Phenolic Composition and Inhibitory Ability of Methanolic Extract from Pumpkin (Cucurbita pepo L) Seeds on Fe-induced Thiobarbituric acid reactive species in Albino Rat’s Testicular Tissue In-Vitro

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ABSTRACT

Pumpkin seed has been associated with myriad of medicinal uses in different part of the world. In this study, phenolic composition and Fe2+ induced thiobarbituric acid reactive species (TBARS) inhibitory ability of methanolic extract from pumpkin seeds in rat’s testes homogenates were determined. The extract was prepared with 80% methanol (v/v) and the radicals [(1,1-diphenyl-2 picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)] scavenging, Fe2+ chelation and ferric reducing abilities of the extract were carried out. The phenolics composition was also investigated using gas chromatography couple with flame ionization detector (GC-FID). The GC analysis revealed the presence of vallinic, coumaric protocatechuic, caffeic, ferulic and sinapinic acids, and apigenin, quercetin, luteolin, kaempferol as the dominant phenolic compounds. The results revealed that the extract inhibited Fe2+-induced TBARS, scavenge DPPH radical and chelate Fe2+ in a dose dependent manner. The extract also scavenged ABTS radical and reduced Fe3+ to Fe2+. Although, the standard used had higher effect compared to the extract, nevertheless, the TBARS inhibitory potential of the extracts clearly gives an insight on the protective potentials against oxidative induced testicular damage that might lead to male infertility if unchecked. These abilities could however be linked to the presence of polyphenolic compounds.

INTRODUCTION

Oxidative stress (OS), which can be defined as increase free radical production or reduce antioxidant defence system has been intensely study and reported as major factor in the pathogenesis of male infertility (Koksal et al., 2000; Ishikawa et al., 2007; Mostafa et al., 2009; Abd-Elmoaty et al., 2010; Akomolafe et al., 2015). Malondialdehyde (MDA), a by-product of lipid peroxidation, is among the major laboratory tests in the measurement of OS, and can be easily measure by the determination of thiobarbituric acid reactive species (TBARs) assay; a major reactive species produced when radicals attack and degrade polyunsaturated fatty acids (PUFAs) (Yagi, 1998; Akomolafe et al., 2015; Adedayo et al., 2015a), and its capable of inducing toxic stress in the cells and form advanced glycation end-products (Nowotny et al., 2015). Transitional metals such as Iron (Fe) has been reported as one of the essential metals, required as the physiological component of many enzymes and proteins (Khan and Awan, 2014), but its free form in the biological system has been reported to cause considerable oxidative damage via induction of radicals production, and biomolecules (lipids, nucleic acids and proteins) oxidation which could result to wide range of impairment to cellular function and integrity (Khan and Awan, 2014). However, consumption of phenolic-rich plants and/or its extracts has been reported to ameliorate OS, and could be due to their richness in natural antioxidant agents such as phenolic compounds (Schiffrin, 2010; Ademiluyi et al., 2014).
The importance of antioxidant compound and the management of several human diseases have attracted much research attention recently (Skotti et al., 2014). According to Farombi and Olatunde (2011) and Atangwho et al. (2013), the use of medicinal plant/extract in the management of some ailments in folklore is a major practice, especially in the developing countries. And several reports have logically linked the nutraceutical values provided by the consumption of plant based food/extracts against several human diseases to the presence of antioxidants and phytochemicals such as vitamins C, α-tocopherol, β-carotene and polyphenols (Fasakin et al., 2011; Adedayo et al., 2015a,b).

_Cucurbita pepo_ Linn (Family: Cucurbitaceae), also known as pumpkin in English and locally called “Elegede” is a popular plant in the Southwest Nigeria (Oloyede, 2012). The young leaf that is locally called “Gboro” is commonly consumed while the pulp of ripe fruits has been reported for its use in the management of intestinal inflammation, stomach and liver disorders and as dietary supplement for vitamin A (Sarkar and Guha, 2008). The seeds, otherwise known as pepitas, are small flat, green edible seeds that are often recommended as dietary supplement and for the management of certain digestive ailments such as constipation and diarrhea in folklore medicine. In this study, we aim to investigate the protective ability of the methanolic extract from the pumpkin seed against Fe-induced TBARs production in albino rat’s testicular tissue _in-vitro_. The ability of the extract to scavenge radicals, chelates Fe$^{2+}$ and reduced Fe$^{3+}$ to Fe$^{2+}$ were also investigated.

Phenolic compositions of the extract were also determined using gas chromatography coupled with flame ionization detector (GC-FID).

**MATERIALS AND METHODS**

**Chemicals and reagent**

All chemicals and reagent used were of analytical grade and glass-distilled water was used. Kenxin refrigerated centrifuge Model KX3400C was use while UV-Visible spectrophotometer (Model 6305; Jenway, Barloworld Scientific, Dunmow, United Kingdom) was used to measure the absorbance.

**Samples collection and preparation of extract**

Fresh Pumpkin fruits were harvested from a local farm in Akure metropolis, Nigeria. Authentication of the sample was carried out at the Department of Plant Science, Ekiti State University, Ado-Ekiti, Nigeria by Mr. Ajayi (the herbarium curator). The voucher specimen number given was UHAE 332. The seeds were carefully removed with table knife, washed with distilled water and dried to constant weight in oven at 40 °C. Thereafter, the seeds was grounded to powder and soaked in hexane to remove the fat and dried. The methanolic extraction was carried using the method of Chu et al., (2002). Ten gramme of the pulverized sample was extracted with 100 mL of absolute methanol and was filtered (Whatman no. 2) under vacuum after 24 h. The filtrate was evaporated using a rotary evaporator under vacuum at 45 °C. The extract was stored under refrigeration for subsequent analysis.

**Determination of total phenol content**

Total phenol content was determined using Folin-Ciocalteau’s reagent method (Singleton et al., 1999). Briefly, appropriate dilution of the extract was oxidized with 2.5 mL of 10% Folin-Ciocalteau’s reagent (v/v) and neutralized with 2.0 mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance was measured thereafter at 765 nm. The total phenol content was subsequently calculated and presented as gallic acid equivalents (GAE).

**Determination of total flavonoid content**

Determination of total flavonoid content was carried out using a slightly modified method of Meda et al., (2005), 0.5 mL of appropriately dilutions of the extract was mixed with 0.5 mL of absolute methanol, 50 µL of 10% AlCl$_3$, 50 µL of 1 M Potassium acetate and 1.4 mL of distilled water. The mixture was incubated at room temperature for 30 min. The absorbance of the mixture was subsequently measured at 415 nm. The total flavonoid content was calculated and presented as quercetin equivalents (QE).

**GC-FID characterization of constituent phenolics in the methanolic extract of pumpkin seeds**

The qualitative-quantitative analysis of the phenolic compounds of the sample was carried out using the method reported by Kelley et al., (1994). The phenolic extract was extracted as described by Kelley et al., (1994) and Provan et al., (1994) and the purified phenolic extracts (1 ml: 10:1 split) were analyzed for composition by comparison with phenolic standards (Aldrich Chemical Co., Milwaukee, WI) on a Hewlett-Packard 6890 gas chromatography (Hewlett-Packard Corp., Palo Alto, CA) equipped with a derivatized, non-packed injection liner, a Rtx-5MS (5% Diphenyl-95% Dimethyl polysiloxane) capillary column (30 m length, 0.25mm film thickness), and detected with a flame ionization detector (FID). The following conditions were employed; injector temperature, 23°C; temperature ramp, 80°C for 5 min then ramped to 250°C at 30°C/min; and a detector temperature of 320 °C

**Lipid peroxidation and Thiobarbituric acid reactions assay**

Wister male albino rat (weighing 205 mg) was decapitated under mild diethyl ether and the testes tissue was rapidly isolated. The tissue was placed on ice, weighed and subsequently homogenized in cold saline (1/10 w/v) with about 10-up-and – down strokes at approximately 1200 rev/min in a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 3000 x g to yield a pellet that was discarded (Belle et al., 2004). Hundred microliter of the supernatant fraction was mixed with a reaction mixture containing 30 µL of 0.1 M pH 7.4 Tris-HCl buffer, extract (0 – 100 µL) and 30 µL of freshly prepared 250 µM FeSO$_4$. The volume was made up to 300 µL with distilled water before incubation at 37°C for 1 h. The colour reaction was
developed by adding 300 μL 8.1% sodium dodecyl sulphate to the reaction mixture. 500 μL of acetic acid/HCl (pH 3.4) mixture and 500 μL of 0.8% thiobarbituric acid was subsequently added. The mixture was incubated at 100°C for 1 h. Ethylenediaminetetraacetic acid (EDTA) was used as control. TBARs produced were measured at 532nm (Ohkawa et al., 1979).

Free radical scavenging ability

Ability of the extract to scavenge DPPH free radical was evaluated (Gyamfi et al., 1999). In brief, appropriate dilution of the extract or Vitamin C (1 mL) was mixed with 1 mL of 0.4 mM methanolic solution of DPPH radical, the mixture was left in the dark for 30 min and the absorbance was taken at 516nm. The DPPH radical scavenging ability was subsequently calculated.

Total antioxidant power

Total antioxidant power of the extract was assessed using the ABTS radical model as described by Re et al., (1999). The ABTS radical was generated by reacting 7 mmol/l of ABTS aqueous solution with 2.45 mmol/l of K$_2$S$_2$O$_8$ solution in the dark for 16 h and adjusting the Abs734 nm to 0.700 with ethanol. Two hundred microliter of the appropriate dilution of the extract was added to 2.0 mL ABTS radical solution and the absorbance was measured at 734 nm after 15 min. The trolox equivalent antioxidant capacity was subsequently calculated.

Fe$^{2+}$ chelating ability

The extract ability to chelate Fe$^{2+}$ was determined using the method of Puntel et al., (2005) with some modifications. A 500 mmol/L of freshly prepared FeSO$_4$ (150 mL) was added to the mixture containing 168 mL of 0.1 mol L Tris-HCl (pH 7.4), 218 mL saline and the extract or ethylenediaminetetraacetic acid (EDTA) (0 -100 μL). The mixture was incubated for 5 min and then 13 mL of 0.25% 1, 10-phenanthroline (w/v) was added. The absorbance was measured at 510 nm in a spectrophotometer. The percentage of chelated Fe was subsequently calculated.

Determination of reducing property

The reducing property was determined by assessing the ability of the extract to reduce FeCl$_3$ solution as described by Oyaizu, (1986). 2.5 mL aliquot was mixed with 2.5 mL 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. and then 2.5 mL of 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. 1 mL of the supernatant was mixed with an equal volume of distilled water and 0.2 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant property was subsequently and presented as ascorbic acid equivalent.

Data analysis

The results of the triplicate experiments were pooled and expressed as mean ± standard deviation (SD). One way analysis of variance and the least significance difference (LSD) was carried out. Significance was accepted at $P<0.05$.

RESULTS

The total phenolic (phenol and flavonoid) content in the methanolic extract from pumpkin seed is presented in Table 1. The total phenol and total flavonoid contents of the extract reported as gallic acid equivalent (GAE) and quercetin equivalent (QE) were 32.90 mg GAE/g and 21.50 mg QE/g respectively. The GC phenolics profile of the extract is presented in Tables 2. As shown in Table 2, the result revealed the presence of some phenolic acids and flavonoids predominantly were P-hydroxybenzaldehyde (6.69 mg/100 g), protocatechuic acid (100.23 mg/100 g), p-coumaric acid (119.68 mg/100 g), vanillic acid (415.35 mg/100 g ), caffeic acid (139.71 mg/100 g), sinapinic acid (45.23 mg/100 g), ferulic acid (111.98 mg/100 g), apigenin (28.99 mg/100 g ), kaempferol (48.81 mg/100 g), luteolin (12.68 mg/100 g), quercetin (60.85 mg/100 g), Myricetin (2.28 mg/100 g).

Table 1: The total phenol and flavonoid contents, reducing property (FRAP) and ABTS radical scavenging ability of methanolic extract from pumpkin seed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenolic content (mg GAE/g)</td>
<td>32.90±3.03</td>
</tr>
<tr>
<td>Total flavonoid content (mg QE/g)</td>
<td>21.50±0.90</td>
</tr>
<tr>
<td>ABTS radical scavenging ability (mmol.TEAC/g)</td>
<td>3.90±0.73</td>
</tr>
<tr>
<td>FRAP (mg AAE/g)</td>
<td>8.67±1.24</td>
</tr>
</tbody>
</table>

Values represent Mean ± Standard deviation of triplicate experiments.

Table 2: The main phenolic constituents of the methanolic extract from pumpkin seeds.

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Amount (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocatechuic acid</td>
<td>100.23±2.12</td>
</tr>
<tr>
<td>P-coumaric acid</td>
<td>119.68±4.41</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>415.35±2.44</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>139.71±1.32</td>
</tr>
<tr>
<td>Sinapinic acid</td>
<td>45.23±1.45</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>111.98±3.21</td>
</tr>
<tr>
<td>Apigenin</td>
<td>28.99±0.89</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>48.81±1.29</td>
</tr>
<tr>
<td>Luteolin</td>
<td>12.68±0.76</td>
</tr>
<tr>
<td>Quercetin</td>
<td>60.85±1.23</td>
</tr>
<tr>
<td>Myricetin</td>
<td>2.28±0.22</td>
</tr>
</tbody>
</table>

Values represent Mean ± Standard deviation of triplicate readings.

As shown in Fig. 1, incubation of testes homogenate with Fe$^{2+}$ solution caused a significant increase in malondialdehyde (MDA) content to 166.89% (Induced) as against the basal (100%) However, addition of the extract (0.83 -1.67 mg/mL) and/or EDTA (1.56-6.25 μg/mL) in a dose dependent manner caused a significant decrease in the MDA content in the testes homogenate. The DPPH and ABTS radicals scavenging abilities of the extract were presented in Fig. 2 and Table 1 respectively. The result revealed that the extract scavenged DPPH radicals in a dose dependent manner at the concentrations tested (50 - 200 μg/mL). The ABTS radical (ABTS$^*$) scavenging ability presented as trolox equivalent antioxidant capacity (TEAC) is presented in Table 1. The extract was able to scavenge ABTS$^*$ (3.90 mmol TEAC/g). The Fe$^{2+}$ chelating ability of the extract and EDTA is presented in Fig. 3. EDTA had higher chelating ability of 89.5% while that of
the extract was 63.7% at the highest concentration used. The result of the ferric reducing antioxidant power (FRAP) of the extract was presented as ascorbic acid equivalent in Table 1. The extract exhibited ferric reducing antioxidant power of 8.67 mg AAE/g.

**DISCUSSION**

Recently, research on polyphenolic compounds has become subject of interest as a result of their numerous health benefits (Skotti et al., 2014). Several reports have linked the antioxidant activities of many plant foods/extracts to the polyphenolic compounds, and its believed to be due to the reduct properties of their polyhydroxyl molecule, which is known for their ROS adsorbing and neutralizing potentials, chelating of transitional metal catalysts and activation antioxidant enzymes activities (Dai and Mumper, 2010; Tsao, 2010; Tulio et al., 2014). The total phenol and flavonoid contents of the extract are presented in Table 1, as gallic acid (GAE) and quercetin (QE) equivalent respectively. The studied extract had higher total phenol content than total flavonoid (Table 1). This study is in consistence with several reports of some tropical plants with similar trends between the total phenol and flavonoid content (Gacche et al., 2010; Handique et al., 2012; Radojkovic et al., 2012). The use of chromatography analysis to quantify and qualify phenolic profile of plant material has proven to be more advantageous over total phenolic content determination using Folin Ciocalteu method, as it reveals accurate information of individual compounds (Nwanna et al., 2016). The result of the phenolic composition using GC-FID revealed the presence of dominant eleven phenolic compounds of which six were phenolic acids while the remaining five were flavonoids (Table 2). This however, suggests that the extract is rich in phenolics. Phenolics are secondary metabolites and their consumption in phenolic-rich plant foods have been linked to numerous health benefits such as anti-bacterial, ant-glycemic, antiviral, carcinogenic, anti-inflammatory and, vasodilatory properties and prevention of lipid peroxidation, a key process in the onset and progression of many degenerative diseases (Dryden et al., 2006; Pandey and Rizvi, 2009; Skotti et al., 2014). Hence, the phenolic constituent in this studied extract could therefore be responsible for the observed biological activities.

One of the major mechanisms of cell injury in aerobic organisms subjected to oxidative stress is lipid peroxidation of biological membranes (Pandey and Rizvi, 2009). The effect of the extract on FeSO₄ - induced TBARS production in the isolated rat testes homogenates is presented in Fig. 1. The result revealed that incubation of the testes tissue homogenates with 250 µM FeSO₄ caused a significant (p<0.05) increase in TBARS content. Several report have shown that Fe²⁺ can catalyze one-electron transfer reactions that generates reactive species, such as OH radical, formed from hydrogen peroxide (H₂O₂) via Fenton’s reaction, which could consequently degrade membrane lipids, generates peroxy and alkoxy radicals, and favours propagation of lipid oxidation (Akomolafe et al., 2015; Adedayo et al., 2015a, b). In this study, the extract caused a remarkable reduction in the TBARS produced, the effect that could be linked to the phenolic contents (Table 1 and 2).

Scavenging/chelation of free radicals and transition metals have been reported as some of the mechanisms by which antioxidant compounds could protect the cells/biomolecules from radical/metal induced oxidative damage (Valko et al., 2005; Flora, 2009; Kedare and Singh, 2011; Khan and Awan, 2014). To unravel some possible mechanisms by which the studied extract inhibits Fe²⁺ induced TBARs production in testicular tissues homogenate, the radicals (DPPH⁻ and ABTS⁻⁺) scavenging and Fe²⁺ chelating abilities of the extract were assessed. Both DPPH⁻ and ABTS⁻⁺ scavenging assay methods are based on spectrophotometry and are commonly used to determine the

**Fig. 1:** Inhibition of Fe²⁺ induced lipid peroxidation in rat testes tissue homogenate by the pumpkin methanolic extract and EDTA. The concentrations of the extract used for the plot of the graph are 0.83, 1.00, 1.25 and 1.67 mg/mL. The concentrations of the EDTA used for the plot of the graph are 1.56, 3.13, 4.69 and 6.25 µg/mL. Values represent mean ± standard deviation of triplicate experiments.

**Fig. 2:** DPPH radical scavenging ability of extract and Vit C. Values represent mean ± standard deviation of triplicate experiments.

**Fig. 3:** Fe²⁺ chelating ability of the extract and EDTA. Values represent mean ± standard deviation of triplicate experiments.
antioxidative ability of natural extracts based on their ability to scavenge/reduce the radical cation (Re et al., 1999; Kedare and Singh, 2011; Skotti et al., 2014). Several reports have revealed that excessive production of free radicals or ROS is one of the causes of male infertility which could be due to their deleterious effect on testes integrity/function (Aitken and Roman, 2008; Sankako et al., 2012; Agarwal et al., 2014). From our results, the assay on DPPH and ABTS radical scavenging abilities revealed that the extract could scavenge radicals. The result further revealed that the extract also chelated Fe²⁺ in a dose dependent manner. The radicals scavenging and Fe³⁺ chelating abilities could be among the mechanism of actions by which the studied extract prevented productions of TBARs, which if left uncheck could induce OS in the body (Adedayo et al., 2015a). The reducing power of the extract, expressed as ascorbic acid equivalent (AAE) could be via electron and/or hydrogen atom transfer ability of the phenolic compounds present in the extract (Chen et al., 2013; Al-Fartosy and Abdulwahid, 2015).

According to Allhorn et al (2005), ability of plant/food extract or natural compound to reduce metals could be a pointer to it potent anti-oxidation defense mechanism. Therefore, the polyphenolic compounds The reducing power of the extract, expressed as ascorbic acid equivalent (AAE) could be via to function as good electron/hydrogen atoms donor. Hence, the reducing power of the extract may have contributed to its protective effect observed.

CONCLUSION

In this study, the extract from the C. pepo fruit’s pulp was able to prevent/inhibit Fe²⁺ induced TBARS production in the rat testes homogenate in vitro. This ability could be as a result of its radicals scavenging and Fe³⁺ abilities, and ability to reduce Fe²⁺ to Fe³⁺. Consequently, it could be linked to the presence of polyphenolic compounds. However, further in vivo and clinical study should be carried out.

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