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Research Article

Evaluation of Membrane Stabilization Activity of Leaf Extract OF Leptadenia Hastata (Pers.) Decne

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Abstract

Leptadenia hastata leaves extracts have demonstrated a positive effect on wound healing and has been used since antiquity amongst people of Eastern Nigerian for that purpose. However, the underlying mechanism for its actions has not been unexplored. The model system employed in many *in vitro* investigations of drug and membrane interactions is the Erythrocyte membrane. The study has the aim of investigating the membrane stabilization potential of *L. hastata* leaves extracts using an *in vitro* hemolytic assay. The extract of leaves of *Leptadenia hastata* was prepared by cold maceration using methanol at room temperature (27 ± 2ºC). The Phytochemical analysis of the extract was also carried out and revealed the presence of typical Phytochemical constituents with relatively high concentrations of Flavonoids and Terpenoids. The effect of the extract on heat-induced and hypotonic solution-induced haemolysis was evaluated. The result indicated that LHE (50,100,200 and 400 µg/ml) exhibited significant ($p < 0.05$) inhibition of hypotonic solution-induced haemolysis but not heat-induced haemolysis. Therefore, it can be inferred that the anti-inflammatory actions of *Leptadenia hastata* may partly be due to membrane stabilization.

Keywords: Membrane Stabilization; *Leptadenia Hastata*; Wound-healing; Heat-Induced Haemolysis; Hypotonicity-Induced Haemolysis

Introduction

Leptadenia hastata is a creeping plant which is known to be a famine food in many parts of Africa. The leaves, flowers and young shoots of *Leptadenia hastata* are edible and have been used as cooked vegetables in soups. Boiled leaves of *Leptadenia hastata* are chopped and mixed with cowpeas among Ugandans [1]. *Leptadenia hastata* ranked 3rd in an interview on the preference for 14 wild herbaceous vegetables held in Burkina Faso in 1999, its taste was considered good, and its tolerance of drought, insects and poor soil conditions as very good [1]. It has acuminate leaves which are up to 10 cm long, mostly ovate, light green. The flowers are cream or yellowish green. The fruits are two-valved, conical, dehiscing to release cottony winged seeds. The stems are light green and the shoots spread into the air with long internodes. When crushed, the plant exudes sticky sap.

Latex gotten from *Leptadenia hastata* is usually applied on wounds and also applied nasally against headaches. Constipation, urethral discharge, gonorrhoea, stomach ache and diarrhoea have been treated with decoctions and macerations of roots and leaves of *Leptadenia hastata* [2]. Traditional healers in Nigerias also use the plant for hypertension, catarrh and skin diseases [3].

L. hastata leaves have shown the presence of phenolic glycosides, tannins, flavonoids, proanthocyanins, alkaloids and saponins. The total phenolic, total flavonoid and proanthocyanidin contents were in the ranges of 17 - 38, 10 -16 and 4 -10 mg/g respectively and this quantity were dependent on the extraction solvent [4].

Findings from a study by Bello., *et al.* showed the potential of *L. hastata* extracts in diabetes mellitus management. In their study, the hypoglycaemic and hypolipidaemic of the fresh leaves *L.*

hastata in normal and alloxan-induced diabetic rat model were evaluated [4,5].

Interestingly, *L. hastata* leaves extracts have demonstrated a positive effect on wound healing and has been used since antiquity amongst people of Eastern Nigerian for that purpose. The results from the Nikiéma., *et al*. study verify the topical use of *L. hastata* latex in wound-healing. However, the mechanism by which it performs this function has not been explored. A popular model system for *in vitro* investigations is the erythrocyte membrane, and this model will be adopted in this study [6]. The aim of the study was to investigate the membrane stabilization potential of *L. hastata* leaves extracts using an *in vitro* hemolytic assay.

Materials and Methods

Fresh leaves of *Leptadenia hastata* were collected from Nnobi, Anambra state, Nigeria. The plant was identified and authenticated by International Centre for Ethnomedicines and Drug Development (INTERCEDD), Nsukka. The plant material was cut into smaller pieces and shade dried at room temperature for 11 days. After drying, exact weights were recorded. The plant material was reduced to coarse powder using an electric blender. The powdered material was maintained at room temperature and protected from light until required for extraction. The powdered plant material (2.4 kg) was extracted by maceration in methanol (7,500 ml) at room temperature (27 ± 2ºC) with intermittent shaking. The mixture was filtered with Muslin cloth followed by whatman filter paper (no.1). The marc was washed with fresh methanol (2,500 ml) and filtered. The filtrate was evaporated to dryness in open air to yield 56.5g of the methanol extract (LHE; 2.57% w/w).

Phytochemical Analysis of Leptadenia hastata leaf extracts

The tests were carried out based on procedures outlined by Trease and Evans, 1996 [7].

Test for Flavonoids

About 10 ml of ethyl acetate was added to 0.20g of the extract and heated on water bath for 3 mins, the mixture was cooled, filtered and the filtrate was used for the following tests:

- **Ammonium test:** About 4 ml of filtrate was shaken with dilute ammonia solution. The layers were allowed to separate and the yellow colour in the ammoniacal layer indicates the presence of flavonoids.
- **1% Aluminium chloride solution test:** Another 4 ml portion of the filtrate was shaken with 1 ml of 1% aluminium chloride solution. The layers were allowed to separate. A yellow colour in the aluminium chloride layer indicates the presence of flavonoids.

Test for Saponins

About 20 ml of distilled water was added to 0.25g of extract and boiled on a hot water bath for two mins. The mixture was filtered, while hot and allowed to cool and filtrate was used to do the following tests;

- **Frothing test:** About 5 ml of the filtrate was diluted with 15 ml of distilled water and shaken vigorously. A stable froth upon standing indicates the presence of saponins.
- **Emulsion test:** To frothing solution was added 2 drops of olive oil and the contents shaken vigorously. The formation of emulsion indicates the presence of saponins.

Test for Tannins

About 1 g of the extract was boiled with 20 ml of water was filtered and used for the following tests;

- **Ferric chloride test:** To 3 ml of the filtrate, few drops of ferric chloride were added. A greenish black precipitate indicates the presence of tannins.
- **Lead acetate test:** To a little of the filtrate was added lead acetate solution. A reddish colour indicates the presence of tannins.

Test for Steroids and Terpenoids

Ethanol (9 ml) was added to 1g of the extract and refluxed for a few minutes and filtered. The filtrate was concentrated to 2.5 ml on a boiling water bath. 5 ml of hot distilled water was added to the concentrated solution. The mixture was allowed to stand for 1h and the waxy matter was filtered off. The filtrate was extracted with 2.5 ml of chloroform using separating funnel. To 0.5 ml of the chloroform extract in a test tube was added 1 ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface shows the presence of steroids.

Another 0.5 ml of the chloroform extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 mins on a water bath. A gray colour indicates the presence of terpenoids

Test for Alkaloids

About 20 ml of 3% sulphuric acid in 50% ethanol was added to 2g of the extract and heated on boiling water bath for 10 mins, cooled and filtered. About 2 ml of the filtered was tested with a few drops of Mayer's reagent (Potassium mercuric iodide solution),

Wagner's reagent (Iodine in Potassium iodide solution), and Picric acid solution (1%). The remaining filtrate was placed in 100 ml separating funnel and made alkaline with dilute ammonia solution. The aqueous alkaline solution was separated and extracted with two 5 ml portion of dilute sulphuric acid. The extract was tested with a few drops of Mayer's, Wagner's, Dragendorff's reagents and picric acid solution. Alkaloids give milky precipitate with few drops of Wagner's reagent; yellowish precipitate with few drops of picric acid and brick red precipitate with few drops of Dragendorff's reagent.

Test for carbohydrates (Molisch test)

About 0.1g of the extract was boiled with 2 ml of distilled water and filtered. To the filtrate, few drops of naphthol solution in ethanol (Molisch's reagent) were added. Concentrated sulphuric acid was then gently poured down the side of test tube to form a lower layer. A purple interfacial ring indicates the presence of carbohydrate.

Test for reducing sugars

A 5 ml of a mixture of equal parts of Fehling's solution I and II were added to 5 ml of aqueous extract and then heated on a water bath for 5 mins. A brick red precipitate shows presence of reducing sugar.

Test for glycosides

Dilute sulphuric acid (5 ml) was added to 0.1 g of the extract in a test tube and boil for 15 mins on water bath. The mixture cooled and neutralized with 20% potassium hydroxide solution. 10 ml of a mixture of equal parts of Fehling's solution I and II was added and boiled for 5 minutes. A more brick red precipitate indicates the presence of glycoside.

Test for Resins (Precipitation test)

About 0.20g of the extract was extracted with 15 ml of 96% ethanol. The alcoholic extract was then poured into 20 ml of distilled water in a beaker. A precipitate occurring indicates the presence of resins.

Test for Proteins (Millon's test)

About 0.50 g of the extract was extracted with 20 ml of distilled water and the filtrate was used. To a little portion of the filtrate in test tube, two drops of millon's reagent were added. A white precipitate indicates the presence of proteins.

Test for fats and oil

About 0.1 g of the extract was pressed between filter paper was observed. A control was also prepared by placing 2 drops of olive oil on filter paper. A translucency of the filter paper indicates the presence of fats and oil.

Test for acidic compound

About 0.1g of the extract was placed in a clear dried test tube with sufficient water added to it. This was warmed in a hot bath and cooled. A piece of water wetted litmus paper was dipped in the filtrate and colour change on the litmus paper was observed. Acidic compounds turn blue litmus paper red.

Preparation of erythrocyte suspension

Fresh Ox blood (20 ml) was collected from an Abattoir at Ikpa market, Nsukka and was placed into EDTA tubes, 10 ml was withdrawn, transferred to centrifuge tubes, and centrifuged at 4000 rpm for 5 min. The supernatant was decanted, and the packed cells were washed three times with equal volume of normal saline, and it was reconstituted to 40% (v/v) suspension of RBC with isotonic buffer solution. The composition of the buffer solution (g/L) was NaH_2PO_4 (0.2 g), Na_2HPO4 (1.15 g) and NaCl (9 g).

Heat induced Haemolysis

The plant extract was dissolved in isotonic buffer containing 0.01% tween and then diluted to give the test concentrations (50,100,200,400 µg/ml). The isotonic buffer solutions (5 ml) containing the extract were put in 4sets (per concentration) into centrifuge tubes.

Control tubes contained 5 ml of the vehicle (0.01% buffered tween). Erythrocyte suspension (0.05 ml) was added to each tube (both standard and control), and gently mixed. A pair of the tubes was incubated at 54ºC in a regulated water bath. The other pair was maintained at 0 - 4ºC in a freezer for 20 min. At the end of incubation, the reaction mixture was centrifuged at 4000 rpm for 5 minutes and the absorbance of the supernatant measured at 540 nm, using spectrum 21D (PEC medicals USA) spectrophotometer and the level of inhibition (%) of haemolysis was calculated using the relation:

Inhibition of haemolysis (%) = 100 [1- (y-x/z-x)]

x = Absorbance of test sample unheated

y = Absorbance of test sample heated

z = Absorbance of control sample.

Hypotonic-solution induced haemolysis

Both isotonic and hypotonic solutions of the extract (50, 100, 200 and 400 µg/ml) were prepared. The prepared extracts (5 ml) were introduced in 2 pairs (each pair containing isotonic and hypotonic solutions) into centrifuge tubes. Control tubes contained 5 ml of the vehicle (0.01% tween in distilled water). Erythrocyte suspension (0.05 ml) was added to each test tube and mixed gently. After incubation for 1h at room temperature (27 ± 2ºC), the mixture was centrifuged for 5 minutes at 4000 rpm and the absorbance of the supernatant measured at 540 nm using spectrum 21D (PEC medicals USA) spectrophotometer. The level of haemolysis was calculated using the relation [8].

Inhibition of haemolysis (%) =100 [1-(y-x/z-x)] where

x=Absorbance of test sample in isotonic solution

y=Absorbance of test sample in hypotonic solution

z=Absorbance of control sample in hypotonic solution

Results and Discussion

Percentage yield= Weight (g) of extract/total weight (g) of plant material extracted x 100/1

% yield = 56.5/2200 x 100/1 = 2.57%

The results of the phytochemical tests revealed the presence of typical phytoconstituents (Table 1).

Phytochemical constituent	Relative presence
Terpenoids	+++
Steroids	+
Alkaloids	+
Flavonoids	++
Acidic compounds	-
Carbohydrates	++
Resins	++
Saponins	+
Proteins	-
Fats and oils	+++
Tannins	-
Reducing sugar	-
Glycosides	+

Table 1: Phytochemical constituents of *Leptadenia hastata*.

Key: Absent

+: Mildly present

++: Moderately present

++: Highly present

++++: Abundantly present.

Effect of extract on hypotonic solution-induced haemolysis of red blood cells

The LHE elicited a dose related and significant inhibition of hypotonic solution-induced haemolysis of ox red blood cells. The inhibition caused by LHE was greater than that of hydrocortisone (50 µg/ml), (Table 2).

Treatment	Concentration (µg/ml)	Absorbance		Inhibition of haemolysis (%)
		Buffered	Un buffered	
LHE	50	0.237 ± 0.006	0.578 ± 0.015*	42.49
	100	0.456 ± 0.016	0.583 ± 0.045*	66.04
	200	0.481 ± 0.140	0.574 ± 0.006*	73.35
	400	0.307 ± 0.004	0.556 ± 0.000*	52.39
Hydrocortisone	50	0.112 ± 0.042	0.525 ± 0.007*	42.48
Distilled water	0	0.372 ± 0.008	0.830 ± 0.007	-
Tween	(0.01%)	0.228 ± 0.015	0.530 ± 0.015	-

Table 2: Effect of extract on hypotonic solution-induced haemolysis of red blood cell.

*Data expressed as Mean ± SEM, n=2; *p < 0.05 compared to Control (ANOVA; LSD posthoc).

Effect of extract on heat-induced haemolysis of red blood cells

The LHE did not elicit any significant inhibition of heat-induced haemolysis of ox red blood cells. However, piroxicam (50 µg/ml) and hydrocortisone (50 µg/ml) showed significant inhibition of heat-induced haemolysis of ox red blood cells, (Table 3).

In this study, it was demonstrated that extracts of leaves of *Leptadenia hastata* at all the concentrations used in the experiment (50,100,200 and 400 µg/ml) showed significant inhibition of hypotonic solution-induced haemolysis of erythrocytes compared to control ($P < 0.05$) but no inhibition of heat-induced haemolysis. The result also highlights that *Leptadenia hastata* demonstrates a linear dose-response relationship in the inhibition of hypotonic solution-induced haemolysis to an extent. The highest inhibition of hypotonic solution-induced haemolysis was observed at 200 µg/ml. During inflammation, lyses of lysosomes occurs which leads to the release their component enzymes that cause a variety of disor-

Treatment	Concentration (µg/ml)	Absorbance readings		Inhibition of haemolysis (%)
		Heated	Unheated	
LHE	50	1.315 ± 0.056	0.170 ± 0.005	-46.79
	100	1.326 ± 0.157	0.290 ± 0.007	-22.31
	200	1.883 ± 0.027	0.539 ± 0.005	-124.75
	400	1.947 ± 0.067	1.121 ± 0.022	-140.55
Hydrocortisone	50	0.099 ± 0.002	0.055 ± 0.006*	95.93
Piroxicam	50	0.080 ± 0.019	0.047 ± 0.047*	96.35
Tween	(0.01%)	0.950 ± 0.046	-	-
Distilled water	0	1.137 ± 0.034	-	-

Table 3: Effect of extract on heat-induced haemolysis of red blood cells.

*Data expressed as Mean ± SEM, n = 2; *p < 0.05 compared to Control (ANOVA; LSD post hoc).

ders. Non-steroidal anti-inflammatory drugs (NSAIDs) exert their beneficial effects by inhibiting the release of lysosomal enzymes or by stabilizing the lysosmal membranes [9]. An exposure of red blood cells to harmful substances such as heat or hypotonic medium results in the lysis of the membranes, accompanied by haemolysis and oxidation of haemoglobin [10]. Since human red blood cell membrane are identical to lysosomal membrane [9], the inhibition of hypotonicity-induced haemolysis is taken to be a measure of the anti-inflammatory activity of leaf extract of *Leptadenia hastata*. The haemolytic effect of hyptonic solution can be attributed to excessive accumulation of fluid within the cell in which leads to the rupturing of its membrane. Injury to red cell membrane makes the cell more susceptible to secondary damage through free radical induced lipid peroxidation [11]. Membrane stabilization leads to prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators [12].

Under stressful conditions such as heat shock, pH shift or hypoxia, increased expression of HSPs protect the cell by stabilizing unfolded proteins, giving the cell time to repair or re-synthesize damaged proteins [13,14]. Heat Shock proteins are generally considered to improve cellular recovery both by either refolding partially damaged functional proteins or increasing delivery of precursor proteins to important organelles such mitochondria and endoplasmic reticulum. Heat shock proteins (especially HSP_{70}) act by protecting key enzymes such as superoxide dismutase, glutathione peroxidase, catalases [13]. Therefore, it can be inferred that the extract does not increase the expression of heat shock proteins.

The Phytochemical analysis revealed the presence of Terpenoids, Steroids, Alkaloids, Flavonoids, Carbohydrates, Resins, Saponins, Fats and oils and Glycosides. The exact underlying mechanism for the membrane stabilizing effect of *Leptadenia hastata* leaf extracts and the chemical constituents responsible for this effect is hitherto not known. However, a number of studies have shown that flavonoids, tannins, saponins and a host of other plant compounds exhibit analgesic and anti-inflammatory effects as a result of their membrane stabilizing activity in various experimental models [14,15]. From the results of the results of the Phytochemical analysis, *Leptadenia hastata* extracts contain moderate amounts of flavonoids, saponins and tannins. The production of free radicals, such as peroxides and superoxide's, are reported to be accountable for cell membrane destabilization [16]. Research has shown that Flavonoids and other phenolic compounds act as effective scavengers of free radicals [17]. Thus, it is not unreasonable to postulate that flavonoids and other phenolic compounds in *Leptadenia hastata* extracts could be responsible for the membrane stabilizing effect in this study.

Conclusion

The extract from *Leptadenia hastata* leaves demonstrated significant inhibition of hypotonic solution-induced haemolysis *in-vitro*. Further studies (preferably *in-vivo*) are required to elucidate the exact mechanism by which *Leptadenia hastata* inhibits hypotonic solution-induced haemolysis but not heat-induced haemolysis and for isolation and purification of the active principles of the plant responsible for the observed biological effects.

Conflicts of Interest

The author declares no conflict of interest.

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Volume 2 Issue 11 November 2018