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Full Length Research Paper

Effect of *Hibiscus sabdariffa* anthocyanins on 2, 4-dinitrophenylhydrazine-induced hematotoxicity in rabbits

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In this study, the 2, 4-dinitrophenylhydrazine-induced biochemical and hematological changes in rabbits were examined under the administrative protocol of anthocyanin extract from *Hibiscus sabdariffa* calyces. Blood levels of reduced glutathione (GSH) and malondialdehyde (MDA) as well as red blood cell counts (RBC), white blood cell counts (WBC), packed cell volume (PCV) and hemoglobin (Hb) concentration were determined as indices of alteration and protection. Relative to control, 2, 4-dinitrophenylhydrazine (2, 4-DNPH) treatment significantly decreased ($P < 0.05$) blood level of GSH and significantly increased blood MDA level .It also significantly ($P < 0.05$) decreased RBC counts, PCV and Hb but increased WBC counts. On the other hand treatment of rabbits with *Hibiscus* anthocyanin extract led to significant ($P < 0.05$) increase in blood GSH, RBC counts, PCV and Hb and a decrease in MDA and WBC counts. These findings indicate that anthocyanin extract from dried calyces of *H. sabdariffa* protects the blood against 2, 4-DNPH lipoperoxidative and hemolytic effects.

Key words: Rabbit, *Hibiscus sabdariffa* , anthocyanin extract, 2,4 dinitrophenylhydrazine, reduced glutathione, malondialdehyde, complete blood count.

INTRODUCTION

A paradox in metabolism is that while the vast majority of complex life requires oxygen for its existence, oxygen is a highly reactive molecule that damage living organisms by producing reactive oxygen species (ROS) (Davies, 1995). Consequently, organisms contain a complex network of antioxidant metabolites such as vitamins C and E and enzymes such as catalase and superoxide dismutase (SOD) that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids (Sies, 1997; Vertuani et al., 2004).

Antioxidants can cancel out the cell-damaging effects of free radicals (Sies, 1997), and people who eat fruits and vegetables rich in polyphenols and anthocyanins have a lower risk of cancer, heart disease and some neurological diseases(Stanner et al., 2004).Antioxidants work in 2 ways first is by chain-breaking which involves breaking the preventive side. Antioxidant enzymes like superoxide dismutase and catalase prevent oxidation by reducing the rate of chain initiation. The antioxidants scavenge initiating radicals and destroy them before oxidation is set in motion (Sies, 1997).

Hibiscus sabdariffa is a plant that finds various uses in traditional medicine. It is used as an antiseptic, diuretic, emollient and purgative agent (Truswell, 1992). It is a remedy for cancer, cough, heart ailments, hypertension and neurosis (Duke, 1985). The dried calyces of *H. sabdariffa* contain the flavonoids gossypetine, hibiscetin, anthocyanins and sabdaretine (Pietta, 2000). Certain amounts of delphinidin-3-monoglucoside and cyanidin-3-monoglucoside which constitute the anthocyanins are also present (Langenhoven et al., 2001). Flavonoids are phenolic compounds, they act as antioxidants in plants

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(Robinson, 1975). There are indications that extracts from the red calyces of *H. sabdariffa Linn* contain antioxi-dant principles (Tseng et al., 1997; Wang et al., 2000; Ologundudu and Obi, 2005; Ologundudu et al., 2006a, b). It is therefore conceivable that the consumption of the extract may provide natural agents against oxidative tissue damage and other free radical-induced disease conditions (Harman, 1984; Wolff et al., 1986).

Phenylhydrazine and its derivative, 2, 4-dinitrophenylhydrazine are toxic agents. Their toxic action has been attributed to their ability to undergo auto oxidation. This increased oxidant potential enables them to oxidize enzymes, membrane proteins and hemoglobin. Phenylhydrazine initiates lipid peroxidation in membrane phosphorlipids (Jain and Hochstein, 1979) while 2,4-dinitrophenylhydrazine induces lipid peroxidation and other oxidative damage in rabbits(Ologundudu and Obi, 2005; Ologundudu et al., 2006a,b) and rats (Maduka et al,2003) . The ability of 2,4-DNPH to induce lipid peroxidation and other free radical damage makes it an appropriate model toxicant for testing the claim that the anthocyanin extract of *H. sabdariffa Linn* calyces could probably protect tissues from oxidative stress-induced changes and other attendant biochemical consequences.

This research was therefore carried out to evaluate the hematoprotective properties of *H. sabdariffa* anthocyanin extract using the model of 2, 4-dinitrophenylhydrazine-induced oxidative stress in rabbits.

MATERIALS AND METHODS

Experimental animals and materials

Male rabbits (weight range 800 – 1000 g and four months old) used for this study were purchased from a local breeder in Benin City, Nigeria. 2,4-dinitrophenylhydrazine,trichloroacetic acid, NaCl and diethyl ether were purchased from BDH Chemical Company (Poole, England), 2,-thiobarbituric acid from Koch-Light Laboratories (England). HCl and absolute ethanol were obtained from WN Laboratories (US) and Chow (Growers mash) was obtained from Bendel Feed and Flour Mills, Ewu, Edo State, Nigeria**.**

Preparation of anthocyanin extract

Anthocyanin extract from *H. sabdariffa calyces* was prepared according to the method described by Hong and Wrolstad (1990a) . 1 kg of *H. sabdariffa* calyces was pulverized and extracted with 10 l 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 1000 rpm. 4 ml 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 samples

Experimental rabbits were divided into 4 groups, 5 rabbits each and housed in standard cages. Rabbits were given free access to feed and water throughout the experiment period that lasted for 28 days. Group 1 and 2 were given a daily twice doses of 2.5 ml H_2O/kg body weight by gavage for 4 weeks. Similar treatment with anthocyanin extract at dose of 100 mg/kg body weight was given to rabbits in groups 3 and 4. After the 21^{st} day of the experiment, rabbits in groups 2 and 4 were intraperitoneally administered with a dose of 28 mg/kg body weight of 2, 4-dinitrophenylhydrazine for 5 consecutive days. By the end of the experimental period, the animals were anaesthetized in a diethyl ether saturated chamber, and then dissected to expose the heart. Blood was obtained via cardiac puncture by means of a 5 ml hypodermic syringe and needle and placed in 2 sets of heparinized bottles. One set was used immediately for hematological analysis while samples in the second set were centrifuged at 3500 rpm (Uniscope model SM 902B Bench centrifuge, Surgifriend Medicals, England) for 10 min each in order to obtain plasma. Plasma samples were collected and kept at -20°C until required.

Hematological analysis

The hematological indices namely red blood cell (RBC) counts and white blood cell (WBC) counts were estimated by visual counting improved by Neubauer counting chambers. Hemoglobin (Hb) and packed cell volume (PCV) were determined using cyanomethemoglobin and microhematocrit methods respectively (Dacie and Lewis, 1997).

Lipid peroxidation assay

Lipid peroxidation was determined spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method as described by Varshney and Kale (1990). Results were expressed in terms of malondialdehyde (MDA) formed per mg protein.

Reduced glutathione

Reduced glutathione concentration in the blood was determined using the method of Jollow et al. (1974).

Statistical analysis

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

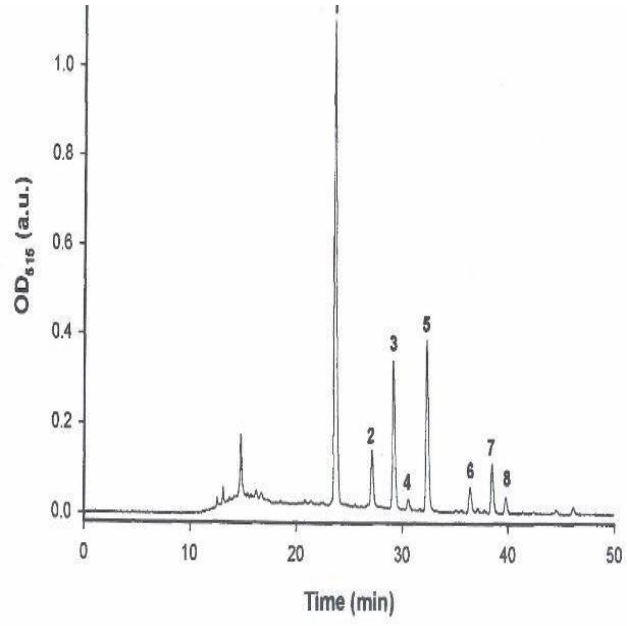

Figure 1. HPLC chromatogram of *H. sabdariffa anthocyanins*.

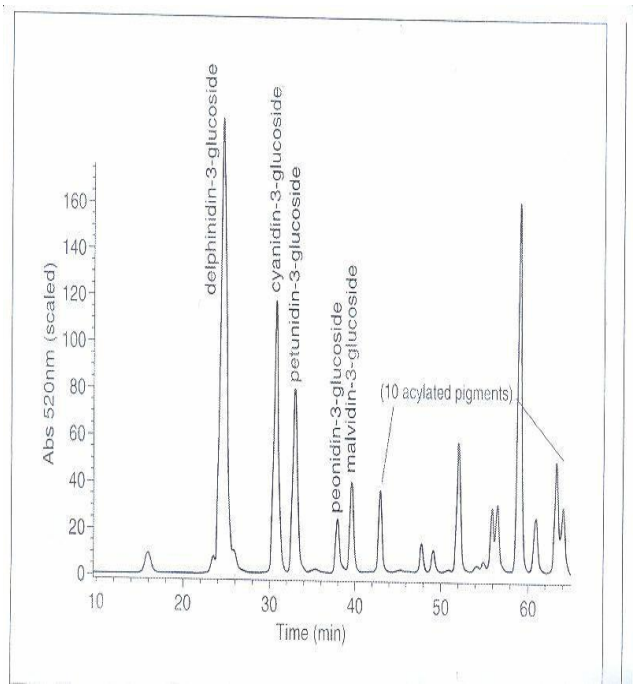

Figure 2. HPLC chromatogram of standard anthocyanins.

RESULTS

Figure 1 shows the result displayed on a multiwavelength 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 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 and cyanidin-3-monoglucoside.

Table 1 shows the effect of administering 2, 4-DNPH and *H. sabdariffa* anthocyanins on the levels of reduced glutathione (GSH) and malondialdehyde (MDA) of the studied rabbits. Intraperitoneal treatment of rabbits in group 2 with 2, 4-DNPH (28 mg/kg body weight) signifycantly ($p < 0.05$) reduced blood content of GSH but increased the MDA level when compared to the control, Group 1.However, treatment of rabbits (Group 3) with anthocyanin extract alone led to significant ($P < 0.05$) increase in blood content of GSH and a decrease in MDA level relative to the values obtained for group 2. Furthermore, treatment of rabbits (group 4) with anthocyanin extract prior to 2, 4-DNPH intoxication maintained at normal, the levels of both MDA and GSH in relation to control.

The changes in red blood cell (RBC) and white blood cell (WBC) counts, packed cell volume (PCV) and hemoglobin (Hb) concentration of rabbits due to the effect of 2, 4- DNPH and *Hibiscus* anthocyanin extract are presented in Table 2. Treatment with 2, 4-DNPH significantly ($P < 0.05$) reduced rabbit blood RBC, PCV, Hb values but caused an increase in WBC counts compared to the control (Group 1). The RBC and WBC counts and Hb concentration of rabbits that received the anthocyanin extract alone (Group 3) and those pretreated with the extract before 2, 4-DNPH administration (Group 4) did not show any significant ($p < 0.05$) difference when compared to the control. However, the PCV of rabbits that were treated with the extract alone (Group 3) was significantly ($p < 0.05$) increased while the PCV of their counterparts that received the extract before 2, 4-DNPH administration (Group 4) did not show any significant ($p < 0.05$) difference when compared to the control.

DISCUSSION

Recently, much attention has focused on the protective biochemical functions of naturally occurring antioxidants in biological systems, and on the mechanisms of their action. Phenolic compounds, which are widely distributed in plants, were considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living systems (Wang et al., 2000; Stanner et al., 2004).

Anthocyanins are phenolic compounds, and their antioxidant effects were investigated in this study. This study demonstrated that *H. sabdariffa* anthocyanins exhibited antioxidant bioactivity in intact cells and *in vivo* systems. As the integrity of cellular membranes is critical to normal cell function, the peroxidative decomposition of membrane lipids is an implication of chemical-induced toxicity.

Table 1. Effect of 2, 4-Dinitrophenylhydrazine and *Hibiscus* anthocyanin on the levels of reduced glutathione (GSH) and malondialdehyde (MDA) of rabbits.

Rabbit Group	Treatment	GSH Concentration (nmol per g protein)	MDA (µmol per mg protein)
1	2.5 ml H_2O /kg bd. wt. (control)	24.84 ± 1.33	1.38 ± 0.02
2	28 mg DNPH /kg bd. wt.	16.39^a ± 0.92	8.50^a ± 0.64
3	100 mg AN /kg bd. wt.	26.27^b ± 0.59	1.07^b ± 0.09
4	100 mg AN /kg bd. wt.+28 mg DNPH /kg bd. wt.	23.53^c ± 0.26	1.44 ± 0.19

Results are means of 5 determinations ±SEM. Values carrying notations are significantly ($p<0.05$) different from control (Group 1). AN; -anthocyanin extract.

Table 2. Effect of 2,4-Dinitrophenylhydrazine and *Hibiscus* anthocyanin on the levels of red blood cells (RBC), white blood cells (WBC), packed cell volume (PCV) and hemoglobin (Hb) of rabbits.

Rabbit Group	Treatment	RBC (Counts/µL)×10^6	WBC (Counts/µL) ×10^3	PCV (%)	Hb concentration (g/dl)
1	2.5 ml H_2O /kg bd. wt. (control)	6.55 ± 0.71	7.4 ± 0.21	34.33 ± 0.88	11.27 ± 0.17
2	28 mg DNPH /kg bd. wt.	3.88^a ± 0.40	11.47^a ± 0.47	26.67^a ± 1.76	7.30^a ± 0.35
3	100 mg AN /kg bd. wt.	6.81 ± 0.34	6.60 ± 0.92	35.33^b ± 1.53	11.93 ± 0.79
4	100 mg AN /kg bd. wt.+28 mg DNPH /kg bd. wt.	5.47 ± 0.21	7.60 ± 0.67	33.33 ± 1.76	11.27± 0.15

Results are means of 5 determinations ±SEM. Values carrying notations are significantly ($p<0.05$) different from control (Group 1).

In the present study, 2, 4-DNPH was shown to enhance lipid peroxidation, cytotoxicity in other words in animal systems. This is in consonance with our earlier reports (Ologundudu and Obi, 2005; Ologundudu et al., 2006a, b).

Evidence from a number of studies *in vitro* and *in vivo* suggests that phenylhydrazine and its derivatives interact with hemoglobin and cytochrome P- 450 in an oxidation reaction, resulting in the generation of destructive free radicals, which are responsible for subsequent hemotoxicity (Itano et al., 1975; Jain and Hochstein, 1979; Maples et al., 1988). The results of the present study showed that *Hibiscus* anthocyanin extract effectively protect the blood from the oxidative damage caused by 2, 4-DNPH.

It is well established that reduced glutathione (GSH), the most important biomolecule protecting against chemically induced cytotoxicity, can participate in the elimination of reactive intermediates by conjugation or by direct free radical quenching. This study showed that 2, 4-DNPH caused a significant reduction in GSH levels in the blood and that a high dose of *Hibiscus* anthocyanins blocked the phenomenon effectively.

The effect of the anthocyanin extract on the hematological parameters in the animals treated with 2, 4-DNPH was also assessed (Table 2). The observed increase in the PCV, RBC counts and Hb concentration in the animals treated with the extract could be explained by the reduced loss of blood cells to lipid peroxidation as a result of the antioxidative properties of the pigments or the erythropoietic potencies already established for antioxidant molecules (Heda and Bhatia, 1986). The reduced PCV, RBC, Hb levels for animals treated with DNPH is a confirmation of the previously established hemotoxic properties exhibited by phenylhydrazine and its derivatives (Maples et al., 1988; Patil et al., 2000). Anthocyanins are known to induce the renal secretion of erythropoietin, the most important signal for differentiation and multiplication of the pluripotent stem cells involved in blood cell formation as reported by (Heda and Bhatia, 1986; Kaur and Kapoor, 2005).

Hemoglobin is a natural constituent of red blood cells and biochemically adapted to carry oxygen in the lungs and deposit it at the tissues for oxidative metabolism. Besides this function, it has been characterized to also play major role in physiologic carbon dioxide removal and acid-base balance. Therefore, an increased production of hemoglobin is an advantage to an organism. This metabolic status can only be ensured by decrease in red blood cell destruction or increased red blood cell production (Ponka, 1997).

White blood cells form part of the immune system in animals working against invading pathogens. The significant increase in WBC counts of the DNPH-treated animals when compared with the control is due to the ability of DNPH to act as hapten, thereby stimulating the production of plasma-cell derivatives of -cells, thus accounting for the increased WBC levels. The prolifera-

tion of WBC by induced maturation of lymphocytes to matured WBC is the first stage of cell-mediated defense in the body in response to the presence of protein antigens and xenobiotics. Pretreatment of animals with anthocyanin extract prior to DNPH intoxication showed a feedback effect, with an observed significant reduction in WBC level. These results attest to the basis for the treatment of leukemia using anthocyanins in folk medicine, although, the biochemical mechanism is still unknown (Kaur and Kapoor, 2005).

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