Pharmaceutical Sciences
June 2018, 24, 112-117
doi: 10.15171/PS.2018.17
http://journals.tbzmed.ac.ir/PHARM

Research Article

Evaluation of the Effect of Aerial Parts of *Scrophularia atropatana* Grossh Total Extracts on Analgesic Activity and Morphine Induced Tolerance in Mice

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Article Info

Article History:
Received: 27 December 2016
Revised: 15 December 2017
Accepted: 30 December 2017
ePublished: 20 June 2018

Keywords:
-Antioxidant activity
-Analgesic
-*Scrophularia atropatana*
-Morphine
-Tolerance

ABSTRACT

Background: *Scrophularia atropatana* contains chemicals activating the GABA system and inhibiting glutaminergic system. As well as we know, drugs inhibiting glutamate system have analgesic effects and reduce tolerance to the analgesic effects of morphine. In the present study, effect of methanol extract of aerial parts of *S. atropatana* on tolerance to the analgesic effects of morphine was evaluated.

Methods: The aerial parts of *S. atropatana* were extracted with methanol by maceration and was further analyzed to determine the content of total phenols, antioxidant activity (DPPH method) and analgesic activity. Several groups of mice received drug regimens for 4 days. Group I: Saline (10 ml/kg, i.p.) + Saline (10 ml/kg, i.p.), group II: Morphine (50mg/kg, i.p.) + Saline (10mg/kg, i.p.) and group III: Morphine (50mg/kg, i.p.) + Extract of *S. atropatana* (100, 200, 400 mg/kg, i.p.)]. On the fifth day the test dose of morphine (9 mg/kg, i.p.) in different groups were assessed. By using hot plate test, the analgesic activity of different doses of the extract was evaluated.

Results: Phytochemical screening indicated the presence of total phenolics content of the extract (152 mg GAE/g) and antioxidant activity (RC_{50} =143µg/ml) in DPPH method. All doses of the extract had analgesic activity. It was found that treatment with extract (100, 200 mg/kg, i.p.) attenuated the development of tolerance to the antinociceptive effect of morphine.

Conclusion: The results of the study demonstrate that *S. atropatana* strongly reduced tolerance to morphine and has analgesic effects. The suggested analgesic effects may be related to the phenolics content and antioxidant activity of the plant alongside inhibition of glutaminergic system.

Introduction

Opioids as one of the most efficient drugs to reduce acute and chronic pains are widely used throughout the world. But the tolerance to the effects of opioids are the most important disadvantage of them.[1] Tolerance (Specially to the analgesic effects) is one of the limiting factors of using opioids in patients suffering from pain. To maintain the chronic effects of opioids numerous methods (for example prescribing increasing doses) have been used during recent years.

Complex intracellular neural mechanisms, including opioid receptor desensitization and down-regulation, are believed to be major mechanisms underlying opioid tolerance. For example, down regulation of µ-receptors seems to have an important role in opioid tolerance. On the other hand, mechanisms like phosphorylation, receptor uncoupling or internalization may result in opioid receptor desensitization.[2,3]

Previous studies have shown some different cellular and molecular mechanisms involved in morphine tolerance. For example role of GABAergic system and GABA transporters,[4-7] Involvement of nitric oxide synthase (NOS)[8] and effect of glutaminergic system on the tolerance.[9,10]

Scrophularia atropatana is a perennial plant with the Persian name of "Gole Meymoni Azari" are widely found in northwest of Iran.[11] Group of compounds present in *S. atropatana* include iridoids, phenylethanoids, flavonoids and etc. Previous studies indicated that *Scrophularia* genus could have agonistic effects on GABAergic system,[12] inhibitory effects on nitric oxide synthase[13] and neuroprotective effects through NMDA receptors in glutaminergic system.[14] The phytochemicals present in *S. atropatana* are very similar to other species for example *S. amplexicaulis* Benth and *S. striata*.[15,16] Therefore we suggest that *S. atropatana* may have the similar

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pharmacologic effects. In the present study, the effect of methanol extract of aerial parts of *S. atropatana* on tolerance to the analgesic effects of morphine was evaluated.

Materials and Methods

Sample preparation and Extraction

Aerial parts of *S. atropatana* Grossh were collected from Ispiran region (East Azerbaijan, Iran) in June-July 2013. The samples were stored at laboratory temperature away from direct sunlight until they became dry.

The air-dried grounded aerial parts of *S. atropatana* (100g) were solvent extracted with 200ml of n-hexane to be defatted. Later on, methanol was used for extraction over a period of 12h in triplicate via maceration at room temperature. The crude extracts were filtered, concentrated and dried at 45°C using a rotary vacuum evaporator. The dried methanol extract was the final extract for injection with suitable solvent.

Total phenolic content (TPC)

Phenolic content of the methanol extract was determined by Folin-Ciocalteu method. In summary, 0.5 ml of the extract solution (1 mg/ml) was mixed with 5 ml of Folin-Ciocalteu 10% (v/v) in distilled water. This mixture was maintained in laboratory for 5 minutes then 4 ml of 1 molar aqueous solution of sodium carbonate was added and after 15 minutes the absorbance of the extract was measured at wavelength of 765 nm. The standard curve was plotted by 25, 50, 100, 200µg/ml solution of gallic acid in methanol:water (50:50 v/v). The total phenolic content was calculated from the calibration curve of gallic acid (mg of gallic acid in 1g of the extract).

Antioxidant activity determination by DDPH method

In this research, antioxidant activity of the extract was evaluated by free radical scavenging method. The free radical DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a dark colored purple compound that turns yellow by receiving an electron from an antioxidant compound. The greater the amount of antioxidant is added, the color change will be more specific.

The stock DPPH solution was prepared with the concentration of 0.08 mg/ml. The initial solution of the extract in methanol was prepared with the concentration of 1 mg/ml. The prepared solution was diluted to half of the initial concentration (6 times). Correspondingly, 2ml of DPPH solution was added to 2ml of each extract concentration. After 30 minutes of incubation time, the absorbances of the samples were measured by a spectrophotometer at a wavelength of 517 nm. The free radical scavenging percentages (R%) were calculated for each concentration as follows:

$$R\ (\%) = 100[[(A\ blank - A\ sample)]/A\ blank] \quad \text{Eq. (1)}$$

RC_{50} (50% radical scavenging concentration), concentration of the methanol extract reducing 50% of the DPPH free radicals, was calculated from the graph of inhibition percentages against different concentration of the extract. Moreover, quercetin was applied as the positive control in this test.

Experimental design and grouping animals

In order to induce tolerance, groups of 8 mice were chosen randomly as follows: group I: Saline (10 ml/kg, i.p.) +Saline (10 ml/kg, i.p.), group II: Morphine (50 mg/kg, i.p.) + Saline (10 mg/kg, i.p.) and group III: Morphine (50 mg/kg, i.p.) + Extract of *S. atropatana* (100, 200, 400 mg/kg, i.p.). Mice were treated by morphine (50 mg/kg) in combination with either the extract (100, 200, 400 mg/kg, i.p.) or saline once a day for four days. To evaluate the degree of tolerance, the antinociceptive effect of a test dose of morphine (9 mg/kg) was measured 24 hours after the last dose of morphine in combination with the extract. The different doses of extract were injected 30 min before daily injection of morphine. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz, Iran.

Hot plate test

To evaluate the analgesic effects of the drug regimen, temperature of the appliance was set to 52±0.5ºc. Then the animal was placed on its surface. Licking the hands was considered as a marker for latency time. If the animal had no response to heat, the procedure would be terminated after 45 seconds (cut off time).

Hot plate response latency is expressed as the percentage of maximal possible effect (MPE%) according to the following equation:

$$MPE\% = [(TL - BL)/ (T_{cut\ off} - BL)] \times 100 \quad \text{Eq. (2)}$$

BL=Base Latency time, TL=Test Latency time, $T_{cut\ off}$ =Time of cut off

Statistical Analysis

Values are presented as Mean ± SEM for each group of studied animals (each group contained 8 mice). Data analysis was performed for all groups using One-way ANOVA parametric test. To identify differences between groups we used Tukey test. The significance level of the differences was considered as P-Value <0.05.

Results

The phenolic content determination

The stock solution of *S. atropatana* was analyzed spectrophotometrically and the data was placed in the equation obtained from calibration diagram of galic acid. The penolic content in this concentration (1 mg/ml) was determined. Eventually, the total phenolic content of the methanol extract equated to 152 mg per each gram of the extract.

Determining the antioxidant activity of the extract using DPPH

The scavenging effect of the methanolic extract of *S. atropatana* on free radicals of DPPH was evaluated. According to the equations acquired from the calibration diagrams, RC_{50} was calculated both for the extract and

quercetin. RC_{50} is the concentration of the extract that is able to reduce 50% of the free radicals. According to the results, the calculated RC_{50} for the methanolic extract and quercetin were 143µg/ml and 2.94 µg/ml, respectively.

Effects of morphine (9 mg/kg, i.p.) on tolerant and nontolerant mice

Figure 1 displays the effects of morphine administration (50 mg/kg, i.p.) in 4 consecutive days on the development of tolerance. To have a similar number of injections in all groups, in the control group there were two injections of saline [saline (10 ml/kg, i.p.) + saline (10 ml/kg, i.p.)], and in the morphine group there were morphine and saline injections [morphine (50 mg/kg, i.p.) + saline (10 ml/kg, i.p.)]. On the fifth day the test dose of morphine (9 mg/kg, i.p.) in different groups were assessed. It was verified that administration of morphine resulted in a significant Reduction in MPE% values compared with the saline group ($p < 0.001$).

Effects of administration of the extract (100, 200, 400mg/kg, i.p.) on morphine-induced tolerance

The effects of pretreatment (30 minutes prior to morphine injection) with different doses of *S. atropatana* extract (100, 200, 400 mg/kg, i.p.) on morphine-induced tolerance are shown in Figure 2.

Results revealed that administration of *S. atropatana* extract (100, 200 mg/kg, i.p.) attenuated the degree of tolerance to morphine in some test times. Furthermore, its effect in 30th minutes was more significant. Additionally, pretreatment with *S. atropatana* extract (400 mg/kg, i.p.) did not decrease the tolerance to morphine.

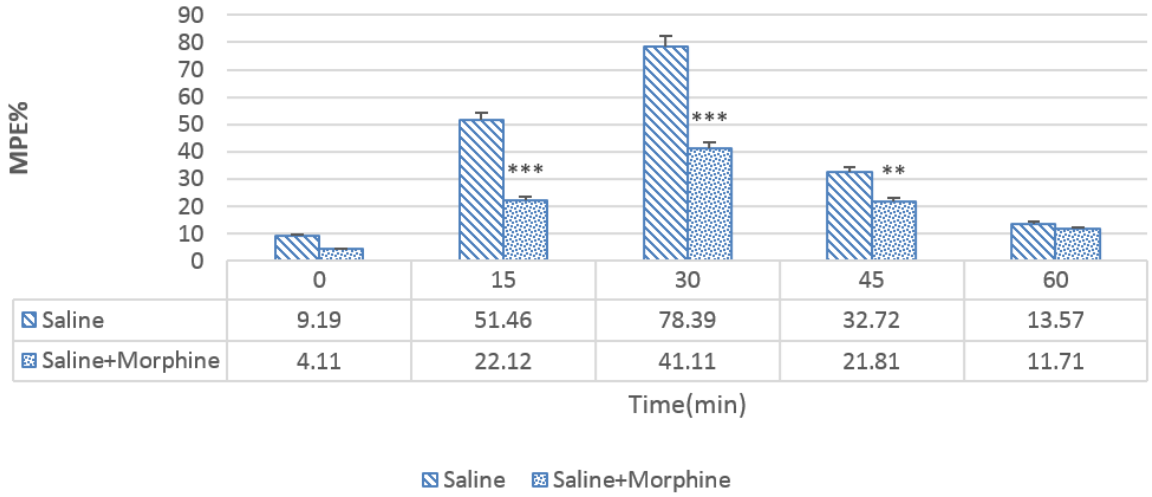


Figure 1. Effects of morphine (9 mg/kg, i.p.) on tolerant and nontolerant mice. Saline group received saline (10 mL/kg, i.p.) + saline (10 mL/kg, i.p.) and the tolerant group received morphine (50 mg/kg, i.p.) + saline (10 mL/kg, i.p.) for 4 days (n = 8 in each group). The test doses of morphine (9 mg/kg, i.p.) in different groups were assessed. Results are expressed as mean ± standard deviation. **p < 0.01 and*** p < 0.001 indicate the significant difference between two groups.

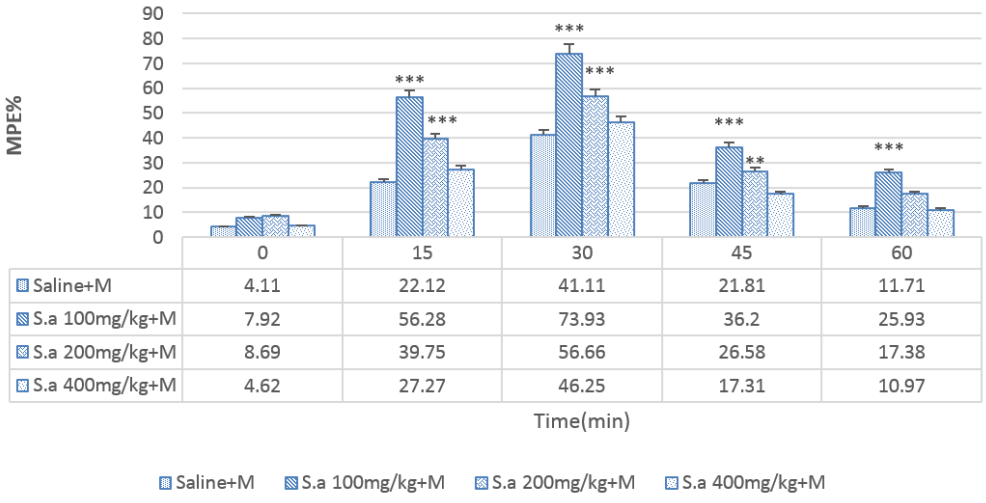
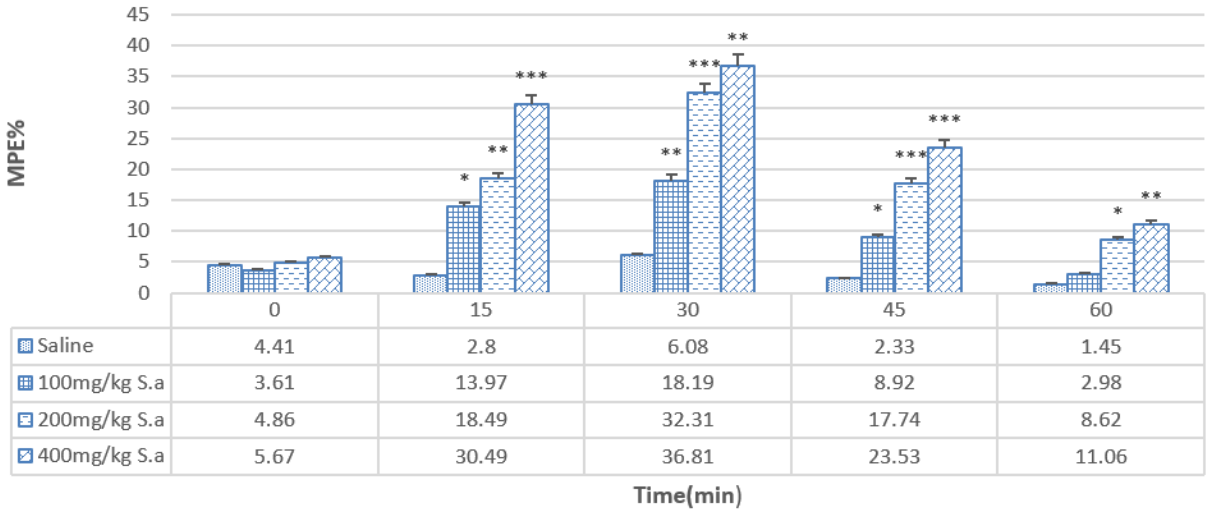


Figure 2. Effects of administration of *S. atropatana* extract (100, 200, 400mg/kg, i.p.) on morphine-induced tolerance. Mice received extract (100, 200, 400 mg/kg, i.p.) + morphine (50 mg/kg, i.p.) for 4 days (n = 8 in each group). On the fifth day the test dose of morphine (9 mg/kg, i.p.) in different groups were assessed. Results are expressed as mean ± SEM. P < 0.01(**), p < 0.001(***) indicate the significantly difference between test group (Saline+ *S. atropatana*) and control group (morphine+saline). S.a: *S. atropatana* extract, M: Morphine.



	0	15	30	45	60
Saline	4.41	2.8	6.08	2.33	1.45
100mg/kg S.a	3.61	13.97	18.19	8.92	2.98
200mg/kg S.a	4.86	18.49	32.31	17.74	8.62
400mg/kg S.a	5.67	30.49	36.81	23.53	11.06

Figure 3. Analgesic effects of the extract (100, 200, 400 mg/kg, i.p.) in hot plate test. MPE% was calculated. Each column represents Mean± SEM for 8 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate the significantly different between Saline and extract (100, 200, 400 mg/kg, i.p.) group.

Analgesic effects of the extract (100, 200, 400 mg/kg, i.p.) in hot plate test

As shown in Figure 3 Injecting various doses of the extract (100, 200, 400 mg/kg, i.p.) resulted in analgesic effects in a dose-dependent manner.

Discussion

The results of this study represent that morphine administration (50 mg/kg, i.p.) for 4 days induce tolerance to analgesic effects of morphine in control groups meaningfully. In previous study, extracts of *Scrophularia* have been shown to have anti-inflammatory activity in different experimental models.[17] The results also proved that various doses of *S. atropatana* extract induce analgesic effects (100, 200, 400 mg/kg, i.p.) and reduce the tolerance to analgesic effects of morphine (100, 200 mg/kg, i.p.). Opioids may activate protein kinase C (PKC) and PKC may phosphorylate glutamatergic receptors, especially N-methyl-D-aspartate (NMDA) receptors.

Opioids can reduce the glutamate transporters activity. Chen and Hung demonstrated that μ receptors activity can increase NMDA receptors activity via PKC in dorsal horn of trigeminal nerves.[18] NMDA receptors activation can increase intracellular Ca^{2+} density, stimulate PKC, exocytosis and finally increase the release of Excitatory amino acids (EAAs).[19]

Elevated extracellular glutamate concentrations would be balanced in neurons and glial cells by glutamate transporters activation. Glial cells stop glutamatergic signals and protect neurons against glutamateric toxicity.[20]

Glutamate transporters gene expression (GLT-1mRNA) in CNS, reduces morphine tolerance and dependence significantly.[21]

Mao et al, proved that chronic intrathecal injection of morphine induces down regulation of glutamate transporters proteins like neuronal glutamate transporter excitatory amino acid carrier-1 (EACC1) and Glutamate Aspartate Transporter (GLAST) in dorsal horn of spinal axis. They demonstrated that increasing glutamate transporters activity reduces morphine tolerance.[22] Furthermore PKC activity has an essential role in morphine tolerance, reducing activity and expression of glutamate transporters[23,24] Results claim that down regulation of glutamate transporters and PKC activity leads to enhancement of EAA concentration in mice treated with morphine.

Previous studies indicated that *Scrophularia* extract has neuroprotective effects via NMDA receptors in glutamatergic system. In fact, iridoid glycosides in genus *Scrophularia* have inhibitory effects in the excitatory neurotransmitters activation process. This mechanism can justify the reduction in morphine tolerance.[14] In this study, administration of *S. atropatana* (100, 200 mg/kg, i.p.) reduced the tolerance to analgesic effects of morphine. Administration of the extract (400 mg/kg, i.p.) did not reduce the tolerance meaningfully. It seems that resultant effects of various chemical molecules in the extract are different with the pathways enhancing the tolerance. The exact mechanisms are still unknown and need further studies. The most effective dose was 100 mg/kg reached its maximum effect within 30 minutes.

Previous studies represent the inhibitory effect of nitric oxide synthase in reducing morphine tolerance, confirming our results.[8] On the other hand, inhibitory effect of *Scrophularia* genus on nitric oxide synthase was shown.[13] *Scrophularia* genus can reduce endogenous nitric oxide resulting in reducing tolerance to analgesic effects of morphine. Therefore some effects of the extract on reducing the tolerance can be caused by some chemical molecules inhibiting nitric oxide synthase.

Previous studies indicate that various compounds are found in *Scrophularia* genus such as flavonoids, phenylpropanoid glycosides, resin glycosides, iridoids and quercetin.[25,26] The analgesic effect of quercetin is applied via inhibition of phospholipase A2, lipoxigenase and nitric oxide synthase. Nitric oxide (NO) is formed in

spinal neurons and has an important role in processing of pain signals.[27] Studies have shown that glycosilated phenylpropanoid compounds extracted from *Scrophularia* genus have analgesic and anti-inflammatory effects.[28] Flavonoids are a group of compounds extracted from *Scrophularia* genus with analgesic effects. Effect of flavonoids on prostaglandins has been conclusively established.[29] On the other hand flavonoids are nitric oxide synthase inhibitors and prevent production of nitric oxide.[30] Since nitric oxide may be a pain mediator, NO reduction may lead to analgesic activity. Studies also show that flavonoids are involved in pain modulation via opioids system.[31]

Scrophularia genus plants have agonistic effects in GABAergic system.[12] This can justify the mechanisms, expressed for the analgesic effects of *S. atropatana* and its effect on morphine tolerance.

The present study indicate that 3 administrated doses of the methanolic total extract (100, 200, 400 mg/kg, i.p.) are able to induce analgesic effects meaningfully. 400 mg/kg dose of the extract was the most efficient dosage reached its maximum effect within 30 minutes.

According to the results, the extract contains large amounts of polyphenols and its antioxidant properties can reduce oxidative stress caused by free radicals. In fact, part of analgesic effects of *S. atropatana* extract may be caused by its antioxidant properties. Botanical extracts are complex mixtures of various compounds causing antioxidant activity. Therefore presence of flavonoids and other phenolic compounds may have a role in sweeping free radicals.[25,26]

According to the results, *S. atropatana* can cause analgesic effects (100, 200, 400mg/kg, i.p.) and reduce the tolerance to analgesic effects of morphine (100, 200 mg/kg, i.p.) meaningfully.

Conclusion

This research demonstrates that *S. atropatana* strongly reduced tolerance to morphine and has analgesic effects. The suggested analgesic effects may be related to the phenolics content and antioxidant activity of the plant alongside inhibition of glutaminergic system.

Conflict of interests

The authors claim that there is no conflict of interest.

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