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Inhibitory effects of *Echinophora platyloba* L. ethanol extract on the development of morphine dependence: An in vivo study using naloxone-precipitated withdrawal symptoms in male mice

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Abstract

Background: Promising results were obtained by using medicinal plants for the treatment and prevention of opioid withdrawal syndrome. A native Iranian plant, *Echinophora platyloba*, has shown analgesic effects that may be mediated through opioid receptors. Thus, the present research evaluated the effects of the ethanolic extract of *E. platyloba* on the acquisition of morphine dependence in mice.

Methods: Thirty-two male mice were randomly allocated into four groups of eight. Morphine dependence was induced in the mice by subcutaneous administration of morphine (50, 50, and 75 mg/kg)×3 days, plus a single dose of morphine (50 mg/kg) on the fourth day. Withdrawal syndrome was precipitated by intraperitoneal injection of naloxone (4 mg/kg) 2 hours after the last dose of morphine. On days of dependence induction and before each morphine dose, the mice received intraperitoneal injections of saline (10 ml/kg) or plant extract (25, 50, and 75 mg/kg). After the injection of naloxone, the symptoms of withdrawal syndrome in each animal were monitored for 30 minutes.

Results: Administering morphine with this method induced morphine dependence in mice that were treated with saline before morphine. However, the mice that received the plant extract showed a significant decrease in the withdrawal symptoms including the number of jumping ($P<0.01$), rearing ($P<0.05$), grooming ($P<0.05$), and diarrhea ($P<0.01$) compared with the saline-treated mice.

Conclusion: Administration of the hydro-ethanolic extract of *E. platyloba* before morphine may inhibit the induction of morphine dependence. Therefore, the plant extract may be considered a therapeutic agent for the prevention of morphine dependence in morphine users.

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Keywords

Morphine dependence
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Highlights

What is current knowledge?

Chronic administration of morphine could induce significant dependence in mice.

What is new here?

The hydro-ethanolic extract of *Echinophora platyloba* can inhibit the development or acquisition of morphine dependence in mice.

Introduction

Opioids, especially morphine, have a widespread application in pain management in various health facilities (1-3). The use of opioids for the treatment of chronic pain may have some adverse consequences, including misuse, tolerance, dependence, and addiction (4-6). Moreover, people may abuse morphine chronically due to its euphorigenic properties. Dependence is also a long-term side effect of morphine administration (7), which is accompanied by physical and psychological symptoms that may manifest with typical withdrawal (8). The symptoms appear in morphine-dependent subjects when morphine administration is stopped abruptly or an antagonist of morphine such as naloxone or naltrexone is injected. These may include but are not limited to anxiety, restlessness, insomnia, yawning, body aches, lacrimation, sweating, rhinorrhea, diarrhea, fever, shaking, and pupillary dilatation (9). These symptoms are so unpleasant that they make the withdrawal syndrome a major obstacle in the way of those who want to quit morphine use.

Nowadays, the use of medicinal plants in the treatment of diseases has been increasing (10). Although this increasing trend might be based on myths or false beliefs (11), it should be noted that a high percentage of current drugs are of plant origin (12, 13). The use of medicinal plants for the treatment of opioid withdrawal syndrome has been suggested in previous studies (14-16), indicating the high potential of medicinal plants for preventing and mitigating morphine withdrawal syndrome.

E. platyloba is a native Iranian plant with different local names, including, Tologh-oti, Khosharizeh, Tigh Masti, and Kuzang. The different local names for this plant indicate its widespread dispersion in different parts of Iran (17). This plant has been traditionally used for the treatment of diarrhea (18), as a food freshener, and as a preservative in paste, pickle, and dairy products (17). In addition, scientific investigations have shown that this plant has some important properties such as in vitro anticancer (19), antibacterial, and anti-fungal effects (20-23). In animal models, the plant extract has shown analgesic (24), wound healing (25), antispasmodic (26), and antioxidant effects (24, 25). Furthermore, in clinical trials, this plant has shown promising effects in relieving premenstrual syndrome (27).

There are some similarities between the physical and psychological signs and symptoms of opioid withdrawal syndrome and premenstrual syndrome (9, 28). Of those common signs in both syndromes, one can refer to the pain in muscles and joints, change in appetite, diarrhea, anxiety, depression, irritability, and suicidal tendencies. Moreover, in both conditions, the levels of β-endorphin (an endogenous opioid) decrease in the brain (29-31). As mentioned previously, *E. platyloba* may have anxiolytic and antidiarrheal effects as well as analgesic properties. On the other hand, pain, anxiety, and diarrhea are among the common morphine withdrawal symptoms. Moreover, the role of opioid receptors in morphine dependence is well documented (32-34), and the plant extract has some phytochemicals that may bind to opioid receptors, thereby changing their activities. Finally, oxidative stress plays an important role in the induction of morphine dependence (35, 36), and *E. platyloba* has shown antioxidant properties (20, 37). Thus, the purpose of this study was to evaluate the effects of ethanol extract of *E. platyloba* on the acquisition (development) of morphine withdrawal symptoms in male mice.

Methods

Animals

The study included 32 male MNRI mice (obtained from the Pasture Institute of Iran. The mice were weighed 22-26 g upon their arrival at the animal house of the University of Maragheh, Iran. The animal house provided the laboratory animals with standard housing conditions including room temperature of 22±2°C, a 12h/12h light-dark cycle, and free access to tap water and rodent pellets. After one week of acclimatization, the mice were randomly allocated into four groups (n=8). During the induction of dependence, the control group received intraperitoneal (i.p.) saline injection (10 ml/kg), while the three experimental groups received the plant extract (25, 50, and 75 mg/kg, i.p.). The

animals in the control and experimental groups were naïve. In the present study, the experimental protocols on laboratory animals were supervised and approved by an institutional Animals Care and Use Ethical Committee at the University of Maragheh, Iran (IR. UM.1400.004). All experiments were conducted according to the ethical guidelines of the local ethics committee. All efforts were made to reduce animal suffering during the tests and to minimize the number of animals used in the research.

Drugs

Morphine sulfate ampoules (Daru Pakhsh Co. Tehran, Iran) and naloxone (Toliddaru Co. Tehran, Iran) were used to induce dependence and withdrawal syndrome, respectively. Different doses of morphine were made by diluting the contents of morphine ampules (10 mg/ml). The vehicle of plant extract, morphine, and naloxone was normal saline.

Plant

E. platyloba was collected in spring from Maragheh (East Azerbaijan, Iran). A botanist at the University of Maragheh conducted authentication of the plant, and a code (UM-DB-001) was assigned to the voucher specimen that was kept in a plant depositor at the Department of Biology of the University of Maragheh.

Preparation of plant ethanol extract

The ethanol extract of *E. platyloba* was prepared according to the method described by Nematian and Mohammadi (24) with brief modifications. In short, 100 g powder of the plant's aerial parts was immersed in 1 liter of hydro-ethanol solvent (80%), and the mixture was placed in a shaker incubator for 72 hours at room temperature. Then, the mixture was filtered using a Whatman filter paper. Next, the solvent (ethanol) was separated using a rotary evaporator. The resulting solution was poured into Petri dishes and kept under a laminar hood for about a week. Desired amounts of the final product with a semi-solid, waxy state were dissolved in normal saline for the preparation of different doses of the plant extract.

Induction of dependence in mice

The animals rendered dependent on morphine according to the method described by Zarrindast (38) as follows: in three consecutive days, each animal received subcutaneous (S.C.) injections of morphine (50, 50, and 75 mg/kg) at 9 a.m., 1 p.m., and 5 p.m., respectively. On the fourth day, the animals received a single dose of morphine (50 mg/kg, S.C.) at 9 a.m.

Induction and assessment of withdrawal signs

For the induction of withdrawal syndrome in morphine-dependent mice, each mouse received an injection of naloxone (4 mg/kg, i.p.) on the test day, 2 hours after the last dose of morphine. For the evaluation of withdrawal signs after naloxone injection, each mouse was separately put for 30 minutes in a glass cylinder (25 cm diameter × 40 cm high), the floor of which was covered by straw paper, and its behavior was recorded with a camera. A person unaware of the experiment conditions counted the number of jumps, rearing, and grooming (including facial and body grooming) in the mice by looking at the videos, using a hand counter (39-41). Moreover, by weighing the filter paper, before and after the test sessions, the weight of stool mass for each animal was calculated. The ratio of the weight of stool mass to 100 g of body weight of each animal was used as a criterion for the evaluation of stool mass (39, 41).

$$Stool\ mass\ (pesent) = \frac{stool\ weight\ (g)}{\text{body weight}\ (g)} \times 100$$

The effects of E. platyloba extract on the development of morphine dependence signs

One hour before each morphine dose, during the first three days of dependence induction, all mice received saline (10ml/kg, i.p.) or the plant extract (25, 50, and 75 mg/kg, i.p.). On the test day, the fourth day of the experiment, 2 hours after the last morphine injection (without administration of the plant extract), the mice received naloxone (4 mg/kg, i.p.). Immediately after the naloxone injection, each animal was put into the cylinder so that its behavior was recorded during the 30-minute test sessions with a camera. Then, naloxone-induced withdrawal signs were evaluated for each animal as measures of dependence on morphine.

Statistical analysis

Data were analyzed with SPSS software (version 18). Animal behaviors (jumping, rearing, grooming), as well as stool mass (as quantitative data), were expressed as mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's post hoc test (in case of significant difference between groups) was used to compare any significant difference between the control and the experimental groups. P-values less than 0.05 were considered statistically significant.

Results

Effects of pretreatment with the E. platyloba extract before morphine on jumping behavior on the test day

The mice that received the plant extract before each morphine administration showed a significant decrease in jumping behavior compared with the saline-treated group [$F(3,28)= 10$, $P<0.001$]. Post hoc tests showed that the extract at doses of 50 mg/kg and 75mg/kg could significantly reduce jumping behaviors ($P<0.01$) (Figure 1).

Effects of chronic administration of the E. platyloba extract with morphine on the ratio of stool weight to body weight on the test day

During the dependence induction, administration of the plant extract before morphine could decrease the stool weight on the test day after naloxone injection [$F (3, 28) = 7.8$, $P<0.01$]. The mice that received doses of 50 mg/kg and 75 mg/kg of the plant extract showed a significant decrease in stool mass compared with the saline group (Figure 2).

Effects of administration of the plant extract before morphine on anxiety-related behaviors during the dependence induction

Administration of the plant extract before morphine during dependence induction could significantly reduce anxiety-related behaviors, namely, rearing [$F (3, 28) = 4.9$, $P<0.01$] and grooming [$F (3, 28) = 5$, $P<0.01$].
Further analysis indicated that only one dose of the plant extract (75 mg/kg, i.p.) significantly reduced both behaviors ($P<0.05$) (Figures 3 and 4).

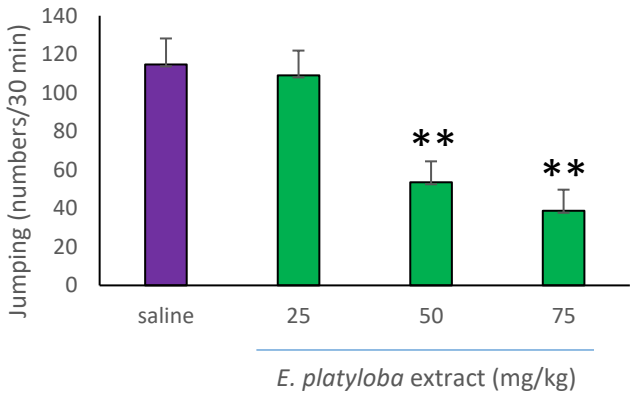

Figure 1. Effects of the extract of *E. platyloba* on the number of jumps in mice. Four groups of mice received saline or plant extract during the induction of morphine dependence. The results showed that the number of jumps decreased in animals who received the plant extract (50 and 75 mg/kg) compared to the saline group. ** indicates a significant difference ($P<0.01$) compared with the saline group.

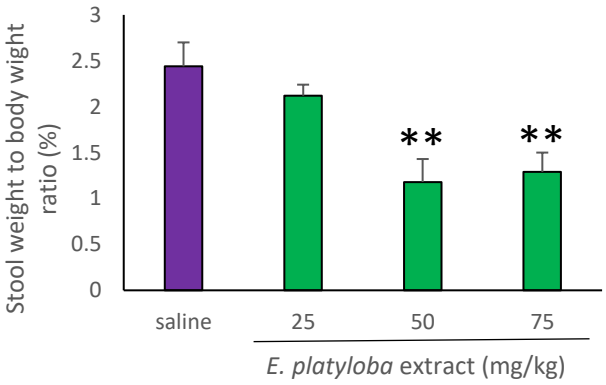

Figure 2. Effects of the extract of *E. platyloba* on the stool weight to body weight ratio in mice. The mice groups that received the plant extract during the dependence induction showed a significant decrease in the weight of stool compared with the saline-treated group. ** indicates a significant difference ($P<0.01$) compared with the saline group.

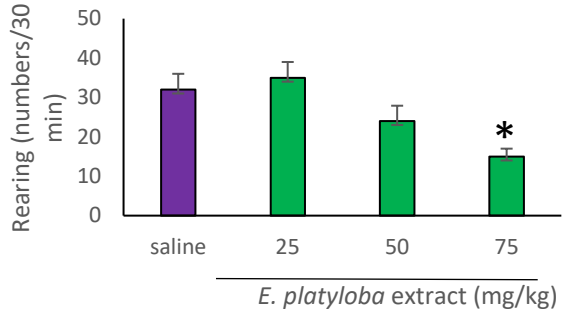

Figure 3. Effects of the *E. platyloba* extract on the number of rearing in mice. Administration of the plant extract during the dependence induction before morphine administration could significantly inhibit the number of rearing in mice. * indicates a significant difference (P<0.05) compared with the saline group.

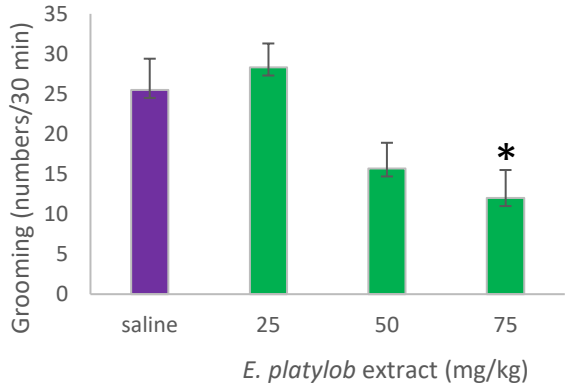

Figure 4. Effects of the *E. platyloba* extract on the number of grooming in mice. The number of grooming decreased significantly in the mice groups that received the plant extract before morphine administration during the dependence induction. * indicates a significant difference (P<0.05) compared with the saline group.

Discussion

The present research showed that morphine administration using the method described by Zarrindast et al (38) could induce a significant dependence in mice. This method was chosen because, in our previous study, it could induce morphine dependence in mice (41). It should be noted that the symptoms of morphine withdrawal are not limited to the ones that were evaluated in the present study. Thus, a limitation of the present research may be the restricted assessment of withdrawal symptoms. However, the reason for selecting these symptoms was that they were more clearly recognized by the observer (we had only one camera for recording the behaviors of the animals). In line with our results, various studies used a similar method and acquired positive results in the induction of morphine dependence (42, 43). The advantage of this protocol over other protocols may be the relatively shorter time (only four days) required for morphine dependence induction (44). More importantly, the present study indicated that the ethanol extract of *E. platyloba* could inhibit the acquisition/development of morphine dependence in mice.

Phytochemical analyses determined the presence of different important metabolites in the plant extract including alkaloids, flavonoids (especially quercetin), stigmasterol, sitosterol, stigmasterol-β-D-glycoside, and saccharose (17, 45, 46). A number of these plant compounds may be involved in inhibitory effects on morphine dependence. The induction of morphine dependence is a complicated process and different mechanisms, brain regions, and neurotransmitter systems may mediate this phenomenon (8, 47). One mechanism that may have an important role in the development of morphine dependence is oxidative stress (35).

Previous studies have shown the antioxidant effects of the plant extract and its flavonoid (quercetin) (20, 37, 48). Therefore, the antioxidant properties of the plant extract may be involved in the prevention of morphine dependence. Another change that may occur during chronic morphine administration is the elevation of the inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukins (49). In contrast, the plant extract, especially its flavonoid components, exerts anti-inflammatory activity through the reduction of TNF-α (50). Thus, parts of the inhibitory effects of the plant extract on the acquisition of morphine dependence may be ascribed to its anti-inflammatory effects.

The role of opioid receptors in the development of morphine dependence has been shown in previous studies (47). On the other hand, Asgari Nematian and Mohammadi showed that the alkaloids of the extract of *E. platyloba* may have analgesic effects by binding to the opioid receptors (51). Therefore, during the dependence induction, administration of the plant extract before morphine may interfere with the binding of morphine to the opioid receptors. This effect may neutralize the effects of morphine thereby decreasing the development of morphine dependence.

Flavonoids are a group of important phytochemicals that may interfere with morphine dependence (52). Moreover, previous studies indicated the inhibitory effects of chronically administered quercetin (the main flavonoid of the plant) on the induction of morphine tolerance and dependence in animal models (53). In our study, administration of the plant extract before morphine could inhibit diarrhea that was induced with the injection of naloxone on test day. By binding to mu-opioid receptors on cholinergic neurons of the enteric nervous system, morphine can inhibit the cholinergic system and the movements of the intestine (54). On the contrary, quercetin, the main flavonoid of the plant, has anticholinesterase effects. Furthermore, chronic administration of quercetin can inhibit loperamide-induced constipation by the regulation of muscarinic acetylcholine receptors; administration of antagonists of these receptors prevents the action of quercetin (55, 56). Therefore, it can be concluded that the plant extract may counteract the effects of morphine on the gut during the induction of dependence by enhancing the acetylcholine activity in the intestine. It is as if the animal had not been given morphine beforehand. As a consequence, on the test day, naloxone injection had no effects on the induction of diarrhea and increase in stool mass. It is also noteworthy to mention that naloxone by itself has no impact on intestinal motility (57). In this regard, previous studies have shown that the agents that could inhibit cholinesterase could suppress the induction of morphine dependence (58).

One theory for the induction of morphine dependence is the cyclic adenosine monophosphate (cAMP) theory. According to this theory, acute injection of morphine decreases the adenylyl cyclase activity and consequently, the intracellular cAMP level reduces. However, in chronic morphine administration, neural adaptations lead to an increase in the activity of adenylyl cyclase, which elevates the cAMP levels (59). One action of quercetin in the neurons is that it can increase intracellular cAMP levels (60). Therefore, administration of the plant extract containing quercetin may neutralize the acute effects of morphine on intracellular cAMP. From this, one may conclude that the plant extract through its flavonoid components prevents the induction of adaptations that may increase the cAMP levels and lead to morphine dependence.

The present study also revealed that the mice who received the plant extract during dependence induction showed decreased rearing and grooming on the test day. Anxiety in laboratory animals may be assessed using rearing and grooming behaviors (61, 62). Thus, the decline in anxiety-dependent behaviors (rearing and grooming) may be the result of chronic anxiolytic effects of the plant extract or its flavonoid (quercetin content) (27, 63).

In line with the present study, some studies indicated the role of different mechanisms in inhibitory actions of medicinal plants in morphine withdrawal in laboratory animals. For instance, Esmaeili and Ebrahimi showed that the extract of *Satureja khozestanica* could decrease morphine withdrawal signs in mice possibly through the inhibition of acetylcholine esterase owing to its flavonoid components or its anti-inflammatory activity (64). Moreover, in another study, administration of L-tetrahydropalmatine, an analgesic alkaloid of *Corydalis yanhusuo*, could reduce morphine withdrawal-induced hyperalgesia in mice through binding to opioid receptors (65). Finally, Kasreifar et al. showed that injection of a hydro-ethanolic extract of aerial parts of *Datura stramonium* could reduce morphine withdrawal symptoms in mice (66); this effect was ascribed to its influence on the cholinergic system and its antioxidant properties.

Although we were more concerned with the role of alkaloids and flavonoids (quercetin) in the effects of the plant extract on morphine dependence, the role of other phytochemicals in the extract should not be overlooked. Research on other metabolites of the plant including stigmasterol, sitosterol, and stigmasterol-β-D-glycoside has shown that these secondary metabolites may change dopamine, Gamma-aminobutyric acid (GABA), and serotonin levels in the brain (67, 68). Each of these neurotransmitters plays an important role in promoting morphine dependence (69); therefore, modulation of the neurotransmitter systems of the brain by these secondary metabolites may be another mechanism involved in the inhibitory properties of the extract of *E. platyloba* on the development of morphine dependence.

Altogether, considering the presence of various secondary metabolites in the plant extract, the exact mechanism through which the extract affects morphine dependence is not clear. Therefore, further studies are required to separately investigate the role of each plant extract constituent on the induction of morphine dependence.

Conclusion

The results of the present study showed that the hydro-ethanolic extract of *E. platyloba* might have preventive effects on the induction of morphine dependence. Because this plant has been widely used in various parts of Iran as an edible plant, it may be possible to use the plant extract as a complementary medicine for the prevention of withdrawal syndrome in people who use morphine. However, there is a need for more preclinical and clinical studies to confirm our findings.

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Ethical statement

This research was conducted according to ethical guidelines approved by the ethics committee of the University of Maragheh (Ethics approval code: IR. UM.1400.004)

Conflicts of interest

The authors declare that there is no conflict of interest.

Author contributions

Dr. Amir Abbas Barzegari designed the experiment, analyzed the data, and wrote the manuscript. Dr. Ahamad Aghaee collected the plant material, identified the plant species, and prepared the plant extract. Kamran Shahabi conducted the experiments on laboratory animals.

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