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ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL EVALUATION OF *PLUMBAGO ZEYLANICA* LINN. *IN VIVO* AND *IN VITRO*

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ABSTRACT

The plant species *Plumbago zeylanica* Linn. (known vernacularly as Chitraka, Chitramulamu, Tellachitramulamu, Agnichela, Agnimaala by its trade or popular names of "Lead wort-white flowered" and "Ceylon Lead wort") belongs to the family Plumbaginaceae, is distributed as a weed in throughout the tropical and subtropical countries of the world. The roots of this plant are the main source of an alkaloid called plumbagin which is traditionally used as germicidal, abortifacient and in treatment of cancer, liver diseases, fever, body pain and inflammation. In the present study callus was raised from the leaf explant of *Plumbago zeylanica*. Maximum callus was obtained on MS medium supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D) at 3.5mg/L. The callus and root extracts were used for primary metabolite quantification and antioxidant activity. Significantly higher antioxidant activity ($105.47 \pm 0.14\%$ at $360 \mu\text{g/ml}$ and phenol content with $2.61 \pm 0.08 \text{ mg/g}$) were observed in *in vivo* grown plant in comparison to *in vitro* raised callus with antioxidant activity ($81.22 \pm 1.06\%$) and phenol content with $1.83 \pm 0.05 \text{ mg/g}$. The greater amount of phenolic compounds leads to more potent radical scavenging effect as shown by crude extracts of root of *Plumbago zeylanica*.

Keywords: *Plumbago zeylanica*, *In vitro*, *In vivo*, Callus, Crude extract, Antioxidant activity.

INTRODUCTION

The medicinal plant *Plumbago zeylanica* belongs to the family Plumbaginaceae, is distributed as a weed in throughout the tropical and subtropical countries of the world. The genus *Plumbago* is represented by 10 species, of which *P. zeylanica* is a potent medicinal plant grown as a perennial herb in most parts of India. However larger scale cultivation was also common in West Bengal and Southern India due to its more therapeutic uses. The roots contain an alkaloid called plumbagin, a natural naphthaquinone (5-hydroxy-2-methyl-1,4-naphthoquinone), possessing various pharmacological activities such as antimalarial¹, antioxidant activity², anticancer, cardiotonic, antifertility action, antibiotic and antineoplastic^{3,4,5}. Its other constituents in roots are chitranone, zeylanone, dihydrosterone, 2-methyl naphthaquin, plumbazeylanone and terpenoids, lupeol and teraxesterol. The plant also contains alkaloids, glycosides, tannin, saponins and steroids⁶. The roots are used extensively in China and other Asian countries for the treatment of cancer, rheumatoid arthritis, dysmenorrhea, and confusion of extremities⁷. Extract of the root is given internally or applied to the ostium uteri, causes abortion^{8,9}.

Pharmaceutical companies largely depend upon material procured from naturally occurring stands which are being depleted rapidly raising concern about possible extinction of the species and providing justification for the development of *in vitro* propagation techniques for medicinal plants. Micropropagation technology through axillary bud proliferation has proven to be a handy tool^{10,11,12}. Presently there is a great demand for the use of plant based medicaments in place of synthetic drugs. *In vitro* techniques offer the possibility of rapid clonal propagation of important plants allowing production of genetically stable and true to-type progeny¹³. An appreciable level of success has been achieved by many investigators in the case of many tropical medicinal plants through micropropagation, for example *Cleistanthus collinus*¹⁵, *Commiphora wightii*¹⁶, *Gymnema sylvestre*¹⁷ and *Hemidesmus indicus*¹⁴. Technique of axillary bud micropropagation has been reported previously for some other species of *Plumbago* such as *Plumbago indica*¹⁸ and *Plumbago rosea*¹⁹. The present study is justifiably planned to culture the callus of valuable medicinal plant *Plumbago zeylanica* Linn. in *in-vitro* condition with various combinations/concentrations of plant growth regulators, and compare the primary metabolites as well as antioxidant activity *in vivo* plant material and *in vitro* cultured callus of *Plumbago zeylanica*.

MATERIALS AND METHODS

Preparation of Media

Murashige and Skoog's medium was used for raising the callus of *Plumbago zeylanica* in *in vitro* condition. The MS medium was prepared by adding required amounts of stock solutions and final volume was made up with distilled water. The pH of the medium was adjusted to 5.8 using 1N NaOH. About 50 ml of the medium was poured into sterile culture flasks. The culture flasks with MS medium was autoclaved at 121°C for 20 min at 15 lb pressure and transferred to the media storage room where they were kept under aseptic condition for further experimental study.

Selection of explants & sterilization

Auxiliary nodes, young leaves and internodes of *Plumbago zeylanica* were used as explants. The explants were first washed with running tap water for 30 min. to remove the soil particles and other extraneous fine particles. The parts of the explant such as nodal segment, stem, leaf and root was cut from the healthy plant of *Plumbago zeylanica* and washed with tap water followed by immersing in a 5% (v/v) solution of detergent (Labolene, Qualigens, India) for 20 min and were subsequently agitated in 0.1% Bavistin (fungicide by BASF India Limited) for 15 min followed by rinsing with distilled water to remove the traces of Bavistin. Then the explants were washed twice thoroughly with sterile double distilled water and transferred to laminar air flow cabinet for further surface sterilization with 0.1% (w/v) HgCl_2 aqueous solution for 2-3 min followed by a thorough rinsing of the material with sterile double distilled water.

Initiation of cultures

There is a high risk of contamination of the MS medium at the time of transfer of the explants into the culture medium. Therefore, surface sterilized explants were transferred aseptically to sterile glass plate. Then undesirable and dead portions of both basal and the top portion of the explants were removed. The nodal explants were placed in an erect position in the culture bottle containing MS medium with the help of sterile forceps. Then lid was closed carefully and sealed with Klin film. The same procedure was used for all the explants. The culture bottles were kept in the growth room at $25 \pm 2^\circ\text{C}$, with a photoperiod of 16 h daylight and 8 h night breaks under the cool white fluorescent light.

Callus induction

Leaf explants were taken from healthy plants of *Plumbago zeylanica* for callus induction. The MS basal medium was supplemented with

concentration ranging between 0.5 to 4.5mgL⁻¹ 2, 4-D (2, 4-Dichlorophenoxyacetic acid). After inoculation with established culture, the culture flasks were sealed properly, labeled and the triplicates were maintained. Then they were transferred to the incubation room and kept in appropriate condition. After two weeks, the callus induction rate was recorded²⁰.

Extraction of callus crude extracts

A known quantity of one-month fresh callus was taken and oven dried at 60°C to constant weight. The callus was finely grinded and extracted with methanol using soxhlet apparatus for 8h and solvent was removed by distillation. The crude extract obtained was used for antioxidant activity studies.

Extraction of plant crude extracts

The dried and powdered leaves (50g) were extracted successively with ethanol as organic solvent (400ml) for 10-12 hrs. through Soxhlet apparatus. The collected extracts were filtered through Whatman No-1 filter paper. The extracts were evaporated to dryness under reduced pressure at 90°C by Rotary vacuum evaporator to obtain the respective extracts and stored at -18°C until used for further analysis.

Primary and Secondary Metabolite

Estimation of carbohydrates

(A) Total soluble sugars: The dried and powdered test sample 50 mg each was macerated in a grinder with 20 ml of ethanol and left for 12 hrs. and mixtures were centrifuged (1200 rpm) for 15min, the supernatants were removed and was concentrated on a water-bath. The volume of these aqueous concentrates were maintained to 50 ml with distilled water and processed further for estimation of soluble sugars by following the method of Loomis and Shull²¹.

(B) Starch: The above residue of each test sample was taken separately for quantitative estimation of starch²².

Quantification of carbohydrates

Aliquot (1 ml) of each of the test sample from above two samples were used to quantify the total carbohydrate using phenol-sulphuric acid method²³. The concentration (mg/gdw) of the total soluble sugars was directly worked out from the regression curve of the standard glucose. Four replicates of each experimental sample were taken and their mean values recorded. The sugar content in terms of glucose equivalent and the use of conversion factor (0.9 to convert the values of glucose to starch) was made in each case.

Quantification of proteins

60 mg of the dried test sample was taken for extraction of protein by the method of Osborne²⁴. In each of 1 ml extract, total protein content was estimated using the standard protocol of Lowry *et al*²⁵. Subsequently, to each of these mixture tubes 0.5 ml of Folin-Ciocalteu reagent diluted with equal volume of distilled water just before use) was rapidly added and after 30 min, the OD of each sample was measured at 750 nm using a spectrophotometer against the blank. Three replicates of each concentration were taken and there mean values were used to compute a regression curve. The total protein content in each sample was calculated by referring the ODs of test sample with the standard curve of BSA. Three replicates were examined in each case and their mean values were recorded.

Extraction and quantification of phenol

1g of each of the dried and milled test sample was taken for extraction of lipids²⁶. The collected lipid was expressed as the weight of total lipids/g of the dried tissue sample. Each of 200 mg dried and milled test samples was homogenized in 80% ethanol (10 ml) for 2 hrs and left over night at room temperature. It was centrifuged, the supernatants were collected individually and the volume of each was raised to 40 ml with 80% ethanol. To estimate total phenols in each of the test sample, the protocol of Bray and Thorpe²⁷ was followed, wherein a standard curve of catechol (a phenol) was taken as standard. Each test sample was processed in this similar manner, ODs were measured and the total level of phenols was calculated

from the mean values (with reference to catechol) by referring the OD of the test sample with the regression curve of the standard.

DPPH radical scavenging assay

Free radical scavenging activity of the extracts was measured using the method of Brand- Williams *et al*.²⁸ with a little modification. 4.3 mg of DPPH (1, 1-Diphenyl -2-picrylhydrazyl) was dissolved in 3.3 ml methanol; it was protected from light by covering the test tubes with aluminum foil. 150 ml DPPH solution was added to 3ml methanol and absorbance was taken immediately at 517nm for control reading. 50 ml of various concentrations of test samples as well as standard compound (Ascorbic acid) were taken and the volume was made uniformly to 150 ml using methanol. Each of the samples was then further diluted with methanol up to 3ml and to each 150 ml DPPH was added. Absorbance was taken after 15 min. At 517nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. The IC50 values for each drug compounds as well as standard preparation were calculated. The DPPH free radical scavenging activity was calculated using the following formula:

$$\% \text{ scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample} \times 100}{\text{Absorbance of control}}$$

The effective concentration of sample required to scavenge DPPH radical by 50% (IC50 value) was obtained by linear regression analysis of dose-response curve plotting between %inhibition and concentrations.

Hydroxyl radical scavenging assay

2-Deoxyribose is oxidized by OH[•] radicals that are formed by Fenton reaction and degraded to Malondialdehyde which can be detected by reacting with Thiobarbituric acid (TBA)²⁹. 100 ml of Riboflavin solution [20 mg], 200 ml EDTA solution [12mM], 200 ml methanol and 100 ml NBT (Nitro-blue tetrazolium) solution [0.1mg] were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer [50mM]. The absorbance of solution was measured at 590nm using phosphate buffer as blank after illumination for 5 min. This is taken as control. 50 ml of different concentrations of test samples as well as standard preparation were taken and diluted up to 100 ml with methanol. To each of these, 100 ml Riboflavin, 200 ml EDTA, 200 ml methanol and 100 ml NBT was mixed in test tubes and further diluted up to 3 ml with phosphate buffer. Absorbance was measured after illumination for 5 min. at 590 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. The IC50 value for each compound as well as standard preparation were calculated by using the following formula

$$\% \text{ scavenging/Inhibition} = \frac{[\text{Absorbance of control} - \text{Absorbance of test sample} / \text{Absorbance of control}] \times 100}{\text{Absorbance of control}}$$

Statistical Analysis

For all the experiments, three samples were analyzed and all the assays were carried out in triplicate. The data are expressed as mean \pm standard deviation.

RESULTS AND DISCUSSION

Callus Growth

The callus growth expressed three distinct phases (Fig. 1). A lag phase of 10d where 10% growth in callus mass was noticed followed by exponential or linear phase with a rapid and significant increase (75%) in formation of callus during 10-15d of culture representing early linear phase. It recorded a growth rate of 15% during late linear growth phase extending from 15th and 30th d of culturing. The growth declined after 30 d representing log phase. Depletion of nutrients, accumulation of toxic products, and other limiting factors might have led to cell death and eventually decline in growth³⁰. Hence, callus was subcultured at 30d interval.

Effect of growth regulators on callus induction

The different concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D) on callus induction from nodal explants was studied in order to determine the optimum concentration of 2, 4-D required in the callusing medium (Table 1).

Table 1: Effect of different concentrations of 2, 4-D on callus induction in *Plumbago zeylanica* from nodal and Leaf explants in MS medium

2,4-D (mg/L)	Percentage of callus induction (Mean \pm SEM)	Type of callus	Degree of callusing at the end of 4 weeks *
0.5	10.07 \pm 0.03	White	+
1	30.13 \pm 0.04	Whitish yellow	+
2	45.27 \pm 0.02	Whitish yellow	++
3	80.24 \pm 0.09	Yellow friable	+++
3.5	95.50 \pm 0.13	Whitish yellow	++++
4	40.39 \pm 0.02	Brownish yellow	++
4.5	30.26 \pm 0.05	Brownish	++

(*)Degree of Callusing: + = Poor; ++ = Fair; +++ = Good; ++++ = Very Good

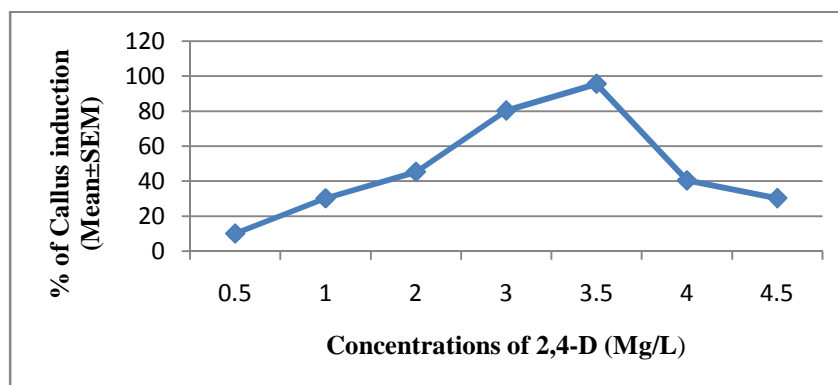


Fig. 1: Effect of different concentration of 2,4-D on callus induction in *Plumbago zeylanica*

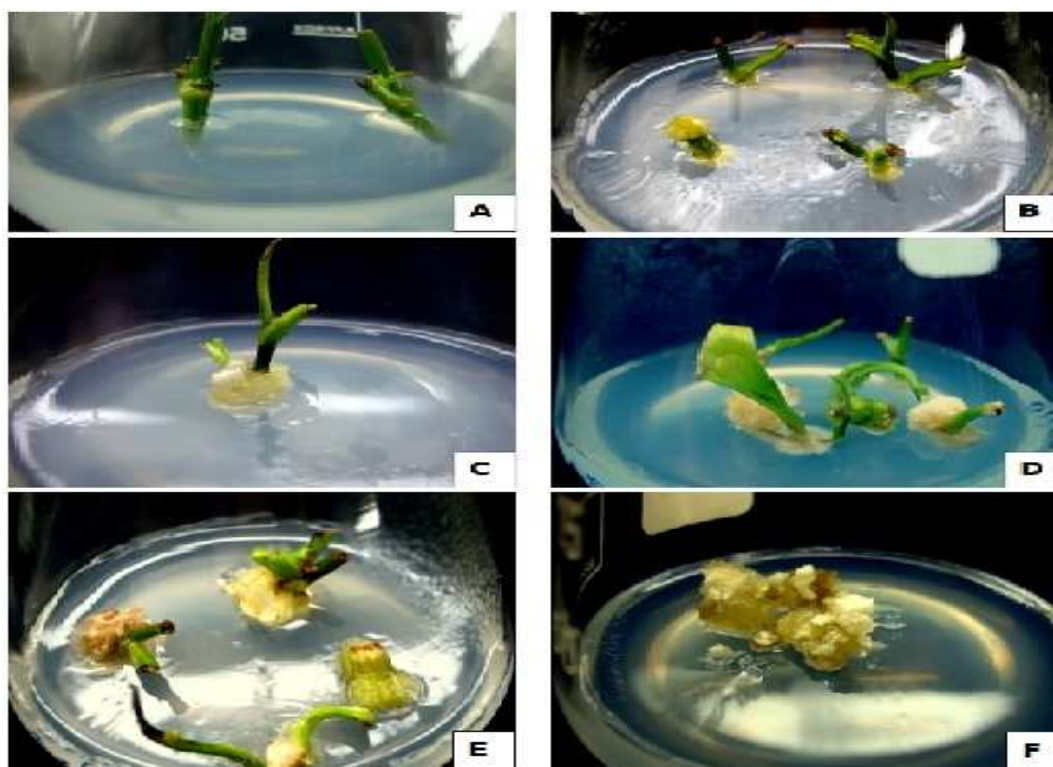


Fig. 2: Different stages of Callus growth

A-1st Day of Inoculation, B,C-Growth after 15 Days, D,E-Growth after 30 Days, F-Growth after 45 Days

However supplementation of the medium with different concentrations of 2, 4-D stimulated callusing at varying degrees. In all the concentrations of 2, 4-D the explants initiated callusing within 3-4 days of inoculation. Subsequently small mass of callus emerged from epidermal part of the explant at different points. In respect of supplementation of 2, 4-D 3.5mg/L was optimal for (95.50 \pm 0.13%) explant callusing (Fig. 2E and 2F). Hence in the subsequent experiments for callus induction this concentration of 2, 4-D i.e.

3.5mg/L was used and the callus development was allowed for 4 weeks, as optimal period. At the end of the 4 week culture period the growing callus pieces were separated from the mother explant and subcultured into the fresh medium with similar composition and maintained through repeated sub-culturing with regular intervals to build up sufficient tissue source for to study organogenesis. It was also observed that increasing the concentration of 2, 4-D to more than 3.5 mg/L have a negative effect on the callus induction. With

further increase in concentration of 2, 4-D there was a low % of callus initiation after 30d and the colors were changed from white to whitish yellow and gradually turned into brown and dark brown. The colors being mainly influenced by location of phenolic secondary metabolites in the cells. Accumulation of the phenolics is in the cytoplasm, it undergoes oxidation and polymerization and the oxidized products appeared brown or dark brown³¹.

Selection of callus culture for antioxidant activity

The amount of sample needed to decrease the initial DPPH concentration (EC₅₀) by 50% is a parameter widely used to measure the antioxidant activity. Higher the EC₅₀ value, lower the antioxidant activity. Antioxidant activity (EC₅₀) was low 70(μg mL⁻¹) in callus during log phase i.e. at 15d. There was an increase in antioxidant activity 35(μg mL⁻¹) in callus obtained during linear phase i.e. up to 30d, which is characterized by accumulation of callus mass indicating the active phase of callus growth. After 30d there was a decrease in antioxidant activity corresponding to the different stages growth phase of callus (Table 3). Maximum accumulation of secondary metabolites probably might be the reason for the antioxidant activity during active growth phase of callus upon 1st subculture as recorded in callus of many plants. Addition of sugars, phosphates, nitrate and calcium which was performed in callus probably might have helped in hyper antioxidant activity as observed in many plant calli^{32,33}. The results of this study are corroborated by the findings in literatures^{34,35}.

Table 2: Antioxidant activity at different stages of growth of callus

Days	% of DPPH scavenging activity (EC ₅₀ value μg mL ⁻¹)
15	70
30	35
45	95

Primary and Secondary Metabolites

A maximum level of soluble sugars (70.23±1.12mg/gdw) was found in callus and minimum level of soluble sugar (55.52±0.14mg/gdw) found in root of *Plumbago zeylanica* Linn. and Phenol (1.83±0.05mg/gdw) where as Maximum level of Phenol (26.10±0.08mg/gdw) of lipid (41.27±0.05mg/gdw) and starch (45.14±1.04mg/gdw), Phenol (26.10±0.08mg/gdw) and protein (42.23±0.12 mg/gdw) were found in crude extracts (Shown in Table 3). The present study (Table 3) shows the phytochemical contents of *Plumbago zeylanica* and also shows that these bioactivities differ between *in vitro* grown callus and *in vivo* grown plants. The callus showed highest soluble sugars but less phenolic contents, starch, lipids and proteins than *in vivo* plant parts. It may be due to addition of Sugar, Phosphate, Nitrate and Calcium in MS media which were performed in *in vitro* grown calli. *In vitro* cells accumulate more sugar due to its easy availability in culture medium and these cells are in highly proliferating stage so they accumulate more primary metabolites than storage metabolites (starch, lipid) and secondary metabolites (phenolic contents).

Table 3: Nutritional Facts of Crude and Callus extracts of *Plumbago zeylanica* Linn.

Nutritional Facts	Crude Extracts (mg/g)	Callus Extracts (mg/g)
Protein	42.23±0.12	31.48±0.22
Starch	45.14±1.04	20.15±1.05
Sugar	55.52±0.14	70.23±1.12
Phenol	2.61. ±0.08	1.83±0.05
Lipid	41.27±0.05	35.14±0.12

Values are represented in Mean ± SD of three replicates

Antioxidant activity of crude extract and callus extract

DPPH radical-scavenging activity

The percentage inhibitions of Ascorbic acid, plant and callus extract is shown in Table 4. The comparison of plant and callus extract is shown in Table 4; which shows that the plant extract is able to inhibit the DPPH 105.47±0.14% in the concentration of 360μg/ml

and the callus is able to inhibit 81.22±1.06%. Callus extract showed significantly lower scavenging potential in comparison to the plant extracts of *Plumbago zeylanica*. The antioxidant and phytochemical content of *in vivo* grown plant was comparatively higher than *in vitro* grown callus^{36,37}.

Table 4: % of inhibition of DPPH by the plant and callus extract with respect to Ascorbic acid as standard

Concentrations (μg/ml)	Ascorbic acid (%)	Plant Extracts (%)	Callus Extracts (%)
40	43.35±0.34	38.36±1.10	10.56±1.54
80	51.52±0.32	48.25±1.08	22.34±0.24
120	58.55±0.11	57.56±0.41	40.12±0.45
160	70.85±0.25	68.12±0.63	51.30±0.65
200	78.41±0.13	74.58±0.25	56.21±1.23
240	86.50±0.56	79.45±1.32	60.24±0.35
280	90.74±0.67	87.11±0.55	65.45±1.33
320	96.20±0.08	92.14±1.22	72.11±0.12
360	112.32±0.75	105.47±0.14	81.22±1.06

Values are represented in Mean ± SD of three replicates

OH-scavenging activity

Among the oxygen radicals, hydroxyl radical is one of the most reactive species and induces severe damage to adjacent biomolecules³⁸. Formation of a highly reactive tissue damaging species like hydroxyl radical is caused by the interaction of iron ions with hydrogen peroxide in biological systems³⁹. All the extracts at different concentrations (40-360μg/ml) of both the extracts showed higher activity in DPPH free radical scavenging assay when compared with that of OH[•] scavenging assay. In Hydroxyl scavenging assay the plant extract is able to inhibit the OH[•] 90.35±0.13% in the concentration of 360μg/ml and the callus is able to inhibit 68.42±0.30% (Table 5).

Table 5: % of inhibition of OH[•] by the plant and callus extract with respect to Ascorbic acid as standard

Concentrations (μg/ml)	Ascorbic acid (%)	Plant Extracts (%)	Callus Extracts (%)
40	37.25±1.18	34.32±0.23	7.33±1.16
80	49.53±0.15	43.20±1.14	12.11±0.22
120	58.44±0.25	50.12±1.26	37.27±0.32
160	65.41±1.36	54.65±0.45	40.25±1.19
200	72.66±0.12	68.63±0.21	45.16±1.35
240	76.40±0.45	71.78±1.11	52.34±0.24
280	82.11±1.25	76.17±0.38	56.50±1.27
320	87.12±0.12	84.16±1.17	61.23±1.44
360	95.52±1.32	90.35±0.13	68.42±0.30

Values are represented in Mean ± SD of three replicates

Several studies are focused on the relationship between antioxidant activities of phenolic compounds, as hydrogen donating free radical scavengers. The antioxidant activity may be due to the presence of phenolic hydroxyl or methoxyl groups, flavones hydroxyl, keto groups, free carboxylic groups and other structural features^{40,41,42}. Although, the interactions between reactive oxygen species and putative antioxidants depend on many biological, physical and chemical parameters, these preliminary results on the *in vitro* DPPH radical scavenging capacity and protection of DNA against oxidative stress emphasized the benefit of the phenolic compounds of medicinal plant extracts.

CONCLUSION

The present study shows the antioxidant activity and phytochemical contents of *Plumbago zeylanica* and *in vitro* grown callus and *in vivo* grown plants. Antioxidant capacity of *in vivo* grown plant was higher than *in vitro* grown calli due to presence of more amounts of secondary metabolites in *in vivo* grown plants. The extracts of *Plumbago zeylanica* callus had the potential to induce antioxidant activity and phenolic metabolites, but it seemed that the undifferentiating cells in callus cultures could be a reason for expressing these desired secondary metabolites in lower ranges.

Nevertheless, this work provides basic information for massive production of phenolic compounds as antioxidants in *Plumbago zeylanica* and indicated the ability to utilize tissue culture techniques towards development of desired bioactive metabolites in *in vitro* culture as an alternative way to avoid using endangered or rare wild plants in pharmaceutical purposes.

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