European Journal of Medicinal Plants

27(1): 1-8, 2019; Article no.EJMP.32348
ISSN: 2231-0894, NLM ID: 101583475

Inhibition of Fe^{2+} Induced Lipid Peroxidation in Rats Brain by Extract of *Euphorbia heterophylla in vitro*

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Authors' contributions

This work was carried out in collaboration among all authors. Author LJB designed the work, author OMO did the statistical analysis. Authors OBO and LO managed the analyses of the study. Author AAO managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2019/v27i130103
Editor(s):
(1) Dr. Daniela Rigano, Department of Chemistry of Natural Compounds, University Federico II of Naples, Italy.
(2) Dr. Marcello Iriti, Professor, Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
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(4) Tanveer Hussain, Mirpur University of Science and Technology (MUST), Pakistan.
Complete Peer review History: http://www.sdiarticle3.com/review-history/32348

Original Research Article

Received 04 September 2017
Accepted 30 December 2017
Published 28 March 2019

ABSTRACT

This study is sought to determine the antioxidant activity and protective ability of aqueous and methanol extractible phytochemicals from *Euphorbia heterophylla* leaves on lipid peroxidation induced in rat brain by pro-oxidant, in vitro. The extracts of the leaves were prepared, and the ability of the extracts is to inhibit 25 µM $FeSO_4$ induced lipid peroxidation in isolated rats' brain, were determined. Thereafter, total phenol content, reducing power (FRAP), Fe (II) chelating, and DPPH* free radical scavenging ability of the extracts was determined and considered as an index of antioxidant activity. The results revealed that the extracts inhibit malondialdehyde (MDA) production in the basal and pro-oxidant induced lipid peroxidised rats in a dose-dependent manner, [methanol 80.11%, aqueous 70.3%] with the methanol extract (MEE) significantly ($P< 0.05$) than that of aqueous extract (AEE). The methanol extract (0.74 ± 0.6 mg/g) had higher total phenol contents than the aqueous (0.57 ± 1.2 mg/g); likewise the methanol extract had higher reducing power (0.08 ± 0.2, 0.03 ± 0.1 mg/g), but had no significant difference in Fe (II) chelating ability

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(EC_{50}= 0.34, 0.36) with DPPH* scavenging ability (EC_{50}=0.075, 0.075). This antioxidant properties and the protective effect of this leaf could be harnessed in the management and prevention of degenerative diseases in association with oxidative stress.

Keywords: Antioxidant activity; Euphorbia heterophylla; lipid peroxidation; reducing power.

1. INTRODUCTION

The element oxygen is imperative for almost all life on Earth. When cells use oxygen to generate energy, free radicals are also created as a result of ATP (adenosine triphosphate) production by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) which result from the cellular redox process. ROS can attack polyunsaturated fatty acids in the cell membrane leading to a chain of chemical reactions called lipid peroxidation. Fatty acid breakdown results in the formation of various oxidative products, which are toxic to cells and are finally converted into stable end products. The rat brain contains large amounts of polyunsaturated fatty acids [1], which maintain its fluidity. Peroxidations of these fatty acids lead to the loss of membrane fluidity and reduction in the activity of membrane enzymes and ion channels. It is possible to measure the extent of peroxide impairment by estimating the stable end products of lipid peroxidation such as malondialdehyde [2].

Fe plays a pivotal role largely for hemoglobin production, myoglobin and cytochrome production, xanthine oxidase formation and the other Fe containing proteins require very small amount of Fe. On the other hand, free Fe in the cytosol and in the mitochondria can cause oxidative damage by elevating superoxide ions. Through Fenton reactions and by activated xanthine oxidase both uric acid (an antioxidant that recycles ascorbic acid in the cell and is therefore vital to the animals that do not produce ascorbic acid, such as primates) and O_2 formed, which causes massive damage either by itself or by reacting it with nitric oxide (NO) to form the powerful peroxy-nitrite (ONOO*) substances. The high level of both Cu and Fe, with relatively low levels of Zn and Mn cause brain cancer and degenerative diseases of the brain like: Parkinson's and Alzheimer's diseases, multiple sclerosis, etc. [3].

E. heterophylla plants are widespread in nature, ranging from herbs and shrubs to trees in tropical and temperate regions all over the world [4]. The leaves of *E. heterophylla* have been reported to contain quercetin [5] and Diterpenoids [6]. Some parts of the plant have been used as a skin irritant, tumor-promoter, anti-tumor/anticancer agent and anti-HIV agent and all these activities are observed in Euphorbia species, which have also been reported in *E. heterophylla* leaf Linn [7]. Therefore, it is expedient to access the mechanism through which this plant extract is used to inhibit the MDA production in the brain tissue, which may be useful in ameliorating neurodegenerative disorders.

2. METHODOLOGY

2.1 Sample Collection and Preparation

Euphorbia heterophylla plant leaves were sourced and harvested from the University farmland (JABU) Ikeji-Arakeji, Osun State, Nigeria. The authentication of the plants was carried out by Dr. Fatunbarin of the Department of Biological Sciences, Joseph Ayo Babalola University, Ikeji Arakeji, Nigeria (JABU/BS/EHL/0045).The leaves were subsequently rinsed under running tap water, air dried under shade and pulverized.

2.1.1 Extract preparation

The extract was prepared by weighing 10g of the powdered leaves and soaked in 100ml of 80% methanol (distilled water for aqueous). The homogenate was filtered through Whatman (No.2) filter paper and later centrifuged at 2000 rpm for 10 min to obtain clear supernatant. The supernatant was then freeze dried into powdered form with the aid of freeze drier. 0.025g of each freeze dried extract was then dissolved in 100ml of distilled water, stored at 4ºC and used for subsequent analysis.

2.1.2 Chemicals and reagent

Chemicals and reagents used, such as Thiobarbituric acid (TBA), 1,10-phenanthroline, deoxyribose, garlic acid, Folin–Ciocalteu's reagent was procured from Sigma-Aldrich, Inc., (St Louis, MO). Trichloroacetic acid (TCA) was sourced from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany), hydrogen peroxide, methanol, acetic acid, thiourea, $CuSO_4.5H_2O$,

H_2SO_4, sodium carbonate, $AlCl_3$, potassium acetate, Tris-HCl buffer, sodium dodecyl sulphate, $FeSO_4$, potassium ferricyanide and ferric chloride were sourced from BDH Chemicals Ltd., (Poole, England), while the water was glass distilled.

2.1.3 Handling of animal

The handling and use of animal for this study was approved by the institution's ethical committee for the use of animals in the laboratory experiments (reference number JABU/CNS/1507). In this study, Wister strain albino rats weighing 200-210g were purchased from the breeding colony of Department of Veterinary Medicine, University of Ibadan, Nigeria. Rats were maintained at 25ºC, on a 12h light/12h dark cycle, with free access to food and water. They were acclimatized under these conditions for two weeks before the experiment.

2.2 Lipid Peroxidation Assay

2.2.1 Preparation of tissue homogenates

Male adult albino rats were immobilized by cervical dislocation and the brain was rapidly isolated and placed on ice and weighed. The brain tissue was subsequently homogenized in cold saline (1:10 w/v) with about 10-up-and-down strokes at approximately 1200 rev/min in a Teflon glass homogenizer. The homogenate was centrifuged for 10min at 3000 × *g* to yield a pellet that was discarded, and a low-speed supernatant (Sl) was kept for lipid peroxidation assay [8].

2.2.2 Lipid peroxidation and thiobarbituric acid reactions

The lipid peroxidation assay was carried out using the modified method of [9]. Briefly 100µl Sl fraction was mixed with a reaction mixture containing 30µl of 0.1M pH 7.4 Tris-HCl buffer, extract (0 - 100µl) and 30µl of 250µM freshly prepared $FeSO_4$. The volume was made up to 300µl with distilled water before incubation at 37^oC for 1hr. The reaction was developed by adding 300µl 8.1% Sodium dodecylsulphate (SDS) to the reaction mixture and that was subsequently followed by the addition of 500µl of acetic acid/HCl (pH 3.4) and 500µl 0.8% Thiobarbituric acid (TBA). The mixture was incubated at 100^oC for 1h and the Thiobarbituric acid reactive species (TBARS) produced was measured at 532nm. Subsequently, the lipid peroxidation was calculated as MDA produced (percentage of control).

2.2.3 Fe^{2+} chelating assay

The Fe^{2+} chelating ability of the extracts was determined using a modified method of [10] with a slight modification by [11]. Freshly prepared 500µM $FeSO_4$ (150µl) was added to the reaction mixture containing 168µl 0.1M Tris-HCl (pH 7.4), 218µl saline and the extracts (0 - 100µl). The reaction mixture was incubated for 5min, before the addition of 13µl 0.25% 1,10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe^{2+} chelating ability was subsequently calculated as percentage (%).

2.2.4 1, 1-diphenyl-2 picrylhydrazyl (DPPH) free radical scavenging ability

The free radical scavenging ability of the extracts against DPPH (1,1-diphenyl-2-picrylhdrazyl) free radical was evaluated as described by [12]. Briefly, appropriate dilution of the extracts (0 - 600µl) was mixed with 1 ml, 0.4 mM methanolic solution containing DPPH radicals, the mixture was left in the dark for 30min and the absorbance taken at 516 nm. The DPPH free radical scavenging ability was subsequently calculated.

2.2.5 Determination of reducing property

The reducing property of the extracts was determined by assessing the ability of the extract to reduce $FeCl_3$ solution as described by [13]. 2.5ml aliquot was mixed with 2.5 ml 20 mM sodium phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated at 50^oC for 20 min. and then 2.5 ml 10% trichoroacetic acid was added. The mixture was centrifuged at 650 × *g* for 10 min. 5ml of the supernatant was mixed with an equal volume of water and 1 ml 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant property was subsequently calculated.

2.2.6 Determination of total phenol content

The total phenol content was determined according to the method of [14]. Briefly, appropriate dilutions of the extracts (200µl) was oxidized with 2.5ml 10% Folin-Ciocalteau's reagent (v/v) and neutralized by the addition of 2.0ml of 7.5% sodium carbonate. The reaction

mixture was incubated for 40min at 45^{o}C and the absorbance was measured at 765nm in the spectrophotometer. The total phenol content was subsequently calculated as gallic acid equivalent.

2.3 Data Analysis

The results of triplicate experiments were pooled and expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was used to analyze the mean and the post hoc treatment was performed using Duncan multiple range test. Significance was accepted at $P<0.05$ [15].

3. RESULTS

The result revealed that methanol extract of Euphobia heterophyla (MEE) had the total phenol content of 72mg/100g and the aqueous extract (AEE) had 40mg/100g (Fig. 1).

The result of incubation of rat's brain in the presence of 250μM Fe^{2+} was presented in Fig. 2, this showed a significant increase ($P<0.05$) in malondialdehyde (MDA) content when compared with basal brain without Fe^{2+} (119.50%). However both extract (MEE and AEE) inhibited the MDA produced in concentration-dependent (0-3.13mg/ml) with MEE having higher inhibition (80.11%) than AEE (70.30%).

Furthermore, the Fe^{2+} chelating ability of MEE and AEE as presented in Fig. 3 revealed the chelating ability of extract of *Euphobia heterophylla*, the extracts chelate in concentration dependent manner (0-5mg/ml).

The free radical scavenging ability of the extracts was revealed in Fig. 4, this was in concentration-dependent manner (0-25mg/ml), MEE had better scavenging ability (75μg/ml) than AEE (42μg/ml).

The result of the ferric reducing antioxidant power (FRAP); of the extract was presented as ascorbic acid equivalent in Fig. 5. The extracts exhibited ferric reducing antioxidant power, MEE (88mg AAE/100g), AEE (44mg AAE/100g).

4. DISCUSSION

The ability of the extracts of *Euphobia heterophylla* (methanol and aqueous) is to contain some reasonable amount of phenolic compound that could be one of the major reason why it is being used in folklore medicine, though this do not possess much phenolic content like mistletoe from Kolanut and Breadfruit trees which has been earlier reported by [16].

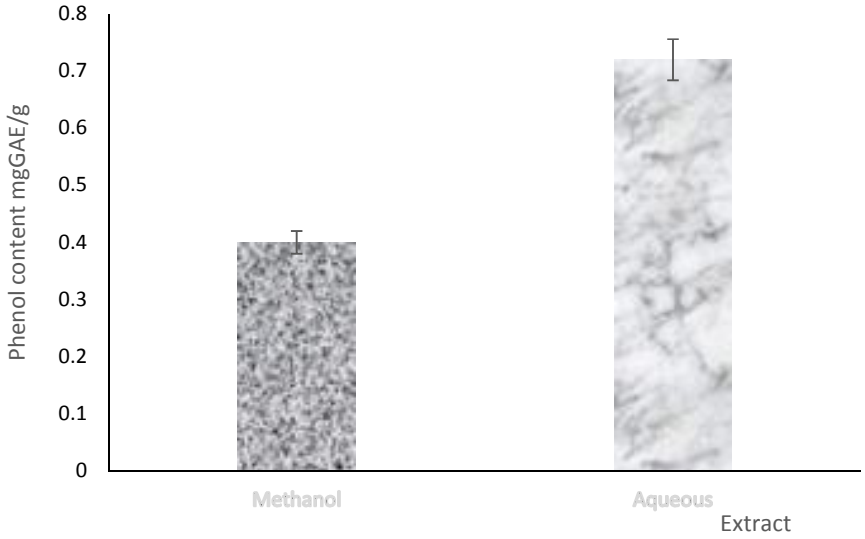
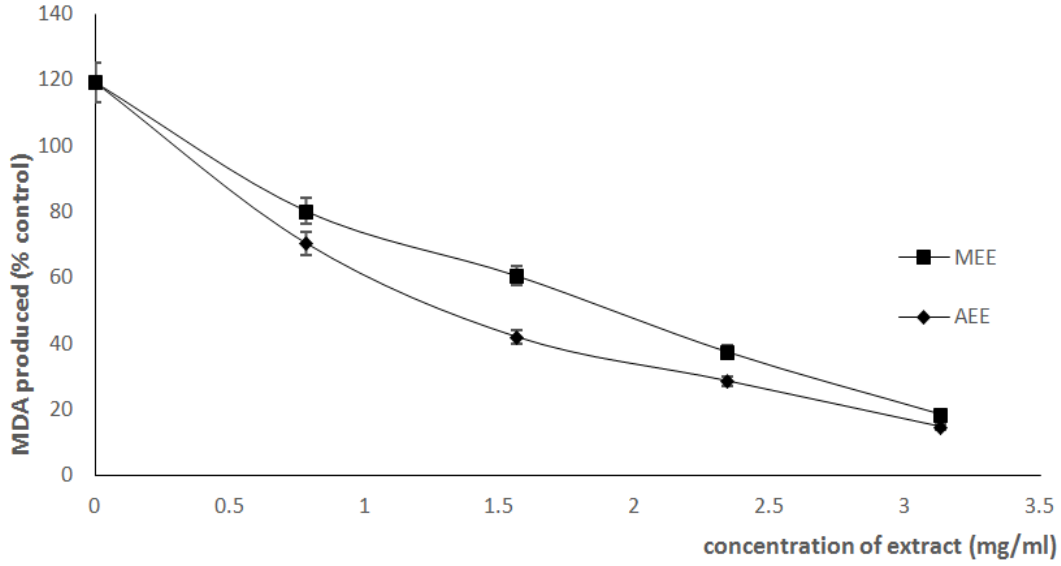


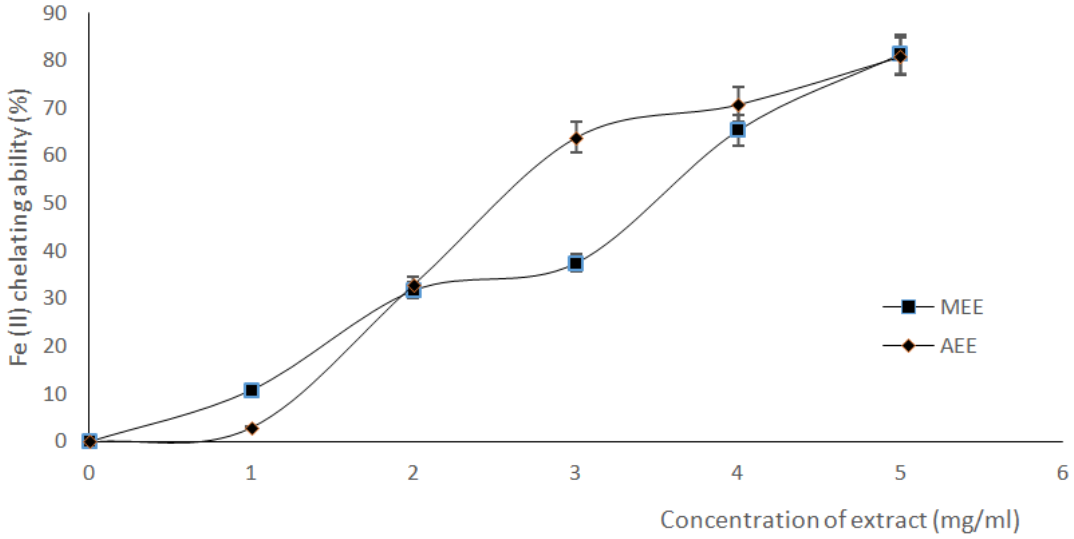
Fig. 1. Total phenol content of aqueous and methanol extract of *E. heterophylla*
Values represent mean ± standard deviation, n = 3



	IC_{50}
MEE	$3.04^{a} \pm 0.21$
AEE	$3.94^{a} \pm 0.10$

Fig. 2. Inhibition of Fe (II) Induced lipid peroxidation in rat's brain by Aqueous and Methanol extract of *E. heterophylla*

Values represent mean ± standard deviation, n = 3



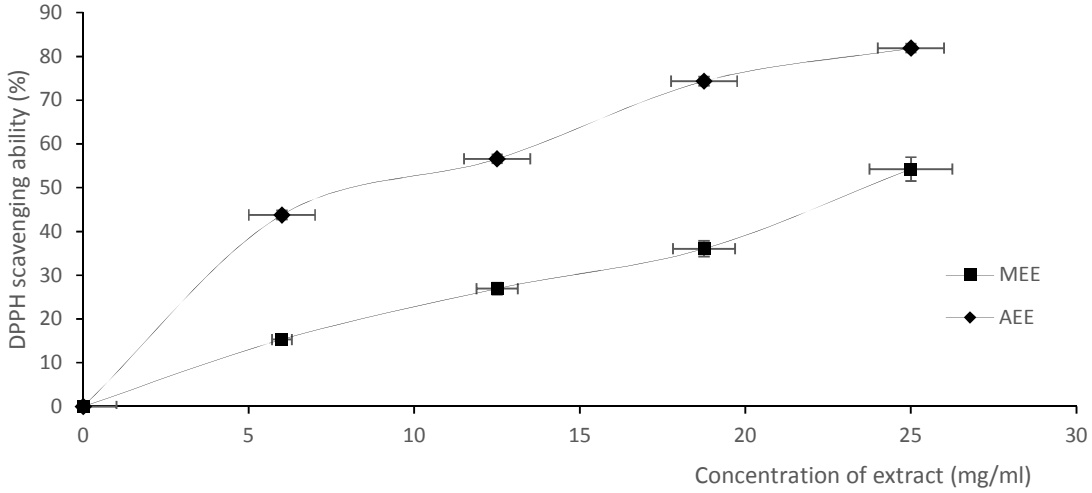
	IC_{50}
MEE	$3.22^{a} \pm 0.14$
AEE	$2.90^{b} \pm 0.05$

Fig. 3. Fe (II) chelating ability of aqueous and methanol extract of *E. heterophylla*

Values represent mean ± standard deviation, n = 3

The result of this study is in agreement with the report of [17], in that Fe^{2+} is a very powerful initiator of lipid peroxidation in the brain (pro-oxidant), the increase in the MDA content of the brain in the presence of Fe^{2+} could be ascribed to the fact that Fe^{2+} can catalyze an electron

transfer reactions which generate reactive oxygen species, such as reactive OH*, which is formed from H_2O_2 through the Fenton reaction. Fe^{2+} also decomposes lipid peroxides, thus generating peroxyl and alkoxyl radicals, which favours the propagation of lipid oxidation [11]; [17]. Elevated Fe^{2+} content in the brain had been linked with Parkinson's disease. Although the etiologies of Parkison's disease remain conceal, of various studies which point to a central role of Fe-induced oxidative stress mechanism. Elevated Fe levels have been localized to deteriorate regions of brains from Parkinson's disease patients, i.e. a finding which also gets demonstrated in animal models of the disease [18].



	IC_{50}
MEE	23.75[a] ± 0.00
AEE	13.25[a] ± 0.10

Fig. 4. DPPH free radical scavenging ability of aqueous and methanol extract of *E. heterophylla*
Values represent mean ± standard deviation, n = 3

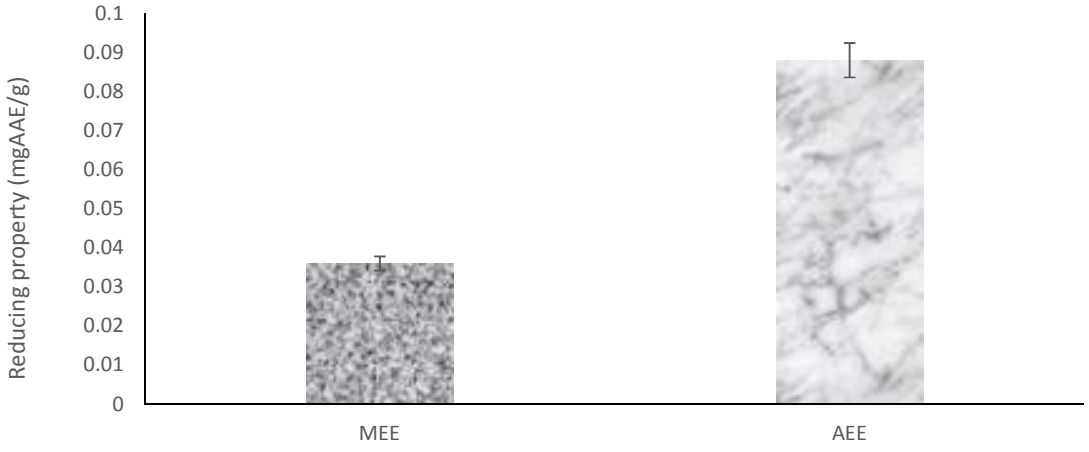


Fig. 5. Reducing power of aqueous and methanol extract of *E. heterophylla*
Values represent mean ± standard deviation, n = 3

However, both aqueous and methanol extract of *E. heterophylla* were able to prevent the progression of lipid peroxidation by inhibiting a reaction in the MDA content of the brain tissue in a concentration dependent manner. The reason for this high inhibition of lipid peroxidation in the brain by extracts from *E. heterophylla* cannot be categorically stated, however, it will not be far afield from the possibility that the extract is rich in phenolics & could form a complexes with Fe^{2+} thereby preventing them from catalyzing the initiation of lipid peroxidation, as well as scavenging the free radicals produced by the Fe^{2+} catalyzed lipid peroxidation reaction [17].

Antioxidant carries out their protective properties on cells either by preventing the production of free radicals or by neutralizing/scavenging free radicals produced in the body or reducing/ chelating the transition metal composition of food [19]. In a bid to explain the main mechanism through which the phenolic extract prevents Fe^{2+} induced lipid peroxidation in the brain, the Fe^{2+}chelating ability was assessed. In this study, the protective ability of the extract of *E. heterophylla* against Fe^{2+} induced oxidative stress by Fe^{2+} chelating process agrees with earlier reports on phenolics, in that one of the process through which they exhibit their antioxidant activity is by forming complex with Fe^{2+} thus preventing the initiation of lipid peroxidation [16]. However, there was no significant different in the chelating ability of both extract, the reason for this cannot be categorically examined.

Researchers have been fascinated with phenolic compounds because they show promise of being powerful antioxidants that can protect the human body from free radicals. The anti-radical activity of phenolics are principally based on the redox properties of their hydroxyl groups and the structural relationships between different parts of their chemical structure [20]. Significantly, both methanol and aqueous extracts of *E. heterophylla* used in this study, scavenged DPPH free radicals; this scavenging ability revealed their antioxidant properties, which is of importance in scavenging excessive free radicals in cells. This finding is in agreement with earlier reports, that there is a correlation between the antioxidant activities and total phenol contents of many plants. Furthermore, the fact that the free radical scavenging ability, Fe^{2+} chelating ability and the inhibition of Fe^{2+} induced lipid peroxidation by the extracts followed the same trend which suggests that, free radical scavenging ability and Fe^{2+} chelation process may be involved in the protective ability of the extracts against Fe^{2+} induced lipid peroxidation in the brain.

There had been a report that the reducing property can be a novel anti-oxidation defense mechanism [21]; this is possibly through the ability of antioxidant compound to reduce transition metals, reduced metals such as Fe^{2+} or Cu (I) rapidly react with lipid hydro-peroxides, leading to the formation of reactive lipid radicals and transformation of reduced metal to its oxidized. The result of this study was gotten by measuring the ability of the extracts to reduce Fe^{3+} to Fe^{2+}. Spectrophotometric quantification used for analyzing the Fe^{2+} production gave the rate of the reduction reaction and thus determine their reductive power. Both extract of *E. heterophylla* were able to reduce Fe^{3+} to Fe^{2+}; a property linked to their rich phenolic content which contributed to their antioxidant properties.

5. CONCLUSION

The lofty protective effect of methanol and aqueous extract of *E. heterophylla* could be due to their high Fe (II) chelating ability, free radical scavenging and reducing power (antioxidant properties). These can help to explain the biochemical rationale which underlying its use in folklore medicine.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/32348