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Antiepileptic potential of *Silybum marianum* seeds in pentylene-tetrazol-induced kindled mice

Huma Waqar[1], Humaira Majeed Khan[1] and Aftab Ahmad Anjum[2]

[1]Institute of Pharmacy, Lahore College for Women University, Lahore 54000, Pakistan; [2]Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

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Abstract

Epilepsy is an abnormality of nervous system showing seizures. Despite, the known antiepileptic activity of plants possessing antioxidant properties, *Silybum marianum* has not been studied yet. Therefore, present experiment was planned to evaluate antiepileptic potential of *S. marianum* (100, 200 and 300 mg/kg) seeds ethanol extract in pentylenetetrazol-induced kindled mice (n = 30). *S. marianum* seed extract (300 mg/kg) provided significant protection against pentylenetetrazol-induced convulsions (seizure intensity, latency and lethality) comparable with reference drug (valproic acid). Furthermore, 300 mg/kg/day dose was effective to prevent oxidative stress causing significant decrease in the lipid peroxidation (1.4 ± 0.4 nmol/mg protein) and increased superoxide dismutase (0.4 ± 0.1 µmol/mg protein) and catalase activity (4.7 ± 0.8 U/mL) of mice brain as compared to induced untreated group ($p<0.05$). It was concluded that antiepileptic activity of *S. marianum* seeds was due to its antioxidant property.

Introduction

Epilepsy is a central nervous system disorder characterized by rapid and recurrent seizures due to synchronized discharge of neuronal network in brain (Malhi et al., 2014). It is the most frequently found neurological disorder after stroke (Scheuer and Pedley, 1990). About 50 per 100,000 of people are suffering from epilepsy in developed country, while ratio of epileptic patients in developing country is 100 per 100,000. It is estimated that in Pakistan, the prevalence of epilepsy is 9.99/1000 population (Khatiri et al., 2003).

The available antiepileptic drugs not only fail to suppress seizure in some patients but also produce adverse effects (Vyawhare et al., 2007). Furthermore, such drugs do not have satisfactory efficacy, tolerance and toxicity.

The trend of using medicinal plants in epilepsy is gaining success by the approval of the World Health Organization due to milder side effects of plants and the diversity of effective constituents (Kiasalari et al., 2013).

Silybum marianum (family: *Asteraceae*) commonly known as milk thistle is native to limited region of Mediterranean and spreads throughout Europe (Das et al., 2008). The plant is naturalized in different areas of Pakistan such as Punjab and Khyber Pakhtoon Khwa (Shah et al., 2011).

The plant has potent antioxidant properties due to which it has been used therapeutically for centuries in the management of liver diseases such as liver cirrhosis, alcoholic hepatitis, liver poisoning, alcoholic fatty liver, and viral hepatitis (Shaker et al., 2010). The active constituents are flavonolignans including silychristin, silydianin and silybin, collectively called as silymarin are also used to treat different neurodegenerative disorders (Valenzuela and Garrido, 1994, Chtourou et al., 2010). Its antioxidant activity also contributed in

cerebral protection as well as prevention in ischemic brain injury due to oxidative stress (Raza et al., 2011).

Oxidative stress is involved in pathogenesis of epilepsy and administration of exogenous antioxidants play role in protection of brain against epileptic seizures (Illhan et al., 2005). Presented study was planned to determine the antiepileptic and antioxidant effect of dried ethanol extract of plant seeds using pentylenetetrazol to induce kindling as well as oxidative stress in mice.

Materials and Methods

Medicinal plant

S. marianum seeds were procured from the local market and authenticated by the Department of Botany, Government College University, Lahore. A voucher specimen (GC Herb. 2895) was deposited in herbarium, Government College University, Lahore, Pakistan.

Extraction

The seeds were dried, grounded and sieved (sieve No 40). Fine powder (500 mg) was soaked in the absolute ethanol (2.5 L) for 7 days. The extract was dried at room temperature and then dissolved in 0.3% carboxymethylcellulose (Raza et al., 2011).

Experimental animals

Thirty six male albino mice, weighing 18-22 g, were kept at the Animal House, Institute of Pharmacy, Lahore College for Women University, Lahore, Pakistan, under 12 hours light/dark cycle. Standard diet and water *ad labitum* were provided to all mice throughout the study.

Drugs and chemicals

Pentylenetetrazol, ketamine, valproic acid, thiobarbituric acid, nitrobluetetrazolium, ethylene diamine tetraacetic acid (EDTA) were obtained from Sigma-Aldrich (USA). Other reagents used were of analytical grade.

Experimental design

Male albino mice were divided into following groups with 5 animals each: Group 1: Control, received 0.3% CMC; Group II: Induced group received pentylenetetrazol (35 mg/kg) intraperitoneally (i.p) on alternate days for 22 days; Group III: Reference group was treated with valproic acid as reference drug (100 mg/kg, p.o) OD for 24 days with pentylenetetrazol (35 mg/kg, i.p) after one hour thrice a week; Group IV, V and VI designated as treatment groups A, B and C received dried *S. marianum* seed extracts at dose level 100, 200 and 300 mg/kg (p.o., OD) respectively for 24 days with pentylenetetrazol (35 mg/kg, i.p.) after one hour of extracts administration thrice a week.

Experimental induction of seizures

Kindling in mice was induced using subconvulsion dose of pentylenetetrazol (35 mg/kg) on every alternate day for 22 days. Mice were observed to record convulsion for 30 min according to Erakovic scale (Erakovic et al., 2001). Level of induced seizure in mice was quantified by scoring system. Zero score was allocated to no responses, 1 to ear and facial twitching, 2 for convulsive waves in body, 3 for myoclonic jerks, 4 to generalized clonic convulsions turning to side position, 5 to tonic extensor status epilepticus and 6 to mortality. Mice were declared kindled after 11 pentylenetetrazol injections or showing three time seizures of score 5 (Ilhan et al., 2006).

Challenge dose

A challenge dose of pentylenetetrazol was administered i.p. (75 mg/kg) to all experimental mice on day 24 and seizures scoring were done using the same Erakovic scale. Latency to tonic clonic seizures (score 5) and lethality was also recorded for 30 min in all groups (Ilhan et al., 2006).

Biochemical assays

After behavioral observation all the animals were decapitated under ketamine (100 mg/kg) anesthesia (Illhan et al., 2006). Brain was separated from each mice, weighed and washed using sterilized normal saline.

Tissue preparation

Mice brains collected were homogenized using chilled tris-HCL buffer (50 mM, pH 7.4) in Teflon homogenizer (Ultra Turrex IKA T18 Basic, USA). Mixtures were cleared from debris by centrifugation at 5,000 xg for one hour (Ilhan et al., 2006). Supernatants were stored in aliquots and later on used to estimate the lipid peroxidation (malondialdehyde assay, MDA), superoxide dismutase (SOD) and catalase activity (CAT).

Estimation of lipid peroxides

Assay for estimation of lipid peroxides was carried out following Mihara and Uchiyama (1978). Phosphoric acid (0.1%; 3 mL) was added in a test tube containing 1 mL of aqueous thiobarbituric acid (0.6%) and 0.5 mL brain homogenate. Suspension was heated for 45 min. N-butanol (4 mL) was added to the mixture and left for 5 min in standing position. Optical density (OD) values were recorded at 520 nm and 535 nm against butanol as a blank. MDA level (nmol/mg protein) was then calculated from difference of two measurements by using the following equation.

Thiobarbituric acid value/MDA value = $[(A_{535} - A_{520}) /15500] \times 10^6$

Superoxide dismutase estimation

Activity units of enzyme superoxide dismutase were measured following the procedure of Beauchamp and

Fridovich (1971). Suspension (0.5 mL) of brain tissue was poured in a test tube having Na_2CO_3 (50 mM; 1 mL), nitrobluetetrazolium (0.4 mL) and EDTA (0.2 mL). The blank was prepared by adding brain homogenate and all other reagents except hydroxylamine hydrochloride. Absorbance of the reaction mixture was measured using 560 nm wavelength against blank. Then, hydroxyl-amine hydrochloride (0.4 mL; 1 mmol) poured in reaction mixture and placed in water bath at 25°C. The absorbance was measured after 5 min. At the same time control was run without test material. The SOD activity was measured by the following equation:

SOD activity = [(V/v) – 1] x dilution factor

Catalase activity

Catalase activity was estimated by a method described by Aebi (1984). Briefly, brain mixture (0.5 mL), phosphate buffer saline (1 mL) and hydrogen peroxide (0.5 mL) were mixed in a test tube. Blank was prepared containing 0.5 mL of 0.15M potassium chloride (KCl) instead of test material and 1 mL phosphate buffer (pH 7.4). Absorbance value was estimated at 240 nm using blank.

Statistical analysis

Data was statistically analyzed using one-way ANOVA followed by Tukey's test.

Results

The effect of different doses (100, 200 and 300 mg/kg) of dried *S. marianum* seed extract and valproic acid (100 mg/kg, reference drug) from 1st day to 21st on pentylenetetrazol induced-kindled mice is expressed in Figure 1. By injecting sub convulsion dose of pentylenetetrazol alternately, progressive increase in convulsion scores was recorded in induced group. The seizure scoring of induced group on day 7, 9, 11, 13, 15, 17, 19 and 21 were found to be 1.6 ± 0.5, 2.0 ± 0.8, 2.8 ± 0.4, 3.1 ± 0.4, 3.5 ± 0.5, 4.0 ± 0.6, 4.5 ± 0.5 and 5.0 ± 0.0 respectively. Pre-treatment with *S. marianum* seed extract (100 mg/kg orally, daily, 1 hour prior to pentylenetetrazol) course of kindling induction in mice was not significantly altered as compared to induced group ($p>0.05$). In the treatment group B, the administration of 200 mg/kg *S. marianum* seed extract caused decrement in seizure intensity on 19th day i.e. 3.3 ± 0.5 as compared to induced group. The administration of *S. marianum* seed extract in doses of 300 mg/kg suppressed seizures significantly in kindled mice to score of 2.1 ± 0.4, 2.1 ± 0.9, 2.8 ± 0.7, 3.0 ± 0.6, 3.8 ± 0.7 on day 13, 15, 17, 19 and 21 (7th, 8th, 9th, 10th and 11th injections) as compared to the induced group. Moreover, the reference drug (valproic acid) was found to be reducing seizure intensity up to 2.8 ± 0.4, 3.3 ± 0.5 and 4 ± 0.5 on day 17, 19 and 21 (9th, 10th and 11th injections)

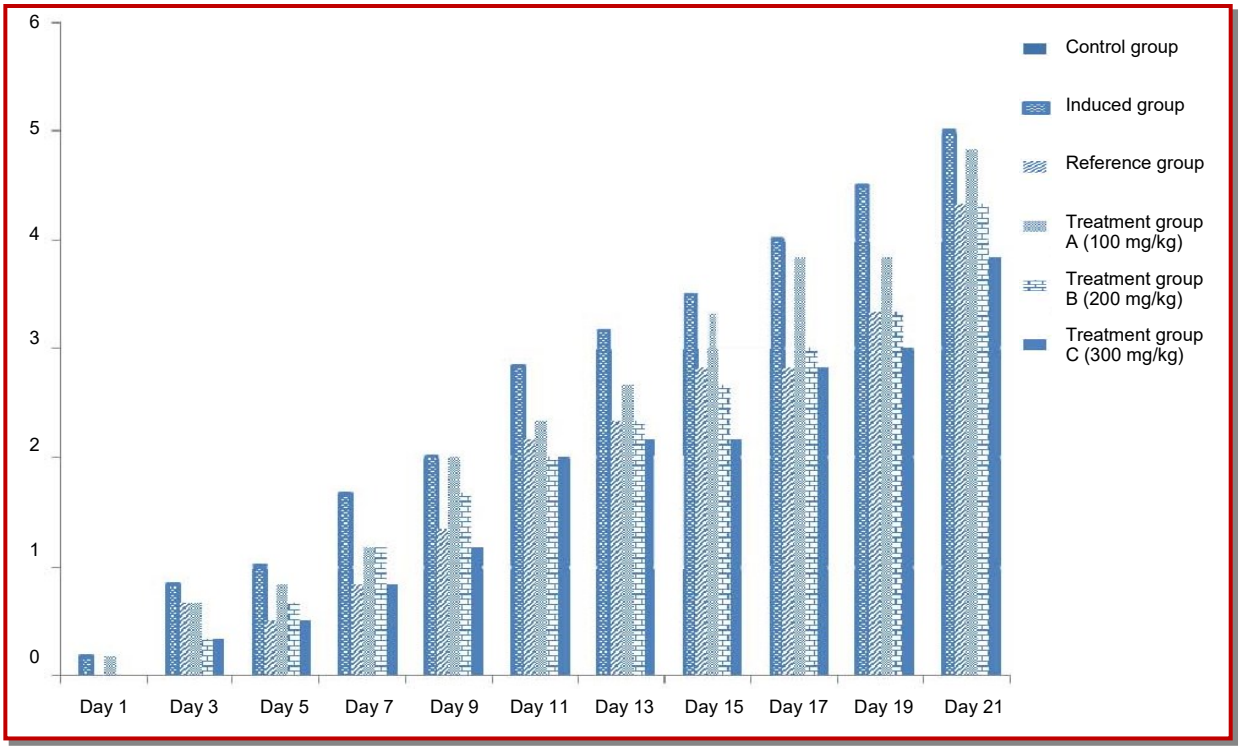

Figure 1: Seizure scoring of control, induced, reference and three treatment groups (A, B and C) from day 1 to 21; Data are mean ± SD ($p<0.05$)

Table I

Comparison between latency time, seizure intensity and lethality after challenge dose of PTZ (75 mg/kg) among different experimental groups on 24th day

Groups	Latency (secs)	Seizure intensity	Lethality
Control group	0.0 ± 0.0	0.0 ± 0.0	0/6
Induced group	56.8 ± 8.2[a]	5.8 ± 0.4[a]	5/6
Reference group	178.3 ± 13.6[ab]	5.0 ± 0.6[ab]	1/6
Treatment group A (100 mg/kg)	67.6 ± 8.1[ac]	5.6 ± 0.5[a]	4/6
Treatment group B (200 mg/kg)	131.1 ± 6.8[abcd]	5.5 ± 0.5[a]	3/6
Treatment group C (300 mg/kg)	187.3 ± 5.2[abde]	5.1 ± 0.4[ab]	1/6

Values are expressed as mean ± SD ($p<0.05$), [a]shows significant difference of control group with induced, reference and treatment groups. [b]shows significant difference of induced group with reference and all treatment groups. [c]shows significant difference of reference group with treatment groups. [d]shows significant difference of treatment group A with treatment group B and treatment group C. [e]shows significant difference of treatment group B with treatment group C.

respectively when compared to induced group ($p<0.05$).

The comparison between latency time, seizure intensity and lethality after challenge dose of pentylenetetrazol (75 mg/kg) among different experimental groups on 24th day is summarized in Table I. These results indicate seizure intensity, lethality and latency to score 5 against challenge dose among kindled mice. The highest seizure scoring was observed in induced group (5.8 ± 0.4). A dose of 300 mg/kg of dried *S. marianum* seed extract and reference drug, valproic acid (100 mg/kg), produced a significant decrease in seizure intensity as compared to induced group i.e. (5.1 ± 0.4 and 5.0 ± 0.6) respectively. The tonic clonic seizures occurred earliest (56.8 ± 8.2 seconds) in induced group. *S. marianum* seed extract (200 mg/kg and 300 mg/kg) significantly delayed the onset of tonic clonic seizures as compared to induced group (131.1 ± 6.8 sec and 187.3 ± 5 sec) respectively. Reference group receiving valproic acid also significantly delayed latency to score 5 as compared to induced group ($p<0.05$) i.e. 178.3 ± 13.6 sec. After challenge dose, five mortalities were observed in induced group, four in treatment Group A (100 mg/kg) and three in treatment Group B (200 mg/kg) respectively. However, only one lethality was reported in reference group and treatment Group C (300 mg/kg).

On 24th day, the isolated brain tissues were processed for biochemical analysis i.e. determination of lipid peroxidation, superoxide dismutase activity and catalase activity (Table II). Induced group had indicated oxidative stress by significant increment of MDA level in brain, as indicator of lipid peroxidation (4.2 ± 1.3 nmol/mg protein) as compared to control group (0.9 ± 0.4 nmol/mg protein). *S. marianum* seed extracts (100, 200 and 300 mg/kg) treatment before pentylenetetrazol challenge significantly attenuated the increased MDA level as compared to induced group ($p<0.05$) i.e. 3.0 ± 0.5 nmol/mg protein, 2.0 ± 0.4 nmol/mg protein and 1.4 ± 0.4 nmol/mg protein respectively. VA also significantly decreased MDA level up to 1.7 ± 0.4 nmol/mg protein as compared to induced group.

There was marked decrease in SOD activity in induced (pentylenetetrazol kindled group) (0.0 ± 0.2 µmol/mg protein) as compared to control group (0.9 ± 0.1 µmol/

Table II

Effect of extract on MDA, SOD and CAT activity in PTZ kindled mice

Groups (n = 6)	MDA (nmole/mg protein)	SOD (µ/mg protein)	CAT (U/ML)
Control group	0.9 + 0.4	0.9 + 0.1	6.2 + 0.9
Induced group	4.2 + 1.3[a]	0.0 + 0.2[a]	3.2 + 0.4[a]
Reference group	1.7 + 0.4[b]	0.3 + 0.1[ab]	3.7 + 0.3[a]
Treatment group A (100 mg/kg)	3.0 + 0.5[abc]	0.2 + 0.7[a]	3.6 + 0.3[a]
Treatment group B (200 mg/kg)	2.0 + 0.4[b]	0.3 + 0.1[ab]	4.9 + 0.4[abcd]
Treatment group C (300 mg/kg)	1.4 + 0.4[bd]	0.4 + 0.1[abc]	4.7 + 0.8[abd]

Values are expressed as mean ± SD ($p<0.05$), **a** shows significant difference of control group with induced, reference and treatment groups. **b** shows significant difference of induced group with reference and all treatment groups. **c** shows significant difference of reference group with treatment groups. **d** shows significant difference of treatment group A with treatment group B and treatment group C. **e** shows significant difference of treatment group B with treatment group C

mg protein). Treatment group A (100 mg/kg), B (200 mg/kg) and C (300 mg/kg) significantly increased SOD activity up to 0.2 ± 0.7 µmol/mg protein, 0.3 ± 0.1 µmol/mg protein and 0.4 ± 0.1 µmol/mg protein respectively when compared to induced group ($p<0.05$). Reference group also significantly increased SOD activity as compared to induced group ($p<0.05$) i.e. 0.3 ± 0.1 µmol/mg protein.

Induced group had shown oxidative stress by significant reduction in catalase activity (3.2 ± 0.4 U/mL) as compared to control group (6.2 ± 0.9 U/mL). Treatment Group A (100 mg/kg) and reference group (100 mg/kg) failed to increase catalase activity when compared induced group ($p>0.05$) i.e. 3.6 ± 0.3 U/mL and 3.7 ± 0.3 U/mL respectively ($p>0.05$). While treatment Group B (200 mg/kg) and treatment Group C (300 mg/kg) significantly increased catalase activity up to 4.9 ± 0.4 U/mL and 4.7 ± 0.8 U/mL as compared to induced group ($p<0.05$).

Discussion

In the present study, the most abundant naturally occurring flavonoid containing *S. marianum* seeds extract has been evaluated as a potential new anti-epileptic and antioxidative agent. Seizures were significantly reduced in groups treated with 200 and 300 mg/kg doses of extracts than pentylenetetrazol kindled group. At lower doses seizures were not reduced. Moreover, 300 mg/kg of dried *S. marianum* seeds extract was able to reduce mortality, seizure intensity as well latency to tonic clonic seizures against challenge dose of pentylenetetrazol i.e. 75 mg/kg. Biochemical studies revealed that pre-treatment with all of the doses of extracts lowered lipid peroxidation. In addition 200 and 300 mg/kg of *SM* increased the SOD and CAT activity. It was concluded from present experiment that pre-treatment with plant extract reduced brain damage caused by oxidative radicals.

Erakovic et al. (2003) reported higher activity of glutamanergic synapses through N-methyl-D-aspartate (NMDA) receptors, higher glutamate extracellular levels and free radicals by the use of pentylenetetrazol in kinling mice.

In pentylenetetrazol kindling mice model, oxidative radicals play key role by damaging brain tissue leading to epilepsy (Jain et al., 2011). In corroboration higher lipid peroxidation and lower antioxidant enzyme level following pentylenetetrazol induced kindling mice or rat models had been reported Erakovic et al. (2003). Results of present study indicated that pentylenetetrazol induced seizures coincide with higher oxidative stress leading to brain tissue injury. There was an increase in MDA and decrease in SOD activity post pentylenetetrazol administration. Current research also showed that pentylenetetrazol administration decreased CAT activity as well.

Numerous studies proved that *S. marianum* is effective antioxidant in treatment as well as in prevention of some neurotoxic and neurodegenerative diseases. Phytochemical studies reveal that silymarin, a flavonolignans isolated from *S. marianum,* has an antioxidant activity as well as free radical scavenging characteristics *in vitro* (Fu et al., 2009). It has shown that the dose of 200 mg/kg of silymarin has been found effective in ameliorating the lipid peroxide and free radical generation against focal cerebral ischemia (Raza et al., 2011). It was observed from present findings that seizures were reduced by using medicinal plant extract. Results were strengthened by antioxidant activities of plants like *Ginkgo biloba* extract (EGb 761) (100 mg/kg po) and *Ferula assa-foetida* gum extract (25, 50 and 100 mg/kg) controlled epileptic seizures against pentylenetetrazol induced seizures in mice (Illhan et al., 2006 and Kiasalari et al., 2013). In addition it was also revealed that *Ginkgo biloba* (EGb 761) (100 mg/kg po) also significantly reduced convulsive behavior (seizure intensity, seizure latency) against same challenge dose (Illhan et al., 2006).

The results of reference drugs were also similar i.e. 100 mg/kg of valproic acid could effectively decrease seizure intensity in pentylenetetrazol kindled mice (Kiasalari et al., 2013). However a difference was found with earlier research that against 75 mg/kg of pentylenetetrazol dose, the pre-treatment of valproic acid did not significantly decrease seizure intensity. This discrepancy could arise from multiple factors i.e. change in laboratory conditions etc. Though increased in latency to score 5 (tonic clonic seizure) was observed similar to previous research (Illhan et al., 2006). In accord findings have been reported by Jain et al. (2011) that valproate inhibited development of kindling. Anti-epileptic agents including phenytoin, carbamazepine and phenobarbitone cause imbalance in oxidation and anti-oxidation levels. Therapy with such drugs had resulted in higher MDA and lower GSH levels in brain tissue (Ilhan et al., 2005). According to findings of present study, plant extract raised CAT level in brain. Although many antiepileptic drugs are being introduced but efforts to develop novel antiepileptic drugs with distinctive mechanism of actions and least side effects are still considered unsuccessful (Loscher, 2011). Research has been initiated for antiepileptic activity in plants which possess multiple mechanisms of actions with minimum side effects (Shenoy et al., 2012).

It is reported that pentylenetetrazol has an important role in development of epileptic seizures via mechanism of blocking $GABA_A$ receptors (Rebrov et al., 2004). The extracts of *S. marianum* seeds are rich in many phytochemicals especially phenols and flavonoids (Atraqchi et al., 2014), which are thought to exert anti-

convulsion effects by modulating channels (Diniz et al., 2015). Therefore, flavonoid compounds with anti-oxidant properties might be an additional candidate by which the anti-convulsant effect of valproic acid is occurred. As Nassiri-Asl et al., (2008) postulated that herbal medicines act as potential therapeutics in management of $GABA_A$ receptors mediated diseases.

Conclusion

S. marianum seeds extract had anti-convulsion and anti-oxidant effects on pentylenetetrazol-kindled seizure.

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Ethical Issue

Experiments were carried out following procedures of care and use of animal for scientific purposes, National Advisory Committee for Laboratory Animal Research.

Conflict of Interest

Authors declare no conflict of interest

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Author Info
Humaira Majeed Khan (Principal contact)
e-mail: humairaphd@hotmail.com