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In Vitro Antioxidant Activity and Nitric Oxide Scavenging Activity of *Citrullus Lanatus* Seeds.

Etim, O.E.[1*], Ekanem, S.E.[1]and Sam,S.M. [2]
1.Department of Biochemistry, Madonna University, Elele, Nigeria.
2.Department of Plant Science and Biotechnology, University of portharcourt, Portharcourt, Nigeria
*Corresponding Author E-mail:okprince25@yahoo.com; okprince25@gmail.com

Abstract
Citrullus lanatus (water melon) is a commonly consumed fruit worldwide .The seeds are often thrown away without considering its potential nutritional and medicinal benefits. In this study, a methanol extract of *citrullus lanatus* seeds was analyzed for the presence of bioactive secondary metabolites and to determine its ability to scavenge 2, 2 - diphenyl - 1 - picrylhydrazyl ($DPPH^{\cdot}$) radical, superoxide anion radical ($O_2^{\cdot -}$), and nitric oxide radical ($NO^{\cdot}$). Quantitative phytochemical analysis was done spectrophotometrically using standard methods. The result showed that the total phenol concentration was 0.96 ± 0.12 mg RE and flavonoid was 9.96 ± 0.86 mg GAE. The extract showed a potent DPPH radical scavenging activity by changing the extract spots from purple to yellow on the thin layer chromatographic (TLC) plate. At the concentration of 1000 µg/ml the extract had its maximum inhibition of 93.85 ± 4.73 % compared to that of ascorbate (95.70 ± 1.71 %).The extract also had a good O_2^{-} anion radical scavenging ability with IC_{50} = 44.65 ± 3.21 µg/mL compared to rutin (IC_{50} = 12.01 ± 2.59 µg/mL). In the 5 mM sodium nitroprusside (SNP) only medium the levels of nitrite and hence nitric oxide ($NO^{\cdot}$) was significantly higher ($p<0.05$) at every time interval compared to the observed levels in the presence of the *citrullus lanatus* seed extract. This therefore suggests that the *citrullus lanatus* seed could be a good source of antioxidants to ameliorate conditions in diseases whose pathogenesis implicates oxidative stress.
Keywords; *Citrullus lanatus,* oxidative stress, antioxidants,Scavenging activity,Metabolites.

1. INTRODUCTION

The use of plants, stem barks and root tubers for various purposes is a very important part of the tradition and culture in African countries. The study of plants particularly as sources of food and medicine for man's well being is one of man's oldest activities from the earliest times. Man had to distinguish between those plants which were poisonous and those which were not and gradually developed a knowledge of naturally occurring drugs which was passed for one generation to another (Trease and Evans, 1989). Traditional medicine is widely spread throughout the world. It is the total combination of knowledge and practice, whether explicable or not, used in diagnosing, preventing or eliminating a physical, mental or social disease.

Watermelon (*Citrullus lanatus)*, from the family of cucumber (*Cucurbitacea*), is a large, oval, round or oblong tropical fruit (Koocheki *et al*., 2007). The skin is smooth, with dark green rind or sometimes pale green stripes that turn yellowish green when ripe. It is a very rich source of vitamins and also serves as a good source of phytochemicals (Perkins-Veazie and Collins, 2004). The therapeutic effect of watermelon has been reported and has been ascribed to antioxidant compounds (Leong and Shui, 2002; Lewinsohn *et al*., 2005). Among them, citrullin and lycopene have been demonstrated to play a prominent role in the treatment and management of ailments such as cancer and cardiovascular diseases (Rimando and Perkins-Veazie, 2005).

Free radicals are unstable molecules that include the hydrogen atom, nitric oxide (NO) and molecular oxygen (O_2). This naturally occurs in the body as a result of chemical reactions during normal cellular processes. Reactive oxygen species (ROS), sometimes called active oxygen species, are various forms of activated oxygen, which include free radicals such as superoxide ions (O_2-) and hydroxyl radicals ($OH^{\cdot}$), nitric oxide radicals as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Halliwell, 1995). Since oxidative stress is reported to be a hallmark in the pathogenesis of some disease conditions, investigations is geared at assessing the free radical scavenging capacity of a methanol extract of *C. lanatus* seeds and its phenolic contents. This is aimed at providing a possible explanation for the ethno-pharmacological claims attributed to this fruit with particular emphasis on the seeds.

2. MATERIALS AND METHODS

2.1. Chemicals:

All the chemicals used in this study were of analytical grade. The solvents; ethanol, ethyl acetate and hexane were purchased from EMD Biosciences (Gibbstown, NJ). L-ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Fluka Chemicals; sulphuric acid, sodium nitroprusside (SNP), sodium nitrite, sulphanilamide, phosphoric acid, naphthylethylenediamine dihydrochloride, acetic acid, ferric chloride ($FeCl_3$), ethylenediaminetetraacetic acid (EDTA), phosphate buffered saline (PBS), polyvinylpolypyrrolidone, riboflavin, Folin-Ciocalteu's reagent (FCR) and trichloroacetic acid (TCA),Iodine crystal, potassium iodide,

mercuric chloride, bismuth carbonate, glacial acetic acid, ammonium solution, aluminium chloride, lead acetate, methanol, alpha-naphthol,chloroform, acetone, hydrogen chloride(HCl), aluminium trichloride, sodium acetate, methionine and NBT) were all purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Preparation of extracts

The *citrullus lanatus* seeds were identified by Dr.S. M.Sam, at the Department of Plant Science and Biotechnology, University of portharcourt, Portharcourt, Nigeria. The seeds were air-dried at room temperature and reduced to fine powder by milling. About 494.08g of the powdered seeds was soaked in 670ml of 80% methanol. The hydromethanolic extracts were concentrated using a rotary evaporator. Final weight was calculated as 4.3g with percentage yield of 0.87%. Extracts were dissolved in the appropriate solvent for the anti-oxidant assays.

2.3. Rapid DPPH radical scavenging assay using dot-blot

Qualitative screening of the methanol seed extracts for anti-oxidant activity (reflective of the phenolic content) was done using DPPH radical according to the method of Soler-Rivas *et al.*,2000 with slight modifications. Briefly an aliquot (5 µl) of each dilution of seed extract and standard anti-oxidant was carefully loaded on a piece of thin layer chromatography (TLC) plates (silica gel 60 F_{254}, Merck) and allowed to air dry. The sheets were then sprayed with DPPH (0.2 % (w/v) in methanol to reveal the anti-oxidant activity of the extract. The intensity of the yellow colour and the rate at which the colour of the extract spots changed from purple to yellow indicated the scavenging potential of the extract (Mosquera *et al.,* 2007) and its phenolic content.

2.4. Qualitative phytochemical analysis

Tests for flavonoids, tannins, carbohydrates/ glycosides, proteins, saponins, resins, steroids, terpenoids and alkaloids were carried out using standard methods (Harborne, 1973; Trease and Evans, 1989).

2.5. Determination of total phenolic contents

Total phenolics were determined using Folin-Ciocalteau reagent (FCR) as described by (Velioglu *et al.*,1998), with slight modifications. FCR consist of a yellow acidic solution containing complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids. Dissociation of a phenolic proton in a basic medium leads to a phenolate anion, which reduces FCR forming a blue coloured molybdenum oxide whose colour intensity is directly proportional to the phenolic contents.

2.6. Determination of tannin contents

Tannin content in each sample was determined using insoluble polyvinyl-polypirrolidone (PVPP), which binds tannins as described by Makkar *et al.*, 1993.

2.7. Determination of flavonoids and flavonols

The flavonoids content was determined according to the method described by (Kumaran and Karunakaran, 2006) with slight modifications. This method is based on the formation of a flavonoid-aluminum complex, which absorbs maximally at 415nm. The absorption of standard rutin solution (0.5 mg/ml) in methanol was measured under the same conditions. The amount of flavonoids in seed extracts in rutin equivalents (RE) was calculated by the following formula:

$$Flavonoid\ content = \frac{A \times m_o}{A_o \times m}$$

Where A is the absorption of seed extract solution, A_o is the absorption of standard rutin solution, m is the weight of seed extract, mg and m_o is the weight of rutin in the solution, mg. The flavonoid content is expressed in mg rutin equivalents/mg plant extract.

The content of flavonols was also determined as described by Kumaran and Karunakaran, 2006 with slight modifications. The amount of flavonols in plant extracts in rutin equivalents (RE) was calculated by the same formula for flavonoids.

2.8. In *vitro* anti-oxidant assays

2.8.1. Qualitative DPPH radical-scavenging assay using thin-layer chromatography

Qualitative screening for anti-oxidant activity was done using the DPPH radical according to the method of Takao *et al.*, 1994.

2.8.2. Quantitative DPPH radical-scavenging assay;

Scavenging activity on DPPH free radicals by the extract was assessed according to the method reported by Gyamfi *et al.*, 1999. DPPH radical scavenging activity was calculated using the equation:

$$\%\ Inhibition\ = 100\ \% \times \left(\frac{A_0 - A_s}{A_0}\right)$$

Where A_0 is the absorbance of the control, and A_s is the absorbance of the tested sample. The IC_{50} value represented the concentration of the extract that caused 50% inhibition of DPPH radical and was calculated by linear regression of plots, where the abscissa represented the concentration of tested sample and the ordinate the average percent of inhibitory activity from three replicates.

2.8.3. Superoxide radical ($O_2^{\cdot-}$)-scavenging assay;

This assay was based on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971) and the method used by Martinez *et al.*, 2001 to determined superoxide dismutase. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes containing reaction mixtures were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was estimated by comparing the absorbance of the control and those of the reaction mixture containing test sample as per the equation:

$$\%\ Inhibition\ = 100\ \% \times \left(\frac{A_0 - A_s}{A_0}\right)$$

Where A_0 is the absorbance of the control, and A_s is the absorbance of the tested sample.

2.8.4. Nitric **oxide radical ($NO^{\cdot}$) scavenging assay;**

Nitric oxide ($NO^{\cdot}$) generated from sodium nitroprusside (SNP) was measured according to the method of Marcocci *et al*., 1994. Briefly, the reaction mixture (5.0ml) containing SNP (5mM) in phosphate buffered saline (pH 7.3), with or without the seed extract at different concentrations, was incubated at 25^{o}C for 180min in front of a visible polychromatic light source (25 Watt tungsten lamp). The NO radical thus generated interacted with oxygen to produce the nitrite ion (NO_2^-) which was assayed at 30 min intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% N-naphthylethylenediamine dihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was measured at 546nm. The nitrite generated in the presence or absence of the seed extract was estimated using a standard curve based on sodium nitrite solutions of known concentrations.

3. Statistical Analysis

Data obtained from this study were analyzed using the statistical package for social sciences (SPSS) version 18.0 for windows. Analysis of variance (ANOVA) were used to compare means, and values were considered significant at $p<0.05$. Posts hoc multiple comparisons for the ANOVA were done using least significant difference (LSD).

4. RESULTS

4.1. Quantitative Analysis on Phytochemical Constituents

The water melon seed extract was found to be rich in bioactive secondary metabolites. The total phenol concentration was 0.96 ± 0.12 mg gallic acid equivalents (GAE) / mg of dry weight extract, non–tannins phenolics 0.14 ± 0.05 mg gallic acid equivalents / mg of dry weight extract, tannins 0.82 ± 0.10 mg gallic acid equivalents (GAE) / mg of dry weight extract. High levels of flavonols and flavonoids were also determined in the seeds (39.56 ± 6.47 and 9.96 ± 0.86 mg rutin equivalents (RE) / g dry weight extract respectively) (Table 1).

Table1; Secondary metabolites in methanol extract of water melon seeds

Phytochemical Constituent	Content
Total Phenolic ‡	0.96 ± 0.12
Non-tannins ‡	0.14 ± 0.05
Tannins ‡	0.82 ± 0.10
Flavonol *	39.56 ± 6.47
Flavonoid *	9.96 ± 0.86

Data represented as Mean ± SD (n = 3).
‡ Expressed as mg gallic acid equivalents / g dry weight extract.
* Expressed as mg rutin equivalents / mg dry weight extract.

4.2. *In vitro* Free Radical Scavenging Activities

4.2.1. Thin Layer Chromatography (TLC) Dot Blot for DPPH Radical Scavenging Capacity of *Citrullus lanatus* Seeds

As shown on the thin layer chromatographic (TLC) plate in Fig.1 below, the water melon seed extract showed a potent DPPH radical scavenging activity by changing the extract spots from purple to yellow. The faster the colour of the spot changes to yellow and the intensity of the yellow spot indicated higher anti-oxidant activity.

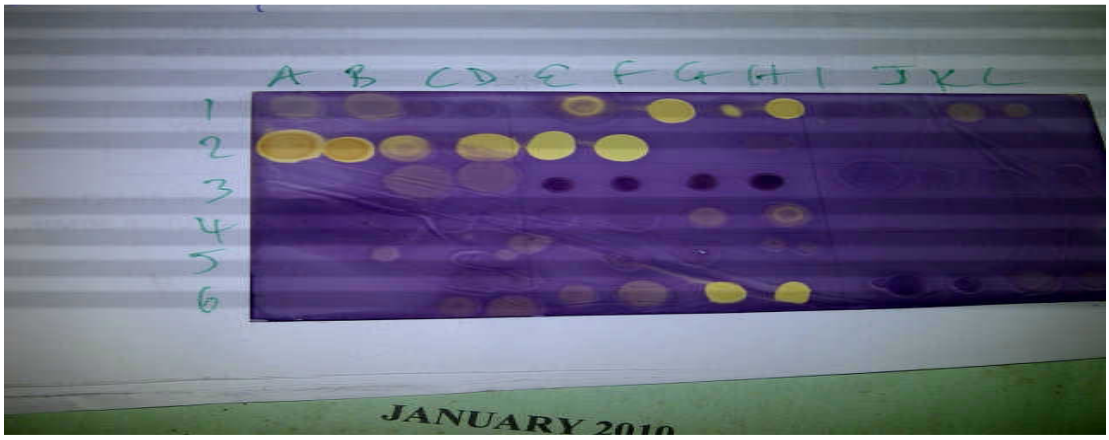

Figure 1: Dot blot of radical scavenging capacity of extracts

4.2.2. Scavenging Effect of Methanol Extract of Water Melon Seeds on DPPH· Radicals

As shown in Fig.2 below, the methanol extract of water melon seed extract inhibited DPPH radical in a concentration dependent manner. At the concentration of 1000 µg/ml the extract had its maximum inhibition of 93.85 ± 4.73 % compared to that of ascorbate (95.70 ± 1.71 %) at the same concentration.

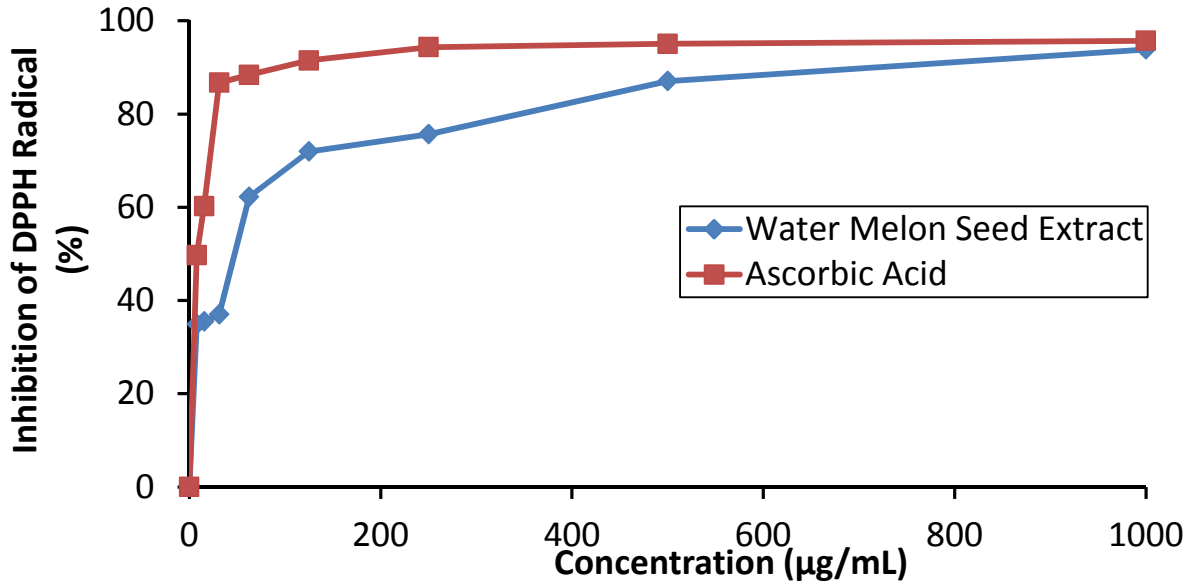

Figure 2: Concentration dependent inhibition of DPPH radical by water melon seed extract compared to ascorbic acid.

4.2.3. Scavenging Effect of Methanol Extract of Water Melon Seeds on Superoxide Anion Radicals ($O_2^{\cdot-}$)

The superoxide anion radical ($O_2^{\cdot-}$) was inhibited by the water melon seed extract and rutin in a dose-related manner as shown in Fig. 3. The extract had a good O_2^{-} anion radical scavenging ability with IC_{50} = 44.65 ± 3.21 µg/mL compared to rutin (IC_{50} = 12.01 ± 2.59 µg/mL). The extract inhibited the radical maximally at a concentration of 500µg/mL with a % inhibition of 87.99 ± 1.74 % compared to rutin 94.00 ± 0.64 %.

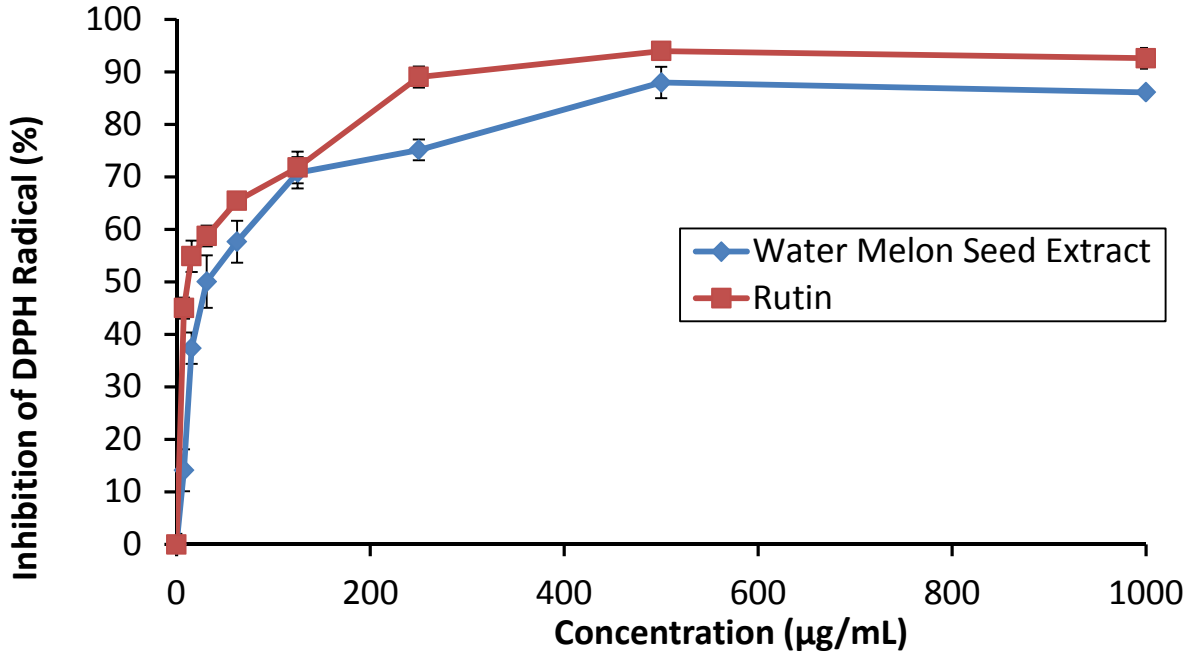

Figure 3: Inhibitory effect of water melon seed extract on superoxide anion radical ($O_2^{\cdot-}$) compared to rutin

Scavenging Effect of Water Melon Seed Extract on Nitric Oxide ($NO^{\cdot}$) Production

The nitric oxide level in all the assay media increased with time as depicted by the increase in nitrite levels. In the 5 mM sodium nitroprusside (SNP) only medium the levels of nitrite and hence nitric oxide ($NO^{\cdot}$) was significantly higher ($p<0.05$) at every time interval compared to the observed levels in the presence of the water melon seed extract (Fig.4).

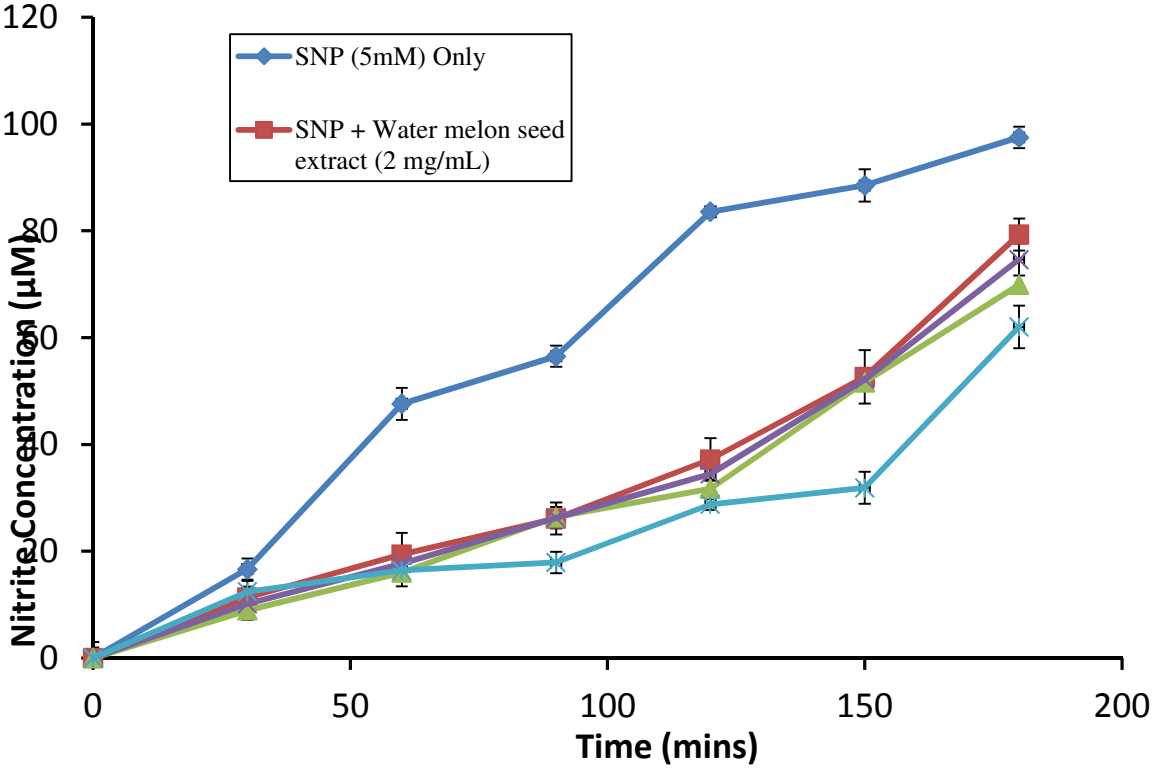

Figure 4: Effect of methanol extract of water melon seeds on the accumulation of nitrite upon decomposition of sodium nitroprusside (SNP; 5 mM) at 25°C.

5. DISCUSSION AND CONCLUSION;

Medicinal plants have become extremely popular all over the world as antioxidants and in recent past several dietary and herbal formulation, which have free radical scavenging potential are relevant in the treatment of chronic diseases (Tiwari and Tripathi, 2007). In biological systems, reactive oxygen species are continuously generated and they play a role in the pathogenesis of many disease conditions by causing extensive damage to biomolecules and tissues (Eze, 2006; Catala, 2009).

The extract showed a potent DPPH radical scavenging potential. The addition of the methanol extract of *C. lanatus* and standard to the DPPH solution caused a rapid decrease in absorbance at 518nm indicating good scavenging capacity of the extract. Phytochemical analysis showed high flavonol contents in the extract (Table 1) suggesting, therefore, that the antioxidant compounds in the extract and standard neutralized the free radical character of DPPH by transferring either electrons or hydrogen atoms to DPPH (Naik *et al*., 2003), thereby changing the color from purple to the yellow colored stable diamagnetic molecule diphenyl picrylhydrazine. Figure 2 also showed that the compounds possessed substantial dose-dependent antioxidant activity with IC_{50} = 36.04 ± 3.14 µg/mL. These therefore suggest that, the extract could be used as a natural antioxidant source to limit free radical damage occurring in the human body.

Superoxide anion (O_2-) radical is also very harmful to cellular components (Muller *et al*., 2007). The seed extract was observed to be an efficient scavenger of superoxide anion radical. The seed extract was observed to be an efficient scavenger of superoxide anion radical. The O_2- radical scavenging activities was inhibited by the seed extract and rutin at 560nm in a dose-related manner as shown in Figure 3. The extract had a good O_2- anion radical scavenging ability with IC_{50} = 44.65 ± 3.21 µg/mL compared to rutin (IC_{50} = 12.01 ± 2.59 µg/mL. The extract inhibited maximally at 500 µg/mL, with a percentage inhibition of 87.99 ± 1.74 % compared to rutin 94.00 ± 0.64%. The scavenging potential may possibly be dependent on the number and locations of the hydroxyl groups in the phenolic compounds present in the extract (Khanduja *et al*., 2006).

The evidence of the radical scavenging potential of the extract was further compared by investigating its ability to scavenge nitric oxide ($NO^{\cdot}$) production. Despite the possible beneficial effects of $NO^{\cdot}$, its contribution to oxidative damage is increasingly becoming evident. This present study shows that the methanol extract of *C. lanatus* seeds has more potent nitric oxide scavenging activity than the standard sodium nitroprusside (SNP) at 546nm. The nitric oxide level in all the assay media increased with time as depicted by the increase in nitrite levels. In Figure 4, the 5mM SNP only medium was significantly higher in nitrite concentrations ($p<0.05$) at every time interval compared to the observed levels in the presence of the *C. lanatus* seed extract.

This study, therefore, shows that the methanol extract of *C. lanatus* seeds exhibited high antioxidant and free radical scavenging activities indicating that the plant is a significant source of natural antioxidant and its potential could, to a greater extent, be attributed to its phenolic contents. This therefore suggests that the water melon seeds though always discarded by many after consuming the flashy part of this fruit could be a good source of antioxidants to ameliorate conditions in diseases whose pathogenesis implicates oxidative stress (Spiteller, 2006).

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