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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. $14.19 \pm 0.5$ 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

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 $25 \pm 2$ °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 $\sigma/S$ and LOQ = 10 $\sigma/S$, where $S$ is the slope of the curve and $\sigma$ 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

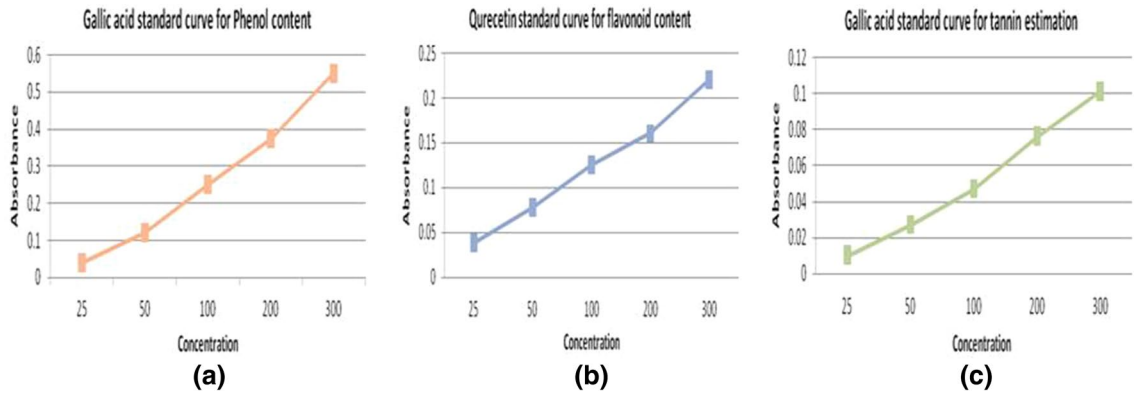


**Fig. 2** **a** Standard curve of gallic acid for phenol estimation. **b** Standard curve of qurecetin for flavonoid estimation. **c** Standard curve of gallic acid for tannin estimation

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 | $C_{20}H_{40}$ |

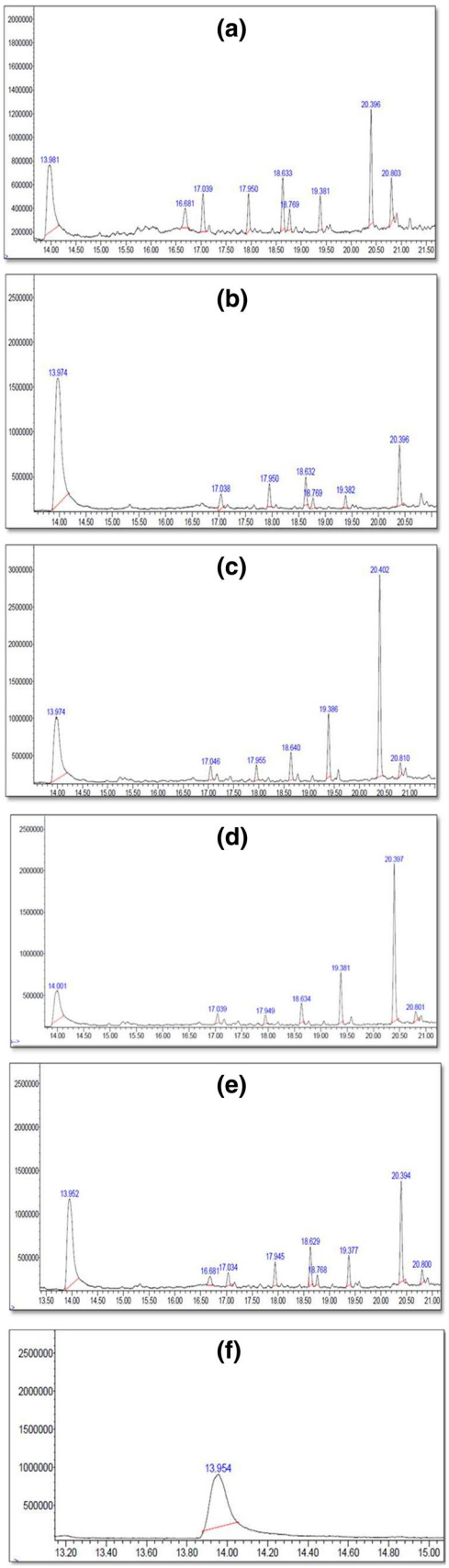


**Fig. 3** GC–MS chromatogram of ethyl acetate extracts of *Plumbago zeylanica* accessions: **a** 439212, **b** 524441, **c** 398891, **d** 539866, **e** 421418 and **f** plumbagin standard

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 $14.19 \pm 0.5$ µg/ml,

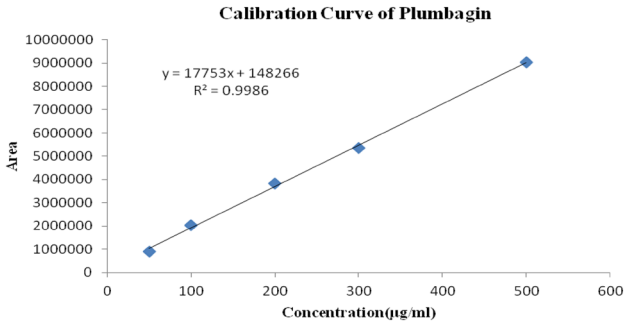


**Fig. 4** Calibration curve of plumbagin

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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