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Antioxidant and hepatoprotective action of the crude ethanolic extract of the flowering top of *Rosa damascena*

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SUMMARY

The hepatoprotective activity of the alcoholic extract of *Rosa damascena* was studied against paracetamol induced acute hepatotoxicity in rats. Liver damage was assessed by estimating serum enzyme activities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and histopathology of liver tissue. Pre- and post-treatment with ethanolic extracts showed a dose-dependent reduction of paracetamol induced elevated serum levels of enzyme activity. The mechanism underlying the protective effects was assayed *in vitro* and the *R. damascena* extracts displayed dosedependent free radical activity using DPPH (IC_{50} = 162.525 μg/ml) and TBA method. The hepatoprotective action was confirmed by histopathological observation. The ethanolic extracts reversed paracetamol induced liver injury. These results suggest that the hepatoprotective effects of *R. damascena* extracts are related to its antioxidative activity.

Key words: Antioxidant; Paracetamol; *Rosa damascene*; Liver enzyme

INTRODUCTION

Rosa damascena Mill. (*Rosaceae*) is a well known flowering plants and cultivated in rose gardens in several places in Bangle, Kashmir and Punjab. Enormous quantity of wild hill roses grows throughout the North West Himalayas and Kashmir (Joshi, 1994). Essential oil, quarcetin, kampferol and cyanidine have been isolated from whole plant (Joshi, 1994). Cyanidin 3, 5-di-glucoside has been isolated from petals and flowers also contain a bitter principle, tannin content, fatty oil and organic acids (Joshi, 1994). The tetrahydroxy-flavanone (kaempferol, 1) isolated from the methanol extract was effective in reducing the maturation of infectious progeny virus apparently due to selective inhibition of the viral protease (Mahmood *et. al*., 1996). On the other hand the pentahydroxyflavone (quercetin, 2) and two 3-substituted derivatives of kaempferol appeared to inhibit HIV-infection by preventing binding of gp120 to CD4 and 2-Phenylethanol-O-(6-O-galloyl)-beta-D-glucopyranoside interacted irreversibly with gp120 and neutralized virus infectivity (Mahmood *et. al*., 1996). The essential oil of *R. damascena* petals

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was evaluated for its antibacterial effects against three strains of *Xanthomonas axonopodis* spp. vesicatoria. (Basim and Basim, 2003) The rose teas were rich in free gallic acid. The highest values of antioxidant activity, total phenols, and gallic acid contents were found. (Vinokur *et al.,* 2006) Achuthan *et al.* reported that *R. damascena* could protect against CCl_4 induced hepatotoxicity, possibly by its free radical scavenging activity. (Achuthan *et al*., 2003) *R. damascena* also has a high ability to inhibit lipid peroxidation in rat model. (Shahriari *et al*., 2006).

As a part of the on-going investigations on Bangladeshi plants for phytochemical, and pharmacological properties (Alam *et al*., 2006a,b) we now report on the hepatoprotective potential of the ethanolic extracts of the flowering tops of *R. damascena* in rat.

MATERIALS AND METHODS

Plant material

The flowering tops of *R. damascena* were collected from the Gazipur, Dhaka, Bangladesh in December 2005 and identified by the experts of Bangladesh National Herbarium, Dhaka and the sample has been preserved in Pharmacology Laboratory, Bangladesh Agricultural University.

Preparation of ethanol extracts

Dried ground flowering tops (400 g) were extracted with 95% of ethanol in a Soxhlet apparatus at an elevated temperature. The extract was concentrated by evaporation under reduced pressure at 40°C using Buchi rotary evaporator to have gummy concentrate of reddish black colour extract (yield appx. 5.6%).

Animals

Swiss albino rat of either sex (120 - 140 g) were obtained from the Animal house of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). The animals were housed under standard laboratory conditions (relative humidity 55 - 65%, r.t. 23.0 ± 2.0°C and 12 h light: dark cycle). The animals were fed with standard diet and water *ad libitum*. The University Animal Research Ethical Committee approved the experimental protocol.

Antioxidant activity by DPPH scavenging assay of *R. damascena*

Antioxidant activity of the ethanolic extract was determined on the basis of their scavenging activity of the stable DPPH free radical. Quantitative assay was performed on the basis of the modified method of Gupta *et al*. (2003). Stock solutions (10 mg/ml) of the plant extracts were prepared in ethanol from which serial dilutions were carried out to obtain concentrations of 1, 5, 10, 50, 100 and 500 mg/ml. Diluted solutions (2 ml) were added to 2 ml of a 0.004% ethanol solution of DPPH, mixed and allowed to stand for 30 min for reaction to occur. The absorbance was determined at 517 nm and from these values corresponding percentage of inhibitions were calculated as

% inhibition=(Blank OD - Sample OD/Blank OD)×100.
(OD = Optical density)

Then % inhibitions were plotted against concentration and from the graph IC_{50} was calculated using probit equation. The experiment was performed in duplicate and average absorption was noted for each concentration. Ascorbic acid was used as positive control.

Estimation of anti-$FeCl_2$-H_2O_2-induced linoleic acid peroxidation

The effect of anti-$FeCl_2$-H_2O_2-induced linoleic acid peroxidation was determined according to the method of Tamura and Shibamoto. (Tamura and Shibamoto, 1991) A mixture (5 ml) containing ethanolic extracts of *R. damascena* (0 - 900 ppm, relative to linoleic acid), linoleic acid (0.1 M), $FeCl_2$ (0.4 mM), H_2O_2 (0.4 mM), and phosphate buffer

(0.2 M, pH 7.4) was incubated at 37°C for 24 h. After incubation, 1.0 ml of thiobarbituric acid (TBA) (1%), and 1.0 ml of HCl (10%) were added to the mixture, which was heated for 30 min on a boiling water bath. After cooling, chloroform (5.0 ml) was added and the mixture centrifuged at 1,000 *g* to give a supernatant. The absorbance of supernatant was measured spectrophotometrically at 532 nm. A low absorbance value indicated a high antioxidant activity.

Paracetamol induced hepatic necrosis protection of the crude extract

The bioassays were conducted according to the World Health Organization guidelines for the evaluation of the safety and efficiency of herbal medicine (WHO, 1992). Four groups of 6 rats (Table 1) received the plant extract by intra gastric gavages at the dose of 1.5 g/kg body weight and 3 g/kg body weight. Tween 80 was used as a carrier of *R. damascena* extract (1.5 and 3 g/kg body weight/day) as well as for paracetamol (3 g/kg body weight), administered per orally three times a week for 14 days. Higher doses have been selected considering the dosing frequency and experimental period. At the end of the experimental period (14 days) the rats were fasted overnight, blood samples for biochemical assay were collected from animals by tail-tip cutting and sacrificed under ether anaesthesia. The liver were collected and after instantaneous washing a part of the liver tissue was kept in 10% formalin solution for further histopathological studies.

Biochemical assays

Activity of aspertate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined using an auto analyser (Reflotron Plus®, Germany).

Histopathological study

Liver immersed in 10% buffer formalin solution, representative blocks of tissue from each lobe were taken. Following over night fixation in 10% formalin, the tissue blocks were gradually dehydrated in ascending concentration of ethyl alcohol. Then it was properly oriented in molten paraffin for making blocks and sectioning. A paraffin bath with a temperature of 60°C to 70°C was used. The melting point of paraffin was 58°C. The tissues were treated in paraffin melted in for 1 h. The tissue was carefully embedded in proper plane. The paraffin was then allowed to harden at room temperature. A properly sharpened microtome knife was used for section cutting. A water bath with a temperature of 45-50°C was used for floatation of the samples. Sections were cut at 6 micron thickness. Ribbons of sections were selected and placed on the water bath. Then sections were taken on glass slides previously smeared with egg

Table 1. Levels of serum AST, ALT, and ALP in control and experimental groups of rats

Group	Treatment	Mean serum AST level (U/L) ± S.D.	Mean serum ALT level. (U/L) ± S.D.	Mean serum ALP level (U/L) ± S.D.
Group-I	Control (Normal food and water only)	27.87 ± 1.98	28.52 ± 1.68	18.21 ± 1.04
Group-II	Treated with paracetamol (3 g/kg body weight)	62.63 ± 1.35[a]	67.73 ± 2.44[a]	22.12 ± 1.34[b]
Group-III	Paracetamol + ethanol extract (1.5 g/kg bd. Wt.)	55.91 ± 3.43[c]	63.40 ± 3.38[d]	22.00 ± 1.41[e]
Group-IV	Paracetamol + ethanol extract (3 g/kg bd. Wt.)	48.34 ± 2.68[a]	57.38 ± 2.98[a]	20.48 ± 0.42[d]

Values are statistically significant at $P < 0.05$. [a]$P < 0.001$, [b]$P < 0.01$, [c]$P < 0.005$, [d]$P < 0.025$, [e]$P < 0.10$ vs control, Student's *t*-test; values are mean ± S.E. (N = 6). Comparison are made as Group I and Group II; Group II and Group III; Group II and Group IV.

albumin. The slides were kept in slanting position for some time to drain-out the water and then allowed to dry at room temperature. Sections of liver were stained using Haematoxylin and Eosin (H & E) stain.

Statistical analysis

Experimental values were expressed as mean ± S.E.M. Independent sample *t*-test was done for statistical comparison. Statistical significance was considered to be indicated by a P value < 0.05 in all cases.

RESULTS

Antioxidant assay

Antioxidant activity of the ethanolic extract of *R. damascena* was performed on the basis of the modified method of Gupta *et al*. (2003). The extract showed excellent antioxidant activity (IC_{50} about 162.525 μg/ml) against DPPH free radical comparable to that of standard drug, ascorbic acid (IC_{50} about 64.307 mg/ml) (Fig. 1). The free radical scavenging property may be one of the mechanisms by which the plants are effective in traditional medicine.

Fig. 2 shows the antioxidant activity of *R. damascena* extract in the linoleic acid peroxidation system, compared with α-tocopherol, induced by $FeCl_2$ and H_2O_2. The antioxidant activity of *R. damascena* extract increased with increasing concentration at the range of 200 - 600 ppm.

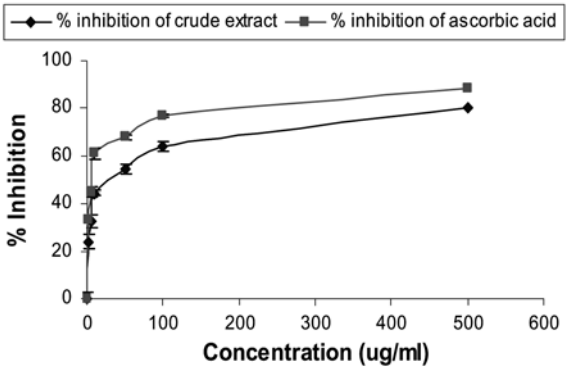

Fig. 1. DPPH scavenging assay of *R. damascene*. Probit equation for % inhibition of ascorbic acid and crude extracts are (y = 0.1047X + 43.267) and (y = 0.1073X + 32.561) respectively. Values are expressed as mean ± S.D.

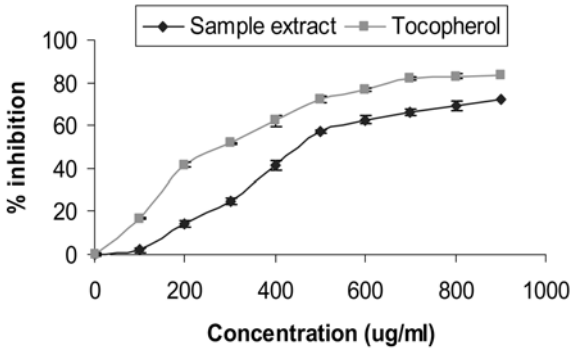

Fig. 2. Antioxidant activity of *R. damascena* extracts and α-tocopherol in the linoleic acid peroxidation system induced by $FeCl_2$ and H_2O_2. The activity was determined by the TBA method. Values are expressed as mean ± S. D.

Biochemical parameters

In order to prove the possible mechanism by which *R. damascena* prevents hepatic damage caused by paracetamol, investigation on levels of AST, ALT, and ALP were carried out. AST, ALT and ALP were found to be elevated after the administration of paracetamol, which was significantly (P < 0.05) reversed by *R. damascena* crude extract (Table 2). These effects were comparable to control group. The control group has the value of AST, ALT, and ALP were 27.87 ± 1.98, 28.52 ± 1.68 and 18.21 ± 1.04 while the paracetamol induced increment of the marker enzyme was 62.63 ± 1.35, 67.73 ± 2.44 and 22.12 ± 1.34. The elevated enzyme level were reversed due to the pre-treatment of the *R. damascena* crude extract at the dose level of 1.5 and 3 g/kg body weight and higher dose more significantly reduced the marker enzyme level (Table 2). The value for AST, ALT and ALP for 1.5 g/kg body weight were 55.91 ± 3.43, 63.40 ± 3.38, 22.00 ± 1.41 and for 3 g/kg body weight were 48.34 ± 2.68, 57.38 ± 2.98 and 20.48 ± 0.42.

Table 2. Effect of methanol extracts (1.5 and 3 g/kg body weight) on liver tissue damaged by paracetamol

Animal group	Observation	Inference
Control (Untreated group) (Group I)	No significant change (Hepatocytes were arranged from central vein in a regular manner)	
Paracetamol treated group (Group II)	Moderated fatty changes (++) Hepatocyte necrosis (++)	Paracetamol caused injury in the liver cells
Paracetamol + ethanol extract (1.5 g/kg body weight)	Moderate fatty changes (++) Hepatocyte necrosis not observed.	Weakly significant activity of paracetamol
Paracetamol + ethanol extract (3 g/kg body weight)	Moderate fatty changes (++) Hepatocyte necrosis not observed	Weakly significant activity of paracetamol

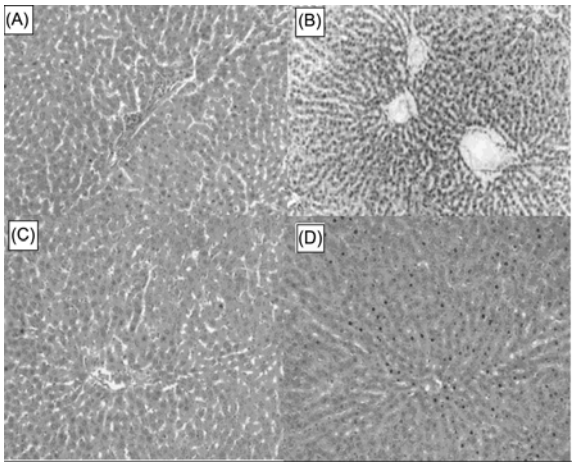

Fig. 3. Liver tissue in control group rat (A) which shows normal architecture but paracetamol treated group (B) shows fatty degeneration and necrosis. Paracetamol + ethanol extract (1.5 g/kg body weight (C)) and paracetamol + ethanol extract (3 g/kg body weight (D)) treated group shows amelioration of the fatty degeneration and normal architecture. (Magnification X 40).

Histopathologic examination

Liver sections of control animals showed normal hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus and central vein (Table 2, Fig. 3). In paracetamol treated animals the sections showed hydropic changes in centrilobular hepatocytes with single cell necrosis surrounded by neutrophils. Congestion of central vein and sinusoids were seen with acute and chronic inflammatory cells infiltrating sinusoids mainly in the central zone. The midzonal and periportal hepatocytes showed mild to moderate degree of fatty change (Fig. 3B). Pretreatment of the animals with crude extract (1.5 and 3 g/kg body weight) showed mild central venous congestion and mild fatty vacuolation (Fig. 3 C and D).

DISCUSSION

Over the past 25 years, epidemiological studies have shown a diminished risk of chronic diseases in populations consuming diets high in fruits and vegetables (Pryo *et al*., 2000). It has been suggested that antioxidants found in large quantities in fruits and vegetables may be responsible for this protective effect (Halliwell, 1994). Generally, food antioxidants act as reducing agents, reversing oxidation by donating electrons and hydrogen ions (Groff and Gropper, 2000). Much attention has been focused on natural antioxidants and some antioxidants isolated from natural sources with high activity have been reported (Kutsuzaki *et al*. 1993; Okamura *et al*., 1993; Parasakthy *et al*., 1996). The antioxidant activity of *R. damascena* extract increased with increasing concentration at the range of 200-600 ppm. Iron salts are thought to react with H_2O_2, called the Fenton reaction, to make hydroxyl radicals, which are the most active free radical formed in biological systems (Hochstein and Atallah, 1988) and known to be able to abstract hydrogen atoms from membrane lipids and bring about peroxidic reactions of lipids (Fong *et a*l., 1973; Kidata *et al*., 1979). From the above results, *R. damascena* extract significantly inhibits the lipid

peroxidation derived from the Fenton reaction, indicating that the extract displayed antioxidant activity in the TBA method.

ALT and AST are two liver enzymes that are associated to the hepatocellular damage. Although both AST and ALT are common liver enzymes because of their higher concentrations in hepatocytes, only ALT is remarkably specific for liver function since AST is mostly present in the myocardium, skeletal muscle, brain and kidneys (McIntyre *et al*., 1987; Witthawaskul *et al.,* 2003). In general with liver disease, serum levels of AST and ALT rise and fall at the same time (Sacher *et al*., 1991). A mild elevation of AST level has been shown to be associated with liver injury or myocardial infarctions (Stroev, 1989). The higher the activity of AST, the larger the infarctions size (Roberts *et al*., 1975; Haweroft, 1987). A typical myocardial infarction gives an AST/ALT ratio greater than 1 while an AST/ALT ratio less than 1 is as a result the release of ALT from the affected liver (Haweroft, 1987). AST/ALT ratio of more than 2 indicates alcoholic hepatitis or cirrchosis (McIntyre *et al.,* 1987). However, crude extracts of *Rosa damascena* shows dose responsive effect and sufficiently decrease the serum level of ALT, AST, and ALP compared to the animal group receiving paracetamol only.

The histopatological study of the liver of different groups of rats showed a normal architecture. Rats treated orally with the extract of *R. damascena* for 7 days, Figure IV showed little abnormalities such as steatosis, clarification and balloning of hepatocytes. These signs not found in the control groups and little change is mostly seen in the rats which received 1.5 and 3 g/kg body weight. The presence of steatosis also in the control groups suggested that this may be caused by diet of the animals. However no necrosis, infiltration, oedema and conjunction, which are the signs of hepatotoxicity, were found. The effect of *R. damascena* seems to have a protective effect on hepatocytes and improves liver architecture due to the presence of antioxidants. In conclusion, this study indicates that the alcoholic extract of *R. damascena* flowering top when taken for long periods of time may prevent paracetamol induced liver damage and may also prevent liver diseases.

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