

Full Length Research Paper

The effect of *hibiscus* anthocyanins on 2, 4-dinitrophenylhydrazine-induced hepatotoxicity in rabbits

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This study examines the effects of anthocyanin extract of the dried calyces of *Hibiscus sabdariffa* Linn on the 2, 4-dinitrophenylhydrazine (2, 4-DNPH)-induced toxic side effects in rabbits liver. The side effects of DNPH which include tissue lipid peroxidation and depletion of antioxidant defenses were induced in rabbits with a single intraperitoneal administration of the toxicant (28 mg kg⁻¹ body weight) at the end of a 14-day treatment with *Hibiscus* anthocyanin extract (100 mg kg⁻¹ body weight twice daily) and after the animals have been fasted overnight. Four hours after the toxicant administration, liver and blood of the experimental animals were analyzed for changes in the activities of hepatic enzyme markers (alanine and aspartate aminotransferases; ALT and AST respectively). The hepatic levels of malondialdehyde (MDA), soluble protein as well as antioxidant molecules, superoxide dismutase (SOD) and reduced glutathione (GSH) were also determined. The anthocyanin extract significantly ($p < 0.05$) lowered the serum levels of the hepatic enzymes ALT and AST and reduced oxidative liver damage. The extract also significantly counteracted the depleting action of DNPH on the liver protein, SOD and GSH levels. Relative to the DNPH treated group, the extract significantly inhibited the formation of MDA in the liver. The data generated in this investigation show that the anthocyanin extract of *H. sabdariffa* dried calyces has potent protective properties against 2, 4-dinitrophenylhydrazine-induced oxidative hepatic toxicity in rabbits.

Key words: Anthocyanin, antioxidant defenses, 2, 4-dinitrophenylhydrazine, hepatic enzyme markers, *Hibiscus sabdariffa*, lipid peroxidation.

INTRODUCTION

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants. Protection against free radicals can be enhanced by ample intakes of dietary antioxidants of which the best studied are vitamins C and E as well as carotenoids

(Vertuani et al., 2004). There is considerable amount of epidemiological evidence revealing an association between diets rich in those vitamins and the decreased risk of cardiovascular disease and certain forms of cancer (Ames, 1983; Block, 1992; Hertog and Feskens, 1993; Wang et al, 2000).

It is generally assumed that the active dietary constituents contributing to these protective effects are the anti-oxidant vitamins. However, recent investigations have revealed that polyphenolic components of plants do exhibit antioxidant properties and do contribute to the anticarcinogenic or car-

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dioprotective actions brought about by diet (Newman, 1992; Wang et al., 2000; Stanner et al., 2004). In particular, some beverages such as red wine and tea have been shown to elicit antioxidant properties both *in vitro* and *in vivo* (Kanner et al., 1994).

Among the 300 species of *Hibiscus* plant is *Hibiscus sabdariffa* Linn which has been shown to have many medicinal uses (Gill, 1992; Morton, 1987). The aqueous extract of the calyces is consumed in the West African sub-region, especially in Nigeria as a local beverage called "Zobo drink". The dried calyces contain the flavonoids-gossypetin, sabdaretin, hibiscetine and anthocyanins (Pietta, 2000). Flavonoids are phenolic substances that act in plants as antioxidants. Antioxidant vitamins such as vitamins C and E along with flavonoids have been shown to be effective in reducing atherosclerosis along with many other diseases (Gaxlane et al., 1994; Jackson et al., 1993; Amin and Buratovich, 2007).

Earlier reports showed that the extracts from the red petals of *H. sabdariffa* contain antioxidant principles (Tseng et al., 1997; Wang et al., 2000; Ologundudu et al., 2006a, b). This research was therefore carried out to evaluate the hepatoprotective properties of *H. sabdariffa*, using the model of 2, 4-dinitrophenylhydrazine-induced oxidative tissue damage in rabbits.

MATERIALS AND METHODS

Experimental animals and materials

Rabbits (800 - 1000 g) used for this study were bred at the Federal College of Agriculture, Akure, Nigeria. The 2, 4-dinitrophenylhydrazine, trichloroacetic acid, sodium chloride and diethyl ether were purchased from BDH chemical company (Poole, England). The 2-thiobarbituric acid from Koch-Light Laboratories, England. Hydrochloric acid and absolute ethanol were obtained from WN Laboratories (USA). Adrenaline and Elman reagent were obtained from Sigma, USA while ALT, AST and protein kits were obtained from Randox Laboratories Ltd, UK. Chow (growers mash) was purchased from BFFM, Ewu, Nigeria.

Preparation of anthocyanin extract

Extraction of anthocyanin was done according to the method described in Hong and Wrolstad, (1990). One kg of *H. sabdariffa* calyces was pulverized and extracted with 10 litres of 0.1% trifluoroacetic acid (TFA) solution for 12 h at 40°C. The extract was filtered through filter paper (Advantech filter paper no. 5C). The filtrate was applied to sepabeads SP-207 resin column (Mitsubishi Chemicals, Japan). The resin was washed with 3 L of water and then eluted with 50% ethanol solution containing 0.1% TFA. The eluate was dried under vacuum at 40°C. The concentrated eluate was then subjected to high-speed liquid chromatography (HPLC) in order to identify its active principles.

HPLC analysis

The HPLC system consisted of a horizontal flow-through planar

centrifuge with a multilayer coil (Pharma-Tech Research Co., Model CCC-1000, MD, USA), a pump (JASCO, 880 - PU), a microflow pH sensor (Broadley-James, Model 14, CA, USA), a manual injection valve with a 20 ml loop and a fraction collector (JASCO, SF-212N). The upper phase, consisting of a mixture of *tert*-butylmethylether: 1-butanol: MeCN: water (2:2:1:5 v/v) containing 0.2% of TFA was used as the stationary phase, while the lower phase was as the mobile phase. A total of 300 mg of crude anthocyanin extract was dissolved in 20 ml of a mixture of the stationary phase: mobile phase (3:1 v/v) and introduced through the injection port. The mobile phase was pumped at 2.5 ml/min, while centrifugation was carried out at 1000 rpm. 4ml of each fraction was collected. A multi-wavelength detector (Waters, 490E) monitored the absorbance of the effluent at 515 nm.

Treatment of animals and collection of blood and liver samples.

The animals were divided into four experimental groups of 3 rabbits each, housed in standard rabbit cages. Rabbits in groups 2 and 3 were given the anthocyanin extract, 100 mg kg⁻¹ body weight by gavage, twice a day for 2 weeks. For the same duration and the same manner, rabbits in groups 1 and 4 were given 2.5 ml H₂O kg⁻¹ body weight. At the end of the 2 weeks all rabbits were fasted overnight. Following the overnight fast, rabbits in groups 3 and 4 received 28 mg DNPH kg⁻¹ body weight in saline. Four hours after DNPH administration, the animals were sacrificed and blood liver tissue samples were taken for biochemical investigations.

Biochemical assay protocol.

AST, ALT and protein levels were determined using standard kits from Randox. MDA was determined based on the method described in Vashney and Kale (1990). While superoxide dismutase (SOD) activity was determined according to the method described in Misra and Fridovich, (1972). Reduced glutathione concentration was determined using the method of Jollow et al., (1974).

Statistical analysis

The data obtained were subjected to standard statistical analysis of variance (ANOVA) procedure of SAS (SAS Institute Inc. 1999). The treatment means were compared using the Duncan procedure of the same software. The significant level was set at P < 0.05.

RESULTS

Figure 1 shows the result displayed on a multi-wavelength detector used to monitor the absorbance of the effluent of *H. sabdariffa* extract at 515 nm. The peaks on the graph indicate the different anthocyanins present in the *H. sabdariffa* extract in form of their glucosides. The anthocyanins were identified by extrapolating from the reference graph shown in Figure 2 which is the HPLC chromatogram of known anthocyanins. The result showed that *H. sabdariffa calyces* contained several anthocyanins but the predominant ones were delphinidin-3-monoglucoside, cyanidin-3-monoglucoside and petunidin-3-monoglucoside.

The effect of *H. anthocyanins* on DNPH-induced changes in alanine amino-transferase (ALT) and aspartate

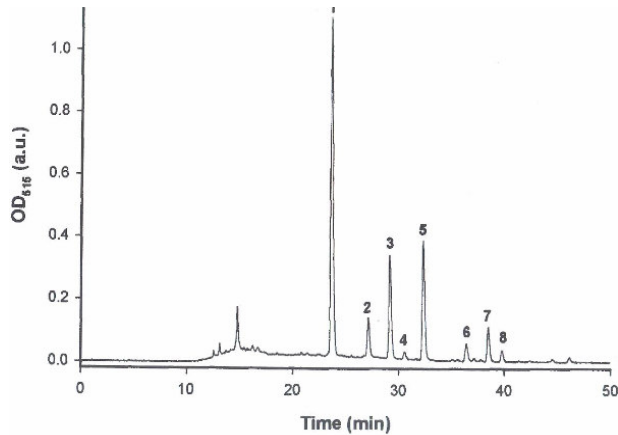


Figure 1. HPLC chromatogram of *H. sabdariffa* anthocyanins.

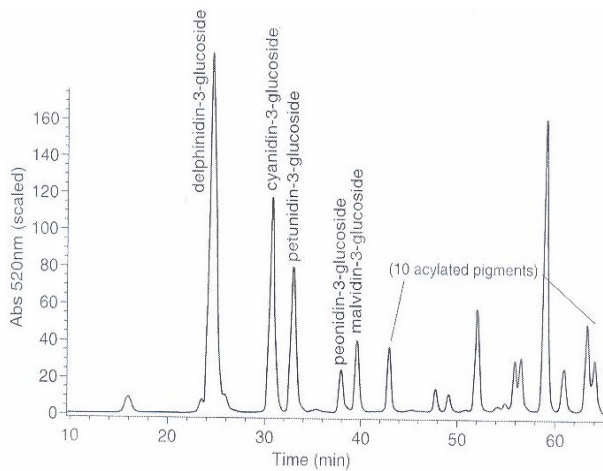


Figure 2. HPLC chromatogram of standard anthocyanins.

amino-transferase (AST) activities in the liver and serum is presented in table 1. These results show that DNPH alone caused a significant reduction in the activities of these enzymes in the liver while their activities in serum were significantly elevated. The results also indicate that the anthocyanin extract significantly blocked enzyme leakage when DNPH treatment was preceded by the administration of the extract.

The effect of *H. anthocyanins* on DNPH-induced changes in malondialdehyde (MDA), soluble protein, SOD and GSH levels in the liver is presented in table 2. These results show that DNPH alone induced significant formation of MDA in the hepatocytes compared to water controls. However, the anthocyanin extract significantly reduced the formation of MDA when DNPH was preceded by oral administration of the extract. The data also shown

that DNPH alone significantly reduced the soluble protein level in the hepatocytes compared to water controls but pretreatment of rabbits with the anthocyanin extract maintained to near normalcy the protein level in the liver when compared to the water controls. DNPH alone caused a significant depletion in the levels of SOD and GSH. However, anthocyanin pretreatment significantly increased the antioxidant status of the liver when DNPH treatment was preceded by the oral administration of the extract.

DISCUSSION

In this investigation, changes in serum and liver activities of ALT and AST together with changes in MDA and protein levels as well as the levels of SOD and GSH in the liver were used to assess both DNPH oxidative damage and protective effect of *H. anthocyanins* against the DNPH-induced damage in rabbits.

Relative to water controls, DNPH caused an increase in serum content of ALT and AST and a corresponding decrease in liver content of both enzymes (Table 1) which is indicative of damage to the liver (Obi and Ozoemena, 1998). The results of this research also indicated that DNPH caused a significant formation of MDA in the hepatocytes of rabbits (Group 4) exposed to the toxicant alone which is indicative of oxidative damage (McCay and Poyer, 1976; Clemens et al., 1984). Furthermore, Table 2 shows that, DNPH caused a significant reduction in the concentration of soluble protein and the concentration of the antioxidant molecules in the liver.

However, treatment of rabbits with 100 mg kg^{-1} body weight of *H. anthocyanins*, twice daily for 2 weeks before DNPH administration significantly impaired its toxic effect on the liver cells. Table 1 showed that the extract has a significant hepatoprotective effect as evidenced by its ability to restore normal levels of the liver and serum activities of ALT and AST when compared to DNPH-treated rabbits. Similar results were obtained for MDA assay (Table 2). The extract significantly impaired the DNPH-induced formation of MDA. Also pretreatment of rabbits with the extract inhibited the ability of DNPH to deplete the protein content of the hepatic cells and the levels of the antioxidant molecules were significantly increased in the extract treated rabbits compared with DNPH-treated extract-free rabbits. DNPH-induced lipid peroxidation has been reported (Clemens et al., 1984; Maduka et al., 2003; Ologundudu and Obi, 2005). Increase of blood and tissue levels of MDA are reliable indices of oxidative stress and lipoperoxidative tissue damage (Clemens et al., 1984; Maduka et al., 2003; Ologundudu and Obi, 2005; Ologundudu et al., 2006a, b). Therefore, the profile of MDA in the hepatocytes of DNPH-treated rabbits is a clear indication that DNPH provokes oxidative stress in the liver.

In vivo, this toxicant is believed to undergo auto oxida-

Table 1. Effect of *H. anthocyanin* (AN) on DNPH-induced alteration of ALT and AST activities in liver and serum.

Treatment	ALT(IU/L)		AST(IU/L)	
	Liver	Serum	Liver	Serum
1 Water control (2.5 ml/kg body weight)	30.87± 0.95	11.71±0.04	39.47±0.52	14.13±0.03
2 AN extract (100 mg/kg body weight)	31.26±0.75	11.80±0.18	39.12±1.06	14.48±0.05
3 AN (100 mg/kg body weight) + DNPH (28 mg/kg body weight)	30.99±0.29	11.90±0.91	38.26±0.12 ^a	14.08±0.16
4 DNPH alone (28 mg/kg body weight)	20.18±0.52 ^a	19.98±0.15 ^a	24.72±1.31 ^b	28.21±0.10 ^a

Tabulated results are means of 3 determinations ± SEM. Statistical comparison is strictly within the same tissue. Values carrying notations are statistically significantly different ($P < 0.05$) from control (group 1) while values carrying different superscripts are statistically significantly different from another.

Table 2. The effect of *H. anthocyanins* on DNPH- induced changes in MDA, protein, SOD and GSH levels in the liver.

Group	Treatment	MDA ($\mu\text{mol}/\text{mg}$ protein)	Soluble Protein (mg/g tissue)	SOD mg/mg Protein	GSH nmol/mg protein
1	Water control (2.5 ml/kg body weight)	1.53±0.01	2.17±0.02	15.81±0.63	23.20±0.01
2	AN extract (100 mg/kg body weight)	1.04±0.22 ^b	2.18±0.01	15.92±1.56	24.83±0.08 ^b
3	AN (100 mg/kg body weight)+DNPH (28 mg/kg body weight)	1.40±0.02 ^c	2.18±0.01	14.98±0.28 ^b	23.33±0.72
4	DNPH alone (28 mg/kg body weight)	13.25±1.48 ^d	1.01±0.03 ^b	10.25±0.56 ^c	17.67±2.43 ^c

Tabulated results are means of 3 determinations ± SEM. Statistical comparison is strictly within the same tissue. Values carrying notations are statistically significantly different ($P < 0.05$) from control (group 1) while values carrying different superscripts are statistically significantly different from another.

tion and becomes a strong oxidant with the ability to initiate lipid peroxidation in membrane phospholipids (Jain and Hochstein, 1979), once the antioxidant defense system has been overwhelmed. The induction of lipid peroxidation is thought to ultimately cause cytotoxic response (Sipes et al., 1977). The lipid oxidation causes disruption of the bilayer and cell integrity accompanied by the leakage of enzymes from the damaged organ into the blood stream. This phenomenon is largely responsible for the changes in the serum and hepatic levels of ALT and AST in rabbits treated with the toxicant (Group 4) in this study.

Table 2 shows that the anthocyanins extract significantly increased the activity of the antioxidant enzyme, SOD and GSH levels compared to DNPH-treated rabbits. Antioxidant enzymes such as catalase, superoxide dismutase and glutathione-s-transferase and glutathione peroxidase are present in oxygen handling cells which are the first line cellular defense against oxidative injury decomposing O_2 and H_2O_2 before they interact to form more reactive radicals (Jainu and Devi, 2004; Amin, 2008). The increase in the activities of the antioxidant enzymes in the extract pretreated rabbits compared to DNPH models is necessary for effective antioxidant activity. It is well established that (GSH), the most important biomolecule protecting against

chemically induced cyto-toxicity, can participate in the elimination of reactive intermediates by conjugation or by direct free radical quenching. This study showed that DNPH reduced GSH levels in the liver, but *H. anthocyanins* blocked the phenomenon effectively.

The mechanism by which *H. anthocyanins* prevent DNPH-induced changes is not clear at this stage. However, it is likely that the extract protected the tissues from damage by blocking DNPH-induced free radical formation. The protection may also be due to the impaired free radical propagation and or complementation of the antioxidant defense system. Further investigation is required to be able to establish the precise mechanism operating here.

As indicated earlier in this report, the mechanism of DNPH-mediated tissue damage suggests an underlying process of oxidation. Therefore the hypothesis on which this investigation was based is that if the anthocyanin extract of dried flowers of *H. sabdariffa* possesses antioxidant properties, therefore, it would prevent lipid peroxidation and other metabolic side effects of DNPH caused by its oxidant action. Present results demonstrated reasonably well that treatment of rabbits with *H. anthocyanins* prior to DNPH intoxication significantly inhibited its cytotoxic and other metabolic side effects in the liver.

Conclusion

It is of interest that the results presented here suggest that this extract consumed by humans as a local soft drink (Zobo) in the West African sub-region contains a potent antioxidant bioactivity. If this property of the extract is also manifested in humans upon consumption it would not only boost the body antioxidant status but also contribute to the prevention of oxidative tissue damage and other related health consequences.

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