

In vitro and In vivo Antioxidant Evaluation and Estimation of Total Phenolic, Flavonoidal Content of *Mimosa pudica* L

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ABSTRACT

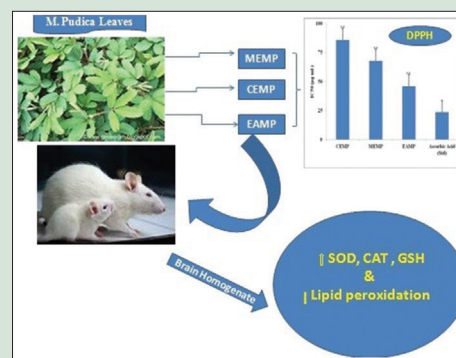
Objective: *Mimosa pudica* Linn. (Mimosaceae) is traditionally used as a folk medicine to treat various ailments including convulsions, alopecia, diarrhea, dysentery, insomnia, tumor, wound healing, snake bite, etc., Here, the study was aimed to evaluate the antioxidant potential of *M. pudica* leaves extract against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (*in vitro*) and its modulatory effect on rat brain enzymes. **Materials and Methods:** Total phenolic, flavonoid contents, and *in vitro* antioxidant potential against DPPH radical were evaluated from various extracts of *M. pudica* leaves. In addition, ethyl acetate extract of *Mimosa pudica* leaves (EAMP) in doses of 100, 200, and 400 mg/kg/day were administered orally for 7 consecutive days to albino rats and evaluated for the oxidative stress markers as thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) from rat brain homogenate. **Results:** The ethyl acetate extract showed the highest total phenolic content and total flavonoid content among other extracts of *M. pudica* leaves. The percentage inhibition and IC₅₀ value of all the extracts were followed dose-dependency and found significant ($P < 0.01$) as compared to standard (ascorbic acid). The oxidative stress markers as SOD, CAT, and GSH were increased significantly ($P < 0.01$) at 200 and 400 mg/kg of EAMP treated animals and decreased significantly the TBARS level at 400 mg/kg of EAMP as compared to control group. **Conclusion:** These results revealed that the ethyl acetate extract of *M. pudica* exhibits both *in vitro* antioxidant activity against DPPH and *in vivo* antioxidant activity by modulating brain enzymes in the rat. This could be further correlated with its potential to neuroprotective activity due to the presence of flavonoids and phenolic contents in the extract.

Key words: *Mimosa pudica*, Flavonoids, 2, 2-diphenyl-1-picrylhydrazyl, Oxidative stress, Brain homogenate

SUMMARY

Total phenolic, flavonoid contents and *in vitro* antioxidant potential were

evaluated from various extracts of *M. pudica* leaves. Again, *in vivo* antioxidant evaluation from brain homogenate on oxidative stress markers as TBARS, SOD, CAT and GSH from rat was investigated. Our findings revealed that *M. pudica* possesses both *in vitro* and *in vivo* antioxidant activity due to presence of phenolics and flavonoids.



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INTRODUCTION

Oxidation is very essential to many living organisms for the production of energy to fuel biological processes. The free radicals and other reactive oxygen species (ROS), which are continuously produced *in vivo*, responsible for oxidation. These ROS and free radicals are byproduct of normal cellular metabolism of the body and are produced during irradiation (ultraviolet [UV] light, X-rays, and γ -rays), inflammation, pollution, and mitochondria-catalyzed electron transport reactions.^[1] ROS may contribute to oxidative damage results to loss of function and even cellular death including various numbers of disorders such as coronary atherosclerosis, ischemia, ageing, diabetes, cancer, immunosuppression, and neurodegenerative disorders.^[2] The interruption of the normal cellular function of lipids, proteins, carbohydrates, enzymes, and nucleic acids is due to an imbalance between pro-oxidants and antioxidants gives rise to oxidative stress.^[3] Moreover, the body can defense to protect against harmful effect of oxygen and nitrogen reactive species using exogenous and endogenous antioxidant enzymes such as catalase (CAT), glutathione

peroxidase, and superoxide dismutase (SOD); and nonenzymatic systems as thiol reduced (glutathione [GSH]), vitamins, minerals, and polyphenols.^[4] Antioxidants are vital substances that possess the ability to protect the body from damage caused by free radical induced oxidative stress. However, sometimes endogenous antioxidants are not able to prevent oxidative damages, requiring the supply of exogenous free radical scavengers. Many synthetic antioxidant agents have been developed to alleviate oxidative stress but have major drawbacks such as

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high cost, lack of availability, and potential to health risk. In this direction, natural antioxidants replaced to synthetic compounds and had potential application in prevention and/or curing of diseases.^[5]

Mimosa pudica Linn. (Mimosaceae) is a common native plant from Central America, Tanzania, South Asia, East Asia, and many Pacific Islands.^[6] Traditionally, the roots and leaves of *M. pudica* are used as a remedy for various diseases such as a headache, migraine, insomnia, diarrhea, dysentery, fever, piles, fistula, epilepsy, and uterine bleeding, etc.^[7-10] Experimental reports indicated that *M. pudica* possess aphrodisiac, purgative, antidropsical, anti-inflammatory, wound healing, hyperglycemic, antimicrobial, antivenom property.^[11,12] Furthermore, *M. pudica* reveals the presence of bioactive constituents such as mimosine, terpenoids, flavonoids, glycosides, alkaloids, quinines, phenols, tannins, saponins, coumarins, D-xylose, D-glucuronic acid 4-O-(3,5 dihydroxybenzoic acid)- β -d-glucuronide, norepinephrine,^[11] and bufadienolide.^[13] Several studies on herbal plants, vegetables, and fruits have indicated the presence of antioxidants such as phenolics, flavonoids, tannins, Vitamin C, Vitamin E, polyphenol, and proanthocyanidins.^[14,15] Thus, the consumption of dietary or other source rich in antioxidant property may support the prevention of oxidative stress-induced degenerative diseases.^[15,16] Based on the traditional and scientific reports, the present study was carried out to evaluate the *in vitro* antioxidant activity of various extracts of *M. pudica* leaves. In addition, the estimation of oxidative stress markers from brain homogenate of ethyl acetate extract of *M. pudica* treated rat was also evaluated.

MATERIALS AND METHODS

Chemicals

2, 2'-diphenyl-1-picrylhydrazyl, phenazine methosulfate, nicotinamide adenine dinucleotide (NADH), Ellman's reagent were obtained from Sigma-Aldrich chemical, Bangalore, India. Carboxymethyl cellulose, thiobarbituric acid, Folin-ciocalteu's reagent, ascorbic acid, trichloroacetic acid, and glacial acetic acid were purchased from Hi-Media Pvt. Ltd., Bombay, India. All other chemicals and reagents used in this study were of analytical grade.

Plant materials and preparation of extract

The leaves of *M. pudica* (Linn) were collected in the month of November from the local places of Berhampur, Odisha, India; and were authenticated by Prof. B. K. Mohanty, Department of Botany, K. K. Autonomous College, Berhampur, Odisha, India. The collected leaves of *M. pudica* were washed thoroughly to remove foreign matter and allowed to dry under shade with a relative humidity of 40–45%. The shade-dried leaves were reduced to coarse powder in roller grinder and then passed through a sieve (No. 40). Then the fine powder were defatted with petroleum ether (Boiling point 40–60°C) and were individually extracted with three different solvents as chloroform, methanol, and ethyl acetate at room temperature by using Soxhlet apparatus for 72 h. The resultant extract was filtered through a muslin cloth and then concentrated in a rotary evaporator under reduced pressure to obtain a thick semisolid brown paste, which was then stored at –20°C until required. The extracts are abbreviated as CEMP, MEMP, and EAMP for chloroform, methanol, and ethyl acetate extract of *M. pudica*, respectively. The yields of these extracts were found to be 10.87, 8.90, and 8.41%, respectively.

Experimental animals

Albino rats (130–160 g) of either sex were obtained from Ghosh enterprises, Calcutta, India. They were randomly housed at an ambient temperature of 25 ± 1°C and 45–55% relative humidity, in polypropylene cages with 12 h light: 12 h dark cycle. The animals were allowed free access to standard food pellets (Rayan's Biotechnologies Pvt. Ltd, Hyderabad, India) and

water *ad libitum*. All the experimental protocols were conducted before the prior permission of Institutional Animal Ethics Committee of Roland Institute of Pharmaceutical Sciences, Berhampur, India for the purpose of control and supervision of experiments on the animal.

Acute oral toxicity study

The acute toxicity study of extract was performed on albino rats according Organisation for Economic Co-operation and Development guidelines -425.^[17] The animals were fasted overnight prior to the experiment with free access to water. The ethyl acetate extracts of *M. pudica* were administered the doses of 50, 300, 500, and 2000 mg/kg/p.o., and the behavioral change was observed up to 24 h. The ethyl acetate extract of *M. pudica* was found to be nontoxic up to the maximum dose of 2,000 mg/kg body weight. Dose selected for *in vivo* antioxidant study were 100, 200, and 400 mg/kg, respectively.

Preliminary phytochemical screening^[18]

The preliminary phytochemical screening was carried out with different extracts of *M. pudica* leaves for the detection of various phytochemicals.

Estimation of total phenolic content^[19] and total flavonoid content^[20]

The total phenolic content (TPC) of extracts was measured using the Folin-Ciocalteu method. Folin-ciocalteu's reagent (1.5 mL, 10% v/v) and 1.2 ml 7.5% w/v Na₂CO₃ were added to the 0.3 mL sample extract. The reaction mixture was thoroughly mixed and was incubated in the dark for 30 min. The absorbance of the reaction mixture was then measured at 765 nm with UV spectrophotometer. TPC was expressed in terms of mg of gallic acid equivalents (GAE) per 100 g fresh material.

The total flavonoid content (TFC) of extracts was measured using the aluminum chloride. Ten milligram of quercetin was dissolved in 80% ethanol and then diluted to 25, 50, and 100 µg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm by a UV-visible spectrophotometer. The amount of 10% aluminum chloride was substituted for the same amount of distilled water in blank. TFC was expressed in terms of mg of quercetin equivalents (QUE) per 100 g fresh material.

In vitro antioxidant by 2, 2-diphenyl-1-picrylhydrazyl scavenging assay^[21]

Free radical scavenging effect of the extracts was assessed by the discoloration of a methanolic solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). DPPH solution was prepared by dissolving 3.2 mg in 100 mL of 82% methanol. A volume of 2.8 mL of DPPH solution was added to glass vial followed by the addition of 0.2 mL of test sample solution in methanol, leading to the final concentration of 1, 5, 10, 25, 50, and 100 µg/mL. A mixture of DPPH and each fraction was shaken well and kept in the dark at controlled room temperature (25–28°C) for 1 h. After incubation, change in color was measured at 517 nm. A mixture of 2.8 mL of 82% methanol and 0.2 mL of methanol were used as blank while 0.2 mL of methanol and 2.8 mL of DPPH solution were taken as control. The percentage inhibition was measured according to following the formula as % of scavenging = [(Abs of control – Abs of sample)/Abs. of control] × 100.

In vivo antioxidant assay

Animal treatment

Twenty-four male albino rats ($n = 6$) were divided into four different groups. Group I served as a control group and treated with vehicle

only (0.5% carboxymethylcellulose sodium). Group II, III, and IV animals were administered orally with 100, 200, and 400 mg/kg of EAMP, respectively, for 7 days. At the end of 7th day, the animals were sacrificed by cervical dislocation and each brain was excised, rinsed in ice-cold normal saline and followed by 0.15 M Tris-hydrochloride. The homogenates were centrifuged at 15,000 × g for 10 min. The supernatants were employed for the following assays.

Lipid peroxidation assay (thiobarbituric acid reactive substances)^[22]

It was evaluated by thiobarbituric acid reactive substances (TBARS) tests during an acid-heating reaction. Aliquots of samples were incubated with 15% trichloroacetic acid and 0.38% thiobarbituric acid. The mixture was heated (1 h) in a boiling water bath. TBARS was determined by reading the absorbance of the pink-colored complex formed in a spectrophotometer at 532 nm.

Superoxide dismutase assay^[23]

This was estimated by the reaction mixture which contained 0.1 mL of phenazine methosulfate (186 µL), 1.2 mL of sodium pyrophosphate buffer (0.052 mL; pH 7.0), 0.3 mL of the supernatant after centrifugation (1,500 × g for 10 min followed by 10,000 × g for 15 min) of homogenate was added to the reaction mixture. Enzyme reaction was initiated by adding 0.2 mL of NADH (780 µM) and stopped after 1 min by adding 1 mL of glacial acetic acid. The amount of chromogen formed was measured by recording color intensity at 560 nm. Results were expressed in units/mg protein.

Catalase assay^[24]

It was determined with reaction solution contained 2.5 mL of 0.05 M phosphate buffers (pH 8.3), 0.7 mL of 0.2 M H₂O₂ and 0.1 mL of tissue homogenate. Changes in absorbance of the reaction solution at 570 nm were determined after 1 min. Results were expressed in units/mg protein.

Reduced glutathione assay^[25]

This was estimated by using dithiobisnitro-benzoate as a substrate. The yellow color developed and read immediately at an absorbance of 412 nm and expressed as µM GSH/g protein.

Statistical analysis

The values were expressed in mean ± standard error of the mean. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison test versus control. $P < 0.05$ and $P < 0.01$ were considered as significant.

RESULTS

Qualitative phytochemical screening

Phytochemical analysis of extracts of *M. pudica* leaves revealed the presence of alkaloids, glycosides, flavonoids, coumarins, lignins, tannins, terpenoids, carbohydrates, protein, fatty acids, and phenolic compounds.

Estimation of total phenolics and flavonoids contents

TPC of CEMP, MEMP, and EAMP was observed at 8.43 ± 1.03 mg, 11.59 ± 1.31 mg, and 15.64 ± 1.31 mg of GAE/100 g, respectively, whereas the TFC of CEMP, MEMP, and EAMP was observed at 0.75 ± 0.10 mg, 1.31 ± 0.01 mg, and 1.97 ± 0.47 mg of QUE/100 g, respectively, as shown in Figure 1. The results suggest that EAMP had a higher level of phenolic and flavonoidal contents as compared to other extracts of *M. pudica*.

In vitro antioxidant activity against 2, 2-diphenyl-1-picrylhydrazyl

The percentage of scavenging effect of DPPH on different extracts of *M. pudica* with a concentration of 1, 5, 10, 25, 50, and 100 µg/mL was compared with ascorbic acid as shown in Figure 2 and found dose-dependent inhibitory antioxidant potential against DPPH. Positive DPPH test suggests that the samples were free radical scavengers. The IC₅₀ values of CEMP, MEMP, EAMP, and ascorbic acid in DPPH assay were 85.97, 67.85, 46.06, and 23.74 µg/mL, respectively, as shown in Figure 3. Among all these extracts, the ethyl acetate extract was found better DPPH scavenging activity with a minimum IC₅₀ value of 46.06 µg/mL as compared to other extracts.

In vivo antioxidant activity of *Mimosa pudica* leaves on rat

Effect of *Mimosa pudica* leaves on body weight and brain weight

No significant changes were observed in body weight of treated and control rats before, and after the administration of the ethyl acetate extract of *M. pudica* for 7 days. Similarly, no considerable differences in the wet weight of the whole brain between control and EAMP treated rats as shown in Table 1.

Effect of *Mimosa pudica* leaves on oxidative markers in rat brain

The oxidative stress markers as lipid peroxidation (TBARS), SOD, CAT, GSH in rat brain homogenate was evaluated. EAMP at 200 and

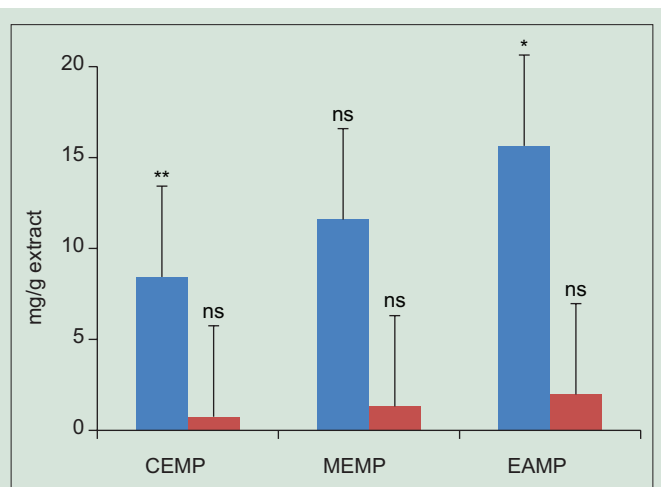


Figure 1: Comparative study of total phenolic content and total flavonoid content of various extracts of *Mimosa pudica* leaves. The values were expressed as mean ± standard error of mean; * $P < 0.05$, ** $P < 0.01$ versus control. ns: Not significant

Table 1: Effect of EAMP on body weight and brain weight in rats

Groups	Treatment	Dose (mg/kg)	Body weight (g)		Wet brain weight (g)
			Before treatment	After treatment	
I	Control	D.W	178.4±3.2	185.5±1.5	1.78±0.02
II	EAMP	100	181.3±2.5*	189.2±1.0*	1.77±0.02*
III	EAMP	200	185.0±3.8*	194.1±1.9*	1.74±0.04*
IV	EAMP	400	183.3±3.4*	195.8±0.8*	1.76±0.04*

All values were expressed as mean±SEM (n=6), where * $P < 0.05$ versus control. EAMP: Ethyl acetate extract of *Mimosa pudica*; SEM: Standard error of mean; D.W: Distilled water

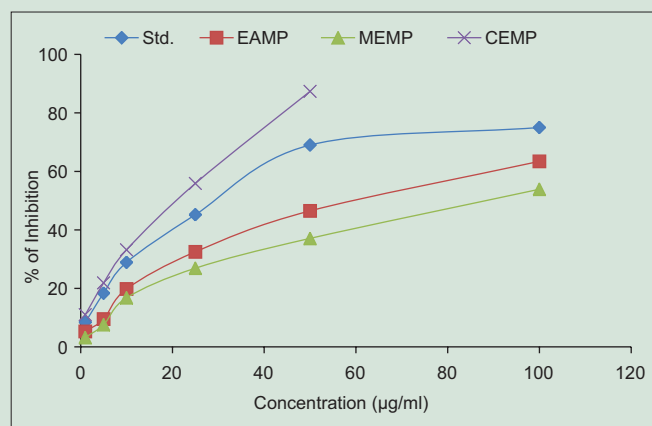


Figure 2: Effect of *Mimosa pudica* extracts on percentage of inhibition against 2, 2-diphenyl-1-picrylhydrazyl. The values were expressed as mean \pm standard error of mean ($n = 6$). CEMP, MEMP, and EAMP were the extracts of chloroform, methanol, and ethyl acetate extract of *Mimosa pudica*, respectively

400 mg/kg showed significantly ($P < 0.01$) and dose-dependently increased the level of antioxidant enzymes such as SOD, CAT, and GSH in brain tissue as compared to control rats as shown in Figure 4. However, EAMP (100 mg/kg) was produced no significant changes in antioxidant enzymes in rat brain tissue. In addition, EAMP at 400 mg/kg dose was significantly ($P < 0.01$) reduced the lipid peroxidation that is TBARS level to 1.22 ± 0.04 as compared to control rats of 1.51 ± 0.04 nM/min/mg protein. No significant change in TBARS level was observed for EAMP (100 and 200 mg/kg) treated rats.

DISCUSSION

Natural antioxidants that are ubiquitous in fruits, vegetables, and medicinal plants have received great attention in recent times since they are effective and lesser toxic than synthetic antioxidants.^[26] Polyphenols and flavonoids act as an antioxidant agent by the property of hydrogen atom donors, singlet oxygen scavengers, and free radical scavenger.^[27,28] Again, flavonoids have been reported as inhibitors of lipid peroxidation^[29] and prevent oxidative damage.^[30] The present investigation observed that the TPCs of crude extracts of *M. pudica* leaves varied from 8.43 ± 1.03 to 15.64 ± 1.31 mg GAE/g, whereas the TFCs varied from 0.75 ± 0.10 to 1.97 ± 0.47 mg QUE/g. All the extracts of *M. pudica* have a significant amount of phenolic and flavonoid components. However, the EAMP extract possessed the higher amount of phenolic and flavonoid content as compared to other extracts. They can be ranked as EAMP > MEMP > CEMP. The presence of flavonoids and phenolic components in ethyl acetate extract of *M. pudica* leaves substantiate the claim for its free radical scavenging activity and further attenuate the progression of oxidative stress induced diseases.

Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation, and by many other mechanisms and thus prevent diseases. Antioxidant activity is expressed in terms of IC_{50} , and a lower IC_{50} value corresponds to a larger scavenging activity.^[31] DPPH is a compound consists of a nitrogen free radical, which is easily quenched by a free radical scavenger. DPPH radicals are reduced into a nonradical form (DPPH-H) in the presence of a proton radical scavenger or hydrogen donating antioxidants.^[32] In fact, DPPH radical has an absorbance at 517 nm, which disappears after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.^[33] The effect of free radical scavenging activity of *M. pudica* extracts on DPPH radicals is thought to be due

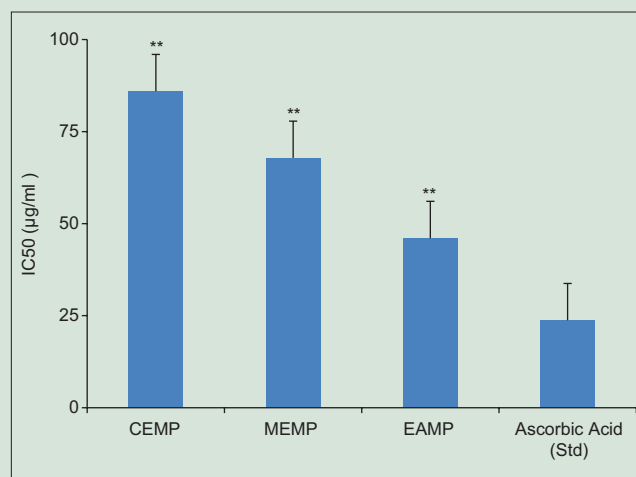


Figure 3: Estimation of IC_{50} value of various extracts of *Mimosa pudica* leaves against 2, 2-diphenyl-1-picrylhydrazyl. The values were expressed as mean \pm standard error of mean ($n = 3$). ** $P < 0.01$ versus standard. CEMP, MEMP, and EAMP were the extracts of chloroform, methanol, and ethyl acetate extract of *Mimosa pudica*, respectively

to their hydrogen donation ability of polyphenols. In the present study, the extracts showed a dose dependent elevation in DPPH scavenging activity of which EAMP showed lower IC_{50} than others. A similar study revealed that the aqueous extract of *M. pudica* showed a significant IC_{50} value (136 ± 0.003) but lower than ascorbic acid.^[34]

Lipid peroxidation is a complex process occurring in aerobic cells which reflects the interaction between molecular oxygen and polyunsaturated fatty acids. The radicals which are responsible for lipid peroxidation, that also causes food deterioration, ageing of organisms, and cancer promotion. These free radicals associated with other relative species cause the oxidation of biomolecules (e.g., protein, amino acids, lipid, and DNA), which leads to cell injury and death.^[3] Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon that can lead to various pathological consequences.^[35] The end products of lipid peroxidation are reactive aldehydes, such as 4-hydroxyl nonenal and malondialdehyde (MDA), many of which are highly toxic to cells.^[36] In addition, the MDA can react with biomolecules and exert cytotoxic, genotoxic, and neurodegenerative disorders. However, the endogenous antioxidants such as SOD and CAT are susceptible to oxidative changes. The enzyme SOD removes the superoxide anion, while CAT,^[37] a heme protein catalyses the reduction of H_2O_2 ^[38] and protects the tissues from highly reactive hydroxyl radicals that could be generated from H_2O_2 . Indeed, GSH provides the first line of defense for the body by scavenging ROS. Oxidative stress readily oxidizes GSH nonenzymatically to glutathione disulfide by electrophilic substances such as free radicals and ROS^[39] and causes depletion of GSH level by inhibiting glutamate-cystine antiporter.^[40] Our results showed that inhibition of MDA formation increases with increase in dose of EAMP that is a reduction in TBARS level. In addition, there is a significant increase of antioxidant enzymes such as SOD and CAT and nonenzymatic GSH level in rat brain homogenate, which is due to the linkage between phenolics and antioxidant enzymatic and nonenzymatic activity.^[40,41] Moreover, literature reveals that leaves of *M. pudica* showed the presence of mimosine, terpenoids, flavonoids, glycosides, alkaloids, quinines, phenols, tannins, saponins, coumarins, d-xylose and d-glucuronic acid 4-O-(3,5-dihydroxybenzoic acid)- β -D-glucuronide,^[11] might have contributed to the antioxidant activity. Several studies were demonstrated by *in vitro* and *in situ* models

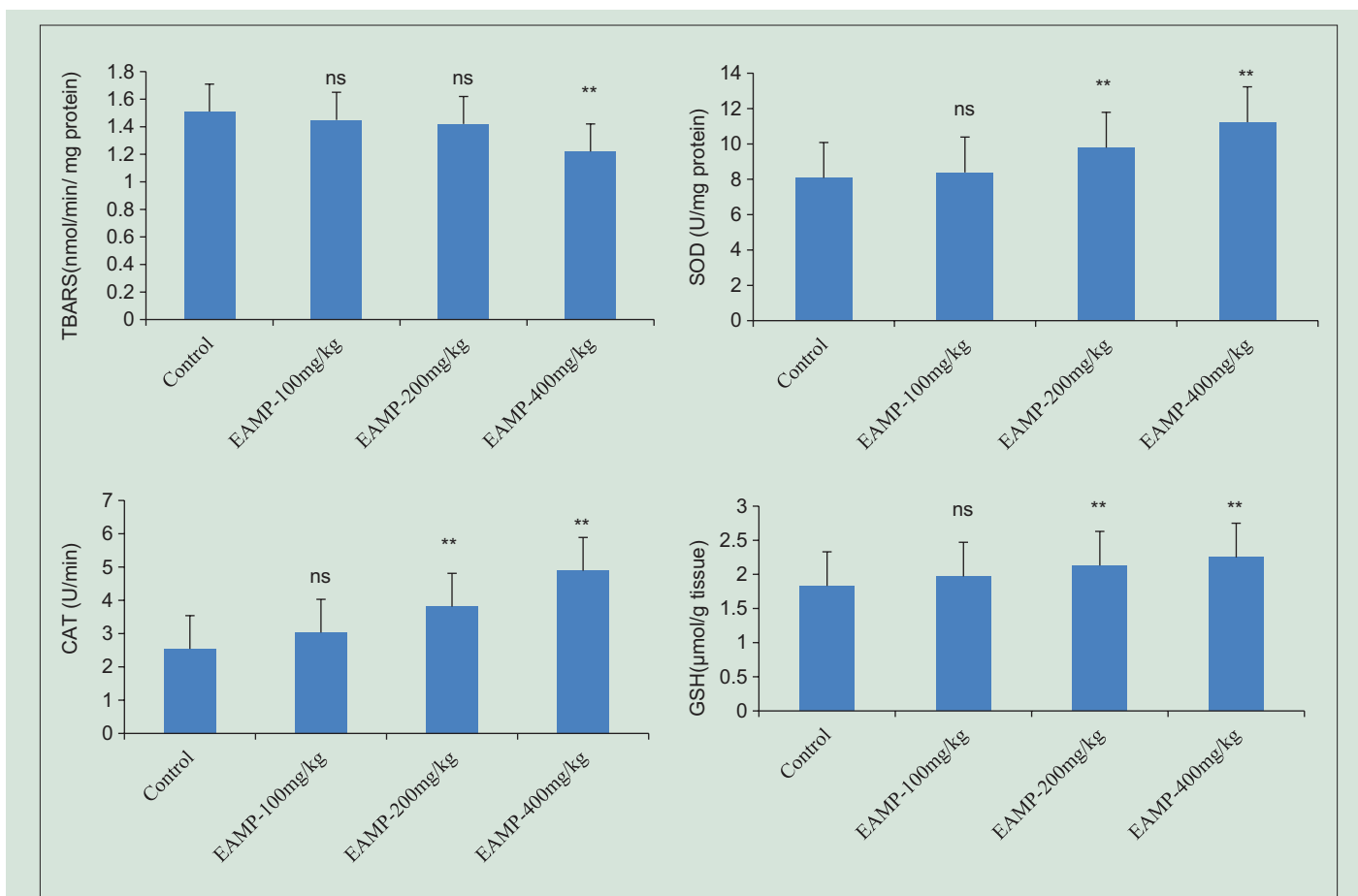


Figure 4: Effect of *Mimosa pudica* leaves on oxidative stress markers of brain in rat. All values were expressed as mean \pm standard error of mean ($n = 6$). ** $p < 0.01$ versus control. ns: Not significant

that, certain flavonoids e.g., quercetin and catechins are capable of penetrate through the blood-brain barrier. Earlier reports found the presence of phenolics, flavonoids, and tannins were found to possess the antioxidant property and attenuate cell death induced by oxidative stress, which supported our present findings.^[40,42]

In summary, the present study revealed that the EAMP showed the highest level of total phenolic, as well as flavonoid compounds, and were capable of initiating, quenching free radicals to terminate the radical chain reaction, and acting as reducing agents. Moreover, a significant and linear relationship was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity. In addition, it has demonstrated that EAMP had a greater potential of the inhibitory effect on cell lipid peroxidation and improved the antioxidant enzymes in rat brain homogenate. The antioxidant and biological activities might be due to the synergistic action of bioactive compounds present in them. The observation of this current investigation strongly suggests the potential antioxidant activity of EAMP leaves potentiates the neuroprotective effect by stimulating brain enzymes. Hence, it may be used as a weapon in various neuroinflammatory and neurodegenerative diseases. Furthermore, studies are warranted for the isolation and the identification of individual phenolic compounds; and also *in vivo* studies are needed for understanding their mechanism of action as an antioxidant better.

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Conflicts of interest

There are no conflicts of interest.

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