

Effect of *Rubia tinctorum* L. Extract on Carrageenan-Induced Paw Edema in Rats

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Abstract

Background: Inflammation is a dynamic process that is elicited in response to various harmful stimulants that may threaten the well-being of the host. Herbs have been traditionally used as anti-inflammatory agents since very early times. *Rubia tinctorum* L. is one of these herbs that have been used for treating inflammatory diseases in Iranian traditional medicine.

Objective: This study aims at considering anti-inflammatory effects of *R. tinctorum* and comparing its extracts in this property.

Methods: The root of *Rubia tinctorum* (Rubiaceae) was collected from Yazd province, in the center of Iran. The root extracts of plant were studied for their anti-inflammatory activity by using carrageenan-induced hind paw edema in Wistar male rats. Indomethacin and normal saline were used as positive and negative controls, respectively. Different concentrations of aqueous, ethanolic, and 50% aq. ethanolic extracts (600, 800, 1000 mg/kg) were given orally before carrageenan injection. The paw thickness was measured at 1, 3 and 5 hours after injection.

Results: Both, aqueous and 50% eq. ethanolic extracts reduced paw edema at 600 and 800 mg/kg concentrations. Moreover, ethanolic extract showed significant effect only in 1st hour after carrageenan injection.

Conclusion: The results indicated that the aqueous extract were exhibited higher anti-inflammatory effect compared with other extracts. Accordingly, the potent anti-inflammatory effect of the root extracts is attributed to the polar compounds which are extracted in the water.

Keywords: *Rubia tinctorum*, Rubiaceae, Anti-inflammatory, Carrageenan, Paw edema



Introduction

Inflammation has been described as the entire complex of tissue changes. When tissue injury occurs, whether caused by any noxious stimuli, multiple substances that cause dramatic secondary changes are released by the injured tissues. This process involves changes in blood flow, increased vascular permeability, destruction of tissues via the activation and migration of leucocytes with synthesis of reactive oxygen derivatives (oxidative burst) [1]. Some of tissue products involved in inflammatory reactions are histamine, bradykinin, serotonin, prostaglandins, and lymphokines. Many of these substances strongly activate macrophages, and within a few hours, the macrophages begin to devour the destroyed tissues. Moreover, macrophages' activity also injure further the still-living tissue cells [2]. Herbs traditionally were used to treat inflammation. The mechanisms by which elicit their activity may be attributed to the variety of secondary metabolites via different procedures [1].

R. tinctorum belongs to the Rubiaceae family, which consists of 6000 species and 500 genera [3]. It is known as "Ronas" in Persian and "madder" in English [4]. This plant is historically originated from Caucasus and Near East. It grows in Mediterranean regions from Spain to Asia, also in the north of Africa and some places in Asia. It cultivated in central and western regions of Iran such as Yazd [5]. A variety of anthraquinones have been found as main constituents of this plant, including purpurin, rubiadin, lucidin and alizarin [6-10]. Different methods were reported for identification and quantification of constituents in madder root, such as electrospray mass spectrometry, spectrophotometric methods, high

performance liquid chromatography (HPLC) and nucleic magnetic resonance (NMR) [11-16]. Previous studies have been showed effects of *R. tinctorum* in treatments of urolithiasis, high blood pressure, coetaneous leshmaniasis and diarrhea [17-20]. Plant's root was incorporated in remedy for acute myeloid leukemia and polycystic ovarian syndrome in Chinese medicine. Besides, in some other studies it shows antimicrobial and antifungal properties [21-23]. This plant was also used by dermatologists for detection of calcium in some coetaneous diseases [24]. Consequently, madder was considered as a potential dye for study of bones [25, 26]. In one of the previous studies in Japan, acute and sub-acute toxicity tests of madder root were surveyed on the mice and no toxic effects was determined [27]. Anti-inflammatory effects of *R. cardifolia*, another member of Rubiaceae family, was demonstrated via inhibition of nitric oxide and carrageenan model [28, 29]. Furthermore, anti-inflammatory effect of this medicinal plant has been attributed to mollugin, a naphthoquinone, one of the active components of these taxa [30, 31].

R. tinctorum has been long used in traditional medicine to cure various ailments, for instance Greek physician had used this plant as a diuretic, treatment of jaundice, sciatica and paralysis [32]. The plant was also applied for treatment of rheumatic disorders in Europe [33]. In the traditional medical texts of Iran, Makhzan-ol-advieh and Tohfat-ol-momenin, the plant was recommended for treatment of inflammatory diseases [34, 35]. According to literature surveys, no study has been done about anti-inflammatory effects of *R. tinctorum* extract *in vivo* or *in vitro*. In the present study, anti-inflammatory activity of different extracts of plant's root was assessed for the first time.

Materials and Methods

Plant material

Rubia tinctorum was collected in Yazd province (central-region of Iran) in October 2011 and identified in pharmacy faculty's herbarium (Tehran University of Medical Sciences, Iran). A specimen was deposited at the herbarium under the reference number 6556-TEH.

Preparation of plant extracts

The shade-dried roots of the plant were powdered, and then 200 g of the powder successively extracted with water; 200 g with ethanol and 200 g with 50% aq. ethanol (3×48h) using percolator apparatus. These extracts were completely concentrated in a rotary evaporator and then freeze dried. The extracts were then kept in opaque containers, under cold storage, until assay.

Chemicals

Carrageenan (Sigma, Germany) was dissolved and indomethacin (Aburaihan Co., Iran) was suspended in isotonic saline. All solvents were purchased from Merck Company (Darmstadt, Germany).

Animals

Male Wistar rats (200-300 g) were obtained from Animal House of Faculty of Pharmacy, Tehran University of Medical Sciences, Iran. The rats were maintained in polyacrylic cages within a temperature-controlled (22 ± 1 °C) room, on a 12 hour light/dark cycle, and were given free access to food and water. Each rat was used only once during the study. All studies were conducted in accordance with the internationally accepted principles for laboratory animal use and care in the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

Carrageenan induced hind paw edema

Anti-inflammatory activity of madder root extracts were determined by a carrageenan-induced paw edema test. In this experiment 77 rats in 11 groups were used for evaluation of anti-inflammatory effect of the plant. Aqueous, ethanolic, and 50% eq. ethanolic extracts of the root were prepared in concentration of 600, 800 and 1000 mg/kg of body weight dissolved or suspended in normal saline solution (NSS). Indomethacin (5 mg/kg) and normal saline were used for positive-control and control groups, respectively. The experimental rats received these doses orally, 90 minutes before subcutaneous injection of carrageenan (50 µL, 1% in NSS) into the plantar surface of the left hind paw. Thickness of the rats paw was measured before carrageenan injection and in 1, 3, and 5 hours after it by digital caliper stainless hardened (China). The paw's thickness difference as an edema criteria and inhibition percentage of each group were calculated and statistical analysis were executed.

Statistical analysis

Calculated values were analyzed with the One-Way ANOVA followed by a Newman-Keuls posttest. Statistical significance was considered as $p < 0.05$.

Results

Searching for novel anti-inflammatory drug is still a very challenging path and herbs seem to be a promising source to explore. In this study, anti-inflammatory effect of *Rubia tinctorum* (madder) was evaluated by carrageenan-induced paw edema model. The madder root was successfully extracted with distilled water, ethanol and 50% aq. ethanol by percolation method. The yields of the crude

extract were 33, 9.74, and 51%, relatively. In the carrageenan-induced paw edema test, aqueous extract (800 mg/kg p.o.) significantly inhibited paw edema 1, 3 and 5 hour after carrageenan injection. Furthermore, aqueous extract (600 mg/kg, p.o.) produced a significant anti-edematous effect 1 hour after carrageenan administration. The 50% aq. ethanolic extracts was effective in all doses 1 hour post carrageenan injection. Ethanolic extract (800 mg/kg, p.o.) also revealed anti-inflammatory effect against experimentally induced paw edema in 1 and 3 h. The ethanolic extract (1000 mg/kg, p.o.) reduced paw edema in the

first hour. Changes in edema thickness (mm) in 1, 3, and 5 h after carrageenan injection following oral administration of drugs are shown in table 1. In comparison to reference drug (i.e. Indomethacin 5mg/kg p.o.), ethanolic extract (1000 mg/kg, 1st h), aqueous extract (800 mg/kg 3rd and 5th h), 50% aq-ethanolic extract (800 mg/kg, 3rd h) has shown significant anti-inflammatory effects but were not significantly different from positive control (Table 1.)

Our results suggested that among all extracts the aqueous extract possessed higher anti-inflammatory activity (Figure 1).

Table 1- Changes in edema thickness (mm) from 1, 3, and 5 h after carrageenan injection following oral administration of normal saline solution (control), indomethacin (indo: 5 mg/kg) and various doses of ethanolic, 50% aq-ethanolic extract, and aqueous extracts of madder root.

Difference in paw edema \pm S.E.M. (mm), Inhibition percent (%)				
Treatment (mg/kg)	N	1h	3h	5h
Control	7	2.42 \pm 0.15	2.09 \pm 0.13	1.69 \pm 0.14
Indomethacin (5)	7	0.51 \pm 0.09, 69.83%	1.11 \pm 0.2, 46.89%	1.27 \pm 0.14, 24.85%
aqueous extract (600)	7	1.18 \pm 0.16***, 51.24%	1.59 \pm 0.07, 23.93%	1.28 \pm 0.11, 24.26%
aqueous extract (800)	7	1.36 \pm 0.15***, 43.80%	1.30 \pm 0.20**, 37.80%	0.86 \pm 0.19**, 49.11%
aqueous extract (1000)	7	1.95 \pm 0.15, 19.42%	2.16 \pm 0.18, -3.34%	2.09 \pm 0.09, -23.66%
50% aq-ethanolic extract (600)	7	1.35 \pm 0.13***, 44.21%	1.91 \pm 0.08, 8.61%	1.92 \pm 0.17, 13.60%
50% aq-ethanolic extract (800)	7	1.72 \pm 0.26**, 28.92%	1.48 \pm 0.18*, 29.19%	1.22 \pm 0.14, 27.81%
50% aq-ethanolic extract (1000)	7	1.57 \pm 0.14**, 35.12%	1.19 \pm 0.18, 43.06%	1.81 \pm 0.14, 7.10%
ethanolic extract (600)	7	1.98 \pm 0.28, 18.18%	1.51 \pm 0.16, 27.75%	1.24 \pm 0.13, 26.63%
Ethanolic extract (800)	7	1.38 \pm 0.13***, 42.98%	1.81 \pm 0.20, 13.40%	1.57 \pm 0.16, 7.10%
ethanolic extract (1000)	7	0.94 \pm 0.16***, 61.16%	1.98 \pm 0.12, 5.26%	2.01 \pm 0.14, -18.93%

* Significantly different compared to NSS ($p < 0.05$)

**Significantly different compared to NSS ($p < 0.01$)

*** Significantly different compared to NSS ($p < 0.001$)

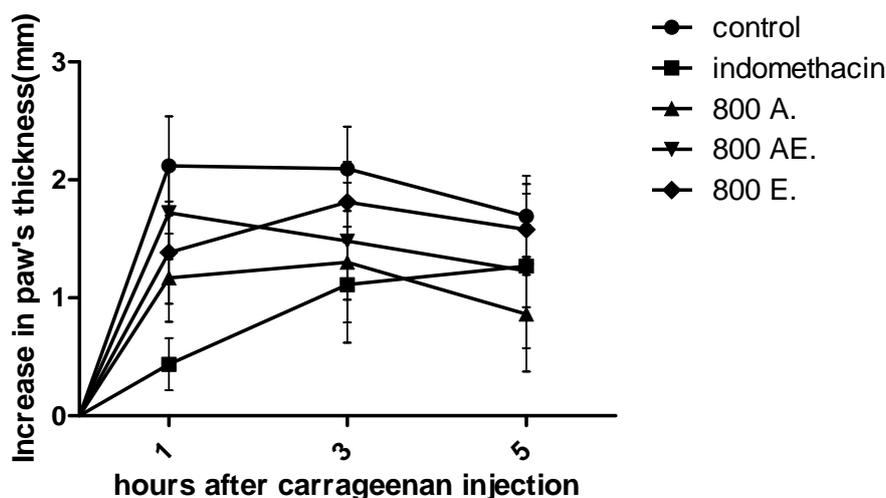


Figure 1- Effect of oral administration of different extracts of *Rubia tinctorum* (800 mg/kg) on carrageenan-induced paw edema in rats. Extracts, normal saline solution and indomethacin were administered 90 min prior to carrageenan (1%) injection, and the rats were evaluated for paw edema at 1, 3, and 5 h post-carrageenan injection. The values represent the mean variation in the paw thickness \pm S.E.M. (n=7) (A:Aqueous, AE:50% aqueous-Ethanol, E: Ethanol, indomethacin: indomethacin 5 mg/kg, control: normal saline solution)

Discussion

Based on results of this study, aqueous extract causes the highest effect on prevention of inflammatory response. This may be attributed to active chemicals of plant which are more soluble in the water. Anthraquinones are prominent ingredients of many medicinal plants including *Rubia tinctorum* [18]. The majority of the anthraquinones present in the madder extracts are glycosides [36]. The importance of these compounds is because of their wide range of biological properties including anti-inflammatory and antioxidant effect [18]. These glycosylated anthraquinones are found more in the aqueous extract and could be responsible for higher anti-inflammatory effect of this fraction. Antioxidant property of anthraquinones has been contributed to different pathways, for instance radical scavenging activity, chelating efficacy on Fe^+ and reducing power [37]. So anti-inflammatory effect of anthraquinones could be related to

their ability to inhibit oxidation pathway in inflammation process.

The development of edema in the rat's paw after injection of carrageenan is a biphasic process. The first phase initiates by mast cells releasing cytoplasmic enzymes, histamine and serotonin in the very first hour resulting in increase of prostaglandin synthetase in inflamed area. The second phase is due to release of interleukin-1 (IL-1) by macrophages to make polymorphonuclear cells (PMNs) accumulate into inflammatory region and release lysosomal enzymes and more prostaglandins in addition to free oxygen radicals (e.g., superoxide). This leads to connective tissue damage and paw swelling. This phase usually occurs 3-5 h post carrageenan administration [38]. Indomethacin as a reference drug can inhibit both phases by preventing serotonin and histamine release during first phase and minimizing prostaglandin's activity and as a result blocking inflammatory mediators release

within 3-5 h [39].

The majority of the madder root extracts exhibited anti-inflammatory effect at first hour after carrageenan injection, so our results indicated that madder root extracts may inhibit histamine and serotonin release. Aqueous extracts were also effective in second phase of induced edema. This could be explained by higher presence of anthraquinones in aqueous fraction and their oxidation inhibition activity.

It is well known that carrageenan stimulates release of many pro-inflammatory cytokines e.g. TNF α and IL-1 β [40]. Multiple sclerosis, arthritis, and inflammatory bowel disease are conditions in which these mediators plays essential role [41-43]. Therefore, *R. tinctorum*, as an inhibitor of carrageenan-induced paw edema, can be considered as a new potential hope to treat these inflammatory diseases.

On the basis of our data, aqueous extract of this plant, which has been long used in Iranian traditional medicine, is more active in inflammatory prohibition. The main difference between anti-inflammatory effects of extracts could be related to the various contents of

active components, which probably have glycosylated anthraquinones structures. Further works should be concentrated on quantification, isolation and structure identification of active compounds in crude extracts as well as underlying mechanisms of their anti-inflammation activity should be of concern.

Conclusion

The results of the present study indicated that the aqueous extract of *R. tinctorum* were exhibited higher anti-inflammatory effect compared with other extracts. Accordingly, the potent anti-inflammatory effect of the root extracts is attributed to the polar compounds which are extracted in the water. Further investigation is required to identification of active constituents of the plant.

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