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Original Article

Rubiadin exerts an acute and chronic anti-inflammatory effect in rodents

A Rubiadina exerce um efeito anti-inflamatório agudo e crônico em roedores

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Abstract:

Rubiadin is identified as a bioactive anthraquinone that exists in some quinone rich plants. The current research was carried out to evaluate the potential anti-inflammatory impact of Rubiadin in acute and chronic inflammation test models in rodents. The anti-inflammatory activity of Rubiadin was examined in cotton pellet-induced granuloma and carrageenan-induced edema as chronic and acute inflammation models in rats. TNF-α level and histopathological changes were assessed using sampled foot tissue of rat in the acute model. Also, the IL-1β level was assessed in the chronic model. One-way ANOVA (post hoc Tukey's) analysis was used for comparing the groups. Rubiadin (0.5 mg/kg, i.p.) induced a significant reduction in TNF α level and the paw edema compared to the control group in carrageenan test. Also, it was observed that the anti-inflammatory activity of Rubiadin (0.5 mg/kg, i.p.) is comparable to mefenamic acid (30 mg/kg, i.p.) as the standard drug. Rubiadin was effective in granuloma induced by cotton pellet concerning the granuloma and transudate formation amount. Rubiadin's anti-inflammatory effects were associated with a significant IL-1β decrease in this model. The results suggest that Rubiadin as a natural compound can possess significant peripheral anti-inflammatory impacts.

Keywords: Rubiadin, anthraquinone, anti-inflammatory, TNF α, IL-1β.

Resumo

A rubiadina é identificada como uma antraquinona bioativa que existe em algumas plantas ricas em quinonas. A presente pesquisa foi realizada para avaliar o potencial impacto anti-inflamatório da rubiadina em modelos de teste de inflamação aguda e crônica em roedores. A atividade anti-inflamatória da rubiadina foi examinada em granuloma induzido por pellet de algodão e edema induzido por carragenina como modelos de inflamação crônica e aguda em ratos. O nível de TNF-α e as alterações histopatológicas foram avaliados usando amostra de tecido do pé de rato no modelo agudo. Além disso, o nível de IL-1β foi avaliado no modelo crônico. A análise ANOVA de uma via (*post hoc* de Tukey) foi usada para comparar os grupos. A rubiadina (0,5 mg / kg, i.p.) induziu uma redução significativa no nível de TNF α e no edema da pata em comparação com o grupo de controle no teste de carragenina. Além disso, foi observado que a atividade anti-inflamatória da rubiadina (0,5 mg / kg, i.p.) é comparável ao ácido mefenâmico (30 mg/kg, i.p.) como o fármaco padrão. A rubiadina foi eficaz no granuloma induzido por pellet de algodão no que diz respeito à quantidade de granuloma e formação de transudato. Os efeitos anti-inflamatórios da rubiadina foram associados a uma redução significativa de IL-1β nesse modelo. Os resultados sugerem que a rubiadina como um composto natural pode ter impactos anti-inflamatórios periféricos significativos.

Palavras-chave: Rubiadina, antraquinona, anti-inflamatório, TNF α, IL-1β.

1. Introduction

Rubiadin; with preferred IUPAC name 1, 3-Dihydroxy-2-methylanthracene-9,10-dione; as a bioactive anthraquinone from the plants belong to the Rubiaceae family possesses antioxidant, nephroprotective, hepatoprotective, and immunomodulatory impacts (Divakar et al., 2010; Rao et al., 2006). Common madder (*Rubia tinctorum* L.) is a traditionally recognized medicinal plant that contains a considerable amount of anthraquinones like Rubiadin in its rhizome and root. The major application of this plant is in dyeing textiles (Derksen and Van Beek, 2002). It has also been used traditionally as a food colorant in most areas of the world. Moreover, the *Rubia* raw extract has been utilized as an anti-bacterial, anti-fungal, and anti-inflammatory agent in folk medicines (Kalyoncu et al.,

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2006). Anthraquinones are commonly known and used in medicine, food chemistry, and the dye industry (Dulo et al., 2021). Particularly, the synthetic and natural derivatives of 9, 10-anthracenedione have shown many useful impacts in humans and mammals, including anti-trypanosomal, anti-neoplastic, and anti-bacterial activities. Other activities like inhibition of intestinal motility, lipid peroxidation, and human telomerase inhibitory have been reported as well. Anthracycline antibiotics, which are considered as the main materials for the treatment of various kinds of cancer, are also 9,10-anthracenedione (Dzierzbicka and Kolodziejczyk, 2005). Various synthetic anthraquinones like doxorubicin are among the most effective factors for therapy of different kinds of cancer like ovarian, uterine, lung, and breast cancer, soft tissue sarcomas in clinical practice, and Hodgkin's disease. Nevertheless, they have shown some disadvantages such as the absence of selectivity and thus toxicity to healthy cells (Granados-Principal et al., 2010). Moreover, the anthraquinones from the *Rubia* genus of the Rubiaceae family indicate significant physiological impacts. These substances have demonstrated renal calculus eliminative, hepatoprotective, anti-inflammatory, immunomodulatory, anti-thrombotic, calcium channel antagonistic impacts, and DNA binding activities in humans and animals (Singh and Chauhan, 2004).

According to the available reference, rubiadin is not a skin and eye irritant and no sensitizing effects known. Additional toxicological information is not included in the list of carcinogenic agents according to the documentation IARC (International Agency for Research on Cancer), NTP (National Toxicology Program), OSHA-Ca (Occupational Safety & Health Administration). Due to the use of medicinal plants contain Rubiadin in traditional medical practices for inflammation and pain treatment and relief, current research aims at evaluating the Rubiadin anti-inflammatory impact and studying its pharmacological activity as an anti-inflammatory agent. Using different standard experimental test models, the anti-inflammatory characteristics of Rubiadin were explored in this work. To our knowledge, it is the first study dealing with the ethnopharmacological characteristics of Rubiadin in an inclusive way.

2. Materials and Methods

2.1. Chemical

Rubiadin, Indomethacin, Diclofenac, and Carrageenan were prepared by Sigma-Aldrich, Darou-Pakhsh Pharmaceutical Company (Iran) and Sigma (USA). TNF-α and IL1 β kits were purchased from eBioscience, USA.

2.2. Animals

Male Wistar albino rats were used as research samples. The weight of subjects was within the range of 150 to 200 g. There were 6 animals that were kept in a standard cage for 12 h in a light/dark cycle at 22 ± 2°C. The animals were supplied with water and food *ad libitum*. The research tests were performed based on the instructions provided for the laboratory animals given by the Faculty of Pharmacy. The experiments were confirmed by the Research and Ethics Committee of Tehran Medical Sciences, Islamic Azad University, Tehran, Iran (IAUPS) (IR.IAU.PS.REC.1397.270). Animals authorized with anesthetic overdose of a combination of Ketamine 300 mg/kg and xylazine 30 mg/kg IP.

2.3. Carrageenan-induced paw edema test

The acute anti-inflammatory effect was investigated based on inhibition of paw edema stimulated by administration of 0.1 mL carrageenan 2% into the rats' hind paw (Winter et al., 1962; Jahandar et al., 2018). Male rats were placed in 4 groups, each group consisting of 6 rats. The groups received mefenamic acid (30 mg/kg, i.p.), Rubiadin (0.3 and 0.5 mg/kg, i.p.), and the control (normal saline: 10 mL/kg, i.p.) separately 30 min prior to carrageenan injection. In the next step, 0.5, 1, 2, 3 and 4 h following the prescription of carrageenan, the paw volume was assessed by a Plethysmometer (model PM 4500, Borj Sanat Company, Iran). The anti-inflammatory activity was indicated as the inhibition percent of the edema compared to the control group. The inhibition percentage of the edema was measured using this equation: edema inhibition percentage = 100 (1-Vt/Vc), where Vc and Vt indicate the edema volume in the control group and test group, respectively.

Enzyme-linked ELISA was used for measuring the TNF-α level in the paw tissues. The gathered samples were homogenized in calcium chloride (1.5%) 4 h after the carrageenan challenge. About 1 mL $CaCl_2$ per 50 mg tissue was used to prepare the supernatants. The supernatants were kept at -70°C. Then, the TNF-α level was measured according to kit brochures provided by the manufacturer (Rat TNF alpha ELISA Kit, eBioscience, USA).

2.4. Histopathological examination

Rats were euthanized after 4 h of carrageenan treatment and their paw tissues were removed. Next, a 10% formaldehyde solution was used for fixing them. The samples were fixated in paraffin wax, and then cut in 5 µm sections and died with eosin and hematoxylin. An area was chosen as the representative area for qualitative light microscopic analysis of the inflammatory tissue response with a ×10 magnification.

2.5. Granuloma induced by cotton pellet

The chronic anti-inflammatory activity of Rubiadin was evaluated based on cotton pellet-induced granuloma in accordance with the approach proposed by Winter and Porter (1957). Six rats were placed in each group. Pellets with a weight of almost 60 mg were made with 5 mm of dental cotton tampons. An autoclave was used for sterilizing the pellets. The pellets were sterilized at 120°C for 30 min under 15 lb pressures. It must be noted that the rats were anesthetized during the experiments. Using a single needle incision, pellets were embedded subcutaneously in the axilla region of the rats. Treatment on the groups was carried out daily lasting for seven days. The rats were treated with indomethacin (5 mg/kg, i.p.), Rubiadin (0.3 and 0.5 mg/kg,

Table 1. Rubiadin activity on the inflammation caused by carrageenan in rat.

Groups	%Paw edema inhibition in various time intervals				
	0.5 hr	1 hr	2 hr	3 hr	4 hr
Mefenamic acid (30 **mg/kg**)	55	42.5	48	43	44
Rubiadin (0.3 mg/kg)	24	17.5	8.3	21	13
Rubiadin (0.5 mg/kg)	48	40	45	61	54

i.p.), and vehicle (normal saline: 10 ml/kg). Next, they were anesthetized again on day 8. The cotton pellets, as well as the granuloma tissues, were separated through surgery, and the extraneous tissues were removed. The weight of the wet pellets was calculated and then they were dried in an incubator for 24 h at 60°C so that a constant weight was achieved. Afterward, the weight of the dried pellets was calculated again. The quantity of the exudates (mg) was measured by subtracting the pellet's constant dry weight from the pellet's immediate wet weight. Granuloma's dry weight was measured following deducing the cotton pellet weight from the pellet's constant dry weight and taken as the amount of granuloma tissue formation. Finally, the inhibition percentage of granuloma tissue formation and exudates were estimated.

IL1-β level in this model evaluated by enzyme-linked ELISA, 8 days after cotton pellet-induced granuloma, the collected blood samples were measured via the heart and IL-1β in serum according to kit brochures provided by the manufacturer (Rat IL-1 beta ELISA Kit).

2.6. Statistical analysis

One-way ANOVA analysis was used for comparing the groups. Then, the post hoc Tukey's test was run. $P < 0.05$ was regarded as a significant difference in means. GraphPad Prism 8 statistical software was utilized for data analysis.

3. Results

3.1. Carrageenan-induced inflammation test

Figure 1 shows the Rubiadin effect (0.3 and 0.5 mg/kg) in rat paw edema caused by carrageenan.

Mefenamic acid (30 mg/kg) and Rubiadin (0.5 mg/kg) caused a significant inhibition ($P < 0.05$) of the carrageenan paw edema formation that was specified at the third hour of the test (peak of edema formation) by 61 and 43% (Table 1).

3.2. Effect of Rubiadin on carrageenan-induced TNFα level in paw tissue

The role of TNFα was considered for evaluating the potential mechanism underlying the anti-inflammatory effects of Rubiadin. About 4 h after the inflammation induced by carrageenan in each treatment group, animals were scarified and then the plantar tissue samples were evaluated based on TNFα. Figure 2 shows the plantar tissue levels of TNF-alpha in animal groups. Tissue levels of TNF-alpha were significantly ($P<0.001$) lower in the mefenamic acid group compared to the control group. Rubiadin (0.5 mg/kg) could cause a significant decrease ($P<0.05$) in TNF levels in the rat tissue after 4 h and the inflammation induced by carrageenan.

Table 2. Comparison of Rubiadin activity on inhibition percent of inflammation induced by a cotton pellet.

Groups	% Inhibition Granuloma	% Inhibition Transudate
Indomethacin (5 mg/kg)	39.37	26.51
Rubiadin (0.3 mg/kg)	34.71	19.76
Rubiadin (0.5 mg/kg)	46.12	38.13

3.3. Histopathological studies

Paw tissue of vehicle-treated rats showed an acute inflammation with extensive damage in the epidermis layer (Figure 3A). Treatments of rats with mefenamic acid (Figure 3B), Rubiadin 0.3mg/kg (Figure 3C), and 0.5mg/kg (Figure 3D) exhibited a significant decrease in the edema and tissue damage.

3.4. Cotton pellet-induced inflammation test

Transudate and granuloma were significantly decreased in the Rubiadin-treated (0.3 and 0.5 mg/kg, i.p.) group in the cotton pellet-induced inflammation test (Figure 4).

The inflammation reduction with Rubiadin (0.5 mg/kg) was 46% compared to the standard drug indomethacin (40%) (Table 2).

3.5. Effect of Rubiadin on serum IL-1β level

The IL-1β level involvement was considered for evaluating the potential mechanism underlying the anti-inflammatory impacts of Rubiadin.

Figure 5 shows the IL-1β serum levels in animal groups. Rubiadin (0.5 mg/kg) could cause significant reduction ($P < 0.05$) in serum IL-1β levels in the cotton pellet-induced granuloma test in the rat. The levels of IL-1β were significantly lower ($p < 0.01$) in the indomethacin group compared to the control group.

4. Discussion

Inflammation, in acute type and especially chronic type, cause numerous clinical problems in patients suffering it. Despite the new and varied methods of treating

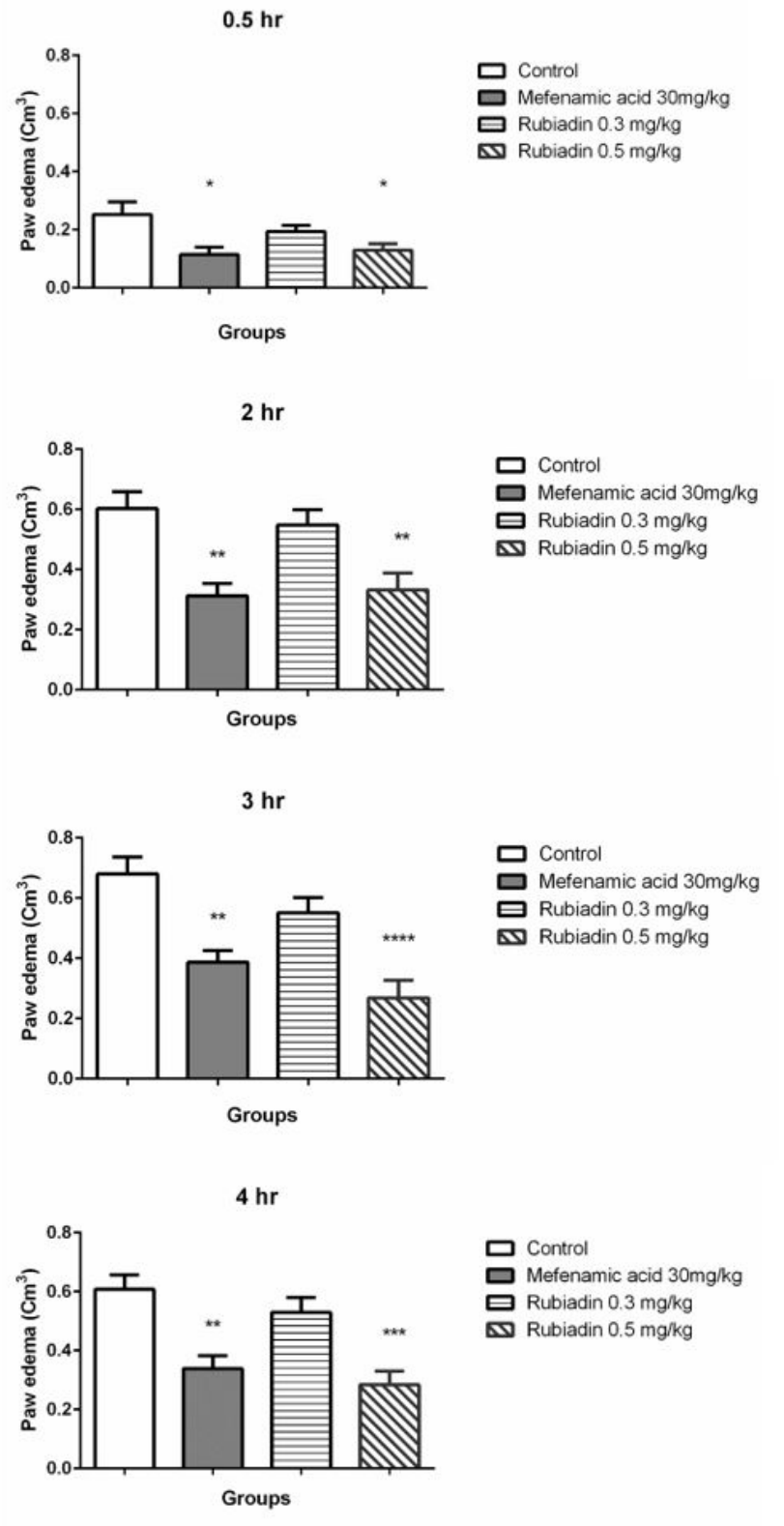


Figure 1. Effect of Rubiadin on the inflammation induced by Carrageenan in rat. Each value represents the mean ± SEM of 6 mice. * P<0.05, ** P<0.01, *** P<0.001, and **** P<0.0001 compared to the control group using one-way ANOVA followd by Turkey's multiple comparison test.

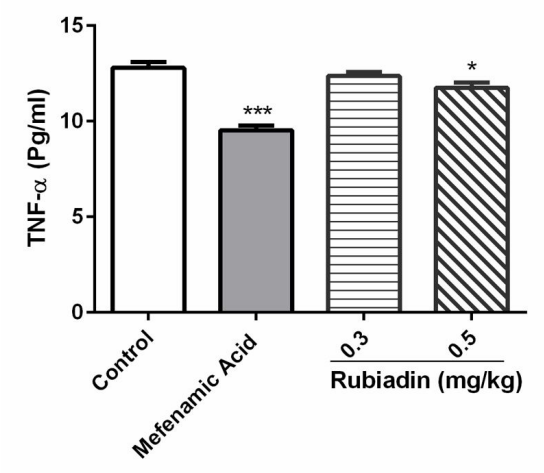


Figure 2. Effect of Rubiadin on carrageenan-induced TNFα in paw tissue at 4 h in rats. The groups received mefenamic acid (30 mg/kg, i.p.), Rubiadin (0.3 and 0.5 mg/kg, i.p.), and the control (normal saline: 10 mL/kg, i.p.) separately 30 min prior to carrageenan injection. Each value represents the mean ± SEM of 6 mice. * P<0.05 and *** P<0.001 compared to the control group using one-way ANOVA followed by Turkey's multiple comparison test.

inflammation, there is a wide research field in this area. These studies are being carried out or underway to create new, better, more effective treatments, and fewer side effects. The present research is the first report that described the anti-inflammatory activity of Rubiadin on chronic and acute inflammation experiment models in rodents.

Our results indicate that Rubiadin (0.5 mg/kg) causes an anti-inflammatory impact in carrageenan-induced inflammation. The inhibitory activity observed in Rubiadin in a 4-h period in carrageenan-induced paw inflammation is more efficient than standard drug (mefenamic acid). The carrageenan test is often used as an acute inflammation model in the experimental animals and shows a high sensitivity to non-steroidal anti-inflammatory drugs. The mechanism of inducing the inflammatory response by carrageenan is a complicated mechanism that requires liberating different mediators of acute inflammation as well as increasing vascular permeability. The rise in the TNF-α, IL-1β, and IL-6 levels, and cytokine has been reported in the inflamed paw (Annamalai and Thangam, 2017). It has been evidenced that TNF-α has a significant role in the formation of edema. It critically plays a role in mechanical allodynia and neutrophil migration after the administration of carrageenan (Rocha et al., 2006).

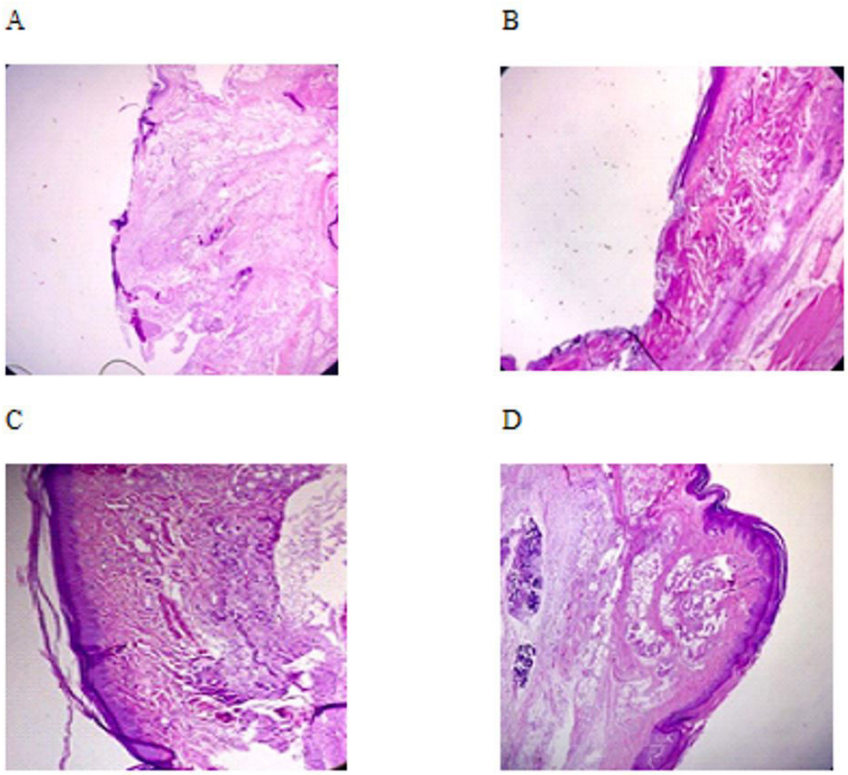
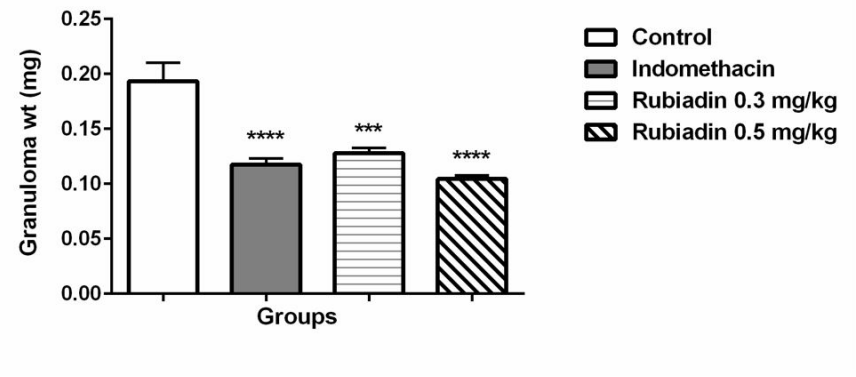


Figure 3. Effect of Rubiadin on histopathological damages induced by carrageenan in paw plantar of the rats. Histopathologic examination of paw tissue of rats treated with Rubiadin, 4 h after injection of carrageenan. (A): Carrageenan-injected paw tissue in the vehicle group. Vasodilatations with edema were observed. (B,C, and D): Carrageenan-injected paw of rats treated with mefenamic acid (30 mg/kg, intraperitoneal) and Rubiadin (0.3 and 0.5 mg/kg) respectively. The edema reduced. Sections were stained with H and E, X10.



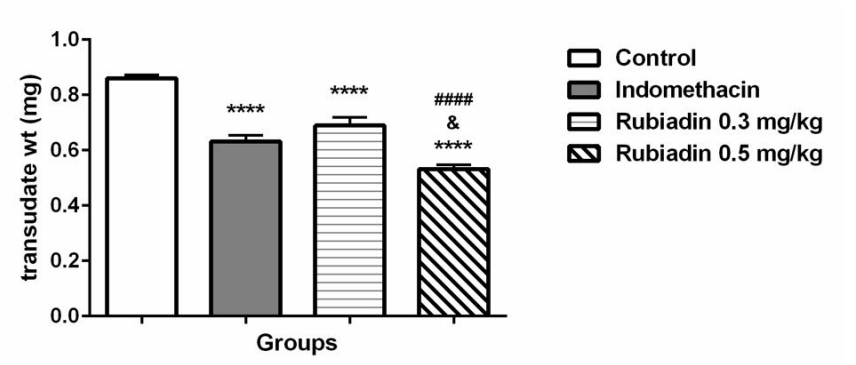


Figure 4. Effect of Rubiadin on the cotton pellet-induced granuloma and transuda in rats
Each value represents the mean ± SEM of 6 mice. *** p<0.001 and**** p<0.0001 compared to the control group, & p<0.05compared to the Indomethacin group, ####p<0.0001 compared to the Rubiadin 0.5 mg/kg using one way ANOVA followed by Tukey's multiple comparison test.

In addition, it was reported that the paw edema induced by carrageenan in rats causes a significant rise in serum TNF-α and NO levels (Kataoka et al., 2012).

Also, the cotton-pellet granuloma test is a commonly used practice for evaluating chronic anti-inflammatory materials (Ismail et al., 1997; Swingle and Shideman, 1972; Cheng et al., 2016; Dzoyem et al., 2017).

There is a correlation between the pellet's dry weight and the amount of granulomatous tissue and a correlation between the wet weight of the pellets and Transudate. Chronic inflammation is observed when the proliferating cells are developed. Proliferating cells might be in granuloma form or in spread form. Rubiadin (0.5 mg/kg) showed a significant anti-inflammatory impact on granuloma induced by cotton pellet. Hence, it is efficient in chronic inflammatory situations and demonstrates potential effectiveness in inhibition of increasing fibroblasts and synthesis of collagen and mucopolysaccharides when granuloma tissue is formed.

Cytokine production is also increased during the inflammatory event. In this regard, Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) critically plays a role

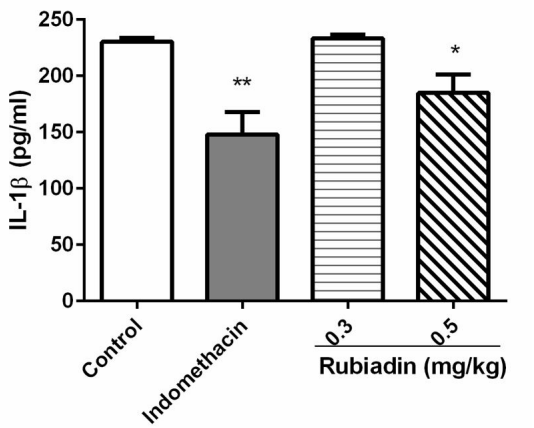


Figure 5. Effect of Rubiadin on cotton pellet-induced IL-1β level in serum in rats. Each The groups received indomethacin (5 mg/kg, i.p.), Rubiadin (0.3 and 0.5 mg/kg, i.p.), and the control (normal saline: 10 mL/kg, i.p.) for 8 days in rats. Value represents the mean ± SEM of 6 mice. * P<0.05 and ** p<0.01 compared to the control group using one-way ANOVA followd by Turkey's multiple comparison test.

in inflammatory profile maintenance (Holtmann and Neurath, 2004). Thus, the IL-1β and TNF-α release in cotton pellet granuloma and carrageenan tests is considered as a reliable model for assessing the anti-inflammatory impact. It was observed that Rubiadin (0.5 mg/kg) is effective in TNF-α and IL-1β level reduction in carrageenan-induced rat paw edema and cotton-pellet granuloma tests. This finding supports the efficacy of Rubiadin in inflammation control through regulating inflammatory reactions. It also shows that Rubiadin is likely involved in arachidonic acid metabolites, producing an edema dependency on neutrophils mobilization. In addition, it is possible that the anti-inflammatory impact of Rubiadin is linked to the scavenging of acute-phase inflammatory mediators, thus reducing edema development. It is probable that Rubiadin plays a role in the regulation of free radical reactions, which unquestionably is involved in inflammatory processes.

Considering previous studies on the pharmacological activity of some species of Rubiaceae genus, they exhibit anti-inflammatory, immunomodulatory, renal calculus eliminative, hepatoprotective, antithrombotic, and calcium channel antagonistic effects and DNA binding activities in humans and animals (Singh and Chauhan, 2004). Previously, anthraquinones were reported to have anesthetic and anti-inflammatory capability in different in vitro and in vivo animal models of inflammation. Anthraquinones have shown the anti-inflammatory impact through inhibiting COX-2 protein (Choi et al., 2013) and shown antioxidant activity (Yen et al., 2000). Additionally, it has been shown that phytochemicals with antioxidant potentials have anti-inflammatory and analgesic impacts regarding plant extracts (Sur et al., 2001).

The toxicant-protective effect of Rubiadin was reported in some organs. Rubiadin as the main constituent of *Rubia cordifolia* Linn. possesses a strong hepatoprotective effect against hepatic damage induced by carbon tetrachloride in rats (Rao et al., 2006). Moreover, according to the reports, the root extract of *R. cordifolia* Linn. has the nephroprotective impact in the ethylene glycol induced urolithiatic model (Divakar et al., 2010). According to the findings of the current research, there are significant anti-inflammatory effects for Rubiadin that reduce all parameters measured in chronic and acute inflammation. Considering the regulation of leukocyte migration by TNF-α and IL-1β, it can be concluded that the leukocyte reduction might be the direct effect of Rubiadin and the indirect effect caused by the reduction in the levels of the cytokines. Overall, the results demonstrate similar actions of Rubiadin and immunomodulators in the reduction of inflammatory mediator production and cell migration. Findings are consistent with some other findings, indicating the inhibitory effect of anthraquinones against cytokine production (Hu et al., 2014; Lu et al., 2013). For example, emodin inhibited IL-6 and TNF-α in mouse bone marrow-derived mast cells (BMMCs) induced by phorbol 12-myristate 13-acetate (PMA) plus the calcium ionophore A23187 (Lu et al., 2013).

In conclusion, Rubiadin effectively and significantly decreased granuloma induced by cotton pellet and paw inflammation induced by carrageenan, implying its impact in the proliferative stage of the inflammation and the acute phase. Rubiadin is introduced as a new drug for the control of inflammation for further research.

The findings of this study show that Rubiadin is biologically active and has considerable activity against chronic and acute inflammations, justifying the use of plants belonging to the *Rubia* genus in relieving inflammation.

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