

Ethanollic Extract of *Moringa Oleifera* Seed Prolongs Blood Coagulation in Wistar albino Rats

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Abstract

The leading cause of death worldwide has been linked to cardiovascular diseases which are mostly associated with thrombosis. It has been estimated that 1 out of 4 people die as a result of thrombotic events, a pathophysiological risk many do not know about. Prevention and treatment of intravascular thrombosis, which still poses a challenge by causing unwanted drug interactions, bleeding risks and incidence of drug resistance requires research. Anti-thrombotic drugs and nutritional supplements that deliver more effective treatment and prevention of intravascular thrombosis are direly needed. In this study, we researched the effect of *Moringa oleifera* ethanollic seed extract on Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT) in vivo using Wistar albino rats. A total of 40 rats were weighed and randomly divided into two groups (I= for PT and II= for aPTT) of 20 rats each (15 rats for test and 5 for control each). Ethanollic extract of *m. oleifera* seed at the dose of 100mg/kg, 200mg/kg and 400 mg/kg were administered to each group using 5 rats per dose for 28 days, PT and aPTT were determined using Quick's method. The data were analyzed using Graph Pad Prism (7.03). There was a statistically significant delay at $P < 0.0001$ of PT and aPTT compared to the control (administered with only distilled water using 5 rats/group). The delay in PT and aPTT is an indication that the seed extract of *m. oleifera* may pose an antagonizing effect on both the intrinsic and extrinsic coagulation pathways, respectively, a property that could be exploited in the prevention and management of thrombotic events.

Keywords: *Moringa oleifera*; Prothrombin Time; Activated Partial Thromboplastin Time; Thrombosis; Coagulation Pathways

Introduction

The intrinsic and extrinsic coagulation pathways contribute significantly to the stabilization of the thrombus [1, 2]. Several important substances that cause or affect blood coagulation have been identified in the blood and body tissues under normal physiological conditions. Some of these substances promote coagulation and are therefore called pro-coagulants, while those that inhibit coagulation are called anticoagulants [3-5].

In the bloodstream, the anticoagulants predominate under physiological conditions, so the blood does not coagulate while in circulation. However, when a blood vessel is ruptured, pro-coagulants from the area of tissue damage become 'activated' and override the anticoagulants, thereby contributing to the clot development through a cascade of activities that result in the formation of prothrombin activator, which is factor Xa that forms a complex with Ca ions, phospholipid and factor V [6, 7]. Generally, Xa is considered to be formed in two ways, although, in reality, the two ways always interact with each other; firstly, by the extrinsic pathway that begins with injury to the vascular wall and surrounding tissues and secondly, by the intrinsic pathway that begins in the blood [8].

The prothrombin time (PT), which measures the extrinsic pathway activities is done to determine the presence or absence of clotting factors IIIa (tissue factor), VIIa, and Xa, while the activated partial thromboplastin time (aPTT), which measures the intrinsic pathway activities, determines the presence or absence of factors XIIa, XIa, IXa, VIIIa and Xa. The formation of Xa which is simultaneous (at different timings) from both pathways results in the activation of prothrombin to thrombin, and thrombin automatically activates fibrinogen to form a more stable fibrin. The coagulation pathways when triggered continues in a cascade of activities which eventually results in bleeding arrest. These clotting factors involved in the cascade activities are sensitive to the presence of circulating anticoagulants and drugs like heparin which may prolong the PT and aPTT in both rats and humans [7, 8]. Pathophysiological events that could lead to thrombosis may include hyperactivity of the coagulation cascades, atherosclerosis which may lead to narrowing of blood vessels, and stagnation of blood flow resulting in immobility. Many of these events are not clinically apparent but still could lead to later problems like myocardial infarction, stroke, deep vein thrombosis and stenosis which affects about 20% of patients admitted to a medical service [9].

Medicinal plants consist of many phytochemical compounds that possess antioxidant activities which are helpful in disease control and prevention [10-12]. There is an increasing interest in the development and evaluation of these naturally available antioxidants and phytochemicals from medicinal plants in the food industry and in the field of preventive medicine [13-15]. *Moringa oleifera* Lam has been reported among those medicinal plants. This promising plant species also known as Moringa or drumstick tree is commonly found in the sub-Himalayan regions of Northwest India with a variety of medicinal uses and high nutritional value [16-17]. The plant is said to contain a mixture of several hydrolytic enzymes, in which proteases are the key enzymes responsible for the observed pharmacological actions [18].

In Nigeria, Hausa/Fulani communities and many other tribes in the North Central region, in particular, have reported that *M. oleifera* has an impressive range of nutritional and medicinal values. It is highly cherished and widely used. In Plateau State, *M. oleifera* is commonly found especially in Langtang North and South Local Government Areas. The locals use the tree to make fences and usually in preparing a local vegetable salad with the boiled leaves mixed with groundnut-cake powder, onions, tomatoes and pepper. The tasty salad is widely known as 'kodon-zogalle', coined from the name of the tree commonly called zogalle in the Hausa language. The seeds are used as coagulants for water purification [19, 20]. Many herbalists and traditional medicine practitioners have utilized various parts of the plant such as the leaves, flowers, seeds, bark and roots to manage ailments such as hypercholesterolemia, hyperglycemia, hepatitis, bacterial and fungal infections, ulcer, and cancer conditions among others [13, 21, 22]. *M. oleifera* roots and seeds have also been prescribed by herbalists for the treatment of snake bites and scorpion stings [23]. It is, therefore, necessary to validate some of these claims using scientific approach [15, 24].

The ability to interpret clotting time, and in particular, the PT and aPTT of an individual can be of great help in the diagnosis of thrombotic events in-vivo. The normal reference ranges for currently used anticoagulants are within 11-16 seconds for PT, and 30-40 seconds for aPTT [31].

This study, therefore, investigated the effect of *M. oleifera* seed on prothrombin time (PT) and activated partial thromboplastin time (aPTT) using Wistar albino rats as a preclinical drug development approach as well as identification of new targets [25].

Materials and Methods

Seed Collection and Authentication

Domestic seeds of *Moringa oleifera* were obtained from Gwafan-Lamingo area of Jos-North in Plateau State Nigeria.

Preparation and Seed Extraction

To prepare the seeds, the coat and wings of the good seeds were removed, and the kernel collected and kept to dry for 5 days at room temperature (25-38°C) in the Department of

Pharmacognosy laboratory. The dried kernel was weighed and a fine powder of the seeds was produced by using the coffee mill attachment of Moulinex domestic food blender (Moulinex Genuine Blender 1.25 Liter, 400 Watt, White - LM2421EG made in France). Seed powder of *M. oleifera* was obtained after blending. The powder was soaked in 4 litres of 70% ethanol at room temperature for 3 days after which it was filtered using filter paper; the bulked filtrate was evaporated using hot air oven at 41°C for 24 hours. Until needed, the powder produced was stored at 4°C. A fresh solution of the seed extract was prepared when needed for the test by dissolving the fine powder of *Moringa oleifera* seed in distilled water in the ratio of 10g to 100ml (to give 100mg/ml concentration) and stirred for two minutes before administration [26].

Experimental Animals

Forty (40) male and female Wistar rats weighing between 150g to 250g were used for the research. The rats were weighed using an electric beam balance at commencement of the experiments and weekly throughout the duration of the experiment. After weighing, the rats were kept in a plastic cage at room temperature with twelve hours a night/dark cycle. They were fed and kept under a hygienic condition in order to prevent infection.

Experimental design

To determine PT, 15 rats were used for the test, and 5 rats were used for control. Similarly, to determine aPTT, 15 rats were used for test and 5 rats were used for the control. For the test (using 15 rats); 100 mg/kg (low dose) of the seed extract was administered to 5 rats, 200 mg/kg (medium dose) was administered to the second group of 5 rats, and 400 mg/kg (high dose) was administered to the third group of rats before determining PT. The same protocol above was used in the determination of aPTT also. The oro-gastric method of intubation was adapted for the administration of seed extract for experimental rats while distilled water was administered to the control groups of 5 rats for both PT and aPTT. These were done in the morning hours before feeding the animals. The rats were intubated daily throughout the period of the experiment (28 days).

Sample Collection

Twenty four hours after the last dose of seed extract was administered, the rats were weighed and then anaesthetized by placing them one at a time in a closed jar containing cotton wool soaked with chloroform, after which they were euthanized by cervical dislocation and blood was collected from the jugular vein into sodium-citrate sample bottles (in the ratio of 4.5ml blood to 0.5ml of sodium-citrate) and the content gently mixed by rolling the bottle. Plasma was immediately separated by spinning in a centrifuge. The blood parameters analyzed were PT and aPTT using Quick's method.

Laboratory Methods

Prothrombin Time Determination

Exactly 4.5ml of blood was collected in a sample bottle

containing 0.5ml sodium-citrate and immediately mixed, then separated to obtain plasma by spinning in a centrifuge. 5 khan tubes were arranged in a rack immersed in the water bath at 37°C. 0.1ml of the PT reagent was pipette into each of the 5 tubes and allowed to warm up to 37°C for 2 minutes. 0.1ml of the test plasma was pipette into the first tube and mixed. At the first appearance of a fibrin clot, the stop watch was stopped and time recorded as PT. The above procedure was repeated for the other four tubes and their PT also recorded (Reference range =11-16 seconds) [27].

Activated Partial Thromboplastin Time Determination

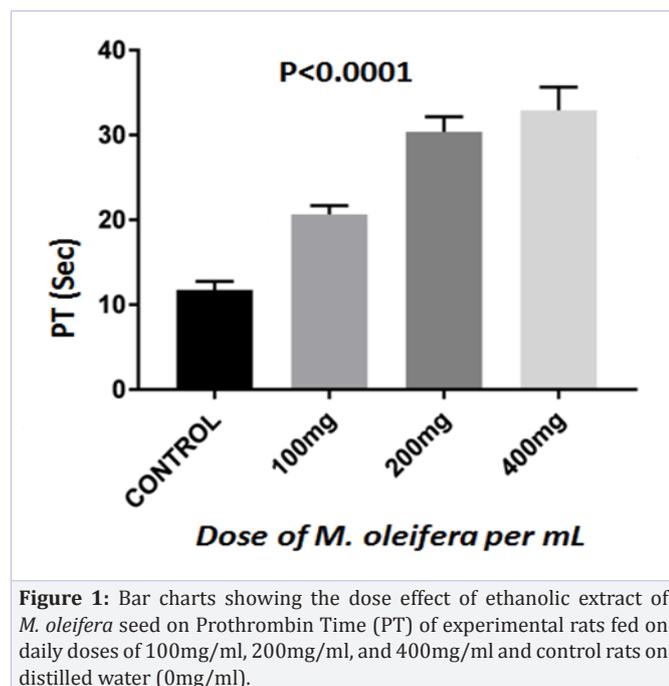
5 khan tubes were arranged in a rack immersed in the water bath at 37°C. 0.1ml of aPTT reagent was incubated in each of the 5 tubes at 37°C for 30minutes. An equal volume (0.1ml) of plasma was put into the tubes. The mixture was thoroughly mixed and left at 37°C for 5minutes.After 5 minutes, 0.1ml of CaCl₂ was added to tube 1 and mixed thoroughly. On addition of CaCl₂, the timer was started, and aPTT was recorded immediately at the sight of a fibrin clot. The procedure above was repeated for each remaining four tubes (Reference range = 30-40 seconds) [28].

Statistical Analysis

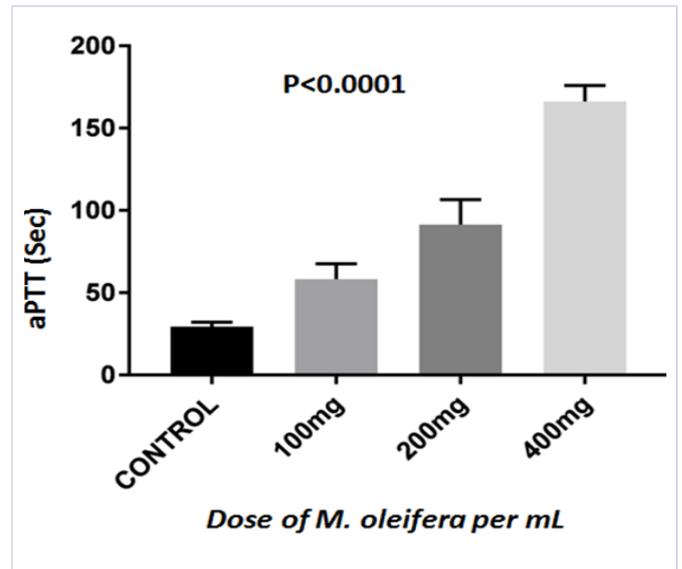
Data collected were expressed as mean ± S.D and analysed using the Graph Pad Prism 7.03 (statistical) software program. One Way analysis of variance (ANOVA) was used to determine the level of significance at 95%, (p-value< 0.05 was considered significant).

Results

The result showed that the mean value of PT and aPTT in control groups fall within the normal range of 11-16s and 30-40s, respectively. At 100mg/ml of the extract however, the mean value of PT and aPTT were 20.65s and 65.22s respectively, at



200mg/ml PT= 30.37s, while aPTT= 89.40s, and at 400mg/ml, PT= 32.91s, while aPTT= 153.30s. The effect of ethanollic extract of *M. oleifera* seed as presented in Figures 1 & 2, were seen to be accompanied by a significant increase in both PT and aPTT with increasing concentration of the dosage of extract in the order of 100mg/ml, 200mg/ml, and 400mg/ml (P<0.0001).



Discussion

Thrombosis has been reported to occur in at least 1 in 1000 people, and those most at risk are the aged and those involved in immobile activities such as hospitalized patients [9]. Antithrombotic drugs in use today though helpful have various side effects, including bleeding and hematoma [29]. Therefore it is necessary to look into the possibility of researching new effective antithrombotic drugs with minimal or no side effects [30-32].

M. oleifera was described as a plant with many medicinal values. Various parts of the plant such as the leaves, seeds, fruits, flowers and bark act as cardiac and circulatory stimulants and antihypertensive, among others [24, 31]. Scientists have been able to prove some of the claims by herbalists about the numerous ailments that can be treated by the use of *Moringa oleifera* seeds [31] some of the claims such as the use of *Moringa oleifera* seeds as antithrombotics are still in the process of being proven [33]. The ability of the seed to reduce platelets count has been reported [34], and the anticoagulant ability of the seeds which helps in water purification has also been reported [24]. Perhaps most importantly, the ability of *M. oleifera* seed to inhibit protease such as thrombin [13] contributes to part of the justification for this work. Similarly, lectin isolated in the seeds of *M. oleifera* has been reported to be responsible for the prolongation of PT and aPTT in vitro [35]. These and many other pieces of evidence including traditional or herbalists claims [31, 36] makes it necessary to

look at its possibility of being a potentially powerful tool for inactivating target proteases which contribute to the pathogenic process that leads to human diseases such as high blood pressure and thrombosis [18, 37].

In this present study, we studied the effect of ethanollic seed extract of *Moringa oleifera* on PT and aPTT in vivo using Wistar albino rats. The result showed that the mean value of PT and aPTT in control groups fall within the normal range of 11-16s and 30-40s, respectively. At 100mg/ml of the extract, the mean value of PT and aPTT were 20.65s and 65.22s respectively, at 200mg/ml, PT= 30.37s, while aPTT= 89.40s, and at 400mg/ml, PT= 32.91s, while aPTT= 153.30s. The effect of ethanollic extract of *M. oleifera* seed as presented in Figures 1 & 2 was seen to be accompanied by a significant increase in both PT and aPTT with increasing concentration of the dosage of extract in the order of 100mg/ml, 200mg/ml, and 400mg/ml ($P < 0.0001$). The study shows that *Moringa oleifera* seed extract may be responsible for the delay in PT and aPTT in Wistar albino rats. Analysis of variance was performed to compare the mean of extract doses and concentrations which reveals significantly ($p < 0.05$) that all the doses (100mg/ml, 200mg/ml, and 400mg/ml) resulted in increased coagulation (haemostatic) time in rats.

Our work supported the report that a coagulant isolated from *m. oleifera* seed called Lectin significantly prolongs activated partial thromboplastin time (aPTT) and prothrombin time (PT), which was carried out in-vitro using human blood [35], but we investigated whole blood PT and aPTT in-vivo using rats. This study also agrees with the findings of Ajibade et al [34], since a reduction in the number of platelets may also mean a decrease in the potential contribution of platelets during haemostatic activities, particularly in the coagulation phase, thereby increasing the time required for the blood to clot as reflected in prolonged PT and aPTT. It could be possible that the reported reduction in the number of platelets may be caused by the ability of the seed extract to cause platelets aggregation thereby hiding or depriving their receptor sites from connecting with vWF during primary haemostasis, an action that is opposed to that of desmopressin a procoagulant [38], and if that is the case, *M. oleifera* seed extract could be said to possess pharmacological activities opposing the action of desmopressin, hence may also be a reasonable replacement for desmopressin antagonist, which means it could be a good replacement of desmopressin antidote in case of overdose or prolonged usage, as overdose or prolonged usage of desmopressin has the potential to eventually result in highly established thrombus formation [39]. The higher the dose the longer it takes for clotting to take place. This means that hypercoagulability (one of the causes of thrombotic events) may be delayed with the right doses of *Moringa oleifera* seed extract since a delay in the coagulation process also means a delay in the formation or activation of thrombin, one of the three required agonists required for the release of vWf, the other two agonists being ADP and collagen [38, 40]. It, therefore, could mean that *M. oleifera* seed extract may possess the ability to reduce the production and release of vWf multimers by alpha-granules

from platelets, an action opposed to that of desmopressin since the clinical efficacy of desmopressin is decrease in bleeding time [40, 41]. Drugs that are used to treat thrombosis are helpful but have a lot of side effects [13]. Even the new anticoagulants such as dabigatran, apixaban and rivaroxaban are said to be responsible for some hemorrhagic complications in patients [14, 15].

There is, therefore, a great need for new medicines with none or minimal side effects. The ability of *M. oleifera* seed extract to prolong the activities of the coagulation pathways (intrinsic and extrinsic at 20.65s and 65.22s respectively) at the dose of 100mg/ml could mean that *M. oleifera* seed extract may have an inhibitory (anticoagulant) effect which increases with an increase in the dose, just as in the case of heparin in rats and humans. These findings could be the reason why *m. oleifera* seeds are used as anti-scorpion, and anti-snake bites by herbalists [18, 23].

The lack of toxicity of the plant and its ability to be metabolized within 24 hours is an encouraging reason for the consideration of *M. oleifera* seed as a potentially good pharmacological agent with respect to anticoagulants [42].

Conclusion

The results of this study showed that *M. oleifera* seed has the ability to delay haemostatic activity for as long as the administered dose lasts, usually within twenty four hours (24hrs.). Since *m. oleifera* works in a similar manner to warfarin, heparin and aspirin including recently developed anticoagulants such as apixaban; it may be a good replacement or another alternative of blood thinners such as aspirin. The seed extract of *M. oleifera* has the advantage of being a nutritional supplement, meaning it has far less side effect if any.

Recommendations

- 1) Developing the seeds of *Moringa oleifera* as a nutritional supplement or even as a drug which could be used in the management of thrombotic events such as deep vein thrombosis (DVT) or its causes such as atherosclerosis, can be very beneficial, especially for patients suffering from an ischemic heart attack or even stroke.
- 2) Further research on the effect of *M. oleifera* seed extract on other haemostatic profiles such as clotting factors, co-factors and vWf, may be able to reveal more on the specific efficacy of this plant seed in arresting thrombotic activities.
- 3) The seed of *M. oleifera* when used as a nutritional supplement at the right dose, can help in the prevention of unnecessary activation of platelets leading to thrombosis, this is because the seed extract has shown to have an antagonizing effect at both the primary and secondary stages of haemostasis.
- 4) A comparative study between *M. oleifera* seed extract and drugs like desmopressin and aspirin may be necessary in order to consider the development of this seed as drug or nutritional supplement, to have a better and more physiologically tolerable drug for the treatment and management of thrombosis.

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