AJMS
(A US National Library of Medicine enlisted journal)
Al Ameen J Med Sci (2011)4(2):175-190
ISSN 0974-1143

ORIGINAL ARTICLE

Spinacia oleracea retards the development of Amygdala kindled epilepsy in rats

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Abstract: The protective role of *Spinacia oleracea* (SO) has been evaluated against the development of Amygdala kindled (AMK) experimental epileptogenesis. Thirty six Holtzman strain adult male albino rats (200-250 g) were equally divided into 1) control, 2) SO, 3) AMK, 4) SO+AMK, 5) DZ+AMK group. After discharge duration (ADD) were used as indices of kindled seizures. In AMK group, seizure stages reached upto stage 4–5 within the second week. EEG tracings showed that pretreatment with SO in AMK group decreased the ADD and seizure stages of SO pretreated rats were limited within stage 1–2 from 1st to 4th week of kindling. Brain monoamine content of Serotonin (5-HT) was decreased in cerebral cortex (CC), cerebellum (CB), caudate nucleus (CN), midbrain (MB) and pons-medulla (PM) of AMK group which was increased by SO pre-treatment. Alteration of Dopamine (DA) and Norepinephrine (NE) in different brain regions of AMK group was also modulated by SO pre-treatment. Thus SO pre-treatment retards the development of amygdala kindled epilepsy in experimental animals by modulating behavioural and neurochemical aspects.
Keywords: Amygdala kindling; ADD; EEG; Brain monoamines

Introduction

Kindling is characterized by a progressive increase in response to the same regularly applied stimulus and this repeated administration of an initially sub convulsive electrical stimulus to specific brain areas results in progressive intensification of seizure activity, culminating in a generalized seizure [1-2]**.** As kindling is a chronic model, it is extensively based on the long-term changes induced by repeated synchronous discharges [3]. It is considered as an animal model of human complex partial and temporal lobe epilepsy [1, 4-5]. Substantial evidence suggests that the amygdala is involved in temporal lobe epilepsy [6], and it may be due to the reason that Amygdala is one of the areas most readily kindled [7] and the basolateral nucleus of the amygdala is a brain area commonly selected to induce kindling [8]. Amygdala kindled (AMK) epileptic model has been established as an important model for the study of epilepsy [9-10] and has become a widely used and more reliable for temporal lobe epilepsy since Goddard *et al*, 1969 [1] who reported that the repeated focal application of initially subconvulsive nonpolarizing electrical brain stimulation to certain brain structures eventually induce a permanent change in the excitability of the stimulated structures. Such a 'kindled' hyperexcitability reflects an enduring alteration in neuronal interaction and also as a modification of the synaptic propagation [11].

Kindling causes dramatic changes in the electrophysiology of amygdala neurons. Spontaneous and evoked epileptiform bursting can be recorded in vitro in amygdala neurons after kindling in vivo [12]. The amygdala is that part of the limbic system most specifically involved with emotional experience. The sensory inflow from various emotional states enters the amygdala by means of a particular set of basolateral nucleus. The basolateral amygdala projects widely to the central nucleus of amygdala. The output nucleus of amygdala makes extensive connection with brain stem areas involved in behavioural and autonomic responses and finally projects to the cerebral cortex [13]. The biological basis of the kindling phenomenon requires being determined, but several studies indicate that alterations in amino acidergic neurotransmission may be involved. Production of epileptiform electrographic discharges and behavioural manifestations in amygdala kindled experimental epilepsy is believed to be mediated by central monoaminergic systems [14-15]. Spontaneous and experimentally induced deficiencies in norepinephrine (NE), dopamine (DA) and/or serotonin (5-HT) have been implicated in the onset and perpetuation of many seizure disorders [14] including the amygdala kindled model of temporal lobe epilepsy.

The advantage of this model is that the efficacy of drug against the progressive process leading to epileptogenesis as well as against the fully kindled state can be determined [16]. Many antiepileptic drugs block kindled seizures while others are effective against development of kindling. Keeping in view of developing an experimental model of epilepsy in rats, it is pertinent to study kindled model, which serves as a useful tool for investigating the efficacy of experimental anticonvulsant agents.

Neuroprotection by herbal therapy is increasingly considered as a promising therapy for preventing and treating epilepsy throughout the world in modern age. India has a rich source of medicinal plants and a great history of using those plants in treatment of wide variety of diseases from time immemorial. So this aspect has evoked a pleothora of investigations of one of such plant of Indian origin which may provide some protection against epileptic seizure.

Spinacia oleracea (SO), commonly called 'spinach', a multipurpose plant found almost all over the Asian countries and are consumed as food by the people. This plant has several medicinal properties. Appearance of spinach in the garden is a welcome sign of spring. This plant has several medicinal properties including anti-tumor properties [17-18], prostate cancer [19], beneficial effect in neurodegeneration [20], showing protection in liver, used as a remedy for urinary calculi. The leaves are used for bowel and lung inflammation, febrile affliction, and cooling [21]. It was reported that SO leaf has CNS depressive effect [22] and can provide protection against seizure by alteration of the brain monoamines which are associated with epilepsy [22]**.** It also offers benefit for humans by acting as antioxidants to prevent oxidative damage in cells and subsequent cancerous growth [23]. Spinach is a dietary powerhouse, full of vitamins and minerals such as vitamin C, and vitamin E [20], vitamin K, vitamin A and Folic acid [24], iron [25], magnesium, manganese, calcium. Spinach is also a ketogenic diet. SO leaves are a storehouse of all essential amino

acids, minerals like phosphorous, magnesium, iron, selenium etc which are believed to improve memory functions by different mechanisms to overcome memory deficit. Parle & Dhingra, 2003 [26] reported that ascorbic acid enhanced memory in aged mice and reversed memory impairment. Considering these criteria the overall purpose of the study is to investigate the antiepileptic properties of SO by an analysis of behaviour pattern as well as seizure pattern, electroencephalographic (EEG) studies & to elucidate the role of essential neurotransmitter in management of epilepsy using SO. Thus the present experiment has been undertaken to observe the herbal modulation of *Spinacia oleracea* on amygdala kindled (AMK) epilepsy.

Materials and Methods

Collection and Preparation of aqueous extract of *Spinacia oleracea* (SO) leaf [22]:

Fresh, young, healthy leaves of *Spinacia oleracea* (SO) were collected from Agricultural Horticulture Society of India, Belvedre, Calcutta and then identified & authenticated (Voucher specimen no.-CNH/ I-I/(239)/2008/Tech.II/278) by Central National Herbarium, Botanical Survey of India (BSI), Govt. of India, Howrah. Then these were kept in the laboratory. After identification *Spinacia oleracea* leaves were cleaned and shade dried, grinded in an electrical grinder to get a free flowing powder and spread over tray with shifting of materials daily to avoid growth of fungus. This powder was subjected to extraction with water (1:3) at room temperature for 24 hr. The extractive solution was filtered with Whatman No.1 filter paper and vacuum dried at 40-50° C to get a brown coloured sticky mass. The extract obtained was stored in the refrigerator and it was dissolved in saline (0.9% NaCl) solution for final use**.**

Animals —- Pure (colony) bred Holtzman Strain adult male albino rats weighing between 200-250 g were chosen.

Maintenance- They were housed individually at an ambient temperature of 25°±1ºC and constant humidity (60%) in a normal photoperiod cycle of 12 hr : 12 hr (light and dark). Standard laboratory diet and drinking water was supplied daily *ad libitum*. Body weight of rats was recorded daily and maintained throughout the experimental period. Before the experiment, the rats were allowed to get accustomed to laboratory conditions (7 days) during which their motor behaviour, food and drinking habits, micturition and fecal output were noted for future comparison. Tail pinch and hand clapping tests were also performed to check any pre-existing epileptogenesis [27-29]. All animal studies were performed in accordance with the rules and regulations of Institutional Animal Ethical Committee.

Chemicals and drugs: Pentobarbitone sodium (PB, Abott India Ltd., 40 mg/kg b.w., i.p.), anesthetic ether (Kobra drugs Ltd., India), diazepam (DZ, Ratiopharm, Germany, 10 mg/kg b.w., i.p.) were used in this study.

Experimental design and grouping of animals: Thirty rats were taken for the experiment. Before the start of the treatment schedule and experiment, the rats were divided into 5 different groups having 6 animals in each.

Group 1: *Control group*- Rats were treated orally with double distilled water (5 ml/Kg body weight), once daily for 14 consecutive days orally by using orogastric cannula between 10:30 am to 11:30 am.

Group 2: *SO group*- Rats were treated orally with aqueous extract of *Spinacia oleracea* leaves at a dose of 400 mg/Kg (standardized in laboratory), once daily for 14 consecutive days orally by using an orogastric cannula between 10:30 am to 11:30 am.

Group 3: *AMK group*- Before preparation of epileptic animals rats were treated orally with double distilled water (5 ml/Kg body weight), once daily for 14 consecutive days orally by using orogastric cannula between 10:30 am to 11:30 am. On the 15^{th} day, stimulation of the basolateral nucleus of amygdala was given by Grass S44 stimulator and continued until the animal had displayed generalized tonic – clonic convulsions.

Group 4: *SO + AMK group*- Amygdaloid kindled epileptic rats were pretreated orally with *Spinacia oleracea* leaves extract (400 mg/Kg), once daily for 14 consecutive days orally by using an orogastric cannula between 10:30 am to 11:30 am. On the 15^{th} day, stimulation of the basolateral nucleus of amygdala was given by Grass S44 stimulator and continued.

Group 5: *DZ + AMK group*- Diazepam (Ratiopharm, Germany) was used as reference drug. Epileptic animals of reference group were treated with diazepam (20 mg/kg body weight) intraperitoneally [27] 1 hour later, the animals were given kindling trials.

Preparation of Amygdaloid kindled epileptic rat model: Prior to surgery all the animals were fasted overnight but had free access to water. Rats were injected with sodium pentobarbitone (PB, Abott India Ltd., 40 mg/kg b.w., i.p.), until a level of anesthesia was achieved where the fore-paw withdrawal reflex was abolished. Anesthetic ether (Kobra drugs Ltd., India) was also used if required.

Surgery by Stereotaxic technique [9, 28-29]:

Anesthetized animals were mounted on stereotaxic instrument (INCO, India Ltd.). Head was fixed in such a position that lambda and bregma sutures were in the same horizontal plane by introducing the incisor bar properly attached to the mouth. Extreme care should be taken in setting the custom-made ear bar to prevent the damage of the tympanic membrane. Care was also taken so that no respiratory disturbance or occlusion occurs. The hair on the scalp was shaved and the skin on the skull was wiped clean with saline and rectified spirit. The scalp was incised posteriorly in the midline and the pericranial muscles and fascia were retracted laterally. After retracting the nuchal musculature, the overlying bone was drilled at the specific loci in basolateral amygdaloid nuclei (-2.6 mm posterior to bregma, 5.3 mm lateral to midline and 9 mm ventral to the duramater) following the coordinates of the stereotaxic atlas of Pellegrino & Cushman, 1967 [30] to make a trephine hole (1-2 mm diameter) in the skull. If any bleeding that was controlled by aseptic bone wax. Total surgery was performed under strict aseptic conditions.

Electrode implantation in Amygdala for Amygdala kindling [9-10, 13] **and in surface cortex for electroencephalographic (EEG) recording of brain waves** [9, 31]:

Through the trephine hole the stimulating electrodes were placed stereotaxically into the basolateral nucleus of the amygdala (-2.6 mm posterior to bregma, 5.3 mm lateral to the midline and 9 mm ventral to the dura). Each stimulating electrode consisted of twisted strands of nichrome wire, 150µm in diameter and insulated everywhere by epoxy resin except the cut tips and the electrode leads were connected to an amphenol microminiature connector strip. For electroencephalographic recording bipolar electrodes were implanted on the surface of the somatosensory cortex through trephine holes. A reference electrode was also implanted over the frontal bone. All electrodes were then soldered to a multiple plug that was fastened to the calvarium with dental acrylic cement that clearly isolated the electrodes from skin and muscle. In control rats implantation of electrodes for EEG recording was same.

Post operative care: Following surgery all the animals were carefully maintained with all the necessary precautions and aseptic measures until they recover from operative stress. For proper antibiotic preventive care 10,000 IU penicillin was given postoperatively to all animals daily for consecutive three days by intramuscular route. Particular care was taken for feeding and for the first two days animals were given intraperitoneal injection of dextrose-saline until the animals become capable of taking standard diet [27]. Animals were allowed a postoperative recovery of 2 weeks before beginning the stimulation paradigm.

Stimulation for Amygdala kindling [9-10, 13]:

Stimulation was delivered from a Grass S44 stimulator through SIU assuring relatively constant current, after discharge thresholds (ADT) were determined using unilateral stimulation train (biphasic square waves, 0.5 msec, 60 Hz, 1-2 sec duration) of ascending intensity. Testing began with 150 µA intensity and was raised in 25-50 µA increments on test spaced 10 min apart until an (AD) after-discharge (neuronal spikes for focal seizure) was elicited. The current was then decreased by 25 µA and depending on whether or not an AD was evoked, it was further decreased or increased, until the lowest threshold stimuli that would generate an AD was reached. Once ADT testing was complete, the first kindling threshold train was administered using the stimulation parameter (200-350 µA, 60 Hz 0.5 msec duration for 1sec) with a fixed supra threshold current. With no further manipulation trials were given once daily until the animal had displayed generalized tonic – clonic convulsions with rearing & falling on two consecutive days which was considered as completion of a full kindling state. Kindling was considered complete after animals developed the final stage of generalized seizures (kangaroo posture and falling back). Stimulation was repeated for five more days to establish fully kindled rats. Seizure stage (convulsive behaviour) and afterdischarge duration (ADD) were used as indices of kindled seizures. ADD was considered as the total time of spikes in the EEG recorded from the site of stimulation as reffered by Potschka *et al* [32]. The ADD was measured daily and the number of kindling trials required to achieve the first generalized convulsion was also determined. From the first day of stimulation after

determining the supramaximal intensity of stimuli, the changes of ADD with the development of amygdala kindled behavioral seizure changes were evaluated in each rat according to the method of Racine, 1972 [33].

Analysis of behaviour features and seizure scoring in experimental animals:

During kindling evolution the following behavioral changes facial movements often associated with rhythmic head nodding, forelimb clonus and falling or generalized convulsion were noted. Kindling was considered complete after animal had displayed generalized tonic–clonic convulsions with rearing and falling on two consecutive days.

Kindled seizures have been classified by Racine, 1972 [33] into five stages:

Immobility, eye closure, twitching of vibrissae, sniffing, facial clonus = 1;

Rhythmic head nodding associated with more severe Facial clonus = 2;

Facial clonus, head nodding and one forelimb clonus = 3;

Facial clonus, head nodding, bilateral forelimb clonus and rearing = 4;

Facial clonus, head nodding, forelimb clonus, rearing, loss of balance & falling accompanied by generalized clonic seizures = 5.

Recording of EEG wave pattern [9, 28, 31]:

Electrical activity was recorded on an 8 channel EEG machine (Recorder and Medicare Systems, India) from cortical and amygdala electrodes. All tests were done by placing the animal in a sound proof box (20×35×25 cm) with observation windows so that the behavioural evidence of wakefulness and visible clinical seizure could be noted along with obtaining the amygdala after discharge (AD) from the first day of stimulation. The chronological changes in the amygdaloid ADD were measured until the first generalized convulsion. Animals' behaviour was critically checked for movement artifacts in the recordings. Epileptiform activity was recorded daily (between 11.00 and 15.00 h) throughout the experimental period. Each recording session lasted for 2-4 hrs without interruption. Occurrence of the epileptiform EEG activity during periods of awake immobility (passive or subdued wakefulness) was taken into account to avoid contamination with movement and other artifacts. Cortical recordings were also obtained from normal animals (not made epileptic by amygdala kindling) to check that there were no animals showing spontaneous epileptiform activity.

After behavioral and electroencephalographic studies were complete animals were sacrificed by cervical dislocation for biochemical estimation of brain monoaminergic system.

Biochemical estimation of brain monoamines (serotonin, dopamine, norepinephrine) [22, 34]:

Collection of brain tissues: For spectrofluorometric estimation of brain monoamines the animals were sacrificed by cervical dislocation (between 11:00 and 12:00 hours). Brain tissues (cerebral cortex, CC; cerebellum, CB; caudate nucleus, CN; midbrain, MB; pons & medulla, PM) were dissected out. The choice of these

areas was based on the selective distribution of the concerned neurotransmitters as well as on the anatomical and the functional connections of the different areas of the brain.

Procedure: Immediately after collection brain tissues were washed in ice-cold saline and homogenized in 10 ml acidified butanol. Tissue homogenate (4 ml) was mixed with 10 ml of 10% heptane and 5 ml of 0.003 *N* HCl. Then the mixture was shaken for 5 minutes and centrifuged at 2000 rpm for 10 minutes. Acid layer (4.5 ml) was eluted and mixed with 100 mg alumina and 1 ml of 2 *M* sodium acetate. Then the mixture was shaken for 5 minutes and centrifuged at 2000 rpm for 10 minutes and the resultant supernatant was taken for the estimation of 5-HT and the precipitate was used for the estimation of NE and DA.

- *Estimation of Serotonin (5-HT)*

To the supernatant, obtained previously, 3 ml of 10% isobutanol and 2 ml of boric acid buffer (pH 10) was added. After shaking the mixture, 2 ml of 10% heptane was added to the butanol phase and 5 ml of 0.1 *N* HCL was added followed by shaking again and 1 ml of 0.3 *N* HCL was mixed. Then it was taken for spectroflurometric reading of 5-HT.

- *Estimation of Dopamine (DA) and Norepinephrine (NE)*

Cold distilled water (5 ml) was added to the precipitate, shaken well and centrifuged at 2000 rpm for 30 seconds. 3 ml of 0.33 *N* acetic acid was added and centrifuged at 2000 rpm for 3 minutes. Supernatant was transferred to glass stoppered centrifuge tube. 1.2 ml of freshly prepared ethylenediamine and ethylene diammonium dihydrochloride mixture (7:5) was added to it and incubated at 50°C for 40 minutes. Mixture was cooled at room temperature and saturated with sodium chloride and 4 ml of 10% isobutanol was added. It was then centrifuged at 2000 rpm for 3 minutes. Supernatant was taken for the estimation of DA and the precipitate was mixed with 4 ml cold distilled water for the estimation of NE.

The fluorescence of 5-HT, DA and NE was measured in the Perkin Elmer MPF 44B Fluorescence spectrophotometer, USA with activation and emission wavelength set at 295 nm and 550 nm (for 5-HT), 320 nm and 370 nm (for DA) and 385 nm and 485 nm (for NE).

Statistical analysis:

All the datas were expressed as MEAN ± S.E.M and analyzed statistically by One-Way Analysis of Variance (ANOVA) followed by multiple comparison 't' test. Difference below the probability level 0.05 was considered statistically significant.

Results

Effect of SO and DZ on seizure stages of amygdala kindled epileptic rats:

Seizure stage (convulsive behaviour) and ADD were used as indices of kindled seizures. In the AMK group, the behavioural seizure stages were increased within the second week and reached a mean seizure stage 4–5. The behavioural stages of SO and DZ treated rats were limited to stage 1–2 from the first week to fourth week. The results of seizure stages from the different groups are summarized in Table 1.

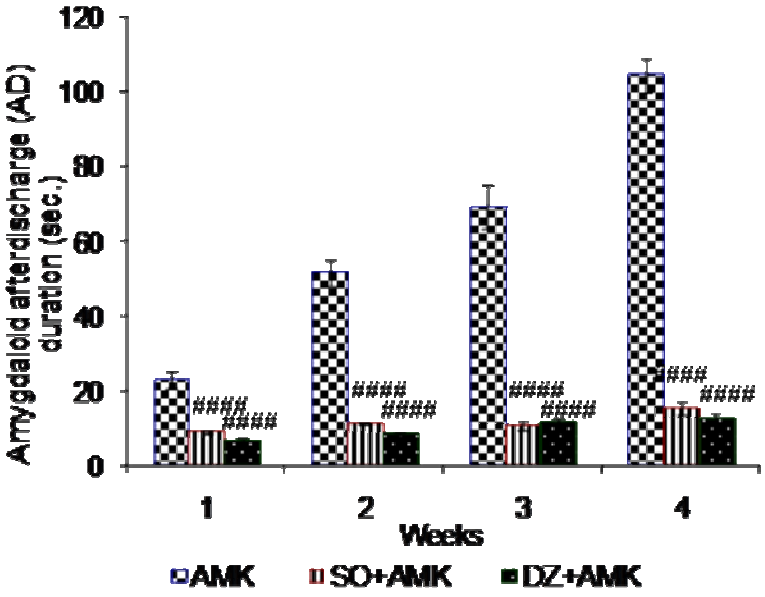
Table-1: Behavioral seizure stages in different groups.

Group	(Behavioral seizure stage/Week)			
	1st Week	2nd Week	3rd Week	4th Week
AMK	3.4±0.27	4.31±0.20	4.86±0.26	5.1±0.40
SO+AMK	1.2±0.33####	1.86±0.42####	1.91±0.45####	1.84±0.32####
DZ+AMK	1.3±0.41###	1.74±0.70###	1.88±0.29####	1.76±0.30####

Values are mean ± SEM, n=6 in each group; ####p<0.001; ###p<0.01 compared to AMK
[**Table 1** shows spontaneous seizure (kindling) developed in kindled rats within second week (stage 4). SO and DZ suppress the progression of behavioral stages, reached up to Stage 1-2 even after fourth week.]

Effect of SO and DZ on ADD of amygdala kindled rats (from EEG tracing) *[Fig 1 and 2]*

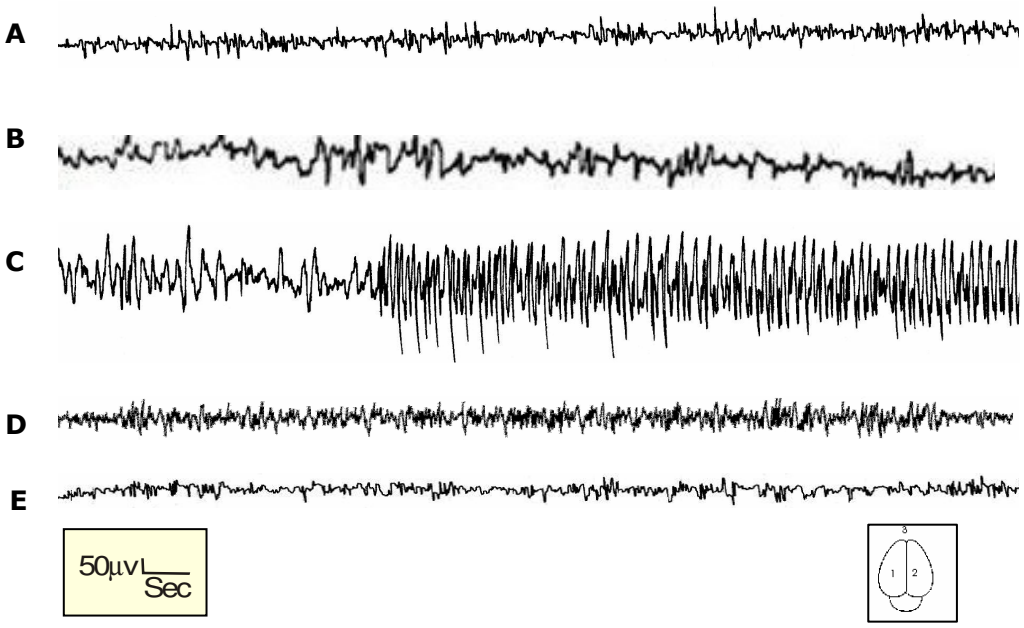
Fig-1: Evolution of amygdaloid after discharge (AD) during kindling procedure.



Values are mean ± SEM, n=6 in each group; ####p<0.001; compared to AMK
[Fig 1 shows Amygdaloid AD developed in AMK groups within second week.
Duration of AD remains decreased in SO and DZ pretreated groups even after fourth week.]

Representative electroencephalographic recordings showing the effect of *Spinacia oleracea* and diazepam on the ADD in amygdala kindled rats. In the control animals, the EEG pattern showed predominance of low voltage fast waves or β waves with few high voltage slow waves or α waves. Treatment with SO (400 mg/kg body weight) showed frequent occurrence of α waves with low β waves. After determining the supramaximal intensity of stimuli, the ADD (not more than 10s duration) at the amygdala (due to focal seizure) were observed in rat (AMK group) from the first day stimulation for kindling when the facial clonus was observed (stage 1).

Fig-2: Effect of SO on EEG recording in Amygdala kindled (AMK) epileptic rats



A- Control group: Predominance of low voltage fast waves or β waves
B- So treated group: Predominance of high voltage slow waves or α waves
C- AMK group: Appearance of high voltage spike waves
D- SO+AMK group: Disappearance of (1) high voltage spike, (2) polyspikes, (3) bursts, with appearance of few isolated sharp positive waves and (4) high voltage slow waves or α waves
E- DZ+AMK group: Abolition of high voltage spike discharges and appearance of sharp positive waves

After second to fourth trials, ADD lasted for more than 15 secs. With subsequent repeated trials, the ADD increased proportionately (stage 2: Rhythmic head nodding associated with more severe Facial clonus). Stage 3 was reached in which unilateral forelimb clonic movement occurred and the ADD appeared to be more than 25 secs. Stages 4 showed bilateral forelimb clonus with rearing and stage 5 were considered by the appearance of generalized fit with clonic and tonic convulsions, when the ADD varied from 40–60 secs or more. The ADD in kindled (AMK) animals was significantly higher (more than 100 secs) after fourth week (28 days) of stimulations. The kindling effect remained markedly decreased in SO and DZ pretreated animals. From the first kindling trials, the SO and DZ treated animals showed very little increase in the ADD (after fourth week, ADD for SO + AMK is about 15 secs and for DZ + AMK about 12 secs) and the kindling phenomena by eliciting convulsive seizure with clonic and tonic convulsion could not be observed even after the fourth week (28 days) of stimulation.

Changes in brain neurotransmitters- *Changes in serotonin (5-HT) level***:**

In SO treated animals, 5-HT level increased in all regions of brain (CC, CB, CN, MB & PM). In AMK group, the 5-HT level decreased in all regions. Following treatment with SO leaves (400 mg/kg, for 14 days) and pretreatment with DZ, 5-HT level increased in all brain regions of amygdala kindled epileptic rats (Table 2).

Table-2: Effect of SO on serotonin (5-HT) level (µg / 100 g of tissue) in discrete brain regions of amygdala kindled (AMK) rats

Groups	CC	CB	CN	MB	PM
Group1 (control)	0.070 ± 0.002	0.363 ± 0.002	0.702 ± 0.004	0.250 ± 0.007	0.561 ± 0.008
Group 2 (SO)	0.427 ± 0.002****	0.831 ± 0.001****	2.662 ± 0.003****	1.965 ± 0.009****	0.747 ± 0.013****
Group 3 (AMK)	0.031 ± 0.002****	0.214 ± 0.004****	0.544 ± 0.003****	0.102 ± 0.011****	0.421 ± 0.010****
Group 4 (SO+AMK)	0.060 ± 0.006####	0.464 ± 0.003####	0.708 ± 0.004####	0.149 ± 0.009###	0.603 ± 0.005####
Group 5 (DZ+AMK)	0.082 ± 0.003####	0.451 ± 0.003####	0.707 ± 0.003####	0.151 ± 0.008####	0.599 ± 0.007####

Values are mean ± SEM, n=6 in each group; **** p<0.001 compared to control; ### p<0.01, #### p<0.001 compared to AMK

Changes in dopamine (DA) level:

In SO treated animals, DA level decreased in all regions of brain (CC, CB, CN, MB & PM). In AMK group, the DA level increased in CC, CB, MB but decreased in CN & PM. Following treatment with SO leaves (400 mg/kg, for 14 days) and pretreatment with DZ decreased the level of DA in all brain regions of amygdala kindled rats (Table 3).

Table-3: Effect of SO on dopamine (DA) level (µg / 100 g of tissue) in discrete brain regions of amygdala kindled (AMK) rats

Groups	CC	CB	CN	MB	PM
Group 1 (control)	0.164 ± 0.010	0.731 ± 0.026	2.645 ± 0.066	0.357 ± 0.005	0.390 ± 0.006
Group 2 (SO)	0.105 ± 0.005****	0.526 ± 0.036****	2.240 ± 0.073****	0.276 ± 0.006****	0.312 ± 0.003****
Group 3 (AMK)	0.343 ± 0.006****	0.942 ± 0.022****	2.436 ± 0.053*	0.420 ± 0.011****	0.294 ± 0.006****
Group 4 (SO+AMK)	0.153 ± 0.003####	0.712 ± 0.028####	2.168 ± 0.070###	0.369 ± 0.009####	0.264 ± 0.006###
Group 5 (DZ+AMK)	0.150 ± 0.003####	0.700 ± 0.027####	2.133 ± 0.051###	0.354 ± 0.008####	0.260 ± 0.006###

Values are mean ± SEM, n=6 in each group; * p<0.05; **** p<0.001 compared to control; ### p<0.01, #### p<0.001 compared to AMK

Changes in norepinephrine (NE) level:

In SO treated animals, NE level decreased in all regions of brain compared to control animals. In AMK group NE level decreased in CC, CB & CN and in MB & PM it increased slightly which was not statistically significant. The level of NE in SO + AMK group and DZ+AMK group showed no significant changes when compared with AMK group (Table 4).

Table-4: Alterations in norepinephrine (NE) level (µg / 100 g of tissue) in discrete brain regions of different groups

Groups	CC	CB	CN	MB	PM
Group 1 (control)	0.045 ± 0.004	0.083 ± 0.003	0.097 ± 0.006	0.064 ± 0.006	0.072 ± 0.005
Group 2 (SO)	0.044 ± 0.006	0.079 ± 0.004	0.094 ± 0.004	0.062 ± 0.006	0.070± 0.004
Group 3 (AMK)	0.032 ± 0.002****	0.080 ± 0.006	0.084 ± 0.006***	0.060 ± 0.005	0.068 ± 0.004
Group 4 (SO+AMK)	0.033 ± 0.001	0.078 ± 0.006	0.077 ± 0.005	0.062 ± 0.005	0.058 ± 0.003
Group 5 (DZ+AMK)	0.030 ± 0.001	0.079 ± 0.006	0.074 ± 0.005	0.062 ± 0.005	0.059 ± 0.003

Values are mean ± SEM, n=6 in each group; ***p<0.01; ****p<0.001 compared to control;

Discussion

The present investigation demonstrated that pretreatment with SO and DZ protected the rats from amygdala kindled epileptic seizure. Repeated subthreshold stimulation with appropriate intervals in basolateral nucleus of amygdala results in increased amygdaloid after-discharge duration (ADD) & complexity of behavioral seizures [33] and the neuronal excitability begins to spread over the large portions of the cortex, can be seen from the EEG tracings of the cortex which corobrates with the observation of Post, 2004 [35].

Neuronal hyperexcitability may play an important role in the modulation of amygdala-dependent behaviours [36]. But SO and DZ attenuated the ADD and delayed the onset and progression of behavioural seizures, indicating that the aqueous extract of *Spinacia oleracea* leaves as well as diazepam exert depressive effects on both seizure generation in the focus and seizure propagation from the focus. All rats in amygdala kindled (AMK) group reached focal seizure (Stage 1 and 2) and these behavioural seizure stages were increased within the second week and reached a mean seizure stage of 4 to stage 5 (generalized seizure). Initially the EEG spike activity increased in length, and the animals displayed seizure activity starting with involvement of forelimbs and the neck region, culminating in a generalized seizure including loss of postural control resembling generalized tonic-clonic epilepsy [33, 37]**.** Once kindling has progressed to this stage, the animal becomes vulnerable for the applied stimulus and this response remains for the rest of the life. But in rats, pretreated with SO and DZ, behavioural stages never reached generalized

seizures and not even the forelimb clonus and the seizure stages remained depressed (within stage 1-2) from the 1st to 4th week of the observation. According to observation at the fourth week, the amygdaloid ADD also remained suppressed at a mere about 15 sec in SO + AMK group whereas that (ADD) was raised to a huge above 100 sec in AMK group. In animals of DZ + AMK group, DZ also lowered the amygdaloid ADD compared to AMK group.

In the present findings it was found that stimulation at the basolateral amygdaloid nuclei led to the development of amygdala kindling followed by a modulation in brain monoaminergic system. The role of monoamines in amygdala kindled epileptogenesis and in recurrent seizure activity is also well documented [14]. During seizure generated by amygdaloid kindling, the 5-HT level is decreased in all brain regions (CC, CB, CN, MB & PM) of rats of AMK group. There are reports of depletion of brain 5-HT after amygdala kindling [38]. Serotonergic control is involved in the regulation of cellular excitability in the limbic brain areas such as the amygdala [36]. Low 5-HT mediates neural hyperexcitability [36] which increased amygdaloid ADD and ultimalely leads to generalized seizure which has been obtained from the present study. But in SO + AMK and DZ + AMK group an elevated level of 5-HT (compared to AMK group) was recognized. There are some evidence that serotonin might modulate kindling [9] and they also reported an increased level of 5-HT in different brain regions (CC, CB, CN and MB) of amygdala kindled rats pretreated with a herbal extract named as *Acorus calamus*. 5-HT mediates an inhibitory tone in the amygdala [36] possibly by enhancing GABA and inhibiting glutamate neurotransmission [39-40]. Thus SO mediated increased level of 5-HT may protects the neurons from excitability, as a result amygdaloid ADD remained depressed and behavioural seizures could not reach generalized tonic clonic seizures. Thus SO may delay the development of kindling possibly through modulating the 5-HT which may act via modulating GABAergic tone.

Simultaneously our result showed that the 5-HT level in group 4 and 5 can't reach the 5-HT level in group 2. This may be due to the rats of group 2 were treated only with herbal extract of SO. They were not made experimentally epileptic. But rats of group 4 (SO+AMK) and 5 (DZ+AMK) were pretreated with SO and DZ respectively and then both these two groups were made experimentally epileptic by amygdala kindling. So they are different experimental conditions as the group 2 is only herb treated group but both group 4 and group 5 are experimental epileptic groups pretreated with herb and standard antiepileptic drug (AED) respectively. Thus the 5-HT level is reduced in group 4 and group 5 due to amygdala kindling though they were pretreated with herb and AED respectively. So the 5-HT level in group 4 and 5 can't reach the 5-HT level in group 2 as it is only the herb treated group.

Together with a depletion in 5-HT, the level of DA is increased in CC, CB & MB and decreased in CN & PM after amygdaloid kindling. This result corroborates with the earlier findings of Bonhaus *et al.,* 1986 [41] who showed an increase in the firing (bursting) of the dopaminergic neurons during a kindled seizure. The enhancement of DA in different brain region also support the studies of Hazra *et al*., 2005 [9], Burnham *et al*., 1981 [42], Sato & Nakashima**,** 1975 [43], Starr, 1996 [44] but

are in contrast with the results of Lewis *et al.,* 1987 [45] who demonstrated no alteration in the brain DA level. Dopamine is believed to act as an inhibitory neurotransmitter in the basal ganglia, but in some other areas of the brain it is possibly excitatory [46]. According to Das, 1995 [47] the inhibitory actions of DA may be mediated through phospholipase C-phosphoinositol pathway to decrease cellular Ca^{++} concentration which inactivates protein kinase C and thereby different enzymes whereas in other brain areas DA activates the adenylate cyclase-cAMP system and thereby activates different enzymes. Both SO and DZ decreased this DA level in all brain regions of amygdala kindled epileptic animals. These findings are inconsistent with previous report that showed amygdaloid release of dopamine abolishes electrical amygdaloid kindling [13]. But Hazra *et al*., 2005 [9] reported that in *Acorus calamus* and DZ treated kindled group DA level had decreased significantly in the CC, CB and CN to combat with amygdala kindled seizure. Thus from the result it is evident that SO may act against the development of amygdala kindled epilepsy through modulating the dopaminergic neurotransmitter or by decreasing its level.

Another essential neurotransmitter, norepinephrine i.e. NE plays also an important role in the amygdala. The studies of Mohr and Corcoran, 1981 [38] revealed that depletion of NE facilitates the development of epileptiform activity in the amygdala kindling model. Facilitation of amygdala kindling in the rat has been demonstrated by transecting the ascending noradrenergic pathways and a number of studies have indicated that catecholamines may play a role in the development of kindling [48]. The inhibitory effect of NE has also been reported in discrete brain areas due to inhibitory receptors at certain neuronal synapses [9]. Aroniadou-Anderjaska *et al*., 2007 [49] reported that NE act against amygdala kindled epilepsy by facilitating GABAergic tone. But in our findings, in AMK group NE level was slightly decreased which was not significant in all brain regions. In SO pretreated animals, NE was decreased insignificantly. The level of NE in SO + AMK group and DZ+AMK group showed no significant changes when compared with AMK group. So, the role of NE in SO pretreated amygdala kindled rats is not clear & undefined.

Besides all these neurotransmitters possibly there is interference of two other neurotransmitters such as gamma-aminobutyric acid (GABA) and glutamic acid in the development of amygdala kindling seizure. Activation of glutamatergic excitatory neurotransmission combined with the collapse of GABA mediated inhibition is currently thought to be the most likely explanation for kindling development [50-52]. But how SO decrease the predominance of AMK induced seizure by the mediation of GABA and glutamate still not recognized though the possible mediation of GABA by SO induced 5-HT has been discussed earlier.
Thus it is tempting to suggest that retardation of the build up of amygdala kindled epilepsy by SO is an effective way to suppress the temporal lobe epilepsy by modulating three essential brain monoamines i.e. 5-HT, DA and NE.

Finally it must be added that treatment with aqueous extract of leaves of *Spinacia oleracea* (400 mg/kg body weight) by modulating brain monoamines, has given a glimpse of a therapeutic approach for management of epilepsy, which may be

beneficially used in contrast to the classical antiepileptic therapeutics. *Spinacia oleracea* has several medicinal property and this plant contains different essential components. But we have investigated for whole leaves rather than any active constituent of *Spinacia oleracea* to search for its antiepileptic property because it belongs to our routine vegetables and we consume the total but not any specific component of it and it may be possible that the protection may be attributed to the combined effects of its constituents rather than any single factor. The significance of aqueous extract is for its normal consumption with aqueous media as it is cooked in water.

Acknowledgement

Ms. Sutapa Das was recipient of fellowship from NTRF (National Tea Research Foundation), Kolkata.

References

1. Goddard GV, McIntyre DC, Leech CK. A permanent change in brain function resulting from daily electrical stimulation. *Exp Neurol* 1969; 25(3): 295-330.
2. McNamara JO. Kindling: an animal model of complex partial epilepsy. *Ann Neurol.* 1984; 16 (suppl 1): S72-S76.
3. Jefferys JGR. The pathophysiology of epilepsies. In: *A textbook of Epilepsy*. 4th edition. Laidlaw J, Richens A, Chadwick D (Eds), Churchill Livingstone. U.K. 1993; p 241-276.
4. Albright PS, Bumham WM. Development of new pharmacological seizure model: effects of anticonvulsants on cortical- and amygdala-kindled seizures in the rat. *Epilepsia.* 1980; 21: 681-689.
5. Wada JA, Sato M, Corcoran ME. Persistent seizure susceptibility and recurrent spontaneous seizures in kindled cats. *Epilepsia* 1974; 15(4): 465-468.
6. Kalviainen R, Salmenpera T, Partanen K, Vainio P, Riekkinen P Sr, Pitkanen A. MRI Volumetry and T2 relaxometry of the amygdala in newly diagonosed and chronic temporal lobe epilepsy. *Epilepsy Res* 1997; 28: 39-50.
7. Cain DP, Desborough KA, McKitrick DJ. Retardation of amygdala kindling by antagonism of NMD-aspartate and muscarinic cholinergic receptors: evidence for the summation of excitatory mechanisms in kindling. *Experimental Neurology* 1988; 100: 179-187.
8. Loscher W, Ebert U, Wahnschaffe U, Rundfeldt C. Susceptibility of different cell layers of the anterior and posterior part of the piriform cortex to electrical stimulation and kindling: comparison with the basolateral amygdala and "area tempestas". *Neuroscience* 1995; 66: 265-276.
9. Hazra R, Ray K, Guha D. The role of *Acorus calamus* in the involvement of monoaminergic and electroencephalographic activities in amygdala kindled rats. *Biogenic Amines* 2005; 19(3): 177-187.
10. Guha D, Maiti AK. Influences of vestibulo-cerebellum on kindling in the cat. *Indian J Med Res* 1989; 90: 275-284.
11. Wada JA, Sato M. Generalized convulsive seizure induced by daily electrical stimulation of the amygdala in cats: correlative electrographic and behavioural features. *Neurology* 1974; 24: 565-567.
12. Rainnie DG, Asprodini EK, Shinnick-Gallagher P. Kindling-induced long-lasting changes in synaptic transmission in the basolateral amygdala. *J Neurophysiol* 1992; 67: 443-454.

13. Lin MT, Wang JJ, Young MS. The protective effect of ditetrahydropalmitine against the development of amygdale kindling seizure in rats. *Neuroscience Letters* 2002; 4: 29-33.
14. Shouse MN, Staba RJ, Ko PY, Saquib SF, Farber PR. Monoamines and seizures: microdialysis findings in locus ceruleus and amygdala before and during amygdala kindling. *Brain Res* 2001; 892(1): 176-192.
15. Shouse MN, Staba RJ, Saquib SF, Farber PR. Long-lasting effects of feline amygdala kindling on monoamines, seizures and sleep. *Brain Res* 2001; 892(1): 147-165.
16. Loscher W, Schmidt D. Which animal models should be used in the search for new antiepileptic drugs? A proposal based on experimental and clinical considerations. *Epilepsy Res* 1988; 2(3): 145-181.
17. Lamson DW, Brignall MS. Antioxidants and Cancer III: Quercetin, *Alternative Medicine Review* 2000; 5:196-208.
18. Lambert JD, Hong J, Yang G, Liao J, Yang CS. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. *Am J Clin Nutr* 2005; 81:284S-291S.
19. McCann SE, Ambrosone CB, Moysich KB, Brasure J, Marshall JR, Freudenheim JL, Wilkinson GS, Graham S. Intakes of selected nutrients, foods, and phytochemicals and prostate cancer risk in western New York. *Nutrition and Cancer* 2005; 53:33-41.
20. Joseph JA, Shukitt-Hale B, Denisova NA, Prior RL, Cao G, Martin A, Taglialatela G, Bickford PC. Long-term dietary strawberry, spinach or vitamin E supplementation retard the onset of age-related neuronal signal-trunsduction and cognitive behavioural deficits. *Neuroscience* 1998; 18(19); 8047-8055.
21. Jain SK, De Fillipps RA. Medicinal Plants of India. Reference Publication. Algoriac, MI. 1991.
22. Das S, Guha D. CNS depressive role of aqueous extract of *Spinacia oleracea* L. leaves in adult male albino rats. *Ind J Exp Biol* 2008; 46: 185-190.
23. Benbrook CM. Elevating Antioxidant Levels in Food through Organic Farming and Food Processing An Organic Center State of Science Review. 2005. <http://www.organiccenter. org/Antioxidant_SSR.pdf>
24. Zhang GF, Storozhenko S, Van Der Straeten D. Lamert WE. Investigation of the extraction behaviour of the main monoglutamate folates from spinach by liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr A* 2005; 1078: 59-66.
25. Wang R, Furumoto T, Motoyama K, Okazaki K, Kondo A, Fukui H. Possible Antitumor Promotors in *Spinacia oleracea* (spinach) and Comparison of their Contents among Cultivars. *Biosci Biotech Biochem* 2002; 66: 248-254.
26. Parle M, Dhingra D. Ascorbic acid: a promising memory enhancer in mice. *J Pharmacol Sci* 2003; 93:129–35.
27. Hazra R, Ray K, Guha D. Inhiitory role of *Acorus calamus* in ferric chloride-induced epileptogenesis in rats. *Human & Experimental Toxicology* 2007; 26: 947-953.
28. Guha D, Biswas D, Purkayastha S. Suppression of penicillin-induced epileptiform activity by *Nigella sativa*: Possible mediation by neurotransmitters. *Biogenic Amines* 2005; 19**:** 309-321.
29. Purkayastha S, Guha D. Monoaminergic correlates of penicillin induced epilepsy. *Biogenic Amines* 2003; 18**:** 29-40.
30. Pellegrino LJ, Cushman AJ. *A stereotaxic atlas of the rat brain*, (Appleton Century Crafts, New York). 1967; 60.
31. Ray K, Guha D. Effect of *Moringa oleifera* root extract on penicillin-induced epileptic rats. *Biogenic Amines* 2005; 19**:** 223-231.

32. Potschka H, Feuerstein TJ, Loscher W. Gabapentin-lactam, a close analogue of the anticonvulsant gabapentin, exerts anticonvulsant activity in amygdala kindled rats. *Naunyn-Schmiedeberg's Arch Pharmacol* 2000; 361: 200-205.
33. Racine RJ. Modification of seizure activity by electrical stimulation II. Motor seizure. *Electroencephalogr Clin Neurophysiol* 1972; 32**:** 281-294.
34. Ray K, Hazra R, Guha D. Central inhibitory effect of *Moringa oleifera* root extract: Possible role of neurotransmitters. *Indian J Exp Biol* 2003; 41: 1279-1284.
35. Post RM. The Status of the Sensitization/Kindling Hypothesis of Bipolar Disorder. *Current Psychosis & Therapeutics Reports* 2004; 2: 135-141.
36. Hughes CR, Keele NB. Phenytoin normalizes exaggerated fear behavior in *p*-chlorophenylalanine (PCPA)-treated rats. *Epilepsy Behav* 2006; 9: 557-563.
37. McNamara JO, Byrne MC, Desheiff RM, Fitz JG. The kindling model of epilepsy: a review. *Prog Neurobiol* 1980; 15: 139-159.
38. Mohr E, Corcoran ME. Depletion of noradrenaline and amygdala kindling. *Exp Neurol*. 1981; 72**:** 507-511.
39. Rainnie DG. Serotonergic modulation of neurotransmission in the rat basolateral amygdala. *J Neurophysiol* 1999; 82: 69-85.
40. Stutzmann GE, LeDoux JE. GABAergic antagonists block the inhibitory effects of serotonin in the lateral amygdala: a mechanism for modulation of sensory inputs related to fear conditioning. *J Neurosci* 1999; 19: RC8.
41. Bonhaus DW, Walters JR, McNamara JO. Activation of substantia nigra neurons: role in the propagation of seizures in kindled rats. *J Neurosci* 1986; 6: 3024.
42. Burnham WM, King GA, Lloyd KG. Extra-focal catecholamine levels in 'kindled' rat forebrain. *Prog Neuropsychopharmacol* 1981; 5**:** 537-541.
43. Sato M, Nakashima T. Kindling: secondary epileptogenesis, sleep and catecholamines. *Can J Neurol Sci* 1975; 2: 439-446.
44. Starr MS. The role of dopamine in epilepsy. *Synapse* 1996; 22(2): 159-194.
45. Lewis J, Westerberg V, Corcoran ME. Monoaminergic correlates of kindling. *Brain Res* 1987; 403: 205-212.
46. Guyton AC. Behavioral and motivational mechanism of the brain the limbic system and the hypothalamus, In: *Textbook of Medical Physiology*. 9th edition. Wornnsiewicz MJ (Ed). Prism Books Pvt Ltd. India. 1991; pp 648-658.
47. Das D. Hormones and Neurotransmitters. In: *Biochemistry* (Academic Publisher, Kolkata, India). 1995; 537.
48. Ehlers CL, Clifton DK, Sawyer CM. Facilitation of amygdala kindling in the rat by transecting ascending noradrenergic pathways. *Brain Res* 1980; 189: 274 –279.
49. Aroniadou-Anderjaska V, Qashu F, Braga MFM. Mechanisms regulating GABAergic inhibitory transmission in the basolateral amygdala: implications for epilepsy and anxiety disorders. *Amino Acids* 2007; 32: 305-315.
50. McNamara JO. Pursuit of the mechanisms of kindling. *Trends in Neurosciences* 1988; 11: 33-36.
51. Morimoto K. Seizure-triggering mechanisms in the kindling model of epilepsy: collapse of GABA-mediated inhibition and activation of NMDA receptors. *Neuroscience & Biobehavioral Reviews*. 1989; 13: 253-260.
52. Burnham WM. The GABA hypothesis of kindling *in* Wada JA (ed.) Kindling 4. Plenum Press, New York. 1989; pp 127-139.

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