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NEUROPROTECTIVE ACTIVITY OF METHANOLIC EXTRACT OF *ALPINEA GALANGA* AGAINST EXCITOTOXICITY PRODUCED BY PTZ-INDUCED KINDLING MODEL IN MICE

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ABSTRACT

Alpinea galanga has been used for years together for its medicinal properties in Chinese medicine system. The use of antiepileptic drugs (AEDs) having neuroprotective action and its possible role in disease modification (i.e., antiepileptogenesis) is gaining interest day by day. The present study aimed to investigate the antiepileptogenic activity of methanolic extract of *Alpinea galanga* rhizomes (MEAG) and subsequently its neuroprotective action against excitotoxicity and reactive oxygen species (ROS) generation in the brain of pentylenetetrazole (PTZ) –induced kindled mice. MEAG was studied for its suppressive action on convulsion and seizure scores (antiepileptogenic effect) and protection of neurons against PTZ-induced oxidative stress injury (neuroprotective effect) when given during kindling acquisition as a pretreatment before every PTZ injection. A major antiepileptic drug Diazepam was also tested for comparison. MEAG and Diazepam showed antiepileptogenic activity as they caused reduction in development of seizure scores and reduced the sensitivity of kindled mice to the convulsive and lethal effects of PTZ and MEAG was found to be superior to Diazepam in preventing seizure scores. MEAG and Diazepam significantly decreased oxidative stress injury in the mice brain as compared to PTZ-kindled group. MEAG showed more neuroprotective action as compared to Diazepam as it enhanced the antioxidant enzyme levels in the mice brain. The results obtained from the study support the hypothesis that neuroprotective action of MEAG not only correlate with its ability to inhibit ROS formation but also for its ability to suppress seizure generation.

INTRODUCTION

A drug with improved anticonvulsant and anti-epileptogenic efficacy is need of the hour as currently available antiepileptic drugs failed to give adequate protection against epileptic seizures in one-third of epilepsy patients as epilepsy still remains one of the major neurological disorders for which safer drugs are awaited. [1]. During seizures prolonged over excitation of neurons leads to neuronal injury and even

death due to some poorly understood underlying biochemical mechanism. The currently available antiepileptic drugs also failed to control progressive epileptogenic consequences like neurodegeneration[2]. Accumulating research evidence supports the hypothesis that oxidative stress during experimental epilepsy leads to abnormal structure alteration of membrane lipids, cellular proteins, DNA and RNA [3] [4]. Molecular oxygen originating from different sources like mitochondria by different biochemical processess gives rise to reactive oxygen species (ROS). Superoxide dismutase (SOD), an intracellular antioxidant enzyme rapidly and specifically reduces O_2^- to hydrogen peroxide (H_2O_2). Another antioxidant enzyme, Glutathione peroxidase (GP_x) acts on H_2O_2 to detoxify it to water [5]. Catalase, the most common antioxidant enzyme found in living organism also catalyzes the decomposition of H_2O_2 to water and oxygen [6]. It is the most common enzyme protecting the cells and neurons from oxidative damage by ROS. One catalase molecule can convert millions of H_2O_2 to water and oxygen every second. The oxidant-antioxidant system works in equilibrium in normal conditions. However an inbalance between the systems causes tissue and neuronal damage by oxidative stress. ROS results in lipid peroxidation by attacking membrane lipids. Lipid peroxidation end product Malondialdehyde (MDA) thus serves as an index for tissue damage by lipid peroxidation. Nitric oxide (NO) role in cellular protection is contraindicatory as sometimes it acts as an oxidant and sometimes as a scavenger of O_2^-[7] [8]. Oxidative injury to brain is the major pathway of neuronal damage in many acute and chronic neuronal disorders like epilepsy, Parkinson's disease and Alzheimer's disease[9][10]. Therefore, the current trend is to look for a novel antiepileptic drug having antioxidant property. The novel drug should give protection against epilepsy, prevent neurodegeneration by ROS and have neuroprotection activity. This will not only give a alteration to the treatment of epilepsy but will also support the hypothesis on free radicals crucial role on the pathogenesis of brain damage by neurotoxins[11].

Alpinea galanga , also called as jujube or Indian date is a commom plant in India belonging to the family Zingiberaceae. Many investigations on its pharmacological activity justify its traditional therapeutic value. It has CNS depressant activity as it is used in many Ayurvedic preparations. Also it is reported to have antioxidant property as it is a rich source of flavonoids[12]. Therefore, the present study was undertaken to evaluate the neuroprotective activity of methanolic extract of *Alpinea galanga* (MEAG) rhizomes on experimentaly induced PTZ-kindled model in mice and also to evaluate whether administration of MEAG gives protection against PTZ induced seizures in mice. Also, by the end of the study we will be able to compare Diazepam with MEAG for its antiepileptic and neuroprotective activity as former is a major antiepileptic drug, biochemically and clinically.

Materials and Methods

Plant

The *Alpinea galanga* rhizomes were purchased from YUCCA Enterprises, MUMBAI. Then it was authenticated in Botanical Survey of India, Deccan Regional Centre, Hyderabad, Government of India. The plant material collected was shade dried to retain its important phytoconstituents and then subjected to size reduction and powder preparation for further extraction process.

Animals

Swiss Albino mice of either sex weighing between 25 to 30 g were procured from Sainath Agencies, Hyderabad. Animals were housed at an ambient temperature of

25±1ºC and 12hr/12hr light dark cycle in polypropylene cages. Animals were acclimatized to lab condition for 7 days before starting of the experiment. Animals were fed with standard diet and water *ad libitum*. All animal studies were performed in accordance to guideline of CPCSEA and Institutional Animal Ethical Committee (IAEC) of Anwarul Uloom College of Pharmacy, Hyderabad (CPCSEA Reg. No. 1534/PO/Re/S/2011 /CPCSEA) and permission letter Ref. No. IAEC/AUCOP/2016/02.

Preparation of Plant Extract

The rhizomes were dried in shade and coarsely powdered. The powder was passed through sieve no. 16. Approximately 200 g of powder was extracted in 60% methanol using a soxhlet apparatus. The extract mass was weighed in a digital balance and the % yield was calculated. The extract was kept in a beaker covered with alluminium foil labeled and kept in freeze for future use. The methanol extracts of *Alpinea galanga* (MEAG) rhizomes were subjected to the following investigations: Preliminary phytochemical screening, Determination of maximum tolerated dose in animals, Antiepileptogenic and Neuroprotective activity.

Preliminary phytochemical screening of extracts

The extracts were subjected to different chemical tests to detect the chemical constituents present in them. 0.5 gm of extract was dissolved in 5 ml of distilled water and filtered. The filtrate was used to determine the presence of various phytoconstituents[13][14].

Determination of Maximum Tolerated Dose

The literature survey showed that the rhizomes of *Alpinea galanga* and their various extract were used of several studies. Hence, on the basis of available information on the plant, limit test for crude methanolic extract of rhizomes of *Alpinea galanga* (MEAG) was conducted at the highest starting dose level i.e. 2000 mg/ kg of body weight. Three female mice were fasted over night with free access to water. Each animal received single dose of MEAG (2000 mg/kg, orally). After administration of the extract, animals were observed for any mortality, morbidity, body weight and signs of toxicity for 14 days (OECD guidelines 423, 2000, for testing of chemicals).

Procedure for induction of kindled seizures

The experiment was performed according to the method described by Erakovic[15]. Swiss Albino mice were grouped into 6, each consisting of 6 animals, weighing 25-30 g each. The first three groups served as non-kindling groups and received normal saline (2 ml/kg) (orally), diazepam (2 mg/kg) (orally) and plant extract (600 mg/kg of MEAG) (orally) respectively on 1,3,5,8,10,12, 15,17,19,22,24 and 26 days of treatment. The next three groups served as kindling groups and received PTZ (35 mg/kg) (s.c.) 30 minutes after treatment. On the 26 th day a PTZ dose of 75 mg/kg s.c. were given to the three kindled groups. Kindling was produced by a total of 11 treatments with PTZ (35 mg/kg) (s.c.) on every second day (Monday, Wednesday and Friday). Animals were observed for 30 minutes after the last drug administration and for an additional 30 minutes for lethality before returning to the home cage. Seizure intensity was evaluated using the following modified scale.

0. No response.
1. Ear and facial twitching.
2. Convulsive waves axially through the body.
3. Myoclonic body jerks.
4. Generalized clonic convulsions, turns over into side position.

5. Generalized convulsions with tonic extension episode and status epilepticus.
6. Mortality.

The animals were considered to be kindled after having received 11 PTZ injections and having reached at least three consecutive stage 4 or 5 seizures. After completion of the study the animals were sacrificed by cervical dislocation and brain will be removed for evaluation of neuroprotective activity.

Sample preparation and biochemical estimation

Brain Homogenate

Animals were sacrificed by cervical dislocation; brains were removed quickly and frozen for biochemical analysis. Brains were homogenized in ten volumes of ice cold Tris-HCl buffer (50 mM, pH 7.4). The homogenate sample was taken for estimation of lipid peroxidation (MDA), nitric oxide (NO). The rest of the homogenate were centrifuged at 1500 rpm for 15 minutes at 4 ºC and the supernatant thus obtained were used for the estimation of SOD and Catalase[16]. Estimation of Glutathione peroxidase (GPx) was done seperately following the procedure given by Paglia[17].

Total Protein

The phenolic group of tyrosine and tryptophan residues (amino acids) in a protein will produce a blue purple colour complex, with maximum absorption in the region of 660 nm wavelength, with Folin-Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Different dilutions of BSA solutions were prepared by mixing stock BSA solutions (5 mg/ml) and water in a test tube and the absorbance was measured at 660 nm to get a standard calibration curve. The concentration of unknown sample was determined from standard graph.[18].

Malondialdehyde (MDA)

MDA reacts with thiobarbituric acid (TBA) to generate a colored product, which can be measured spectro photometrically. 1 ml of sample was added to 1 ml of 40% TCA followed by addition of 2 ml of 0.67% TBA. The mixture was kept 10 minutes in a boiling water bath, cooled immediately in ice-cold water bath, centrifuged at 6,000 rpm for 30 seconds and absorbance of supernatant was recorded at 530 nm. Then MDA was calculated based on molar extinction coefficient (offered by 1M solution) i.e. (1.54×10^5 $M^{-1}cm^{-1}$) and the formula A=KCT [19].

Superoxide Dismutase (SOD)

This method was based on the ability of superoxide dismutase to inhibit the auto oxidation of pyrogallol. For Control 2.9 ml of Tris buffer, 0.1 ml of pyrogallol solution was added and mixed, and reading was taken at 420 nm exactly after 1 minute 30 seconds and 3 minutes 30 seconds. For Sample 2.8 ml of Tris buffer, 0.1 ml of sample solution was added and mixed, and the reaction was started by adding 0.1 ml of pyrogallol solution. Reading was taken same as control and calculation was done using the formula. Units of SOD/3 ml of assay mixture = (A-B) x 100/A x 50, where A and B are initial and final absorbance reading of control and sample[20].

Catalase

The decomposition of H_2O_2 can be followed directly by decrease in absorbance at 240 nm. The difference in absorbance (A) per unit time is a measure of catalase activity. For sample 1 ml of the homogenized tissue was mixed with 2 ml of the H_2O_2 solution and mixed well, and for blank 1 ml of the phosphate buffer was

mixed with 2 ml of H_2O_2 solution. The reaction was started by addition of H_2O_2 and the change in absorption was noted from 0 to 60 seconds. Catalytic activity will be calculated by using the formula Z = ΔA x V x 1000/€ x d x T[21].

Glutathione Peroxidase (GP_x)

Glutathione peroxidase activity will be measured by NADPH using a coupled reaction system consisting of reduced glutathione, glutathione reductase and hydrogen peroxide. The brain tissue was homogenized in 5 volumes of phosphate buffer and centrifuged at 13,000 rpm for 10 minutes. The supernatent was considered as the sample. In a tube 600 µl of potassium phosphate buffer, 300 µl of glutathione reduced and 300 µl of NADPH was added. 300 µl of the sample was added to the tube. 900 µl of distilled water was added to make the volume 2700 µl. The reaction was started by adding 300 µl of 12 mM H_2O_2. Absorbance was measured at 340 nm for 3 minutes. Glutathione peroxidase activity will be calculated by using the formula U = ΔA x V x 1000/€ x d x T [22].

Nitric Oxide (NO)

This assay was based on the enzymatic conversion of nitrate to nitrate reductase. The reaction was followed by a spectrophotometric detection of nitrite as an azo dye product of the Griess reaction, which absorbs light at 540 nm. 100 µL of Griess reagent, 300 µL of the nitrite containing sample and 2.6 µL of deionized water will be mixed in a spectrophotometer cuvette. The mixture was incubated for 30 minutes at room temperature and absorbance was noted. The concentration was determined from a graph [23].

Statistical Analysis

The results will be expressed as Mean±SEM. Statistical analysis of the values observed in all the experimental methods will be performed by ANOVA followed by Dunnett's multiple comparison test. For statistical analysis Microsoft Office Excel and Graphpad Prism version 6.0 will be used. * $p < 0.05$ will be considered as statistically significant.

Results

Phytochemical Examination of Extracts

Preliminary phytochemical analysis of methanolic extract of *Alpinea galanga* rhizomes showed presence of flavonoids, steroids, terpenoids, tannins, carbohydrates, proteins, fixed oils and fats.

Maximum Tolerated Dose (MTD)

The animal does not show any signs of toxicity even after 7 days of drug administration and no mortality were observed. Based on the observation 2000 mg/kg of the extract was found to be safe and it is taken as the maximum tolerated does. Three test doses 200 mg/kg, 400 mg/kg and 600 mg/kg were selected arbitrarily based on the maximum tolerated does (MTD) calculation.

Effect of Diazepam and MEAG on induction of kindling by PTZ

All mice in the entire groups survived without any complications at the end of the kindling period. In the PTZk group, repeated administration of a subconvulsant dose of PTZ (35 mg/kg, s.c.) on every second day (for 24 days, 11 injections) resulted in increasing convulsive activity leading to generalized clonic-tonic seizure score of more than 5. Administration of Diazepam at the dose of 2 mg/kg and MEAG 600 mg/kg respectively did not modify the course of kindling induced by PTZ. However, treatment with MEAG suppressed the kindled seizure as seen in the decreases in mean seizure scores on the

study days (Fig.1). On the test day (PTZ, 75 mg/kg s.c.), PTZ-kindled mice developed a classical pattern of limbic type motor seizures with a mean seizure score 6 (Table 1). The convulsive response consisted of first twitch, short-lasting episodes of clonic seizures, and then continuous clonic seizures with wild running ending with falling of the animal and tonic seizures. Pretreatment with Diazepam and MEAG did not prevent the development of seizure. Diazepam and MEAG pretreated PTZ kindled animals produced a seizure intensity which was less than the saline-treated PTZ kindled mice. Apart from this, the Diazepam and MEAG PTZk group showed a higher latent period as compared to the saline treated PTZk group (p=<0.0001). On the test day (day 26), after the treatment of PTZ 75 mg/kg s.c. all the 6 animals in the saline treated PTZk died while 2 out of 6 animals survived in the Diazepam PTZk group. All the 6 animals survived in the MEAG PTZk group.

Kindling was induced by a total of 11 treatments with 35 mg/kg PTZ s.c. on every second days (Monday, Wednesday, and Friday). Diazepam pretreatment did not alter the course of kindling induced by PTZ. There was no statistically difference for mean seizure scores between the PTZk groups and Diazepam PTZk group. However, treatment with MEAG suppressed the kindled seizure significantly, as none of the animals could achieve a score of 4 with 11 injections of PTZ. At the end of the study the Diazepam and MEAG PTZk group attained a mean seizure score of 5 and 3.5 respectively while saline treated group attained a score of 6.

Biochemical estimation of oxidant/anti-oxidant stress markers

Fig.2- a to e shows the brain levels of oxidant/antioxidant stress markers in the kindled and non-kindled mice. Superoxide dismutase activity was significantly decreased in the brain tissue in the PTZk group in comparison with other groups. PTZ kindling-induced decrement in the SOD activity was prevented by MEAG (p=<0.0001). . The PTZ induced kindling stress also decreased the Catalase activity in the brain of mice. Pretreatment with MEAG significantly brought the Catalase level back to normal (p=<0.0001) as compared to the saline treated PTZk group. There was a significantly lower level of GSH-Px in the PTZk group as compared with the other groups. MEAG-treated PTZk groups showed significantly higher levels of GSH-Px compared with the PTZk group (p=0.0013). PTZ kindling produced a significant increase in the brain tissue MDA content, an index for lipid peroxidation, when compared with other groups. PTZk-induced increment in MDA content of the tissue was significantly preventd by MEAG treatments (p=<0.0001). Therefore, Diazepam and MEAG treated PTZk groups showed significantly lower levels of MDA as compared with the PTZk group. The tissue MDA contents in MEAG group remained near the control value. PTZ kindling produced a significant decrease in the NO activity in brain tissue when compared with other groups. MEAG administration caused a significant increase in the NO levels in comparison with the PTZk group (p=0.0003).

DISCUSSION

The results from the present study demonstrated that the MEAG clearly have antiepileptic property against the development of seizures in PTZ-kindled mice model. MEAG also showed significant effect in controlling clonic-tonic seizures and lethality in PTZ-kindled mice model. Moreover, the experimental results demonstrated that the MEAG was very much effective as a neuroprotective agent against PTZ-kindled seizures by means of its antioxidant actions in controlling the free radicals generated due

to excitotoxicity[24]. In recent years, plants are investigated extensively for their medicinal properties in the light of scientific developments due to their potent pharmacological activities, low toxicity and economic viability. MEAG is a very effective free radical scavenger showing potent antioxidant activity and protecting the neurons against free radical damage. Therefore, MEAG can be used in diseases in which free radicals are involved, eg. Anoxia, ischaemia of heart and brain, arteriosclerosis, cancer and rheumatism [25]. An imbalance between higher levels of cellular reactive oxygen species (ROS) (eg. O_2^-, $^{\cdot}OH$, $NO^{\cdot}$ and $ONOO^-$) and cellular antioxidant defense results in oxidative stress. Different defense mechanisms exist in the brain like enzymatic (Catalase, SOD,GP_x), non-enzymatic (Glutathione) and dietary (Vitamin A, E, C, β-carotene, quinones and flavonoids) to scavenge the reactive oxygen species and act as antioxidants. The vulnerability of brain tissue to ROS is very high because blood perfusion and aerobic metabolism is very high in brain and it has a relatively poor enzymatic antioxidant defense [26]. Brain is also very rich in lipids that are highly susceptible to oxidative damage and the adult damaged neuronal DNA cannot be effectively repaired since there is no DNA replication [27]. PTZ-kindling model, one of the most important rodent experimental epilepsy model shows oxidative stress in the central nervous system [28]. The PTZ-kindled model is characterized by an increased susceptibility to seizures after a single or repeated subconvulsive dose of PTZ. PTZ acts by blocking the chloride ionophore complex of the GABA-A receptor. Single or reapeated dose administration of PTZ leads to decrease in GABAergic function [29].

It also modifies the density and sensitivity of different glutamate receptors subtypes in the brain regions [30]. PTZ also stimulates a variety of biochemical processess in the brain including activation of membrane phospholipases, nucleases and proteases. Marked alterations in metabolism of the membrane phospholipids results in the liberation of free fatty acids (FFA_s), free radicals, diacylglycerols and lipid peroxides [31]. We have selected the PTZ kindling model instead of single dose PTZ model basing on the study results. Different study had shown the difference between single dose PTZ model and PTZ kindling model on the changes of free fatty acid contents in different parts of brain [32]. Their finding suggested that PTZ induced kindling distinctively impaired antioxidant defence in mice brain which a single dose of PTZ failed to do the same. For example a single dose of PTZ did not alter any SOD activity in frontal cortex, but PTZ kindling caused a marked decrease in SOD activity. The present study supports the hypothesis that PTZ induced seizures actively enhances oxidative stress in brain tissue. The primary and most important antioxidant enzyme SOD, rapidly scavenges the superoxide anion radicals to hydrogen peroxide which is less toxic than superoxide anion. In our study there was significant lower level of SOD in PTZ_k mice. Treatment with MEAG significantly increased the SOD level in MEAG+ PTZ_k mice, indicating neuroprotective and antioxidant activity. Catalase is an enzyme which converts the byproduct H_2O_2 formed by scavenging of superoxide anion into less toxic water and oxygen. In our experiment it is observed that the catalase level in PTZ_k group is significantly low. The treated group MEAG+ PTZ_k showed significant increase in catalase level indicating antioxidant and neuroprotective activity. Glutathione peroxidase, an endogenous antioxidant reacts with the free radicals and prevents the generation of hydroxyl radicals, the most toxic form of free radicals. During this defensive process, reduced glutathione is converted to its oxidised form by GP_x. The decreased GP_x activity in PTZ_k mice in our study indicates increased generation of free

radicals in kindled model and its depletion during the process of combating oxidative stress. Treatment with MEAG in MEAG+ PTZ_k group significantly increases GP_x level and thus indicates antioxidant and neuroprotective activity. The elevated level of MDA, a marker of lipid peroxidation indicates a increased generation of free radicals in PTZ_k mice. Treatement with plant extract, i.e. MEAG significantly decreased the level of MDA in PTZ_k mice. Therefore, the significantly lower levels of MDA in MEAG+ PTZ_k mice as in comparision with PTZ_k mice indicate attenuation of lipid peroxidation and free radical generation. In our present study, NO level was reduced after kindling seizures in PTZ_k group. The role of NO in the pathophysiology of seizures remains unclear and debatable. Experiments using nonselective NOS inhibitors yielded conflicting results. Therefore, authers have proposed both pro and anticonvulsant roles of NO [33]. More research is required for an adequate conclusion on the role of NO.

Fig.1- Effects of Diazepam and MEAG pretreatment on the development of PTZ-kindled seizures

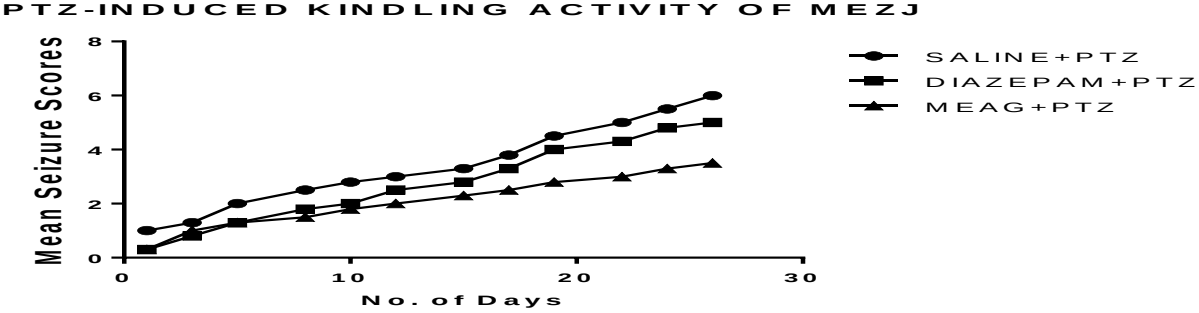

Table-:1- Comparision of the convulsive and lethal effects of PTZ (75mg/kg) (s.c.) in kindled mice on day 26.

Groups	Dose (mg/kg)	Latent period in seconds	Median seizure score	Lethality
Control	Saline(2ml/kg)(p.o.) + PTZ(75mg/kg)(s.c.)	50.33±12.06	6.0±0.1	6/6
Standard	Diazepam(2mg/kg)(p.o.) + PTZ(75mg/kg)(s.c.)	416.2±35.57*** (p=<0.0001)	5.0±0.2** (p=0.0012)	2/6* (p=0.0101)
Test	MEAG(600mg/kg)(p.o.) + PTZ(75mg/kg)(s.c.)	591.8±43.62***,# (*p=<0.0001) (#p=0.0109)	3.5±0.2***,### (*p=<0.0001) (#p=0.0003)	0/6*** (*p=<0.0001) (#p=0.1440)

Values are (Mean±SEM); n=6 in each group; p<0.05, ** p< 0.01 and *** p<0.001 in comparison with control group; # p<0.05, ### p<0.001 in comparison with standard group.*

Fig.2 (a)

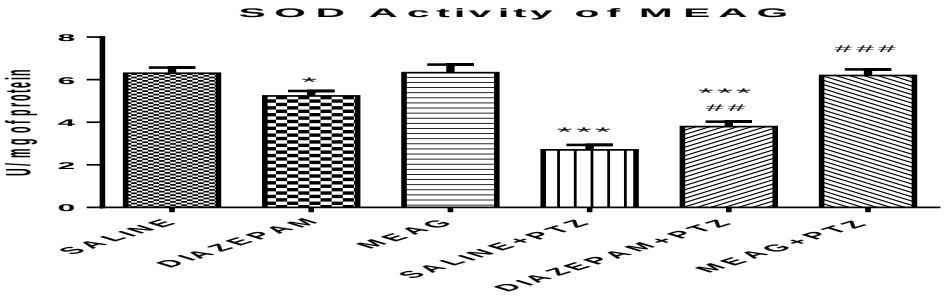

Fig.2 (b)

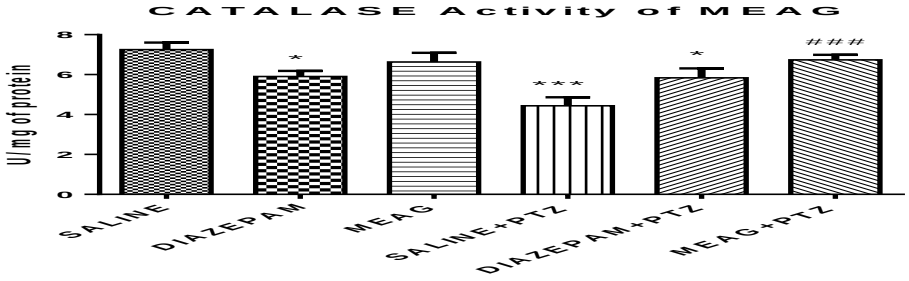

Fig.2(c)

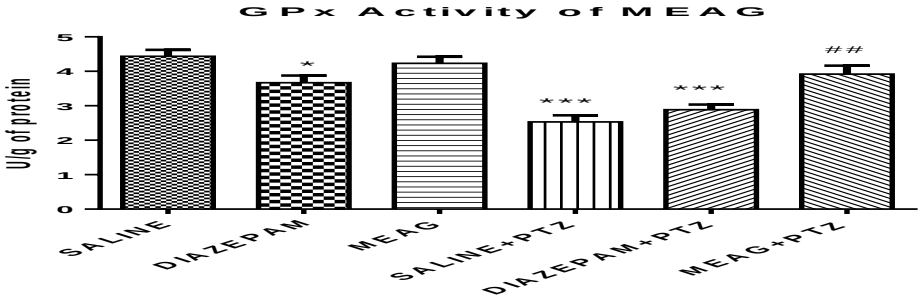

Fig.2 (d)

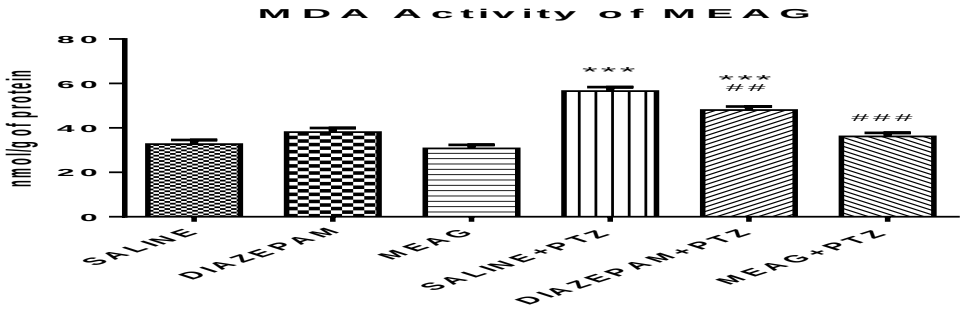

Fig. 2(e)

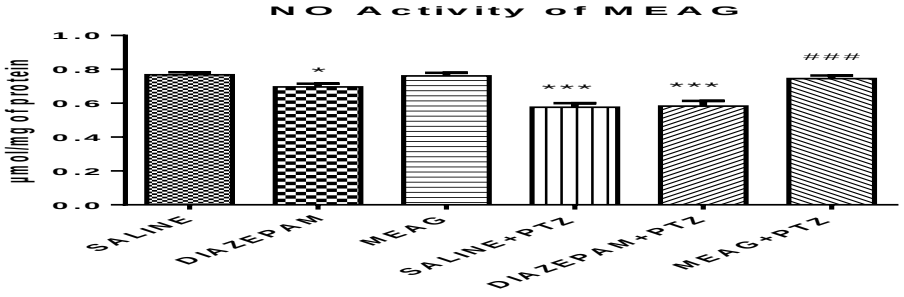

*Fig. 2 (a-e) Brain MDA, SOD, Catalase, GPx and NO activities in each group. Values are (Mean±SEM); n=6 in each group; * p<0.05, ** p<0.01 and *** p<0.001 in comparison with saline treated non-kindled group; # p<0.05, ## p<0.01 and ### p<0.001 in comparison with saline treated kindled group.*

Treatment with MEAG in MEAG+ PTZ_k group significantly increased NO level which may have some antiepileptic as well as neuroprotective activity. A significant decrease of MDA level and enhancement of SOD, Catalase, NO and GP_x level in MEAG+ PTZ_k group over other PTZ_k group indicates its neuroprotective role by antioxidant activity.

CONCLUSION

The present study demonstrates that MEAG significantly prevented PTZ-kindled seizures in mice as compared to Diazepam and attenuated oxidative stress induced by PTZ-kindling rendering neuroprotective action to brain by its antioxidant activity. Therefore it can become a potential drug which can be approached in arresting or inhibiting the seizure genesis caused by excitotoxic agents.

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