Evaluation of Antioxidant and Hepatoprotective Activity of Fruit Rind Extract of Garcinia dulcis (Roxburgh) Kurz

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ABSTRACT
Background: Garcinia spp. belongs to the family Clusiaceae has been traditionally used for the treatment of many ailments including the liver damage. Garcinia dulcis found in North Eastern region of Assam; India can be a potential candidature to combat different ailments. Objective: The present work has been designed in such a way to appraisal the antioxidant and hepatoprotective activity of fruit rind extract of this plant. Materials and Methods: The antioxidant activity was investigated through the various in vitro models, namely, 2,2-diphenyl-1-picrylhydrazine, 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, nitrite oxide. Phytochemical investigation for total phenolic and flavonoids contents were carried out by standard protocol. For the evaluation of hepatoprotective activity, albino Wistar rats were divided into five groups, five animals per group and activity was determined by measuring the contents of liver function marker enzymes such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (ALP), and biochemical parameter, that is, Bilirubin and total protein. Histopathology observation of liver sections was conducted. Results: Phytochemical investigation revealed the presence of both phenolic and flavonoid groups in the extract in a significant amount. Antioxidant activity of the plant extract was observed in all models and percentage of inhibition was dose-dependent. Intoxicated with carbon tetrachloride, elevated the liver function enzymes, bilirubin, and suppressed the production of total protein. Pretreatment with the extract decreased the SGOT, SGPT, ALP, and bilirubin level significantly and increased the production level of total protein in a dose-dependent manner. The histopathological observation supported the hepatoprotective potentiality of the extract. Conclusion: The results indicate that fruit rind part of G. dulcis is nontoxic and the plant can utilize as an antioxidant source. The plant has a protective agent for liver damages and other diseases caused by free radicals. Key words: Antioxidant, free radicals, Garcinia dulcis, hepatoprotective.

SUMMARY
• In vitro antioxidant and in vivo hepatoprotective activity was evaluated
• Methanolic extract was subjected to quantify the both phenolic and flavonoids contents. The extract showed the significant amount of both phenolic and flavonoids contents. The extract showed the free radical scavenging activity in 2,2-diphenyl-1-picrylhydrazine, 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid and nitrite oxide models
• The extract was administrated against the carbon tetrachloride intoxicated animal models to evaluate the hepatoprotective activity by determining the level of liver marker enzymes such as serum glutamate pyruvate transaminase, SGOT, alkaline phosphatase and biochemical parameter such as protein and bilirubin. Pretreatment with the extract reversed the elevated level of the enzymes and increased the protein level in a dose-dependent manner
• The histopathological observations of the liver sections supported the hepatoprotective activity of the extract
• The present study revealed that the Garcinia dulcis extract is a good candidate for preventing liver damage and other disease caused by free radicals.

INTRODUCTION
Free radicals are produced in the human bodies which cause different disorders such as liver injury, Alzheimer’s diseases, atherosclerosis, cardiovascular diseases, diabetes, and rheumatoid arthritis. However, human body developed their own defense mechanism to remove or neutralized the free radicals with the help of antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase.¹⁻³ However, due to heavy exposure to external environmental pollutants, such as smoke, ultraviolet (UV) radiation, and pesticides, the self-defense mechanism is sometimes insufficient to remove the excessive free radicals. Therefore, supplementation of phytochemicals...
with antioxidant properties has become a considerable attention at present scenario.\[4\]

The liver is the largest and most vital organ of the human body and involved in almost all the biochemical pathways such as in secretion, growth, supply of nutrient, energy, and reproduction. The liver is the primary site of intense metabolism and excretion. The major function of liver is the metabolism of carbohydrate, fats and protein; and storage of energy for future use. Therefore, protection of liver from the toxic products is a serious concern for well-being. However, due to consumption of alcohol, exposure of environmental toxic, over drugs consumption lead the liver in various ailments like hepatitis, cirrhosis, etc.\[3,4]\ Carbon tetrachloride (CCl\(_4\)) is a widely used hepatotoxic chemical to induce the liver and tissue damage in laboratory animal experiment.\[7,8\] The main principle of CCl\(_4\) induced liver damage is the lipid peroxidation and decreases the antioxidant enzyme system by producing free radicals.\[9\] Many synthetic drugs used in the treatment of liver disorder are inadequate and sometimes create critical side effect.\[10\] Therefore, nowadays, there is growing interest in evaluation of hepatoprotective activity of traditionally used herbal medicine in a scientific manner and their treatment is safe because they are natural.\[11\] Garcinia Linn. belongs to the family of Clusiaceae, (Thekera, in Assamese, India) is traditionally used for cure of several diseases such as dysentery, liver damage, after childbirth complex and fever.\[12\] The pericarp extract of this family is rich in the both phenolic and flavonoids contents and plays a significant role in the scavenging of free radicals in the different in vitro antioxidant models.\[13\] Garcinia dulcis (Tepor tenga, in Assamese, India) was reported from Dibrugarh, Assam, India by Begum et al.\[14\] which contains considerable medicinal properties, as per traditional knowledge system.

Therefore, the present study was undertaken to evaluate the in vitro antioxidant potential and hepatoprotective activity of fruit rind extract of G. dulcis against the CCl\(_4\) induced albino rats.

**MATERIALS AND METHODS**

Plant material collection and preparation of extract

Fresh fruits were collected from Jokai Botanical Garden, Dibrugarh, Assam, India, in the month of September to November 2014 and identified by Department of Life Sciences, Dibrugarh University. A reference specimen has been retained in the herbaria in the Department of Life Sciences for future reference.

The fruit rind was dried in hot air oven by maintaining the temperature between 25°C and 35°C. The oven-dried sample was then powdered and 50 g of the sample immersed in 100 ml of 80% methanol in a shaking flask condition for 24 h (at 25°C) and later filtered through Whatman No. 1 filter paper. The extract was then concentrated with the help of the rotary evaporator under pressure, and finally, extract was stored in the refrigerator for further experiment.

**Chemicals**

All the chemicals and solvents of analytical grade and procured from the Merck Millipore. 2,2-diphenyl-1-picyrylhydrazine (DPPH), ascorbic acid, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), naphthyl ethylenediamine dihydrochloride were collected from the Sigma-Aldrich (Germany). Other chemicals such as Silymarin, CCl\(_4\), were from Merck Millipore. Standard kits for SGOT, serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphate (SALP), and total bilirubin were purchased from the Span Diagnostics Ltd., India.

**Phytochemical investigation**

Plant extract was subjected toward the quantification of total phenolic and flavonoids contents. Gallic acid and Rutin were considered standard compounds, respectively and the result was expressed in standard equilibrium.

**Estimation of total phenolic content**

Half milliliter of each plant extract (1000 µg/ml) was mixed with the 2.5 ml of Folin–Ciocalteu reagent (diluted 1:10 ratio with distilled water) and 1.5 ml of sodium carbonate (20% w/v). The mixtures were shaken thoroughly using vortex and made up to the volume of 10 ml with double distilled water. The mixtures were then kept for 90 min. The absorbance was measured at 760 nm using UV - Spectrophotometer. Different concentration (10–50 µg/ml) of Gallic acid was used for the preparation of the standard curve. The total phenol content was expressed in Gallic acid equivalent in mg/g of plant extract.\[15\]

**Estimation of total flavonoids content**

Half milliliter of each plant extract (1000 µg/ml) was mixed with 1.5 ml of methanol (75% v/v), 0.1 ml (1 M) of potassium acetate and keep for 1 min. After 1 min incubation, 0.1 ml (10% w/v) aluminum chloride was added and made up the volume 3 ml with double distilled water and kept the mixture for 30 min in dry and shady place. The absorbance was measured at 415 nm. Rutin was used as standard flavonoids for the preparation of calibration curve and total flavonoids was expressed as rutin equivalent in mg/g of plant extract.\[16\]

**Evaluation of antioxidant activity**

Antioxidant activity was evaluated in different in vitro assay models.

2,2-diphenyl-1-picyrylhydrazine radical scavenging assay

DPPH is a stable free radical which is usually converted into 1, 1 dihydroxyl 2-picryl hydration. Whenever it reacts with antioxidant, the degree of conversion can be measured by spectrophotometric method. A volume of 2 ml of different concentration (100, 500, 1000 µg/ml) of the extract and different concentration (100, 500, 1000 µg/ml) of standard solution (Ascorbic acid) were added to the freshly prepared 2 ml of DPPH solution (0.2 mM). After incubation of 30 min in the dark place, absorbance was taken in 517 nm using UV-spectrophotometer.\[17\] The experiment was performed in triplicate and percentage of inhibition was calculated by the following formula.

\[
\text{Absorbance of control - Absorbance of test sample} \times 100% = \text{Percentage of inhibition (})
\]

2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical scavenging assay

The ABTS radical scavenging assay was carried out by the method of Re et al.\[18\] with minor modification. The ABTS radical was formed by the oxidation of ABTS with potassium persulfate. The ABTS cation solution was prepared by mixing of 5 ml ABTS (7 mm) with 100 µl of potassium persulfate and incubated for 12 h. For the preparation of working solution, a necessary amount of the previous solution was added to the phosphate buffer saline (PBS) solution till the absorbance was 0.70 ± 0.001 at 734 nm. The freshly prepared working solution was then kept in the dark for 30 min. One milliliter of different concentrations of plant extract was mixed with 1 ml of working solution and mixed thoroughly; kept in the dark for 10 min. The absorbance was measured at 734 nm. The percentages of inhibition were calculated by above-mentioned formula.
NABAJYOTI GOGOI, et al.: Antioxidant and Hepatoprotective Activity of Garcinia dulcis

**Nitrite oxide radical inhibition assay**

Nitric oxide was generated by dissolving the sodium nitroprusside in an aqueous solution which was measured by Griess reaction. Scavengers of nitric oxide compete with oxygen to help reduce the production of nitrite, oxide.\[^{[19]}\] The reaction (6 ml) mixer was prepared by mixing of sodium nitroprusside (10 mM, 4 ml), 1 ml of different concentration of sample or standard (dissolved in dimethyl sulfoxide) and PBS (1 ml PH 7.4) and kept the mixer for reaction at 25°C for 150 min. After the reaction, 0.5 ml of reaction mixer was removed and transferred to the Griess reagent and incubated for 30 min at diffused light. Griess reagent was prepared by mixing of sulfanilic acid (1 ml, 0.33% w/v) and naphthylethlenediamines dihydrochloride (1 ml, 0.1% w/v). The absorbance was measured at 540 nm.\[^{[20]}\] The percentage of inhibition was calculated above mention formula.

**In vivo animal experiment**

**Experimental animals**

Wistar albino female rats, weighting 80–120 g were used for hepatoprotective experiment. Animals were maintained under standard condition (12 h light/dark cycle; 25°C), fed with standard feed and water ad libitum, at the Central Animal House of Dibrugarh University. Animals were acclimatized to the animal house conditions for 15 days before conducting the experiment. The experimental protocol was reviewed and approved by Dibrugarh University animal ethical committee (approved no. IAEC/DU/98, Regd. no. 1576/GO/a/11/CPCSEA) and animals were cared and handled with before the regulation of CPCSEA.

**Acute toxicity study**

Acute toxicity of methanol fruit rind extract was of G. dulcis was conducted on Albino rats, according to the OECD guideline No. 425.\[^{[21]}\] Animals were fasted for the 12 h, and the extract of 2000 mg/kg (single dose) was administrated orally, and observed for initially for 6 h and conducted the experiment for 14 days to understand mortality, general behavior changes, discomforts and nervous manifestations.

**Carbon tetrachloride induced hepatoprotective activity**

Albino rats were divided randomly into the five groups and containing six animals. The experiment was conducted for the 7 days.

- **Group I:** Positive control (normal) group was administrated with normal saline, 0.9% NaCl (5 ml/kg body weight, po), for 7 days.
- **Group II:** Negative controlled CCl\(_4\) induced group, was administrated with a single dose of normal saline daily, 0.9% of NaCl (5 ml/kg, po) and CCl\(_4\)/olive oil (1:1 v/v, 1 ml/kg, ip) on the alternate days for 7 days.
- **Group III:** Standard group, was administrated with a single daily dose of Silymarin (50 mg/kg, po) and CCl\(_4\)/olive oil (1:1 v/v, 1 ml/kg, ip) on every alternate days for 7 days.
- **Group IV:** Low-dose test group, was administrated with methanolic extract (200 mg/kg, po) single dose daily and CCl\(_4\)/olive oil (1:1 v/v, 1 ml/kg, ip) in the alternate days of 7 days.
- **Group V:** High-dose test group, was administrated with methanolic extract (400 mg/kg, po) single dose daily and CCl\(_4\)/olive oil (1:1 v/v, 1 ml/kg, ip) in the alternate days for 7 days.\[^{[22]}\]

**Assessment of hepatoprotective activity**

**Biochemical estimation**

On the 8 days, after 12 h administration of the last dose, blood were collected from the retro-orbital plexus using ether as anesthesia. The animals were then sacrificed by cervical decapitation. Blood was also withdrawn by intracardiac puncture. Serum was separated from the fresh blood by centrifugation at 5000 rpm for 10 min and stored at 4°C until use. The conventional liver biochemical test from the blood serum, namely, Serum Glutamate Pyruvate Oxaloacetate Transaminase (SGOT), SGPT were estimated by the methods of Reitman and Frankel\[^{[23]}\] and SALP was estimated by the methods of King and Kind\[^{[24]}\] using standard kit from Span Diagnostics Ltd., India. Total protein and bilirubin contents were estimated by Lowry et al. and Malloy and Evelyn methods, respectively.\[^{[25,26]}\]

**Histopathological studies**

After collection of blood by intracardiac puncture, liver was excised from the different group, washed with normal saline and dried with blotting paper. It was fixed with 10% formalin. The fixed liver was then dehydrated in graded alcohol (30%–100%) and embedded in paraffin. Microtome sections (0.5 µ) were prepared and stained with hematoxylin-eosin dye and finally examined under microscope (×40) for histopathological changes for the hepatoprotective assessment.\[^{[27]}\]

**Statistical analysis**

The data were expressed in mean ± standard error, (\(n = 3, 6\)). All data were analyzed by one-way ANOVA followed by Tukey test. The graphical representations were prepared using Sigma Plot software (Systal software Inc. version 13, San Jose, USA).

**RESULTS**

**Phytochemical investigation**

**Total phenolic and flavonoids contents**

The quantification of total phenolic and flavonoids contents of G. dulcis is summarized in Table 1. The results were expressed in Gallic acid equivalent (mg/g of extract) and Rutin equivalent (mg/g extract), respectively.

**Antioxidant activity investigation**

**2,2-diphenyl-1-picrylhydrazine radical scavenging assay**

The inhibition percentage of DPPH radical scavenging activity of three concentrations of the rind extract is presented in Figure 1. The rind extract exhibited dose-dependent inhibition on DPPH models. The inhibition of DPPH free radicals was increased with increasing the concentration of the plant extract. The maximum inhibition of the extract was 56.44% ± 3.45%, while ascorbic acid was 231.86% ± 2.18% at 1000 µg/ml.

**2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical scavenging assay**

The inhibition percentage ABTS radical scavenging of the three concentrations of plant extract is presented in Figure 2. As illustrated in Figure 2, the scavenging activity of the extract was dose dependent and increased the percentage of inhibition with increasing the concentrations. The high inhibition was found at 1000 µg/ml concentration, i.e.; 64.09% ± 1.21%, while it was found 123.1% ± 2.10% in the case of Ascorbic acid.

**Table 1:** Estimate value of total phenolic and flavonoids contents of Garcinia dulcis

| Plants extract | Total phenolic content (GAE mg/g dry extract) | Total flavonoids content (RE mg/g dry extract) |
|----------------|---------------------------------------------|-----------------------------------------------|
| Garcinia dulcis | 56.52±2.94                                  | 84.88±3.91                                    |

Values are mean±SEM of three replicates. GAE: Gallic acid equivalent; RE: Rutin equivalent; SEM: Standard error mean.
Nitrite oxide radical inhibition assay
Nitrite oxide radicals inhibition of the rind extract was also found to be dose-dependent. The inhibition was increased with increasing of concentrations of the plant extract. The maximum inhibition was recorded 56.15% ± 0.972%, while in ascorbic acid, it was 418.99 ± 5.35, at 1000 µg/ml. The result is presented in Figure 3.

In vivo animal experiment
Acute toxicity
There is no mortality among the animals and recorded no toxicity, and abnormal behavior observed at the dose level 2000 mg/kg. The results suggest that the rind extract is safe at the dose of 2000 mg/kg body weight. Therefore, administrated dose was selected at 200, 400 mg/kg, po for further experiments.

Effect of rind extract on serum marker enzyme, total protein, and bilirubin
The serum marker SGOT, SGPT, SALP, and bilirubin were significantly elevated in the content of CCl₄ intoxicated animals. However, the total protein level was reduced in CCl₄ intoxicated animals. In contrast, pretreatment with rind extract (200, 400 mg/kg) and Silymarin (50 mg/kg) exhibited a reversed parameter level significantly in a dose-dependent manner. The results are presented in Figures 4-8.
Histopathological observation

Histopathological observation of liver sections of the different groups supports the hepatoprotective activity of the rind extract. The control group animal exhibited normal hepatic cell with defined cytoplasm, prominent nucleus, and normal hepatic vein [Figure 9a]. The CCl₄ intoxicated group showed loss of total hepatic architecture with necrosis, inflammation, crowding central vein in many areas [Figure 9b]. The others group pretreatment with Silymarin and rind extract (200, 400 mg/kg) showed the liver protection by minimizing inflammation, less necrosis and reducing crowding of central vein in a dose-dependent manner [Figure 9c-e].

DISCUSSION

Phytochemicals are usually recognized as antioxidant which contain bioactive polyphenols, such as phenolic acid and flavonoids. Phenolic acid contains the major phenol containing groups which are served as a primary source of antioxidant or free radical scavenger, while polyphenols act as an antioxidant terminator by scavenging free radicals to form stable one.[28,29] In current findings, it was confirmed that phenol and flavonoids are present in G. dulcis rind extract, which are responsible for scavenging free radicals in various antioxidant models.
DPPH evolves free radicals whenever it puts in solvents. The DPPH system is widely accepted tools to determine the free radical scavenging activities of antioxidants; it accepts an electron or hydrogen radical as stable molecules. The deductive capability of free radicals in DPPH system can be determined by decreasing absorbance at 517 nm, experimentally. The present findings revealed that G. dulcis rind extract has the potential to scavenge the DPPH free radicals in a dose-dependent manner.

The ABTS is relatively a recent free radical scavenging method which produces more drastic radicals, often used for screening complex antioxidant from the mixture such as plant extract, biological beverage, and fluids. In this method, the antioxidant potentiality is measured by loss of blue-green color of ABTS$^+$ when the antioxidant is putted into it. The antioxidant reduces ABTS$^+$ to stable ABTS and decolorize. Our finding regarding on removal of ABTS$^+$ free radicals is dose-dependent. Nitride oxide produces continuously inside the human body, intermediating the regulation of various metabolic processes. Excess production of NO• may cause several diseases such as inflammation, cancer, etc. NO• is reactive under aerobic conditions and usually react with oxygen to produce stable product, i.e., nitrate and nitrite by producing intermediates such as NO$_3$-, N$_2$O$_4$, and N$_2$O$_5$. These free radicals cause alternating the functional behavior of cellular component. The free radical scavenging principle in NO• models is that the antioxidants present in extract compete with oxygen to react with nitride oxide and inhibits the production of intermediate compounds, Hence, protect the cellular components. Nitride oxide can be generated by incubation of sodium nitroprusside in PBS at 25°C. The present investigation on NO• scavenging potentiality of G. dulcis rind extract was found to be dose dependent.

Our results showed that the methanolic extract of the G. dulcis possess significant antioxidant activity. The involvement of free radicals in the damage of liver has been investigated for many years. The free radical scavenging potentiality of the extract was also accompanied by the hepatic protection induced by CCl$_4$. In, liver CCl$_4$ is metabolically activated by cytochrome P450 mixed oxidase in endoplasmic reticulum to form CCl$_4$• free radicals which bind covalently to unsaturated lipid membrane, elevation of bilirubin level, reduction of total protein of serum and followed by liver enzyme marker level such as SGOT, SGPT, SALP, and finally hepatocyte damage.

The assessment of liver protection can be evaluated by determining the serum enzyme. Hepatocellular necrosis or cell membrane injury leads to elevate of serum SGOT and SGPT, released from liver to blood circulation. The assessment of SGPT is considered as a high index because it is found mainly in the liver and catalase conversion of alanine to pyruvate and glutamate, and release as similar manner to the circulation. Hence, it covered the 90% of total enzyme. Slight elevation of this enzyme is considered as myocardial infarction, jaundice, etc., SALP is related to functioning hepatocyte and elevation in the serum is indicated the biliary pressure. Our results show increased the level of serum enzymes in the negative group than the normal group. Administration of the methanolic fruit rind extract of G. dulcis and silymarin significantly reverses the elevated level of these enzymes, in a dose-dependent manner. Silymarin is well-known hepatoprotective agent derived from Silybum marianum. Hence, silymarin was used as the standard drug. The result indicated that the methanolic fruit rind extract of G. dulcis can ameliorate hepatic function of liver damage by CCl$_4$.

Alteration of total protein has been considered as one of the major factor associated with the hepatic dysfunction. The present study showed that the protein level was decreased in CCl$_4$ induced group compared to control group, which restored almost the normal range pretreatment with the G. dulcis extract a dose-dependent manner. Bilirubin usually removed from the blood by liver through conjugation and secreted in the bile. Breakdown of the red blood cells and elevation of this is indicating the hepatic dysfunction. The current study found that the bilirubin concentration was elevated in CCl$_4$ induced group, compared to the control group and pretreatment with the extract restored the level of bilirubin in a dose-dependent manner.

CONCLUSION

The present finding revealed that the fruit rind extract of G. dulcis contains both phenolic and flavonoids compounds, possesses potent antioxidant activity in vitro models and a significant hepatoprotective activity. These findings indicate that the fruit can be used as functional fruit with any toxicity, and their uses should be considered as an adjunct therapeutic strategy to combat different hepatic disorder and other diseases caused by free radicals. In addition, further studies to separate and characterized the active compounds of G. dulcis and their mechanism toward the hepatoprotective are in progress.

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Conflicts of interest
There are no conflicts of interest.

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