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Histomorphological and Thrombogenic Status of Wistar Rats Fed with *Allium sativum* (GARLIC) in Oxidative Stress Induced with Carbon Tetrachloride

I. K. Aromose[1], M. O. Ebeye[1], I. T. Ekwere[1], M. O. Odigie[1*], P. R. C. Esegbue[2] and J. C. Igweh[1]

[1]Department of Human Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Delta State University, Abraka, Delta State, Nigeria.
[2]Department of Human Physiology, Faculty of Basic Medical Sciences, Olabisi Onabanjo University, Shagamu, Ogun State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author IKA carried out the bench work, author MOE managed the literature searches, author ITE performed the statistical analysis, authors PRCE and MOO wrote and monitored the first draft of the manuscript, and author JCI managed and supervised the experimental protocol. All authors read and approved the final manuscript.

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ABSTRACT

Though concerns over the safety of medicinal drugs are following rife consumption of some herbal preparations, the underlying pathogenesis remains however cryptic. Awareness of the far-reaching effects of herbal preparations is germane to their continued use in traditional medicine. This study investigated in Wistar rats [fed with *Allium sativum* (garlic)], the status of their thrombogenic indices due to oxidative stress induced by carbon tetrachloride. Thirty (30) Wistar rats were randomly assigned into five groups of six animals each (*n* = 6): G1, G2, G3, G4 and G5. While G1 (normal control) received 1 ml/kg of groundnut oil, G2 (negative control) received a single dose of 1 ml/kg of

**Corresponding author: E-mail: osgiedeprof@yahoo.com;*

Carbon tetrachloride (CCl_4) for 2 weeks. G3 and G4 respectively got 250 and 500 mg/kg of *Allium sativum* extract twice daily for 2 weeks and then treated with a single dose of 1ml/kg CCl_4. G5 received 150 mg/kg of Vitamin E twice daily for 2 weeks and then treated with a single dose of 1 ml/ kg of CCL_4 to induce oxidative stress. The rats were then euthanized with blood samples collected for haematological analysis. Selected organs were also harvested and observed for histomorphological changes. Evaluation of data for statistical significance was done, using one-way analysis of variance (ANOVA). While kidney had some loss of nuclei, increased cytoplasmic eosinophilia, and congested blood vessel, hepatocytes showed some round to oval nuclei interspersed with sinusoids. Also, though garlic administration did not alter blood calcium concentration, it caused a decreased globulin but increased platelets, fibrinogen, bleeding and clotting time. Garlic in this study, therefore, was seen to be associated with a favourable dose-dependence improvement and maintenance of thrombogenic indices.

Keywords: Oxidative stress; Allium sativum; carbon tetrachloride; thrombogenicity.

1. INTRODUCTION

Thrombus formation and propagation depend on the presence of abnormalities of blood flow, blood vessel wall, and blood clotting components, known collectively as Virchow triad [1]. The processes that trigger venous thrombosis are not obvious. However, it is clear that the mechanisms initiating venous thrombosis are very different to those initiating arterial thrombosis [2] with endothelial imbalance possibly playing an important role [3].

Venous thromboembolism has a multifactorial origin and occurs in the context of complex interactions between environmental and genetic predisposing factors. Being an important mediator of abnormal platelet function and dysfunctional endothelium-dependent vasodilation, Oxidative stress plays a vital role in the physiopathology of venous thrombosis [4]. Recent studies have shown that vascular endothelial surfaces create a non-thrombogenic structure, such that endothelial injury that occurs relative to factors like anoxia, mechanical stress, free radicals, cytokines, and thrombin leads to increased free oxygen radicals, lipid peroxidation, and platelet activation and coagulation [5].

Apart from having a history of human consumption and use for over 7,000 years, Garlic (*Allium sativum*) has been known to oppose oxidative stress through its anti-oxidant and anti-haemorrhage activities. This food spice is native to central Asia, and has long been a staple in the Mediterranean region, as well as a frequent seasoning in Asia, Africa, and Europe [6-10].

Over time, Garlic has been used for medicinal purposes. Louis Pasteur, for instance, noted garlic's antibacterial activity in 1858. Subsequently, garlic was used as an antiseptic to prevent gangrene during World War I and World War II [11]. The potency of onion (*Allium cepa*) and garlic as medicinal plants is due to their high content of vitamins, trace elements, amino acids and several organo-sulphur compounds [12]. These "*magic*" drugs are well known for their: fibrinolytic effects [13], hemodynamic and haemostatic effects [14,15], platelet effects [16], immunologic effects [16], lipid-lowering effects [17], anti-atherosclerotic effects [18], anti-oxidative effects [19], anti-cancer effects [20], vascular effects [7], anti-microbial effects [21] and hepato-protective effects[9] among other health benefits.

From a clinical standpoint, if biomarkers that reflect the extent of oxidative stress were available, such markers would be useful for physicians to gain an insight into the pathological features of various diseases and assess the efficacy of drugs.

1.1 Aim of Study

A study aimed at examining the histo-architectural and thrombogenic changes that may occur in Wistar rats fed with *Allium sativum.* Specifically, the study attempted to:

i. Investigate the histo-morphological alterations in the kidney, following dose-dependent administration of A*llium sativum*
ii. Ascertain the ameliorative effect of A*llium sativum* administration on the thrombogenic properties of blood, following carbon tetrachloride, induced oxidative stress
iii. Examine the effect of *Allium sativum* on plasma proteins (fibrinogen, albumin, and globulin).

2. METHODOLOGY

2.1 Scope of Study

The study was conducted in the Department of Human Physiology, Faculty of Basic Medical Sciences, Delta State University, Abraka, Delta State, Nigeria. Due to the sensitive and invasive nature of the study, Wistar rats were used as a choice of experimental model.

2.2 Study Design

The experimental rats were randomly divided into five groups of six rats (G1, G2, G3, G4 and G5). Each group of rats were separately housed in standard cages. The first group, G1 (standard control) was given 1ml/kg of groundnut oil. While G2 (negative control) was treated with a single dose of 1ml/kg CCl_4 [15] once after 2weeks, G3 and G4 were respectively administered 250 and 500mg/kg of *Allium sativum* extract orally twice daily (morning and evening) for 2weeks and then treated with a single dose of 1ml/kg CCl_4 once after 2 weeks garlic administration. G5 got 150 mg/kg of Vitamin E twice daily for 2weeks and then treated with a single dose of 1ml/kg of CCL_4 to induce oxidative stress.

2.3 Materials and Methods

2.3.1 Procurement and preparation of animals

30 adult female wistar rats of approximately the same age and a body weight of 100–250 g were obtained and kept under a 12:12 hr light-dark cycle at room temperature in the animal house of Delta State University, Abraka, Delta State, Nigeria. All animals were allowed to adapt to the environment for two weeks after their arrival before the experiment started. All animals were housed in standard cages in a clean and neat surrounding with *ad libitum* access to water and standard rat diet. Animal handling was performed about CPCSEA guidelines, and Delta State University, Abraka, Nigeria rules.

2.3.2 Preparation of plant's extract

Fresh garlic extract was prepared on a daily basis prior to administration. The extract was made by crushing garlic, following 1 g to 10 ml of water for the low dose and 2 g to 10 ml of water for the high treatment. An aqueous extract was prepared by homogenising the bulbs in pestle and mortar using distilled water. It was then filtered with the help of muslin cloth and Wattmann filter paper. The extract was administered immediately after preparation as it is known that aqueous garlic lose some of its active components if left for a long time without refrigerating at 4^0c.

2.3.3 Ethical clearance

All experimental procedures were performed in strict accordance with the recommendations and Guide for the Care and Use of Laboratory Animals of the Delta State University (Delsu). Ethical clearance was sought and approved by the Research and Ethics committee of Delsu. The study also adhered to the code of conduct stipulated by the Institute for Laboratory Animal Research.

2.3.4 Acute toxicity study

The dose selection of *Allium Sativum* was based on acute toxicity studies, carried out according to OPPTS (Office of Prevention, Pesticide and Toxic Substance) following the limit test procedure. The influence of garlic extract on the chronic toxicity test was examined orally in Wistar rats for 6 months. There were no toxic symptoms due to garlic extract even at a dose level of 2000 mg/kg for 5 times a week. The animals were fasted overnight prior to the studies. Mice were divided into two groups of three each. The test dose of 2 g/kg body weight and 5 g/kg body weight were given orally to either group of mice. Mice were observed for 72 h for mortality.

2.3.5 Induction of oxidative damage with carbon tetrachloride (CCl_4)

Carbon tetrachloride (CCl_4) in its concentrated form was obtained from a local laboratory in Lagos State, Nigeria. CCl_4 was diluted in groundnut oil prior to administration to neutralise its hepatotoxicity level. CCl_4 was dissolved in groundnut oil in the ratio 1:1 v/v. Oxidative damage was induced in rats at a dose of 1mL/kg CCl_4 [15].

2.4 Procedure

2.4.1 Preparation of stock solutions of garlic extract

Low dose (250 mg/kg): 1 g of garlic was weighed with electronic weighing balance, homogenised in pestle and mortar using 10 ml of distilled water and filtered with Wattmann filter paper. This gave stock solutions of 100 mg/ml.

High dose (500 mg/kg): 2 g of garlic was weighed with electronic weighing balance, homogenised in pestle and mortar using 10 ml of distilled water and filtered with Wattmann filter paper. This gave stock solutions of 200 mg/ml.

2.4.2 Blood sample collection

At the end of the 14 days of administration, animals were euthanized by cervical dislocation, a procedure that involves giving a sharp blow in the neck within the region of the first two cervical vertebrae (C1 and C2). Blood samples were then collected from the superior vena cava, using a 5ml syringe. The sample was centrifuged at 3000rpm for 15minutes and the sera collected were stored frozen (at -20^{o}C) and later used for haematological analysis. Worth mentioning is that for each group of rats, before euthanizing, a lancet was used to inflict minimal injury, and the bleeding and clothing time were obtain with the aid of a stopwatch.

2.4.3 Platelet count determination

Hemocytometer was cleansed, and its glass was cover with a piece of cotton; saturated with alcohol and let air dry. Diluting pipette was then filled with blood sample (from rats) until sufficient blood has welled up on the fingertip to the 0.5 mark on the pipette by using the mouthpiece. Excess blood from the tip of the pipette was then wiped, followed by immersion of the pipette tip in the platelet diluting fluid (Dameshek's solution); and while holding the pipette vertically, diluting fluid was sucked exactly to the 11 mark. Dilution was done very quickly and precisely to prevent clotting of the blood and to insure accuracy. Thus, a 20-fold dilution was obtained with the volume of the mixing chamber being 11 µl–1 µl=10 µl, containing 0.5 µl of blood. The ends of the pipette were then closed with the thumb and middle finger and the pipette was shook for 5 minutes. After 20 minutes of wait (with hemolysis achieved), hemocytometer was then placed in a light microscope and examined with low-power objective (10 x) and weak light. Platelets count was then done in 100 rectangles considering the cells inside the squares and from two sides.

2.4.4 Organ collection

The animals were dissected and the testes, kidneys, as well as and liver were removed, cleared of adherent tissues and weighed immediately using an electronic weighing balance. Of each animal euthanized, one organ each was used for histological studies.

2.4.5 Analytical approach

Results of the study were presented as mean ± Standard error of mean (SEM) of sample size. Mean values among and between groups were compared statistically by one way analysis of variance (ANOVA), followed by post hoc Turkey's test for multiple comparison using Statistical package for social sciences (SPSS version 20). $p < .05$ was considered to be statistically significant. Data was further subjected to least significant difference (LSD) post hoc test and differences between means accepted significant at $p < .05$.

3. RESULTS

This study showed the effect of aqueous extract of *Allium sativum* on the histo-architecture and thrombogenic properties of wistar rats, following administration of Carbon Tetrachloride (CCl_4).

4. DISCUSSION

This study examined the histo-morphological and thrombogenic changes in carbon tetrachloride-induced oxidative stress in wistar rats fed with *Allium sativum.* Garlic from the study was observed to cause a decrease in plasma globulin levels; but increased platelets, fibrinogen, bleeding and clotting time in rats induced with oxidative stress.

Chart I (above) shows platelet count (PC) for CCl_4 administered rats (oxidative stress induced) following treatment with *Allium sativum*. As shown, compared to control, a dose-dependent, insignificant increase ($p <. 05$) in PC was observed after treatment with *allium sativum*.

This implies that *Allium sativum* has the potential to increase plasma platelet count and boost up the process of platelet plug formation in situations of haemorrhage, leading to swift clotting. This outcome is synergistically in line with those of Chart II where CCl_4 caused a decrease in bleeding time, while aqueous administration of *Allium sativum* caused a dose-dependent increase in bleeding time of the rats. Also, CCl4 significantly ($p < .05$) increased clotting time. In contrast, *Allium sativum* decreased the clotting time in a dose-dependent manner with

statistical significance; indicating that decrease clotting time implies an increase in platelet count and clotting factors. Vitamin E significantly ($p < .05$) increased clotting time, attenuate the supposed effect of CCl4administration.

Charts III and IV respectively shows the Effect of *Allium sativum* administration on Fibrinogen and albumin, globulin levels of rats treated with Carbon Tetrachloride (CCl_4). As observed, CCl4 caused an insignificant increase in albumin levels of rats, while *Allium sativum* significantly decreased albumin level ($p<0.05$); though not in a dose-dependent manner. Also, *Allium sativum* significantly ($p < .05$) increased globulin level in a dose-dependent manner. Vitamin E caused a significant decrease in globulin level of the CCl4 treated rats. The implication of this is that, Alluium sativum acted antagonistically in reducing albumin levels in the blood, which physiologically indicates a decrease in the transport modalities of proteins and very important biomolecules and metabolites. The same cannot be said for fibrinogen, as fibrinogen is also implicated in blood clotting processes. Also, Compared to control, CCl4 decreased the fibrinogen concentration with no significance. *Allium sativum* and Vitamin E administration increased the fibrinogen level with statistical significance ($p<0.05$).

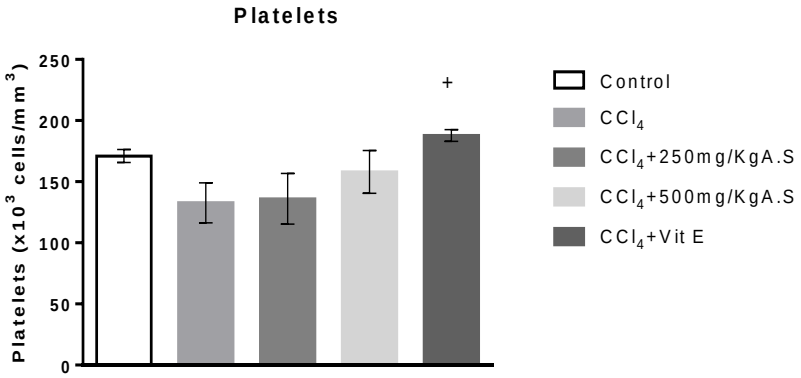

Chart 1. Status of platelet count (PC) in rats treated with carbon tetrachloride (CCl4) induced oxidative stress after administration of *Allium sativum*

Insignificant increase in PC ($p < .05$) as compared to rats treated with CCl_4. A.S = Allium sativum, CCl_4 = Carbon Tetrachloride

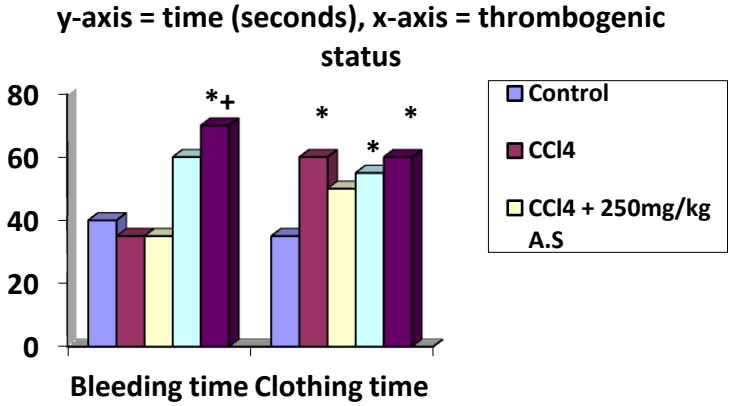

Chart 2. Effect of *Allium sativum* on bleeding and clotting times of rats treated with carbon tetrachloride (CCl_4)

CCl4 decreased the bleeding time, while aqueous administration of Allium sativum caused a dose dependent increase in bleeding time of the rats. Also, CCl4 significantly ($p < .05$) increased clotting time. In contrast, Allium sativum decreased the clotting time in a dose dependent manner with statistical significance. Vitamin E significantly ($p < .05$) increased clotting time. A.S = Allium sativum, CCl_4 = Carbon Tetrachloride

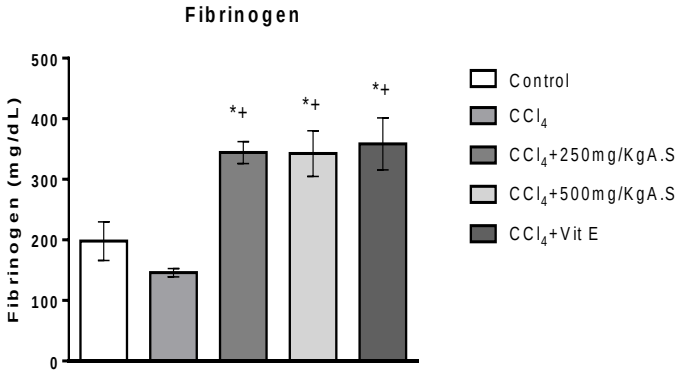

Chart 3. Effect of *Allium sativum* on fibrinogen of rats treated with carbon tetrachloride (CCl4)

Compared with control, CCl4 decreased the fibrinogen concentration with no significance. Allium sativum and Vitamin E administration increased the fibrinogen level with statistical significance (p<0.05). A.S = Allium sativum, CCl_4 = Carbon Tetrachloride

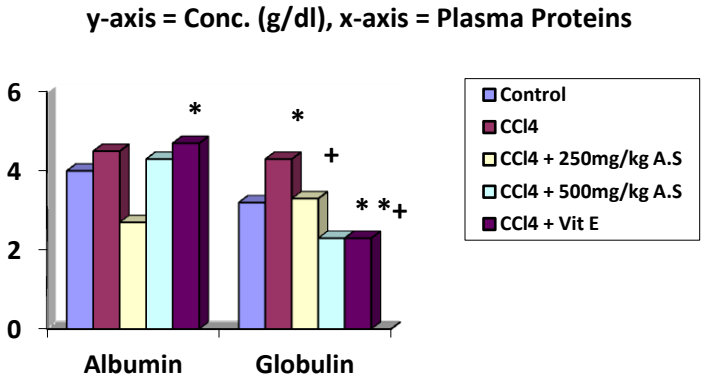

Chart 4. Effect of *Allium sativum* on albumin and globulin levels of rats treated with carbon tetrachloride (CCl_4)

CCl4 caused an insignificant increase in the albumin level of rats, while Allium sativum significantly decreased albumin level (p<0.05); though not in a dose dependent manner. A.S = Allium sativum, CCl_4 = Carbon Tetrachloride. Also, Allium sativum significantly (p < .05) increased globulin level in a dose dependent manner. Vitamin E also caused a significant decrease in globulin level of the CCl4 treated rats

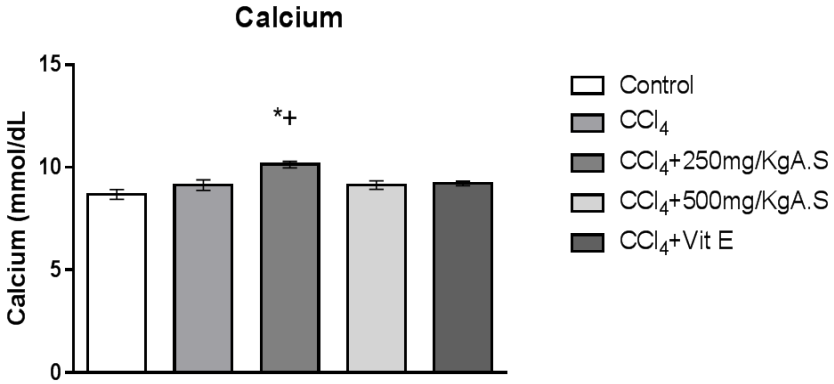

Chart 5. Effect of *Allium sativum* on calcium level of rats treated with carbon tetrachloride (CCl4)

Compared to control, CCl4 caused an insignificant increase in calcium level of rats. 250 mg/Kg of Allium sativum caused a significant (p<0.05) increase in Calcium level, which was attenuated in higher dose (500 mg/Kg). A.S = Allium sativum, CCl_4 = Carbon Tetrachloride.

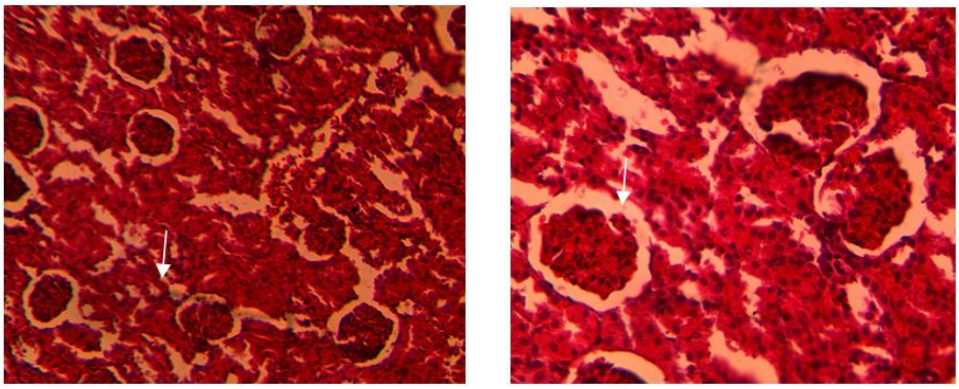

Plate I **Plate II**

Plate I. Normal control rat kidney composed of glomeruli (Arrow head) with H & E staining x 200

Plate II. Normal control rat kidney composed of renal tubules (Thin Arrow) with H & E staining x 400

Fig. 1. Comparing photomicrograph of kidney histology for Normal control (Group 1) rats

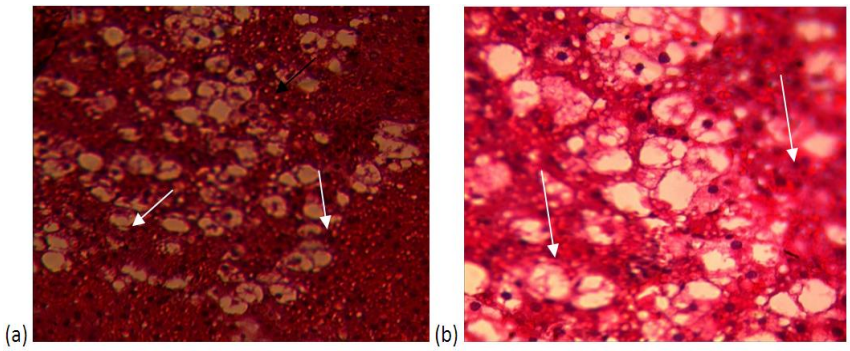

Plate III **Plate IV**

Above figure shows hepatocytes containing fat vacuoles with clear intracytoplasmic spaces (thin arrow) H & E staining (a) x 200 and (b) x400 magnifications

Fig. 2. Showing photomicrograph of hepatocytes histology for rats treated with 1 ml/kg CCl4 (G_2) after 2 weeks

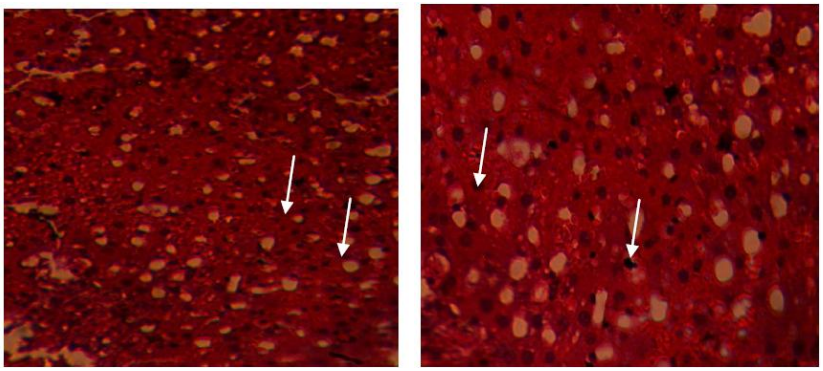

Plate V **Plate VI**

(a) (b)

Above figure shows hepatocytes containing fat vacuoles with clear intracytoplasmic spaces (thin arrow). Haematoxylin and eosin. (a) x 200 and (b) x400 magnification

Fig. 3. Showing photomicrograph of hepatocytes histology for rats treated with 250 mg/kg (G_3) of *Allium sativum* extract daily for 2 weeks

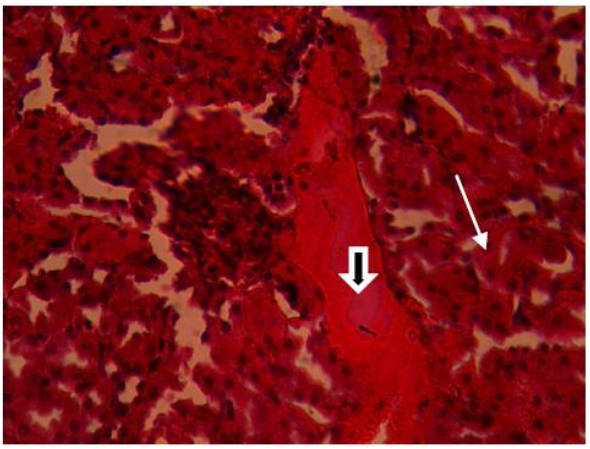

Plate VII

Plate VII. Group II *(*Negative control) rats kidney composed of glomeruli (thin arrow), tubules (thick arrow) and congested blood vessel with H & E staining x 400

Fig. 4. Showing photomicrograph of kidney histology for group rats treated with single dose of 1 ml/kg of CCl_4 after 2 weeks

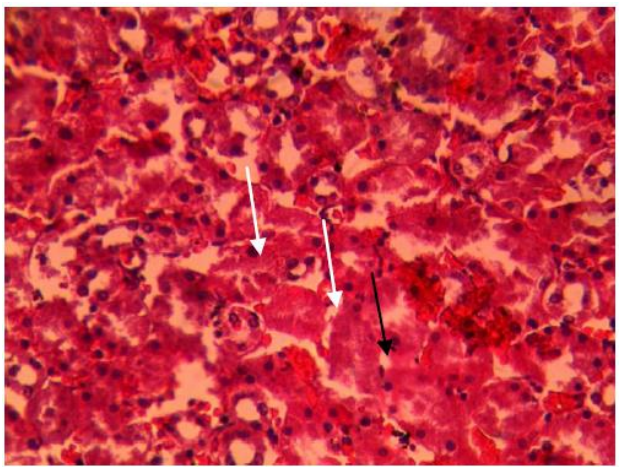

Plate VIII

Plate VIII. Kidney of Group III rats composed of tubules with loss of nuclei and increased cytoplasmic eosinophilia (thin arrow) and congested blood vessel. H & E staining x 400

Fig. 5. Showing photomicrograph of kidney histology for group 3 treated rats (250 mg/kg *Allium sativum* extract daily for 2 weeks)

For plasma calcium levels, Chart V shows the Effect of *Allium sativum* on Calcium level of rats treated with Carbon Tetrachloride (CCl4). Worth stressing is that calcium had been implicated in several physiological processes in the body, and in blood clotting processes. From presented results in Chart V, Compared to control, CCl4 caused an insignificant increase in calcium level of rats. 250 mg/Kg of *Allium sativum* caused a significant ($p<0.05$) increase in Calcium level, which was attenuated in higher dose (500 mg/Kg). The implication of this is that, serum calcium concentration is dose-dependent and/or prolonged administration of allium sativum. This can be attenuated in higher doses.

Figs. 1 and 2 Shows Comparisons of photomicrograph of kidney histology for Normal

control (Group 1) rats and rats treated with 1 ml/kg CCl4 (G_2) for 2 weeks. Upon carful observation, hepatocytes containing fat vacuoles with clear intracytoplasmic spaces (thin arrow) was seen in Group 2 animals as against those in Group 1. This indicates that fat vacuole obviously came up, following administration of CCl4 to group II animals; implicative of alterations in renal histo-architecture as a result of CCl4 administration. For Fig. 3 however, a similar observation was seen in the hepatocytes (liver cells) of group 3 rats, which were administered 250 mg/kg of CCl4 for two weeks. Fat vacuoles with clear intracytoplasmic spaces were seen, implicating CCl4 as having a potential to alter histomorphology of the liver. Renal tubules with loss of nuclei and increased cytoplasmic eosinophilia and congested blood vessels were observed in the histo-architecture of groups 3 rats who were treated with CCl4 (Fig. 5). Though possible mechanism(s) for this alteration is yet to be fully understood, it is believed that congestion of renal vessels (in this case) is a function of increased renal blood flow, resulting from increased ultrafiltration and/or selective reabsorption.

Before now, studies have shown that garlic has a protective action against decrease in fibrinolytic activity and increase in clotting time in patients with myocardial infarction and coronary artery disease [16]. Normally, fibrinolysis occurring in the body maintains the fluidity of the circulating blood by dissolving and removing deposits of fibrin. Perhaps an important function of fibrinolysis is to remove very minute clots from tiny peripheral vessels that eventually would become occluded were there no way to cleanse them. Defects in coagulation and fibrinolytic activity are main factors for the development of thrombus. Garlic, when used continuously produces constant lytic activity, probably due to decrease in cholesterol and an increase in the plasminogen activity [16].

Fibrinolysis releases fibrin degradation products which have anticoagulant activity. Increased plasmin may lower factor V, VIII, IX and can cause increase in clotting time [17]. The commonly used thrombolytic agents like streptokinase and urokinase are expensive, antigenic, and effective only on parenteral use and cannot be used for a long time. These drawbacks are absent with garlic therapy [17]. In the present study, we found that ingestion of raw garlic for two months had brought about an increase in fibrinolytic activity and increase in clotting time in normal individuals. No side effect was noted with garlic. Also, it is cheap and can be taken orally. So, daily use of simple dietary substance like garlic may be useful for prevention of thromboembolic phenomenon.

Although variable data were obtained in the study, results taken together imply that aged garlic extract (AGE) probably exerts its inhibitory effect on platelet aggregation, either by suppressing the influx of calcium ions by chelating calcium within platelet cytosol or by altering other intracellular second messengers within the platelets [2]. Some preparations appear to be anti-oxidative, whereas others may stimulate oxidation. These additional biological effects attributed to AGE may be due to compounds, such as S-allylcysteine, *S*-allylmercaptocysteine, N^{α}-fructosyl arginine and others, formed during the extraction process. Although not all of the active ingredients are known, ample research suggests that several bioavailable components likely contribute to the observed beneficial effects of garlic [7,8].

These results suggest that the anticoagulant action of rich garlic oil was due to inhibition and/or inactivation of thrombin. In addition, rich garlic oil benefits blood anticoagulation factors, which might further prevent the development of thrombus formation[17]. More so, Garlic caused increase in fibrinogen level. This is consistent with findings of Kung-chi in 2007 [22]. This researchers earlier found out that the intake of garlic oil at high dose significantly increases plasma fibrinogen concentration and affected the levels of several haematological parameters such as erythrocyte count, haemoglobin and platelets.

Thus, AGE exerts selective inhibition on platelet aggregation and adhesion; platelet functions that may be important for the development of cardiovascular events such as myocardial infarction and ischemic stroke.

Increased platelet aggregation plays a pivotal role in the etiology of cardiovascular disease. Upon platelet aggregation, an increase in free cytoplasmic Ca^{2+} results in the inhibition of soluble guanylyl cyclase (sGC) and adenylyl cyclase (AC), leading to a decrease in cyclic guaninosine-5′-monophosphate (cGMP) and cAMP, respectively. This leads to the activation of the glycoprotein IIb/IIIa (GPIIb/IIIa) fibrinogen receptor, resulting in platelet shape change. Aged garlic extract (AGE) decreases platelet

aggregation; however, the mechanisms involved are not clearly defined [22,23]. These results indicate that AGE inhibits platelet aggregation by increasing cyclic nucleotides and inhibiting fibrinogen binding and platelet shape change [23].

4.1 Benefit of Study

From this study, the following can be deduced:

i. Garlic has high fibrinolytic activity and can maintain blood in fluid state, and therefore useful as nutritional supplement for patients suffering from Diabetes and or prone to cardiovascular disorders
ii. Garlic has both prophylactic and protective effects on haemostasis
iii. Garlic has a protective action against alteration of thrombogenic parameters in oxidative stress.

5. CONCLUSION

This study has shown, that compared to control, Carbon Tetrachloride (CCl_4) caused an insignificant increase in calcium level of rats, while *Allium sativum* significantly increased it. This was however attenuated at higher dose. More so, a significant and an insignificant increase were respectively seen in the plasma globulin and albumin levels following administration of CCl_4. However, fibrinogen levels and bleeding time had a significant decrease, with platelet counts and clothing times increasing after administration of *Allium sativum*.

6. RECOMMENDATIONS

From this study, the following recommendation is advice for the furtherance and improvement of this work.

i. Effects of the constituents of Garlic on thrombogenic indices.
ii. Effect of *Garlic* on thrombogenic indices in diseases that induce oxidative stress.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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