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# Effects of the Hydroalcoholic Extract of *Peganum harmala* Against the Venom of the Iranian *Snake Naja naja oxiana* in Mice

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## ABSTRACT

*Peganum harmala* contains pharmacologically active compounds and has been utilized for various purposes over the years. Due to public health concerns about snakebite envenoming, this study aimed to assess the potential antagonistic effects of this plant against the lethal impact of snake (*Naja naja oxiana*) venom. This study used five protocols and 56 adult albino mice in seven equal groups (A, B1, B2, C, D, E, and F). In protocol I (control), group A received only 4 mg/kg of venom, while groups B1 and B2 received the *P. harmala* extract at doses of 15 and 30 mg/kg, respectively. In protocol II, group C was simultaneously administered 15 mg/kg of the extract and 4 mg/kg of venom. In protocol III, group D received 4 mg/kg of venom, followed by the administration of 15 mg/kg of the extract after 20 min. In protocol IV, group E was treated with venom-extract pre-incubated for 20 min at the same doses. In protocol V, group F received 30 mg/kg of the extract orally 60 min before the injection of venom at 4 mg/kg. The route of injection was IP. The average time of death after venom injection was 31 ± 5 min. Groups B1 and B2 survived, while the animals in group C died after 29 ± 7 min, group D after 18 ± 4 min, group E after 17 ± 5 min, and group F after 22 ± 3 min. In conclusion, *P. harmala* does not protect against *Naja naja* venom and accelerates its lethal effect in an unknown way.

**Keywords**

*Snakebite, Peganum harmala, Naja naja oxiana, Venom, Synergist*

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## Abbreviations

*IP: Intraperitoneal*
*WHO: World Health Organization*
*LD50: Lethal dose 50%*
*IV: Intravenously*
*PLA2: Phospholipase A2*
*LD100: Lethal dose 100%*
*MAO: Monoamine oxidase*



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## Introduction

Snakebite envenomation is a global public health issue recognized by WHO, especially in tropical and subtropical regions. Over five million people are affected annually, resulting in 135,000 deaths. Survivors often face long-term disabilities, exceeding the fatality rate [1-3].

Iran, a temperate region, exhibits an impressive biodiversity with a notable presence of 81 snake species. Among these, 25 are venomous and hold significant medical importance. In a period spanning from 2002 to 2011, Iran witnessed a substantial number of reported snakebite incidents, which reached a staggering figure of 53,787 cases, resulting in 67 deaths.

The Caspian cobra (*Naja naja oxiana*) (Figure 1), belonging to the Elapidae family, is primarily found in the northeastern part of Iran, mainly in Khorasan province [4-7]. It is one of the most venomous snakes in Iran and in some neighboring countries. Its venom contains a highly potent neurotoxin with an exceptionally low LD50, making it more deadly than other cobra venoms. Studies have indicated that its LD50 is 10 µg/mouse when administered IV and recorded after 24 hours [8, 9]. Mortality due to Iranian cobra bites is 70%-75% if not treated, which is the highest rate among cobras, especially the Naja genus [10]. While N. n. oxiana is responsible for numerous fatal snakebites in Iran, the exact number of individuals envenomed by this snake has not been officially reported.

Antivenoms are considered the most conventional and effective treatment for venomous bites, as they can neutralize the toxins present in the venom and alleviate its effects. They play a crucial role in reducing the severity of envenomation, preventing complications, and promoting recovery.

However, it is important to acknowledge that antivenoms also have certain disadvantages. These include the risk of allergic reactions, such as early potentially life-threatening anaphylactic reactions and delayed reactions of the serum sickness type. Limited availability, high cost, specificity to particular types of venom, time limitations for administration, and the potential for other side effects should be noted [11-13]. Moreover, antivenoms have demonstrated limited efficacy in effectively treating the local destructive effects induced by venoms. Consequently, there exists a continuing and significant medical need pertaining to venomous bites and stings. It is paramount for scientists to persist in their endeavors to explore and develop more potent alternatives, as well as make advancements in the field of antivenom research.

In regions with a high frequency of snakebite incidence and limited access to medical facilities, the utilization of herbal medicines may be the only hope for saving the lives of victims [14, 15].

One illustrative example involves the employment of Indian Rauvolfia serpentina within traditional Ayurvedic medicine to address snakebite complications. Another notable plant is *Andrographis paniculata*, also known as the "King of Bitters," which has a longstanding history in traditional medicine for its use in treating snakebites and venomous envenomation. *Azadirachta indica*, commonly referred to as Neem, can be topically applied to mitigate the swelling and inflammation triggered by snakebites. *Aloe barbadensis*, commonly recognized as aloe vera, offers pain alleviation and support in wound healing when applied topically to the site of snakebite. Moreover, Curcuma longa, also known as turmeric, possesses curcumin, which exhibits considerable anti-inflammatory and antioxidant properties that can facilitate wound healing by reducing inflammation when topically administered [16]. Certain traditional medicines, that contain natural PLA2 inhibitors, possess potent anti-snake venom properties. For instance, the ashwagandha plant (*Withania somnifera*) has been found to neutralize the venom of the speckled cobra (*Naja naja*) [17], and the leaf extract of *Acalypha indica* has been shown to have the potential to neutralize the venom of the Russell's viper [18].

The scientific investigation of herbal antidotes for snake venom holds significant importance in the management of snakebites, while the effectiveness of this traditional treatment approach remains largely unproven in most cases.

*Peganum harmala L.* (*P. harmala*) (Figure 1), a perennial plant, is commonly known as Syrian Rue, harmala, or Espand in Iranian traditional folklore, and belongs to the family Zygophyllaceae [19]. While its primary origin is central Asia, it has scattered and now grows in various regions, including Australia, northern Africa, and southwestern America. *P. harmala* thrives in semiarid conditions similar to those found in Iran [20, 21].

Recent research has shown that *P. harmala* contains numerous *phytoconstituents* largely in its seed [22]. The bioactive alkaloids, including harmine, harmaline, harmol, vasicine, vasicinone, deoxyvasicine, and deoxyvasicinone are responsible for their therapeutic functions, such as anticancer, antidiabetic, antimicrobial, anti-inflammatory, antiviral, antidiarrheal, antiemetic, antidepressant, anthelmintic, and antioxidant properties, which have been vastly documented [23-25].

In Iran, the most popular traditional use of P. *harmala* seeds is as a disinfectant by burning the seeds and producing smoke through direct heat. While there are reports of individuals in various regions utilizing *P. harmala* to treat snake bites, these claims lack

scientific and research-based evidence.

This study was conducted to assess the potential of *P. harmala* as a remedy for snakebites, representing the first investigation into its efficacy in countering the lethal effects of snake venom. Accordingly, we aimed to investigate the possible antagonistic impact of *P. harmala* on the venom of the Iranian cobra, *N. n. oxiana.*

## Results

### *Evaluation of Peganum Hermala extract for its antivenom activity*

*Protocol I, acute toxicity study*

Prior to the main tests, several pilot studies were conducted to determine the LD100 of Naja naja oxiana venom in mice. Control groups, were including Group A, B1, and B2. In Group A, all mice were administered a dose of 4 mg/kg of *N. n. oxiana* venom alone and animals in group B1 and B2 received only the *Peganum Hermala* extract at doses of 15 mg/kg and 30 mg/kg, respectively. The mortality rate in group A was 100%, and the average time to death was 31 ± 5 minutes (Figure 2). Conversely, all mice in Groups B1 and B2, survived. This observation indicates a lack of toxic effects of the extract at the concentrations tested (Table 1).

*Protocol II, the effect of Peganum Hermala extract were injected simultaneously with the venom of N. n. oxiana*

All mice in Group C were treated with 15 mg/kg of *Peganum Hermala* extract along with 4 mg/kg of venom simultaneously. In this group, the mortality rate was 100%, and the average time to death was 29 ± 7 minutes. These values were not significantly different from the time to death of animals in Group A. (Figure 1) (Table 1).

*Protocol III, the effect of Peganum Hermala extract were injected 20 min after the venom of N. n. oxiana*

In this protocol, animals in Groups D received 15 mg/kg of *Peganum Hermala* extract, 20 minutes after being treated with 4 mg/kg of venom. The average time to death in Group D was 18 ± 4 minutes, which was significantly different from the time to death of animals in Group A ($p < 0.001$) (Figure 2) (Table 1).

*Protocol IV, effect of a mixture of N. n. oxiana venom and Peganum Hermala extract*

In this protocol, group E was treated with a mixture of 4 mg/kg of venom and 15 mg/kg of *Peganum Hermala* extract were incubated for 20 min. The average time to death in this group was 17 ± 5 minutes, which was significantly different from the time to death of animals in group A ($p < 0.01$) (Figure 2) (Table 1).

*Protocol V, effect of oral administration Peganum Hermala extract against N. n. oxiana venom*

In this protocol, group F was treated with 4 mg/kg of venom and 30 mg/kg of *Peganum Hermala* extract orally. The average time to death in group F was 22 ± 3 minutes, which was significantly different from the time to death of animals in group A ($p < 0.01$) (Figure 2) (Table 1).


**Figure1.**
A) Peganum harmala seeds, B) Iranian snake Naja naja oxian (Prepared by B. Fathi)

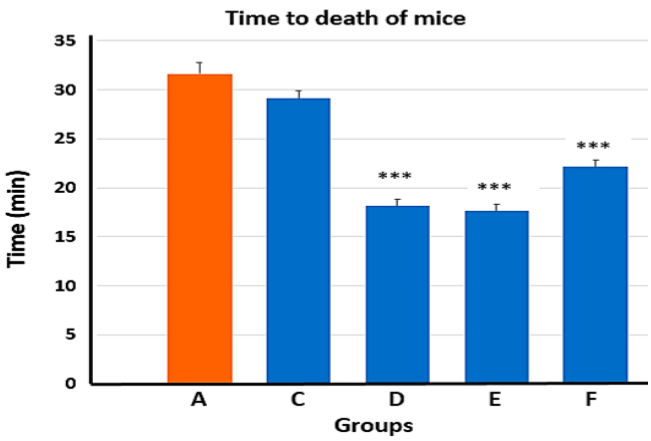


**Figure 2.**
Time to death of mice after application of venom (V) and Peganum Hermala extract in different experimental protocols.
Protocols: I (groups A): Only venom was injected at dose of 4 mg/kg (control). II (group C): Venom at 4 mg/kg and plant extract at 15 mg/kg have been injected simultaneously. III (group D): The plant extract has been injected 20 minutes after the venom injection at the pervious doses. IV (group E): Venom and plant extract have been incubated for 20 min prior to being injected at the pervious doses. V (group F) treated with venom at 4 mg/kg (ip) 60 minutes after administration of plant extract at 30 mg/kg orally. The level of significance considered was $p < 0.05$.

**Table 1.**
Application of different protocols and summary of the experiment results

| Protocols | NO of mice | Venom Mg/kg | P. Harmala Mg/kg | Average time to death |
|---|---|---|---|---|
| A | 8 | 4 | - | 31 ± 5 |
| B1 | 8 | - | 15 | Live |
| B2 | 8 | - | 30 | Live |
| C | 8 | 4 | 15 | 29 ± 7 |
| D | 8 | 4 | 15 | 18 ± 4 |
| E | 8 | 4 | 15 | 17 ± 5 |
| F | 8 | 4 | 30/orally | 22 ± 3 |

## Discussion

There is limited scientific research available regarding the specific interaction between *P. harmala and* cobra venom. The results of the present study showed that *P. harmala* extract does not have a protective effect and increases the speed of the deadly effect of the *N. n. oxiana* venom in an unknown way. In other words, it has a synergistic effect on this venom.

The *N. n. oxiana* venom exhibits neurotoxic properties and also possesses cytotoxic effects [26]. The inhibitory effect of cobra venom on nicotinic acetylcholine receptors results in the prevention of post-synaptic neurotransmitter connections, ultimately leading to respiratory muscle paralysis, particularly preventing the crucial function of the diaphragm, which clinically is the reason for victim death [27, 28].

*P. harmala* contains a variety of chemical compounds, including amino acids, such as phenylalanine, valine, histidine, and glutamic acid; flavonoids, such as coumarin, tannins, and sterols; and is rich in toxic alkaloids of the β-carboline type, such as Harmine, Harmaline, Harmol, and Harmalol [29-31]. In several studies on traditional herbal treatments, the toxicity and interactions of this plant have been identified [31]. Beta-carbolines bind to receptors, such as serotonin, muscarinic, histamine, and beta-adrenergic. Therefore, it seems that it cannot interfere with the blocking of acetylcholine receptors by venom. In other words, *P. harmala* extract does not have an inhibitory effect on specific receptors of active substances of *N. n. oxiana* venom.

According to a report, *P. harmala* has been associated with the clinical symptoms of intoxication. Animals experiencing intoxication exhibit various manifestations, including increased excitability, trembling, muscle stiffness, and an unsteady gait. Following a short narcotic state and heightened activity, animals may also encounter difficulties in breathing, mydriasis, hypothermia, and urinary problems [32, 33]. In severe cases, paralysis, depression of the central nervous system, dyspnea, and arterial hypotension have been observed. However, it is important to note that in this particular study, the administration of two different doses of *P. harmala* (15 and 30 mg/kg) did not result in any signs of intoxication. This lack of intoxication could potentially be attributed to the low concentration of *P. harmala* used in the study. The LD50 (lethal dose required to kill 50% of test subjects) of its alkaloid harmane in mice has been reported as 50 mg/kg [29]. Therefore, we assumed that the toxic effects of *P. harmala*, even at low doses, manifest by intensifying the toxic effect of cobra venom. Furthermore, previous studies have demonstrated that *P. harmala* seed extract possesses antispasmodic effects, inducing a myorelaxant effect on rabbit and guinea pig smooth muscles in vitro [29]. It is probable that the synergistic effect between *P. harmala* and *N. n. oxiana* venom, which accelerates the paralysis caused by the venom, is associated with these toxic activities.

The results showed that the venom incubated with the *P. harmala* extract (group E) kills the animals at a faster rate compared to the control group A and group C. It can be concluded that the P. harmala extract can react with the venom molecules and change the structure of these molecules so that they can react more easily to their receptors or more receptors are caught in an unknown way. However, such facilitation has been also observed in group D, in which the admin-

istration of extract and venom had a 20-min interval. Moreover, the time to death in group C, in which extract and venom were administrated simultaneously, was very close to the control group. This may indicate that extract interaction is different in vitro and in vivo.

Other possibilities may help to explain this synergic effect. *P. harmala* affects the cardiovascular system, reducing blood pressure that may accelerate the time to death of animals [34]. In severe cases, paralysis, CNS depression, dyspnea, hypothermia, and low blood pressure occur. Moreover, its notable alkaloids encompass beta-carbolines (e.g., harmaline, harman, harmalol, and harmine), as well as quinazoline derivatives (vasicine and vasicinone) that have mild hallucinogenic effects and are known for their ability to inhibit monoamine oxidase enzyme which breaks down certain neurotransmitters, namely serotonin, dopamine, and noradrenaline. Inhibiting MAO can lead to increased levels of these neurotransmitters, which can have various effects on the body [35-39].

Therefore, it is reasonable that the MAO-inhibiting properties of *P. harmala* may disrupt the metabolism of venom toxins. Such disruption has the potential to augment the concentration of toxins, thereby raising the severity and duration of venom-induced toxic effects. As a consequence, accelerated and intensified neurotoxic and cardiotoxic effects may occur within the organism. However, it should be noted that the interactions between MAO inhibitors and venom toxins can be complex and depend on multiple factors, including the specific venom toxins, concentration of MAO inhibitors, and the individual's physiological responsiveness. Regarding the route of administration, although it has been reported that the alkaloids of *P. harmala* are readily absorbed by the digestive system [32], the findings of the current study indicated no significant difference in the time of animal mortality when administering *P. harmala* extract orally or intraperitoneally.

### Conclusion

The results of the present study indicated that the extract of *P. harmala* not only lacks a protective effect against *Naja naja* venom but also enhances the speed of the venom's lethal effect in an unknown manner. Although the extract alone had no toxic effect, interaction with venom exhibited a synergistic effect with the venom. The extract may increase the susceptibility of animals to this venom in an unclear way. These findings suggested that the plant extract has either a lower competitive ability with the neurotoxin or no competitive ability at all. Further investigation, particularly at the molecular level, is essential to enhance result clarity and gain a deeper understanding of the underlying mechanisms involved in these interactions.

## Materials & Methods

### Venom

The freeze-dried crude venom of *N. n. oxiana* was kindly provided by the Razi Vaccine and Serum Research Institute, Karaj, Iran. The Lyophilized venom was stored at 4ºC and freshly prepared by dissolving it in a sterile physiological saline solution (0.9 % NaCl) to a final volume of 500 µl before injection into the animals.

### Preparation of P. harmala extract

The fresh plant was harvested during early summer from agricultural fields located in the vicinity of Sabzevar city (36°12'45"N and 57°40'35"E) in the western region of Razavi Khorasan province, Iran. The plant specimen was precisely identified as *P. harmala* at the Ferdowsi University of Mashhad Herbarium (13613-FUMH). Subsequently, the plant material was subjected to drying in a dark room at a temperature of 28°C ± 4°C for two weeks. Following the drying process, the black seeds were carefully separated and finely ground into a powder. methanolic extract of *P. harmala* was prepared in the Department of Pharmacognosy at the Pharmacy College of Ferdowsi University of Mashhad.

A total of 100 g of powder was dissolved in 300 ml of 70% methanol and left to stand for 48 h. The solution was stirred for 30 min at room temperature and then filtered through Whatman filter paper no. 1. An additional 200 ml of methanol was added to the remaining mixture, and the process was repeated three times. The resulting solution was protected from light by placing it in an aluminum-covered glass container. Using a vacuum rotary evaporator (IKARV 10, Germany) set at 50°C and 60 rpm, methanol evaporated from the solution. The resulting solution was a highly viscous, dark red honey-like liquid. The solution was poured onto a plate and transferred to an oven for one week until it dried into a solid form. Afterwards, it was covered with aluminum foil and stored in a refrigerator at 4°C until use.

To dissolve the extract, we conducted tests using various solutions, including saline solution, a mixture of saline solution, and 2-3 drops of DMSO 0.01%. In addition, we employed diverse methods, such as heating, shaking, and centrifugation in attempts to dissolve the extract. However, none of these approaches yielded successful results. Ultimately, we achieved dissolution by using 2 normal HCL while adjusting pH to 7.5 with NaOH.

### Animals

For this study, 56 adult albino mice of both sexes aged 8-10 weeks and weighing 28-40 g were purchased from the Animal House of Mashhad University of Medical Sciences. The animals were housed in the animal facility of the Faculty of Veterinary Medicine under controlled environmental conditions, including a 12:12 light-dark cycle, a temperature of 23ºC ± 2ºC, and a relative humidity of 55% ± 10%. They were kept in standard rodent cages and provided with food and water ad libitum. The experimental protocol was conducted following the guidelines of the Animal Ethics Committee of the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad. The protocol was approved by this Ethics Committee with the code IR.UM.REC.1401.171.

### Experimental protocols

The ability of *P. Harmala* extract to antagonize the lethal effects of *N. n. oxiana* venom was investigated using five different protocols (I, II, III, IV, and V) (Table 1).

For this study, 56 mice were divided into seven equal groups (A, B1, B2, C, D, E, and F).

In protocol I (controls), group A received *N. n. oxiana* venom only at a dose of 4 mg/kg and groups B1 and B2 received only P. harmala extract at the doses of 15 and 30 mg/kg, respectively.
In protocol II, group C was treated simultaneously with 15 mg/kg of *P. harmala* extract, along with 4 mg/kg of venom.
In protocol III, group D was treated with 15 mg/kg of *Peganum Harmala* extract, 20 min after the administration of *N. n. oxiana* venom at 4 mg/kg.

In protocol IV, group E was treated with the mixture of venom and *P. harmala* extract which was preincubated for 20 min at room temperature (26°C ± 2°C) prior to injection into animals. The route of administration in this study was IP.

It has been reported that the main route of administration for *P. harmala* is the oral route, as its alkaloids are well absorbed by the digestive system (Tahri et al., 2011). Therefore, in protocol VI, group F received the extract orally at a dose of 30 mg/kg after 24 h of food deprivation. After one hour, they received a dose of 4 mg/kg of venom. The survival time of each animal (in minutes) after the injection of venom, extract, and venom/extract was recorded and statistically compared with the control groups.

### *Statistical analysis*

The data are presented as mean ± SEM and all the results were analyzed using SPSS-22 (SPSS Inc., Chicago, Illinois). One-way analysis of variance was used to analyze the data, followed by a post-hoc analysis using a Tukey test. The level of significance was considered $p < 0.05$.

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## Conflict of interest

The authors declare that there is no conflict of the interest

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**Online supplemental material**

[Describe available supplemental material here, including brief legends for these materials, if applicable, for example Figure S1., Movie M1, …]

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