Evaluation of the Antioxidant Effects of Hibiscus Sabdariffa Calyx Extracts on 2, 4-Dinitrophenylhydrazine-Induced Oxidative Damage in Rabbits

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Abstract

Aqueous and anthocyanin-rich extracts of dried calyces of Hibiscus sabdariffa Linn, have been evaluated for antioxidant bioactivity. They were assessed for antioxidant activity based on their ability to impair 2, 4-dinitrophenylhydrazine (DNPH)-induced oxidative damage in selected organs of the toxicant exposed rabbits. The organs examined were the blood and liver. Exposure of rabbits to DNPH (28 mg/kg body weight) caused significant (P<0.05) adverse biochemical and hematological changes relative to controls. Pretreatment with (100 mg/kg body weight) whole aqueous extract of H. sabdariffa (HS) and HS calyx anthocyanins, provided varying degrees of protection against DNPH-induced biochemical and hematological changes. Relative to the controls, whole aqueous extract and anthocyanin-rich extract treatments increased the levels of PCV, hemoglobin and RBC and decreased WBC count significantly (P<0.05). The dried calyces contain the flavonoids – gossypetin, sabdaretine, hibiscetine and anthocyanins (Pietta, 2000). 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 (Gaxlane et al., 1994; Jackson et al., 1993). Previous reports showed that the extract from the red calyces of Hibiscus sabdariffa contain potent antioxidant principles (Tseng et al., 1997; Wang et al., 2000; Ologundudu and Obi, 2005; Ologundudu et al., 2006a, b; Ologundudu et al., 2009a,b; Ologundudu et al., 2010). The aim of this research therefore is to evaluate this claim further to determine the active principles.

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 (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 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 (Newmark, 1992; Wang et al., 2000). 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 is Hibiscus sabdariffa Linn, family Malvaceae, which has many medicinal uses (Morton, 1987; Gill, 1992; Hirunpanich et al., 2006; Alarcon-Aguilar et al., 2007). The dried calyces contain the flavonoids – gossypetin, sabdaretine, hibiscetine and anthocyanins (Pietta, 2000). 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 (Gaxlane et al., 1994; Jackson et al., 1993). Previous reports showed that the extract from the red calyces of Hibiscus sabdariffa contain potent antioxidant principles (Tseng et al., 1997; Wang et al., 2000; Ologundudu and Obi, 2005; Ologundudu et al., 2006a, b; Ologundudu et al., 2009a,b; Ologundudu et al., 2010). The aim of this research therefore is to evaluate this claim further to determine the active principles.

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 0C 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 extract from Hibiscus sabdariffa calyces was prepared according to the method described by Hong and Wrolstad, (1990a). One kilogram of Hibiscus sabdariffa calyces was pulverized by means of Binatone blender and extracted with 10 litres of 0.1 % trifluoroacetic acid (TFA) aqueous solution for 12 hours at 20 °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 °C. A portion of the concentrated eluate was then subjected to high-pressure liquid chromatography (HPLC) in order to identify its anthocyanin components while the remaining was freeze-dried using a freeze-dryer. The dried sample obtained was resuspended in distilled water and kept in the refrigerator until required for oral administration and biochemical investigation.

Identification of H. sabdariffa anthocyanins using HPLC:

The HPLC system (Pharma-Tech Research Co., Model CCC-1000, MD, USA), consisted of a horizontal flow-through planar centrifuge with a multilayer coil, 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 at 1000 rpm. Four ml of each fraction was collected. A multi-wavelength detector (Waters, 490E) monitored the absorbance of the effluent at 515 nm.

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: Aqueous 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: 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 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: Aqueous 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.
Group 6: 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.

Blood sample collection and hematology:

The ventral sides of the rabbit's ears were sterilized by swabbing with cotton wool soaked in methylated spirit to dilate the veins. The blood was collected using 1 ml hypodermic syringe and needle. The blood samples were collected into EDTA bottles.

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 (Dacie and Lewis, 1997).

Biochemical determinations:

The L-alanine aminotransferase (L-ALT), L-aspartate aminotransferase (L-AST) activities and total protein were estimated using assay kits obtained from Randox Laboratories, UK.

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

Results

The changes in the red blood cell (RBC) and white blood cell (WBC) counts, packed cell volume (PCV)
and hemoglobin (HB) concentration of rabbits due to 2, 4-dinitrophenylhydrazine (DNPH), aqueous extract of H. sabdariffa (HS) and H. sabdariffa anthocyanin extract (AN) treatments are presented in Table 1. Treatment with DNPH alone significantly (p< 0.05) reduced the rabbit RBC, PCV and Hb values but caused an increase in WBC counts compared to control (Group 1). The RBC and WBC and Hb concentration of rabbits that received the whole aqueous extract and anthocyanin extract separately (Groups 2 and 3) and those pretreated with the extracts before DNPH administration (Groups 5 and 6) did not show any significant (p>0.05) difference when compared with the control. However, the PCV of rabbits that were treated with both extracts alone (Groups 2 and 3) was significantly (p<0.05) difference relative to the control.

The effects of DNPH, Aq extract of HS, and HS anthocyanin extract on the levels of L-alanine aminotransferase (L-ALT) and L-aspartate aminotransferase (L-AST) activities in the liver and serum are presented in Table 2. These results show that treatment with DNPH alone (Group 4) caused a significant (pThe effects of DNPH, aqueous extract of HS and HS anthocyanins on the levels of malondialdehyde (MDA) in the liver, brain and serum of rabbits is presented in Table 3. The data show that DNPH treatment (Group 4) significantly (p

**Discussion**

The observed increase in PCV of the animals treated with aqueous extract of H. sabdariffa and anthocyanin extract could be attributed to reduced loss of blood cells to lipid peroxidation. The increase could also be due to the erythropoietic ability of antioxidant molecules (Heda and Bhatia, 1986). The erythropoietic property can be due to anthocyanin-associated increase in absorption of iron, a property that has been widely reported in ascorbate administration (Ponka, 1997). Iron has only been reported to be present in significant amount in the whole extract of H. sabdariffa, but anthocyanins themselves are known to induce the synthesis and release of erythropoietin by the kidney (Ponka, 1997; Photis, 1998). There was a reduced PCV level for animals treated with DNPH. This is a further evidence in support of the previously established hemotoxic ability of DNPH and its derivatives (Patil et al., 2000). The ability of the aqueous extract of Hibiscus sabdariffa and anthocyanin extract to individually ameliorate the toxic effect of DNPH on the erythrocyte was also assessed. They, individually showed ability to prevent the toxic effect of DNPH as indicated by a significantly increased (pHemoglobin is a natural constituent of red blood cells and biochemically adapted to carry oxygen from the lungs and transport it to tissues for oxidative metabolism. Besides this function, it has been structurally prepared to also play major roles physiologically in carbon dioxide removal and acid-base balance. Therefore, an increased production of hemoglobin is an advantage to an organism. This advantage can only be ensured if there is decreased red blood cell destruction and/or increased red blood cell production (Ponka, 1997). Treatment of animals with aqueous extract of H. sabdariffa and anthocyanin extract revealed that they both have the same pattern of effects on hemoglobin as they had on PCV. There was a statistically significant (p White blood cells are known for defense against invading pathogens and xenobiotics. The significant (pAqueous extract of H. sabdariffa and anthocyanin isolate caused higher red blood cell counts when compared with the water control (pTo fully assess the protective effect of the different treatments on the cellular function and integrity, there was the need to monitor the serum non-secretory, non-specific enzymes which are native mainly to the liver. ALT and AST are enzymes produced in hepatocytes, the major cell types in the liver. The level of these enzymes in the blood is increased in conditions in which hepatocytes are damaged or die. As the cells are damaged, the enzymes leak out into the bloodstream. All types of hepatitis (viral, alcoholic, drug-induced, etc.) cause hepatoocyte damage that can lead to elevations in the serum activity of the enzymes. The serum level of the enzymes is also increased in cases of liver cell death resulting from other causes, such as shock. Biochemically, alanine aminotransferase is involved in ammonia metabolism in the liver and its transportation from other tissues by reversible transamination reaction involving an amino acid and pyruvate, resulting in the formation of alanine and a metabolizable alpha-keto acid derivative of the initial amino acid in the PLP-dependent pathway. The time course of appearance of this enzyme in the serum gives a predictive indication of progression of injury to tissues. Groups administered aqueous extract of H. sabdariffa and anthocyanin extract of H. sabdariffa showed no significant (PPrior administration of aqueous extract of H. sabdariffa and anthocyanin extract of H. sabdariffa followed by treatment with DNPH resulted in significant decrease in their respective serum ALT and AST activities compared with those treated with DNPH only. These values are statistically significant (PMalondialdehyde (MDA) is one of the prominent by-products released when
membrane lipids are peroxidized. This product originates from the free radical attack on the poly-unsaturated fatty-acyl group esterified to the C-2 hydroxyl group of glycerol back-bone of phospholipids. The reaction involves scission of the double bonds in these molecules which is accompanied with the release of carbonyl compounds together with malondialdehyde.

Malondialdehyde formation in biological tissues is secondary to the primary effect of the free radical attack. Since polyunsaturated fatty acyl groups are found on the membrane lipids, their break-down portends an assault on membrane integrity and fluidity. These two 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 cell membrane, and a possible necrotizing tissues induced by oxidative properties of attacking free radicals (Das and Nair, 1980; Flynn et al., 1983).

The water control levels of MDA in the brain, serum and liver were high compare to that of groups treated with the extracts. The biochemical reason for this observation is the residual free radicals generated during catabolic reactions and oxidative phosphorylation which involves electron transport chain. Free radicals generated during normal metabolic processes can be scavenged by dietary antioxidants assimilated in the liver via the hepatic portal vein (Kuhnau, 1976; Mazza and Miniati, 1993; Mazza et al., 2003; Youdim et al., 2002). Also bilirubin which is being conjugated by the liver cells is also known to absorb free radicals and the additive effect is expected to result in reduction of malondialdehyde levels. In group comparison between each of the extracts, anthocyanin isolate administration resulted in lower amount of MDA in the tissues. This finding agrees with the report of Youdim et al. (2000). There was no significant difference between the MDA levels obtained for the groups treated with the anthocyanin and aqueous extract but significantly lower than the water control. The biochemical basis for this is the presence of anthocyanin and anthocyanidin in both the isolate and the aqueous extract of H. sabdariffa. The intra-transmembrane movement of anthocyanidin allows the molecules to interdigitate with the membrane phospholipids, also in glycoside form-anthocyanin; it functions as antioxidant in polar environment of the cell (Felgines et al., 2002). Also, the ability of anthocyanidin to induce the expression of antioxidant enzymes play significant role in reducing the MDA levels of groups administered with anthocyanin-based preparation. Administration of DNPH resulted in significant increase in malondialdehyde level in the tissues. The values recorded for the brain were slightly higher than those of the liver indicating a better protection offered by bilirubin, dietary antioxidants and ability of the liver to metabolize DNPH better than the brain as earlier indicated by Galli et al. (2002). Pretreatment of the rabbits with the anthocyanin-rich extracts prior to DNPH treatment effectively blocked the effect of DNPH. This claim was confirmed by reduction of malondialdehyde level below the values recorded for water control and the similarity in the values obtained when the animals were treated with each of the extracts only. This finding agrees with the previously published data of Tsuda et al. (1999), Matsumoto et al. (2001), Passamonti et al. (2003), Ologundudu et al. (2010).

Conclusion

It is of interest that the results of this work showed similarity in antioxidant effects of both the whole aqueous and anthocyanin-rich extracts of Hibiscus sabdariffa, though anthocyanin isolate appeared to be more effective in this capacity. This finding points to the fact that anthocyanins, the main constituents of the anthocyanin-rich extract are likely to be responsible for the similar effects presented by the whole aqueous extract. The less potency of the latter may be due to the heterogeneity of its constituents. Therefore, Hibiscus sabdariffa calyces contain potent antioxidant principles which are likely anthocyanins.

References


### Illustrations

#### Illustration 1

Table 1: Effects of DNPH, Aq extract of Hibiscus sabdariffa (HS) and HS anthocyanins on the levels of red blood cells (RBC), white blood cells (WBC), packed cell volume (PCV) and hemoglobin (Hb) of rabbits.

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatment</th>
<th>RBC (counts/µL) x 10^6</th>
<th>WBC (counts/µL) x 10^3</th>
<th>PCV (%)</th>
<th>Hb (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5 ml H_2O/kg bd wt. (control)</td>
<td>4.55 ± 0.71</td>
<td>7.80 ± 0.21</td>
<td>34.33 ± 0.08</td>
<td>10.80 ± 0.10</td>
</tr>
<tr>
<td>2</td>
<td>100 mg AqE/kg bd wt.</td>
<td>5.71 ± 0.35</td>
<td>7.83 ± 0.52</td>
<td>36.33 ± 1.86</td>
<td>12.43 ± 0.83</td>
</tr>
<tr>
<td>3</td>
<td>100 mg AN/kg bd wt.</td>
<td>5.81 ± 0.30</td>
<td>7.60 ± 0.02</td>
<td>35.90 ± 1.33</td>
<td>11.25 ± 0.50</td>
</tr>
<tr>
<td>4</td>
<td>28 mg DNPH/kg bd wt.</td>
<td>3.80 ± 0.44</td>
<td>13.40 ± 0.22</td>
<td>26.67 ± 1.76</td>
<td>8.30 ± 0.35</td>
</tr>
<tr>
<td>5</td>
<td>100 mg AqE/ + 28 mg DNPH/kg bd wt.</td>
<td>5.18 ± 0.04</td>
<td>7.60 ± 0.67</td>
<td>33.00 ± 0.58</td>
<td>11.00 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>100 mg AN+ 28mg DNPH/kg bd wt.</td>
<td>4.70 ± 0.21</td>
<td>6.93 ± 0.44</td>
<td>33.33 ± 1.10</td>
<td>11.10 ± 0.60</td>
</tr>
</tbody>
</table>

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 from each other while values with different superscripts are significantly different from one another. DNPH: 2, 4-dinitrophenylhydrazine, AN: anthocyanin, AqE: aqueous extract.
### Illustration 2

Table 2: Effects of DNPH, Aq extract of HS and HS anthocyanins on L-ALT and L-AST activities in the liver and serum.

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatment</th>
<th>L-ALT (IU/L)</th>
<th>L-AST(IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Serum</td>
</tr>
<tr>
<td>1.</td>
<td>2.5ml H₂O/kg bd wt. (control)*</td>
<td>30.78 ± 0.30</td>
<td>11.54 ± 0.20</td>
</tr>
<tr>
<td>2.</td>
<td>100 mg AqE/kg bd wt.</td>
<td>30.46 ± 0.12</td>
<td>11.88 ± 0.17</td>
</tr>
<tr>
<td>3.</td>
<td>100 mg AN/kg bd wt.</td>
<td>30.98 ± 0.15</td>
<td>11.75 ± 0.04</td>
</tr>
<tr>
<td>4.</td>
<td>28 mg DNPH/kg bd wt.</td>
<td>24.36 ± 0.73</td>
<td>17.42 ± 0.23</td>
</tr>
<tr>
<td>5.</td>
<td>100 mg AqE + 28 mg DNPH/kg bd wt.</td>
<td>28.99 ± 0.40</td>
<td>12.51 ± 0.34</td>
</tr>
<tr>
<td>6.</td>
<td>100 mg AN + 28 mg DNPH/kg bd wt.</td>
<td>29.45 ± 0.46</td>
<td>11.95 ± 0.13</td>
</tr>
</tbody>
</table>

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.
Illustration 3

Table 3: Effects of DNPH, Aq extract of HS and HS anthocyanins on the levels of malondialdehyde (MDA) in the liver, brain and serum of rabbits.

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatment</th>
<th>MDA (µmol/mg protein)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Brain</td>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>2.5 ml H₂O/kg bd wt. (control)*</td>
<td>7.40 ± 0.08</td>
<td>7.61 ± 0.38</td>
<td>3.35 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>100 mg AqE/kg bd wt.</td>
<td>5.62a ± .58</td>
<td>7.73 ± 0.06</td>
<td>3.34 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>100 mg AN/kg bd wt.</td>
<td>5.36a ± 0.65</td>
<td>7.26 ± 0.09</td>
<td>3.07 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>28 mg DNPH/kg bd wt.</td>
<td>22.12b ± 0.20</td>
<td>24.23a ± 0.37</td>
<td>10.25a ± 0.34</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>100 mg AqE + 28 mg DNPH/kg bd wt.</td>
<td>6.55 ± 0.21</td>
<td>6.67 ± 0.67</td>
<td>4.50 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>100 mg AN + 28 mg DNPH/kg bd wt.</td>
<td>6.35 ± 0.20</td>
<td>6.43 ± 0.96</td>
<td>3.54 ± 0.28</td>
<td></td>
</tr>
</tbody>
</table>

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.
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