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(RESEARCH ARTICLE)

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Biochemical and histological analysis of ethanol peel extract of *Citrus sinensis* on mercury chloride-induced prefrontal cortex damage in Wistar rats

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Abstract

Mercury is one of the heavy metals which is found extensively in many environmental and certain occupational settings. It is the third most dangerous heavy metal and its toxicity causes serious risks to health through unfavorable pathological and biochemical effects. Sweet orange is the fruit of the citrus species (*Citrus sinensis*) in the family Rutaceae.. Over time there has been increasing interest in the utilization of these peels for both nutritional and medicinal purposes. Studies have reported the antioxidant potential of orange peel oil in laboratory rodents, indicating its protective effect against oxidative stress-mediated ailments. The study evaluated the effect of ethanolic extract of *Citrus sinensis* peel on mercury chloride-induced changes in the prefrontal cortex of adult Wistar rats. Twenty-five Wistar rats were purchased and divided into five groups containing five animals in each group. Group 1 was administered with distilled water, Group 2, Group 3, Group 4, and Group 5 were administered with 5 mg/kg of mercury chloride only, 5 mg/kg of mercury chloride and 140 mg/kg Silymarin, 5 mg/kg mercury chloride and 1000 mg/kg of ethanoic extract of orange peel, and 5 mg/kg mercury chloride and 1500 mg/kg of ethanoic extract of orange peel. All administration was done orally and the administration lasted for 14 days. The animals were sacrificed humanely using 75 mg/kg ketamine intraperitoneally and prefrontal was dissected out. Part was homogenized in 0.1M phosphate buffered saline for the estimation of superoxide dismutase (SOD), and catalase (CAT) activities, and Malondialdehyde (MDA) level and the other part was fixed by immersion in 10 neutral buffered formal-saline for histological evaluation. The results indicate that the MDA level in control Group(13.29±1.183 µmol) is statistically significantly lower compared to the Group 2 (21.88±1.96 µmol), group 3 (27.44±3.52 µmol), Group 4 (22.20±1.16 µmol) and Group 5 (25.40±3.13 µmol). No statistically significant different that was observed in SOD activity. It CAT activities in control group (53.51±6.83 nmol/ml) is statistically significantly lower compared to the group 2 (26.21±3.70 nmol/ml), Group 3 (27.17±5.32 nmol/ml), Group 4 (25.30±4.24 nmol/ml) and Group5 (26.59±4.22 nmol/ml). Protective role of the extract was observed from the histological evaluation. This study suggests that ethanolic extract of *Citrus sinensis* peel have protective effects on mercury-induced changes in prelimbic part of the prefrontal cortex of adult Wistar rats.

Keywords: Prelimbic; Catalase; Superoxide dismutase; Intraperitoneally

1. Introduction

The medicinal properties of orange peel have been documented in several studies. It has been used for centuries in the traditional Chinese medicine to treat indigestion and to improve inflammatory syndromes of the respiratory tract (Huang & Ho, 2010). Erukainure et al. (2012) reported the antioxidant potential of orange peel oil in laboratory rodent, indicating its protective effect against oxidative stress-mediated ailments. These reported medicinal properties can be attributed to the phytochemical constituents of the peel. Flavonoids, consisting mainly of polymethoxylated flavonoids

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(PMFs), terpenoids, such as limonene and linalool, and other volatile oils make up the major phytochemical constituents of orange peel. The chemo preventive potential of these

Over time there have been increasing interests in the utilization of these peels for both nutritional and medicinal purposes. The peel has been reported to be edible and mostly consumed when there was a scarcity of resources and maximal nutritional value was desired (Erukainure et al., 2012). It has an increased vitamin C and fiber contents but with high concentrations of pesticides. However, increased dietary vitamin A is required when consuming orange peel due to the presence of citral, an aldehyde that antagonizes the action of vitamin A (Ashraf et al., 2019).

Human mercury exposures occur chiefly through inhalation of elemental mercury vapor via occupational or dental amalgam exposure or through ingestion of mercury bonded to organic moieties (methyl, dimethyl, or ethyl mercury), primarily from seafood. Most human metallic mercury exposure comes from mercury vapor outgassing from amalgam fillings, at a rate of 2 to 28 micrograms per facet surface per day, of which about 80% is absorbed, according to the World Health Organization and Berglund et al. (1988). A less common source of mercury vapor is spilled mercury (Zeitz et al., 2002).

Methyl and dimethyl mercury (organic mercury) usually originate from biological sources, chiefly fresh or salt water fish. Over three thousand lakes in the United States have been closed to fishing due to mercury contamination (Berlin et al., 2007) and many species of ocean fish are also tainted with considerable concentrations of mercury .

Mercury toxicity in humans varies with the form of mercury, the dose and the rate of exposure. The target organ for inhaled mercury vapor is primarily the brain (Berlin et al., 2007). Mercurous and mercuric salts chiefly damage the gut lining and kidney (Berlin et al., 2007), while methyl mercury is widely distributed throughout the body (Berlin et al., 2007). Toxicity varies with dosage: large acute exposures to elemental mercury vapor induce severe pneumonitis, which in extreme cases can be fatal (Berlin et al., 2007). Low-grade chronic exposure to elemental or other forms of mercury induces subtler symptoms and clinical findings, as discussed hereinafter.

2. Materials and Methods

Twenty-five (25) Wistar rats weighing 150g-180g each was procured from the animal house of the Human Anatomy Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University (ABU), Zaria, Kaduna, Nigeria and housed in new wired cages in the same facility, and were allowed to acclimatize for few days before the commencement of the experiments. The rats were categorized into control and treated groups, all given food (rat chow) and water ad libitum.

Plant materials : Citrus sinensis fruit was obtained from Samaru market, Zaria, Nigeria and was taken to the Herbarium unit of Department of Biological Sciences, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria, the fruit was identified and authenticated by comparing it with the existing voucher number of ABU0990. Afterwards, the peel was separated from the fruit and was taken for extraction process.

2.1. Drugs and chemical Mercury

Fifty (50) grams of mercuric chloride, a white powdered substance was used to induce hepato- renal toxicity. The product was manufactured by British Drug Houses (BDH) Chemicals, Poole, England. Silymarin (Silybon-70®) was obtained from a Pharmaceutical Store, Dialogue Pharmacy, Kaduna, and used as standard (reference) antioxidant drug. Each tablet is film coated and contains 70 mg of silymarin. The product was manufactured by Micro Labs Limited 92, Sipcot, Hosur-635 126, India with CAS number; 65666-07-1.

2.1.1. Animal feed

Pelletized Vital Feed manufactured by Grand Cereals and Oil Mills Limited (GCOML), Plateau State, Nigeria was obtained and used to feed the rats for the experiment

2.1.2. Other materials

Other materials used in the study include the following; Light Microscope, Amscope, Computer, Tissue Processing Reagents, Cages, Animal feed, Distilled water, Weighing Balance (Kitchen and Digital weighing scale), 1ml needle and syringe, sacrificing chamber, Bouin's fluid, Gloves, Dissecting tray, Dissecting kit, Gloves, Sacrificing chamber, Centrifuge Machine, Ketamin Injection, Pipette, Glass slide, Cover slip.

2.2. Methods

2.2.1. Plant extraction

The peel was separated from the fruit and dried under laboratory temperature and pressure and grounded to powder with the aid of a pestle and mortar. The powder was used for the extraction using a Soxhlet extraction method in the Department of Pharmacognosy and Drug Development ABU, Zaria.

2.2.2. Phytochemical screening

Qualitative phytochemical screening for secondary metabolites contained in ethanol peel extract of Citrus sinensis was conducted according the method of Trease and Evans (2002) in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria.

2.2.3. Acute Toxicity Study

The acute toxicity (LD50) ethanol peel extract of Citrus sinensis was determined using the Lorke's method. This method was in two phases; phase 1 and 2.

2.3. Phase 1

Nine (9) rats which were divided into three (3) groups of three (3) animals each. The various groups were administered the extract at different doses of 10, 100 and 1000 mg/kg. The rats were observed closely for 24 hours, no sign of toxicity, behavioral changes or occurrence of mortality was observed.

2.4. Phase 2

This phase involves the use of three (3) rats which were divided into three (3) groups of one animal each. The rats were administered higher doses of the extract at 1600, 2900 and 5000 mg/kg. The rats were then placed under observation for 24 hours for signs of toxicity, behavioral changes as well as mortality of which none was recorded.

The LD50 was then calculated using the formula $LD50 = \sqrt{(Do \times D100)}$.

D0 = Highest dose that gave no mortality D100 = lowest dose that produced mortality

2.5. Dose preparations

The dose preparation of ethanol peel extract of Citrus sinensis was carried as follows: One hundred grams (62 g) of the fraction was measured and dissolved in 800 ml of distilled water. This makes up the working solution from which little portion was measured out for the administration of the rats. Different doses of fraction were administered to rats by gavage using orogastric tube for the experimental period.

The dose preparation of standard drug (Silymarin) is as follows: ten grams (10 g) of Silymarin was dissolved in 300 ml of distilled water to form a solution which aided drug administration to the rats by gavage.

The dose preparation of mercury is as follows: Fifteen grams (15 g) of mercuric chloride was dissolved in 300 ml of distilled water to form a solution which aided drug administration to the rats by gavage.

2.6. Experimental Design

Mercuric chloride was purchased from Steve Moore chemicals Limited Samaru, Zaria. The oral LD50 of Mercury chloride in rats for oral administration is adopted from Sadeeq et al. (2013) as 166mg/kg body weight.

Twenty-five (25) male Wistar rats were divided into five (5) groups of five (5) rats each. Group I served as control and was administered 2 ml/kg distilled water. Group II was administered mercury chloride ($HgCl_2$) 5 mg/kg (12.5% LD50 as reported by Sadeeq et al. (2013)). Group III was administered silymarin 140 mg/kg following the method of Ahmed et al. (2010) followed by 5 mg/kg $HgCl_2$, while groups IV and V were administered 1000 mg/kg and 1500 mg/kg of ethanol peel extract of Citrus sinensis followed by 5 mg/kg $HgCl_2$ respectively. The administrations were via oral route and lasted for a period of 2 weeks.

All experimental procedures were carried out in the Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria, except otherwise specified.

2.6.1. Animal sacrifice

At the end of the experimental period, the rats were euthanized under ketamine anesthesia. The skull was cracked open and the brain was harvested and divided into two hemispherical halves, one half was weighed and homogenized in a solution of 0.1M phosphate-buffered saline pH 7.5. The total homogenate was centrifuged at 1000 rev/min in a refrigerated centrifuged for 7 minutes. The supernatants were used for the estimation of biochemical parameters, while the other hemisphere was fixed in Bouin's fluid. The fixed tissues were taken to the histology laboratory of the Human Anatomy Department, Ahmadu Bello University, Zaria for tissue processing.

2.6.2. Body and organ weights

Before euthanasia, rats' absolute body weights were measured using a digital weighing scale (Kerro BL 20001, China, 0.1 g) at the beginning (Initial Weight), across the study period and, at the end (Final Weight) of the experiment. Percentage weight change was computed as described by Agbon et al. (2021) and means were compared between the groups.

Harvested brains were weighed using a digital scale (Acculab VICON, VIC-303, USA, 0.001 g). Relative organ weight (Organosomatic index) was computed as described by Agbon et al. (2021) [(organ weight/ final body weight) ×100] and values obtained were analyzed and compared between the groups.

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2.7. Biochemical assay

Malondialdehyde assay : Malondialdehyde (MDA) levels in brain samples were measured by the method of Uchiyama & Mihara (1978). This method depends on the formation of MDA as an indicator of lipid peroxidation, which reacts with thiobarbuturic acid producing thiobarbuturic acid reactive substances (TBARS), measured spectrophotometrically at 532 nm.

Superoxide dismutase (SOD) activity assay: The activity of SOD enzyme was evaluated by the method of Sun et al. (1988). The analysis of SOD was based on the principle in which xanthine reacts with xanthine oxidase to produce superoxide radicals. The level of suppression of this reaction measured the SOD activity. Results were expressed as U/mg protein.

Catalase activity assay: Catalase enzyme activity was evaluated using a spectrophotometric test based on the yellow complex with molybdate and hydrogen peroxide, which was described in detail by Goth (1991) in plasma was expressed with 1, 1, 2, 2,-tetramethoxypropane as the standard (nmol/ml).

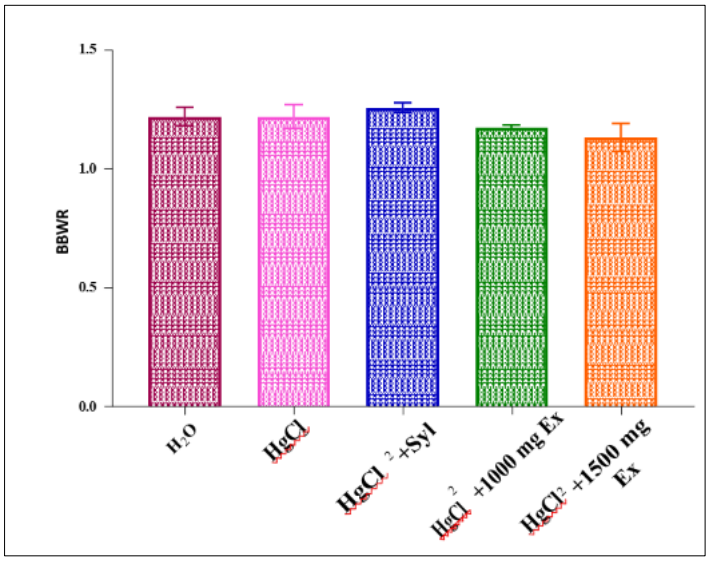
2.8. Histological and histochemical studies

Harvested brains were fixed in Bouin's fluid and processed using histological techniques for light microscopic examination. Processed histological (prefrontal cortex) sections were stained with Hematoxylin and Eosin (H&E) to demonstrate general histoarchitectural features of the prefrontal cortex layers. Histological tissue processing was carried out in the Histology Unit of the Department of Human Anatomy, ABU, Zaria

3. Results

Table 1: Phytochemical Constituents of Citrus sinensis peel

Secondary metabolites	Impression
Reducing sugars	+
Saponins	+
Anthracene glycosides	+
Deoxy-sugar cardiac glycosides	+
Tannins	+
Flavonoids	+
Alkaloids	-



HgCl2=Mercury chloride (5 mg/kg), Syl=silymarin (100 mg/kg), Ex= Ethanol extract of *Citrus sinensis*

Figure 1 Brain-body Weight Ratio of Wistar Rats Following Treatment with $HgCl_2$ and Ethanol Peel Extract of Citrus sinensis.n= 5, mean ± SEM, one-way ANOVA, $p>0.05$ when compared across the groups.

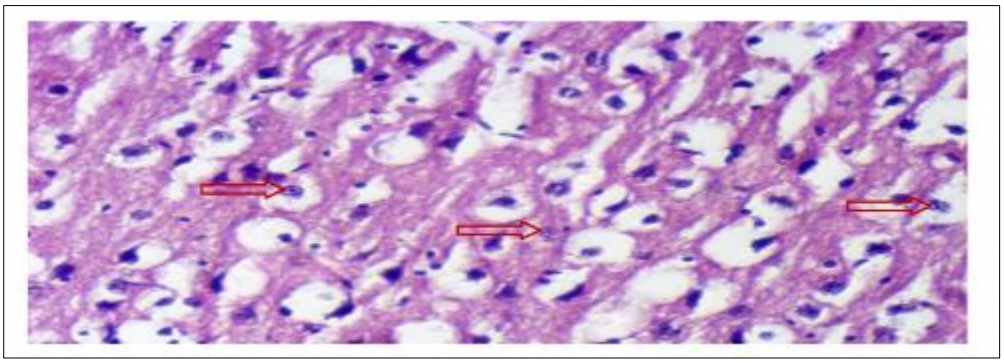


Figure 2 Photomicrograph of prelimbic part of the prefrontal cortex of Wistar rat in control group administered with distill water with preserved histoarchitectural of the neurons. *Mag:* x400

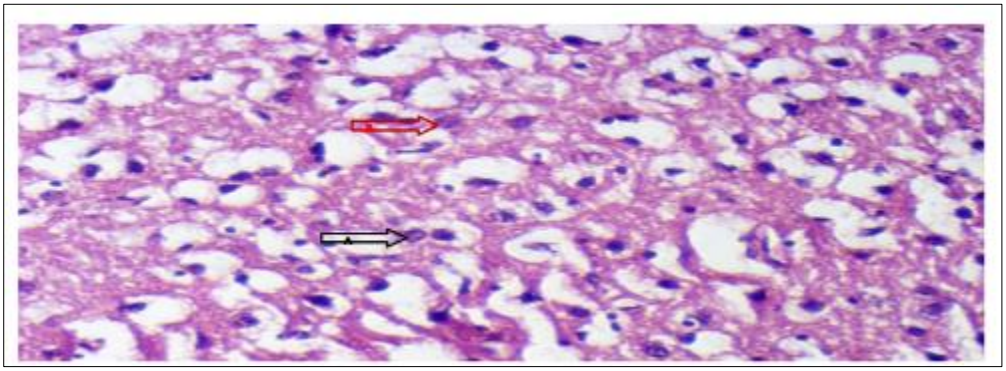


Figure 3 Section of prelimbic part of the prefrontal cortex of Wistar rat in control group administered with 5 mg/kg body weight with karyorrhexis (B) and pyknosis (A). *Mag:* x400

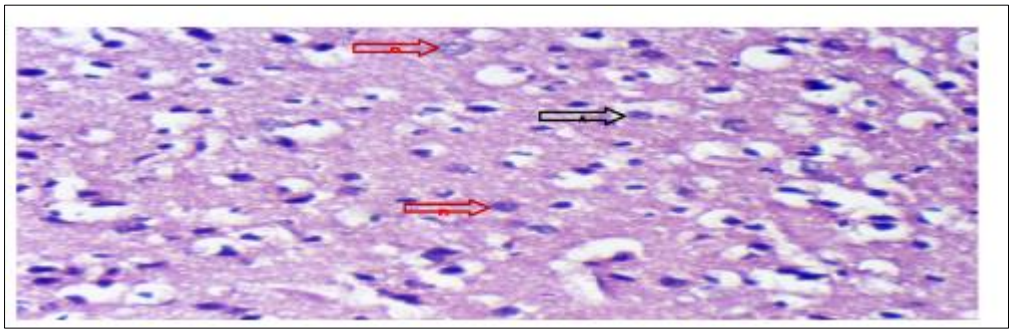


Figure 4 Section of prelimbic part of the prefrontal cortex of Wistar rat in control group administered with Silimarin 140mg/kg + Mercury Chloride 5 mg/kg mg/kg body weight with karyolyis (B) and pyknosis (A). Mag: x400

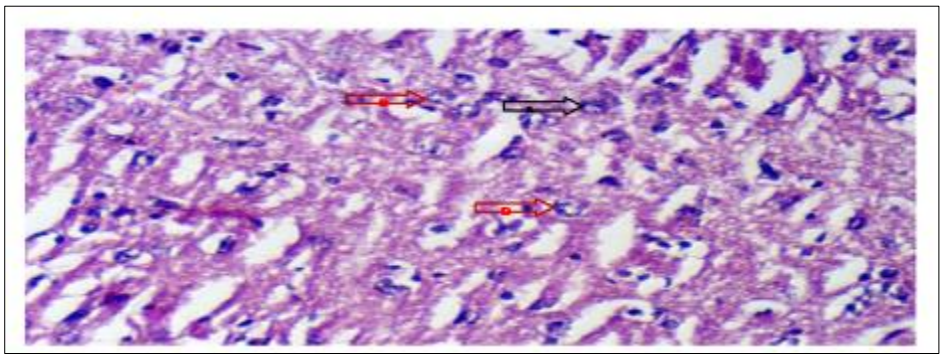


Figure 5 Section of prelimbic part of the prefrontal cortex of Wistar rat in control group administered with Citrus sinensis L. peel Extract 1500 mg/kg + Mercury Chloride 5 mg/kg body weight with karyorrehexis and vacuolation (B), and pyknosis (A). Mag: x400

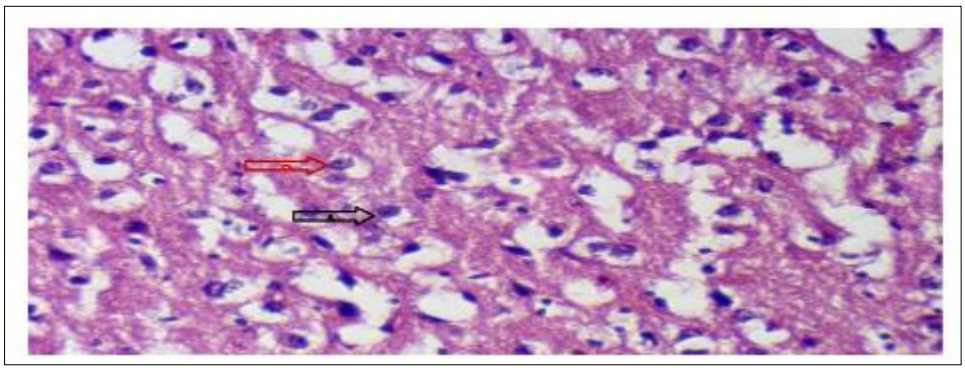


Figure 6 Section of prelimbic part of the prefrontal cortex of Wistar rat in control group administered with distill water with preserved histoarchitectural of the neurons (B) and pyknosis (A). Mag: x400

MDA level increased significantly (p<0.05) in the $HgCl_2$-treated group when compared to the control group, similarly in the 1000 mg/kg *Citrus sinensi* group when compared to the control group, which is indicative of lipid peroxidation. Lower (p>0.05) level of MDA was observed in the silymarin + $HgCl_2$ and 1500 *Citrus sinensi* mg/kg group when compared to $HgCl_2$-treated group.

- SOD results revealed an increase (p>0.05) in the extract treated groups when compared to the $HgCl_2$-treated group.
- CAT results revealed a decrease (p>0.05) in all treatment groups when compared to the control group.

4. Discussion

Heavy metals have harmful effects on human health, and exposure to these metals has been increased by industrial and anthropogenic activities and modern industrialization. Contamination of water and air by toxic metals is an environmental concern and hundreds of millions of people are being affected around the world. Food contamination with heavy metals is another concern for human and animal health. Concentration of heavy metals in water resources, air, and food is assessed with this regard (Mousavi *et al.*, 2013). Metals among the other environmental pollutants may also occur naturally and remain in the environment. Hence, human exposure to metals is inevitable, and some studies have reported gender differences in the toxicity of metals (Balali- Mood *et al.*, 2021).

An important requirement in toxicological experiments is the ability to assess the effects of xenobiotics on specific organs. For many organs, this is done through macroscopic examination of the organs, measuring organ weight, and histopathologic examination of the tissue. Organ weight can be the most sensitive indicator of an effect of an experimental compound, as significant differences in organ weight between treated and untreated (control) animals may occur in the absence of any morphological changes (Georgieff *et al.*, 2015). The comparison of the organ weights of treated animals with untreated animals is often complicated by differences in body weights between groups. Therefore, other parameters that are commonly used for analysis of organ weight are the ratio of the organ weight to body weight. The results obtained in this study showed no differences in the brain-body weight ratio of the experimental animals, this reveals that the administration of orange peel extract followed by mercuric chloride have no significant effect onthe organ-body weight ratio. This finding is similar to the report by Fiati Kenston *et al. (*2018) who reported that administration of heavy metals such as lead have no significant effect on the organ-body weight of several organs including brain.

Brain tissues are more liable to oxidative damage because of high concentration of polyunsaturated fatty acids that are predominantly susceptible to lipid peroxidation that plays a key role in necrosis and cell death (Arora, M., & Kaur, P. 2013). The results obtained in the study revealed an increase in the level of malonaldehyde (MDA) in the Wistar rats of all the groups administered with mercuric chloride groups when compared to the control group. This is in agreement with the work where it was reported that, methyl mercury administration reduced non-enzymatic and enzymatic antioxidants such as SOD and GSP, that caused lipid, protein, and DNA oxidative damage and enhanced neurocyte apoptosis in cerebral cortex (Liu *et al*., 2013). Additionally, the damage of cerebral neurons may occur by singlet oxygen, mostly due to oxidation of essential amino acids. Furthermore, they found that heavy metals restrict lipid metabolism in the brain of animals and affect the metabolism of cholesterol, total lipids and triglycerides leading to imbalance between the lipids synthesis and breakdown (Apostoli, 2006). Mercuric chloride is believed to generate ROS in tissues

and increasing MDA levels which indicating oxidation of unsaturated fatty acids in the brain with subsequent alteration in the anti-oxidative defence system both in experimental animals and humans . In a separate study which investigated the ameliorative effect of melatonin on mercuric chloride-induced neurotoxicity in rats concluded that mercuric chloride in both low and high doses increased significantly the oxidative stress by decreasing antioxidants including SOD, catalase and GSP enzymes and increased MDA compared to control group (Aryal *et al*., 2020).

5. Conclusion

This study has demonstrated

- Increase in the level of MDA and decrease in the activities of catalase enzymes in the brain of Wistar rat following mercuric chloride administration but these effects are protected against by co-administration with ethanol peel extract of *Citrus sinensis*
- Histoarchitecture distortion including neurosis, vacuolation, pyknosis and karyorrhexis in the pre-limbic part of the rat's prefrontal cortex following mercuric chloride administration which was ameliorated to some extent by co-administration with ethanol peel extract of *Citrus sinensis.*

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The Ethical Review Committee on animal studies, Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria Nigeria, approved the study and assigned approval Number.

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