Research Journal of Pharmacognosy (RJP) 4(1), 2017: 59-66
Received: Aug 2016
Accepted: Oct 2016

Original article

Morphine withdrawal attenuating effect, toxicity and alkaloid composition of *Sophora alopecuroides* L. var. *alopecuroides*

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Abstract

Background and objectives: The seeds of *Sophora alopecuroides* L. var. *alopecuroides* may benefit treatment of opioid dependence. Therefore, the plant alkaloid composition, toxicity and effects on morphine withdrawal were studied. **Methods:** The alkaloid composition was determined by GC and GC/MS analysis. Mice were made dependent by morphine injected 3 times a day for 3 days. The withdrawal jumping and diarrhea were induced by administration of naloxone 2 h after the 10th injection of morphine on the day 4. The ethanol 90% extract (100, 200, 300 mg/kg), alkaloid fraction (5, 10, 20 mg/kg), morphine (50 mg/kg) or saline were injected 30 min before naloxone. All drugs were injected subcutaneously to groups each consisting of 10 mice. To assess toxicity, different doses of the ethanol or aqueous extracts dissolved in normal saline were gavaged once to groups each consisting of 30 mice. Afterward, the numbers of dead animals within 72 h after gavage were counted and LD_{50} was calculated. **Results:** Matrine, cytisine, sophoridine, *n*-methyl cytisine, sophocarpine and sophoramine were the major alkaloids. All doses of the total extract, alkaloid fraction and morphine decreased jumping and diarrhea significantly compared to the saline (p<0.001). The effects of the total extract and alkaloid fraction were not significantly different from morphine (p>0.05). The ethanol and aqueous extracts LD_{50} were 355 mg/kg and 540 mg/kg, respectively. **Conclusion:** The plant inhibited opioid withdrawal with efficacy comparable to morphine. The alkaloids may be involved in the effect. The ethanol and aqueous extracts are moderately and slightly orally toxic, respectively.

Keywords: addiction, alkaloid, *Sophora alopecuroides*, toxicity

Introduction

Opioid dependence is a worldwide health problem that has economic, personal and public health consequences. There are three major approaches to pharmacologic treatment of opioid dependence: opioid detoxification, agonist maintenance and antagonist maintenance. Opioid detoxification is utilized mainly for transition into or out of a maintenance program over a very short period of time. Methadone, buprenorphine, α_2-adrenoceptor agonists and adjunct medications are used for the opioid detoxification. In antagonist maintenance, naltrexone (an opioid antagonist) is used. The pharmacologic agents with proven efficacy in the agonist maintenance are buprenorphine, buprenorphine/naloxone and methadone [1,2]. The pharmacologic agents used in the treatment of opioid dependence have important limitations in efficacy and safety.

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Novel pharmacologic options with more efficacy and better safety profile are needed for opioid dependence treatment [3,4].

Plants can be a source of new opioid dependence pharmacotherapies [5,6]. *Sophora alopecuroides* L. var. *alopecuroides* (*S. alopecuroides*), (Leguminosae) is a perennial herb growing in Western Asia including Iran and Central Asia. The plant seeds have been traditionally used for treatment of many disorders including pain, inflammation, diarrhea, fever, bacterial infections, chronic liver diseases, heart failure and hypertension [7]. Moreover, decoction of the plant seed has been administered orally for detoxification and maintenance treatment of opium and heroin addicts in Iran. The plant and its alkaloids have been described as poisonous [8]. Quinolizidine alkaloids are responsible for the therapeutic and adverse effects of *S. alopecuroides*. The alkaloids are very soluble in ethanol and water. The alkaloids have shown a variety of pharmacological effects on the immune, nervous, gastrointestinal and cardiovascular systems and also anticancer and antimicrobial activities [9,10]. One of the alkaloids called cytisine and its analog varenicline have been used to treat nicotine addiction [11]. There has been no study on the phytochemical characteristics of the *S. alopecuroides* from Iran. Moreover, the toxicity and effects of *S. alopecuroides* on the opioid withdrawal have not been evaluated so far. Therefore, the present study was undertaken to examine these subjects.

Experimental

Plant material

The seeds of *S. alopecuroides* were collected from Kerman Province, Iran at the fruiting stage (November, 2015). A voucher specimen of the plant was deposited at the Herbarium of the Institute of Medicinal Plants, ACECR, Karaj, Iran.

Extraction

The plant seeds were dried, powdered (500 g) and macerated with 90% ethanol solution or water for 3 days with three changes of the solution. The resulting extract was filtered and evaporated under vacuum into a dried powder (30 g, 6% and 13 g, 2.6% for ethanol and aqueous extracts, respectively).

Extraction of alkaloids

Alkaloid extraction was carried out as described by Kamada *et al.* [12]: 200 mL of $CHCl_3$- Me OH- NH_4OH (15: 5: 1) was added to 600 mg of 90% ethanol extract and sonicated for 10 min. After filtration, the residue was washed with 200 mL of solution twice. The pooled filtrate was evaporated to dryness. Five mL of $CHCl_3$ and 2 mL of 1 N H_2SO_4 were added to the residue and the solution was mixed. The $CHCl_3$ phase was removed and the H_2SO_4 phase was adjusted to pH 10 with 28% NH_4OH. Alkaloids were extracted once with 2 mL and twice with 1 mL of $CHCl_3$ from the solution. The combined extracts were filtered after adding anhydrous Na_2SO_4 and were evaporated to dryness at 40 °C (265 mg).

GC (gas chromatography) and GC/MS (gas chromatography-mass spectrometry) analyses

The extraction of alkaloids was analyzed on a Younglin Acm 600 instrument with an FID detector operated with a split/splitless injector (Younglin, Korea) and DB-5 capillary column, 30 m×0.25 mm i.d., 0.25 μm film thickness (Agilent, USA). Carrier gas: helium, linear velocity: 30 cm/sec, flow: 0.8 mL/min. Injection temperature: 290 ^oC. Injection volume: 1.0 μL. Injection mode: split (1:50). Temperature program: 50 ^oC for 5 min, rising at 3 ^oC/min to 240 ^oC, then rising at 15 ^oC/min to 300 ^oC, held at 300 ^oC for 3 min. FID (290 ^oC): H_2 flow: 50 mL/min; air flow: 400 mL/min. GC/MS analysis was performed on an Agilent 6890/5973 N instrument and DB-5 capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness). Carrier gas: helium, Linear velocity: 32.4 cm/sec, flow: 0.8 mL/min; injection temperature: 290 ^oC; injection volume: 1.0 μL. Injection mode: split (1:10); temperature program: 50 ^oC, for 5 min, rising at 3 ^oC/min to 240 ^oC, then rising at 15 ^oC/min to 300 ^oC, held at 300 ^oC for 3 min; MS interface temperature: 290 ^oC, MS mode: EI,

Ionization voltage: 70 eV; mass range: 40-500 u; scan speed: 3.18 scans/sec; interval: 0.50 sec (2 Hz). Data handling was conducted using a Chem. Station (Agilent, USA).

Identification of the components
The compounds from the alkaloids extract were identified by comparison of their retention indices which were calculated by using the retention times of injected *n*-alkanes (C_8–C_{28}) (obtained from Fluka, USA) with the same chromatographic conditions, along with the fragmentation patterns of the mass spectra with those reported in the literatures and the published mass spectra or WILEY library (13). The percentage of the identified compounds was calculated based on GC peak areas without any correction factors as described previously [13-16].

Drugs
Morphine sulfate was purchased from the Darou Pakhsh pharmaceutical company (Iran). Naloxone hydrochloride was obtained from the Sigma-Aldrich company (USA). All drugs and extracts were dissolved in normal saline. The drugs and extracts were prepared immediately prior use and injected subcutaneously in a volume of 5 mL/kg. The doses of total extract, alkaloid fraction and morphine were as follows. Total extract: 100, 200 and 300 mg/kg; alkaloid fraction: 5, 10 and 20 mg/kg; morphine: 50 mg/kg.

Animals
Male albino mice weighing 25-30 g from our own breeding colony (Institute of Medicinal Plants, ACECR, Karaj, Iran) were used. The mice were maintained at a temperature of 22-25 ºC on a 12 hr dark-light cycle. The animals had access to standard rodent feed and water *ad lib*. Ten animals were used for each dose of the extracts or drugs. All animals were used only once.

LD_{50} (median lethal dose) in mice
To determine toxicity, different doses of the total ethanol or aqueous extracts dissolved in normal saline were gavaged in the groups each consisting of 30 mice (15 males and 15 females) in a volume of 5 mL/kg. Afterward, the numbers of dead animals within 72 h after gavage were counted and LD_{50} was calculated by the graphical method of Miller and Tainter as described previously [17,18]. The administered doses were 12.5, 25, 50, 100, 200, 300, 400, 500, 750 and 1000 mg/kg.

Effects on opioid withdrawal in mice
The effects on morphine withdrawal were evaluated using a method described previously [19]. To induce morphine dependence in the mice, morphine was given with the following dosage schedule. Morphine was injected thrice a day at 9:30 am, 1:30 pm and 5:30 pm, using the doses 50, 50 and 75 mg/kg respectively for 3 days. The higher afternoon dose was aimed to minimize overnight withdrawal. Moreover, a 50 mg/kg dose of morphine was given in morning of the 4^{th} day (2 h before naloxone injection). Hyperactivity and Straub tail effect were noted after morphine injection.
Naloxone (2 mg/kg) was given 2 h after the last injection of morphine in the 4^{th} day. Subsequently, the animals were placed singly on a piece of blotting paper in a cylindrical glass (25 cm in diameter, 40 cm height) for 30 min. Naloxone immediately caused morphine withdrawal signs as jumping and diarrhea. The number of jumps and feces weight during the 30 min period was recorded for each animal. The total extract and alkaloid fraction as active treatments, morphine (positive control) or saline (negative control) were injected 30 min before naloxone. The ethics committee of the Institute of Medicinal Plants (ACECR, Karaj, Iran) approved this study (No. 256).

Statistical analysis
The animal study results were presented as mean±standard error of the mean (SEM). One way analysis of variance (ANOVA) followed by Tukey *post hoc* test was used for data analysis. $p<0.05$ was considered as significant.

Results and Discussion
Alkaloid extraction was analyzed by GC and

GC/MS and the main compounds were determined. Matrine (23.19%), cytisine (20.9%), sophoridine (17.16%), *n*-methyl cytisine (13.39%), sophocarpine (9.13%) and sophoramine (1.2%), were identified as the main components in the alkaloid extract. The lack of nicotine in the *S. alopecuroides* from Iran is in contrast to the Chinese *S. alopecuroides* which contained nicotine [20]. Nicotine is a toxic substance [21]. Thus, lack of nicotine in the *S. alopecuroides* from Iran decreases the plant toxicity. LD_{50} of the total ethanol and aqueous extracts were 355 mg/kg and 540 mg/kg, respectively. There was no significant difference between mortalities of male and female animals. Substances having LD_{50} values in the range of 50-500 mg/kg and 500-5000 mg/kg were considered moderately and slightly toxic, respectively [17]. The 90% ethanol and aqueous extracts of the plant administered orally, were moderately and slightly toxic, respectively in the mice. Moreover, the LD_{50} of the aqueous extract was 52% higher than that of the 90% ethanol extract, showing less toxicity of the aqueous extract compared to the 90% ethanol extract. The seeds of *S. alopecuroides* have been used orally as a crude drug in China for thousands of years. The Chinese *S. alopecuroides* seeds have shown little toxicity [22,23]. Traditional use of the plant in Iran and the results of the present study suggest that the Iranian plant is not more toxic than the Chinese plant.

All doses of the total extract and alkaloid fraction as well as morphine decreased jumping significantly compared to saline (p<0.001) (figures 1, 2). The effects of the total extract (200 mg/kg, 300 mg/kg, SC) and alkaloid fraction (10 mg/kg, 20 mg/kg, SC) were not significantly different from morphine (50 mg/kg, SC) (P>0.05).

All doses of the total extract and alkaloid fraction as well as morphine decreased diarrhea significantly compared to saline (p<0.001) (figures 3,4). The effects of the total extract (200 mg/kg, 300 mg/kg, SC) and alkaloid fraction (10 mg/kg, 20 mg/kg, SC) were not significantly different from morphine (50 mg/kg, SC) (p>0.05).

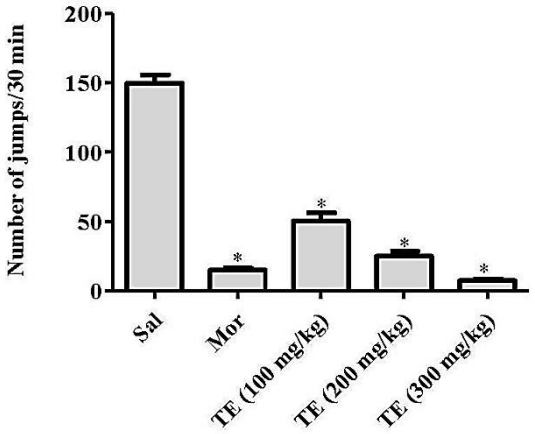

Figure 1. Effects of saline (Sal), morphine (Mor) (50 mg/kg, SC), *S. alopecuroides* total extract (TE) (100 mg/kg, 200 mg/kg, 300 mg/kg, SC) on jumping induced by naloxone (2 mg/kg, SC) in groups each consisting of 10 morphine dependent mice. Data are given as mean+SEM. p<0.001 for all asterisked columns compared to the saline. The effects of the total extract (200 mg/kg, 300 mg/kg) were not significantly different from morphine (p>0.05).

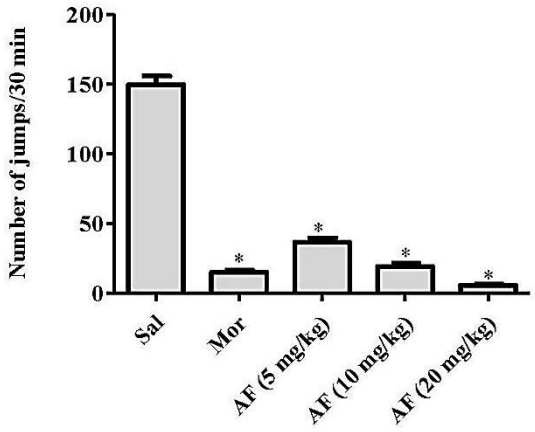

Figure 2. Effects of saline (Sal), morphine (Mor) (50 mg/kg, SC), *S. alopecuroides* alkaloid fraction (AF) (5 mg/kg, 10 mg/kg, 20 mg/kg, SC) on jumping induced by naloxone (2 mg/kg, SC) in groups each consisting of 10 morphine dependent mice. Data are given as mean+SEM. p<0.001 for all asterisked columns compared to the saline. The effects of the alkaloid fraction (10 mg/kg, 20 mg/kg) were not significantly different from morphine (p>0.05).

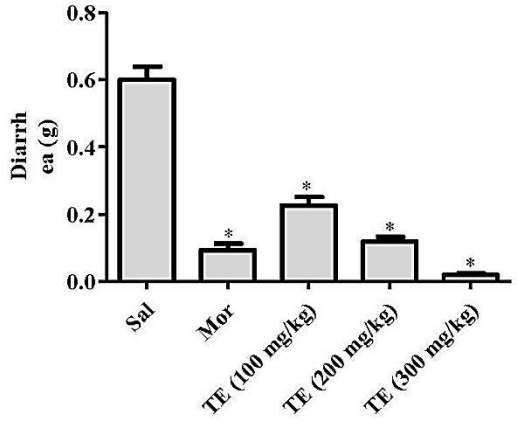

Figure 3. Effects of saline (Sal), morphine (Mor) (50 mg/kg, SC), *S. alopecuroides* total extract (TE) (100 mg/kg, 200 mg/kg, 300 mg/kg, SC) on diarrhea induced by naloxone (2 mg/kg, SC) in groups each consisting of 10 morphine dependent mice. Data are given as mean+SEM. $p<0.001$ for all asterisked columns compared to the saline. The effects of the total extract (200 mg/kg, 300 mg/kg) were not significantly different from morphine ($p>0.05$).

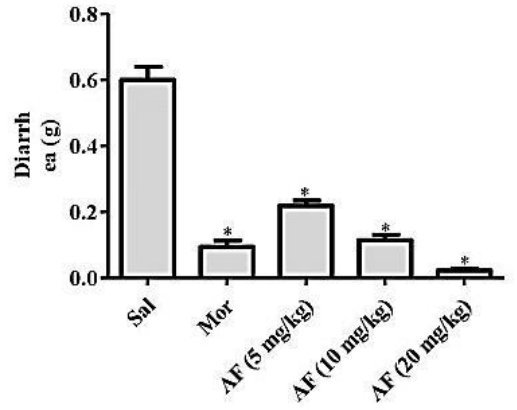

Figure 4. Effects of saline (Sal), morphine (Mor) (50 mg/kg, SC), *S. alopecuroides* alkaloid fraction (AF) (5 mg/kg, 10 mg/kg, 20 mg/kg, SC) on diarrhea induced by naloxone (2 mg/kg, SC) in groups each consisting of 10 morphine dependent mice. Data are given as mean+SEM. $p<0.001$ for all asterisked columns compared to the saline. The effects of the alkaloid fraction (10 mg/kg, 20 mg/kg) were not significantly different from morphine ($p>0.05$).

The *S. alopecuroides* total extract and alkaloid fraction considerably attenuated the signs of morphine withdrawal. The inhibitory effects of the total extract (200 mg/kg, 300 mg/kg, SC) and alkaloid fraction (10 mg/kg, 20 mg/kg, SC) on the opioid withdrawal were comparable to morphine (50 mg/kg, SC). Morphine as slow-release oral morphine (SROM) is an effective alternative to methadone for opioid detoxification and maintenance. SROM is available in some European countries and may be an alternative treatment for patients not responding to or tolerating methadone because of side effects such as QTc prolongation (cardiac effects) [24-26]. The present results confirm the traditional use of the plant for the treatment of opioid dependence in Iran. The effects of plants from numerous families have been examined in opioid dependence and withdrawal syndrome so far [5,6]. However, there has been no study regarding the family Leguminosae and the genus *Sophora*. Further, the effects of the compounds in *S. alopecuroides* in the animal models and clinical trials of opioid dependence have not been evaluated. The mechanisms of the inhibitory effects of the *S. alopecuroides* total extract and alkaloid fraction on the morphine withdrawal were not investigated in the present study. The effects of the plant cannot be totally attributed to matrine, and the other alkaloids; besides matrine may also be involved in the plant effects. Cytisine is an agonist of nicotinic receptors [27,28] and agonists of nicotinic receptors can suppress opioid withdrawal [29,30]. Thus, the nicotinic receptor agonistic action of the *S. alopecuroides* cytisine may have some role in the morphine withdrawal inhibitory effect of *S. alopecuroides*. Several studies have reported involvement of μ and κ opioid receptors in the analgesic effect of matrine in mice [31-33]. However, a study concluded that matrine showed no affinity for μ, κ and δ opioid receptors *in vitro* and its analgesic effect in mice might be through cholinergic activation rather than acting on opioid receptors directly [34]. Therefore, matrine present in *S. alopecuroides* may alleviate morphine withdrawal by activation of opioid and/or nicotinic receptors and/or via other

mechanism(s). In another study, the plant total extract did not cause Straub tail effect in mice. This indicates that it did not activate μ_2 opioid receptors and thus may not produce the opioid adverse effects caused by μ_2 receptor activation (physical dependence, respiratory depression and constipation) [35]. The same mentioned study suggested that the plant could be regarded as a non-opioid agent. In practice, there has also been no report showing the addictive potential of the plant despite its use for several thousand years. Notably, the plant may have significance considering the paucity of medications approved for opioid detoxification and relapse prevention particularly non-opioid medications [1]. Also, the effectiveness of the plant comparable to morphine (as shown in the present study), confirmed its possible clinical value. Finally, the promising results of the current study warrant conduction of clinical trials regarding the efficacy and safety of the plant in opioid detoxification and maintenance treatment. Further studies addressing identification of the components and mechanisms mediating the inhibitory effect of the plant on the opioid withdrawal are needed.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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