

Anti-inflammatory Potential of *Euphorbia helioscopia* Extracts against RAW264.7 Macrophages

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ABSTRACT

Background: *Euphorbia helioscopia*, commonly known as sun spurge, is one of the largest species of an annual herb in the family Euphorbiaceae, which is extensively distributed worldwide. In folk medicine, it is used to treat various ailments such as malaria, osteomyelitis, and gastrointestinal ailments. The anti-inflammatory potential of *Euphorbia helioscopia* extracts on LPS-stimulated RAW264.7 macrophages was investigated in this work. **Results:** The ethanol extract was found to markedly inhibit the pro-inflammatory cytokines (IL-6 and TNF α) production compared to the aqueous extract treated cells. Moreover, extracts downregulated the nitric oxide levels. Finally, in this study, we have shown that *Euphorbia helioscopia* exerts a potent pharmacological effect on reducing the inflammatory response in macrophages. **Conclusion:** The results of the experiment revealed that the *Euphorbia helioscopia* extracts used in this study have anti-inflammatory characteristics, with the ethanolic extract having the most. The ethanolic extract had the strongest anti-inflammatory impact in terms of greatest reduction of proinflammatory indicators including IL-6, TNF-, and NO.

Keywords: *Euphorbia helioscopia*, Anti-inflammatory, ELISA, IL-6, TNF- α ,

BACKGROUND

Pain, redness, swelling, and the sensation of heat all are signs of inflammation, which would be a complicated immune system defense mechanism. Usually, in controlled manner inflammation is valuable to the host; however, its exaggerated response can be detrimental and leads to tissue injuries, and contributes to the development of several inflammatory ailments such as rheumatoid arthritis, diabetes, hypertension, obesity, chronic obstructive pulmonary disease, inflammatory bowel disease, inflammatory bowel disease atherosclerosis, multiple sclerosis and asthma, etc.^[1-2] Chronic inflammation of any region of the body or organs can also have serious consequences for a person's health. Most medications, such as corticosteroids and NSAIDs, reduce inflammation but have negative side effects when used long-term.^[2] Hence, there is a need for novel medicines of natural origin to treat inflammatory disorders with higher efficacy and fewer adverse effects.

Lipopolysaccharide Component of the cell wall of Gram-negative bacteria, stimulates macrophages and initiates many cellular signaling pathways by producing pro-inflammatory cytokines like TNF-, IL-6, and other inflammatory mediators like nitric oxide.^[3] Inflammatory mediators such as IL-6, IL-1 β , TNF, and nitric oxide produced by the TLR4 axis in response to LPS stimulation induce NF-kB

transcription factor activation, contributing to the development of chronic inflammatory disorders. Inhibition of aberrant macrophage activation could be useful in the treatment of inflammation-related diseases. As a result, LPS-induced inflammation in macrophages has become the most extensively used model for studying lead candidates' anti-inflammatory potential and mechanism.

E. helioscopia (*Euphorbia helioscopia*) is a medicinal plant broadly spread globally. For decades, it has been mainly used to treat numerous illnesses, including phlegm, edema, osteomyelitis, scab cough, malaria, tuberculosis, fistula, dysentery, and cancer.^[4-5] A study has indicated that *E. helioscopia* ethanol extract possesses antitumor activity in both *in vitro* and *in vivo* study.^[5-6] Nevertheless, the role of *E. helioscopia* extract on inflammation is not elucidated yet. The goal of our research was to see how anti-inflammatory *E. helioscopia* aqueous and ethanol extracts affected LPS-stimulated RAW 264.7 cells.

MATERIALS AND METHODS

Chemicals and reagents

Ethanol and aqueous extracts of *Euphorbia helioscopia* were obtained from the cold extraction of the aerial parts of *Euphorbia helioscopia*.

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Griess reagent and Dulbecco's Modified Eagle Medium (DMEM) were procured from sigma, TNF α and IL-6 ELISA kits were purchased from Invitrogen India. Lipopolysaccharide (LPS) *E. coli* serotype, and pen-strep, Phosphate Buffer Saline (PBS), and FBS were obtained from Gibco. MTT dye was procured from Himedia.

Plant Material

Aerial parts of the plant of *Euphorbia helioscopia* were collected from CSIR-IIIM, Field Station, Chatha, Jammu. The plant was known and authenticated by a plant taxonomist, Dr. Mohd Akhter of Taxonomy, University of Kashmir. A voucher specimen (voucher no. 1080) was placed in Kashmir University Herbarium (KASH).

Preparation of extracts

The total weight of collected plant material was 12kgs. The aerial part of the plant was cleaned properly and then processed for drying in a well-ventilated shade drier where fresh air was flowing continuously (drying room, NPC Division, IIIM Jammu.) with proper temperature (20–25°C) and humidity (74–76%). The dried plant components were mechanically ground at IIIM Jammu's c.GMP Division using a commercial electrical stainless-steel blender. After 5 days of preparation, the powdered plant material was stored in an airtight container at 4°C until extraction. The dried substance weighed around 1100 g. The powdered plant material was divided into two equal 550g parts, one for each of the two extractions. Cold extraction was performed on one section of the 550g powdered material using ethanol as the solvent, while cold extraction was performed on the other portion using water as the solvent.

Extraction; 550gms of processed plant material was kept in two large conical flasks and four Litre of a solvent such as ethanol and water were further added to each flask individually. The opening of the flask was closed with aluminum foil and kept in a reciprocating shaker for 24 hr at 150 rpm to ensure full mixing and complete elucidation of active ingredients to dissolve in the appropriate solvent. This process was repeated continuously for four consequent cycles.

The extract was then filtered via muslin cloth, Whatman no 1 filter paper, and a vacuum and pressure pump (AP-9925 Auto Science). A rotating vacuum evaporator RE52 with a water bath temperature of 50°C was used to remove the solvent from the extract. The residues were then collected and put to use in the experiment. The weight of the extracts obtained from the Ethanol and aqueous extraction was 55.67gms and 185gms, respectively.

Cell viability assay

Ethanol and aqueous extract of *E. helioscopia* was dissolved in DMSO. Cultured cells in 96 well plate were stimulated with 10 μ M, 5 μ M and 1 μ M concentrations of both the extracts for 48 hr. MTT determined the cell viability of treated cells. MTT solution 100 μ L (2.5mg/ml) was added to the treated cells before 4 hr of termination. Then, to dissolve the formazan, 100 μ L DMSO was added to each well. The absorption value was measured by Plate reader (Tecan infiniteM200) at 570 nm wavelength.

Quantification of Nitrite by Griess reagent

Raw 264.7 macrophages were treated with various concentrations of EA and AE for 1 hr before being stimulated for 24 hr with LPS (1 μ g/ml). The total inorganic nitric oxide released in the supernatant was then calculated using Griess reagent (0.1% N-1-naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid). The supernatant has been collected by centrifugation at 1100 rpm and kept at room temperature for 5 min with an equal volume of Griess reagent. A Tecan infinite M200 plate reader was used to detect absorbance at

540 nm. The formula was used to calculate NO % inhibition, with the nitrate level of the LPS stimulated group served as the control.

$$\text{Nitric oxide Inhibition \%} = \frac{\text{NO}_2 (\text{CONTROL})^* - \text{NO}_2 (\text{SAMPLE})}{\text{NO}_2 (\text{control})^*} \times 100$$

*Control: LPS induced group

Cytokine Production in RAW 264.7 cells

The inhibitory effect of Ethanol and aqueous extract of *E. helioscopia* on IL-6 and TNF α cytokine production in cell culture media was calculated by ELISA kit (Invitrogen). RAW 264.7 cells were seeded at a concentration of 1x10⁶ in each well and incubated overnight. After that, the cells were treated with both extracts, followed by a 1 hr LPS treatment to produce inflammation. Culture supernatant was collected and analyzed for IL-6 and TNF α produced in each sample.

Statistical Analysis

All of the studies were done in triplicate, and the findings are provided as the mean SD of three independent experiments, with *p*-values (***p*<0.001, ***p*<0.01, **p*<0.05) indicating significance. For the one-way ANOVA analysis, graph pad prism programme was used, followed by Dennett's test.

RESULTS

Effect of *Euphorbia helioscopia* extracts on Cell Viability

The cell viability study from MTT assay showed a minimum of 90.28% and 91.5% cell survival at a dose of 10 μ M of Ethanol and Aqueous extracts of *Euphorbia helioscopia* and maximum cell survival of 94.16% 95.26% at a dose of 1 μ M of Ethanol and Aqueous extracts (AE) (Figure 1).

Effect of *Euphorbia helioscopia* extracts on Nitric oxide determination

In a dose-dependent way, the *Euphorbia helioscopia* extract was able to reduce NO generation in RAW 264.7 cells. Ethanol and Aqueous extracts treatment at a concentration of 10, 5, 1 μ M showed 46.61, 38.22, and 30.13 percent of NO inhibition in Ethanol extract and 40.13, 31.64, and 28.34 for aqueous extract of *Euphorbia helioscopia*, respectively (Figure 2).

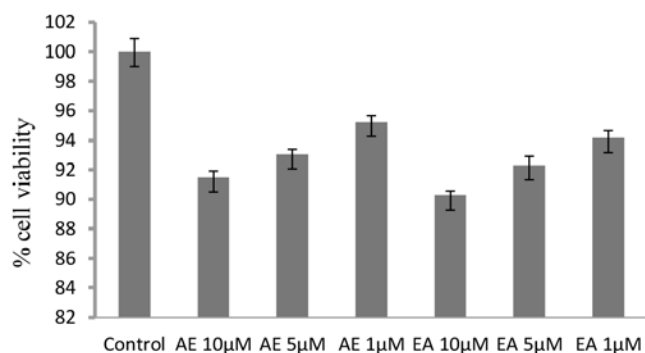


Figure 1: Percentage cell viability at different concentrations of EA and AE by MTT assay.

Data expressed as Mean \pm SD from three independent assays. Control indicates no treatment.

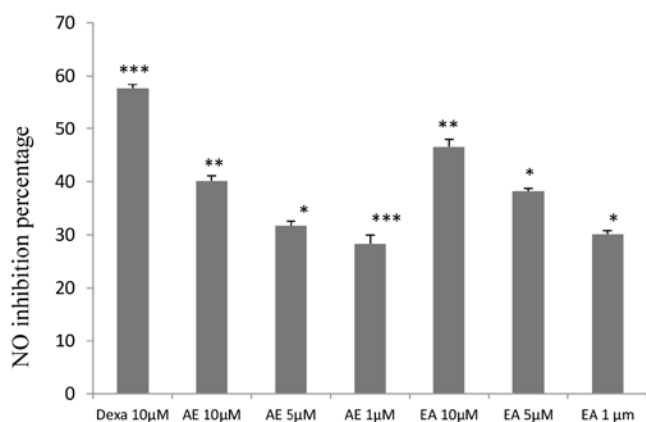


Figure 2: Effect of EA and AE treated cells on NO production in LPS stimulated RAW264.7 macrophages.

Data expressed as Mean \pm SD along with significance at p value *** p <0.001, ** p <0.01, * p <0.05 versus untreated LPS stimulated cells.

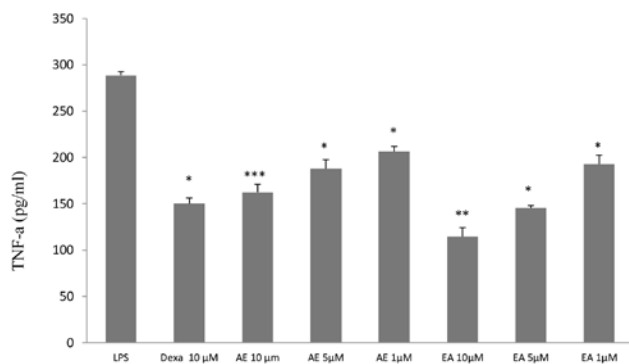


Figure 3: TNF- α production in untreated LPS stimulated, and AE and EA extracts treated (10, 5 and 1µM) LPS stimulated RAW264.7 macrophages by ELISA.

Data expressed as Mean \pm SD along with significance at p value *** p <0.001, ** p <0.01, * p <0.05 versus untreated LPS stimulated cells.

Effect of *Euphorbia helioscopia* extracts on TNF- α production

Effect of Ethanolic and Aqueous extracts of *E. helioscopia* on LPS induced production of TNF- α . Treatment of cells with Ethanolic and Aqueous extracts decreased the production of TNF- α in a dose dependent manner in comparison to LPS treated cells. Treatment of cells with 10, 5, and 1µM of Ethanol and Aqueous extracts of *Euphorbia helioscopia* showed 114.42, 145.31, 192.81 and 162.5, 187.8, and 206.6pg/ml production of TNF- α respectively, which is significantly less than LPS treated Cells (Figure 3).

Effect of *Euphorbia helioscopia* extracts on IL-6 production

The concentration of IL-6 released by LPS stimulated RAW264.7 cells decreases with the increase in the concentration of *E. helioscopia* extracts. (Figure 4) indicates that both Ethanol and aqueous extract decreased the IL6 production in RAW264.7 macrophages being 134.9, 205.8 and 310 Pg/ml for 10, 5 and 1µM of EA and 173.69, 291.19 and 370 pg/ml for 10, 5 and 2.5uM of AE respectively. The values indicate that extracts

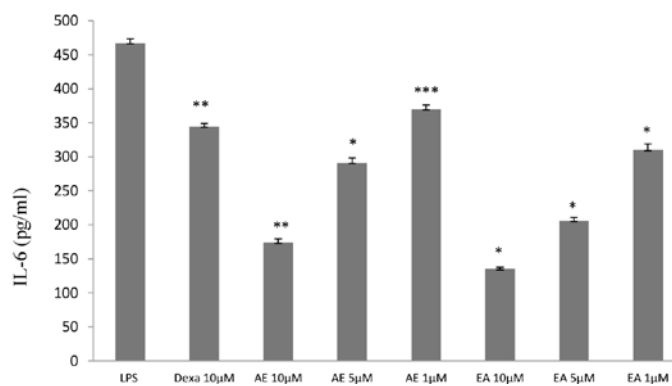


Figure 4: IL-6 production in LPS stimulated RAW macrophages and treatment with AE and EA extracts at different concentrations (10, 5 and 1µM). Data expressed as Mean \pm SD along with significance at p value *** p <0.001, ** p <0.01, * p <0.05.

treatment in LPS stimulated cells significantly inhibited the levels of IL-6. Maximum inhibition was observed in 10uM of EA.

DISCUSSION

The need for discovering new anti-inflammatory drugs with fewer side effects and a broader efficacy stems mostly from the undesirable effects of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). Plant-based molecules appear to be potential prospects for developing drugs that are free of these restrictions. The current research work is focused to evaluate the anti-inflammatory activity of ethanol and aqueous extract of aerial part of *Euphorbia helioscopia* on LPS stimulated RAW264.7 macrophages. LPS is a mitogen that increases the production of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6, as well as inflammatory mediators iNOS and COX-2, by activating NF- κ B via Toll-like receptor 4 (TLR4).^[7] It is well documented that NF- κ B is one of the most important transcription factors involved in the modulation of pro-inflammatory cytokines and inflammatory mediators.^[8]

Both extracts showed significant percent cell viability on RAW264.7 cells, cytotoxicity assay was done to evaluate the safety level of extracts. RAW264.7 cells were cultured with extracts (1µM-10µM) for 48 hr and the cell viability percentage is shown in Figure 1. The result shows that in comparison to the normal cells, ethanol extracts showed significant percent cell viability.

NO plays a key role in the pathophysiology of inflammation as a mediator and signaling molecule. Inducible NO synthase (iNOS) is the principal enzyme involved in NO synthesis during the inflammatory process, and its expression is boosted by inflammatory reactions and cytokines. As a result, neutrophils, macrophages, and other cells produce excessive amounts of NO.^[9]

In this experiment, we found that extracts of *E. helioscopia* exhibited a dose-dependent reduction in NO levels in cultured macrophages. RAW 264.7 macrophages treated with ethanol extract at 10uM produced the lowest amount of NO, indicating a 46.61 percent reduction of NO generation (Figure 2).

Small proteins produced by several number of cells, including macrophages, neutrophils, and endothelial cells, are hypothesized to be orchestrators of inflammation.^[10] TNF- is an important inflammatory mediator and signaling that molecule that has been discovered as a crucial therapeutic target for inflammatory disorders such as spondylitis, rheumatoid arthritis, psoriasis, inflammatory bowel disease, and others.^[11] In compared to LPS-treated cells, extract-treated cells showed a significant drop in TNF- in a dose-dependent manner, according to our findings.

TNF production was determined to be lowest at 10 M EA (Figure 3). The inflammatory process and signaling are both aided by IL-6. It is linked to inflammatory disorders like cartilage metabolism, cancer, angiogenesis, chronic anaemia, arthritis, and the acute-phase response, among others.^[12] Treatment with *Euphorbia helioscopia* extracts reduced the generation of IL-6 in RAW 246.7 macrophages in a dose-dependent manner, with a substantial difference compared to LPS-treated cells, according to our findings (Figure 4). At 10M, Ethanol extract had the lowest production.

In summary, our data indicated that *Euphorbia helioscopia* possesses significant anti-inflammatory activity. Extracts of *Euphorbia helioscopia* suppress several pro-inflammatory mediators and cytokines, in *in vitro* model. Besides Isolation and characterization of molecules from extracts, as well as exploration of the signaling cascade involved, could have further justified the molecule's anti-inflammatory properties, indicating potential study limitations.

CONCLUSION

In this study, we extracted *E. helioscopia* using aqueous and ethanol extracts and tested their anti-inflammatory activity in an *in vitro* model utilizing raw 264.7 cells. In a dose-dependent manner, the extracts were able to suppress the levels of pro-inflammatory mediators such as IL-6, TNF, and NO. These mediators are thought to be orchestrators of inflammation and could be used as therapeutic targets in the future. Our findings suggested that an ethanol extract of *Euphorbia helioscopia* could reduce pro-inflammatory mediators; therefore, isolated molecules from extracts and elucidating the underlying molecular mechanism will be a step forward in this approach.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

iNOS: Inducible NO synthase; **E. helioscopia:** *Euphorbia helioscopia*; **EA:** Ethanolic extract; **AE:** Aqueous extract; **LPS:** Lipopolysaccharide;

NO: nitric oxide; **IL-6:** Interleukin 6; **NF-Kb:** nuclear factor kappa B; **COX:** cyclooxygenase; **IL1-β:** Interleukin 1 beta; **ELISA:** Enzyme linked immune sorbent assay; **TLR:** Toll like receptor; **TNFα:** tumor necrosis factor alpha; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; **NSAIDs:** Non-steroids anti-inflammatory drugs.

REFERENCES

- Gupta S, Ahsan AU, Wani A, Khajuria V, Nazir LA, Sharma S, et al. The amino analogue of β-boswellic acid efficiently attenuates the release of pro-inflammatory mediators than its parent compound through the suppression of NF-κB/IKBα signalling axis. *Cytokine*. 2018;107:93-104. doi: 10.1016/j.cyt.2017.12.004, PMID 29229421.
- Mani S, Duraipandian C, Chidambaram SB. Analgesic, anti-inflammatory and acute oral toxicity profile of leaf and bark extracts of *Albizia procera*. *BMC Complement Med Ther*. 2022;22(1):50. doi: 10.1186/s12906-021-03497-7, PMID 35216561.
- Singh A, Chibber P, Kolimi P, Malik TA, Kapoor N, Kumar A, et al. Rohitukine inhibits NF-κB activation induced by LPS and other inflammatory agents. *Int Immunopharmacol*. 2019;69:34-49. doi: 10.1016/j.intimp.2019.01.015, PMID 30665042.
- Yang Y, Chen X, Luan F, Wang M, Wang Z, Wang J, et al. *Euphorbia helioscopia* L.: A phytochemical and pharmacological overview. *Phytochemistry*. 2021;184:112649. doi: 10.1016/j.phytochem.2020.112649, PMID 33440297.
- Wang ZY, Liu HP, Zhang YC, Guo LQ, Li ZX, Shi XF. Anticancer potential of *Euphorbia helioscopia* L extracts against human cancer cells. *Anat Rec (Hoboken)*. 2012;295(2):223-33. doi: 10.1002/ar.21517, PMID 22190452.
- Cheng J, Han W, Wang Z, Shao Y, Wang Y, Zhang Y, et al. Hepatocellular Carcinoma Growth Is Inhibited by *Euphorbia helioscopia* L. Extract in Nude Mice Xenografts. *Biomed Res Int*. 2015;2015:601015. doi: 10.1155/2015/601015, PMID 26090427.
- Kim JY, Park SJ, Yun KJ, Cho YW, Park HJ, Lee KT. Isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* inhibits LPS-induced iNOS and COX-2 expression via the attenuation of NF-kappaB in RAW 264.7 macrophages. *Eur J Pharmacol*. 2008;584(1):175-84. doi: 10.1016/j.ejphar.2008.01.032, PMID 18295200.
- Roman-Blas JA, Jimenez SA. NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. *Osteoarthritis Cartilage*. 2006;14(9):839-48. doi: 10.1016/j.joca.2006.04.008, PMID 16730463.
- Ahmad G, Hassan R, Dhiman N, Ali A. Assessment of Anti-inflammatory Activity of 3-Acetylmyricadiol in LPS Stimulated Raw 264.7 Macrophages. *Comb Chem High Throughput Screen*. 2022;25(1):204-10. doi: 10.2174/1386207324666210319122650. PMID 33745430.
- Wojdasiewicz P, Poniatowski LA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm*. 2014;2014:561459. doi: 10.1155/2014/561459, PMID 24876674.
- Bradley JR. TNF-mediated inflammatory disease. *J Pathol*. 2008;214(2):149-60. doi: 10.1002/path.2287, PMID 18161752.
- Sharma JN, Al-Omran A, Parvathy SS. Role of nitric oxide in inflammatory diseases. *Inflammopharmacology*. 2007;15(6):252-9. doi: 10.1007/s10787-007-0013-x, PMID 18236016.

GRAPHICAL ABSTRACT



SUMMARY

Inflammation is an innate immune response of the body, however, exaggerated inflammatory response leads to the genesis of various diseases. The conventional therapy used to treat inflammation has various side effects. Plant-based therapy seems to be an alternative. *Euphorbia helioscopia* is a medicinal plant that possesses various pharmacological activities. Examined for its anti-inflammatory potential in Raw264.7 macrophages, the ethanolic extract of *Euphorbia helioscopia* at 10uM demonstrated a strong anti-inflammatory activity.

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