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Full Length Research Paper

Effect of *Hibiscus sabdariffa* anthocyanins on 2, 4-dinitrophenylhydrazine-induced tissue damage 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 cytotoxic effects in rabbits. Twenty male adult rabbits used for the study were divided into four groups. Group 1, the control took only water while animals in groups 2 and 4 received 100 mg/kg body weight of the anthocyanin extract of *H. sabdariffa* once daily for 28 days. After the 22nd day of treatment, the rabbits in groups 3 and 4 received 28 mg/kg body weight of DNPH for the remaining 5 days of treatment, after which the animals were sacrificed. Relative to control, DNPH caused significant ($p < 0.05$) increase in the formation of malondialdehyde (MDA) in serum, liver and brain and decreased the levels of reduced glutathione (GSH) in liver and brain. Also, DNPH caused a significant ($p < 0.05$) elevation in the activity of glucose-6-phosphate dehydrogenase (G6PD) in the serum and liver. However, pretreatment with *H. sabdariffa* anthocyanin extract significantly ($p < 0.05$) reduced MDA formation, increased the levels of GSH and maintained at normalcy the activity of G6PD in the tissues, thereby effectively ameliorated the toxic effects of DNPH. These findings indicate that anthocyanin extract from dried calyces of *H. sabdariffa* protects the rabbit against 2, 4 -DNPH lipoperoxidative and cytotoxic effects.

Key words: Anthocyanin extract, 2, 4-dinitrophenylhydrazine, glucose-6-phosphate dehydrogenase, *Hibiscus sabdariffa*, rabbit, reduced glutathione, malondialdehyde.

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 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 (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 antioxidant nutrients such as α - tocopherol and β - 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 (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 in both *in vitro* and *in vivo* systems (Kanner et al., 1994). Among the more than 300 species of *Hibiscus* plant is *Hibiscus sabdariffa L.*, which has many medicinal uses (Morton, 1987; Gill, 1992). The dried calyces contain the flavonoids - gossypetin, sabdaretin, hibiscetin 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

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diseases (Jackson et al., 1993; Gaxlane et al., 1994; Amin and Buratovich, 2007). There are indications that the extract from the red calyces of *H. sabdariffa* possess antioxidant principles (Tseng et al., 1997; Wang et al., 2000; Ologundudu and Obi, 2005; Ologundudu et al., 2006a, b; Ologundudu et al., 2009a, b). This research was therefore carried out to evaluate the protective effect of *H. sabdariffa* anthocyanins using the model of 2, 4 - dinitrophenylhydrazine-induced oxidative stress in rabbits.

MATERIALS AND METHODS

Experimental animals and materials

Male rabbits (weight range 800 - 1000g and four months old) used for this study were purchased from a local breeder in Benin City, Nigeria. 2, 4 -Dinitrophenylhydrazine, trichloroacetic acid, sodium chloride and diethyl ether were purchased from BDH Chemical Company (Poole, England), 2,-thiobarbituric acid from Koch-Light Laboratories (England). Hydrochloric acid and absolute ethanol were obtained from WN Laboratories (US) and glucose- 6 - phosphate dehydrogenase kit was obtained from Randox Laboratories, UK. 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 at 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

Experimental rabbits were divided into four 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. Rabbits in group 1 were given a daily dose 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 2 and 4. After the 22nd day of the experiment, rabbits in groups 3 and 4 received a dose of 28 mg/kg body weight of 2, 4 -dinitrophenylhydrazine intraperitoneally, for 5 consecutive days.

By the end of the experimental period, the animals were anaesthetized in a diethyl ether saturated chamber. While under anesthesia the abdominal regions were opened exposing the heart and liver. Blood was obtained by cardiac puncture by means of a 5 ml hypodermic syringe and needle and placed in heparinized bottles, centrifuged at 3500 rpm for 10 min (SM 9026B bench centrifuge, Surgifriend Medicals, England). From each rabbit, liver and brain samples were also obtained and 1 g portion of each homogenized in ice-cold saline (1:4, ^{w}/v) and centrifuged at 3500 rpm for 10 min to obtain a clear supernatant.

Biochemical assay protocol

Lipid peroxidation was determined spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method as described in Varshney and Kale (1990). Results were expressed in terms of malondialdehyde (MDA) formed per mg protein. Reduced glutathione concentration in the blood was determined using the method of Jollow et al. (1974). The total activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was determined using assay kit from Randox.

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 $P < 0.05$.

RESULTS

Figure 1 shows the profile displayed on a multiwave length 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 main ones were delphinidin-3-monoglucoside, cyanidin-3-monoglucoside and petunidin-3-monoglucoside.

As shown in Table 1, the results obtained in this study indicated that intoxication with a single dose of 28 mg/kg DNPH (Group 3) daily for 5 consecutive days resulted in a significant increase ($p < 0.05$) in plasma, liver and brain malondialdehyde (MDA) concentrations compared to control. However, pretreatment with 100 mg/kg body weight of anthocyanin extract of *H. sabdariffa* for 22 days before DNPH intoxication (Group 4) significantly ($p<0.05$)

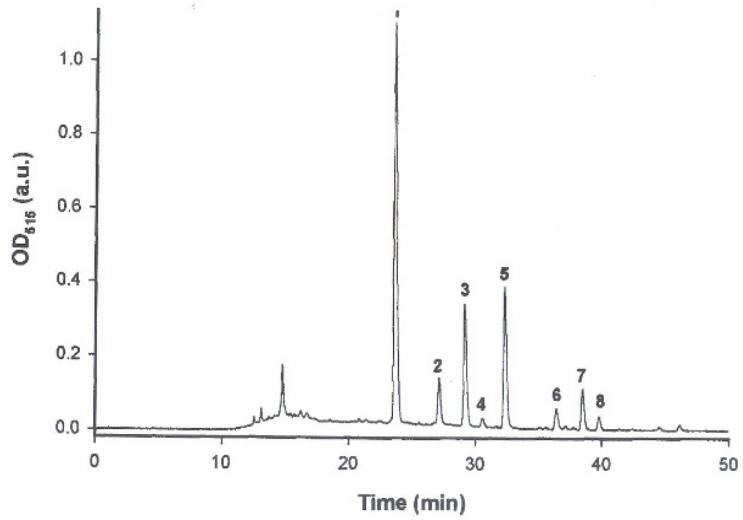

Figure 1. HPLC chromatogram of *Hibiscus sabdariffa* anthocyanins.

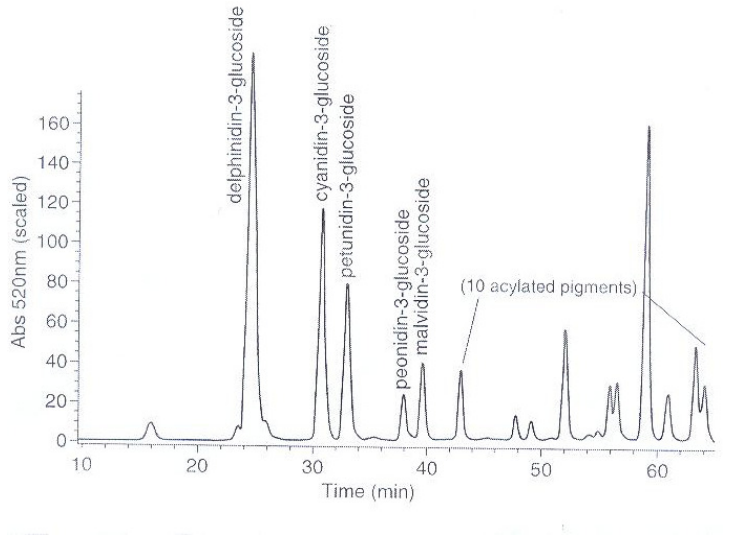

Figure 2. HPLC chromatogram of standard anthocyanins.

decreased by 22.5, 9.2 and 27.2% plasma, liver and brain respectively the MDA concentration compared to control. DNPH administration without pretreatment with anthocyanins extract produced a 51.6, 17.5 and 31.5% significant rise in plasma, liver and brain respectively in MDA concentration compared to control while a non-significant increase was observed in plasma, liver and brain MDA level in group 4 (AN+DNPH) compared to control.

Table 2 shows that DNPH intoxication significantly ($p < 0.05$) reduced the liver and brain levels of reduced glutathione (GSH) by 34.0 and 28.6% respectively compared to control while treatment with *H. sabdariffa* anthocyanins extract for 28 days without DNPH intoxication (group 2) increased significantly ($p < 0.05$) the GSH levels (by 8.6%) in the liver compared to control. Pretreatment with *H. sabdariffa* anthocyanins prior to DNPH intoxication (group 4) caused a significant restoration (almost to control level) of the DNPH depleted GSH concentration in both liver and brain.

Table 3, shows that the activity of glucose-6-phosphate dehydrogenase (G6PD) was significantly ($p < 0.05$) elevated in the serum following DNPH intoxication as observed when group 3 is compared to control. However, treatment with *H.* anthocyanins alone (Group 2) had no significant ($p > 0.05$) effect on G6PD activity in the serum and liver compared to control. Pretreatment with the extract prior to DNPH administration (Group 4) maintained

Table 1. Effect of 2, 4 -dinitrophenylhydrazine and *Hibiscus* anthocyanins on the tissue levels of malondialdehyde (µmol per mg protein) of rabbits.

Rabbit group	Treatment	Serum	Liver	Brain
1	2.5 ml H_2O/kg bd. wt. (control)	1.38 ± 0.02	2.71 ± 0.19	3.24 ± 0.13
2	100 mg AN/kg bd. wt.	1.07 ± 0.9[a]	2.46 ± 0.20	2.36 ± 0.35[a]
3	28 mg DNPH/kg bd. wt.	8.50 ± 0.64[b]	8.45 ± 0.78[a]	13.43 ± 0.48[b]
4	100 mg AN/kg bd. wt. + 28 mg DNPH/kg bd. wt	1.44 ± 0.19	3.70 ± 0.12	3.33 ± 0.38

Results are means of 5 determinations ± SEM. Statistical comparison is strictly within the same tissue. Values carrying notations are statistically ($p < 0.05$) significantly different from control (group 1) while values carrying different superscripts are statistically significantly different from another.

Table 2. Effect of 2, 4-dinitrophenylhydrazine and *Hibiscus* anthocyanins on the tissue levels of reduced glutathione (nmol per g protein) of rabbits.

Rabbit group	Treatment	Liver	Brain
1	2.5 ml H_2O/kg bd. wt. (control)	24.84 ± 1.33	24.48 ± 0.21
2	100 mg AN/kg bd. wt.	26.39 ± 0.25[a]	24.71 ± 0.69
3	28 mg DNPH/kg bd. wt.	16.39 ± 0.92[b]	17.48 ± 0.19[a]
4	100 mg AN/kg bd. wt. + 28 mg DNPH/kg bd. wt	23.53 ± 0.26	24.10 ± 0.38

Results are means of 5 determinations ± SEM. Statistical comparison is strictly within the same tissue. Values carrying notations are statistically ($p < 0.05$) significantly different from control (group 1) while values carrying different superscripts are statistically significantly different from another.

Table 3. Effect of 2, 4 -dinitrophenylhydrazine and *Hibiscus* anthocyanins on the tissue levels of glucose- 6 -phosphate dehydrogenase (µmol per min. per mg protein) of rabbits.

Rabbit group	Treatment	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	28 mg DNPH/kg bd. wt.	4.50 ± 0.98[a]	34.07 ± 0.06[a]
4	100 mg AN/kg bd. wt. + 28 mg DNPH/kg bd. wt.	1.21 ± 0.00	33.40 ±0.23

Results are means of 5 determinations ± SEM. Statistical comparison is strictly within the same tissue. Values carrying notations are statistically ($p < 0.05$) significantly different from control (group 1) while values carrying different superscripts are statistically significantly different from another.

at normalcy the levels of the enzyme in the liver and serum.

DISCUSSION

An explosion of interest in examining the involvement of free radicals in carcinogenesis has led to the use of dietary antioxidant treatments in quenching free radical-mediated attacks, hence promoting general human health. The antioxidants may act as free radical scavengers, reducing agents, chelating agents for transition metals, quenchers of singlet oxygen molecules and activators of antioxidative defense enzyme systemsto suppress free radical damage in biological systems (Satue-Gracia et al., 1997; Aviram and Fuhrman, 2002).

In this study, malondialdehyde (MDA), reduced glutathione (GSH) and glucose-6 -phosphate dehydrogenase (G6PD) activity were used to indicate the degree of tissue damage following 2, 4 -dinitrophenylhydrazine (DNPH) intoxication and the levels of protection against such damage offered by pretreatment with *H. sabdariffa* anthocyanins prior to DNPH intoxication.

MDA concentration in serum, liver and brain tissue homogenates in our investigations significantly increased after 5 consecutive days of DNPH administration. Increase in 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., 2009a, b). Therefore,

the profile of MDA in the tissues of DNPH-treated rabbits is a clear indication that DNPH provokes oxidative stress in rabbits. *In vivo*, this toxicant is believed to undergo auto oxidation 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 leakage of cellular content from the damaged organ into the blood stream. However, this phenomenon was effectively blocked when the animals were pretreated with *H. anthocyanin* extract before DNPH intoxication.

In accord with our earlier reports (Ologundudu et al., 2009a,b), the results presented in this study (Table 2) show that DNPH administration caused a statistically ($p < 0.05$) significant decrease in reduced glutathione (GSH) concentration in the liver and brain. It is well established that 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. So the decrease in the levels of GSH in these tissues results in the accumulation of free radicals leading to increased rate of lipid peroxidation in these tissues. This study however, showed that pretreatment with anthocyanin extract of *H. sabdariffa* prior to DNPH intoxication significantly increased the concentration of reduced glutathione in the liver and brain, thus reducing the accumulation of free radicals and decreased rate of lipid peroxidation.

Table 3 shows that DNPH administration significantly ($p < 0.05$) increased both serum and liver activity of G6PD relative to control (Group 1) and anthocyanin extract-treated DNPH-free rabbits (Group 2). This change in G6PD activity is a toxic response to DNPH intoxication. Pretreatment with *H.* anthocyanins however effectively ameliorated this change in both serum and liver (group 4). G6PD is a widely distributed enzyme in tissues and its role as the anchor of reductive metabolism is well established. It does not only represent the major regulatory enzyme of the pentose phosphate pathway but also its metabolic products: NADPH and phosphogluconolactone are involved in reductive anabolism and nucleotide synthesis respectively. The deficiency of this enzyme has been implicated in favism and hemolytic anemia. Its determination in this research was necessitated by the fact that it represents the chief enzyme that provides the reduced glutathione with hydride ion (H^-) in form of NADPH for sustenance of free radical detoxification (Bergmeyer et al., 1974).

The mechanism by which *H. sabdariffa* 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 calyces 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 tissues.

Conclusion

Antioxidant activity of *H.* anthocyanins seems to play a critical role against the 2, 4 -dinitrophenylhydrazine-induced tissue damage in rabbits.

REFERENCES

Ames BN (1983). Dietary carcinogens and anticarcinogen: Oxygen radicals and degenerative diseases. Science 221: 1256-1264.

Amin A, Buratovich M (2007). The Anti-cancer Charm of Flavonoids: A cup-of-tea will do! Recent Pat. Anti-Cancer Drug Discov. 2(2): 109-117.

Aviram M, Fuhrman B (2002). Wine flavonoids protect against LDL oxidation and atherosclerosis. Ann. NY. Acad. Sci. 957: 146-161.

Bergmeyer HU, Bernt E, Schmidt F, Stork H (1974). D-Glucose: determination with hexokinase and glucose-6-phosphate dehydrogenase. In: Methods of enzymatic analysis 2nd English ed. Bergmeyer, H. U. (Ed) Harcourt Brace New York, NY. 1974: 1196-1201.

Block G (1992). The data support a role for antioxidants in reducing cancer risk. Nutr. Rev. 50: 207-213.

Clemens MR, Reinmer H, Waller HD (1984). Phenylhydrazine-induced lipid peroxidation of red blood cells *in vitro* and *in vivo*: Monitoring by the production of volatile hydrocarbons. Biochem. Pharmacol. 53(11): 1715-1718.

Gaxlane JM, Mansion JE, Hennekens CH (1994). Natural antioxidants and cardiovascular disease. Observational epidemiologic studies and randomized trials. In: B. Frel (Ed), Natural antioxidants in human health and disease. Academic press, San Diego pp. 387-409.

Gill LS (1992). Ethnomedicinal uses of plants in Nigeria. University of Benin Press, Benin City p. 132.

Hertog MGL, Feskens EJM (1993). Dietary antioxidants flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. Lancet 342: 1007-1011.

Hong V, Wrolstad RE (1990a). Use of HPLC separation/photodiode detection for characterization of anthocyanins. J. Agric. Food Chem. 38: 708-715.

Jackson RL, Ku G, Thomas CE (1993). Antioxidant, a biological defense mechanism for the prevention of atherosclerosis. Med. Res. Rev. 13: 161-182.

Jain S, Hochstein P (1979). Generation of superoxide radicals by hydrazine: Its role in phenylhydrazine-induced hemolytic anemia. Biochem. Biophys. Acta 586: 128-136.

Jollow DJ, Mitchel JR, Zampaghonic A, Gillette JR (1974). Bromobenzene-induced live necrosis; protective role of glutathione and evidence for 3, 4-bromobenzeneoxide as the hepatotoxic metabolite. Pharmacol. 11: 151-169.

Kanner J, Frankel E, Granit R, German B, Kinsella J (1994). Natural antioxidants in grapes and wines. J. Agric. Food Chem. 42: 64-69.

Maduka HCC, Okoye ZSC, Eje A (2003). The influence of *Sacoglottis gabonensis* stem bark extract and its isolate bergenin, Nigerian alcoholic additive, on the metabolic and hematological side effects of 2, 4-dinitrophenylhydrazine-induced tissue damage. Vascular Pharmacol. 39: 317-324.

Morton JF (1987). Rosella. In: Fruits of Warm Climates. Darling, C.F. (Ed). Media Inc. Green-shore, pp. 281-286.

Ologundudu A, Lawal AO, Adesina OG, Obi FO (2006a). Effect of ethanolic extract of Hibiscus sabdariffa L on 2, 4-dinitrophenylhydrazine-induced changes in blood parameters in rabbits. Global J. Pure Appl. Sci. 12(3): 335-338.

Ologundudu A, Lawal AO, Adesina OG, Obi FO (2006b). Effect of ethanolic extract of Hibiscus sabdariffa L on 2, 4-dinitrophenylhydrazine-induced low glucose level and high malondialdehyde levels in rabbit brain and liver. Global J. Pure Appl. Sci. 12(4): 525-529.

Ologundudu A, Obi FO (2005). Prevention of 2, 4-dinitrophenylhydrazine-induced tissue damage in rabbits by orally administered decoction of dried flower of *Hibiscus sabdariffa L* J. Med. Sci. 5(3): 208-211.

Ologundudu A, Ologundudu AO, Ololade IA, Obi, FO (2009a). Effect of *Hibiscus sabdariffa* anthocyanins on 2, 4-dinitrophenylhydrazine-induced hematotoxicity in rabbits. Afr. J. Biochem. Res. 3(4): 140-144.

Ologundudu A, Ologundudu AO, Ololade IA, Obi FO (2009b). The effect of *Hibiscus* anthocyanins on 2, 4-dinitrophenylhydrazine-induced hepatotoxicity in rabbits. Int. J. Phys. Sci. 4(4): 233-237.

Pietta PG (2000). Flavonoids as antioxidants. J. Nat. Prod. 63(7): 1035-1042.

SAS Institute Inc. (1999). SAS/STAT User's Guide. Version 8 for Windows. SAS Institute Inc., SAS Campus Drive, Cary, North Carolina, USA.

Satue-Gracia MT, Heinonen M, Frankel E (1997). Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems. J. Agric. Food Chem. 45: 3362-3367.

Sipes IG, Krishna G, Gillette JR (1977). Bioactivation of carbon tetrachloride, chloroform and bromotrichloromethane: role of cytochrome $P\text{-}_{450}$. Life Sci. 20: 1541-1548.

Stanner SA, Hughes J, Kelly CN, Butriss J (2004). A review of the epidemiological evidence for the antioxidant hypothesis. Pub. Health Nutr. 7(3): 407-422.

Tseng TH, Kao ES, Chu FP, Lin-Wa HW, Wang CJ (1997). Protective effect of dried flower extracts of *Hibiscus sabdariffa L* against oxidative stress in rat primary hepatocytes. Food Chem. Toxicol. 35(12): 1159-1164.

Varshney R, Kale RK (1990). Effect of calmodulin antagonist on radiation induced lipid peroxidation in microsomes. Int. J. Rad. Biol. 58: 733-743.

Vertuani S, Augusti A, Maufredina S (2004). The antioxidants and pro-oxidants network: An overview. Cur. Pharm. Des. 10(14): 1677-1694.

Wang CJ, Wang JM, Lin WL, Chu CY, Chou FP, Tseng TH (2000). Protective effect of *Hibiscus* anthocyanins against tert-butyl hydroperoxide-induced hepatic toxicity in rats. Food Chem. Toxicol. 38: 411-416.