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Research Article
Calycosin Ameliorates Inflammatory Paw Edema in Mice via Inhibiting NF-κB Activation

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Abstract

Background and Objective: Calycosin is recognized to exhibit a variety of pharmacological properties. This study investigated the anti-inflammatory effects and mechanisms of calycosin. **Materials and Methods:** Mice were randomly separated into six groups: Control, carrageenan (Car), calycosin (12.5, 25 and 50 mg kg^{-1}) and indomethacin (10 mg kg^{-1}). All groups were treated orally once per day for a week. One hour after the final treatment, paw edema was induced by using carrageenan in all groups except for the control group. The volumes of the paws were recorded 1, 3 and 5 h after carrageenan treatment and paw histopathological changes were assessed. Myeloperoxidase (MPO) activity and IL-1β and TNF-α levels were analyzed by using ELISA. The expression of p-IκBα in the cytoplasm and NF-κB p65 in the nucleus were quantified using Western blotting. **Results:** Calycosin (25 and 50 mg kg^{-1}) significantly decreased paw edema and had protective effects on paw histopathological changes caused by carrageenan in mice. Further investigation demonstrated that calycosin (25 and 50 mg kg^{-1}) also decreased MPO activity, IL-1β and TNF-α levels, expression of p-IκBα in the cytoplasm and NF-κB p65 in the nucleus. **Conclusion:** Collectively, these data indicated that calycosin displayed anti-inflammatory bioactivity through down-regulation of NF-κB activation.

Key words: Calycosin, carrageenan, paw edema, anti-inflammation, bioactivity, NF-κB activation, myeloperoxidase activity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Inflammation is considered to be a pathophysiological feedback mechanism from the body towards numerous stimuli, including burns, microbial infections, allergens and mechanical injuries[1]. Although such a reaction is defensive, inflammatory mediators and cytokines involved in the inflammatory cascade can often lead to pathological reactions including organ dysfunction and death[2]. Macrophages play an important role during the development of an inflammatory response. Activated macrophages produce inflammatory mediators and cytokines that may cause damage[3,4]. Therefore, compounds that control these factors can have anti-inflammatory properties. Non-steroidal anti-inflammatory drugs (NSAIDs) are considered to be the most common treatment for inflammatory diseases. The primary pharmacological effect of NSAIDs is prevention of prostaglandin synthesis by inhibition of the enzymatic function of COX. However, the applications of NSAIDs as anti-inflammatory drugs are limited by their adverse effects, such as gastric injury and ulceration, nephrotoxicity and bronchospasm[5]. Therefore, investigation of safer and less toxic drugs as alternatives to NSAIDs is urgently required.

Flavonoids are small molecules that exist in vegetables, fruit and medicinal plants. Previous studies have demonstrated that flavonoid compounds are bioactive in a variety of ways, including exhibiting anti-inflammatory[6], antibacterial[7], antioxidant[8] and anticancer[9,10] properties. Depending on their structure, flavonoid compounds can be categorized as flavonoids, isoflavones, chalcones, anthocyanins and flavanones, etc. Calycosin is a type of isoflavone and represents the active ingredient extracted from the traditional herb *Radix astragali*. Like many other flavonoid compounds, it has displayed strong anti-inflammatory activity in previous studies[11]. It was shown to inhibit the production of pro-inflammatory cytokines in LPS-induced bone marrow-derived dendritic cells and rheumatoid arthritis synovial fibroblasts *in vitro*[12,13]. Additionally, calycosin exhibited an anti-inflammatory effect by suppressing the expression of NO in LPS-induced RAW 264.7 cells[14]. Calycosin was shown to attenuate cerulein-induced acute pancreatitis *in vivo* through the MAPK and NF-κB signaling pathways in mice[15]. Calycosin can also ameliorate DSS-induced acute colitis via regulation of the NF-κB and JNK signaling pathways[16]. In addition, recent reports have found that calycosin can attenuate diabetes-induced renal inflammation through inhibition of NF-κB activation both *in vitro* and *in vivo*[17]. However, the ability for calycosin to attenuate carrageenan-induced paw edema remains unexplored. This study was aimed to investigate the anti-inflammatory properties of calycosin, the anti-inflammatory effects and mechanism of calycosin using an animal model of carrageenan-induced paw edema.

MATERIALS AND METHODS

Chemicals and materials: This research was conducted from March 6, 2018-October 10, 2018 at the Key Laboratory of Chemical Drugs, Shandong Academy of Pharmaceutical Sciences (Jinan, Shandong, China). Calycosin (purity>98%) was kindly provided by Xian Qinghe Biological Science and Technology Co., Ltd. (Xian, China). Indomethacin was purchased from Xian Bohua Pharmaceutical Co., Ltd. (Xian, China). Carrageenan was provided by Shanghai Hongshun Technology Co., Ltd. (Shanghai, China). The MPO, IL-1β and TNF-α kits were purchased from Kejing Bio-Tech (Nanjing, China). The NF-κB p65 and p-IκBα antibodies were bought from Abcam (Cambridge, MA, USA).

Animals: Sixty male C57BL/6J mice (20-22 g) were obtained from Beijing Huafukang Animal Facility (Beijing, China). All mice were housed in standard conditions of ambient temperature (20±2°C) and humidity (50%-70%) by using a 12 h light/dark cycle. Mice had free access to food and water were acclimated to the conditions for 7 days prior to experimentation. This study was carried out in compliance with the regulations approved by the Animal Ethics Committee of Shandong Academy of Pharmaceutical Sciences (Serial number: IACUC-2018-009).

Experimental procedure: Animals were randomly allocated into 6 groups: Control, carrageenan, indomethacin (10 mg kg^{-1}) and calycosin (12.5, 25 and 50 mg kg^{-1}) groups of 10 mice each. All groups were treated orally once per day for a week in accordance with their grouping, with mice in the control and carrageenan groups treated with 1% carboxymethylcellulose sodium (10 ml kg^{-1}). One hour after the final treatment, paw edema was induced by subplantar injection of 30 μL 1% carrageenan in normal saline into the right-hand paw, except for the control group. Paw volumes were measured by using a digital micrometer prior to and 1, 3 and 5 h after carrageenan injection. The percentage increase in paw volume was calculated by using the equation[18]:

$$\text{Increase } (\%) = \frac{Vc - Vt}{Vt} \times 100\%$$

where, Vc and Vt were pre and post-injection mouse paw volumes, respectively.

Measurement of biochemical parameters: The MPO activity, IL-1β and TNF-α levels in paw tissue samples were measured

by ELISA according to methods described previously[19]. Animals were sacrificed 5 h after injection of carrageenan then paw samples were obtained from the carrageenan-injected and control groups. The tissue was then prepared for quantification of IL-1β and TNF-α levels and MPO activity by ELISA.

Histopathological analysis: Paw tissue samples were collected and fixed in 10% formalin for 3 days, then decalcified in 10% nitric acid for 24 h. Tissue samples were dehydrated, fixed in paraffin and cut into 5 μm sections. Hematoxylin and eosin (H and E) staining was performed to evaluate pathological changes.

Western blot analysis: Protein was extracted from the cytoplasms and nuclei of cells from 100 mg of paw tissue using an ExKine Protein Extraction Kit (AmyJet Scientific, Wuhan, China). Protein concentration was quantified by using a BCA kit (Cwbio, Beijing, China). Nucleoprotein and cytoplasmic proteins were separated using SDS-PAGE then transferred onto nitrocellulose membranes. Membranes were blocked using 5% skim milk and incubated with primary antibodies against p65 and p-IκBα overnight at 4°C. After three washes with TBS-T, membranes were incubated with secondary antibodies for 1 h at room temperature. Finally, membranes were impregnated with ECL detection reagents after washing with TBS-T. Bands were visualized by using an LAS-4000 Image Reader (Fuji Film, Tokyo, Japan). Band intensities were quantified using Image Analysis Software (Rockville, MD, USA).

Statistical analysis: The SPSS (version 19.0) was used to process data, which are represented as Mean±SD. Significance of differences were calculated using one-way ANOVA. The $p<0.05$ was deemed significant.

RESULTS

Effect of calycosinon paw edema: Pre-treatment with calycosin (25, 50 mg kg^{-1}) reduced the percentage increase in paw volume considerably following the injection of carrageenan ($p<0.01$), indicating that calycosin suppressed carrageenan-induced paw edema. Indomethacin (10 mg kg^{-1}) also clearly inhibited paw edema after injection of carrageenan. The effect of calycosin (50 mg kg^{-1}) was a little weaker than indomethacin (Fig. 1).

Effect of calycosinon histopathological changes: The effect of calycosin on paw histopathological changes was investigated as shown in Fig. 2a-f. The results demonstrated

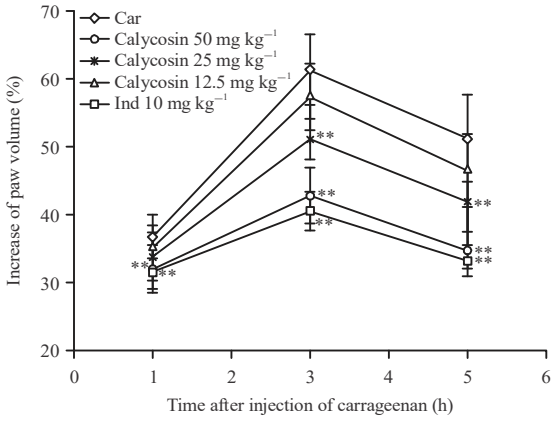

Fig. 1: Effect of calycosin on carrageenan-induced paw edema
**p<0.01 vs. the carrageenan group, Car: Carrageenan, Ind: Indomethacin

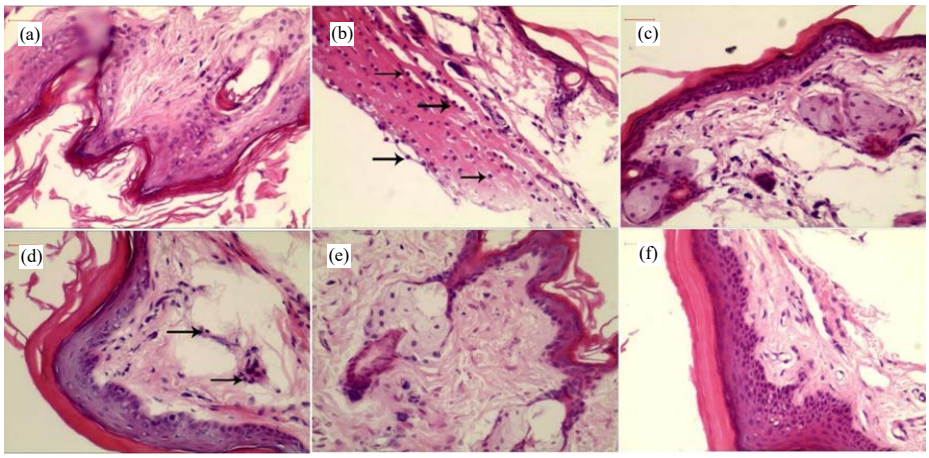

Fig. 2(a-f): Effect of calycosin on paw histological changes (H and E stain, magnification 200×), (a) Control group, (b) Carrageenan group, (c) Indomethacin group, (d-f) Calycosin (12.5, 25 and 50 mg kg^{-1}) groups
Arrows indicate site of inflammatory cell infiltration

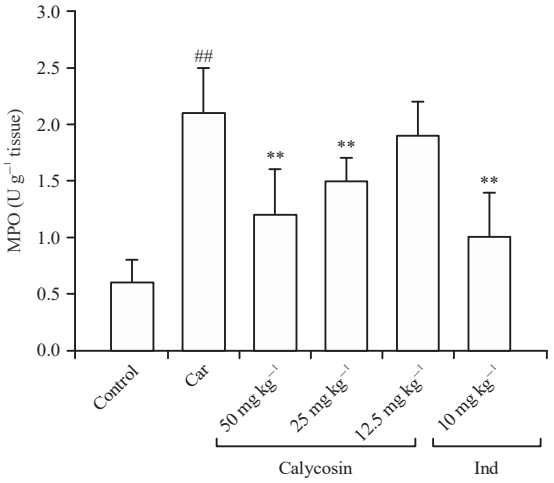

Fig. 3: Effect of calycosin on MPO activity

##p<0.01 vs. the control group, **p<0.01 vs. the carrageenan group, Car: Carrageenan, Ind: Indomethacin

that the paw tissue from the control group presented a healthy tissue structure. Conversely, significant paw histopathological changes including the infiltration of neutrophils and lymphocytes were observed in the Car group (Fig. 2b). Inflammatory cells could be clearly observed in the calycosin (12.5 mg kg^{-1}) group (Fig. 2d). However, both indomethacin and calycosin (25, 50 mg kg^{-1}) significantly reduced inflammatory cell infiltration indicating that they induced a protective effect on carrageenan-induced paw histopathological changes (Fig. 2c, e, f).

Effect of calycosin on MPO activity: The MPO activity was significantly upregulated by carrageenan induction. Compared with the Car group, calycosin (25 and 50 mg kg^{-1}) reduced MPO activity ($p<0.01$). Indomethacin (10 mg kg^{-1}) also clearly suppressed MPO activity ($p<0.01$). Calycosin at 50 mg kg^{-1} exhibited effects that were comparable to those of indomethacin (Fig. 3).

Effect of calycosin on IL-1β and TNF-α levels: Both IL-1β and TNF-α levels were significantly greater in the Car group. Compared with the Car group, calycosin (25, 50 mg kg^{-1}) caused a notable decrease in the levels of IL-1β and TNF-α. Indomethacin (10 mg kg^{-1}) also induced a considerable decrease in IL-1β and TNF-α levels. Calycosin at 50 mg kg^{-1} exhibited effects comparable to those caused by indomethacin (Fig. 4).

Effect of calycosin on NF-κB p65 and p-IκBα expression: The expression of NF-κB p65 nucleoprotein was markedly

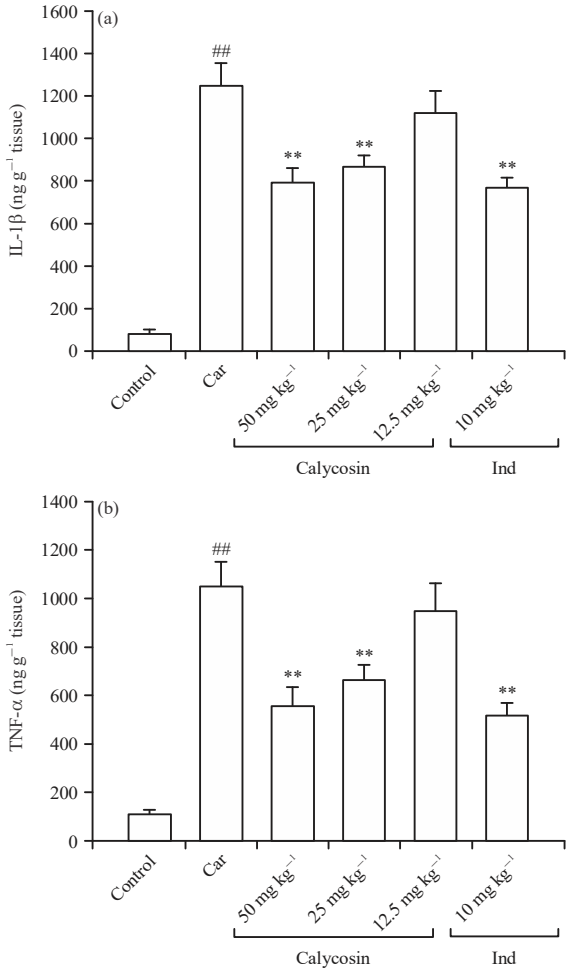

Fig. 4(a-b): Effect of calycosin on IL-1β and TNF-α levels, (a) IL-1β and (b) TNF-α levels were measured by ELISA

##p<0.01 vs. control group, **p<0.01 vs. carrageenan group, Car: Carrageenan, Ind: Indomethacin

increased in the Car group. In comparison, calycosin (25, 50 mg kg^{-1}) and indomethacin significantly reduced this expression ($p<0.01$). The expression of cytoplasmic p-IκBα was also significantly greater in the Car group. Calycosin (25, 50 mg kg^{-1}) and indomethacin demonstrated considerable inhibition of the expression of p-IκBα in the cytoplasm in comparison to the Car group (Fig. 5).

DISCUSSION

This study investigated the anti-inflammatory effects and the possible mechanisms of action of calycosin using a carrageenan-induced mouse paw edema model. The results

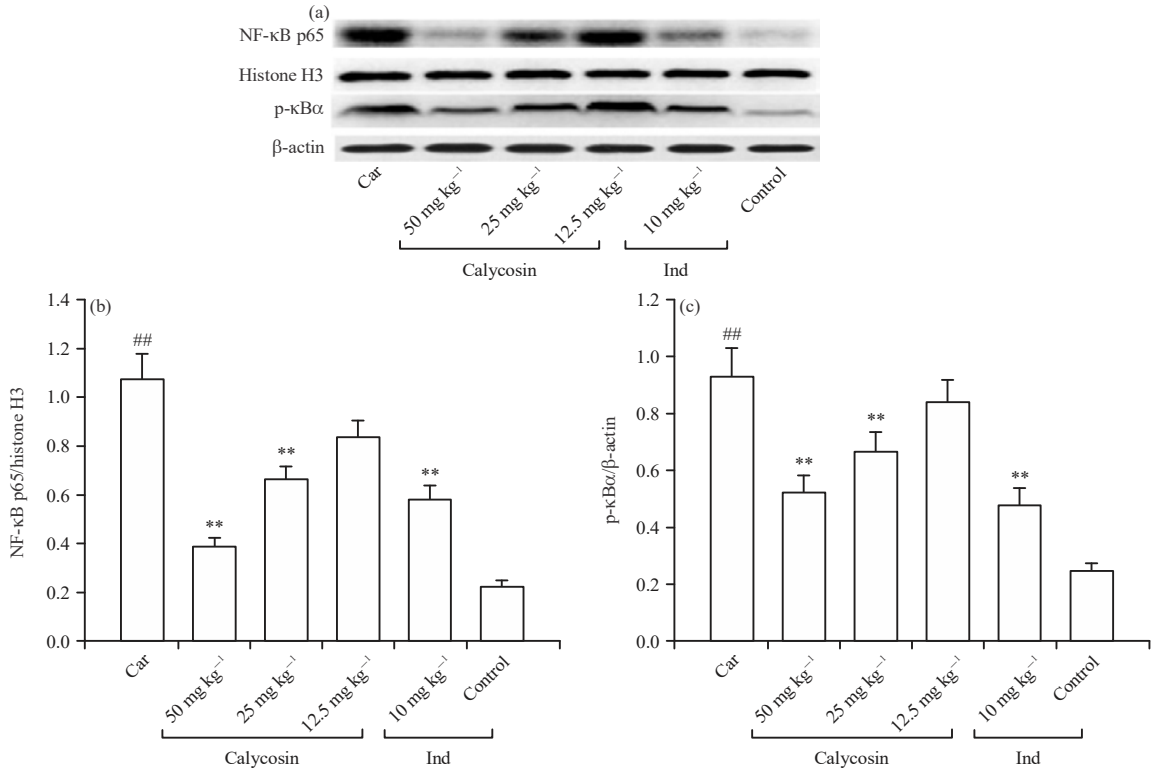

Fig. 5(a-c): Effect of calycosin on NF-κB p65 and p-IκBα expression, (a) Western blot analysis, (b) Expression of NF-κB p65 normalized against Histone H3 and (c) expression of p-IκBα normalized against β-actin

##p<0.01 vs. the control group, **p<0.01 vs. the carrageenan group, Car: Carrageenan, Ind: Indomethacin

demonstrated that calycosin significantly reduced paw edema and inhibited paw histopathological changes induced by carrageenan. In addition, calycosin significantly decreased MPO activity, IL-1β and TNF-α levels, the expression of p-IκBα in the cytoplasm and NF-κB p65 in the nucleus in the paw tissues of mice.

The carrageenan-induced inflammatory paw edema animal model is one which is highly reproducible that has been widely used to evaluate anti-inflammatory agents[18]. The model is believed to be effective in the study of inflammation-related mediators and cytokines produced during an inflammatory response[20,21]. The inflammatory model is considered to be a biphasic event. The final phase, post-1 h is principally caused by the infiltration of neutrophils that secrete pro-inflammatory mediators and cytokines after the 0-1 h early phase[22]. This study demonstrated that calycosin effectively reduced paw edema induced by carrageenan suggested that calycosin exhibits an anti-inflammatory action. In this regard, consistent with the results described above, previous findings have demonstrated that calycosin can exert a fine anti-inflammatory effect by inhibiting NO production in macrophages[14]. In addition, previous studies have reported that calycosin was also found to attenuate cerulein-induced acute pancreatitis and DSS-induced acute colitis[15,16].

The anti-inflammatory effect of calycosin might be related to the reduction in neutrophil infiltration and production of pro-inflammatory cytokines. Leukocytes, especially neutrophils (PMN), act in a pivotal role in the inflammatory response. Myeloperoxidase (MPO) is an enzyme that catalyzes the reduction of peroxides, the most reliable index for evaluating aggregation and infiltration of PMN in tissue. Therefore, the degree of MPO activity in local tissues can reflect the degree of PMN aggregation and infiltration, qualitatively[23]. The results demonstrated that 25 and 50 mg kg^{-1} of calycosin significantly decreased MPO activity, which suggested that calycosin could reduce neutrophil infiltration at the site of an inflammatory response. The results above mirrored previous studies[15,16]. Pro-inflammatory cytokines are increased during an inflammatory response and play key roles in the development of various inflammatory diseases[24]. The TNF-α can activate macrophages and T cells leading to an immune response. The TNF-α is also able to stimulate the release of other inflammatory cytokines.

Similarly, IL-1β is regarded as the most important inflammatory cytokine produced by macrophages. The release of IL-1β can cause cell death or tissue damage during an inflammatory response[25]. To investigate the effects of calycosin on inflammatory cytokines, its anti-inflammatory effects were evaluated using a paw edema model. The results demonstrated that 25 and 50 mg kg^{-1} calycosin clearly decreased the levels of IL-1β and TNF-α in a dose dependent manner. In this regard, previous studies have demonstrated that calycosin was able to suppress the expression of pro-inflammatory cytokines in LPS-induced bone marrow-derived dendritic cells and rheumatoid arthritis synovial fibroblasts[12,13].

Many studies have revealed that the control of inflammation is closely related to the nuclear transcription factor kappa B(NF-κB) signalling pathways[26,27]. The NF-κB consists of five components: RelA (p65), RelB, p50, p52 and cRel. The main form of NF-κB comprises homodimers and heterodimers of p50 and p65 subunits. Previous studies have shown that activated NF-κB triggers transcriptional upregulation of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α. Therefore, the regulation of NF-κB signaling pathway activation should reduce the severity of inflammatory damage. In a resting state, the inhibitory subunit of the IκB protein can tightly bind to NF-κB dimers, which further capture NF-κB in the cytoplasm. IκBα can be phosphorylated by the IκB kinase (IKK) complex. Phosphorylated IκB proteins are degraded by proteasomes that trigger the ingress of NF-κB to the nucleus. Once NF-κB enters, the transcription of various inflammatory cytokines is induced to generate an inflammatory response in local tissues[28-30]. In this study, the expression of p-IκBα and NF-κB p65 in the cytoplasm/nucleus were closely monitored and studied. Results demonstrated that calycosin at 25 and 50 mg kg^{-1} significantly inhibited the expression of p-IκBα in the cytoplasm and NF-κB in the nucleus in comparison with the Car group. This suggested that the phosphorylation of IκBα in the cytoplasm is decreased and the nuclear translocation process of NF-κB is suppressed, preventing NF-κB entering the nucleus to induce the production of pro-inflammatory factors. A number of previous studies supported the observation that calycosin can inhibit the activation of NF-κB[17,31,32]. Although, the present research demonstrated that calycosin exhibits strong anti-inflammatory activity, demonstrating prodigious potential as a therapeutic drug, the anti-inflammatory effects and mechanism of action of calycosin should be further studied in other inflammatory models.

CONCLUSION

The findings of this study demonstrated that calycosin possessed anti-inflammatory activity with a mechanism that was possibly mediated by the inhibition of NF-κB activation. These results support the pharmacological basis of calycosin for the treatment of various inflammatory illnesses.

SIGNIFICANCE STATEMENT

This study has discovered the anti-inflammatory effect of calycosin in a carrageenan-induced paw edema animal model that can be beneficial for the treatment of various inflammatory illnesses. This finding may help researchers find more effective anti-inflammatory drugs by structural modification of calycosin.

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