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Oral Acute & Sub-Chronic Toxicity Assessment of Ethanol Leaf Extract of *Simarouba glauca*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SDEOE and NEJO designed the study. Authors SDEOE performed the statistical analysis. Authors SEDOE and NEJO wrote the protocol, and wrote the first draft of the manuscript. Authors EKIO and FOA managed the analyses of the study. Author SDEOE managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Traditional herbal medicine and their preparations have been widely used for thousands of years and are still in use in developing and developed countries owing to their medicinal values and their presumed relative safety. This belief that medicinal plants are not toxic or are with less side effect due to their natural origin is debatable; hence this study was conducted to evaluate the safety and (or) toxicity of Ethanol leaf extract of *Simarouba glauca* (EESG) on liver, kidney and heart functions of *Wistar* rats. The oral acute toxicity of EESG was evaluated in line with Lorke's method. The sub-chronic toxicity of EESG was carried out according to the OECD guidelines with modification and using a total of twenty-four (24) male *Wistar* rats; divided into four groups of six rats each, following a two-week acclimatization. Test rats were orally administered EESG at doses of 500, 1000 and 2000 mg/kg body weight respectively daily for thirty (30) days, while the control was given only feed and water *ad libitum*. At the end of the experiment, the rats were fasted overnight and sacrificed under chloroform anesthesia; relevant biochemical and histopathology analyses were carried out.

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The data obtained from the oral acute test indicate that the LD_{50} was above 5000 mg/kg and there was no death recorded. There were significant increases ($P < 0.05$) in percentage (%) body weight of rats administered respective doses of EESG. There were significant reductions ($P<0.05$) in mean liver: body weight ratio of rats administered EESG 500 and 2000 mg/kg respectively, significant reductions ($P<0.05$) in mean kidney: body weight ratios of rats given EESG 1000 and 2000 mg/kg respectively; significant reductions ($P<0.05$) in mean heart: body weight ratios of test rats administered EESG 2000 mg/kg; whereas others were not significantly different ($P>0.05$) relative to their respective control. Plasma ALT and GGT activities of rats administered respective dose of EESG were significantly reduced ($P<0.05$); plasma ALP activities were significantly elevated ($P<0.05$) relative to the control after 30 days. There were no significant differences ($P>0.05$) in plasma total proteins and albumin levels. Plasma total and unconjugated bilirubin of rats administered respective dose of EESG were not significantly different ($P>0.05$); whereas, rats given EESG recorded significant reduction in plasma conjugated bilirubin. Plasma urea was significantly elevated ($P<0.05$) in rats administered EESG 1000 and 2000 mg/kg respectively. Test rats given EESG 500 and 1000 mg/kg respectively recorded significant elevations in plasma creatinine and rats given EESG 2000 mg/kg recorded significant decrease in plasma creatinine levels; others were not significantly different relative to the control. Plasma chloride and potassium ion levels of rats administered respective doses of EESG were not significantly different ($P>0.05$); significant reduction ($P<0.05$) in plasma sodium ions concentration in all group compared to the control. Plasma calcium ion levels in all group were not significantly different ($P>0.05$); whereas there were significant reductions ($P<0.05$) in plasma bicarbonate ion levels relative to their respective controls. Although plasma ALP activity were significantly elevated, there were no elevations in specific liver function enzymes and no visible hepatocellular damage. Furthermore, the conspicuous elevations observed in plasma urea and creatinine levels do not exclusively indicate EESG-induced organ injury. Therefore, it is suggestive that EESG was not significantly toxic to the to the liver, kidney and heart respectively and may be administered at lower doses in further studies.

Keywords: Leaf extract; toxicity; Simarouba glauca.

1. INTRODUCTION

World Health Organization [1] describes traditional herbal medicines as natural occurring plant-derived substances with minimal or no industrial processing used to prevent and treat illness within local or regional healing practices [2]. Traditional herbal medicine and their preparations have been widely used for thousands of years in developing and developed countries owing to their natural origin and presumed less side effects [3]. Physiological and Pharmacological actions exhibited by a variety of plants can be attributed to chemical compounds synthesized by these plants. The traditional application of plants for prevention and treatment purposes predates recorded history and all forms of allopathic medicine; including clinical and chemical pharmacological approaches. Modern day synthetic pharmacological agents were hitherto prepared as crude drugs such as tinctures, teas, powders, and other herbal formulations [4] and with several active drugs derived directly from their respective plant sources. These included, such as aspirin (from willow bark), digitoxin (from foxglove), morphine (from the opium poppy), quinine (from cinchona bark), and pilocarpine (Jaborandi) [5]. The W.H.O [1] reports have revealed that the use of herbal remedies throughout the world is on the increase, and indeed exceeding the use of conventional drugs by two to three times. In fact, herbal remedies are fast becoming the lifeblood for primary health care of approximately 75 – 80 % of the world population that resides mainly in the developing countries of Africa. This is because herbal preparations are cheap and locally available; and are thought to have no side effects [6]. However, huge concerns have been raised about the safety of herbal drugs, although reports of injury or death arising from adverse reactions to plants supplements are scanty [7]. Several pharmacological compounds such as alkaloids, anthraquinone glycosides, pyrrolizidine alkaloids amongst other synthesized by various plants have been implicated in toxicity and damage to vital organs [8,9,10]. These findings further underscore the need for thorough safety evaluation of herbal preparations.

Simarouba glauca, commonly known as “Paradise tree” or “Laxmitaru” belongs to the family *Simaroubaceae*. The plant is also known by other common names such as bitter ash,

bitter damson princess tree and others [11]. *Simarouba glauca* is a medium-sized tree that grows up to 20 m high, with a trunk 50 to 80 cm in diameter. It produces bright green leaves 20 to 50 cm in length, small white flowers, and small red fruits. The name *glauca* refers to "bluish green" foliage derived from the Greek word "glaukos" which means bluish [12]. The plant is native to the Amazon rainforest and other tropical areas of Mexico, Cuba, Haiti, Jamaica, and North and Central America [13]; exotic to India, Sri Lanka, Phillippines, Myanmar and Nigeria [12]. It can adapt to a wide range of range of temperatures (30-45°C) and altitudes up to 1000 meters above sea levels [14]; it is cultivated by seed and can be grown in soils outside these regions.

In 2007, it was introduced to Nigeria, Ubiaja, Esan South East Local Government Area of Edo state by Blessing Akele (Ph. D) and Osagie-Eweka; cultivated in *Cercobela* Farms®.

S. glauca has a long history of herbal medicine having many pharmacological properties that have been documented. In Cuba, it is called gavilan, an infusion of the leaves or bark is considered to be astringent and used as a digestion and menstrual stimulant and an anti-parasitic remedy, it is taken internally for diarrhea, dysentery, malaria and colitis. It is also used externally for wounds and sores. In Belize, the tree is called negrito or dysentery bark; the bark (and occasionally the root) is boiled in water to yield a powerful astringent and tonic used to wash skin sores and to treat dysentery, diarrhea, stomach and bowel disorders, hemorrhages and internal bleeding [11]. In Brazil, it is employed much the same way against fever, malaria, diarrhea, dysentery, intestinal parasites indigestion and anemia [11]. In Brazilian herbal medicine, *Simarouba glauca* bark has long been the most highly recommended (and most effective) natural remedy against chronic and acute dysentery. Bark and leaf of *S. glauca* contain triterpenes useful in curing amoebiasis, diarrhea and malaria. Chemicals present in leaf, fruit, pulp and seed of *S. glauca* have been reported to possess analgesic, antimicrobial, antiviral, astringent, emmenagogue, stomachic, tonic, vermifuge properties [15]. *Simarouba glauca* extract is used for reducing patchy skin pigmentation [16]. The plant extract is subject of one Patent, where its water extract was found to increase skin keratinocyte differentiation and to improve skin hydration and moisturization (17,18]. The major active groups of phytochemicals in *Simarouba glauca* are the quassinoids, which belong to the triterpene chemical family. Ailanthinone, glaucarubinone and holacanthone are considered some of the main active quassinoids in *Simarouba*. Other chemicals include benzoquinone, canthin, dehydroglaucarubinone, glaucarubine, glaucarubolone, melianone, simaroubidin, simarolide, simaroubin, simarubolide, sitosterol and tirucalla [19]. Some of these active compounds have demonstrated *in-vitro* anti-tumor activity [20,21,22,23], *in-vitro* anti-viral activity [24], *in-vitro* anti-amebic activity [25,26,27], *in-vitro* anti-bacteria activity [28], *in-vivo* anti-malaria activity [29,30,31] amongst others. The therapeutic effects are attributed to quassinoids inherent in *S. glauca* [20,32,33].

Image 1. Young paradise tree (*S. glauca*) growing in Cercobela Farms® (Osagie-Eweka Photo Library)

In view of the renewed interest in *S. glauca* and its several biological activities, it is has become imperative to assess the toxicity safety of the plant leaf as a prelude to further therapeutic studies. There is as yet only scanty information on the toxicity of *S. glauca* leaf and hence, the study which seeks to bridge the gap in knowledge.

2. MATERIALS AND METHODOLOGY

2.1 Collection of *S. glauca* Leaf and Preparation of Ethanol Extract

Leaves of *S. glauca* were obtained (harvested) from Cercobela Farms®, Ubiaza, Esan South East Local Government Area of Edo State,

Nigeria. Fresh plant specimen was authenticated and voucher specimen obtained at the Department of Plant Biology and Biotechnology Herbarium, University of Benin, Benin City. Nigeria with voucher N0. UBH_S382. The leaves were rinsed with tap water and air-dried at the Department of Biochemistry' Laboratory for twenty-eight (28) days at room temperature. Leaves were pulverized and sieved off a mesh to obtain fine particles at the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin. A 500 g pulverized leaf was soaked in a 2.5 L absolute ethanol (99 % purity; analytical grade) and stirred at intervals for 24 hours. The procedure was repeated for another 24 hours to obtain filtrate. The filtrate was freeze-dried to obtain dried ethanol extract (EESG); as previously reported by [34].

2.2 Reagents Test kits

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin and direct bilirubin, total protein, albumin, urea, creatinine, calcium, sodium, chloride, potassium and bicarbonate test kits were purchased from Randox Laboratory (United Kingdom)

2.3 Experimental Animals

Male *Wistar* albino rats weighing between 167.00 and 266.33 g were used for the acute and sub-chronic toxicity studies. The animals were housed in metabolic cages; were fed a normal commercial pelleted diet (Livestock Feeds® PLC), water *ad libitum* and maintained under laboratory conditions of 12 h light/12 dark cycle with a two-week acclimatization prior to commencement of studies. The research was conducted in accordance with the internationally acceptable guidelines for use of animals for laboratory experiment.

2.4 Acute Toxicity Studies (*In Vivo*)

Acute toxicity evaluation was conducted by the methods previously reported by [35] to determine LD_{50}. In Phase I, a total of nine (9) male *Wistar* rats were used after a two-week (2) acclimatization. *Wistar* rats were divided into three groups of n = 3 with each group receiving 10, 100 and 1000 mg/kg body weight respectively of Ethanol leaf extract of *S. glauca* (EESG) and observed for 24 hours for signs of behavioral changes and (or) death. Post administration in the Phase II, a total of three (3) male rats were used and divided into three (3) groups of n = 1. Each group was administered doses of 1600, 2900 and 5000 mg/kg EESG respectively and observed for another 24 hours for signs of behavioral changes associated with toxicity and (or) mortality (Table 1 & 2).

The lethal dose (LD_{50}) of EESG leaf was calculated as shown below:

$$LD_{50} = \frac{\sqrt{D_0 + D_{100}}}{2} \quad (1)$$

D_0 = Highest dose that result in no death; D_{100} = Lowest dose that resulted to death.

2.5 Sub-Chronic Toxicity Studies (*In-Vivo*)

The Sub-Chronic toxicity study was conducted as prescribed in [36] test guidelines, N0. 425; described by [37,38]. A suitable dose range was adopted for the sub-chronic toxicity study. A total of twenty-four (24) male *Wistar* rats were utilized in this phase of the study and were allowed access to food and drinking water *ad libitum*. The rats were distributed into four (4) groups of n = 6. Test animals received 500, 1000 and 2000 mg/kg body weight respectively of EESG daily for thirty (30) days, while the control group received only rat pellets and drinking water *ad libitum*.

2.6 Collection of Data and Specimen

At the end of the study, the rats were fasted overnight, anesthetized using a chloroform saturated chamber and sacrificed. Under anesthesia, the thoracic and abdominal regions were opened up and blood was withdrawn from the hepatic portal vein or thoracic aorta using a 5ml syringe; emptied into a 5 ml heparinized specimen bottles. The blood was then centrifuged at 3,500 rpm for 15 minutes to obtain a clear supernatant (Plasma) that was stored at -18^{0}C until required for biochemical analyses; all of which were carried out within a few days. A 1 g tissue of liver, kidney and heart respectively was excised and homogenized in a homogenizer. The homogenate was centrifuged in a refrigerated centrifuge at 3500 rpm/10 minutes and the supernatant decanted for relevant biochemical evaluations. The liver and

kidney organs were harvested and stored in formal saline solution (0.9 g of NaCl in 90 mL of distilled water and mixed with 10 ml of 40 % formalin to obtain a final volume of 100 ml) for histopathology evaluation.

2.7 Biochemical Analyses

Liver function tests which include aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), Plasma Total protein and Albumin, total bilirubin and direct bilirubin were done using methods described by [39,40,41,42,43,44] respectively. Kidney function test which include urea, creatinine, calcium, sodium, chloride, potassium and bicarbonate were done using colorimetric methods described by [45,46,47,48,49,50,51] respectively and with the aid of commercially available test kits; product of Randox® Laboratories (United Kingdom).

2.8 Histopathology Evaluation of Sectioned Liver Tissue

The excised organ fixed in formal saline were trimmed into 5 mm thick and dehydrated with graded concentrations of ethanol (70, 95 and 99 %: absolute ethanol), cleared in xylene and embedded in paraffin wax. The embedded tissues (Liver, Kidney and Heart) were sectioned at 6 μm thickness stained with hematoxylin and eosin (H & E) and examined under the light microscope according to the method described by [52,53]. The sections were photographed at X100 and 400 magnifications respectively with the Vanox-T Olympus photographic microscope.

2.9 Statistical Analyses

Data obtained from the study are expressed as mean and standard deviation (mean ± SD) where applicable. Statistical differences between means of test group were evaluated by paired t-test and one-way analysis of variance (ANOVA); while the post-hoc comparison tests were carried out using the Turkey's multiple comparison test. Differences in means were considered significant at $P < 0.05$ and not significant at $P > 0.05$. All statistical analyses were conducted using GraphPad prism®, version 7.

3. RESULTS

3.1 Acute Toxicity Study of Ethanol Leaf Extract of *S. glauca* (EESG) in Rats

The data obtained from the acute toxicity evaluation show that EESG administered to Wistar rats was relatively safe; no death was recorded after phase I & II of the study. This suggests that the LD_{50} of EESG exceeded 5000 mg/kg body weight (Tables 1 & 2).

Table 1. Acute toxicity studies of ethanol leaf extract (EESG) administered to male *Wistar* rats (Lorke' Method Phase I)

Group	Weight of rats (g)	Extract	Dose	Observations			
				Behavioral change	Eating habit	Sleep	Mortality
I	171.33 ± 1.5	**EESG**	10 mg/kg	NØ	NØ	NØ	NØ
II	177.00 ± 1.0	**EESG**	100 mg/kg	NØ	NØ	NØ	NØ
III	167.00 ± 1.7	**EESG**	1000 mg/kg	NØ	NØ	NØ	NØ

Weights are mean ± SD, n = 3, NØ = No Significant Observation, EESG (Ethanol Leaf Extract of S. glauca)

Table 2. Acute toxicity studies of ethanol leaf extract (EESG) administered to male *Wistar* rats (Lorke' Method Phase II)

Group	Weight of rats (g)	Extract	Dose	Observations			
				Behavioral change	Eating habit	Sleep	Mortality
I	212	**EESG**	1600 mg/kg	NØ	NØ	NØ	NØ
II	196	**EESG**	2900 mg/kg	NØ	NØ	NØ	NØ
III	197	**EESG**	5000 mg/kg	NØ	NØ	NØ	NØ

Weights are mean ± SD, n = 1, NØ = No Significant Observation, EESG (Ethanol Leaf Extract of S. glauca)

3.2 Sub-Chronic Toxicity Study of Ethanol Leaf Extract of *S. glauca* (EESG) in *Wistar* Rats

3.2.1 Effect of EESG on body weight changes

The data presented in Fig. 1a indicate that there were significant increases ($P < 0.05$) in final mean body weights of test animals administered 500, 1000 and 2000 mg/kg EESG respectively; including the control after 30 days when compared with their respective initial mean body weights taken before the commencement of the administration of EESG. Fig. 1b clearly show percentage (%) weight gain which indicate that rats administered respective doses of EESG recorded significant increase in weight ($P < 0.05$) when compared to the control. Although, the increase in weight observed in rat given EESG 1000 mg/kg body weight was not significantly different ($P > 0.05$) relative to the control; whereas, rats administered EESG 500 and 2000 mg/kg respectively gained highest weight. There was significant reduction ($P < 0.05$) in liver/body weight ratio of rats administered EESG 500 and 2000 mg/kg respectively, whiles rats given EESG 1000 mg/kg was not significantly different ($P > 0.05$) compared to the control (Fig. 2). The rats administered EESG 1000 and 2000 mg/kg respectively recorded significant reduction ($P > 0.05$) in kidney/body weight ratio, whereas rat given EESG 500 mg/kg was not significantly different ($P > 0.05$) relative to the control (Fig. 2). There was also significant reduction ($P < 0.05$) in heart/body weight ratio of rats given EESG 2000 mg/kg, while the heart/body weight ratio of rats administered EESG 500 and 1000 mg respectively were not significantly different ($P > 0.05$) relative to the control (Fig. 2).

3.3 Effect of EESG on Liver Function Parameters and Total Proteins

There were no significant differences ($P > 0.05$) in plasma ALT activities of *Wistar* rats administered respective doses of EESG when compared with the plasma ALT activity of the control (Fig. 3a). The plasma AST activities of test rats administered EESG 500, 1000 and 2000 mg/kg respectively were significantly lower ($P < 0.05$) relative to the plasma AST activity of the control (Fig. 3a). Plasma ALP activities were significantly elevated ($P < 0.05$) in all groups of test rats given varying doses of EESG when compared with the ALP activity of the control; ALP activity decreased as doses increased (Fig. 3a). There was significant reduction ($P < 0.05$) in plasma GGT activities of rats administered respective doses of EESG compared to the plasma GGT activity of the control (Fig. 3a). The liver, heart and kidney ALT activities of rats administered respective doses of EESG were not significantly different ($P > 0.05$) when compared to their respective control (Fig. 3b). The liver and heart GGT activities of rats administered respective doses of EESG were likewise not significantly different ($P > 0.05$) relative to their respective controls (Fig. 3c). Similarly, plasma total proteins and albumin concentration of rats given respective doses of EESG were not significantly different ($P > 0.05$) when compared to their respective controls (Fig. 4).

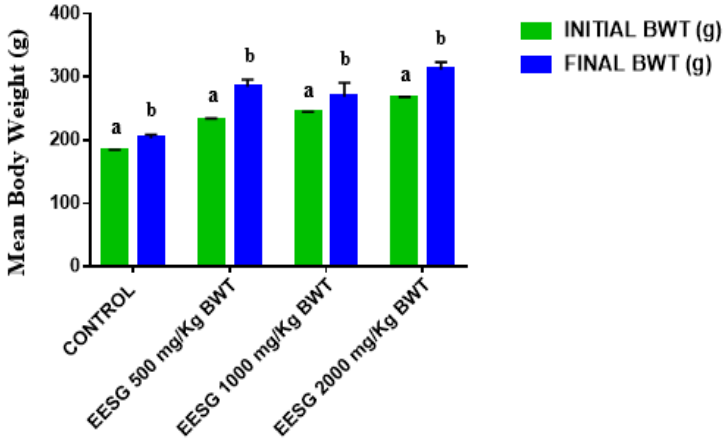

Fig. 1a. Effect of varying dose of ethanol leaf extract of *S. glauca* (EESG) on Body Weight (g) of Male *Wistar* Rats after 30 days

Data with similar lower case alphabet are not significantly different (p > 0.05); data with different lower case alphabets are significantly different (p < 0.05). (Data are Mean ± SD)

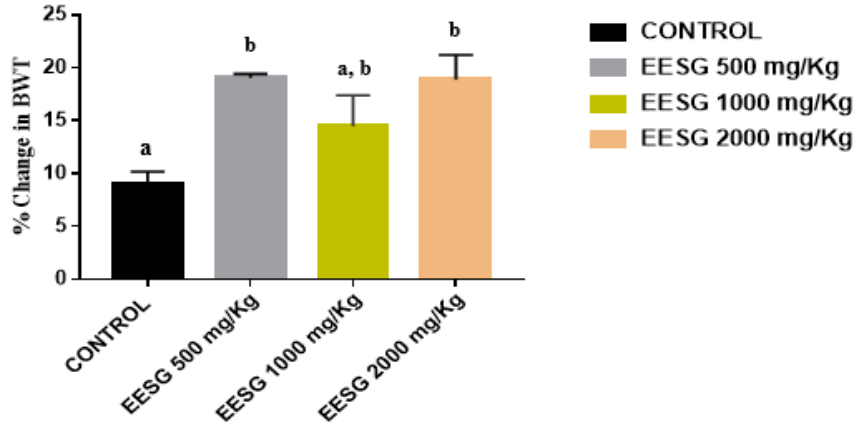

Fig. 1b. Percentage Changes in body weight of Male *Wistar* Rats administered respective doses of EESG after 30 days

Data with similar lower case alphabet are not significantly different (p> 0.05); data with different lower case alphabets are significantly different (p < 0.05). (Data are Mean ± SD)

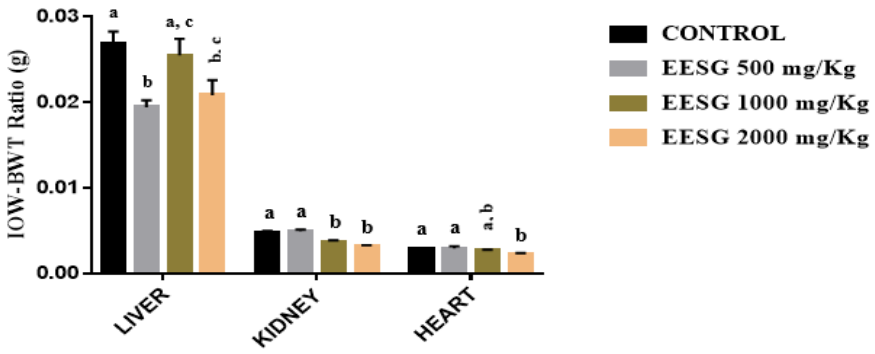

Fig. 2. Effect of varying dose of EESG on Internal Organ/Body Weights (IOW/BWT) Ratios of Male *Wistar* Rats after 30 days

Data with similar lower case alphabet are not significantly different (p > 0.05); data with different lower case alphabets are significantly different (p < 0.05). Data are Mean ± SD

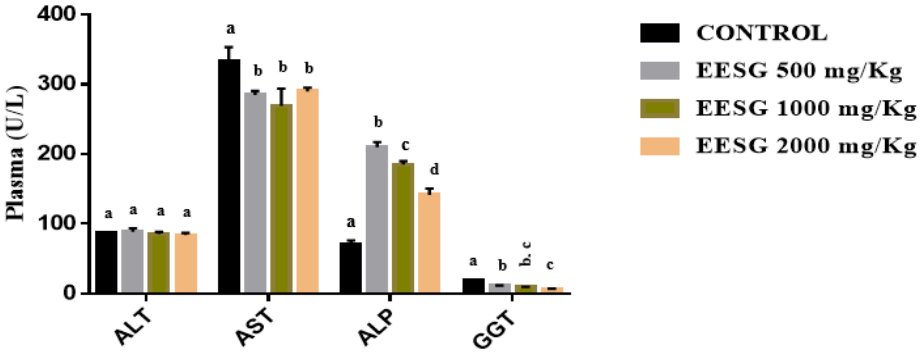

Fig. 3a. Effect of varying doses of EESG on Plasma ALT, AST, ALP and GGT activities of Male *Wistar* Rats

Data with similar lower case alphabets are not significantly different (P > 0.05); data with different lower case alphabets are significantly different (P < 0.05). Data are Mean ± SD

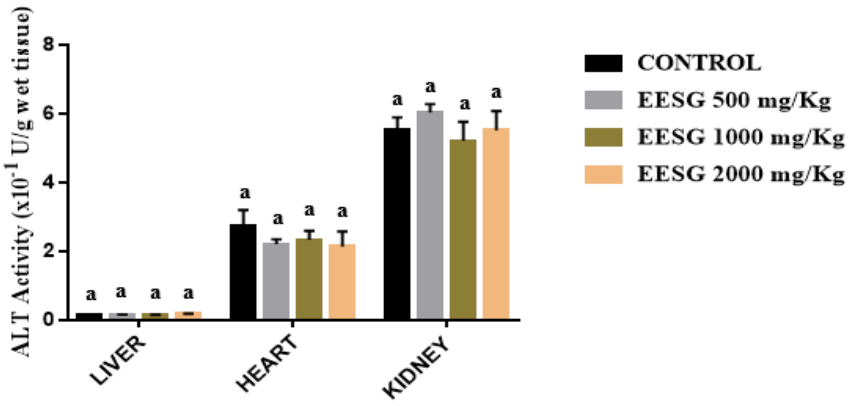

Fig. 3b. Effect of varying doses of EESG on liver, heart & kidney ALT activities of male *Wistar* Rats

Data with similar lower case alphabets are not significantly different (P > 0.05); data with different lower case alphabets are significantly different (P < 0.05) Data are Mean ± SD

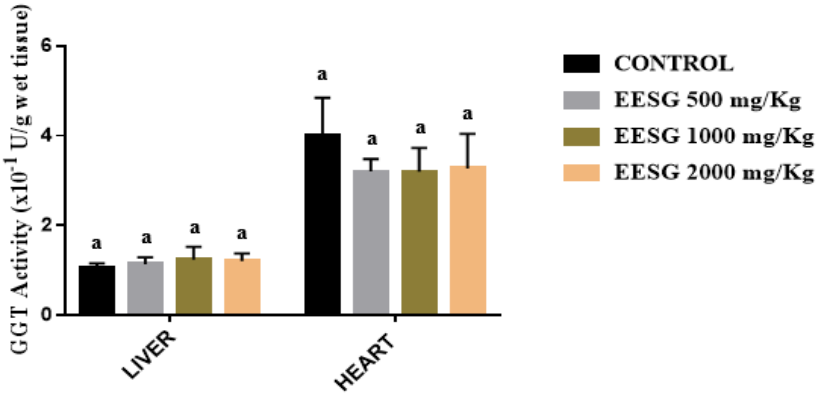

Fig. 3c. Effect of varying doses of EESG on Liver & Heart GGT activity of Male *Wistar* Rats

Data with similar lower case alphabets are not significantly different (P > 0.05); data with different lower case alphabets are significant different (P < 0.05). Data are Mean ± SD

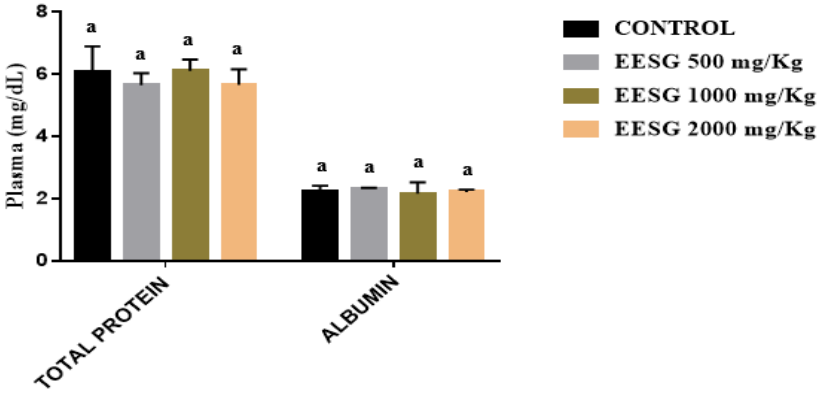

Fig. 4. Effect of varying doses of EESG on plasma total protein & albumin levels of male *Wistar* Rats after 30 days

Data with similar lower case alphabets are not significantly different (P > 0.05); data with different lower case alphabets are significant different (P < 0.05). Data are Mean ± SD

The plasma total and unconjugated bilirubin of rats administered respective doses of EESG were not significantly different (*P>0.05*) compared to the plasma total and unconjugated bilirubin respectively of the control after 30 days (Fig. 5). However, there was significant reduction (*P<0.05*) in plasma conjugated bilirubin of rats administered EESG 500 mg/kg; whereas the conjugated bilirubin of rats given EESG 1000 and 2000 mg/kg were not significantly different (*P>0.05*) relative to the control (Fig. 5).

3.4 Effect of EESG on Kidney Function Parameter

There was significant elevation (*P < 0.05*) in plasma urea concentration of rats administered EESG 1000 and 2000 mg/kg respectively; whereas the plasma urea concentration of rat given EESG 500 mg/kg was not significantly different (*P > 0.05*) relative to the plasma urea concentration of the control (Fig. 6). The plasma creatinine levels of rats administered EESG 500 and 1000 mg/kg respectively were significantly elevated (*P < 0.05*) while rat given EESG 2000 mg/kg recorded significant reduction (*P < 0.05*) in plasma creatinine compared to the plasma creatinine level of the control (Fig. 6). There was significant reduction (*P < 0.05*) in plasma Na^+ concentration of test rats administered respective doses of EESG relative to the control (Fig. 7). Whereas, there were no significant differences (*P > 0.05*) in plasma Cl^- and K^+ concentrations of rats given respective doses of EESG relative to their control (Fig. 7). There were no significant differences (*P > 0.05*) in plasma "corrected" calcium levels of rats given respective doses of EESG compared to the control (Fig. 8); whereas, there were significant reduction (*P < 0.05*) in plasma HCO_3^- concentration of rats administered EESG 500 and 1000 mg/kg respectively; rats given EESG 2000 mg/kg recorded plasma HCO_3^- level that was not significantly different (*P > 0.05*) relative to the plasma HCO_3^- level of the control after 30 days (Fig. 8).

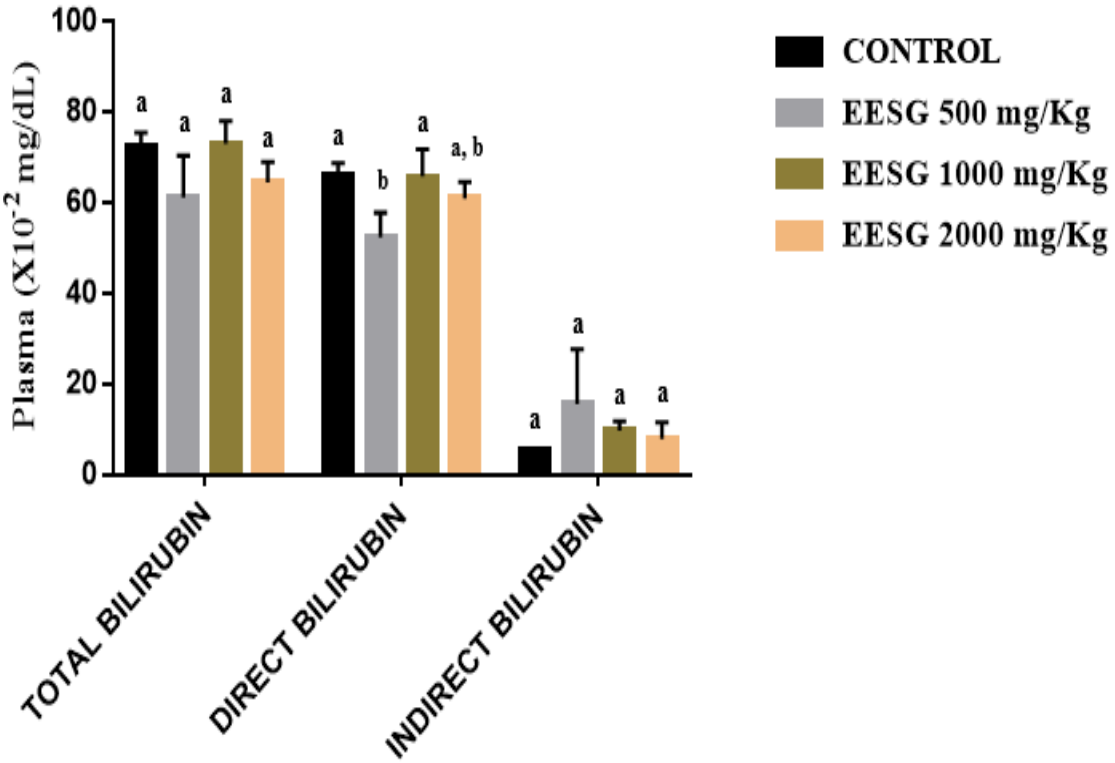

Fig. 5. Effect of varying doses of EESG on plasma total, direct & indirect bilirubin of male *Wistar* Rats after 30 days

Data with similar lower case alphabets are not significantly different (P > 0.05); data with different lower case alphabets are significantly different (P < 0.05). Data are Mean ± SD

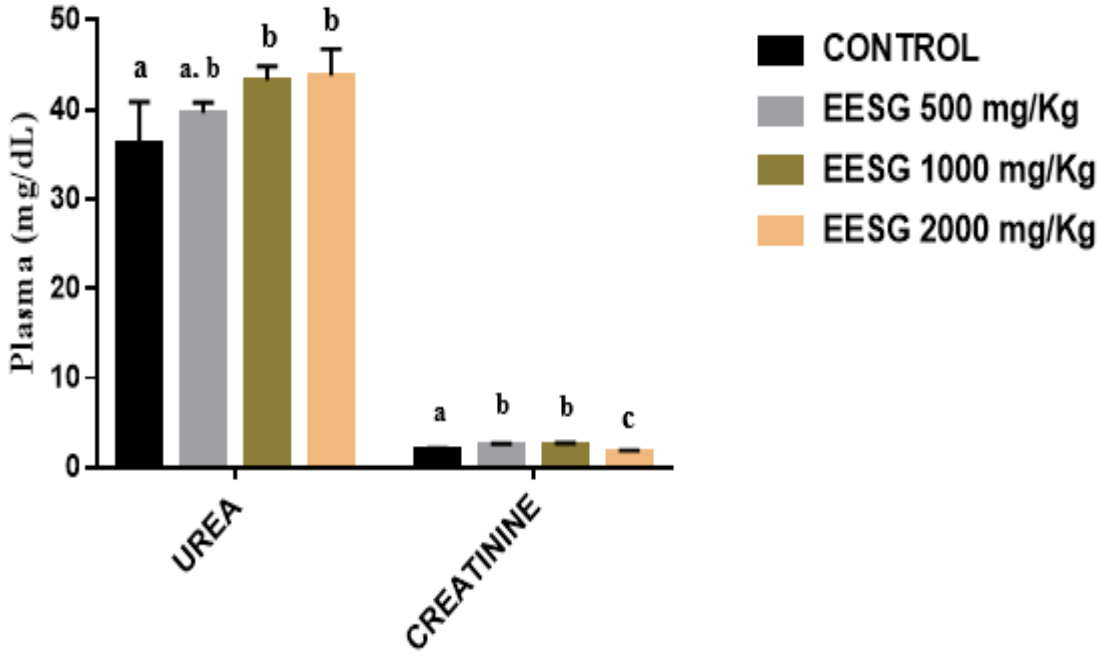

Fig. 6. Effect of varying doses of EESG on plasma urea and creatinine levels of male *Wistar* Rats

Data with similar lower case alphabets are not significantly different (P > 0.05); data with different lower case alphabets are significantly different (P < 0.05). Data are Mean ± SD

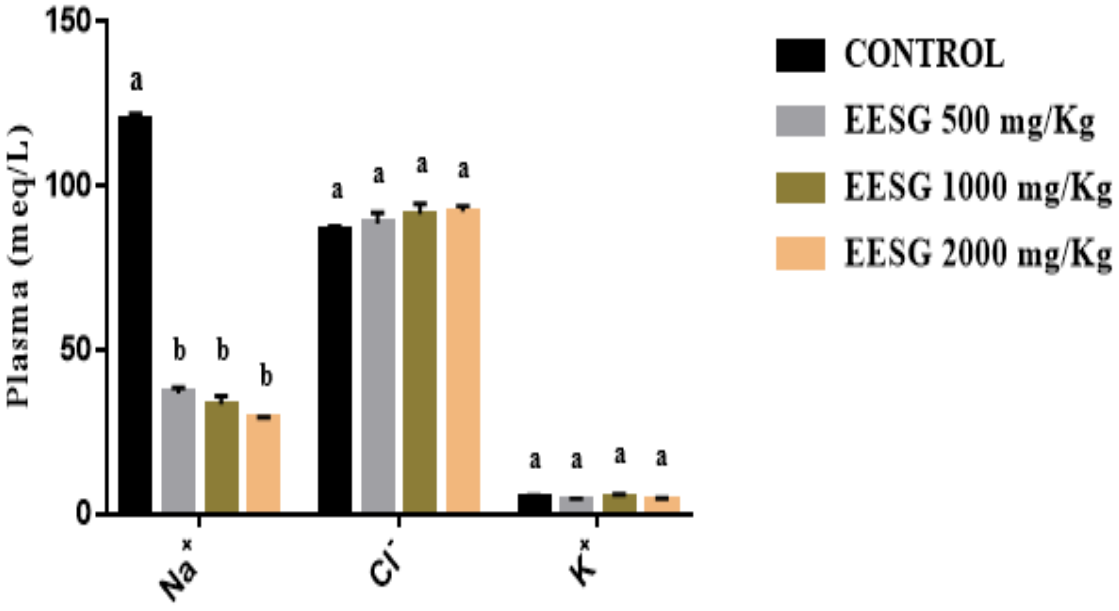

Fig. 7. Effect of varying doses of EESG on Plasma Sodium, Chloride and Potassium ion concentration of male *Wistar* Rats

Data with similar lower case alphabets are not significantly different (P > 0.05); data with different lower case alphabets are significantly different (P < 0.05). Data are Mean ± SD

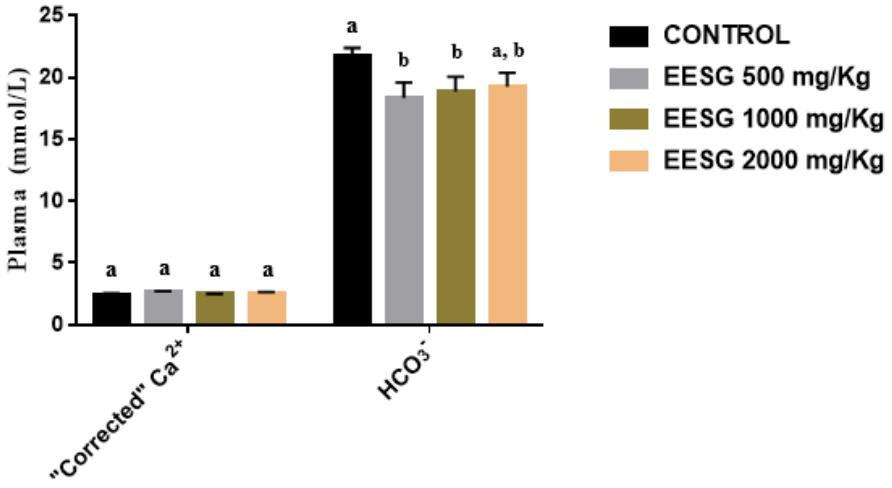

Fig. 8. Effect of varying doses of EESG on Plasma "Corrected" Ca^{+2} and HCO_3^- Concentration of Male *Wistar* Rats

Data with similar lower case alphabets are not significantly different (P > 0.05); data with different lower case alphabets are significantly different (P < 0.05). Data are Mean ± SD

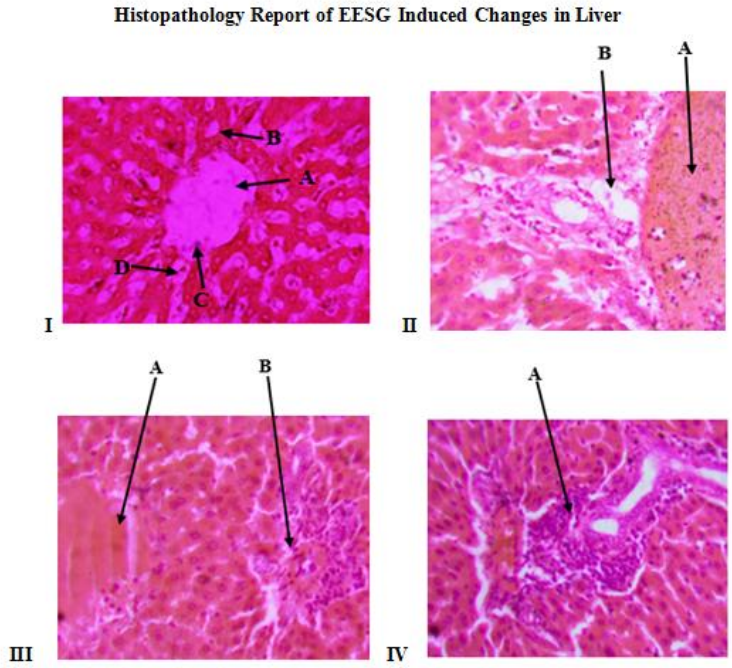

Plate I. Photomicrograph of sectioned liver of Control rats with normal/clear central vein, CV; (A), Normal Hepatic Artery, HA (B), Anastomosing plates of hepatocytes surrounds the portal tract (C), and hepatic sinusoids (D); normal lobular architecture. Plate II: Photomicrograph of sectioned liver of rats administered EESG 500 mg/kg indicates congested central vein, CV (A); congested hepatic Sinusoid (B), quite distinct steatosis; normal lobular architecture. Plate III: Photomicrograph of sectioned liver of rats administered EESG 1000 mg/kg indicates congested central vein, CV (A); inflamed periportal spaces (B); normal lobular architecture. Plate IV: Photomicrograph of sectioned liver of rats administered EESG 2000 mg/kg indicates inflamed periportal spaces (A); normal lobular architecture

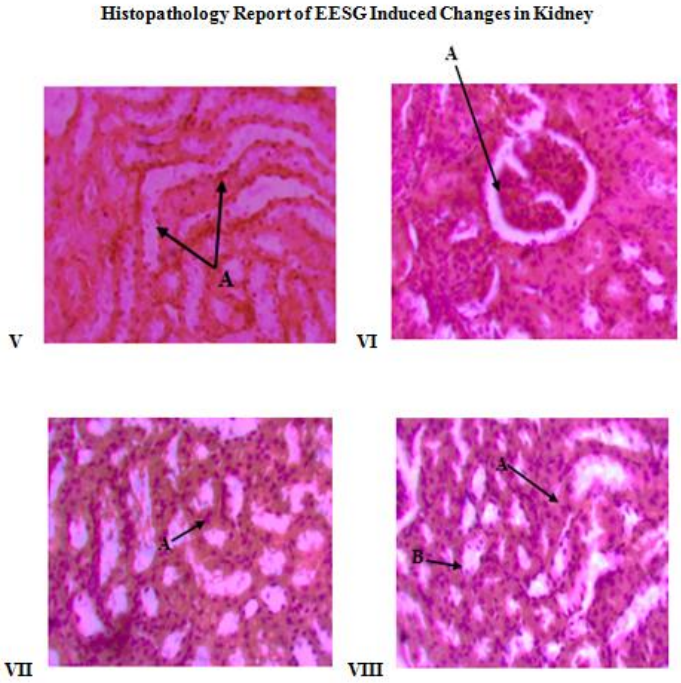

Plate V. Sectioned Kidney of Control rats shows normal tubules and normal glomerulus (A), Plate VI. Indicates that sectioned kidney of rats administered EESG 500 mg/kg with normal tubules and glomerulus (A). Plate VII. Indicate sectioned kidney of rats administered EESG 1000 mg/kg had normal tubules and glomerulus (A). Plate VIII. Shows sectioned kidney of rats administered EESG 2000 mg/kg with normal tubules (A) and glomerulus (B)

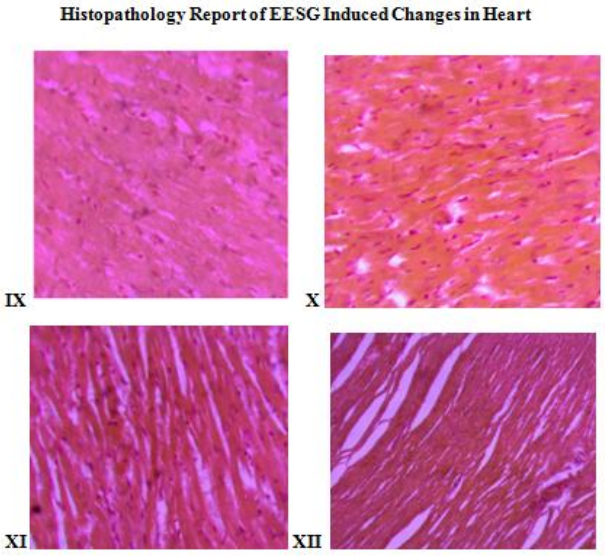

Plate IX. Photomicrograph of sectioned heart of control rats indicates normal myocardial fibril. Plate X. Sectioned heart of rats administered EESG 500 mg/kg indicates normal myocardial fibril. Plate XI. Sectioned heart of rats administered EESG 1000 mg/kg also indicates normal myocardial fibril. Plate XII. Sectioned heart of rats administered EESG 2000 mg/kg also indicates normal myocardial fibril

4. DISCUSSION

Oral acute toxicity study was carried out to evaluate the lethal dose (LD_{50}); possible immediate side effects as well as sub-chronic toxicity of ethanol leaf extract of *S. glauca* on male rats at varying doses. This approach is often adopted when testing the efficacy and (or) toxicity threshold of the proposed therapeutic agent. It is however imperative to ascertain the agonistic, antagonistic and toxic effect of plants on biochemical, histological and gravimetric parameters. A substance if poisonous, oftentimes will exhibit its effect within minutes; with the more poisonous substance eliciting toxic effect at relatively low doses [36]. Plants contain medicinal and anti-medicinal compounds; these may exist in varying proportion and hence should be evaluated in both regards according to [36] guidance document (N0. 425) on acute oral toxicity testing and considering the [35].

In this study, toxicity of ethanol leaf extract of *S. glauca* was tested. Several studies on acute toxicity of a number of related and unrelated plants have been reported [37,54,55]. [38] also reported that stem bark ethanol extract of *S. versicolor* (which belongs to the same family, *Simaroubaceae*) when administered to *Wistar* rats for 30 days; did not result to any observable signs of toxicity or mortality. In the present study, oral acute administration of EESG to rats at doses up to 5000 mg/kg was endured and did not result to fatality as no death of animal was recorded. As such, EESG is relatively safe with little or no noticeable immediate toxic effect. Therefore, in view of the outcome of acute toxicity assessment, dose up to 2000 mg/kg body weight was adopted for the sub-chronic toxicity study.

A decrease in body weight of animals on exposure to certain substance(s) over a period of time may portend the harmful nature of that test compound [56]. This is more so when there are observed deleterious changes in organ:body weight of vital organs such as the liver, kidney and heart respectively. In the present sub-chronic toxicity study, the increase in body weight of test rats orally administered respective doses of EESG indicate that the phyto-compounds inherent in EESG was not harmful; did not cause loss in body weight of rat (Figs. 1a & b). Although, it was observed that the increase in body weight of rats given EESG 1000 mg/kg was statistically not significant relative to the control. The presence of essential vitamins and minerals in their right proportion in a healthy state may stimulate appetite and increase body weight. Therefore, the increase in body weight of EESG-treated rats may not be unconnected with the vitamin content (Vitamin B1, B2, B3 and Vitamin C and A) of *S. glauca* [57]. Contrariwise, there was significant reduction ($P < 0.05$) in the liver: body weight ratio of rats given EESG 500 and 2000 mg/kg respectively, reduction ($P < 0.05$) in kidney: body weight ratio of rats administered EESG 1000 and 2000 mg respectively and a significant reduction in heart: body weight ratio of rats given EESG 2000 mg/kg (Fig. 2).

A large number of herbal preparations do not have drug regulatory approval to demonstrate their safety and efficacy [58]; therefore, it is obligatory to evaluate the safety of medicinal plants preparations and decoction via toxicological assessment studies on vital organs. Alterations, particularly increase in organ to body weight ratio may indicate toxicity-induced organ damage [59], tissue hyperplasia and enlargement of organs which are often signs of organ stress.

The data from the current study indicate reductions in liver, kidney and heart: body weight ratios at certain doses; whereas others were respectively not significantly different relative to their control (Fig. 2). The findings of the present study are at variance with the observations and report of organ (tissue) hyperplasia concomitant with toxicants [59]. Therefore, it is suggestive that *S. glauca* did not elicit significant aberration capable of architectural alteration of vital organs such as the liver, kidney and heart respectively (Plates I-XII). This is consistent with the findings of [37] who reported that oral dose of expeller extracted (EESO), solvent extracted (SESO) and refined (RSO) *Simarouba glauca* seed oil administered to test rats neither altered the vital organs' architecture nor affected the organ weight. Although there were mild congestion and inflammation in hepatic central vein and periportal spaces respectively of rats administered respective doses of EESG; this may be attributable to lengthy administration-induced toxicity of ethanol leaf extract at rather very high doses (Plates II-IV).

Elevations in ALT, AST and GGT activities in plasma or serum are often secondary to tissue damage [60]. The degree of liver damage induced by chemical compounds or otherwise may be evaluated by determining the level of

specific biochemical markers of liver function such as Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP) and Gamma-Glutamyl Transferase (GGT) respectively [61]. ALT is specific to the liver while AST is associated with the liver and heart. These enzymes are mainly found in the cytoplasm of animal cells [62,63] are subject to alterations in cases related to liver dysfunction and injury. Furthermore, increase in the activities of these enzymes in plasma implies rupture to cell membrane and (or) possible secretion of these enzymes into the bloodstream.

In the present study, the respective doses of EESG orally administered to test rats did not result to conspicuous clinically significant liver lesion (Plates II-IV). This is further validated by the observations that there were no significant changes in plasma ALT levels of test rats given varying doses of EESG relative to control (Fig. 3a); significant reduction ($P < 0.05$) in plasma AST and GGT activities of test rats given respective doses of EESG (Fig. 3a). It is worthy of note that plasma GGT activity is the most reliable liver function test (LFT); it has demonstrated a more sensitive liver diagnostic marker relative to other liver specific enzymes [64]. European document for ecotoxicology and toxicology has stated that the biological significance of the decrease in specific liver enzyme activity was unclear; as such, was typically dismissed as being of no toxicological relevance [65]. Contrariwise, the plasma ALP activities were significantly elevated ($P < 0.05$) in all group of test rats administered respective doses of EESG suggesting that such elevation in ALP activities may connote toxicological consequences of the use of *S. glauca*. However, several iso-enzymes of ALP exist in the liver, bones, placenta, kidneys and intestine. The activity of this enzyme is elevated in many clinical states; the most important being bone and liver diseases [55]. Significant elevations in plasma ALP activity without hepatic lesions have been reported linked to cholestasis [66].

In the study being reported, there were no significant elevations in plasma ALT, AST and GGT activities. This is further strengthened by the data presented in Figs. 3b & c which indicate that ALT and GGT measured in the liver, kidney and heart respectively were not decreased; not significantly different ($P > 0.05$) compared to their respective control. The data further suggests that EESG (*S. glauca*) is hepato-protective and there were no significant organ lesions recorded (Plates I-XII respectively); as such, these enzymes did not increase in the plasma (Fig. 3a). Therefore, with the normal levels of plasma ALT remaining normal and decrease in GGT activities, it stands to suggest that EESG did not aggravate on the overall, significant hepatocellular lesions especially at these test doses. The findings of the study being reported is at variance with the earlier report that elevated serum or plasma ALT and (or) GGT strongly indicate liver cell damage [64].

The significant increase in plasma ALP activities observed in the current study may have been due to secretions from other tissues capable of synthesizing iso-enzymes of ALP (tissue nonspecific ALP, TNSALP) [67,68,69] (Fig. 3a). With elevated plasma ALP and reduced GGT, it is unlikely that the increases in plasma ALP could have been of hepatic origin. Typically, increase in plasma ALP of hepatic origin is often accompanied by a simultaneous immediate rise in plasma GGT [64]; this therefore suggest that elevations in plasma ALP activity in plasma may not be unconnected with suspected biliary duct obstruction [66]. As earlier mentioned, increase in ALP activity may also be associated with bone disease; elevated serum or plasma ALP levels without concomitant increase in GGT activity points more to bone disorders, rather than liver diseases [55]. It is also important to note that the prominent congestions observed in the liver (Plates II-IV) of rats administered respective doses of EESG may have elicited hepatobiliary obstruction suggesting that ALP isoenzyme of the biliary duct (TNSALP) may have been secreted into the bloodstream (Fig. 3a). This may also have contributed at least in part to the overall increase in plasma ALP activity; the magnitude of this contribution nevertheless remains uncertain.

Quite a large number and amount of biological proteins are dissolved in the plasma; measurement of dissolved protein provides useful diagnostic information regarding the state or wellbeing of a subject. Evaluation of relevant fractions of total protein further reveals clinical information for the treatment and management of chronic diseases and conditions. Albumin makes up more than half of the total protein present in serum. Approximately 30 to 40% of the body's total albumin pool is found in the intravascular compartment. The remainder is extravascular and is located in the interstitial spaces, mainly of the muscles and skin [43]. Albumin does not diffuse freely through intact vascular

endothelium; hence, it is the major protein providing the critical colloid osmotic or oncotic pressure that regulates passage of water and diffusible solutes through the capillaries. Albumin accounts for 70% of the colloid osmotic pressure [43]. Albumin is predominantly synthesized in the liver; serves to transport bilirubin, hormones, metals, vitamins, and drugs and the rate of protein synthesis under normal condition is constant. Hyperproteinemia, hyperalbuminemia and hyperglobulinemia are indicative of pathological and toxicological conditions concomitant with aberrations in protein albumin and globulin fractions.

In the present study; given that there were no significant changes or alterations recorded in plasma total proteins and albumin concentrations of test rats administered varying doses of EESG (Fig. 4), it is therefore suggestive of non-interference with protein synthesis; that EESG did not impair the synthesizing function of the liver.

Bilirubin exists in conjugated and unconjugated forms. The residual circulating amount of conjugated bilirubin in the plasma of healthy individual is very minimal and as such, increase in plasma conjugated bilirubin strongly suggest hepatocellular dysfunction; whereas, is not the case with unconjugated bilirubin [70]. However, increase in unconjugated bilirubin (≥ 90 %) is indicative of acute hemolysis of red blood cells or Gilbert syndrome [70] and increased degradation of heme.

In the present study, the plasma total and unconjugated bilirubin were obviously not significantly different (Fig. 5) which indicates that respective doses of EESG leaf did not elicit visible significant hepatotoxic effect capable of compromising the liver's integrity. The significant reduction ($P < 0.05$) in plasma conjugated bilirubin recorded in test rats administered EESG 500 mg/kg further indicate that *S. glauca* did not alter the liver's excretory function particularly related to bilirubin conjugation; whereas others were not significantly different ($P > 0.05$) relative to their respective control (Fig. 5). Although, the liver histopathology reports presented in plates II-IV shows portal congestion, albeit, did not show hepatobiliary obstruction and hepatocellular lesions capable of hindering the liver's conjugative function. In fact, the reduction in conjugated bilirubin observed in the study being reported may further strengthen the suggestion of possible hepato-protective potential of *S. glauca*. That there were no significant alterations in plasma ALT at all doses of EESG investigated suggest that both the liver's architecture and function may well be intact; that at no dose was liver damage visible (Fig. 5).

The Kidney's function is evaluated by its capacity to effectively remove toxic waste products from the blood and regulate plasma electrolytes. Estimated urea and creatinine levels are a reliable acute kidney marker; may assist in diagnosis of kidney impairment [71]. Urea is a product of protein and purine metabolism; it's regarded as toxic when it exceeds allowable limits. Creatinine is an endogenously synthesized compound from creatine and phosphocreatine in skeletal muscles; its excretion from the blood is entirely dependent on the kidney's filtration capacity and thus, significant elevations in creatinine levels of serum or plasma may indicate glomeruli dysfunction.

In the present study, plasma creatinine levels increased significantly at 500 & 1000 mg/kg respectively. Likewise, there was significant increases in plasma urea levels as doses increased (Fig. 6). The implication of the data obtained in the study strongly suggests that the kidney's functional capacity may have been compromised by oral administration of EESG particularly at the aforementioned doses with respect to elevations in plasma creatinine levels. It has been reported that oral administration of leaf extracts of *S. glauca* to test animals is capable of stimulating increase in plasma urea and creatinine [72]; likely due to the presence of chemotherapeutic principles inherent in leaf of *S. glauca* (quassinoids glaucarubinone and alianthinone) [11] Therefore, the proportionate elevation in plasma urea as doses increased (Fig. 6) supports the earlier claim of [72]; may have resulted from increase in amino acids metabolism [18,20]; perhaps contributed to increase in plasma urea levels, necessitated by the effect of therapeutic compounds present in the plant [11]. The increase in plasma creatinine levels may not be unconnected with complication elicited by the EESG leading to poor glomerular filtration capacity. Albeit, the histopathology photomicrographs presented in Plates VI-VIII did not reveal significant tubular and (or) glomerular lesions. Nevertheless, elevations in urea and creatinine levels observed in the study being reported indicate that the kidney's detoxifying function was impaired.

Sodium ion (Na^+) is a major cation of the extracellular fluid; it contributes to the regulation of body fluid and osmotic pressure at the

extracellular and intracellular fluid compartments. Sodium may be ingested as sodium chloride available in food [73]. Hypernatremia may be experienced in hyperadrenalism, prolonged hyperpnea (increased breathing to meet oxygen and metabolic demand especially during physical exercise), increased renal sodium conservation in hyperaldosteronism, dehydration and excessive saline infusions amongst others [64] whereas, hyponatremia may be observed during vomiting, diarrhea, increased perspiration, natriuretics, diuretics abuse or slat-loss nephropathy, renal tubular acidosis, amongst others [64]. Chloride ion (Cl^-) is a major anion of the extracellular fluid compartment that contribute to electrolyte regulation and balance including proper hydration, osmotic pressure and acid/base balance [64]. Elevated serum chloride ion level is associated with dehydration, renal tubular acidosis, acute renal failure, diabetes insipidus, metabolic acidosis concomitant with prolonged diarrhea and loss of $NaHCO_3$, Salicylate intoxication, respiratory alkalosis; amongst others. Low serum chloride ions level is linked to excessive sweating, prolonged vomiting, persistent gastric secretion, salt-losing nephritis, aldosteronism, metabolic acidosis related to increased secreted organic anions, respiratory acidosis, potassium depletion associated with alkalosis and many others (Wu, 2006). Potassium ion (K^+) is a vital cation of the intracellular fluid compartment; also, an important constituent of the extracellular fluid due to its influence on muscle activity, acid-base regulation and osmotic pressure in body fluids [73]. Hyperkalemia is often associated with renal derangement, dehydration shock or adrenal insufficiency [64].

The data obtained from the present study shows that EESG administered at respective doses resulted to significant reduction ($P < 0.05$) in plasma sodium; while plasma chloride and potassium ion concentrations respectively were not significantly altered relative to the control (Fig. 7); that the electrolyte regulatory function of the kidneys was not compromised (Plates V-VIII). Interestingly, it was albeit obvious that EESG demonstrated natriuretic effect at respective doses of EESG; as plants capable of lowering sodium ion in the system are perceived as possible natriuretic agents.

Plasma HCO_3^- ion exist in plasma or serum as dissolved CO_2 and bicarbonate anion; available as physiological buffering system to maintain the internal environment regulated by the kidney. Plasma bicarbonate ion is elevated in compensatory response to metabolic alkalosis and respiratory acidosis whereas, it's lowered in response to metabolic acidosis and respiratory alkalosis respectively [74]. Ca^{2+} is a major element of physiological and biochemical importance. It's the most prevalent cation present in mammals; it functions in skeletal muscle mineralization, blood clot, neural conduction, regulation of skeletal and cardiac muscle tone, stimulatory secretion of exocrine glands amongst others [75]. Elevated calcium ion level is associated with primary and tertiary hyperparathyroidism, malignant disorders linked to bone diseases, carcinoma of breast, lungs or kidney, multiple myeloma, lymphoma and leukemia amongst others whereas low or reduced calcium ion level (hypoparathyroidism) is associated with vitamin D deficiency (Nutritional malabsorption), chronic renal failure, magnesium deficiency, osteomalacia, proximal and distal tubule diseases; amongst others [64].

In the present study, plasma "corrected" Ca^{+2} level was not significantly different ($P > 0.05$) (Fig. 8); while interestingly, there was significant reduction in plasma HCO_3^- at respective doses of EESG (Fig. 8) signifying compensatory response to renal tubular (metabolic) acidosis [75]; perhaps concomitant with hyponatremia (Fig. 7) [64].

5. CONCLUSION

The EESG orally administered to test rats was relative safe at LD_{50} above 5000 mg/kg. Although, EESG appeared to have elicited alterations in plasma ALP; perhaps contributed by TNSALP isoenzymes, apparently triggered by EESG-induced portal congestion; alterations in the kidney's functional capacity to effectively clear urea and creatinine respectively. Nonetheless, it did not result to elevations in specific liver enzymes and non-enzymes parameters; there were no hepatocellular, tubular and glomeruli or myofibril damages. The outcome suggests that EESG may have induced some biochemical alterations; albeit, it was neither significantly toxic to the liver, kidney nor heart and may be administered at lower doses in further studies.

ETHICAL APPROVAL

Animal Ethic committee approval was obtained to carry out this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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