

HEPATOPROTECTIVE EFFECT OF SEPHADEX G50 RED FRACTION OF *H. ROSASINENSIS* PETAL ANTHOCYANIN ADMINISTERED TO RATS IN ETHANOL

FREDERICK O. OBI, IGNATIUS I. ICHIDE, ANDREW A. OKORORO, OGBAKPAH ESIRI and STEPHEN OGHRE

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ABSTRACT

The hepatoprotective effects of the two sephadex G50 fractions of the anthocyanin obtained from the petals of *H. rosasinensis* were studied. Treatment of rats with 8.40 µg of the red fraction (G50 RF) in 5% aqueous ethanol/kg body weight 5 days/week for 4 weeks before carbon tetrachloride resulted in significantly ($P < 0.05$) less hepatotoxicity than with carbon tetrachloride alone, as measured by plasma L-alanine aminotransferase activity and liver malondialdehyde levels 18 hr after carbon tetrachloride. When similar treatment was given to rats using the second fraction, the purple fraction (G50 PF) there was no significant ($P > 0.05$) decrease in hepatotoxicity relative to those treated with carbon tetrachloride alone. These data suggest that it is the red rather than the purple pigment of *H. rosasinensis* anthocyanin that is protective against carbon tetrachloride-induced liver injury.

Key Words: Anthocyanin; G50RF; G50PF; Hepatotoxicity; Carbon tetrachloride

INTRODUCTION

The plant *Hibiscus rosasinensis* (family, Malvaceae) also known as garden hibiscus is a common shrub in West Africa. It flowers all year round but it does so copiously during the rainy season months (April to October) when its red petals, are very noticeable. The flowers of *H. rosasinensis* have been known for its antifertility efficacy and use in correcting menstrual disorders. The decoction of the flower is also used as remedy for influenza, cough and asthma, while the decoctions of the stem, leaves and flowers are used as a remedy for tertiary syphilis (Gill, 1992).

Anthocyanins are water soluble glycosides of anthocyanidins and are part of the C₁₅ phenolics known collectively as flavonoids with typical A ring (benzoyl) and B-ring (hydroxy cinnamoyl) system along with an oxygen - containing heterocyclic ring (Fig. 1).

They are responsible for the brilliant orange, pink, scarlet, red, mauve, violet and blue colours of flower petals and fruits of higher plants (Harborne, 1967; Strack and Wray,

1989). Not much is known about the physiological and biochemical functions of anthocyanins inspite of the continuous interest in their distribution and chemistry (Takeda *et al*, 1994; Tatsuzawa *et al*, 1994).

Previous reports (Obi *et al*, 1998; Obi and Ozoemena 1998) show that *H. rosasinensis* anthocyanin extract protects the liver against carbon tetrachloride (CCl₄) - induced liver injury. Carbontetrachloride is believed to cause tissue damage via lipid peroxidation (Reinke *et al*, 1988; Sipes *et al*, 1977).

In the process of desalting a solution of the anthocyanin on sephadex G50 column, we discovered that the deep red anthocyanin

FREDERICK O. OBI, Department of Biochemistry, Faculty of Science, University of Benin, P. M. B. 1154, Benin City, Nigeria
IGNATITUS I. ICHIDE, Department of Biochemistry, Faculty of Science, University of Benin, P. M. B. 1154, Benin City, Nigeria
ANDREW A. OKORORO, Department of Biochemistry, Faculty of Science,, Delta State University, Abraka, Nigeria
OGBAKPAH ESIRI, Department of Biochemistry, Faculty of Science,, Delta State University, Abraka, Nigeria
STEPHEN OGHRE, Department of Biochemistry, Faculty of Science,, Delta State University, Abraka, Nigeria

contains two distinct fractions. A red fraction (G50 RF) which elutes first from the column, closely followed by a purple fraction (G50 PF). Therefore, the present study was designed to ascertain which of the two components prevents the CCl_4 - induced liver injury using malondialdehyde level and plasma L-alanine

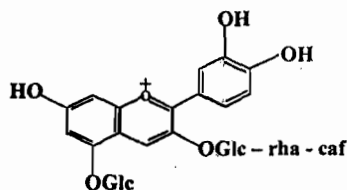


Fig. 1: Structure of anthocyanin. The anthocyanin shown is cyanidin 3-(4-caffeoylrutinoside) -5 glucose from *Silene dioica* (Tatsuzawa *et al.*, 1994) caf, caffeic acid; glc, glucose; rha, rhamnose

aminotransferase activity as the biochemical indicators of toxic injury.

MATERIALS AND METHODS

Experimental animals and materials:

White albino rats (130 - 160g) bred in the Animal Unit of the school of Pharmacy, University of Benin, were used in this study. They were divided into seven experimental groups of four rats per group, housed in standard rat cages (Griffin and George Modular Cage System, model YSM 580 cage base and YSM 600 - 540 cage top) and left to acclimatise to laboratory conditions for two weeks before the commencement of the experiment. Ethanol and formic acid were purchased from BDH Chemical (Poole, England), Carbon tetrachloride and chloroform were from May and Baker (Dagenham,

England). 2-Thiobarbituric acid from Merck Chemicals (Merck, Darmstadt). Sephadex G-50 for column chromatography, particle size 20 - 80 μ , was purchased from Pharmacia Fine Chemicals (AB Uppsala, Sweden), L-alanine aminotransferase kit (Ref. 99 75 02) was obtained from QCA (Spain). Other materials include corn oil (Mazola; produced for CPC, UK) and rat pellets (Pfizer, Nigeria).

Extraction and purification of anthocyanin from *H. rosasinensis* petals.

The anthocyanin from *H. rosasinensis* petals was extracted as described previously (Obi *et al.*, 1998) and the ethylacetate and petroleum ether (bp 40 - 60°C) "washed" extract treated as follows: The extract was mixed with 10% lead nitrate solution (1 ml extract: 2 ml lead nitrate). Lead precipitate of the red anthocyanin was obtained, decanted and the residue dissolved in 20% sodium chloride solution. The lead ions were released from the residue as lead chloride and filtered under gravity through Whatman 541 filter paper (hardened ashless) and clear red filtrate was obtained. The filtrate was desalted on Sephadex G50 columns (12 x 3.5cm and 50 x 1.0 cm). The sephadex was swollen in distilled water at 4°C for 4-6 hr and the columns were packed and washed with distilled water (3 bed volumes). The top of the bed was covered with a thin layer of glass wool. Five and 2 ml of the filtrate were applied onto the 12 x 3.5 and 50 x 1.0 cm columns respectively and eluted with distilled water. Three ml fractions were collected and the contents of appropriate tubes pooled and kept at 4°C until required.

Table 1: Effect of anthocyanin on liver lipid in CCl_4 hepatotoxicity^a

Group	Treatment	Total lipid level (mg/g wet wt) x 10 ² Mean \pm SEM (n)	Difference (%) ^b	
			1	2
1.	-AN - CCl_4	6.0 \pm 1.0 (2) ^c	-	-
2.	-AN + CCl_4	3.3 \pm 0.8 (2) ^b	-45	-
3.	+AN ₁ + CCl_4	5.5 \pm 0.8 (4) ^c	-8.3	+67
4.	+AN ₂ + CCl_4	7.8 \pm 0.6 (4) ^c	+30	+136
5.	+AN ₃ + CCl_4	5.7 \pm 1.3 (3) ^c	-5.0	+73
6.	+AN ₄ + CCl_4	7.3 \pm 1.0 (3) ^c	+22	+121
7.	+AN ₅ + CCl_4	3.3 \pm 0.3 (4) ^d	-45	0.0

^aAN = anthocyanin; AN₁ = ethylacetate and petroleum ether "washed" anthocyanin extract;

AN₂ = AN₁ precipitated with $\text{Pb}(\text{NO}_3)_2$, redissolved in NaCl and filtered; AN₃ = AN₂ desalted on sephadex G50 column (12 x 3.5 cm); AN₄ = Red fraction from 50 x 1.0 cm sephadex G50 column chromatography of AN₂; AN₅ = Purple fraction of AN₂ from the same 50 x 1.0 cm sephadex column; n = number of rats (in groups with less than 4, rats were lost in course of vehicle or anthocyanin administration by gavage or following CCl_4 treatment).

^b Percentage difference from groups 1 and 2 respectively.

^c Values with different superscripts are significantly different from each other (P<0.5)

^d See footnote C.

Table 2: Effect of anthocyanin on liver malondialdehyde level in CCl₄ hepatotoxicity^a

Group	Treatment	Malondialdehyde (MDA) (MDA Unit/mg lipid)10 ⁻² Mean ± SEM (n)	Difference (%) ^b	
			1	2
1.	-AN - CCl ₄	2.5 ± 1.7 (2) ^c	-	-
2.	-AN + CCl ₄	9.3 ± 1.9 (2) ^d	+269	-
3.	+AN ₁ + CCl ₄	4.7 ± 1.4 (4) ^c	+216	-41
4.	+AN ₂ + CCl ₄	2.2 ± 0.3 (4) ^c	-14	-77
5.	+AN ₃ + CCl ₄	4.9 ± 0.5 (3) ^c	+95	-47
6.	+AN ₄ + CCl ₄	4.2 ± 1.1 (3) ^c	+78	-52
7.	+AN ₅ + CCl ₄	9.5 ± 0.8 (4) ^d	+276	+1.9

^a See table 1 footnote for the interpretation of abbreviations

^b Percentage difference from groups 1 and 2 respectively.

^c Values with different superscripts are significantly different from each other (P<0.05).

^d See footnote C.

Treatment of Animals

The various anthocyanin samples – the ethylacetate and petroleum ether “washed” (AN₁), lead precipitated and resolubilized filtrate (AN₂), the 12 x 3.5 cm sephadex column pooled eluate (AN₃), the respective 50 x 1.0 cm sephadex column pooled red (AN₄), and purple (AN₅) eluates, were quantified spectrophotometrically (Francis, 1985). All were diluted with distilled water to give 3.34µg/ml and then mixed with absolute ethanol to give a solution of the various samples in 5% aqueous ethanol (v/v). From AN₁, AN₂, AN₃, AN₄ and AN₅ (now in 5% aqueous ethanol), 8.40µg/kg body weight was given orally by intubation to rats in groups 3, 4, 5, 6 and 7 respectively. The control rats in groups 1 and 2 received 2.5 ml of 5% aqueous ethanol/kg body weight. Rats in all the groups received these treatments 5 days/week for 4 weeks. After the daily anthocyanin administration, the rats were maintained on chow and water *ad libitum*. Following the last anthocyanin treatment, rats were fasted for 12 hours. Liver injury was thereafter induced by CCl₄ as described by Hase *et al* (1996). A mixture of CCl₄ in corn oil (1:1) was injected s.c. with a dose of 6ml/kg body weight to rats in groups 2 (control) and those in groups 3, 4, 5, 6 and 7 (test). The CCl₄ free control rats (group 1) were injected s.c. with CCl₄-free corn oil (6ml/kg body weight).

Collection of Plasma and Liver Samples:

Eighteen hours after CCl₄ treatment, each rat was anaesthetised in a chloroform saturated chamber. The thoracic and abdominal regions were opened to expose the heart and liver. Blood was obtained via heart puncture by means of a 5 ml hypodermic

syringe and needle and placed in ice-cold 5 ml heparinized tubes. The plasma samples were separated from the red cells by centrifugation (5000 rpm for 10 min) and both components left in ice until required. The liver of each rat was excised and left standing on ice in separate 50ml glass beakers.

Extraction and Estimation of Liver Total Lipid:

Liver total lipids was extracted as described by Weil and Stetten (1947). One gram of liver was homogenized in 10ml chloroform: methanol (1:1/v/v) the extraction solvent, in a mortar containing 2g acid washed sand. The homogenate was transferred into a stoppered glass tube and left standing for 48 hr at room temperature. The organic phase was separated and stored at 4°C. Five millilitres of the extraction solvent were added to the residue and left standing for another 24 hr and the organic phase separated. The two organic phases were pooled and made up to 25 ml with the extraction solvent. For the quantitative estimation of total lipid in the extract, the 25 ml of lipid extract was transferred to a clean, dry, 50ml beaker placed on a mild hot plate and left until the volume decreased to about 5 ml. The beaker was then kept in an oven at 80°C and left to dry for 1 hr. Ten millilitres of hexane were added, swirled thoroughly and filtered into a dry and pre-weighed 25 ml beaker. The original beaker and filter paper were rinsed with hexane and transferred into the 25ml beaker. The beaker was placed on a mild hot plate until the volume of the hexane was reduced to 5ml and thereafter it was transferred to an oven at 80°C for 1 hr. The beaker and its content were allowed to cool and weighed. The difference in weight between the weight of the

beaker plus lipid residue and the weight of the beaker alone represents the weight of the total lipid in grams.

Biochemical Assay Protocols and Statistical Analysis:

(i) Enzyme assay and malondialdehyde estimation.

Plasma L-alanine aminotransferase activity was assessed at 37°C using the colorimetric method of Reitman and Frankel 1957 as described by Bergmeyer and Erlich (1974). Liver malondialdehyde was assayed by the method of Hunter *et al* (1963) as modified by Gutteridge and Wilkins (1982). An aliquot (0.6 ml) of 20% liver homogenate in ice-cold physiological saline was added to 3 ml glacial acetic acid and 3 ml of 1% thiobarbituric acid (TBA) in 0.5M NaOH solution in a centrifuge tube. The mixture was transferred to a boiling water bath for 15 min, allowed to cool and centrifuged at 5000 rpm for 5 min. The absorbance of the clear pink coloured product was read at 532 nm.

(ii) Statistical analysis:

The data are presented as means \pm SEM. The mean values of the various treatment groups were compared using

ANOVA and the least square difference test (Lapin, 1978). The significance level was set at $P < 0.05$.

RESULTS

The elution profile of the sodium chloride resolubilized Pb-precipitate of *H. rosasinensis* petal anthocyanin is shown in Fig. 2. The first fraction G50 RF was closely followed by the second G50 PF and so the contents of the tubes in which they were distinctly and separately eluted were pooled (tubes 3 to 6 for G50 RF and tubes 8 to 15 for G50 PF). The hepatoprotective effect of the two sephadex G-50 fractions of this anthocyanin and that of the unfractionated extracts at different levels of purification, have been examined. The results are shown in Tables 1, 2 and 3. In normal rat liver, total lipid level was 6×10^2 mg/g wet weight while malondialdehyde level was 2.5×10^{-8} MDA units/mg lipid (Tables 1 and 2). In plasma, the normal ALT level was 62 IU/L (Table 3). The malondialdehyde and ALT levels were increased to 9.3×10^{-8} MDA units/mg lipid and 518 IU/L, respectively (Table 2 and 3), while the lipid level was decreased to 3.3×10^2 mg/g

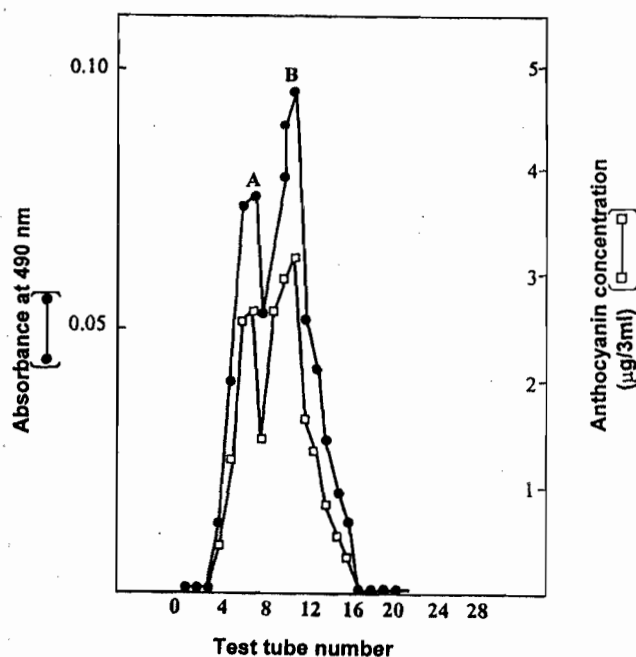


Fig. 2: Elution and concentration profiles of *H. rosasinensis* petal anthocyanins fractionated on a 50 x 1cm sephadex G50 column.

The fractions were eluted with distilled water (39 ml, tubes 3 to 15). The eluates in tubes 3 to 6 and 8 to 15 were pooled and constitute the red (G50 RF) and the purple (G50 PF) fractions respectively. Peak A = G50 RF; Peak B = G50 PF.

Table 3: Effect of anthocyanin on plasma L-alanine aminotransferase activity in CCl₄ hepatotoxicity^a

Group	Treatment	ALT Activity (IU/L) Mean ± SEM (n)	Difference (%) ^b	
			1	2
1.	-AN - CCl ₄	62 ± 1.5 (2) ^c	-	-
2.	-AN + CCl ₄	518 ± 54 (2) ^d	+736	-
3.	+AN ₁ + CCl ₄	131 ± 11 (3) ^{c,e}	+113	-75
4.	+AN ₂ + CCl ₄	211 ± 60 (4) ^{c,e}	+240	-59
5.	+AN ₃ + CCl ₄	225 ± 50 (3) ^{c,e}	+263	-57
6.	+AN ₄ + CCl ₄	226 ± 3.0 (2) ^{c,e}	+265	-56
7.	+AN ₅ + CCl ₄	327 ± 66 (4) ^{d,e}	-427	-37

^a See Table 1 footnote for the interpretation of abbreviations

^b Percentage difference from groups 1 and 2.

^c Values with different superscripts are significantly different from each other (P<0.05).

^{d,e} See footnote C.

wet weight (Table 1) 18 hr after CCl₄ administration. AN₁, AN₂, AN₃, AN₄ significantly increased liver lipid level relative to AN-free CCl₄ treated group but AN₅ did not (Table 1). The anthocyanin samples with the exception of AN₅ also significantly decreased the liver malondialdehyde and plasma ALT levels (Tables 2 and 3); when compared to the AN-free CCl₄ treated group.

DISCUSSION

In this investigation, plasma ALT activity and liver malondialdehyde levels were used to measure both CCl₄-induced hepatotoxicity and protection of sephadex G50 red and purple fractions of *H. rosasinensis* petal anthocyanin in particular, against the same effect of CCl₄ in rats. In agreement with the reports of previous workers, our results show that CCl₄ caused an elevation in blood content of ALT (Hase *et al*, 1996; Reinke *et al*, 1988) and liver malondialdehyde levels which is indicative of damage to the liver and other organs of the body (Ngaha *et al*, 1989; Reinke *et al*, 1988; Lin and Wang, 1986). Treatment of rats with 8.40 µg of various anthocyanin samples/kg body weight 5 days/week for 4 weeks before CCl₄, caused less hepatotoxicity than with CCl₄ alone (Tables 2 and 3) as evidenced by decreased plasma content of ALT and liver malondialdehyde level relative to CCl₄ treated anthocyanin-free group. The present data not only corroborate our earlier reports that *H. rosasinensis* anthocyanin protects against CCl₄-induced hepatic injury when administered in water (Obi and Ozoemena, 1998) or in aqueous ethanol (Obi *et al*, 1998), they also provide strong indication that this anthocyanin, has two pigments, a red and purple pigment. Furthermore, and of particular interest is the demonstration that the red pigment is the active hepatoprotective

anthocyanin.

It would appear based on the results presented in Tables 1 and 2 that AN₂ affords better hepatoprotection than AN₄ (the sephadex G50 red fraction) in view of their percentage differences relative to group 2 (see column 4 subcolumn 2 of Tables 1 and 2). The results in Table 3 though, indicate that AN₁ rather than AN₂ is a better liver protector than AN₄ (see Table 3 groups 3 and 6 under percentage difference relative to group 2). Statistical evaluation however, shows that the percentage differences in hepatoprotective action of AN₁, AN₂ and AN₄ are not significantly different from each other (Tables 1, 2 and 3, column 3). This is not unexpected in view of the fact that the same concentration of the active hepatoprotective agent (the red anthocyanin pigment) was present in all the samples.

AN₃ is the desalted whole anthocyanin with the red and purple pigments intact. It differs from AN₅ since the latter lacks the purple pigments. The results in Table 1, 2 and 3 show that AN₃ is hepatoprotective while AN₅ is not. Thus the present findings suggest that in AN₃ and in the other anthocyanin samples where the purple pigment was coadministered with the red, the purple pigment did not contribute to the hepatoprotective action.

In our previous discussion (Obi *et al*, 1998) a number of mechanisms were proposed for CCl₄-induced liver damage. One of the mechanisms is that trichloromethyl radical (*CCl₃) is produced from carbon tetrachloride by reductive dechlorination of CCl₄. The trichloromethyl radical abstracts a hydrogen atom from a fatty acid to form chloroform and a lipid radical. The lipid radical may then react with molecular oxygen to initiate lipid peroxidation which is thought to ultimately cause the cytotoxic response (Sipes *et al*,

1977; Recknagel, 1983; Brattin *et al.*, 1985; Reinke *et al.* 1988). This mechanism suggests an underlying process of oxidation. On the basis of this we hypothesized (Obi *et al.*, 1998;

Obi and Ozoemena, 1998) that if anthocyanin possesses antioxidant action, it would prevent lipid peroxidation and by extension membrane damage. Our earlier reports could only show that the presence of *H. rosasinensis* anthocyanin ensured that the integrity of liver cell (s) and organelle membranes were not entirely compromised following CCl₄ administration. The idea of lipid peroxidation and antioxidant potency was not adequately addressed. However, the results of the present investigation show that CCl₄ does induce lipid peroxidation since its administration to rats led to increased liver level of malondialdehyde, one of the strong indicators of lipid peroxidation (Reinke *et al.*, 1988). Therefore, *H. rosasinensis* anthocyanin, particularly the red pigment, is a potent naturally occurring antioxidant.

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