Current Botany 2025, 16: 220-225
doi: 10.25081/cb.2025.v16.9428
https://updatepublishing.com/journal/index.php/cb

Research Article

ISSN: 2220-4822

Comparative *in vitro* antioxidant analysis of leaf, stem and root extracts of *Plumbago zeylanica* L. (Plumbaginaceae)

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ABSTRACT

Medicinal plants have become increasingly popular in modern life because of their numerous beneficial uses in nutrition, healthcare, medication and cosmetics. One such medicinally important plant is *Plumbago zeylanica* L. which grows in all districts of Kerala, belonging to the family Plumbaginaceae and its Sanskrit name is "Chitrak". The leaves and roots of the plant contain an alkaloid called Plumbagin. The root yield pigments like3, 3- biplumbagin, 3-chloroplumbagin, binaphthoquinone, droserone, isozeylanone, elliptinone, and zeylanone. The current investigation concentrated on the preliminary evaluation of phytochemical and comparative *in vitro* antioxidant analysis of leaf, stem and root of *P. zeylanica*. Screening of phytochemicals showed the presence of various secondary metabolites like alkaloids, glycosides, terpenoids, steroids, flavonoids, phenols and tannins which were present in the root methanolic extract than that of the stem and leaf extracts. The comparative free radical scavenging activities were assessed using a variety of assays, including DPPH, superoxide anion scavenging and nitric oxide scavenging. In the DPPH assay, maximum scavenging activity of the root methanolic extract was observed to be 94.24±1.76% whereas the methanolic leaf and stem extract were found to be 92.65±1.08% and 82.45±0.89% respectively at 200 μg mL^{-1} concentration. As compared to the IC_{50} values of the standard drug ascorbic acid, the root methanolic extract showed a lower IC_{50} value. The lower IC_{50} value indicates higher free radical scavenging activity. In the superoxide anion scavenging assay, maximum activity was found to be 83.23±2.12% at 200 μg mL^{-1} concentration of methanolic root extract and the IC_{50}values exhibited by root methanol extract (39.38±1.03 μg mL^{-1}) were comparatively less than that of standard drug ascorbic acid (48.89±1.67 μg mL^{-1}). In the nitric oxide scavenging assay, the root methanolic extract showed the highest capacity (79.23±2.11%) to decrease the synthesis of nitrite ions than that of leaf and stem extracts. The IC_{50} value exhibited by root methanol extract (42.17±2.42 μg mL^{-1}) was approximately closer to that of standard drug ascorbic acid (39.38±1.03 μg mL^{-1}). The outcome indicates that *P. zeylanica* root extract has the highest potential for antioxidants than compared to leaf and stem extracts. The secondary metabolites contained in roots might be contributing to this maximum antioxidant activity and it may be an excellent source of natural antioxidant.

KEYWORDS: *Plumbago zeylanica*, DPPH, Superoxide anion, Nitric oxide, Antioxidant

Received: January 17, 2025
Revised: August 20, 2025
Accepted: August 21, 2025
Published: August 28, 2025

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INTRODUCTION

Oxidation reactions produce unintended consequences depending on where they occur. If they occur in the food system, then the food spoilage; if they occur in a living system, the cell is damaged or killed (Ajay *et al.*, 2019). The addition of antioxidants is required to control the oxidative deterioration (Apeteng *et al.*, 2016). Phytochemicals known as antioxidants that stop or reduce the cell damage brought on by unstable free radicals (Gangabhagirathi & Joshi, 2015). Reactive oxygen species like superoxide radical, hydrogen peroxide, nitric oxide radical, hydroxyl radical and other peroxides from lipids make up around 5% of the oxygen that is inhaled by the human body. These react with various lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage. Oxidative stress plays a major role in the development of many conditions, including Alzheimer's disease, Parkinson's disease, macular degeneration, certain cancers, alcoholism, cardiovascular disease, emphysema and inflammatory diseases (Edwin *et al.*, 2009; Apeteng *et al.*, 2016; Dev *et al.*, 2018).

In the human body, several components (both endogenous and exogenous in origin) function collaboratively and synergistically when antioxidants are introduced into the food system as a defense mechanism against reactive oxygen species. Antioxidant supplementation is now being recognized as an important means

of improving free radical protection as part of a healthy lifestyle and a well-balanced diet (Srinivasan *et al*., 2016). Numerous bioactive substances with antioxidant properties can be found in Plants like flavonoids, steroids, phenols, alkaloids, carotenoids etc. (Halliwell & Gutteridge, 2015). One such significant medicinal plant is *P. zeylanica*.

P. zeylanica grows in all districts of Kerala, Tamil Nadu, Karnataka and Andhra Pradesh, common, wild or in cultivation due to its therapeutic use. The sap of the plant causes discoloration of the skin resembling the colour of the lead accounting for its Latin name *Plumbago* and the popular name leadwort. It is called 'Chitrak' in Sanskrit. The other synonyms include vellakoduveli, chitramoolam, thumpakoduveli, etc. (Jain, 2019). It is a rambling sub-scandent perennial herb with white flowers. The leaves and roots contain an alkaloid called Plumbagin (Roy & Bharadvaja, 2017). The root contains pigments like 3-chloroplumbagin, 3, 3-biplumbagin, binaphthoquinone, isozeylanone, zeylanone, elliptinone and droserone (Sharma & Kaushik, 2014).

P. zeylanica contains a variety of secondary metabolites like flavonoids, steroids, alkaloids, glycosides, saponins, tannins, triterpenoids, coumarins, phenolic compounds, fixed oils and napthoquinones. Napthoquinones present in the plant are plumbagin, plumbagic acid, chloroplumbagin and elliptone. (Budavari *et al*., 1996). In Kerala, the Kurumas, Kurichyas and Paniyas tribes of Wayanad use plant roots for the remedy of the digestive system like weak digestion, grahni, piles and abdominal pain (Marjana *et al*., 2018). Root and root bark are stomachic, carminative, antihelminthic, and used to treat troubles in intestine, itching, piles, inflammation, dysentery, bronchitis, leucoderma, and liver problems. Stem is useful against cough, anaemia, epilepsy, asthma, jaundice, piles, leprosy, scabies, urinary calculi, vaginal discharge, elephantiasis, migraine, and laryngitis. Leaves are used against dysentery, and its paste is applied directly to the rheumatic painful areas or itching skin (Oyedapo, 1996). Typically, not much biological data is accessible regarding the therapeutic qualities and antioxidant capacities of this priceless ethno medicinal plant. Therefore, based on the exhaustive literature survey, the current study aims the evaluation of phytochemical and *in vitro* antioxidant analysis helps the sustainable utilization of this medicinal plant.

MATERIALS AND METHODS

Plant Extracts

Plant materials were gathered from several locations within the Kollam district. Botany Department of TKMCAS conducted the botanical identification of the plant and its herbarium (TKMCAS/KOL/XII/2024/NR/01) was deposited in college herbarium for future reference. Whole plant materials were collected, cleaned and then their leaves, stem and root were weighed individually, dried in the shade and then oven drying. 50 grams of powdered materials were extracted (hot extraction method in a Soxhlet apparatus) separately with petroleum ether for the removal of wax content and then the defatted materials were again extracted with methanol for 72 h. Crude methanol extracts (leaves, stem and root) were used for preliminary phytochemical studies and *in vitro* antioxidant analysis.

Preliminary Phytochemical Studies

In this study, the concentrated methanol extract of leaf, stem and root of *Plumbago zeylanica* L. were subjected to the detection of various phytoconstituents, using standard chemical tests (Majouli *et al*., 2017).

In vitro Antioxidant Property

DPPH assay (2, 2-diphenyl -1-picryl hydrazyl assay)

The scavenging effect of DPPH free radicals were measured using the procedure outlined by Blios in 1958. In this assay, various concentrations of plant extracts like 12.5, 25, 50, 100 and 200 μg mL^{-1} were standardised and each concentration was then made up to 40 μL using Dimethyl Sulfoxide. To each sample, 2.96 mL of 0.1 mM DPPH solution was added and then incubated at room temperature in dark condition for 20 minutes. Absorbance was read at 517 nm. 3 mL of DPPH was used as control. Different concentration of ascorbic acid was used as standard.

Calculation formula: % of scavenging activity (DPPH) = (OD of the control-OD of the Sample/OD of the control) ×100.

The antioxidant properties of the extracts were expressed in the form of IC_{50}.

Superoxide radical scavenging assay

The nitroblue tetrazolium reduction procedure was selected for the evaluation of scavenging activity of superoxide radical (Yen & Duh, 1994). 2 μM riboflavin, 3 μg sodium cyanide, 6 μM ethylene diamine tetraacetic acid, 50 μM nitroblue tetrazolium, 67 mM phosphate buffer and various concentrations of extracts were taken as reaction mixture. The tubes with reaction mixture were illuminated continuously with an incandescent light for 15 minutes and the OD was measured at 530 nm before and after the process of illumination. Ascorbic acid was used as a standard. The percentage of inhibition was calculated by using the following formula:

Scavenging activityof Superoxide anion (%) = (OD of the control-OD of the Sample/OD of the control) ×100.

The antioxidant properties of the extracts were expressed in the form of IC_{50}.

Nitric oxide scavenging assay

In this assay, varying quantities of the test sample made in methanol were combined with 5 mM^{-1} sodium nitro prusside

dissolved in phosphate buffer of saline pH 7.4, and the mixture was incubated at 25 °C for 30 minutes. Methanol without test ingredient was used as control. 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid, 0.1% N-1-naphthyl ethylene diamine dihydrochloride) was then added to 1.5 mL of incubated solution. The percentage of scavenging activity was computed using the standard ascorbic acid as a reference and absorbance was measured at 546 nm (Rachh *et al.*, 2009).

Statistical Analysis

Three duplicates of each of the above-mentioned experiments were conducted. GraphPad InStat DTCG was used to calculate the Mean±standard deviations for the experimental assay data. According to the Tukey-Kramer Multiple Comparisons Test, analysis of variance was conducted.

RESULT AND DISCUSSION

Preliminary Phytochemical Evaluation of *P. zeylanica*

Phytochemicals are plant-derived bioactive chemical compounds with wide ranging benefits to the health of mankind. Phytochemical screening facilitates chemo profilers, which identify the main pharmacologically and therapeutically valuable components in plant extracts (Rafa *et al.*, 2016). The phytochemical screening of *P. zeylanica* showed the presence of most of the secondary metabolites like glycosides, flavonoids, alkaloids, terpenoids, steroids, phenols and tannins were present in the root methanolic extract than that of stem and leaf extracts. The results were shown in Table 1.

Plants always produce a complex mixture of phytochemicals like glycosides, tannins, flavonoids, steroids, polyphenols, terpenoids and alkaloids (Ibrahim *et al.*, 2018). Most of the secondary metabolites exhibit some biological and toxicological activities (Rafat *et al.*, 2010). Glycosides were reported to exhibit antidiabetic properties. In the preliminary phytochemical analysis, glycosides were found to be present in the leaf, stem and root methanolic extracts of *P. zeylanica*. Flavonoids have many pharmacological properties like anti-inflammatory, antioxidant, antimicrobial and anticancer activity (Ajayi *et al.*, 2011). In the present study, flavonoids were found to be noticed in the leaf and root methanolic extracts of *P. zeylanica*. Many of the alkaloids are neuromodulators or neurotoxins and have many pharmacological properties like antiprotozoal, cytotoxic, antidiabetic and anti-inflammatory, etc. and mainly evolved for defence against herbivores. In this preliminary phytochemical study, alkaloids were found to be present in the leaf, stem and root methanolic extracts of *P. zeylanica*. Terpenoids (isoprene units) are used against skin diseases and as antiseptics as well as respiratory ailments. In this study, terpenoids are present only in the leaf and root extracts.

Phenols and tannins werre noticed in all the methanol extracts (leaf, stem and root) of *P. zeylanica*. Tharmaraj & Antonysamy (2013) reported the presence of steroids, alkaloids, phenols, cardiac glycosides, tannins, terpenoids, coumarins and sterols in the ethanolic extracts of *P. zeylanica* aerial parts collected from Tenkasi.

Table 1: Preliminary phytochemical evaluation of *P. zeylanica*

Phytochemicals	Crude methanolic extracts		
	Leaf	Stem	Root
Glycosides	+	+	+
Flavonoids	+	-	+
Alkaloids	+	+	+
Terpenoids	+	-	+
Steroids	-	-	+
Phenols	+	+	+
Tannins	+	+	+

In vitro Antioxidant Property

An antioxidant is a chemical which can stop the activity of free radicals. This study analysed the free radical scavenging activity of *P. zeylanica*'s leaves, stem and roots using a variety of assays like DPPH, Superoxide anion scavenging, and Nitric oxide scavenging.

DPPH assay (2, 2-diphenyl -1-picrylhydrazyl assay)

A purple-coloured stable free radical is DPPH (2,2-diphenyl-1-picrylhydrazyl) which changed into yellow when scavenged which results in a decrease in absorbency. The degree of colour change reveals the radical elimination potential of the antioxidant compounds or extracts as well as their ability of hydrogen donation (Ajayi *et al.*, 2019). In the current study, it was observed that *P. zeylanica* root extract exhibited a greater scavenging effect on the DPPH radical compared to that of the stem and leaf extracts, which increased as the sample concentration increased. Each value represents the mean±SD of triplicate measurements. The maximum scavenging ability of the root extract was observed to be 94.24±1.76% whereas the leaf and stem extracts were found to be 92.65±1.08% and 82.45±0.89% respectively (Table 2 & Figure 1).

Comparative IC_{50} values of ascorbic acid and crude methanolic extracts of *P. zeylanica* are shown in Table 3. As compared to the IC_{50} values of the standard drug ascorbic acid, root methanolic extract showed a lower IC_{50} value. Higher free radical scavenging activity is indicated by a lower IC_{50} value. Thus, compared to leaf and stem extract, root methanolic extract of *P. zeylanica* exhibited significantly high free radical scavenging activity. A same type of DPPH scavenging activity was reported in methanolic root extracts of *Plumbago capensis* (Lakshmanan *et al.*, 2016). Jain *et al.*

Table 2: Effect of crude methanolic extracts of *P. zeylanica* in DPPH assay

Sample concentration ($\mu g\ mL^{-1}$)	% of scavenging			
	Ascorbic acid (Standard)	Crude methanolic extracts		
		Leaf	Stem	Root
12.5	17.84±0.34	11.23±0.26	5.34±1.34	12.68±0.01
25	36.12±1.23	29.00±1.23	18.45±2.11	34.64±1.21
50	54.36±0.86	52.12±0.26	46.12±0.35	60.62±1.98
100	72.85±1.22	76.15±1.45	60.15±0.54	83.16±0.45
200	83.35±0.95	92.65±1.08	82.45±0.89	94.24±1.76

Table 3: Comparative IC_{50} values of ascorbic acid and crude methanolic leaf, stem and root extract of *P. zeylanica* in DPPH assay

S. No.	Samples	IC_{50} value ($\mu g\ mL^{-1}$)
1	Standard Drug-Ascorbic acid	45.34±2.57
2	Crude methanolic leaf extract	48.34±3.74
3	Crude methanolic stem extract	65.13±2.08
4	Crude methanolic root extract	41.67±3.33

Table 4: Effect of crude methanolic extracts of *P. zeylanica* in superoxide anion scavenging assay

Sample concentration ($\mu g\ mL^{-1}$)	% of scavenging			
	Ascorbic acid (Standard)	Crude methanolic extracts		
		Leaf	Stem	Root
12.5	25.12±0.56	20.32±1.56	16.28±2.15	26.45±2.13
25	38.18±3.98	36.24±2.45	29.31±3.89	38.98±3.01
50	52.17±2.19	54.10±3.12	40.18±2.98	56.23±2.56
100	65.98±1.35	67.22±2.41	52.31±3.17	71.13±1.02
200	71.25±2.13	75.81±2.15	72.31±2.01	83.23±2.12

Table 5: Comparative IC_{50} values of ascorbic acid and crude methanolic leaf, stem and root extract of *P. zeylanica* in superoxide anion scavenging assay

S. No.	Samples	IC_{50} value ($\mu g\ mL^{-1}$)
1	Standard Drug-Ascorbic acid	48.89±1.67
2	Crude methanolic leaf extract	43.07±2.45
3	Crude methanolic stem extract	95.47±3.16
4	Crude methanolic root extract	39.38±1.03

(2019) reported the *in vitro* antioxidant activity of the ethanolic root extract of *P. zeylanica* in 2006. Nahak & Sahu, (2011) reported the antioxidant activity in the root extracts of *P. zeylanica* and *P. indica* comparatively by the DPPH assay.

Superoxide radical scavenging assay

Superoxide radicals are very harmful to cellular components, important reactive oxygen free radicals with weak chemical activity, cannot penetrate lipid membranes and immediately converted to H_2O_2 by superoxide dismutase. The extract's ability to prevent formazone production by scavenging the superoxide anions produced in the riboflavin light NBT system served as the basis for the superoxide anion scavenging experiment (Stief, 2003). The superoxide anion was significantly scavenged by the plant extracts, and this action increased as concentrations rose from 12.5 to 200 µg mL^{-1} (Table 4 & Figure 2). Maximum superoxide radical scavenging activity was found to be 83.23±2.12 % at 200 µg mL^{-1}concentration of methanolic root extract. Comparative IC_{50} values of ascorbic acid and crude methanolic extracts of *P. zeylanica* were shown in Table 5. IC_{50}values exhibited by root methanol extract (39.38±1.03 µg mL^{-1}) was comparatively less than that of standard drug ascorbic acid (48.89±1.67 µg mL^{-1}) which indicated that root extract possessed significantly higher superoxide scavenging activity than that of leaf and stem extracts of *P. zeylanica*. Same type of scavenging activity was reported on the aqueous leaf extract of *Indigofera tinctoria*

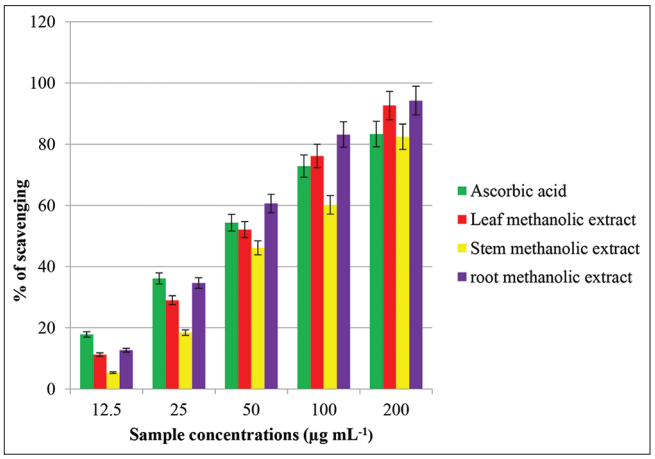

Figure 1: Effect of crude methanolic extracts of *P. zeylanica* in DPPH Assay

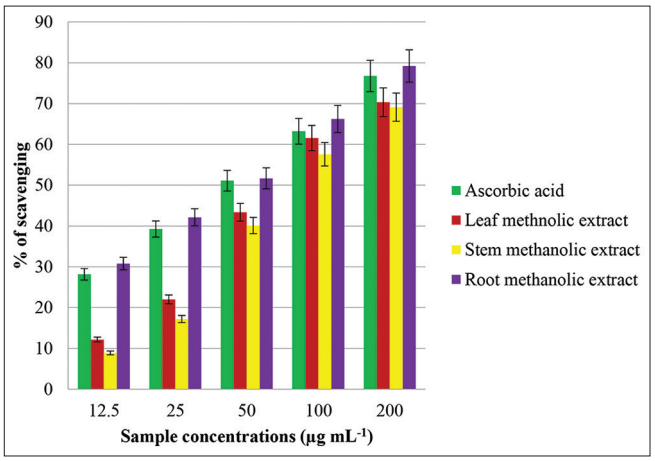

Figure 2: Effect of crude methanolic extracts of *P. zeylanica* in superoxide anion scavenging assay

(Srinivasan *et al.*, 2016). In this study of *I. tinctoria*, 250 µg mL^{-1} aqueous extract exhibited 26.79% of optimum inhibition and the IC_{50} value was 46.59 µg mL^{-1}. The result of *P. zeylanica* has a more powerful scavenging effect on superoxide anion than that of *I. tinctoria*.

Nitric oxide scavenging assay

In aqueous solution, sodium nitroprusside continuously produces nitric oxide, which then combines with oxygen to produce nitrite ions, which can be measured using the Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Large amounts of nitric oxide may cause tissue injury (Gincy & Sasikumar, 2007). In the current investigation, methanolic root extract exhibited highest capacity (79.23±2.11%) to react with oxygen and inhibit the production of nitrite ions than that of leaf and stem extracts of *P. zeylanica* (Table 6 & Figure 3). Comparative IC_{50} values of ascorbic acid and crude methanolic extracts of *P. zeylanica* were shown in Table 7. The IC_{50} value exhibited by root methanol extract (42.17±2.42 µg mL^{-1}) was very much similar to that of standard drug ascorbic acid (39.38±1.03 µg mL^{-1}). A similar type

Table 6: Effect of crude methanolic extracts of *P. zeylanica* in nitric oxide scavenging assay

Sample concentration ($\mu g\ mL^{-1}$)	% of scavenging			
	Ascorbic acid (Standard)	Crude methanolic extracts		
		Leaf	Stem	Root
12.5	28.17±1.23	12.17±0.03	8.89±2.12	30.78±0.23
25	39.27±0.18	22.01±1.33	17.23±1.11	42.12±1.34
50	51.11±1.21	43.35±2.89	40.10±0.14	51.67±2.11
100	63.23±2.11	61.56±1.11	57.56±1.06	66.23±1.43
200	76.78±0.23	70.34±0.98	69.13±0.08	79.23±2.11

Table 7: Comparative IC_{50} values of ascorbic acid and crude methanolic leaf, stem and root extract of *P. zeylanica* in nitric oxide scavenging assay

S. No.	Samples	IC_{50} value ($\mu g\ mL^{-1}$)
1	Standard Drug-Ascorbic acid	39.38±1.03
2	Crude methanolic leaf extract	65.23±3.78
3	Crude methanolic stem extract	80.35±1.67
4	Crude methanolic root extract	42.17±2.42

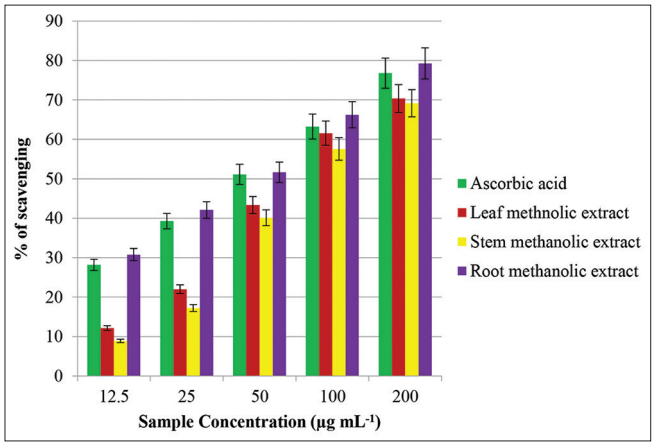

Figure 3: Effect of crude methanolic extracts of *P. zeylanica* in nitric oxide scavenging assay

of nitric oxide scavenging activity was noticed on petroleum ether leaf extract of *Boerhavia diffusa* (Vasundhara & Devi, 2015) which was low as compared to *P. zeylanica*.

CONCLUSION

The findings from the current investigation, root extract of *P. zeylanica* has the highest potential for antioxidant than that of the leaf and stem extracts. The secondary metabolites especially flavonoids, phenols and tannins present in roots might be contributing to this highest antioxidant activity. This plant's sustainable use will benefit from this research. To assess and identify the bioactive components causing this antioxidant action, more research is required.

ACKNOWLEDGEMENTS

The laboratory support for this study was provided by the Department of Botany at T.K.M College of Arts & Science in Kollam, Kerala, for which the authors are grateful.

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