Journal of Anatomical Sciences

Email:anatomicaljournal@gmail.com

J Anat Sci 12 (1)

Effect of Ethanolic Extract of *Psidium Guajava* Leaves on the Cerebellar cortex of Adult Male Wistar Rats Treated with Mercuric Chloride

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ABSTRACT

Mercury toxicity has been reported to cause distortion of gait and motor co-ordination due to deficit in the activity of cerebellum. Chelation therapy used in heavy metal toxicity is expensive and has been associated with adverse side effects. Therefore, the present study was aimed at evaluating the effect of ethanolic extract of *Psidium guajava* leaves on the Cerebellar cortex of adult male Wistar rats treated with Mercuric Chloride. Twenty adult Wistar rats were divided into four groups of five rats each. Group 1 served as control and received 1 ml/kg of distilled water, Group 2 received 41.5 mg/kg of Mercuric chloride and 1ml/kg of distilled water, Group 3 received 41.5 mg/kg of Mercuric chloride and 500mg/kg body weight of ethanolic extract of *Psidium guajava* leaves and Group 4 received 41.5 mg/kg of Mercuric chloride and 1000 mg/kg of ethanolic extract of *Psidium guajava* leaves. Motor coordination was evaluated in experimental animals using beam walking test. At the end of the experiment, brain tissues were carefully harvested and fixed in Bouin's fluid, processed and stained for histological studies. The result revealed a significant increase ($p<0.05$) in the meantime taken by the rats to cross the beam, neuronal degeneration and depletion of Nissl substance in the cerebellar cortex of Wistar rats exposed to Mercuric chloride when compared to the control and groups treated with ethanolic extract of *Psidium guajava* leaves. Ethanolic extract of *Psidium guajava* leaves reversed motor deficits, neurodegeneration and neurotoxicity induced by Mercuric Chloride in adult male Wistar rats.

Keywords: *Psidium guajava*, Mercuric Chloride, Cerebellar cortex, Motor co-ordination, Neurotoxicity.

INTRODUCTION

Humans are exposed frequently to ecological pollutants via numerous routes. While air pollution is a major route of human exposure to toxicants[1], exposure via ingestion of contaminated water or food also represents an important route of human exposure to toxins[2]. These pollutants include heavy metals and chemicals derived from natural processes like outflows from volcanoes and anthropogenic processes, particularly from coal-fires, residential heating systems and power stations[3]. Mercury one of these heavy metals is a highly toxic metal present in every facet of the environment inferable from its non-biodegradability and persistence in the soil. Several studies have reported the deleterious effect of mercury exposure in several organ systems with the central nervous system its principal target[4,5]. A wide scope of neurological disorders including autism spectrum disorders, Alzheimer's disease, Parkinson's disease, epilepsy, depression, mood disorders, tremor, memory impairment and motor deficit have been associated with exposure to Mercury[6,7]**.** Many other symptoms associated with prolonged exposure to inorganic mercury include; skin rash and dermatitis, mood swings, memory loss, mental disorders and muscle weakness [8].

Although adults and children are at risk of mercury exposure, studies however have shown that women who are pregnant and children most especially between 0-59 months are more vulnerable to mercury exposure[9]. Despite reports on the health hazard associated with exposure to mercury, its usage is still on the rise. In many countries today, skin lightening products containing mercury has been banned, notwithstanding, there are reports that this item are as yet publicized on the web and are accessible for buyers particularly in low and middle income countries[10].

Prolonged exposure to mercury during gold mining and consumption of contaminated fish and shell fish by inhabitants of coastal towns in riverine areas like Niger Delta has also increased the risk of mercury poisoning in Nigeria[11]. Therefore, it is apparent that mercury exposure remains a considerable occupational and public health problem. Current treatments available for treatment of mercury toxicity involve the use of chelation therapy using chelating agents. These chelating agents chelates mercury but do not treat tissue damage and disturbances in normal biological activities

instigated by mercury exposure[12]. These agents are also expensive to acquire, possess side effects and have been reported to chelate essential elements important for normal biological processes in the body [13,14] hence the need to look for natural remedies that are cheap, readily available and efficient.

Psidium guajava generally known as guava is an average sized tree native to tropical and subtropical countries[15]. Also, every part of this plant has been reported to possess a wide spectrum of biological properties such as; antibacterial,[16] antidiarrhoeal, anti-inflammatory[17] and antioxidant properties[18]. Some countries use the leaves as antibiotic in the form of decoction for injuries, ulcers and tooth aches, others use the extract from the leaves in management of diabetes, hypertension as well as gargle for sore throats, swelling of the mouth and laryngitis[19]. Phytochemical screening assay of the ethanolic extract of *Psidium guajava* revealed the presence of many rich antioxidants like flavonoid, tannins, alkaloids, saponins and steroid glycosides [20] and findings from toxicological studies in animal models and controlled human studies indicate the safety of the leaves and fruit without any side effects[21]. Due to the presence of antioxidants in *Psidium guajava* and its ability to elicit potential effect in the prevention of degenerative changes, the plant is receiving more attention from researchers[22]. Therefore, this study investigated if ethanolic extract of Psidium guajava leaves could reverse neurotoxicity induced by mercuric chloride in the cerebellar cortex of adult male Wistar rats

MATERIALS AND METHODS

Experimental Animals: Thirty-two (32) apparently healthy adult male Wistar rats (150-180g) used in the present study were obtained from Animal House of the Department of Human Anatomy, Ahmadu Bello University, Zaria-Nigeria. The rats were housed in clean cages with wood shavings as their beddings. They were fed with pelletized vital feed manufactured by Grand Cereals and Oil Mills, Plateau State, Nigeria and were allowed access to clean water throughout the experiment.

Acquisition of Plant Materials: Fresh leaves of *Psidium guajava* were gotten from the premises of the Post graduate school, Ahmadu Bello University, Zaria. The leaves were identified and authenticated with a voucher number 3253 in Department of Biological Science Herbarium, Ahmadu Bello University, Zaria.

Preparation of Plant Extract: The extraction of *Psidium guajava* leaves was carried out in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. in accordance to the method of maceration described[23] for the preparation of ethanol extracts. A sample of 1000g of guava leaves was placed in 1.5 L pure ethanol (purity 94.0%) for 4 days at room temperature. After 4 days, the solution was filtered using filter and funnel. The filtrate was then poured into the evaporating dishes and evaporated on the water bath (Digital thermostatic water bath Mc Donald Scientific international – 220volt, 50 Hz and 10A) at a temperature of 80^0C. With reference to the powdered sample, a yield of 14.2 % of the extracts was obtained. To obtain the testing sample, the solid mass was dissolved with distilled water. A final concentration of 500mg/kg body weight and 1000mg/kg body weight was used for the experiment.

Acute Toxicity Study: Acute toxicity study to determine the oral median lethal dose (LD_{50}) of ethanol extract of *P. guajava* leaves (EEPGL) was carried out according to the method of[24]. A total of 12 Wistar rats was used for this study. The study was conducted in two (2) phases. In the first phase, nine (9) Wistar rats were divided into three (3) groups with three (3) Wistar rats per group. In this phase, Wistar rats in each group were given ethanolic extract of *Psidium guajava* at doses of 10, 100 and 1000mg/kg bwt respectively. They were observed for 24 hours to monitor their behaviour as well as if mortality will occur. After twenty-four (24) hours observation, since no mortality was recorded, the second phase of the experiment was conducted. Three (3) Wistar rats were used for the second phase with one (1) Wistar rats in each group. Wistar rat in each group was administered higher doses (1600, 2900 and 5000mg/kg bwt) of ethanolic extract of *Psidium guajava* respectively. They were also observed for 24 hours for behavioural changes as well as mortality. The LD_{50} was calculated using the formula;

$LD_{50} = \sqrt{(D_0 \times D_{100})}$

Where,

D_{100} = highest dose that gave no mortality

D_0 = lowest dose that produced mortality

Chemical Substance: Mercuric Chloride manufactured by May and Bakers Chemical Laboratory Limited Dagenham England wit-N202 was obtained from Steve Moore Laboratory store, Samaru-Zaria, Kaduna State.

Experimental Protocol: Based on the LD_{50} of ethanol extract of *Psidium guajava* leaves for Wistar rats which is greater than 5000mg/kg body weight, doses of 10% (500mg bwt) and 20% (1000mg bwt) of the LD_{50} was used in the study. Earlier studies have established the LD_{50} of Mercuric Chloride to be 166 mg/kg body weight[25,26]. 25% of the LD_{50} (41.5mg/kg bwt) was administered in this study.

Experimental Design: The rats were separated randomly into four (4) groups of five (5) rats each. The animals in Group 1 received 1ml /kg distilled water from day 1 to day 42; Group 2 received 41.5mg/kg body weight of Mercuric chloride from day 1 to day 21 and 1ml/kg distilled water day from day 22 to day 42, Group 3 was administered with 41.5mg/kg body weight

of Mercuric Chloride from day 1 to day 21 and 500mg/kg body weight of ethanolic extract of *Psidium guajava* leaves from day 22 to day 42 and Group 4 received 41.5mg/kg body weight of Mercuric chloride from day 1 to day 21 and 1000mg/kg body weight of ethanolic extract of *Psidium guajava* leaves from day 22 to day 42. All administration were carried out orally.

Beam Walking Test: Motor coordination and balance was evaluated by measuring the ability of the experimental animals to traverse a narrow beam to reach an enclosed safety platform [27]. The test comprised of elevated platforms linked by a 100 cm long wooden beam with a width of 3 cm. The beam was placed horizontally, with one end mounted on a narrow support and the other end attached to an enclosed hamster box (20 x 20 x 20 cm), the beam-walking ability was measured using a 6-point scale. The rats were trained for seven (7) days on the beam walking apparatus before the onset of administration**.** To begin testing, the rats were lifted from the home cage at the tail, supported around the neck, and gently at the start point of the beam with their faces directed towards the goal box. Beam walking ability was recorded in seconds; the time in which the animal spent on the platform, the time spent to traverse the beam, the time taken (latency) until the animal entered the goal box (up to 60s) was recorded. The apparatus was cleaned with 70% alcohol after each trial and the rats were returned to their cages carefully.

Animals Sacrifice and Histology: After the last administration, the rats were humanely sacrificed under anaesthesia with ketamine at a dose of 75mg/kg IP[28]. The brain of the rats was assessed and excised by making a mid-sagittal incision through the skull. The excised brain was fixed in Bouin's fluid for a period of 48 hours for proper fixation. Afterwards, they were routinely processed and stained for histological studies at the Human Anatomy Department, Ahmadu Bello University, Zaria. After staining with H and E and Cresyl violet[29], microscopic slides were viewed under the microscope using X250 magnifications and photomicrographs were taken using MD900 Amscope® digital camera.

Data analysis: Data obtained from the study was analyzed using SPSS version 23 and was expressed as Mean ± SEM (Standard error of mean). One-way analysis of variance (ANOVA) was employed to compare the mean differences between experimental groups in the neurobehavioral assay. A value of $P<0.05$ was considered significant.

RESULTS

Acute Toxicity Study: No lethality was observed when ethanolic extract of *Psidium guajava* leaves was administered orally in Wistar rats even at doses as high as 5000mg/kg bwt. There was no sign of toxicity after 48 hours and 14 day observation in the experimental group except from weakness noticed in animals that took 5000mg/kg. The absence of death at 5000mg/kg bodyweight showed that the LD_{50} of the extract is greater than 5000mg/kg body weight.

Physical Observation: Wistar rats in Group I (Control) were very active with no visible physical changes observed. Rats in group 2 showed decreased physical activity, gnawing, restlessness and grooming, in contrast to rats in group 3 and group 4 which showed progressive improvement in physical activity.

Motor Co-ordination Assessment: The result of motor co-ordination using beam walking test revealed initial significant increase ($p<0.05$) in time taken to cross the beam in the second, third and fourth week in all experimental groups when compared to control. Significant increase ($p<0.05$) in time taken to cross the beam was also observed in the fifth and sixth week in Wistar rats in group 2 and 3 when compared with rats in control. However, this increase in time taken was insignificant ($p>0.05$) in the fifth and sixth week in Wistar rats in Group 4 when compared with control (Table 1)

Table 1: Effect of mercury chloride exposure, *Psidium guajava* leaves on motor coordination

	Training	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Group 1	15.02±3.20	4.90±1.33	4.89±0.89^{abc}	3.58±0.57^{cde}	3.66±0.69^{bcd}	3.81±0.73^{bc}	3.15±0.54^{bc}
Group 2	18.86±1.94	9.57±2.82	14.0±3.51^{a}	25.04±3.80^{c}	29.87±6.45^{b}	26.80±6.58^{b}	25.55±6.52^{b}
Group 3	28.34±4.04	13.94±0.87	19.60±0.34^{b}	28.43±0.91^{d}	29.11±0.98^{c}	21.28±2.78^{c}	16.76±2.04^{c}
Group 4	21.44±5.98	16.21±2.83	22.62±6.91^{c}	27.80±1.14^{e}	20.93±1.90^{d}	17.31±1.75	7.08±1.03
P	0.279	0.068	<0.001	<0.001	0.028	0.031	0.024

Values expressed as mean ± S.E.M. One way ANOVA followed by Tukey's post hoc test. Group 1 = (Distilled H2O), Group 2 = (41.5mg/kg HgCl2 + Distilled H2O), Group 3 = (41.5mg/kg HgCl2 + 500mg/kg bwt EEPGL), Group 4 = (41.5mg/kgHgCl2+1000mg/kg bwt EEPGL). Means in a column sharing superscripts are significantly different from one another. EEPGL – Ethanolic extract of *Psidium guajava*.

Histological Observation: Wistar rats in Group 1 orally administered distilled water throughout the experiment showed normal cytoarchitecture of the Purkinje cell layer of the Cerebellar cortex with the Purkinje cells appearing normal (Figure 1). The photomicrograph of rats in Group 2 administered Mercuric Chloride and distilled water showed distortion of the Purkinje cell layer with presence of degenerated Purkinje cells and cell loss (Figure 1). Rats in Group 3 administered Mercuric Chloride and ethanolic extract of *Psidium guajava* leaves at a dose of 500mg/kg bwt showed normal orientation of the layers in the Cerebellar cortex, normal Purkinje cells and evidence of degenerating cells (Figure 1) whereas the rats in Group 4 administered Mercuric Chloride and treated with ethanolic extract of *Psidium guajava* leaves a higher dose of 1000mg/kg bwt showed normal orientation of the layers of the Cerebellar cortex similar to what was observed in control (Figure 1). Result from Cresyl violet staining for Nissl substance for Wistar rats in Group 1 revealed intense staining for Nissl substance within the perikaryon of the Purkinje cells (Figure 2). A very weak staining intensity was observed in Wistar rats in Group 2 administered Mercuric chloride followed by treatment with distilled water (Figure 2). Rats in Group 3 administered Mercuric chloride followed by treatment with lower doses of the extract revealed better staining intensity when compared with rats in Group 2 (Figure 2). Staining intensity similar to what was observed in control was observed in Wistar rats in Group 4 administered Mercuric chloride followed by treatment with higher doses of the extract (Figure 2).

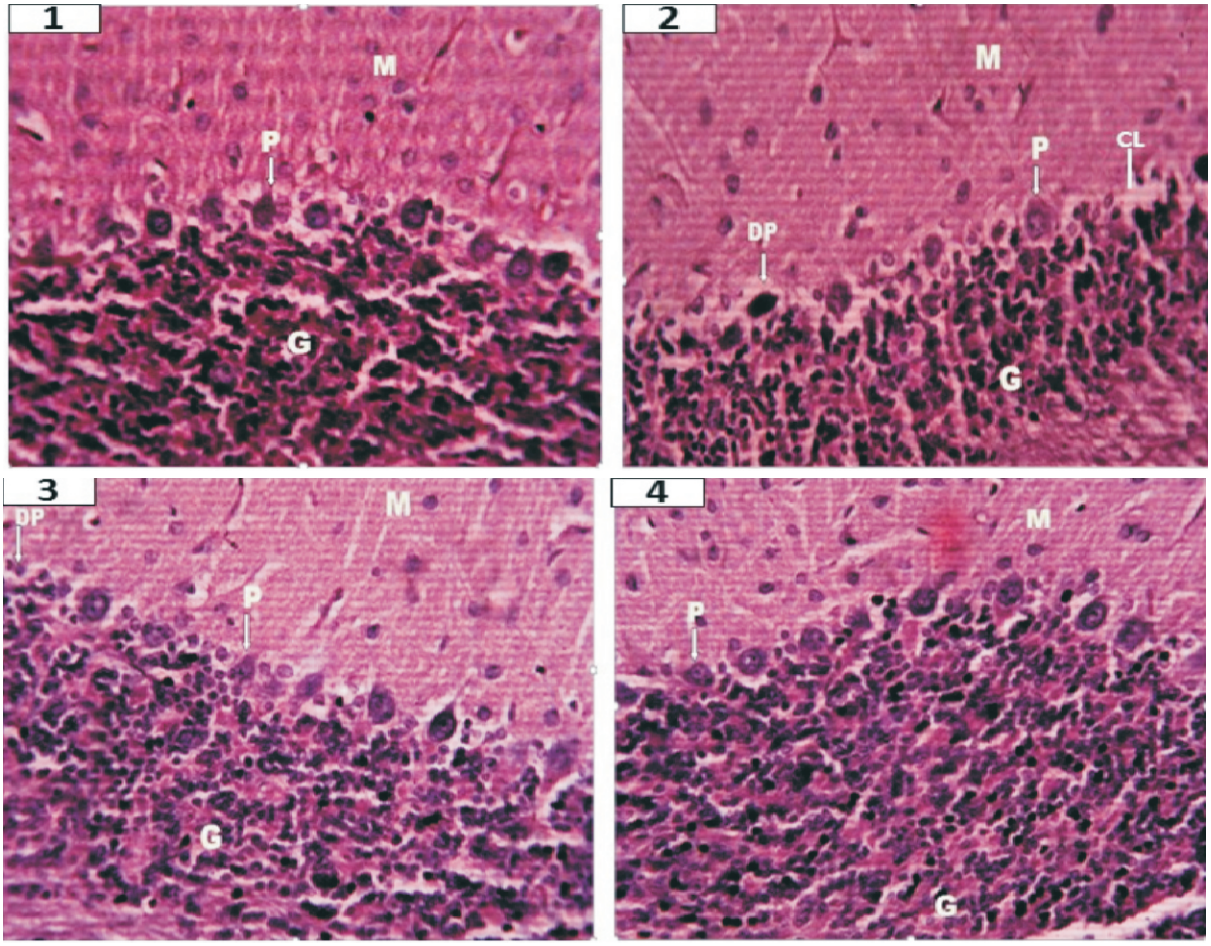

Figure 1: Photomicrograph of the cerebellar cortices of adult male Wistar rats (H and E Mag X250). Group 1 = (Distilled water), Group 2 = (41.5mg/kg $HgCl_2$ + distilled water), Group 3 = (41.5mg/kg $HgCl_2$ + 500mg/kg bwt EEPGL), Group 4= (41.5mg/kg $HgCl_2$+ 1000mg/kg bwt EEPGL). M – Molecular Layer, P – Purkinje Cell Layer, G – Granular Layer, CL - Cell loss, DP –Degenerating Purkinje Cells. EEPGL – Ethanolic extract of *Psidium guajava*

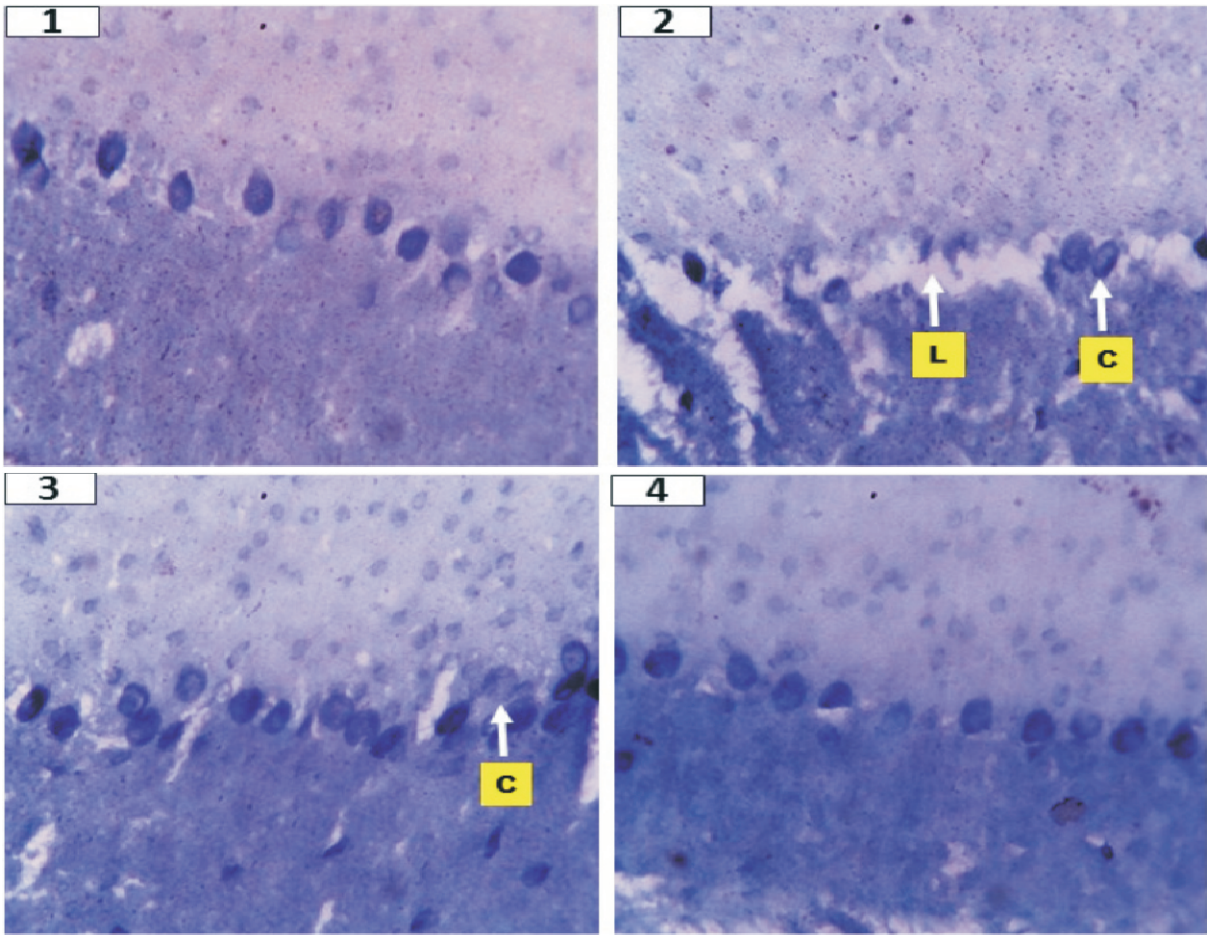

Figure 2: Photomicrograph of the cerebellar cortices of adult male Wistar rats (Cresyl violet Mag X250).). Group 1 = (Distilled water), Group 2 = (41.5mg/kg $HgCl_2$ + distilled water), Group 3 = (41.5mg/kg $HgCl_2$ + 500mg/kg bwt EEPGL), Group 4= (41.5mg/kg $HgCl_2$+ 1000mg/kg bwt EEPGL). C- Chromatolysis, L- Cell Loss. EEPGL – Ethanolic extract of *Psidium guajava*

DISCUSSION

The cerebellum is the major integrative center for the coordination of muscular activity, facilitation of movement and motor planning Serious injury to the cerebellar cortex can prompt numerous issues some of which include; ataxia, dysmetria and oculomotor impairment[30]. When toxins get into the cerebellar cortex, they target several cells one of which is the Purkinje cells that play a fundamental role in controlling motor movement. Abnormal functioning or impairment of the Purkinje cell has been reported as a principal contributor in several neurological diseases[31].

Beam walking test is used to evaluate motor functions in experimental animals. The longer the time taken by experimental animals to cross the beam in the beam walking test is suggestive of motor functions deficit[32]. In the present study, Wistar rats exposed to Mercuric chloride showed significant increase in time taken to cross the beam. This could be associated with motor impairment which may be as a result of neuronal degeneration, distortion in the general morphology of the Purkinje cells as observed in the result from the histological studies of this study. This is in agreement to the works of [33]and [7]who reported that exposure to mercury led to changes in the central nervous system leading to behavioral changes, tremors, incoordination, irritability, fatigue and in some cases even death. Better performance in crossing the beam was observed in groups treated with ethanolic extract of *Psidium guajava* as similar time taken was observed in groups treated with the highest dose of the extract when compared to the control in the second and third week of administration of the extract that on the fifth and sixth week of the experiment, indicative of the ameliorative ability of the extract. Improved motor co-ordination observed in the group treated with higher doses of the extract suggest that the extract is more potent at higher doses. The result observed could be due to the ability of the extract to scavenge for free radical as well as reverse the distortion in the cytoarchitecture elicited by mercury exposure. The action observed could also be due to synergism of quercetin a type of flavonoid with other vital component of the extract which has played a major role in protecting the tissues from injury[34]. A study by [35]and [36] indicated that plants rich in

antioxidants could help to reduce oxidative stress and to some extent protect tissues from damages.

Histopathological assessment is worthwhile in giving sufficient data about intense or constant impacts of poisonous substances that may not be distinguished by other biomarkers[37]. The results from the present study revealed distortion of the Purkinje cell layer, cell loss and neuronal degeneration in the Mercuric chloride exposed rats. The observed changes in the cerebellar cortex of rats exposed to Mercuric chloride could interfere with motor activity and many other important motor functions such as loss of fine movement, loss of grasping, maintenance of equilibrium and loss of regulation of muscle tone which are modulated by the spinal cord and brain stem mechanisms involved in postural control. Degeneration of the cells could be due to the ability of mercury to cause increase lipid peroxidation, thus distorting normal activity of the cell[35]. This could also be due to the ability of mercury to cause disruption of the mitochondria, thus distorting the energy production and increasing oxidative stress[5]. This is similar to the work of [38] who reported distortion in the cerebellar cortex of Wistar rats after exposure to 52mg/kg of mercuric chloride for three weeks, [39] also reported that exposure to toxins including heavy metals such as mercury causes cerebellar impairment and the sustained use or exposure to this toxin may result in irreversible damages.

Nissl substances are the centers of intense protein synthesis important in the activity of neurotransmitters[40]. They appear as appear as basophilic granular areas within the perikaryon of neurons with Cresyl Violet staining in light microscopy[41]. The Purkinje cells in the groups treated with mercuric chloride alone followed by treatment with distilled water showed chromatolysis with weak staining of the Nissl substance when compared to the control and other treatment groups. Nissl substance degeneration in mercuric chloride treated groups may lead to insufficient synthesis of neurotransmitters. This is consistent with the work of [42], who reported loss of Nissl substance in cerebellar neurons following mercury induced toxicity. Improved staining observed in groups treated with ethanolic extract of *Psidium guajava* may suggest that *Psidium guajava* was able to protect against Nissl substance degeneration thus improving motor co-ordination and activity observed in the beam walking test in this study.

CONCLUSION

The present study revealed that treatment with ethanol extract of *Psidium guajava* leaves *was able to* reverse neurotoxicity, neurodegeneration and distortion in Wistar rats treated with mercuric chloride. Therefore, ethanolic extract of this plant should be encouraged for consumption by individuals exposed to mercury poisoning.

ACKNOWLEDGEMENTS

The authors are grateful for the support and encouragement given by the staff of the Department of Human Anatomy, Ahmadu Bello University, Zaria.

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