Journal of Applied Pharmaceutical Science Vol. 6 (09), pp. 115-120, September, 2016
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2016.60917
ISSN 2231-3354 

# 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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**ARTICLE INFO**

*Article history:*
Received on: 24/12/2015
Revised on: 11/04/2016
Accepted on: 07/06/2016
Available online: 26/09/2016

***Key words:***
Pumpkin seed, oxidative stress, testicular disease, Phenolic compounds.

**ABSTRACT**

Pumpkin seed has been associated with myriad of medicinal uses in different part of the world. In this study, phenolic composition and $Fe^{2+}$ 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, $Fe^{2+}$ 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 $Fe^{2+}$-induced TBARS, scavenge DPPH radical and chelate $Fe^{2+}$ in a dose dependent manner. The extract also scavenged ABTS radical and reduced $Fe^{2+}$ to $Fe^{3+}$. 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 induce 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 (Köksal *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).

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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 i*n-vitro.* The ability of the extract to scavenge radicals, chelates $Fe^{2+}$ and reduced $Fe^{2+}$ to $Fe^{3+}$ 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 powdered 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, W1) 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 FeSO4. 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 $K_2S_2O_8$ 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 $^oC$ 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*-hydroxybenzaldhyde (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.

| Parameter | Extract |
|---|---|
| Total phenolic content (mg GAE/g) | 32.90±3.03 |
| Total flavonoid content (mg QE/g) | 21.50± 0.90 |
| ABTS radical scavenging ability (mmol.TEAC/g) | 3.90±0.73 |
| FRAP (mg AAE/g) | 8.67±1.24 |

Values represent Mean ± Standard deviation of triplicate experiments.

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

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

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.

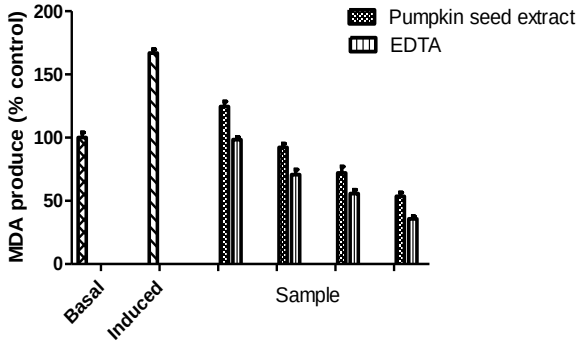


**Fig. 1:** Inhibition of $Fe^{2+}$ induced lipid peroxidation in rat testes tissue homogenate by the pumpkin methanolic extract and EDTA. The concentrations of the extract used for the plot of the graph are 0.83, 1.00, 1.25 and 1.67 mg/mL. The concentrations of the EDTA used for the plot of the graph are 1.56, 3.13, 4.69 and 6.25 µg/mL. Values represent mean ± standard deviation of triplicate experiments.

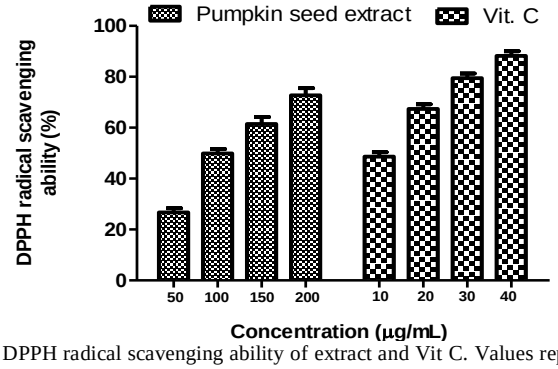


**Fig. 2:** DPPH radical scavenging ability of extract and Vit C. Values represent mean ± standard deviation of triplicate experiments.

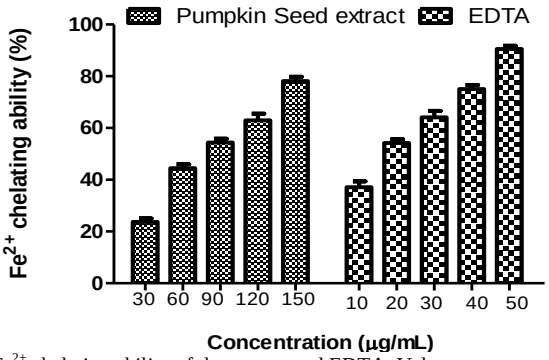


**Fig. 3:** $Fe^{2+}$ chelation ability of the extract and EDTA. Values represent mean ± standard deviation of triplicate experiments.

## 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 redox 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 $FeSO_4$ - 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 $FeSO_4$ caused a significant ($p$<0.05) increase in TBARS content. Several report have shown that $Fe^{2+}$ can catalyze one-electron transfer reactions that generates reactive species, such as OH radical, formed from hydrogen peroxide ($H_2O_2$) via Fenton's reaction, which could consequently degrade membrane lipids, generates peroxyl and alkoxyl radicals, and favour 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 $Fe^{2+}$ induced TBARs production in testicular tissues homogenate, the radicals ($DPPH^*$ and $ABTS^{*+}$) scavenging and $Fe^{2+}$ 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

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 $Fe^{2+}$ in a dose dependent manner. The radicals scavenging and $Fe^{2+}$ 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 $Fe^{2+}$ induced TBARS production in the rat testes homogenate *in vitro*. This ability could be as a result of its radicals scavenging and $Fe^{2+}$ abilities, and ability to reduce $Fe^{2+}$ to $Fe^{3+}$. Consequently, it could be linked to the presence of polyphenolic compounds. However, further in vivo and clinical study should be carried out.

**Financial support and sponsorship:** Nil.

**Conflict of Interests:** There are no conflicts of interest.

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**How to cite this article:**

Akomolafe SF, Oboh G, Oyeleye SI, Molehin OR, Ogunsuyi OB. 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. J App Pharm Sci, 2016; 6 (09): 115-120.