

## In Vitro Antioxidant Potentials of Ethanol Extract of Unripe and Ripe *Dennetia tripetala* (DT) Fruits.

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### ABSTRACT

*Dennetia tripetala* fruit is a popular Nigerian fruit from the family of plant known as Annonaceae. The whole fruit (flesh and seed) is usually consumed as snacks and it is oftentimes consumed with local gin (ufop in Ibibio or kaikai in Igbo) or added to dishes as spice due to its peculiar strong pepperish taste and sweet aroma. The present study is aimed at evaluating the antioxidant potentials of ethanol extract of ripe and unripe DT fruit *in vitro*. The antioxidant activity of the ethanol extract of DT was evaluated spectrophotometrically using various *in vitro* models like 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and hydrogen peroxide scavenging activity; metal chelating activity and reducing power. Vitamin C was used as the standard antioxidant. Unripe and ripe DT fruits, as well as vitamin C showed a competitive inhibition of DPPH and H<sub>2</sub>O<sub>2</sub> free radicals. As concentration of the extracts increased from 20 to 100 µg/ml, the % scavenging activity for vitamin C increased from 87.86 ± 0.11 to 90.66 ± 0.07 and for ripe DT fruits from 15.15 ± 0.24 to 25.52 ± 0.23, while for unripe, fruits values increased from 12.09 ± 0.35 to 23.06 ± 0.12. The IC<sub>50</sub> values was highest in unripe (549.23) followed by ripe (276.63) and lowest in vitamin C (12.92) indicating that vitamin C was the best scavenger of DPPH radical. Similar trend was obtained for H<sub>2</sub>O<sub>2</sub> scavenging activity as well as reducing power. Unripe DT fruit extract was more potent at chelating metal ions (IC<sub>50</sub> was 95.38), followed by the standard ascorbic acid with IC<sub>50</sub> of 97.03 and was lowest in ripe DT fruit extract with IC<sub>50</sub> value of 124.66. Unripe and ripe DT are potent antioxidants in nature and may be used to supplement our diets as rich sources of natural antioxidants for health protection.

**Key words:** Antioxidant, DPPH, hydrogen peroxide, metal chelating activity, reducing power

### 1. INTRODUCTION

Plants remain a common and readily available source of food and medicine either in the form of traditional concoction or pure active agents. Much of the observed therapeutic effects of plants have been linked to their potential antioxidant activity (Kasote et al. [1]). It is documented that two-thirds of the World's plant species have medicinal value [2] and almost all of the plants have potent antioxidant potentials [3]. According to literature, plants have a very efficient complex enzymatic and non-enzymatic antioxidant defense system to prevent free radical mediated toxic effects. Enzymatic antioxidant defense system includes glutathione peroxidase, catalase and superoxide dismutase; while non-enzymatic antioxidant include glutathione, vitamins A, E and C [4]. These antioxidants in plants play dual functions as they help in the performance of their normal physiological functions and protection from microbes, as well as help plants cope with environmental stress [5].

Plant phenolics perform antioxidant roles by acting as electron donors. This is due to the presence of multiple hydroxyl ions; they are capable of donating hydrogen ions to stabilize unstable free radicals. Phenolics also have reducing power, metal ion chelating activity [6] and the ability to act as signaling molecules in cells through the modulation of protein kinase and lipid

kinase signaling pathways [7,8]. In phyto research, *in vitro* investigations are often made for two reasons namely; to screen and attribute antioxidant potential to plants as their secondary metabolites and to understand the possible mechanism of action of plant antioxidants [9]. Various assays are used to evaluate plants abilities as electron donor, reducing agents and metal chelators. Example of these assays include DPPH scavenging potential, metal chelating activity, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, ferric reducing potentials etc.

Pepper fruit (*Dennettia tripetala*) is widely cultivated by the people of Cameroun, Ivory Coast and Southern Nigeria. In Nigeria, it is called mmimi in Igbo, Ako in Edo, Ata Igberere in Yoruba and nkarika in Ibibio.



**Figure 1: (A) *Dennettia tripetala* tree with leaves and unripe fruits. (B) Ripe (red) and unripe (green) *Dennettia tripetala* fruit [28]**

Pepper fruits are green when unripe and red when ripe. Phytochemical screening of the fruits revealed the presence of tannins, flavonoids, terpenes, alkaloids, cardiac glycosides etc. It is traditionally used for the treatment of cough, toothache, fever, diabetes and nausea during pregnancy. Its antioxidant, anti-hyperglycemic, anti-inflammatory and anti-inociceptive effects have been established *in vivo* [10,11,12]. The aim of this study was to evaluate the anitioxidant potentials of ethanol extract of unripe and ripe DT fruits.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

Chemicals used in the study were ascorbic acid, butylated hydroxy toluene(BHT), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ferric chloride, sodium phosphate, ammonium molybdate, ferrous ammonium sulphate, ethylenediaminetetraacetic acid (EDTA), dimethyl sulphoxide(DMSO), ammonium acetate, glacial acetic acid, trichloroacetic acid, sodium dihydrogen phosphate, disodium hydrogen phosphate and ferrozine. All the chemicals were purchased from British Drug House (BDH) England and all solvents used were of analytical grade.

### 2.2 Collection and Preparation of Plant Sample

Ripe and unripe fruits of *Dennettia tripetala* were obtained between March to April 2018 from Marian Market, Calabar, Cross River State. The samples were authenticated by a Botanist in the Department of Botany and Ecological Studies, University of Uyo, Akwa Ibom State. Unripe fruits of *Denettia tripetala* were ground and dried at room temperature. The same was done for the ripe fruits of *Denettia tripetala*. The ground sample was stored in an air-tight container until required for use.

### 2.3 Extraction of *Dennettia tripetala* fruits

Ground fruits of ripe and unripe *Dennettia tripetala*(500 g) were extracted by macerating in 2125ml of 80% ethanol for 72 hr and filtered. The filtrate was concentrated in a water-bath at 45°C to dryness. It was weighed and stored in a refrigerator at -4°C until required for use.

### 2.4 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) Determination

Free radical scavenging activities of ethanol extract of *Dennettia tripetala* fruit and vitamin C were prepared at concentrations of 20, 40, 60, 80 and 200 µg/mL and were determined in accordance with the Shimada *et al.* [13] method, which is based on the principle of scavenging the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical. DPPH was added to the solutions prepared and stirred vigorously. The mixtures were kept in the dark for 30 min and the absorbance was measured at 517 nm against a blank [13]. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging activity(\%)} = [A_0 - A_1] / A_0 \times 100 \text{ where}$$

$A_0$  is the absorbance of the control reaction and  $A_1$ =absorbance in the presence of the sample of ripe and unripe DT.

## 2.5 Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) Scavenging Activity

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch *et al.* [14] A solution of H<sub>2</sub>O<sub>2</sub> (40 mM) was prepared in phosphate buffer (50 mM, pH 7.4). 0.4ml hydrogen peroxide was added to ethanol extract (20-100 µg/ml) and absorbance value of the reaction mixture was recorded at 230 nm after 10 mins. Blank solution contained phosphate buffer without H<sub>2</sub>O<sub>2</sub>. The percentage of hydrogen peroxide scavenging was calculated thus:

$$\text{H}_2\text{O}_2 \text{ scavenging activity(\%)} = [(A_0 - A_t) / A_0] \times 100 \text{ where}$$

$A_0$  is the absorbance of control and  $A_t$  is the absorbance of test.

## 2.6 Reducing Power Assay

The reducing power ability of the extracts of ripe and unripe DT fruits were evaluated by the method described by Oyaizu [15]. The reaction mixture contained 1.0 mL of various concentrations of extracts (20–100 µg/mL), 2.5 mL of 1% potassium ferricyanide and 2.5 mL of 0.2 mol/L of 0.2 M sodium phosphate buffer. The mixture was incubated at 50°C for 20 mins, cooled rapidly and the reaction ended by the addition of 2.5 mL of 10% trichloroacetic acid, followed by centrifugation at 3000 rpm mins, for 10 min. Then, 2.5 mL of the upper layer was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride. After 10 the absorbance was measured at 700 nm against blank that contained distilled water and phosphate buffer. Increase in absorbance indicated increased reducing power of the sample. Vitamin C was used as standard.

## 2.7 Chelating Ability on Iron (III) Ions

The iron (III) ion (Fe<sup>3+</sup>) chelating potential of the extracts were evaluated by the method of Dinis *et al.* [16] with slight modifications. The reaction mixture contained 2.0 mL of various concentrations of the extracts (20–100 µg/mL) and 0.1 mL of 2 mM/L FeCl<sub>3</sub>. The reaction was initiated by the addition of 0.2 mL of 5 mM/L ferrozine, shaken vigorously and allowed at room temperature for 10 mins and the absorbance of the reaction mixture was measured at 562 nm against a reagent blank. A lower absorbance of the reaction mixture indicated a higher iron (III) ion (Fe<sup>2+</sup>) chelating ability. The control contained all the reagents except sample. Ascorbic acid was used as standard. The percentage inhibition of ferrozine–Fe<sup>2+</sup> complex formation was calculated by using the formula given below:

$$\text{Ferrous ion (Fe}^{2+}\text{) chelating activity (\%)} = [1 - (\text{Absorbance of test} / \text{Absorbance of control})] \times 100$$

## 2.8 Determination of IC<sub>50</sub> Value

The inhibitor concentration against the percentage activity was plotted ([I]-activity % graph). Using the linear (y=mx+c) equation on the graph, for y=50 value, x point became the IC<sub>50</sub> value.

## 2.9 Statistical Analysis

Data were expressed as Mean ± Standard error of mean (SEM) (n = 3). The 50% inhibitory concentration (IC<sub>50</sub>) of ethanol extract of unripe and ripe DT fruits were estimated from regression analysis of concentration-inhibition curves using EXCEL.

## 3. RESULTS AND DISCUSSION

The results of the *in vitro* antioxidant potentials of ethanol extracts of unripe and ripe pepper fruits are presente in tables 1-4. Results showed that both fruits scavenged DPPH and H<sub>2</sub>O<sub>2</sub> free radicals; chelated iron metals and reduced Fe<sup>3+</sup> in a concentration-dependent manner. The IC<sub>50</sub> which is the concentration of extracts that is able to inhibit 50% of the free radicals

generated was used to measure the potency of the unripe and ripe fruit extracts. Notably, the values of the IC<sub>50</sub> were inversely proportional to the potency of the plant material.

Free radical scavenging activities are **important** due to the detrimental roles of free radicals in foods and in biological systems. Various methods are currently used to evaluate the antioxidant activity of plant polyphenols. Most chemical assays are based on the ability to scavenge synthetic free radicals, using a variety of radical-generating systems and methods for detection of the oxidation end-point. DPPH and H<sub>2</sub>O<sub>2</sub> radical scavenging methods are common spectrophotometric procedures for examining the antioxidant potency of plant extracts.

**Table 1: Effect of ethanol extract of unripe and ripe DT fruits on 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity**

Conc. (µg/ml)	UDT (%)	RDT (%)	Vitamin C (%)
20	12.09 ± 0.35	15.15 ± 0.24	87.86 ± 0.11
40	14.32 ± 0.23	15.67 ± 0.32	88.46 ± 0.11
60	20.30 ± 0.34	23.66 ± 0.03	89.46 ± 0.70
80	22.31 ± 0.23	24.18 ± 0.40	90.14 ± 0.04
100	23.06 ± 0.12	25.52 ± 0.23	90.66 ± 0.07
IC <sub>50</sub>	549.23		12.92

Data presented as (Mean ± SEM), n=3. UDT-unripe *Dennetia tripetala*, RDT-ripe *Dennetia tripetala*

**Table 2: Effect of ethanol extract of ripe and unripe DT fruit on hydrogen peroxide scavenging activity.**

Conc. (µg/ml)	UDT (%)	RDT (%)	Vitamin C (%)
20	8.18 ± 1.82	24.74 ± 0.58	24.06 ± 0.28
40	17.73 ± 1.55	27.47 ± 0.73	26.45 ± 0.34
60	22.01 ± 0.98	28.15 ± 1.16	27.46 ± 1.29
80	23.04 ± 0.10	27.48 ± 0.38	28.15 ± 0.94
100	28.16 ± 0.28	29.18 ± 0.36	30.03 ± 0.72
IC <sub>50</sub>	205.69	179.06	167.79

Data presented as (Mean ± SEM) n=3. UDT-unripe *Dennetia tripetala*, RDT-ripe *Dennetia tripetala*

**Table 3: Effect of ethanol extract of ripe and unripe DT fruit on reducing power**

Conc. (µg/ml)	UDT (%)	RDT (%)	Vitamin C (%)
20	0.73 ± 0.00	0.70 ± 0.00	1.00 ± 0.00
40	0.74 ± 0.00	0.71 ± 0.00	1.00 ± 0.00
60	0.76 ± 0.00	0.76 ± 0.00	1.07 ± 0.00
80	0.78 ± 0.00	0.84 ± 0.00	1.14 ± 0.00

100                      0.82 ± 0.00                      1.00 ± 0.00                      1.38 ± 0.00

161 Data presented as (Mean ± SEM), n = 3. UDT-unripe *Dennetia tripetala*, RDT-ripe *Dennetia tripetala*

163 **Table 4: Effect of ethanol extract of ripe and unripe DT fruit on metal chelating activity**

Conc. (µg/ml)	UDT (%)	RDT (%)	Vitamin C (%)
20	24.00 ± 0.36	55.52 ± 0.42	26.61 ± 0.36
40	56.32 ± 0.91	57.02 ± 0.32	35.74 ± 0.10
60	59.34 ± 0.23	61.03 ± 0.32	36.45 ± 0.30
80	66.97 ± 0.42	64.16 ± 0.23	40.97 ± 0.17
100	68.98 ± 0.23	68.67 ± 0.21	47.29 ± 0.35
IC <sub>50</sub>	95.38	124.66	97.03

165 Data presented as (Mean ± SEM), n = 3. UDT-unripe *Dennetia tripetala*, RDT-ripe *Dennetia tripetala*

166 DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances [17]. In the  
 167 DPPH assay (Table 1), the antioxidants in the unripe and ripe fruit extracts were able to reduce the stable radical DPPH to the  
 168 yellow coloured diphenyl-picrylhydrazine by hydrogen-donation to form non-radical DPPH-H [9,18]. Table 1 showed that the ext  
 169 IC<sub>50</sub> values of the extracts for DPPH scavenging activity were effective in the orders: Vitamin C < ripe DT fruits < unripe DT  
 170 fruits, thus suggesting a more potent DPPH free radical scavenging action for ripe DT fruit compared to unripe DT fruit. This  
 171 finding does not corroborate the findings of Adedayo *et al.* [19] who reported a higher DPPH scavenging activity for the unripe  
 172 DT fruits compared to the ripe fruits.

173  
 174 Hydrogen peroxide has strong oxidizing properties. It can be produced endogenously by many oxidizing enzymes such as  
 175 superoxide dismutase. It can cross membranes and may slowly oxidize lipids and proteins. The ability of ethanol extract of  
 176 unripe and ripe DT fruits to scavenge hydrogen peroxide has been shown in Table 2 and compared with that of vitamin C as  
 177 standards. These results showed that the ripe DT fruit extract had an effective hydrogen peroxide scavenging activity than the  
 178 unripe fruit. The IC<sub>50</sub> of the H<sub>2</sub>O<sub>2</sub> activity decreased as follows: Vitamin C < ripe DT fruits < unripe DT fruits. Hydrogen peroxide  
 179 gives rise to hydroxyl radical (OH·)- a very reactive free radical which can damage the cell membrane by initiating lipid  
 180 peroxidation[20]. Therefore, the ability of ripe and unripe extract to scavenge hydrogen peroxide is very important mechanism for  
 181 cell protection from damage by xenobiotics.

182  
 183 The ability to reduce Fe<sup>3+</sup> indicates the capacity of a plant extract to donate electrons, which is an important mechanism of plant  
 184 polyphenol's antioxidant action [21, 22]. The results in Table 3 showed that the ripe extract had a better Fe<sup>3+</sup> reducing power  
 185 than the unripe, however, the standard vitamin C was the best. The antioxidant components present in the ethanol extracts of  
 186 ripe and unripe DT fruit may have resulted in the reduction of Fe<sup>3+</sup>/ferricyanide complex to its iron(II) ion form (Fe<sup>2+</sup>) thus,  
 187 demonstrating its reducing power ability. This work is in contrast with the work of Adedayo *et al.*[19] who reported a higher  
 188 reducing power for unripe DT fruits. There is a positive relationship between reducing power and the presence of phenolics and  
 189 flavonoids. IHEMEJE *et al.* [23] reported that both ripe and unripe DT fruits contain flavonoids; this may have been responsible for  
 190 their reducing power.

191  
 192 In nature iron is found in two states: iron(II) ion (Fe<sup>2+</sup>) or iron(III) ion (Fe<sup>3+</sup>). Iron(III) ion is abundant in foods and has the ability to  
 193 combine with vitamin C to form iron(II) ion. Iron(II) ion can in turn combine with oxygen to form hydrogen peroxide and hydroxyl  
 194 ion free radicals which are capable of causing oxidative damage to biomolecules [24,25]. Table 4 shows the iron(II) ion (Fe<sup>2+</sup>)  
 195 chelating activities of ripe and unripe ethanol extracts of DT fruits. The IC<sub>50</sub> values of the extract for metal chelating activity were  
 196 effective in the orders: unripe DT fruit < vitamin C < unripe DT fruit. This implies that the unripe fruit was a better Fe<sup>2+</sup> chelator  
 197 than the standard vitamin C. The iron(II) ion chelating effect of DT fruits may confer protection against oxidative damage by  
 198 inhibiting production of reactive oxygen species (ROS) and lipid peroxidation[26]. In this reaction, ferrozine quantitatively formed  
 199 complexes with Fe<sup>2+</sup>. In the presence of chelating agents, the complex formation was interrupted, resulting in a decrease in the  
 200 red colour of the complex. Measurement of colour reduction, allowed the estimation of the metal chelating activity of the chelator  
 201 [27] Absorbance of the plant extracts was inversely proportional to the metal chelating activity. Adedayo *et al.*[19] reported that  
 202 the unripe fruits had higher metal chelating activity than the ripe DT fruit.

203  
 204 Bassey *et al.* [28], reported on the possible beneficial effect of DT in boosting spermatogenesis by reducing the concentration of  
 205 prostate specific antigen (PSA) in male albino Wistar rats. This may have been due to DT's potent antioxidant potentials as  
 206 observed in this study. Khaki *et al.* [29] reported that antioxidants can protect the DNA and vital molecules from oxidative  
 207 damage; and can improve spermatogenesis thereby promoting fertility rate in men.



## 4. CONCLUSION

There is a surge of interest in the antioxidative properties of plants due to their possible uses as natural agents to replace artificial ones. The results of the free radical scavenging potential of ethanol extract of unripe and ripe DT fruits exhibited potent antioxidant activities. The extracts IC<sub>50</sub> values were effective in the orders: % DPPH activity- unripe DT fruit < ripe DT fruit < Vitamin C; % H<sub>2</sub>O<sub>2</sub> activity- unripe DT fruit < ripe DT fruit < vitamin C; reducing power- Vitamin C < unripe DT < ripe DT; % metal chelating activity-ripe DT fruit < vitamin C < unripe DT fruit. Therefore, the present data suggest that ethanol extract of ripe and unripe DT fruits may be used to supplement our diets as rich sources of natural antioxidants for health protection. In food industries, it may be used to reduce or prevent lipid oxidation in food products, thereby promoting and maintaining nutritional quality, as well as extension of shelf life of foods and pharmaceuticals. Further studies should be done to isolate bioactive compounds in order to establish their pharmacological potentials.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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