

Antinociceptive effects of *Peganum harmala* L. alkaloid extract on mouse formalin test.

Hamid Reza Monsef, Ali Ghobadi, Mehrdad Iranshahi
Department of Pharmacognosy, Faculty of Pharmacy

Mohammad Abdollahi

Department of Toxicology & Pharmacology, Faculty of Pharmacy, and Laboratory of Toxicology, Pharmaceutical Sciences Research Centre, Tehran University of Medical Sciences, Tehran, Iran

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Abstract. PURPOSE. To evaluate the effect of *Peganum harmala* (Syrian rue) a wild-growing flowering plant belonging to the family Zygophyllaceae and found abundantly in Iran on formalin-induced pain response in mice. **METHODS.** Total alkaloid extract was prepared from dry seeds of *Peganum harmala*. All doses of extract were dissolved in normal saline and administered intraperitoneally 30 minutes before formalin injection to the mouse paw. Nociception was recorded 0-5 (early phase, A) and 15-40 (late phase, B) minutes after formalin injection. The alkaloid extract was subjected to silica gel column chromatography using a linear gradient with a CHCl₃-MeOH system and different fractions collected. The effective fraction in formalin test were further purified and isolated by preparative thin layer chromatography (TLC) and identified on the basis of nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis. **RESULTS.** Alkaloid extract in doses (mg/kg) used induced significant reduction in pain response when compared to control as follow: 16 (28.63%), 20 (59.15%), 24 (80.75%), 28 (90.14%) and 30 (100%) in the early phase and 20 (24.67%), 24 (59.93%), 28 (78.52%) and 30 (100%) in late phase. Observed responses in both phases of A and B were dose-dependent with r^2 of 0.93 and 0.99 respectively. ED₅₀ for phases of A and B were 27.87 and 24.63 mg/kg respectively ($p < 0.001$ for all groups). **CONCLUSION.** Harmaline, the last step of extraction is the main effective antinociceptive agent of the *Peganum harmala* alkaloid extract.

INTRODUCTION

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. Thus, study of plant species that traditionally have been used as pain killers should still be seen as a logical search strategy, in research for new analgesic drugs [1-4]. The *Peganum harmala* L. (Syrian rue) is a wild-growing flowering plant belonging to the Zygophyllaceae family and is found abundantly in Middle East and North Africa [5]. From ancient times, it has been claimed to be an important medicinal plant. Its seeds are known to possess hypothermic, and hallucinogenic properties [6,7]. It has been used traditionally as an emmenagogue and an abortifacient agent in the Middle East and North Africa [8]. There are several reports in the literature indicating a great variety of pharmacological activities for *Peganum harmala* L such as anti-bacterial, antifungal and MAO-inhibition [9]. It has also been known to interact with α_2 -Adrenoceptor subtypes [10] and have hallucination potency and be effective in the treatment of dermatosis [11], hypothermic [12] and cancer [13]. Among the several models of persistent nociception, formalin has been well established as a valid model to study central sensitization events at the spinal level after peripheral inflammatory states. In this test, two types of nociception were postulated; a short-lasting nociception caused by a direct effect on nociceptors followed by a long-lasting nociception due to inflammation. Since the formalin test measures the response to a long-lasting nociceptive stimulus, it has a closer resemblance to clinical pain. [14-16].

The objective of this study was firstly to determine the *Peganum harmala* analgesic and anti inflammatory properties and secondly to find the active antinociceptive ingredient(s) of the extract.

Corresponding Author: Mohammad Abdollahi, Associate Professor of Pharmacology and Toxicology, Department of Toxicology and Pharmacology, Faculty of Pharmacy, and Laboratory of Toxicology, Pharmaceutical Sciences Research Centre, Tehran University of Medical Sciences, Tehran, PO Box 14155-6451, Iran mohammad.abdollahi@utoronto.ca

MATERIALS AND METHODS

Plant material

The seeds of Syrian rue, collected from local market of Tehran province, in May 1999, were used in this investigation.

Extract preparation

The dry seeds of Syrian rue (100 g) were grinded and then were extracted with 80% ethanol for 24 hr in a continuous extraction (soxhlet) apparatus (Iranian Scientific and Industrial Research Center, Tehran). The extract was filtered, and ethanol was evaporated on a rotatory evaporator under vacuum at a temperature of 45°C to a small volume. Then a small amount of NH₃ (25%) was added to make pH of 9. Subsequently, 100 ml of chloroform was added and slowly shaken for 10 minutes until alkaloids separated from water and enter to the chloroform phase. This was repeated for three times and then total chloroform phase was evaporated, yielding a total alkaloid extract.

Animals

Male albino N-MRI mice from Institute de Pasteur of Tehran weighing 25-32 g were used in the experiments. All experiments were performed according to "The Animal Welfare Act" (Act P.L. 99-198) considering all ethical circumstances. The animals were housed in standard stainless steel cages in a temperature controlled room (22±2°C) with a 12-12 hr light-dark cycle. The mice were randomly distributed into groups of seven as control and test subjects. All animals had access to food and water throughout the experiments. For antinociception recording, mice were allowed to acclimatize for 30 minutes before any injection.

Preparation of doses

The doses of 12, 16, 20, 24, 28 and 30 mg/kg of the alkaloid extract were used. Doses were selected on the basis of extract dry weight and being at the range of 0.1 of extract's LD₅₀ [17]. Sodium chloride 0.9% was used as solvent. All doses were administered intraperitoneally 30 min before formalin injection to animals.

Formalin test

Each mouse received 25 µl of formalin (0.5%) subcutaneously into the dorsal surface of the right hind paw

using a microsyringe with a 26-gauge needle. Immediately after formalin injection, animals were placed individually in a glass cylinder (20 cm wide, 25 cm long) on a flat glass floor and a mirror was arranged at an angle of 45°C under the cylinder to allow clear observation of the paws of the animals. Only licking or biting of the injected paw was defined as a nociceptive response. The total time of the response was measured during periods of 0-5 min (early phase) and 15-40 min (late phase) [16].

Isolation and identification of the effective alkaloids

The alkaloid extract (5 g) was chromatographed on a silica gel column (3.5×90 cm), using a linear gradient with a CHCl₃-MeOH system, and collected in 5 fractions (9.5-0.5, 9-1, 8.5-1.5, 8-2, 7.5-2.5). All fractions was concentrated in room temperature, then examined for antinociceptive activity using the formalin test. The effective fraction of alkaloid extract in terms of antinociception in formalin test was purified again and isolated with precoated silica gel plates used for TLC. The solvent system for the TLC was CHCl₃-MeOH-NH₃ (50:50:3). ¹³C NMR and ¹H NMR were further used to determine the effective component of the alkaloid [18].

Statistical analysis

Comparison between groups was made by one-way analysis of variance (ANOVA) followed by Newman-Keul's test. Differences with P<0.05 between experimental groups were considered statistically significant. Microsoft Excel software was used to examine the dose dependency and ED₅₀ of data presented in figure 1.

RESULTS

Antinociceptive induced pharmacological activity with different doses of alkaloid extract on the formalin-test in mice are shown in figure 1. Alkaloid extract in all doses (mg/kg) used induced significant reduction in pain response (P<0.01) in comparison to control as follow: 16 (28.63%), 20 (59.15%), 24 (80.75%), 28 (90.14%), and 30 (100%), in the early phase and 20 (24.67%), 24 (59.93%), 28 (78.52%) and 30 (100%), in the late phase. Observed responses in both phases of A and B were dose-dependent with r² of 0.93 and 0.99 respectively. ED₅₀ for phase of A and B were 27.87 and 24.63 mg/kg respectively. In comparison to saline, no significant differences were observed in animals treated by a dose of 12 mg/kg of extract (P>0.05). Table 1

shows that among the 5 fractions obtained from the alkaloid extract, the fourth fraction demonstrated greatest antinociceptive (100% inhibition of pain response in both phases of formalin test, $P < 0.001$).

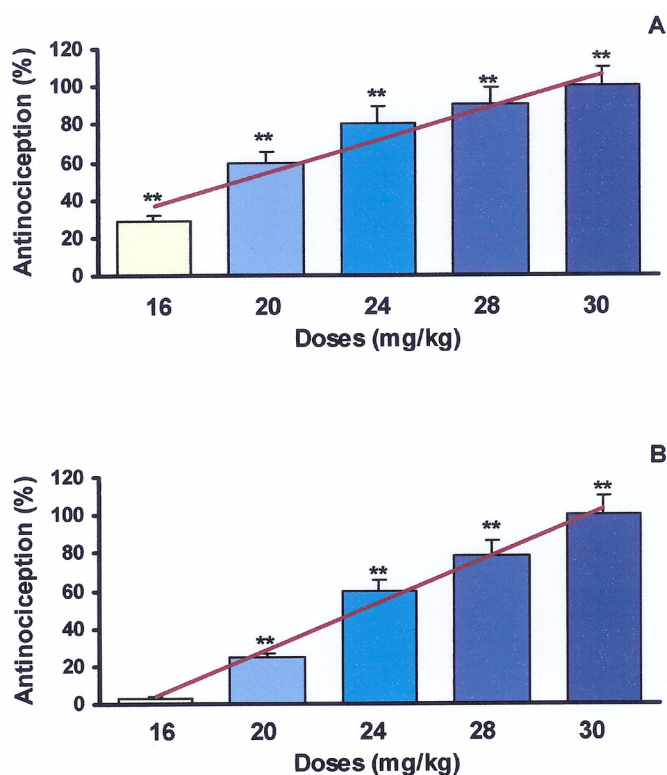


Figure 1: Antinociceptive effects of *Peganum harmala* extract on mouse formalin test.

All doses of extract were dissolved in normal saline and administered intraperitoneally 30 minutes before formalin injection to the mouse paw. Nociception was recorded 0-5 (early phase, A) and 15-40 (late phase, B) minutes after formalin injection. Each point is the mean \pm SE of seven animals that represent percent of inhibition of nociception response in respect to control. Mean \pm SE of control values for phase A and B were respectively (215 ± 19.2 and 310 ± 29.6). **Difference between control & treated groups is significant at $P < 0.001$. Antinociception in both phases of A ($r^2 = 0.93$) & B ($r^2 = 0.99$) are dose-dependent. ED₅₀ in phases of A & B are 27.87 & 24.63 mg/kg respectively.

TLC separation of the fraction 4 demonstrated two bands with R_f of 0.33 & 0.63. The compound with R_f of 0.33 induced 96% ($P < 0.001$) and 87% ($P < 0.001$) inhibition of pain response in acute and chronic phases of formalin test respectively (Table 2). The active component of fraction 4 was identified as harmalin by ¹³C NMR and ¹H NMR as follows: mp = 229° c, ¹H NMR (CDCl₃): δ 8.05 (bs, 1H, H-1), 7.47 (d, $J = 8.8$ Hz, 1H, H-9), 6.85 (d, $J = 2.4$ Hz, 1H, H-12), 6.82 (dd, $J = 8.8$ Hz, $J = 2.4$ Hz, 1H, H-10), 3.86 (s, 3H, OCH₃), 3.8 (t, $J = 8.4$ Hz, 2H, H-5), 2.82 (t, $J = 8.4$ Hz, 2H, H-6), 2.34

(s, 3H, CH₃). ¹³C NMR (CDCl₃): 158.3 (C-3), 157 (C-11), 137 (C-13), 128.5 (C-2), 128 (C-8), 120.5 (C-9), 116.5 (C-7), 110.5 (C-10), 92 (C-12), 54.5 (OCH₃), 48 (C-5), 21.9 (CH₃), 19.4 (C-6).

Table 1: Effect of five isolated fractions from total alkaloid extract of *Peganum harmala* on mouse formalin test.

Fraction Number	Yield of fractionation (%)	Dose (mg/kg)	Antinociception (%)	
			Phase A	Phase B
1	24	7.20	7.5 \pm 0.10	0.0 \pm 0.00
2	9.0	2.70	5.0 \pm 0.09	0.0 \pm 0.00
3	3.5	1.05	9.0 \pm 0.14	4.2 \pm 0.05
4	38.5	11.55	100.00**	100.00**
5	2.5	0.750	3.3 \pm 0.07	0.0 \pm 0.0

The alkaloid extract (5 g) was chromatographed on a silica gel column (3.5 \times 90 cm), using a linear gradient with a CHCl₃-MeOH system, and collected in 5 fractions (9.5-0.5, 9-1, 8.5-1.5, 8-2, 7.5-2.5). All fractions was concentrated in room temperature. Doses were selected based on the ratio of weight percentage for each fractions considering 30 mg/kg as the most effective dose of total alkaloid extract. All doses of extract were dissolved in normal saline and administered intraperitoneally 30 minutes before formalin injection to the mouse paw. Nociception was recorded 0-5 (early phase, A) and 15-40 (late phase, B) minutes after formalin injection. Each point is the mean \pm SE of seven animals that represent percent of inhibition of nociception response in respect to control. Mean \pm SE of control values for phase A and B were respectively (215 ± 19.2 and 310 ± 29.6). **Difference between control and treated groups is significant at $P < 0.001$.

Table 2: Effects of isolated fraction 4 from total alkaloid extract of *Peganum harmala* on mouse formalin test.

Isolated compounds (R_f)	Yield of further fractionation (%)	Injected dose (mg/kg)	Antinociception (%)	
			Phase A	Phase A
0.33	5.75	1.72	95.6 \pm 8.8**	87 \pm 9.1**
0.63	3.25	0.97	8.0 \pm 13.1	0.0 \pm 0.0

The effective fraction number 4 (see table 1) was purified and isolated with precoated silica gel plates and then examined by preparative TLC and also identified on the basis of NMR and MS spectroscopic analysis. The solvent system were CHCl₃-MeOH-NH₃ (50:50:3). Two bands with R_f of 0.33 and 0.63 were isolated and based on formalin test result (table 1), the effective alkaloid was in band with R_f of 0.33. Doses were selected based on the ratio of weight percentage for each fractions considering 30 mg/kg as the most effective dose of total alkaloid extract. All doses of extract were dissolved in normal saline and administered intraperitoneally 30 minutes before formalin injection to the mouse paw. Nociception was recorded 0-5 (early phase, A) and 15-40 (late phase, B) minutes after formalin injection. Each point is the mean \pm SE of seven animals that represents percent of inhibition of nociception response in respect to control. Mean \pm SE of control values for phase A and B were respectively (215 ± 19.2 and 310 ± 29.6). **Difference between control and treated groups is significant at $P < 0.001$.

DISCUSSION

The results of the present experiments demonstrates the significant antinociceptive effects of an alkaloid extract in both phases of the formalin test at doses of 16, 20, 24 and 28 mg/kg. In the formalin test, the initial pain (early phase) is explained as a direct stimulation of nociceptors and the late phase is thought to be secondary to the inflammatory reactions [14-16]. We further isolated the effective alkaloid fraction using column chromatography and TLC. The effective fraction was purified by preparative TLC and then analyzed by NMR and GC-MS indicating that suggests that the effective alkaloid is harmalin. In the formalin test, several mediators such as histamine, kinin, serotonin and prostaglandins are released from damaged cells which take part in the inflammatory response and are able to stimulate nociceptors and induction of pain [19]. To our knowledge based on a search of the literature no studies have been conducted on the interactive effects of Syrian rue with these mediators. Among several β -carbolines (harmine, harmaline, harmalol, harman, vasicine and vasicinon) derived from *Peganum harmala* extract, harmaline has been found to be the major active alkaloid. Harmaline (harmidine, $C_{13}H_{14}N_2O$) in moderate doses has been reported to cause tremors and clonic convulsions without increasing spinal reflex excitability [20]. Harmaline acts as a reversible MAOI [9] and in common with other beta-carbolines bind to 5-HT receptors [12]. Harmaline has also been reported to induce spasmolytic effects on guinea-pig isolated trachea with interaction to muscarinic, histaminic and β -Adreno-receptors [21]. Harmaline also inhibits MK-801 (noncompetitive NMDA channel blocker) binding to the NMDA receptor in rabbit brain [22]. In addition, β -carbolines generally have an affinity for the opioid delta and mu receptors [23]. All of these pharmacological properties of harmaline may conceivably be responsible for antinociceptive effects of *Peganum harmala* extract. In conclusion, this study demonstrates analgesic activity of an extract of Syrian rue which parallels the traditional use of this extract as an analgesic and antiinflammatory medicine. The mechanisms) of action of harmaline remain to be elucidated by further studies.

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