Central nervous system activity of acute administration of ethanol extract of *Punica granatum* L. seeds in mice

Sokindra Kumara, Kamal Kishore Maheshwari & Vijender Singha
Department of Pharmacy, MJP Rohilkhand University, Bareilly, 243 006 India

Received 20 March 2008; revised 1 September 2008

Role of ethanolic extract of *P. granatum* seeds on central nervous system (CNS) in animal models of elevated plus maze test, barbiturate-induced sleeping time, tail suspension test, hot-plate and tail-flick test was studied. *P. granatum* (PG) extract was administered to young and aged mice at single doses of 100, 250 and 500 mg/kg, perorally while diazepam (1 mg/kg), morphine (5 mg/kg) and imipramine (30 mg/kg) were used intraperitoneally as standard drugs. The results showed that PG extract at all dose levels significantly exhibited the anxiolytic activity. In another study PG extract (250 and 500 mg/kg) significantly increased the sleeping latency and reduced the sleeping time. Tail suspension test showed that PG extract (250 and 500 mg/kg) was able to induce a significant decrease in the immobility time, similar to imipramine, a recognized antidepressant drug. Tail-flick and hot-plate tests exhibited antinociceptive property of PG extract, similar to morphine, a recognized antinociceptive agent. Phytochemical investigation of ethanol extract for the presence of phenolic compounds, flavonoids, tannins, anthocyanins, sugars and saponins was also carried out. Phytochemical screening and measurement of reducing power revealed the CNS activity of ethanol extract of PG seeds may be due to its antioxidative profile.

**Keywords:** Antioxidant, Flavonoids, Imipramine, Morphine, *Punica granatum*

It is by now commonly accepted that under situations of oxidative stress, reactive oxygen species such as superoxide (O2·-), hydroxyl (OH·-), and peroxyl (-OOH, ROO-) radicals are generated. These reactive oxygen species play an important role in degenerative or pathological processes, such as aging\(^1\), cancer, coronary heart disease, Alzheimer’s disease\(^2,3\), neurodegenerative disorders, atherosclerosis, cataracts, and inflammation\(^4\). Oxidative damage was considered a likely cause of age associated brain dysfunction because the brain is believed to be particularly vulnerable to oxidative stress due to a relatively high rate of oxygen free radical generation without commensurate levels of antioxidative defenses\(^5,6\). The use of traditional medicine is widespread, and plants are still a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical scavenging mechanism as part of their activity\(^7,8\). In searching for novel natural antioxidants, some plants have been extensively studied in the past few years for their antioxidant and radical scavenging components. The antioxidant properties of green tea extract\(^10\), blueberry, spinach or straw berry\(^11\), *Allium sativum*\(^12\) and *Glycyrrhiza glabra*\(^13\) have already proven beneficial in reducing oxidative tissue injury. Plant derived flavonoids exhibit variety of biological properties\(^14\). Certain phenolic antioxidants attenuate neuronal cell death induced by oxidative stress\(^15\).

The pomegranate tree, *Punica granatum* L. (family Punicaceae) especially its fruit, possesses a vast ethnomedical history and represents a phytochemical reservoir of heuristic medicinal values\(^16,20\). The phytochemical analysis of an ethanolic extract from *P. granatum* seeds revealed the presence of a wide variety of constituents such as flavonoids, glycosides, tannins, anthocyanins, and ascorbic acid\(^21,22\). Pomegranate fruit possess powerful antioxidant\(^23,24\), anti-inflammatory\(^25\), antidiabetic\(^26\), and neuroprotective\(^27\) properties in various in vitro models. However, there is no report on central nervous system (CNS) activity of *P. granatum* seeds. Therefore, the present study has been undertaken to
evaluate the action of the ethanolic extract of *P. granatum* seeds in young and aged animal models using a set of pharmacological profile of CNS.

**Materials and Methods**

*Plant material and preparation of extract—*Seeds of *P. granatum* were purchased from locally and plant material was identified and authenticated at Department of Pharmacognosy, Hamdard University, New Delhi. A voucher specimen (201) was deposited in the department. The seeds were extracted successively with petroleum ether, chloroform followed by ethanol in Soxhlet extractor. The ethanol extract was evaporated under reduced pressure to obtain dry masses. The extract was then stored in a desiccator.

*Drugs and chemicals—*Diazepam (Ranbaxy, India), morphine (Sigma Chemicals, USA), imipramine (Torrent, India), pentobarbital (Neon, India), butylated hydroxytoluene (Merk, Germany), trichloroacetic acid (CDH, India), potassium ferricyanide (S D Fine Chemicals, India) and Tween 80 (CDH, India) were used.

*Phytochemical screening—*Phytochemical investigation of ethanolic extract for the presence of phenolic compounds, flavonoids, tannins, anthocyanins, sugars and saponins was carried out using the methods described by Trease and Evans28.

**Determination of antioxidant activity—**The antioxidant activity of different extracts was assessed on the basis of reducing power. The reducing power of the extracted samples (dissolved in ethanol) or butylated hydroxytoluene (BHT, dissolved in ethanol) were determined as per Jayaprakasha et al29. Different concentrations (12.5, 25, 50, 100, 200 and 400 µg/ml) of each extract, were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. To reduce ferricyanide into ferrocyanide, the mixture was incubated at 50°C for 20 min. Thereafter, an equal volume of 1% trichloroacetic acid was added to the mixture and centrifuged at 6,000 rpm for 10 min. The upper layer of the solution was collected and mixed with distilled water and 0.1% ferric chloride at a ratio of 1:1:0.2. Absorbance was measured to determine the amount of ferric ferrocyanide (Prussian Blue) formed at 700 nm against a blank in a Shimadzu UV 160A spectrophotometer. The BHT was used for the positive control in ethanolic extracts. The reducing power tests were run in triplicate. Increase in absorbance of the reaction indicated the reducing power of the samples.

*Animals—*Male Swiss albino mice (Mus musculus albinus), 3 months (young) and 14 months old (aged), weighing 20-25 g and 38-42 g, respectively, were obtained from Central Animal House, Jamia Hamdard, New Delhi, India. They were housed in an environmentally regulated room on a 12:12 hr L:D cycle with 25°C ± 2°C and had free access to food and water. The experimental protocol was approved by the Institutional Animal Ethical Committee and experiments conducted according to the CPCSEA, India guidelines on the use and care of experimental animals. Experiments were carried out between 0900 and 1800 hr.

*Experimental protocol—*The animals were tested during the light period and observed in a closed room with constant temperature and poorly illuminated room with a red light. All tests were performed in different days with distinct groups of animals. The ethanolic extract of *P. granatum* seeds was suspended in 5% Tween 80 and was administered to young and aged mice at single doses of 100, 250 and 500 mg/kg perorally, while diazepam (1 mg/kg), morphine (5 mg/kg) and imipramine (30 mg/kg) were used intraperitoneally as standard drugs.

*Elevated plus maze test—*The elevated plus maze test was used to evaluate antianxiety activity, as per Itoh et al30. Briefly, the apparatus consisted of two open arms (16×5 cm²) and two enclosed arms (16×12×5 cm³). The arms extended from a central platform (5×5 cm²), and maze was elevated to a height of 25 cm from the floor. The seed extract was administered, po, in varying doses 60 min before the evaluation of antianxiety activity. At the time of experiment, each mouse was placed at the center of maze, facing one of the enclosed arms. During a 5 min test period the time spent on open arms and in closed arms was recorded.

*Effect on sleeping time—*After 60 min of oral administration of PG extract (100, 250 and 500 mg/kg, po) or vehicle (Tween 80), all groups received sodium pentobarbital (40 mg/kg, ip). The time elapsed between the administration of pentobarbital until the loss of the righting reflex was recorded as the sleep latency and the time elapsed between the loss and voluntary recovery of the righting reflex is recorded as sleeping time31.

*Effect on tail suspension test—*Antidepressant activity was assessed by tail suspension test as per Steru et al32. For the test, mice were suspended on the edge of a shelf, 58 cm above the ground with adhesive
tape placed approximately 1 cm from the tip of the tail. The duration of immobility was recorded for a period of 5 min after the 60 min of PG extracts and 30 min of imipramine administration.

Effect on motor activity—Locomotor activity of mice was evaluated by means of the actophotometer apparatus. The animals were placed individually in the actophotometer immediately subsequent to administering, and ambulation was recorded at 30, 60 and 120 min. The locomotor activity was expressed in terms of total photobeam count/5 min/animal.

Antinociceptive activity—The antinociceptive activity of PG extract was performed in mice by tail-flick and hot-plate responses, i.e., the reaction time (in seconds) of each animal, was measured at 60 min after extract administration, and 30 min after morphine (5 mg/kg, ip) administration which served as positive control.

Tail-flick test—The painful stimulus of tail was produced by a heating wire and cut-off time was 15 sec using tail-flick analgesiometer.

Hot-plate test—The parameter evaluated was the latency time for licking of legs and jumping responses after exposure on the hot-plate surface. The hot-plate temperature was kept at 55°C ± 0.5°C and the cut-off time was 30 sec.

Statistical analysis—Data are presented as the mean ± SE. ANOVA was applied for the analysis of the result and Scheffe was used as a post-hoc test. Differences between groups were regarded as significant at a level of P<0.05.

Results

Phytochemical screening—Phytochemical screening of ethanolic extract revealed the presence of phenolic compounds, flavonoids, tannins, anthocyanins, sugars and saponins.

Determination of antioxidant activity—The reducing capacity of P. granatum seeds extracts was investigated by measuring the Fe³⁺–Fe²⁺ conversion. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. All three extracts exhibited the reducing powers and were concentration dependent. The reducing powers (expressed as µg extract/ml) of samples studied have been shown in Fig. 1. It was found that the ethanolic extract had a higher degree of reducing power followed by chloroform and petroleum ether extract.

Elevated plus maze test—The PG extracts (100, 250 and 500 mg/kg, po) and diazepam (1 mg/kg, ip) induced significant (P<0.01) increase in the occupancy in the open arms. The PG extract and diazepam showed a decreased preference for the closed arms. However, the PG extract was found to be more effective in aged animals as compared to young animals (Table 1).

Effect on sleeping time—The absolute values of sleep latency and sleeping time (Fig. 2) demonstrate that animals treated with PG extract (250 and 500 mg/kg), 60 min before the injection of pentobarbital, presented an significant (P<0.05) increase in the sleep latency and reduction of pentobarbital-induced sleeping time in mice. However, PG (100 mg/kg) did

![Graph](image_url)

Fig. 1—Antioxidative activities of different P. granatum seed extracts as measured by the reducing power method. Absorbance values represent means of triplicates of different extract samples analyzed.

<table>
<thead>
<tr>
<th>Treatments (mg/kg)</th>
<th>Time spent in open arms (sec)</th>
<th>Time spent in closed arms (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Aged</td>
</tr>
<tr>
<td>Control</td>
<td>35.56 ± 6.25</td>
<td>39.36 ± 7.59</td>
</tr>
<tr>
<td>PG 100</td>
<td>60.36 ± 7.23</td>
<td>65.86 ± 8.94</td>
</tr>
<tr>
<td>PG 250</td>
<td>63.87 ± 8.50</td>
<td>70.22 ± 9.50</td>
</tr>
<tr>
<td>PG 500</td>
<td>109.5 ± 8.25</td>
<td>103.24 ±10.5</td>
</tr>
<tr>
<td>Diazepam 1</td>
<td>125.45 ± 8.52</td>
<td>130.82 ± 7.50</td>
</tr>
</tbody>
</table>

Table 1—Effects of P. granatum (PG) extract (100, 250 and 500 mg/kg, po) and diazepam (1 mg/kg, ip) on elevated plus maze test in young and aged mice after 60 min of extract administration. [Values are expressed as mean±SE from 10-12 animals in each group]

Statistical analysis was done using ANOVA followed by Scheffe test .
not alter the sleeping latency and sleeping time in young and aged mice.

Effect on tail suspension test—In this test, PG extract in both the doses (250 and 500 mg/kg) significantly ($P<0.05$) decreased the immobility time in both young and aged mice, as compared to respective controls. PG at 100 mg/kg dose failed to decrease the immobility time in mice. On the other hand, the mice treated with imipramine (30 mg/kg), as expected of an antidepressant drug, was able to decrease the immobility time in both young and aged mice (Fig. 3).

Effect on motor activity—In order to check the general locomotor performance of aged and young mice, a 5 min activity test was performed for each mouse. No significant alteration in the total locomotor activity was observed in aged and young mice after 60 min of administration of PG extract.

Antinociceptive activity—The reaction time to nociceptive effect produced by hot-plate or tail-flick test was significantly ($P<0.05$) increased under the administration of PG extract (250 and 500 mg/kg, po) at 60 min or morphine (5 mg/kg, ip) at 60 min. PG extract at 100 mg/kg failed to induce antinociceptive effect at this dose level. The antinociception caused by PG extract was lower compared with morphine in both young and aged mice (Table 2).

Discussion

In the present study, the effects of ethanolic extract of *P. granatum* seeds were studied in several behaviour animal models such as elevated plus maze test, barbiturate-induced sleeping time, motor activity test, tail suspension test, hot-plate and tail-flick test to investigate its possible central activity in both young and aged mice. These tests are classical models for screening central nervous system (CNS) actions providing information about anxiolytic, psychomotor

Table 2—Effects of *P. granatum* (PG) extract (100, 250 and 500 mg/kg, po) and morphine (5 mg/kg, ip) on tail-flick and hot-plate test in young and aged mice after 60 min of extract administration

<table>
<thead>
<tr>
<th>Treatments (mg/kg)</th>
<th>Reaction time (Seconds)</th>
<th>Tail-flick</th>
<th>Hot-plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Young</td>
<td>Aged</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>8.0 ± 1.80</td>
<td>7.62 ± 2.06</td>
</tr>
<tr>
<td>PG 100</td>
<td></td>
<td>8.23 ± 1.55</td>
<td>9.97 ± 2.0</td>
</tr>
<tr>
<td>PG 250</td>
<td>12.893 ± 1.07ª</td>
<td>13.66 ± 1.39ª</td>
<td></td>
</tr>
<tr>
<td>PG 500</td>
<td>13.22 ± 0.69ª</td>
<td>13.85 ± 1.05ª</td>
<td></td>
</tr>
<tr>
<td>Morphine 5</td>
<td>15.00 ± 0.75ª</td>
<td>14.12 ± 1.12ª</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis was done using ANOVA followed by Scheffe test. $^aP<0.05$ vs vehicle control mice.
performance, myorelaxant activity, anti-depressant and analgesic activity. The results of the present study are in agreement with earlier reports that antioxidant flavonoids attenuate oxidative stress induced brain disorders in animals. In light of this, it is interesting to note that pomegranate juice has a significant antioxidant capacity. Glutathione is a well-characterized reactive nitrogen and oxygen species scavenger in the brain, and catechins (another constituent of pomegranate juice) have been shown to increase intracellular glutathione levels and modulate glutathione peroxidase activity. Although these compounds (interestingly present in dark chocolate as well) possess potent antioxidant capacities, they also increase superoxide dismutase activity and have been shown to protect against brain lipid peroxidation. Despite the widespread traditional use of PG for treating various disorders, there are very few scientific evaluations of its CNS activities. The present work demonstrated that the ethanolic extract of PG has anxiolytic effects in both young and aged mice. This action of PG extract represents the functional similarity to benzodiazepines which are widely used as anxiolytic agents. The benzodiazepines are known to act through the BZD-GABA receptors. The role of GABA in anxiety is well established. The mechanism of anxiolytic action of PG extract may involve an action on GABAergic transmission; however, further studies are needed to ascertain this.

Earlier reports on the chemical constituents of plants and their pharmacology suggest that plant containing flavonoids, saponins, and tannins possess activity against many CNS disorders. Phytochemical tests of ethanolic extract of PG seeds revealed the presence of flavonoids, tannins, and saponins. It is possible that the mechanism of anxiolytic action of PG extract could be due to the binding of any of these phytochemicals to the GABA\(_A\)-BZDs complex.

Different doses of PG extract and imipramine in mice were able to induce antidepressant effect in tail suspension test (Fig. 3). The tail suspension test is considered one of the most widely validated tests for assaying new antidepressant agents. This was supported by other workers who found that flavonoids and tannins were found to have antinociceptive and antioxidant activity. Pentobarbital induced sleeping time test was also used to evaluate the possible antidepressive-like effects observed with PG extract in this study (Fig. 2). Increase in sleep latency and decrease in sleeping time are classically related to CNS stimulant drugs. The antinociceptive effect of PG extract and morphine was assessed by the use of two common tests, tail-flick, specific for spinal reflex and hot-plate, which reflects a more complex, centrally integrated process. Both PG extract and morphine showed antinociceptive activity (Table 2). The PG extract may involve an action on opioid receptors; however, further studies are needed to ascertain this mechanism. The antinociceptive activity of PG extract (500 mg/kg) was almost equivalent to that of morphine in mice at spinal and supra spinal level.

In conclusion, the earlier reports and the CNS activities demonstrated by the ethanolic extract may be due to the presence of antioxidants that were present in the extract. Further investigations of the mechanism(s) of action of the plant extract, and the active substance(s) responsible for its biological actions, are necessary.

References