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Original Research

Anthocyanins fraction of red radish (*Raphanus sativus* L) protects hepatic damage induced by carbon tetrachloride in albino rats

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Silymarin

Abstract

Objective: We investigated hepatoprotective and antioxidant activity of extracted anthocyanins fraction of red radish for the first time.

Methods: Anthocyanins fraction of red radish (*Raphanus sativus*; AFRS) was selectively extracted by employment of polymeric ion-exchange resin. AFRS was evaluated for antioxidant and hepatoprotective activity against carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. The animals were divided into seven groups of six animals each. Group I (control) received vehicle. Group II (drug control) received AFRS. Group III (toxicant) received CCl_4. Group IV, V and VI received AFRS at doses of 50, 100 and 150 mg/kg *po*, respectively. Group VII (standard) received silymarin. Various biochemical parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TB) and direct bilirubin (DB) levels in serum as well as the glutathione (GSH) and malondialdehyde (MDA) levels in the liver were determined. Histopathological changes in the liver were also studied. The activity of AFRS was compared with the reference drug silymarin.

Results: The yield of AFRS was found to be 0.084% (w/w). AFRS treated group did not show any significant change in the activity of serum ALT, AST, ALP, TB, DB, MDA and GSH level compared to control group. CCl_4 significantly raised the serum level of all biochemical parameters (except GSH) in the toxicant group. The pre-treatment of AFRS for seven days had reversed the alteration of biochemical parameters towards normal, and the effects were comparable to standard drug (silymarin 100 mg/kg). The animals received pre-treatment of AFRS showed amelioration in necrotic zones and hepatocellular degeneration.

Conclusion: This study demonstrates the antioxidant and hepatoprotective activity of anthocyanins fraction isolated from *Raphanus sativus* and thus scientifically supports the usage of it as food colorant and also justifies the use of the crude extracts of radish to treat various liver ailments in Indian folk medicine.

INTRODUCTION

The liver is the key organ of metabolism and excretion. It is often exposed to a variety xenobiotics and therapeutic agents. Until today, people have not yet found an actual curative therapeutic agent for liver disorders. In fact, most of the available remedies help the healing or regeneration of the liver [1]. Natural remedies from medicinal plants are considered to be effective and safe alternative treatments for hepatotoxicity [2]. In view of this, the present study was undertaken to investigate the hepatoprotective activity of anthocyanins fractions of red radish (*Raphanus sativus* L) against carbon tetrachloride (CCl_4)-induced hepatotoxicity in albino rats.

The roots of *Raphanus sativus* L, fam. Cruciferae, contains a significant quantity of anthocyanins, which are widely used as natural food colorants, because of the high stability and characteristics similar to synthetic Food Red No. 40 [3]. The anthocyanins of red radish are a mixture of 12 acylated pelargonidin glycosides

with a combination of *p*-coumaric acid, ferulic acid, malonic acid [4, 5]. Anthocyanins are water-soluble natural pigments belong to the flavonoids class of compounds, which act as a powerful antioxidants helping to protect plant tissue from UV irradiation [6]. Autumn leaves produce anthocyanins to prevent photo-oxidative damage as leaves senesce [7]. Anthocyanins were found in one study to have the strongest antioxidant power out of 150 flavonoids [8]. Pelargonidin is reported to protect the amino acid tyrosine from the highly reactive oxidant peroxynitrate [9]. Findings also support the photo protective function of pelargonidin and kaempferol by their ability to quench free radicals like superoxide [10]. Research shows antioxidant and radical scavenging activity of Leaves and stem of *R.sativus* [11] and acylated pelargonidin derivatives extracted from red radish [12]. Crude extract of *R.sativus* is shown to possess hepatoprotective activity against zearalenone-induced peroxidative hepatic damage in Balb/c mice [13]. *R.sativus* cultivated with sulfur (sulfur-radish extract) is shown to possess hepatoprotective activity in mice [14]. It has been reported that aqueous extracts of *R.sativus* possess antiurolithic activity [15].

There are no reports found on the systematic investigation to access the hepatoprotective activity of active constituents from *R.sativus*, either in a pure form or in the fraction. For this purpose, the hepatoprotective and antioxidant activity of the extracted anthocyanin's fraction from the root of *R.sativus* were assessed against CCl_4-induced hepatic damage in albino rats and compared with clinically available drug, *i.e*. silymarin.

MATERIALS AND METHODS

Materials

Gum Acacia was purchased from Qualigens Fine Chemicals (Mumbai, India). Diethyl ether and CCl_4 were purchased from Merck (Mumbai, India). Reagent kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin were procured from Crest Biosystem (Goa, India). Silymarin was sourced from Micro Labs (Bangalore, India). Thiobarbituric acid, glutathione (GSH), Ellman's reagent {5,5'-dithiobis-(2-nitrobenzoic acid)}, Diaion HP-20 and Diaion SP-207 resins were obtained from Sigma-Aldrich (Bangalore, India). All other chemicals were of analytical grade. The fresh red radish was purchased from a local vegetable market in Cuttack, Orissa, India, and authenticated in the department of Botany by Dr. Mrutyunjay Pradhan, Revenshaw University, Cuttack, India, and a voucher specimen was deposited in the department herbarium (voucher number RUBD 2486).

Extraction of anthocyanins fraction

The pigments in the red radish root (10 kg) were extracted with 0.05 M sulfuric acid (v/v). The extract was partitioned on polymeric resin (Diaion SP-207) and eluted with 70% aqueous (aq.) methanol containing 1% citric acid. Methanolic fraction was dried in a rotary evaporator under vacuum at 40ºC. The pigment (65 g) was loaded onto an open glass column (60 x 500 mm) packed with preconditioned Diaion HP-20 resin in 0.1% aq. trifluoroacetic acid and eluted stepwise with 5, 10, 20, 30, 50 and 70% aq. methanol containing 0.1% trifluoroacetic acid, and then 100% methanol containing 0.1% trifluoroacetic acid. The 70% aq. methanol fraction was collected and evaporated to dryness under reduced pressure as described by Otsuki *et al* [4]. About 8.4 g of the anthocyanin's fraction were obtained as the residue of which yield was found to be 0.084%w/w using following formula:

$$\textbf{Yield of AFRS} = \frac{\textbf{Grams of AFRS powder}}{\textbf{Grams of red radish root}} \textbf{ x 100}$$

Animals

The experiment conducted according to Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines for laboratory animal facility (1053/ac/10/CPCSEA). Male albino rats with an initial weighing of 143-182 g were used for the study. The animals were housed in polypropylene cages and maintained on a standard pellet diet and water *ad libitum*. They were exposed to a 12 h light/dark cycle and at a temperature of 24 ± 2ºC and were acclimatized to the environment for two weeks prior to the study.

Acute toxicity study

An acute oral toxicity study was carried out as per OECD - 423 guidelines. Swiss albino rats were divided into six groups of six animals each. The animals were fed with pellets rat chow and had free access to drinking water *ad libitum*, but starved for 12 h prior to testing. Gum Acacia mucilage (4% w/v) in distilled water was used as the vehicle [16]. First group served as a control, which receives the vehicle at a dose of 1 ml/kg, po. Anthocyanins fraction of *R.sativus* (AFRS) dispersed in the vehicle was administered as a single dose to five other groups at the dose level of 100, 200, 500, 1000, 2000 mg/kg *po*. All animals were observed for their behavioral changes, toxic symptoms and mortality up to 72 h.

Study design

The animals were divided into seven groups of six animals each. The animals were kept in a fasted state 12 h before the study, but were free accessed to standard diet and water throughout the study. Group I (control) received vehicle at 1 ml/kg *po* once daily for seven days. Group II (drug control) received AFRS

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dispersed in the vehicle at a dose of 100 mg/kg *po* once daily for seven days. Group III (toxicant) received vehicle at 1 ml/kg *po* once daily for seven days. Group IV, V and VI received AFRS dispersed in the vehicle at a dose of 50, 100 and 150 mg/kg *po* once daily, respectively, for seven days. Group VII (standard) received silymarin dispersed in the vehicle at a dose of 100 mg/kg *po* once daily for seven days. On the 7th day, after 30 min of pretreatment of AFRS or silymarin to animals, CCl_4 diluted with liquid paraffin (1:1) was administered to group III, IV, V, VI and VII at a dose of 0.1 mg/kg *po*.

Sample collection

On the 8th day, *i.e.* twenty-four hours after the administration of the CCl_4, animals were anesthetized with diethyl ether. Blood was collected from the retroorbital plexus. Subsequently, the animals were sacrificed, and their livers were removed and immediately immersed in 10% buffered formalin for histopathological examinations. For biochemical determination, the livers were homogenized in ice-cold potassium perchlorate (150 mM). The ratio of tissue weight to homogenization buffer was 1:10. From the latter, suitable dilutions for determination of the levels of GSH, lipid peroxidation product malondialdehyde (MDA) were prepared in their respective buffers depending on the assay that was run. To obtain serum, collected blood samples were allowed to clot at 37ºC for 30 min and centrifuged at 3500g for 10 min.

Biochemical analysis

In serum, ALT and AST were determined according to the method described by Reitman and Frankel [17], ALP uses the method described by Kind and King [18], total and direct bilirubin (TB and DB) was measured using the method described by Jendrassik and Grof [19]. The GSH content in the liver homogenate was estimated using the method described by Moron *et al* [20] and assay for the MDA formation as described in the method by Stocks and Dormandy [21]. Total serum proteins were determined according to the method described by Lowry *et al* [22] using bovine serum albumin as a standard.

Histopathological studies

For the histological examination, pieces of liver were fixed at 10% neutral phosphate buffer formalin and the hydrated tissue sections, 5 µm in thickness, were stained with hematoxylin and eosin. The sections were examined under a light microscope.

Hepatoprotective activity

The hepatoprotective activity, expressed as percentage hepatoprotection (*H*) for individual parameters (ALT, ALP, AST, DB, TB and MDA) was calculated as follows:

$$H = 1 - \left(\frac{M - C}{T - C}\right) \times 100$$

M is the mean value for an individual biochemical parameter in AFRS + CCl_4 groups, *T* is the mean value of an individual biochemical parameter of the toxicant group (CCl_4) alone, and *C* is the mean value of a parameter particular biochemical in the control group (vehicle).

Statistical analysis

Results were analyzed using SigmaPlot statistical software (version 12), and were expressed as mean ± standard error of the mean (SEM). The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Statistical P value < 0.05 was considered to be significant.

RESULTS

Acute toxicity study

In acute toxicity studies, AFRS did not show any toxicity and mortality up to 2000 mg/kg. As per the ranking system European Economic Community (EEC) for acute oral toxicity, the LD_{50} dose of 2000 mg/kg and above is categorized as unclassified (EC Directive 83/467/EEC, 1983). There were no changes in behavioral activities such as restlessness, respiratory distress, diarrhea, convulsions, and coma of the animals which received AFRS in different doses. However, the animals showed reduced locomotor activity and mild depression observed at a dose of 500 mg/kg and above but these changes were persisted up to 2 h, and later animals became normal. Hence based upon such effect, AFRS was tested at doses of 50, 100 and 150 mg/kg *po* for preliminary screening of hepatoprotective and antioxidant activity.

Effect of AFRS on ALT, AST, ALP and bilirubin levels

The results of the effect of the AFRS on transaminase, phosphatase and bilirubin levels are presented in Table 1. The drug control group did not show any significant change in the activity of serum ALT level compared to control group. CCl_4 significantly ($P < 0.001$) raised the serum ALT level in the toxicant group. This increase was about 8 times compared to the control group. Pretreatment with AFRS reversed the alteration in ALT level towards normal significantly ($P < 0.001$) in a dose-dependent manner and the decrease in the serum ALT level was 46.97, 58.14 and 72.29%, in groups pretreated with AFRS 50, 100 and 150 mg/kg *po*, respectively, compared to the toxicant group ($P < 0.001$). Silymarin pretreated group also showed significant ($P < 0.001$) decrease in the serum ALT level by 70.31% compared to the toxicant group.

Table 1. Effect of AFRS and silymarin on serum ALT, AST, ALP and bilirubin levels in rats after CCl_4 intoxication

Groups	Dose (mg/kg)	ALT (U/l)	AST (U/l)	ALP (U/l)	TB (mg/dl)	DB (mg/dl)
I[a]	1	34.8 ± 0.3	87.2 ± 0.6	474.2 ± 1.2	0.722 ± 0.004	0.362 ± 0.003
II[b]	100	34.5 ± 0.4	82.7 ± 0.6[*a]	471.3 ±0.5	0.72 ± 0.004	0.358 ± 0.003
III[c]	0.1	267.8 ± 0.5[*ab]	222.3 ± 0.7[*ab]	1250 ± 1.9[*ab]	2.363 ± 0.008[*ab]	1.072 ± 0.003[*ab]
IV[d]	50	142.0 ± 0.3[*cefg]	154.3 ±0.6 [*cefg]	810.5 ±1.0[*cefg]	2.013 ± 0.002[*cefg]	0.967 ± 0.003[*cefg]
V[e]	100	112.1 ± 0.5[*cdfg]	123.3 ± 0.7 [*cdfg]	716.3 ± 2.3 [*cdf,**g]	1.923 ± 0.004[*cdfg]	0.915 ± 0.004[*cdfg]
VI[f]	150	74.2 ±1.2[*cdeg]	108.5 ± 0.4[*cde]	701.3 ±1.9 [*cdeg]	0.7 ± 0.006[*cdeg]	0.7 ± 0.006[*cdeg]
VII[g]	100	79.5 ± 1.2[*cdef]	116.7 ± 0.8[*cde]	711.3 ± 1.3[*cdf,**e]	0.75 ± 0.005[*cdef]	0.75 ± 0.005[*cdef]
ANOVA	F	12789.527	5852.171	27970.958	19906.394	4775.047
	P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Group I: control (vehicle); **group II:** drug control (AFRS); **group III:** toxicant (CCl_4); **group IV:** 50 mg/kg AFRS + CCl_4; **group V:** 100 mg/kg AFRS + CCl_4; **group VI:** 150 mg/kg AFRS + CCl_4; **group VII:** standard (silymarin) + CCl_4. **ALT:** alanine aminotransferase; **AST:** aspartate aminotransferase; **ALP:** alkaline phosphatase; **TB:** total bilirubin; **DB:** direct bilirubin. Statistical analysis by ANOVA followed by Tukey post hoc test; significant at *****$P < 0.001$ and ******$P < 0.01$. Values are expressed as mean ± SEM; n = 6 rats per group. Duration of pretreatment of AFRS and the standard is up to 7 days.

A significant ($P < 0.001$) decrease in activities of serum AST level in drug control was observed compared to control group. CCl_4 significantly ($P < 0.001$) increased serum AST in the toxicant group by 3 times compared to control group. This increase in AST level was significantly ($P < 0.001$) reversed by AFRS and silymarin. The decrease in serum AST level by 30.58, 44.53 and 51.19%, occurred respectively in AFRS 50, 100 and 150 mg/kg *po* pretreated groups compared to the toxicant. AST level was significantly ($P < 0.001$) decreased in silymarin pretreated group compared to the toxicant group by 47.5%.

Rats receiving only AFRS, *i.e.* in the drug control group, did not show any significant changes in serum ALP level compared to control group. In the toxicant group ALP level raised by 3 times compared to the control group. ALP levels in AFRS and silymarin pretreated groups were significantly ($P < 0.001$) lower than CCl_4 treated group. The decrease in serum ALP levels were 35.16, 42.69 and 43.89%, respectively in 50, 100 and 150 mg/kg *po* AFRS pretreated groups compared to toxicant group. Silymarin pretreated group also reduced the ALP level significantly ($P < 0.001$) by 43.09% compared to the toxicant group.

As such AFRS administered alone did not produce any changes in total bilirubin and direct bilirubin level compared to control group. A significant increase ($P < 0.001$) in the total bilirubin and direct bilirubin levels occurred in 327.3 and 296.13% respectively in CCl_4 treated group compared to control group after 24 h of exposure to CCl_4. However, bilirubin levels were significantly ($P < 0.001$) reversed to normal level in AFRS protected groups in a dose-dependent manner. The decrease in total bilirubin and direct bilirubin level were 16.13 and 12.09% respectively in 50 mg/kg, 19.88 and 16.81% respectively in 100 mg/kg, and 70.83 and 36.36% respectively in 150 mg/kg *po* AFRS pretreated groups compared to CCl_4 alone treated group. Similarly, in silymarin treated group significant ($P < 0.001$) decrease in total bilirubin and direct bilirubin were observed, *i.e.* 68.75 and 31.81% respectively, compared to CCl_4 treated group.

Effect of AFRS on MDA and GSH levels

The effect of AFRS on MDA and GSH status on rat livers after CCl_4 administrations are presented in Figs.1&2. No changes in MDA and GSH levels were observed in AFRS alone treated group in comparison to control group. Highly significant ($P < 0.001$) increase in MDA by 151.87% and decrease in GSH by 57.37% occurred in CCl_4 treated group compared to control group. There was a decrease in MDA and increases in GSH level were observed in AFRS and silymarin pretreated groups. The percentage decreased with MDA and an increase in GSH levels were 14.99 and 37.14% respectively in 50 mg/kg, 39.2 and 85.71%, respectively in 100 mg/kg, and 42.14 and 88.57% respectively in 150 mg/kg *po* AFRS pretreated groups compared to the toxicant group. Silymarin pretreated group also significantly ($P < 0.001$) raised the MDA level by 41.16% and lowered the GSH level by 85.71% compared to the toxicant group.

Hepatoprotective activity of AFRS

The percentages of hepatoprotection exhibited by different doses of AFRS, estimated by the above given formula, are shown in Table 2. The percentage hepatoprotection were increased in a dose-dependent manner between AFRS pretreated groups. AFRS 100 mg/kg group gave 23.76% more protection for ALT compared to the AFRS 50 mg/kg group. In the same manner 24.34% hepatoprotection increased for ALT in the AFRS 150 mg pretreated group compared to AFRS 100 mg/kg pretreated group. Hepatoprotection for AST was increased 45.57% in the AFRS 100 mg/kg

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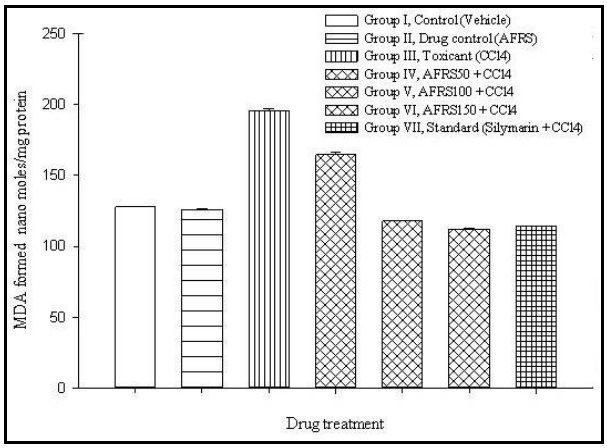

Figure 1. Effect of AFRS and silymarin on malondialdehyde (MDA) levels. Values are expressed as mean ± SEM; n = 6 rats per group. Significant at P < 0.001; group I *vs* group III, group III *vs* group IV. Duration of pretreatment of AFRS and standard is up to 7 days

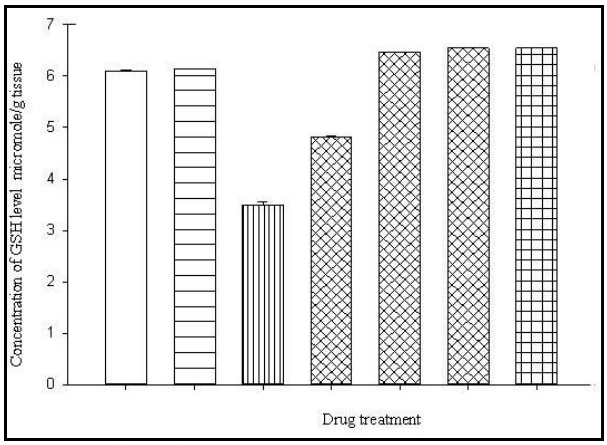

Figure 2. Effect of AFRS and silymarin upon glutathione (GSH) status. Values are expressed as mean ± SEM; n = 6 rats per group. Significant at P < 0.001; group I *vs* group III, group III *vs* group IV. Duration of pretreatment of AFRS and the standard is up to 7 days.

group compared to AFRS 50 mg/kg group and 14.95% increase in hepatoprotection in the AFRS 150 mg pretreated group compared to AFRS 100 mg/kg pretreated group. AFRS 100 mg/kg group gave 21.42% more protection for ALP compared to the AFRS 50 mg/kg group. However, in the AFRS 150 mg/kg pretreated group, the protection was similar to AFRS 100 mg/kg pretreated group. Only a 2.8% increase in hepatoprotection observed. Hepatoprotection for TB was increased 25.75% in the AFRS 100 mg/kg group compared to AFRS 50 mg/kg group and 278% increase in hepatoprotection in the AFRS 150 mg pretreated group compared to AFRS 100 mg/kg pretreated group. Hepatoprotection for DB was increased 49.59% in the AFRS 100 mg/kg group compared to AFRS 50 mg/kg group and 136.95% increase in hepatoprotection in the AFRS 150 mg pretreated group compared to AFRS 100 mg/kg pretreated group. AFRS 100 mg/kg group gave 161.51% more protection for MDA compared to the AFRS 50 mg/kg group. However, in the AFRS 150 mg/kg pretreated group, the protection was similar to AFRS 100 mg/kg pretreated group. Only a 7.48% increase in hepatoprotection observed. AFRS 100 mg/kg group gave 130.76% more protection for GSH compared to the AFRS 50 mg/kg group. However, in the AFRS 150 mg/kg pretreated group, the protection was similar to AFRS 100 mg/kg pretreated group. Only a 3.33% increase in hepatoprotection observed. AFRS pretreated group showed almost equal and in some parameters better protection compared to silymarin pretreated group.

Histopathological examination

Histopathological studies also provided supportive evidence for the biochemical analysis. The control group showed the normal parenchymal architecture (+) with cords of hepatocytes, portal tracts, and central veins (Fig.3). CCl_4 treatment induces ballooning degeneration, centrilobular necrosis, bridging necrosis and apoptosis in hepatocytes (Fig.4). The toxin mediated changes in the liver of rats pretreated with 100 mg/kg *po* AFRS were of much lesser intensity than those observed in the livers of CCl_4 treated rats, which showed remarkable recovery on ballooning degeneration but not on hepatocytes apoptosis. Centrilobular and bridging necrosis were occasionally present (Fig.5).

Changes in liver weight to the body weight ratio

Data in Table 3 shows changes in liver weight:body weight. The drug control group gained the highest weight while the CCl_4 treated group gained the lowest. The liver to body weight gain ratio for the CCl_4 treated group had the highest value. Weight gains observed in AFRS pretreated groups were higher than the silymarin pretreated group.

Table 2. Hepatoprotection offered by AFRS and silymarin against CCl_4 induced hepatotoxicity in rats (in %)

Treatment	Dose	ALT	AST	ALP	TB	DB	MDA	GSH
AFRS + CCl_4	50 mg/kg	53.99%	50.33%	56.65%	21.32%	14.78%	43.89%	50%
AFRS + CCl_4	100 mg/kg	66.82%	73.27%	68.79%	26.81%	22.11%	114.78%	115.38%
AFRS + CCl_4	150 mg/kg	83.09%	84.23%	70.72%	101.34%	52.39%	123.37%	119.23%
Silymarin	100 mg/kg	80.81%	78.16%	69.43%	98.29%	45.35%	120.51%	115.38%

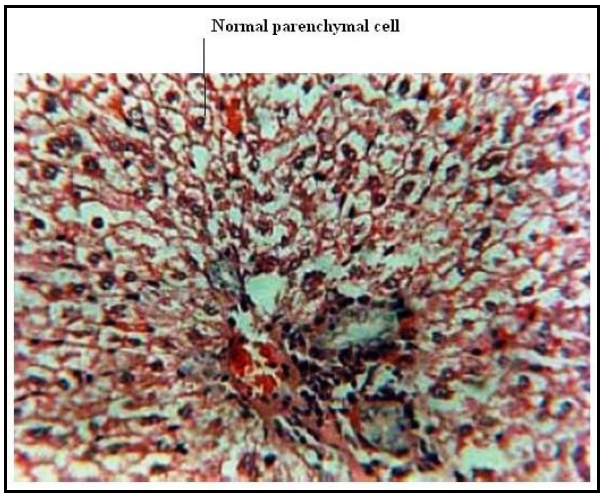

Figure 3. Liver sections from control rat showing normal hepatic architecture (+) (H&E, 400x).

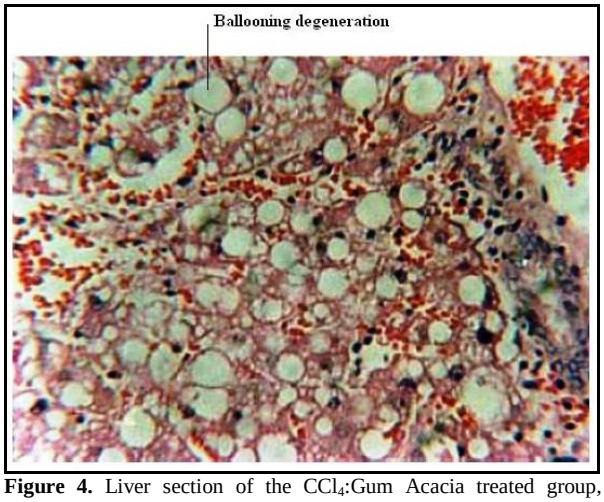

Figure 4. Liver section of the CCl_4:Gum Acacia treated group, showing numerous ballooned hepatocytes and centrilobular necrosis (++++) (H&E, 400x).

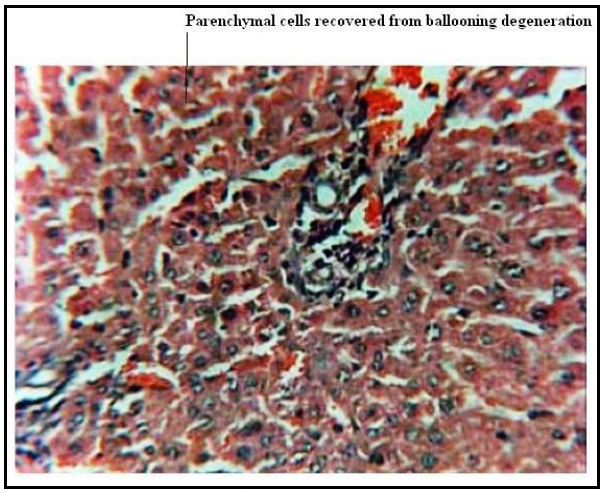

Figure 5. Liver section of the CCl_4:AFRS treated group, showing recovery from ballooning degeneration with occasional presence of centrilobular necrosis (++) (H&E, 400x).

Table 3. Changes in body weight and liver weight

Groups	Percentage of weight gained	Liver to body weight gained ratio
I	2.92	0.886
II	2.74	0.849
III	0.36[b]	1.114[a]
IV	1.97	0.853
V	2.50	0.872
VI	2.40	0.872
VII	2.29	0.845

Group I: control (vehicle); **group II:** drug control (AFRS); **group III:** toxicant (CCl_4); **group IV:** 50 mg/kg AFRS + CCl_4; **group V:** 100 mg/kg AFRS + CCl_4; **group VI:** 150 mg/kg AFRS + CCl_4; **group VII:** standard (silymarin) + CCl_4 (n= 6 rats per group). Significantly ($P < 0.01$) [a]higher or [b]lower than the control value.

DISCUSSION

Methanol is the most commonly used solvent for extraction of anthocyanins pigment, as with other classes of flavonoid compounds because its low boiling point allows for rapid concentration of the extracted material. However, the resultant extract contains low-polarity contaminants, and further purification may be necessary. Acidic environment is maintained during extraction of anthocyanins to prevent their conversion into the pseudo-base form [23]. We employed cation exchange resin (in two stages) which forms strong hydrogen bonds with the proton of the phenolic hydroxyl group, in order to isolate anthocyanins pigment followed by the anthocyanins fraction as described by Otsuki *et al* [4]. The red radish pigments (available as a red amorphous powder) are being increasingly used as a food colorant.

The data in Table 1 shows that the high levels of serum ALT, AST, ALP and bilirubin in the toxicant group is the consequence of CCl_4-induced liver dysfunction and denote the damage to the hepatic cells. Pretreatment with different doses of AFRS reversed biochemical parameters towards normal and led to a significant inhibition of ALT, AST, ALP and bilirubin levels. Significant decrease in respective serum enzyme levels among AFRS pretreated groups were observed in a dose-dependent manner compared with CCl_4 treated group. The effect produced was comparable to that of silymarin. This was supported by the data in Table 3, that the liver to body weight gained ratio for the CCl_4 treated group had the highest value, indicating that CCl_4 increased liver weight: an evidence of inflammation, with minimal gain in body weight [24]. Data from Table 2 demonstrate that treatment with ARFS (100 and 150 mg/kg *po*) showed significant hepatoprotective and antioxidant activity, and was comparable with the standard silymarin.

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The hepatic damage induced by CCl_4 is well known to be mediated by its free radical metabolites such as trichloromethyl (CCl_3•) and peroxytrichloromethyl (Cl_3COO•), which readily reacts with unsaturated membrane lipids to produce lipid peroxidation and/or with other critical cellular macromolecules leading to cell damage [25]. Lipid peroxidation is expressed in terms of thiobarbituric acid reacting substances; the thiobarbituric acid reaction with MDA is generally considered to be an indicator of the secondary breakdown products of oxidized polyunsaturated fatty acids [26]. As it can be observed from Fig.1, MDA formation was significantly increased in the toxicant group. However, MDA formation was dramatically reduced in AFRS 100 and 150 mg/kg pretreated groups. The protection offered by AFRS was comparable to that of silymarin. The reduction in the formation of hepatic MDA in the AFRS treated rats demonstrates their membrane stabilizing and free radical scavenging properties [27].

Glutathione constitutes the first line of defense against free radicals [28]. CCl_4 intoxication caused a significant depletion in GSH contents of the rat livers (Fig.2). Pretreatment with AFRS prevented depletion of GSH level and promoted the conversion of oxidized glutathione (GSSG) into GSH by the reactivation of hepatic glutathione reductase enzyme after the CCl_4 treatment step [29]. The availability of sufficient amount of GSH thus increased the detoxification of active metabolites of CCl_4 through the involvement of glutathione peroxidase (GPx). The restoration of the GSH to normal tissue levels shows its hepatoprotective activity [30]. This clearly demonstrates the fact that, AFRS have hepatic cell membrane stabilizing and cell regeneration capability, which is due to their anti lipid peroxidation and/or free radical-quenching activity. Silymarin is also known to exert its hepatoprotective action through a similar mechanism of action [31]. Incidentally both these substances are polyphenolic compounds and belong to the flavonoids family. Thus, it can be safely concluded that the hepatoprotective actions of the red radish anthocyanins fraction are due to their antioxidant and free radical scavenging properties common to flavonoids.

In conclusion, AFRS demonstrated significant hepatoprotective and antioxidant activity on rat livers against CCl_4 toxicity as indicated by the biochemical parameters and confirmed by the histopathological studies, which scientifically supports its usage as natural colorants in food, beverage and pharmaceutical industry. The present study further justifies the use of the crude extracts of radish to treat various liver ailments in Indian folk medicine. Furthermore, the consumption of red radish in the diet should provide considerable health benefits.

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COMPETING INTERESTS

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper**.**

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