

COMPARISON OF SOME *in-vitro* ANTIOXIDANT PROPERTIES OF *Cola acuminata* AND *Cola nitida* FRUIT TESTAS

ABSTRACT

Testas of *Cola* fruit are usually considered as agricultural waste by many farmers as their focus is always on the seed because of its economic importance. This study therefore aimed at evaluating and comparing the antioxidant potentials of *C. acuminata* and *C. nitida* fruit testas. The methanol extract of the fruit testas of *C. acuminata* and *C. nitida* were evaluated and compared for their antioxidant properties such as: their scavenging abilities on hydroxyl radicals, ABTS (2, 2- Azino-bis-3-ethylbenzothiazoline-6-sulphonic Acid), DPPH (2, 2-diphenyl-2-picrylhydrazyl) and nitric oxide. Also their ferric reducing ability and total antioxidant were investigated. In all the antioxidant parameters assayed, the fruit testas of *C. acuminata* and *C. nitida* compared favorably well with the standard (Butylated Hydroxytoluene) as they both showed positive correlation with the standard. The antioxidant ability of the methanol extract of *C. nitida* was significantly lower compared with that of *C. acuminata* in all the parameters tested. This can be attributed to the hydrogen atom donating potentials of the testas which can be as a result presence of vital secondary metabolites in therapeutic mopping up of free radicals in form of reactive oxygen species (ROS) and reactive nitrogen species (RNS)

Keywords: Antioxidants, Hydroxyl radicals, DPPH, testas, therapeutic.

1.0 INTRODUCTION

Many health related problems such as some pathological conditions which present cardiovascular disease, cancer, neurological disorder, diabetes, other diseases and aging have been attributed to oxidative stress. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the major free radicals causing harm to the bio-system as they are directly or indirectly responsible for several of these degenerative ailments in human [1]. Diseases such as diabetes are responsible for the generation of reactive oxygen species which is as a result of the complications developed such as neuropathy, retinopathy, cardiovascular disease. This may result in cell damage through oxidative stress [1][2].

Blocking the process of oxidative stress requires the engagement of antioxidants which are well-known for their activity in neutralizing the effect of the presence of free radicals in the bio-system. This is achieved by the interruption of the chain reactions where free radicals changes other less-harmful molecules to free radicals like themselves [3]. Oxidative enzymes like superoxide dismutase, catalase, some chemicals like ascorbic acid, carotenoids glutathione are known to be responsible for protection of the bio-system against cell damage from free radicals and when their functions are impaired, it results in disease and accelerated aging [4]. Highly reactive oxygen and nitrogen species damages human health by breaking the bonds in DNA molecules hence, altering the cell's genetic apparatus and subsequently resulting in proliferation of cancerous cells.

In recent times, many plant wastes from agricultural processes are now found to be of economic importance. For instance, the kolanut husk is proven to be good raw material for the formulation of animal feeds [5]. Many plant products are good natural source of many antioxidants at varying quantities, examples of which include: phenols, quinines, tocopherols, gallic acid, gallates e.t.c. [6][7].

Cola which has its origin from West Africa, is from the family sterculiaceae and the fruit has a shape like capsule, fleshy with irregular-shaped pink, red or white seeds. The seeds may turn brown when dried [8]. *Cola* is consumed culturally both by individuals or group settings and often for ceremonies such as weddings, funerals and entertainment of tribal chiefs in many parts of Western and sub-Saharan Africa precisely North and Eastern Nigeria, Ivory Coast, Sierra Leone [8][9]. In nutrition, the seed has been used as soft drink additives while in ethnomedicine, the seed is used in managing digestive impairment, food poisoning and even sometimes asthma [10][11].

For several decades the seeds of *Cola* have been the center of attraction in *Cola* fruit because of its wide usage and economic value. There is need to explore other part of the fruit which most times have been regarded as waste in the process of post-harvest handlings. Hence, this study's objective is to determine and compare the antioxidant properties of the fresh *C. acuminata* and *C. nitida* fruit testas.

2.0 METHODS:

2.1 Sample Preparation: The pods of *C. acuminata* and *C. nitida* fruits were opened after which the seeds were removed, and then washed with distilled water to remove unwanted particles. The seeds were soaked in water for few days then the testas were removed and air-dried at room temperature under the shade to avoid loss of bioactive components through irradiation. The dried testas were pulverized to fine powder using high speed blender and finally extracted using methanol as solvent. 10g of the pulverized testa was mixed with 100ml of methanol at room temperature for 24hours after which the supernatant was removed. The residue was re-extracted under the same conditions until the solvent became colorless. The supernatant was then filtered through whatman No. 1 filter paper. The filtrate was made to pass through a rotary evaporator to remove the methanol solvent at 40 °C. The methanolic extract was dried and stored in the refrigerator for further analysis.

2.2 Determination of 2, 2-diphenyl-2-picrylhydrazyl Scavenging Ability: The determination of DPPH (2, 2-diphenyl-2-picrylhydrazyl) radical scavenging activities was evaluated based on the method described by Singh *et al.* [12]. Methanol extracts of the *Cola* testas (1ml) of the different concentrations (i.e., 25, 50, 100, 200, 400, 800, and 1000 $\mu\text{g}/\text{mL}^{-1}$) made by reconstituting in respective solvents were added to DPPH solution (5mL, 0.1mM) in methanol and mixed. After 20 minutes of reaction at 25°C, the absorbance was measured at 517nm against a blank (methanol) in a UV-Vis spectrophotometer. Methanolic DPPH solution (5ml) without antioxidant was used as control. The DPPH scavenging activity of the extract was expressed as IC₅₀ (inhibitory concentration), that is, the concentration of the extract at which DPPH radicals were scavenged by 50%. Butyl hydroxy toluene (BHT) was used as standard antioxidant.

The percentage quenching of DPPH was calculated as follows:

$$\text{DPPH Quenching Capacity (\%)} = \frac{(\text{Abs}_{\text{control}}) - (\text{Abs}_{\text{sample}})}{(\text{Abs}_{\text{control}})} \times 100$$

Where: Abs_{sample} is the absorbance of the sample (extract and standard antioxidant) and Abs_{control} is DPPH solution without the added extract.

2.3 Determination of Scavenging Capacity on Nitric Oxide Radical

Nitric oxide (NO) generated from sodium nitroprusside (SNP) in aqueous solution at physiological pH was estimated by the use of the Griess reaction [13]. The reaction mixture (3ml) containing SNP (10mM, 2ml), phosphate buffer saline (0.5ml, pH 7.4), and the extracts (0.5ml) at different concentrations (25, 50, 100, 200, 400, 800, and 1000 $\mu\text{g}/\text{mL}^{-1}$) were incubated at 25°C for 150min. After incubation, 0.5mL of the incubated solution containing nitrite was pipetted and mixed with 1mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1ml of N-(1-naphthyl) ethylenediamine dihydrochloride was added, mixed, and allowed to stand at 25°C for 30 min. The absorbance of pink colored chromophore formed during diazotization was measured at 540nm. The NO scavenging activity of the extract was expressed as

IC₅₀ (inhibitory concentration), that is, the concentration of the extract at which NO radicals were quenched by 50%. Butyl hydroxy toluene (BHT) was used for comparison.

$$\text{Nitric Oxide Scavenging Capacity (\%)} = \frac{(\text{Abs}_{\text{control}}) - (\text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100$$

Where: Abs sample is absorbance of the sample and Abs control is absorbance of control.

2.4 Determination of Ferric Reducing Ability

Ferric reducing capacity was determined according to Oyaizu [14]. From the solution (25, 50, 100, 200, 400, 800, and 1000 μgml^{-1}) of the *Cola testas* extract, 0.5ml portion was mixed with 2.5ml of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5mL of potassium ferricyanide [$\text{K}_3\text{Fe}_3(\text{CN})_6$] (1%) in test tubes. The resulting solution was mixed and incubated at 50°C for 20 min. Then, 2.5ml of trichloroacetic acid (TCA) (10%, w/v) was added to all tubes and the solutions were centrifuged at 3000 $\times g$ for 10 min. The aqueous solution of FeCl_3 (1%, 1 ml) was diluted by adding deionized water (5 ml). To this solution, the upper layer of the centrifuged solution (5 ml) was mixed and incubated at 35°C for 10 min. Absorbance of the developed color was measured at 700nm. BHT was used as standard. Reducing capacity of the *Cola testas* extracts towards Fe^{3+} was interpreted from relation of absorbance and reducing capacity: when absorbance increases, reducing capacity also increases.

2.5 Determination of Hydroxyl Radical Scavenging Ability

The hydroxyl radical scavenging assay was performed by the method described by Klein *et al.* [15]. The testas methanolic extracts and BHT were dissolved separately in DMSO and water. 1 ml of each extracts with various concentrations was mixed with 1 ml of iron-EDTA solution (0.1% ferrous ammonium sulfate and 0.2% EDTA in a ratio of 1:1). After mixing, 0.5ml of 0.02% EDTA solution and 1ml of DMSO were added.

The reaction was initiated by adding BHT (0.2%) and incubated at 90° C in a water bath for 20 min. Then, 2ml of 15% TCA was added to the termination of the reaction. 2ml of Nash reagent (ammonium acetate (75g), 3ml of glacial acetic acid, and 2 ml of acetylacetone were mixed, and volume was made up to 1L with distilled water) was added and kept at room temperature for 20 min. The reaction mixture without the testas extracts served as control, and BHT was used as standard. All the experiments were done in triplicates. A UV-VIS spectrophotometer measured the absorbance of the reaction mixture at 412 nm against a blank solution. The percentage inhibition was calculated from the standard, and the IC₅₀ value was determined.

2.6 Determination of 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6 Sulphonic Acid (ABTS) Scavenging Ability

The ABTS radical scavenging ability of *Cola acuminata* and *Cola nitida* fruit testas extracts were determined by method used by Zhou *et al.* [16]. A solution of ABTS radical cation was prepared by reacting 7mM ABTS with 2.45 mM potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) at room temperature in the dark for 16 hours. The mixture was then diluted with deionized water to obtain an absorbance of 0.700 ± 0.005 at wavelength of 734nm. The sample solution (100 μl) at a range of concentrations was mixed with the ABTS⁺ solution (100 μl) in 96-well plates. After incubation at 30 °C for 30 min in the dark, the absorbance at wavelength of 734 nm was immediately recorded. BHT was used as control. The activity of ABTS⁺ radical scavenging was calculated.

2.7 Determination of Total Antioxidant Capacity

The total antioxidant capacities of the testas were determined using the thiocyanate method [17]. Briefly, 10mg of the methanolic extract of the *Cola acuminata* and *Cola nitida* fruit testas were dissolved in 10ml ethanol. Then, 100 or 250 $\mu\text{g/ml}$ of each testas extract or a standard sample in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5ml of linoleic acid emulsion in

potassium phosphate buffer (0.04 M, pH 7.0). Alternatively, a 5ml control solution was prepared consisting of 2.5ml linoleic acid emulsion and 2.5ml potassium phosphate buffer (0.04 M, pH 7.0). Each solution was incubated at 37 °C in a glass flask in the dark. After reaction with FeCl₂ and thiocyanate, the peroxide values were determined at 3 minutes intervals by reading the absorbance at 500nm in a spectrophotometer. During the linoleic acid oxidation, peroxides form and oxidize Fe²⁺ to Fe³⁺. The Fe³⁺ ions form a complex with SCN⁻ which has a maximum absorbance at 500nm. Therefore high absorbance indicates high linoleic acid oxidation. The solutions without extract were used as blanks. All antioxidant activity data were reported as the average of triplicate analyses. The inhibition of lipid peroxidation in % was calculated by following equation:

$$\% \text{ Inhibition} = 100 - [(A_1/A_0) \times 100]$$

Where; A₀ was the absorbance of the control reaction and A₁ was the absorbance in the presence of the testa extract.

2.8 Statistical Analysis

All the experimental results were expressed as mean ± SEM of three replicates. Data were evaluated using one way variance analysis. *P* values < 0.05 were regarded as significantly different.

3.0 RESULT AND DISCUSSION

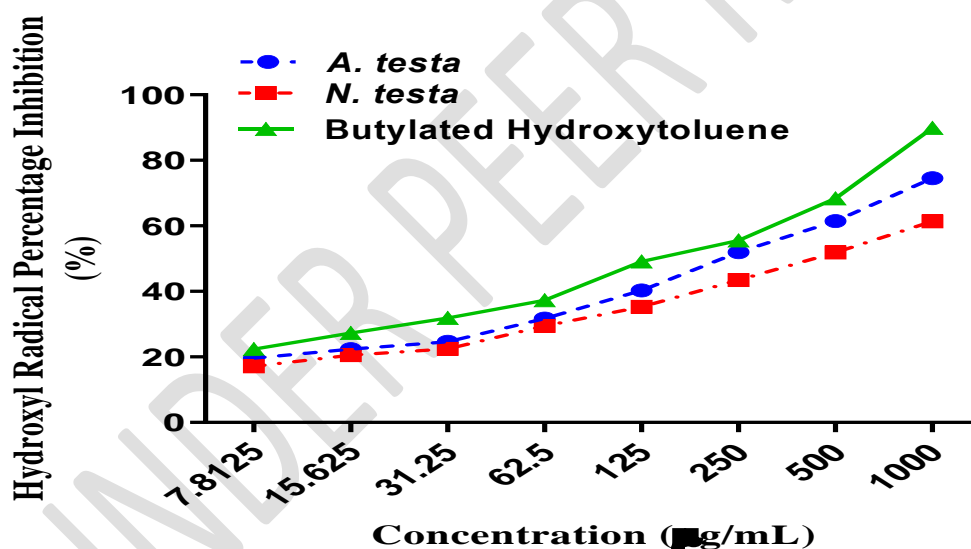


Figure 1: In-vitro Hydroxyl Radical Scavenging Ability of testas of *Cola acuminata* and *Cola nitida* Fruits

Values are expressed as mean of three replicates ± Standard Error of Means

Key: *A. testa* = *Cola acuminata* seed testa

N. testa = *Cola nitida* seed tetsa

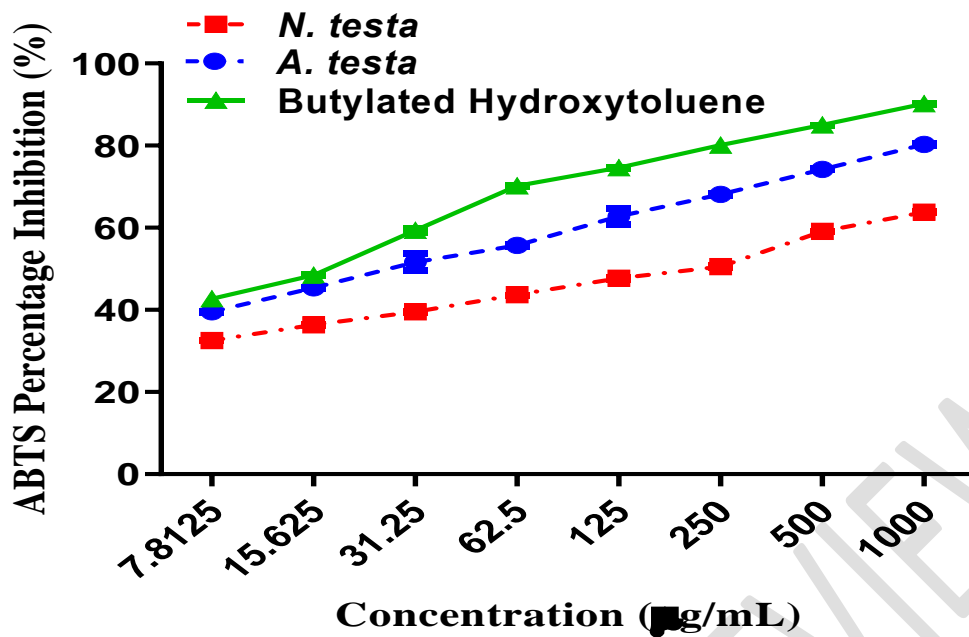


Figure 2: ABTS (2,2¹-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) Scavenging Ability of testas of *Cola acuminata* and *Cola nitida* Fruits

Values are expressed as mean of three replicates \pm Standard Error of Means

Key: A. testa = *Cola acuminata* seed testa

N. testa = *Cola nitida* seed tetsa

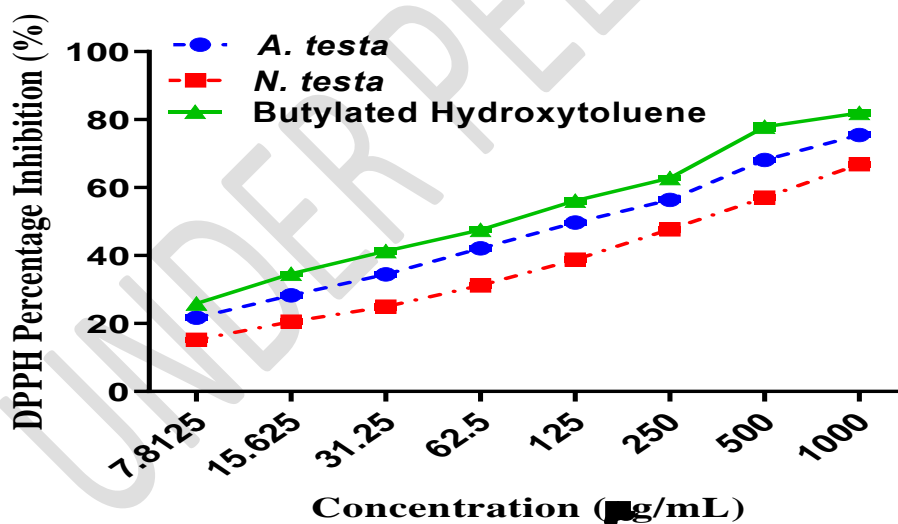


Figure 3: DPPH (2, 2-diphenyl-2-picrylhydrazyl) Scavenging Ability of testas of *Cola acuminata* and *Cola nitida* Fruits

Values are expressed as mean of three replicates \pm Standard Error of Means

Key: A. testa = *Cola acuminata* seed testa

N. testa = *Cola nitida* seed tetsa

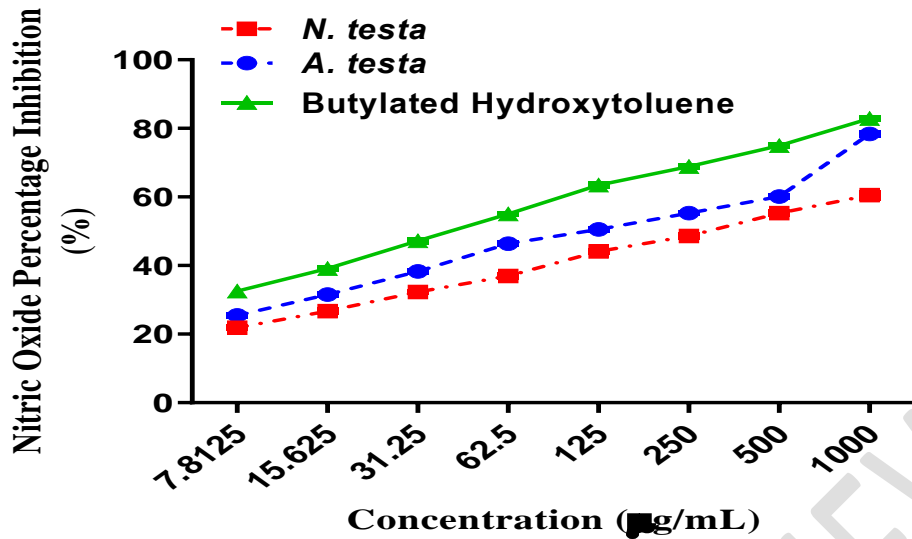


Figure 4: Nitric Oxide Scavenging Ability of *Cola acuminata* and *Cola nitida* Fruits

Values are expressed as mean of three replicates \pm S.E.M

Key: A. testa = *Cola acuminata* seed testa

N. testa = *Cola nitida* seed tetsa

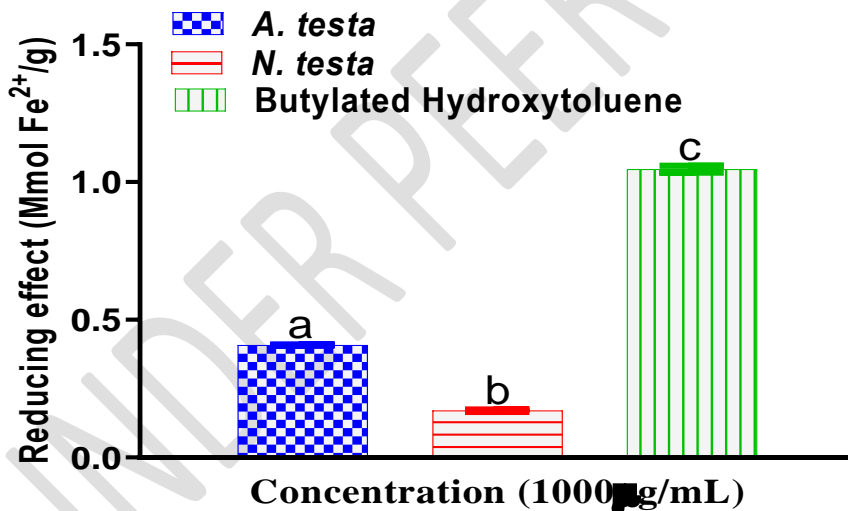


Figure 5: Ferric Reducing Ability of testas of *Cola acuminata* and *Cola nitida* Fruits

Values are expressed as mean of three replicates \pm S.E.M and bars with different alphabets are significantly different ($P < 0.05$) from each other.

Key: A. testa = *Cola acuminata* seed testa

N. testa = *Cola nitida* seed tetsa

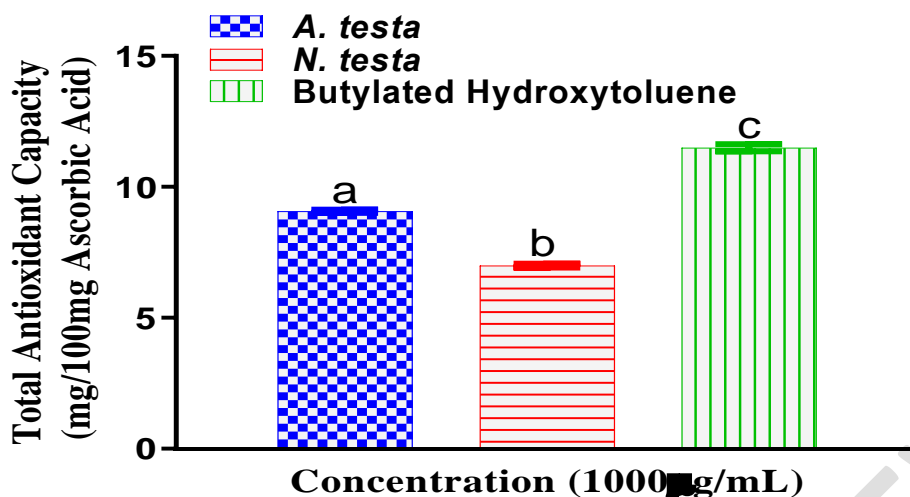


Figure 6: Total Antioxidant Capacity of testas of *Cola acuminata* and *Cola nitida* Fruits

Values are expressed as mean of three replicates \pm S.E.M and bars with different alphabets are significantly different ($P < 0.05$) from each other.

Key: A. testa = *Cola acuminata* seed testa
N. testa = *Cola nitida* seed tetsa

The antioxidant parameters; *in-vitro* inhibitory capacity of the testas of *C. acuminata* and *C. nitida* fruits on Hydroxyl Radical, ABTS (2,2¹-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid), DPPH (2, 2-diphenyl-2-picrylhydrazyl) and Nitric Oxide investigated in this study were found to have a correlating progression with that of the Butylated Hydroxytoluene standard likewise their ferric reducing and total antioxidant capacities. Although the testas inhibitory effects on these parameters are not significantly as high as that of the standard Butylated Hydroxytoluene but testa of *C. acuminata* showed significant increase in all the parameters assayed for when compared with that of *C. nitida*. Hydroxyl radical is important reactive oxygen specie which can react with some other biomolecules such as polyunsaturated fatty acid units in phospholipids of the cell membrane subsequently leading to cell damage and responsible for lipid peroxidation, protein oxidation and disruption of the DNA [18][19]. The hydroxyl radical scavenging power of these testas significantly increases with increase in their concentrations.

ABTS is commonly known for its use by many food and agricultural research based industries to estimate the antioxidant potentials of foods because the radical cation reacts to most antioxidants such as phenolic, thiol and vitamin C also for determining the antioxidant capacity of substances such as phenolics, carotenoids e.t.c [20][21][22]. DPPH is one of the most important molecules for the *in vitro* antioxidant assays. It is known to have hydrogen acceptor capability. The hydrogen atom donating capability of the extracts of the testas was estimated by the decolorization of methanol solution of DPPH. Nitric oxide on the other hand is important for the physiologic role it plays in the generation of reactive nitrogen species which is known for its cell injury or death causing ability by induction of nitrosative stress [23]. Nitric oxide reacts with superoxide anion radical ($O_2^{\cdot -}$) to produce peroxynitrite anion ($ONOO^-$) a Reactive Nitrogen Species. The inhibitory effect of the testas of both *C. acuminata* and *C. nitida* on the hydroxyl radical, DPPH, ABTS and Nitric oxide compared

favourably well with the results of some of the positive antioxidant properties as reported by Olorunfemi *et al.*[24] and Sudha *et al.*[25].

4.0 CONCLUSION:

From the present study, the antioxidant properties of the extracts of *C. acuminata* and *C. nitida* fruit testas showed a positive correlation with the standard they were compared with which is the butylated hydroxytoluene. This may be attributed to strong hydrogen donating ability and scavenging ability of the testas on free radicals which are important precursors for the generation of reactive oxygen species and reactive nitrogen species. Hence, these testas can serve as potential precursor for discovery of novel drugs in the pharmaceutical industry.

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