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RESEARCH ARTICLE

Cell Membrane Stabilizing Effect and Serum Biochemical Analysis of Rats Treated with Methanolic Leaf Extracts of *Sida acuta* and *Crotalaria pallida* var *obovata*

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ABSTRACT

In an attempt to scientifically evaluate the mechanism of action of anti-inflammatory effects of *Sida acuta* and *Crotalaria pallida* var *obovata* which are medicinal plants used by the traditionalists, the present study was carried out to investigate the cell membrane stabilizing activities of methanolic extracts of the plants on rat red blood cell at five different concentrations; 1mg/ml, 2mg/ml, 4mg/ml, 6mg/ml and 8mg/ml. Indomethacin 0.10mg/ml was used as standard reference cell membrane stabilizing agent for comparison. Red blood cell of Wistar strain albino rats in hypotonic saline was used for the study. Also, the toxicity of the two plants in rats was investigated by treating the animals orally with the extracts for 28 days after which their serum biochemical profiles were analysed. The extractives inhibited hypotonic- induced haemolysis of erythrocytes *in vitro*; the methanolic extract of *Sida acuta* leaves demonstrated 30.6%, 34.7%, 37.5%, 44.4% and 41.7% inhibition while *Crotalaria pallida* var *obovata* demonstrated 16.7%, 19.4%, 36.1%, 30.6%, and 31.9% inhibition against 1mg/ml, 2mg/ml, 4mg/ml, 6mg/ml and 8mg/ml respectively, and the inhibition is in dose-dependent manner. The rats administered with *Crotalaria pallida* var *obovata* extract showed significant changes of an increase in aspartate aminotransferase and gamma-glutamyl transferase activities, decrease in total protein, and albumin levels while *Sida acuta* showed no changes. From these results, it can be suggested that the anti-inflammatory activities of these plants may be due to their membrane stabilizing effects, *Crotalaria pallida* var *obovata* is toxic to the rats at the dose-levels used.

KEYWORDS

Sida acuta, *Crotalaria*, Erythrocytes, Haemolysis, Membrane, Toxicity

INTRODUCTION

The use of medicinal herb in the treatment and prevention of diseases is attracting the attention of scientists worldwide (Sofowora, 1982). This is corroborated by World Health Organization in its quest to bring primary health care to the people. The plant kingdom has long served as a prolific source of useful drugs, food, additives, flavoring agents, colorants, binders and lubricants. As a matter of fact, it has been estimated that about 25% of all prescribed medicines today are substances derived from plants (Gamaniel, 2000).

Olajide (2003) reported that Nigerian vegetation is naturally endowed with arrays of floristic composition of different plant forms including trees, shrubs, herbs and other non-wood forest resources and these could be used for the betterment of the population especially for the treatment of various human and animal ailments. Herbal plants constitute one of the many

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resources of the forest in which the health of the average rural people in Nigeria depends on. They serve as the repository of healing materials and are known to have minimum or no side effects (Gbile and Adesina, 1986).

Since time immemorial, mankind believing plants are the drugs to cure various types of health problems. Moreover, herbal drugs are playing a major role in the world because of their safety, efficacy and cost effectiveness (Agarwal, 2001; Saleem *et al.*, 2010).

Currently used anti-inflammatory drugs are associated with some severe side effects; therefore, the development of potent anti-inflammatory drugs with fewer side effects from plants is necessary (Saha and Ahmed, 2009).

Sida acuta Burm.f. commonly called wireweed belong to the family Malvaceae. It is a terrestrial, perennial erect shrub believed to have originated in Central America, but today has a pantropical distribution and is considered a weed in some areas (Parsons and Cuthbertson, 2001). The plant is used by the traditionalists in western part of Nigeria for the treatment of various diseases of human and animals among which are; malaria, ulcer, fever, gonorrhea, breast cancer, inflammation, bleeding, sore wound, and pyrexia (Kayode, 2006; Edoga *et al.*, 2005). Studies have demonstrated some biological activities exhibited by this plant. The Guatemala and Nicaragua used the whole plant for the treatment of asthma, renal disorders, inflammation, cold, fever, headache, ulcer, and worm (Cacares *et al*, 1987; Coee and Anderson 1996). The Ghats use the paste of the leaves mixed with oil for the treatment of dandruff and also for straightening hair (Ignacimuthu *et al*, 2006; Malairajan *et al*, 2006). The phytochemical characterization of the plant shows the presence of carbohydrates, alkaloids, saponin, fixed oils, tannins and flavonoids (Palaksha and Ravishankar, 2012).

Crotalaria pallida var. *obovata* G.Don otherwise known as smooth rattlebox is a perennial non climbing shrub of Fabaceae family. It is pantropical in Africa and principally in the West African coastal region, across the Congo basin and then common all around the Great lakes and rivers in their vicinity, but also cultivated in Zimbabwe and Southern Mozambique. The plant has been reported to contain alkaloids, flavonoids, terpenoids, saponins, phenols, steroids and tannins (Govindappa *et al*, 2011). The traditionalists use the plant for the treatment of inflammatory related diseases. Stirling and Urquhart 1962 reported Crotalaria to contain pyrrolizidine alkaloid which is toxic to animals. This study was carried out to determine the effects of extracts of *Sida acuta* and *Crotalaria pallida* var *obovata* on the osmotic fragility of red blood cells and their level of toxicity with regards to the biochemical analysis of the blood serum.

MATERIAL AND METHODS

Plant Materials

Fresh leaves of *Sida acuta* Burm. f. were collected from Gwagwalada, Federal Capital Territory, Abuja where it grows as weed. Fresh leave of *Crotalaria pallida* var *obovata* were collected from Kaura Namoda in Kaura Namoda Local Government of Zamfara State. Confirmatory identification of the plants was done in the Department of Botany, University of Abuja.

The leaves were washed with water to remove dirt and then shade-dried at room temperature. After drying, the leaves were milled into powder using mortar and pestle, sieved and then stored in an air tight container for extraction.

Extraction of the Leaves

Cold extraction method was employed. Portion of 150g for *Sida acuta* of the powdered sample was weighed into a conical flask. Pure methanol (1000ml was used for extraction of the active components of the plant leaves.) was added and left for 48hours with intermitted shaking. The procedure was repeated for 12 hours. The solution was filtered and the filtrate was concentrated using vacuum rotary evaporator (IKA, Germany) at an optimum temperature of 40–50°C. The percentage yield of the extract was 6.27%. The concentrate was subjected to activity study. The same procedure was also employed for *Crotalaria pallida* var *obovata*. Portion of

180g for *Crotalaria pallida* var *obovata* of the powdered sample were weighed into a conical flask. The percentage yield of the extract was 8.06%. A fresh 10% (w/v) solution of the extracts was prepared with normal saline to make appropriate dosage required for the study.

Preparation of Animals

Adult Wistar albino rats of both sexes weighing 120–200g, obtained from the animal house of the Faculty of Veterinary Medicine, University of Abuja, Abuja, Nigeria, were used. They were housed in metal steel cages and acclimatized for seven days before the experiments. They were given free access to water and fed with growers mash bought from the local market.

Preparation of Solutions

Standard Drug

0.10mg/ml of Indomethacin was prepared in isotonic saline (0.85% NaCl) to make the concentration required for the study.

Red Blood Cell Suspension

10% (v/v) of rat red blood cell suspension was prepared with normal saline and kept in refrigerator at 4^0C as stock erythrocytes and the membrane stabilizing activity of the extract was assessed using hypotonic solution-induced rat erythrocyte hemolysis.

Test Solution

4.5ml of test solution consists of 2ml of hypotonic saline (0.25%w/v); 1ml of phosphate buffer (pH 7.4); 1ml of test extract (1mg/ml - 8mg/ml) in normal saline and 0.5ml of rat red blood cells in isotonic saline.

Test Control

4.5ml of test control consists of 2ml of hypotonic saline (0.25% w/v); 1ml of phosphate buffer (pH 7.4); 1ml of isotonic saline and 0.5ml of rat red blood cells in isotonic saline

Standard Drug Solution

4.5ml of standard solution consists of 2ml of hypotonic saline (0.25%w/v); 1ml of phosphate buffer (pH 7.4); 1ml of Indomethacin (0.1mg/ml) and 0.5ml rat red blood cells in isotonic saline.

Procedure

Modified method of Shinde *et al.*, (1999) used to screen and study drugs, chemicals and herbal preparations that exhibit anti-inflammatory properties or potentials was employed. Whole blood was obtained from the inner canthus of the rat's eye using heparin tube into a sample bottle, centrifuged and supernatant was carefully collected. The remaining packed cells were washed three times with equal volume of isotonic buffered solution (154mM NaCl in 10mM sodium phosphate buffer), and the packed cells were been centrifuged each time at 3000 rpm for 20 min. 10% (v/v) of erythrocyte suspension was prepared as stock erythrocytes.

1 ml of varying concentrations of the extracts (1, 2, 4, 6 or 8mg/ml) or 0.10 mg/ml of indomethacin in the case of standard drug solution, was mixed with 1 ml of phosphate buffer, 0.5 ml of stock erythrocytes and 2 ml of the hypotonic solution was added to make 4.5 ml. The test control consists of 2ml of hypotonic saline (0.25%w/v), 1ml of phosphate buffer (pH 7.4), 1ml of isotonic saline and 0.5ml of rat red blood cells in isotonic saline, the reaction mixtures were incubated at 37°C for 30 min. The absorbance of the supernatant solution was measured with spectrophotometer at 540 nm. Each experiment was carried out in triplicate and the average was taken.

The percentage inhibition of hemolysis or membrane stabilization was calculated according to modified method described by Shinde *et al*, (1999).

% Inhibition of hemolysis = $[(A_1-A_2)/A_1] \times 100$

Where A_1 is the Optical density of test control and A_2 is the Optical density of test sample.

Thirty animals divided into 5 groups of 6 animals per group were used for toxicological study. The first two groups were administered with 500 and 1000 mg/kg of the extract of *Sida acuta*, the other two groups were administered with 500 and 1000 mg/kg of the extract of *Crotalaria pallida* var *obovata*, while the fifth group was administered with 3 ml/kg of distilled water respectively. All the administrations were done

orally for 28 days after which the animals were anaesthesized with ether and blood was obtained from their inner canthus of the eye into a sample bottles, placed in a slanting position and allowed to coagulate. The blood was then centrifuged and the serum was collected for analysis.

Determination of Serum Biochemical Parameters

Total protein was measured using biuret reaction while albumin was measured by colorometric estimation using the sigma diagnostics albumin reagent (Sigma Diagnostic, U.K) which contained bromocresol green (BCG). Globulin was obtained from the difference between total protein and albumin. Aspartate aminotransferase (AST) was determined by monitoring the concentration of oxaloacetate hydrazine formed with 2,4-dinitrophenyl hydrazine (Rietmans and Frankel, 1957), Alanine aminotransferase (ALT) was determined by monitoring the concentration of pyruvate hydrazine formed with 2,4-dinitrophenyl hydrazine (Rietman and Frankel, 1957), while Gamma glutamyltransferase (GGT) was measured using modified method of Szasz procedure (Szasz, 1974) which measured the quantitative formation of 5-amino-2-nitrobenzoate.

Statistics

All values were expressed as mean ± SD. The data collected were statistically analyzed by using one-way ANOVA and difference between means was assessed by Duncan's new multiple range, $p<0.05$ was considered statistically significant.

RESULTS

The result of the effect of methanolic extract of *Sida acuta* on hypotonic solution induced hemolysis in rat red blood cells is as shown in table 1.

Table 1: Effect of extract of *Sida acuta* Burm. f., on hypotonic solution induced hemolysis of rat erythrocyte

Samples	Concentration	Optical Density	% Inhibition of Hemolysis
Control	50 mM	0.72±0.016	- - -
Extract	1mg/ml	0.50±0.008	30.6±8.2*
Extract	2mg/ml	0.47±0.007	34.7±0.8**
Extract	4mg/ml	0.45±0.009	37.5±0.9**
Extract	6mg/ml	0.40±0.013	44.4±1.2***
Extract	8mg/ml	0.42±0.014	41.7±1.4**
Indomethacin	0.10mg/ml	0.36±0.013	50.0±1.3***

* = 0 ≤ 0.3, ** = 0.5 ≥ 1, *** = 1 ≥ 1.5. The values are presented as mean ± S.D. (standard deviation).

All the concentrations used inhibit the hemolysis with 6mg/ml having the highest inhibition of 44.4±1.2% which is comparable with the inhibition produced by 0.10mg/ml of indomethacin which is 50.0±1.3%. 1mg/ml of the extract showed the lowest inhibition of 30.6±8.2%, followed by 2mg/ml and 4mg/ml which are 34.7±0.8% and 37.5±0.9% respectively, the inhibition is in dose dependent manner.

The extract of *Crotalaria pallida* var *obovata* also showed significant increase in inhibition of hemolysis with 4mg/ml concentration producing the highest inhibition of 36.1±1.5% lower than indomethacin with inhibition of 50.0±8.2%. 1mg/ml of the extract showed the least inhibition of 16.7±1.6% followed by 2mg/ml which shows 19.4±1.6% while 6mg/ml and 8mg/ml produced 30.6±8.2% and 31.9±1.6% inhibition respectively (table 2).

The methanolic extract of *Crotalaria pallida* var *obovata* produced a significant decrease ($p<0.05$) in serum albumin at 500mg/kg doses, while 1000mg/kg insignificantly ($p<0.05$) decreased the total proteins and globulin. The ALT, AST and GGT are all significantly ($p<0.05$) increased as the extract of *Sida acuta* did not produce any significant changes in the serum biochemistry (table 3)

Superscripted items indicate statistically significant values. Total protein, albumin and globulin were measured in g/l. ALT, AST and GGT were measured in IU/L.

Table 2: Effect of extract of *Crotalaria pallida* var *obovata* on hypotonic solution induced hemolysis of rat erythrocytes

Samples	**Concentration**	**Optical density**	**% Inhibition of Hemolysis**
Control	50 mM	0.72±0.016	- - -
Extract	1mg/ml	0.60±0.082	16.7±1.6*
Extract	2mg/ml	0.58±0.016	19.4±1.6**
Extract	4mg/ml	0.46±0.017	36.1±1.5**
Extract	6mg/ml	0.50±0.008	30.6±8.2***
Extract	8mg/ml	0.49±0.016	31.9±1.6**
Indomethacin	0.10mg/ml	0.36±0.016	50.0±8.2***

* = 0 ≤ 0.3, ** = 0.5 ≥ 1, *** = 1 ≥ 1.5. The values are presented as mean ± S.D. (standard deviation).

Table 3: Effects of the extracts of *Sida acuta* and *Crotalaria pallida var obovata* on the serum biochemical parameters of rats

Treatment	Dose (mg/kg)	T. Prot.	Alb	Glob	ALT	AST	GGT
Sida acuta	500	39.8±1.7	25.6±4.3	14.2±0.8	28.3±0.27	26.1±2.2	31.2±1.8
Sida acuta	1000	45.3±2.8	29.3±2.7	16.0±1.6	27.4±4.3	33.5±3.7	35.7±3.5
C. obovata	500	25.7±1.3	15.9±3.4[a]	9.2±1.4	31.1±3.0	37.4±2.5	56.7±3.8
C. obovata	1000	27.5±3.1	17.2±1.8	12.3±0.5	59.9±3.1[b]	62.3±0.6[b]	98.3±4.3[b]
Distilled Water	3ml/kg	37.8±1.3	23.6±2.1	14.2±1.8	29.8±0.5	28.3±1.6	32.4±2.7

DISCUSSION

It has been established that the vitality of cells depends on the integrity of their membrane (Ferrali *et al.*, 1992) and exposure of cells to injurious substances such as hypotonic solution results in lysis of such cell and elaboration of its contents into the surrounding environment resulting into different kinds of reactions. The hemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell which lead to the rupturing of its membrane, such injury to red blood cell membrane will further render the cell more susceptible to secondary damage through free radical-induced lipid peroxidation (Augusto *et al*., 1982; Ferrali *et al*., 1992). The red blood cell membrane resembles the lysosomal membrane and as such, the effect of drugs on the stabilization of red blood cell membrane could be extrapolated to the stabilization of lysosomal membrane (Omale and Okafor, 2008) therefore, as the membrane stabilizes, it interfere with the release and or action of mediators like histamine, serotonin, prostaglandins, luekotrienes etc (Shinde *et al*., 1999).

Also, those substances with membrane-stabilizing activities are known for their ability to interfere with the early phase of inflammatory reactions, such as the prevention of the release of phospholipases that trigger the formation of inflammatory mediators (Aitadafoun *et al*., 1996) thereby preventing inflammatory reactions.

Both *Sida acuta* and *Crotalaria pallida* var *obovata* have been reported to possess anti-inflammatory activities. Oboh and Onwukame, (2005) investigated the analgesic and anti-inflammatory properties of the crude extract of *Sida acuta* in mice by using tail immersion and mouse ear oedema model. The crude extracts exhibited significant analgesic and anti-inflammatory activities in mice and was dose dependent, it may therefore be suggested that part of the anti-inflammatory effect of *Sida acuta* may be as a result of its cell membrane stabilizing activities which prevent the release of inflammatory mediators from cells, as its inhibitory effect to hemolysis is less when compared to that of Indomethacin. Membrane stabilizing activities for both *Sida acuta* and *Crotalaria pallida* var *obovata* observed using

the above model were performed on the rat erythrocyte membrane and the membrane stabilizing activity of the extracts increased significantly in dose-dependent manner although the activity of *Crotalaria pallida* var *obovata* is less than that of *Sida acuta* which in turn is less than that of Indomethacin.

The mode of action of the extracts and Indomethacin could be as a result of their binding to the erythrocyte membranes with subsequent alteration of the surface charges of the cells. This might have prevented physical interaction with aggregating agents or promote dispersal by mutual repulsion of like charges of those substances involved in the hemolysis of red blood cells.

The methanolic crude extract of *Crotalaria pallida* var *obovata* caused a significant decrease to the level of albumin while the decrease or elevation seen in other serum biochemical parameters is insignificant at 500mg/kg. The 1000mg/kg produced a significant elevation in alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT). Albumin is a protein synthesized in the liver, thus low level of albumin can be an indication of liver damage or disease. Decreased albumin may also suggest kidney disease or disorder such as glomerulonephritis with loss of albumin in the urine leading to decreased serum protein levels. ALT is present in the liver and other cells. It is used to identify liver damage such as those arising from liver cell inflammation or necrosis (Kim *et al*, 2008). Although ALT is widely distributed, significant increases in its plasma activity are rarely seen other than in liver damage, it is therefore particularly useful in measuring hepatic necrosis especially in small animals (Cornelius 1989). Since it is one of the specific assayable liver enzymes, its elevated level in this study may indicate hepatic damage caused by *Crotalaria pallida* var *obovata* extract. AST is an enzyme found in high amount in liver, heart and muscle cells and it is also found in lesser amounts in other tissues. A very high level of AST is frequently seen with acute hepatitis (Han *et al*., 2012), it may be normal to moderately increased with chronic hepatitis, blocked bile ducts, cirrhosis and liver cancer. The increase in AST in this study may be an indication of liver damage caused by *Crotalaria pallida* var *obovata* extract used. Serum GGT analysis may be used to determine the cause of alkaline phosphatase (ALP) elevation, both ALP and GGT are elevated in disease of the bile ducts and in some liver diseases, but only ALP will be elevated in bone disease, in general, an increased GGT level indicates that the liver is being damaged (Tietz 1994). This also signified that the *Crotalaria pallida* var *obovata* extract used in this study damaged the liver. This corroborate the report of Stirling and Urquhart 1962 which states that Crotalaria contains pyrolizzidine alkaloids which are hepatotoxic.

CONCLUSION

Based on these results, it could be said that *Sida acuta* and *Crotalaria pallida* var *obovata* contained principles that are capable of stabilizing rat red blood cells membrane against hypotonic-induced lysis, *Crotalaria pallida* var *obovata* is hepatotoxic while *Sida acuta* is non-toxic to the rats at the doses used. The results thus suggests that methanolic extracts of the leaf of *Sida acuta* and *Crotalaria pallida* var *obovata* may offer some beneficial effects in the management of inflammatory conditions, serve as a useful supplementary therapy in hemolytic disease, and also in free radical mediated oxidative cell injury conditions. *Crotalaria pallida* var *obovata* on the other hand may cause more injury to the animals than its beneficial effect so caution must be taken anytime it is involved in the treatment of ailments.

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