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Effect of Stress on Anxiety in Streptozotocin Induced Diabetic Mice Using *Moringa oleifera* Lam. Pods



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ABSTRACT

Objective: This study was designed to investigate the effect of stress on anxiety in STZ-induced diabetic mice using ethanolic extract of *Moringa oleifera* Lam pod. **Materials and methods:** The elevated plus maze paradigm, hole board apparatus, open field test and light/dark box paradigm were used to assess the anxiolytic activity of ethanolic extract of *M. oleifera* lam. Pods (100, 200mg/kg, orally), Diazepam (1mg/kg, i.p.) were administered 30 min. prior the test and stress was applied using forced swim test, by providing empty drinking bottle, acute stress, water immersion (hot, cold), etc. at different time intervals. **Results:** The results showed that ethanolic extract of *M. oleifera* in presence of stress condition, in the elevated plus maze, the extract significantly increased exploration to open arm in same way to that of Diazepam. At the dose of 200mg/kg, the extract significantly increased both the entries into and time spent in the open arm by mice. In the hole board test, the extract significantly increased the number and duration of head poking. Further in open field test, the ethanolic extract of *M. oleifera* Lam. Pods significantly increased rearing, and number of squares traversed. In the light/dark paradigm, the extract produced significantly increase in time spent in lightbox as compared to control group. **Conclusion:** The results of present study suggest that the ethanolic extract of *M. oleifera* lam. pods extract may possess an anxiolytic effect.

INTRODUCTION

The stressed condition causes physiological changes, as anxiety or panic attacks, irritability, sadness or depression, the secretion of stress hormones (glucagon, catecholamine, cortisol and Growth Hormone) and especially, cortisol increases during the acute stress and emotional stimuli^[1]. Anxiety disorders are the most common forms of mental illness of the adult population. Use of animal models for anxiety has made an important contribution to clinical and pharmacological anxiety research as well as basic research of the mechanisms involved. Animal models of anxiety have been used in psychopharmacology mostly in relation to the success or failure of a given model in predicting the clinical anxiolytic potency of pharmacological agents.^[2] Animal models of anxiety in psychopharmacology are models of the effect of benzodiazepines (BZ), which mainly function via specific BZ-receptors in the brain. Animal models make a fundamental contribution to the area of anxiety research at the clinical, industrial and scientific level. The relationship between anxiety and stress is point of much interest. Chronic stress induces mood disorder like behavior in mammals including humans and it may be main factor in the development of anxiety.^[3] Exposure to various types of stress results in anxiogenic behavior in the animals.

Pods of *M. oleifera*, commonly referred to as "Shevaga" in Marathi is the most widely cultivated species of the genus *Moringa*, which is the only genus in the family Moringaceae. All parts of this plant are renewable sources of tocopherols (γ and α), phenolic compounds, β -carotene, vitamin C and total proteins, including the essential sulfur amino acids, methionine and cysteine. Unsaturated fatty acids, especially oleic acid, carbohydrates and minerals are present in the seed in reasonable amounts, while the leaves have appreciable amounts of saponins (80g/kg), besides low quantity of phytates (21g/kg) and tannins (12g/kg).^[5] *Moringa oleifera* is the most nutrient-rich plant yet discovered. *Moringa* provides a rich and rare combination of nutrients, amino acids, antioxidants, antiaging and anti-inflammatory properties used for nutrition and healing.^[6] The phytochemical reports on *M. oleifera* Lam. indicates that the pods contain, Alkaloids, Carbohydrates, Glycosides, Saponins, Flavonoids. A survey of the literature on *M. oleifera* revealed no or little investigative reports were found on its CNS activity; therefore, we undertook this study to determine the anxiolytic potential of *M. oleifera* by using different preclinical models for anxiety based on exploratory behavior. The goal of this study was to determine the

possible link between the stress, diabetes and anxiety. Short water immersions and other methods were used as the stressor. Its effects on serum cortisol level (SCL) in diabetic rats were observed.

MATERIALS AND METHODS

Extract preparation

The pods were collected from a local area in Aurangabad and authenticated at Botanical Dept. of Aurangabad; voucher specimen (No.0773) of the product has been preserved in same department for future reference. The pods were dried under shade and powdered by using grinder mixer. The powdered material (150g) was soaked in Petroleum ether (60 – 80⁰C) to remove lipids, filter it and filtrate was discarded, residue extracted with 95% ethanol by soxhlet for 72hr. After extraction the solvent should filter and evaporated in a vacuum, whatever residue may be obtained was evaporated on water bath to obtain solid brown coloured dry mass. For administration purpose, dried mass dissolved in distilled water.

Phytochemical screening

Phytochemical investigation of the extract for the presence of Alkaloid, Carbohydrates, Glycosides, Saponins, Flavonoids was done using methods which was described by Kokate (1994) and Trease and Evans (1997).

Animals

Swiss albino mice of either sex weighing between (25-40g) were used. They were maintained at temperature of 25 ± 2°C and relative humidity of 45 to 55% and under standard environmental conditions (12 h. light /12 h. dark cycles). The animals had free access to food and water. All the experiments were carried out between 9 to 18 hrs. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Y.B. Chavan College of Pharmacy Aurangabad (Approval number- CPCSEA/IAEC/P'col-20/2011-12/44).

Animals are randomly divided as, i) Normal Control (distilled water 0.1ml/10gm body wt. i.p.), ii) Diabetic control (distilled water 0.1ml/10gm body wt. i.p.), iii) Diabetic Stress (distilled water 0.1ml/10gm body wt. i.p.), iv) Diabetic Stress+ Metformin (120mg/kg, oral), v) Diabetic Stress +

Diazepam (1mg/kg, i.p.), vi) Diabetic Stress+ EMO (100mg/kg,oral), vii) Diabetic Stress+ EMO (200mg/kg, oral).

Chemicals

Diazepam (Ranbaxy Lab. Ltd. Mumbai) was used as standard drug for this study. Petroleum ether and Ethanol were purchased locally and of analytical grade, Glucose, Metformin (Sohan healthcare Pvt. Ltd.). Apparatus as Glucometer, Hole-board, Elevated plus-maze, Open field, Light/dark exploration apparatus.

Acute toxicity studies

Mortality and signs of any toxicity were observed and recorded after the administration of a dose of 50, 100, 200, 500, 1500, 2000 and 3000mg/kg orally and i.p. of ethanolic extract of *Moringa oleifera* in different groups of mice. The mortality rate was observed and recorded for 24hr.

Experimental procedures for stress

Experiments were performed in the morning between 08:00 and 12:00 h.^[9] Water immersion and other stressors program developed at our laboratory were used randomly to provoke the stress as it produces sense of fear of drowning and asphyxia. This stressor model was developed in our laboratory. Water immersion and forced swimming cause mainly a psychic stress (fear of drowning and asphyxia), which in turn causes a physical stress (vigorous physical activity). After the experiments, the rats were sacrificed by decapitation in the morning (8–10 h)^[10] and blood samples were collected, serum was separated and stored at -20°C until analysis.^[11,12] Then, serum cortisol level was measured by radioimmunoassay.^[10,13,14]

Some behavioural parameters which are used to test the anxiolytic activity-

Elevated plus-maze test (EPM): The EPM consisted of two open arms ($35 \times 5\text{cm}$) crossed with two closed arms ($35 \times 5 \times 20\text{cm}$). The arms were connected together with a central square of $5 \times 5\text{cm}$. The apparatus was elevated to the height of 25cm in a dimly illuminated room. Mice were treated with EMO (100, and 200mg/kg), diazepam (1mg/kg, i.p), or vehicle 30 min before being placed individually in the centre of the EPM, facing toward a closed arm. The time spent in both the open and closed arms was recorded for 5 min. The numbers of entries into the open and

closed arms were also counted during the test. An entry was defined as having all four paws within the arm.^[15]

Hole-board test: The hole-board apparatus consists of a wooden box (40 × 40 × 25cm) with 16 holes (each of diameter 3cm) evenly distributed on the floor. The apparatus was elevated to the height of 25cm. Mice were treated with the EMO (100 and 200mg/kg, Orally) or vehicle 30 min before they were placed in the apparatus. The numbers of head pokes during a 5-min period were recorded. Diazepam (1mg/kg, i.p.), an anxiolytic agent was used as a standard drug.^[8]

Open field test: The apparatus consisted of a wooden box (60 × 60 × 30cm). The floor of the box was divided into 16 squares (15 × 15cm). The apparatus was illuminated with a 40-W lamp suspended 100cm above. Mice were treated with EMO (100 and 200mg/kg) or vehicle. After 30 min, they were placed individually in one of the corner squares; the number of rearing, assisted rearing (forepaws touching the walls of the apparatus), and the number of squares crossed were counted for 5 min. Diazepam (1mg/kg, i.p.) was used as the positive control drug.^[8,9]

Light/dark exploration test: The apparatus consisted of two boxes (25 × 25 × 25cm) joined together. One box was made dark by covering its top with plywood, whereas a 40-W lamp illuminated the other box. The light source was placed 25cm above the open box. The mice were placed individually in the centre of the lit box and observed for the next 5 min for the time spent in the lit and dark boxes. The mice were treated with EMO (100 and 200mg/kg), diazepam (1mg/kg, i.p.), or vehicle 30 min before being placed in the lit box.^[16]

Statistical Analysis

All observations are given mean ± SEM and data were analysed using One way ANOVA followed by Dunnett's- test using INSTAT GraphPad .

RESULTS

Phytochemical Screening

Phytochemical screening of ethanolic extract of *Moringa oleifera* (MO) Lam. Pods show the presence of Alkaloid, Carbohydrates, Glycosides, Saponins, Flavonoids.

Acute toxicity studies

No mortality and no signs of any toxicity were evidence after the administration of a limit dose of 3000mg/kg ethanolic extract of *Moringa oleifera* in acute oral toxicity test hence, for oral administration the doses selected were 100mg/kg and 200mg/kg.

Serum cortisol levels

The STZ-treated stressed mice have increased cortisol level (8.00±1.19) when compared with control group. EMO at 100, and 200mg/kg brought about a significant ($p < 0.05$) and dose-dependent decrease in the cortisol level when compared with diabetic stress animals. Serum cortisol levels are listed in [Table 1]

Table 1. Effect of EMO treatment on Cortisol level in bloodstream of mice

Group	Treatment	Cortisol level (µg/DL)
A	Control	4.64±1.09
B	Diabetic control	5.97±0.09
C	Diabetic Stress (DS)	8.00±1.19*
D	DS + EMO (100mg/kg)	4.60 ±1.52*
E	DS+ EMO (200mg/kg)	4.09±1.09*

Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05 vs respective diabetic stress group.

Elevated plus-maze test

The vehicle-treated mice spent 28.11±5.30 s in the open arm and 241.20±6.44 s in the closed arm, with 6.50±0.99 entries into the open arm and 12.83±0.87 entries into the closed arm in EPM. EMO (100mg/kg) and diazepam (1mg/kg) induced significant ($P < 0.01$) increase in the occupancy in the open arm compared with diabetic stress group. EMO in the dose 200mg/kg did not cause a significant decrease in the time spent in the closed arm, whereas EMO at a dose of 100mg/kg and diazepam brought about a significant ($P < 0.01$) decrease in the time spent in the closed arm. The animals treated with diazepam and EMO (100mg/kg) showed a decreased preference for the closed arm and significantly ($P < 0.01$) increased entries into the open arm.

EMO at 200mg/kg did not produce any significant increase in open arm entries. [Table 2] [Fig.1].

Table 2. Effect of EMO treatment on animals stay in the open and enclosed arm on EPM

Group	Treatment	No. of entries in open arm	No. of entries in enclosed arm	Time spent in Open arm (sec.)	Time spent in enclosed arm (sec.)
A	Control	6.50±0.99	12.83±0.87	28.11±5.30	241.20±6.44
B	Diabetic control	2.33±0.55	15.16±0.54	21.02±2.50	247.63±5.87
C	Diabetic Stress(DS)	1.00±0.00	16.00±2.04	17.62±0.50	272.43±2.08
D	DS + Metformin (120mg/kg)	6.50±1.05**	12.33±0.88	47.96±17.60*	241.02±7.30
E	DS + EMO (100mg/kg)	10.50±0.76**	6.83±0.87**	100.50±2.71**	119.55±12.52**
F	DS+ EMO (200mg/kg)	6.00±1.03**	3.00±0.57**	30.48±2.41	258.95±5.74
G	DS+ Diazepam (1mg/kg)	11.00±0.96**	6.00±0.97**	140.21±1.88**	131.40±1.89**

Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05, and **P < 0.01 vs Respective diabetic stress group.

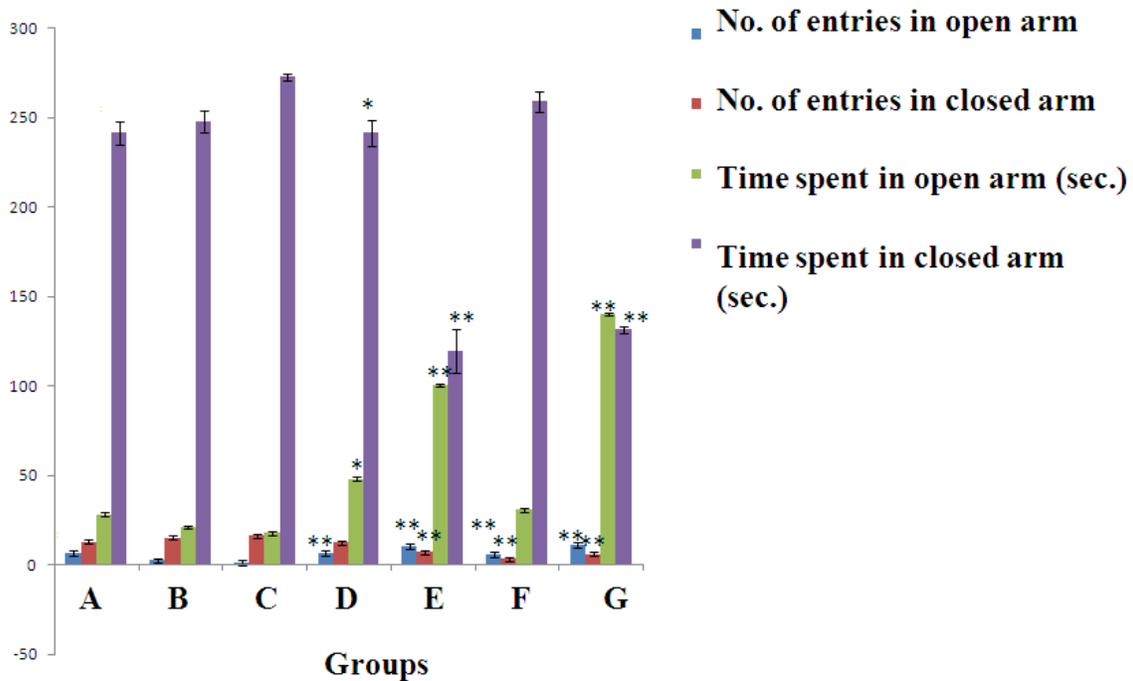


Fig. 1 Effect of EMO treatment on animals stay in the open and enclosed arm on EPM

Results are expressed as Mean \pm SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05 ,and **P < 0.01 vs Respective diabetic stress group.

Hole-board test

On the hole-board apparatus and the number of head pokes and the no. of square crossed were noted. With the dose of 120mg/kg of Metformin there was significant (P< 0.05) increase in number of head pokes when compared with diabetic stress. EMO at 100 and 200mg/kg, orally increased the number of head pokes significantly (P< 0.01) and dose-dependently. Diabetic and diabetic stress group showed non-significant decrease in number of head poking and square crossed. The reference standard (diazepam, 1mg/kg, i.p.) treated group showed significant increase in exploratory activity (P< 0.01). [Table 3][Fig.2]

Table 3. Effect of EMO treatment on no. of poking and no. of square crossed in Hole board test.

Group	Treatment	No. of poking	No. of square Crossed
A	Control	28.33±4.27	13.33±2.10
B	Diabetic control	24.83 ±1.75	5.16±0.47
C	Diabetic Stress (DS)	22.00 ±2.56	3.50±0.61
D	DS + Metformin (120mg/kg)	33.66 ±1.52 *	14.00±0.57
E	DS + EMO (100mg/kg)	31.83 ±0.79	15.50±0.84
F	DS+ EMO (200mg/kg)	36.33 ±1.08 **	13.66±1.30
G	DS+ Diazepam (1mg/kg)	36.83 ±4.11 **	19.66±2.21

Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05 ,and **P < 0.01 vs Respective diabetic stress group.

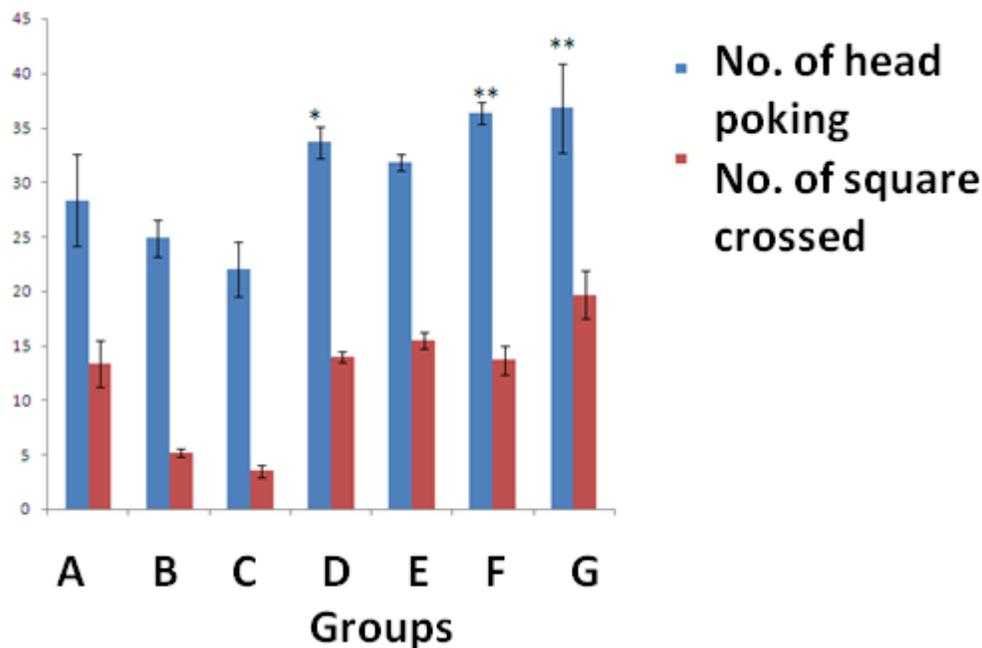


Fig. 2. Effect of EMO treatment on no. of poking and no. of square crossed in Hole board test.

Results are expressed as Mean \pm SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05, and **P < 0.01 vs Respective diabetic stress group.

Open field test

The STZ-treated stress mice traversed 33.83 \pm 7.06 squares and showed 11.83 \pm 3.77 rearing during the test interval of 5 min. EMO at 100 and 200mg/kg, Metformin and diazepam brought about a significant (P< 0.01 and P< 0.05) and dose-dependent increase in the number of squares traversed. The rearing was significantly (P< 0.05 and P< 0.01, respectively) increased by EMO (100 and 200mg/kg) and diazepam when compared with diabetic stress animals. [Table 4][Fig.3]

Table 4. Effect of EMO treatment on no. of square crossed and rearing in open field test.

Group	Treatment	No. of square crossed	Rearing
A	Control	63.33 \pm 9.00	18.50 \pm 3.45
B	Diabetic control	35.50 \pm 7.16	12.66 \pm 3.69
C	Diabetic + Stress (DS)	33.83 \pm 7.06	11.83 \pm 3.77
D	DS + Metformin (120mg/kg)	63.00 \pm 10.27*	27.83 \pm 2.53**
E	DS + EMO (100mg/kg)	83.83 \pm 4.23**	26.33 \pm 2.67*
F	DS+ EMO (200mg/kg)	83.83 \pm 1.53**	26.83 \pm 2.02**
G	DS+ Diazepam (1mg/kg)	87.00 \pm 4.78**	27.83 \pm 3.00**

Results are expressed as Mean \pm SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05 ,and **P < 0.01 vs Respective diabetic stress group.

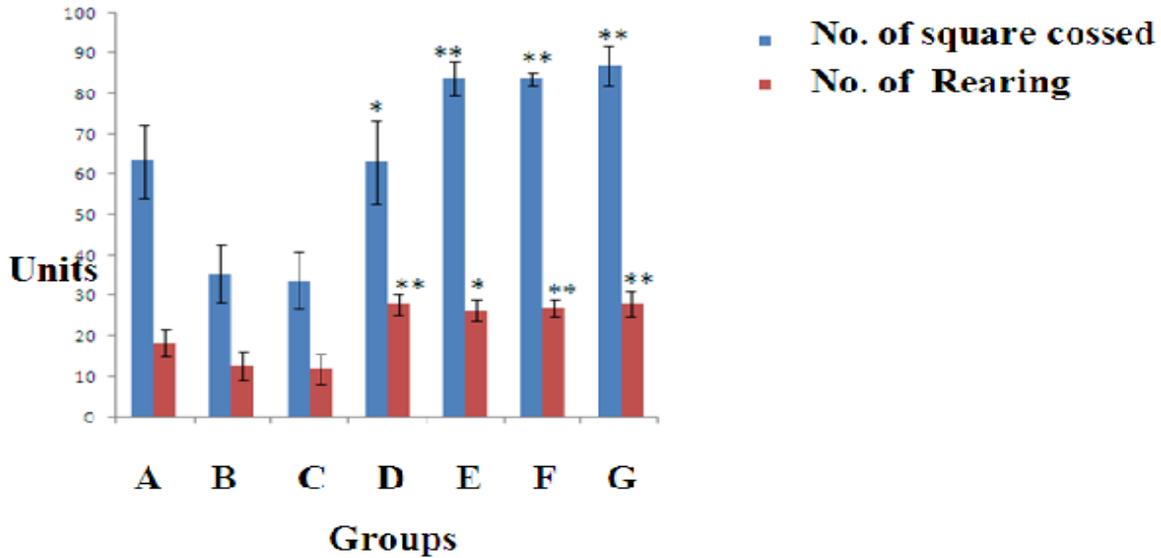


Fig. 3. Effect of EMO treatment on no. of square crossed and rearing in open field test.

Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05, and **P < 0.01 vs Respective diabetic stress group.

Light/dark exploration test

The animals treated with diazepam (1mg/kg) showed significant (P< 0.05 and P< 0.01) increase in the time spent in the lighted box and decrease in the time spent in the dark box. EMO (100mg/kg) and Metformin (120mg/kg) showed non-significant increase in the time spent in the lighted box and decrease the time spent in the dark box when compared with diabetic stress animals. EMO (200mg/kg) failed to produce any significant change in the transfer latency [Table 5] [Fig.4]

Table 5. Effect of EMO treatment on time spent and transfer latency in light/dark exploration model.

Group	Treatment	Time spent in lighted box (sec)	Time spent in dark box (sec)
A	Control	102.86±4.09	197.13±4.09
B	Diabetic control	77.66±12.86	222.33±12.89
C	Diabetic + Stress (DS)	75.00 ±12.74	226.00±12.89
D	DS + Metformin (120mg/kg)	93.27±9.73*	205.72±10.56
E	DS + EMO (100mg/kg)	98.48 ±13.77*	201.52±13.77
F	DS+ EMO (200mg/kg)	110.76 ±4.33*	189.23±4.33*
G	DS+ Diazepam (1mg/kg)	203.67±2.09**	170.49±22.28*

Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05, and **P < 0.01 vs Respective diabetic stress group.

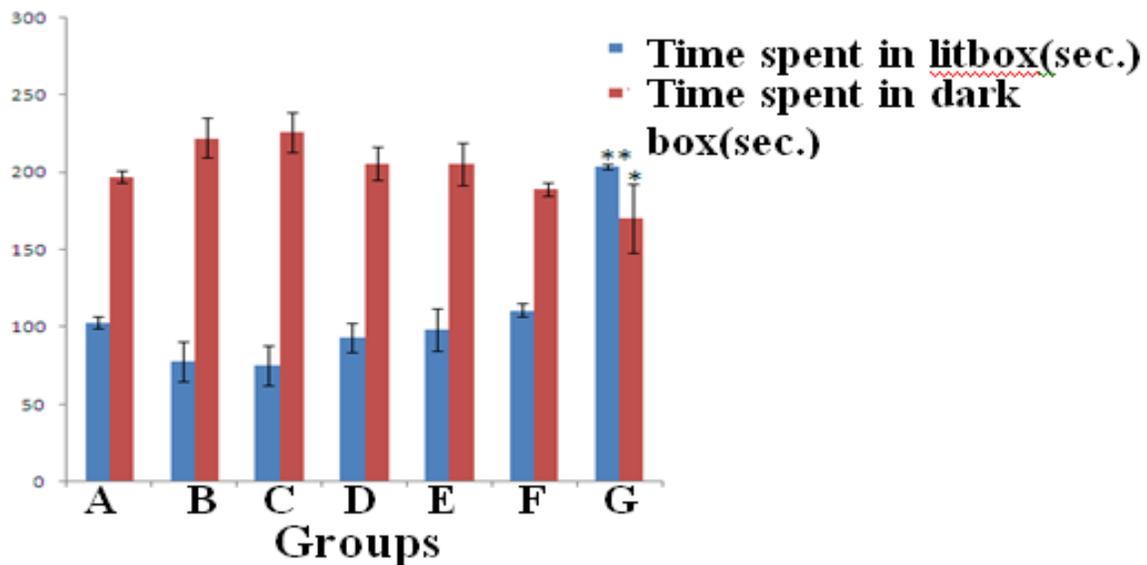


Fig. 4. Effect of EMO treatment on time spent and transfer latency in light/dark exploration model.

Results are expressed as Mean \pm SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05, and **P < 0.01 vs Respective diabetic stress group.

DISCUSSION

Research shows there are strong links between anxiety disorders and depression and diabetes.^[17] The benzodiazepines (BZDs) are relatively safe and are widely used anxiolytic agents. These agents are known to act through the BZD-GABA receptors. The role of GABA in anxiety is well established.^[18]

The present results showed that the stress caused by water immersion and other methods caused a significant increase in Serum Cortisol level in streptozotocin diabetic rats and it is responsible for anxiogenic behavior in animal.

The present study demonstrated that the ethanolic extract of *M. oleifera* Lam. Pods had anxiolytic effects in mice in several behavioural parameters, like the elevated plus-maze, hole-board, open field, and light/dark paradigms and also have anti-stress activity^[19] because it decreases the serum cortisol level in EMO treated diabetic animals. The anxiolytic activity of some agents have been assessed by using the hole-board test.^[20] A significant increase in the exploratory head-dipping behaviour was observed after treatment with 100 and 200mg/kg of *M. oleifera* Lam. Pods extract, thus reinforcing the hypothesis that it has anxiolytic-like activity.

The EPM is one of most useful animal test for research on behavioral pharmacology of anxiety. In EPM, mice will normally prefer to spend much of their time in the closed arms. This preference appears to reflect an aversion towards open arms that is generated by fear of open spaces. Drugs that increase open arm exploration are considered as anxiolytics.^[21] In this study, we observed that EMO (100 and 200mg/kg) induced significant increase in the both the number of entries and time spent in the open arms. The number of entries and the time spent in the closed arms were reduced in the extract-treated group as compared to the diabetic stress and control group. The results obtained in the open field test showed that EMO administration significantly increased rearing, and number of squares traversed, which supports the anxiolytic-like activity of EMO.

The light and dark paradigm is based on the natural aversion of mice to brightly lit places. Anxiolytics reduce the natural aversion to light and increase the time spent in the lit compartment. In this model, compared to diabetic stress group, EMO produced significant increase in the time spent in the lighted box and decrease in the time spent in the dark box, thus demonstrating its anxiolytic-like activity.

The chemical constituents of plants and their pharmacology suggest that plants containing flavonoids, saponins, and alkaloids possess activity against many CNS related disorders. Phytochemical tests of EMO revealed the presence of flavonoids and saponins. It is possible that the mechanism of anxiolytic action of ethanolic extract of *Moringa oleifera* could be due to the binding of any of these chemicals to the GABA_A-BZD complex. In support of this, it has been hypothesized that flavones, which are present in *M. oleifera* Lam pods, bind with high affinity to the BZD site of the GABA_A receptor.^[19]

The results obtained in this study suggest that the ethanolic extract of *Moringa oleifera* lam. Pods possess the anti-stress and anxiolytic activity which is possibly mediated through GABA_A-BZD mechanism. Thus, *M. oleifera* has potential clinical application in the management of anxiety and stress. Further investigation of mechanism of action of the plant extract as well as the active substance responsible its biological action is necessary.

CONCLUSION

The results of present study suggest that the ethanolic extract of *M. oleifera* lam. pods extract may possess an anxiolytic effect.

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