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In-silico analysis of ethanol peel extract of *Citrus sinensis* on mercury chloride-induced prefrontal cortex damage in Wistar rats

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Abstract

This 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 chemical structure of sixteen compounds from *Citrus sinensis* peel obtained from GC-MS analysis reported in the literature were retrieved from the PubChem compound database and the crystal structure of human peroxisome proliferator- activated receptor gamma (PPARγ; PDB:ID: 2P4Y) reported to increase the expression of catalase gene, human extracellular copper-zinc superoxide dismutase enzyme (PDB ID: 2JLP) and mammalian 15-lipoxygenase - 1 enzyme (PDB ID: 2P0M) responsible for the production of lipid peroxidation were retrieved from the PDB database. Ligands and protein preparation, docking and MM-GBSA relative binding free energy calculation were done in Maestro 2023.1. Data obtained was analyzed using the IBM SPSS (*version 25)* using one-way ANOVA and *p*-value < 0.05 was considered statistically significant. 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 difference was observed in SOD activity. The CAT activities in control group (53.51±6.83 nmol/ml) was 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).The present study suggests, 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: Silymarin; Ketamine; Perillaldehyde; Lipid peroxidation; Malondialdehyde

1. Introduction

Mercury is a known toxic heavy metal, noted for inducing public health disasters in Niger State Nigeria (Sadeeq *et al.*, 2013). It exists in both organic and inorganic forms: which includes metallic mercury and mercury vapor (Hg^0) and mercurous ($Hg2^{++}$) or mercuric (Hg^{++}) salts; while organic mercury, which includes compounds in which mercury is bonded to a structure containing carbon atoms (methyl, ethyl, phenyl, or similar groups). The biological behavior, pharmacokinetics, and clinical significance of the various forms of mercury vary with chemical structure. There is some

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interconversion in vivo between the various forms of mercury. Inhaled elemental mercury vapor, for example, is easily absorbed through mucus membranes and the lung and rapidly oxidized to other forms (but not so quickly as to prevent considerable deposition of elemental mercury in the brain) (Bernhoft, 2012). Methyl mercury is easily absorbed through the gut and deposits in many tissues, but does not cross the blood-brain barrier as efficiently as elemental mercury (Clarkson and Magos, 2006).

Orange is the fruit of the citrus species (*Citrus sinensis*) in the family Rutaceae (Amber *et al.*, 2020). It is also called sweet orange, to distinguish it from the related *Citrus aurantium*, refer to as bitter orange. Citrus fruits are mainly used by juice processing industries while the peels are generally wasted in the industries. A very large amount of oranges byproduct wastes, such as peels which are formed every year. From waste materials, there is always an increased attention in bringing useful products and citrus wastes are no exceptions. Suitable methods have to be adopted to utilize orange peel and pulp for the conversion into value-added products (Arora & Kaur, 2013).

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).

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 (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 secondary metabolites especially PMFs in antimutagenic and antitumor properties has been reported (Lu *et al.*, 2020).

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 (LD_{50}) 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

2.4.1. 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.

2.5. Experimental Design

Mercuric chloride was purchased from Steve Moore chemicals Limited Samaru, Zaria. The oral LD_{50} 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% LD_{50} 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.

2.5.1. 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.

2.5.2. 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.

In-silico Analysis and Antioxidant System

2.6. Target and ligands retriever

The chemical structure of sixteen compounds from *Citrus sinensis* peel obtained from GC-MS analysis reported by Qiao *et al.* (2008), Cholke *et al.* (2017), and Uraku *et al.* (2020), with silymarin (as a standard drug) were retrieved in sdf format from the PubChem compound database and the crystal structure of human peroxisome proliferator-activated receptor gamma (PPARγ; PDB:ID: 2P4Y) reported to increase the expression of catalase gene (Okuno *et al.*, 2010), human extracellular copper-zinc superoxide dismutase enzyme (PDB ID: 2JLP) and mammalian 15- lipoxygenase - 1 enzyme (PDB ID: 2P0M) responsible for the production of lipid peroxidation (Armstrong *et al.*, 2016) was retrieved from the PDB database at www.rscb.org.

2.6.1. Protein and ligands preparation

The protein preparation was carried out using a protein preparation wizard in Maestro version 21.8. In the preprocess tab, bond order was assigned, hydrogens were added, zero-order bonds to metal and disulfide bonds were created and lastly "het states" using Epik at 7.0±1.0 pH was generated. Waters and other chains not involved in the binding were removed in the review and modified tab. The H-bond assignment was optimized and restrained minimization of the target was done using the OPLS4 force field.

The retrieved ligands and the standard drugs were prepared using LigPrep in Maestro, generate a possible state at the target was set at a pH of 7.0±1.0 while the setting was used in default.

2.6.2. Grid generation and molecular docking

Receptor grid generation in Glide was used to generate the grid for molecular using the co- crystalline ligand to define the binding site and excluding it from the grid generation with other settings used in default. Molecular docking analysis was carried out using Ligand Docking in Glide with default settings.

MM/GBSA binding- free energy calculation

The prime MMGBSA method (Prime Version 4.8) exhibited the relative binding-free energy

(ΔG bind) of each ligand molecule using the formula below:

$$\Delta G(\text{bind}) = \Delta G(\text{solv}) + \Delta E(\text{MM}) + \Delta G(\text{SA})$$

where:
Δ*G*solv = difference in GBSA solvation energy of the protein-ligand complex and the sum of the solvation energies for unliganded protein and ligand.

Δ*E*MM = difference in the minimized energies between protein-ligand complex and the sum of the energies of the unliganded protein and ligand.
Δ*G*SA = difference in surface area energies of the complex and the sum of the surface area energies for the unliganded protein and ligand.
Prime MM-GBSA calculates the energy of optimized free receptors, free ligand, and a complex of the ligand with a receptor. It also calculates the ligand strain energy by placing the ligand in a solution that was autogenerated by VSGB 2.0 suit. The prime energy visualizer presented the visualization of energy.

ADME (Absorption, Distribution, Metabolism, Excretion) properties prediction

Swiss ADME server is available at http://www.swissadme.ch/index.php, a service provided by the Swiss Institute of Bioinformatics was used to compute the physicochemical and pharmacokinetics properties, drug-likeness and medicinal chemistry friendliness of the ligands.

2.7. Data Analysis and Presentation

All data obtained was analyzed using the IBM SPSS (*version 25)* and the results were expressed as Mean ± SEM. A one-way analysis of variance (ANOVA) was used to compare across the group. LSD post hoc test was used where necessary and a *p*-value less than 0.05 was considered statistically significant.

3. Results

Table 1 Molecular Docking and MM/GBSA Score of Mammalian 15-Lipoxygenase - 1 Enzyme and *Citrus Sinensis* Peel Extract Ligands

S/N	Hit Ligands	Docking score (kcal/mol)	MM/GBSA ΔG Bind (kcal/mol)
1	Perillaldehyde	-4.365	-24.83
2	Limonene	-4.269	-22.22
3	D-Limonene	-4.269	-22.22
4	Nonanal	-1.619	-22.06
5	β-cis-Ocimene	-3.116	-21.8
6	γ-Terpinene	-5.454	-21.22
7	Hexahydronaphthalene	-5.565	-20.83
8	1,1,1,5-Tetrachloropentane	-4.112	-20.03
9	Myrcene	-2.485	-20.00
10	Linalool	-3.489	-19.75
11	Silymarin	-5.184	33.21

Table 2 Molecular Docking and MM/GBSA Score of Human Extracellular Copper-Zinc Superoxide Dismutase Enzyme and Ligands Present in Citrus sinensis peel

S/N	Hit Ligands	Docking score (kcal/mol)	MMGBSA ΔG Bind (kcal/mol)
1	3-Chloro-2-nitrobenzyl alcohol	-4.98	-21.41
2	α-terpineol	-3.451	-18.05
3	Germacrene D	-3.117	-14.59
4	Hexahydronaphthalene	-3.221	-14.51
5	Linalool	-2.105	-13.85

6	2-Pyridinecarboxaldehyde	-4.052	-13.76
7	Glutamic Acid	-3.543	-13.46
8	2,4-Decadienal	-0.777	-11.87
9	Perillaldehyde	-3.507	-10.45
10	Octanal	-0.224	-9.95
11	Silymarin	-	-

Table 3 Molecular Docking and MM/GBSA Score of Human Peroxisome Proliferator- Activated Receptor Gamma (Pparγ) and Ligands Present in Citrus Sinensis Peel Extract

S/N	Hit Ligands	Docking score (kcal/mol)	MMGBSA ΔG Bind (kcal/mol)
1	Silymarin	-8.85	-71.25
2	hexahydronaphthalene	-7.461	-44.42
3	delta-Amorphene	-7.365	-43.21
4	Citronellyl acetate	-2.594	-41.37
5	AKOS024319444	-5.229	-40.59
6	Dodecanal	0.521	-40.42
7	AKOS022101751	-6.182	-39.43
8	Linalool	-4.175	-38.96
9	Neryl acetate	-5.091	-38.94
10	2,4-Decadienal	-3.629	-38.86
11	Nootkatone	-5.272	-38.11

4. Discussion

The mechanism of heavy metals toxicity functions in similar pathways usually via reactive oxygen species (ROS) generation, enzyme inactivation, and suppression of the antioxidant defense. However, some of them cause toxicities in a particular pattern and bind selectively to specific macromolecules (Balali-Mood *et al.*, 2021). Moreover, increased mercury exposure has been associated with coronary heart disease, hypertension and carotid artery atherosclerosis due to formation of reactive oxygen species (ROS) which are responsible for production of oxidized low density lipoprotein with subsequent atherosclerosis(Asgary *et al.*, 2017).

The Oxidant/antioxidant imbalance is the major mechanism by which heavy metals including mercuric chloride induces its neurotoxicity. Superoxide dismutase; a key enzyme in the antioxidant system and plays very crucial role in inactivation of reactive oxygen species by dismutation of superoxide free radicals $O2^{-}$ (Fernández *et al*., 2016). In the present study, administration of mercuric chloride in all the treatment groups reveals no statistically significant changes in the activities of SOD. This observation is dissimilar to the findings of Liu *et al*. (2013); it reported significant inhibition of SOD enzymes following mercury administration. This disparity could be due to differences in dosage and duration of administration.

Similar to the present work, a previous study demonstrated that the neurotoxic effects of mercury chloride in rats were characterized by morphological changes for example neuronal loss, perivascular and pericellular edema with degeneration of astrocytes. These neuropathological changes accompanied by oxidative stress and increased lipid peroxidation which caused neuronal cell death (C. K., 2015).

5. Conclusion

Perillaldehyde as a ligand present in the ethanol peel extract of *Citrus sinensis* as a potential antagonist of mammalian 15-lipoxygenase - 1 enzyme to slow down the rate of MDA formation.

3-Chloro-2-nitrobenzyl alcohol compound as a potential human extracellular copper- zinc superoxide dismutase enzyme agonist to enhance the activities of SOD enzyme.

Silymarin as a potential agonist of human peroxisome proliferator- activated receptor gamma to up-regulate the activities of catalase enzymes.

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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