

Active *Cousinia thomsonii* Extracts Modulate Expression of Crucial Proinflammatory Mediators/ Cytokines and NFκB Cascade in Lipopolysaccharide-Induced Albino Wistar Rat Model

This article was published in the following Dove Press journal:
Journal of Inflammation Research

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Introduction: Chronic inflammation is implicated in a multitude of diseases, including arthritis, neurodegeneration, autoimmune myositis, type 2 diabetes, rheumatic disorders, spondylitis, and cancer. Therefore, strategies to explore potent anti-inflammatory regimens are pivotal from a human-health perspective. Medicinal plants represent a vast unexplored treasure trove of therapeutically active constituents with diverse pharmacological activities, including anti-inflammatory properties. Herein, we evaluated *Cousinia thomsonii*, an edible medicinal herb, for its anti-inflammatory/immunomodulatory properties.

Methods: Soxhlet extraction was used to obtain different solvent extracts (hexane, ethyl acetate, ethanol, methanol, and aqueous extract) in increasing order of polarity. In vitro anti-inflammatory assays were performed to investigate the effects of extracts on protein denaturation, proteinase activity, nitric oxide surge, and erythrocyte-membrane stabilization. The most effective extracts, ie, ethyl acetate (CTEA) and ethanol (CTE) extracts (150–200 g) were selected for further in vivo analysis using albino Wistar rats. Wistar rats received varying concentrations of CTEA and CTE (25, 50, and 100 mg/kg) for 3 weeks, followed by a single subplantar injection of lipopolysaccharide. Dexamethasone served as positive control. Blood was obtained from the retro-orbital plexus and serum separated for estimation of proinflammatory cytokines (IL6, IL1β, IFNγ and TNFα). Western blotting was performed to study expression patterns of crucial proteins implicated in the NFκB pathway, ie, NFκB p65, NFκB1 p50, and NFκB2 p52. Histopathological examination was done and gas chromatography–mass spectrometry (GC-MS) carried out to reveal the identity of compounds responsible for ameliorating effects of *C. thomsonii*.

Results: Among five tested extracts, CTEA and CTE showed marked inhibition of protein denaturation, proteinase activity, nitric oxide surge and erythrocyte-membrane hemolysis at 600 μg/mL ($P<0.001$). Both these extracts showed no toxic effects up to a dose of 2,500 mg/kg. Extracts exhibited concentration-dependent reductions in expression of IL6, IL1β, IFNγ, TNFα, NFκB-p65, NFκB1, and NFκB2 ($P<0.05$). Healing effects of extracts were evident from histopathological investigation. GC-MS analysis revealed the presence of important anti-inflammatory compounds, notably stigmast-5-en-3-ol, oleate, dotriacontane, ascorbic acid, *n*-hexadecanoic acid, and α-tocopherol, in *C. thomsonii*.

Conclusion: *C. thomsonii* possesses significant anti-inflammatory/immunomodulatory potential by virtue of modifying levels of proinflammatory cytokines/markers and NFκB proteins.

Keywords: chronic inflammation, interleukin, cytokine, immunoresponse, nitric oxide, compounds

Introduction

Inflammation is a complex biological response to harmful stimuli, such as microbial infection, injury, or toxins. This process is regulated by multiple factors, eg, proinflammatory mediators/cytokines, enzymes, vasodilators, and immune cells. Acute inflammation is triggered by pathogenic infections or injuries and usually lasts a few weeks, while chronic inflammation is triggered by self-damaging autoimmune reactions or due to infections from persistent antigens, and usually lasts months or years. Normally, acute inflammation displays a protective role by eliminating infectious agents, thereby initiating the healing process, but uncontrolled/chronic inflammation proves destructive, since it triggers continuous synthesis of proinflammatory mediators like prostaglandins, kinins, leukotrienes, IFN γ , TNF α , IL1 β , and IL6, as well as free radicals that cause severe tissue damage.¹ Chronic inflammation is implicated in many dreadful human diseases, notably arthritis, ulcerative colitis, asthma, Crohn's disease, cardiovascular diseases, stroke, neurodegenerative diseases, and cancer.^{2,3} Worldwide, three in five mortalities are associated with chronic inflammatory diseases.⁶ Arthritis alone affects 15% of the Indian population, ie, >180 million people. Psoriasis has a prevalence of 0.44%–2.8% in India.⁷ The overall estimated inflammatory bowel-disease population in India is approximately 1.4 million, second only to the US (with 1.64 million).⁸ As per reports, there were roughly 2.5 core cases of asthma patients in India during the year 2001.⁹ The estimated prevalence of inflammatory diseases in the Western world is 5%–7%.⁴ The prevalence of ulcerative colitis in adults in the US is 238 per 100,000 people and that of Crohn's disease 201 per 100,000 people.¹⁰ Gout is the most prevalent form of inflammatory arthritis. It impairs the quality of a person's life through severe pain and inflammation of acute gout attacks from deposits of monosodium urate crystals in the joints. It is more common in men, and is strongly age-related. Studies performed at the Royal College of General Practitioners between 1994 and 2007 revealed that the mean annual incidence of gout was 18.6 per 10,000 people.¹⁰ Pelvic inflammatory disease (PID) is a common ailment among females, and is characterized by infection of pelvic organs, including the uterus, fallopian tubes, ovaries, and cervix. More than 750,000 new cases of PID are diagnosed every year in the US. In modern industrialized countries, the annual incidence of PID in women aged 15–39 years seems to be 10–13 per 1,000 women, with peak incidence of about 20 per 1,000 in the age-group 20–24 years.^{11,12}

Medicinal herbs and formulations thereof have been used for treating inflammatory diseases for centuries. For instance, arthritis, oral inflammation, eye inflammation, postsurgical inflammation, edema, and rheumatism are frequently treated in Ayurvedic and Unani medicine.¹³ In this study, we evaluated *Cousinia thomsonii* for its anti-inflammatory/immunomodulatory activities. *C. thomsonii*, commonly known as Thomson's thistle, is widely distributed in the Himalayan belt, spreading from Afghanistan to west Nepal and Tibet, and is conventionally used to treat different inflammatory disorders. The plant is edible and traditionally used for treating arthritis, bodyache, cough, asthma, dermatitis, swelling, and joint pain.^{14–17} With important medicinal properties, the herb faces huge threats in its natural environment.¹⁸ To the best of our knowledge, this study is the first to report the effectiveness of *C. thomsonii* on expression patterns of crucial inflammatory proteins/markers, ie, IL1 β , IL6, IFN γ , TNF α , NF κ Bp65 (RelA), NF κ B1 p50, and NF κ B2 p52 in lipopolysaccharide (LPS)-induced albino Wistar rat model. It is worth mentioning that LPS is a component of the outer cell wall of Gram-negative bacteria that acts as a strong endotoxin when injected into body. It binds to certain receptors present on the membrane of immune cells and stimulates the discharge of endogenous proinflammatory factors, such as IFN γ , TNF α , IL1 β , and IL6 as well as free radicals.¹⁹ IL6 is an important interleukin that plays an imperative role in the recruitment of monocytes at the site of injury or infection. Monocytes then differentiate into macrophages that perform phagocytosis during the inflammatory cascade. IL6 stimulates many pathways, including NF κ B, thus promoting transcriptional activation of several genes, which leads to synthesis of inflammatory cytokines. Soon after its production at the primary site of inflammation, IL6 enters the bloodstream and reaches the liver, where it induces synthesis of serum amyloid A, CRP, fibrinogen, α_1 -antichymotrypsin, and haptoglobin.²⁰ Evidence suggests that IL6 has potential implications in the development of neuropathic pain in post-peripheral nerve damage. IL1 β is another cytokine that activates the cJun N-terminal kinase, p38MAPK, and NF κ B pathways.²¹ Switching on such pathways results in the transcriptional activation of various genes that promote the expression of chemokines, adhesion molecules, cytokines, and lymphokines.²² TNF α is secreted by a diverse range of immune cells, eg, T cells, monocytes/macrophages, adipocytes, smooth-muscle cells, and fibroblasts. TNF α activates signaling events that trigger apoptosis or necrosis. It is also implicated in developing

resistance to infections and cancer. TNF α triggers production of prostaglandinE₂ and collagenase enzymes in synovial cells, thus damaging joints, especially during arthritis. TNF α can also induce fever by stimulating the synthesis of IL1 β and PGE₂.²³ IFN γ is secreted by various immune cells like CD8⁺ cytotoxic lymphocytes, natural killer cells, CD4⁺T_H1 lymphocytes, and antigen-presenting cells. IFN γ promotes inflammatory responses by activating the PI3K and NF κ B pathways. Reports suggest that IFN γ plays an important role in inducing chronic lung inflammation.²⁴ During atherosclerosis, excessive secretion of IFN γ by T cells stimulates macrophages to initiate phagocytosis and also enhances the expression of adhesion molecules on the wall of smooth muscle. Such events not only sustain the inflammatory event but also encourage the formation of plaques, thus worsening the state of inflammation.²⁵ The NF κ B family consists of five proinflammatory proteins: NF κ B1 p50, NF κ B2 p52, RelA p65, RelB, and cRel. These proteins play a central role in inflammatory events by forming transcription factors in the form of heterodimers or homodimers.⁵ Such dimers are involved in the activation of various proinflammatory genes like *IL1B*, *TNFA*, *IL8*, and *IL6*. During unstimulated conditions, these NF κ B hetero/homodimers are retained in the cytoplasm by the action of certain inhibitory proteins, eg, I κ B α , I κ B β , I κ B ϵ , I κ B δ , and I κ B γ .^{26–28} Elevated levels of proinflammatory NF κ B dimers is the characteristic feature of many inflammatory disorders, notably psoriasis, rheumatoid arthritis, and inflammatory bowel disease.

Methods

Collection and Identification of Plant Material

C. thomsonii (whole plant) was obtained from higher altitudes of Sankoo in the Kargil region of Ladakh (India). The plant was identified at the Centre of Plant Taxonomy, Department of Botany, University of Kashmir (India) and preserved in its herbarium under voucher specimen number 2620-KASH.

Preparation of Extracts

Whole plant was shade-dried at 25°C \pm 2°C for 2 weeks and later crushed to powder using a grinder. The powder was then extracted using different solvents, ie, hexane, ethyl acetate, ethanol, methanol, and water in increasing order of polarity via a soxhlet extractor (60°C–80°C). The solvent extracts were then concentrated in a rotary evaporator

at low pressure. Concentrated extracts were stored in a refrigerator at 4°C for further in vitro and in vivo investigations.

In Vitro Anti-inflammatory Analysis

Protein-Denaturation Assay

This assay was performed according to the method described previously by Djuichou-Nguemngang et al,²⁹ with some modifications. The reaction mixture consisted of *C. thomsonii* extracts with varying concentrations, ie, 100, 300, and 600 μ g/mL added to 5% aqueous solution of BSA. Each reaction mixture was incubated at 37°C for 20 minutes, and then denaturation of protein (BSA) was induced by placing the mixture in a water bath for 15 minutes at 70°C. The temperature of the reaction mixture was allowed to decline at room temperature (23°C \pm 2°C) and turbidity measured by checking absorbance at 660 nm via ultraviolet-visible spectrophotometry. The solution of BSA in distilled water was used as the control. Acetylsalicylic acid (aspirin) was used as the positive control. Each test was performed three times and inhibition of protein denaturation shown by each extract calculated:

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{(\text{Absorbance of control})} \times 100$$

Antiproteinase Assay

This assay was performed according to a method described earlier.³⁰ Briefly, the reaction mixture contained 0.06 mg trypsin, 1 mL 20 mM Tris HCl buffer (pH 7.4), and 1 mL test samples of different concentrations (100, 300, and 600 μ g/mL). The mixture was incubated at 37°C for 5 minutes, followed by addition of 1 mL 0.8% (w/v) casein. The mixture was again incubated at 37°C for 20 minutes. Perchloric acid (70%, 2 mL) was added to seize the reaction, and the cloudy suspension was centrifuged. Absorbance of supernatant was read at 210 nm. Tris HCl buffer (20 mM, pH 7.4) was taken as blank. The control contained all components of reaction mixture, except the plant extract or aspirin. The experiment was repeated three times and proteinase inhibition calculated:

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{(\text{Absorbance of control})} \times 100$$

HRBC Membrane–Stabilization Assay

This test was carried out as per the protocol described in an earlier study.³¹ A fresh intravenous human blood sample was voluntarily provided by one of the contributing authors with written informed consent. The volunteer was healthy and not taking any anti-inflammatory or other medications. This blood collection was performed as per the Declaration of Helsinki and formally approved by the Institutional Research Ethics Committee/Board of Research Studies. The blood sample was mixed with Alsever's solution (0.8% sodium citrate, 2% dextrose, 0.42% sodium chloride, and 0.05% citric acid in water). The mixture was centrifuged for 10 minutes at 3,000 rpm, and cells packed at the bottom of the centrifuge tube were subjected to triple washing with 0.85% sodium chloride solution (ie, isosaline). The final packed-cell volume was reconstituted as 10% v: v suspension with isosaline. The ability of *C. thomsonii* extracts to stabilize HRBC membrane was tested through hypotonic solution–mediated hemolysis of human erythrocytes. The test mixture consisted of 5 mL 50 mM NaCl (hypotonic solution) and 0.50 mL stock erythrocyte suspension in 10 mM sodium PBS (pH 7.4) containing varying concentrations of *C. thomsonii* extracts (100, 300, and 600 µg/mL). Diclofenac sodium was used as positive control for comparing results. A buffered hypotonic saline solution mixed with 0.5 mL RBCs was taken as control. Incubation of test mixtures was carried out at room temperature for about 10 minutes, followed by centrifugation at 3,000 g for 10 minutes. Supernatant was collected in separate tubes and tested for absorbance at a wavelength of 560 nm. Inhibition of erythrocyte hemolysis was calculated:

Percentage inhibition =

$$\frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{(\text{Absorbance of control})} \times 100$$

Nitric Oxide Assay

This assay was performed according to the protocol described earlier.³² Briefly, three concentrations (ie, 100, 300, and 600 µg/mL) of each solvent extract were prepared and gallic acid taken as standard. Griess reagent was prepared manually in the laboratory by adding equal amounts of 0.1% naphthylethylene diamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid immediately before use. Sodium nitroprusside (0.5 mL, 10 mM) in PBS (pH 7.3) was mixed with 1 mL varying concentrations of the *C. thomsonii* extracts and incubated at room temperature (23°C±2°C) for 3 hours. An equal volume of

Griess reagent was added to the extract. Control samples contained no extracts, but contained PBS (pH 7.3). A volume of 150 µL reaction mixture was pipetted into a 96-well plate and absorbance recorded at a wavelength of 546 nm using an ELISA microplate reader. The NO scavenger gallic acid was used as positive control. The experiment was repeated three times and inhibition of nitric oxide (NO) calculated:

Nitric oxide scavenged(%) =

$$\frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{(\text{Absorbance of control})} \times 100$$

In Vivo Studies

Experimental Animals

Adult male albino Wistar rats (6–8 weeks old) weighing 150–200 g were procured from the Department of Pharmaceutical Science, University of Kashmir, India. A total of 72 male Wistar rats were used for the study (18 rats for acute safety study arranged in three groups of six rats per group and 54 rats for anti-inflammatory study arranged in nine groups of six rats per group). All animal studies were performed according to the Declaration of Helsinki and the guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals, government of India, established under chapter 4, section 15 (1) of the Prevention of Cruelty to Animals Act 1960. Our In vivo study was approved by the Institutional Animal Ethics Committee, Department of Pharmaceutical Sciences, University of Kashmir, Srinagar, India (wide approval F [IAEC approval] KU/2018/10, October 31, 2018). Experimental animals were acclimatized for 2 weeks before in vivo experimentation began. Rats were treated humanely and fed properly with food and water ad libitum. The environment at the institutional animal house was well maintained with a 12-hour light/dark cycle, relative humidity 50%±20%, temperature 23°C±2°C, and ventilation of 10–15 air changes/hour.

Acute Safety of Plant Extracts

Effects of CTEA and CTE extracts on general behavior and safety of the rats was investigated as per the guidelines of the Organisation for Economic Co-operation and Development (OECD). Briefly, experimental animals were given a single dose of 2,500 mg/kg CTE and CTEA via oral gavage. Immediately after dosing, the animals were monitored continuously for 4 hours for any toxic symptoms. The number of survivors was noted

after 24 hours, and the animals were kept under keen observation for 13 days. Any change in general body weight, behavior, mortality, or other physiological activity was carefully observed. On the 14th day, blood was collected from the retro-orbital plexus of the rats and assayed for various hematological parameters. The rats were killed through cervical dislocation and the weight of different vital organs evaluated for any change.

In Vivo Anti-inflammatory Study

Wistar rats were categorized into nine groups of six rats per group: group 1 normal saline only, group 2 LPS only, group 3 LPS + 25 mg/kg CTE, group 4 LPS + 50 mg/kg CTE, group 5 LPS + 100 mg/kg CTE, group 6 LPS + 25 mg/kg CTEA, group 7 LPS + 50 mg/kg CTEA, group 8 LPS + 100 mg/kg CTEA, and group 9 LPS + 0.5 mg/kg dexamethasone. Group 1 received normal saline only for 21 days. Group 2 received a single dose of 100 μ L LPS (0.5 mg/kg) via a subplantar route into the right hind paw on the 22nd day. Groups 3–8 received plant extracts though oral gavage for 21 days and a single dose of 100 μ L LPS (0.5 mg/kg) on the 22nd day. Group 9 received dexamethasone (0.5 mg/kg) 1 hour before LPS injection on the 22nd day. Blood collection and tissue extraction was done 3 hours after LPS injection on the 22nd day.

Determination of Serum Levels of IFN γ , TNF α , IL6, and IL1 β

Fine-glass capillaries were used to obtain blood samples from the rats (through the retro-orbital plexus) into red-top vacutainers. Vacutainers were later centrifuged at 3,000 rpm for 6 minutes to obtain serum. Serum samples were pipetted into pyrogen-free Eppendorf tubes and preserved at -20° C for cytokine estimation. Rat-specific ELISA kits purchased from Cloud-Clone were used for expression analysis of various cytokines, ie, IFN γ , TNF α , IL6, and IL1 β , and readings were measured on an ELISA plate reader (BioTek 800TS).

Western Blotting and Protein Expression

In vivo expression analysis of proinflammatory proteins NF κ B1, NF κ B2, and NF κ B p65 was done using Western blotting. Paw-tissue samples were extracted from the rats after killing them (via cervical dislocation). Samples were incubated on ice with lysis buffer containing 50 mM Tris HCl (pH 8), 2 mM EDTA (pH 8), 10 mM NaF, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.25% sodium

deoxycholate, 1%NP40, 10% glycerol, 10 μ L/mL protease-inhibitor cocktail, and 0.1% SDS. Following incubation for 40 minutes, the samples were centrifuged at 10,000 rpm for 10 minutes at 4° C. The supernatant was collected in separate Eppendorf tubes and the pellet discarded. Protein in each sample was quantified via Bradford assay. Equal amounts of sample were loaded into wells during SDS-PAGE. Then, proteins were transferred from polyacrylamide gel to methanol-activated polyvinylidene fluoride (PVDF) membrane. Blocking of PVDF membrane was done with 5% non-fat-dry milk prepared in PBS-Tween 20 (PBST) solution. Blocked PVDF membrane was incubated overnight with the primary antibodies (purchased from Abcam) and dissolved in the blocking solution with recommended dilutions at 4° C. Following overnight incubation, the PVDF membrane was washed three times with PBST. Ultimately, the PVDF membrane was incubated with secondary antibody (purchased from Abcam) diluted with PBST solution at room temperature for 1 hour. The secondary antibody was horseradish peroxidase-conjugated. Chemiluminescence reaction was employed for detecting protein signals.

Histopathology

For histopathological analysis, paw tissue was extracted from the rats after killing them through cervical dislocation. Formalin 10% was used for fixing isolated samples. These were dehydrated by passing them through a graded sequence of alcohol solutions, and then the dehydrated samples were entrenched in paraffin blocks. An automated microtome was used to cut 3–5 μ m-thick sections of paraffin-embedded tissue. Tissue sections were subsequently stained with hematoxylin and eosin. Sections were mounted with distereene phthalate xylene and finally observed under microscopy (Olympus).

Gas Chromatography–Mass Spectrometry Analysis

Gas chromatography–mass spectrometry (GC-MS; Shimadzu QP2010 Ultra) was performed for identifying the types of compounds present in *C. thomsonii*. Specifications used for GC were column-oven temperature 80° C, injector temperature 250° C, injection mode split-column flow 1 mL/min, flow-control mode linear velocity, purge flow 3 mL/min, and split ratio 5. The temperature was set to 80° C, then increased by 3° C/min to 200° C with further increments of 10° C– 200° C and held for 5 minutes. Specifications for MS were ion-source temperature 240° C, interface temperature

250°C, solvent-cut time 2 minutes, scan range 45–900 m/z, event time 0.3 seconds, starting time 3.5 minutes, and ending at 51 minutes. GC-MS of the extract ran for 51 minutes. Interpretation of GC-MS spectra was done using the National Institute Standard and Technology database. Unknown spectra were compared with known compound spectra of already in the database.

Statistical Analysis

Each value obtained in the study is expressed as the mean \pm SD of three independent experiments. Evaluation of results was done using SPSS 12.0 and Origin 8.1. Normal distribution for quantitative variables was assessed with the Kolmogorov–Smirnov test. For comparing the measured data between different groups, one-way ANOVA and Tukey multiple-comparison tests were used. $P < 0.05$ were considered statistically significant.

Results

Effect on Protein Denaturation

Denaturation is an important process that alters the secondary and tertiary structure of proteins, thus affecting their normal biological functions. Inflammatory processes often involve destruction of normal protein function, due to denaturation.

At a concentration of 600 $\mu\text{g/mL}$, CTEA exhibited maximum suppression of protein denaturation, with inhibition of $78.72\% \pm 3.93\%$ ($P < 0.001$) followed by CTE ($70.20\% \pm 3.86\%$, $P < 0.001$). Methanol extract showed inhibition of $59.03\% \pm 3.05\%$ ($P < 0.05$), aqueous extract $47.14\% \pm 2.85\%$ ($P < 0.05$), and hexane extract $36.81\% \pm 1.99\%$, $P > 0.05$ at 600 $\mu\text{g/mL}$. Aspirin, which served as the positive control, showed maximum inhibition ($86.54\% \pm 4.32\%$, $P < 0.001$) at 600 $\mu\text{g/mL}$. CTEA and CTE showed IC_{50} of 170.96 ± 8.54 $\mu\text{g/mL}$ and 217.50 ± 10.87 $\mu\text{g/mL}$, respectively, while IC_{50} was 820.63 ± 41.03 – 449.36 ± 22.46 $\mu\text{g/mL}$ in the rest of the extracts. The positive control showed the lowest IC_{50} : 63.04 ± 3.15 $\mu\text{g/mL}$ (Table 1).

Effect on Proteinases

Proteinases are implicated in causing severe tissue damage during inflammatory cascades, and using proteinase inhibitors is believed to provide a considerable level of protection against this damage. While investigating the antiproteinase potential of various solvent extracts of *C. thomsonii*, we observed a significant dose-dependent reduction in proteinase activity due to CTEA treatment, achieving the highest inhibition of $77.37\% \pm 3.61\%$ at 600 $\mu\text{g/mL}$ ($P < 0.001$, Table 2). CTE also showed marked inhibition (70.4%

Table 1 Inhibition of protein denaturation shown by *Cousinia thomsonii* extracts at varying concentrations and their IC_{50} values

Treatment	% Inhibition at different concentrations			IC_{50} ($\mu\text{g/mL}$)
	100 $\mu\text{g/mL}$	300 $\mu\text{g/mL}$	600 $\mu\text{g/mL}$	
Aqueous extract	$22.18 \pm 1.10^{\text{ns}}$	$33.13 \pm 1.65^{\text{ns}}$	$47.14 \pm 2.85^*$	$652.46 \pm 32.62^{\text{ns}}$
Hexane extract	$10.33 \pm 0.51^{\text{ns}}$	$28.26 \pm 1.41^{\text{ns}}$	$36.81 \pm 1.99^{\text{ns}}$	$820.63 \pm 41.03^{\text{ns}}$
Ethanol extract	$43.16 \pm 2.15^*$	$55.31 \pm 2.76^*$	$70.20 \pm 3.86^{**}$	$217.50 \pm 10.87^{**}$
Methanol extract	$26.72 \pm 2.03^{\text{ns}}$	$41.97 \pm 2.59^*$	$59.03 \pm 3.05^{**}$	$449.36 \pm 22.46^*$
Ethyl acetate extract	$45.10 \pm 2.29^*$	$58.89 \pm 2.94^{**}$	$78.72 \pm 3.93^{***}$	$170.96 \pm 8.54^{**}$
Aspirin	$50.25 \pm 3.04^*$	$70.78 \pm 3.58^{***}$	$86.54 \pm 4.32^{***}$	$63.04 \pm 3.15^{***}$

Notes: Each value represents the mean \pm SD of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\text{ns}}P > 0.05$.

Table 2 Inhibition of proteinase activity exhibited with different concentrations of *Cousinia thomsonii* extracts and their IC_{50} values

Treatment	% Inhibition at different concentrations			IC_{50} ($\mu\text{g/mL}$)
	100 $\mu\text{g/mL}$	300 $\mu\text{g/mL}$	600 $\mu\text{g/mL}$	
Ethanol extract	$43.56 \pm 2.62^*$	$59.16 \pm 2.95^{**}$	$70.4 \pm 3.52^{***}$	$186.26 \pm 9.31^{***}$
Aqueous extract	$31.47 \pm 1.57^{\text{ns}}$	$43.52 \pm 2.17^*$	$50.83 \pm 2.54^*$	$547.7 \pm 27.38^{\text{ns}}$
Methanol extract	$40.25 \pm 2.01^*$	$50.45 \pm 2.52^*$	$58.15 \pm 2.90^*$	$344.28 \pm 17.21^*$
Hexane extract	$21.02 \pm 1.05^{\text{ns}}$	$32.27 \pm 1.61^{\text{ns}}$	$43.63 \pm 2.18^*$	$729.69 \pm 36.48^{\text{ns}}$
Ethyl acetate extract	$48.05 \pm 2.81^*$	$63.35 \pm 3.15^{**}$	$77.37 \pm 3.61^{***}$	$109.35 \pm 5.46^{***}$
Aspirin	$50.0 \pm 3.13^*$	$68.93 \pm 3.54^{**}$	$81.1 \pm 4.05^{***}$	$57.65 \pm 2.88^{***}$

Notes: Each value represents the mean \pm SD of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\text{ns}}P > 0.05$.

$\pm 3.52\%$, $P < 0.001$) at 600 $\mu\text{g/mL}$. Inhibition exhibited by aqueous and methanol extract was $50.83\% \pm 2.54\%$ ($P < 0.05$) and $58.15\% \pm 2.90\%$ ($P < 0.05$), respectively. Hexane showed the lowest inhibition: $43.63\% \pm 2.18\%$ ($P > 0.05$). Aspirin (positive control) exhibited maximum inhibition of $81.1\% \pm 4.05\%$ ($P < 0.001$) at 600 $\mu\text{g/mL}$. CTEA and CTE extracts showed the lowest IC_{50} among all tested extracts, ie, $109.35 \pm 5.46 \mu\text{g/mL}$ for CTEA and $186.26 \pm 9.31 \mu\text{g/mL}$ for CTE, while in the other extracts IC_{50} was 729.69 ± 36.48 – $344.28 \pm 17.21 \mu\text{g/mL}$. Aspirin showed IC_{50} of $57.65 \pm 2.88 \mu\text{g/mL}$.

Effect on HRBC-Membrane Stabilization

Membrane destruction is an important phenomenon during inflammatory responses that releases proinflammatory enzymes, ie, PLA_2 . This enzyme subsequently activates an arachidonate pathway that synthesizes important inflammatory mediators, thus triggering an inflammatory response. In order to test the efficacy of *C. thomsonii* extracts in preventing membrane destruction, we performed HRBC membrane-stabilization assays. Almost all tested extracts prevented HRBC-membrane hemolysis in a dose-dependent manner; however, at a concentration of 600 $\mu\text{g/mL}$ CTEA showed the highest inhibition of RBC hemolysis ($80.5\% \pm 4.09\%$, $P < 0.001$), followed by CTE ($73.93\% \pm 3.99\%$, $P < 0.001$). The aqueous extract showed inhibition of $62.17\% \pm 3.10\%$ ($P < 0.05$). Methanol extract showed inhibition of $64.07\% \pm 3.35\%$ ($P < 0.01$) at 600 $\mu\text{g/mL}$. Hexane extract exhibited least membrane-stabilizing potential ($49.67\% \pm 2.48\%$, ($P > 0.05$). Diclofenac sodium (positive control) showed inhibition of $87.22\% \pm 4.36\%$ ($P < 0.001$) at a dosage of 600 $\mu\text{g/mL}$. The efficacy of CTEA and CTE extracts was also evident in their IC_{50} values — $40.80 \pm 2.04 \mu\text{g/mL}$ in CTEA and $52.09 \pm 2.60 \mu\text{g/mL}$ in CTE — while IC_{50} was 582.66 ± 29.13 – $172.34 \pm 8.61 \mu\text{g/mL}$ for the other extracts. Diclofenac sodium showed lowest IC_{50} : $34.09 \pm 1.70 \mu\text{g/mL}$ (Table 3).

Effect on Nitric Oxide Production

Among all the tested extracts, CTEA showed the highest nitrite-scavenging capacity ($77.49\% \pm 3.87\%$) at 600 $\mu\text{g/mL}$, followed by CTE which showed inhibition of $73.23\% \pm 3.66\%$ at 600 $\mu\text{g/mL}$ ($P < 0.001$, Table 4). Aqueous, methanol and hexane extracts showed inhibition of $58.63\% \pm 2.93\%$ ($P < 0.05$), $66.63\% \pm 3.33\%$ ($P < 0.01$), and $52.72\% \pm 2.63\%$ ($P < 0.05$), respectively, at 600 $\mu\text{g/mL}$. Gallic acid (positive control) showed inhibition of $89.6\% \pm 4.08\%$ ($P < 0.001$) at maximum concentration. CTEA and CTE showed IC_{50} of $70.52 \pm 3.52 \mu\text{g/mL}$ and $119.26 \pm 5.96 \mu\text{g/mL}$, respectively, while this was 526.38 ± 26.31 – $233.86 \pm 11.69 \mu\text{g/mL}$ in the rest of the extracts. Gallic acid showed lowest IC_{50} : $39.70 \pm 1.98 \mu\text{g/mL}$.

Acute Safety Study

We observed no adverse effects or mortality due to administration of single doses of 2,500 mg/kg CTEA and CTE. During this period, rats showed no changes in normal behavior. Though we observed some alterations in daily water and feed intake, such changes were insignificant in comparison to the control group. Body weight of the rats was monitored every day and mean body weight calculated for each group. There was no significant change in the overall body weight of rats in treated groups compared to the control group. Body weight was 175 ± 0.77 to $181 \pm 0.21 \text{ g}$. Table 5 depicts the relative organ weights of the treated and control groups. Livers, hearts, kidneys, spleens, and lungs of CTEA- and CTE-treated rats showed no significant changes compared to the normal/control group. Table 6 demonstrates the hematological parameters of control and treated rats. Hb, RBC count, and PCV exhibited no significant changes between control and treated animal groups. When compared to the normal/control group, the change in the WBC count of tested rats was insignificant. We observed no significant change in MCH, MCV, MCHC, platelet count, or differential leukocyte counts of treated animals when compared to control/normal animals

Table 3 Inhibition of HRBC-membrane hemolysis due to varying concentrations of *Cousinia thomsonii* extracts and their IC_{50} values

Treatment	% Inhibition at different concentrations			IC_{50} ($\mu\text{g/mL}$)
	100 $\mu\text{g/mL}$	300 $\mu\text{g/mL}$	600 $\mu\text{g/mL}$	
Ethyl acetate extract	$50.0 \pm 3.52^*$	$71.0 \pm 3.88^{***}$	$80.5 \pm 4.09^{***}$	$40.80 \pm 2.04^{***}$
Aqueous extract	$43.42 \pm 2.17^*$	$53.94 \pm 2.69^*$	$62.17 \pm 3.10^{**}$	$246.78 \pm 12.33^*$
Methanol extract	$45.86 \pm 2.73^*$	$57.15 \pm 3.15^*$	$64.07 \pm 3.35^{**}$	$172.34 \pm 8.61^{**}$
Hexane extract	24.67 ± 1.23^{ns}	39.14 ± 1.95^{ns}	$49.67 \pm 2.48^*$	582.66 ± 29.13^{ns}
Ethanol extract	$48.34 \pm 3.43^*$	$68.67 \pm 3.73^{**}$	$73.93 \pm 3.99^{***}$	$52.09 \pm 2.60^{***}$
Diclofenac sodium	$52.0 \pm 3.65^*$	$72.5 \pm 3.96^{***}$	$87.22 \pm 4.36^{***}$	$34.09 \pm 1.70^{***}$

Notes: Each value represents the mean \pm standard deviation of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{ns}P > 0.05$.

Table 4 Inhibition of nitric oxide production due to varying concentrations of *Cousinia thomsonii* extracts and their IC₅₀ values

Treatment	% Inhibition at different concentrations			IC ₅₀ (μg/mL)
	100 μg/mL	300 μg/mL	600 μg/mL	
Hexane extract	34.18±1.70 ^{ns}	41.63±2.08*	52.72±2.63*	526.38±26.31 ^{ns}
Aqueous extract	37.63±1.88 ^{ns}	46.45±2.32*	58.63±2.93*	391.34±19.56 ^{ns}
Methanol extract	40.61±2.49*	57.76±2.88*	66.63±3.33**	233.86±11.69*
Ethanol extract	43.25±3.20*	69.60±3.48**	73.23±3.66***	119.26±5.96***
Ethyl acetate extract	46.21±3.31*	72.72±3.63**	77.49±3.87***	70.52±3.52***
Gallic acid	50.0±3.50*	77.47±3.77**	89.60±4.08***	39.70±1.98***

Notes: Each value represents the mean ± standard deviation of three independent experiments. **P*<0.05; ***P*<0.01; ****P*<0.001; ^{ns}(*P*>0.05).

Table 5 Relative organ weight (g/100 g body weight) of CTEA- and CTE-treated rats during acute-toxicity study

Organ	Control group	CTEA (2,500 mg/kg)	CTE (2,500 mg/kg)
Heart	0.375±0.020	0.353±0.011 ^{ns}	0.329±0.050 ^{ns}
Kidney	0.339±0.015	0.341±0.018 ^{ns}	0.346±0.025 ^{ns}
Liver	2.602±0.053	2.400±0.31 ^{ns}	2.59±0.061 ^{ns}
Lung	0.513±0.073	0.521±0.092 ^{ns}	0.517±0.073 ^{ns}
Spleen	0.250±0.039	0.237±0.011 ^{ns}	0.242±0.081 ^{ns}

Notes: CTEA, *Cousinia thomsonii* ethyl acetate; CTE, *C. thomsonii* ethanol. Each group contained six rats. ^{ns}*P*>0.05.

either. These results suggested that administration of CTEA and CTE up to 2,500 mg/kg body weight in the rats resulted in no mortality, and hence was considered safe as per the OECD-423 guideline. However, considering the efficacy of any drug, we selected lower doses of CTEA and CTE up to 100 mg/kg body weight for further in vivo studies.

Cytokine Expression

Injection of LPS resulted in manifold increases in serum levels of the proinflammatory cytokines IL6, IL1β, TNFα, and IFNγ in comparison to the normal group. IL6, IL1β, TNFα, and IFNγ levels were 82.3±4.11 pg/mL, 65.78±3.28 pg/mL, 72.47±3.62 pg/mL, and 136.69±6.83 pg/mL,

respectively, in the group treated with LPS only. For the untreated/normal group, concentrations were 11.5±0.575 pg/mL (IL6), 10.75±0.5375 pg/mL (IL1β), 18.6±0.93 pg/mL (IFNγ), and 18.35±0.9175 pg/mL (TNFα; *P*<0.001). Both CTEA and CTE extracts demonstrated a dose-dependent reduction in cytokine levels. Comparatively, CTEA was found to be more dominant in downregulating expression of cytokines than CTE. At a concentration of 100 mg/kg, CTEA reduced IL1β levels from 65.78±3.28 pg/mL to 15.68±0.78 pg/mL (*P*<0.001, Figure 1A), IL6 from 82.3±4.11 pg/mL to 22.18±1.20 (*P*<0.001, Figure 1B), TNFα from 72.47±3.62 pg/mL to 25.19±1.25 pg/mL (*P*<0.001, Figure 1C), and IFNγ from 136.69±6.83 pg/mL to 50.55±2.52 pg/mL (*P*<0.001, Figure 1D). Dexamethasone (positive control) caused a significant reduction in the of cytokine levels to 16.47±0.8235 pg/mL (IL6), 13.33±0.66 pg/mL (IL1β), 37.8±1.89 pg/mL (IFNγ), and 18.35±0.91 pg/mL (TNFα; *P*<0.001).

Protein-Expression Analysis

Western blotting showed concentration-dependent decreases in expression of NFκB1 p50 (50 kDa), NFκB2 p52 (52 kDa), and NFκB p65/RelA (65 kDa) in CTEA- and CTE-treated groups compared to the LPS only-treated

Table 6 Hematological parameters of albino wistar rats treated with CTEA and CTE during acute-toxicity study

	Control group	CTEA (2,500 mg/kg)	CTE (2,500 mg/kg)
RBC (10 ⁶ /μL)	9.30±0.19	9.21±0.15 ^{ns}	9.19±0.1 ^{ns}
Hb (g/dL)	15.90±0.55	15.37±0.17 ^{ns}	15.53±0.0 ^{ns}
PCV (%)	40.96±0.01	41.90±0.09 ^{ns}	40.01±0.78 ^{ns}
MCV (fL)	52.10±0.68	51.67±0.13 ^{ns}	52.18±0.09 ^{ns}
MCH (pg)	18.60±0.10	18.0±0.10 ^{ns}	18.50±0.16 ^{ns}
MCHC (g/dL)	36.88±0.74	35.09±0.10 ^{ns}	36.02±0.40 ^{ns}
Platelet count (10 ³ /μL)	1267.29±30.8	1251.41±33.8 ^{ns}	1213.8±35.07 ^{ns}
WBCs (10 ³ /μL)	13.12±0.49	12.34±0.1 ^{ns}	12.72±0.36 ^{ns}

Notes: CTEA, *Cousinia thomsonii* ethyl acetate; CTE, *C. thomsonii* ethanol. Each group contained six rats. ^{ns}*P*>0.05.

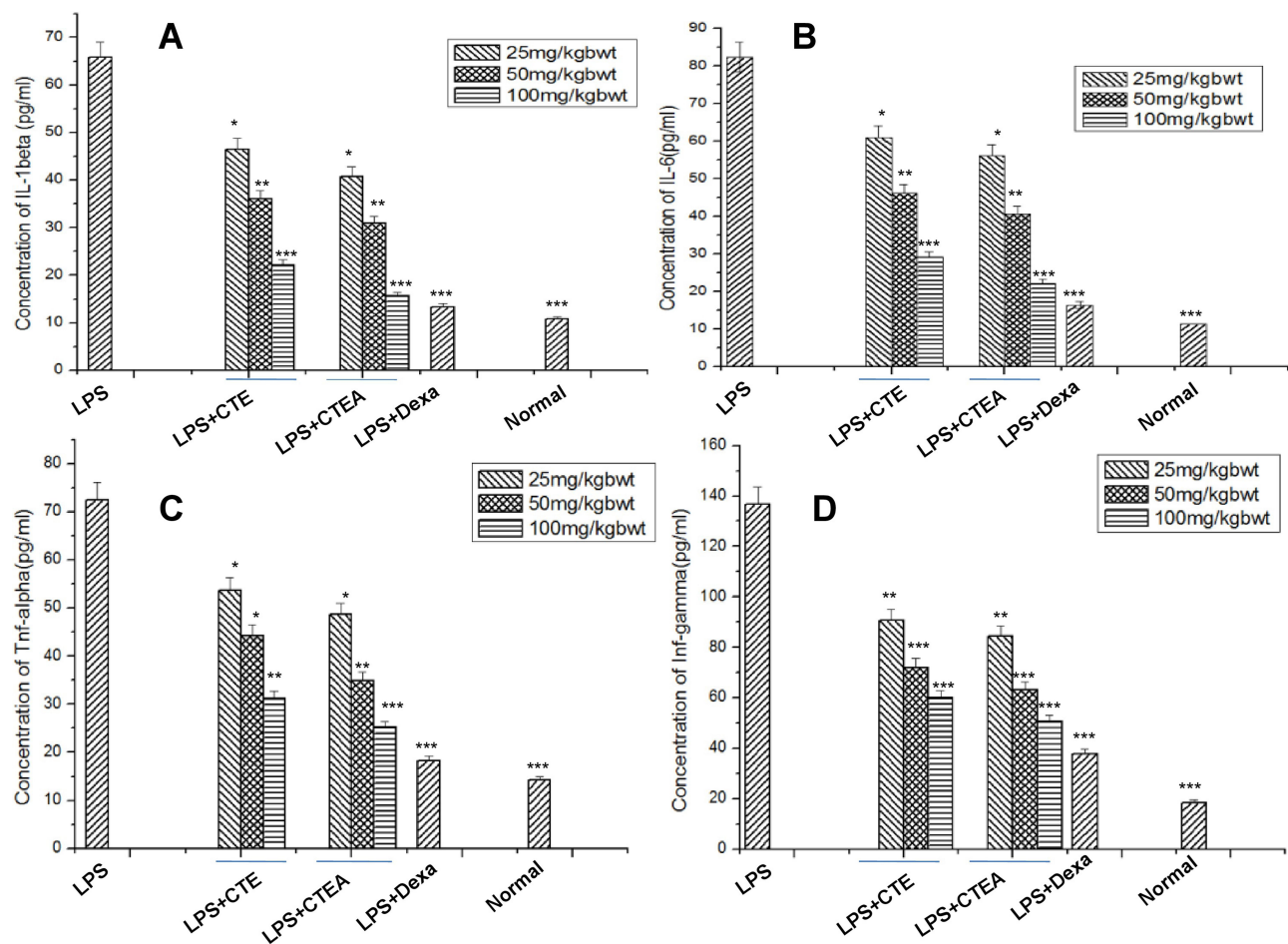


Figure 1 Effect of *Cousinia thomsonii* CTE and CTEA extracts on levels of serum proinflammatory cytokines. (A) IL-1 β (pg/mL). (B) IL-6 (pg/mL). (C) TNF α (pg/mL). (D) IFN γ (pg/mL). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

group. β -actin (42 kDa) was used as loading control. Results shown by groups treated with 100 mg/kg CTEA and CTE were almost comparable to the positive-control group (Figures 2 and 3).

Effect on Paw Histopathology

Subplantar injection of LPS into the right hind paw of the rats triggered acute inflammatory reactions. The initial phase of inflammatory reaction involves the production of mediators like histamine, bradykinins, and cyclooxygenase products, while the later phase is associated with migration of neutrophils and synthesis of arachidonic metabolites, such as prostaglandins, prostacyclins, thromboxanes, and leukotrienes. Histopathological examination of the normal/control group showed normal appearance of dermis and epidermis, and there were no lesions. There was no leukocyte infiltration or edema (Figure 4A). The group treated with LPS only showed prominent vasodilatation with edema in the epidermal and dermal layers. There was

migration of neutrophils into intercellular spaces, between collagen fibers, and around vessels (Figure 4B). The groups that received CTEA (Figure 4C and E) and CTE (Figure 4F and H) exhibited less edema and reduced infiltration of polymorphonuclear cells in a concentration-dependent manner. Besides, there was less vascular congestion and epidermal thickening (Figure 4C and H). The anti-inflammatory effects exhibited at the highest concentration (100 mg/kg) of active extracts were almost comparable to the dexamethasone-treated group (Figure 4I).

GC-MS Analysis

GC-MS is an extensively used spectroscopic technique employed for the identification of compounds in a sample based on their mass:charge (m/z) ratio. Altitudes of diverse peaks depict abundance that varies from case to case. Locations of peaks demonstrate time of elution for each peak, which is different for different compounds due to dissimilarity in their structures. The GC-MS spectra of

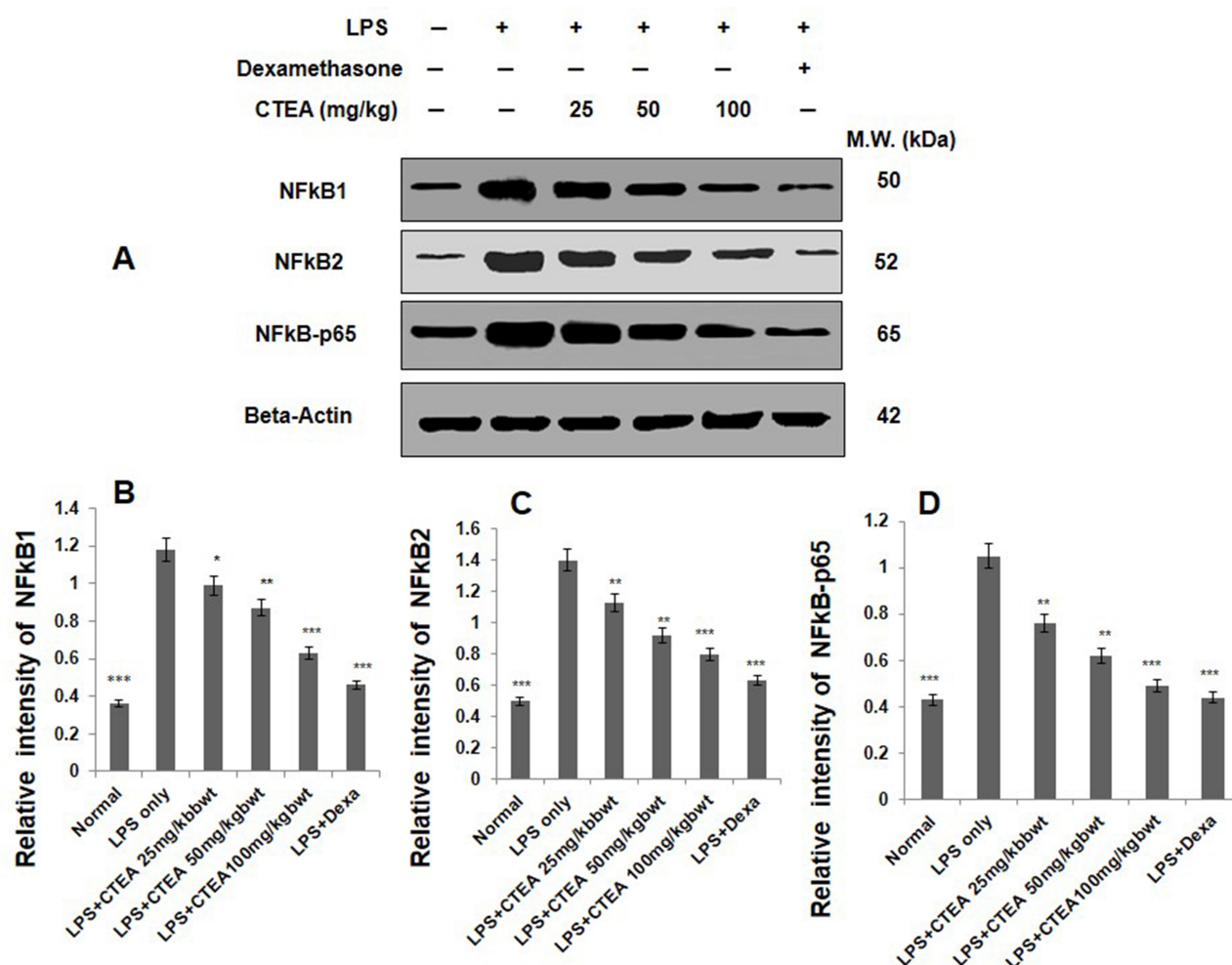


Figure 2 Western blotting images. **(A)** Effect of CTEA on the protein expression of NFκB1 p50, NFκB2 p52, and NFκB p65. Lane 1 normal, lane 2 LPS only, lane 3 LPS + 25 mg/kg CTEA, lane 4 LPS + 0.5 mg/kg CTEA, lane 5 (LPS + 100 mg/kg CTEA, lane 6 LPS + dexamethasone. **(B)** Relative intensity of NFκB1 (n=6 per group). **(C)** Relative intensity of NFκB2 (n=6 per group). **(D)** Relative intensity of NFκB-p65 (n=6 per group). * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

C. thomsonii revealed the presence of 15 major compounds: 9-octadecenoic acid, *N*-hexadecanoic acid (palmitic acid), phytol acetate, α -tocopherol, hexanoic acid, 3-hydroxy-methyl ester, 2-propenoic acid pentadecyl ester, stigmast-5-en-3-ol-oleate, ascorbic acid, 2,6-dihexadecanoate, dotriacontane, *N,N*-dimethyl-*O*-(1-methyl-butyl)-hydroxylamine, and 1-dodecanol (Table 7, Figures 5 and 6). Most of the compounds identified in *C. thomsonii* have well-documented anti-inflammatory properties.

Discussion

Denaturation of proteins is a well-recognized phenomenon implicated in arthritic reactions and tissue injury during inflammatory insult.³³ Protein denaturation generates auto-antigens during inflammatory conditions like rheumatic arthritis, diabetes, and cancer.³⁴ Many NSAIDs act by

preventing protein denaturation.³⁵ Therefore, inhibition of protein denaturation is considered an important strategy to restrain inflammation. In this study, we found dose-dependent inhibition of protein denaturation due to administration of CTEA and CTE, which indicates the anti-inflammatory potential of these extracts. Many studies have also reported inhibitory effects of medicinal plant extracts on protein denaturation. Kumar et al observed a strong inhibitory effect on protein denaturation while investigating the anti-inflammatory potential of stem-bark extract of *Semecarpus anacardium*.³⁵ Similarly, Umapathy et al reported significant inhibition of protein denaturation due to *Albuca setosa* aqueous extract.³⁶ Also, Govindappa et al found marked inhibitory effects of *Wedelia trilobata* aqueous extract on protein denaturation while investigating its anti-inflammatory efficacy.³⁷

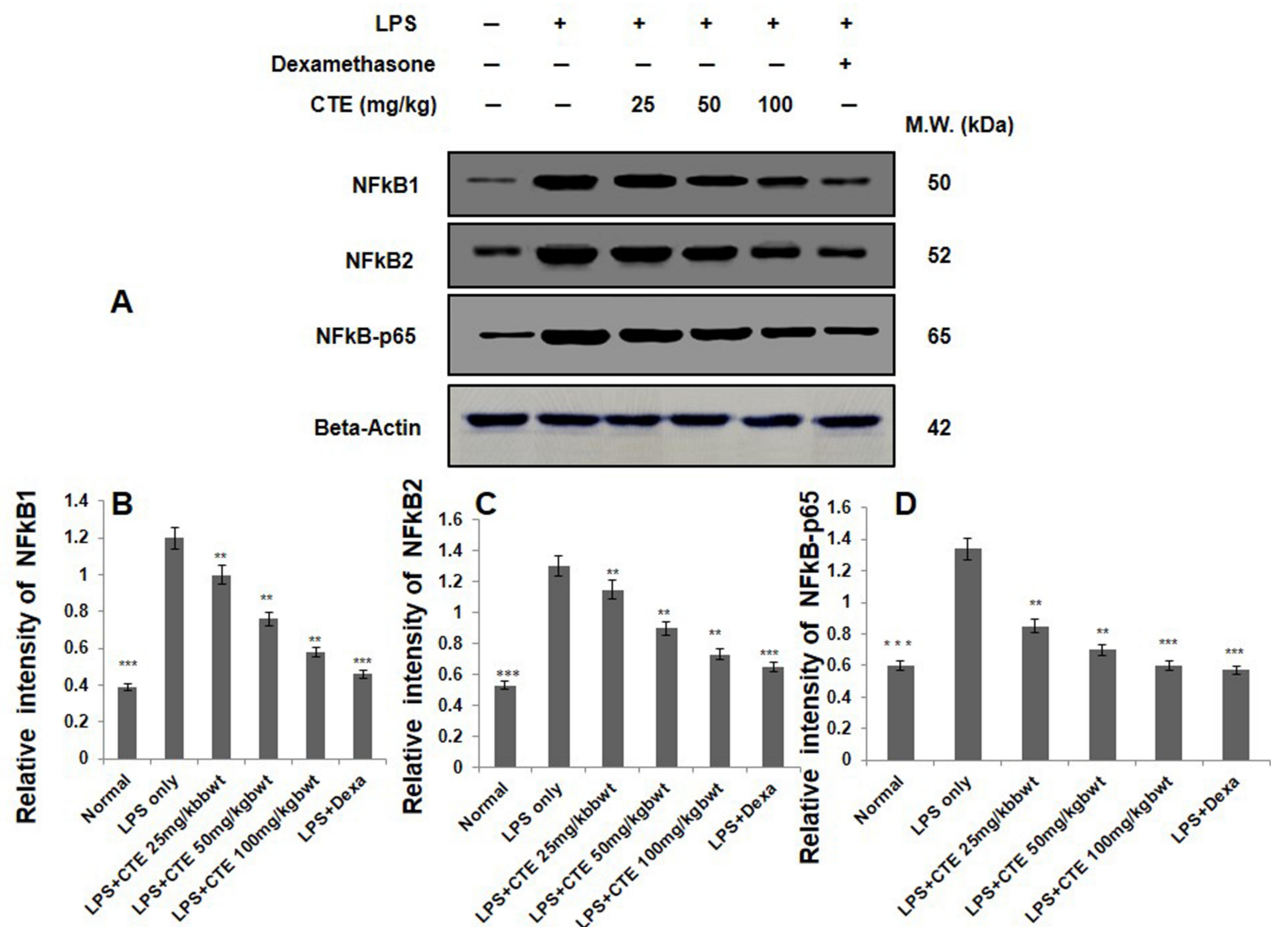


Figure 3 Western blotting images. (A) Effect of ethanol extract (CTE) extract on the protein expression of NFκB1 p50, NFκB2 p52, and NFκB p65. Lane 1 normal, lane 2 LPS only, lane 3 LPS + 25 mg/kg CTE, lane 4 LPS + 50 mg/kg CTE, lane 5 LPS + 100 mg/kg CTE, lane 6 (LPS + dexamethasone). (B) Relative intensity of NFκB1 (n=6 per group). (C) Relative intensity of NFκB2 (n=6 per group). (D) Relative intensity of NFκB p65 (n=6 per group). ** $P < 0.01$; *** $P < 0.001$).

Leukocyte infiltration is an important defensive mechanism that occurs during inflammatory reactions. Infiltrated cells secrete lysosomal contents containing proteases and bactericidal enzymes that help in neutralizing infectious foreign agents. However, prolonged/uncontrolled inflammation encourages collateral cellular and tissue damage. This includes damage to the cellular membranes, making them prone to secondary damage, due to free radicals generated by the process of lipid peroxidation. Membranes of erythrocytes have properties similar to lysosomal membrane. The ability of medicinal agents, including plant extracts, to inhibit RBC hemolysis is considered the measure of its anti-inflammatory potential. When injured, lysosomal membrane releases PLA₂, which hydrolyzes membrane phospholipids into free fatty acids (eg, arachidonic acid) and lysophospholipids.³⁶ Lysophospholipids and arachidonic acid in turn act as precursors for various proinflammatory factors. The arachidonic acid pathway

produces leukotrienes and prostaglandins, while the lysophospholipid pathway synthesizes platelet-activating factor.³⁸ As such, the strategy to inhibit PLA₂ will in turn inhibit COX and LOX enzyme action, thereby blocking the inflammatory process.³⁹ In other words, we can say that stabilizing the lysosomal membrane averts its lysis and release of cytoplasmic contents, thus preventing tissue injury and surge of inflammatory response.³⁶ We found CTEA followed by CTE exhibited strong potential to stabilize erythrocyte membrane by preventing its lysis than other extracts, suggesting that these two extracts may effectively prevent leukocyte-membrane lysis and subsequent release of the PLA₂ enzyme, thus blocking inflammatory surge. Furthermore, CTEA and CTE caused prominent inhibition of protease activity, thus averting proteolytic destruction of proteins. Upregulation of proteases is a marked characteristic of many inflammatory diseases, including arthritis. For instance, the role of matrix

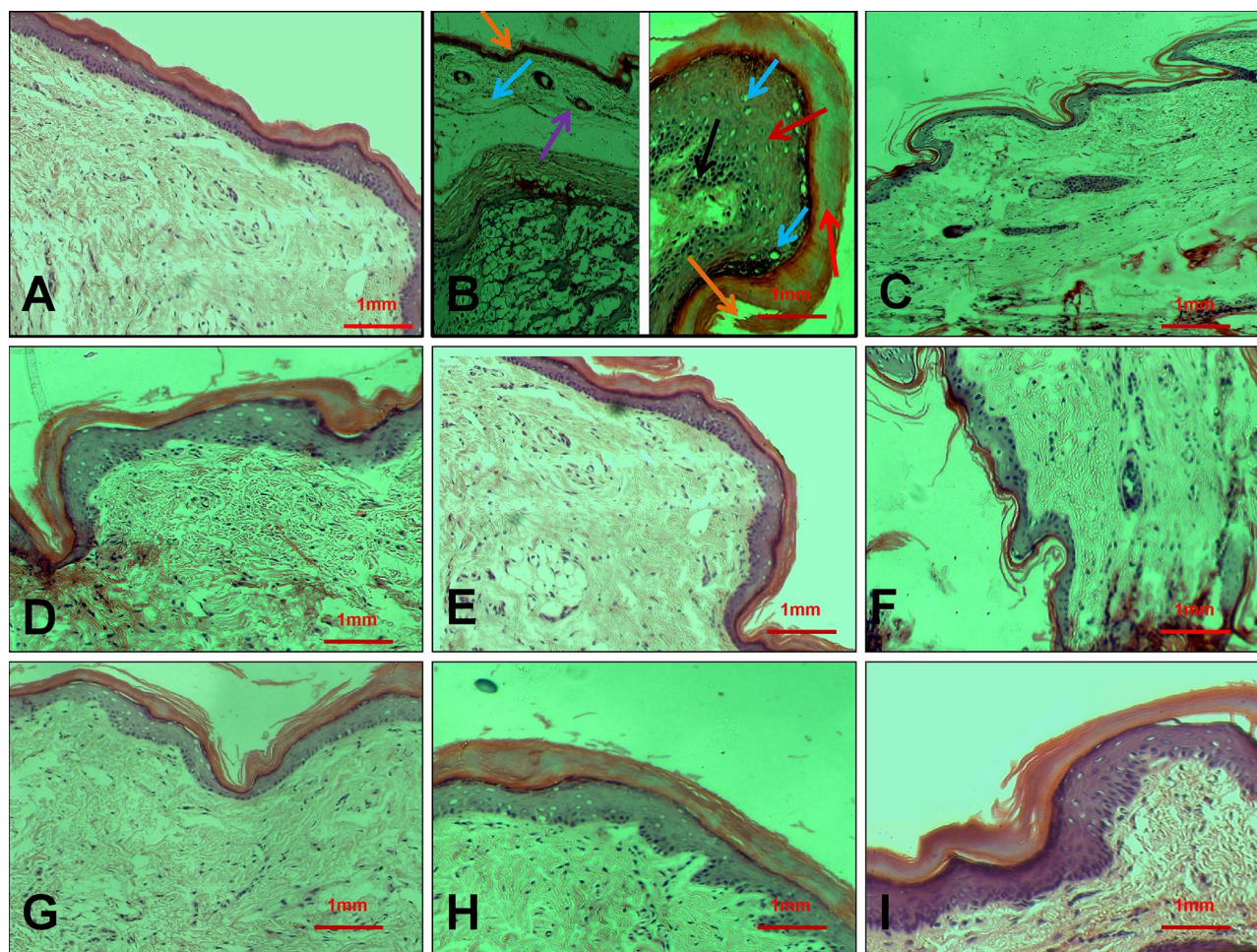


Figure 4 Histopathological images showing ameliorating effect of *Cousinia thomsonii* on Wistar rat-paw tissue in LPS-induced edema model. (A) Control/normal group. (B) LPS alone group. (C) LPS + 25 mg/kg CTEA. (D) LPS + 50 mg/kg CTEA. (E) LPS + 100 mg/kg CTEA. (F) LPS + 25mg/kg CTE. (G) LPS + 50 mg/kg CTE. (H) LPS + 100 mg/kg CTE. (I) LPS + 0.5 mg/kg dexamethasone.

Notes: (B) Red arrows indicate inflamed dermis and epidermis due to LPS induction, black arrow shows subepidermal zone infiltrated by inflammatory cells, orange arrows show formation of lesions in inflamed epidermis, purple arrow shows vascular congestion due to migration of inflammatory cells, and blue arrows indicate cytoplasmic vacuolation due to breakdown of cellular components.

metalloproteinases (eg, MMP2 and MMP9) and serine proteases (eg, human neutrophil elastase) are well implicated in inflammatory reactions.

NO, the gaseous free radical, has many regulatory functions in cellular physiology, but its uncontrolled synthesis due to prominent expression of inducible NO synthase can result in catastrophic collapse of important cellular functions, along with synergistic discharge of inflammatory factors. NO can interact with many other free radicals, resulting in the generation of highly damaging peroxynitrite, which can oxidize low-density lipids and instigate irreparable injury to the cellular membrane.⁴⁰ We found a prominent dose-dependent reduction in levels of NO production due to CTEA and CTE, which might possibly have been due to the presence of NO-inhibiting constituents in *C. thomsonii*.

Inflammation involves diverse genes, proteins, transcription factors, and B-cell/T-cell populations that form part of many pathways. The final products of these inflammatory pathways are known as mediators, notably IL6, IFN γ , TNF α , and IL1 β .^{41,42} Their expression is upregulated multiple times during an inflammatory cascade. During chronic inflammation, such cytokines as TNF α , IL1 β , IL6, and IFN γ trigger apoptosis, necrosis, ion-channel expression, neuron excitation, neurotransmitter release, demyelination, glial and neuronal function loss, and neurodegeneration.^{43,44} TNF α is an important mediator that activates NF κ B, AP1, and IRF, which in turn regulate the expression of several other genes that encode cytokines.⁴⁵ Our study observed considerable decline in serum levels of IL1 β , TNF α , IFN γ , and IL6 due to treatment with CTEA and CTE in LPS-induced rats, thus pointing at its

Table 7 GC-MS analysis of compounds identified in *Cousinia thomsonii*

Sample	R _t (minutes)	% Area	Molecular formula	MW	Compound name
1	43.710	1.63	C ₂₂ H ₄₂ O ₂	338	Phytol acetate
2	50.605	9.64	C ₄₇ H ₈₂ O ₂	678	Stigmast-5-en-3-ol, oleate
3	50.239	6.63	C ₃₂ H ₆₆	450	Dotriacontane
4	50.065	2.37	C ₁₈ H ₃₄ O ₂	282	9-octadecenoic acid
5	46.942	10.83	C ₂₉ H ₅₀ O ₂	430	α -tocopherol
6	37.645	7.78	C ₃₈ H ₆₈ O ₈	652	Ascorbic acid 2,6-dihexadecanoate
7	37.645	1.69	C ₁₆ H ₃₂ O ₂	256	n-hexadecanoic acid
8	28.462	10.71	C ₁₈ H ₃₄ O ₂	282	2-propenoic acid, pentadecyl ester
9	20.201	4.63	C ₁₂ H ₂₆ O	186	1-dodecanol
10	6.502	6.34	C ₇ H ₁₄ O ₃	146	Hexanoic acid, 3-hydroxy-, methyl ester
11	3.894	1.94	C ₇ H ₁₇ NO	131	N,N-dimethyl-O-(1-methyl-butyl)-hydroxylamine

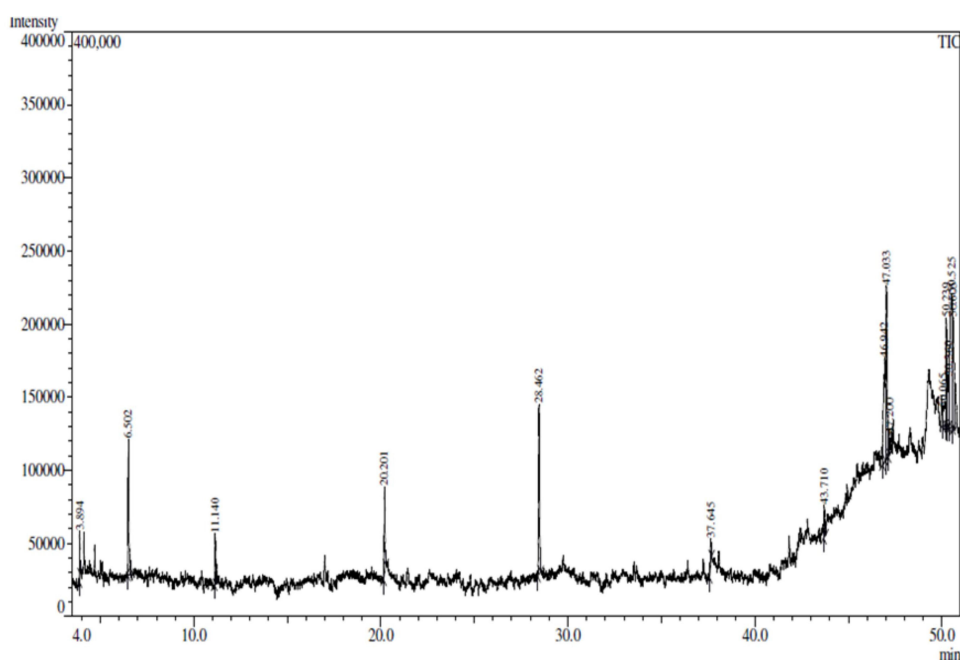
Abbreviations: R_t, retention time; MW, molecular weight.

potential role in modulating the expression of these crucial proinflammatory factors at the molecular level.

We observed a dose-dependent decrease in the expression of NF κ B1 p50, NF κ B2 p52, and NF κ B p65/RelA due to administration of active *C. thomsonii* extracts (CTEA and CTE) in LPS-induced rat. This could probably be due to the presence of certain bioactives that prevent the release of NF κ B dimers from I κ Bs, probably by blocking the interaction between I κ K and I κ Bs, thus blocking I κ B phosphorylation. Presumably, I κ Bs remain continuously bound to NF κ B dimers and do not allow their nuclear translocation. Studies have also reported that

phytoconstituents can inhibit I κ Ks, thus preventing phosphorylation or ubiquitination of I κ Bs and retrieving NF κ B dimers in cytoplasm.³⁴

Inflammatory reactions involve degranulation and subsequent release of various inflammatory mediators and free radicals. These chemical entities lead to respiratory burst by consuming both glucose and oxygen. Harmful factors like proteinases, reactive nitrogen species, and reactive oxygen species released during prolonged inflammatory reaction not only destroy invading pathogens but also damage local tissue.¹³ Histopathological examination of paw tissue of group2 (LPS-treated only) clearly

**Figure 5** Typical chromatogram showing peaks corresponding to various compounds present in *Cousinia thomsonii*.

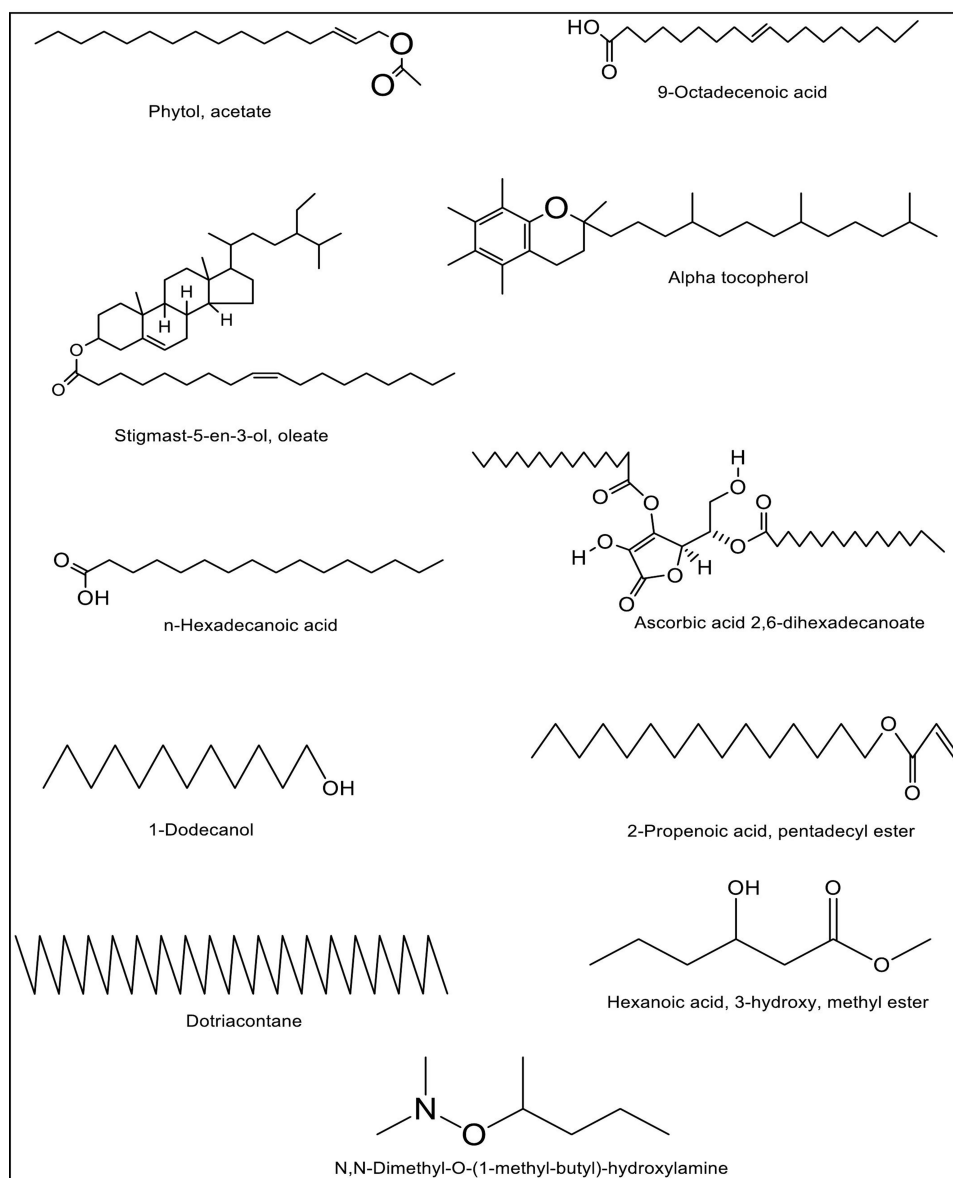


Figure 6 Structures of compounds identified in *Cousinia thomsonii*.

demonstrated leukocyte infiltration, vasodilatation, edema, and necrosis. Ameliorating effects of active *C. thomsonii* extracts was evident from tissue sections that showed dose-dependent rescue from inflammatory damage. Maleki et al observed similar results while investigating the efficacy of *Stachys inflata* hydroalcoholic extract. Ben et al reported similar outcomes while investigating anti-inflammatory effects of *Pistacia lentiscus*.^{46,47}

GC-MS analysis showed *C. thomsonii* contains several potential anti-inflammatory compounds that act through diverse mechanisms. For instance, phytol acetate, an acyclic diterpene alcohol, possesses anti-inflammatory properties.⁴⁸ Phytol inhibits the infiltration of neutrophils

and total leukocytes, as well as inhibiting IL1 β activity and TNF α expression.⁴⁹ Phytol also reduces the levels of proinflammatory mediators like histamine, serotonin, PGE₂, and bradykinins.⁵⁰ β -sitosterol inhibits phosphorylation of NF κ B.⁵¹ Prieto et al promulgated the anti-inflammatory potential of β -sitosterol under in vivo oxazolone-induced delayed hypersensitivity and came to the conclusion that this compound ameliorates edema without affecting arachidonic acid pathways.⁵² Loizou et al reported that β -sitosterol has inhibitory action on vascular adhesion and ICAM-1 in TNF α -stimulated human aortic endothelial cells.⁵³ A recent study by Yuan et al showed that β -sitosterol reduced the expression of

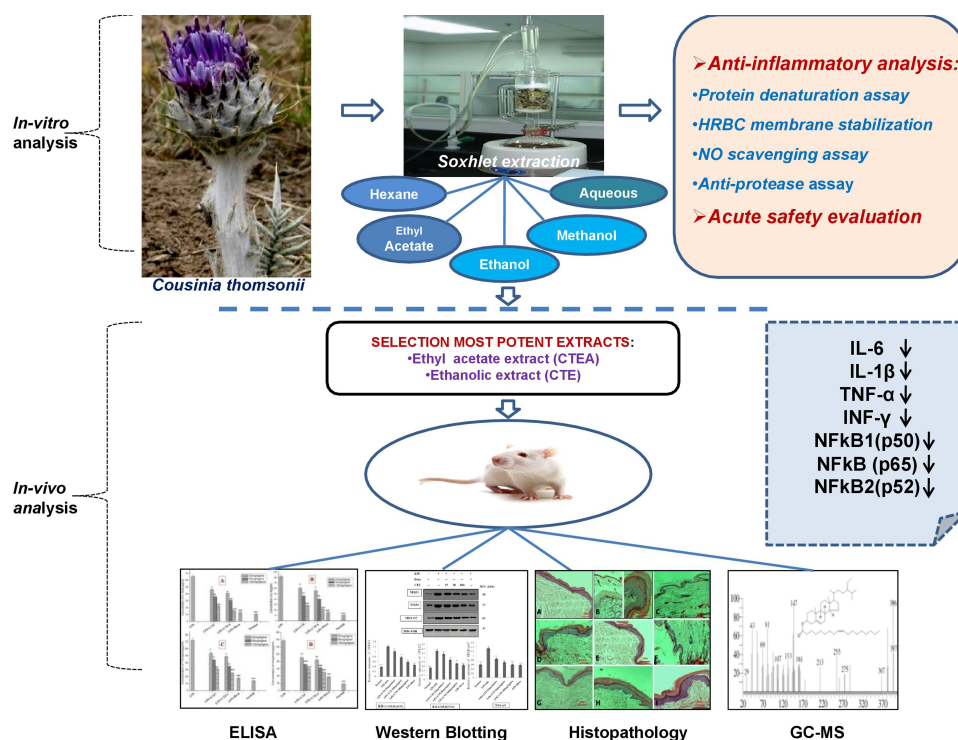


Figure 7 Possible anti-inflammatory/immunomodulatory mechanisms exhibited by *Cousinia thomsonii*.

proinflammatory cytokines and chemokines like TNF α . They also found that β -sitosterol acted as a potent blocker of NF κ B translocation to nuclei.⁵⁴ Stigmasterol, a phytosterol thwarts IL1 β -triggered NF κ B cascade via inhibition of I κ B degradation. Stigmasterol blocks the gene-level expression of *MMP13* and *MMP3* in IL1 β -stimulated human and mouse chondrocytes.⁵⁵ A derivative of ascorbic acid (vitamin C), namely 2,6-dihexadecanoate, also possesses anti-inflammatory activity.⁵⁶ Studies have shown that ascorbic acid blocks inflammation by decreasing expression of IL6, hsCRP, and TNF α . Moreover, ascorbic acid has also been reported to upregulate the expression of anti-inflammatory cytokines IL10 and IL4.^{57–59} Release of fatty acids due to hydrolytic action of PLA₂ on cell membranes is considered an important step in initiation of the inflammatory process. *N*-hexadecanoic acid, commonly known as palmitic acid, is a saturated fatty acid that acts as an important inhibitor of PLA₂, thus preventing inflammation.⁶⁰ Dotriacontane shows anti-inflammatory potential by reducing prostaglandins through the inhibition of cyclooxygenase.⁶¹ Tocopherol (vitamin E) suppresses IL1 and IL6 in rat models. Tocopherol is a strong antioxidant that neutralizes free radicals that stimulate NF κ B signaling, thereby

preventing the transcriptional activation of various bone-resolving cytokines, notably IL1 and IL6. Furthermore, tocopherol has also been reported to stall proinflammatory expression of the COX2 enzyme.⁶² While performing GC-MS analysis of *Bruguiera cylindrica*, Eldeen et al noticed that a fraction containing 2-propenoic acid pentadecyl ester as its main constituent possessed strong selective inhibitory action on the COX2 enzyme.³³

Conclusion

C. thomsonii possesses significant anti-inflammatory/immunomodulatory potential, which is evident from its property to avert protein denaturation, proteinase activity, NO surge, and HRBC-membrane hemolysis. The herb shows a marked ameliorating effect on the expression pattern of critical interleukins (TNF α , IL1 β , IL6, and IFN γ), as well as NF κ B1 p50, NF κ B2 p52, and NF κ B p65/RelA, thus highlighting its potential role in blocking the inflammatory surge possibly due to these molecular mechanisms (Figure 7). Several immunomodulatory and anti-inflammatory compounds identified in it might be acting individually or in a synergistic manner to ameliorate inflammatory response.

Abbreviations

CTEA, *Cousinia thomsonii* ethyl acetate; CTE, *C. thomsonii* ethanol; GC-MS, gas chromatography–mass spectrometry; PID, pelvic inflammatory disease; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; PVDF, polyvinylidene fluoride.

Acknowledgments

The authors are extremely thankful to the Department of Science and Technology Science and Engineering Research Board (DST-SERB), government of India for providing financial assistance to carry out this research work under grant EMR/2016/004958.

Disclosure

The authors report no conflicts of interest in this work.

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