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Research Article
Amelioration of Lead-Induced Neurotoxicity by *Cucurbita pepo* (Pumpkin) Fruit Extract in Wister Rats

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Abstract

Background and Objective: Lead toxicity is one of the most significant heavy metal toxicities ravaging biotic life especially human and animals. This study aimed to investigate the ameliorative potential of *Cucurbita pepo* fruit against lead acetate-induced neurotoxicity in rats. **Materials and Methods:** Rats were orally administered lead acetate (100 mg kg^{-1} body weight) with or without the fruit extract (200 mg kg^{-1} body weight) or resveratrol (3.57mg kg^{-1} body weight) co-administration for 2 weeks. The activity of brain acetylcholinesterase (AchE) was significantly increased among lead acetate-induced animals but decreased significantly in animals administered with pumpkin extract and resveratrol. Similarly, a decreased in glutathione level (GSH) and increased level of hippocampal nitric oxide (NO) was observed in group 2. This was reversed in groups 3 and 4 administered pumpkin fruit extract and resveratrol respectively. **Results:** Molecular docking studies revealed mild inhibition of AchE and neuronal nitric oxide synthase (nNOS) by 2 compounds of the fruit and resveratrol through weak interaction. Para aminobenzoic and γ-aminobutyric acid have the highest binding affinity to AchE with docking score of -7.1 and -4.1 kcal mol^{-1}, respectively, while γ-aminobutyric acid exhibit higher binding affinity to nNOS than para aminobenzoic acid with docking scores of -3.5 and -2.8 kcal mol^{-1}, respectively. The levels of liver biomarkers increased significantly in group 2 but decreased in group 1, 3 and 4. Similarly, significant increase in urea, creatinine and Na^+ with a concomitant reduction in potassium and bicarbonate ions in group 2 compared to other groups were observed. **Conclusion:** The study revealed that administration of *C. pepo* fruit extracts has the potential to ameliorate lead acetate-induced neurotoxicity and better liver and kidneys functions.

Key words: Lead toxicity, molecular docking, nephrotoxicity, neurotoxicity, pumpkin fruit

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lead toxicity has been one of the most significant heavy metal toxicities, destroying lives (man and animals) due to its ubiquitous nature, through series of biochemical and morphological changes[1]. Lead binds albumin and get translocated to various organs and tissues, where it induces oxidative stress-chronic inflammation malicious cycle, which can lead to oxidative damage in organs and tissues[2]. Oxidative damage has been associated with cardiovascular, reproductive, neurological, cognitive, liver, kidney diseases among others[2,3].

According to Institute for Health Metrics and Evaluation (IHME) 2019, long term exposure to lead accounts for 900 thousand deaths and 21.7 million years of disability-adjusted life years (DALYS), with the highest burden being in low and middle-income countries[4]. Statistically, 62.5% of the global burden of developmental intellectual disability, 8.2% of hypertensive heart disease, 7.2 % of ischemic heart disease and 5.65% of stroke has been attributed to lead toxicity[4].

At present, treatment of lead-induced neurotoxicity focuses principally on chelation therapy, which employs the administration of chelation agents such as meso-2,3-dimercaptosuccinic acid and ethylenediaminetetraacetic acid, that bind to facilitate the excretion of lead. Although, this intervention effectively diminishes lead level in circulation, it has proven to demonstrate no improvement in neuropsychological function for children[5]. Hence, new and better treatment is required.

Recently, plants-derived constituents have been shown to detoxify or ameliorate diseases caused by oxidative damage due to xenobiotics, including heavy metals toxicities. The utilization of medicinal plants and plant-derived products for therapeutic purpose is rapidly increasing and gaining attention globally because they exhibit little or no safety concern and their bio-friendliness compared to synthetic medications. *Cucurbita pepo* (Pumpkin) is one of the highly nutritionally, important fruit-bearing vegetable belonging to the family *Cucurbitaceae*, with a reservoir of variety of nutrients and bioactive compounds such as unsaturated fatty acids, micro and macro minerals, α and γ tocopherols, β-carotene, proteins (albumin and globulins), vitamins and phytosterols[6]. This study aimed to investigate the ameliorative potentials of *Cucurbita pepo* (pumkin) fruit against lead induced neurotoxicity in rats.

MATERIALS AND METHODS

Chemicals and kits: The chemicals used in this study include: Lead acetate, disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl) and ethanol 95% and isoflurane (manufactured by Fluka, Switzerland). All chemicals used were of analytical grade.

All kits used were obtained from Solarbio Life Science Limited, Beijing China. These include, reduced glutathione (GSH) Catalog Number: BC1175), Acetylcholinesterase (AchE) Assay Kit Catalog Number: BC2020, nitric oxide assay kit and kits for liver function and kidney function.

Animals: Twenty-four adults Wister albino male rats (250-265 g body weight) were obtained from National Veterinary Research Institute Vom, Jos, Plateau State, Nigeria.

Ethical approval was obtained from Ethical Committee for Animal Studies, Nasarawa state University, Keffi. The animals were handled in accordance with principles of Planning Research and Experimental procedures on Animals: The rats were housed in the animal's house of the Department of Biochemistry and Molecular Biology, Nasarawa State University, Keffi. They were maintained on standard rat vital feed and water which were made available *ad libitium* for 14 days. The ambient temperature was between 28-30°C, humidity of 55±5 %, approximately 12 hrs of light (06:30 to 18:30 hrs) and 12 hrs of darkness (18:30 to 06:30 hrs) to acclimatize the laboratory condition prior to the commencement of the research work.

Collection and extraction of pumpkin fruit: Pumpkin fruits were obtained from a farmland in Nasarawa Local Government Area, Nasarawa State, Nigeria. The plant was authenticated by Prof. Onovo J. at the Department of Plant Science and Biotechnology, Nasarawa State University, Keffi. Voucher specimen was deposited at the Nasarawa State University herbarium. The Pumpkin fruits were then washed, pilled, cut into pieces and allowed to air-dry for 5 days. Thereafter, converted into powder and preserved in a refrigerator at -4°C in plastic container. The preserved powder was used when required by suspending it in a distilled water to prepare fresh stock solutions of 0.1 and 0.5 mg mL^{-1}. The volume of the solution that corresponds with the dose to be administered to each animal based on animal weight was obtained using the equation:

$$\text{Volume (mL)} = \frac{\text{Dosage (mg kg}^{-1}\text{) body weight in kg}}{\text{Concentration (mg mL}^{-1}\text{)}}$$

Experimental design: After the acclimatization, the animals were weighed and divided into 4 groups with 6 animals each per group.

- **Group 1:** Control-received feed and water throughout the period of 2 weeks
- **Group 2:** Received Pb salt in water only 100 mg kg^{-1} body weight once a day and haphazardly for the period of 2 weeks
- **Group 3:** Received Pb salt in water 100 mg kg^{-1} and *C. pepo* extract 500 mg kg^{-1} body weight twice a day for the period of 2 weeks
- **Group 4:** Received Pb salt in water 100 mg kg^{-1} and Resveratrol supplement extract 3.57 mg kg^{-1} body weight twice a day for the period of 2 weeks

The treatments were administered orally and with the aid of a feeding tube and all the animals were allowed access to feed and water *ad libitum* throughout the treatment period.

Collection of blood sample: After the administration for 2 weeks, the rats were anaesthetized with diethyl ether and blood sample was collected with the aid of capillary tube via an ocular vein puncture into plain bottle for biochemical analysis.

Biochemical analysis: Standardized diagnostic kits (Randox® by Randox laboratories ltd. United Kingdom) were used to determine liver and kidney function parameters according to modified conventional methods as described by the kit's manufacturer[7].

Determination of nitric oxide level: Nitric oxide formation was measured in hippocampal homogenate by assaying nitrite spectrophotometrically using the Griss reagent [1% sulfanilamide in 5% phosphoric acid (sulfanilamide solution) and 0.1% N-1-naphthylenediamine dihydrochloride in bidistilled water (NED solution)], as described previously[8].

Determination of acetylcholinesterase (AchE) activity: AchE catalyzes Ach hydrolysis to generate choline and choline reacts with 5,5-dithiobis(2-nitrobenzoic acid) to form 5-mercapto nitrobenzoic acid (TNB) which absorbs at 412 nm. AchE activity was expressed as U g^{-1} of brain tissue as described by the kit manufacturers[9].

Determination of total reduced glutathione (GSH): Glutathione (GSH) is a natural three peptide containing sulfhydryl (SH). It is made up of glutamic acid, cysteine and glycine, Glutathione reacts with 5,5'-dithiobis-2-nitrobenoic acid (DTNB) to give a yellow product that absorbs maximum ally at 412 nm. The amount of GSH was determined as GSH mg dL^{-1} of blood[10].

Molecular docking

Collection and preparation of ligands and proteins: Chemical compositions of *C. pepo* fruit characterized using GC-MS were obtained according to the method described by Badr *et al.*[11] and Berroukche *et al.*[12] 3-dimensional structures of the chemical compounds from *C. pepo* were retrieved in structure-data file (SDF) formats from PubChem database, minimized in PyRx virtual screening tool and converted to AutoDock ligands (pdbqt) for the docking analysis using Universal Force Field at 200 steps.

Crystal structure of acetylcholinesterase and neuronal nitric oxide synthase (nNOS) with PDB ID: 3LII and 4EUX respectively, were obtained from protein data bank. Cocrystallized ligand and interfering crystallographic water molecules complexed with the proteins were removed, chain B and A, respectively, of the proteins were minimized at 200 steepest decent steps at 0.02 Å with conjugate gradient steps of 10 at 0.02 Å and 10 update intervals using UCSF Chimera 1.14[13].

Active site determination and docking analysis: The active sites of the proteins were determined using Computed Atlas of Surface Topography of proteins (CASTp 3.0)[14]. Multiple docking of the phytochemical compounds (ligands) of the sample was performed using Autodock Vina in PyRx software according to Johnson *et al.*[15] by targeting the amino acids at the active site of the proteins. Interactions between amino acid residues of the proteins and compounds with the lowest docking score was visualized with UCSF Chimera 1.14 and Biovia Discovery Studio 20.1 and the 3D and 2D representation presented, respectively. Docking analysis was done using resveratrol as standard.

RESULTS

Table 1 shows the effects of pumpkin and Resveratrol on Brain AchE, GSH and NO against lead induced toxicity in rats. AchE activity significantly ($p<0.05$) increased in all groups (2, 3 and 4) compared to the control. However, groups 3 and 4 significantly revealed decreased AchE activities compared to group 2.

The brain reduced GSH concentrations were significantly ($p<0.05$) higher in groups 3 (32.87 ± 2.80 mg dL^{-1}) and 4 (33.52 ± 2.41 mg dL^{-1}) compared to 2 and non-significantly higher than group 1. The result of NO analysis revealed a significant elevation ($p<0.05$) across all the groups when compared to the control (group 1) but decreased in groups 3 and 4 (0.67 ± 0.80 and 0.44 ± 0.34 µmole mg^{-1}) significantly ($p<0.05$) compared to group 2 (1.53 ± 0.42 µmole mg^{-1}).

Table 1: Effects of pumpkin and Resveratrol on Brain Acetylcholinesterase, GSH and NO

Groups	AchE (UI L^{-1})	GSH (mg dL^{-1})	NO (µmole mg^{-1})
Control	0.07±0.02	31.20±1.78	0.32±0.24
Pb	0.71±13[a]	18.74±2.16[a]	1.53±0.42[a]
Pb+pumpkin	0.61±08[b]	32.87±2.80[b]	0.67±0.80[ab]
Pb+resveratrol	0.13±08[b]	33.52±2.41[b]	0.44±0.34[b]

The data represent Mean±SEM (n = 6), [a]Indicate a significant difference ($p<0.05$) compared to the control group, values with b are significantly different compared to group 2, Pb: lead acetate, AchE: Acetylcholinesterase, GSH: Reduced Glutathione, NO: Nitric Oxide

Table 2: Effects of pumpkin fruit and resveratrol on lead acetate-induced hepatotoxicity

Treatments	AST (U L^{-1})	ALT (U L^{-1})	Total protein (g dL^{-1})	Total bilirubin (mg dL^{-1})	Direct bilirubin (mg dL^{-1})
Control	10.95±1.29	5.14±0.99	30.38±0.66	6.75±2.03	10.91±0.93
Pb	19.89±1.29[a]	11.99±2.13[a]	72.64±5.10[a]	30.29±0.58[a]	17.47±0.93[a]
Pumpkin+Pb	14.87±2.19[b]	6.23±1.38[b]	37.09±16.19	12.76±2.30[ab]	13.57±2.26[ab]
Resveratrol+Pb	10.80±0.48[b]	4.75±0.97[b]	33.55±2.28	10.35±1.29[ab]	11.61±0.95[ab]

The data represent Mean±SD (n = 6). [a]Indicate a significant difference ($p<0.05$) compared to the control group, values with b are significantly different compared to group 2, Pb: Lead acetate, ALT: Alanine amino transferase, AST: Aspartate amino transferase

Table 3: Effects of pumpkin fruit and resveratrol on lead acetate-induced nephrotoxicity

Treatments	Urea (mg dL^{-1})	Creatinine (mg dL^{-1})	Na^+ (mmol L^{-1})	K^+ (mmol L^{-1})	HCO_3^- (mmol L^{-1})
Control	23.85±2.35	3.54±3.99	186.37±2.83	52.58±52.55	2.10±0.39
Pb	34.75±1.73[a]	4.69±0.82[a]	195.79±3.30[a]	9.40±1.034[a]	1.03±0.28[a]
Pumpkin+Pb	13.21±1.89	1.69±0.82[ab]	195.79±3.30[a]	11.24±2.82[ab]	0.47±0.18[a]
Resveratrol+Pb	14.60±2.76	1.38±.37[a,b]	184.21±2.13[b]	14.26±0.43[ab]	1.13±0.15

The data represent mean±SD (n = 6), a: indicate a significant difference ($p<0.05$) compared to the control group, values with b are significantly different compared to group 2, Pb: lead acetate

From Table 2, The levels of AST, ALT, TP, TB, DB were significantly ($p<0.05$) elevated across group 2 compared to other groups (3 and 4) and the control. Treatment with pumpkin and resveratrol significantly decreased the levels compared to the control.

Table 3 shows the effects of Pumpkin Fruit and Resveratrol on Nephrotoxicity. The concentration of urea and creatinine increased significantly ($p<0.05$) in groups 2 compared to 3, 4 and the control. However, the groups treated with pumpkin and resveratrol (3 and 4) exhibit a significant reduction ($p<0.05$) in level of serum urea and creatinine compared to lead acetate only in Group 1. Whereas Na^+ remain unchanged, K^+ decreased significantly ($p<0.05$) in group 2 compared to control and other groups (3 and 4). The level of bicarbonate decreased significantly ($p<0.05$) in all the groups compared to the control. However, group 3 showed significantly decreased ($p<0.05$) bicarbonates level compared to all the groups.

C. pepo fruit has been found to contain several bioactive compounds. Out of the 31 compounds identified from *C. pepo* fruit and docked with the enzymes AchE and nNOS, only 2 revealed the highest binding affinities. Based on the molecular docking studies, para aminobenzoic and γ-aminobutyric acid have the highest binding affinity to the active site of AchE with docking score of -7.1 and -4.1 kcal mol^{-1}, respectively (Table 4), while γ-aminobutyric acid exhibit higher binding affinity to nNOS active site than para aminobenzoic acid with docking scores of -3.5 and -2.8 kcal mol^{-1}, respectively. The result revealed that the two compounds exhibit lower binding affinity compared to resveratrol (control). The molecular docking studies showed a mild inhibition of the activity of AChE by the compounds paraaminobenzoic acid and γ-aminobutyric acid found in the fruit of *C. pepo* through weak interactions with the amino acids (Serine 203 and Histidine 447) at the active site of AChE (Fig. 1).

Table 4: Docking score of top compounds in *c. pepo* fruit with AchE and nNOS

	Binding affinity (kcal mol^{-1})	
Compound	AchE	nNOS
Para aminobenzoic acid	-7.1	-2.8
γ-aminobutyric acid	-4.1	-3.5
Resveratrol (control)	-6.9	-3.8

Furthermore, molecular docking results revealed that there was an interaction between para aminobenzoic and γ-aminobutyric acid of the fruit extract with the active site amino acid residues (Tyrosine 588) of nNOS via conventional hydrogen bond formation (Fig. 2).

DISCUSSION

Acetylcholinesterase (AchE) is one of the two cholinesterase enzymes mainly found at postsynaptic neuromuscular junctions and cholinergic brain synapses, that catalytically hydrolyses the neurotransmitter acetylcholine into acetic acid and choline. This is mainly responsible for the

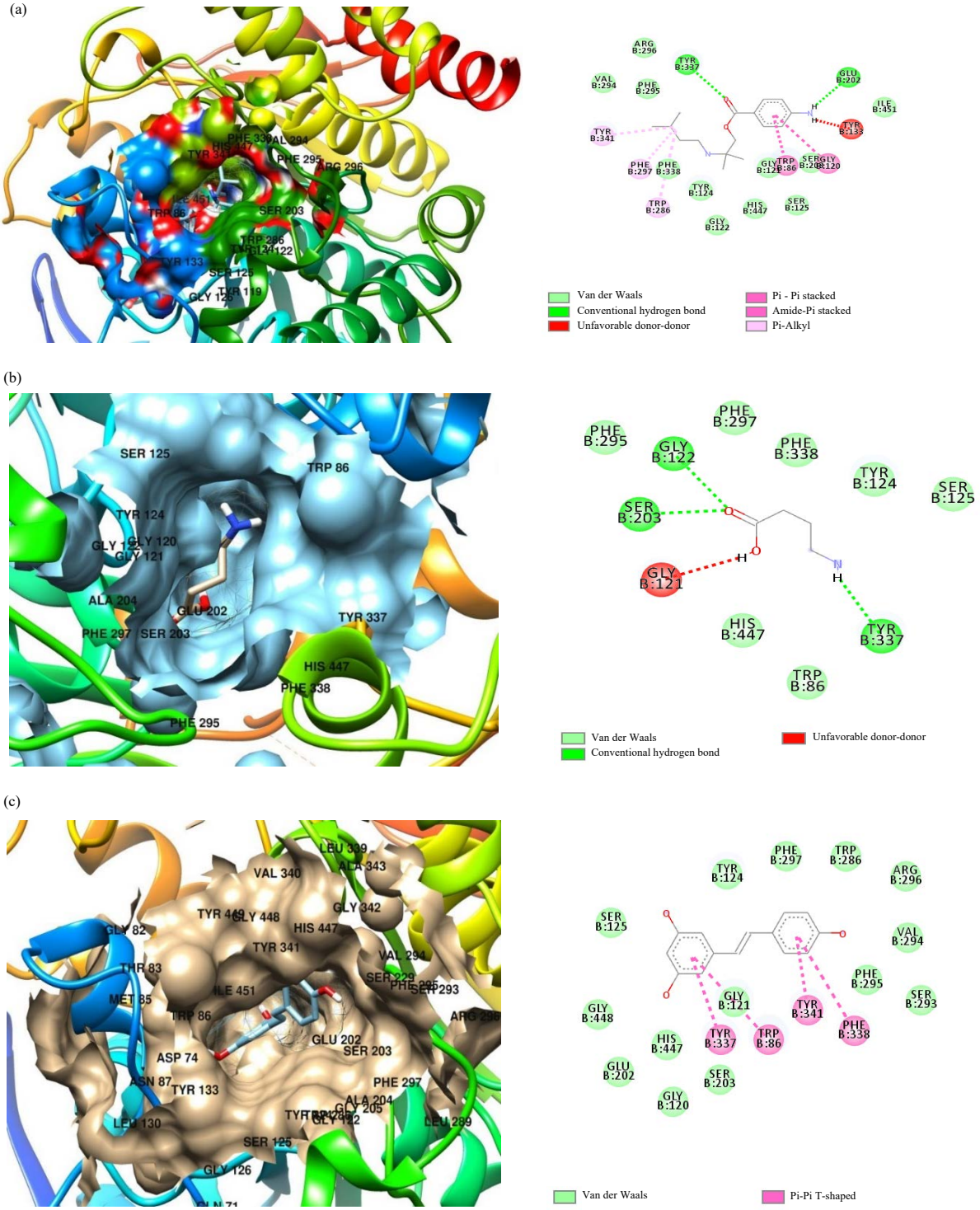

Fig. 1(a-c): 3D (left) and 2D (right) views of Molecular Interactions of (a) Para aminobenzoic acid, (b) γ-aminobutyric acid and (c) resveratrol with AchE

termination of the nerve impulse transmission at the cholinergic synapses. Continuous hydrolysis of acetylcholine can accelerate the aggregation of β-amyloid peptides leading to neurovegetative disorder[16]. Lead exposure has been

reported to induce neurotoxicity in children due to its high rate of absorption and increased penetration across the blood-brain barrier, leading to impairment in central nervous system function[17].

This study demonstrates a significant elevation in the activity of AchE enzyme induced by lead acetate exposure. Over activation of AchE has been observed in certain neurological condition such as Alzheimer's disease, causing significant diminish in acetylcholine, increases uptake of choline and decreases the proportion of nicotinic receptors, hence, leading to weakened neurotransmission, impairment of cognition and memory loss[18]. Treatment with C. *pepo* fruit extract and resveratrol ameliorated the hyperactivation of AchE induced by lead acetate.

The molecular docking studies showed a mild inhibition of the activity of AchE by the compounds para aminobenzoic acid and γ-aminobutyric acid found in fruit of *C. pepo* through weak interaction with the amino acids (Serine 203 and Histidine 447) at the active site of AchE (Fig. 1). This could have eventually lead to restoration of AchE activity to optimum level and prevent Alzheimer's like symptoms. Although, strong inhibition of AchE can result in cholinergic poisoning, however, weak inhibition of AchE has been shown to have relevance in the therapy of Alzheimer's disease, myasthenia gravis and glaucoma[19].

The present investigation shows a significant reduction in GSH level induced by lead acetate. However, this was restored by *C. pepo* fruit across the group treated with the fruit extract and resveratrol. This finding agrees to the previous studies where lead acetate caused decrease in brain GSH level of rats[20]. The mechanism of lead-induced reduction in GSH antioxidant system is via its ability to bind to the thiol group of GSH[21].

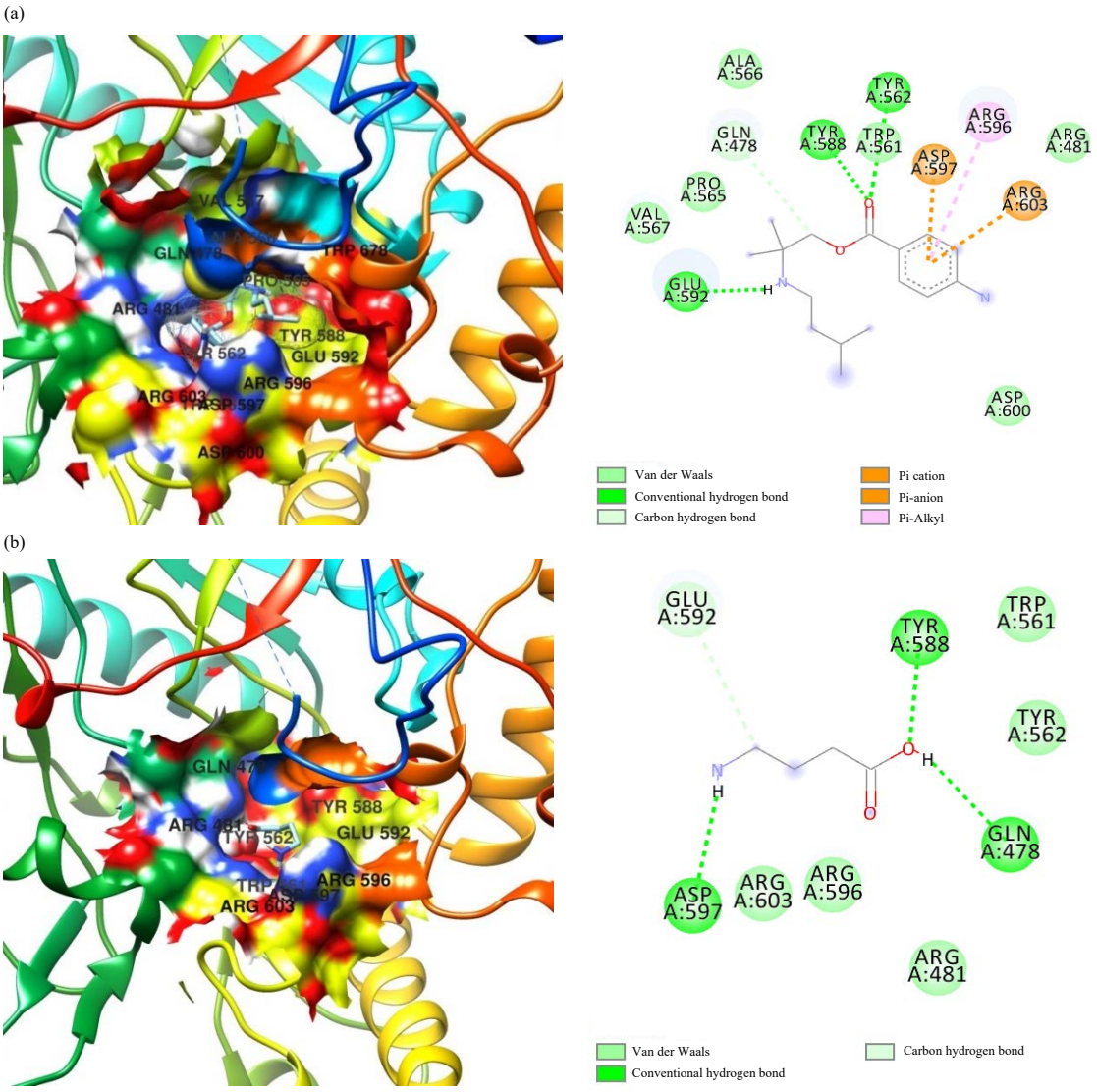

Fig. 2(a-c): Continue

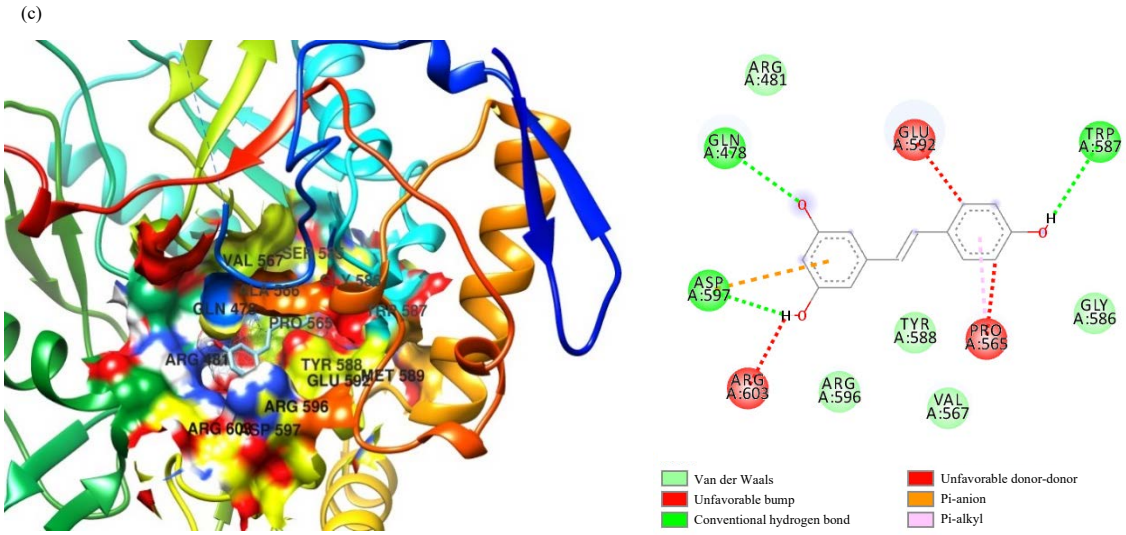

Fig. 2(a-c): 3D (left) and 2D (right) views of Molecular Interactions of (a) Para aminobenzoic acid, (b) γ-aminobutyric acid and (c) resveratrol with nNOS

The significant increase in NO concentration of hippocampal homogenate induced by lead acetate was bettered in the group administered *C. pepo* fruit extract. NO is an endogenous gaseous molecule synthesized by oxidation of the amino acid L-arginine through enzymatic action of nitric oxide synthases (NOS) isoforms, that function as a retrograde neurotransmitter in synapses and play a crucial role in intracellular signaling in nerve cells[22]. Low concentration of NO in the cell exhibit protective or regulatory effects while high concentration is toxic[23]. The reversal of increased hippocampal NO level by *C. pepo* in this study, could be through inhibition of nNOS activity. This is evidently demonstrated by the molecular docking studies; where para aminobenzoic acid and γ-aminobutyric acid of the fruit extract interact with active site amino acid residue (Tyrosine 588) of nNOS via conventional hydrogen bond formation (Fig. 2).

The elevation in the level of ALT, AST, urea, creatinine and Na^+, with a concomitant reduction in K^+ ion and bicarbonate levels has been reported in similar research[24]. Liver is the prime target of toxicants due to biotransformation, an autopsy study of humans exposed to lead revealed bioaccumulation of 33%, followed by kidney, then other soft tissues[25]. Elevation in liver enzymes could be attributed to lead induced cell lysis, leading to leakage of cytoplasmic enzymes into systemic circulation. In this study, administration of *C. pepo* fruit to lead induced rats showed a profound decrease in level of ALT, AST, urea, creatinine and Na^+. This collectively, indicate hepatoprotective and nephroprotective potentials of the extract and resveratrol. This could have been achieved through regeneration and/or maintenance of cellular and structural integrity of the damaged liver tissues.

CONCLUSION

This study revealed that *C. pepo* fruit extracts and resveratrol can ameliorate lead acetate-induced toxicity by normalizing the levels of AChE, GSH and nNOS which correlate positively with the docking studies. The docking studies showed moderate inhibition of AChE and nNOS by two compounds of the fruit.

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