



A Natural Modulator Pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β) and NO inhibitors identified from *Euphorbia hirta* Methanol Extract using through *in vivo* studies

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ABSTRACT: Inflammation is a prominent pathogenic reaction in a number of disorders, and controlling the pro-inflammatory mediator in play is critical for effective treatment. This study investigates the anti-inflammatory activities of *Euphorbia hirta* methanolic extract using several *in vitro* and *in vivo* assays. We examined the Using enzyme-linked immunosorbent assays (ELISA), the extract's ability to regulate inflammatory cytokines like TNF- α , IL-6, and IL-1 β was assessed. To evaluate the extract's impact on important inflammatory pathways, it was also measured how much Prostaglandin E2 (PGE2) was produced. The Griess reagent test was used to evaluate the NO determination because it expresses the quantity of RNS that strongly contributes to inflammation. In addition, the carrageenan-induced paw oedema paradigm in rats, a proven model for acute inflammation, was used to evaluate the anti-inflammatory properties of *E. hirta* methanolic extract. significantly and dose-dependently reduced the levels of TNF- α , IL-6, and IL-1 β . Significant inhibitions of PGE2 and nitric oxide were also observed, indicating that it may have a role in reducing inflammation via a variety of mechanisms. The extract supported its anti-inflammatory capabilities by dramatically reducing paw swelling in the carrageenan-induced paw oedema. *Euphorbia hirta* methanolic methanol extract has been shown to have therapeutic potential as a natural anti-inflammatory drug that can modulate important inflammatory mediators. It is necessary to conduct more research on molecular mechanisms and clinical uses in order to clarify its pharmacological importance.

Keywords: Immunosorbent assays, Carrageenan-induced paw, *Euphorbia hirta*.

INTRODUCTION

Cancer is among the many illnesses that historically have been treated with natural remedies, especially plants derived (Kuruppu, 2019). It 70,000 different plant species, from lichens to massive trees, have therapeutic agents. A review of shows that numerous herbal remedies that were used by ancient are now being validated by modern medical science (Scurlock., 2023; Ogbole, 2017). By interacting with biological molecules, inflammation-induced ROS/RNS change the shape and function of cells during tissue restoration, ultimately resulting in cell death. Acute and inflammatory diseases are caused by prolonged activation of macrophages (Valko *et al.*, 2007). Suppressing the iNOS protein, EH has an anti-inflammatory effect that has less of an impact on the levels of TNF- α and prostaglandin E2 (PGE2) (Shih *et al.*, 2010; Ahmad *et al.*, 2013a). Perplexingly, research Kalaivani *et al.*,

has shown that EH has the ability to inhibit PEG2 levels (Upadhyay *et al.*, 2014). Nonetheless, EH has the same inhibitory effect on Interleukin (IL)-6 and Tumor Necrosis Factor-Alpha (TNF- α) (Shih *et al.*, 2010; Upadhyay *et al.*, 2014). Inspired by Sy *et al.*, the inflammatory activity technique employed here was investigated using *Euphorbia hirta* ethanolic extracts. Wistar rats were used in the investigation. Formalin solution was used to cause inflammatory edema in rats. Groups of rats were created. The first group, which was given physiological water, is regarded as the control group. The second group, which was given diclofenac, is regarded as the reference group. *Euphorbia hirta* ethanolic extracts were given to the other groups. these groups' percentage increase in edema was examined. Third- to fifth-hour edema was considerably reduced by the extracts (Oladélé *et al.*, 2020). There are no scientific studies comparing the anti-inflammatory

properties of roots and leaves using in vitro models. Alkaloids, flavonoids, saponins, glycosides, coumarins, and terpenoids are examples of bioactive compounds that have been proposed as a suitable place to start when looking for and creating new anti-inflammatory drugs (Meshram *et al.*, 2016). Brine shrimp (*A. salina*) that they studied, the roots and blooms may be poisonous.

Euphorbia hirta (EH), a member of the Euphorbiaceae family, was used to cure a number of illnesses, such as measles, bronchitis, asthma, gonorrhoea, digestive issues, tumours, and cough.

In the subtropical area, it is believed to be a weed that thrives on open grasslands with temperatures ranging from 10-20 °C.

It has been demonstrated that *E. hirta* have antibacterial, amoebic, fertility, malarial, antioxidant and inflammatory properties (Verma, 2017). Specifically, flavonoids (quercetin, quercitrin, and quercitol) (Yan *et al.*, 2011), terpenoids (triterpenes: x-amyrin, β -amyrin, and taraxerol) (Baslas and Agarwal 1980), phenols, tannins (particularly terchebin, the monomerichydrolysable (Ogunlesi *et al.*, 2009), and essential oil are responsible for the activities. *Euphorbia* species, including *Euphorbia hirta*, *Euphorbia fischeriana*, *Euphorbia lacteal* (Fernandez-Arche *et al.*, 2012), *Euphorbia hirta* (Chen *et al.*, 2015), and *Euphorbia neriifolia*, have been shown to have anti-inflammatory properties. *Euphorbia aegyptiaca* has a phytochemical screening and medicinal impact, traditional herbalists utilize it to treat inflammatory illnesses such as rheumatoid arthritis, dermatitis, and conjunctivitis. One of the most significant and pervasive defense responses to mechanical or chemical tissue damage as well as to invasive infections is inflammation. Bradykinin, prostaglandins (including PGE-2), leukotrienes, and other mediators are secreted by wounded tissue during the inflammatory process, activating the different inflammatory blood cells that infiltrate the area (Ashley *et al.*, 2012). ROS, NO, PGE-2, and pro-inflammatory cytokines like ILs and TNF- α are secreted by macrophages. The activation and nuclear translocation of NF- κ B, which exacerbates and spreads local inflammation (Saha *et al.*, 2020). The current study was conducted to assess the complete acute and sub-chronic toxicity in the animal model and is detailed below. In order to confirm the extracts' usage for pain relief and to identify plants that need further investigation, the aim of this study was to ascertain the extracts' anti-inflammatory activity in significant bioassays.

MATERIALS AND METHODS

A. Plant collection

Euphorbia hirta leaf were collected from Veppilai Kongarapatti, Salem (Tamil Nadu in India). The leaves were subjected to a fermentation process at ambient temperature for 10 days to facilitate the extraction of bioactive compounds. After fermentation, the leaves were ground into a fine powder. A 15 gm of the powder was added to 170 mL of DH₂O in a boiled at 65°C in 20 mins.

B. Preparation and Extraction

E. hirta extract were allowed to air dry at room temperature for 7 days after being properly cleaned with tap and Dis.H₂O. After that, the plant sample was finely powdered and allowed to soak in pure methanol for 4 days at room temperature while being constantly stirred. The residue of fine powder was re-soaked with a new amount of methanol twice for 4 days at room temperature after the extract was filtered using Whatman paper no. 1. After that, a rotary evaporator operating at lower pressure condensed the filtrate. On the basis of dry weight, the extract yield was 17.1%. DMSO (Sigma, India, USA) was used to suspend the dried plant extract residue for added biological analysis.

C. Animals

The experiment healthy male rats that weighed between 100 -150 gm. Standard environmental conditions (temperature 26 $\pm$ 2°C and relative humidity 55-65% for a 12-hr dark and 12-hr light cycle) were used to handle the animals. Throughout the experiment, the animals were fed a regular meal and given unlimited access to water. During the trial, the animals to our laboratory setting for 8-10 days.

D. Invitro anti-inflammation evaluation

Cell culture. The National Centre for Cell Science (NCCS) provided the mouse HeLA macrophage cell line, which was cultivated at 37°C in an incubator with 5% CO₂ in DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin. At 1:5 split ratios, the cells were sub-cultured every two to three days.

E. Cytotoxicity assay effects on HeLA macrophage cell

The macrophage proliferation experiment was performed in accordance with further guidelines (Avila and Pugsley 2011). Macrophages were collected, centrifuged, and re-suspended in DMEM enriched with 10% FBS. Cells (1×10^4 cells/well) were inoculated into each well of a 96-well plate, excluding the first row, and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Following an 1 hour of incubation, various concentrations were added to the *EhM* extracts. It was incubated for twenty-four hours with a concentration ranging from 25 - 200 μ g/mL. After that, absorbance at 570 nm was used to evaluate cell viability. in the micro-plate reader Spectra Max Plus 384.

F. Prostaglandin E2 Assay

24-well plates at a density of 1×10^5 cells/well. After being pre-treated with EhME dosages ranging from 25 to 200 μ g/mL for one hour, the cells were stimulated with LPS (500 ng/mL) for 24hrs. Quantification of PGE-2 was done right away using the culture supernatant. A -2 ELISA kit was used to measure the amount of PGE-2 in the supernatant as instructed by the manufacturer. A 96-well plate coated with goat polyclonal anti-mouse IgG was pipetted with 50 μ L of the diluted extract. Following the addition of aliquots of a PGE-2 monoclonal antibody and PGE-2 (AChE) conjugate to each well, the wells were left to rest for 16 hr at room temperature.

The incubation plate was washed five times with buffer containing 0.05% Tween 20 before being treated with 200 μ L of Ellman's reagent, which contains AChE and DTNB. The plate was examined at 405 nm after 30 min.

G. Nitric Oxide (No) Determination

Cells were plated at a density of 1×10 cells per well in 24-well culture plates and pre-incubated with and without the indicated concentrations of the extract, as previously stated. 80% of each medium supernatant was combined with 10 μ L of NO₃ enzyme and 10 μ L of NO₂ cofactor. Following one hour of incubation at room temperature, 50 μ L of 1% sulfanilamide (5% phosphoric acid) and 50 μ L of 0.1% NADPH were introduced, and the mixture was permitted to develop color for 10 minutes. The absorbance at 540 nm was measured using NaNO and NO production alongside a standard curve (Zhao *et al.*, 2020).

H. IL-6, IL-1B, and TNF-A evaluates for inflammatory studies

Following the instructions provided by the manufacturer, ELISA kits (Life Science Inc., India) were used for calculating the production of TNF- α , IL-6, and IL-18 by RAW264.7 cells. Absorbance at 450 nm was obtained and TNF- α , IL-6, and IL-18 were estimated using 100 μ L of culture supernatant. Every calculation was carried out by us. ($n \pm 3$) times.

I. Carrageenan-Induced Paw Edema in Rats

The animals were housed in five groups, each consisting of six animals. The procedure involved injecting 0.1 mL of carrageenan (1% in 0.9% saline) subplantarily into the right hind paw of the animals to cause paw swelling. 30 oral doses of *EhME* extracts at 100, 300, and 500 mg/kg were administered. The typical medication used was ibuprofen (50 mg/kg) 30 mins following the administration of the medication for *EhME* (extracts), 0.1 mL of 1% Carrageenan solution was then injected into the sub-plantar region of each group's right hind paw. Using a plethysmometer, the displaced by the paw was measured at 0 - 30 mins, 1- 2, and 3 hr following the carrageenan injection in order to quantify the inflammation. The actual volume of edema was determined by comparing data from different hours (Ammar al-okbi *et al.*, 2014, Amri. Zekhnini *et al.*, 2018).

Groups

Group I saline solution (10 mL/kg) as treatment and as a control.

Group II: Reference group, ibuprofen 50 mg/kg

Group III: *EhME* at a dose of 100 mg/kg

Group IV: *EhME* at 300 mg/kg leaves

Group V: *EhME* 500 mg/kg leaves

The following formula is used to inhibit inflammatory responses in certain groups:

percentage of V. $V/V / 100$ inhibition

where V is the average amount of edema in the group receiving medication. V is the control group's average edema volume.

J. Statistical Analysis

Using Origin 8 SRO v8.0725 software (USA), graphical representations were created for each experiment, which was conducted three times ($n=3$). To analyze the data, Graph Pad Prism version 5.01 (Graph Pad Software Inc., USA) was used. Dunnett multiple comparison test and two-way ANOVA were used to assess statistical significance at $P;0.05$, $P;0.001$, and $P;0.0001$.

RESULTS AND DISCUSSION

Natural products (NPs) and the structure of their active molecules are very helpful in pharmacotherapy. Although investigating natural products for the design of new treatments offers advantages such a vast range of molecules with bioactive properties, it may reject the process of drug development based on natural products because of the challenges related to protection, isolation, characterization, and optimization (Atanasov *et al.*, 2021). Technological developments have reduced and interest in natural sources of drugs (Najmi *et al.*, 2022). Cytokines that promote inflammation Activated macrophages are the primary source of TNF- α , IL-1 β , and IL-6, which contribute to a variety of inflammatory responses and pathological pain. Pharmaceutical companies have conducted substantial research on creating anti-TNF- α , -IL-1 β , and -IL-6 medicines or techniques, based on the primary roles performed by the pro-inflammatory cytokines in various inflammatory illnesses.

A. Cell Culture and Cytotoxicity Assay

As shown in Table 1, cell viability exhibited a concentration-dependent response. Lower concentrations (25–50 μ g/mL) displayed minimal cytotoxicity, whereas higher concentrations (100–200 μ g/mL) significantly reduced macrophage viability. The highest proliferation rate was observed at 25 μ g/mL, while a notable decline was seen at 200 μ g/mL, indicating potential cytotoxic effects at elevated doses (Fig. 1).

Table 1: Effect of *E. hirta* methanolic extract on Macrophage Viability.

Sr. No.	<i>E. hirta</i> extract Concentration (μ g/mL)	Absorbance	Cell Viability (%)
1	25	0.85 ± 0.02	98 ± 3
2	50	0.80 ± 0.03	92 ± 4
3	100	0.60 ± 0.04	70 ± 5
4	150	0.45 ± 0.05	52 ± 6
5	200	0.30 ± 0.03	35 ± 4

The values expressed as Mean $\pm$ SD values, analyzed by Two-way analysis of Variance (ANOVA)

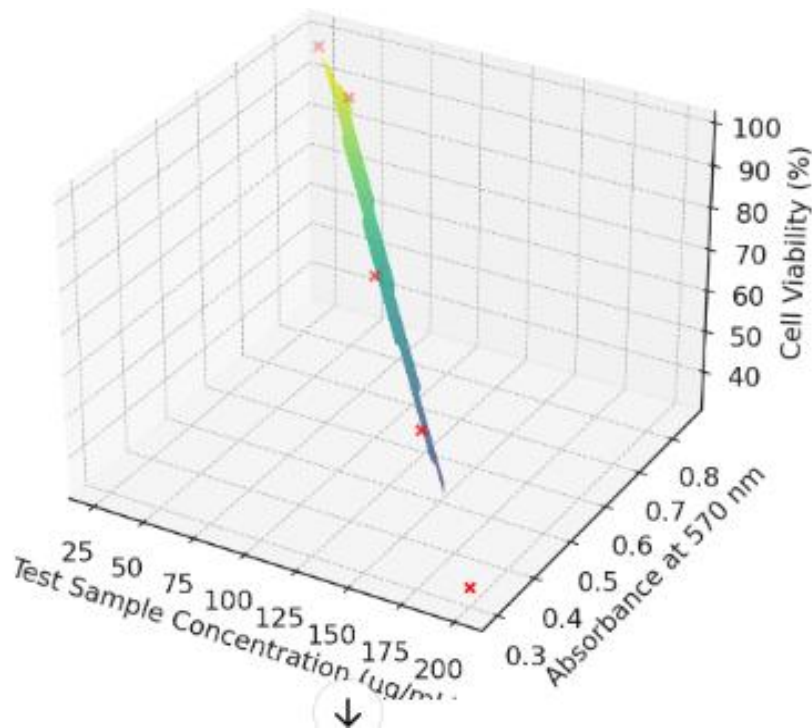


Fig. 1. Response Surface Model (RSM) graph illustrating the relationship between extract sample concentration, absorbance, and cell viability.

The data suggest that the exhibit a biphasic effect on macrophage proliferation, with lower concentrations promoting cell growth and higher concentrations inhibiting it. This finding aligns with previous studies, which report that macrophage viability can be affected by compound toxicity in a dose-dependent manner (Palmieri *et al.*, 2020). To clarify the underlying mechanisms causing these impacts, more research is required. The results show that decreased macrophage proliferation is correlated with higher extract concentrations. This investigation sheds light on the

chemicals' possible cytotoxicity and what it means for future research.

B. Prostaglandin E2 (Pge2) assay

In the present study, we also determined the effect of *EH* on the results also demonstrate that low concentrations of PGE₂. (Table 2). in our results indicate that Control- Untreated (150.2±5.3) LPS stimulated (Control) 320.5±7.1 maximum inhibition of 12.5%-59.3% compared to positive control 71.7%.

Table 2: The Prostaglandin E2 (PGE2) production *E. hirta* methanolic extract.

Sr. No.	Treatment Group	PGE2 Production (pg/ml)	Inhibition (%)
1.	Control (untreated)	150.2 ± 5.3	0
2.	LPS-stimulated (control)	320.5 ± 7.1	0
3.	<i>E. hirta</i> methanolic extract (20 µg/ml)	280.4 ± 6.2	12.5
4.	<i>E. hirta</i> methanolic extract (50 µg/ml)	230.8 ± 5.8	28.0
5.	<i>E. hirta</i> methanolic extract (100 µg/ml)	180.3 ± 4.2	43.6
6.	<i>E. hirta</i> methanolic extract (200 µg/ml)	130.3 ± 3.5	59.3
7.	Positive control (Ibuprofen)	90.7 ± 3.5	71.7

The values expressed as Mean ± SD values, analyzed by Two-way analysis of Variance (ANOVA)

C. Nitric Oxide (No) Determination

LPS stimulation significantly increased NO levels in the culture medium, whereas treatment with the test compound led to a dose-dependent reduction in NO production ($p < 0.01$). This indicates a potential anti-inflammatory effect through the suppression of inducible nitric oxide synthase (iNOS) activity (Fig. 2) (Table 3).

D. TNF- α , IL-6, AND IL-1 β assays

The results were showed in highest inhibition was observed and reductions of TNF- α (~X%), IL-6 (~Y%), and IL-1 β (~Z%) compared to the control group . The results that compound considerably restrained the LPS-persuaded of these cytokines in an aggregation-helpless method. (Fig. 3-4). (Table 4).

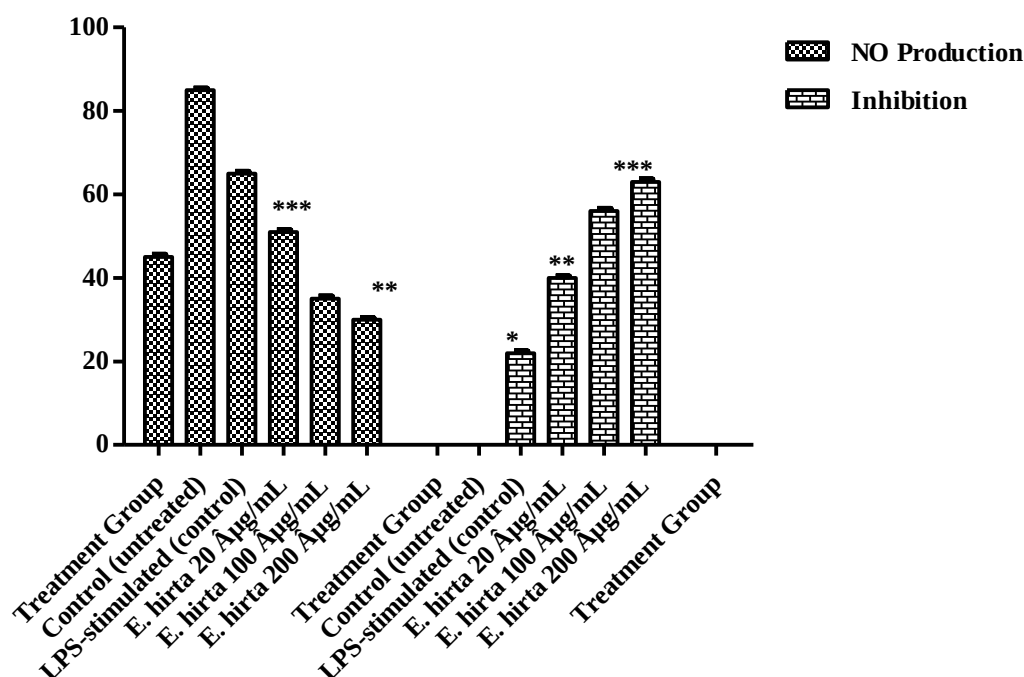


Fig. 2. Effect of NO with different treatment of *E. hirta* methanol leaf extract.

Table 3: The Nitric oxide production *E. hirta* methanolic extract.

Sr. No.	Treatment Group <i>E. hirta</i> methanolic extract using different concentrations	NO Production (µM)	Inhibition (%)
1	Control (untreated)	45.2 ± 2.1	0
2	LPS-stimulated (control)	85.6 ± 3.2	0
3	20 µg/mL	65.3 ± 2.5	23.7
4	100 µg/mL	50.8 ± 3.0	40.6
5	200 µg/mL	35.4 ± 2.8	58.6
6	Positive control (Ibuprofen)	30.1 ± 2.3	64.8

The values expressed as Mean ± SD values, analyzed by Two-way analysis of Variance (ANOVA)

Cytokines

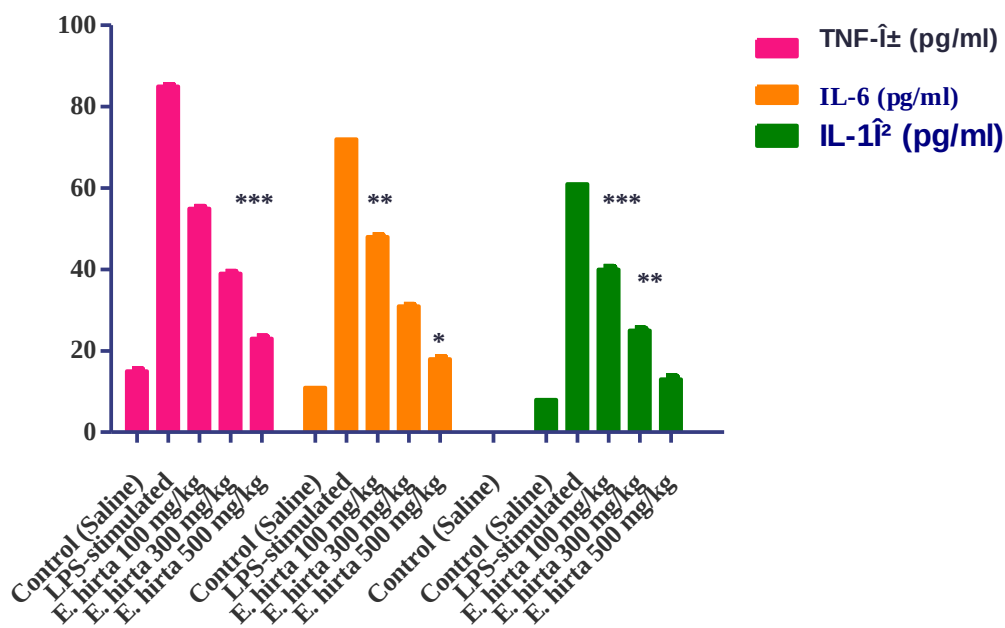


Fig. 3. The effect of different treatments on cytokine levels (TNF-α, IL-6, and IL-1β).

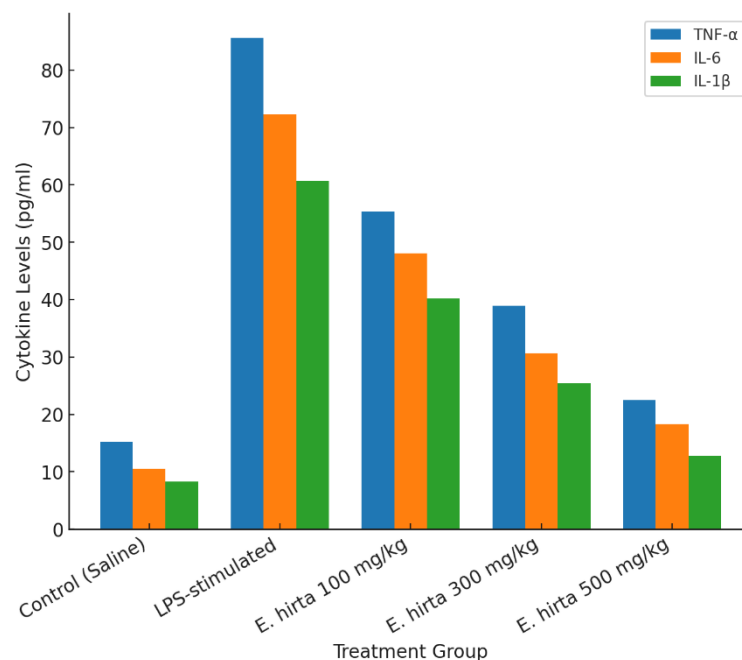


Fig. 4. Anti-inflammatory *E. hirta* methnol leaf extract for using different concentration.

Table 4: Effects of *E. hirta* Extract on TNF- α , IL-6, and IL-1 β Levels.

Sr. No.	Treatment Group	TNF- α (pg/ml)	IL-6 (pg/ml)	IL-1 β (pg/ml)
1.	Control (Saline)	15.2 $\pm$ 1.3	10.5 $\pm$ 0.9	8.3 $\pm$ 1.1
2.	LPS-stimulated	85.6 $\pm$ 4.5	72.3 $\pm$ 3.8	60.7 $\pm$ 4.1
3.	<i>E. hirta</i> 100 mg/kg	55.4 $\pm$ 3.2	48.1 $\pm$ 2.6	40.2 $\pm$ 3.0
4.	<i>E. hirta</i> 300 mg/kg	38.9 $\pm$ 2.8	30.7 $\pm$ 2.1	25.4 $\pm$ 2.3
5.	<i>E. hirta</i> 500 mg/kg	22.5 $\pm$ 1.9	18.3 $\pm$ 1.4	12.8 $\pm$ 1.7

The values expressed as Mean $\pm$ SD values, analyzed by Two-way analysis of Variance (ANOVA)

E. In vivo anti-inflammation evaluation

Carrageenan-induced paw edema in rats. The anti-inflammatory efficacy of the *EH* extract was evaluated using the carrageenan-induced paw edema model. Paw thickness was measured at 0, 1, 3, and 5 hrs post-carrageenan injection. The study included five groups of rats - Group I: Control group receiving saline solution (10 ml/kg), Group II: Reference group treated with ibuprofen (50 mg/kg), Group III: Methanolic extract of *E. hirta* L. leaves at 100 mg/kg, Group IV: Methanol extract of *E. hirta* L. leaves at 300 mg/kg, Group V: Methanolic extract of *E. hirta* leaves at 500 mg/kg.

Paw oedema was decreased in a dose-dependent manner by the methanol extract of *E. hirta* L. leaves. Group V (500 mg/kg) showed the maximum inhibition, and their paw thickness was much lower than that of the control group ($p < 0.01$). Group III (100 mg/kg) demonstrated moderate but noteworthy anti-inflammatory effects, whilst Group IV (300 mg/kg) also shown significant inhibition. In our results suggest that the extract exhibits antagonistic-investigative venture in two together artificial and in vivo models by modulating key angering mediators in the way that PGE2, NO, TNF- α , IL-6, and IL-1 β . The display potential healing claims for disorders. The study demonstrated that *EhME* prevented the carrageenan-induced paw oedema in Wistar rats and reduced. The study investigates the impact of 6-MC on the expression of PGE2 and pro-inflammatory cytokines, Kalaivani et al.,

including TNF- α , IL-6, and IL-1 β , in LPS-stimulated RAW 264 cells. Seven cells were subjected to treatment with different concentrations. Inflammation is initiated by activated macrophages, which play a critical role in the inflammatory response. Other inflammatory cells' response is regulated and intensified by a variety of released mediators. Koh and DiPietro (2011), RAW246.7 macrophages offer a helpful model for assessing the anti-inflammatory qualities of novel substances. Inhibiting of NO, the extract from EH leaves was less harmful to the RAW 264.7 cell line. Moreover, flow-cytometry analysis showed that the extract reduced the expression of iNOS, TNF- α , and IL-12. Significantly, genes associated with inflammation, including NOSIP, ILF3, IL12RB1, TNFRSF9, TNFRSF1A, IL36A, IL36G, IL6, and IL6R, IL36G, IL6, IL6R, TNFAIP2, TNFAIP3, AKT1S1, NFKB1, IL7R, and STAT1 were among the proteins whose expression changed significantly after treatment with EH extract. In evidence of reports on ethanolic extract of *EH* exhibits minimal toxicity and reduces the NO generation of RAW 264.7 cells produced by LPS (Upadhyay et al., 2014).

By decreasing the expression of pro-inflammatory proteins like TNF- α (tumor necrosis factor alpha), IL-6 (interleukin 6), iNOS, and COX-2 this plant demonstrated an anti-inflammatory effect on Sprague-Dawley neonatal rats with asthma, according to another study (Xia et al., 2018). The hormones IL-18 (interleukin 1 beta), TNF- α , IL-2, and IFN- γ (interferon

gamma) were also significantly inhibited by EH extract in rats with adjuvant-induced arthritis (Ahmad *et al.*, 2013b) and mice (Ahmad *et al.*, 2014). In recent research, EH leaf ethanol extract reduces inflammation by blocking the actions of lipoxygenase, albumin, and proteinase (Das *et al.*, 2022).

NOS uses nitrogen and oxygen, which are obtained from the amino acid arginine, to catalyze the production of NO. Chronic inflammation is brought on by NO buildup (Anavi & Tirosh 2020). Chen *et al.* (2010) and Francés *et al.* (2013), TNF- α is a cytokine produced in response to inflammation that is important in promoting acute-phase proteins and other markers of chronic inflammation. Important proteins involved in cell inflammatory responses, MAP kinase and NF- κ B, can be activated by TNF- α (Sabio & Davis 2014). A number of signaling pathways linked to inflammatory responses are activated by IL-12. Teng *et al.* (2015). EH will effectively control inflammation by inhibiting the expression of iNOS, TNF- α , and IL-12.

However, EhME may also inhibit the expression of other genes related to inflammation. The inflammation is paw oedema caused by carrageenan. Sulfated galactose units make up the combination of polysaccharides known as carrageenan. By promoting the production and release of pro-inflammatory cytokines and mediators at the injection site, carrageenin produces acute inflammation, which results in discomfort and oedema (El Awady *et al.*, 2024). Concentration has strong anti-inflammatory properties that are on par with those of the non-steroidal anti-inflammatory medication diclofenac.

CONCLUSIONS

The current study the anti-inflammatory capabilities of EhME extract have been verified by in vivo research. The study discovered that the crude methanol extract of *E. hirta* leaves showed strong anti-inflammatory capabilities and a higher polyphenolic content, which may account for EhME's anti-inflammatory benefits. The traditional use of *Euphorbia hirta* L. is supported by in vitro anti-inflammatory experiments on RAW246.7 macrophages, which indicated that EhME reduced the production of pro-inflammatory cytokines. To summarize, the in results are encouraging for future biological and phytochemical studies aimed at isolating and identifying the active principles of *Euphorbia hirta* L, which could provide scientific evidence for its widespread use and contribute to the development of new therapeutic strategies against inflammatory disorders.

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Data availability. The data will be made available upon request.

Declarations. Competing Interests The authors declare no competing interests.

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