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# Pharmacological Investigation of *Calotropis gigantea* L. for Anti-asthmatic and Anti-inflammatory Activities

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## Abstract

**Background:** Asthma is a chronic inflammatory airway disease characterized by airflow obstruction, bronchial hyperresponsiveness, and excessive mucus production. Although current therapies improve symptom control, long-term management remains challenging, encouraging the search for plant-based alternatives. *Calotropis gigantea* L. (*Asclepiadaceae*) has been traditionally used to treat respiratory and inflammatory disorders. **Aim:** To evaluate the anti-asthmatic and anti-inflammatory activities of root bark extracts of *C. gigantea* and identify the principal bioactive constituent through bioactivity-guided fractionation. **Methods:** Petroleum ether, chloroform, ethanolic, and aqueous root bark extracts (100 and 150 mg/kg, i.p.) were evaluated using milk-induced leukocytosis and eosinophilia, egg albumin-induced mast cell degranulation, and carrageenan-induced paw oedema models. The most active ethanolic extract was fractionated by silica gel column chromatography into six fractions (F1–F6). Fractions were screened using the clonidine-induced catalepsy model in mice with chlorpheniramine maleate as the reference. The most active fraction was purified, and the isolated compound was characterized by UV, IR, MS, and $^{1}H/^{13}C$ NMR spectroscopy. **Results:** The ethanolic extract exhibited the strongest anti-asthmatic and anti-inflammatory activity, significantly reducing leukocytosis, eosinophilia, mast cell degranulation, and paw oedema. Fraction F4 showed the highest activity, producing inhibition of clonidine-induced catalepsy comparable to chlorpheniramine. Phytochemical characterization identified stigmasterol as the major bioactive constituent. **Conclusion:** *C. gigantea* root bark possesses significant anti-asthmatic and anti-inflammatory properties, validating its traditional use. The pharmacological effects are primarily attributed to stigmasterol, highlighting its potential as a natural therapeutic candidate for asthma and inflammatory disorders.

**Major Finding:** Bioactivity-guided fractionation identified Fraction F4 as the most active fraction, and stigmasterol was isolated as the principal compound responsible for the anti-asthmatic and anti-inflammatory effects.

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**Keywords:** Asthma, *Calotropis gigantea,* Eosinophilia, Inflammation, Leukocytosis, Mast Cell Stabilisation, Stigmasterol

**Abbreviations:** ECGR – Extracts of *Calotropis gigantea* Root Bark; PECGR – Petroleum Ether Extract of *Calotropis gigantea* Root Bark; CECGR – Chloroform Extract of *Calotropis gigantea* Root Bark; EECGR – Ethanolic Extract of *Calotropis gigantea* Root Bark; AECGR – Aqueous Extract of *Calotropis gigantea* Root Bark; CPM – Chlorpheniramine Maleate; OECD – Organisation for Economic Co-operation and Development; IL – Interleukin; TNF-α – Tumour Necrosis Factor-alpha; NF-κB – Nuclear Factor kappa B

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## 1. Introduction

Asthma is defined as a persistent inflammatory condition affecting the respiratory airways. Its pathogenesis involves a diverse array of cellular components, including but not limited to eosinophils, mast cells, neutrophils, T lymphocytes, epithelial cells, and macrophages. This cellular involvement contributes to the hallmark features of asthma: airflow obstruction resulting from inflammation, bronchospasm, and hypersecretion of mucus within the airways[1]. Globally, approximately 300 million individuals are currently affected by asthma, with projections indicating an additional 100 million cases by 2025. The global epidemiology of asthma exhibits significant geographical variability in terms of prevalence, severity, and mortality rates. High-income nations report a higher incidence of asthma, whereas the majority of asthma-related mortalities occur in low- and middle-income countries[2].

The aetiology of asthma is multifactorial, arising from intricate interactions between environmental triggers and genetic predispositions. The underlying pathophysiology encompasses intermittent airflow limitation, bronchial hyperresponsiveness (BHR), chronic airway inflammation, and structural remodelling of the bronchial airways. Current therapeutic strategies for asthma primarily involve the administration of bronchodilators and anti-inflammatory agents, with treatment intensity tailored to disease severity. Despite these established treatments, there remains a critical need for enhanced asthma control. Consequently, herbal remedies are being investigated as potential alternative or adjunctive therapies to conventional synthetic medications[3].

*Calotropis gigantea* Linn., belonging to the family *Asclepiadaceae*, has been recognised in diverse traditional medicinal systems, including *Ayurveda, Siddha, Unani*, and Chinese medicine[4]. This plant, commonly referred to as Crown Flower or Giant Indian Milkweed, is geographically distributed across Africa, South Asia, and India[5].

Preclinical and clinical evaluation study claiming the use of ethanolic extract of *C. gigantea* L. root bark (ECGR) for the treatment of asthma is scarce, according to a review of the literature. Thus, the goal of the current study was to support the conventional assertion that ECGR is beneficial for asthma[6].

The current study aimed to assess the anti-asthmatic effect of ECGR against milk-induced leukocytosis and eosinophilia, egg albumin-induced mast cell degranulation, and carrageenan-induced acute paw oedema in rodent models because natural anti-inflammatory, leukotriene inhibitor, and antioxidant products are effective in controlling bronchial asthma[7]. The present study aimed to evaluate the potential protective effects of various solvent extracts of *C. gigantea* root bark against clonidine-induced catalepsy as a primary screening model for antiasthmatic activity. The study aims to establish a clear link between pharmacological activity and chemical identity, thereby providing a scientifically grounded basis for the antiasthmatic potential of *C. gigantea* L. root bark.

## 2. Materials and Methods

### 2.1 Procurement, Authentication, and Extraction of *Calotropis gigantea* L. Root Bark

A voucher specimen of *C. gigantea* L. root bark was collected from the local flora of Nashik, Maharashtra, India, and its taxonomic identity was authenticated by Dr. Hemantkumar A. Thakur, Department of Botany, GES's HPT Arts and RYK Science College, Nashik. The

authenticated specimen (Herbarium No. PS-01/2016) has been deposited as a reference for ongoing and future botanical research. For subsequent phytochemical investigations, the dried root bark was comminuted using a mechanical pulveriser. Extraction was carried out using the Soxhlet apparatus with a series of solvents, including petroleum ether, chloroform, ethanol, and water. The obtained extracts were concentrated under reduced pressure using a rotary evaporator and then lyophilised. The yields of the freeze-dried extracts of *C. gigantea* L. root bark (ECGR) were calculated, and these samples were further analysed for the presence of bioactive constituents[8].

## 2.2 Reagents and Chemicals

Egg albumin, aluminium hydroxide, Evans blue, disodium cromoglycate, dexamethasone, RPMI-1640 medium, toluidine blue, carrageenan, and indomethacin were procured from HiMedia Laboratories Private Limited, Mumbai, Maharashtra, India.

## 2.3 Experimental Animals

Adult male Wistar albino rats (150–200 g) and young adult Swiss albino mice (6–8 weeks, 20–25 g) were utilised for *in vivo* studies. The animals were maintained under standardised laboratory conditions (25°C ± 1°C, 45–55% relative humidity, 12-hour light/dark cycle) with free access to food and water. Before experimentation, animals were acclimatised to laboratory conditions for 7–10 days. Experimental protocols (MSG/PC/CPCSEA/II/2017/02) were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of GES's Sir Dr. M. S. Gosavi College of Pharmaceutical Education and Research, Nashik, following the guidelines set by the Committee for Control and Supervision of Experiments on Animals[9].

## 2.4 Methodology for Experimental Investigation

### *2.4.1 Study on Acute Toxicity*

An acute toxicity assessment of ECGR was performed in compliance with OECD Guideline 425. The principal objectives of this study were to estimate the median lethal dose ($LD_{50}$) and to define suitable dose ranges for further experimental evaluations. Rats received oral administrations of ECGR formulated in a 1% carboxymethyl cellulose (CMC) suspension, with subsequent observation for a period of 72 hours to monitor clinical signs of toxicity and record any mortality[10].

## 2.5 Investigation of Anti-asthmatic and Anti-inflammatory Potency of ECGR

### *2.5.1 Milk-induced Leukocytosis and Eosinophilia*

Mice (n = 60) were systematically randomised into ten distinct experimental cohorts, each comprising six animals. Blood samples were acquired via the retro-orbital plexus for haematological analysis. The control group received a vehicle solution (1% Tween-80, 5 mL/kg, intraperitoneal [i.p.] administration). The i.p. route was selected for administration of plant extracts to ensure rapid and consistent systemic absorption while minimising variability associated with gastrointestinal absorption. Experimental groups were administered ECGR at dosages of 100 and 150 mg/kg, i.p., respectively. The positive control group received dexamethasone (50 mg/kg, i.p.). Thirty minutes following treatment administration, all groups received a subcutaneous injection of boiled and cooled milk (4 mL/kg) to induce an inflammatory response. Total leukocyte and eosinophil counts were quantified in each cohort both prior to treatment and 24 hours post-milk injection. The differential change in these cellular populations was subsequently calculated to evaluate the anti-inflammatory efficacy of ECGR[11].

### *2.5.2 Egg Albumin-induced Mast Cell Degranulation*

Mice were randomly assigned to ten experimental groups, each comprising six animals. A three-day administration schedule was employed. The control group received an intraperitoneal injection of vehicle (1% Tween-80 solution, 5 mL/kg). The experimental groups were administered ECGR at intraperitoneal doses of 100 mg/kg and 150 mg/kg, respectively. A positive control group was treated with the reference anti-allergic agent sodium cromoglycate (50 mg/kg, i.p.). On the fourth day, all mice were subjected to an intraperitoneal injection of saline solution. Peritoneal fluid was harvested after a five-minute interval, and mast cells were isolated by centrifugation. The isolated cells were washed with RPMI-1640 medium and subsequently exposed to egg albumin (100 mg/mL) for

a 10-minute incubation at 37°C. Following incubation, the cells were stained with 1% toluidine blue and examined microscopically at 10x magnification. Mast cell degranulation, identified by morphological characteristics indicative of cell rupture, was assessed by enumerating 100 cells per sample. The percentage of degranulated cells in each group was calculated to evaluate the inhibitory potential of the respective treatments[12].

### 2.5.3 Carrageenan-induced Acute Paw Oedema

Rats were randomly allocated into ten groups, with each group comprising six animals. A treatment schedule spanning three days was implemented. The control group was given a vehicle consisting of a 1% Tween-80 solution at a dose of 5 mL/kg, administered intraperitoneally. The experimental groups received ECGR intraperitoneally at doses of 100 mg/kg and 150 mg/kg, respectively. For the reference group, the standard anti-inflammatory agent, indomethacin, was administered orally at a dose of 10 mg/kg. Indomethacin was administered orally (p.o.) because its anti-inflammatory efficacy in the carrageenan-induced paw oedema model is conventionally validated through oral administration, thereby facilitating comparison with published studies. Thirty minutes after extract administration, a 0.1 mL injection of 1% (w/v) carrageenan solution was administered subplantarly into the right hind paw of each rat[13]. The untreated left hind paw served as a control. Paw volumes were measured in both paws at 1, 2, and 3 hours following carrageenan injection in the control, standard, and extract-treated groups. The percentage inhibition of oedema for each rat and group was determined using the following formula:

$$\% \text{ Inhibition of oedema} = [(VC - VT) / VC] \times 100$$

Where: VC: increase in paw volume of the control group, and VT: increase in paw volume of the test group.

## 2.6 Bioactivity-guided Fractionation and Compound Isolation

The ethanolic extract of *C. gigantea* L. root bark (EECGR) was subjected to bioactivity-guided fractionation using silica gel-based column chromatography with an n-hexane: chloroform (10:1) solvent system. Fractions were combined based on Thin Layer Chromatography (TLC) behaviour to yield six primary fractions, F1 through F6. The percentage yield (*w/w*) of each fraction was calculated. Fractions were evaluated using the clonidine-induced catalepsy model[14]. Catalepsy was induced by subcutaneous administration of clonidine (1 mg/kg). Test fractions (50 mg/kg, i.p.) were administered 30 minutes before clonidine injection. Chlorpheniramine maleate (CPM, 10 mg/kg, i.p.) served as the reference standard. Duration of catalepsy was recorded at 30-minute intervals for 180 minutes. The most active fraction was subjected to repeated column chromatography using a hexane: ethyl acetate (10:0 to 6:4) gradient solvent system. Final purification was achieved with hexane: ethyl acetate (80:20), yielding a single compound. The isolated compound was characterised by UV spectroscopy, IR spectroscopy, mass spectrometry, and $^{1}H$ and $^{13}C$ NMR spectroscopy.

## 2.7 Data Collection and Analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using a one-way analysis of variance (ANOVA) in GraphPad Prism version 9.0. To explore differences between groups, a post-hoc Dunnett's multiple comparisons test was applied. Statistical significance was defined as $p < 0.05$.

# 3. Results

## 3.1 The Percentage Yield of the Extract and Bioactive Constituent's Analysis

Bioactive constituents were found in several extracts of *C. gigantea* L. root bark (ECGR), as indicated in Table 1. The petroleum ether (PECGR) and chloroform (CECGR) extracts predominantly contained alkaloids and tannins, indicating enrichment of relatively nonpolar constituents. In contrast, the ethanol (EECGR) and aqueous (AECGR) extracts showed the presence of steroids, saponins, glycosides, flavonoids, and carbohydrates, reflecting effective extraction of polar and semi-polar phytochemicals. Proteins were detected only in trace amounts in the aqueous extract. This polarity-dependent distribution suggests that ethanol was the most efficient solvent for extracting multiple bioactive classes relevant to anti-inflammatory and anti-allergic activity. The percentage yields of freeze-dried

*C. gigantea* L. root bark extracts in the petroleum ether (PECGR), chloroform (CECGR), ethanol (EECGR), and aqueous (AECGR) were determined to be 5.7, 5.0, 9.0, and 15.0 % w/w, respectively.

## 3.2 Acute Toxicity Study

Oral administration of PECGR, CECGR, EECGR, and AECGR extracts suspended in a 1% carboxymethyl cellulose (CMC) solution at a 1% (w/v) concentration for 72 hours did not result in any fatalities or observable toxic effects in the test animals. Although the OECD guidelines set a maximum tolerated dose of 2000 mg/kg for *Calotropis* extracts, the selected doses (100 and 150 mg/kg) were considered appropriate to study the potential anti-asthmatic and anti-inflammatory effects of *Calotropis* extracts.

## 3.3 Investigation of Anti-asthmatic and Anti-inflammatory Potency of ECGR

### *3.3.1 Milk-induced Leukocytosis*

Administration of milk significantly increased total leucocyte count in control animals (Table 2). Treatment with EECGR at both tested doses (100 and 150 mg/kg, i.p.) produced a statistically significant reduction in leucocyte count compared with the control group ($p < 0.01$). The effect of EECGR was comparable to that of dexamethasone, the standard reference drug. PECGR, CECGR, and AECGR showed moderate reductions in leucocyte count; however, these changes did not reach statistical significance at the tested doses. No clear dose-dependent trend was observed for PECGR or CECGR.

### *3.3.2 Milk-induced Eosinophilia*

As shown in Table 3, milk administration induced a marked increase in eosinophil count in control animals. EECGR treatment significantly suppressed eosinophilia at both 100 and 150 mg/kg doses ($p < 0.01$), demonstrating a consistent inhibitory effect. Dexamethasone also produced a significant reduction, validating the experimental model. Other extracts exhibited marginal reductions without statistical significance. Although variability was observed in some treatment groups, the trend consistently favoured EECGR as the most effective extract in attenuating eosinophil elevation.

### *3.3.3 Egg Albumin-induced Mast Cell Degranulation*

Egg albumin challenge caused pronounced mast cell degranulation in control mice (Table 4). EECGR significantly reduced the number of degranulated

**Table 1.** The bioactive constituents' analysis of extracts of *Calotropis gigantea* L. root bark (ECGR)

| Phytochemical Class | Test | PECGR | CECGR | EECGR | AECGR |
|---|---|---|---|---|---|
| Steroids | Liebermann–Burchard | – | – | + | + |
| Saponins | Foam test | – | – | + | + |
| Alkaloids | Dragendorff's | + | + | – | – |
| | Mayer's | + | + | – | – |
| | Wagner's | + | + | – | – |
| | Hager's | + | + | – | – |
| Glycosides | Modified Borntrager's | – | + | + | + |
| | Legal's | – | + | + | + |
| | Keller–Killiani | – | + | + | + |
| Flavonoids | Shinoda | – | + | + | – |
| | Alkaline reagent | – | + | + | – |
| Tannins | Ferric chloride | + | – | + | + |
| Proteins | Biuret | – | – | – | + (trace) |
| Amino acids | Ninhydrin | – | + | – | – |
| Carbohydrates | Molisch | – | – | + | + |

(+) present; (–) absent. PECGR: Petroleum ether extract of *Calotropis gigantea* L. root bark; CECGR: Chloroform extract of *Calotropis gigantea L.* root bark; EECGR: Ethanol extract of *Calotropis gigantea* root bark; AECGR: Aqueous extract of *Calotropis gigantea* L. root bark.

**Table 2.** Effect of extracts of *Calotropis gigantea* L. root bark (ECGR) on milk-induced leucocytosis in mice

| Treatment | Dose (mg/kg, i.p.) | Leucocyte Count Difference |
|---|---|---|
| Control (1% Tween-80) | 5 ml/kg | 4572 ± 15.63 |
| PECGR | 100 | 3325 ± 27.29 |
| | 150 | 3766.67 ± 31.61 |
| CECGR | 100 | 3642 ± 13.66 |
| | 150 | 3931 ± 17.94 |
| EECGR | 100 | 2632.33 ± 28.74** |
| | 150 | 2700 ± 11.47** |
| AECGR | 100 | 3732 ± 16.45 |
| | 150 | 3841 ± 14.36 |
| Dexamethasone | 50 | 2572 ± 16.70** |

Data were expressed as mean ± standard error of the mean (SEM) and analysed by a one-way analysis of variance (ANOVA) and a post-hoc Dunnett's multiple comparisons test; n = 6 in each group; **p < 0.01 significant compared with the control group.

**Table 3.** Effect of extracts of *Calotropis gigantea* L. root bark (ECGR) on milk-induced eosinophilia in mice

| Treatment | Dose (mg/kg, i.p.) | Eosinophil Count Difference |
|---|---|---|
| Control (1% Tween-80) | 5 ml/kg | 140.48 ± 2.09 |
| PECGR | 100 | 132.14 ± 1.65 |
| | 150 | 135.54 ± 1.43 |
| CECGR | 100 | 123.13 ± 2.81 |
| | 150 | 126.16 ± 1.78 |
| EECGR | 100 | 103.15 ± 1.21** |
| | 150 | 104.03 ± 1.48** |
| AECGR | 100 | 135.00 ± 1.52 |
| | 150 | 137.00 ± 1.23 |
| Dexamethasone | 50 | 101.21 ± 1.62** |

Data were expressed as mean ± standard error of the mean (SEM) and analysed by a one-way analysis of variance (ANOVA) and a post-hoc Dunnett's multiple comparisons test; n = 6 in each group; **p < 0.01 significant compared with the control group.

**Table 4.** Effect of extracts of *Calotropis gigantea* L. root bark (ECGR) on egg albumin-induced mast cell degranulation in mice

| Treatment | Dose (mg/kg, i.p.) | Degranulated Mast Cells | Mast Cell Protection (%) |
|---|---|---|---|
| Control | 5 ml/kg | 84.17 ± 1.04 | – |
| PECGR | 100 | 62.43 ± 1.21 | 25.8 |
| | 150 | 67.53 ± 1.43 | 19.8 |
| CECGR | 100 | 72.84 ± 1.73 | 13.5 |
| | 150 | 75.72 ± 1.32 | 10.0 |
| EECGR | 100 | 34.32 ± 1.05** | 59.2 |
| | 150 | 35.04 ± 2.03** | 58.4 |
| AECGR | 100 | 53.61 ± 1.82 | 36.3 |
| | 150 | 57.02 ± 1.03 | 32.3 |
| Sodium cromoglycate | 50 | 32.93 ± 1.53** | 60.9 |

Data were expressed as mean ± standard error of the mean (SEM) and analysed by a one-way analysis of variance (ANOVA) and a post-hoc Dunnett's multiple comparisons test; n = 6 in each group; **p < 0.01 significant compared with the control group.

mast cells at both doses tested ($p < 0.01$), resulting in a high percentage of mast cell protection. The protective effect of EECGR was comparable to that of sodium cromoglycate, a standard mast cell stabiliser. AECGR showed moderate protection, whereas PECGR and CECGR exhibited relatively lower protective effects. These findings indicate that ethanol-soluble constituents of *C. gigantea* root bark effectively stabilise mast cell membranes.

### *3.3.4 Carrageenan-induced Acute Paw Oedema*

Carrageenan injection produced progressive paw oedema in control rats over the 3-hour observation period (Table 5). EECGR significantly reduced paw oedema at all-time points (1, 2, and 3 h) compared with control animals ($p < 0.01$). The percentage inhibition of oedema produced by EECGR was substantial and approached that of indomethacin, the standard anti-inflammatory drug. PECGR, CECGR, and AECGR

**Table 5.** Effect of extracts of *Calotropis gigantea* L. root bark (ECGR) on Carrageenan-induced acute paw oedema in rats

| Treatments | Dosage (i. p.) | Paw volume (ml) Mean ± SEM | | | % Inhibition of oedema | | |
|---|---|---|---|---|---|---|---|
| | | 1 hr | 2 hr | 3 hr | 1 hr | 2 hr | 3 hr |
| Control (1% Tween-80) | 5 ml/kg | 0.54 ± 0.01 | 0.62 ± 0.02 | 0.63 ± 0.04 | - | - | - |
| PECGR | 100 | 0.45 ± 0.03 | 0.54 ± 0.02 | 0.50 ± 0.04 | 16.67 | 12.90 | 20.63 |
| | 150 | 0.47 ± 0.01 | 0.57 ± 0.01 | 0.52 ± 0.02 | 12.96 | 8.06 | 17.46 |
| CECGR | 100 | 0.43 ± 0.02 | 0.53 ± 0.01 | 0.58 ± 0.01 | 14.52 | 14.52 | 7.94 |
| | 150 | 0.44 ± 0.01 | 0.55 ± 0.01 | 0.59 ± 0.01 | 18.52 | 11.29 | 6.35 |
| EECGR | 100 | 0.29 ± 0.42** | 0.30 ± 0.03** | 0.32 ± 0.04** | 46.30 | 51.61 | 49.21 |
| | 150 | 0.30 ± 0.40** | 0.31 ± 0.04** | 0.34 ± 0.05** | 44.44 | 50.00 | 46.03 |
| AECGR | 100 | 0.48 ± 0.08 | 0.57 ± 0.01 | 0.55 ± 0.01 | 11.11 | 8.06 | 12.70 |
| | 150 | 0.49 ± 0.05 | 0.58 ± 0.01 | 0.56 ± 0.02 | 9.26 | 6.45 | 11.11 |
| Indometha-cin | 10 mg/ kg, p.o. | 0.22 ± 0.02** | 0.28 ± 0.03** | 31 ± 0.02** | 59.26 | 54.84 | 50.79 |

Data were expressed as mean ± standard error of the mean (SEM) and analysed by a one-way analysis of variance (ANOVA) and a post-hoc Dunnett's multiple comparisons test; n = 6 in each group. **p<0.01 significant compared with the control group.

**Table 6.** Yield of EECGR fractions

| Fraction | Yield (g) | Yield (% w/w) |
|---|---|---|
| F1 | 0.10 | 0.14 |
| F2 | 1.92 | 2.50 |
| F3 | 3.58 | 3.90 |
| F4 | 5.84 | 8.75 |
| F5 | 0.48 | 0.72 |
| F6 | 2.73 | 4.09 |

produced only mild to moderate inhibition of oedema, which was less consistent across time points and doses.

## 3.4 Bioactivity-guided Fractionation

Among the various screening models employed to assess antiasthmatic potential, the ethanolic extract of *C. gigantea* L. root bark (EECGR) exhibited the most pronounced protective effect contrast to other extracts. On the basis of these findings, EECGR was selected for further bioactivity-guided fractionation. EECGR was subjected to silica gel column chromatography using an n-hexane: chloroform solvent system (10:1). Based on similarities in thin-layer chromatography profiles, the eluted fractions were pooled to yield six distinct fractions, designated F1 to F6. The respective yields of these fractions are summarised in Table 6. Fraction F4 constituted the highest yield both in absolute mass and percentage recovery, suggesting enrichment of major bioactive constituents within this fraction.

## 3.5 Effect of EECGR Fractions on Clonidine-induced Catalepsy

Fractions F2 to F6 were evaluated for their antiasthmatic potential using the clonidine-induced catalepsy model in mice. Subcutaneous administration of clonidine (1 mg/kg) induced a sustained cataleptic response lasting up to three hours in the control group. Clonidine administration in the vehicle (1% Tween-80 solution)-treated control group produced sustained catalepsy lasting up to 180 minutes. Treatment with fraction F4 resulted in a marked and statistically significant reduction in catalepsy duration across all observation time points, with effects comparable to CPM. Other fractions showed only marginal or moderate inhibition (Table 7).

## 3.6 Isolation and Characterisation of the Bioactive Compound

Given its superior antiasthmatic activity, fraction F4 was subjected to repeated column chromatography over silica gel using a gradient system of hexane: ethyl acetate (10:0 to 6:4). Final purification was achieved

**Table 7.** Effect of EECGR fractions on clonidine-induced catalepsy

| Group | Dose (mg/kg) | 30 min | 60 min | 90 min | 120 min | 150 min | 180 min |
|---|---|---|---|---|---|---|---|
| Control | Vehicle | 96.40 ± 4.10 | 95.72 ± 3.05 | 101.85 ± 4.96 | 99.18 ± 3.42 | 98.02 ± 4.88 | 97.76 ± 4.33 |
| F2 | 50 | 89.92 ± 2.83 | 90.46 ± 3.12 | 97.24 ± 2.74 | 95.10 ± 2.98 | 94.36 ± 2.11 | 95.48 ± 2.86 |
| F3 | 50 | 86.31 ± 3.92 | 82.47 ± 2.06 | 91.38 ± 1.84 | 88.26 ± 3.77 | 85.04 ± 2.18 | 86.72 ± 1.96 |
| F4 | 50 | 34.82 ± 1.68** | 37.05 ± 2.22** | 45.12 ± 3.21** | 42.96 ± 1.54** | 40.73 ± 1.81** | 41.89 ± 1.66** |
| F5 | 50 | 82.25 ± 2.12 | 84.57 ± 1.48 | 83.29 ± 2.12 | 85.46 ± 2.21 | 82.28 ± 2.10 | 83.64 ± 2.16 |
| F6 | 50 | 83.67 ± 2.14 | 86.25 ± 2.61 | 85.38 ± 3.29 | 87.41 ± 2.47 | 83.10 ± 1.96 | 84.02 ± 1.58 |
| CPM | 10 | 31.94 ± 2.81** | 33.88 ± 3.52** | 41.76 ± 2.19** | 44.65 ± 1.18** | 39.22 ± 1.44** | 40.87 ± 1.29** |

Data were expressed as mean ± standard error of the mean (SEM) and analysed by a one-way analysis of variance (ANOVA) and a post-hoc Dunnett's multiple comparisons test; n = 6 in each group. **p < 0.01 significant compared with the control group.

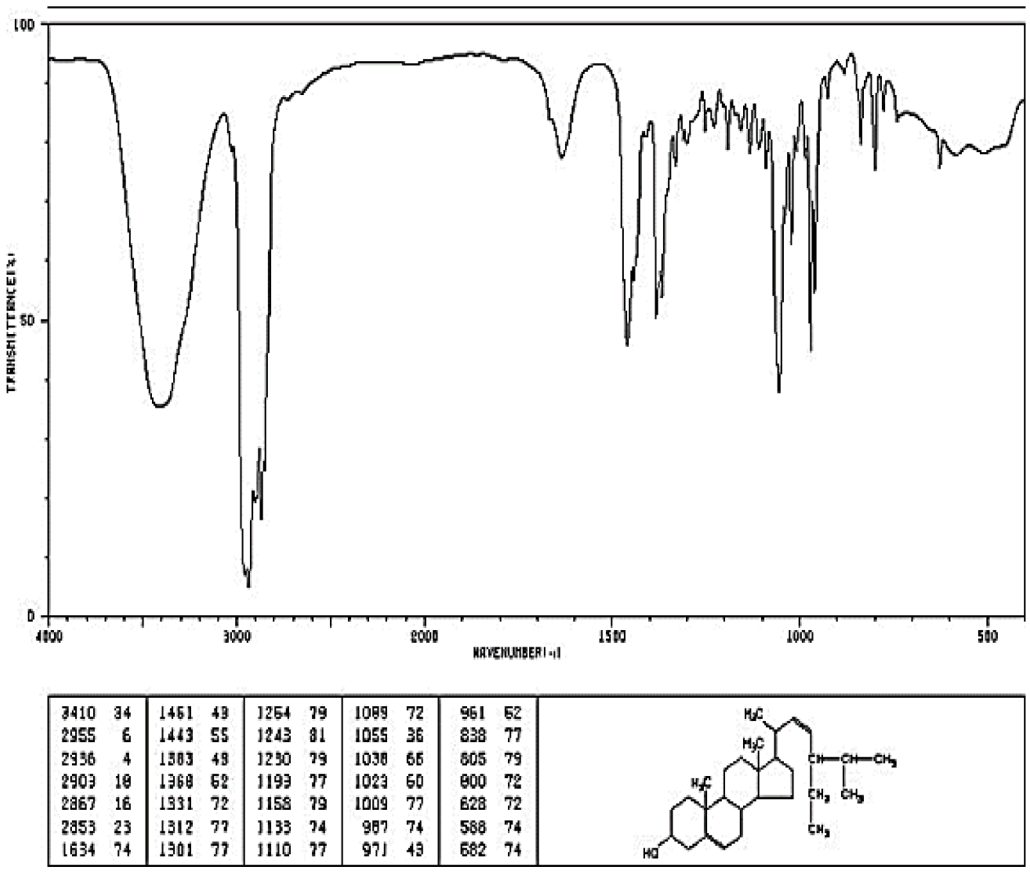



**Figure 1.** IR spectrum of the compound isolated from fraction F4.

using hexane: ethyl acetate (80:20), yielding a single compound with an $R_f$ value of 0.58. The isolated compound crystallised as white needle-shaped crystals with a melting point range of 160–164 °C.

### *3.6.1 Spectral Characterisation*

Infrared spectroscopy revealed a broad absorption band at 3428 $cm^{-1}$, indicative of hydroxyl group stretching. Peaks at 2937 and 1465 $cm^{-1}$ corresponded to methyl C–H stretching and bending vibrations, while absorptions at 1642 and 881 $cm^{-1}$ confirmed the presence of olefinic C=C and unsaturated C–H bonds. The vibrations of -$CH_3$ were observed at 2937 $cm^{-1}$ and at 1465 $cm^{-1}$, and vibrations of =$CH_2$ were seen at 2852 $cm^{-1}$ and at 1460 $cm^{-1}$, respectively. The C-C vibration peak was demonstrated at 1053

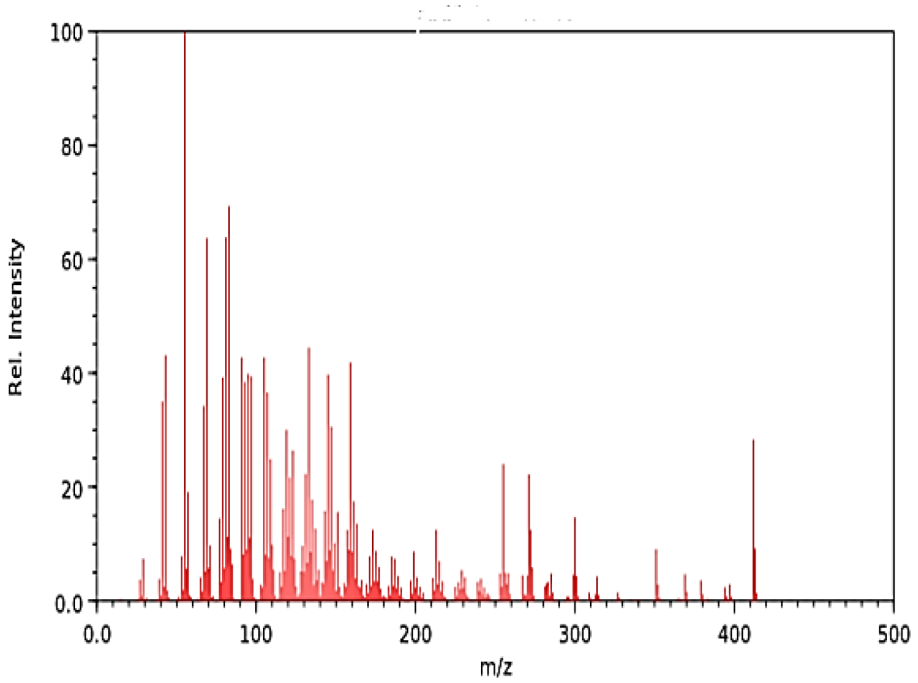



**Figure 2.** Mass spectrum of compound isolated from fraction F4.

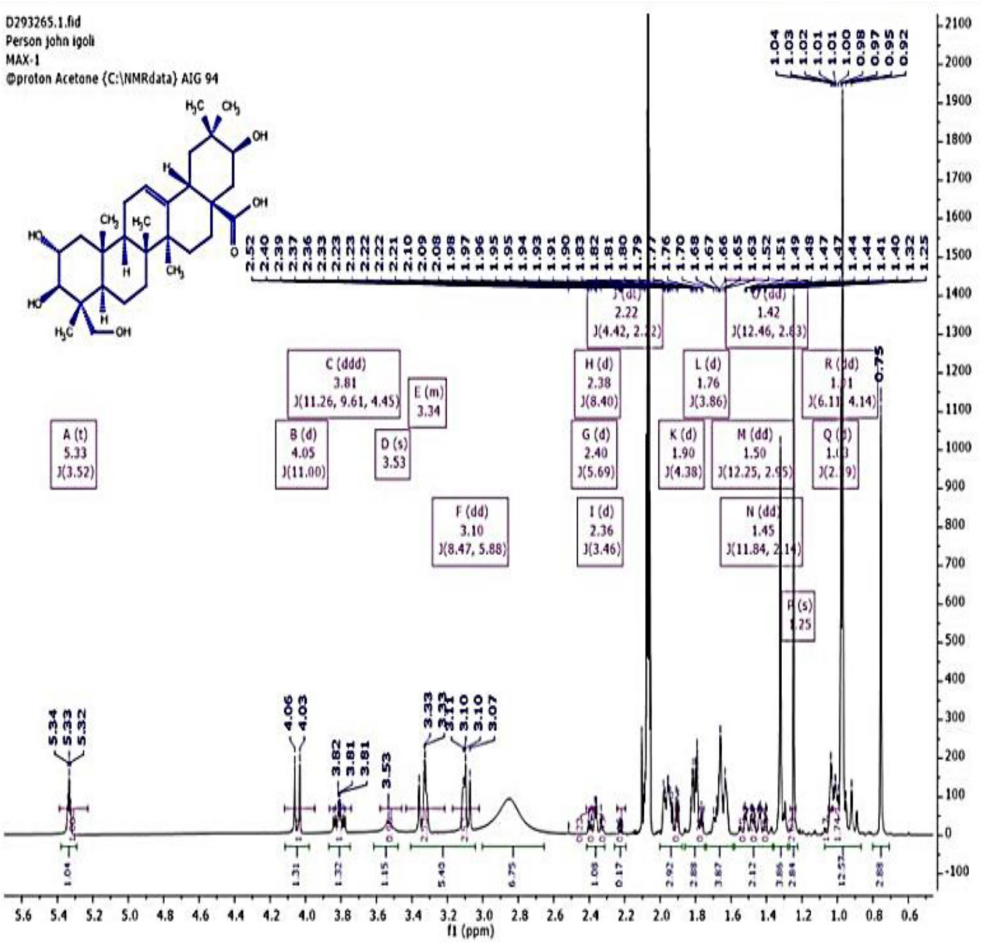



**Figure 3.** $^{1}$H NMR spectrum of the compound isolated from fraction F4.

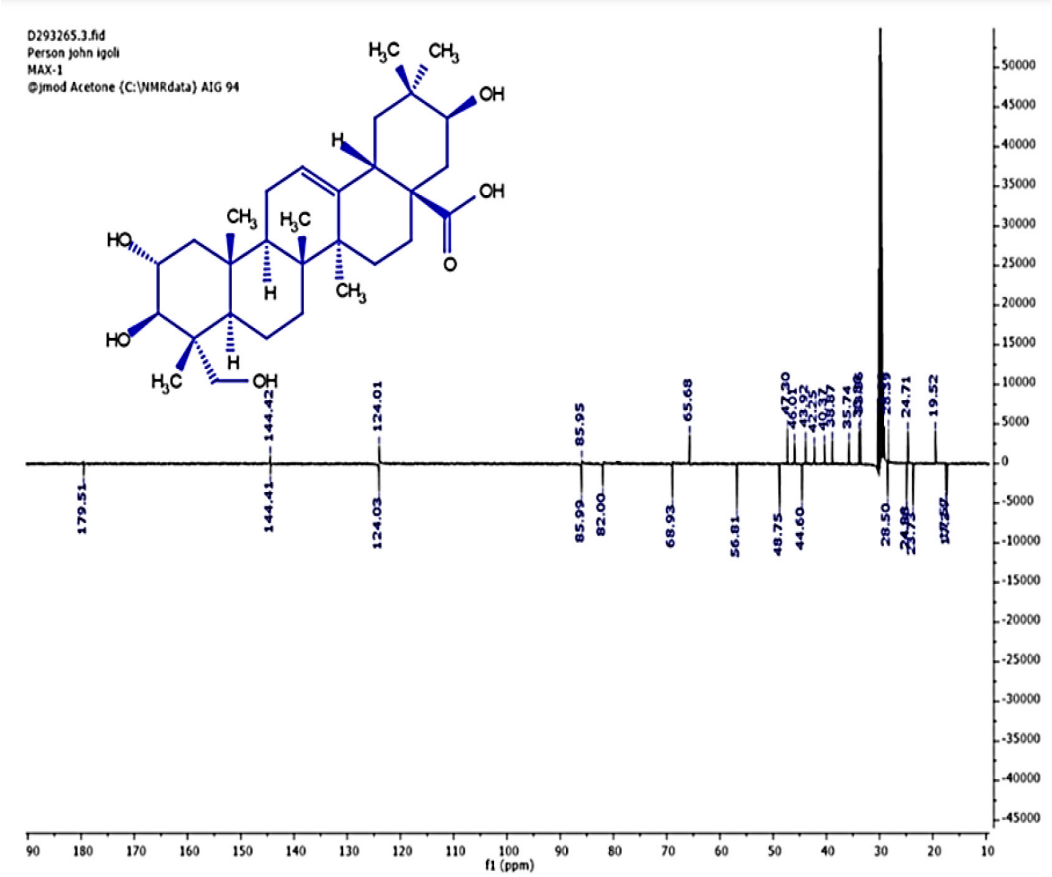



**Figure 4.** $^{13}C$ NMR spectrum of compound isolated from fraction F4.

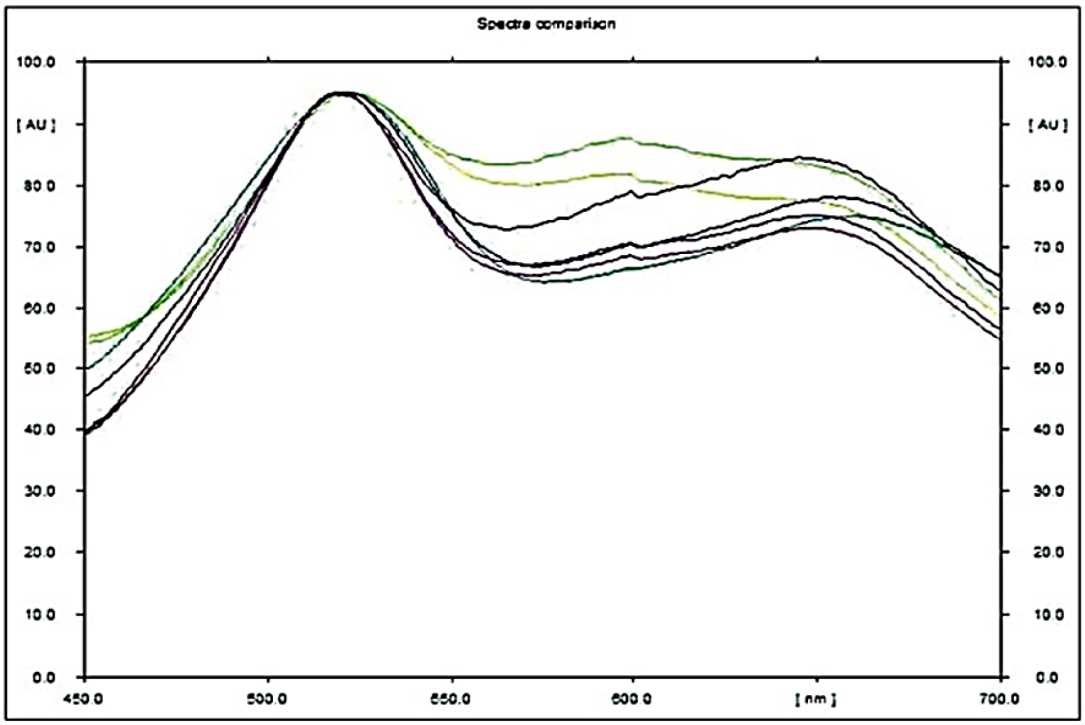



**Figure 5.** UV-visible spectrum of the purified compound isolated from fraction F4 showing $\lambda_{max}$ at 257 nm.

$cm^{-1}$, which confirms the structure of stigmasterol (Figure 1).

Mass spectrometric analysis showed a molecular ion peak at m/z 412 [$M^+$], consistent with the molecular formula of stigmasterol (Figure 2).

The $^1H$ NMR spectrum exhibited characteristic signals including a multiplet at δ 5.14 for the olefinic H-6 proton and a triplet of double doublets at δ 3.25 for the H-3 proton attached to the hydroxyl-bearing carbon. Additional olefinic protons at δ 4.14 and 4.61, respectively, identical with the chemical shift of H-22 and H-23 of stigmasterol. Six methyl group signals appeared between δ 0.91 and 1.26 (Figure 3).

The $^{13}C$ NMR spectrum showed resonances at δ 140.8 and 121.0 ppm corresponding to C-22 and C-6 double bonds. Signals at δ 130.1 and 129.1 ppm indicated additional unsaturation, while the signal at δ 71.6 ppm confirmed the presence of a β-hydroxylated carbon. Collectively, these spectral features conclusively identified the compound as stigmasterol (Figure 4).

The UV spectrum displayed a maximum absorption at 257 nm, further supporting the steroidal framework (Figure 5).

## 4. Discussion

This study assessed the therapeutic potential of different freeze-dried extracts of *C. gigantea* L. root bark (ECGR) administered intraperitoneally at doses of 100 and 150 mg/kg for mitigating asthma-like symptoms in rodent models of asthma and inflammation. Asthma is a chronic inflammatory disease characterised by airway inflammation, excessive mucus production, and heightened airway hyperresponsiveness. The milk-induced leukocytosis and eosinophilia model is a well-established experimental paradigm used to simulate the pathophysiological processes of asthma[15,16]. Leukocytes and eosinophils are key contributors to airway inflammation in asthma, as they release an array of pro-inflammatory mediators, including cytokines, histamine, and major basic protein, which collectively exacerbate airway inflammation. Additionally, leukocytes generate reactive oxygen species, contributing to oxidative stress and tissue injury[17,18]. Eosinophils are particularly implicated in mucus hypersecretion and bronchial hyperreactivity. In this study, treatment with the ethanolic extract of *C. gigantea* L. root bark (ECGR) significantly attenuated milk-induced elevations in both leukocyte and eosinophil counts. These results indicate that EECGR may exert anti-inflammatory effects, potentially through attenuation of oxidative stress and modulation of immune cell responses.

Anaphylaxis represents a severe, life-threatening hypersensitivity reaction characterised by the rapid release of histamine and pro-inflammatory cytokines. Common triggers include allergens such as egg albumin. EECGR demonstrated marked anti-allergic properties by inhibiting the release of these inflammatory mediators and stabilising mast cells, thereby preventing their degranulation. These effects may be attributed to the presence of bioactive constituents such as saponins and flavonoids within EECGR. Saponins have been documented for their mast cell-stabilising activities[19,20], while flavonoids, including apigenin and luteolin, are known to suppress the release of histamine and other pro-inflammatory mediators from immune cells[21,22]. The findings of the present study suggest that EECGR significantly reduces mast cell degranulation, indicating its potential efficacy in managing type I hypersensitivity reactions and highlighting its promise as a candidate for further therapeutic development.

The carrageenan-induced rat paw oedema model is a widely recognised experimental system for evaluating the anti-inflammatory efficacy of pharmacological agents[23,24]. Carrageenan induces paw oedema by stimulating the release of various pro-inflammatory mediators, including prostaglandins, leukotrienes, histamine, bradykinin, and tumour necrosis factor-alpha (TNF-α). The inflammatory response is biphasic[25]. The early phase, occurring within the first few hours, is characterised by the release of histamine, serotonin, and kinins, whereas the late phase, beginning approximately 2 to 3 hours post-injection, is driven primarily by prostaglandin-like substances. The late phase is particularly responsive to both steroidal and non-steroidal anti-inflammatory drugs. Pre-treatment with EECGR significantly reduced paw oedema volume and increased the percentage inhibition of oedema formation. These results collectively support the anti-inflammatory potential of EECGR.

The clonidine-induced catalepsy model serves as a reliable indicator of antihistaminic and antiallergic activity relevant to asthma. The $\alpha_2$-adrenergic agonist clonidine induces a dose-dependent state of catalepsy in mice. Experimental evidence demonstrates that this

effect is blocked by $H_1$ receptor antagonists, but not by $H_2$ antagonists, suggesting that the response is specifically mediated through $H_1$ pathways. This observation is further supported by findings that intracerebroventricular administration of histamine produces a similar cataleptic effect, which is also neutralised by $H_1$ blockers. Since clonidine stimulates the release of histamine from mast cells present within the mouse brain, clonidine-induced catalepsy is likely a direct consequence of mast cell-derived histamine activating central $H_1$ receptors[26,27].

The marked inhibition of catalepsy by fraction F4 suggests the presence of compounds capable of modulating histaminergic pathways or stabilising inflammatory mediators. Isolation and identification of stigmasterol provide a mechanistic rationale for the observed activity, as phytosterols are known to exhibit anti-inflammatory and immunomodulatory effects[28-30]. These findings demonstrate that enrichment of bioactive constituents through chromatographic fractionation significantly enhances pharmacological efficacy.

## 5. Conclusion

The ethanolic extract of *C. gigantea* L. root bark (EECGR) contains a range of bioactive phytoconstituents, including steroids, saponins, and flavonoids, which collectively contribute to its demonstrated anti-asthmatic and anti-inflammatory activities. These effects were evidenced by significant mitigation of milk-induced leukocytosis and eosinophilia, inhibition of mast cell degranulation triggered by egg albumin in mice, and suppression of carrageenan-induced acute paw oedema in rats. The present study confirms the antiasthmatic potential of *C. gigantea* L. root bark. Bioactivity-guided fractionation of EECGR identified stigmasterol as a principal active constituent effective in ameliorating clonidine-induced catalepsy in mice. The results support stigmasterol as a promising natural lead molecule for asthma management.

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## Author Contribution

Raosaheb Y. Ghegade: Conceptualisation, supervision, and manuscript review; Vishal B. Jadhav: Investigation, data collection, and original draft preparation; Chandrashekhar D. Patil and Ganesh B. Sonawane: Data analysis, manuscript writing, and editing; Deepak B. Somavanshi and Yogesh S. Ahire: Phytochemical and pharmacological investigations; Sujit A. Jadhav and Durgesh T. Gautam: Review, editing, and supervision. All authors have read and agreed to the published version of the manuscript.