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Cytotoxicity and Free Radical Scavenging Activities of Hexane Fractions of Nigeria Specie of African Pear *(Dacryodes edulis)*

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ABSTRACT

The cytotoxicity using Brine shrimp lethality assay and free-radical scavenging activities of fractions obtained from chromatographic separation of *Dacryodes edulis* (G.Don) H.J. Lam (African Pear) were investigated. The hexane extract was separated into smaller fractions by using a mixture of hexane, ethylacetate and methanol and silica gel 70-230 mesh as adsorbent in a gradient elution-column chromatographic technique. The *in vitro* antioxidant assay involved determination of the effect on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and spectrophotometric determination of *D. edulis* scavenging effect on hydrogen peroxide carried out at 285 nm. The free radical scavenging activities were compared with the activities of known antioxidants such as Ascorbic Acid (AA), Butylated hydroxyanisole (BHA) and α-Tocopherol. Fractions obtained were pooled based on R_f values obtained from Thin Layer Chromatography (TLC). Cytotoxicity analysis revealed that out of the five pooled fractions (D_1-D_5) screened, fractions D_1, D_3, and D_4 were not toxic as their LC_{50} values were all greater than 1,000 μg mL^{-1}, D_2 (LC_{50} = 0.0000 μg mL^{-1}) and D_5 (LC_{50} = 116.6931 μg mL^{-1}) were toxic at varied degrees with reference to 1,000 μg mL^{-1}. None of the fractions showed significant free radical scavenging activity when compared to standards Butylated hydroxylanisole (BHA), Ascorbic acid (AA) and α-Tocopherol. At 1.0 mg mL^{-1} when screened with 2,2-diphenyl-1-picrylhydrazyl radical, D_4 (17.7%) and D_5(17.6%) both scavenged better than α-Tocopherol(15.4%). Fractions D_2(14.2%), D_4 (14.8%) and D_5 (17.5%), at 500 μg mL^{-1} exhibited better activities than α-Tocopherol (12.4%) and at 250 μg mL^{-1}, only D_4 (14.0%) was better than α-Tocopherol (12.4%), these %inhibition values are however lower than values obtained for BHA and AA. None of the samples scavenged better than α-Tocopherol (12.1%) at 125 μg mL^{-1} and only D_4(10.6%) exhibited greater scavenging capacity than α-Tocopherol (10.4%) at the least concentration of 62.5 μg mL^{-1}. The activity was also low with scavenging effects on Hydrogen peroxide (H_2O_2) measured at 285 nm. Interestingly, α-Tocopherol showed an exceptionally high antioxidant activity in scavenging OH radical when compared to its activity in DPPH. The ability to scavenge hydroxyl radical which is known to cause cellular damage justifies the ethno medicinal application of *D. edulis*.

Key words: Free-radical, cytotoxicity, *Dacryodes edulis,* 2,2-diphenyl-1-picrylhydrazyl radical, hydrogen peroxide.

INTRODUCTION

Medicinal plants in Nigeria were considered by several researchers to form an important component of the natural wealth of the country (Iyamabo, 1990). The tropical rainforest of Nigeria

has been described as a reservoir of phytomedicines. Many of these plants contain substances that can be used for therapeutic purposes if used by man. One of such plant is *Dacryodes edulis* G. Don. Lam (Local pear) of the family Burseraceae. It is an evergreen tree attaining a height of 18-40 m in the forest but not exceeding 12 m in plantation. It grows mostly in the tropics. It is cultivated in most rural communities by the peasant farmers for its fruits. The fruit is red, turning blue-black when ripe with unpleasant turpentine smell (Leakey, 1999; Gill, 1992; Iwu *et al.,* 1999; Sofowora, 2008). It is claimed to have many folklore uses. *D. edulis* stern bark is used in treating cough (Igoli *et al.*, 2005). The exudates have also been used in traditional medicines for treatment of wounds and parasitic skin diseases, as antibacterial agent and incense and for treatment of respiratory diseases (Ekpa, 1993; Hutchinson *et al.*, 1963).

Much biological work has been carried out on *D. edulis*. The phytochemical screening of the exudates of the plant showed that they contain bioactive compounds comprising saponins, alkaloids, tannins, flavonoids and phenolic compounds. The plant has also been reported to contain carbohydrate, lipids and protein, vitamins and minerals (Okwu *et al.*, 2005). The anti-inflammatory activities due to the presence of saponins in *D. edulis* have been reported (Igoli *et al.*, 2005). The plant has been used for various antimicrobial activities (Ekpa, 1993). The essential oils from the leaves, fruits, stems and root-back of the plant and their constituents has been reported (Onocha *et al.*, 1999), so also is the chemical composition, antioxidant and antimicrobial properties of the essential oil of the plant's resin (Obame *et al.*, 2008). The objective of this research is to determine the cytotoxicity of fractions obtained from chromatographic separation of *D. edulis* using Brine shrimp lethality assay and also the free-radical scavenging activity of the fractions by subjecting the extract to *in vitro* antioxidant assays. This involved determination of the effect on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). DPPH radical gives strong absorption at 517 nm (deep violet colour) in visible spectroscopy. The absorption vanishes or is decolourized as the electron becomes paired off in the presence of a free radical scavenger. Other *in vitro* antioxidant assay employed was spectrophotometric determination of *D. edulis* scavenging effect on hydrogen peroxide carried out at 285 nm. The free radical scavenging activities were compared with the activities of known antioxidants such as Ascorbic Acid (AA), Butylated hydroxyanisole (BHA) and α-Tocopherol.

MATERIALS AND METHODS

Reagents and chemicals: Hexane, ethyl acetate, methanol, butanol and chloroform, hydrochloric acid, ammonia solution, naphthol, bismuth nitrate, potassium iodide, sodium hydroxide, sodium chloride, copper sulphate pentahydrate, sodium potassium tartarate, potassium chloride, glacial acetic acid, disodium hydrogen phosphate and dihydrogen potassium phosphate were all BDH general purpose chemicals and distilled prior to use. Dimethylsulphoxide (M and B, England), hydrogen peroxide and silica gel 70-230 microns (Merck, Germany) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, butylated hydroxylanisole (BHA) and α tocopherol were obtained from Sigma Chemical Co. (St Louis, MO).

Equipment and apparatus: Soxhlet apparatus, Mettler analytical balance H80 (UK), Water Bath (Gallenkamp), Rotavapor RII0 (Buchi, England), silica gel GF_{254} (precoated aluminium sheets-Merck Germany), pH meter (Jenway model), UV-Visible spectrophotometer (Unico1200 and Perkin Elmer lambda 25 models), Glass Column chromatographic materials and fraction collectors.

Plant material: Fresh leaves of *D. edulis* were collected in September, (2009) in the premises of University of Ibadan, Nigeria and specimens were identified and authenticated at Botany and Microbiology Department of University of Ibadan. The leaves were air-dried under mild sunshine for 2 weeks and ground into fine powder with a Hammer Mill (Ashai 7500) and subjected to solvent extraction. The research activities were conducted at the Department of Chemistry and Central Science Laboratory, University of Ibadan, Nigeria.

Extraction and fractionation procedure: Hexane (10 L) was successively used to extract 1 kg of *D. edulis*. The mixture obtained was filtered to remove the marc. The combined filterates were evaporated to dryness in a rotary evaporator at 37°C and stored in desiccators prior to further analysis. Thin Layer Chromatography (TLC) was employed using silica gel 60 F_{254} precoated plates and solvent system: Ethyl acetate/methanol (8:2) to detect antioxidant activity by using DPPH as a spray reagent. Yellow coloration on the spots on the TLC plates though not immediate indicates that the extract of *D. edulis* has antioxidant activity. Ten grams of the hexane extract of *D. edulis* was pre-adsorbed and added to the column in the dry state. The extract was thoroughly mixed with 5 g of silica gel in a mortar. The mixture was allowed to dry until there was no trace of solvent. Seventy grams of silica gel was packed into the column and the adsorbed hexane mixture was added on top. The mobile phase was gradiently introduced to elute the material. The proportion of the more polar solvent was increased gradually in the non-polar one. This produced the stock solutions used with the gradient mixer. Ten milliliter each of the eluent were collected in the fraction collectors and analysed by TLC. Solvent system used was hexane, EtOAc and methanol and silica gel $60F_{254}$, 70-230 mesh was used as adsorbent. Different solvent systems employed for TLC included 100% (EtOAc), EtOAc: MeOH (9:1), EtOAc: MeOH (7:3), EtOAc: MeOH (5:5), Hexane: EtOAc (6:4), Hexane: EtOAc (2:8) and adsorbent for TLC was silica gel $60F_{254}$ precoated aluminium plates. The retention factor R_f obtained from TLC analysis of the 90 fractions obtained was used as the basis for bulking the fractions accordingly thus: 1-25 (D_1), 26-36 (D_2), 37-50 (D_3), 51-55 (D_4), 56-60 (D_5), 61-90 (D_6). Thereafter, cytotoxicity test using Brine shrimp lethality assay and free radical scavenging activity test were carried out on the bulked fractions D_2, D_3, D_4 and D_5 using the following spectrophotometric experiments; scavenging effect on DPPH and scavenging effect on hydroxyl radical generated by hydrogen peroxide. D_1 was mainly fatty acid substances and did not show antioxidant activity at preliminary screening but the remaining fractions were subjected to quantitative antioxidant screening.

Cytotoxicity analysis

Brine shrimp lethality test: The brine shrimp lethality test (BST) was used to predict the presence in the fractions, cytotoxic activity (Meyer *et al*., 1982). The shrimp's eggs were hatched in sea water for 48 h at room temperature. The nauplii (harvested shrimps) were attracted to one side of the vials with a light source. Solutions of the extracts were made in DMSO, at varying concentrations (1000, 100 and 10 μg mL^{-1}) and incubated in triplicate vials with the brine shrimp larvae. Ten brine shrimp larvae were placed in each of the triplicate vials. Control brine shrimp larvae were placed in a mixture of sea water and DMSO only. After 24 h the vials were examined against a lighted background and the average number of larvae that survived in each vial was determined. The concentration at 50% mortality of the larvae (LC_{50}) was determined using the Finney computer programme.

Free radical scavenging activity

Scavenging effect on DPPH: A 3.94 mg of 2, 2-diphenyl-1-picryhydrazyl radical (DPPH), a stable radical was dissolved in methanol (100 mL) to give a 100 μm solution. To 3.0 mL of the methanolic solutions of DPPH was added 0.5 mL of each of the bulked fractions with doses ranging from 1.0 to 62.5 μg mL^{-1} (Lugasi *et al.*, 1999; Mutee *et al.*, 2010; Oloyede *et al.*, 2010). The decrease in absorption at 517 nm of DPPH was measured 10 minutes later. The actual decrease in absorption was measured against that of the control and the percentage inhibition was also calculated. The same experiment was carried out on butylated hydroxylanisole (BHA), ascorbic acid (AA) and α-tocopherol which are known antioxidants. All test and analysis were run in triplicates and the results obtained were averaged.

Scavenging effect on hydrogen peroxide: Spectrophotometric determination of the hexane fractions of *D. edulis* was carried out at 285nm. A solution of 2 mM hydrogen peroxide was prepared in phosphate buffered-saline (PBS) pH 7.4. The fractions at the following concentrations; 0.1, 0.05, 0.025, 0.0125 and 0.00625 mg mL^{-1} was added to the H_2O_2 solution. Decrease in absorbance of H_2O_2 at 285 nm was determined spectrophotometrically 10minutes later against a blank solution containing the test extract in PBS without H_2O_2. All tests were run in triplicates and averaged (Soares *et al.*, 1997; Oloyede and Farombi, 2010).

Statistical analysis: Data (Absorbance measurements) are expressed as mean absorbance±SD. of triplicate analysis. Statistical analysis was performed by a one-way analysis of variance (ANOVA) for more than two means while Student's t-test was used for comparison between two means. Values of $p<0.05$ were taken to be statistically significant. The LC_{50} after 48 h was determined by probit analysis tested using the Finney computer programme.

RESULTS AND DISCUSSION

Cytotoxicity analysis: Cytotoxicity analysis as determined by Brine shrimp lethality test revealed that fractions D_1, D_3 and D_4 were not toxic as their LC_{50} values were all greater than 1,000 μg mL^{-1}, D_2 (LC_{50} = 0.0000 μg mL^{-1}) and D_5 (LC_{50} = 116.6931 μg mL^{-1}) were all toxic at varied degrees with reference to 1,000 μg mL^{-1}. The toxicity results obtained shows that it has medicinal importance as it has been established by other workers that secondary metabolites from plants which are active medicinally are most times toxic to Brine shrimp larvae *Artermia salina* nauplii which is a living organism with no advance nervous system (Aiyelaagbe *et al.*, 2009; Oloyede and Farombi, 2010). Some of the fractions being non-toxic showed significant antioxidant activity at scavenging free radicals. They also showed significant activity in scavenging hydroxyl radical which are known to cause cellular damage. The ability of the fractions to be used as primary antioxidant is significant for no harm is done to organism cells. Other fractions which are toxic also showed significant antioxidant activity but their use at high dose should be properly monitored (Potterat, 1997; Mensor *et al.*, 2001). Also this cytotoxic and antioxidant activities displayed by this plant confirms the presence in this plant diverse secondary metabolites suggesting a wide range of biological application of the plant (Ramya *et al.*, 2010; Mothana *et al.*, 2010).

Free radical scavenging activities: Scavenging activities of all the bulked samples (semi-pure compounds) of the hexane fractions of *D. edulis* on 2,2-diphenyl-1-picrylhydrazyl

radical (DPPH) and hydrogen peroxide (H_2O_2) are as shown in Table 1-4. Their free radical scavenging activities were compared with the activities of known antioxidants such as Ascorbic Acid (AA), Butylated hydroxyanisole (BHA) and α-Tocopherol. The activities were also determined as a function of their %Inhibition (%I) which was calculated using the formula:

$$\%I = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Scavenging effects on DPPH: The result of analysis revealed that percentage inhibition decreases with decrease in concentration of the samples; the scavenging activity of *D. edulis* on DPPH was however not promising because the % inhibition at all concentration was lower than that of BHA and AA. However, at 1.0 mg mL^{-1}, D_4 (17.7%) and D_5 (17.6%) both scavenged better than α-Tocopherol (15.4%). Fractions D_2 (14.2%), D_4 (14.8%) and D_5 (17.5%), at 500 μg mL^{-1} exhibited better activities than α-Tocopherol (12.4%) and at 250 μg mL^{-1}, only D_4 (14.0%) was better than α-Tocopherol (12.4%). None of the samples scavenged better than α-Tocopherol (12.1%) at 125 μg mL^{-1} and only D_4 (10.6%) exhibited greater scavenging capacity than α-Tocopherol (10.4%) at the least concentration of 62.5 μg mL^{-1} (Table 1, 2).

Scavenging effects on hydrogen peroxide (H_2O_2): The scavenging activities of fractions collected from chromatographic separation of *D. edulis* and Ascorbic Acid (AA), Butylated hydroxyanisole (BHA) and α-Tocopherol on H_2O_2 is shown in Table 3 and the % inhibition is shown in Table 4. Scavenging effects on H_2O_2 was measured in triplicates after 10 min of

Table 1: Scavenging effects of *D. edulis*, AA, BHA and α-T on DPPH Absorbance *517 nm

	Concentration				
Sample	A	B	C	D	E
D_2	0.6533±0.480	0.0797±0.003	0.0513±0.002	0.0537±0.005	0.9562±0.033
D_3	0.6480±0.011	0.7860±0.060	0.8477±0.043	0.7797±0.015	0.7843±0.047
D_4	0.7143±0.083	0.7873±0.020	0.8030±0.052	0.8317±0.027	0.8293±0.035
D_5	0.4923±0.038	0.7027±0.029	0.7740±0.053	0.7690±0.043	0.8020±0.048
AA	0.0843±0.010	0.2893±0.128	0.2977±0.124	0.320±0.0820	0.5147±0.015
BHA	0.0370±0.006	0.0460±0.006	0.0483±0.002	0.0490±0.002	0.0650±0.003
α-T	0.6800±0.029	0.7040±0.003	0.7047±0.007	0.7070±0.007	0.7207±0.012

*The scavenging activity of the hexane fractions and known antioxidants on DPPH was measured at 517 nm and each value represents the mean ± standard deviation of triplicate analysis. A: 0.1 mg mL^{-1}, B: 500 μg mL^{-1}, C: 250 μg mL^{-1}, D: 125 μg mL and E: 62.5 μg mL^{-1}. AA: Ascorbic acid, BHA: Butylated hydroxyanisole, α-T = α-Tocopherol

Table 2: %Inhibition of samples and known antioxidants at $Absorbance_{517\,nm}$ on DPPH

	Sampel						
Concentration	D_2	D_3	D_4	D_5	AA	BHA	α-T
A	15.2	11.3	17.7	17.6	90.9	95.4	15.4
B	14.2	08.0	14.8	17.5	68.7	94.3	12.4
C	07.5	07.6	14.0	11.3	67.8	94.0	12.4
D	05.3	05.2	12.0	09.9	65.4	93.9	12.1
E	04.2	04.6	10.6	09.1	44.3	91.9	10.4

*A = 0.1 mg mL, B = 500μg mL^{-1}, C = 250 μg mL^{-1}, D = 125 μg mL^{-1} and E = 62.5 μg mL^{-1}. AA = Ascorbic acid, BHA = Butylated hydroxyanisole and α-T = α-Tocopherol

Table 3: Scavenging effects of *D. edulis*, AA, BHA and α-T on H_2O_2 Absorbance *285 nm

	Concentration				
Sample	A	B	C	D	E
D_2	3.0229±0.004	3.0498±0.003	3.2123±0.001	3.2855±0.004	3.3548±0.004
D_3	3.0667±0.006	3.1681±0.005	3.2038±0.005	3.3983±0.057	3.5191±0.008
D_4	3.2803±0.003	3.3486±0.033	3.6185±0.004	3.7435±0.003	3.7783±0.010
D_5	3.0735±0.001	3.1047±0.001	3.3367±0.053	3.4003±0.037	3.5294±0.006
AA	0.1952±0.001	0.2078±0.012	1.2645±0.119	2.7586±0.049	2.9236±0.211
BHA	0.0413±0.016	0.0617±0.019	0.0740±0.015	0.0947±0.003	0.1126±0.014
α-T	0.0321±0.045	0.0633±0.032	0.1552±0.061	0.1807±0.015	0.4940±0.017

*The scavenging activity of the hexane fractions and known antioxidants on H_2O_2 was measured at 285 nm and each value represents the Mean±SD of triplicate analysis. A = 0.1 mg mL^{-1}, B = 500 μg mL^{-1}, C = 250 μg mL^{-1}, D = 125 μg mL^{-1}, E = 62.5 μg mL^{-1}. AA: Ascorbic acid, BHA: Butylated hydroxyanisole, α-T: α-Tocopherol

Table 4: %Inhibition of samples and known Antioxidants at $Absorbance_{285nm}$ on H_2O_2

	Sample						
Concentration	D_2	D_3	D_4	D_5	AA	BHA	α-T
A	19.8	18.6	13.0	18.5	94.8	98.9	99.1
B	19.1	15.9	11.2	17.6	94.5	98.4	98.3
C	14.8	15.0	04.0	11.5	66.5	98.0	95.9
D	12.8	09.8	00.7	09.8	26.8	97.5	95.2
E	11.0	06.6	00.2	06.4	22.4	97.0	86.9

A = 0.1 mg mL^{-1}, B = 500 μg mL^{-1}, C = 250 μg mL^{-1}, D = 125 μg mL^{-1} and E = 62.5 μg mL^{-1}. AA: Ascorbic acid, BHA: Butylated hydroxyanisole, α-T: α-Tocopherol

incubation at 285 nm. H_2O_2 has only a weak activity to initiate lipid peroxidation, but its activity as an active- oxygen specie comes from its potential to produce the highly reactive hydroxyl radical. Interestingly, one of the known antioxidants (α-T) showed an exceptionally high antioxidant activity in scavenging OH radical when compared to its activity in scavenging DPPH. The results of antioxidant activities obtained in this research is in agreement with that obtained for the antioxidant properties of the essential oil of the plant's resin by Obame *et al.* (2008). The plant though not very effective as a proton donor in the experiment involving DPPH, it however showed significant activity as a hydroxyl radical scavenger. The two tests has therefore revealed the potential ability of *D. edulis* in scavenging free radicals (Lugasi *et al.*, 1999; Potterat, 1997).

CONCLUSION AND RECOMMENDATION

The results obtained from investigating the cytotoxicity and antioxidant activities of *D. edulis* has proven that the semi-pure compounds present in the fractions are useful potential source of useful drugs. The cytotoxic ability of this plant makes it a valuable entity in the therapy of diseases involving cell or tumour growth.

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