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Evaluating the Mood and Memory-enhancing Effects of *Punicagranatum* Seeds (Pomegranate)Extract using an Animal Model

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Abstract

The research aimed to elucidate the neurological benefits of Punicagranatum seeds (Pomegranate), which have been traditionally touted for their therapeutic properties. To understand their impact, the study systematically examined the seeds' effects on key neurological functions: anxiety, depression, memory, and motor coordination. Through a series of experiments, the seeds were observed to significantly reduce anxiety levels, alleviate depressive symptoms, and enhance memory performance. The efficacy of these seeds might be attributed to their rich content of bioactive compounds, including isoflavones, triterpenes, and antioxidants. Since, there is an increasing demand for natural remedies and a shift towards holistic wellness, the potential therapeutic applications of Punicagranatum seeds cannot be understated. If these promising results can be consistently replicated in more extensive clinical trials, the seeds could be harnessed to develop innovative, natural treatments for neurological disorders.

Keywords: Anxiety, Depression, Memory, *Punicagranatum*, Mice.

1. Introduction

The seeds of *Punicagranatum*(Pomegranate) are known to contain high levels of polyphenols, such as flavonoids, anthocyanin's, hydroxybenzoic acids, hydroxycinnamic acids, and tannins (Venkata *et al*., 2011). Additionally, these seeds serve as rich sources of various fatty acids, particularly unsaturated fatty acids, including linoleic, oleic, palmitic, stearic, linoleic, arachidic, and palmitoleic acids (Elfalleh *et al.*, 2011). Several studies have provided evidence of the diverse health benefits associated with *Punicagranatum* seeds, including in vivo anti-inflammatory, anti-cancer, antimicrobial, and antioxidant properties (Fourati *et al*., 2020). Furthermore, *Punicagranatum* seeds have a notable magnesium content (14mg/100g), which has been observed to function as an N-methyl-D-aspartate (NMDA) receptor blocker, effectively relieving nerve pain and exhibiting a euglycemic effect on diabetes by reducing the occurrence of type 2 diabetes in healthy individuals (Dumlu and Gürkan, 2007). Furthermore, research has indicated that the daily consumption of 400 mg of magnesium oxide can function as a muscle relaxant, potentially easing anxiety by inducing relaxation in patients (Guerra, Volpe and Mao, 2009).

Dementia specifically impairs a person's ability to carry out daily activities, thereby affecting decision-making. To gauge the extent of dementia, it is customary to assess an individual's ability for new learning and memory utilizing specific models. Learning is the initial stage of forming new memories in the brain, built upon past experiences. It encompasses processes like encoding, storing, retrieving, and retaining information in memory, as well as the possibility of forgetting it (Okano *et al*., 2000). Short-term memory pertains to temporarily holding information without the need for repetition, whereas long-term memory involves retaining data over an extended period through repetition. The typical learning process relies on neurotransmitters like dopamine, acetylcholine, and 5-hydroxytryptamine (5HT), which stimulate various brain regions, including the hippocampus, amygdala, and sensory, visual, and auditory cortices of the cerebral cortex(Wiltgen *et al.*, 2006). Additionally, certain anxiolytic and antidepressant medications can influence learning, memory, or induce amnesia.

The primary treatment options for managing dementia are cholinesterase inhibitors and dopamine agonists(O'Luanaigh and Lawlor, 2008). These medications are widely acknowledged as effective approaches. Among various neurodegenerative disorders, Alzheimer's disease (AD) is characterized by progressive dementia, often stemming from the loss of cholinergic neurons or reduced acetylcholine levels. In the management of AD, the primary approach involves the use of acetylcholinesterase (AChE) inhibitors, which work by

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elevating acetylcholine levels in the brain. Herbal remedies have been traditionally preferred in medical practices to address various ailments, as they contain bioactive compounds known to provide therapeutic benefits.

Depression refers to a psychological state marked by enduring and contrasting indicators of unhappiness, guilt, exhaustion, diminished focus, lack of interest, reduced self-assurance, and disturbances in eating or sleeping habits. For a diagnosis to be made, these symptoms of depression need to persist for a period longer than two weeks. Each type exhibits unique characteristics and may necessitate specific approaches to treatment and overall management. The Diagnostic and Statistical Manual recognizes various forms of depression, such as persistent depressive disorder, major depression, situational or seasonal affective disorder, postpartum depression, bipolar depression, psychotic depression, premenstrual dysphoric disorder, and atypical depression (Rihmer and Gonda, 2011).

The importance of this research line lies in the potential ability of *Punicagranatum* to act either as a dietary supplement or as an adjuvant to the existing therapies for mood and dementia disorders. Therefore, the objective of the current work is to evaluate the anxiolytic, antidepressant and the memory enhancing properties of *Punicagranatum* seeds using various animal models.

2. Materials and Methods

2.1. Collection of Seeds and Extraction

In May 2020, the *Punicagranatum* seeds were gathered from Irbid, located in North of Jordan. The plant material was authenticated by a botanist. To ensure future reference, a voucher specimen was sent to the Herbarium at Hashemite University in Zarqa, Jordan, where it was assigned the Herbarium number HU.No. 6257. To prepare the seeds of *Punicagranatum* for further use,500g of seeds was soaked in 1.5 L of ethanol for a duration of 21 days. During the maceration process, the seeds were kept in airtight ambered bottles. Subsequently, the solvent was filtered and evaporated, resulting in an extract. This extract was then stored at 4°C until it underwent freeze-drying for future utilization.

2.2. Experimental Design

The study involved a group of healthy 12-weeks age male mice weighing 25-30gbred at the Department of Biology in Hashemite University's animal house. Following a one-week acclimatization period, the mice were separated into six groups, each comprising 10 mice. They were housed in polycarbonate cages with food and water freely available at a constant temperature of 25 ± 2°C. The control group received 5% Dimethyl sulfoxide (DMSO), while two other groups were given standard drugs: diazepam at a dose of 3 mg/kg(Cheng *et al.*, 2013)and imipramine at a dose of 30 mg/kg(Podolan *et al.*, 2019). The remaining test groups were orally administered ethanol extracts of Punicagranatum at doses of 50, 100, and 200 mg/kg for 21 days by oral gavage. All administrations of DMSO, standard drugs, and seed extracts took place once daily between 12 am and 1 pm. To assess the behavioral impact, different models for anxiety, depression, and memory were employed on days 8 and 21 of the study. The behavioral sessions were recorded using a mobile camera running the Android operating system.

2.3. Models for Memory

2.3.1. Passive Avoidance Test

As a rapid method for evaluating fear-based avoidance memory in mice, the Passive Avoidance Test (PAT) is employed. The setup consists of both light and dark compartments, including a grid floor and a guillotine gate. The dark area is connected to a mild electric current source for delivering mild electric shocks(Cho *et al.*, 2003). In the habituation phase, the mice are introduced to the illuminated section for 300 seconds on the first and second days. They faced the dark area with the guillotine door open. If the mice enter the dark compartment during this phase, the door is closed, and a 0.6 mA foot shock lasting 0.5 seconds is given through the grid floor.

In the testing phase, the mice are reintroduced to the apparatus after specific intervals: eight days and twenty-one days. This allows for the assessment of short-term and long-term memories. During testing, the mice are placed in the illuminated section with the gate open, giving them unrestricted access to the dark avoidance section. The duration taken for the mice to re-enter the dark area while successfully avoiding the electric shock is recorded, with a maximum cutoff time of 300 seconds.

2.3.2. Morris Water Maze Test

The aim of this test was to evaluate spatial learning and memory acquisition, specifically those dependent on the hippocampus, in both short-term and long-term contexts. The experimental apparatus used was a rectangular water pool measuring 60 cm × 30 cm, with a fixed central platform of size 15 cm × 13 cm submerged in the water and hidden by a non-transparent material. The water temperature was maintained at 25°C. During the training sessions, the platform was visible by keeping the water level below it. The mice were placed into the pool and allowed to find the platform. Four training sessions were conducted on consecutive days, enabling the mice to gradually improve their ability to locate the platform within a shorter time. On the eighth and twenty-first days, the mice were tested again to assess their spatial learning capabilities. Improvement in spatial learning was determined by a decrease in the time taken to find the platform(Gallivan and Schmitzer-Torbert, 2018).

2.4. Motor Skilled Learning and Coordination

2.4.1. Effects on Motor Coordination through Rotarod

In the present investigation, the impact of anxiety-reducing drugs on motor coordination was evaluated using a rotarod apparatus. This apparatus comprises a plastic rod measuring 8 cm × 3 cm, enclosed for safety purposes. Initially, mice were subjected to training on stationary rods, involving four consecutive trials. The test sessions occurred on the 8th and 21st days after administering ethanol extracts, at two different speeds: 10 and 30 rpm. The time taken for the mice to remain on the rotating rod before falling off, known as "latency time," was recorded, with a maximum limit of 180 seconds. An increase in the duration spent on the rotating rod indicated an enhancement in muscular activity. It is important to mention that drugs with an impact on neuromuscular

coordination, like diazepam, typically lead to a reduction in the latency time for mice to stay on the rotating rod.(Asgharzade *et al*., 2015).

2.4.2. Stationary Rod Test

The educational capacity was assessed using the Stationary Rod Test (SRT), which involved elevated steel rods with a netted base. The mice were initially trained by walking on the elevated rod with the netted stage. Daily training was conducted over four consecutive days, with each trial lasting 120 seconds. After completing the training, DMSO (a solvent), standard drugs, and *Punicagranatum* seed extracts were orally administered for 21 days. On the eighth and twenty-first days, the time taken by the animals to reach the stage was observed to evaluate short and long-term motor skill learning(Lundquist *et al.*, 2022).

2.5. Models for Anxiety

2.5.1. Light and Dark Test

To evaluate the anxiolytic effects of seed extracts, this study employed a model using the preference of animals to occupy the lighter compartment as an indicator. The experimental setup comprised two identical in volume compartments, each measuring 20 cm × 20 cm× 35 cm (height). One compartment was made brighter by illuminating it with a 100 V bulb, while the other compartment was made darker using dark glasses. The mice were initially introduced to the darker zone and allowed to freely move between the two compartments through a small gate placed in between. Test sessions lasting 5 minutes were conducted, during which the mice could freely explore both compartments. The researchers measured the time spent by the mice in the light compartment, calculated the percentage of time spent there, and counted the number of transitions made between compartments. An increase in the percentage of time spent in the light area was considered an indication of anxiolytic activity(Bourin and Hascoët, 2003).

2.5.2. Elevated Plus Maze (EPM)

Afterwards, mice underwent Elevated Plus Maze (EPM) testing on the eighth and twenty-first days of dosing. The EPM apparatus consisted of two open arms measuring 50 cm × 10 cm and two closed arms measuring 50 cm × 10 cm × 38 cm, all connected to a central area measuring 10 cm × 10 cm. The maze was elevated to a height of 50 cm from the ground. Mice were placed in the central area facing one of the open arms, and their behavior was observed and recorded during a 5-minute test session. The recorded behaviors included the number of entries into both the open and closed arms, as well as the time spent by the mice in each arm.

An increase in the time spent in the open area and the number of entries into the open arm were considered indicative of anxiolytic activity.(Contreras *et al.*, 2014).To calculate the anxiety index, the researchers used the following formula:

Anxiety Index = 1 - (time in open arm / total time) + (entries of open arm / total entries).

If the anxiety index result fell between 0.6 and 1.0, this indicates the presence of anxiety in mice, while a decrease in the index reflects an anxiolytic effect.

2.5.3. Head Dip Test

The test employed a white square wooden box with dimensions of 35 cm × 45 cm × 45 cm, featuring three equally spaced and sized holes on each side. Mice were placed inside the box for a 5-minute session, and the number of head dips through the holes was recorded on the 8th and 21st days of the study (Kliethermes and Crabbe, 2006). An increase in the number of head dips in this model indicated a rise in anxiety, while a decrease in head dipping pointed to the anxiolytic effect of the extracts(Solangi and Najam, 2013).

2.5.4. Open Field Test Apparatus

The open field test (OFT) is a widely used apparatus for evaluating various behaviors in rodents, such as anxiety, memory, exploration, and depression. It consists of a transparent plexiglass cube measuring 75 cm × 75 cm × 40 cm, with a marked floor divided into 25 boxes, each measuring 15 cm × 15 cm. The central area is a 30 cm × 30 cm square. During the test, mice are placed in the center of the apparatus and allowed to explore for a 10-minute session(Crupi *et al.*, 2010; Russo *et al.*, 2013). Several parameters were measured during the test to assess the behavior of the mice, including the total distance covered, the number of entries into the central area, the duration of time spent in the central area, the frequency of rearing (standing on hind legs), and the duration of rearing. The test is informative as it provides insights into the mouse's motor activity, anxiety levels, exploratory behavior, and cognitive functions. An increase in the time spent in the center or the number of center entries suggests anxiolytic (anti-anxiety) activity. Moreover, an increase in the frequency or duration of rearing behavior indicates heightened exploratory and cognitive behavior, as well as a reduction in anxiety. Recent research has also associated an increase in rearing behavior with potential antidepressant effects(Bee and Maheshwari, 2019).

2.6. Models for Depression

2.6.1. Forced Swim Test

The aim of the test was to assess the antidepressant effects of seed extracts on the 21st day. For the experiment, a rectangular plexiglass container measuring 46 cm × 20 cm was used. Depression was induced in mice during the initial 2-minute session using the learned helplessness phenomenon. The last 4 minutes of immobility time exhibited by the mice were recorded as an indicator of antidepressant activity(Aiello *et al.*, 2015; Planchez *et al.*, 2019). Immobility was defined as the absence of movement in mice, except for instances where mice needed to keep their heads out of the water. The percentage reduction in immobility time was used as a measure to evaluate the antidepressant effect of the extracts(Adachi *et al.*, 2022).

2.6.2. Tail Suspension Test

On the 21st day, the tail suspension method was utilized to assess the antidepressant effect. The animals were suspended upside down using tape attached to their tails for a duration of 6 minutes(Steru *et al.*, 1985). During the initial 2 minutes, depression was induced through the learned helplessness phenomenon. The immobility time observed during the last 4 minutes was indicative of antidepressant activity. Immobility time was measured by

evaluating the absence of movement in mice attempting to upright themselves. The percentage reduction in immobility time was used to gauge the degree of antidepressant effect caused by the extracts(Planchez *et al.*, 2019; Adachi *et al.*, 2022).

2.7. Statistical Analysis

The statistical analysis of the results was conducted using the SPSS statistical software package version 26. All values were presented as Mean ± SEM (Standard Error of the Mean). To analyze the data, a one-way analysis of variance (ANOVA) was initially employed. Subsequently, a post hoc Dunnett test was conducted to compare specific groups to the control group. The significance levels for the post hoc analysis were set at $P < 0.05$ and $P < 0.1$, indicating statistically significant differences between groups.

3. Results

3.1. Effect on Memory and Learning

3.1.1. Passive Avoidance Test

Figure 1 presents findings related to the impact of *Punicagranatum* seed extract on memory and learning, assessed through the Passive Avoidance Test (PAT). The 50 mg/kg dose of *Punicagranatum* showed a significant increase in latency time on the 8th day. The 100 mg/kg dose displayed a notable increase in reaction time on the 8th day and the 21st day. Furthermore, the 200 mg/kg dose resulted in a significant increase in latency time on the 21st day, and remarkably, an extremely significant increase on the 8th day. In contrast, the administration of diazepam at 3 mg/kg led to a significant decrease in latency time on both the 8th and 21st days. Conversely, imipramine at 30 mg/kg showed a significant increase in latency time on the 8th and 21st days.

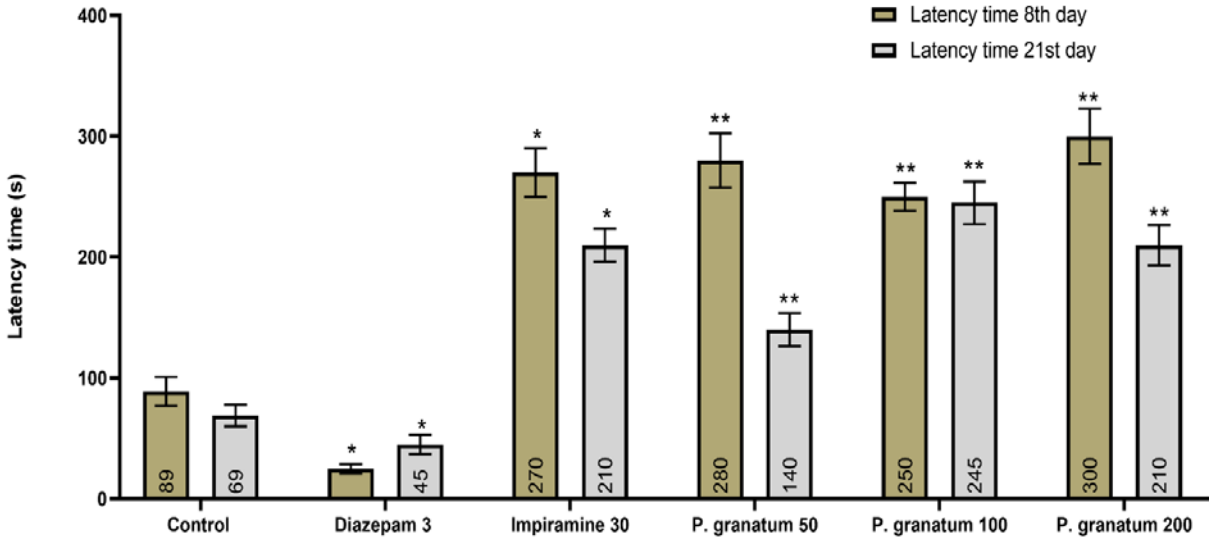

Figure 1: Impact of *Punica granatum* seeds' extract on memory (passive avoidance test). *P < 0.05, indicating a substantial difference compared to the control group, and **P < 0.01, indicating a highly substantial difference compared to the control group.

3.1.2. Water Maze Test

Figure 2 presents the effects of *Punicagranatum* seed extract on memory and learning, as evaluated through the water maze test. The results indicated that doses of *Punicagranatum* at 50 mg/kg and 100 mg/kg significantly reduced the time taken to reach the central hidden stage on the 8th day and the time taken to reach the central stage on the 21st day. Moreover, the 200 mg/kg dose of *Punicagranatum* also led to a significant reduction in the time to reach the stage on the 8th day and a substantial reduction on the 21st day. Conversely, the group treated with Diazepam at 3 mg/kg showed a significant increase in the time taken to reach the central hidden stage on both the 8th and 21st days. Meanwhile, the group given imipramine at 30 mg/kg exhibited a notable decrease in the time taken to reach the central hidden stage on the 8th and 21st days, compared to the untreated group.

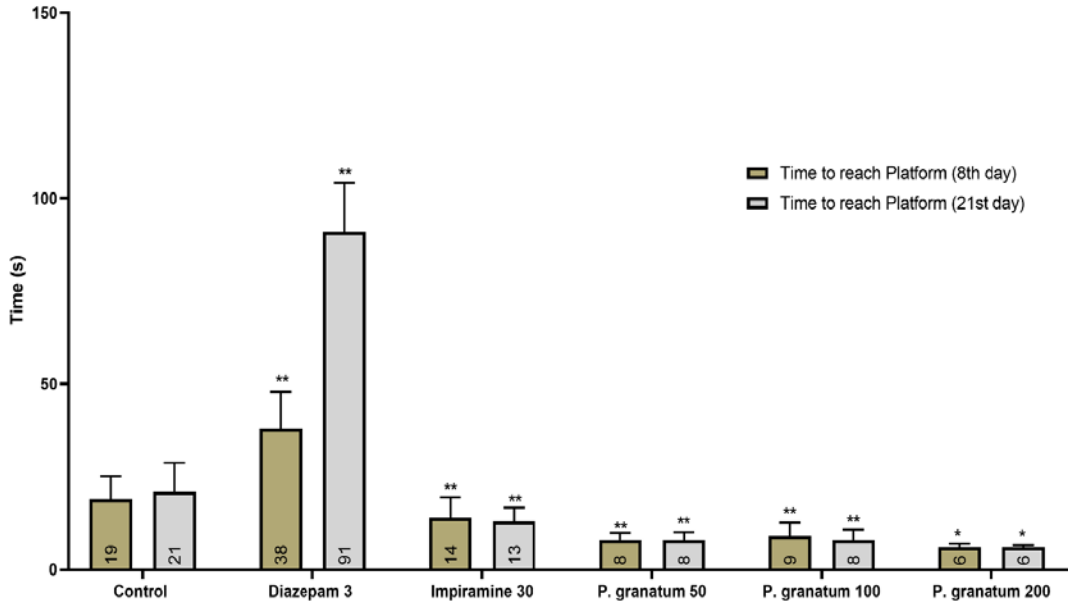

Figure 2: Impact of ***Punica granatum*** seeds' extract on the memory (water maze test). *P < 0.05, indicating a substantial difference compared to the control group, and **P < 0.01, indicating a highly substantial difference compared to the control group.

3.3. Effects on Motor Coordination and Motor Skilled Learning

3.3.1. Rotarod Test

According to table 1, the administration of Punicagranatum at doses of 100 mg/kg and 200 mg/kg resulted in a significant increase in fall time on the 21st day at low speed. Additionally, these Punicagranatum groups exhibited a highly significant increase in fall time on both the 8th and 21st days at high speed, in comparison to the untreated mice.

Table 1. Impact of *Punica granatum* seeds' extract on motor coordination by rotarod.

Groups/doses (mg/kg)	Fall time(s) on 10 rpm		Fall time(s) on 30 rpm	
n=10	8th day	21st day	8th day	21st day
Control	131.5 ± 5.3	121.5 ± 8.1	7.35 ± 1.1	5.22 ± 0.4
P. granatum 50mg/kg	152.2 ± 6.7	157.3 ± 7.9	22.1 ± 1.4**	28.3 ± 1.3**
P. granatum 100mg/kg	169.1 ± 7.2	172.1 ± 9.3*	26.4 ± 0.9**	29.9 ± 2.7**
P. granatum 200mg/kg	162.2 ± 4.9	163.4 ± 7.4*	36.1 ± 1.7**	36.5 ± 3.1**
Diazepam 3*mg/kg*	20.4 ± 2.1*	37.3 ± 3.2**	5.1 ± 0.3*	4.8 ± 0.5**
Imipramine 30*mg/kg*	128.1 ± 6.3	150.1 ± 5.3	27.4 ± 1.4*	29.3 ± 2.5**

The results were reported as average ± SEM (Standard Error of the Mean). The statistical significance was denoted as follows: *P < 0.05, indicating a substantial difference compared to the control group, and **P < 0.01, indicating a highly substantial difference compared to the control group.

On the other hand, Diazepam at 3 mg/kg demonstrated a highly significant reduction in fall time on the 8th day at both speeds (rpm), and there was also a highly significant reduction in fall time on the 21st day at both speeds.

Furthermore, imipramine at 20 mg/kg showed a highly significant increase in fall time on both the 8th and 21st days at high speed.

3.3.2. Stationary Rod Test

Figure 3 provides insights into the effects of *Punicagranatum* seed extract on memory and learning, evaluated through the Stationary Rod Test. The group administered *Punicagranatum* at a dose of 50 mg/kg showed a significant reduction in the time taken to reach the elevated stage on the 8th day, and a substantial reduction on the 21st day. Similarly, the group receiving *Punicagranatum* at a dose of 100 mg/kg exhibited a highly significant reduction in the time to achieve the elevated stage on both the 8th and 21st days.

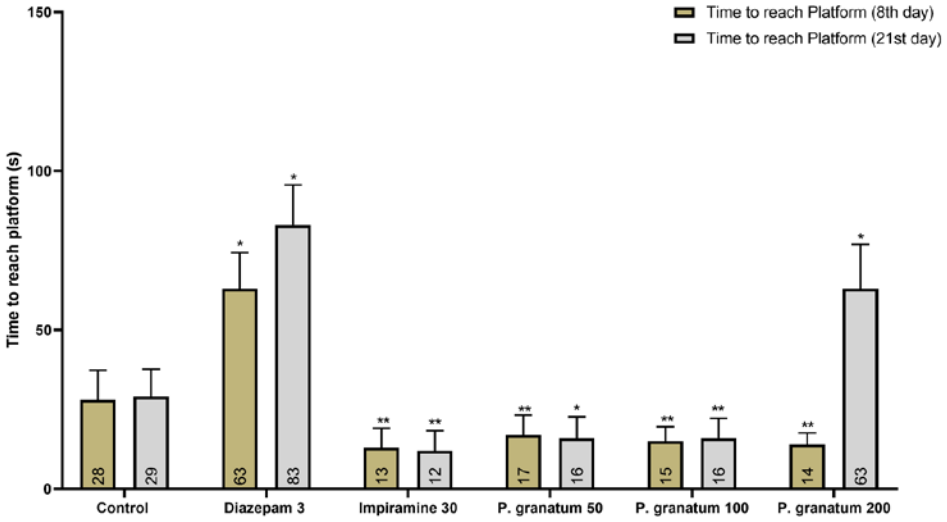

Figure 3: Impact of *Punica granatum* seeds extract on motor skilled learning and memory (stationary rod). *P < 0.05, indicating a substantial difference compared to the control group, and **P < 0.01, indicating a highly substantial difference compared to the control group.

In contrast, *Punicagranatum* at a dose of 200 mg/kg showed a significant reduction in the time to reach the stage on the 8th day but a notable increase in the time to reach the stage on the 21st day, in comparison to the reference group.

Animals treated with diazepam at 3 mg/kg demonstrated a substantial increase in the time taken to reach the elevated stage on both the 8th and 21st days, while those given imipramine at 30 mg/kg exhibited a significant reduction in the time to reach the elevated stage on both the 8th and 21st days, compared to the control group.

3.4. Effect on Anxiety

3.4.1. Light and Dark Model

In terms of the effects on anxiety, table 2 presents information on the impact of *Punicagranatum* seed extract in the light and dark models.

Punica granatum seed extract at a dose of 50 mg/kg demonstrated a highly significant increase in transitions on the 8th and 21st days, and it also exhibited a significantly increased percentage of time spent in the light area on the 21st day compared to the reference group.

Punicagranatum at doses of 100 and 200 mg/kg showed an extremely substantial increase in the percentage of time spent in the light area on both the 8th and 21st days. Furthermore, the group administered *Punicagranatum* at a dose of 100 mg/kg demonstrated a highly significant increase in transitions on the 8th day compared to the untreated group.

Animals treated with diazepam at 3 mg/kg exhibited a greatly substantial increase in the percentage of time spent in the light area and transitions on both the 8th and 21st days.

Additionally, animals given imipramine at 30 mg/kg showed a significant increase in transitions on the 8th day.

These results suggest that *Punicagranatum* seed extract may have anxiolytic effects, as indicated by increased time spent in the light area and increased transitions, particularly at higher doses. Diazepam also demonstrated anxiolytic effects, while imipramine showed mixed effects on anxiety-related behaviors.

Table 2. The impact of *Punica granatum* seeds' extract on anxiety by light and dark model.

Groups/doses (mg/kg)	Number of transition		Time in light (%)	
n=10	8th day	21st day	8th day	21st day
Control	1.4 ± 0.3	2.2 ± 0.4	6.3 ± 0.9	9.5 ± 1.3
P. granatum 50mg/kg	5.8 ± 0.7**	6.6 ± 1.1**	32.2 ± 5.1	37.7 ± 2.1**
P. granatum 100mg/kg	4.5 ± 0.4**	4.4 ± 0.5	54.4 ± 4.7**	51.5 ± 5.1**
P. granatum 200mg/kg	3.4 ± 0.4	3.2 ± 0.3	39.4 ± 6.5**	40.2 ± 3.2**
Diazepam 3*mg/kg*	4.8 ± 0.5	0.9 ± 0.4	27.7 ± 3.1**	49.9 ± 4.9**
Imipramine 30*mg/kg*	5.1 ± 0.7*	3.2 ± 0.7	12.1 ± 1.5	7.2 ± 1.2

The results were reported as average ± SEM (Standard Error of the Mean). The statistical significance was denoted as follows: *P < 0.05, indicating a substantial difference compared to the control group, and **P < 0.01, indicating a highly substantial difference compared to the control group.

3.4.2. Elevated plus Maze

Table 3 presents the findings regarding the impact of *Punicagranatum* seed extracts on anxiety using the elevated plus maze test.

The administration of *Punicagranatum* at doses of 100 and 200 mg/kg resulted in a highly significant and significant increase in the amount of time spent in the open area on the 8th and 21st days, respectively. Moreover, these two groups displayed a significant increase in transitions on the 8th day. Additionally, the 100 and 200 mg/kg doses exhibited a significantly reduced amount of time spent in the closed arms on the 8th and 21st days, respectively, along with a substantial decrease in transitions. Furthermore, the anxiety index was notably reduced on the 8th and 21st days with the administration of *Punicagranatum* at 100 and 200 mg/kg, respectively.

Table 3. The impact of *Punica granatum* seeds' extract on anxiety (elevated plus maze).

Groups/doses (mg/kg)	Open arm's time (seconds)		Closed arm's time (seconds)		Closed-arm entries (times)		Open arm entries (times)		Anxiety index	
n=10	8th day	21st day	8th day	21st day	8th day	21st day	8th day	21st day	8th day	21st day
Control	10 ± 1.4	25 ± 4.1	290 ± 33.2	275 ± 31.7	2.5 ± 0.3	3.9 ± 1.1	3.5 ± 0.5	3.7 ± 0.6	0.73	0.69
P. granatum 50mg/kg	60 ± 6.2	20 ± 2.1	240 ± 18.7	280 ± 13.1	4.1 ± 0.7	3.1 ± 0.3	7.1 ± 0.8	3.9 ± 0.8	0.51	0.75
P. granatum 100mg/kg	182 ± 18**	85 ± 5.6*	118 ± 11.2**	215 ± 20.2*	4.2 ± 0.2	4.1 ± 0.6	9.1 ± 0.8**	6.7 ± 0.9	0.32	0.58
P. granatum 200mg/kg	208 ± 23**	89 ± 9.2*	92 ± 7.2**	211 ± 22.1*	2.6 ± 0.2	4.5 ± 0.5	8.1 ± 0.6**	8.5 ± 0.7	0.31	0.49
Diazepam 3*mg/kg*	175 ± 32.1**	197 ± 22.1**	125 ± 8.3**	103 ± 9.5**	2.7 ± 0.3	5.3 ± 0.4*	4.9 ± 0.5	9.1 ± 1.1*	0.48	0.26
Imipramine 30*mg/kg*	30 ± 5.1	42 ± 13.3	270 ± 24.7	258 ± 15.6	4.9 ± 0.6	4.8 ± 0.3	6.2 ± 0.3	7.2 ± 0.7	0.6	0.68

The results were reported as average ± SEM (Standard Error of the Mean). The statistical significance was denoted as follows: *P < 0.05, indicating a substantial difference compared to the control group, and **P < 0.01, indicating a highly substantial difference compared to the control group.

Animals treated with diazepam at a dose of 3 mg/kg showed a significantly higher amount of time spent in the open arm on the 8th and 21st days, while also displaying an increased number of transitions on the 21st day.

Imipramine at a dose of 30 mg/kg resulted in an increased amount of time spent in the open arm compared to the reference group.

Overall, the seed extract of *Punicagranatum* at doses of 100 and 200 mg/kg on the 8th day exhibited a significant reduction in the anxiety index, following the established standard. The effects of *Punicagranatum* seed extracts on anxiety using the elevated plus maze test were summarized in Table 6.

3.4.3. Head Dip Test

Table 4 provides a summary of the impact of *Punicagranatum* seed extract on the number of head dips.

The administration of *Punicagranatum* at a dosage of 100 mg/kg resulted in a highly significant decrease in the number of head dips on the 21st day. Similarly, the group receiving *Punicagranatum* at a dosage of 200 mg/kg exhibited an extremely substantial reduction in head dips on both the 8th and 21st days.

Animals treated with diazepam at a dosage of 3 mg/kg showed a significant reduction in head dips on the 8th day, as well as a greatly substantial reduction on the 21st day.

Furthermore, animals administered imipramine at a dosage of 30 mg/kg demonstrated a highly significant reduction in head dips compared to the untreated group.

These results suggest that *Punicagranatum* seed extract may have an effect on exploratory behavior, as indicated by the reduction in head dips. Diazepam and imipramine also showed significant effects on head dipping behavior in the mice.

Table 4. The impact of *Punica granatum* seeds' extract on head dips.

Groups/doses (mg/kg)	Number of head dips	
n=10	8th day	21st day
Control	32.1 ± 1.3	30.3 ± 1.2
P. granatum 50mg/kg	27.8 ± 1.7	26.3 ± 0.9
P. granatum 100mg/kg	25.3 ± 1.3	16.2 ± 0.4**
P. granatum 200mg/kg	23.4 ± 1.8*	16.7 ± 0.7**
Diazepam 3*mg/kg*	40.5 ± 1.5*	22.2 ± 0.8**
Imipramine 30*mg/kg*	20.1 ± 1.3**	16.3 ± 0.3**

The results were reported as average ± SEM (Standard Error of the Mean). The statistical significance was denoted as follows: *P < 0.05, indicating a substantial difference compared to the control group, and **P < 0.01, indicating a highly substantial difference compared to the control group.

3.4.4. Open Field Test

Table 5 presents the effects of *Punicagranatum* seed extract on anxiety using the open field test (OFT).

When administered at a dose of 50 mg/kg, *Punicagranatum* showed a highly significant increase in the total distance traveled on the 8th day. Additionally, there were significant and highly significant increases in center entries on the 8th and 21st days, respectively. Moreover, the duration of rearing exhibited significant and highly significant increments on the 8th and 21st days compared to the reference group.

Punicagranatum at doses of 100 and 200 mg/kg demonstrated an extremely substantial increase in the total distance traveled on the 8th day. Furthermore, there were highly significant increments in center entries, center time, as well as the number and duration of rearing on both the 8th and 21st.

Animals treated with diazepam at a dose of 3 mg/kg displayed a significant reduction in the total distance traveled on the 21st day. Additionally, there was a highly significant increase in center entries and center time on the 21st day, along with a significant reduction in the number and duration of rearing.

Imipramine at a dose of 30 mg/kg exhibited an extremely significant reduction in center entries on the 21st day, and an extremely significant increase in the number of rearing on both the 8th and 21st days.

Table 5. Outcome of *Punica granatum* seeds' extract in open field test.

Groups/doses (mg/kg)	Total distance travelled (cm)		No. of center entries		Centre time(s)		No. of rearing		Duration of rearing (s)	
n=10	8th day	21st day	8th day	21st day	8th day	21st day	8th day	21st day	8th day	21st day
Control	1910 ± 75	2311 ± 20	2.5 ± 0.2	2.6 ± 0.2	3.1 ± 0.4	3.2 ± 0.4	4.1 ± 0.3	10 ± 0.9	4.4 ± 0.2	12 ± 0.8
P. granatum 50mg/kg	3025 ± 18**	2150 ± 16	5.6 ± 0.3*	8.3 ± 0.7**	4.9 ± 0.9	7.5 ± 1.3	5.1 ± 0.8	11 ± 0.8	15.1 ± 1.3**	20.5 ± 1.4*
P. granatum 100mg/kg	2701 ± 30*	2480 ± 22	7.1 ± 0.9**	8.9 ± 0.8**	13.4 ± 1.2**	14.1 ± 0.8**	15.2 ± 1.2**	22.1 ± 1.3**	16.2 ± 0.8*	30.1 ± 2.2**
P. granatum 200mg/kg	2855 ± 21**	2472 ± 30	6.9 ± 0.8**	10.5 ± 1.1**	14.3 ± 0.9**	18.1 ± 0.7**	22.3 ± 2.1**	22.4 ± 2.4**	13.5 ± 1.2**	31.3 ± 4.2**
Diazepam 3*mg/kg*	2533 ± 25	1387 ± 18*	6.4 ± 0.4	11.1 ± 1.3**	5.2 ± 0.3	18.1 ± 0.7**	3.1 ± 0.1	2.2 ± 0.3**	3.1 ± 0.2	6.5 ± 0.8*
Imipramine 30*mg/kg*	1871 ± 17	1618 ± 19	3.2 ± 0.2	5.9 ± 0.6**	4.3 ± 0.2	21.2 ± 1.9**	14.2 ± 1.1**	18.9 ± 1.2**	9.2 ± 1.1	12.1 ± 2.4

The results were reported as average ± SEM (Standard Error of the Mean). The statistical significance was denoted as follows: *P < 0.05, indicating a substantial difference compared to the control group, and **P < 0.01, indicating a highly substantial difference compared to the control group.

3.5. Effect on Depression

Two models were utilized in the assessment of the antidepressant research. In the course of this evaluation, all the mice involved in the study demonstrated survival. Not a single mouse experienced drowning when subjected to the forced swim test or the tail flick method.

3.5.1. Forced Swim Test

Table 6 presents the findings regarding the impact of *Punicagranatum* seed extract on immobility time in the forced swim test (FST) as a measure of antidepressant activity.

Punica granatum seed extract at doses of 50, 100, and 200 mg/kg exhibited remarkably significant reductions in immobility time on the 21st day, resulting in percentage reductions of 22%, 29%, and 40%, respectively, compared to the untreated group.

Furthermore, imipramine at a dose of 30 mg/kg displayed the highest antidepressant activity among the treatments, with a 47% reduction in immobility time compared to the control group in the forced swim test (FST).

3.5.2. Tail Suspension Test

Table 6 presents the findings regarding the impact of *Punicagranatum* seed extract on immobility time in the forced swim test (FST) as a measure of antidepressant activity.

In comparison to the control group, *Punica granatum* seed extract at doses of 50, 100, and 200 mg/kg exhibited highly significant reductions in immobility time on the 21st day. The percent reduction in immobility time was measured at 25%, 27%, and 37%, respectively.

Similarly, Imipramine administered at a dose of 30 mg/kg demonstrated an extremely substantial reduction in immobility time, reaching a percent reduction of 47% on the 21st day when compared to the reference group.

Table 6. Effect of *Punica granatum* seeds' extract on depression by forced swim test and tail suspension test.

Groups/doses (mg/kg)	Forced swim test		Tail suspension test	
n=10	Immobility time (seconds) 21st day	Reduction immobility (%)	Immobility time (seconds) 21st day	Reduction immobility (%)
Control	199 ± 3.1	----------	209 ± 5.3	----------
P. granatum 50mg/kg	155 ± 8.1**	22	156 ± 2.1**	25
P. granatum 100mg/kg	143 ± 7.1**	29	153 ± 2.7**	27
P. granatum 200mg/kg	120 ± 6.1**	40	132 ± 4.3**	37
Diazepam 3*mg/kg*	210 ± 12.3	6	220 ± 5.2	5
Imipramine 30*mg/kg*	105 ± 7.3**	47	110 ± 6.6**	47

The results were reported as average ± SEM (Standard Error of the Mean). The statistical significance was denoted as follows: *P < 0.05, indicating a substantial difference compared to the control group, and **P < 0.01, indicating a highly substantial difference compared to the control group.

4. Discussion

Herbal medicines are becoming increasingly important in the prevention and treatment of various disorders due to their rich content of secondary metabolites. In this context, the present study aimed to explore the pharmacological potential of *Punicagranatum* seeds. The focus was on its ability to alleviate anxiety and depression, as well as its impact on memory improvement.

To assess the antidepressant effects of *Punicagranatum* seeds in mice, the forced swimming and tail suspension tests were employed. The results showed that the extracts demonstrated their most potent antidepressant effects at a dose of 200 mg/kg, comparable to the effects of imipramine, a well-known antidepressant medication. This positive impact could be attributed to the presence of active metabolites like polyphenols and β-carotene, which are known to influence the central nervous system and play a role in alleviating depressive symptoms. These findings suggest the potential of *Punicagranatum* as a natural alternative for treating depression. (CNS)(Kistlera *et al.*, 2015).

According to Bekir *et al.*, (2013), the ethanol seeds of *Punica granatum* seeds extract has been identified as a cholinesterase inhibitor, implying its potential to provide neuroprotective effects. This is significant as cholinesterase is associated with cognitive impairment through the breakdown of acetylcholine. Increased acetylcholinesterase (AChE) activity in the brain accelerates acetylcholine breakdown, elevating the risk of dementia progression. As a result, there is growing interest in developing cholinesterase inhibitors to manage cognitive impairment. In addition, there is a desire for agents that inhibit butyrylcholinesterase (BChE) activity in humans. The disruption of monoaminergic neurotransmission by monoamine oxidase (MAO) inhibitors has been linked to neurodegenerative diseases such as Parkinson's and Alzheimer's (Youdim and Riederer, 2004; Bolea *et al.*, 2013). Therefore, the inhibition of MAO activities through suitable agents, particularly from plants, presents a potentially beneficial therapeutic approach for addressing cognitive impairment. Prior studies have consistently demonstrated the significant inhibitory effects of plant extracts on MAO activity. The present study suggests that the tested *Punicagranatum* extracts may also possess this ability to inhibit MAO activity(Yildiz *et al.*, 2014), thus contributing to their potential neuroprotective properties. These findings underscore the promise of *Punicagranatum* as a natural source for the development of neuroprotective compounds.

In a study conducted by Arora *et al.*, (2020), *Cucurbita moschata* seed extracts exhibited significant anxiolytic activity at a dose of 200 mg/kg. This effect was comparable to the standard drug alprazolam in both experimental models. Furthermore, the ethanol seeds at the same dose led to changes in motor coordination. The suggested mechanism for these effects was an increase in chloride ion influx, indicating a potential involvement of γ-Aminobutyric acid type A (GABAA) receptors in the extracts' action. Given these findings, it is reasonable to speculate that *Punica granatum* seeds might produce a similar pattern of effects, potentially showing anxiolytic properties and influencing motor coordination. Yet, this needs more investigation.

The memory-suppressing impact of antidepressant and anxiolytic medications has been previously noted (Nicolas and Ruby, 2020). The focus has shifted towards exploring alternative treatments that offer beneficial outcomes without undesirable consequences. Thus, this particular investigation sought to assess the cognitive-enhancing properties of *Punica granatum* seeds through three distinct models: the stationary rod, passive avoidance, and water maze tests. In all three models employed, the seeds derived from *Punica granatum* seeds displayed a noteworthy improvement in memory retrieval, both in the short-term and long-term, when compared to the control group.

Punica granatum seeds widely recognized for its effectiveness in improving various diseases, primarily due to the presence of diverse secondary polyphenolic compounds like quercetin(Saparbekova *et al.*, 2023). These metabolites have been attributed to multiple biological activities associated with *Punicagranatum seeds.* Notably, *Punica granatum* seeds exhibits strong antioxidant properties, which can be attributed to its significant content of β-carotene. This compound not only boosts immunity but also helps reduce the risk of developing conditions like cancer and the progression of heart disease.

Previous research has indicated the potential benefits of utilizing pomegranate peel to enhance cognitive functions and provide neuroprotection(Adiga *et al.*, 2010; Harakeh *et al.*, 2020). For instance, in a study conducted by Adigaet al. (2010), supplementation of rat diets with pomegranate peel seeds (at a dosage of 100 mg/kg) for a duration of two weeks resulted in improved spatial learning and memory, even in the presence of impairment caused by diazepam. Similarly, Harakeh*et al.* (2020) found that administering a solution of pomegranate peel seeds (at a dosage of 50 mg/kg) along with ellagic acid for a period of four weeks led to improvements in memory deficits and degenerative changes in a rat model of Alzheimer's disease induced by $AlCl_3$. Another study conducted by H. Ahmed *et al.*, (2014) demonstrated that pomegranate seeds at dosages of 100 and 200 mg/kg exhibited cognitive-enhancing effects, potentially through the reduction of Amyloid-β Precursor **(**Aβ) aggregates in the hippocampus. Additionally, Fatima *et al.* (2017), demonstrated that oral consumption of *Punica granatum* seeds at a dosage of 500 mg/kg showed ameliorating effects on scopolamine-induced cognitive dysfunction, particularly in the passive avoidance response.

Although the findings of this study are of value, some have limitations that should be mentioned. The results of this study are behavioral observations; some markers should be measured in the brain homogenate such as biogenic amines to validate the effect of seeds extract on these markers. Also, same group of mice were assessed at two different time points, different group should be assessed at each time point.

5. Conclusion

This study sheds light on the considerable potential of Punicagranatum seeds in addressing neurological concerns. Not only do they offer relief from anxiety and depression, but they also bolster memory function. These

outcomes are possibly due to the seeds' abundance in bioactive compounds.

6. Future work and recommendations

While this research has shown promising results, it would be beneficial to extend the experiments to a more diverse group of test subjects. This includes different age groups, ethnic backgrounds, and health conditions, to ascertain the universality of the benefits. Furthermore, a longitudinal study observing the prolonged intake of Punicagranatum seeds and its impact on neurological functions would offer insights into its long-term benefits and potential side effects. Moreover, comparing the effects of Punicagranatum seeds with established pharmaceutical treatments for anxiety, depression, and memory-related issues will help to contextualize their efficacy in the broader medical landscape. Additionally, it would help to investigate if combining Punicagranatum seeds with other natural remedies can enhance or modify their neurological benefits.

7. Data Availability

The supporting data for this research article can be obtained by making a reasonable request to either the corresponding author or the first author of the study.

8. Ethical Approval

This study received approval from the Institutional Review Board at the Hashemite University, Zarqa, Jordan, with the assigned approval number IRB No.: 18/2020/2021.

9. Conflicts of Interest

The authors of this research article declare that they have no conflicting interests.

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