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Research article

Neuroprotective Potential of *Citrullus lanatus* Seed Extract and Vitamin E Against Mercury Chloride Intoxication in Male Rat Brain

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ABSTRACT

Mercury chloride toxicity continues to be relevant in the advent of increased interest in mining activity in Nigeria. The neuroprotective potential of Citrullus lanatus seed extract (CLSE) (Watermelon seed) and vitamin E (VIT E) on mercury chloride intoxication on the frontal cerebral cortex of male rats was investigated. Forty two (42) male rats were randomized into six groups of 7 rats each. Group 1: control group received food and water; Group 2: received CLSE (200 mg/kg); Group 3: received VIT E (500 mg/kg); Group 4: received HgCl2 (4 mg/kg); Group 5: received HgCl2 (4 mg/kg) + VIT E (500 mg/kg) and Group 6: received HgCl2 (4 mg/kg) + CLSE (200 mg/kg). Treatment lasted 14 days and on 15th day of the experiment, gross morphometric, behavioural tests and brain tissue processing using paraffin wax technique were done. While gross body and brain morphometric evaluations were not significantly different, behavioural studies show that CLSE and VIT E significantly ($p<0.05$) increased the number of lines crossed relative to control. Histology showed that HgCl2 caused degeneration of neurons of the frontal cerebral cortex when compared with the control. Co-treatment of HgCl2 with CLSE and VIT E showed histological features of protection of cerebral neurons from mercury damage. CLSE and VIT E mitigated HgCl2–induced degeneration of frontal cerebral cortical neurons thus demonstrating their neuropotential capacity to protect cerebral cortex neurons from mercury toxicity..

Keywords: *Citrullus lanatus extract, mercuric chloride, Vitamin E, rat frontal cortex, cortical*

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INTRODUCTION

Mercury is one of the heavy metal pollutants present in the environment due in part to industrial activities like increased mining, high rate of fossil fuel burning and wide spread of raw materials containing mercury. Most human exposure to mercury is caused by outgassing of mercury from dental amalgam, ingestion of contaminated fish, occupational exposure and coal burning among others (Boylan *et al*., 2003). Atmospheric elemental mercury settles in water, where it is converted by microorganisms into organic (methyl or ethyl) mercury, which is ingested by smaller creatures which are eventually consumed by larger fish and it is known that fish at the top of the food chain (e.g., tuna, swordfish, or shark) may concentrate considerable mercury in their tissues (Burger *et al*., 2011). The chief target organ of mercury vapor is the brain, but peripheral nerve function, renal function, immune function, endocrine and muscle function, and several types of dermatitis have been described. In particular, mercuric chloride has been reported to damage rats' cerebral cortex (Owoeye and Farombi, 2015; Owoeye and Arinola, 2017). The mechanism of mercury toxicity has been associated with oxidative stress (Abdel Moneim, 2015) and disruption of DNA repair (Crespo-Lopez *et al*., 2009) which may account for why it poisons cellular functions by altering the tertiary and quaternary structure of proteins and by binding with sulfhydryl and selenohydryl groups. Compounds or substances with antioxidant activity will therefore be useful in ameliorating mercury toxicity. These may be synthetic or natural plant products, example of which is Citrullus lanatus. Citrullus lanatus (watermelon) belongs to the family of Cucurbitaceae and the fruit can be eaten raw, contains 6% sugar by weight and 92% water and helps in boosting

antioxidant level because it is exceptionally rich in carotenoids such as lycopene, lutein and B-carotene (Chandrika *et al*., 2009). It is also rich in magnesium, calcium, potassium, iron, phosphorus and zinc (El-Adawy and Taha, 2001). Watermelon is a rich natural source of lycopene, a carotenoid of great interest because of its antioxidant capacity and potential health benefits (Mandel *et al*., 2005) which might be due to its possession of phenolics and glycosides among others (Rahman *et al*., 2013). Erhirhie and Ekene (2013) reported the use of the seed as a demulcent, tonic, hypotensive and as treatment for urinary tract infections, while Varghese *et al*. (2013) reported it's hypoglycaemic and antioxidant effect. In Nigeria, Citrullus lanatus is known among the Yoruba as "Eso bara or Elegede"; among the Igbo as "Anyu", while the Hausas call it "Guna, Kankana or Shaman".

Vitamin E (□-tocopherol) is a liposoluble antioxidant that protects body tissue from damage caused by free radicals, which can harm cells, tissues, and organs. Vitamin E is also important in the formation of red blood cells and it helps the body use vitamin K, helps to widen blood vessels and prevents intravascular blood clotting. It is the primary membrane bound, lipid-soluble, chain-breaking antioxidant that protects cell membranes against lipid peroxidation (Bulger and Maier, 2003). Vitamin E treatment has been reported to be beneficial in: preventing formaldehyde-induced tissue damage in rats (Gulec *et al*. 2006); radiation-induced cerebellar injury in rats (Owoeye *et al*., 2011) and reduction of mercury-induced oxidative stress in rat lung (Celikoglu *et al*., 2015).

The frontal lobe of the brain is associated with executive and cognitive functions such as self-control, planning, reasoning, and abstract thought (Kandel *et al*., 2000; Kiernan, 2009; Bigos *et al*., 2015). Important nerve tracts associated with the frontal cortex include the corticospinal tract, corticomesencephalic tract, corticopontine tract and cortico-bulbar tract. The corticospinal tracts are involved in control of movement of muscles of the contralateral part of the body while corticobulbar tracts are involved in movement of muscles of the head and neck. Corticobulbar tracts are involved in swallowing, phonation, and movements of the tongue, however, all functions of the corticobulbar tract involve inputs from both sides of the brain (Afifi and Bergman, 2005).

In view of the important functions which the frontal cerebral cortex performs, the present study was designed, using the rat model, to investigate the possible protective effect of Citrullus lanatus seed extract (CLSE) against mercuric chloride-induced frontal cortex damage using vitamin E as a standard antioxidant. The outcome will enable us answer the research question on whether Citrullus lanatus seed extract can protect rat's cerebral cortex from mercuric chloride injury.

MATERIALS AND METHODS

Experimental Animals: Forty two adult male Wistar rats weighing between 130 g-150 g were procured and maintained in the Animal House of the College of Health Sciences, Bowen University, Iwo, Nigeria. They were housed in netted wooden cages having dimensions 43 cm × 40 cm × 29 cm and soft wood shavings employed as bedding at room temperature in a 12 hour light/dark cycle. They were allowed to acclimatize for a week before randomization into different experimental groups. They were fed with rodent pellet diet and water ad libitum within the duration of acclimatization. Animal experiments were done in accordance with the guidelines for use of research animals and all animals received humane care in accordance with the principle of humane care and use of laboratory animals (Public Health Service, 1996).

Plant materials: Dried Citrullus lanatus seeds were purchased from a local market in Jos, Nigeria. The authentication was done at the University of Ibadan herbarium (March, 2016) with the voucher number UIH-22504, and a voucher specimen was deposited. The dried Citrullus lanatus seeds were air dried and then pulverized, the powder was soaked in absolute n-hexane for 4 days, filtered with Whattman filter paper and concentrated using rotary evaporator to obtain a residue which after drying weighed 2.1 kg a percentage yield of 15%. The extract was termed Citrullus lanatus seeds extract (CLSE) and was dissolved in propylene glycol before administration. The acute oral toxicity studies carried out for n-hexane extracted Citrullus lanatus seed oil showed that the extract was safe up to a dose of 2,000 mg/kg body weight according to Madhavi *et al*. (2012). The dose used in this study, 200 mg/kg body weight, a tenth of the published safe dose was based on previous study (Omigie and Agoreyo, 2014).

Preparation and administration of mercuric chloride solution: Dry powder of Mercuric chloride (HgCl2, 99% purity) manufactured by Loba Cheme PVT Ltd, Mumbia, 40005, India, was purchased from Julimark Enterprises, Yemetu, Ibadan, Nigeria. Using a digital weighing balance, 100 mg of HgCl2 was measured and dissolved in 20 mL of distilled water, stirred thoroughly with a glass rod and then preserved in a specimen bottle for administration. From this stock solution, HgCl2 was administered to each animal as 4 mg/kg body weight.

Administration of vitamin E: Vitamin E (VIT E) 100 mg capsules were purchased from Adewole Medicine and Supermarket store, Ponkuku Area, Iwo, Nigeria with batch number G150466, S14C117, manufactured by Gujarat Liquid Pharmacaps Limited, Gujarat India. Each soft gelatin capsule containing 100 mg of DL- α-tocopherol acetate as 100 mg vitamin E acetate was punctured with a new size 21G needle (Hypojet, Spain) attached to a new 1 ml hypothermic syringe (Becton Dickinson, La Portde Clair, France). The oily formulation of vitamin E was then neatly and completely aspirated out with the syringe measuring approximately 0.2 ml containing 100 mg of DL-α-tocopherol. The insulin syringe was attached to a clean intra-gastric gavage through which each rat was administered orally the measured dose of 500 mg/kg.

Research design and animal grouping: The rats were randomized into 6 groups with a minimum of 6 rats in each group.

Group 1: control group given food and water only

Group 2: CLSE (200 mg/kg)

Group 3: VIT E (Vitamin E) (500 mg/kg)
Group 4: HgCl2 (4 mg/kg)
Group 5: HgCl2 (4 mg/kg) + VIT E (500 mg/kg)
Group 6: HgCl2 (4 mg/kg) + CLSE (200mg/kg)

All drugs were administered through oropharyngeal cannula and lasted 14 days. Dosages were based on published reports: CLSE (200 mg/kg, Omigie and Agoreyo, 2014); HgCl2 (4 mg/kg, Sheikh *et al*., 2013) and VIT E (500 mg/kg, Viana *et al*., 2003).

Behavioural tests: On the day 15, the animals were subjected to behavioural tests i.e. Open field test (Olopade *et al*., 2012). Open field test: A wide box approximately 120 cm by 120 cm with an open roof was used. The box painted white had lines drawn horizontally and vertically on its floor forming square grids. The animal was placed in the centre square quadrant and then left free to move around. The parameters examined included frequency of grooming, rearing and transitions (line crossing). Each animal was subjected to this test for a period of 5 minutes, after which the box was cleaned with 70% alcohol and dried before introduction of the next animal so as to avoid possible biasing effect due to odour clues left by previous rats.

Tissue extraction, processing, histology and histomorphometry: After completing the behavioural tests, the animals were monitored till the 15th day of experiment after which they were weighed and then euthanized by cervical dislocation. The brains were carefully dissected out, rinsed, blotted dry, weighed and then fixed in 10% formalin. The fixed brains were then processed with routine paraffin wax techniques. Serial sections of 5µm thickness were cut using a rotary microtome (Leitzer Wetzler, Germany) and sections were then stained using Haematoxylin and Eosin (H&E) according to published methods (Bancroft and Gamble, 2008). Images were acquired from the histological slides using an Olympus (Japan) microscope using Sony Cybershot DSC W610 camera at different magnifications. Applying a modification of the technique of Zhen and Doré (2007), we counted the number of non-viable (pyknotic eosinophilic neurons) pyramidal cells of the external pyramidal layer (EPL) of the frontal cerebral cortex under a light microscope of all the observed fields in each group at 40x objective lens with final magnifications of 768x.

Statistical analysis: All data were presented as means ± standard error of mean. Data were analyzed using one way analysis of variance (ANOVA) with Microsoft Office Excel 2011 and GraphPad Prism software version 5.01. Results were considered statistically significant when P-value was <0.05.

RESULTS

General observation: Rats that received HgCl2 only were weak in the first week but gradually regained strength. There was no significant difference in the final body weight and the brain weight ratio as shown in Table 1 and Figure 1. Although the weight differences were insignificant, animals in the control, CLSE and VIT E groups recorded higher values: 26 %, 24% and 20% respectively compared with HgCl2 treated groups viz: 15% and 13% in HgCl2 and HgCl2 + VIT E groups respectively. The only exemption was the HgCl2 + CLSE (24 %).

Behavioural tests evaluation: As shown in Figure 2, treatment with CLSE and VIT E significantly ($p<0.05$) increased line crossing and rearing, while VIT E significantly ($p<0.05$) increased grooming when compared with control group. However, treatment with $HgCl_2$ + CLSE significantly reduced the frequency of line crossing and grooming when compared with the $HgCl_2$ group

Table 1:
Effect of treatments on the weight of animals

Group	Initial weight (g)	Final weight (g)	Difference in weight (g)	% difference
Control	133 ±3.6	168 ±5.2	35	21
CLSE	140 ±2.4	174 ±7.1	34	21
VIT E	130 ±2.1	156 ±3.2	26	17
$HgCl_2$	137 ±2.1	157 ±5.6	20	14
$HgCl_2$+VIT E	140 ±2.0	158 ±3.6	18	13
$HgCl_2$+CLSE	136 ±4.8	164 ±5.2	32	18

Values were expressed as mean ± SEM (N= 6). CLSE = Citrullus lanatus seed extract, $HgCl_2$ = mercuric chloride, VIT E = Vitamin E.

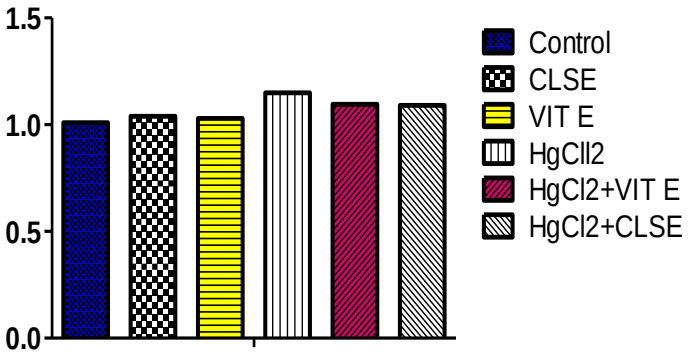

Figure 1:
The effects of treatments on the relative brain weight of rats. Values are presented as mean ± SEM (N= 6). The brain/body weight alterations were insignificant. CLSE = Citrullus lanatus seed extract, HgCl2 = mercuric chloride, VIT E = Vitamin E.

Histological examination of cerebral cortex
Shown in Figure 3 is the histology of external pyramidal layer of the frontal cerebral cortex of rats. The representative photomicrographs of the different groups show that some of the cortical neurons in the $HgCl_2$ group have degenerated with some exhibiting dark soma, hyperchromatic nuclei and some angulated while some show normal features (Figure 3D).

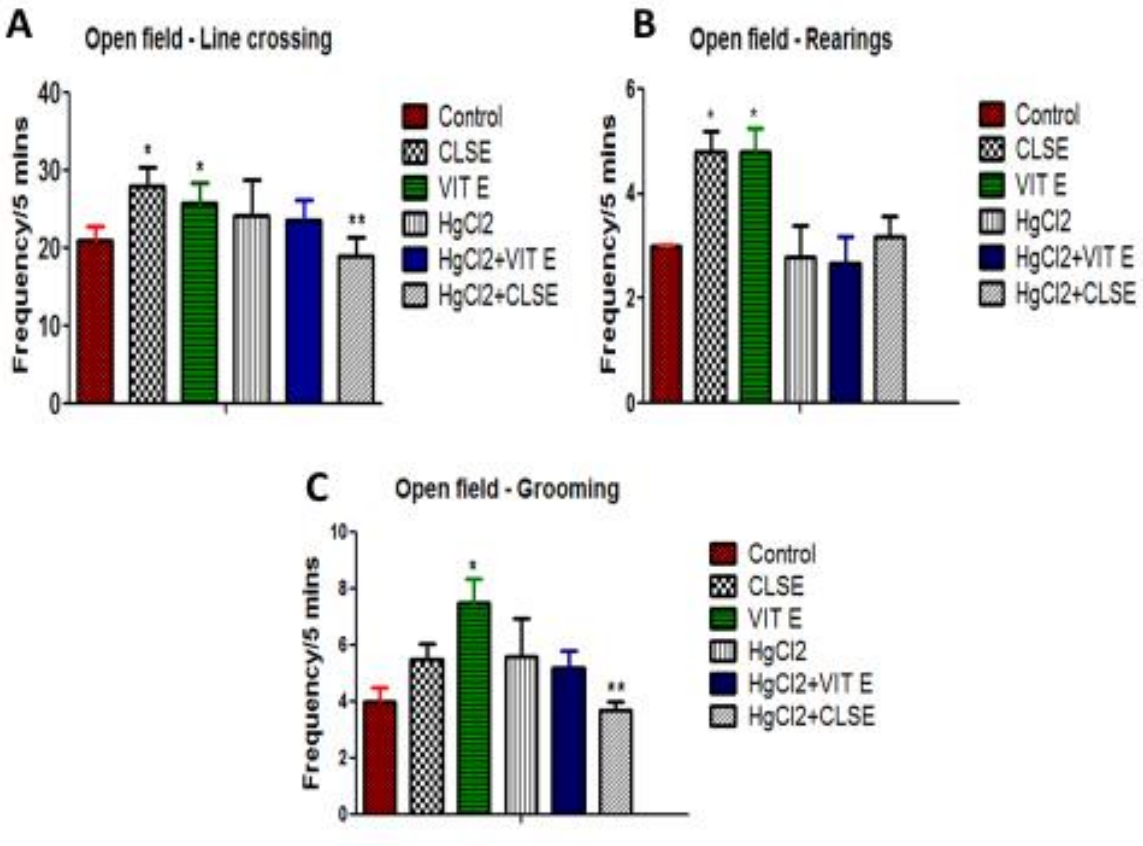

Figure 2:
Histogram of behavioural tests in the open field experiments in control and treated groups. (A) Horizontal movements (B) Vertical movements (rearings) (C) Grooming. Movements were significantly increased by CLSE and VIT E treatments compared control while treatment with $HgCl_2$+CLSE significantly reduced line crossing and grooming relative to $HgCl_2$ alone. Values are expressed as mean ± standard error of mean (N=6). CLSE = *Citrullus lanatus* seed extract, $HgCl_2$ = mercuric chloride, VIT E = Vitamin E. *P<0.05 against control, **P<0.05 against $HgCl_2$.

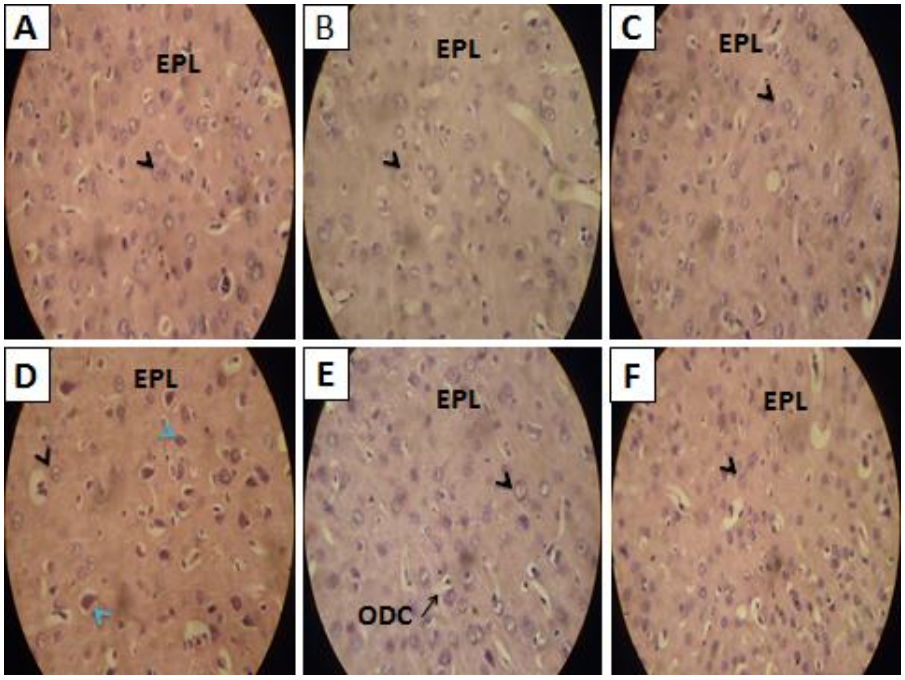

Figure 3:
Representative photomicrographs of the frontal cerebral cortex.
(A) Control, (B) CLSE, (C) Vit. E, (D) $HgCl_2$, (E) $HgCl_2$ + Vit E, (E) $HgCl_2$ + CLSE. CLSE = *Citrullus lanatus* seed extract, $HgCl_2$ = mercuric chloride, VIT E = Vitamin E, EPL= External pyramidal layer, Black arrowhead = normal neuron, Blue arrowhead = degenerated neuron, ODC= Oligodendrocyte. H & E – stained tissue sections. Final magnifications: 768x.

In contrast, the neurons of all other groups show clear soma with distinct margins with nucleoli distictively observed. Among the glia cells, oligodendrocytes with their typical "perinuclear halo" are visible

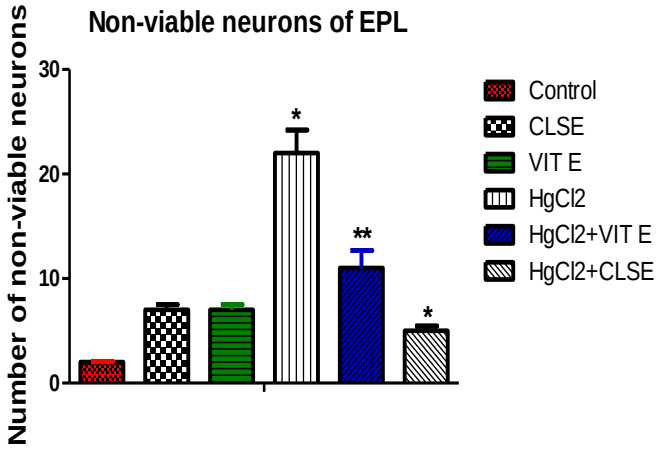

Figure 4: Effect of *Citrullus lanatus* seed extract and mercuric chloride on viability of pyramidal cells of the external pyramidal layer (EPL) of the frontal cerebral cortex.

Values are expressed as mean ± standard error of mean (N=6). The number of non-viable neurons of the EPL was counted under a light microscope. Pyknotic eosinophilic neurons indicated early neuronal damage. Neuronal damage was expressed as the mean number of pyknotic neurons in the EPL of all the observed fields in each group at 40x objective lens. The number of non-viable neurons in $HgCl_2$ was significantly higher than that of control, CLSE and VIT E groups. There were significant differences between $HgCl_2$+VIT E and $HgCl_2$+CLSE groups when compared with the $HgCl_2$ group. CLSE = *Citrullus lanatus* seed extract, $HgCl_2$ = mercuric chloride, Vit. E = Vitamin E. *P<0.05 against control, **P<0.05 against $HgCl_2$

Morphometric evaluation of neurons of the external pyramidal layer of frontal cerebral cortex

The non-viable neurons indicated by pyknotic eosinophilic neurons were counted. The number of non-viable neurons in HgCl2 was significantly higher ($p<0.05$) than that of control, CLSE and VIT E groups as shown in Figure 4. However, the values for HgCl2+VIT E and HgCl2+CLSE groups were significantly lower ($p<0.05$) when compared with the HgCl2 group**.**

DISCUSSION

This study investigated the potential neuroprotective effect of Citrullus lanatus seed extract (CLSE) and Vitamin E (VIT E) on mercuric chloride (HgCl2) intoxication in the brain of rats. Although there was greater body weight increases in control rats and those in the CLSE and VIT E groups than those treated with HgCl2, these were insignificant suggesting that despite its toxicity HgCl2 did not cause sufficient organ or tissue necrosis which might have led to significant reduction in the present study (Rossi *et al*., 2003). Similarly the absence of a significant reduction or increase in the relative brain body weight ratio along the groups suggested the absence of significant tissue inflammation among the surviving rats or possession of anti-inflammatory effect by CLSE and VIT E which agreed with the findings of Rossi *et al*. (2003) and Madhavi *et al*. (2012).

When administered alone, CLSE improved the number of line crossing, number of rearing and number of grooming all of which suggested an increase in the locomotive, exploratory and absence of anxiety in the rats in agreement with the reports of Olopade *et al*. (2012). Similarly, VIT E gave comparable results with CLSE by improving all these parameters. Interestingly, HgCl2 also improved exploratory activities suggesting that the rats overcame the initial weakness following mercury administration but reduced grooming suggested the occurrence of anxiety (Ajao and Akindele, 2013). The microanatomy of the frontal cortex of rats in the control, CLSE and VIT E groups were normal showing cortical neurons with distinct cellular outlines, large soma with large nuclei showing visible nucleoli and oligodendroglia cells surrounded by the characteristic "perinuclear halo" (Young *et al*., 2006). However, the histology of the frontal cortex of rats treated with HgCl2 demonstrated evidence of toxicity as shown by degenerating neurons (Figure 2). Some of these neurons were pyknotic while others were angulated which were evidences of onset of neuronal degeneration which more in the HgCl2 group quantitatively as shown in Figure 4 (Stevens and Lowe, 2001). The neuronal degeneration elicited by HgCl2 is in agreement which published reports that the cerebral cortex is often affected by mercury intoxication (Owoeye and Farombi, 2015; Owoeye and Arinola, 2017). The vulnerability of the central nervous (CNS) to mercuric chloride toxicity has been attributed to varying factors like oxidative stress due to free radical generation, neurotransmitter disruption and stimulation of the neural excitoxins, resulting in damage to many parts of the brain, its influence on DNA repair mechanisms and direct interaction with DNA molecules all of which may lead to genotoxicity (Crespo-López *et al*., 2009; Bernhoft, 2012). The ability of mercuric chloride to be converted to methyl mercury which can easily cross the blood brain barrier and accumulate in the brain at much higher concentrations also promotes neurotoxicity (Clarkson and Magos, 2006).

The consequence of degeneration of frontal cerebral cortex neurons will include the inability of the animal or human to perform executive functions such as self-control, planning, reasoning, attention, decision making, judgments, overall control of motor function and abstract thought (Kandel *et al*., 2000; Kiernan, 2009; Bigos *et al*., 2015). In rats, the acute implication would include reduction in locomotor and exploratory abilities buttressed by our results stating the HgCl2-treated rats were very weak initially until they later gained strength suggesting a form of recovery. There could also be upper motor neuron lesion manifestations since the corticospinal tract and some other important corticofugal projection fibres are associated with the frontal cortex (Afifi and Bergman, 2005), although we did not demonstrate these in these experiments.

That the histology of the cortex of HgCl2 + CLSE and HgCl2 + VIT E showed scanty degenerating neurons relative to the toxicant group (HgCl2) as shown in Figures 2E and 2F is

an evidence that CLSE and VIT E mitigated the damaging effect of HgCl2. This finding is supported by the fact that substances with antioxidant capabilities can neutralize or reduce the oxidative damage of the toxic effect of HgCl2. Both CLSE and VIT E have demonstrated antioxidant properties according to published reports (Mandel *et al*., 2005; Rahman *et al*., 2013; Bulger and Maier, 2003; Owoeye *et al*., 2011). The potency of CLSE might be supported by the report that, compared with other solvent extract, the hexane extract of *Citrulus lanatus* seed which was used in this experiment was the most powerful anti-oxidant extract (Rahman *et al*., 2013). There is no doubt that CLSE and VIT E have prevented frontal cortex neuronal damage due to their antioxidant abilities which mitigated the damage and hence prevented all the possible consequences associated with such lesions.

Taken together, HgCl2 caused histologically demonstrated damage to the neurons of the frontal cerebral cortex of rats. Co-treatment of HgCl2 with CLSE and VIT E demonstrated histological improvement of the neurons suggesting that they mitigated HgCl2 damage. Although it has been reported that changes in organ weight induced by toxicants is a reliable marker of toxicity (Elias and Nelson, 2012), the relative brain weight of rats in this study did not indicate the toxicity which the histology demonstrated.

In conclusion, results from this study showed that HgCl2 was toxic to the frontal cerebral neurons. However, CLSE and VIT E demonstrated neuroprotection by ameliorating the observed toxicity at the concentration at which both were administered. Since watermelon is readily available and affordable, it is suggested that it be further investigated so that workers exposed to HgCl2 may benefit from its neuroprotective capability.

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