American Journal of Biomedical Research, 2013, Vol. 1, No. 4, 86-92
Available online at http://pubs.sciepub.com/ajbr/1/4/4
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DOI:10.12691/ajbr-1-4-4

Anticoagulant and Antioxidant Activities of *Dracaena arborea* Leaves (Wild.) Link

Nwaehujor Chinaka O.[1,*], Ode Julius O.[2], Nwinyi Florence C.[2], Madubuike Stella A.[3]

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria
[2]Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Abuja, Nigeria
[3]Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria
*Corresponding author: chinaka_n@yahoo.com

Received October 04, 2013; Revised October 16, 2013; Accepted October 20, 2013

Abstract The crude methanol extract of *Dracaena arborea* leaves induced significant ($p<0.01$) increase in the clotting times of 21 ± 0.54 sec and 25 ± 1.1 sec at 5% and 10% concentrations of the extract respectively compared to the baseline clotting time of 7 ± 0.63 sec for the blood sample. The extract also exhibited potent *in vivo* and *in vitro* anticoagulant activities. Increased doses (100 and 200 mg/kg) of the extract, heparin (0.75 and 1.5 mg/kg) and aspirin (1.0 and 2.0 mg/kg) were found to have significantly ($p < 0.01$) prolonged the mean bleeding times with respect to the baseline in rabbits. However, in thrombin-induced clotting assay, the extract demonstrated a reduced potency compared to heparin. DPPH (1, 1-Diphenyl-2-picrylhydrazyl) and FRAP (Ferric reducing/antioxidant power) spetrophotometric assays revealed that the crude leaf extract possesses appreciable high antioxidant potentials. *Dracaena arborea* leaves (Wild.) Link could be a source of novel anticoagulant and antioxidant compounds for the management of various hematological disorders.

Keywords: *anticoagulant, Dracaena arborea, antioxidant, clotting time, stroke, thrombin*

Cite This Article: Nwaehujor Chinaka O., Ode Julius O., Nwinyi Florence C., and Madubuike Stella A., "Anticoagulant and Antioxidant Activities of *Dracaena arborea* Leaves (Wild.) Link." *American Journal of Biomedical Research* 1, no. 4 (2013): 86-92. doi: 10.12691/ajbr-1-4-4.

1. Introduction

Heart attacks and strokes resulting from thrombosis and intravascular blood clots are increasingly becoming the most common cause of debilities and untimely deaths worldwide. Prevention of these diseases has been a major health challenge over decades because of inefficacies associated with most of the readily available drugs. Antithrombotic agents include anticoagulants, antiplatelets and thrombolytics that decrease clots in the body by dissolving already formed clot or prevent clot formation [1]. Anticoagulants are indicated for strokes, transient ischaemic attacks, deep vein thrombosis and pulmonary embolism [2]. Oral anticoagulants have been used in the management of atherothrombotic stroke treatment [3] which accounts for 61% of all strokes and have been relied upon for prevention and treatment for several decades. These drugs however, produce a highly variable anticoagulant effect in patients, requiring their effect to be measured by special blood tests and their dose adjusted according to the results [4]. Frequent development of immune thrombocytopenia, haemorrhages and idiosyncratic adverse reactions during treatment of patients with the drugs [5] are some of the limitations encountered in anticoagulant therapy. The oral direct thrombin inhibitors (e.g., dabigatran) and oral direct inhibitors of factor Xa (e.g., rivaroxaban, apixaban) were recently produced as alternatives to oral vitamin K antagonists for stroke prevention in atrial fibrillation. But each of these drugs has distinct pharmacological properties that could influence optimal use in clinical practice [6]. There is critical need to explore for effective and alternative anticoagulants from natural products with minimal side effects.

Fortunately, plants offer prospect as sources of various medicaments and potent chemotherapeutic agents. *Dracaena arborea* (Asparagaceae) commonly called tree Dracaena is a popular plant in Nigerian tradomedical practice for its use in the treatment of various diseases. It is locally known as *ope kannakanna, peregun, akuku* in *Yoruba* dialects of western Nigeria and *ogirisi* in *Igede* (Middle belt, Nigeria) and in *Ibo* (Eastern Nigeria). The roots, leaves and fruits are used for effective treatment of skin infections, stomach ache and dental caries in western Nigeria [7]. The plant leaves are also employed locally by the *Ibo* people in Nsukka community to relieve hypertension, stroke and body pains. The leaves boiled in palm nut soup with the fern, *Nephrolepis biserrata* and *Dissotis multiflora*, is used to strengthen pregnant women and those delivered of their babies in Ghana [8]. In a bid to establish the antihypertensive effects and other folkloric uses, the anticoagulant and antioxidant activities of the plant leaves were investigated. Increased oxidative stress generates reactive oxygen species (ROS) which are implicated in a variety of pathologies including heart attacks, strokes, renal failure, diabetes, neurodegenerative

diseases, etc. [9]. Presently, there is paucity of information on the *in vitro* antioxidative activities of *D. arborea* leaves. In a previous study, the ethyl acetate and aqueous fractions of the leaf extract demonstrated insecticidal activity against *Sitophilus zeamais* (Motsch.) and *Callosobruchus maculatus* (Fab.) [10] and offered protection to stored grains.

The present study focused on evaluation of the anticoagulant and antioxidant effects of the methanol extract of *D. arborea* (Willd.) Link leaves using standard models.

2. Materials and Methods

2.1. Experimental Animals

Matured inbred albino rats of both sexes weighing between 80-190 g and locally bred albino rabbits (2.0-2.2 kg) were obtained from the Laboratory Animal Unit of the Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria. The animals were kept in different cages in the same room with a temperature varying between 28 and 30°C; lighting period was between 15 and 17 h daily. The rats were kept in stainless steel wire mesh cages which separated them from their faeces to prevent coprophagy. They were supplied clean drinking water and fed standard feed (Grower mash pellets, Vital feeds®, Nigeria). Rabbits were given fresh forages *ad libitum.* The animals were allowed two weeks to acclimatize prior to commencement of the experiments. The laboratory animals were used in accordance with laboratory practice regulation and the principle of laboratory animal care as documented by Zimmerman [11].

2.2. Chemicals, Reagents, Drugs and Equipment

Freshly prepared solutions, analytical grade chemicals and reagents were used in the experiments. Methanol, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), Ferric reducing/antioxidant power (FRAP) reagents, Ferric sulphate ($FeSO_4$ x $7H_2O$), Ascorbic acid (vitamin C), bovine thrombin purchased from Sigma Aldrich, USA; heparin (Mayne Pharma, Espana), soluble aspirin tablets 75 mg (Bristol laboratories Ltd, UK), Sodium phosphate (BDH, Poole, England), Spectrophotometer (Spectrumlab 752 S., B. Bran Scientific and instrument company, England) were used for the tests.

2.3. Plant Collection and Identification

Fresh leaves of *D. arborea* were collected from Obukpa village, in Nsukka Local Government Area of Enugu State, Nigeria in April, 2013. The plant roots were authenticated by Mr A.O. Ozioko, a taxonomist with Bioresources Development and Conservation Programme (BDCP), Aku road, Nsukka, Enugu State, Nigeria.

2.4. Preparation and Extraction of Plant Material

The plant leaves were dried under mild sunlight, and then reduced to coarse particles with mortar and pestle before been pulverized into fine particles using a laboratory hammer mill. The plant material was exhaustively extracted by cold maceration in 80% methanol with intermittent shaking at 2 h intervals for 48 h. The extract was filtered and concentrated *in vacuo* using a vacuum rotary evaporator. The concentration and percentage yield of the extract were determined. The concentrated *D. arborea* extract, subsequently denoted as DAE was stored in a refrigerator at 4^0C until further use.

2.5. Acute Oral Toxicity Studies

Acute toxicity studies were conducted using the method described by Lorke [12]. Thirty (30) matured Wistar rats of both sexes were marked with 10% picric acid, weighed and randomly separated into 6 groups (A-F) of 5 rats each. Groups A–E were given varying oral doses (150; 300; 600; 1200 and 1500 mg/kg) of DAE respectively, while group F (6^{th} group) received an equivalent volume (10 ml/kg) of distilled water. All treatments were given orally by gastric intubation. The rats were observed for signs suggestive of toxicity within 72 h. The animals that survived were further monitored for two weeks for toxic effects. The test was terminated after two weeks and all the animals were humanely sacrificed and postmortem examinations carried out on them.

2.6. Blood Clotting Time

The ability of *D. arborea* to inhibit coagulation of blood *in vitro* was quantitatively assayed following the procedure adopted by Koffuor and Amoateng [13]. A fixed volume (1.0 ml) of whole blood drawn from the marginal ear vein of rabbits was added to 0.2 ml of *D. arborea* (5 and 10% w/v) in test tubes placed in a water bath at 37°C. The time taken for the blood samples to clot was recorded. Five determinants were made. A similar determination was carried out using heparin (1.5 µL of 5% w/v) as a reference anti-coagulant. Controls were set up using distilled water (0.2 ml) and blood, and blood alone (baseline clotting time).

2.7. Rabbit Bleeding Time

The *in vivo* anticoagulant activity of *D. arborea* at 200 and 400 mg/kg was investigated as described by Elg *et al.* [14]. Rabbits were pre-treated orally with DAE for 30 min. Pricking a small vein in the margin of the ear induced bleeding. The bleeding vein was gently blotted with filter paper every 5 s till cessation of bleeding (when no more bleeding was observed for 60 s). The observation time was limited to 10 min. Care was taken that no pressure was exerted on the ear tips that could affect homeostasis. The above procedure was repeated using aspirin (1-2 mg/kg), distilled water and after intravenous administration of heparin (0.75-1.5 mg/kg). A baseline bleeding time was determined before any drug administration.

2.8. Thrombin-induced Clotting Time Assay

This assay measures the prolongation of thrombin generation. When human plasma is incubated with a compound which inhibits blood coagulation, the time taken for the clot formation will be prolonged as compared to the control (no inhibitor added). In the assay, 100 µl of human plasma (pre-incubated at 37°C for 5 min

before use) was incubated with different concentrations of the extract for 5 min at 37°C. Heparin was used as a positive control while buffer served as the negative control. One hundred µl of bovine thrombin (2.5 U/ml, Sigma) was then added to initiate the reaction. The test was carried out in triplicates. The time for clot formation was recorded and compared with the reference (heparin) values [15].

2.9. Antioxidant Capacity of *D. Arborea* Leaf Extract Using the 1, 1- diphenyl-2-picrylhydrazyl Radical (DPPH) Spectrophotometric Assay

The method of Mensor *et al.* [16] was adopted. Two (2) ml of the test extract at concentrations ranging from10 µg/ml to 400 µg/ml was each mixed with 1 ml of 0.5 mM DPPH (in methanol). Absorbance at 517 nm was taken after 30 min incubation in the dark at room temperature. The concentrations were prepared in triplicates. The percentage antioxidant activity was calculated as follows:

$$\%[AA] = 100 - (\frac{\left[\begin{matrix} absorbance\ of\ sample \\ - absorbance\ of\ blank \end{matrix}\right]}{Absorbance\ of\ control} * 100)$$

1ml of methanol plus 2 ml of the extract was used as blank while 1ml of 0.5 mM DPPH solution plus 2 ml of methanol was used as control. Ascorbic acid was used as the reference standard.

2.10. Ferric Reducing/Antioxidant Power (FRAP) Assay

The total antioxidant potential of the extract was determined using a ferric reducing ability of plasma (FRAP) assay of Benzie and Strain [17] as a measure of "antioxidant power". FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{11}-tripyridyltriazine compound from colorless oxidized Fe^{111} form by the action of electron donating antioxidants. Standard curve was prepared using different concentrations (100-1000 µmol/L) of $FeSO_4$ x $7H_2O$. All solutions were used on the day of preparation. In the FRAP assay the antioxidant efficiency of the extract under the test was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. The results were corrected for dilution and expressed in µmol Fe^{11}/L. Vitamin C was measured within 1 h after preparation. The sample to be analyzed was first adequately diluted to fit within the linearity range. All determinations were performed in triplicate. Calculations were made by a calibration curve.

$$FRAP\ value\ of\ sample\ (mM) = \frac{chang\ in\ abs\ \ from\ 0-4\ \min}{chang\ in\ abs\ of\ \ std\ 0-4\ \min} \times \underline{FRAP\ value\ of\ \ std}$$

3. Statistical Analysis

All data were expressed as Mean ± SEM. Data were analyzed using One way analysis of variance (ANOVA) at 5% level of significance. Dunnet's test was used to detect the difference among the treatment groups.

4. Results

4.1. Description of the Extract

The methanol extract of *D. arborea* leaves was light brownish in colour. The extraction process gave a yield of 10.23 % w/w.

4.2. Acute Toxicity Study of the Extract in Rats

DAE did not cause death in the experimental rats by the oral route even at the highest test dose of 1500 mg per kg body mass. There were also no changes in faecal consistency of the animals within the period. At post mortem, there was no observable gross lesion in the liver, gastro-intestinal tract, spleen, heart and kidneys of the experimental rats.

4.3. Clotting Time

DAE at concentrations of 5% and 10% significantly ($p < 0.01$) produced increased clotting times of 21 ± 0.54 and 25 ± 1.1 s respectively compared to the baseline clotting time of 7 ± 0.63 s for the blood sample (Table 1). However, the blood added to heparin failed to clot.

Table 1. The *in vitro* effect of DAE (5 and 10%) on clotting time of blood taken from the marginal ear vein of rabbits in a clotting time test

Sample	Volume	Mean clotting time (s)
Blood	1.0 ml	7 ± 0.63^{a}
Blood + vehicle	1.2 ml	11 ± 0.63^{a}
Blood + 5% DAE	1.2 ml	$21 \pm 0.54^{b**}$
Blood + 10% DAE	1.2 ml	$25 \pm 1.1^{b**}$

Values are mean ± SEM (n=5). ^{b**} Significant ($p<0.01$) difference between the samples and the control (blood alone).

4.4. Rabbit Bleeding Time

DAE was observed to have significantly ($p<0.01$) prolonged the bleeding time with respect to the baseline. At a dose of 100 mg/kg, the extract induced a bleeding time of 61.8 ± 1.4 s but when the dose was doubled (200 mg/kg), the bleeding time became elevated to 125.1± 1.0 s which was proportionately a more than a double increase. Heparin (0.75 and 1.5 mg/kg) and aspirin (1.0 and 2.0 mg/kg) also significantly ($p<0.01$) induced increased bleeding times compared to the baseline. The bleeding time of 125.1± 1.0 s at the highest test dose (200 mg/kg) of the extract was seen to be a significant ($p<0.01$) increase relative to 61.0 ± 1.7 s with heparin (1.5 mg/kg) and 70.02 ± 0.0 s with aspirin (2.0 mg/kg) (Figure 1).

4.5. Thrombin-induced Clotting Time Assay

A comparison of the *in vitro* activity of crude extract of *D. arborea* leaves with heparin in thrombin-induced clotting time is presented in Figure 2. DAE produced a significant ($p<0.01$) reduction in thrombin-induced

clotting times compared to heparin. At 100 µg/ml, DAE produced clotting at 50.3 ± 0.6 s relative to 330.8 ± 2 s for heparin. Similarly, DAE (1000 µg/ml) induced clotting at 170.5 ± 0.5 s but the same concentration of heparin prolonged the effect to occur at 460.1 ± 0.8 s.

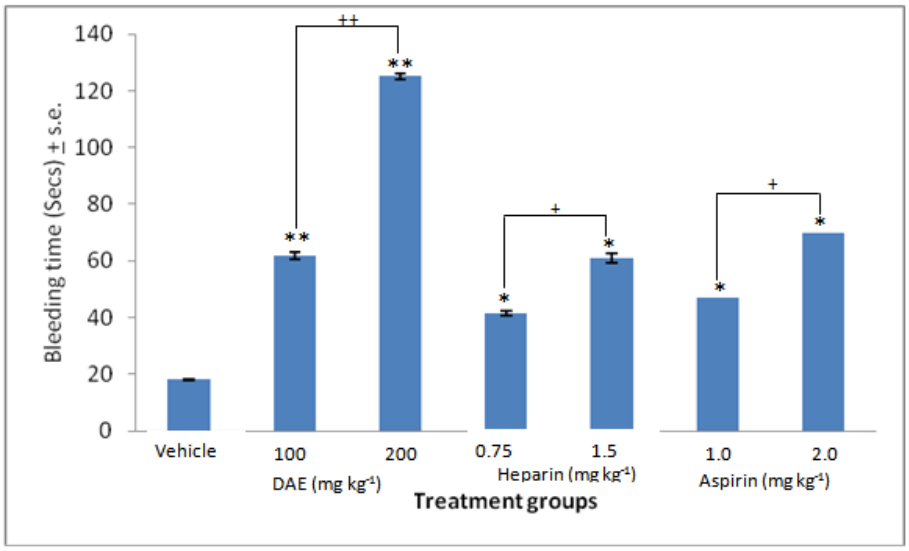

Figure 1. The effect of DAE (100-200 mg/kg), heparin (0.75-1.5 mg/kg) and aspirin (1-2 mg/kg) on the bleeding time in rabbits. Values are Means ± SEM (n=5). **p<0.01, *p<0.05 compared to vehicle treated group. $^{++}$p<0.01, $^{+}$p<0.05 significant differences between dose levels

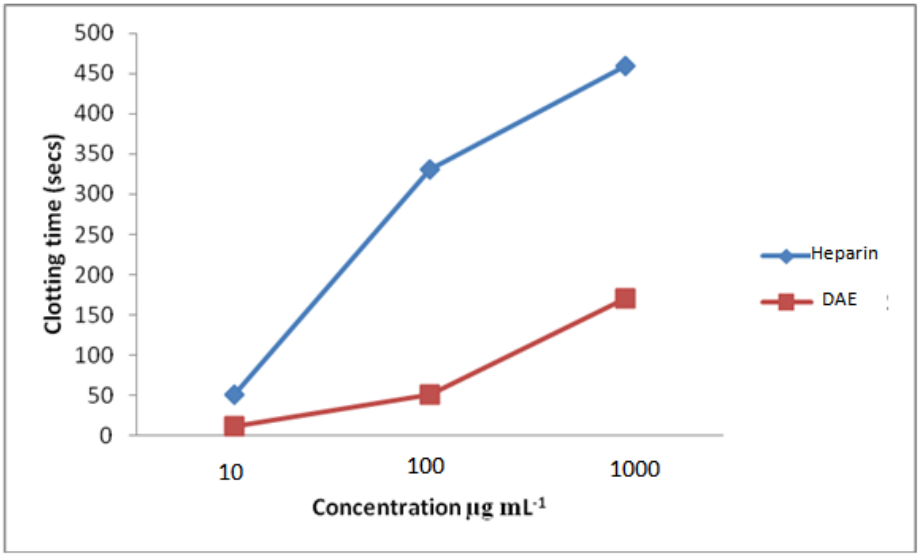

Figure 2. A comparison of the *in vitro* activity of DAE and heparin in thrombin-induced clotting time

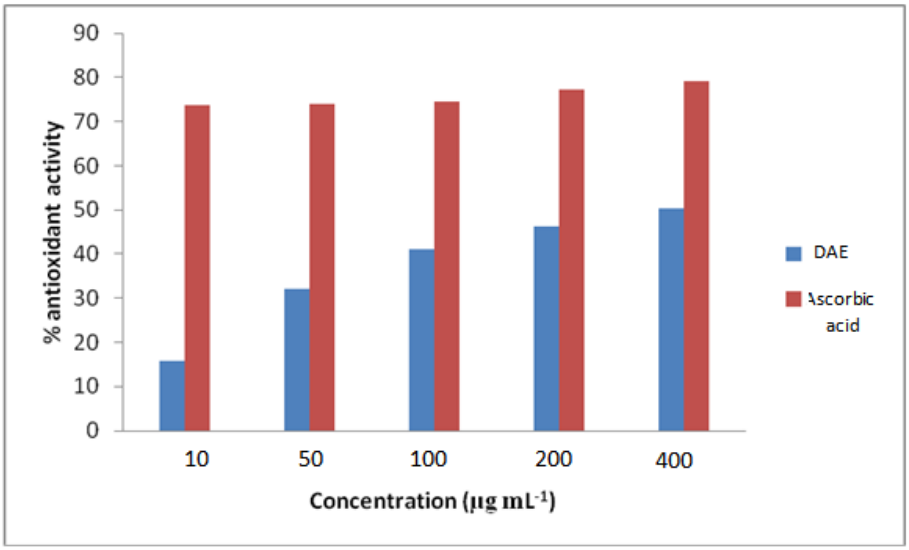

Figure 3. The antioxidant activity of DAE with DPPH spectrophotometric assay

4.6. DPPH Spectrophotometric Assay

The crude methanol extract of *D. arborea* leaves demonstrated appreciable but reduced antioxidant activity relative to the reference values of ascorbic with DPPH radical scavenging method. At the lowest concentration (10 µg/ml), the extract exhibited a mean antioxidant activity of 15.7 percent while ascorbic acid produced 73.6 percent (Figure 3). However, at the highest concentration (400 µg/ml), the extract had 50.3 percent antioxidant activity compared to 79.2 percent with ascorbic acid at the same concentration.

4.7. Ferric Reducing/Antioxidant Power Assay (FRAP)

The ferric reducing ability of plasma, a measure of the antioxidant ability, showed that the methanol extract of *D. arborea* produced a dose dependent antioxidant effect. At 10 µg/ml, the mean antioxidant power (FRAP value) was 0.12 mM; this increased to 0.43 mM at 100 µg/ml and then 1.16 mM at 400 µg/ml. DAE had antioxidant power of 1.35 mM at 1000 µg/ml compared to ascorbic acid with a standard FRAP value of 2 mM at the same maximum concentration (Figure 4).

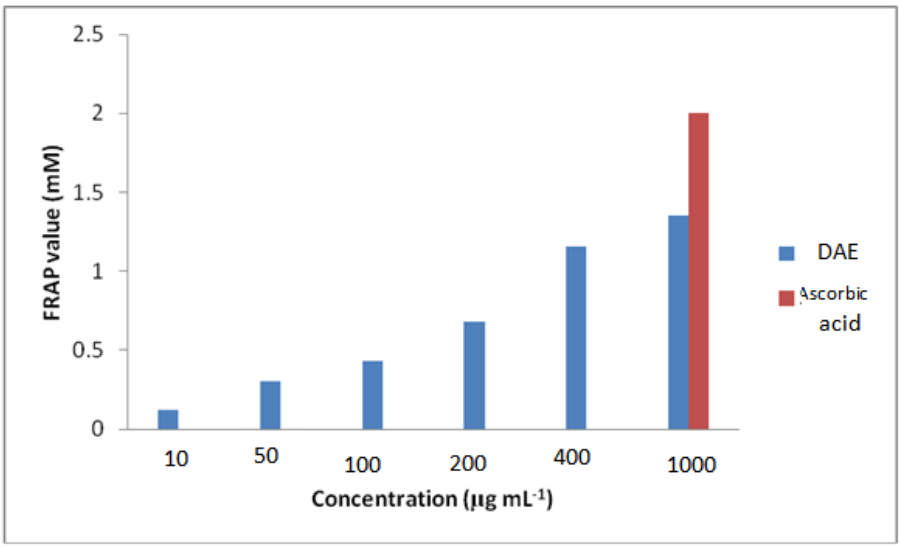

Figure 4. The antioxidant activity of *D. arborea* leaf methanol extract determined by the FRAP assay

5. Discussion

The extract was tolerated by the experimental rats at the highest oral test dose of 1500 mg/kg; hence there were no observable mortalities. The methanol leaf extract of *D. arborea* significantly increased the *in vitro* time of blood clotting (Table 1) but along with heparin, and aspirin, the *in vivo* effect shows prolongation in the bleeding times (Figure 1). The high doses (100-200 mg/kg) of the extract exerted the most profound bleeding effect compared to heparin or aspirin. The extract however displayed a comparatively lower potency than heparin in thrombin-induced clotting time. A given concentration (1000 µg/ml) of the extract produced clotting at 170.5 ± 0.5 s while heparin at the same concentration prolonged the clotting time to 460.1 ± 0.8 s. This is logical since heparin is a pure compound but the extract is a crude and mixture of many compounds. The mechanism by which the extract exhibited anticoagulant activity is not understood but chelating agents, heparin and vitamin K antagonists are known to interfere with blood clotting processes.

Chelating agents including trisodium citrate, sodium oxalate and ethylene diaminetetraacetic acid (EDTA) bind with calcium ions and render them unavailable to facilitate coagulation reactions [18]. Heparin, contained in mast cells is released into circulation when mast cells are disrupted during inflammation. Heparin can inhibit clotting factor IXa, XIa and thrombin but its action on factor Xa accounts for its potency as an anticoagulant [19]. Heparin can inhibit both the generation of thrombin and also the formed thrombin. The synthesis of clotting factors II, VII, IX and X in the liver depends on adequate amounts of vitamin K. Coumarin and inadedione group of oral anticoagulant drugs antagonizes the synthesis of non functional forms of coagulant proteins and thereby prevents blood clot formation [20].

Blood coagulation is an important part of homeostasis in which platelets; a non nucleated cell derived from megakaryocyte plays a prominent role to prevent excessive bleeding [21]. Platelets change shape and adhere to damaged endothelium or aggregate to themselves resulting in plug formation which is strengthened by fibrin containing clot to stop bleeding and for repair of damaged vessel to begin. Disorders of coagulation are known to cause haemorrhage or thrombosis [22]. Fibrin is the more important component of clots that form in veins but platelets are the major component of clots that form in arteries where they can cause heart attacks and strokes by blocking the flow of blood in the heart and brain, respectively [4].

The crude extract produced a significant ($p < 0.05$) concentration dependent antioxidant activity with a maximal effect of 50.3 percent against 79.2 percent with ascorbic acid at 400 µg/ml in DPPH photometric assay. DPPH assay reveals the ability of the extract to scavenge free radicals. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [23]. The reduction capability of DPPH is determined by the decrease in the absorbance at 517 nm induced by antioxidants. This system is valid for

the primary characterization of the scavenging potential compound [24]. DAE's ability to concentration dependently reduce DPPH forming yellowish coloured diphenylpicrylhydrazine suggests that, it is a free radical scavenger and probably acts so by donating electron or hydrogen radical.

The ferric reducing antioxidant power (FRAP) of the extract was assayed using the method described by Benzie and strain [17]. The principle of this method is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous form in the presence of antioxidants. Other authors have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts and have associated this effect with the presence of reductones [25, 26]. Reductones are reported to react with precursors of peroxides thus preventing peroxide formation. However, activity of antioxidants has been attributed to various mechanisms including prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [27]. The FRAP values of DAE at 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml were significant at $p < 0.05$. There was also a concentration dependent increase in the FRAP values of the extract. The FRAP values of the extract at 400 µg/ml and 1000 µg/ml were 1.20 mM and 1.34 mM respectively while ascorbic acid has 2 mM at 1000 µg/ml (Figure 2). This revealed a high antioxidant capacity of the crude extract. ROS are oxygen-centered molecules which include the non-radicals, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl anion (HO) and single Oxygen (O_2); and the radicals, superoxide anion (O_2^-), hydroxyl radical (HO^-), and nitric oxide (NO^-) [28]. ROS is known to play important roles in the aetiology of many diseases [9,29]. The high antioxidant capacities of the extract revealed a measure of its ability to scavange and neutralize toxic radicals with potential to cause stroke and heart attacks. This may be the rationale behind the folkloric use of the plant material in the treatment of diverse health challenges including hypertension.

Research is on-going to determine the constituents and the specific mechanism of action of the extract in preventing blood coagulation.

6. Conclusion

The results of the study demonstrated that the crude extract of *D. arborea* leaf possesses pharmacologically active anticoagulant and antioxidant principles that could be isolated and evaluated for clinical uses.

Acknowledgements

The authors wish to sincerely thank Mr. A.O. Ozioko of Bioresources Development and Conservation Programme (BDCP), Nsukka, Enugu State, Nigeria for identifying the plant sample.

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