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Antioxidant and Anticoagulant Properties of *Phyllanthus fraternus* GL Webster (Family: Euphorbiaceae)

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ABSTRACT

The aim of this study was to investigate the antioxidant and anticoagulant activities of the ethanolic extract of *Phyllanthus fraternus*. The antioxidant and anticoagulant activity of an ethanolic extract of *Phyllanthus fraternus* (gulf leaf-flower) was evaluated using *in vitro* and *in vivo* experimental models. The results obtained indicate that the extract of *P. fraternus* (PFE) exhibits antioxidant activity by significantly scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, concentration-dependent reducing capacity and inhibition of lipid peroxidation. Detection of phenols in the extract gave preliminary evidence of its possible antioxidant activity which correlated with the total antioxidant capacity. The extract significantly increased *in vitro* clotting time and bleeding time in rabbits. Heparin and aspirin exhibited similar effects. Since thrombus formation and oxidative stress are known to be main risk factors contributing to stroke, the anticoagulant and antioxidant activity in the plants makes it useful as adjuncts in the management of stroke and other neurodegenerative disorders.

Key words: *Phyllanthus fraternus* extract (PFE), reducing power, scavenging effect, total antioxidant capacity, thrombus formation

INTRODUCTION

Phyllanthus fraternus GL Webster (Family: Euphorbiaceae) is an annual herb commonly occurring in gardens, waste places and roadsides. In Ghanaian traditional medicine, the leaves of the plant have been used extensively for many years in the treatment of a broad spectrum of diseases. These diseases include diabetes, alcohol-induced liver damage, jaundice, malaria, kidney stones, hypertension, stroke, pain, genito-urinary tract and intestinal infections, anaemia, liver cancer, abdominal pains and diarrhea (STEPRI and CSIR, 2007). *Phyllanthus fraternus* was reported to possess antidiabetic (Okoli *et al*., 2010; Kushwah *et al*., 2010), antiviral (Ogata *et al*., 1992), anti-inflammatory activities and analgesic properties (Calixto *et al*., 1998; Santos *et al*., 1994). Increased oxidative stress generates various degenerative diseases such as hypertension and cardiovascular problems (Khairunnuur *et al*., 2010). Antioxidative activity of five *Phyllanthus* species (excluding *P. fraternus*) has been reported elsewhere (Kumaran and Karunaaran, 2007). As far as our literature survey could ascertain, no information is available on the *in vitro* antioxidative activities of the *P. fraternus*.

In an attempt to shed light on the possible mechanism(s) by which *P. fraternus* exerts its anti-stroke effect, the anticoagulant activity was investigated. Oral anticoagulant are used in the

management of atherothrombotic stroke (Donnan *et al.*, 2008) which accounts for 61% of all strokes and has become the mainstay of stroke treatment and prevention for several decades. The high cost and significant side effects (such as gastrointestinal bleeding and skin rash) of these oral anticoagulants have however, become a major limitation in their use (Hankey *et al.*, 2000; Taylor *et al.*, 1996). Thus, the quest to find alternative anticoagulants from natural products with fewer side effects is warranted.

Therefore, the aim of this study was to investigate the antioxidant and anticoagulant activities of the ethanolic extract of *Phyllanthus fraternus*.

MATERIALS AND METHODS

Fruit sample collection: Fresh leaves of *Phyllanthus fraternus* were collected in September, 2008, from the herbal garden of the Pharmacognosy Department, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST and identified by Mr. G.H. Sam of the Department of Pharmacognosy, KNUST.

Preparation of extract: The leaf samples were sun dried for seven days and milled into a dry powder. A 285 g quantity of the powder was soxhlet extracted with 96% ethanol. The extract obtained was concentrated under reduced pressure, evaporated to dryness and stored in a desiccator. This is subsequently referred to as PFE or the extract.

Animals: Locally bred rabbits with weights ranging from 2.0-2.5 kg were obtained from the animal house of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST. The rabbits were kept in standard metallic in the animal house under ambient humidity conditions. They were allowed access to normal rabbit feed (GAFCO, Tema) and clean water during the period of the experiment.

Drugs: The drugs and chemicals used in this study include: heparin (Mayne Pharma, Espana), soluble aspirin tablets 75 mg (Bristol laboratories Ltd., UK), Folin-Cicocalteu's phenol reagent (EMD Chemicals Inc., Gibbstown, NJ., USA), sodium bicarbonate, sulfuric acid, sodium phosphate, ammonium molybdate, potassium ferricyanide, tannic acid, ferric chloride, n-propyl gallate, linoleic acid (BDH, Poole, England), trichloroacetic acid, thiobarbituric acid (Sigma-Aldrich, St. Louis, MO., USA).

Total phenol assay: The total soluble phenols present in PFE (0.5-2.5 mg mL^{-1}) was quantitatively determined by colorimetric assay using the Folin-Cicocalteu's phenol reagent (Slinkard and Singleton, 1977) with some modifications. Tannic acid (0.05-0.25 mg mL^{-1}) was used as the reference drug.

Experimental procedure: PFE (1 mL) was added to 1 mL of Folin-Cicocalteu's phenol reagent (diluted five folds in distilled water). The content of the test tube was mixed and allowed to stand for 5 min at 25°C in an incubator. A 1 mL portion of 2% sodium bicarbonate solution was added to the mixture. The reaction mixture was added to the mixture. The reaction mixture was then incubated at 25°C. It was then centrifuged at 3000 rpm for 10 min to obtain a clear supernatant. The absorbance of the supernatant was then determined at 760 nm using a Cecil CE 2040 spectrophotometer (Cecil Instrument Limited, Milton Technical Centre, England). Distilled water

(1 mL) was added to 1 mL Folin-Cicocalteu's phenol reagent and the mixture is processed similarly as the test drugs and used as the blank. All measurements were done in triplicates. Data obtained for the tannic acid was analyzed as linear regression of the absorbance against concentration from which tannic equivalents were obtained and the data obtained expressed graphically as column graphs of concentration against the tannic acid equivalent (Total phenol content).

Total antioxidant capacity assay: The antioxidant activity of PFE (0.5-2.5 mg mL^{-1}) was evaluated by the phosphomolybdenum method (Prieto *et al.*, 1999) with modifications. Ascorbic acid (0.05-0.25 mg mL^{-1}) was used as the reference drug.

Experimental procedure: PFE (0.3 mL) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. The absorbance of the supernatant was then determined at 695 nm using a Cecil CE 2040 spectrophotometer (Cecil Instrument Limited, Milton Technical Centre, England) against blank after cooling to room temperature. Distilled water (0.3 mL) is processed as the extract and used as the blank. All measurements were done in triplicates. Data obtained for the ascorbic acid was analyzed as linear regression of the absorbance against concentration from which ascorbic acid equivalents were obtained and the data obtained expressed graphically as column graphs of concentration against the ascorbic acid equivalents (Total antioxidant capacity).

Reducing power: The reducing capacity of PFE (0.1-3 mg mL^{-1}) in methanol was determined using the method of Fe^{3+}- reduction to Fe^{2+} (Oyaizu, 1986) as previously described (Amarowicz *et al.*, 2005) with modifications, using n-propyl gallate (0.001-0.3 mg mL^{-1}) as a reference antioxidant. PFE (1 mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and the 2.5 mL of 1% potassium ferricyanide solution in a test tube. The mixture was incubated at 50°C for 20 min. Following this, 1.5 mL of 10% Trichloroacetic acid solution (TCA) was added to the incubated mixture and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was then mixed with 2.5 mL distilled water and 0.5 mL of the 0.1% ferric chloride solution ($FeCl_{3(aq)}$) in a test tube. The absorbance was then measured at 700 nm. Distilled water was used in place of the test drug and this was used as the blank. Data was presented as concentration-absorbance curves and the IC_{50} (concentration that gives 50% of maximal response) determined.

Free radical scavenging assay: The experiment was carried out as previously described with a few modifications (Govindarajan *et al.*, 2003).

Experimental procedure: PFE (0.5-2.5 mg mL^{-1} in methanol) was compared with n-propyl gallate (0.001-0.03 mg mL^{-1} in methanol) as a reference free radical scavenger. The extract was centrifuged at 3000 rpm for 10 min and the supernatant collected. The supernatant (1 mL) was added to 3 mL of methanolic solution of DPPH (20 mg L^{-1}) in a test tube. The reaction mixture was kept at 25° C for 1 h in an orbital shaker (BoroLabs, Aldermaston Berkshire, EC.). The absorbance of the residual DPPH was determined at 517 nm in a Cecil CE 2040 spectrophotometer (Cecil Instrument Limited, Milton Technical Centre, England). A 1 mL quantity of methanol (99.8%) was added to 3 mL DPPH solution, incubated at 25°C for 1 h and used as control. Methanol

was used as the blank. Three replicates were used. The results were expressed as percentage DPPH scavenging effect against concentration and the IC_{50} determined.

Linoleic acid autoxidation: The method as previously described was used with modifications (Yen *et al*., 2003) PFE (0.5-2.5 mg mL^{-1} in 96% ethanol) was compared with n-propyl gallate (0.001-0.03 mg mL^{-1} in 96% ethanol) as a reference antioxidant. A 2 mL quantity of the PFE mixture and 2.05 mL of 2.51% linoleic acid (in 96% ethanol) was mixed in a test tube. Four millilitres of 0.05 M phosphate buffer (pH 7.0) and 1.9 mL of distilled water were added. The test tube was screw cap and placed in an oven at 40°C in the dark for seven days. To 2 mL of the PFE mixture prepared and incubated (described above), 2 mL of 20% trichloroacetic acid (aq) solution and 1 mL of 0.6% thiobarbituric acid (aq) solution were added. This mixture was placed in boiling water bath for 10 min and after cooling, was centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured.

The % inhibition of linoleic acid autoxidation was calculated as follows:

$$\%\,\text{Inhibition} = \frac{(C_0 - C_1) - (D - D_0 - C_0)}{(C_0 - C_1)} \times 100$$

where, C_0 (Full Reaction Mixture) is the degree of lipid peroxidation in the absence of antioxidant. C_1 is the underlying lipid peroxidation before the initiation of lipid peroxidation. D is any absorbance produced by the extract being tested. D_0 is the absorbance of the extract alone.

Blood clotting time: The ability of PFE to inhibit coagulation of blood *in vitro* was quantitatively assessed. Briefly, 1.0 mL quantities of blood, drawn from the marginal ear vein of rabbits were added to 0.2 mL of PFE (5 and 10% w/v) in test tubes placed in a water bath at 37°C. The time taken for the blood to clot was recorded. Five determinations 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 vehicle (0.2 mL) and blood and blood alone (baseline clotting time).

Rabbit bleeding time: An *in vivo* anticoagulant effect of PFE (1.5 and 3.0 mg kg^{-1}) was investigated as previously described with modifications (Elg *et al*., 2001). In this determination, rabbits were pre-treated with PFE 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 sec till cessation of bleeding (no rebleeding for 1 min) was noted. 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 heparin (0.75-1.5 mg kg^{-1}), aspirin (1-2 mg kg^{-1}) or vehicle (distilled water). A baseline bleeding time was determined before any drug administration.

Statistical analysis: GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA., USA.) was used for all statistical analyses. Data are presented as Mean±SEM and analyzed by One-way ANOVA followed by Newman-Keuls multiple comparisons test (post test). The $p \leq 0.05$ was considered statistically significant in all analysis. The graphs were plotted using Sigma Plot for Windows Version 11.0 (Systat Software Inc., Germany).

RESULTS

Total phenol assay: The phenol content of tannic acid (0.01-0.3 mg mL^{-1}) increased with increasing concentration (r^2 = 0.9901). PFE (0.5-2.5 mg mL^{-1}) also showed a concentration dependent increase in phenolic content expressed as tannic acid equivalent (mg mL^{-1}) from 0.0225 to 0.3551 (Fig. 1).

Total antioxidant capacity: The total antioxidant capacity of ascorbic acid (0.01-0.3 mg mL^{-1}) increased with increasing concentration (r^2 = 0.9035). PFE (0.3-10.0 mg mL^{-1}) also showed an increase in total antioxidant capacity expressed as ascorbic acid equivalent (mg mL^{-1}) from -0.0356 to 0.0702 (Fig. 2).

Correlation between the Total Phenol Content (TPC) and the Total Antioxidant Capacity (TAC): The TPC of PFE correlated with the TAC (r^2 = 0.9811) (Fig. 3).

Reducing power: We observed a concentration dependent reducing activity by both PFE (0.3-10 mg mL^{-1}) and n-propyl gallate (0.001-0.03 mg mL^{-1}) with IC_{50} values (mg mL^{-1}) of 1.168 and 0.116, respectively (Fig. 4). The n-propyl gallate however, exhibited 10-fold potency in reducing power compared to PFE.

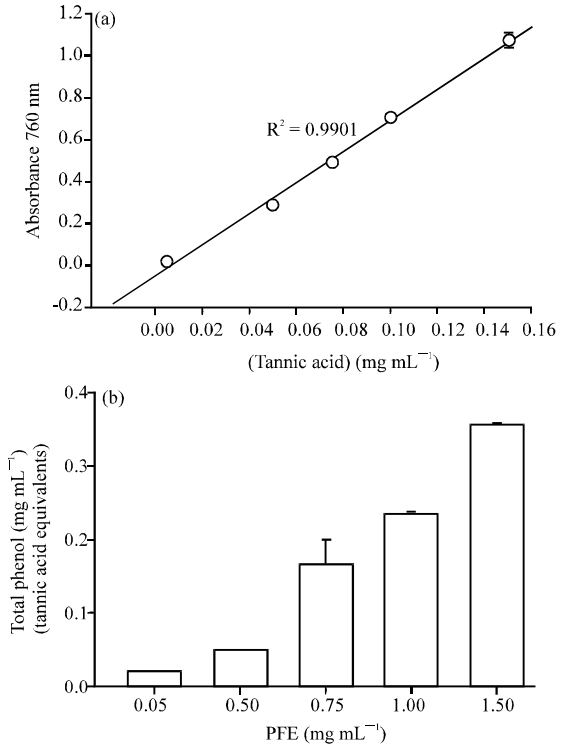

Fig. 1(a-b): Total phenol content; (a) A relationship between the absorbance and concentration of tannic acid (0.01-0.3 mg mL^{-1}) and (b) total phenolic substances present in various concentrations of PFE (0.3-10 mg mL^{-1}) expressed as tannic acid equivalent. Each point in a and columns in b represent the Mean ±SEM (n = 3)

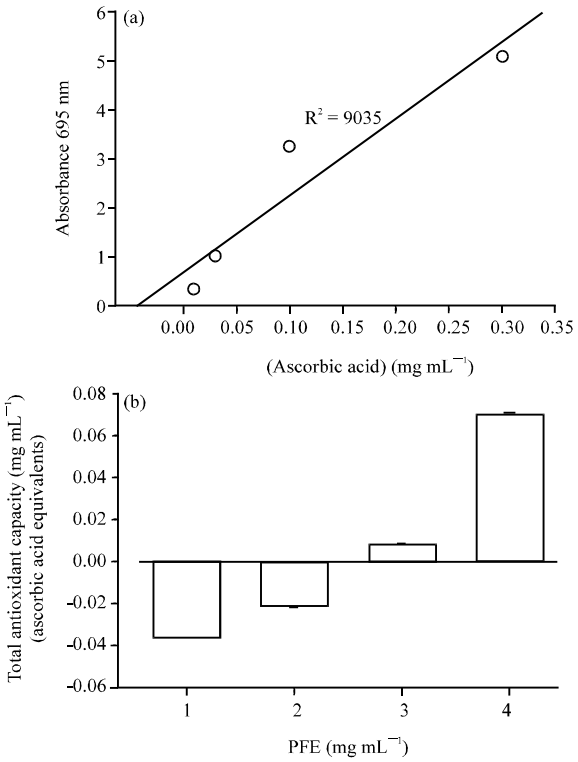

Fig. 2(a-b): Total antioxidant capacity; (a) A relationship between the absorbance and concentration of ascorbic acid (0.01-0.3 mg mL^{-1}) and (b) total phenolic substances present in various concentrations of PFE (0.1-10 mg mL^{-1}) expressed as ascorbic acid equivalent. Each point in a and columns in b represent the Mean±SEM (n = 3)

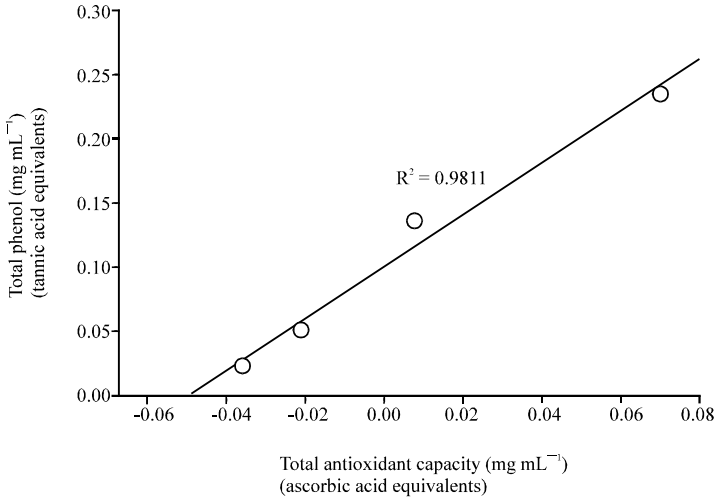

Fig. 3: A relationship and comparison between the Total Phenol Content (TPC) and total antioxidant capacity of PFE (0.25-2.5 mg mL^{-1})

Free radical scavenging effect: The DPPH assay is used to establish the ability of an agent to scavenge free radicals. PFE showed a concentration-dependent scavenging activity in a similar

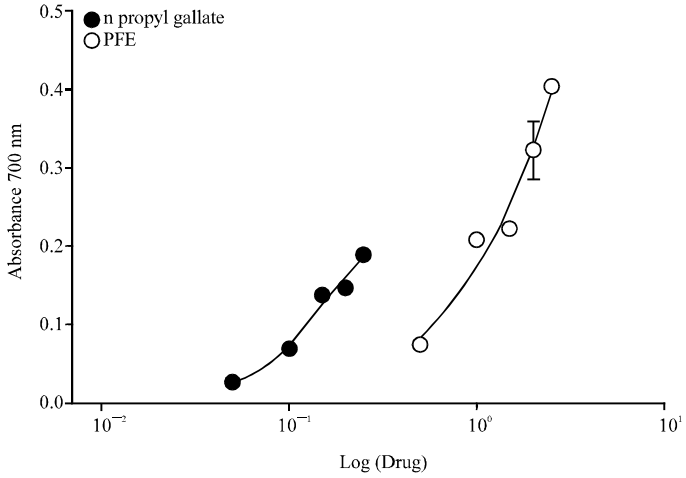

Fig. 4: Reducing power of PFE (0.5-2.5 mg mL^{-1}) compared to n-propyl gallate (0.05-0.25 mg mL^{-1}). Each point represents the Mean±SEM (n = 3)

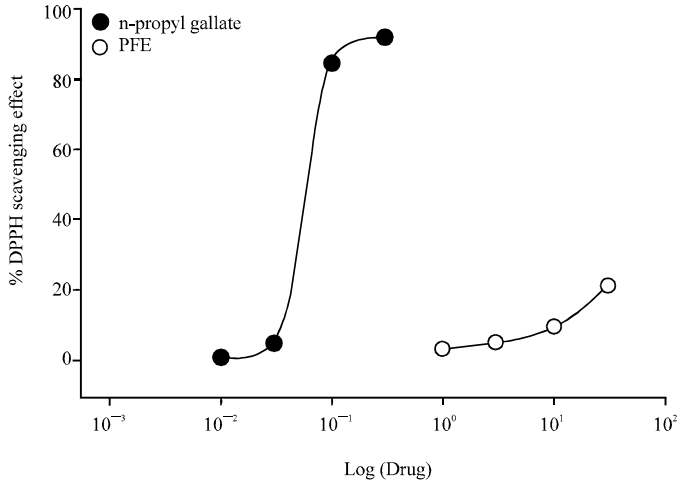

Fig. 5: Free radical scavenging ability of PFE (0.3-10 mg mL^{-1}) compared to *n*-propyl gallate (0.01-0.3 mg mL^{-1}) in the DPPH radical assay. Each point represents the Mean±SEM (n = 3)

manner to n-propyl gallate. The IC_{50} values (in mg mL^{-1}) of 11.404 and 0.062 for PFE and n-propyl gallate, respectively were obtained (Fig. 5), thus n-propyl gallate is 180 times potent than PFE.

Linoleic acid autoxidation assay: All the test samples used, n-propyl gallate (0.001-0.3 mg mL^{-1}) and PFE (0.3-10 mg mL^{-1}) showed concentration dependent ability to inhibit the autoxidation of linoleic acid (Fig. 6). The n-propyl gallate with an IC_{50} value of 0.006 was found be more potent than PFE, with an IC_{50} value of 1.081.

Clotting time: PFE at concentrations of 5% w/v and 10% w/v increased clotting time significantly ($p \leq 0.001$) with respect to the baseline clotting time. Blood added to heparin did not clot. A summary of the effects of PFE as compared to the baseline clotting time is as shown in Fig. 7.

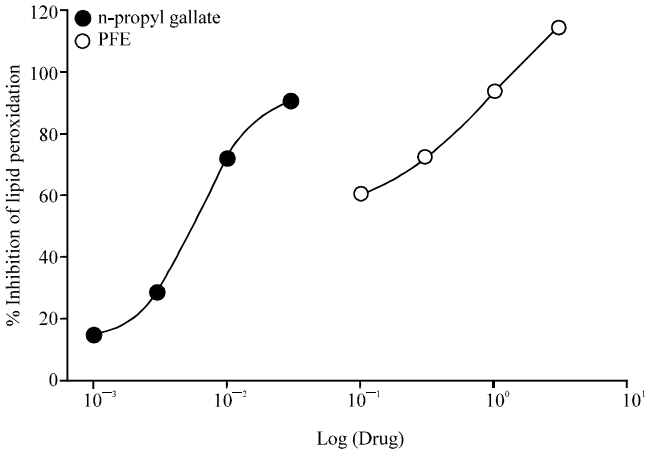

Fig. 6: Percentage inhibition of linoleic acid autoxidation by PFE (0.3-10.0 mg mL^{-1}) compared to n-propyl gallate (0.001-0.3 mg mL^{-1}). Each point represents the Mean±SEM (n = 3)

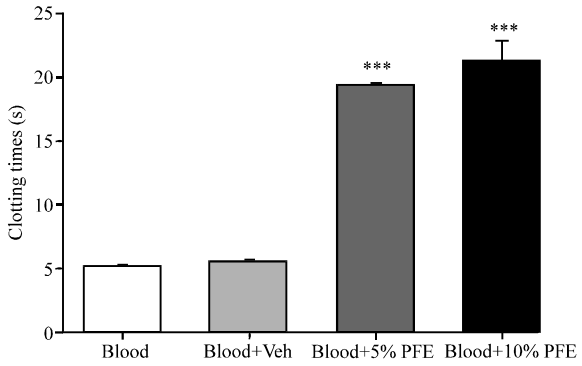

Fig. 7: The effect of PFE (5 and 10% w/v) on clotting time of blood taken from the marginal ear vein of rabbits in a clotting time test. Values are Mean±SEM (n = 5). ***$p \leq 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post hoc test)

Rabbit bleeding time: PFE at a concentration of 1.8 mg mL^{-1} increased the bleeding time significantly ($p \leq 0.01$) with respect to the baseline bleeding time. Doubling the concentration of PFE administered (i.e., 3.6 mg mL^{-1}) about doubled the bleeding time ($p \leq 0.001$). Aspirin (1.0 and 2.0 mg kg^{-1}) and heparin (0.75 and 1.5 mg kg^{-1}) also significantly ($p \leq 0.05$-$p \leq 0.01$) increased bleeding time. The differences in dose effects were very significant ($p \leq 0.001$) for all drugs used. A summary of the effects of PFE and the reference drugs as compared to the baseline bleeding time is as shown in Fig. 8.

DISCUSSION

The present study signifies the antioxidant and anticoagulant scavenging effects of the hydro-ethanolic extract of *P fraternus*. The phytochemical principles detected in *P fraternus* includes alkaloids (securine and related alkaloids); lignans (e.g., phyllanthine and hypophyllantine); tannins; flavanoids (e.g., quercetin, rutin); methyl salicylate; carboxylic acid; saponins (STEPRI and CSIR, 2007). The flavonoids (such as quercetin and rubin) are known to be potent free radical terminators (Boots *et al*., 2008; Chung and Woo, 2001; Utkina, 2009;

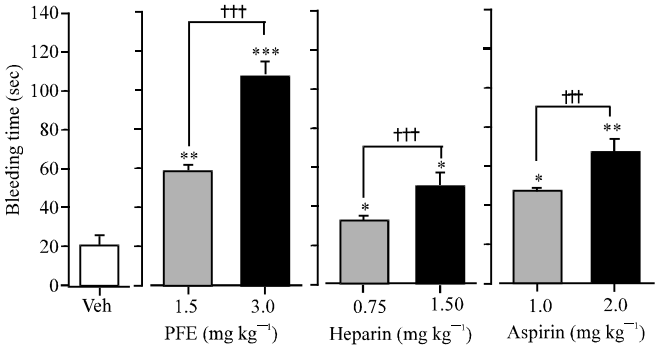

Fig. 8: The effect of PFE (1.5-3.0 mg kg^{-1}), heparin (0.75-1.5 mg kg^{-1}) and aspirin (1-2 mg kg^{-1}) on the bleeding time in rabbits. Values are Means±SEM (n = 5). ***$p \leq 0.001$; ** $p \leq 0.01$; *$p \leq 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post hoc test). $^{\dagger\dagger\dagger}p \leq 0.001$ comparison between dose levels used (Two-way ANOVA followed by Bonferroni's post hoc test)

Cibin *et al.*, 2006; Ouattara *et al.*, 2011) and can help reduce atherosclerosis leading to heart attacks or stroke (Knekt *et al.*, 2000). Thus the antioxidant and anticoagulant properties exhibited by *P. fraternus* may be related to the presence of these constituents.

The detection of phenols in the *P. fraternus* extract was a preliminary evidence of its possible antioxidant activity and this increased with increasing concentration of PFE. Polyphenols have the ability to undergo electron-donation reactions with oxidizing agents producing stable species (Kang *et al.*, 2005) and thus inhibiting or delaying the oxidation of different biomolecules (Amarowicz *et al.*, 2005; Seidel *et al.*, 2000; Bahri-Sahloul *et al.*, 2009). Hence, various plant phenols such as Vitamin E (α-tocopherol), exhibit antioxidant properties (Kang *et al.*, 2005; Ozgova *et al.*, 2003; Seidel *et al.*, 2000; Gupta *et al.*, 2007; Balakumar *et al.*, 2010). Phenolic antioxidants are potent free radical terminators and their presence is a good marker of potential antioxidant activity. The high potential of phenolic compounds to scavenge free radicals may be explained by their phenolic hydroxyl groups. Polyphenols therefore, have Reactive Oxygen Species (ROS) quenching and inhibition of lipid peroxidation and exerts anticarcinogenic, antimutagenic, antitumor, antibacterial, antiviral and anti-inflammatory effects (Ozgova *et al.*, 2003; Ranjbar *et al.*, 2007; Hasani-Ranjbar *et al.*, 2010). The total phenol was assayed based on the reduction of phosphomolybdate-phosphotungstate salts to form a blue complex that is detected quantitatively at 700 nm.

PFE also showed a concentration dependent total antioxidant capacity. The phosphomolybdenum method of assay of the total antioxidant capacity used was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm. Chemical constituents such as carotenoids, flavonoids and cinnamic acid derivatives are known to be responsible for showing antioxidant effect in this assay (Taga *et al.*, 1984; Nakazono *et al.*, 2006; Al-Rejaie, 2009). In addition, a high correlation was obtained between the total antioxidant capacity and the total phenol content suggesting that the phenols detected may also be responsible for the antioxidant effect found in PFE.

P. fraternus also concentration dependently scavenged DPPH radicals *in vitro*. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al.*, 1997). The reduction capability of DPPH is determined by the decrease in the

absorbance at 517 nm induced by antioxidants. This test system is valid for the primary characterization of the scavenging potential of compounds (Krings and Berger, 2001). PFE's ability to concentration-dependently reduce DPPH forming yellowish-colored diphenylpicrylhydrazine suggest that it is a free radical scavenger and probably acts so by donating an electron or hydrogen radical.

PFE showed a concentration dependent increase in its reducing power. This measurement, as described by Oyaizu (1986), investigates the ability of an agent to transform Fe^{3+} to Fe^{2+}. 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 (Duh, 1998; Tanaka *et al*., 1998). Reductones are also reported to react with certain precursors of peroxides, thus preventing peroxide formation. However, activity of antioxidants has been attributed by various mechanisms, among which some of them are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997).

P. fraternus showed an ability to inhibit lipid peroxidation in linoleic acid autoxidation assay. In biological systems, lipid peroxidation generates a number of degradation products, such as malondialdehyde and it is found to be an important cause of cell membrane destruction and cell damage (Yoshikawa *et al*., 1997; Oboh, 2008). Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates the aforementioned degradation and is considered a marker of oxidative stress (Janero, 1990).

The ability of PFE to scavenge free radicals, possessing reducing capacity and inhibiting lipid peroxidation suggests that PFE is an antioxidant that can be classified as preventive, scavenging or chain breaking.

P. fraternus extract, heparin and aspirin significantly increased the time of blood clotting *in vitro* and the bleeding time *in vitro*. Blood coagulation is an important part of haemostasis wherein damaged blood vessels are covered by platelets and fibrin containing clot to stop bleeding and begin repair of the damaged vessel (Owren and Stormorken, 1973). Disorders of coagulation have been known to cause hemorrhage or thrombosis (Rapaport, 1993). Coagulation begins with platelet aggregation and plug formation (primary haemostasis) followed by the secondary homeostasis characterized by formation of fibrin as a result of a complex cascade involving coagulation factors, thus strengthening the platelet plug (Furie and Furie, 2005). Anticoagulants and antiplatelet agents, such as heparin and aspirin (Tohgi *et al*., 1992), respectively, have been used in the management of thrombosis, a major risk factor in stroke and other complications of the cardiovascular disorders (Handoll *et al*., 2002; McCardel *et al*., 1990). Blood clotting and bleeding time measurements are generally used in the preliminary detection of agents that affect the coagulation systems (Dejana *et al*., 1979; Van Genderen *et al*., 1997). PFE induced anticoagulant activity probably through inhibition of the platelet plug formation or by interfering with the coagulation cascade mechanisms. Further investigations are warranted to ascertain the exact mechanisms by which PFE exerts these effects. Nevertheless, these findings lend some information to the use of PFE traditionally in the management of stroke.

CONCLUSION

From the results obtained, the ethanolic extract of *P. fraternus* exhibited antioxidant and anticoagulant effects, thus supporting its traditional uses in the management of conditions where oxidative stress is implicated and thromboembolic stroke. A product formulated from this plant could be beneficial as adjunct therapy in cardiovascular disorder management in Ghana.

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