Indian Journal of Natural Products and Resources
Vol. 8(2), June 2017, pp. 132-139

Antioxidant and hepatoprotective activity of fruit rind extract of *Garcinia morella* (Gaertn.) Desr.

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Received 05 August 2016; Revised 04 May 2017

The present work was undertaken to determine the *in vitro* antioxidant and *in vivo* hepatoprotective activity of traditionally used *Garcinia morella* (Gaertn.) Desr. (*Kuji thekera* in Assamese). Fruit rind extract of *G. morella* was studied to examine the antioxidant activity of different concentrations (100, 500, 1000 µg/mL) in various *in vitro* models *viz;* 2,2-diphenyl-1-picrylhydrazine (DPPH), 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Nitric Oxide (NO). Percentage Inhibition was exhibited in a dose dependent manner in all the models. Hepatoprotective activity was determined by measuring the activity of liver function enzymes such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and biochemical parameter like bilirubin, total protein in albino rats. Albino rats administrated with CCl_4 elevated the liver function enzymes and bilirubin, but suppressed the production of total protein. Pretreatment with the extract decreased the AST, ALT, ALP, and bilirubin level significantly and increased the production level of total protein in a dose dependent manner. The histopathological observation of liver sections supported the hepatoprotective activity of the extract. Phytochemical investigation revealed that both phenolic and flavonoids groups were present in the plant. Toxicological study confirmed that the plant is non toxic. The present study indicates that the methanolic fruit rind extract of *G. morella* may be an effective protective agent against the diseases caused by free radicals and it is a viable candidate for treating liver disorder.

Keywords: Antioxidant, Free radicals, *Garcinia morella* (Gaertn.) Desr., Hepatoprotective.

IPC code; Int. cl. (2015.01)- A61K 36/00, A61K 36/38

Introduction

Free radicals usually produced inside the human bodies increase the risk factor of diseases like liver injury, Alzheimer's, atherosclerosis, cardiovascular, diabetes, rheumatoid arthritis. Human body developed its own mechanism to prevent the cascade reaction of free radicals by producing antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase[1-3]. But, sometimes due to exposure of heavy pollutants, such as smoke, UV radiation, and pesticides, the self mechanism is unable to neutralize the excessive free radicals from the body. Therefore, consumption of functional fruit, diet with antioxidant properties has gained considerable attention in the present scenario[4].

The liver is the largest and the most vital organ of human body. It is involved in almost all the biochemical pathways such as growth, supply of nutrient, energy, and reproduction. The liver is the primary site of intense metabolism and excretion and the major function includes 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 to environmental toxins, over drug consumption lead the liver in various ailments like hepatitis, cirrhosis and the like[5]. Carbon tetrachloride (CCl_4) is a widely used hepatotoxic chemical to induce the liver and tissue damage in laboratory animal experiment[6]. The main principle of CCl_4 induced liver damage is the lipid peroxidation and decreased reduction in the production of antioxidant enzyme system by producing free radicals[7]. Many synthetic drugs used in the treatment of liver disorder are inadequate and sometimes produce critical side effects[8]. Therefore, now-a-days there is a growing interest in the evaluation of hepatoprotective activity of traditionally used herbal medicine in a scientific manner.

Garcinia morella (Gaertn.) Desr. (*Kuji thekera* in Assamese) belongs to the family Clusiaceae and is traditionally used for the cure of several diseases such as

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liver damage, dysentery, after childbirth complication, and fever[9]. The methanolic extract of this family is rich in both, the phenolic and the flavonoids contents and play a significant role in the scavenging of free radicals in the different *in vitro* antioxidant models[10]. *G. pedunculata* has the potential activity against the liver damage[11]. Therefore, the present study was undertaken to evaluate the *in vitro* antioxidant potential and hepatoprotective activity of the fruit rind extract of *G. morella* against the CCl_4 induced albino rats.

Materials and Methods

Collection of plant material and extract preparation

Fresh fruits were collected from homestead garden of Lakhimpur District, Assam, India, during August to October, 2014. The fruits were identified at Department of life sciences, Dibrugarh University and a reference specimen (No. DUL.Sc.460) was deposited in the herbaria of the department.

The fruit rind was separated from the seed portion and dried under hot air oven in between 25 to 35 °C. The dried sample was powdered in Motor Grinder and 100 g of the sample was immersed in 200 mL of 80 % methanol and was kept under shaking conditions at 25 °C for 24 h. The extract was then filtered through Whatman No. 1 paper and concentrated with the help of rotary evaporator under pressure. The concentrated extract was stored in the refrigerator for further experiment.

Chemicals

Chemicals and solvents were of analytical grade. DPPH (2, 2' diphenyl -1 –picrylhydrazine), ascorbic acid, ABTS (2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphanic acid), napthylethylenediamine dihydrochloride were purchased from the Sigma Aldrich (Germany). Other chemicals such as silymarin, CCl_4 were purchased from Merck Millipore. Standard kits for AST, ALT, ALP, and total bilirubin were purchased from the Span Diagnostics Ltd, India.

Phytochemical investigation

The plant extract was subjected to quantification of total phenolic and flavonoids contents. Gallic acid and rutin were considered as standard compounds, respectively and the results were expressed in standard equilibrium.

Estimation of total phenolic content

About 0.5 mL of the plant extract (1000 µg/mL) was mixed with the 2.5 mL of Folin- Ciocalteu reagent (diluted in 1:10 ratio with distilled water) and 1.5 mL of sodium carbonate (20 % w/v). The mixture was shaken thoroughly and the volume was made up to 10 mL with double distilled water. The mixture was then kept for 90 min and the absorbance was measured at 760 nm using UV- Spectrophotometer. Calibration curve was prepared with different concentration (10-50 µg/mL) of gallic acid and total phenol content was expressed in gallic acid equivalent in mg/g of plant extract[12].

Estimation of total flavonoids content

About 0.5 mL of the plant extract (1000 µg/mL) was mixed with 1.5 mL methanol (75 % v/v), 0.1 mL (1 M) of potassium acetate and kept for 1 min. Then, 0.1 mL aluminium chloride (10 % w/v) was added and the final volume was made up to 3 mL with double distilled water. The mixture was kept for 30 min in a dry and shaded place and then, the absorbance was measured at 415 nm. Rutin was taken as standard flavonoids for the preparation of calibration curve and the total flavonoid was expressed as rutin equivalent in mg/g of plant extract[13].

Antioxidant activity

The antioxidant activity was evaluated by different *in vitro* assay models.

DPPH radical scavenging assay

DPPH is a stable free radical that 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. About 2 mL of different concentration (100, 500, 1000 µg/mL) of the extract and different concentration (100, 500, 1000 µg/mL) of standard (Ascorbic acid) were added to the freshly prepared 2 mL of DPPH solution (0.2 mM). The solution was kept in dark for 30 min and then, the absorbance was taken at 517 nm by using UV- spectrophotometer[14]. The experiment was performed in triplicate and the percentage of inhibition was calculated by following formula.

$$\%\,\text{inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

ABTS radical scavenging assay

The ABTS radical scavenging assay was carried out by the method of Re *et al.*[15] with minor modification. The ABTS radical was formed by the oxidation of ABTS with potassium per-sulphate. The ABTS cation solution was prepared by mixing 5 mL ABTS (7 mM) with 100 µL of potassium per-sulphate and was incubated for 12 h. For working solution, a necessary amount of above mentioned solution was added to the phosphate buffer saline solution till the absorbance was

0.70±.001 at 734 nm. The working solution was then kept in the dark for 30 min. About 1 mL of different concentrations of plant extract was mixed with 1 mL of working solution and mixed thoroughly. It was kept in the dark for 10 min and the absorbance was measured at 734 nm. The percentages of inhibition were calculated by the above mentioned formula.

Nitric oxide radical inhibition assay

Nitric oxide was generated by dissolving the sodium nitroprusside in aqueous solution which was measured by Griess reaction. Scavengers of nitric oxide compete with oxygen to help reduce the production of nitric oxide[16]. The reaction mixture (6 mL) was prepared by mixing of sodium nitroprusside (10 mM, 4 mL), 1 mL of different concentration of sample or standard (dissolved in DMSO) and phosphate buffer saline (PBS, 1 mL PH 7.4) and kept it for reaction at 25 °C for 150 min. After reaction, 0.5 mL of reaction mixture was removed and transferred to the Griess reagent and incubated for 30 min at diffused light. Griess reagent was prepared by mixing sulphanilic acid (1 mL, 0.33 % w/v) and napthylethylenediamine dihydrochloride (1 mL, 0.1 % w/v). The absorbance was measured at 540 nm[17]. Percentage of inhibition was calculated using the above mentioned formula.

***In vivo* animal experiment**

Experimental animals

Wistar albino female rats, weighting 80-120 g were used for investigating the hepatoprotective activity. Animals were kept 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 fifteen days before conducting the experiment. Experimental protocol was reviewed and approved by Dibrugarh University animal ethical committee (No. IAEC/DU/98). Animals were cared and handled according to the regulation of CPCSEA.

Acute toxicity study

Acute toxicity of fruit rind extract of *G. morella* was conducted according to the OECD guideline No. 425[18]. Animals were fasted for the 12 h and the extract of 2000 mg/kg (single dose) was administrated orally, and observed initially for 6 h and experiment was conducted for 14 days to understand mortality, general behaviour change, discomfort, and nervous manifestation.

CCl_4 induced hepatoprotective activity

Albino rats were divided into five groups containing six animals per group. Group I (Normal group)- administrated with normal saline, 0.9 % NaCl (5 mL/kg body weight, po), daily for 7 days; Group II (Negative group)- administrated with single dose of normal saline (0.9 % of NaCl, 5 mL/kg, po) daily and CCl_4/olive oil (1:1 v/v, 1 mL/kg, ip) on the alternate days for 7 days; Group III (Standard group)- administrated with a single dose of Silymarin (50 mg/kg, po) daily and CCl_4/olive oil (1:1 v/v, 1 mL/kg, ip) on the alternate days for 7 days; Group IV (Low dose group) administrated with single dose of methanolic extract (200 mg/kg, po) daily and CCl_4/olive oil (1:1 v/v, 1 mL/kg, ip) on the alternate days for 7 days; and Group V (High dose group) administrated with single dose of methanolic extract (400 mg/kg, po) daily and CCl_4/olive oil (1:1 v/v, 1 mL/kg, ip) on the alternate days for 7 days[19].

Assessment of hepatoprotective activity

Biochemical estimation

The day after the last dose was administered; blood was collected from each group by retro-orbital plexus technique using ether as anaesthesia. The animals were then sacrificed by cervical decapitation. Fresh bloods were subjected to centrifugation at 5000 rpm for 10 min to separate the serum and serums were stored at 4 °C for further experiment. The conventional biochemical test for liver function was determined by the activity of liver function enzymes such as Aspartate transaminase (AST) and Alanine transaminase (ALT) by the method of Reitman and Frankel[20]. However, Alkaline Phosphate (ALP) was estimated by the method of King and Kind[21] by using standard kit from Span Diagnostics Ltd, India. Total protein and bilirubin contents were estimated by standard methods[22,23].

Histopathological studies

Livers were excised immediately after sacrifice, washed with normal saline and dried with blotting paper. It was then fixed with 10 % formalin. The fixed livers were dehydrated in graded alcohol (30-100 %) and embedded with paraffin. Microtome sections (0.5 μ) were prepared and stained with haematoxylin- eosin dye and finally examined under microscope (40 x) for histopathological changes[24].

Statistical analysis

The results were expressed in mean±standard error, (n= 3, 6) and the data were analyzed by one way ANOVA followed by Tukey test in SPSS (Version 18).

Results

Phytochemical investigation

Total phenolic and flavonoids contents

The quantification of total phenolic and flavonoids contents of *G. morella* is given in Table 1. The results were expressed in gallic acid equivalent (mg/g of extract) and rutin equivalent (mg/g extract), respectively.

Antioxidant activity

DPPH radical scavenging assay

The percentage of inhibition in DPPH models of the extract is given in Fig. 1. The inhibition was dose dependent and the highest inhibition was observed by extract having concentration of 1000 µg/mL.

ABTS radical scavenging assay

The percentage of inhibition in ABTS model of the extract is given in Fig. 2. The scavenging activity of the extract was dose dependent and the highest inhibition was exhibited by extract having concentration of 1000 µg/mL.

Nitric oxide radical inhibition assay

The percentage of inhibition of the extract was also found to be dose dependent in NO_2 model. The maximum inhibition was recorded in 1000 µg/mL than other two concentrations. The results are presented in Fig. 3.

Table 1 — Estimate value of Total phenolic and flavonoids contents of *G.morella*

Plants extract	Total phenolic content GAE mg/g dry extract	Total flavonoids content RE mg/g dry extract
G. morella	54.11±0.78	44.25±0.35

Values are mean±SEM of three replicates; GAE- Gallic acid equivalent; RE- Rutin equivalent; SEM- Standard error mean.

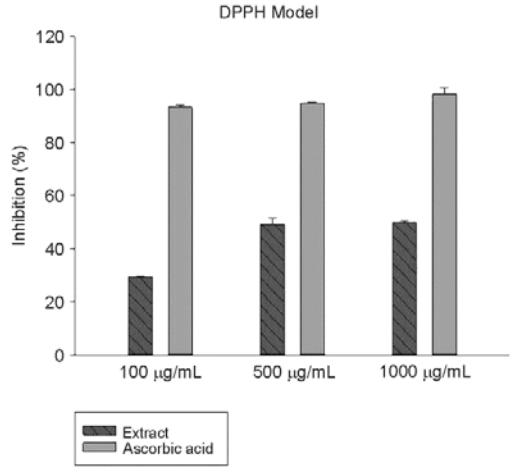

Fig. 1 — Effect of *G. morella* fruit rind extract and known antioxidant (ascorbic acid) in DPPH model. Values are mean±SEM of three replicates.

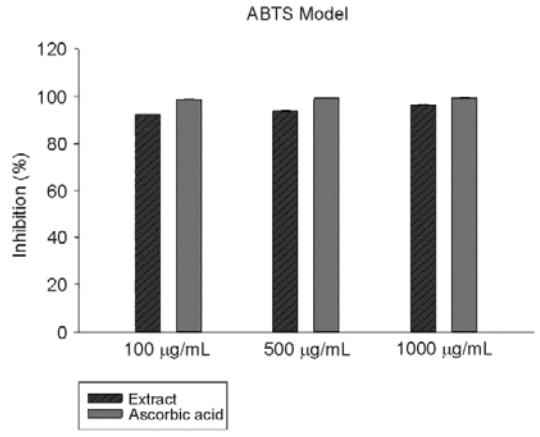

Fig. 2 — Effect of *G. morella* fruit rind extract and known antioxidant (ascorbic acid) in ABTS model. Values are mean±SEM of three replicates

***In vivo* animal experiment**

Acute toxicity

No mortality, abnormal behavior, discomfort, etc. were observed among animals of the group when they were administrated orally a dose of 2000 mg/kg. The result revealed that the rind extract was safe up to the level 2000 mg/kg body weight. Therefore, for hepatoprotective experiment, the doses were fixed at 200 and 400 mg/kg body weight as low dose and high dose, respectively.

Effect of rind extract on serum marker enzyme, total protein, and direct bilirubin

Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline Phosphate (ALP), and direct bilirubin were elevated in the negative controlled group. The total protein was reduced in the negative group. In contrast, pretreatment with rind extract (200, 400 mg/kg) and silymarin (50 mg/kg) reversed the biochemical parameter to almost the control level. The results are given in Fig. 4-6.

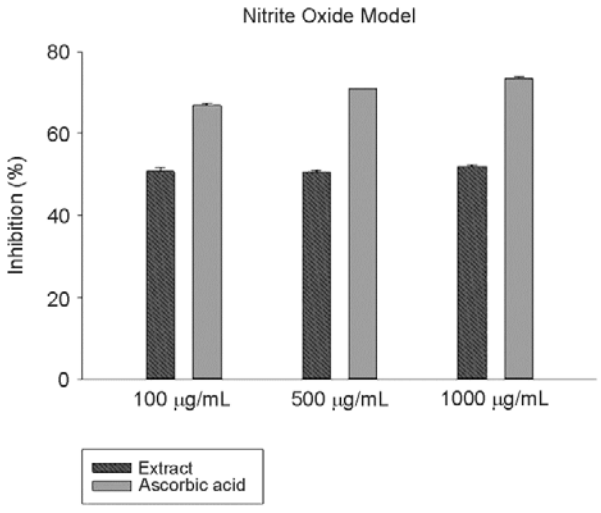

Fig. 3 — Effect of *G. morella* fruit rind extract and antioxidant (ascorbic acid) in Nitric Oxide model. Values are mean±SEM of three replicates.

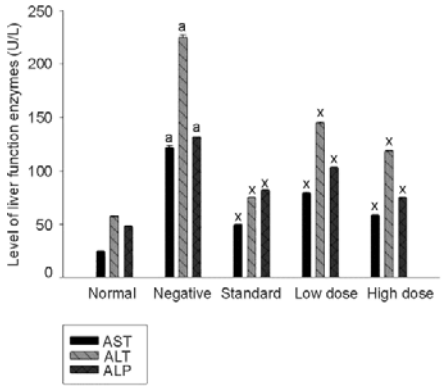

Fig 4 — Effect of *G. morella* extract on serum liver function marker enzymes in experimental animal model. *p* values: ^{a}<0.001, when negative group is compared with normal group whereas ^{x}<0.001 when experimental groups are compared with the negative group.

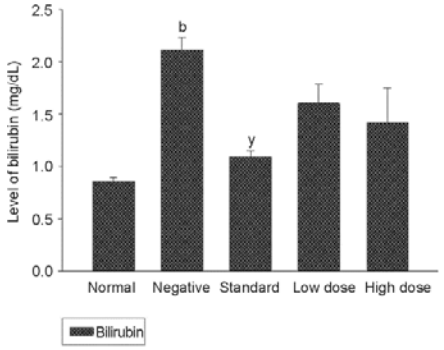

Fig. 5 — Effect of *G. morella* extract on serum bilirubin in experimental animal model. *p* values: ^{b}<0.05 when negative group is compared with Normal group, whereas ^{y}<0.05 when experimental groups are compared with the negative group.

Histopathological observation

Histopathological observation of liver sections of different groups supported the hepatoprotective activity of the rind extract. The control group animal exhibited normal hepatic cell with defined cytoplasm, prominent nucleus, and normal hepatic vein (Plate 1a). The CCl_4 intoxicated group showed loss of the total hepatic architecture with necrosis, crowding central vein in many areas (Plate 1b). In other groups, the liver improvement was observed by minimizing inflammation, less necrosis, and reducing crowding of central vein in a dose dependent manner (Plate 1c-e).

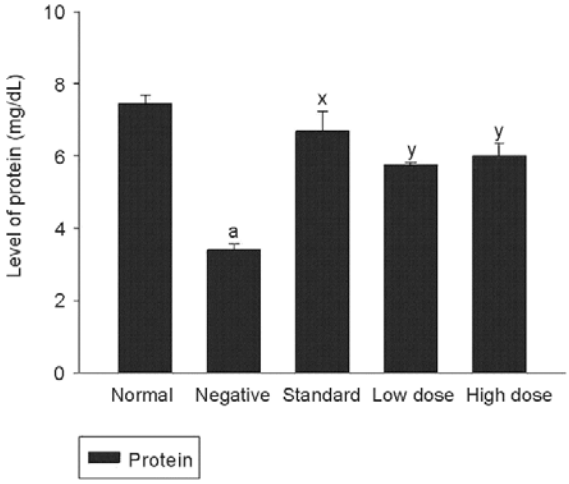

Fig. 6 — Effect of *G. morella* extract on protein level in experimental animal model. *p* values: ^{a}<0.001 when negative group is compared with normal group, whereas ^{x}<0.001, ^{y}<0.05 when experimental groups are compared with the negative group.

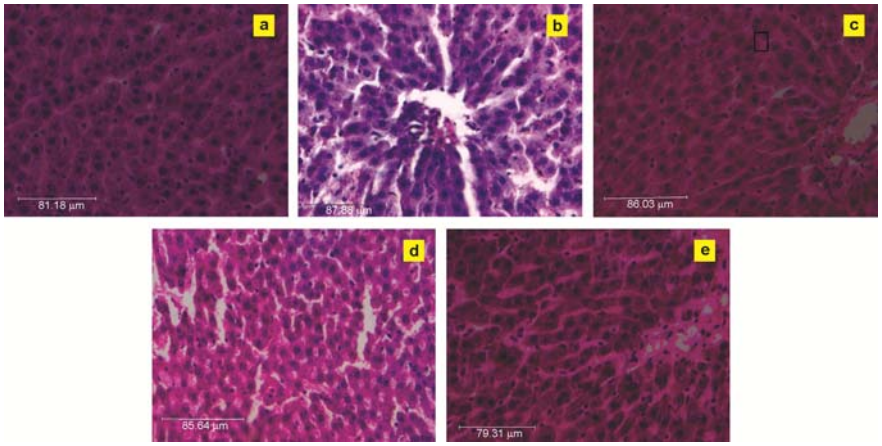

Plate 1 — Effect of methanolic fruit rind extract of *G. morella* on liver. [Liver section, 0.5 µm size, H α E, 40x, a) Control group, exhibiting the normal liver architecture with normal hepatic cells, with define cytoplasm, prominent nucleus and normal central vein, b) Negative group, exhibiting total loss of hepatic architecture with necrosis, inflammation, crowding of central vein, c) Standard group, exhibiting normal hepatic architecture with minimal necrosis, normal central vein, d) Low dose test group (200 mg/kg) showing a pattern of reduced necrosis, inflammation, and e) High dose group, (400 mg/kg), is showing normal architecture with reduced inflammation and normal central vein.

Discussion

Plants having medicinal properties are considered as antioxidant due to the presence of bioactive polyphenols, such as phenolic and flavonoids. Major phenol groups usually present as polyphenols serve as an antioxidant by scavenging free radicals to stable form[25]. Both phenolic and flavonoids are found in the extract. Therefore, the extract scavenged the free radicals in different models.

The DPPH system is a widely accepted tool to determine the free radical scavenging activities of the antioxidants. The delocalization of the spare DPPH molecules gives rise deep violet color in solvent. The antioxidant potentiality can be measured by deduction of absorbance at 517 nm with loss of violet color[26]. The present findings reveal that the *G. morella* rind extract has the potential to scavenge the DPPH free radical in a dose dependent manner. In an earlier experiment, Gogoi *et al.*[27], reported significantly similar findings. The ascorbic acid showed more inhibition than the plant extract in both the investigations.

The ABTS is a relatively recent *in vitro* free radical scavenging model involving chemically produced huge amount of radicals which often utilized for screening antioxidant potentiality of complex mixture such as plant extract, biological beverage, and fluids[28]. The *G. morella* extract showed potent antioxidant activity in ABTS model in a dose dependent manner, which may be due to the presence of phenolic and flavonoids group.

NO• radical generated in animal tissue by the activation of enzyme nitric oxide syntheses, which is later involved in metabolizing of arginine to citrulline with the formation of NO• through five different electron oxidative reaction[29]. Excess generation of NO• may sometimes cause several diseases such as inflammation and cancer. Scavengers of nitric oxide compete with oxygen leading to the reduced production of nitric oxide and its intermediate components. Present investigation found that the inhibition percentage of *G. morella* rind extract was dose dependent in the experimental model.

The present results show that the methanolic extract of the *G. morella* possesses 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 dependent oxidase in endoplasmic reticulum to form CCl_3• free radical, that combine with cellular lipid and protein in the presence of oxygen to induce lipid peroxidation. Lipid peroxidation changes endoplasmic reticulum structure, other membrane, and loss of metabolic enzyme activation leading to the loss of liver function that further shows elevation of AST, ALT, ALP enzymes level and bilirubin level and ultimately shows reduction of total protein in serum[30,31].

The assessment of liver function can be evaluated by determining the serum enzyme. Hepatocellular necrosis or cell membrane injury leads to elevation of serum AST and ALT released from liver to blood circulation. The assessment of ALT is considered as high index, because it is found mainly in the liver and catalase conversion of alanine to pryuvate and glutamate. Hence, it covers 90 % of total enzyme[32]. Slight elevation of this enzyme is considered as myocardial infarction, jaundice, etc. Elevation of ALP in the serum indicates the dysfunction of hepatocyte and the biliary pressure[33]. The present study showed increased level of serum enzymes in the negative group than the normal groups. Administration of the methanolic fruit rind extract of *G. morella* significantly reversed the elevated level of these enzymes, in a dose dependent manner. This indicated that the methanolic fruit rind extract of *G. morella* can ameliorate hepatic function of liver damage by CCl_4. The earlier investigation on *Randia dumetorum*, a medicinal plant, traditionally used in Assam showed similar results[34].

Alternation of total protein has been considered as one of the factor associated with the hepatic dysfunction[35]. Present study showed that the protein level decreased in the negative group compared to the control group, which restored almost to the normal range by pretreatment with the *G. morella* extract in a dose dependent manner.

Bilirubin is usually removed from the blood by liver through conjugation and secreted in the bile. It breaks down the red blood cells and an elevation of this indicates the hepatic dysfunction[36]. Current study found that the bilirubin concentration was elevated in the negative group as compared to the control group and pretreatment with the extract restored the level of bilirubin in a dose dependent manner.

Conclusion

The present findings revealed that the fruit rind extract of *G. morella* contains both phenolic

and flavonoids compounds, possesses potential antioxidant activity *in vitro* models and a significant hepatoprotective activity. The present investigation indicated that the fruit of *G. morella* could be used as functional fruit without toxicity, and this 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. morella* and their mechanism towards the hepatoprotective are in progress.

Acknowledgment

The authors express sincere gratitude to ICMR, New Delhi for financial support (No. 59/14/2011/BMS/TRM dt. 25.03.2012). The authors also thank Prof. A. K. Rai and Banasri Mech, Research Scholar, Department of Life Sciences, Dibrugarh University for help and providing Microtome facility.

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