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RESEARCH ARTICLE

Antinociceptive Activity of Various Extracts of *Peganum harmala* L. and Possible Mechanism of Action

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ABSTRACT

The seeds of *Peganum harmala* L. (*Pgh*) (Zygophyllaceae) have been used in Moroccan traditional medicine to treat various diseases. The objective of this study was to investigate the analgesic effect of ethyl acetate (EAE), butanolic (BE) and aqueous (AqE) extracts of seeds of *Peganum harmala* and to elucidate the possible action mechanism of each extract. The antinociceptive action was assayed in experimental models of writhing, formalin, tail flick and hot plate tests in mice. The EAE, BE and AqE (i.p or p.o. routes) showed significant reduction in acetic acid-induced writhings in mice with a maximum effect of 35.12% reduction for BE (25 mg/kg, i.p.). In the formalin test, the pre-treatment with AEA and BE (12.5 and 25mg/kg, i.p.) caused marked dose-related inhibition of formalin-induced licking in both phases, whereas the AqE (25mg/kg, i.p.) reduced the nociception response only in the second phase of formalin test. Hot plate and tail flick tests showed a significant central acting analgesic properties of AEA and BE. The EAE and BE contain active analgesic components acting both centrally and peripherally. Preliminary phytochemical screening of the extracts showed the presence of alkaloids, flavonoids, sterols and saponin. The extracts' antinociceptive effect has been avoided by naloxone (1 mg/kg) in the first phase of formalin and hot plate tests indicating that these extracts act partly through an opioid-mediated mechanism. In conclusion, our results demonstrated that the different extracts of *Peganum harmala* had both central and peripheral antinociceptive activities that may be mediated by opioid receptors.

Keywords: *Peganum harmala* L., Rats, Mice, Antinociceptive activity, Opioid

In analysis the research during the last decades, it is estimated that the analgesics are one of the highest therapeutic categories on which research efforts are concentrated [1]. Analgesic compounds available in the market, still present a wide range of undesired effects [2] leaving an open door for new and better compounds. Natural products are believed to be an important source of new chemical substance with potential therapeutic applicability. Several plant species traditionally used as analgesics [3]. There are reports about analgesic effects of medicinal plants in the literatures [4-10]. *Peganum harmala* (L.) is a member of the family Zygophyllaceae [11] commonly known as '*Harmal'* which grows spontaneously in semiarid and predesertic regions of south-east Morocco and distributed in North Africa and the Middle East [12]. In Moroccan traditional medicine, seeds of *Peganum harmala* were used as powder, decoction, maceration or infusion for fever, diarrhoea, abortion and subcutaneous tumours and is widely used as a remedy of dolorous events (rheumatic pain, painful joint and intestinal pain) [13]. It is also used for treatment of asthma, jaundice, lumbago and many other human ailments [14, 15]. There are several reports in the literature indicating a great variety of pharmacological activities for *Peganum harmala* L. such as anti-bacterial, anti-fungal and monoamine oxidase inhibition [16]. It was effective in the treatment of dermatosis [17], hypothermia [18] and cancer [19].

Considering the popular use of this plant to relieve some pains, we focused in this report to investigate the antinociceptive effect of ethyl acetate, butanolic and aqueous extracts using chemical and thermal nociception models. On the other hand, an attempt was conducted to determine the participation of the opioid system in the antinociceptive effect of these extracts using naloxone (a non-selective opioid antagonist). In

addition, preliminary phytochemical screening was conducted in order to determine the phyto-constituents in the tested extracts.

MATERIALS AND METHODS

Animal Models and Habituation

Male *Sprague-Dawley* rats and male mice, weighing 180-230 and 25-30 g, respectively, were used in this study. Animals were housed in groups of three rats or six mice per standard makrolon cage, on 12-h light/12-h dark cycle; and air temperature was maintained at 22 ± 2°C. They were offered food and water ad libitum.

Experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigation of experimental pain in conscious animals [20].

Plant Material

The seeds of *P. harmala* were collected in the month of July 2004 from Marrakech (Haouz, Morocco). Samples of the plant were identified and stored in the Herbarium of faculty of Science Semlalia Marrekech (voucher number 4229).

Preparation of Extracts

The powdered dry seeds of *Peganum harmala* (210 g) were extracted with methanol for 24 h in a continuous extraction soxhlet apparatus. This methanolic extract was concentrated and then partitioned successively between water and organic solvents of increasing polarities to afford the new extracts: hexane (hexanic extract), dichloromethane (dichloromethane extract), ethyl acetate (ethyl acetate extract) and *n*-butanol (butanolic extract), in this order, as well as aqueous extract, which is the water-soluble remaining extract. The extracts obtained were concentrated using a rotary evaporator, and dissolved and made up to appropriate volume with 0.9% NaCl just before experiment. Chemistry procedures allowed obtaining the following yields: 21.9% methanolic extract, 1.8% ethyl acetate extract (EAE), 6.7% butanolic extract (BE) and 6.1% aqueous extract (AqE), respectively.

Determination of Acute Toxicity

Acute toxicity was determined as described by Lorke [21]. Mice were treated intraperitoneally (12.5, 25 and 50 mg/kg) and orally (25, 50 and 100 mg/kg) with AEA, BE and AqE, whereas the control groups received normal saline orally or intraperitoneally. Each experimental group contained eight animals (four males and four females). The general symptoms of toxicity were observed for 24 h and mortality was recorded at the end of this period.

Antinociceptive Tests

Writhing Test

Abdominal contraction, induced by i.p. injection of acetic acid 0.6%, consisted of a contraction of the abdominal muscle together with a stretching of the hind limbs [22]. The animals were pre-treated with 0.1ml/10g EAE, BE or AqE of *Peganum harmala* (12.5 or 25mg/kg, i.p.) 30 min before, or the extracts (25mg/kg, p.o.) 1h before, acetic acid injection. The control groups received the same volume of normal saline (0.1ml/10g). The resulting writhe and stretching were observed and counted every 5 min over a period of 30 min after acetic acid injection. Five minutes after the administration of the acid, the number of writhes and stretching movements (contraction of the abdominal musculature and extension of hind limbs) was counted over a 5 min for a period of 30 min. The stretching of elicited analgesic effect was compared to that of an effective dose of acetylsalicylic acid (ASA, 200 mg/kg, i.p.).

Formalin Test

The method used in the present study was similar to that described previously by De Miranda [23] with slight modifications. It consists briefly of subcutaneously injection of 20µl of 20% formalin into the right posterior paw of mice placed in a transparent enclosure. Throughout 5 min prior to this procedure; each mouse was allowed to adapt the testing box and left freely moving and exploring (habituation). The formalin-induced licking of the paw was considered as indicative of the nociceptive behaviour. Using a chronometer; the total time spent in licking and biting the injected paw is recorded, quantifying the nociceptive behaviour. Formalin test in rodent consists of two successive phases [24]. The nociceptive response normally peaked 5 min after formalin injection (early-phase) and 15-30 after formalin injection (late-phase), representing the tonic and inflammatory pain responses, respectively.

The animals were pre-treated intraperitoneally with EAE, BE or AqE of *P. harmala* (12.5 and 25mg/kg), or with morphine (10mg/kg) or ASA (200mg/kg), 0.5h beforehand. On the other hand, to investigate the participation of opioid system in the antinociceptive effect of the *P. harmala* extracts, animals were pretreated with naloxone (1mg/kg) subcutaneously (s.c.) 15 min before administration of extracts according to the method described by Abdel-Fattah et al. [25].

Hot-plate test

In this test, animals were placed in a glass cylinder on heated metal plate maintained at 55 ± 2°C. The latency of nociceptive responses such as licking or shaking one of the paws or jumping was recorded as the reaction time. The animals were pretreated with saline (10ml/kg), morphine (10mg/kg, i.p.) or EAE, BE or AqE (12.5 and 25mg/kg, i.p.), and they were put later at 0, 0.5h on the heated surface of the plate at 55 ± 2°C. In order to determine the involvement of opioid system on the antinociceptive effect, naloxone (1mg/kg, s.c.) was administered 15 min before treatment with morphine (10mg/kg, i.p.) EAE, BE or AqE (25mg/kg, i.p.) [26,27].

Table 1. Effect of ethyl acetate, butanolic and aqueous extracts of *Peganum harmala* seeds on the acetic acid-induced writhing behaviour in mice

Treatment groups	*Dose (mg/kg)*	*Number of writhes (during 30min)*	*% of writhes inhibition*
Control (i.p.)		102.50 ± 13.55	-
EAE (i.p.)	12.5	93.63 ± 6.3	8.65
	25	84.50 ± 8.19**	17.56
BE (i.p.)	12.5	86.13 ± 4.52**	15.97
	25	66.50 ± 7.45***	35.12
AqE (i.p.)	12.5	93.00 ± 5.53	9.26
	25	86.63 ± 6.07**	15.48
Control (p.o.)		99.88 ± 7.66	-
EAE (p.o.)	25	88.38 ± 11.22*	11.51
BE (p.o.)	25	80.25 ± 5.92***	19.65
AqE (p.o.)	25	88.75 ± 10.66*	11.14
ASA (i.p.)	200	35.38 ± 3.2***	65.48

Each value represents mean S.E.M, n = 8. Statistical significant test with control was done by Student's *t*- test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. EAE = Ethyl acetate extract; BE = Butanolic extract; AqE = Aqueous extract; ASA = Acetyl Salicylic Acid.

Tail Immersion Test

The procedure consisted of immersing the base of the animal's tail in 55°C heated water [28]. The nociceptive response to this thermal stimulation is indicated by the reflex withdrawal of the animal's tail. Using a chronometer, the latency time was recorded before normal saline or extracts treatment and at 30, 60, 90, 120, 180 or 240 min after treatment. Rats were treated with EAE, BE or AqE (12.5 and 25mg/kg, i.p.).

Phytochemical Screening

Preliminary phytochemical properties of the ethyl acetate, butanolic and aqueous extracts of *Peganum harmala* using standard procedures to identify the constituents, alkaloids with H_2SO_4 and Dragendorff's reagents, flavonoids with the use of Mg and HCl, tannin with $Fecl_3$ solution, anthocyanes with HCl, sterols and/or terpenes with acetic anhydride and H_2SO_4, quinons with HCl and ammoniac and saponin with ability to produce suds [29-33].

The Ulcerogenic Activity of Extract

The ulcerogenic activity of the extract was investigated using the method of Mimura et al. [34]. Mice of either sex were fasted for 18 h with access to water. At the end of the fasting period, the mice were treated intraperitoneally (12.5 and 25mg/kg) and orally (25 mg/kg) with AEA, BE or AqE and ASA (200mg/kg, i.p.), whereas the control groups were received normal saline orally or intraperitoneally. Eight hours after drug administration, animals were sacrificed and the stomachs were opened along the greater curvature. The stomach mucosa was examined for ulcer lesions using a hand lens (×20 magnification). The length of lesions on the glandular portion were estimated and summed up to calculate the ulcer index [34].

Statistical Analysis

The mean ± SEM response was calculated, and comparisons between the experimental groups were made using *Student's t*-test. The *p* values less than 0.05 were considered significant.

RESULTS

Oral administration (25, 50 and 100mg/kg) and intraperitoneal administration (12.5 and 25mg/kg) of the AEA, BE and AqE did not cause any death and did not show any toxic symptoms or change in general behaviour or other physiological activities of mice. However, an intraperitoneal dose of 50 mg/kg of all extracts induced abdominal writhing, body tremors and slight decrease in locomotor activity.

Writhing Test

As shown in Table 1, the EAE, BE or AqE of *P. harmala*, given by i.p. or p.o. (12.5 and 25 mg/kg) routes, caused a dose-related inhibition of acetic acid-induced visceral nociceptive responses in all of the analysed periods. Furthermore, i.p. administration of extracts was more potent than p.o. Such effects were observed in mice pre-treated by ASA (65.48%). The most potent effect was contributed to the BE (35.12% of writhes inhibitions), as compared with those of the EAE (17.56%) and AqE (15.48%).

Formalin test

Pre-treatment of animals with ethyl acetate or butanolic extracts promoted a significant inhibition of formalin-induced licking in the early and in the late phases only at higher doses (Fig 1), While only the second phase of nociceptive response was significantly reduced by 25mg/kg of aqueous extract ($p<0.001$). When tested in the formalin induced-pain as a reference, ASA caused marked inhibition of licking responses in the second phase ($p<0.001$) whereas morphine acted throughout both phases ($p<0.001$).

As shown in Fig 2, naloxone reversed significantly the antinociceptive effect of EAE and BE in the early phase. However, there is no significant effect on the nociceptive effect of AqE. In the late phase, subcutaneously-administered naloxone did not show any significant effect on the *Peganum harmala* extracts antinociception.

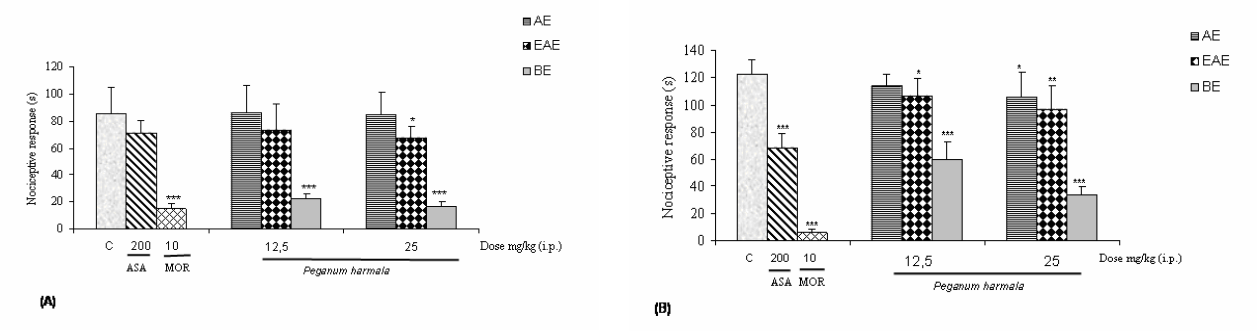

Fig 1. Effect of *Peganum harmala* extracts on nociceptive response in first (**A**) and second (**B**) phases of formalin test. Ethyl acetate, butanolic and aqueous extracts (12.5 and 25 mg/kg; i.p.), morphine (10mg/kg, i.p.), ASA (200mg/kg, i.p.) and normal saline were administered 30min before the start of the test. Each Histogram represents the mean latencies (± S.E.M.) for a testing group of eight mice. *p<0.05; **p<0.01 and ***p<0.001 compared to control using *Student's t*-test. EAE = Ethyl acetate extract; BE = Butanolic extract; AqE = Aqueous extract; Mor = Morphine; ASA = Acetyl Salicylic Acid; C = Control.

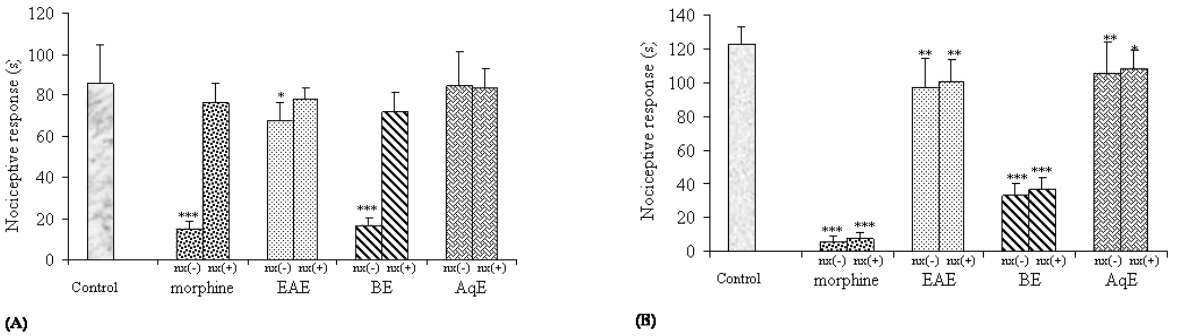

Fig 2. Effect of *Peganum harmala* extracts on nociceptive response in first (**A**) and second (**B**) phases of formalin test in absence (-) and presence (+) of naloxone (1mg/kg, s.c.). Ethyl acetate, butanolic and aqueous extracts (25 mg/kg, i.p.) and morphine (10mg/kg, i.p.) were administered 30 min before formalin injection. Each Histogram represents the mean latencies (± S.E.M.) for a testing group of eight mice. *p<0.05; **p<0.01 and ***p<0.001 compared to control using *Student's t*-test. EAE = Ethyl acetate extract; BE = Butanolic extract; AqE = Aqueous extract; MOR = Morphine; nx = naloxone.

Tail Immersion Test

The pre-treatment of animals with ethyl acetate and butanolic extracts delayed significantly (p<0.05) the reaction time to the nociceptive stimulus (Fig 3). This effect that was recorded 60 min after extracts administration was potent about 1h and decreased at 2h after this pre-treatment. On the other hand, no difference in the time latencies was observed when the control rats were compared with those pre-treated by aqueous extract.

Hot Plate Test

The results are reported in Table 2. EAE (p<0.05) and BE (p<0.01) (25mg/kg, i.p.) of *Peganum harmala* and morphine (10mg/kg, i.p.) (p<0.001) significantly increased the reaction time to the nociceptive response in the hot plate. AqE and ASA had no effect on this test. On the other hand, these results also showed that pre-treatment with a non-selective opioid receptor antagonist (naloxone, 1mg/kg, s.c.) reversed significantly the antinociceptive effect of the morphine (10mg/kg, i.p.), EAE and BE (25mg/kg, i.p.) in the hot plate test.

Phytochemical Screening

The preliminary phytochemical screening of various extracts of *Peganum harmala* revealed the presence of alkaloids, flavonoids, saponins, sterols, terpenes and quinines as presented in Table 3. In particular, the EAE and BE were highly positive for the alkaloids and flavonoids but aqueous extract was only positive for alkaloids. Saponin, sterols and terpenes were detected only in the aqueous extract and quinone was positive only for butanolic extract.

DISCUSSION

The results of present study show that the ethyl acetate, butanolic and aqueous extracts of *Peganum harmala*, administered either intraperitoneally or orally, produces significant antinociceptive action against chemical (acetic acid-induced visceral pain or formalin-induced nociception) and thermal (hot-plate test or tail-flick) models of nociception in mice and rats. These results also showed that butanolic extract was the most effective and more potent than the ethyl acetate and aqueous extracts.

This work for the first time shows that the EAE, BE or AqE of *Peganum harmala*, when given intraperitoneally or orally, produces dose-related and significant antinociception according to assessment of the abdominal constrictions elicited by acetic acid, a model used to evaluate the potential analgesic activity of drugs.

Table 2. Effect of ethyl acetate, butanolic and aqueous extracts from *Peganum harmala* on the nociceptive response in the hot plate test

Treatment (mg/kg)	*Time after treatment (min)*	
	0	*30*
Control (saline)	8.84 ± 1.09	8.36 ± 0.97
EAE	9.25 ± 0.72	9.46 ± 0.47*
BE	8.38 ± 1.02	11.16 ±1.68**
AqE	8.54 ± 0.7	9.16 ± 1.11
EAE (25) + nx	8.46 ± 0.63	8.55 ± 0.69
BE (25) + nx	8.70 ± 1.11	8.74 ± 1.25
AqE (25) + nx	8.53 ± 0.62	8.99 ± 10.1
Morphine 10	9.80 ± 1.18	18.60 ± 2.09***
Morphine (10) + nx	9.50 ± 0.91	8.99 ± 1.48
ASA 200	10.25 ± 1.72	9.40 ± 1.82

Each value represents mean S.E.M, n = 8. Statistical significant test with control was done by Student's *t*- test. *p<0.05, **p<0.01, ***p<0.001. All injections were i.p. EAE = Ethyl acetate extract; BE = Butanolic extract; AqE = Aqueous extract; nx = naloxone; ASA = Acetyl Salicylic Acid.

Table 3. Phytochemical screening of extracts of *Peganum harmala*

Constituents	Ethyl acetate extract	Butanolic extract	Aqueous extract
Alkaloids	+++	+++	++
Flavonoids	+++	++	−
Tanins	−	+	−
Saponins	−	−	+++
Quinones	−	+++	
Sterols	−	−	+++
Terpenes	−	−	+++
Anthocyanes	−	−	−

Phytochemical test: - negative and + positive, +++ quantitative presence

It has been suggested that acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons [22]. It is sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) and to narcotics and other centrally acting drugs [22, 35, 36]. The results of the present study confirm previous data by demonstrating that morphine (a narcotic drug) and aspirin (a non-steroid anti-inflammatory drug) cause significant inhibition of acetic acid-induced pain. Furthermore, the analgesic effects of the extracts were more prominent than that of ASA, but it was lower when compared to morphine in inhibiting the acetic acid-induced visceral nociceptive response.

The acetic acid-induced writhing method is widely used for the evaluation of peripheral antinociceptive activity [37]. Also called as the abdominal constriction response, it is very sensitive and able to detect antinociceptive effects of compounds and dose levels that may appear inactive in other method like tail-flick test [38]. Local peritoneal receptors are postulated to be partly involved in the abdominal constriction response [39]. The method has been associated with prostanoids in general, e.g. increased levels of PGE_2 and PGE_2 in peritoneal fluids [40] as well as lipoxygenase products by some researchers [41, 42].

An important disadvantage of acetic acid-induced writhing method model is that other classes of drugs such as adrenergic antagonists and muscle relaxants can reveal the same effect, [43], favouring possible false positive results. Due to its lack of specificity, it is usual to analyse positive results in the writhing test in combination with the results of other tests. For this reason, the formalin test was performed as well. The test has a distinctive biphasic nociceptive response termed early and late phases. Drugs that act primarily on the central nervous system inhibit both phases equally while peripherally acting drugs inhibit the late phase [44, 45]. Our results showed that the time spent in licking the injured paw was significantly reduced by intraperitoneal administration of the EAE and BE in both phases, while the treatment with aqueous extract (i.p.) protects only the late phase. In this test, the early phase is probably a direct result of stimulation of nociceptors in the paw and reflects centrally-mediated pain while the late phase is due to inflammation with a release of serotonin, histamine, bradykinin and prostaglandins [46] and at least to some degree, the sensitization of central nociceptive neurons [46-48]. In fact, the effect of the EAE and BE on both phases showed that they contain

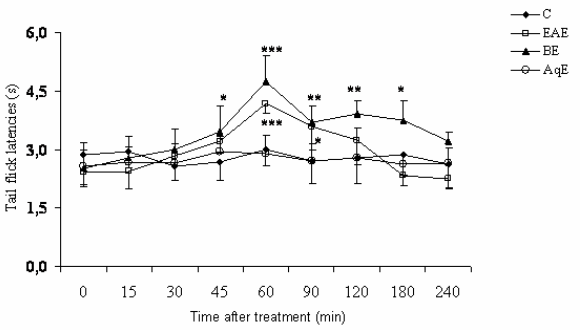

Fig 3. Effect of the ethyl acetate, butanolic and aqueous extracts of *Peganum harmala* on the nociceptive response in tail immersion test. The tested extracts (25mg/kg, i.p.) and normal saline were administered 30min before testing. Each point represents the mean latencies (± S.E.M.) for a testing group of six rats. *p<0.05; **p<0.01 and ***p<0.001 compared to control (C) using *Student's t*-test. EAE = Ethyl acetate extract; BE = Butanolic extract; AqE = Aqueous extract; C = Control.

active analgesic principles acting both centrally and peripherally, while, the active constituents of aqueous extract act rather peripherally.

On the other hand, our results showed that naloxone reversed the antinociceptive effect of ethyl acetate and butanolic extracts in the first phase but not in the second phase of formalin test. This finding clearly suggests that ethyl acetate and butanolic extracts involve at least partially the opioid system in their antinociceptive action. Furthermore, this result indicated that the ethyl acetate and butanolic extracts contains more than one active compound, and at least, one of their polar components is acting through the opioid system. Besides this suggestion, also a peripheral mechanism seems partly implicated, since the ethyl acetate and butanolic extracts antinociceptive effect in the inflammatory phase was not reversed by naloxone.

In the hot-plate test, a central model that has a selectivity for opioid-derived analgesics [49], intraperitoneal administration of ethyl acetate and butanolic extracts exerts a potent antinociceptive action confirming the central activity of these extracts. It is also interesting to note in this test that pre-treatment with naloxone reversed this antinociceptive activity confirming that antinociceptive action is produced by activation of the opioid system. Furthermore, the central analgesic effect of ethyl acetate and butanolic extracts may be supported by the results recorded in the tail immersion test which is a selective method to screen centrally-acting opiate analgesic drugs. Indeed, the EAE and BE showed marked inhibition on the reaction time to the thermal nociceptive stimulus.

Another interesting result of the current study was the fact that oral administration of the extracts of *Peganum harmala* presented some efficacy effect like intraperitoneal administration in preventing the acetic acid-induced pain but this action was significantly more efficacious when administered by intraperitoneal route, concordant with the fact that pre-systemic metabolic may reduce the concentration of active components of these extracts.

According to these findings, it can be suggested that the central and peripheral effects may result in two classes of extracts compounds. Polar compound(s) which is acting centrally on opioid system is highly present at the ethyl acetate and butanolic extracts. The second class of compounds which is acting peripherally is present at all three extracts. In our phytochemical experiments, we have shown that *Peganum harmala* seeds extracts contain alkaloids, flavonoids, saponin, terpenes, sterols and quinons.

The peripheral antinociceptive effect may be attributed to alkaloids because of their presence at the three extracts. In addition, previous work had reported the antinociceptive effect of alkaloids extract of *Peganum harmala* in formalin test wich is due to the alkaloids [10]. Several flavonoids isolated from medicinal plants have been discovered to possess significant antinociceptive and/or anti-inflammatory effects [50]. It is, therefore, possible that both the anti-nociceptive and anti-inflammatory effects observed with ethyl acetate and butanolic extracts may be attributable to its flavonoids.

In conclusion, this study has shown that ethyl acetate, butanolic and aqueous extracts of *Peganum harmala* possess significant anti-nociceptive effect in laboratory animals at the doses investigated. The butanolic extract which is effective and potent in the same way of the analgesic reference drug (ASA) acts partly through an opioid-mediated mechanism. Furthermore, the results support the traditional use of this plant in relieving painful conditions. The analgesic ctivity can be related to phytochemicals such as alkaloids, flavonoids, terpenes, saponin and sterols reported in the seeds extracts. Further studies are in fact currently underway to isolate and characterize the active principle(s) of the crude extracts.

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