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The Antidepressant Effects of *Melilotus officinalis* Fruit Ethanolic Extract: A Mouse Model Study

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ABSTRACT

Melilotus officinalis (*M. officinalis*) is widely known for its anti-inflammatory, antioxidant, anticonvulsant, and anti-anxiety properties. The present study focused on evaluating the antidepressant potential of the ethanolic extract of *M. officinalis* fruit in a murine model. To assess toxicity, the Loreck method was used to determine the acute toxicity of the extract. Adult male mice were treated with fluoxetine, saline, or the ethanolic extract of *M. officinalis*. Behavioral assessments, including the forced swim test and tail suspension test, were performed, along with an open-field test to measure locomotor activity. The results showed that the lethal dose (LD50) of *M. officinalis* extract was higher than 5000 mg/kg. Treatment with the extract (excluding the 25 mg/kg dose in the forced swim test) significantly reduced the immobility time in both behavioral tests, while also increasing the time spent swimming without altering climbing behavior. Furthermore, no significant changes in locomotor activity were observed in the open-field test. These findings suggest that *M. officinalis* has a low toxicity profile and exhibits antidepressant effects comparable to fluoxetine. Further research is needed to clarify the underlying mechanisms and assess the long-term safety of the extract.

Keywords: *Melilotus officinalis*, Antidepressant effects, Ethanolic extract, Fruit, Behavioral tests

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Introduction

Major depressive disorder (MDD) is a widespread mental health condition that impacts around 17% of individuals at some point in their lifetime [1, 2]. The causes of depression are multifactorial, involving altered brain chemical levels, particularly serotonin and noradrenaline, as well as disruptions in the hypothalamic-pituitary-adrenal (HPA) axis, inflammation, oxidative stress, neurodegeneration, and suppressed neurogenesis [3-5].

While various antidepressant medications are available, their prolonged onset of action, suboptimal effectiveness, and numerous side effects have led to a decrease in their use. As a result, there is a growing interest in exploring natural treatments, such as herbal remedies, which are often considered simpler and more effective alternatives [6, 7]. Numerous plants have been utilized in traditional medicine for their potential antidepressant effects [8]. Common antidepressants, such as fluoxetine (an SSRI), reboxetine (an SNRI), and selegiline (an MAOI), generally work by increasing the concentration of brain neurotransmitters in the synaptic cleft [3].

Melilotus officinalis (*M. officinalis*), also known as sweet clover, is an edible plant from the legume family that resembles fenugreek and alfalfa. Its crescent-shaped, fragrant fruit has been traditionally used for treating conditions like rheumatic and migraine pain. Studies have shown that *M. officinalis* possesses multiple

pharmacological properties, including analgesic, anticonvulsant, anti-inflammatory [9], antioxidant, and anti-anxiety effects [10]. Moreover, recent research emphasizes the significant role of oxidative stress and inflammation in the development of depression [11-13].

The tail suspension test (TST) and forced swim test (FST) are widely recognized as the most reliable and frequently used methods for assessing depression in laboratory mice. While FST is relatively inexpensive, fast, and reliable, it does have some limitations, including its sensitivity to acute treatments and stress related to swimming. In contrast, the TST is simpler and more cost-effective, with the added benefit of not inducing swimming or temperature-related stress. Like FST, it is also responsive to acute treatments [14-16].

Considering the limited research on the antidepressant effects of *M. officinalis* extract, this study aimed to evaluate the antidepressant potential of the ethanolic extract of *M. officinalis* fruit using both FST and TST. Additionally, the locomotor activity was assessed through the open-field test in male mice.

Materials and Methods

Preparation of ethanolic extract of M. officinalis fruit

The fruit of *M. officinalis* was first prepared, identified, and approved by a botanist before further use. The ethanolic extract was prepared using the percolation method. A total of 100 grams of powdered, dried fruit was placed in a decanter funnel with 900 ml of 80% ethanol, divided into three equal portions of 300 ml each, and left for 48 hours. Afterward, the decanter valve was opened to collect the liquid extract, and the ethanol was removed using a rotary evaporator at 40°C. The concentrated extract was then dried, stored in a refrigerator, and kept away from light until further use.

Animals

For this experiment, adult male mice weighing between 20 and 30 grams were used. These animals were sourced from the university's animal breeding facility and housed under standard laboratory conditions, which included a 12-hour light and 12-hour dark cycle and a controlled temperature of 23 ± 2 °C. The mice were provided with free access to water and standard food, except during the experimental tests. All procedures followed ethical guidelines for animal handling and care, as stipulated by the relevant ethics committee and regulations for laboratory animal protection.

Drugs

Fluoxetine hydrochloride was the drug used in this study. All drugs and extracts were administered to the mice via intraperitoneal injections at a volume of 10 ml/kg. The extracts and drugs were dissolved in 9% normal saline.

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

Twelve male mice were used for the acute toxicity assessment of the ethanolic extract. The mice were divided into groups and given the following doses intraperitoneally:

1. Three mice received 10 mg/kg.
2. Three mice received 100 mg/kg.
3. Three mice received 1000 mg/kg.

If no deaths occurred and the animals exhibited no signs of severe distress, higher doses were administered as follows:

1. One mouse received 1600 mg/kg.
2. One mouse received 2900 mg/kg.
3. One mouse received 5000 mg/kg [17].

The mice were monitored for 24 hours post-injection to observe for signs of toxicity, including death, tremors, abdominal cramps, constipation, changes in motor activity, or other unusual behaviors.

Animal grouping

A total of 60 mice were randomly assigned to 10 groups, with six mice in each group, for further experimental procedures.

FST test setup
In the forced swim test (FST), the first group, acting as the negative control, received a 10 ml/kg dose of normal saline. The second group, as the positive control, was administered fluoxetine at 20 mg/kg. The third through fifth groups were given various doses of the ethanolic extract of *M. officinalis*, specifically 50, 100, and 200 mg/kg. Thirty minutes following the administration of these substances, the mice were subjected to the FST.

FST procedure
The FST measures antidepressant effects by observing changes in the duration of immobility, swimming, or climbing. After receiving the treatments, each mouse was placed individually in a 5-liter beaker (dimensions: 8 × 12 × 25 cm) filled with water at a temperature of 25 °C. The time spent immobile (when the mouse stopped moving), swimming (when the mouse swam in circles), and climbing (when the mouse attempted to climb the beaker's walls) were recorded. The total duration of the test was 6 minutes, with the first 2 minutes allocated for the mice to acclimate to the environment, and the last 4 minutes used to measure the behaviors of interest, all timed in seconds by an observer unaware of the group assignments [14, 15, 18].

TST test setup
The tail suspension test (TST) followed a similar design as the FST. However, separate mice were used for this test. Group 1, as the negative control, was given normal saline at 10 ml/kg, and group 2, the positive control, received fluoxetine at 20 mg/kg. Groups 3 through 5 were administered different doses of *M. officinalis* ethanolic extract: 50, 100, and 200 mg/kg. The TST was conducted 30 minutes after the substances were injected.

TST procedure
For the TST, two vertical metal stands were positioned 70 cm apart, with a 50 cm length of string stretched between them. Each mouse's tail was attached to the string using a strap, and the mouse's behavior was observed. Immobility time was recorded, which referred to the period when the mouse was inactive. This test also lasted for 6 minutes, with the first 2 minutes allocated for acclimatization, and the remaining 4 minutes measured for immobility [14, 15, 18].

Open field test
To assess the effect of *M. officinalis* ethanolic extract on locomotor behavior, each mouse was placed in a Plexiglass box (40 × 50 × 60 cm). The number of crossings and rearing events (when the mouse stands on its hind legs) were recorded with a counter. This test lasted for 5 minutes, with 1 minute for acclimatization and the following 4 minutes used to record the animal's movements [14, 15, 18].

Statistical analysis
The results are presented as mean ± standard deviation, based on six mice per group. To assess differences between the treatments, one-way ANOVA was performed, followed by the Newman-Keuls post hoc test. Graphical data representation was done using Excel 2016, while statistical analysis was conducted using GraphPad Prism 9. A significance level was set at $P < 0.05$.

Results and Discussion

Acute toxicity of ethanolic extract of M. officinalis
The toxicological assessment revealed that no mortality occurred across all tested doses (10–5000 mg/kg). However, higher doses, particularly those above 1000 mg/kg, led to symptoms such as tremors, impaired movement, and withdrawal reactions. According to the Loreck method, the LD50 for the ethanolic extract of *M. officinalis* exceeded 5000 mg/kg, indicating the plant is non-toxic.

Antidepressant effects in the FST: changes in immobility duration
Analysis showed a significant variation in immobility duration across the treatment groups ($F[(4, 25)] = 140$, $P < 0.0001$). As illustrated in **Figure 1**, the post hoc analysis indicated that the 100 and 200 mg/kg doses of *M. officinalis* extract significantly reduced immobility time compared to the control. Fluoxetine also decreased immobility time, although it was more effective than the extract ($P < 0.05$).

Antidepressant effects in the FST: changes in swimming duration

The variance analysis revealed a significant difference in the swimming duration between the groups ($F[(4, 25)] = 104$, $P < 0.0001$). **Figure 2** shows that the 100 and 200 mg/kg doses of *M. officinalis* extract significantly increased swimming time when compared to the control. Fluoxetine also enhanced swimming duration but was found to be more potent than the plant extract ($P < 0.05$).

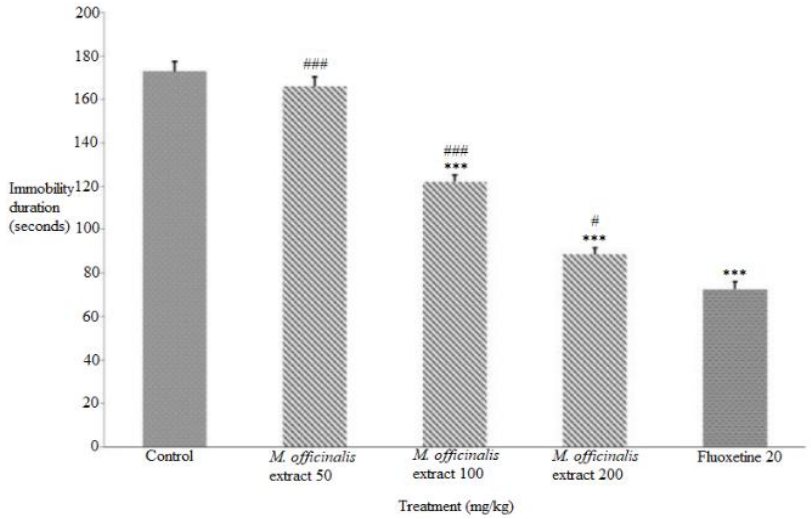

Figure 1. Effect of ethanolic extract of *M. officinalis* fruit on immobility duration in the forced swim test; *indicates a significant difference compared to the normal saline (negative control) group ($P < 0.001$); #represents a significant difference compared to fluoxetine (positive control) group ($P < 0.05$); ###denotes a significant difference at $P < 0.001$; the results are presented as mean ± standard deviation for six mice in each group.

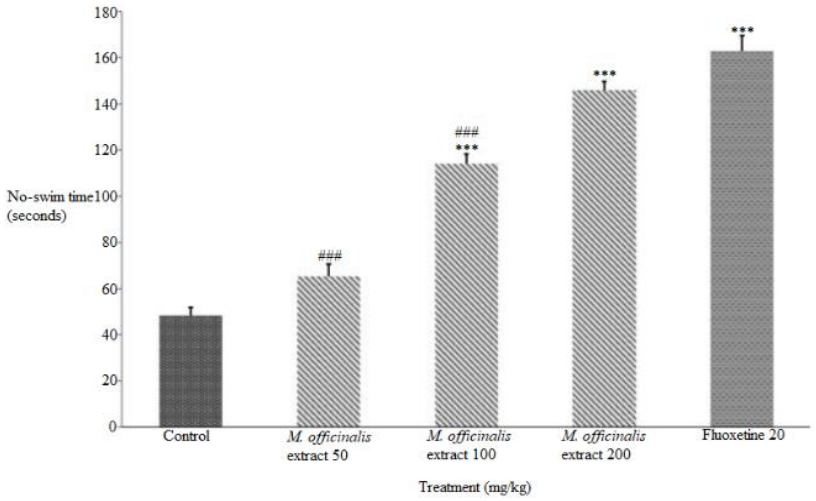

Figure 2. Effect of ethanolic extract of *M. officinalis* fruit on swimming duration in the forced swim test; *indicates a significant difference compared to the normal saline (negative control) group ($P < 0.001$); ###represents a significant difference compared to the fluoxetine (positive control) group ($P < 0.001$); the results are presented as mean ± standard deviation for six mice in each group.

Effect of ethanolic extract of M. officinalis fruit on climbing duration in the forced swim test (FST)

As shown in **Figure 3**, none of the doses of the ethanolic extract of *M. officinalis*, norfluoxetine, produced a significant increase in climbing duration during the FST ($P > 0.05$).

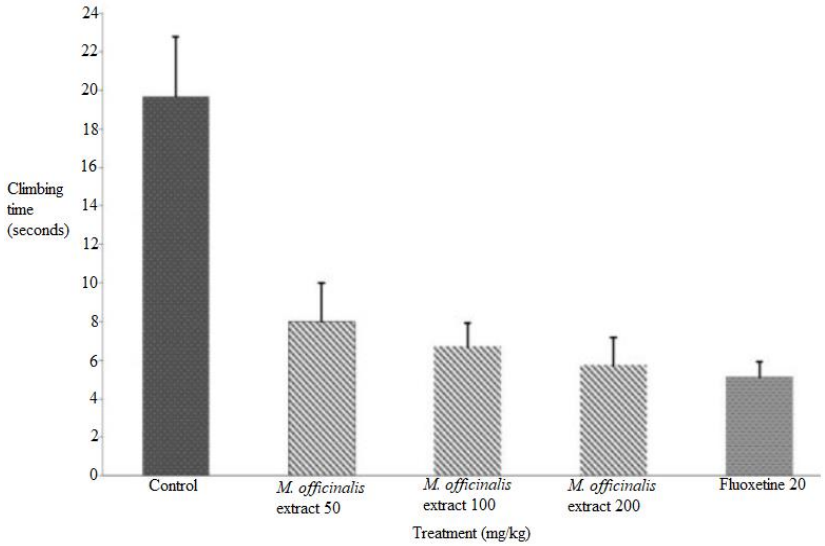

Figure 3. Impact of ethanolic extract of *M. officinalis* fruit on climbing time in the FST; results are expressed as mean ± standard deviation for 6 mice per group.

Effect of ethanolic extract of M. officinalis on immobility duration in the tail suspension test (TST)

The analysis of variance revealed significant differences in the duration of immobility in the TST among different treatments ($F [(4, 25) = 96.9, P < 0.0001]$). As depicted in **Figure 4**, all tested doses of the M. officinalis extract (50, 100, and 200 mg/kg) notably reduced the immobility time when compared to the control group. Fluoxetine also decreased immobility, although it was less effective than the extract in this test ($P < 0.05$).

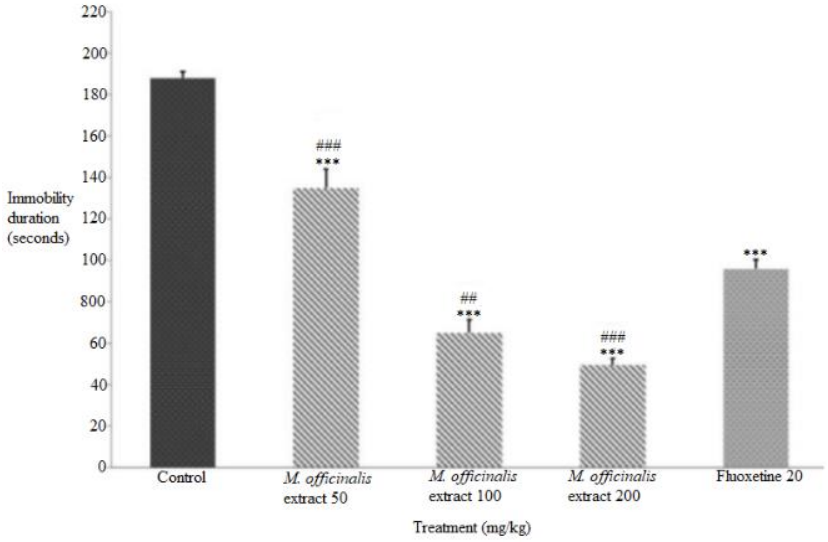

Figure 4. Influence of the ethanolic extract of *M. officinalis* on immobility time in the TST; *** indicates a significant difference compared to the normal saline group (negative control) with $P < 0.001$; # shows a significant difference compared to fluoxetine (positive control) with $P < 0.05$; and ### marks $P < 0.001$; results are expressed as mean ± standard deviation for 6 mice per group.

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

Table 1 illustrates that none of the administered doses of the M. officinalis ethanolic extract significantly influenced the number of times the mice crossed the squares or stood on their hind legs when compared to 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

In this study, the acute toxicity of the ethanolic extract from *M. officinalis* fruit was assessed using Lorke's method. Additionally, the antidepressant effects of this extract were evaluated through the forced swim test (FST) and tail suspension test (TST), while the extract's influence on motor activity was observed using the open-field test. The acute toxicity results showed no fatalities even at a high dose of 5000 mg/kg, indicating that the extract is essentially non-toxic in terms of toxicological classification. This finding is in agreement with previous biochemical and pathological research, which also found no toxic effects from *M. officinalis* extract [19].

In the FST, the 100 and 200 mg/kg doses of the ethanolic extract notably reduced immobility time compared to the control group. These doses also significantly increased swimming time; however, none of the extract doses significantly altered climbing time. The effects observed with fluoxetine were similar to those of the extract, although fluoxetine was more potent in reducing immobility. Similar results were seen in earlier studies, where fluoxetine both reduced immobility and increased swimming time without affecting climbing time [18].

The TST results indicated that all three doses of the extract, as well as fluoxetine, reduced immobility duration. However, fluoxetine showed weaker effects than the 100 and 200 mg/kg doses of the extract, which may be attributed to differences between the FST and TST. Overall, the antidepressant effects of *M. officinalis* extract were comparable to those of fluoxetine, suggesting that its action may be similar to that of serotonergic drugs.

Additionally, no significant changes in the mice's locomotor activity were observed in the Open-field test following administration of the extract. The number of square crossings and instances of standing on two legs were not significantly altered by any dose of the extract. This is consistent with previous research showing that antidepressants typically do not affect locomotor activity in the open field test [18]. In contrast, substances like stimulants or opioids may induce false positive results by increasing these behaviors. Therefore, any observed reductions in immobility or increases in locomotor activity are likely due to stimulant effects rather than antidepressant actions [20].

Research on the chemical composition of *M. officinalis* has identified two major categories of phenolic compounds, hydroxycoumarins and flavonoids, such as kaempferol [21]. These compounds have been linked to antidepressant activity in several studies [22-24]. For example, coumarin derivatives have been shown to inhibit monoamine oxidase enzymes [25]. Drugs like selegiline, which act as monoamine oxidase inhibitors, prevent the breakdown of neurotransmitters such as serotonin, dopamine, and norepinephrine, thereby contributing to their antidepressant effects [3].

Flavonoids, including kaempferol, have similar antidepressant effects, acting by boosting serotonin, dopamine, and norepinephrine levels while inhibiting serotonin breakdown [26]. In addition, *M. officinalis* extract, which contains a wealth of phenolic compounds, has been shown to reduce depressive behaviors in mice as observed in the forced swim test (FST). In this study, the extract exhibited antidepressant effects at doses of 300 mg/kg in acute use and between 30 to 300 mg/kg in subacute use, consistent with previous research indicating that the extract works through serotonergic pathways to decrease immobility and enhance swimming [27].

Additionally, *M. officinalis* extract has been found to improve oxidative stress levels, demonstrating strong antioxidant properties [9]. Given that oxidative stress plays a significant role in depression [12], it is plausible that the extract's antioxidant effects contribute to its antidepressant properties. Oxidative imbalances in depression lead to a reduction in superoxide dismutase (SOD) activity and an increase in nitric oxide production. Studies have indicated that *M. officinalis* extract reduces nitric oxide levels [28], which could be a mechanism through which it alleviates depression symptoms in mice. Notably, many antidepressants, such as fluoxetine, also possess antioxidant properties that help balance oxidative stress [12].

Inflammatory processes are also central to depression's development. Elevated levels of pro-inflammatory cytokines are commonly seen in individuals with depression and animal models [11]. Research has demonstrated

that *M. officinalis* reduces inflammatory cytokines like IL-1β, TNF-α, IL-6, and NF-kB in aged mice [29]. This anti-inflammatory effect may further contribute to the antidepressant effects of *M. officinalis*.
Given the diversity of bioactive compounds present in *M. officinalis*, it seems likely that the extract exerts its antidepressant effects through multiple pathways, including antioxidative, serotonergic, and anti-inflammatory mechanisms. However, further studies are required to clarify the exact pathways involved.

Conclusion

This study explored the antidepressant potential of the ethanolic extract from *M. officinalis* fruit in mice. The results indicate that *M. officinalis* is non-toxic and exhibits antidepressant effects comparable to fluoxetine. Therefore, *M. officinalis* may serve as a viable therapeutic option for depression. However, further research is necessary to fully understand the underlying mechanisms of its antidepressant action and to assess its long-term safety during chronic use.

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Ethics Statement: None

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