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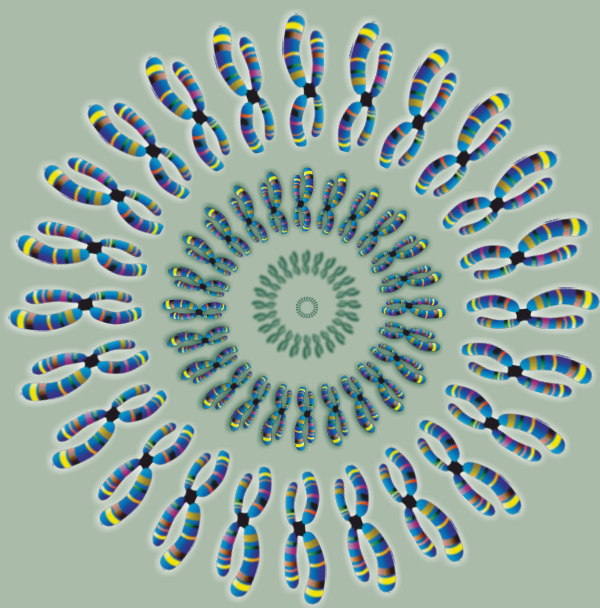

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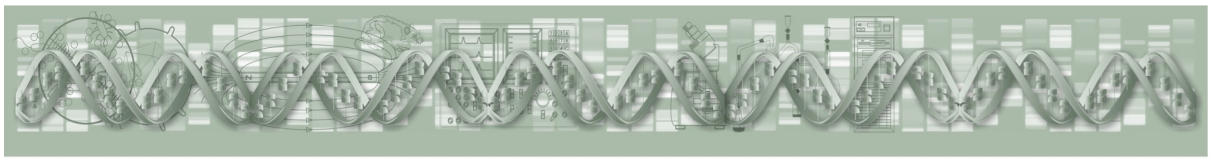

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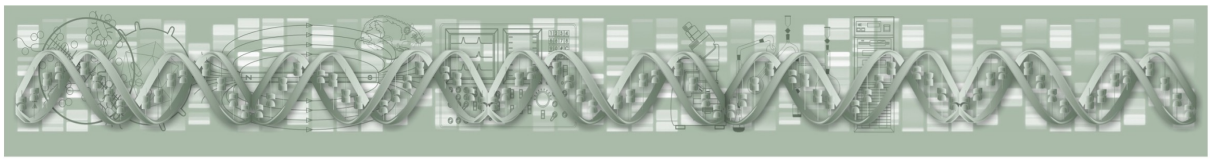

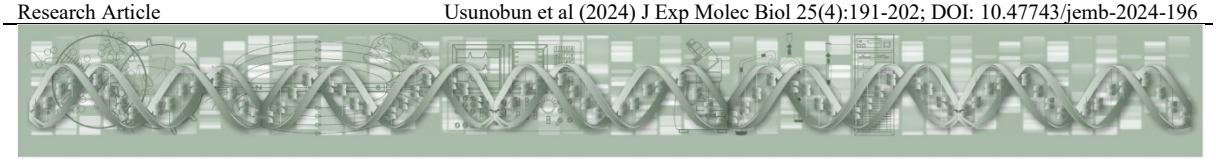

EFFICACY OF *CHROMOLAENA ODORATA* SUPPLEMENTATION ON METHOTREXATE-INDUCED NEPHROPATHY AND OXIDATIVE STRESS

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Abstract

We carried out this research to investigate the nephroprotective efficacy of aqueous leaf extract of *Chromolaena odorata* (AEOC) on kidney toxicity in methotrexate (MTX)-induced rats. Five groups of six albino wistar male rats each were used. Groups III-V were administered MTX at a dose of 7 mg/kg B.W. intraperitoneally from the 8th day for three days consecutively, while groups II and IV also received 250 mg/kg B.W. of AEOC orally once daily for ten days. Group I received normal saline and served as normal control. Creatinine and urea were analyzed in the serum. Superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), histopathological analysis were assessed using the kidney tissues, while blood collected in EDTA bottles was used for hematology. The obtained results revealed that three days of consecutive methotrexate administration caused a significant rise in kidney function parameters as well as severe oxidative damage and oxidative stress, as shown by a significant rise in creatinine and urea, an increased level of MDA and a significant drop in SOD and CAT. However, AEOC attenuated methotrexate-induced biochemical alterations and oxidative stress in the kidney. Also, AEOC alone or in combination with methotrexate showed an increase in packed cell volume (PCV), red blood cells (RBC), hemoglobin (HB), white blood cells (WBC), and platelet levels compared to the group treated with methotrexate alone, which showed degenerative levels in the analyzed hematological parameters. Showing corroboration, histopathological analysis showed deterioration and collapse of the kidney tubules in rats treated with methotrexate alone, whereas AEOC groups showed considerable nephroprotection and restoration towards normalcy. The results of this study revealed that the AEOC has a nephroprotective efficacy against methotrexate-induced kidney toxicity and damage via scavenging of free radicals and stabilization and/or enhancement of endogenous antioxidant status.

Keywords: *Chromolaena odorata,* kidney, methotrexate, nephroprotection, oxidative stress

Introduction

In vivo exposure to toxins and poisons represents the major cause of nephropathy (Mahi-Birjand et al. 2020), which is attributed to the use of medications such as methotrexate (MTX). Methotrexate is a drug commonly employed for the treatment of cancer and inflammatory diseases such as arthritis, systemic lupus erythematosus, and dermatomyositis (Dalaklioglu et al. 2013). MTX-mediated anticancer and immunosuppressive effects are owing to its function

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as an antagonist for folic acid (competitively inhibits dihydrofolate reductase, the enzyme that catalyzes the conversion of dihydrofolate to tetrahydrofolate), which in turn prevents cellular mitosis by inhibiting thymidylate, purines, and folic acid required for DNA synthesis (Gibson et al. 2019), preventing cells from dividing (Liu et al. 2019). Prolonged MTX use generates organ toxicities and damages. Reports have previously shown that oxidative stress and the free radicals released induce hepatotoxicity (Cetinkaya et al. 2006, Usunobun et al. 2022a; Ikponmwosa and Usunobun 2022). In addition to free radicals and reactive oxygen species (ROS) generation and accumulation, an insufficient antioxidant defense status promotes hepatotoxicity (Uraz et al. 2008, Usunobun et al. 2022a, Ikponmwosa and Usunobun 2022). The kidney, a crucial organ, is responsible for filtering the blood to remove wastes, maintaining water and electrolyte homeostasis, and regulating blood pressure (Jerman and Sun 2017). Because ~90% of administered MTX is excreted unchanged through the kidney, MTX is more likely to damage the kidneys than other organs by crystal nephropathy and/or tubular toxicity as a consequence of the overwhelming production of reactive oxygen species within the kidney (Dabak and Kocaman 2015). Thus, decreasing MTX toxicities and, in turn, restricting the discontinuation of MTX therapy is a current therapeutic challenge.

Herbal medicines have been used for decades for the treatment and management of various diseases by improving immunity and general health (Babich et al. 2020, Pelvan et al. 2022). Medicinal plants are rich in phytochemicals exhibiting antioxidant activity (Iuchi et al. 2003). *Chromolaena odorata* (L.) King & Robinson originated in North America and is also very common in Nigeria. It is known as Siam Weed, Awolowo, independent weed, Christmas Bush, Devil Weed, etc. (Lalith 2009, Ngozi and Osuji 2014). The fresh leaves or decoction of *C. odorata* (L.) King & Robinson has been widely used for wound treatment and healing due to its anti-microbial effects. Numerous investigations have demonstrated its effect against dermatological problems, diarrhea, fever, toothache, diabetes, and colitis (Odugbemi 2006, Akinmoladun and Akinloye 2007). Leaves of *C. odorata* possess insecticidal properties and are used as a green manure as well as for the preservation of dead bodies (Ukwueze et al. 2013). Other pharmacological properties of *C. odorata* leaves include antimalarial (Ongkana 2003), analgesic (Chakraborty et al. 2011), antipyretic, antispasmodic (Oludare et al. 2000), anti-gonorrhoeal (Caceres et al. 1995), fungicidal, diuretic (Gopinath et al. 2009), and blood coagulating (Triaratana et al. 1991). Some specific phenolic compounds that account for medical values have been identified and isolated from leaves of *C. odorata* (Metwall and Ekejinba 1981, Usunobun and Ewere 2016). The objective of this study was to elucidate the nephroprotective efficacy of aqueous leaf extract of *Chromolaena odorata* (AEOC) against nephrotoxicity induced by methotrexate in Wistar albino male rats.

Materials and Methods

Chemicals and Reagents

MEDVALIK Pharmaceuticals Limited, Lagos, Nigeria, was the source of the used methotrexate (liquid), 50 mg/2mL (Zuvius Life Sciences, India). All the other chemicals and reagents used in this research study were of analytical grade.

Collection and Identification of plant material

C. odorata leaves were obtained from Edo State University Uzairue in Iyamho, Edo State, Nigeria, and identification was carried out at Edo State University Uzairue, Department of Plant Biology and Biotechnology Herbarium with voucher number EUH/00066.

Preparation and Extraction of plant material

The obtained *C. odorata* leaves were well rinsed, air-dried at room temperature for four weeks, pulverized, and crushed into a fine powder using an electric blender. To prepare the aqueous extract, 1 kg of the powdered plant was soaked in 5 liters of double-distilled water for 48 hours

at room temperature with daily stirring for thorough extraction. Upon completion of 48 hours, filtration was carried out first with Whatman filter paper No. 42 (125 mm) and then with cotton wool. The collected filtrate was concentrated in a rotary evaporator at 40 ^{o}C to one-tenth its original volume and then reduced in a water bath to a solid form, which was weighed and dissolved in normal saline for administration to the rats on each experiment day.

Experimental Animal and Design

A total of thirty (30) Wistar male rats with a weight ranging from 180 to 200 g were obtained from Ambrose Alli University, Ekpoma, Edo State, Nigeria, and kept in a wood-gauze cage, an adequately ventilated room, and a well-lit animal house of the Department of Biochemistry, Edo State University, Uzairue, Nigeria. All the rats were given free access to water and fed with standard laboratory pellets. The Ethics Committee of the Faculty of Basic Medical Sciences at Edo State University Uzairue gave ethical clearance and approval for this study, and guidelines for ethical conduct in the care and use of non-human animals in research were rightly respected and followed (APA 2012).

After acclimatization for seven days, the rats were weighed and distributed into groups as follows: Group I orally received normal saline daily and was the normal control. Group II received AEOC (250 mg/kg B.W.) orally once daily for ten days. Group III: received MTX (7 mg/kg B.W.) intraperitoneally from the 8th day for three consecutive days. Group IV received AEOC (250 mg/kg B.W.) orally once daily for ten days and then administered MTX (7 mg/kg B.W.) intraperitoneally from the 8th day for three consecutive days. Group V received Vitamin C (100 mg/kg B.W.) orally once daily for ten days and then administered MTX (7 mg/kg BW) intraperitoneally from the 8th day for three consecutive days. The chosen MTX dose was based on the study of Aladaileh et al. (2019), while the chosen AEOC dose was based on the study of Ijioma et al. (2014).

Upon completion of the experiment, twenty-four (24) hours after the last administration, the rats were weighed, sacrificed, and blood taken in EDTA bottles for hematological analysis and in plain tubes to obtain serum. The blood collected in plain tubes was centrifuged at 4000 rpm for 30 min after being allowed to stand for 45 minutes to obtain serum for the determination of urea and creatinine. The kidney was carefully removed, washed in ice-cold saline, and weighed while a portion was fixed in 10% phosphate-buffered formalin for histopathological examination. The remaining portion was stored at -20 ^{o}C and a 10 % kidney tissue homogenate was prepared using a phosphate buffer solution at pH 7.34 for the assessment of superoxide dismutase (SOD), malondialdehyde (MDA), and catalase (CAT).

Biochemical Parameters

Urea was assessed using the RANDOX kit (United Kingdom) following the Fawcett and Scott (1960) method, while creatinine was assessed using the Jaffe' method. Malondialdehyde (MDA) was determined as postulated by Ohkawa et al. (1979). Superoxide dismutase (SOD) activity was assessed using the Misra and Fridovich (1972) method, while the Cohen et al. (1970) method was used to assess the activity of catalase (CAT).

Hematological parameters

Hematological analyses on parameters such as hemoglobin, white blood cells, red blood cells, platelets, and packed cell volume were carried out at the hematology laboratory, Irrua Specialist Teaching Hospital, Edo Central, Edo State, Nigeria.

Histopathological studies

After the euthanasia of the rats, kidney tissues were removed and rinsed in normal saline. The removed kidneys were thereafter fixed in 10% buffered formalin and sent to the Chemical Pathology Laboratory, University of Benin Teaching Hospital, Nigeria, for histopathological examination.

Statistical Analyses

All results were expressed as mean ± standard error of the mean, and statistically, SPSS and one-way analysis of variance (ANOVA) were used, with *P<0.05* being considered significant.

Results and discussions

Kidney Function Assessment

The efficacy of kidney function assessment as shown in Table 1 indicates a significant rise ($p<0.05$) in urea and creatinine in rats treated with 7 mg/kg MTX for three consecutive days, compared to the control group and groups treated with AEOC. However, rats given MTX and treated with AEOC or Vitamin C showed a significant drop ($p<0.05$) in urea and creatinine levels compared to rats given 7 mg/kg MTX alone for three consecutive days.

Table 1. Efficacy of aqueous leaf extract of *Chromolaena odorata* (AEOC) on kidney function assessment in methotrexate (MTX)-induced Wistar male rats

Treatment groups	Creatinine (mg/dl)	Urea (mg/dl)
Control	$0.80^{a} \pm 0.06$	$27.21^{a} \pm 2.79$
AEOC alone (250 mg/kg B.W.)	$0.81^{a} \pm 0.12$	$25.42^{a} \pm 1.70$
MTX alone (7 mg/kg B.W.)	$5.20^{b} \pm 1.03$	$89.12^{b} \pm 2.33$
MTX (7 mg/kg B.W.) + AEOC (250 mg/kg B.W.)	$1.59^{a} \pm 0.56$	$40.12^{c} \pm 1.63$
MTX (7 mg/kg B.W.) + Vitamin C (100 mg/kg B.W.)	$1.31^{a} \pm 0.19$	$38.41^{d} \pm 1.60$

Results are expressed as mean ± standard Error of the mean, n = 4. On the column, values with different superscripts (a, b, c, d) differ significantly ($p<0.05$)

Plasma infiltration and metabolic homeostasis maintenance remain important functions of the kidney. Nephrotoxicity has the capacity to retard kidney excretory activities and alter kidney physiology and structure (Zhang and Sun 2015, Shang and Falah 2019). Medicinal plants known to be easily available and cost-friendly remain best remedy for combating nephrotoxicity and hepatotoxicity caused by drug overdose (Yousuf et al. 2022). In this study, AEOC at a dose of 250 mg/kg B.W. ameliorated methotrexate (MTX)-induced kidney toxicity in Wistar male rats. As observed in this study in the group of rats administered MTX for three consecutive days, kidney impairment and damage are indicated by a buildup of creatinine and urea in the blood (Airaodion et al. 2020). The kidney toxicity could likely occur in the MTX-treatment group by precipitating MTX in kidney tubules and decreasing glomerular filtration rate. The findings of this work agree with similar reported works by Usunobun et al. 2022b, Usunobun et al. 2023). However, administration of AEOC or Vitamin C exhibited ameliorative effects, reducing the blood concentration of urea and creatinine in alignment with the works of Usunobun et al. 2022b, Usunobun et al. 2023. It is likely that the AEOC with its previously reported bioactive agents including tannins, flavonoids, alkaloids, and phenols (Usunobun and Ewere 2016) improved the cell membrane permeability and increased the clearance of creatinine and urea by the kidney.

Lipid peroxidation and antioxidant status assessment

To establish the antioxidant efficacy of AEOC, MDA concentration and SOD and CAT levels were assessed in tissue homogenates of the kidney, as shown in Table 2 below. Our study showed a significant rise *(P < 0.05)* in MDA concentration and a significant drop in SOD and CAT levels in the kidney of the MTX group. On the other hand, administration of AEOC caused

a significant drop *(P < 0.05)* in MDA concentration and noticeable improvement in levels of SOD and CAT in the tissue homogenate of the kidney.

Table 2. Efficacy of aqueous leaf extract of *Chromolaena odorata* (AEOC) on oxidative stress parameters of kidney in methotrexate (MTX)-induced Wistar male rats

Treatment groups	SOD (U/mg protein)	MDA (µmol/mg protein)	CAT (U/mg protein)
Control	$90.08^{a} \pm 3.15$	$1.83^{a} \pm 0.33$	$2.28^{a} \pm 0.20$
AEOC alone (250 mg/kg B.W.)	$96.63^{a} \pm 3.42$	$1.90^{a} \pm 0.27$	$2.42^{a} \pm 0.19$
MTX (7 mg/kg B.W.)	$45.66^{b} \pm 2.08$	$14.93^{b} \pm 2.09$	$0.50^{b} \pm 0.11$
MTX (7 mg/kg B.W.) + AEOC (250 mg/kg B.W.)	$69.89^{c} \pm 3.38$	$5.38^{c} \pm 0.99$	$1.51^{c} \pm 0.17$
MTX (7 mg/kg B.W.) + Vitamin C (100 mg/kg B.W.)	$73.55^{c} \pm 2.59$	$6.09^{c} \pm 1.05$	$1.83^{c} \pm 0.21$

Results are expressed as mean ± standard error of the mean, n = 4. On the column, values with different superscripts (a, b, c, d) differ significantly ($p<0.05$). SOD = superoxide dismutase; MDA = malondialdehyde; CAT = catalase

Indicating an increase in oxidative damage and oxidative stress, results from our study showed a marked decline in the levels of CAT and SOD as well as a marked rise in MDA in animals administered MTX alone compared to the animals in the control and extract groups (Table 2), similar to previous studies of Usunobun et al. 2022a, Ikponmwosa and Usunobun 2022. This could be because increased lipid peroxidation, as seen with the increased MDA, decreases membrane fluidity, thus altering the function of the plasma membrane and compromising its integrity (Wong-Ekkabut et al. 2007). However, AEOC or Vitamin C showed efficacy as treatment significantly alleviated MTX-induced oxidative damage and oxidative stress, as reflected by a reduction of MDA and enhanced endogenous SOD and CAT activities compared to the rats administered MTX alone similar to previous studies of Usunobun et al. 2022a, Ikponmwosa and Usunobun 2022). As observed in this study, AEOC enhanced endogenous antioxidant enzymes and acted as a scavenger of free radicals.

Hematological profile

The results of the efficacy of AEOC on hematological parameters on methotrexate (MTX)-induced Wistar rats are as shown in Figures 1-5 below. As observed in the figures, three days of consecutive 7 mg/kg MTX administration and subsequent toxicity showed a significant reduction in packed cell volume (PCV), red blood cell (RBC), hemoglobin (HB), white blood cells (WBCs), platelet levels when compared to the control and AEOC or Vitamin C group. However, AEOC or Vitamin C showed enhancement of hematological parameters compared to MTX degeneration.

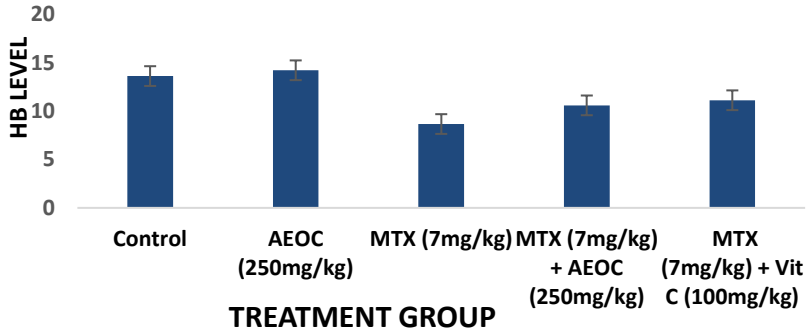

Figure A. Efficacy of the aqueous leaf extract of *C. odorata* (AEOC) on hemoglobin (HB) levels in methotrexate (MTX)-induced Wistar rats

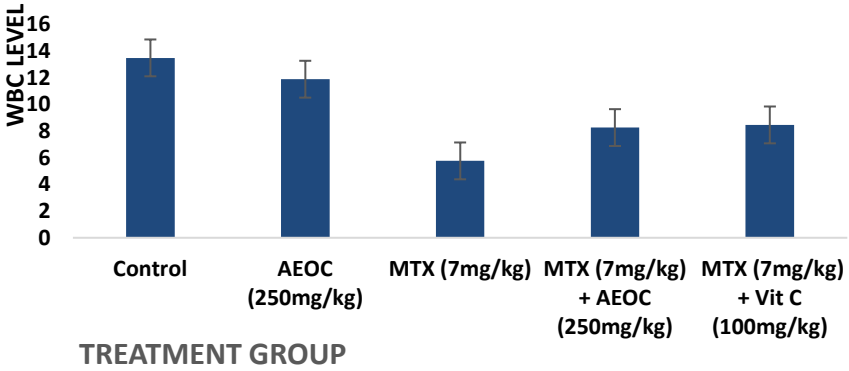

Figure B. Efficacy of the aqueous leaf extract of *C. odorata* (AEOC) on white blood cell (WBC) count in methotrexate (MTX)-induced Wistar rats

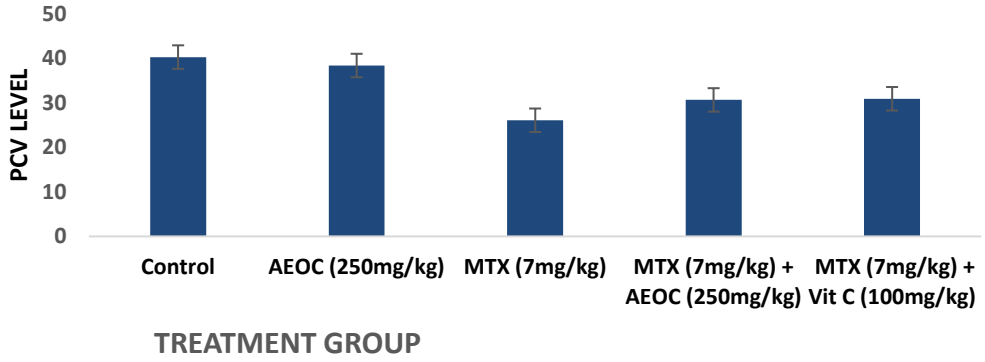

Figure C. Efficacy of the aqueous leaf extract of *C. odorata* (AEOC) on packed cell volume (PCV) in methotrexate (MTX)-induced Wistar rats

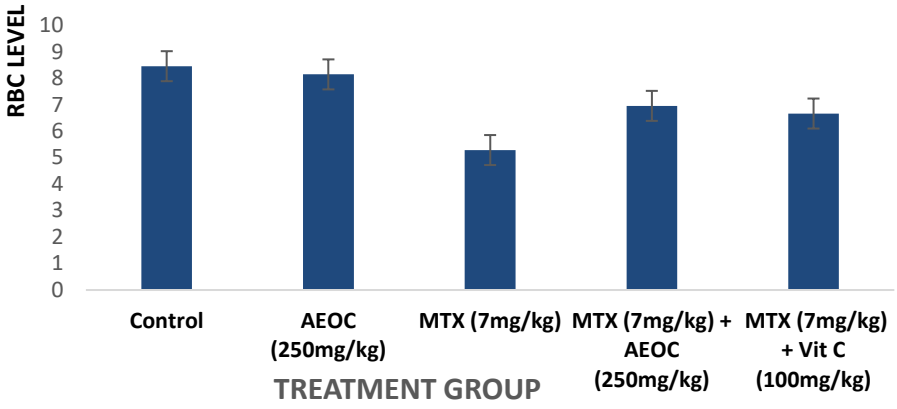

Figure D. Efficacy of the aqueous leaf extract of *C. odorata* (AEOC) on red blood cells (RBC) in methotrexate (MTX)-induced Wistar rats

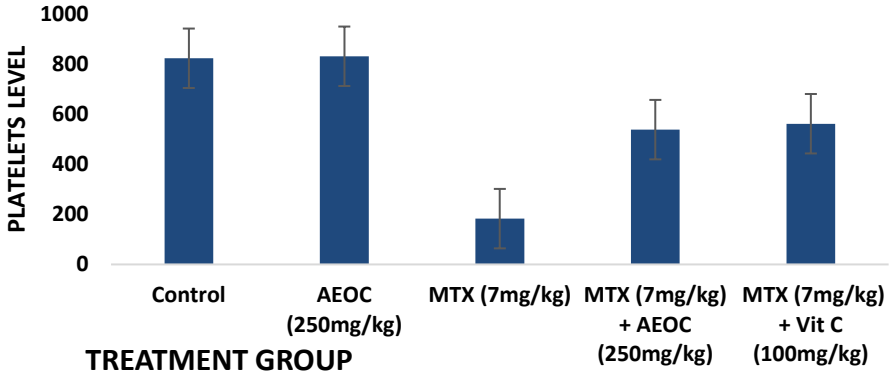

Figure E. Efficacy of the aqueous leaf extract of *C. odorata* (AEOC) on platelets (PLTS) in methotrexate (MTX)-induced Wistar rats

RBCs function in hemoglobin transport (Woo et al. 2012). As observed in this study in MTX-treated rats, a significant decline in RBC, HB, and PCV was observed, which is indicative of anemia in the rats. The observed significant decline in RBC count following MTX administration for three consecutive days imply slowed-down erythropoiesis as well as the destruction or death of mature RBCs. The trend in RBCs and WBC counts in this study aligns with Ohbayashi et al. (2010) and Rofe et al. (1994). However, administration of AEOC showed a significant increase in the aforementioned parameters, similar to those of Doğan (2018). The efficacy of AEOC on hematology can be attributable to secondary metabolites with antioxidant potential.

Histopathological assessment

The examination of the kidneys of control rats showed Malpighian corpuscles with glomeruli, which have Bowman's capsule surrounding them. The AEOC (250 mg/kg B.W.) treatment group had similar glomerular cell distribution as the control. However, the kidneys of MTX-treated rats showed congestion and necrosis in the tubules, marked glomerular collapse, and dilatation. The kidneys of rats administered AEOC and MTX showed fewer inflammatory cells around the glomerulus and improvement of the glomerular architecture. The kidneys of rats administered vitamin C and MTX also revealed massive recovery.

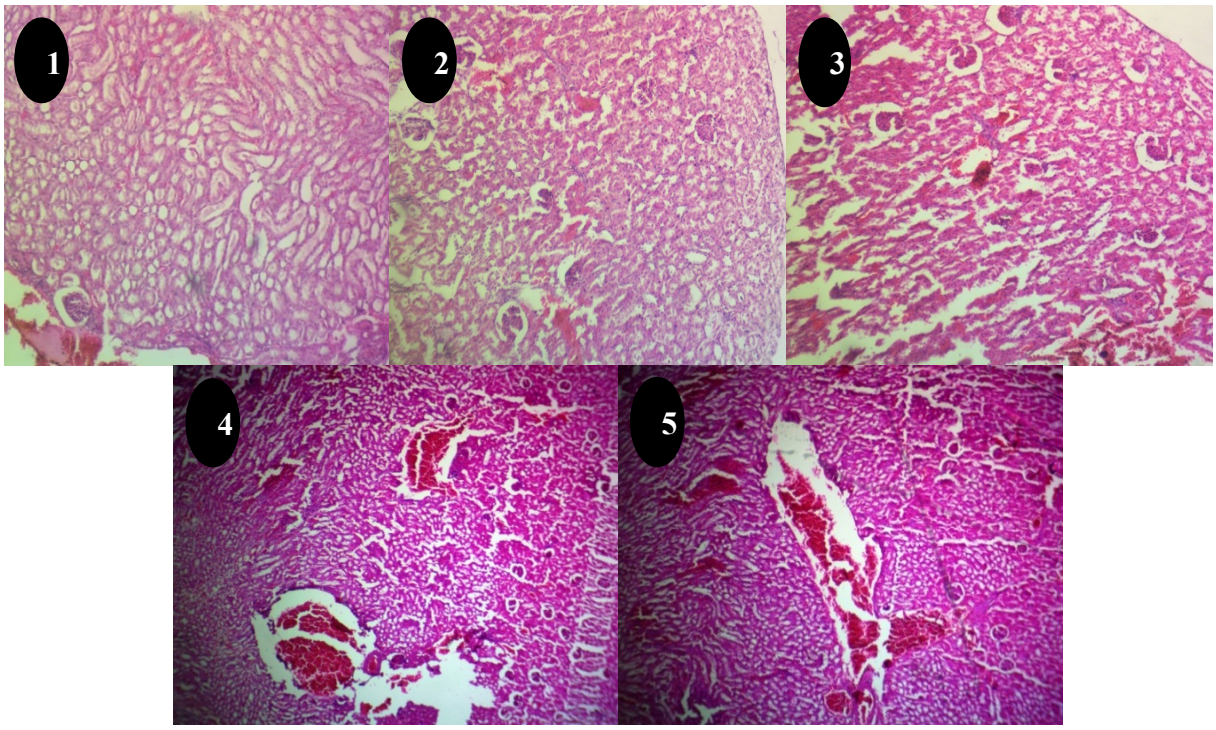

Figure 1. A photomicrograph of a rat kidney treated with normal saline solution (control) and sacrificed on the 11th day, showing Malpighian corpuscles with glomeruli surrounded by Bowman's capsule.
Figure 2. A photomicrograph of a rat kidney treated with AEOC alone and sacrificed on the 11th day showing normal architecture and similar glomerular cell distribution as the control.
Figure 3. A photomicrograph of a rat kidney administered methotrexate alone for three consecutive days and sacrificed on day 11 showing congestion, inflammation, marked glomerular collapse, dilatation, and necrosis in the tubules.
Figure 4. A photomicrograph of a rat kidney administered methotrexate for three consecutive days and AEOC and sacrificed on the 11th day, showing fewer inflammatory cells around the glomerulus and improvement in glomerular architecture.
Figure 5. A photomicrograph of a rat kidney administered methotrexate for three consecutive days and vitamin C and sacrificed on the 11th day showing reduced inflammation and massive recovery.

Our observed histological disturbances were corroborated by our observed increased serum urea and creatinine, both renal function indicators, whose above-normal increases indicate damage to the function of nephrons.

Conclusions

Bioactive agents in AEOC such as flavonoids, alkaloids, tannins, and phenols and their enhancement of endogenous enzymatic antioxidants improved AEOC potency and efficacy in reducing the effect of methotrexate-induced nephrotoxicity. This study established that AEOC possesses nephroprotective effects against methotrexate-induced kidney damage in rats via histopathology, biochemical parameters, improved endogenous enzymes (SOD and CAT), decreased oxidative stress and lipid peroxidation (MDA), improved hematopoiesis, and free radical scavenging properties. The mechanism of action of AEOC against methotrexate-induced nephrotoxicity can be attributed to the plant`s ability to fight and scavenge free radicals

as well as enhance and/or maintain endogenous antioxidant status, which defends the kidneys against oxidative damage and stress tension, thus motivating kidney healing and anti-inflammatory mechanisms.

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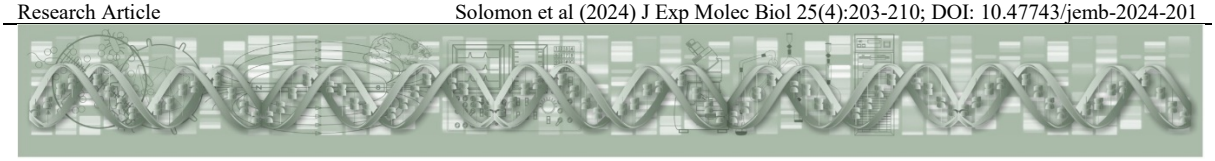

GROWTH PERFORMANCE AND SEMEN CHARACTERISTICS OF MONGREL RABBIT BUCKS FED DIETARY LEVELS OF GARLIC (*ALLIUM SATIVUM*) MEAL

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Abstract

This study was designed to evaluate the effect of garlic meal supplementation on growth performance and semen characteristics in rabbit bucks. Twenty-four (24) mixed breed rabbit bucks were used for this study. The bucks were allowed to acclimatize for two weeks before the commencement of the study, during this period, they were fed formulated diet and Calopogonium ad-libitum. Feed and clean drinking water were offered ad libitum to the experimental animals. Four different experimental diets were formulated with varying levels of 0.00, 0.50, 1.00 and 1.50% garlic meal as supplement and were coded as T1, T2, T3 and T4 respectively, with as control. The 24 bucks were weighed and randomly allotted into the four experimental treatments and replicated thrice with two rabbit bucks per replicate in a complete randomized design (CRD) for a period of 8 weeks. At the end of the feeding period, semen collection was done using a specially designed artificial vagina for rabbits. The data obtained were subjected to one-way analysis of variance (ANOVA) procedure using IBM Statistical Package for Social Science (SPSS) version 21. The result showed that garlic meal had significant effect ($P<0.05$) on final weight, total and daily feed intake. Garlic significantly influenced ($p<0.05$) sperm volume, motility, concentration and total cells per ejaculate however, did not affect sperm morphology. In conclusion, garlic meal supplementation at 1.0% (10g/kg) can improve some growth and seminal parameters in male rabbits.

Keywords: garlic, supplements, spices, sperm profile, semen, growth, performance

Introduction

The use of antibiotics as growth promoters over the years by farmers to improve the growth performance in livestock and also for the treatment and prevention of certain livestock diseases and infections have been banned in many Countries due to a reduced social acceptance by consumers of animal products for the fear of residual deposits. However, there has been a search for alternative replacements of antibiotics with natural feed additives (Onunkwor et al. 2022).
According to Lee et al. (2013), it has been known from ancient times that garlic possess biological, antibacterial, antifungal and antioxidants properties. Garlic as natural plant products are excellent growth promoter (Khan et al. 2012a) having no resistance and residues (Jin 2010) are used to increase feed efficiency. Garlic are readily available, less hazardous and affordable for financially disadvantaged farmers and this have attracted increasing interest as an alternative feeding strategy to replace antibiotics growth promoters.
Garlic as natural feed additives are commonly incorporated into the diets of livestock particularly rabbits and poultry, for the prevention of diseases, improve weight gain, feed

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efficiency, flavor, palatability and above all to exert a positive effect on rabbit buck's fertility (Abou-Elkhair et al. 2014). This is done by stimulating secretion of digestive enzymes, leading to aid digestion and absorption (Rehman and Munir 2015). According to Salah et al. 2014, garlic increased sperm concentration and improved male reproductive functions.
The aim of this study was to investigate the effects of dietary supplementation of garlic meal on growth performance and semen characteristics in mongrel rabbit bucks.

Materials and Methods

Experimental Site

The study was conducted at the Rabbitry unit of the Teaching and Research Farm, Department of Animal Science, University of Uyo, Uyo, Akwa-Ibom State. The study site as described by Solomon et al. (2023) is located at 50 1′N; 70 35′E with a mean annual temperature of between 26°C and 28°C, with mean annual rainfall ranging from 2000 mm – 3000 mm.

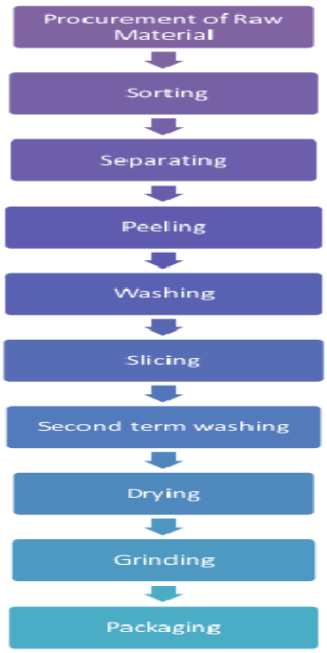
Experimental animals and management

Twenty-four (24) mixed-breed rabbit bucks were purchased and used for this study. The rabbit bucks were allowed to acclimate for a period of two weeks before the study commenced. During this period, the bucks were fed formulated diet and Calopogonium ad-libitum. Oxytetracycline 20% and ivermectine injection were administered intramuscularly and subcutaneously respectively against bacteria, internal and external parasites according to manufacturer's recommendation before the commencement of the study, which lasted for 8 weeks (56 days). The animals were housed in a wire-meshed rabbit hutch in the course the study. Fresh feed and clean drinking water were made available ad libitum to the bucks.

Sample Preparation

The fresh garlic cloves were purchased from Itam market, in Uyo Local Government Area of Akwa Ibom State, Nigeria. They were thoroughly washed under running water, peeled, cut into small sizes and air- dried for 6 days. The dried Garlic was ground into powder, stored in a bottle jar under room temperature and used as garlic meal in the study.

Sample preparation (Garlic Powder Meal)



Experimental Diets

Four different experimental diets were formulated with varying levels of 0.00, 0.50, 1.00 and 1.50% garlic meal as supplement and were coded as T1, T2, T3 and T4 respectively and represent in Table 1, with T1 as control diet.

Table 1. Experimental Diets

Ingredients	**T1 (0.00% DGM)**	**T2 (0.50% DGM)**	**T3 (1.00% DGM)**	**T4 (1.50% DGM)**
Maize	41.20	41.20	41.20	41.20
Soybean mean	12.80	12.80	12.80	12.80
Wheat offals	42.00	42.00	42.00	42.00
Bone meal	3.00	3.00	3.00	3.00
Common salt	0.25	0.25	0.25	0.25
Lysine	0.25	0.25	0.25	0.25
Methionine	0.25	0.25	0.25	0.25
Premix	0.25	0.25	0.25	0.25
Total	100.00	100.00	100.00	100.00
Calculated Composition				
Metabolizable Energy (Kcal/kg)	2675.50	2675.50	2675.50	2675.50
Crude protein (%)	16.68	16.68	16.68	16.68
Crude fibre (%)	6.23	6.23	6.23	6.23
Ether extract (%)	3.54	3.54	3.54	3.54

Data Collection

The bucks were weighed using an electronic kitchen scale with 1g sensitivity at the commencement of the study, to obtain their initial weights. They were subsequently weighed weekly to monitor weight changes and recorded. The individual feed intake was obtained as the as the difference between the feed offered and left over after a 24-hour feeding.

Weight gain per rabbit was calculated as the difference between final weight and initial weight. The feed conversion ratio was computed by dividing the total weight gain by the total feed intake as follows:

Feed conversion ratio = feed intake/weight taken

Semen Collection

A specially designed artificial vagina (AV) for rabbits was used to collect semen collection from the bucks, by introducing a rabbit doe as a teaser into the buck's cage. The buck was careful observed, and the AV placed gently at the vulva of the doe as the buck and mounted the doe, so as to direct the penis into the AV for penetration and ejaculation.

Semen evaluation

The semen samples obtained were examined using a compound microscope as described by Shinkut (2015), and adopted by Solomon et al. (2023). This method entails the visual or gross examination of the semen sample immediately after collection to assess the volume and colour. microscopic examination for motility, concentration, percentage live spermatozoa and morphological abnormalities was carried out thereafter.

Experimental Design

The bucks were weighed and randomly assigned into four experimental groups and replicated thrice to have two rabbit bucks per replicate, in a completely randomized design (CRD) for a period of 8 weeks.

Statistical Analysis

The data obtained from the study were subjected to one-way analysis of variance (ANOVA) procedure using IBM Statistical Package for Social Science (SPSS) version 20. Were significant differences were observed, the means were separated using Duncan of the software.

Results

Growth Performance of rabbit buck fed diets supplemented with garlic meal

The results on growth performance of mongrel rabbit bucks fed varied levels of dietary supplementation of garlic meal are presented in Table 2. The result showed that garlic meal had a significant effect ($P<0.05$) on final weight, total and daily feed intake. Total and daily weight gains and feed conversion ration were also influenced ($p<0.05$) by garlic meal supplementation in the bucks' diets. Higher final weights were observed in rabbit groups whose diets were supplemented with garlic meal except those of rabbit bucks in T4 that had similar statistical value ($p>0.05$) with bucks in the control group. Final weights observed in the study were 1593.50, 1658.50, 1789.50 and 1653.33 g for T1, T2, T3 and T4 respectively. Rabbit bucks with 1.00% (T3) and 1.50% (T4) garlic in their diets recorded significantly higher ($p<0.05$) total and daily feed intake compared to those with 0.00% (T1) and 0.50% (T2) garlic supplementation in their diets respectively. The total feed intake was 4154.00 and 4154.83g for bucks in T3 and T4 respectively, while bucks in T1 and T2 recorded total feed intake of 3976.00 and 4086.00 g respectively. Garlic meal supplementation did also affect the feed conversion ratio, as values observed in the study varied statistically ($p<0.05$) in all treatment groups.

Table 2. Growth Performance of Mongrel Rabbit Bucks Fed varied levels of Garlic powder Meal

Parameters	T1 (Control)	T2 (0.5% GMP)	T3 (1.0% GMP)	T4 (1.5% GMP)	SEM
Initial weight (g)	1147.67	1146.33	1147.83	1143.50	13.83
Final weight (g)	1593.50^{b}	1658.50^{ab}	1789.50^{a}	1653.33^{ab}	32.05
Total weight gain (g)	445.83^{b}	517.17^{ab}	641.67^{a}	509.83^{ab}	33.47
Daily weight gain (g)	7.96^{b}	9.15^{ab}	11.46^{a}	9.11^{ab}	0.60
Total Feed intake (g)	3976.00^{c}	4086.00^{b}	4154.00^{a}	4154.83^{a}	22.72
Daily feed intake (g)	71.00^{c}	72.97^{b}	74.18^{a}	74.20^{a}	0.41
Feed Conversion Ratio	9.18^{a}	8.13^{ab}	6.53^{b}	8.79^{b}	0.59

a, b, c - Means on the same row but with different superscripts are significant at $P< 0.05$; GMP-Garlic Powder Meal

Semen Characteristics of rabbit bucks fed diets supplemented with garlic meal.

The results on semen profile of rabbit bucks administered supplemental levels of garlic meal are presented in Table 3. The results revealed that garlic significantly influenced ($p<0.05$) sperm volume, motility, concentration and total cells per ejaculate. However, garlic meal did not affect sperm morphology as percentage normal and abnormal sperm cells were statistically similar ($p>0.05$) in all treatment levels.

Table 3. Semen characteristics of Rabbit bucks fed diets supplemented with garlic meal

Parameters	T1 (Control)	T2 (0.5% GMP)	T3 (1.0% GMP)	T4 (1.5% GMP)	SEM
Volume (ml)	0.59^{b}	0.58^{b}	0.71^{a}	0.68^{a}	0.02
Colour	Milky	Milky	Milky	Milky	
Motility (%)	66.00^{c}	70.67^{b}	71.00^{b}	75.33^{a}	1.06

Concentration (x 10^6/ml)	75.67^b	81.00^b	96.33^a	94.33^a	2.75
Normal cells (%)	71.00	73.33	80.33	77.67	2.19
Abnormal cells (%)	29.00	26.67	19.67	22.33	2.19
Live cells (%)	81.67	84.67	85.33	84.33	1.30
Dead cells (%)	18.33	15.33	14.67	15.67	1.30
Total cells/ejaculate) (X 10^6/ml)	44.65^c	46.96^c	68.36^a	64.11^b	3.16

a, b, c - Means on the same row but with different superscripts are significant at (P< 0.05); GMP-Garlic Powder Meal, SEM – Standard error of mean

Semen volume was higher in treatments 3 (0.71ml) and 4 (0.68ml) respectively compared to those of bucks in T1 (0.59 ml) and T2 (0.58 ml). Garlic meal supplementation in the bucks' diets did not affect (p>0.05) the semen colour as all treatments recorded milky colour. This study observed a significant increase (p<0.05) in sperm motility with garlic meal supplementation in bucks' diets. Values observed were 66.00, 70.67, 71.00 and 75.33% for T1, T2, T3 and T4 respectively. Garlic meal supplementation also increased semen concentration in T3 and T4 when compared to T1 and T2. Bucks in T3 recorded sperm concentration of 96.33 X 10^6/ml while bucks in treatment group 1, 2 and 4 recorded values of 75.67, 81.00 and 94.33 X 10^6/ml respectively. Live and dead sperm cells were not influenced by garlic meal supplementation in the bucks' diets. Live cells percentage observed were 81.67, 84.67. 85.33 and 84.33% for T1, T2, T3 and T4 respectively. Garlic meal supplementation significantly increased (p<0.05) total sperm cells per ejaculate with higher values recorded in T3 (68.36 X 10^6/ml) followed closely by T4 (64.11 X 10^6/ml) while T1 and T2 had similar (p>0.05) values of 44.65 and 46.96 X 10^6/ml respectively.

Discussions

The significant result observed in final weights in this study agrees with the statement of Abdelnour et al. (2018), who highlighted the use of plant-based supplements in livestock production to improve growth performance. This therefore implies that incorporating such plant-based supplements into rabbit feeds may enhance nutrient absorption and utilization as documented by Földešiová et al. (2015). Reports by Olaleye et al. (2006), and Panggabean et al. (2019), described garlic as a herb which acts as growth promoter with ability to reduce the degradation of amino acids, thereby causing improved weight gain due to the availability of more amino acids. The increased daily and total feed intake of mongrel rabbit buck fed diets containing 0.50, 1.00 and 1.50% GPM respectively resulted in the increase in bucks' weight when compared to the control. This improvement could be as a result of the bioactive constituents of the garlic powder meal on the digestibility process as reported by Mandey et al. (2019) and Suliman et al. (2021). There was a significant improvement in feed intake of bucks in this study, particularly in rabbit bucks fed diets containing 1.00% garlic meal which could be due to enhanced appetite, increased pancreatic and digestive enzymes secretion, and improved intestinal digestibility as noted by Arif et al. (2022). Earlier reports (Dhama et al. 2015, Sapsuha et al. 2021), again, noted that the presence of garlic meal could deactivate the growth of pathogenic bacteria, enhance the multiplication of beneficial bacteria, and accelerate nutrient metabolism and absorption in the digestive tract which may result in the overall improved body weight of rabbit bucks

Better feed conversion ratio (FCR), was observed in bucks fed diets containing 1.00% garlic supplementation in their diets, implying that the feeds were better utilized due to the enhanced

production of endogenous enzymes, pancreatic juices, and bile salt triggered by the synergetic effects of the bioactive components of the garlic when compared to the control according to Adu et al. (2020) and Sapsuha et al. (2021).

Effect of garlic meal supplementation on semen characteristics of rabbit bucks

The results on semen profile of rabbit bucks in this study showed significant increase in semen volume at 10 and 15 g/kg (T3 and T4 respectively) supplementation in the diets. This however, differs from the result of Shinkut et al. (2016), who recorded reduced semen volume with garlic meal at 2.5 and 5.0% supplementation in their study. This variation may be due to higher doses used in comparison with this study. The authors also attributed the observed increase in mean ejaculate volume of the control when compared to the treated groups to possibility of contamination of the semen by urine as reported by Jordi et al. (2005), who noted a 13% chance of contamination of rabbits' semen by urine during collection when using artificial vagina. Earlier report (Campos et al. 2014), put the normal ranges of semen volume between 0.3-0.6 ml, which is below the values observed in T3 and T4. This observation is however similar to those of Ekuma et al. (2017), who observed that the supplementation of garlic alone, combination of garlic and vitamin E significantly increases the sperm motility and spermatozoa proportion in their study. Higher motility was observed in bucks fed diets with garlic supplementation than in those without garlic supplementation. This observation however, disagrees with the report of Shinkut et al. (2016), and Omotoso et al. (2012), who in their respective studies, observed no significant difference in sperm motility in rabbit bucks, and a decrease in sperm motility in Wister rats administered dietary levels of garlic.

The higher sperm concentration observed in the study is similar to earlier reports (Salah et al. 2014, Shinkut et al. 2016, Ekuma et al. 2017). Ajao and Ola (2022), stated that the general positive impact of spice supplementation on sperm parameters can be attributed to the spices' antioxidant properties and androgenic effects as supported by Ramandeep et al. (2013) and Morakinyo et al. (2010), which they noted have the ability to enhance sertoli cells activities in maintaining sperm production. Normal and abnormal sperm cells as well as live and dead sperm cells were not affected by garlic supplementation in this study. These results, however, vary from the report of Ajao and Ola (2022) and Ekuma et al. (2017), who observed significant differences in these parameters in their respective studies but, similar to the report of Shinkut et al. (2016), who also observed no significant effect of garlic supplementation on these parameters. Garlic meal supplementation significantly increased the total sperm cells per ejaculate in bucks fed T3 diet, followed by bucks fed T4 diet. However, bucks fed the control diet T1 and T2 had similar values.

Conclusions

The findings of this study showed that garlic meal had a significant effect on final weight, total and daily feed intake. Garlic meal supplementation can also increase semen volume, motility, concentration and total sperm cells per ejaculate. Hence, supplementation of garlic meal in rabbit bucks' diets at 1.0% (10 g/kg), can improve final weight, feed intake and semen parameters.

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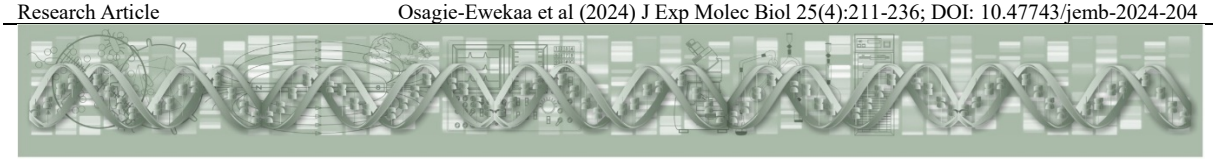

PROPHYLACTIC ANTIHYPERTENSIVE EFFECT OF EXTRACT OF *SIMAROUBA GLAUCA* ON SALT-LOAD INDUCED HYPERTENSION IN NORMOTENSIVE MALE WISTAR RAT

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Abstract

Simarouba glauca has been reported to demonstrate a wide range of medicinal properties; including folklore management of hypertension disorder. The current study focused on the application of aqueous leaf extract of *Simarouba glauca* (AESG) as a potential prophylactic anti-hypertensive agent in male *Wistar* rats, following salt-load induced hypertension. A total of 15 experimental adult male *Wistar* rats weighing between 184 and 244 g were used for the study. The rats were allotted into five (5) groups of 25, 50, and 100 $mgkg^{-1}$ body weight AESG; group that received 8 % NaCl for one week to induce hypertension; replaced with 0.9 % NaCl daily in drinking water for 4 weeks; the normotensive group, received food and water only *ad libitum*. Body weights and relevant hemodynamics were obtained weekly for four weeks, using the non-invasive (tail-cuff) MRBP system according to the method described by Bunag and Butterfield. Biochemical evaluation and histopathology investigation were conducted on blood plasma and relevant tissues respectively after 4 weeks according to previously established and reported methods; data were analyzed with *GraphPad* Prism, version 9 and presented as mean ± Standard Deviation. The results indicated that salt-load elicited significant weight loss; elevated hemodynamics; particularly, systolic and diastolic blood pressures; altered relevant biochemical indicators of hepatic and renal functions. Inversely, groups pre-treated with respective dose of AESG exponentially gained weight, significantly prevented alterations of hemodynamics and mitigated relevant biochemical indicators and pathological changes in relevant organs. Pre-treatment with AESG; particularly at 50 $mgkg^{-1}$, remarkably demonstrated significant anti-hypertensive potential.

Keywords: medicinal plants, blood pressure, antihypertensive, *Simarouba glauca*

Introduction

Hypertension is a disorder of public health importance characterized by elevated systolic blood pressure of ≥ 140 mmHg and a diastolic Blood Pressure of ≥ 90 mmHg after repeated visits to a qualified health practitioner. The World Health Organization has estimated that high blood pressure causes one in every eight deaths; reported that approximately seven hundred million people are currently living with untreated hypertension; making hypertension the third leading cause of mortality in the world. The health Organization further stated that the number of people living with hypertension has doubled to 1.28 billion since 1990 as at the time of the report (Campbell et al. 2022). Globally, there are one billion hypertensive people; four million people die annually as a direct result of complications associated with hypertension (Campbell et al. 2022).

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Hypertension is categorized in two types, primary or essential (90-95 % of hypertensive cases) and secondary (5-10 %) hypertension. Although, the specific cause of primary hypertension is yet to be linked to any known or diagnosed related cause; is yet to be explicated. However, pathophysiological contributors such as stress, dysregulation of the sympathetic nervous system, over-stimulation of vasoconstrictors, dysregulation of the Renin-Angiotensin-Aldosterone system (RAAS); resulting to excessive sodium ions retention; decreased production of vasodilators such as prostacyclin, nitric oxide (NO), and natriuretic peptides; resulting from endothelial dysfunction, oxidative stress-induced vascular remodeling; obesity; and diabetes mellitus, atherosclerosis and amongst others are speculated to be responsible for the development of essential hypertension; whereas, secondary hypertension has been strongly reported to be associated with known underlying factors such as apnea, drug-induced, neurological, and/or endocrine dysfunction (such as aldosteronism) (National Clinical Guideline Centre (UK) 2011). Secondary hypertension develops less rapidly, when compared with primary or essential hypertension. It has been reported that non-essential or secondary hypertension has been linked to identifiable; modifiable causes; when not properly managed can provoke cardiovascular complications; multi-organ dysfunction and failure such as peripheral arterial diseases, cardiac dysfunction and cardiomegaly, renal failure, retinal hypertension; amongst others (British Heart Foundation 2015).

Blumenthal et al. (2015) also reported that lifestyle modifications, such as exercises, weight reduction, Dietary Approach to Stop Hypertension (DASH); which includes low-salt diets have demonstrated a 70 % prospect to lowering blood pressure, enhance antihypertensive drug efficacy and decrease risk of cardiovascular complications.

The reports of Campbell et al. (2022) and Lovibond et al. (2011), have equally revealed that the cost of managing hypertension with allopathic medicine such as angiotensin II receptor antagonists, alpha blockers, angiotensin converting enzyme inhibitors, Beta blockers, calcium channel blockers, diuretics, direct rennin inhibitors, vasodilators has raised concern for decades. This is coupled with comorbidities and the adverse effects such as muscle cramps, dizziness, extreme tiredness, dehydration, blurred vision, abnormal heart rate, skin rash, cough, vomiting, kidney failure, fever, sore throat, diarrhea, fatigue, headache, diarrhea, constipation, edema, swollen ankle, weakness, depression, hallucinations, insomnia, impotence, palpitation and a host of others associated with the application of antihypertensive drugs (Lovibond et al. 2011, Park et al. 2017).

The recent cross-sectional studies reported by Zhao et al. (2020), Lorbeer et al. (2017) and Qian et al. (2016) have implicated the Non-Alcoholic Steatohepatitis as an independent risk factor to the onset of hypertension and related cardiovascular complications; as well as demonstrated clinical evidence that hypertension may promote the onset of the Non-Alcoholic Fatty Liver Disease (NAFLD) and the progression of liver injury; amongst others. Studies have equally reportedly suggested that NAFLD may promote hypertension by initiating systemic inflammation, capable of triggering triglyceride and lipid intermediate accumulation in the liver that may result to hepatocellular lipotoxicity and (or) hepatocyte damage (Qian et al. 2016, Lorbeer et al. 2017, Zhao et al. 2020).

The study of Landazuri et al. (2017) reported the application of numerous pharmacological agents of medicinal plants origin. The study revealed that inherent biological compound(s) in medicine plants have prospects in management and possible treatment of hypertension. According to the 1977 World Health Assembly (WHA) report, approximately 70-80% of the world's population rely on non-conventional medicine mainly from herbal sources for primary health care, particularly in the developing countries where the cost of consulting orthodox medical practitioners and the price of antihypertensives are beyond the reach of a majority of the affected category; hence, the experimental application of potential bioactive compounds inherent in *Simarouba glauca* leaf. Considering the cost implication of managing hypertension

and related complications; the adverse effects of allopathic agents; the paucity of data on the cardiovascular potential effect of *Simarouba glauca* (*S. glauca*), the study seek to evaluate the potential anti-hypertensive efficacy of *S. glauca* on experimental salt-load induced hypertensive of male *Wistar* rat hitherto pre-treated with respective dose of *Simarouba glauca* aqueous leaf extract; as well as the pathophysiological impact of induced-hypertension on liver metabolism and the electrolyte homeostatic function of the kidney.

Patil and Gaikwad (2011) reported that *Simarouba glauca*, commonly known as "Paradise tree" or "Laxmitaru" belongs to the family Simaroubaceae. *S. glauca* has a long history of herbal medicine applications, considering its numerous pharmacological properties. Joshi and Joshi (2002), equally reported that the bioactive chemicals present in leaf, fruit, pulp and seed of *S. glauca* have demonstrated analgesic, antimicrobial, antiviral, astringent, emmenagogue, stomachic, tonic, vermifuge properties. The major active groups of phytochemicals in *S. glauca* are the quassinoids, which belong to the triterpene chemical family. Ailanthinone, glaucarubinone and holacanthone are considered as some of the main active quassinoids available in genus, *Simarouba*. Other chemicals include benzoquinone, canthin, dehydroglaucarubinone, glaucarubine, glaucarubolone, melianone, simaroubidin, simarolide, simaroubin, simarubolide, sitosterol and tirucalla (Technical Data Report 2002).

Materials and Methods

Collection of *S. glauca* leaves and Aqueous Extraction of Compounds

Fresh leaves of *S. glauca* was harvested from Cercobela Farms® located at Ubiaza, Esan South East Local Government Area of Edo State, Nigeria. A fresh plant specimen was deposited at the Department of Plant Biology and Biotechnology Herbarium, University of Benin, Benin City, Nigeria and authenticated with a voucher N0. UBH$_S$382. The leaves were rinsed with distilled water and air-dried at room temperature at the Department of Biochemistry, University of Benin, for twenty-eight (28) days. According to the extraction method previously described by Osagie-Eweka et al. (2016), the leaves were pulverized and sieved at the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, to obtain fine a fine powder. A 500 g leaf powder was soaked in 2.5 L of distilled water and stirred at intervals for 24 h, and filtered. The extract plant material was re-macerated in another portion of 2.5 L of distilled water and stirred at intervals for another 24 h. Both filtrate portions were pooled and freeze-dried at the Department of Biochemistry, Adekunle Ajasin University, Akungba-Akoko, Ondo State to obtain a fine powdered aqueous leaf extract (AESG); with a yield of 6 % w/w extraction. The extract was stored in a sterile bottle in the refrigerator at 18°C until required for analyses.

Plate 1.1. *S. glauca* plant in its natural habitat (Cercobela Farms®, Ubiaja), Osagie-Eweka photo library (2014)

Experimental Animals

A total of 15 experimental adult male *Wistar* rats weighing between 184 and 244 g were used for the study. The animals were housed in metabolic cages; fed and were maintained under laboratory conditions of 12 h light / 12 h dark cycle and were acclimatized for two weeks prior

to commencement of studies. *Wistar* rats in this study were handled in accordance with the international, natural and institution guidelines for care and use of laboratory animals in Biochemical and Biomedical research as promulgated by the Canadian Council of Animal Care (1984). The study protocols on animal use were approved by the Faculty of Pharmacy, University of Benin Ethics Committee with reference number EC/FP/021/11.

Hypertension induction

Hypertension was induced in accordance with the method described by Simchon et al. (1991). Eight (8) % NaCl solution was prepared and administered to test rats with the calibrated (150 mL) drinking bottles. Drinking bottles were washed daily and refilled with 8 % NaCl for one (1) week, after which rats were placed on Normal saline (0.9 % NaCl) for the duration of the study to sustain the attained hypertensive state in test rats.

Administration of AESG & Antihypertensive Prophylactic Study

The experiment rats were orally administered AESG as prescribed in the OECD (2010), No. 425 test guidelines; as described by Oliveira et al. (2016) and Rout et al. (2014). The rats were randomly allotted into five (5) groups of n = 3. Test rats received doses of 25, 50, and 100 $mgkg^{-1}$ body weight respectively of AESG, the untreated group received 8 % to induce hypertension and subsequently received 0.9 % NaCl in drinking water daily for 4 weeks to sustain hypertensive state; while the control group received only water *ad libitum*. The initial body weights of rats were obtained before commencement of the study; weekly for 4 weeks. Baseline systolic (SBP), diastolic Blood Pressures (DBP), mean arterial pressure (MAP) and heart rate (HR) were obtained using the non-invasive (tail-cuff) blood pressure measurement system (MRBP, IITC LIFE SCIENCE). Experimental rats were pre-administered AESG 25, 50 & 100 mg/kg body weight respectively for one week, after which experimental rats were exposed to 8 % NaCl load (induced hypertension) (Simchon et al. 1991) for one week, as treatment with extract continued. After one week, 8 % NaCl was withdrawn and replaced with 0.9 % NaCl to sustain hypertension for 4 weeks.

Collection of data and Samples/Specimen

Test rats in each group were weighed weekly; change in body weight was recorded. SBP, DBP, MAP and HR of rats exposed to 8 % NaCl (Induced Hypertension) and (or) already hypertensive rats were measured weekly and recorded. At the end of the *in vivo* experimental study; on the 30th day, final hemodynamic variables were obtained; rats were fasted overnight. The following day, rats were anesthetized with 1.5 g/kg intraperitoneal injection of urethane according to a method previously described by Bilanda et al. (2019). A 5 ml sample of venous blood was withdrawn into heparinized specimen bottles. The blood samples were centrifuged at 3,500 rpm for 10 min to obtain plasma samples which were stored at 18 ℃ and used for biochemical analyses within a few days. The liver and kidneys were harvested from each rat, cleared off connective tissues and fixed in formal saline (0.9 g of NaCl salt in 90 mL of distilled water, mixed with10 mL of 40 % formalin to obtain a final volume of 100 mL) for histological evaluation. The fixed-excised organs were trimmed into 5 mm thickness; dehydrated with graded concentrations of ethanol (70, 95 & 99 %, absolute ethanol); cleared in xylene and embedded in paraffin wax. The embedded tissues were further sectioned into 6 µm thickness, stained with haematoxylin and Eosin (H & E); examined under the light microscope; according to the methods described by Windsor (1994) and Gurr (1959). The sections were photographed at a magnification of x400 with the Vanox-T Olympus photographic microscope.

Blood Pressure Measurement

Principle

Blood pressure measurements were obtained weekly according to the principle of Nikolai Korotkov; method described by Bunag and Butterfield (1982). Each rat was subjected to acclimatization in the restrainer for 1-2 h / day for one (1) week before the commencement of

blood pressure measurement. This was essential to reduce movement artifact, improve pulse and produce desired recordings/electronic traces.

Biochemical analyses

Alanine Amino Transferase (ALT) was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine, Aspartate Amino Transferase (AST) was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine, Alkaline Phosphatase (ALP) was measured by monitoring the concentration of P-nitrophenol formed when P-nitrophenylphosphate is hydrolyzed by ALP in the presence of H_2O, Gamma Glutamyl Transferase (γGT) was measured by monitoring the absorbance values of 5-amino-2-nitrobenzoate produced by the reaction of L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine in the presence of γGT in the specimen/sample, Lactate was measured in the plasma sample; according to the methods described by Reitman and Frankel, Englehardt et al., Tietz, Deutsche Geselleschaft fur Klinische Chemie respectively using commercial test kits (Randox® Laboratories, United Kingdom) (Reitman and Frankel 1957, Englehardt 1970, Weisshaar 1975, Teitz 1987). The Total Proteins, Albumin and Globulins were estimated according to the methods described by Tietz, Doumas et al. and calculated by subtraction of albumin from the total proteins respectively using commercial test kits (Randox® Laboratories, United Kingdom) (Doumas et al. 1971, Tietz 1995). Lipid profile tests which include total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) were done using colorimetric methods and calculative method described by Roeschlau et al., Jacobs and Van Denmark, Friedewald et al. respectively using commercial test kits (Randox® Laboratories, United Kingdom) (Jacob and VanDenmark 1960, Friedewald et al. 1972, Roeschlau et al. 1974); the cardiac risk ratio was calculated using relevant lipid profile indices by dividing the total cholesterol value by the HDL-C value. The Total Bilirubin and Conjugated (Direct Bilirubin) were estimated according to a colorimetric method described by Jendrassik and Grof (1938); whereas, the unconjugated (indirect) bilirubin was calculated by subtracting the value of the conjugated bilirubin from the total bilirubin value, using commercial test kits (Randox® Laboratories, United Kingdom). The plasma sodium, chloride ions were estimated according to the methods described by Maruna (1958), Tietz et al. (1986), respectively using commercial test kits (Randox® Laboratories, United Kingdom). The plasma Urea and Creatinine were estimated according to the methods described by Weatherburn (1967) and Bartels and Bohmer (1972).

Statistical Analysis

Data are expressed as mean ± SD (standard deviation). Differences between means of test groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$. All statistical analyses were conducted using GraphPad Prism®, version 9.

Results and discussions

Results

Prophylactic Effect of varying dose of AESG on Body Weight (g) of Male *Wistar* Rats Exposed to 8 % NaCl, Normal Saline (N.S) for 4 weeks

The data presented in Figure 1 indicate significant reduction in body weights of experimental rats pre-treated with varying dose of Aqueous Leaf Extract of *S. glauca* (AESG); including the untreated Hypertensive group at week 2, upon exposure to 8% NaCl to induce hypertension. Within week 2-4, there was observed reversal of weight loss; significant increases in body weights of experimental rats following continuous administration of varying dose of AESG; including untreated hypertensive group, although there was observed weight loss, upon

replacement of the 8% NaCl with 0.9% NaCl to sustain desired hypertensive state. Statistical analysis of the data obtained for weight of rats indicate significant mean weight gain at 11.43±1.35, 16.45±0.98, 11.60±0.57% of rats administered 25, 50 & 100 mgkg^{-1} body weight of AESG respectively after 4 weeks against a significant loss in mean body weight of -6.72±0.01% of untreated hypertensive group vis-à-vis the normotensive group which equally gained 18.00±0.07% mean body weight after 4 weeks. The group pre-treated with AESG 50 mgkg^{-1} demonstrated the highest gain in percentage body weight when compared with other pre-treated groups vis-à-vis the untreated hypertensive group. The group pre-treated with AESG 50 mgkg^{-1} competed favorably with the normotensive group.

Prophylactic Effect of varying doses of AESG on Hemodynamics Indices of Male *Wistar* Rats Exposed to 8 % NaCl, Normal Saline (N.S) for 4 weeks.

The data presented in Figure 2 indicate significant ($P \leq 0.05$) exponential increases in systolic blood pressure (SBP) of untreated hypertensive group through week 1 (123.500 ± 2.8 mmHg), week 2 (142.59 ± 1.9 mmHg), week 3 (147.17 ± 7.5 mmHg); particularly at week 4 (148.58 ± 2.5 mmHg); when compared to the Normotensive group with SBP through week1 (126.67 ± 1.2 mmHg), week 2 (127.38 ± 5.6 mmHg), week 3 (131.0 ± 6.7 mmHg) and week 4 (130.33 ± 3.9 mmHg). Accordingly, the data presented in Figure 2 further revealed groups pre-treated with 25, 50 or 100 mgkg^{-1} AESG significantly prevented systolic hypertension through week 2 to 4, when compared with untreated hypertensive group. The Diastolic Blood Pressure (DBP) and the Mean Arterial Pressure (MAP) data presented in Figures 3 and 4 respectively show that 25 and 50 mg/kg AESG demonstrated significant DBP and MAP lowering effect; compared favorably with the normotensive group; particularly at week 2 and 3 respectively; when compared to the untreated hypertensive group's week 2 and 3.

Remarkably, pre-treatment with respective doses of AESG (25, 50 and 100 mgkg^{-1}) significantly prevented SBP elevations at week 2 of 8 % NaCl high salt load; when compared with the untreated hypertensive group. Notably, AESG 50 mgkg^{-1} demonstrated most effective feature; which significantly ($P \leq 0.05$) prevented elevation of SBP at week 2 (127.75 ± 7.5 mmHg); further significantly ($P \leq 0.05$) reversed SBP elevation at week 3 (115.08 ± 5.6 mmHg), when compared to AESG 25 & 100 mgkg^{-1}; the untreated hypertensive group respectively. The data presented in Figure 5 indicate obviously that there was no significant difference ($P \geq 0.05$) in the Heart Rates (HRs) of groups pre-treated with respective dose of AESG, when compared with the HR of untreated hypertensive group through week 2 - 4.

Furthermore, the data presented in Figures 6-10 illustrate the SBP, DBP, MAP and HR recorded polygraph charts of normotensive, and untreated hypertensive groups vis-à-vis groups pre-treated with 25, 50 or 100 mgkg^{-1} AESG.

Prophylactic Effect of varying Dose of AESG on Liver Function of Male Wistar Rats exposed to 8 % NaCl, Normal Saline for 4 weeks

The data presented in Figure 11 indicate that induced hypertension (8 % NaCl load) elicited significant ($P \leq 0.05$) elevation in plasma Alanine transaminase (ALT) activity; followed by significant ($P \leq 0.05$) reduction in plasma Aspartate transaminase (AST) and ɣ-glutamyl transferase (GGT) activities respectively; whereas, the plasma Alkaline Phosphatase (ALP) and Lactate dehydrogenase (LDH) activities appeared non-significantly ($P \geq 0.05$) affected by the induced hypertension; when compared with the enzyme activities of the normotensive group. Consequently, the group pre-treated with AESG 50 mgkg^{-1} significantly ($P \leq 0.05$) prevented elevated plasma ALT activity; when compared with the enzyme activity of the untreated hypertensive group; whereas, the groups pre-treated with AESG 25 & 100 mgkg^{-1} respectively exhibited observed elevated ($P \leq 0.05$) plasma ALT activities; when compared with the enzyme activity of the untreated hypertensive group.

The groups pre-treated with AESG 50 & 100 mgkg^{-1} respectively significantly ($P \leq 0.05$) prevented and further reduced plasma AST activity; when compared with the plasma AST activity of untreated hypertensive group. Neither the plasma ALP activity of the untreated

hypertensive group nor the groups pre-treated with varying dose of AESG were altered by induced hypertension (8 % NaCl load); whereas, the plasma LDH activity of the groups pre-treated with 25 and 50 $mgkg^{-1}$ respectively was significantly ($P \leq 0.05$) elevated; when compared with the plasma LDH activity of the untreated hypertensive group. The data presented in Figure 12 illustrate the liver synthesizing functional ability; which featured plasma Total Proteins, Albumin and Globulins. The data highlight significant ($P \leq 0.05$) reductions in plasma total proteins and globulins concentrations; significant ($P \leq 0.05$) elevation in plasma Albumin concentrations of untreated hypertensive group; when compared with the normotensive group. Strikingly, it was observed that the groups pre-treated with AESG 25 & 50 $mgkg^{-1}$ respectively significantly ($P \leq 0.05$) attempted to prevent the lowering of plasma total proteins and globulins concentrations respectively, when compared with the untreated hypertensive group; whereas, the group pre-treated with AESG 100 $mgkg^{-1}$ demonstrated a non-significant ($P \geq 0.05$) and significant ($P \leq 0.05$) effects at preventing the lowering of plasma total proteins and plasma globulin concentrations respectively; when compared with the untreated hypertensive group. The group pre-treated with varying dose of AESG (25, 50 and 100 $mgkg^{-1}$) demonstrated significant ($P \leq 0.05$) effect at preventing and further lowing the plasma albumin concentrations; when compared with the untreated hypertensive group.

Prophylactic effect of varying Doses of AESG on Plasma Lipid Profile & Cardiac Risk Ratio of Male *Wistar* Rats Exposed to 8 % NaCl, Normal Saline for 4 Weeks.

The data presented in Figure 13 indicate that the untreated hypertensive group featured significant ($P \leq 0.05$) elevations in plasma Total Cholesterol (TC), High-Density Lipoprotein Cholesterol (HDL-C), Low-Density Lipoprotein Cholesterol (LDL-C), Triglycerides (TG) concentrations; a non-significant ($P \geq 0.05$) increase in the plasma Cardiac Risk Ratio (CRR), when compared with the of the normotensive group. Pre-treatment with varying dose of AESG; particularly, the 25 & 50 $mgkg^{-1}$ significantly ($P \leq 0.05$) prevented and further reversed elevations in plasma TC, LDL-C and TG concentrations respectively, when compared with the untreated hypertensive group; with a corresponding significant ($P \leq 0.05$) elevation in the HDL-C concentrations of the groups pre-treated with AESG 25 & 50 $mgkg^{-1}$; when compared with the untreated hypertensive group.

Whereas, the group pre-treated with AESG 100 $mgkg^{-1}$; however, demonstrated poor prophylactic effect; evidenced by significant ($P \leq 0.05$) elevations in TC, LDL-C; a significant ($P \leq 0.05$) reduction in HDL-C concentrations when compared with the untreated hypertensive group. Furthermore, the group pre-treated with AESG 100 $mgkg^{-1}$ demonstrated poor prophylactic effect, although significantly ($P \leq 0.05$) prevented further elevations in plasma TG concentrations when compared to the untreated hypertensive group.

The plasma CRR level of the untreated hypertensive group was quite elevated ($P \geq 0.05$); when compared with the plasma CRR level of the normotensive group. Impressively, groups pre-treated with AESG 25 and 100 $mgkg^{-1}$ prevented CRR; characterized by significantly ($P \leq 0.05$) lower CRR levels; when compared with the untreated hypertensive. Whereas, the group pre-treated with AESG 100 $mgkg^{-1}$ featured elevated CRR levels, when compared with the untreated hypertensive group.

Prophylactic Effect of varying Dose of AESG on Plasma Total Bilirubin, Conjugated & Unconjugated Bilirubin of Male Wistar Rats Exposed to 8 % NaCl, Normal Saline for 4 weeks

The data presented in Figure 14 indicate that the untreated hypertensive group demonstrated significantly ($P \leq 0.05$) elevated plasma conjugated bilirubin; markedly ($P \leq 0.05$) lowered plasma Total Bilirubin and unconjugated bilirubin concentrations; when compared with the normotensive group. However, pre-treatment with varying dose of AESG (25, 50 and 100 $mgkg^{-1}$) significantly ($P \leq 0.05$) prevented elevated plasma conjugated bilirubin levels;

prevented and further significantly ($P \leq 0.05$) reduced plasma total bilirubin and unconjugated bilirubin levels respectively; when compared to the untreated hypertensive group.

Prophylactic Effect of varying Dose of AESG on Plasma Sodium & Chloride ions of Male Wistar Rats Exposed to 8 % NaCl, Normal Saline for 4 weeks

The data presented in Figure 15 indicate that induced hypertension, that is the untreated hypertensive group demonstrated significant ($P \leq 0.05$) elevation and reduction in plasma sodium and chloride ions concentrations respectively; when compared with the normotensive group. Furthermore, it was observed that pre-treatment with varying dose of AESG (25, 50 and 10 $mgkg^{-1}$) did not prevent elevations in plasma sodium ion concentration. That is, there were significant ($P \leq 0.05$) elevations of plasma sodium ions concentrations in groups pre-treated with AESG 25, 50 and 100 $mgkg^{-1}$ respectively; when compared with the sodium ion concentration of untreated hypertensive group. Likewise, groups pre-treated with AESG 25 and 50 $mgkg^{-1}$ respectively demonstrated corresponding increases ($P \leq 0.05$) in plasma chloride ion concentrations, a non-significant corresponding increase in chloride ion concentration of group pre-treated with AESG 100 $mgkg^{-1}$; when compared with the chloride ion concentration of the untreated group.

Prophylactic Effect of varying Dose of AESG on Plasma Urea & Creatinine levels of Male Wistar Rats Exposed to 8 % NaCl, Normal Saline for 4 weeks

The data presented in Figure 16 feature significant ($P \leq 0.05$) elevation and reduction of plasma Urea and Creatinine concentrations respectively in untreated hypertensive group, when compared with the normotensive group. Whereas, pre-treatment with respective doses of AESG (25, 50 and 100 $mgkg^{-1}$) significantly ($P \leq 0.05$) prevented elevated plasma urea concentration; when compared with the urea concentration of untreated hypertensive group. Similarly, groups pre-treated with AESG 25 and 50 $mgkg^{-1}$ respectively, significantly ($P \leq 0.05$) prevented and further lowered the creatinine concentrations, when compared with the creatinine level of the untreated hypertensive group. Contrariwise, the group pre-treated with AESG 100 $mgkg^{-1}$ demonstrated significant ($P \leq 0.05$) rise in creatinine level; when compared with the creatinine level of the untreated hypertensive group.

Discussions

Hypertension, being a condition characterized by asymptomatic cardiovascular complications; is often not unconnected with comorbidities, such as obesity, diabetes *mellitus*, likely compromised liver functioning, cardiac disorder, renal dysfunction; amongst others; perhaps arising from underlying conditions. Hence, the study equally examined relevant biochemical indicators; taking under consideration, the preliminary investigative study on blood pressure lowering potential of the aqueous leaf extract of *Simarouba glauca* (Osagie-Eweka et al. 2023). The study adopted the salt-sensitive hypertensive model, characterized by salt-load induced Na^+ and water retention (Guyton 1992, Lifton et al. 2001, Rodriguez-Iturbe et al. 2007); consequently, resulted to elevated systolic (SBP) and diastolic blood pressure (DBP); accompanied with alterations in mean arterial pressure (MAP) and heart rates (HR) of experimental animals. Although, scientific review has reported contrary findings, that salt-resistant subject may not often present with alterations in relevant hemodynamic indicators due to their extra ability to pass out high salt load from system circulation, when compared to the salt-sensitive subject (Guyton 1990).

The study highlighted the remarkable impact of Na^+-load induced hypertension on body weight of experimental models. The outcome of the study revealed significant weight loss in the untreated hypertensive group. While the data of the present study is at variance with considered epidemiological and experimental studies respectively (Guyton 1990, Guo et al. 2017, Iida et al. 2019), which indicated that high salt-induced hypertension did not elicit hypertension-

related weight loss. Conversely, pre-treatment with respective doses of AESG; particularly at 50 $mgkg^{-1}$ remarkably prevented hypertension induced weight loss, associated with high salt load on experimental models. The present study indicates that prophylactic management of salt-load induced hypertension strongly correlated with geometric increase in the body weight of experimental models administered respective doses of AESG. In other words, it demonstrated convincing findings that pre-treatment with AESG strongly mitigated high salt-load induced weight loss in experimental models.

According to the World Health Organization Joint News Report in Geneva, Switzerland, a subject is said to be hypertensive, after several ambulatory blood pressure measurement of relevant hemodynamics, of elevated SBP of ≥ 140 mmHg and DBP of ≥ 90 mmHg (Campbell et al. 2022). In the study being reported, experimental animal exposed to 8 % NaCl load demonstrated marked elevation in SBP and DBP of 142.59 ± 1.9 mmHg and 91.79 ± 6.8 mmHg respectively, which obviously indicated that salt-load elicited desired hypertension (see Figures 2, 3 & 7 (MRBP Polygraph)). The elevated hemodynamics were sustained in the experimental models by withdrawal of 8 % NaCl and prompt replacement with 0.9 % NaCl through week 2-4. The outcome of the salt-load, which resulted in hypertension in the present study is consistent with the reports of Rodriguez-Iturbe et al. (2007), Lifton et al. (2001) and Guyton (1992) perhaps suggestively attributed to high salt-load sensitivity of experimental animals. Furthermore, it is imperative to state that 8 % salt load, resulted to enlarged coronary artery of the heart, as presented in micrograph Figure 19b; which is consistent with a prominent feature associated with hypertension-induced vascular alterations. Interestingly, the experimental groups pre-treated with respective doses of AESG; particularly, the AESG 25 and 100 $mgkg^{-1}$ respectively demonstrated remarkable reduction in systolic, diastolic and mean arterial blood pressures when compared to the untreated hypertensive model. Studies have reportedly implicated the potential antihypertensive effect of biologically active phytocompounds in experimental models; coupled with natriuretic agonistic effect. A similar study equally reported the potent anti-hypertensive properties of triterpenoids isolated from the *reishi* or *lingzhi* mushroom of the *G. lucidum* (Boh et al. 2007, Onyema-Iloh et al. 2018). Alkaloid (Aloperine) from plant source has been reported to demonstrate a vasorelaxant effect on isolated smooth muscle tissue indicating a possible hypotensive agent (Yang et al. 2018). Likewise, the Aloperine isolated from *S. flavescens* root strongly indicated KCNQ5 potassium channel activation; which demonstrated both voltage-dependent and voltage-independent; vascular-expressed KCNQ5 opening; subsequently reversed blood volume/pressure (Manville *et al*., 2019). Previous study reported from our laboratory has equally revealed the abundant presence of bioactive phytomedicinal compounds in *S. glauca* (Osagie-Eweka et al. 2016); supported by the report of Gurupriya et al. (2017). The data of the present study clearly indicate that AESG possess potential antihypertensive effect; perhaps attributed to inherent phytocompounds such as alkaloid(s), as previously reported (Gurupriya et al. 2017).

The data presented in Figure 11 indicate that untreated hypertensive group demonstrated elevated plasma alanine aminotransferase (ALT); with apparently normal levels of plasma aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), ɣ-glutamyl aminotransferase (GGT) and Lactate dehydrogenase (LDH); likewise, the data presented in Figure 12 show that plasma total proteins, albumin and globulin levels of untreated hypertensive group appeared equally unaltered. Studies conducted by Ikewuchi (2013) and Ayalogu et al. (2011) reported significant elevations in biomarkers of liver enzymes; particularly, the plasma ALT in salt load induced hypertensive models. Considering that other relevant indicator enzymes of liver function, such as GGT and others were not altered in the present study; including the total proteins, albumin and globulins; there was no obvious, significant pathological liver damage capable of compromising its integrity and function. It's most likely and strongly adduced that the observed elevated plasma ALT in untreated hypertensive group and other groups pre-treated

with respective dose of AESG, may have resulted from inflammatory hepatic stenosis; arising from high salt load-induced hypertension, as evidenced in micrograph Figures 17b-e. Additionally, It is important to note that pe-treatment with respective dose of AESG did not demonstrate significant hepato-protective effect from high salt-load induced inflammatory stenosis. In a similar evaluation of the pathological and biochemical conditions associated with high-salt activated hypertension, it is observed in the data presented in Figure 14 that untreated hypertensive group presented with hyper-conjugated bilirubin, which obviously indicate that salt load elicited inflammatory stenosis; may have resulted in constricted bile excretion, leading to accumulated conjugated bilirubin in the blood. Previous studies conducted by Ikewuchi (2013) and Ayalogu et al. (2011) reported indicate that high salt-induced hypertension elicited markedly elevated serum conjugated bilirubin; which agrees with the data of the present study. The observed inflammation may have equally resulted to complications linked with symptoms of steatohepatitis. Interestingly, respective dose of AESG effectively prevented elevation of plasma conjugated bilirubin, characterized by inflamed stenosis; suspected to have been evoked by high salt-induced hypertension.

The data presented in Figure 13 indicate marked elevation of plasma total cholesterol, low density lipoprotein-Cholesterol and triglycerides; including increased cardiac risk ratio in untreated hypertensive group. The outcome of the present study clearly demonstrated that high-salt load was associated with mechanisms capable of eliciting disturbances in cholesterol homeostasis in hypertensive state; considering the role of the liver in cholesterol metabolism and the observed inflammatory stenosis presented in micrographs Figures 17b-e, there are convincing indications that elevated cholesterols may not be unconnected with the state of the liver. Prevention of elevated levels of cholesterols; particularly, the triglycerides is key to the management of hypertension related cardiovascular complications such as vascular hardening and arteriosclerosis. The review report of Nordestgaard and Varbo, underscores the role of triglycerides in cardiovascular diseases. The review further implicated triglycerides in hypertensive condition pointing that “the cholesterol content of triglyceride-rich lipoprotein (remnant cholesterol) is more likely to be the cause of atherosclerosis and cardiovascular disease rather than raised triglycerides” (Nordestgaard and Varbo 2014). The review reported by Zhao et al. (2020), highlighted the pathophysiological contribution of Non-Alcoholic Fatty Liver disorder (NAFLD) to the progression of hypertension related cardiovascular conditions. The review also emphasized the possibility of hypertension induced NAFLD-linked to steatohepatitis; resulting to symptomatic biochemical alterations and complications associated with multisystemic adverse effects (Zhao et al. 2020). The data of the present study agrees with the report of the review published by Zhao et al., with respect to the possibility of hypertension-elicited steatohepatitis (Zhao et al. 2020). However, the outcome of the present study revealed that pre-treatment at 25 & 50 $mgkg^{-1}$ significantly prevented elevations in total cholesterol, low-density lipoprotein cholesterols and triglycerides; with marked reduction in cardiac risk ratio at 25 & 50 $mgkg^{-1}$ respectively. The marked reduction in plasma lipoprotein cholesterols and triglycerides; low cardiac risk observed, may not be unconnected with the extract’s ability to potentially mitigate the predisposing effect of dyslipidemia associated with the etiology of hypertension and cardiovascular diseases at low doses. Although, previous study in our laboratory reported that oral administration of aqueous leaf extract at higher doses of 500, 1000 and 2000 $mgkg^{-1}$ respectively elicited hyperlipidemia (Osagie-Eweka et al. 2021). However; at lower doses in the present study, respective doses of AESG significantly mitigated hypertension-induced dyslipidemia. Review and several studies on the roles of bioactive compounds of medicinal plants origin, have reported evidence of anti-hyperlipidemic effect; the regulatory potential of these biologically active compounds on lipid metabolism; particularly at the level of cholesterol synthesis and re-uptake at the liver and enterocytes respectively (Cicero et al. 2007, Derosa et al. 2013, Gil-Ramírez et al. 2016, Hu et al. 2016, Patti et al. 2018). The review report of Ji et

al. (2019) highlighted the possible regulatory mechanisms capable of preventing hypercholesterolemia and the potential anti-dyslipidemic activities of numerous bioactive compounds of medicinal plants origin; ranging from Curcumin, a polyphenol isolated from *Curcuma longa Linn* which inhibits cholesterol absorption through its binding to the Niemann-Pick C1 like1 (NPC1L1-related transporter) (a transmembrane protein which facilitates the transfer of cholesterol in diets and bile from the lumen into the brush border of the membrane of the enterocytes) (Altmann et al. 2004, Feng et al. 2010, Feng et al. 2017). Free cholesterol (FC) is esterified to cholesterol esters (CEs) by acyl CoA: cholesterol acyltransferase-2 (ACAT)-2. ACAT2 can catalyze the formation of cholesteryl ester in intestinal epithelial cells [61]. Triterpenic acid (oleanolic acid (OA) and ursolic acid (UA)) isolated from hawthorn; berberine (extracted from Coptis chinensis) are responsible for the cholesterol-lowering effect of these plants by inhibiting intestinal ACAT activity (Lin et al. 2011, Wang et al. 2014). Inhibition of microsomal triglyceride transfer protein (MTTP) leads to decreased apolipoprotein B (APOB) secretion and chylomicron assemblage; a number of in vitro studies have demonstrated that several flavonoids, such as taxifolin, quercetin, naringenin and tangeretin have Microsomal Triglyceride Transfer Protein (MTTP) inhibitory activities (Wilcox et al. 2001, Casaschi et al. 2002, Casaschi et al. 2004, Kurowska et al. 2004). The review of Ji et al. (2019) further highlighted numerous herbal medicinal plants with bioactive compounds such alkaloids (berberine), saponins (ginsenoside), polyphenols (pomegranate) and flavonoids (taxifolin, quercetin), that have significantly demonstrated anti-hypercholesterolemic and anti-dyslipidemic properties and potentials in attenuating atherosclerosis-related cardiovascular complications. Interestingly, previous study conducted in our laboratory reported the significant presence of numerous phytocompounds in the leaf extracts of *S. glauca*; some of which have been implicated in cholesterol homeostasis and lipid regulation (Osagie-Eweka et al. 2016). Therefore, the data presented in Figure 13 clearly indicate that respective doses of AESG demonstrated significant lipid and lipoprotein cholesterol-lowering activity; which may likely be attributed to the availability of relevant phytocompounds.

Electrolyte homeostasis and fluid volume is tightly regulated by the kidneys; strictly monitored under hypertensive condition. The overall assessment of the optimum functionality of the kidneys is directly proportional to the amount of sodium and water balance either excreted or reabsorbed. The role of the kidneys in regulation of blood volume; by extension, blood pressure can't be over emphasized. The increased reabsorption of sodium by the kidneys is accompanied by corresponding retention of water which increases blood volume or blood pressure. The homeostatic regulatory mechanism of the kidneys equally maintains required levels of chloride ions and other relevant ions necessary to regulate electrolyte-water balance. The measurement of creatinine concentration in blood or plasma is a functional assessment of the integrity and the filtration capacity of the glomerulus (Glomerular filtration rate, GFR); the experimental and clinical significance of elevated concentration of measured creatinine in plasma or serum is indicative of poor filtration capacity or compromised renal function. Urea is a non-protein nitrogenous waste (NPN) obtained from deaminated amino acids of breakdown of proteins; which is available as ammonia, converted to urea by the liver enzymes activities; excreted by the kidneys. Therefore, characteristically elevated levels of urea in blood equally indicates suspected compromised renal functioning associated with poor glomerular filtration capacity (Salazar 2014). In the present study, the data presented in Figure 15 reveals marked elevations in plasma sodium ions in untreated hypertensive group; including groups pre-treated with respective doses of AESG. However, the chloride ion concentrations indicate non-significant alterations in untreated hypertensive group and groups pre-treated with respective doses of AESG. The elevated plasma sodium ion is attributed to salt-load induced hypertension on experimental models; leading to increased sodium absorption and water retention; whereas, it

appeared there was a corresponding efflux of chloride ions that resulted to non-significant elevations in plasma chloride ions. The measured plasma creatinine levels of untreated hypertensive and other groups pre-treated with respective doses of AESG where not elevated, which indicate that salt-load induced hypertension did not impact on the kidney's ability to effectively clear creatinine from the blood; whereas, there was observed elevated urea concentration in the untreated hypertensive group; which indicate that the kidney's urea excretory function was impaired; perhaps attributed to inflammation observed in the histological evaluation of the renal tubules. Interestingly, groups pre-treated with respective doses of AESG demonstrated reno-protective anti-inflammatory responses; effectively attempted to prevented elevated plasma urea concentrations.

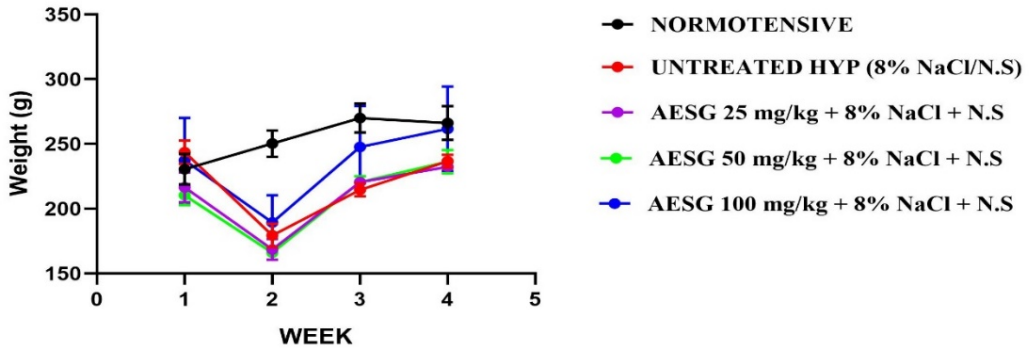


Figure 1. Prophylactic effect of varying doses of AESG on Mean Body Weight (g) of Male *Wistar* Rats Exposed to 8 % NaCl, Normal Saline (N.S) for 4 weeks against Normotensive & Hypertensive groups respectively. Data are Mean ± SD (n ≥ 3). Data are Mean ± SD (n ≥ 3)

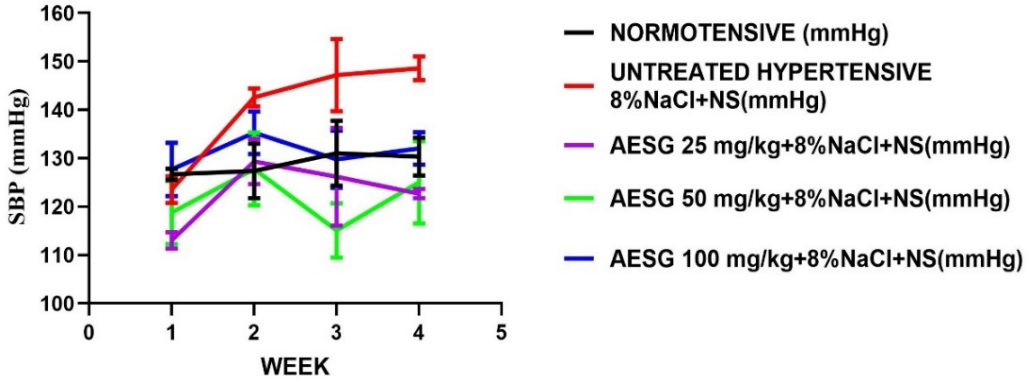


Figure 2. Prophylactic effect of varying doses of AESG on Mean SBP (mmHg) of Male *Wistar* Rats Exposed to 8 % NaCl, Normal Saline (N.S) for 4 weeks against Normotensive & Hypertensive groups respectively. Data are Mean ± SD (n ≥ 3). Data are Mean ± SD (n ≥ 3)

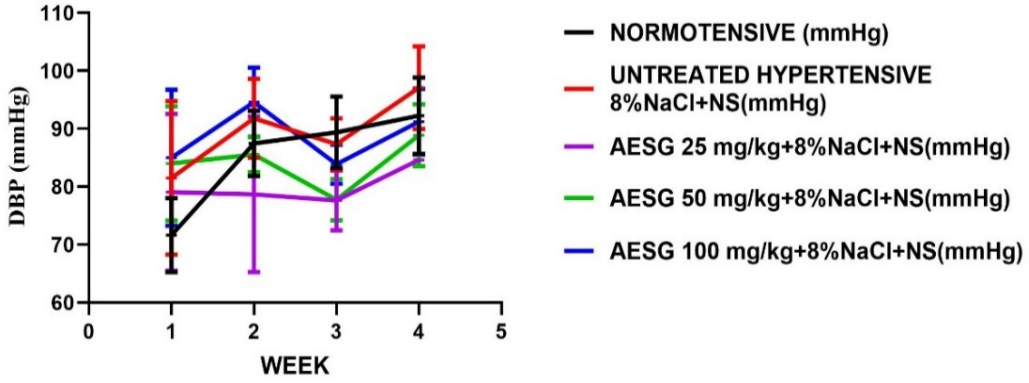


Figure 3. Prophylactic effect of varying doses of AESG on Mean DBP (mmHg) of Male *Wistar* Rats Exposed to 8 % NaCl, Normal Saline (N.S) for 4 weeks against Normotensive & Hypertensive groups respectively. Data are Mean ± SD (n ≥ 3). Data are Mean ± SD (n ≥ 3)

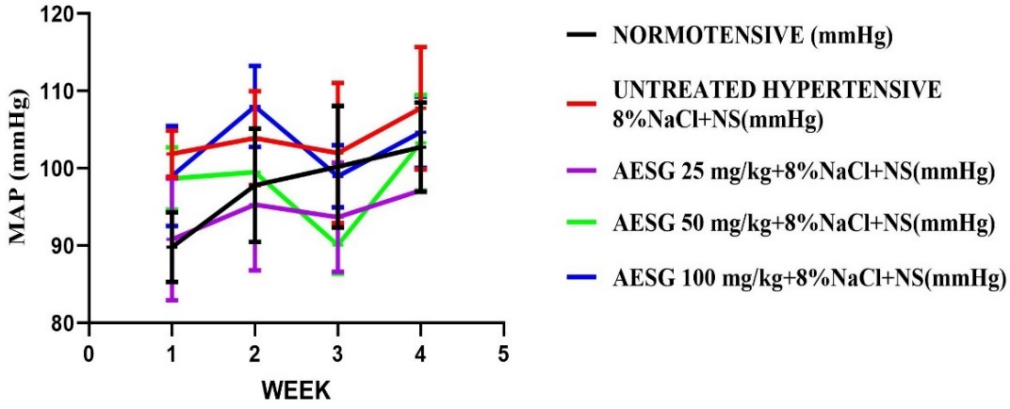


Figure 4. Prophylactic effect of varying doses of AESG on MAP (mmHg) of Male *Wistar* Rats Exposed to 8 % NaCl, Normal Saline (N.S) for 4 weeks against Normotensive & Hypertensive groups respectively. Data are Mean ± SD (n ≥ 3). Data are Mean ± SD (n ≥ 3)

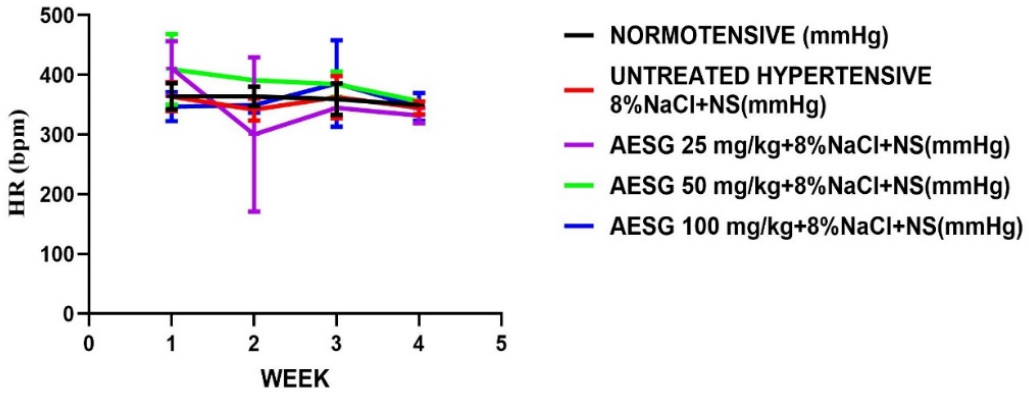


Figure 5. Prophylactic effect of varying doses of AESG on Mean Heart Rate (HR) (bpm) of Male *Wistar* Rats Exposed to 8 % NaCl, Normal Saline (N.S) for 4 weeks against Normotensive & Hypertensive groups respectively. Data are Mean ± SD (n ≥ 3). Data are Mean ± SD (n ≥ 3)

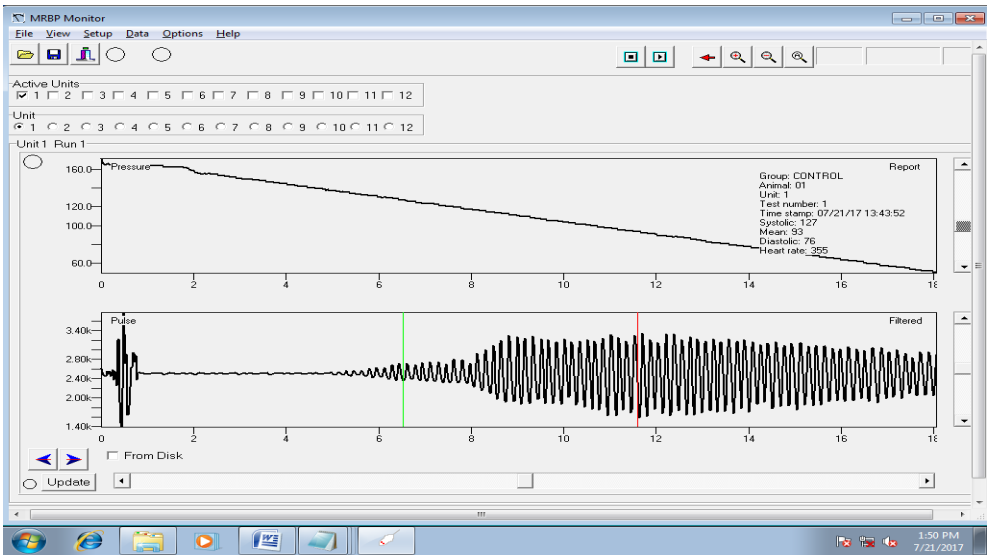


Figure 6. MRBP Record polygraph chart of Normotensive *Wistar* Rat

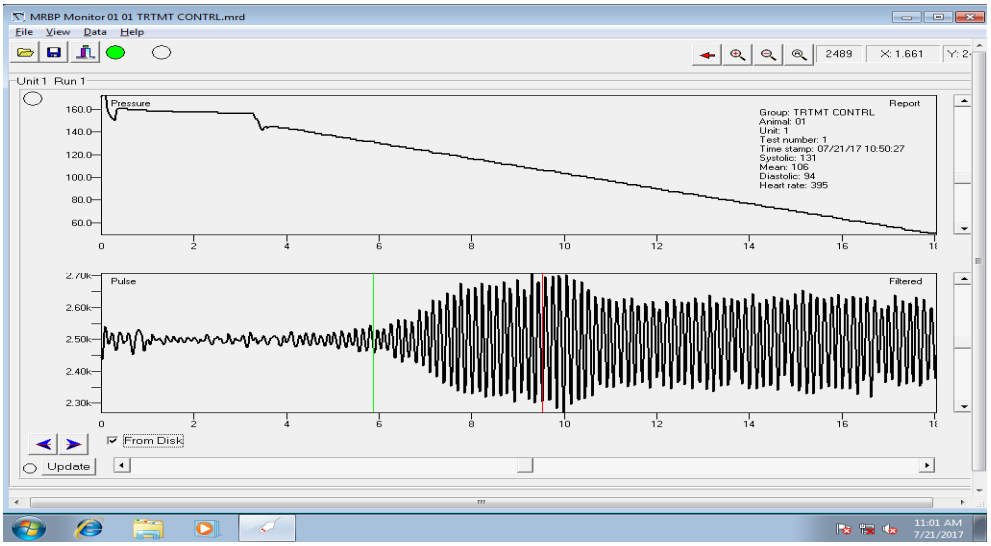


Figure 7. MRBP Record polygraph chart of untreated Hypertensive *Wistar* Rat

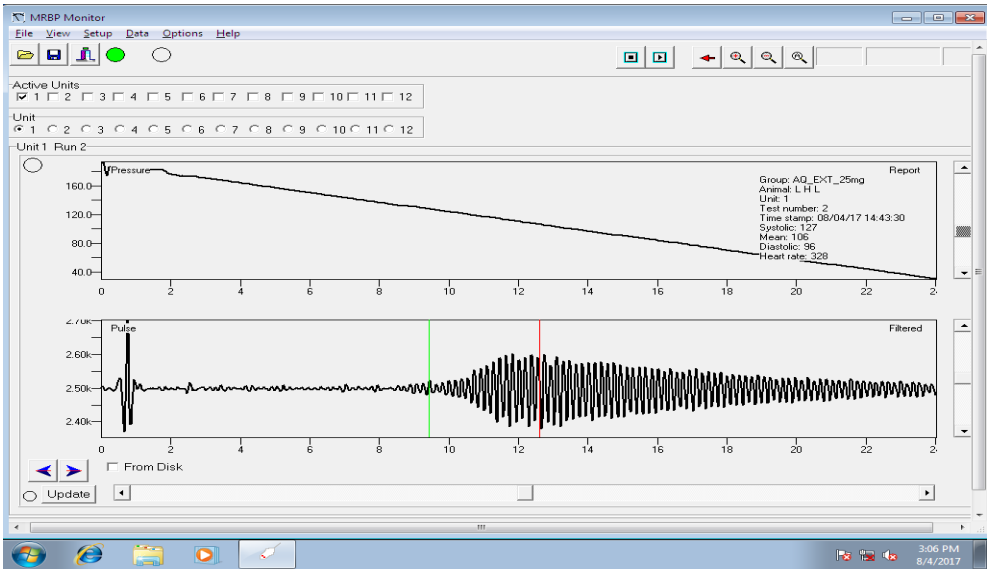


Figure 8. MRBP Polygraph chart of *Wistar* Rat Administered AESG 25 $mgkg^{-1}$

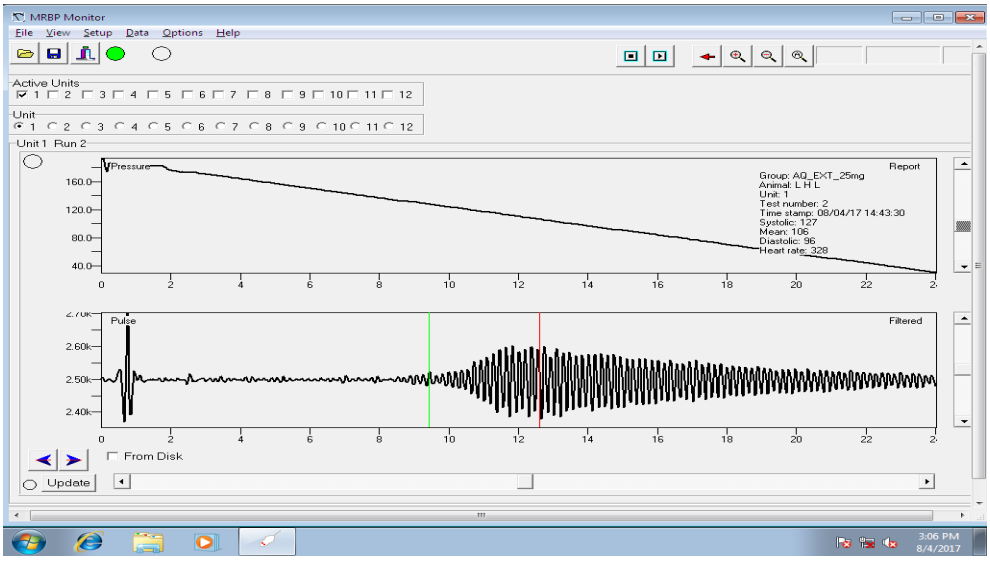


Figure 9. MRBP Polygraph chart of *Wistar* Rat Administered AESG 50 mgkg^{-1}

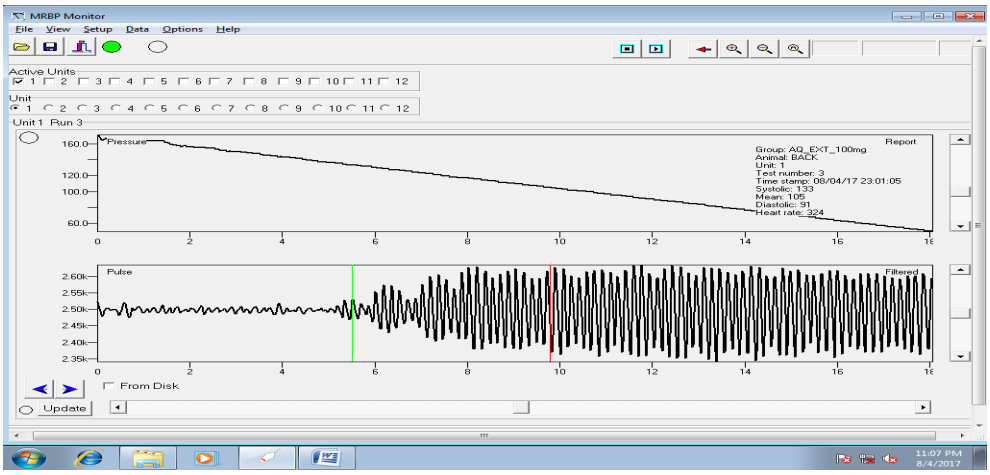


Figure 10. MRBP Record polygraph chart of *Wistar* Rat Administered AESG 100 mgkg^{-1}

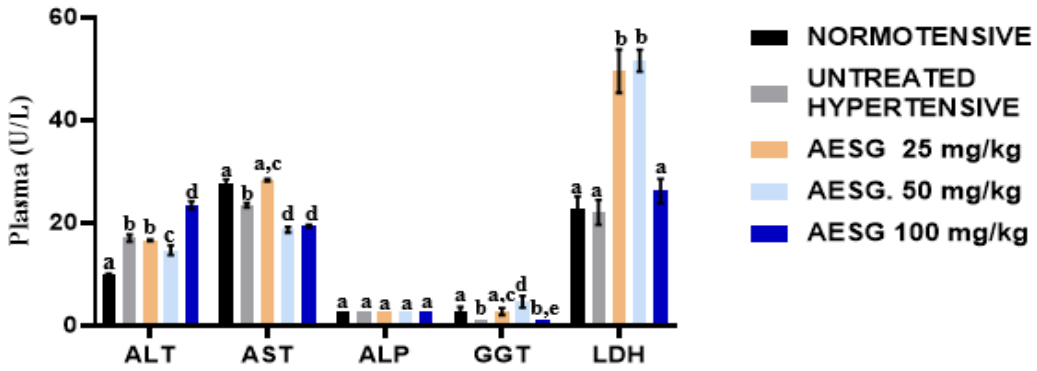


Figure 11. Prophylactic effect of varying Doses of AESG on Liver Function of Male Wistar Rats Exposed to 8 % NaCl, Normal Saline for 4 weeks, against the Normotensive & Hypertensive groups respectively. Data with similar lowercase alphabets are not significantly different amongst mean ($P \geq 0.05$); whereas, data with different lowercase alphabets are significantly different amongst mean ($P \leq 0.05$). Data are Mean ± SD ($n \geq 3$)

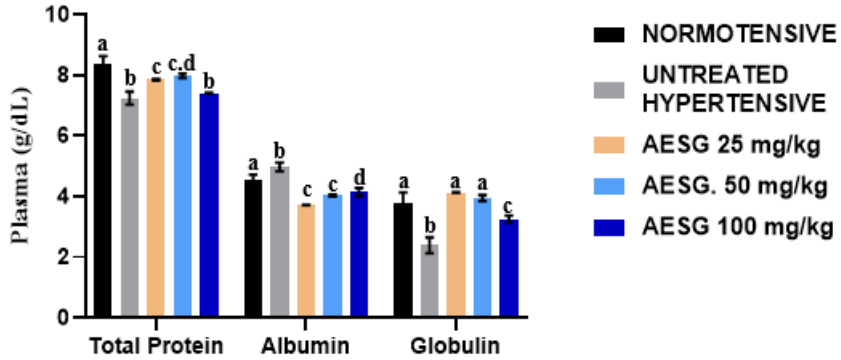


Figure 12. Prophylactic effect of varying Doses of AESG on Liver synthesizing Function of Male Wistar Rats Exposed to 8 % NaCl, Normal Saline for 4 weeks, against the Normotensive & Hypertensive groups respectively. Data with similar lowercase alphabets are not significantly different amongst mean ($P \geq 0.05$); whereas, data with different lowercase alphabets are significantly different amongst mean ($P \leq 0.05$). Data are Mean ± SD ($n \geq 3$)

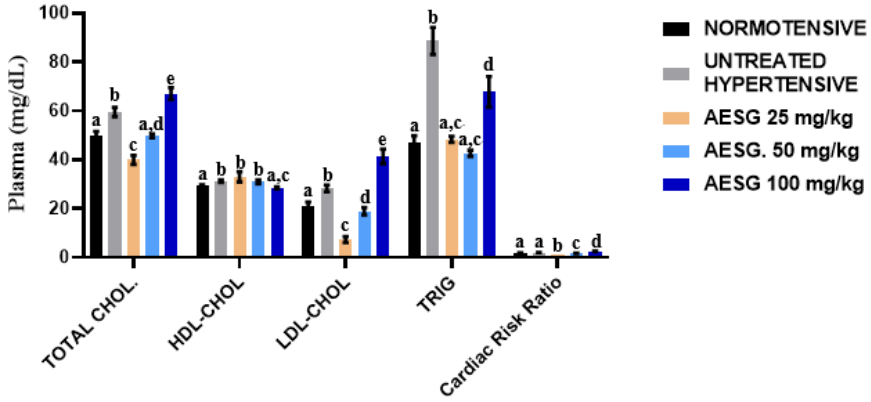


Figure 13. Prophylactic effect of varying Doses of AESG on Plasma Lipid Profile & Cardiac Risk Ratio of Male Wistar Rats Exposed to 8 % NaCl, Normal Saline for 4 weeks, against the Normotensive & Hypertensive groups respectively. Data with similar lowercase alphabets are not significantly different amongst mean ($P \geq 0.05$); whereas, data with different lowercase alphabets are significantly different amongst mean ($P \leq 0.05$). Data are Mean ± SD ($n \geq 3$)

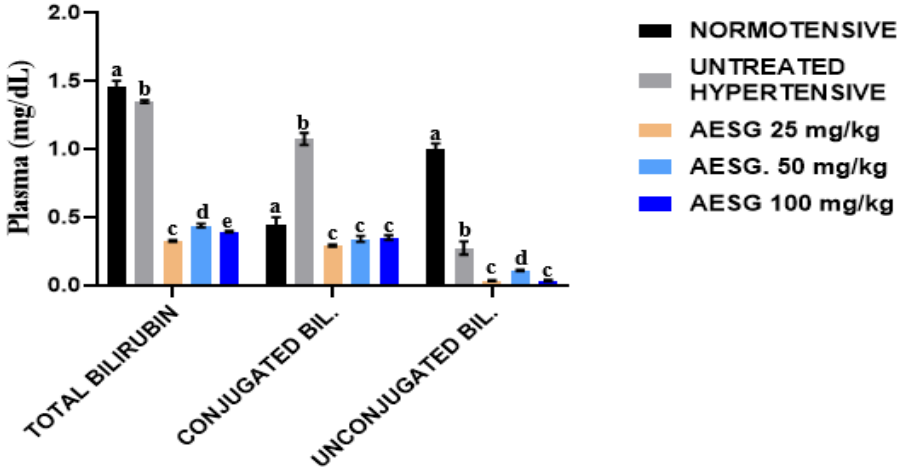


Figure 14. Prophylactic Effect of varying Doses of AESG on Plasma Total Bilirubin, Conjugated & Unconjugated Bilirubin of Male Wistar Rats Exposed to 8 % NaCl, Normal Saline for 4 weeks, against the Normotensive & Hypertensive groups respectively. Data with similar lowercase alphabets are not significantly different amongst mean ($P \geq 0.05$); whereas, data with different lowercase alphabets are significantly different amongst mean ($P \leq 0.05$). Data are Mean ± SD ($n \geq 3$)

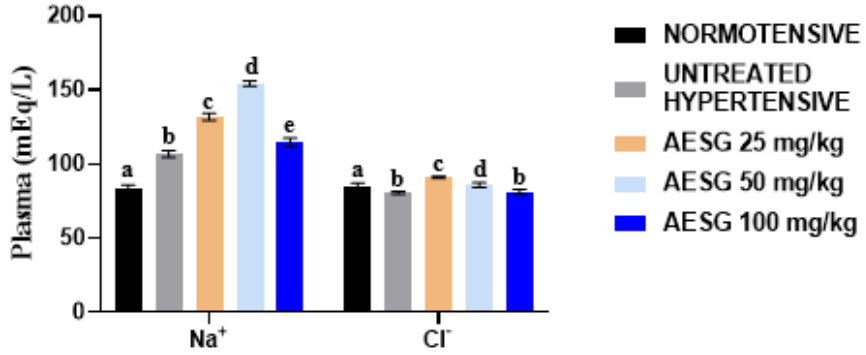


Figure 15. Prophylactic Effect of varying Doses of AESG on Plasma Sodium & Chloride ions of Male Wistar Rats Exposed to 8 % NaCl, Normal Saline for 4 weeks, against the Normotensive & Hypertensive groups respectively. Data with similar lowercase alphabets are not significantly different amongst mean ($P \geq 0.05$); whereas, data with different lowercase alphabets are significantly different amongst mean ($P \leq 0.05$). Data are Mean ± SD ($n \geq 3$)

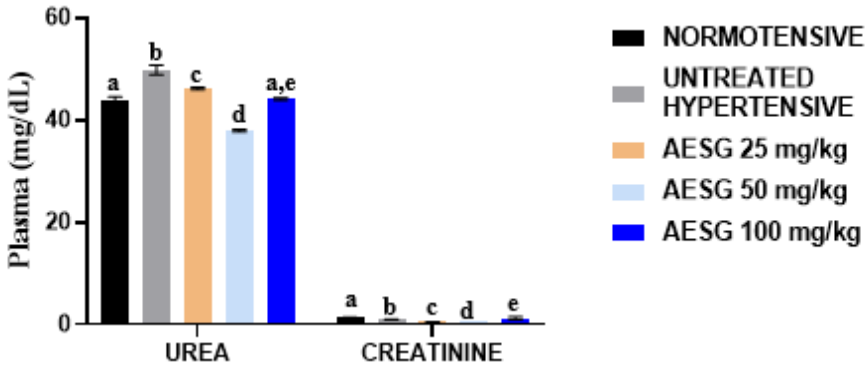
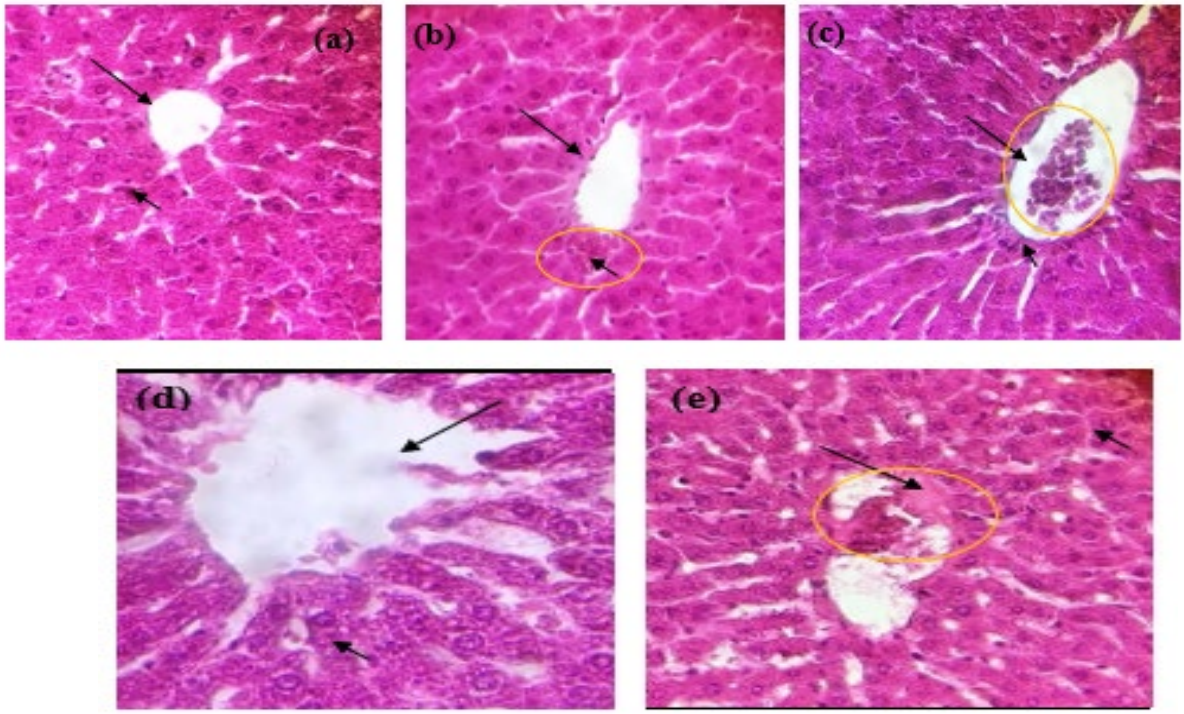


Figure 16. Prophylactic Effect of varying Doses of AESG on Plasma Urea & Creatinine levels of Male Wistar Rats Exposed to 8 % NaCl, Normal Saline for 4 weeks, against the Normotensive & Hypertensive groups respectively. Data with similar lowercase alphabets are not significantly different amongst mean ($P \geq 0.05$); whereas, data with different lowercase alphabets are significantly different amongst mean ($P \leq 0.05$). Data are Mean ± SD ($n \geq 3$)

Histology Evaluation
Liver Tissue

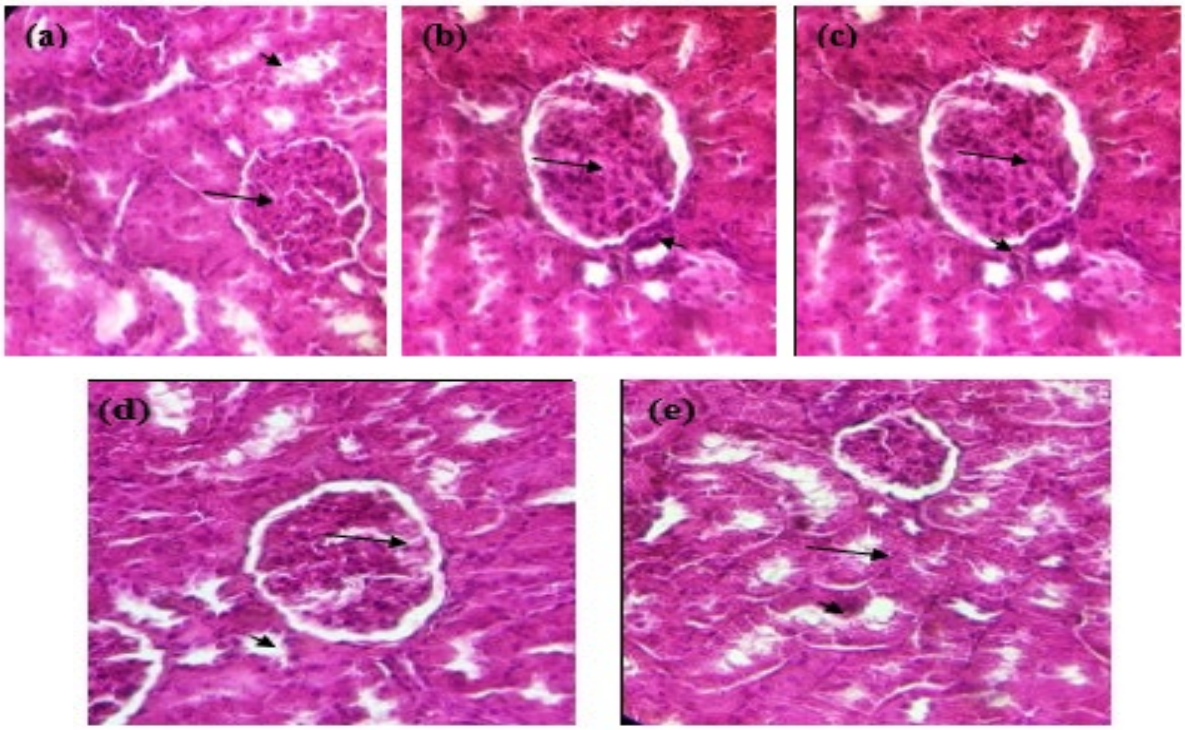


(a) Normotensive group: Illustrates distinct central venule (**long arrow**), hepatocytes with quite healthy nucleus (**short arrow**); well fenestrated sinusoids.
(b) Untreated Hypertensive group: Illustrates distinct central venule (**long arrow**) with noticeable inflammatory cells surrounding it (**short arrow**); the hepatocytes show mild stenosis.
(c) AESG 25 mgkg-1 group: Illustrates centriole with thickened wall surrounding by inflammatory cells (**long arrow**). There is quite a stenosis (**short arrow**)
(d) AESG 50 mgkg^{-1} group: Illustrates distinct centriole (**long arrow**). There is prominent stenosis of fatty changes (**short arrow**)
(e) AESG 100 mgkg^{-1} group: Illustrates slightly congested central venule (**long arrow**). There is prominent stenosis and mild fatty changes (**short arrow**)

Figure 17a. Normotensive group: Illustrates distinct central venule (long arrow), hepatocytes with quite healthy nucleus (short arrow); well fenestrated sinusoids. **Figure 17b.** Untreated Hypertensive group: Illustrates distinct central venule (long arrow) with noticeable inflammatory cells surrounding it (short arrow) the hepatocytes show mild stenosis. **Figure 17c.** AESG 25 mgkg^{-1} group: Illustrates centriole with thickened wall surrounding by inflammatory cells (long arrow). There is quite a stenosis (short arrow). **Figure 17d.** AESG 50 mgkg^{-1} group: Illustrates distinct centriole (long arrow). There is prominent stenosis of fatty changes (short arrow). **Figure 17e.** AESG 100 mgkg^{-1} group: Illustrates slightly congested central venule (long arrow). There is prominent stenosis and mild fatty changes (short arrow).

Kidney Tissue

Fig. 18. Kidney x400 (H & E)

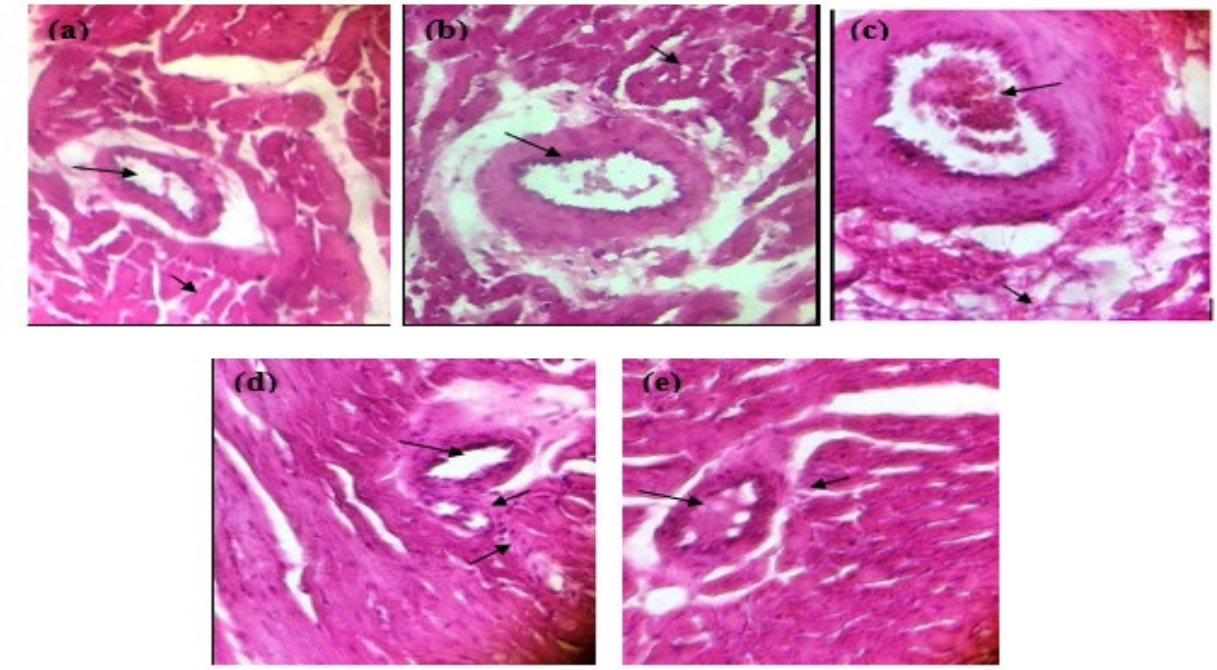


(a) Normotensive group: reveals visible renal corpuscle (**long arrow**) and distinct interstitial space (**short arrow**) and tubules.
(b) Untreated Hypertensive group: reveals visible renal corpuscle (**long arrow**) with mild inflammatory cells surrounding it (**short arrow**); the tubules appeared not so distinct.
(c) AESG 25 mgkg^{-1} group: reveals renal corpuscles with slight granulation (**long arrow**). There is mild distortion in the tubules (**short arrow**)
(d) AESG 50 mgkg^{-1} group: reveals renal corpuscles with slight granulation (**long arrow**). There is visible interstitial and tubules (**short arrow**)
(e) AESG 100 mgkg^{-1} group: reveals visible atrophied renal corpuscle (**long arrow**) with distortion in the tubules (**short arrow**).

Figure 18a. Normotensive group: reveals visible renal corpuscle (long arrow) and distinct interstitial space (short arrow) and tubules. **Figure 18b.** Untreated Hypertensive group: reveals visible renal corpuscle (long arrow) with mild inflammatory cells surrounding it (short arrow) the tubules appear not so distinct. **Figure 18c.** AESG 25 mgkg^{-1} group: reveals renal corpuscles with slight granulation (long arrow). There is mild distortion in the tubules (short arrow). **Figure 18d.** AESG 50 mgkg^{-1} group: reveals renal corpuscles with slight granulation (long arrow). There is visible interstitial and tubules (short arrow). **Figure 18e.** AESG 100 mgkg^{-1} group: reveals visible atrophied renal corpuscle (long arrow) with distortion in the tubules (short arrow).

Heart Tissue

Fig. 19. Heart x400 (H & E)



(a) Normotensive group: illustrates visible bundles of myofibrils (**short arrow**); interstitial space with prominent coronary artery (**long arrow**)
(b) Untreated Hypertensive group: reveals bundles of myofibrils (**short arrow**); interstitial space and visibly enlarged coronary artery (**long arrow**)
(c) AESG 25 mgkg^{-1} group: reveals enlarged congested coronary artery (**long arrow**) with indistinct bundles of myofibrils (**short arrow**).
(d) AESG 50 mgkg^{-1} group: reveals prominent coronary artery (**long arrow**), with visible myofibrils (**short arrow**); interstitial space with mild inflammatory infiltrates (**short arrow**)
(e) AESG 100 mgkg^{-1} group: reveals visibly congested coronary artery (**long arrow**) with not so distinct myofibrils and interstitial; characterized by mild inflammation and fatty changes (**short arrow**).

Figure 19a. Normotensive group: illustrates visible bundles of myofibrils (short arrow); interstitial space with prominent coronary artery (long arrow). **Figure 19b.** Untreated Hypertensive group: reveals bundles of myofibrils (short arrow); interstitial space and visibly enlarged coronary artery (long arrow). **Figure 19c.** AESG 25 mgkg^{-1} group: reveals enlarged congested coronary artery (long arrow) with indistinct bundles of myofibrils (short arrow). **Figure 19d.** AESG 50 mgkg^{-1} group: reveals prominent coronary artery (long arrow), with visible myofibrils (short arrow); interstitial space with mild inflammatory infiltrates (short arrow). **Figure 19e.** AESG 100 mgkg^{-1} group: reveals visibly congested coronary artery (long arrow) with not so distinct myofibrils and interstitial; characterized by mild inflammation and fatty changes (short arrow).

Conclusions

Considering the analyzed data, the outcome of the study revealed that pre-treatment with respective doses of AESG prevented salt-load induced weight loss; elevated systolic, diastolic blood pressures, mean arterial blood pressure and heart rates. We equally observed that the plant extract did not cause injury on the liver and kidney. Prophylactic treatment with respective doses of AESG prevented dyslipidemia and cardiovascular risk associated with elevated

lipoprotein cholesterols in salt-load induced hypertensive experimental models. On the overall, it was noted that the AESG demonstrated a significant antihypertensive effect; although, with little or no observed adverse effect on relevant metabolic organs and tissue considered in the study. Therefore, it is strongly recommended that further studies be conducted on other extracts of *S. glauca* to ascertain its antihypertensive efficacy; possible isolation of compounds and mechanism(s) of action.

Acknowledgements: The invaluable contributions of Osarenotor Osayomwanbo, *Ph.d* and Olusanya Olasehinde, *Ph.d* in correcting and suggesting the use of appropriate terms in the body of the work and assisting to put the references in order are acknowledged.

Authors' Contributions

SD.E, designed the work, conducted the analysis and interpretation of the data; reported the outcome of the analyzed data obtained from the study. **N.E.J** contributed to the concept of the study and approved the submission of the corrected version.

Conflict of Interest Declaration

The Author(s) declares and disclose that there are no competing or conflicts of interests before, during the course of the work and after.

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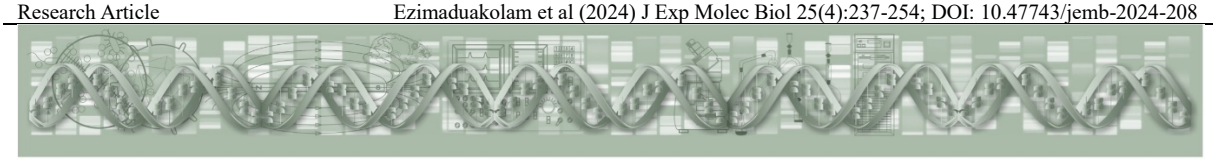

PHYTOCHEMICAL ANALYSIS AND TOXICITY STUDIES OF ETHANOL LEAVES EXTRACT OF *AZADIRACHTA INDICA*, *ANACARDIUM OCCIDENTALE* AND *MORINGA OLEIFERA* IN RATS

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Abstract

Medicinal plants are important natural products in drug discoveries due to their pharmacological activities. *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* have been used in traditional medicine practices for the treatment of many diseases. Various pharmacological activities of these plants have been reported and documented. Beside the medicinal value and pharmacological activities of these plants their phytoconstituents and safety for consumption should also be considered. This study aims at evaluating the phytochemicals composition and toxicity profile of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* in rats. The phytochemicals analyses were performed using standard analytical methods. Acute and sub-acute toxicity tests were conducted using Lorke's method and OECD guidelines, respectively. This finding revealed that ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* demonstrated the presence of significant amount of many phytochemicals. In acute toxicity test, the LD_{50} value of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* obtained was 3807.89 mg/kg, 4505.55 mg/kg, and above 5000 mg/kg, respectively. In sub-acute toxicity test, ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* demonstrated an increase in the levels of ALT, AST, total bilirubin, and globulin, while the *Moringa oleifera* exhibited a significantly ($p < 0.05$) decrease in the levels of ALT, AST, and total bilirubin coupled with increase in total protein and albumin levels. The concentrations of urea, creatinine, sodium, potassium, calcium, chlorides, and HCO_3^- were significantly ($p < 0.05$) increased in ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* while decreased in ethanol leaves extract of *Moringa oleifera* in rats. However, ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* demonstrated a significant ($p < 0.05$) decrease in RBC count and PCV while *Moringa oleifera* exhibited a significant ($p < 0.05$) increase in RBC count and PCV. Ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* contain significant amount of phytochemicals which might be responsible for their pharmacological activities. Ethanol leaves extract of *Moringa oleifera* at 5000 mg/kg dose is relatively non-toxic and safe for gavage administration while ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* are slightly toxic for oral administration.

Keywords: *Azadirachta indica*, *Anacardium occidentale*, *Moringa oleifera*, phytochemicals, toxicity

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Introduction

Medicinal plants have been recommended for the treatment of diseases and their safety measures have been documented (WHO 2000). Medicinal plants contain bioactive compounds known to demonstrate pharmacological activities and medicinal properties. Bioactive compounds include phytochemicals that have significant application in drug discovery and development (Abubakar et al. 2021). Phytochemicals from many plants have been reported to demonstrated various pharmacological activities including anti-diabetic (Ebong et al. 2008), anti-hypertensive (Mamta et al. 2013, ACS 2000), anti-cancer (Madhuri and Pandey 2009, Shaikh et al. 2016), anti-inflammatory (Patra et al. 2009), antioxidants (Lobo et al. 2009, Adamu et al. 2017, Ibrahim et al. 2024), anti-ulcer (Abubakar et al. 2020a, 2020b, Abubakar et al. 2021), antipyretic activities (Patra et al. 2009, Chandra et al. 2010), and anti-microbial (Mahesh and Satish 2008, Falowo et al. 2018).

Azadirachta indica commonly known as neem, is a medicinal plant belonging to the family Meliaceae, found abundantly in the tropical and subtropical countries of the world (Mohammad 2016). In Nigeria, *Azadirachta indica* is commonly called Atu yabasi in Igbo, Maina in Hausa, and Dongoyaro' in Yoruba. Neem plant has many therapeutic applications such as traditional medicine, modern medicine, nutraceuticals, food supplements, folk medicine, pharmaceutical intermediates and chemical entities for synthetic drugs (Mahima et al. 2013, Kumar et al. 2015). Different parts of *Azadirachta indica* have been used for the treatment of various diseases including inflammation, viral infections, hypertension, and fever and gastrointestinal diseases (Mossini and Kemmelmeier 2005, Shah et al. 2009, Talpur et al. 2013, Kaur et al. 2020). The parts of the plants have been reported to demonstrated antifungal, antibacterial, antiviral, and antioxidant activities (Adamu et al. 2017).

Anacardium occidentale L. is an evergreen perennial plant found abundantly in tropical countries and belongs to the family Anacardiaceae. The plant is popularly known as cashew and locally called kashu (Igbo), Fisa (Hausa), and Kaju (Yoruba) in Nigeria. Different parts of *Anacardium occidentale* have been used in treatment of many diseases including diarrhea, dysentery, pain, ulcer, diabetes, inflammation, dermatitis, headache, and infectious (Lizcano et al. 2010, Baptista et al. 2018, Gandji et al. 2018). It has been reported that cashew nuts demonstrated anti-diabetic activity due to its low sugar and high fiber contents (Bes-Rastrollo et al. 2007). Aqueous ethanol extract of leaves and nuts of *Anacardium occidentale* L. were reported to demonstrated anti-ulcerogenic activity in rats (Konan and Bacchi 2007, Behravan et al. 2012). Study showed that the acetone extract of cashew stem bark in rodents contains antibodies, and exhibited anti-inflammatory and antinociceptive activities (Vanderlinde et al. 2009). Onasanwo et al. (2012) reported that dichloromethane leaves extract of *Anacardium occidentale* L. demonstrated analgesic activity in rats. Aqueous extract leaves of *Anacardium occidentale* L. exhibited anti-diabetic activity in rats (Sokeng et al. 2001). Studies showed that petroleum ether and ethanolic extracts of cashew leaves demonstrated antimicrobial activities (Dahake et al. 2009, Doss and Thangavel 2011, Onasanwo et al. 2012).

Moringa oleifera Lam. (Family Moringaceae) is an arboreal plant found in many regions in the world mainly in tropical areas (Estrada-Hernández et al. 2016). The plant has been used daily as food vegetable and has nutritional and medicinal value (Ahmad et al. 2014, Santos 2014). *Moringa oleifera*, commonly called Moringa, is widely cultivated in various parts of Nigeria and is locally called Odudu oyibo in Igbo, Zogale in Hausa, and Aweigbale in Yoruba. *Moringa oleifera* has been used in the treatment of many diseases which include skin infections, anaemia, anxiety, asthma, blackheads, blood impurities, bronchitis, catarrh, chest congestion, and cholera (Khawaja et al. 2010, Hamza 2010, Singh and Sharma, 2012). Different parts of *Moringa oleifera* have been reported to demonstrate many pharmacological activities including anti-diabetic, anti-inflammatory, anti-spasmodic, anti-hypertensive, anti-tumor, anti-oxidant, anti-

pyretic, anti-ulcer, anti-epileptic, reported antibacterial, antifungal, diuretic, cholesterol lowering, renal, cardioprotective, and hepatoprotective activities (Lai et al. 2010, Paliwa et al. 2011, Huang et al. 2012, Sharma et al. 2012, Kou et al. 2018). The plant has been used in the manufacture of different pharmaceutical products (Ariana et al. 2022). Leaves, flowers, pods, and seeds of the plant have been used in production of drugs against bacteria, fungi, viruses, and other pathogens in humans (Falowo et al. 2018, Ariana et al. 2022).

Safety is one of the major criteria in the selection of medicinal plants of research interest. Efficacy of plant extracts should not only be considered but also their safety for consumption. Consumption of medicinal plants without knowledge of their toxic level could lead to damaging of organs especially liver and kidney due to their roles in metabolism and excretion of substances. Thus, screening of plant extracts for treatment of disease is couple with the knowledge of their toxicity profile. Research interest on pharmacological activities and medicinal properties of medicinal plants has been increase worldwide. High percentage of the people in the world relies on plants and herbs for remedies than the use of synthetic drugs. Conventional treatments produce side effects and are expensive for higher population of people in the world. Plants and herbs have fewer side effects and are less expensive than synthetic drugs. *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* are common medicinal plants familiar in the tropical and subtropical countries. These plants have been locally used for therapeutic purposes and they are availability, less cost and widely distributions in almost every community. Because of these the interest of researchers on these plants has drastically increased. This study aims at evaluating the phytochemicals composition and toxicity profile of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* in rats.

Materials and Methods

Plant Materials

Fresh leaves of *Anacardium occidentale*, *Azadirachta indica*, and *Moringa oleifera* were obtained from the Umudike forest, Ikwuano Local Government Area, Abia state, Nigeria. The plants samples were collected in the last quarter of the rainy season in October, 2023. The samples were immediately transported to the Herbarium unit of Michael Okpara University of Agriculture, Umudike, Abia for identification. The *Anacardium occidentale* (MOUAU/CVM/VPP/HERB/16/008), *Moringa oleifera* (MOUAU/CVM/VPP/HERB/16/009) and *Azadirachta indica* (MOUAU/CVM/VPP/HERB/16/010) samples were identified and authenticated by a taxonomist at the Department of Forestry, College of Natural Resources and Environmental Management, Michael Okpara University of Agriculture, Umudike, Abia, Nigeria.

Preparation of Plant extracts

The leaves of the plants were air dried under a shade for 14 days. The dried leaves were pulverized to fine powders using fabricated grinding machine. Preparation of the plant extracts was carried out using the method described by Abubakar et al. (2022a) with little modification. The fine powder (500 g) of each plant sample was extracted with 1.5 L of ethanol for 48 hours with intermittent stirring at 2 hour interval period. Each of the plant extract was filtered using Whatman No. 1 filter paper and concentrated using rotary evaporator under reduced pressure at a temperature 40^0C for three hours. For all the three plant samples a solid dark-green extract was obtained with a weight 100.66 g, 140.18 g and 110.94 g and percentage yield 20.13%, 28.04% and 22.19% for *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* leaves, respectively. The extracts were stored in desiccators until further used.

Experimental Animals

A total of 32 Wistar rats (8 – 10 weeks) of either sex weighing between 180 – 200 g were used in this study. The animals were obtained from the laboratory animal production unit of the

Zoology and Environmental Biology, Michael Okpara University of Agriculture, Umudike. The animals were housed in aluminum cages (5 rats per cage) under standard conditions temperature 23±2°C, relative humidity 30-70%, and 12/12 hours light-dark cycle. The animals were fed with standard grower pellets (Chikkun Finisher Mash, Chikkun Feeds Ltd) and had access to water *ad libitum* for seven days of acclimatization before the commencement of the experiment.

Qualitative Determination of Phytochemicals

Test for the Presence of Alkaloids

The presence of alkaloids in the ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* were determined using Wagner's test method described by Trease and Evans (1989) and Abubakar et al. (2020a, 2022). The plant extracts (3 mL) was added into a test tube containing 3 ml of 1% HCl and then heated at 60°C for 20 minutes. The contents were allowed to cool and then Wagner's reagent (1 mL) was added drop by drop into the test tube. The presence of alkaloids was detected by the formation of reddish-brown precipitate.

Test for the Presence Flavonoids

Flavonoids in the plant extracts were identified by sodium hydroxide test described by Mosa et al. (2012) and Abubakar et al. (2022, 2020a). The plant extracts (3 ml) was added into a test tube containing 1 ml NaOH 10% solution. The deep yellow colour which became colourless following addition of dilute HCl solution indicated the presence of flavonoids.

Test for Tannins

Ferric chloride test was used for qualitative determination of tannins in the three plant extracts as described by Trease and Evans (1989). The plant extracts (1 mL) was added into a test tube containing two milliliters of 5% $FeCl_3$ solution. The presence of tannins was detected by the formation of black or blue-green colour.

Tests for Glycosides

Glycosides in the plant extracts were identified by Salkowski's test method described by Trease and Evans (1989) and Abubakar et al. (2022, 2020a). The plant extracts (5 mL) was added into a test tube containing 25 mL of 1% sulphuric acid and then boiled for 15 minutes. The contents were allowed to cool and then neutralized with 10% NaOH and followed by addition of 5 mL of Fehling's solution A and B. The presence of glycosides was observed by the formation of brick red precipitate of reducing sugars.

Test for Cardiac Glycosides

Keller-Killani test was employed for the screening of cardiac glycosides as described by Mosa et al. (2012) and Abubakar et al. (2020a, 2022). The glacial acetic acid (2 mL) containing one drop of ferric chloride solution was added into each test tube containing 5 mL of the respective plant extracts. One mile of concentrated sulphuric acid was added into the test tube. The formation of brown ring at the interface, violet colour below the brown ring, and greenish colour throughout the acetic acid layer indicated the presences of cardiac glycosides.

Test for Steroids

The presence of steroids in the plant extracts was determined using the method of Trease and Evans (1989). Chloroform (5 mL) and sulphuric acid (5 mL) were added into a test tube containing 500 µl of the plant extracts. The presence of steroids was observed by the formation of violet colour which changed to blue-green.

Test for Saponins

The plant extracts was analyzed for the presence of saponins using Froth test as described by Trease and Evans (1989) and Abubakar et al. (2020a, 2022). The plant extracts (3 mL) was added into a test tube containing 3 mL of distilled water. The contents were thoroughly mixed by vigorous shaken of test tube for about 30 seconds and allowed to stand for 30 minutes. The presence saponins was detected by the formation of stable persistent froth.

Test for Phenols

The qualitative determination of phenols in the plant extracts was carried out in accordance to method described by Trease and Evans (1989) with little modification. Ethanol (5 mL) and ferric chloride (5 mL) was added into each test tube containing 5 mL of plant extracts. Phenols in the plant extracts were identified by the formation of an ink blue color.

Test for Terpenoids

Terpenoids screening in the plant extracts was carried out using the method described by Trease and Evans (1989) and Abubakar et al. (2020a, 2022). The plant extract (1 mL) was added into a test tube containing ethanol (1 mL) and acetic anhydride (1 mL) and then thoroughly shaked. Concentrated sulphuric acid (10 mL) was added into the test tube. The presence of terpenoids was observed by the formation of pink color.

Quantitative Determination of Phytochemicals

Determination of Alkaloids Content

The quantity of alkaloids in each of the plant extracts was determined using the method of Harborne (1973) with little modification. Five grams of each of the plants dried extract was dissolved in 200 mL of 10% acetic acid and 50 mL of ethanol. The contents were allowed to stand for 2 hours, filtered using Whatman No. 1 filter paper and then concentrated using water bath. Concentrated NH_4OH solution was added to the extract in drops and the precipitate formed was collected, washed with dilute NH_4OH solution and then filtered using Whatman No. 1 filter paper. The final alkaloid residue was dried and weighed and the alkaloids content was obtained.

Determination of Flavonoids Content

The flavonoids content in each of the plant extracts was determined using the method described by Harborne (1973) with some modifications. The plants dried extract (5 mg) was boiled (100°C) in 50 ml of 2M HCl solution for 30 minutes under reflux. The content was allowed to cool and then filtered using Whatman No. 1 filter paper. The ethyl acetate (50 mL) was added into the mixture in drops and then to the volume. The mixture was filtered using filter paper and then dried in oven at 6ºC. The dried flavonoids residue was weighed and the quantity of flavonoids in the plant extracts was estimated.

Determination of Saponins Content

Quantitative determination of saponins in each of the plant extracts was carried out using the method of El-Olemylet al. (1994) with a little modification. Five grams of the plants dried extract was dissolved in 150 mL of 50 % ethanol. The contents were boiled for 30 minutes and then filtered using Whatman filter paper. One gram of charcoal was added to the filtrate and the content was heated at 100°C for 30 minutes. The content was filtered using Whatman filter paper and then allowed to cool at room temperature. Acetone (150 mL) solution was added into the filtrate and the content was filtered using Whatman filter paper. The filter paper was quickly introduced into desiccator containing anhydrous calcium chloride. The saponins residue was concentrated to dryness in oven at 6°C. The dried saponins residue was weighed and the saponins content in the plant extracts was determined.

Determination of Steroids Content

The quantitative determination of steroids content in the grapefruits aqueous extract was carried out according to the method described by Evans (1996) with some modifications. Each of the plant extracts (1 mL) was introduced into 10 mL volumetric flask followed by addition of sulphuric acid (2 mL) and iron chloride (2 mL). The contents were treated with 2 mL of potassium hexacyanoferrate (III) solution and then incubated at 70°C for 30 minutes with constant shaking. Absorbance of the sample against the blank was read at 780 nm wavelength using spectrophotometer. The amount of steroids in the plant extracts was determined.

Determination of Tannin Content

Tannin in each of the plant extracts was quantitatively determined using the method of AOAC (1999) with some modifications. The tannic acid standard solution was prepared by dissolving 10 mg of tannic acid in 100 mL of distilled water. Different concentrations of tannic acid standards (0 – 2.5 ml aliquots) were prepared in the respective 25 mL volumetric flasks followed by the addition of Folin-Denis reagent (2.5 ml) and sodium carbonate solution (1.25 mL) and then the content was made up to the volume. After 30 minutes absorbance was measured at 760 nm wavelength using spectrophotometer. The dried powder extract (1 g) of each of the plants was boiled in distilled water (80 mL) for half hour. The contents were allowed to cool and then diluted in 100 mL volumetric flask. The contents were filtered using Whatman filter paper No. 1 and the amount of tannin in the filtrate was determined. The tannin content in the plant extracts was obtained using tannic acid standard curve and expressed as mg/g of the extract.

Determination of Cardiac Glycosides

Quantitative determination of cardiac glycosides in the plant extracts was performed using the method of Harborne (1973). One gram of each of the plants dried powder extract was dissolved in 20 mL of distilled water and then 2.5 mL of 15% lead acetate was added. The mixture was filtered using Whatman filter paper. A volume of 2.5 mL chloroform was added into the filtrate and the content was vigorously shaken. The lower layer was collected and evaporated to dryness in oven at 6°C. The residue was dissolved in 3 mL of glacial acetic acid followed by addition of 0.1 mL of 5% ferric chloride and 0.25 mL of concentrated H_2SO_4. The contents were shaken and incubated for 2 hours in the dark. Absorbance of the sample against the blank was measured at 530 nm spectrophotometrically.

Determination of Terpenoids Determination

Terpenoids content in each of the plant extracts was analyzed using the method of Harborne (1973). The dried plant extracts (1 g) were dissolved in 50 mL of ethanol and then filtered using Whatman filter paper. The filtrate was treated with 2.5 ml of 5% aqueous phosphomolybdic acid and 0.25 mL of concentrated H_2SO_4 gradually. The contents were allowed to stand for 30 minutes. Absorbance of the sample against the blank was read at 700 nm using spectrophotometer.

Determination of Phenols

The quantitative analysis of phenols in each of the plant extracts was carried out using the method described by Santhi and Sengottuvel (2016). One gram of each of the plant extracts was dissolved 50 mL of ether and boiled for 15 minutes. The plant extracts was pipetted out into a 50 mL conical flask and then 10 mL of distilled water, 2 mL of NH_4OH, and 5 mL of conc. amyl alcohol was added into the flask. The contents were shaked and incubated for 30 minutes at room temperature. The absorbance of the sample against the blank was measured using spectrophotometer at 505 nm wavelength.

Determination of Glycosides

Glycosides in the each of the plant extracts were determined using the method of Nbaeyi-Nwaoha and Onwuka (2014). The plant extracts (1 g) was added into the respective test tubes containing 2.5 mL of 15% lead acetate. The contents were mixed thoroughly and then filtered using Whatman filter paper. The chloroform (2 mL) was added into the filtrate, mixed thoroughly and allowed to settle at room temperature. The lower portion was collected and evaporated to dryness in oven at 60°C. The dried lower portion was mixed with 3 mL of glacial acetic acid, 0.1 mL of 5% ferric chloride and 0.25 mL of conc. H_2SO_4 and then allowed to stand for 3 hours. Absorbance of the sample against the blank was read at 568 nm wavelength using spectrophotometer and the amount of glycosides was obtained.

Acute Toxicity (LD_{50}) Study

The acute toxicity test of each plant extracts was carried out in using the Lorke's method (1983). The method involves two phases. In the first phase, nine rats were divided into three groups; three rats per group. The animals in group 1, 2, and 3 were orally administered with 10, 100 and 1000 mg/kg doses of the plant extracts per body weight of the rats, respectively. In the second phase, three rats were used. The animals were treated with 1600, 2900 and 5000 mg/kg doses of the plant extracts per body weight, respectively. All the animals were observed for any sign of toxicity and lethality for 24 hours. The animals were initially observed for 24 hours and up to 7 days. The LD_{50} value of each of the plant extracts was calculated using the following formula:

$LD_{50} = \sqrt{D0 \text{ X } D100}$

Where; D_0= highest dose that produced no mortality, D_{100} = lowest dose produced mortality.

Sub-acute Toxicity Study

Sub-acute toxicity test was conducted using the Organization for Economic Co-operation and Development (OECD) guidelines 407 (2008) with some modifications. A high dose (800 mg/kg) of each of the three plant extracts was selected based on the results of the acute toxicity test. The Wistar rats (8 – 10 weeks, 180 – 200 g, n = 20) were divided into 4 groups each of 5 rats. Group 1 (normal control) was treated with 10 ml/kg/day b.w. of normal saline by gavage. Group 2, 3, and 4 were administered orally with the 800 mg/kg/day b.w. of the ethanol leaves extract of *Anacardium occidentale*, *Azadirachta indica*, and *Moringa oleifera*, respectively. The repeated dose **(**800 mg/kg) extract was administered daily for 28 days. Signs of toxicity and mortality were observed, and body weight of the animals was evaluated weekly. At the end of the administration period, the animals were fasted for 12 hours, anaesthetized using chloroform and then euthanized by cervical dislocation. Blood samples were collected by cardiac puncture into EDTA and plain tubes for haematological and biochemical analysis, respectively. For the biochemical analysis the blood samples were allowed to clot and retract within two hours and then centrifuged at 10,000 x g for 10 minutes. The sera were separated for the various biochemical analyses.

Biochemical analysis

The analysis of biochemical parameters total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) alkaline phosphatase (ALP), total bilirubin, globulin, urea, creatinine, sodium, potassium, calcium, chlorides, and HCO_3^- was conducted spectrophotometric/colometric method using standard diagnostic kits (Sigma-Aldrich) according to the manufacturer's instructions.

Hematological analysis

The hematological counts parameters white blood cells (WBC), red blood cells (RBC), haemoglobin (Hb), platelet count (PLT), packed cellular volume (PCV) mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were analyzed using hematology analyzer (Sysmex).

Statistical Analysis

The results were expressed as mean ± SD. Statistical Package for Social Sciences (SPSS) Statistics version 22 software (IBM Corp., Armonk, NY, USA) was used for data analysis. Significant differences were determined using one way analysis of variance (ANOVA) at confidence level (95%) by Dunnett's test. Mean differences were considered statistically significant at $p < 0.05$.

Results

Qualitative Phytochemical Screening of the Plant extracts

Table 1 shows the result of qualitative phytochemical screening of the ethanol leaves extract of *Anacardium occidentale*, *Azadirachta indica*, and *Moringa oleifera*. The result showed that all the plant extracts contain various phytochemicals including alkaloids, flavonoids, saponins, glycosides, tannins, terpenoids, steroids, cardiac glycosides, and phenols (Table 1). However, the *Azadirachta indica* and *Anacardium occidentale* leaves extract demonstrated high amount of alkaloids and saponins while *Moringa oleifera* leaves extract exhibited moderate amount of alkaloids and saponins. The *Azadirachta indica* and *Moringa oleifera* leaves extract contained moderate amount of tannins, glycosides, and phenols which were fairly found in the *Anacardium occidentale* leaves extract. The moringa, *Anacardium occidentale*, and *Azadirachta indica* leaves extract exhibited high, moderate, and low amount of flavonoids, respectively. Moderate amount of terpenoids and steroids were found in the *Azadirachta indica* and *Anacardium occidentale* leaves extract while *Moringa oleifera* leaves extract demonstrated low amount of terpenoids and steroids. All the three plant extracts demonstrated low amount cardiac glycosides (Table 1).

Table 1. Qualitative Phytochemicals Screening of the Plant extracts

Phytochemical	*Ao* Extract	*Ai* Extract	*Mo* Extract
Alkaloids	+++	+++	++
Tannins	+	++	++
Flavonoids	++	+	+++
Phenols	+	++	++
Terpenoids	++	++	+
Saponins	+++	+++	++
Glycosides	+	++	++
Steroids	++	++	+
Cardiac glycosides	+	+	+

+++ = High amount, ++ = Moderate amount, + = Low amount. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo)

Quantitative Phytochemicals Composition of the Plant extracts

The quantitative phytochemicals composition of the ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* is shown in Figure 1. The ethanol leaves extract of *Azadirachta indica* demonstrated high significant amount of alkaloids (60.49 mg/g), saponins (35.45 mg/g), and tannins (13.79 mg/g), than the *Anacardium occidentale* (35.11 mg/g, 28.55 mg/g, 1.99 mg/g), and *Moringa oleifera* (15.34 mg/g, 25.19 mg/g, 12.84 mg/g), respectively. High quantity of flavonoids (12.84 mg/g) and phenols (19.07 mg/g) was recorded in *Moringa oleifera* ethanol leaves extract compared to the amount demonstrated by the *Azadirachta indica* (5.44 mg/g, 9.93 mg/g) and *Anacardium occidentale* (10.32 mg/g, 7.12 mg/g), respectively. *Anacardium occidentale* ethanol leaves extract exhibited high value of glycosides (7.90 mg/g), steroids (11.14 mg/g), cardiac glycosides (5.62 mg/g), and terpenoids (10.60 mg/g) than the *Azadirachta indica* (4.82 mg/g, 3.92 mg/g, 3.32 mg/g, 7.72 mg/g) and *Moringa oleifera* ethanol leaves extract (6.62 mg/g, 5.66 mg/g, 2.69 mg/g, 6.33 mg/g), respectively (Figure 1).

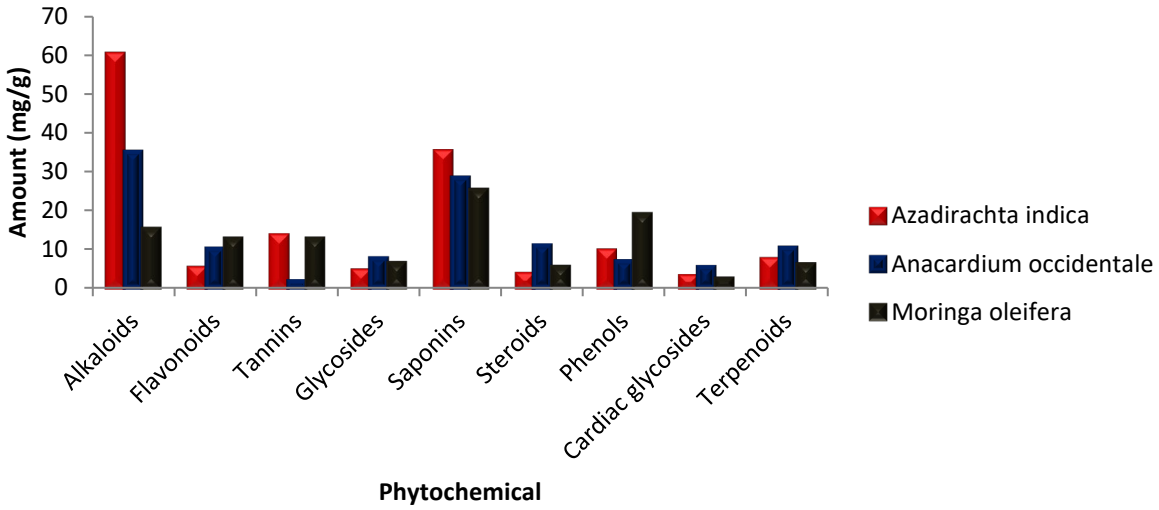

Figure 1. Quantitative Phytochemicals Composition of the Plant extracts
Values are expressed as mean ± SD (n = 3)

Acute Toxicity Test

For the ethanol leaves extract of *Azadirachta indica*, no sign of toxicity was observed and no mortality recorded at all the doses administered in the first phase (10, 100 and 1000 mg/kg). In the second phase, 1600 mg/kg and 2900 mg/kg doses of the extract the animals did not show any sign of toxicity and no mortality found. However, at 5000 mg/kg of the extract, rats were initially calm and depressed, and 100% mortality was recorded before the end of 24 hours period of administration. Thus, the LD_{50} value of the ethanol leaves extract of *Azadirachta indica* was 3807.89 mg/kg. In the first phase of acute toxicity test of ethanol leaves extract of *Anacardium occidentale* there was no sign of toxicity observed nor mortality recorded at all the administered doses (10, 100 and 1000 mg/kg). In the second phase, administration of 1600 mg/kg and 2900 mg/kg produced no mortality while at 5000 mg/kg, 33.33% mortality was recorded and 100% mortality rate was recorded at 7000 mg/kg of the extract. Thus, the LD_{50} value of ethanol leaves extract of *Anacardium occidentale* was 4505.55 mg/kg body weight. The ethanol leaves extract of *Moringa oleifera* at all the doses in phase 1 (10, 100 and 1000 mg/kg) and phase 2 (1600, 2900, and 5000 mg/kg) did not produce mortality or any sign of toxicity in rats during the observation period. Thus, the LD50 value of ethanol leaves extract of *Moringa oleifera* was greater than 5000 mg/kg.

Sub-acute Toxicity Studies

Effect of the Plant extracts on Body Weight of Rats

Figure 2 shows the effect of the plant extracts on body weight of rats. In the third week of the plant extracts administration, ethanol leaves extract of *Azadirachta indica* demonstrated significant ($p < 0.05$) decrease in the body weight of rats as compared with the control. In comparison with the control, administration of ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* significantly ($p < 0.05$) decrease the body weight of rats in week 4. However, the ethanol leaves extract of *Moringa oleifera* exhibited significant ($p < 0.05$) increase in the body weight of rats as compared with the control in week 4 (Figure 2).

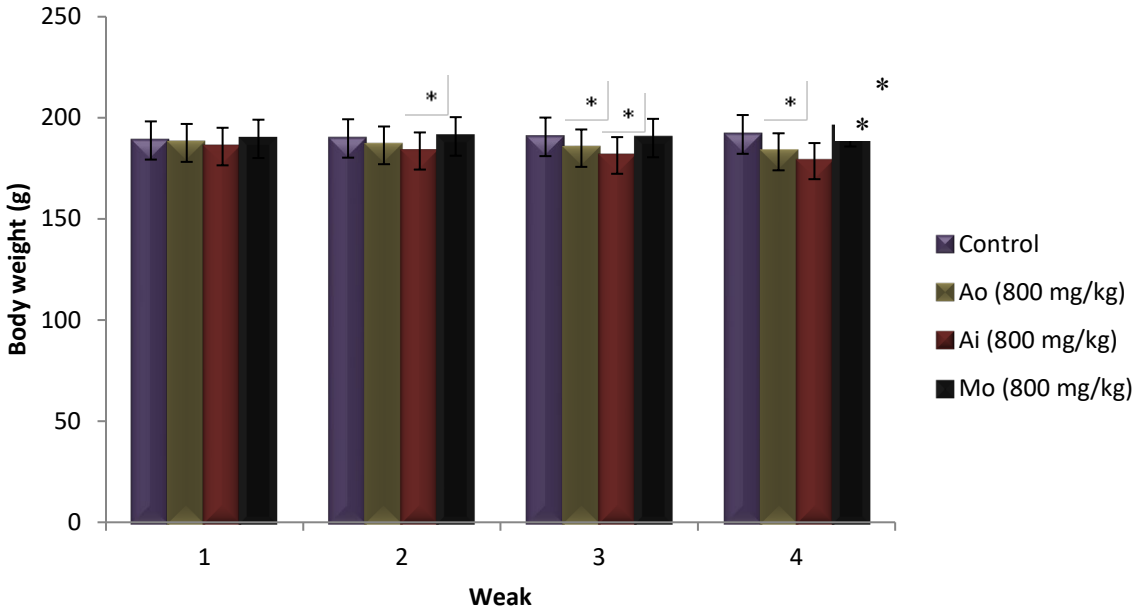

Figure 2. Effect of the plant extracts on the body weight of rats
Data are expressed as mean ± SD (n = 5).
*$p < 0.05$ statistically significant compared with control (One-way ANOVA) followed by Dunnett's multiple comparison test. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo)

Effect of the Plant extracts on Biochemical Parameters in Rats

Table 2 shows the effect of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale* and *Moringa oleifera* on liver function parameters in rats. The ethanol leaves extract of *Moringa oleifera* demonstrated significant ($p < 0.05$) increase in total protein levels in the rats compared with the control. The concentration of albumin in the rats was significantly ($p < 0.05$) decrease following administration of the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* when compared with the control. In comparison with the control, the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* exhibited significant ($p < 0.05$) increase in the level of ALT, AST and total bilirubin in the rats. The ethanol leaves extract of *Azadirachta indica* demonstrated a significant ($p < 0.05$) decrease in the level of ALP compared with control and the other treated groups. However, the result also showed that ethanol leaves extract of *Azadirachta indica* and *Moringa oleifera* demonstrated significant ($p < 0.05$) increase in globulin levels in the rats compared with the control (Table 2).

Table 2. Effect of Plant extracts on Liver Function Parameters in Rats

Group	Control	*Ao* (800 mg/kg)	*Ai* (800 mg/kg)	*Mo* (800 mg/kg)
Total protein (g/dl)	5.78±0.11^{a}	5.65±0.15^{a}	5.81±0.28^{a}	6.19±0.10^{b}
Albumin (g/dl)	3.29±0.04^{a}	3.18±0.09^{ab}	3.09±0.07^{b}	3.39±0.07^{b}
Globulin (g/dl)	2.49±0.13^{a}	2.48±0.08^{a}	2.72±0.23^{ab}	2.90±0.09^{b}
ALT (u/l)	35.00±1.00^{a}	39.33±1.16^{b}	43.00±3.61^{b}	34.14±3.00^{a}
AST (u/l)	42.33±2.52^{a}	45.00±4.58^{b}	44.67±2.52^{b}	41.00±1.73^{a}

ALP (u/l)	81.33±4.73[a]	79.33±4.51[a]	76.67±6.11[b]	80.00±9.17[a]
TB (mg/dl)	0.69±0.04[a]	0.83±0.09[b]	0.85±0.06[b]	0.66±0.04[a]

Data are expressed as mean ± SD (n = 5)
Values with different letters are statistically significant ($p < 0.05$) (One-way ANOVA) followed by Dunnett's multiple comparison test. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo), standard deviation (SD), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB)

The effect of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale* and *Moringa oleifera* on the renal function parameters in rats is shown in Table 3. All the three plant extracts exhibited non-significant ($p > 0.05$) changes in the levels of urea, creatinine, potassium, calcium, chlorides, and HCO_3^- when compared with the control. Although the no significant changes were observed, the levels of urea, creatinine, potassium, calcium, chlorides, and HCO_3^- were increased following administration of the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* and decreased by the administration of the ethanol leaves extract of *Moringa oleifera* in rats. However, in comparison with the control, the ethanol leaves extract of *Anacardium occidentale* demonstrated significant ($p < 0.05$) increase in concentration of sodium in the rats.

Table 3. Effect of the Plant extracts on Renal Function Parameters in Rats

Group	Control	*Ao* (800 mg/kg)	*Ai* (800 mg/kg)	*Mo* (800 mg/kg)
Urea (mg/dl)	20.25±0.47[a]	21.34±1.84[a]	20.93±0.79[a]	19.87±1.12[a]
Creatinine (mg/dl)	0.76±0.06[a]	0.81±0.09[a]	0.79±0.03[a]	0.74±0.06[a]
Na+ (mEq/L)	130.73±1.96[a]	135.57±2.31[b]	132.20±2.86[a,b]	129.67±2.93[a]
k+ (mEq/L)	4.36±0.15[a]	4.47±0.14[a]	4.50±0.11[a]	4.54±0.18[a]
Cl^- (mEq/L)	89.60±0.70[a]	90.93±0.94[a]	92.20±2.27[a]	89.83±1.01[a]
HCO_3^- (mmol/L)	19.87±0.31[a,b]	20.3±0.10[b]	20.23±0.15[b]	19.73±0.35[a]

Values are expressed as mean ± SD (n = 5)
Values with different letters are statistically significant ($p < 0.05$) (One-way ANOVA) followed by Dunnett's multiple comparison test. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo), standard deviation (SD), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB)

Effect of the Plant extracts on Haematological Parameters

Table 4 shows the effect of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale* and *Moringa oleifera* on the haematological parameters in rats. The ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* demonstrated significant ($p < 0.05$) decrease in RBC count and PCV compared with the control. In comparison with the control, the ethanol leaves extract of *Moringa oleifera* exhibited non-significant ($p > 0.05$) increase in RBC count and PCV (Table 4). Hemoglobin content was significantly ($p < 0.05$) decrease in rats administered with the ethanol leaves extract of *Azadirachta indica*. All the three plant extracts demonstrated significant ($p < 0.05$) increase in WBC count (Table 4). In comparison with control, ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* exhibited significant ($p < 0.05$) decrease and increase in PLT and MCH content, respectively (Table 4).

Table 4. Effect of the Plant extracts on Haematological Parameters in Rats

Group	Control	*Ao* (800 mg/kg)	*Ai* (800 mg/kg)	*Mo* (800 mg/kg)
RBC (x10^6/mm^3)	7.07±0.38^a	6.57±0.14^b	6.41±0.21^b	7.16±0.11^a
PCV (%)	44.00±1.22^a	41.00±1.50^b	40.33±2.08^b	44.04±1.00^a
Hb (g/dl)	16.73±0.46^a	16.07±0.12^{a,b}	15.73±0.70^b	16.80±0.26^a
WBC (x10^3/mm^3)	8.95±0.45^a	9.73±0.08^{b,c}	10.16±0.28^c	9.56±0.15^b
PLT (x10^3/mm^3)	234.00±2.61^a	227.67±2.52^b	229.00±2.19^b	233.33±3.16^a
MCV (fl)	62.31±2.15^a	62.43±0.22^a	63.02±4.29^a	61.48±0.52^a
MCH (pg)	23.69±0.63^a	24.47±0.40^b	24.55±0.31^b	23.47±0.25^a
MCHC (g/dl)	38.03±0.53^a	39.20±0.73^a	39.10±0.30^a	38.19±0.35^a

Values are expressed as mean ± SD (n = 5)
Values with different letters are statistically significant ($p < 0.05$) (One-way ANOVA) followed by Dunnett's multiple comparison test. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo), standard deviation (SD), packed cell volume (PCV), haemoglobin (Hb), white blood cell (WBC).

Discussions

Result of this study showed that ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* contained many phytochemicals including alkaloids, flavonoids, tannins, glycosides, saponins, steroids, cardiac glycosides, phenols, and terpenoids. Phytochemicals have been reported with many pharmacological activities and have medicinal importance (Oghenejobo 2017, Abubakar et al. 2022). It has been reported that flavonoids isolated from different plant extracts exhibited antioxidant activity scavenging activity, anti-cancer, anti-malarial, antihypertensive and anti-ulcer activity (Ballard and Marostica 2018). Brewer (2011) reported that alkaloids isolated from different plant extracts demonstrated analgesic activity. Saponins are source of steroidal hormones which have been shown to demonstrated anticholesterolmia activity (Kar 2007). Tannins isolated from plant extracts have been used as wound healing agents, astringents agents and in treatment of gastrointestinal diseases (De-Bruyne et al. 1999, Kar 2007). Denwick (2002) reported that cardiac glycosides isolated from different plant extracts have been used in management of cardiovascular diseases and including congestive heart failure and cardiac arrhythmia. Steroids are important precursors for biosynthesis of sex hormones and steroidal drugs such as corticosteroid (Majeed et al. 2004). Phenols from plant extracts demonstrated many pharmacological activities including anti-ulcer, anti- inflammatory, cytotoxic and antitumor, anti-spasmodic, and anti-depressant activities (Ghasemzadeh et al. 2010).

Acute toxicity test (LD_{50}) provides basic information for classification and hazard assessment of new chemical substances which can be used for safety statements on labels of potentially poisonous substances (OECD 2001). Lethal dose 50 % (LD_{50}) is an indicator of the potential of substance to produce harmful effect and can be used to obtained therapeutic dose and therapeutic index of test substance (Barile, 2010). In this study, the LD_{50} value of ethanol leaves extract of *Azadirachta indica* (3807.89 mg/kg) and *Anacardium occidentale* (4505.55 mg/kg) was less than 5000 mg/kg; while that of *Moringa oleifera* was greater than 5000 mg/kg. Substances with an LD_{50} value greater than 5000 mg/kg through gavage administration are regarded as being safe (Abubakar et al. 2020a; OECD, 2001). It has been reported that substance with $LD_{50} \leq 50$ mg/kg, $LD_{50} = 50 - 300$ mg/kg, $LD_{50} = 301 - 2000$ mg/kg, $LD_{50} =$

2001 - 5000 mg/kg, and LD_{50}> 5000 mg/kg is regarded as highly toxic, toxic, moderately toxic, slightly toxic, and non-toxic, respectively (Loomis and Hayes 1996, Erhirhie et al. 2018). This indicated that the ethanol leaves extract of *Moringa oleifera* at 5000 mg/kg dose is relatively non-toxic and safe for gavage administration. This also suggested that the ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* are slightly toxic for oral administration.

Change in body weight is important indicator of adverse effects of a substance (Abubakar et al. 2020a). In this study, ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* demonstrated significant decrease in the body weight of rats while *Moringa oleifera* exhibited significant increase in the body weight of rats. Decrease in body weight is associated with toxic effect of a chemical substance (Teo et al. 2002, Abubakar et al. 2020a). Liver and kidney are major organs affected by the toxic effect of substances (Auerbach et al. 2012, Awe and Banjoko 2013). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are important markers of liver functions and used as biomarkers to evaluate toxic effect of a substance (Rahman et al. 2000). High levels of AST and ALT in the blood are attributed to destruction of liver parenchymal cells (Abubakar et al. 2020a). High level of AST and ALP is an indicator of bile duct, lungs, kidneys and liver damage (Awe and Banjoko 2013, Mujahid et al. 2017). In the present study, the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* demonstrated an increase in the levels of ALT, AST, total bilirubin, and globulin. This indicated that liver cell functions in the rats might be affected by the oral administration the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica*. However, the ethanol leaves extract of *Moringa oleifera* demonstrated a decrease in the levels of ALT, AST, and total bilirubin coupled with increase in total protein and albumin levels. This suggests that repeat-dose oral administration of the *Moringa oleifera* did not caused changes in the liver cell functions in the rats.

Urea and creatinine are waste products passed into the bloodstream and excreted by the kidney. Urea and creatinine levels are vital renal functions biomarkers (Rahman et al. 2000). High levels of urea and creatinine in the blood is an indicator of renal function impairment (Cameron and Greger 1998). In the present study, administration of the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* increased the levels of urea, creatinine, sodium, potassium, calcium, chlorides, and HCO_3^- while decreased by the administration of the ethanol leaves extract of *Moringa oleifera* in rats. This suggested that sub-acute administration of the *Moringa oleifera* extract does affected normal renal functions while *Anacardium occidentale* and *Azadirachta indica* extract could cause renal impairment.

In this study, ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* demonstrated significant decrease in RBC count and PCV while *Moringa oleifera* exhibited significant increase in RBC count and PCV. Hemoglobin content was significantly decrease by the ethanol leaves extract of *Azadirachta indica*. All the three plant extracts demonstrated significant increase in WBC count. High rate of hematopoiesis renewal has been considered as a sensitive target for toxicity (d'Yvoire et al. 2012). Increase in the rate of hematopoiesis is associated with increase in erythrocytes and leukocytes. In conditions such as dehydration and blood loss, more cells are produced in biological system to meet out the demand for more mature cells (d'Yvoire et al. 2012). Thus, increase in WBC counts by the plant extracts could be attributed to any such factors. Haemoglobin (Hb) is responsible for the transport of oxygen into the tissues and carbondioxide out of the tissues and absorbs nutrients used to release energy for normal body function (Enechi et al. 2019). Presence of phytochemicals is associated with elevated level of red blood cell and hemoglobin due to their potential to activate the production of erythropoietin that stimulates the stem cells of the haemopoietic tissue to produce more red blood cells (Arunsi et al. 2020). Phytochemicals demonstrated inhibitory effect on red blood cells hemolysis which elevates the serum levels of bilirubin resulting to harmful effects on the hematological system (Fevery 2008). White blood cells (WBC) play an important role in the

immune system by fighting infections and inflammation in the body. Thus, the non-significant changes in WBCs may indicate that the extracts did not increase or suppression the immune response of treated animals (Tousson et al. 2011). This suggested that the ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* could affect the erythropoiesis process.

Conclusions

The ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* contain several phytochemicals. The presence of these bioactive metabolites implies that the plants have medicinal properties. The ethanol leaves extract of *Moringa oleifera* at 5000 mg/kg dose is relatively non-toxic and safe for gavage administration while ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* are slightly toxic for oral administration. The repeat-dose oral administration of the *Moringa oleifera* did not caused changes in the liver and kidney cell functions in the rats while repeat-dose oral administration of the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* might affect the normal liver and kidney cell functions. This study also suggests that ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* could affect the erythropoiesis process. Further studies should be done to evaluate chronic toxicity profile of the plants and to investigate medicinal properties and pharmacological effects of the plants.

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Conflict of Interest
The authors declare no conflict of interest.

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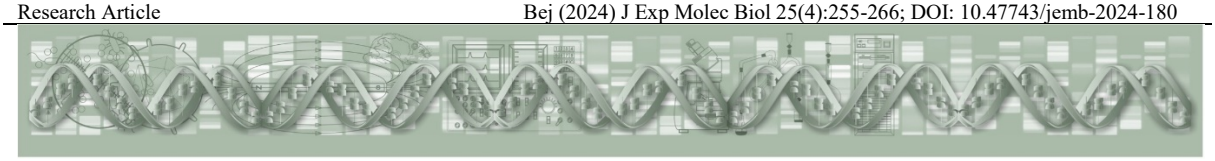

MOLECULAR CLONING AND QUANTITATIVE mRNA EXPRESSION OF *sox9* GENE IN THE GONADAL DEVELOPMENTAL PERIOD OF THE FISH COBALTCAP SILVERSIDE *HYPOATHERINA TSURUGAE*

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Abstract

Sox9 is a transcription factor of high mobility group (HMG) box family DNA binding domain. It plays a crucial role in gonadogenesis during embryonic developmental period. 1454 bp of *sox9* mRNA transcript of *Hypoatherina tsurugae* (D. S. Jordan & Starks) was cloned and sequenced. It consists of an open reading frame (ORF) of 1436 bp, that encodes a 479 aa protein, found to be identical to the HMG box of other fish species. A phylogenetic tree was constructed by comparing the mRNA sequence of 50 different fishes across various taxa available in the NCBI database and using as outgroup *Acipenser sinensis*. The tree shows a high homology of *sox9* from *H. tsurugae* with that from *Maelanotaenia boesemani*, the two forming a single clade. The expression of *sox9* was studied in *amhy*+ (male) individuals. It begins from baseline at 0 wah (week after hatching) and is expressed in an increasing fashion. In *amhy*- (female) individuals it is highly expressed at initial stage (0 wah) and the expression reaches its peak at 2 wah then declines, indicating the low expression needed for differentiation of the female sex organs. The histological sections of gonads were studied in different stages of biweekly collected larvae during the sex determination/differentiation period and it showed that differentiation of gonads male/female is decided at 6 wah. In this stage the primary oocytes are clearly recognized and it correlates with the expression of *sox9* genes. These finding add to the knowledge for a better understanding of molecular mechanisms of sex determination and differentiation period in fishes.

Keywords: Atheriniformes, *sox9*, *Hypoatherina tsurugae*, gonadal development

Introduction

It has been reported that the *amhy* gene (Y chromosome-linked anti-Müllerian hormone) has a critical role in male sex determination of an old world Silverside, *Hypoatherina tsurugae* (D. S. Jordan & Starks) (Bej et al. 2017). Many other genes/transcription factors have also been described as master sex determining genes in various fishes (Matsuda et al. 2002, Myosho et al. 2012, Yano et al. 2012, Hattori et al. 2013, Takehana et al. 2014). From these references, it is clear that the genetic machinery of fishes which control gonadal development is very diverse and is not limited to a particular gene/transcription factor as most interestingly reported for the *sdY*, an immune related gene that can crosstalk as sex determining gene in Salmonidae (Yano et al. 2012).

Sox (Sry related high mobility group box) are a gene family of transcription factors that possess an important role in reproduction and development of gonads (Hu et al. 2021). *Sox9* is a member of Sox-family that serves a crucial role in testis formation besides other function like cartilage formation (Healy et al. 1999, Jakubiczka et al. 2010). This is a potential candidate gene in fish

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that may drive downstream development of gonads after being triggered by master sex determining gene during gonadal differentiation period. Sekido and Lovell-Badge (2008) believed this gene is to be the main effector of *sry* gene to regulate the downstream pathway. Studies show that the mutation in *sox9* gene results in abnormal bone formation and even sex reversal (Jakubiczka et al. 2010, Georg et al. 2010).

Hypoatherina tsurugae, commonly called cobaltcap silverside, has very little information about its reproductive biology and sex differentiation. In this species besides the *amhy* gene (Bej et al. 2017), the expression of other genes has not been studied during the gonadal determination/differentiation period. So, the objective of this paper is to study the expression pattern of *sox9* gene in this species and its role during the gonadal development period.

Materials and Methods

About 100 matured wild cobaltcap silversides were collected using a hand net and were subsequently reared in a 500 liter tank to obtain gametes and offsprings for experiments. The tanks were supplied with filtered natural sea water at a rate of 100 ml/min. Larvae were fed rotifers *Branchionus rotundiformis* and *Artemia* nauplii from the first day to satiation twice daily and gradually weaned into powdered marine fish food (AQUEON, Franklin, WI).

Genomic DNA was isolated from caudal fin tissue following a protocol described by Aljanabi and Martinez (1997). The genotyping of larvae to know their sex (male / female) were performed using primers Amh 613 F and Amh 35 R (Bej et al. 2017).

Cloning of *sox9* gene

For cloning, total RNA was isolated from *amhy*+ individuals testis using TRIzol (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instruction. 1 µg of total RNA per sample was reverse transcribed using SuperScript III (Thermo Fisher Scientific) with Oligo-(dT) primers (Merk Millipore, Darmstadt, German) in 20 µl reactions. The PCR was performed according to the following conditions: 3 min at 94ºC, 30 cycles of 30 sec at 94ºC, 45 sec at 60ºC and 2.5 min at 72ºC, then followed by a final elongation for 5 min at 72ºC. PCR products were electrophoresed in 1% agarose gel, purified, and sequenced in an ABI PRISM 3100 capillary sequencer (Life Technologies, Carlsbad, CA) using the BigDye Terminator method. Sequences were analyzed with GENETYX version 11.0 (GENETYX, Tokyo, Japan). All primers are listed in Table 1.

Table 1. List of Primers used in cloning and qRT-PCR (designed for this study)

Sl.No	Name of Primers	Sequences
1	Sox1 F	5'-TTCGCATGAATCTCCTCGACC-3'
2	Sox last R	5'-TCCTCAGGGCCTGGACACAG -3'
3	*sox* RT 809 F	5'-GGTGAGCTGAGCA GCGAGGT-3'
4	*sox* RT 935 R	5'-TGCAGGTTGAAGGAGCCGTA-3'
5	*β-actin*Fw17	5'- GCCTGAAACCGGTTCCCTT-3'
6	*β-actin*Rv1838	5'-TTTTCGGAACACATGTGCACT-3'

7	*β-actin* RT F	5'-GTGCTGTCTTCCCCTCCATC-3'
8	*β-actin* RT R	5'-TCTTGCTCTGGGCTTCATCA-3'

Real-Time/Quantitative PCR (qRT-PCR)

For expression studies, total RNA was isolated from *amhy*+ and *amhy*- individuals every two weeks after hatch (wah), namely 0 wah, 2 wah, 4 wah, 6 wah, 8 wah and 10 wah. The expression level of mRNA transcripts was analyzed by qRT-PCR using specific RT primers designed for *sox9* locus using conditions from a previous study (Bej et al. 2017). The β-*actin* gene was taken as an endogenous control because of its stability during sex determination/differentiation period, using the primers from (Chapman et al. 2015) (Table 1).

Sequence analysis

The multiple alignment software Clustal W was used for the alignment of nucleotide sequences and their deduced amino acid sequences. The phylogenetic tree was constructed using MEGAX with Maximum Likelihood, the initial tree inferred with the Neighbour-Joining and the BioNJ algorithms, and the Tamura-Nei model**.** The model was determined also using MEGAX. The Confidence in the tree topology was assessed with 1,000 bootstrap replicates.

Statistical analysis

In qRT-PCR expression studies, per each time point five to seven samples were taken. The differences in gene expression between groups were analyzed by ANOVA followed by a Tukey test using GraphPad prism (v.6.0; GraphPad software, San Diego, CA). Differences in gene expression were considered as statistically significant at $p < 0.05$.

Histological analysis of gonadal sex differentiation

Trunk samples were dehydrated through an ascending ethanol series (70%, 90%, 99%, and 100%), cleared in xylene, embedded in Paraplast Plus (McCormick Scientific, St. Louis, MO), sectioned serially with a thickness of 5 μm, and stained with hematoxylin and eosin. Stages of gonadal sex differentiation were determined by light microscopy using histological criteria for another atheriniform, the pejerrey *Odontesthes bonariensis* (Ito et al. 2005).

Data Accessibility

DNA sequences: GenBank accessions; *Hypoatherina tsurugae sox*9 [PP108745], *Acanthochromis polyacanthus sox9* [XM_022211835.2], *Amphiprion ocellaris sox9* [XM_023286694.3], *Anabas testudineus sox9* [XM_026358727.1], *Anarrhichthys ocellatus sox9* [XM_031867589.1], *Anoplopoma fimbria sox9* [XM_054606768.1], *Astatotilapia calliptera sox9* [XM_026164918.1], *Chelmon rostratus sox9* [XM_041957848.1], *Cololabis saira sox9* [XM_061712632.1], *Cottoperca gobio sox9* [XM_029437533.1], *Dicentrarchus labrax sox9* [XM_051389381.1], *Epinephelus fuscoguttatus sox9* [XM_049561363.1], *Etheostoma cragini sox9* [XM_034894690.1], *Etheostoma spectabile sox9* [XM_032544694.1], *Haplochromis burtoni sox9* [XM_005936187.2], *Hippoglossus hippoglossus sox9* [XM_034593479.1], *Kryptolebias marmoratus sox9* [XM_017425448.3], *Larimichthys crocea sox9* [MH996432.1], *Lates calcarifer sox9* [KR492508.1], *Mastacembelus armatus sox9* [XM_026325302.2], *Maylandia zebra sox9* [XM_004559402.2], *Melanotaenia boesemani sox9* [XM_041970542.1], *Micropterus salmoides sox9* [XM_038701099.1], *Morone saxatilis sox9* [XM_035655588.1], *Nematolebias whitei sox9* [XM_037689002.1], *Neolamprologus brichardi sox9* [XM_006791412.2], *Odontesthes bonariensis sox9* [AY319415.4], *Oncorhynchus mykiss sox9* [AB006448.1], *Oreochromis niloticus sox9* [XM_003450119.4], *Oryzias latipes sox9* [NM_001105086.1], *Oryzias melastigma sox9* [XM_024272555.2], *Paralichthys olivaceus sox9* [KY924902.1], *Perca fluviatilis sox9* [XM_039825773.1], *Plectropomus leopardus sox9* [XM_042504339.1], *Poecilia formosa sox9* [XM_007556363.2], *Pseudoliparis swirei sox9* [XM_056407289.1],

Pundamilia nyererei sox9 [XM_005744343.1], *Sander lucioperca sox 9* [XM_031312361.2], *Scatophagus argus sox9* [XM_046415919.1], *Seriola dumerili sox9* [XM_022752333.1], *Simochromis diagramma sox9* [XM_040049522.1], *Siniperca chuatsi sox9* [XM_044181768.1], *Solea senegalensis sox9* [XM_044024558.1], *Stegastes partitus sox9* [XM_008303357.1], *Thunnus albacares sox9* [XM_044332285.1], *Thunnus maccoyii sox9* [XM_042393607.1], *Toxotes jaculatrix sox9* [XM_041062045.1], *Trematomus bernacchii sox9* [XM_034143142.1], *Xiphias gladius sox9* [XM_040123617.1] and *Acipenser sinensis sox9* [KJ526295.1]

Results

Sequence analysis of *sox9*

The isolated *sox9* cDNA was 1454 bp with an open reading frame (ORF) of 1436 bp, encoding a 479 aa protein (GenBank Accession number – PP108745) (Figure 1). It shows a close similarity at nucleotide level to the HMG box of *sox9* gene of *Melanotaenia boesemani* (96.42%), *Stegastes partitus* (92.43%), *Odontesthes bonariensis* (92.42%), *Xiphias gladius* (91.01%), *Seriola dumerili* (90.98%), *Lates calcarifer* (90.52%), *Dicentrarchus labrax* (90.51%) and *Oreochromis niloticus* (89.40%). By using the Clustal W software, the 479 amino acid sequence of *H. tsurugae* was aligned with nine other fish species. The results showed that the homology was high: *Melanotaenia boesemani* (98.54%), *Odontesthes bonariensis* (96.66%), *Lates calcarifer* (96.86%), *Xiphias gladius* (96.25%), *Dicentrarchus labrax* (95.82%), *Plectropomus leopardus* (95.41%), *Perca flavescens* (95.2%) and *Oreochromis niloticus* (92.99%) (Figure 2).

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atgaatctcctcgacccatacctgaagatgacagaagaacaggagaagtgtcactctgac
 M  N  L  L  D  P  Y  L  K  M  T  E  E  Q  E  K  C  H  S  D
gctcccagcccaagcatgtctgaggactccgcaggctcgccgtgcccgtccggctccggg
 A  P  S  P  S  M  S  E  D  S  A  G  S  P  C  P  S  G  S  G
tcggacactgaaaacacccggccgtccgacaaccacctcctcggaggtcctgactacaag
 S  D  T  E  N  T  R  P  S  D  N  H  L  L  G  G  P  D  Y  K
aaggagaacgaagaagaaaagtttcccgtgtgcatcagagacgcggtgtcccaggtattg
 K  E  N  E  E  E  K  F  P  V  C  I  R  D  A  V  S  Q  V  L
aagggttatgactggacgctggtgcccatgccggtgcgcgtcaacggttcaagcaaaagc
 K  G  Y  D  W  T  L  V  P  M  P  V  R  V  N  G  S  S  K  S
aaacctcacgtcaaaagacccatgaacgcgttcatggtgtgggctcaagcagctcggagg
 K  P  H  V  K  R  P  M  N  A  F  M  V  W  A  Q  A  A  R  R
aaactggcagatcaataccctcatttgcacaacgcagaactcagcaaaaccctgggaaaa
 K  L  A  D  Q  Y  P  H  L  H  N  A  E  L  S  K  T  L  G  K
ctttggagattgctcaatgaggtagagaagcgaccgtttgtggaggaagctgagcgactg
 L  W  R  L  L  N  E  V  E  K  R  P  F  V  E  E  A  E  R  L
agagtgcaacataagaaggatcaccccgactacaaatatcagccaaggcgaagaaaatca
 R  V  Q  H  K  K  D  H  P  D  Y  K  Y  Q  P  R  R  R  K  S
gtcaagaacggtcagagcgagtccgaggacggcgagcaaactcacatctctccaaatgcg
 V  K  N  G  Q  S  E  S  E  D  G  E  Q  T  H  I  S  P  N  A
atcttcaaggctctgcagcaggccgactctccggcctccagcatgggcgaggttcactca
 I  F  K  A  L  Q  Q  A  D  S  P  A  S  S  M  G  E  V  H  S
ccaggagaacattcaggtcaatcacagggcccgccaacacccccaaccacccccaagaca
 P  G  E  H  S  G  Q  S  Q  G  P  P  T  P  P  T  T  P  K  T
gatctcccttccagcaaagctgacctaaaacgtgaggggcgccccatgcaggagggctcc
 D  L  P  S  S  K  A  D  L  K  R  E  G  R  P  M  Q  E  G  S
agccgccagctcaacatagactttggagctgtggacatcggtgagctgagcagcgaggtc
 S  R  Q  L  N  I  D  F  G  A  V  D  I  G  E  L  S  S  E  V
atctccaacatgggaagcttcgatgttgatgagtttgatcagtacctgccccctcacagc
 I  S  N  M  G  S  F  D  V  D  E  F  D  Q  Y  L  P  P  H  S
catgccggggtgactggcgcagcccccgctggctacactggcagctacggtatcaacagc
 H  A  G  V  T  G  A  A  P  A  G  Y  T  G  S  Y  G  I  N  S
tcctcggttggccaggcagccaacgttggagcccacgcctggatgtccaaacagcagcag
 S  S  V  G  Q  A  A  N  V  G  A  H  A  W  M  S  K  Q  Q  Q
cagcagcattctctgaccaccctgggtggagcaggagaacaaggccagcagggtcagcag
 Q  Q  H  S  L  T  T  L  G  G  A  G  E  Q  G  Q  Q  G  Q  Q
agagccacccagattaagacagagcagctgagccccagtcactacagcgagcagcagggc
 R  A  T  Q  I  K  T  E  Q  L  S  P  S  H  Y  S  E  Q  Q  G
tccccacagcatgtcacctacggctccttcaacctgcagcactacagcacctcctcttac
 S  P  Q  H  V  T  Y  G  S  F  N  L  Q  H  Y  S  T  S  S  Y
ccctccatcacaagagcacagtatgactattcagaccaccaaagtggtgccaactcatac
 P  S  I  T  R  A  Q  Y  D  Y  S  D  H  Q  S  G  A  N  S  Y
tacagccatgcagctggtcagggctccagcctgtactccaccttcagctatatgagcccc
 Y  S  H  A  A  G  Q  G  S  S  L  Y  S  T  F  S  Y  M  S  P
agccagaggccgatgtacaccccgattgctgacagcaccggggtgccctctgtgccgcag
 S  Q  R  P  M  Y  T  P  I  A  D  S  T  G  V  P  S  V  P  Q
```

Figure 1. *sox9* gene of *Hypoatherina tsurugae* with complete CDS

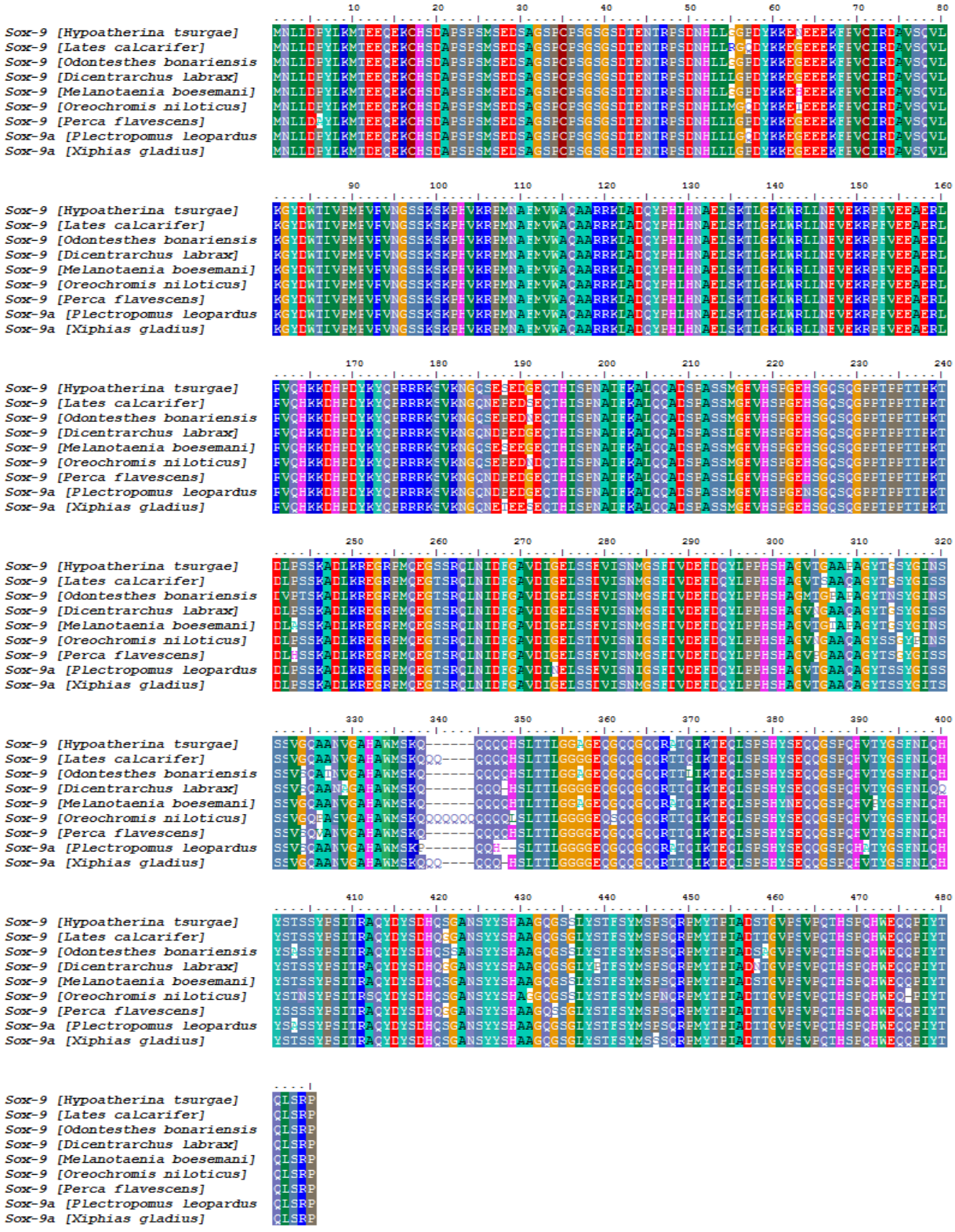

Figure 2. Deduced amino acids sequence of *H. tsurugae sox9* gene aligned with other ortholog sequences

A phylogenetic tree was constructed by comparing the mRNA sequence of 50 different fish species across various taxa available in the NCBI database and taking *Acipenser sinensis* as outgroup (Figure 3). The tree shows a high homology of *H. tsurugae sox9* with *Maelanotaenia boesemani sox9*, the two being sister taxa as they both belong to the order Atheriniformes.

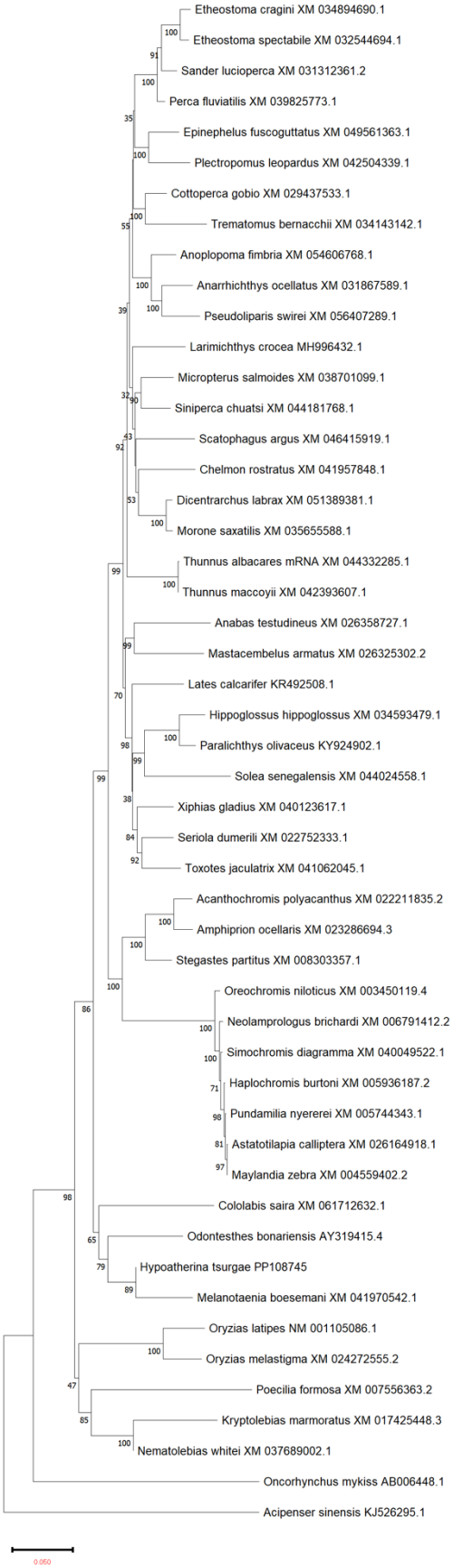

Figure 3. Phylogenetic tree retrieved with the *sox9* mRNA sequence of 50 different fish species along with *H. tsurugae*

Gene expression analysis

The result of qRT-PCR illustrated that in *amhy*- (female) individuals the expression was quite high at 2 wah then sharply decreases whereas in *amhy*+ (male) individuals it begins from baseline at 0 wah and gradually increases in an exponential fashion until complete differentiation of gonads occur (Figure 4).

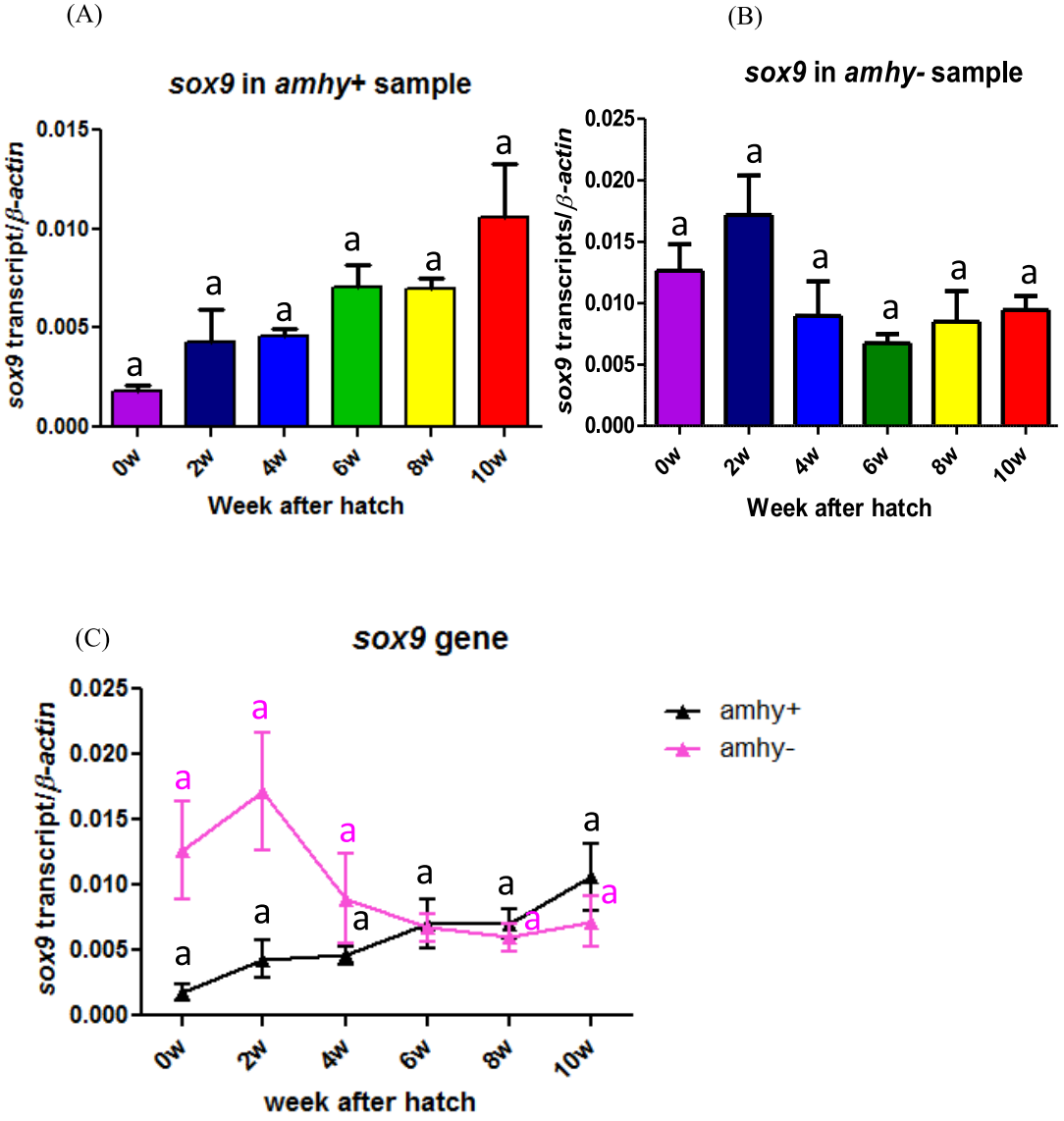

Figure 4. Quantitative mRNA expression of *sox9* gene (a) in *amhy*+ individual (male) (b) *amhy*- individual (female) (c) Both in *amhy*+ and *amhy*- plotted in same graph. Values represent the mean ± SEM of 5-7 fish per time point

The histological sections of gonads in different larval stages showed that differentiation of gonads male/female is decided at 6 wah. In this stage the primary oocytes are clearly recognized (Figure 5) which is also correlated with expression of *sox9* gene.

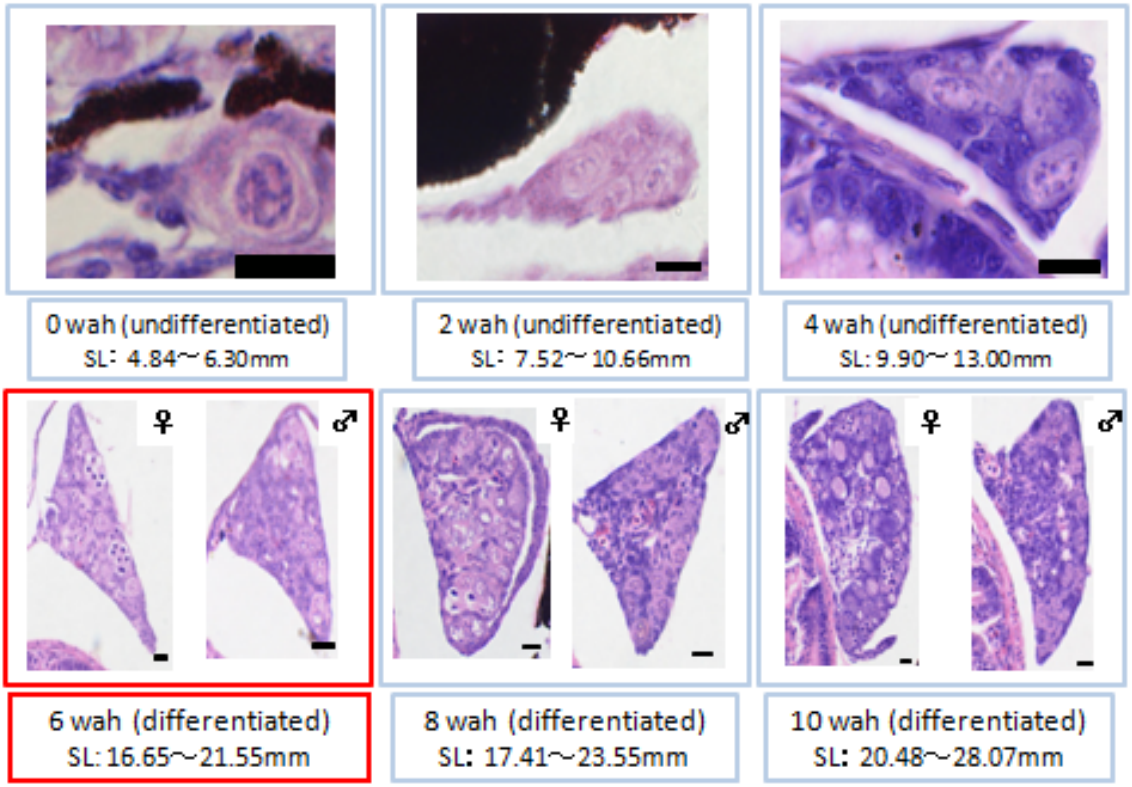

Figure 5. Histological differentiation of gonad *amhy*+ (male) and *amhy*- (female) analyzed every two weeks after hatch

Discussions

In the present study, the *sox*9 gene of *H. tsurugae* has been successfully cloned and sequenced. The *sox*9 mRNA is 1454 bp encoding a 479 aa protein. The *sox*9 gene has very close similarity with *sox*9 gene of *Melanotaenia boesemani*, *Stegastes partitus*, *Odontesthes bonariensis*, *Xiphias gladius*, *Seriola dumerili*, *Lates calcarifer*, *Dicentrarchus labrax*, and *Oreochromis niloticus*. The number of *sox*9 subtypes is not the same in different species (Luo et al. 2010). There is only one type of *sox*9 gene found in higher vertebrates but usually two subtypes are found in teleosts, likely due to genome duplication (Meyer et al. 2005, Hu et al. 2021). It is presumed that there may be two types of *sox*9 genes in *H. tsurugae* but only one type was cloned in this study. The phylogenetic analysis revealed that our sequence forms a clade with another Atheriniformes, *Maelanotaenia boesemani*.

In this study, focus is given to the expression pattern of the *sox*9 gene in gonads of *H. tsurugae*. From the qRT-PCR result, the expression of the *sox*9 gene is correlated with the expression of the *amhy* gene that significantly reached a peak at 6 wah, then decreases (Bej et al. 2017). The expression of *amhy* was detected from before the appearance of first signs of histological differentiation in presumptive Sertoli cells surrounding germ cells in the undifferentiated gonads. Similarly, the expression of *sox9* in *amhy*+ (male) begins from baseline at 0 wah and is expressed in an increasing fashion needed for the male developmental pathway for testis differentiation. In *amhy*- (female) individuals though highly expressed in the initial stage (0 wah) and the expression reaches a peak at 2 wah it declines afterwards which indicates the low expression needed for differentiation of female gonads, the ovary. It has been reported in the expression profile of the Zebra fish that the *sox9* gene reaches a peak at 18 days post hatch which is significantly different from male sex individuals and after 18 days after hatch it decline abruptly (Jorgensen et al. 2008). The expression of *sox9a* and *sox9b* was also studied in medaka fish and it is expressed in testis and ovary during the developmental period of gonads (Klüver

et al. 2005). However, the study of the expression of *sox9* gene in the medaka fish gave inconsistent results (Yokoi et al. 2002, Nakamoto et al. 2005). In the rice field eel, *Monopterus albus*, the *sox9* gene is also expressed in both testis and ovary during the developmental period (Zhou et al. 2003). The *sox9* gene is over expressed in testicles as compared to the ovary in *Acipeneser sturio* and *Acipenser fulvescens* (Berbejillo et al. 2012, Burcea et al. 2018). It has been reported that *sox9* is involved in many signaling pathways during sex determination/differentiation period and in gonad development of embryo and adult.

Conclusions

From the above study, it may be concluded that expression of *sox9* is essential for development and differentiation of male sex organ testis and it is assumed that after the trigger of sex determining gene *amhy* switches on, the next cascade is performed by *sox9* gene and other sex related genes to differentiate the gonad during sex differentiation period for *H. tsurugae*. However, more functional experiments are necessary to understand the mechanisms of downstream pathways of gene regulation during gonadal differentiation period of this species.

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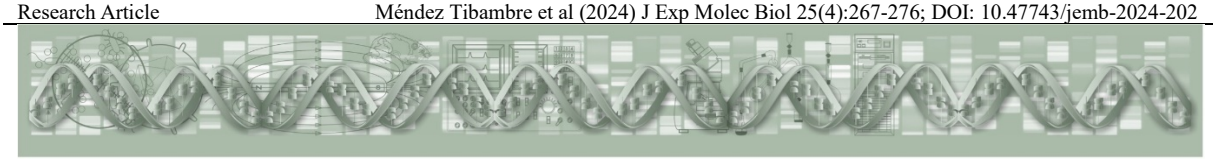

ENHANCING POTATO VIRUS X DIAGNOSIS: CHELEX™ 100 RESIN RNA EXTRACTION METHOD EVALUATION

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Abstract

Potato Virus X (PVX) is a prevalent pathogen affecting potato crops, leading to substantial yield losses. This study investigates the efficacy of Chelex™ 100 resin RNA extraction method for PVX detection, through RT-qPCR analysis. We evaluate RNA yield, sensitivity, and detection limits of this method compared with Phenol-chloroform RNA extraction protocol. Our results demonstrate that Chelex™ 100 resin extraction yields superior RNA concentrations and exhibits greater sensitivity compared to ELISA immunoassay diagnostic test. RT-qPCR successfully detects PVX RNA in dilutions up to 1:100 using Chelex™ 100 resin extraction. These findings highlight the potential of Chelex™ 100 resin extraction coupled with RT-qPCR as a rapid and reliable method for PVX detection.
Keywords: PVX, Chelex, molecular diagnostics, plant virus

Introduction

Potato (*Solanum tuberosum* L.) is one of Colombia's most important crops, with a cultivated area spanning approximately 113,000 hectares and yielding an average of 22.78 tons per hectare (FEDEPAPA 2023). However, the presence of numerous viral pathogens, particularly Potato virus Y (PVY), Potato leafroll virus (PLRV), Potato yellow vein virus (PYVV), and Potato virus X (PVX), is associated with a negative impact on crop production (Guzmán et al. 2010, Campos and Ortiz 2020, Kumar et al. 2022). Among them, PVX is one of the most widespread and economically damaging. It is transmitted through various means, including pollen, true seeds, contaminated farming equipment, and direct contact between healthy and infected foliage or roots (Kreuze et al. 2020, Verchot 2022).

Incidence studies in Colombia have shown that yields can decrease by 3% to 50%, depending on the viral load (Sierra et al. 2021, García et al. 2023). This reduction is attributed to the successive planting of contaminated seeds, climatic conditions, and the response of different potato varieties to viral infection. The impact of PVX on crops generates the need of early and accurate diagnosis for effective disease management and mitigation of economic losses (Devi et al. 2024). Traditional methods for the detection of PVX, such as enzyme-linked immunosorbent assay (ELISA), have been the standard for diagnosis for decades (Boston and Haliloğlu 2011, Boonham et al. 2014, Qamar et al. 2016, Raigond et al. 2022). While ELISA offers established protocols and reliable results, it is slow and laborious. Furthermore, for this reason, there is a need to develop alternative diagnostic methods that can offer greater sensitivity, specificity, speed, and practicality in the context of PVX detection (Khurana and Marwal 2016, Hema and Konakalla 2021). Given these requirements, the diagnosis of PVX has increasingly involved qPCR detection of nucleic acids.

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Developing more effective diagnostic tools for PVX relies on a thorough understanding of its molecular characteristics. PVX belongs to the genus *Potexvirus* within the family *Alphaflexiviridae*, and is characterized by a genome composed of a single positive-sense RNA strand approximately 6.4 kilobases in length (Grinzato et al. 2020). The PVX genome encodes at least five open reading frames (ORFs), including the viral replicase (RdRp), movement proteins located in the Triple gene block (Tgb), and the viral capsid protein (CP). A methylguanosine cap is situated at the 5' end of the genome, while the 3' end is polyadenylated (Verchot 2022). To enhance the sensitivity and specificity of diagnostic assays, previous research has focused on targeting for quantitative reverse transcription polymerase chain reaction (RT-qPCR) various regions of the PVX genome such as the capsid region, the RdRp gene, and other conserved sequences (Abbas and Hameed 2012, Ruíz et al. 2016, Kumar et al. 2021).

However, for RT-qPCR detection to be effective, it is imperative to develop a simplified nucleic acid extraction method designed for PVX detection. Traditional extraction techniques, such as commercial kits or the Phenol-chloroform extraction method, pose inherent challenges, including complexity and reliance on hazardous chemicals, making their widespread adoption difficult, especially in resource-limited settings (Bhat and Rao 2020). Therefore, this study focuses on exploring the efficacy of Chelex™ 100 resin extraction, a simpler and safer alternative (Shiaw et al. 2010, Shetty 2020), to isolate high-quality nucleic acids from PVX-infected potato samples. Integrating Chelex™ 100 resin extraction with PCR-based diagnostic assays offers a rapid, reliable, and easy-to-use approach for PVX detection.

Materials and Methods

Plant material and virus isolates

For the diagnosis of PVX, symptomatic *Solanum tuberosum* plants were collected from a pool of *in vitro* specimens originated from crops located in Ventaquemada, Boyacá, Colombia. The plants were screened for PVX using double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) with polyclonal antibodies from Agdia (Indiana, USA), following the manufacturer's protocol. Ten potato plants confirmed as PVX positive by ELISA were used for RNA extraction and molecular analysis by RT-qPCR.

RNA extraction

In this study, two distinct RNA extraction protocols were employed to assess their efficacy in isolating viral RNA from potato *in vitro* plants. The first method utilized the Phenol-chloroform technique (method I), a traditional approach widely used in RNA extraction, serving as a benchmark against which the effectiveness of the second method was compared. The method II employed Chelex™ 100 resin. This choice was made due to the known advantages of Chelex™ 100, including its rapid protocol and ease of use (Seufi and Galal 2020, Gautam 2022). The starting material for RNA extraction in both methods was represented by 50 mg of infected potato plant tissue, ground into a fine powder inside a 2 mL tube by using liquid nitrogen and a thin metal rod.

Phenol-chloroform method

Total RNA was extracted from samples using method I, modified from the protocol described by Kingston, 2010. The ground plant material was resuspended in 1 ml of lysis buffer (pH 8.2), containing 1% Sodium Dodecyl Sulfate (SDS), 0.18 M Tris, 4.5 mM EDTA, and 0.09 M Lithium Chloride. Then, 300 µL of phenol equilibrated with TLE solution (0.2 M Tris, 0.1 M Lithium Chloride, 5 mM EDTA) were added. The mixture was vortexed for 2 minutes, followed by the addition of 600 µL of chloroform, and incubated at 50°C for 20 minutes. Post-incubation, the tubes were centrifuged at 13,000 rpm for 20 minutes at 4°C. The clarified aqueous phase was extracted twice with 600 µL of phenol equilibrated with TLE solution and 600 µL of

chloroform. RNA precipitation was achieved by adding a 1:10 ratio of 3 M Sodium Acetate to the sample volume, an equal volume of isopropanol, followed by 12 to 16 hours incubation at -20°C. Subsequently, the tubes were centrifuged at 13,000 rpm for 20 minutes at 4°C. The resulting pellet was washed twice with 70% ethanol, air-dried, and resuspended in 50 µL of RNase-free water.

Chelex™ 100 resin method

The method II was modified from Dreskin et al. (2022), where 50 mg of potato plant ground tissue was homogenized in a 0.5 mL microtube containing 120 µl solution of 6% Chelex™ 100 resin. The mixture was vortexed for 1 min, incubated at 95 °C for 10 min and then refrigerated for 3 min at -20 °C. After, the samples were centrifuged at 13,000 rpm for 1 minute at 4 °C. Approximately 20 µl of the supernatant containing the RNA was transferred to a clean 0.5 mL microtube (Figure 1).

RNA Quantification and Storage

The RNA samples were quantified using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA concentration measurements were repeated three times for each sample. Subsequently, the samples were stored at -80°C for further RT-qPCR analysis.

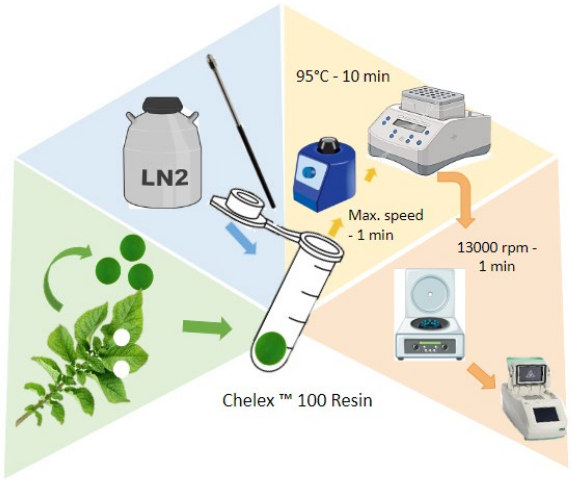

Figure 1. Diagram of rapid protocol applied for RNA extraction using Chelex™ 100 Resin on PVX infected potato plants

RT-qPCR assay

The RT-qPCR was performed in single-tube reactions with a total volume of 10 µL. Each contained 2 µL of the RNA template, 5 µL of (2x) qScript XLT One-Step RT-qPCR ToughMix (Quantabio, Germany), 0.25 µL of each primer (10 µM), 1.5 µL of BSA (20 mg/mL), 0.5 µL of DMSO and 0.5 µL ddH2O for a final volume of 10 µL. The extracted RNA was normalized to 100 ng/µL, and 1:10, 1:20, 1:50, and 1:100 dilution series were prepared to evaluate the sensitivity of the test. The primers PVX-F (5'-TGGAAGGACATGAARGTGC-3') and PVX-R (5'-CGAATTTGTGCTCAGGCTTG-3') were used to amplify a 282 bp fragment from the capsid protein (ORF5) gene (Yan, 2021). Real-time PCR was conducted on a CFX96 Real-Time System (Bio-Rad, USA), with the following thermal cycling conditions: 55 °C for 10 min, 95 °C for 3 min, then 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The PCR products were analyzed by gel electrophoresis on a 3% agarose gel. Products were visualized using a Bio-Rad ChemiDoc XRS Gel Photo Documentation System (Bio-Rad, USA) and compared to the HyperLadder 50 bp DNA ladder (Bioline, USA).

Results and discussions

RNA Quantity Assessment

This study focused on streamlining the RNA extraction process for PVX detection, thereby enabling a more efficient process achieving optimal sensitivity using quantitative reverse transcription polymerase chain reaction. We evaluated two distinct extraction methods. The method I, is a widely described standard protocol known for its efficiency albeit labor-intensive nature and safety concerns (Toni et al. 2018). The method II uses the Chelex™ 100 resin recognized for its ability to preserve nucleic acid integrity while offering expedited processing (Walsh et al. 1991, Sajib et al. 2017, Gautam 2022).

Samples subjected to extraction method I exhibited RNA concentrations between 100 to 140 ng/µL, whereas those processed using method II displayed concentrations exceeding 500 ng/µL (Table 1). This difference highlights the superior RNA yield achieved with method II, exceeding the minimum concentration threshold for PCR development, which is approximately 10 ng/µL (Lorenz 2012).

Chelex™ resin is a styrene-divinylbenzene copolymer, which has been reported as a chelating agent of divalent cations like magnesium and calcium (Gautam 2022). The chelation mechanisms inhibit nuclease activity, ensuring the preservation of nucleic acids during the extraction process in complex samples and facilitating the release of nucleic acids from cells or tissues (Singh et al. 2018). The polar resin binds polar cellular components while DNA and RNA remain in the water solution above Chelex™ (Panda et al. 2019). Moreover, the method utilizes thermal denaturation to extract nucleic acids from plant tissues. Raising the temperature of the sample promotes the binding of the resin to the ions and facilitates the release of the nucleic acids from the cells (Guan et al. 2021, Lim et al. 2022).

Table 1. Total RNA concentration obtained from 50 mg of plant tissue, using extraction method I and method II. Each value represents the average of the replicates of each sample and the standard deviation

Sample No.	Average Total RNA Concentration ± S.D. (ng/µL)	
	Phenol-chloroform (method I)	Chelex™ 100 resin (method II)
1	115 ± 3.7	532 ± 7.8
2	113 ± 4.5	550 ± 5.7
3	118 ± 2.8	524 ± 6.9
4	125 ± 4.9	567 ± 8.2
5	132 ± 4.1	511 ± 6.3
6	138 ± 3.8	546 ± 4.6
7	114 ± 5.3	520 ± 9.1
8	107 ± 5.1	563 ± 7.6
9	121 ± 6.0	507 ± 5.4
10	137 ± 4.7	578 ± 9.5

Our results demonstrate that method II is simpler and faster when it is compared with method I. Additionally, it does not require the use of organic solvents, making it safer and easier to handle. However, Chelex™-resin is generally less effective at removing contaminants and may yield lower purity of RNA than method I.

RT-qPCR for RNA Detection

The RT-qPCR amplification targeted the capsid protein (ORF5) gene of PVX. The size of the fragment obtained was 282 base pairs (Figure 2), consistent with the expected result. The bands obtained from the amplification of the RNA extracted with method II were thicker and more pronounced compared to the bands obtained from RNA extracted with method I. This observation could suggest a more effective RNA extraction using the method II, resulting in visibly stronger bands indicating successful amplification of the ORF5 gene. This band intensity can be attributed to the efficiency of the Chelex™ 100 resin as a chelating agent, which allows preserving the integrity of the nucleic acid during the extraction process and its subsequent analysis.

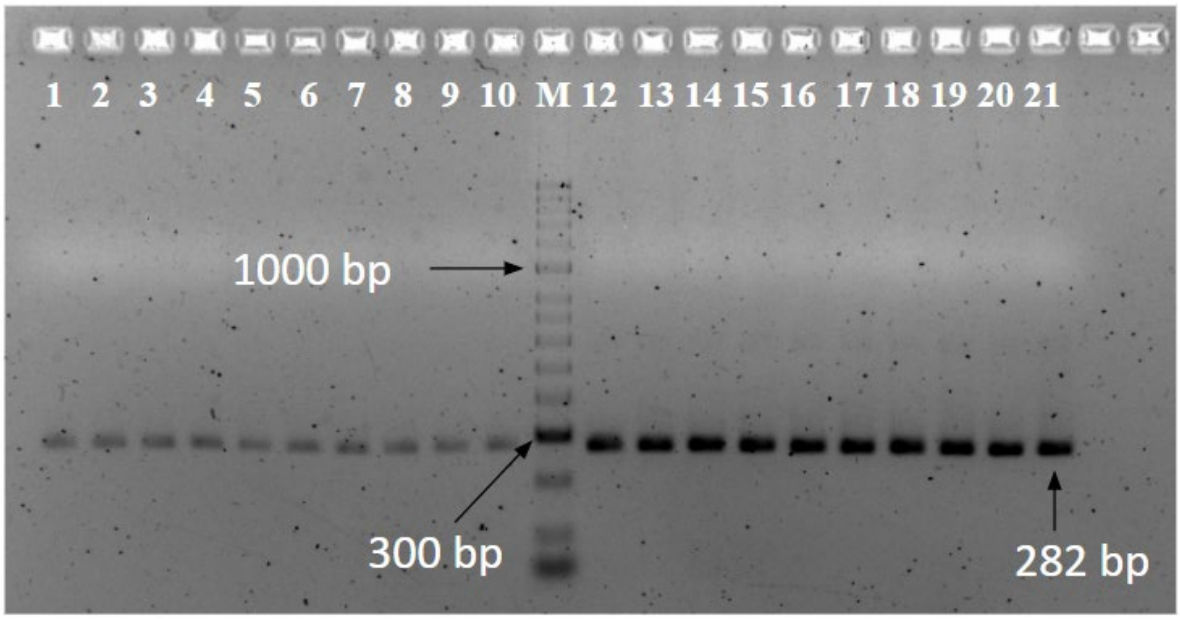

Figure 2. Agarose gel electrophoresis of amplimers of the capsid protein (ORF5) gene from PVX-infected potato plants, obtained using RNA extracted with the method I, lanes 1-10 and the method II, lanes 12-21. Lane M shows the molecular marker: Hyperladder 50 bp

Detection sensitivity comparison for PVX

For sensitivity evaluation, the RNA extracted with method II from PVX-infected tissue was diluted at ratios of 1:10, 1:20, 1:50, and 1:100, and then analyzed using RT-qPCR. Results demonstrated that RT-qPCR can detect the 1:10, 1:20, 1:50 dilutions between 25 to 26 Ct values with a standard deviation of ± 2.1, indicating robust sensitivity within this dilution range. However, the fluorescence level decreased at the 1:100 dilution (Figure 3A), but PVX RNA was still detectable, indicating a reduction in the sensitivity. The analysis of the denaturation curves allowed identification of a single melting temperature (Tm) value among the analyzed samples: Tm =79.5±1°C, indicating the occurrence of a single variant of this virus in the potato plants analyzed (Figure 3B).

The RT-qPCR analysis was also conducted with dilutions derived from nucleic acids extracted from method I to compare the sensitivity in detecting the PVX gene in the RNA obtained from both evaluated extraction methods (data not shown). The amplification results of the dilutions were confirmed by gel electrophoresis, where PCR products of 282 base pairs were obtained for each dilution of each extraction method, as depicted in Figure 4.

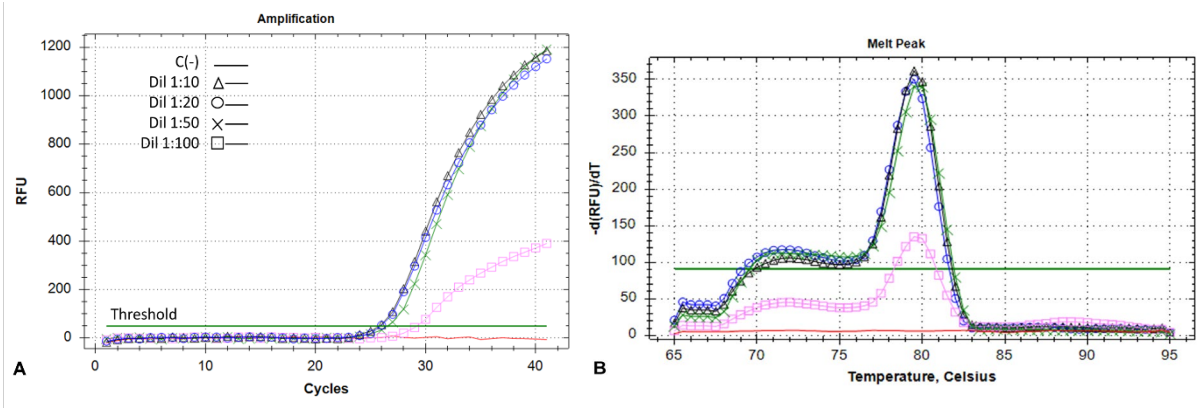

Figure 3. (A) Amplification curves by RT-qPCR for dilutions 1:10, 1:20, 1:50 and 1:100 using the SYBR Green I system and the primers for the detection of PVX in infected potato tissues. (B) Profiles of the denaturation curves of PVX-specific amplicons obtained by RT-qPCR in the different dilutions

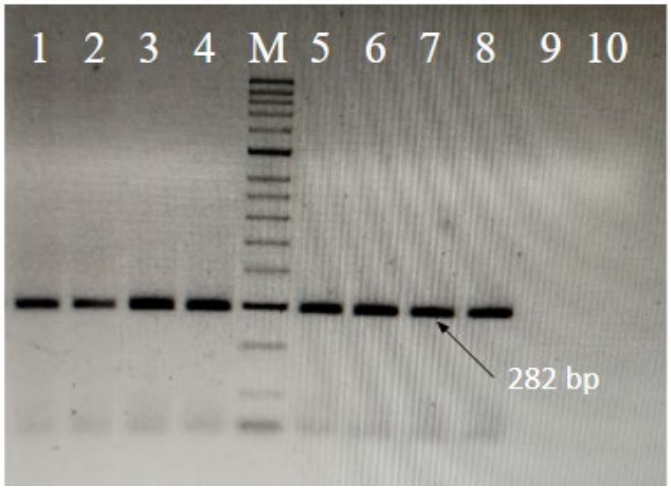

Figure 4. Sensitivity analysis of PVX detection by RT-qPCR from RNA isolated using Method I (lanes 1-4) and Method II (lanes 5-8). Lanes 1 and 5 show the 1:10 dilution, lanes 2 and 6 the 1:20 dilution, lanes 3 and 7 the 1:50 dilution, and lanes 4 and 8 the 1:100 dilution for each method. Lane M represents the Hyperladder 50 bp molecular marker, and lanes 9-10 are negative controls

From a previous study, data on sensitivity limits were obtained from the conventional immunoassay ELISA used for the diagnosis of PVX (Yan, 2021), with the objective of comparing the detection limits with the RT-qPCR technique in different dilution ranges (Table 2). The sensitivity results showed that RT-qPCR, from both RNA extraction methods, demonstrated higher sensitivity for PVX detection compared to reported ELISA assays, detecting up to a 1:100 dilution, whereas ELISA was limited to detect up to a 1:20 dilution.

Table 2. Comparison of the detection sensitivity of ELISA (Yan 2021) and RT-qPCR for PVX with both extraction methods applied. "+" indicates the positive detection of the virus and "-" indicates a negative result for the detection

Dilutions	**ELISA**	**RT-qPCR**	
		Phenol-chloroform (method I)	**Chelex™ 100 resin (method II)**

1:10	+	+	+
1:20	+	+	+
1:50	-	+	+
1:100	-	+	+

Furthermore, the RT-qPCR technique offers advantages in terms of sensitivity and speediness of results. RT-qPCR exponentially amplifies specific nucleic acid sequences within a short timeframe, facilitating the detection of even small quantities of viral RNA; this high sensitivity is particularly crucial in the context of crop infectious diseases, where early detection is desirable for containment and mitigation efforts (Jeong et al. 2014).
Additionally, ELISA assays could incur higher costs and are less efficient compared to PCR-based methods (Boonham et al. 2014). ELISA procedures typically require approximately 8 hours to yield results, while Chelex™ RNA extraction coupled with RT-qPCR provides more precise results in just 3 hours, significantly reducing time and labor costs. In addition, ELISA immunoassay may include the risk of false negative results, since it relies on antibody-antigen interactions, which may not be as sensitive in detecting low viral concentrations. This is particularly evident when testing is conducted during the early stages of infection when viral antigen concentrations are low (Cassedy et al. 2021), compromising the accuracy of the diagnosis.

Conclusions

The study establishes the efficacy of the Chelex™ 100 resin RNA extraction method in optimizing the extraction process for PVX detection via RT-qPCR. While the Phenol-chloroform method remains reliable, the Chelex™ 100 resin method offers expedited processing, higher RNA yield, and comparable sensitivity. These findings not only contribute to the optimization of PVX diagnostic protocols but also underscore the importance of RNA extraction methodologies in molecular biology research.

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