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Original Research Paper

Anatomy

COMPARING THE NEUROPROTECTIVE AND ANTIOXIDANT EFFECT OF THE AQUEOUS AND ETHANOLIC LEAF EXTRACTS OF VERNONIA AMYGDALINA ON THE CEREBELLUM OF MERCURY CHLORIDE INTOXICATED ADULT MALE WISTAR RATS.

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ABSTRACT

This study compared the neuroprotective and antioxidant effects of the aqueous and ethanolic extracts of *Vernonia amygdalina* (VA) (bitter leaf) on the cerebellum of mercury chloride intoxicated adult male Wistar rats. Forty two rats used in this study were divided into six groups of seven animals each. All the animals were given water and feed ad libitum. Group A served as the negative control and had food and water only. Group B served as positive control and were given 0.5mg/kg/bw of mercury chloride for 14 days. All the experimental groups also received 0.5mg/kg/bw of mercury chloride for 14 days. All mercury chloride administration was via intraperitoneal route. Groups C1 and D1 received aqueous extract of the leaf of VA at 200mgkkg/bw and 400mg/kg/bw respectively for 14 days, while C2 and D2 were given ethanolic extract of VA at 200mg/kg/bw and 400mg/kg/bw respectively. All bitter leaf administration was done orally. At the end of 2 weeks the rat were subjected to the hanging wire neurobehavioural test, after which they were sacrificed and their brains harvested; two were fixed in 10% formal saline for histological studies while the remaining five were refrigerated and processed for antioxidant assay. Our result showed increase in MDA levels in the positive control (Group B) compared to the negative control (Group A). Also, there was highly significant reduction in levels of SOD, GSH and CAT in the Group B compared with the control. However following administration of the aqueous and ethanolic extracts of VA, there were statistically significant improvements in levels of SOD, CAT and GSH in the experimental groups, although the groups that received the aqueous extracts showed better outcomes than their ethanol extract counterparts. Histological study of the control group shows the normal cerebellar cytoarchitecture. However group C1 to D2 that were given the extracts showed noticeable improvements when compared to the positive control, although the group treated with aqueous extract of *Vernonia amygdalina* produced better outcomes than the group treated with the ethanolic extract. We therefore conclude that the leaf extracts of VA can confer some protection (in a dose dependent manner) against the effects of mercury chloride toxicity on the cerebellum of Wistar rats and that the aqueous extract produces better neuroprotective and antioxidant outcomes than the ethanolic extract.

KEYWORDS : *Vernonia amygdalina,* mercury chloride, malondiadehyde (MDA), catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), neuroprotection, cerebellum, antioxidant.

INTRODUCTION

Mercury chloride is the chemical compound of mercury and chlorine with the formula of $Hgcl_2$. This white crystalline solid is a laboratory and a molecular compound. Once used in the treatment of syphilis, it is no longer used for medicinal purposes because of mercury toxicity and the availability of superior treatments. Mercury chloride is highly toxic, both acutely and as a cumulative poison. It attacks the gastrointestinal tract and renal systems (EPA, 1998). Compounds of mercury tend to be much more toxic than either the elemental or the salts. These compounds have been implicated in causing brain and liver damage. Symptoms of mercury poisoning depend upon the type, dose, method and duration of exposure. They may include muscle weakness, poor coordination, and numbness in hands and feet, skin rashes, anxiety, memory problems, difficulty in speaking, difficulty in hearing and trouble seeing, acute renal failure, ulcerative colitis, anaemia, disseminated intravascular coagulation, chronic sepsis and severe weight lost. In this study therefore, mercury chloride was used to induce toxicity to the brain in other to test the potency of bitter leaf.

The use of herbal products for medicinal benefits has played an important role in nearly every culture on earth. Herbal medicine was practiced by ancient people of Africa, Asia, Europe and the Americans. Over 50% of all modern drugs are of natural product origin, and natural products play an important role in drug development programs of the pharmaceutical industry. The consumption of a variety of local herbs and vegetables by man is believed to contribute significantly to the improvement of human health, in terms of prevention, and cure of diseases because plants have for long served as a useful and rational source of therapeutic agents (Jisaka *et al,* 1993). *Vernonia amygdalina* (VA) commonly called bitter leaf due to its bitter taste is a widely used local plant in Nigeria for both therapeutic and dietary purposes. The bitter taste is said to be due to anti-nutritional factors such as alkaloids, saponins, tannins and glycosides (Song *et al,* 2005). The macerated leaves of the plants are consumed as vegetables and condiments while the water extract serves as tonic for prevention of certain illnesses (Izevbigie *et al.,* 2003). Bitter juice has been used over centuries to treat fevers especially related to malaria (Kokwaro *et al.,* 2009).

V. amygdalina also provide anti-oxidant benefits (Iwolakun et al., 2006), thus they help to reduce risk for serious diseases associated with oxidative stress like cancer, heart disease, stroke, aging, diabetes, arthritis, fibromyalgia, Parkinson's Disease, Alzheimer's, autoimmune diseases, cognitive decline, and eye conditions like macular degeneration. V. amygdalina extracts may also strengthen the immune system through many cytokines (including NFκB, pro inflammatory molecule) regulation (Coldtiz *et al.,* 1995). Studies also show that bitter leaf decreased blood glucose by 50% compared to untreated diabetic animals. Bitter leaf extract decreased bad cholesterol (LDL) by nearly half, while simultaneously raising good (HDL) cholesterol levels in an animal

study (Nwanjo, 2004). The use of bitter leaf to treat various skin diseases like eczema and ringworm could be due to its anti-inflammatory effects. Bitter leaf juice is used by local woman in Guinea-Bissau to contract the uterus after childbirth (Huffman *et al.,* 1989).

The cerebellum plays important roles in motor control. It also contributes to coordination, precision and accurate timing. It receives input from sensory systems of the spinal cord and from the other part of the brain, and integrates these inputs to fine-tune motor activity. Cerebellar damage produces disorders in fine movement, equilibrium, posture and motor learning.

MATERIALS AND METHODS

Bitter leaves were purchased from ekeoma market in Elele Rivers State, Nigeria. It was identified and authenticated by the head of pharmacognosy department, Madonna University Nigeria, Elele campus.

Experimental animals

Forty two (42) adult male Wistar rats weighing 160-180g were separated into six (6) groups of seven rats each. They were purchased from the animal house of Abia State University Uturu, Nigeria. They were housed in standard cages and left to acclimatize for seven days under natural condition in the animal house of Abia State University before the commencement of the experiment. The animals were maintained on pelletized growers feed obtained from vital feeds.

Preparation of plant material for analysis

Fresh bitter leaves were air dried at room temperature for 14 days to remove sufficient moisture. The dried sample were cut into smaller pieces and further pulverized with the aid of manual mill. 400g each of the macerated plant was soaked in 200ml of distilled water and 200ml of 99.8% ethanol (analytical grade) for aqueous and ethanolic extractions respectively for 48 hours and then filtered with whatman (No.1) filter paper. The marc (shaft) was rewashed until all the extractable constituents were completely washed out and then filtered. Both filtrates were combined and concentrated at 45^{o}c until a dried extract was obtained. Chemicals used for this study were of analytical grade.

Histological studies

At the conclusion of the neurobehavioural studies, the rats were sacrificed and the brains were harvested. Two of the brains from each group were fixed in 10% formal saline for histological studies. The other five brains were individually homogenized and centrifuged at 10 000 rpm to separate the supernatant from the residue. The supernatant were then used for biochemical analysis of antioxidant parameters (MDA, SOD, CAT and GSH). After 24 hours, the fixed tissues were subjected to haematoxylin and eosin (H&E) histological procedure.

Neurobehavioural Studies - Hanging Wire Test

This test is used to determine muscle strength and motor coordination to test for cerebellar function. The rat is suspended on its forelimbs to a thin rod suspended at a height. The time spend on this suspension is then recorded. Maximum duration is five minutes, after which the animals can be manually removed.

Data Analysis

Data were reported as Means ± SD of 7 rats in each group. Data analysis was carried out using Microsoft excel and ezanova. The means were compared with ANOVA.

RESULT

TABLE 1: Result Of Antioxidant Studies.

GROUP	MDA	SOD	CAT	GSH
A	**0.32**±0.16	**0.40**±0.17	**3.23**±0.58	**7.43**±0.77
B	**0.55**±0.10*	**0.15**±0.07*	**1.74**±0.36*	**3.72**±0.54**
C1	**0.40**±0.09	**0.31**±0.11	**2.90**±0.49	**5.18**±0.70**
C2	**0.43**±0.06	**0.31**±0.08	**2.59**±0.70	**4.30**±0.62**
D1	**0.36**±0.06	**0.39**±0.20	**3.09**±0.25	**6.89**±0.93
D2	**0.40**±0.12	**0.33**±0.08	**2.90**±0.35	**4.95**±0.36**

Data is presented as Mean ± Standard Deviation of 7 rats in each group.

*indicates statistical significance at P≤0.05
**indicates high statistical significance at P≤0.05.

The results of the antioxidant studies presented in Table 4 above indicated that malondialdehyde (MDA) levels were increased across all experimental groups compared to the control. However, only group B was statistically significant. Superoxide dismutase (SOD) was reduced in all the experimental groups B, C1, C2, D1 and D2 compared to the control group A. The differences however were not statistically significant except in groups B and C1. Catalase (CAT) also showed statistically significant reduction in the experimental groups B, C1 and D1. Although the others were also reduced compared to the control, their differences did not attain statistically significance. Glutathione peroxidase (GSH) was highly significantly reduced in all experimental groups when compared to the control except group D2.

TABLE 2: Result of Hanging wire Test

GROUP	INITIAL	FINAL
A	4.38±.1.10	4.46±0.40
B	4.20±0.65	1.14±0.25**
C1	4.36±0.45	2.14±0.45**
C2	4.43±0.45	1.56±0.53**
D1	4.19±0.50	2.43±0.86*
D2	4.23±0.57	2.21±0.48**

Data is presented as Mean ± Standard deviation of 7 rats in each group. Results were calculated in minutes. * Indicates statistical significance at P≤0.05. **Indicates high statistical significance at P≤0.05.

Table 2 shows the results of the hanging wire test carried out on 7 rats in each of the 6 groups used for the experiment. INITIAL represents the data collected prior to experimentation (after acclimatization), while FINAL represents data collected at the end of the experimental period of 14 days. There was no statistically significant difference between INITIAL and FINAL for group A (control) which received only food and water. There was a high statistically significant difference in the means of the INITIAL AND FINAL data for group B. For the treatment groups C1, C2, D1 and D2 we also see high statistically significant differences in the means between INITIAL and FINAL. However, C1 and D1 showed the highest average rise in means in the C and D groups respectively.

TABLE 3: Comparing the results of the Hanging wire Test for Initial and final readings across groups

GROUP	A	B	C1	C2	D1	D2
INITIAL	4.38± 1.10	4.20± 0.65	4.36± 0.45	4.43± 0.45	4.19± 0.50	4.23± 0.57
FINAL	4.46± 0.40	1.14± 0.25*	2.14± 0.45*	1.56± 0.53*	2.43± 0.86*	2.21± 0.48*

Data is presented as Mean ± Standard Deviation of 7 rats in each group.

*indicates statistical significance at P≤0.05. **indicates high statistical significance at P≤0.05

Table 3 presents the results of the hanging wire test, comparing the INITIAL data across the groups as well as the FINAL data across the groups. For the INITIAL means, there was no statistically significant difference in the experimental groups when compared to the control. However, all experimental groups B to D2 were statistically significantly lower than the control group A for FINAL. Out of all, D1

was the highest for the D groups and in general while C1 was the highest among the C groups.

4.2 HISTOLOGICAL STUDIES

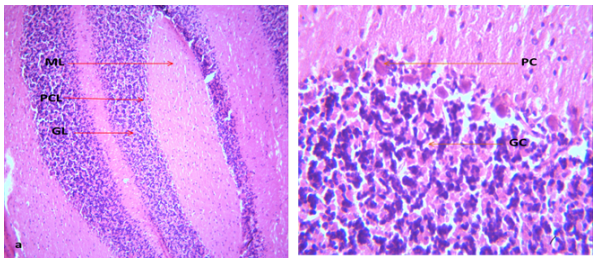

Plate 1: Representative photomicrograph of rat cerebellum in group A showing normal cerebellar cytoarchitecture; a molecular layer (ML), Purkinje cell layer (PCL),and granular layer (GL); b purkinje cell (pc) and granular cell (GC).

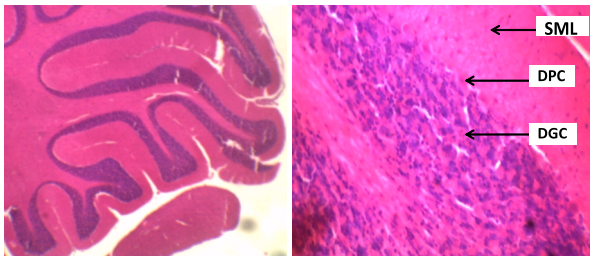

Plate 2: Representative photomicrographs of rat cerebellum in group B showing: scanty molecular layer (sml), dying purkinje cells (dpc) and dying molecular cells (dmc).

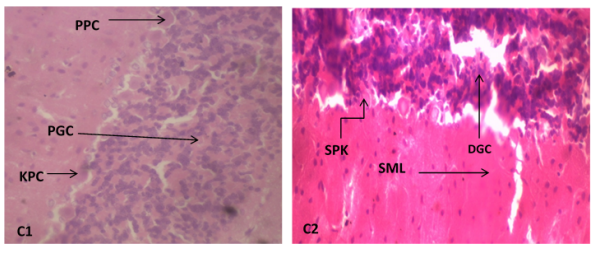

Plate 3: Representative photomicrographs of rat cerebellum in group C1 showing Pyknotic purkinje cells (PPC), karyorhetic purkinje cells (KPC) and pyknotic granular cells (PGC) and C2 showing shrinking purkinje cells (SPK), dying granular cells (DGC) and scanty molecular layer (SML).

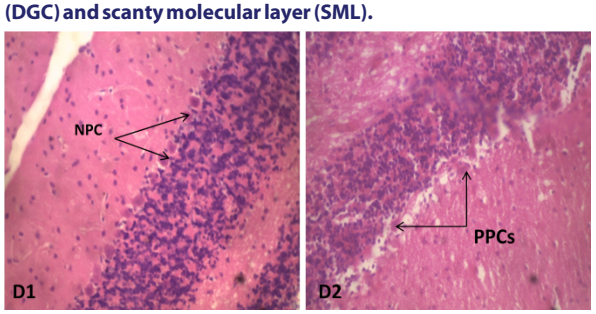

Plate 5: Representative photomicrographs of rat cerebellum in group D1 showing Normal purkinje cell (NPC) and D2 Cerebellum showing normal cells and tissue arrangement in with a few Pyknotic purkinje cells (PPCs).

DISCUSSION

Our result shows that mercury chloride as low as 0.5mg/kg b.w administered intraperitoneally twice a week produced signs of toxicity on the rats. This was manifested through several physical signs of weakness including reduction in size, reduced food consumption and reduced general agility. Toxicity was further evident in the result of the antioxidant studies presented in Table 1 as well as the histology and neurobehavioural results. This is supported by the works of several researchers: Barregard, (1991) reported on the toxic effect of mercury on erythrocyte and leucocyte count; Rao et al., (2010) on the liver; Hallee, (1969) and Goyer, (1991) on the lungs; Rice et al., (1990) and Clarkson, (2003) reported on the gastrointestinal tract, including bleeding gums, loosening of teeth, mouth and throat ulceration, excessive salivation and foul breath; Pambor et al., (2007) and Goh, (2008) reported on erythemas and dermatitis following contact with skin; Thurtson et al., (2007) and Altman, (2008) reported on the heart and cardiovascular system; Booth, (2005) reported skin rashes, dim vision and clamminess; Rao & Sharma, (2001) and Baltimore, (2006) reported on menstrual disturbances and pregnancy complications following exposure to mercury through different routes. Halas et al., (2007) also reported that mercury chloride administration cause zinc deficiency in the brain of rats. Adamu (2013) reported that mercury intoxication reduces the concentration of copper in the brain. Zacconi et al., (2007) in the same vein reported that decrease in copper concentration in the brain can interfere with the modulatory role of dopamine receptors and consequently lead to motor function deficits similar to what is seen in patients of Parkinsonism.

Reports from the Agency for toxic substances and Disease Registry (ATSDR) (1999) reports that exposure to mercury causes a wide variety of cognitive, personality, sensory, and motor disturbances. They also reported symptoms such as tremors, nervousness, memory loss and neuromuscular changes including muscle atrophy and weakness (ATSDR, 1999). Rats under such health conditions will not perform well in the hanging wire test, hence supporting the outcome of our research. The poor result of the hanging wire test presented in Table 2 and 3 reveal that the rats had muscle weakness and possible muscular atrophy.

Also, the result of the biochemical analysis presented in table 1 show that the rats in the experimental group B which received mercury chloride only were under oxidative stress. Here, there was a significantly higher level of MDA which is an indicator of lipid peroxidation. High MDA level is fallout of oxidative damage which could be linked to the generation of free radicals, resulting in the peroxidation of membrane lipids (Bartsch and Nair, 2000). The decrease in this endogenous antioxidant in our experimental groups B to D2 supports the presence of oxidative stress. Also, there were reduced levels of catalase (CAT) and super oxide dismutase (SOD) and reduced glutathione (GSH). GSH is an endogenous antioxidant which plays a vital role in the detoxification of xenobiotics and scavenging of free radicals in cells (Bartsch and Nair, 2000). A decline in cellular level has been considered to be indicative of oxidative stress. Therefore, the increase in MDA levels and corresponding decrease in SOD, CAT and GSH leave us with no doubt that mercury chloride administered twice a week intraperitoneally at 0.5mg/kg body weight of rat induced oxidative stress in the rats in the experimental group, especially when placed in the light of the results from the control group.

Our result showed that the two extracts of bitter leaf used for this research showed antioxidant properties in a dose dependent fashion. This is inferred following our results which reveal that for all the tests carried out, the D groups which received 400mg/kg/b.w of *Vernonia amygdalina* performed better than the C groups which received 200mg/kg/b.w. This is supported by the work of Nwanjo 2005 which reported that the efficacy of bitter leaf increased with increasing doses. The level of lipid peroxidation (MDA) decreased in the experimental groups C1 to D2 following administration of the different extracts of *Vernonia amygdalina* compared to group B.

It also showed that the values for MDA in the D groups were lower than those of the C groups. This represents a dose-dependent pattern of action. This is supported by the work of God'swill et al., (2010) which showed that aqueous extracts of VA prevented lipid peroxidation. Odukoya et al., (2007) also reported the antiperoxidative effect of leaves of *Vernonia amygdalina*. Another study by Nwanjo, (2005) on diabetic rats showed that the aqueous

extracts of VA decreased the levels of serum malondialdehyde. A study by Owolabi et al. (2008) further showed that both the ethanolic and aqueous extracts of VA have potent antioxidant abilities. Furthermore, the levels of the antioxidant enzymes which were significantly depleted in the experimental group B started to rise with *Vernonia amygdalina* administration in a dose-dependent manner. We are therefore convinced by these results in line with the finding of other researchers that *Vernonia amygdalina* is a potent antioxidant.

But we noticed from our result that the aqueous extract conferred better resistance to oxidative stress than the ethanolic extracts. Levels of MDA were the least in the aqueous groups C1 and D1 compared to their ethanolic (C2, D2) counterparts. Owolabi et al., (2008) however reported that the ethanolic extraction confers higher antioxidant activity than aqueous extract. This is contrary to the findings of our research.

The cerebellum showed evidence of toxicity as it manifested histopathological changes, including various degrees and stages of cell death, distortion of the Purkinje cell layer, infiltration of cells in the granular cell layer and general disorientation of the architecture of the Purkinje cell layers. There was sparse distribution of Purkinje cells of the Purkinje cell layer especially in group B which received mercury chloride without treatment. The Purkinje cell is the sole motor output of the cerebellar cortex. Reduction in its number, size or efficiency could lead to interferences with the motor functions such as loss of fine movement, loss of grasping, loss of equilibrium and loss of regulation of muscle tone. These signs were evident from our results in the hanging wire test. Fuyuta et al,. (1978) reported abnormal cytoarchitecture of the brain in infants prenatally exposed to mercury. Mercury is known to bind to microsomal and mitochondrial enzymes, resulting in cell injury and death (Barr et al., 1973). Gagelli *et al., (*2011) had shown that cell sizes and numbers were decreased in mice treated orally with inorganic mercury at high doses for a week. Our results also show decrease in cell sizes and cell numbers in the various experimental groups.

The hanging wire test was carried out to test for muscular strength and by extension cerebellar function. In this case the animals were suspended on a hanging wire using their forelimbs. The rationale is to determine how long they can suspend there which by extension is an indicator of muscle strength and motor activity. The results were compared with those taken before mercury chloride and *Vernonia amygdalina* administration. So, the initial records are here referred to as INITIAL while the final data collected at the end of the experiment were referred to as FINAL. Our result as presented in tables 2 and 3 shows statistically significant reduction for FINAL when compared to INITIAL in all the experimental groups (B, C1, C2, D1& D2). In the control group however, there was no statistically significant difference between INITIAL and FINAL. In fact, FINAL was even higher than INITIAL. This could be owing to the fact that the rats were already aware of the procedure and could device means of lasting longer than at their first exposure. The reduction in the FINAL for the experimental groups is an indication of weakness as well as reduced muscular strength. This result is supported by the histology results which show varying degrees and stages of necrosis following mercury chloride intoxication as discussed earlier on. Gagelli *et al., (*2011) reported an association between motor function disorders and reduced copper & dopamine concentration as seen in patients with Parkinson's. It has been shown that exposure to high concentration of mercury vapor causes tremor initially affecting the hand and then spread to the other part of the body (Dolbec *et al.,* 2000; Yoshida *et al.,* 2011). This is expected for group B as previous studies have documented mercury chloride toxicity on different parts of the brain. However, we observe that the groups treated with the aqueous extract of *Vernonia amygdalina* had longer time of suspension compared to those that received the ethanolic extract. This was evident in both the C and D groups.

These results therefore suggest that the aqueous extract of *Vernonia amygdalina* had more protective outcomes than the ethanolic extract. This may be attributed to the reported water solubility of some active phytochemicals in bitter leaf like flavonoids and phenols. Several researchers have reported that flavonoids and other phenolic compounds are potent water soluble antioxidants and free radical scavengers, which prevent oxidative cell damage, and have strong anticancer activity (Okwu, 2004). Torel et al., (1986) reported that flavonoids are good antioxidants, and luteolin (a flavonoid found in VA) has been reported to be a strong antioxidant.

CONCLUSION

Mercury is known to accumulate in the human body and so may manifest its toxic effects after many years. Therefore, toxicity might predispose to chronic ailments over time and eventually lead to reduced life expectancy. The World Health Assembly in 1989, adopted among its resolutions, the support of national traditional medicine program, drawing attention to herbal medicines as being of great importance to the health of individuals and communities. Presently, more than 50% of all modern clinical drugs are of plant origin (Suffness and Douros, 1982). Vernonia amygdalina has been a plant of choice for the treatment or management of several ailments.

Although many synthetic drugs exist which are used as antioxidants, reports have shown that the users are also exposed to other dangers. Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are suspected to be tumorigenic (Ito et al., 1985). This is the reason for preference of natural alternatives, and this research goes ahead to confirm that bitter leaf is one of such natural alternatives.

Recommendation

We recommend the continuous incorporation of bitter leaf in our diet as it has been proven to be very nutritious and also medicinal. We also recommend that the numerous researches on African herbs like bitter leaf be converted into useful end products for the betterment of the people instead of having them pile up in journals and libraries. Finally, we recommend that further comparative researches be done with other pure solvents as well as solvent mixtures like ethanol and water to ascertain the best combination that gives the best phenolic yield and that the protective properties of bitter leaf against environmental toxicants be extended to other organs and systems of the body.

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