IGBINEDION UNIVERSITY OKADA
JOURNAL OF PHARMACEUTICAL SCIENCES

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ANTIOXIDANT POTENTIALS OF ETHANOL LEAF EXTRACT OF *MILICIA EXCELSA* (WELW.) C. C. BERG. (MORACEAE)

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ABSTRACT

Background and aim: Previous studies have demonstrated the antipsychotic, antidepressant and antiamnesic effects of *Milicia excelsa* leaf extract in various brain disorders. However, there are scanty reports on the antioxidant effect of the leaf despite the involvement of oxidative stress in the pathogenesis of brain disorders.
Methods: This study assessed the antioxidant potential of ethanol leaf extract of *Milicia excelsa* in mice at 250, 500 and 100 mg/kg via *in vivo* assay and 0.078 to 10 mg/ml via *in vitro* assay using standard methods.
Results: Extract showed *in vitro* antioxidant potential in all model assays. It showed no significant ($p>0.05$) increase in malondialdehyde level, but significantly ($p<0.05$) elevated antioxidant indices of superoxide dismutase, catalase and reduced glutathione.
Conclusion: This study therefore, lend credence to the antioxidant potential of *M. excelsa* leaf to justify its various reported uses in literature.

Keywords: *Milicia excelsa*, ethanol extract, leaf, antioxidant activities

INTRODUCTION

Oxidative stress manifests when the body antioxidant defense capacity cannot counteract the free radicals produced in the body [1]. It plays an indispensable role in the pathogenesis of several human diseases which may include autoimmune diseases, such as rheumatoid arthritis, cellular aging, cancer [2], respiratory diseases [3], cardiovascular diseases [4], neurodegenerative disorders [5], ocular disease [6], and renal diseases [7], amongst others [1].

Free radicals occur as a consequence of normal cellular metabolic functions, resulting in production of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. Production of these free radicals may be amplified as a consequence of both the external and internal factors [8, 9]. The external factors may include but not limited to environmental pollution, pesticide, cigarette smoke and toxic metals [8] while the internal factors may be as a consequence of impaired intracellular metabolism [9] or xenobiotics [10]. Free radicals may cause destruction of cellular molecules such as nucleic acid, lipids, and proteins, leading to their loss of functions and subsequently to disease states in living organism [11].

Both natural and synthetic antioxidants have been reported to scavenge free radicals [12]. In addition, synthetic antioxidants have been implicated as promoters of carcinogenesis, and consequently have found limited use in food, cosmetics, and pharmaceutical products [13]. Plant-derived antioxidants possessing free radical scavenging benefits could be helpful in brain disorders such as Parkinson disease [14], Alzheimer disease [15], epilepsy [16], stress [17], schizophrenia, depression and dependencies [18] due to vulnerability of the brain to oxidative damage. Medicinal plants have been an integral part of folkloric medicines over centuries with high potential for cure with nominal side effects and their use in preventive medicines [19].

Milicia excelsa (Welw.) C. C. Berg (Moraceae), popularly referred to as "Iroko tree" or African teak, is a large deciduous tree growing up to 50 m tall in its natural habitat, the humid forests of West Africa [20]. Its various plant parts are employed in indigenous medicine to treat mental illnesses [21], rheumatism, asthma, oedema, dysmenorrhoea, among other uses [22]. The antipsychotic [23], anticonvulsant [24], antidepressant [25] and anti-amnesic effects [26] of the leaf have been reported.

Due to implication of oxidative stress in the pathogenesis of diverse diseases as well as documented studies on the potential restorative effects of several medicinal plants [12, 15, 16], we evaluated antioxidant effects of *M. excelsa* leaf ethanol extract in mice using *in vivo* (brain homogenate) and *in vitro* models, to provide scientific basis for its usage in traditional medicine.

MATERIALS AND METHODS

Plant material and extraction

Leaves of *M. excelsa* were collected from the main campus of Obafemi Awolowo University (OAU) Ile-Ife, Osun State, Nigeria and authenticated (IFE- 17482) by Mr. G. A. Ademoriyo of the Herbarium Unit, Department of Botany OAU, Ile-Ife. Briefly, the leaves were air-dried for 14 days at room temperature and mechanically ground into fine powder. Extract was prepared as previously described [23], and 1.0 kg of fine powder macerated in 5L flat-bottomed flask with 70% ethanol for 3 days (72 h), filtered and filtrate concentrated *in vacuo* at 40^{o}C to

yield 70 g residue (7.0% w/w) which was stored in a desiccator prior to use.

Drugs and chemicals

DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate), ascorbic acid, 2-thiobarbituric acid, Tween 20 (Sigma Aldrich, USA), and other chemicals used were of analytical grade.

Animals

Adult mice of both sexes (18 - 25 g) were purchased from the Animal House of Faculty of Pharmacy, OAU, Ile-Ife. They were kept under a 12-hour daylight/ 12-hour dark natural cycle and sustained with standard commercial diet (Guinea feeds brand, Bendel Feeds Nigeria) and free access to clean drinkable water. Experimental protocols employed in this investigation were as approved by the Igbinedion University Okada Ethical Committee vide the approval number IUO/ETHIC/22/07 which is in conformity with the National Institute of Health (NIH, 1985) on the use of experimental animals in the laboratory.

Pharmacological experiments

General preliminary in vitro antioxidant assay: Preliminary *in vitro* antioxidant assays of ethanol leaf extract of *M. excelsa* (EME) were carried out using DPPH [27], inhibition of nitric oxide radical [28] and the ferrous ion-chelating FIC) [29] assay models at 0.078, 0.156, 0.31, 0.63, 1.25, 2.50, 5.00 and 10.00 mg/ml extract. Ability of EME to scavenge free radical was assessed using the stable radical DPPH. The antioxidant agent donates hydrogen which reacts with DPPH and causes its reduction accounting for its colour change from deep violet to light yellow when measured spectrophotometrically (UV–VIS spectrophotometer, PEC MEDICA, USA) at 517 nm. Nitric oxide radical inhibitory assay involves the interaction of extract with Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) (NED). Absorbance of chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with NED was read at 546 nm using a UV–VIS spectrophotometer (PEC MEDICA, USA). Ferrous Ion Chelating (FIC) assay measures the capacity of extract to chelate free ferrous ions in solution thereby inhibiting Fe (II) binding to ferrozine which generates a highly colored complex measured spectrophotometrically (UV–VIS spectrophotometer, PEC MEDICA, USA) at 562 nm.

General experimental design for in vivo antioxidant determination: Adult Swiss albino mice of either sex (18-25 g) were sorted into 4 groups (n=5). Group 1 mice were orally fed with 2% Tween 20 in normal saline (10 mg/kg, p.o.) and served as the control. Groups 2 to 4 received 250, 500 and 1000 mg/kg EME dissolved in 2% v/v Tween 20 in normal saline respectively. Mice in Groups 1 to 4 were fed for 7 consecutive days as above.

Tissue preparation for in vivo antioxidant assays: On day 8, 24 hours after the last treatment, mice were mildly anesthetized with anesthetic ether, sacrificed and their whole brain collected. Each fresh whole brain was weighed and homogenized in an ice bath with a glass homogenizer, after addition of 10 times their weight of normal saline (0.9 % NaCl). Resultant homogenate was further centrifuged at 3000 rpm for 10 minutes, and resulting cloudy supernatant liquid used for various antioxidant assays [26].

Determination of malondialdehyde (MDA): Thiobarbituric acid (TBA) procedure [30] which quantifies the MDA reactive product was employed to estimate lipid peroxidation in EME treated mice brains. To brain sample

of 0.5 ml was added 0.5 ml phosphate buffer (0.1 M, pH 8.0), 0.5 ml 24% trichloroacetic acid and mixture incubated at room temperature for 10 minutes. Product was subsequently centrifuged at 2000 rpm for 20 minutes. To 1.0 ml of resulting supernatant was added 0.25 ml 0.33% TBA in 20% acetic acid, boiled at 95ºC for 1 hour. Pink coloured product formed was cooled and absorbance read at 532 nm using a spectrophotometer (PEC MEDICA, USA). Values were calculated using molar extinction coefficient of MDA $\varepsilon 532 = 1.53 \times 10^5\ M^{-1}\ cm^{-1}$, MDA reactive product expressed in µM.

Determination of Superoxide Dismutase (SOD): The method employed in the estimation of SOD from brain samples of EME treated mice was as previously described [31]. Briefly, to 200 µL of brain sample homogenate was added 2.5 ml 75 mM of Tris–HCl buffer (pH 8.2), 30 mM EDTA and 300 µL of 2 mM pyrogallol were added and absorbance recorded at 420 nm for 3 minutes using spectrophotometer. Activity of SOD is expressed as units/L.

Evaluation of Catalase (CAT) activity: CAT activity was estimated on the brain samples as earlier described [32]. Briefly, to 50 µL of brain sample was added 450 µL phosphate buffer (0.1M, pH 7.4) and 500 µL 20 mM H_2O_2 in a cuvette and absorbance determined at 240 nm for 1 min using a spectrophotometer (PEC MEDICA USA). Molar extinction coefficient of H_2O_2 0.043.6 mM cm^{-1} was used to estimate CAT activity. One unit of activity is equal to 1 mmol of H_2O_2 degraded per minute which is expressed as units/ml.

Brain glutathione (GSH) estimation: The method employed in the estimation of GSH from the brain sample of EME-treated mice was as earlier reported [33]. To 1.0 ml of brain supernatant obtained after centrifugation of each mouse brain homogenate was added 0.5 ml 10 mM Ellman's reagent and 2.0 ml 0.2 M phosphate buffer (pH 8.0). Resultant yellow coloured-product was spectrophotometrically (PEC MEDICA USA) determined at 412 nm against a blank containing 3.5 ml phosphate buffer. A series of standards were also treated similarly. Estimated amount of GSH in brain samples was expressed in µM.

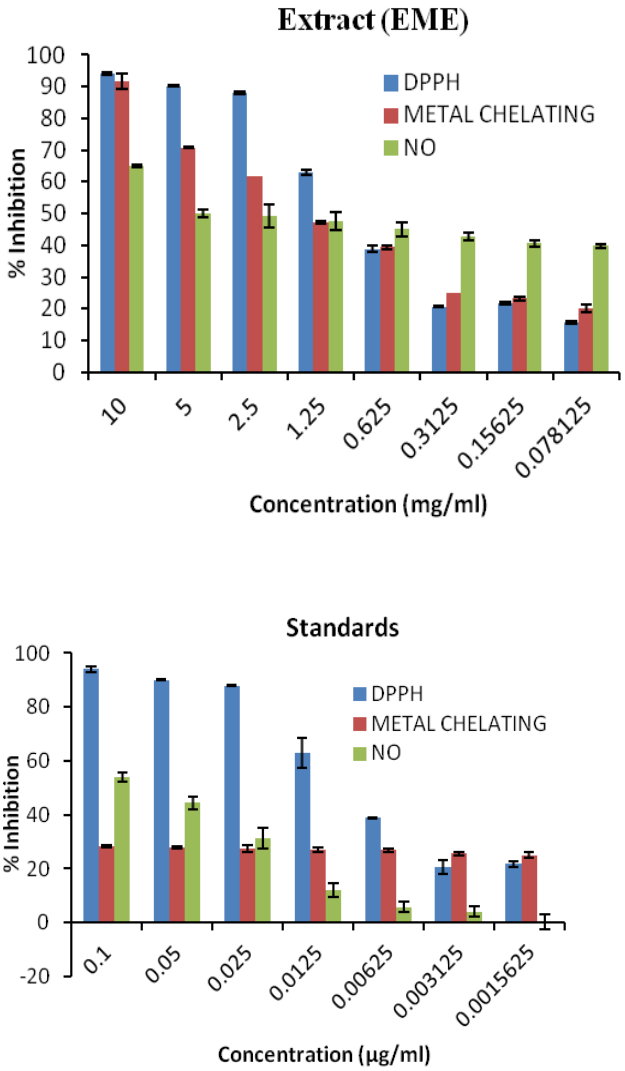
Statistical analysis

Data were presented as means ± SEM, analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Statistical significance level was set at $p<0.05$.

RESULTS

***In vitro* antioxidant activity**

The EME and the various standards showed *in vitro* antioxidant effect in a dose-dependent manner [Figure 1 Panel A] and [Figure 1 Panel B] respectively. The extract did not give complete inhibition over the entire concentration range used (0.078125 - 10 mg/ml) (Figure 1A). With DPPH assay which appeared to be the most potent, 87.9 – 94.0 % inhibition was recorded at 2.5-10 mg/ml. Order of potency of antioxidant assay models in this study was: DPPH > metal chelating > NO at higher concentrations (1.25-10 mg/ml) of extract, and NO > metal chelating > DPPH at lower concentrations (0.078125 - 1.25 mg/ml). Inhibitory concentration (IC50) for DPPH assay was 1.11 mg/ml compared to ascorbic acid standard (0.012 mg/ml), and 1.63 mg/ml for metal chelating assay compared to ascorbic acid standard (0.013 mg/ml). However, IC50 for NO scavenging assay was 3.17 mg/ml compared to EDTA standard (0.08 mg/ml) [Figure 2].



Figures 1. A-B: *In vitro* antioxidant assessment of EME (Panel A) and standards (Panel B).

Each bar represents mean ± S.E.M, n=3. EME; ethanol leaf extract of *Milicia excelsa* extract, Standards: ascorbic acid for DPPH and nitric oxide while EDTA as standard for metal chelating assay (Panel B).

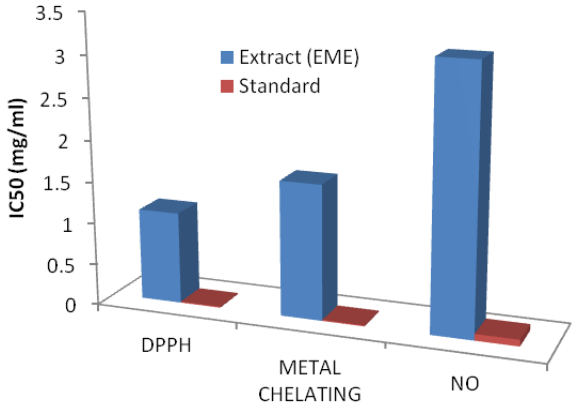


Figure 2: Inhibition (IC_{50}) of *in vitro* antioxidant assays of EME and various standards

Each bar represents mean of triplicate measurements (n=3). EME; ethanol leaf extract of *Milicia excelsa* extract. Standards: ascorbic acid for DPPH and nitric oxide; EDTA as standard for metal chelating assay

In vivo antioxidant effects of ethanol leaf extract of *Milicia excelsa*

According to Figure 3, extract had no significant ($p>0.05$) effect on MDA, but showed significant ($p<0.05$) elevation of

SOD and CAT activities compared to control group. EME also significantly ($p<0.05$) increased GSH concentration in mouse whole brain homogenate when compared with control group [Panel A-D].

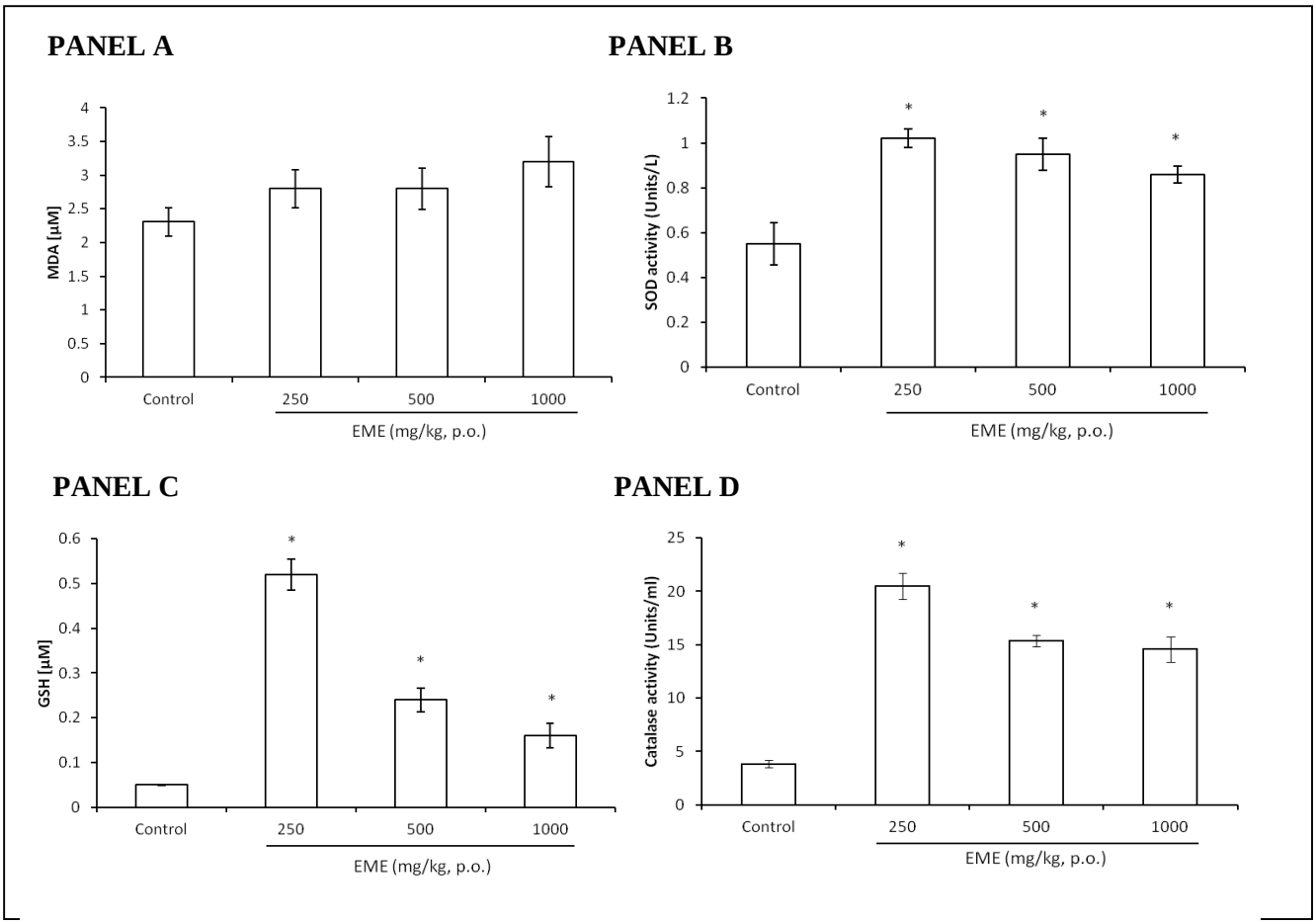


Figure 3. A-D: Effects of EME on Malondialdehyde [Panel A], Superoxide dismutase [Panel B], Glutathione [Panel C], and Catalase [Panel D] in mice whole brain. Values are Mean ± SEM, n=5. *p<0.05 compared to control.

DISCUSSION

The findings of this study showed that ethanol leaf extract of *M. excelsa* (EME) may possess antioxidant effect in both *in vitro* and *in vivo* models. The plant extract demonstrated free radical scavenging potentials in the *in vitro* antioxidant assays models (DPPH, FIC and NO) used which adds to existing knowledge of medicinal plants such as *Ornithogalum sintenisii* and *Schotia latifolia* reported to exhibit free

radical scavenging effects in vitro models [12, 34]. Several phytoconstituents present in medicinal plant extracts have shown *in vitro* antioxidant potential but a few have demonstrated therapeutic usefulness in intact animals as a result of their interaction with pharmacokinetic processes in animals [35]. This provided basis to evaluate the *in vivo* effect in mice whole brain homogenate using MDA, GSH, SOD, and CAT as antioxidant indices.

From our findings, EME did not induce lipid peroxidation suggesting that it did not induce oxidative stress and membrane damage to mouse brain since increased MDA levels

have been implicated as an index of oxidative stress and membrane damage [36]. Moreover, EME-treated mice showed elevated reduced glutathione (GSH) in mice whole brain homogenate indicative of protective antioxidant role against oxidative stress via the upregulation of GSH since GSH protects against the toxic and detrimental effects of lipid peroxidation [37].

Furthermore, observed increase in enzymatic activities of both SOD and CAT is consistent with antioxidant properties. SOD stimulates scavenging of superoxide radicals via conversion to hydrogen peroxide (H2O2) and molecular oxygen (O2) [38], while CAT is responsible for the reduction of hydrogen peroxides and protects higher tissues from the deleterious effect of highly reactive hydroxyl radicals [39]. Therefore, EME may exert its antioxidant effects by offering protection against free radicals, since free radical scavenging enzymes like SOD and CAT protect the biological system from oxidative stress [40].

It is worth mentioning that observed dose-dependent increase in MDA and dose-dependent decrease in SOD, CAT, and GSH may suggest toxic effect of EME on the antioxidant defense system at higher doses. Hence, caution should be exercised in the chronic use of EME at higher doses, despite an earlier scientific documentation of the oral acute toxicity (LD_{50}) of leaf extract to be at least 5000 mg/kg in mice [31].

In summary, *in vitro* and *in vivo* antioxidant potentials of *M. excelsa* leaf extract according to this study, possibly supports earlier scientific claims as an antipsychotic [23], anticonvulsant [24], antidepressant [25], anti-amnesic and cognitive-enhancing [26] agent. Moreover, antioxidants have been demonstrated to have a positive impact in reducing the risk of brain damage caused by oxidative stress in a variety of neurological illnesses [14-18], including neurodegenerative disorders [5].

CONCLUSION

This study concludes for the first time, that *M. excelsa* leaf ethanol extract may possess antioxidant effects. However, this work may be complemented by further antioxidant studies in different disease states for which the extract has been reported.

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