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Comparative Evaluation of Analgesic Activity of Solenanthus circinatus Ledeb. Root Extract and Fractions in Rat Models of Pain

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Abstract

Solenanthus circinatus root, commonly called "Azaar Choob" in persian medicine, has a very long history of use in relieving pain and inflammation in muscle contusion and bone bruises and fractures. In this study, the analgesic effect of root extract and various fractions of *S. circinatus* were assessed in male rats using tail flick and formalin tests. Powdered roots of the plant was extracted with ethanol in a soxhlet apparatus and then fractionated with solvents of increasing polarity including petroleum ether, chloroform, ethyl acetate and n-butanol in a liquid-liquid extractor. Ethanolic extract and fractions were then chromatographed on silica gel TLC plates using various solvent systems. The spots on the chromatograms visualized using colour reagents which showed the presence of various compounds. In order to evaluate analgesic effect, rats were treated with 200 mg/kg of extract and fractions intraperitoneally and verified by tail-flick and formalin tests and compared with diclofenac group (25 mg/kg) as a standard drug. Extract and almost all fractions revealed significant analgesic effects compared to the control group. Based on the results, analgesic activity of root extract and fractions of *S. circinatus* were comparable with that of diclofenac. Petroleum ether fraction displayed higher pain relieving activity than diclofenac at 25 mg/kg in tail-flick test. Interestingly the analgesic efficacy of petroleum ether fraction surpassed other fractions at 60 min interval after injection. Ethyl acetate and petroleum ether were found to be superior to other fractions in manifestation of analgesic activity. The root extract is currently under further detailed investigation.

Keywords: Solenanthus circinatus, Analgesic, Tail-flick test, Formalin test.

1. Introduction

Pain is one of the classic manifestations of the inflammatory conditions (1). The non-steroidal anti-inflammatory drugs (NSAIDs) are the most prescribed medications for the treatment of inflammatory diseases (2). The potential side effects of these drugs has led to increasing interest in natural anti-inflammatory and herbal products particularly those with long history of traditional usage. The root of *S. circinatus* is called "Azaar Choob" in Persian folk medicine which has been used for relieving pain and topical inflammation particularly in bone fractures since the age of Avicenna (3). *Solenanthus*, is a genus of flowering plants in the family Boraginaceae with white flowers which grows in warm regions of the world. There are four species of the genus in Iran while ten species distributed throughout the tropical Asia (4). Azaar Choob is frequently used as an externally applied poultice for the treatment of bone contusion, tendon damages and to reduce joint inflammation in persian folk medicine (5). Azaar Choob is the main part of a formula which is a combination with *Curcuma longa*, *Glycyrrhiza glabra* and yolk to treat

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sprained or fractured bone prescribing by the folks and traditional practitioners successfully. Careful literature search, revealed a single report on the analgesic efficacy of *S. circinatus* extract while no studies was observed on its phytchemical analysis. This has prompted us to assess the analgesic activity of *S. circinatus* ethanolic root extract (ESC) and various fractions. Hence this is considered to be the first study of the phytochemical and analgesic properties of this plant.

Preliminary literature search on other plants of Boraginaceae family clearly revealed that the roots of the genera close to *Solenanthus* have so far elaborated biologically active substances such as phenolic acids, saponins, phytosterols, alkaloids and particulaly naphthoquinones and allantoin (6,7). Although no phytochemical analysis have been reported so far, the roots of Boraginaceae plants are known to elaborate structurally diverse naphthoquinones as the major constituents, capable of demonstrating analgesic properties which is unique to this plant family (8,9). Roots of other Boraginaceae species such as *Symphytum officinalis* (comfrey) is known for its topical application in the form of extract, poultice and ointment in treatment of joint inflammation, wounds, bone fractures, muscle and joint pains and thrombophlebitis (10). A survey of the uses of plants of Borage family declared that the topical application of *Symphytum officinalis* in the United Kingdom, is rated the most effective treatment for fractures and the post-operative surgical wounds and the problems with tendons, ligaments, and muscle injuries (11). Extraction and quantification of several compounds such as allantoin, p-hydroxybenzoic acid, rutin, hydrocaffeic acid, rosmarinic acid, chlorogenic acid, and shikonin have been reported from the roots of Boraginaceae plants including *Alkanna*, *Lithospermum*, *Echium*, *Borago*, *Symphytum*, *Onosma* and *Nonea* (12, 13).

Based on traditional evidences, the analgesic and anti-nociceptive activity of the ethanol extract and fractions of *S. circinatus* root, including petroleum ether, chloroform, ethyl acetate and n-butanol fractions from the hydroalcoholic extract were studied in male rats.

2. Material and Methods

2.1. Collection and authentication of plant material

Aerial parts of *S. circinatus* were collected from Darab, southern Iran and authenticated by the plant taxonomist of the Dept. of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences. A Voucher specimen of the plant (MPRCH-93-01) has been deposited in the herbarium of Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz- Iran.

2.2. Extraction and Fractionation

The freshly collected roots of *S. circinatus* were minced and exhaustively extacted (100 g) with ethanol 96% for 4 h using a Soxhlet apparatus. The extract was filtered and the solvent was removed under reduced pressure to afford a light brown gummy residue as ethanolic extract. The extract was further suspended in water and fractionated successively with petroleum ether, chloroform, ethyl acetate and n-butanol in a liquid-liquid extractor. The individual fractions washed with water in a separatory funnel and the solvent was evaporated to dryness at 50°C under reduced pressure. The dried extract and fractions further concentrated in a speed vacuum and freeze dried and finally stored at -20 °C prior to pharmacological screening.

2.3. Preliminary Phytochemical Screening

The methanolic solution of *S. circinnatus* ethanol extract was assessed for the presence of phytochemical components, using the standard reagent methods (14). The results of preliminary phytochemical screening are presented in Table 1.

2.4. Animals

The experiments were conducted on adult male Sprague-Dawley rats weighing 180-220 g (n=72). The animals were fed a standard laboratory diet and water ad libitum and kept at 25±2 °C with a 12 h light/dark cycle. During the entire period of study the rats had access to standard pellets diet and tap water *ad libitum*. Animals were acclimatized to laboratory conditions before the test (n=5 in each experimental group in the study).

Table 1. Reagent tests with ethanolic root extract of *S. circinatus* Ledeb.

Types of Tests	Phytochemical constituents	EtOH Extract
Ferric Chloride	Phenols	+
Vanillin HCl	Flavonols	+
Frothing	Saponins	-
Anisaldehyde /H_2SO_4	Phenyl propanoids	+
Shinoda	Flavonoids	+
Zinc HCl	Flavonoids	+
Dragendorff	Alkaloids	-
Mayer	Alkaloids	-
Wagner	Alkaloids	-
Salkowski	Terpenoids	+
Liebermann	Steroids	+
Vanillin/ H_2SO_4	Terpenoids	+

The handling and treatment of animals were carried out in accordance with the ethical guidelines the protocols approved for the care and use of laboratory animals by the Ethics Committee of Shiraz University of Medical Sciences.

2.5. Evaluation of in vivo analgesic activity

In this study, two types of painful stimuli were used to induce pain in the rats by direct stimulation of pain receptors and they included: thermally (tail-flick test) and chemically (formalin) induced pain. The antinociceptive effects of ehanolic extract and various fractions of *S. circinatus* root were further compared to sodium salycilate as control.

2.5.1. Formalin Test

In this model, in negative control group, the animals received vehicle (DMSO 300 µL/kg, i.p.) and in positive control group rats received the standard drug diclofenac sodium 25 mg/kg, i.p. Analgesic activity of *S. circinatus* hydroalcoholic extract and fractions at dose of 200 mg/kg, i.p. was assessed by observing the reaction time in the test groups. 60 minutes after vehicle, diclofenac sodium and extracts administration, 50 µL of 2.5% formalin (v/v in distilled water) was subcutaneously injected into the plantar surface of the animals' right hind paw. Nociception was rated using the original formalin test protocol (15). Briefly, the pain scoring measurement was as follows: 0, no response behavior of the injected paw; 1, limping during locomotion or resting the paw lightly on the floor; 2, elevation of the injected paw; 3, licking or biting of the injected paw, or grooming.

Behavioural responses were observed and recorded for 1 h after the formalin injection. The first 5 min was considered as the early phase and the period between 20 and 60 min as the second phase. Following subcutaneous intraplantar injection of formalin, the animals were immediately placed in a chamber with a mirror placed under it, with a 45 °C angle underneath the floor to allow an unobstructed view of the formalin injected paw. All animals were brought to the test chamber 1 h prior to the experiment.

2.5.2. Tail-flick Test

Tail-flick (Borje Sanat, Tehran, Iran) was employed for assessment of acute analgesic activity, 70% of light intensity and sensitivity were used. A 15 second cut-off was imposed to prevent tail injury. Duration of time in tail-flick latency was measured as a response to pain. All samples including EtOH extract and individual fractions of *S. circinatus* root were tested. In this study, rats were randomly placed into nine groups of 5 rats each. The positive control group were given an i.p. injection of 25 mg/kg of diclofenac sodium. The negative control group received 300 µl/kg,i.p. dose of DMSO. Animals in groups 3 to 7, received a 200 mg/kg i.p. dose of hydroalcoholic exract, pe-

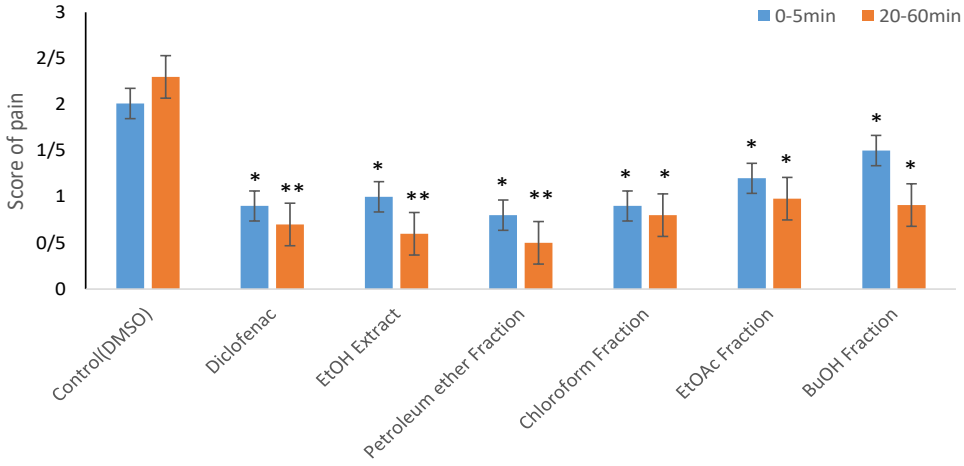

Figure 1. The analgesic activities of *S. circinnatus* Ledeb. root extracts in the formalin test in rats.
Data are given as Mean±SD for the animals.
*Indicates significantly lower scores compared to those of the control group (P<0.05)
**Indicates significantly lower scores compared to those of the control group (P<0.01)
aindicates a significant difference as compared with the Diclofenac group (P<0.05).

troleum ether, chloroform, ethyl acetate and butanol fractions respectively. Analgesic activity was assessed by observing the reaction time. Following the administration of vehicles, diclofenac, extract and fractions, the reaction time was noted at 30 and 60 min intervals (16).

3. Results

Since the only report in the literature describes the analgesic activity of ehanolic extract of *S. circinatus* root and no detailed study has been reported earlier, therefore this study was undertaken to locate the analgesic fraction of the ethanolic extract. The analgesic screening was conducted using the tail-flick and formalin tests.

3.1. Formalin test

The effect of EtOH extract and individual

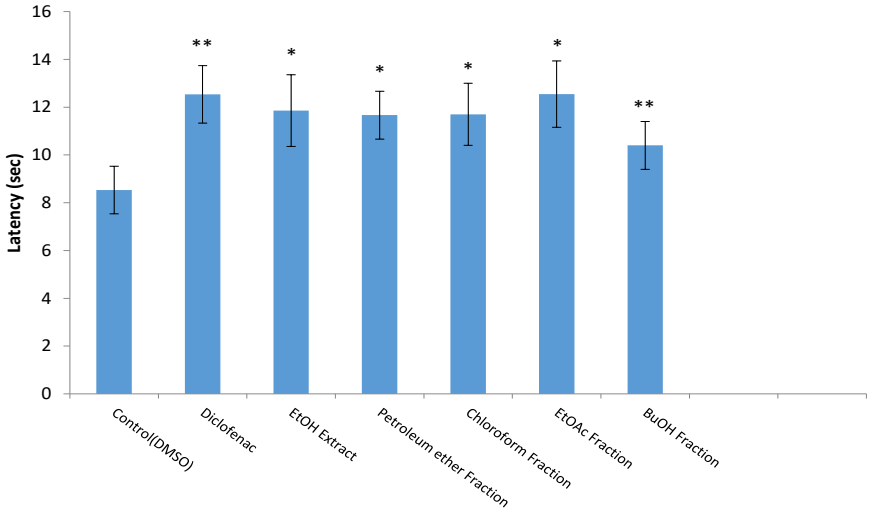

Figure 2. The effect of *S. circinatus* Ledeb. roots extract and fractions administration on the latency time in the tail-flick test after 30 min.
Data are expressed as Mean±SD for the animals.
*Indicates a significant difference as compared with the control group (P<0.05)
**Indicates a significant difference as compared with the control group (P<0.01).

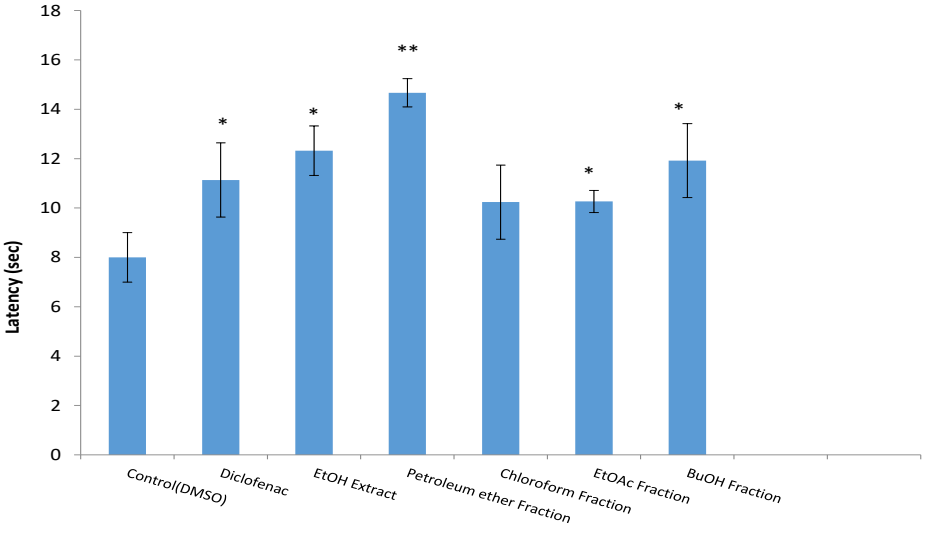

Figure 3. The effect of *S. circinatus* Ledeb. roots extract and fractions administration on the latency time in the tail-flick test after 60 min.

Data are expressed as Mean ± SD for the animals.

*Indicates a significant difference as compared with the control group (P<0.05)

**Indicates a significant difference as compared with the control group (P<0.01).

fractions in early (0-5 min) and late (20-60 min) phases of formalin test are shown in Figure 1. In the both early and late phases of formalin test doses of 200 mg/kg EtOH extract and fractions were shown to have significant effect on the nociception compared to that of control group. In the late phase of formalin test, EtOH extract and petroleum ether fraction were found to exhibit higher analgesic activity (P<0.01) as compared with the control.

3.2. Tail-flick test

As shown in Figure 2, injection of 200 mg/kg of EtOH extract and fractions to rats significantly enhanced the tail flick reaction time compared to control group (DMSO) in 30 min after injection. The antinociceptive effect of ethyl acetate at 30 min after injection was significantly higher than other fractions. The ethyl acetate fraction was found to demonstrate a close degree of activity with diclofenac group.

As shown in Figure 3. a 200 mg/kg; i.p. injection of petroleum ether to the rats, caused a significant increase in time of latency compared to control group within 60 min after injection. Therefore the antinociceptive effect of this fraction was found to be superior to diclofenac in the tail-flick test in the present study.

Table 2. The analgesic activities of *S. circinatus* Ledeb. root extracts in the formalin test in rats.

Time (min) Groups	0-5	5-10	10-15	15-20	20-25	25-30	30-35	35-40	40-45	45-50	50-55	55-60
Control Negative	2.17 ±0.2	1.83 ±0.6	2.03 ±0.6	2.46 ±0.5	2.32 ±0.5	2.17 ±0.4	2.36 ±0.4	2.14 ±0.3	2.35 ±0.5	2.28 ±0.4	2.46 ±0.5	2.38 ±0.5
Diclofenac (25mg/kg)	1.7 ±0.1*	1.08 ±0.5*	0.64 ±0.5**	0.48 ±0.4**	1.03 ±0.5**	0.92 ±0.5**	1.46 ±0.4**	1.20 ±0.5**	1.1 ±0.4 **	0.85 ±0.4**	0.70 ±0.3**	0.93 ±0.5**
Total extract (200mg/kg)	1.7 ±0.5*	0.46 ±0.2**+	0.36 ±0.2**	0.73 ±0.5**	1.08 ±0.4**	0.72 ±0.4**	0.85 ±0.4**+	0.75 ±0.5**+	0.65 ±0.4**+	0.74 ±0.4**	0.71 ±0.4**	0.52 ±0.3**

Data are Mean±SD (n=5 in each group).

*Significant difference with the control group (P<0.05)

+Significant difference with diclofenac group (P<0.05).

**Significant difference with the control group (P<0.01)

4. Discussion

Non-steroidal analgesics are currently used to treat bone fracture pain. While this class of pain relieving drugs are effective in reducing musculoskeletal pain, reports indicate that they retard the process of bone healing in mice and humans. Therefore, NSAIDs and cyclooxygenase-2 inhibitors can slow down the process of bone formation at the fracture site (17). Therefore, the need for developing new sources of natural pain releiving agents directed our attention towards a plant which is widely used for relieving pain and inflammation in Iranian traditional system of medicine.

The severe adverse effects of chemical drugs and the issue of return to the use of natural remedies are still highlighted (18). In order to search for new pain medicines with limited side effects, the study of traditional herbal species still remain to be an efficient research strategy for the development of new analgesic drugs (19). Pain is a response that includes sensational, emotional or sentimental parts. Disease, inflammation, and damage to the central and peripheral nervous system cause significant changes in pain pathways such as increased irritability, altered gene expression and formation of the new molecules such as neurotransmitters, enzymes and receptors. Having the feeling of pain in the long term can have adverse psychological effects in human. For this reason, human beings have always sought to find a solution for pain management or to eliminate the painful stimuli and reduce pain (20). The analysis of recent research indicates that, special attention have been paid in natural analgesic drugs. Since the analgesic drugs available in the pharmaceutical market show a wide range of adverse effects, for instance, aspirin can cause damage to the digestive tract, kidneys, and central nervous system, while reported to be devoid of effectiveness in some patients (21). The opioid analgesics may cause drug resistance, dependence, euphoria, and abuse and addition (22). According to previous studies, the most commonly used assays for evaluation of antinocicepting activity is the formalin test. The test is a popular chemical assay which consists of two phases. The acute phase is being related to neurogenic or non inflammatory pain, in which the pain message is transmitted through specific neural pathways and the chronic phase is related to inflammatory pain, in which transmission of the pain message is due to the inflammatory response induced by formalin. Medications that can affect the nervous system are usually effective in the acute phase, and anti-inflammatory drugs often modulate pain in the chronic phase of the formalin test (23) The analgesic agents may act quite differently in the first and second phases. Therefore, this test was used to determine the possible antinociceptive mechanism of the ESC. Drugs such as opioids, function centrally and inhibit both phases (24) while peripherally acting analgesics like aspirin and indomethacin and dexamethasone inhibit the delayed phase. It seems that the late phase is an inflammatory response associated with inflammatory pain that can be inhibited by anti-inflammatory drugs (25, 26).

The roots of *S. circinatus* has been used as part of various medicinal formulations to relieve bone fracture pain and inflammation and reduce bone or tissue inflammation in Iranian traditional medicine (27). Anti-inflammatory and pain relieving effect have been reported in plants of Boraginaceae family (28, 29). This may indicate that, certain group of compounds such as polyphenols or naphthoquinones and their hydroxy derivatives that are biosynthesized in the roots of this family might be involved partially or collectively in the manifestation of antinociceptive activity.

In the tail-flick test, the rats in treated groups received an intraperitoneal dose of 200 mg/kg of extract and fractions. The positive control group received sodium diclofenac at 25 mg/kg/day while the negative control received DMSO. Results compared through the statistical analysis performed and $P<0.05$ was considered as significant. Antinociceptive effect were detected at 30 and 60 minutes intervals of time following i.p. administration of extract and fractions. The results of treated group showed significant differences with those of positive and negative control groups. In the formalin test, treatment of animals with extract and fractions were also found to exhibit satisfactory results when compared with positive control (diclofenac 25 mg/kg) and the statistical analysis were found to be at significant level ($P<0.05$). There was no significant difference

between the treated and control groups in formalin test in the first phase of pain (5 min) while in the second phase (15-60 min), the *S. circinatus* extract declared a significant antinociceptive effect (Table 2). The results of previous research showed that S.circinatus ethanolic root extract significantly decreased pain in the tail flick test when compared with the group that received sodium salicylate. All doses of *S. circinatus* hydroalcoholic extract, i.e. 100, 200 and 300 except 50 mg/kg, significantly inhibited the increasing inflammation in foot paw in male Sprague Dawley rats. There was also significant differences among the treated and the control group, received a dose of 300 mg/kg. Previous research has shown that hydroalcoholic extract of *S. circinatus* inhibited the inflammatory response induced by carrageenan (27). The overall results indicated that the petroleum ether and ethyl acetate fractions had powerful analgesic activity in preliminary investigation.

To establish the chemical profiles of *S. circinatus* root extract (ESC), thin-layer chromatography was performed as a qualitative chemical test. The results indicated the presence of chlorogenic acid together with compounds like triterpene saponins. These groups of compounds have been isolated from the plants of Boraginaceae family, but no report was found on chemical constituents of *S. circinatus* root in previous studies (28, 29). It appears that among the chemical components of the root, naphthoquinones and their hydroxylated derivatives such as shikonin and alkannin or their modified structures are among the major components of the root (30). Therefore the antinociceptive and anti-inflammatory effect of ESC, may partly be attributed to the presence of these compounds in the ethanolic extract. The diverse structural features of these compounds may justify their individual or synergistic analgesic activity. As derived from the results of both hot plate and tail-flick tests, it may be concluded that the mechanism of action of *S. circinatus* root in manifestation of analgesic activity is supposed to be through inhibiting central and peripheral pathways. Based on the types of phytoconstituents of *S. circinatus* root and the presence of allantoin and the the topically absorbable phenolic acids like rosmarinic and chlorogenic acids, it has been elucidated that the mechanism involved in the manifestation of analgesic activity might be through the inhibition of cyclooxygenase-2 in the prostaglandin pathway (31).

Significant analgesic effects was observed for petroleum ether fraction at 60 minutes after i.p. injection, using the tail-flick model. However, the effectiveness of this fraction at 30 minutes interval was not found to be significant when compared to the negative control. The results of the chromatographic analysis of this fraction reveals the presence of compounds of phenolic nature and therefore led us to conclude their possible contributions to the antinociceptive effects. Additional evidences for this assumption came from the earlier reports on anti inflammatory and antinociceptive characters of phenylpropanoids (32, 33).

The results of antinociceptive effect of chloroform fraction at 200 mg/kg in tail-flick model, did not show significant differences at 30 and 60 min. intervals compare to positive control. The analgesic properties of ethyl acetate fraction was studied in similar conditions to other fractions. In the tail-flick model, this fraction showed significant antinociceptive effect at the dose of 200 mg/kg at 30 and 60 min after i.p. injection ($P<0.05$). Based on the results of thin-layer chromatography, the presence of compounds of higher polarity like steroids or triterpenoids and their glycosidic counterparts, may play a significant role in partial contribution of this fraction to antinociception activity of the extract.

The antinociceptive properties of n-butanolic root fraction of *S. circinatus* was evaluated by using tail-flick method at a dose of 200 mg/kg and compared to positive (diclofenac 25 mg/kg) and the negative control groups, which observed to be at significant level ($P<0.05$). Efficacy of this fraction at 30 and 60 minutes was significantly higher than negative control. However, this fraction declared a lower degree of effectiveness at 30 minutes interval compare to than standard control. Examination of the type of chemical constituents in this fraction, which was deduced from the observation of the chromatogram, revealed the presence of steroids and terpenoids with glycosidic structures may be responsible for the activity. Polar groups of herbal compounds have been report-

ed to exhibit antinociceptive effects (34).

The present study shows that petroleum ether fraction of *S. cicinatus* root had more pronounced pain relieving effects compared to total extract and other fractions. Therefore, the non-polar compounds of the extract was found to contribute to the antinociception properties of *S. cicinatus* root. It is obvious that the separation of each of these compounds and their separate evaluation of their pharmacological effects will help in the determination of the active chemical constituents that require the separation and identification of these components using conventional chemical methods as well as instrumental analysis.

Comparing the efficiency of different fractions of ethanolic extract, it may be concluded that the petroleum ether fraction of *S. circinatus* root extract declares higher degree of antinociceptive and antiinflammatory characteristics. In other words, the lipophilic compounds of the *S. circinatus* root share the activity with polar compounds of phenolic nature and allantoin, a 5-ureidohydantoin compound and a major metabolic intermediate that resides in the ethyl acetate fraction here. The evidence in support of possible antinociceptive and antiinflammatory role of allantoin is that, the genesis of this nitrogen-rich compound is specific to Boraginaceae roots which has been demonstrated so far significant antinociceptive effect (35).

The active chemical constituents of ethanol root extract of *S. circinatus* and particularly those of pet.ether and ethyl acetate fractions including alkaloids, hydroxyquinones and steroids, may remain specific in the onset of antinociceptive and antiinflammatory effects that possibly exert through inhibition of synthesis, release or the action of inflammatory mediators such as prostaglandin, serotonin, protease and histamine (36). Moreover the synergistic analgesic effects of these constituents should not be ignored.

5. Conclusion

S. circinatus root have been an indispensable alternative treatment in ameliorating inflammation and skeletal pain in Persian traditional medicine. Experimental studies revealed that the degree of antinociceptive activity showed variability among the extract as well as fractions. According to our findings, the ethanol extract of the roots of the plant produced an antinociceptive and anti-inflammatory effect when assessed in rat models of nociception. In conclusion, the results of present study provide acceptable anti-nociceptive effects in rats. As an overall evaluation, the analgesic and antinociceptive characteristics observed either for the ethanolic extract or fractions may remarkably support the traditional uses of *S. circinatus* root in management and treatment of bone and muscle contusion, bruising and fracture pains and inflammation.

Statistical Analysis

The results obtained were expressed as mean values±SD and statistical comparison of data was performed using one way analysis of variance (ANOVA) followed by Dunnett's test. All levels of significance were set at $P<0.05$.

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Conflict of Interest

None declared.

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