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Comparative Effects of Orange Blossom, Violet, and Marjoram Extracts and Lorazepam on Sleep Deprivation-Induced Anxiety in Mice

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Sleep deprivation leads to various disorders, and abnormal anxiety is one of the most important among those. Although hypnotics, such as lorazepam, are effective in this regard, the respective agents have numerous side effects. Nutritional treatments (such medicinal herbs) are sometimes better than chemical medicine and can be recommended for this purpose. In our study, we compared the effects of ethanolic extracts of three medicinal herbs (orange blossom, violet, and marjoram) and lorazepam on the level of anxiety related to sleep deprivation in mice. Fifty male mice were assigned to five groups, the control (C), lorazepam (L), orange blossom (OB), violet (V), and marjoram (M). Animals in each group were treated orally with these herbal extracts, Lorazepam, or water for 10 days. The sleep deprivation protocol was then performed for all groups using a water column set with platforms. Twenty-four hours after the start of the sleep deprivation protocol, the effects of the above agents on the anxiety level were compared using the elevated plus maze, light/dark box, and marble burying tests. In addition, the effects of these agents on the plasma cortisol level were analyzed. It was found that extracts of orange blossom and marjoram were more effective than the violet extract and lorazepam solution in reducing anxiety caused by sleep deprivation. Among the herbal extracts, the orange blossom extract was the most effective in this regard.

Keywords: mice, anxiety, extracts, orange blossom, marjoram, violet, lorazepam, behavioral tests.

INTRODUCTION

Sleep plays an important role in maintaining stable body conditions and adequate functioning of the brain [1]. Sleep deprivation leads to pathologically high anxiety, cognitive disorders, and other psychological problems [2]. As aging progresses, side effects of sleep deprivation can be exacerbated due to the increased susceptibility to stressors [3]. Irritability, a predisposition to violence, cognitive impairment, and high anxiety are among the most common side effects of sleep deprivation among the elderly [4]. Anxiety is a diffused, unpleasant, and vague feeling of worry and fear with an unknown origin, which involves uncertainty, helplessness, and physiological excitation [5]. To understand the anxiety caused by sleep deprivation in our study, male mice were selected, and the effects of orange blossom, violet, and marjoram extracts recommended for anxiety treatment in traditional medicine and also the analogous effects of lorazepam were evaluated in these animals. Insomnia was induced using the water platform model. The effectiveness of herbal extracts and lorazepam was measured by behavioral tests. Since abnormal anxiety caused by sleep deprivation is accompanied by increases in the plasma cortisol levels, hormonal measurements were also conducted on blood samples taken from experimental mice.

METHODS

Animals. Experiments were carried out on 50 male mice with a mean body mass of 30–35 g, which were assigned to five groups of 10 animals in each; the mice were kept and fed in special cages. During the treatment, animals were kept under an illumination regimen of 12/12 h light and darkness at 25°C. The floor of cages was covered with sterile straw. The mice had easy access to water and food.

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Preparation of Herbal Extracts and Lorazepam Solution. First, medicinal herbs required for the preparation of extracts were purchased, and their authenticity was confirmed before use. The required amount of each herb material was separately ground with an electric mill. Then, 50 g of the resulting powder was weighed and divided into two 25-g parts. Each part was added to 300 ml of 80% ethanol, and the flasks were mixed on a shaker at 200 rpm for 24 h. The resulting mixtures were filtered. Then, ethanol was removed using a rotary evaporator. The content was poured into Petri dishes and placed in an oven at 50°C for 48 h. The obtained contents (dry matter) were scraped and weighed. The dry matter of each herb extract was kept in a freezer at –50°C. The solutions prepared from each extract and lorazepam were kept in lidded glass containers at –20°C until application of the treatments.

Treatment with the Extracts. All mice (five groups of 10) were perorally treated with herbal extracts, lorazepam, or water for 10 days using a gavage feeding syringe. Group L was treated with lorazepam solution (1.4 mg/kg), group C was treated with water, and three groups were treated with the extracts namely violet (V, 100 mg/kg), orange blossom (OB, 130 mg/kg), and marjoram (M, 100 mg/kg).

Sleep Deprivation Protocol. After 10 days of gavage with the mentioned solutions, the sleep deprivation protocol was used employing a water column model [6]. Animals of each group were put in a container filled with water at room temperature. The containers had 10 platforms in two rows, which made it possible for the mice to freely move from one platform to another. After the mice entered the REM phase of sleep, they experienced muscle relaxation and, therefore, fell into water and woke up.

Anxiety-Related Behavior. Twenty-four hours after the induction of insomnia, the mice were gently dried using a towel and transferred to an animal behavior assessment room. The following three behavioral tests were performed to determine the level of anxiety caused by sleep deprivation and stress and to find out how effective the extracts and lorazepam were in reducing the anxiety level.

Elevated Plus Maze (EPM) Test. The test used an elevated plus-shaped device (50 × 10 cm) made of wood, with two open and two enclosed arms. There were 40-cm-long walls on the two sides and at the ends of the closed arms. To prevent the fall of animals, 1-cm edges were embedded on the sides and ends of open arms. Four arms reached a central square (10 × 10 cm). The maze was kept at a height of 50 cm from the ground [5]. To perform the test, the animals were separately placed at the center of the maze and allowed to freely search around for 10 min. During this period, their behaviors were recorded by a video camera installed on the top of the maze. The parameter used for investigating the anxiety-like behavior was the duration of stay in the open arms (increased duration of presence in these arms indicated lower levels of anxiety).

Light/Dark Box Test. This test is based on the intrinsic aversion of rodents to bright light when searching in a new environment. The test box (46 × 27 × 30 cm) consisted of two chambers, the small one as the dark area and the larger one as the light area. The two chambers were connected to each other through a hole (7.5 × 7.5 cm) at the bottom of the dark part [7]. Each mouse was individually placed at the center of the larger chamber in front of the hole, and its behavior was recorded by a video camera installed on the top of the box. The parameter used for investigating anxiety-related behavior was the duration of stay in the light chamber (increased duration of presence in this chamber was regarded as a lower level of anxiety).

Marble Burying Test. This test is based on defensive burying behavior of rodents in response to potentially dangerous stimuli; marble balls may induce the above type of behavior [7]. To perform this test, animals were individually put for 30 min in standard cages with 15 glass marbles evenly placed in each cage. The floors of the cages were covered with sterile straw to a depth of 5 cm. Defensive burying behavior was studied by counting the number of buried marbles. Higher numbers of buried marbles indicated greater levels of anxiety.

Serum Cortisol Measurement. After performing behavioral tests, the serum cortisol level was measured to evaluate systemic anxiety characterized by the level of plasma corticosteroids. Animals were anesthetized, and a blood sample was taken from the heart using a special syringe. The samples were centrifuged, and their serum parts were separated using a sampler. The serum samples were analyzed in an immunoassay analyzer in a medical diagnostic laboratory (in strict accordance with the producer's instruction), and the cortisol concentrations were measured. Since the plasma cortisol level increases with increased anxiety, higher concentrations of plasma cortisol were regarded as correlates of the greater levels of anxiety.

Statistical Analysis. Quantitative data were analyzed using SPSS-19. Mean values in different groups after treatment, indices obtained in behavioral tests, and plasma corticosteroid measurement results were compared. ANOVA (in the case of equality of the variances) and the Welch test (in the case of inequality of the variances) were used to compare different groups in terms of the effectiveness of herbal extracts and lorazepam in reducing anxiety caused by sleep deprivation. The results are reported below as means ± s.e.m. The selected level of significance in intergroup comparisons was $P < 0.05$. If the ANOVA and Welch test gave statistically significant results, paired comparisons were also made. Since the sample size was smaller than 50, the Shapiro-Wilk test was used to examine the normal distribution of the data.

RESULTS

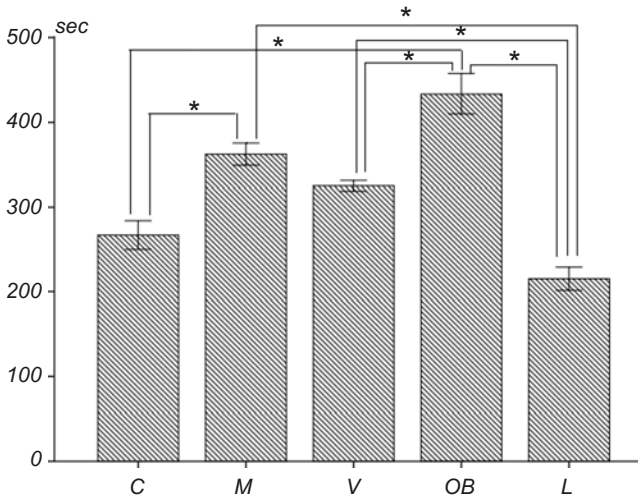
Elevated Plus Maze (EPM). The Shapiro-Wilk test confirmed the normality of the data distribution ($P > 0.05$), and the Levene's test rejected the equality of variances ($P < 0.05$). The Welch test showed that there were significant differences between the groups in the mean time spent in the open arms of the maze (F (4.3/66) = 22.085, $P < 0.05$).

Results of the Dunnett's test (Fig. 1) demonstrated that the times spent in the open arms of the EPM by animals of groups OB and M were significantly longer than others ($P < 0.05$). The duration of presence in the open arms of the EPM in the V group was significantly longer than that observed in the L group but smaller than that in the OB group ($P < 0.05$). The differences between other groups were statistically insignificant ($P > 0.05$).

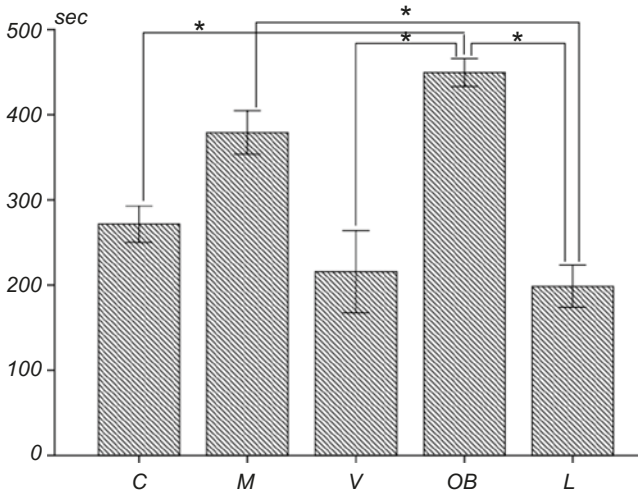
Light/Dark Box Test. The normality of the data distribution was confirmed ($P > 0.05$; Shapiro-Wilk test), and the Levene's test rejected the equality of variances ($P < 0.05$). The Welch test showed that there were significant between-groups differences in the mean time spent in the light chamber (F (4.13/214) = 21.017, $P < 0.05$).

Results of the Dunnett's test (Fig. 2) indicated that the mean time spent in the light chamber by mice of the OB group was significantly longer than that shown by mice of the C group ($P < 0.05$). In addition, the mean time spent in the light chamber by animals of the V and L groups was significantly shorter than that of the OB group ($P < 0.05$). There were no significant differences between the other groups ($P > 0.05$).

Marble Burying Test. The Shapiro-Wilk test confirmed the normality of the data distribution ($P > 0.05$), and the Levene's test confirmed the equality of variances ($P > 0.05$). ANOVA showed that there were significant differences between the experimental groups in the mean number of buried marbles (F (4.33) = 17.554, $P < 0.05$). The results of the Tukey's test (Fig. 3) demonstrated that the mean number of marbles buried by the OB group



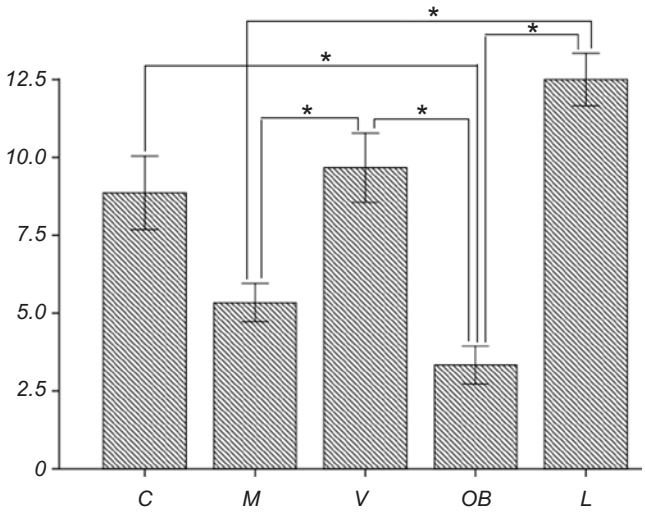
F i g. 1. Comparison of the time (sec) spent in the open arms of the elevated plus maze. Designation of the groups: control (C) and treated with marjoram (M), violet (V), and orange blossom (OB) extracts and with lorazepam (L). The data presented in diagrams are means ± s.e.m. Asterisks denote significant differences between the compared groups ($P < 0.05$).



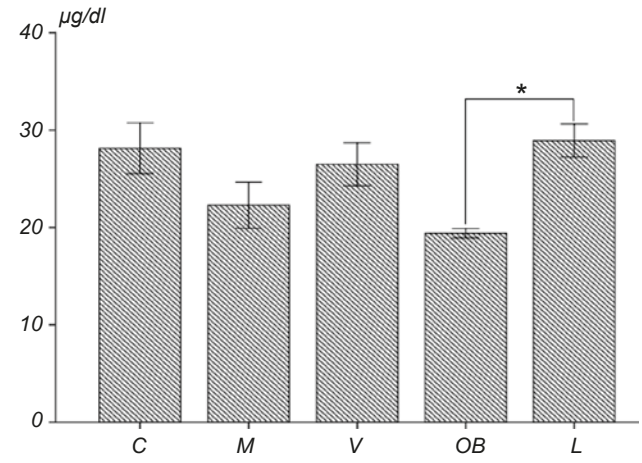
F i g. 2. Comparison of the mean time (sec) spent in the illuminated area in the light/dark box test. Designations are similar to those in Fig. 1.

was significantly smaller than those in the C, V, and L groups ($P < 0.05$). However, no significant differences were observed between the other groups ($P > 0.05$).

Measurement of the Serum Cortisol Level. The Shapiro-Wilk test confirmed the normality of the data distribution ($P > 0.05$), and the Levene's test rejected the equality of variances ($P < 0.05$). ANOVA results demonstrated that there were significant differences between the groups in the serum level of cortisol (F (4, 12,357) = 9.979, $P < 0.05$). Results of the Dunnett's test showed that the serum cortisol concentration in the OB group was significantly lower than that in the L group ($P < 0.05$). As shown in Fig. 4, the cortisol level in the OB group was lower than those in other groups, but the differences did not reach the significance level ($P > 0.05$).



F i g. 3. Comparison of the mean number of marbles buried by animals of different groups during 30 min in the burying test. Designations are similar to those in Figs. 1 and 2.



F i g. 4. Comparison of the mean serum cortisol levels between the experimental groups. Designations are similar to those in Figs. 1–3.

DISCUSSION

In the EPM test, extracts of orange blossom and marjoram caused a significant increase in the time spent by mice in the open arms compared to that in the L group and in the control. Since a longer duration of the presence in the open arms indicates a lower level of anxiety, it can be stated that extracts of orange blossom and marjoram exerted clear positive effects in reducing anxiety.

In the light/dark box test, extracts of orange blossom and marjoram also caused a significant increase in the time spent in the light chamber compared to those in animals treated with lorazepam and water. This also suggests the effectiveness of orange blossom and marjoram extracts in reducing anxiety.

In the marble burying test, the orange blossom extract caused a significant reduction in the number of marbles buried during 30 min compared to the effects of other extracts, lorazepam, and water. Since the higher number of buried marbles is indicative of higher anxiety levels, it can be concluded that the orange blossom extract significantly decreased manifestations of anxiety. Therefore, the orange blossom extract in the dose used was more effective than other extracts and lorazepam in lowering the anxiety levels.

Our results also demonstrated that the orange blossom extract caused a significant reduction in the serum cortisol level; the drop was greater than in the case of action of lorazepam. However, the reduction was not statistically significant compared to the control. Therefore, although the orange blossom extract at the dose of 130 mg/kg was effective in cortisol reduction, higher doses of this herbal extract should probably be recommended.

According to the published data, the orange blossom (*Citrus aurantium* L.) extract has often been used to treat anxiety. Woelk et al. [8] studied the antianxiety effects of the orange peel essence on the results of the EPM test in rats. Their findings indicated that the duration of presence in the open arms of the maze increased noticeably after a period of treatment with this extract.

Antianxiety and sedative effects of the orange blossom extract and orange essential oil on rats

were investigated in another study [7]. In these experiments, the orange blossom extract was administered to the animals orally for 30 min, or the orange blossoms essential oil was injected once a day for 15 days. Then the light/dark box test and marble burying test were performed on the animals. Based on the results of the light/dark box test, a single administration of the essential oil to mice increased their time of presence in the light chamber, whereas daily injection of the essential oil did not cause any significant change in this duration. However, both single and daily treatments with the essential oil suppressed the defensive burying behavior.

In a study of the effects of orange blossom essential oil on anxiety-related behavior and on its interaction with the GABAergic pathways, such oil was i.p. injected into male rats at different doses for 5 days, and 0.1 mg/kg of lorazepam solution was also injected 30 min before the essential oil injection on the fifth day. Evaluations of anxiety-related behaviors using the EPM test indicated that the mean duration of presence in the open arms was significantly longer in animals treated with 2.5 and 5% orange blossom essential oil ($P < 0.05$). The findings suggested that this essential oil could reduce manifestations of anxiety-related behavior in male rats through the action on the GABAergic pathways [9].

One of the active compounds in orange blossoms is limonene; in general, it reduces the activity of neurons in the CNS. After reaching the brain, this compound attacks GABA receptors and provides reduction of reduces their anxiety-related activities. Another study showed that limonene reduced anxiety levels by reducing the effects of GABA through influencing GABA-A receptors. Therefore, limonene is one of the basic compounds of orange blossom with antianxiety properties [9].

Coumarin is another compound in orange blossom, which exhibits similar sedative effects. Pereira et al. [10] showed that injection of coumarin into the prefrontal cortex and hippocampus of rats could increase secretion of GABA into the prefrontal cortex by affecting GABA-A receptors [9].

Linalool is another compound in orange blossoms that exhibits inhibitory effects on the CNS through modulation of presynaptic inhibition and acetylcholine release prevention. Linalool is an antagonist of glutamate receptors that prevents epilepsy and its subsequent anxiety by affecting the above receptors [9].

Other major compounds in orange blossom are flavonoids that cause a tranquil feeling by increasing contact with benzodiazepine receptors. In the study on rats, Mahmoudi et al. [11] showed that flavonoid compounds in orange blossom could play a considerable role in reducing anxiety in rats. Flavonoids are believed to be ligands for GABA-A receptors and, as reported in many studies, possess characteristics and mechanisms of action similar to those of benzodiazepines. Thus, a number of flavonoids are called herbal benzodiazepines [9].

The sedative and antianxiety effects of marjoram (*Origanum majorana* L.) and diazepam on two groups of male Wistar rats were compared earlier [12]. Members of one animal group were given the marjoram extract at doses of 100, 200, and 400 mg/kg, while other experimental rats were treated with 1.2 mg/kg of diazepam plus dimethyl sulfoxide. About 30 min after the induction of REM sleep by 40 mg/kg of ketamine, the antianxiety effects of marjoram and diazepam were evaluated using the EPM test. As was found, there were significant increases in the sleep duration induced by ketamine and in the duration of presence in the open arms by rats treated with the marjoram extract compared to the respective indices in the group receiving diazepam. Moreover, the marjoram extract exhibited greater sedative and antianxiety effects at the dose of 200 mg/kg [12].

In another study [13], the antianxiety effects of European marjoram (*Origanum vulgare* L.) were compared with those of normal saline in two groups of rats. One group received the extract at doses of 50, 100, and 200 mg/kg, while the control group received the same volume of normal saline. Results of the EPM test demonstrated that there was a significant increase in the duration of presence in the open arms for rats treated with the marjoram extract compared to control animals.

There are several species of marjoram, the chemical compounds of which differ from each other depending on the species, harvest time, and plant growth stages. Phenolic and aromatic compounds, including carvacrol and thymol, are the main active components in these plants. These compounds affect neuronal and neuroendocrine activities by influencing calcium-dependent potassium channels in the CNS. Since phenolic compounds and ursolic acid in the marjoram extract are able to cross the blood-brain barrier and exert effects preventing oxidative stress in the nervous system, these agents were believed to have antianxiety properties. One

of the compounds in marjoram extract is borneol, an agent exhibiting analgesic effects and increasing activity of GABA receptors. Considering the existence of flavonoids in the marjoram extract, the sedative and antianxiety effects of the latter have been attributed to its influence on benzodiazepine receptors connected to GABA-A receptors [9].

In a study on 50 patients with chronic insomnia, the sedative and hypnotic effects of violet oil were investigated. To this end, drops of violet oil were administered intranasally before sleeping at night for one month. The patients were asked to fill out the questionnaire of the Insomnia Severity Index (ISI) before and one month after the treatment. Results demonstrated a substantial improvement in the sleep quality and indices of ISI in patients after treatment with this oil ($P < 0.05$). In addition, administration of violet oil caused side effects only in single patients [13].

Our study indicated that orange blossom extract (300 mg/kg) and marjoram extract (100 mg/kg) were more effective than violet extract (100 mg/kg). The orange blossom and marjoram extracts were found to be more effective than lorazepam solution (1.4 mg/kg) in reducing the anxiety level caused by sleep deprivation in mice.

All applicable international, national, and/or institutional guidelines for the care and experimental use of animals were observed in the present study. .

The authors, T. Hajjar, M. Arhami, and M. R. Vaezi Kakhki, declare the absence of any conflict in commercial or financial relations, relationships with organizations or persons that in any way could be related to the study, and also in interrelations of the co-authors.

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