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Article

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Effect of *Combretum micranthum* Methanol leaf extract against exposure to Lead on behavioral activities in Mice

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ABSTRACT

This study examined the relationship between behavioural alterations in lead exposure and possible protective role of *Combretum micranthum* methanol leaf extract in mice. Thirty six (36) Swiss Albino mice (19 - 22g body weight) were used for the study, randomized equally into six groups and treated for the period 14days. The behavioural models employed in the study are: Open field test, hole board assay, mouse beam walk assay, and the staircase test. Result of the study indicated significant protective effect of the *Combretum micranthum* methanol extract against lead acetate, in terms of the behavioural tests carried out (0.01; $p<0.05$) when co-administered together. Findings of the present study suggested *Combretum micranthum* to be a potential plant in preventing the brain functional damage induced by lead administration.

Keywords: lead acetate, behavioural alterations, protection, brain, toxicity.

INTRODUCTION

Effects and implications of heavy metals intoxication causes chronic diseases, leading to increased stress and risk to the behavioral activities of both plants and animals.[1-5] Among heavy metals, lead represents a main environmental poison. Lead is a soft, grey-blue heavy metal which is a common cause of poisoning in domestic animals throughout the world.[6] Lead metal exist in both organic (Tetraethyl lead) and inorganic (lead acetate, lead chloride) forms in the environment.[7] It has been used in paintings, pipes, ammunition and alloys for welding storage materials for chemical reagents.[8] Exposure to lead mainly occurs through respiratory and gastrointestinal systems, where a small quantity excreted in urine and the rest accumulates in various body organs.[9] The cellular and intercellular levels, may result in behavioral alterations that can remain even after lead level has fallen.[10] An estimated number of about 400 children died from lead intoxication in Nigeria and laboratory testing later confirmed high levels of lead in the blood of the surviving.[11]

Combretum micranthum (figure 1) is a shrub species belonging to the family of Combretaceae, commonly called

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kinkeliba (health tree) in Benin, Senegal, Gambia and across multiple regional dialects of West Africa.[12,13] Ethanol extract of *Combretum micranthum* leaf is a widely used in West Africa for treating several conditions such as fatigue, liver ailments, headache, convalescence, blood disease, weight loss, cancer and sleep problems.[14]

Figure 1. The plant of *Combretum micranthum (with enlarged snap of leaves).*

The present study aimed at investigating the preventive effects of *Combretum micranthum* methanol leaf extract on behavioural impairment induce by lead acetated in mice.

MATERIALS AND METHODS

Materials

Collection and Identification of Plant

Fresh leaf of *Combretum micranthum* plant were obtained from Shira Local Government Area (N 11^{O} 27' 29" and E 10^{O} 2' 48") of Bauchi State in Nigeria. The plants were identified in the Herbarium Unit, Department of Biological Sciences, Bayero University, Kano. And a voucher specimen number of BUKHAN 0272 was issued.

Chemicals, Reagents and Equipment

All the chemicals and reagents used for this work were of analytical grade and purchased from reputable chemical manufacturers, e.g. SIGMAALDRICH-FLUKA. The laboratory equipment used, were also of standard quality.

Animals Care

Swiss albino mice (19 - 22g body weight) were used for this study. The animals were obtained from the animal facility in the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. They were housed in standard metal animal cages at room temperature in the animal house of Pharmacology and Therapeutics, Bayero University, Kano. They were allowed to acclimatize for a week prior to use. The protocol of the study was guided by the best International Guidelines in animal care .[15-18]

Ethical Clearance

Ethical clearance for the research was granted by the Bayero University, College of Health Sciences Research Ethics Committee with a reference number BUK/CHS/HREC/VII/62.

METHODS

Preparation of the Extract

The leaf of *Combretum micranthum* plant were air dried under the shade, powdered to get a coarse powder and stored in a well closed container. The dried coarse powder was subjected to Microwave Assisted Extraction by method described by Waghmare *et al.*,[19] and finally extracted with methanol following the method described by Harborne *et al*.[20]

Experimental Design

Mice were randomized to six groups of 6 each:

(a) Group-I: Normal Control: administered with Distilled water only.
(b) Group-II: Negative Control: Lead acetate was administered orally at a dose of 40 mg/kg b.w.[21]
(c) Group-III: A dose of 40 mg/kg lead acetate and 100 mg/kg b.w. extract was administered simultaneously
(d) Group-IV:A dose of 40 mg/kg lead acetate and 50 mg/kg b.w. extract was administered simultaneously
(e) Group V: A dose of 40 mg/kg lead acetate and 25 mg/kg b.w. extract was administered simultaneously.
(f) Group VI: Positive Control: A dose of 40 mg/kg lead acetate and a standard drug diazepam 0.5 mg/kg [22] was administered simultaneously.

The treatment period for the animals was carried out for two weeks. [21] After which the animals in each group were subjected to behavioral test.

Behavioral Test

The behavioral test carried out in this study are; Mouse Beam Walking Assay, Open Field Test, Hole Board Test and Staircase Test. The tests were conducted twice: on the 7th and 14th day of administration, so as to monitor progression.

Mouse Beam Walking Assay: The method described by Stanley *et al*.[23] was used for this study. The beam was made of wood (8 mm in diameter and 60 cm long) elevated 30 cm above the bench by metal supports. Mice were trained to walk from a start platform along a ruler (80 cm long and 3 cm wide) elevated 30 cm above the bench by metal supports to a goal box. Three trials were made for each mouse, such that the mice tested would be aware that there was a goal box that could be reached. Thirty minutes post-treatment, each mouse was placed on the beam at one end and allowed to walk to the goal box. Mice that fall were returned to the position they fell from, mouse was allowed to spend a maximum of 60 second on the beam. The number of foot slips, which is a sensitive measure of motor coordination deficit was recorded for each mouse.[23] The beam-

walking test was carried out under proper conditions of silence and illumination.

Open Field Test: The method of Brown *et al.*[24] was adopted in this study. The arena of the open field consists of 72 x 72 cm wooden box of 36 cm high in which the floor is divided into 16 squares (18 x 18 cm). At the beginning of test each mouse was placed into the centre of arena or one of the four corners of the open field and allowed to explore the apparatus for 5 minutes. Open field was carefully cleaned with 70% ethanol solution after every test. The activities in the tests was recorded by a digital video camera (resolution of 30 samples per second) mounted centrally 150 cm above the open field and then analysed. The behaviours scored for each mouse are: Line Crossing (Frequency with which the mice crossed one of the grid lines with all four paws) and Rearing (Frequency with which the mice stood on their hind legs in the maze).

Staircase Test: This test was conducted according to the method described by Simian *et al.*[25] The staircase was enclosed in transparent Perspex vertical walls (45 cm x 12 cm x 25 cm) with 5 identical steps 2.5 cm high, 10 cm wide and 7.5 cm deep. After Thirty (30) minutes of post- treatment, each mouse of every group was place on the floor of the Perspex box (with its back to the staircase). The behavior of each mouse videotaped and number of upward steps climbed and rearing were recorded over a 5 min period. A step was considered climbed, if a mouse placed all its four paws on it. Rearing was counted when a mouse rose on its hind limb both against the wall and on a step. The staircase was wiped with 70% ethyl alcohol and allowed to dry after each trial to avoid modification in behavior due to olfactory cues.

The Hole-Board Test: The apparatus used was a wooden board (60 cm x 30 cm) with 16 evenly spaced holes (1cm diameter x 2 cm depth)[26] After Thirty (30) minutes of post-treatment, each mouse of every group was placed at a corner of the board and the number of head dips on the hole was were counted using a tally counter during a 5 minute period. A head dip was considered when the mouse dipped its head into the hole to the level of the eyes.

Statistical Analysis

Results were expressed as Mean ± SEM (standard error of the mean). A 0.05 level of probability was used as the criterion of significance in all cases. The significant differences were carried out using One-Way Analysis of Variance (ANOVA) followed by Post-Hoc test (Tukey) and Independent T- test. All statistical analyses were carried out using SPSS Software and Microsoft Excel Spread Sheet.

RESULTS

Mouse Beam Walking Assay

The number of footslips where observed in this assay (Figure 2).There was no significant difference of treatment within the groups during the first week of trail in number of foot slip in the beam walk. However, Independent T- test analysis indicated a significant effect between the two test days (Day 7 and Day14) in terms of the number of foot slip in the beam walking assay ($F(0.05) = 0.014$, $p<0.05$). ANOVA showed that there was a significant protective effect of the extract on the lead acetate in terms of motor coordination carried out on Day 14 ($F(22.57) = 0.01$; $p<0.05$). And Post hoc analysis also revealed that, mice administered doses (25mg/kg, 50mg/kg and 100mg/kg) of the extract elicited a general significant decrease ($P< 0.05$) in number of foot slips on the beam as compared to those treated with 40mg/kg lead acetate alone, but there was no significant difference as compared with animals in the normal control and positive control treatment groups.

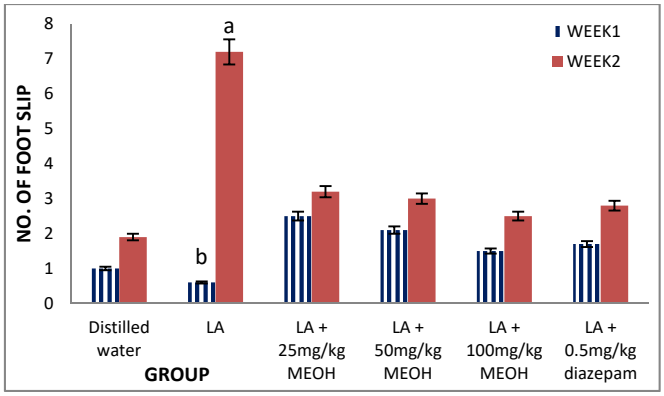

Figure 2: Effects of *C. micranthum* methanol extract against lead acetate (LA) on the number of foot slips in the Mouse Beam Walk test. Data are expressed as mean ± S.E.M, n = 6 at $P < 0.05$. The superscript ([a]) represent significant difference of negative control with other groups while the superscript ([b]) represent significant between day7 and day14 within a group.

Open Field Test

The Total Distance Covered (number of line crosses) and the frequency of rearing were scored in this test (figure 3 and figure 4). The test was carried out twice (Day 7 and Day14) . ANOVA shows a statistical significant difference of treatment within the groups only in the Frequency of Rearing carried out on Day 14 in the open field, ($F(9.67) = 0.01$, $p<0.05$). However, Independent t-test analysis indicated a significant effect between the two days (Day 7 and Day14) in terms of the total distance covered ($F(0.18) = 0.02$, $p<0.05$). Post hoc analysis revealed that, mice administered doses (25mg/kg, 50mg/kg and 100mg/kg) of the extract elicited a general significant increase ($P< 0.05$) in frequency of rearing as compared to those treated with 40mg/kg lead acetate alone, but there was no significant difference of the mice administered doses of the extract with animals in the normal control and positive control treatment groups.

Staircase Test

Two parameters were measured in the staircase test, number of steps climbing and number of rearing, and the test was carried out twice (Day 7 and Day14) as shown in figure 5 and 6 below. ANOVA shows significant difference of treatment within the groups during Day14 trials both in number of steps climbing and number of rearing in the staircase ($F(6.41) = 0.17$ for number of rearing and $F(84.06) = 0.01$ for number of step climbing; $p<0.05$) respectively. However, Independent T- test

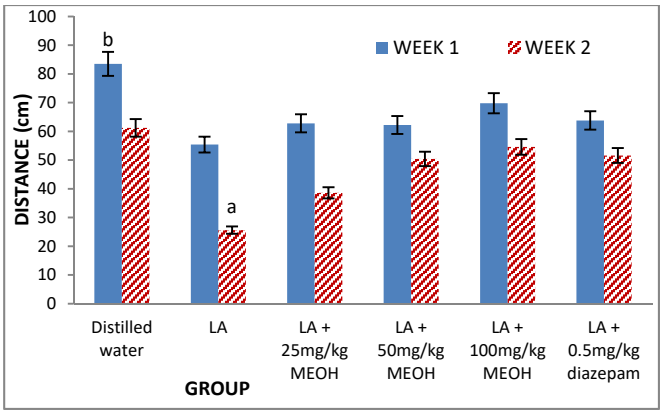

Figure 3: Effects of *C. micranthum* methanol extract against lead acetate (LA) on the total distance covered in the Open Field test. Data are expressed as mean ± S.E.M, n = 6 at P < 0.05. The superscript ([a]) represent significant difference of negative control with other groups while the superscript ([b]) represent significant between day7 and day14 within a group.

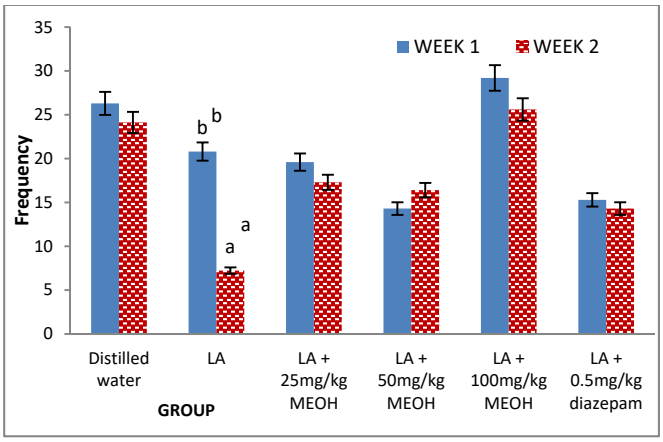

Figure 4: Effects of *C. micranthum* methanol extract against lead acetate (LA) on the number of rearing in the Open Field test. Data are expressed as mean ± S.E.M, n = 6 at P < 0.05. The superscript ([a]) represent significant difference of negative control with other groups while the superscript ([b]) represent significant between day7 and day14 within a group.

analysis indicated no significant effect between the two days (Day 7 and Day14) in terms of number of rearing and number of steps climbing. Post hoc analysis also revealed that, mice administered 100mg/kg dose of the extract elicited a general significant increase (P< 0.05) both in the number of step climbing and number of rearing in the staircase test as compared to those treated with 40mg/kg lead acetate alone, but there was no significant difference with animals in the normal control and positive control treatment groups, indicating protection against motor coordination and exploratory deficit by the extract.

Hole Board Test

The number of head dipping was the only parameter measured in the hole-board test (figure 7). Statistics analysis revealed that, mice administered 100mg/kg dose of the extract elicited a general significant increase (P< 0.05) in the number of head dipping as compared to those treated with 40mg/kg lead acetate alone, but there was no significant difference with animals in the normal control and positive control treatment groups, indicating protection against exploratory deficit by the extract.

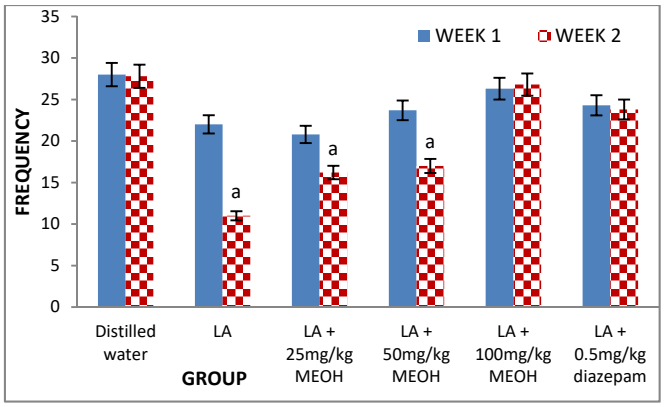

Figure 5: Effects of *C. micranthum* methanol extract against lead acetate (LA) on the number of step climbing in the Staircase test. Data are expressed as mean ± S.E.M, n = 6 at P < 0.05. The superscript ([a]) represent significant difference of negative control with other groups while the superscript ([b]) represent significant between day7 and day14 within a group.

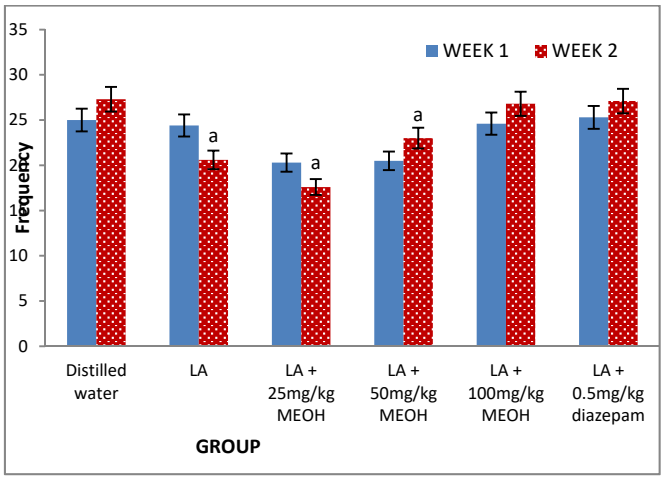

Figure 6: Effects of *C. micranthum* methanol extract against lead acetate (LA) on the number of rearing in the Staircase test. Data are expressed as mean ± S.E.M, n = 6 at P < 0.05. The superscript ([a]) represent significant difference of negative control with other groups while the superscript ([b]) represent significant between day7 and day14 within a group.

Discussion

Treatment of mice with lead acetate for 14 consecutive days caused a significant decrease in the body weight of mice. The results are in agreement with several other studies[27], which suggested that the reduced growth was due to reduced food consumption via lead effects on the satiety set-point. Banu *et al.*, reported that lead acetate, given in low (160mg/kg/days) and high (320mg/kg/days) doses, cause dose-dependent significant

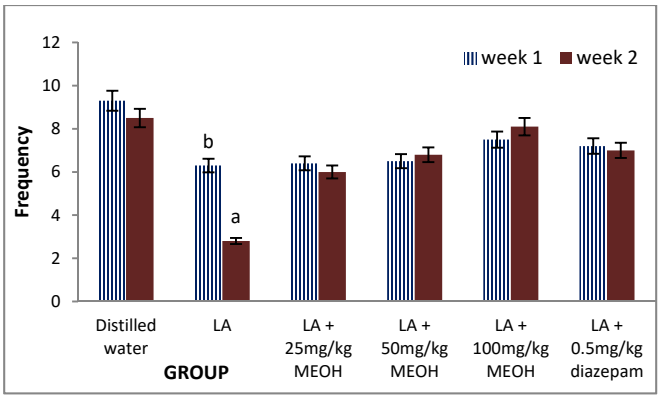

Figure 7: Effects of the extract and diazepam (DZ) against lead acetate (LA) in mg/kg on the number of head dipping in the Hole Board test. Data are expressed as mean ± S.E.M of six animals in each group. Significance at $P < 0.05$ with one bearing superscript (^{a}) as significant difference from other groups.

Table 1: Showing Change in Body Weight of Mice Before and After Administration Period

Group	Before (g)	After (g)
Distilled Water	19.83 ±1.22	23.40±1.25^a
Lead Acetate	21.67±1.56^a	16.75±3.28^b
Lead Acetate + 100mg/kg Ex.	19.83±0.87	22.17±1.17^a
Lead Acetate + 50mg/kg Ex.	22.67±0.61	23.50±1.71^a
Lead Acetate + 25mg/kg Ex.	19.00±0.63	19.20±1.53
Lead Acetate + 0.5mg/kg Dz.	20.33±0.45	21.50±1.54^a

Values are Mean ± Standard Error of Mean with those bearing different superscripts within the same column and row being significantly ($P< 0.05$) different. N = 6.

decrease in body weight as compared to control animals.[28] Several other investigators confirm these findings, that at low and high doses of lead acetate administeration to rat orally for days, causes an observable significant decrease in body weight. Similar changes were observed by Antnio Graca *et al.*[29] This change in the body weight was prevented from occurring when the mice were concurrently treated with the methanol extract of *Combretum micranthum* at doses of 100 mg/kg, 50 mg/kg and 25 mg/kg body wt orally for the similar time-period (Table 1).

Brain is an essential organ of the body which is reported to be impaired on exposure to lead with respect to time. Research, shows that the Central Nervous System (CNS) is an important target organ for lead toxicity, since subchronic/chronic exposures to lead are highly neurotoxic in adults and particularly to the developing CNS.[30] The work of Chao *et al.*[31] also supported the fact that lead exposure is detrimental to brain function. The present study shows that the methanol leaf extract of *Combretum micranthum* shows a good protective effect on the central nervous system against lead toxicity. This action was demonstrated by its effects on beam-walk assay, open field test, stair-case assay and hole-board test. Diazepam used in the study is to illustrate the active property of drug in the central nervous system.[32] Beam-walking assay is used to test the effect of substance on motor coordination in laboratory animals.[23] The number of foot slips in the beam walking assay is a sensitive measure in detecting lead-induced motor deficit in mice and may be more useful in predicting doses that could prevent such effect. The open field test, stair-case test and hole-board test are experimental methods used in scientific research to measure anxiolytic activities (determined by the frequency of rearing in the both open field and staircase test), exploratory (determined by the number of line crossing and head dipping in open field test and hole board test respectively) and locomotion activities (determined by the number of step climbing in staircase test).[24-26] The more these behaviors occur, the less anxious the animal is. On the other hand, if the animal doesn't show these behaviors then it is more anxious.[24-26]

Result from co-administration of *Combretum micranthum* methanol extract (100mg/kg, 50mg/kg and 25mg/kg) with lead acetate in mice for 14days in the present study, showed induced improvement in the anxiolytic effects, motor coordination activities, locomotive activities and exploratory activities, as opposed to the effects observed in the mice treated with 40mg/kg lead acetate for the same period. However, the results seems to indicate that the 100mg/kg dose of *Combretum micranthum* methanol extract has more modulatory effect as compared to the other doses (50mg/kg and 25mg/kg).

The significant effect of the extract at highest dose and diazepam on the animals in the beam walking, staircase test, open field test and hole board test respectively, revealed the protective activity of the extract against lead toxicity. The ability of the extract to potentiate diazepam in the behavioural assays showed that, the activities of the extract may be acting via the central mechanisms of the brain.[33]

The *C. micranthum* might have produced its protective effects through activation of receptors of endogeneous neurotransmitters such as dopamine, serotonin, histamine, neuropeptides, norepinephrine or gamma amino butyric acid (GABA). Thus, since the extract potentiates the motor coordination, exploration and locomotive properties of diazepam, it may act by interacting with GABA – mediated synaptic transmission. [34] GABA is known to be an important inhibitory neurotransmitter in the brain. Diazepam acts at the level of the limbic, thalamic and hypothalamic regions of the CNS through potentiation of GABA at the $GABA_A$ receptor.[35]

CONCLUSION

The findings of the present study showed that *Combretum micranthum* methanol leaf extract may be responsible for the observed pharmacological activities. This could have greater importance as therapeutic agent in preventing or slowing related disorders associated with lead toxicity on the brain, which can result to behavioural impairment in animals. Hence *Combretum micranthum* is a potential plant in preventing behavioral defect induced by heavy metals toxicity.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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