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HPTLC fingerprint of 85% methanolic extract of the root bark of *Plumbago zeylanica* Linn and it's lethal dose estimation in mice

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ABSTRACT
The root bark of *Plumbago zeylanica* Linn has potent analgesic and anti-inflammatory activity which has been used in many formulations of Indian system of medicine. The present studies investigated the quality of different solvent extracts from this plant and analyzed the safety of methanolic extract in mice. The qualitative studies for phytochemical constituents done on the extracts revealed the presence of more constituents such as polyphenols, sterols, CHO and proteins in the methanolic extract of this plant among other extracts and also this extract yield more. The HPTLC fingerprint of this extract shows the presence of seven compounds with high concentration at R_f 0.85. The methanolic extract was screened for acute toxicity study in rats and mortality was not found up to the dosage of 4000 mg/kg b.wt. By Litchfield and Willcoxan method, LD50 estimated more than 4000 mg/kg and ED50 found from 200 mg/kg to 400 mg/kg b.wt.

Keywords: *Plumbago zeylanica* Linn, Methanolic extract, Phytochemicals, Fingerprinting, Acute toxicity.

1. Introduction
Plumbago zeylanica (Plumbaginaceae), commonly known as Lead Wood is a perennial herb grows up to 1.5 m having ovate and subacute leaves with elongated spikes, white slender corolla, and oblong pointed capsule [1]. It is distributed throughout India and other Asia countries [1]. The root and root bark of this herb have much therapeutic values having bitter sharp taste, stomachic, and carminative action, and astringent to the bowels, anti-helmintic, and alterative action and cures intestinal troubles, dysentery, leucoderma, inflammation, piles and bronchitis. The leaves are caustic, vesicant, aphrodisiac and good for scabies externally [2]. In Siddha system of medicine, the root bark of *P. zeylanica* is extensively used as an ingredient in many Sastric formulations such as *Rasagandhi mezhugu*, *Nandhi mezhugu*, *Poora mathirai*, etc., in treating Cancerous pain [3]. Plumbagin, a compound isolated from this plant is said to be very effective in Cancer management [4]. The objectives of this study were to access which type of solvent extract of *Plumbago zeylanica* gives better yield and has more active constituents and profiling HPLC finger print and estimating the median lethal dose of the particular extract.

2. Materials and methods
2.1 Collection and authentication of plant material
The whole fresh parts of *Plumbago zeylanica* were collected from TAMPCOL farm, Chennai. Macroscopic and microscopic characterization was observed using standard methods described by Trease and Evans, 1958 [5] and got authentication (Voucher No. 000568) from the botany department of Captain Srinivasa Murthi Drug Research Institute, Dept. of AYUSH, Chennai. Moreover, the plant marker compound Plumbagin was identified for authentication. The hydro alcoholic extract was fractionated with chloroform. The chloroform extract was concentrated *in vaccuo* and subjected to column chromatography in the silica gel of the size 60-120 mesh and eluted using solvents of increasing polarity (Hexane→Hexane : Chloroform→Chloroform). The compound obtained in the mobile phase was compared with authentic standard Plumbagin obtained from Hi-Media.

2.2 Extracts preparation
The collected plants were shade dried for a period 10 days and then coarsely powdered using a pulverizer. The pulverized herbs were then stored in airtight containers until further use. The coarsely powdered plant drugs were subjected to successive soxhlet extraction with various solvents of increasing polarity and stored in the refrigerator. The extracts were concentrated *in-vaccuo* and percentage of yields was calculated.

2.3 Qualitative and quantitative phytochemical analyses
The different extracts obtained by the above method were subjected to qualitative phytochemical tests following the standard protocol of Harborne, 1989 [6]. Each test was repeated thrice. The quantification was done in raw air dried plant material as well as in the hydro alcoholic extract and the values were estimated by the mean of five replicates. The total phenolic was estimated using Folin-Ciocalteau reagent by the method of Bray HG *et al* [7]. The amount of total tannin was estimated using Folin-Denis reagent by the method of AOAC [8]. The amount of total carbohydrate was estimated using Phenol-Sulphuric acid by the method of Dubois *et al* [9]. The amount of Vitamin C and E were estimated using dinitrophenyl hydrazine by the method of Sarojini *et al*. [10] and dipyridyl method by Jayasree *et al.* [11] respectively.

2.4 HPTLC Finger printing
Chromatography was performed on a 10 x 3 cm pre-coated HPTLC Silica gel $60F_{254}$ plate as stationary phase. The plates were washed with methanol and activated at 60 ^{0}C for 5 min prior chromatography. 10 µl of hydro alcoholic extracts prepared at a concentration of 1 mg/ml in alcohol were applied to the plate of 6mm band using CAMAG Linomat V applicator. The slit dimension was kept at 5 mm x 0.45 mm and 20 mm/sec scanning speed was employed. The mobile phase Toulene: Ethyl acetate (6: 4) was chosen after trial and error and 10 ml of the mobile phase was used for chromatography. Linear ascending development was carried out in 20 x 10 cm twin glass chamber saturated with the mobile phase. The plates were photo documented at 254 nm and 366 nm. The chromatogram of the developed plates was scanned at 265 nm.

2.5 Animal procurement and maintenance
Swiss strain Albino mice of either sex, weighing 20-30 g were purchased from The Tamil Nadu Veterinary University Animal House, Chennai, India and they were acclimatized in SASTRA University Animal University, Tanjore, India at 21-23 ºC. Animal ethical guidelines of CPCSEA, Ministry of Animal Husbandry and Welfare, Govt. of India were strictly followed for the care and maintenance of procured animals. The animals were fed on standard rodent pellet and RO water was provided *ad libitum*. The animals were kept for overnight fasting before experimentation.

2.6 Acute Toxicity protocol
The animals were divided into 10 groups of each containing 8 rats per group. The methanolic extract was suspended in 2 ml, 5% Tween 80 and administered orally as a single dose ranging from 100 to 4000 mg/kg body weight respectively to all groups. The median lethal dose, LD_{50} and effective dose ED_{50} value of the extract were calculated according to the method of Litchfield and Willcoxan, 1949 [12] by plotting the log dose against the probit values.

3. Results and discussion
3.1 Authentication of Plant: The stem was terate, angular and smooth. T.S of the stem shows the following features. Epidermis show single cell layered with cuticles. The epidermal cells are short and broad. The cortex cells are parenchymatous in which some of the cells contain chloroplast, starch grains and phenols. The chlorenchyma cells are broad and arranged in rings. Phloem fibres are present with single cell thickness, but in some places the fibres are present with 3-4 cell thickness. Phloem cells containing starch grains are seen. Cambium shows two cell thickness and these cells are narrow and rectangular in shape. Xylems are endarch with several cell thicknesses which are broad and polygonal. The xylem vessels are also broad. The pith regions show parenchymatous, large polygonal cells arranged without intercellular spaces. T.S of root shows the following the features. Periderm shows two cell thickness with thick cell walled. Lenticels are present in some region. Epidermis show 2-3 cell thickness and they are rectangular in shape. The cortex cells are spherical contains starch grains. Phloem fibers are present with 1-2 cell thickness with lignified cell walls. The phloem cells are 7-8 thickness and they are short and compactly arranged cells. Starch grains are present in the phloem cells. Cambium shows 2-3 cell thickness. Xylems are exarch with several cell thickness and the vessels are broad. The pith cells are parenchymatous. Some of the cells contain starch grains and calcium crystals. An orange crystalline compound obtained in the mobile phase was identified as Plumbagin by comparison with an authentic sample at 2.74 RT.

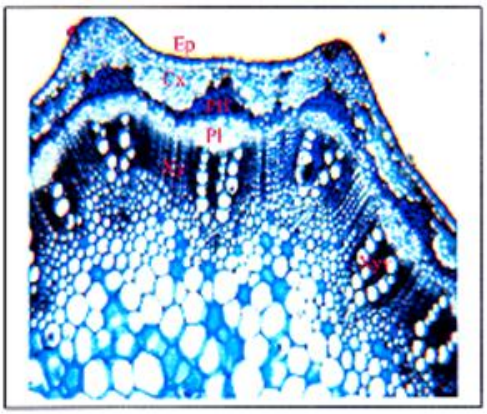

Plate 1: Transverse section of stem

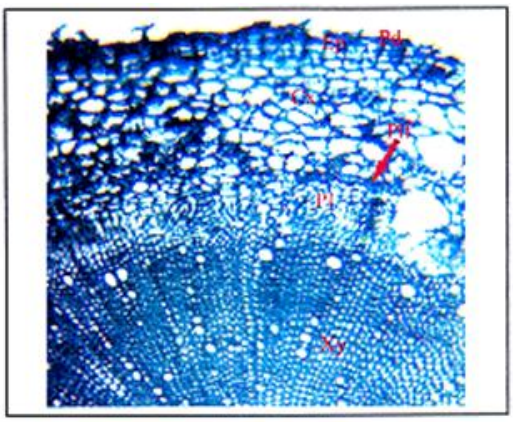

Plate 2: Transverse section of stem
(A portion enlarged)

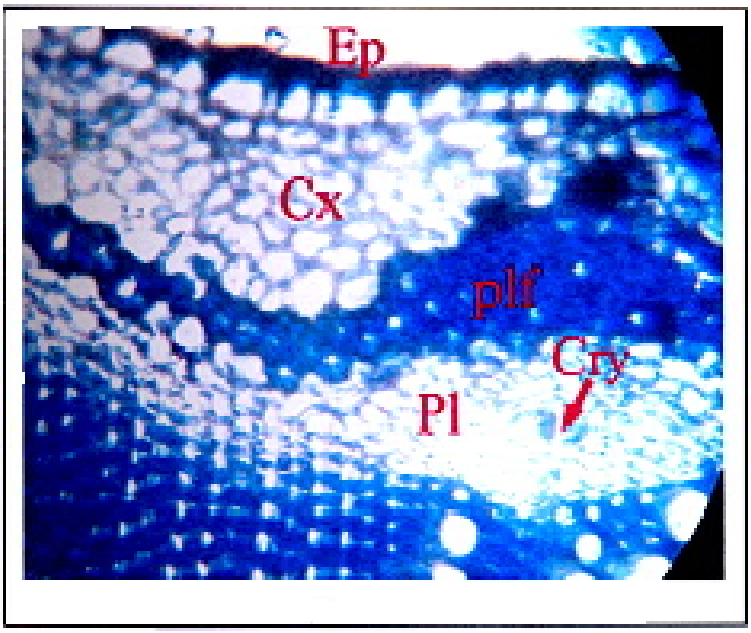

Plate 3: Transverse section of root

3.2 Qualitative and quantitative phytochemical analyses

The extract yielded by the solvent 85% methanol in both cold and hot method show more yield 7% rather than other extract. The results were shown in table 1. The qualitative phytochemical analyses (Table 2) show the presence of polyphenol, phytosterol, carbohydrate, protein and amino acids in the methanolic extract among other extract. The result of concentration of active constituents in raw material and methanolic extract was shown in table 3. Methanolic extract possesses high yield and more phytoconstituents, this extract is further analyzed for chromatographic finger print and acute toxicity study. The methanolic extract shows efficacy against skin diseases and has hepatoprotective action [1].

Table 1: % Yield of Extracts

% Yield									
Hexane		**Chloroform**		**Ethyl acetate**		**85% Methanol**		**70% Ethanol**	
Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot
2.45	2.68	2.96	3.02	1.78	2.08	6.78	6.86	1.36	1.54

Table 2: Qualitative analysis of different extracts

Phytoconstituents	Hexane	Chloroform	Ethyl acetate	85% Methanol
Alkaloids	-	-	-	-
Flavanoids	-	-	-	-
Polyphenolics	+	+	-	+
Phytosterol	+	+	+	+
Saponins	-	-	-	-
Fixed oils and fats	-	-	-	-
Carbohydrates	-	-	-	+
Amino acids and proteins	-	-	-	+

Table 3: Concentration of active constituents

Active compound	% Concentration of raw plant	% Concentration in extract proportionate to yield from raw plant	% Concentration expected in extract
Phenols	2.34±0.27	0.0107±0.0008	0.79±0.07
Tannins	1.68±0.34	0.0589±0.0012	0.79±0.21
Carbohydrates	3.8±0.45	0.0089±0.0002	4.66±1.21
Vitamin C	1.44±0.98	0.0520±0.0005	1.84±0.43
Vitamin E	0.89±0.08	0.0115±0.0006	0.85±0.01

3.3 HPTLC profile of Methanolic extract

The HPTLC finger print shows peaks at maximum R_f values 0.1, 0.15, 0.19, 0.39, 0.51, 0.78 and 0.85 (Fig. 1) The peak at an R_f value of 0.85 is found to have a greater area (12927.8) among the observed peaks. The photodocumentation report at 366 nm and 254 nm were shown in plate 4.

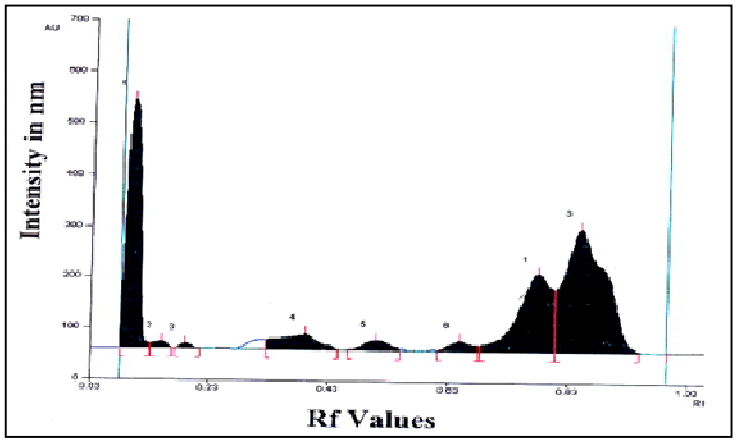

Fig 1: HPTLC Finger printing of Methanolic extract of *Plumbago zeylanica*

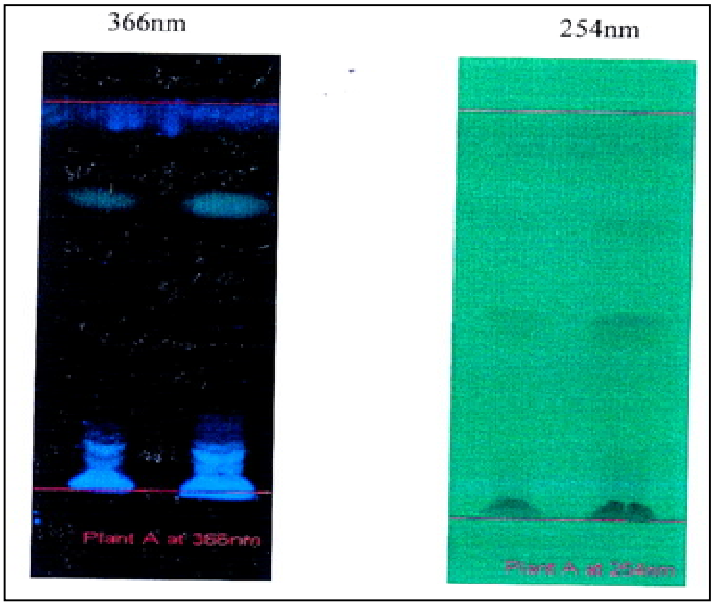

Plate 4: Photo documentation report of Methanolic extract of *Plumbago zeylanica*

3.4 Determination of LD_{50} and ED_{50} values of Methanolic extract

The methanolic extract exhibited no lethal effect on mice up to the dosage of 4000 mg/kg b.wt. The effective dose value found to fall between 200-400 mg/kg b.wt. No other signs of toxicity were observed in the acute toxicity study. The data obtained from the study is shown in the table 4.

Table 4: Acute Toxicity Data in Mice for Methanolic Extract

Group	Dose mg/kg	Log dose mg/kg	Dead /Total	Dead %	Corrected %	Probit
1	100	2.0	0/8	0	3.12	3.19
2	200	2.301	0/8	0	3.12	3.19
3	300	2.477	0/8	0	3.12	3.19
4	500	2.698	0/8	0	3.12	3.19
5	700	2.845	0/8	0	3.12	3.19
6	900	2.954	0/8	0	3.12	3.19
7	1100	3.041	0/8	0	3.12	3.19
8	2000	3.3.1	0/8	0	3.12	3.19
9	3000	3.4771	0/8	0	3.12	3.19
10	4000	3.6021	0/8	0	3.12	3.19

Note: Correction for 0% Dead was calculated using the formula 100 x (0.25 / n) = 100 (0.25/8) = 3.12

4. Conclusion

The authors conclude that the HPTLC fingerprint of the methanolic extract of the root bark of *P. zeylanica* shall be used as standard in accessing the quality of this plant. Further, this extract has good safety profile shall be consumed even more than 4000 mg/kg body weight.

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