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Allium sativum extract affects medial prefrontal cortical cytoarchitecture

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Abstract

Allium sativum, a medicinal plant is reported with antioxidant ability. However, toxicity has also been reported, which warranted this investigation into its effects on the medial prefrontal cortex of adult male Wistar rats. A toxicity test of the extract on mice was carried out, and thereafter 24 male Wistar rats were divided into 4 groups (n = 6); the control group, and 78 mg/kg, 156 mg/kg and 312 mg/kg *Allium sativum* extract groups. The administration was orally lasting for 14 days. On day 15, the animals were anaesthetized with 50 mg/kg ketamine hydrochloride (i.p), and perfusion-fixed. The medial prefrontal cortex was then processed for histology and some immunohistochemical studies. Results showed that LD_{50} of *Allium sativum* extract was approximately 650 mg/kg body weight. The prefrontal cortical sections showed adverse Nissl substance distribution with the presence of dark nuclei and significantly ($p<0.05$) higher Nissl staining in the 312 mg/kg group. Enolase-2 and GFAP were expressed more in the 312 mg/kg group, all indicating brain tissue damage processes. In conclusion, *Allium sativum* causes alterations in cellular integrity, and the expression of enolase-2 and GFAP in the prefrontal cortex, whose effects were dose dependent.

Introduction

Herbs are considered very important in all cultures because of it medicinal roles [1]. This is further buttressed by the belief that drugs from plant extracts are generally less harmful with little or no side effects unlike the synthetic ones [2]. Studies on green tea, milk thistle, red grapes and turmeric revealed that these plants possess anti-inflammatory properties, and are also thought to be anti-carcinogenic and anti-mutagenic due to the presence of polyphenols; a naturally occurring compound found in them [3,4]. Another widely investigated herbal plant is *Allium sativum*; commonly known as garlic.

Allium sativum is a spicy flavoring agent widely cultivated and consumed as food in many countries and has been widely used as a popular remedy for various disorders for thousands of years [5]. In local Nigerian languages it is called ayim mbakara in Ibibio, ayuu in Ibo and ayo in Yoruba. Garlic belongs to the *Allium* family, which also constitute onions, shallots and leeks. Its preparations are available commercially in the form of garlic oil, garlic powder and pills, and are widely used for certain therapeutic purposes. This is based on the various therapeutic functions of the plant including its role as a protective agent against the deleterious effects of metal poisoning [4,6].

Studies have also shown that garlic serves as an anti-diabetic drug where it significantly reduces serum glucose in streptozotocin-induced diabetic rats while increasing serum insulin in non-diabetic rats [7]. It also reduces total cholesterol, triglycerides, urea and uric acid in serum [7,8]. Garlic modulate the activity of transferases and cytochrome P_{450} isozymes, both *in vitro* and *in vivo* [9], plays an anti-canceric role in colon and prostate cancers [10,11], as well as colorectal adenomas [12], and is also a potent analgesic and anti-nociceptive agent [13].

In the brain, studies have shown that garlic serves as a neuro-protective and neuro-rescue agent against reactive oxygen species [14], improves spatial memory [15], protects hippocampal neurons against lead-induced neural damage [16], prevents memory impairment [17], neuroprotects against traumatic brain injury [18] and also improve visual memory and attention [19]. Notably, these actions of garlic are carried out via its antioxidant, anti-apoptotic and anti-atherogenic properties [20].

Despite these numerous beneficial roles of garlic, there are reports that consumption of garlic can lead to perturbations on Sertoli cell junctions and dilatation of rough endoplasmic reticulum which can further lead to apoptosis in testicular germ cells [21]. It also causes allergic reactions, alteration of platelet function and coagulation [22], and alteration of cellular architecture of such organs as the heart, liver, kidney and stomach mucosa [23-25].

It is also reported that *Allium sativum* affects cognitive processes and cause cellular changes in the medial prefrontal cortex [26,27]. However, what changes could be affected? This therefore led to this study on the effects of administration of *Allium sativum* on the prefrontal cortex of adult Wistar rats.

Materials and methods

Twenty four adult male albino Wistar rats of body weights 150 g -180 g were obtained and housed in 14 cages (40 cm × 35 cm) of 3 rats each in the Animal House facility of the Faculty of Health Sciences, University of Uyo, Uyo, Nigeria. The animals were allowed 12 hours light and 12 hours dark cycles at 27°C -30°C room temperature. They

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were fed standard rat pelletized diet (Grand Cereals Ltd, Nigeria) and water *ad libitum*. Ethical approval was obtained from the Faculty of Basic Medical Sciences Ethics Committee and handling of the animals followed the guidelines of National Institute of Health of the United States. The rats were randomly divided into groups 1-4 (n = 6). Group 1 served as the control and groups 2-4 were the test groups (Table 1).

Preparation of the extract

Fresh garlic bulbs were obtained from a local market in Uyo, Akwa Ibom State of Nigeria, and was identified and authenticated by the Curator of the University of Uyo Herbarium. Each fresh clove was peeled, pounded with mortar and pestle and blended with a blender (Century, China). Extraction of garlic components ensued by suspending the pulp in 60% ethanol for 30 minutes after which it was evaporated in a water bath at 40°C to reduce water content without losing the needed components. This was followed by reconstitution in distilled water; after which it was preserved in the refrigerator at 4°C.

Lethal dose determination

The up and down method was used in the determination of the lethal dose (LD_{50}). This method does not use death of the animal as end point but an estimate of LD_{50} is derived by an increase or decrease in dosage of the extract administered to the animal one at a time. For this research, 21 albino mice were used (3 mice per dosage). First administered dosage was 400 mg/kg, and was steadily increased by 50 mg/kg after 2 days if each mouse survived the initial dosage. The highest administered dosage was 700 mg/kg. The surviving animals were monitored for delayed death for a total of 7 days and they were observed for physical signs of toxicity such as writhing, gasping, decreased respiratory rate, body limb and death [28,29].

Administration of the garlic extract

Group 1 which was the control received 5ml of distilled water per kilogram body weight (kg b.w.) as placebo, while groups 2, 3 and 4 received 78 mg, 156 mg and 312 mg of *Allium sativum* extract per kg b.w., respectively for 14 days. Oral administration of the garlic extract and water was done in the mornings (8-9 am).

Termination of the experiment

On day 15, the animals were anaesthetized intraperitoneally with 50 mg of ketamine hydrochloride per kg b.w. (Rotex M`edica, Germany), and immediately followed with intracardial perfusion of phosphate-buffered saline (1M, pH 7.35) via their thoraco-abdominal walls, and perfusion-fixed with 10 % neutral buffered formalin. The brains of the animals were then removed and post-fixed in 10 % neutral buffered formalin for 48 hours. The prefrontal cortex is located anteriorly in the frontal lobe and is defined as its agranular part [30]. Serial sections of the medial prefrontal cortex were processed for histology by the Cresyl fast violet method and immunolabelled with anti-enolase and glial fibrillary acidic protein.

Table 1. Groupings of the experimental animals

Groups (n=6)	Dosage	Duration (Days)
1 (Control)	Distilled water (5ml/kg)	28
2	78 mg/kg *Allium sativum* extract	14
3	156 mg mg/kg *Allium sativum* extract	14
4	312 mg/kg *Allium sativum* extract	14

Briefly, the tissues were routinely processed for paraffin wax embedding. 10 µm thick paraffin sections on slides were then routinely processed for histomorphology with Cresyl violet staining. Representative serial paraffin sections on slides were brought to water and antigen retrieval was performed using citrate buffer (pH 6.0) in a microwave oven for 5 minutes, followed by protein block using 3% hydrogen peroxide for 10 minutes. Sections were thereafter preincubated in 2% normal goat serum for 30 minutes and incubated for an hour at room temperature (20-23°C) each in monoclonal mouse anti-enolase-2 (Novocastra, Leica Biosystems, 22C9, 1:100) for neuron specific enolase and mouse monoclonal anti GFAP (Novocastra, Leica Biosystems, NCL-L-GFAP-GA5, 1:100) for GFAP, followed by incubation in goat anti-mouse secondary antibody (1:100) for an hour. Detection of reaction was by means of the avidin-biotin complex with diaminobenzidine as the chromogen. Sections were then counterstained with Harris haematoxylin, dehydrated, cleared and cover slipped with DPX. Processed slides were viewed under the light microscope (Olympus) and photomicrographs obtained using a computer assisted digital microscope's camera (Amscope).

Cellular density was determined manually by means of ImageJ® software. Briefly, images of the whole prefrontal cortex was obtained for each section and randomly mapped with the ImageJ® gridlines. Counting of cell nuclei was done manually taking into consideration the nuclei on the upper and right borders of the mapped areas.

Statistical analysis

One way analysis of variance was used to analyze all the data, followed by a post hoc Tukey's test. All analysis was done using Graphpad Prism for Windows (version 5.01, San Diego California, USA). Data at probability level $P < 0.05$ was regarded as significant and are presented as Mean ± Standard Error of Mean.

Results

Lethal dose (LD_{50}) Determination

From this investigation, using the up and down method, the LD_{50} of *Allium sativum* on mice was estimated to be 650 mg/kg (Table 2).

Histology

Sections of the medial prefrontal cortex of the control group showed well-stained Nissl substance throughout the cortical layers (outer granular, outer pyramidal and inner granular layers). In comparison with the control group, the medial prefrontal cortex of the 78 mg/kg *Allium sativum* extract group also showed well-stained Nissl substance throughout the cortical layers. The 158 mg/kg *Allium sativum* group showed well-stained Nissl substance in some of the neurons throughout the cortical layers compared with the control group, while the 312 mg/kg *Allium sativum* had well-stained Nissl substance with small size and significantly ($p < 0.05$) higher population compared with the 78 mg/kg *Allium sativum* and the control groups. However, there was no difference in population between the 158 mg/kg *Allium sativum* group and the control group (Figure 2 and 3).

Immunohistochemistry

Neuron Specific Enolase (NSE): The section of the medial prefrontal cortex of the control group animals showed the neurons with less expression of enolase-2 throughout the cortical layers. The groups that received 78 mg/kg and 156 mg/kg of *Allium sativum* extract also showed less expression of enolase-2 throughout the cortical layers, while 312 mg/kg group showed increased expression of the *Allium sativum* extract compared with the control group (Figure 3).

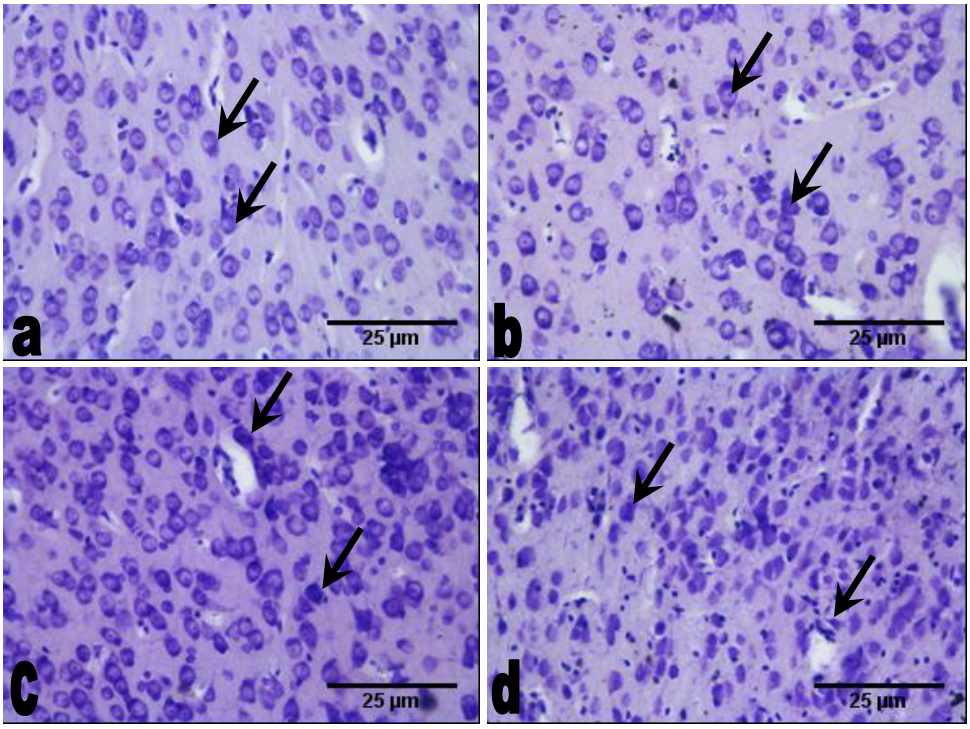

Figure 1. Sections of the medial prefrontal cortex at the end of the experimental period (Cresyl fast violet, × 200).
a. The control group animals with well-stained Nissl substance (arrow) in the cortical layers.
b. The 78 mg/kg 14 days group showing well-stained Nissl substance (arrow) throughout the cortical layers.
c. The 156 mg/kg 14 days group with well-stained Nissl substance (arrow) in throughout the cortical layers with high density.
d. The 312 mg/kg 14 days group with well-stained Nissl substance (arrow), having high density with reduce size.

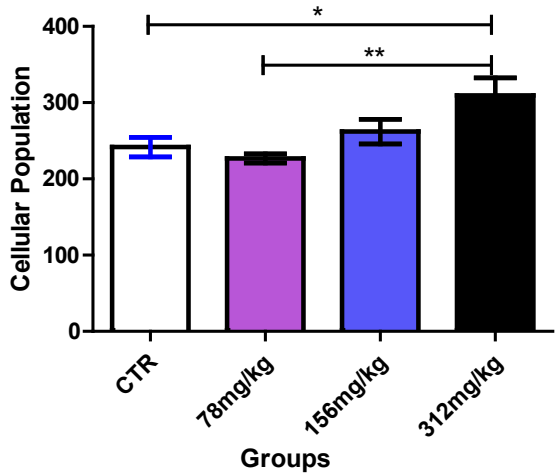

Figure 2. Cellular population in the prefrontal cortex after 14 days administration of *Allium sativum*
* - Significantly different from the control group at $p < 0.05$
** - Significantly different from the 312 mg/kg *Allium sativum* group at $p < 0.01$

Table 2. Lethal dose (LD_{50}) determination result

Average weight (g)	Dosage (mg/kg)	Remark
22	400	Survived
22	450	Survived
21	500	Survived
22	550	Survived
22	600	Survived
21	650	Died
22	700	Died

Glial Fibrillary Acidic Protein (GFAP): The section of the prefrontal cortex of the control group animals showed less expression of glial fibrillary acidic protein (GFAP) throughout the cortical layers. This was also observed in the group that received 78 mg/kg *Allium sativum* extract compared with the control group. However groups 3 and 4 that received 156 mg/kg and 312 mg/kg *Allium sativum* extract showed slight increase expression of GFAP especially in the astrocytic processes throughout the cortical layers compared with the control group (Figure 4).

Discussion

The effects of sub-chronic administration of *Allium sativum* extract on the medial prefrontal cortex of adult male Wistar rats were investigated in this study. The study focused on the histology arrangement of Nissl and some structural proteins in the outer granular, outer pyramidal and inner granular layers of the medial prefrontal cortex.

The acute toxicity of *Allium sativum* result showed that *Allium sativum* had an oral median lethal dosage of 650 mg/kg in mice, an indication that *Allium sativum* is very toxic to the body if taken at this dose. This report differs from previous studies [31-33], probably due to the *Allium* specie type or its constituents, or the animal species or route of administration. This is because the route of administration, the specie type of the plant and the specie of the animal is known to differ in toxicity exposure [34]. This toxic property corroborates already reported toxic effects of *Allium sativum* in different tissues of the body [21,23-27].

The Nissl substance results revealed no difference in staining intensity in the test groups compared with the control. There was a

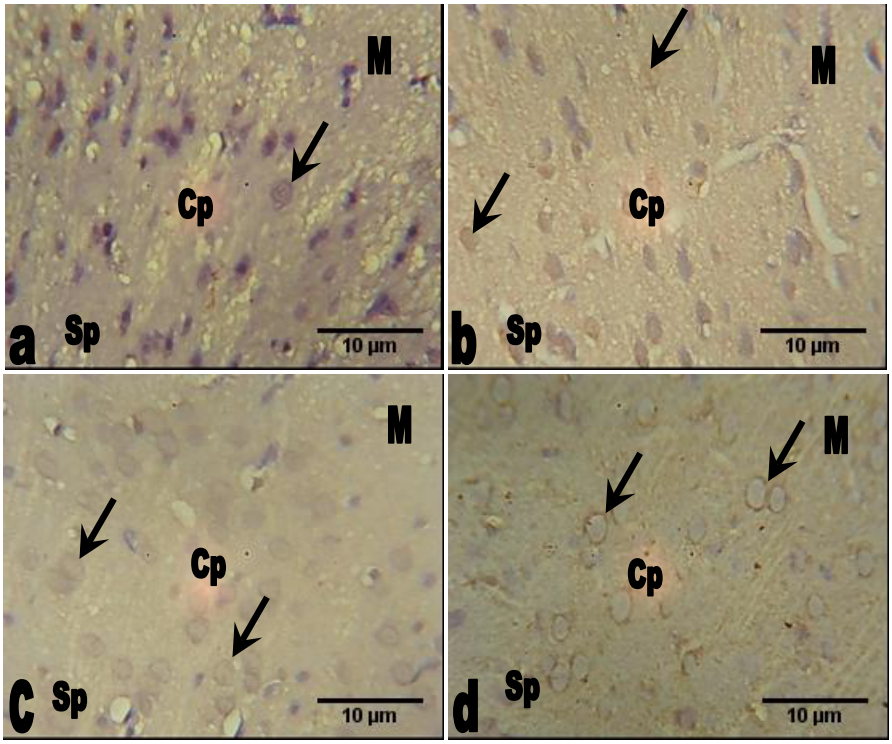

Figure 3. Photomicrograph of the sections of the prefrontal cortex of the control and groups 2, 3 and 4 animals (neuron specific enolase, NSE, Mag. ×400).
a. The medial prefrontal cortex of the control group showing the neurons with little expression of enolase-2 (arrow) throughout the cortical layers.
b. The medial prefrontal cortex of 78 mg.kg *Allium sativum* group showing the neurons with little expression of enolase-2 (arrow) throughout the cortical layers.
c. The medial prefrontal cortex of the 156 mg/kg *Allium sativum* group showing the neurons with little expression of enolase-2 (arrow) throughout the cortical layers.
d. The medial prefrontal cortex of the 312 mg.kg *Allium sativum* group showing the neurons with increase expression of enolase-2 (arrow) throughout the cortical layers.

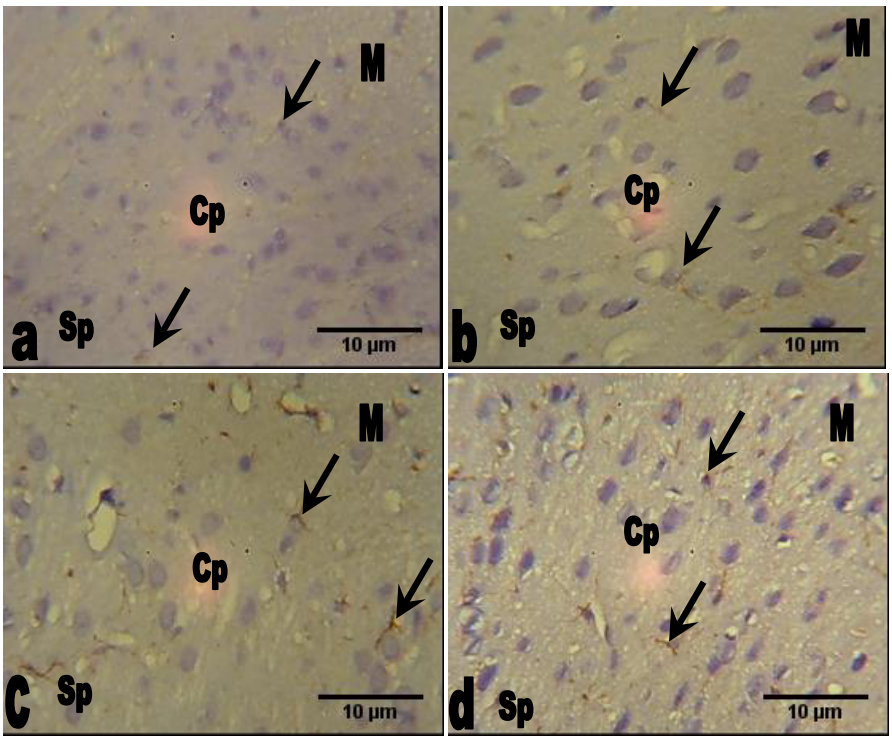

Figure 4. Photomicrograph of the sections of the medial prefrontal cortex of the control and groups 2, 3 and 4 animals (GFAP, Mag. ×400).
a. The section of the medial prefrontal cortex of the control group animals showing little expression of glial fibrillary acidic protein (GFAP) (arrow) throughout the cortical layers.
b. The medial prefrontal cortex of the 78 mg/kg *Allium sativum* animals showing little expression of GFAP (arrow) throughout the cortical layers.

higher cellular population in the 312 mg/kg *Allium sativum* group. As there was no difference in Nissl substance, this indicates that the given doses of *Allium sativum* may not affect Nissl distribution. Nissl substances are rough endoplasmic reticulums that stain due to the presence of the anionic macromolecules, DNA and ribosomal RNA, thus, serves as site for protein synthesis [35]. Hence, the general protein synthesizing ability of these cells may not have been affected.

Nissl technique stains the entire population of neurons and glial cell types in the same section [36-38]. Thus, a higher cellular population is indicative of proliferation of these cells, which may be physiological or pathological [39]. This increase may be due to gliosis as the prefrontal cortex is not known to undergo adult neurogenesis. Gliosis usually results as a protective mechanism for the neural tissues [40], and this may have been the case in the present study. The prefrontal cortex of the 78 mg/kg *Allium* group did not show a difference with the control, an indication that this dosage and duration did not adversely affect Nissl distribution.

The 312 mg/kg *Allium sativum* group showed an increase in the expression of enolase-2, while the rest of the test groups showed less expression of the protein compared with the control group. Neuron-specific enolase plays a role in the survival of neurons and can provide neuroprotective effects via binding to neurons in a calcium-dependent manner [41]. Increased NSE expression indicates neuronal damage [42], which ultimately may lead to neuronal cell death. *The increase in the expression of enolase-2 in the 312 mg/kg Allium group is an indication of the damage to the neurons of the prefrontal cortex of this group. This result supports the dark nuclei observed in the Nissl stain study of this same group*. The prefrontal cortex of the 78 mg/kg *Allium* group did not show apparent difference with the control, an indication that this dosage and duration did not adversely affect enolase-2 expression.

Allium sativum extract caused alterations in the expression of glial fibrillary acidic protein (GFAP). GFAP expression was observed in the prefrontal cortex of the test groups, which was higher in the 156 mg/kg and 312 mg/kg Allium sativum groups compared with the control group. Increased expression of GFAP indicates astroglial activation and gliosis, often resulting from neurodegeneration [43], due to the astrocytes need to mop-up the resultant debris formed [44]. This may be a reason for the increased expression of GFAP in these test groups. Although the expression of GFAP in the present study appear protective, improper GFAP regulation has been linked to various disorders and injuries which can cause glial cells to react in detrimental ways; for example, glial scarring which is partially caused by up-regulation of GFAP [45,46], further providing an enabling environment for *neurodegeneration*. The prefrontal cortex of the 78 mg/kg *Allium* group did not show apparent difference with the control, an indication that this dosage and duration did not adversely affect GFAP expression.

The prefrontal cortex of the brain is involved in cognitive processes, and damage or improper regulation of its cellular and structural proteins may adversely affect the prefrontal cortical functions [47,48]. As *Allium sativum* extract, especially the high dose group showed some neurodegenerative processes, it may therefore alter this function, which may trigger other consequences.

Conclusion

The administration of ethanolic extract of *Allium sativum* did not influence Nissl distribution, but cause cellular proliferation and increased NSE and GFAP expressions of prefrontal cortex in the high dosage groups, which may indicate neurodegenerative processes. Although the specific neurons affected cannot be elucidated, further research is necessary.

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Conflict of interest and sources of funding statement

Authors funded the research, and hereby declare no conflict of interest.

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