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ARTICLE

Studies on saponins of leaf of *Clerodendron thomsonae* Balfour

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ABSTRACT In this study, the phytochemical screening of the leaf extracts of *Clerodendron thomsonae* Balfour were performed to ascertain one of its secondary metabolite constituents, saponin. The results revealed the presence of cardiac glycosides, flavonoids, alkaloids, glycosides and saponin glycosides. However, the plant extract was devoid of tannins, phlobatannins and anthracenes.The presence of saponins was detected by the formation of long-lasting foams and haemolysis of red blood cells. Phosphate-buffered saline (PBS) extract foamed with a height of 9.25 ± 0.4 mm and a foaming time of 20.93 ± 0.56 h while the methanol/phosphate buffered saline (M/PBS) extracts possessed a foam height of 3.75 ± 0.11 mm and disappeared fully after 27.37 ± 0.04 h. Generally, the foaming time was not proportional to the foam height. The melting point of both the aqueous and methanol crude extracts ranged between 283 to 315°C and 125 to 240°C, respectively. PBS and M/PBS extracts possessed haemolytic activities against human erythrocytes (ABO) but at varying degrees. The haemolytic activities for full and partial haemolysis of human red blood cells ranged between 1 to 2^8 and 2^3 to 2^{11}, respectively.
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KEY WORDS

phytochemical screening
secondary metabolites
saponins
foam-forming
full haemolysis
partial haemolysis
haemolytic activity

Saponins are a diverse group of compounds widely distributed in the plant kingdom (Güclü-Ustündag and Mazza 2007). They occur constitutively in a great many plant species, in both wild plants and cultivated crops. In cultivated crops, the triterpenoid saponins are generally predominant, while steroid saponins are common in plants used as herbs or for their health-promoting properties (Fenwick et al. 1991). They are stored in plant cells as inactive precursors but are readily converted into biologically active compounds by plant enzymes (Mert-Türk 2005). They derive their name from their ability to form stable, soaplike foams in aqueous solutions (Shi et al. 2004).

In chemical terms, saponins are glycosylated compounds that are widely distributed in the plant kingdom and can be divided into three groups; a triterpenoid, a steroid, and a steroidal glycoalkaloid. Triterpenoid saponins are found primarily in dicotyledonous plants. They are also found in some monocots, whereas steroid saponins occur mainly in monocots, such as the Lilliaceae, Amaryllidaceae and in certain dicots, such as foxglove (Hostettmann and Marston 1995). Oats (Avena spp.) are unusual because they contain both triterpenoid and steroid saponins (Price et al. 1987). Steroidal glycoalkaloids are found primarily in members of the family Solanaceae, which includes potato and tomato (Osbourn 1996).

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Saponins are attracting considerable interest as a result of their diverse properties both deleterious and beneficial (Shi et al. 2004). The pharmacological reputation of the saponins used to be like all detergents which on injection, causes lysis of the blood cells, and are highly toxic. This rather negative reputation for saponins has been transformed since the 1960s by closer observation of the action of many herbal remedies, and in particular of the remedy ginseng. It is now accepted that these rather elusive substances are responsible for quite astonishing properties, and in some ways can claim to have challenged the whole edifice of orthodox pharmacology (Herbs2000.com 2006). Because of the presence of both hydrophilic and hydrophobic regions, saponins are excellent emulsifiers and foaming agents, and provide functional roles in foods. A high saponin diet can be used in the inhibition of dental caries and platelet aggregation, it is also used in the treatment of hypercalciuria in humans, and as an antidote against lead in epidemiological studies. The ability of saponins to form emulsions in the intestine have lead to the investigation into their role in lowering serum cholesterol in humans (MacDonald et al. 2005). Their toxicity is related to their activity in lowering surface tension (Birk 1969). Saponins have a bitter taste and when ingested orally are practically non-poisonous to warm blooded animals. When injected directly into the blood stream, however, they are dangerous and quickly dissolve red blood cells. Saponins normally break down in the digestive system and must enter the blood

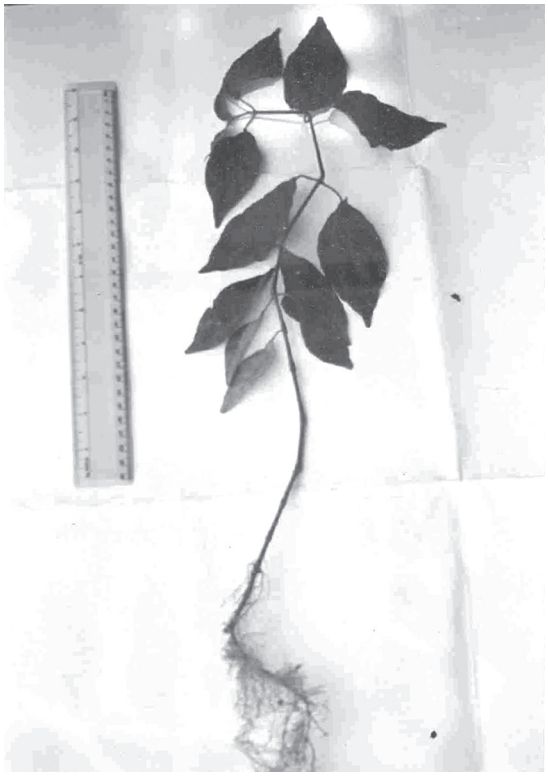

Figure 1. The plant *Clerodendron thomsonae* Balfour.

stream to be toxic (Elpel 2000), but fish assimilate saponin directly into their blood stream via their gills. Fish poisoned by saponins become stupefied and float to the surface where they can easily be collected. Saponins have been shown to inhibit cholinesterase; trypsin and proteinase activities (George 1965). The biological activity of saponin such as anticancer and anti cholesterol activity has led to the emergence of saponins as commercially significant compounds with expanding applications in food, cosmetics, and pharmaceuticals sectors (Güclü-Ustündag and Mazza 2007). They have also been reported to reduce the more harmful LDL-cholesterol selectively in the serum of rats, gerbils and human subjects (Potter 1993; Harris et al. 1997; Matsuura 2001). They are soluble in ethanol, methanol, water or mixture of water with methanol or ethanol (Gennario 1985). Some are exceptions to these characteristics e.g soybean do not form complexes with cholesterol. Ladino clover saponin is not toxic to fish and does not haemolyse red blood cells (Walter et al. 1955). Saponins occur in more than 90 families (Chandel and Rastogi 1980) and at least 500 genera of plants (Basu and Rastogi 1967). A certain plant and even part of the same plant may contain different saponins which can differ in bulbs, blossoms and fruits (Fenwick and Oakenfull 1981). Saponins have been found in legumes, soybean (Fenwick and Oakenful 1981; Shi et al. 2004), sugar beet, spinach asparagus and horse chestnut (Fenwick and Oakenful 1981), Millet (*Pennisetum typhoideum*), bitter Kola (*Garcinia kola*), Cocoyam (*Colocosia esculenta*), guinea corn (*sorghum vulgare*), groundnut (*Arachis hypogeae*), sweet potatoes (*Ipomea batata*) and cassava (*Manihot esculenta*; Sodipo and Arinze 1985). The neem, (*Azadirachta indica*; Sodipo and Tizhe 1991).

The biological effects of saponins arise from their steroids and proteins (Dourmashkin et al. 1962). Saponins affect ATPase activity owing probably to a gentle alteration of the erythrocyte membrane. Aescin and whole extracts of horse chestnuts inhibits and reduce the development of atherosclerosis in rabbits and rats (Birk 1969). Ginseng saponin stimulates the renal nuclear and cytoplasmic RNA synthesis and it is therefore suggested that RNA and protein in rat kidneys are stimulated by those saponin which ultimately improve the functions of the kidney (Chandel and Rastogi 1980). By their ability to lower surface tension and possession of emulsifying properties, saponins tend to alter permeability of the cell wall thereby exhibiting a general toxicity on all organised tissues (Basu and Rastogi 1967). Saponins are known to decrease blood lipids, lower cancer risks, and lower blood glucose response (Petit et al. 1993; Kim et al. 1998; Lee et al. 2000; Yoshikawa et al. 2001). Saponins have also been shown to have an inverse relationship with the incidence of renal stones (Shi et al. 2004).

Certain saponin drugs have long been known as fish poisons and for killing fish by artesanal fishermen. They are also considered to be the active components of many traditionally used fish poison (Francis et al. 2001). Saponins have been reported to be highly toxic to fish because of their damaging effect on the respiratory epithelia (Roy et al. 1990; Rooj 1993). Fish have also been shown to exhibit stress reactions to the presence of saponins in water (Francis et al. 2002). Rooj (1993) discovered in the bark and leaves of *Teminalia tomentosa*, a common Indian plant to contain juice saponin consisting of arjunolic acid as its ingredient. This acid is known to be toxic to fish.

The potentials of the class of plant glycoside in the control of schistosomiasis (Bilharziasis) have also been discussed (Lemma 1965; Dorsaz et al. 1988; Kishor and Sati 1990; Sodipo 1998; Apers et al. 2000). The molluscicidal activity of the saponins according to Francis et al. (2002), may be due to their characteristic detergent effect on the soft body membranes of the molluscs.

The plant, *Clerodendron thomsonae* Balfour (Fig. 1) is of the genus Clerodendron consisting of about 400 herbs, vines, shrubs and tress of the tropics and many of which are grown as garden plants. It belongs to the family Verbenaceae, Order Lamiales. A world wide but mainly tropical grouping of about 100 genera and over 3,000 species, some of which are important for their flowers. The plant is common in the coastal region of Nigeria. It is mainly used by fishermen in killing fish in a primitive method of fishing. This is achieved

by macerating the leaf of the plant after which it is sprinkled in the river thereby causing marked behavioural change in the fish. This is characterized by restlessness, fast swimming, occasional jumping and surfacing responses in the fish. The fish die after about 15 minutes of the application of the plant in water. Among the Ikweres in the south – south region of Nigeria, the plant is called "Egwaro".

Materials and Methods

Collection of plant materials

The plant leaf of *Clerodendron thomsonae* Balfour. was collected from two different localities: "Alakahia" and "Aluu" in Rivers State, Nigeria. The plant samples were thoroughly examined to ensure that they were disease-free and then authenticated by the Herbarium section of the Plant Science and Biotechnology Department of the University of Port Harcourt. Nigeria. The identity of the plant was also confirmed at the forestry Research Institute of Nigeria, Jericho, Nigeria.

Treatment of plant material

After the separation of the plant into various parts, the leaves were subjected to standard heat treatment using the Cosair oven at 80°C for 10 mins prior to rapid drying at 60°C (Joslyn 1970). Further drying was carried out in the sun until it was completely dried. The drying was carried out in order to inactivate the glycosidases present in the leaves which are capable of destroying the saponin activity in the fresh plant tissues. The dried leaves were ground with a clean mortar and pestle and further milled into a fine powder using an electric blender (Moulinex) and made to pass through a 0.25 mm sieves (Ende-cotts (Test sieves) Ltd, England).

Preparation of methanol extract

Aliquots of the ground sample was weighed into whatman paper thimbles (60 mm x 26 mm) and plugged with glass wool. Each aliquot of the sample and glass wool was then covered by a strip of aluminum foil and held in place by a strip of copper wire. The sample (50 g in all) was extracted by glass soxhlet (intermittent with Allihn type of condenser) in 500 ml of methanol (b. pt. 64°C - 65°C) for 24hr.

Preparation of water extract

50g of the plant sample was extracted with 500 ml portion of distilled water using the cold extraction method in a percolator (2L), the tap was plugged with a glass wool and left overnight for extraction with occasional shaking. The extract was then collected by running off the extract through the tap.

Preparation of phosphate-buffered saline (PBS) extract

0.5 g of the plant sample was extracted by macerating with 25 ml PBS using glass mortar and pestle and boiled in boiling water for 30 min. The temperature of the water bath was maintained at 100°C. It was then filtered into 100 ml conical flask, stoppered with cotton wool. The PBS extract was stored at 4°C in a refrigerator until use.

Preparation of methanol/phosphate buffered saline (M/PBS) extract

0.5 g portion of the plant sample was extracted by macerating with 25 ml methanol using a glass mortar and pestle and then transferred into a 100 ml conical flask, stoppered with cotton wool. It was boiled in boiling water for 10 min at 65°C with occasional shaking at 5 min intervals. The temperature of the water bath was maintained at 65°C. After 10 min, it was filtered hot and the methanol filterate evaporated slowly and the residue resuspended in 25 ml PBS. The resulting extract (M/PBS) was then stored at 4°C until use.

Foam forming activities of PBS and M/PBS extracts

The foam forming activity was determined in quadruplicates using a stoppered test tube (17 mm x 175 mm; O'Dell et al. 1959; Oyedapo et al. 1999).

Treatment of erythrocytes

Fresh venous blood was collected from healthy donors into a clean sterile tube containing EDTA (5 mg/8 ml blood) and used within 24 hr. Centrifugation was carried out at 3,000 rpm for 5 min. The supernatant was discarded using Pasteur pipette. Packed cells were washed five (5) times with PBS at 2% (v/v) and stored at 4°C until use (Ralston 1976).

Haemolytic assay

The method used was an adaptation of the Haemagglutination procedure of Gordon and Marquardt (Gordon and Marquardt 1974). Using micro titre plates (Sever 1962) and then modified by Sodipo (Sodipo and Tizhe 1991). Haemolytic assay were conducted by a two – fold serial dilution of the different extracts (PBS and M/PBS) using U-shaped bottom micro titre plates with 2% (v/v) erythrocytes suspension (ABO). PBS (25 µl) was added to each hole 2 to 12, and 50 µl of PBS or M/PBS extract to hole 1, using a dropper. Serial 1:2 dilution was then carried out using micro-dilution by transferring 25 µl from hole 1 into hole 2 through to hole 11 leaving 12 as control and undiluted. The micro diluters were washed with distilled water, rinsed with PBS and dried on a piece of tissue before carrying out fresh titration. The diluter was twiddled and drawn up the side of the hole in hole 1 to dislodge any sample on the outer side of the diluter and then put into hole 2, twiddling and drawing up the side of the hole 1. This procedure was repeated in all the holes up to hole 11, leaving hole 12 in each case undiluted, serving as the control. 25 µl

Table 1. Results of phytochemical screening.

Test	Results
Alkaloids:	
Wagner's reagent	+
Mayer's reagent	+
Tannins	-
Phlobatannins	-
Anthraquinones	-
Combined – Anthraquinones	-
Anthracene	-
Flavonoids:	+
Glycosides	+
Saponin glycosides	+
Lieberman-Burchard	+
Salkowski's	+
Keller-Killian's	-
Foam formation	+
Emulsion	+
$SbCl_3$	-

Table 2. Foam-forming activity of PBS and M/PBS extract of *Clerodendron thomsonae*.

Extract	Foam-forming Activity	
	Foam height (mm)	Foaming time (hr)
PBS	9.25 ± 0.40	20.93 ± 0.56
M/PBS	3.75 ± 0.11	27.37 ± 0.04

Results are the mean value of 4 determinations in each case ± SEM

of sample was transferred by micro diluter and mixed in holes with 25 µl PBS, the dilution for example in holes 2 to 6 was 1:2; 1:4; 1:8; 1:16 and 1:32 respectively. The dilution in hole 11 was 1:1024. After dilution, a drop of treated erythrocyte was added into each hole (holes 1 to 12) and incubated at room temperature to give a sedimentation pattern. The sedimentation patterns of the erythrocyte in the undisturbed plates were read after incubating for 2 hr at room temperature (30°C) to determine the titre. A positive pattern indicating full haemolysis (FH) appeared as a circular big spot of red solution surrounded by clear zone (if any), while a negative pattern indicating no haemolysis appeared as a uniform small spot of erythrocytes at the bottom of the well, surrounded by a big concentric clear zone. In some micro titrations where a positive pattern was observed, the clump of erythrocytes was rather large and non-uniformed spot formed. In such cases, a partial haemolysis (PH) was recorded.

Phytochemical screening

The methanolic extract fraction was assayed for the presence of secondary metabolites using standard procedures (Sofowora 1993; Oyedapo et al. 1999). (a) For alkaloids, 0.1 g of the extract was stirred in 10%(v/v) HCl on a steam bath followed by filtration. The filtrate (1ml) was mixed with a few drops of Mayer's reagent. To another 1ml of the filtrate was added few drops of Wagner's reagent. The mixtures were observed for turbidity or formation of precipitate. (b) Saponins were screened by dissolving 0.1 g of the extract in 2 ml of distilled water, with vigorous shaking until froth appeared. The tubes were warmed for 10 min. in a water bath. The presence or absence of frothing was noted after warming. (c) For tannins, 0.1 g of the extract was taken up in 10 ml distilled water, and filtered. Then, a few drops of ferric chloride reagent were added to the filtrate. The mixture was observed for the formation of blue, blue-black, green or green-black colouration or precipitate. (d) Tests for flavonoids involved (i) suspending 0.1 g of the extract in 5ml ethanol, followed by shaking and filtering. To 1ml of the filtrate was added a few drops of 0.5N alcoholic KOH .The mixture was observed for yellowish suspension or precipitate. (ii) (0.1 g) of the extract was suspended in 5 ml of ethylacetate, shaking vigorously and filtered. To 1ml of the filtrate was added few drops of dilute ammonia solution. The alkaline layer was observed for turning light or deep brown. (e) Cardiac glycosides were screened by dissolving (0.1 g) of the extract in 5 ml chloroform followed by filtration. Concentrated sulphuric acid was carefully layered at the bottom of the tube without disturbing the solution. It was observed for the formation of a sharp brown ring at the chloroform/sulphuric acid interface

Determination of melting point

A few milligram of the sample was forced down through the open end of a clean capillary tube towards the sealed end. The packed tube was then inserted into the heating bore of a melting point apparatus. Having regulated the temperature rise to be gradual, the temperature switch was switched on. The sample went through various stages of physical changes such as:

First change of sign (darkening)
First liquid formation
Meniscus formation and finally
Complete liquid formation.

All these changes were viewed through a magnifying glass. The temperature range over which this physical changes occurred was determined and recorded as the melting point.

Results

Phytochemical screening of the leaf of *Clerodendron thomsonae* Balfour showed the presence of secondary metabolites (Table 1). However, it was devoid of tannins, Phlobatannins, Anthraquinones, combined- anthraqunones and Anthracene (Table 1).

Foam- forming activity carried out revealed that the PBS extract had a higher foam height when compared with the

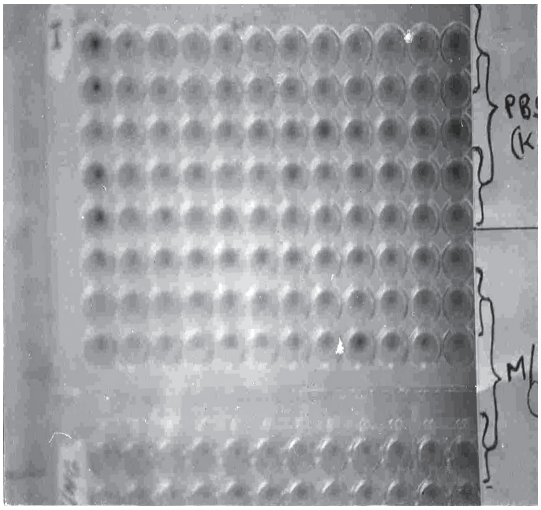

Figure 2. The Haemolytic pattern of PBS and M/PBS of *Clerodendron thomsonae* Balfour.

Table 3. Determination of haemolytic activity of crude saponin extracts (PBS and M/PBS).

Extract	Pattern of Haemolysis	Titre		
		A	B	O
PBS	FH	2^8	2^7	2^0
	PH	2^9	2^9	2^{10}
M/PBS	FH	2^4	2^6	2^0
	PH	2^9	2^{10}	2^3

Titre is defined as the reciprocal of greatest dilution at which haemolysis occurred.
All values are mean of duplicate determination.
FH – Full haemolysis, PH – Partial haemolysis.

Table 4. Results of determination of melting point.

Extract	°C
Aqueous	288-315
Methanol	125-240

M/PBS extract. However, the M/PBS extract showed a longer foaming time when compared to the PBS extract (Table 2). The results of the haemolytic assay carried out on both the PBS and M/PBS extract of the leaf of the plant *Clerodendron thomsonae* Balfour. were found to posses haemolytic activity with different human erythrocytes (Table 3). Partial and Full haemolysis for both PBS and M/PBS extracts were observed with human erythrocytes (Fig. 2). The result obtained from haemolytic activity of the PBS extract revealed that blood group O recorded the least titre while blood group A recorded the highest titre with respect to full haemolysis (FH). However, in the partial haemolysis result of PBS extract, blood group O recorded the highest titre, whereas the same but lower titre was recorded for both blood group A and B (Table 3). The M/PBS extract results obtained for full haemolysis (FH) showed that blood group B recorded the highest titre while blood group O recorded the lowest titre. Blood group B also recorded the highest titre with respect to partial haemolysis (PH) while blood group O recorded the lowest titre (Table 3).

The results of the determination of melting point (m. pt) showed the melting point range of both methanol and aqueous extract of the leaf of *Clerodendron thomsonae* Balfour. The result revealed that the aqueous extract had a higher melting point range than the methanol extract (Table 4).

Discussion

The phytochemical screening of the leaf extract of *Clerodendron thomsonae* Balfour. was studied for the presence of various secondary metabolite among which is saponin. The result obtained from this research showed the presence of saponin in the leaf. Further confirmatory test for saponins were achieved in view of the positive responses to Fehlings solution. The sugar portion were most probable those of saponins, since it is known that a particular saponin can contain as many as twelve different sugar or saccharic acid units (Kitagawa et al. 1975). These sugars are regarded as united and in a straight chain with the terminal unit attached to a OH guinyl of the sapogenin through glycosidic linkage (Birk 1969).

The result of the study showed that the PBS and M/PBS extract of the leaf of *Clerodendron thomsonae* Balfour. not only foamed copiously but also formed foams that lasted for a considerable length of time. The PBS extracts had a higher foam height of 9.25 ± 0.4 mm while that of the M/PBS extract was 3.75 ± 0.11 mm. The foaming time for PBS extract was 20.93 ± 056 while M/PBS extract had a foaming time of 27.37 ± 0.04 hr. This result is in agreement with Sodipo and Mohammed (1990), Sodipo and Tizhe (1991) that the foaming time is not always proportional to the foaming height.

Furthermore, the PBS and the M/PBS extract of the leaf were found to posses haemolytic activity using different human erythrocytes. Generally, the results obtained with blood group O showed a very mild activity. However, results of the haemolytic activity observed with both PBS and M/PBS extract could be attributed to the saponin content of the plant extract (James 1964; Newberne 1980; Khalil and El-Adawy 1994). This toxicologically interesting property of many saponins to bring about haemolysis *i.e.* the release of haemoglobin from erythrocytes is as a result of change in membrane permeability. This is considered to be influenced by the affinity of the aglycone to cholesterol in cell membranes (Glauert 1962; Frobbe 1992). 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 Khalil and El-Adawy (1994) and Oda et al. (2000) where different saponins showed different levels of haemolytic activities. This observation can also be correlated to the findings of Fenwick and Oakenfull (1981) that a certain plant and even part of the same plant may contain different saponins which can differ in biological features. However, this haemolytic activity only takes place on parenterial administration since by mouth there is usually only limited absorption. Walter et al. (1955) reported that Ladino Clover saponin is not toxic to fish and does not haemolyse red blood cells. This could have been the reason for the titre result obtained in full haemolysis of both PBS and M/PBS extract of blood group O where a titre of 2^0 (no haemolysis) was recorded.

The results of determination of melting point (m.pt) revealed that the aqueous extract had a higher melting point than the methanol extract. The observation simply showed that results were obtained as mixtures of various compounds hence the melting point range between 288 to 315°C and 125 to 240°C for both aqueous and methanol extract respectively. This observation is in agreement with the findings of Shi et al. (2004) that saponins constitute a complex and chemically diverse group of compounds. Melting points normally indicate the extent of purity of substances. A pure sample usually has a sharp and definite melting point while an impure or mixed sample will have a melting point range due to the differences in melting points of the substances present in the mixture.

The ability of the leaf of *Clerodendron thomsonae* to kill fish on application in water in a similar manner to *Teminalia tomentosa* could also be attributed to the presence of arjunolic acid in the saponins of leaf of *Clerodendron thomsonae* Balfour.

The presence of saponin earlier detected by the ability of the aqueous extract to form a stable froth when vigorously shaken and also the ability to haemolyse red blood cells was eventually confirmed in view of the positive responses to Fehling's solution. Again, the steroidal portions were detected by the Lieberman-Burchard's and Salkowski's reagents. The haemolysin responsible for the haemolytic activity of the plant extract could be attributed to the presence of saponin. However, for a more conclusive and unambiguous identification of saponins, purification and structural elucidation exercises should be carried out. These should form major components of future research efforts.

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