

**Research Paper**

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ISSN 0189-6016©2008PROTECTIVE EFFECTS OF *PUNICA GRANATUM* SEEDS EXTRACT AGAINST AGING AND SCOPOLAMINE INDUCED COGNITIVE IMPAIRMENTS IN MICE**Sokindra Kumar^{1*}, Kamal Kishore Maheshwari², Vijender Singh³**^{1,3} Department of Pharmacy, Ram-Eesh Institute of Vocational & Technical Education, 3-Knowledge Park I, Kasna Road, Greater Noida, UP, India 201306.² Department of Pharmacy, MJP Rohilkhand University, Bareilly, UP, India.***Email:** sokindra_kumar@yahoo.co.in**Abstract**

Dementia is one of the age related mental problems and characteristic symptom of various neurodegenerative diseases including Alzheimer's disease. This impairment probably is due to the vulnerability of the brain cells to increased oxidative stress during aging process. Many studies have shown that certain phenolic antioxidants attenuate neuronal cell death induced by oxidative stress. The present work was undertaken to assess the effect of ethanolic extract of *Punica granatum* seeds on cognitive performance of aged and scopolamine treated young mice using one trial step-down type passive avoidance and elevated plus maze task. Aged or scopolamine treated mice showed poor retention of memory in step-down type passive avoidance and in elevated plus maze task. Chronic administration (21 days) of *Punica granatum* extract and vitamin C significantly ($p < 0.05$) reversed the age induced or scopolamine induced retention deficits in both the paradigms. *Punica granatum* extract also significantly lowered lipid peroxidation level and increased antioxidant glutathione level in brain tissues. *Punica granatum* preparations could be protective in the treatment of cognitive disorders such as dementia and Alzheimer's disease.

Key words: *Punica granatum*, Cognitive deficits, Vitamin C, Scopolamine, Antioxidants**Introduction**

With an increasing aging population, Alzheimer's disease (AD) represents a significant healthcare issue, which is likely to gain in prevalence. AD is a chronic neurodegenerative disorder characterized by progressive memory loss, diminished cognitive ability and behavioral disturbances with advancing age (Foster *et al.*, 1994). Recent reports suggest the involvement of free radicals in the pathophysiology of AD. 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 (Brewer, 1998; Sohal *et al.*, 1990). In AD, β -amyloid peptide has been implicated in oxidative stress and free radical production (Pappolla *et al.*, 1997; Hardy and Higgins, 1992; Suh, 1997). Furthermore, oxidative stress appears to mediate β -amyloid peptide toxicity by free radical production, suggesting a pathophysiological link between β -amyloid peptide and imbalance between reactive oxygen production and protective system.

Several studies have shown that plant derived flavonoids exhibit variety of biological properties (Hollman *et al.*, 1996). It has been demonstrated that certain phenolic antioxidants attenuate neuronal cell death induced by oxidative stress (Schroeter *et al.*, 2000). Many studies suggest that supplementation with antioxidants may delay the development of Alzheimer's disease (Stocker, 1994). *Punica granatum* (Pomegranate) is an ancient fruit with exceptionally rich ethnomedical applications. The phytochemical analysis of an ethanolic extract from *Punica granatum* seeds has demonstrated the presence of a wide variety of constituents such as flavonoids, glycosides,

tannins, anthocyanins, ascorbic acid (Cerdea *et al.*, 2003; Prez-Vicente *et al.*, 2002; Noda *et al.*, 2002). Many studies have shown that Pomegranate is a powerful antioxidant (Chidambara Murthy *et al.*, 2002; Plumb *et al.*, 2002; Seeram *et al.*, 2005), anti-inflammatory (Ahmed *et al.*, 2005), antidiabetic (Huang *et al.*, 2005), and neuroprotective (Loren *et al.*, 2005). Earlier we have shown the antidepressant effect of pomegranate seeds extract (Sokindra *et al.*, 2007)

In the current experiment, we studied the effect of chronic ethanolic extract of *Punica granatum* on cognitive parameters of young and aged mice. Learning and memory parameters in these mice were evaluated using one trial step-down type passive avoidance task and elevated plus-maze test. Oxidative stress namely, malondialdehyde (measure of lipid peroxidation) and the antioxidant glutathione level in brains of mice were analyzed following the behavioral paradigms. Results suggest a protective effect of pomegranate extract and its possible use in the treatment of cognitive disorders.

Material and Methods

Plant material and extraction

Seeds of *Punica granatum* were purchased from herbal drug store, New Delhi and plant material was identified and authenticated at Department of Pharmacognosy, Hamdard University, New Delhi. A voucher specimen (2204) was deposited in the department. The plant material was extracted successively with petroleum ether, chloroform followed by ethanol in Soxhlet extractor. The ethanol extract was evaporated under reduced pressure to give an average yield of 35% w/w. The extract was then stored in a desiccator. Phytochemical screening was done in accordance with standard protocol.

Isolation and purification of compounds

A sample (200 g) of extract was subjected to column chromatography (Silica Merck 60-100 mesh, 120 X 5 cm) and was eluted with a series of gradient of solvents in increasing polarity order. Each dried fraction was analyzed by various spectroscopic techniques. More details regarding the methodology of isolation and chemical identification of the active compounds of *Punica granatum* will be published elsewhere.

Animals

Swiss albino mice of either sex of 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 five per cage, with free access to food and water, and maintained under standard laboratory conditions. 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 09:00 and 18:00 h.

Drugs and treatment schedule

Vitamin C (Loba chemicals, India), Scopolamine (Merck, USA), Thiobarbituric acid (Glaxo, India), DTNB (Sigma-Aldrich, St. Louis, USA), Trichloro acetic acid and Sodium dodecyl sulfate (CDH, India).

To assess the effect of chronic treatment of ethanolic extract of *Punica granatum*, the following drug regimens were employed. The aged mice were randomly distributed into four groups. The first group of animals received only vehicle treatment perorally for a period of 21 days. Subsequent three groups of animal received varying doses of pomegranate extract (250 and 500 mg/kg perorally once a day) for a period of 21 days. The extract was suspended in 5 % tween 80. The last group received vitamin C (250 mg/kg, p.o.) as a standard drug. Similar drug treatment was also performed in different groups of young mice. In young animals, amnesia was induced by administration of scopolamine (1 mg/kg, i.p.) on 21st day and the Step down latency (SDL) and Transfer latency (TL) recorded. Retention was recorded after 24 h. On 21st day, after 60 mins of administration of doses, scopolamine was administered and SDL and TL noted after 30 mins. On the next day (day 22), animals were tested for their retention using passive avoidance and elevated plus maze task. After recording the cognitive parameters, mice were sacrificed and their brains were immediately processed for biochemical estimations.

Passive Avoidance Test

A step-down type passive avoidance test apparatus was used to evaluate the effects of pomegranate extract on learning and memory as described by Reddy and Kulkarni (1998) and Raghavendra *et al* (1999). The apparatus consisted of an electric grid with a centrally located shock free platform (10 X 7 X 30 cm). During the training session, each mouse was gently placed on platform, as the mouse turned down the platform foot shock (2mA) was delivered for 2 seconds. The mouse was removed from the enclosure immediately after receiving the shock to their respective home cages. The Step down latency (SDL) was recorded from the time the mouse was placed on platform until it stepped down to the platform. The retention test was carried out 24 h after training, in the similar manner, except that the electric shocks were not applied to grid floor. Short latencies indicate poor retention compared to significantly longer latencies.

Elevated Plus Maze Test

Acquisition and retention of memory was evaluated by using the elevated plus maze learning task, which measures spatial long-term memory (Reddy and Kulkarni, 1998). Transfer latency (TL) (the time in which the animal moves from the open arm to the enclosed arm) was utilized as an index of learning and memory processes. The procedure was basically identical to that described by Itoh *et al.* (1991). The elevated plus maze consisted of two open arms (16 X 5 cm) and two enclosed arms (16 X 5 X 12 cm) with an open roof. The maze was elevated to a height of 25 cm from the floor. The animals were placed individually at the end of either of the open arms and the transfer latency was noted on the first day. To become acquainted with the maze, the animals were allowed to explore the plus maze for 20 seconds after reaching the closed arm. On the second day, 24 h after the first exposure, transfer latency was again noted. A long latency period to reach enclosed arm indicates poor retention compared to significantly shorter latencies.

Locomotor activity test

The animal's locomotor behavior was monitored using actophotometer (Inco, India). Before subjecting the animals to cognitive tasks, they were individually placed in actophotometer and the ambulatory activity registered for five-minute period. The locomotor activity was expressed in terms of total photobeam count/5 minute per animal

Measurement of lipid peroxidation

MDA which is a measure of lipid peroxidation was measured as described by Ohkawa *et al* (1979). Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 sodium phosphate buffer (pH 7.4). The reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulfate (8.1%) were added to 0.1 ml of processed tissue sample. The mixture was then heated at 100 °C for 60 mins. The mixture was cooled with tap water and 5 ml of n-butanol: pyridine (15:1 % v/v), and 1 ml of distilled water were added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 mins, the organic layer was withdrawn and absorbance was measured at 532 nm using spectrophotometer. 1, 1, 3, 3-Tetramethoxypropane was used to prepare the standard curve.

Measurement of glutathione

Glutathione was estimated according to the method of Ellman (1959) with slight modification. Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 sodium phosphate buffer (pH 7.4). This homogenate was then centrifuged with 5 % trichloroacetic acid to centrifuge out the proteins. To 0.1 ml of this homogenate, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5'5' dithiobis (2-nitrobenzoic acid) (DTNB) and 0.4 ml of double distilled water was added. The mixture was vortexed and the absorbance read at 412 nm within 15 mins.

Statistical analysis

Results are expressed as means \pm S.E.M. The data were analyzed using one-way ANOVA followed by Dunnet's test. The criterion for statistical significance was $p < 0.05$.

Results

Effect of pomegranate on passive avoidance performance of aged and scopolamine induced amnesia in young mice

Vehicle treated aged mice showed significant ($p < 0.05$) decrease in step down latency (SDL) on second day in comparison to young mice, indicating age related dementia in aged mice. Chronic administration (21 days) of pomegranate extract (250 and 500 mg/kg, p.o.) and vitamin C (250 mg/kg, p.o.) significantly increased the SDL on second day (Figure 1A). Scopolamine (1mg/kg, i.p.) significantly ($p < 0.05$) decreased the SDL (second day) in young mice. Scopolamine induced cognitive deficit in second day was significantly reversed by pomegranate extract in young mice (Figure 1 B)

Effect of pomegranate on elevated plus maze performance of aged and scopolamine induced amnesia in young mice

Vehicle treated young mice exhibited short transfer latency (TL) into enclosed arms on second day compared with vehicle treated aged mice (data not shown), indicating poor retention ability of aged mice. Chronic treatment of pomegranate extract and vitamin C significantly ($p < 0.05$) decreased the TL in aged and scopolamine treated young mice compared with respective controls (Figures 2A, 2B).

Table 1: Effect of chronic administration of *Punica granatum* (PG) extract (250 and 500 mg/kg) and vitamin C (250 mg/kg) on Lipid peroxidation and Glutathione level in aged mice and in young mice. Values are expressed as mean \pm S.E.M. # $p < 0.05$ Vs vehicle treated mice, n = 8-10 animals.

Treatment (mg/kg)	Lipid peroxidation (nmol/mg protein)	Glutathione (nmol/mg protein)
Young mice		
Vehicle	0.24 \pm 0.03	2.96 \pm 0.06
PG 250	0.19 \pm 0.04	2.88 \pm 0.07
PG 500	0.18 \pm 0.03	3.08 \pm 0.08
Vitamin C	0.20 \pm 0.04	3.12 \pm 0.08
Aged mice		
Vehicle	0.36 \pm 0.04	1.80 \pm 0.03
PG 250	0.22 \pm 0.03 [#]	2.28 \pm 0.04 [#]
PG 500	0.18 \pm 0.03 [#]	2.49 \pm 0.04 [#]
Vitamin C	0.19 \pm 0.04 [#]	2.41 \pm 0.30 [#]

Effect on lipid peroxidation level

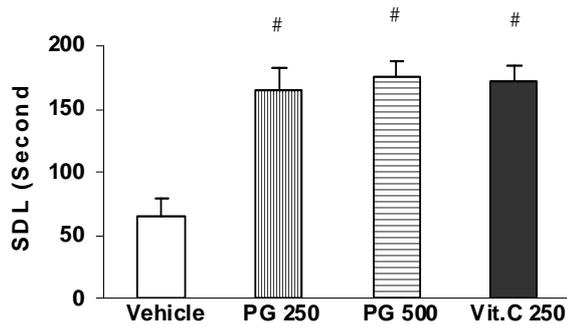
The MDA level was estimated on day 22. The vehicle treated aged mice showed significantly ($p < 0.05$) rise in level of MDA as compared to vehicle treated young mice. Chronic treatment of pomegranate extract (250 and 500 mg/kg, p.o.) and Vitamin C (250 mg/kg, p.o.) significantly decreased the MDA level in aged mice as compared to vehicle treated aged mice (Table 1). In young mice, no significant effect on MDA level was observed.

Effect on glutathione level

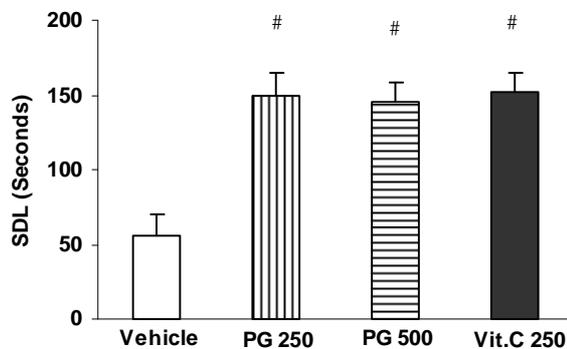
Chronic administration of pomegranate extract (250 and 500 mg/kg, p.o.) and Vitamin C (250 mg/kg, p.o.) significantly ($p < 0.05$) increased the glutathione level in aged mice as compared to vehicle treated aged mice. In young mice, no significant effect on glutathione level was observed (Table 1).

Effect on locomotor activity

In order to check the possible interference of general sensorimotor function, and motor behavior during cognitive tasks, mice were tested with an actophotometer. There was no significant effect on locomotor activity observed in any animal (data not shown)



A



B

Figure 1: Effect of chronic administration of *Punica granatum* (PG) extract (250 and 500 mg/kg) and vitamin C (Vit.C 250 mg/kg) on Step down latency (SDL) in aged mice (A) and in young mice (B). Values are expressed as mean \pm S.E.M.

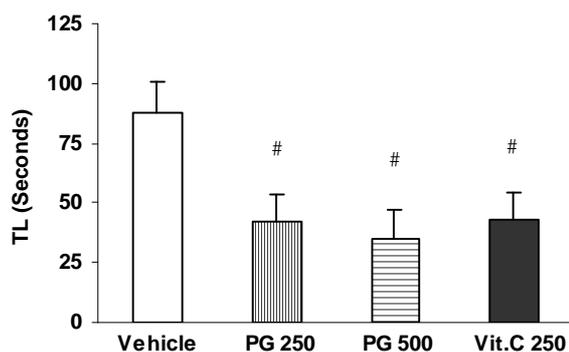
$p < 0.05$ Vs vehicle treated mice, n = 8-10 animals.

Discussion

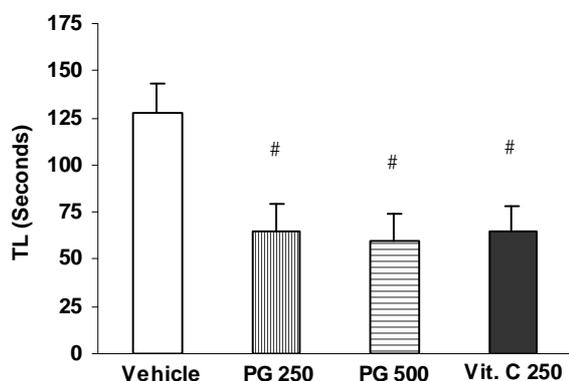
Cognitive dysfunction is one of the most striking age-related impairment seen in human beings and animals. This impairment probably is due to the vulnerability of the brain cells to increased oxidative stress during aging process. Oxidative stress is defined as a cytological consequence caused by a mismatch between the

production of free radicals and the ability to scavenge them (Simonian and Coyle, 1996; Loren et al., 2005). Lipid peroxidation is a measure of free radical generation. The gradual increase in levels of malondialdehyde (MDA) the end product of lipid peroxidation in the brain of aged mice shows an increase in levels of lipid peroxidation. Also, there was a simultaneous decrease in the glutathione levels. Glutathione is an essential tripeptide, and antioxidant found in all animal cells. It reacts with the free radicals and can protect cells from singlet oxygen, hydroxyl radical and superoxide radical damage (Sharma et al., 2000).

Several studies have shown that plant derived flavonoids exhibit variety of biological properties (Hollman et al., 1996). It has been demonstrated that certain phenolic antioxidants attenuate neuronal cell death induced by



A



B

Figure 2: Effect of chronic administration of *Punica granatum* (PG) extract (250 and 500 mg/kg) and vitamin C (Vit.C 250 mg/kg) on Transfer latency (TL) in aged mice (A) and in young mice (B). Values are expressed as mean \pm S.E.M.

[#] $p < 0.05$ Vs vehicle treated mice, $n = 8-10$ animals.

oxidative stress (Schroeter et al., 2000). Many studies suggest that supplementation with antioxidants may delay the development of Alzheimer's disease (Stocker, 1994).

The results of this study clearly indicate that chronic administration of *Punica granatum* seeds extract and vitamin C to aged mice and young mice reverse impairment of memory retention in one trial step down avoidance and elevated plus maze task. Furthermore, chronic treatment of *Punica granatum* and vitamin C also protected the brain cells against increased oxidative stress induced during aging. Considering the fact that the amnesia associated

with increased brain oxidative stress during brain aging and its reversal by antioxidants (Carney et al., 1991; Socci et al., 1995; Clausen and Nielsen, 1989). Our results suggest that the anti-amnesic effect of *Punica granatum* seeds extract in present study could be due to its antioxidant action.

In summary, we show that chronic treatment of *Punica granatum* seeds extract alleviates age-dependent and scopolamine induced cognitive deficit of mice on passive avoidance and elevated plus maze tasks, which could be due to its antioxidant action. Hence, it may be concluded that *Punica granatum* may be protective in cognitive dysfunctions.

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