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 (1H and 13C NMR), Infra red and mass spectroscopy. The lethal concentration (LC50) value was observed for crude extract, betasitosterol, gugultetrol-18-ferrurate 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. 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, chitrannone, 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
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 perennial 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 naphthaquinone showing a broad range of pharmaceutical activities.

Pharmacological effects of plumbagin have been investigated on anti-cancer, anti-leishmanial, anti-plasmodial, anti-bacterial, anti-fungal, anti-inflammatory, antihyperglycemic, hypolipidaemic, 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 anticoagulant 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<sub>254</sub> percolated on alumina 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. Celegihini), to check the purity of isolated compound and crude extract of dichloromethane, High resolution HPLC was performed using shimidzu LC –10AT up chromatograph provided with isocratic pump and UV visible detector. The crude dichloromethane extracts were filtered through 2 µ -membrane filters and used for analysis. Column of C<sub>18</sub> 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 (CPSEA Registration No: 512/01/a/2001/CPSECA), (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 for analysis. Column of C<sub>18</sub> 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.

**Pharmacological effects of plumbagin have been investigated on anti-cancer, anti-leishmanial, anti-plasmodial, anti-bacterial, anti-fungal, anti-inflammatory, antihyperglycemic, hypolipidaemic, 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).

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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.

Experimental design

For anti-inflammatory study, rats weighing (80 - 120 g) 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.

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

<table>
<thead>
<tr>
<th>S.NO</th>
<th>TEST</th>
<th>OBSERVATION</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Terpenoids</td>
<td>Appearance of blue</td>
<td>presence</td>
</tr>
<tr>
<td>2.</td>
<td>Flavanoids</td>
<td>Appearance of Yellow</td>
<td>Presence</td>
</tr>
<tr>
<td>3.</td>
<td>Steroids</td>
<td>Absence of pale green color</td>
<td>Absence</td>
</tr>
<tr>
<td>4.</td>
<td>Carbohydrate</td>
<td>Absence of pale green to cherry red</td>
<td>Absence</td>
</tr>
<tr>
<td>5.</td>
<td>Alkaloids</td>
<td>Absence of orange color</td>
<td>Absence</td>
</tr>
<tr>
<td>6.</td>
<td>Tannins</td>
<td>Absence of blue color</td>
<td>Absence</td>
</tr>
</tbody>
</table>

Percent inhibition of inflammation was calculated using the formula:

$$\text{Percent Inhibition} = \frac{V_c - V_t}{V_c}$$

Where, $V_c$ and $V_t$ represents average paw volumes in control and treated group, respectively.

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 determined (Meyer et al., 1982; McLaughlin, 1991). The $LC_{50}$ values were obtained from the best-fit line plotted concentration versus percentage lethality.

RESULTS AND DISCUSSIONS

The phytochemical screening of dichloromethane extract of *P. zeylanica* root confirmed the presence of terpenoids, flavanoids and absence of steroids, Carbohydrate, Alkaloids and Tannins. The results are given in Table 1. The dichloromethane extract of *P. zeylanica* root confirmed the presence of terpenoids, flavanoids and absence of steroids, Carbohydrate, Alkaloids and Tannins. The results are given in Table 1. The dichloromethane extract of *P. zeylanica* root confirmed the presence of terpenoids, flavanoids and absence of steroids, Carbohydrate, Alkaloids and Tannins. The results are given in Table 1. The dichloromethane extract of *P. zeylanica* root confirmed the presence of terpenoids, flavanoids and absence of steroids, Carbohydrate, Alkaloids and Tannins.
Table 2. Effect of methanol extract of *P. zeylanica* in carrageenin induced rat paw oedema in rats.

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

N = 6 (Student ‘t’ test), ** Significant at p < 0.01

The graph revealed a known pattern (Graph 1), whereas the cytotoxicity 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
(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 to 18.98 - 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
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


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


