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Effects of *Hibiscus sabdariffa* calyx anthocyanins and ascorbate on 2, 4-dinitrophenylhydrazine-induced changes in the activities of antioxidant enzymes in rabbits.

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Research Article

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ABSTRACT

The effects of anthocyanin-rich extract of the calyces of *Hibiscus sabdariffa* (HS) Linn and ascorbate on the 2, 4-dinitrophenylhydrazine (DNPH)-induced changes in the levels of antioxidant enzymes of rabbits were evaluated in this study. The organs examined were the blood, brain and liver. Thirty male adult rabbits used for the study were divided into six groups. Group 1, the control took only water while animals in groups 2,3,5 and 6 received 100 mg/kg body weight of the extracts once daily for 28 days. After 22nd day of treatment, the rabbits in groups 4, 5 and 6 received 28 mg/kg body weight of DNPH for the remaining 5 days of treatment, after which the animals were sacrificed. Exposure of rabbits to DNPH (28 mg/kg body weight) caused significant ($P<0.05$) increase in catalase and superoxide dismutase activities relative to the DNPH-free group. The activity of glucose-6-phosphate dehydrogenase was also significantly ($p<0.05$) elevated in the serum following DNPH treatment when compared to control. However, pre-treatment with (100 mg/kg body weight) HS anthocyanins and ascorbate separately provided varying degrees of protection against DNPH-induced biochemical changes. Relative to the controls, the extract and ascorbate treatments significantly ($P<0.05$) decreased the activities of the antioxidant enzymes. Examined separately and compared, both treatments appeared to have offered effective protection against DNPH-induced oxidative damage, though the anthocyanin isolate appeared to be more effective in this capacity. Our findings show that *Hibiscus sabdariffa* anthocyanins are probably more potent antioxidants than ascorbate.

INTRODUCTION

Hibiscus sabdariffa Linn (Roselle) belongs to the family of *Malvaceae*, which is native to old World tropics, probably in the East Indies; now cultivated throughout the tropics [5]. The vegetable is widely grown and commonly used as port herb or soup in the northern part of Nigeria. In Nigeria especially in the northern part, the extract of the red calyces is consumed as a beverage known as zobo.

Ethnobotanical information regarding *Hibiscus sabdariffa* reveals the following medicinal uses: diuretic, diaphoretic, antibacterial agent, antifungal agent, mild laxative, sedative, antihypertensive, gastrointestinal disorder treatment, hypercholesterolemia treatment, kidney stone treatment, liver damage treatment, agent for decreasing the viscosity of the blood, and agent for treating the after effects of drunkenness [1,7]. Among the chemical constituents of the flower are the flavonoids, gossypetine, hibiscetine, anthocyanin and sabdaretine [20]. Certain amounts of delphinidin-3-monoglucoside and cyaniding-3-monoglucoside which constitute the anthocyanins are also present [9].

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Some studies have reported that *Hibiscus sabdariffa* is effective for decreasing the levels of total lipids, cholesterol and triacylglycerol, suggesting the possibility that *Hibiscus sabdariffa* functions as hypolipidemic agent [7,13]. Studies on the effect of *Hibiscus sabdariffa* calyx extract on the activities of antioxidant enzymes are however, scanty. The present study is therefore aimed at evaluating the effect of *Hibiscus sabdariffa* anthocyanins as compared to ascorbate, on 2,4-dinitrophenylhydrazine-induced changes in the activity of antioxidant enzymes in rabbits.

Phenylhydrazine and its derivatives 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 protein and hemoglobin. Phenylhydrazine is able to initiate lipid peroxidation in membrane phospholipids [8] while 2,4-dinitrophenylhydrazine has been shown to be capable of inducing lipid peroxidation and other oxidative damage in rabbits [14,15,16] and rats [10]. 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 extract of *Hibiscus sabdariffa* Linn calyces can protect tissues from oxidative stress-induced changes and other attendant biochemical changes.

MATERIALS AND METHODS

Plant material

Fresh calyces of *H. sabdariffa* were harvested from Botanical Gardens University of Ilorin, Kwara State, Nigeria. They were dried under continuous air-flow maintained at 25 ^{0}C 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.

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 (100cmX40cmX30cm) 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.

Preparation of anthocyanin-rich extract from plant materials

Anthocyanin-rich extract from *Hibiscus sabdariffa* calyces was prepared according to the method described in our previous reports (Ologundudu et al., 2009a, b).

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-DNPH 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/kg body weight of 2, 4-dinitrophenylhydrazine administered intraperitoneally daily from day 24 (5 days 2,4-DNPH treated) before sacrifice.

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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/kg body weight of 2, 4-dinitrophenylhydrazine administered intraperitoneally daily from day 24 (5 days 2,4-DNPH treated) before sacrifice.

Biochemical determinations

Catalase activity was determined by the method of Sinha (1971) by following its decomposition of H_2O_2. The superoxide dismutase activity was determined by the method of Misra and Fridovich (1972). The activity of glucose-6-phosphate dehydrogenase was determined using assay kit obtained from Randox Laboratories, UK. The method is as described in the manual/leaflet.

Statistical analysis

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

RESULTS

The effects of DNPH, HS anthocyanins and ascorbate on the activities of G6PD in the serum and liver of rabbits are presented in Table 1. Administration of DNPH (Group 4) significantly ($p<0.05$) increased the level of G6PD in the serum. Rabbits that received the anthocyanin-rich extract and ascorbate separately before DNPH administration (Groups 5 and 6) did not show altered level of the enzyme in both serum and the liver when compared with control but were significantly reduced in the serum when compared with DNPH only group.

Table 1: Effects of DNPH, HS anthocyanins and ASB on the activities of G6PD in the serum and liver of rabbits

Group #	Treatment	Glucose-6-phosphate dehydrogenase activity(µmol/min/mg protein)	
		Serum	Liver
1.	2.5 ml H_2O/kg bd wt. (control)*	1.27 ± 0.22	33.33 ± 0.37
2.	100 mg AN/kg bd wt.	1.10 ± 0.13	33.83 ± 0.44
3.	100 mg ASB/kg bd wt.	1.35 ± 0.33	33.63 ± 0.07
4.	28 mg DNPH/kg bd wt.	4.50^a ± 0.98	33.07^a ± 0.06
5.	100 mg AN + 28 mg DNPH/kg bd wt.	1.21 ± 0.01	33.40 ± 0.23
6.	100 mg ASB + 28 mg DNPH/kg bd wt.	1.82 ± 0.56	33.67 ± 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. DNPH: 2, 4-dinitrophenylhydrazine, AN: anthocyanin, ASB: Ascorbate.

Table 2: Effects of DNPH, HS anthocyanins and ascorbate on serum, liver and brain catalase activities.

Group #	Treatment	Specific activity of catalase (n mole H_2O_2 decomposed/min/mg protein)		
		Serum	Liver	Brain
1.	2.5 ml H_2O/kg bd wt. (control)*	2.55 ± 0.08	5.89 ± 0.35	5.60 ± 1.05
2.	100 mg AN/kg bd wt.	2.88 ± 0.67	8.05^a ± 0.25	6.60 ± 1.10
3.	100 mg ASB/kg bd wt.	2.90 ± 1.17	6.40 ± 0.20	6.10 ± 0.23
4.	28 mg DNPH/kg bd wt.	2.10^a ± 0.05	12.50^b ± 0.11	14.45^a ± 1.20
5.	100 mg AN + 28 mg DNPH/kg bd wt.	2.75 ± 0.01	7.90^a ± 0.57	6.01 ± 0.55
6.	100 mg ASB +28 mg DNPH/kg bd wt.	2.74 ± 0.72	7.10 ± 0.50	10.10^b ± 1.50

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 effects of DNPH, HS anthocyanins and ascorbate on the specific activities of catalase in the serum, liver and brain of rabbits is presented in Table 2. Administration of DNPH (Group 4) caused

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significant ($p<0.05$) depletion of serum catalase activity but caused a significant increase in the level of the enzyme in both the brain and the liver when compared with control (Group 1).Relative to control, treatment with anthocyanin extract alone (Group 2) caused a significant rise in the liver level of the enzyme. Rabbits that received anthocyanin alone (Group 5) and those that received ascorbate alone (Group 6) before DNPH administration showed a significant increase in serum level of catalase and a significant decrease in its levels in the liver and brain when compared with those exposed to DNPH only (Group 4).

The effects of DNPH, HS anthocyanins and ascorbate on the specific activities of SOD in the serum, liver and brain of rabbits is presented in Table 3. DNPH treatment significantly ($p<0.05$) reduced the activity of SOD in the serum but caused a significant increase in its activity in liver and brain relative to control (Group1). Groups treated with anthocyanin and ascorbate separately before DNPH administration (Groups 5 and 6) did not show any significant difference in the activities of the enzyme in the tissues when compared with control.

Table 3: Effects of DNPH, HS anthocyanins and ASB on the specific activities of SOD in the serum, liver and brain.

Rabbit group	Treatment	Specific activity of SOD (nmole/mg protein)		
		Serum	Liver	Brain
1.	2.5 ml H_2O/kg bd wt (control)*	2.20 ± 0.00	5.50 ± 0.31	1.92 ± 0.50
2.	100 mg AN/kg bd wt.	2.29 ± 0.13	6.00 ± 0.51	1.90 ± 0.25
3.	100 mg ASB/kg bd wt.	2.25 ± 0.50	6.39 ± 0.61	1.95 ± 0.35
4.	28 mg DNPH/kg bd wt.	2.18^a ± 0.08	6.98^a ± 0.50	2.45^a ± 0.26
5.	100 mg AN + 28 mg DNPH/kg bd wt.	2.26 ± 0.06	5.86 ± 0.67	1.95 ± 0.61
6.	100 mg ASB +28 mg DNPH/kg bd wt.	2.22± 0.02	6.60 ± 0.37	2.25 ± 0.27

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.

DISCUSSION

Recently, attention has been focused on the protective role of naturally occurring antioxidants in biological systems, and on the mechanisms of action. Phenolic compounds, which are widely distributed in plants, are currently believed to be antioxidants capable of preventing oxidative damage in living systems (Wang *et al*, 2000; Stanner *et al*, 2004). Anthocyanins are phenolic compounds, and their antioxidant roles, as compared to ascorbate were investigated in this study.

Glucose-6-phosphate dehydrogenase

Tissue toxicity was induced by administration of DNPH and the toxicity was established by the significantly increased serum specific activity of glucose-6-phosphate dehydrogenase[6,18]. This property of DNPH has been well characterized and it stems from its cellular disruption and oxidative damage resulting in hemolysis. The integrity of erythrocyte membrane is maintained by reduced glutathione whose level in turn depends on the cellular level of NADPH, a metabolic product of the reaction of glucose-6-phosphate dehydrogenase in pentose phosphate pathway. Therefore the observed significant increase in the specific activity of glucose-6-phosphate dehydrogenase under the condition of DNPH administration was a toxic response, which was necessary for the maintenance of erythrocyte membrane integrity and prevention of oxidative damage [18].

Treatment with each of ascorbate and anthocyanin isolate did not show any significant effect on the level of G6PD in liver and serum, but prophylactic administration of each of them before DNPH treatment offered significant protection as evidenced by the significant reduction in the serum levels of specific activity of glucose-6-phosphate dehydrogenase of rabbits pretreated with each of the extract and ascorbate prior to DNPH treatment, when compared with group administered with DNPH alone [18,22]. This indicated that the antioxidant preparations exhibit prophylactic-type of protection against chemical damage in the blood cells. Also there was no significant difference between the two treatment groups (Groups 5 and 6) which indicate that the effectiveness of both anthocyanins and ascorbate in counteracting the DNPH-induced changes in the activity of G6PD is comparable. The liver is a rich but not absolute source of glucose-6-phosphate dehydrogenase, but its activity in the liver homogenate can provide an insight into

likely basal free radical generation during course of normal cellular metabolism in the liver and the response of the cells to the generated free radicals. Also since some rabbits were treated with DNPH, there is a need to understand how liver cells manage the oxidative damage and its rippling effects.

The recorded increased specific activity of glucose-6-phosphate dehydrogenase in the liver, as a result of anthocyanins and ascorbate administration as compared to the water control may point to the ability of the preparations to individually induce the expression and the specific activity of the enzyme in the liver cells [17,19].

Administration of DNPH resulted in decreased activity of glucose-6-phosphate dehydrogenase in liver homogenate. This property of DNPH has been well characterized and it stems from its cellular disruption and oxidative damage [8,15,16]. These factors result in cell necrosis and subsequent loss of intracellular glucose-6-phosphate dehydrogenase and the pH changes as the enzyme leaks out of the hepatocytes into the blood, thus, accounting for the reduced specific activity of glucose-6-phosphate dehydrogenase under condition of DNPH administration in the liver. This latter phenomenon could also be responsible for the elevated level of the enzyme observed in the serum of animals treated with DNPH [12,16]. Prophylactic administration of each of the antioxidant preparations and subsequent treatment of rabbits with DNPH revealed similar pattern for ascorbate and anthocyanin isolate indicating the higher ability of anthocyanin to scavenge free radicals produced by DNPH administration and reduce the expression of the genes for glucose-6-phosphate dehydrogenase compared with ascorbate [4].

Catalase

DNPH administration resulted in significant ($P<0.05$) increase in the specific activity of catalase in liver and brain compared to the water control. This can be accounted for by the cellular need to detoxify the increased hydrogen peroxide produced during DNPH toxicity. 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 of cellular defense against oxidative injuries decomposing O_2 and H_2O_2 before they interact to form more reactive radicals [2,8,14,16]. The increase in the activity of catalase in the DNPH-treated models is necessary for effective protection. The significant reduction in serum activity of catalase is a result of hemotoxic action of DNPH which caused the depression of the enzyme production at gene level and consequent depletion of the enzyme level in the serum.

Prior administration of anthocyanin extract and vitamin C, followed by treatment with DNPH resulted in significantly decreased specific activity of catalase in liver and brain compared with those treated with DNPH alone while the activity of the enzyme in the serum was significantly increased relative to DNPH- treated rabbits, and maintained at control levels. Again the results obtained for the brain under this treatment, showed that anthocyanins may be more effective in counteracting the oxidant effect of DNPH than ascorbate as shown by the significant difference in the catalase activity of the liver and brain of Groups 5 and 6.

It is also noteworthy that comparing the activities of catalase in the three tissues, the serum has the lowest. This low activity could possibly be due to other available agents for detoxifying hydrogen peroxide, these include the presence of dietary antioxidants and bilirubin which is a product of heme degradation [8] aptly present in the blood cells and circulation.

Superoxide dismutase (SOD)

Like glucose-6-phosphate dehydrogenase, superoxide dismutase is also a widely distributed enzyme in tissues and its role as one of the early enzymes catalyzing detoxification of superoxide has been well documented [3]. It does not only represent the first major enzyme of superoxide metabolizing enzyme, its deficiency has been linked to life-threatening pathologic conditions [6]. Its determination in this research was significant because it represents the chief enzyme that prevent peroxidation initiated when superoxide radicals attack the unsaturated fatty acyl groups of phospholipids; a reaction which heralds other downstream reactions like hemolysis in red blood cells and necrosis of tissues [22].

The significant reduction in the specific activity of SOD in the serum of rabbits treated with DNPH alone is due to the fact that DNPH induces hemolysis in the red blood cells. Cell lyses is occasioned principally by free radical reactions on membrane lipids which ultimately deplete and thus, lowering serum specific activity of superoxide dismutase [16].

The liver and brain of groups treated with anthocyanin extract (Group 2) and ascorbate (Group 3) showed no significant difference in the specific activity of superoxide dismutase when compared with each other and the water control. However, Group 2 showed a higher specific activity of SOD than Group 3, even though the difference is insignificant. On the other hand, treatment with DNPH resulted in significantly increased specific activity of superoxide dismutase compared to the water control ($P<0.05$). The accumulation of DNPH in the liver must have significantly increased the rate of superoxide generation possibly higher than what the basal enzyme activity could cope with. Therefore, to salvage the cells from the peroxidative action of superoxide and tissue necrosis, an SOS response was induced to increase the cellular expression of superoxide dismutase thus, accounting for the significant increase in the superoxide dismutase activity of liver and brain under DNPH administration. Prophylactic administration of the anthocyanin extract and vitamin C, followed by treatment with DNPH resulted in significantly decreased specific activity of superoxide dismutase compared with those treated with DNPH alone. ANOVA however showed no significant difference between the two groups. The results, therefore, show that the anthocyanin extract and ascorbate showed comparable potencies probably due to their similar polarities.

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