European Journal of Medicinal Plants
7(1): 16-25, 2015, Article no.EJMP.2015.063
ISSN: 2231-0894

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An Evaluation of the Sub Acute Toxicity and Haemostatic Effects of Leaves Extract of *Achyranthes aspera* in Mice and Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BEO and EEE conceived the work. Authors BEO, EEE and CFP designed the work and decided the experimental protocols. Author BEO collected the plant and prepared the extract. All the authors participated in the various experiments. Authors BEO and CFP drafted the manuscript while Author EEE proof read the manuscript and made technical and grammatical inputs. The statistical analysis was done by author CFP while author MHO did the literature search. Author CFP was responsible for correspondence. All the authors read and approved the final manuscript for submission.

Article Information

DOI: 10.9734/EJMP/2015/7043
Editor(s):
(1) Shanfa Lu, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, China.
(2) Marcello Iriti, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Anonymous, Brazil.
(2) Anonymous, Brazil.
(3) Anonymous, India.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1017&id=13&aid=8066

Original Research Article

Received 23rd September 2013
Accepted 6th December 2014
Published 6th February 2015

ABSTRACT

Background: *Achyranthes aspera* is used mostly in Nigerian and other folklore medicines to stop bleeding from wounds and in the treatment of other conditions such as dog bite, scorpion bite, gonorrhoea, obstetric disorders and diabetes mellitus.
Objectives: The present study evaluated the toxicological and haemostatic effects of the leaves extract of *Achyranthes aspera* in adult Wistar albino rats and Swiss mice using experimental

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models.
Methods: The acute toxicity profile of the methanol leaves extract, skin irritation test as well as the bleeding and clotting times in experimentally induced wounds were studied in Swiss mice and Wistar albino rats using standard methods. The phytochemical screening of the extract was undertaken using standard methods.
Results: The intra-peritoneal LD_{50} of the extract was found to be 1224.7mg/kg and the topical skin irritation was negative. Phytochemical screening revealed the presence of alkaloids, saponins, tannins, flavonoids, anthraquinones, cardiac glycosides and terpenes. Administration of the extract for 21 days orally to the animals caused a significant (*P=.05*) decrease in clotting and bleeding times, with bleeding time in a dose-dependent manner compared to the control animals. Topical administration of the extract also caused a significant (*P=.05*) decrease in bleeding time compared to control animals with a less significant decrease when compared to orally administered extract.
Conclusion: The results have shown that *Achyranthes aspera,* a traditional folklore medicinal plant has haemostatic effect which may provide therapeutic potential capable of arresting bleeding and contain some biologically active principle(s) which are responsible for the haemostasis.

Keywords: Achyranthes aspera; haemostasis; bleeding; clotting; rats.

1. INTRODUCTION

Trauma is a common cause of death in most people between the ages of 5 years to 45 years [1]. Each year world-wide, about three million people die as a result of trauma [1], many after reaching hospital. Among trauma patients who survive to reach hospital, ex-sanguination is a common cause of death, accounting for nearly half of in-hospital trauma deaths in some settings [2]. Bleeding may occur following trauma, surgery, gynaecological disorders, treatment with antiplatelet, anticoagulant or thrombolytic therapy [3]. The common cause of blood loss in our environment is trauma from road traffic accident [4]. This may be disastrous even with minor blood loss, due to pre-existing anaemia, which is very common in the tropical countries [4]. Uncontrolled haemorrhage is the commonest cause of death from trauma of combat [5]. An estimated 1000 - 2000 bleeding cases are admitted to the University of Uyo Teaching Hospital (UUTH) in Akwa Ibom State, Nigeria annually for treatment [6]. The common causes of blood loss are injuries from road traffic accidents (RTA) with 32% of the total cases, other causes are surgery (22%), obstetric (21%), gynaecology (20%) and penetrating wounds (gun-shots) (5%) (University of Uyo Teaching Hospital record, 2010) [6].

Therefore, controlling haemorrhage will always remain a top priority in trauma care. The search and development of haemostatic agents for better bleeding control will be of immense benefit. An ideal agent should be universally effective, has consistent therapeutic outcome, low toxicity profile, easy to use, readily available, cheap, easy to administer, has a long half-life, logistically superior and suitable for prophylaxis [3].

Approximately 30,000 - 50,000 people are involved in road traffic accident, violent and other form of injuries resulting in between 15,000 to 20,000 deaths as a result of bleeding in Nigeria every year [4]. Each year, 600,000 injured patients bleed to death world-wide. World Health Organization (WHO) figures show that nearly six million (6,000,000) people die each year from injuries, which account for 10% of the world's deaths. Most of those injury deaths occur in developing countries, where deaths from road traffic crashes and homicides have been steadily increasing. Almost half of those deaths are caused by bleeding [7].

Blood transfusion, despite its restraints in recent times because of the dreaded complication of HIV/AIDS, hepatitis B - virus transmission and religious beliefs, particularly among the Jehovahs witnesses [8,9] is a major requirement for treating life threatening loss of blood , because of the dearth of synthetic haemostatic medications in our hospitals [10].

Hence, novel haemostatic medications are urgently needed to overcome these hindrances and shortcomings by continued search for an ideal agent that is easy to use, inexpensive, and readily available with no storage requirements, non-immunogenic, versatile and biologically inert with minimal side effects [11]. A large number of medicinal plants have been reputed for treating bleeding episodes in folk medicine, for example, the leaves and roots of *Achyranthes aspera* – a

magic herb in folk medicine [12], which hold a reputed position as medicinal herb in different systems of medicine in India and Akwa Ibom State (South Eastern Nigeria). Different parts of the plant form ingredients in many native prescriptions and in combination with more active remedies [13].

Achyranthes aspera Linn (*Amaranthaceae*) is an annual stiff erect herb found commonly as a weed in tropical and subtropical regions of India, Baluchistan, Srilanka, Tropical Asia, Africa, Australia, Ceylon and America [14]. It is known as prickly chaff flower in English. In Nigeria, it is known variously as “udok mbiot” in Ibibio, “Agukwu” in Igbo, “Aboro” in Yoruba and “Kaimin kadangaru” in Hausa [15].

The morphological features of *Achyranthes aspera* have been fully described. In folk medicine, different parts of the plant are used as expectorant, stomach tonic, laxative, diuretic, linthontriptic, sudorific, demulcent, anti-inflammatory and haematinic [16], dog bite [17]' haemorrhage [18], diuretic, diaphoretic and antisyphilitic [19], urinary calculi, rashes, influenza, otorrhoea, renal dropsy, gonorrhoea and abdominal pain [20,21,22,23,24], scorpion bite [25], gynaecological and obstetric disorders [26], typhoid [27]. The hypoglycaemic [28], cancer chemoprevention [29], hepatoprotective [30], analgesic and antipyretic [31,32,33,34,35], anti-inflammatory and anti-arthritic [36], antimicrobial [37,38,39,40,41], antioxidant [42,43], antidepressant [44,45], bronchoprotective [46], anti-allergic [47], wound healing [43], immunomodulatory [48], hypolipidemic [49], spermicidal activity [50,51,52,53,54], larvacidal effects on mosquitoes [55], antifertility [56]. Earlier reports showed that it contains flavonoids, alkaloids, glycosides, saponins, phytosteroids, triterpenoids, tannins, carbohydrates and anthraquinones, betaine, achyranthine, amino acids, β-ecdysone, hentriacontane, lauric acid, myristic acid, β-sistosterol, stigmasterol and oleonolic acid and fatty oil [40,57,58,59,60]. An elemental analysis of the stem material revealed a total of fifty elements that composed of five macro elements, twenty-seven micro-elements and eighteen heavy metals with high concentration of potassium, calcium, chloride and phosphorus elements [61].

There is no pharmacological report on the ethnomedicinal uses of this plant in the management of bleeding disorders to the best of our knowledge. This stimulated our interest to evaluate the haemostatic effects of the leaf extract on bleeding disorders.

2. MATERIALS AND METHODS

2.1 Plant Materials

Fresh *Achyranthes aspera* leaves were collected in June, 2012 from Etinan and Ikono Local Government Areas of Akwa Ibom State, Nigeria and authenticated by Dr. (Mrs) Uduak Essiett of the Department of Botany, University of Uyo, Akwa Ibom State, Nigeria. A voucher specimen (Herbarium No.:218 was deposited in the Herbarium of the Department of Pharmacognosy and Natural Medicine, Faculty of Pharmacy, University of Uyo. The leaves were shed dried and pulverised to powder using pestle and mortar. The leave powder (900g) was extracted with methanol by cold maceration for 72 hours to obtain the methanol extract (ME). The extract was filtered, decanted and evaporated to dryness using a water bath at 40°C to obtain methanol extract (ME). The extract was weighed and percentage yield calculated.

2.2 Phytochemical Screening

The methanol extract was screened for the presence of alkaloids, tannins, phlobatannins, cardiac glycosides, cyanogenic glycosides, saponins, anthraquinones, flavonoids and terpenes using standard methods [62].

2.3 Experimental Animals

Wistar albino rats and Swiss mice of both sexes were obtained from the laboratory animal facility of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, Nigeria. The animals were maintained at standard laboratory conditions with natural lighting cycles (12 hours light and 12 hours darkness). They were fed with standard pellet feed (Guinea feed, Nigeria) and water ad libitum during an acclimatization period of at least 7 days before experimentation. All animal experiments were in compliance with the National Institute of Health Guide lines for care and use of laboratory animal (Pub. No.: 85-23, revised 1985).

2.4 Acute Toxicity and Lethality (LD_{50}) Test

The acute toxicity and lethality (LD_{50}) of methanol extract (ME) was determined in mice using Lorke's method with slight modification [63]. Animals in group of three were administered by the intraperitoneal route 500, 750, 1000, 1500 or 2000 mg/kg of the methanol extract suspended in Tween 80 (30%). They were observed for 24 h for any overt signs of toxicity or death and further 14 days for any signs of delayed toxicity. The LD_{50} was calculated as the geometric mean of the highest non-lethal dose (1000 mg/kg) and the lowest lethal dose (1500 mg/kg).

2.5 Acute Skin Irritation Study

The primary irritation test was performed on Wistar rats using standard method [64]. Twenty animals (150-180 g) were divided into two groups of ten animals each, One group served as the control and the other as test group respectively. Dorsal hairs of the rats were shaved a day to the commencement of the study. 100 µl of the extract (50 mg/ml) was applied over one square centimetre area of the shaven dorsal skin of the test animals. 50 µl of Formalin (0.8 %) was applied as standard irritant. The animals were observed for 7 days for any sign of oedema and erythema.

2.6 Bleeding Time

The effects of the extract on bleeding from experimental induced fresh wounds were evaluated using rat model of the tail-tip transection method [65]. Thirty six Wistar rats were randomly allocated into 6 groups of 6 animals each. The extract was suspended in 30 % tween 80 and administered either orally or topically. Control animals received Tween 80 (30%) either orally or topically. Groups 1 to 3 were pre-treated with extract orally at the doses of 122.5 mg/kg, 244.9 mg/kg and 367.4 mg/kg while group 4 ($control_1$) received 10 ml/kg of 30 % Tween 80 for 21 days. On day 22, the tail of each rat was restrained by an assistant and 5mm length from the tip of the tail was measured with a plastic ruler and transected with a surgical blade. At the same time a stopwatch was started and the cut dabbed with a small piece of filter paper at 15 seconds interval until the paper no longer stained red with blood oozing from the cut. Bleeding time was taken as the time at which the first drop of blood showed, to the time when the filter paper stopped showing blood stain[72]. The animals in Group 5 were applied the extract topically, while the animals in group 6 ($control_2$) were applied Tween 80 (30% v/v) topically. The tail of each rat in group 5 and group 6 was transected with a surgical blade. Immediately, 100 µl of the extract (50 mg/ml) dropped on the cut area of the animals in group 5 and 50 µl of 30 % Tween 80 was dropped on the cut area of animals in group 6. At the same time a stopwatch was started. The cut was dabbed with a small filter paper at 15 second intervals until the paper no longer stained red with blood oozing from the cut. Bleeding time was taken as the time at which the first drop of blood showed to the time when the filter paper stopped showing blood-stain [66].

2.7 Coagulation or Clotting Time

The effect of the extract on coagulation of fresh blood was evaluated using the capillary tube method [67]. Twenty four Wistar rats were randomly allocated into 4 groups of 6 animals each. Each group received the extract suspended in Tween 80 (30% v/v), while the control group received Tween 80 (30% v/v). Groups 1 to 3 were pre-treated with the extract orally at doses of 122.5 mg/kg, 244.9 mg/kg and 367.4 mg/kg while group 4 (control) received 10 ml/kg of Tween 80 (30% v/v) for 21 days. On day 22, each rat was restrained by an assistant, the tail was briefly warmed for 1 minute in a water bath at 40°C, then dried and 5mm length from the tip was measured with a plastic ruler and transected with a surgical blade. About 25µl sample of blood was collected into a micro-haematocrit glass capillary tube. The stopwatch was started when the blood first made contact with the tube. The blood was left to flow by gravity between the two marks of the tube, 45mm apart by tilting the tube alternately to $+60^o$ and -60° angles with respect to the horizontal plain until blood ceased to flow (reaction point). The interval between the introduction of the blood in the tube and the time of clot formation was taken as the coagulation time [66].

2.8 Statistical Analysis

Data are presented as mean±SEM or simple percentage. The INSTAT statistical package was employed. Data were analysed using unpaired Students'-test, paired Students' t-test and one way analysis of variance (ANOVA). The results of the treated groups were compared with the negative control and were considered significant at $P=.05$.

3. RESULTS

3.1 Phytochemical Analysis

The maceration of the powdered plant material in methanol gave a total extract yield of 1.42%. The methanol extract (ME) gave positive reactions for saponins, alkaloids, tannins, free and combine anthraquinones, flavonoids, cardiac glycosides and terpenes, with abundance saponins. This extract gave a negative reaction for phlobatannins and cyanogenic glycoside (Table 1).

3.2 Acute Toxicity and Lethality Test

The acute toxicity testing of the methanol extract (ME) in mice gave an intraperitoneal LD_{50} value of 1224.7mg/kg (Table 2).

The effective doses of the extract and the therapeutic index are presented in Table 3.

3.3 Effect of Extract on Acute Skin Irritation of Wistar Rats

The acute irritation study of the extract showed negative for the extract on the intact skin of the rats (Table 4)

3.4 Effect of Extract on the Bleeding Time in Wistar Rats

Oral administration of the extract to the rats at the doses of 122.5 mg/kg, 244.9 mg/kg and 367.4 mg/kg for 21 days, dose dependently reduced the bleeding time with percentage inhibition of bleeding of 65.8 %, 50.8 % and 33.0 % at the doses of 367.4, 244.9 and 122.5 mg/kg respectively (Table 5).

Topical treatment of the animals with the extract at100 µg/ml reduced the bleeding, with a percentage inhibition of 24.0 % (Table 6).

3.5 Effect of Oral Administration of Extract on the Coagulation Time in Wistar Rats

Oral administration of the extract at the doses of 122.5, 244.9 and 367.4 mg/kg for 21 days reduced the coagulation time with percentages of 24 %, 23 % and 21% at doses of 367.4, 244.9 and 122.5 mg/kg respectively (Table 7).

Table 1. Phytochemical Analysis

S/N	Chemical constituents	Test	Inference
1	Saponins	Foam test	+++
		Fehling's test	+++
2	Alkaloids	Mayer test	++
		Dragendorffs test	++
3	Tannins	Ferric chloride test	++
		Bromine water test	++
4	Phlobatannins	Dilute HCl test	--
5	Anthraquinones	Borntrager's test	
	Free		++
	Combine		++
6	Flavonoids	Magnesium metal test	++
7	Glycosides		
	Cardiac glycosides	Keller-Killiani test	++
		Salkowski test	++
		Lieberman's test	++
	Cyanogenic glycoside		--

++Positive (Moderate amount), ++ + Positive (copious amount), -- Absent

Table 2. Acute Toxicity and Lethality Test

Dose (mg/kg)	Number of deaths Per group within 24 hours
2500	3/3
2000	3/3
1500	3/3
1000	0/3
750	0/3
500	0/3

Number of animals per group = 3. The numerator indicates the number of death animal (s) per group

Table 3. Effective doses (ED_{50}) and therapeutic dose

LD_{50}	1224.7mg/kg
1/10 LD_{50}	122.5mg/kg
2/10 LD_{50}	244.9mg/kg
3/10 LD_{50}	367.4mg/kg
Therapeutic index	3-10

Table 4. Effect of extract on acute skin irritation in rats

Substance	Result
Formalin (0.8%)- 50 µl	+
Extract (100 µl of 50 mg/ml)	-

+ = Positive indicating presence of skin irritation and - = Negative indicating absence of skin irritation

Table 5. Effect of Orally Administered extract or tween 80 (30% v/v) for 21 Days on Bleeding Times

Treatment	Bleeding Time (Mean±SEM) in seconds	Percentage inhibition of bleeding
Control:Tween 80 (10 ml/kg)	157.2 ± 3.47	-
ME(367.4 mg/kg)	53.8 ± 2.6*	65.8
ME(244.9mg/kg)	77.3 ± 1.8*	50.8
ME (122.5mg/kg)	105.3 ± 19*	33.0

*ME = Methanolic extract, Values are Mean ± SEM (n = 6 animals per group). *P=.05, compared to control group (student t-test)*

Table 6. Effect of Topically Administered extract or tween 80 (30%v/v) on Bleeding Times

Treatment	Bleeding Time (Mean±SEM) in seconds	Percentage inhibition of bleeding
Tween 80 (30%v/v) (control =50 µl)	160 ± 24.99	-
ME (100 µg/ml)	115 ± 8.64	28.0

ME= Methanolic extract, Values are Mean ± SEM (n = 6 animals per group). P=.05 compared to control group (student t-test)

Table 7. Effect of Orally Administered ME or Tween 80 (30%v/v) for 21 Days on Coagulation (Clotting) Time (second)

Treatment	Bleeding time (Mean±SEM) in seconds	Percentage inhibition
Tween 80 (10 ml/kg)	110.8±2.06	-
ME(367.4 mg/kg)	84.3±2.64*	24
ME(244.9 mg/kg)	85.7±4.27*	23
ME (122.5 mg/kg)	87.3±3.86*	21

*Values are Mean ± SEM (n=6 animals in each group). *P=.05 compared to control group (student t-test). M E (Methanolic extract)*

4. DISCUSSION

A scale proposed by Lorke in1983, roughly classifies substances according to their LD_{50} as follows [69]: very toxic - (LD_{50}<1.0mg/kg), toxic - (LD_{50} up to 10.0mg/kg), less toxic-(LD_{50} up to 100.0mg/kg) and only slightly toxic (LD_{50} up to 1000.0mg/kg). Substances with LD_{50} values greater than 5000 mg/kg are practically non toxic. Based on this proposal, with an intraperitioneal LD_{50} of 1224.7mg/kg, the extract used in this study is relatively safe. The absence of skin irritation may be due to the protective effect of the extract in preventing the release of preformed mediators from the granules of eosinophills, thereby preventing hypersensitivity reactions of the skin [46]. The study was carried out to evaluate the potential of *Achyranthes aspera* on the haemostatic mechanism with primary interest on how it affects bleeding time

and clotting times. The methanol leaf extract of *Achyranthes aspera* exhibits haemostatic activities by decreasing bleeding and clotting times. Our studies on rats demonstrated that topically and orally administered *Achyranthes aspera* extract have haemostatic effects. The bleeding time evaluates the primary haemostatic system, which involves the platelet and blood vessel wall, while the clotting time evaluates their effect on the secondary haemostatic system that involves the coagulation system. These indices are measures of blood coagulation, while clotting time measure the intrinsic pathway, the bleeding time measure the extrinsic pathway. Clotting time test is a qualitative measurement of factors involved in the intrinsic pathway [68]. Coagulation studies are of great importance considering the role of blood in life. Arrest of bleeding (haemostasis) from small blood vessels in the skin and elsewhere usually involves vasoconstriction and the formation of haemostatic plugs through platelet aggregation. The significant reduction in bleeding time obtained, however suggest that the extract may possibly be acting in part on the integrity of the blood vessels to produce this effect. Another suggestive mechanism for its reduction of bleeding time may possibly be via modulation of platelet functions by increasing the intensity of platelet plug formation through platelet aggregation. The ability of the extract to have effect on the clothing time implies the possible involvement of blood cells in the haemostatic effect of the extract. This involves other mechanisms, such as prothrombin activation with subsequent convertion to thrombin and which in turn converts fibrinogen to insoluble fabrins. The reduction of coagulation time by the extract is an indication that the extract may also interfere with the blood coagulation pathways. Thus haemostatic effect of the extract may derive from acceleration of the coagulation process with consequent reduction in clothing time, as well as vasoconstnction and platelet plug formulation with a resultant decreased in bleeding time, which are necessary in limiting blood loss from damaged vessels. Phytochemical analysis of the extract revealed the presence of flavonoids, alkaloids, glycosides, saponins, anthraquinones and terpenes. Some of these organic compounds have been identified as haemostatic principles from plants, terpenes [69], tannins and alkaloids [62], and saponins [70]. *Achyranthes aspera* exhibits significant nutritional promise to maintain health life [71] and has been reported possess potential to cure skin diseases [72].

5. CONCLUSION

The results of the present study supports the traditional use of *Achyranthes aspera* and suggest that the plant extract possesses compounds with haemostatic properties that can be used as haemostatic agents in new drugs for the therapy of bleeding disorders. Further studies are required to isolate the active principles and carry out pharmacological evaluation.

ACKNOWLEDGEMENT

The authors acknowledge the technical assistance of the laboratory staff of the Departments of Pharmacology and Pharmacognosy, Faculty of Pharmacy of University of Uyo, Nigeria.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All the animal experiments were in compliance with the National Institute of Health Guide lines for care and use of laboratory animal (Pub. No.: 85-23, revised 1985). An approval for the use of animals' experimental protocols was secured from the University of Uyo Committee of ethical and appropriate use of animals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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