

# Antithrombotic activities of *Newbouldia laevis* (P. Beauv) seem. ex Bureau leaves

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## ABSTRACT

The study evaluated anticoagulant properties of the methanol extract of *Newbouldia laevis* leaves using blood clotting time, bleeding time and thrombin-induced clotting assay as standard procedures. Oral acute toxicity studies showed that the extract had a high safety margin, up to 2000 mg/kg in Wistar rats. The methanol leaf extract of *N. laevis* significantly ( $p < 0.05$ ) prolonged blood clotting times from the baseline value of  $11.0 \pm 0.6$  s for the blood sample to  $18.0 \pm 0.7$  s and  $32.0 \pm 1.0$  s at 5 % and 10 % concentrations respectively. The crude extract also exhibited appreciable *in vivo* and *in vitro* anticoagulant potency. High doses of the extract were most significant ( $p < 0.01$ ) in inducing rabbit bleeding which became prolonged to  $55.8 \pm 1.4$  s and  $73.1 \pm 0.8$  s at 100 and 200 mg/kg respectively compared to the baseline ( $18.0 \pm 0.2$  s) and effects of the reference anticoagulants. Aspirin was found to have produced bleeding intervals of  $47.0 \pm 0.3$  s and  $70.1 \pm 0.2$  s at 1.0 and 2.0 mg/kg while heparin (0.75 and 1.5 mg/kg) induced bleeding times of  $41.6 \pm 0.8$  s and  $61.0 \pm 1.7$  s respectively. The vehicle (distilled water) induced a transient baseline bleeding time of  $18.0 \pm 0.2$  s. However, the leaf extract of *N. laevis* also potentiated elevation in thrombin-induced clotting time in a dose dependent manner but at a reduced potency compared to heparin. Phytochemical analysis revealed the presence of reducing sugars, alkaloids, tannins, flavonoids, resin, phenols, proteins and acid compounds in the crude extract. The results demonstrated that the methanol leaf extract of *N. laevis* possesses pharmacologically active anticoagulant principles that could be isolated and evaluated for clinical or physiological purposes.

## INTRODUCTION

Anti-thrombotics include anticoagulants, anti-platelets and thrombolytics that decrease the rate of blood clotting in the body by dissolving already formed ones or prevent clot formation (Webster, 2001). Most of the synthetic agents including heparin, non-steroidal anti-inflammatory drugs (e.g. aspirin), warfarin, ethylenediaminetetraacetic acid (EDTA), and citrate have been found valuable in preservation of blood samples, management of heart attacks and other complications of cardiovascular disorders (Handoll *et al.*, 2002; Tohgi *et al.*, 1992., McCardel *et al.*, 1990). Anticoagulants are particularly indicated for strokes, transient ischaemic attacks, deep vein thrombosis and pulmonary embolism (Holbrook *et al.*, 2005).

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Oral anticoagulants have been used in the management of atherothrombotic stroke treatment (Donnan *et al.*, 2008) which accounts for 61% of all strokes and have been relied upon for prevention and treatment for several decades. Physiological anticoagulants like heparin are not known to exert maximal preservative property on whole blood because its anticoagulant potentials are readily neutralized in plasma (Cheesbrough, 2000). EDTA is adjudged to be toxic to platelets and generally considered unsafe to be used in preserving blood for transfusion (Forscher *et al.*, 1985).

Progressive cellular degeneration with compromised haematological profiles have been acknowledged over time in stored whole blood with various synthetic anticoagulants (Ahmed and Orakah, 2009; Lipp *et al.*, 2005). This development underscores the rationale to exploit for a novel anticoagulant with physiological and therapeutic activities. Over the past decade, natural products derived from plant sources have proved to be useful sources of 'lead compounds' and new therapeutic agents.

Some herbal plants including *Zingiber officiale*, *Ginkgo biloba*, *Allium sativum*, *Panax ginseng* and *Synclisia scabrida* have been studied and found to have marked anticoagulant properties (Afonne *et al.*, 2000; Tattelman, 2005; Mousa, 2010). *Newbouldia laevis* commonly called African border tree or boundary tree is known locally as *Aduruku* in Hausa, *Ogirisi* in Igbo and *Akoko* in Yoruba languages of Northern, Eastern and Western Nigeria, respectively.

The plant is valued for many medicinal properties in various African tropical catalogues. Extracts from different parts of the plant (leaves, stem bark and roots) have been shown to possess antimicrobial, anti-malarial, antioxidant, nociceptive and anti-inflammatory properties (Gofner *et al.*, 1997; Akunyili, 2000). The aqueous and ethanol leaf extracts displayed uterine contractile effects (Bafor and Sanni, 2009).

Preparation from the plant leaves is popular and highly acclaimed locally for anticoagulant effects among other healing activities by traditionalists in Ovoko community, Nsukka Local Government Area (L.G.A), Enugu State, Nigeria. The plant is considered sacred and used as a symbolic marker for sacred spots by the Yoruba tribe of Western, Nigeria. The present study sought to investigate the anticoagulant activities of *N. laevis* leaves using standard experimental models.

## MATERIALS AND METHODS

### Experimental animals

Matured inbred albino rats of both sexes weighing between 80-190 g and locally bred 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, 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 use of the animals for experimentation complied with the 'Principles of Laboratory Animal Care' (NIH publication No. 85-23, revised 1985); all experiments were subjected to assessment and approval by the Laboratory Animal Ethics Committee of the University of Calabar, Nigeria and also in conformity with the standard guidelines of Helsinki Declaration, 1964.

### Plant Materials and Extraction

Fresh leaves of *Newbouldia laevis* were collected from Ovoko community, Nsukka L.G.A. of Enugu State, Eastern Nigeria in April, 2011. The plant material was identified by Mr. A. O. Ozioko, a taxonomist with International Centre for

Ethnomedicine and Drug Development (InterCEDD) Nsukka, Enugu State, Nigeria. The leaves were dried at room temperature and pulverized into coarse powder using hammer mill. A 500 g of the powdered leaves was extracted by cold maceration in 80 % methanol with intermittent and vigorous shaking every 2 h for 48 h. The extract was filtered with Whatman No. 1 filter paper; the filtrate concentrated *in vacuo* at 40 °C to dryness with a rotary evaporator, was denoted as *Newbouldia laevis* methanol leaf extract (NLMLE). The concentration and percentage yield of the extract were determined.

### Phytochemical analysis

Tests for the presence of alkaloids, flavonoids, tannins, phenols, carbohydrates (Roopashree *et al.*, 2008), reducing sugars, resin (Mushore and Matuvhumye, 2013), proteins (Fenk *et al.*, 2007) and acidic compounds were conducted based on standard procedures (Trease and Evans, 1989).

### Acute toxicity test

Thirty (30) matured albino rats of both sexes were weighed and randomly allocated to 5 groups (A–E) of 6 rats per group. Groups A–D were administered varying oral doses (300, 600, 1000, and 2000 mg/kg) of the leaf extract (NLMLE), respectively but group E (5<sup>th</sup> group) was given an equivalent volume of distilled water (10 ml/kg). The rats, allowed access to feed and water *ad libitum* were observed for signs of toxicity and death for 72 h.

### Blood clotting time

The ability of *N. laevis* extract to inhibit *in vitro* coagulation of blood was quantitatively assayed following a standard procedure adopted by Koffuor and Amoateng (2011). Briefly, 1.0 ml of whole blood drawn from the marginal ear vein of rabbits was added separately to 0.2 ml of 5 % and 10 % w/v of NLMLE in test tubes placed in a water bath at 37 °C. The time taken for the blood to clot in the separate concentration of the extract was recorded.

A repeat of each test was performed to obtain five determinants in coagulation times exerted with differences in concentration of the extract. Controls were coagulation time for blood in 0.2 ml distilled water or blood alone as baseline clotting times.

### Rabbit bleeding time

*In vivo* anticoagulant activity of *N. laevis* leaf extract was investigated as described by Elg *et al.* (2001). Rabbits were pre-treated orally with 100 and 200 mg/kg of the extract and then allowed 30 min. before pricking a small vein in the margin of the ear to induce bleeding.

The site of bleeding was gently blotted with filter paper every 5 s. till cessation of bleeding (no bleeding 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 heparin (0.75 and 1.5

mg/kg), aspirin (1 and 2 mg/kg) and distilled water, respectively. A baseline bleeding time was already determined before any drug administration. Five determinations were taken after which a mean bleeding time for each treatment was obtained and compared.

### Thrombin-induced clotting time assay (TT)

This assay measures the prolongation of thrombin generation. When human plasma is incubated with a compound which inhibits blood coagulation, the time taken for clot formation will be prolonged compared to the control (test devoid of inhibitor). In this assay, 200  $\mu$ l of human plasma (pre-incubated at 37 °C for 5 min before use) was incubated with different concentrations (10, 100 and 1000  $\mu$ g/ml) of the extract for 5 min at 37 °C; buffer and normal plasma served as the controls. Replicated concentrations (10, 100 and 1000  $\mu$ g/ml) of heparin were used as the reference anticoagulant. A fixed concentration (100  $\mu$ l) of bovine thrombin (2.5 U/ml, Sigma) was added to each sample to initiate reaction.

The time for clot formation was recorded accordingly (Dong *et al.*, 1998). Results were expressed as a prolongation time relative to controls.

### Data analysis

All data were expressed as Mean  $\pm$  SEM. Data were analyzed using one way analysis of variance (ANOVA) with Dunnett's test as the post hoc. Mean differences among treatment groups at  $p < 0.5$  was considered significant.

## RESULTS

### Description of the extract

The methanol extract of *Newbouldia laevis* leaves was light greenish brown in colour with no peculiar odour. The extraction process gave a yield of 13.47 % w/w.

### Phytochemical analysis

The results revealed the presence of alkaloids, reducing sugars, tannins, flavonoids, resins, phenols, proteins and acidic compounds in the crude methanol extract of *N. laevis* leaves.

### Acute toxicity studies

Oral acute toxicity studies showed that the methanol leaf extract of *N. laevis* did not cause mortality or overt pathological lesions in the experimental rats even at the highest test dose (2000 mg/kg) within 72 h of the investigation. The rats moved freely, fed normally with no deviation in the consistency of the droppings relative to the control.

### Blood clotting time

Blood clotting times with 5% and 10% w/v of extract (NLMLE) were significantly ( $p < 0.05$ ) prolonged compared to control values with distilled water or blood alone. The higher concentration (10%) of extract induced the sampled blood to clot

at  $32.00 \pm 1.0$  s; 5% extract produced a mean blood clotting time of  $18.00 \pm 0.7$  s while distilled water or blood alone produced clotting within a limited of  $11.00 \pm 0.4$  s and  $7.00 \pm 0.6$  s respectively (Table 1).

**Table 1:** *In vitro* effects of 5% and 10% *Newbouldia laevis* methanol leaf extract on rabbit blood clotting time.

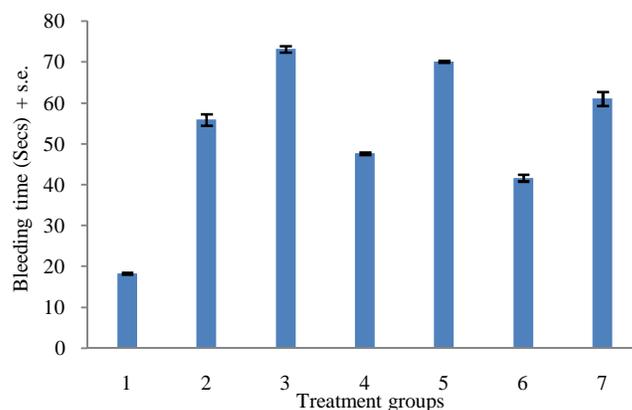
Concentration	Mean Clotting time (s)
Blood alone	$7.00 \pm 0.6$
Blood + distilled water	$11.00 \pm 0.4$
Blood + 5% extract (NLMLE)	$18.00 \pm 0.7^*$
Blood + 10% extract (NLMLE)	$32.00 \pm 1.0^*$

Values are mean  $\pm$  SEM (n=5); \* $p < 0.05$  compared to control groups; NLMLE= *Newbouldia laevis* methanol leaf extract

### Rabbit bleeding time

The duration of bleeding in rabbits treated with 100 and 200 mg/kg of *N. laevis* extract was found to have significantly ( $p < 0.01$ ) increased to  $55.8 \pm 1.4$  s and  $73.1 \pm 0.8$  s respectively from the baseline bleeding time of  $18.0 \pm 0.2$  s produced by the vehicle (distilled water).

Aspirin also induced increased bleeding time values of  $47.0 \pm 0.3$  s and  $70.1 \pm 0.2$  s at 1.0 and 2.0 mg/kg while heparin caused the same effect (bleeding) for  $41.6 \pm 0.8$  s and  $61.0 \pm 1.7$  s at 0.75 and 1.5 mg/kg respectively (Fig. 1). Even though, the bleeding times produced with the doses of extract became prolonged, there was no significant ( $p > 0.05$ ) difference in the effects with those of aspirin or heparin.



**Fig. 1:** *In vivo* effects of *Newbouldia laevis* leaf extract (100-200 mg/kg), aspirin (1-2 mg/kg) and heparin (0.75-1.5 mg/kg) on bleeding time in rabbits. Values are means  $\pm$  S.E. (n=5). \*\* $p < 0.01$  compared to vehicle-treated group. † $p < 0.05$  significant differences between dose levels

### Thrombin-induced clotting time assay (TT)

A comparison of the *in vitro* anticoagulant activity of NLMLE with heparin in thrombin-induced clotting time is presented in Table 2. The leaf extract of *N. laevis* significantly ( $p < 0.01$ ) potentiated elevation in thrombin-induced clotting time in a dose dependent manner but at a reduced potency compared to heparin. At 10, 100 and 1000  $\mu$ g/ml, the extract prolonged thrombin-induced clotting time of 40.2 s produced with buffer to 97.3, 172.3 and 251.4 s respectively but relative to 280.2, 300.8 and 330.1 s with heparin at the same concentration.

**Table 2:** Comparison of the *in vitro* activity of *Newbouldia laevis* extract with heparin in Thrombin-induced clotting time.

Compound/extract	Concentration (µg/ml)	Prolongation time (s)
<i>N. laevis</i> extract	10	97.3 ± 0.5**
<i>N. laevis</i> extract	100	172.3 ± 0.7**
<i>N. laevis</i> extract	1000	251.4 ± 1.2**
Heparin	10	280.2 ± 0.4**
Heparin	100	330.8 ± 0.6**
Heparin	1000	330.1 ± 0.2**
Negative control (buffer)		40.2
Normal plasma	-	15.7

Values are mean ± SEM (n=5); \*\* p < 0.01 compared to negative control (buffer)

## DISCUSSION

The crude leaf extract of *N. laevis* was tolerated by the experimental rats hence; the highest oral test dose (2000 mg/kg) produced no observable mortality within 72 h of the investigation. The methanol extract exhibited marked anticoagulant activity and this could be the reason for the prolonged *in vitro* time of blood clotting observed with the test samples (Table 1). The *in vivo* effects of the extract in relation to that of aspirin and heparin resulted in increased bleeding times (Figure 1). The high doses (100-200 mg/kg) of the extract however, exhibited the most profound bleeding effect compared to either aspirin or heparin. The extract displayed a comparatively lower potency compared to heparin in thrombin-induced clotting time. This was demonstrated when 1000 µg/ml of the extract produced clotting at 251.4 ± 1.2 s but heparin at the same concentration prolonged the clotting time to 330.1 ± 0.2 s. This is quite logical since heparin is a pure compound while the extract is crude with many contaminants. The mechanism by which the extract mediated its anticoagulant activity is not fully understood but chelating agents, heparin, aspirin and vitamin K antagonists are known to interfere with blood clotting processes using diverse mechanisms.

Chelating agents including trisodium citrate, sodium oxalate and ethylene diaminetetraacetic acid (EDTA) bind with calcium ions and render them unavailable to facilitate coagulation reactions (Rowell, 1969). Heparin, contained in mast cells is released into circulation when mast cells degranulate during inflammation. Heparin can inhibit clotting factor IXa, XIa and thrombin but its action on factor Xa accounts for its potency as an anticoagulant (Melvin, 1977).

Heparin can inhibit both the generation of thrombin and also the formed thrombin. Non-steroidal anti-inflammatory drugs including aspirin inhibit cyclooxygenase enzymes (COX) of prostaglandin and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) biosynthesis from arachidonic acid. Repeated doses of aspirin can irreversibly inhibit COX-1-dependent TXA<sub>2</sub> formation. Thromboxane induce platelet aggregation and also acts as amplification signal for other, more potent platelet agonists such as thrombin and adenosine diphosphate (Brunton *et al.*, 2008). The synthesis of clotting factors II, VII, IX and X in the liver depends on adequate amounts of vitamin K. Coumarin and inadenedione group of oral anticoagulant drugs antagonizes the synthesis of non functional forms of coagulant proteins and thereby prevents blood clot

formation (Schneeweiss *et al.*, 2012). The *N. laevis* leaf extract prolonged thrombin-induced clotting time suggesting that its anticoagulant effect might be linked to its ability to inhibit thrombin generation and fibrin formation. The phytochemical screening revealed the presence of reducing sugars, alkaloids, tannins, flavonoids, resin, phenols, proteins, and acid compounds in the crude extract. A bioactive phyto-constituent may be responsible for the observed anticoagulant activities of the leaf extract.

## CONCLUSION

The prolonged *in vitro* time of blood clotting coupled with *in vivo* effects that culminated in increased bleeding times and the potentiated thrombin-induced clotting time with the extract demonstrated that the methanol leaf extract of *N. laevis* possesses pharmacologically active anticoagulant principles that could be isolated and evaluated for clinical and physiological applications.

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