CNS depressive role of aqueous extract of *Spinacia oleracea* L. leaves in adult male albino rats

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Treatment with *Spinacia oleracea* extract (SO; 400 mg/kg body weight) decreased the locomotor activity, grip strength, increased pentobarbitone induced sleeping time and also markedly altered pentylenetetrazole induced seizure status in Holtzman strain adult male albino rats. SO increased serotonin level and decreased both norepinephrine and dopamine levels in cerebral cortex, cerebellum, caudate nucleus, midbrain and pons and medulla. Result suggests that SO exerts its CNS depressive effect in PTZ induced seizure by modulating the monoamines in different brain areas.

**Keywords:** Anticonvulsant, Epilepsy, Neurotransmitters, PTZ, *Spinacia oleracea*

*Spinacia oleracea* L. (SO), commonly named as spinach (family Amaranthaceae) is an annual plant having medicinal property native to central and southwestern Asia. It is cultivated for the sake of its succulent leaves and was introduced in Europe in the 15th century and is probably of Persian origin. It is a favorite food vegetable among Indians in winter season and is a dietary powerhouse, full of vitamins and minerals such as vitamin C, iron, and vitamin E. It also contains magnesium, manganese, calcium, vitamin K, vitamin A and folic acid. Presence of different carotenoids such as lutein, β-carotene, violaxanthin and 9´-(Z)-neoxanthin has also been reported in organically grown spinach. Spinach leaves contain several active components, including various flavonoids, which exhibit antioxidant, antiproliferative and antiinflammatory properties in biological systems. Quercetin is the most abundant flavonoid of spinach, which possesses antihistaminic property. Spinach shows some beneficial effect in neurodegeneration due to presence of certain phytochemicals in it.

In preliminary study the beneficial role of spinach in retarding functional age-related CNS and cognitive behavioral deficits have been reported. Since the plant parts have not been examined for their central nervous system (CNS) depressive action, the present study has been undertaken to evaluate the CNS depressive action of SO leaves in an experimental rat model of epilepsy.

**Materials and Methods**

**Animals**—Pure (colony) bred Holtzman strain adult male albino rats weighing between 200-250 g were caged individually at an ambient temperature of 25±1°C 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. Experiments were carried out after the approval of Animal Ethical Committee of the institute.

**Preparation of extract**—Fresh, young, healthy leaves of SO were collected from the local market and identified and authenticated by the Botanical Survey of India, Howrah. SO leaves were 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 was dissolved in double distilled water for final use.

**Experimental protocol**

**Schedule I**

The rats (48) were divided into control group (Group I) and treatment group (Group II, III, IV, V, ...
VI, VII and VIII), comprising 6 rats each. Group I rats were fed with saline (5 ml/kg body weight) for 14 days consecutively. Rats of Group II, III, IV, V, VI, VII and VIII were treated orally with aqueous extract of SO at a dose of 150, 200, 250, 300, 350, 400, 450 mg/kg body weight respectively by orogastric cannula for 14 days daily between 0900-1100 hrs. On the 14th day locomotor activity and pentobarbitone induced sleeping time were recorded for selection of the effective dose (400 mg/kg) and further experiments were continued with the selected dose.

On the 14th day, grip strength was measured in control group and SO treated group (400 mg/kg). On 15th day, animals of control group and SO treated group (400 mg/kg) were sacrificed by cervical dislocation (between 1100-1200 hrs) for biochemical estimation of brain monoamines such as serotonin (5-HT), dopamine (DA), norepinephrine (NE) in different regions of brain, e.g. cerebral cortex (CC), cerebellum (CB), caudate nucleus (CN), midbrain (MB) and pons and medulla (PM).

Schedule II
Twelve rats were divided into saline pretreated (5 ml/kg, po) control epileptic (Group A) and experimental epileptic rats (Group B) pretreated with SO extract (400 mg/kg body weight). Rats of both the groups were treated once daily with their respective doses for consecutive 14 days. On the 15th day all animals were made epileptic through intraperitoneal administration of pentylenetetrazole (PTZ) to evaluate the role of SO on PTZ induced seizure parameters.

Locomotor activity (open-field test)—Open field test was studied for recording the locomotor activity. The apparatus (1 m x 1 m) was made up of plywood, surrounded by 40 cm high wall with inside surface painted black. It was strongly lit by two 150 W lamps from 150 cm above. The surface of the floor was equally divided into 25 squares. The open field experiment was performed in a sound proof room by placing the square shaped box in that room. The animals were placed gently in the centre of the apparatus one after another, where they were free to walk and to get adapted to the new environment. After completion of their training individually, the animals were treated with saline (control) or SO extract (150, 200, 250, 300, 350, 400 and 450 mg/kg body weight) and 30 min later the animals were placed individually in the apparatus and the number of squares traversed in 5 min was considered as locomotor score. The floor of the box was cleaned after every trial. The same protocol was followed for 14 days.

Pentobarbitone (PB) induced sleeping time and changes in latency period—Pentobarbitone (PB) (Neon Laboratory Ltd. India,) at a dose of 40 mg/kg body weight was administered intraperitoneally to measure pentobarbitone induced sleeping time. The PB induced sleeping time was measured as the time interval between the loss and regain of the righting reflex. The sleep latency is the duration of the time between the administration of the pentobarbitone and loss of righting reflex. The righting reflex was considered to be lost when the animal was placed on its back and failed to regain its normal posture within 10 sec. The experimental rats were treated with aqueous extract of SO, 4 hr before the intraperitoneal administration of PB and control rats received only PB.

Motor coordination test by grip-strength—Grip strength was measured by using the rotarod. This apparatus was made up of a metallic rod rotating at a speed of 10 rotations/min by the stimulation of electrical stimulator. On the day of experiment rats of control group and SO treated group (400 mg/kg body weight) were fed with their respective doses 1 hr prior to the experiment. Rats were placed on the rod with the body axis perpendicular to the rod’s long axis and with the head direction against the direction of rotating rod. To observe the behavioral strategy used by an animal to maintain motor coordination, rats were placed on the rod individually. Duration of the time, the subject can exist on that rotating rod was recorded as grip strength.

Drug and dose—Pentylenetetrazole was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was dissolved in isotonic saline solution. It was administered intraperitoneally at a dose of 40 mg/kg body weight.

Anticonvulsant activity
Pentylenetetrazole (PTZ) induced seizure—PTZ (40 mg/kg body weight) was injected to both control group and SO treated (400 mg/kg body weight) group. In SO treated group SO extract (400 mg/kg body weight) was fed to the rat 1 hr before the intraperitoneal administration of PTZ. After application of PTZ rats were transferred to a square shaped box, made up of cardboard and were observed for every 15 min for 2 hr. Onset of seizure, ictal phase, interictal phase, seizure score and percentage of protection from seizure were recorded.
Observation procedure—Seizure score was recorded according to the following modified scale of Patel et al.\textsuperscript{12}:

no response = 0, gustatory movement = 1, tremor = 2, head bobbing = 3, forelimb clonus = 4, rearing, falling and clonus = 5

Biochemical estimation of serotonin (5-HT), dopamine (DA) and norepinephrine (NE)—The animals were sacrificed by cervical dislocation (between 1100-1200 hrs). Brain tissues were dissected out, washed in ice-cold saline and homogenized in 10 ml acidified butanol. Homogenate (4 ml) was mixed with 10 ml 10% heptane and 5 ml 0.003 N HCl and then shaken for 5 min and centrifuged at 2000 rpm for 10 min. Acid layer (4.5 ml) was eluted and mixed with 100 mg alumina and 1 ml of 2 M sodium acetate. The mixture was shaken for 5 min and centrifuged at 2000 rpm for 10 min.

Supernatant was taken for estimation of 5-HT and precipitate was used for estimation of DA and NE.\textsuperscript{13}

Cold distilled water (5 ml) was added to the precipitate, shaken well and centrifuged at 2000 rpm for 30 sec. 3 ml of 0.33 N acetic acid was added and centrifuged at 2000 rpm for 3 min. Supernatant was transferred to glass stoppered centrifuge tube. 1.2 ml of freshly prepared ethylenediamine and ethylenediamine dihydrochloride mixture (7:5) was added to it and incubated at 50°C for 40 min. Mixture was cooled at room temperature and saturated with sodium chloride and then 4 ml 10% isobutanol was added. It was centrifuged at 2000 rpm for 3 min. The supernatant was taken for estimation of DA and to the precipitate 4 ml of distilled water was added. This was taken for estimation of NE.\textsuperscript{8}

The fluorescence of 5-HT, DA and NE was measured in the Perkin Elmer MPF 44B Fluorescence spectrophotometer with activation and emission wavelength set at 295 and 550 nm (for 5-HT), 320 and 370 nm (for DA) and 385 and 485 nm (for NE).\textsuperscript{13}

Statistical analysis—The values were evaluated statistically by using Students-t test for determination of level of significance in various groups of animals. The values given in the table are mean ± SE (n=6). P value less than 0.05 was considered statistically significant.

Results

Locomotor activity—The aqueous extract of S. oleracea inhibited the locomotor activity of rat in a dose dependent manner. At the dose of 150 and 200 mg/kg the decrease in locomotor activity was not statistically significant. Locomotor activity was decreased significantly at higher doses (250, 300, 350, 400 and 450 mg/kg) reaching its peak at 400 mg/kg (P<0.001; Table 1).

Pentobarbitone (PB) induced sleeping time—SO decreased the latency period of sleep. The latency period was decreased at all the doses of SO but the dose, 150 mg/kg showed no significant result. At 300, 350, 400 and 450 mg/kg latency period decreased significantly (P<0.001) reaching its maximum at 400 mg/kg. SO extract (250, 300, 350, 400 and 450 mg/kg) increased PB induced sleeping time significantly with gradual increase of dose whereas at 150 and 200 mg/kg results were insignificant. The level of significance (P<0.001) was same for all of rest doses (Table 2).

PTZ-induced epileptic seizure—SO (400 mg/kg)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Locomotor activity (No. of squares crossed in 5 min)</th>
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<tbody>
<tr>
<td>Gr. I (control)</td>
<td>37.33 ± 3.46</td>
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<tr>
<td>Gr. II (150 mg/kg)</td>
<td>33.17 ± 3.88</td>
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<tr>
<td>Gr. III (200 mg/kg)</td>
<td>30.17 ± 2.21</td>
</tr>
<tr>
<td>Gr. IV (250 mg/kg)</td>
<td>25.00 ± 1.86\textsuperscript{a}</td>
</tr>
<tr>
<td>Gr. V (300 mg/kg)</td>
<td>19.17 ± 2.66\textsuperscript{a}</td>
</tr>
<tr>
<td>Gr. VI (350 mg/kg)</td>
<td>15.33 ± 1.28\textsuperscript{c}</td>
</tr>
<tr>
<td>Gr. VII (400 mg/kg)</td>
<td>11.50 ± 1.06\textsuperscript{c}</td>
</tr>
<tr>
<td>Gr. VIII (450 mg/kg)</td>
<td>13.83 ± 1.91\textsuperscript{c}</td>
</tr>
</tbody>
</table>

P values: \textsuperscript{a}< 0.02, \textsuperscript{b}< 0.01, \textsuperscript{c}< 0.001

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sleep latency (sec)</th>
<th>Sleeping time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. I (control)</td>
<td>244.5 ± 3.40</td>
<td>173.33 ± 2.42</td>
</tr>
<tr>
<td>Gr. II (150 mg/kg)</td>
<td>235.83 ± 3.93</td>
<td>181.33 ± 2.78</td>
</tr>
<tr>
<td>Gr. III (200 mg/kg)</td>
<td>229.17 ± 3.54\textsuperscript{a}</td>
<td>181.83 ± 2.61</td>
</tr>
<tr>
<td>Gr. IV (250 mg/kg)</td>
<td>202.17 ± 3.38\textsuperscript{a}</td>
<td>186.17 ± 2.10\textsuperscript{a}</td>
</tr>
<tr>
<td>Gr. V (300 mg/kg)</td>
<td>177.00 ± 3.12\textsuperscript{c}</td>
<td>193.33 ± 4.51\textsuperscript{b}</td>
</tr>
<tr>
<td>Gr. VI (350 mg/kg)</td>
<td>121.83 ± 2.76\textsuperscript{c}</td>
<td>252.50 ± 2.74\textsuperscript{c}</td>
</tr>
<tr>
<td>Gr. VII (400 mg/kg)</td>
<td>91.00 ± 1.86\textsuperscript{c}</td>
<td>378.17 ± 2.36\textsuperscript{c}</td>
</tr>
<tr>
<td>Gr. VIII (450 mg/kg)</td>
<td>92.5 ± 2.39\textsuperscript{c}</td>
<td>355.83 ± 2.61\textsuperscript{c}</td>
</tr>
</tbody>
</table>

P values: \textsuperscript{a}< 0.02, \textsuperscript{b}< 0.01, \textsuperscript{c}< 0.001
can prolong onset of seizure significantly ($P<0.001$) and it can also increase interictal phase significantly ($P<0.001$) as compared to control group. But ictal phase decreased by SO (significance level $P<0.001$). Seizure scoring was also inhibited by SO extract in comparison to control value ($P<0.001$). Thus $S$. oleracea can give approximately 72.76% protection against PTZ-induced seizure (Table 3).

**Alteration in brain monoamines level**—The level of serotonin (5-HT) in different brain regions (CC, CB, CN, MB, PM) was enhanced significantly by the application of SO as compared to control. Both dopamine (DA) and norepinephrine (NE) level decreased significantly in CC, CB, CN, MB and PM (Table 4).

**Discussion**

The present study was attempted to delineate CNS depressive role of aqueous extract of Spinacia oleracea leaves in adult male albino rats. To evaluate this property SO induced alterations in behavioral activities including locomotor activity, PB induced sleeping time, grip strength, PTZ-induced seizure status with subsequent modulation in brain monoamines such as 5-HT, DA and NE in various regions of brain were examined. The pilot experiment with SO in the doses of 150, 200, 250, 300, 350, 400 and 450 mg/kg showed that 400 mg/kg was the most significant effective dose. Higher dose above 400 mg/kg failed to produce any further change. Thus 400 mg/kg was selected for the entire study. Although rotarod test results may indicate both central nervous system (CNS) activity and peripheral nervous system (PNS) activity but locomotor activity and PB induced sleeping time both are important parameters for the determination of CNS activity. Rotarod result shows grip strength (seconds) of 486.67±6.79 in control rats whereas this value was reduced to 137.83±3.88 after the treatment with SO. Maintenance of grip strength on the rotarod without falling was significantly ($P<0.001$) decreased in SO treated rats as compared to control. Loss of grip strength may be due to motor incoordination which was influenced by SO. The results indicate CNS depressive activity of $S$. oleracea (SO) as evidenced by reduced locomotor score and potentiation of PB induced sleeping time. Seizure study results also revealed that pretreatment with SO extract reduced duration of ictal phase and seizure score produced by PTZ. On the other hand interictal phase and onset of seizure were lengthened. These findings were in line with an increase in 5-HT and decline in DA and NE after SO treatment.

Generally, a balanced ratio exists among three essential brain monoamines (5-HT, DA and NE) to maintain a delicate balance between excitation and inhibition in physiological system. Biochemical

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**Table 3**—Effect of SO on Pentylenetetrazole induced seizure parameters (onset of seizure, ictal phase, interictal phase, seizure score & percentile protection)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Onset of seizure (sec)</th>
<th>Ictal phase (sec)</th>
<th>Interictal phase (sec)</th>
<th>Seizure score (every 15min)</th>
<th>Percentile protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (control)</td>
<td>31.5 ± 2.14</td>
<td>180.67 ± 3.34</td>
<td>60.67 ± 2.28</td>
<td>208.17 ± 5.78</td>
<td>0</td>
</tr>
<tr>
<td>Group B (400 mg/Kg)</td>
<td>186.17 ± 4.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41.17 ± 2.50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>300.50 ± 6.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.83 ± 2.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>72.76&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*P value: <sup>c</sup>< 0.001

**Table 4**—Effect of SO on 5-HT, DA & NE level (µg / 100 g of tissue) in different regions of rat brain

<table>
<thead>
<tr>
<th>Group</th>
<th>5-HT (µg / 100 g of tissue)</th>
<th>DA (µg / 100 g of tissue)</th>
<th>NE (µg / 100 g of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CB</td>
<td>CN</td>
</tr>
<tr>
<td>Gr. I (control)</td>
<td>0.067</td>
<td>0.350</td>
<td>0.714</td>
</tr>
<tr>
<td>Gr. VII (400 mg/kg)</td>
<td>0.005&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.034&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.069&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*P values: <sup>b</sup>< 0.01, <sup>c</sup>< 0.001

CC = Cerebral cortex, CB = Cerebellum, CN = Caudate nucleus, MB = Midbrain, PM = Pons and Medulla
estimation of essential brain monoamines (5-HT, DA and NE) showed an alteration in various parts of brain after treatment with aqueous extract of SO. Level of 5-HT was increased significantly in the cerebral cortex (CC), cerebellum (CB), caudate nucleus (CN), midbrain (MB) and pons and medulla (PM). Serotonin acts as an inhibitory neurotransmitter in higher regions of nervous system. Serotonin is secreted from median raphe nuclei that originate from reticular activating system (RAS) and thereby project mainly to the dorsal horn of the spinal cord and hypothalamus. A large number of fibres are also received by cerebral cortex, which come from brain stem (midbrain, pons and medulla). The aqueous extract of S. oleracea inhibited the locomotor activity of rat in a dose dependent manner whereas SO decreased the latency period of sleep and increased PB induced sleeping time in the same manner. Locomotor activity of rat decreased significantly at higher doses (250, 300, 350, 400 and 450 mg/kg) in comparison to control values but at the dose of 150 and 200 mg/kg the decrease in locomotor activity was not statistically significant. The more effective dose being 400 mg/kg. In case of sleep latency period, SO extract (150 mg/kg) showed no significant result. At 300, 350, 400 and 450 mg/kg dose of SO extract sleep latency period was decreased significantly (P<0.001) but more effect was observed at 400 mg/kg. SO extract (250, 300, 350, 400 and 450 mg/kg) increased PB induced sleeping time significantly with gradual increase of dose whereas at 150 and 200 mg/kg results were statistically insignificant. The level of significance (P<0.001) was same for 250, 300, 350, 400 and 450 mg/kg but more effective dose being 400 mg/kg. There were no further remarkable change at the dose above 400 mg/kg. So, the effective dose was selected at 400 mg/kg. According to Beninger depressants act via reduction of locomotion and rearing. Reduction of locomotor activity may be a sign of CNS depression as it was evidenced by Ray et al. According to Pal and Dandiya, decrease in rearing as well as locomotion is also suggestive for depression. 5-HT plays important role in animal behaviour such as locomotor depression. From the present results it may be assumed that increased level of 5-HT in various brain regions may be responsible for decreased locomotor activity. SO potentiated PB induced sleeping time. Potentiation of sleeping time is also suggestive for CNS depressant effect of particular drug. The exact mechanism by which SO potentiated PB induced sleeping time is not known. 5-HT mediated stimulation of reticular activating system (RAS) may be implicated in the events that led to prolongation of pentobarbitone induced sleeping time and the reduction of sleep latency by the SO extract. In the present study, SO significantly decreased grip strength as it was measured by duration of time that the rat can remain on rotating rod. Grip strength is a measurement of motor coordination. Motor incoordination probably indicates impairment of motor neurons. This hypothesis was compensated by decreased grip strength after pretreatment with SO which may produce motor incoordination and it may impair motor neurons as an anticonvulsant activity of SO. In the present study PTZ induced seizure parameters were studied to focus only anticonvulsant activity of SO. SO decreased seizure score and duration of ictal phase. SO increased onset of seizure and interictal phase. Thus it may give protection against PTZ induced seizure. Serotonin is known to be major endogenous anticonvulsants. SO may exert its anticonvulsant activity by elevation of 5-HT level.

In the present study SO decreased the level of NE in various regions of brain such as CC, CB, CN, MB, and PM. Therefore SO can also enhance sleeping time possibly by altering the level of NE. Anticonvulsant property of SO and decreased rate of locomotion and grip strength also may be due to declination of excitatory brain monoamine NE. Dopamine (DA) receptors are associated with the alteration of movement. DA is secreted from substantia nigra and transported to basal ganglia. Dopamine is inhibitory in nature but in some specific regions it acts as excitatory. Dopamine level is decreased in all parts of brain such as CC, CB, CN, MB and PM by the treatment of aqueous extract of SO. After SO treatment modulation of these central neurotransmitters may be related to decreased locomotion and sleep prolongation. Grip strength in SO treated rat may be decreased by modulation of DA. It is well known that blockade of dopaminergic neurotransmission reduces convulsive threshold and potentiates the action of convulsants. Thus SO can delineate PTZ induced seizure by down regulation of dopaminergic neurotransmission.

From the overall explanations it may be said that aqueous extract of Spinacia oleracea may produce CNS depressive role by modulation of balance between 5-HT, DA and NE. Thus it may alter the
ratio between excitation and inhibition in general physiological system.

It is hereby concluded that SO has potent CNS depressive property.

Acknowledgement

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References


