A study was conducted to assess the protective effects of Combretum micranthum methanol leaf extract on lead-induced brain damage in Swiss Albino mice. Thirty-six (36) mice were randomly divided into six groups and treated with lead acetate for 14 days. The extract showed normal neurons compared to those in the negative control group. The extract also reduced malondialdehyde (MDA) levels, enhanced GABA levels, and protected the neuronal cell membrane.

**ABSTRACT**
Combretum micranthum is traditionally used for many therapeutic purposes such as fatigue, liver ailments, headache, sway, blood disease, weight loss among others. Studies suggested that free radical production may be a main reason behind stress induces neurochemical changes that include neurotransmitter imbalance and histopathological alterations in the brain cell. Thus, this study examined the relationship between brain histopathological alterations and neurotransmitter imbalance (gamma aminobutyric acid - GABA) in lead exposure, and possible neuroprotective 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 14 days: a) Normal Control (Distilled water), b) Negative control (40 mg/kg lead acetate), c) Lead acetate + 100 mg/kg b.w. extract, d) lead acetate + 50 mg/kg b.w. extract, e) lead acetate + 25 mg/kg b.w. extract and f) positive control (lead acetate + diazepam 0.5 mg/kg b.w.). The study assayed for protective potential of Combretum micranthum on neuronal cell membrane through determination of the brain cells malondialdehyde (MDA) levels, morphology and GABA levels. Result of the study showed a significantly increase level of GABA in the brain of mice administered doses of the extract with a decreased level of MDA, as compared to those mice treated with 40 mg/kg lead acetate alone, which showed decreased level in GABA (310.83 pg/ml), but an increased level of MDA (30.03 µmol/TBARS/mg). Also, the mice administered doses of the extract showed normal neurons while intensive neuronal necrosis was observed on those mice treated with 40 mg/kg lead acetate alone. However, mice administered with the doses of the extract shows no any significant difference with those in the normal control and positive control treatment groups. Also the Combretum micranthum methanol leaf extract was found, using GC-MS, to contain propargylamine, a compound known to possess anti-apoptosis functions, and also those with GABAergic functions 3,4-dichlorophenylhydrazine, guanidine and aminoxyacetic acid. Findings of the present study has proven Combretum micranthum to be a potential plant in managing and preventing brain structural damage induced by lead administration.

**Keywords:** Lead, Combretum micranthum, GABA, malondialdehyde (MDA) and histopathology

**INTRODUCTION**
Effects and implications of heavy metals intoxication cause chronic respiratory disease, leading to increased stress and risk to the nervous system (Mameli et al., 2001). Among heavy metals, lead represents a main environmental poison. Lead which is a soft, grey-blue heavy metal is a common cause of poisoning in domestic animals throughout the world (Khan et al., 2008). Lead is a poisonous metal, which exist in both organic (Tetraethyl lead) and inorganic (lead acetate, lead chloride) forms in the environment (Shalan et al., 2005).
Exposure to lead mainly occurs through the respiratory and gastrointestinal systems, where a small quantity is excreted in urine and the rest accumulates in various body organs, resulting to neurotransmitters imbalance and some morphological alterations in the cellular and intercellular levels (Ibrahim et al., 2012). Exposure of animals to lead caused cerebellar edema, cerebral satellitosis and encephalomalacia in the brain, as well as impairments in cortex, hippocampus and cerebellum (El-Neweshy and ElSayed, 2011). Stress-induced changes affect both neuronal and glial metabolism that can affect GABA levels (Kiss et al., 2008).

*Combretum micranthum* is a shrub species belonging to the family of Combretaceae, commonly called kinkeliba (health tree) in Benin, Senegal, Gambia and across multiple regional dialects of West Africa (Welch et al., 2017). It is known locally as Géézà in (Hausa), Okan (Yoruba) and Nza otego (Igbo) in Nigeria and often found in bushes and on hills in West Africa (Burkill, 1985). Ethanol extract of *Combretum micranthum* leaf is reported to be rich in polyphenols (tannins, flavonoids and other components) and is a widely known ethnomedicinal plant used in West Africa for treating several conditions such as fatigue, liver ailments, headache, convalescence, blood disease, weight loss, cancer and sleep problems (Welch et al., 2017). The present study aimed at evaluating the protective effects of *Combretum micranthum* methanol leaf extract on brain cell damage induced by lead acetate in mice.

**MATERIALS AND METHODS**

**Collection and Identification of Plant**
Fresh leaf of *Combretum micranthum* plant were obtained from Shira Local Government Area (N 11° 27' 29" and E 10° 2’ 48") of Bauchi State in Nigeria. The plant was identified in the Herbarium Unit, Department of Biological Sciences, Bayero University, Kano, and a voucher specimen number of BUKKHAN 0272 was issued.

**Chemicals, Reagents and Equipment**
All chemicals and reagents used for the research were of analytical grade and purchased from reputable chemical manufacturers. 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 Department, Aminu Kano Teaching Hospital, AKTH. 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 (IRAC, 1983; NRC, 1997, NIH, 2008 and OECD, 1999).

**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.

**Preparation of the Extract**
The leaves of *Combretum micranthum* plant were shade-dried, powdered to get a coarse powder and stored in a well closed container. The dried coarse powder was subjected to Microwave Assisted Extraction Method (HiNaRi Microwave Appliance: Model No.: MX 120BTC, 240V, 2450MHz, 1350Watts made in Korea). For Microwave-Assisted Extraction (MAE) of the *Combretum micranthum*, the powdered samples were mixed thoroughly with a suitable modifier (MeOH – H₂O in ratio of 4:1) as needed for the specific experiment. Sufficient time was allowed for the powder to absorb the modifier and get saturated. The saturated powder was then placed into the extraction vessel, and an appropriate amount of the extracting solvent was added. Different time of irradiation with the microwave extractor operating at an appropriate power level (850watts) is needed for MAE. The samples was treated in an intermittent way, i.e., irradiation–cooling–irradiation under microwave maintaining a particular ratio of irradiation and cooling time (5:5) (Waghmare et al., 2015). The samples was filtered and concentrated to 1/10 volume (<40°C). this was followed by acidifying it with 2M Tetraoxosulphate (vi) acid (H₂SO₄), then extracted with chloroform (CHCl₃ x3) giving two layers: CHCl₃ layer containing nonpolar compounds (Terpenoids) and the aqueous acidic layer containing highly polar compounds (flavonoids). The aqueous layer was then purified by basifying to pH 10 with sodium hydroxide (NaOH) and further extracted with chloroform-methanol (3:1) twice followed by extraction with chloroform. The aqueous basic layer was then concentrated and finally extracted with methanol (Harborne, 1989).
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 administered orally at a dose of 40 mg/kg b.w. (Wang et al., 2007).
(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 (Wolfman et al., 1994) was administered simultaneously.

The treatment period for the animals was carried out for two weeks (Wang et al., 2007). After which the animals in each group were sacrificed according to the world standard ethics (IRAC, 1983; NRC, 1996, NIH, 1996 and OECD, 2006).

Tissue Collection and Preparation of Tissue Homogenates
The mice from each group were sacrificed and dissected after the treatment period. Brains of the mice were quickly taken out and divided into two portions: one part was inserted into phosphate buffer for use in MDA and GABA levels determination and the other into formalin solution for use in histopathology analysis. All procedures were carried out in ice cold conditions.

Determination of Lipid Peroxidation (Cellular integrity)
Lipid peroxidation was determined as thiobarbituric acid reactive substance according to Ohkawa et al., (1979) with slight modification by Atawodi et al., (2005) using 15% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA). Lipid peroxidation generates peroxide intermediates which upon cleavage release malondialdehyde, a product which reacts with thiobarbituric acid. The product reaction is a coloured complex which absorbs light at 532nm and can hence be measured.

Histopathology
The tissues were immediately fixed in 10% formal saline after removal from the animal for 48 hours. After the fixing process was completed the tissues were processed by passing them through ascending grades of methanol from 70% to 90% and 100% methanol for a period of 12 hours to properly dehydrate them after which they were cleared in xylene for 2 hours and then infiltrated and embedded in liquid paraffin wax. The tissues were then cut using rotary microtome at 5 micron thickness and the section was stained using haematoxylin and eosin staining technique. After dehydration and cleaning, sections were finally viewed under light microscope (Harris, 2000).

Determination of Gamma-Aminobutyric Acid (Neurotransmitter) assay
Gamma-aminobutyric acid (GABA- a major neurotransmitter) levels was determined using Rat Gamma-aminobutyric acid (GABA) ELISA kit (Shanghai Koon Biotech Co., Ltd., China), based on the principle of biotin antibody sandwich technology enzyme linked immunosorbent assay (ELISA).

Phytochemical Screening by GC-MS
Methanol extract (0.5g) was dissolved 95% methanol. The extract was filtered through microfilter 0.45 µm, then 2 µl of this solution was employed for GC/MS screening. GC-MS screening was carried out on a Shimadzu GCMS-QP2010Ultra system comprising a gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100% Dimethyl poly siloxane), helium (99.9%) was used as carrier gas at a Flow Control Mode: Pressure 100.0 kPa, Total Flow: 17.6 mL/min, Column Flow: 1.33 mL/min, Linear Velocity: 43.0 cm/sec, Purge Flow: 3.0 mL/min, Split Ratio:10.0, injector temperature 220°C; ion-source temperature 200°C. The oven temperature was programmed from 100°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 220°C, ending with a 9 min isothermal at 220°C.
Mass spectra were taken at 70 eV, then the time required for sample chromatography was 20 minutes (Poaranthaman et al., 2012). Phytochemicals were identified using MassHunter\Library\NIST14.L at Multi-User Science Research Laboratory Ahmed Bello University Zaria.

**Statistical Analysis**

All the assays were conducted in triplicates and results were expressed as Mean ± SEM (standard error of the mean). Data were analyzed using One Way Analysis of Variance (ANOVA) for the Biochemical Analysis while Post Hoc Test (Tukey) was performed to compare difference within the treatment groups. A 0.05 level of probability was used as the criterion of significance in all cases. All the statistical analysis were carried out using SPSS Software and Microsoft Excel Spread Sheet.

**RESULTS AND DISCUSSION**

**Malondialdehyde (MDA) Level:**

The brain Malondialdehyde levels in the normal and all the experimental groups are presented in Table 1. Mice administered doses (25 mg/kg, 50 mg/kg and 100 mg/kg) of the extract elicited a general significant decrease (P< 0.05) in level of malondialdehyde (MDA) as compared to those treated with 40 mg/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.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>19.03±0.63&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>40 mg/kg Lead Acetate</td>
<td>30.03±1.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lead Acetate + 100 mg/kg extract</td>
<td>18.99±1.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lead Acetate + 50 mg/kg extract</td>
<td>24.75±1.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lead Acetate + 25 mg/kg extract</td>
<td>23.14±0.78&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lead Acetate + 0.5 mg/kg Diazepam</td>
<td>20.27±1.48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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

**Histopathological findings**

Results from histopathological studies of the brain samples of treatment groups are given in plate 1. The results showed Intense Neuronal Necrosis (NN) changes in morphology of the brain structure induced by lead through oxidative stress in mice exposed to 40 mg/kg lead acetate (negative control) orally for 14 days, as was observed in the transverse section of the brain (plate 1B). However, there were no alterations in brain histology in animals that were treated with only distilled water (normal control) and those treated with lead acetate + 100 mg/kg *Combretum micrantum* extract (plate 1A and plate 1C respectively), but slight neuronal necrosis and atrophy was found on those treated with Lead Acetate + 50 mg/kg *Combretum micrantum* extract and Lead Acetate + 25 mg/kg *Combretum micrantum* extract (plate 1D and plate 1E respectively), while neuronal pigmentation was seen on those treated with lead acetate + 0.5 mg/kg diazepam (positive control) (plate 1F) during the same treatment period.
Plate I: Transverse section of the brain of lead induced mice administered different doses of *C. micrantum* leaf extract for 14 days (Haematoxylin and Eosin Staining X 400).

A: showing Normal Neurons (N), B: showing Intense Neuronal Necrosis (NN), C: showing Normal Neurons (N), D: showing Slight Neuronal Necrosis (SN) and Atrophy (NA), E: showing Slight Neuronal Necrosis (SN) and Atrophy (NA) and F: showing Neuronal Pigmentation (NP)

**Gamma-Aminobutyric Acid (GABA) level**
The brain GABA contents in the normal and all the experimental mice are presented in Table 2. ANOVA indicated a significantly (*p<0.05*) increase in GABA level in the brain of mice administered 100 mg/kg dose of the extract as compared to those treated with 40 mg/kg lead acetate alone, but it shows no significant difference with animals in the normal control and positive control treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>GABA Content (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>387.92±20.21b</td>
</tr>
<tr>
<td>40mg/ml Lead Acetate</td>
<td>310.83±5.52a</td>
</tr>
<tr>
<td>Lead Acetate + 100 mg/kg Extract</td>
<td>387.49±16.31b</td>
</tr>
<tr>
<td>Lead Acetate + 50 mg/kg Extract</td>
<td>339.78±8.60</td>
</tr>
<tr>
<td>Lead Acetate + 25 mg/kg Extract</td>
<td>334.56±10.64</td>
</tr>
<tr>
<td>Lead Acetate + 0.5 mg/kg Diazepam</td>
<td>374.44±19.53b</td>
</tr>
</tbody>
</table>

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

**Phytochemical Composition by GC-MS Analysis**
In GC-MS analysis, 6 bioactive phytochemical compounds were identified in the methanol leaf extract of *Combretum micrantum* as shown in Table 3. The identification of phytochemical compounds is based on peak area, molecular weight and molecular formula from the library data of corresponding compounds.
Table 3: Phytocomponents identified in the Methanol extract of *Combretum micranthum* by GC-MS.

<table>
<thead>
<tr>
<th>Number of peaks</th>
<th>Retention time</th>
<th>Peak area %</th>
<th>Compound name</th>
<th>Molecular weight</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43.64</td>
<td>3.73</td>
<td>2-dimethylAcetic acid</td>
<td>261</td>
<td>C₇H₉NO₃</td>
</tr>
<tr>
<td>2</td>
<td>36.98</td>
<td>0.91</td>
<td>(aminoxy)-Ethanamine</td>
<td>2417</td>
<td>C₉H₈N₂O</td>
</tr>
<tr>
<td>3</td>
<td>40.53</td>
<td>1.48</td>
<td>Propargylamine</td>
<td>158</td>
<td>C₇H₇N</td>
</tr>
<tr>
<td>4</td>
<td>43.64</td>
<td>3.73</td>
<td>3,4-dichlorophenylhydrazine</td>
<td>277</td>
<td>N₂H₄</td>
</tr>
<tr>
<td>5</td>
<td>36.98</td>
<td>0.91</td>
<td>(aminoxy)-Acetic acid</td>
<td>2406</td>
<td>C₇H₅NO₃</td>
</tr>
<tr>
<td>6</td>
<td>88.09</td>
<td>0.92</td>
<td>Guanidine</td>
<td>730</td>
<td>HNC(NH₂)₂</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Reactive oxygen species attack cellular components containing polyunsaturated fatty acid residues to produce peroxy radicals which undergo a cyclization reaction to form endoperoxides and eventually trans-4-hydroxy-2-nonenal and MDA (Piccoli *et al.*, 2012). The decrease in the brain MDA levels in animals administered doses of the extracts together with 40mg/kg lead acetate for 14 days as compared to animals administered 40mg/kg lead acetate alone through same period as observed in the study, clearly indicated the protective action of the extract against state of stress in the brain tissues, that may possibly be caused by the lead toxicity. Histopathological examination of brain sections also shows no alterations in brain histology in animals that were administered the extract (100mg/kg) together with lead acetate orally for 14 days as compared to the intense neuronal necrosis observed in the brains of mice exposed to 40 mg/kg lead acetate alone for same days. This can be associated with increased MDA as lipid peroxidation indicators (Dewanjee *et al.*, 2013). The anti-necrotic activity of the *Combretum micranthum* methanol leaf extract could be attributed to its phytocomponents such as propargylamine compound, which serves as a functional moiety for neurodegenerative disorders by stabilizing mitochondrial membrane potential and induced anti-apoptotic Bcl-2.4 (Zindo *et al.*, 2015). Also, the significantly (p<0.05) increased in GABA content in the brain of the mice administered with 40 mg/kg lead acetate and the extract for 14 days as compared to mice administered 40 mg/kg lead acetate alone, is in line with previous report (Adonaylo and Oteiza, 1999), that Pb and Ca²⁺ may competitively interact at the sites involved in the release of neurotransmitters, resulting in the inhibition of Ca-dependent stimulus-coupled release. Disrupting of calcium homeostasis and inhibition of thiol-containing enzymes leads to inhibition of GABA. The GABAergic activities of the plants could be attributed to its bioactive compounds (hydrazine, guanidine and aminoxyacetic acid) that are capable of enhancing GABA activity (Valenti *et al.*, 2012). Hydrazine functions in elevating effect of GABAn the brain (Matsuyama *et al.*, 1983). Aminoxyacetic acid is a compound that serves as GABAergic agent by inhibiting aminobutyrate aminotransferase activity *in-vivo*, thereby raising the level of gamma-aminobutyric acid in tissues (Bingham *et al.*, 2001). Guanidine compounds act as ion channel modulators. Guanidine exhibits characteristics consistent with a positive allosteric modulator for the human GABA-A receptor (Heather and Eric, 2016). Hence the possible neuroprotection observed in the groups administered *Combretum micranthum* methanol leaf extract against lead toxicity, could be attributed to its phytocomponents (N-Propargylamine, hydrazine, guanidine and aminoxyacetic acid).

**CONCLUSION**

The findings of the present study showed that compounds present in *Combretum micranthum* methanol leaf extract have beneficial effect in modulating and maintaining brain cells integrity which could have greater importance as therapeutic agent in preventing or slowing neurodegenerative related diseases. Hence *Combretum micranthum* is a potential plant in preventing cell membrane neuronal damage induced by heavy metals like lead.

**Conflict of interests**

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

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