

HISTOMORPHOLOGICAL AND THROMBOGENIC STATUS OF WISTAR RATS FED WITH *ALLIUM SATIVUM* (GARLIC) IN OXIDATIVE STRESS INDUCED WITH CARBON TETRACHLORIDE

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 1ml/kg of groundnut oil, G2 (negative control) received a single dose of 1ml/kg of Carbon tetrachloride (CCl_4) for 2 weeks. G3 and G4 respectively got 250 and 500mg/kg of *Allium sativum* extract twice daily for 2 weeks and then treated with a single dose of 1ml/kg CCl_4 . G5 received 150mg/kg of Vitamin E twice daily for 2 weeks and then treated with a single dose of 1ml/kg of CCl_4 to induce oxidative stress. The rats were then euthanised 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

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 triad¹. 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 thrombosis² with endothelial imbalance possibly playing an important role³.

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 thrombosis⁴.

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 coagulation⁵.

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 Europe⁶⁻¹⁰.

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 II¹¹. 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 Compounds¹². These “magic” drugs are well known for their: fibrinolytic effects¹³, hemodynamic and haemostatic effects^{14&15}, platelet effects¹⁶, immunologic effects¹⁶, lipid-lowering effects¹⁷, anti-atherosclerotic effects¹⁸, anti-oxidative effects¹⁹, anti-cancer effects²⁰, vascular effects⁷, anti-microbial effects²¹ and hepatoprotective effects⁹ 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.

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 *Allium sativum*
- ii. Ascertain the ameliorative effect of *Allium 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).

Methodology

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.

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 CCl₄¹⁵ 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 CCl₄ once after 2weeks garlic administration. G5 got 150mg/kg of Vitamin E twice daily for 2weeks and then treated with a single dose of 1ml/kg of CCl₄ to induce oxidative stress

Materials and Methods

Procurement and Preparation of Animals

30 adult female wistar rats of approximately the same age and a body weight of 100–250g were obtained and kept under a 12:12hr 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.

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 1g to 10ml of water for the low dose and 2g to 10ml 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 4⁰c.

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.

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.

Induction of Oxidative Damage with Carbon tetrachloride (CCl₄)

Carbon tetrachloride (CCl₄) in its concentrated form was obtained from a local laboratory in Lagos State, Nigeria. CCl₄ was diluted in groundnut oil prior to administration to neutralise its hepatotoxicity level. CCl₄ was dissolved in groundnut oil in the ratio 1:1 v/v. Oxidative damage was induced in rats at a dose of 1mL/kg CCl₄¹⁵.

Procedure

Preparation of Stock Solutions of Garlic Extract

Low dose (250mg/kg)

136 1g of garlic was weighed with electronic weighing balance, homogenised in pestle and mortar
137 using 10ml of distilled water and filtered with Wattmann filter paper. This gave stock
138 solutions of 100mg/ml.

139 **High dose (500mg/kg)**

140 2g of garlic was weighed with electronic weighing balance, homogenised in pestle and mortar
141 using 10ml of distilled water and filtered with Wattmann filter paper. This gave stock
142 solutions of 200mg/ml.

143 **Blood Sample Collection**

144 At the end of the 14 days of administration, animals were euthanised by cervical **dislocation,**
145 **a procedure that involves giving a sharp blow in the neck within the region of the first two**
146 **cervical vertebrae (C1 and C2).** Blood samples were then collected from the superior vena
147 cava, using a 5ml syringe. The sample was centrifuged at 3000rpm for 15minutes and the
148 sera collected were stored frozen (at -20°C) and later used for haematological analysis. Worth
149 mentioning is that for each group of rats, before euthanising, **a lancet was used to inflict**
150 **minimal injury, and the bleeding and clotting time were obtained with the aid of a stopwatch.**

151 **Platelet count Determination**

152 **Hemocytometer was cleansed, and its glass was covered with a piece of cotton; saturated with alcohol**
153 **and let air dry. Diluting pipette was then filled with blood sample (from rats) until sufficient blood has**
154 **welled up on the fingertip to the 0.5 mark on the pipette by using the mouthpiece. Excess blood from**
155 **the tip of the pipette was then wiped, followed by immersion of the pipette tip in the platelet diluting**
156 **fluid (Dameshek's solution); and while holding the pipette vertically, diluting fluid was sucked exactly**
157 **to the 11mark. Dilution was done very quickly and precisely to prevent clotting of the blood and to**
158 **insure accuracy. Thus, a 20-fold dilution was obtained with the volume of the mixing chamber being**
159 **11 µl–1 µl=10 µl, containing 0.5 µl of blood. The ends of the pipette were then closed with the thumb**
160 **and middle finger and the pipette was shaken for 5 minutes. After 20 minutes of wait (with hemolysis**
161 **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.

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.

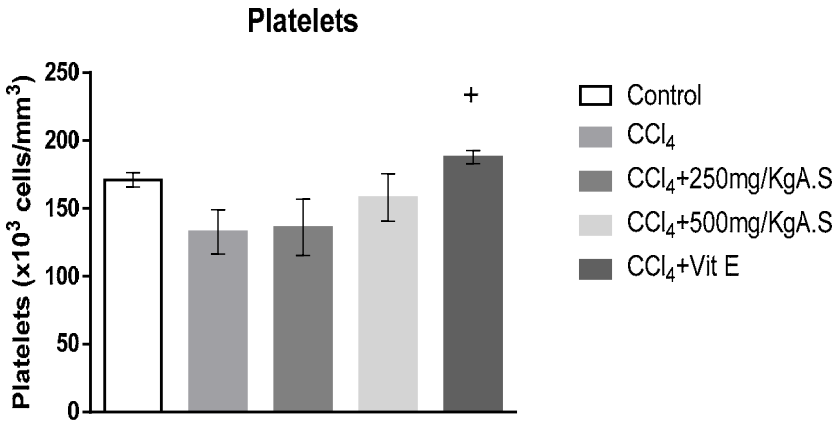
Analytical Approach

Results of the study were presented as mean $\pm$ 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 $p < .05$

Results

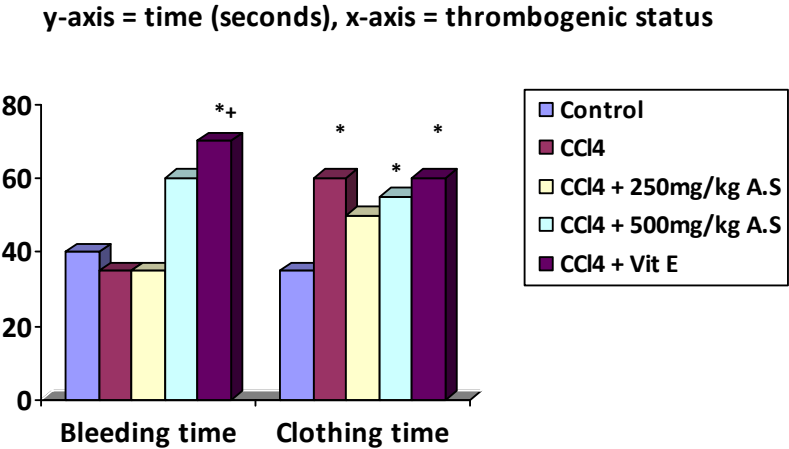
This study showed the effect of aqueous extract of *Allium sativum* on the histo-architecture and thrombogenic properties of wistar rats, following administration of Carbon Tetrachloride (CCl_4)

CHART 1: Status of Platelet count (PC) in rats treated with Carbon Tetrachloride (CCl_4) induced oxidative stress after administration of *Allium sativum*



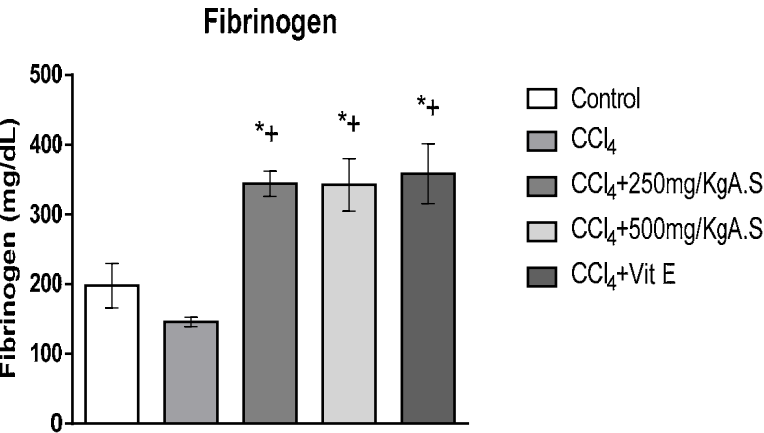
Insignificant increase in PC ($p < .05$) as compared to rats treated with CCl_4 . A.S = Allium sativum, CCl_4 = Carbon Tetrachloride

CHART 2 Effect of Allium sativum on Bleeding and Clotting times of rats treated with Carbon Tetrachloride (CCl_4)



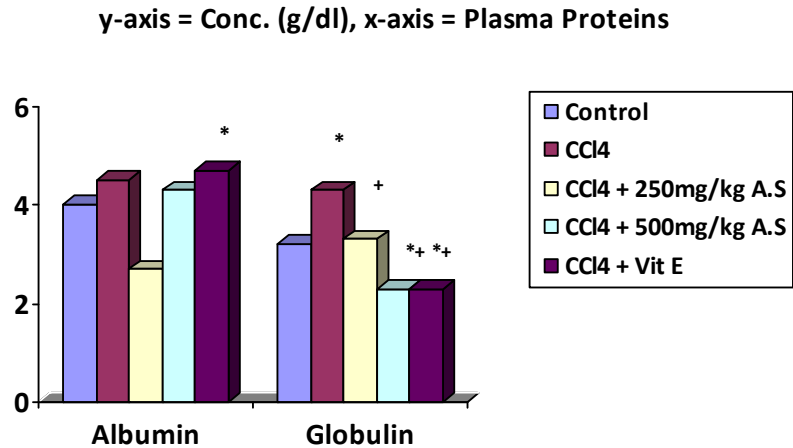
CCl_4 decreased the bleeding time, while aqueous administration of Allium sativum caused a dose dependent increase in bleeding time of the rats. Also, CCl_4 significantly ($p < .05$) increased clotting time. In contrast, Allium sativum decreased the clotting time in a dose dependent manner with statistical 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 (CCl_4)



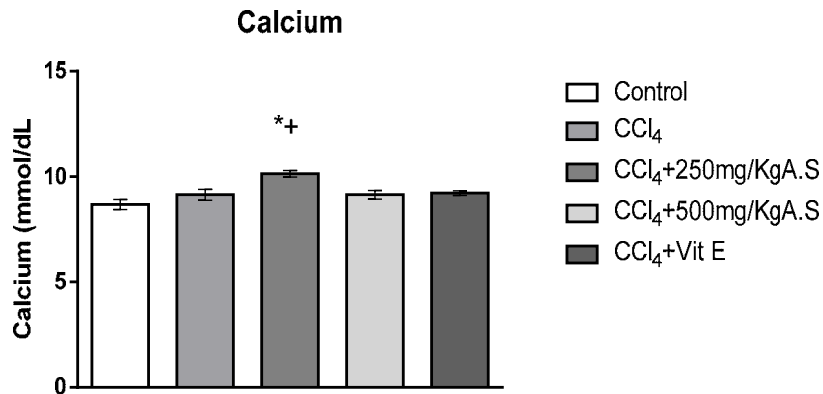
Compared with control, CCl_4 decreased the fibrinogen concentration with no significance. Allium sativum and Vitamin E administration increased the fibrinogen level with statistical significance ($p < 0.05$). A.S = Allium sativum, CCl_4 = Carbon Tetrachloride

CHART 4: Effect of *Allium sativum* on Albumin and Globulin Levels of rats treated with Carbon Tetrachloride (CCl₄)



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*, CCl₄ = 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. 250mg/Kg of *Allium sativum* caused a significant ($p<0.05$) increase in Calcium level, which was attenuated in higher dose (500mg/Kg). A.S = *Allium sativum*, CCl₄ = Carbon Tetrachloride.

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

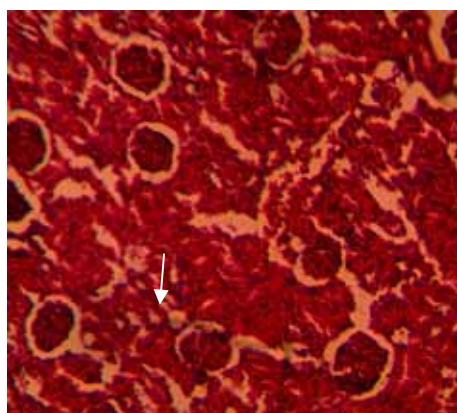


PLATE I

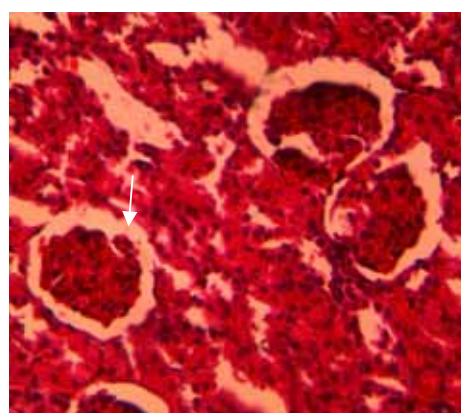
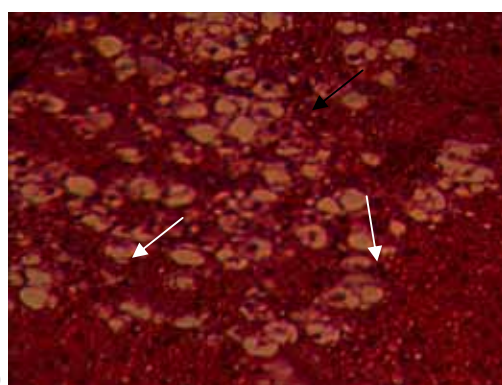


PLATE II

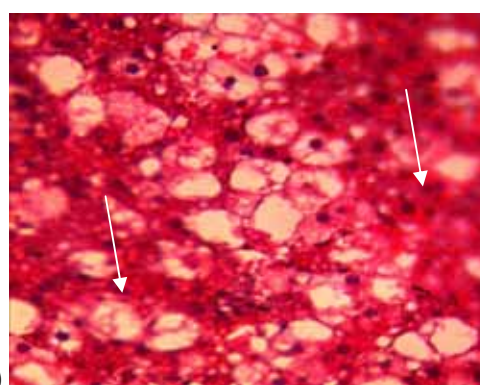
PLATE I: Normal control rat kidney composed of Glomeruli (Arrow head) with H & E staining x 200

PLATE II: Normal control rat kidney composed of Renal Tubules (Thin Arrow) with H & E staining x 400.

FIGURE 2: Showing photomicrograph of hepatocytes histology for rats treated with 1ml/kg CCl₄ (G₂) after 2weeks.



(a)



(b)

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

FIGURE 3: Showing photomicrograph of hepatocytes histology for rats treated with 250mg/kg (G₃) of *Allium sativum* extract daily for 2weeks.

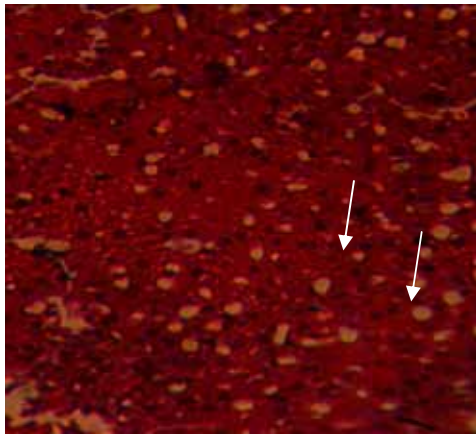


PLATE V

(a)

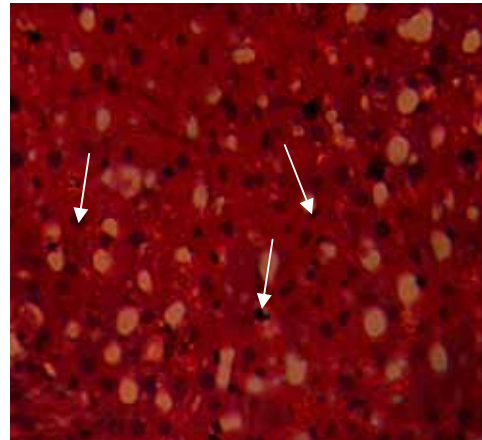


PLATE VI

(b)

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

FIGURE 4: Showing photomicrograph of kidney histology for group rats treated with single dose of 1ml/kg of CCl₄ after 2weeks.

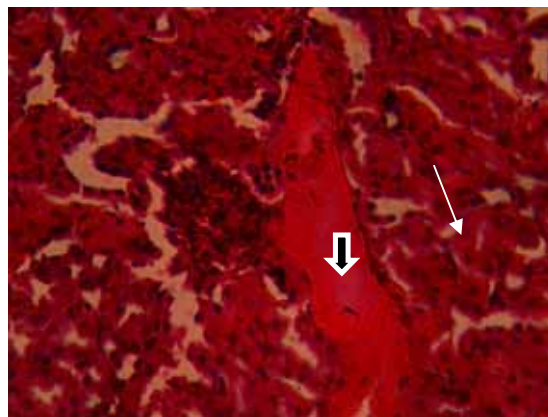


PLATE VII

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

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

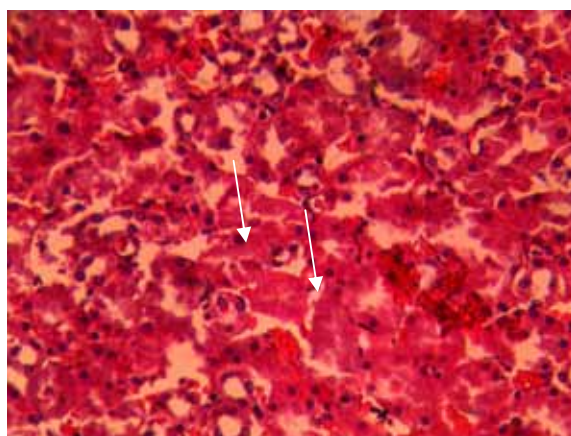


PLATE VIII

PLATE IV: 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.

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 CCl₄ 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 CCl₄ 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 CCl₄ administration.

Charts III and IV respectively shows the Effect of *Allium sativum* administration on Fibrinogen and albumin, globulin levels of rats treated with Carbon Tetrachloride (CCl₄). 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, Allium 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 ($p < 0.05$).

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. 250mg/Kg of Allium sativum caused a significant ($p < 0.05$) increase in Calcium level, which was attenuated in higher dose (500mg/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.

Figures I and II Shows Comparisons of photomicrograph of kidney histology for Normal control (Group 1) rats and rats treated with 1ml/kg CCl₄ (G₂) for 2 weeks. Upon careful 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 figure III however, a similar observation was seen in the hepatocytes (liver cells) of group 3 rats, which were administered 250mg/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 (Figure V). 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 disease¹⁶. 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 activity¹⁶. Fibrinolysis releases fibrin degradation products which have anticoagulant activity. Increased plasmin may lower factor V, VIII, IX and can cause increase in clotting time¹⁷. 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 therapy¹⁷. 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 platelets². 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 formation¹⁷. More so, Garlic caused increase in fibrinogen level. This is consistent with findings of Kung-chi in 2007²². 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 guanosine-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 change²³.

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.

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 clotting times increasing after administration of *Allium Sativum*.

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

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