In Vivo Analgesic and Anti-Inflammatory Activities of the ethanolic extract from *Otostegia limbata* Leaves through Classic Models in Mice and Rats

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Abstract: Pain and inflammation have a direct impact on both human and animal health. A natural substance with a number of biological functions is resveratrol *Otostegia limbata*. OL's in vivo analgesic and anti-inflammatory effects are being assessed in the current investigation. The analgesic outcomes demonstrated that OL may greatly reduce the frequency of writhes and raise the pain threshold and time in mice standing on a hot plate. The anti-inflammatory outcomes demonstrated that OL could reduce mouse ear oedema. *Otostegia limbata* dramatically reduced the formation of NO, increased the activity of SOD in serum, and greatly inhibited WBC and pleurisy exudates in an acetic acid-induced pleurisy test. OL was able to decrease the expression of TP, PGE2, NO, and MDA as well as lower the level of MDA and increase T-SOD activity in the carrageenan-induced synovitis test. *Otostegia limbata* (OL) has demonstrated anti-inflammatory and anti-nociceptive properties. Numerous inflammatory diseases depend heavily on the enzymes cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), and cytokines. The goal of this study was to look into the mechanisms underlying the anti-inflammatory effects of OL. The findings demonstrated that intraplantar injection of carrageenan caused the development of peripheral inflammation in a time-dependent manner, increasing levels of tumour necrosis factor (TNF-), interleukin 1 (IL-1), nitric oxide (NO), prostaglandin E2 (PGE2), as well as the expression of the proteins iNOS and COX-2 in the affected paw. However, systemic treatment of *Otostegia limbata* (1–30 mg/kg, i.p.) could lessen edoema in infrared-aimed rat paws in a dose-dependent manner, with an ED50 value of 8.41 (5.26–14.76) mg/kg. It reduced the levels of NO, PGE2, aspartate aminotransferase, and alanine aminotransferase in the serum, suppressed the protein expression of iNOS and COX-2 enzymes, and reduced the production of PGE2, TNF-, and IL-1 in infected paw tissue. We also showed

that OL dramatically reduced the liver's level of malondialdehyde (MDA) five hours after carrageenan injection. Additionally, histological studies showed that indomethacin and EA both markedly reduced the migration of polymorph nuclear leukocytes into the region of inflammation.

Key word: Acetic Acid-Induced Abdominal Writhing Test, Hotplate, Carrageenan, ellagic acid, Inducible nitric oxide synthase, prostaglandin E2, Rat, *Otostegia limbata*.

1. Introduction

Inflammation and pain are involved in almost all human and animal diseases, and they are typically brought on by physical, pharmacological, or biological stressors, alone or in combination [1]. Redness, swelling, heat, discomfort, and malfunction are the usual signs of inflammation. Therefore, inflammation and pain always interact with one another. Analgesics are a class of drugs that can generally reduce pain perception. Traditional analgesics are crucial for treating pain, but when used in clinics, they frequently have negative side effects [2]. Nonsteroidal anti-inflammatory medicines (NSAIDs), like analgesics, are the main treatment for illnesses with a chronic inflammatory response; nonetheless, long-term usage frequently results in significant side effects, such as cardiovascular and gastrointestinal problems that restrict their development [3, 4]. There is an urgent need for new analgesic and anti-inflammatory drug research. Many plants with analgesic properties have been found in recent years, and a variety of herbal formulations are being recommended as analgesics [5]. There are several reports on the anti-inflammatory properties of ingredients from Chinese traditional medicine, such as alkaloids, saponins, flavonoids, terpenoids, volatile oils, coumarin, aldehydes, and ketones, due to the wide spectrum of pharmacological activity with few adverse effects [6, 7]. Redness, swelling, pain, and heat are common manifestations of inflammation, which is the body's reaction to microorganisms that have invaded the area. Numerous studies have demonstrated that inflammation has a role in the development of a number of diseases, including ageing, cancer, cardiovascular dysfunction, and other serious and disabling conditions [8, 9, 10]. Free radical overproduction, complicated enzyme activity, and the release of a number of inflammatory and pro-inflammatory mediators are all components of acute inflammation. A well-known acute model of inflammation that is frequently used to screen new anti-inflammatory drugs is the carrageenan-induced paw edoema. A biphasic edoema was brought on by injecting carrageenan into the subplantar surface of rat paws. While the delayed phase (after 1 h) is attributed to

neutrophil infiltration and the continuation of prostaglandin production, the early phase, which is observed around 1 h, is related to the release of histamine, serotonin, bradykinin, and to a lesser extent prostaglandins produced by cyclooxygenase enzymes (COX) [11]. The delayed phase of carrageenan-induced acute inflammation is also accompanied by the release of neutrophil-derived free radicals, nitric oxide (NO), and pro-inflammatory cytokines such tumour necrosis factor (TNF-), and interleukin-1 (IL-1) [12]. Different observations imply that medications that target the COX enzyme, free radical production, and expression of pro-inflammatory proteins (such as inducible nitric oxide synthase; iNOS) may be able to manage inflammatory states more effectively than currently available therapeutic agents [13]. Plant polyphenols are significant components of human diet. Plant polyphenols are crucial to human nutrition. More and more research is showing that dietary phenolic compounds have biological benefits, such as antioxidant, anti-inflammatory, anti-cancer, and anti-atherosclerotic effects [14]. Commercially accessible dietary supplements include extracts from red raspberry leaves or seeds, pomegranates, or several other sources that have high quantities of EA. This substance demonstrates anti-oxidative, anti-carcinogenic, Based on the information provided above, the purpose of this study was to evaluate the mechanisms underlying the anti-inflammatory effects of EA on rat paw edoema brought on by carrageenan [15, 16].

Fig 1: *Otostegia limbata* (Benth.) Boiss

2. Materials and Methods

2.1 Collection and authentication of plants

The plant sample was collected from district (Mansehra) during session June 2021 and Identified by Prof. Dr Ghulam Mujtaba Shah, Chairman, Department of Botany, Hazara University Mansehra KP, Pakistan. After identification the voucher Number (15060) was assigned to the plant species and specimen were deposited in the Herbarium of Hazara University (HUP) for permanent record. The plant materials were washed with tap water, separated and dried in shade for 15 days. These materials were used afterward for phyto- 5 chemical and Pharmacological activities of in-vivo biological screening i-e analgesic and anti-inflammatory activity. The plant material was powdered with the help of electrical grinder. The Whatman filter paper was used after the muslin cloth to filter the extracts. Rotary evaporation will be used at 40°C to remove extra solvent from the filtrate. Until further examination, the extract was kept in a container of amber colour.

2.2 Extraction of plants material

Plant extracts were prepared using microwave extraction technology, according to a previously reported procedure. The microwave's power setting was set at 9000 W. There are three basic stages to this process. In the first step, 750 mL of ethanol and 100 g of each plant powder were added to separate beakers in a 1000 mL container. The microwave was on for 2 minutes, then off for 30 seconds while the beakers were in it. Five times these procedures were carried out. The same process will be used to complete two additional cycles. The muslin cloth will be used to filter the extracts first, and then Whatman filter paper. Rotary evaporation at 40°C was used to remove extra solvent from the filtrate. The extracts will be kept till further analysis in a container of amber colour [17].

2.3 Analgesic Assay

The acetic acid-induced writhing and hot plate tests were used to evaluate the analgesic effects. The xylene-induced mouse ear oedema, the acetic acid-induced rat pleurisy, and the carrageenan-induced rat synovitis tests, respectively, were used to assess the anti-inflammatory effects [18].

Acetic Acid-Induced Abdominal Writhing Test

Five groups of fifty mice were created at random (containing an equal number of both males and females). Indomethacin (2 mg/kg), physiological saline, a high (30 mg/kg), a medium

(10 mg/kg), and a low (3 mg/kg) dose of OL were administered to the mice, respectively. Once daily for four days, the mice received an oral treatment. The mice in each group received an intraperitoneal injection of 0.7% AC (10 mL/kg) on the fourth day following administration for 1 hour. The number of writhes (writhe reactions involving abdominal contractions, stretching of the rear paws, writhing of abdominal muscles, and times of hips up) within the next 20 minutes was then noted [2].

The analgesic percentage was calculated as follows:

$$\textbf{Inhibition rate}\% = \frac{\text{number of writhes (control)} - \text{number of writhes (treated)}}{\text{number of writhes (control)}} \times \mathbf{100}\%$$

Hot Plate Test

The hot plate holding the female mice had a constant temperature of 55.5 0.5 C. Activities like lifting, licking the backs of the animals, and even jumping were thought to be antinociceptive signs. Female mice that had a pain threshold (the amount of time needed for a mouse to first display one of the antinociceptive indications on the hot plate) of less than 5–30 s were eligible for the test. Five groups of fifty qualified mice were assigned at random to receive physiological saline, indomethacin (2 mg/kg), and RSV (30, 10, and 3 mg/kg), respectively. For four days, the mice received an oral administration once each day. The pain threshold of mice in each group was measured at 30, 60, 90, and 120 minutes on the fourth day following oral treatment. If the mouse failed to exhibit an antinociceptive indication within 60 seconds of the test's start, the pain threshold was determined to be 60 seconds, and the animal was removed right away.

Animals

Adult male Wistar rats (160–200 g) were housed at controlled temperature (22 ± 2°C) and allowed free access to food and drinking water. Testing took place in the middle of the light period of a 12 h/12 h light/dark cycle. All animal experiments were carried out in accordance with the All animal experiments were carried out in accordance with the Pharmacological lab, Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, Pakistan. The animals were used only once and then euthanized.

2.4 Anti-Inflammatory Assay

Carrageenan-induced mouse paw edema model was used for induction of inflammation [19].

Animals

Adult male Wistar rats (160–200 g) were housed at controlled temperature (22 ± 2°C) and allowed free access to food and drinking water. Testing took place in the middle of the light period of a 12 h/12 h light/dark cycle. All animal experiments were carried out in accordance with the Pharmacological lab, Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, Pakistan. The animals were used only once and then euthanized.

Carrageenan-induced Rat Paw Edema

Each rat received an intraplantar injection of 100 l of 1% (suspension in saline) lambda carrageenan to cause paw edoema in the right hind paw. 30 minutes prior to the carrageenan injection, OL (1, 3, 10 and 30 mg/kg) and indomethacin (5 mg/kg) were delivered intraperitoneally. Rats' paw volumes were measured using a plethysmometer (Ugo Basile, Varese, Italy) at various time intervals before and after carrageenan administration (1, 2, 3, 4, and 5 h). The volume of the right hind paw before and after carrageenan treatment, respectively, was used to calculate the degree of edoema that was caused. This calculation was done using the formula a b. The carrageenan-induced edoema feet were dissected and kept at 80°C after the mice were euthanized after 5 hours. To measure malondialdehyde, the entire liver tissue was cleaned in ice-cold normal saline and then immediately homogenized in a cold KCl solution (1.5% concentrations) (MDA). In order to measure the amounts of PGE2 and NO, blood was also collected and the serum extracted from the clot was maintained at a temperature of 80°C.

Statistical Analysis

The means and standard errors of the means for 6–8 animals per group are provided for all experimental results. One-way ANOVA was used for the statistical analysis, and Tukey's post-hoc test was then run. Using GraphPad Prism 5.0 software, linear regression was used to analyse data from individual studies to establish the ED50 value for the anti-inflammatory action of EA and its associated 95% confidence intervals (San Diego, CA, USA).

3. RESULTS AND DISCUSSION

Inflammation and pain is now the subject of nearly all human and animal diseases, and existing analgesics and NSAIDs have a number of side effects while being used to treat these conditions [20, 21]. The use of medicinal plants as a source of medications for therapeutic purposes has been a result [22]. In the current work, numerous established animal models were developed to demonstrate the analgesic and anti-inflammatory effects of RSV. Because AC

releases endogenous mediators (prostaglandins) that can stimulate peripheral nociceptor(s) and neurons responsive to NSAIDs, opioids, and other centrally acting medications, it is known that abdominal constriction generated by AC is a nonselective model [23]. The number of writhes in the indomethacin-treated and OL-treated groups was all significantly reduced (P 0.01) when compared to the control group, and the inhibition rates in the high, medium, and low doses of OL-treated groups (22.22%, 50.14%, and 25.35%, respectively) were all lower than those in the indomethacin-treated group (58.12%), as shown in Table 1. According to the results, both indomethacin and OL had strong analgesic effects, with indomethacin displaying higher activity than OL. This outcome was in line with research on OL's analgesic effect using capsaicin and glutamate models [24]. The hot plate test is important for assessing centrally acting analgesics, which are always used to raise mice's pain thresholds for heat [25, 26]. Table 2's findings reveal that, in comparison to the control group, the indomethacin-treated and OL-treated groups all had significantly higher pain thresholds at various time periods (P 0.05), particularly at 60 min (P 0.01). This result demonstrated that OL had potent analgesic effects by showing that OL could clearly extend the time that the mice stood on a hot plate and could enhance the mice's capacity to withstand heat.

Since the OL's significant analgesic function had been established, we also looked at its anti-inflammatory effects. To test for anti-acute inflammatory activity and to assess the anti-inflammatory effects of steroids, xylene-induced oedema in the ears is used [27, 28]. Significant enlargement of the right ear could be noticed in our investigation after the administration of xylene to the both sides. The DXM-treated group had an inhibition rate of 40.87%, while the high, medium, and low dosages of OL-treated groups had inhibition rates of 17.06%, 24.61%, and 30.15%, respectively, when compared to the control group for the degree of ear oedema in the rats. It was claimed that both OL and DXM have anti-inflammatory properties, with DXM outperforming OL. However, this might work better for acute inflammatory disorders than for anti-acute inflammatory disorders. This outcome was pertinent to the earlier discovery that OL might reduce inflammation at comparable doses [29].

Analgesic activity

According to the analgesic results, OL may increase the time and pain threshold of mice standing on hot plates while also significantly lowering the frequency of writhes. The inhibition rates in the high, medium, and low doses of OL-treated groups (22.22%,

50.14%, and 25.35%, respectively) were all lower than those in the indomethacin-treated group (58.12%), as shown in the table below. The number of writhes in the indomethacin-treated and OL-treated groups was all significantly reduced (P 0.01) when compared to the control group (Table 1 and 2).

Table 1. Analgesic effects of OL on writhing test induced by acetic acid. n = 10 ($x \pm s$).

Groups	Dosage	Number of writhes	Inhibition rate%
Control	-	56.40 ± 7.64	-
Indomethacin	2mg	26.50 ± 4.41b	58.12
OL-L	100mg	43.40 ± 3.72b	22.22
OL-M	200mg	30.17 ± 6.13b	48.14
OL-H	300mg	39.57 ± 2.15b	24.35

OL-H, OL-M, and OL-L represent the groups treated with high, medium, and low dose of resveratrol, respectively. In each line, different digits indicate significant statistical difference. b $P < 0.01$ versus blank control.

Table 2. Analgesic effects of OL on hot plate test. n = 10 ($x \pm s$).

Group	Dosage	Pain threshold on different time points			
		30min	60min	90min	120min
Control	-	24.64± 0.3 b	27.81± 0.1 a	29.12± 0.4b	32.61± 0.6a
Indomethacin	2mg	39.34± 0.1 b	48.37± 0.5 a	52.70± 0.1 b	56.67± 0.3 a
OL - L	100mg	45.66± 0.5 b	47.56± 0.3 a	49.26± 0.5 b	52.89±0.1 a
OL- M	200mg	51.09± 0.6 b	53.36± 0.4 a	57.16± 0.3 b	57.60± 0.5 a
OL -H	300mg	45.36± 0.4 b	49.76± 0.6 a	53.04± 0.6 b	55.72± 0.4 a

OL-L, OL-M and OL-H represent the groups treated with high, medium, and low dose of OL respectively. In each line, different digits indicate significant statistical difference. a $P < 0.05$; b $P < 0.01$ versus blank control.

Anti-inflammatory activity

The findings of this study demonstrated that *Otostegia limbata* (OL) significantly reduced the edoema in the rat hind paws caused by an intraplantar injection of carrageenan, demonstrating significant anti-acute inflammatory efficacy. To clarify the underlying mechanisms at play in this animal model, the anti-inflammatory effect of OL was further assessed in the current study. We proved that the effect may be brought on by a reduction in the release of pro-inflammatory cytokines, the expression of inflammatory enzymes (iNOS and COX-2), as well as the production of their byproducts (NO and PGE2). Additionally, we demonstrated how OL's anti-oxidative mechanisms could protect the liver from harm caused by carrageenan. The carrageenan-induced paw edoema is a well-established model of acute inflammation that involves numerous inflammatory mediators in the development of the condition. It has been extensively used to assess the anti-edematous effect of naturalI the current work, we demonstrated that dose-dependent anti-inflammatory effects of OL were produced in carrageenan-induced rat paw edoema. Our findings supported earlier research that showed OL has a pronounced anti-inflammatory effect in animal models [30]. On the other hand, it is well known that the inflammation brought on by carrageenan in the hind paw is significantly aided by neutrophil infiltration [31]. According to a histological analysis of our results, OL caused a noticeable decrease in the number of neutrophils that infiltrated the paws that had been treated with carrageenan. An increasing body of research has shown that triterpenoids, phenolic acids, and flavonoids have anti-inflammatory and antinociceptive properties in animal models [32]. The considerable antinociceptive and anti-inflammatory effects of flavonoids such luteolin, quercetin, and rutin have also been observed in studies [33]. As a result, it was postulated that OL's phenolic content might be responsible for its antioxidant and anti-inflammatory properties.

Effects of Ellagic Acid on Carrageenan-induced Rat Paw Edema

Carrageenan intraplantar injection in rats led to a time-dependent rise in paw volume [Figure 2]. However, at 1, 2, 3, 4, and 5 h following a carrageenan injection, OL therapy reduced the paw edoema in a dose-dependent manner. The anti-inflammatory impact of OL was measured by ED50 values with 95% confidence intervals of 8.41 (5.26-14.76) mg/kg. As anticipated, post-carrageenan edoema was significantly inhibited by the reference medication, indomethacin (5 mg/kg) [Figure 2].

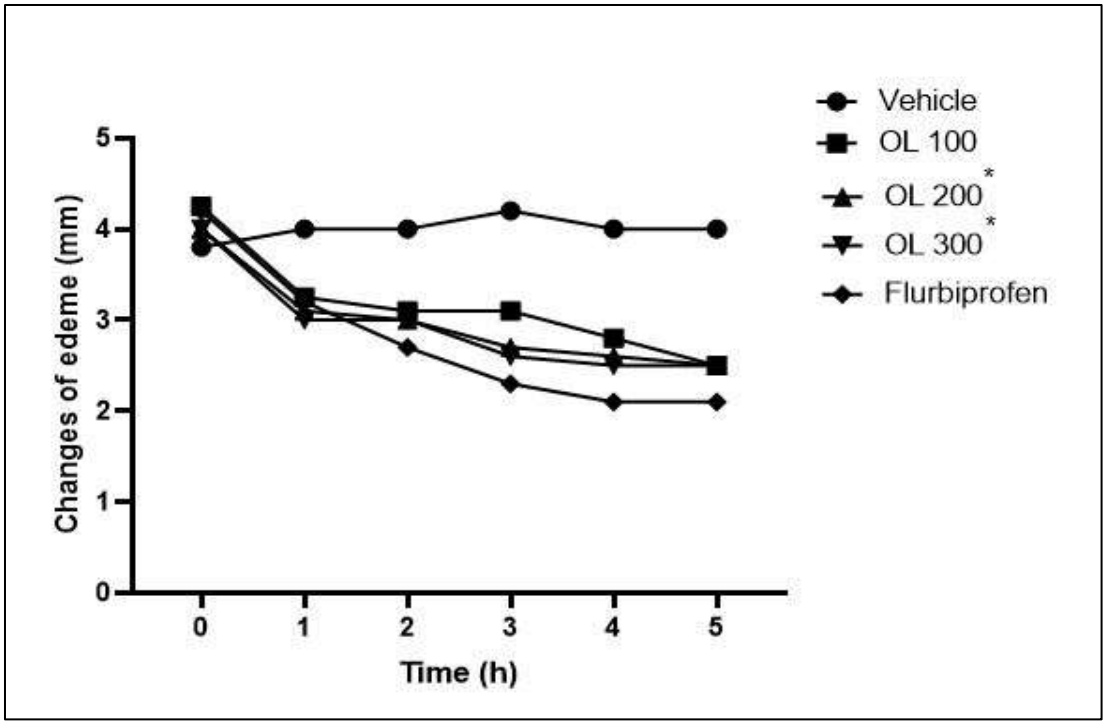

Figure 2: Effects of *Otostegia limbata* (OL) on hind paw edema induced by carrageenan (Carr) in rats. Each value represents the mean ± standard error of the mean. *P < 0.05 as compared to the Carr group (one-way ANOVA followed by Tukey's test). VEH: Vehicle, OL: *Otostegia limbata.* (1–30 mg/kg, intraperitoneal [i.p.]), Indo: Indomethacin (5 mg/kg, i.p.

CONCLUSIONS

Shortly, these results indicated that OL had potent analgesic and anti-inflammatory activities and could be a potential new drug candidate for the treatment of inflammation and pain. Collectively, the anti-infl ammatory mechanisms of OL might be related to the decrease in the level of MDA, iNOS, and COX-2 in the edema paw via the suppression of pro-infl ammatory cytokines (TNFα, IL1 β), NO and PGE2 overproduction.

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