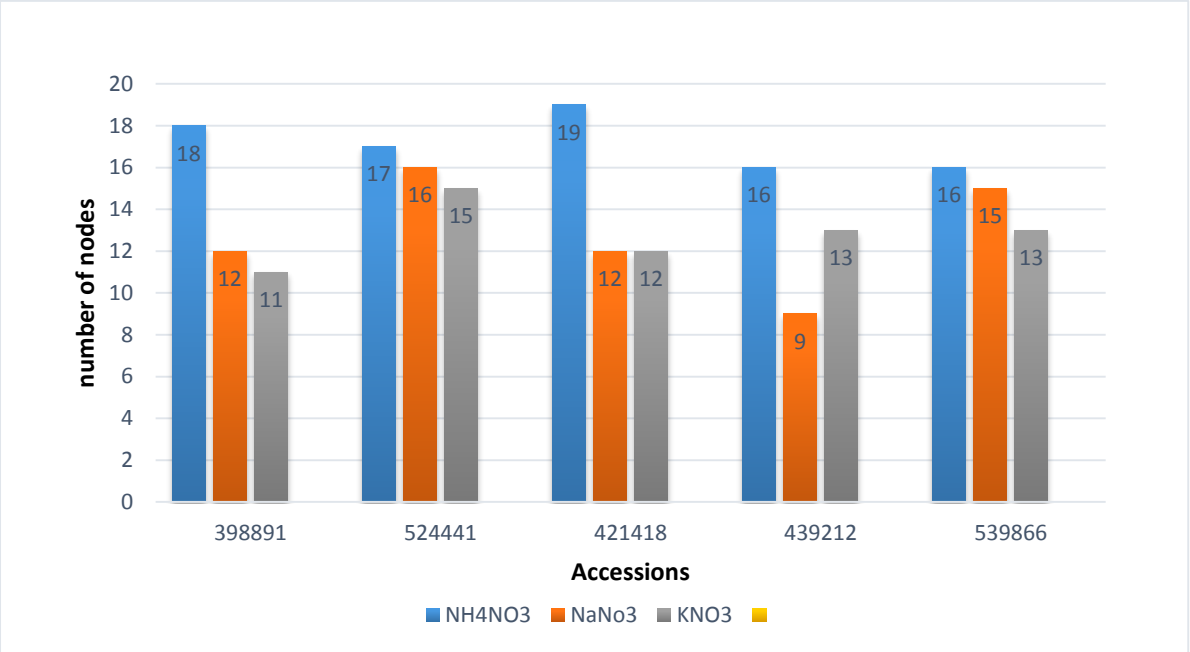


Figure-5: Effect of different nitrogen sources on nmber of nodes in five different accessions of *Plumbago zeylanica.*

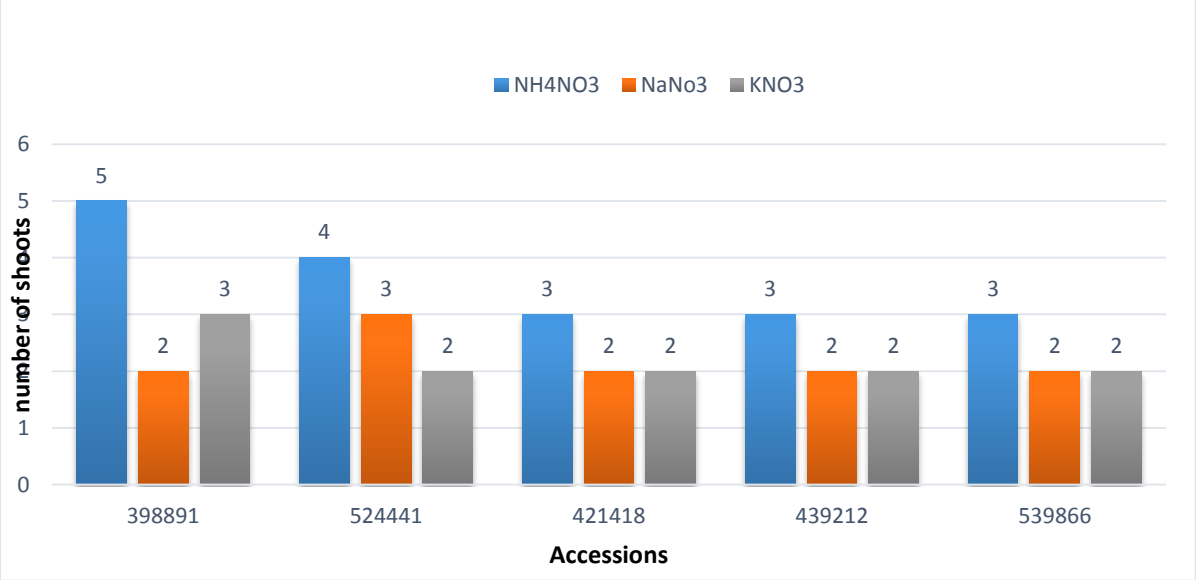


Figure-6: Effect of different nitrogen sources on number of shoots in five different accessions of *Plumbago zeylanica.*

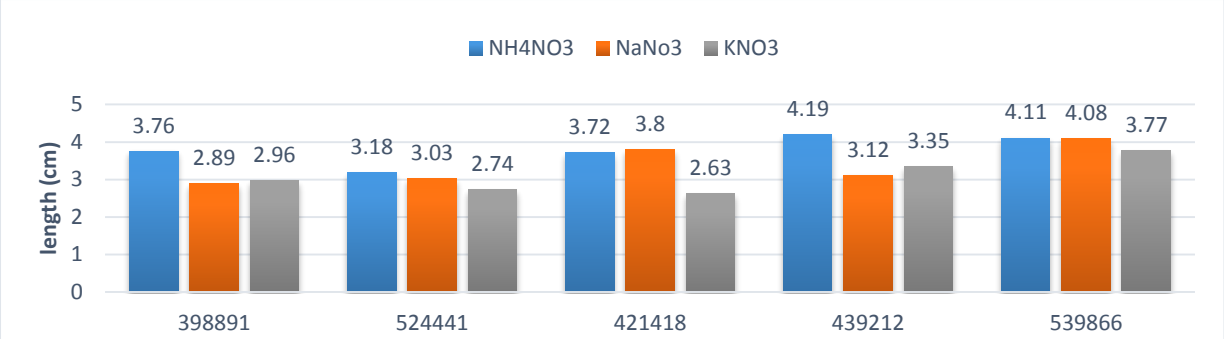
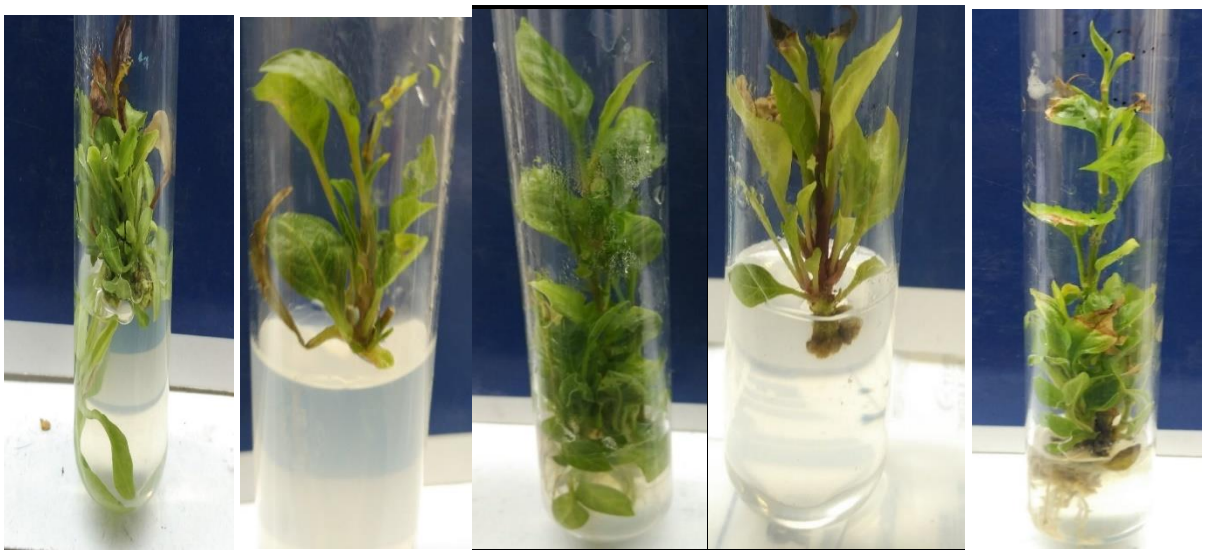


Figure-7: Effect of different nitrogen source on length of shoots in five different accessions of *Plumbago zeylanica*



Acc. 398891 Acc. 524441 Acc. 421418 Acc. 439212 Acc. 539866

Figure-8: *In vitro* culture of five different accession of *Plumbago zeylanica* in MS media containing ammonium nitrate as nitrogen source (After eight weeks of inoculation)

C. Phytochemical analysis of the different accessions of the explant *P. zeylanica*

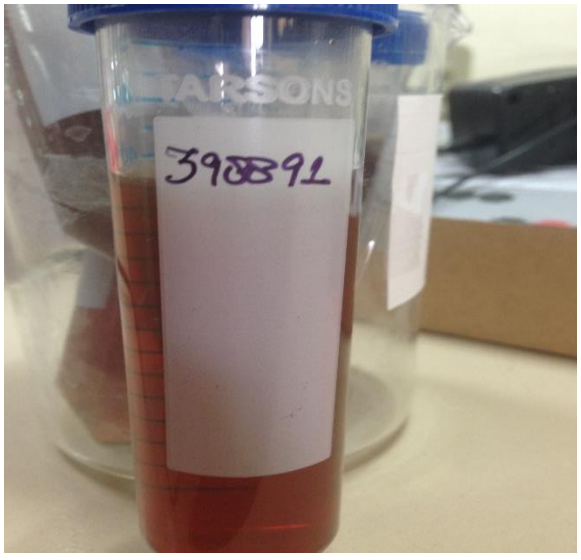
Five different accessions of *P. zeylanica* were screened for presence of phyto-chemicals such as reducing sugars, flavanoids, alkaloids, tannins and terpenoids using aqueous extract of plant leaf. Reddish brown color indicated the presence of reducing sugar in the plant extact of each accessions. In case of flavanoids, light red color was observed which indicated that each accession of plant contains flavanoids. The presence of alkaloids was determined by achievement of turbid solution of aqueous extract of each plant. No color change was observed in the experiment of confirmation of tannins which concluded that tannins are not present in the five different accession tested. A reddish brown ring like layer was observed in the test for terpenoids which confirms the presence of them. Tyagi and Menghani, 2014 also reported that the presence of reducing sugars, flavanoids, alkaloids and terpenoids and absence of tannins in aqueous extract of *P. zeylanica.*

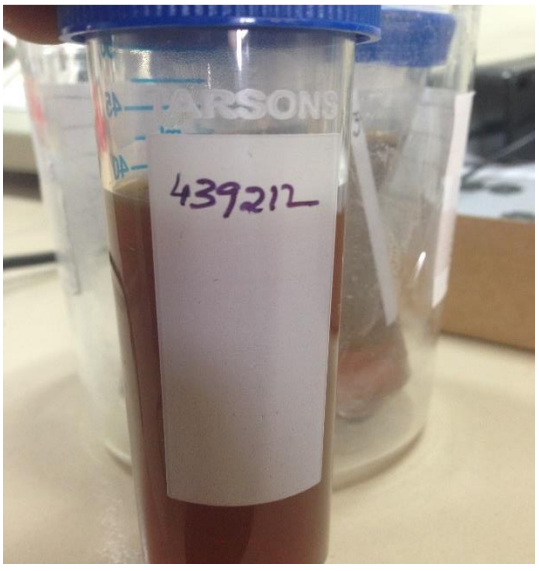
Table-3: Details of presence and absence of phyto-chemicals in *P. zeylanica.*

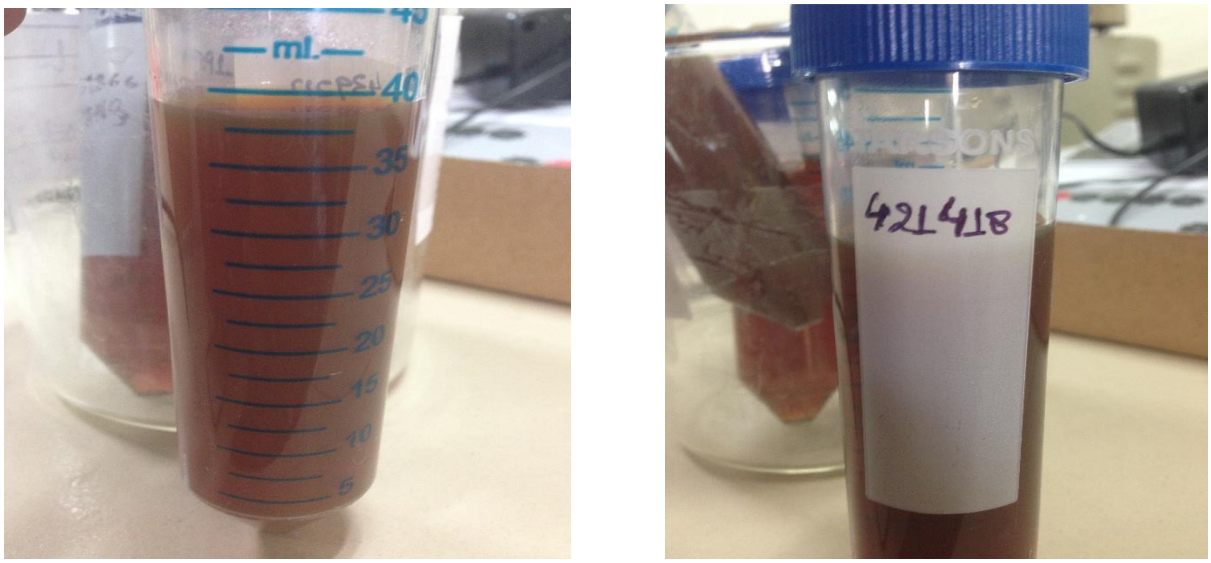
Extract	Accession Number	Phytochemicals				
		Reducing Sugars	Flavanoids	Alkaloids	Tannins	Terpenoids
Aq.	398891	+	+	+	-	+
	524441	+	+	+	-	+
	421418	+	+	+	-	+
	439212	+	+	+	-	+
	539866	+	+	+	-	+

D. Biosynthesis of Silver Nanoparticle

All five accession were tested for their potential to form silver nano-particle by using aqueous extract of whole plant. Change in the colour of the solution from slightly yellow to dark brown after overnight incubation the solution of silver nitrate confirms the potential of extract of the plant to form silver nano-particles. Characterisation of silver nano-particle was done by using UV-Vis spectrophotometer and Scanning electron microscopy (SEM). UV-Vis analysis was done to check the formation of silver nano-particles based on the absorbance at 350 to 450 nm which indicates the presence of silver nano-particle. The peaks of silver nano-particles form by each accession were found approximately at 440-450 nm at absorbance of 1.8 to 2.0 which corresponds to Plasmon excitation of Silver nano-particles. SEM was performed to give an account of morphology of silver nano-particle synthesized. Malar T. & Johnson M. 2015 also characterised the silver nano-particles prepared from leaf extract of *P. zeylanica* by using UV-Vis. They have also reported the absorbance lying at the range of 430 to 470 nm.







Figeure-9: Silver nitrate solution mixed with leaf extract after overnight incubation.

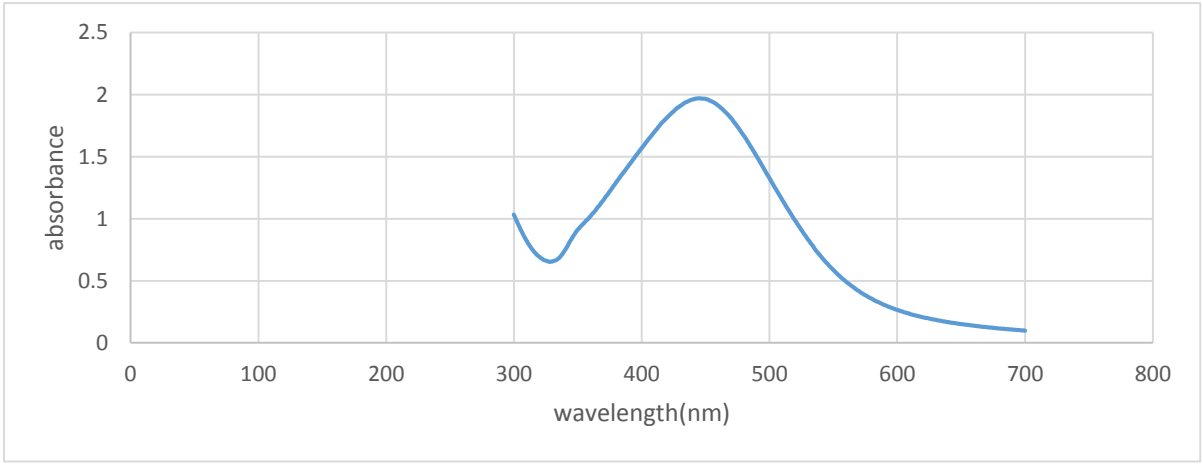


Figure 10- UV-Vis spectroscopy for the silver nano-particle from Accession no. 398891

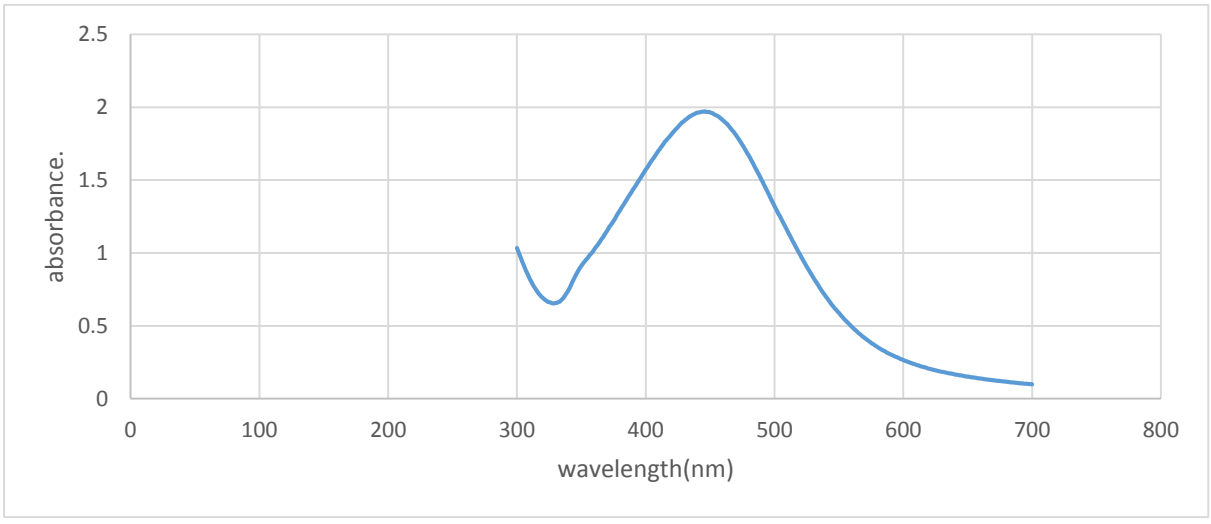


Figure 11- UV-Vis spectroscopy for the silver nano-particle from Accession no. 421418

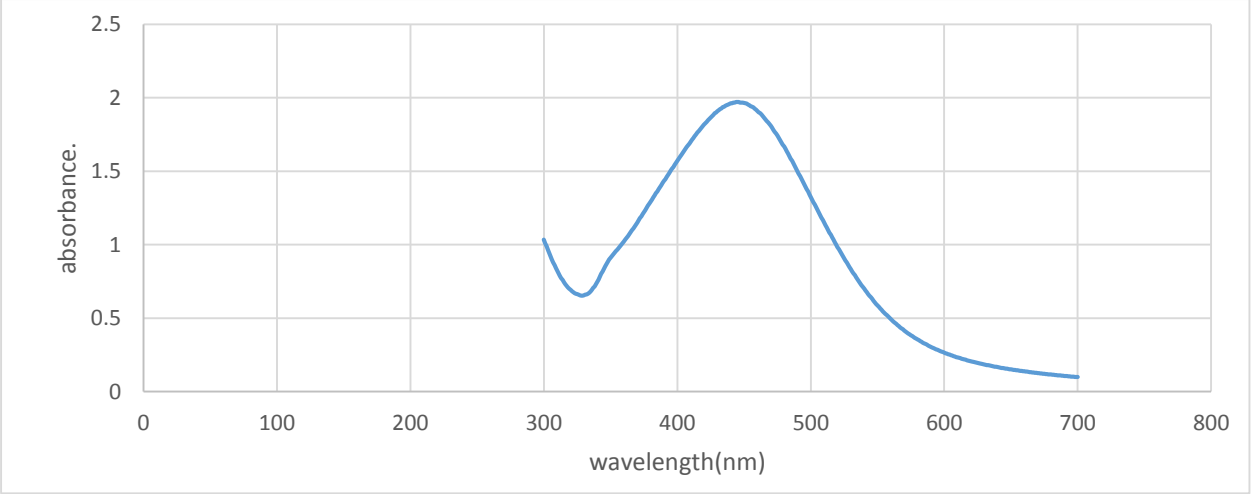


Figure 12- UV-Vis spectroscopy for the silver nano-particle from Accession no. 439212.

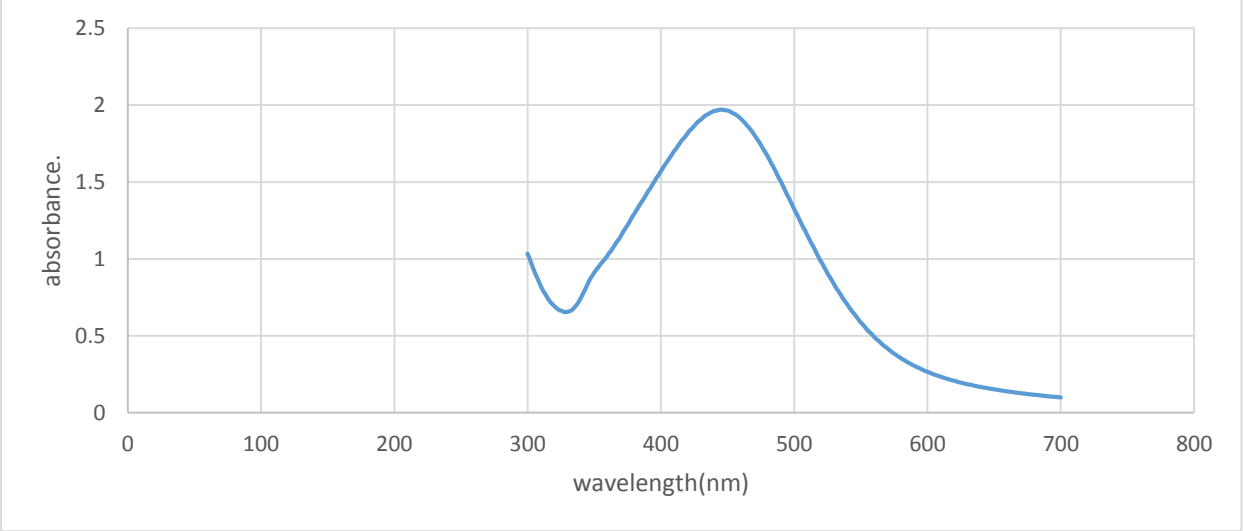


Figure 13- UV-Vis spectroscopy for the silver nano-particle from Accession no. 524441.

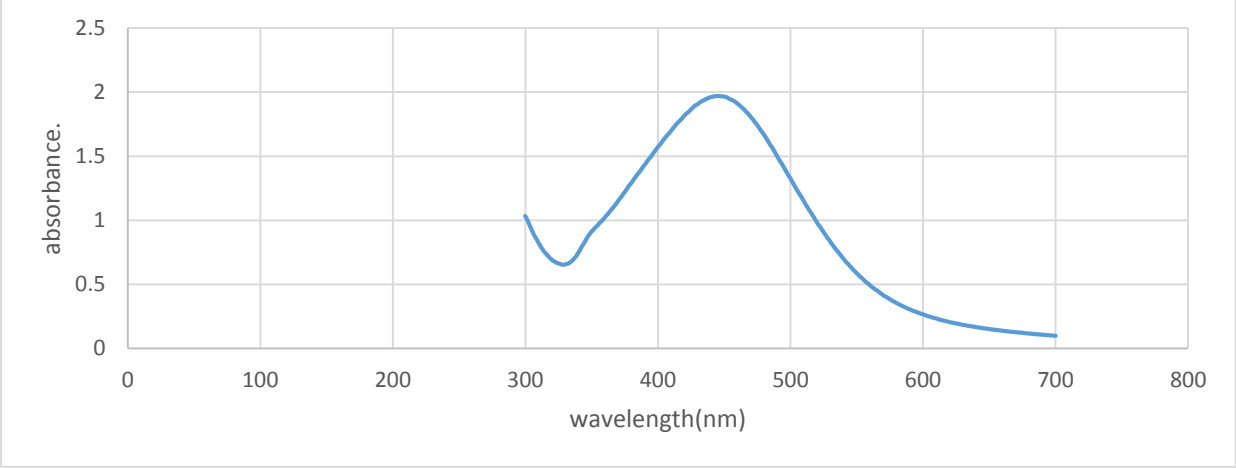
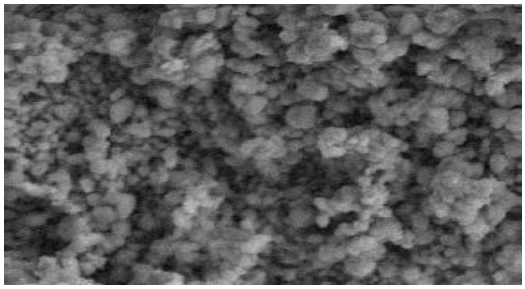
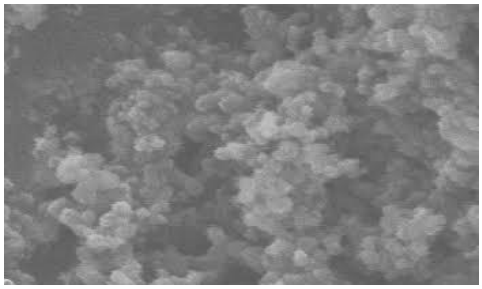


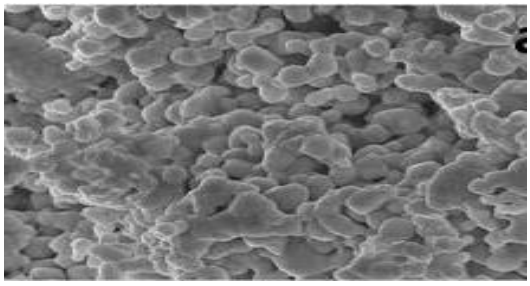
Figure 14- UV-Vis spectroscopy for the silver nano-particle from Accession no.539866



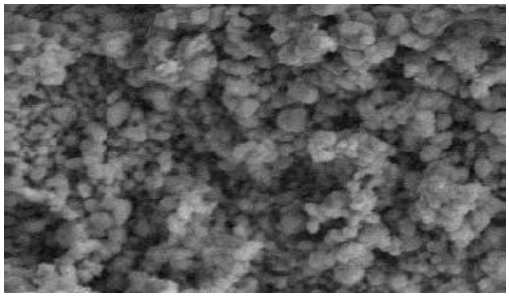
Acc. 398891



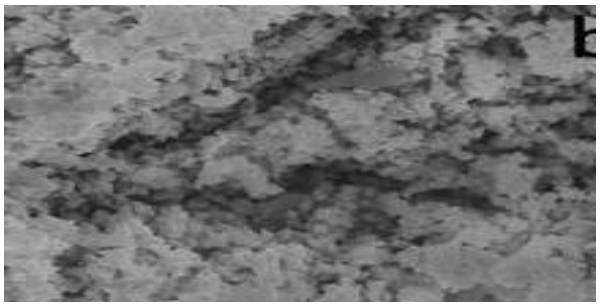
Accc. 524441



Acc. 439212



Acc. 421418



Acc no. 539866

Figure-15: SEM analysis of silver nano-particles of five different accessions.

F- Qualitative estimation of β-sitosterol in potential accession 421418 and 524441 using Gas Chromatography:

Presence of β-sitosterol was confirmed with the help of Gas chromatography technique. This analysis was done for two different accession 421418 which was grown on yeast extract used as elicitor and 524441 grown on ammonium nitrate as nitrogen source. These accessions 421418 and 524441were chosen based on their growth after eight weeks of inoculation on MS media with Yeast extract as elicitor and MS media with Ammonium nitrate as nitrogen source respectively. The analysis shows the presence of β- sitosterol in each accession at a retention time of 10.5min with an average quality of 96% of the compound present in each sample. The presence of β- sitosterol is confirmed by the library search report which shows the area, retention time, compound name and quality of the sample.

1. GC Analysis of leaf extract of *P. zeylanica* accession number 421418:

Sample-1

Library Search Report

Data Path : D:\MassHunter\GCMS\1\data\DTU\phytosterol\
Data File : 421418.D
Acq On : 23 May 2016 16:28
Operator :
Sample : 421418 base
Misc :
ALS Vial : 1 Sample Multiplier: 1

Search Libraries: C:\Database\NIST11.L Minimum Quality: 0

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	10.564	100.00	C:\Database\NIST11.L			
			.beta.-Sitosterol trimethylsilyl e ther	233648	002625-46-9	96
			.beta.-Sitosterol trimethylsilyl e ther	233651	002625-46-9	87
			Stigmastan-3,5-diene	210541	1000214-16-4	25

PHYTOSTEROL 260-310.M Mon Jun 13 14:27:23 2016

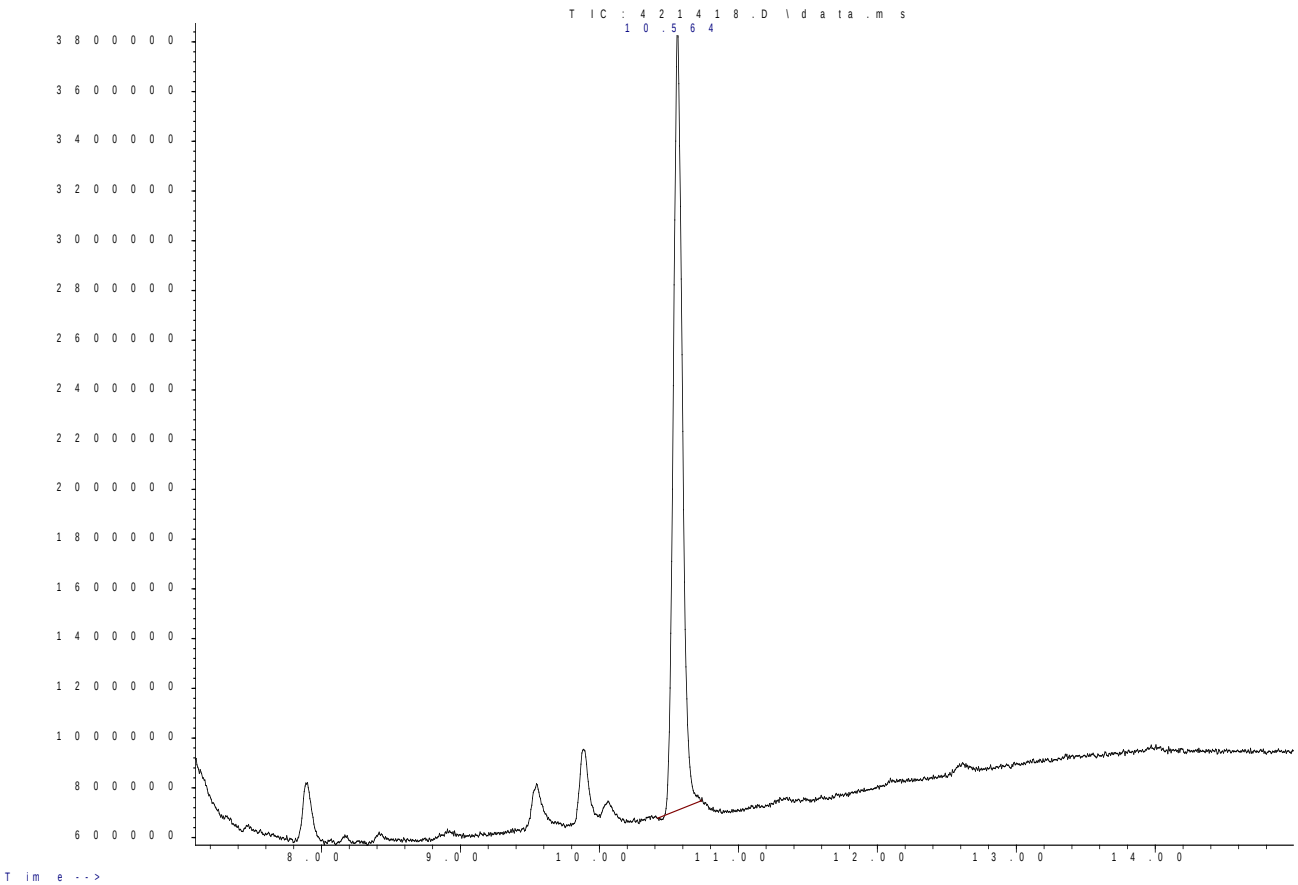


Figure-16: Chromatogram of β-Sitosterol from leaf extract of P. zeylanica Accession no.-421418 grown on yeast extract used as an elicitor.

Sample-2

Library Search Report

Data Path : D:\MassHunter\GCMS\1\data\DTU\phytosterol\
Data File : 421419ye.D
Acq On : 23 May 2016 16:47
Operator :
Sample : 421418ye
Misc :
ALS Vial : 1 Sample Multiplier: 1

Search Libraries: C:\Database\NIST11.L Minimum Quality: 0

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	10.539	100.00	C:\Database\NIST11.L			
			.beta.-Sitosterol trimethylsilyl e ther	233648	002625-46-9	96
			.beta.-Sitosterol trimethylsilyl e ther	233651	002625-46-9	94
			Stigmastan-3,5-diene	210541	1000214-16-4	44

PHYTOSTEROL 260-310.M Mon Jun 13 14:32:03 2016

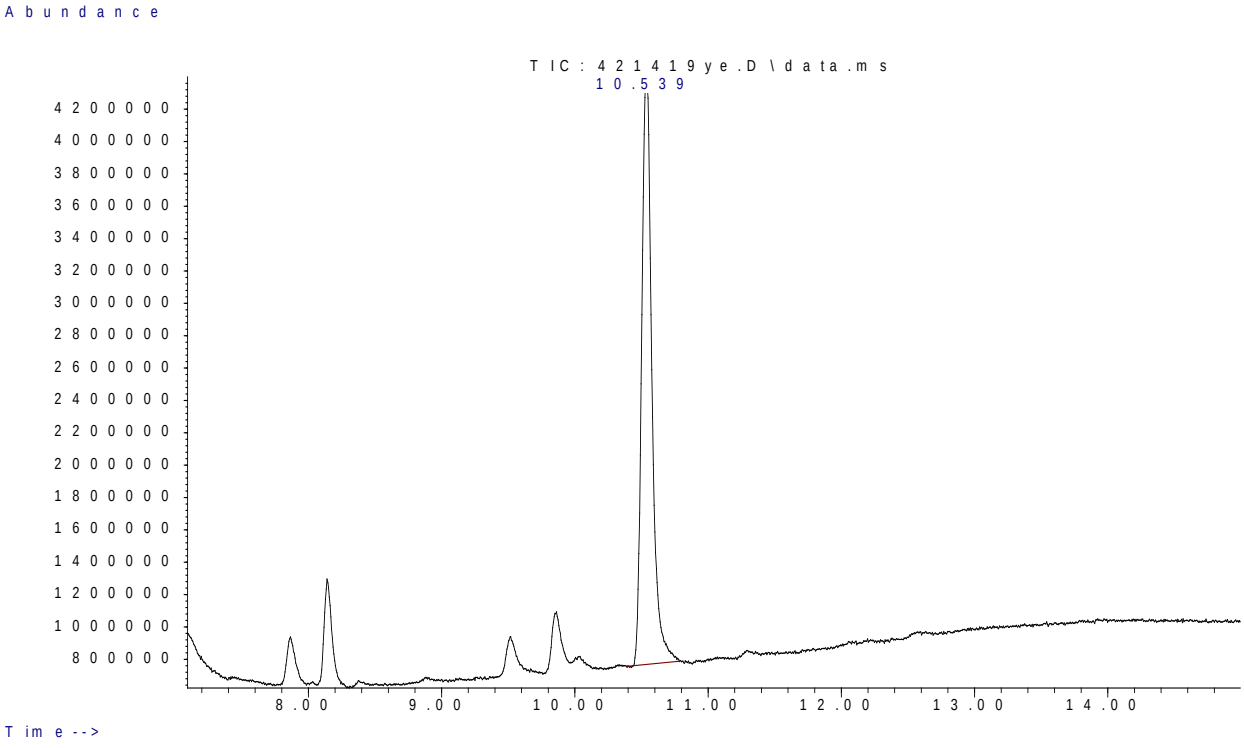


Figure-17 Chromatogram of β-sitosterol from leaf extract of P. zeylanica Accession no.-421418 grown on yeast extract used as an elicitor.

Sample-3

```
  Data Path : D:\MassHunter\GCMS\1\data\DTU\phytosterol\
  Data File : 421420ye1.D
  Acq On    : 23 May 2016  17:04
  Operator  :
  Sample    : 421418ye1
  Misc      :
  ALS Vial  : 1   Sample Multiplier: 1

  Search Libraries:   C:\Database\NIST11.L              Minimum Quality:   0

  Unknown Spectrum:   Apex
  Integration Events: ChemStation Integrator - autoint1.e
```

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	8.148	11.95	C:\Database\NIST11.L			
			(+)-.alpha.-Tocopherol, O-trimethylsilyl-	235573	002733-26-8	99
			.alpha.-Tocopherol, trimethylsilyl ether	235572	052760-33-5	94
			1,4-Bis[3-[4-trifluoromethylphenyl]-1,2,4-oxadiazolyl]benzene	235444	040067-74-1	37
2	10.541	88.05	C:\Database\NIST11.L			
			.beta.-Sitosterol trimethylsilyl ether	233648	002625-46-9	96
			.beta.-Sitosterol trimethylsilyl ether	233651	002625-46-9	87
			Stigmastan-3,5-diene	210541	1000214-16-4	44

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PHYTOSTEROL 260-310.M Mon Jun 13 14:32:58 2016
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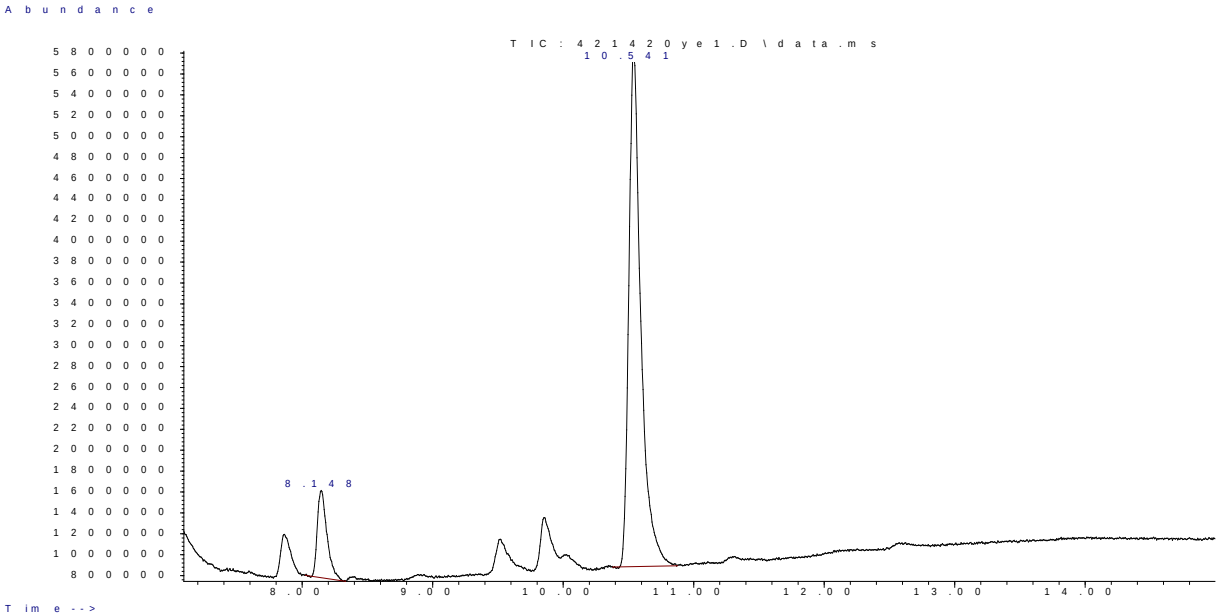

Figure-18 Chromatogram of β-sitosterol from leaf extract of P. zeylanica Accession no.-421418 grown on yeast extract used as an elicitor.

GC Analysis of leaf extract of *P. zeylanica* accession number 524441

Sample-1

Library Search Report

Data Path : D:\MassHunter\GCMS\1\data\DTU\phytosterol\
Data File : 524441a.D
Acq On : 23 May 2016 18:24
Operator :
Sample : 524441 NH4NO3-2a
Misc :
ALS Vial : 1 Sample Multiplier: 1

Search Libraries: C:\Database\NIST11.L Minimum Quality: 0

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
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			.beta.-Sitosterol trimethylsilyl e ther	233651	002625-46-9	91
			Ergosta-4,6,22-trien-3.alpha.-ol	210533	096790-32-8	44

PHYTOSTEROL 260-310.M Mon Jun 13 14:41:45 2016

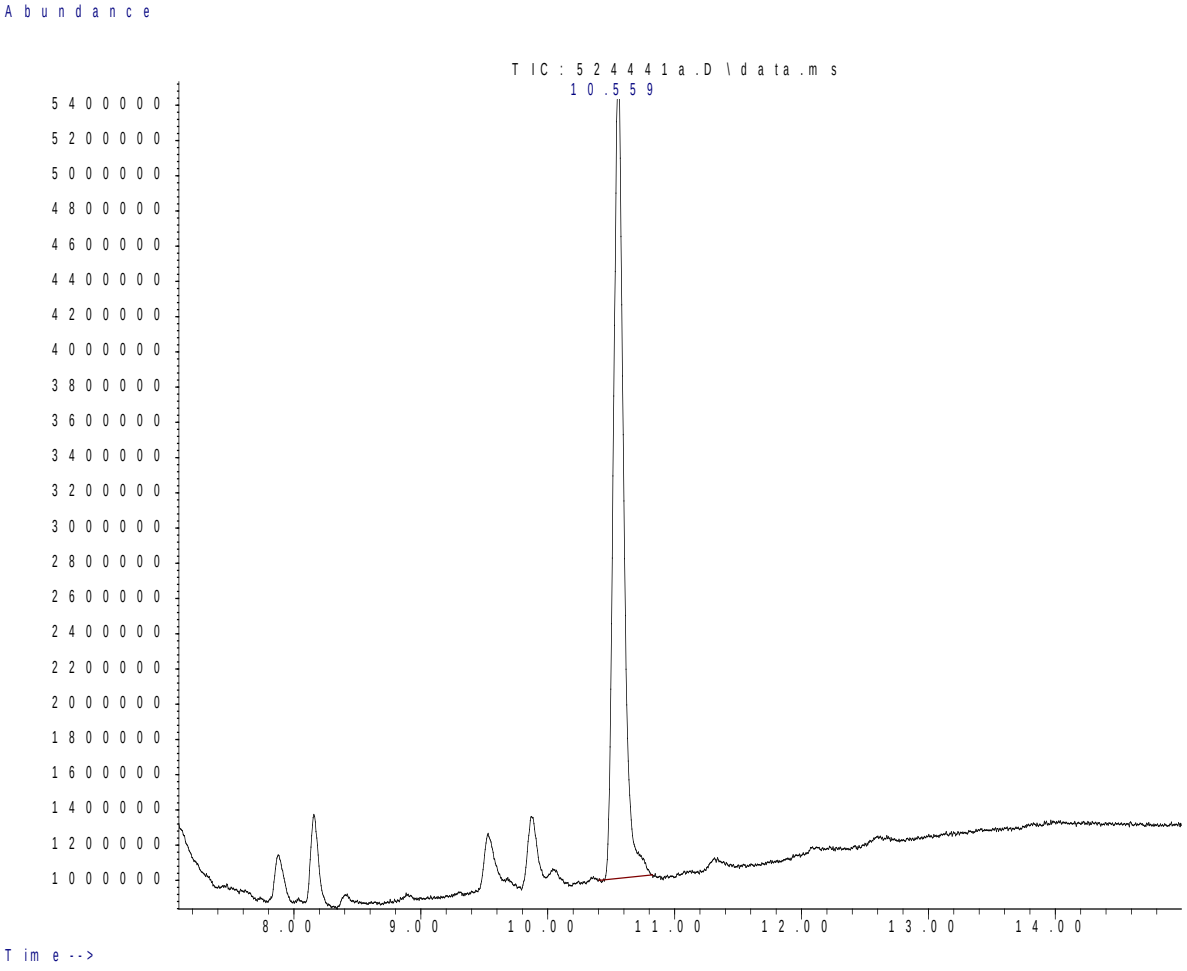

Figure 19- Chromatogram of β-sitosterol from leaf extract of P. zeylanica accession number 524441 grown on NH_4NO_3 as nitrogen source.

Sample-2

Library Search Report

Data Path : D:\MassHunter\GCMS\1\data\DTU\phytosterol\
Data File : 524441b.D
Acq On : 23 May 2016 18:43
Operator :
Sample : 524441 NH4NO3b
Misc :
ALS Vial : 1 Sample Multiplier: 1

Search Libraries: C:\Database\NIST11.L Minimum Quality: 0

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
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			.beta.-Sitosterol trimethylsilyl e ther	233648	002625-46-9	96
			.beta.-Sitosterol trimethylsilyl e ther	233651	002625-46-9	91
			Stigmastan-3,5-diene	210541	1000214-16-4	45

PHYTOSTEROL 260-310.M Mon Jun 13 14:44:11 2016

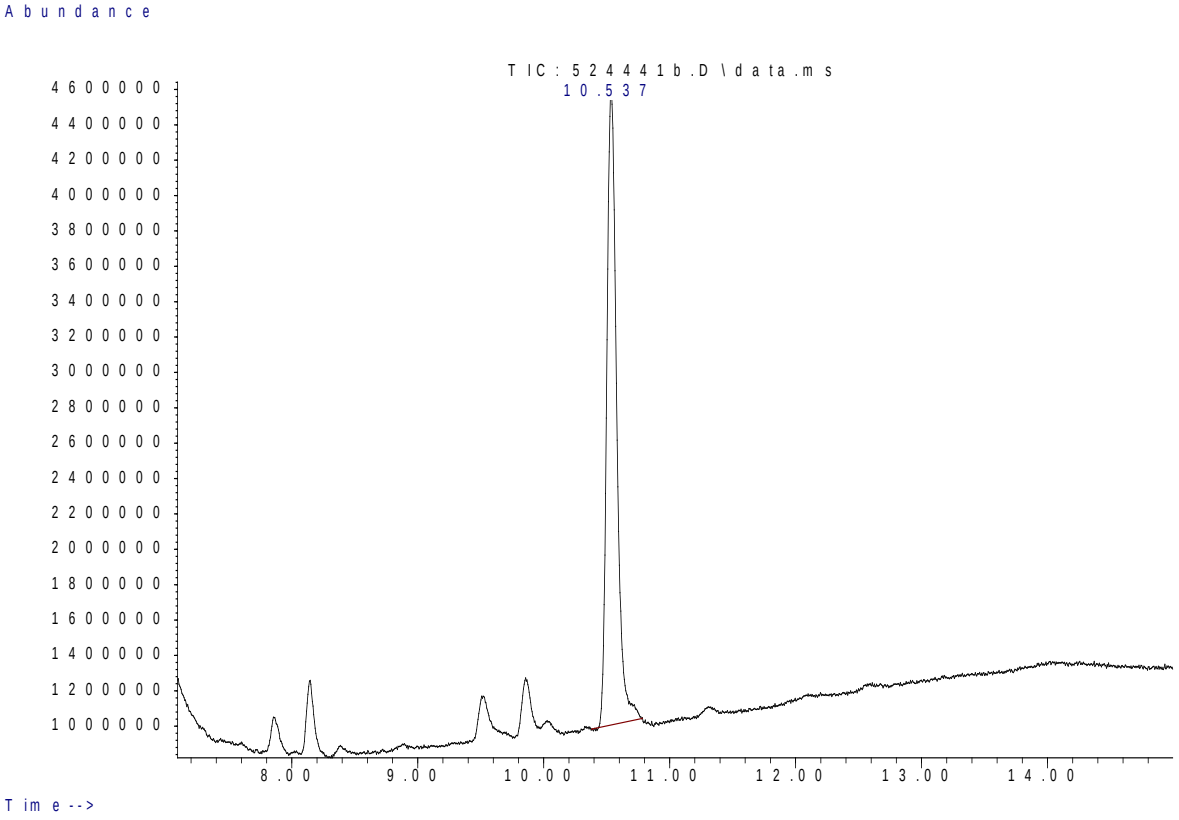

Figure 20- Chromatogram of β-sitosterol from leaf extract of P. zeylanica accession number 524441 grown on NH_4NO_3 as nitrogen source.

Sample-3

Library Search Report

Data Path : D:\MassHunter\GCMS\1\data\DTU\phytosterol\
Data File : 5244421.D
Acq On : 23 May 2016 18:01
Operator :
Sample : 524441 NH4NO3
Misc :
ALS Vial : 1 Sample Multiplier: 1

Search Libraries: C:\Database\NIST11.L Minimum Quality: 0

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
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			.beta.-Sitosterol trimethylsilyl e ther	233648	002625-46-9	96
			.beta.-Sitosterol trimethylsilyl e ther	233651	002625-46-9	87
			Stigmastan-3,5-diene	210541	1000214-16-4	44

PHYTOSTEROL 260-310.M Mon Jun 13 14:46:10 2016

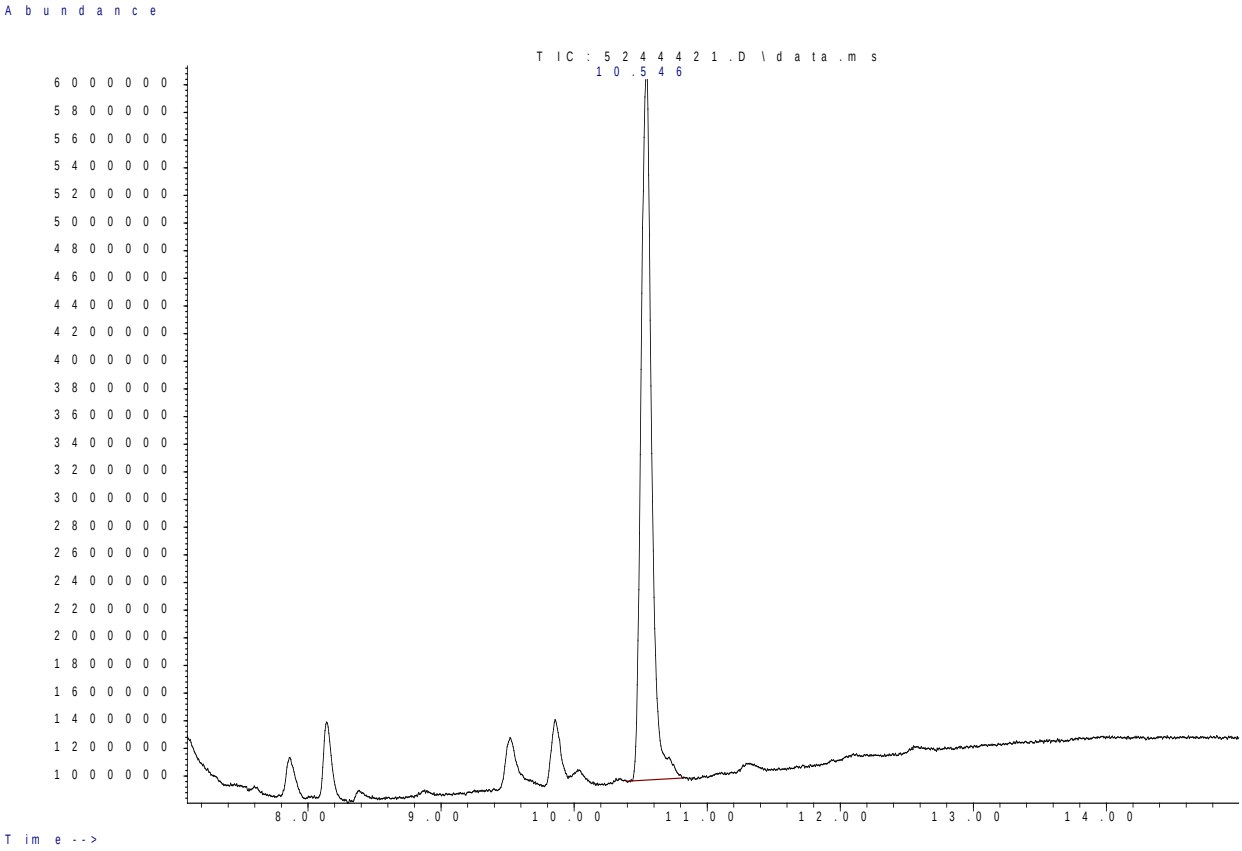

Figure- 21 Chromatogram of β-sitosterol from leaf extract of P. zeylanica accession number 524441 grown on NH_4NO_3 as nitrogen source.

Conclusion

In this study effect of different elicitors and different nitrogen source on the growth and shoot proliferation of five different accessions of *P. zeylanica* was recorded. It has been concluded that yeast extract and ammonium nitrate were found to be potential substrates for the growth and shoot multiplication. Accession number 421418 was found potential accession in case of yeast extract as plant elicitor and accession number 524441was found to be potential in case of ammonium nitrate. Along with these investigations potential of five different accession for silver nano-particle synthesis was tested and it has been recorded that each accession has the potential for silver nano-particle synthesis. Sterol analysis of potential accession 421418 and 524441 was also done with the help of Gas Chromatography. Both these accessions shown the presence of β-sitosterol.

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Richa Tyagi Ekta Menghani Phytochemical screening of Plumbago zeylanica: A potent **Herb** Suresh Gyan Vihar University, Jaipur .India 2. JECRC University, Jaipur .India International Journal of Pharma Sciences and Research (IJPSR) ISSN: 0975-9492 Vol 5 No 03 Mar 2014, 71-72

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