Hepatoprotective effect of *Hibiscus hispidissimus* Griffith, ethanolic extract in paracetamol and CCl₄ induced hepatotoxicity in Wistar rats

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*Hibiscus hispidissimus* Griff. is used in tribal medicine of Kerala, the southern most state of India, to treat liver diseases. In the present study, the effect of the ethanolic extract of *Hibiscus hispidissimus* whole plant on paracetamol (PCM) - induced and carbon tetrachloride (CCl₄) - induced liver damage in healthy Wistar albino rats was studied. The results showed that significant hepatoprotective effects were obtained against liver damage induced by PCM and CCl₄ as evidenced by decreased levels of serum enzymes, glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (SAKP), serum bilirubin (SB) and an almost normal histological architecture of the liver of the treated groups compared to the toxin controls. The extract also showed significant antilipid peroxidant effects *in vitro*, besides exhibiting significant activity in quenching 1, 1-diphenyl – 2-picryl hydrazyl (DPPH ) radical, indicating its potent antioxidant effects.

**Keywords**: Carbon tetrachloride, DPPH quenching, Hepatoprotection, *Hibiscus hispidissimus*, Lipid peroxidation, Paracetamol.

*Hibiscus hispidissimus* Griffith (syn. *Hibiscus furcatus* DC. non Willd., *Hibiscus aculeatus* Roxb. non.Walter.), belonging to the family Malvaceae is known as ‘Comfort root’ or ‘Big Thicket *Hibiscus*’ or ‘Pine *Hibiscus*’ in English and “Uppanacham” in Malayalam. It is distributed throughout India. It is an erect, suffrutescent, rambling, prickly herb, 60-150 cm in height. It is used by tribal healers of Kerala to treat liver diseases. The leaves are acidic and are eaten after cooking. They are said to improve digestion and are considered anthelmintic¹. The juice of the leaves mixed with honey is applied in eye diseases¹. An infusion of roots in water is used as a cooling drink in hot weather and a decoction of the root bark is given as a remedy for poisons, swellings and for cleansing the kidneys¹. The petroleum ether extract of the leaves of *H. hispidissimus* yielded triterpene components like friedelin, beta-sitosterol and taraxerol². Hepatoprotective effects of *H. hispidissimus* have not been validated. In the present study, the hepatoprotective effect of *H. hispidissimus* whole plant against CCl₄ and PCM induced liver damage in Wistar rats is reported.

**Materials and Methods**

**Plant material and preparation of the extract**—The whole plants of *H. hispidissimus* were collected from Palode, Thiruvananthapuram district, Kerala. They were authenticated by the plant taxonomist of the Institute and a voucher specimen (TBGT 57006 dated 04/01/06) was deposited at the Institute’s Herbarium. The plants were washed thoroughly in tap water, shade-dried and powdered. The powder (100 gm) was successively extracted with 1000 ml of ethanol overnight, at room temperature with constant stirring. The extract was filtered and the filtrate concentrated under reduced pressure to yield 800 mg of the crude extract (0.8% with respect to the dried plant material). This crude extract was referred to as HH. It was reconstituted in 0.5% Tween-80, to desired concentrations and used for the experiments.

**Animals**—Wistar albino rats, males (270-300 g) and Swiss albino mice, males (25-30 g) were obtained from the Institute’s Animal House. They were housed under standard conditions (temperature 24°-28°C, relative humidity 60-70% and 12 hr dark-light period).
cycles), fed commercial rat feed (Lipton India Ltd., Mumbai, India) and boiled water ad libitum. All experiments involving animals were carried out according to National Institute of Health guidelines, after getting the approval of the Institute’s Animal Ethics Committee.

**PCM induced hepatotoxicity**—Rats were divided into 6 groups, each containing 6 rats. Groups 1 and 2 were the normal control and toxin control groups respectively and both received 0.5% Tween-80 (2 ml po), for 6 days. Groups 3, 4 and 5, were administered HH (100, 200 and 300 mg/kg po), for 6 days and group 6, was given Silymarin (Sigma Chemical Company, USA), the known hepatoprotective agent (100 mg/kg po) for 6 days. Paracetamol (Acetaminophen-PCM-2.5 g/kg in 0.5% Tween-80, 1.5 ml po) was administered to groups 2-6 on the fifth day.

**CCl₄ induced hepatotoxicity studies**—Rats were divided into six groups, each containing six rats. Group 1, the normal control group was administered po, a single daily dose of 0.5 % Tween-80 (1ml) on all 5 days and olive oil (1 ml/kg) sc, on days 2 and 3. Group 2, the CCl₄ control group was administered a single daily dose of 0.5 % Tween-80 (1 ml) po, on all 5 days and on the second and third day they were administered sc, 2 ml/kg of CCl₄: olive oil (1:1).Groups 3, 4 and 5 were administered HH (100, 200 and 300 mg/kg), p.o for all 5 days and a single dose of CCl₄: olive oil mixture (2 ml/kg) sc, on days 2 and 3, 30 min after HH administration. Group 6 was administered Silymarin, at a dose of 100 mg/kg p o, on all 5 days and a single dose of CCl₄– olive oil mixture (2 ml/kg) sc, on days 2 and 3, 30 min after Silymarin administration.

**Biochemical and histopathological studies**—48 hr after PCM or 24 hr after CCl₄ intoxication, the animals were sacrificed by mild ether anaesthesia. Blood samples were collected for evaluating the biochemical parameters and liver tissue slices were collected for histopathological studies. Biochemical parameters like serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (SAKP) and serum bilirubin (SB) were assayed according to standard methods. Seven µm thick paraffin sections of buffered formalin-fixed liver samples were stained with haematoxylin - eosin for photomicroscopic observations of the liver histological architecture of the control and treated rats.

**Assessment of lipid peroxidation**—The antilipid peroxidant effect of HH was studied in vitro, following modified methods. Briefly, 0.5 g of the rat liver tissue was sliced and homogenized with 10 ml of 150 mM KCl-Tris-HCl buffer (pH 7.2). The reaction mixture was composed of 0.25 ml of liver homogenate, Tris-HCl buffer (pH 7.2), 0.1 mM ascorbic acid (AA), 4 mM FeCl₂ and 0.05 ml of various concentrations of HH extract. The mixture (in triplicate) was incubated at 37°C for 1 hr in capped tubes. Then, 0.1 N HCl, 0.2 ml of 9.8% sodium dodecyl sulphate (SDS), 0.9 ml of distilled water and 2 ml of 0.6% thiobarbituric acid (TBA) were added to each tube and vigorously shaken. The tubes were placed in a boiling water bath at 100°C for 30 min. After cooling, the flocculent precipitate was removed by adding 5 ml of n-butanol and they were centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured at 532 nm.

**DPPH radical scavenging activity**—DPPH radical scavenging activity was measured by the spectrophotometric method. To a methanolic solution of DPPH (200 µM), 0.05 ml of the test compound dissolved in ethanol were added at different concentrations (100 - 500 µg/ ml). An equal amount of ethanol was added to the control. After 20 min, the decrease in the absorbance of test mixture (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition calculated by using the formula given below:

\[
\text{Inhibition} \% = \frac{\text{control} - \text{test}}{\text{control}} \times 100
\]

**Behavioural and toxic effects**—Seven groups of ten mice were treated with HH extract, (100, 200, 400, 800, 1600, 3200 and 6400 mg/kg p.o). They were observed continuously for 1 hr for any gross behavioural changes, symptoms of toxicity and mortality if any and intermittently for the next 6 hr and then again, 24 hr after dosing with HH extract.

**Statistical analysis**—This was carried out using the Student’s t-test.

**Results**

**Effect of HH on serum hepatic enzyme levels**—Both the hepatotoxins (PCM, CCl₄) significantly produced severe liver damage as indicated by a marked increase
in SGOT, SGPT, SAKP and SB values of the toxin groups. Treatment with HH (100, 200 and 300 mg/kg) caused significant reduction of these values in both the cases (Tables 1 and 2) and the hepatoprotective effect was almost comparable to that of silymarin, the known hepatoprotective agent. No significant enhancement of the hepatoprotective effect was observed by increasing the dosage of HH beyond 100 mg/kg in both the cases.

**Histopathology**—Histopathological observations basically supported the results obtained from serum enzyme assays. The liver of PCM - treated rats showed fatty degeneration of hepatocytes with nuclear pyknosis and karyolysis. The liver of CCl4 – treated rats showed massive fatty changes, gross necrosis and broad infiltration of lymphocytes and Kupffer cells around the central vein and loss of cell boundaries. The histopathological observations of the liver of rats pretreated with HH and subsequently given PCM/CCl4 showed a more or less normal histological architecture of the liver, having reversed to a large extent, the hepatic lesions produced by the toxins, almost comparable to the normal control and silymarin groups (Figs 1a - 1g).

**Effect of HH on in vitro lipid peroxidation**—HH showed very potent inhibition of FeCl2-AA stimulated rat liver lipid peroxidation in vitro at concentrations of 100 and 200 µg/ml. There was a significant increase of malondialdehyde (MDA) in FeCl2-AA treated rat liver homogenate, compared to normal control without FeCl2-AA (Table 3).

**Effect of HH on DPPH free radical scavenging activity**—HH showed maximum inhibition (88.77%) of DPPH free radical at 400 µg/ml. 25 µg/ml dose failed to evoke significant response and it was observed that the free radical was scavenged in a concentration dependent manner upto 400 µg/ml. (Table 4).

**Toxicity studies**—In the acute toxicity studies, the HH extract was found to be toxic (5/10 mice died) at the dose of 6400 mg/kg, but it was found to be safe (non lethal) upto 3200 mg/kg. The LD50 of HH was therefore 6400 mg/kg po, in mice (data not shown).

**Discussion**

In recent years, many studies have been undertaken with traditional medicines, in an attempt to develop new drugs for hepatitis. In the present study, we used two mechanistically different models including CCl4 and PCM for liver damage induction to investigate whether the plant extract HH could decrease efficiently the toxicity produced by these hepatotoxicants.

Paracetamol is a well known antipyretic, which produces hepatic necrosis at higher doses. This hepatotoxicant is primarily metabolised by sulfation
and glucuronidation to reactive metabolites and then activated by the Cytochrome P-450 system to produce liver injury. Its mode of action is by covalent binding of its toxic metabolite, n-acetyl p-benzo quine - amine to tissue macromolecules, resulting in cell necrosis. Protection against paracetamol-induced toxicity has been used as a test for a potential hepatoprotective agent by several investigators. Damage to the structural integrity of the liver by paracetamol is reflected by an increase in the levels of serum transaminases, within 48 hr of exposure. These enzymes are cytoplasmic in location and are released into circulation after cellular damage.

Our studies indicated that pretreatment of rats with 100, 200 and 300 mg/kg p.o. of HH extract before paracetamol administration resulted in a significant protection of paracetamol-induced elevation of serum marker enzymes. This was also supported by liver biopsy which showed that histopathological damage caused by PCM was improved in rat liver treated with HH.

CCl₄ has been one of the most intensively studied hepatotoxicants to date and provides a relevant model for other halogenated hydrocarbons that are used widely. A single exposure to CCl₄ can lead to severe centrilobular necrosis and steatosis. The changes associated with CCl₄-induced liver damage are similar to that of acute viral hepatitis. CCl₄ is transformed by Cytochrome P-450 system to produce trichloromethyl free radicals. These free radicals may again react with oxygen to form trichloromethyl peroxyl radicals which may attack lipids on the membrane of endoplasmic reticulum to elicit lipid peroxidation, finally resulting in cell necrosis and consequent cell death. Marked increase in release of hepatic enzymes into the blood stream is often associated with massive necrosis of the liver. CCl₄ is known to cause marked elevation of serum enzymes. In the present study, a significant increase in activity of SGOT, SGPT, SAKP and SB was observed within 24 hr of exposure to CCl₄ indicating considerable hepatocellular injury. Our results indicated that HH at all the doses tested significantly prevented the increased serum enzyme activity induced by CCl₄, indicating improvement of the functional status of the liver by this herb, which was also supported by the histopathological findings.

The recovery towards normalisation of serum enzymes and histological architecture caused by HH was almost similar to that caused by silymarin in the present study. Silymarin is a known hepatoprotective compound, protecting the plasma membrane of hepatocytes. Similar results have also been reported.

FeCl₂ - ascorbic acid mixture is known to stimulate lipid peroxidation in microsomes of rat liver in vitro. Table 3—Inhibitory effect of ethanolic extract of Hibiscus hispidissimus whole plant (HH) on FeCl₂ - ascorbic acid (AA) induced lipid peroxidation in rat liver homogenate in vitro. [Values are mean ± SD of 3 animals]

<table>
<thead>
<tr>
<th>Groups</th>
<th>HH Concentration (µg/ml)</th>
<th>MDA (nmol/g wet liver)</th>
<th>MDA inhibition (%)</th>
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<td>Normal control</td>
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<td>1.35 ± 0.60</td>
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<td>FeCl₂ - AA+HH</td>
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<td>1.38 ± 0.01</td>
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**P ≤ 0.01, compared to FeCl₂ - AA Control

Table 4—Effect of ethanolic extract of Hibiscus hispidissimus whole plant (HH) on DPPH radical scavenging activity [Values are mean of 3 experiments]

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**Fig. 1—Effect of Hibiscus hispidissimus whole plant methanolic extract (HH) on PCM/CCl₄ induced liver damage in rats. (a). Control - well defined nuclei and cytoplasm (× 300); (b) PCM treated - necrosis of hepatocytes with nuclear pyknosis (↑), congestion, vacuolar degeneration and karyolysis (× 300); (c). Liver pretreated with HH (100 mg/kg) before PCM administration – lesser necrosis compared to (b) (× 300); (d) Liver pretreated with silymarin (100 mg/kg ) prior to paracetamol administration, showing normalcy of hepatic cells with mild necrosis (× 300); (e) CCl₄ treated showing fatty changes (↑), gross necrosis, broad infiltration of lymphocytes and Kupffer cells around the central vein and loss of cell boundaries (× 300); (f) Liver pretreated with HH (100 mg/kg) prior to CCl₄ administration - The lesser necrosis compared to (e) (× 300); (g) Liver pretreated with silymarin (100 mg/kg) prior to CCl₄ administration, showing normalcy of hepatic cells with mild fatty changes and necrosis (× 300).**
In the present study, HH prevented the rise of lipid peroxides (MDA production), showing its significant anti lipid peroxidant effects.

DPPH radical is widely used as the model system to investigate the scavenging activities of several natural compounds. DPPH is scavenged by antioxidants through the donation of proton forming the reduced DPPH which can be quantified by its decrease of absorbance. HH significantly quenched DPPH radicals indicating its potent free radical scavenging activity, in the present study.

The results also revealed the non-toxic nature of HH upto 6400 mg/kg in mice and was not surprising as it is extensively used in tribal medicine of Kerala to treat various ailments. HH is a rich source of triterpenes like hibiscatin, gossypitrin etc and flavonoids which have been shown to possess probable mechanism of action of Hibiscus hispidissimus appears to be its effect as a free radical scavenger and inhibitor of lipid peroxidation of the liver plasma membrane. Further studies are in progress in our laboratory to explore its chemical constituents and detailed mechanism of action.

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References


