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Comparative Study on the Effect of *Hibiscus sabdariffa* Calyx Anthocyanins and Ascorbate on 2,4-Dinitrophenylhydrazine-induced Damage in Rabbits

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Abstract The antioxidant bioactivities of the whole isolated anthocyanins from *Hibiscus sabdariffa (HS)* calyx were evaluated and compared with the bioactivity of vitamin C as a standard water soluble antioxidant vitamin. They were assessed for antioxidant activity based on their ability to impair 2, 4-dinitrophenylhydrazine (DNPH)-induced oxidative tissue damage in toxicant exposed rabbits. Exposure of rabbits to DNPH (28 mg/kg body weight) caused significant ($p<0.05$) reduction in packed cell volume (PCV), hemoglobin level and red blood cell (RBC) counts but increased white blood cell (WBC) counts relative to the DNPH-free rabbits. L-alanine aminotransferase (L-ALT) and L-aspartate aminotransferase (L-AST) activities were significantly ($p<0.05$) increased in the serum of DNPH-exposed rabbits compared to the DNPH-free rabbits. There were corresponding decreases in the liver status of both enzymes. DNPH exposure also caused significant change in malondialdehyde levels in serum and liver relative to DNPH-free controls. However, pre-treatment with 100 mg/kg body weight of the anthocyanin extract and vitamin C separately provided varying degrees of protection against DNPH-induced biochemical and hematological changes. Relative to the controls, HS calyx anthocyanins and vitamin C treatments increased the levels of PCV, hemoglobin and RBC and decreased WBC counts significantly ($p<0.05$) and so, effectively ameliorated the DNPH-induced hemotoxicity. The same treatments significantly lowered the activities of L-ALT and L-AST in the serum relative to the DNPH-exposed group, while maintaining their levels in the liver. The treatments also significantly ($p<0.05$) lowered the level of malondialdehyde in the serum and liver. However, when examined separately and compared, the HS calyx anthocyanins appeared to have offered the more effective protection than vitamin C, against the DNPH-induced oxidative damage and so *H. sabdariffa* calyces possess potent antioxidant principles which are likely to be anthocyanins.

Keywords *Hibiscus sabdariffa,* antioxidant bioactivity, anthocyanins, biochemical and hematological changes, vitamin C

1. 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 and carotenoids. There is a considerable amount of epidemiological evidence revealing an association between diets rich in fruits and vegetables and a decreased risk of cardiovascular disease and certain forms of cancer [2,8]. It is generally assumed that the active dietary constituents contributing to these protective effects are antioxidant nutrients such as α - tocopherol and β - carotene. However, recent investigations highlight the role of polyphenolic components of higher plants that may act as antioxidants or via other mechanisms contributing to the anticarcinogenic or cardioprotective actions[14,31]. In particular some beverages such as red wine and tea have been shown to elicit antioxidant properties in both *in vitro* and *in vivo* systems[10].

Among the more than 300 species of Hibiscus is *Hibiscus sabdariffa Linn* which has many medicinal uses[9,1]. The dried calyces contain the flavonoids – gossypetin, sabdaretine, hibiscetine and anthocyanins[24]. Flavonoids are phenolic substances. They act in plants as antioxidants. It is thought that in humans absorbed flavonoids and their metabolites may display an *in vivo* antioxidant activity. Antioxidant vitamins such as vitamins C and E along with flavonoids have been shown to be effective in reducing atherosclerosis[6]. Our previous reports showed that the extract from the red calyces of *Hibiscus sabdariffa* contain potent antioxidant principles[15-20]. The aim of this re-

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search therefore was to evaluate this claim further by comparing the antioxidant potency of HS anthocyanins with that of ascorbate, a standard water soluble antioxidant, since anthocyanins are known to be water soluble polyphenolic compounds.

2. Materials and Methods

2.1. Plant Materials

Fresh calyces of *H. sabdariffa L.* were harvested from Botanical Gardens, University of Ilorin, Kwara State, Nigeria. They were dried under continuous air-flow maintained at 25℃ until constant weight. Identification and taxonomical classifications were done at herbarium of the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo-State, Nigeria.

2.2. Animals

Thirty (30) rabbits (*Oryctolagus cuniculus*) used for this research work were obtained from a private breeder in Benin City. The animals weighed 800-1000 g on purchase and were in very good state of health as confirmed by a veterinary physician. The animals were housed in twos (same sex) in improvised rabbit cages composed of wire mesh (100 cm X 40 cm X 30 cm) under 14 hr/10 hr light/dark regimen. They were fed with growers mash (obtained from Bendel Flours and Feed Mill, Ewu, Edo State, Nigeria) and water *ad libitum*. The animals were protected from parasite infestation by proper veterinary management throughout the duration of the treatment.

2.3. Preparation of Anthocyanin-rich Extract from Plant Materials

Preparation of anthocyanin-rich extract from *Hibiscus sabdariffa* calyces was carried out according to the method described in our previous reports[19-20].

2.4. Biochemical Assay Protocols

Hematological indices namely red blood cell (RBC) and white blood cell (WBC) counts were estimated by visual counting, improved by Neubauer counting chambers. Hemoglobin (Hb) concentration and packed cell volume (PCV) were determined using cyanomethemoglobin and microhematocrit method respectively[3]. The L-alanine aminotransferase (L-ALT) and L-aspartate aminotransferase (L-AST) activity in the serum and liver was estimated by the method of Reitman and Frankel[26], while lipid peroxidation was determined spectrophotometrically by the thiobarbituric acid reactive substances (TBARS) method as described by Varshney and Kale[30], and was expressed in terms of malondialdehyde (MDA) formed per mg protein. Measurement of absorbance was done at 532nm.

2.5. Experimental Design

Thirty (30) rabbits weighing 800-1000 g were used for this research work. They were randomly selected into six (6) experimental groups as shown below. The experiment lasted for 28 days.

Group 1: Water treated control. Each rabbit was given distilled water, 2.5 ml/kg body weight.

Group 2: Anthocyanin-rich extract of *H. sabdariffa* was administered at a dose of 100 mg/kg body weight, to each rabbit in this group by gavage.

Group 3: Ascorbate was administered at a dose of 100 mg/kg body weight, to each rabbit in this group by gavage.

Group 4: 2, 4-Dinitrophenylhydrazine was administered at a dose of 28 mg/kg body weight intraperitoneally to each rabbit in this group during the last 5 days of the 28-day study period, before sacrifice.

Group 5: Anthocyanin-rich extract of *H. sabdariffa* was administered at a dose of 100 mg/kg body weight for 28 days to each rabbit in this group accompanied with 28 mg of 2, 4-dinitrophenylhydrazine per kg body weight administered intraperitoneally daily from day 24 (5 days 2,4-DNPH treatment) before sacrifice.

Group 6: Ascorbate was administered at a dose of 100 mg/kg body weight for 28 days to each rabbit in this group accompanied with 28 mg of 2, 4-dinitrophenylhydrazine per kg body weight administered intraperitoneally daily from day 24 (5 days 2,4-DNPH treatment) before sacrifice.

2.7. Statistical Analysis

The data obtained were subjected to standard statistical analysis of variance (ANOVA) using the SAS software[27]. Treatment means were compared using the Duncan procedure of the same software. The significance level was set at $P<0.05$

3. Results

The effects of DNPH, HS anthocyanins and ascorbate on the levels of red blood cell (RBC) and white blood cell counts (WBC), packed cell volume (PCV) and hemoglobin (Hb) concentration in rabbits are presented in Table 1. Treatment with DNPH alone significantly ($p< 0.05$) reduced the rabbit RBC counts, PCV level, Hb concentration but caused a significant ($p<0.05$) increase in WBC counts compared to control (Group 1). The RBC and WBC counts, PCV level and Hb concentration of rabbits that received the anthocyanin-rich extract and ascorbate separately (Groups 2 and 3) and those pretreated with them before DNPH administration (Groups 5 and 6) did not show significant ($p>0.05$) alteration when compared with the control.

The effects of DNPH, HS anthocyanins and ascorbate on the activities of L-ALT and L-AST in the liver and serum are presented in Table 2. These data show that DNPH alone (Group 4) caused a significant ($p<0.05$) reduction in the activities of these enzymes in the liver while their activities in the serum were significantly ($p<0.05$) elevated. The results also indicate that relative to DNPH group, prior exposure of rabbits to anthocyanin-rich extract and ascorbate

before DNPH treatment led to impaired DNPH-induced L-ALT and L-AST alterations (Groups 5 and 6). When compared, rabbits treated with anthocyanin extract (Group 5) showed a significantly higher level of L-ALT in the liver and lower level in the serum than those in Group 6 that received ascorbate prior to DNPH administration. Same trend was obtained for L-AST.

The effects of DNPH, HS anthocyanins and ascorbate on the levels of MDA in the liver and serum of rabbits are presented in Table 3. The group that received DNPH alone (Group 4) showed a significant increase in MDA levels in the liver and serum when compared with control (Group 1). The respective groups treated with anthocyanin extract alone (Group 2) and ascorbate alone (Group 3) showed significant ($p<0.05$) reduction in liver MDA levels. Relative to control, the groups treated with anthocyanin (Group 5) and ascorbate before DNPH intoxication (Group 6) did not show any significant difference in MDA levels in the tissues but the MDA values were significantly reduced when compared with the MDA level of the group treated with DNPH only.

4. Discussion

Anthocyanin-rich extract and ascorbate groups showed an increase in PCV, RBC and Hb compared to the water control. The observed increase in PCV, RBC and Hb of the animals treated with the anthocyanin extract and ascorbate could be explained as the resultant effect of reduced loss of blood cells to lipid peroxidation following the antioxidant activities of the extracts or the erythropoietic potencies already established for antioxidant molecules[7]. The erythropoietic property can be due to the increased absorption of iron which has been widely reported in ascorbate administration[7]. Also, iron has been reported to be present in significant amount in the whole extract of *H. sabdariffa,* in addition to the ability of anthocyanin to induce the synthesis and release of erythropoietin by the kidney[7]. The significant decrease in the PCV, RBC and Hb levels of animals treated with DNPH alone was a confirmation of the previously established hemotoxic properties exhibited by DNPH and its derivatives[19,22]. Both treatments individually showed the ability to prevent the toxic effect of DNPH as indicated by significantly increased ($p<0.05$) PCV, RBC and Hb levels when compared with the group treated with DNPH only, while rabbits that received the anthocyanin-rich extract and ascorbate separately (Groups 2 and 3) and those pretreated with them before DNPH administration (Groups 5 and 6) did not show significant ($p>0.05$) alteration when compared with the control. This observation is possibly explained by the ability of the different treatments to reduce hemolysis induced by peroxidative reaction of DNPH and its metabolic derivatives and to potentiate erythropoiesis; thereby bringing about replenishment of the destroyed red blood cells (RBCs) due to DNPH treatment. Ascorbate is known to aid intestinal absorption of iron[23] and iron is known to induce erythropoiesis in animals and humans[23,25]. 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[7,11].

Table 1. Effects of 2, 4- DNPH, HS anthocyanins and ascorbate (ASB) on the levels of red blood cells (RBC), white blood cells(WBC), packed cell volume (PCV) and hemoglobin (Hb) of rabbits

Group #	Treatment	RBC (counts/μL) x 10^6	WBC (counts/μL) x 10^3	PCV (%)	Hb (g/dl)
1.	2.5 ml H_2O/kg bd wt. (control)	4.55 ± 0.71	7.4 ± 0.21	34.33 ± 0.88	11.27 ± 0.17
2.	100 mg AN/kg bd wt.	5.81 ± 0.34	7.60 ± 0.02	35.33± 1.53	11.93^b ±0.17
3.	100 mg ASB/kg bd wt.	5.16 ± 0.04	5.43 ± 0.58	35.00± 0.58	11.33 ± 0.09
4.	28 mg DNPH/kg bd wt.	3.88^a ± 0.40	11.47^a ± 0.22	26.67^a±1.76	7.30^a ± 0.35
5.	100 mg AN + 28 mg DNPH/kg bd wt.	5.47± 0.21	6.93 ± 0.44	33.33± 1.76	11.27 ± 0.15
6.	100 mg ASB +28 mg DNPH/kg bd wt.	5.33 ± 0.23	7.13 ± 0.03	33.33 ± 0.33	11.20 ± 0.12

Results are presented as means ±SEM of five (5) determinations. Statistical comparison is strictly within the same tissue. Values carrying superscripts differ significantly ($p<0.05$) from control (Group 1). Values with same superscript do not differ significantly while values with different superscripts are significantly different from one another. DNPH: 2, 4-dinitrophenylhydrazine, AN: anthocyanin, ASB: Ascorbate.

Table 2. Effects of DNPH, HS anthocyanins and ASB on L-ALT and L-AST activities in liver and serum

Group #	Treatment	L-ALT (IU/L)		L-AST(IU/L)	
		Liver	Serum	Liver	Serum
1.	2.5 ml H_2O/kg bd wt. (control)*	30.78 ± 0.30	11.54 ± 0.20	39.70 ± 0.17	14.66 ± 0.28
2.	100 mg AN/kg bd wt.	30.98 ± 0.15	11.75 ± 0.04	39.36 ± 0.31	13.81 ± 0.18
3.	100 mg ASB/kg bd wt.	31.13 ± 0.40	11.74 ± 0.12	39.36± 0.21	14.80± 0.14
4.	28 mg DNPH/kg bd wt.	24.36^a ± 0.73	17.42^a ± 0.23	35.16^a ± 0.16	22.02^a ± 0.14
5.	100 mg AN + 28 mg DNPH/kg bd wt.	29.45^b± 0.46	11.95 ± 0.13	38.97 ± 0.44	14.89 ± 0.11
6.	100 mg ASB +28 mg DNPH/kg bd wt.	28.74^c ± 0.54	12.05^b ± 0.06	37.21^b ± 0.47	16.31^b ± 0.22

Results are presented as means ± SEM of five (5) determinations. Statistical comparison is strictly within the same tissue. Values carrying superscripts differ significantly ($p<0.05$) from control (Group 1). Values with same superscript do not differ significantly while values with different superscripts are significantly different from one another. *See Table 1 footnote for interpretation of abbreviations.

Table 3. Effects of DNPH, HS anthocyanins and ASB on the levels of MDA in the liver and serum of rabbits

Group #	Treatment	MDA (μmol/ mg protein)	
		Liver	Serum
1.	2.5 ml H_2O/kg bd wt. (control)*	7.40 ± 0.08	1.38 ± 0.02
2.	100 mg AN/kg bd wt.	5.36^a ± 0.65	1.01 ± 0.09
3.	100 mg ASB/kg bd wt.	5.57^a ± 0.24	1.04 ± 0.01
4.	28 mg DNPH/kg bd wt.	22.12^b ±0.20	8.50^a ± 0.64
5.	100 mg AN + 28 mg DNPH/kg bd wt.	5.35 ± 0.20	1.44 ± 0.19
6.	100 mg ASB +28mg DNPH/kg bd wt.	6.66 ± 0.90	1.95 ± 0.03

Results are presented as means ±SEM of five (5) determinations. Statistical comparison is strictly within the same tissue. Values carrying superscripts differ significantly ($p<0.05$) from control (Group 1). Values with same superscript do not differ significantly while values with different superscripts are significantly different from one another. *See Table 1 footnote for interpretation of abbreviations

The white blood cells are known for defense against invading pathogens and transforming native cells. A significant increase in the WBC of the DNPH-treated rabbits when compared with the control and the pretreated groups is possibly due to the ability of DNPH to act as hapten thereby stimulating the production of plasma-cell derivatives of β-cells, thus accounting for the increased WBC levels. The proliferation 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[12,19]. Pretreatment of rabbits with anthocyanin extract and ascorbate before DNPH treatment showed a feed-back effect, with an observed significant reduction (to control level) in WBC level. This finding probably laid credence for the use of anthocyanins in the treatment of leukemia in folk medicine, although the biochemical mechanism is still unknown[11].

The groups treated with anthocyanin extract and ascorbate showed no significant difference in serum and liver levels of AST and ALT compared to the water control. This is an indication of reduced toxicity of both the anthocyanin extract and ascorbate to the liver. When DNPH-treated group was compared with the water control, there was a significant increase ($P<0.05$) in the serum activities of the enzymes with corresponding decrease in their activities in the liver. The increased activity of these enzymes in the serum is a clear indication of the toxicity of DNPH on the muscle and liver. The toxicity is sequel to the free radical generation and repression of protein synthesis in these tissues[20]. Prior administration of anthocyanin extract and ascorbate followed by treatment with DNPH resulted in significant decrease in the serum activities and an increase in the liver activities of the enzymes compared with those treated with DNPH only. Since the toxicity of DNPH is related to its ability to generate free radicals, it follows therefore that the prophylactic administration of these extracts built up defense and thus reducing significantly the oxidative damage provoked by DNPH administration. Tissue necrosis that usually characterizes oxidative damage is therefore minimized and the tissue activities of ALT and AST are preserved and therefore, their serum activities normalized. When comparing Group 5 with Group 6, rabbits treated with anthocyanin extract before DNPH treatment (Group 5) showed a significantly higher level of ALT in the liver and lower level in the serum than the group that received ascorbate prior to DNPH administration (Group 6). Same trend was obtained for L-AST. This indicates that anthocyanins offer better protection on the liver than ascorbate. This is particularly possible because anthocyanins, being polyphenols, are well furnished with free hydroxyl (OH^-) groups. The role of these hydroxyl groups in biological activities, including antioxidant properties of anthocyanins is well documented[28,17]. Anthocyanins have the potential to reduce cell destruction by scavenging free radicals thereby reducing cell loss due to necrosis and ultimately preserving the enzymes in their native environment rather than leaking into the blood stream as a result of necrotizing cells or tissues[29].

Malondialdehyde formation in biological tissues is secondary to the primary effect of the free radical attack. Since polyunsaturated fatty acyls are found on the membrane lipids, their break-down is a breach on the membrane integrity and cellular fluidity. These two latter factors are central to cellular and tissue functions in all forms of cellular and multicellular organisms. A high level of this product would then indicate loss of membrane integrity, and a possible necrotizing tissues induced by oxidative properties of attacking free radicals[4,5]. This undoubtedly explains why administration of DNPH resulted in significant increase in malondialdehyde levels in the tissues. Pretreatment of rabbits with the anthocyanin-rich extract and ascorbate prior to DNPH treatment effectively blocked this effect of DNPH. This claim is confirmed by reduction of malondialdehyde level below the values recorded for water control and similarity in the values obtained when the animals were treated with each of the extract and ascorbate only. This finding agrees with the reports of Matsumoto et al.[13] and Passamonti et al.[21].

5. Conclusions

It is of interest that the findings of this work show that *Hibiscus sabdariffa* calyx contains potent antioxidant principles which are likely anthocyanins and with potencies well comparable with that of ascorbate, a well-established water soluble antioxidant. In fact, it does appear from this research that anthocyanins are more powerful antioxidants than ascorbate.

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