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EFFECT OF *SYNSEPALUM DULCIFICUM* BERRY EXTRACT ON OXIDATIVE STRESS AND HEPATOTOXICITY INDICES, FOLLOWING SUBACUTE ADMINISTRATION IN NORMAL RATS

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ABSTRACT

Toxicological evaluation of *Synsepalum dulcificum* berry extract was carried out by assessing its effect on oxidative stress and hepatotoxicity markers in rats administered 25-200 mg/kg of the extract for 14 days. Total bilirubrin level was decreased in animals administered the extract compared to untreated ones ($P < 0.05$). Extract at 50 mg/kg significantly reduced serum aminotransferase and alkaline phosphatase activities, produced no significant change in aspartate aminotransferase activity but significantly increased catalase activity. Glutathione-S-transferase activity was significantly ($P < 0.05$) increased in the 50-200 mg/kg administered groups. Malondialdehyde levels were reduced in groups administered 25-100 mg/kg of extract but significantly increased in the 200 mg/kg group. Glutathione level and activities of catalase and hepatic glutathione peroxidase were reduced in the 200 mg/kg group ($P < 0.05$). These results suggest that extract at concentrations of 50-100 mg/kg may boost antioxidant defense and demonstrate hepatoprotective property while higher concentrations may be pro-oxidative.

Keywords: antioxidative, hepatoprotective, toxicological evaluation, *Synsepalum dulcificum*

INTRODUCTION

Synsepalum dulcificum (Schumach. and Thonn.) Daniell, a shrub of the Sapotaceae family, is indigenous to tropical West Africa. It is known as "agbayun" among the Yorubas of south-west Nigeria. *Synsepalum dulcificum* berry is popularly known as "miracle fruit" because of its unique taste-modifying property of causing sour edibles to taste sweet after the mouth has been exposed to the fruit's mucilaginous pulp. This taste-modifying property has been known to local people in West Africa for centuries, where the fruit has been used to sweeten sour foods and beverages such as Koko and Kenkey made from fermented maize and millet, and palm wine.

The active principle in the fruit responsible for the taste-modifying effect has been identified as a glycoprotein named miraculin (Kurihara and Beidler, 1968) and has been reported to be useful in controlling obesity when administered orally (Giroux and Henkin, 1974). It has also been reported that oral administration of miracle fruit to fructose-rich chow fed rats, caused the reversal of the raised value of the glucose-insulin index and improved insulin sensitivity (Chen et al., 2006).

There are indications that miracle fruit contains an appreciable amount of polyphenols (Buckmire and Francis, 2006) (Table 1). Phenolic compounds and flavonoids which are widely distributed in plants are known to exert multiple biological effects including antioxidant, free radical scavenging, anti-inflammatory and anticarcinogenic effects (Miller, 1996). Natural antioxidants especially phenolics and flavonoids from tea, wine, fruits, vegetables and spices are already exploited commercially either as antioxidant additives or as nutritional supplements (Schuler, 1990).

Table 1: Characterized compounds from miracle fruit

Chemical Class		Name
Flavonoid		
	Anthocyanin	Cyanidin-3-monogalactoside
		Cyanidin-3-monoglucoside
		Cyanidin-3-monoarabinoside
		Delphinidin-3-monogalactoside
		Delphmidin-3-monoarabinoside
	Flavonol	Quercetin-3-monogalactoside
		Kaempferol-3-monoglucoside
		Myricetin-3-monogatactoside
	Quercetin aglycones	Kaempferol
		Myricetin
	Glycoprotein	Miraculin

(Giroux and Henkin, 1974; Buckmire and Francis, 2006).

Apart from some reports on its taste-modifying property, there is a dearth of data on the pharmacological and toxicological properties of the fruit. In the present study, the toxicological evaluation of *Synsepalum dulcificum* methanolic fruit extract has been carried out by investigating the effect of its subacute administration on some biomarkers of oxidative stress and hepatotoxicity in rats.

MATERIALS AND METHODS

Chemicals

Sodium dodecyl sulphate (SDS), 5, 5′- dinitrobis-2-nitrobenzoic acid (DTNB), sulphosalicyclic acid

(SSA), glutathione (GSH) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals and reagents used in this study were of analytical grade.

Preparation of extract

The fresh fruits were collected from a farm in Isua-Akoko, Ondo State, Nigeria. Authentication was carried out at the Forestry Research Institute of Nigeria, Ibadan, Nigeria and voucher specimen was deposited at their herbarium (voucher number: 108903). The fresh fruits of *S. dulcificum* were weighed, deseeded and homogenized (613 g) in 80% methanol (1000 ml). The mixture containing the fruits' outer covering and mucilaginous pulp was left for 48 h before filtrate collection. The filtrate was concentrated using a rotary evaporator and then lyophilized to get the extract (50.1 g) used for the study. The percentage yield of the extract was 8.2.

Screening and assay for phytochemical components

Phytochemical screening

Phytochemical screening of the extract was carried out as previously described (Sofowora, 1993; Trease and Evans, 1989). The screening involves simple chemical tests to detect the presence of alkaloids, flavonoids, terpenoids, saponins, tannins, anthraquinones, and cardiac glycosides.

Total phenolic content

The total phenolic content of extract was determined using the Folin–Ciocalteu method described by Singleton et al., (1999) as modified by Dewanto et al. (2002). To 125 µl of suitably diluted extract was added 0.5 ml of distilled water and 125 µl of Folin–Ciocalteu reagent. The mixture was allowed to stand for 6 min before the addition of 1.25 ml of 7% Na_2CO_3 solution. The mixture was allowed to stand for 90 min and the absorbance was read at 760 nm. The amount of total phenolics was expressed as mg/g tannic acid equivalent.

Total flavonoid content

The estimation of total flavonoid content was carried out as described by Dewanto et al. (2002). To 0.25 ml of suitably diluted extract, 75 µl of a 5% $NaNO_2$ solution, 0.150 ml of a freshly prepared 10% $AlCl_3$ solution, and 0.5 ml of 1 M NaOH solution were added. The final volume was adjusted to 2.5 ml with distilled water. The mixture was then allowed to stand for 5 min and the absorbance was measured at 510 nm. The amount of total flavonoids was expressed as mg/g quercetin equivalent.

Animal handling and treatment

Male Wistar albino rats weighing 200-220 g were purchased from the Animal Colony of the Physiology Department, University of Ilorin, Nigeria. The animals were kept in standard laboratory conditions under natural light–dark cycle and maintained on normal laboratory chow and water ad libitum. Animals were handled and used in accordance with the international guide for the care and use of laboratory animals (NRC, 1996).

The animals were randomly divided into five groups with five animals in each group. Group I served as the control and received distilled water

throughout the duration of the experiment. Group II-V received oral administration of 25-, 50-, 100- and 200 mg/kg S. dulcificum extract respectively, once daily for 14 consecutive days. Twenty-four hours after the last administration, animals were sacrificed by cervical dislocation. Blood samples were collected and sera were prepared by centrifugation at 3000 g for 15 min and used for the estimation of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities and total bilirubrin level.

Livers were immediately removed, washed in ice–cold 1.15% KCl and 10% tissue homogenates were prepared with 50 mM Tris-HCl buffer, pH 7.4. The homogenates were centrifuged at 10000 g for 15 min to obtain the supernatant fractions which were used for the estimation of total protein content, levels of malondialdehyde (MDA) and GSH as well as the activities of catalase (CAT), glutathione-S-transferase (GST) and glutathione peroxidase (GPx).

Biochemical estimations

Antihepatotoxic indices

Total protein content, serum activities of AST, ALT and ALP and the total bilirubrin level were assayed spectrophotometrically using commercially available assay kits (Randox Laboratories Ltd., UK) following the instructions of the manufacturer.

Antioxidative indices

MDA level

Assay for MDA level, an important biomarker of lipid peroxidation was carried out according to the method of Ohkawa et al. (1979). This is based on the reaction of the chromogenic substrate 2-thiobarbituric acid and malondialdehyde to yield a coloured complex with maximum absorbance at 532 nm. Briefly, the homogenate (300 µl) was taken into test tubes and 300 µl of 8.17% SDS and 500 µl of acetic acid were added. The solution was then incubated at 100¬oC for 1h after which the absorbance was read at 532 nM.

GSH level

GSH level was assayed by measuring the rate of formation of the chromophoric product in a reaction between 5,5′- dinitrobis-2-nitrobenzoic acid (DTNB) and free sulphydryl groups at 412 nm as described by Beutler et al., (1963). The homogenate (0.2 ml) and 3 ml of 4% SSA were mixed, allowed to stand for 5 min and then filtered. To 1 ml of filtrate, 4 ml of 0.1M phosphate buffer, and 0.5 ml of Ellman's reagent were added. The absorbance was read at 412 nm.

GPx activity

GPx activity was measured by the method of Ellman (1953). The reaction mixture containing 0.5 ml of 0.04M phosphate buffer pH 7.4, 0.1 ml of 10 mM sodium azide, 0.5 ml of tissue homogenate, 0.2 ml glutathione, 0.1 ml of 0.02M H_2O_2 and 0.6 ml of distilled water was incubated at 37^{o}C for 3 min. The reaction was stopped by the addition of 0.5 ml of 10% TCA and the resulting mixture centrifuged at 3000 rpm for 5 min. To 0.5 ml of the supernatant obtained, 2 ml of K_2HPO_4 and 1 ml of Ellman's reagent (0.04 g of DTNB in 100 ml phosphate buffer) were added and the absorbance was read at 412 nm. GPx

activity was expressed as µmol GSH consumed/min/mg protein.

Catalase activity

Catalase activity was determined by following the decomposition of H2O2 according to the method of Sinha (1972). Properly diluted sample (1 ml) was rapidly mixed with an assay mixture containing 4 ml of H_2O_2 solution and 5 ml of 0.1M phosphate buffer (pH 7.0) in a conical flask. A 1 ml portion of the reaction mixture was withdrawn and added to 2 ml of dichromate/acetic acid reagent at 60 s intervals. The H_2O_2 content of the withdrawn sample was determined by taking the absorbance at 570 nm. Catalase activity was expressed as µmol H_2O_2 consumed/min/mg protein

GST activity

GST activity was determined according to the procedure described by Habig et al. (1974). The method is based on the rate of conjugate formation between GSH and 1-chloro-2,4-dinitrobenzene (CDNB). Briefly, 30 µl of GSH was introduced into the blank and test tubes. This was followed by the addition of 150 ml of enzyme substrate (1-chloro-2, 4-dinitrobenzene) to each of the tubes. Then 30 ml of enzyme source was added to the test tube alone, and the tubes were made up to total volume of 3.0 ml with 0.1 M phosphate buffer, pH 6.5. The reaction was allowed to run for 60 s at 31^{o}C b-efore the absorbance was read against the blank at 340 nm.

Statistical analysis

All values were expressed as mean ± SD (n = 5). Statistical analysis was done with SPSS 11.09 for windows using one way analysis of variance (ANOVA) followed by Duncan's multiple range test. The significance level was set at $P < 0.05$.

RESULTS

Phytochemical screening of the extract of *Synsepalum dulcificum* fruit showed the presence of tannins, flavonoids, steroids and cardiac glycosides. The total phenolic content and total

flavonoid content were 0.262 mg/g tannic acid equivalent and 0.672 mg/g quercetin equivalent respectively (Table 2).

Table 2. Phytochemical groups detected in *S. dulcificum* fruit extract

Phytoconstituent	Result
Alkaloids	-
Flavonoids	+
Anthraquinones	-
Saponins	-
Tannins	+
Steroids	+
Cardiac glycosides	+
Total phenolic content	0.262 mg/g tannic acid equivalent
Total flavonoid content	0.672 mg/g quercetin equivalent

+ : detected in extract; - : not detected in extract

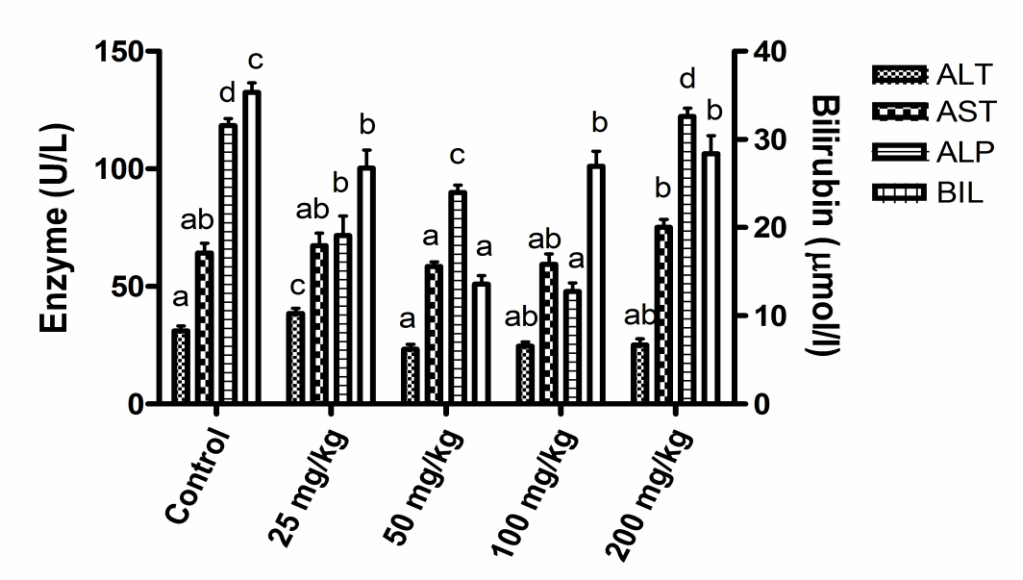

Figure 1: Effect of *S. ducificum* extract on serum bilirubin level and ALT, AST, and ALP activities.

Results are expressed as means ± SD (n = 5). For each parameter, bars with different letters are significantly different (P < 0.05).

ALT, AST and ALP activities together with serum bilirubin level are shown in Figure 1.

S. dulcificum extract at 50 mg/kg caused significant reductions in both serum ALT and ALP activities (P < 0.05) while no concentration

of the extract produced any significant change in serum AST activity, compared to the control. All concentrations of the extract significantly decreased serum bilirubrin level ($P < 0.05$) with the highest reduction occurring in the 50 mg/kg S. dulcificum - treated group.

Treatment with 25-, 50-, and 100 mg/kg of extract reduced hepatic MDA level by 27%, 29%, and 23% respectively while 200 mg/kg caused an increase of 41% compared with the control ($P < 0.05$) (Figure 2). There was a slight increase in GSH level in the 50 mg/kg-treated group ($P > 0.05$) while GSH level in the 100 mg/kg- and 200 mg/kg-treated groups were decreased ($P < 0.05$) compared with the control (Figure 2).

Figure 3 indicates that there was a significant increase in catalase activity in the 50 mg/kg group while no significant change was produced at other concentrations compared to the control. GPx activity in the 25- and 50 mg/kg groups were slightly increased ($P > 0.05$) but significantly reduced in the 200 mg/kg group compared with the control (Figure 3). As shown in Figure 3, the extract at 50-, 100-, and 200 mg/kg produced significant increases in GST activity compared with the control while the increase produced at 25 mg/kg was not significant.

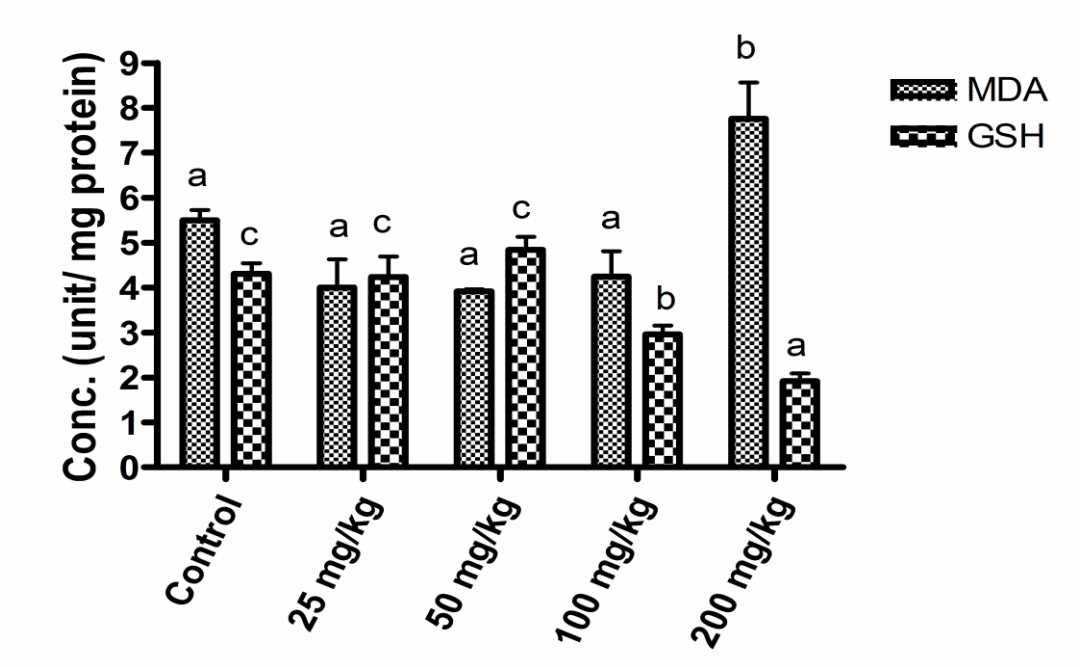

Figure 2: Effect of *S. ducificum* extract on hepatic malondialdehyde and reduced glutathione levels.

Values are expressed as means ± SD (n = 5). For each parameter, values with different letters are significantly different ($P < 0.05$).

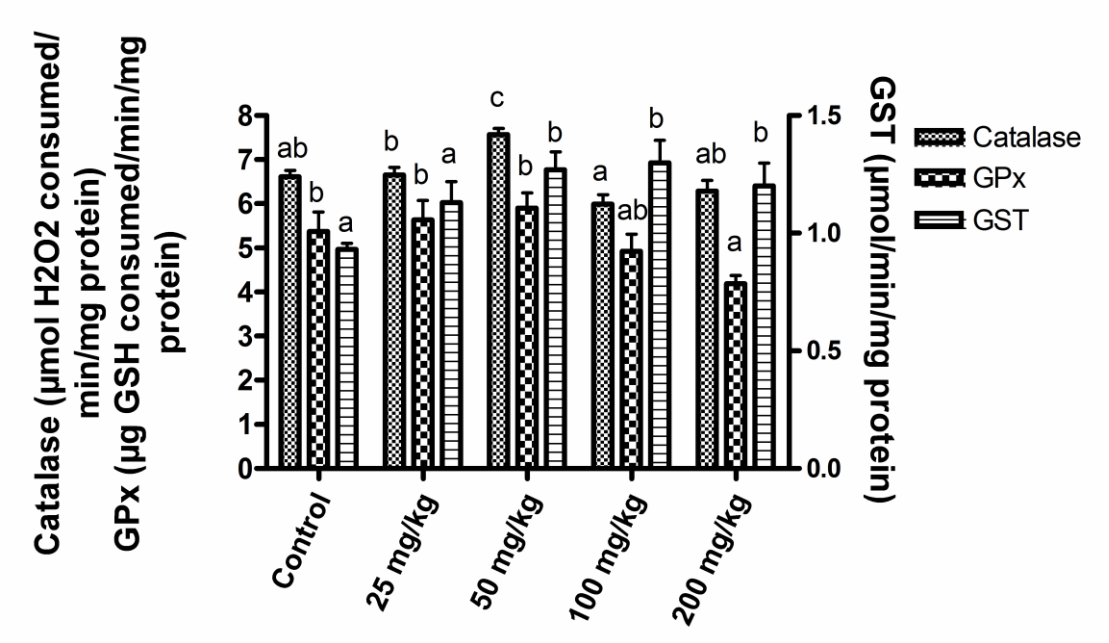

Figure 3: Catalase, GPx and GST activities in experimental groups.

Values are expressed as means ± SD (n = 5). For each parameter, values with different letters are significantly different ($P < 0.05$).

DISCUSSION

In recent years dietary plants with antioxidative property have been the center of focus in many pharmacological and nutraceutical investigations. It is believed that these plants can prevent or protect tissues against damaging effects of free radicals (Osawa and Kato, 2005). The phytochemicals present in these plants have been credited with the medicinal properties demonstrated by the plants. Although many plants are popularly used in traditional medicine, there is a growing concern among health care professionals about their safety since toxicological evaluations have not been carried out on many of them.

The presence of tannins, flavonoids, terpenoids and cardiac glycosides in *Synsepalum dulcificum* fruit is suggestive of its potent bioactivity. Tannins have been reported to possess antidiarrhoec and antihaemorrhagic property (Asquith and Butler, 1986). The antioxidant and multiple bioactivities of flavonoids are well documented. Cardiac glycosides have been used for over two centuries as stimulants in cases of cardiac failure (Trease and Evans, 1996). Terpenes and terpenoids are the primary constituents of essential oils which are used in traditional and alternative medicines such as aromatherapy. The total phenolic content and total flavonoid content (0.262 mg/tannic acid equivalent and 0.672 mg/g quercetin equivalent respectively) are indicative of the antioxidant

potential of the berry. It has been recognized that phenolic compounds and in particular, flavonoids show antioxidant activity and their effects in human nutrition and health are considerable. Many flavonoids have been characterized from *S. dulcificum* berry (Table 1).

ALT and AST are among the most sensitive markers employed in the diagnosis of hepatic damage since they are cytoplasmic in location and are released into circulation after cellular damage (Sallic et al., 1991). The primary importance of measuring alkaline phosphatase (ALP), a marker enzyme for the plasma membrane of tissues including liver, is to check the possibility of bone or liver diseases (Trevor, 2001). The membrane bound enzymes like ALP and γ-glutamyltransferase are released unequally into bloodstream depending on the pathological phenomenon (Sillanaukee, 1996). Extract (50- and 100 mg/kg) - induced reduction in levels of liver function biomarkers (ALP and ALT) may signify protective effect against damage to hepatocyte membrane integrity.

Evaluation of serum bilirubin level is a very sensitive test employed in the diagnosis of hepatic diseases as it provides useful information on how well the liver is functioning (Saravanan et al., 2006). Decrease in total bilirubrin level caused by the extract may be an additional indicator of its hepatoprotective potential.

The observed increases in GST, CAT and GPx activities (25- and 50 mg/kg) and the level of GSH (50 mg/kg) of *Synsepalum dulcificum* extract treated rats may signify extract-dependent boost in the antioxidant defense system at the given concentrations. This suggestion is also supported by the observed reductions in hepatic MDA level (25 mg/kg: 27% reduction, 50 mg/kg: 29% reduction, and 100 mg/kg: 23% reduction). Reduction in MDA levels is commonly used to ascribe protective antioxidant values to drugs (Viani et al., 1991; Adaramoye. 2010), since lipid peroxidation is thought to be directly related to oxidative damage.

On the other hand, the 200 mg/kg extract-induced increase in MDA and attendant decrease in GSH levels and GPx activity ($P < 0.05$) are indicative of ensuing oxidative stress and a pro-oxidative tendency. Levels of MDA reflect free radical-mediated cell membrane damage (Viani et al., 1991). Depletion of hepatic GSH may result in the accumulation of free radicals that can initiate membrane damage by lipid peroxidation.

The present study revealed the antioxidant potential of *Synsepalum dulcificum* berry (miracle fruit). It also suggests that extract at lower dose range of < 100 mg/ kg may boost the antioxidant defense and exert hepatoprotective properties while higher doses may be pro-oxidative. Studies are ongoing in our laboratories to evaluate the bioactivity of *Synsepalum dulcificum* fruit in specific oxidative stress-mediated pathologies with a view to possibly isolating and characterizing novel pharmacological principles from it.

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