BIOTECHNOLOGICAL APPROACHES FOR THE PRODUCTION OF PLUMBAGIN FROM *PLUMBAGO ZEYLANICA*

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By

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June, 2020

Dedicated to My Family

DECLARATION

I, Arpita Roy, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out under the supervision of Dr. Navneeta Bharadvaja (Assistant Professor, Department of Biotechnology, Delhi Technological University, Delhi, India).The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

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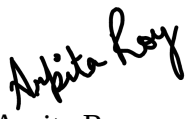

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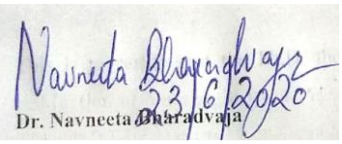
This is to certify that the Ph.D. thesis entitled **"Biotechnological approaches for the production of plumbagin from *Plumbago zeylanica*"** submitted by **Arpita Roy** (Roll No: 2K16/PhD/BT/03) to the Delhi Technological University, Delhi for the award of the degree of **Doctor of Philosophy** in **Biotechnology** is based on the original work carried out under my supervision. It is further certified that the work embodied in this thesis has neither partially nor fully submitted to any other University or Institution for the award of any degree or diploma.



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ABSTRACT

Demand for medicinal plants has drastically enhanced due to the presence of therapeutically important compounds and is continuously rising in national and international markets. Exploring elite accession among numerous accessions, collected from different locations is an alternative approach to satisfy the increase demand of medicinal plants. Biotechnological approaches are reliable source for production of therapeutically important compound. It also provides long-term utilization of plants.

Plumbago zeylanica, a pharmaceutically important medicinal plant has been explored in the present study. *In vitro* shoot culture was established for five accessions of *Plumbago zeylanica*. Four different plant tissue culture media, three different carbon sources and three different nitrogen sources were tested for five accessions of *P. zeylanica* to evaluate optimum culture condition based on growth. The accession which showed maximum shoots number was chosen for further investigation. Accession-based study of *in vitro* shoot culture showed that accession number IC 524441 is one of the elite accessions and chosen for further studies.

Adventitious root suspension culture was explored for enhanced production of plumbagin. Optimization of adventitious root suspension culture showed that highest plumbagin production was obtained in ½ strength MS liquid media having 3% sucrose with 2 g/L of inoculum density. Further elicitation with yeast extract (150 mg/L) increases threefold plumbagin production as compared to control one and highest plumbagin production was 90.96±0.51 µg/mL.

Further cell suspension culture was also explored for enhanced production of plumbagin. Optimization of cell suspension culture showed that MS medium having 1 mg/L NAA with 3 g/L inoculum density and 150mg/l yeast extract at pH 5.8 was optimal for plumbagin production.

Maximum plumbagin production was enhanced up to 3.3 times as compared to control one and maximum amount of plumbagin production was 83.30±0.18 µg/mL.

Biochemical analysis of thirteen different accessions of *Plumbago zeylanica* were performed where concentration of therapeutically important compounds such as total plumbagin, total flavonoids content, total phenol content, total tannin content and antioxidant activity were evaluated and results showed that IC-524441 is an elite accession. Same thirteen accessions were assessed for genetic diversity analysis using CBDP and SCoT marker. Genetically diverse accessions can be utilized by plant breeders for the generation of elite accessions which have high quantity of pharmaceutically important secondary metabolites.

Further residual plant material of *P. zeylanica* was used for silver nanoparticles synthesis and its application in antibacterial and dye degradation were investigated. Biochar was also derived from residual shoot and root culture and its application in removal of cadmium and chromium were studied.

Role of plumbagin against different cancer receptor using *in silico* method was also evaluated. The ligand plumbagin was docked against three different cancer receptors i.e. COX-2, EGFR and TACE to evaluate its potential effect on different cancer. *In-silico* studies showed that plumbagin is a potential therapeutic agent for treatment of cancer and same can be established through *in vivo* studies and clinical trials to confirm its efficiency in patients.

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LIST OF ABBREVIATIONS

2,4-D	2,4- dichlorophenoxy acetic acid
AMOVA	Analysis of molecular variance
BAP	6-Benzylaminopurine
CBDP	CAAT box-derived polymorphism
COX2	Cyclooxygenase 2
DPPH	2,2-diphenyl-1-picrylhydrazyle
EGFR	Epidermal growth factor receptor
EY	Eosin Yellow
FCC	Face Centred Cubic
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
Kn	Kinetin
MB	Methylene Blue
MO	Methyl Orange
MR	Methyl Red
MS	Murashige and Skoog
NAA	Naphthalic Acetic Acid
NBPGR	National Bureau of Plant Genetic Resources
NB	nutrient broth
NMPB	National Medicinal Plant Board
PR	Phenol Red
SA	Salicylic Acid
SCoT	Start Codon Targeted Polymorphism
TACE	Tumor necrosis factor alpha converting enzyme
UPGMA	Unweighed Pair-Group Method with Arithmetic Means
WHO	World Health Organization

CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1. Introduction

Plants produce diverse groups of bioactive compounds that are involved in defense mechanisms against stress conditions. Understanding the metabolic processes and use of plants for human benefits has augmented the scope of therapeutic plant utilization. Almost 50% of pharmaceuticals that are available in the market are obtained from natural materials. Market demand for medicinal plants are high due to the presence of several bioactive compounds that possess wide range of pharmaceutical applications. WHO stated that more than 80% of world's population depends principally on natural drugs for their fundamental medicinal needs. Due to the wide range of medicinal properties of plants, Indian market for medicinal plant is expected to grow around 22% during 2017-2022. This creates an opportunity for the researchers to generate huge revenue from a growing market.

Continuous supply of raw material is a great concern for process industries. Production of safe and nontoxic compounds is important for any drug development process. Drugs that are derived from natural resources interact favorably with the human body and produce beneficial effects. Today various important drugs that are available in market derived from plants, which are presently used in various countries around the world. Increasing demand for bioactive compounds in recent years resulted in the exploration of tissue culture technology for the production of valuable bioactive compounds. Bioactive compounds often accumulate in special tissues and are therefore difficult to extract, isolate and purify. Usually, these phytocompounds have complicated structures therefore chemical synthesis is not advantageous. This has encouraged extraction of

phytocompounds from naturally growing plants to meet the commercial demand due to which there is continuous exploitation of plant from natural environment.

P. zeylanica commonly called Chitrak, belongs to *Plumbaginaceae* family. It is a perennial herb, having a semi woody stem with numerous branches, and found in Bengal and Southern India region. Genus *Plumbago* contains three species i.e. *Plumbago indica L., P. zeylanica L.,* and *P. capensis L.* Among these *Plumbago zeylanica* having most therapeutic usage is a highly cultivated plant. *P. zeylanica* has multi-purpose medicinal property and contains various phytocompounds such as alkaloids, steroids, tri-terpenoides, tannins, coumarins, flavonoids and napthoquinones (Ming at al., 2011). Naphthoquinones present in the plant includes plumbagin, 3-biplumbvagin, chitranone, elliptone, chloroplumbagin, plumbagin acid, etc. Plumbagin is the most important bioactive compounds and possesses several pharmacological activities i.e. anti-cancer activity, anti-fungal, anti-oxidant, cardio-protective, anti-malarial, anti-hyperglycemic (Roy and Bharadvaja, 2018). It was reported that in Indian subcontinent plumbagin requirement is about 7 metric tonnes/year (Bhattacharya, 2008). Growing demand in domestic as well as international markets leads to overexploitation of *P. zeylanica* from its natural environment. Additionally, synthetic approach to produce plumbagin is not commercially promising (Wurm and Gurka, 1986). Slow growth of the plant and excessive harvesting leads to the enormous demand of this plant and plumbagin by pharmaceutical industries that results in depletion of wild population of the plant. Therefore, to overcome this situation and effective method of plumbagin production and conservation of the plant is required.

Conventional method of whole plant cultivation for production of bioactive compounds possesses several challenges such as low yield, time consumption, etc. Biotechnological approaches are the alternative way for commercial production of bioactive compounds. Biotechnology possesses an

opportunity to utilize organ, cell, or tissue, by growing them *in vitro* conditions and make them produce pharmaceutically important compounds. Various biotechnological methods for secondary metabolite production includes root culture, shoot culture, and cell suspension culture (Jaisi *et al.*, 2013; Silja *et al.*, 2014). For clonal multiplication of desired plant, direct shoot formation from suitable explant is of great interest. Proliferation of shoot is similar to conventional propagation method. Similarly, root culture is another alternative method for secondary metabolite production. Plant roots that are formed during normal and stressed conditions from any non-root tissue are referred to as adventitious roots. Adventitious roots growing in suitable medium supplemented with growth regulators under sterile condition shows rapid growth and production of secondary metabolite. Adventitious roots are natural, show vigorous growth in media supplemented with phytohormone and has incredible abilities of valuable secondary metabolites accumulation. Callus culture is the dedifferentiated cell that is produced on media with high auxin concentration. Cell suspension cultures have been utilized for the production of various compounds. Biotechnological method has several advantages which include bioactive compound production under controlled environment, enhanced production, chances of less microbial contamination, etc. It is a substitute for conservation and provides fast growth of elite genotype which is independent of environmental variations. Plant cells are totipotent and therefore use of biotechnological methods for production of high-value compound is an attractive method.

Presence of biologically active compounds, their production and composition are highly influenced by environmental factors. Accessions that are belongs to different agro-climatic zones contain diverse amount of phytocompounds. Environmental changes can lead to reduction of total yield but the overall therapeutic potential may be significantly improved by increasing the bioactive compounds. Therefore, it is significant to analyse the presence of diverse compounds in

the plants collected from various locations (different accessions). Plants can produce various bioactive compounds that have therapeutic activity such as flavonoids, tannins and phenolic acids. They are the major source of antioxidants. Therefore, it is important to evaluate biochemical analysis among different accessions. Information on accession-based study can be used by plant breeders for production of elite accession with highest amount of bioactive compound. Further elite accession can be provided to farmers or pharmaceutical companies for large scale production. Further evaluating genetic diversity of accessions provides valuable traits to species in demand. Molecular markers are used for genetic diversity analysis and they are very reliable due to their high-resolution power. Cross between species with desirable traits contribute to crop improvement. Further utilization of residual plant material for effective synthesis of nanomaterial provides an economical approach. Nanoparticles possess unique physicochemical and optoelectronic properties and used in various applications such as molecular diagnostics, drug delivery, imaging, catalysis or sensing. Green synthesis evolves use of biological material for nanoparticles synthesis. Plant material possesses various advantages as it is safer, eco-friendly, cheaper, easy and relatively fast as compared to microbe assisted synthesis. Among various nanoparticles, silver nanoparticles gain lot of attention as it possesses various applications such as antibacterial, antifungal, antimalarial, anti-acne, anti-plasmodial, anticancer, etc and it also has significant dye degradation efficiency. Apart from dyes, heavy metals are another detrimental contaminant which are toxic in soluble as well as elemental forms. High level of heavy metals can damage fertility of soil and also affects productivity (Hooda and Alloway, 1994). Traditional method for heavy metal elimination from wastewater is costly, therefore cost-efficient and environment friendly method is of prime requirement. Biochar is a carbon-rich product which plays an important role in heavy metals removal from wastewater. Various studies reported the efficient production of biochar from plant

material and its use in removal of different heavy metals. Utilization of bio char derived from residues of *in-vitro* grown plant is a cheap and useful method for elimination of poisonous heavy metals from wastewater.

Pharmaceutical role of *P. zeylanica* is well recognized, but the detailed mode of action against specific disease targets is still not well known. Utilization of computational approaches can help in the identification of specific target of a particular disease in a cost-effective manner with less time. In-silico method is extensively used to find out the information at molecular level. Computational results help in explaining the molecular interactions and suggest probable mechanisms that are involved in the process. Treatment of many diseases like cancer is only possible when it is identified at initial stages. Phytocompound acts as ligand against disease target and specific computational tools can be used to analyse the stability of complex. Therefore, to make this perception clear, role of plumbagin was evaluated against three cancer receptors i.e. COX-2, EGFR and TACE was evaluate.

1.2. Objectives

In the present investigation following objectives are undertaken;

1. Biotechnological approaches for plumbagin production
    a) In-vitro shoot culture
    b) Adventitious root suspension culture
    c) Cell suspension culture
2. Biochemical and Genetic diversity analysis of *P. zeylanica* accessions
3. Utilization of residual plant material of *P. zeylanica* for potential phytoremediation activity
    a) Silver nanoparticles synthesis and its application

b) Bio-char preparation from root and shoot of *P. zeylanica* for cadmium and chromium degradation

4. *In-silico* analysis of plumbagin against different cancer receptors

1.3. Structure of thesis

*First chapter**:*** This chapter deals with the introduction and objectives of the study.

Second chapter: A detailed review of literature on different topics relevant to the present study.

Third chapter: This chapter deals with the complete protocol, results and discussion of work carried out for shoot culture establishment, optimization of culture parameter and plumbagin production.

Fourth chapter: This chapter deals with the complete protocol, results and discussion of work carried out for root suspension culture establishment, optimization of culture parameter and production of plumbagin.

Fifth chapter: This chapter deals with the complete protocol, results and discussion of work carried out for callus and cell suspension culture establishment, optimization of culture parameter and plumbagin production

Sixth chapter: This chapter deals with the biochemical and genetic diversity analysis of different accession collected from different locations.

Seventh chapter: This chapter deals with the utilization of residual material of *P. zeylanica* for silver nanoparticles synthesis and its potential role in antibacterial and dye degradation activity.

Eighth chapter: This chapter deals with the utilization of residual shoot and root material of *P. zeylanica* for biochar production and used of biochar for cadmium and chromium removal.

Ninth chapter: This chapter deals with the *in-silico* analysis of plumbagin against three different cancer receptors.

Tenth chapter: This chapter deals with conclusions of entire work drawn from the outcome.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Overview of *Plumbago zeylanica*

P. zeylanica, a medicinal plant which acts as a rejuvenator which promotes strength, intelligence and longevity. It has dark green leaves which are placed alternatively on the stem. Flowers are white with diameter of 1/2 to 3/4 inch, pentamerous, regular and bisexual with a pleasant fragrance existing in a cluster or bunches. Corolla of the flower is tubular and slender in shape have white colour while the calyx is enclosed with stalked and is densely arranged, various gland is present secreting mucous like substance thus making the flower sticky. The flower possesses a basal, single ovule, pentagonous and superior ovary (Kumar *et al.,* 2009). It has long roots that are slightly branched and fresh roots are light yellow colour whereas dried one are reddish brown colour (Kumar *et al.,* 2009).

2.2 Therapeutic properties of *Plumbago zeylanica*

P. zeylanica possesses a variety of therapeutic properties like anti-cancer, anti-diabetic, anti-inflammatory, anti-malarial, anti-fertility, anti-bacterial, anti-oxidant, etc. Ahmed *et al.,* (2007) reported anti-bacterial activity of alcoholic extract of *P. zeylanica* against multi-resistant strains of *Shigella* and *E. coli.* Devi and Krishna (2012) found that *P. zeylanica* root (methanolic extract) possess anti-bacterial activity against *B. subtilis*. Shweta and Dubey (2015) studied the role of crude leaves extract of *P. zeylanica* against *E. coli, Staphylococcus aureus, B. cereus,* and *Candida* and found that they show potential anti-microbial activity. Zarmouh *et al.,* (2010) studied anti-diabetic property of *Plumbago zeylanica* against diabetic rats and found that there was an increase in hexokinase and decrease in glucose-6-phosphate when ethanolic extract (100mg) was given.

Various reports suggest anticancer activity of *P. zeylanica* against cancer cell lines. A study reported that in Ehrlich Ascites Carcinoma in animal model, *P. zeylanica* (ethanolic extract) possess significant anti-cancer activity and reduces increased level of lipid peroxidation (Hiradeve *et al.*, 2010). Sankara *et al.*, (2013) reports that *P. zeylanica* methanolic extract resulted in moderate anti-cancer activity against MCF-7 and HT-29.

2.3 Chemical composition

Every herbal plant present on the earth consists of various bioactive compounds which are very important as these bioactive compounds have the ability to act against a variety of diseases. Similarly, *Plumbago zeylanica* being a medicinal herb also contains a variety of bioactive compounds which display an imperative activity against various diseases. *P. zeylanica* contains variety of secondary metabolites and out of which naphthoquinones play significant pharmaceutical activities. Various naphthoquinones are present in the plant out of which plumbagin is the most important compound.

2.4 Plumbagin

Plumbagin (C_{11}-H_8-O_3) (Figure 2.1) is a napthoquinone which is mostly present in *Plumbago* species (Arunachalam *et al.*, 2010). Plumbagin shows several pharmacological activities i.e. anti-cancer activity, anti-fungal, anti-oxidant, cardio-protective, anti-malarial, anti-hyperglycemic (Roy and Bhardvaja, 2018). Plumbagin is a high value phytocompound, and its demand is increasing continuously. Presently, roots of *Plumbago* spp. (*Plumbago zeylanica*, *Plumbago indica (P. rosea* (1763) is a later synonym of *P. indica* (1754), *Plumbago capensis* (*P. capensis* (1794) is a later synonym of *P. auriculata* (1786)) are the mostly exploited for plumbagin. In India, the estimated annual trade of *Plumbago zeylanica*, *Plumbago indica,* and *Plumbago capensis* are reported to be 500-1000, 100-200 and below 10 million tonnes, respectively (Roy and Bharadvaja,

2018). Quantity of phytocompounds produced from plants is affected by several factors, which includes environment changes, species variations and pathogens. Cultivation of some medicinal plants is also difficult because of their low germination rate. Various plants contain plumbagin and list of them were mentioned in table 1.

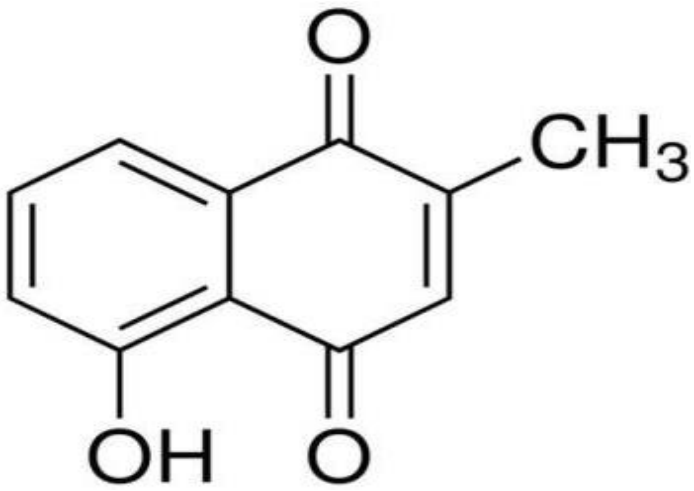


Figure 2.1: Chemical structure of plumbagin

Table 2.1- List of plant containing plumbagin

Plant Name	Plumbagin % (g DW)	Part	References
Plumbago zeylanica	0.26		De-Paiva *et al.*,2003
	1.34	Roots	Hsieh *et al.*,2005
	0.247		Dorni *et al.*,2007
Plumbago rosea	0.9-1.0	Roots	Dinda *et al.*,1997
	0.569		Dorni*et al.*,2007
Plumbago capensis	0.429	Roots	Dorni *et al.*,2007

	0.15		Itoigawa *et al.*,1991
Plumbago angustifolia	1.5-2.5	Roots	Nahalka*et al.*,1996
Dorosophyllumcapensis	0.0004	Whole plant	Crouch *et al.*,1990
Dorosophyllumlusitanicum	0.242	Whole plant	Grevenstuk *et al.*,2008
Dorosophyllumnatalensis	0.223	Whole plant	Crouch *et al.*,1990
Nephenthesthorelii	0.092	Roots	Likhitwitayawuid *et al.*,1998
Nephentheskhasiana	1.3	Roots	Raj *et al.*,2011
Nephenthesventricosa	0.51	Leaves	Shin *et al.*,2007

2.5 Chemistry of plumbagin

Plumbagin (5-hydroxy-2-methyl-1, 4- naphthoquinone) is a stirring yellow pigment that patently appears in the family *Plumbaginaceae* (Padhye *et al.*, 2010) and is also present in *Nephenthes* and *Droseraceae* (Crouch *et al.,* 1990; Shin *et al.*, 2007). It was first isolated in 1829 (D'Astafort, 1829) and it was recognized to have a quinone like character by Roy and Dutt in 1928. Roy and Dutt (1928) established that on distillation of plumbagin over zinc dust it showed presence of acidic hydroxyl group, and they obtained 8-methylnaphthalene and naphthalene. Plumbagin synthesis occurs through acetate-malonate pathway in *Plumbago* species (Mallavadhani*et al.* 1998). Quantity of plumbagin is influenced by various factors such as growth, age and flowering of the plants, its locality, soil conditions and season. Higher plumbagin content was reported in older plants grown in dry soil (Agarval and Gosh, 1985). Plumbagin is soluble in organic solvents which include chloroform, acetone, alcohol, benzene and acetic acid. This compound is exceedingly corrosive with toxic properties (Budavari *et al.,* 1996). Synthesis of plumbagin is shown in Figure 2.2.

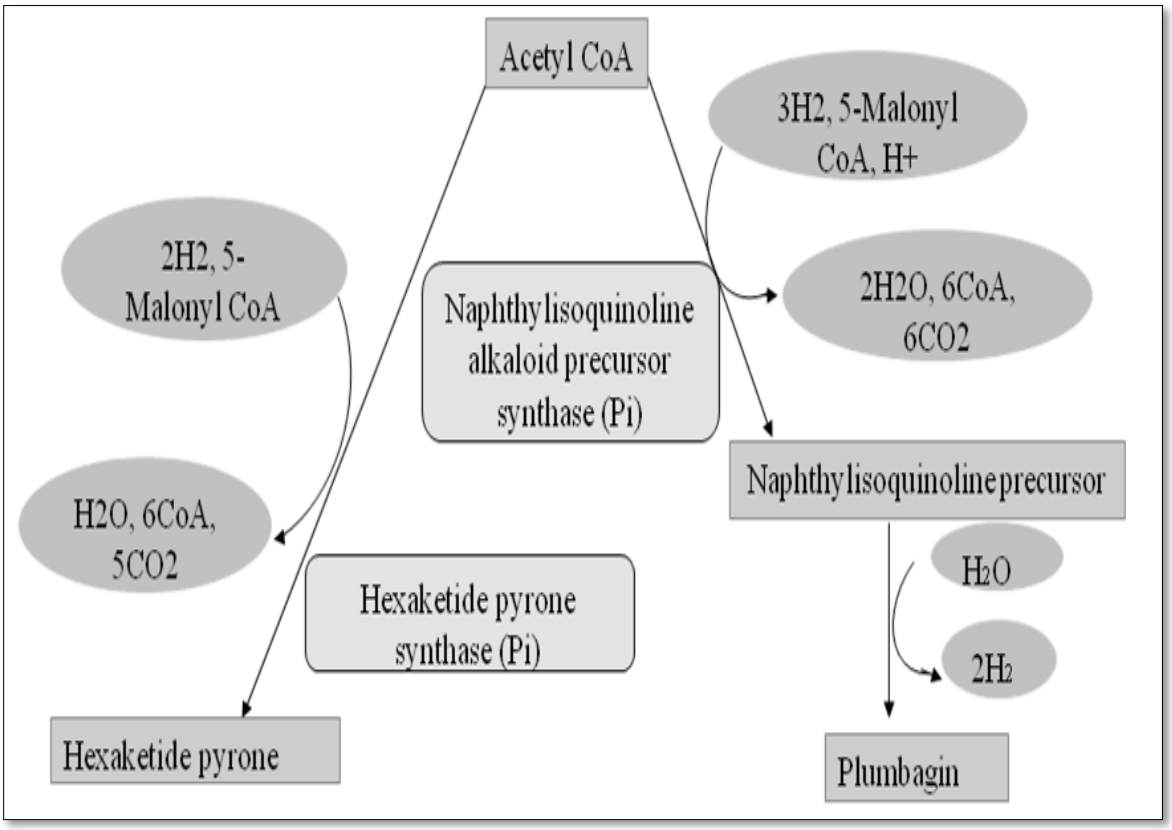


Figure 2.2: Synthesis of plumbagin (Nayak *et al.*, 2015)

2.6 Pharmaceutical activities of plumbagin

Most of the data available on plumbagin are related to its anticancer activity against several cancers which include lung, ovarian, cervical, prostate, melanoma and breast cancer (Roy and Bhardvaja, 2018). Plumbagin inactivates Akt/NF-κB, MMP-9 and VEGF signalling pathway which play significant role in invasion, metastasis, and angiogenesis (Sandur *et al.*, 2006). Plumbagin possess specific activity towards cancer cells without damaging the normal cells (Nazeem *et al.*, 2009). It is known to have anti-angiogenic, proapoptotic and anti-metastatic effects in cancerous cells. Plumbagin also inhibits NF-κB, PKCε, JNK, and STAT-3 (Yan *et al.*, 2015). Ahmad *et al.* (2008) reported that plumbagin significantly down regulates NF-κB-DNA binding activity in breast cancer cells. Plumbagin suppresses activation of NF-κB in tumor cells and therefore affects the

biological function of leukocytes (Checker at al., 2009). Sakunrangsit *et al.* (2016) investigated the influence of plumbagin on growth of human endocrine resistant breast cancer cells and observed that at micro-molar concentrations plumbagin possess cytotoxic effects. Plumbagin also showed neuroprotective effects in neonatal Sprague Dawley rats by suppressing isoflurane-induced neuronal apoptosis by regulating ERK/JNK and BDNF-TrkB-PI3/Akt signalling (Yuan *et al.,* 2017). Cao et al. (2018) reported that plumbagin inhibited the growth of KYSE150 and KYSE450 ESCC cell lines (esophageal cancer cells) and induced massive apoptosis in ESCC cells. This antitumor effect may be due to the stopping of STAT3-PLK1-AKT signalling by plumbagin. Apart from its anti-cancer activity, it shows numerous other potential roles such as anti-fertility, anti-microbial, anti-diabetic activity, anti-atherosclerotic, hypo-cholesterolemic activity, etc (Roy and Bharadvaja, 2018). Plumbagin also possess anti-gonorrheal and anti-mycobaterial activity (Kuete *et al.,* 2009). Dzoyem *et al.,* (2007) reported anti-fungal activity of plumbagin against twelve different strains of yeast and filamentous fungi and found that plumbagin showed potential antifungal activity. Nair *et al.,* (2008) reported antimicrobial activity of plumbagin against *C. albicans* and *S. aureus* and validated antimicrobial activity by using an *ex-vivo* porcine skin model. A study also reported potential of plumbagin in anti-malarial activity against 3D7 and K1 *Plasmodium falciparum* clones (Sumsakul *et al.,* 2014).

2.7 Biotechnological approaches

2.7.1 Shoot culture

P. zeylanica is cultivated vegetatively and traditional methods of propagation are difficult and not effective. Also, the production of bioactive compound is very less in wild-grown plants. Hence, to minimize the time period required for growth as well as bioactive compound production, *in-vitro* cultivation is an efficient tool. Various studies reported establishment of shoot culture of *P.*

zeylanica. Selvakumar *et al.,* (2001) reported induction of shoot from nodal explant where MS media contains 27.2µM Adenine Sulphate + 2.46 µM IBA. Verma *et al.,* (2002) reported shoot induction in MS media supplemented with 8.87mmol/l BAP+ 0.49mmol/l IBA. Chaolot *et al.,* (2006) reported that MS+4.4mg/l BAP+ 1.4mg/l IAA showed maximum shoot growth of *P. zeylanica.* Another study reported highest shooting in MS+1mg/l BAP+0.5mg/l IBA+2mg/l Adenine Sulphate (Sivanesan *et al.,* 2009). Chandravanshi *et al.,* (2014) found that MS+13.3µM BA+ 135.74 µM Adenine Sulphate provides maximum shoot growth. Chatterjee *et al.,* (2015) reported maximum shoot growth in MS media having 2mg/l BA and 0.2mg/l NAA.

2.7.2 Cell suspension culture

Cell suspension culture has become a well-established platform for natural compound production. It is an important tool and is independent of various constraints like seasonal and geographical variations. Additionally, it removes dependence on wild sources, thereby preventing its depletion. Commercialization of natural products produced by this method has also increased significant consumer acknowledgment because they come under non-GMO (Fischer et a., 2015). Plant cell suspensions are normally derived from callus that is developed on solidified media. Transfer of friable callus bunches to liquid medium and agitating on rotary shakers results in formation of single cells or small aggregates. Various studies reported production of plumbagin through cell suspension culture. Nahálka *et al.,* (1996) reported production of plumbagin in cell suspension culture of *Drosophyllum lusitanicum* Link. They reported the influence of different media and hormones in plumbagin production. The maximum amount of plumbagin obtained was 1.5 g/l. Komaraiah *et al.,* (2002) reported enhanced plumbagin production (15.18 mg/g) from cell suspension culture of *P. rosea* where chitosan (150 mg/l) was used as an elicitor. Silja *et al.,* (2016)

reported enhanced plumbagin production (12.05 mg/g) from cell suspension culture of *Plumbago rosea.*

2.7.3 Root culture

Adventitious root culture is considered to be a good source of crude material for pharmaceutical industries. Adventitious root cultures are an alternative and effective way to produce high value phytocompounds. They possess fast growth, biochemical stability and relatively high secondary metabolites production. Accumulation of plumbagin occurs mainly in roots of *Plumbago* species, due to which culturing of roots provides a substitute method for enhanced production of plumbagin. Limitations associated with establishing root cultures include low survival rate, high chances of contamination and slow growth rate. Panichayupakaranat and Tewtrakul (2002) studied adventitious root cultures of *Plumbago indica* using various concentrations of NAA (0.5-2.0 mg/l) and kinetin (0.1-0.5 mg/l) in B5 media. They found the highest concentration of plumbagin (0.016 ± 0.0030% dry weight) in a media supplemented with 1 mg/l NAA and 0.1 mg/l Kn. Jaisi *et al.* (2013) reported higher production of plumbagin (1.04 mg/g Dry weight) from gamma ray treated root cultures of *Plumbago indica.* Silja and Satheeshkumar (2015) also established adventitious root culture from *Plumbago indica*leaf explants and found maximum production in media supplemented with 0.5 mg/L IBA and 0.1 mg/L GA3. Jaisi *et al.* (2016) reported enhanced plumbagin production in root cultures of *Plumbago indica* by application of biotic and abiotic elicitors. They have found 10.6mg/g dry weight of plumbagin in a culture treated with 150mg/l chitosan and 7.6 mg/g dry weight of plumbagin in a culture treated with 100 µM $AgNO_3$. Jaisi and Panichayupakaranant (2020) reported enhanced production of plumbagin i.e. 14.62 mg/g DW in chitosan and L-alanine treated root culture of *Plumbago indica*.

2.8 Biochemical and Genetic diversity analysis

Analysis of important bioactive compounds in different accessions is essential to find out the best one as therapeutic value of medicinal plants depends on presence of bioactive compounds. Different pharmacological activities depend upon the presence and concentration of various bioactive compounds such as flavonoids, tannins, phenolic compounds, etc. Various studies reported antioxidant activity of *P. zeylanica*. Tilak *et al.,* (2004) assessed antioxidant activity of root extract of *P. zeylanica* using DPPH method and obtained maximum antioxidant activity of 298 ± 19 (µmol/l). They observed that antioxidant activity is due to the production of phytocompounds by the plant. Abera *et al.,* (2015) also assessed the antioxidant activity of root extract of *P. zeylanica* and found maximum antioxidant activity of 156.00±0.048 mg/g using DPPH method. Alam et al. (2012) reported that total phenol content significantly influences the antioxidant activity of plant. Tilak *et al.,* (2004) evaluated total phenol content and total flavonoid content in *P. zeylanica* plants and found maximum total phenol content of 523 ± 3.5 µmol/l and total flavonoid content of 112 ± 5.9 µmol/l. Jaradat *et al.,* (2016) reported evaluation of total tannin content from leaves of *Plumbago europaea* and *Plumbago auriculate*. They found 29.58 ± 0.24 mg/g of tannin content in case of *P. europaea* and 9.55 ± 0.2 mg/g in case of *P. auriculate*.

Genetic diversity analysis among various accessions can be done by using different molecular markers. Accessions which are genetically varied can be utilized for improvement of crops. Genetic diversity is the total number of genetic characteristics in the genetic makeup of a species. Around 30 to 90 % of genome comprises repetitive DNA and they are highly polymorphic. These regions are vulnerable for induction of mutations which is responsible for evolution. These DNA regions form the base of various markers, which can be used for analysis of plant genome.

Molecular markers are not able to find out particular gene activity but able to analyze diversity among accessions. They are not influenced by environmental parameters and therefore produce reliable results. Better quality and high yielding accessions can be used by farmers and pharmaceutical industries to enhance production of compounds and drug development. Therefore, genetic diversity analysis is a vital method for characterization and production of elite accessions to obtained high quality natural drugs.

2.9 Silver nanoparticles synthesis and biochar production

Further utilization of residual plant material for effective synthesis of nanomaterial provides an economical approach. Nanotechnology basically deals with nanomaterial synthesis which ranges from 1-100nm. Nanoparticles possess extraordinary properties because of their specific potentials like shape, size, and distribution compared to larger particles. They exhibit high surface to volume proportion with decreasing size. In materials science, green synthesis has gained wide consideration due to its advantages like eco-friendly, reliable and sustainable protocol for synthesizing wide range of nanomaterials (Sunkar and Nachiyar, 2012). Nanoparticle synthesis using biological method utilizes micro-organisms, enzyme and plant extract. However, plant extract possesses numerous advantages over other methods such as simple method, utilization of non-toxic materials, cost-effective and biocompatibility. Various nanoparticles i.e. Ag, Pt, Au, and Pd were synthesized using plant extract. Among them, silver has a potential role in biological systems and pharmaceuticals. Therefore, silver nanoparticles synthesis from residual material of *P. zeylanica* was investigated and its biological and environmental application were evaluated. Similarly, utilization of residual plant material for effective production of biochar provides an economical approach. As traditional method for heavy metal elimination is costly for large volumes, therefore cost-efficient and environment friendly method are required. Biological

methods are cost-effective and environmentally friendly. Various approaches have been investigated for efficient heavy metals removal using bio-sorbents such as fly ash, peat, microbial biomass and agricultural byproducts which include soya bean hulls, sugarcane bagasse, cotton seed hulls, walnut hulls and corn cobs (Al-Qahtani *et al.,* 2016). Recently, few reports have come up with the utilization of bio char for heavy metal degradation. Biochar is prepared by biomass heating under low oxygen or even absence of it. Due to its high aromaticity, it is considered as an effective absorbent for both organic and inorganic pollutants. Bio char has several advantages like eco-friendly, economical and aesthetically acceptable. Utilization of bio char derived from residual plant material is a cheap and useful method for elimination of poisonous heavy metals from wastewater. Therefore, bio-char production from residual material of *P. zeylanica* was investigated and its application in cadmium and chromium removal were evaluated.

The literature review been published in "**Current Pharmaceutical Biotechnology**", (Bentham Science Publishing)

Arpita Roy and Navneeta Bharadvaja "Biotechnological Approaches for Production of Pharmaceutically Important Compound: Plumbagin", Current Pharmaceutical Biotechnology, 2018, 19(5), 372-381. IF-1.516

CHAPTER 3

ESTABLISHMENT OF IN VITRO SHOOT CULTURE OF *PLUMBAGO ZEYLANICA* FOR PLUMBAGIN PRODUCTION

3.1 Introduction

Conventional propagation method using seed is not a reliable and influence by environmental factors. Therefore *in-vitro* propagation offers an efficient way for rapid mass propagation and conservation of elite genotype which are independent from seasonal changes. *In vitro* propagation possesses various advantages such as higher multiplication and growth rate, production virus free plants, etc. Various factors influence growth and shoots multiplication of plants these includes culture media carbon source, nitrogen source, etc. Carbon source maintains osmotic potential and provides energy to the cells (Durand *et al.,* 2018). Different media contains different salt concentration which are required for optimum growth. Similarly, nitrogen sources are also affecting the growth of plants. Quantity of bioactive compounds depend upon geographical environment of an accession (Yang *et al.,* 2018). Various factors like plant's age, specific part of plant and climate conditions affects phytocompound production and possess significant challenges in order to identify a potential accession. Objective of this experiment was to optimize nitrogen source, carbon source and culture medium which promotes growth of different accessions. Media which provides maximum growth in all the accessions were consider for further experiments. Ethyl acetate of leaves were used to analyzed the plumbagin content in all the accessions.

3.2 Materials and methods

3.2.1 Plant material collection and culture conditions

Accession number - IC-421418, IC-539866, IC-398891, IC-524441 and IC- 439212 of *Plumbago zeylanica* were obtained from NBPGR, New Delhi.

For initial establishment of aseptic culture semisolid MS basal medium with 3% sucrose and 0.8% agar was used. Medium pH was adjusted to 5.8 before autoclaving. For carbon source optimization three different sources i.e. glucose, sucrose and fructose (at 3% w/v) were used. For nitrogen source optimization three different sources i.e. sodium nitrate, ammonium nitrate, and potassium nitrate were used. Culture media optimization was performed using four different medias i.e. MS, White, Gamborg's B5, and Nitsch medium. Media was supplemented with 1 mg/l BAP for all the experiments. Nodal explants from each accession were aseptically transferred to media and incubated at 25±2°C with 16h photoperiod using 36W fluorescent lamps.

Results were expressed in mean values ± standard error (SE) with replicates of three each containing three explants. Effect of various treatments on shoot growth was compared to detect significant differences among the treatments using ANOVA at 5 % probability level.

3.2.2 GC-MS analysis and plumbagin estimation

Preparation of ethyl acetate extract: - - Freshly *in-vitro* grown plant materials were air dried at room temperature and then grounded with mortar pestle. 1gm plant material was then macerated in 10ml of ethyl acetate for 48 hours and then filtered for further analysis.

Plumbagin estimation- 10 mg of plumbagin standard (Sigma Aldrich) was dissolved in 10ml of ethyl acetate (1mg/ml stock). From stock solution, further dilutions were done and 1µl solution was injected into the system. GC-MS (Agilent GC (7890B GC)) analysis was done using 5MS (5% phenyl, 95% methyl syloxan) capillary column where temperature of injection port and detector was 270 °C and 280 °C respectively. Flow rate of 1.0 ml/min and split ratio of 1:20 were

used. Sample volume of 1µl was used with direct injection mode and compounds were identifyed using NISTII library database.

3.3 Results and Discussion

3.3.1 Effect of different carbon source on *Plumbago zeylanica* accessions

Carbon source is vital for in vitro culture conditions, it enhances the growth of plantlets. 2–4 % sucrose is most favorable for any propagation system and therefore 3% of carbon sources were used in this experiment. Influence of carbon sources on multiplication of shoot was observed (Table 3.1). After three weeks of inoculation, MS media containing 3% sucrose showed shoot initiation. Highest shoot length and number was observed in accession number IC-524441 i.e. 4.2±0.54 cm and 5.2±0.44 respectively whereas minimum shoot number and length was observed in accession number IC-539866 i.e. 2.2±0.44 and 3.0±0.29cm respectively. In case of 3% glucose supplemented media accession number IC-524441 showed maximum shoot number (3.2±0.44) and shoot length. Minimum growth was observed in fructose supplemented media. Carbohydrates helps in morphogenesis which is accountable for enlargement and cell wall composition (Baskaran & Jayabalan 2005). Buah *et al.* (2000) reported that in case of tissue cultured banana plants**,** sucrose containing media produced more leaf number as compared to media containing glucose and fructose. They also reported that at high temperatures fructose released a toxic substance increased hyperhydricity and decreased water potential in leaves which cause leaves enlargement. Baskaran & Jayabalan (2005) reported that in case of *Eclipta alba*, growth of plantlet was maximum in medium contains 3 % glucose. In case of *Solanum nigrum* maximum shoot length was observed in 4% sucrose containing media whereas maximum shoot number was in media containing 4% fructose (Sridhar and Naidu, 2011). In case of *Arnica montana,* it was found that 3% sucrose provided optimum growth (Petrova *et al.,* 2015). Kundu *et al.,* (2016) also observed

that 3% sucrose containing media gives maximum shoot multiplication in case of *Centella asiatica*.

Table 3.1: Effect of carbon sources on *Plumbago zeylanica* accessions

Experiment detail	Accession Number	Shoot Number (M±SE)	Shoot Length (cm) (M±SE)
Sucrose	IC-524441	5.2±0.44[a]	4.2±0.54 [a]
	IC-398891	2.8±0.45[b]	3.4±0.76 [b]
	IC-439212	2.4±0.54 [b]	3.2±0.44 [b]
	IC-539866	2.2±0.44 [b]	3.0±0.29 [b]
	IC-421418	4.6±0.54 [a]	3.5±0.41 [b]
Glucose	IC-524441	3.2±0.44[a]	3.4±0.27[a]
	IC-398891	2.2±0.45 [b]	3.2±0.44 [a]
	IC-439212	1.6±0.54 [c]	2.8±0.22 [a]
	IC-539866	1.8±0.45[c]	3.3±0.44 [a]
	IC-421418	2.4±0.54 [b]	3.1±0.38 [a]
Fructose	IC-524441	2.1±0.45 [a]	2.7±1.27 [b]
	IC-398891	1.8±0.45 [a]	2.3±1.13 [b]
	IC-439212	1.7±0.54 [a]	2.5±0.72 [b]
	IC-539866	1.4±0.54 [b]	3.6±0.68 [a]
	IC-421418	1.2±0.45 [b]	3.3±0.65 [a]

3.3.2 Effect of nitrogen sources on *Plumbago zeylanica* accessions

Nitrogen, a vital element for growth also influences shoot multiplication. Its availability and form in which it is present are important for growth of plant in culture media. Nitrate has been considered as an important source of nitrogen for tissue culture. To optimize nitrogen source, different source of nitrogen i.e. sodium nitrate, ammonium nitrate and potassium nitrate were used (Table 3.2). After three weeks of inoculation, MS media containing ammonium nitrate showed shoot initiation. Maximum shoot length and number (4.1±0.56) was observed in accession number IC-524441 i.e. 3.179±0.21cm and 4.1±0.56 respectively where media was containing ammonium nitrate. Minimum shoot number and length was obtained in case of accession number IC-539866 i.e. 4.111±0.39cm and 2.2±0.42 respectively. Sodium nitrate followed by potassium nitrate showed less growth in all accessions. Mohamed (2011) studied influence of various nitrogen source in case of *Zea mays* and observed that ammonium sulphate nitrate enhanced growth as compared to other source of nitrogen. Chandravanshi *et al.*, (2014) reported highest shoot growth in case of *P. zeylanica* where MS media containing ammonium nitrate.

Table 3.2: Effect of different nitrogen sources on *Plumbago zeylanica* accessions

Experiment detail	Accession Number	Shoot Number (M±SE)	Shoot Length (cm) (M±SE)
NH_4NO_3	IC-524441	4.1±0.56[a]	3.179±0.21[b]
	IC-398891	3.3±0.67[b]	3.759±0.41[b]
	IC-439212	2.3±0.48[c]	4.188±0.05[a]
	IC-539866	2.2±0.42[c]	4.111±0.39[a]
	IC-421418	3.4±0.51[b]	3.719±0.80[b]

	IC-524441	3.0±0.47 [a]	3.033±0.36 [a]
	IC-398891	1.8±0.42 [b]	2.883±0.23 [b]
$NaNO_3$	IC-439212	2.2±0.42 [b]	3.194±0.13 [a]
	IC-539866	1.9±0.57 [b]	4.083±0.66 [a]
	IC-421418	2.3±0.48 [b]	3.8±0.78 [a]
	IC-524441	2.8±0.42 [a]	2.744±0.35 [b]
	IC-398891	2.1±0.31 [a]	2.963±0.25 [b]
KNO_3	IC-439212	2.2±0.42 [a]	3.35±0.18 [a]
	IC-539866	1.7±0.48 [b]	3.766±0.28 [a]
	IC-421418	2.7±0.48 [a]	2.633±0.24 [b]

3.3.3 Effect of different culture medias on *Plumbago zeylanica* accessions

Culture media play a significant role in shoot multiplication. Influence of various media on number of shoots in case of all the accessions is furnished in Table 3.3. Shoot length and shoot number was highest in MS media in case of accession number IC-524441 (3.839±0.7 cm and 6.0±0.47). Among different media, best growth was recorded in MS medium which supports maximum shoot multiplication in all the accessions of *P. zeylanica* followed by Nitsch, B5, and White media. Chandravanshi *et al.,* (2014) reported highest multiplication of shoot in MS media containing 13.3µM BAP+135.74µM Adenine Sulphate in case of *P. zeylanica*. Another study reported that MS+ 4.44µM BAP provide highest shoot growth in *P. zeylanica* (Vijay *et al.,* 2016).

Table 3.3: Effect of different media on *Plumbago zeylanica* accessions

Experiment detail	Accession Number	Shoot Number (M±SE)	Shoot Length (cm) (M±SE)
WHITE Media + 1 mg/l BAP	IC-524441	3.2±0.42[a]	3.244±0.108 [a]
	IC-398891	2.9±0.56 [b]	2.822±0.2 [b]
	IC-439212	2.6±0.51 [b]	2.486±0.22 [b]
	IC-539866	2.0±0.47[b]	3.588±0.73 [a]
	IC-421418	3.0±0.47 [a]	2.664±1.37 [b]
NITSCH Media + 1 mg/l BAP	IC-524441	3.3±0.48 [a]	3.688±0.55[a]
	IC-398891	2.5±0.52 [b]	3.969±0.5 [a]
	IC-439212	2.7±0.57 [b]	1.9±0.18[c]
	IC-539866	2.1±0.56 [b]	2.572±0.25 [b]
	IC-421418	3.2±0.42 [a]	2.836±0.14 [b]
MS Media + 1 mg/l BAP	IC-524441	6.0±0.47 [a]	3.839±0.7 [a]
	IC-398891	5.2±0.42 [b]	3.341±0.13 [a]
	IC-439212	4.9±0.56 [b]	2.928±0.26[b]
	IC-539866	3.9±0.56[c]	3.236±0.64 [a]
	IC-421418	5.3±0.48 [a]	3.694±0.22 [a]
B5 Media + 1 mg/l BAP	IC-524441	4.7±0.48 [a]	2.922±0.49 [b]
	IC-398891	2.7±0.48[c]	3.48±0.2 [a]

IC-439212	1.6±0.51[d]	3.933±1.65 [a]
IC-539866	1.2±0.53[d]	2.966±1.12[b]
IC-421418	3.2±±0.42[b]	2.5±0.52 [b]

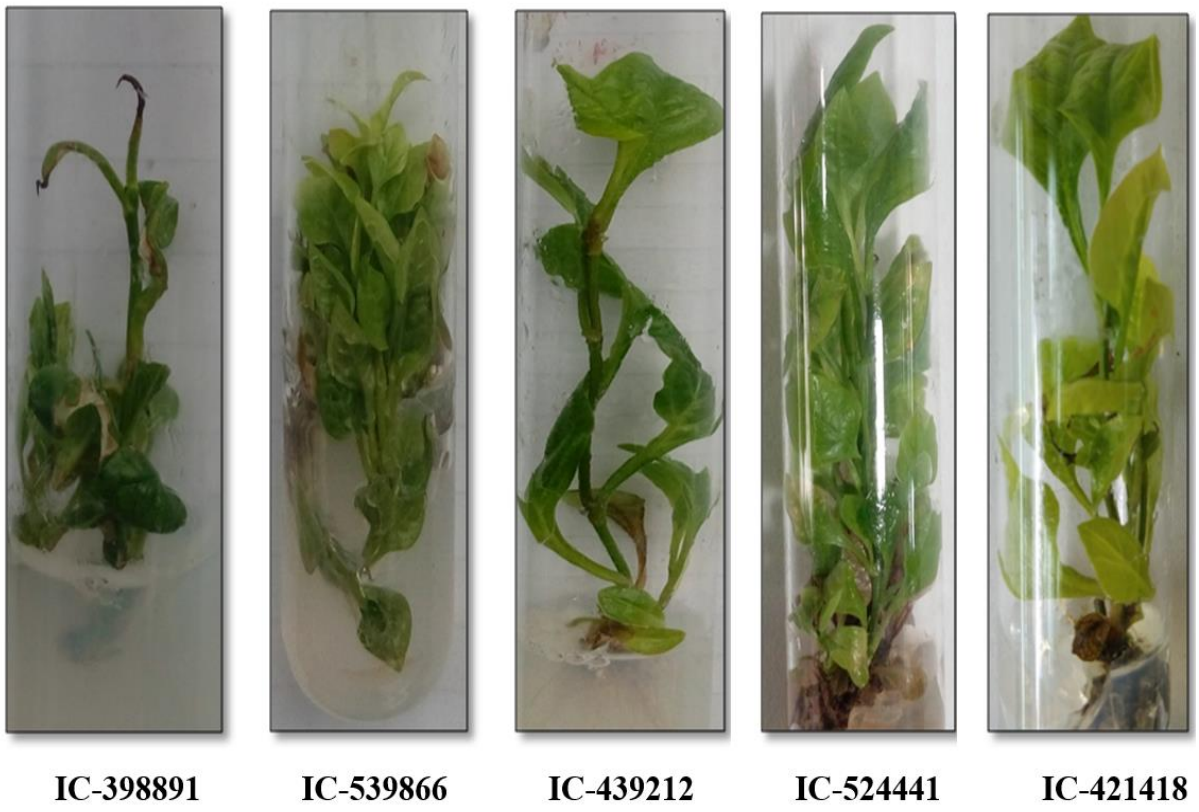


Figure 3.1: Shoot culture of *Plumbago zeylanica* accessions grown in MS medium with ammonium nitrate and 3% sucrose

3.3.4 Plumbagin estimation

Plumbagin estimation was done by comparing the peaks of samples with plumbagin standard (Sigma-Aldrich). Peak of plumbagin was obtained at retention time 13.939-14.001 (Figure 3.2). From Figure 3.4 it is clear that accession number IC-524441 contains maximum plumbagin content.

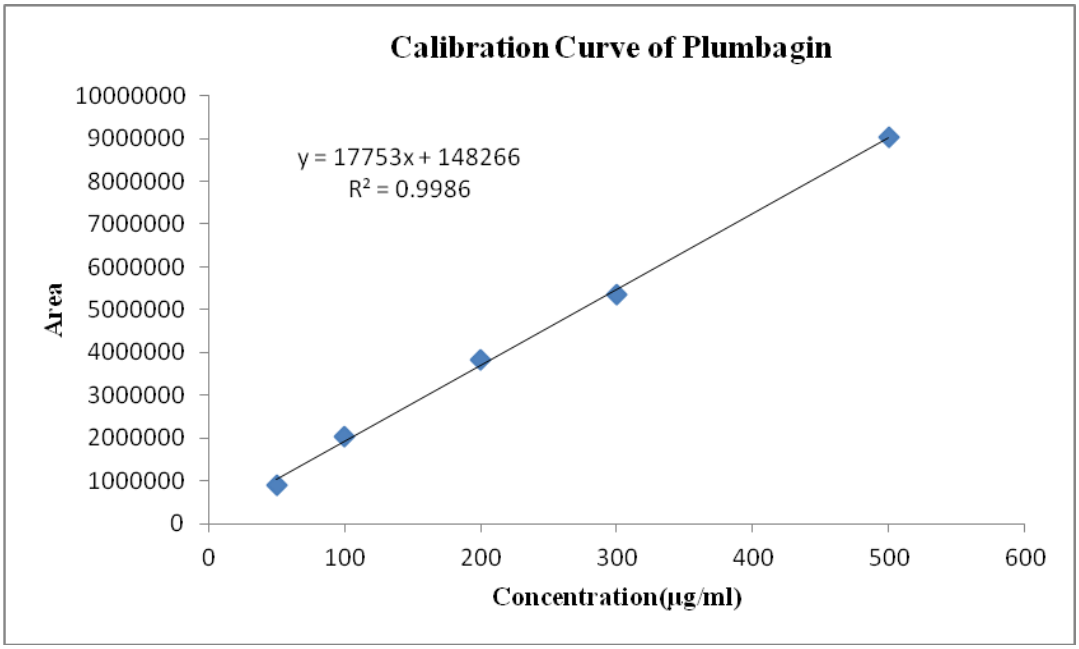


Figure 3.2: Calibration curve of Plumbagin

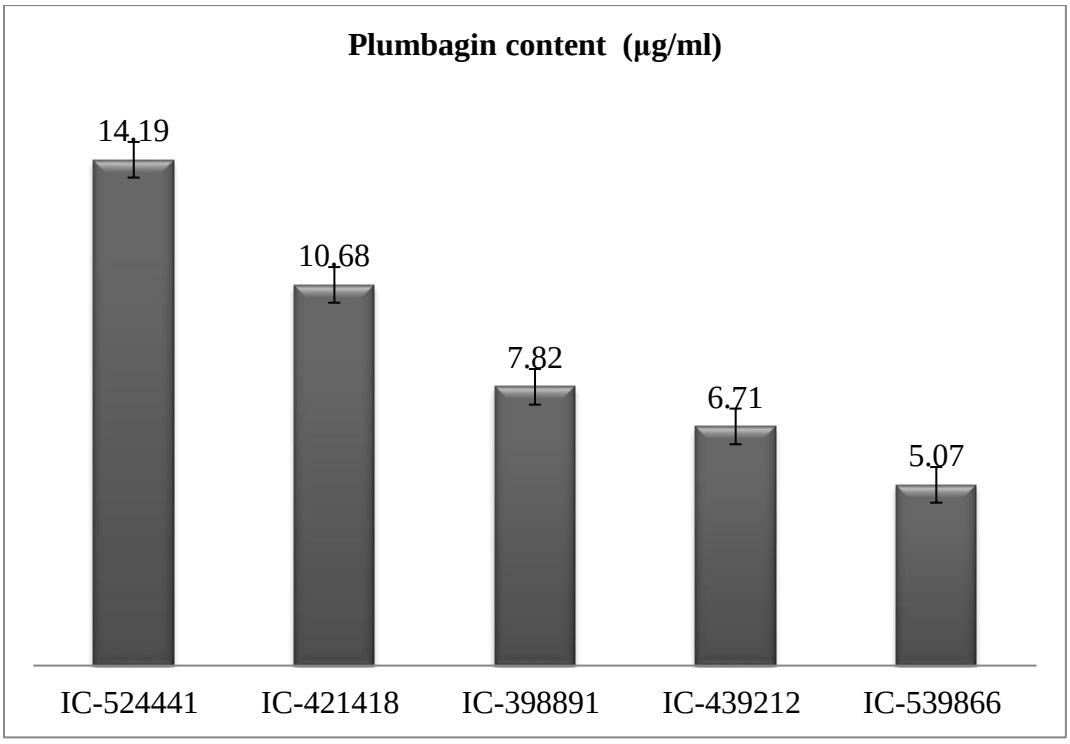
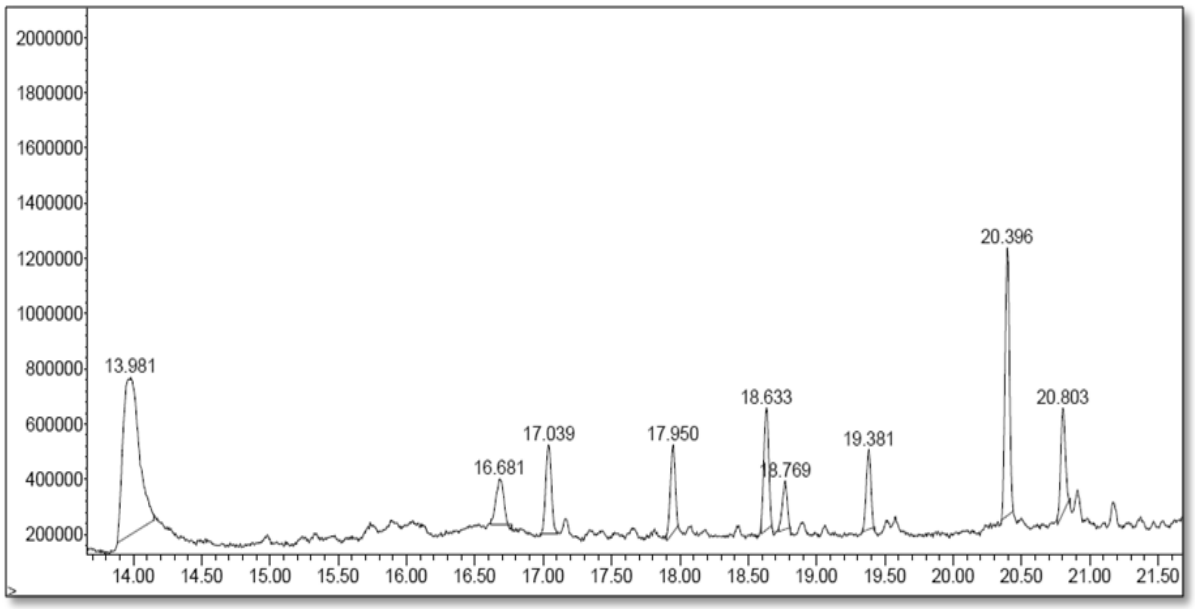


Figure 3.3: Plumbagin production from shoot culture of different accessions of *P. zeylanica*

3.3.5 GC-MS analysis

GC-MS analysis of all the accessions showed presence of various compounds (Table 3.4 and Figure 3.2). Results showed the presence of 1, 4-Naphthalenedione, o-Anisic acid, 5-Eicosene, Phthalic acid, 1-Octadecene, Thioctic acid, 2-Benzoyl-1, 3-Eicosene, 2-dihydro-1-isoquinoli necarbonitrile, Silane, Octadecanal, 5-t-Butyl-cycloheptene, 3-Methoxy-2-methyl-2-(1-phenyl-ethylamino)-propionic acid and 1-Nonadecene. Identification of compounds was done using NISTII library. Accession number IC-524441 contains higher amount of 1, 4-Naphthalenedione i.e. plumbagin as compared to other three accessions. A study reported 1, 4-Naphthalenedione presence in *Plumbago zeylanica* roots (Rajakrishnan *et al.*, 2017). Similarly, another study reported presence of 2-Pentadecanone, 1-Heptadecene, Tetracontane, Tetradecanoic acid, Isophytol in *P. zeylanica* (Sharma *et al.*, 2015).



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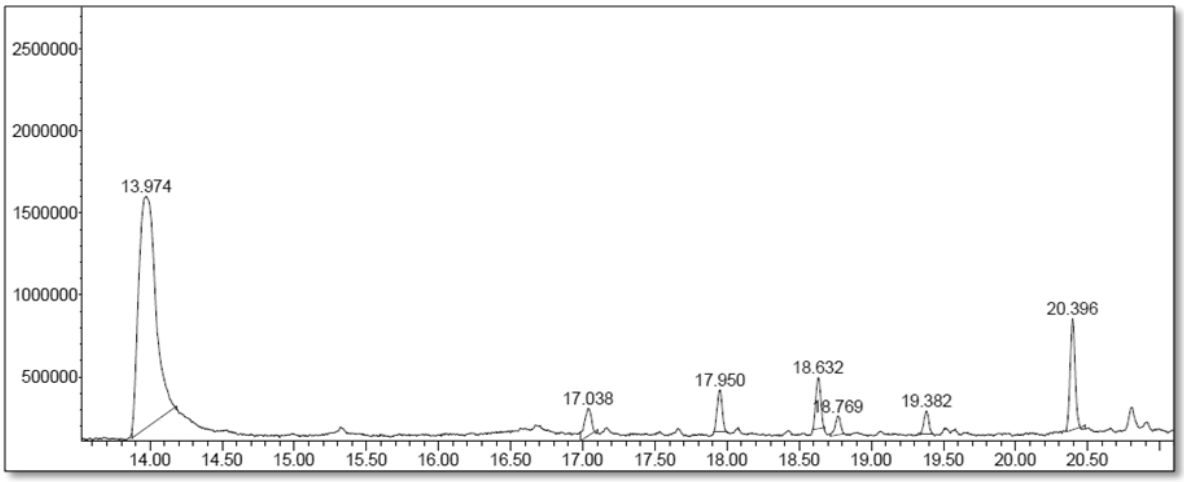
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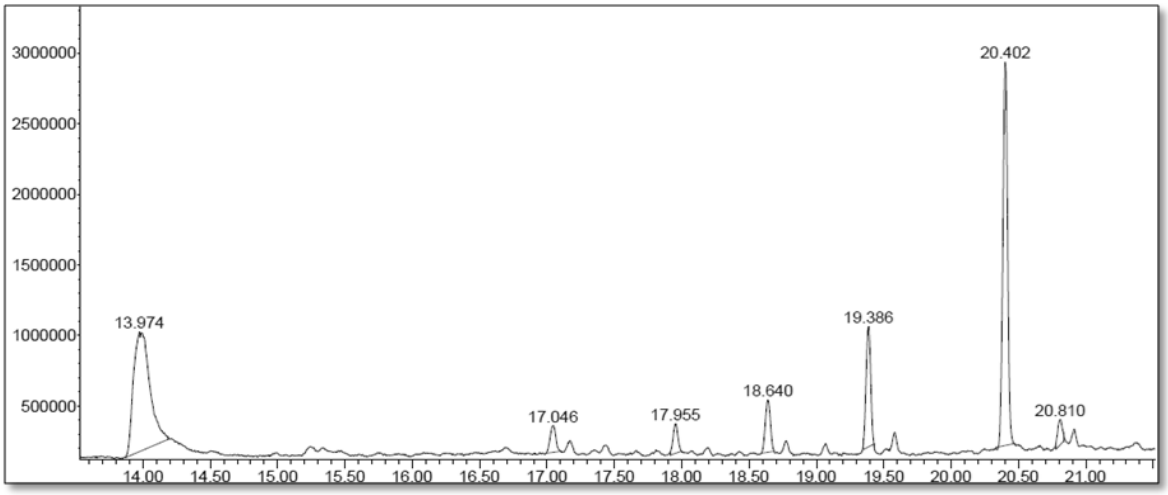
(b)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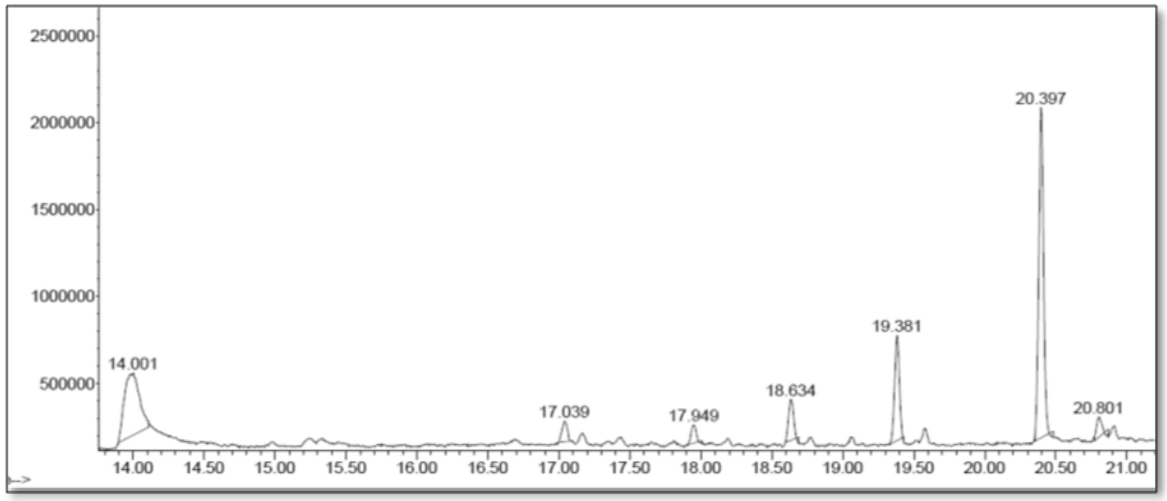
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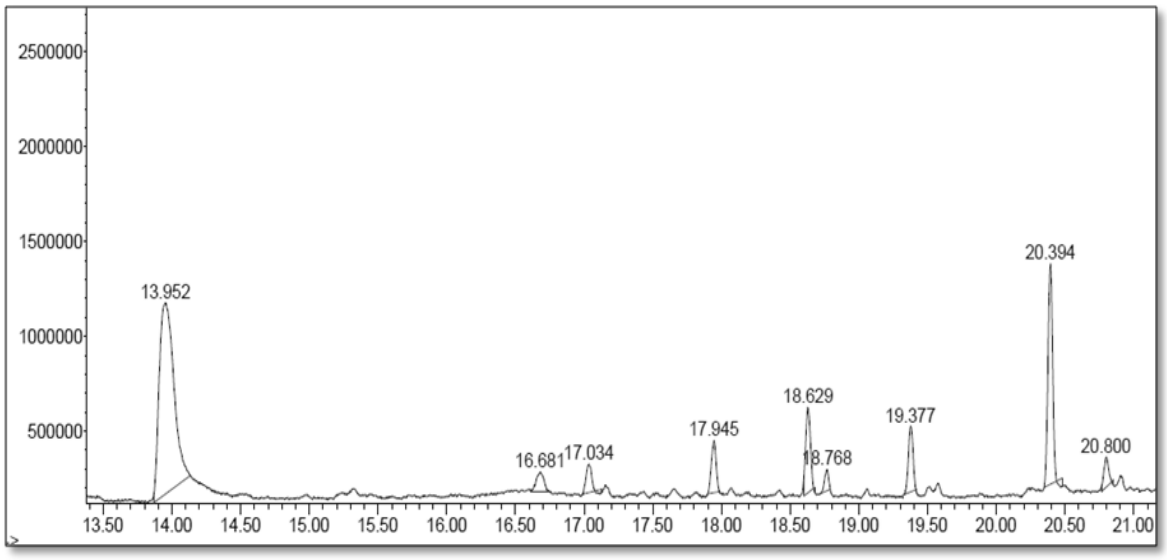

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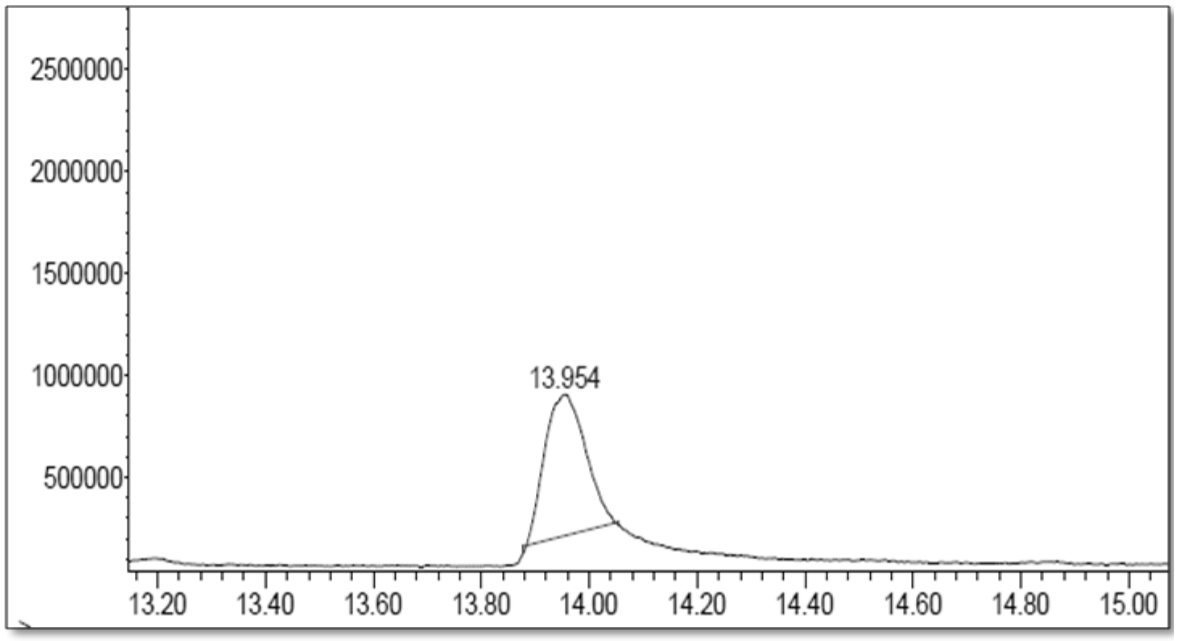

(f)

Figure 3.4: GC-MS chromatogram of *Plumbago zeylanica* accessions: (a) IC-439212, (b) IC-524441, (c) IC-398891, (d) IC-539866, (e) IC-421418 and (f) Plumbagin Standard

Table 3.4: Phytocompounds in aerial part of *P. zeylanica* accessions

Peak	Retention Time	Area %	Compound	Molecular formula	Nature of compound
IC-421418					
1	13.952	52.839	1,4-Naphthalenedione	$C_{10}H_6O_2$	Naphthalene derived organic compound
2	16.681	2.816	o-Anisic acid	$C_8H_8O_3$	Carboxylic acid
3	17.043	2.916	Cetene	$C_{16}H_{32}$	Oily hydrocarbon
4	17.945	4.582	Phytol	$C_{20}H_{40}O$	Acyclic diterpene alcohol
5	18.629	7.683	Phthalic acid	$C_6H_4(COOH)_2$	Aromatic dicarboxylic acid
6	18.768	1.563	3,7,11-Trimethyl-2,4-dodecadiene	$C_{15}H_{28}$	Alkadienes
7	19.377	5.703	Bicyclo[3.3.0]octan-3-one	$C_{10}H_{14}O$	Ethyl pentyl ketone
8	20.394	19.247	Phthalic acid	$C_6H_4(COOH)_2$	Aromatic dicarboxylic acid
9	20.800	2.651	1-Eicosene	$C_{20}H_{40}$	Acyclic Alkenes
IC-439212					
1	13.974	39.207	1,4-Naphthalenedione	$C_{10}H_6O_2$	Naphthalene derived organic compound
2	17.046	3.016	5-Eicosene	$C_{20}H_{40}$	Acyclic Alkenes
3	17.955	2.841	Silane	SiH_4	Saturated chemical compounds
4	18.638	5.325	5-(3-Methylbutyl)-2-pyridinecarboxylic acid	-	Carboxylic acid

5	19.386	11.002	2-Benzoyl-1,2-dihydro-1-isoquinolinecarbonitrile	-	-
6	20.402	36.322	Phthalic acid	$C_6H_4(COOH)_2$	Aromatic dicarboxylic acid
7	20.810	2.286	3-Eicosene	$C_{20}H_{40}$	Acyclic Alkenes
IC-539866					
1	13.974	73.804	1,4-Naphthalenedione	$C_{10}H_6O_2$	Naphthalene derived organic compound
2	17.038	3.429	1-Octadecene	$C_{18}H_{36}$	Long-chain hydrocarbon and an alkene
3	17.950	3.885	5-t-Butyl-cycloheptene	$C_{11}H_{20}$	Cycloalkene
4	18.632	4.656	Phthalic acid	$C_6H_4(COOH)_2$	Aromatic dicarboxylic acid
5	18.769	1.909	Octadecanal	$C_{18}H_{36}O$	Long chain fatty aldehyde
6	19.832	2.004	3-Methoxy-2-methyl-2-(1-phenyl-ethylamino)-propionic acid	-	Carboxylic acid
7	20.396	10.273	Phthalic acid	$C_6H_4(COOH)_2$	Aromatic dicarboxylic acid
IC- 524441					
1	14.010	74.529	1,4-Naphthalenedione	$C_{10}H_6O_2$	Naphthalene derived organic compound
2	17.048	2.149	3-Eicosene	$C_{20}H_{40}$	Acyclic Alkenes
3	17.954	2.180	Phytol	$C_{20}H_{40}O$	Acyclic diterpene alcohol
4	18.638	4.086	Di-sec-butyl phthalate, 1,2-Benzenedicarboxylic acid	$C_{16}H_{22}O_4$, $C_8H_6O_4$	Esters of *phthalic* acid, Carboxylic acid
5	19.387	3.504	Bicyclo[3.3.0]octan-3-one, Glycine	$C_{10}H_{14}O$, $C_2H_5NO_2$	Ethyl pentyl ketone, amino acid

6	20.400	10.606	Phthalic acid	$C_6H_4(COOH)_2$	Aromatic dicarboxylic acid
7	20.805	2.948	5-Eicosene, 1-Nonadecene	$C_{20}H_{40,}C_{19}H_{38}$	Acyclic Alkenes, Long-chain hydrocarbon and an alkene
IC-398891					
1	13.981	37.817	1,4-Naphthalenedione	$C_{10}H_6O_2$	Naphthalene derived organic compound
2	16.681	5.281	o-Anisic acid	$C_8H_8O_3$	Carboxylic acid
3	17.039	7.612	5-Eicosene	$C_{20}H_{40}$	Acyclic Alkenes
4	17.950	6.377	Cyclopropaneoctanal	C_3H_6	Aldehyde
5	18.633	9.002	Phthalic acid	$C_6H_4(COOH)_2$	Aromatic dicarboxylic acid
6	18.769	3.463	Thioctic acid	$C_8H_{14}O_2S_2$	Saturated fatty acid
7	19.381	5.479	2-Benzoyl-1,2-dihydro-1-isoquinolinecarbonitrile	-	-
8	20.396	17.713	Phthalic acid	$C_6H_4(COOH)_2$	Aromatic dicarboxylic acid
9	20.803	7.257	1-Nonadecene	$C_{19}H_{38}$	Long-chain hydrocarbon and an alkene

3.4 C**onclusion**

P. zeylanica used in several medicines and contains pharmaceutically important secondary metabolite i.e. plumbagin. Plant growth and bioactive compound production is influenced by genotype and environmental conditions. Therefore, it is essential to select a particular accession for enhanced plumbagin production. In this experiment, effect of various parameters such as nitrogen source, carbon source and culture medias were assessed for growth. It was observed that all the factors significantly influenced the growth of accessions. MS media having ammonium nitrate and sucrose promotes growth of all the accessions. GC analysis showed the occurrence of 7-9 peaks in all accessions and accession number IC-524441 showed highest plumbagin content.

This study has been published in "**Acta Physiologiae Plantarum**", (Springer)

Arpita Roy and Navneeta Bharadvaja "Effect of Various Culture Condition on Shoot Multiplication and GC-MS Analysis of *Plumbago zeylanica* Accessions for Plumbagin Production", Acta Physiologiae Plantarum, 2018, 40 (11): 190. IF-1.608

CHAPTER 4

ROOT SUSPENSION CULTURE ESTABLISHMENT FOR ENHANCED PLUMBAGIN PRODUCTION

4.1 Introduction

Production of plumbagin in wild plants are low due to the slow growth of plants. Exploitation of plants growing in natural habitats for plumbagin production for commercial purpose leads to depletion of plant population. To enhance the biomass and bioactive compound, culturing of roots is an efficient method which is considered to be more consistent than other culturing techniques. Root culture is a substitute method for enhance production of bioactive compounds which are of medicinal interest without hampering the natural habitat. Various studies reported production of phytocompounds using adventitious root culture such as *Rubia tinctorum* (Aşci *et al.,* 2018), *Oplopanax elatus* (Han *et al.,* 2019), *Stevia rebaudiana* (Ahmad *et al.,* 2020), *Morinda coreia* (Kannan *et al.,* 2020), etc. Root culture provides production of bioactive compounds at large scale that are naturally present in plant roots. Suspension cultures are appropriate for increases production of bioactive compounds (Kannan *et al.,* 2020). Although effect of different auxin concentration was reported in *in vitro* root culture of *P. zeylanica* (Sivanesan and Jeong, 2009; Saxena *et al.,* 2000), however limited work has been done regarding plumbagin production (Jaisi *et al.,* 2013; Silja and Satheeshkumar, 2015). Growth of root culture and plumbagin production are affected by several parameters such as culture media, media strength, hormone concentration, etc. (Silja and Satheeshkumar, 2015; Mahajan and Kapoor, 2017; Espinosa-Leal *et al.,* 2018). Accession (IC-524441) was chosen for this experiment due to its high plumbagin content obtained from previous study. Adventitious roots were induced from nodal explant and culture optimization was done for biomass and plumbagin production.

4.2 Materials and methods

4.2.1 Effect of auxins and culture media on root induction

Nodes were cultured on MS media supplemented with variable concentration of IAA (1.0, 1.5 and 2.0 mg/L), IBA (1.0, 1.5 and 2.0 mg/L), and NAA (1.0, 1.5 and 2.0 mg/L). Cultures were incubated at 25±2 ºC with 16/8 h photoperiod. Auxin that showed maximum growth of root was selected for further studies.

Four culture media i.e. MS, Nitsch, Gamborg's B5 and Schenk & Hildebrandt containing 3% sucrose +1mg/L IBA were used for induction of roots.

4.2.2 Establishment of root suspension culture

Roots (1-2 cm) that were emerging from node segments were cut off after 21 days and inoculated on liquid MS medium with 1 mg/L IBA. Cultures were incubated on orbital shaker at 90rpm without any light and sub-culturing was done after every three weeks interval onto fresh medium with same IBA concentration.

4.2.3 Effect of culture parameters on root suspension culture

Different MS medium strength (¼, ½, ¾ and 1), inoculum density (1, 2, 3 and 4 g/L) and sucrose concentration (1–5%) were used for culture parameter optimization.

Growth index was measured as

Growth index (GI for fresh roots) = (Final weight – Initial weight)/Initial weight

4.2.4 Effect of elicitor on production of plumbagin

For elicitation study, stock solutions of salicylic acid, jasmonic acid, yeast extract and malt extract were prepared. Jasmonic acid and salicylic acid (50, 100, and 150 µM) were prepared in ethanol whereas yeast extract and malt extract (10 mg/ml) were prepared in distilled water. Fresh roots (2

g/L) from 21 days old culture were inoculated onto ½ liquid MS medium having 1 mg/L IBA. Aseptically, elicitors were added and flasks without elicitor were kept as control. Cultures were incubated on orbital shaker and harvested after 21 days.

4.2.5 Plumbagin quantification

Roots (10 mg) were extracted with methanol (1 ml) (72hr maceration) and filtered with 0.22-µm syringe filter. HPLC analysis was done where C18 column with methanol and water (80:20) as mobile phase was used. Flow rate of 1.0 ml/min and detection wavelength of 272 nm was used. Plumbagin calibration curve was plotted using plumbagin standard (Sigma, USA) over concentration range 100–500 µg/ml (Figure 4.1).

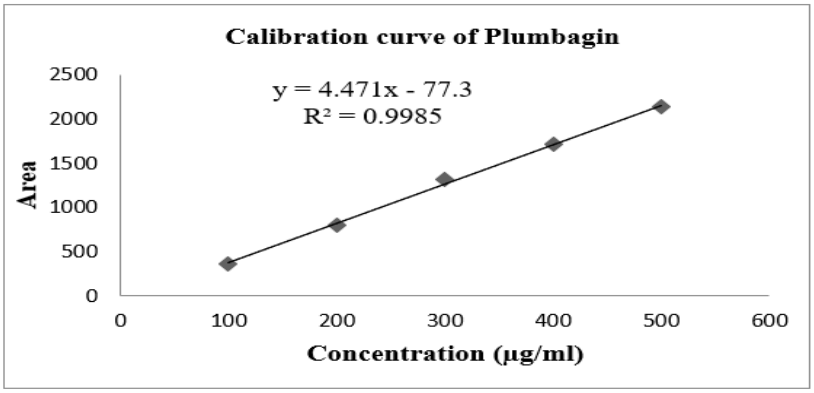

Figure 4.1: Calibration curve of plumbagin

*Statistical analysis***:** ANOVA was used to compare the significance of treatments and Tukey's range test at 5% probability was done using SPSS for Windows.

4.3 Results and discussion

4.3.1 Effect of auxins on root induction

Roots were initiated after 2 weeks of incubation. Root induction was observed in MS media having IAA and IBA (Table 4.1 and Figure 4.2). Root growth significantly varied with IAA and IBA concentrations. Maximum root number was observed in MS media containing 1 mg/L IBA i.e. i.e.

20.1±0.73 whereas 2 mg/L IAA showed maximum root length i.e. 4.33±0.53 cm. Therefore, media containing 1 mg/L of IBA was chosen for root suspension culture establishment. Various studies reported adventitious root induction in plants like *Gynura procumbens* (Saiman *et al.,* 2012), *Hypericum perforatum* (Gaid *et al.,* 2016), *Oplopanax elatus* (Han *et al.*, 2019), *Stevia rebaudiana* (Ahmad *et al.,* 2020), etc. however, growth regulators were different for different cases. A study reported that in case of *Panax notoginseng* MS media supplemented with IBA showed maximum root induction (Gao et al. 2005). Martin *et al.,* (2008) reported used shoot, leaf, and inter-node for adventitious root induction in *O. prostrata* and media having IBA/NAA alone or BAP/KIN combination provides root induction. Dohare *et al.,* (2012) reported that in case of *P. zeylanica* highest root number (7.00±0.98) and root length (6.99±0.89 cm) was observed in MS media having 1 mg/L IBA.

Table 4.1: Effect of auxin on root induction

Auxin	Concentration (mg/L)	Explant cultured	Root induction %	Response	Number of roots	Length of root(cm)
IBA	0.5	10	70	Root	10.10 ± 0.56^{e}	3.34 ± 0.24^{b}
	1.0	10	90	Root	20.10 ± 0.56^{a}	3.95 ± 0.33^{a}
	1.5	10	80	Root	15.30 ± 0.67^{b}	3.06 ± 0.42^{bc}
	2.0	10	70	Root	10.50 ± 0.70^{de}	2.74 ± 0.35^{c}
IAA	0.5	10	80	Root	6.50 ± 0.70^{g}	1.20 ± 0.42^{ef}
	1.0	10	90	Root	9.10 ± 0.73^{f}	1.75 ± 0.24^{d}
	1.5	10	70	Root	11.20 ± 0.63^{d}	1.68 ± 0.36^{de}
	2.0	10	70	Root	13.10 ± 0.73^{c}	4.33 ± 0.45^{a}
NAA	0.5	10	0	Callus	-	-
	1.0	10	0	Callus	-	-
	1.5	10	0	Callus	-	-
	2.0	10	0	Callus	-	-

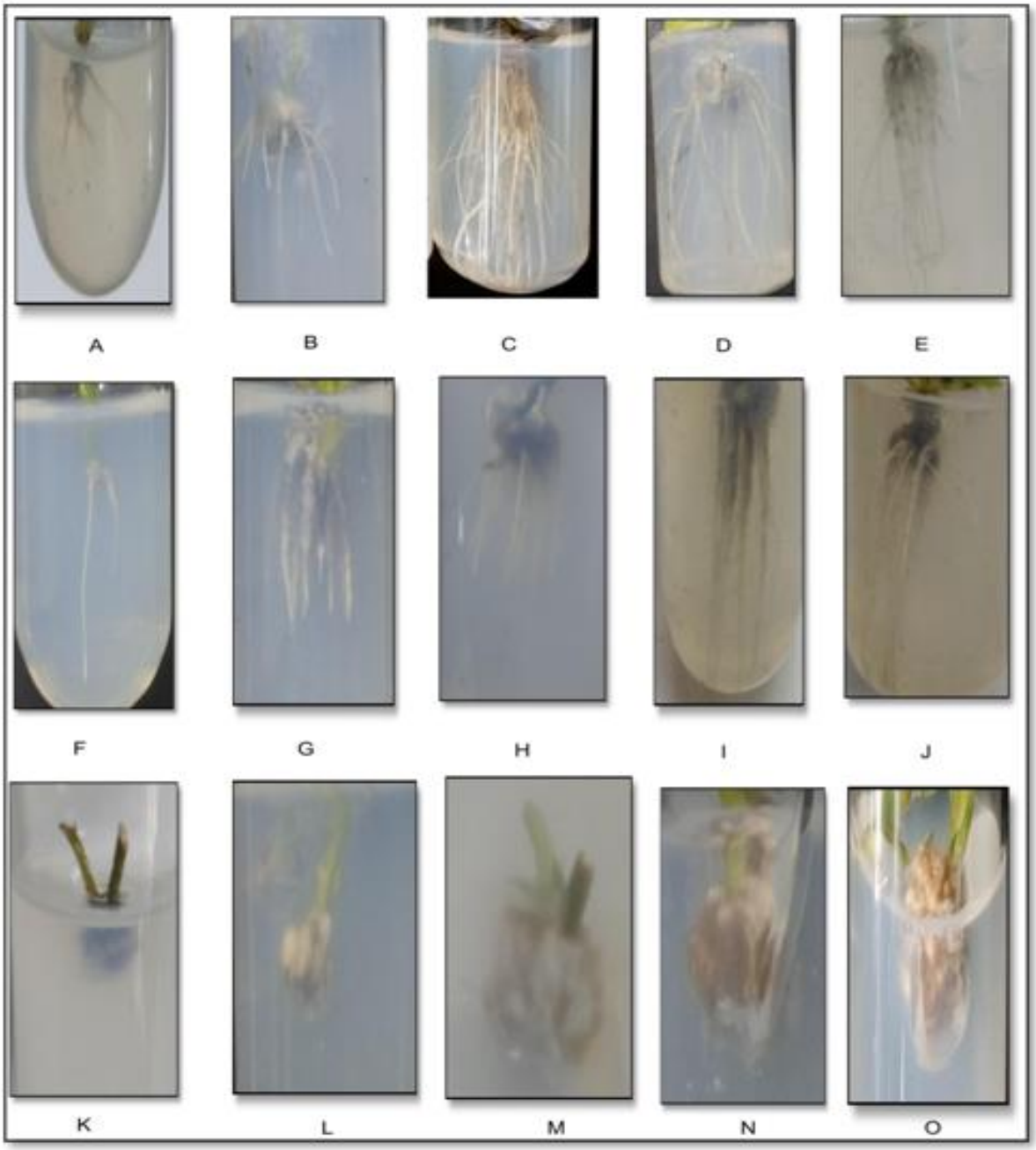

Figure 4.2: Root induction from in vitro nodal explants of *P. zeylanica* cultured on modified Murashige and Skoog (MS) media supplemented with different concentrations of plant growth regulators (Indole-3-butyric acid (IBA), Indole-3-butyric acid (IAA) and 1-Naphthalene acetic acid (NAA)). A) 0.1 mg/L IBA B) 0.5 mg/L IBA C) 1.0 mg/L IBA D) 1.5 mg/L IBA E) 2.0 mg/L IBA, F) 0.1 mg/L IAA G) 0.5 mg/L IAA H) 1.0 mg/L IAA, I) 1.5 mg/L IAA J) 2.0 mg/L IAA, K) 0.1 mg/L NAA L) 0.5 mg/L NAA M) 1.0 mg/L NAA, N) 1.5 mg/L NAA and O) 1.5 mg/L NAA

4.3.2 Effect of different culture media on root induction

Variation in culture media play important role in induction of roots. Effect of various media on root induction is mentioned in Table 8. Maximum root induction and root length was observed in MS media i.e. 22.1±0.73 and 3.95±0.45 cm followed by Nitsch, B5 and Schenk & Hildebrandt (Table 4.2 and Figure 4.3). A study reported that B5 media supplemented with 1 mg/L NAA and 0.1 mg/L Kn was optimum for root induction in *Plumbago rosea* (Panichayupakaranant and Tewtrakul, 2002). Another study reported that in case of *Scopolia parviflora,* Gamborg's B5 media showed highest root growth (Min et al. 2007). Sivanesan and Jeong (2009) reported that in case of *Plumbago zeylanica,* MS medium showed maximum growth of roots followed by B5 and Schenk & Hildebrandt media. Praveen and Murthy (2010) found that MS media possess highest root growth in case of *Withania somnifera*. Lee *et al.,* (2011) used B5, MS and Schenk & Hildebrandt medium for root induction in *Aloe vera* whereas Sujatha and Ranjitha (2012) used the same three media for root induction in *Artemisia vulgaris* and both the studies found that MS media provides maximum induction of root.

Table 4.2: Effect of different media on root induction

Media	Explant cultured	Root induction percentage	Number of roots	Length of root (cm)
MS+1 mg/L IBA	10	90	22.10±0.67[a]	3.95±0.48[a]
Nitsch+1 mg/L IBA	10	80	18.30±0.78[b]	3.50±0.38[a]
B5+1 mg/L IBA	10	80	14.80±0.63[c]	2.80±0.46[b]
Schenk & Hildebrandt+1 mg/L IBA	10	60	5.50±0.53[d]	1.80±0.45[c]

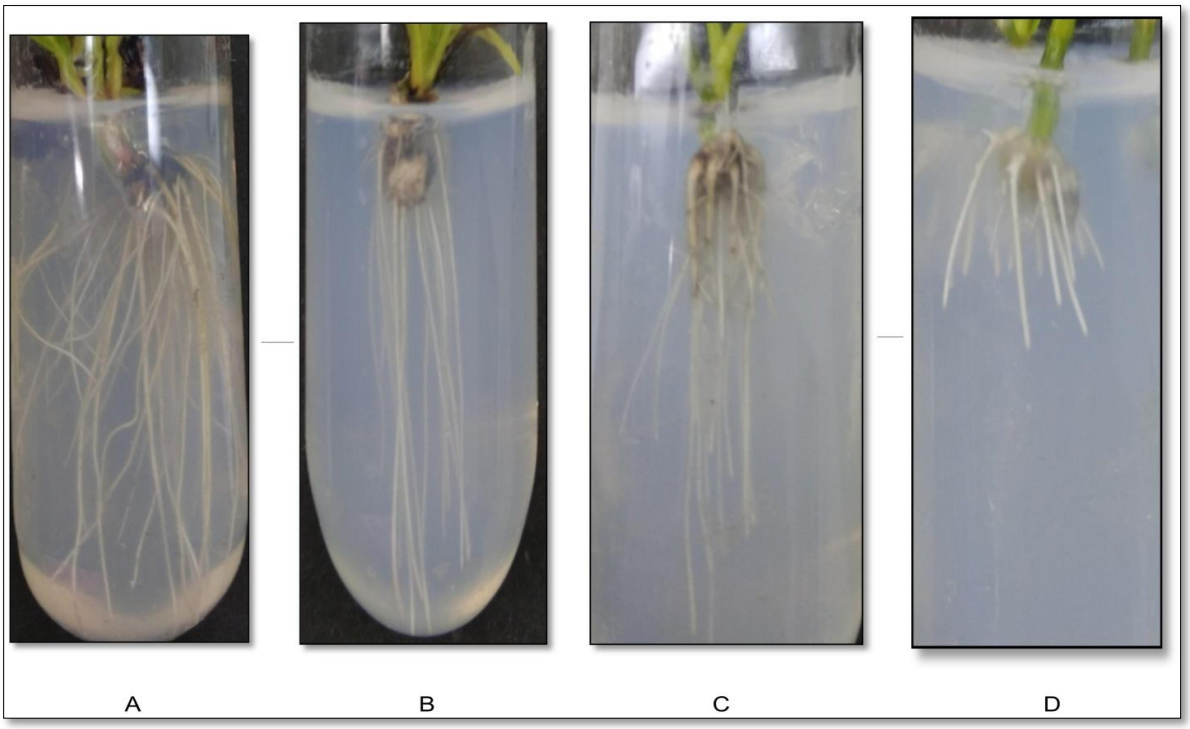

Figure 4.3: Root multiplication of *P. zeylanica* in different media A) MS+1 mg/L IBA B) Nitsch+1 mg/L IBA C) B5+1 mg/L IBA, D) Schenk & Hildebrandt+1 mg/L IBA

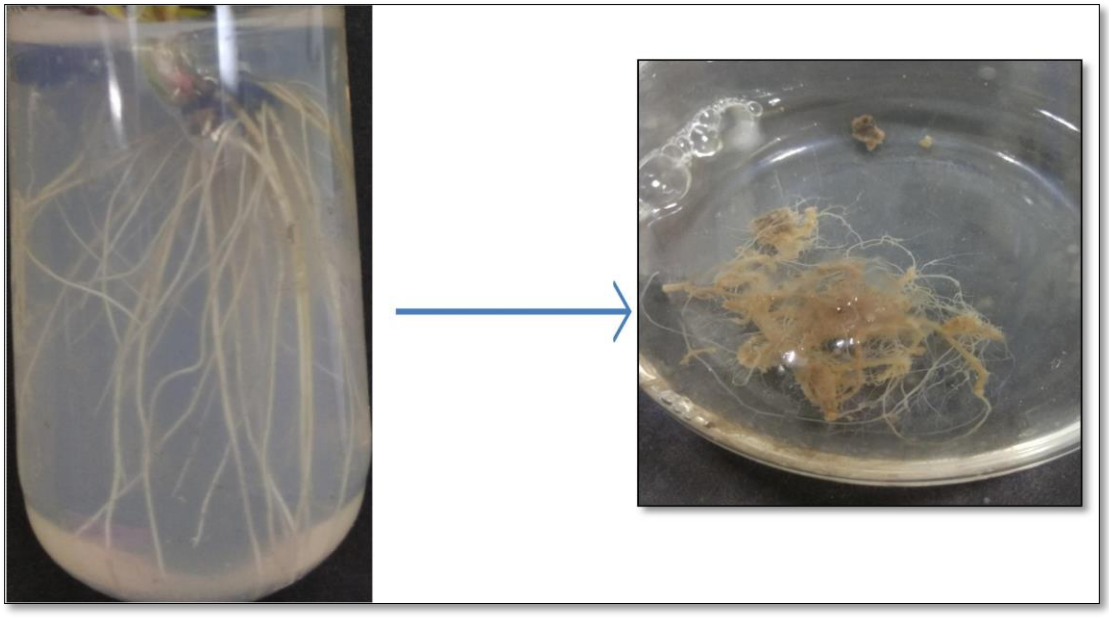

Figure 4.4: Establishment of root suspension culture of *P. zeylanica*

4.3.3 Effect of MS media strength, inoculum density and sucrose concentration on root suspension culture and plumbagin production

All the four strengths showed elongated roots formation. ½ strength MS media provides maximum growth and plumbagin production i.e. 11.54 ±0.16 and 26.65 ± 0.55 µg/ml whereas minimum plumbagin production was observed in ¼ strength media i.e. 21.96±0.78 µg/ml (Table 4.3). Media strength optimization enhanced biomass and secondary metabolites production in various plants such as *Echinacea angustifolia* (Wu *et al.*, 2006), *M. citrifolia* (Baque *et al.*, 2010) and *P. sepium* (Yin *et al.*, 2013). A study reported that ½ strength MS media containing IBA provides maximum root induction in *Plumbago zeylanica* (Verma *et al.*, 2002). Sivanesan and Jeong (2009) reported maximum rooting in ½ MS + 0.5 mg/L NAA whereas Ceasar *et al.*, (2013) obtained maximum roots in ½ MS + 1 mg/L IBA in case of *P. zeylanica*. Lower salt concentration of media enhances root growth by increasing the ions availability to roots, high concentration of salt inhibits the root growth due to lower water potential which further inhibits mineral and water absorption from culture media (Cui *et al.*, 2010). In *E. angustifolia* root culture ¼ and ½ strength MS showed maximum phenols and flavonoids production however ½ strength MS gives better biomass production (Wu et al. 2006). Rajesh *et al.*, (2014) reported that in case of *Podophyllum hexandrum*, maximum biomass and podophyllotoxin production was observed in ½ MS medium. Similarly in in case of *Ophiorrhiza mungos*, ½ strength MS medium showed maximum phytocompound production (Deepthi and Satheeshkumar, 2017).

Inoculum density also affects root culture performance. In this study, inoculum density of 2 g/L roots provides highest plumbagin production i.e. 27.18 ±0.51 µg/ml and therefore it was chosen as optimum and used for further experiments (Table 4.3). It was reported that inoculum density affects byproduct accumulation (Wu *et al.*, 2006) which was also observed in our study. For

effective tissue culture system inoculum density optimization is a fundamental factor (Lee and Shuler, 2000). A study reported that inoculum density of 5 g/L showed maximum biomass and ginsenoside production in Ginseng root culture (Wu *et al.*, 2008). Reis *et al.,* (2011) reported that in case of *Stevia rebaudiana* root culture, 0.2 g/flask inoculum density was optimum. Silja and Satheeshkumar (2015) reported that 2 g/L inoculum density provides highest plumbagin production in *Plumbago rosea* root culture and similarly for the *Ophiorrhiza mungos root culture* (Deepthi and Satheeshkuma, 2017).

Table 4.3: Effect of different culture parameter on plumbagin production

MS strength	**Biomass (GI)**	**Production (µg/ml)**
¼	2.43±0.16 ^{d}	21.96±0.78 ^{b}
½	11.54±0.16 ^{b}	26.65±0.55 ^{a}
¾	14.84±0.21^{a}	23.99±0.48 ^{b}
1	4.15±0.21 ^{c}	22.40±0.45^{c}
Inoculum density (g/L)		
1	11.75±0.15^{a}	26.52±0.55^{ab}
2	7.75±0.23 ^{b}	27.18±0.51 ^{a}
3	3.29±0.10^{c}	25.20±0.48^{bc}
4	2.05±0.10^{d}	23.77±0.47 ^{c}
Sucrose concentration (%)		
1	4.33±0.16^{c}	20.67±0.52^{c}
2	5.65±0.13^{b}	26.36±0.30^{ab}
3	8.22±0.25^{a}	27.17±0.71^{a}
4	5.03±0.15^{b}	26.43±0.54^{ab}
5	3.44±0.12^{d}	19.44±0.45^{c}

Sucrose concentration also affects root growth significantly. Among various sucrose concentrations, 3% showed maximum root growth and plumbagin production. Lower sucrose concentrations (1–3%) were found to be more effective for root growth. Plumbagin production at 3% sucrose (27.17±0.71)) was higher as compared with other concentrations (Table 4.3). A study reported that 3% sucrose containing medium resulted better plantlets in *Lycopersicon esculentum* (Gubis et al. 2005). Cui *et al.,* (2010) reported that in *Hypericium perforatum*, ½ MS containing 3% sucrose was optimal for biomass production. Yin et al. (2013) reported that in case of *Pseudostellaria heterophylla*, 4% sucrose provided highest biomass and saponin production. Kusuma *et al.,* (2017) reported that 5% sucrose concentration increased root biomass in *Gynura procumbens*.

4.3.4 Effect of elicitors on production of plumbagin

Elicitors are substances which generate stress responses and induce or improve production of specific compounds (Han *et al.,* 2019). Elicitors induce different defence responses in plants which leads to hypersensitive response, reactive oxygen species generation and phytoalexins production. Elicitors are categorised as biotic and abiotic depending upon their nature. Elicitor type and its concentration influence growth and plumbagin production in *P. zeylanica* root culture. Elicitors were added in the medium before root inoculation and roots were harvested after twenty-eight day of incubation period. Treatments with yeast extract, methyl jasmonate, and salicylic acid significantly improved production of plumbagin as compared with untreated root cultures. Cultures treated with 150 mg/L yeast extract showed maximum biomass and plumbagin production i.e. 10.12 ± 0.35 and 90.96 ± 0.51 µg/mL respectively, which was nearly three times as compared with control culture i.e. 27.39 ± 0.41 (Table 4.4 and Figure 4.5). Various studies reported that concentration of elicitor is a crucial factor, because higher amount of elicitor may induce

hypersensitive response which leads to cell death (Namedo, 2007). In case of *P. ginseng* yeast extract (0.5–3.0 g/L) improved production of saponin (Lu *et al.*, 2001). In case of *Adhatoda vasica*. yeast extract (50 and 100 mg/L) enhanced 0.336% vasicine (Bhambhani *et al.*, 2012). A study reported that 50µM of jasmonic acid treatment enhanced plumbagin production up to 1.23% DW in *Plumbago rosea* (Silja and Satheeshkumar,2015).

Table 4.4: Effect of elicitors on production of plumbagin

	Elicitor		**Biomass (GI)**	**Production(µg/ml)**
Control	-	-	8.22 ± 0.25^{b}	27.39 ± 0.41^{f}
Biotic	Malt Extract	50 mg/L	5.08 ± 0.11^{e}	24.01 ± 0.43^{h}
		100 mg/L	3.33 ± 0.15^{g}	23.54 ± 0.50^{h}
		150 mg/L	2.17 ± 0.15^{h}	19.45 ± 0.27^{j}
	Yeast Extract	50 mg/L	5.10 ± 0.2^{e}	51.30 ± 0.60^{c}
		100 mg/L	7.64 ± 0.15^{c}	63.47 ± 0.36^{b}
		150 mg/L	10.12 ± 0.35^{a}	90.96 ± 0.51^{a}
Abiotic	Salicylic acid	50 µM	4.45 ± 0.13^{f}	21.43 ± 0.21^{i}
		100 µM	5.42 ± 0.10^{de}	32.36 ± 0.22^{e}
		150 µM	4.88 ± 0.16^{ef}	41.08 ± 0.66^{d}
	Methyl Jasmonate	50 µM	5.67 ± 0.15^{d}	31.89 ± 0.23^{e}
		100 µM	7.9 ± 0.18^{bc}	25.80 ± 0.18^{g}
		150 µM	3.08 ± 0.13^{g}	20.87 ± 0.37^{i}

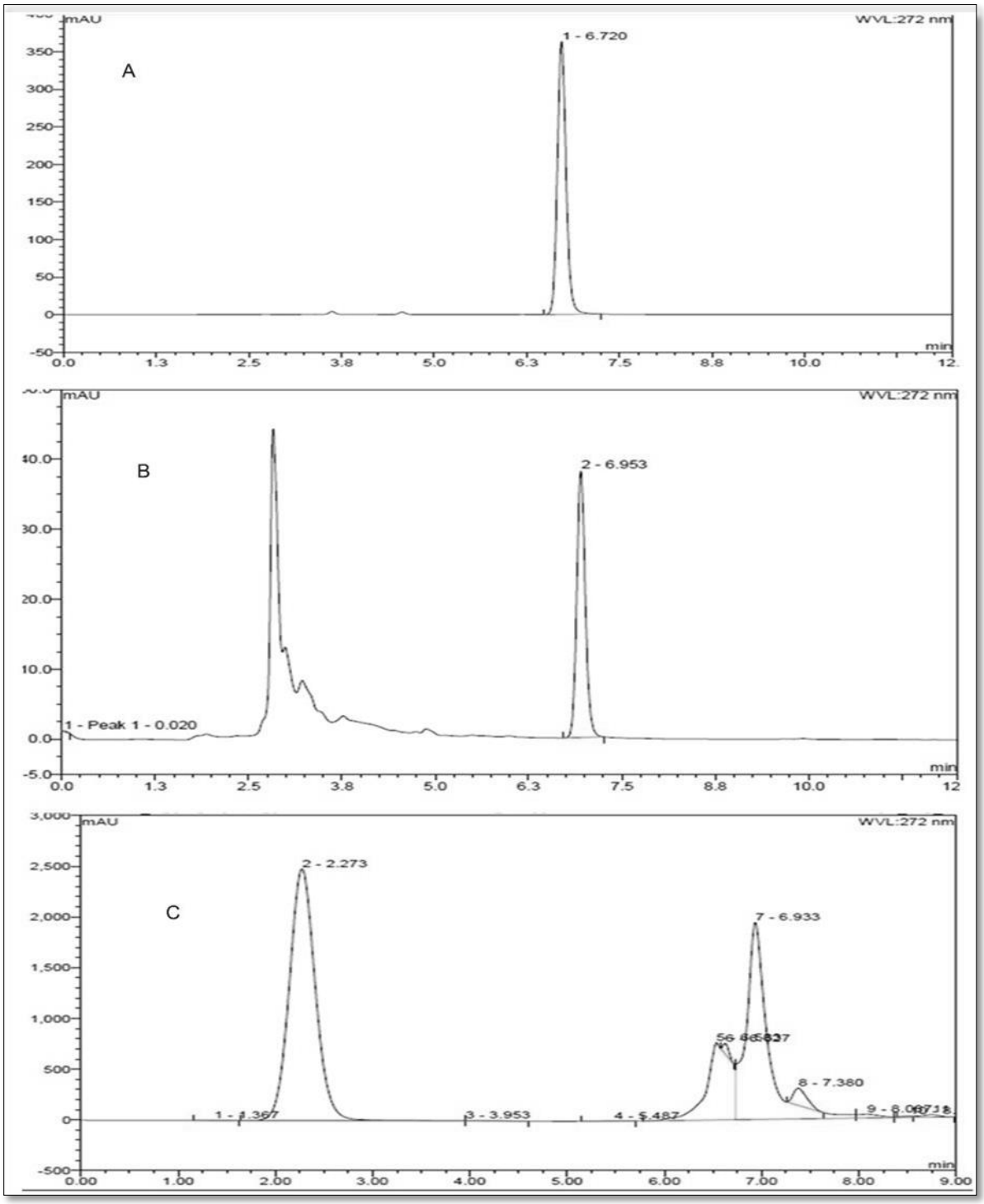

Figure 4.5: HPLC chromatogram of a) Plumbagin standard, b) Control culture and c) yeast extract treated culture

4.4 Conclusion

Root growth and bioactive compound production is influenced by various culture conditions. Therefore, it is essential to optimize culture parameters for enhance plumbagin production. Influence of different auxin concentrations and culture media showed that MS media with 1 mg/L IBA provides maximum root induction. Further culture parameter optimization showed, ½ MS medium with 3% sucrose and 2 g/L inoculum density was optimal for plumbagin production. Furthermore, yeast extract supplemented culture showed three times enhanced production of plumbagin.

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CHAPTER 5

ESTABLISHMENT OF CELL SUSPENSION CULTURE OF *PLUMBAGO ZEYLANICA* AND ENHANCED PRODUCTION OF PLUMBAGIN

5.1 Introduction

Single cell cultures offer an excellent way to explore the potential of plant cells**.** Plumbagin production using cell suspension cultures provides an alternative method. Cell suspension provides constant secondary metabolites production by overcoming the influence of unexpected environment circumstances and diseases in wild plants. Cell suspension cultures are appropriate for plumbagin production and could be easily scaled up for commercial production. Various studies have been conducted to establish cell suspension culture of different plants which includes *Plumbago rosea* (Komaraiah *et al.*, 2001), *Citrus sinensis*(Gerolino *et al.*, 2015), *Parkia biglobosa* (Abbas *et al.,* 2018), *Thevetia peruviana (*Arias *et al.,* 2016*).* One of the vital parameters in production of bioactive compounds is role of stresses. Often, plant stress response induces production of desired chemical compounds in the plant, this process is called elicitation. Elicitors are chemical compounds, when supplemented to cell cultures in minute amount generates morphological and physiological responses and phytocompound accumulation. Different elicitors induce different plant defense response including reactive oxygen species production, hypersensitive response and phytoalexins production. Elicitors can be abiotic, biotic or chemical elicitors, they are either exogenous or endogenous. Numerous biotechnological approaches have been used to increase productivity and elicitation is one of them. Use of different elicitors (chitosan, salicylates, jasmonates, methyl jasmonate, yeast extract, and filtrate of fungal culture) in cell suspension cultures were reported as an efficient method for increased phytocompound production (Komaraiah et al. 2002; Silja *et al.,* 2015; Mendoza *et al.*, 2018; Pourianezhad *et al.*,

2019). In the present investigation, callus culture was induced using *P. zeylanica* leaf explants and then cell suspension culture was established. Further effect of different culture parameters such as media strength, pH and inoculum density on plumbagin production was optimized. To study the effect of different elicitors, cell suspension culture was treated with biotic and abiotic elicitors which resulted in enhanced production of plumbagin.

5.2 Materials and methods

5.2.1 Role of different auxin and medias on callus induction

For induction of callus, leave explants (20×20 mm) of accession number IC-524441 was cultured on MS media. Media pH was adjusted to 5.8±0.2 ahead of autoclaving at 121º for 21 min. Different concentration of auxins were supplemented to the media (0.5, 1.0, 1.5 and 2.0 mg/L of NAA, IBA and 2, 4-D). Cultures were incubated at 25±2ºC with 16/8h light/dark supply. Influence of auxin concentrations on callus induction was recorded based on visual observations. Auxin that provides best callus response was selected for further study, where effect of different media (MS, B5, Nitsch and Schenk & Hildebrandt) on callus induction was optimized.

5.2.2 Establishment and growth analysis of cell suspension culture

MS media supplement with 1 mg/L NAA was utilized for establishment of cell suspension culture. Sub-culturing of cell suspension was done onto fresh media once in every three weeks interval with same hormonal concentration.

Callus induction frequency was calculated as:

Frequency of callus induction = Number of explants producing callus/Total number of explants × 100

For growth curve, cultures were harvested at different time period, fresh and dry weight of filtered cell biomass were determined. Cultures were harvested in triplicate and growth index was calculated as Growth Index (GI) = (Final weight- Initial weight)/ Initial weight

5.2.3 Effect of different culture parameters on biomass and plumbagin production

Role of pH, media strength and inoculum density on production of plumbagin and biomass was studied. Callus was inoculated at different pH (5, 5.4, 5.8, 6.2), media strength (full, half, quarter, double) and inoculum density (1, 2, 3 and 4 g/L).To study the role of different elicitors i.e. salicylic acid, yeast extract, jasmonic acid and malt extract were used at three different concentrations. Elicitors were supplemented to individual flasks and flasks without elicitors were used as a control.

5.2.4 Quantification of plumbagin

For quantitative analysis plumbagin standard (Sigma Aldrich) was prepared in methanol. For sample preparation, 10 mg of cell biomass was taken and mixed with 1ml methanol and kept for 48hrs maceration. After this samples were filtered with 0.22-micrometer filter.

Chromatographic conditions: Plumbagin was estimated using HPLC (Thermo Fisher HPLC system) where analytical column was C18 and methanol: water (80:20) was used as mobile phase. Flow rate and detection wavelength were 1.0 ml/min and 272 nm respectively.

Statistical analysis: Data collection was done after incubation of three weeks. Results were expressed as mean ± SD with three replicates. Statistical analysis was carried out using ANOVA and Tukey's range test at 5% probability using SPSS was done.

5.3 Result and discussion

5.3.1 Influence of auxin and medias on callus induction

The *in-vitro* callus cultures were successfully established from leaf explants of *P. zeylanica*. Among the different concentrations of auxins tested, the maximum frequency of callus growth was achieved on the medium containing 1 mg/L NAA (Table 5.1, (Figure 5.1). Therefore, this media combination was further used for regular sub-culturing of callus. Media without any hormones showed no symptom of callus growth, which suggests that growth regulator is a vital factor for callus induction and plant cell development. The type and concentration of auxin influences both growth and product formation in cultured plant cells (Nagella and Murthy, 2010). Amoo and Ayisire (2005) reported successful callus induction in MS media where only 2, 4-D was supplemented whereas Saika *et al.,* (2013) found that maximum callus induction was obtained in MS media containing NAA (3 mg/L) + BAP (0.5 mg/L). Arunkumar *et al.,* (2014) obtained maximum callus induction in MS media containing 2 mg/L NAA in case of *Caesalpinia* sappan whereas Sen *et al.,* (2014) found that MS media containing both NAA (0.5 mg/L) + 2, 4-D (2 mg/L) showed highest callus induction in *Achyranthes aspera.* Malayaman *et al.,* (2017) reported highest rate of callus induction in MS media containing 1.5 mg/L NAA+0.5 mg/L BAP in case of *Phyllanthus debilis*. Das *et al.,* (2018) reported that in case of *Coscinium fenestratum* maximum callus induction was obtained in MS media containing 2, 4-D (0.1 mg/L) + kinetin (2.0 mg/L). Mahendran *et al.,* (2018) obtained maximum friable callus in 2, 4-D (2.0 mg/L) + NAA (0.5 mg/L) in case of *Gloriosa superba L* whereas Hu *et al.,* (2019) found maximum callus growth in NAA(0.5 mg/L) +2, 4-D (0.1 mg/L)+BA(1.5 mg/L) supplemented media in case of *L. japonica* .

Evaluation of different media for callus induction is an important factor. Therefore, in this study, four different plant tissue culture media i.e. MS, Nitsch, B5, and Schenk & Hildebrandt with 1

mg/L NAA were used to optimize media for best callus induction. It was found that MS media showed highest callus growth followed by B5, Nitsch, and Schenk & Hildebrandt (Table 2). According to Mederos-Molina (2004), MS media is very rich in nutrients especially macronutrients which could be the possible reason for maximum callus induction. Saika *et al.*, (2013) tested two types of culture media i.e. Woody Plant Medium and MS for callus induction in *Aquilaria malaccensis* and found that MS media was more effective. Verma *et al.,* (2016) utilized four media i.e. B5, Linsmaier and Skoog, MS, and Chee and Pool for callus induction in *Crocus* species and found that MS media provides highest callus induction frequency. Sharma *et al.*, (2017) tested three culture media i.e. MS, B5 and Woody Plant Medium for callus induction in *Crataeva tapia* and observed that MS media showed maximum callus growth. Similarly, Açıkgöz, (2020) reported that MS media provides maximum callus growth in case of *Ocimum bacilicum.*

Table 5.1: Effect of auxin on callus induction

Auxins	**Concentration (mg/l)**	**Explant cultured**	**Callus induction percentage**	**Nature of response**
Control	-	10	0	No response
NAA	0.5	10	80	Callus induction
	1.0	10	100	Callus induction
	1.5	10	90	Callus induction
	2.0	10	90	Callus induction
2,4-D	0.5	10	70	Callus induction
	1.0	10	90	Callus induction
	1.5	10	80	Callus induction
	2.0	10	70	Callus induction

IBA	0.5	10	0	Root induction
	1.0	10	0	Root induction
	1.5	10	0	Root induction
	2.0	10	0	Root induction

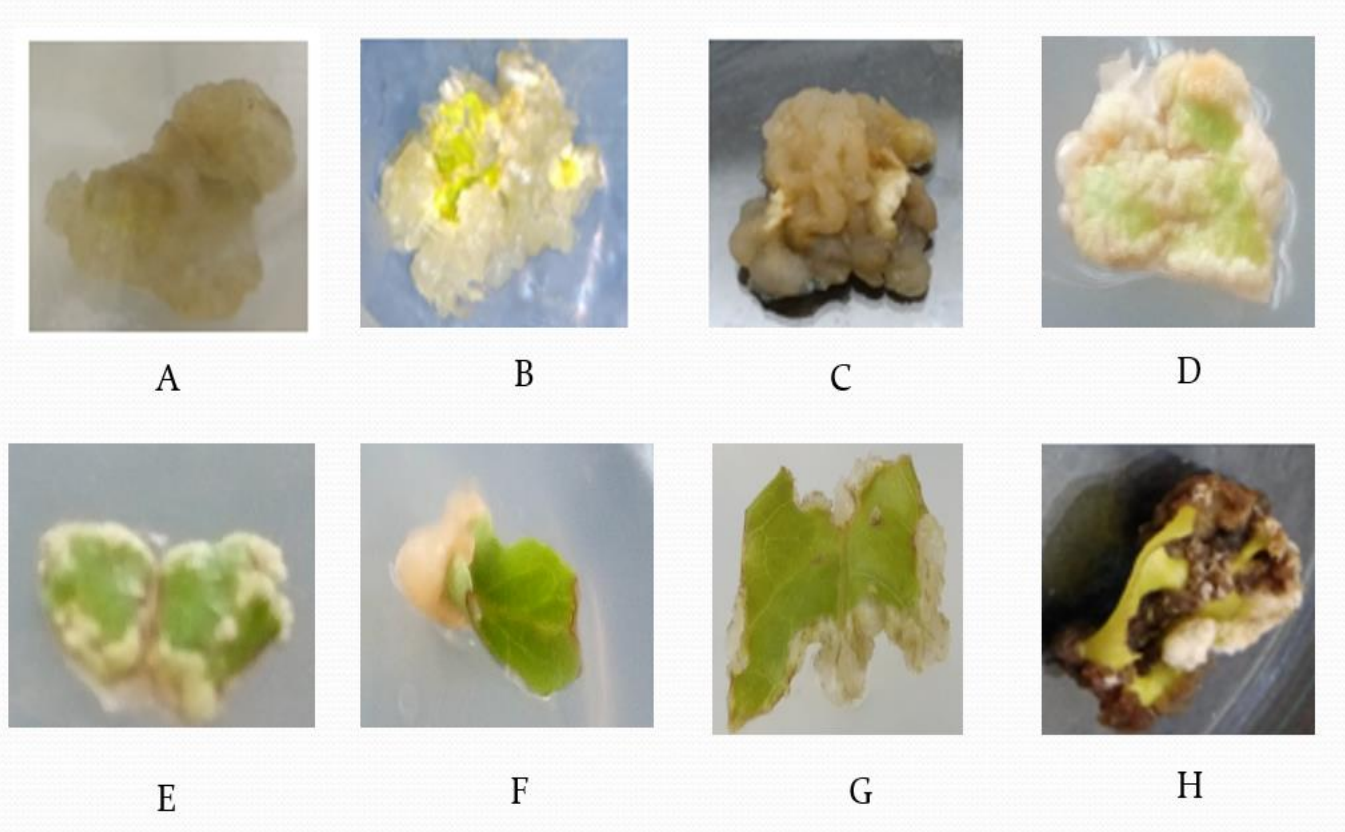


Figure 5.1: Callus induction in different hormone supplemented media A) 0.5 mg/L NAA, B)1.0 mg/L NAA, C) 1.5 mg/L NAA, D)1.5 mg/L NAA, E) 0.5mg/L 2,4-D, F)1.0 mg/L 2,4-D, G) 1.5 mg/L 2,4-D and H) 2.0 mg/L 2,4-D

Table 5.2: Effect of different media on Callus induction

Media	Explants	Explant cultured	Callus induction %	Nature of response
MS+1 mg/l NAA	Leaves	10	100	++++
Nitsch+1 mg/l NAA	Leaves	10	80	+++
B5+1 mg/l NAA	Leaves	10	70	+++
Schenk & Hildebrandt+1 mg/l NAA	Leaves	10	50	++

5.3.2 Establishment of cell suspension culture

Cell suspension culture was successfully established in liquid MS medium containing 1 mg/L NAA (Figure 5.2). Cell suspension culture was initially whitish and forms cell aggregates. After four weeks of incubation period, suspension culture turns into yellowish. Several studies reported that sub-culturing period possesses a critical role in secondary metabolite production (Namedo, 2007; Hussain *et al.,* 2012). Therefore, it was important to optimize the sub-culturing period by analysing the growth kinetics of cell culture. In this study, we observed four days of lag phase and after this cell entered in log phase where they were dividing, increase in biomass production was observed until twenty days (Figure 5.3). After log phase, decline phase started due to lack of nutrients or oxygen in the medium. Fresh as well as dry weight of cells decreased during death phase. Maximum growth index of 1.0g/50ml was obtained in log phase. In case of *P. rosea* cell suspension culture, stationary phase was observed after 25 days of growth (Satheeshkumar and Seeni, 2002). Deo *et al.,* (2010) reported that suspension cultures of *Colocasia esculenta* were able to double in two weeks. Secondary metabolites production is influenced by growth phase of cell

culture (Bourgaud et al. 2001). Cell suspension culture possesses higher cell multiplication which is an important feature of meristematic cells and this minimizes time required for culture to go in stationary phase, which is vital for secondary metabolite production (Thorat *et al.*, 2017). Ali *et al.*, (2013) obtained maximum cell growth on 27th days in case of *Artemisia absinthium L.* cell suspension cultures. Silja *et al.*, (2014) reported that in case of *P. rosea* cell suspension culture maximum growth of cells obtained on eighth day whereas Hu *et al.*, (2019) found that maximum cell growth was obtained on 10th day in case of *L. japonica* cell suspension culture.

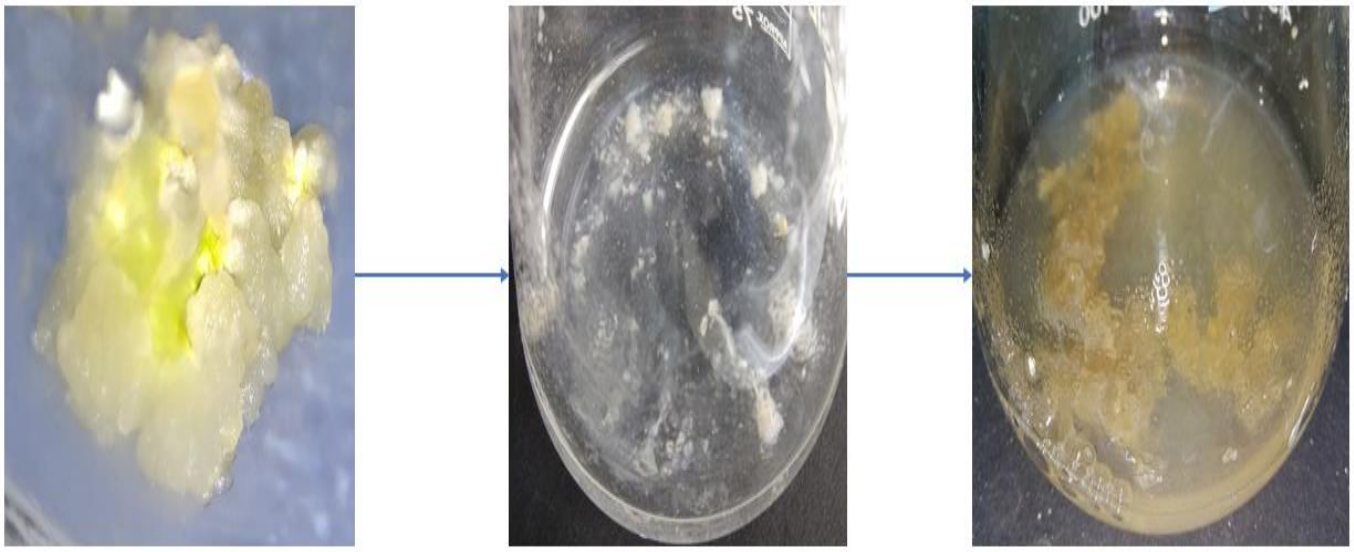
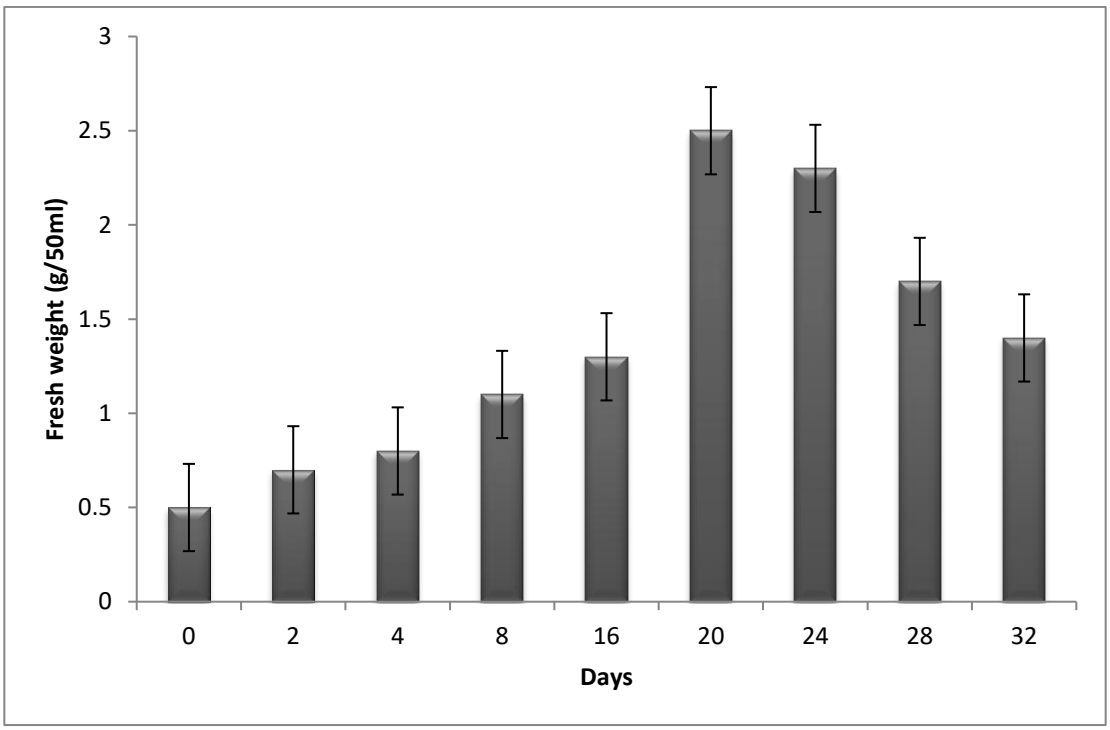


Figure 5.2: Establishment of cell suspension culture of *P. zeylanica*



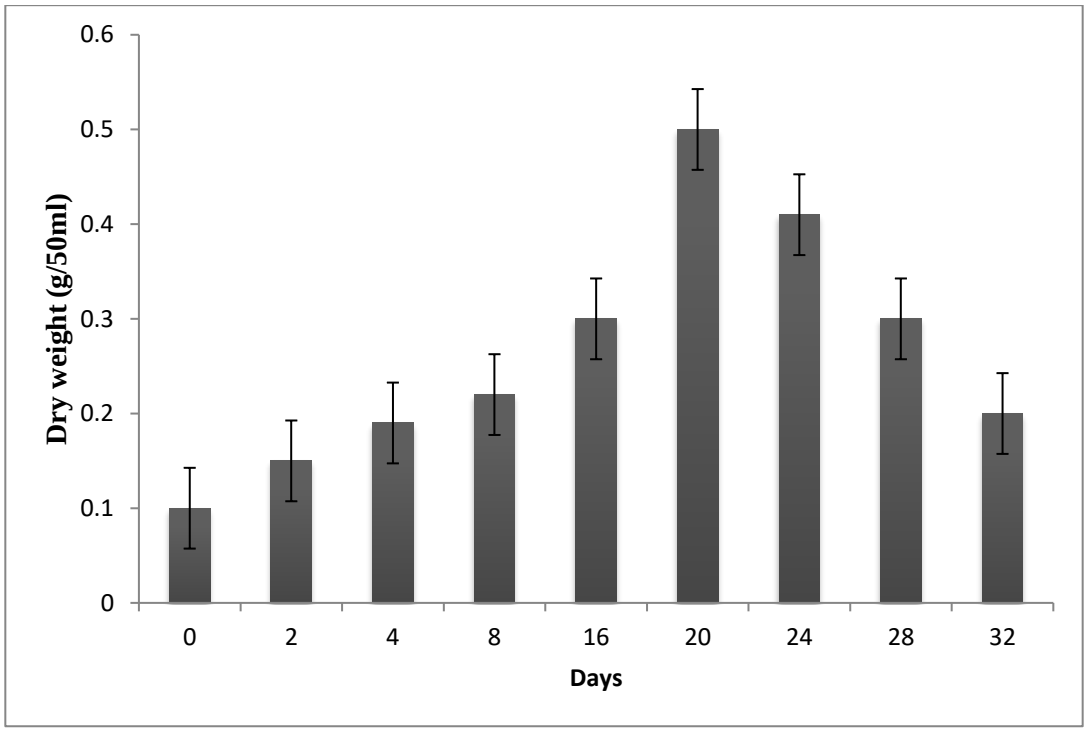


Figure 5.3: Growth curve of cell suspension cultures of *P. zeylanica* with time. Data represents mean ± SE

5.3.3 Role of medium strength, inoculum density and pH on plumbagin production

Optimum nutrient concentration is vital aspect of cell growth and secondary metabolites production. Four different MS media strength were used for plumbagin production and cell growth. Full strength MS media possess highest plumbagin production i.e. 20.12±0.38 µg/mL whereas maximum growth index was obtained in double strength media i.e. 15.67±0.42. Lowest amount of plumbagin production was observed in ¼ strength media i.e. 15.78±0.48 µg/mL(Table 5.3). Lower salt concentration decreases cell growth whereas higher salt concentration increases cell growth. Lian *et al.,* (2002) reported that full and half strength medium showed ginsenoside production and cell growth in *Panax ginseng*, while 2X strength suppressed both ginsenoside production and cell growth. Lattanzio *et al.,* (2009) reported that full strength MS medium enhanced antioxidant activity and biomass of *Origanum vulgare* shoot culture as compared with half strength. Aly *et al.,* (2010) found that full strength MS media provides highest accumulation of alkaloid in cell suspension cultures of *Hyoscyamus muticus.* Nagella and Murthy (2010) also reported that full strength MS media favored biomass and withanolide A production in *Withania somnifera* cell suspension cultures. Masoumian *et al.,* (2011) found that full strength media showed highest total flavonoids production in *Hydrocotyle bonariensis.*

Inoculum density is a vital parameter which affects cell suspension culture. Plumbagin content was highest in cell suspension culture inoculated with 3 g/L cells i.e. 25.17±0.71 µg/ml. As inoculum density of 3 g/L possess maximum production of plumbagin therefore it was chosen as optimum and utilized for further experiments. Inoculum density affects byproduct accumulation (Wu *et al.,* 2006) which was also observed in this study. Therefore, inoculum density optimization is an essential parameter for determining successful tissue culture (Pillai *et al.,* 2015). Pan *et al.,* (2000) found that inoculum density of 1.5–3.0 g/L supports total taxol production in *Taxus*

chinensis cell culture. Zhao *et al.,* (2001) reported that inoculum density of 3g/L is optimum for Jaceosidin production in *Saussurea medusa* cell suspension culture. Zhang *et al.,* (2002) reported that inoculum density of 50g/L provides highest taxol production in cell culture of *Taxus yunnanensis.* Nagella and Murthy (2010) reported that maximum biomass and withanolide A production was found with 10 g/Linoculum density in *Withania somnifera*. Similarly, 10 g/L inoculum density was also optimum for biomass production in cell suspension culture of *Stevia rebaudiana* (Mathur and Shekhawat, 2013). Li *et al.,* (2016) tested different inoculum density (25 to 125 g/L) for cell suspension culture of *Lonicera macranthoids* and found that inoculum density of 50 g/L was optimum for biomass production and chlorogenic acid production was higher when inoculum density was 50 and 75 g/L.

pH of culture medium is generally adjusted to 5-6 before autoclaving and hydrogen ions concentration in the medium changes during culture period. Optimum pH is a vital factor which directs growth of cell and secondary metabolites production. Medium pH influence nutrient uptake, cellular growth, plant gene expression, etc. (Chen *et al.,* 2014). Effect of four different culture pH i.e. 5.0, 5.4, 5.8, 6.2 were evaluated for biomass and plumbagin production. pH had moderate effect on biomass production, maximum production of biomass was obtained at pH 5.4 i.e. 14.04±0.25 whereas pH 5.8 showed highest plumbagin production i.e. 25.51±0.33 µg/mL. Lowest amount of plumbagin production was recorded in pH 5.0 i.e. 19.34±0.35 µg/mL. Change in medium pH is generally associated with uptakes of inorganic nitrogen source by different cells (Sarasketa *et al.,* 2006). Malik *et al.,* (2008) reported that pH 5.75 showed highest biomass production whereas pH 9.50 favored acetylshikonin production in cell suspension culture of *Arnebia euchroma*. Nagella and Murthy (2010) reported that higher and lower pH did not support biomass and withanolide A production and optimum pH was 5.8 in case of *Withania somnifera*.

Li *et al.,* (2016) reported that pH of the medium showed moderate effect on biomass production and maximum biomass was obtained at pH 5.5 and 6.0 on the other hand, maximum chlorogenic acid production was found at pH 5.5 in *Lonicera macranthoids.* Mishra *et al.,* (2019) reported thatmedium pH significantly affects the biomass and phytocompound production, maximum total alkaloid production was obtained at pH 5.82 in case of *Catharanthus roseus*.

Table 5.3: Effect of different culture parameter on plumbagin production

MS strength	**Biomass (GI)**	**Production (µg/ml)**
¼	5.83±0.15^{d}	15.78±0.48 ^{d}
½	10.59±0.32 ^{c}	18.66±0.56^{b}
1	14.20±0.33^{b}	20.12±0.38^{a}
2	15.67±0.42 ^{a}	17.73±0.25^{c}
Inoculum density (g/L)		
1	8.13±0.61^{c}	20.61±0.58^{cd}
2	7.78±0.18 ^{d}	21.15±0.58 ^{c}
3	14.09±0.21^{a}	25.02±0.24^{a}
4	8.60±0.35^{b}	22.40±0.22 ^{b}
pH		
5	10.6±0.31^{d}	19.34±0.35^{c}
5.4	14.04±0.25^{a}	21.37±0.31^{ab}
5.8	13.58±0.57^{b}	25.51±0.33^{a}
6.2	11.16±0.20^{c}	20.44±0.55^{ab}

5.3.4 Role of elicitors on production of plumbagin

In this study, influence of different elicitor and their concentration on growth as well as plumbagin production was observed. It was found that plumbagin production was very much influenced with elicitor supplementation. Treatment of *P. zeylanica* suspension cultures with yeast extract induced higher production of plumbagin. Addition of yeast extract improved accumulation of plumbagin and possess no effect on cell growth. Among the three concentrations of yeast extract tested, maximum plumbagin production i.e. 83.30 µg/mL was obtained from cells grown at 150 mg/L concentration. It was also observed that with increase concentration of yeast extract, plumbagin also increased whereas growth of the biomass decreased. Maximum of 3.3-fold enhancement in plumbagin was observed as compared to control culture.A study reported that elicitation at high concentration may leads to hypersensitive response which may cause cell death (Namedo, 2007). Supplementation of elicitors in optimum concentrations improves secondary metabolite production (Krzyzanowska et al. 2012). Similar kind of results was reported in case of ajmalicine and taxol production (Yari Khosroushahi *et al.,* 2006). Biomass and phytocompound production were affected by type and concentration of elicitor (Salehi *et al.,* 2018), which was also observed in this study. Influence of yeast extract on accumulation of plumbagin has been also reported in cell culture of *P. rosea, U. tomentosa* and *S. baicalensis*. Addition of yeast extract in *Panax ginseng* cell suspension culture also enhanced saponin production (Lu et al. 2001). Komaraiah et al. (2002) reported role of yeast extract on production of plumbagin in *P. rosea* cell suspension culture and found enhancement of 2.4 times of plumbagin production with 1.5% of yeast extract treatment. Bhambhani et al. (2012) reported that addition of 50 and 100 mg/L yeast extract increased the production of vasicine up to 0.391 and 0.336 % DW and two times biomass enhancement in cell cultures of *Adhatoda vasica*. Stimulation ofplumbagin production by elicitors

such yeast extract, jasmonic acid and chitosan has also been observed in *Plumbago rosea* (Silja *et al.*, 2014). Roy and Bharadvaja (2019) also reported role of yeast extract on plumbagin production in root suspension culture of *P. zeylanica* where three times enhancement in plumbagin production was observed. Açıkgöz, (2020) reported that 50 mg/L yeast extract treatment enhanced rutin and isoquercetin production in cell suspension culture of *Ocimum bacilicum.*

Table 5.4: Effect of elicitors on production of plumbagin

	Elicitor		**Biomass (GI)**	**Production(µg/ml)**
Control	-	-	13.58 ± 0.57^{a}	25.26 ± 0.39^{g}
Biotic	Yeast Extract	50 mg/L	8.24 ± 0.23^{c}	46.64 ± 0.30^{c}
		100 mg/L	9.49 ± 0.14^{b}	59.48 ± 0.37^{b}
		150 mg/L	13.27 ± 0.20^{ab}	83.30 ± 0.18^{a}
	Malt Extract	50 mg/L	5.69 ± 0.10^{gh}	19.67 ± 0.16^{j}
		100 mg/L	4.67 ± 0.13^{h}	18.14 ± 0.25^{jk}
		150 mg/L	3.24 ± 0.27^{i}	16.87 ± 0.37^{k}
Abiotic	Methyl Jasmonate	50 µM	6.71 ± 0.14^{e}	32.56 ± 0.35^{e}
		100 µM	7.38 ± 0.17^{d}	26.48 ± 0.76^{g}
		150 µM	6.07 ± 0.29^{ef}	20.12 ± 0.40^{i}
	Salicylic acid	50 µM	5.33 ± 0.18^{g}	23.43 ± 0.21^{h}
		100 µM	6.09 ± 0.23^{f}	30.36 ± 0.23^{f}
		150 µM	7.11 ± 0.20^{de}	39.08 ± 0.66^{d}

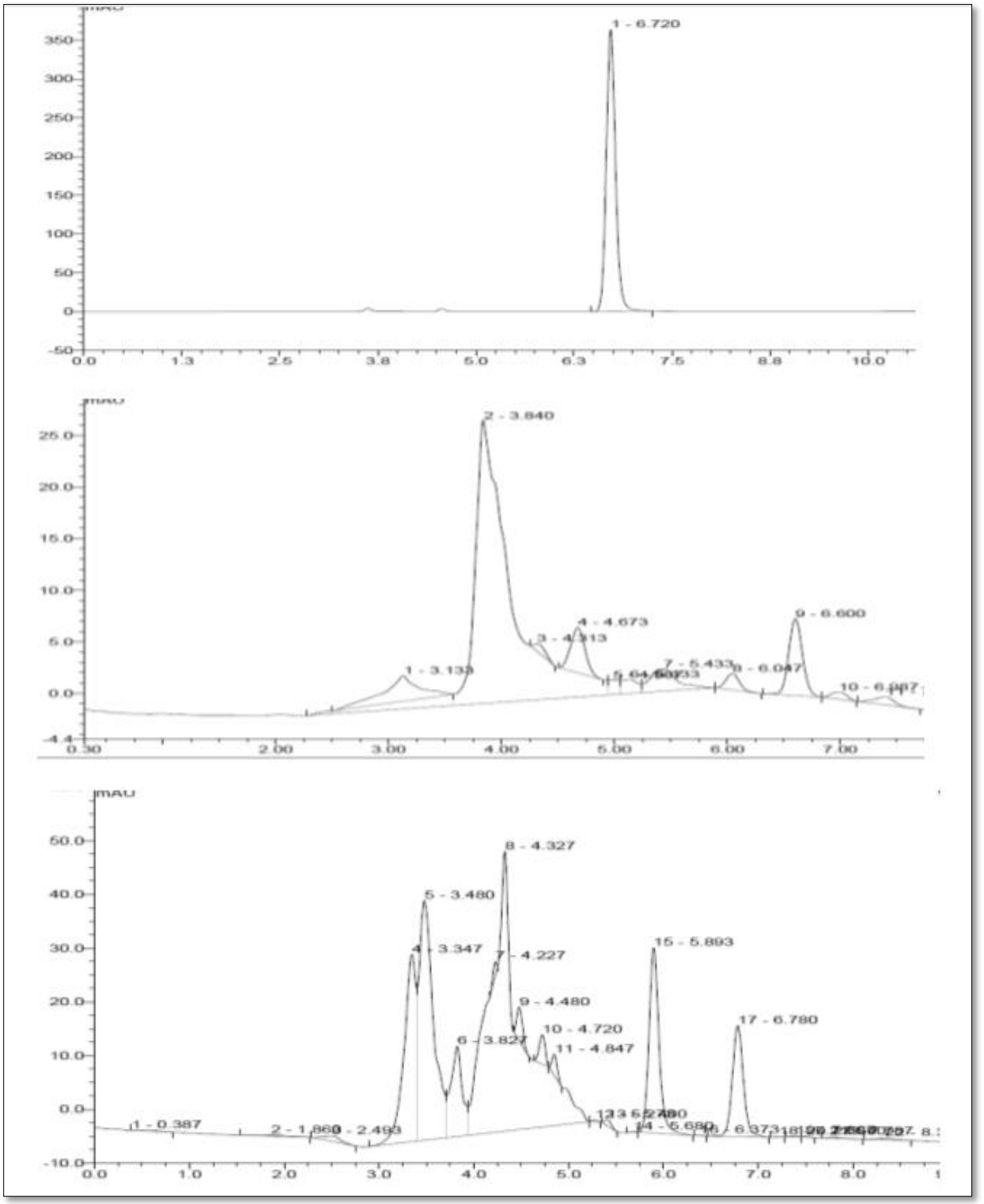


Figure 5.4: HPLC chromatogram of a) Plumbagin standard, b) Control culture and c) yeast extract treated cell suspension culture

5.4 Conclusion

In conclusion, *P. zeylanica* cell suspension culture was successfully established. This study suggested that various culture parameters effects production of plumbagin in cell suspension cultures of *P. zeylanica*. Leaf explant inoculated on MS medium supplemented with 1 mg/L NAA induced maximum callus growth and therefore utilized for the establishment of cell suspension culture. MS medium+1 mg/L NAA+ 3 g/L inoculum density provides maximum plumbagin production. Treatment of suspension culture with yeast extract (150mg/L) resulted in enhancement of plumbagin production up to 3.3 times as compared to the control culture. These results showed that yeast extract can be used effectively for plumbagin production in suspension culture of *P. zeylanica.*

The manuscript of this study is under communication

CHAPTER 6

BIOCHEMICAL AND GENETIC DIVERSITY ANALYSIS OF *PLUMBAGO ZEYLANICA* ACCESSIONS

6.1 Introduction

Medicinal plants that are growing in diverse locations contain different concentration of phytocompounds. Various environmental conditions influence the bioactive compound production. Medicinal potential of plants is due to the presence of phytocompounds including flavonoids, tannins, phenolic compounds, etc. These compounds help in human defense mechanism and fight against various problems (Atoui *et al.,* 2005). Due to their therapeutic importance there is an increase demand of natural medicine in both domestic and international market. This reason validates the biochemical analysis of *P. zeylanica*. Production of bioactive compounds is influenced by environmental factors, therefore studying different accessions for biochemical analysis provides the details about best accession. Best accession can be further utilized by pharmaceutical industry for production of quality drugs. Various studies reported antioxidant activity, total flavonoid content, total phenolic content and total tannin content of *P. zeylanica* (Tilak *et al.,* 2004; Abera *et al.,* 2015; Jaradat *et al.,* 2016). However, none of them utilized different accessions to estimate the presence of different phytochemical contents.

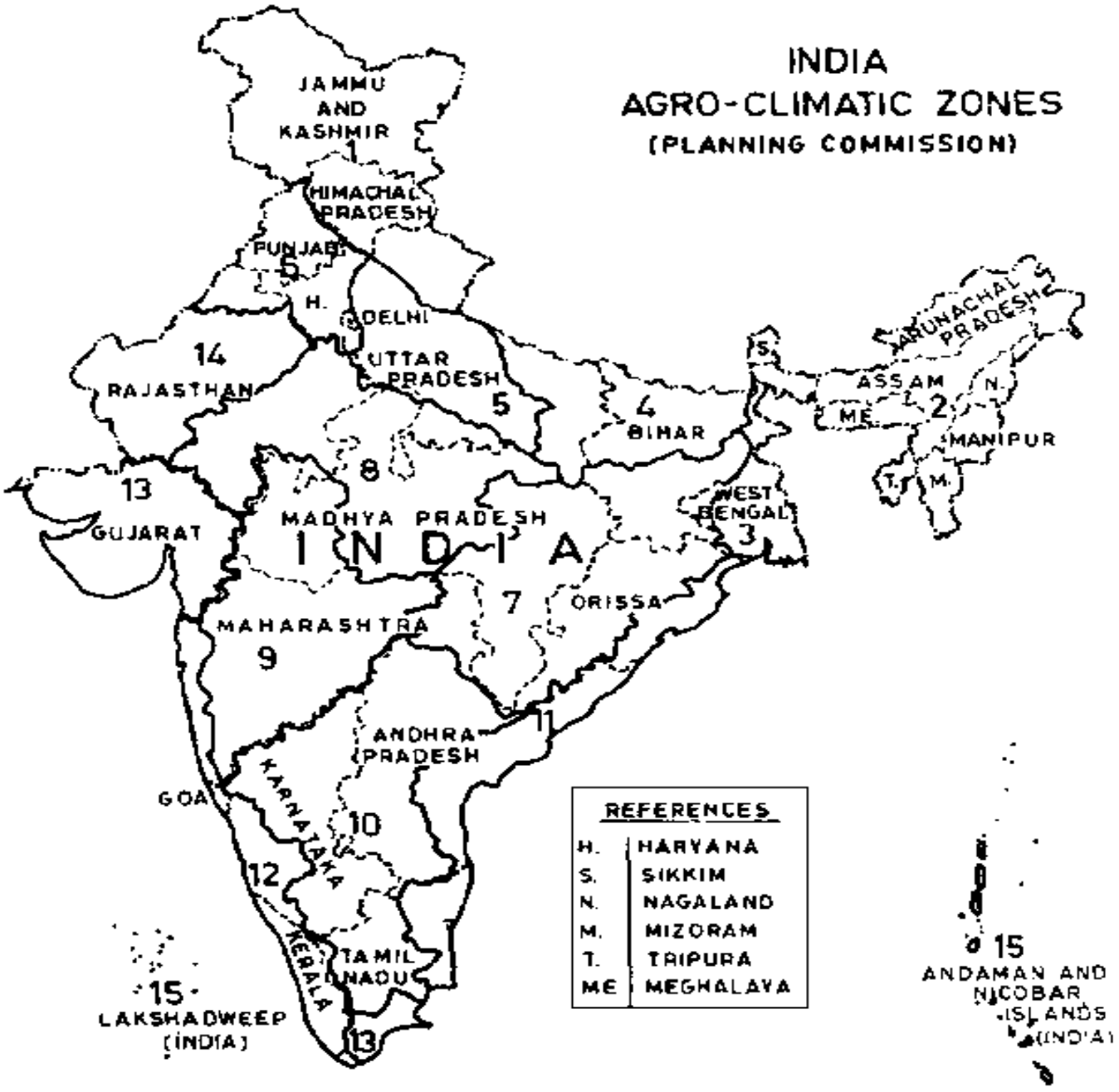
Genetic diversity among accessions shows the genetic relationship among various accessions. Genetic diversity analysis is essential for conservation, germplasm collection and breeding programs. Different accessions can be used to develop new varieties. Molecular genetic markers represent an effective technique to survey the role of different factors on hereditary diversity and population structure (Kumar and Agrawal, 2019). Diverse marker systems are available for genetic diversity analysis. Among them, SCoT and CBDP have been proved to be a helpful for population

genetic diversity studies. They can be used to screen breeding material as marker-based selection is independent of environmental conditions. These markers have characteristic functional domains which corresponds to conserved DNA sequences within genes. Collard and Mackill (2009) developed start codon targeted (SCoT) polymorphism primers which were designed from the conserved area encompassing translation initiation codon, ATG (Gupta *et al.,* 2019). It is single 18 nucleotides which are utilized as both forward and reverse primer for PCR. SCoT marker examination has been effectively utilized in various genetic diversity studies such as *Elymus sibiricus, Trichosanthes dioica, Andrographis paniculate,* rose varieties, etc. (Zhang *et al.,* 2015, Tiwari *et al.,* 2016; Kumar and Agrawal, 2019; Agarwal *et al.,* 2019). etc. CBDP also utilizes single primer for PCR. CBDP primers are 18 nucleotides in length and comprise of a central CCAAT nucleotides core flanked by filler sequence towards 5′ end and di- or trinucleotides towards 3′ end (Singh *et al.,* 2014). Genetic diversity in *Plumbago zeylanica* using different markers such as SSR, ITS, ISSR, RAPD, etc. has been reported in literature (Hajia *et al.,* 2014; Panda *et al.,* 2015). However, no study has been reported till now to assess the genetic diversity among *P. zeylanica* accessions using SCoT and CBDP markers. Therefore, in the present study an attempt has been made to analyze the biochemical content and genetic diversity of *P. zeylanica* accessions.

6.2 Material and Method

6.2.1 Plant material collection

Thirteen accessions of *P. zeylanica* (provided by NBPGR) collected from different locations of India were used for this study (Table 6.1 and Figure 6.1).



1. Western Himalayan Region
2. Eastern Himalayan Region
3. Lower Gangetic Plains Region
4. Middle Gangetic Plains Region
5. Upper Gangetic Plains Region
6. Trans-Gangetic Plains Region
7. Eastern Plateau & Hills Region
8. Central Plateau & Hills Region
9. Western Plateau & Hills Region
10. Southern Plateau & Hills Region
11. East Coast Plains & Hills Region
12. West Coast Plains & Ghats Region
13. Gujarat Plains & Hills Region
14. Western Dry Region
15. The Islands Region

Figure 6.1: Agro-climatic zones of India (Ahmad *et al.*, 2017**)**

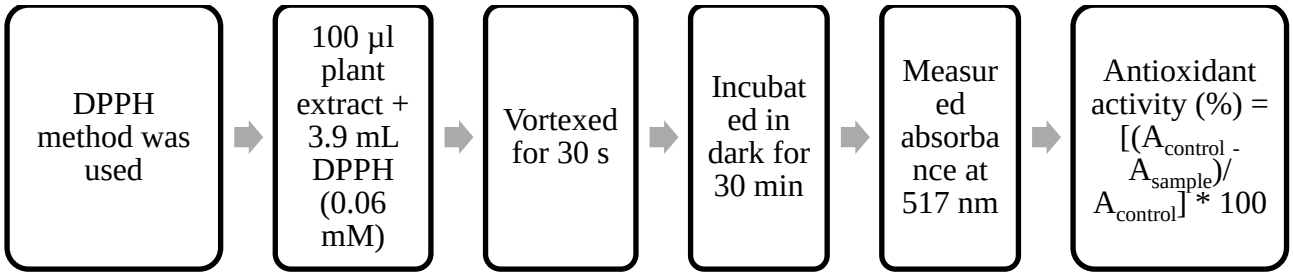
Table 6.1: List of *P. zeylanica* accessions used in the study
(Refer Figure 6.1 for agro-climatic zones of India)

S. No.	Accession No.	Collection State	Agro-climatic Zone
1	IC-539866	Uttar Pradesh	5
2	IC-211317	Delhi	5
3	IC-524440	Solan, Himachal Pradesh	6
4	IC-524441	Solan, Himachal Pradesh	6
5	IC-524444	Solan, Himachal Pradesh	6
6	IC-539867	Shimla,Himachal Pradesh	6
7	IC-256070	Shimla,Himachal Pradesh	6
8	IC-421418	Bastar, Chhatisgarh	7
9	IC-398891	Gumal, Jharkhand	7
10	IC-439214	Ranchi, Jharkhand	7
11	IC-340668	East Godavari, *Andhra Pradesh*	10
12	IC-0624282	Kadapa, Andhra Pradesh	10
13	IC-0624281	Ananthapur, Andhra Pradesh	10

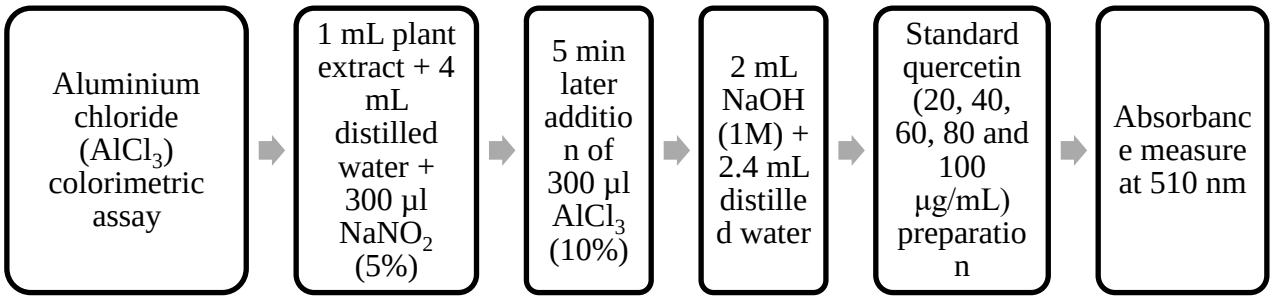
6.2.2 Biochemical analysis

For estimation of plumbagin, antioxidant activity, total phenol content, total flavonoid content and total tannin content, freshly *in-vitro* grown plant materials were dried and grounded with mortar pestle. 1.0 gm of plant material was macerated in 1 ml methanol for 48 hours and plant extract was filtered and used for further analysis.

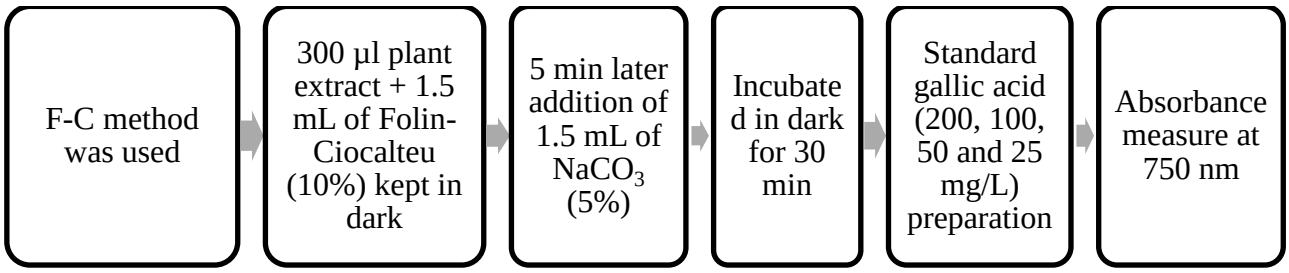
6.2.2.1 Antioxidant activity



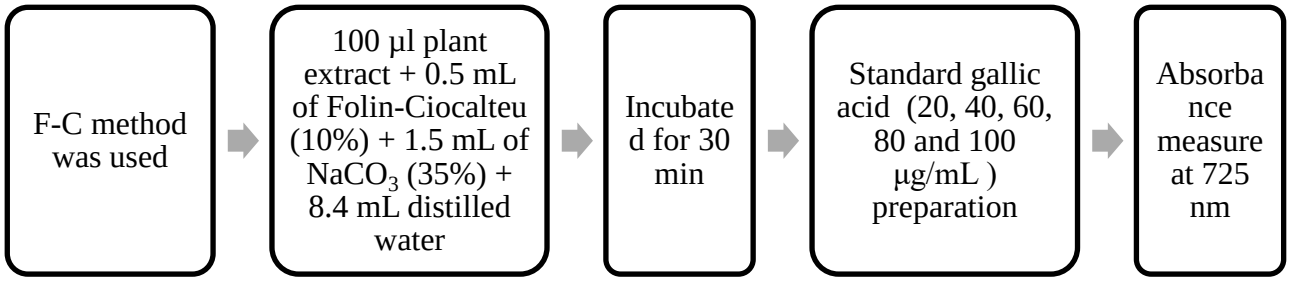
6.2.2.2 Total flavonoid estimation



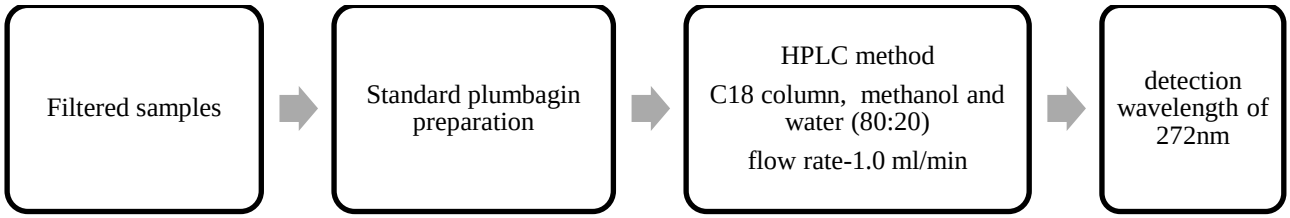
6.2.2.3 Total phenolic estimation



6.2.2.4 Total tannin estimation



6.2.2.5 Plumbagin estimation



6.2.3 Genetic diversity analysis

6.2.3.1 Extraction of DNA

Genomic DNA of all accessions was extracted from fresh leaves. CTAB method with some minor modifications was used for extraction of DNA. Approximately 200 mg of leaves were crushed with liquid nitrogen and mixed with 2 ml of CTAB (2% pre-warmed CTAB containing 0.2% β-mercaptoethanol) and incubated at 66 °C for one hour in water bath. 1ml of chloroform: isoamyl alcohol (24:1) was added before centrifugation (10 mins at 10,000 rpm). Supernatant was taken into a fresh tube and equal volume of ice-cold isopropanol was added and incubated on 4 ºC overnight and centrifugation was done (10,000 rpm at 10 mins) to form a pellet. 70% ethanol was used to wash pellet wit and then air dried. Final DNA pellet was dissolved in 100µl TE. After this RNAse treatment was given to the samples and final DNA samples were stored at -20 °C. DNA quality was verified using spectrophotometry at 260 nm and 280 nm and electrophoresis (0.8% agarose gel).

6.2.3.2 SCoT and CBDP markers analysis

Fifteen SCoT primers and twenty CBDP primers (Sigma Chemicals, USA) were used to study genetic diversity analysis in *P. zeylanica* (Figure 6.2 and 6.3).

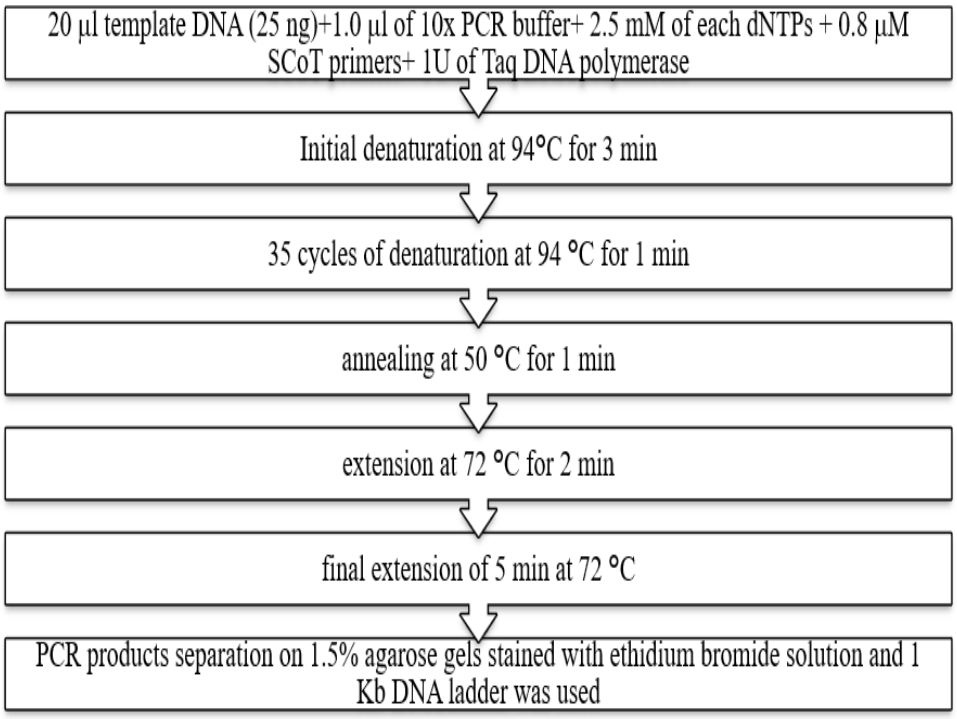


Figure 6.2: PCR mixture and amplification steps for SCoT

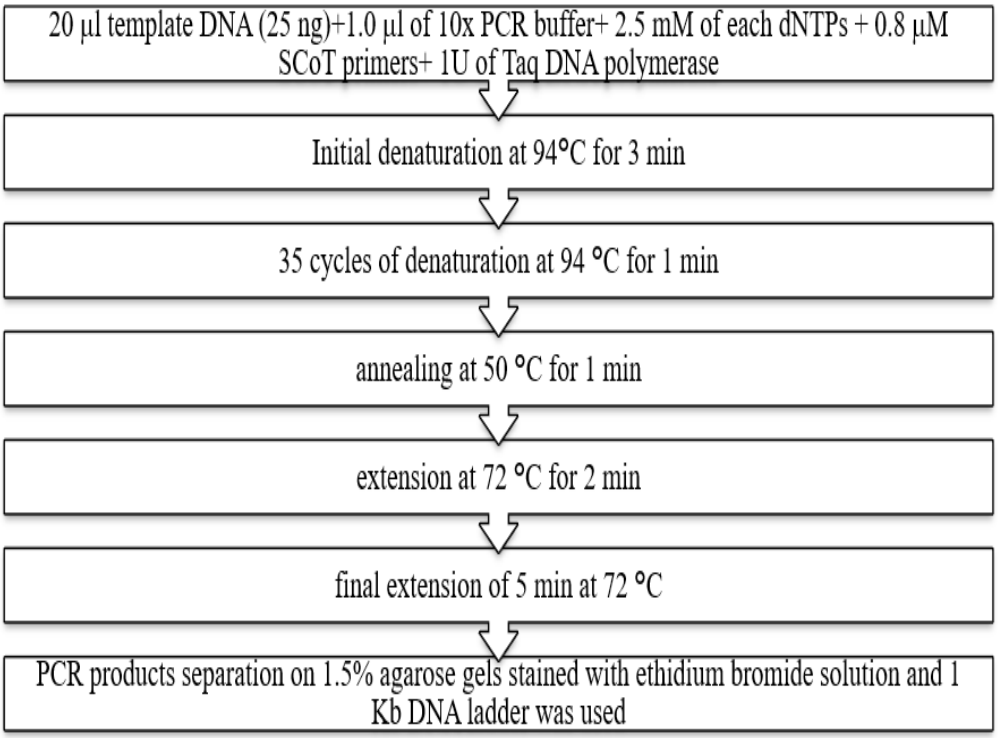


Figure 6.3: PCR mixture and amplification steps for CBDP

PCR products was scored visually on thirteen accessions of *Plumbago zeylanica*. Only clear noticeable bands were considered for analysis. Band presence was scored as "1" and band absence as "0" to make a binary data sheet from gel picture. Polymorphic information content (PIC) where PIC value was calculated as PIC = 2*fi*(1 − fi) where fi is frequency of amplified allele (band present) and (1 − fi) is the frequency of the null allele (band absent) (Roldan-Ruiz *et al.*, 2000). UPGMA clustering and AMOVA were done.

6.3 Results and Discussion

6.3.1 Biochemical analysis

DPPH free radical scavenging effect of different accession of *P. zeylanica* ranges from 15.67-134 µg/ml (Table 6.2). All the extracts have notable effect on scavenging free radicals and IC-524441 showed highest scavenging activity (134.17±0.76 µg/ml) while lowest scavenging activity was observed in IC-539867 (15.67±0.58 µg/ml). This could be due to highest content of total polyphenolic compounds which include anthraquinones, flavonoids, xanthones, anthocyanidins and tannins (Panche *et al.,* 2016).

Total phenolic content of samples ranges from 30.50 to 80.47 µg/ml (Table 6.2). IC-524441 possesses highest amount of phenolic content (80.47±0.64 µg/ml) whereas lowest content was found in IC-539867 (30.50±0.70 µg/ml). Significant differences in phenolic content among the accession may be due to various parameters that are associated with the accessions like ecological factors, temperature, etc. Presence of different phenolic compounds may be the cause of variation of total phenolic content in the plant extracts. Polyphenols are vital dietary antioxidants as they possess strong free radical scavenging activity. Various reports suggested that polyphenols protect against numerous diseases (Cory *et al.,* 2018). These compounds possess significant role in growth and reproduction, and protect against damaging pathogens. Sharma *et al.*, (2014) reported that

methanolic extract of *P. zeylanica* leaves also showed presence of phenolic content i.e. 28.25±0.001 µg/ml.

Total flavonoid content ranges from 18.40-68.63 µg/ml (Table 2). IC-524441 possess maximum amount of flavonoid content (78.63±0.78) while minimum content was found in IC-539866 (18.40±0.78 µg/ml). Flavonoids are potential scavengers of most oxidizing molecules which include singlet oxygen and several other free radicals involved in various diseases such as cancer (Kumar and Pandey, 2013). It suppresses reactive oxygen formation, chelating trace elements and protects antioxidant defenses (Kurutas, 2015). Sharma *et al.,* (2014) reported flavonoid content in stem of *P. zeylanica* i.e. 72.3µg/ml

Total tannin content ranges from 50.60-109.50µg/ml (Table 6.2). Highest amount was observed in accession number IC-524441 i.e. 109.50±0.50 µg/ml and lowest in accession number 539866 which is 50.60±0.60 µg/ml. Tannins are water-soluble compounds, widely found in nature and possibly found in all plants (Pizzi, 2019). Tannins possess wide range of pharmaceutical activities such as antimicrobial, antioxidant, free radical scavenging and anti-ulcerogenic activities (Shad *et al.,* 2012).

Plumbagin amount in plants were estimated by comparing samples with standard plumbagin solution. Plumbagin concentration varied between 5.3-25.54 µg/ml (Table 6.2). Results showed that accession numbers IC-524441 contained maximum amount of plumbagin i.e. 25.54 ±0.58 µg/ml and minimum in accession number 539867 which is 5.3±0.55 µg/ml. Accession number IC-524441 possesses maximum phenolic content, flavonoid content, tannin content, antioxidant activity and plumbagin production.

Table 6.2: Biochemical analysis of 13 accessions of *P. zeylanica*

Accession No	Antioxidant activity (µg/ml)	Total phenolic content estimation(µg/ml)	Total flavonoid estimation (µg/ml)	Total tannin Estimation (µg/ml)	Plumbagin Estimation (µg/ml)
IC-524440	89.20±0.72[c]	69.23±0.78 [c]	55.70±0.96[b]	94.53±0.50 [b]	22.44±0.71[b]
IC-524441	134.17±0.76[a]	80.47±0.64 [a]	78.63±0.78a	109.50±0.50 [a]	25.54 ±0.58[a]
IC-524444	109.50± 0.87[b]	76.97±0.55[b]	62.17±0.86 a	94.97±0.85 [b]	24.17±0.58[ab]
IC-539866	19.13±0.81[h]	40.13±0.71[e]	18.40±0.78[f]	50.60±0.60 [f]	7.79±0.64 [i]
IC-539867	15.67±0.58 [h]	30.50±0.70 [f]	30.47±0.55 [d]	73.07±0.70[d]	5.3±0.55 [j]
IC-256070	29.50±0.50[g]	61.57±0.55[c]	50.33±0.76 [b]	89.00±0.50[c]	7.98±0.61 [i]
IC-211317	78.33±0.58 [d]	45.00±0.50 [e]	51.30±0.70 [b]	51.47±0.55	16.89±0.55[d]
IC-421418	79.57±0.75[d]	55.13±0.71 [d]	43.87±0.78[c]	86.00±0.40 [c]	21.18±0.76[c]
IC-340668	54.67±0.58[f]	34.97±0.35 [f]	25.13±0.61[e]	66.93±0.40 [e]	8.74±0.96[h]
IC-0624282	74.30±0.61 [d]	66.67±0.49 [c]	29.03±0.55 [e]	55.20±0.62[f]	10.74±0.55 [f]
IC-0624281	65.17±0.76[e]	70.80±0.98[b]	49.33±0.67 [c]	78.07±0.80 [d]	10.07±0.64 [f]
IC-398891	59.63±0.64 [f]	53.03±0.55[d]	47.17±0.38 [c]	54.97±0.85 [f]	9.64±0.71[g]
IC-439214	64.87±0.81[e]	49.00±0.50 [e]	31.30±0.62[d]	60.70±0.62[e]	13.44±0.58[e]

6.3.2 Genetic diversity analysis

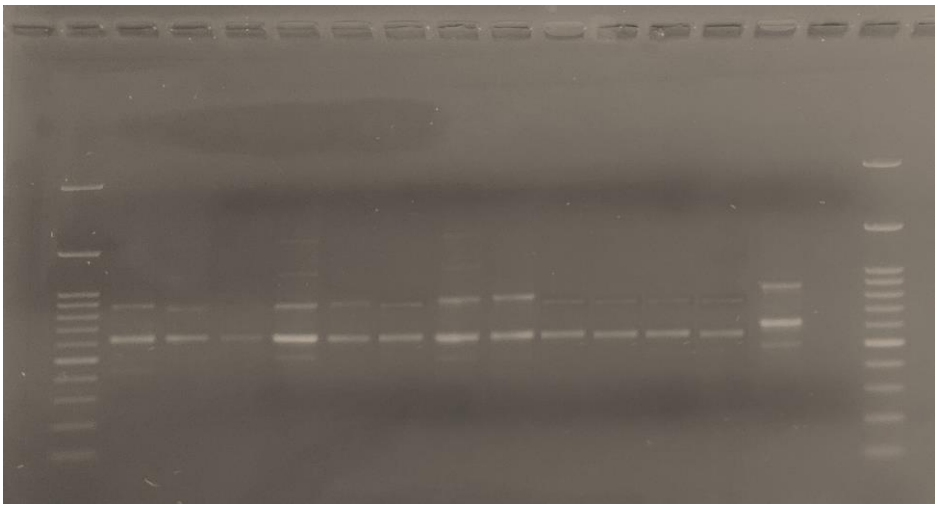
To evaluate genetic diversity among13 accessions of *P. zeylanica*, two different molecular markers i.e. CBDP and SCoT were used. SCoT and CBDP are gene targeted markers and used to find our best marker system. From the literature survey it was found that only few studies reported use of SCoT markers to characterize *P. zeylanica* where no reports were found on CBDP markers. This

is first report where gene-based markers were used for genetic diversity analysis of different *P. zeylanica* accessions.

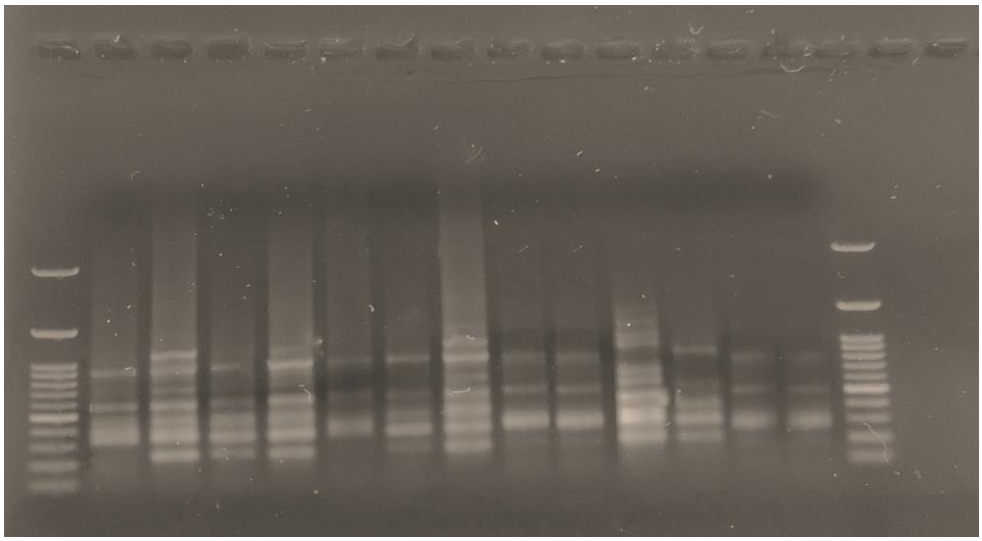
Fifteen SCoT primers were used for DNA amplification. All 15 primers generated polymorphic DNA amplification products (Table 6.3. These fifteen SCoT primers produce 86 amplicons and amplicon number varies from 3(SCoT-4) to 10(SCoT-15) and average is 5.73 amplicons/primer. Polymorphic bands vary from 1 to 8 and average of 4.45 amplicons/primer. Polymorphism percentage varies from 33.33% to 100% with average of 76.33%/primer. PIC values vary from 0.12 to 0.37 and average is 0.25/primer. Similarly, in case of *Tinospora cardifolia,* 102 amplicons (5.3 amplicons/primer) were obtained with nineteen SCoT primers (Paliwal et al. (2013). They reported that average polymorphic amplicons were 4.74/ primer and average polymorphism and PIC value were 87.02% and 0.49/ primer. Tiwari *et al.,* (2016) reported that in case of *Andrographis paniculata,* 20 SCoT primers generate 132 amplicons (6 amplicons/primer) and average polymorphic amplicons of 4.5/ primer. The percentage of polymorphism was 75% and average PIC value was 0.34/ primer. These outcomes indicated that SCoT primers showed high amplification potential and polymorphism (Figure 6.4).

Very few genetic diversity analysis have been conducted with CBDP markers. However, this is the first study in *P. zeylanica* using CBDP markers. Twenty CBDP primers generated 110 amplicons (5.5 amplicons/primer (Table 6.4). Polymorphic amplicons vary from 2(CAAT-5) to 8 (CAAT-7) and average is 4.7 amplicons/ primer. Polymorphism percentage vary from 60.00% to 100% with average of 84.9%/primer. PIC values vary from 0.15 (CAAT-19) to 0.59 (CAAT-2), average is 0.26/primer. Similarly, using seventeen CBDP primers, a study reported gender specific diversity in jojoba genotypes where 133 amplicons in male and 126 amplicons in female (Heikrujam *et al.,* 2015). They reported that in female Jojoba average PIC was 0.42/ CBDP primer.

Singh et al. (2014) reported used twenty-five CBDP markers in Jute and obtained 82% polymorphism and average PIC value of 0.41/primer. In another study where Chickpea genotypes was used, the average PIC value was 0.45/primer with CBDP markers (Hajibarat *et al.*, 2015).



(a)



(b)

Figure 6.4: PCR amplified product of 13 accessions of P. *zeylanica* (a) CBDP (primer 1) and (b) SCoT (primer 7) with 100bp ladder

Table 6.3: SCoT primers sequence analysis

Primer	Primer Sequence	% of GC	Total no of amplicon	No. of monomorphic amplicon	No. of polymorphic amplicon	Polymorphism %	PIC
SCoT1	ACGACATGGCGACCACGC	66.67	4	1	3	75	0.27
SCoT2	ACCATGGCTACCACCGAC	61.11	4	1	3	75	0.27
SCoT3	ACCATGGCTACCACCGAG	61.11	4	1	3	75	0.31
SCoT4	ACCATGGCTACCACCGGG	66.67	3	2	1	33.33	0.12
SCoT5	ACCATGGCTACCACCGTG	61.11	7	1	6	85.71	0.32
SCoT6	CCATGGCTACCACCGGCC	72.22	7	2	5	71.43	0.23
SCoT7	CCATGGCTACCACCGCAG	66.67	7	3	4	57.14	0.23
SCoT8	CAACAATGGCTACCACCA	50	6	1	5	83.33	0.29
SCoT9	CAACAATGGCTACCACCC	55.56	4	0	4	100.00	0.28
SCoT10	CAACAATGGCTACCACCG	55.56	6	1	5	83.33	0.27
SCoT11	CAACAATGGCTACCACCT	50	5	2	3	60	0.22
SCoT12	CAACAATGGCTACCACGT	50	5	1	4	80	0.18
SCoT13	CAACAATGGCTACCAGCA	50	7	0	7	100	0.19
SCoT14	ACGACATGGCGACCAACG	61.11	7	1	6	85.71	0.37
SCoT15	CCATGGCTACCACCGGCG	77.78	10	2	8	80	0.22

Table 6.4: CBDP primers sequence analysis

Primer	Primer Sequence	% of GC	Total no of amplicon	No. of monomorphic amplicon	No. of polymorphic amplicon	Polymorphism %	PIC
CAAT1	TGAGCACGATCCAATACC	50.00	5	2	3	60.0	0.18
CAAT 2	TGAGCACGATCCAATAAG	44.44	9	2	7	77.8	0.59
CAAT 3	TGAGCACGATCCAATCAG	50.00	5	1	4	80.0	0.16
CAAT 4	TGAGCACGATCCAATGAT	44.44	6	0	6	100.0	0.26
CAAT 5	TGAGCACGATCCAATTGC	50.00	3	1	2	66.7	0.21
CAAT 6	TGAGCACGATCCAATGAG	50.00	4	1	3	75.0	0.31
CAAT 7	TGAGCACGATCCAATGCG	55.56	8	0	8	100.0	0.32
CAAT 8	TGAGCACGATCCAATTGA	44.44	4	1	3	75	0.35
CAAT 9	TGAGCACGATCCAATTCA	44.44	5	0	5	100	0.36
CAAT 10	CTGAGCACGATCCAATAG	50.00	5	1	4	80	0.23
CAAT 11	CTGAGCACGATCCAATAC	50.00	8	2	6	75	0.22
CAAT 12	CTGAGCACGATCCAATAT	44.44	5	0	5	100	0.27
CAAT 13	CTGAGCACGATCCAATCA	50.00	7	1	6	85.71	0.24
CAAT 14	CTGAGCACGATCCAATGG	55.56	4	1	3	75	0.17
CAAT 15	CTGAGCACGATCCAATGT	50.00	4	1	3	75	0.25
CAAT16	TGAGCACGATCCAATAGC	50.00	4	0	4	100	0.23
CAAT17	TGAGCACGATCCAATAAT	38.89	4	0	4	100	0.23
CAAT18	TGAGCACGATCCAATGTT	44.44	5	0	5	100	0.33
CAAT19	TGAGCACGATCCAATATA	44.44	8	1	7	87.5	0.15
CAAT20	CTGAGCACGATCCAATAG	50.00	7	1	6	85.71	0.25

6.3.2.1 Cluster analysis

Cluster analysis was done to estimate the genetic diversity among *P. zeylanica* accessions. In case of SCoT data analysis, three clusters were obtained in dendrogram. In cluster 1 and cluster 2 only 2 accessions were clustered while cluster 3 contains nine accessions. Cluster 3 was further grouped in two subcluster. Paliwal et al. (2013) also found three clusters using SCoT data in case of *T. cardifolia*. In cluster1 and 3 three and four accessions were present while in cluster 2, thirty-two accessions were grouped (Figure 6.5). In case of CBDP data analysis, three clusters were obtained in dendrogram. In cluster 1, only 1 accession were grouped while cluster 2 and cluster 3, six accessions were present in each cluster. Cluster 2 was further grouped in five subcluster where in Subculster 1, 2 accessions were present and other four subcluster contains 1 accession in each. Similarly, Cluster 3 was further grouped in five subcluster where in Subculster 1, 2 accessions were present and other four subcluster contains 1 accession in each. Similar kind of genetic similarity was reported in Jojoba (Heikrujam et al. 2015) and Linseed (Singh et al. 2014) where CBDP markers were used.

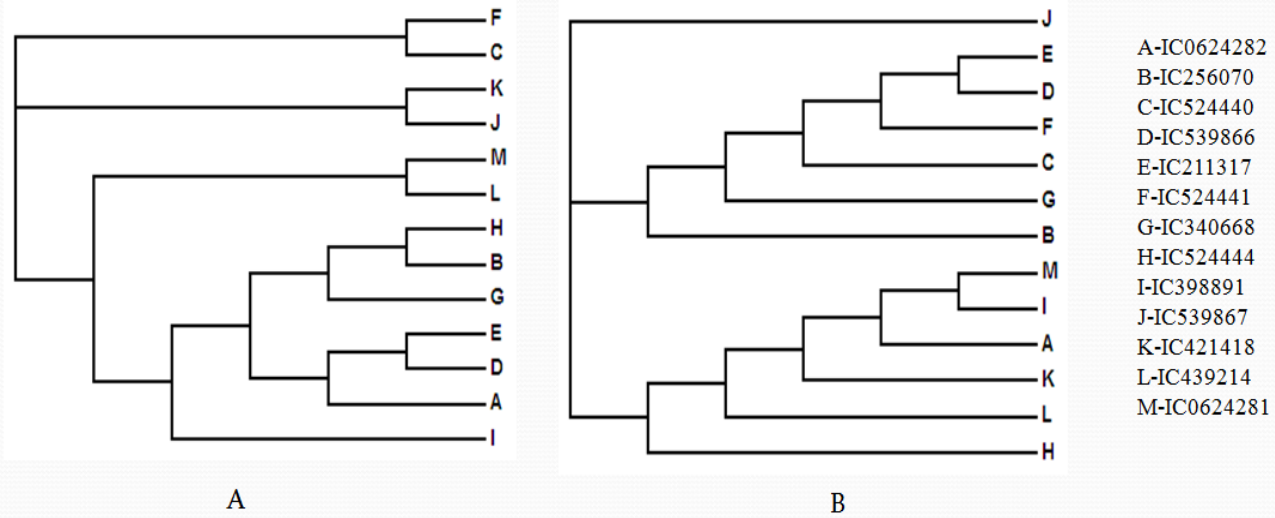


Figure 6.5: UPGMA clustering of 13 accessions of *P. zeylanica* for SCoT and CBDP markers data

6.3.2.2 AMOVA study

AMOVA was done to estimate the difference in *P. zeylanica* accessions. AMOVA analysis of SCoT marker showed variation of 53% among individuals, 39% within individuals and 8% among agro-ecological regions (Figure 6.6). Similarly, AMOVA analysis of CBDP marker showed maximum variation of 57% within individuals, 38% among individuals and 5% among agro ecological regions (Figure 6.7). SCoT and CBDP markers are able to provide information about DNA polymorphism. A study reported high level of genetic diversity in case of *Breonandia salicinia* population i.e. 17% with 14 ISSR markers (Gaafar *et al.,* 2014). Gorji *et al.,* (2011) studied twenty-four varieties of tetraploid potato using SCoT, RAPD and ISSR markers and performed AMOVA analysis, they observed less than 10% of total genetic variation. Tiwari *et al.,* (2016) reported that AMOVA analysis for CBDP marker showed maximum variation of 10% whereas in case SCoT markers it was 2%.

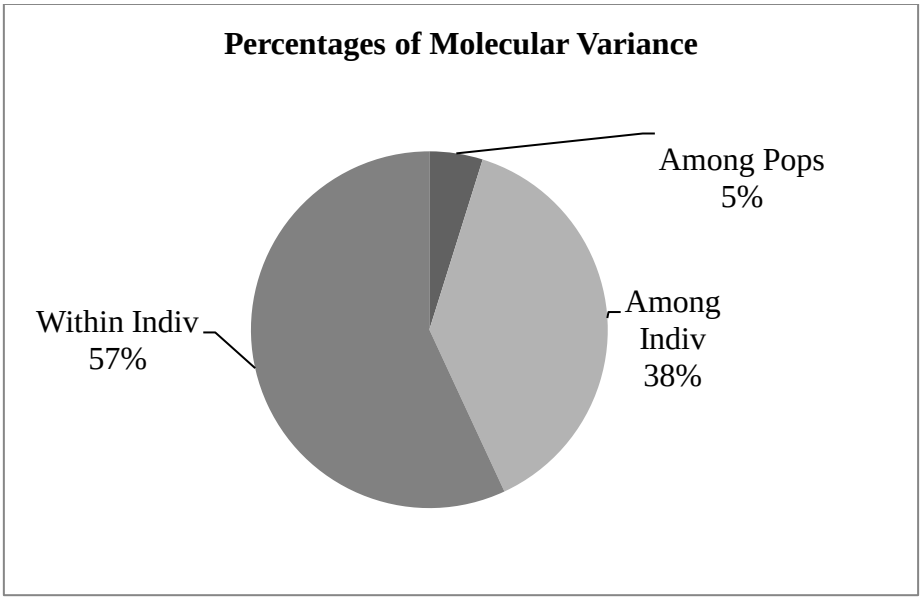


Figure 6.6: Analysis of Molecular Variance (AMOVA) with CBDP marker

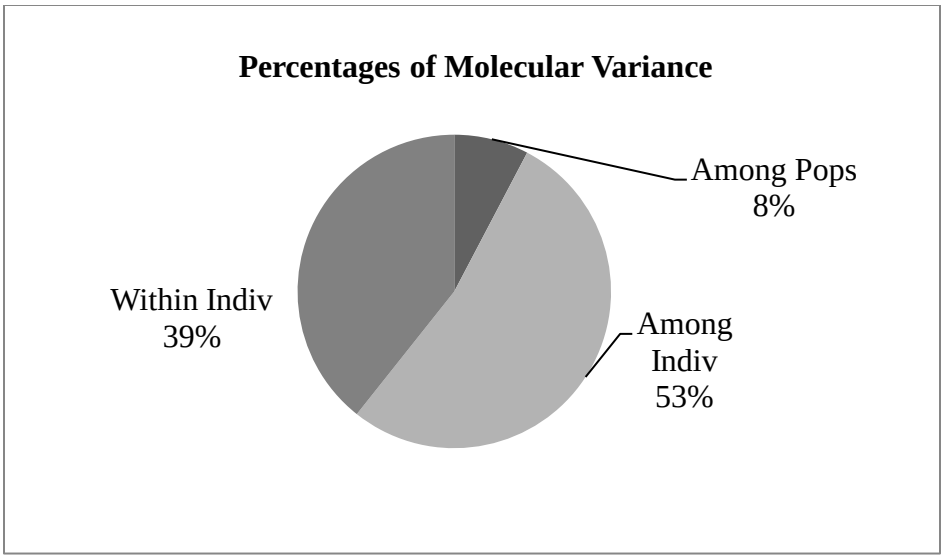


Figure 6.7: Analysis of Molecular Variance (AMOVA) with SCoT marker

6.4 Conclusion

Production of bioactive compounds are influenced by various factors, therefore studying different accessions for biochemical analysis provides the details about best accession. In this study it was found that IC-524441 provides maximum antioxidant activity, phenolic content, flavonoid content, tannin content and plumbagin content. SCoT and CBDP marker analysis were effectively done to evaluate genetic diversity among *P. zeylanica* accessions. Polymorphism shown by SCoT and CBDP can be utilized for molecular study of *P. zeylanica* accessions, which provides valuable information for germplasm management, improve breeding strategies, and genetic resources conservation. Both the markers provide higher reproducibility and can be used for any plant species. Therefore, based on the requisite and plant species, several numbers of primers can be designed. The method has numerous advantages and it is comparatively easy to carry out as there is no requirement of previous genome sequence information.

The manuscript of this study is under preparation

CHAPTER 7

GREEN SYNTHESIS OF SILVER NANOPARTICLES FROM *P. ZEYLANICA* AND ITS ANTIBACTERIAL AND DYE DEGRADATION ACTIVITY

7.1 Introduction

Nanotechnology deals with nanomaterial synthesis which ranges from 1-100nm. Nanoparticles have extraordinary properties due to their particular potentials like shape, size, and distribution compared to larger particles. Various nanoparticles that are frequently examined includes Ag, Au, Cu, Pt, Pd, etc. and among them silver play important role in living forms, biological systems, and pharmaceuticals (Gurunathan *et al.,* 2009). Mostly, nanoparticles are synthesized using physical, chemical and biological methods. Chemical and physical methods have various problems such as low yield, use of toxic chemical agents, expensive, etc. (Mallick *et al.,* 2004). So eco-friendly method is required to solve this issue. Biological method uses micro-organisms, plant extract and enzymes, they have various advantages such as simple method, cost-effective and biocompatibility (Kouvaris *et al.,* 2012). Earlier report suggested that presence of neumerous compounds in *P. zeylanica* act as reducing and capping agent (Nayak *et al.,* 2016). Use of residual plant material for nanoparticles synthesis has various advantages such as independent of environmental variations, constant supply of plant material, etc. Synthetic dyes are one of the toxic pollutants that come from numerous industrial processes such as printing, textile, leather etc. They are considered as recalcitrant pollutants which contain composite structure and cannot be easily degraded (Chung and Cerniglia, 1992). Therefore, an efficient method to eliminate these pollutants from environment is of high demand. In this experiment residual material of *P. zeylanica* was used for silver nanoparticles synthesis. Further antibacterial activity of nanoparticles and dye degradation potential were assessed.

7.2 Materials and Methods

7.2.1 Aqueous extract preparation

Residual plant material was air dried for a week. 500mg of air-dried plant material were boiled in 50 ml of distilled water for 10 minutes. Then extract was cool down and filtered.

7.2.2 Optimization of silver nanoparticles synthesis and its characterization

1mM of $AgNO_3$ solution was prepared by measuring 33.974 mg of $AgNO_3$ dissolving it in 200ml of distilled water. 1ml of aqueous extract was added to 9ml of $AgNO_3$ (1:10). Nanoparticles synthesis was done at different temperatures (25, 30, 50 and 70 °C), time interval (0 to 96 hours), $AgNO_3$ concentration (1 to 5 mM) and plant extract concentration (1-5 ml). Bio-reduction was observed by color change from colorless to yellowish brown and detected by using UV–Vis spectrophotometer (Pekin Elmer). Detection wavelength of silver nanoparticles was 300 to 700nm Purification of nanoparticles solution was done by repeated centrifugation at 4,500 rpm for 10 min. For characterization of synthesized nanoparticles FTIR, SEM, TEM and XRD were done.

7.2.3 Antibacterial Activity

Synthesized silver nanoparticles were tested against five bacteria bacterial i.e. *Escherichia coli, Swenella putrifaciens, Staphylococcus aureus, Serratia marcescens* and *Alcaligenes faecalis*. pH of nutrient broth and nutrient agar was adjusted to 7 before autoclaving (15 lbs pressure at 121°C temperature for 15 min). Each bacterium was grown in 20 ml NB and incubated overnight at 37°C and further used for experiment. Antibacterial activity was done using disc diffusion method. 100µL of each culture was spread uniformly on agar plates. 100µl of different concentration of silver nanoparticles solution were loaded onto disc and plates were incubated overnight at 37°C and zone of inhibition was recorded.

7.2.4 Degradation of different dyes

Photocatalytic activity of synthesized silver nanoparticles in dye degradation was performed using different dyes i.e. EY, MO, MB, MR and PR. 1 ml respective dye (1 mM) was mixed with 1ml of synthesized silver nanoparticles and volume made up to 5 ml using distilled water. All the solutions were kept in Sunlight. For catalytic degradation 1ml respective dye (1mM) was mixed with 1ml of 0.1M $NaBH_4$. Then 1ml of synthesized silver nanoparticles was added and volume made up to 5ml using distilled water. The change in color intensity were measured using UV–Vis spectrophotometer in the range of 300-700nm.

7.3 Results and Discussion

7.3.1 Effect of culture parameters on silver nanoparticles synthesis

7.3.1.1 *Effect of different temperature*

All the four incubation temperatures showed color change from light yellow to brown. SPR band of synthesized silver nanoparticles were found at 446, 425, 431 and 423 nm for 25, 30, 50, and 70°C temperature respectively (Figure 7.1). Maximum AgNPs synthesis was obtained at 70°C. Nayak *et al.,* (2016) obtained maximum silver nanoparticles synthesis at 80°C. Zhang *et al.*, (2013) reported silver nanoparticles synthesis at room temperature in case of *Aloe vera.* Oluwaniy *et al.,* (2016) also found that in case of *Thevetia peruviana*, lower temperature (i.e. 30°C) provides rapid silver nanoparticles synthesis.

7.3.1.2 *Effect of different time interval*

For optimization of time interval, nanoparticles formation was estimated from 24h to 96h. SPR band of nanoparticles was found at 432, 435, 434 and 437 nm for 24h, 48h, 72h and 96h time interval respectively (Figure 7.1). Shankar *et al.,* (2004) found 90% metal ions reduction within

four hours. A study reported maximum synthesis of silver nanoparticles from *Melissa officinalis* after 72hr of incubation period (de Jesús Ruíz-Baltazar *et al.*, 2017).

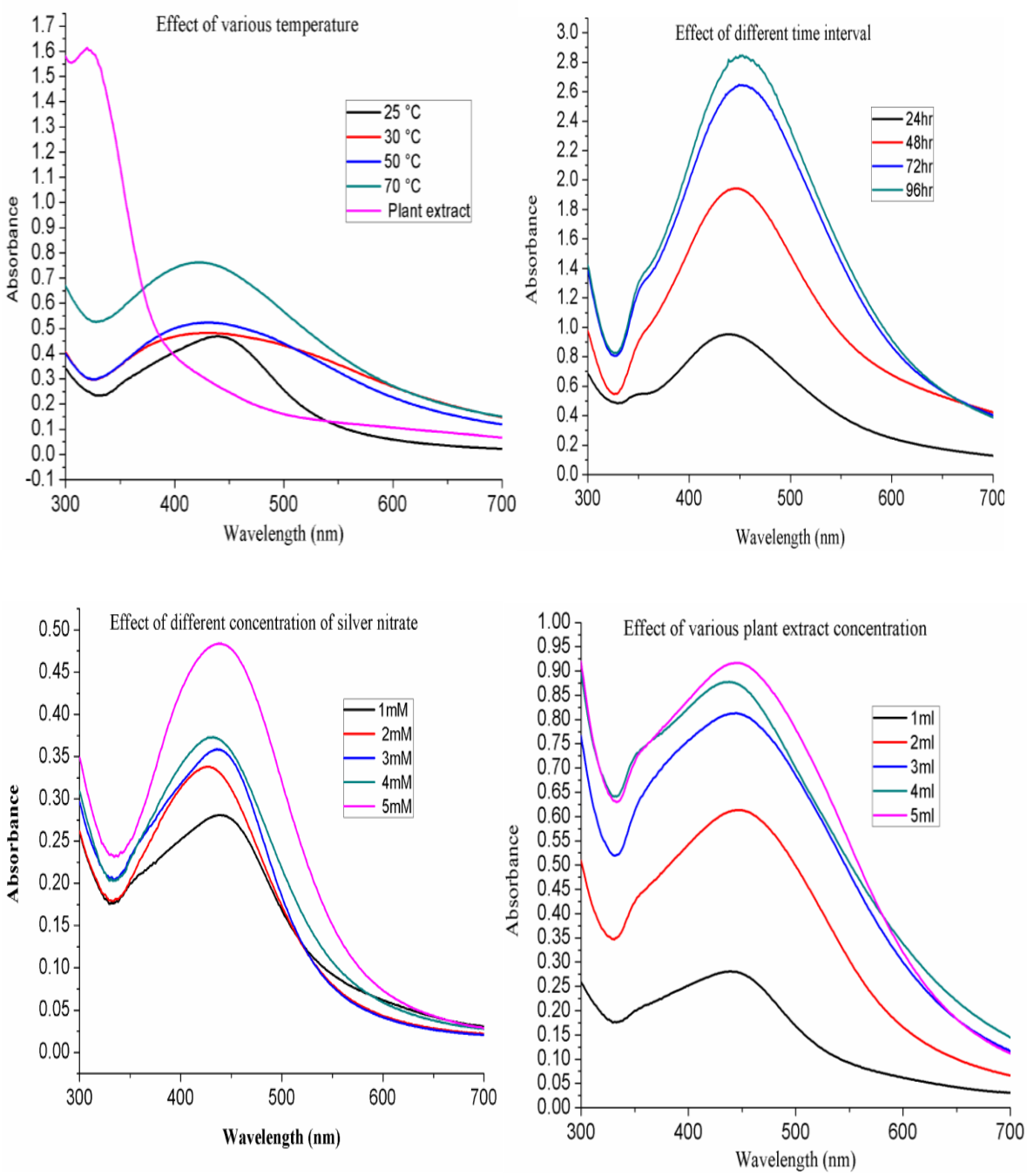


Figure 7.1: UV–Vis spectra of synthesized silver nanoparticles at different temperature, time interval, silver nitrate concentration and plant extract concentration

7.3.1.3 *Effect of various concentration of silver nitrate*

It was observed that silver ion intensity increases with increase concentration of silver nitrate (Figure 7.1). Rate of synthesis was significantly depending on concentration of silver nitrate. Oluwaniy *et al.,* (2016) also found highest nanoparticles synthesis at 5mM silver nitrate concentration in case of *Thevetia peruviana*. Salunke *et al.,* (2014) reported that in case of *P. zeylanica,* maximum nanoparticles synthesis at 0.7mM concentration. Ramesh *et al.,* (2015) reported that 1mM concentration of $AgNO_3$ was optimum for silver nanoparticles synthesis from *Emblica officinalis.*

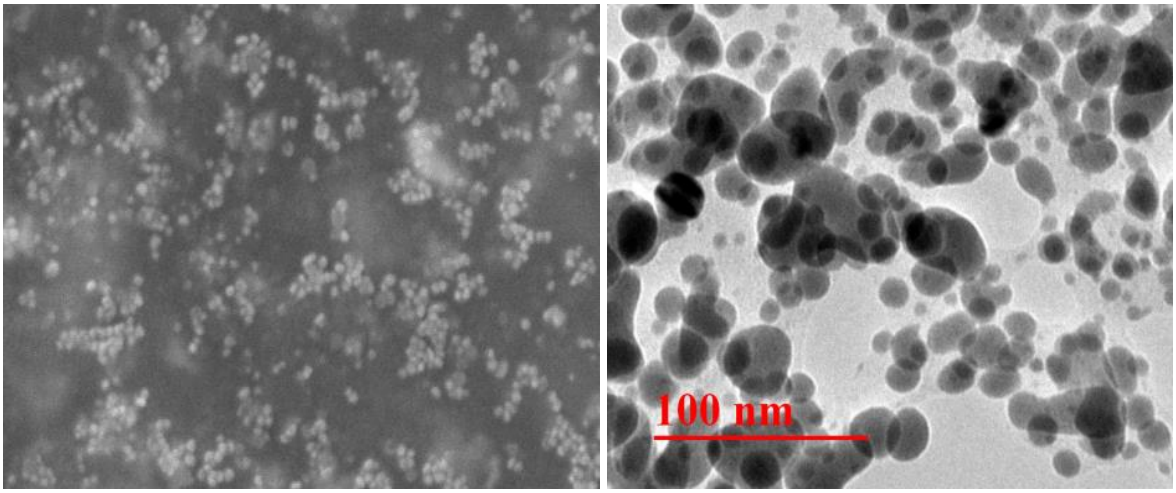
7.3.1.4 *Effect of various plant extract concentration*

For optimization of plant extract concentration, 1-5ml of plant extract was used. Absorption spectra reveals that 5ml concentration of plant extract showed maximum nanoparticles synthesis at 441 nm (Figure 7.1) whereas 1ml plant extract concentration showed minimum synthesis. A study reported that 5 ml of *P. zeylanica* extract possess maximum silver nanoparticles synthesis (Salunke *et al.,* 2014). Similarly, another study reported that 10 ml *Emblica officinalis* extract was optimum for silver nanoparticles synthesis (Ramesh *et al.,* 2015).

7.3.2 SEM and TEM Analysis

SEM analysis provides surface morphology of silver nanoparticles. It was observed synthesized silver nanoparticles were uniform and spherical in shape (Figure 7.3a). TEM analysis reveals the size and morphology of nanoparticles. TEM image clearly indicates that synthesized nanoparticles were spherical in shape (Figure 7.3). It was found that synthesized nanoparticles were in 50–110 nm (average size 55nm) range size. A study reported that *P. zeylanica* synthesized silver nanoparticles were 60nm size (Salunke *et al.,* 2014). In case of *Emblica officinalis* synthesized silver nanoparticles, the size ranges from 10 to 70 nm and shape was spherical (Ramesh *et al.,*

2015). Bagherzade *et al.,* (2017) reported that *Crocus sativus* synthesized silver nanoparticles was spherical shape and 20 nm in size.



(a) (b)

Figure 7.2: a) SEM image and b) TEM images

7.3.3 FT-IR analysis

FT-IR analysis provides the details about presence of biologically active molecules on the surface of silver nanoparticles. Spectrum of plant extract (Figure 7.2) showed presence of six major peaks i.e. 1212.4 cm^{-1}, 1368.6 cm^{-1}, 1633.7 cm^{-1}, 1739.9 cm^{-1}, 2341.4 cm^{-1} and 3355.0 cm^{-1}. Silver nanoparticles spectrum (Figure 7.2) showed five peaks at 1214.0 cm^{-1}, 1370.4 cm^{-1}, 1642.1 cm^{-1}, 1739.9 cm^{-1} and 3341.1 cm^{-1}. The peak at 1633.7$cm^{-}1$ in plant extract was due to the presence of amide I vibration and it was shifted to 1642.1 cm^{-1} in case of silver nanoparticles because of the proteins that probably bind to silver nanoparticles through amine groups**.** Peak at 1212.4 cm^{-1}, 1368.6 cm^{-1} and 2341.4 cm^{-1} show the presence of C-O-C, O-H and C-O group respectively. Peak at 1740.2 cm^{-1} signifies C=O stretch which indicated ketones, aldehydes, esters, or carboxylic acids presence (Salunke *et al.,* 2014). Peak at 3355.0 cm^{-1} shows N–H bond which indicated the primary and secondary amines presence. Presence of similar peaks in plant extract as well as in

silver nanoparticles shows the existence of biomolecules attachment in the extract to nanoparticles. From the result it was seen clearly that different functional groups play important role in nanoparticles synthesis (Song and Kim, 2009). A report suggested that flavonoids presence in *Tephrosia purpurea* leaf extract may act as reducing agent and carboxylate group attach on the surface of nanoparticles helps in stabilization of nanoparticles during the synthesis (Ajitha *et al.,* 2014).

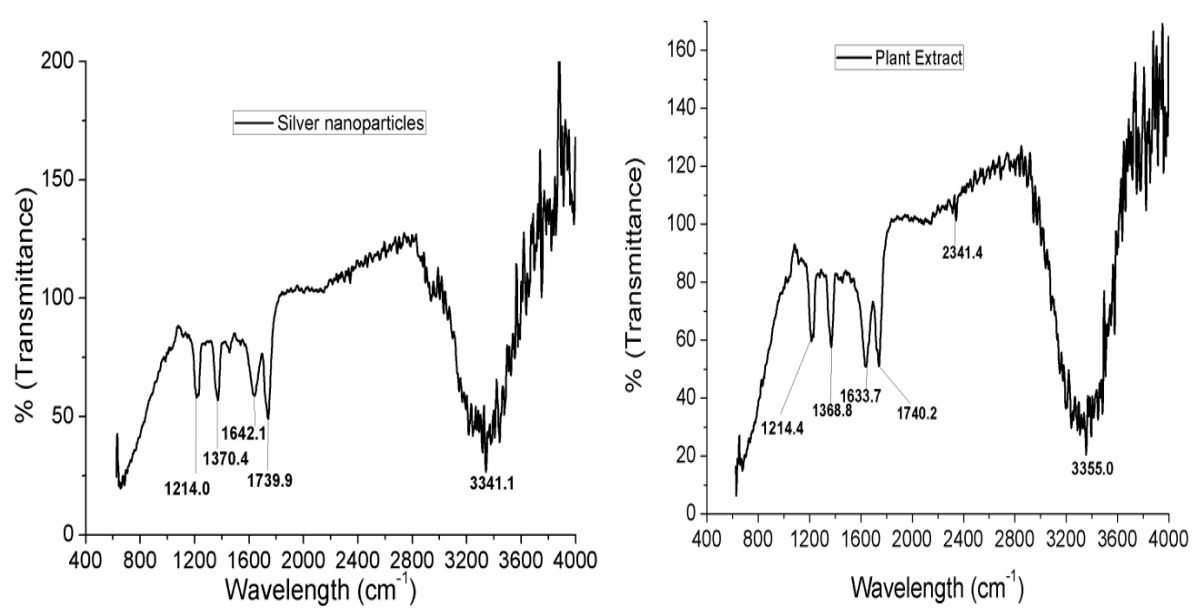


Figure 7.3: FT-IR spectrum of silver nanoparticles and plant extract XRD analysis

7.3.4 XRD analysis

XRD pattern of silver nanoparticles (Figure 7.4) was compared with standard JCPDS data no. 04-0783 which confirms crystalline FCC nature, as indicated by the peaks at 2θ values (10-80°) of 24.88, 44.4, 64.86 which can be indexed to 111, 200, 220 planes. The predominant peak was obtained at 200. Several unassigned peaks were also seen which might be due to bioorganic phases crystallization that appears on the synthesized nanoparticles surface (Ponarulselvam *et al.,* 2012). But these peaks are comparatively weaker than those of silver which suggested that silver is the

main element in the composite. MeenaKumari and Philip (2015) reported that in case of *Punica granatum* synthesized silver nanoparticles, predominant orientation was obtained at 111. Nayak *et al.,* (2016) found XRD peaks at 111, 200, 220, 311 and 222 orientations in case of *F. benghalensis* and *A. indica* and predominate orientation was at 200.

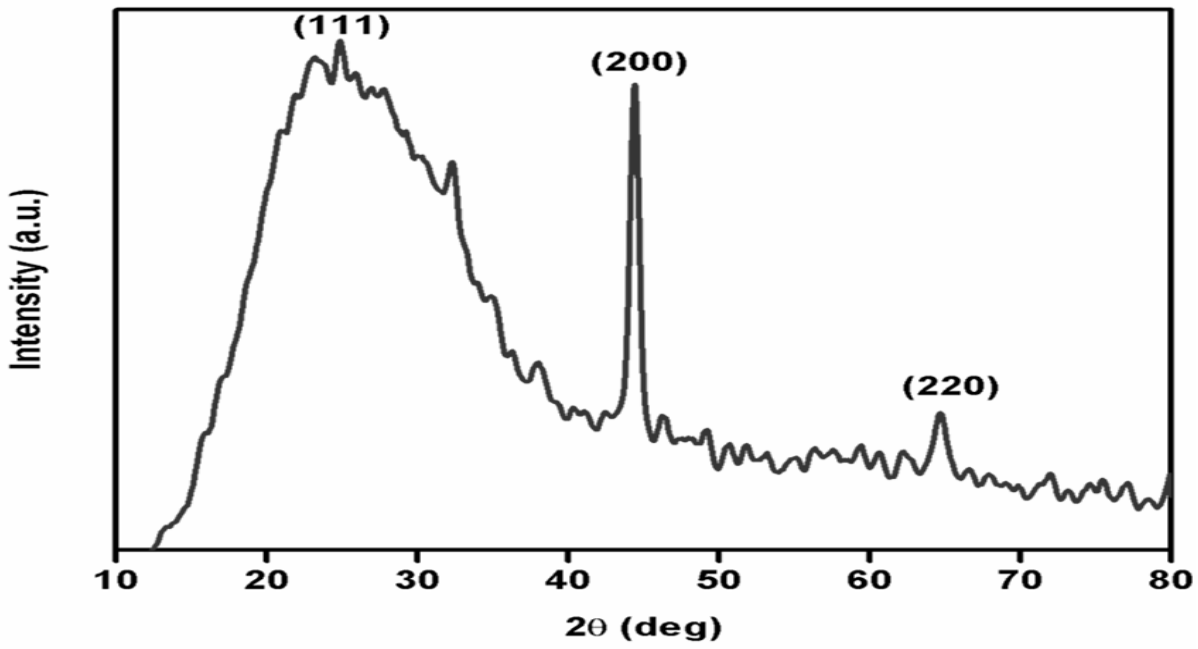


Figure 7.4: XRD pattern of silver nanoparticles

7.3.5 Antibacterial studies

Antibacterial activity of silver nanoparticles was evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Alcaligenes faecalis, Serratia marcescens and Swenella putrifaciens* and result showed that of silver nanoparticles possesses potential activity against all bacteria except *Swenella putrifaciens* (Table 7.1; Figure 7.5). Antibacterial activity of of silver nanoparticles was highest against *Serratia marcescens* with 28±0.57mm zone of inhibition. Though silver nanoparticles show prominent antimicrobial activity but its mode of action is still not clear. AgNPs has capability to attach with the cell membrane of bacterial which leads to structural changes and

leads to cell death. Ramesh *et al.,* (2015) reported antibacterial activity of AgNPs against *E. coli*, *K. pneumonia*, *S. aureus* and *B. subtilis.* Nayak *et al.,* (2016) studied antibacterial potential of AgNPs against *Bacillus* sp, *E. coli*, and *Pseudomonas* sp. Salunke *et al.,* (2014) studied antibacterial activity of *P. zeylanica* synthesized AgNPs against *S. aureus, Acinetobacter baumannii* and *E. coli*. Similarly, Velammal *et al.,* (2016) reported that antibacterial activity of *P. zeylanica* synthesized silver nanoparticles against *S. aureus, B. subtilis, P.aeruginosa,* and *E. coli.*

Table 7.1: Zone of inhibition of silver nanoparticles against some microorganisms

Microorganisms	**Zone of inhibition (mm)**				
	Kanamycin (+ control)	**Distilled water (- control)**	**Silver nanoparticles (100µg)**	**Silver nanoparticles (500µg)**	**Silver nanoparticles (1000µg)**
E. coli	23±1	0	13±0.57	15±1	20±0.57
S. aureus	24±0.57	0	15±1	18±0.57	21±1
S. putrifacians	9±1.15	0	0	0	0
Alcaligenes faecalis	26±0.57	0	15±0.57	19±1	22±0.57
Serratia marcescens	35±1	0	22±0.57	25±0.57	28±0.57

Mean values ± SD

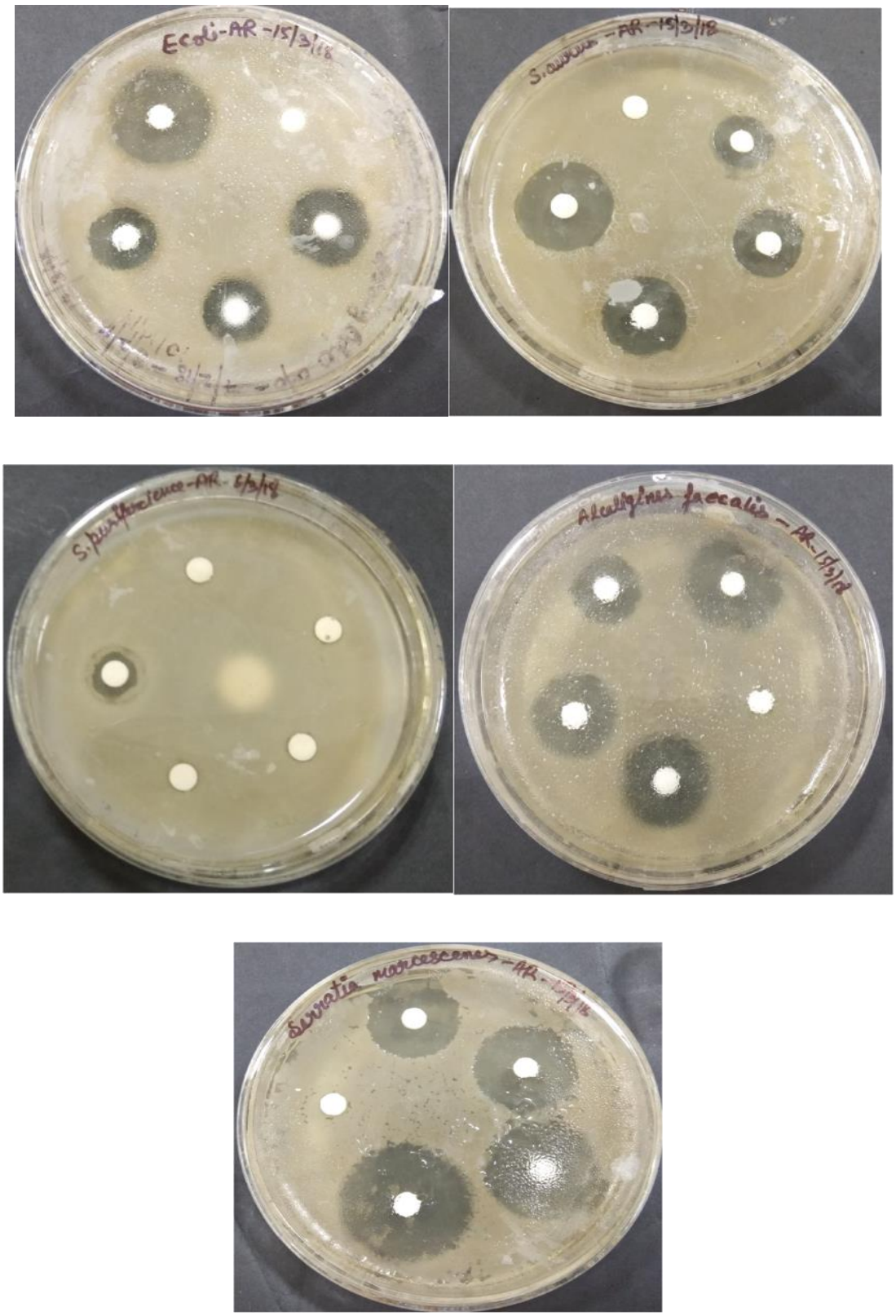
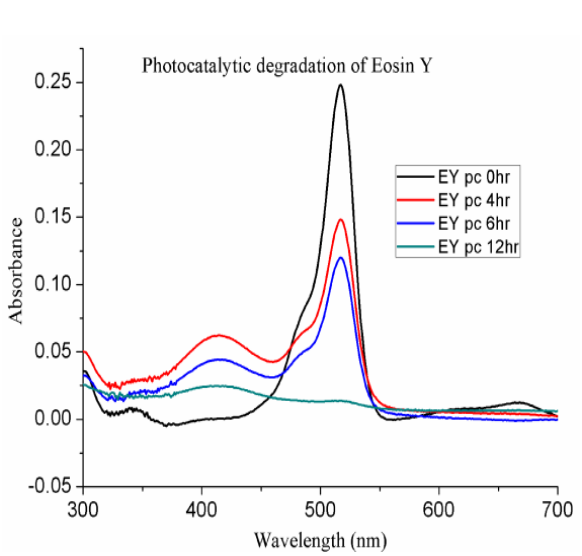


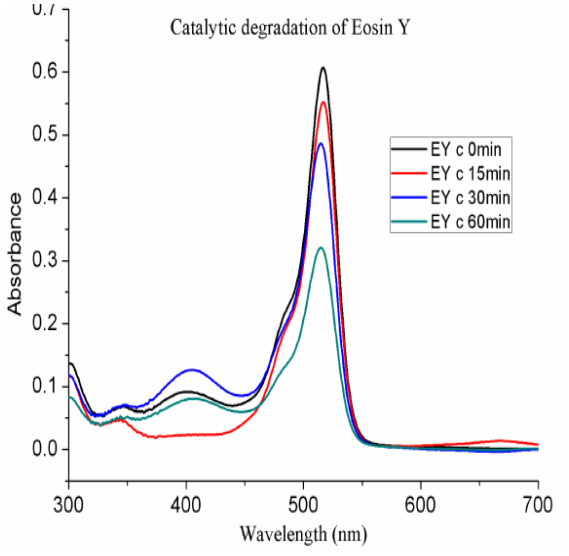
Figure 7.5: Antimicrobial activity of silver nanoparticles

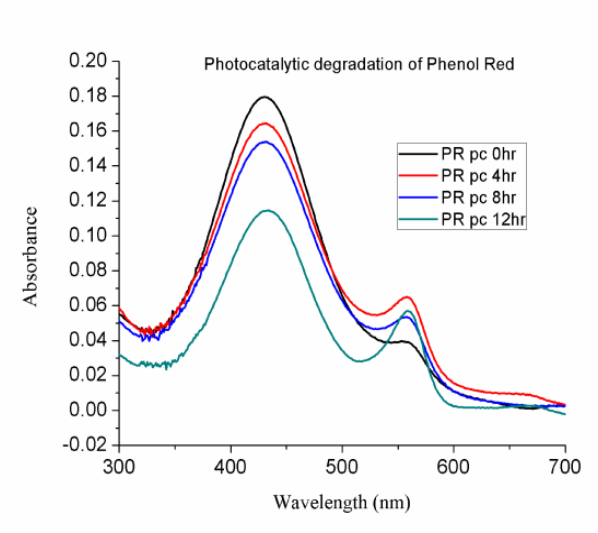
7.3.6 Degradation of dyes

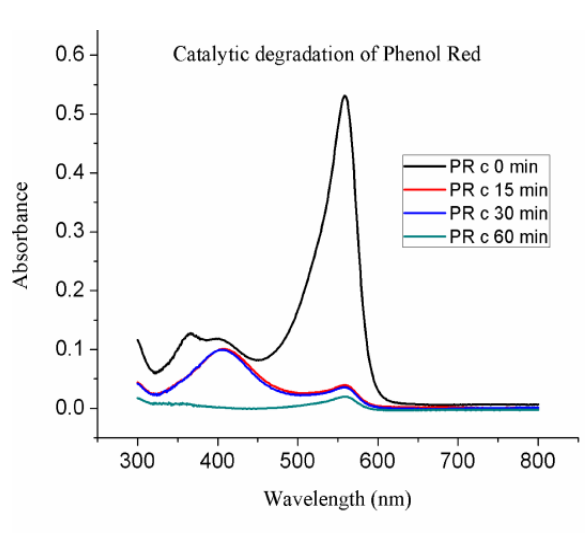
Silver nanoparticles acts as a catalyst and possess potential role in dyes degradation. MB is phenothiazine cationic dye and used in paper coloring, cottons, wool dyeing etc. It causes breathing problems, diarrhoea, vomiting and nausea (Velammal *et al.,* 2016). MO is majorly found in the textile and food industry effluent and carcinogenic in nature (Qurat-ul-ain *et al.,* 2016). EY is anionic dye which exhibit yellow-green fluorescence and utilized in printing, dyeing, printing ink and fluorescent pigment industries (Erjaee *et al.,* 2017). PR used as a bromination catalysts, estrogenic properties, pH indicator and screening test (Ghaedi *et al.,* 2012). *MR* is used for identification of bacteria producing stable acids during mixed acid glucose fermentation.

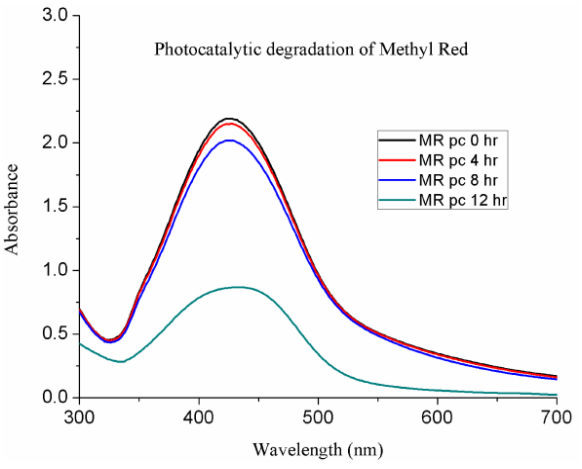
Catalytic activity of nanoparticles depends on its size, composition and shape. MR, MO, EY, MB and PR UV–Vis spectral band in water normally appears at 410nm, 465nm, 515nm, 664nm and 560nm respectively. $NaBH_4$ addition gradually decreased the absorption intensity, which shows the dye reduction. Figure 7.6 shows UV–Vis spectra of dye degradation in nanoparticles presence which was recorded at uniform interval of time. In case of nanoparticles absence there was almost no reduction in the solutions whereas there was an increase in degradation rate when nanoparticles solution was added. Catalytic activity of silver nanoparticles was highest in PR followed by MB, MR, MO and EY. Decrease in dyes absorbance was observed with time, as the time proceeded, degradation rate slow down, this could be due to the degraded products which adhering onto the surface of nanoparticles. Several studies reported the use of various other plants which were obtained from the wild source for AgNPs synthesis and their dye degradation activity against different dyes. MeenaKumari and Philip (2015) reported the catalytic degradation of MO, MB and EY using silver nanoparticles synthesized from *Punica granatum.*

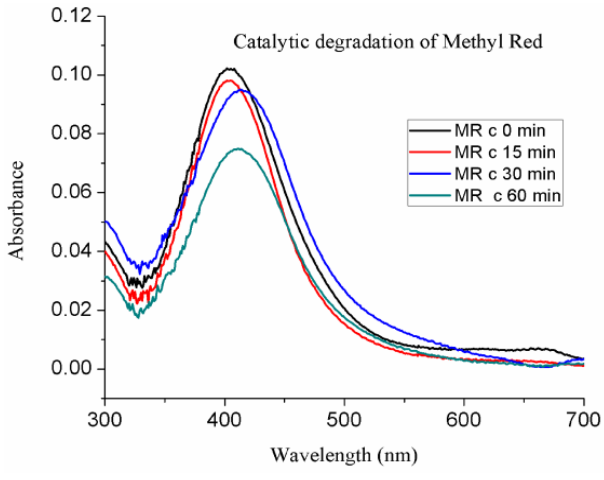

Photocatalytic degradation of Eosin Y
Absorbance
Wavelength (nm)
EY pc 0hr
EY pc 4hr
EY pc 6hr
EY pc 12hr


Catalytic degradation of Eosin Y
Absorbance
Wavelength (nm)
EY c 0min
EY c 15min
EY c 30min
EY c 60min


Photocatalytic degradation of Phenol Red
Absorbance
Wavelength (nm)
PR pc 0hr
PR pc 4hr
PR pc 8hr
PR pc 12hr


Catalytic degradation of Phenol Red
Absorbance
Wavelength (nm)
PR c 0 min
PR c 15 min
PR c 30 min
PR c 60 min


Photocatalytic degradation of Methyl Red
Absorbance
Wavelength (nm)
MR pc 0 hr
MR pc 4 hr
MR pc 8 hr
MR pc 12 hr


Catalytic degradation of Methyl Red
Absorbance
Wavelength (nm)
MR c 0 min
MR c 15 min
MR c 30 min
MR c 60 min

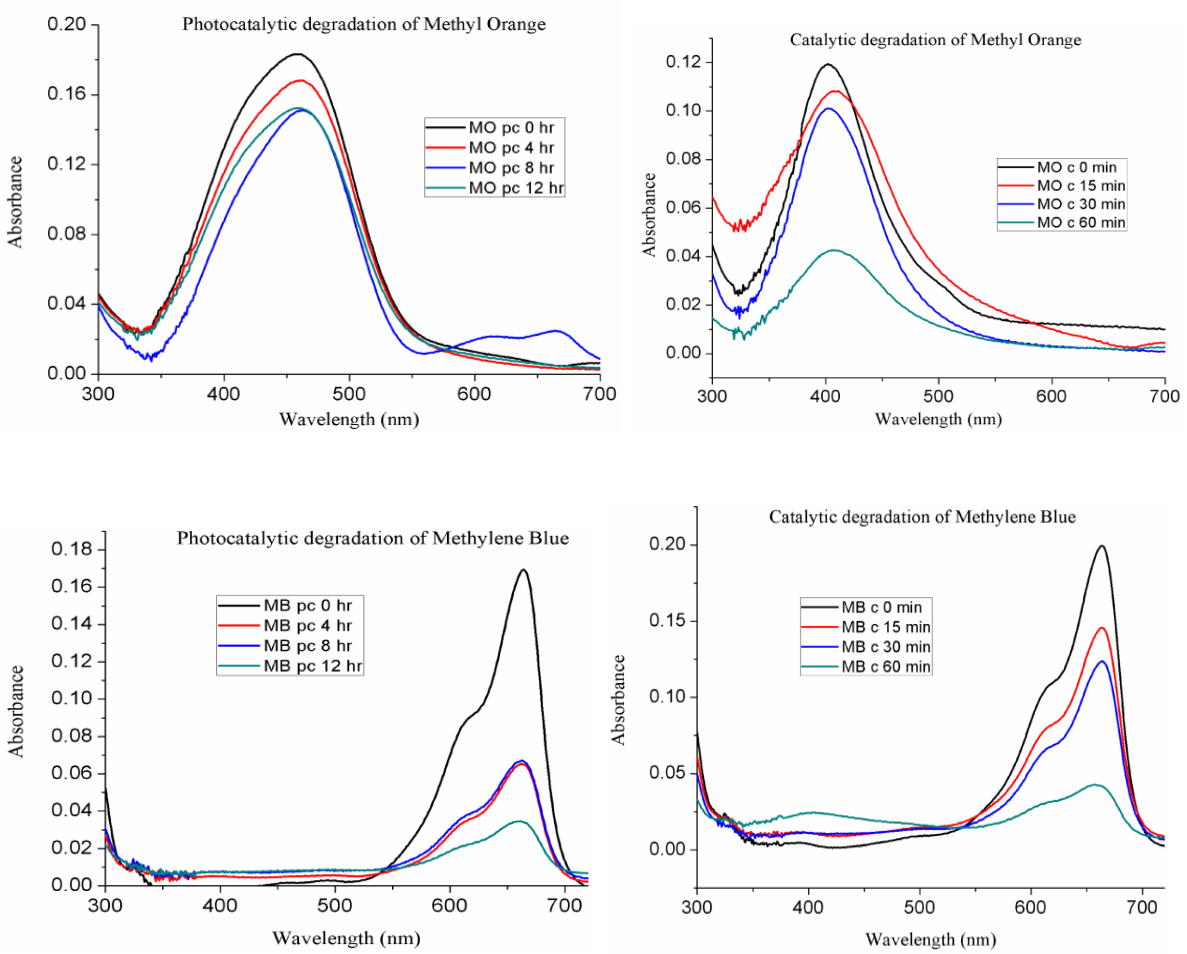


Figure 7.6: UV–visible absorption spectra for degradation of EY, PR, MR, MO and MB

Photocatalytic activity of silver nanoparticles was also evaluated against the same five dyes (Figure 7.6). Degradation of dyes observed by decrease in peak intensity within 12 h of incubation, complete dye degradation was not observed in any case. Photocatalytic activity of silver nanoparticles was highest in EY followed by MB, MR, PR and MO. A study reported that silver nanoparticles were highly efficient and stable photocatalysts under proper temperature condition with visible light source for organic dye degradation (Sumi *et al.*, 2017). Photocatalytic efficiency of silver nanoparticles in visible light is due to SPR excitation (Garcia, 2011). A study reported

that sunlight was faster for dye degradation in presence of metal catalyst as compared to other irradiation methods (Kansal *et al.,* 2007).

7.4 Conclusion

Silver nanoparticles synthesis using *P. zeylanica* showed maximum peak at 446 nm. SEM and TEM image reveal the spherical shape and average size of 55 nm. FT-IR data provides information about functional groups responsible for synthesis which includes phenolic compounds, alkaloids, amino acids etc. XRD data showed that synthesized silver nanoparticles were crystalline structure with FCC geometry. Furthermore, antibacterial activity of AgNPs was evaluated against different microorganisms and it showed desirable antibacterial effect against *A. faecalis, E. coli, S. marcescens* and *S. aureus*. Dye degradation capacity of silver nanoparticles showed that nanoparticles were active in degrading all the five dyes under both the conditions. Catalytic degradation showed maximum degradation efficiency in PR whereas photocatalytic degradation showed maximum degradation efficiency in EY. In case of MO both the degradation method showed lower degradation capacity as compared to the other dyes. Present study showed the potential of *P. zeylanica* as AgNPs synthesis agent and its application in antibacterial and dye degradation activity.

This study has been published in "**Bioinspired, Biomimetic and Nanobiomaterials**", (ICE Publishing)

Arpita Roy and Navneeta Bharadvaja "Silver Nanoparticles Synthesis from *Plumbago zeylanica* and its Dye Degradation Activity", Bioinspired Biomimetic and Nanobiomaterials, 2019, 8(2), 130-140. IF-0.973

CHAPTER 8

*PLUMBAGO ZEYLANICA*DERIVED BIO CHAR FOR REMOVAL OF CHROMIUM AND CADMIUM

8.1 Introduction

Heavy metals are detrimental contaminants which are toxic in soluble as well as elemental forms. Diverse activities which include development of industries, absurd management of waste, defective landfill operations, manufacturing and mining, etc. leads to increase contamination of metals in soil as well as in water. High level of heavy metals can damage fertility of soil and also affects productivity (Hooda and Alloway, 1994). Heavy metals that are found in wastewater include Cd, Cr, Ni, As, Pb, etc. Permeable limit of heavy metals for ingestion is 5 g/cm^3 and beyond this limit it causes negative effects on human health (Beyersmann and Hartwig 2008). Heavy metals have an affinity to get accumulated in food chain and create hazard for higher tropic levels (Kumar *et al.,* 2013). Water is an important resource for living being and consumption of heavy metal contaminated water leads to various disorders. Therefore, removal of these metals from water is of great importance.

Cadmium (Cd) is commonly used in different processes and toxic in nature. Main commercial utilization of cadmium includes production of alloys, pigments, soldering, and batteries. Human exposure to cadmium occurs through various sources which includes use of metals in industries, consumption of contaminated food, cigarettes smoking, and functional activities in cadmium-contaminated areas and contaminated water (Tchounwou *et al.,* 2012). Prolong utilization of water or food that is contaminated with Cd even low concentration can lead to renal damage. Chromium (Cr) is another heavy metal which enters into environment (air, water, and soil) from various sources. Chromium possess detrimental effects on human health and can cause cancer of digestive

tract and lung, damage of nervous system, etc. (Singh *et al.,* 2011). It is released from various industries which includes tannery, chromate production, metal processing and pigment production. Traditional method for heavy metal elimination from wastewater includes reverse osmosis, chemical precipitation, ion exchange filtration, evaporation, membrane technology, etc. But cost of these methods is occasionally high for large volumes, therefore cost-efficient and environment friendly method is of prime requirement (Hanif *et al.,* 2007). Biological methods are cost-effective and eco-friendly. Phytoremediation utilizes plant for remediation of heavy metals from contaminated soil and water. It is an emerging technology, cheaper, effective, and has long-term applicability. Various methods have been investigated for efficient heavy metals removal using biosorbents such as fly ash, peat, microbial biomass and agricultural byproducts which include soya bean hulls, sugarcane bagasse, cotton seed hulls, walnut hulls and corn cobs (Al-Qahtani *et al.,* 2016). Recently, few reports have come up with the utilization of bio char for heavy metal degradation. Biochar is prepared by biomass heating under low oxygen or even absence of it (Ippolito et al. 2012). Due to its high aromaticity, it is considered as an effective absorbent for both organic and inorganic pollutants (Bain *et al.,* 2013). Bio char has several advantages like eco-friendly, economical and aesthetically acceptable in nature. Utilization of bio char derived from *in-vitro* grown plant is a cheap and useful method for elimination of poisonous heavy metals from wastewater. *In-vitro* grown culture possesses advantage like production of large biomass in small time period which is beneficial for commercial scale. Danh and Veticon, (2010) and Roongtanakiat *et al.,* (2009) reported removal of iron, manganese, zinc and copper from industrial wastewater and contaminated soil using *Vetiveria zizanoides*. Similarly, Waoo *et al.,* (2015) used *in-vitro* culture of *Lantana camera* for degradation of Lead. *Lantana camera* cultures were exposed to different concentration of lead (0.1-50 mg/l) and accumulation capability of culture has shown

survival up to 50 mg/l Lead containing media, beyond this survival rate was decreased which indicates phytoremediation ability of *Lantana camera.* Beesley *et al.,* (2014) carried out pot experiments in green house for degradation of arsenic in soil-water solution and observed reduction in phytotoxicity of arsenic. Plants that are soil grown possess limited longevity while in-vitro cultures can be available throughout the year. Hence, it is beneficial to use *in-vitro* cultures for remediation at commercial scale. Various reports on single or mixed metal removal using different plants have been successfully done (Shukla et al. 2007; Barthwal *et al.,* 2008; Olowoyo *et al.,* 2012). In this investigation, bio char was prepared from residual shoot and root material of *P. zeylanica.* Further biochar was utilized for removal of chromium and cadmium from synthetic solution and wastewater sample.

8.2 Materials and Methods

8.2.1 Biochar preparation

In-vitro grown shoot and root waste part of plant were air-dried and crushed. Concentrated H_2SO_4 was added to the crushed samples (1.8ml of H_2SO_4 in 1gm of sample) then samples were placed in oven at 170˚C for 5 hours followed by repeated centrifugation at 10000 rpm (Eppendrop) for 15 minutes to remove excess acid. After this samples were dried at 110˚C for 3-4 hours in hot air oven until complete removal of moisture. Samples were sieved through 95µm sieve and yield of biochar was calculated.

Yield of biochar (%) = 100 × Yield (in grams)/Mass of raw material Yield (in grams)

8.2.2 Physiochemical properties analysis of biochar

Bulk density

Bulk density (g/ml) was calculated using a measuring cylinder which was filled to the mark with prepared biochar. The cylinder was tapped for 1– 2 min to compress the biochar to a stable volume. The compressed material was taken out of the cylinder and measured.

Bulk density=Mass (in grams)/Volume(ml)

Moisture content and Ash content

Moisture content (%) and ash content (%) were calculated using the following formula

Moisture content (%) = 100 × (Sample before dry-sample after dry)/ Sample before dry

Ash content (%) = 100 × Mass of ash/Mass of wet sample

Conductivity

500mg of biochar was placed in a beaker having 50 ml distilled water. It was mixed and then allowed to stay for about 1 h. Conductivity was measured using conductivity meter.

8.2.3 Effect of different conditions on cadmium and chromium removal

Synthetic chromium and cadmium solutions were prepared by using potassium dichromate and cadmium chloride respectively. Five different concentration (100-500 ppm) of standard cadmium and chromium solution was used to prepare the standard curve. Spectrophotometer was first calibrated using various concentration of chromium and cadmium solution. Concentration of Cd and Cr were evaluated using UV-Vis spectrophotometer and absorbance was measured at 283 nm and 425 nm respectively. A straight-line calibration curve was obtained which was used to determine the concentration of chromium and cadmium.

Heavy metal removal experiments were done in the laboratory incubated shaker by changing various conditions. Effect of time, pH, heavy metal concentration and biochar concentration were studied at 25˚C. Biochar concentration was varied from 1 to 4mg/ml by keeping all other parameters constant. Initial cadmium and chromium concentration varied from 100 to 500 ppm, pH varied from 4-8and time period were varied from 1 hr to 6 hr. Experiment was conducted in batch wise and flasks were agitated at 110 rpm using orbital shaker and observation was recorded. 1 mL from solution was withdrawn and biochar from the solution was separated immediately by using centrifugation at 10000 rpm for 10 minutes. Instrument was calibrated before experiment conducted for accuracy of results.

Percent removal of chromium and cadmium was calculated as:

$$\% \text{ Removal} = 100 \times \frac{(C_i - C_f)}{C_i}$$

Where C_f is the final concentration and C_i is the initial concentration of metal of metal in ppm

8.2.4 Effect of biochar on removal of chromium and cadmium from wastewater

Wastewater containing effluents from metal industries was collected from Yamuna River near Yamuna Vihar Area, Delhi, India. Concentration of Cd and Cr were estimated using UV-Vis spectrophotometer. Experiment was conducted in batch manner where 60mg of bio char derived from shoot as well as root was added in 30 ml of each sample solution and flasks were agitated at 110 rpm using orbital shaker and observation was recorded from 0-6hours.

8.3 Results and Discussion

Studies on effluent treatment having heavy metals have revealed that adsorption is a useful process for heavy metal remediation from wastewater and activated carbon are extensively utilized for this

purpose (Hegazi *et al.*, 2013). In spite of its widespread use in wastewater treatment industries, it remains an expensive material. Therefore, there is a requirement of safer and inexpensive method for degradation of heavy metals from contaminated water. Various reports on utilization of sugarcane bagasse, saw dust, rice husk, coconut husk, neem bark, etc. for heavy metal degradation from wastewater have been investigated (Hegazi *et al.*, 2013). Gawronski *et al.*, (2018) reported that *Plumbaginaceae* family is tolerant to lead and most commonly cultivated in lead polluted sites. This may be because of the capability of *Plumbaginaceae* family to degrade heavy metals and provides ability to grown in heavy metal contaminated areas. By acknowledging this fact about *Plumbaginaceae* family, in this investigation bio char has been prepared from both *in-vitro* grown root and shoot culture. Characteristics of prepared bio char from shoot culture and root culture are mentioned in Table 8.1.

Table 8.1: Characteristics of prepared bio char from *Plumbago zeylanica*

Parameters	**Value**	
	Shoot biochar	**Root derived biochar**
Bulk Density (g/ml)	0.65	0.55
Moisture (%)	0.80	0.75
Ash (%)	0.90	0.86
Conductivity µS/cm	150	180
Size of Particles (µm)	≤90	≤90

8.3.1 Effect of time, heavy metal concentration, pH and biochar concentration on removal of cadmium and chromium

Absorption of metal ion was higher in case of bio char derived from shoot culture as compared to bio char derived from root culture. Effect of bio char derived from shoot and root culture on

chromium and cadmium removal with time was recorded (Table 8.2). As time of adsorption is changed from 1 to 6 hours, efficiency increases and afterwards no change was observed. This is due to the larger surface area of biochar accessible at initial phase for the adsorption of chromium and cadmium. Adsorption process reached equilibrium in six hours, after which no more cadmium and chromium adsorbed from solutions due to saturation of active sites of adsorbent (Kannan *et al.,* 2013). Various studies have reported use of *in vitro* cultures for heavy metal degradation. A study reported removal of arsenic using *in vitro* culture of *Vetiveria zizanioides* (Singh *et al.,* 2017). They exposed *V. zizanioides* plantlets to different concentration of arsenic for two different time period i.e. 7 and 14 days and found that *V. zizanoides* plants effectively degrade arsenic and maximum degradation (65%) obtained at 200 μM after 14 days.

Efficiency of biochar was studied at different concentration of chromium and cadmium by keeping other factors constant. Initial concentration was of cadmium and chromium was varied from 100 to 500 ppm. Bio char derived from shoot culture showed 80-26% removal of cadmium and 63-23% removal of chromium over 100-500ppm concentration range. Similarly, bio char derived from root culture showed 61-24% cadmium removal and 40-22% chromium removal over the same concentration range. It was observed that at low metal concentration, removal (%) was high and increase concentration of heavy metal leads to decrease in removal percentage (Table 8.2). At 100 ppm, cadmium and chromium showed highest removal percentage which could be due to the availability of surface area on the adsorbent (Banat *et al.,* 2000). When the concentration of metal ions was high, the surface area of adsorbent becomes less effective and percentage of removal get reduced. Tangjuank *et al.,* (2009) also reported that at lower concentration of heavy metals i.e. lead and cadmium, percentage of removal was higher. A study reported that with increasing initial metal concentration, adsorption efficiency of metals decreased in case of cadmium and zinc (Wu

et al. 2008). El-Ashtoukhy et al. (2008) described that at lower concentrations, metals get adsorbed at specific sites, whereas with increase concentrations saturation takes place and exchange sites are packed which leads to less adsorption efficiency. Singanan *et al.,* (2007) reported removal of 95-97% of chromium from water collected from tannery using activated carbon prepared from *Tridax procumbens*. Similarly, Dula *et al.,* (2014) also reported absorption of Cr (VI) by *Oxytenanthera abyssinica* derived activated carbon and found 98.39 % absorption at 318K. Senthil *et al.,* (2016) reported that bio char prepared from eucalyptus seeds showed absorbance of toxic Zn ion from contaminated water and adsorption capacity was 80.37 mg/g.

pH is a vital factor for adsorption of heavy metals. Metal ions binding with surface is significantly depends on pH (Doke and Khan, 2017). To find out the role of pH, pH of solution ranging between 4 and 8 were used. It was observed that maximum removal efficiency of chromium and cadmium are higher at neutral pH. As the pH was increased from 4 to 8 efficiency decreases as shown in the Table 8.2. Optimum pH was at 7.0 and further experiments were done at this pH. Similar observation was obtained in a study where authors found maximum nickel adsorption at pH7 in case of biochar obtained from *Hyphaene thebaica* seed coat (El-Sadaawy and Ola Abdelwahab, 2014). pH depends on both surface properties of biochar and heavy metal species present in the solution. Bansal *et al.,* (2009) reported that at lower pH, surface of adsorbent closely linked with hydronium ionthat limits the access to ligands by metal ions due to repulsive forces and at higher pH, binding efficiency decreases due to low metal solubility and its precipitation. Sekher *et al.,* (1998) observed that with pH increase, negatively charge ligands got exposed which result in enhanced positively charged metal ions attraction.

Effect of biochar concentration at four different concentration (1, 2, 3 and 4 mg/ml) on adsorption of cadmium and chromium were observed whereas other factors remain constant i.e. time, pH, and

initial concentration of cadmium and chromium. As the biochar concentration increases removal efficiency initially increases and reaches to maximum and after this it decreases. Maximum removal percentage was observed at 2mg/ml of biochar concentration (Table 8.2). At low biochar concentration number of active sites is higher therefore maximum absorption taking place (Thavamani and Rajkumar 2013). With the increase in biochar concentration particles aggregation take place, as a result efficiency of chromium and cadmium uptake decreases. Komkiene and Baltrenaite (2015) also obtained similar observations in case of cadmium, copper, lead, zinc removal from aqueous solution using biochar.

Table 8.2 Effect of time heavy metal concentration, pH and biochar concentration on percentage removal of chromium and cadmium

Parameters	**Removal percentage of chromium from synthetic water using shoot derive biochar**	**Removal % of cadmium from synthetic water using shoot derive biochar**	**Removal percentage of chromium from synthetic water using root derive biochar**	**Removal percentage of cadmium from synthetic water using root derive biochar**
Time (in hour)				
0	0	0	0	0
1	4	9	2	8
2	12	20	12	12
3	21	32	20	26

4	34	51	27	45
5	60	76	38	59
6	62	79	40	61
Concentration (ppm)				
100	63	80	40	61
200	42	69	36	59
300	37	46	30	40
400	30	37	25	33
500	23	26	22	24
pH				
4	0	0	0	0
5	19	27	13	12
6	32	39	25	30
7	76	62	57	49
8	60	52	50	45
Biochar concentration				
1	55	45	43	37
2	80	66	61	52
3	60	51	48	45
4	45	35	22	25

8.3.2 Effect of biochar on removal of chromium and cadmium from wastewater

Wastewater from Yamuna canal which is located near Wazirabad area was collected. Further characterization of wastewater sample was performed (Table 8.6). Shoot culture derived bio char showed 60% and 71% removal of chromium and cadmium from wastewater. Similarly, root culture derived bio char showed 52% and 60% removal of chromium and cadmium from wastewater sample (Table 8.7 and 8.8). Removal (%) of chromium and cadmium is associated with time and concentration. Studies on effluent treatment having heavy metals have revealed that adsorption is a useful method for heavy metal removal from wastewater. Activated carbon is extensively utilized as an adsorbent (Hegazi *et al.,* 2013), in spite of its widespread use in wastewater treatment industries, activated carbon remains a costly material. Therefore, there is a requirement of safer and cost-effective method for degradation of heavy metals from contaminated water. This leads to encourage investigation of alternative material which is low cost as well as environment friendly. Plants are an alternative source for remediation of heavy metals, but it is dangerous when heavy metal contaminated plants are taken as food or therapeutic agent. Various reports on utilization of sugarcane bagasse, saw dust, rice husk, coconut husk, neem bark, etc. for heavy metal degradation from wastewater have been investigated (Hegazi *et al.,* 2013).

Table 8.3: Characteristics of Yamuna wastewater from Wazirabad area

Parameters	**Value**
pH	6.4
Electrical Conductivity/ Total Dissolved Solids	847 µS/cm
Concentration of chromium	240-ppm

Concentration of cadmium 450-ppm

Table 8.4: Effect of *in- vitro* shoot and root culture derived bio char on cadmium concentration in wastewater

Time	**Bio char derived from shoot culture against Cadmium**		**Bio char derived from root culture against Cadmium**	
	Concentration (in ppm)	**% removal**	**Concentration (in ppm)**	**% removal**
0hr	450	0	450	0
1hr	412	8	432	4
2hr	364	19	361	20
3hr	330	27	330	27
4hr	306	32	276	39
5hr	177	60	218	52
6hr	130	71	180	60

Table 8.5: Effect of *in- vitro* shoot and root culture derived bio char on chromium a concentration in wastewater

Time	**Bio char derived from shoot culture against Chromium**		**Bio char derived from root culture against Chromium**	
	Concentration (in ppm)	**% removal**	**Concentration (in ppm)**	**% removal**
0hr	220	0	220	0

1hr	191	13	194	12
2hr	164	25	183	17
3hr	126	43	153	30
4hr	111	50	134	39
5hr	92	58	121	45
6hr	86	60	101	52

8.4 Conclusion

In the present investigation bio char prepared from *in-vitro* grown shoot culture of *P. zeylanica* showed more potential results over *in-vitro* grown root culture for degradation of chromium and cadmium. As root of this plant is pharmaceutically important source for secondary metabolite production so *in-vitro* grown root can be used for metabolite production and shoot can be utilized for heavy metals remediation. This will provide complete utilization of *in-vitro* grown plant at commercial scale without affecting the natural habitat of plant. *In vitro* cultures provide several advantages over soil grown culture as it is independent of geographical variations and grows in less time. Easily manageable, cost effective and eco-friendly nature is the key feature which possesses vital role in *in-vitro* culture selection for degradation of pollutants. It was also observed that percentage of heavy metal removal was depends on initial concentration of cadmium and chromium, time period, pH of solution and biochar. This study showed that *P. zeylanica* shoot derived bio char can be used for degradation of cadmium and chromium from waste water in a cost-effective manner.

The manuscript of this study is under communication

CHAPTER 9

IN-SILICO ANALYSIS OF PLUMBAGIN AGAINST DIFFERENT CANCER RECEPTORS

9.1 Introduction

In-silico method is extensively used in pharmacology and chemistry in order to find out the information at molecular level. Computational results help in explaining the molecular interactions and suggest probable mechanisms that are involved in the process. Structure-based virtual screening and post-screening analysis methods are an important step in finding lead molecule. Treatment of many diseases like cancer is only possible when it is identified at initial stages. Medicinal plants are widely utilized for treatment of various diseases across the world therefore they are used for the development of new drugs. They contain wide range of phytocompounds which includes alkaloids, flavonoids, sterols, gylcosides, etc. Medicinal plants have the capability to suppress cancerous cells due to the presence of various cancers inhibiting compounds like taxols, phytosterols, napthoqunoes etc. Additionally, significance of plant derived drugs for cancer treatment is growing at a rate of 10–40% (Molassiotis *et al.,* 2006). Utilization of plant secondary metabolites having anticancer properties is preferred choice for cancer therapy (Grover *et al.,* 2012).

Current situation demands effective therapies for cancer, which can target cancer cells or processes that are involved in metastasis. Various molecular targets that are responsible for cancer have been recognized, one of the potential targets to inhibit various diseases is cyclooxygenases commonly referred to as COXs. It belongs to myeloperoxidases family, and present at the luminal side of endoplasmic reticulum and nuclear membrane (Chandrasekharan and Simmons, 2004). *Cyclooxygenase 2 (*COX-2) (prostaglandin H2 synthatase-2) has two isoforms COX-2 and COX-

1. They are different from each another in a way that COX-1 is found in almost all cell type. It is a housekeeping enzyme, has a constant level under normal condition. COX-2 is inducible in nature which is regulated by growth factors and various cytokines and thus over expressed during inflammation (Ramsay *et al.,* 2003). COX-2 is absent in normal conditions and present in high amount in diseased state. COX-2 has a vital role in proliferation of cell and cancer (Bakhle, 2001). Epidermal growth factor receptor (EGFR) is a member of tyrosine kinase receptor family and over-expression of EGFR causes various human cancers such as oral cancer (Forastiere and Burtness, 2007). At molecular level, EGFR stimulation induces intrinsic tyrosine kinase activity and cellular signaling which results in development and proliferation of cells. Alteration in expression of protein and tyrosine kinases activity is responsible for development of various cancers (Noolvi *et al.,* 2011). Therefore, inhibiting EGFR linked signalling poses a great potential to combat various diseases.

Tumor necrosis factor alpha converting enzyme (TACE) is another target for cancer, it belongs to the metalloproteinase and disintegrin containing enzyme family. It produces a soluble form of TNF-α i.e. pro TNF-α. TNF-α is mainly a pro-inflammatory and immune-modulatory cytokine which is synthesized as membrane anchored precursor. Pro-inflammatory mediators are vital for continuous operation of immune system. On the other hand, when it is overproduced it causes tissue or organ damage (Szollosi *et al.,* 2016) and enhanced production of TNF-α is accountable for autoimmune diseases and infections.

The present investigation explores the role of plumbagin against three cancer receptors i.e. COX-2, TACE and EGFR. Molecular docking analysis was performed to identify the binding sites. Further molecular dynamics simulation was done for the protein-ligand complex which possess maximum negative binding energy.

9.2 Materials and methods

9.2.1*Preparation of protein and ligand*

Three-dimensional structures of TACE (1BKC), EGFR (PDB: 1M17) and COX-2 (PDB: 3LN1) were retrieved from Protein Data Bank (PDB). Structure of plumbagin (ligand) (CID: 10205) was retrieved from NCBI – PubChem.

9.2.2 *Preparation of receptors*

To prepare receptors for docking analysis, co-crystallized water molecules, nonpolar hydrogens, lone pairs, small molecules and non-standard residues were omitted, and polar hydrogens and Gasteiger charges were added.

9.2.3 *Molecular docking*

AutoDock4 is commonly utilized tools for docking studies, it has the efficiency to predict fast and precise ligand to targets binding confirmations. It is a grid-based process which is used for rapid estimation of binding energy of test confirmations. It initiates with processing of ligand by removing coordinates and water molecules from PDB file. For conformational searching Lamarckian genetic algorithm was used. Main residues were placed in a grid of 60 Å × 60 Å × 60 Å (x, y, z). Finally, protein-ligand interaction viewed in discovery studios (2019).

9.2.4 *Molecular dynamic simulation (MDS)*

MDS of best target proteins with Plumbagin were performed using non-commercial Desmond version 2019.4 (D. E. Shaw Research, New York, 2019; Schrödinger). For description of water molecules, TIP3P model was used. The neutralization of each complex was done using salt concentration of 0.15 M. The standard relaxation protocol and other standard settings given in Desmond module package were used. For production runs of 1ns, 5ps time steps with NPgT

ensemble at 300K was deployed for simulating each system prepared. For each simulation, Root mean square deviation (RMSD), Potential energy, and Root mean square fluctuations (RMSF) values were recorded and observed for stability of the complex.

9.3 Results and Discussion

9.3.1 Molecular Docking

Molecular docking score of plumbagin with TACE was –5.81 kcal/mol, which shows a good binding affinity of TACE with plumbagin. Docked complex formed H-bond with Ile277, Gln278 and Arg473 and showed hydrophobic interactions with Asp259 residues (Figure 9.1). Docked complex possesses molecular interaction with histidine and glutamate residues which have a vital function in proteolytic reactions. TNF-α is an important pro-inflammatory mediator which is produced in higher amount in case of inflammatory diseases such as multiple sclerosis, rheumatoid arthritis, cancer, diabetes, etc. (Murumkar *et al.,* 2010). Various studies reported role of TACE in colon cancer (Ramirez *et al.,* 2007). In colon cancer, TNF-α is present in higher amount and inhibiting the production of TNF-α can block the signals that are accountable for producing mature TNF-α. Various proteases involve in the process of pro-TNF-α and TNF-α converting enzyme (TACE) is the main component. As this enzyme has a vital role in conversion of TNF-α to its soluble form, therefore choosing this enzyme can be effective to overcome the increased production of TNF-α. Pu *et al.,* (2015) reported that a quinazoline derivative was able to treat arthritis by blocking TNF- α production mediated by TACE. Niu *et al.,* (2015) reported that activation of TACE in stressed condition results in blockade of TNF- α secretion, which in turn leads to congestive heart failure. By taking into account the facts that plumbagin binding with TACE interferes with the binding process of substrate and TACE and this shows that plumbagin is a potential TACE inhibitor.

Molecular docking score of plumbagin with EGFR was –5.97 kcal/mol and docked complex formed H-bond with lys721 and Ala719. It showed hydrophobic interactions with Ile720, Glu738, Ile765, Asp831residues (Figure 9.2). Liu *et al.,* (2006) reported that quinazoline rings of the compound show lipophilic interactions with hydrophobic residues Val702, Lys721, Met742, Leu764 and Thr766. Yousuf *et al.,* (2017) found that residues Leu768, Met769, Leu694, Gly772, and Leu820 of EGFR receptor formed considerable interactions with inhibitor.

Molecular docking score of plumbagin with COX-2 was –6.67 kcal/mol, which shows a good binding affinity of COX-2 with plumbagin. Docked complex formed H-bond with Tyr371 and Ser516 showed hydrophobic interactions with Phe367, Leu- 370, Ser339, Phe504, Val 509, Gly512 (Figure 9.3). Various studies have reported the role of various mitogens and growth factors in COX-2 induction (Smith *et al.,* 2000). Therefore, COX-2 has a significant role in cell proliferation and cancer (Bakhle, 2001). A study reported that COX-2 has been involved in angiogenesis, apoptosis inhibition and tumor development, and inhibitors which are specifically used for COX-2 can stop cancer development (Khan and Lee, 2009). Arora *et al.,* (2017) reported COX-2 inhibition by using *Operculina turpathum* extract suppressed oral squamous carcinoma cell lines. The anticancer potential of plumbagin against COX-2 in melanoma patients was reported by Gowda *et al.,* (2017) and it reduce cellular apoptosis and increase proliferation (Gowda *et al.,* 2017). Plumbagin can suppress cancer cells malignancy through various mechanisms which include growth inhibition, invasion, metastasis, apoptosis induction and anti-angiogenesis (Cao *et al.,* 2018). Llorens *et al.,* (2002) reported that Ibuprofen and Naproxen interact with active site of COX-2 enzyme with Arg120 and Tyr355 residues. Sidhu *et al.,* (2010) reported that mefenamic acid interacts with Tyr385 residue of COX-2. D'Mello et al, (2011) reported that active site of COX-2 possesses three important regions i.e. hydrophilic region (Glu524, Arg120 and Tyr355),

hydrophobic pocket (Trp387, Phe518, Tyr385, Ala201, Tyr248 and Leu352) and side pocket (His90, Arg513 and Val523). Krishna *et al.*, (2013) reported that cyclprodigiosin interacted with Tyr324, Phe487 and Arg89 residues whereas prodigiosin interacted with Leu321 and Tyr324 residues. Plumbagin particularly binds to the catalytic site and interaction of ligand–protein complex is stable.

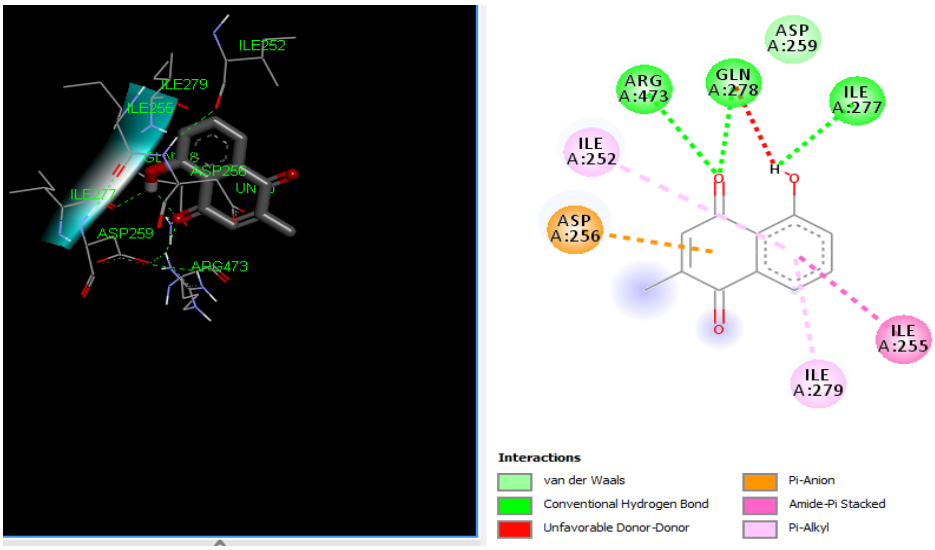


Figure 9.1: Docking of TACE with Plumbagin

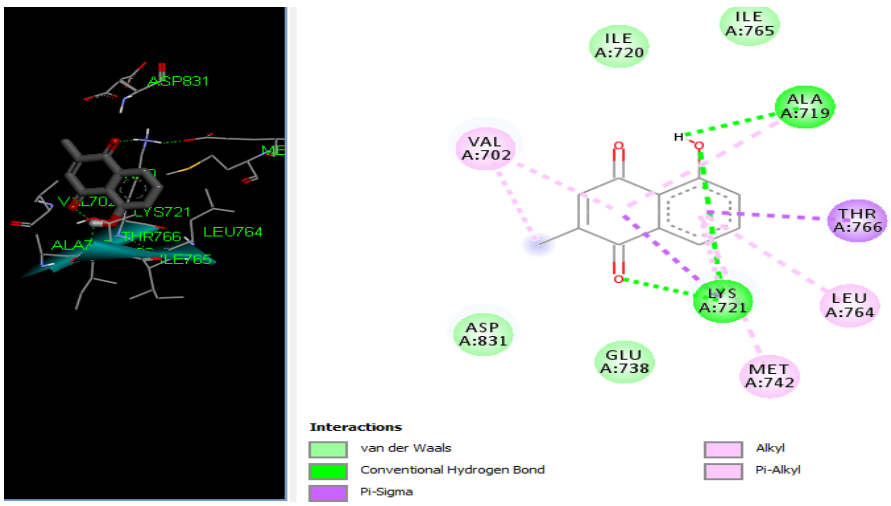


Figure 9.2: Docking of EGFR with Plumbagin

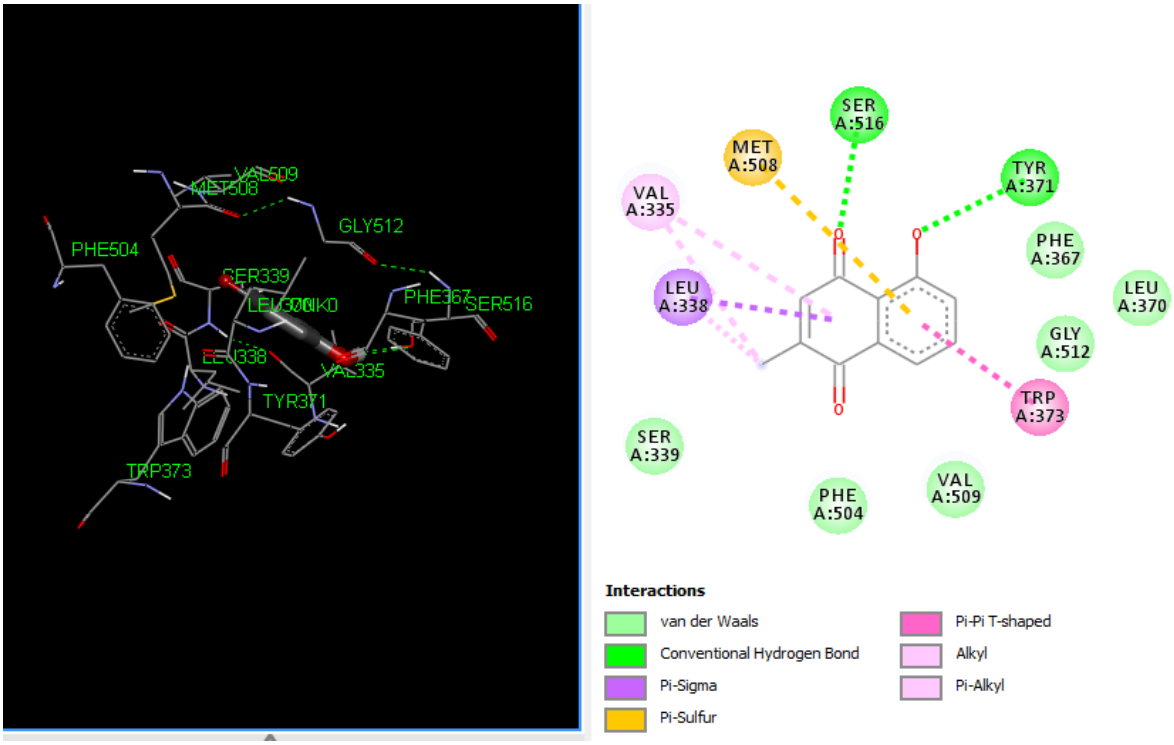
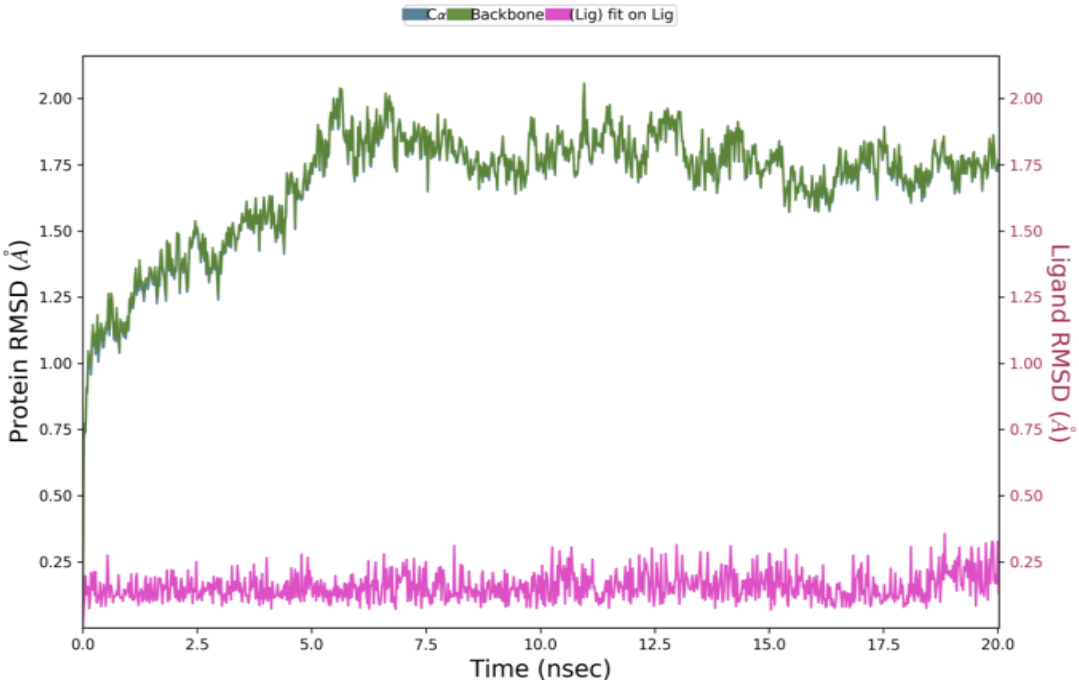


Figure 9.3: Docking of COX-2 with Plumbagin

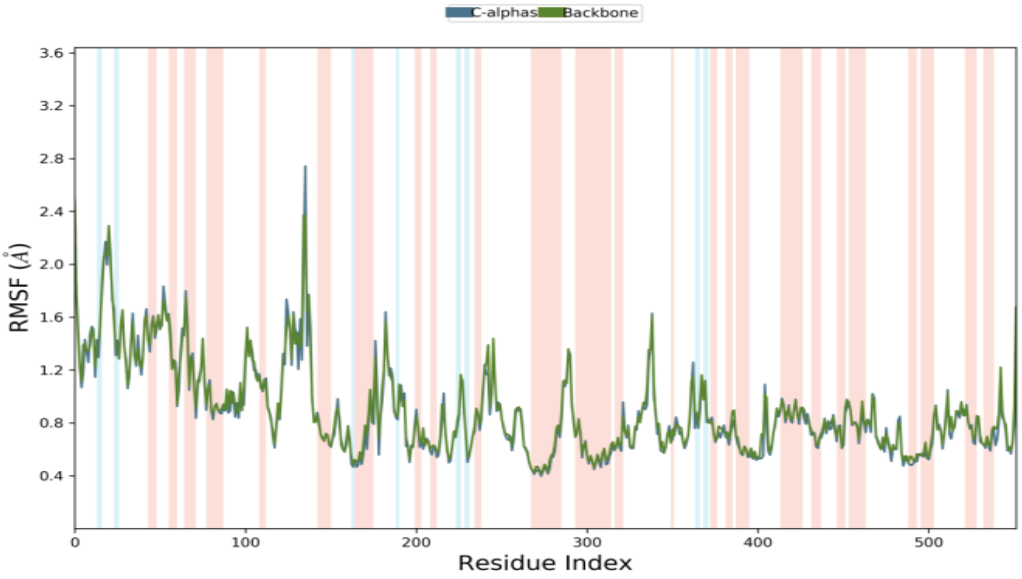
9.3.2 Molecular dynamic simulation

MDS results of 3ln1 showed quite stable complex with lower RMSD values. RMSD values for 3ln1 Cα lies within the range 1.0- 1.75 Angstrom and plumbagin within 4.0-5.6 A RMSF value depicts the extent of fluctuations in each residue of the protein during the simulation period of 20 ns (Figure 4). The RMSF values of 3ln1 was Cα- 0.5-2.8 A and thus, lower RMSD and RMSF values depicts the stability of the complex, minute rearrangements and conformational changes during the simulation period around the binding site residues. The average potential energy of 3ln1 and complexes was -159724.720 kcal/mol (Figure 5). MDS result reproduced the major docking interactions during the simulation period of 20 ns. Active site residues like SER 516, TYR 371, TYR 341 and SER 339 shows hydrogen bonding as well as hydrogen bonding through water bridges. ARG 106 also interacted with direct hydrogen bonding as well as water bridges. During

complete simulation period, six residues in total shows hydrogen bonding either direct (ARG106, SER339, TYR 341, TYR371, and SER516) or through water bridges (ARG106, SER339, TYR 341, TYR371, VAL509, and SER516). TYR371 and VAL509 form stable hydrogen bonds for more than 50% of the simulation period. Other residues show hydrophobic interactions with plumbagin namely VAL335, LEU338, PHE504, VAL509, ALA513 and LEU517. Our results are in well conjunction with the results obtained by Razzaghi-Asl *et al.*, (2018) where they also used COX-2 protein and serious of compounds as COX-2 inhibitor. They obtained average RMSD value of 2.23 A with ZINC_1130464 ligand which is larger than the RMSD value we obtained in our case i.e. 1.68 A. The RMSF fluctuations of binding residues of our complex were also similar with their study (results obtained by Razzaghi-Asl *et al.*, (2018) Gly512, Ala513, Ser339, Val509 and Leu338 0.56, 0.58, 0.64, 0.59 and 0.69 Å, respectively and in case of our study Gly512, Ala513, Ser339, Val509 and Leu338 0.56, 0.57, 0.96, 0.50, and 0.59 respectively.



(a)



b)

Figure 9.4: (a) RMSD plot for 3ln1 b) RMSF plot for 3ln1

9.4 Conclusion

In the present scenario development of novel compound having biological activity is required. This study showed that inhibition of COX-2 a potential cancer target can be done by a natural compound plumbagin and it was efficiently inhibited by plumbagin. These showed minimal energy on docking against target hence suggesting the compound's stability. It was also found that plumbagin efficiently interact with the active sites of target. Molecular dynamics simulation study suggested that plumbagin has the potential able to inhibit COX-2. Further evaluation of pharmacokinetics and pharmacodynamics of plumbagin can be done.

CHAPTER 10

CONCLUSION AND FUTURE PROSPECTS

Medicinal plants are vital source of therapeutically active compounds which prevents diverse diseases. Pharmaceutical industries exploit medicinal plants for production of herbal medicine which lead to extinction of these plants. Therefore, there is requirement of an alternative technique which can conserve them as well as enhanced the production of important phytocompounds. Biotechnological approaches like shoot culture, root culture, and cell suspension culture have shown their potential for production of desired compound in a small period of time and also removes the chances of endogenous contamination. In addition, biotechnological approaches also provide the opportunity to optimize various culture conditions to enhance the bioactive compound production. *P. zeylanica* an important medicinal plant was selected for the study. *P. zeylanica* was propagated through nodal segment and widely utilized in many ayurvedic preparations. This plant contains a bioactive compound i.e. plumbagin, which possess several pharmacological activities such as anticancer, antimicrobial etc. Various reports are available on *in vitro* cultures for plumbagin production. However, detailed experiments with accession-based study on shoot culture, adventitious root and cell suspension cultures combined with elicitor treatments for enhancing production of plumbagin from *P. zeylanica* are not reported till date. In this investigation an attempt was done to find out a suitable biotechnological approach for production of plumbagin using shoot, adventitious root and cell suspension cultures of *P. zeylanica.* Pharmaceutical demand of useful bioactive compounds such as plumbagin has enhanced significantly during last two decades. Therefore, for enhanced production of plumbagin, it is significant to optimize culture parameters such as culture media, inoculum density, media strength, elicitors, etc.

Environmental circumstances influence pharmaceutical potential of any plant. Therefore, it is significant to study different accessions which belong to different locations. Different accessions contain varied amount of bioactive compound. Phytochemical analysis reveals that accessions number IC- 524441 is a best accession in terms of bioactive compound production.

Silver nanoparticles synthesis using residual plant material and its role in antibacterial and dye degradation activity reveals the potential of *P. zeylanica* for its environmental application. Further formation of bio-char using *P. zeylanica* showed the capability of degrading cadmium and chromium. Further *in-silico* analysis revealed that plumbagin efficiently interact with the active sites of target and has the potential to inhibit COX-2.

Medicinal plants are gaining lot of scientific attentions due to its various pharmaceutical activities. They are utilized by the pharmaceutical industries for the development of various drugs which leads to the overexploitation of these plants from their natural habitat. Therefore, alternative methods for conservation and sustainable use of them for phytocompound production is essential. Though, significant work has been carried out by researchers for production of various phytocompounds but still there is more research required to meet the current demand.

Accession based study enables us to find out better-quality accession for higher production of therapeutically important compound. Further role *P. zeylanica* in green synthesis of nanoparticles and heavy metal remediation possess its importance in environmental applications. In future, metabolic engineering or application of precursors can be utilized for enhanced production of pharmacologically active compound.

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APPENDIX

MEDIA COMPOSITIONS

MS Media

Salts	Concentration (mg/L)
Major Salts	
Sodium nitrate	1650
Potassium nitrate	1900
Calcium chloride.$2H_2O$	440
Magnesium sulphate.$7H_2O$	370
Potassium phosphate monobasic	170
Minor Salts	
Boric acid	6.2
Cobalt chloride hexahydrate	0.025
Copper sulphate pentahydrate	0.025
EDTA disodium salt dihydrate	37.3
Ferrous sulphate heptahydrate	27.8
Manganese sulphate monohydrate	16.9
Molybdic acid (sodium salt)	0.214
Potassium Iodide	0.83
Zinc sulphate heptahydrate	8.6
Vitamins	
Thiamine (HCl)	0.1
Niacine	0.5
Glycine	2.0
Pyrodoxine (HCl)	0.5
myo-Inositol	100

Sucrose - 30 g/L, **pH** – 5.8, **Agar –** 0.8%

Gamborg B5 Media

Salts	Concentration (mg/L)
Major Salts	
Ammonium sulphate	134 .00
Calcium chloride	113.23
Magnesium sulphate	122.09
Potassium nitrate	2500.00
Sodium phosphate monobasic	130.42
Minor Salts	
Boric acid	3.0
Cobalt chloride hexahydrate	0.025
Copper sulphate pentahydrate	0.025
EDTA disodium salt dihydrate	37.3
Ferrous sulphate heptahydrate	27.8
Manganese sulphate monohydrate	10.0
Molybdic acid (sodium salt)	0.213
Potassium Iodide	0.75
Zinc sulphate heptahydrate	2.0
Vitamins	
Thiamine (HCl)	10.0
Niacine	1.0
myo-Inositol	100.0
Pyrodoxine (HCl)	1.0

Sucrose - 20 g/L, **pH** – 5.8, **Agar –** 0.8%

Nitsch Media

Salts	Concentration (mg/L)
Major Salts	
Ammonium nitrate	720.00
Calcium chloride	113.23
Magnesium sulphate anhydrous	90.34
Potassium nitrate	950.00
Manganese sulphate.H_2O	18.94
Minor Salts	
Boric acid	10.0
Copper sulphate pentahydrate	0.025
Ferrous sulphate heptahydrate	27.85
Molybdic acid (sodium salt)	0.25
Zinc sulphate heptahydrate	10.0
Vitamins	
Thiamine (HCl)	0.5
Niacine	5.0
myo-Inositol	100.0
Pyrodoxine (HCl)	0.5
Folic acid	0.5
Biotin	0.05

Sucrose - 20 g/L, **pH** – 5.8, **Agar –** 0.8%

Schenk and Hilderbrandt Media

Salts	Concentration (mg/L)
Major Salts	
Potassium phosphate monobasic	300.00
Calcium chloride.$2H_2O$	113.23
Magnesium sulphate anhydrous	195.34
Potassium nitrate	2500.00
Manganese sulphate.H_2O	10.00
Minor Salts	
Boric acid	5.0
Cobalt chloride hexahydrate	0.1
Copper sulphate pentahydrate	0.2
EDTA disodium salt dihydrate	20.0
Ferrous sulphate heptahydrate	15.0
Zinc sulphate.$7H_2O$	1.0
Molybdic acid (sodium salt)	0.01
Potassium Iodide	1.0
Vitamins	
Thiamine (HCl)	5.0
Niacine	5.0
myo-Inositol	1000.0
Pyrodoxine (HCl)	0.5
Folic acid	0.5
Biotin	0.05

Sucrose - 20 g/L, **pH** – 5.8, **Agar –** 0.8%

White Media

Salts	Concentration (mg/L)
Major Salts	
Calcium nitrate	221.96
Potassium nitrate	80.00
Potassium chloride	65.00
Magnesium sulphate	360.00
Sodium phosphate monobasic	18.98
Sodium sulphate	200.00
Manganese sulphate.H_2O	5.04
Minor Salts	
Boric acid	1.5
Copper sulphate pentahydrate	0.010
Ferrous sulphate heptahydrate	2.5
Molybdenum trioxide	0.001
Potassium Iodide	0.75
Zinc sulphate heptahydrate	2.67
Vitamins	
Thiamine (HCl)	0.1
Niacine	0.5
Glycine	3.0
Pyrodoxine (HCl)	0.1
myo-Inositol	100

Sucrose - 30 g/L, **pH** – 5.8, **Agar –** 0.8%

LIST OF PUBLICATIONS

1. Arpita Roy and Navneeta Bharadvaja "Biotechnological Approaches for Production of Pharmaceutically Important Compound: Plumbagin", Current Pharmaceutical Biotechnology, 2018, 19(5), 372-381.

2. Arpita Roy and Navneeta Bharadvaja "Effect of Various Culture Condition on Shoot Multiplication and GC-MS Analysis of *Plumbago zeylanica* Accessions for Plumbagin Production", Acta Physiologiae Plantarum, 2018, 40 (11): 190.

3. Arpita Roy and Navneeta Bharadvaja "Silver Nanoparticles Synthesis from *Plumbago zeylanica* and its Dye Degradation Activity", Bioinspired Biomimetic and Nanobiomaterials, 2019, 8(2), 130-140.

4. Arpita Roy and Navneeta Bharadvaja "Establishment of root suspension culture of *Plumbago zeylanica* and enhanced production of plumbagin", Industrial Crops and products. 2019, 137, 419-427.

5. Enhanced production of plumbagin through cell suspension culture of *Plumbago zeylanica* (under communication).

6. *Plumbago zeylanica* derived bio char for removal of chromium and cadmium (under communication).

7. Biochemical and molecular analysis of *plumbago zeylanica* accessions (under preparation).

CONFERENCES ATTENDED

1. **Arpita Roy** and Navneeta Bharadvaja, "Phytochemical analysis of two medicinally important plants: *Plumbago zeylanica* and *Centella asiatica*". **International Conference on the Green Planet Past, Present and Future**, Kolkata, 21st–23rd December 2016.

2. **Arpita Roy** and Navneeta Bharadvaja, "A review on multi-purpose medicinal plant Plumbago zeylanica and in-vitro production of plumbagin". **National Conference on Pharmacognosy-Scope of Phytochemically Unexplored Medicinal Plants**, New Delhi, 12th January 2017.

3. **Arpita Roy**, and Navneeta Bharadvaja, "Synthesis of silver nanoparticles from medicinal plants and its biological application: A review"**. BIOTIKOS 2017-National Conference on Nanobiotechnology** (28th September-29th September 2017).

4. **Arpita Roy**, and Navneeta Bharadvaja, "Application of nano-biotechnology in various fields" **National conference on Thieme Chemistry: Science of synthesis**, 28th September 2018.

5. **Arpita Roy**, and Navneeta Bharadvaja, "Production of plumbagin from suspension culture of *Plumbago zeylanica*" **International Conference on Advances in Biosciences and Biotechnology**, 31st January-2nd February 2019

6. **Arpita Roy**, and Navneeta Bharadvaja, "Qualitative analysis and anti-bacterial investigation of *Plumbago zeylanica*" **International conference on Plant Biology and Biotechnology**, 11-13th March 2019, Singapore.

OTHER PUBLICATIONS

1. **Arpita Roy** and Navneeta Bharadvaja "Venom-Derived Bioactive Compounds as Potential Anticancer Agents: A Review", International Journal of Peptide Research and Therapeutics. 2020. (Accepted)
2. Vedant Sachdeva, **Arpita Roy,** Navneeta Bharadvaja "Current prospects of nutraceuticals: A review, Current Pharmaceutical Biotechnology, 2020, 21. (Accepted)
3. Swati Raina, **Arpita Roy**, Navneeta Bharadvaja "Degradation of dyes using biologically synthesized silver and copper nanoparticles", Environmental Nanotechnology, Monitoring & Management, 2020, 13, 100278.
4. Shruti Ahuja, **Arpita Roy,** Lakhan Kumar and Navneeta Bharadvaja "Media optimization using Box Behnken Design for enhanced production of biomass, beta-carotene and lipid from *Dunaliella salina"*, Vegetos, 2020, 31–39.
5. M. Laxmi Krishnan, **Arpita Roy** and Navneeta Bharadvaja "Elicitation effect on the production of asiaticoside and asiatic acid in shoot, callus and cell suspension culture of *Centella asiatica*", Journal of applied Pharmaceutical Science. 9(6), 067-074.
6. **Arpita Roy,** Nupur Jauhari and Navneeta Bharadvaja, "Medicinal plants as a potential source of chemo preventive agents", Anticancer Plants: Natural Products and Biotechnological Implements, 109-139, Springer International, Singapore. ISBN 978-981-10-8064-7.
7. **Arpita Roy** and Navneeta Bharadvaja, "Removal of Toxic Pollutants using microbial fuel cells", Removal of Toxic Pollutants through Microbiological and Tertiary Treatment-New Perspective, Elsevier. ISBN 9780128210147 August 2020.

8. Ayushi Verma, **Arpita Roy** and Navneeta Bharadvaja, "Remediation of heavy metals using nano phytoremediation", Advance Oxidation Processes for Effluent Treatment Plant, Elsevier. ISBN 9780128210116 July 2020.

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REVIEW ARTICLE

Biotechnological Approaches for the Production of Pharmaceutically Important Compound: Plumbagin

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Abstract: ***Background***: Increased demand for compounds that are derived from natural source are gaining more and more importance. Plumbagin is a plant naphthoquinone which is present in several families, including Iridaceae, Plumbaginaceae, Ebenceae, Drosophyllaceae, Nepenthaceae and Droseraceae. Plumbagin possesses high therapeutic efficacy and minimal side effects. It has various pharmaceutical activities which include anticancer, antibacterial, anti-inflammatory, antioxidant, antifungal, neuroprotective and hypolipidemic activities. In natural habitat, production of plumbagin is low due to species variations and environmental changes, considering importance of this bioactive compound, alternative techniques for its enhanced production needs to be devised. In the present review, various production techniques and scale-up strategies for plumbagin production are discussed.

Objectives: Aim of this review is to provide an insight into the chemistry of plumbagin, its pharmaceutical activities, perspective of cell suspension culture, root culture, hairy root culture and scale up strategies for its production.

Methodology: All the data compiled and presented here were obtained from various E-resources like Pubmed, Science Direct, and Google Scholar up to February 2018.

Result: This review comprises isolation, extraction and quantification method for plumbagin, its pharmaceutical activities, various tissue culture production techniques and scale-up strategies for enhanced production.

Conclusion: Plumbagin is an important phytocompound which shows potential towards treatments of various diseases. Demand for the production of plumbagin continuously increasing worldwide due to its pharmacological properties. To fulfil commercial demand of plumbagin alternative technologies need to be investigated. Biotechnological approaches like cell suspension culture, root suspension culture and hairy root culture are alternative techniques for plumbagin production. These techniques provide continuous supply of bioactive compounds. However, research on various aspects of tissue culture production techniques is in preparatory stage and requires culture and process optimization for development of a commercially practical process.

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1. INTRODUCTION

Plants produce diverse groups of secondary metabolites which are involved in defence mechanisms against stress conditions. Increasing knowledge of metabolic processes and impact of plants on human physiology has augmented the scope of therapeutic plants utilization. Almost 50% of pharmaceuticals that are available in the market are obtained from natural materials. Interestingly, market demand for medicinal plants is going to be high because a large number of active ingredients in medicinal plants are yet to be discovered [1]. As indicated by World Health Organization more than 80% of world's population depends principally on natural drugs for their fundamental medicinal services prerequisites. Due to the wide range of medicinal properties of these plants, the Indian medicinal plant extract market is expected to grow around 22% during 2017-2022 [2]. The global market for herbal medicinal product represents absolute opportunity of US$ 8,858.3 million in 2018 over 2017 and incremental opportunity of US$ 142.07 billion between 2017 and 2027. In terms of value, this market is likely to reach US$

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272.64 billion in 2027 [3]. As a result of this, there is an increased investment as well as significant demand for medicinal extracts in international markets. This creates an opportunity for the researchers to generate huge revenue from a growing market [2].

Continuous supply of raw materials, reduction in hazardous material, health and environmental protection are a great concern for process industries. The desire for production of valuable products that are safe and nontoxic in nature during and after their use is an important parameter for the development of any product. Drugs that are derived from natural resources interact favourably with the human body and produce beneficial effects [4]. About 50% of the drugs approved by US Food and Drug Administration are phytogenic compounds or derivatives thereof [5]. Today several distinct chemicals derived from plants are important drugs, which are currently used in one or more countries around the world [6]. The evolving commercial demand of secondary metabolites in recent years resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology. Secondary metabolites often accumulate in special tissues and are therefore difficult to extract, isolate and purify. Usually these phytocompounds have complicated structures [7] therefore organic synthesis is not cost effective. This has encouraged extraction of phytocompounds from field grown plants to meet the commercial demand due to which there is continuous exploitation of the plant from natural habitat.

Naphthoquinones constitute one of the diverse groups of plant secondary metabolites which show various activities [8] that include anti-feedant [9], antimicrobial [10], and allelopathic activities [11]. Plumbagin (C_{11}-H_8-O_3) is a napthoquinone which is mostly present in *Plumbago* species [12]. Plumbagin shows several pharmacological activities *i.e*. anti-cancer activity [13], anti-fungal [14], anti-oxidant [15], cardio-protective, anti-malarial, anti-hyperglycemic [16]. Plumbagin is a high value phytocompound, and its demand is increasing continuously. Presently, roots of *Plumbago* spp. (*Plumbago zeylanica, Plumbago indica (P. rosea* (1763) is a later synonym of *P. indica* (1754)*, Plumbago capensis* (*P. capensis* (1794) is a later synonym of *P. auriculata* (1786)) are the most exploited source of plumbagin. In India, the estimated annual trade of *Plumbago zeylanica*, *Plumbago indica,* and *Plumbago capensis* are reported to be 500-1000, 100-200 and below 10 million tonnes, respectively [17]. Quantity of phytocompounds produced from plants is affected by several factors, which includes environment changes, species variations and pathogens. Cultivation of some medicinal plants is also difficult because of their low germination rate. In literature plumbagin production using plant tissue culture has been reported [18-21]. Yield of plumbagin in cell and callus culture has been observed to be minute [21], whereas to some extent immobilized cell culture provides better yield [22]. Plant cell culture is a promising technology for the production of pharmaceutically useful compounds. However only few plant cell cultures are successfully established for commercial purposes [23]. One of the major difficulties associated with production of secondary metabolites is that they are produced by specialized cells at specific developmental stages and compounds are not produced if cells remain undifferentiated [24]. Due to this problem a new route for phytocompound production is utilized which involves the process of transformation of desired plant species using a natural vector system *Rhizobium rhizogenes. Rhizobium rhizogenes* is a gram negative soil bacterium which infects dicotyledonous plants that produce chemicals as a food source for *Rhizobium.* This leads to the emergence of hairy roots at the infection site of the plant [25]. These hairy roots are capable of unlimited growth in hormone free media. Hairy root cultures are able to produce phytocompounds in the same amount as natural roots and therefore provide a promising system for production of phytocomponds [26]. These transformed roots have the ability to regenerate whole plants (viable) and also maintain genetic stability during subculturing. Hairy roots provide the ability to transfer Ri plasmids into plant cells, which provides a tool to study gene expression. Various advantages of hairy root culture make it a promising tool for mass production of phytocompounds.

One of the vital factors in production of bioactive compounds in medicinal plants is the role of stresses. Often, plant stress response induces production of desired chemical compounds in the plant, this process is called elicitation. Elicitors are substances which trigger stress responses when applied in small quantity to a living system. They induce or improve the biosynthesis of specific compounds which have an important role in plants' adaptations to stressful conditions [27]. Several biotechnological strategies have been hypothesized and applied for productivity enhancement. Elicitation is recognized as the most practically feasible strategy for enhancing production of desirable secondary compounds from cell, organ, and plant systems [28]. This review comprises chemistry of plumbagin, its pharmaceutical activity, and perspectives of cell suspension, hairy root culture and scale-up strategies for its production.

2. CHEMISTRY OF PLUMBAGIN

The oldest reference about *Plumbaginaceae* family is found in ancient Indian Ayurvedic texts of "Charaka" [29]. *Plumbaginaceae* contains ten genera and around 300 species. It is mostly found in the Mediterranean and western Asia region [30]. The plumbagin-producing subfamily Plumbaginoideae (that comprises only four genera and 30 species) is also strongly represented in Africa and Madagascar. The vast majority of species in the family (subfam. Staticoideae) is devoid of plumbagin. Three species of *Plumbaginaceae* family are found in India which includes *Plumbago zeylanica*, *Plumbago capensis* and *Plumbago indica*. They are differentiated by their flower colour, *Plumbago zeylanica* has white flowers, *Plumbago indica* red flowers and *Plumbago capensis* blue flowers [31]. Plumbagin (Fig. **1**) is a 5-hydroxy-2-methyl-1, 4- naphthoquinone having molecular formula C_{11}-H_8-O_3. It is a stirring yellow pigment that patently appears in the family *Plumbaginaceae* [31] and is also present in *Nephenthes* and *Droseraceae* [14, 18]. Plumbagin was first isolated in 1829 [32] and it was recognised to have a quinone like character by Roy and Dutt in 1928. Roy and Dutt [33] established that on distillation of plumbagin over zinc dust it shows the presence of an acidic hydroxyl group, and they obtained 8-methylnaphthalene and naphthalene. Madinaveitia and Gallego [34] established the correct molecular

formula of plumbagin. It was successfully synthesized through a chemical process [35]. Plumbagin is soluble in organic solvents which include acetone, chloroform, alcohol, benzene and acetic acid. This compound is exceedingly corrosive with toxic properties [36]. Solubility of plumbagin in supercritical carbon dioxide was determined at 40 °C and 8-18 MPa pressure [37]. Synthesis of plumbagin is shown in Fig. (**2**).

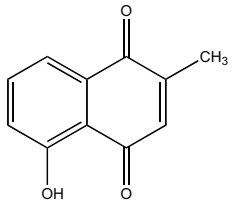

Fig. (1). Chemical structure of plumbagin.

3. ISOLATION, EXTRACTION AND QUANTIFICATION METHOD

Crouch *et al.* [18] reported an HPLC method for isolation of plumbagin from *Drosera* sp. Gupta *et al.* [38] developed a rapid, accurate and simple HPLC method for plumbagin quantification. Budzianowski [39] isolated plumbagin and some rare naphthoquinone glucosides from *Drosera gigantea* shoots. Isolation of plumbagin from *P. capensis* was achieved by using hexane: ethyl acetate: methanol: water (40:10:10:2, v/v) as solvent system in centrifugal liquid-liquid partition chromatography [40]. Madhavan *et al.* [41] used HPTLC for isolation of plumbagin from *Drosera burmannii.* Different extraction techniques were reported for plumbagin from different plant sources. Solvent extraction was used in the case of *P. scandens* for the extraction of plumbagin [40]. De Paiva *et al.* [42] analyzed a crude chloroform extract of *Plumbago capensis* by counter current chromatography to check the presence of plumbagin presence. The effect of pH on plumbagin content in *Dionaea muscipula* was studied by Babula *et al.* [43] using HPLC coupled with diode array detection. It was observed that with increase in acidity up to pH 5 there was an increase in plumbagin content. Plumbagin concentration was negligible in culture media with pH below 3 [43]. Unnikrishnan *et al.* [44] used HPTLC for quantification of plumbagin in *P. zeylanica* and *P. indica* roots where hexane: ethyl acetate was used as a mobile phase and it was found that plumbagin content was higher in *P. indica* as compared to *P. zeylanica.* A Thin Layer Chromatography method was used for plumbagin determination in hairy root cultures of *P. indica* [45]. Muhammad *et al.* [46] used HPLC for plumbagin quantification in *P. europaea* leaves and roots and it was found that roots contain 1.9% of plumbagin and leaves contain 1.5% of plumbagin. Ariyanathan *et al.* [47] also used HPTLC method for quantification of plumbagin and reported 0.04% in *P. capensis,* 0.17% in *P. indica* and 0.01% in *P. zeylanica*. Chellampillai *et al.* [48] used cold maceration for the purpose of isolation and extraction of plumbagin from *P. zeylanica* roots. Roots were cold macerated in chloroform: dichloromethane (1:1) mixture and the extract was successively washed with water. Cold maceration yielded 1.2% of fine crystalline plumbagin UV absorption, mass spectra and NMR confirmed the structure of the isolated compound. Gangopadhyay *et al*. [49] estimated plumbagin using HPLC by employing an isocratic linear solvent system of water and acetonitrile (20:80). Pillai *et al.* [50] used chloroform for extraction of plumbagin and HPLC was used for detection. Similarly, Silja and Stheeshkumar [51] utilized HPLC for estimation of plumbagin in *Plumbago indica* adventitious root culture. They used methanol and acetic acid (80:20) buffered with trimethyl amine (pH 3.5) as a mobile phase. Jaisi *et al*. [52] extracted plumbagin in ethanol, evaporated and dissolved crude extract in methanol followed by quantification of plumbagin using HPLC where methanol: aqueous acetic acid (5%) (80:20) was used as a mobile phase.

4. PHARMACEUTICAL ACTIVITIES OF PLUMBAGIN

Most of the data available on plumbagin is related to its anticancer activity against various cancers such as ovarian [53], lung [54], prostate [55], cervical [56], melanoma [57] and breast cancer [58]. Plumbagin possesses anticancer ac-

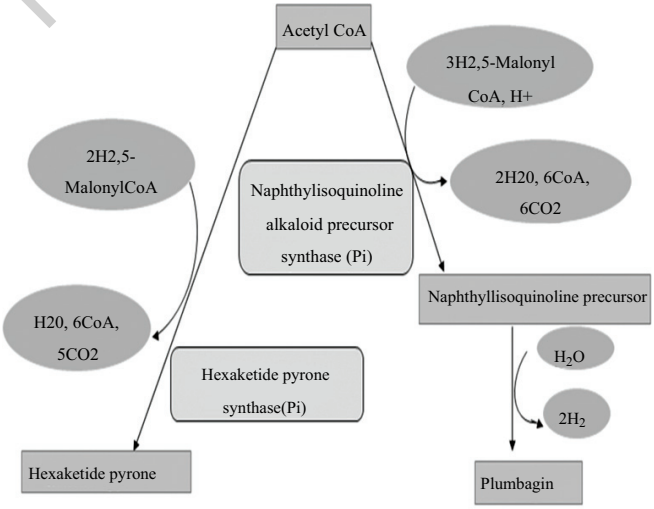

Fig. (2). Synthesis of plumbagin.

tivity by Akt/NF-κB signaling pathway inactivation as well as inactivation of MMP-9 and VEGF pathways which are considered important for invasion, metastasis, and angiogenesis [59]. Plumbagin possess selectivity towards cancer cells and does not damage normal epithelial, lung and cervical cells, which is a desirable attribute [59-61]. Plumbagin also shows inhibitory effects on intestine carcinogenesis and cause cytogenetic changes in mouse, it also possesses an anti-proliferative activity in human cervical cancer cells [13, 62]. It is known to have anti-angiogenic, proapoptotic and anti-metastatic effects in cancerous cells. Plumbagin also inhibits NF-κB, PKCε, JNK, and STAT-3 [63].

Plumbagin has biological effects in human keratinocytes. This compound was highly cytotoxic in nature and a strong inducer of reactive oxygen species. It induced oxidative DNA damages and accumulated DNA strand breakage. EGFR activation by plumbagin was attenuated by superoxide dismutase mimics, which indicates mechanisms related to reactive oxygen species [64]. NF-κB plays key role in regulating various processes that include expression of immunoregulatory genes, cellular proliferation, and induction of apoptosis in leukocytes, during innate and adaptive immune responses [65]. Ahmad *et al.* [66] reported that plumbagin significantly down regulates the NF-κB-DNA binding activity in breast cancer cells. It has been reported that plumbagin suppresses the activation of NF-κB in tumor cells and therefore affects the biological function of leukocytes participating in various immune responses [67]. Sakunrangsit *et al.* [68] investigated the effect of plumbagin on growth of human endocrine resistant breast cancer cells and found that at micro-molar concentrations plumbagin exhibits cytotoxic effects and combination of plumbagin and tamoxifen at fixed low concentrations showed increased growth inhibition in endocrine resistant cells. Li *et al.* [69] reported that plumbagin suppressed BAX, BCL-2, pro-caspase-3 expression and cleaved caspase-3 in gastric cancer cells which inhibit apoptosis in human gastric cancer cells that may be due to the ability of plumbagin to suppress STAT3 and Akt phosphorylation. Cao *et al*. [70] reported that plumbagin inhibited the proliferation and survival of KYSE150 and KYSE450 ESCC cell lines (esophageal cancer cells) in a dose-dependent manner. It also induced mitotic arrest and massive apoptosis in ESCC cells, this antitumor effect may be due to the stopping of STAT3-PLK1-AKT signalling by plumbagin. Plumbagin also showed neuroprotective effects in neonatal Sprague Dawley rats by suppressing isoflurane-induced neuronal apoptosis by regulating ERK/JNK and BDNF-TrkB-PI3/Akt signalling [71].

Apart from its anti-cancer activity, it shows numerous other potential roles such as anti-fertility, anti-microbial [72], anti-diabetic activity [73], anti-atherosclerotic [74], hypocholesterolemic activity [74], *etc*. Sunil *et al*. [73] reported that plumbagin significantly reduced blood glucose level and change all other biochemical parameters to nearly normal. Further, this compound enhanced the hexokinase activity and decline glucose-6-phosphatase and fructose-1, 6-bisphosphatase activities in diabetic rats. After treatment with plumbagin enhanced protein and GLUT4 mRNA expression were observed. The results indicated the anti-diabetic potential of plumbagin. Sharma *et al*. [74] reported that plumbagin reduced the serum cholesterol and LDL- cholesterol in hyperlipidaemic rabbits. It reduced cholesterol/phospholipid ratio by 45.8% and raise the decline HDL- cholesterol level. Further it also prevented cholesterol and triglycerides accumulation in liver and aorta. This indicated hypo-cholesterolemic activity of plumbagin. Recently a new potential of plumbagin has been explored which includes anti-malarial activity against 3D7 and K1 *Plasmodium falciparum* clones [75]. Plumbagin isolated from *Diospyros canaliculata* and *Diospyros crassiflora* showed anti-gonorrheal and anti-mycobaterial activity [76] Gurumurthy *et al.* [77] reported bactericidal effects of plumbagin against *M. smegmatis* and various *M. tuberculosis* strains which suggest that plumbagin possesses potential role against tuberculosis. Mahoney *et al.* [78] evaluated anti-fungal activity of four napthoquinones *i.e.* juglone, 1, 4-naphthoquinone, menadione, and plumbagin. Napthoquinones delayed germination of *Aspergillus flavus* and at higher concentration it completely inhibits the growth. Curreli *et al.* [79] reported that plumbagin inhibits the growth of white rot basidiomycetes *Pleurotus sajor-caju* in malt agar plates. Anti-fungal activity of plumbagin against twelve different strains of yeast and filamentous fungi *viz*., *Aspergillus flavus, Aspergillus niger, Alternaria* sp*., Candida albicans, Candida krusei, Candida glabrata, Cryptococcus neoformans, Candida tropicalis, Cladosporium* sp., *Geotrichum candidum, Fusarium* sp*.,* and *Penicillium* sp. was demonstrated by Dzoyem *et al.* [80]. This study showed potential role of plumbagin as an antifungal agent as compared to the ketoconazole which was used as a control standard. Nair *et al.* [81] reported the antimicrobial activity of plumbagin against *Candida albicans* and *Staphylococcus aureus* with minimum inhibitory concentration of 5µg/ml. Validation of antimicrobial activity was done by using an *ex-vivo* porcine skin model.

5. PLANT TISSUE CULTURE TECHNIQUES FOR PLUMBAGIN PRODUCTION

5.1. Cell Suspension Culture

Cell suspension culture has become a well established platform for natural compound production. It is an important tool and is independent of various constraints like seasonal and geographical variations. Additionally, it removes dependence on wild sources, thereby preventing its depletion. Further utilization of plant cell culture reduces the requirement of precious water resources which is associated with the contemporary agriculture production. Commercialization of natural products produced by this method has also increased significant consumer acknowledgment because they come under non-GMO [82]. Plant cell suspensions are normally derived from callus that is developed on solidified media. Transfer of friable callus bunches to liquid medium and agitating on rotary shakers results in formation of single cells or small aggregates. Plumbagin presence in *in-vitro* shoot culture*,* root culture and cell suspension culture has been reported [20, 83, 84]. Production of plumbagin in cell suspension culture systems is given in Table **1** [85, 86]. Cell suspension culture has several problems in the enhancement of metabolite production such as it is very slow and takes a long time period to get suitable roots for further use [87]. Also, various studies suggested that cell suspension culture accumulates compounds during tissue or organ differentiation, due to which there is no active production of phytocompounds. Due

Table 1. Cell suspension culture for plumbagin production.

Culture System	Plant Name	Explants	Culture Condition	Plumbagin Content (mg/g Dry Weight)	References
Cell suspension Culture	*Drosophyllum lusitanicu*	Nodal	-	1.5	[85]
Callus/Cell suspension Culture	*Plumbago rosea*	Leaf	-	4.5	[20]
Cell suspension Culture	*Plumbago rosea*	Leaf	Chitosan (150 mg/l)	15.18	[20]
Cell Culture	*Plumbago rosea*	-	Immobilized cell ($CaCl_2$)	92.13	[22]
Cell suspension Culture	*Plumbago rosea*	Inter-node	IBA (1.5mg/L) +IAA (1.0 mg/L)	12.05	[51]
Cell suspension Culture	*Plumbago rosea*	Leaf	100µM Jasmonic Acid	1.0	[86]

to these problems, a new route for the production of phytocompounds is utilized which involves the process of transformation of desired plant species using a natural vector system, *Agrobacterium rhizogenes.* This leads to hairy roots emergence at the infection site of the plant [25].

5.2. Root Culture

Adventitious root culture of medicinal plants is considered to be a good source of crude material for pharmaceutical industries. Adventitious root cultures are an alternative and effective way to produce high value phytocompounds due to their biochemical stability, faster growth and relatively high secondary metabolites production. Accumulation of plumbagin occurs mainly in roots of *Plumbago* species, due to which culture of roots provides an alternative method for enhanced production of plumbagin. Limitations associated with establishing root cultures include low survival rate, high chances of contamination and slow growth rate. The highest amount Lenora *et al.* reported the plumbagin content from intact plant (13.3mg/g DW in *P. indica* roots) which were grown conventionally [88]. Panichayupakaranat and Tewtrakul [83] studied adventitious root cultures of *Plumbago indica* using various concentrations of α- naphthalene acetic acid (NAA) (0.5-2.0 mg/l) and kinetin (0.1-0.5 mg/l) in B5 media. They found the highest concentration of plumbagin (0.016 ± 0.0030% dry weight) in a media containing 1 mg/l NAA and 0.1 mg/l Kinetin. Jaisi *et al.* [89] reported higher production of plumbagin (1.04 mg/g Dry weight) from gamma ray treated root cultures of *Plumbago indica.* Silja *et al.* [86] also established adventitious root culture from *Plumbago indica* leaf explants. They established root culture in media containing 1.5 mg/l IAA and 1mg/l IBA and found that without elicitation maximum production was obtained in media containing 0.5 mg/L IBA and 0.1 mg/L GA3. Jaisi *et al.* [90] also reported enhanced plumbagin production in root cultures of *Plumbago indica* by using biotic and abiotic elicitors. They have found 10.6mg/g dry weight of plumbagin in a culture treated with 150mg/l chitosan and 7.6 mg/g dry weight of plumbagin in a culture treated with 100 µM $AgNO_3$.

5.3. Hairy Root Culture

Hairy root culture is a substitute method to enhance secondary metabolites production. Hairy roots result from T-DNA transfer to the genome of plants by bacterial Ri-plasmids which code for auxin synthesis [91]. T-DNA contains genes which provide the ability to synthesize opine, this T-DNA is utilized by *R. rhizogenes* to tap nitrogen and carbon sources for further development. Products from virulence genes located on the Ri-plasmid (non transferred segment) are responsible for T-DNA excision and transfer into plant cells for chromosomal integration inside the recipient cell [92]. High levels of lateral branching of roots and absence of geotropism are characteristics of hairy roots and they grow faster than normal roots due to their extensive branching [93] and do not require growth hormones in the media [94]. They have the capability of secondary metabolite synthesis specific to the plant species from which they have been developed [95]. They possess biochemical stability which makes them grow faster and they have the ability to produce secondary metabolites [96, 97]. Hairy root culture has numerous advantages over cell suspension culture such as biochemical and genotypic stability, hormone free medium and cytodifferentiation. These parameters play an important role in the production of phytocompounds. Low doubling time, easier maintenance, faster growth, and continuous source for phytocompound production are advantages of hairy root culture [98]. Numerous phytocompounds produced from hairy root cultures have been reported in literature [94]. It has been observed that hairy roots synthesise novel phytocompounds which are not present in normal tissue [99]. In some cases, where phytocompounds accumulate only in the plant's aerial part, hairy root culture has appeared to accumulate these metabolites [100]. Production of plumbagin in hairy root cultures from different *Plumbago* sp. has been achieved successfully as given in the Table **2** [101-104].

6. SCALE UP STRATEGIES FOR PLUMBAGIN PRODUCTION

Scaling up of suspension culture or hairy root culture in bioreactor systems provides the best conditions possible for growth of cells and formation of product. The engineering challenge for mass cultivation lies in the scale-up of the plumbagin production process. Extensive literature survey reveals non-availability of any report on large-scale plumbagin production under controlled bioreactor conditions. It would be interesting to study *Plumbago* sp. cultivation in

Table 2. Hairy root culture for plumbagin production.

Plant Name	Microbes Used	Explants	Culture Condition	Plumbagin Content (% Dry Weight)	References
Plumbago zeylanica	A4	Leaf	-	0.042	[101]
Plumbago rosea	ATCC 15834	Leaf	Different *A. rhizogenes* cultures	-	[102]
Plumbago indica	ATCC 15834	Leaf	Chitosan (200mg/l) + Methyl Jasmonate (80μM)	1.196	[49]
Plumbago indica	ATCC 15834	Leaf	NAA (0.5mg/l)	0.79	[49]
Plumbago rosea	A4M70GUS	Leaf and inter-node	Methyl Jasmonate (50μM)	5.0	[103]
Plumbago rosea	ATCC 40357	Shoots	Inoculum density (1g/l)	1.325	[50]
Plumbago indica	Wild A4	Shoots	-	0.253	[104]

bioreactor to increase plumbagin production. Scaling up of plant cultures from laboratory to industrial scale level is an important aspect and also a critical step that is often rate limiting for commercial production of natural compounds. Scaling up from shake flasks to bioreactors is not straightforward as it affects the cell growth environment in terms of hydrodynamic shear forces and rheological properties [105]. These problems can be overcome with the help of improving bioreactor design and optimising crucial system and culture parameters such as adequate mixing, reduction in mass transfer, low shear stress, and availability of nutrients [105, 106]. Large scale plant cells culturing was reported to be conducted in various bioreactors which includes stirred tank bioreactors, bubble column bioreactors, hollow fiber bioreactors, airlift bioreactors, membrane bioreactors and rotating drum bioreactors [106, 107]. Most widely used bioreactor is stirrer tank bioreactor which provides easy scale up, good amount of oxygen transfer, better mixing abilities and easy compliance. Hairy root culture provides a stable secondary metabolite production source which draws the attention of the scientific community to its exploitation. Scale up of hairy root culture in a suitable bioreactor which provides best conditions for growth and formation of product is necessary for any phytocompound production, although the need for development of suitable bioreactors for cultivation of hairy roots has long been recognized [108]. Due to the complex structure of hairy roots, analysis of their growth and large scale culture development is difficult. Growth of hairy roots is not homogeneous and affects reactor performance. Furthermore, morphology of hairy root is quite plastic as it responds to local environment changes. Morphological changes include change in length and density of hairy root which directly affect phytocompound production [109]. Different reactor configurations have been reviewed for hairy root cultures [50, 110, 111]. Suitability of the particular type of reactor, impeller and operation condition are required for the hairy root culture of various plant species for plumbagin production. Different kinds of bioreactors were utilized for enhanced production of plumbagin in hairy root culture systems [49, 50, 104]. An extensive survey of literature showed that there is no such report available on large scale production of plumbagin under controlled conditions in a bioreactor. Therefore, study on the plumbagin production in bioreactors would provide an opportunity for further development of a suitable bioreactor system. Parameters controlling the growth and product formation are important aspect of bioreactor design. Controlling altogether of bioreactor output includes various control parameters such as pH, temperature, carbon dioxide, dissolved oxygen and other gases. Challenges in mass cultivation of hairy roots in bioreactors include technological barriers of mixing, aeration rate, and adhesion and shear sensitivity. Mechanical agitation causes wounding of hairy roots which creates callus formation and branching of roots which lead to formation of an interlock matrix that resists nutrient flow. All these hurdles need to be tackled accurately at bioreactor level for effective plumbagin production. The envisaged strategy for enhanced production of plumbagin is sketched in Fig. (**3**).

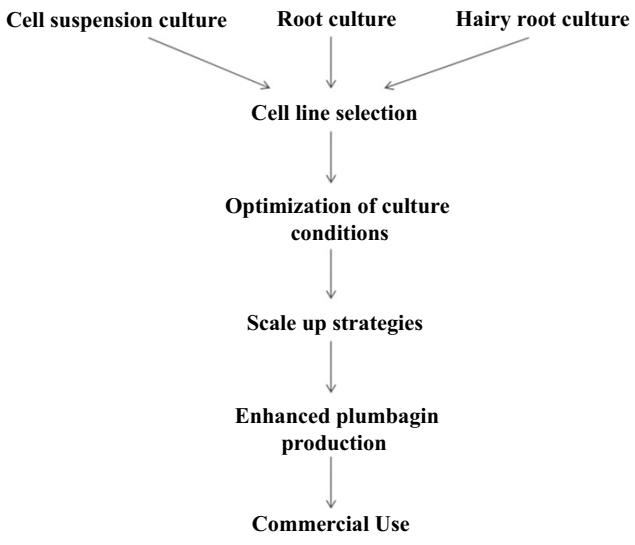

Fig. (3). Strategy for enhance production of plumbagin.

CONCLUSION

Demand of herbal products is increasing day by day for which phytocompounds need to be produced continuously and in higher amount. Medicines derived from herbal origin have been used since the dawn of mankind, indeed. They have provided opportunities to researchers for future research and development in this field. In modern cancer treatment, chemotherapy is an important option, plant derived agents used in chemotherapy provide fewer side effects to the patients as compared to the chemical agents. To cure cancer there is a need to find a better way to treat this disease by finding anti-cancer compounds from herbal sources. Phytocompounds are natural and have advantages over synthetic compounds, such as easy availability in nature, since they are natural products, the problem of acquiring resistance against these compounds is also minimized to a great extent. Medicinal plants synthesize a variety of bioactive compounds which possess anticancer activity. They inhibit cancer growth by modulating the activity of specific enzymes and hormones and protect the body by enhancing detoxification functions. Plumbagin is one of the important compounds which show potential towards treatment of several cancers in breast, lung, colon, prostate, liver, *etc*. in cell culture as well as in animal models. Plumbagin is a simple plant secondary metabolite which is present in several families, including Iridaceae, Plumbaginaceae, Ebenceae, Drosophyllaceae, Nepenthaceae and Droseraceae. Anti-cancer activity of plumbagin is due to its ability to inhibit abnormally expressed growth factors and thereby inhibit cancer cell growth. Due to the importance of plumbagin in anti-cancer activity there is a need for its large scale production. Demand for the production of plumbagin is continuously increasing worldwide due to its various pharmacological properties. Plumbagin production from wild resources makes it a labour intensive and expensive process and also the use of wild plants for its production leads to depletion of natural resources. Presently, plumbagin production in cell culture is in an initial stage and there is a long way to establish a process which is commercially viable. Hairy root culture provides new openings for *in-vitro* production of valuable secondary metabolites from plants. It provides good prospects for the commercial production of high value products from plants. For large cultivation of hairy roots, various parameters like inoculum density, medium, growth measurement, effects of different physical factors on growth, recovery of product and result reproducibility need to be assessed before the commercialization of the technology. In this review, an effort has been made to compile the different cultures for the enhanced production of plumbagin and scale-up strategies for plumbagin production.

FUTURE PROSPECTS

Utilization of plant-derived compounds for various disease treatments is attracting more and more attention worldwide due to their effectiveness and low impact on normal cells. Naturally derived products offer great opportunity to evaluate not only new chemical classes of anticancer agents, but also potentially relevant mechanisms of action. There is no such report available which shows the use of drug-containing plumbagin for treatment of cancer. Till now the investigations which were done in plumbagin for its anticancer activity are on a laboratory scale only. Also, production of plumbagin from roots of wild *Plumbago* species is laborious, expensive and leads to overexploitation of these plants. To meet with the increasing demand of plumbagin, other alternatives need to be investigated. Although research into plumbagin cell suspension, root and hairy root culture are in beginning stage, there are miles to go before establishment of an economically viable process. Improvement in culture technology by optimizing all parameters could help in developing a promising alternative for plumbagin production.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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ORIGINAL ARTICLE

Effect of various culture conditions on shoot multiplication and GC–MS analysis of *Plumbago zeylanica* accessions for plumbagin production

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Abstract

Plumbago zeylanica, a pharmaceutically important medicinal plant, contains a wide range of phytocompounds. Culture parameters like carbon source, nitrogen source, and culture media are essential for the development and growth of explants. In this investigation, the influence of various carbon sources (sucrose, glucose, and fructose at 3% concentration), nitrogen source (ammonium nitrate, sodium nitrate, and potassium nitrate) and plant tissue culture media (MS medium, Gamborg's B5 medium, White medium and Nitsch medium) on shoot multiplication of five different accessions was studied. Optimum growth of all five accessions was observed in MS media containing 3% sucrose and ammonium nitrate as a source of carbon and nitrogen. Out of five accessions, IC-524441 showed the highest shoot multiplication. Further, methanolic extracts of all accessions (grown in MS media containing 3% sucrose and ammonium nitrate as nitrogen source) were prepared and comparison of extracts in DPPH assay indicated that accession number IC-524441 was the most effective free radical scavenging agent. Total phenolic, flavonoid and tannin content ranges were from 20 to 70 µg/ml, 40 to 100 µg/ml and 55 to 120 µg/ml, respectively, and the highest amount was found in accession number IC-524441. Sucrose and ammonium nitrate content may be responsible for increased antioxidant activity, flavonoids content, phenolic content, and tannin content in accession number IC-524441. GC–MS of ethyl acetate extract of all five accessions of *P. zeylanica* was conducted (grown in MS media containing 3% sucrose and ammonium nitrate as nitrogen source). GC–MS analysis of the aerial part showed the presence of various phytocompounds, which include 1,4-naphthalenedione, 3-eicosene, 5-eicosene, phthalic acid, *o*-anisic acid, thioctic acid, 1-octadecene, 5-*t*-butyl-cycloheptene, 2-benzoyl-1,2-dihydro-1-isoquinolinecarbonitrile, octadecanal, silane, 3-methoxy-2-methyl-2-(1-phenyl-ethylamino)-propionic acid, and 1-nonadecene. Accession number IC-524441 contains the highest amount of plumbagin, i.e. 14.19 ± 0.5 µg/ml as compared to the others.

Keywords Accessions · Carbon source · Antioxidant activity · Phenolic content · GC–MS analysis

Abbreviations

ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
F–C	Folin–Ciocalteu
LOD	Limit of detection
LOQ	Limit of quantification
MS	Murashige and Skoog medium
NBPGR	National Bureau of Plant Genetic Resources
SD	Standard deviation

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Introduction

Plumbago zeylanica (Chitrak) is a perennial herbaceous plant which belongs to *Plumbaginaceae* family and found in Bengal and Southern India region (Sharma and Singh 2015). It is widely utilized for the treatment of skin diseases, diarrhoea, piles, rheumatism and leprosy (Roy and Bharadvaja 2017). It contains a wide variety of secondary metabolites like steroids, glycosides, saponins, flavonoids, triterpenoids, alkaloids, coumarins, tannins, phenolic compounds, carbohydrates, fats, proteins, and naphthoquinones (Ming et al. 2011) which are responsible for its medicinal

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activities. It possesses antibacterial, antifungal (Uma Devi et al. 1999) anticancer (Roy et al. 2018), antiplasmodal, antitumour, stimulatory activity, anti-inflammatory, antihyperglycaemic, central nervous system and hepatoprotective activity (Kumar et al. 2009). Previously identified and isolated compounds from this plant include plumbagin, chitranone, elliptinone, seselin, suberosin, etc (Lin et al. 2003; Jetty et al. 2010; Roy and Bharadvaja 2017). Due to its wide range of medicinal activities, this plant is overexploited. Medicines currently available in market from this plant are Chitrak Capsules (Dr Wakde's Organic), Chitrak Haritaki (Dabur) and Chitrak powder (Herbal hills) (Chitrak products source 2018). Conventional method of propagation using seed is not a reliable approach because of poor seed setting and germination. In vitro propagation provides an alternative method of conservation and rapid growth of elite genotype which is independent of seasonal changes as well as production of virus-free plant species. The advantages of in vitro propagation are higher growth and multiplication rate. The growth and multiplication of shoots are influenced by various parameters (Israeli et al. 1996), one of which is a carbon source addition in culture media which is necessary for the growth of the plants, because the autotrophic ability of plants is limited in in vitro conditions. Addition of carbon source provides energy and also maintains the osmotic potential of cells (Yaseen et al. 2013). Different media and variation in nitrogen sources are also important parameters that affect growth and the biochemical composition of tissue-cultured plants. Till now, there have been no studies regarding the effect of various carbon sources, nitrogen source and media in different accessions of *P. zeylanica*. However, the influence of various sources of carbon (glucose, sucrose and maltose) at various concentrations (1%, 3%, 5%, 7%, and 9%) on the growth of hairy root of *Arnica montana* L. has been reported by Petrova et al. (2015). Similarly, Sridhar and Naidu (2011) also reported the impact of fructose, glucose, maltose and sucrose on *Solanum nigrum* growth. Mohamed (2011) reported the effect of different nitrogen sources (Nitrophoska, urea, ammonium sulphate nitrate and ammonium sulphate) in *Zea mays*. Roy et al. (2016) reported the influence of various media (MS, B5 and Nitsch) on the multiplication of shoot in *Centella asiatica*. Variation in carbon source also influences the amount of phytochemicals present in different accessions (Bruni and Sacchetti 2009; Petrova et al. 2015). The quantity of pharmaceutically important compound depends upon the geographical conditions of that accession (Bruni and Sacchetti 2009; Kundu et al. 2016). Identification of potential accessions of medicinal plants poses a significant challenge, because various factors such as genetic composition of the plant, climate variations, age of the plant or harvesting period and specific part of plants harvested for processing influence the phytocompounds (Bruni and Sacchetti 2009). The objective of this study is to investigate a suitable carbon source, nitrogen source and culture media that promote shoot multiplication and growth of different accessions. Variation of phytochemical (antioxidant activity, total phenolic, flavonoid and tannin contents) amount among accessions was also investigated. Further, ethyl acetate leaf extracts of all accessions were analysed using GC–MS (gas chromatography–mass spectrometry). This study helps to identify the best source of carbon, nitrogen and culture media for the highest growth and presence of various compounds in all the five accessions.

Materials and methods

Collection of plant material

Plumbago zeylanica accessions—IC-421418, IC-524441, IC-439212, IC-398891 and IC-539866—were collected from NBPGR, New Delhi. These accessions were further maintained in MS media (0.2 mg/l BAP) in our Plant Tissue Culture Laboratory, DTU.

Culture medium and parameters

For shoot culture establishment, semisolid MS basal medium ($CaCl_2{\cdot}2H_2O$, $CuSO_4{\cdot}5H_2O$, $CoCl_2{\cdot}6H_2O$, KNO_3, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, H_3BO_3, KI, $Na_2MoO_4{\cdot}2H_2O$, glycine, $FeSO_4{\cdot}7H_2O$, Na_2EDTA, NH_4NO_3, $MnSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, nicotinic acid, meso-inosito, pyridoxine hydrochloride), sucrose and agar were purchased from Himedia Pvt. Ltd., Mumbai, India. For various experiments, MS media were prepared from stock solutions (major, minor, vitamins) by varying the carbon source (sucrose, glucose and fructose at 3% concentration), nitrogen source (ammonium nitrate, sodium nitrate and potassium nitrate) and culture media (MS medium, Gamborg's B5 medium, White medium and Nitsch medium); 0.8% agar was used for all the cultures. For all the experiments, the medium was supplemented with 1 mg/l BAP. Nodal explants from each accession were aseptically transferred to media and 25 ± 2 °C was used for incubation with 16 h photoperiod using 36 W fluorescent lamps (Philips, Kolkata, India).

Statistical analysis

Results were expressed as mean ± SD with three replicates each containing three explants. The effect of various treatments on shoot multiplication was compared to detect significant differences among the treatments using ANOVA at 5% probability level.

Phytochemical studies

Methanolic extract preparation

Fresh in vitro-grown plant material MS media containing 3% sucrose and ammonium nitrate as nitrogen source was dried at room temperature and ground with mortar and pestle. 1.0 gm of the plant material was macerated in 10 ml methanol for 48 h and the plant extract was filtered and stored at 4 °C for further investigation.

Antioxidant activity

DPPH assay was done to estimate the antioxidant activity (Brand-Williams et al. 1995). Stock solutions of plant extract (1 mg/ml) were prepared in methanol and from this 50 µg/ml was used. Similarly, 1 mM DPPH stock solution was prepared in methanol and from this 0.06 mM DPPH was used. 100 µl plant extracts + 3.9 ml of DPPH solution was mixed by vortexing for 30 s and left in the dark for 30 min incubation. Methanol and DPPH solution were used as blank and negative control. Sample absorbance was determined at 517 nm.

Antioxidant activities were calculated as:

$$\text{Antioxidant activity } (\%) = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100.$$

Total flavanoid estimation

Aluminium chloride ($AlCl_3$) colorimetric assay was done to estimate the flavanoids (Lamaison and Carnat 1990). 1 ml extract + 4 ml DW (distilled water) + 300 µl $NaNO_2$ (5%) was added. Five minutes later, 300 µl $AlCl_3$ (10%) + 2 ml NaOH (1M) + 2.4 ml DW was added. Different concentrations of standard quercetin (100, 80, 60, 40 and 20 µg/ml) were prepared. Sample absorbance and quercetin standards were determined using UV–visible spectrophotometer at 510 nm.

Total phenolic content estimation

Total phenolic content was estimated using the F–C method (Singleton and Rossi 1965). 200 µl plant extract + 1.5 ml F–C (10%) reagent was mixed and kept in dark for 5 min. After this, 1.5 ml $NaCO_3$ (5%) was added and mixed properly. The final mixture was kept in dark for 30 min. The calibration curve of gallic acid standard solutions (200, 100, 50 and 25 mg/l) was prepared for quantification of total phenolic content. Absorbance was recorded at 750 nm.

Total tannin estimation

The F–C method was used to determine the tannin content (Singleton et al. 1999). 100 µl plant extract + 0.5 ml F–C (10%) reagent + 1 ml $NaCO_3$ (35%) + 8.4 ml DW were added in a test tube. The entire reaction mixture was mixed well and kept for 30 min. Various concentrations of standard gallic acid (100, 80, 60, 40 and 20 µg/ml) were used. The absorbance of samples and gallic acid was recorded at 725 nm.

GC–MS analysis

Ethyl acetate extract preparation Fresh in vitro-grown plant material (MS media with 3% sucrose) was dried at room temperature and then ground with mortar and pestle. 1.0 gm of plant material was then macerated in 10 ml of ethyl acetate for 48 h. Plant extracts were filtered with 0.45-µm filter.

Plumbagin estimation and instrumentation For quantitative analysis of plumbagin, 10 mg of plumbagin standard (Sigma Aldrich) was dissolved in 10 ml of ethyl acetate, i.e. astock solution of (1 mg/ml) was prepared. From the stock solutions, further dilutions were prepared by diluting the required solution volume and 1 µl solution was injected into the system.

GC–MS analysis was conducted using an Agilent GC (7890B GC) system. The analytical column was 5MS (5% phenyl, 95% methyl siloxane) capillary column. The injection port and detector port temperature were 270 °C and 280 °C. 1 ml/min flow rate and 1:20 split was used (Rajakrishnan et al. 2017). The sample injection volume was 1 µl and direct injection mode was used. Compound identification was carried out using the database of NISTII library (McLafferty and Stauffer 1989).

Validation Validation of the method was done using parameters such as linearity, correlation coefficient (r), LOD and LOQ.

Linearity Linearity study was done by serially diluting the stock solution of the standard (1 mg/ml) to a given concentration range as 50, 100, 200, 300 and 500 µg/ml. The calibration curve was made after triplicate analysis of plumbagin standard by plotting the peak area against concentration (µg/ml). The correlation coefficient (r) was determined by calculating $\sqrt{R^2}$.

LOD is the minimum amount of analyte present in a sample which can be detected but not necessarily quantified.

LOQ is the minimum amount of analyte present in a sample which can be quantified.

LOD and LOQ studies were done to detect and quantify the limits of the method to check the presence of any impurities using the following equations:

LOD = 3.3 σ/S and LOQ = 10 σ/S, where S is the slope of the curve and σ is SD.

Results and discussion

Effect of culture conditions on shoot multiplication

Effect of different carbon sources

Carbon source is essential for in vitro culture conditions; addition of carbon source in culture medium enhances plantlet growth. In this study, 3% of different carbon sources were used. Shoot initiation started after 3 weeks of their inoculation on MS media containing sucrose as a carbon source. Comparative analysis showed that the highest shoot length (4.2 ± 0.54 cm) and maximum shoot number (5.2 ± 0.44) were observed in accession number IC-524441 when 3% sucrose was used as carbon source in media (Table 1). Accession number IC-539866 showed the minimum number of shoots (2.2 ± 0.44) and minimum length of shoot (3.0 ± 0.29 cm). Glucose was the second most productive carbon source for multiplication of shoots. At 3% glucose level, accession number IC-524441 showed maximum shoot number (3.2 ± 0.44) and shoot length (3.4 ± 0.27 cm) (Table 1). Fructose showed least growth in all accessions. The requirement of carbon source depends on the developmental stage of the culture and there can be variations based on carbon source type (Thompson and Thorpe 1987). In in vitro conditions, plants are not completely autotrophic in nature and, therefore, there is requirement of carbon source in culture media which supports proliferation of shoots, induction of roots and overall plant growth. Various types of carbon sources, i.e. non-reducing and reducing sugars, are utilized in the medium. The morphogenetic potential of in vitro cultures can be influenced by manipulating the carbon source type and concentration. Sucrose is a most widely utilized source of carbon and energy; it is a disaccharide, highly water-soluble substance which works as a molecule transporter and is able to transport through the plasma membrane (Petrova et al. 2015). A study reported that media containing sucrose generated more number of leaves as compared to media containing fructose and glucose in banana plants (Buah et al. 2000). They also recorded that at higher temperature, fructose released a toxic substance, i.e. 5-hydroxymethyl-2-furaldehyde which increased hyperhydricity as well as declined the water potential in leaves, causing leaf enlargement. Gubis et al. (2005) also reported that media containing 30 g/l of sucrose generated healthy plantlets in *Lycopersicon esculentum* as compared to other types and concentrations of carbon source. Baskaran and Jayabalan (2005) reported that the relative growth rate of plantlet was best found in medium containing 30 g/l of glucose in *Eclipta alba*. Sridhar and Naidu (2011) reported that the highest shoot number was obtained at 4% fructose and maximum shoot length at 4% sucrose in case of *S. nigrum*. Petrova et al. (2015) reported that the carbon source influences the growth of *A. montana* and optimum growth was found at 3% sucrose. Similarly, Kundu et al. (2016) also reported that 3% sucrose along with 1.5 mg/l BAP in a nutrient media gives the highest multiplication of shoots in case of *C. asiatica*.

Table 1 Effect of different carbon sources on the number and length of shoots in five different accessions of *Plumbago zeylanica* after 8 weeks of inoculation

Experiment detail	Accession number	Number of shoots (M ± SD)	Length of shoots (cm) (M ± SD)
Sucrose	524441	5.2 ± 0.44	4.2 ± 0.54
	398891	2.8 ± 0.45	3.4 ± 0.76
	439212	2.4 ± 0.54	3.2 ± 0.44
	539866	2.2 ± 0.44	3.0 ± 0.29
	421418	4.6 ± 0.54	3.5 ± 0.41
Glucose	524441	3.2 ± 0.44	3.4 ± 0.27
	398891	2.2 ± 0.45	3.2 ± 0.44
	439212	1.6 ± 0.54	2.8 ± 0.22
	539866	1.8 ± 0.45	3.3 ± 0.44
	421418	2.4 ± 0.54	3.1 ± 0.38
Fructose	524441	2.1 ± 0.45	2.7 ± 1.27
	398891	1.8 ± 0.45	2.3 ± 1.13
	439212	1.7 ± 0.54	2.5 ± 0.72
	539866	1.4 ± 0.54	3.6 ± 0.68
	421418	1.2 ± 0.45	3.3 ± 0.65

Values are expressed as mean ± standard deviation (M ± SD)

Effect of different nitrogen sources

Nitrogen is a crucial element for growth. Nitrogen availability and the form in which it is present are of great importance. Nitrate is considered to be an essential form of nitrogen for tissue culture. To identify the best source of nitrate, different sources of nitrogen, i.e. ammonium nitrate, sodium nitrate and potassium nitrate, were tested on accessions of *P. zeylanica*. Data on shoot multiplication are presented in Table 2. Shoot initiation started 3 weeks after inoculation on MS media containing ammonium nitrate as a nitrogen source. Comparative analysis showed that the highest shoot length (3.179 ± 0.21 cm) and maximum shoot number (4.1 ± 0.56) were observed in accession number IC-524441 when ammonium nitrate was added to the media. Accession number IC-539866 showed the minimum number of shoots (2.2 ± 0.42) and minimum shoot length (4.111 ± 0.39 cm). Sodium nitrate was the second most efficient nitrogen source for multiplication of shoots and potassium nitrate showed least growth in all accessions. In plant tissue culture medium, nitrate ions, ammonium salt, amino acids and complex organic compounds supply nitrogen. Nitrate ions and ammonium salts are good sources of nitrogen because they can be absorbed and metabolized easily by cells, which further leads to seed breaking, root branching and dormancy of bud and stops apical dominance. Hence, nitrogen in the form of ammonium ion and nitrate ion is a dominant mineral nutrient in most plant tissue culture formulations. Optimization of these compounds stimulates regeneration in recalcitrant cultivars (Benson 2000). Mohamed (2011) reported the effect of different nitrogen sources in *Zea mays* and found that ammonium sulphate nitrate increased the growth as compared to other nitrogen sources. Similarly, Chandravanshi et al. (2014) recorded maximum multiplication of shoots in *P. zeylanica* in MS media containing ammonium nitrate and 13.3 µM BAP and 135.74 µM adenine sulphate. Several studies have shown that tissue culture growth is possible on a medium having ammonium as the sole source of nitrogen. Since ammonium nitrate has been banned due to its explosive nature (New York explosion chelsea 2016), sodium nitrate can be utilized as a substitute in culture media to supply the nitrogen source.

Effect of different culture media

Various factors influence optimum shoot multiplication. Variations in external factors such as culture media are an important factor which may influence the multiplication of shoots (Staba 1980). The effect of various media on shoot number in accessions of *P. zeylanica* (IC-524441, 398891, 439212, 539866, and 421418) is furnished in Table 3. Plant height (3.839 ± 0.7 cm) and maximum shoot number (6.0 ± 0.47) was higher in MS media for accession number IC-524441 followed by Nitsch, B5 and White media, respectively. Accession number IC-524441 was the best among all accessions, followed by 398891, 421418, 539866 and 439212 (Table 3). Chandravanshi et al. (2014) also reported maximum shoot multiplication in MS media supplemented by 13.3 µM BAP and 135.74 µM adenine sulphate. Vijay et al. (2016) reported that 4.44 µM BAP gave maximum shoot multiplication in *P. zeylanica*. Similarly, Roy et al. (2016) reported that MS media containing 1 mg/l BAP showed the highest shoot multiplication in *C. asiatica*.

Table 2 Effect of different nitrate sources on the number and length of shoots in five different accessions of *Plumbago zeylanica* after 8 weeks of inoculation

Experiment detail	Accession number	Number of shoots (M ± SD)	Length of shoots (cm) (M ± SD)
NH_4NO_3	524441	4.1 ± 0.56	3.179 ± 0.21
	398891	3.3 ± 0.67	3.759 ± 0.41
	439212	2.3 ± 0.48	4.188 ± 0.05
	539866	2.2 ± 0.42	4.111 ± 0.39
	421418	3.4 ± 0.51	3.719 ± 0.80
$NaNO_3$	524441	3.0 ± 0.47	3.033 ± 0.36
	398891	1.8 ± 0.42	2.883 ± 0.23
	439212	2.2 ± 0.42	3.194 ± 0.13
	539866	1.9 ± 0.57	4.083 ± 0.66
	421418	2.3 ± 0.48	3.8 ± 0.78
KNO_3	524441	2.8 ± 0.42	2.744 ± 0.35
	398891	2.1 ± 0.31	2.963 ± 0.25
	439212	2.2 ± 0.42	3.35 ± 0.18
	539866	1.7 ± 0.48	3.766 ± 0.28
	421418	2.7 ± 0.48	2.633 ± 0.24

Values are expressed as mean ± standard error (M ± SE)

Table 3 Effect of different media on number and length of shoots in five different accessions of *Plumbago zeylanica* after 8 weeks of inoculation

Experiment detail	Accession number	Number of shoots (M ± SD)	Length of shoots (cm) (M ± SD)
White media	524441	3.2 ± 0.42	3.244 ± 0.108
	398891	2.9 ± 0.56	2.822 ± 0.2
	439212	2.6 ± 0.51	2.486 ± 0.22
	539866	2.0 ± 0.47	3.588 ± 0.73
	421418	3.0 ± 0.47	2.664 ± 1.37
Nitsch media	524441	3.3 ± 0.48	3.688 ± 0.55
	398891	2.5 ± 0.52	3.969 ± 0.5
	439212	2.7 ± 0.57	1.9 ± 0.18
	539866	2.1 ± 0.56	2.572 ± 0.25
	421418	3.2 ± 0.42	2.836 ± 0.14
MS media	524441	6.0 ± 0.47	3.839 ± 0.7
	398891	5.2 ± 0.42	3.341 ± 0.13
	439212	4.9 ± 0.56	2.928 ± 0.26
	539866	3.9 ± 0.56	3.236 ± 0.64
	421418	5.3 ± 0.48	3.694 ± 0.22
B5 media	524441	4.7 ± 0.48	2.922 ± 0.49
	398891	2.7 ± 0.48	3.48 ± 0.2
	439212	1.6 ± 0.51	3.933 ± 1.65
	539866	1.2 ± 0.53	2.966 ± 1.12
	421418	3.2 ± ± 0.42	2.5 ± 0.52

Values are expressed as mean ± standard error (M ± SE)

Phytochemical studies

Methanol was used for the extraction process as it shows a broad range of solubility properties for low molecular and relatively polar substances which include antioxidant active compounds.

In the present study, DPPH free radical scavenging effect of different extracts ranged from 18 to 185 µg/ml and were in the order of IC-524441 > IC-421418 > IC-398891 > IC-439219 > IC-539866 (Table 4). IC-524441 exhibited the highest scavenging activity (185 ± 0.22 µg/ml), while lowest scavenging activity was observed in IC-539866 (18 ± 0.44 µg/ml). This may be due to the presence of high polyphenolic compounds which possess scavenging free radicals, hydroxyl and superoxide radical by single electron transfer (Czapecka et al. 2005).

Total phenolic content of samples ranged from 20 to 70 µg/ml (Table 4). Among all the accessions, IC-524441 showed the highest phenolic content (70 ± 0.54 µg/ml), while the lowest was observed in IC-539866 (20 ± 0.45 µg/ml). Considerable differences between the results of phenolic content may be due to various factors associated with the accessions such as environmental factors, location and temperature. Polyphenols are important dietary antioxidants due to their radical scavenging activity. Various in vitro investigations have reported the antioxidant role of polyphenols which protects against many diseases (Matkowski et al. 2008). These compounds have a vital role in growth and reproduction, and provide protection against harmful predators and pathogens. A study reported that methanolic extract of *P. zeylanica* leaves have 28.25 ± 0.001 µg/ml of phenolic content (Sharma et al. 2014) (Fig. 1).

Table 4 Phytochemical studies in different accessions of *Plumbago zeylanica*

Accession number	Antioxidant activity (µg/ml)	Total phenolic content (µg/ml)	Total flavanoid content (µg/ml)	Total tannin content (µg/ml)
524441	185.18 ± 0.22	70 ± 0.54	100 ± 0.54	120 ± 0.55
421418	120.81 ± 0.45	65 ± 0.66	69 ± 0.5	105 ± 0.71
398891	66.67 ± 0.54	50 ± 0.45	53 ± 0.4	75 ± 0.8
439212	85.18 ± 0.54	35 ± 0.54	49 ± 0.5	69 ± 0.55
539866	18.51 ± 0.44	20 ± 0.45	40 ± 0.66	55 ± 0.8

Fig. 1 In vitro culture of five different accessions of *Plumbago zeylanica* in MS medium with sucrose as carbon source and ammonium nitrate as nitrogen source

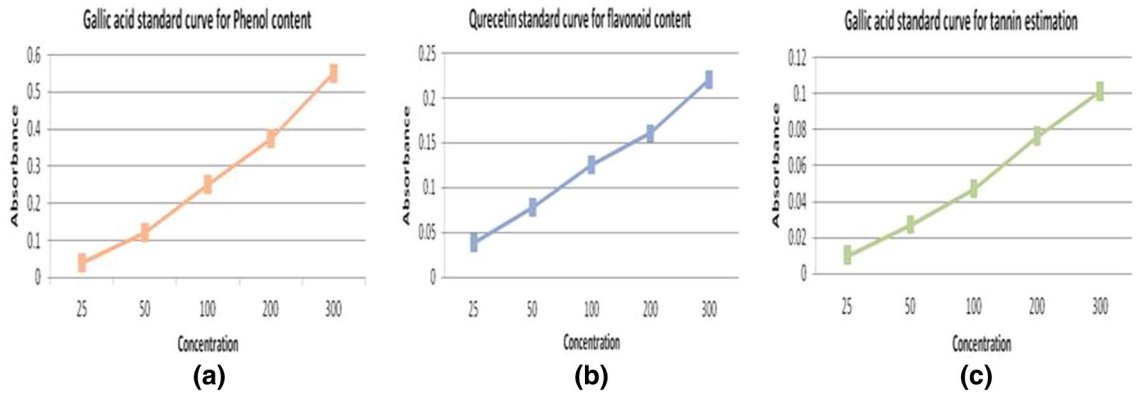

Fig. 2 **a** Standard curve of gallic acid for phenol estimation. **b** Standard curve of qurecetin for flavonoid estimation. **c** Standard curve of gallic acid for tannin estimation

The total flavonoid content varied between 40 and 100 µg/ml (Table 4; Fig. 2b). Among all the accessions, IC-524441 showed the highest amount of flavonoid content (100 ± 0.8 µg/ml), while the lowest content was observed in IC-539866 (40 ± 0.7 µg/ml). Flavonoids include flavanols, flavones and condensed tannins. Antioxidant activity of these compounds depend on the presence of free OH group, especially 3-OH. Flavonoids are polyphenolic compounds which possess in vitro antioxidant activity and act as in vivo antioxidants (Shimoi et al. 1996). Flavonoids (water-soluble compound) are potential scavenging molecules and possess strong anticancer activity (Salah et al. 1995). Flavonoids suppress reactive oxygen formation and increase antioxidant defences (Agati et al. 2012). A study reported the presence of flavonoid content in stem and leaves of *P. zeylanica*, i.e. 72.3 µg/ml and 32.433 µg/ml (Sharma et al. 2014).

Total tannin content was determined using gallic acid as standard; the maximum amount was found in accession number IC-524441, 120 ± 0.55 µg/ml, and the minimum in accession number 539866, 55 ± 0.8 µg/ml. (Table 4; Fig. 2c). Tannins are widespread in nature and probably present in all plants. These are amorphous, astringent substances and water-soluble phenolic compounds (Elgailani and Ishak 2014). Tannins show several biological activities such as protection against oxidative stress and degenerative diseases. Tannins are able to heal wounds and inflamed mucous membranes. Tannins possess high antioxidant, antimicrobial, free radical scavenging and antiulcerogenic activities; moreover in heart mitochondria, they show potent lipid peroxidation inhibition (Shad et al. 2012).

Recently, much consideration has been given to natural phytocompounds and their medical advantages. Plants are major source of antioxidants and are able to produce various secondary metabolites that have therapeutic potential. Medicinal plants contain phenolic compounds such as flavonoids, tannins and phenolic acids. The difference in antioxidant activity among accessions was due to the variation in phenolic, flavonoid and tannin content which may

Table 5 List of phytochemicals identified by GC–MS in ethyl acetate extract of the aerial part of different accessions of *P. zeylanica*

Peak	Retention time	Area percentage	Compound	Nature of compound	Molecular formula
Accession number 398891					
1	13.981	37.817	1,4-Naphthalenedione	Naphthalene-derived organic compound	$C_{10}H_6O_2$
2	16.681	5.281	*o*-Anisic acid	Carboxylic acid	$C_8H_8O_3$
3	17.039	7.612	5-Eicosene	Acyclic alkenes	$C_{20}H_{40}$
4	17.950	6.377	Cyclopropaneoctanal	Aldehyde	C_3H_6
5	18.633	9.002	Phthalic acid	Aromatic dicarboxylic acid	$C_6H_4(COOH)_2$
6	18.769	3.463	Thioctic acid	Saturated fatty acid	$C_8H_{14}O_2S_2$
7	19.381	5.479	2-Benzoyl-1,2-dihydro-1-isoquinolinecarbonitrile	–	–
8	20.396	17.713	Phthalic acid	Aromatic dicarboxylic acid	$C_6H_4(COOH)_2$
9	20.803	7.257	1-Nonadecene	Long-chain hydrocarbon and an alkene	$C_{19}H_{38}$
Accession number 539866					
1	13.974	73.804	1,4-Naphthalenedione	Naphthalene-derived organic compound	$C_{10}H_6O_2$
2	17.038	3.429	1-Octadecene	Long-chain hydrocarbon and an alkene	$C_{18}H_{36}$
3	17.950	3.885	5-*t*-Butyl-cycloheptene	Cycloalkene	$C_{11}H_{20}$
4	18.632	4.656	Phthalic acid	Aromatic dicarboxylic acid	$C_6H_4(COOH)_2$
5	18.769	1.909	Octadecanal	Long chain fatty aldehyde	$C_{18}H_{36}O$
6	19.832	2.004	3-Methoxy-2-methyl-2-(1-phenyl-ethylamino)-propionic acid	Carboxylic acid	–
7	20.396	10.273	Phthalic acid	Aromatic dicarboxylic acid	$C_6H_4(COOH)_2$
Accession number 439212					
1	13.974	39.207	1,4-Naphthalenedione	Naphthalene-derived organic compound	$C_{10}H_6O_2$
2	17.046	3.016	5-Eicosene	Acyclic alkenes	$C_{20}H_{40}$
3	17.955	2.841	Silane	Saturated chemical compounds	SiH_4
4	18.638	5.325	5-(3-Methylbutyl)-2-pyridinecarboxylic acid	Carboxylic acid	–
5	19.386	11.002	2-Benzoyl-1,2-dihydro-1-isoquinolinecarbonitrile	–	–
6	20.402	36.322	Phthalic acid	Aromatic dicarboxylic acid	$C_6H_4(COOH)_2$
7	20.810	2.286	3-Eicosene	Acyclic alkenes	$C_{20}H_{40}$
Accession number 524441					
1	14.010	74.529	1,4-Naphthalenedione	Naphthalene-derived organic compound	$C_{10}H_6O_2$
2	17.048	2.149	3-Eicosene	Acyclic alkenes	$C_{20}H_{40}$
3	17.954	2.180	Phytol	Acyclic diterpene alcohol	$C_{20}H_{40}O$
4	18.638	4.086	Di-sec-butyl phthalate 1,2-Benzenedicarboxylic acid	Esters of phthalic acid, carboxylic acid	$C_{16}H_{22}O_4$, $C_8H_6O_4$
5	19.387	3.504	Bicyclo[3.3.0]octan-3-one, glycine	Ethyl pentyl ketone , amino acid	$C_{10}H_{14}O$, $C_2H_5NO_2$
6	20.400	10.606	Phthalic acid	Aromatic dicarboxylic acid	$C_6H_4(COOH)_2$
7	20.805	2.948	5-Eicosene, 1-nonadecene	Acyclic alkenes, long-chain hydrocarbon and an alkene	$C_{20}H_{40}$, $C_{19}H_{38}$
Accession number 421418					
1	13.952	52.839	1,4-Naphthalenedione	Naphthalene-derived organic compound	$C_{10}H_6O_2$
2	16.681	2.816	*o*-Anisic acid	Carboxylic acid	$C_8H_8O_3$
3	17.043	2.916	Cetene	Oily hydrocarbon	$C_{16}H_{32}$
4	17.945	4.582	Phytol	Acyclic diterpene alcohol	$C_{20}H_{40}O$
5	18.629	7.683	Phthalic acid	Aromatic dicarboxylic acid	$C_6H_4(COOH)_2$
6	18.768	1.563	3,7,11-Trimethyl-2,4-dodecadiene	Alkadienes	$C_{15}H_{28}$
7	19.377	5.703	Bicyclo[3.3.0]octan-3-one	Ethyl pentyl ketone	$C_{10}H_{14}O$
8	20.394	19.247	Phthalic acid	Aromatic dicarboxylic acid	$C_6H_4(COOH)_2$
9	20.800	2.651	1-Eicosene	Acyclic alkenes	$C_{20}H_{40}$

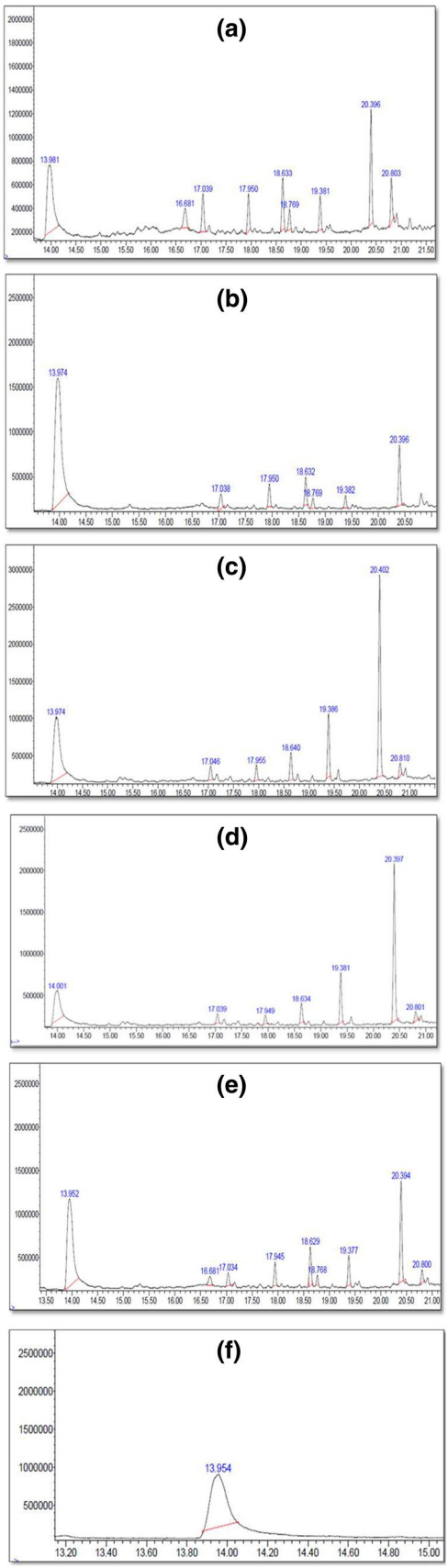

Fig. 3 GC–MS chromatogram of ethyl acetate extracts of *Plumbago zeylanica* accessions: **a** 439212, **b** 524441, **c** 398891, **d** 539866, **e** 421418 and **f** plumbagin standard

be influenced by the carbon and nitrogen source. Accession number IC-524441 showed maximum growth and possesses maximum content of total phenolic, flavonoid, tannin and antioxidant activity when grown in MS media containing sucrose and ammonium nitrate as carbon and nitrogen source. Similar observations were noticed on other four accessions. This may be because sugars induce some signals which affect metabolism, growth, development and gene expression in plants and also affect phytocompound production (Petrova et al. 2015).

GC–MS analysis

GC–MS of different accessions of *P. zeylanica* (Table 5; Fig. 3) revealed the presence of 1,4-naphthalenedione, 3-eicosene, 5-eicosene, phthalic acid, *o*-anisic acid, thioctic acid, 1-octadecene, 5-*t*-butyl-cycloheptene, 2-benzoyl-1,2-dihydro-1-isoquinoli necarbonitrile, octadecanal, silane, 3-methoxy-2-methyl-2-(1-phenyl-ethylamino)-propionic acid and 1-nonadecene. Compound identification was carried out using the NISTII library. In accession number IC-524441, 1,4-naphthalenedione was present in higher amount, whereas in accession number IC-398891, phthalic acid was in higher amount. Rajakrishnan et al. (2017) reported the presence of 1,4-naphthalenedione in roots of *P. zeylanica*. Similarly, Sharma et al. (2015) reported the presence of tetradecanoic acid, 2-pentadecanone, 1-heptadecene, tetracontane, isophytol, etc. in *P. zeylanica*.

Linearity for plumbagin was determined by plotting calibration graph of peak area against the standard concentrations (Fig. 4). Calibration curve shows that plumbagin had a range between 50 and 500 µg/ml. Linear regression equation for plumbagin is $y = 17{,}753x + 148{,}266$, $R^2 = 0.9986$, where x is standard plumbagin concentration and y is peak area. LOQ was 0.101 µg/ml and LOD was 0.033 µg/ml. A linear relationship was obtained for plumbagin concentration (50–500 µg/ml) and the correlation coefficient (r) was 0.99.

Plumbagin estimation Plumbagin concentration in the plant extracts was obtained by comparing samples with standard plumbagin (Sigma-Aldrich) solution. On GC analysis of ethyl acetate extract, chromatogram showed peak at retention time of 13.939–14.001. Results showed that accession numbers IC-524441, IC-421418, IC-398891, IC-439219 and IC-539866 contained 14.19 ± 0.5 µg/ml,

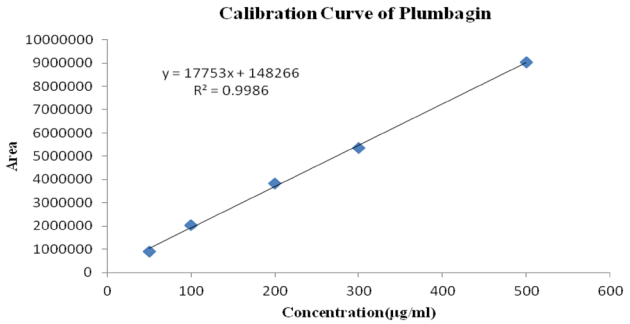

Fig. 4 Calibration curve of plumbagin

10.68 ± 0.5 µg/ml, 7.82 ± 0.5 µg/ml, 6.71 ± 0.5 µg/ml and 5.07 ± 0.5 µg/ml of plumbagin respectively (Fig. 3). The data clearly revealed that accession number IC-524441 contained the highest amount of plumbagin. This is the first report on plumbagin estimation from various in vitro-grown accessions using GC–MS.

Conclusion

Plumbagin is the most important phytocompound of *P. zeylanica*. Plant growth and production of secondary metabolite is affected by interactions between genotype and different environmental conditions. Hence, screening and selection of particular accession for increase in plumbagin production is essential. In the present study, various contributory factors like carbon, nitrogen source and plant tissue culture media were used for the assessment of the best carbon source, nitrogen source and media for higher growth. It was found that all the three parameters used in this study affected plant growth. MS media containing sucrose and ammonium nitrate promote in vitro growth of *P. zeylanica* accessions. It is interesting that total flavonoid content, phenolic content and tannin content correlate well with the results of DPPH test, and findings of this study support the fact that some medicinal plants commonly consumed in India are promising sources of potential antioxidants. The highest amount of phytochemical presence could be due to the influence of sucrose and ammonium nitrate which results in the highest shoot multiplication rate. Gas chromatography of ethyl acetate fraction revealed the presence of 7–9 peaks in five different accessions of *P. zeylanica*. Accession number IC-524441 showed the highest amount of plumbagin content. This is the first study on the effect of various culture parameters on growth of different accessions and plumbagin content. This information can be utilized for more comprehensive studies on influences of various other parameters for the production of important phytocompounds present in *P. zeylanica*.

Author contribution statement AR: designed and performed all the experiments and wrote the manuscript with support from Dr NB. NB: supervised Arpita Roy for conducting the experiments successfully.

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Establishment of root suspension culture of *Plumbago zeylanica* and enhanced production of plumbagin

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ABSTRACT

Plumbagin found in the roots of *Plumbago zeylanica* L. possesses various biological activities like anticancer, antibacterial, anti-inflammatory, etc. In this investigation, roots were initiated from *P. zeylanica* nodal explants, highest root number (24.1 ± 0.73) was achieved in Murashige and Skoog MS media + 1 mg/L indole-3-butyric acid (IBA) whereas maximum root length (3.33 ± 0.53 cm) was observed in MS media + 2 mg/L indole-3-acetic acid (IAA). MS liquid medium containing 1 mg/L IBA was used for establishing root suspension culture. Optimization of culture parameters reveals that half strength liquid MS media + 3% sucrose + 2 g/L inoculum density resulted in highest plumbagin production. Application of different concentrations of abiotic (yeast extract and malt extract) and biotic elicitors (methyl jasmonate and salicylic acid) enhanced plumbagin content significantly. Up to threefold increase in plumbagin concentration was achieved by treating the root suspension culture with 150 mg/L yeast extract. Results indicate that utilization of elicitor can enhance plumbagin production as well as other phytochemicals in *P. zeylanica* root culture.

1. Introduction

Secondary metabolites of medicinal plants are utilized as fragrances, essential oils, feedstock, pigments, and pharmaceuticals. Secondary metabolites are synthesized in small amount and possess various pharmacological actions. In several plants, these metabolites are produced and concentrated in roots. *Plumbago zeylanica* (*Plumbaginaceae*) commonly known as 'Chitrak' is rich in plumbagin and is commonly used for the treatment of diarrhea, piles, anasarca, rheumatism and leprosy (Roy and Bharadvaja, 2017a, 2017b). Plumbagin inhibits cell proliferation by undergoing autophagic cell death and down regulates NF-κB regulated proliferative expression, antiapoptotic and angiogenic gene products, which further cause apoptosis (Nayak et al., 2015). Roots of *Plumbago* species are main source of plumbagin, however due to slow grow of plant, extraction of plumbagin is limited (Roy and Bharadvaja, 2018a,). Pharmaceutical industries heavily depend on materials that are procured from natural condition which leads to rapid depletion, raising concern about probable extinction. In field grown plant, quality and quantity of secondary metabolites is affected by biotic and abiotic factors, which results in low yield of metabolite and also changes medicinal property of active constituent. Therefore, alternative production system that is not affected by environmental conditions must be identified for plumbagin production. To enhance the biomass and biochemical content of plant, *in-vitro* root culture is an efficient strategy which can produce secondary metabolites at higher concentration. Adventitious root culture is an alternative system for enhancement of bioactive substances of pharmaceutical interest without depleting natural plant population.

Adventitious root culture for secondary metabolite production has been reported in many plant species which include *Panax ginseng* (Lu et al., 2001), *Withania somnifera* (Sivanandhan et al., 2012), *Glycyrrhiza uralensis* (Yin et al., 2014), etc. Root culture is an important method for large scale secondary metabolites production of compounds that naturally accumulates in roots of plants. Suspension cultures are suitable for biomass production and can be easily scaled up to provide raw material for pharmaceutical industries (Thiruvengadam et al., 2016). Adventitious roots are produced by direct or indirect organogenesis due to mechanical damage of the tissue (Silja and Satheeshkumar, 2015). These cultures show high proliferation rate and active metabolism. Previous studies have reported the effect of different auxins concentrations on *in-vitro* rooting induction in *P. zeylanica*, however, none of their reports are in light of plumbagin production (Saxena et al., 2000; Sivanesan and Jeong, 2009). Multiplication of roots and plumbagin production is influenced by various parameters which includes plant tissue culture media, hormone concentration, strength of media, elicitors, etc.

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Phytochemical analyses shows the presence of plant constituents, which have been identified to demonstrate therapeutic and physiological activities. Medicinal plants are rich source of compounds such as phenolic acids, tannins, flavonoids, etc which are accountable for their therapeutic properties. Therefore, estimation of these compounds is necessary to evaluate the therapeutic importance of plant. Quantity of phytocompounds depends upon geographical conditions of a plant accession. Therefore, identification of elite accession possesses a significant challenge because of various factors like climate variations, genetic composition of plant, plant's age or harvesting period (Kundu et al., 2016; Bruni and Sacchetti, 2009; Roy et al., 2016). In the previous study conducted by the authors (Roy and Bharadvaja, 2018b), five accessions of *P. zeylanica* were optimized for multiplication of shoots and plumbagin production. Out of five accessions, best accession (IC-524441) based on plumbagin production was chosen for this study. In the present investigation, adventitious roots have been induced from nodal explants and culture condition has been optimized for establishment of root culture and elicitation to increase the production of plumbagin. Further phytochemical analysis was done for yeast extract elicited root culture and non elicited root culture for accessing therapeutic value of this accession.

2. Materials and methods

2.1. Effect of auxins on root induction

For root induction, nodal explants were cultured on MS (Murashige and Skoog) media (3% sucrose, 0.8% agar) supplemented with varying concentration of (0.1, 0.5, 1.0, 1.5 and 2.0 mg/L) IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), and Naphthalene acetic acid (NAA). Incubation of cultures was done at 25 ± 2 °C with 16/8 h photoperiod. Visual observations were recorded and auxin that resulted in maximum root growth was selected for further studies.

2.2. Effect of different culture media on root induction

Four different culture media i.e. MS, Gamborg's B5, Nitsch, and Schenk & Hildebrandt media containing 3% sucrose were utilized for root induction. Nodal explants were aseptically transferred to medias supplemented with 1 mg/L IBA.

2.3. Establishment of root suspension culture

For establishing root suspension culture, direct adventitious root (1–to 2 cm) emerging from nodal explants (21 days old) were inoculated on MS liquid medium containing 1 mg/L IBA and incubated on orbital shaker at 90 rpm without any light. Sub-culturing of roots was done after every three weeks interval onto fresh liquid medium with same concentration of IBA.

2.4. Effect of culture parameters on production of plumbagin

Roots were inoculated in different strength of MS medium (¼, ½, ¾ and 1) containing 1 mg/L IBA. For optimizing the inoculum density 1, 2, 3 and 4 g/L of fresh weight root segments were inoculated onto half strength MS. For optimizing sucrose concentration on plumbagin production, 1–5% of sucrose was used in MS liquid media.

2.5. Effect of elicitor on production of plumbagin

For elicitation, stock solutions of jasmonic acid, salicylic acid, yeast extract and malt extract were prepared. Salicylic acid and jasmonic acid (50, 100, and 150 μM) was prepared in ethanol. Yeast extract and malt extract solution (10 mg/mL) were prepared in distilled water and autoclaved. Fresh roots (2 g/L) from 21 days old culture were inoculated onto fresh ½ liquid MS medium containing 1 mg/L IBA. Elicitors were added aseptically to the individual flasks. Flasks without any elicitor were kept as control. Cultures were incubated on orbital shaker and harvested after 21 days. Water was used for harvesting and fresh weight was recorded. Growth index was measured as

Growth index (GI for fresh roots) = (Final weight – Initial weight)/ Initial weight

2.6. Plumbagin quantification

Plumbagin estimation was done using HPLC as per method given by Silja and Satheeshkumar, 2015 with some modification. C18 column where methanol and water (80:20) were used as mobile phase, flow rate was 1.0 ml/min. UV detector was used with detection wavelength of 272 nm. Calibration curve of plumbagin was plotted using plumbagin standard (Sigma, USA) over concentration range 100–500 μg/mL. Plumbagin peak area showed linearity over selected concentration ranges and linear equation was $y = (-77.3) + 4.471x$ ($r^2 = 0.99$) (Fig. 3).

Sample preparation: Roots (10 mg) were extracted with methanol (1 mL) (72 h maceration) and filtered with 0.22-micrometer syringe filter. All the extracts were subjected to HPLC analysis. Experiments were conducted twice and each treatment had five replicates. Root suspension culture experiment was conducted in triplicates and values regarding biomass and plumbagin content are presented as a mean ± sd.

2.7. Statistical analysis

Data was recorded after eight weeks of incubation in case of solid cultures and four weeks of incubation in case of liquid cultures. Statistical analysis was done using analysis of variance (ANOVA) to compare significance of treatments and Tukey's range test at 5% probability was done by using Statistical Package for the Social Science (SPSS) for Windows.

3. Phytochemical studies

3.1. Antioxidant activity

Antioxidant activity was estimated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method given by Brand-Williams et al. (1995). Methanolic root extracts solution of 50 μg/mL was prepared. Antioxidant activity was calculated as: Antioxidant activity (%) = $[(A_{control} - A_{sample})/ A_{control}] * 100$ Fig. 5.

3.2. Total flavanoid estimation

Total flavonoid content was determined using aluminum chloride ($AlCl_3$) colorimetric method as given by Lamaison and Carnat, 1990 Fig. 6.

3.3. Total phenolic estimation

Total phenolic content was estimated using Folin-Ciocalteu method as given by Singleton and Rossi, 1965 Fig. 7.

3.4. Total tannin estimation

Total tannin content was estimated using Folin-Ciocalteu method as given by Singleton et al., 1999 Fig. 8.

4. Results and discussion

4.1. Effect of auxins on root induction

Auxins are generally used for adventitious root induction from

Table 1
Adventitious root induction from nodal explants of *Plumbago zeylanica* in different concentrations of IBA, IAA and NAA in Murashige and Skoog media after a period of eight weeks. Data represents mean ± standard deviation. Different letters indicate significant differences ($P < 0.05$) within groups using one-way ANOVA.

Auxin	Concentration (mg/L)	Explant cultured	Root induction percentage	Nature of response	Number of roots	Length of root (cm)
Indole-3-butyric acid	0.1	10	60	Root	3.40 ± 0.51^{h}	1.30 ± 0.48^{de}
	0.5	10	70	Root	10.10 ± 0.56^{e}	3.34 ± 0.24^{b}
	1.0	10	90	Root	20.10 ± 0.56^{a}	3.95 ± 0.33^{a}
	1.5	10	80	Root	15.30 ± 0.67^{b}	3.06 ± 0.42^{bc}
	2.0	10	70	Root	10.50 ± 0.70^{de}	2.74 ± 0.35^{c}
Indole-3- acetic acid	0.1	10	50	Root	2.30 ± 0.48^{i}	0.72 ± 0.18^{f}
	0.5	10	80	Root	6.50 ± 0.70^{g}	1.20 ± 0.42^{ef}
	1.0	10	90	Root	9.10 ± 0.73^{f}	1.75 ± 0.24^{d}
	1.5	10	70	Root	11.20 ± 0.63^{d}	1.68 ± 0.36^{de}
	2.0	10	70	Root	13.10 ± 0.73^{c}	4.33 ± 0.45^{a}
Naphthalene acetic acid	0.1	10	0	–	-	-
	0.5	10	0	Callus induction	—	-
	1.0	10	0	Callus induction	–	-
	1.5	10	0	Callus induction	–	-
	2.0	10	0	Callus induction	—	-

several medicinally important plants. Emergence of adventitious roots on nodal explants varied according to the type and concentration of auxins supplemented in the media. Rooting was initiated at the cut end of nodal explants after two weeks of incubation period. Induction of roots was observed in all the media supplemented with IBA and IAA. However, optimal induction of roots was observed on MS media having IBA and IAA (Table 1 and Fig. 1). Root induction and root number significantly varied with varying concentration of IBA and IAA. MS media containing 1 mg/L IBA induced maximum root number i.e. 20.1 ± 0.73 whereas 2 mg/L IAA showed maximum length of root i.e. 4.33 ± 0.53 cm. Callus induction was observed in media containing NAA. Since the aim of experiment was to obtain root line that produces

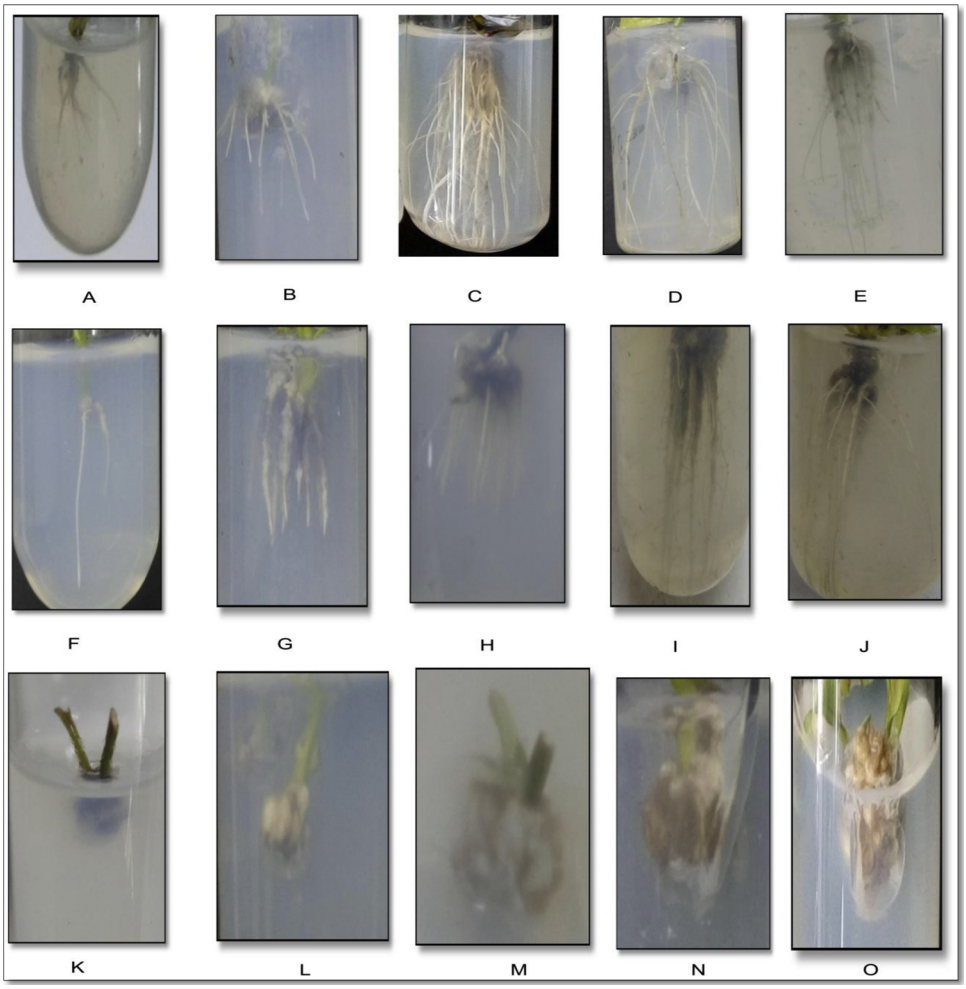

Fig. 1. Root induction from in vitro nodal explants of *P. zeylanica* cultured on modified Murashige and Skoog (MS) media supplemented with different concentrations of plant growth regulators (Indole-3-butyric acid (IBA), Indole-3-butyric acid (IAA) and 1-Naphthalene acetic acid (NAA)). A) 0.1 mg/L IBA B) 0.5 mg/L IBA C) 1.0 mg/L IBA D) 1.5 mg/L IBA E) 2.0 mg/L IBA, F) 0.1 mg/L IAA G) 0.5 mg/L IAA H) 1.0 mg/L IAA, I) 1.5 mg/L IAA J) 2.0 mg/L IAA, K) 0.1 mg/L NAA L) 0.5 mg/L NAA M) 1.0 mg/L NAA, N) 1.5 mg/L NAA and O) 1.5 mg/L NAA.

Table 2
Adventitious root induction from nodal explants of *Plumbago zeylanica* in different media supplemented with indole butyric acid after a period of eight weeks[1] .

Media	Explants	Explant cultured	Root induction percentage	Nature of response	Number of root	Length of root(cm)
Murashige and Skoog + 1 mg/L Indole-3-butyric acid	Node	10	90	Root	22.10 ± 0.67[a]	3.95 ± 0.48[a]
Nitsch + 1 mg/L Indole-3-butyric acid	Node	10	80	Root	18.30 ± 0.78[b]	3.50 ± 0.38[a]
Gamborg's B5 + 1 mg/L Indole-3-butyric acid	Node	10	80	Root	14.80 ± 0.63[c]	2.80 ± 0.46[b]
Schenk & Hildebrandt + 1 mg/L Indole-3-butyric acid	Node	10	60	Root	5.50 ± 0.53[d]	1.80 ± 0.45[c]

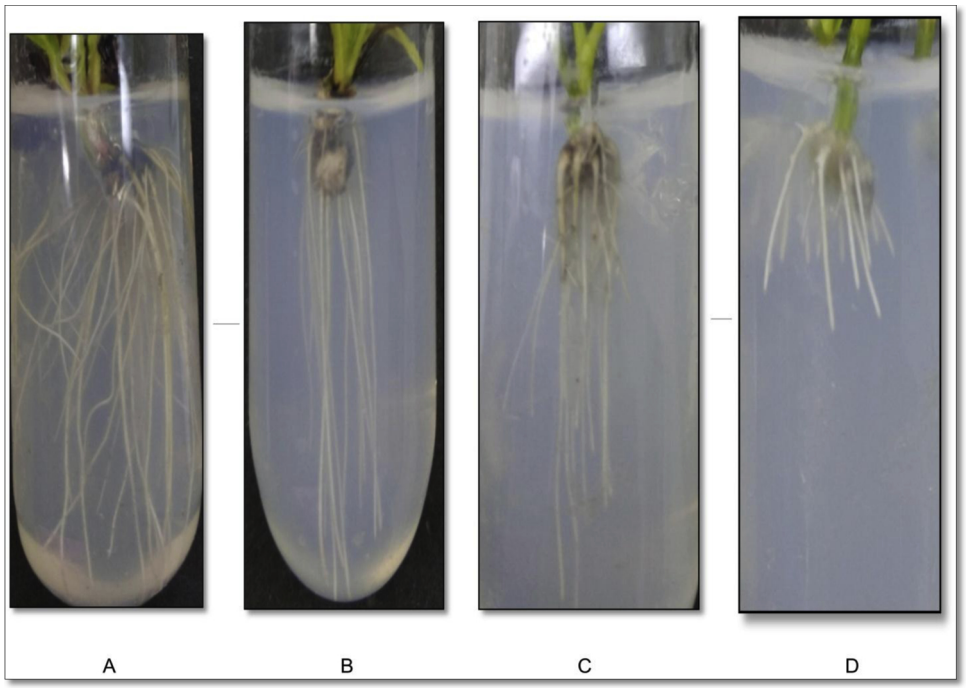

Fig. 2. Root multiplication of *P. zeylanica* in different media A) Murashige and Skoog (MS) + 1 mg/L indole-3-butyric acid B) Nitsch + 1 mg/L indole-3-butyric acid C) Gamborg's B5 + 1 mg/L indole-3-butyric acid, D) Schenk & Hildebrandt + 1 mg/L indole-3-butyric acid.

high biomass and plumbagin therefore roots induced in media containing 1 mg/L IBA was selected for establishment of root suspension culture. Hormones that are given exogenously can trigger differentiation process and induction pathways. Researchers have reported induction of adventitious roots in plants like *Withania somnifera* (Praveen and Murthy, 2010), *Periploca sepium* (2013b), *Prunella vulgaris* (Fazal et al., 2014), *Plumbago rosea* (Jaisi et al., 2013), *Hypericum perforatum* (Gaid et al., 2016) and *Oldenlandia umbellata* (Krishnan and Siril, 2018), however growth regulators for adventitious root induction were different for different species. Gao et al. (2005) reported that 2, 4-D supplemented media resulted in the formation of callus whereas media supplemented with IBA forms adventitious root development in *Panax notoginseng*. Martin et al. (2008) reported adventitious root induction from shoot, leaf, and inter-node, where media containing IBA/NAA alone or BAP/KIN combination was used in *O. prostrata*. Praveen and Murthy (2010) reported that IBA was a potential auxin for adventitious root development in case of *Withania somnifera*. Dohare et al. (2012) reported highest number of root formation in MS media supplemented with 1 mg/L IAA (7.00 ± 0.98) and highest root length in MS media supplemented with 1 mg/L IBA (6.99 ± 0.89 cm) in *P. zeylanica*.

4.2. Effect of different culture media on root induction

Presence of difference components in different media influences growth of root culture. Therefore, assessment of different media for root growth is important. Among tested four medias, maximum root induction (22.1 ± 0.73) and highest root length (3.95 ± 0.45 cm) was obtained in MS media followed by Nitsch, B5 and Schenk & Hildebrandt (Table 2 and Fig. 2). Difference in root growth may be due to presence of various nutrients in the culture media which strongly influences growth. Panichayupakaranant and Tewtrakul (2002) used Gamborg's B5 media supplemented with 1 mg/L NAA + 0.1 mg/L Kinetin for root induction in *Plumbago rosea*. Min et al. (2007) reported that Gamborg's B5 media was optimum for *Scopolia parviflora* adventitious root

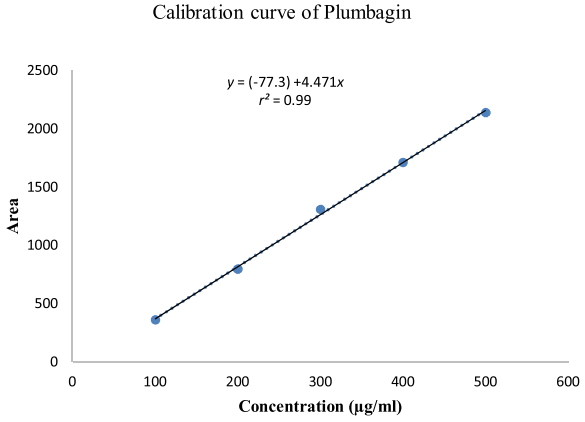

Fig. 3. Calibration curve of Plumbagin standard.

[1] Data represents mean ± standard deviation. Different letters indicate significant differences ($P < 0.05$) within groups using one-way ANOVA.

Table 3
Effect of different culture parameter on biomass and plumbagin production of adventitious root cultures of *Plumbago zeylanica*. Data represents mean ± standard deviation. Different letters indicate significant differences ($P < 0.05$) within groups using one-way ANOVA.

Murashige and Skoog strength	Biomass (GI)	Production (μg/mL)
¼	2.43 ± 0.16 ^{d}	21.96 ± 0.78 ^{b}
½	11.54 ± 0.16 ^{b}	26.65 ± 0.55 ^{a}
¾	14.84 ± 0.21^{a}	23.99 ± 0.48 ^{b}
1	4.15 ± 0.21 ^{c}	22.40 ± 0.45^{c}
Inoculum density (g/L)		
1	11.75 ± 0.15^{a}	26.52 ± 0.55^{ab}
2	7.75 ± 0.23 ^{b}	27.18 ± 0.51 ^{a}
3	3.29 ± 0.10^{c}	25.20 ± 0.48^{bc}
4	2.05 ± 0.10^{d}	23.77 ± 0.47 ^{c}
Sucrose concentration (%)		
1	4.33 ± 0.16^{c}	20.67 ± 0.52^{c}
2	5.65 ± 0.13^{b}	26.36 ± 0.30^{ab}
3	8.22 ± 0.25^{a}	27.17 ± 0.71^{a}
4	5.03 ± 0.15^{b}	26.43 ± 0.54^{ab}
5	3.44 ± 0.12^{d}	19.44 ± 0.45^{c}

cultures. Wu et al. (2008) reported maximum root growth in MS media for Ginseng. Sivanesan and Jeong (2009) reported that MS medium showed highest root growth followed by B5 and Schenk & Hildebrandt media in case of *Plumbago zeylanica*. Praveen and Murthy (2010) tested MS, Nitsch and Nitsch, B5 or Chu media for adventitious root culture of *W. somnifera* and found that MS media was the most efficient medium for root growth.Lee et al. (2011) also used MS, B5 and Schenk & Hildebrandt medium for adventitious root culture of *Aloe vera*. Sujatha and Ranjitha, 2012tested MS, B5 and Schenk & Hildebrandt media for adventitious root culture of *Artemisia vulgaris* and reported that MS media showed maximum root induction. Jaisi et al. (2013) obtained highest root growth in B5 media containing 1 mg/L IBA in case of *Plumbago indica*.

4.3. Effect of MS media strength on biomass and plumbagin production

Optimum concentration of nutrients is an essential factor which controls growth of roots and secondary metabolites production. All the four different strength showed formation of elongated roots. Half strength MS media showed maximum growth and plumbagin production i.e. 11.54 ± 0.16 and 26.65 ± 0.55 μg/mL whereas minimum plumbagin production was recorded in ¼ strength media i.e. 21.96 ± 0.78 μg/mL (Table 3). MS media with lower salt strength increase root growth (Silja and Satheeshkumar, 2015), whereas, higher salt strengths decrease the root growth. This may be due to the fact that low water potential inhibits nutrient absorption from the media (Cui et al., 2010). In case of ¼ MS strength, roots grew and matured quickly, but did not reach sufficient biomass levels, this could be due to a deficiency of nutrients. At ¾ MS highest growth index was obtained i.e. 14.84 ± 0.21. Media optimization increases biomass as well as secondary metabolites production in *E. angustifolia* (Wu et al., 2006), *M. citrifolia* (Baque et al., 2010) and *P. sepium* (2013b). Roots were induced in ½ strength MS media containing IBA in *P. zeylanica* (Verma et al., 2002). Ceasar et al. (2013) reported maximum roots in ½ MS + 1 mg/L IBA whereas highest rooting in ½ MS + 0.5 mg/L NAA was reported by Sivanesan and Jeong (2009) in *P. zeylanica*. Optimum macroelement and microelement concentration is important for plant growth. Full and half strength MS media produce maximum biomass in adventitious root culture of *P. ginseng* whereas higher secondary metabolite production was observed in full strength MS (Yu et al., 2000). In *E. angustifolia* root culture, ¼ strength MS showed only maximum total flavonoids and phenols production whereas ½ strength MS showed better accumulation of biomass along with total flavonoids and phenols production (Wu et al., 2006). Baque et al. (2010) reported that ¼ MS media was beneficial for biomass and secondary metabolite production in *M. citrifolia* root culture. Rajesh et al. (2014) reported maximum biomass and podophyllotoxin production in ½ MS medium in *Podophyllum hexandrum*. Deepthi and Satheeshkumar (2017) reported that ½ strength MS medium was optimum for *Ophiorrhiza mungos* root culture.

4.4. Effect of inoculum density on biomass and plumbagin production

Inoculum density is a crucial factor which influences growth and plumbagin production under *in-vitro* culture condition. Results reveal that lower inoculum density was favored as compared with higher one. Plumbagin content was high in root culture which was inoculated with 2 g/L roots i.e. 27.18 ± 0.51 μg/mL. Since inoculum density of 2 g/L showed maximum plumbagin production, it was utilized for further experiments (Table 3). Optimization of inoculum density is a fundamental parameter for achieving a successful tissue culture (Su and Lei, 1993; Lee and Shuler, 2000). Wu et al. (2008) reported that 5 g/L inoculum density provides maximum biomass accumulation as well as ginsenoside production in roots of ginseng. Reis et al. (2011) reported that fresh weight of 0.2 g/flask inoculum size was optimum for *Stevia rebaudiana* adventitious root cultures whereas fresh weight of 0.15 g/flask was optimum for secondary metabolite and biomass production in *H. perforatum* (Cui et al., 2011). Silja and Satheeshkumar (2015) reported that 2 g/L inoculum density showed maximum plumbagin production in case of *Plumbago rosea* root culture. Deepthi and Satheeshkumar (2017) reported that 2 g/L root inoculum was optimum for *Ophiorrhiza mungos* adventitious root cultures.

4.5. Effect of sucrose concentrationon biomass and plumbagin production

Carbohydrates concentration in culture medium affects development and growth of *in-vitro* cultures. Sucrose concentration is one of the important factors which affect root growth and secondary metabolite significantly. Inoculation of roots onto medium containing different sucrose concentrations showed significant change in biomass and plumbagin production. Among them, 3% sucrose showed maximum root growth as well as plumbagin production. Lower concentrations of sucrose (1–3%) were found to be better for proliferation and elongation of roots. Plumbagin production in 3% sucrose (27.17 ± 0.71) was highest as compared with other concentrations (Table 3). When sucrose concentration was increased or decreased from 3% biomass and plumbagin production decreased. Sucrose is most commonly utilized carbon and energy source which affects medium's osmotic pressure. Therefore, change in sucrose concentration leads to osmotic imbalance which directly regulates secondary metabolism in organ cultures. 2018b) reported advantage of sucrose over fructose and glucose in shoot culture of *P. zeylanica*. Optimum concentration of sucrose for root induction varies with different plant species. Gubis et al. (2005) reported that medium containing 3% sucrose resulted in better plantlets in case of *Lycopersicon esculentum*. Lee et al. (2006) reported that 3% sucrose showed highest dry root biomass in case of *Gymnema sylvestre* root culture. Cui et al. (2010) reported that ½ MS containing 3% sucrose was optimum for accumulation of biomass in case of adventitious root cultures of *Hypericium perforatum*., 2013b)Yin et al., (2013a) reported that 4% sucrose showed maximum biomass and saponin production in *Pseudostellaria heterophylla*. Reports have also shown that 5% sucrose concentration increase the adventitious root biomass in case of *Gynura procumbens* (Kusuma et al., 2017) and *Ophiorrhiza mungos* (Deepthi and Satheeshkumar, 2017).

4.6. Effect of elicitors on biomass and plumbagin production

Elicitors are substances which trigger stress responses when used in small amount. It induces or improves production of specific compounds which helps plant in adapting to stressful situation (Radman et al.,

Table 4
Effect of elicitors on biomass and plumbagin production of adventitious root cultures of *Plumbago zeylanica* [2] .

	Elicitor		Biomass (GI)	Production(μg/mL)
Control	-	-	8.22 ± 0.25^{b}	27.39 ± 0.41^{f}
Biotic	Yeast Extract	50 mg/L	5.10 ± 0.2^{e}	51.30 ± 0.60^{c}
		100 mg/L	7.64 ± 0.15^{c}	63.47 ± 0.36^{b}
		150 mg/L	10.12 ± 0.35 ^{a}	90.96 ± 0.51^{a}
	Malt Extract	50 mg/L	5.08 ± 0.11 ^{e}	24.01 ± 0.43^{h}
		100 mg/L	3.33 ± 0.15^{g}	23.54 ± 0.50^{h}
		150 mg/L	2.17 ± 0.15^{h}	19.45 ± 0.27^{j}
Abiotic	Methyl Jasmonate	50 uM	5.67 ± 0.15 ^{d}	31.89 ± 0.23^{e}
		100 uM	7.9 ± 0.18^{bc}	25.80 ± 0.18^{g}
		150 uM	3.08 ± 0.13^{g}	20.87 ± 0.37^{i}
	Salicylic acid	50 uM	4.45 ± 0.13^{f}	21.43 ± 0.21^{i}
		100 uM	5.42 ± 0.10 ^{de}	32.36 ± 0.22^{e}
		150 uM	4.88 ± 0.16^{ef}	41.08 ± 0.66^{d}

2003). Elicitors induce different defence responses in plants, leading to production of reactive oxygen species and phytoalexins. Their application in root suspension culture induces defence response which leads to enhanced production of secondary metabolite. Methyl jasmonate results in stress induce production of secondary metabolite. Salicylic acid works by generating phenolic free radical which inhibits ascorbate and catalase peroxidase in the cells. Elicitation using yeast extract in *Plumbago indica* has also been reported by different investigators (Komaraiah et al., 2003; Jaisi and Panichayupakaranant, 2016) but exact mechanism has not been explained yet. The type of elicitor (biotic and abiotic) and their concentration affects growth and plumbagin production in *P. zeylanica* root culture. Elicitors were applied in the medium prior to root inoculation and roots were harvested after twenty eight day of incubation period. Yeast extract and methyl jasmonate treated cultures showed significant biomass accumulation as well as plumbagin production. Production of plumbagin might stimulated from the early defense mechanism which caused by the various elicitors. Treatments with yeast extract (50, 100 and 150 mg/L), methyl jasmonate (50 μM), and salicylic acid (100 and 150 μM) significantly enhanced plumbagin production compared with untreated root cultures. Maximum biomass (10.12 ± 0.35 g/L) was obtained in cultures treated with 150 mg/L yeast extract and maximum amount of plumbagin i.e. 90.96 ± 0.51 μg/mL was achieved in the same condition which was approximately three times more as compared with control culture i.e. 27.39 ± 0.41 (Table 4 and Fig. 4). The tested elicitors showed no negative effect on the growth of root culture except malt extract that distinctly reduced root biomass by 60% compared with control cultures. Studies show that elicitor concentration is a critical parameter in elicitation process, as its higher amount may induce hypersensitive response leading to cell death (Namedo, 2007). Elicitation for increased production of secondary metabolite in root suspension culture were reported in various plants which include *Valeriana amurensis* (Cui et al., 2012), Fagonia indica (Khan et al., 2017) and *Panax ginseng* (Le et al., 2018). Elicitation using yeast extract (0.5–3.0 g/L) increased saponin production in *P. ginseng* (Lu et al., 2001). Komaraiah et al. (2002) reported that 1.5% yeast extract enhance plumbagin production upto 2.4-fold in *P. rosea* cell suspension culture. Bhambhani et al. (2012) reported that yeast extract (50 and 100 mg/L) increase 0.336% vasicine production and 2-fold biomass in *Adhatoda vasica*. Silja and Satheeshkumar (2015) reported that 50μM jasmonic acid treatment enhanced the plumbagin content up to 1.23% DW in adventitious root suspension culture of *Plumbago rosea*.

[2] Data represents mean ± standard deviation. Different letters indicate significant differences ($P < 0.05$) within groups using one-way ANOVA. GI is Growth index and measure by (Final weight – Initial weight)/Initial weight.

4.7. Phytochemical studies

Phytochemicals are bioactive compounds found in plants that are formed during normal metabolic process. Studies of these compounds reveal the presence of plant constituents which have therapeutic applications. Therefore, estimation of these compounds is required for pharmaceutical applications of the plant. Concentration of phytocompounds varies from plants to plants and accessions to accessions. It can also be enhanced by elicitation of *in-vitro* cultures. In the present study antioxidant activity, total phenol, flavonoid and tannin content of elicited and non elicited root culture of *P. zeylanica* was estimated in order to access the therapeutic value of this plant. Antioxidants are reactive oxygen species scavengers, therefore, estimation of these compounds is important. DPPH is one of the simple, cost effective, and rapid method used to estimate antioxidant activity of any plant extracts (Kim et al., 2007). DPPH scavenging activity of methanolic extracts of elicitor treated and non treated adventitious roots culture was evaluated. It was found that yeast extract elicited root culture possess more antioxidant activity i.e. 188.2 ± 0.83 μg/mL than non-elicited culture i.e. 65.8 ± 0.83 μg/mL. This could be due to enhanced accumulation of flavonoids and phenolics, as they are potential scavengers of free radicals. Tilak et al. (2004) reported that boiled ethanolic extract of *P. zeylanica* root showed 341 ± 18 μmol/L of DPPH activity., Roy and Bharadvaja 2018b) reported that methanolic extract of *P. zeylanica* shoot possess 185 ± 0.22 μg/mL of DPPH activity. Total phenolic, flavonoid and tannin content was also higher in case of yeast extract elicitor treated root cultures i.e. 100.8 ± 0.83 μg/mL, 99 ± 0.70 μg/mL and 120.6 ± 0.54 μg/mL than non elicitor treated root culture i.e. 75 ± 0.70 μg/mL, 49.8 ± 0.84 μg/mL, 68.4 ± 0.54 μg/mL. This may be because of enhanced production of total polyphenolic compounds in the elicitor treated root culture. Plant phenolics possess various pharmaceutical activities such as anti-mutagenic, anti-carcinogenic, anticancer, cardiovascular, antibacterial and antifungal activity (Matkowski et al., 2008). Flavonoids are most important natural phenolics and they are highly diverse and widespread group of compounds which possess various pharmaceutical activities (Salah et al., 1995). Tannins are extensively present in the nature and possess antioxidant, free radical scavenging, antimicrobial, and antiulcerogenic activities (Shad et al., 2012). A study reported phenolic (28.25 ± 0.001 μg/mL) and flavonoids (32.433 μg/mL) content in methanolic extract of leaves of *P. zeylanica* (Sharma et al., 2014). Similarliy, Tilak et al. (2004) reported that ethanolic extract of *P. zeylanica* root showed 112 ± 5.9 μmol/L of flavonoid content and aqueous extract showed 523 ± 3.5 μmol/L of phenolic content. Antioxidant activity and presence of various phytocompounds in root culture may be responsible for therapeutic role of *Plumbago zeylanica* which can be further enhanced by elicitation.

5. Conclusion

P. zeylanica is utilized for treatment of various diseases and contains a pharmaceutically important secondary metabolite i.e. plumbagin. Various culture parameters influence plant growth and plumbagin production. Root suspension culture is an effective system for plumbagin production. In this investigation, effect of varying concentration of auxins and plant culture media on root induction of *Plumbago zeylanica* was studied. Root growth depends on the varying concentration of auxin and media, media supporting highest root growth was used for root suspension culture establishment. It was found that in vitro culture conditions greatly influence the root growth and plumbagin production in adventitious root culture of *P. zeylanica*. Optimization of various culture parameters leads to increased production of biomass as well as plumbagin in shake flask cultures. ½ MS liquid medium + 1 mg/L IBA + 3% sucrose + 2 g/L inoculum density was optimum for plumbagin production. Further production of plumbagin increased up to three times in yeast extract treated culture. This is believe to be the first

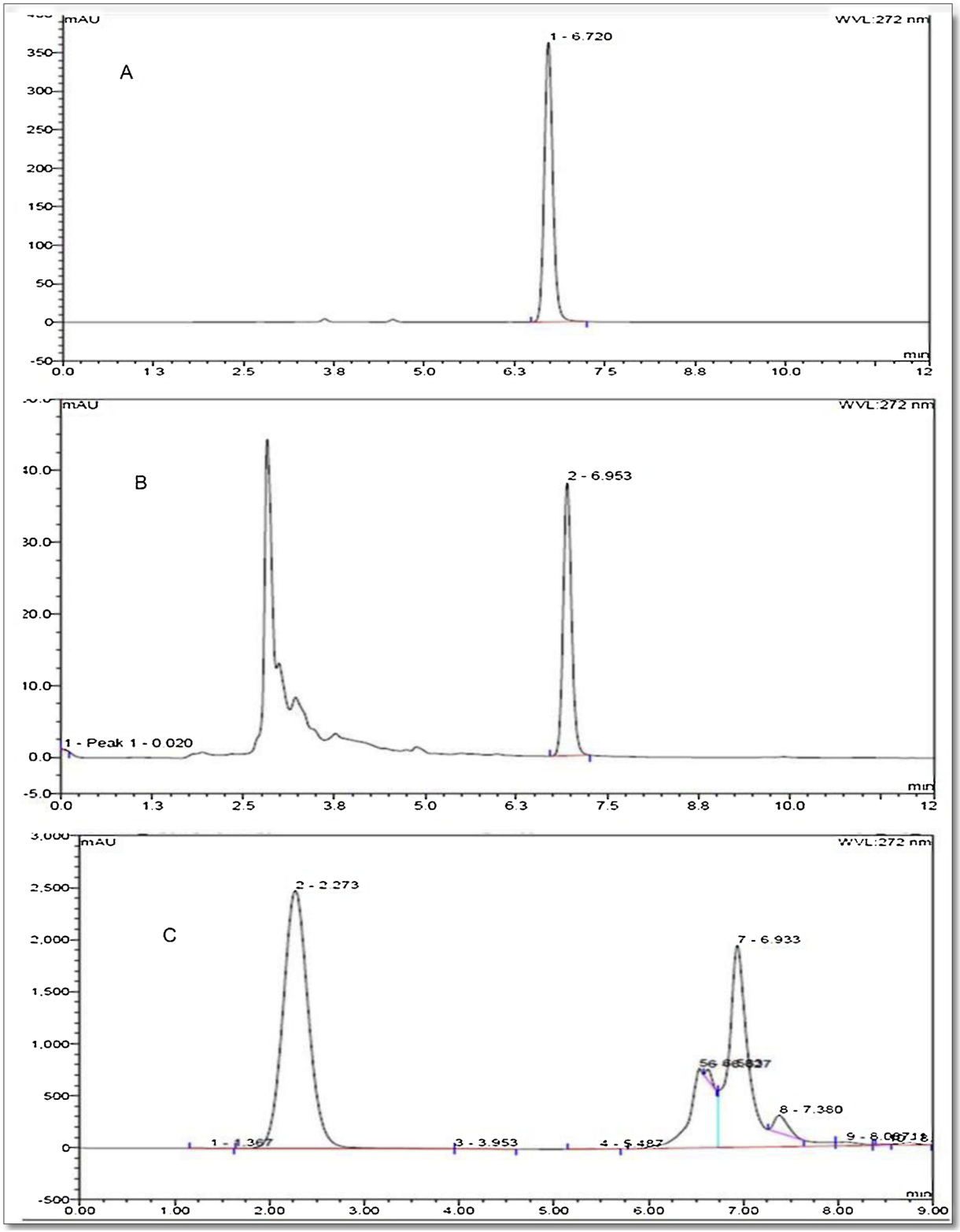

Fig. 4. HPLC chromatogram of a) Plumbagin standard, b) Control culture (Murashige and Skoog liquid medium + 1 mg/L indole butyric acid + 3% sucrose + 2 g/L inoculum density) and c) yeast extract treated culture (Murashige and Skoog liquid medium + 1 mg/L indole butyric acid + 3% sucrose + 2 g/L inoculum density + 150 mg/L yeast extract).

Fig. 5. Protocol for DPPH method.

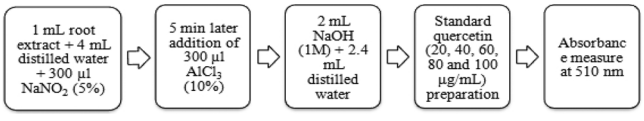

Fig. 6. Protocol for total flavanoid estimation.

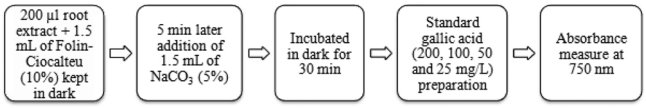

Fig. 7. Protocol for total phenolic estimation.

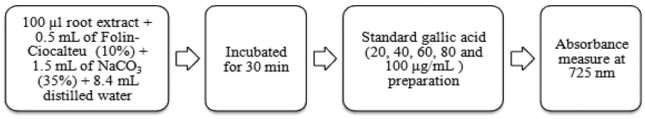

Fig. 8. Protocol for total phenolic estimation.

report on culture parameter optimization for root suspension culture of *P. zeylanica* and enhanced plumbagin production. Since adventitious root culture is an effective way of plumbagin production, it can be utilize for large-scale production.

Conflict of interest

Authors have no conflict of interest.

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Silver nanoparticle synthesis from *Plumbago zeylanica* and its dye degradation activity

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Silver nanoparticle synthesis using plants has great potential as it is cost-effective, environment-friendly, reproducible and energy saving compared to chemical or physical methods. The present investigation deals with influence of various conditions on silver nanoparticle synthesis from in vitro-grown *Plumbago zeylanica*. Synthesized nanoparticles were characterized using ultraviolet–visible (UV–Vis) spectroscopy, scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy, transmission electron microscopy (TEM) and X-ray diffraction (XRD). UV–Vis spectroscopy confirmed the formation of nanoparticles, FT-IR spectroscopy provided information about functional groups attached to the nanoparticles' surface; XRD analysis illustrated their crystalline structure. The shape (spherical) and size (55 nm) of synthesized nanoparticles was confirmed by using SEM and TEM. The antibacterial activity of silver nanoparticles was evaluated against five bacteria using the disk diffusion method, and a positive result was obtained against four bacteria. The catalytic and photocatalytic degradation efficiency of silver nanoparticles was also evaluated, and it was found that the catalytic efficiency of silver nanoparticles in the case of phenol red, methylene blue and methyl red is higher than in the case of eosin Y, whose photocatalytic degradation showed higher efficiency. This indicates that synthesized silver nanoparticles are capable of degrading organic dyes. This approach can be utilized for degradation of organic dyes at a large scale.

1. Introduction

Nanotechnology deals with synthesis and design of nanomaterials, which range from 1 to 100 nm. Nanoparticles have extraordinary properties compared to larger particles because of their particular qualities such as shape and size distribution. Nanoparticles exhibit a higher surface-to-volume ratio with diminishing size. Nowadays the most frequently examined nanoparticles are those that are produced using noble metals – for example, silver (Ag), platinum (Pt), gold (Au) and palladium (Pd). Among these nanoparticles, silver plays a huge role in biological systems, living forms and pharmaceuticals.[1] Mostly, nanomaterials are synthesized utilizing chemical, physical and biological techniques. The physical strategy has the problem of low yield, and the synthetic (chemical) strategy utilizes different chemical agents that are lethal in nature and the procedure is additionally expensive.[2] Salunke *et al.*[3] reported that synthetically synthesized silver nanoparticles (AgNPs) possessed low antibacterial activity compared to nanoparticles that are biologically synthesized. Thus, eco-friendly synthesis techniques are required to address the issues related to physical and chemical syntheses. The biological method of nanoparticle synthesis using microorganisms,[4] enzymes and plant extract provides various advantages over other methods, which include simplicity, utilization of green solvents and non-toxic materials, cost-effectiveness and biocompatibility.[5] *Plumbago zeylanica*, commonly known as 'chitrak', belongs to the Plumbaginaceae family. It is found in Uttar Pradesh, West Bengal and Maharashtra.[6,7] Usage of plant material for synthesis of nanoparticles can be worthwhile over other natural procedures because of the ease of maintaining plant cultures.[8] Different studies on AgNP synthesis utilizing plants are found in the literature.[9–11] Bankar *et al.*[12] utilized banana peel for AgNP synthesis. Earlier studies suggest that numerous compounds present in *P. zeylanica* possess a vital role as reducing and capping agents in the reaction.[11] The environment-friendly technique of utilizing in vitro plant cultures for AgNP synthesis has an advantage due to the ease of growing them in a culture medium and it is independent of geographical and seasonal variations. It provides a continuous supply of plant material of uniform quality that can be used for nanoparticle synthesis.

Organic dyes are commonly utilized in various industries, including the paper, food, plastic, textile, cosmetic and pharmaceutical industries.[13] Discharge of these dyes from industries causes environmental pollution. Various studies have shown that dyes are mutagenic, carcinogenic and harmful to the surroundings.[14] Therefore, there is a requirement of an effective method for eliminating these contaminants from the environment. The process of metal nanoparticle reduction in the presence of sodium borohydrate is a new and rapid technique for removing pollutants from water.[15] *Edison and Sethuraman*[16] reported synthesis of AgNPs by utilizing the fruit extract of *Terminalia chebula* and evaluated their catalytic efficiency on methylene blue reduction. Until now, there have been no such studies on synthesis of AgNPs from in vitro-grown *P. zeylanica* and their role in catalytic and photocatalytic degradation. In the present study, the influence of various parameters (temperature, time interval, silver nitrate ($AgNO_3$) concentration and aqueous extract concentration) on AgNP synthesis was investigated using in vitro-grown *P. zeylanica* aerial extract. The antibacterial activity of nanoparticles against *Escherichia coli*, *Alcaligenes faecalis*, *Serratia marcescens*, *Staphylococcus aureus* and *Swenella putrefaciens* and their dye degradation potential against five dyes

(eosin yellow, methylene blue, methyl orange, methyl red and phenol red) were assessed.

2. Materials and methods

Nutrient agar (NA), nutrient broth (NB) and kanamycin were bought from HiMedia (Mumbai, India). Silver nitrate, methyl red, methyl orange, methylene blue, eosin yellow and phenol red were bought from Fisher Scientific. Distilled water was used throughout the experiment.

2.1 Aqueous extract preparation

P. zeylanica (aerial part) plant material grown in in vitro condition[17] was air-dried for a week. The air-dried plant material (500 mg) was boiled in 50 ml of distilled water for 10 min. Then, the extract was left at room temperature to cool down and was filtered and stored at 4°C.

2.2 Optimization of synthesis of AgNPs and their characterization

Silver nitrate solution (1 mM) was prepared by weighing 33·974 mg of silver nitrate in 200 ml of distilled water. Aqueous extract (1 ml) was added to 9 ml of silver nitrate (1:10). Nanoparticle synthesis was performed at various temperatures (25, 30, 50 and 70°C), time intervals (0–96 h), silver nitrate concentrations (1–5 mM) and plant extract concentrations (1–5 ml). The bioreduction of Ag^{+} to Ag^{0} (color changes from colorless to yellowish brown) was observed and detected by using an ultraviolet–visible (UV–Vis) spectrophotometer (PerkinElmer). The wavelength range for detection of AgNPs was 300–700 nm, and the presence of reduced silver ions (Ag^{+}) was indicated by an absorption peak in the range 350–500 nm. Purification of nanoparticle solution was done by centrifugation at 4500 revolutions per minute for 15 min. This process was repeated for three to four times to make sure that any adsorbed substances on surface of nanoparticles were removed. Possible functional groups that were responsible for silver ion reduction and capping of biologically reduced nanoparticles were determined using Fourier transform infrared (FT-IR) spectroscopy (Thermo Fisher). FT-IR spectra of nanoparticles and plant aqueous extract was analysed. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to determine the surface morphology and size of the nanoparticles. To investigate the purity and phase formation, X-ray diffraction (XRD) was done.

2.3 Antibacterial activity

P. zeylanica-synthesized AgNPs were tested against five different bacterial cultures, which include *E. coli*, *S. putrefaciens*, *S. aureus*, *S. marcescens* and *A. faecalis*. The pH of NA and NB was adjusted to 7 before autoclaving. Each bacterial culture was grown in 20 ml NB and incubated at 37°C overnight and further used for experiments. Antibacterial activity was performed using the disk diffusion method. One hundred microliters of each culture was spread uniformly with the help of a glass spreader on agar plates. One hundred microliters of different concentrations of AgNP solution was loaded onto a disk using micropipettes, and plates were incubated overnight at 37°C. After incubation, the mean diameter of the zone of inhibition around the disk was recorded.

2.4 Degradation of different dyes

The photocatalytic activity of synthesized AgNPs in dye degradation was performed using different dyes, namely eosin yellow, methyl orange, methylene blue, methyl red and phenol red. One milliliter of the respective dye (1 mM) was mixed with 1 ml of synthesized AgNPs, and the volume made up to 5 ml using distilled water. All the solutions were kept in sunlight. For catalytic degradation, 1 ml of the respective dye (1 mM) was mixed with 1 ml of 0·1 M sodium borohydride ($NaBH_4$). Then, 1 ml of synthesized AgNPs was added and the volume made up to 5 ml using distilled water. The change in color intensity was measured in the range 300–700 nm using a UV–Vis spectrophotometer.

3. Results and discussion

3.1 UV–Vis analysis

UV–Vis analysis was performed to determine AgNP formation. It is the most efficient method for detecting nanoparticle formation. Conversion of silver ions from silver nitrate to reduced nanosilver was observed as a change in color. An AgNP peak was found roughly at 446 nm, which corresponds to plasmon excitation of AgNPs. Widening of the peak demonstrates the polydispersed nature of particles. The absorbance peak at 446 nm provides suitable spectroscopic information for AgNP synthesis.[18,19] Logeswari *et al.*[20] reported AgNP synthesis from *Ocimum tenuiflorum*, *Syzygium cumini*, *Solanum trilobatum*, *Centella asiatica* and *Clonorchis sinensis* extract and characterized the synthesized nanoparticles by UV–Vis spectroscopy and found maximum absorbance at 420 nm. Salunke *et al.*[3] reported maximum absorbance at 440 nm in *P. zeylanica*-synthesized nanoparticles. Ponarulselvam *et al.*[21] reported maximum absorbance at 400 nm in the case of *Catharanthus roseus*-synthesized AgNPs.

3.2 Effect of different temperatures

Four different temperatures, namely 25, 30, 50 and 70°C, were used to determine the AgNP formation. All of the temperatures resulted in a change in the color of solution from light yellow to brown. The surface plasmon resonance (SPR) band of *P. zeylanica*-synthesized AgNPs was found at 446, 425, 431 and 423 nm for temperatures of 25, 30, 50 and 70°C, respectively (Figure 1). The outcome demonstrates that, at lower temperatures, the SPR peak shows a bathochromic shift compared to that at the higher temperature (i.e. 70°C). The bathochromic shift demonstrates that the mean diameter of AgNPs diminished.[18] Maximum AgNP synthesis was obtained at 70°C. Nayak *et al.*[11] obtained maximum AgNP synthesis at 80°C. Zhang *et al.*[22] reported AgNP synthesis from aloe (leaf) at room temperature. Oluwaniyi *et al.*[19] also reported that a lower temperature (i.e. 30°C) provides rapid AgNP synthesis in the case of *Thevetia peruviana*.

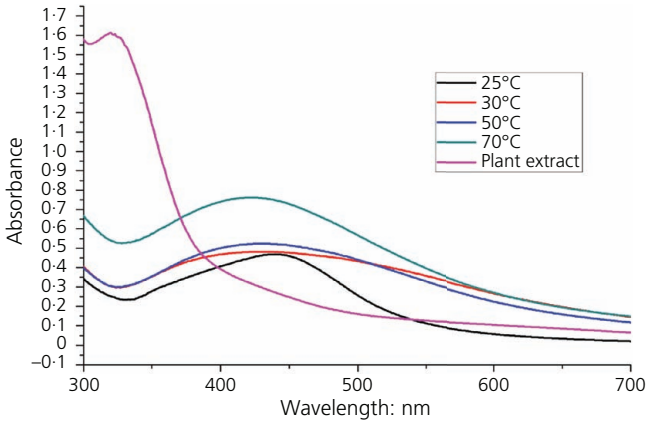

Figure 1. UV–Vis spectra of synthesized AgNPs at different temperatures

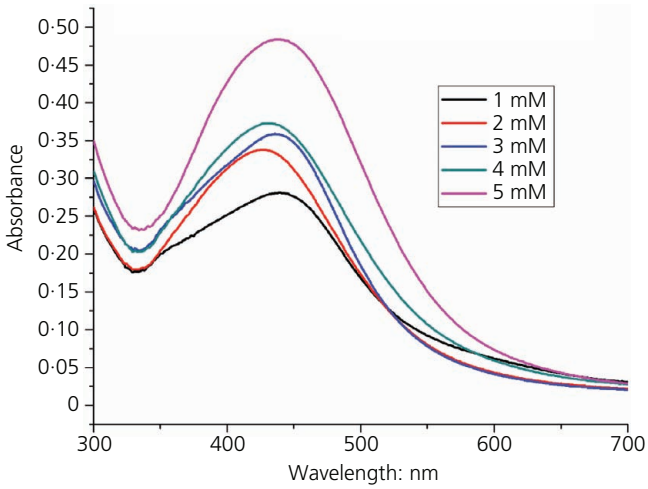

Figure 3. UV–Vis spectra of synthesized AgNPs at different silver nitrate concentrations

3.3 Effect of different time intervals

To estimate the effect of time interval on nanoparticle synthesis, formation of nanoparticles was observed from 24 to 96 h. The SPR band of nanoparticles was found at 432, 435, 434 and 437 nm for 24, 48, 72 and 96 h time intervals, respectively (Figure 2). De Jesús Ruíz-Baltazar *et al.*[23] reported maximum synthesis after a 72 h incubation period in the case of *Melissa officinalis*, which was comparable with the authors' result. Shankar *et al.*[24] reported AgNP synthesis with 1 mM silver nitrate and found 90% metal ion reduction within 4 h. The variation in bioreduction rates may be because of the difference in enzymatic activity present in the *P. zeylanica* extract.

3.4 Effect of various concentrations of silver nitrate

Absorption spectra obtained at various concentrations of silver nitrate were measured (Figure 3). Silver ion intensity increases with increase in silver nitrate concentration; the SPR peak was at 460 nm for all concentrations. The synthesis rate was observed to depend significantly on the silver nitrate concentration, which agrees well with previous reports.[25,26] At higher concentrations, the particle size increases, which increase the spectrum intensity.[18] Oluwaniyi *et al.*[19] also obtained maximum nanoparticle synthesis at 5 mM concentration of silver nitrate in the case of *T. peruviana*. Salunke *et al.*[3] reported maximum nanoparticle synthesis at 0·7 mM concentration in the case of *P. zeylanica*. Ramesh *et al.*[27] reported AgNP synthesis from *Emblica officinalis* using 1 mM concentration of silver nitrate.

3.5 Effect of various plant extract concentrations

The effect of various concentrations of plant extract (1–5 ml) on AgNP synthesis was investigated (Figure 4). UV–Vis spectra

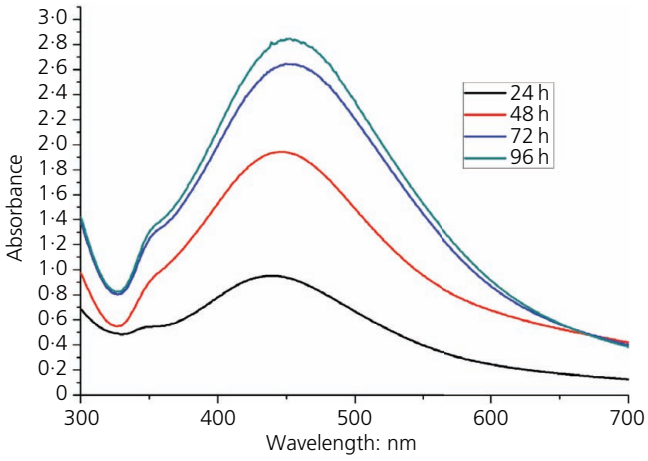

Figure 2. UV–Vis spectra of synthesized AgNPs at different time intervals

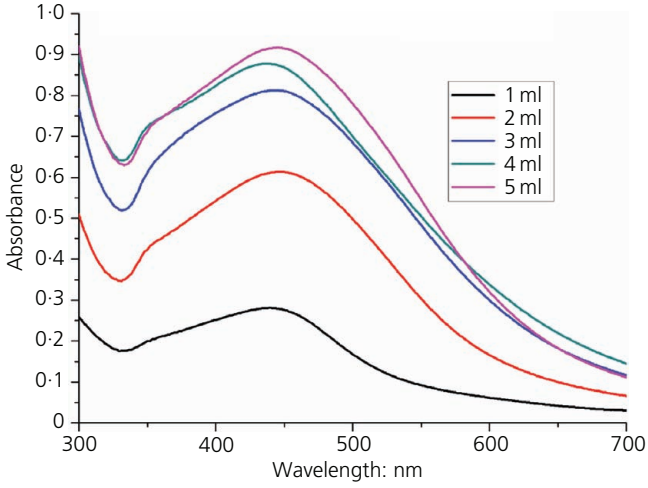

Figure 4. UV–Vis spectra of synthesized AgNPs at different concentrations of plant extract

showed that 5 ml of *P. zeylanica* aqueous extract solution gives maximum formation of nanoparticles with absorbance at 441 nm. Salunke *et al.*[3] also utilized 5 ml of *P. zeylanica* extract for AgNP synthesis. Ramesh *et al.*[27] reported that the optimum concentration of plant extract for AgNP synthesis was 10 ml in the case of *E. officinalis* and there was a minor increase in the SPR band intensity from 5 to 10 ml.

3.6 FT-IR analysis

FT-IR spectroscopy was done to determine the presence of biologically active molecules that specifically bind onto the surface of AgNPs and the local molecular environment of the capping agent. The plant extract spectrum (Figure 5) shows six major peaks, which are at 1212·4, 1368·6, 1633·7, 1739·9, 2341·4 and 3355·0 cm^{-1}. The AgNP spectrum (Figure 5) shows five peaks at 1214·0, 1370·4, 1642·1, 1739·9 and 3341·1 cm^{-1}. The peak at 1633·7 cm^{-1} for the plant extract was due to the amide I vibration presence, and this was shifted to 1642·1 cm^{-1} for AgNPs because of the proteins that possibly bind to AgNPs through amine groups. The peaks at 1212·4, 1368·6 and 2341·4 cm^{-1} show the presence of C–O–C, O–H and C–O groups, respectively. The peak at 1740·2 cm^{-1} signifies the symmetric stretch of C=O, which indicates the presence of aldehydes, ketones, esters or carboxylic acids.[3] The peak at 3355·0 cm^{-1} shows the N–H bond (presence of primary and secondary amines). The presence of similar peaks for the plant extract as well as for AgNPs indicates the presence of biomolecule attachment in the extract to nanoparticles. From the results, it is clear that different functional groups play an important role in the synthesis of AgNPs.[28] The exact mechanism for AgNP synthesis is still not clear. A report suggested that the presence of flavonoids in leaf extract of *Tephrosia purpurea* may perform as a reducing agent and the attachment of the carboxylate group on the nanoparticles' surface helps in stabilization during the synthesis.[29]

3.7 SEM and TEM analyses

The SEM image visualizes the surface morphology of AgNPs. It showed that crystalline AgNPs were uniform and relatively spherical in shape (Figure 6). Aggregation of nanoparticles was also observed, which might be induced by solvent evaporation during preparation of the sample. These could impact on particle size variation.[25] TEM analysis provides the size as well as morphology of biosynthesized AgNPs. TEM images of AgNPs at two different magnifications were employed (Figure 6). The TEM images clearly reveal that the synthesized nanoparticles are spherical in shape. The size of the nanoparticles was in the 50–110 nm range (average size 55 nm). The size of synthesized AgNPs was similar to those from earlier reports on *Aloe vera*[30] and *Crocus sativus*.[31] Nayak *et al.*[11] reported that the sizes of *Ficus benghalensis*- and *Azadirachta indica*-synthesized AgNPs were 85·95 and 90·13 nm. Salunke *et al.*[3] reported that *P. zeylanica*-synthesized AgNPs were 60 nm in size. Ramesh *et al.*[27] reported that the size of AgNPs synthesized from *E. officinalis* was 10–70 nm and these were spherical in shape.

3.8 XRD analysis

The XRD pattern of AgNPs (Figure 7) was compared with standard Joint Committee on Powder Diffraction Standards data number 04-0783, which confirms the crystalline face-centered cubic nature of AgNPs, as indicated by the peaks at 2θ values (10–80°) of 24·88, 44·4 and 64·86°, which can be indexed to the 111, 200 and 220 planes. The predominant peak was obtained at 200. Many unassigned peaks were also observed in the XRD data, which might be because of crystallization of bioorganic phases that appear on the synthesized nanoparticles' surface.[21] However, these peaks are comparatively weaker than those of silver, which suggests that silver is the main element in the composite. The result was comparable with that of Nayak *et al.*,[11] who obtained XRD peaks at 111, 200, 220, 311 and 222 orientations and predominant orientation at 200 in the case of

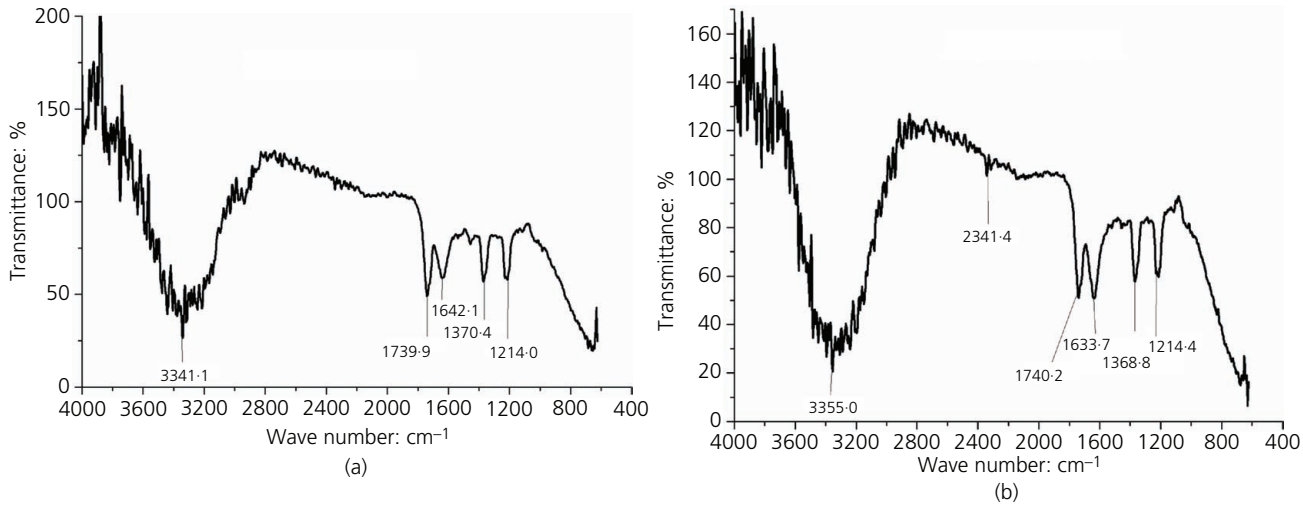

Figure 5. FT-IR spectra of (a) AgNPs and (b) plant extract

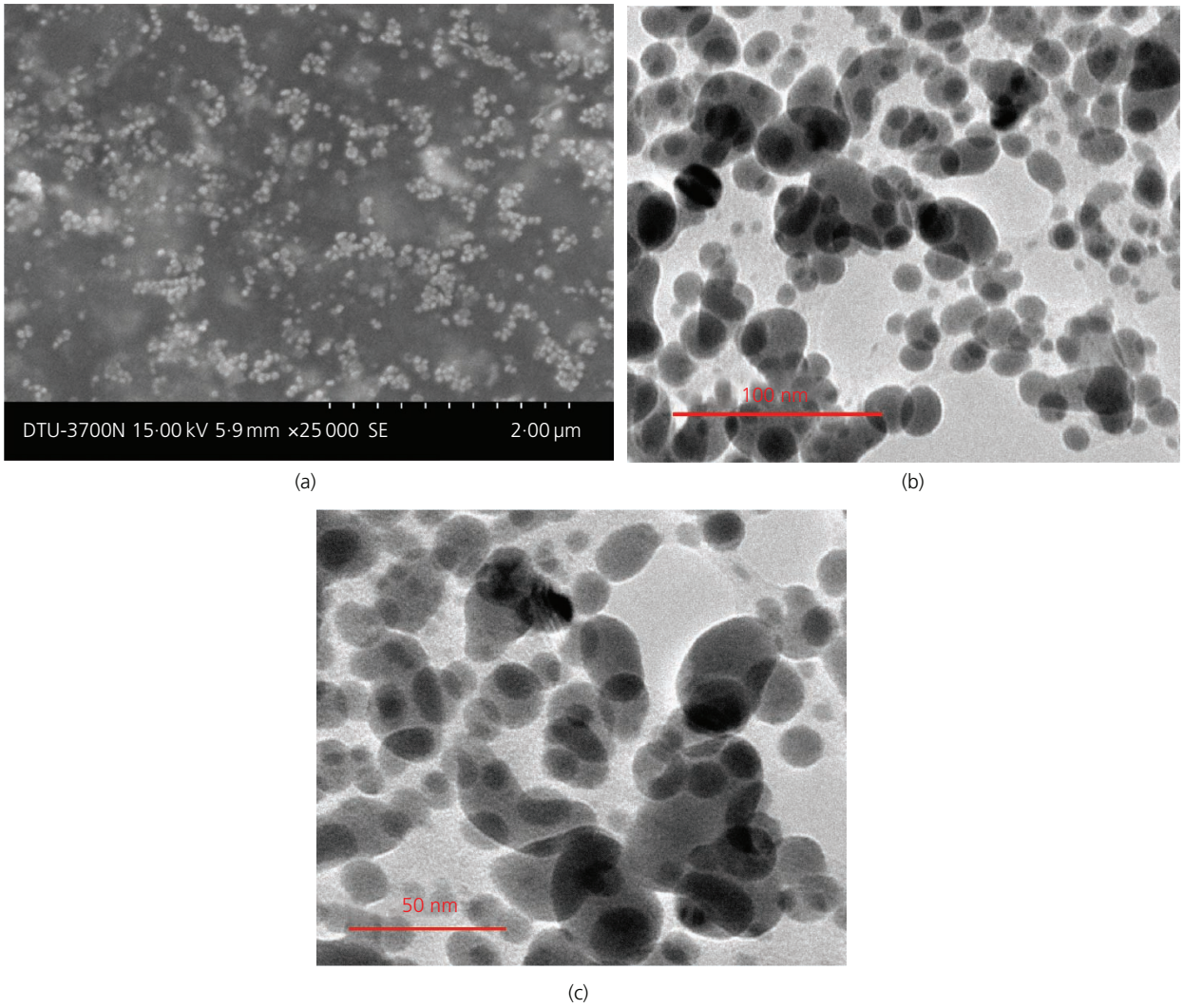

Figure 6. AgNPs synthesized from *P. zeylanica*: (a) SEM micrograph; (b, c) TEM micrograph images (scale bars representing 100 and 50 nm)

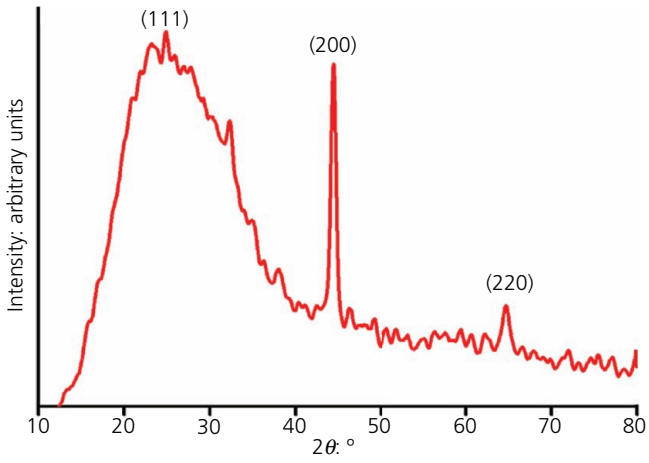

Figure 7. XRD pattern of AgNPs

F. benghalensis and *A. indica*. MeenaKumari and Philip[32] reported predominant orientation at 111 in the case of *Punica granatum*-synthesized AgNPs.

3.9 Antibacterial studies

The antibacterial activity of *P. zeylanica*-synthesized AgNPs was tested against *E. coli*, *S. aureus*, *A. faecalis*, *S. marcescens* and *S. putrefaciens*, and results showed that AgNPs possess potential activity against all bacteria except *S. putrefaciens* (Table 1; Figure 8). Kanamycin and different concentrations of synthesized AgNPs were loaded on the sterile disk introduced in the agar plates. Distilled water exhibits no zone of inhibition, whereas kanamycin and AgNPs do. The antibacterial activity of AgNPs was the highest against *S. marcescens*, with a 28 ± 0·57 mm zone of inhibition. The cell wall of gram-positive bacteria contains a firm network made out of three macromolecular concentric shells, which makes it impervious to mechanical break, whereas

Table 1. Antibacterial activity of AgNPs

Microorganism	Zone of inhibition: mm				
	Kanamycin (+ control)	Distilled water (– control)	AgNPs (100 µg)	AgNPs (500 µg)	AgNPs (1000 µg)
E. coli	23 ± 1	0	13 ± 0·57	15 ± 1	20 ± 0·57
S. aureus	24 ± 0·57	0	15 ± 1	18 ± 0·57	21 ± 1
S. putrefaciens	9 ± 1·15	0	0	0	0
A. faecalis	26 ± 0·57	0	15 ± 0·57	19 ± 1	22 ± 0·57
S. marcescens	35 ± 1	0	22 ± 0·57	25 ± 0·57	28 ± 0·57

Mean values ± standard deviation

gram-negative bacteria have a single particle thick network of lipoprotein and lipopolysaccharides.[33,34] It was reported that AgNPs have the capability to attach to the cell membrane of bacteria, which results in structural changes and development of 'pits' on the cell surface, followed by aggregation of nanoparticles.[35] Li *et al.*[36] showed that AgNPs enter bacterial cells and condense deoxyribonucleic acid (DNA), which results in inhibition of DNA replication and cell reproduction. Salunke *et al.*[3] reported the antibacterial activity of *P. zeylanica* (root)-synthesized AgNPs against *Acinetobacter baumannii*, *S. aureus* and *E. coli* and found maximum activity against *A. baumannii* and *S. aureus*. Similarly, Velammal *et al.*[37] reported *P. zeylanica* (bark)-synthesized AgNPs' antibacterial activity against *S. aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *E. coli.* In both studies, the authors utilized wild sources of *P. zeylanica* (root/bark) for nanoparticle synthesis, whereas, in the present study, aerial in vitro-grown parts of *P. zeylanica* were used for nanoparticle synthesis. The synthesized AgNPs possess a higher antibacterial activity against, namely, *S. aureus* (24 ± 0·57 mm) and *E. coli* (23 ± 1 mm) compared to the previously synthesized nanoparticles from *Plumbago* bark (8 mm for *S. aureus* and *E. coli*). Ramesh *et al.*[27] synthesized AgNPs from *E. officinalis* (fruit) and investigated its antibacterial activity against *E. coli*, *S. aureus*, *Klebsiella pneumoniae* and *B. subtilis*. Similarly, Nayak *et al.*[11] investigated the antibacterial potential of synthesized AgNPs (*F. benghalensis* and *A. indica* bark) against *E. coli*, *Bacillus* sp and *Pseudomonas* sp. Qurat-ul-ain *et al.*[38] synthesized AgNPs from five different plants (*Curcuma longa*, *S. cumini*, *Eucalyptus camaldulensis*, *Mangifera indica* and *Viola betonicifolia*) and reported their antibacterial activity against *E. coli* and *B. subtilis*. They found the highest antibacterial activity of AgNPs (synthesized from *S. cumini*) against *E. coli* (32 ± 0·28 mm). Erjaee *et al.*[39] reported AgNP synthesis from *Chamaemelum nobile* extract and investigated its antibacterial potential against *E. coli*, *S. aureus*, *B. subtilis* and *Salmonella typhimurium*. They found the highest zone of inhibition in the case of *E. coli* – that is, 15·1 ± 0·2 mm. Plant material that is obtained from root, bark and fruit sources requires more time to grow, which is not suitable for commercial requirement, whereas, in the case of in vitro (aerial)-grown material, this time is less.

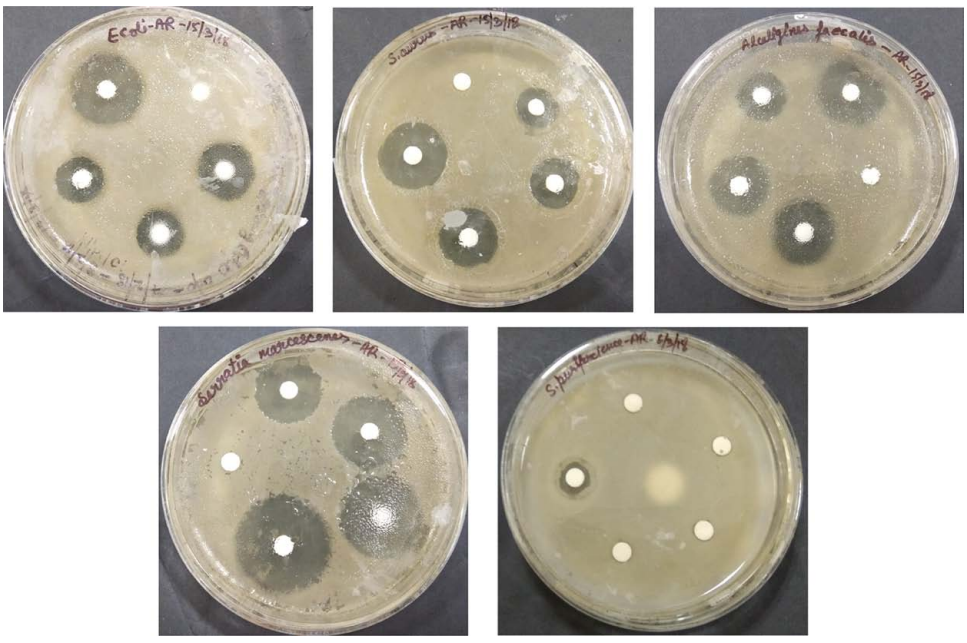

Figure 8. Antibacterial activity of AgNPs

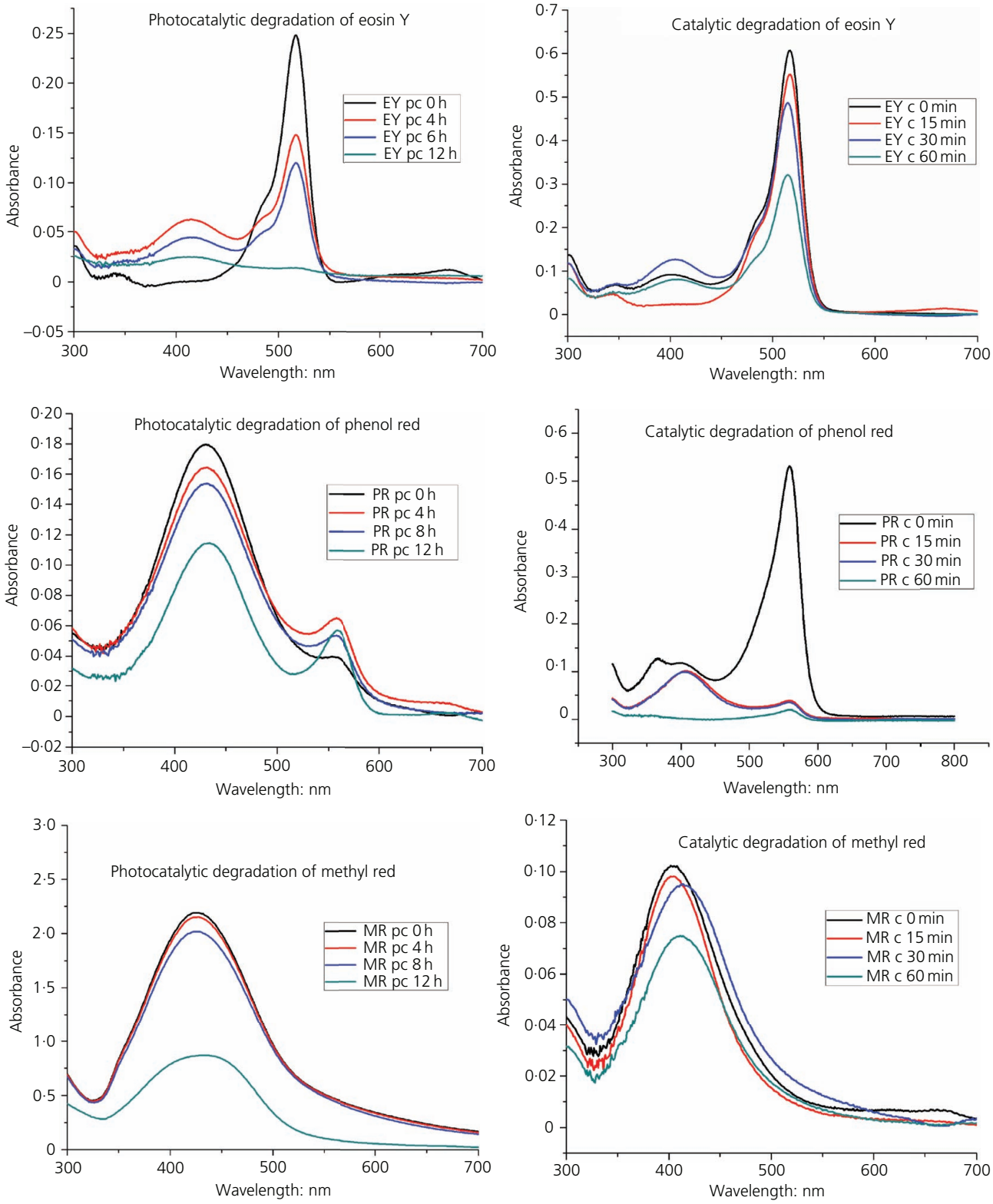

Figure 9. UV–visible absorption spectra for degradation of eosin Y (EY), phenol red (PR), methyl red (MR), methyl orange (MO) and methylene blue (MB). c, catalytic; pc, photocatalytic (continued on next page)

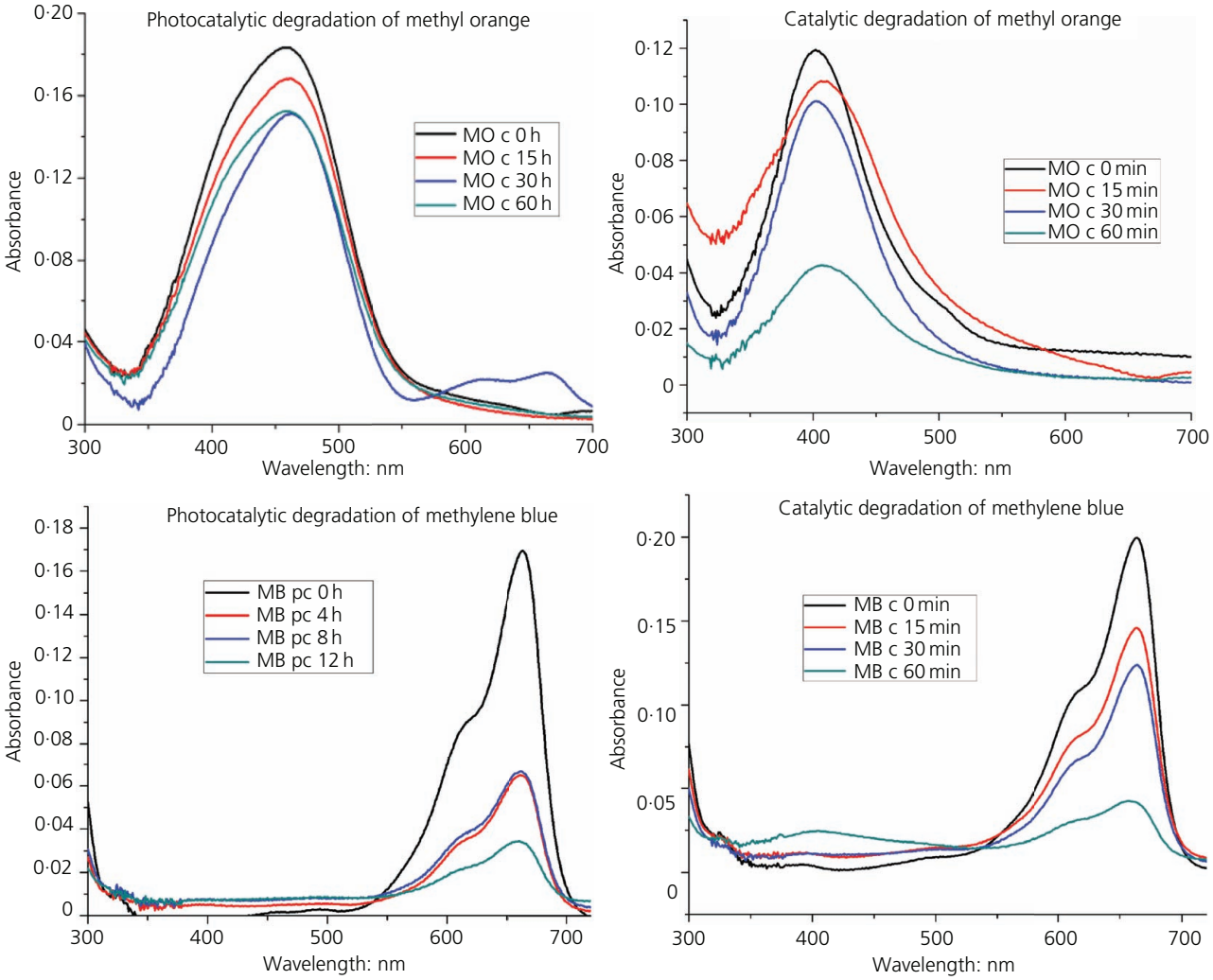

Figure 9. Continued

Various researchers have reported synthesis of nanoparticles using different extracts; however, there has been no report on utilization of in vitro cultures for AgNP synthesis. In vitro plant cultures possess various advantages such as cost-effectiveness, easily manageable biomaterial, eco-friendliness, non-pathogenicity and independence from geographical and seasonal variations. They also provide continuous production of plant material of uniform quality throughout the year, which can be beneficial for synthesis of nanoparticles at the commercial scale.

3.10 Degradation of dyes

An important utilization of AgNPs is for catalyzing degradation of organic dyes that are toxic in nature. Methylene blue is a phenothiazine cationic dye used in paper coloring, cottons, wool dyeing and so on. It causes damage to human beings such as breathing difficulties, diarrhea, vomiting and nausea.[40] Methyl orange is found mainly in the textile and food industries' effluent and is carcinogenic in nature.[41] Eosin Y is an anionic dye that exhibits yellow-green fluorescence and is utilized in the printing, dyeing, printing ink and fluorescent pigment industries.[42] Phenol red is used as a bromination catalyst, as a pH indicator and in screening tests and has estrogenic properties.[43] Methyl red is used for identification of bacteria producing stable acids during mixed acid glucose fermentation.

The catalytic activity of nanoparticles depends on their size, composition and shape. The catalytic degradation efficiency of synthesized AgNPs was explored using five dyes, namely methyl red, phenol red, methylene blue, methyl orange and eosin Y. The UV–Vis spectral bands of methyl red, methyl orange, eosin Y, methylene blue and phenol red in water normally appear at 410, 465, 515, 664 and 560 nm, respectively. Sodium borohydride addition gradually decreases the absorption intensity, which indicates reduction of dyes. Because of a substantial kinetic barrier and a lower encounter probability, the degradation rate of dyes in water is very slow and complete disappearance of the absorption

peak has not been observed[30] in any case. Figure 9 shows the UV–Vis spectra of dye degradation in the presence of nanoparticles, which was recorded at uniform intervals of time. In the case of the absence of nanoparticles, there was almost no reduction in the solutions, where as there was an increase in the degradation rate when nanoparticle solution was added. The catalytic activity of AgNPs was in the order phenol red > methylene blue > methyl red > methyl orange > eosin Y. A decline in the dyes' absorbance was observed with time, and, as time progressed, the degradation process slowed down; this could be because of degraded products that adhered onto the nanoparticles' surface. To the best of the authors' knowledge, this is the first report of a comparative study of catalytic and photocatalytic degradation of dyes using AgNPs synthesized from in vitro-grown *P. zeylanica*. Several studies reported dye degradation using AgNPs synthesized from different plants (collected from wild sources). Vidhu and Philip[44] reported catalytic degradation of methyl orange, methylene blue and eosin using AgNPs synthesized from seeds of *Trigonella foenum-graecum*. Jyoti and Singh[45] reported catalytic degradation of safranin O, methyl orange, methyl red and methylene blue using *Zanthoxylum armatum*-synthesized AgNPs. MeenaKumari and Philip[32] reported the catalytic degradation of methyl orange, methyl blue and eosin Y using AgNPs synthesized from *P. granatum*.

The photocatalytic efficiency of AgNPs was also tested using the same five dyes (Figure 9). Degradation of dyes was observed as a peak intensity decrease within 12 h of incubation; complete degradation of dyes was not observed in any case.[32] The photocatalytic activity of AgNPs was in the order eosin Y > methylene blue > methyl red > phenol red > methyl orange. Sumi *et al.*[46] reported that AgNPs were very effective and stable photocatalysts under the proper temperature condition with a visible light source for organic dye degradation. The photocatalytic efficiency of AgNPs in visible light is due to SPR excitation.[47] Kansal *et al.*[48] reported that sunlight was found to be faster for dye degradation in the presence of a metal catalyst compared to other irradiation methods. Kumar *et al.*[49] investigated photocatalytic degradation of methyl orange using *Ulva lactuca*-synthesized AgNPs. Vanaja *et al.*[50] reported photocatalytic degradation of methylene blue using *Morinda tinctoria*-synthesized AgNPs and observed degradation in 60 min. Wang *et al.*[51] reported sunlight-driven catalysis for methyl orange and Coomassie Brilliant Blue degradation using *Psidium guajava*-synthesized AgNPs and observed degradation up to 10 and 6 h, respectively. Various researchers have reported dye degradation activity of AgNPs synthesized using different plant materials; however, there is no report on utilization of in vitro cultures of *Plumbago* for synthesis of AgNPs and their role in catalytic and photocatalytic dye degradation.

4. Conclusion

Recently, focus on medicinal plant research has gained more attention. Synthesis of AgNPs using the aqueous extract of *P. zeylanica* provides an efficient, simple and eco-friendly method. Water-soluble components present in the plant extract act as a reducing agent and stabilize nanoparticles by attaching onto them. In vitro-grown *P. zeylanica* extract for AgNP synthesis has not been studied until now. In the present investigation, it was found that in vitro-grown plants are a potential source for nanoparticle synthesis compared to plant material collected from wild sources. Silver has shown an excellent antimicrobial activity. In this study, it was found that synthesized AgNPs act as a potent antibacterial agent against *E. coli*, *A. faecalis*, *S. marcescens* and *S. aureus*. A comparative study of photocatalytic and catalytic degradation of five different dyes was done, and it was found that catalytic degradation in the case of phenol red, methyl red and methylene blue was more potent than photocatalytic degradation. Therefore, it has been concluded that in vitro-grown *P. zeylanica* is a potential source for synthesis of AgNPs.

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