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Original Research Article

# Assessment of Acute Toxicity and Analgesic Activity of Organic and Aqueous Fractions from *Retama Monosperma* Stems

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ABSTRACT

*Retama monosperma*, an endemic Mediterranean plant, is traditionally used for various illnesses and various biological activities due to its bioactive phytochemicals. The current research study investigated the acute toxicity and analgesic activity of the fractions from the stems of *Retama monosperma*. These activities were conducted *in vivo* using animal models (Swiss albino mice and Wistar Albino rats), focusing on the four fractions obtained from the stems of *Retama monosperma* (hexane, ethyl acetate, ethanol, and aqueous), through Soxhlet extraction. Acute oral toxicity was assessed in mice, while analgesic activity was evaluated using the acetic acid-induced writhing test for peripheral effects and the tail immersion test for central effects. The fractions were confirmed to be nontoxic at doses of 50 and 2000 mg/kg, with the lethal dose exceeding 50 mg/kg for the ethanol fraction and 2000 mg/kg for the other fractions. The fractions studied inhibited the pain in a dose-dependent manner in the acetic acid-induced writhing test, especially for the ethyl acetate fraction. However, in the tail immersion test, the fractions did not exhibit significantly enhanced effects. The analgesic activity of *Retama monosperma* stems could be ascribed to the secondary metabolites detected in this plant. This study demonstrated the safety of fractions of *R. monosperma* in acute toxicity and highlights their potential for peripheral analgesic activity. Future investigations, including the isolation of active compounds, are necessary to complete this work and improve the application of this species in pain-associated diseases.

***Keywords*:** *Retama monosperma*, Acute toxicity, Pain, Analgesic activity, Peripheral analgesic, Central analgesic

## Introduction

The traditional application of medicinal plants and plant- derived products has been a practice since ancient times as a natural reservoir of treatments for healing diverse ailments, utilizing diverse formulations.[1] Plants contain numerous chemical compounds that serve as the foundation for medical therapies worldwide, owing to their healing properties and minimal adverse effects.[2] Additionally, medicinal plants can play vital roles in preventing chronic diseases, as they exhibit a various pharmacological and therapeutic properties, such as antioxidant, antibacterial, anticancer, and anti-inflammatory.[3] In fact, many drugs used in the pharmaceutical industry are derived from natural resources, especially medicinal plants.[4,5]
Morocco, a Mediterranean country, is renowned for its diverse climatic conditions, including humid and sub-humid climates.[6] The flora of Morocco stands as among the most varied and plentiful in the world, with around 4200 species. For centuries, a wide variety of species have been utilized by the Moroccan populace, particularly in rural regions, for medicinal purposes in folk remedies, employing traditional preparation methods.[7]

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*Retama monosperma*, locally referred to as *R'tem*, is a leguminous (Fabaceae) shrub. It is distinguished by its small white flowers, deciduous leaves, and photosynthetic cladodes. This species spontaneously and abundantly grows in numerous Moroccan natural forests and coastal areas with sandy soils.[8] Communities across North Africa and the Mediterranean Basin have historically used these plants in their traditional medicine for the treatment of a broad spectrum of diseases. These natural remedies are applied in skin care, alleviating joint pain, and reducing inflammation. Furthermore, these plants are employed in the treatment of healing in circumcisions, eczema, and rheumatism.[9,10]Numerous studies in the chemical composition of various sections of *R. monosperma*, reported the abundance of this species with diverse bioactive compounds. The main components found in this species are dipiperidine and quinolizidine alkaloids, notably Sparteine, Ammodendrine, Cytisine, and Anagyrine.[11] Pinitol, a type of cyclitol, has also been found.[12] Additionally, this species contains flavonoids, both aglycones and glycosides, such as Kaempferol, Genistin, Genistein, Daidzin, and Rutin.[13] Previous pharmacological studies have shown that extracts from various sections of *R. monosperma* possess diverse pharmacological activities. the alkaloids within these extracts have been shown to exhibit antifungal and antibacterial activities.[14-16] Additionally, these extracts have demonstrated antioxidant capabilities *in vitro*,[15,17] anticancer effects against cervical cancer cell lines, anti-aging capacity,[13] and *in vivo* anti-inflammatory capacity.[18] Many analgesic agents pose notable risks of toxicity after acute and chronic use, leading to potential side effects such as gastrointestinal upset, gastric ulcers, bleeding, and liver damage. Therefore, considerable research is directed towards exploring medicinal plants known for their analgesic properties, mainly due to their various biological compounds, which offer

reduced side effects, cost-effectiveness, and widespread availability.[19-21]

This study aims to investigate, for the first time, both the acute toxicity and analgesic activity of fractions from *Retama monosperma* stems through the tests: acetic acid-induced writhing test and tail immersion test.

## Materials and Methods

*Study area and plant identification*

Stems of *Retama monosperma* were collected from the Mehdia forest located in the Rabat-Sale-Kenitra region of Morocco during July 2021 (geographical coordinates: 34°12'43.7"N 6°41'30.8"W). The identification of the plant specimens was verified by a botanist at the Herbarium of the Botany Department, at the Scientific Institute of Rabat, Morocco, where they were assigned the Voucher Specimen code RAB113533 and subsequently deposited. The stems of *Retama monosperma* were cleaned, dried at ambient temperature, finely ground into powder, and stored in opaque glass bottles. This storage method was selected to protect the powdered stems from exposure to light and moisture, preserving them for future research purposes.

*Extraction of Plant materials*

Stems of the plant, *R. monosperma*, were initially air-dried in a shaded area before being finely ground. Then, 50 g of the obtained powder were placed into a cotton cellulose cartridge for the extraction. The extraction process employed a Soxhlet extractor and involved the sequential use of 400 mL of various solvents: starting with hexane (from Sigma-Aldrich), followed by ethyl acetate (from Solvachim) derived from the stems' pomace, and ending with ethanol (from Biosmart). Furthermore, the pomace was macerated by distilled water with a volume of 1000 mL for 8 h, kept in darkness at ambient temperature. The resulting fractions were then filtered through Whatman filter paper and concentrated under diminished pressure at a water bath temperature of 35-40°C, using a rotary evaporator. Subsequently, the concentrated aqueous extract was frozen at -80°C for 24 h before undergoing freeze-drying to transform it into a powdered form. The fractions produced were preserved at 4°C for future use.[15]

*Animals*

To assess the pharmacological effects of the four fractions (hexane, ethyl acetate, ethanol, and aqueous) from *R. monosperma* stems, Swiss albino mice, with a weight range of 20 - 30 grams, and Wistar Albino rats, weighing between 160 - 250 grams, were employed in the study. The animals were sourced from the animal facility at the Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Morocco. They were kept in conditions with a temperature maintained at 22 ± 2 °C, under a cycle of 14 h of light and 10 h of darkness, with unrestricted access to food and water.

All experimental methods adhered to the "Principles of Laboratory Animal Care" and followed the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Sciences, receiving approval from the National Institutes of Health. All animals were given a precise dosage based on their weight and respecting the administration method used.

*Acute toxicity test*

The evaluation of acute oral toxicity of *R. monosperma* stems extracts was carried out on female Swiss albino mice, following the protocols established by the Organization for Economic Cooperation and Development.[22] For each fraction, three female mice with body weights ranging from 20 to 30 grams, non-pregnant and nulliparous, were subjected to 4-hour fasting period but had free access to water. They were individually housed in sterile cages. Then, *R. monosperma* fractions, at a single dose, were administered orally to each mouse using an esophageal probe. The administration dosage was 2000 mg/kg for the hexane, ethyl acetate, and aqueous fractions, and 50 mg/kg for the ethanol fraction. Following treatment, the animals were monitored for the initial 30 min for mortality and clinical signs such as salivation, convulsions, diarrhea, coma, and death. Throughout a 14-day period, changes in body weight and the mortality count among the mice were carefully observed and documented.[23]

*Peripheral analgesic activity by acetic acid-induced writhing test*

The acetic acid-induced writhing test is one of several methods used to measure pain. We used it to evaluate the analgesic activity of each fraction of *R. monosperma*, based on the procedure recommended by Koster et al in 1959.[24] Male albino mice were weighed and then randomly divided into 10 groups each containing 5 mice. The control group, the first group, did not receive any form of treatment. The subsequent groups were administered the various fractions orally, at dosages of 300 mg/kg and 500 mg/kg, and the ethanol extract administered at dosages of 20 mg/kg and 40 mg/kg. Aspirin was administered at a dosage of 125 mg/kg. Thirty minutes following the administration of the fractions, each mouse was intraperitoneally injected with a 3 % acetic acid (from Solvachim) solution was used at a dosage of 3.75 mL/kg body weight to induce pain perception (nociception). Ten minutes after the acetic acid injection, the number of writhes produced by each mouse was observed and recorded for a period of 10 min. The percentage reduction in abdominal writhing was determined using the following formula:

$$\% \ Inhibition = 1 - \frac{The\ number\ of\ contortions\ observed\ in\ mice\ from\ the\ treated\ group}{The\ number\ of\ contortions\ observed\ in\ mice\ from\ the\ negative\ control\ group} \times 100$$ ----------Equation 1

*Central analgesic activity by tail immersion test*

The central analgesic activity of fractions from *R. monosperma* on centrally mediated pain was assessed using the tail immersion test.[25] Wistar rats with body weights ranging from 160 to 250 grams, were used for this test. The remaining experimental animals were randomly allocated, following the arrangement used in the abdominal writhing test and received treatment: the model group (which did not receive any form of treatment), the positive control group (treated with 0.1 mg/kg of Morphine), and the treatment groups (which received doses of 500 mg/kg for the hexane, ethyl acetate, and aqueous fractions, and 40 mg/kg for the ethanol fraction). At intervals of 0, 30, 60, and 120 min after treatment, the lower 6 cm section of each rat's tail was submerged in warm water kept at a temperature of 55.0 ± 0.5 °C to record the incubation period of the uncomfortable reaction. The tail was kept in the water for no more than 10 seconds to prevent scalding the rats.

*Statistical analysis*

Statistical evaluation and comparison of means were conducted using a one-way analysis of variance (ANOVA), followed by the Tukey test. A *p*-value of less than 0.05 was considered to indicate statistically significant differences among the mean values. These analyses were carried out utilizing GraphPad Prism version 8.

## Results and Discussion

*Acute toxicity test*

During the acute toxicity assessment of *R. monosperma* fractions, conducted according to OECD guideline 423, all animals were closely monitored for the emergence of any signs of toxicity initially for 30 min and during 14 days after administration of the fractions. The doses were set at 2000 mg/kg for hexane, ethyl acetate, and aqueous fraction, and at 50 mg/kg for the ethanol fraction. Over the acute oral toxicity assessment of the fractions, no deaths were recorded among the animals, nor were there any signs or symptoms indicative of severe toxicity. Additionally, no abnormal behaviors in feed and water intake were noted in the mice throughout the study period in both control and treated groups. The fractions were deemed to be safe at doses of 50 and 2000 mg/kg, and these results mean that the lethal dose ($LD_{50}$) was higher than 50 mg/kg for the ethanol fraction and 2000 mg/kg for the hexane, ethyl acetate, and aqueous fractions. Over the 14 days of the test period, the mean body weight of the three mice in the control and the treatment groups remained largely unchanged, except for the

group receiving the aqueous fraction. This group experienced a decrease in weight between days 8 and 11, after which their weight returned to normal, as illustrated in Figure 1.

Generally, toxicity assessment of extracts from medicinal plants and examining the adverse effects associated with acute toxicity in animal models are crucial steps in determining the safety of these plants. Similarly, this study was designed to investigate the acute oral toxicity of both the organic and aqueous extracts from the stems of the *R. monosperma*, when administered orally in a single dose to the Swiss albino mice model. In this current study discovered that oral administration of the hexane, ethyl acetate, and aqueous fractions at a dose up to 2000 mg/kg, as well as the ethanol fraction at 50 mg/kg, did not lead to death or exhibit sings of severe toxicity, indicating its non-toxicity and safety in Swiss albino mice throughout the observation period. All treated animal groups exhibited a normal increase in body weight compared to the control group, this observation confirms the suggestion that the *R. monosperma* fractions did not result in toxic effects in mice model. Body weight serves as a crucial indicator for identifying the potential toxic effects of fractions. Variations in body weight act as an indicator of adverse side effects as surviving animals should not experience a reduction in weight greater than 10 % of their initial body weight.[26] Furthermore, assessing the consumption of water and feed by animals plays a significant role in evaluating the safety of formulations intended for therapeutic use.

This is because the adequate nutrient absorption is essential for maintaining the physiological state of the animals and ensuring an accurate and appropriate response to the experimental drug, as opposed to a distorted response resulting from inadequate nutritional conditions.[27] Consequently, the median lethal dose ($LD_{50}$) for the fractions is greater than 2000 mg/kg of body weight for the hexane, ethyl acetate, and aqueous fractions, whereas it is greater than 50 mg/kg for the ethanol fraction. To our knowledge, there are no scientific reports on the toxicity assessment of *R. monosperma*. Therefore, this research serves as the first report to investigate and assess the safety of this medicinal species.

In addition, the toxicity of methanol extract from the fruit of *Retama raetam* was assessed in Sprague-Dawley rats (both female and male). This study demonstrated that the $LD_{50}$ of this extract is 1995 mg/kg.[28] However, the results of the acute toxicity test involving aqueous extract from the leaves of *Retama sphaerocarpa* in female rat model indicated that no death was observed at 200, 500, and 1000 mg/kg body weight. Furthermore, no toxic signs were revealed in rats that received 200 and 500 mg/kg body weight.[29]

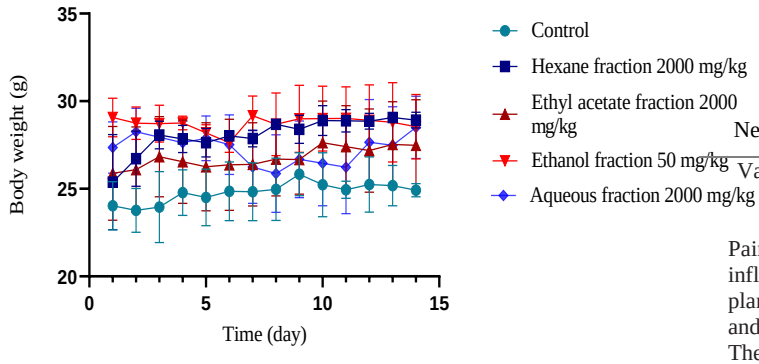


**Figure 1:** Effects of *R. monosperma* fractions on body weight changes during the acute toxicity test in mice. The data is shown as the mean weight of mice (n=3).

*Analgesic activity (Peripheral and Central analgesic activity)*

The potential analgesic activity of the four fractions was assessed through animal models to elucidate the role of peripheral pain mechanisms in the effects observed following acetic acid injection. The results of the potential analgesic activity of the four fractions of *R. monosperma* are shown below in Table 1. The ethyl acetate fraction at 300 mg/kg resulted in a 36 % decrease in abdominal writhes, with the highest reduction observed at 500 mg/kg, achieving a 49 % decrease. This showed a significant difference of all fractions studied (hexane, ethanol, and aqueous) compared to aspirin, besides, the hexane fraction showed the lowest analgesic activity, and a dose-dependent response was also noted.

The central analgesic potential of the studied fractions, especially in terms of central pain mechanisms, was further evaluated through the tail immersion test. The activity of the four fractions from the stems of *R. monosperma* on average reaction time, as measured by tail immersion test in Wistar Albino rats, is presented in the Table 2. The outcomes of the tail immersion test (at 0, 30, 60, 90, and 120 min) following the oral administration of the hexane, ethyl acetate, and aqueous fractions in 500 mg/kg, and the ethanol fraction at 40 mg/kg. And intraperitoneal administration of the morphine at a dose of 0.1 mg/kg. Compared to the model group, the tail dumping latency of rats treated with the *R. monosperma* fractions and morphine was significantly increased. The group treated with morphine exhibited the highest mean reaction time, demonstrating significant differences compared to the fractions derived from *R. monosperma*. The response times to pain triggered by thermal stimuli in the tail immersion test did not show significantly improved changes with the studied fractions. However, the most notable results showed by the ethyl acetate fraction.

**Table 1:** Analgesic activity in acetic acid-induced writhing test of *R. monosperma* fractions in mice.

| Extracts | Dose (mg/kg) | Number of writhings | Inhibition (%) |
|---|---|---|---|
| Hexane fraction | 500 | 31.80 ± 2.38$^{abef}$ | 14.42 % |
| | 300 | 35.00 ± 2.12$^{abj}$ | 5.81 % |
| Ethyl acetate fraction | 500 | 18.80 ± 1.64$^{ci}$ | 49.41 % |
| | 300 | 23.75 ± 2.98$^{dgi}$ | 36.08 % |
| Ethanol fraction | 40 | 27.75 ± 1.71$^{dgi}$ | 25.32 % |
| | 20 | 30.00 ± 2.00$^{aefh}$ | 19.26 % |
| Aqueous fraction | 500 | 24.80 ± 2.28$^{dfghi}$ | 33.26 % |
| | 300 | 27.80 ± 1.92$^{efgh}$ | 25.18 % |
| Aspirin | 125 | 21.66 ± 1.90$^{cdgi}$ | 41.71 % |
| Negative Control | | 37.16 ± 1.78$^{bj}$ | - |

Values within the same column that have differing superscript letters signify significant differences ($p < 0.05$).

Pain often accompanies a variety of clinical diseases, including inflammation, cancer, and gout.[30] It was noted that polyphenols of plants have various biological properties, not only anti-inflammatory and antipyretic effects, but also exhibit enhanced analgesic activity. The analgesic activity of the four fractions obtained from the stems of *R. monosperma* was screened and assessed through two different animal models. This evaluation aimed to determine the involvement of peripheral and central pain mechanisms in the observed effects. The acetic acid-induced writhing test is associated with peripheral mechanisms, while the tail immersing test is concerned with the central acting mechanisms.

Mice subjected to the test exhibit writhing responses due to local inflammation induced by an intraperitoneal injection of acetic acid at 0.1 %. In the writhing test, acetic acid initiates an inflammatory process that leads to the release of mediators including TNF-α and Il-6, in addition, IL-1$\beta$, IFN-γ, NF-kB and cyclooxygenases.[31] In this way, these mediators are responsible for inducing pain by stimulating the increased release of substance P and excitatory amino acids at

presynaptic terminals.[32] Peripherally acting analgesics act by inhibiting the production at pain chemoreceptors.[33]
However, the tail immersion test evaluates the complex response to acute nociceptive stimuli that are non-inflammatory in nature.[34] The tail-flicking response is controlled by a spinal reflex. Drugs can reduce peripheral pain by blocking signal transduction in spinal nerve fibers.[35] Analgesics that act on the central nervous system (CNS) not only raise the threshold for pain, they also modify the physiological response to pain and remove the patient's apprehension and anxiety.[36]
In the current research, administration of *R. monosperma* stems fractions showed potential in reducing of the number of abdominal contortions in mice, thus possesses a peripheral analgesic activity that is dependent on the dosage (dose dependent manner). The observed activity might be ascribed to the secondary bioactive compounds present in the examined fractions, such as flavonoids and saponins,[15,18] additionally, this could be due to the synergistic actions of these secondary metabolites. In addition, the variation in peripheral analgesic activity among the fractions may be due to differences in their chemical composition. However, the fractions did not show a significantly improved changes in the tail immersion test.
Based on a literature review, the analgesic activity of *R. monosperma* has not been evaluated. Therefore, this current study is the first to unveil the analgesic activity of *R. monosperma*, introducing new insights into its potential medicinal uses. Moreover, there have been numerous publications describing the analgesic activity of various plant in the Retama genus. Similar results were observed with the extract of the seeds of *Retama reatam*, obtained with methanol, showed an analgesic activity at a dose of 50 mg/kg, with a percentage reduction of writhing of 34.5 %.[37] Besides the isoflavones isolated from the aerial parts of *R. reatam* (genistein, 6-hydroxygenistein, 3' methylorobol, and pratensein) showed inhibition from 21.40 % to 86.19 % at 1 mg/kg.[38]

**Table 2:** Analgesic activity of *R. monosperma* fractions on nociceptive response in the tail immersion test.

| Treatment | Dose (mg/kg) | Reaction time (second) | | | | |
|---|---|---|---|---|---|---|
| | | 0 min | 30 min | 60 min | 90 min | 120 min |
| Hexane fraction | 500 | $2.13 \pm 0.29^{a}$ | $3.68 \pm 0.36^{abd}$ | $3.34 \pm 0.23^{abcf}$ | $3.59 \pm 0.33^{ac}$ | $3.70 \pm 0.09^{acd}$ |
| Ethyl acetate fraction | 500 | $3.02 \pm 0.44^{a}$ | $3.51 \pm 0.22^{abd}$ | $4.26 \pm 0.61^{abcd}$ | $4.42 \pm 0.37^{bd}$ | $4.74 \pm 0.34^{b}$ |
| Ethanol fraction | 40 | $2.26 \pm 0.31^{a}$ | $3.33 \pm 0.59^{abd}$ | $3.29 \pm 0.23^{abcf}$ | $3.37 \pm 0.31^{ac}$ | $3.33 \pm 0.45^{acdf}$ |
| Aqueous fraction | 500 | $2.69 \pm 0.52$ | $4.44 \pm 0.51^{ab}$ | $4.5 \pm 0.54^{db}$ | $4.57 \pm 0.32^{bd}$ | $3.55 \pm 0.27^{acd}$ |
| Morphine | 0.1 | $2.53 \pm 0.43^{a}$ | $6.64 \pm 0.13^{c}$ | $6.75 \pm 0.12^{e}$ | $7.15 \pm 0.15^{e}$ | $7.70 \pm 0.18^{e}$ |
| Negative Control | - | $2.35 \pm 0.32^{a}$ | $2.62 \pm 0.39^{ad}$ | $2.63 \pm 0.47^{acf}$ | $2.25 \pm 0.39^{f}$ | $2.03 \pm 0.31^{cf}$ |

Values within the same column that have differing superscript letters signify significant differences ($p < 0.05$).

## Conclusion

In the current study, the acute toxicity and the analgesic activity were validated in Swiss albino mice and Wistar Albino rats. The acute oral toxicity test, 14 days after administration of 2000 mg/kg body weight for hexane, ethyl acetate, and aqueous fractions and 50 mg/kg body weight for the ethanol fraction (at a single dose), showed that these doses are safe in mice model. Furthermore, the fractions studied demonstrated peripheral analgesic activity acting dose-dependently. These findings confirm the traditional uses of *R. monosperma*. Future investigations will delve into the mechanisms and pharmacological compounds of *R. monosperma*.

## Conflict of Interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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