Investigating the Antidepressant Impact of Ethanolic Extract of *Melilotus officinalis* Fruit in Mouse Models

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ABSTRACT

Melilotus officinalis (*M. officinalis*) has potent anti-inflammatory, anti-anxiety, antioxidant, and anticonvulsant activities. Considering the above, the current study aimed to investigate the antidepressant impact of ethanolic extract of *M. officinalis* fruit in mouse models. In the current study, the acute toxicity of the ethanolic extract of *M. officinalis* was investigated by the Loreck method. Adult male mice were treated with fluoxetine, normal saline, or ethanolic extract of *M. officinalis,* and then Forced swim and Tail suspension tests were performed on them. The locomotor activity of mice was also investigated in the open-field test. The findings showed that the median lethal dose of *M. officinalis* extract was more than 5000 mg/kg. Intraperitoneal administration of this extract (except for the dose of 25 mg/kg in the Forced swim test) decreased the time of immobility in all groups in the Tail suspension and Forced swim test. This extract also enhanced the time of swimming without significant changes in the climbing time in the Forced swim test. The locomotor activity of rats in the open field test was not affected by the ethanolic extract of *M. officinalis*. The findings of this study show that *M. officinalis* is a non-toxic plant and has an antidepressant impact similar to fluoxetine. Thus, this plant has a therapeutic effect on depression. However, further studies are essential to investigate the exact mechanism of antidepressant impacts and the absence of adverse effects in chronic administration.

Key Words: *Antidepressant effect, Fruit, Melilotus officinalis, Ethanolic extract*

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INTRODUCTION

Major depressive disorder is a common and debilitating psychiatric disorder that affects approximately 17% of people at some point in their lives [1, 2]. Studies revealed that changes in brain monoamine levels (such as serotonin and noradrenaline), dysfunction of the HPA (Hypothalamic Pituitary Adrenal Axis), inflammatory-immune processes, oxidative and nitrative stresses, neurodegeneration, and inhibition of neurogenesis play a role in the depression pathogenesis [3-5].

Although many antidepressant drugs are available, their slow therapeutic effects (weeks to months), poor response, and even high side effects have led to a decrease in their use and a greater shift to the use of less complicated and highly effective drugs (especially herbal medicines) [6, 7]. Today, medicinal plants and their extracts are used as natural therapies with high potential for the treatment of

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different diseases (including depression and anxiety). In this regard, hundreds of plants are used for this purpose in traditional medicine [8]. Common antidepressants consist of SSRIs (selective serotonin reuptake inhibitors) such as fluoxetine, SNRIs (selective norepinephrine reuptake inhibitors) such as reboxetine, and MAOIs (monoamine oxidase inhibitors) such as selegiline. Most of these drugs act by increasing the concentration of brain monoamines in the synaptic cleft [3].

Melilotus officinalis (*M. officinalis*) is an edible plant belonging to the legume family. The plant closely resembles fenugreek and alfalfa. The fruit of this plant is fragrant and crescent-shaped and has been traditionally used to treat rheumatic and migraine pain. Previous studies have reported the analgesic and ulcerogenic, anticonvulsant, antioxidant, antitumor, anti-inflammatory [9], and anti-anxiety [10] effects of *M. officinalis*. Studies have also revealed that oxidative stress and inflammation play a vital role in depression pathogenesis [11-13].

The TST (tail suspension test) and FST (forced swim test) are the most valid and common tests for examining depression in laboratory mice. Compared to TST, FST is inexpensive and a fast and reliable tool. FST is sensitive to acute treatment, and its disadvantages include poor levels and swimming stress. The most important advantages of TST are its simplicity and cheapness, and the absence of swimming and temperature stress. Like FST, this test is also sensitive to acute treatment [14-16].

Given the lack of studies on the antidepressant impacts of *M. officinalis* extract, the current study aimed to investigate the antidepressant impact of *M. officinalis* fruit ethanol extract in FST and TST tests and to investigate locomotor activity in the open field test in male mice.

MATERIALS AND METHODS

Preparation of ethanolic extract of M. officinalis fruit

First, the fruit of *M. officinalis* was prepared and after examining the appearance and approval of the botanist, it was used for the next steps. The percolation method was used to prepare the ethanolic extract. For this purpose, 100 grams of powdered and dried *M. officinalis* fruit was placed in a decanter funnel with 900 ml of 80% ethanol (in 3 stages with 300 ml each time) for 48 hours. After opening the decanter valve and collecting the internal liquid, the ethanol in the extract was separated using a rotary device (temperature 40 °C). After concentration and drying, the extract was stored in a refrigerator away from light and away from light until use.

Animals

In this experimental study, adult male mice with an average weight of 20 to 30 grams were utilized. The animals were obtained from the university animal breeding center and kept under standard laboratory conditions, including a light cycle of 12 hours of light and 12 hours of darkness, at a temperature of 23 ± 2 °C. The mice had free access to water and prepared food except during the test period. In the present study, the principles of handling laboratory animals were followed by the rules for the protection and maintenance of laboratory animals and the statements of the ethics committee.

Drugs

In this study, fluoxetine hydrochloride was used. In this study, all drugs and extracts were injected intraperitoneally into the animals in a specific volume of 10 ml/kg. 9% normal saline was also used to dissolve the drugs and extracts.

Acute toxicity study of ethanolic extract of M. officinalis using Lorke's method

For this purpose, 12 male mice received different doses of ethanolic extract as follows: 1- Three mice received a dose of 10 mg/kg intraperitoneally. 2- Three mice received a dose of 100 mg/kg intraperitoneally. 3- Three mice received a dose of 1000 mg/kg intraperitoneally.

If no mortality is observed in this section and the mice show resistance, higher doses of the extract will be used as follows: 1 - One mouse will receive a dose of 1600 mg/kg intraperitoneally. 2 - One mouse will receive a dose of 2900 mg/kg intraperitoneally. 3 - One mouse will receive a dose of 5000 mg/kg intraperitoneally [17].

After the injection of the above doses, the mice were monitored for 24 hours for any signs of toxicity, including the number of deaths, abdominal contractions, tremors, constipation, motor activity, etc.

Animal grouping

In this section of the study, a total of 60 mice were divided into 10 groups of 6 in a completely random manner.

FST test section

The first group or negative control group received normal saline at a dose of 10 ml/kg. The second group or positive control group received fluoxetine at a dose of 20 mg/kg. The third to fifth groups received different doses of ethanolic extract of *M. officinalis*, namely 50, 100, and 200 mg/kg. In each group, 30 minutes after the injection of the drug or extract, the mice were subjected to the FST test.

FST test

In this test, the reduction in the immobility duration or the increase in the duration of swimming or climbing is considered an antidepressant effect. After receiving the drugs or ethanolic extract, the animals were placed individually in a 5-liter beaker with the specifications (8 × 12 × 25) containing water at a temperature of 25 °C. In this

test, the cessation of arm and leg movements was recorded as the duration of immobility, the circular movements of the mouse around the cylinder as the duration of swimming, and the climbing of the walls of the beaker as the duration of climbing. The time interval of this test was 6 minutes, the first 2 minutes were considered for the animal's habituation to the environment, and in the last 4 minutes, the above behaviors were recorded by a timer or stopwatch in seconds and by a person who did not know the groups [14, 15, 18].

TST test section

The experimental design of this group was similar to the FST test, but separate mice were tested as follows: Group 1, or the negative control group received normal saline at a 10 ml/kg dose. Group 2 or the positive control group received fluoxetine at a dose of 20 mg/kg. Groups 3 to 5 received different doses of *M. officinalis* ethanolic extract, namely 50, 100, and 200 mg/kg. In this method, mice were also subjected to the TST test 30 minutes after the injection of the compounds.

TST test

In this animal test, metal stands 70 cm high were used. A 50 cm long string was stretched between the two metal stands in a longitudinal direction and the mouse's tail was fixed with a strap. Then, the test began with the mouse's motor activity. The time interval during which the mouse was inactive and immobile was recorded as the duration of immobility with a stopwatch in seconds. This test also lasted 6 minutes, with the first 2 minutes being used for the animal's adaptation to the environment and the remaining 4 minutes being recorded as immobility [14, 15, 18].

Open field test

To study the impact of ethanolic extract of *M. officinalis* on locomotor activity such as walking and locomotion, the animals were placed separately in a Plexiglas box (40 × 50 × 60 cm). For this purpose, the number of times the mice passed through each square and stood on their feet was recorded using a counter. The duration of this test was also 5 minutes, with 1 minute being used for the animal's adaptation to the environment, and the animal's behavior was recorded in the next 4 minutes [14, 15, 18].

Statistical analysis

In this study, the results were expressed as the mean ± standard deviation for 6 mice in each group. One-way variance analysis was used, followed by the Newman-Keuls post hoc test. Excel 2016 was used for graphing and GraphPad Prism 9 was used for data analysis. In all calculations, $P < 0.05$ was considered a level of significance.

RESULTS AND DISCUSSION

Acute toxicity of ethanolic extract of M. officinalis

The results demonstrated that the doses studied (10 to 5000 mg/kg) did not cause mortality in mice, but in the group treated with doses above 1000 mg/kg, symptoms such as spasms, gait disturbance, and withdrawal were observed. Therefore, according to the Lorek method, the LD50 of *M. officinalis* was above 5000 mg/kg and this plant is practically non-toxic in terms of toxicological classification.

Antidepressant impact of ethanolic extract of M. officinalis fruit in FST test on immobility duration

The findings of the variance analysis demonstrated that there was a significant difference in immobility duration between treatments $F [(4, 25) = 140, P < 0.0001]$. According to **Figure 1**, the results of the Newman-Keuls post hoc test indicate that doses of 100 and 200 mg/kg of *M. officinalis* extract significantly decreased immobility duration in the FST test compared to the control group. Fluoxetine also reduced immobility duration as a standard drug. Of course, fluoxetine was more potent than different doses of the extract in reducing immobility duration in this test ($P < 0.05$).

Antidepressant impact of ethanolic extract of M. officinalis fruit in FST test on swimming duration

The findings of the variance analysis demonstrated that there was a significant difference in swimming duration between treatments $F [(4, 25) = 104, P < 0.0001]$. According to **Figure 2**, doses of 100 and 200 mg/kg of ethanolic extract of *M. officinalis* significantly increased swimming time in the FST test than the control group. Fluoxetine, as a standard drug, also increased swimming time. However, fluoxetine was more potent than the different doses of the extract in increasing swimming time in this test ($P < 0.05$).

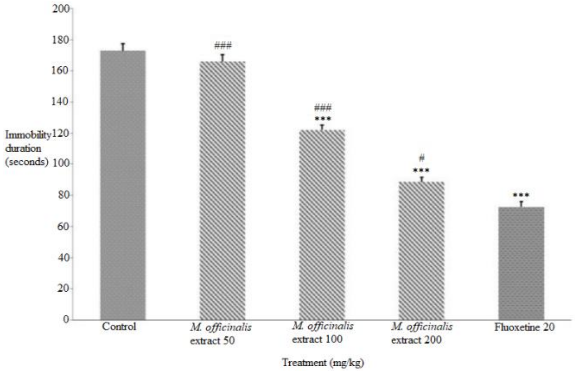

Figure 1. Impact of ethanolic extract of *M. officinalis* fruit on immobility time in the forced swim test. ***Significant difference with normal saline group (negative control) with $P > 0.001$; #Significant

difference with fluoxetine (positive control) with P > 0.05; and ### P < 0.001. Findings are expressed as mean ± standard deviation for 6 mice in each group.

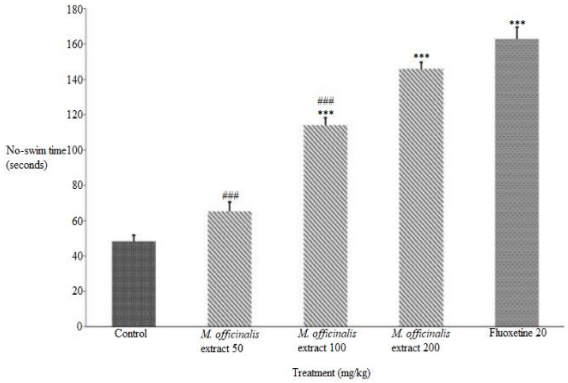

Figure 2. Impact of ethanolic extract of *M. officinalis* fruit on swimming time in FST test. *** Significant difference with normal saline group (negative control) with P < 0.001. ### Significant difference with fluoxetine (positive control) with P < 0.001. Findings are expressed as mean ± standard deviation for 6 mice in each group.

Antidepressant impact of ethanolic extract of M. officinalis fruit on climbing time in the FST test

According to **Figure 3**, none of the doses of ethanolic extract of *M. officinalis* and even fluoxetine could significantly increase climbing time in the FST (P > 0.05).

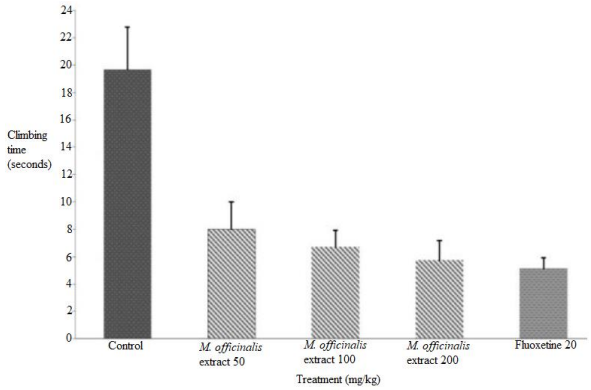

Figure 3. Impact of ethanolic extract of *M. officinalis* fruit on climbing time in the FST. Results are expressed as mean ± standard deviation for 6 mice per group.

Antidepressant impact of ethanolic extract of M. officinalis in TST on immobility duration

Based on the results of a one-way analysis of variance, there is a significant difference in immobility duration in TST between treatments F [(4, 25) = 96.9, P < 0.0001]. The results of **Figure 4** show that all three doses of *M. officinalis* extract (50 to 200 mg/kg) significantly reduce immobility duration in TST than the control group. Fluoxetine also reduces immobility duration. However, unlike FST, fluoxetine was weaker than the different doses of the extract in reducing immobility duration in this test (P < 0.05).

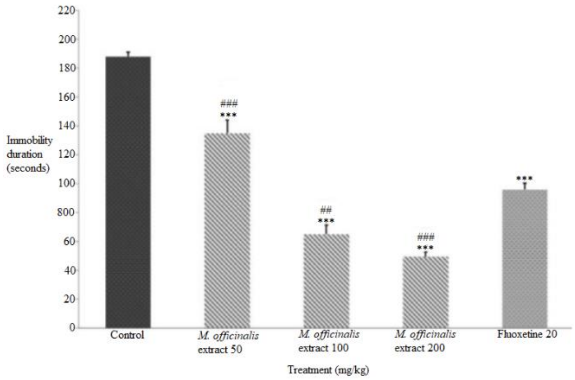

Figure 4. Impact of ethanolic extract of *M. officinalis* fruit on immobility time in TST test. *** Significant difference with normal saline group (negative control) with P < 0.001; # Significant difference with fluoxetine (positive control) with P < 0.05; and ### P < 0.001. Findings are expressed as mean ± standard deviation for 6 mice in each group.

Impact of ethanolic extract of M. officinalis on activity of locomotor in the open field test

The results of **Table 1** show that none of the doses of ethanolic extract of *M. officinalis* caused a significant alteration in the number of crossings of the square and standing on two legs than the control group (P > 0.05).

Table 1. Impact of ethanolic extract of *M. officinalis* fruit on locomotor activity or number of square crossings and standing on two legs in the open field test.

Group	Dose (intraperitoneal injection) (ml/kg)	Number of crossings through the square	Number of standing on two legs
Normal saline (control)	10	30.34 ± 67.3	10.70 ± 50.2
***M. officinalis* plant extract**	50	80.28 ± 26.2	8.832 ± 1.19
***M. officinalis* plant extract**	100	25.80 ± 2.15	50.9 ± 12.1
***M. officinalis* plant extract**	200	30.25 ± 80.1	83.9 ± 1.17

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In the current study, the acute toxicity of the ethanolic extract of the fruit of *M. officinalis* was investigated using Lorke's method. Also, the antidepressant impact of this extract was studied in the FST and TST tests and the effects of the extract on the animal's motor activity were investigated in the Open-fielded test. The findings of the acute toxicity section revealed that the ethanolic extract of the fruit of *M. officinalis* did not cause mortality even at a dose of 5000 mg/kg; therefore, this extract is practically non-toxic in terms of toxicological classification. Consistent with our findings, biochemical and pathological studies have reported that the extract of *M. officinalis* does not have toxic effects [19].

The findings of the FST test section indicated that doses of 100 and 200 mg of ethanolic extract of *M. officinalis* decreased the immobility time than the control group. Of course, these doses significantly enhanced the swimming time in the FST test, but none of the extract doses changed, or rather, significantly increased, the climbing time. On the other hand, the effects of the standard drug (fluoxetine) were also exactly similar to *M. officinalis* extract. However, all three doses of *M. officinalis* extract caused a weaker reduction in immobility time compared to fluoxetine. On the other hand, in agreement with previous results, the findings of the current study demonstrated that fluoxetine significantly enhanced swimming time and decreased immobility time. However, climbing time did not show a significant increase in fluoxetine [18].

The findings of the TST test also revealed that all three doses of the extract and fluoxetine decreased the immobility duration in this test, although fluoxetine in this test was weaker than the doses of 100 and 200 mg of the extract in reducing the immobility duration, which could be due to the difference in the types of FST and TST tests; therefore, according to the findings of the current research and previous research, the antidepressant effects of *M. officinalis* extract are similar to fluoxetine, or in other words, serotonergic drugs.

Our results also showed that different doses of the extract did not affect the animal's locomotor activity. In other words, in the open field test, the number of crossings through the square and standing on two legs did not increase or decrease significantly with any of the extract doses. In line with our findings, it has been reported that antidepressants do not cause significant changes in the animal's locomotor activity in the open field test [18]. However, some drugs such as mental stimulants pentobarbital, opioids, etc. cause false positive responses and significant increases in the number of crossings through the square and the number of standing on two legs in this test. Therefore, in the case of these drugs, the reduction in immobility and the increase in locomotor activity are not due to antidepressant effects, but rather to their stimulant effects [20].

Analysis of the compounds present in the *M. officinalis* plant has shown the presence of two significant groups of phenolic compounds, namely - hydroxycoumarin and flavonoids (such as kaempferol) [21]. Studies have stated the antidepressant impact of phenolic compounds [22-24]. In other words, a study has shown that coumarin compounds inhibit the monoamine oxidase enzyme [25]. Monoamine oxidase inhibitory drugs (such as selegiline) inhibit this enzyme and prevent the breakdown of neurotransmitters such as serotonin, dopamine, and norepinephrine in the brain, and exert their antidepressant effect through this mechanism [3].

Flavonoids also have antidepressant properties. In other words, flavonoids (such as kaempferol) exert their antidepressant effect by increasing the amount of serotonin, dopamine, and norepinephrine, and reducing serotonin metabolism in the brain [26]. It has also been stated that the *M. officinalis* extract is rich in phenolic compounds and causes antidepressant effects in the FST test in mice. The findings of the current research demonstrated that this extract showed antidepressant impacts in acute use at a dose of 300 mg/kg and subacute use at a dose of 30 to 300 mg/kg. The results of this study revealed that this extract has an effect through the serotonergic mechanism and decreases the duration of immobility and increases the duration of swimming, which is completely consistent with the findings of the current study [27].

In addition to the above findings, it has been shown that *M. officinalis* extract significantly improves oxidative stress and has a good antioxidant effect [9]. Previous studies have also revealed that oxidative stress plays a vital role in depression and its pathogenesis [12]. Oxidative imbalance in depression causes a decrease in the activity of the enzyme Superoxide dismutase (SOD) and an increase in the production of Nitric oxide. In this regard, previous studies have shown that *M. officinalis* extract causes a decrease in the production of Nitric oxide [28]. Therefore, it is likely that *M. officinalis* causes antidepressant effects in mice by decreasing the production of Nitric oxide. Of course, antidepressant drugs also reduce depression symptoms in patients by balancing the processes of oxidative stress. For example, fluoxetine, as an antidepressant, has antioxidant properties [12].

Of course, inflammation also plays a vital role in the pathogenesis of depression. In other words, previous research has shown that the amount of pro-inflammatory cytokines such as is increased in depressed individuals and even in laboratory mice [11]. *M. officinalis* has also reduced inflammatory cytokines such as IL-1β, TNF-α, IL-6, and NF-Kb in an aged mouse model [29]. Therefore, the

anti-inflammatory effect of *M. officinalis* can also play a vital role in reducing depression caused by it in depressed mice.

Based on the discussion, since there are numerous bioactive compounds in *M. officinalis* extract, it seems that this extract can exert its antidepressant effects through multiple mechanisms including antioxidant, serotonergic, and anti-inflammatory pathways, although the precise determination of the mechanism requires further studies.

CONCLUSION

The current research aimed to study the antidepressant impact of ethanolic extract of *M. officinalis* fruit in mouse models. The findings of the current study demonstrate that *M. officinalis* is a non-toxic plant and has an antidepressant effect similar to fluoxetine. Thus, this plant has a therapeutic effect on depression. However, more studies are essential to investigate the exact mechanism of antidepressant effects and the absence of adverse effects in chronic administration.

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