

***In vitro* ANTIOXIDANT, ANTI-INFLAMMATORY AND THROMBOLYTIC PROPERTIES OF LEAF EXTRACT AND FRACTIONS OF *Pterocarpus mildbraedii* HARMS**

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ABSTRACT

The study was undertaken to evaluate the *in vitro* antioxidant, anti-inflammatory and thrombolytic properties of methanolic extract (PMME) and fractions of the leaf of *Pterocarpus mildbraedii* with a view to exploring the therapeutic potentials of the plant especially as far as treatment and management of oxidative, inflammatory and heart related disorders are concerned. The study involved collection, identification, extraction with 80% methanol, fractionation of hydromethanolic extract of *P. mildbraedii* leaves with n-hexane, ethyl acetate and n-butanol; evaluation of the contents for total phenolics, flavonoids as well as vitamins C and E. Evaluation of the antioxidant and anti-inflammatory potentials of the plant using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing power, hydrogen peroxide reduction, total antioxidant capacity (TAC), and stabilization of erythrocyte membrane and inhibition of albumin denaturation were also carried out. The total phenolic content ranged between 13.31 ± 0.12 and 234.99 ± 2.64 GAE (gallic acid equivalent)/g, flavonoid content ranged between 44.30 ± 0.74 and 228.74 ± 7.44 QE (quercetin equivalent)/g, vitamin C content was 11.98 ± 0.04 mg/g while vitamin E content was 8.62 ± 0.29 ig/g. The fractions exhibited potent and significant antioxidant and anti-inflammatory activities which are concentration-dependent, as well as appreciable thrombolytic activities. Moreover, the extract and fractions compared favourably with various reference drugs used in the study. It was observed that the extract and its fractions possessed bioactive components with beneficial effects in the management of oxidative, inflammatory and heart-related conditions.

Keywords: *Pterocarpus mildbraedii* methanolic extract, Antioxidant, Anti-inflammatory, Thrombolytic, Membrane stability, Haemoglobin, Denaturation.

INTRODUCTION

Oxidative stress is a disturbance in the equilibrium between the production of free radicals (reactive oxygen and nitrogen species, RONS) and the endogenous defense mechanisms (antioxidants) in favour of the production of oxidant species. The body generates free radicals (oxidants) as part of normal metabolic processes, regulation of body functions and signal transduction (Sharma, 2014). This includes generation of energy in the mitochondria through oxidative phosphorylation, detoxication of xenobiotics by cytochrome P₄₅₀ using oxidizing enzymes, apoptosis of defective cells, regulation of cellular function, acting as second messengers for destruction of microorganisms by macrophages (Pham-Huy *et al.*, 2008; Sharma, 2014). Free radicals so generated are capable of damaging important cellular components like protein, DNA, carbohydrates and membrane lipids, as well as causing damage to endothelial cells and leading to a cascade of inflammatory reactions.

The body counteracts oxidative stress by producing enzymatic and non-enzymatic antioxidants, which are compounds/molecules that delay autoxidation and propagation of free radicals by one (or more) of several mechanisms which includes (i) scavenging species that initiate peroxidation (ii) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides (iii) quenching superoxide ions thus preventing formation of peroxides (iv) breaking the auto-oxidative chain reaction and/or (v) reducing localized O₂ concentrations. Antioxidants also donate their electrons to stabilize free radicals and make them stable compounds so as to minimize their harmful effects (Brewer, 2011; Obeagu, 2018). Oxidative stress has been implicated in a number of pathological conditions such as cancer, diabetes, neurological disorders, metabolic disorders and cardiovascular diseases (Qazi and Molvi, 2018).

Many plant products (fruits and vegetables) have been reported to possess antioxidants capable of eliminating, inhibiting or stabilizing free radicals (Pisoschi and Negulescu, 2011). Some of such plants found in sub-Saharan Africa include *Cleome nutidosperma*, *Emilia coccinea*, *Euphorbia heterophylla*, *Physalis angulata*, *Scopania dulcis*, *Richardia bransitensis*, *Spigella antbelmia*, *Tridax procumbens*, *Blighia sapida*, *Erythrophleum suaveolens*, *Lecaniodiscus cupanioides*, *Moringa oleifera*, *Nauclea diddericbii*, *Pterocarpus soyauii* (Edeoga et al., 2005; Lawal et al., 2010).

P. mildbraedii (commonly called Red sandal wood) is classified as part of the family Papilionaceae, grows in the lowland rainforest, dry evergreen and riverine forests in Nigeria, Sierra Leone, Ghana, Cameroon, Equatorial Guinea and Tanzania. It is a semi-wild tree which mostly exists in the wild but scantily seen in households and seldom cultivated by farmers (Okoli et al., 2015). The plant grows to about 15 - 25 m tall and having stem diameter of up to 20 m. It has smooth brown bark and slowly releases red gum when cut (Usunobun and Igwe, 2016). The young and tender leaf of *P. mildbraedii* is widely consumed for its therapeutic and medicinal values and is recommended for use to prevent diabetes and treatment of headaches, pains, fever and convulsions (Ucheghu et al., 2015). While few studies have suggested that the plant contains phytochemicals such as phenolics and flavonoids which indicate that it could serve as an antioxidant, hypocholesterolemic, anti-microbial or cardioprotective agent (Ucheghu et al., 2015; Usunobun and Igwe, 2016), this study seeks to further investigate the antioxidant, anti-inflammatory and thrombolytic activities of the plant to ascertain the value of its use as a therapeutic agent.

MATERIALS AND METHODS

Reagents and Chemicals

All reagents and chemicals used for this study were of analytical grade from various manufacturers.

Preparation of Methanol Extract of *P. mildbraedii*

Young fresh leaves were collected from Ajebandele, Ile-Ife, Nigeria. The plant was identified and authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo

University (OAU), Ile-Ife, where specimen number (IFE-17722) was given. The methanolic extract was prepared according to the method of Oyedapo and Amos (1997). The leaves were cut into bits, air-dried, pulverized and suspended in 80% (v/v) methanol for 72 hr at room temperature with occasional stirring and then filtered. The residue was further extracted with the same solvent until the extract became colourless. The extracts were pooled together, filtered and concentrated *in vacuo* to slurry under reduced pressure at 40 °C on rotary evaporator (Rotavapor RII, BUCHI Switzerland). The slurry was dispensed into clean petri dishes and exposed to air to obtain dark brown residue termed *P. mildbraedii* methanol extract (PMME) and kept in the desiccator for further use.

Fractionation of Methanol Extract using Solvent Partition Technique

The methanol extract was partitioned according to a procedure that was based on earlier reported method (Oyedapo et al., 2010) using solvents of increasing polarities. PMME (21 g) was suspended in hot distilled water (100 ml), allowed to dissolve, filtered and partitioned sequentially in a separating funnel with n-hexane (500 ml x 6). The hexane fractions were pooled together and concentrated to dryness under reduced pressure to yield hexane fraction (HF). The aqueous layer was partitioned with ethyl acetate (500 ml x 13). The ethyl acetate fractions were pooled together and concentrated to dryness under reduced pressure to yield ethyl acetate fraction (EAF). The aqueous layer was further partitioned with n-butanol (500 ml x 20), fractions pooled together and concentrated to dryness under reduced pressure to yield butanol fraction (BF). The aqueous layer remaining was concentrated to dryness under reduced pressure to yield aqueous fraction (AqF).

Biochemical Analyses

A: Evaluation of Antioxidant Potentials

Spectrophotometric methods were used to evaluate the antioxidant potentials of the methanol extract and fractions: total phenolics (mg TAE/g extract) (Singleton et al., 1999), total flavonoid (mg QE/g extract) (Sun et al., 1999), vitamin C (mg/g) (Japota and Dani 1982) and vitamin E ($\mu\text{g/g}$) (Santhosh et al., 2013).

B: Evaluation of Antioxidant Activities

The DPPH-radical scavenging activities of the extract and its fractions were carried out according to the procedure of Blois (1985). The percentage DPPH radical scavenging activity was calculated using the expression:

Percentage Scavenging Activity =

$$\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

The reducing power was measured according to the procedure of Oyaizu (1986), increased absorbance of the reaction mixture indicated increased reducing power; total antioxidant capacity (TAC) was based on those of Prieto *et al.* (1999) and Aliyu *et al.* (2012). The antioxidant capacity of the extract and fractions was expressed as the number of milligram equivalent of ascorbic acid (mg AAE).

The hydrogen peroxide radical scavenging assay activity was carried out according to a procedure that was based on the method reported by Archana and Vijayalakshmi (2018). Ascorbic acid was used as standard and the percentage inhibition was calculated using the expression below:

Percentage Inhibition =

$$\frac{Abs_{test} - Abs_{control}}{Abs_{test}} \times 100$$

C: Evaluation of Anti-inflammatory Activities

Evaluation of the anti-inflammatory activities was carried out using

(i): inhibition of albumin denaturation according to the procedure of Mizushima and Kobayashi (1968) and Aina and Oyedapo (2013). The percentage inhibition was calculated as:

Quantity of protein left =

$$\frac{Abs_{test} - Abs_{control}}{Abs_{test}}$$

Percentage of inhibition =

$$\frac{\text{Quantity of protein left}}{\text{Total protein}} \times 100$$

(ii): membrane stabilizing activity assay of the extract and fractions was based on an earlier procedure (Oyedapo *et al.*, 2010). Ibuprofen (an

anti-inflammatory drug) was used as standard and the percentage membrane stability was calculated as:

Percentage membrane stability = [100 -

$$\frac{Abs_{drug\ test} - Abs_{drug\ control}}{Abs_{blood\ control}}] \times 100$$

The blood control represented 100% lysis.

D: Assay of Thrombolytic Activity

The thrombolytic activity was carried out to assess the *in vitro* clot lysis potential of the *P. mildbraedii* methanolic extract and its fractions according to the method reported by Reyad-ul-Ferdous *et al.* (2014). Briefly, venous human blood (500 μ l) was collected into sterile pre-weighed Eppendoff tubes (M_1), left for 45 min to coagulate at room temperature, centrifuged at 3000 rpm for 5 min and the serum was completely removed. The blood clots were weighed (M_2), the extract and fractions (100 μ l, 1 mg/ml) were added and allowed to stand for 1½ hr at 37 °C in a water bath. The released fluid was removed after incubation and tubes containing the blood clot was re-weighed (M_3). Aspirin (1 mg/ml) was used as standard.

Percentage clot lysis was calculated using the formula:

Percentage of clot lysis =

$$\frac{\text{weight of released clot } (M_3 - M_1)}{\text{clot weight } (M_2 - M_1)} \times 100$$

Statistical Analysis

Data was analysed using Microsoft Excel 2010 and Graph Pad Prism 5, and expressed as Mean \pm SEM, n=5.

RESULTS

A: Concentrations of Antioxidant Potentials in PMME and Fractions of *P. mildbraedii*

Table 1 gives a summary of the antioxidant potentials (phenolics, flavonoids, vitamin C and vitamin E) of the extract and different fractions of leaf of *P. mildbraedii*. It was noted that the plant is very rich in vital bioactive metabolites that

possess and exhibit potent antioxidant and anti-inflammatory activities. Also, in table 2 is the summary of the total antioxidant capacities of the extract and its fractions revealing that all the fractions possess and exhibit significant antioxidant capacities. The aqueous fraction has the highest antioxidant capacity.

B: Antioxidant Activities of PPME and its Fractions

Figures 1-3 depict the antioxidant activities of the leaf extract and fractions of *P. mildbraedii* on DPPH - radical scavenging, ferric reducing antioxidant power, and hydrogen peroxide inhibition activities. It was observed that the PPME and its fractions exhibit potent and significant activities. The antioxidant activity was found to be in the following order; ethyl acetate fraction > aqueous fraction > methanolic extract > butanol fraction > hexane fraction for the three models. Moreover, the activities were concentration-dependent and compared favourably with those of the standard reference (ascorbic acid).

C: Anti-inflammatory Activities of PPME and its Fractions

Figures 4 and 5 depict the anti-inflammatory activities of the PPME and its fractions using inhibition of denaturation of albumin and red blood cell membrane stabilizing activity exposed to both heat and hypotonic-induced lyses models. The percentage inhibition of albumin denaturation of the PPME, its fractions and

aspirin (a standard non-steroidal anti-inflammatory drug) was aspirin ($32.77 \pm 1.43\%$), PPME ($73.46 \pm 0.49\%$), hexane fraction ($41.82 \pm 2.42\%$), ethyl acetate fraction ($84.91 \pm 0.19\%$), butanol fraction ($73.26 \pm 0.48\%$) and aqueous fraction ($68.16 \pm 0.28\%$). The albumin denaturation inhibition was observed to be concentration-dependent which implied that as concentration increases percentage inhibition also increased proportionally.

Figure 5 is a summary of the stabilizing effect of the extract and fractions on red blood cell membrane exposed to both heat and hypotonic lyses. It was observed that ethyl acetate fraction exhibited the highest range of activity (11.38 ± 0.55 and $74.23 \pm 1.06\%$), PPME (30.74 ± 14.77 and $73.83 \pm 1.21\%$), hexane fraction (56.45 ± 2.91 and $60.28 \pm 0.23\%$), aqueous fraction (11.22 ± 0.45 and $30.47 \pm 11.42\%$), and butanol fraction the least activity (3.06 ± 1.69 and $13.69 \pm 1.13\%$) as against ibuprofen, the standard anti-inflammatory drug (8.38 ± 0.88 and 17.73 ± 4.62). The mode of protection exhibited by PPME, its fractions and ibuprofen was monophasic.

D: Thrombolytic Activity

Figure 6 depicts the thrombolytic activities of the PPME and its fractions as expressed in percentage of clot lysis. These results reveal that the leaf extract of the plant and its fractions could be good thrombolytic agents and good candidates for the development of agents in the management and treatment of cardiovascular disorders.

Table 1: Antioxidant Potentials of Methanol Extract and Fractions of *P. mildbraedii*

Molecule	Methanol Extract	Hexane Fraction	Ethyl acetate Fraction	Butanol Fraction	Aqueous Fraction
Total Phenolics (mgGAE/g)	189.07 ± 0.94	13.31 ± 0.12	234.99 ± 2.64	94.99 ± 0.60	101.19 ± 0.40
Total Flavonoids (mgQE/g)	49.85 ± 1.34	44.30 ± 0.74	228.74 ± 7.44	63.93 ± 0.37	108.00 ± 1.28
Vitamin C (mg/g)	11.98 ± 0.04				
Vitamin E ($\mu\text{g/g}$)	8.62 ± 0.29				

Each value represented the Mean \pm SEM of 5 determinations
GAE- gallic acid equivalent; QE- quercetin equivalent

Table 2: Total Antioxidant Capacity of Methanol Extract and Fractions of *P. mildbraedii*

Extract/Fraction	Total Antioxidant Capacity (mgAAE/g)
Methanol	103.94 ± 1.85
Hexane	278.94 ± 10.03
Ethyl acetate	436.81 ± 1.60
Butanol	430.79 ± 9.22
Aqueous	503.47 ± 11.02

Each value represented the Mean ± SEM of 5 determinations AAE (Ascorbic Acid Equivalent)

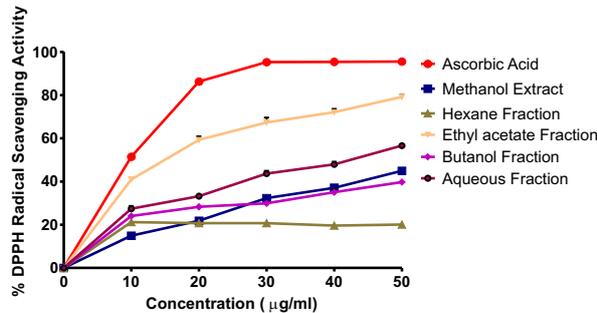


Figure 1: DPPH Scavenging Activities of PPME and Fractions of Leaf of *P. mildbraedii*
Each value represented the Mean ± SEM of 5 determinations

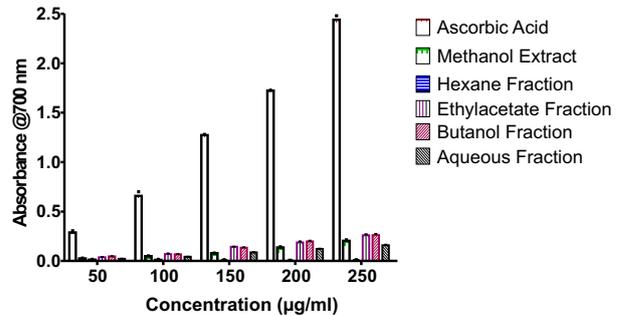


Figure 2: Ferric Reducing Antioxidant Power of PPME and Fractions of *P. mildbraedii*
Each value represented the Mean ± SEM of 5 determinations

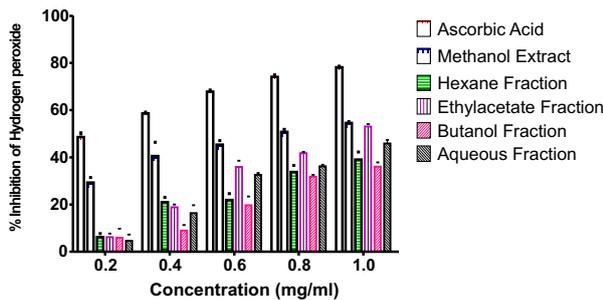


Figure 3: Inhibition of Hydrogen Peroxide by PPME and Fractions of *P. mildbraedii*
Each value represented the Mean ± SEM of 5 determinations

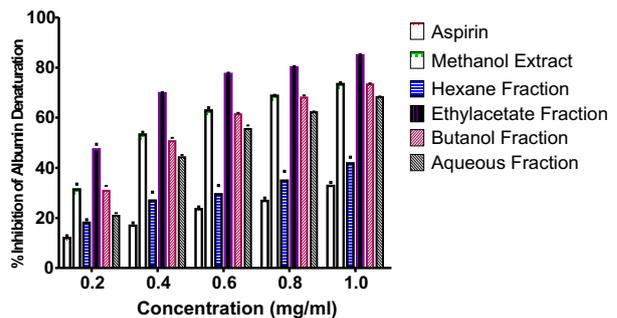


Figure 4: Inhibition of Albumin Denaturation of PPME and Fractions of *P. mildbraedii*
Each value represented the Mean ± SEM of 5 determinations

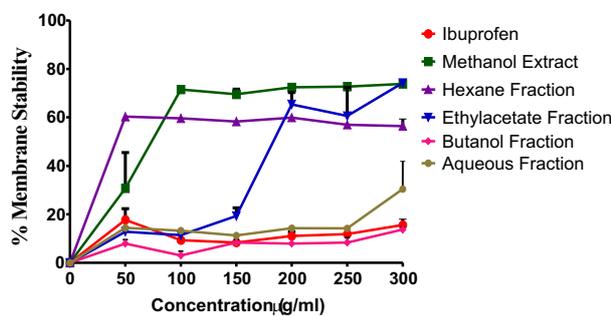


Figure 5: Membrane Stabilizing Potential of PPME and Fractions of *P. mildbraedii*
Each value represented the Mean ± SEM of 5 determinations

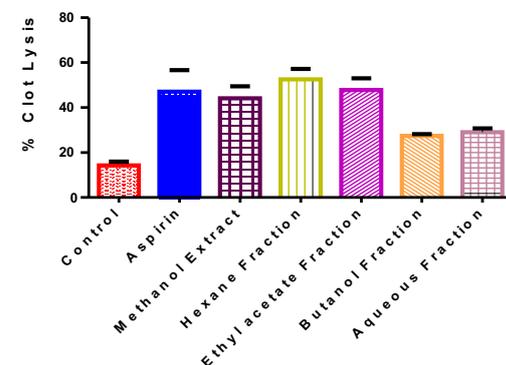


Figure 6: Thrombolytic Effect of Methanol Leaf Extract of *P. mildbraedii* and its Fractions
Each value represented the Mean ± SEM of 5 determinations

DISCUSSION

Naturally occurring phenolics found in fruits and vegetables have been found to possess and exhibit different properties ranging from antioxidants, anti-inflammatory, anti-proliferative, anti-cancer, anti-spasmodic, anti-depressant, anti-ulcer and protection against chronic and degenerative diseases such as cardiovascular diseases (Yadav *et al.*, 2016; Ravimannan and Nisansala, 2017).

The antioxidant potentials of the *P. mildbraedii* extract and the fractions were determined by DPPH scavenging activity, ferric reducing antioxidant power, hydrogen peroxide scavenging activity and total antioxidant capacity and compared with standard ascorbic acid. DPPH is a stable free radical and widely employed to test the ability of compounds/agents to scavenge radicals. It is the easiest, fastest and most convenient ways of evaluating antioxidants and radical scavenging activity of plant extracts (Usunobun and Igwe, 2016; Archana and Vijayalakshmi, 2018).

The percentage DPPH scavenging power of the extract and fractions was concentration-dependent, which implies that as concentrations of the extract and fractions increase the percentage radical scavenging increased. It was observed that the ethyl acetate fraction was the most potent and comparable to the standard ascorbic acid. The results of this study agreed with the previous reports of Usunobun and Igwe (2016) and Yembeau *et al.* (2018) who observed concentration-dependent DPPH scavenging activities for extracts of *P. mildbraedii* and *Theobroma cacao* respectively.

Ferric reducing antioxidant power (FRAP) is another model employed to assess antioxidant properties of phytochemicals and is based on the reddish-brown colour of Fe^{3+} being changed to green when reduced, with increasing absorbance representing an increase in reducing ability of an agent or compound (Oyaizu, 1986). The reducing ability of PMME and fractions was also concentration-dependent and in agreement with earlier results of Usunobun and Igwe (2016) which reported that the reducing ability of aqueous extract of *P. mildbraedii* leaf was exerted by the donation of a hydrogen atom in breaking the free radical chain.

Olarewaju *et al.* (2018) reported that the ferric reducing antioxidant power of aqueous leaf extracts of *Solanum macrocarpon*, *Amaranthus viridis* and *Telfairia occidentalis* were also concentration-dependent. The total antioxidant capacity (TAC) of the PMME and fractions shows that the vegetable can be employed as a source of both water-soluble and fat-soluble antioxidants (Aliyu *et al.*, 2012). This observation is in agreement with the report of Olarewaju *et al.* (2018) which stated that TAC reflects the synergistic interactions of compounds present in a vegetable and also an index that describes ability of the antioxidants to neutralize preformed free radicals. Potential *et al.* (2016) also reported that the TAC of a vegetable is basically due to the flavonoids and other polyphenol contents in the plant.

Hydrogen peroxide, a weak oxidizing agent which inactivates some enzymes by the oxidation of essential thio (-SH) groups, can sometimes be toxic to the cell when it crosses the cell membrane giving rise to hydroxyl radicals through the Fenton reaction (Archana and Vijayalakshmi, 2018). The inhibition of hydrogen peroxide ability of the extract and its fractions is in agreement with a previous study which reported dose-dependent inhibition of hydrogen peroxide by phloroglucinol, a polyphenol component of Laminariaceae family (Archana and Vijayalakshmi, 2018). It is surmised that consumption of *P. mildbraedii* could be beneficial in preventing oxidative stress related degenerative disorders.

Inflammation is a normal complex response to tissue injuries to stimuli triggered by physical microbial infections, heat and chemical injuries (Ashley *et al.*, 2012; Akinwunmi and Oyedapo, 2015). It is a protective attempt by the body to eliminate injurious stimuli as well as initiate the healing process for the tissues (Ashley *et al.*, 2012). Denaturation of protein is one of the causes of rheumatoid arthritis. The mechanism of denaturation probably involves alteration of the bonds associated with the tertiary structure of proteins. It has been well established that denaturation of tissue proteins leads to inflammatory disorders such as arthritis (Williams *et al.*, 2008).

Studies have revealed that steroidal and non-

steroidal anti-inflammatory drugs usually employed in the treatment and management of inflammatory conditions are not devoid of adverse effects such as ulcer and gastric irritation (Oyedapo *et al.*, 2010; Sakat *et al.*, 2010). Plant products that exhibit abilities to prevent or inhibit protein denaturation would serve as an anti-inflammatory agent and be considered for the development of anti-inflammatory agents (Osman *et al.*, 2016). It was observed from the study that the extract and fractions exhibited a dose-dependent inhibition of albumin denaturation, with ethyl acetate fraction showing the highest activity. The inhibition of heat-induced denaturation of albumin was observed to be concentration-dependent and compared with the standard drug ibuprofen. It implied that anti-inflammatory activity of PMME and fractions could be due to the presence of phenolic and related molecules in them. Other plants which have been reported to exhibit anti-inflammatory properties through inhibition of albumin denaturation include *Monodora myristica* (Akinwunmi and Oyedapo, 2015) and *Oxalis corniculata* (Sakat *et al.*, 2010). This anti-inflammatory activity might be through the inhibition of the release of lysosomal content of neutrophils at the site of inflammation (Sakat *et al.*, 2010; Akinwunmi and Oyedapo, 2015).

The vitality of cells depending on the integrity of their membranes is an established factor and the exposure of red blood cells to injuries such as heat and hypotonic medium leads to the lysis of their membrane associated with hemolysis (Aina and Oyedapo, 2013; Tantary *et al.*, 2017). Stabilization of erythrocyte membrane, an analogue of lysosomal membrane is important in mimicking the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophils like proteases and bactericidal enzymes which cause further tissue damage. Injury caused to erythrocyte membrane would further render the cell more susceptible to secondary damage through free radical-induced lipid peroxidation. Stabilization of erythrocyte membrane exposed to both hypotonic and heat-induced lyses by the extract and its fractions as investigated in this study revealed that *P. mildbraedii* leaf showed a dose-dependent membrane stabilizing activity over the concentration ranges

tested. This result compared favourably with the activity of ibuprofen.

The potency of the extract could be as a result of synergistic activities of its many constituents and that of ethyl acetate fraction due to the high content of its polyphenol constituents (Oyedapo *et al.* 2010; Umapathy *et al.*, 2010). This is in agreement with earlier studies (Aina and Oyedapo, 2013; Akinwunmi and Oyedapo, 2015) who reported the abilities of extract of seeds of *Monodora myristica* and *Cyclosorus afer* to stabilize erythrocyte membrane exposed to both heat and hypotonic-induced lyses. The activities were dose-dependent and due to the presence of naturally occurring flavonoids. Sakat *et al.* (2010) also reported the dose-dependent stabilizing activity of *Oxalis corniculata* on erythrocyte membrane exposed to heat induced lysis.

Thrombosis is the formation of a typical mass (blood clot) in the artery of a living vascular system linked with myocardial infarction and stroke, while thrombolysis is the breakdown of blood clots through pharmacological means involving thrombolytic drugs which dissolve clots (Tanvir *et al.*, 2015, Jakaria *et al.*, 2017). The most commonly used thrombolytic drug is aspirin, whose use is limited and which has to be administered in low dose as a result of attendant side effects due to inhibition of prostanoids which have protective effects on the gastrointestinal mucosa. It is therefore desirable to find natural products which will exert thrombolytic effect with little or no unwanted side effects. In this study, it was observed that *P. mildbraedii* extract and its fractions exhibited substantial and significant thrombolytic potentials which compared favourably with the reference standard drug (aspirin). This is an important finding which may have significant implication in cardiovascular health, particularly in atherothrombotic patients (Reyad-ul-Ferdous *et al.*, 2014; Tanvir *et al.*, 2015). It will however be necessary to study the structural properties of the active compounds responsible for this activity and investigate their mechanism of action.

CONCLUSION

The results of our investigations reveal that the leaves of *P. mildbraedii* contain vital bioactive

metabolites including phenolic compounds with significant antioxidant and anti-inflammatory activities which could be helpful in the management of oxidative and inflammatory disorders as well as serving as potent thrombolytic agent.

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