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Original Article

Anti-Inflammatory Activity Test of *Dendrophthoe glabrescens* (Blakely) Barlow Leaf Extract on Mice (*Mus musculus*) Induced with Carrageenan

Uji Aktivitas Antiinflamasi Ekstrak Daun Benalu Jeruk (*Dendrophthoe glabrescens* (Blakely) Barlow) terhadap Mencit (*Mus musculus*) yang Diinduksi Karagenan

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Abstract

Inflammation is a natural defense mechanism of the body against injury or disease; however, excessive inflammation can lead to various chronic conditions. Prolonged use of synthetic anti-inflammatory drugs is associated with adverse effects, creating a need for safer natural alternatives. One such candidate is the citrus *benalu* leaf (*Dendrophthoe glabrescens* (Blakely) Barlow), a parasitic plant rich in flavonoids, saponins, and tannins. This study aimed to evaluate the anti-inflammatory activity of ethanol extract from citrus *benalu* leaves in carrageenan-induced mice. A laboratory experimental design using a Randomized Pre- and Post-Test Control Group approach was employed with 25 mice divided into five groups: negative control (CMC-Na 0.5%), positive control (sodium diclofenac), and three treatment groups receiving citrus *benalu* leaf extract at doses of 100, 200, and 400 mg/kgBW, respectively. The extract was administered orally 30 minutes after carrageenan induction, and paw edema was measured every 30 minutes for 180 minutes. One-way ANOVA revealed significant differences among groups, and LSD post hoc analysis indicated that all treatment groups differed significantly from the negative control ($p < 0.05$) but not from the positive control ($p > 0.05$). The 400 mg/kgBW dose demonstrated the greatest efficacy, reducing paw edema to 3.08%. These findings suggest that citrus *benalu* leaf extract has promising potential as a natural anti-inflammatory agent, offering an alternative to synthetic drugs with fewer side effects.

Abstrak

Peradangan (inflamasi) merupakan mekanisme pertahanan alami tubuh terhadap cedera atau penyakit; namun, peradangan berlebihan dapat memicu berbagai penyakit kronis. Penggunaan obat antiinflamasi sintetis dalam jangka panjang sering menimbulkan efek samping, sehingga diperlukan alternatif alami yang lebih aman. Salah satu kandidat adalah daun benalu jeruk (*Dendrophthoe glabrescens* (Blakely) Barlow), tanaman parasit yang kaya akan flavonoid, saponin, dan tanin. Penelitian ini bertujuan mengevaluasi aktivitas antiinflamasi ekstrak etanol daun benalu jeruk pada mencit yang diinduksi karagenan. Penelitian eksperimental laboratorium ini menggunakan rancangan *Randomized Pre- and Post-Test Control Group* dengan 25 ekor mencit yang dibagi menjadi lima kelompok: kontrol negatif (CMC-Na 0,5%), kontrol positif (natrium diklofenak), serta tiga kelompok perlakuan yang menerima ekstrak daun benalu jeruk dengan dosis 100, 200, dan 400 mg/kgBB. Ekstrak diberikan secara oral 30 menit setelah induksi, kemudian pembengkakan pada telapak kaki kanan mencit diukur setiap 30 menit selama 180 menit. Analisis one-way ANOVA menunjukkan perbedaan yang signifikan antar kelompok, dan uji post-hoc LSD menunjukkan bahwa semua kelompok perlakuan berbeda signifikan dengan kontrol negatif ($p < 0,05$), tetapi tidak berbeda dengan kontrol positif ($p > 0,05$). Dosis 400 mg/kgBB menunjukkan efektivitas tertinggi dengan penurunan edema hingga 3,08%. Temuan ini menegaskan potensi ekstrak daun benalu jeruk sebagai agen antiinflamasi alami yang berpeluang menjadi alternatif pengganti obat sintetis dengan risiko efek samping yang lebih rendah.

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INTRODUCTION

Inflammation is the body's initial defense mechanism against injury caused by internal or external factors, such as physical trauma, chemical agents, microbial infections, and cancer.[1] It is characterized by erythema, hyperthermia, pain, and edema, and involves immune cells such as neutrophils, mast cells, and macrophages, as well as key enzymes including cyclooxygenase (COX) and lipoxygenase (LOX).[1–5] Inflammation is closely linked to oxidative stress, which plays a critical role in initiating and exacerbating various degenerative

diseases.[6] According to the World Health Organization (WHO, 2024), degenerative diseases account for approximately 41 million deaths annually, underscoring their significant global health burden.[7]

Inflammation can be managed by alleviating pain or preventing tissue damage through the use of steroidal and non-steroidal drugs.[8] Both classes of anti-inflammatory agents function by inhibiting the release of prostaglandins at sites of tissue injury.[9] However, prolonged use of synthetic anti-inflammatory drugs is associated with significant adverse effects, including gastrointestinal disorders, peptic ulcers, and renal impairment. Steroidal drugs may also suppress immune function and lead to complications such as hypertension, moon face, and osteoporosis.[10] Consequently, there is a growing need for alternative treatments with minimal side effects, one of which involves the use of medicinal plants. In response to this need, the "back to nature" movement has gained traction, particularly in Indonesia, a country rich in biodiversity. The World Health Organization (WHO) has endorsed the use of traditional medicine derived from natural sources, citing its potential to reduce the side effects commonly associated with synthetic drugs.[11]

Bali Province, located in Indonesia, is home to several citrus production centers, including Jembrana, Tabanan, Badung, Gianyar, Klungkung, Bangli, Karangasem, Buleleng, and Denpasar City. Among these, Bangli Regency records the highest citrus yield, producing 93,162.3 tons annually from a harvest area of 38,140.21 hectares, with an average yield of 24.42 kw per hectare.[12] Local citrus farmers frequently encounter parasitic plants known as *benalu* (Balinese), which hinder citrus tree growth and reduce crop yields. These parasites are typically eradicated due to their detrimental effects. One such parasitic plant is the citrus *benalu* leaf, scientifically identified as *Dendrophthoe glabrescens* (Blakely) Barlow, which survives by attaching to citrus trees as its host.[13]

Despite its parasitic nature, the citrus *benalu* leaf exhibits potential as a medicinal plant. Its secondary metabolite profile varies depending on the host plant and includes alkaloids, steroids, triterpenoids, flavonoids, saponins, and tannins.[13] Among these, flavonoids, saponins, and tannins are recognized for their anti-inflammatory properties. A study by Neman et al.,[14] demonstrated that *benalu* leaves from kersen trees possess anti-inflammatory activity in formalin-induced rat edema, attributing this effect to the presence of flavonoids, saponins, and tannins. Additionally, research has shown that kersen *benalu* leaves exhibit strong antioxidant activity, with an IC_{50} value of 21.70 µg/mL.[15] Similarly, citrus *benalu* leaves have demonstrated potent antioxidant activity, with an IC_{50} value of 54.49 µg/mL.[13] Further support for the anti-inflammatory potential of citrus *benalu* leaves comes from Gas Chromatography–Mass Spectrometry (GC-MS) analysis, which identified additional bioactive compounds with anti-inflammatory properties.

This study employed mice (*Mus musculus*) induced with carrageenan to evaluate the anti-inflammatory efficacy of citrus *benalu* leaf extract. Carrageenan, a polysaccharide derived from seaweed species such as *Eucheuma*, *Chondrus*, and *Gigartina*, is commonly used to induce inflammation due to its irritant properties.[16] It offers several advantages as an inducer, including non-residual effects, minimal tissue damage, and heightened sensitivity to anti-inflammatory agents.[17] These characteristics make carrageenan a widely accepted model for evaluating anti-inflammatory activity.[18]

Based on the above considerations, this study aims to assess the effectiveness of citrus *benalu* leaf extract (*Dendrophthoe glabrescens* (Blakely) Barlow) in reducing carrageenan-induced edema in mice (*Mus musculus*), and to identify the bioactive compounds present in the extract through GC-MS analysis, thereby exploring its potential as a natural alternative to synthetic anti-inflammatory drugs.

RESEARCH METHODS

Tools. Analytical balance (Ohaus Pioneer PA 224C), Rotary evaporator (BUCHI R-300), oral sonde, glass jar, 100 ml beaker glass (PYREX), oven (MEMMERT), drinking bottle, plethysmometer (Orchid).

Materials. Citrus *benalu* leaf taken from Manikliyu village, Kintamani, Bali, carrageenan (PT. Brataco, Indonesia), distilled water, standard feed (Br-551), 96% ethanol (PT. Brataco, Indonesia), diclofenac sodium tablets (Kimia Farma), CMC Na (PT. Brataco, Indonesia).

Research Procedure

Preparation of Citrus *Benalu* Leaf Extract

The leaves of citrus *benalu* (*Dendrophthoe glabrescens* (Blakely) Barlow) were thoroughly washed, air-dried, and subsequently oven-dried at 50 °C for two days until completely desiccated. The dried leaves were then ground into a fine powder. A total of 100 g of powdered simplicia was subjected to maceration using 96% ethanol at a material-to-solvent ratio of 1:10 for three days. The resulting macerate was filtered through flannel cloth and concentrated using a vacuum rotary evaporator to obtain a thick extract. This maceration process was repeated twice to ensure optimal extraction.[13] The plant sample was taxonomically identified at the Biological Research Center, National Research and Innovation Agency (BRIN), Eka Karya Botanical Garden, Bedugul, under transaction number 1617-80983-1, dated February 27, 2023.

Preparation of Citrus *Benalu* Leaf Extract Test Solution

For the 100 mg/kg body weight (BW) dose, 1.8 mg of citrus *benalu* leaf extract was dissolved in 1% carboxymethyl cellulose (CMC) to a final volume of 10 mL. For the 200 mg/kg BW dose, 3.6 mg of extract was similarly dissolved in 1% CMC to a total volume of 10 mL. For the 400 mg/kg BW dose, 7.2 mg of extract was dissolved in 1% CMC, also adjusted to a final volume of 10 mL.

Preparation of 1% CMC-Na Suspension

A 1% carboxymethyl cellulose sodium (CMC-Na) suspension was prepared by dissolving 1 g of CMC in 10 mL of hot distilled water. The mixture was stirred until homogeneous, after which additional distilled water was added to reach a final volume of 100 mL.[19]

Preparation of Diclofenac Sodium Suspension

A 50 mg diclofenac sodium tablet was ground, and 45.410 mg of the resulting powder was taken. The powder was placed in a mortar, then mixed with a 0.5% CMC suspension until homogeneous, and the volume was adjusted to 10 mL.[16]

Preparation of 1% Carrageenan Induction

A total of 0.1 g of carrageenan was weighed and then dissolved in 0.9% sodium chloride in a 10 mL volumetric flask.[16]

GC-MS Testing

A total of 0.5 g of citrus *benalu* leaf powder was placed into a microtube containing 1.5 mL of methanol. The mixture was vortexed for 1 minute and centrifuged at 9,000 rpm for 3 minutes. The resulting supernatant was collected and used for Gas Chromatography–Mass Spectrometry (GC-MS) analysis. The GC-MS analysis was conducted over a 60-minute run time, with the injector temperature set at 260 °C, the detector temperature at 250 °C, and the column temperature at 325 °C. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. Bioactive compounds were identified based on chromatographic peaks and mass spectral (MS) data, which revealed the molecular weights of the detected compounds.[20]

Anti-inflammatory Testing

This study was conducted on 25 mice (Mus musculus) weighing between 20 and 30 g. The animals were randomly divided into five groups, each consisting of five mice. Ethical approval for the study was obtained from the Health Research Ethics Committee of Poltekkes Denpasar (Approval No.: DP.04.02/F.XXXII.25/0930/2024). Prior to testing, the mice were fasted for 18 hours. Each mouse was then induced with 0.2 mL of 1% carrageenan via subplantar injection in the paw.[16] Citrus *benalu* leaf extract was administered orally 30 minutes after carrageenan induction. Edema was measured over a six-hour period following the injection, with assessments taken at 0, 30, 60, 90, 120, 150, and 180 minutes using a plethysmometer.

The experimental groups were as follows:

1. **Group I** (Negative Control): Received 1% CMC-Na

2. **Group II** (Positive Control): Received sodium diclofenac
3. **Group III** (Treatment): Received citrus benalu leaf extract at 100 mg/kg BW
4. **Group IV** (Treatment): Received citrus benalu leaf extract at 200 mg/kg BW
5. **Group V** (Treatment): Received citrus benalu leaf extract at 400 mg/kg BW

Test Parameters

The parameter used in this study is the percentage of inhibition, which is calculated from the paw volume data of mice using the following formula:

$$\%\ edema = \frac{Vkt - Vko}{Vko} x\ 100\%$$

Description:
Vkt = Volume of mice paw at time x (ml)
Vko = Volume of mice paw at time 0 (ml)

Data Analysis

The spectra obtained from GC-MS analysis were processed using the instrument's integrated database library, generating chromatogram outputs. From these chromatograms, retention times were used to identify compounds present in the sample, while percentage area data were utilized to estimate the relative concentration of each compound.

Inflammation data were collected by measuring the thickness of edema in the hind paws of female mice (Mus musculus) induced with carrageenan. The data were categorized into control and treatment groups and analyzed statistically using a Completely Randomized Design (CRD). One-way Analysis of Variance (ANOVA) was employed to determine significant differences among groups. The ANOVA test was considered valid if the data met the assumption of normality ($p > 0.05$). If a significant difference was detected ($p \leq 0.05$), further analysis was conducted using a post-hoc test to identify specific group differences.

RESULTS AND DISCUSSION

Gas Chromatography–Mass Spectrometry (GC-MS) is a powerful analytical technique that combines gas chromatography for the separation of volatile compounds under high vacuum and low pressure with mass spectrometry for determining molecular weight, molecular formula, and generating charged molecular fragments.[21,22] GC-MS is widely used for the separation and analysis of complex mixtures, including plant secondary metabolites, due to its high sensitivity and ability to detect compounds at very low concentrations. The results are presented in the form of chromatograms and mass spectra, allowing for precise identification of bioactive constituents.[20,23]

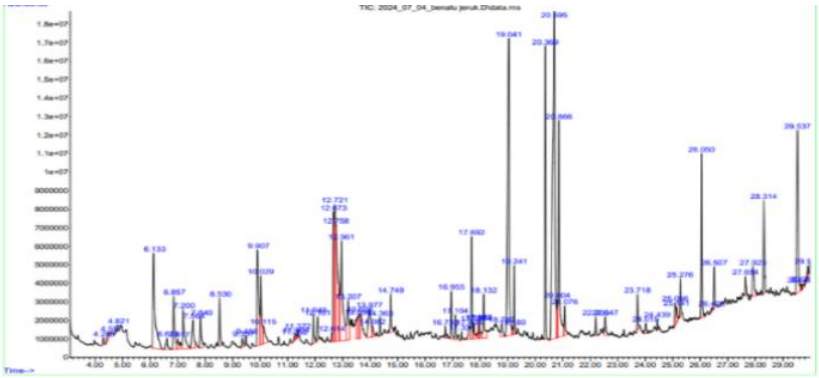

Figure 1. Chromatogram Graph of Citrus *Benalu* Leaf Extract Samples

In this study, GC-MS analysis of citrus *benalu* leaf extract revealed a total of 70 peaks in the chromatogram (**Figure 1**). Four of the most dominant peaks were selected based on their percentage area, as shown in **Table 1**. The most abundant compound was **9,12,15-Octadecatrienoic acid**, representing 15.14% of the total area with a retention time of 20.695 minutes. Also known as linolenic acid, this carboxylic acid

contains 18 carbon atoms and three cis double bonds. It is an essential fatty acid of plant origin, crucial to human health. Previous studies have associated 9,12,15-Octadecatrienoic acid with a range of pharmacological activities, including anti-inflammatory, antioxidative, anticancer, anti-obesity, antimetabolic, and cardiovascular-protective effects.[24] The second most prominent compound was **Octadecanoic acid**, accounting for 5.30% of the total area with a retention time of 20.866 minutes. Also referred to as stearic acid (not oleic acid, which is cis-9-Octadecenoic acid), Octadecanoic acid has the molecular formula $C_{18}H_{36}O_2$ and belongs to the saturated fatty acid group. Its straight-chain structure contributes to its solid form at room temperature.[25] Octadecanoic acid has been reported to exhibit antioxidant and anti-inflammatory properties,[26] and also plays roles in biological systems as an antibacterial and antifungal agent.[27] Both 9,12,15-Octadecatrienoic acid and Octadecanoic acid are bioactive compounds that may contribute to the anti-inflammatory potential of citrus *benalu* leaf extract. While these compounds are commonly found in flavonoid-rich plant matrices, they are classified as fatty acids rather than flavonoids.[28]

Table 1. Active Components Based on the Highest Outer Percentage in Citrus *Benalu* Leaf Extract Samples

Compound	Area (%)	Retention Time	Qual
9,12,15-Octadecatrienoic acid	15,14 %	20,695	97
Octadecanoic acid (CAS)	5,30 %	20,866	99
Phytol	4,69 %	20,369	91
1,2,3-Benzenetriol (CAS)	4,49 %	12,758	97

The third most abundant compound identified was **Phytol**, with a percentage area of 4.69% and a retention time of 20.363 minutes. Phytol is an acyclic diterpene alcohol derived from terpenoids.[29] It is naturally found in various foods, including fish, ruminant meat, green vegetables, and dairy products, and is a key component of chlorophyll, vitamin E, and vitamin K.[30] Phytol has demonstrated anti-inflammatory effects, including significant reduction of carrageenan-induced paw edema in animal models. It also inhibits edema induced by histamine, PGE_2, serotonin, and bradykinin, and suppresses leukocyte recruitment. Furthermore, phytol has been shown to reduce levels of myeloperoxidase (MPO), interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and malondialdehyde (MDA), while increasing glutathione (GSH) levels during acute inflammation.[30–32]

The fourth most abundant compound was **1,2,3-Benzenetriol**, also known as **pyrogallol**, a phenol derivative with strong anti-inflammatory and antimicrobial properties.[26,33] Pyrogallol has been identified as a major constituent in various plant parts[34] and is known for its ability to combat bacterial pathogens and reduce inflammation through its antioxidant activity.[33,35,36]

Based on the presence of these dominant compounds—9,12,15-Octadecatrienoic acid, Octadecanoic acid, Phytol, and 1,2,3-Benzenetriol—the citrus *benalu* leaf extract demonstrates promising potential as an anti-inflammatory and antioxidant agent. Anti-inflammatory activity is closely linked to antioxidant capacity, as oxidative stress plays a key role in the inflammatory process.[37,38] Antioxidants help neutralize reactive oxygen species (ROS), which, when present in excess, can damage cellular components such as lipids, proteins, and nucleic acids, leading to membrane instability and hemolysis.[33,39]

Inflammation testing was conducted by inducing mice with 0.2 mL of 1% carrageenan via subplantar injection. This method is known to stimulate an increase in cyclooxygenase-2 (COX-2) levels, a key enzyme involved in the inflammatory response.[40] The use of 0.2 mL of 1% carrageenan has been shown to reliably induce paw edema in mice, consistent with previous studies.[41]

The anti-inflammatory efficacy of the extract was evaluated using the paw edema method, with inflammation volume measured using a plethysmometer filled with mercury. This device operates on the principle of Archimedes' law,[42] allowing precise measurement of volume displacement caused by paw swelling. Although edema is typically monitored for six hours at 30-minute intervals,[16,43] in this study, the swelling had subsided by the 180-minute mark.

Carrageenan-induced inflammation occurs in two distinct phases. The first phase involves the release of histamine, serotonin, and bradykinin, while the second phase is characterized by the overproduction of prostaglandins, along with elevated levels of bradykinin, proteases, and lysosomal enzymes.[44] Edema generally persists for up to five hours post-injection and gradually resolves within 24 hours.[45] The percentage of edema observed in each group is presented in **Table 2** and **Figure 2**.

Table 2. Percentage of Paw Edema in Mice Following Carrageenan Induction

Average	Pretest (%)	After Induction (%)	30 Minutes (%)	60 Minutes (%)	90 Minutes (%)	120 Minutes (%)	150 Minutes (%)	180 Minutes (%)
Negative control	0.00	64.29	44.90	42.86	43.88	31.63	1.63	31.63
Positive control	0.00	119.18	131.51	73.97	34.25	24.66	10.96	2.74
Extract dose 100 mg/kgBW	0.00	148.21	119.64	107.14	98.21	71.43	51.79	26.79
Extract dose 200 mg/kgBW	0.00	125.86	77.59	67.24	68.97	50.00	37.93	10.34
Extract dose 400 mg/kgBW	0.00	110.77	69.23	81.54	58.46	40.00	26.15	3.08

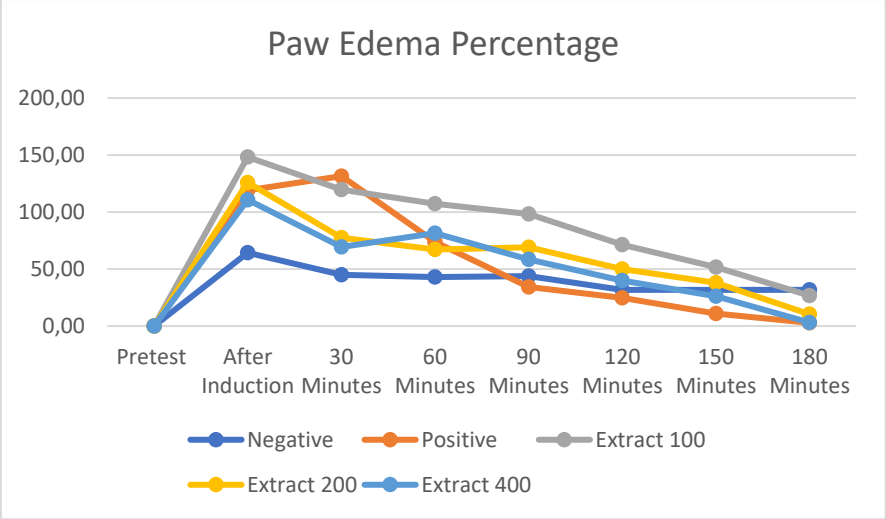

Figure 2. Percentage of Paw Edema in Mice Over Time Following Carrageenan Induction and Treatment with Citrus *Benalu* Leaf Extract

Following carrageenan induction, a significant increase in paw edema was observed across all experimental groups. The negative control group, which received 1% CMC-Na, exhibited consistently elevated edema levels throughout the 180-minute observation period. This outcome is attributed to the absence of anti-inflammatory activity in CMC-Na, leaving the inflammatory response entirely dependent on the animals' innate immune mechanisms.[46] Statistical analysis began with a normality test using the Shapiro–Wilk method, which yielded a significance value of $p > 0.05$ for the percentage reduction in edema from 30 to 180 minutes. This result confirms that the data were normally distributed. A homogeneity test using Levene's method also produced a significance value of $p > 0.05$, indicating that the data were homogeneously distributed across groups.[47] Given that both assumptions of normality and homogeneity were met, parametric analysis was conducted using one-way ANOVA.

The one-way ANOVA revealed statistically significant differences in the mean percentage reduction of edema among groups at all time points ($p < 0.05$, and in some cases $p < 0.001$), indicating that treatment with citrus *benalu* leaf extract significantly affected the inflammatory response compared to the negative control. Post hoc analysis using the Least Significant Difference (LSD) method confirmed that the negative control group had the highest edema percentage, significantly greater than all treatment groups.

The positive control group, which received diclofenac sodium, showed a marked reduction in edema compared to the negative control ($p < 0.05$), demonstrating the rapid and effective anti-inflammatory action of the standard drug.[48] Diclofenac sodium exerts its effect by non-selectively inhibiting cyclooxygenase (COX) enzymes and reducing the bioavailability of arachidonic acid.[49] Its selection for this study was based on its known pharmacokinetics, including high tissue penetration in inflamed regions such as the plantar surface of the paw.[50]

Treatment groups receiving citrus *benalu* leaf extract at varying doses exhibited dose-dependent anti-inflammatory effects. Notably, the group administered 400 mg/kg BW showed a significant reduction in edema, with results approaching those of the positive control group ($p > 0.05$), particularly between 60- and 180-minutes post-induction. These findings suggest that 400 mg/kg BW is the most effective dose, consistent with previous studies reporting optimal anti-inflammatory activity at this concentration.[51]

Flavonoids exert anti-inflammatory effects primarily by inhibiting key pro-inflammatory enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX), which are involved in the biosynthesis of prostaglandins and leukotrienes.[52,53] Additionally, flavonoids suppress neutrophil degranulation, thereby reducing the release of arachidonic acid and limiting the production of inflammatory mediators.[54,55] They also inhibit histamine release from mast cells and reduce leukocyte infiltration, contributing to the attenuation of the inflammatory response.[56]

Saponins demonstrate anti-inflammatory activity by inhibiting exudate formation and reducing vascular permeability, which helps to limit tissue swelling and leukocyte migration.[57,58] Certain saponin compounds have also been shown to modulate the NLRP3 inflammasome pathway, further suppressing the release of inflammatory cytokines such as IL-1β and IL-18.[59]

Tannins possess potent antioxidants and anti-inflammatory properties. Their antioxidant activity is linked to the inhibition of reactive oxygen species (ROS) production by neutrophils, monocytes, and macrophages, thereby reducing the formation of hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and hydroxyl radicals (-OH).[60] These actions help stabilize cellular membranes and prevent oxidative damage during inflammation.

The anti-inflammatory efficacy of the ethanol extract of citrus *benalu* leaves (*Dendrophthoe glabrescens* (Blakely) Barlow) is likely attributable to its bioactive constituents, including flavonoids, saponins, and tannins, which are known to modulate inflammatory pathways. However, a limitation of this study is the duration of observation, which was restricted to 180 minutes. While standard protocols often extend to 360 minutes,[61,62] the decision to limit the test duration was based on the observation that edema had nearly resolved by 180 minutes. Extending the test beyond this point could result in measurements that exceed baseline values, potentially confounding the interpretation of anti-inflammatory effects.

CONCLUSION

This study demonstrates that the ethanol extract of *Dendrophthoe glabrescens* (Blakely) Barlow, commonly known as citrus *benalu*, exhibits significant anti-inflammatory and antioxidant activities. GC-MS analysis identified key bioactive compounds—namely 9,12,15-octadecatrienoic acid, octadecanoic acid, phytol, and 1,2,3-benzenetriol—which are known to mitigate oxidative stress and modulate inflammatory pathways. In vivo evaluation using a carrageenan-induced mouse model confirmed the extract's anti-inflammatory potential, with the 400 mg/kg BW dose achieving a notable reduction in paw edema to 3.08%. These findings suggest that *D. glabrescens* leaf extract holds promise as a natural candidate for the development of alternative anti-inflammatory and antioxidant therapies. To advance its translational potential, further research is warranted, including comprehensive pharmacological profiling, toxicity assessments, and clinical trials to establish its safety, efficacy, and consistency in therapeutic applications.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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