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Determination of Effect of Esfand (Peganumharmala) Seed Extract on Fear Behavior in Male Rats with varying Ages

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ABSTRACT

The present study was formulated in order to determine effect of inhalation of Esfand seeds extract on fear behavior in the rats with varying ages (i.e. 20-, 35-, 50-, and 20-35-50-day old). The extract was provided from the Esfand collected from Damghan Desert. The rats were subjected to inhalation of the extract at 12 mg/kg. Elevated-plus maze was adopted in order to assess fear in the rats. The results showed that inhalation of Esfand seeds extract increased the time spent and the number of visits to closed arm and the area between closed arm and neutral area in test rats as compared to control rats. This was considered as indicative of fear behavior. This effect was attributed to the neurotransmitters harmaline and harmine in the extract.

KEYWORDS: Esfand, fear, harmaline, harmine, elevated-plus maze.

1- INTRODUCTION

Fear is a feeling induced by a threat and causes a swift reaction in a person or animal. Simply put, fear is defined as the ability of recognizing danger in order to confront or escape from it; however, a paralytic response may also occur during harsh fears. Fear is a mental physiologic response to an acute danger and it consists of autonomic, neuroendocrine, and behavioral responses. Stress is considered a warning sign preparing the creature to encounter the threat. Generally speaking, fear and stress can be defined as responses to acute and chronic dangers, respectively (Boissy, 1995).

During fear, a few acute reactions occur in body which makes it ready for fighting against the acute threats. A French physiologist Walter Bradford Cannon (1929) first defined such reactions: as a result of activation of sympathetic system of body, epinephrine, norepinephrine, and steroidal hormones are released in body; these hormones result in increased heartbeat, respiration rate, metabolism, responsiveness of brain to stimulators, as well as pupillary dilation; these events prepare the body to confront the potential threat. On the contrary, fear is not always caused by direct perception. Fears sometimes are conditional trends; that is, provided we face with a sign of threat, the same reactions will occur in body no matter there is no color, odor, or voice of the threat.

Amygdala can be called the center of fear in brain. Amygdala is a part of limbic system placed in temporal part of brain and it is attached to anterior hippocampus which decodes and fixates fear-causing experiences. Amygdala receives data from visual and auditory cortexes and somatosensory part and sends response to hypothalamus after data processing. Hypothalamus controls hormone production and homeostasis as well as autonomous parts of body in brain stem; amygdala can produce fear responses through hypothalamus. If the proteins formed in amygdala during decoding and fixing scary memories can be prevented in a way from being produced, a medicine is in fact produced to cease fear. Such medicine can be considered as the most useful and principle stress-reducing medication.

Various animal models have been proposed to measure fear-induced behaviors such as staircase test, light/dark test, and elevated plus maze (EPM). Nowadays, the most common test to measure fear and stress in rats is EPM. The set used in the test consists of two closed arm (6 cm wide, 30 cm long, and 10 cm high) and two open arms (6 cm wide and 30 cm long) as well as a short wall (2 cm high). They are placed perpendicularly forming a square (8×8 cm) in the conjunction of these two arms. The areais placedat the height of 40 cm from the ground. In this method, height induces fear in rats. Therefore, due to feeling more secure in closed arm, the rat tends to stay there. However, if the fear is removed by any reason, there will be no difference between open and closed arms for the rats. The test is, furthermore, capable of assessing such motor activities of rats as the time spent in open and closed section, number of visits to open part, number of visits to closed part, and overall number of visits to arms (Pellow and Filos, 1986).

Hallucinogens alter mind work and influence on central neural network. Hallucinogens, when consumed, interfere with the communication system related to receptors in brain resulting in interference in 5 senses. During a few years, chemists have been able to identify approximately one hundred hallucinogen compounds in

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variety of plants and fungi. Some of such compounds have been isolated and analyzed. Recent investigations have proven that some of these compounds cause fear. Esfand (*Peganumharmala*) has been found to contain such compounds.

Active compounds of Esfand are alkaloids mostly accumulating in seeds and root (Lobe et al. 1985). Beta-Carbolines such as harmaline and harmine form over 60% of seed alkaloids. Moreover, quinazolines such as vasicine and vasicinoneare other alkaloids found in Esfand (Glasby, 1978).

Psychoactive effects of Esfand seeds are rooted in inhibitory effect of monoamine oxidase (MAO) of beta-carbolinealkaloids which stimulates brain and causes visual hallucination (Udenfriend et al. 1958). Effects of Espand and its beta-carboline alkaloids on cardiovascular system mostly involve reduction blood pressure, bradycardia, and arrhythmia (Aarons et al. 1977). Authors have shown that Esfand extract has considerable fungicidal and bactericidal effects which are mostly attributed to harmine (El-Rifaie, 1980; Al-Sharma et al., 1981; Ross et al., 1980; Ahmad et al., 1992).

The present paper was formulated so as to determine the effect of Esfand extract on fear behavior in male rats with varying ages.

2- MATERIALS AND METHODS

2-1- Esfand collection

Esfand was collected from Damghan Desert(15 km off Damghan City) on July 15, 2012 at around 2 p.m. Sampling site's soil was also sampled and taken to a soil lab (Khak-Azmay-e-Gorgan) for analysis.

2-2 Extraction

40 gr of collected Esfand seeds was weighed, rinsed, and ground following drying. 150 cc methanol (Merck Co.) was added to the ground seeds and exposed to 50°C in bain-marie so that alkaloids were dissolved in methanol and active materials are extracted. This was performed for four times each for 1h to ensure complete extraction. The final extract was put in rotary at 90 rpm and 60°C to completely remove the solvent. Afterwards, 1000 cc hydrochloric acid 2% was added to the dried extract.

Hydrochloric acid 2% was derived from hydrochloric acid 37 as follows:

$N.V=N.V$

$V \times 37 = 1000 \times 2$

$V= 54$

Then, 54 cc of hydrochloric acid was dissolved in 946 cc distilled water. The sample was filtered by use of vacuum pump and Buchner funnel. Then, 1000 cc of petroleum ether was added to the filtered sample in two phases. Petroleum formed the upper phase due to being lighter than alkaloid phase and consequently, the lower phase was used for analyses. Subsequently, NH_4OH was added until the pH 10. Then, 1000 cc chloroform was added to the sample in 4 steps each 250 cc and alkaloids were isolated by means of a decanter. Being neutral, alkaloids were again placed in the upper phase. The solvent was again removed by rotary and the alcoholic extract was finally extracted from the seeds.

2-3- Harmaline determination via HPLC

HPLC[1] method was adopted in order to determine amount of harmaline. Standard consumption rate for harmalinewas first calculated as follows:

$20 = \frac{x}{7.9} \times 1000$ x = 0.158 gr

0.158 gr harmalinewas provided by HPLC methanol in a 10-ml flask. As density of the solvent methanol is 0.79, ppm is a w/w unit. Therefore, methanol weight was considered in calculations.

2-4- Respiratory chamber

Respiratory chamber was designed and made for inhalation experiments for rats. A glass chamber (thickness: 10 mm; length: 30 cm, width: 30 cm, height: 40 cm) was insulated by aquarium glue. A 1 cm wide hole was made in order to place the special hose for nebulizer mouth. Much precision was devoted in insulation to control rats inside the chamber.

2-5- Rats

Rats with varying weights and ages were adopted. 5 male and 15 female rats were bought from Pastor Institute and kept in the laboratory of Islamic Azad University of Damghan in order to obtain rats in three age groups (i.e. 20-, 35-, and 50-day old). The rats were kept in standard status (12h dark and 12h light) at 24+2°C and they were fed with special food and tap water.

2-6- Nebulizer

[1]HPLC model: Agilent 1200
Detector: UV vis VWD Agilent 1200 series, 330 nm
Column type: Agilent eclipse XDB C18. 5µm 4.6×150 mm
Method: Isocratic method (2-propanol, CAN, water, formic acid) (100, 100, 300, 0.3)
Flow: 0.75 ml/min

Nebulizer is a medical set which turns a medicine from liquid to aerosol. The aerosol can reach depth of lungs through inhalation. Nebulizer consists of a compressor, a white filter made of polyethylene (5µm holes), outlet valve (a 100cm long and 1cm wide plastic tube), and drug chamber (the main part of nebulizer placed at the end of the plastic tube). As air enters nebulizer, the liquid in drug chamber turns to aerosol. Nebulizer is able to spray 2 ml/min of the drug at 2.1-2.5 bar. The final aerosols are 0.53-13 µm wide.

2-7- The test process

Inhalation was decided to be performed at 9 a.m. when they are not exposed to any environmental stress (e.g. starvation, etc.). Afterwards, the rats were weighed and sorted in terms of weight and age. The rats were moved to the respiratory chamber through the valve. Insulation was repeated each time. Then, a given amount of the extract was poured into the drug chamber. Spraying was performed after nebulizer was turned on. This took 15 min in all tests when the rats inhaled the aerosols. The set was subsequently turned off and rats were kept inside the respiratory chamber for full inhalation. The rats were then put in a box for 5 min in order to eliminate any stress caused by being exposed to spraying. Finally, the rats were subjected to elevated-plus maze for behavioral test.

2-8- Behavioral determination by elevated-plus maze

Elevated-plus maze was adopted in order to assess fear. The set was made from wood and it consisted four arms in the form of +. Open arm was dark while closed arm was designed to be light. The arms were 5×10cm. Two ends of the closed arm were set at the height of 40 cm. A 1cm high glass edge was set at two ends of open arms to prevent the rats from falling down. Four arms reach a central area (10×10cm) called neutral area. The maze was placed at the height of 50 cm from the ground by a few legs. The rats were put in the neutral area so that their heads were toward closed arm. Proper lighting was provided by use of a 100v light placed at the height of 120cm from the maze center. The animal was let to walk freely in different parts of the maze for 5 min. the following parameters were measured during the experiments:

- Number of visits to open arms
- Number of visits to closed arms
- Time spent at the open arm
- Time spent at the closed arm
- Overall number of visits to arms

2-9- Determination of moisture percentage in the extract

Dry weight of the extract was assigned in order to assign the proper dose of Esfand extract. Some of the extract was first weighed. Another weighing was performed after drying in oven. The weight difference was considered as dry matter of the extract:

Dry weight: 1.750 mg Extract weight: 3000 mg

Dry weight (x) = 58 mg Extract weight: 100 mg

Moisture percentage in the extract: 100-58=42%

The dosage at 12 mg/kg was calculated as follows:

Dry weight (x) = 58 mg Extract weight: 100 mg

Used dose: 12 mg Extract weight (x) = 20.68 mg

Therefore, 12 mg effective material exists per 20.68 ml of the extract. 20.68 ml of the extract per each kg rats' bd. wt. was dried in oven and then, the sample (5 cc) was provided with saline.

2-10- Statistical analysis

Statistical analyses were performed via SPSS software through one-way ANOVA and Tukey Test at $p>0.05$ ($n<6$). The p-value for 20-day old group was estimated by Mann-Whitney Test because the data for this group were not normal according to Shapiro-Wilk Test.

Visiting open or closed arms were considered when whole rat body was in the arms. The time spents in arms was calculated this way. Percentage of number of visits and time spent in open arm were calculated as follows:

$$\text{Percentage of visit to open arm} = \frac{numberofvisitstoopenarm}{numberofvisitstoclosedarm+numberofvisitstoopenarm} \times 100$$

$$\text{Percentage of the time spent in open arm} = \frac{thetimespentinopenarm}{thetimespentinclosedarm+thetimespentinopenarm} \times 100$$

The mean visit for each group was calculated according to the time spent and the results were recorded. Furthermore, total visits to arms were considered as locomotion.

3- RESULTS

3-1- Soil

EC and pH of paste were found to be 10.5 and 7.6, respectively. Clay, silt, and sand were also found to be 18%, 12%, and 70%, respectively. The soil sample can thus be said to be S-L type.

3-2- Harmaline in the extract

Harmaline in the extract was estimated through HPLC method and it was found to be 21.244 mg/kg.

3-3- Comparing inhalation effect of Esfand seeds extract at 12 mg/kg on the time spent in open and closed arms as well as neutral area in 20-, 35-, 50-, and 20-35-50-day old rats in Elevated-plus maze

	Closed arm		Open arm		Neutral area	
	mean	Standard deviation	mean	Standard deviation	mean	Standard deviation
Test group	270.00	8.37	9.17	8.61	20.83	5.85
	263.83	15.82	17.50	6.31	27.83	11.20
	271.67	6.83	4.83	1.60	23.50	5.65
	267.50	16.36	8.50	8.12	24.00	9.72
Control group	226.00	14.42	34.83	7.57	39.17	13.23
	263.83	15.82	50.33	14.84	37.50	5.39
	170.83	11.14	84.17	7.36	45.00	7.07
	155.83	4.92	95.00	6.32	49.17	5.85

3-4- Comparing inhalation effect of Esfand seeds extract at 12 mg/kg on the number of visits to open and closed arms as well as neutral area in 20-, 35-, 50-, and 20-35-50-day old rats in Elevated-plus maze

	Closed arm		Open arm		Neutral area	
	mean	Standard deviation	mean	Standard deviation	mean	Standard deviation
Test group	3.67	0.52	1.33	1.37	3.83	0.98
	3.67	0.82	1.33	0.52	4.33	1.03
	4.17	0.41	1.00	-	4.33	0.82
	3.67	0.52	1.33	1.37	4.00	1.79
Control group	6.67	1.37	9.83	1.33	7.33	2.07
	6.17	0.75	4.33	0.52	8.17	0.98
	6.50	0.84	7.83	1.17	8.33	1.37
	6.67	1.37	9.83	1.33	9.67	1.03

3-5- Comparing inhalation effect of Esfand seeds extract at 12 mg/kg on the time spent between closed arm and neutral area as well as between open arm and neutral areas in 20-, 35-, 50-, and 20-35-50-day old rats in Elevated-plus maze

	Closed arm		Open arm	
	mean	Standard deviation	mean	Standard deviation
Test group	270.00	8.37	9.17	8.61
	263.83	15.82	8.33	6.31
	271.67	6.83	4.83	1.60
	267.50	16.36	8.50	8.12
Control group	226.00	14.42	34.83	7.57
	212.17	15.63	50.33	14.84
	170.83	11.14	84.17	7.36
	155.83	4.92	95.00	6.32

3-6- Comparing inhalation effect of Esfand seeds extract at 12 mg/kg on the number of visits to between closed arm and neutral area as well as between open arm and neutral areas in 20-, 35-, 50-, and 20-35-50-day old rats in Elevated-plus maze

	Closed arm		Open arm	
	mean	Standard deviation	mean	Standard deviation
Test group	3.67	0.52	1.33	1.37
	3.67	0.82	1.33	0.52
	4.17	0.41	1.00	-
	3.67	0.52	1.33	1.37
Control group	6.67	1.37	9.83	1.33
	6.17	0.75	4.33	0.52
	6.50	0.84	7.83	1.17
	6.67	1.37	9.83	1.33

3-7- Comparing locomotion in 20-, 35-, 50-, and 20-35-50-day old rats

	Locomotion	
	Test group	Control group
20-day old	5	16.5
35-day old	5	10.5
50-day old	5.17	14.33
20-35-50-day old	5	16.5

4- DISCUSSION

The present paper aimed at determining the effect of Esfand seeds extract inhalation at 12 mg/kg in 20-, 35-, 50-, and 20-35-50-day old rats. The results showed that the time spent in arms was higher in the rats inhaled the extract than control group. As staying in closed arm is a sign of fear, it can be concluded that inhalation of Esfand seeds extract resulted in higher fear in the rats. Also, a considerable reduction was detected in the number of visits to the arms and neutral area, especially to open arm, in the rats inhaled the extract. This is in agreement with the results acquired by Vaezi et al. (2012) who studied effect harmaline on stress in rats.

That harmaline and harmine exist in Esfand seeds extracts has been well documented (Berlin et al., 1993; Chatterjee&Ganguly, 1968; Nettleship&Slaytor, 1974; Pinner et al., 1995; Pan et al., 1997; Kartal et al., 2003; Herraiz et al., 2010). When harmaline connects to 5HT2A receptor in cell surface, arachidonic acid is produced. This compound is considered as one of effective intermediates in N-Methyl-D-Aspartat (NMDA) receptor. This receptor has been proven to play role in appearance of defensive behaviors such as fear.

Moreover, the time spent between closed arm and neutral area in the rats inhaled the extract was higher than control group; however, control rats spent more time between open arm and neutral area. This is also indicative of effect of Esfand seeds extract on fear behavior. The test rats visited open arm and spent more time there while reverse was found for closed arm. This, also, indicates the effect of Esfand seeds extract on fear behavior in the tested rats.

Fear induction in the present study can be traced in changed premotor system and consequently muscle vibration. Presumably due to the role of cholinergic system in fear behavior at 12 m/kg, fear occurs. In other words, the system inhibits muscarinic receptors. Such inhibition is competitive enzymatic system and activation occurs by priendoles (beta-carbolines with the aromatic ring C). This indicates that beta-carbolines at lower doses reduce fear behavior.

Future studies are directed toward determination of other neurotransmitters on fear behavior and comparison between them. Also, it is recommended to adopt fear-inhibiting drugs in order to assign defeating mechanisms affecting the extract performance. Finally, it is suggested to study the effect of Esfand seeds extract in other models and if possible, on humans.

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The authors declare that they have no conflicts of interest in the research.

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