



## **Herbology, Antibacterial and Antihemorrhagic Effect of *Allium sativum* Linn (*Lilliaceae*) Extracts against Bacterial Meningitis Pathogens**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author KBT designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AAA and ODO managed the analyses of the study. Authors YJ and USU managed the literature searches. All authors read and approved the final manuscript.*

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### **ABSTRACT**

Bacterial Meningitis pathogen (BMP) causes cellular hemorrhagic sepsis, metabolic, endocrine and neurologic disorders in human systems. This burden of the disease is highest in the developing countries and especially in the immunity compromised rural populations in Sub-Saharan Africa. The *A. sativum* bulb extracts (JEAS, EEAS and AEAS) contain valuable pharmacologically active principles for antihemorrhagic and antibacterial properties against bacterial meningitis pathogens. It extracts were evaluated in Wistar rats to provide scientific basis for ethnomedicinal uses. The

phytochemical screening was conducted using standard method prescribed. Anti-haemorrhagic activity was evaluated using sterilized blade lancet to cut 2mm long and 2mm deep in the rat groin model. The coagulative effects of time were significantly at  $P < 0.05$  reduced by the bulb extracts applied as compared to that of the control. The phytochemical screening revealed the presence of alkaloids, cardiac glycosides, carbohydrates, flavonoids, saponins, steroids and tannins. The result of this study revealed that *A. sativum* bulb extracts possessed good secondary metabolites with bioactive principles, biological and pharmacological functions. This provide scientific basis for the plant in the management of haemorrhagic sepsis and infectious disease.

**Keywords:** *Phytochemicals; anti-haemorrhage; A. sativum bulb; bacterial meningitis; folk medicine.*

## 1. INTRODUCTION

Haemostasis is the process of blood clot formation to stop bleeding at the site of vascular injury. The haemostatic system maintains the integrity of the vascular complex network of cellular, ligand receptors and interacts enzymatically. The agent used to keep the blood within a damaged blood vessel is called antihaemorrhagic to prevent bleeding and save from shock i.e. it antagonizes haemorrhage (bleeding). It is essential that blood remain fluid within the circulation but clots at sites of vascular injury to prevent blood loss and general shock [1].

Bacterial meningitis (BM) is a devastating air-borne infectious disease of humans, and it is of huge health burden as it can easily kill or maim when meningeal pathogens invaded blood stream. The BM causes cellular hemorrhagic sepsis, metabolic, endocrine and neurologic disorders. This burden of the disease is highest in the developing countries and especially in the immunity compromised rural populations [2].

It is mostly endemic during the hot dry season typical of the tropical Sub-Saharan African countries, the hot climate of which favours the breeding of the meningococcal organisms that infect the meninges [1,2]. Inflammation of the meninges causes leakage of the infected cerebrospinal fluid (CSF) and alteration of the brain system (cognitive deficit) giving rises to meningism (stiff neck, severe headache, fever, rashes, shortness of breath or noisy breathing, hydrocephalus (swelling or oedema of the brain microglia and astrocytes due to inappropriate antidiuretic hormone secretion etc) [1].

Meningococcal-induced haemorrhage is a complication of malnutrition and a serious health burden of economic and societal especially in the impoverished rural communities of Sub-Saharan Africa and has actually been noted to be of high impact in the Northern States of Nigeria;

probably with regards to the exceptionally hot dry weather and climate change of this part of the country [3].

Traditional medicine constitutes an important source of drugs for ethnopharmacological investigation. Various medicinal food-plants and animal products-supplements are available for use in certain immune-deficiency disease conditions related to malnutrition such as hemorrhagic sepsis. Furthermore, phytotherapy still remains a habitual part of health care system wholly or in part especially in rural communities [4].

The aim of this research is to evaluate the antihaemorrhagic and antibacterial activities of the fresh juice (JEAS), ethanolic (EEAS) and aqueous (AEAS) extracts of *A. sativum* on bacterial meningitis pathogens. *Allium sativum* (AS) is one of such medicinal food-plant that has a long history of use as food condiment and it also has a high value of use traditionally for many ailments including typhoid fever, ulcer, cholera, dysentery etc and thus, there is a need to investigate its specific *in vitro* antibacterial activity on meningeal bacterial organisms as well as its *in vitro* antihaemorrhagic activity in condition of meningococcal-induced cellular injuries having been known to boost the immune system [5].

Thousands of people worldwide also die of cerebrospinal meningitis fever as a result of disseminated intravascular coagulation (DIC) of the blood cells often caused by certain meningococcal organisms and the associated inability of the blood cells (haemoglobin) to transport oxygen round the body (methaemoglobinaemia) [6].

*A. sativum* (AS) has a long history of use as food condiment and also a high value of use traditionally for many ailments including antimicrobial effects. However, its specific activity on meningeal bacteria as well as

antihaemorrhagic activity in condition of meningococcal-induced cellular injuries has actually not been reported in literature. Also, a scientific validation of this utilization has not been previously made to the best of my knowledge [6].

The prevalence of meningeal bacterial organisms, the potential of epidemic virulence of meningeal bacteria organism in Northern Nigeria varies with the geographical location, time of season, climate and pathogenic serotype strains of the causative organism [7]. Six subspecies serotypes of *meningococci* (A, B, C, W-135, X and Y) have been clinically recognized in Sub-Saharan African countries of which serotypes A and C *meningococci* were found to be more prevalent and predominant among other serotypes which often occur as co-morbid infections [7]. The epidemics of Sudan in 2006 was of serotypes W-135 and X [8]. The most prevalent meningeal bacteria organism in Nigeria is of the serotype A and C strains of *Neisseria meningitides* seen abundantly during the harmattan period when humidity is very low, usually around November-June and which is the breeding time of the organisms [7]. *Neisseria meningitides* is the most implicated bacterial organism that causes meningitis and constitutes about 80% of the epidemic cases in Africa [9]. Other bacterial organisms (*Haemophilus influenzae* type b, *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*) also cause meningitis to a lesser extent (about 20% of the epidemic cases) [1,9]. Thus, these five bacterial organisms are those mostly known to be involved in meningitis, systemic septic-inflammations and hemorrhage (brain septic-haemorrhage) in Sub-Saharan Africa including Nigeria [9,10]. Bacterial meningitis caused by *H. influenzae* and *meningococci* have a better prognosis than of *S. pneumoniae* and *E.coli* that mainly attack neonates and growing children. The group B *streptococci* (subtype III) occur mainly during the first week of life (newborns), while *Escherichia coli* and *Klebsiella pneumoniae* are of both newborns and growing children less than five years of age. *Haemophilus influenzae* type B, *Neisseria meningitides* (meningococci) and *Streptococcus pneumoniae* affect mostly the adults, but people over 50 yrs have increased risk of *Escherichia coli* [11].

### 1.1 Folkloric and Ethnomedical Uses

*A. sativum* has an ancient use as antiseptic, antibacterial, carminative, bleeding of the nose, diaphoretic, anthelmintic, antimicrobial, antifatulence, and analgesic. It is also used

locally for confluent smallpox, typhoid fever, dropsy or congestive heart failure, pulmonary tuberculosis or phthisis and for eye and ear infections [12]. It has also been reported to have antitumour and anticholesterolaemia properties whereby it is used to decrease low density lipoprotein and / or increase high density lipoprotein as to lower blood pressure. In China and Arabia, it has a history of use as energizer or palliative and it is believed to have a natural nutritional healing power for cough, hay fever, pile or dysentery and also to promote digestion (dyspepsia) [11,12].

### 1.2 Treatment of Haemorrhage and Bacterial Meningitis

Haemostasis is the process of blood clot formation to stop bleeding at the site of vascular injury. The haemostatic system maintains the integrity of the vascular complex network of cellular, ligand receptors and interacts enzymatically [1]. The agent used to keep the blood within a damaged blood vessel is called antihaemorrhagic to prevent bleeding and save from shock i.e. it antagonizes haemorrhage (bleeding). It is essential that blood remain fluid within the circulation but clots at sites of vascular injury to prevent blood loss and general shock [1,2].

#### 1.2.1 Steps in haemostasis

- It is the first stage of wound healing. This involves blood changing from a liquid to a gel. Intact blood vessels are central to moderating blood's tendency to clot. The endothelial cells of intact vessels prevent blood clotting with a heparin-like molecule thrombomodulin, and prevent platelet aggregation with nitric oxide and prostacyclin.
- When endothelial injury occurs, the endothelial cells stop secretion of coagulation and aggregation inhibitors and instead secrete von Willebrand and factor which initiate the maintenance of haemostasis after injury.
- Haemostasis has three major steps:
  1. Vasoconstriction (vascular spasm).
  2. Temporary blockage of a break by a platelet plug, and
  3. Blood coagulation or formation of a fibrin clot.

These processes seal the hole until tissues are repaired [2].

### 1.2.2 Antihæmorrhagic agents

Amicar, Instat, Ethicon 360, Evicel fibrin sealant, Vitamin K and Povidone iodine

#### 1.2.3 Amicar (aminocaproic acid)

It is a derivative of amino acid lysine can either be injected, oral solution and tablets. It also acts as antifibrinolytic agent by inhibitory plasminogen activators which have fibrinolytic properties. The inhibitory effects of amino caproic acid appear to be exerted at the site of vascular injury to set vascular spasm.

*Mechanism of Action:* It binds reversibly to the kringle domain of plasminogen and block binding of plasminogen to form fibrin and its activation to plasmin thereby promotes hæmostasis (stops bleeding) or it inhibits activation of plasminogen activators which reduces blood loss [2].

#### 1.2.4 Instat (avitene, microfibrillar bovine derived)

It is a collagen absorbable hæmostat that promotes hæmostasis and wound healing, prevents bleeding and fastens clotting mechanism.

*Mechanism of Action:* The contact of instat with a bleeding surface collagen hæmostat attracts platelets. Then adhere to its fibrils and undergo the release of its content. This triggers aggregation of the platelets into thrombin to form fibrous mass; initiating the formation of a biochemical and physiologic plate plug [2].

#### 1.2.5 Ethicon 360 (hæmostatic matrix surgical)

It is an absorbable hæmostat. It is a plant-base product used to control bleeding either surgical operation or accidental trauma with wide fresh wound. It interacts with the phospholipid around the injured area. It has astringent property and promotes hæmostasis to form clot and seal the vascular tissue injured gap.

*Mechanism of Action:* It bind the injured vascular tissue, initiates and accelerates hæmostasis, seal the injured gap with clot, stops bleeding and promote wound healing [2].

#### 1.2.6 Evicel fibrin sealant (human glue)

It consist fibrinogen and thrombin contained in two syringes, the tips of which for a common

port. It allows delivery of the two components to a bleeding point where fibrinogen converts to fibrin at a rate determined by the concentration of the thrombin. The fibrin glue can be used to secure surgical hæmostasis, on a large raw and fresh wide wound surface. It also prevents external oozing of blood in patient.

*Mechanism of Action:* It is an adjunct to hæmostasis for use in patient to prevent bleeding and control wound healing [2].

#### 1.2.7 Vitamin K (coagulation vitamin, phytomenadione)

It is essential for normal coagulation. Vitamin K<sub>1</sub> (phylloquinone) is widely distributed in plants and K<sub>2</sub> includes vitamin synthesized in the alimentary tract by bacteria to form menaquinones. Therefore, vitamin K is necessary for the final stage in the syntheses of six coagulation-related proteins in the liver. The coagulant factors are II (Prothrombin), VII, IX and X with two others committed steps.

*Mechanism of Action:* vitamin K is an essential cofactor for the gamma – carboxylase enzymes which promotes the hepatic formation of active prothrombin (factor II). *Therapeutic Uses:* For the treatment of hæmorrhage condition in infants, antidote for coumarin anticoagulant). Phytomenadione (Konakion) is preferred for its more rapid action, dose regimen vary according to the degree of urgency [13].

#### 1.2.8 Pyodine, butadine, pharmadine (povidone-iodine)

It is a stable chemical complex of polyvinylpyrrolidone (povidone, PVP) and elemental iodine. It contains from 5.0% to 12.0% available iodine. Povidone-iodine is a broad spectrum antiseptic and astringent for topical application in the treatment and prevention of infection in wounds. Maybe used as first aid for minor cuts, grazes, burns, abrasions and blisters. It is an effective broad-spectrum bactericide and effective against yeasts, moulds, fungi, viruses and protozoans.

*Mechanism of action:* Povidone-iodine is a broad spectrum microbicide that destroys microbial protein and DNA by penetrate the cell wall of organism and exert astringent. It also inhibits neuraminidases and receptor binding inhibition [14].

## 2. MATERIALS AND METHODS

### 2.1 Collection and Identification of Plant Material

The bulbs of *A. sativum* were obtained as fresh farm products from Okene market, in Kogi State, Nigeria in April, 2020. The plant's identification was authenticated by Mr. Ayegba Ojochele Sule at the Herbarium Unit of the Department of Biological Sciences, Faculty of Sciences, Kogi State University, Anyigba, and voucher specimen number of KSU/BS/058 was deposited for future reference.

### 2.2 Extraction of Plant Material

The collected bulbs of *A. sativum* (600 g) were washed and air dried under shade for 2 hours and the dry scaly outer covering was peeled-off to obtain the fresh garlic cloves which were then divided into three parts of 200 g each. These three portions were crushed separately for cold extraction according to the method of Abdullahi et al. [15]. The first portion was homogenized and poured into a muslin cloth to squeeze out the juice, while second and third portions were homogenized and submerged into 500 ml of 96% ethanol and 500 ml of distilled water respectively for 24 hours and both filtered after thorough shaking. The first and second portions were freeze dried, while the third portion was evaporated over water bath at 50°C to obtain the powdered yield. The three samples obtained were then stored in separately labeled air-tight container for later use. The percentage (%) extract yield was calculated as follows: % yield = weight of extract (g) / weight of garlic cloves (200 g) x 100

### 2.3 Solutions

Working concentrations to be used for the study were prepared from the obtained extracts (JEAS, EEAS and AEAS) using distilled water as a diluent. The required various concentrations to be used for each experiment were always freshly prepared from the stock solution of the extracts.

### 2.4 Laboratory Animals

Male and female adult wistar rats weighing (160 – 200 g), in-bred in the Animal House, College of Health Sciences, Kogi State University, Anyigba, Kogi State were used. The animals were fed with

a standard diet and tap water *ad libitum* and kept at room temperature and were handled according to standard protocols for the use of laboratory animals.

### 2.5 Phytochemical Screening of *Allium sativum*

The methods of Abdullahi et al. [15] and Kokori et al. [16] were used to screen for the presence of various phytochemical constituents in the *A. sativum* extracts. Phytochemical constituents screened include alkaloids, anthraquinones, cardiac glycosides, carbohydrates, fat and oils, flavonoids, saponins, steroids, terpenoids and tannins. 2.0g of powders of each of the three extracts were dissolved in 100 ml of distilled water for this screening experiment.

### 2.6 Determination of Blood Clotting Time (Antihemorrhagic) Effect of *Allium sativum* Extracts (Haemostatic and Styptic Properties)

The concentrations of the three extracts used throughout the studies were deduced from the pilot studies for both antihemorrhagic effects and antibacterial activities. This experiment was carried out according to Mielke's method described by Mark et al. [17] with standard rat blood clotting time of 2-5 minutes. Twelve adult wistar rats of both sexes were used to determine the effect of the three extracts of *A. sativum* under investigation (JEAS, EEAS and AEAS) on the blood clotting time shortening. The animals were divided into 4 groups of 3 rats per group as control, JEAS, EEAS and AEAS. Sterilized scalpel blades were used to lacerate wounds of about 1mm deep and 2 mm long below the groins (thighs) of the hind legs of the rats in all the groups using one hind leg each in two alternate days. Thus, both hind legs of the three rats in each group were used to obtain a total of six experiments per group (n = 6). The 4 groups wounded rats were then given an immediate treatment by topical application of standard antiseptic microbicidal astringent (5%Wosan Jawa Povidone Iodine), 5 mg/ml of JEAS, 5 mg/ml of EEAS and 5 mg/ml of AEAS respectively and observed for clotting time (the time bleeding stops). The mean blood clotting time for the 3 rats in each group for the duplicate experiments were then calculated and compared with the control group.

## 2.7 Determination of Antibacterial Activity of *Allium sativum* Extracts

The bacterial cultures were prepared by transferring with sterile wire loop, each bacterium cell into a Nutrient Broth Medium which was incubated at 37°C for 24 hours as described by WHO [12] to obtain a colony of the bacterial cells. The cells were maintained in Nutrient Broth Media in universal bottles labeled for each bacterium. Three tubes (1, 2, 3) each containing 9 ml of normal saline were set up for 3-fold serial dilution (1:1000) for the gram-positive bacterium, in which 1 ml of the overnight culture was transferred into the first test tube and mixed; and 1ml of this then taken into the second test tube from which another 1 ml was removed into the third tube which was then deemed to be  $1 \times 10^3$  colony forming unit / ml (CFU/ml). The same serial dilution, but in a fourth tube containing 4.5 ml of normal saline and 4-fold dilution (1:5000) was also performed for the gram-negative bacteria to the last concentration of  $1 \times 10^5$  CFU/ml. The last diluted concentrations (5 test tubes of the bacterial organisms) were incubated at 37°C for 24 hours after which, the bacterial organisms (suspension) were inoculated on prepared Molten sterile Mueller-Hinton agar plates by taking 2 ml of each of the organisms and flooding it over the agar surface by agar well diffusion method of Sharma and Aneja, [18]. Four different concentrations (10, 15, 20 and 25 mg/ml) and 5 mg/ml of the standard drug (cefuroxime) were used to study the growth inhibitory effect for each of the three extracts of *A. sativum*. Wells were bored on the agar dishes

and a drop of the molten agar was used to seal the bottoms of the bored wells prior to filling them with 0.2 ml of each the various drug concentrations. The plates were then kept for 1 hour to diffuse and then incubated at 37°C for 24 hours. The experiment was performed in duplicate and the zones of growth inhibition around the two wells for each drug concentrations were measured in millimeter using a ruler. The mean of the duplicate experiments for each concentration of the extracts were then calculated and recorded as the growth inhibitory zone of the extract concentrations for each of the organisms [19,20].

## 2.8 Statistical Analysis

Results obtained were recorded as mean  $\pm$  SEM and subjected to one way analysis of variance (ANOVA). Where significant differences exist, Waller Duncan post hoc test was performed using Statistical Analysis System (SAS, software version 2002). The results were regarded as significant at  $P < 0.05$  or lower.

## 3. RESULTS

### 3.1 Phytochemical Screening of the Extract

The Table 1 is a summary of the phytochemical components or secondary metabolites of the extract. From the obtained result, anthraquinone and tannins were not present in the extract of the bulbs.

**Table 1. The phytochemical constituents of *Allium sativum* (AS) bulb extracts (JEAS, EEAS and AEAS)**

Phytochemical components	Inference		
	JEAS	EEAS	AEAS
Alkaloids	Present	Present	Present
Anthraquinone	Absent	Absent	Absent
Carbohydrates	Present	Present	Present
Cardiac glycosides	Present	Present	Present
Fats and Oils	Present	Present	Present
Flavonoids	Present	Present	Present
Saponins	Present	Present	Present
Steroidal ring	Present	Present	Present
Terpenoids	Present	Present	Present
Tannins	Absent	Absent	Absent

Note: JEAS= juice extract, EEAS= ethanolic extract and AEAS=aqueous extract

**Table 2. Effect of blood clotting time of *A. sativum* extracts in wistar rats' wounds and arranged according to waller duncan ranking in descending order**

Treatment	Clotting time (minutes)
5% Povidone Iodine (control)	1.05± 0.01
5mg/ml JEAS	0.94± 0.01 <sup>a</sup>
5mg/ml EEAS	0.99± 0.04 <sup>b</sup>
5mg/ml AEAS	1.20± 0.04

Values are presented as Mean ± SEM, and triplicated in values for each of the extracts, a and b = P < 0.05 compared to the standard drug (control) - One Way ANOVA followed by Waller Duncan Post Hoc Test, Df =3, N=6

**Table 3. Inhibitory effect of *A. sativum* extracts on the growth of clinical bacterial isolates**

Test extracts	Zone of growth inhibition (mm) of the extracts at drug concentrations (mg/ml)					Bacterial orgs
	10	15	20	25	5(cefuroxime)	
JEAS	20.5±0.50	25.5±0.50	29.5±0.50	30.5±0.50 <sup>a</sup>	35	<i>Streptococcus pneumoniae</i>
EEAS	-	-	-	12.5±0.50	35	
AEAS	-	-	-	-	35	
JEAS	28.0±0.00	33.5±0.50 <sup>a</sup>	34.0±0.00 <sup>a</sup>	36.0±0.50 <sup>a</sup>	35	<i>Neisseria meningitides</i>
EEAS	-	-	14.5±0.50	17.5±0.50	35	
AEAS	-	-	-	-	35	
JEAS	23.0±0.00	26.5±0.50	28.0±0.00	30.5±0.50 <sup>a</sup>	35	<i>Haemophilus influenza</i>
EEAS	-	15.5±0.50	18.5±0.50	20.0±0.50	35	
AEAS	-	-	-	-	35	
JEAS	29.0±0.00	31.5±0.50 <sup>a</sup>	32.0±0.00 <sup>a</sup>	35.5±0.50 <sup>a</sup>	35	<i>Escherichia coli</i>
EEAS	-	-	-	25.5±0.50 <sup>a</sup>	35	
AEAS	15.5±0.50	16.0±0.00	19.0±0.00	20.5±0.50	35	

- = No zone of inhibition; Values are presented as Mean ± SEM, a=P < 0.05 compared to the standard drug (control) - One way ANOVA followed by Waller Duncan Post Hoc Test, Df=4 and N=15

### 3.2 The Blood Clotting Time Effect of *Allium sativum* Extracts in Rats

The result obtained showed that *A. sativum* extracts showed significant reduction (p<0.05) in bleeding time and shortened clot time at the site of the inflicted injuries on wistar rats compared with that of standard drug (control) (See Table 3).

### 4. DISCUSSION AND CONCLUSION

The phytochemical screening of the bulb extracts (JEAS, EEAS and AEAS) for bioactive constituents observed in *A. sativum* extracts revealed phytoconstituents with biological activities (Table 1). Some of these compounds are responsible for the characteristic odours like allylmethylsulphate (AMS) as garlicophobia, pungencies and colour of the plants while others give a particular plant its culinary, medicinal or poisonous virtue [21]. The result of the phytochemical screening showed that extracts were found to contain saponins, fats and oils, flavonoids, alkaloids, carbohydrates, cardiac glycosides and terpenoids. The antihemorrhagic

and antibacterial properties of these extracts can be linked to the presence of alkaloids, cardiac glycosides, carbohydrates, fats and oils, flavonoids, saponins, terpenoids, vitamin K and iodine. Indeed, members of these phytoconstituents are known to have antihemorrhagic and antibacterial activities coupled with primary metabolites (vitamins and antioxidants) [12,22]. Alkaloids, saponins, cardiac glycosides, oils, fats and terpenoids were also reported to have demonstrated activity against bacteria; have antidropsy effect and reduced inflammo-pathologic responses in the systemic and central nervous system [14]. Alkaloid, saponins, volatile sulphurous oil and flavonoids in this plant are known to form complexes with peptidoglycans, sterols and other cell wall components of bacteria resulting to cell leakage, cellular apoptosis and finally death of bacteria [13].

Presence of saponin, volatile organo-sulphur compounds, terpenoids, alkaloids and flavonoids in all the extracts (Table 1) suggested their ability to play such role as antibacterial,

antihaemorrhagic agents similar study has reported these phytoconstituents as having potential antimicrobial and coagulative (shorter blood clotting time) properties e.g wellawel or Siam weed (*Chromolaena odorata*) which showed promising antimicrobial and coagulative activities [22]. The presence of vitamin K, oils, organo-sulphur-iodine and saponins was also reported to hasten the healing of wounds and neuro-inflamed vasogenic injuries similar to results of JEAS and EEAS [21].

The results of this study showed that bulbs extracts of *A. sativum* possessed active phytochemicals analysis with organosulphur volatile oil substances, which can be used as novel antibacterial agents. It exhibited potent antihemorrhagic and antibacterial activities which fight microbes due to its phytoconstituents, antioxidant properties, and dietary medicines coupled with zeoliteherbal supplement. Moreover, *A. sativum* extract can interact with the entire system. It has a vital role in human health, human nutrition as integrative medicine against bacterial meningitis pathogens.

#### **ETHICAL APPROVAL**

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The studies were conducted according to the ethical guidelines and good laboratory practice for animal care and use in Kogi State University, Anyigba [23].

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#### **COMPETING INTERESTS**

Authors have declared that no competing interests exist.

#### **REFERENCES**

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