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Full Length Research Paper

Anti-inflammatory and cytotoxic effects of extract from *Plumbago zeylanica*

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***Plumbago species* (Family-Plumbaginaceae) are one of the most important medicinal plants which are used for anti- inflammatory, antimicrobial diseases. Our work involves the study of anti inflammatory and cytotoxic effects of *Plumbago zeylanica.* The root of *P. zeylanica* extracted with methanol was used for determining the anti inflammatory effects. The methanolic extracts at 300 and 500 mg/kg produced 31.03 and 60.3% inhibition of acute inflammation, respectively, in Carrageenin induced raw paw oedema confirming that *P. zeylanica* roots are effective against acute inflammation. For the evaluation of cytotoxicity, the crude dichloromethane extract was subjected to silica gel column chromatography and 120 fractions were collected. Their structures were elucidated with the help of spectroscopic techniques. High performance liquid chromatography (HPLC) was performed to determine the purity of gugultetrol-18-ferrulate in crude extract and the structure of betasitosterol and gugultetrol-18-ferrulate was identified using nuclear magnetic resonance spectroscopy analysis (^{1}H and ^{13}C NMR), Infra red and mass spectroscopy. The lethal concentration (LC50) value was observed for crude extract, betasitosterol, gugultetrol-18- ferrulate and it was found to be 90, 75 and 65 ppm, respectively. The use of *Plumbago* species as an effective anti inflammatory agent and its cytotoxic effects have been ascertained and proved.**

Key words: *Plumbago zeylanica,* anti inflammatory, cytotoxicity, betasitosterol, gugultetrol-18-ferrulate, high performance liquid chromatography.

INTRODUCTION

Today, Ayurvedic, Hoemoeo and Unani Physicians utilize numerous species of medicinal plants. (Narayana and Thamanna, 1987). Many compounds used in today's medicine have a complex structure and synthesizing these bioactive compounds chemically at a low price is not easy (Mujumdar et al., 2000; Madhava, 1998). The increasing awareness about side effects of drugs had made the western pharmaceutical industries to turn towards the plant based Indian and Chinese medicine (Balandrin and Klocke, 1988). *Plumbago* popularly known as chittiramulam, in Tamil and white leadwort in English. *Plumbaginaceae* is distributed as a weed throughout the tropical and subtropical countries of the world. The family *Plumbaginaceae* consists of 10 genera and 280 species.

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The genus *Plumbago* includes 3 species, namely *Plumbago indica. L, Plumbago rosea. L, Plumbago capensis. L, and Plumbago zeylanica .L,* which are distributed in several parts of India. In *P. zeylanica* root and bark is an active part used as a traditional herbal medicine to treat several diseases. Compounds isolated from *P. zeylanica L.* are composed of naphthoquinone, such as plumbagin, 3- biplumbagin, 3- chloroplumbagin, chitranone, elliptinone, isoshinanolone and coumarins such as seselin, 5-methoxyseselin, suberosin, and xanthyletin. Other compounds such as 2, 2-dimethyl-5-hydroxy-6-acetylchromene, plumbagin acid have also been isolated and identified (Yuan-Chuen Wang 2005; Michael, 1956). The whole plant and its root have been used as a folk medicine in Taiwan for the treatment of rheumatic pain, menostasis, carbuncle and injury by bumping (Okoli and Akah, 2005). Roots and root barks of this plant are the most frequently used plant parts which have traditionally

Figure 1. Plant of *P. Zeylanica*

been used for the treatment of various ailments, such as dyspepsia, piles, diarrhoea, skin diseases, leprosy and also reported to posses antibacterial, antifungal, (Uma et al., 1999) and vesicant diuretic properties and further may be used as a substitute for cantharides (Nguyen et al., 2004). The pharmacological importance of this peren-nial shrub lies in its ability to produce a napthoquinone, called plumbagin (Ayo, 2007), mainly in its roots. Recent discoveries of the tumor inhibitory (Krishnaswami and Puroshothaman, 1980; Roober, 1996) and radiomodifying effects (Uma et al., 1999) . Plumbagin (2-methyl-5-hydroxy- 1,4-naphtho- quinone) is a natural naphtha-quinone showing a broad range of pharmaceutical activities.

Pharmacological effects of plumbagin have been inves-tigated on anti-cancer, anti-leishmanial, anti-plasmodial, anti-bacterial, anti-fungal, anti-inflammatory, antihyperglycemic, hypoli-pidaemic, antiatherosclerotic, antiallergic, central nervous system stimulatory, cytotoxic and anti-insecticidal property. (Yuan-chuen et al., 2005; Yen- Ju et al., 2006; Vanisree et al., 2004). Plumbagin was also reported to be an effective chitin-synthetase inhibitor (Sleet et al., 1983; Renata, 2001).

The root of *P. zeylanica* has been reported to be a powerful poison when given orally or applied to ostium uteri, causes abortion (Azad et al., 1982). The acute toxicity studies of *P. zeylanica* in albino rats revealed that the oral LD50 of the drug is 65 mg/kg body weight and in the dead animals, the post mortem revealed a profuse bleeding in the viscera (Premakumari et al., 1977). The structure of the plumbagin has been reported to closely resemble the vitamin K and the anticoagulant property of *P. zeylanica* might be similar to coumarin derivative, the hemorrhage may be due to the competitive inhibition of vitamin K activity, needed for the synthesis of clotting factors (Santhakumari and Rathinam, 1978). The anti-coagulant activity of the *P. zeylanica* was reported after an hour exposure and the effect of *P. zeylanica* on platelets and coagulation profile lead to the development of an antithrombotic drug (Shahbiren and Nayak, 2004). The root of *P. Zeylanica* has been reported in the literature for its biological activity such as antiparasitic (Ramachandra and Ravishankar, 2002) and insect antifeedant (Alluri and Rao, 2005).

MATERIALS AND METHODS

Plant extraction

The plant *P. zeylanica* root was collected, shade dried and powdered (Zaheer and Ahsana, 2008). The powdered 1 kg of the material was soaked in solvent dichloromethane (4000 ml) for 48 h and repeats the process for three times to get complete extraction. The solvent was removed in a rotary vacuum and stored the extract in refrigerator for further study. Figure 1 shows the whole plant and root of *P. zeylanica*. Phytochemical analysis was done to check for the presence of steroids, alkaloids, flavonoids and terpenoids etc.

Thin layer chromatography (TLC)

TLC is performed using silica gel 60 F_{254} percolated on alumna sheets. The metabolites were applied point wise as different spots on TLC plates and must be eluted with different solvent system. The plate was viewed under ultra violet (UV) lamp at 254 nm. For further clarity the plates were derivatised, using PUNCAL-D solution (A solution of Cerisulphate (1.6 g) and Ammonium hepta molybdate (21.6 g) Conc. Sulphuric acid (50 ml) in 450 ml of water. Spraying the reagent on TLC plate followed by drying and heating did derivitisation at 130°C in a hot air oven. Blue colored spots appear indicates the presence of organic molecules.

High performance liquid chromatography (HPLC)

(Renata M. S. Celeghini), to check the purity of isolated compound and crude extract of dichloromethane, High resolution HPLC was performed using shimadzu LC –10AT up chromatograph provided with isocratic pump and UV visible detector. The crude dichloro-methane extracts were filtered through 2 μ -membrane filters and used for analysis. Column of C_{18} ODS, Gemini 5 μ, 110A of dimensions 250 x 4.5 mm with mobile phase 70:30:1 (methanol : water : acetic acid), was used at flow rate of 0.5 ml / min. The detection wavelength was 254 nm and injection volume was 20 μl.

Anti-inflammatory activity

The experimental design was approved by the ethical committee of central research institute for Siddha, (CCRAS), Chennai – 600 106 (TN). {Ethical approval No. 37/IAEC/Pharma/CRIS/2006} wister rats were obtained from department of Laboratory Animal Sciences (CPCSEA Registration No: 512/01/a/2001/CPCSEA), (Winter et al., 1962) Animals were housed in groups of three and two in two standard suspended polycarbonate cages with top grill having facilities for feed and drinking water in glass bottles with stainless steel sipper tubes. The environmental conditions were maintained

Table 1. Chemical test results for *P. zeylanica* constituents.

S.NO	TEST	OBSERVATION	RESULT
1.	Terpenoids	Appearance of blue color	presence
2.	Flavanoids	Appearance of Yellow color	Presence
3.	Steroids	Absence of pale green color	Absence
4.	Carbohydrate	Absence of pale green to cherry red	Absence
5.	Alkaloids	Absence of orange color	Absence
6.	Tannins	Absence of blue color	Absence

at 21 ± 2°C, and relative humidity of 50 - 55% with a 12 h light/dark cycle. The animals had free access to sterile feed of standard composition containing all macro and micronutrients. Aqua guard water filter-cum-purifier was provided. 100 g of root were extracted with 500 ml of methanol and then filtered. The filtrate was concentrate to dryness in a rotary vacuum evaporator less than 40°C and used for animal study.

Adult albino rats of either sex (80 – 120 gm) were divided into four groups, each consisting of six animals. 0.1 ml of freshly prepared suspension of carrageenin (1 in 0.5% CMC) was injected under planter aponeurosis of right hind paw of the rat according to the method of Winter et al. 1962. Four groups were treated with two dose levels of 300 and 500 mg/kg.

One group received only Tween 80 r (vehicle), which served as control while another group received Diclofenac in the dose of 10 mg/kg to serve as standard drug for comparison. The paw volume was measured by plethysmograph before and after three hours of carrageenin treatment (Charles, 2006). The increase in paw volume has been expressed as percent increase over control recorded in terms of unit.

Percent inhibition of inflammation was calculated using the formula:

$$\text{Percent Inhibition} = \frac{Vc - Vt}{Vc}$$

Where, Vc and Vt represents average paw volumes in control and treated group, respectively.

Experimental design

For anti- inflammatory study, rats weighing (80 -120 g) were selected. The data were subjected to statistical analysis as per (Srinivas, 2000), by using the test of significance at 1 and 5% levels. All the parameters were statistically analyzed by student's 't' test.

Cytotoxicity test

Cytotoxicity test was carried out using the standard procedure as described by Meyer et al. (1982),(McLaughlin (1991) and Parra et al. (2001) . Samples were prepared by dissolving in (3 ml) of DMSO. From this solution, the concentrations of 500, 250, 100, and 50 ppm were prepared by serial dilution. Each concentration was tested for cytotoxicity in triplicates, using DMSO as control brine shrimp eggs (*A. salina* Leach) were hatched in a hatching chamber, filled with fresh sea water. Ten larvae of brine shrimps were transferred to each sample test-tube using disposable pipettes. The test tubes were maintained under illumination. Survivors were counted after 24 h and the percentage death at each concentration was deter-mined (Meyer et al., 1982; McLaughlin, 1991). The LC_{50} values were obtained from the best-fit line plotted concentration verses percentage lethality.

RESULTS AND DISCUSSIONS

The phytochemical screening of dichloromethane extract of *P. zeylanica* root confirmed the presence of terpe-noids, flavanoids and absence of steroids, Carbohydrate, Alkaloids and Tannins. The results are given in Table 1. The dichloromethane extract of *P. zeylanica* root and pure fraction was subjected to TLC using Hexane : Ethyl acetate (7:3) and Methanol: Ethyl acetate (2:8) as mobile phase. The dried plates were viewed under UV lamp at 254 nm. For further clarity the plates were derivatised using PUNCAL-D solution {a solution of Cerisulphate (1.6 g) and Ammonium hepta molybdate (21.6 g) in 50 ml of Conc. Sulphuric acid}. Appearance of blue colored spots indicates the presence of organic molecules.

This fraction was checked for its purity as single spot by HPTLC. To check the purity of isolated compound and crude extract of dichloromethane the compounds were subjected to HPLC. The result obtained by gradient chromatography on C-18 column with UV detection at 254 nm and eluted with 70:30:1 (methanol: water: acetic acid). Guggultetrol-18-ferrulate content was examined with the above HPLC method for the 14 different sources of sample of *P. zeylanica.* The results shown in (Figure 2) shows HPLC chromatogram of dichloromethane extract of *P. zeylanica* as gugultetrol-18-ferrlutate. The retention time for the 14 different samples in the crude extract was given with the gugultetrol-18-ferrlutate retention time as 4.913 min. The mean increase in paw volume in methanol extract of *P. zeylanica at* 300 and 500 mg/kg and Diclofenac treated groups was 0.41 ± 0.061, 0.23 ± 0.083 and 0.20 ± 0.02 ml, respectively, as compared to 0.58 ± 0.098 in control group. The methanolic extract of *P. zeylanica* roots were tested for its anti-inflammatory effects at 300 and 500 mg/kg concentrations produced 31.03 and 60.30% ($P \leq 0.01$) inhibition of acute inflame-mation, respectively; in carrageenen induced rat paw oedema proves that methanolic extracts of root are effective against acute inflammation (Table 2). Cytotoxi-city of dichloromethane extract of *Plumbagoz zeylanica*

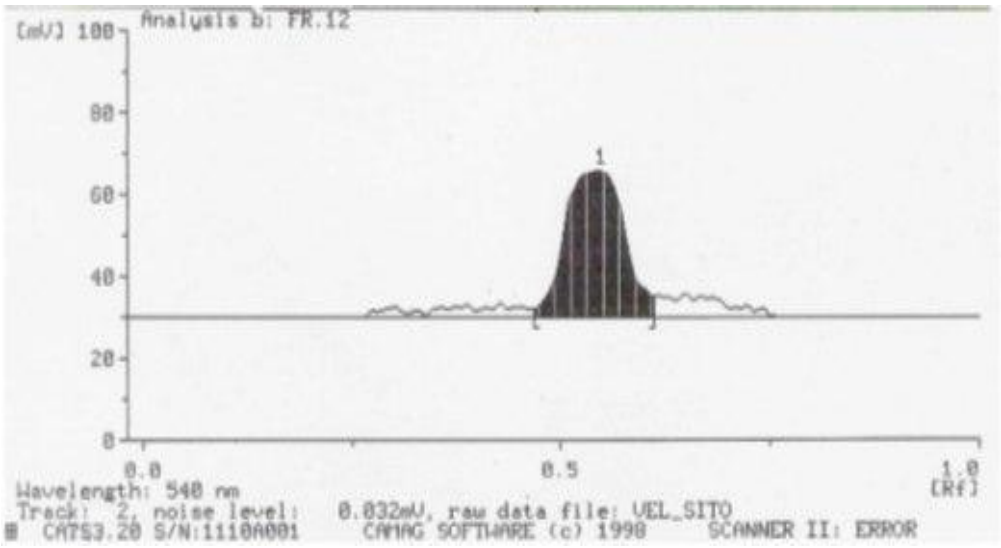
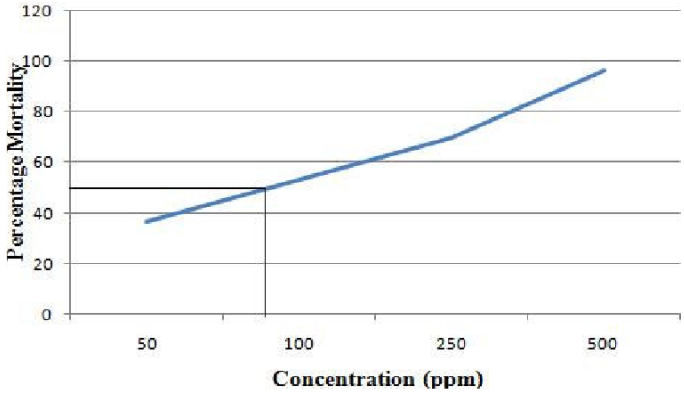


Figure 2. HPTLC Chromatogram of beta sitosterol.

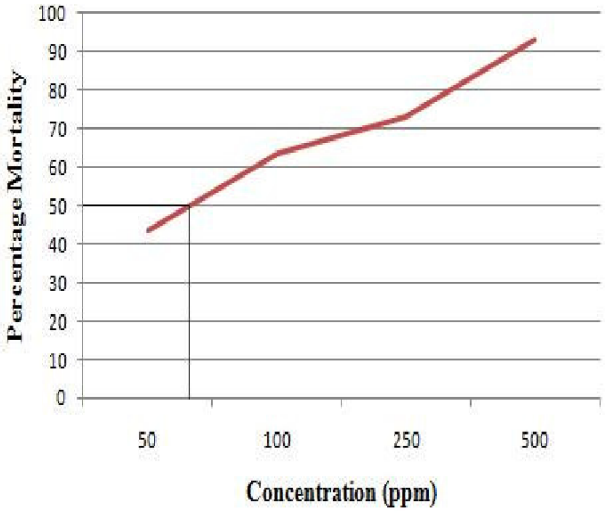
Table 2. Effect of methanol extract of *p. zeylanica* in carrageenin induced rat paw oedema in rats.

S.No	Groups	Increase in paw volume (ml) Mean ± S.E	Inhibition of inflammation (%)
1.	*P. zeylanica* (300 mg/kg)	0.41**± 0.061	31.03
2.	*P. zeylanica* (500 mg/kg)	0.23** ± 0.083	60.30
3.	Diclofenac	0.20** ± 0.02	65.55
4.	Control	0.58 ± 0.098	Nil

N = 6 (Student 't' test), ** Significant at p ≤ 0.01



Graph 1. Cytotoxicity of dichloromethane extract of *p. zeylanica*(graph for Lc50).



Graph 2. Cytotoxicity of beta sitosterol isolated from *P. Zeylanica* (Graph for LC50).

*revealed a known pattern (*Graph 1*),whereas the c*ytoto-xicity of beta sitosterol isolated from *Plumbago Zeylanica showed distinct similarity with the previous result(*Graph 2*).* Cytotoxicity of Guggultetrol-18-ferrulate was found to be normal (Graph 3).

The fraction 12 was eluted using 2% Ethanol/ Hexane (300 mg) and another fraction 84 was eluted using IPA/ Ethanol (100 mg) with traces of impurity. High performance thin layer chromatography was performed for both fractions 12 and 62 - 65 in Hexane: ethyl acetate

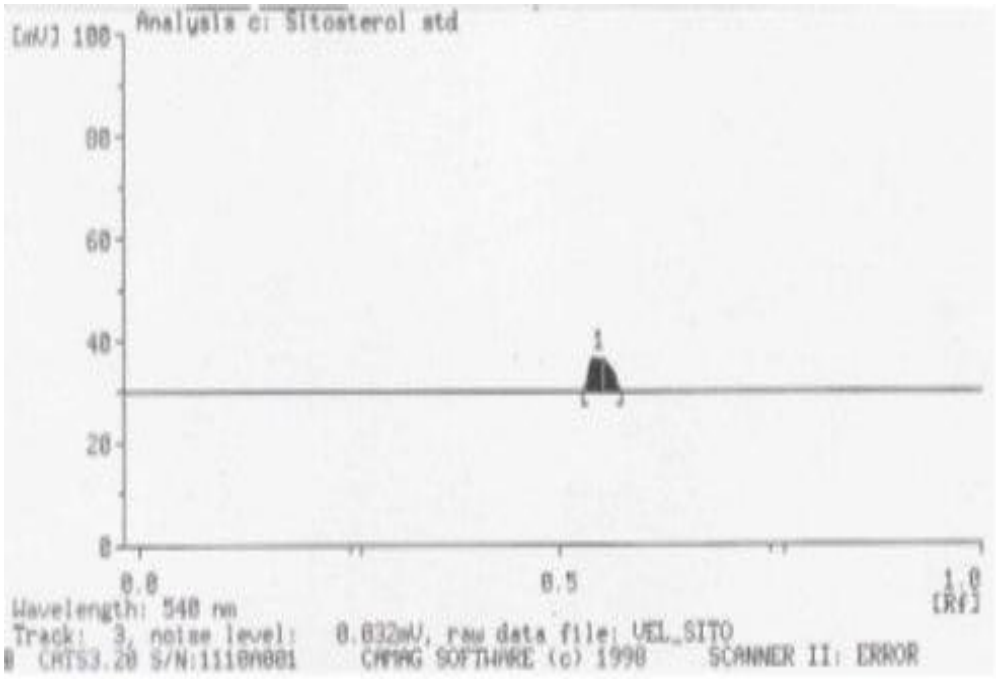
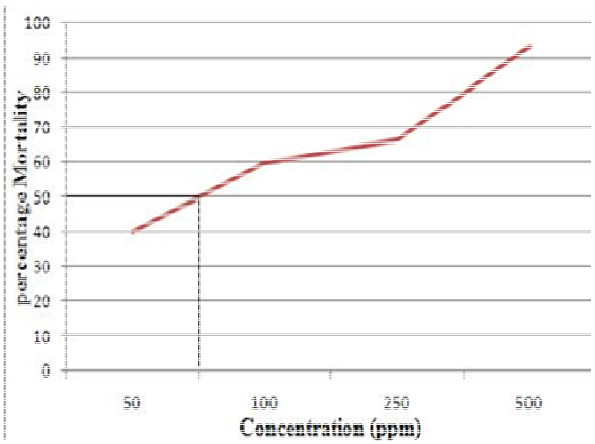


Figure 3. HPTLC Chromatogram of beta sitisterol standard



Graph 3. Cytotoxicity of Guggultetrol-18-ferrulate (Graph for LC50).

(7:3) and methanol: ethyl acetate (2:8) respectively. It was found that the fraction 12 has the R_f value of 0.5 cm and the fraction 62 - 65 has the R_f value of 0.7 cm, respectively, (Figure 3). The high purity of guggultetrol-18-ferrulate in crude extract was confirmed by high performance liquid chromatogram with a retention time of 4.86 and 4.91 min, respectively.

The structural analysis of isolated compound was identified using ^{1}H and ^{13}C NMR, Infra red and mass spectroscopy. Through ^{1}H NMR analysis, the CH_3 group is assigned in the range 0.83 - 1.00 ppm. The CH_2 peak corresponds to this 1.10 - 1.50 ppm. The CH group is assigned to the 1048 - 1.82 ppm. IR spectrum shows 2937 cm^{-1}. The olefin double bond peaks appear at 5.36 ppm whose IR peaks appear at 1464 cm^{-1}. The hydroxyl group appear in the ^{1}H NMR at 3.53 ppm which corresponds to the secondary alcohol. IR peaks at 3435 $cm^{-1.}$ From the ^{13}C NMR analysis CH_3 peaks at 12.12 - 18.98 ppm. The CH_2 peaks are assigned to18.98 - 28.24 ppm.

The CH peak is assigned to 33.95 - 56.77 ppm. The double bond peaks at 140.76 - 121.72 ppm. The secondary hydroxyl group at 71.81 ppm. IR peaks CH_2, CH appears at 2868 cm^{-1}. The molecular peaks at m/e: $414(M)^{+}$ and molecular formula is $C_{29}H_{50}O$, respectively. From the above discussion the structurally possible compound is assigned as beta sitosterol (Figure 4). ^{1}H NMR the CH_3 group is assigned to 0.92 ppm all CH_2 peaks corresponded to the 1.29 - 1.34 ppm. Secondary hydroxyl group assigned to 3.362 - 3.59 ppm, IR spectrum shows CH_3, CH_2, at 2925, 2852 cm^{-1}. Aromatic proton at 6.82 - 7.30 ppm, IR peaks at 1466 cm^{-1}. Methoxy group at 3.52 ppm as a singlet double bond at 5.4 and 5.58 ppm in IR hydroxyl group at 3400 cm^{-1}. at 63.26 - 79.53 ppm.

The carboxyl group peaks at 174.59 ppm. The double bond peaks at 149.29 and 101.20 ppm. Methoxy group peaks at 56.76 ppm. The molecular peaks at m/e is $494(M)^{+}$ and molecular formula is $C_{28}H_{46}O_7$. From the above discussion the possible compound is assigned as guggultetrol-18- Ferrulate (Figure 5) . In the crude extract the LC_{50} values was found to be 90 ppm. In the sitosterol the LC_{50} value was found to b 75 ppm. And LC_{50} value for

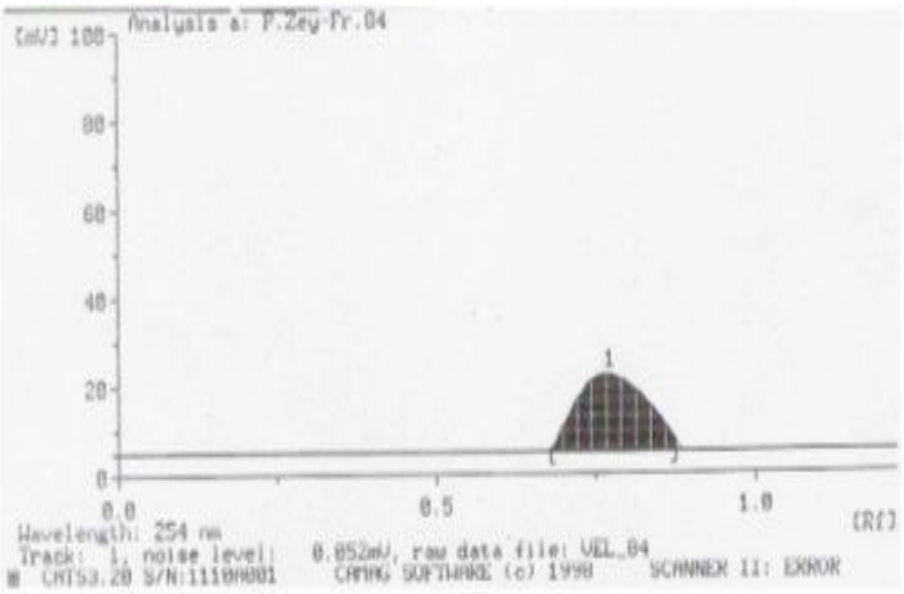


Figure 4. HPTLC Chromatogram of gugultetrol-18-ferrulate

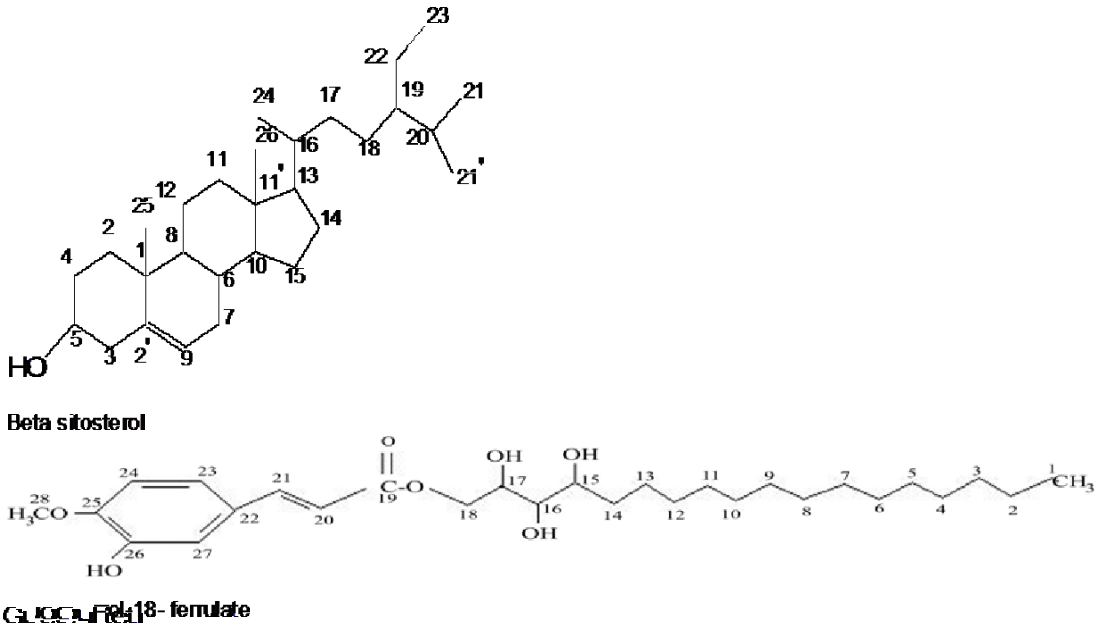


Figure 5. Structure of isolated compounds.

guggultetrol-18-ferrulate was found to be 65 ppm. The crude extract (dichloromethane extract) of *P. zeylanica* and purified extract containing betasitosterol and guggultetrol-18-ferrulate was found to have high cytotoxic effects. Thus, the use of effective and safe plant extract preparation by rural communities needs to be promoted among the people that are likely to benefit from such applications.

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REFERENCES

Alluri KV, Tayi RVN, Dodda S, Mulabagal V (2005). Assessment of bioactivity of Indian medicinal plants using brine shrimp (artemia salina) lethality assay, 32: 125-134.

Ayo RG, Amupition JO, Yimin Z (2007). Cytotoxicity and antimicrobial studies of 1, 6, 8-trihydroxy-3-methyl-anyhraquinone (emodin) isolated from the leaves of cassia nigricans. Vahl, 6: 1276-1279.

Azad C, Sushanta KC, Azadkhan AK (1982). Antifertility activity of *Plumbago zeylanica* Linn.root, Indian J. Med. Res., 76: 99-101.

Balandrin MJ, klocke JA (1988). Medicinal aromatic and industrial materials from plants. *Springer Verlag*, Berlin, Heidelberg, 4: 1-36.

Charles dorni AI, Vidyalakshmi KS, Hannah VR (2006). Anti-inflammatory activity of plumbago capensis. Pharmacognosy magazine. 2: 17-19.

Krishnaswamy M, Purushothaman KK (1980). Plumbagin. A study of its anticancer, antibacterial and antifungal properties, Indian J. Exp. Biol., 18: 876-877.

Madhava C (1998). Pharmacognostic studies of *Plumbago Zeylanica L.* (chitreka, chitramulamu), dissertation, post graduate diploma in plant drugs, S.V. University, tirupati, India.

McLaughlin JL (1991). Assays for bioactivity. In: Hostettmann K (ed). Methods in Plant Biochemistry. Academic Press: London, 6: 1-33.

Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLauglin JL (1982). Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med., 45: 31-34.

Michael AS, Thompson CG, Abramovitz M (1956). Atremia salina as a test organism for a bioassay. Science, pp.123, 464.

Mujumdar AM, Naik DG, Dange CN, Puntambekar HM (2000). Anti-inflammatory activity of curcuma amada roxb. In albino rats. J. pharmacol., 32: 375-377.

Narayana R, Thammanna K (1987). Medicinal plants of tirumala hills, department of garden, tirumala tirupati devasthanams, triupati, India.

Nguyen AT, Malonne H, Duez P, VanhaelenFastre R, Vanhaelen M, Fontaine J (2004). Cytotoxic constituents from Plumbago zeylanica. Int J. Pharm., 75: 500-4.

Okoli C, Akah O, Ezugworie (2005). U. Anti-inflammatory activity of extracts of root bark of securidaca longipedunculata fres (*polygalaceae*). Afr. J. Trad. CAM, 3: 54-63.

Parra AL, Yhebra RS, Sardinas IG, Buela LI (2001). Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD50 value) in mice, to determine oral acute toxicity of plant extracts. Phytomed., 8: 395-400.

Premakumari K, Rathinam S, Santhakumari G (1977). Antifertility activity of *Plumbagin.*. I J. Pharmacol., 43: 120-122.

Ramachandra RS, Ravishankar GA (2002). Plant cell cultures: chemical factories of secondary metabolites, Biotech. Advances, 20: 101-153.

Renata MS, Celeghini Janete HY, Vilegas FM, Laneas (2001). Extraction and Quantitative HPLC Analysis of Coumarin in Hydroalcoholic extracts of Mikania glomerata Spreng. (“guaco”) Leaves. J. Braz. Chem. Soc., 12(6): 706-709.

Roober JE, Speedie MK, Tyler VE (1996). Pharmacognosy and pharmacobiotechnology, Williams and wilkins, Baltimore, Md.,

Santhakumari S, Rathinam K (1978). Anticoagulant activity of *Plumbagin*, Indian J. Exp. Biol., 16: 485-487.

Shah BN, Nayak, BS, Seth AK, Jalalpure SS, Patel KN (2004). Search for medicinal plants as a source of anti-inflammatory and antiarthritic agents-A review. Pharmacognosy magazine.pp. 344-50.

Sleet RB, Brendel K (1983). Improved methods for harvesting and counting synchronous populations of artemia nauplii for yse in development toxicology. Ecotoxicol. Environ. Safety, 7: 435-446.

Srinivas K, Rao MEB, Rao SS (2000). Anti-inflammatory activity of heliotropium indicum linn. And leucas aspera spreng. In albino rats. J Pharmacol., 32: 37-38.

Srinivas K, Rao MEB, Rao SS (2000). Anti-inflammatory activity of heliotropium indicum linn. And leucas aspera spreng. in albino rats. J Pharmacol., 32: 37-38.

Uma DP, Soloman FE, Sharda AC (1999). Indian medicinal plants and their roots. Pharmaceut Biol., 37: 231-236.

Vanisree M, Hsin-Sheng T (2004). Plant cell culture- an alternative and efficient source for the production of biologically important secondary metabolites, Int. J. Appl. Sci. Eng., 2: 29-48.

Vieira RF, Skorupa LA (1993). Brazilian medicinal plants gene bank, Acta. Hortic., 330: 51-58.

Winter CA, Risely EA, Nuss GW (1962). Carregeenan-induced oedema in the hind paw of rat as an assay for anti-inflammatory activity. Prosoc. Exp. Biol., 111: 544-547.

Yen-Ju H, Lei-Chwen L, Tung-Hu T (2006). Measurement and pharmacokinetic study of plumbagin in a conscious freely moving rat using liquid chromatography/tandem mass spectrometry. J Chromatography, 844: 1-5.

Yuan-Chuen Wang, Tung-Liang Huang. (2005). High-performance liquid chromatography for quantification of plumbagin, an anti-helicobacter pylori compound of plumbago zeylanica L. J. Chromatogr. A 1094: 99-104.

Zaheer A, Ahsana D (2008). Advances in Natural products Importance in health and Economy.