

## *Panchagavya Ghrita*, an Ayurvedic formulation attenuates seizures, cognitive impairment and oxidative stress in pentylenetetrazole induced seizures in rats

Joshi R<sup>1</sup>, Reeta KH<sup>1</sup>, Sharma SK<sup>3</sup>, Tripathi M<sup>2</sup> & Gupta YK<sup>1\*</sup>

<sup>1</sup>Neuropharmacology Laboratory, Department of Pharmacology; <sup>2</sup>Department of Neurology,  
All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India

<sup>3</sup>Department of AYUSH, Ministry of Health and Family Welfare, Government of India, India

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*Panchagavya Ghrita* (PG), according to Ayurvedic formulary of India (AFI), is used to treat epilepsy (*apasmara*), fever (*jvara*), mania (*unmade*) and jaundice (*kamala*). In the present study, we examined its effect on convulsions, oxidative stress and cognitive impairment in pentylenetetrazole (PTZ) induced seizures in rats. PG @ 250, 500, 1000, 2000 and 4000 mg/kg was administered orally for 7 days to male Wistar rats. On day 7, PTZ (60 mg/kg) was injected intraperitoneally 2 h after the last dose of PG. Sodium valproate (300 mg/kg) was used as positive control. Latency to myoclonic jerks, clonus and generalized tonic clonic seizures (GTCS) were recorded for seizure severity. Cognitive impairment was assessed using elevated plus maze and passive avoidance tests. Malondialdehyde and reduced glutathione levels were measured in rat brain. The results have shown that pretreatment with PG @ 500, 1000, 2000 and 4000 mg/kg exhibited 16.6, 33.3, 50 and 100% protection against occurrence of GTCS. The pretreatment with PG has significantly improved cognitive functions and the oxidative stress induced by seizures demonstrating its protective effect against PTZ induced seizures, and further, use of PG as an anticonvulsant in Ayurvedic system of medicine.

**Keywords:** Anticonvulsant, Antiepileptic drugs, Clonus, Convulsions, Epilepsy, GTCS, PTZ

Epilepsy, a chronic neurological disorder, is characterized by recurrent seizures and requires long-term treatment with antiepileptic drugs (AEDs). The objective of AEDs therapy for epilepsy is freedom from seizures with minimal side effects. However, in AED therapy, one third of the patients remain unresponsive to treatment<sup>1</sup>. Cognitive impairment is an important issue associated with long-term use of AEDs. In addition, oxidative stress has been implicated in the pathogenesis and progression of seizures, which adds to the cognitive deficit in patients with epilepsy<sup>2</sup>. Furthermore, 30% of the patients are refractory to AEDs, which leads to increased morbidity and overall reduced quality of life<sup>3</sup>. Hence, there is a need to develop novel drugs with varied mechanisms of action, better efficacy and lesser side effects.

Complementary/alternative medicines (CAM) have been used since ancient times for the treatment of epilepsy. Ayurvedic medicines account for 43% of

patients on monotherapy and 38% in combination with other CAM therapies in epilepsy<sup>4</sup>. *Panchagavya Ghrita* (PG) is an Ayurvedic medicine mentioned in *Charaka Samhita* for the treatment of epilepsy. It contains five components viz. cow milk, ghee and curd from cow milk, cow urine and fresh cow dung juice in equal proportions. Traditionally, it is claimed to be useful against mania, epilepsy, fever and jaundice<sup>5</sup>. Hepatoprotective<sup>6</sup> and immunomodulatory activity<sup>7</sup> of PG has also been demonstrated. The antioxidant<sup>8</sup> and anticancer<sup>9</sup> activity of cow urine have been reported already. The anticonvulsant effect of some polyherbal ayurvedic formulations containing PG as one of the components had also been demonstrated against experimental model of seizures<sup>10</sup>. Here, we explored the effect of PG on seizures and seizure-induced oxidative stress and cognitive impairment in PTZ experimental model of seizures to substantiate the claims made by traditional healers.

### Materials and Methods

**Animals**—Male Wistar rats (150-200 g) obtained from the Central Animal Facility of the All India Institute of Medical Sciences, New Delhi, India, were

Correspondence:

Ph: +91 11 26593282, 26593684; Fax: +91 11 26588663

E-mail: yk.ykgupta@gmail.com (YKG);

rupajoshi@iims@gmail.com (RJ)

housed in polyacrylic cages and maintained under standard laboratory conditions with a natural dark and light cycle. The standard dry rat pellet diet and tap water was provided *ad libitum*. The rats were deprived of food 12 h before behavioral testing, as this is known to enhance their motivation to perform the test<sup>12</sup>. The study protocol was approved by the Institutional Animal Ethics Committee (568/IAEC/2010 dated 12<sup>th</sup> November, 2010).

**Materials—Panchagavya Ghrita (PG)** manufactured by *Arya Vaidya Sala*, Kottakkal, Kerala, India, was purchased from their pharmacy outlet in Delhi. The details of the medicine included batch no. 140563, date of manufacturing-May 2011, date of expiry-May 2013 and manufacturing licence no. 1/25D/76. Each 10 g of PG constituted 10.5 ml each of *Ghritam* (cow ghee), *Gomaya svarasar* (fresh cow dung), *Gomutram* (cow urine), *Dadhi* (curd from cow milk) and *Ksiram* (cow milk). The formulation is prepared and standardized as per method described in Ayurvedic Formulary of India (AFI)<sup>13</sup>. This light yellow colored semi-solid preparation was melted by heating in water bath (35-37 °C) before administration to rats. The prescribed dose of PG as mentioned in the AFI<sup>13</sup> is 12 g daily in divided doses. Different studies on PG have used a wide range of doses, 100 mg to 4000 mg/kg<sup>10,11</sup>. In the present study, 500 mg/kg dose of PG was selected to evaluate its effect on PTZ induced seizures. Since, some protective effect was observed with this dose, a lower dose (250 mg/kg) and higher doses were selected to study its dose dependent effect. Sesame oil purchased from local market was used as vehicle control<sup>11</sup>. Pentylenetetrazole (PTZ), 5'5-dithiobis (2-nitrobenzoic acid) (DTNB) and reduced glutathione (GSH) were purchased from Sigma (Sigma Chemical Co., USA). Sodium valproate was used as the standard drug. All other reagents were of analytical grade and were obtained from Qualigens, India. Fresh drug solutions were prepared on each day of experimentation. Drugs/vehicles were given orally by gavage in a volume not greater than 1.0 ml/100 g body weight.

**Experimental groups**—Animals were randomly divided into 10 groups each containing 6 animals. Group I served as normal control where no active treatment was given. In group II, PTZ was injected @ 60 mg/kg, intraperitoneally (i.p.). Group III received vehicle (sesame oil) @ 2 ml/kg. Group IV served as positive control and received single dose of valproate (300 mg/kg, i.p.). Groups V to IX were

given PG orally @ 250, 500, 1000, 2000 mg/kg once daily and 4000 mg/kg in two divided doses for 7 days, respectively. On the 7<sup>th</sup> day, PTZ was injected 2 h after the last dose of PG/vehicle and 30 min after valproate to group III. Group X served as *per se* group where only PG was administered orally @ 4000 mg/kg.

**Experimental induction of seizures**—Pentylenetetrazole (PTZ) solution was prepared afresh in normal saline. On 7<sup>th</sup> day, sodium valproate and PG was administered 30 min and 2 h, respectively before seizure induction by PTZ (60 mg/kg, i.p.). This dose of PTZ had been standardized earlier in our laboratory as the dose that produces convulsions in all the rats without causing any mortality<sup>14</sup>. The latency to myoclonic jerks and the incidence of generalized tonic-clonic seizures (GTCS) with loss of righting reflex were noted. Animals were observed for 30 min after PTZ challenge.

**Cognitive function assessment**—Assessment of cognitive functions by elevated plus maze and passive avoidance tests were performed before induction of seizures. Seizures were induced after 2 h of PG administration. The behavioral parameters were done again 24 h after the seizure induction by PTZ.

**Elevated plus maze test**—Acquisition and retention of memory processes were evaluated as described previously<sup>15</sup>. The elevated plus maze (EPM) consists of two closed arms and two open arms forming a cross, with a quadrangular center, placed 50 cm above the floor. The rat was placed at the end of one open arm facing away from the central platform and the time that elapsed between placement of the rat on plus maze and crossing of its four limbs in the enclosed arm was recorded as transfer latency. In this experiment, if the rat did not enter the enclosed arm in 60 s, it was gently pushed on the back into the enclosed arm and the transfer latency was noted as 60 s. The rat was allowed to move freely in the plus maze regardless of open and closed arms for 10 s after measurement of transfer latency. The retention transfer latency was measured 24 h after seizures induction.

**Passive avoidance test**—Memory retention was evaluated with passive avoidance apparatus (Ugo Basile, Italy) according to the method described earlier<sup>16</sup>. The passive avoidance apparatus had two separate chambers connected by a guillotine door. One chamber was lit using a bulb, and the other was dark. The floor in both the chambers consisted of steel grids, used to deliver electric shocks. On the

acquisition trial, each rat was placed in the lighted chamber. After 30 s of habituation, the guillotine door separating the light and dark chambers was opened, and the initial latency to enter the dark chamber was recorded. Immediately after the rat entered the dark chamber, the guillotine door was closed and an electric foot shock (75 V, 0.2 mA, 50 Hz, 0.2 s) was given to the floor grids for 0.2 s. The rat was removed from the dark chamber 5 s later and returned to its home cage. The retention latency was measured in the same way as in the acquisition trial, but the foot shock was not given.

**Biochemical estimations**—After the behavioral assessment, animals were rapidly decapitated under ether anesthesia and the brains were dissected out. The whole brain of each rat was used to assess biochemical parameters by preparing 10% (w/v) homogenate with ice-cold 0.1 M phosphate buffer (pH 7.4).

**Reduced glutathione (GSH) levels**—Reduced glutathione was measured according to the method of Ellman<sup>17</sup>. The homogenate was mixed with equal quantity of 10% trichloroacetic acid and centrifuged to precipitate out the proteins. To the 100  $\mu$ l of protein free supernatant thus obtained, 2 ml of 0.3 M phosphate buffer (pH 8.4), 0.5 ml of 0.04% DTNB in 1% tri-sodium citrate and 0.4 ml of double distilled water were added in succession. A parallel standard GSH was run to determine the concentration of GSH in test samples. The absorbance was read in a spectrophotometer at 412 nm within 15 min. The concentration of reduced glutathione is expressed as  $\mu$ g/g-wet tissue.

**Lipid peroxidation levels**—Lipid peroxidation was assessed using malondialdehyde (MDA), as an indicator<sup>18</sup>. About 1.5 ml of 20% (v/v) acetic acid

(pH 3.5), 1.5 ml of 0.8% (w/v) of thiobarbituric acid and 0.2 ml of 8.1% (w/v) of sodium dodecyl sulphate were added to 0.1 ml of brain homogenate, and then heated at 95°C for 60 min. After cooling the mixture, 5 ml of n-butanol/pyridine (15:1) was added and vortexed. The organic layer was separated by centrifugation at 4000 rpm for 10 min and its absorbance was measured at 532 nm using a double beam spectrophotometer. The concentration of MDA is expressed in nmol/g-wet tissue.

**Statistical analysis**—Data were expressed as means  $\pm$  SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. The *P* value <0.05 were considered as significant. All statistical analyses were performed using the SPSS statistical software package, version 16.0.

## Results

**Pentylentetrazole-induced seizures**—Pretreatment with PG produced significant dose-dependent protection against PTZ-induced seizures. PG @ 500, 1000, 2000 and 4000 mg/kg significantly increased latency to myoclonic jerks compared to PTZ group. The latency to clonus and GTCS also increased significantly at all doses whereas duration of GTCS only decreased significantly @ 1000 and 2000 mg/kg. Additionally, at the dose of 4000 mg/kg it showed 100% protection against PTZ-induced GTCS whereas the doses of 500, 1000 and 2000 mg/kg produced 16.6, 33.3 and 50% protection against GTCS, respectively. Sodium valproate provided complete protection against seizures (Table 1).

### Effect on cognitive impairment

**Elevated plus maze**—There was no significant difference in initial transfer latency among the groups,

Table 1—Effect of *Panchagavya Ghrita* (PG) on latency to myoclonic jerks, clonus and generalized tonic clonic seizures (GTCS) and duration of GTCS in PTZ-induced seizures in rats. Sodium valproate (VPA) served as positive control. Sesame oil was used as vehicle control.

Groups	Latency to Myoclonic jerks (s)	Latency to clonus (s)	Latency to GTCS (s)	Duration of GTCS (s)	% protection against GTCS
PTZ (60 mg/kg)	52.6 $\pm$ 2.1	61.7 $\pm$ 3.5	67.7 $\pm$ 3.2	18.3 $\pm$ 2.6	0 (0/6)
Vehicle (2 ml/kg)	53.5 $\pm$ 2.8	60.7 $\pm$ 2.7	67.8 $\pm$ 1.9	19.0 $\pm$ 2.7	0 (0/6)
VPA (300 mg/kg)	-	-	-	-	100** (6/6)
PG 250 mg/kg	66.0 $\pm$ 2.5	81.2 $\pm$ 6.2*	104.0 $\pm$ 4.25	12.7 $\pm$ 2.3	0 (0/6)
PG 500 mg/kg	75.7 $\pm$ 3.8*	109.8 $\pm$ 11.1**	151.0 $\pm$ 11.9***	10.8 $\pm$ 1.1	16.6 (1/6)
PG 1000 mg/kg	88.3 $\pm$ 4.2 **	115.5 $\pm$ 7.2***	190.3 $\pm$ 6.7***	8.25 $\pm$ 1.1*	33.3 (2/6)
PG 2000 mg/kg	104.3 $\pm$ 4.1 ***	121.3 $\pm$ 5.2***	239.0 $\pm$ 6.7***	6 $\pm$ 0.58*	50 (3/6)
PG 4000 mg/kg	155.3 $\pm$ 4.8***	161.8 $\pm$ 3.65**	-	-	100** (6/6)

Data presented as mean  $\pm$  SEM (n=6). \**P* <0.05, \*\**P* <0.01, \*\*\**P* <0.001, as compared to PTZ group. -, Not detectable

whereas significant differences in retention transfer latency was observed between the groups [F (9, 50) = 15.58,  $P < 0.001$ ] as revealed by one way ANOVA. The retention transfer latency was significantly ( $P < 0.001$ ) increased in the PTZ group as compared to vehicle control group, indicating thereby impairment of memory in the PTZ group (Fig. 1A). PG @ 2000 and 4000 mg/kg significantly improved memory retention as shown by decreased retention transfer latency compared to PTZ control group which was also comparable to that of the sodium valproate group.

**Passive avoidance test**—There were no significant differences in initial latency among the groups, whereas retention latency differed significantly [F (9,50) = 36.77,  $P < 0.001$ ]. Post hoc analysis revealed that retention latency was significantly decreased in the PTZ group ( $P < 0.001$ ) as compared to vehicle control. PG pretreatment @ 2000 ( $P < 0.05$ ) and 4000 mg/kg ( $P < 0.001$ ), significantly increased the retention latency in comparison to PTZ group.

Similarly, retention latency in the valproate group also showed significant increase ( $P < 0.001$ ). However, there was no significant difference in retention latency between PG 250, 500 and 1000 mg/kg group and PTZ group (Fig. 1B).

**Biochemical measurements**

**Reduced glutathione levels**—Levels of reduced glutathione (GSH) differed significantly between the groups [F (9, 50) = 34.05,  $P < 0.001$ ]. They were significantly decreased in the PTZ group ( $P < 0.001$ ) as compared to vehicle control group. PG pretreatment reversed the PTZ-induced decrease in GSH levels @ 2000 and 4000 mg/kg. However, there was no significant difference between PG treated groups @ 250, 500 and 1000 mg/kg as compared to PTZ group. The increased GSH level in PG pretreated groups was found to be comparable to that of the valproate group (Fig. 2A).

**Malondialdehyde levels**—There was a significant difference in brain MDA levels between the groups

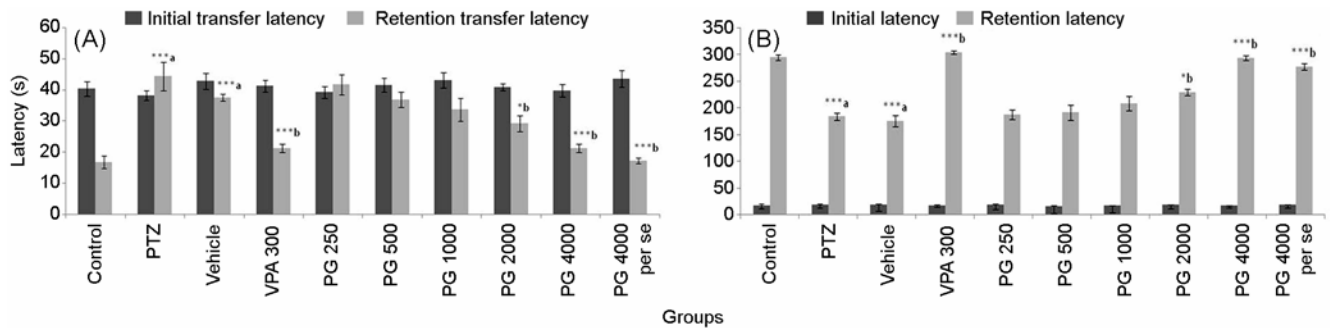


Fig. 1—Effect of *Panchagavya Ghrita* (PG, mg/kg) on initial and retention transfer latencies in the (A) elevated plus maze test; and (B) passive avoidance test in the pentylenetetrazole (PTZ, mg/kg) seizure model. Sodium valproate (VPA) served as positive control. Sesame oil was used as vehicle control (2 ml/kg). [Data represent mean  $\pm$  SEM (n=6). \* $P < 0.05$ , \*\*\* $P < 0.001$ ; a, as compared with the normal control group; and b, as compared with the PTZ group].

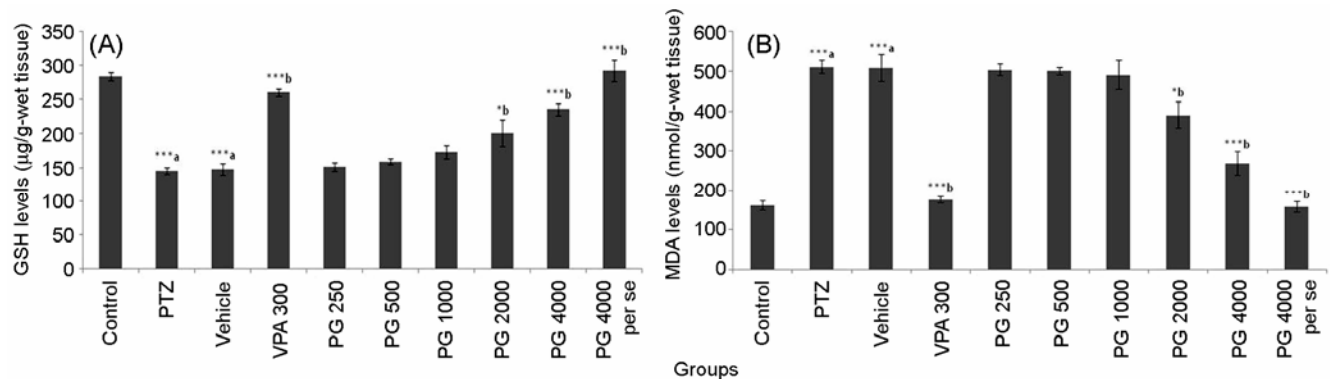


Fig. 2—Effect of *Panchagavya Ghrita* (PG, mg/kg) on whole-brain (A) GSH levels; and (B) MDA levels in the pentylenetetrazole (PTZ, 60 mg/kg) induced seizures in rats. Sodium valproate (VPA) served as positive control. Sesame oil was used as vehicle control (2 ml/kg). [Data represent mean  $\pm$  SEM (n=6), \* $P < 0.05$ , \*\*\* $P < 0.001$ ; a, as compared to normal control group; and b, as compared with the PTZ group].

[ $F(9,50) = 45.85, P < 0.001$ ]. There was a significant increase in MDA levels in the PTZ group as compared to the control group. However, pretreatment with PG significantly reduced the increased MDA levels @ 2000 and 4000 mg/kg as compared to PTZ group and the values were found to be comparable to that of the sodium valproate group (Fig. 2B). There was no significant reduction in the MDA levels in PG treated groups @ 250, 500 and 1000 mg/kg.

### Discussion

Antiepileptic drugs are the mainstay for treatment of epilepsy. Unfortunately, seizures in up to 30% do not respond to them. This treatment gap has motivated investigations into the traditional ayurvedic formulations to treat seizures. In the present study, the anticonvulsant effect of PG was investigated using PTZ induced seizure model in rats. PG exhibited dose-dependent protection against PTZ-induced seizures. PG @ 4000 mg/kg produced complete (100%) protection against GTCS. It also improves seizures induced cognitive impairment and oxidative stress in rats.

*Panchagavya Ghrita* contains cow milk, clarified butter and curd from cow milk, cow urine, and fresh juice of cow dung<sup>13</sup>. Cow milk contains carbohydrate, calcium chloride, casein, phosphorus, sodium citrate, iron, vitamins A, carotene and riboflavin<sup>19</sup>. Cow urine has sulphur, ammonia, copper, iron, urea, uric acid, sodium, potassium, magnesium, calcium, vitamins A-E, lactose, enzymes and creatine. Clarified butter from cow milk contains phospholipids, diglycerides, triglycerides and beta carotene with known antioxidant properties<sup>11</sup>. In addition, Sayyah *et al.*, had demonstrated the antiepileptic activity of beta carotene and vitamin A in PTZ kindling model of epilepsy in mice<sup>20</sup>. Further, cow urine which contains purine derivatives<sup>21</sup> is known to play a role in seizures<sup>22</sup>.

Association of cognitive impairment with seizures is well documented<sup>23,24</sup>. In the present study also, seizure induced cognitive impairment was observed in PTZ control group. PG pretreatment significantly ameliorated the cognitive impairment induced by seizures. These observations are well in alignment with the earlier studies on herbal drugs with antioxidant potential in ameliorating the cognitive impairment associated with seizures by reducing lipid peroxidation in rat models of seizures<sup>25,26</sup>

The role of oxidative stress in epilepsy is now well established. Seizure activity increases the levels of free radicals and decreases the antioxidant defense mechanisms in the brain<sup>27</sup>. Imbalance between oxidant and antioxidant system in rat brain was also observed in the present study which was reversed by pretreatment with PG. Neuroprotective effect of medicinal plants/Ayurvedic preparations with antioxidant property<sup>26,28</sup> including the *in-vitro* antioxidant activity of PG have been already reported<sup>29</sup>. Similarly, presence of volatile fatty acids in cow urine and its possible role in the antioxidant potential has also been discussed by many<sup>9,30,31</sup>. Usage of cow urine concoction in treatment of convulsions is reported from Nigeria<sup>32</sup>. These existing reports explain the antioxidant potential of PG as observed in this study in preventing seizures, seizures associated cognitive impairment and oxidative stress.

Since epilepsy is a chronic disorder, patients use multiple complementary/alternative medicines (CAM) for its treatment. The present study has authenticated the PG as a potential anticonvulsant agent and hence scientifically approves its use in the treatment of epilepsy, with the additional benefit of protecting against seizure-associated cognitive impairment and oxidative stress.

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### Conflict of interest

None of the authors have any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this study is consistent with those guidelines.

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