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Evaluation of Anti-epileptic Effect of *Sinapis alba* using Maximal Electroshock Seizure Model

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Epilepsy is a prevalent neurological disorder, prompting an ongoing quest for new therapeutic agents. *Sinapis alba*, commonly known as yellow mustard, has garnered interest for its potential medicinal properties. This study aimed to assess the anti-convulsant potential of Sinapis alba in rats. Wistar albino rats were categorized into 5 distinct experimental groups (six each) : a normal control, a disease control, a group administered *Sinapis alba* seed oil (200mg/kg body weight), another given sodium valproate (300mg/kg body weight), and a combination group receiving both *Sinapis alba* seed oil and sodium valproate (150mg/kg body weight each). Antioxidant markers were subsequently extracted from the brain samples, and cresyl violet staining was employed to discern pathological changes. The findings revealed a significant diminution in the durations of flexion, clonic convulsion, and stupor in the test, standard, and combination groups in contrast with the disease control. Additionally, the duration of tonic hind limb extension (THLE) noted a substantial decrease in the *Sinapis alba* group, sodium valproate group, and combination group. Moreover, the administration of *Sinapis alba* seed oil led to an elevation in antioxidant levels and a concomitant reduction in lipid peroxide levels. Intriguingly, a synergistic effect on generalized tonic-clonic seizures was observed upon integrating mustard oil with sodium valproate. Our research suggests that *Sinapis alba* seed extract demonstrates promising anti-epileptic properties and stands as a potential supplementary drug for managing generalized tonic-clonic seizures.

Keywords: Epilepsy; Pharmacognosy; Plant Products; Seizures; Yellow Mustard.

Mustard is one of the oldest widely-grown condiments, dating back to 3000 BC. The most commonly used mustard varieties include *Sinapis alba* (yellow mustard), *Brassica nigra* (black mustard), and *B. juncea* (Indian mustard). Mustards belong to the *Brassicaceae* family. *Sinapis alba,* also known as yellow or white mustard, is used both as a spice and for medicinal

purposes in various conditions.[1] The seeds contain a bland fixed oil of about 23-25%, sinalbin (a crystalline compound), sinapin-sulphocyanide, mucilage, lecithin, myrosin, and 4% ash, which consists of potassium, calcium, and magnesium phosphates. They also contain vitamin A, thiamine (B1), riboflavin, proteins, and water.[2] The phenolic groups found abundantly in white mustard are sinapic acid and p-hydroxybenzoic acid, which contribute to its antioxidant activity.[3] S. alba has a pungent, bitter, sharp, unctuous, and hot flavor. While white mustard contains glucosinolate-sinalbin, black mustard contains sinigrin. Their hydrolyzed products differ, which is responsible for the pungency and sharp taste. Among these, black mustard is more pungent.[4]

According to literature on its use in traditional medicine, *S. alba* has been employed to treat cholera, dysmenorrhea, amenorrhea, whooping cough, high fever, headaches, and swollen joints. It also possesses emetic and diuretic effects.[2,5] They are potent natural antioxidants.[3] Yellow mustard calms vata and kapha and is beneficial for ailments related to the head and ear. It is applied in the treatment of skin diseases due to its antimicrobial and anti-inflammatory activities.[6] The high levels of glucosinolates present make it valuable in anti-cancer therapy.[7] It aids in regulating irregular heartbeats, cholesterol, and blood sugar levels and provides relief from respiratory congestion, largely due to the magnesium in it.[3]

Ayurvedic literature mentions the use of these seeds in the treatment of nerve disorders such as hysteria, delirium, and epilepsy, proving effective when taken internally.[2] There's also evidence of its use for epilepsy in German Renaissance herbals.[8] While there have been some studies on the anti-epileptic effect of *B. nigra* showing positive results, there are none conducted with yellow mustard seeds to date.[9, 10] Hence, this research was embarked upon to evaluate the efficacy of *Sinapis alba* in treating epilepsy. We hypothesize that the potential neuroprotective action of S. alba could be attributed to its potent antioxidant properties, which help in reducing neuronal oxidative damage during epileptogenesis. In this study, the efficacy of *Sinapis alba* seed oil was examined using the MES model in Wistar rats.

MATERIALS AND METHODS

Materials

Sodium valproate (SV) (Encorate 300mg tablet) was indented from Radha Medicals, Manipal. Cold-pressed yellow mustard seed oil –Brand Planton was obtained from Green Trade Company, New Delhi.

Experimental animals

The study was conducted as per the guidelines set by the Committee for Control and Supervision of Experiments on Animals (CCSEA). Thirty albino Wistar rats of 200 -300 grams weight were sanctioned for the experiment and were housed at the Central Animal House, Manipal. Housing conditions were as follows: - 3 rats per cage with 12:12 hours of light: dark lighting conditions, the temperature maintained at 25Ú±3ÚC and humidity was 50%. Normal rodent diet pellet (VRK Nutritionals, Maharashtra) and water ad libitum were provided.

Procedure

Adult albino female Wistar rats weighing 200-300 grams were utilized in this study. After acclimatization for 1 week, rats were divided into five groups. Each group had 6 rats each (random grouping was done as shown in Table 1) and treated for 14 days as follows:

Dose of yellow mustard oil and sodium valproate was obtained from previous studies. [11,12,13] Sodium valproate was freshly prepared daily. A gap of half an hour was provided between the dosing of yellow mustard seed oil and valproate. After 14 days of treatment as shown in Table 1, generalized tonic-clonic seizures were induced.

Seizure induction: Grand mal or generalized tonic-clonic seizures were induced using an electro-convulsive meter (Inco Company, Ambala City, India). Electric stimulation of intensity 150mA, 0.2 seconds (50-60 Hz frequency) via ear clip electrodes was used to induce seizures on the 14th day of the experiment.[14] Induction was done after 1 hour of dosing with oil, valproate, and distilled water.

The duration of all the following stages was noted down: -

- Flexion
- Tonic hind limb extension (THLE)

• Clonic convulsions
• Stupor

Death/recovery after twenty-four hours of induction was recorded.

THLE was the endpoint (backward extension of hindlimb- > 90° against the plane of body axis). Percentage inhibition of convulsions and percentage protection were calculated. On the 15th day, rats were euthanized with thiopentone sodium (120 mg/kg i.p.). Rats were dissected, and the brain was removed; ice-cold saline (ICS) was used to clean. Brain samples were stored in phosphate buffer saline (PBS) for biochemical estimations and in 10% formalin for histopathological analysis.

Parameters

The parameters assessed in the MES model were body weight, duration of seizures, latency to seizure onset, recovery or death, seizure score/stage, and death after 24 hours.

Assessment of biochemical parameters

Oxidative stress markers

Brain homogenate was prepared, and the levels of superoxidase dismutase (SOD), malonaldehyde (MDA),

Nitrite and glutathione (GSH) were estimated.

Histopathological analysis

The brain tissue was stored in 10% formalin after dissection. Cresyl violet staining technique was used to assess the extent of neuronal degeneration. Serial coronal brain sections of 30-50µm thickness were taken using a cryostat at frozen condition (-18°C). Sections were hydrated with xylene, 100%, 90%, and 70% alcohol. The sections were stained with Cresyl violet at 37-50 °C. This stain is helpful since it stains neuronal cell bodies, and degenerating or pyknotic neurons can easily be identified under a light microscope. Dehydration of sections was carried out using 70 %, 95%, and 100% alcohol grades and later washed with xylene. Mounting was done using mountant- DPX. Cresyl violet stained sections were then observed under a light microscope for the qualitative analysis of pyramidal neurons of the hippocampal CA3 region.

Statistical analysis

Statistics were carried out using GraphPad Prism 8.0.1. One-way ANOVA followed by "Post Hoc Tukey's multiple comparison test" was performed for biochemical and behavioural parameters. For body weight, two-way ANOVA was performed, after which post hoc Bonferroni's test was carried out. All the data were expressed as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

The results of the study are mentioned below:

Gas chromatography-mass spectroscopy (GC-MS) analysis of yellow mustard (*Sinapis alba*) seed oil

Analytical Research & Metallurgical Laboratories Pvt Ltd, Bangalore, performed GC-MS of cold-pressed *Sinapis alba* seed oil. The identified compounds are depicted in Table 2. A total of 21 compounds were identified, of which major proportion was "9-Octadecenoic acid, methyl ester (20.74%), 6,8-Difluoro-2,2,4,4,6,7,7,8,9,9-decamethyl-[1,3,5,2,4,6,7,8,9]trioxahexasilonane (14.87%), 9,12-Octadecadienoic acid (Z,Z)-, phenylmethyl ester(5.4%), 9-Octadecenoic acid, 1,2,3-propanetriyl ester (4.83%), Hexadecanoic acid, methyl ester (4.74%), 9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl) oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester (3.61%)."

Weight of rats

The total body weights of animals on Day 1, day 14, and day 15 is shown in Table no. 3. On the first day, the mean weights of different

Table 1. Different groups along with the treatment doses

Groups	Treatment
Group 1 Normal control	Distilled water (10 mL/kg, p.o.)
Group 2 Disease control	Distilled water (10 mL/kg, p.o.)
Group 3 Test drug	Yellow mustard oil (200 mg/kg) (p.o.)
Group 4 Standard drug	Sodium valproate (300 mg.kg) (p.o.)
Group 5 Test + standard	Yellow mustard oil (200 mg/kg) (p.o.) + sodium valproate (150 mg/kg) (p.o.)

group were not statistically different. In 2 weeks after treatment, there was an increase in the weight of rats of group 3 (oil 200 mg/kg) and group 5 (oil 200 mg/kg + SV 150 mg/kg), which was significant (p-value <0.05 and p<0.01 respectively) as compared to control.

On the 15th day (i.e., 24 hours after MES induction), we could observe a significant difference (p-value <0.05) in weight of rats administered with oil 200 mg/kg (group 3) and oil 200 mg/kg + SV 150 mg/kg (group 5) when compared to disease control (where the weight reduced post-induction).

Anti-epileptic effect of *Sinapis alba* in Maximal electroshock model

The average duration of flexion, THLE, clonic-convulsions and stupor are presented in Table 4, along with the percentage inhibition of

Table 2. List of compounds identified from the yellow mustard (*Sinapis alba*) oil sample along with its retention time and %area.

Peak	Retention Time (min)	Area	Area %	Name
1	3.157	39756	3.61	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester
2	4.859	112809	1.04	1-Butene, 4-isothiocyanato [1]
3	9.177	55508	0.51	Tetradecane
4	14.736	130209	1.2	N-Benzylisatoic anhydride
5	15.055	56654	0.52	Phthalic acid, 3,5-dimethylphenyl 2-isopropylphenyl ester
6	16.033	1608701	14.87	6,8-Difluoro-2,2,4,4,6,7,7,8,9,9-decamethyl-[1,3,5,2,4,6,7,8,9] trioxahexasilonane
7	16.506	525131	4.85	Hexadecanoic acid, methyl ester
8	18.215	2243696	20.74	9-Octadecenoic acid, methyl ester, (E)
9	18.571	52857	0.49	Oleyl Alcohol
10	19.992	512335	4.74	9-Hexadecenoic acid, methyl ester, (Z)
11	20.182	91401	0.84	Eicosanoic acid, methyl ester
12	21.448	583762	5.4	9,12-Octadecadienoic acid (Z,Z)-, phenylmethyl ester
13	21.633	2206619	20.4	9-Octadecenoic acid, methyl ester, (E)
14	22.18	143823	1.33	Phosphine imide, P,P,P-triphenyl[1]
15	23.85	64822	0.6	1,5-Di-p-tolyl-anthraquinone
16	24.492	522751	4.83	9-Octadecenoic acid, 1,2,3-propanetriyl ester
17	27.486	97870	0.9	Ergosta-8(14),15,22-trien-3-ol, (3.beta.,5.alpha.,22E)
18	28.25	206902	1.91	beta.-Sitosterol
19	28.536	58037	0.54	5-(p-Aminophenyl)-4-(p-tolyl)-2-thiazolamine
20	29.447	496285	4.59	gamma. –Sitosterol
21	30.125	656236	6.07	2,4-Di-tert-butyl thiophenol

Table 3. Body weight of different groups measured at day 1, 14 and 15

No.	Groups	Body weight in grams		
		Day 1	Day 14	Day 15
1	Normal control	271.66±6.78	279.16±7.28	281±7.21
2	Disease control	258±4.78	293.5±5.32	275.33±6.46
3	Oil 200mg/kg	277.5±12.48	324.33±11.15*	317.5±13.22a
4	SV 300mg/kg	284.5±11.17	306.83±12.10	309.83±11.53
5	Oil 200mg/kg + SV 150mg/kg	267.83±9.69	327.83±9.86a	324.5±11.53*, a

Two-way ANOVA. Post hoc - Bonferroni's multiple comparisons test. Data expressed as Mean±SEM.
*p <0.05 vs normal control (group 1), a p-value <0.05 vs disease control (group 2)

THLE in comparison to MES disease control. There was a significant reduction in the duration of flexion, clonic convulsion and stupor period in test, standard and combination groups compared to disease control. The duration of THLE in group 3, group 4 and group 5 was significantly low ($p<0.05$). THLE was completely inhibited in sodium valproate 300mg/kg. SV 300mg/kg showed 100% protection from THLE, while the mustard oil 200mg/kg and combination of mustard oil 200mg/kg + SV 150 mg/kg showed 33.33% and 66.6% protection respectively. There was no mortality recorded after 24 hours of induction in any of the groups.

Effect of *Sinapis alba* seed oil on brain oxidative stress

The levels of MDA were expressed as nmol/gram protein. There was an elevation in MDA levels in MES control compared to the normal control ($p<0.05$). There is a significant reduction ($p<0.05$) in the MDA levels in oil 200mg/kg, SV 300mg/kg, and oil 200mg/kg + SV 150mg/mg compared to disease control. MDA levels of treatment groups 4 and 5 were comparable with normal ($p>0.05$).

Glutathione levels decreased significantly in disease control ($p<0.05$) compared to normal control. In comparison with disease control, there was a significant difference in GSH (reduced form) in those treated with either oil 200mg/kg, SV 300 mg/kg or oil 200mg/kg + SV 150 mg/kg ($p<0.05$). GSH levels of group 4,5 were comparable with normal control ($p>0.05$).

Nitrite levels in disease control increased significantly when compared to the normal group ($p<0.05$). There was a decrease in group 3,4,5

Table 4. Effect of yellow mustard seed oil (*Sinapis alba*) in MES-induced epilepsy in rats

No.	Groups	Flexion (Duration in seconds)	Tonic hind limb extension (THLE) (Duration in seconds)	Clonic convulsions (Duration in seconds)	Stupor (Duration in seconds)	% Inhibition of convulsions (THLE)
1	Normal	-	-	-	-	-
2	Disease	10.02±1.48	9.97±0.69	33.71±4.07	130.1±10.35	0
3	Oil 200mg/kg	5.987±1.19[a]	5.48±1.13[a,b]	21.25±5.82	72.33±9.39[a]	33.33
4	SV 300 mg/kg	4.63±0.41[a]	0[a]	14.37±1.83[a]	58.33±8.71[a]	100
5	Oil 200mg/kg+ SV 150 mg/kg	5.67±0.45[a]	3.84±1.31[a,b]	16.02±2.21[a]	59.83±10.96[a]	66.66

All values are expressed as Mean±SEM. One-way ANOVA, post hoc - Tukey's multiple comparison test was performed. All the values were compared with disease control (group 2).
[a]$p<0.05$ vs disease control
[b]$p<0.05$ vs SV 300mg/kg

Table 5. Effect of *Sinapis alba* seed oil on brain oxidative stress status (MES model)

Groups	MDA (nM/g)	GSH (µM/mg)	Nitrite (mM/L)	SOD (U/mg)
Normal	45.35±5.57	10.68±0.09	31.99±3.41	52.14±1.24
Disease	101.9±5.4*	8.7±0.19*	66.08±4.12*	36.54±2.05*
Oil 200mg/kg	71.04±8.19*,[a]	9.71±0.06*, [a,b]	47.20±2.19*,[a]	46.7±2.54 [a]
SV 300 mg/kg	50.72±1.79[a]	10.32±0.22[a]	36.75±3.58[a]	51.81±1.25[a]
Oil 200mg/kg + SV 150 mg/kg	59.19±2.54[a]	10.26±0.21[a]	40.81±1.26[a]	48.41±1.24 [a]

One way ANOVA and then post hoc Tukey's multiple comparison test. Values expressed as Mean±SEM.
*$p<0.05$ vs normal control
[a]$p<0.05$ vs disease control
[b]$p<0.05$ vs sodium valproate 300mg/kg

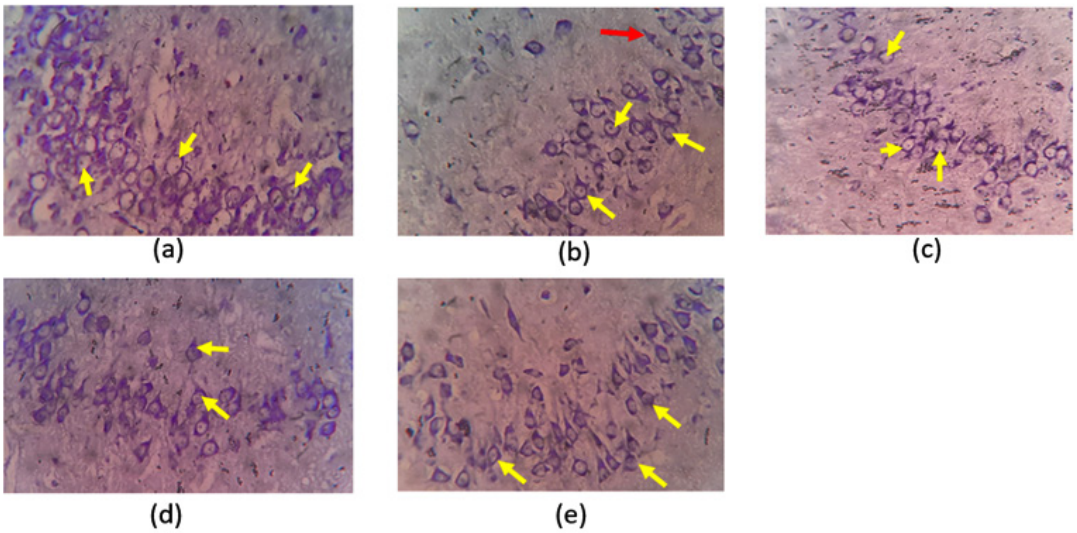

Fig. 1. Representative photomicrographs of Cresyl violet stained hippocampal CA3 region of rats of different groups. (a)group 1 (normal control); (b) group 2 (Disease control); (c) group 3 (Treated with 200 mg/kg yellow mustard oil); (d) group 4 (Treated with 300mg/kg Sodium valproate); and (e) group 5 (Treated with 150mg/kg sodium valproate + 200mg/kg yellow mustard oil). [Magnification: 40x10 X]

nitrite levels when compared to group 2(disease control). Group 4 (SV 300mg/kg) nitrite levels reduced significantly ($p<0.05$). Nitrite levels in brain of rats treated with Oil 200mg/kg (group 3) alone and in combination with sodium valproate 150 mg/kg (group 5) were comparable to sodium valproate 300mg/kg (group 4).

Superoxide dismutase (SOD) levels reduced significantly in disease control when compared to normal ($p<0.05$). In SV 300mg/kg (group 4), the SOD levels increased significantly with respect to disease control ($p<0.05$) and almost matched normal levels. The levels of group 3 and group 5 increased significantly ($p<0.01$) compared to MES control and were comparable to group 4 (Table 5).

Qualitative analysis of Cresyl violet stained pyramidal neurons of hippocampal CA3 region

Cresyl violet stained pyramidal neuronal cell bodies of the hippocampal CA3 region of rats of group 1 (normal control) showed normal healthy neurons (indicated by the yellow arrow in figure 1) with healthy-looking cell membrane, clear cytoplasm, and prominent nucleus. Cresyl violet stained hippocampal CA3 region of disease group 2 (disease control group) also showed predominantly healthy neurons and very few degenerating, flame-shaped, pyknotic cell bodies of pyramidal neurons (indicated by the red arrow in Figure 1). The flame-shaped cells that look deeply basophilic indicate karyopyknosis of the hippocampus's neurons. Based on this observation, it can be stated that the acute model of epilepsy is unable to elicit structural changes in the hippocampal CA3 region. Further, it can be noted that hippocampal CA3 neurons of group 3 (treated with 200 mg/kg yellow mustard oil), group 4 (treated with 300mg/kg Sodium valproate), and group 5 (treated with 150mg/kg sodium valproate + 200mg/kg yellow mustard oil) looked healthy and normal (indicated by the yellow arrow in figure 1). It can be noted that all the groups exhibited normal hippocampal structures with predominantly healthy neurons (indicated by the yellow arrow). However, Group 2 showed very few degenerating pyknotic neurons (indicated by the red arrow).

It can be noted that the neurons that look characteristically flame-shaped are relatively more in group 2 when compared to those of other groups. Groups 3, 4, and 5 showed predominantly healthy neurons (indicated by yellow arrow) when compared to group 2. The neurons of groups 4 and 5 looked almost at par with the normal control group.

Sinapis alba (yellow/white mustard) is a widely used spice in India and has valuable medicinal properties. Yellow mustard has anti-

microbial, anti-inflammatory, anti-proliferative, antioxidant, analgesic and immunomodulatory properties.[6,7,15,16] It is also known for its stimulant, expectorant, rubefacient, appetizing, diuretic, emetic, carminative, digestive, and diaphoretic properties.[3] Ayurvedic literature mentions the use of *S. alba* in the treatment of diseases involving the nervous system, such as hysteria and epilepsy.[2,17] Even it is present in 16-17th century Renaissance period literature. It is mentioned in the list of the herbal plants used in epilepsy therapy along with other species belonging to the family i.e., *Brassica nigra* during the 16 – 17th century Renaissance period.[8] There are two studies already done on *B. nigra* (black mustard) which belongs to same family, conducted using PTZ model in mice and penicillin model in rabbits. And it has been proven to have anti-convulsant property.[9,10] Though *S. alba* is extensively used in alternative medicine, there are no published scientific reports regarding the same. So, in this study we evaluated the effect of *Sinapis alba* seed oil (yellow mustard) in maximal electroshock-induced epileptic rat models. Several studies have been carried to check the efficacy of anti-epileptic drug sub-therapeutic dose combinations with products of plants with antioxidant properties in different epileptic models.[13, 18] Therefore, we wanted to check if the combination of *S. alba* oil with sub-therapeutic dose of sodium valproate had some synergistic effect.

In the MES model, the standard sodium valproate had 100% protection, while yellow mustard seed oil showed 33.33% protection and a combination of half-dose sodium valproate (150mg/kg) and yellow mustard oil 200 mg/kg showed 66.66% protection. Prevention of THLE correlates with reduced seizure spread to the brain and hence inhibits neuronal death.[14] Histopathological results showed no significant difference since this was an acute model; there was not much of structural changes in the CA3 region of the hippocampus. However, when compared, the MES-disease control showed some flamed pyknotic cells, while the test, standard and combination group was at par with the normal control. This suggests that mustard oil and its combination was able to prevent neuronal loss.

The biochemical tests conducted showed raised MDA and nitrite levels, levels of SOD and GSH (reduced form) declined in the MES control, this is in agreement with previous studies conducted.[19] Our study evidently reduced MDA and nitrite levels and enhanced SOD, GSH levels suggesting the anti-oxidative action of *S. alba* seed oil. The oxidative enzyme levels almost matched the normal levels in animals administered with standard sodium valproate and combination of half-dose SV and yellow mustard oil. The overall result obtained conveys that combination of yellow mustard oil (200 mg/kg) and sub-therapeutic dose valproate (150mg/kg) has some additive effect in protection against THLE and controlling GTCS, since the data was comparable with the standard.

Antioxidants are ideal in amelioration of the diseases like Alzheimer's, epilepsy, stroke etc.[20] Metabolic oxidative stress creates imbalance between levels of pro-oxidants and antioxidant species. ROS, reactive nitrogen species and lipid peroxides form the pro-oxidative agents, while catalase, SOD, GSH helps in neutralizing these. Brain consumes higher amount of oxygen to cope up with the extensive metabolic processes. Hence, it undergoes a lot of oxidative damage.[21] Oxidative stress with marked increase in generation of free radicals is observed after seizure insult, which further suppresses the neuronal system and attributes to epileptogenesis, followed by precipitation of spontaneous recurrent seizures after a few months to years.[22] Frantseva et al., have shown that free radicals are generated upon incidence of seizure, and they are linked to seizure-induced neuronal cell death mainly in the hippocampal region.[23] However, it is also observed that antioxidants can inhibit the lipid peroxidation reactions and reduce cell loss in the hippocampus.[23] In vivo study conducted earlier showed that mustard seed can enhance the levels of SOD, GSH, catalase and reduced the levels of lipid peroxide marker-MDA.[15,24-26]

CONCLUSION

The current study shows that *Sinapis alba* oil has demonstrated anti-epileptic effect in MES model and it can be considered as an add-on drug to the current anti-epileptic regimens. Hence, this study adds to the scientific literature and provides a platform for detailed molecular studies on *Sinapis alba* extract in different forms of epilepsy.

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None.

Conflict of Interest

There is are no conflict of interest.

Ethical clearance

The protocol was approved by (IAEC/KMC/65/2021) by the Institutional Animal Ethics Committee, KMC, MAHE, Manipal. The study was conducted as per Committee for Control and Supervision of Experiments on Animals (CCSEA) guidelines.

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