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Effect of *Punica granatum* peel extract on learning and memory in rats

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ABSTRACT

Objective: To evaluate potential memory enhancing effect of *Punica granatum* peel extract on rats. **Methods:** Healthy adult male albino rats of Wistar strain were used. Each group of 6 rats were administered either distilled water or 50 mg/kg of extract or 100 mg/kg of extract for 15 days and subjected to passive avoidance test or T-maze test. In the next phase rats were administered distilled water or 100 mg/kg of extract for 15 days and the rats were given injection diazepam before subjecting them to the tests. **Results:** The overall performance was better in test groups compared to control groups. Among the test groups, 100 mg/kg rats performed better than 50 mg/kg. The effect on spatial learning parameters like mean number of alternations and mean percentage bias was more marked compared to retention testing parameters like latency. 100 mg/kg Punica extract treated group also improved performance of diazepam treated rats. **Conclusions:** There is a definite trend of memory improvement by *Punica granatum* peel with effects being more marked on spatial learning tendency and long term memory than on retention capacity.

1. Introduction

Pomegranate (*Punica granatum*) is an ancient fruit and a known rich source of bioactive compounds. Although it has been used in the folklore medicine for centuries[1], it is only recently that modern scientists have systematically evaluated the fruit for its various medicinally useful properties. The fact is evident by significantly increased number of published literature on medicinal and nutritional values of the plant in recent times. Different parts of fruit have been successfully evaluated for antiulcer, anthelmintic, anti-inflammatory, diuretic, cardio protective, antidiarrheal, anticancer, antidepressant and also as a retroviral entry inhibitor[2-8]. The notable properties are its profound antioxidant capacity which is among the maximum in plants and is of the level of the green tea and red wine[9]. This antioxidant capacity is attributed to abundance of flavanoids which are known to have beneficial effects on various biological systems and it is the antioxidant property of the fruit which mainly accounts for cardio-protection, improvement in gastric ulcer and enhancement of cognition. In a comparative *in vitro* study of pulp versus peel, the peel was found to possess significantly higher antioxidant capacity than the pulp[10, 11]. Therefore it is reasonable to believe that the peel could be even more beneficial in variety of disorders where oxidative stress has been implicated, including degenerative diseases like Alzheimer's dementia.

This study aims to evaluate potential memory enhancing activity of the peel in normal rats as well as diazepam treated rats.

2. Materials and methods

2.1. Animals

Healthy adult male inbred albino rats of Wistar strain weighing around 200 g at intake were housed 3-4 per cage in temperature and humidity controlled environment under reverse day-night cycle. They were given water *ad libitum* and fed with commercial food pellets. The protocol was approved by institutional animal ethics committee, KMC Manipal on 20th December 2008 and care of the animals was taken as per standard guidelines (IAEC/KMC/19/2008-2009).

2.2. Aqueous extract preparation

The dried coarse pieces of pomegranate peel (Pg) were obtained from local ayurvedic shop and authenticity

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confirmed by a phytochemist. The particles were finely powdered in a mixture followed by filtering to remove large sized remnants. The separated powder was transferred to a round bottom flask and 1.5 liters of distilled water was added and soaked for 2 hours followed by boiling for 3–4 hours. The procedure was repeated twice to obtain the liquid extract. The clear supernatant was evaporated on a water bath and the remaining liquid was dried in a desiccator to obtain a thick paste (yield 16.8%).

2.3. Acute toxicity study

Five groups of 2 rats each were used for the acute toxicity study. The animals were fasted for 24 hours prior to drug treatment. Two rats were given 10 mg/kg of extract orally. After administration, the animals were observed frequently for the next four hours and then after 24 and 48 hours. Irwin's test was conducted where the animals were observed for gross behavioral changes and observed for a period of 48 hours for any toxic effects[12]. The subsequent doses were then increased by a factor of 1.5 on a log scale if the dose was tolerated.

2.4. Experimental protocol

The study was divided into two phases. In phase one, two doses were evaluated for their effects on memory against control and the better performing dose was further tested in diazepam induced amnesia model in phase two. Three groups were used in phase one namely control (distilled water), *Punica granatum* peel extract 50 mg/kg and 100 mg/kg. In phase two, the groups compared were control, control with diazepam and Pg(100 mg/kg) with diazepam. The rats were administered with the oral doses of drug for 15 days before subjecting them to the tests and total volume of fluid administered was kept constant across all the treatment groups. In phase two, diazepam (1 mg/kg ip) was injected 30 minutes before the exploration. All the experiments were conducted between 9 am to 12 am in a noise free, temperature and humidity controlled environment.

2.5. Assessment of learning and memory

2.5.1. Passive avoidance test

The apparatus consists of a rectangular box with 70.0 cm× 12.5 cm grid floor and 17.5 cm high walls. In one wall, there is 7.5 cm ×7.5 cm opening connecting large compartment to a small 25.0 cm×12.5 cm box with dark walls, electrifiable grid floor and removable ceiling. The connection between the two compartments can be closed with a sliding door. Illumination is provided with a 100 W bulb placed 100 cm above the centre of the large compartment. A rat in an open field tends to enter any recesses in the walls and to hide there. When placed in a large box, connected through a narrow opening with a small dark compartment, the rat rapidly finds the entrance to the dark chamber, enters into it and spends most of total exploration time there.

The experiment was performed in 3 stages[13].

1)Exploration: The animal was placed in the center of the large box facing away from the entrance to the small compartment. The door between the 2 compartments was kept open. The rat was allowed to explore the apparatus for 3 minutes and then placed back in the home cage.

2)Learning: The next day, the time when rat entered the small compartment was measured with a stop watch. The sliding door between the two parts was closed and electric shock (1.5 mA, 50 Hz) was applied. The ceiling was opened and the rat was returned to the home cage. Retention was tested after 24 hours.

3)Retention testing: The time when rat entered the small compartment was measured with a stop watch. Maximum 180 seconds were given to a rat to enter the dark compartment. If it doesn't enter, latency value was recorded as 180 seconds and the rat was returned to the home cage.

Usually the parameter measured in passive avoidance test is latency to enter dark compartment on retention. If a rat has remembered a 24 hours old shock, it is likely to remember it during the next 180 seconds it spends in the apparatus and is not likely to enter the dark compartment. In such a case the latency value would be 180 seconds. On the other hand the rat which does not remember the shock is likely to enter the dark compartment at the same time as learning day (which is usually less than 20 seconds). Therefore in a particular group there would be extreme values and usage of mean or even median for statistical analysis would not yield valid results. Therefore another qualitative parameter was designed. That is number of rats in each group exhibiting "memory of shock behavior" The rat was considered to have remembered the shock if it didn't enter dark compartment or immediately came out after entering or sitting at the entrance of light compartment and resisting attempts to close the door. The assumption here is that rats have different ways of expressing their fear of shock. Increase in the mean latency of the group or more number of rats exhibiting "memory of shock behavior" in a group were considered as indices of improved retention.

2.5.2. Spatial learning test (T-Maze test) [14]

The T-maze consists of a stem 35 cm×12 cm and two arms 35 cm×12 cm each, at the ends of which, are the goal areas 15 cm×12 cm each, containing food pellets. The side walls are 40 cm in height. The apparatus is kept in a sound attenuated room. To assess the spatial learning ability, rats were subjected to spontaneous alternation test for four days followed by rewarded alternation tests for four days. Before subjecting them to the test, they were deprived of food for two days to motivate them for food reward with body weight maintained at 85% of pretest and given two days of exploration to acclimatize them to the maze environment.

2.5.3. Spontaneous alternation test

On the following 4 days, 6 trials were given daily. In each trial, the rat was placed in the start area (the end of the stem) with the help of a wooden board. In each trial, the arm chosen by the rat and the number of alternations made were noted. When a rat chooses opposite arms in 2 consecutive trials then one alternation is said to have occurred. The inter trial interval was 1 minute. The rat was deemed to have entered a particular arm when it entered the arm with all four limbs. Percentage bias was calculated for each rat using following formula:

$$\% \text{ bias} = \frac{\text{Total number of choices of more frequently chosen side} \times 100}{\text{Total number trials}}$$

More number of alternations and less percentage bias was considered as an index of improved learning ability.

2.5.4. Rewarded alternation test

This test was done after completion of spontaneous alternation test. Test consisted of 6 trials per day, for 4 consecutive days. Each trail had two runs viz., forced run and choice run. In the forced run, the rat was forced to one of the arms by blocking the other arm and was allowed to consume the pellet there. In the choice run, the forced arm was kept empty and the pellet was placed in the opposite arm. Both the arms were free for the rat to run. Now the rat had to enter the arm opposite to the forced arm if it had to be considered a "correct response". The forced arm was predetermined and it was same for all the rats on a given day and was changed on subsequent days. Percentage of correct response was calculated as:

$$\%\ \text{correct response} = \frac{\text{Total number of choices of correct response} \times 100}{\text{Total number trials}}$$

Increase in the mean % correct response was considered as improved learning capability.

2.5. Statistical analysis

One way analysis of variance(ANOVA) with Bonferroni's post-hoc was used for calculation of variance in all tests and chi-square test for comparison of rats with memory of shock. The analysis was carried out using SPSS 16 for windows (statistical software for social sciences).

3. Results

In acute toxicity study the oral administration of aqueous extract of *Punica granatum* peel produced no mortality up to 1 000 mg/kg dose. However, signs of apathy and mental dullness were observed during first 24 hours of observation at 1 000 mg/kg. The doses for main study were selected as 50 mg/kg body weight and 100 mg/kg body weight based on the acute toxicity studies and doses used in previous studies[5]. The pase 1 passive avoidance results are depicted in Table 1. Exploration performance of all three groups was same with regards to mean latency as well as number of rats with memory of shock. While in retention testing, the mean latency of the extract treated rats were higher as compared to control and also the number of rats exhibiting "memory of shock behavior" was maximum in 100 g/kg Pg treated rats. However, the differences were statistically insignificant ($P>0.05$). The T maze performance in phase 1 is given in Table 2 .The spontaneous alternation test results showed significantly better performance by extract treated rats as compared to control with regard to both mean number of alternations and mean percentage bias ($P<0.05$). In rewarded alternation test, performance increased with increasing dose albeit the difference was statistically insignificant ($P>0.05$).

The passive avoidance behavior in phase two is also shown in Table 1. The exploration performance of all the groups was same with regard to mean latency and number of rats with "memory of shock behavior". During retention testing the mean latency time was significantly higher ($P<0.05$) in rats treated with diazepam and extract as compared to diazepam alone. The diazepam control group showed a lower latency time. The number of rats exhibiting memory of shock behavior was significantly more in test group as compared to control ($P<0.05$). In phase 2 T maze (Table 2), the extract treated group performed significantly better than diazepam control group with regard to mean percentage bias ($P<0.05$). Although the performance of test group was better in terms of mean number of alternations and mean number of correct entries, the difference was found to be insignificant ($P>0.05$).

Table 1

Passive avoidance test results ($n=6$).

	Group	Exploration performance		Retention performance	
		Latency to enter dark compartment (in seconds)	Number of rats with memory of shock	Latency to enter dark compartment (in seconds)	Number of rats with memory of shock
Phase 1	Control	13.83 ± 2.01	0	37.00 ± 28.65	1
	Pg(50 mg/kg)	13.80 ± 3.37	0	89.67 ± 35.09	3
	Pg(100 mg/kg)	13.33 ± 2.47	0	92.67 ± 35.53	5
Phase 2	Control	13.83 ± 2.01	0	37.00 ± 28.65	1
	Diazepam + control	12.30 ± 2.28	0	17.17 ± 4.17	0
	Diazepam + Pg	12.50 ± 1.65	0	65.83 ± 36.11*	3*

Latency analyzed with ANOVA and shock memory with Chi square test, *$P<0.05$ significant vs diazepam control.

Table 2

T- maze test results($n=6$).

	Groups	Total No of trials	Spontaneous alternation performance		Rewarded alternation performance
			Mean no of alternations	Mean percentage of bias	Mean percentage of correct entries
Phase 1	Control	144	11.83 ± 0.63	64.58 ± 0.93	76.39 ± 5.34
	Pg(50 mg/kg)	144	12.00 ± 1.05	61.80 ± 1.99	85.42 ± 4.27
	Pg(100 mg/kg)	144	14.67 ± 0.80*	55.56 ± 2.56*	90.28 ± 3.86
Phase 2	Control	144	11.83 ± 0.63	64.58 ± 0.93	76.39 ± 5.34
	Diazepam+ control	144	9.80 ± 1.64	72.22 ± 3.82	85.42 ± 1.78
	Diazepam+ Pg	144	12.00 ± 0.73	59.72 ± 2.06**	90.28 ± 2.56

*$P<0.05$ signifant vs control, **$P<0.05$ significant vs diazepam control (ANOVA with bonferroni post hoc, $P<0.05$).

4. Discussion

Dementia is a disorder of cognitive impairment affecting mainly memory and also other higher mental functions like language, visuospatial ability, calculation, and judgment etc[15]. Imbalance of oxidative stress and natural antioxidant system is considered to be an important pathophysiological feature of almost all types of dementias including Alzheimer's disease[16]. Oligomeric A-beta, characteristic feature of Alzheimer's disease, confers oxidative insult to neurons and glial cells and initiate changes in synaptic plasticity and this happens long before their deposition to form the amyloid plaques[17]. Therefore antioxidant compounds can potentially halt further damage of neurons and progression of disease. Several rich sources of polyphenols like green tea, blueberry fruit, gingko biloba, grape wine and curcumin have been successfully shown to enhance learning and memory in various animal models. Although their action on learning and memory has been attributed to their antioxidant and anti inflammatory properties, it is becoming recognized that phenolic compounds may also have specific action on intracellular signaling pathways[18].

It is now an established fact that there are multiple types of memory and they are mapped to different anatomical circuits in the brain. Although some characteristics of human memory are lacking in animals, taxonomy of animal memory is fairly similar to the human memory. There are number of evidences showing that nonhuman animals do segment the world into objects and although they can't verbally express, they can display through their behavior that they know about the objects[19, 20].

Different models have been developed to evaluate different components of cognition, which includes models like Morris water maze, Radial maze, elevated plus maze etc. Passive avoidance test is a widely used model for testing retention capacity in animals and has given dependable and reproducible results in screening agents that affect learning and memory[21].

T-maze behavior makes use of the characteristic of animals to explore the environment and change their behavior accordingly. T-maze and also Y-maze and radial maze are among the commonly used models to assess long term memory of animals. Because the correct choice is trial dependant, these mazes also assess spatial working memory. In our study, performances in both the models were better with test groups as compared to control group with regard to almost all the parameters. The drug also reversed the amnesia caused by diazepam. However, the improvement of spatial working memory was more marked among the two components of memory. The possible reason for inability to achieve statistical significance in some parameters can be explained on the basis of smaller sample size per group. The improvement in cognition may be attributed to the profound antioxidant capacity of the peel.

The study demonstrates a definite trend towards cognitive improvement by *Punica granatum* peel. However, larger scale studies using different animal models are required to conclusively determine the amplitude of its effect and its role in specific components and types of memories.

Conflict of interest statement

We declare that we have no conflict of interest.

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