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ANTIOXIDANT AND NEUROPROTECTIVE EFFECTS OF LEUCAS INDICA ON IRON INDUCED LIPID PEROXIDATION IN RAT BRAIN *IN VITRO*

T. RAVI KIRAN*

Department of Microbiology and Biotechnology, Bangalore University, Jnana Bharathi Campus, Bangalore – 560 056

ABSTRACT

Neurodegenerative diseases have been linked to oxidative stress arising from peroxidation of membrane biomolecules and high levels of iron have been reported to play an important role in neurodegenerative diseases. The present study compares the antioxidant and neuroprotective effects of aqueous and methanolic extract of *Leucasindica*(L.*indica*)on iron induced lipid peroxidation in rat's brain *in vitro*. The total phenol,total flavonoids, vitamin C content,reducing property and hydroxyl radical scavenging ability were determined. 4-hydroxy isopthalic acid was the major phenoliccompound identified by HPLC-VWD analysis. The results of the study revealed that methanolic extract of *L. indica* had significantly higher vitamin C, flavonoid content, reducing power and DPPH scavenging ability than the Aqueous extract. However, both extracts significantly inhibited (p<0.05) Fe(II) induced lipid peroxidation in the rat brain in a dose-dependent manner. In conclusion, the methanolic extract was more potent inhibitor of lipid peroxidation in the rat'sbrain and this is probably due to its higher phenol content and reducing power.

KEY WORDS: ***Leucasindica*, Brain, Lipid peroxidation, 4-hydroxy isopthalic acid, HPLC.**

T. RAVI KIRAN
Department of Microbiology and Biotechnology, Bangalore University, Jnana Bharathi Campus, Bangalore – 560 056

INTRODUCTION

Free radicals and reactive oxygen species (ROS) such as superoxide(O_2^-), hydroxyl ($^{\cdot}OH$)and peroxyl radicals (ROO $^{\cdot}$) are normal by products of aerobic metabolism produced *in vivo* during oxidation.[1] Overproduction of ROS can result in oxidative damage to various biomolecules including lipids, proteins, DNA and cell membranes.[2] Oxidation of biological molecules has been postulated to inducea variety of pathological events such asatherogenesis, carcinogenesis,Parkinson's disease and aging.[3]Oxidative stress results from either a decrease of natural cell antioxidant capacity or an increased amount of ROS in organisms. However, consumption of foods rich in antioxidant phytochemicals may help fight degenerative diseases caused by oxidative stress by improving body's antioxidant status.The human brain is particularly susceptible to oxidative stress due to its high concentration of polyunsaturated fatty acids, which are potential substrates for lipid peroxidation, its low levels of antioxidant defence enzymes, its high consumption of total oxygen and its elevated levels of transition metals that can catalyse radical formation.[4,5,6] Iron is the most important metal in the brain and is vital for normal neuronal metabolism.[7] However, high contents of iron in tissues can augment neuron degeneration through free radical processes by different mechanisms, including participation in Fenton's reaction, as well as the development of membrane lipid peroxidation, which leads to cell membrane fluidity, thereby jeopardising cell viability.[8,9] In recent years, there has been a great interest in finding natural antioxidants from plant materials. Numerous crude extracts and pure natural compounds from plants were reported to have antioxidant and radical-scavenging activities. [10,11,12,13] Among the antioxidant compounds, phenolics and flavonoids which are largely distributed in nature, have been studied more comprehensively.[14,15,16] Since ancient times, therapeutic records have existed for several species of Lavandula (*Lamiaceae*) and are one of the most popular medicinal plants with high economic value. The neuroprotective, anti-inflammatory, antioxidant and antibacterial properties of Lavandula species were also demonstrated.[17, 18, 19,20]*Leucasindica* belonging to the family of *Lamiaceae*, is an herbaceous, erect, slender annual, 30-50cm high plant with dense foliage which is widely distributed throughout Bangladesh, India, Sri Lanka, Nepal, China and tropical American countries.[21] Although studies have reported on the chemical characterization of phytoconstituents and antioxidant properties of *L.indica*,[22,23] there is still limited information on its potential use in the management/prevention of neurodegenerative diseases associated with oxidative stress. Therefore the present study was designed to identify the phenolic compounds and to investigate antioxidant properties of *L.indica extracts* against Fe^{2+}-induced lipid peroxidation in rat brain homogenates.

MATERIALS AND METHODS

Chemicals and reagents

Thiobarbituric acid, 1,1, Tetramethoxy propane, gallic acid, ellagic acid, quercetin and 4-HIA (4-hydroxy isopthalic acid) were obtained from sigma(St.Louis, MO, USA). All organic solvents and general chemicals were of analytical grade and purchased from local companies.

Plant material and extraction procedure

Fresh samples of L.*indica*were collected from Savandurga forest, Magadi, Karnataka. Authentication of the plants was carried out in the Department of Botany, Bangalore University, Bengaluru and vocher specimen was deposited in the herbarium (BUB no. 2224). The leaves were washed thoroughly under running tap water and shade dried at room temperature (RT) and finely powdered using blender. The aqueous (Aq) and methanolic (MeOH) extracts were prepared by soaking the powder in water and methanol respectively for about 24 hours. The mixtures were filtered and concentrated by using rotary evaporator at 40 $^{\circ}$C. The extracts were stored at-20 $^{\circ}$C until further use.

Animal care

Animal experiments were approved by the Institutional Animal Ethics Committee (IAEC), Bangalore University, Bengaluru, India (BUB/Zoo/IAEC/Anim/2011-15). The experiments were carried out in accordance with the guidelines set by CPCSEA (Committee for the purpose of control and supervision of experiments on animals), India. Male *Wistar* rats (3 months old), weighing between 150-170 grams were procured from Sri Raghavendra Enterprises, Bangalore. Animals were housed three per cage in polypropylene cages fitted with steel mesh and were maintained at a temperature of 23± 2 $^{\circ}$C , relative humidity of 55-65% and daily exposure of 12 h light and 12 hr dark cycle. Animals had free access to standard food (Amruth feeds, India) and tap water *ad libitum*.

Preparation of brain homogenates

On the day of the experiments, the rats were sacrified by decapitation and the whole brain was rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold phosphate buffer (pH 7.4, 1/10 w/v). The homogenate was stored at -20 $^{\circ}$Cuntil further use.

Determination of vitamin C

Vitamin C content of the extracts was determined using the method of Benderitteret al.[24] Briefly, 75 µl DNPH (2,4- dinitrophenyl hydrazine, 230 mg thiourea and 270 mg $CuSO_4.5H_2O$ in 100 ml of 5 M H_2SO_4) were added to 500 µl reaction mixture [300 µl of appropriate dilution of the extracts with 100 µl 13.3% trichloroacetic acid (TCA) and water]. The reaction mixture was subsequently incubated for 3 h at 37 $^{\circ}$C, then 0.5 ml of 65% H_2SO_4 (v/v) was addedto the mediumand the absorbancewas measured at 520 nm.

The vitamin C content of the extracts was subsequently calculated.

Determination of phenol content

The total phenol content was determined using the method reported of Singleton et al.[25] Appropriate dilutions of the extracts were mixed with 2.5 mL of 10% Folin-Ciocalteu reagent (v/v) and neutralized by 2.0 mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45 ˚C and the absorbance was measured at 765 nm. The total phenol content was subsequently calculated using gallic acid as standard.

Determination of flavonoid content

The flavonoid content was determined according to the method of Medaet al.[26] Briefly, 0.5 mL of appropriately diluted sample was mixed with 0.5 ml methanol, 50 µl of 10% $AlCl_3$, 50 µl of 1M potassium acetate and 1.4 ml water and allowed to incubate at RT for 30 min. Thereafter, the absorbance of the reaction mixture was subsequently measured at 415 nm. The total flavonoids was calculated using quercetin as standard.

Antioxidant assay (DPPH assay)

DPPH assay was carried out according to the procedure of Shon et al.[27] Briefly, 0.5 ml of the sample extracts and nano encapsulated extract were added to 1 ml of 2,2 DPPH solution (0.1 mMin 95% ethanol) and incubated at RT for 20 min. The absorbance was read at 517 nm against blank.

$$\text{Scavenging effect (\%)} = \frac{\text{Abs}_{\text{Control(517)}} - \text{Abs}_{\text{Sample (517)}}}{\text{Abs}_{\text{Control (517)}}} \times 100$$

Where Abs control = absorbance of the control (reacting mixture without the test sample) and, Abs test sample = absorbance of reacting mixture with the test sample.

Identification and quantification of phenolic compounds by HPLC analysis

High performance liquid chromatography (HPLC-VWD) was performed with the HPLC system (Agilent Technologies, Germany). Reverse phase chromatographic analyzes were carried out in gradient condition using C 18 column (5 µm, 250 × 4 mm I.D) packed with 5 µM diameter particles, the mobile phase consisted of 2% acetic acid in water (solvent A) and (solvent B) with the following gradient: 80% A for 10min, to 20% A in 4 min, to 0%A in 4min and continuing at 0% A untill completion of run. The mobile phase was filtered through a 0.45 µM membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stocks solutions of standard references of gallic acid, ellagic acid, 4-hydroxy isophthalic acid were prepared in HPLC grade methanol at a concentration of 25 parts per million. All solutions and samples were filtered through 0.45 µM membrane filter. Quantification was carried out by the integration of the peaks using external standards method at 278 nm. The flow rate was 1.5ml/min and the injection volume was 20 µl. The chromatography peaks were confirmed by comparing their retention time in VWD-UV spectrum with those of reference standards. All chromatographic separations were carried out at ambient temperature.

Lipid peroxidation and thiobarbituric acid reactive substances

The lipid peroxidation assay was carried out using the modified method of Ohkawaet al.[28]Briefly, 1ml of homogenate containing {1mM ascorbate (100 µl) 100 µM EDTA (100 µl), 1mM iron sulphate (100 µl) and 100 µM H_2O_2 (100 µl)} was incubated at 37 ˚C in a oscillating water bath for 1 hour. At the end of the incubation period, 0.5 ml butylated hydroxyltoluene (BHT)(mg/ml in ethanol) and 1 ml of 15% TCA was added. The tubes were sealed and heated for 10 min in boiling water bath to release protein bound Malondialdehyde (MDA), the product of lipid peroxidation. To avoid adsorption of MDA to insoluble proteins, the sample were cooled to 4 ˚C and centrifuged at 200×g for 20 min. Following centrifugation, 2ml of protein free supernatant was removed from each tube and 0.5 ml of 0.33% TBA was added to this fraction. All tubes were heated for 1 hr at 95 ˚C in a water bath. After cooling, the TBA – MDA complexes were extracted with 2 ml butanol and centrifuge at 2000 x g for 15 minutes, supernatant was taken for OD at 532 nm and MDA levels were determined from a standard curve generated from 1,1,3,3 tetra methoxy propane. The results were expressed as nmoles MDA / mg tissue.

Degradation of deoxyribose (Fenton's reaction)

The ability of the extracts to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the method ofHalliwell and Gutteridge.[29] Briefly, different concentrations of extracts were added to a reaction mixture containing 120 µl 20 mM deoxyribose, 400 µl 0.1 M phosphate buffer, 40 µl 20 mM hydrogen peroxide and 40 µl 500 µM $FeSO_4$, and the volume wasmadeupto 800 µl with distilled water. The reaction mixture was incubated at 37 ˚C for 30 min, and the reaction was stopped by the addition of 0.5 ml of 2.8% TCA, followed by the addition of 0.4 ml of 0.6% TBA solution.The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at 532 nm.

$$\text{Percentage OH radical scavenging ability (\%)} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{test sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

Where Abs control = absorbance of the control (reacting mixture without the test sample) and, Abs test sample = absorbance of reacting mixture with the test sample.

Reducing property

The reducing property of the extracts was determined by assessing the ability of the extract to reduce a ferric chloride ($FeCl_3$)solution as described by Pulido et al.[30] A 2.5 ml aliquot was mixed with 2.5 ml, 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml, 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, and then 2.5 ml, 10% TCA was added. This was then centrifuged at 650 x g for 10 min. Also, 5 ml of the supernatant was mixed with an equal volume of water and 1 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm and higher absorbance indicates greater reducing power.

Statistical Analysis

Values are expressed as mean ± SE. Statistical analysis was performed by one and two way ANOVA, followed by Tukey's test when appropriated. The results were considered statistically significant for $p<0.05$.

RESULTS AND DISCUSSION

Vitamin C content

The vitamin C concentration was significantly ($p<0.05$) higher in MeOH extract by 78% over the Aqextract(Table- 1). Vitamin C has been reported to contribute to the antioxidant activity of the plant. It is a good reducing agent and exhibits its antioxidant activities by electron donation.[31,32]

Table 1

Vitamin C, Phenols and Flavanoid contents of L. indica extracts.

Samples	Vitamin C(mg/ml)	Phenol (mg/100g)	Flavanoid(mg/100g)
Aqueous extract	0.516±0.072[a]	15.08±4.977[a]	8.5±0.5[a]
Methanolic extract	2.08±0.142[b]	17.66±2.803[a]	11.16±0.6[b]

Values are represented as mean ± S.E (n = 3). Significance between the extracts is represented in lower case (p<0.05).Those not showing the same letters are significantly different.

Total phenol and total flavonoid

Antioxidants from plant origin have always been tagged with possibilities in treating and lowering the risk of various diseases such as inflammation and cancer. Phenolic compounds can protect the human body from free radicals, whose formation is associated with the normal natural metabolism of aerobic cells. Natural polyphenols are capable of removing free radicals, chelate metal catalysts, activating antioxidant enzymes, reducing tocopherol radicals, and inhibiting oxidases.[33,34] The total phenol and flavonoid content in MeOH and Aq extracts is represented in Table 1. The phenolic content was insignificant between the extracts. MeOH extract showed significantly higher flavonoid content (27%) than Aq extract. The higher phenolic and flavonoid content in MeOH extract of *L. indica*may contribute to its potential antioxidant property and curative ability adsorbing and neutralizing free radicals. The results are in agreement with the studies of Ramani et al,[24] wherein higher phenolic and flavonoid content was obtained in alcoholic extracts of different *Leucas*species.

DPPH radical scavenging activity

The DPPH assay is used as a tool for the *in vitro* evaluation of extracts and fractions, and its results can indicate the presence of phenolic and flavonoid compounds in plant extracts .[35]The DPPH radical scavenging activity of the extracts is represented in Fig-1. The MeOH and Aq extracts of *L. indica* exhibited concentration dependent antiradical activity. The results revealed that MeOH extract was significantly ($p< 0.05$) more effective free radical scavenging agent than Aq extract due to its high content of phenolic and flavonoid compounds. Our results are in agreement with the studies of Vinayagam and Sudha,[23]and Ramani et al.[24]

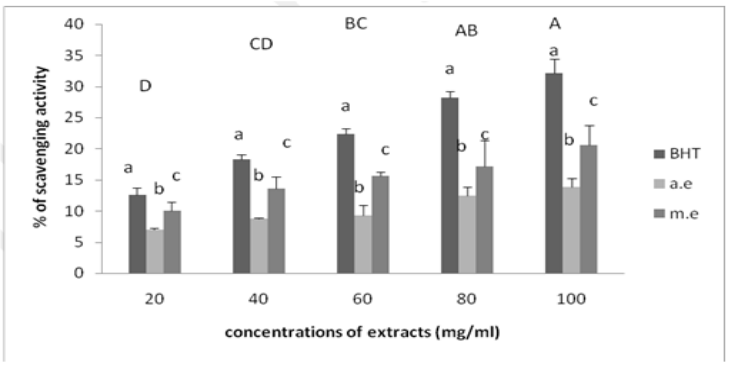

Figure 1

DPPH scavenging activity of L. indica

Aqueous extract (a.e), methanolicextract (m.e)extract and BHT- Butylated hydroxytoluene.Values are represented as mean ± S.E (n = 3). Significance between the groups means is represented in upper case and between extracts in lower case (p<0.05). Those not sharing the same letters are significantly different.

HPLC Analysis

The fingerprint chromatogram (Fig 2a, 2b and 2c) obtained by the HPLC identified three peaks with their retention time at 4.7, 13.3 and 15.4 minutes corresponding to gallic acid, ellagic acid and 4-HIA respectively. The 4-HIA was the major phenolic compound (1.9mg/g extract) followed by ellagic acid and gallic acid. The other unidentified peaks were also obtained.

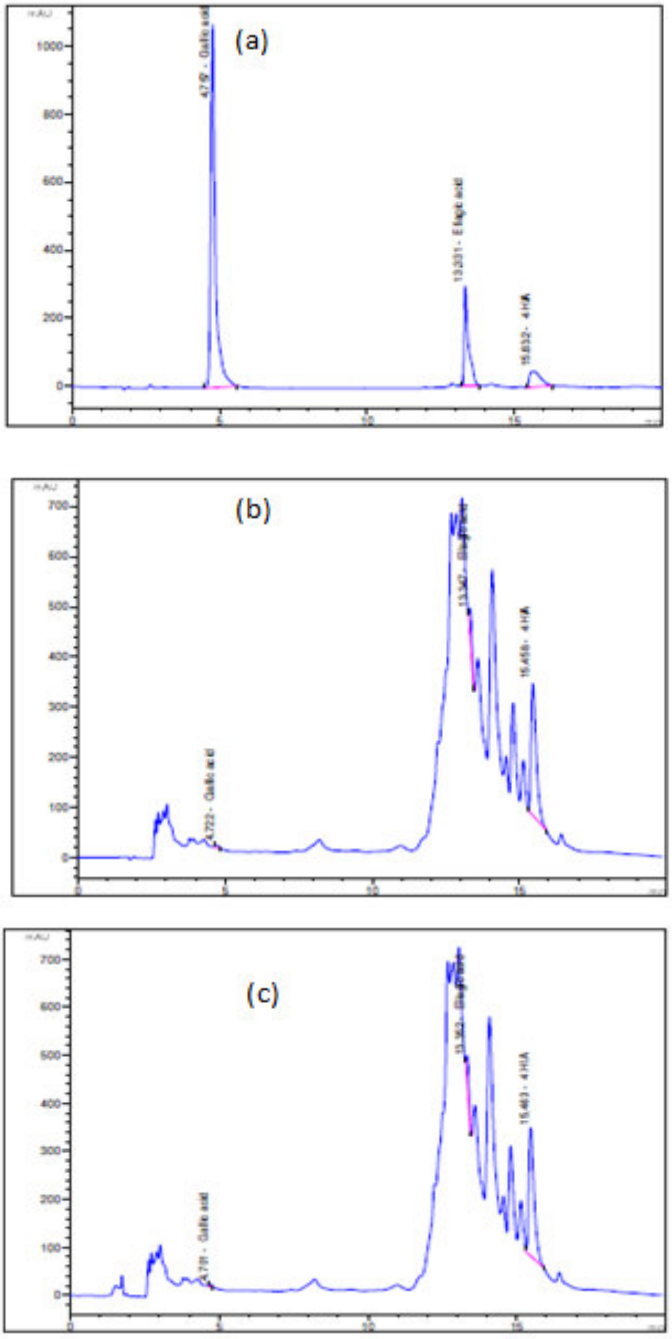

Figure 2

HPLC profile of (a) Standard, (b) aqueous and (c) methanolic extracts showing peaks of Gallic acid, Ellagic acid and 4- Hydroxyisopthalic acid at different Retention times.

4 HIA, the major compound present in the extracts of *L. indica,* is a hydroxyl aromatic acid. This compound has several interesting biological activities, including its role as an antioxidant and cytoprotectant.[36] Although, ellagic acid and gallic acid is present in low quantities in the extracts compared to 4-HIA, its antioxidant potential has been previously demonstrated, suggesting the possibility that this compound also contributes to the antioxidant activity displayed by the extract.[37]

Lipid peroxidation and thiobarbituric acid reactions

Figure 3 represents the interaction of *L.indica* extracts with Fe(II)–induced lipid peroxidation in rat's brain. The incubation of rat brain with Fe(II) significantly ($p< 0.05$) increased the MDA content by 53% when compared to the control. Both the extracts of *L. indica*significantly inhibited ($p< 0.05$) Fe(II) induced lipid peroxidation in the rat's brain in a dose-dependent manner. The results clearly indicate that

Fe^{2+} is a potent pro-oxidant, causing an increase in the MDA content in the rat brains when compared with basal brain tissue (without extract and pro-oxidant). This result is in accordance with the studies of Obohetal.[38]Other species belonging to the *Lamiaceae* family have revealed protective effects againstFe^{2+}-induced lipid peroxidation. [39]The increased lipid peroxidation in the presence of Fe^{2+} could be attributed to the fact that Fe^{2+} can catalyze one-electron transfer reactions that generate ROS, such as the reactive ·OH, which is formed from H_2O_2 through the Fenton reaction.[8]

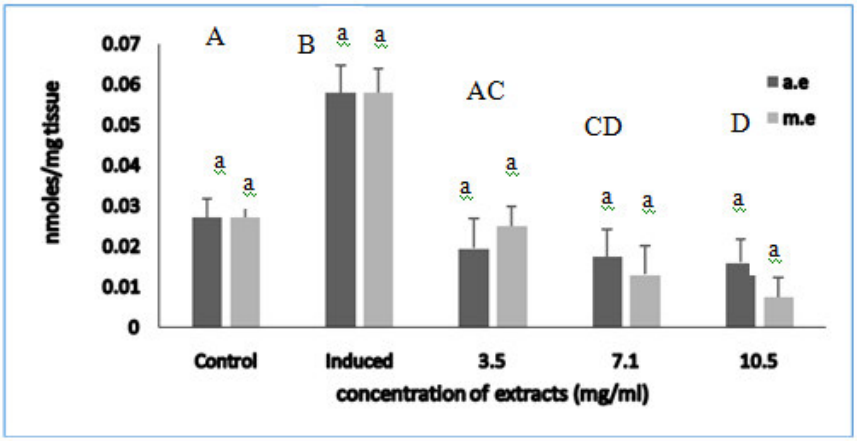

Figure 3

Inhibition of Fe^{2+}-induced Lipid peroxidation in rat brain by aqueous(a.e) and methanolic (m.e) extracts of L. indica.

Control = lipid peroxidation without Fe^{2+} as pro-oxidants and no extracts; Induced = lipid peroxidation with Fe^{2+} as pro-oxidants and no extracts.Values are represented as mean ± S.E (n = 3). Significance between the groups means is represented in upper case and between extracts in lower case (p<0.05). Those not sharing the same letters are significantly different.

Reducing property

The reducing power of the *L. indica* extracts is represented in Figure. 4. The reducing ability of the extracts showed a significant dose-dependent trend increasing with increases in the concentrations of the extracts. The MeOH extract had significantly higher (p<0.05) reducing power than the Aq extracts. Allhornet al.[40] reported that the reducing property can be a novel antioxidation defense mechanism, possibly through the ability of the antioxidant compound to reduce transition metals. The higher reducing power of the MeOH extract may be related to the extractable antioxidant phytochemicals present in it which is higher than that of the Aq extract.

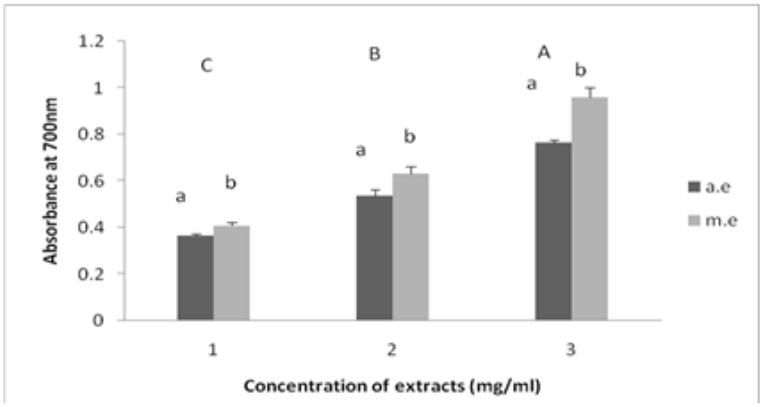

Figure 4

Reducing power of aqueous(a.e) and methanolic (m.e) extracts of L. indica.

Values are represented as mean ± S.E (n = 3). Significance between the groups means is represented in upper case and between extracts in lower case (p<0.05). Those not sharing the same letters are significantly different.

Fenton's reaction

The ability of the MeOH and Aq extracts of *L. indica* to inhibit Fe (II)/H_2O_2- induced decomposition of deoxyribose as an index for hydroxyl radical scavenging ability is represented in Fig. 5. The results revealed that there was no significant difference between the extracts and concentrations to scavenge OH• induced decomposition of deoxyribose. The scavenging effects demonstrated by the extracts could be due to the presence of antioxidant compounds.

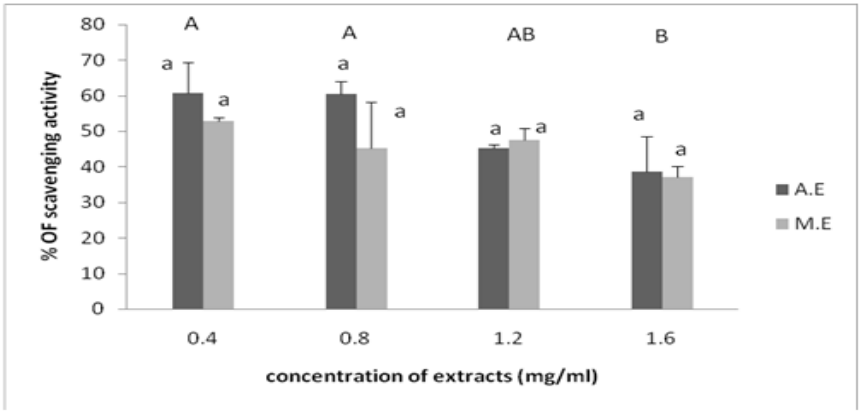

Figure 5
Hydroxyl radical scavenging ability of aqueous (A.E) andmethanolic (M.E) extracts of L.indica.

Values are represented as mean ± S.E (n = 3). Significance between the groups means is represented in upper case and between extracts in lower case (p<0.05). Those not sharing the same letters are significantly different.

In conclusion, the study demonstrated that both the extracts of *L. indica* possess strong antioxidant properties and could inhibit Fe^{2+}-induced lipid peroxidation in brain. However, MeOH extract had a significantly higher (p<0.05) ability to inhibit the Fe^{2+}-induced lipid peroxidation in rat brain, which could be attributed to its higher antioxidant activity of phytochemicals in the extract. These results indicated that the *L. indica* has many chemical compounds which were able to scavenge free radicals. Therefore, the plant has promising compounds to be tested as potential antioxidant drugs for the treatment of diseases resulting from oxidative stress. Further *in vivo* studies with *L. indica* on iron metabolism are needed for its beneficial therapeutic use.

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