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ESTIMATION OF ANTIOXIDANT PROPERTIES OF *Combretum micranthum* METHANOL LEAF EXTRACT AS AN INDEX FOR NEUROPROTECTION AGAINST LEAD INDUCED TOXICITY IN MICE

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Abstract

Combretum micranthum is a medicinal plant used for the treatment of various diseases in the North-Eastern part of Nigeria with little or no scientific basis. It is thus the aim of this research to validate antioxidant capacity of *Combretum micranthum* (CM) methanol leaf extracts as an index for its neuroprotective potential against lead induce toxicity in the brain of mice. Thirty six (36) Swiss Albino mice (19 - 22g body weight) were used for the study, randomized equally into six groups of six animals each and treated for the period of 14days: Normal Control (Distilled water), Negative control (40mg/kg lead acetate), Group III (lead acetate + 100 mg/kg b.w. extract), Group IV (lead acetate + 50 mg/kg b.w. extract), Group V (lead acetate + 25 mg/kg b.w. extract) and Positive control (lead acetate + 0.5 mg/kg b.w diazepam). The study assayed for the neuroprotective potential of *Combretum micranthum* by following in vitro tests (DPPH, Metal Chelating, and Reducing Power assays) and in-vivo tests (levels of glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD)). The study indicated a significant neuroprotective effect of the *Combretum micranthum* methanol extract in terms of its IC50 values (DPPH: 0.467 mg/ml, Metal Chelating: 0.290 mg/ml, and Reducing Power: 0.004 mg/ml) in the *in vitro* tests and also indicated a significant increase of GSH, CAT and SOD levels in the brain of mice administered doses of the extract, as compared to those mice treated with 40 mg/kg lead acetate alone, which showed decreased level in the antioxidants (GSH: 23.34±1.43µG/ML, CAT: 8.65± 0.77U/mg and SOD: 10.93±0.30 U/mg) in the in vivo tests. Also, the extract was found, using GC-MS, to contain propargylamine, guanidine and acetic acid, compounds known to possess antioxidant functions. Thus, findings of the present study suggested *Combretum micranthum* to be a potential plant in preventing oxidative stress and brain damage that may be induced by lead administration.

INTRODUCTION

Among heavy metals, lead represents a main environmental poison. This pollutant causes well documented neurological impairment [1]. Lead is a toxic heavy metal widely distributed in the environment and chronic exposure to levels of lead has been a matter of public health concern in many countries [2]. Nigerian Federal Ministry of Health states the discovery of 355 cases of lead poisonings in Zamfara State, Nigeria with 46 percent

proving fatal, which led to the deaths of at least 163 people between March and June 2010, including 111 children [3]. An estimated number of about 400 children died from lead intoxication in Nigeria, Laboratory testing later confirmed high levels of lead in the blood of the surviving children [4]. One possible molecular mechanism involved in lead neurotoxicity is the disruption of the antioxidant balance, which can lead to brain injury via oxidative damage to critical biomolecules, such as lipids, proteins and DNA [5].

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 and often found in bushes and on hills in West Africa [6,7]. 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 [7]. In the present study, the effect of *Combretum micranthum* methanol leaf extract against the toxicity of lead acetate on brain tissues of mice were investigated by examination of its antioxidant efficiency.

MATERIALS AND METHODS

Plant Material Collection and Identification

Fresh samples of the plants *Combretum micranthum* (CM) were obtained from Shira Local Government Area (N 11O 27' 29" and E 10O 2' 48") of Bauchi State in Nigeria. The plants were identified at the Herbarium Unit, Department of Biological Sciences, Bayero University Kano (B.U.K.). A voucher specimen (No. BUKHAN 0272) was deposited and preserved in Herbarium Department of Botany, Bayero University Kano for further reference.

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.

Ethical Clearance

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

Preparation of *Combretum micranthum* Extract

The leaves of the plants *Combretum micranthum* used for the study after collection 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 – H_2O in a 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 times of irradiation with the microwave extractor operating at an appropriate power level (850 watts) was used for MAE. The sample was treated intermittently, i.e., irradiation–cooling–irradiation under microwave maintaining a particular ratio of irradiation and cooling time (5:5) minutes in 5 cycles [8]. The samples were further filtered and concentrated under vacuum using a Rotary Evaporator. The filtrate was then separated for flavonoids by evaporating the filtrate to 1/10 volume (<40 ^oC) followed by acidifying it with 2 M Tetraoxosulphate (VI) acid (H_2SO_4), then extracted with chloroform ($CHCl_3$ x3) giving two layers: $CHCl_3$ layer containing non-polar 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 is then concentrated and finally extracted with methanol [9].

In Vitro Antioxidant Tests of *Combretum micranthum* Samples

DPPH scavenging activity

The DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) assay has been widely used to determine the free radical-scavenging activity of various plants and pure compounds. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals were monitored according to Manzocco et al., [10]. The sample extract (0.2 mL) is diluted with methanol and 2 mL of DPPH solution (0.5 mM) was added at different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0ml). After 30 min, the absorbance is measured at 517 nm. The percentage of the DPPH radical scavenging is calculated using the equation: % inhibition of DPPH radical = (AB – AS)/AB x 100 (where AB is the absorbance blank and AS is the absorbance test sample has taken place).

Metal chelating activity

The chelation of ferrous ions is estimated using the method of Dinis et al.,[11]. Exactly 0.1 mL of the extract at different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0ml) is added to a solution of 0.5 mL ferrous chloride (0.2 mM). The reaction is started by the addition of 0.2 mL of ferrozine (5 mM) and

incubated at room temperature for 10 min and then the absorbance is measured at 562 nm.

Percentage of inhibition (%) of Metal chelating activity = (AB – AS)/AB x 100 (where AB is the absorbance blank and AS is the absorbance test sample has taken place).

Reducing power method (RP)

In the method described by Oyaizu [12]. About 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of K3Fe (CN)6(1% w/v) are added to 1.0 mL of sample dissolved in distilled water at different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0ml). The resulting mixture is incubated at 50 C for 20 min, followed by the addition of 2.5 mL of Trichloroacetic acid (10% w/v). The mixture is centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of FeCl3 (0.1%, w/v). The absorbance is then measured at 700 nm against a blank sample.

Experimental Design

Animals were equally randomized to six groups of 6 animals:

(a) Normal Control: administered with Distilled water only.
(b) Negative Control: Lead acetate was administered orally at a dose of 40 mg/kg b.w [13].
(c) A dose of 40 mg/kg lead acetate and 100 mg/kg b.w. *C. micranthum* was administered simultaneously
(d) A dose of 40 mg/kg lead acetate and 50 mg/kg b.w. *C. micranthum* was administered simultaneously
(e) A dose of 40 mg/kg lead acetate and 25 mg/kg b.w. *C. micranthum* was administered simultaneously.
(f) Positive Control: A dose of 40 mg/kg lead acetate and a standard drug diazepam 0.5 mg/kg [14] was administered simultaneously.

The treatment period for the animals was carried out for two weeks [13]. After which the animals in each group were sacrificed according to the world standard ethics [15-18].

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, inserted into phosphate buffer and used for the in-vivo antioxidants levels determination. All procedures were carried out in ice-cold conditions.

Measurement of Antioxidant Activities:

Reduced glutathione (GSH) assay

Reduced Glutathione (GSH) assay was done according to Ellman [19] as described by Rajagopalan et al., [20]. To 150 uL of serum (in phosphate pH 7.4), 1.5 ml of 10% TCA was added and centrifuged at 1500g for 5 minutes. Thereafter, 1 ml of the supernatant was treated with 0.5 ml of Ellman'sreagent. A blank was prepared with 1ml of the diluted precipitating agent and 0.5 ml of Ellman's reagent. Reduced glutathione, GSH, is proportional to the absorbance at 412 nm.

Catalase activity (CAT)

Catalase activity was measured using Abei's method [21]. Exactly 10 uL of serum was added to the test tube containing 2.80 ml of 50 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1 ml of freshly prepared 30 mM H_2O_2 and the decomposition rate of H_2O_2 was measured at 300 nm for 5 minutes on a spectrophotometer.

Superoxide dismutase (SOD) activity

The level of SOD activity was assayed by the method described by Fridovich [22]. The principle works based on superoxide dismutase to inhibit auto-oxidation of adrenaline at pH 10.2. About 0.1 ml of the serum was diluted in 0.9 ml of distilled water to make 1:10 dilution of serum. An aliquant mixture of 0.2 ml of the diluted serum was added to 2.5 ml of 0.05 M Carbonate buffer. The reaction was started with the addition of 0.3 mM Adrenaline. The reference mixture contained 2.5 ml of 0.05 M Carbonate buffer, 0.3 ml 0.3 mM of distilled water. Absorbance was measured over 30 s up to 150 s at 450nm.

Phytochemical Screening by GC-MS

About 0.5g of the methanol extract was dissolved in 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 polysiloxane), 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 [23]. Phytocomponents were identified using MassHunter\Library\NIST14.L at

Multi-User Science Research Laboratory of Ahmadu 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) and Post - Hoc Test (Tukey) was performed to compare differences within the treatment groups. A 0.05 level of probability was used as the criterion of significance in all cases. All the statistical analysis was carried out using SPSS Software and Microsoft Excel Spread Sheet.

RESULTS

In Vitro Tests

As presented in Figures 1, Figure 2 and Figure 3, DPPH, Metal chelating and Reducing power inhibition values are dose-dependent, whereby they increased in the range of the tested concentrations, for the extract and the standard control used (Ascorbic acid). Also, the DPPH, Metal chelating radical inhibition decreased in the order Ascorbic acid > Methanol extract while Reducing power decreased in the order of methanol extract > Ascorbic acid as determined from their IC50 values presented in Table 1.

In Vivo Analysis

The brain Reduced Glutathione (GSH), Catalase (CAT) and Superoxide dismutase (SOD) levels in the normal and all the experimental mice are presented in Table 2. ANOVA indicated a significant increase in the level of Superoxide dismutase (SOD) and Catalase (CAT) in the brains of mice administered the extract (Lead Acetate + 100mg/kg Methanol) as compared to those treated with 40mg/kg lead acetate alone, but it shows no significant difference with animals in the normal control and positive control treatment groups. Also, it revealed a significantly increased level of Reduced Gluthathione (GSH) but only to animals in Lead Acetate + 100mg/kg Methanol treatment group as compared to those animals in the other groups.

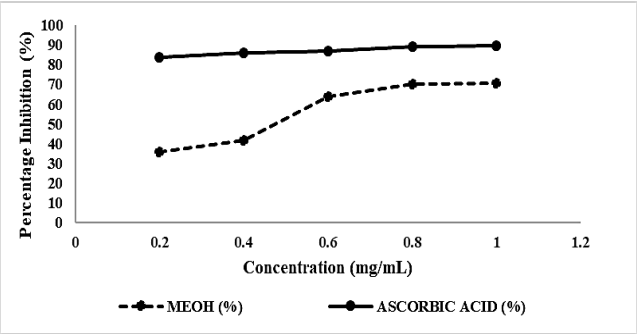

Figure. 1. Effect of different concentration of *Combretum. micranthum* extract and ascorbic acid (standard) on DPPH activity.

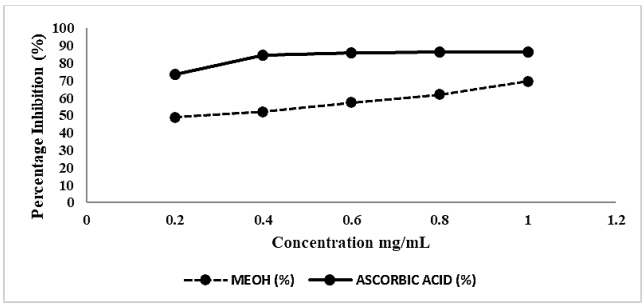

Figure 2. Effect of different concentrations of *Combretum micranthum* extract and ascorbic acid (standard) on metal chelating activity.

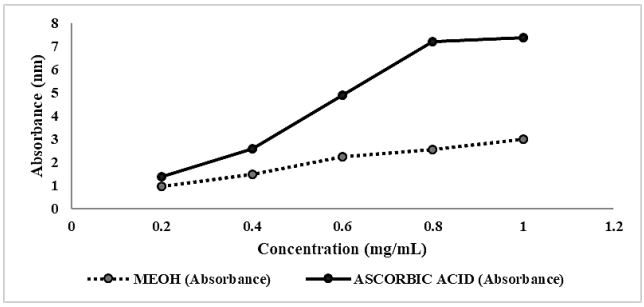

Figure 3. Effect of different concentrations of *Combretum micranthum* extracts and ascorbic acid (standard) on reducing power activity.

Table 1: Antioxidant activity of the extracts (IC_{50}) in terms of DPPH, metal chelating and reducing power, protection assay

Extracts	DPPH (mg/ml)	Metal Chelating (mg/ml)	Reducing Power (mg/ml)
Methanol	0.467	0.290	0.004
Ascorbic Acid	-4.484	-1.811	0.096

Phytochemical Composition by GC-MS Analysis

Combretum micranthum ethanol extract revealed the presence of medicinal active constituents. In the GC-MS analysis, 6 bioactive phytochemical compounds were identified in the methanol extract of *Combretum micranthum* as shown in Table 3. The identification of phytochemical compounds is based on the peak area, molecular weight and molecular formula from the library data of corresponding compounds.

Table 2: Results of antioxidants (superoxide dismutase, reduced gluthathione and catalase) levels in the brains of the experimented mice

Group	SOD (U/mg)	GSH (ug/ml)	CAT (U/mg)
Distilled Water	13.63±1.30	25.28± 1.90^{b}	10.73 ± 0.43^{b}
4mg/ml Lead Acetate	10.93±0.30^{a}	23.34±1.43^{b}	8.65 ± 0.77^{a}
Lead Acetate + 100mg/kg MEOH	15.80±1.32^{b}	35.75±0.44^{a}	10.86 ± 0.21^{b}
Lead Acetate + 50mg/kg MEOH	12.73±0.22	26.45±0.50^{b}	9.82 ± 0.35
Lead Acetate + 25mg/kg MEOH	11.46±0.35	27.13±1.73^{b}	9.39 ± 0.26
Lead Acetate + 0.5mg/kg Diazepam	14.08±1.44	27.22±0.90^{b}	10.59 ± 0.34 ^{b}

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

Table 3: Bioactive components identified in the methanol extract of *Combretum micranthum* by GC-MS

Number of peaks	Retention	Peak area (%)	Compound name	Molecular weight	Formula
1	36.98	0.91	Acetic acid	2403	CH_3COOH
2	40.53	1.48	Propargylamine	158	C_3H_5N
3	40.53	1.48	Propanenitrile	156.5	C3H5N
4	44.00	1.51	2-Propenal	167	CH_2=CHCHO
5	66.503	39.50	2-methyl-Methional	4799	$CH_3SCH_2CH_2CHO$
6	88.09	0.92	Guanidine	730	$HNC(NH_2)_2$

DISCUSSION

Ability of the plants to neutralized free radical effects can be supported by in vitro antioxidants (radical scavenging) activities [24]. The scavenging activity of DPPH, Metal chelating and Reducing power by the present plant extract was found to be appreciable. The radical scavenging activities of the plants could be attributed to the total flavonoids contents, compounds that are capable of donating hydrogen to a free radical to remove odd electron which is responsible for radical's reactivity [25].

The brain is an essential organ of the body which is reported to be impaired on exposure to free radicals, generated by heavy metals (lead) with respect to time [26]. To understand the clinical syndromes of heavy metal-induced human diseases, it is important to use in vivo animal models [27]. Heavy metal-induced oxidative stress has also been the focus of toxicological research for the last decade to evaluate their possible mechanism of toxicity [28]. Cells try to counter oxidative stress using the first line defence system such as radical-scavenging enzymes like SOD, CAT, GST and GPx [29]. Reactive oxygen species attack cellular components containing polyunsaturated fatty acid residues to produce peroxyl radicals that undergo a cyclization reaction to form endoperoxides [30]. Superoxide dismutase (SOD) and catalase (CAT) are endogenous antioxidant enzymes responsible for the detoxification of deleterious oxygen radicals and their activities are used to assess oxidative stress in cells [31]. The first line of defense to the cells is provided by the existence of a mutually supportive relationship between metalloenzyme SOD, which accelerates the dismutation of endogenous cytotoxic superoxide radicals to H_2O_2, and CAT, which converts the deleterious peroxide radicals into water and oxygen [28, 31].

In the present study, the activity of the brain SOD and CAT levels was increased in animals co-administered with the extract as compared to those administered 40mg/kg lead acetate alone. The induction of these antioxidant enzymes may indicate an adaptive response to counter the damaging effect of oxidative stress possibly generated during metabolism. Similar results have been also reported by Lakshmi et al, [30] and Dewanjee et al, [31]. Glutathione (GSH), also an antioxidant in plants, is capable of preventing damage to important cellular components caused by reactive oxygen species by playing a pivotal role in the scavenging of hydroxyl radical and singlet oxygen directly as well as in the detoxification of hydrogen peroxides and lipid hydro-peroxides by the activity of GSH [28, 32]. The observed decrease in the brain GSH level (for 40mg/kg lead acetate treatment group) in the present study may suggest an increased demand or overutilization of GSH by the cell possibly to combat ROS generation in the mice.

The antioxidant activities of the Combretum micranthum methanol leaf extract could be attributed to the

compounds present in it (Table 3), which is capable of removing electrons that are responsible for radical's reactivity. Acetic acid is involved in the mechanisms of converting free radicals to a safer product. The oxidative addition of acetic acid to alkenes by two equivalents of Mn(OAc)3 in AcOH at reflux to give γ-lactones [33].The novel class of N-propargylaminenitroxyls, JSAKs, has an ability to either block or reduce the progression of neurotoxic cascade of brain damage, where ROS mediate deleterious effects [34]. Guanidine, aminoguanidine and methylguanidine have direct scavenging activities against H_2O_2, HOCl, hydroxyl radical and peroxynitrite [35]. Hence the possible significance increased antioxidant parameters (SOD, GSH, CAT) levels in the groups administered *Combretum micranthum* methanol leaf extract could be attributed to the presence of these phytocomponents (propargylamine, guanidine and acetic acid).

CONCLUSION

In conclusion, this study is evident that Lead caused oxidative stress in brain tissues. We observed more reduction in SOD, CAT and GHS activities in the group administered only lead compound. However, the groups administered, combination of *Combretum micranthum* methanol leaves extract and the lead compound, have normal/increased SOD, CAT and GHS activities. Thus, findings of the present study showed that compounds present in *Combretum micranthum* methanol leaves extract have a beneficial effect in modulating the antioxidant enzymes in the brain of mice, this could have greater importance as therapeutic agents in preventing or slowing oxidative stress-related degenerative diseases. Hence *Combretum micranthum* is a potential plant in preventing the oxidative stress induced by lead toxicity.

CONFLICT OF INTERESTS

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

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