Evaluation of the antinociceptive effect of hydroethanolic extract of *Rhaponticum repens* (L.) Hidalgo in Wistar rats

Running title: Antinociceptive effect of *Rhaponticum repens*

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Abstract

Background: Chemical analgesics often have limitations, including adverse effects and insufficient efficacy. Therefore, there is a pressing need to investigate novel compounds with enhanced efficacy and a more favorable side-effect profile.

Methods: This experimental study aimed to evaluate the analgesic properties of *Rhaponticum repens* (RR) extract, a natural compound, using the formalin test in Wistar rats. Twenty-one male Wistar rats were randomly divided into three groups (n=7): group 1 received normal saline, group 2 received morphine (10 mg/kg), and group 3 received *RR* (100 mg/kg). Thirty minutes post-injection, formalin (2.5%) was injected into the hind paw of each rat to induce pain. Licking time and pain scores were recorded during both the acute and chronic phases of the test. Data were analyzed using one-way ANOVA with Prism software, with statistical significance set at $P \leq 0.05$.

Results: The RR extract significantly reduced licking time and pain scores compared with the control group ($P \leq 0.01$ in the early phase, $P \leq 0.001$ in the late phase, $P \leq 0.05$and $P \leq 0.001$ in the pain scores). Additionally, the extent of hind paw edema was significantly reduced in both the morphine and RR extract groups ($P \leq 0.001$).

Conclusion: These findings suggest that the hydroalcoholic extract of RR exhibits analgesic effects on both acute and chronic formalin-induced pain, making it a promising candidate as a potential alternative to chemical painkillers. However, further research is necessary to fully elucidate its therapeutic potential.

Keywords: Formalin test, Pain, *Rhaponticum repens*, Morphine

Introduction

Pain is an unpleasant sensation that arises from damage to various tissues and organs of the body (1). However, pain serves as a warning signal that helps prevent accidents and serious injuries, contributing to the maintenance of homeostasis through protective reflexes (1,2).

Since becoming aware of pain, humans have sought to identify its causes and develop methods for alleviation. Understanding pain and its mechanisms is essential for accurately classifying patients. Throughout history, various treatments have been employed to relieve pain, including opioid analgesics, steroid anti-inflammatory drugs, nonsteroidal anti-inflammatory drugs (NSAIDs), as well as local and general anesthetics (3,4).

Despite advances in pharmaceutical science and the development of chemical drugs for pain relief, the use of herbal medicines is recommended due to their fewer complications, easier accessibility, and cost-effectiveness (5,6).

Considering the adverse effects associated with chemical drugs in recent decades, scientists and researchers have increasingly focused on the therapeutic potential of medicinal plants.

Rhaponticum repens (RR) is indigenous to Iran and often grows as a weed in fields, gardens, and barren lands. It possesses anti-parasitic and antibacterial properties and is utilized in the treatment of constipation, the prevention of excessive gallbladder secretions, the reduction of blood pressure, and the prevention of hemorrhoidal bleeding and intestinal ulcers (6,7).

Phytochemical analyses conducted on this plant have identified six primary compounds: camphor, myrcene, α-cubenene, caryophyllene oxide, heptadecane are found in various parts of the plant (7,8). Camphor plays a significant role in pain reduction through TRPV1 channel activation and subsequent desensitization (9).

Considering the analgesic and anti-inflammatory properties of plants from the *Asteraceae*, alongside the side effects associated with many synthetic drugs, a study on the analgesic and anti-inflammatory effects of these plants is warranted.

In this study, we aimed to investigate the analgesic potential of an alcoholic extract of RR in formalin-induced pain in Wistar rats, aiming to identify a more effective and potentially safer alternative to conventional chemical pain relievers (10,11).

The formalin test is a standard test for assessing the efficacy of chemical and herbal pain relievers, first introduced by Duboyson and Denis in 1977. The injection of diluted formalin induces a two-stage pain response. The first stage is characterized by acute and rapid pain occurring within the first 5 minutes of the test.10 to 15 minutes after the injection, a relatively painless phase emerges, following the activation of neurophysiological pathways that inhibit pain. Finally, the second stage of pain, characterized by chronic pain, begins and persists until 60 minutes after the injection. Transient pain in the first stage (Acute) occurs due to the direct stimulation of pain receptors by formalin. In contrast, the second stage of pain (Chronic) arises from inflammatory reactions following tissue damage, which involves the release of inflammatory mediators such as histamine, prostaglandin, and bradykinin, resulting from formalin injection (12). Given the adverse effects of synthetic analgesics such as addiction, gastrointestinal complications, and central nervous system disturbances associated with long -term use, this study aimed to explore the potential of RR extract as a natural alternative with possibly fewer side effects. The investigation focused on evaluating both early antinociceptive properties of the extract using the formalin test model, which allows simultaneous assessment of neurogenic and inflammatory pain pathways. This research contributes to the growing body of evidence supporting the use of phytochemicals in pain management while addressing the critical need for safer analgesic options.

Methods

This experimental study was conducted on 21 male Wistar rats, weighing 200-250 g, were obtained from the Laboratory Animal Center of Bu-Ali Sina University. Animals were housed in polypropylene cages (4 rats per cage) under standard laboratory conditions (12-hour light/dark cycle, temperature of 22 ± 2°C, and humidity of 50 ± 10%). In all stages of the study, the international regulations approved by the ethics committee of Bu-Ali Sina University (IR.BASU.REC.1401.008) regarding the treatment of laboratory animals were observed. Male Wistar rats were selected to avoid hormonal fluctuations associated with the estrous cycle in female rats (13).

Rats were randomly divided into three groups (n=7 per group):

• Group I: Received an intraperitoneal (i.p.) injection of saline (100 µL) followed by a subcutaneous (s.c.) injection of formalin (2.5%,100 µL) into the left hind paw (14).

• Group II: 30 minutes after an intraperitoneal injection of morphine (10 mg/kg), a subcutaneous injection of formalin was administered into the left hind paw.

• Group III: The extract was administered (i.p.) (100 mg/kg) and 30 minutes later, formalin was injected (s.c.) into the left hind paw.

The dose of RR extract was selected based on previous studies demonstrating the efficacy of similar sesquiterpene-rich plant extracts in pain models (15).

The formalin test was employed to assess the analgesic effects of RR extract. Thirty minutes post-administration of saline, morphine, or RR extract, 100 µL of 2.5% formalin solution was injected subcutaneously into the plantar surface of the left hind paw. Subsequently, animals were placed individually in transparent glass observation chambers.

The formalin test comprises two distinct phases:

Acute Phase (0-5 minutes): Characterized by immediate pain response, primarily manifested as licking and flinching behavior.

Chronic Phase (15-60 minutes): Associated with a delayed, inflammatory response, characterized by persistent licking and biting of the injected paw.

Pain behaviors were scored by an observer using a 4-point scale:

0: No response

1: Slight flinching or licking

2: Moderate flinching or licking

3: Severe flinching, biting, or lifting of the paw

The duration of pain behaviors (licking and flinching) and pain scores were recorded during both phases (14,16). The formalin test was chosen for its ability to evaluate both acute (Neurogenic) and chronic (Inflammatory) pain phases, providing a more comprehensive assessment than single-mechanism tests like hot plate or tail flick (17).

RR leaves were collected from local agricultural fields and orchards in Hamedan. The collected leaves were air-dried in a dark, well-ventilated room. Dried leaves were then ground into a fine powder using a mechanical grinder and sieved through a 1-mm mesh.

A 100 g portion of the powdered leaves was macerated with 1000 mL of 80% ethanol for 48 hours at room temperature with constant stirring.

The resulting extract was filtered through Whatman No. 1 filter paper, and it was placed in an incubator for 48 hours at a temperature of 40 °C to remove the solvent.

The concentrated extract was further dried in a fume hood to obtain a dry powder.

The formalin-induced pain scores were recorded individually for each rat. The mean and standard deviation were calculated for each group of seven rats. One-way ANOVA was used to compare the means between different treatment groups using Prism software. Statistical significance was set at $P \leq 0.05$.

Results

The level of inflammation in the hind paw of rats was measured after the administration of the RR. The results indicated that inflammation levels in the group receiving both the extract and morphine were significantly reduced compared to the group I receiving saline. This suggests that the RR exhibited notable anti-inflammatory properties. Figure 1 presents a sample of the inflamed hind paw of rats.

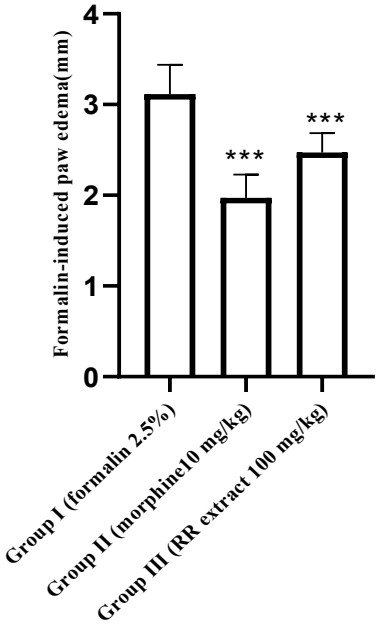

Figure 1. Effects of intraperitoneally administered RR extract and morphine on formalin-induced paw edema. Significant differences between group I and other groups are indicated by *** ($P < 0.001$)

Licking time

Early Phase: Licking time was measured in three groups, with results indicating that pain levels were similar across morphine and RR extract groups showed a significant reduction in licking time compared to the group I ($P \leq 0.01$) (Figure 2-a).

Late Phase: The results from this phase demonstrated that the extract, similar to morphine, significantly reduced licking time stimulated by formalin. This indicates that the extract effectively alleviated pain compared to the group I (Figure 2-b).

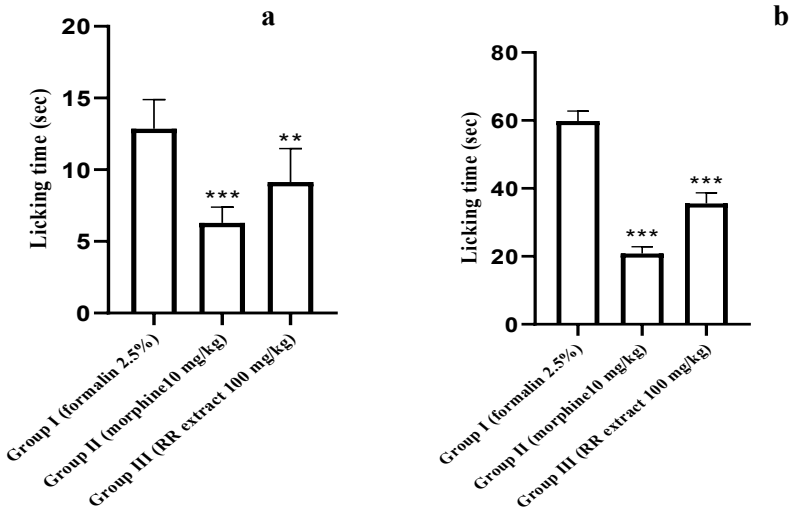

Figure 2. Effects of intraperitoneally administered RR extract and morphine on the early (a) and late (b) phases of formalin-induced paw-licking in rats. Significant differences between groups are indicated by ** ($P < 0.01$), *** ($P < 0.001$)

Pain score

In this study, the total number of reactions exhibited by rats following formalin injection was evaluated in both acute and chronic phases. The total movements were quantified and assigned as a pain score for each rat.

As shown in Figure 3, the average pain score in group I was approximately 13.1, significantly higher than that in the other groups.

Thus, both morphine injection and the extract yielded significant reductions in pain scores within these groups. Similarly, during the chronic phase, the results paralleled those observed in the acute phase. Both the morphine and RR extract groups exhibited significantly lower pain scores than the group I.

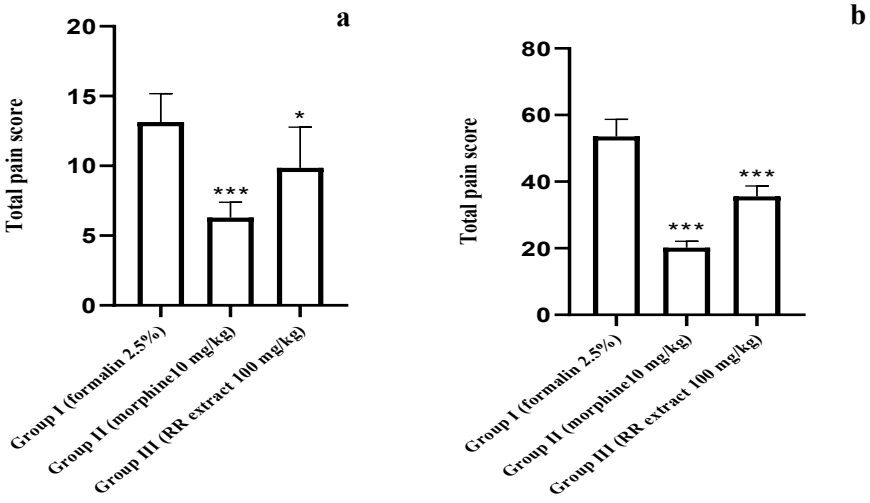

Figure 3. Effects of intraperitoneally administered RR extract and morphine on pain score, during both the early (a) and late (b) phases. Significant differences between groups are indicated by * ($P < 0.05$), *** ($P < 0.001$)

Discussion

One of the most well-known complications in medicine that has captured the attention of researchers is pain. Pain is a physiological response of the body to a harmful stimulus, whether physical, chemical, or otherwise. It is an experience that all individuals encounter throughout their lives, and a primary reason people seek medical attention is the presence of pain in a specific area of the body (18,19).

Today, pain relievers are often used to reduce pain. However, these medications, which are metabolized in the liver, can have numerous complications, including central nervous system disorders, respiratory issues, cardiovascular problems, smooth muscle contractions, gastrointestinal disorders, decreased alertness and drowsiness, and addiction (20). In ancient traditional medicine, numerous medicinal plants, including *Matricaria chamomilla*, *Teucrium polium*, *Vitis vinifera* fruits and leaves, *Glycyrrhiza glabra* root, *Valeriana officinalis*, and *lavandula angustifolia*, as well as herbal products like RR extract, have been used as analgesics and sedatives (10,19). Due to the complications associated with chemical drugs and the inefficacy of some treatments, this study aimed to investigate the analgesic effect of RR extracts as a natural alternative on Wistar rats.

The first factor examined in this study was the rate of inflammation in the hind paw of the rats in study groups. The results of the study showed that the rate of inflammation in the rat groups receiving the extract and morphine was significantly reduced compared to the group I. In other words, both the RR extract and morphine prevented the recruitment of inflammatory cells to the site of formalin injection, thereby inhibiting the inflammatory response.

Research has shown that RR contain isoflavonoid compounds that inhibit cyclooxygenase (COX) activity in human monocytes. These compounds reduce the synthesis of prostaglandins, specifically prostaglandin E2 and thromboxane B2, by inhibiting the COX enzyme. This inhibition of COX is one of the primary mechanisms underlying the anti-inflammatory effects of these plants (21-23). Flavonoids are actually natural Polyphenol compounds in plants that reduce inflammation and pain in humans (22).

In this case, Hoodgar et al. (2011) found that the hydroalcoholic extract of *Securigera securidaca L.* seed exhibited anti-inflammatory effects in mice. They attributed this effect to flavonoids and tannins in the plant (15).

Other anti-inflammatory compounds reported in the RR extract include sesquiterpene lactones. These compounds, found in plants of the Salvia L. genus, inhibit tissue inflammation by suppressing the cyclooxygenase pathway (Leading to reduced prostaglandin and thromboxane production) and the lipoxygenase pathway (Leading to reduced leukotriene production) (24,25).

The second objective of this study was to investigate the analgesic effects of the RR extract and morphine using the formalin test in rats. First, the duration of licking behavior was measured in three groups during the early phase of the test. The results indicated a significant decrease in pain-related licking time in both group III and morphine group II compared to the group I ($P \leq 0.05$). Similarly, during the late phase of the test (15-60 minutes post-formalin injection), both the RR extract and morphine significantly reduced formalin-induced licking behavior.

Extensive research has been conducted on the analgesic effects of medicinal plant extracts, both in Iran and globally. This growing body of evidence underscores the importance of natural compounds as potential pain management strategies among researchers.

In this case, Eidy (2014) investigated the analgesic effects of sage extract using the acetic acid, formalin, and hot plate methods in NMRI mice. They reported that the *Salvia aethiopis* extract, at a concentration of 50 mg/kg, significantly reduced pain in mice during the chronic phase (26).

However, in this study demonstrated that the RR extract significantly reduced pain in rats during both the primary and secondary phases of the test. This suggests that the RR extract may possess stronger analgesic properties compared to *Salvia aethiopis* extract.

Mahmoudi et al. (2013) used the tail-flick and writhing tests to investigate the analgesic effects of the hydroalcoholic extract of *Hedera helix* in male rats. The results demonstrated that doses of 200 and 300 mg/kg of the extract significantly reduced pain compared to the control group. They proposed that this analgesic effect may be mediated by the activation of the opioid pathway (27).

Alimohammadi et al. (2015) employed the hot plate test to investigate the analgesic effects of the hydroalcoholic extract of *Scrophularia striata* Boiss. They demonstrated that hydroalcoholic extract of *Scrophularia striata* Boiss possesses analgesic properties and may serve as a viable alternative to chemical analgesic drugs. They attributed the hydroalcoholic extract of *Scrophularia striata* Boiss analgesic effects to the presence of flavonoid compounds, glycotropenoids, and phenylpropanoid glycosides, which are believed to reduce edema and inhibit cellular inflammation (28).

Dashti et al. (2009) investigated the analgesic effects of cinnamon extract in Wistar rats using the formalin test. Their findings revealed that doses of 50 mg/kg and 100 mg/kg of cinnamon extract did not exhibit significant analgesic properties. However, a higher dose of 500 mg/kg significantly reduced pain in the tested rats (29). The present study, revealed that the RR extract induced analgesic effects at a lower concentration (100 mg/kg) compared to cinnamon extract (500 mg/kg). This suggests that the RR extract may contain higher concentrations of analgesic compounds than cinnamon. Camphor, a major analgesic constituent of RR extract, binds to its numerous receptors on nociceptive neurons, thereby mitigating pain sensation (9, 30).

Additionally, numerous studies investigating the analgesic mechanisms of medicinal plants have identified compounds such as carotenoids, vitamin C, phytosterol and calcium as potential pain relievers (31-33). It is essential to note that the dosage of herbal extracts is a critical factor in analgesic testing. In all the studies mentioned, a direct correlation was observed between the dose of the extract and its analgesic properties. In other words, higher doses of the extract were associated with greater pain reduction.

Taheriran et al. (2004) demonstrated a dose-dependent analgesic effect of *Coriandrum sativum* (CS) extract in the formalin test. A dose of 100 mg/kg reduced pain by 82%, while a dose of 200 mg/kg reduced pain by 92% in mice (34).

Conclusion

The results of this study demonstrated that the RR extract possesses anti-inflammatory properties, reducing inflammation induced by formalin injection in the rat hind paw. Additionally, the acute and chronic analgesic effects of the RR extract were investigated using the formalin test. A concentration of 100 mg/kg of the extract was found to significantly reduce pain in both acute and chronic phases, suggesting its potential as a promising analgesic agent.

However, further pharmacological and toxicological research is necessary to evaluate the safety and efficacy of the RR extract as a therapeutic agent. A comprehensive understanding of the underlying physiological mechanisms of action would contribute to the development of more effective pain management strategies.

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Ethical Statement
The study was approved by the Ethics Committee of Bu-Ali Sina University (IR.BASU.REC.1401.008).

Conflicts of Interest
All authors declare that there is no conflict of interest.

Author Contributions
M. R: Writing - Original draft, Investigation, Data curation. S.Y: Writing - Review and Editing, Validation, Supervision, Resources, Investigation, Formal analysis, Conceptualization. R. K: Writing - Review and Editing, Validation. A. S: Writing - Original draft, Investigation, Data curation, Formal Analysis.

Data Availability Statement
Data will be made available on request.

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