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Phytochemical screening and haemolytic activities of crude and purified saponins of aqueous and methanolic extracts of leaves of *Tephrosia vogelii* Hook. F

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ABSTRACT

The phytochemical screening of the aqueous and methanolic extracts of leaves of Tephrosia vogelii Hook.f. was carried out to ascertain the presence of saponins and further studies carried out on the crude and purified saponins. The results of phytochemical screening using aqueous(with a yield of 16.396%) and methanol (with a yield of 12.316%) extracts revealed the presence of cardiac glycosides, alkaloids, saponins and phlobatannins. However, the plant was devoid of anthraquinones and tannins. The presence of saponins was further detected by the haemolysis of red blood cells. Crude PBS extract possessed haemolytic activities against human erythrocytes (A,B,O and AB) but at varying degrees. The haemolytic activities for full and partial haemolysis(FH and PH) with human red blood cells A, B, O and AB ranged between $2^0$to 2^4, 2^4, 2^6 and 2^4 respectively. The haemolytic activity of the purified saponins(PS) was also determined. The results of the haemolytic activity of crude aqueous extract and purified saponins using erythrocytes of blood group O were the same (PS_32^1 FH, 2^3PH) and (PS_52^1 FH, 2^3 PH).

Key words: Secondary metabolites, saponins, *Tephrosia vogelii* hook. f., full haemolysis, partial haemolysis.

INTRODUCTION

Saponins are glycosides of 27 carbon atom steroids or 30 carbon atom triterpenes in plants. They are often referred to as “natural detergent” because of their foamy texture. They derive their name from the Latin word “ Sapo” which means that the plant consists of frothing agent when diluted in aqueous solution. They comprise polycyclic aglycones and sugar moieties (hexoses, pentoses and saccharic acids). The sapogenin or aglycone part is either a triterpene or steroid. They are found in different plants such as soap-wort, soapberry, soapbark, soaproot, soyabeans, vegetables and herbs. Commercial saponins are mainly extracted from *Quillaja saponaria* and *Yucca schidigera.* Saponins as the sapogeninaglycone have also been identified in the animal kingdom in snake venom, starfish and sea cucumber. Saponins can be easily extracted using hot water, ethanol or methanol because of their solubility in them. They are characterized by form- forming, haemolytic activities and bitter taste, and occur in small quantities in nature [2]. Some toxic saponins are known as sapotoxins. They have been known to be toxic to cold- blooded creatures like snake and fish [12]. Some examples of saponins are ginsenosides of ginsen, titogenin and digitogenin. They are used as detergents for cleaning and foam as well as shampoo producers. In some cases, saponins from different parts of the same plant have been found to exhibit different properties. For example; *Spinaciaoleracea* plant root contains spina-saponins A and B, which show potent antibiotic activities, but the leaves are practically free of saponins. The saponins from the stem of *Guaicum Officinale* do not possess any biological activity whereas the leave saponins have marked heamolytic actions [1]. Again, sometimes, the saponins from different plants have been found to exhibit the same biological properties for example; bark of *Schima mertersiana* contains piscisidal saponins, so also the fruits, roots and bark of *Balanites aegyptiaca*[16]. Differences in saponins contents have been observed in some cases when the same varieties of plants are collected from different localities [1]. Saponinsare natural antibiotics, cholesterol- lowering agent [4] as well as immune and energy boosters.

A medicinal plant is a plant which one or more of its organs contains substances that may be used for therapeutic purposes or as precursors for the synthesis of useful drugs (WHO 1998). Several plants have advanced to clinical use in modern times [9] in the treatment of chronic diseases such as diabetes [7], hypertension and cardiovascular diseases. One of such plants is *Tephrosia vogelii* Hook. f**.** The plant is native to Nigeria, Kenya, South Africa, Zambia, Tropical America, Southeast Asia and Malaysia as a cover crop.

MATERIALS AND METHODS

Treatments of plant part

The identified leaves were washed to remove particles and dust. The washed leaves were heated at 80^0C for 10min and 60^0C for 30min to deactivate enzymes(glycosidases) which may break down saponins[7]. Further drying using atmospheric air was carried out for complete dryness. The dried leaves were ground into powder using a mortar and pestle before been separately milled into fine powder using an electric blender to pass through a 0.3mm sieve (BS 410 Endecotts Ltd London). The sample was sieved with 0.3mm sieve made of brass material.

Preparation of extracts

A 50g portion of the sieved sample was weighed into a 500ml conical flask and 300ml of distilled water added. It was thoroughly shaken and covered with a piece of thin foil paper and allowed to stand overnight. It was shaken intermittently for maximum extraction. The aqueous extract was carefully decanted into another conical flask. Another 300ml of distilled water was added and the mixture treated the the way. The extraction was carried out for a period of seven days until the supernatant was colourless. The supernatant was then transferred into a weighed, cleaned, dry aluminium plate and the distilled water evaporated at low temperature using a hot plate and residue stored at room temperature until required. The sample residue from aqueous extraction was re-extracted with methanol to produce the crude methanolic extract.

Phytochemical screening

Phytochemical screening was carried out using aliquots of both methanol and aqueous extracts using the following tests

Tests for alkaloids

A 0.1g sample of extract was stirred with 5ml of 1% aqueous HCl on a steam bath and filtered. 1ml of filtrate was then treated with a few drops of Mayer's reagent and another 1ml portion similarly treated with Wagner's reagent. The precipitation of both or either of these reagents was taken as evidence of alkaloids [14][20].

Tests for anthraquinones

A 1.0g portion of extract was shaken with 5ml benzene, filtered and 1ml of 10% ammonia solution added to the filtrate. The mixture was shaken and the presence of free anthraquinones was observed(Borntrager's test) [14]. For the combined anthraquinones, 1.0g of aqueous extract was boiled with 5ml aqueous concentration of H_2SO_4 and filtered. After shaking with 3ml benzene, the benzene layer was separated and half its own volume of 10% ammonia solution added. A pink, red or violet colouration in the ammoniacal lower phase indicated the presence of anthraquinone derivatives [20].

Test for Tannins

A 1.0g portion of extract was stirred with 10ml of distilled water and filtered. Ferric chloride was added to the filtrate. A blue-black, green or blue-green precipitate was taken as evidence of tannins [20].

Test for phlobatannins

The deposition of red precipitate when an aqueous extract of the plant part was boiled with 1% aqueous HCl was taken as evidence for the presence of phlobatannins[20].

Liebermann-Buchard's test

A 0.5g portion of the extract was dissolved in 1ml of acetic anhydride and cooled in ice and concentrated sulphuric acid then carefully added. A colour change from violet to blue green indicated the presence of steroidal nucleus (i.e. aglycone of the cardiac glycosides) [15].

Salkowski's test

A 0.5g portion of extract was dissolved in 2ml chloroform and concentrated sulphuric acid added to form a lower layer. A reddish-brown colour at the interface indicated the presence of steroidal ring(i.e. aglycone portion of the cardiac glycoside) [19].

Keller Kiliani's test
A 0.5g portion of the extract was dissolved in 2ml of glacial acetic acid containing one drop of 5% ferric chloride solution and 1ml sulphuric acid(conc.) was then carefully added. A brown ring at the interface indicated the presence of deoxy sugar which is characteristic of cardenolides [20].

Test for saponins
The ability to produce foam in aqueous solutions and haemolyse red blood cells using crude aqueous extract and purified saponins were used as screening tests for saponins.

Purification of saponins from *Tephrosia vogelii* leaves
The reverse phase chromatography column (Octadecylsilane(C-18) bonded to silica gel-J. T. Baker) was chosen to separate and purify the saponins from the crude extracts. The saponins solution was diluted to 6.7mg/ml and stored at 4^0C until required.

Determination of haemolytic activities of partially purified and crude aqueous extract
Haemolytic assay
Fresh human blood was collected from healthy donors of four blood groups (O,A,B and AB) using 5ml sterile syringes with needles by venepunctures. The blood collected was transferred into appropriately labelled clean sterile sample bottles containing Ethylenediaminetetraacetic Acid (EDTA) (3mg/5ml of blood) and used within 24 hr. The blood samples (O,A,B and AB) were transferred into calibrated centrifuge and centrifuged using the bench centrifuge for 5min at maximum speed (3000rpm). The supernatant was discarded using Pasteur pipette and the packed cells were washed five times with PBS (pH 7.2). The washed erythrocytes were suspended in PBS at 2% v/v and stored at 4^0C until use[13]. The method used was an adaptation of the haemagglutination procedure of[5] using micro titre plates [13] and modified [17]. Haemolytic assay was conducted by a two-fold serial dilution of different extracts (crude aqueous extract and purified saponins) using U-shaped bottom micro-titre plates with 2% v/v of the erythrocytes suspension in PBS. A 25µl portion of PBS was added to wells 2 to 12, and 50µl of prepared aqueous extract to well 1 using a micro diluter by transferring 25 µl from well 1 into well 2 through to well to well 11 leaving well 12 undiluted as control. The micro diluters were washed with distilled water, rinsed with PBS and dried on a piece of tissue paper before carrying out fresh dilution. After dilution, a drop (25 µl) of treated erythrocyte was added to each of the wells (wells 1 to 12) and incubated for 30 min at room temperature for the sedimentation patterns to take place. The sedimentation patterns of erythrocyte suspension in the undisturbed plate were read after incubating for 30 min at room temperature (30^0C) to determine the titre(defined as the reciprocal of the greatest dilution at which haemolysis occurred). Apositive pattern indicating full haemolysis (FH) appeared as a big circular spot of red solution surrounded by small clear zone. A negative pattern indicating no haemolysis (NH) appeared only as a uniform small spot of erythrocytes at the bottom of the well, surrounded by a big concentric clear zone. In some micro dilutions or titrations where a positive pattern was observed, the clump of erythrocytes appeared as a rather large and non-uniform spot. In such cases, a partial haemolysis (PH) was recorded[18].

Statistical Analysis
Data obtained from this study were analysed using one way analysis of variance (ANOVA) at $P < 0.05$.

RESULTS AND DISCUSSION

The phytochemical screening of the extracts of the leaves of *Tephrosia vogelii* Hook.f. was carried out using aliquots of both aqueous and methanolic extracts for the test of cardiac glycosides, alkaloids, saponins, phlobatannins, tannins and anthraquinones. The extracts revealed the presence of cardiac glycosides, alkaloids, saponins and phlobatanninsas shown in Table 1. However, from Table 1 the plant extract was devoidof tannins and anthraquinones. These were carried out by the method of [20]. The haemolytic activities of crude aqueous extract and purified saponins of leaves of the plant were carried out using fresh human blood groups O, A, B and AB. The results showed that the plant part possessed haemolytic activity with different human erythrocytes. Full and partial haemolysis for these samples were observed with human erythrocytes. With respect to full haemolysis (FH) using crude aqueous extract, blood group O recorded the highest titre (2^3) while both blood groups A and B recorded the least titre (2^1) as shown in table 2. With respect to partial haemolysis (PH) using crude aqueous extract blood group O recorded the highest titre (2^6) whereas, the same but lower titre (2^4) was recorded for blood groups A, B and AB. With respect to full haemolysis (FH) using purified saponins (PS_3) and (PS_5), all the blood groups recorded the same titre of (2^1) (Table2). Also, with respect to partial haemolysis (PH) using purified saponins (PS_3 and PS_5), all the results of the blood groups recorded the same titre of (2^3) as shown in table 2. Results of the haemolytic activity observed with both crude aqueous extract and its purified saponins could be attributed to the saponins content of the plant [6]; [10]; [8] (Table2). This toxicologically interesting property of many saponins to bring about haemolysis i.e. the release of haemoglobin from erythrocytes is as result of change in membrane permeability. This is

considered to be influenced by the affinity of the aglycone to cholesterol in cell membranes [3]. The ability of the plant extracts to lyse red blood cells at varying degrees could be attributed to the presence of different types of saponins as was reported in the findings of [8] and[11](Table 2) where different saponins showed different levels of haemolytic activity. This observation can also be correlated with the findings of [18] that a certain plant and even part e.g. leaves of the same plant may contain different saponins which can differ in biological features. However, this haemolytic activity only takes place with parenteral administration since in oral administration there is usually only limited absorption.[21] reported that *Ladino clover* saponins is not toxic to fish and does not haemolyse red blood cells.

Table 1: Phytochemical screening of aqueous and methanol extracts of leaves of *Tephrosia vogelii* hook. f.

Test	Aqueous Extract	Methanolic Extract
Alkaloids		-
Magner's test	+	-
Wagner's test	+	+
Saponins	+	-
Anthraquinones	+	+
Tannins	-	+
Phlobatannins	+	-
Keller-Killiani's test	+	+
Leibermann-Burchard's test	+	+
Salkowski's test	+	+

\+ = *positive Result*
\- = *Negative Result*

Table 2: Haemolytic activities of crude aqueous extract and purified saponins of leaves of *Tephrosia vogelii* hook. f.

EXTRACT/SAMPLE	*TITRE							
	BLOOD TYPE (GROUP)							
	O		A		B		AB	
	FH	PH	FH	PH	FH	PH	FH	PH
AQUEOUS	2^3	2^6	2^1	2^4	2^1	2^4	2^2	2^4
PS_3	2^1	2^1	2^1	2^3	2^1	2^3	2^1	2^3
PS_5	2^1	2^1	2^1	2^3	2^1	2^2	2^1	2^3

**Titres are the reciprocal of the highest dilution at which haemolysis occurred.*
Concentration of crude aqueous extract is 0.1g/ml = 100mg/ml of PBS
Concentration of purified saponins are 0.0320g/5ml = 6.4mg/ml and 0.0351g/5ml = 7.02mg/ml of PBS. 5µl was taken from each of them.
FH = Full haemolysis
PH = Partial haemolysis
PS_3 = (30% fraction) = Purified saponins$_3$
PS_5 = (50% fraction) = Purified saponins$_5$

CONCLUSION

The results of this study have revealed that the leaves of the plant *Tephrosia vogelii* Hook. f. contained saponins which were responsible forits haemolytic activities against blood group O, A, B and AB.

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