Attenuation of expression of cytokines, oxidative stress and inflammation by hepatoprotective phenolic acids from Thespesia populnea Soland ex Correa stem bark

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Abstract

Recently a highly active hepatoprotective phenolic acids fraction (PF) has been isolated from Thespesia populnea stem bark. This fraction showed hepatoprotective action against toxic chemicals such as carbon tetrachloride, acetylamophen and thioacetamide. It also showed in vitro anti-Hepatitis B viral activity in cultured cells. In the present study, follow up investigations were carried out to determine the effect of PF on the expression of TNF-alpha, IL-6 and IL-10 in acetylamophen challenged rats. Further, the anti-inflammatory and in vitro antioxidant activities of PF were assessed. Acetaminophen administration resulted in liver damage and elevated levels of TNF-alpha, IL-6 and IL-10. PF (2 mg/kg) administration prevented, to a large extent, acetaminophen-induced increase in the levels of these cytokines and liver injury. PF administration attenuated carrageenan induced paw edema in mice and rats. It showed potent in vitro antioxidant activity. Thus, the present study shows for the first time that PF from T. populnea attenuates toxic chemical-induced up-regulation of the cytokines and possesses antioxidant and anti-inflammatory properties. PF did not exhibit any conspicuous toxic symptoms in the preliminary subacute toxicity evaluation in rats. Therefore, PF is very promising for drug development.

Keywords: Hepatoprotection, TNF-alpha, IL-6, IL-10, anti-inflammatory, Thespesia populnea Soland ex. Correa, phenolic acids, antioxidants, rats, mice

Introduction

Thespesia populnea Soland ex. Correa, a plant of the Malvaceae family, is used in folk-medicine in India for the treatment of liver diseases. The decoction of the bark is used in Ayurveda for the treatment of skin and liver diseases (Varier, 1994). The bark and flower of T. populnea possess several pharmacological activities including hepatoprotective, antioxidant, memory improving property and cholesterol lowering property. Previously, we have reported hepatoprotective activity of ethanol extract of T. populnea stem bark (Yuvaraj and Subramoniam, 2009). The plant bark extract has been reported to have anti-inflammatory properties in rodent models (Vasudevan and Parle, 2006). Further, the methanol extract of T. populnea (bark) possesses antioxidant property against carbon tetrachloride induced oxidative stress in rats (Ilavarasan et al., 2003). Several hepatoprotective, anti-inflammatory, antieptic, neuroprotective, antiarteriosclerosis, antidiabetes and anticancer drugs have recently been shown to have antioxidant and/or antiradical scavenging mechanism as part of their activity (Repetto and Llesuy, 2002). It is not known whether or not antioxidant, anti-inflammatory and hepatoprotective properties are exerted by the same phytochemical(s) or different compounds.
Recently, we have isolated from the alcohol extract of stem bark of *Thespesia populnea*, a hepatoprotective fraction containing three closely related phenolic acids (Yuvraj et al., 2012). In this context, one of the aims of the present study is to test whether or not the same hepatoprotective phenolic acids have anti-inflammatory and antioxidant properties also.

It has been demonstrated that various inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-1 beta (IL-1β) produced during drug-induced liver injury are involved in promoting tissue damage (Blazka et al., 1996; Ishida et al., 2002; Bourdi et al., 2002). The production of TNF-α is one of the earliest events in the hepatic inflammatory response that triggers the release of other cytokines. TNF-α also directly induces cytotoxicity and has been implicated in hepatocyte apoptosis and necrosis (James et al., 2005). In this context, it was thought of interest to determine whether or not the hepatoprotective phenolic acids from *T. populnea* attenuate acetaaminophen-induced elevation of cytokines (TNF-α, IL-6 and IL-10).

Further, the phenolic acid fraction was subjected to preliminary subacute toxicity evaluation in rats.

**Materials and Methods**

**Plant materials, preparation of extract and fractions**

*T. populnea* stem bark was collected from Thodupuzha, Kerala, India in the month of March. The specimens were identified and voucher specimens of plants were deposited in the herbarium (TTP-876) in the Department of Pharmacognosy, Nagarjuna Herbal Concentrates Ltd, Kerala, India. Ethanol extract of the dried and coarsely powdered stem bark of *T. populnea* was subjected to solvent fractionation and an active ethyl acetate fraction of the ethanol extract was obtained as described recently (Yuvraj et al., 2012). The yield of ethanol extract was about 23% of the stem bark powder. The yield of ethyl acetate fraction was about 14% of ethanol extract.

The active fraction/component (3 closely related phenolic acids) was isolated from the ethyl acetate fraction by column chromatography and the purity was confirmed by HPLC (Yuvraj et al., 2012). The yield of phenolic acid-fraction was about 53% of ethyl acetate fraction.

**Animals**

Male Swiss albino mice 25 ± 5 g body weight and male albino rats of Wistar strain weighing 190 ± 10g were used in this study. Animals were housed in polypropylene cages under standard conditions (23 ± 2°C, humidity 60-70%, 12 h light/dark cycles) and fed with standard pellet diet (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. The protocol was approved by Institutional Animal Ethics Committee constituted as per CPCSEA guidelines.

**In vitro antioxidant assay**

**HPPH assay**

1. 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was carried out as described (Burits and Bucar, 2000). Briefly, 1 ml of methanol solution of 0.25 mM DPPH was added to 1 ml of different concentrations of the phenolic acid-fraction (PF) of *T. populnea* (dissolved in 80% methanol) and standard (ascorbic acid) solution. 1 ml of deionised water/methanol served as control. After a 20 min incubation period at room temperature in dark, the absorbance was read at 517 nm. Radical scavenging activity was expressed as the inhibition % and was calculated as % radical scavenging activity = (control OD - sample OD)/control OD X 100.

**Assay of total antioxidant potential**

2. 2′-azinoisobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay was done essentially as described (Re et al., 1999). Briefly, ABTS was dissolved in deionised water to 1.8 mM concentration. To 10 µl of different concentrations of PF or standard (ascorbic acid) and 1.99 ml of ABTS solution was added and kept in dark for 15 min and red at 734 nm. This was compared with control where 10 µl of the solvent was added to 1.99 ml of ABTS solution. PF was dissolved in 80% methanol. Assay was performed in triplicate. Anti-oxidant activity was expressed as the % of ABTS radical reduction. Radical scavenging activity was expressed as the inhibition % and was calculated as % radical scavenging activity = (control OD - sample OD)/control OD X 100. IC₅₀ was calculated from the plot of inhibition (%) against PF concentration.

**Lipid peroxidation inhibition assay**

Lipid peroxidation induced by Fe⁺⁺/ascorbate system in rat liver homogenate was estimated by TBA reaction method (Ohkawa et al., 1979). The reaction method contained rat liver homogenate 0.1 ml in Tris-HCl buffer; KCl (30 mM); FeSO₄ (NH₄)₂SO₄·6H₂O (0.16 mM); ascorbic acid (0.06 mM) and various concentrations of PF in a final volume of 0.5 ml. The reaction mixture was incubated for 1 h at 37°C. After the incubation, 0.4 ml was removed and treated with 0.2 ml of 8.1% SDS, 1.5 ml of 0.8% TBA and 1.5 ml (20%) acetic acid. The total volume was made up to 4 ml with distilled water and then kept in a water bath at 95-100°C for 1 h. After cooling, 1 ml of distilled water and 5 ml of n-butanol were added to the reaction mixture, shaken and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance at 532 nm was measured. Inhibition of lipid peroxidation was determined by comparing the OD. of the treatments with that of control. Catechin was used as a standard.
Anti-inflammatory activity of *T. populnea* active component against carrageenan-induced paw oedema

**Carrageenan-induced mice paw oedema (topical application)**

Swiss albino male mice were divided into two groups, each group contained six animals. All mice were injected with 0.1 ml of 1% (w/v) freshly prepared suspension of carrageenan solution into sub-planter region of the right hind paw. 1% (w/v) of *T. populnea* phenolic acids fraction (PF) in coconut oil was applied on the paw of treatment group of mice after carrageenan injection, whereas only the coconut oil was applied on the paw of control mice. The animals were killed after 3 h of carrageenan injection and the paws were cut identically at the ankle joints. The oedema of the injected paw was found by subtracting from its weight that of the control paw (Subramoniam, et al., 2012).

**Carrageenan-induced mice paw oedema (oral administration)**

Eighteen male Swiss albino mice were divided into 3 groups. All mice were injected with 0.1 ml of 1% (w/v) freshly prepared suspension of carrageenan solution into sub-planter region of the right hind paw. *T. populnea* PF (2 mg/kg) and indomethacin (10 mg/kg) in 5% Tween 80 solution were administered orally to group 2 and group 3 mice, respectively, 30 min before the injection of carrageenan. The control mice (group 1) were administrated 5% Tween 80 solution only. The animals were killed after 3 h of carrageenan injection and the paws were cut identically at the ankle joints. The oedema of the injected paw was found by subtracting from its weight that of the control paw (Singh, et al., 1984).

**Carrageenan-induced paw oedema in rats**

Eighteen rats were divided into three groups, each group consisting of six animals. Oedema was induced by sub-planter injection of 0.1 ml of 1% carrageenin into the right hind paw of each rat. The paw volume was measured before (0 h) and at 1, 2, 3, and 4 h after the injection of carrageenan using a plethysmometer. *T. populnea* PF (2 mg/kg) or indomethacin (10 mg/kg) in 5% Tween 80 solution was administrated orally to the test groups, 30 min before the injection of carrageenan. The control group of rats received 5% aqueous Tween 80 solution (5 ml/kg) orally (Kulkarni, et al., 1986).

**Effect of *T. populnea* PF on serum cytokine levels in mice**

In this experiment, 18 mice were randomly divided into 3 groups of 6 mice in each group. Normal control mice (group 1) were administrated single daily dose of 5% Tween 80 in distilled water (2 ml/kg body weight, p.o.) for 3 days and a single dose of phosphate buffered saline (1 ml/kg body weight, i.p.) on day 3. Acetaminophen (AA) control mice (group 2) were administrated single daily dose of 5% Tween 80 in distilled water (2 ml/kg body weight, p.o.) for 3 days and a single dose of AA (300 mg/kg body weight, i.p.) on day 3 after 60 min of vehicle administration (James, et al., 2005). AA was dissolved in phosphate buffered-saline). Experimental mice (group 3) were administrated single daily dose of *T. populnea* PF (2 mg/kg body weight, p.o.) in 5% Tween 80 for 3 days and a single dose of AA (300 mg/kg body weight, i.p.) on day 3 after 60 min of PF administration.

After 24 h of AA administration, all the animals in different groups were sacrificed by cervical decapitation under pentobarbitone sodium (60 mg/kg body weight) anesthesia. Blood was collected and serum was separated by centrifugation and used for various biochemical estimations.

**Preliminary toxicity evaluation**

In this experiment, a total of 24 rats were used. The rats were randomly divided into 4 groups of 6 rats in each group. Normal control rats (group 1) were administrated single daily dose of 5% Tween 80 in distilled water (2 ml/kg body weight). p.o. for 28 days. Group 2 rats were administrated single daily dose of *T. populnea* PF (2 mg/kg body weight), p.o. in 5% Tween 80 for 28 days. Group 3 rats were administrated single daily dose of *T. populnea* PF (8 mg/kg body weight), p.o. in 5% Tween 80 for 28 days. Group 4 rats were administrated single daily dose of *T. populnea* PF (16 mg/kg body weight), p.o. in 5% Tween 80 for 28 days.

The behavior of the animals was observed daily for 1 h for 28 days. Initial and final body weights, water and food intake, and state of stool were observed. After 28 days, all the animals in different groups were sacrificed by cervical decapitation under pentobarbitone sodium (60 mg/kg body weight) anesthesia. Blood was collected and serum was separated by centrifugation and used for various biochemical estimations. The organs were collected and observed for pathological and morphological changes.

**Biochemical analysis**

Serum AST, ALT, alkaline phosphatase bilirubin, glucose, total protein and albumin were determined with a spectrophotometric diagnostic kits (SPAN Diagnostics Ltd, Surat, India). The plasma levels of IL-6, IL-10 and TNF-alpha were measured by ELISA using cytoscreen immunoassay kits (BioSource International, Camarillo, CA). Samples were run according to manufacturer’s instructions. The sensitivity of assay with the lower limit was at 5 nmol/l for IL-6 and IL-10, and 10 nmol/l for TNF-alpha. Hemoglobin was measured using hemoglobinometer following standard methods. Urea, uric acid, creatinine, cholesterol and lipids were determined by conventional methods (Jam, 1986).

**Statistical analysis**

The values are expressed as the mean ±SD and the significance between different groups were determined by one way analysis of variance (ANOVA) coupled with Duncan’s Multiple Range Test (DMRT), taking p < 0.05 as significant.
**Results**

*In vitro antioxidant activity*

The capacity of *T. populnea* PF (phenolic acids-fraction) to scavenge DPPH radicals is shown in Table 1. PF dose dependently reduced DPPH radicals. At a low concentration range of 2-10 µg/ml, PF scavenged DPPH radicals and had an IC₅₀ value of 16.53 ± 0.12 µg/ml; ascorbic acid had an IC₅₀ value of 18.59 ± 0.72 µg/ml.

In ABTS assay (assay for total antioxidant potential) also, PF showed antioxidant activity. The IC₅₀ value of PF was 12.38 ± 0.14 µg/ml whereas that for ascorbic acid was 15.31 ± 0.16 µg/ml. Thus, the PF was better than ascorbic acid in ABTS assay (Table 1).

**Effect of PF on carrageenan-induced paw oedema in mice (topical drug application)**

For the acute phase of inflammation, carrageenan-induced paw-volume increase, and the effects of PF on the same were evaluated. Table 2 shows that paw weight of control group of mice increased after 3 h of carrageenan treatment. PF (1 %) when externally applied in coconut oil reduced paw weight by 29.4%.

**Effect of PF on carrageenan-induced paw volume in mice (oral drug administration)**

Oral administration of PF also inhibited carrageenan induced paw oedema in mice. It reduced the paw weight (3 h after carrageenan administration) by 17.2% whereas indomethacin inhibited the same by 20% (Table 2).

**Effect of PF on carrageenan-induced paw oedema in rats**

Anti-inflammatory activity of PF was evaluated by carrageenan induced rat paw oedema model also. Sub-planter injection of 0.1 ml of 1% carrageenan produced increase in paw volume (oedema) of all the animals. The onset of action was evident from 1 h in carrageenan treated groups. The increase in paw volume was the maximum at the 3rd h (Table 3). Pretreatment with PF (2 mg/kg body weight, p.o.) or indomethacin (10 mg/kg) had significant anti-inflammatory effect in comparison with control group. PF (2 mg/kg) inhibited carrageenan-induced paw oedema by 71 % while indomethacin inhibited the same by 77% at 3h after carrageenan injection. The effect of indomethacin was more pronounced at 1st and 2nd h compared to the effect of PF at 1st and 2nd h after carrageenan injection (Table 3).

**Effect of *T. populnea* PF on the levels of cytokines in acamptophen-challenged mice**

Administration of acetaminophen (300 mg/kg, i.p.) induced a significant (p< 0.05) increase in the levels of ALT (up to 3.5 fold) and AST (2.5 fold) as compared to the normal controls (Figure 1) indicating liver damage. However, pretreatment with *T. populnea* PF (2 mg/kg, p.o.) prevented acetaminophen-induced increase in the activities of ALT and AST; the enzyme values in this group were almost comparable to the enzyme values in normal control group.

![Figure 1: Effect of T.populnea phenolic acids fraction on serum ALT and AST levels in acetaminophen-induced hepatotoxicity in mice](image)

**Figure 1: Effect of *T. populnea* phenolic acids fraction on serum ALT and AST levels in acetaminophen-induced hepatotoxicity in mice**

TP: *T. populnea* phenolic acids fraction; Values are mean ±SD., n=6, Values in acetaminophen group are significantly different from corresponding values in normal control group. p < 0.001; values in TP + acetaminophen group are not significantly different from those of normal control group.

![Figure 2: Effect of *T. populnea* phenolic acid fraction on serum IL-10, IL-6 and TNF-alpha levels in acetaminophen-induced hepatotoxicity in mice](image)

**Figure 2: Effect of *T. populnea* phenolic acid fraction on serum IL-10, IL-6 and TNF-alpha levels in acetaminophen-induced hepatotoxicity in mice**

TP: *T. populnea* phenolic acids fraction; Values are mean ±SD., n=6, Values in acetaminophen group are significantly different from corresponding values in normal control group. p < 0.001; values in TP + acetaminophen group are not significantly different from those of normal control group.

Acetaminophen treatment resulted in a marked increased in the levels of TNF-alpha, IL-6 and IL-10. Administration of PF (2 mg/kg) to mice prior to acetaminophen intoxication attenuated, to a large extent, acetaminophen-induced increase in the levels of these cytokines (Figure 2).
Table 1: *In vitro* antioxidant activities of *T. populnea* phenolic acids fraction

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>DPPH radical scavenging (IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml))</th>
<th>ABTS radical scavenging (IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml))</th>
<th>Inhibition of lipid peroxidation (IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. populnea</em> (phenolic acid fraction)</td>
<td>16.53 ± 0.12</td>
<td>12.38 ± 0.14</td>
<td>150.27 ± 12.56</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>18.59 ± 0.72</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catechin</td>
<td>-</td>
<td>-</td>
<td>180.34 ± 13.42</td>
</tr>
</tbody>
</table>

Values are mean ± SD. The values were obtained from three different experiments performed in duplicate.

Table 2: Effect of *T. populnea* phenolic acids-fraction on carrageenan-induced paw oedema in mice

<table>
<thead>
<tr>
<th>Carrageenan control</th>
<th><em>T. populnea</em> (2 mg/kg) + Carrageenan</th>
<th>% oedema inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrageenan control</td>
<td>255.0 ± 6.0</td>
<td>180.0 ± 13.7*</td>
</tr>
<tr>
<td>Oral administration, weight of paw (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrageenan control</td>
<td>252.4 ± 10.3</td>
<td>209.8 ± 10.2*</td>
</tr>
<tr>
<td><em>T. populnea</em> (2 mg/kg) + Carrageenan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indomethacin (10 mg/kg) + Carrageenan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% oedema inhibition</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.2</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 6 mice in each group. Values marked with asterisk are significantly different from control values, *p < 0.05* (DMRT).

Table 3: Inhibition of carrageenan-induced oedema in rats by *T. populnea* phenolic acids fraction or indomethacin

<table>
<thead>
<tr>
<th>Groups</th>
<th>Oedema produced in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Carrageenan Control</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td><em>T. populnea</em> PF (2 mg/kg) + Carrageenan</td>
<td>0.08 ± 0.01* (27)</td>
</tr>
<tr>
<td>Indomethacin (10 mg/kg) + Carrageenan</td>
<td>0.06 ± 0.01* (45)</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 6 rats in each group. Values marked with asterisk are significantly different from control values, *p < 0.05; **p < 0.01* (DMRT).

Values in parentheses indicate percentage of oedema inhibition.
Table 4: Effect of *T. populnea* phenolic acid fraction on body weight, food intake and water intake in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TP-PF(2mg/kg)</th>
<th>TP-PF(8 mg/kg)</th>
<th>TP-PF(16 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>165.00 ± 7.07</td>
<td>173.33 ± 5.77</td>
<td>173.33 ± 5.77</td>
<td>171.66 ± 7.63</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>206.50 ± 4.94</td>
<td>213.33 ± 7.63</td>
<td>215.33 ± 8.66</td>
<td>213.33 ± 7.63</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>41.50 ± 2.12</td>
<td>40.00 ± 5.00</td>
<td>41.66 ± 2.88</td>
<td>41.66 ± 2.88</td>
</tr>
<tr>
<td><strong>Food and water intake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food intake (g/day/rat)</td>
<td>16.80 ± 1.20</td>
<td>15.52 ± 2.20</td>
<td>17.20 ± 2.65</td>
<td>14.54 ± 2.86</td>
</tr>
<tr>
<td>Water intake (ml/day/rat)</td>
<td>28.46 ± 3.11</td>
<td>25.02 ± 2.62</td>
<td>26.98 ± 2.88</td>
<td>26.79 ± 2.15</td>
</tr>
</tbody>
</table>

TP-PF – *Thespesia populnea* phenolic acids fraction. Values are mean ± SD for 6 rats in each group. Values are not significantly different from control values, at *p* < 0.05 (DMRT).

Table 5: Effect of *T. populnea* PF on serum biochemical parameters in rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>TP-PF (2mg/kg)</th>
<th>TP-PF (2mg/kg)</th>
<th>TP-PF (2mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (IU/L)</td>
<td>55.83 ± 4.72</td>
<td>54.38 ± 1.57</td>
<td>54.12 ± 2.3</td>
<td>56.15 ± 1.79</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>97.12 ± 6.13</td>
<td>94.05 ± 2.56</td>
<td>97.39 ± 2.84</td>
<td>95.64 ± 2.73</td>
</tr>
<tr>
<td>ALP (KA unit)</td>
<td>59.10 ± 1.45</td>
<td>58.78 ± 3.01</td>
<td>60.68 ± 2.63</td>
<td>59.88 ± 2.15</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.51 ± 0.01</td>
<td>0.51 ± 0.11</td>
<td>0.51 ± 0.05</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl)</td>
<td>0.20 ± 0.03</td>
<td>0.19 ± 0.05</td>
<td>0.20 ± 0.03</td>
<td>0.21 ± 0.3</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>7.04 ± 0.25</td>
<td>7.16 ± 0.13</td>
<td>7.16 ± 0.30</td>
<td>6.96 ± 0.36</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.26 ± 0.26</td>
<td>4.44 ± 0.16</td>
<td>4.29 ± 0.22</td>
<td>4.24 ± 0.31</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>82.02 ± 1.58</td>
<td>80.52 ± 1.58</td>
<td>78.65 ± 5.29</td>
<td>79.40 ± 4.23</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>58.41 ± 1.79</td>
<td>57.46 ± 0.44</td>
<td>57.77 ± 1.79</td>
<td>56.82 ± 1.44</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>46.15 ± 4.53</td>
<td>48.14 ± 2.35</td>
<td>49.42 ± 0.81*</td>
<td>49.35 ± 0.73*</td>
</tr>
<tr>
<td>VLDL (mg/dl)</td>
<td>11.68 ± 0.35</td>
<td>11.49 ± 0.08</td>
<td>11.55 ± 0.35</td>
<td>11.36 ± 0.08</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>24.18 ± 3.30</td>
<td>20.89 ± 1.31*</td>
<td>17.67 ± 6.47**</td>
<td>18.46 ± 4.02**</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>92.76 ± 1.27</td>
<td>94.72 ± 1.62</td>
<td>88.68 ± 5.03</td>
<td>89.59 ± 3.94</td>
</tr>
<tr>
<td>Hb g%</td>
<td>12.28 ± 0.40</td>
<td>12.42 ± 1.18</td>
<td>13.02 ± 0.65</td>
<td>12.48 ± 2.17</td>
</tr>
<tr>
<td>TBARS (nanomoles)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>malondialdehyde/ml)</td>
<td>1.69 ± 0.11</td>
<td>1.59 ± 0.32</td>
<td>1.5 ± 0.38</td>
<td>1.51 ± 0.23</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>26.68 ± 1.92</td>
<td>26.44 ± 1.03</td>
<td>26.19 ± 3.60</td>
<td>26.84 ± 1.53</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>1.81 ± 0.22</td>
<td>1.82 ± 0.13</td>
<td>1.81 ± 0.24</td>
<td>1.69 ± 0.16</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>34.87 ± 6.89</td>
<td>33.50 ± 5.10</td>
<td>32.25 ± 6.53</td>
<td>32.00 ± 1.73</td>
</tr>
</tbody>
</table>

TP - PF *Thespesia populnea*-phenolic acids fraction. Values are mean ± SD of 6 rats in each group. Values marked with asterisk are significantly different from control values, *p < 0.05; **p < 0.01 (DMRT). ALT - alanine aminotransferase; AST-aspartate aminotransferase; ALK-alkaline phosphatase
**Preliminary toxicity evaluation**

*T. populnea* PF administration (p.o.), at the doses of 2 mg/kg, 8 mg/kg and 16 mg/kg body weight p.o. for 28 days did not result in any obvious toxic symptoms. The general behavior of the animals was not changed. Food and water intake of animals were not significantly altered by the treatment compared to control group (Table 4). Body weight gains of animals were similar to those of control group after 28 days of PF administrated (Table 4). State of the fecal droppings was not changed in any of the groups.

The effects of *T. populnea* PF on serum biochemical parameters are shown in Table 5. Serum ALT, AST and alkaline phosphate (ALP) levels were not significantly changed in all the 3 PF treated groups. Serum protein, albumin, bilirrubin, glucose, haemoglobin, urea, uric acid and creatinine levels were also not significantly changed by PF administration at all doses studied. The serum lipid profiles levels were not substantially changed. However, at higher doses (8 and 16 mg/kg), PF administrated to rats resulted in an increase in the levels of HDL whereas LDL levels were decreased. The serum TBARS levels showed a decreasing trend in *T. populnea* PF treated groups (Table 5).

**Discussion**

The present study shows that hepatoprotective phenolic acid fraction (3 closely related phenolic compounds) has in vitro antioxidant and in vivo anti-inflammatory properties also. In our previous studies, we have shown the in vivo antioxidant properties of PF (Yuvaraj et al., 2012). Phenolic compounds have been proved to be more potent antioxidants than Vitamins E and C and carotenoids (Takebayashi et al., 2006). In the present study, the in vitro free radical scavenging activity of the phenolic acids fraction (PF) was found to be better than vitamin C. The DPPH and ABTS scavenging ability of PF may be attributed to its hydrogen donating ability higher than ascorbic acid. A number of chronic diseases are indeed characterized by an intense increase in lipid peroxides. PF effectively inhibited the lipid peroxidation at very low concentration compared to catechin. So, one of the mechanisms of its hepatoprotective activity could be antioxidant activity. Although antioxidant, anti-inflammatory and hepatoprotective properties of *T. populnea* are known, this study shows for the first time that all these 3 properties are due to the hepatoprotective phenolic acids.

During inflammation, macrophages and neutrophils secrete a number of mediators (cyclooxygenoids, oxidants, cytokine and lytic enzymes) responsible for initiation, progression and persistence of inflammation (Lefkowitz et al., 1999). Prostaglandin E2 (PGE2) and nitric oxide (NO) are most important among these mediators. These are produced in macrophages by cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), respectively (Harris et al., 2002; MacMicking et al., 1997). Likewise, the neutrophils too produce oxidants and release granular constituents comprising of lytic enzymes (Yoshikawa and Naito, 2000). Inhibition in the release of these mediators is a potential strategy to control inflammation and is implicated in mechanism of action of a number of anti-inflammatory drugs including the representative ones like dexamethasone (Bourke and Moynagh, 1999).

To investigate the effects of drugs on the acute phase of inflammation, carrageenan-induced paw oedema is an established model (Campos et al., 1995). Carrageenan, a mucopolysaccharide, produces maximal oedema in 3 h (Leme et al., 1973). Carrageenan activates cyclo-oxygenase pathway. However, the early phase of the carrageenan response is due to the release of serotonin and histamine (DiRosa et al., 1971).

The carrageenan model is typically associated with activation of the cyclooxygenase and lipoxygenase enzyme pathways and is sensitive to glucocorticoids and prostaglandin synthesis antagonists. There are biphasic effects in carrageenan-induced edema. The first phase begins immediately after injection and diminishes within 1 h. The second phase begins at 1 h and remains through 3 h (Garcia-Pastor et al., 1999; Daniel and Jamil, 2004). The early phase of the carrageenan response is due to the release of serotonin and histamine (GamaChe et al., 1986; Kulkarni et al., 1986). On the other hand, the delayed phase of carrageenan-induced edema results mainly from the potentiating effects of prostaglandins on mediator release, especially of bradykinin (Kulkarni et al., 1986). Both *T. populnea* PF and indomethacin started to block inflammation in the first measurement (1 h) and also remarkably reduced edema in late phase (after 3 h). This indicates that PF may inhibit histamine and/or serotonin release in the first phase of acute inflammation and can also effectively inhibit prostaglandin and bradykinin in the second phase.

Hydrocortisone and some NSAIDs strongly inhibit the second phase of carrageenan-induced oedema, but some others are effective against both phases (Kulkarni et al., 1986). Indomethacin is a well-known inhibitor of histamine, serotonin and prostaglandin in acute inflammation (Gepdiremen et al., 2004).

The specific mechanisms of action of PF may depend on the inhibition of the formation of several inflammatory mediators. Detailed studies are needed to clarify the mechanism(s) of action of PF. Anti-inflammatory activity of PF may at least partially, be involved in its hepatoprotective activity against toxic chemicals.

There is growing evidence that the initial aceterminophen-induced hepatocyte damage may lead to activation of innate immune cells within the liver, thereby stimulating hepatic infiltration of inflammatory cells (Hsu et al., 2006). Activated
cells of the innate immune system produce a range of inflammatory mediators, including cytokines, chemokines, and reactive oxygen and nitrogen species that contribute to the progression of liver injury. Some of these mediators, such as tumor necrosis factor-alpha (TNF-\(\alpha\)), interferon-gamma (IFN-\(\gamma\)), and interleukin-1 beta (IL-1 \(\beta\)), Fas or Fas ligand, are directly involved in causing liver damage. On the other hand, the innate immune cells also represent a major source of hepatoprotective factors, as it has been demonstrated that transgenic mice deficient in IL-10 or IL-6 are more susceptible to AA-induced liver injury (Blazka et al., 1995; Blazka et al., 1996; Ishida et al., 2002; Bourdi, et al., 2002; Masubuchi et al., 2003; Reilly et al., 2001). The present study shows that acetaminophen induced upregulation of TNF-\(\alpha\) is prevented by PF treatment. This may counteract the levels of other cytokine elevated by acetaminophen administration.

The depletion of glutathione also enhances the expression of TNF-\(\alpha\) (Agarwal and Pierseco, 1994), which primes phagocytic NADPH oxidase to enhance the production of oxygen free radicals (Gupta et al., 1992). In addition, the breakdown of the GSH-dependent antioxidant defensive system increases the intracellular flux of oxygen free radicals creating an oxidative stress and initiation of cell death.

The rise in serum levels of AST and ALT has been attributed to the damaged structural integrity of the liver cells because those are cytoplasmic in location and are released into circulation after cellular damage (Sallie et al., 1991). T. populnea seems to preserve the structural integrity of the hepatocellular membrane as evident from the significant reduction of acetaminophen-induced rise in serum enzymes in mice. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes (Ahmed and Khater, 2001).

The activated innate immune cells also produce hepatoprotective cytokines like IL-6 and IL-10 in order to prevent damage and regenerate the cells at damaged site (Ju et al., 2002). Thus, it is reasonable to observe upregulation of IL-6 and IL-10 in response to acetaminophen-induced hepatic damage. Several studies have also been demonstrated that mice with deficiency of IL-10 or IL-6 are more susceptible to acetaminophen-induced liver injury (Ishida et al., 2002; Masubuchi et al., 2003). In this context, we also observed that acetaminophen administration caused the elevation of TNF-alpha as well as IL-6 and -10 whereas administration of T. populnea PF down regulated TNF-alpha, which might consequently, at least in part, diminish inflammatory and endothelial injury and consequently reduced the level of IL-6 and -10. Since IL-6 and IL-10 are involved in hepatocyte proliferation and regeneration at the site of injury, these levels remain normal in acetaminophen intoxicated rats treated with T. populnea PF suggest that PF might protect the hepatocytes either directly by inhibition of inflammatory reactions or indirectly by sparing antioxidant activity/improvement of antioxidant defense system especially glutathione dependent system. It is of interest to note that PF did not show any toxic effects in the preliminary toxicity evaluation.

As reported by others (Kim et al., 1997; Gamal et al., 2003; Song et al., 2004), AA increased serum ALT and AST activities, exacerbated oxidation damage, enhanced inflammatory reaction and elevated cytokines release.

Growing evidence shows that overdose of acetaminophen leads to overproduction of toxic intermediate NAPQI, which induces hepatocyte damage and consequently activates innate immune cells within the liver, thereby stimulating hepatic infiltration of inflammatory cells (James et al., 2005; Masubuchi et al., 2005). The arachidonic acid cascade is highly activated during inflammation, resulting in the formation of eicosanoids, and it is mediated by cyclooxygenase (COX-2) and 5-lipoxygenase enzymes (Jain et al., 2001), which involve release of a wide variety of inflammatory mediators such as prostaglandins, thromboxanes and leukotrienes (Insel, 1996).

Overdoses of acetaminophen depletes glutathione stores, leading to accumulation of NAPQI, mitochondrial dysfunction (Parmar et al., 1995), and the development of acute hepatic necrosis.

It has been indicated that proinflammatory cytokine, TNF-alpha, is the central mediator for the regulation of several biomarkers such as C-reactive protein and von Willebrand factor in inflammation, especially in acute phase response (Hsu et al., 2006; Tomita et al., 2004). In the present study, AA treatment caused the elevation of this proinflammatory cytokine. It was found that PF down regulated TNF-alpha, which might have consequently, partially diminished inflammatory and endothelial injury.

The four-week toxicity study of T. populnea PF in rats did not identify any conspicuous toxic effects, but T. populnea produced beneficial effects such as marginal increase in the levels of HDL and decrease in LDL especially at high doses. Overall, this study strongly recommends using T. populnea PF for the development of safe medicine for liver diseases.

**Conclusion**

The study shows that T. populnea hepatoprotective phenolic acids-fraction effectively inhibits acute inflammatory reactions and oxidative stress and prevents acetaminophen-induced up regulation of the pro-inflammatory cytokine (TNF-alpha). In the preliminary study, T. populnea PF did not show any toxicity in rats. T. populnea PF is a potent natural agent to treat liver diseases.
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Conflict of interest
We declare that we have no conflict of interest.

References


