

Phytochemical screening and comparative study of the effects of serial extracts of *solanum aculeastrum* seeds on serum lipid profile of testosterone propionate induced benign prostatic hyperplasia in male wistar rats

Joseph Anna Ubon^{1*}, Monday I. Akpanabiatsu², Justina I. Udotong³ and Udeme Ededem Okon⁴

^{1*}Research Scholar ²Professor of Biochemistry ³Senior Lecturer ⁴Research Scholar

^{1*} Department of Biochemistry, Faculty of Basic Medical Sciences, College of Health Sciences, University of Uyo, Akwa Ibom State, P.M.B. 1017, Uyo, Akwa Ibom

State, Nigeria.

^{2,3}Department of Biochemistry, Faculty of Science, University of Uyo, Akwa Ibom State, P.M.B. 1017, Uyo, Akwa Ibom State, Nigeria.

⁴Department of Clinical Biochemistry, Faculty of Natural and Applied Sciences, Michael Okpara University of Agriculture, Umudike, Abia State, P.M.B. 7267, Umuahia, Abia State, Nigeria.

ABSTRACT

The phytochemical analysis and serum lipid profile of male Wistar rats following oral administration of serial extracts of *solanum aculeastrum* seeds were evaluated and compared. Finely ground *Solanum aculeastrum* seeds (1000 g) were extracted with hexane, chloroform, benzene, ethylacetate and ethanol respectively using serial exhaustive extraction technique. Male Wistar rats weighing 280 ± 20 g were injected with 10 mg/kg body weight of Testosterone Propionate through intraperitoneal route for twenty eight days to induce BPH. The animals were divided into eight (8) groups of six (6) rats each. Group 1 (Normal control) was not induced with BPH and served as normal control, group 2 was induced and not treated and served as BPH control, group 3 (Finasteride control) was induced and received standard drug, finasteride while groups 4 (Hexane extract treated group), 5 (Chloroform extract treated group), 6 (Benzene extract treated group), 7 (Ethylacetate extract treated group) and 8 (Ethanol extract treated group) were induced and treated orally with 300 mg/kg body weight of hexane, chloroform, benzene, ethylacetate and ethanol extracts respectively for twenty eight days. The animals were sacrificed and blood collected through cardiac puncture. Biochemical studies were conducted using standard procedures. Steroids, alkaloids, flavonoids, phenols, tannins, cardiac glycosides, coumarins, terpenoids, proteins and reducing sugars were detected. The yield (g/ 100 g) of flavonoids was maximum (11.07), followed by phenols (0.327). TC, TG and VLDL-C levels were not significantly altered except in chloroform extract where significant increase in LDL-C levels was observed. Hexane extract exhibited the best effect compared to the standard drug. These results suggest that all the serial extracts may be safe for use in the management of BPH and support its therapeutic use by the herbalists in treating inflammatory diseases.

Keywords: *Solanum aculeastrum*, BPH, Lipid profile, Phytochemicals, Wistar rats.

1. INTRODUCTION

Natural products have proven to be a rich source of therapeutic agents both in folk medicine and scientific ethno-botanical studies due to their phytochemicals. Phytochemicals are potent bioactive compounds found in medicinal plant parts and are precursors for the synthesis of useful drugs (Sofowora, 1993). Herbal medications work through the synergicity of its phytochemicals. The qualitative and quantitative estimation of the phytochemical constituents of a medicinal plant is considered to be an important step in medicinal plant research (Kokate, 1994). Progress in phytochemical research has been aided enormously by the development of rapid and accurate methods of screening plants for particular chemicals (Banso and Adeyemo, 2007).

Solanum aculeastrum (Solanaceae) commonly known as *Omotobo* by the *Abagusii* community of Kenya is also known as soda apple or goat bitter apple or poison apple (Laban *et al.*, 2015). In Nigeria, the *Efik/Ibibios*, the fourth largest ethnic group in the country, it is commonly referred to as *Nditot Ekpo* or *Nkejhe nditot*. The species name *aculeastrum* refers to the thorns that adorn most parts of the shrub. It is a shrub or small tree native to tropical Africa down to South Africa. It grows in a wide range of soil terrain and climatic conditions (Koduru *et al.*, 2006b; Iweala and Ogidigo, 2015a). It occurs naturally in grassland, woodland and in forest margins. It has also been recorded from gentle to steep slopes on various soil types such as sandy soils, reddish brown clay-loam and brown sandy loam (Aboyade *et al.*, 2009; Aboyade *et al.*, 2010). The petals are white to pale violet and the flower has a bitter, sour smell. At maturity, the fruits or berries are about 4 to 5 cm in diameter, egg-shaped, becoming greenish-yellow when ripe (Wanyonyi *et al.*, 2003; Laban *et al.*, 2015). The fruits, both mature and immature, contain the alkaloid solanine (Hutchings *et al.*, 1996). The leaves and berries of *Solanum aculeastrum* contain mainly straight-chain aliphatic hydrocarbons (Koduru *et al.*, 2006a). Among the *Abagusii* community of Nyamira County of Kenya, the fruits and leaves of *Solanum aculeastrum* are used fresh, dried, boiled, or charred (ashed) for the treatment of jigger infestations and wounds (*Tungiasis*),

swollen joints in fingers, gangrene, toothaches, gonorrhoea, bronchitis, rheumatism and in ringworm in cattles (Koduru *et al.*, 2006a; Koduru *et al.*, 2007a; Laban *et al.*, 2015). They are also used as eyewash (Laban *et al.*, 2015). A decoction of the root bark is used in Kenya for the treatment of sexually transmitted bacterial diseases, including gonorrhoea as well as acne (Kokwaro, 2009). The *Efik/Ibibios* of Nigeria use decoction of the ripe berries for the treatment of splenomegaly (Ubon, 2019). Ethnobotanical survey revealed that the berries are used in the treatment of breast cancer (Koduru *et al.*, 2006a; Koduru *et al.*, 2007). Methanol and aqueous extracts of the berries have been shown to have moderate antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aureginosa* and *Bacillus subtilis* bacteria (Wanyonyi *et al.*, 2002; Wanyonyi *et al.*, 2003; Wabwoba *et al.*, 2010).

II MATERIALS AND METHODS

Plant Materials

Samples of ripe fruit berries of *Solanum aculeastrum* Dunal were obtained from locations in Itu Local Government Area of Akwa Ibom State in Nigeria between November, 2017 and January 2018, and authenticated by a taxonomist at the Department of Botany and Ecological Studies, University of Uyo, Uyo, Nigeria. A voucher specimen with number 'Ubon, UUH 2687 'Itu' was deposited in the herbarium of the University of Uyo, Uyo, Nigeria. The samples were washed under clean gently running tap water to remove dirt on the fruits. After the fruits were kept for 2 hrs for the water to dry off, a sharp stainless steel knife was used to cut open the fruits, in order to remove the seed. The seeds were freed from the mesocarp and pericarp and air-dried at room temperature (25 ± 2 °C) until a constant weight was obtained. After drying, the seeds were ground using a desk top grinder (Model No: QBL-18L40, Turinar Corp, Shang-Hai, China) into fine particles and stored in different plastic containers with screw cap.

Preparation of Extracts

The *Solanum aculeastrum* seeds extracts were prepared through serial exhaustive extraction technique using the modified methods of Nidal *et al.* (2015), Pandey and Tripathi (2014) and Azmir *et al.* (2013). The finely ground *Solanum aculeastrum* Dunal seeds (1000 g) were soaked in 1000 ml n-hexane at 25 °C for 24 hours in a 2000 ml separating funnel with continuous shaking. After that, the filtrate was obtained by running the tap of the separating funnel. The sample residue in the separating funnel was re-extracted with another 1000 ml n-hexane. The combined filtrate was collected and kept in a labeled pre-weighed volumetric flask at room temperature. The residue was air-dried and the process of extraction was repeated as described four more times with chloroform, n-benzene, ethylacetate and finally with ethanol. The filtrates of each solvent extraction was collected and kept in labeled weighed volumetric flasks at room temperature. The different filtrates collected in weighed volumetric flasks were separately placed in a Büchi rotary evaporator at 40 °C in order to recover the solvents, and to obtain the crude extracts. The weights of the crude extracts were determined by calculating the difference in the weights. The extracts were kept in different sterile brown bottles and stored at -4 °C in the refrigerator.

Animal Treatment

Forty eight (48) matured male Wistar rats weighing $200 - 280 \pm 20.0$ g were used in this work. The animals were obtained from the animal house, Biochemistry Department, University of Uyo, Uyo, Akwa Ibom State. The animals were housed in well ventilated cages in the experimental room at a temperature of 25 ± 4 °C and relative humidity of 65 ± 5 % with an alternating 12 hours light and dark cycle for three days to acclimatize. They were allowed access to food (grower's mash from Vital Feeds, Jos, Plateau State, Nigeria) and water *ad libitum*. All animals handling and experiments were carried out in line with the guidelines of institutional animals' ethical committee as approved by the Post-Graduate School, University of Uyo, Nigeria. Sacrifice of animals was performed under full anaesthesia and the carcasses were properly disposed by burying.

BPH Induction

Adult male Wistar rats weighing $200 - 280 \pm 20.0$ g were induced with BPH by intraperitoneal injection of testosterone propionate (10 mg/kg body weight) for twenty eight (28) days (Ejike and Ezeanyika, 2011; Iweala and Ogidipo, 2015b; Mbaka *et al.*, 2017; Cai *et al.*, 2018).

Experimental Design

The animals were weighed and randomly selected into eight (8) groups of six (6) animals each and treatment regimen conducted as shown in Table 1. All treatment lasted for twenty eight (28) days. The animals had free access to feed and water *ad libitum* throughout the period of experiment and their body weights were measured weekly throughout the period of the experiment.

Table 1: Animal Grouping and Treatment

| Group | Name | Treatment |
|-------|--|--|
| 1. | Normal Control (NC) | Normal animals + 0.40 ml Olive oil |
| 2. | BPH Control (BPHC) | BPH induced rats without treatment |
| 3. | Finasteride Control (FinC) | BPH + finasteride (5 mg/kg b. wt.). |
| 4. | Hexane Extract Treated group (HETG) | BPH + hexane extract (300 mg/kg body wt.). |
| 5. | Chloroform Extract Treated group (CETG) | BPH + chloroform extract (300 mg/kg body wt.). |
| 6. | Benzene Extract Treated group (BETG) | BPH + n-benzene extract (300 mg/kg body wt.). |
| 7. | Ethylacetate Extract Treated group (EaETG) | BPH + ethylacetate extract (300 mg/kg body wt.). |
| 8. | Ethanol Extract Treated group (OHETG) | BPH + ethanol extract (300 mg/kg body wt.). |

Animal Sacrifice and Preparation of Sera for Analysis

All experimental animals were anaesthetized using chloroform fumes 24 hours after the last administration of the extract. Blood samples for sera preparation was collected by cardiac puncture into sterile plain tubes and EDTA (0.77M) bottles for

haematological analysis. The liver, kidneys and prostates were harvested from scarified rats, washed with ice-cold saline solution (0.9% w/v), blotted, and weighed. Serum samples were extracted from the clotted blood into sterile plain tubes after centrifugation at 2000 rpm for 10 minutes using a bench top centrifuge (MSE Minor, England). The sera were stored in the refrigerator for analyses while the whole blood samples were used in determining haematological indices.

Drugs and Chemicals

All chemicals and reagents used for this research were of analytical grade and were obtained from Sigma-Aldrich, St. Louis, USA. Testosterone Propionate (TP) was obtained from Tokyo Chemical Industry, Tokyo, Japan.

Statistical Analysis

Statistical analysis was carried out using window SPSS version 23.0. One way analysis of variance (ANOVA) was adopted for comparison and results were subjected to post hoc test using Turkey multiple comparison test. The data were expressed as means \pm standard error of the mean (SEM) and values with $p < 0.05$ were considered significant.

Qualitative Phytochemical Analysis

Chemical tests for the identification of bioactive chemical constituents in *Solanum aculeastrum* Dunal were carried out in the different extracts using the standard procedures as described by Kokate (2005), Sofowara (1993), Trease and Evans (1989) and Harborne (1973).

Test for Saponins

Froth Test: About 0.5 grams of each of the extracts were separately shaken vigorously with 2 ml of distilled water in different test tubes and allowed to stand for 15 minutes. Persistent foaming or the appearance of creamy mist of small bubbles (froth) showed the presence of saponins (Harborne, 1973; Sofowara, 1993; Tiwari *et al.*, 2011).

Test for Alkaloids

About 0.5 grams of each of the extracts were successively dissolved in 10 ml of 2 % dilute hydrochloric acid (0.054M HCl), heated for 5 minutes and then filtered. Each of the filtrates (1 ml) was pipette into a test-tube and used to test for the presence of alkaloids as follows:

Mayer's Test: Mayer's reagent (Potassium mercuric iodide solution) was added successively to 1 - 2 ml of each of the filtrates and observed for 15 minutes. Formation of yellow precipitate showed the presence of alkaloids.

Wagner's Test: Wagner's reagent (Solution of iodine in potassium iodide) was added successively to 1 - 2 ml of each of the filtrates and observed for 15 - 20 minutes. The formation of brown or reddish brown precipitate indicates the presence of alkaloids.

Tannic Acid Test: About 2 - 3 drops of 10 % Tannic acid solution (0.200 g tannic acid/1000 ml of deionized water) reagent was successively added to 1.0 ml of each of the extract. The appearance of amorphous or crystalline precipitate showed the presence of alkaloids.

Test for Flavonoids

Preparation of Test solution: About 500 mg of each extract was dissolved in 100 ml of the respective solvent and then filtered through whatman filter paper No.1. Thus, the filtrates obtained were used as test solutions for the following preliminary screening tests.

Sulphuric Acid (H₂SO₄) Test: Dilute aqueous ammonia (5 ml) was added to 2 ml of each of the extracts followed by 1 ml of 2M H₂SO₄. An orange or yellow colouration that disappears on standing for 10 - 15 minutes indicates the presence of flavonoids.

Lead Acetate Test: About 2 ml of 10 % lead acetate solution was successively added to 2 ml of extracts. Appearance of a yellow colour precipitation indicates the presence of flavonoids.

Sodium Hydroxide (NaOH) Test: About 2 ml of each of the extracts was taken in a test tube and 1 ml of 1N NaOH solution was added. The formation of yellow colour which decolourizes on addition of few drops of 0.1M HCl indicates the presence of flavonoids (Santhi and Sengottuvel, 2016).

Test for Phenols

Ferric Chloride (FeCl₃) Test: About 0.5 ml of FeCl₃ (w/v) solution was successively added to 2 ml of the extracts and the formation of bluish black colour indicates the presence of phenol (Santhi and Sengottuvel, 2016).

Lead Acetate Test: About 0.5 ml of 10 % lead acetate (w/v) solution was successively added to 2 ml of the extracts and the formation of a yellow coloured precipitate indicates the presence of phenol.

Test for Tannins

Ferric Chloride (FeCl₃) Test: About 0.2 g of each of the extracts were dissolved in 10 ml of the respective solvents and then filtered through Whatman filter paper No.1. To about 5 ml of each of the extracts in a test tube, 1 ml of 5 % FeCl₃ in pyridine was added. The formation of dark green colour indicates the presence of tannin (Santhi and Sengottuvel, 2016).

Lead Acetate Test: To about 2 - 3 ml each of the extracts in a test tube, 2 ml of 1 % lead acetate solution were successively added. The formation of a yellowish precipitate indicates the presence of tannin.

Test for Cardiac Glycosides

Keller-Killani Test: About 0.2 g of each of the extracts were dissolved in 10 ml of the respective solvent and then filtered through Whatman filter paper No.1. Glacial acetic acid (1 ml) was carefully added to 2 ml of each of the filtrates obtained and cooled. Thereafter, 1 ml of 1 % FeCl₃ solution was added and the contents were transferred carefully to a test tube containing 2 ml of concentrated H₂SO₄. A reddish brown ring was observed at the junction of two layers.

Test for Anthraquinones

Borntrager's Test: About 0.5 g of each of the extracts was successively boiled with 5 ml of 10 % H₂SO₄ and filtered. Each of the filtrates was shaken with 2.5 ml of benzene, the benzene layer was separated and half its own volume of 10 % NH₄OH added. A pink, red, or violet colouration in the ammonia phase showed the presence of anthraquinones.

Test for Cyanide

Picric acid Test: About 0.5 g of each of the extracts were dissolved in 10 ml of their respective solvents and then filtered through Whatman filter paper No.1. Few drops of 40 % picric acid (a yellow compound) were added to 2 ml of each of the filtrates obtained. The formation of a reddish brown precipitate of isopurpuric acid indicates the presence of volatile hydrogen cyanide (HCN).

Test for Steroids

Salkowski's Test: About 0.5 g of each of the extracts were dissolved in 10 ml of their respective solvents and then filtered through Whatman filter paper No.1. Chloroform (2.0 ml) and 2.0 ml of concentrated H₂SO₄ was carefully added to 2.0 ml of each of the filtrates and shaken. The formation of a red colour by the chloroform layer and a greenish yellow fluorescence by the acid layer indicates the presence of steroids.

H₂SO₄ Test: About 0.5 g of each of the extracts were dissolved in 10 ml of their respective solvents and then filtered through Whatman filter paper No.1. Acetic anhydride (2.0 ml) was added to 2.0 ml of each of the filtrates followed by 2.0 ml of a 0.5M H₂SO₄. A change in colour from violet to blue or green indicates the presence of steroids.

Test for Terpenoids

Salkowski's Test: About 0.5 g of each of the extracts were dissolved in 10 ml of their respective solvents and then filtered through Whatman filter paper No.1. Thereafter, about 2.0 ml of chloroform and 3.0 ml of concentrated sulphuric acid (2M H₂SO₄) was carefully added. The formation of a reddish brown layer in the inner phase indicates the presence of terpenoids.

Test for Glycosides

Borntrager's Test: About 0.5 g of each of the extracts was successively boiled with 5 ml of 10 % H₂SO₄ and filtered. Each of the filtrates was shaken with 2.5 ml of benzene, the benzene layer was separated and half its own volume of 10 % NH₄OH added. The formation of a pink colour indicates the presence of glycosides.

Test for Coumarins

Sodium Hydroxide (NaOH) Test: About 0.5 g of each of the extracts were dissolved in 10 ml of their respective solvents and then filtered through Whatman filter paper No.1. Thereafter, about 3 ml of 10 % NaOH was successively added to 2 ml of each of the filtrates. The formation of a yellow colour indicates the presence of coumarins.

Test for Anthocyanins

About 0.5 g of each of the extracts were dissolved in 10 ml of their respective solvents and then filtered through Whatman filter paper No.1. Thereafter, 2 ml of 2N HCl and aqueous ammonia was successively added to 2 ml of each of the filtrates. The formation of pink red colour which turns blue violet indicates the presence of anthocyanins.

Test for Proteins

Biuret Test: To about 0.5 mg of each of the extracts, equal volumes of 40 % NaOH solution and two drops of 1 % copper sulphate solution was added. The formation of a violet colour indicated the presence of protein.

Xanthoproteic Test: To about 1 ml of each of the extracts was added 1 ml of concentrated H₂SO₄. The formation of a white precipitate which turns to yellow on boiling and to orange on addition of NH₄OH indicated the presence of protein.

Ninhydrin Test: To about 0.5 mg of each of the extracts, two drops of 0.25 % Ninhydrin reagent (indane-1,2,3-trione) was added and heated for few minutes. The formation of a pink or purple colour indicated the presence of proteins.

Test for Carbohydrates

Fehling's Test: Equal volumes of Fehling's solutions A (copper sulphate in distilled water) and B (potassium tartarate and sodium hydroxide in distilled water) were successively added to 2 ml of each of the extracts in a test tube which was placed in a boiling water bath for few minutes. Upon boiling, the contents of the test tubes were mixed together. The formation of a brick red precipitate was considered as an indication for the presence of reducing sugars (carbohydrates).

Benedict's test: About 2 ml of Benedict's reagent (an alkaline solution of sodium carbonate, sodium citrate and copper sulphate) was added to 2 ml of each of the extracts and mixed properly. The test tube was placed in a boiling water bath for 5 minutes and allowed to cool. The formation of a reddish brown precipitate was considered as an indication of the presence of carbohydrates.

Tollen's Test: About 2 - 3 ml of Tollen's reagent (ammoniacal silver nitrate solution) was added to 2 ml of each of the extracts in a test tube and kept in a boiling water bath for ten minutes. The formation of a shining white silver mirror at the sides of the test tube indicates the presence of (a reducing sugar) carbohydrates.

Quantitative Phytochemical Analysis**Estimation of Alkaloids**

Alkaloid determination was done using the method of Harborne (1973). The powdered sample (1.0 g) was weighed into a 250 ml beaker and 200 ml of 10 % acetic acid in ethanol was added and covered and allowed to stand for 4 hours. It was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated NH₄OH was added by drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH₄OH and then filtered. The residue is the alkaloid, which was dried and weighed.

Calculation:

$$\text{Gram \% of alkaloid} = \frac{\text{Weight of alkaloid}}{\text{Weight of sample used}} \times \frac{100}{1}$$

Determination of Saponins

The ground sample (2.0 g) was placed in a 500 ml flask. 300 ml of 50 % alcohol were added and boiled under reflux for 30 minutes and filtered while hot through a filter paper. 2.0 g of charcoal were added to the filtrate, boiled and filtered while hot.

The filtrate was cooled and an equal volume of acetone was added to completely precipitate the saponin. The precipitated saponin was collected by decantation and dissolved in small amount of boiling 95 % alcohol and filter while hot. The filtrate was cooled

to room temperature to separate the saponin in a relatively pure form. The clear supernatant fluid was decanted and the saponins suspended in about 20 ml of alcohol and filtered. The filter paper was transferred to a dessicators containing anhydrous calcium chloride and left to dry and weighed.

Calculation

$$\text{Gram \% of saponin} = \frac{\text{Weight of saponin}}{\text{Weight of sample used}} \times \frac{100}{1}$$

Estimation of Flavonoids

The powdered sample (2.0 g) was repeatedly extracted with 100 ml of 80 % aqueous methanol at room temperature. The mixture was filtered through a Whatman No.1 filter paper into a pre-weighed 250 ml beaker. The filtrate was transferred into a water bath and allowed to evaporate to dryness and the residue was weighed (Krishnaiah *et al.*, 2009).

Calculation:

$$\text{Gram \% of flavonoids} = \frac{\text{Weight of flavonoids}}{\text{Weight of sample used}} \times \frac{100}{1}$$

Determination of Total Tannins by Spectrophotometric Method

The total tannins were determined by Folin-Ciocalteu method (Alhakmani *et al.*, 2013). About 0.1 ml of the sample extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of FolinCiocalteu phenol reagent, 1 ml of 35 % sodium carbonate solution and diluted to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 minutes. A set of reference standard solutions of tannic acid (20, 40, 60, 80, 100 µg/ml) were prepared in the same manner as described earlier. Absorbance for test and standard solutions were measured against the blank at 700 nm with an UV/Visible spectrophotometer. The estimation of the tannin content was carried out in triplicate. The tannin content was expressed in terms of mg of tannic acid equivalents/grams of dried sample.

Calculation

$$\text{Tannin (mg/100g)} = \frac{\text{Abs. of sample}}{\text{Abs. of std}} \times \frac{\text{Conc. of std}}{\text{Wt. of sample}} \times \text{dilution factor} \times \frac{100}{1}$$

Estimation of Total Phenols by Spectrophotometric Method

The powdered sample (2.0 g) was defatted with 100 ml of diethylether using a Soxhlet apparatus for 2 hours. The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. Five ml of the extract was pipetted out into a 50 ml flask, then 10 ml of distilled water was added. Two ml of NH₄OH solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark and left to react for 30 minutes for colour development and the absorbance was read at 505 nm.

Calculation

$$\text{Phenol (mg/100g)} = \frac{\text{Abs. of sample}}{\text{Abs. of std}} \times \frac{\text{Conc. of std}}{\text{Wt. of sample}} \times \text{dilution factor} \times \frac{100}{1}$$

Determination of Cardiac Glycosides

The plant extract (8.0 ml) was transferred to a 100 ml volumetric flask and 60 ml of H₂O and 8 ml of 12.5 % lead acetate were added, mixed and filtered. Fifty (50) ml of the filtrate was transferred into another 100 ml flask and 8 ml of 47 % Na₂HPO₄ were added to precipitate excess Pb²⁺ ion. This was mixed and completed to volume with water. The mixture was filtered twice through same filter paper to remove excess lead phosphate. 10 ml of purified filtrate was transferred into clean Erlenmeyer flask and treated with 10 ml Baljet reagent (95 ml picric acid and 5 ml aqueous sodium hydroxide solution). A blank titration was carried out using 10 ml distilled water and 10 ml Baljet reagent. This was allowed to stand for one hour for complete colour development. The colour intensity was measured colorimetrically at 495 nm.

Calculation:

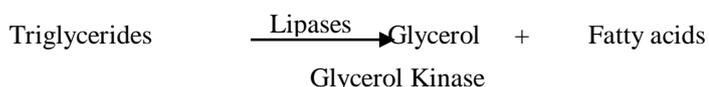
$$\text{Cardiac glycosides (grams \%)} = \frac{\text{Abs.}}{77} \times \frac{100}{1}$$

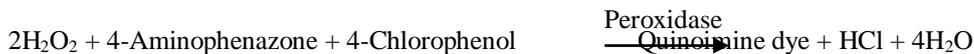
Biochemical Estimation of Triacylglycerol Using Randox Laboratories Assay Kit

Triacylglycerol in the serum were estimated based on the method described on the manufacturer's manual.

Assay Principle

Triacylglycerol in the sample were hydrolysed by the enzyme lipase. The glycerol concentration was determined by enzymatic assay coupled with Trinder reaction that terminates in the formation of quinoneimine dye formed from H₂O₂, 4-aminophenazone and 4-chlorophenol under catalytic influence of peroxidase. The amount of dye formed as determined by its absorbance at 546 nm, is directly proportional to the concentration of triacylglycerol in the sample.





Procedure

The reagents provided were pipetted into three test tubes marked blank, standard and test. The components were mixed and incubated at 37 °C for 5 minutes and the absorbance of sample (A_{sample}) and standard (A_{standard}) against blank reagent measured within 60 minutes read at 520 nm. The amount of triacylglycerol in the sample was determined using the relationship.

$$\text{Triacylglycerol (mmol/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \frac{\text{Conc.of Std}}{1}$$

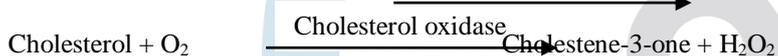
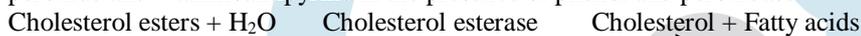
Where standard concentration = 2.23 mmol/l

Biochemical Estimation of Total Cholesterol Using Randox Laboratories Assay Kit

Serum total cholesterol concentrations in the samples were estimated based on the enzymatic endpoint method described on the manufacturer's manual.

Assay Principle

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.



Procedure

The samples and reagents provided were pipetted into three test tubes marked blank, standard and test. The components were mixed and incubated at 37 °C for 5 minutes and the absorbance of sample (A_{sample}) and standard (A_{standard}) against blank reagent measured within 60 minutes read at 564 nm. The amount of total cholesterol in the sample was determined using the relationship.

$$\text{Cholesterol (mmol/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \frac{\text{Conc.of Std}}{1}$$

Where standard concentration = 5.44 mmol/l

Estimation of High density lipoprotein (HDL) cholesterol using Randox Laboratories Assay Kit

Serum high density lipoprotein cholesterol concentrations in the samples were estimated based on the method described on the manufacturer's manual.

Assay Principle

Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL (high density lipoproteins) fraction which remains in the supernatant is determined.

Procedure

The samples, standard and diluted precipitant were pipetted into three centrifugation tubes, mixed and allowed to sit for 10 minutes at room temperature (25 °C). They were centrifuged for 10 minutes at 4000 rpm. The clear supernatants were separated within two hours. About 500 µl of the cholesterol standard was added to 50 µl each of the blank, standard and sample supernatant mixed and incubated at 37 °C for 5 minutes. The absorbance of sample (A_{sample}) and standard (A_{standard}) against blank reagent measured within 60 minutes read at 500 nm. The amount of HDL Cholesterol in the sample was determined using the relationship.

$$\text{HDL Cholesterol (mmol/l)} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \frac{\text{Conc.of Std}}{1}$$

Where standard concentration = 2.27 mmol/l

Estimation of LDL and VLDL Cholesterol

Low density lipoproteins (LDL) and very low density lipoprotein (VLDL) Cholesterol were estimated by the standard formula:

$$\text{VLDL} = \frac{\text{Triacylglycerol}}{5}$$

$$\text{LDL cholesterol} = \text{Total cholesterol} - (\text{HDL cholesterol} + \text{VLDL cholesterol})$$

III. RESULTS

Qualitative Phytochemical Analysis

The results of the qualitative studies of the fractions show that eleven (11) phytochemicals: saponins, steroids, alkaloids, flavonoids, phenols, tannins, cardiac glycosides, coumarins, terpenoids, proteins and reducing sugars were detected in all the fractions (Table 1). Cyanide, anthroquinones and anthocyanins were not detected in any of the fractions. Saponins was absent in chloroform, benzene and ethylacetate fractions. Amino acids and proteins were not detected in hexane, chloroform, benzene and ethylacetate fractions.

Table 1: Qualitative phytochemical screening of serial extracts of *Solanum aculeastrum* seeds

| Phytochemicals | Hexane | Chloroform | Benzene | Ethylacetate | Ethanol |
|--|--------|------------|---------|--------------|---------|
| Saponin | | | | | |
| a. Froth's test | + | - | - | - | + |
| Alkaloids | | | | | |
| a. Mayer's test | + | + | + | + | + |
| b. Wagner's test | + | + | + | + | + |
| c. Tannic acid test | + | + | + | + | + |
| Flavonoids | | | | | |
| a. H ₂ SO ₄ test | + | + | + | + | + |
| b. Lead Acetate test | + | + | + | + | + |
| c. NaOH test | + | + | + | + | - |
| Phenols | | | | | |
| a. FeCl ₃ test | + | + | + | + | + |
| b. Lead Acetate test | + | + | + | + | + |
| Tannin | | | | | |
| a. FeCl ₃ test | + | + | + | + | + |
| b. Lead Acetate test | + | + | + | + | + |
| Cyanide | | | | | |
| a. Picric acid test | - | - | - | - | - |
| Steroids | | | | | |
| a. Salkowski's test | + | + | - | - | + |
| b. Conc. H ₂ SO ₄ test | + | + | - | - | + |
| Cardiac glycosides | | | | | |
| a. Keller-Killani's test | - | + | + | + | + |
| Glycosides | | | | | |
| a. Borntrager's test | + | + | + | + | + |
| Coumarins | | | | | |
| a. Cupric Acetate test | + | + | + | + | + |
| Anthocyanins | | | | | |
| Terpenoids | + | + | + | + | + |
| Proteins | | | | | |
| a. Xanthoproteic test | - | - | - | - | - |
| b. Ninhydrin test | - | + | - | - | + |
| c. Biuret's test | - | - | - | - | - |
| Carbohydrate | | | | | |
| a. Fehling's test | + | + | + | + | + |
| b. Benedict's test | + | + | + | + | + |
| c. Tollen's test | - | - | + | - | - |
| Anthroquinone | | | | | |
| a. Borntrager's test | - | - | - | - | - |

+ = detected; - = absent or below detectable concentration.

Quantitative Phytochemical Analysis

The air dried seeds of *Solanum aculeastrum* contains a significant amount of alkaloids, flavonoids, phenols, saponins, cardiac glycosides and tannin content (Table 2). The flavonoids contents (g/100 g) was the highest (11.07), followed by phenols (0.327), tannins (0.310), saponins (0.236), alkaloids (0.0974) and cardiac glycosides (0.0511).

Table 2: Quantitative phytochemical analysis of *Solanum aculeastrum* seeds

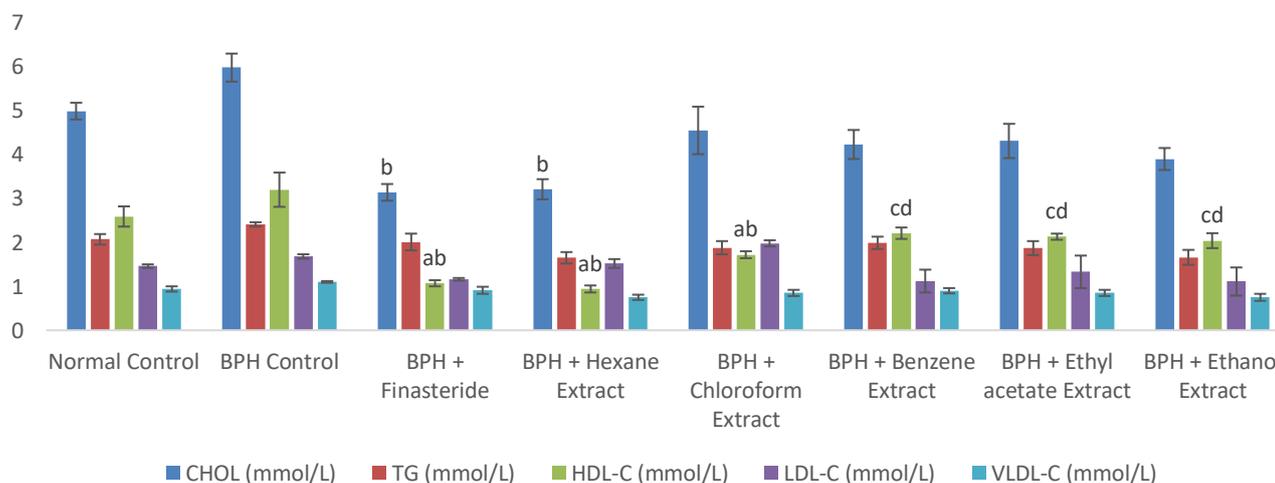
| Phytochemicals | Percentage composition (g /100 g) |
|----------------|--------------------------------------|
|----------------|--------------------------------------|

| | |
|--------------------|--------|
| Alkaloids | 0.974 |
| Saponins | 0.236 |
| Flavonoids | 11.07 |
| Tannins | 0.310 |
| Phenols | 0.327 |
| Cardiac glycosides | 0.0511 |

Effects of Serial Extracts of *Solanum aculeastrum* Seeds on Serum Lipid Profile of Testosterone Propionate Induced BPH in Male Wistar Rats

The results of the effects of serial extracts of *Solanum aculeastrum* seeds on serum lipid profile of testosterone propionate induced BPH in male Wistar rats are presented in figure 1. The result shows that there was a significant ($p < 0.05$) decrease in serum cholesterol concentration only in the finasteride and hexane extract treated group compared to the BPH control. There were no significant changes in serum TG, LDL-C and VLDL-C concentration in all the test groups compared to both the BPH control and normal control. However, the results indicate a significant decrease in HDL-C concentration in the finasteride, hexane and chloroform extract treated groups compared to both the BPH control. The results also revealed a significant increase in serum HDL-C levels in the benzene, ethylacetate and ethanol extracts treated groups compared to the finasteride treated group.

Figure 1: Effects of serial extracts of *Solanum aculeastrum* seeds on the lipid profile of testosterone propionate induced BPH in male Wistar rats.



Values are expressed as Mean \pm SEM, $n = 6$; a = $p < 0.05$ (Test groups compared with normal control); b = $p < 0.05$ (Groups 3, 4, 5, 6, 7 and 8 compared with group 2); c = $p < 0.05$ (Groups 4, 5, 6, 7 and 8 compared with group 3); d = $p < 0.05$ (Test groups compared with group 4); e = $p < 0.05$ (Test groups compared with group 5); f = $p < 0.05$ (Test groups compared with group 6); g = $p < 0.05$ (Test groups compared with group 7).

IV. DISCUSSION

Phytochemical constituents of medicinal plants are believed to have numerous therapeutic activities such as anti-HIV, anti-plasmodial, anti-diarrheal, anti-septic, anti-bacterial, anti-viral, anti-inflammatory, anti-microbial, hypoglycemic, antioxidant, analgesic and hepatoprotective properties as well as other physiological activities (Sofowora, 1993; Cushnie and Lamb, 2005; Ebana *et al.*, 2005; Evans, 2005; Akpanabiatu, 2012). They exhibit structure related biochemical and pharmacological actions capable of reducing the risk of multiple diseases (Savage, 1993; Karimi *et al.*, 2013). Flavonoids have been utilized to improve human health via their multiple biological functions including anti-inflammatory (Yamamoto and Gaynor, 2001), antimicrobial (Cushnie and Lamb, 2005), antioxidant (Iweala and Ogidipo, 2015b), anticancer activities (Ishiola *et al.*, 2018) and the prevention of osteoporosis (Selvaraj *et al.*, 2014). Alkaloids have been used as an analgesic, antispasmodic or bactericidal agents (Frantisek, 1991). They are known to inhibit certain mammalian enzymic activities such as those of phosphodiesterase, prolonging the action of cAMP. They also affect glucagons and thyroid stimulating hormones (Okaka *et al.*, 1992). Saponins have been reported to be useful in reducing inflammation of upper respiratory passage and also chiefly as foaming and emulsifying agents and detergents (Frantisek, 1991). Tannins have astringent properties that hasten the healing of wounds and prevention of decay. Tannin compounds have antimicrobial activities and are responsible for preventing and treating urinary tract infections and other bacterial infections (Ebana *et al.*, 2005). The result of the present study indicated that seed extracts of *Solanum aculeastrum* contains some biologically active compounds which could serve as potential source of bioactive agents in herbal medicine.

Lipid profile are useful in monitoring health status of the cardiovascular system. The results in figure 1 revealed that oral administration of serial extracts of *Solanum acculeastrum* did not significantly alter the serum TG, LDL-C and VLDL-c levels in

all the treatment groups compared to the BPH control and normal control groups. However, there was a significant decrease in the finasteride and hexane extract treated group compared to the BPH control. Again, there was a significant decrease in HDL-C concentration in the finasteride, hexane and chloroform extract treated groups compared to both the BPH control.

The results suggest that oral administration of serial extracts of *Solanum aculeastrum* may not have adverse effect on the cardiovascular system. The significant increase in HDL-C levels in the benzene, ethylacetate and ethanol extracts treated groups compared to the finasteride treated group corroborates a possibility of improving the cardiovascular system. This result agrees with that of Ruiz-Roso *et al.* (2010) and may be due to the presence of plant proteins and antioxidant compounds including flavonoids, carotenoids, and other phenolic compounds (Hanrahan *et al.*, 2009; Charles, 2013).

V. CONCLUSIONS

The present study showed that the seed extracts of *Solanum aculeastrum* contains phytochemicals which may possess pharmacological potentials. The polar solvents - hexane, chloroform and ethanol were the most effective extraction solvents for the phytochemicals investigated. The results of serum lipid profile of serial extracts of *Solanum aculeastrum* seeds indicates that extracts are cardioprotective, highly safe and may be used for medicinal purposes. However, further toxicity studies are recommended.

VI. ACKNOWLEDGEMENT: Authors wish to thank Mr Etop Samuel Obot and Aniekan Michael Ana who assisted with the collection of plant materials.

VII. DECLARATION OF CONFLICTING INTEREST

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Aboyade, O. M., Yakubu, M. T., Grierson, D. S. and Afolayan, A. J. (2009). Safety Evaluation of Aqueous Extract of Unripe Berries of *Solanum aculeastrum* in Male Wistar Rats. *Bulletin of Chemical Society of Ethiopia*, 17: 61 - 66.
2. Aboyade, O. M., Yakubu, M. T., Grierson, D. S. and Afolayan, A. J. (2010). Safety Evaluation of Aqueous Extract of Unripe Berries of *Solanum aculeastrum* in Male Wistar Rats. *African Journal of Pharmacy and Pharmacology*, 4: 90 - 97.
3. Akpanabiutu, M., Otitoju, O., Edet, E., Ndem, J., Uwah, A. and Ufot, F. (2012). Vitamin E Supplementation with *Rauwolfia Vomitoria* Root Bark Extract Improves Hematological Indices. *North American Journal of Medical Sciences*, 4(2): 86 - 89.
4. Alhakmani, F., Kumar, S. and Khan, S. A. (2013). Estimation of Total Phenolic Content, *In-Vitro* Antioxidant and Anti-Inflammatory Activity of Flowers of *Moringa Oleifera*. *Asian Pacific Journal of Tropical Biomedicine*, 3(8): 623 - 627.
5. Azmir, J., Zaidu, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. and Omar, A. K. (2013). Techniques for Extraction of Bioactive Compounds from Plant Materials: A Review. *Journal of Food Engineering*, 117(4): 426 - 436.
6. Bansa, A. and Adeyemo, S. (2007). Phytochemical and Antimicrobial Evaluation of Ethanolic Extract of *Dra-caena manni* Bark. *Nigerian Journal of Biotechnology*, 18 (1-2): 27 - 32.
7. Cai, H., Zhang, G., Yan, Z. and Shang, X. (2018). The effect of *Xialiqi* Capsule on testosterone-induced benign prostatic hyperplasia in rats. *Evidenced-Based Complementary Alternative Medicine*, 5: 1 - 9.
8. Charles D. J. (2013). *Antioxidant Properties of Spices, Herbs and Other Sources*. 1st Edition. Springer. New York, USA: 125 - 127.
9. Cushnie, T. and Lamb, A. J. (2005) Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, 26(5): 343 - 356.
10. Ebana, M. C., Ellong, A. and Owona, D. (2005). L'amblyopie chez le Strabique en Milieu Camerounais. *Bulletin of the Belgian Society of Ophthalmology*, 297: 39 - 44.
11. Ejike, C. E. C. C. and Ezeanyika, L. U. S. (2011). Inhibition of the experimental induction of benign prostatic hyperplasia: a possible role for fluted pumpkin (*Telfairia occidentalis* Hook f.) seeds. *Urologia Internationalis*, 87(2): 218 - 224.
12. Evans, W. C. (2005). *Trease and Evans Pharmacognosy*. 15th edition, Elsevier, A Division of Reed Elsevier India Private Limited, New Delhi, India, 20 - 22.
13. Frantisek, S. S. (1991). *The Natural Guide to Medicinal Herbs and Plants*. United Kingdom: Tiger Barks Cast, Twinkemhan, 1 - 5.
14. Hanrahan, C., Rebecca, J. F. and Fundukian, L. J. (2009). *The Gale Encyclopedia of Alternative Medicine*. 3rd Edition Volume 3. Gale, Detroit, Mich, USA. 15 - 18.
15. Harborne, J. (1973). *Phytochemical Methods*. In: Harborne, J. B. (Ed.). *A Guide to Modern Techniques of Plant Analysis*. London: Chapman and Hall, 279.
16. Hutchings, A., Scott, A. H., Lewis, G. and Cunningham, A. B. (1996). *Zulu Medicinal Plants, An inventory*. University of Natal Press, Pietermaritzburg. Ishola, I. O., Yemitan, K. O., Afolayan, O. O. Anunobi, C. C. and Duojaie, T. E. (2018). Potential of *Moringa oleifera* in the Treatment of Benign Prostate Hyperplasia: Role of Antioxidant Defence Systems. *Journal of Medical Principles and Practice*, 27: 15 - 22.
17. Iweala, E. E. J. and Ogidipo, J. O. (2015a). Prostate Specific Antigen, Antioxidant and Hematological Parameters in Prostatic Rats Fed *Solanum macrocarpon* L. Leaves. *Asian Journal of Biological Sciences*, 8: 30 - 41.

18. Iweala, E. E. J. and Ogidigo, J. O. (2015b). Effect of *Celosia argentea* F. Cristata (L.) Schinz. on Prostate Specific Antigen, Antioxidant Status and Hematological Parameters in Rats Induced with Benign Prostate Hyperplasia. *Asian Journal of Biochemistry*, 10: 42 - 51.
19. Karimi, E., Jaafar, H. Z. and Ahmad, S. (2013) Antifungal, Anti-Inflammatory and Cytotoxicity Activities of Three Varieties of *Labisia Pumila* Benth from Microwave Obtained Extracts. *BMC Complementary and Alternative Medicine*, 24: 13 - 20.
20. Koduru, S., Asekun, O. T., Grierson, D. S. and Afolayan, A. J. (2006a). Isolation of Volatile Compounds from *Solanum aculeastrum* (Solanaceae). *Journal of Essential Oil Bearing Plant*, 1: 65 - 69.
21. Koduru, S., Grierson, D. S. and Afolayan, A. J. (2006b). Ethnobotanical Information of Medicinal Plants used for the Treatment of Cancer in the Eastern Cape Province, South Africa. *Current Science Journal*, 92: 906 - 908.
22. Koduru, S., Grierson, D. S., Van de Venter, M. and Afolayan, A. J. (2007a). Anticancer activity of steroid alkaloids isolated from *Solanum aculeastrum*. *Pharmaceutical Biology*, 45: 613 - 618.
23. Koduru, S., Jimoh, F. O., Grierson, D. S. and Afolayan, A. J. (2007b). Antioxidant activity of two steroid alkaloids extracted from *solanum aculeastrum*. *Journal of Pharmacology and Toxicology*, 2: 160 - 167.
24. Kokate, C. (1994). *Practical Pharmacognosy*, 4th edition. Vallabh Prakashan, New Delhi, India. 112 - 120.
25. Kokate, C. K., Purohit, A. P. and Gokhale, S. B. (2005). *Pharmacognosy*. 33rd edition. Nirali Prakashan, New Delhi, India. 108 - 109.
26. Kokwaro, J. O. (2009). *Medicinal Plants of East Africa*. (3rd ed). Nairobi: East African Publishing Bureau.
27. Krishnaiah, D., Devi, T., Bono, A. S. and Sarbatly, R. (2009). Studies on the Phytochemical Composition of Six Malaysian Plants. *Journal of medicinal Plants Research*, 3(2): 67 - 72.
28. Laban, L. T., Anjili, C. O., Mutiso, J. M., Ingonga, J., Kiige, S. J., Ngedzo, M. M. and Gicheru, M. M. (2015). Experimental Therapeutic Studies of *Solanum aculeastrum* Dunal on *Leishmania major* Infection in BALB/c mice. *Iran Journal of Basic Medical Sciences*, 18(1): 67 - 71.
29. Mbaka, G., Anunobi, C., Ogunsina, S. and Osiagwu, D. (2017). Histomorphological changes in induced benign prostatic hyperplasia with exogenous testosterone and estradiol in adult male rats treated with aqueous ethanol extract of *Secamone afzelii*. *Egyptian Journal of Basic and Applied Sciences*, 4: 15 - 21.
30. Nidal, A. J., Mahdi, M. A. and Mahmoud, M. A. (2015). Novel serial Extraction Method for Antibacterial and Antifungal Evaluation of the entire *Eryngium campestre* L. Plant from Jerusalem and Palestine. *Journal of Chemical and Pharmaceutical Research*, 7(3): 905 - 913.
31. Okaka, J. C., Enoch, N. J. and Okaka, N. C. (1992). *Human Nutrition: An Integrated Approach*. Enugu: ESUT Publications, 57 - 58.
32. Pandey, A. and Tripathi, S. (2014). Concept of Standardization, Extraction and Pre-phytochemical Screening Strategies for Herbal Drugs. *Journal of Pharmacognosy and Phytochemistry*, 2(5): 115 - 119.
33. Ruiz-Roso B., Quintela J. C., de la Fuente E., Haya J., Pérez-Olleros L. (2010). Insoluble carob fiber rich in polyphenols lowers total and LDL cholesterol in hypercholesterolemic subjects. *Plant Foods for Human Nutrition*, 65(1): 50 - 56.
34. Santhi, N. K. and Sengottuvel, R. (2016). Qualitative and quantitative phytochemical analysis of *Moringa concanensis*. *International Journal of Current Microbiology and Applied Sciences*, 5(1): 633 - 640.
35. Savage, G. P. (1993). Saponins. In: Macre, R., Robinson, R. K. and Sadler, M. J (Eds.), *Encyclopedia of Food Science, Food Technology and Nutrition*. London, Academic Press. 3998 - 4001.
36. Selvaraj, G., Kaliamurthi, S., Ekambaram, R. and Thirugnanasabandan, R. (2014). Qualitative and Quantitative Phytochemical Studies of *Acanthus ilicifolius*. *Research Journal of Phytochemistry*, 8: 133 - 138.
37. Sofowora, A. (1993). *Medicinal Plants and Traditional Medicine in Africa*, 2nd ed., Ibadan, Nigeria: Spectrum Books Limited (Publisher), 134 - 156.
38. Tiwari, P. Kumar, B., Kaur, M., Kaur, G. and Kaur, H. (2011). Phytochemical Screening and extraction: A Review. *Internationale Pharmaceutica Scientia*, 1(1): 98 - 104.
39. Trease, G. and Evans, W. (1989). *A Textbook of Pharmacognosy*, 13th ed., London: Bailliere Tindall Ltd., 19 - 21.
40. Ubon, J. A., Akpanabiatu, M. I. and Udotong, J. I. R. (2019). Effect of Serial Extracts of *Solanum Aculeastrum* Seeds on Some Biochemical Indices of Testosterone Propionate Induced Benign Prostatic Hyperplasia in Male Wistar Rats. Unpublished Ph.D Seminar Paper, Presented at the Postgraduate Seminar Presentation held at the Department of Biochemistry, University of Uyo, Uyo, Nigeria.
41. Wabwoba, B., Anjili, C. O., Ngeiywa, M. M., Ngure, P. K., Kigonde, E. M. and Ingonga, J.(2010). Experimental Chemotherapy with *Allium sativum* (Liliaceae) Methanolic Extract in Rodents Infected with *Leishmania major* and *Leishmania donovani*. *Journal of Vector Borne Diseases*, 47: 160 - 167.
42. Wanyonyi, A. W., Chhabra, S. C., Mkoji, G., Udo, E. and Njue, W. N. (2002). Bioactive Steroidal Alkaloid Glycosides from *Solanum aculeastrum*. *Phytochemistry*, 59: 79 - 84.
43. Wanyonyi, A. W., Chhabra, S. C., Mkoji, G., Njue, W. and Tarus P. K. (2003). Molluscicidal and Antimicrobial Activity of *Solanum aculeastrum*. *Fitoterapia*, 74: 298 - 301.
44. Yamamoto, Y. and Gaynor, R. B. (2001). Therapeutic Potential of Inhibition of the NF-Kb Pathway in the Treatment of Inflammation and Cancer. *Journal of Clinical Investigation*, 107(2):135 - 142.