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Antioxidant Properties of *Hibiscus sabdariffa* calyces anthocyanins against 2, 4-dinitrophenylhydrazine (DNPH)-induced damage in *Rattus norvegicus* (Albino Rats)

Bada, S. O.[1] and Dada, I.B.O.[2]
[1,2] *Biochemistry and Toxicology unit, Department of Science Laboratory Technology, Rufus Giwa Polytechnic, P.M.B 1019, Owo, Ondo State, Nigeria.*

ABSTRACT

Hibiscus sabdariffa anthocyanins antioxidant roles against 2, 4-dinitrophenyIhydrazine (DNPH) induced changes on the levels of antioxidant enzymes of rats were investigated in this study. The organs examined were the blood and liver. Exposure of rats 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 while the level of reduced glutathione (GSH) was significantly reduced following DNPH treatment when compared to control. However, pre-treatment with (100 mg/kg body weight) H. sabdariffa calyx anthocyanins provided varying degrees of protection against DNPH-induced oxidative damages. Relative to the controls, the extract treatments significantly (P<0.05) decreased the activities of the antioxidant enzymes. From the results obtained, the extract appeared to have offered effective protection against DNPH-induced oxidative damage. So, H. sabdariffa calyx possesses potent antioxidant values which are probable to be anthocyanins.

***Keywords:* Hibiscus sabdariffa, anthocyanin-rich extract, antioxidant enzymes, 2, 4-dinitrophenyIhydrazine**

1. 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 [1]. 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. The dried calyces contain the flavonoids-gossypetin, sabdaretine,hibiscetine and anthocyanins [2]. It is thought that in humans absorbed flavonoids and their metabolities may display an *in vivo* antioxidant activity. 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 [3]; [4]. Anthocyanins are phenolic compounds, and their antioxidant roles were investigated in this study. The distribution of these anthocyanin types in *H. sabdariffa* agrees with earlier reports of [5] who found that cyanidin, delphinidin, and pelargonidin are the most common anthocyanins in nature with cyanidin glycosides reportedly present in nearly 90% of all fruits [6]. 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 [7]; [8]; [3].

It is generally assumed that the active dietary constituents contributing to these protective effects are antioxidant nutrients such as - tocopherol and - carotene. However, recent investigations have revealed that polyphenolic components of plants do exhibit antioxidant properties and do contribute to the anticarcinogenic or cardioprotective actions brought about by diet [3]; [4]. In particular some beverages such as red wine and tea have been shown to elicit antioxidant properties in both *in vitro* and *m vivo* systems [9]. Antioxidant vitamins such as vitamins C and E along with flavonoids have been shown to be effective in reducing atherosclerosis [10].

Phenylhydrazine and its derivatives 2, 4-dinitrophenylhydrazme 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 [11] while 2,4-dmitrophenylhydrazme has been shown to be capable of inducing lipid peroxidation and other oxidative damage in rabbits [12]; [13]; [14] and rats [15]. The ability of 2,4-DNP 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.

The present investigation is therefore aimed at evaluating the effect of *Hibiscus sabdariffa* calyces anthocyacins on 2, 4-dinitrophenylhydrazine-induced changes in the activity of antioxidant enzymes in rats.

2. MATERIALS AND METHODS

2.1 Plant material

Fresh calyces of *H. sabdarijfa* were harvested from Amurin village in Owo, Ondo State, Nigeria and air dried under the shade. Identification and taxonomical classifications were done at herbarium of the Enviromental Biology unit of Science Laboratory Technology Department, Rufus Giwa Polytechnic, Owo, Nigeria.

2.2 Animals

The animals were protected from parasite infestation by proper veterinary management throughout the duration of the treatment. Twenty (20) Albino rats (*Rattus norvegicus*) weighed (200-300 g) used for this research work were obtained from federal medical centre (FMC), Owo, Nigeria. The animals were housed in cages, fed with animal mash and water *ad libitum.*

2.3 Preparation of anthocyanin-rich extract from plant material

Anthocyanin extract from *Hibiscus sabdariffa* calyces was prepared according to the method described by [16]. Briefly, one kilogram of *Hibiscus sabdariffa* calyces was pulverized using laboratory blender. Extraction was performed with 10 litres of 0.1% trifluoroacetic acid (TFA) aqueous solution for 12 hours at 20 ^{0}C on an orbital shaker. The extract was filtered through filter paper (Advantech number 5C). A portion of the filtrate (10 ml) was applied to Sepabeads SP-207 resin column (Mitsubishi Chemicals, Japan). The resin was washed with 3 litres of water and then eluted with 50% ethanol solution containing 0.1 %TFA. The eluate was dried under vacuum at 20 ^{0}C and then freeze-dried. The dried sample obtained was re-suspended in distilled water and kept in the refrigerator until required for oral administration and biochemical investigation.

2.4 Experimental design

Twenty (20) male Albino rats weighing 200-300 g were used for this research work. They were randomly selected into four (4) experimental groups as shown below. The experiment lasted for 28 days.

Group 1: Water treated control. Each rat was given distilled water, 2.0 ml/kg body weight

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

Group 3: 2. 4-Dinitrophenylhydrazine was administered at a dose of 28 mg/kg body weight intraperitoneally to each rat in this group 6 hours prior to sacrifice.

Group 4: Anthocyanin-rich extract H. *sabdariffa* was administered at a dose of 100 mg/kg body weight for 28 days to each rat in this group followed with 28 mg/kg body weight of 2, 4-dinitrophenylhydrazine administered intraperitoneally 6 hours prior to sacrifice.

2.5 Biochemical determinations

The activity of glucose-6-phosphate dehydrogenise was determined using assay kit obtained from Randox Laboratories, UK. The method is as described in the manufacturer's leaflet procedure.

Catalyse activity was determined using Aebi's method [17]. An aliquot (10µl) of each tissue supernatant was added to cuvette containing 1.99 µl of 50mM phosphate buffer (pH 7.0). reaction was started by addition of 1000 µl of freshly prepared 30mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometric at 240nm. Activity of catalyse was expressed as U/mg of protein.

The superoxide dismutase activity was measured according to method of [18]. Briefly, assay mixture contained 0.1 mL of supernatant, 1.2 mL of sodium pyrophosphate buffer (pH 8.3; 0.052 M), 0.1 mL of phenazine methosulphate (186 µM), 0.3 mL of nitrobluetetrazolium (300 µM) and 0.2 mL of NADH (750 µM). NADH was added to the mixture and after incubation at 30ºC for 90 s, the reaction was stopped by addition of 0.1 mL of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 mL of n-butanol. Colour intensity of the chromogen in the butanol was measured spectrophotometrically at 560 nm. One unit of enzyme activity was defined as that amount of enzyme which caused 50% inhibition of NBT reduction/mg protein.

GSH in tissue homogenate was determined by the method described by [17] with slight modifications. An aliquot of 0.5 mL of each tissue homogenate was precipitated with 0.5 mL of trichloroacetic acid (10% w/v). The precipitate was removed by centrifugation. 0.8 mL of the filtered sample was mixed with 0.3 DTNB (4 mg/mL) and 0.9mL phosphate buffer (0.1 M, pH 7.4). The yellow colour developed was read at 412 nm. Reduced glutathione was expressed as µg/mg of protein.

2.6 Data analysis

The results were analysed by one-way ANOVA, using SPSS Microsoft Excel package version 22.0. All data is expressed as Mean ± SE (Mean of 5 determinations). The significance level was set at P<0.05

3. RESULTS AND DISCUSSION

The effects of DNPH and *H.sabdariffa* anthocyanins on the activities of G6PD in the serum of rats are presented in Table 1. Administration of DNPH (Group 3) significantly (p<0 05) increased the level of G6PD in the serum. Rats that received the anthocyanin-rich extract before DNPH administration (Group 4) did not show altered level of the enzyme in serum when compared with control but were significantly reduced when compared with DNPH treated group only.

Table 1: Effect of DNPH and HS anthocyanins on the activities of G6PD in the serum during DNPH induced oxidative stress in rats

Groups	Treatments	Glucose -6-phosphate dehydrogenase activity in serum (µmol/min/mg protein)
1	2.5 ml H_20/kg- bd wt. (control)	1.27±0.22
2	100mg AN/kg bd wt.	1.10±0.13
3	28 mg DNPH/kg bd wt.	4.50 ±0.98
4	100 mg AN + 28 mg DNPH/kg bd wt.	1.21±0.01

Results are presented as means ±SEM of five (5) determinations.
DNPH: 2, 4-dinilrophenythydrazine, AN: anthocyanin

It has been well-known that tissue toxicity can be induced by administration of DNPH and the toxicity was documented to have significantly increased serum specific activity of glucose-6-phcsphate dehydrogenase [19] and [20]. This property of DNPH stems from its cellular disruption and oxidative damage resulting in haemolysis. 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 [19]; [21]. Treatment with anthocyanins did not show any significant effect on the level of G6PD in serum but prophylactic administration of anthocyanins 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 rats pretreated with the extract prior to DNPH administration, when compared with group administered with DNPH alone [22]; [19]. This indicated that the extract exhibit protection against chemical damage in the blood cells. [23] and [13]; [14] reported that administration of DNPH resulted in cell necrosis and subsequent loss of intracellular glucose-6-phosphate dehydrogenase 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 observable fact could also be responsible for the elevated level of the enzyme observed in the serum of animals treated with DNPH.

The effect of DNP and HS anthocyanins on the specific activities of catalase in the liver of rats is presented in Table 2. Administration of DNPH (Group 3) caused a significant (p<0.05) increase in the level of the enzyme in 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.

Table 2: Effect of *H. Sabdariffa* anthocyanins on the liver catalase during DNPH induced oxidative stress in rats

Groups	Treatments	Specific activity of catalase (n mole H_2O_2 decomposed/min/mg protein)
1	2.5 ml H_20/kg- bd wt. (control)	3.33±0.37
2	100mg AN/kg bd wt.	7.02±0.45
3	28 mg DNPH/kg bd wt.	18.62±0.36
4	100 mg AN + 28 mg DNPH/kg bd wt.	7.19±0.87

Results are presented as means ±SEM of five (5) determinations.
DNPH: 2, 4-dinilrophenythydrazine, AN: anthocyanin

According to [24] cells are always under the threat of oxidative injury caused by hydrogen peroxide. Hydrogen peroxide can be produced in the cell by wide varieties of metabolic pathways by both endogenous and exogenous substrates [24] and [25]. The cells are shielded from the oxidative effect of hydrogen peroxide by the enzymatic activity of catalase and glutathione peroxidase. DNPH administration resulted in significant (P<0. 05) increase in the specific activity of catalase in liver 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 0_2 and H_20_2 before

they interact to form more reactive radicals [26]; [27]; [13]; [14]. The increase in the activity of catalase in the DNPH-treated models may be necessary for effective protection. The results above also revealed that rats treated with anthocyanin-rich extract showed significant increased levels of catalase in the liver. This observation is likely due to the ability of anthocyanins to induce expression of catalase in the liver as earlier explained.

The effects of DNPH and *H. Sabdariffa* anthocyanins on the specific activities of Suproxide dismutase (SOD) in the serum of rats is presented in **Table 3.** DNPH treatment significantly ($p<0.05$) reduced the activity of SOD in the serum. Superoxide dismutase is a widely distributed enzyme in tissues and its role as one of the early enzymes catalyzing detoxification of superoxide has been established [24]; [25]. It represents one of the first major superoxide metabolizing enzymes and its deficiency has been linked to serious pathologic conditions [21] and [20]. The significant reduction in the specific activity of SOD in the serum of rats treated with DNPH alone could be linked to the fact that DNPH induces haemolysis in the red blood cells and exhaustion of the enzyme as a result of oxidative stress caused by DNPH. Cell lyses is occasioned principally by free radical reactions on membrane lipids which ultimately deplete and thus, lowering serum specific activity of superoxide dismutase [19]; [21]; [14].

Table 3: Effect of DNPH and *H. Sabdariffa* anthocyanins on the specific activities of SOD in the serum during DNPH induced oxidative stress in rats

Groups	Treatments	Specific activity of SOD (nmole/mg protein)
1	2.5 ml H_20/kg- bd wt. (control)	0.18±0.14
2	100mg AN/kg bd wt.	0.26±0.10
3	28 mg DNPH/kg bd wt.	0.12±0.01
4	100 mg AN + 28 mg DNPH/kg bd wt.	0.23±0.08

Results are presented as means ±SEM of five (5) determinations.
DNPH: 2, 4-dinilrophenythydrazine, AN: anrhocyanin

However, Group 2 showed a higher specific activity of SOD than Group 3, even though the difference is insignificant. Prophylactic administration of the anthocyanin extract brought SOD activities to near normal in rats pretreated with the extract prior to DNPH-treatment.

The effects of DNPH and HS anthocyanins on the level of GSH in the liver of rats are presented in **Table 4.** The group that received DNPH alone (Group 3) showed a significant ($p < 0.05$) decrease in the level of GSH in rats when compare with control.

Table 4: Effect of DNPH and *H.Sabdariffa* anthocyanins on the hepatic levels of reduced glutathione (GSH) during DNPH induced oxidative stress in rats

Groups	Treatments	GSH (µmol/mg protein)
1	2.5 ml H_20/kg- bd wt. (control)	24.84±1.33
2	100mg AN/kg bd wt.	26.27±0.59
3	28 mg DNPH/kg bd wt.	16.39±0.92
4	100 mg AN + 28 mg DNPH/kg bd wt.	23.55±0.72

Results are presented as means ±SEM of five (5) determinations.
DNPH: 2, 4-dinilrophenythydrazine, AN: anrhocyanin

GSH is an intra-cellular reductant and plays major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds [28]. Deficiency of GSH in the lens leads to cataract formation. Glutathione also plays an important role in the kidney and takes part in a transport system involved in the reabsorption of amino acids. In this study there was a decrease in liver reduced glutathione (GSH) level in rats treated with DNPH alone, but a dramatic rise in the level of liver GSH was observed in rats pre-treated with the extract. The stabilization of this enzyme by the *H.Sabdariffa* anthocyanins is an indication of the improvement of the functional status of the liver. This might be related to the chemopreventive effects of *H.Sabdariffa* anthocyanins.

4. CONCLUSION

It is cleared from this study that, the plant part indicated contain positive antioxidant potentials as revealed in various parameters studied in this work. Consequently, consumption of *H.Sabdariffa* decoction known as “zobo” in Nigeria may be encouraged on the account of its natural antioxidant bio-constituents, and also justify the use of *H.Sabdariffa* anthocyanins in the prevention of chemically induced toxicity.

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