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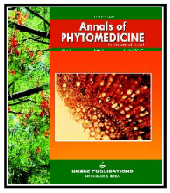

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Anticonvulsant effects of *Argemone mexicana* L. against maximal electroshock and pentylenetetrazole induced seizure in rats

Vinod Gahlot and Dinesh Kumar Yadav◆

SGT College of Pharmacy, SGT University, Gurugram-122505, Haryana, India

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Abstract

After stroke and Alzheimer's disease, epilepsy is the third most common neurological disorder. Medicinal plants possess enormous importance for the management of various diseases, traditionally. In this study, the anticonvulsant effect of the hydroalcoholic extract of *Argemone mexicana* L. (HAEAM) was evaluated in maximal electroshock (MES) and pentylenetetrazole (PTZ) seizure tests. The HAEAM was orally administered and the occurrence of tonic seizures induced by MES and clonic seizures induced by PTZ were monitored. In addition, biochemical parameters such as antioxidant enzymes and AChE level were analyzed in the brain and also evaluate the histological architecture of the hippocampus and cortex. In the MES test, the HAEAM showed a significant (p<0.05) anticonvulsant effect by decreasing the duration of hind limb extension (extensor phase), as compared to control. In the PTZ test, HAEAM showed a significant (p< 0.05) effect as compared to control by delaying the onset of convulsions. The HAEAM treatment significantly improved the enzymes (AChE, CAT, SOD, and GSH) level in the brain. Histological examination of the treated group showed amelioration of damaged hippocampus and cortex tissues, hence confirmed the antiepileptic efficacy of HAEAM. The outcomes of the study suggested that HAEAM might be a promising source for anticonvulsant activity.

1. Introduction

About 70 million people engaged with epilepsy globally and among them, approximately 12 million are estimated to be inherent in India. The total part of the engaged population with epilepsy, one-sixth part occupied of the global burden. The study also estimated an average frequency of 1.54% and 1.03% for rural and urban in developing countries (Amudhan *et al*., 2015). It is a life-threatening condition and carries a risk of premature mortality. It is somewhat higher in men than women which mostly appeared in the elderly stage due to the higher frequency of stroke and neurodegenerative disorders (Beghi, 2020). Psychosis is a state which is characterized by a mental distortion of normal thinking or reality with the following signs such as delusions, hallucinations, catatonia, impaired social cognition, or disruption of normal thoughts (Amudhan *et al*., 2015). Psychosis is generally more conceived by the patients associated with bipolar disorder, schizophrenia, and Parkinson's sickness. Although, numerous newly developed drugs with their exclusive advantages have been familiarized till date, still due to unsatisfactory side effects, they are being failed to avail reasonable therapy to treat even control seizures in the patients. Moreover, roughly 30% of the patients who are taking treatment with modern antiepileptic drugs, do not reach satisfactory treatment with such pathogenesis and they are still engaged with several mental unfitness (Ffytche *et al*., 2017). Taking all these into consideration, the paucity of the patients is extended to other novel effective drugs with the least side effects for the treatment of epilepsy.

The biomedical evidence claim that resulting from the persistent generation of oxidative stress (OS) is the possible mechanism involved in epileptogenesis with liberal distortion of targeted and non-targeted neuronal cell inhabitants and protein masses. ROS includes a diversity of radicals especially hydrogen peroxide, superoxide radical and hydroxyl radical oxygen, which are generally produced during normal cellular metabolism. The production of ROS is increased stressed metabolism or cellular stress. Antioxidant enzymes inside the body scavenge the overproduction of ROS by the antioxidant enzymes; namely, glutathione peroxidase (GSH), superoxide dismutase (SOD), catalase (CAT), *etc*. Evenly, the remaining surplus ROS as nitric oxide is further neutralized by the antioxidant defensive mechanism. The brain is predominantly exposed to oxidative stress due to its high consumption or supply of oxygen than other organs (Diniz *et al*., 2015). The brain is comprised of even protected with the different types of poly unsaturated fatty acids that are susceptible to lipid peroxidation which can be catalyzed to hydroxyl radical development (Birben *et al*., 2012).

Herbal medicine and its derived products are considered as the potential sources for the development of new drugs or even the treatment of various illnesses. Herbal medicines greatly reflect the important role of traditional medicine, and they have been pronounced in different pharmacopeias, globally (Yuan *et al*., 2016). *Argemone mexicana* (*A. mexicana*) is one of the traditional Indian herbal medicine, used for the treatment of various disorders such as antipyretic, anti-inflammatory, sedatives, diabetes, and many more,

Corresponding author: Dr. Dinesh Kumar Yadav

Associate Professor, SGT College of Pharmacy, SGT University, Gurugram-122505, Haryana, India

E-mail: dineshnbri08@gmail.com

Tel.: +91-7042348251

especially in the Asian population (Tariq *et al*., 2015). According to our extensive literature reviews up to the time of this work proposal, no prior reports were found looking into the effects of these noteworthy plants for the management of epilepsy.

Therefore, considering the above describe facts, our objective is to reveal the pharmacological potential of *A. mexicana* for the management of epilepsy, using maximal electroshock seizure (MES) and pentylenetetrazole (PTZ)-induced epileptic model with another bioassay anticholinesterase and antioxidant activity.

2. Materials and Methods

All chemicals and reagents were of analytical grade used in the study and acquired from SRL and Himedia Laboratories Ltd., Mumbai. Acetylcholinesterase and other commercial reagent kits (ROS, GPx, GSH, NOS) were used for the assessment of biochemical studies which were obtained from Amplicon Biotech, Delhi, India.

2.1 Plant material

The plant material was collected from the local region of Delhi NCR and authenticated by CSIR-National Institute of Science Communication and Information Resources. The plant specimen was submitted to the Raw Material Herbarium and Museum with the authentication number NISCAIR/RHMD/Consult/2020/3660-61.

2.2 Preparation of hydroalcoholic extract of *A. mexicana* (HAEAM)

Accurately, weighed 500 g of coarsely powdered whole plant material of *A. mexicana* and transferred it in a round bottom flask containing ethanol and distilled water in 7: 3 v/v ratio (2.8 l) and reflux method for extraction was performed at 60ºC temperature. After extraction, the extract was filtered, concentrated to dryness and stored in an airtight container for further use (Mukherjee *et al*., 2008).

2.3 Estimation of total phenols, flavonoids and DPPH free radical scavenging activity

The total phenol, flavonoids content and free radical scavenging analysis for HAEAM were estimated by the Folin Ciocalteu (FC), aluminum chloride and DPPH method with some modifications in the described protocol (Gaurav *et al*., 2020). The content in HAEAM was expressed as mg gallic acid/rutin equivalent /gm of the sample.

2.4 *In vitro* AChE inhibition activity

AChE inhibition activity was performed as per standard protocol with little modification (Hasnat *et al*., 2013). Briefly, 10 µl of different concentrations of polyherbal formulation (200-1000 µg/ml) was mixed with 150 µl of 0.1 M sodium phosphate buffer (pH 8.0), followed by the addition of 20 µl of enzyme solution (0.09 units/ml) and 10 µl of DTNB (10 mM) solution in a 96 wall plate and incubated for 15 min at room temperature. The reaction was initiated by the accumulation of substrate (10 µl of acetylthiocholine iodide (ATCI), 14 mM solution). Further, the developed color was measured spectrophotometrically at 410 nm wavelength after 10 min.

2.5 Experimental animals

Wistar albino rats weighing 175-200 gm will be procured from the SGT Medical College and University (1401/PO/Re/S/10/CPCSEA) with the approval number Pharma/FMHS/SGTU/791. The animals were housed in polypropylene cages and provided free access to water and food at 21 ± 1°C and 12 h light/dark cycle. The experimental procedure was processed as per the standard protocol of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

2.6 Establishment of the epilepsy rat model

Fifty rats were taken for the study which was alienated into five groups as follows: Group I control saline group. Group II (MES and PTZ control group): rats administered an intraperitoneal injection to prompt the chronic epilepsy model (Abdel-Rahman *et al*., 2015). Groups III-IV rats were receiving protective doses of the extract (low 200 mg/kg, p.oand high 400 mg/kg, p.o., respectively of HAEAM) along with PTZ. Group V: received standard. Importantly, the selected doses of the extract were determined according to those reported in previous studies either for the same plant or for closely related plants belonging to the same family.

2.7 Determination of anticonvulsant activity

2.7.1 Maximal electroshock seizure (MES) test

The anticonvulsions potential of HAEAM was determined through maximal (tonic hind limb extension) electroshock seizure (MES) test in the control and drug-treated animals. In this method, supramaximal stimulation was conceded through the trans-auricular introduction of copper electrodes with a fixed strength of the current 150 mA for 2 seconds in the rats. The hind limbs tonic extension was noted (Abdel-Rahman *et al*., 2015).

2.7.2 PTZ-induced seizure test

The anticonvulsant effect of the HAEAM was determined through the induction of seizure by PTZ injection. The ability to reduce the onset of PTZ by the test drug was considered as a positive response by the drug. In brief, 85 mg/kg ip dose of PTZ was administered to the animals and the convulsive effect was recorded after 30 min of drug administration. The mortality percentage for positive and negative treatment was noted as per the defined protocol (Abdel-Rahman *et al*., 2015).

2.8 *In vivo* antioxidant capacity of HAEAM

The antioxidant capacity of HAEAM on the brain was determined by isolating the cortex and hippocampus part of each animal's brain. The isolated part of the brain tissues was homogenized PBS (pH = 7.4), carefully. Further, the obtained homogenate was centrifuged at 3000 g for 15 min at 4°C. Thereafter, the obtained supernatant was separated from each centrifuged homogenates and the level of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH) was determined spectrophotometrically using an ELISA reader at 405 nm (Ibrahim *et al*., 2021).

2.9 Assessment of AChE activity in the brain

The AChE assay on brain homogenates was accomplished according to the standard protocol with slight modifications (Hasnat *et al*., 2013). In brief, 0.1 ml of homogenates supernatant, 2 ml of 0.1% BSA sodium phosphate buffer, 0.1 ml DTNB and 0.05 ml of acetylthiocholine iodide (AChI) were mixed and remained for a period till the change of color. The variation in absorbance based on

the color intensity was recorded spectrophotometrically at 412 nm for 2 min at the interval. Acetylcholinesterase activity was represented as per the cited protocol.

2.10 Histopathology of the brain (hippocampus and cortex)

Brain tissue was collected from each positive and negative treated animal and stored in buffered formalin were used for histopathology study. Each tissue was proceeded to produce blokes by fixing in paraffin and 5 µm thickness thin transverse sections of the hippocampus and cortex region were taken using a microtome (Auti and Kulkarni, 2019). Hematoxylin and eosin (H & E) were used to stain each section and examined under a digital microscope at 40X after complete slide formation.

2.11 Statistical analysis

Results are represented as the mean ± standard deviation (SD) using one-way ANOVA, followed by Tukey multiple comparison tests. The statistical comparison was done by comparing the control group to the negative control and drug-treated group. The statistical significance values were considered as $p<0.05$. The analysis was carried out using Graph Pad Prism 5, software.

3. Results

The extraction process for *A. mexicana* hydroalcoholic extract was done successfully. The obtained yield was found as 13.27% (w/w) which was used for further studies.

3.1 Estimation of total phenols, flavonoids and DPPH free radical scavenging activity

Total phenols and flavonoids in aqueous extract of HAEAM were determined successfully. The resulted outcomes were expressed statistically in terms of Mean ± SD. Each measurement was taken in triplicate for the determination of statistical differences. The outcomes of total phenols and flavonoids content estimation in HAEAM were found as 11.363 ± 0.754 and 1.929 ± 0.0365 equivalents to mg of gallic acid and rutin/gm of extract. Further, in DPPH antioxidant activity, the IC_{50} of HAEAM showed 263.61 ± 2.879 µg/ml while IC_{50} of ascorbic acid showed 80.07 ± 2.144 µg/ml.

3.2 AChE inhibitory activity

The AChE inhibitory activity by HAEAM was tested using Ellman's colorimetric method in a 96-well microplate.. The outcomes revealed that HAEAM showed significant ($p<0.005$) and dose-dependent AChE inhibitory potential. Galantamine was used as the positive control which showed comparatively high AChE inhibitory potential then sample. The resulted outcomes as inhibition curves have been presented in Figure 1.

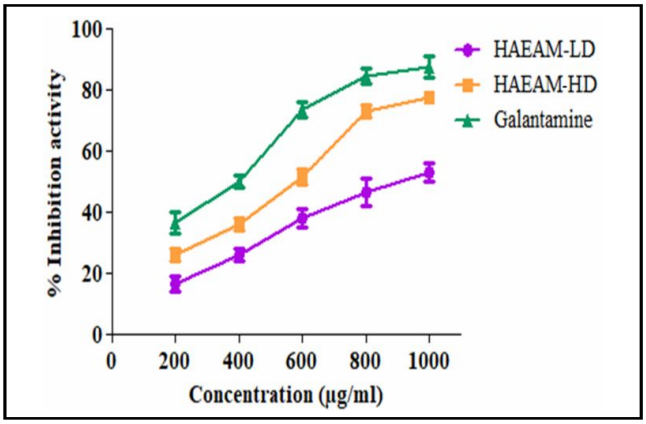

Figure 1: Evaluation of AChE activity of extract by Ellman assay.

3.3 Maximal electroshock seizure (MES) test

The protective potential of HAEAM against seizure-induced by MES was evaluated on rats using two different doses. Treatment of rats with the HAEAM (200 mg/kg/p.o.), did not show any significant effect against seizure-induced by MES while at a higher dose (400 mg/kg/p.o.), it produces significant protection. The obtained results revealed that 100 % of the negative control rats showed hind limb tonic extensions seizure. In the case of the positive control group, 80% protection against MES seizures was exhibited by diazepam. In addition, the low and high doses of HAEAM provided 50% and 70% protection, respectively (Table 1).

Table 1: Anticonvulsant activity of HAEAM in MES-induced seizure in rat

Treatment	No. of animals exhibiting seizure	Protection against seizures (%)
NC	10/10	0
HAEAM-LD	5/10	50
HAEAM-HD	3/10	70
Diazepam	2/10	80

The results are expressed ratio and percentage (n =10), NC-Normal control; TC - Toxic control; HAEAM-LD - Hydroalcoholic extract of *A. mexicana* 200 mg/kg/b.w - HAEAM-HD - Hydroalcoholic extract of *A. mexicana* 400 mg/kg/b.w.

3.4 Pentylenetetrazole (PTZ)-induced seizures test

In this study, the outcomes of the study were made based on the protective strength of HAEAM against PTZ-induced convulsions/ lethality in rats. The results have been represented in Table 2. The resulted data showed that both doses of HAEAM hindered the PTZ-induced threshold seizure latency up to 116.4 ± 3.86 and 144.7 ± 4.34 s, respectively, compared with normal controls (56.24 ± 2.85). Diazepam (7.5 mg/kg) as positive control produced significant ($p <$ 0.05) inhibition of tonic seizures and the number of animal death. In comparison, to low dose HAEAM, high dose of HAEAM showed more promising protection in contradiction of seizure (50%) and mortality (90%). HAEAM showed comparable protective potential in contradiction of seizure-induced by PTZ and barred the death ratio as like standard diazepam (Table 2).

3.5 Determination of antioxidant parameters in brain

The effect of oxidative stress on the brain was determined by assessing the brain antioxidant enzymes. The homogenate of the

brain was used to assess the antioxidant potential of HAEAM. Treatment of HAEAM-HD exhibited remarkable (p<0.05) improvement in the level of CAT, SOD, and GSH activities than the groups administered with HAEAM-LD. Interestingly, treatment with HAEAM-LD was seemed to comparable antioxidant effect as like standard. The outcomes of the study have been represented in Table 3.

Table 2: Anticonvulsant activity of HAEAM in PTZ-induced seizure in rats

Treatment	No. of animals exhibiting seizure	Latency(s)	Protection against seizures (%)	Protection against lethality (%)
NC	10/10	056.24 ± 02.85	0	0
HAEAM-LD	5/10	116.40 ± 3.86*	50	60
HAEAM-HD	4/10	144.70 ± 4.34*	60	90
Diazepam	0/10	ND	100	100

The results are expressed as Mean ± SD and %, n = 10 rats/group; * indicate significance compared to normal control group at p< 0.05; ND = not determined. NC-Normal control; TC - Toxic control; HAEAM-LD - Hydroalcoholic extract of *A. mexicana* 200 mg/kg/b.w; HAEAM-HD- Hydroalcoholic extract of *A. mexicana* 400 mg/kg/b.w.

Table 3: Effect of orally administration of HAEAM in biochemical parameters of rat brain antioxidant status

Parameters	NC	TC	HAEAM-LD	HAEAM-HD	Diazepam
CAT(U/min)	015.31 ± 0.93	008.43 ± 1.95$^{###}$	14.39 ± 1.22**	16.99 ± 1.10***	17.83 ± 1.50***
SOD(U/mg protein)	019.66 ± 1.95	010.08 ± 1.79$^{###}$	16.14 ± 2.30	18.63 ± 3.44*	21.33 ± 2.89*
GSH (nmol/min/mg protein)	169.73 ± 6.01	140.66 ± 4.46$^{##}$	190.02 ± 6.01*	211.51 ± 5.27**	220.4 ± 5.95**

Data are expressed as mean ± SD (n = 6). Values with superscripts (#) are significantly different between normal control vs toxic control and superscripts (*) is significantly different between toxic control vs treatment groups. The value p< 0.05 considered significant. The symbol represents the significance level such as #/* (p<0.05); ##/** (p< 0.01) and ###/***(p<0.001). NC-Normal control; TC-Toxic control; HAEAM-LD - Hydroalcoholic extract of *A. mexicana* 200 mg/kg/b.w; HAEAM-HD- Hydroalcoholic extract of *A. mexicana* 400 mg/kg/b.w.

3.6 Detection of AChE in brain

AChE inhibition potential of the HAEAM was determined on homogenates of different treated group rat brain by Ellman assay method. The outcomes of the study revealed that significant AChE level increased in the toxic control (TC) group which was found significantly decreased in the groups treated with diazepam and HAEAM. These findings revealed that *A. mexicana* possess good inhibitory activity of AChE as assessed by *in vivo* and *in vitro* methods. The outcomes of the study have been represented in Figure 2.

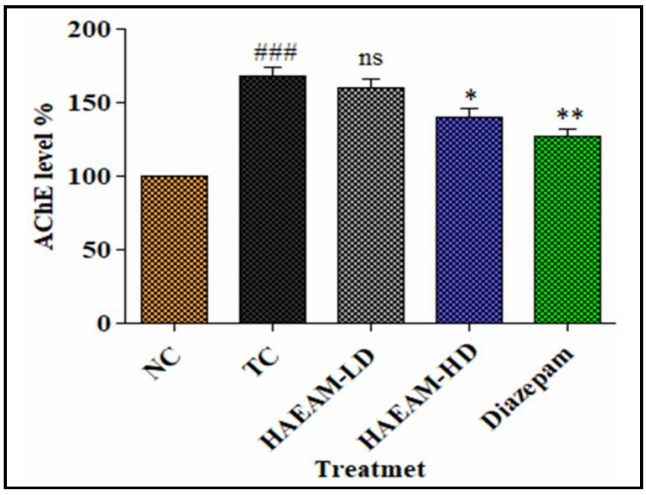

Figure 2: Effect of extract treatment on brain AChE activities. Data are presented as Mean ± SD. ###p<0.05, p<0.01 and p<0.001 vs normal control group; ***p<0.05, p<0.01 and p<0.001 vs toxic control group.

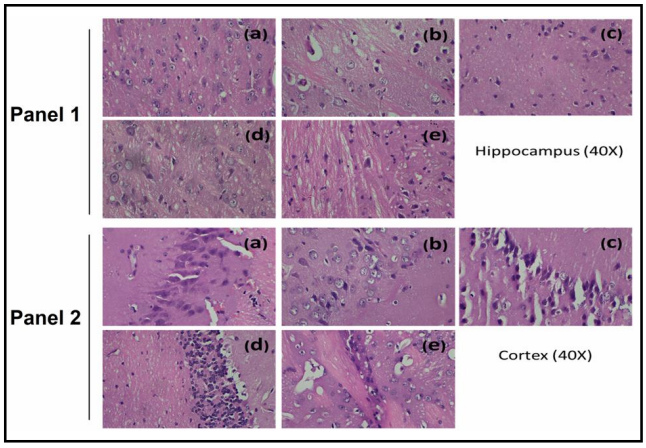

Figure 3: Effect of HAEAM on H.E. stained hippocampus (panel 1) and cortex tissue (panel 2) at 40X magnification. (a) Normal control group: Hippocampus and cortex: showing normal histology, normal neuronal cell, (b) toxic control group: Hippocampus and cortex: showing neuronal degeneration with pyknotic nuclei, a reduced layer of the neuronal cell, (c) HAEAM-LD: Showing neuronal degeneration with pyknotic nuclei, the reduced layer of the neuronal cell, (d) HAEAM-HD group: Showing a focal mild neuronal cell with pyknotic nuclei and (e) Diazepam (standard group: Showing normal histology, the focal mild neuronal cell with pyknotic nuclei.

3.7 Histopathology of the brain (hippocampus and cortex)

Histopathological investigation of hippocampus and cortex were examined successfully. The toxic control group (Figure 3b) for

hippocampus and cortex revealed various histopathological alterations like multifocal restrained neuronal degeneration with pyknotic nuclei, a multifocal restrained reduced layer of a neuronal cell in hippocampus and cortex as compared to the normal control group (Figure 3a). Treatment with different doses of *A. mexicana* (HAEAM-LD and HAEAM-HD) depicted a reduction in neuronal distortion and normal histology. The layers of neuronal cells were observed normally in the disease control group (Figure 3 c and d). The results of HAEAM-HD showed comparable outcomes as found in the group treated with diazepam (Figure 3c).

4. Discussion

Despite the use of biological models for screening of the anticonvulsant effect of HAEAM, the MES and PTZ-induced seizure models persist to consider more specific in the primary stages of treatment as well as the generation of a new entity to cure or treat epilepsy which heavily relies on the animal models (Löscher, 2017). In this study, the different dosage of HAEAM was subjected to screen their protective effect against MES and PTZ-induced seizure in rats.

The present study revealed that HAEAM (200 and 400 mg/kg/b.w) have a significant anticonvulsant effect against MES and PTZ-induced seizures. In the MES test, 100 % of the controlled rats showed hind limb tonic extension (HLTE) seizure. Rahimi *et al*. (2019) stated the tested and standard drug exhibited anticonvulsant activity and offered significant protection against electroshock-induced HLTE (Rahimi *et al*., 2019). In the MES, a significant protective effect of HAEAM was envisaged against HLTE seizures. In addition, the protection against HLTE in MES-induced seizures represents the ability of extracts to either nourish or even decrease the discharge of the seizure effect induced due to distorted ability of normal function of brain cells (Rahimi *et al*., 2019). Moreover, seizure of MES can be congested either by hindering the voltage-dependent Na+ channels or by obstructive glutamatergic excitation arbitrated by the N-methyl-D-aspartate (NMDA) receptors (Farber *et al*., 2002). Therefore, extracts disclosed antiepileptic effect might be due to hindering the voltage-dependent Na+ channels or by obstructive the glutamatergic neurotransmission arbitrated by NMDA receptors. Therefore, the significant effect of HAEAM against the convulsion may be due to the occurrence of huge potential bioactive compounds like flavonoids, phenols, *etc*. (Diniz *et al*., 2015).

In the case of the PTZ test, the standard diazepam and extracts exert a significant anticonvulsant effect. The ability of HAEAM to interrupt the onset of convulsions and/or abbreviate the active onset time of convulsions was deliberated as a positive indication of its anticonvulsant effect. Interestingly, it was suggested that bioactive molecules present in the extract are active in the suppression of seizures induced by PTZ and which was found positively overlapped in the drug-treated group against seizure-induced by the MES model (Fisseha *et al*., 2021). Presented data revealed standard diazepam is more therapeutically active against the seizure induced by PTZ than MES seizures which make us formalized to understand the fact that PTZ is a GABA-A receptor antagonist and blocks the main GABAergic inhibitory pathways in the central nervous system (Treiman, 2001). Diazepam shows its effects by increasing inhibition mediated by GABA receptors in the brain (Nicholson *et al*., 2018). Moreover, PTZ induced seizures can also be obstructed by dropping T-type Ca2+ currents (Masicampo *et al*., 2018). Therefore, the anticonvulsant activities of the extract against PTZ induced seizures can be due to induction in GABA neurotransmitters in the central nervous system, inhibiting T-type Ca2+ currents, or hindering the glutamatergic neurotransmission arbitrated by NMDA receptors (Abdel-Rahman *et al*., 2015).

The irregularities in cholinergic transmission are accompanying with the sternness of epilepsy and other brain disorders. Acetylcholine-sterase (AChE) works as the key enzyme intricate in the acetylcholine hydrolysis in the neuromuscular junction and synapse which is responsible for the termination of the nerve impulse (Colovic *et al*., 2013). Moreover, one of the characteristic variations is that occur in brain disorders is an increase in AChE activity, the enzyme accountable for acetylcholine hydrolysis from both non-cholinergic and cholinergic neurons of the brain (Ferreira-Vieira *et al*., 2016). However, AChE activity is augmented within and from place to place of amyloid plaques to endorse the assemblage of amyloid beta-peptides into fibrils and to upsurge the peptides cytotoxicity (Carvajal and Inestrosa, 2011). Recent literature reported that due to the multifactorial pathogenesis of brain disorders, plant extract containing a huge number of bioactive compounds will be favored as an operative therapeutic approach against brain disorders (Mathew *et al*., 2013).

The body has an effective defense mechanism to prevent oxidative stress by a set of antioxidant enzymes such as CAT, SOD, and GSH (Ferdinando Giacco and Michael Brownlee, 2011). Though, *A. mexicana* and its bioactive constituents could effectively limit the oxidant stress. Our findings for *in vitro* antioxidant activity are strongly related to the previous reports (Srivastava *et al*., 2012). Similarly, oral intake of different doses of HAEAM restores oxidative stress by increasing the levels of SOD, CAT, and GSH. Amelioration of the antioxidant ability indicates the efficacy of the *A. mexicana* extract attenuates oxidative stress in epileptic rats. Hematoxylin and eosin-stained brain tissue showed improved neurodegeneration with pyknotic nuclei observed in the disease control group. *A. mexicana* ameliorates worsening changes in the region of the brain hippocampus and cortex due to its neuroprotective effect (Amin *et al*., 2013). It has been described that accretion and accumulation of amyloid-beta (Aβ) are the main malefactors in the severe pathogenesis of brain disorders that characterize to neurode-generation (Zhang *et al*., 2018). AChE endorses the accumulation of neurofibrillary tangles and amyloid-beta plaques, which indicates pathogenesis of the brain such as epilepsy (Gnatek *et al*., 2012). So far, it can be considered as the treatment with HAEAM showed significant amelioration of AChE levels in the brain.

5. Conclusion

HAEAM is enriched in phenols and flavonoids which may exhibit a protective role against MES and PTZ-induced seizures. It also showed an antiepileptic effect *via* regulation of AChE level as well as improves antioxidant enzymes in the brain which tend to signify a decrease in oxidative damage *via* quenching free radicals and histological architecture of the hippocampus and cortex. As there are inadequate approaches for epilepsy management, HAEAM provides an economic, safe, and effective therapeutic alternative for the treatment of epileptic disease.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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