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ORIGINAL ARTICLE

# Anti-inflammatory Activity of *Zanthoxylum rhetsa* Bark Fractions via Suppression of Nuclear Factor-kappa B in Lipopolysaccharide-Stimulated Macrophages

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## ABSTRACT

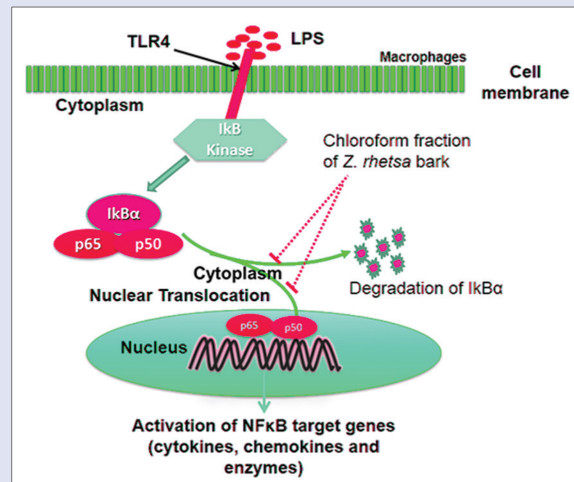
**Background:** *Zanthoxylum rhetsa* is a plant used in traditional medicine and is known to possess health benefits such as antibacterial, antidiabetic, and anti-diarrheal activities. **Objectives:** The objective of this study was to explore and demonstrate the anti-inflammatory activity of various solvent fractions of *Z. rhetsa* bark. **Materials and Methods:** The effect of crude methanolic extract and its fractions (hexane, chloroform, ethyl acetate, and butanol) on the levels of pro-inflammatory cytokines (interleukin-1β, tumor necrosis factor-alpha, and interleukin-6) and inflammatory factors was tested via targeting nuclear factor-kappa B (NF-κB) signaling pathways in lipopolysaccharide (LPS)-stimulated mouse RAW 264.7 macrophages. **Results:** Treatment with all the solvent fractions at various concentrations (50, 100, and 200 μg/ml) suppressed pro-inflammatory cytokine levels in a dose-dependent manner. Among the fractions, the chloroform fractions exhibited a significant inhibition of LPS-induced inflammation. Moreover, these fractions effectively suppressed the expression of various NF-κB signaling targets, including NF-κB, nitric oxide synthase, and cyclooxygenase-2 as well as inhibited the degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha (inhibitor of kappa B alpha [IκBα]). This anti-inflammatory effect was mediated by the prevention of IκBα degradation, as this protein prevents the translocation of NF-κB from the cytoplasm to the nucleus, initiating the transcription of pro-inflammatory genes. **Conclusion:** Thus, the *Z. rhetsa* chloroform fraction may be an effective natural anti-inflammatory agent against inflammation-associated diseases.

**Key words:** Inflammation, natural products, pro-inflammatory cytokines, RAW 264.7 cells, *Zanthoxylum rhetsa*

## SUMMARY

- Chloroform fraction of *Z. rhetsa* bark extract was the best among all the solvent fractions as it efficiently reduced the level of pro-inflammatory cytokines and mediators in LPS-stimulated RAW 264.7 macrophages.

**Abbreviations used:** ANOVA: Analysis of variance; AR: Ankyrin repeat; ATCC: American Type Culture Collection; BCA: Bicinchoninic acid; BSA: Bovine serum albumin; Carr: Carrageenan; COX-2: Cyclooxygenase-2; DMEM: Dulbecco's modified minimal essential medium; DMSO: Dimethylsulfoxide; ELISA: Enzyme-linked immunosorbent assay; HRP: Horseradish peroxidase; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; iNOS: Inducible nitric oxide synthase; IκBα: Inhibitor of kappa B alpha; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; MDA: Malondialdehyde; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB: Nuclear factor-kappa B; NO: Nitric oxide; NSAIDs: Nonsteroidal anti-inflammatory drugs; PBST: Phosphate-buffered saline-Tween-20; PGE2: Prostaglandin E2; PVDF: Polyvinylidene fluoride; SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-alpha.

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## INTRODUCTION

Inflammation is an important immune response initiated against the abnormalities in the body, and it is essential to repair the damage. Inflammatory processes are triggered by any stimuli, i.e., physical (e.g., tissue injury), chemical (histamine), or biological (e.g., pathogen infection). Inflammation is a non-specific and highly regulated defense mechanism to localize and remove the invading "nonself" agent from the

site and promote healing.[1] Chronic inflammation is a significant cause of various human diseases, including cancer, diabetes, allergy, autoimmune disorders, neurodegenerative diseases, colitis, and arthritis.[2]

The initial step in an inflammatory is the dilation of blood vessels to enhance blood flow to the affected area. This is mainly for increasing the number of leukocytes and other blood components in the affected area in order to establish an effective immune response. In response to the stimuli, macrophages, which are present in abundance at the affected site, secrete inflammatory mediators such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and pro-inflammatory cytokines (tumor necrosis factor-alpha [TNF-α], interleukin-1 beta [IL-1β], and interleukin-6 [IL-6]). The nuclear factor-kappa B (NF-κB) pathway is essential for the activation of genes encoding pro-inflammatory proteins. Lipopolysaccharides (LPSs) are endotoxins that have been used as stimuli to activate the immune system via Toll-like receptor 4 in various experimental models.[3,4] On simulation of the immune system by LPS, various signaling pathways are activated, specifically the NF-κB pathway, which is responsible for the induction of pro-inflammatory and inflammatory proteins.[5]

The control of inflammation has become an important topic of research because it is the cause of many illnesses. Currently, nonsteroidal anti-inflammatory drugs (NSAIDs) are the most common type of therapeutics used to reduce pain caused by inflammation; however, they commonly affect the gastrointestinal tract and can cause bleeding in the stomach and gut.[6] Thus, there is an urgent need for safer alternatives, particularly compounds from edible plants, which are believed to be minimally toxic. In line with this demand, in this study, we selected a wild edible plant *Zanthoxylum rhetsa*, which has been used in many parts of the world. For example, the leaves are used as a flavoring agent by the Mizos of Mizoram, India.[7]

*Z. rhetsa* (Family: Rutaceae) is a medium-sized tree with recurved prickles on its branches and trunk. Plants of the genus *Zanthoxylum* synthesize various secondary metabolites with numerous health benefits. In general, they have been used in the perfume, textile, food, aquatic, and pharmaceutical industries.[8] *Z. rhetsa* is believed to be a promising therapeutic candidate as previous studies have described its various pharmacological activities such as UV protective,[9] antihelminthic,[10] antibacterial,[11] antidiabetic,[12] Anti-diarrheal,[13] anticestodal,[14] and antioxidant activities.[15] Since there are no reported studies on the anti-inflammatory potential of *Z. rhetsa* fractions and the molecular mechanism of its anti-inflammatory action, we explored the anti-inflammatory potential of *Z. rhetsa* fractions in an *in vitro* experimental model, RAW 264.7 macrophages.

## MATERIALS AND METHODS

### Cell culture

RAW 264.7 murine macrophages were purchased from the American Type Culture Collection (USA), cultured in Dulbecco's modified minimal essential medium containing 10% (v/v) of heat-inactivated fetal bovine serum and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and maintained at 37°C in a humidified incubator with 5% $CO_2$. Cultures that reached 80% confluency were subcultured and used for further experiments.

### Preparation of *Zanthoxylum rhetsa* extract and its fractions

The bark material of *Z. rhetsa* was collected from Pangkor Island (Malaysia), air-dried, and ground, and the powdered material (910 g) was subjected to ultrasound-assisted extraction with 100% methanol and dried under vacuum at 40°C to yield the crude methanolic extract (65 g). The extract was then subjected to liquid–liquid partitioning using organic solvents such as hexane, chloroform, ethyl acetate, and butanol. The resulting fractions were dried under vacuum, lyophilized, and stored at −20°C for future *in vitro* analysis.[16]

### Cytotoxicity assay

Cytotoxicity assay was conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to identify the non-toxic range for further analysis. RAW 264.7 cell culture at 70%–80% confluence was seeded into 96-well plates ($1 \times 10^4$ cells/well) and incubated overnight to facilitate attachment to the plate. After incubation, the seeded cells were treated with various concentrations (15.62, 31.25, 62.5, 125, 250, 500, and 1000 μg/ml) of extract and different *Z. rhetsa* fractions (methanol, hexane, chloroform, ethyl acetate, and butanol) for 24 h, followed by incubation with 5 mg/ml of MTT solution for 4 h at 37°C. Next, 100 μl of DMSO was added to each well to dissolve the insoluble formazan crystals. The formazan was quantified by measuring the absorbance at 570 nm, and finally, the percentage of cell viability in each treatment group was calculated by comparing with untreated group.

### Measurement of nitric oxide production

The nitrite level was determined based on the Griess reaction method to identify the level of nitric oxide (NO) production. The RAW 264.7 cells were seeded in 6-well plates ($5 \times 10^5$ cells/well) and grown overnight. On the following day, the seeded cells were treated with different concentrations (50, 100, and 200 μg/mL) of test fractions and dexamethasone as a positive control drug. On treatment, the cells were incubated for 2 h, and all the wells were treated with LPS (1 μg/mL) to induce inflammation, except the negative control group that had only fresh media without any treatment. After incubation for 24 h, the culture supernatant was collected and assayed for nitrite by mixing with the Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride, and 5% phosphoric acid). The mixture was stored for 10 min in the dark, and absorbance was measured at 550 nm. The nitrite concentration was determined using sodium nitrite as a standard.

### Measurement of pro-inflammatory cytokine production

The pro-inflammatory cytokines in the supernatant collected from the treated cells were quantified using the same protocol used for nitrite analysis. For quantification of IL-1β, TNF-α, and IL-6, the collected cell culture supernatant was transferred into three separate 96-well plates coated with the capture antibody against the respective cytokine providing in the mouse-specific enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA), and the measurement was performed according to the manufacturer's instructions.

### Protein expression analysis

Cells were harvested after treatment with various concentrations of the chloroform fraction, positive control drug, dexamethasone, and negative control. Ice-cold RIPA mammalian protein extraction lysis buffer with protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland) was added to the harvested cells for protein extraction. The protein concentrations in the sample were determined by bicinchoninic acid protein assay kit, with bovine serum albumin (BSA) as a standard. Western blotting was performed to quantify the expression of various protein targets. Briefly, equal concentrations of protein samples were added and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% polyacrylamide gel, and the bands were transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was then blocked with 5% BSA, washed with phosphate-buffered saline-Tween-20 (PBST), and incubated with

specific primary antibodies overnight at 4°C with shaking. After incubation, the membranes were washed with PBST and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for about 1 h at room temperature on a shaker. Following incubation, the membranes were washed with PBST, and the chemiluminescence substrate (Thermo Scientific, Rockford, IL, USA) was added just before viewing the blots using a Chemidoc™ XRS (Bio-Rad). The intensity of the specific bands was analyzed using a Bio-Rad Lab image software.

### Statistical analysis

Results were analyzed using one-way analysis of variance followed by a *post hoc* Tukey's test. The experiments were performed in triplicate, and data are presented as the mean ± standard deviation. Differences were considered statistically significant when the *P* values were 0.05 or less.

## RESULTS

### Effect of *Zanthoxylum rhetsa* extract and solvent fractions (butanol, ethyl acetate, chloroform, and hexane) on the viability of RAW 264.7 cells

To select the optimal drug concentrations for the cell culture experiments, we investigated the effect of *Z. rhetsa* extract and solvent fractions on cell viability after incubation for 24 h by MTT reduction assay. As seen in Figure 1, exposure to concentrations of 250 μg/ml and below did not significantly influence cell viability, even though there was a slight decrease in viability as the concentration increased. Significantly higher cytotoxicity was observed in groups treated with 500 μg/ml and 1000 μg/ml than in the groups treated with 250 μg/ml and lower concentrations. Thus, 50, 100, and 200 μg/ml were selected as non-toxic concentrations of the extract and fractions for further cell culture experiments.

### Inhibitory effect of *Zanthoxylum rhetsa* extract and solvent fractions on nitric oxide production in lipopolysaccharide-stimulated mouse RAW 264.7 macrophages

We investigated the effect of *Z. rhetsa* extract and its solvent fractions on the production of NO in LPS-stimulated RAW 264.7 cells. All the active fractions significantly inhibited the LPS-induced NO production in a dose-dependent manner [Figure 2]. The cells treated with the chloroform and ethyl acetate fractions showed a greater inhibitory effect than the LPS-alone treated group and the groups treated with the other fractions or the standard drug dexamethasone.

### Inhibitory effect of *Zanthoxylum rhetsa* extract and solvent fractions on the production of the pro-inflammatory cytokines tumor necrosis factor-α, interleukin-6, and interleukin-1β in lipopolysaccharide-stimulated mouse RAW 264.7 macrophages

Treatment of the RAW 264.7 cells with LPS alone induced inflammation by producing high levels of TNF-α, IL-6, and IL-1β. To investigate the effect of the extract and solvent fractions on the production of pro-inflammatory cytokines in LPS-stimulated macrophages, ELISA was used. The extract and its solvent fractions effectively suppressed pro-inflammatory cytokine production in a dose-dependent manner. The chloroform fraction exerted the highest inhibitory effect on TNF-α production, followed by the ethyl acetate, methanol, hexane, and butanol fractions [Figure 3a]. The highest reduction in IL-6 production was achieved by the chloroform fraction, and the effect was almost similar to that dexamethasone [Figure 3b]. For IL-1β production [Figure 3c], the 200 μg/ml chloroform fraction and the 100 and 200 μg/ml ethyl acetate fractions did not show a major difference in the reduction achieved, and the reduction was similar to that achieved by dexamethasone. Thus, the chloroform fraction induced the most significant reduction in pro-inflammatory cytokine production and was chosen for further protein expression studies.

### Inhibitory effect of *Zanthoxylum rhetsa* chloroform fraction on the expression of inducible nitric oxide synthase, cyclooxygenase-2, inhibitor of kappa B alpha, and nuclear factor-kappa B in lipopolysaccharide-stimulated mouse RAW 264.7 macrophages

The NF-κB pathway is a vital signaling pathway that controls the production of inflammatory markers. Therefore, we investigated the

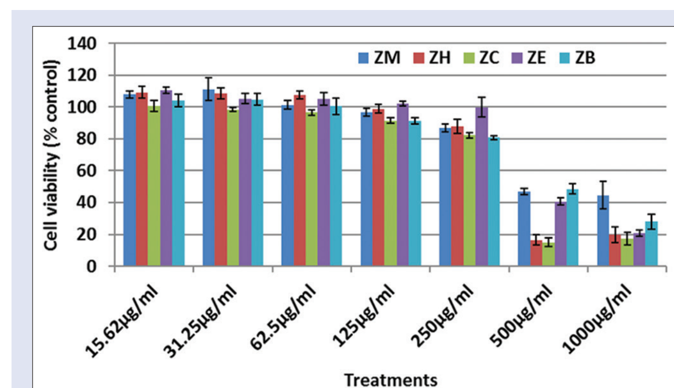


**Figure 1:** Effect of solvent fractions of *Zanthoxylum rhetsa* extract on viability of mouse RAW 264.7 macrophages. Cell viability following incubation with indicated concentrations of methanolic extract and fractions (butanol, ethyl acetate, chloroform, and hexane) for 24 h was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability is expressed as a percentage of untreated cells. Results shown in the graphs are mean ± standard deviation obtained from triplicate experiments

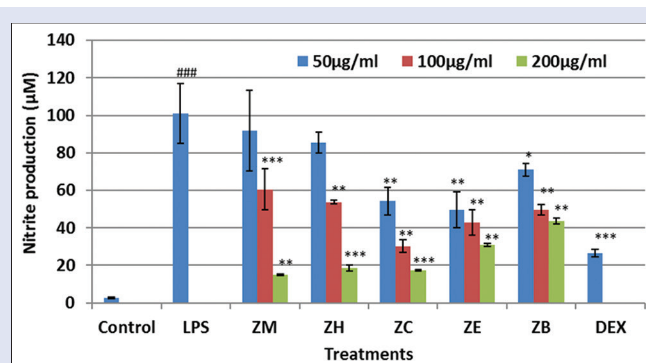


**Figure 2:** Effects of solvent fractions of *Zanthoxylum rhetsa* extract on nitric oxide production in lipopolysaccharide-stimulated mouse RAW 264.7 macrophages. Nitric oxide production after incubation with indicated concentrations of fractions (methanol, butanol, ethyl acetate, chloroform, and hexane) for 24 h was quantified using Griess reagent. Nitric oxide levels are expressed in μM, and the values shown in the graphs are mean ± standard deviation obtained from triplicate experiments. ###$P < 0.001$, lipopolysaccharide-treated group versus control; **$P < 0.01$ and *$P < 0.05$, significant difference from the lipopolysaccharide-treated group

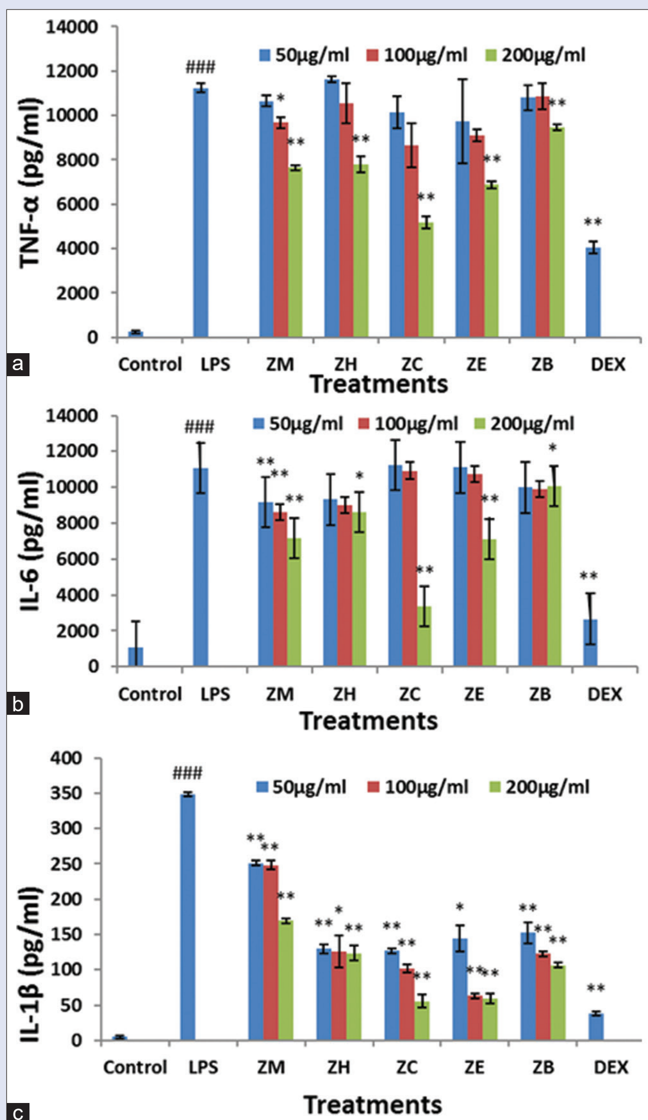


**Figure 3:** Effect of solvent fractions of *Zanthoxylum rhetsa* extract on the production of pro-inflammatory cytokines (a) tumor necrosis factor-alpha, (b) interleukin-6, and (c) interleukin-1 beta in lipopolysaccharide-stimulated mouse RAW 264.7 macrophages. Cytokine production following incubation with the indicated concentrations of the various fractions (methanol, butanol, ethyl acetate, chloroform, and hexane) for 24 h was determined by enzyme-linked immunosorbent assay. Tumor necrosis factor-alpha, interleukin-6, and interleukin-1 beta levels are expressed in pg/ml, and the values shown in the graph are mean ± standard deviation obtained from triplicate experiments. ###*P* < 0.001, lipopolysaccharide-treated group versus control; ***P* < 0.01 and **P* < 0.05, significant difference from the lipopolysaccharide-treated group

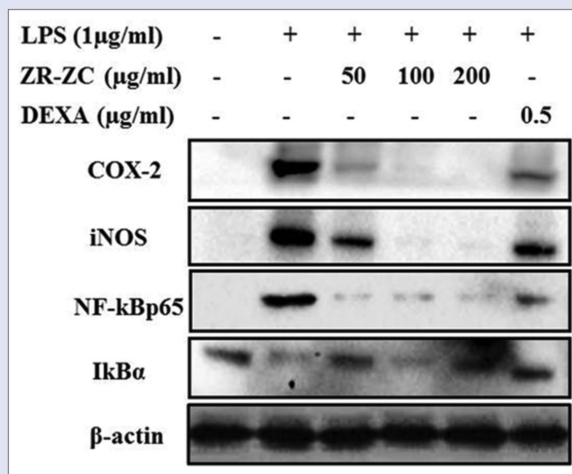


**Figure 4:** Effect of chloroform fraction of *Zanthoxylum rhetsa* extract on the expression of inflammatory mediators cyclooxygenase-2, inducible nitric oxide synthase, nuclear factor-kappa B p65, and inhibitor of kappa B alpha in lipopolysaccharide-stimulated mouse RAW 264.7 macrophages. Cells were treated with indicated concentrations of the fraction for 24 h, followed by protein extraction and separation by electrophoresis. The expression was quantified by Western blot analysis. Experiments were conducted in triplicate and images shown are representative of three experiments

effect of the chloroform fraction on the expression of iNOS, COX-2, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (inhibitor of kappa B alpha [IκBα]), and NF-κB. Expression of iNOS and COX-2 was suppressed by the chloroform fraction in a dose-dependent manner through Western blotting analysis [Figure 4], and the trend was similar to that observed for NO production [Figure 2].

Expression of IκBα and activated NF-κB (p-NF-κB p65), following LPS stimulation, seemed to be antagonistic. In LPS-stimulated RAW 264.7 cells, phosphorylated IκBα releases the active subunit for the successful translocation of p-NF-κβ p65 into the nucleus. Pretreatment with the chloroform fraction reversed the effect by preventing the phosphorylation of IκBα and the activation p-NF-κB p65 in a dose-dependent manner.

## DISCUSSION

Inflammation, particularly in a chronic state, is linked with various ailments which include diabetes, cardiovascular diseases, rheumatoid arthritis, and development of tumors including their metastasis.[17,18] Currently, NSAIDs are the most common group of drugs used to treat such conditions, but they pose many side effects, especially of the gastrointestinal tract, and are also unsuitable for patients with different medical backgrounds. Recently, many biomedical researchers have focused on natural products to find safer and effective anti-inflammatory compounds. Thus, in this study, we have investigated the anti-inflammatory mechanism of *Z. rhetsa* bark fractions in LPS-stimulated macrophages as an alternative anti-inflammatory candidate.

The innate immune system generically defends against "nonself" material such as exposure to LPS, which initiates endotoxin shock in cells that mimic septic shock or severe sepsis in the human body. Macrophages are one of the most important immune cells involved and triggered in an immune response; therefore, LPS-induced RAW 264.7 macrophages were selected for this study. Macrophages are classified as macrophage 1 or macrophage 2 and are distinguished according to their byproducts of NO and trophic polyamines, respectively.[19] The macrophages involved in this study were of the type macrophage 1 and their byproduct NO is involved in the pathogenicity of inflammation and acts as a messenger in the destructive activity of inflammation. Due to this fact, levels of NO and iNOS were examined. Another important gene, COX-2 was also included as it is responsible for the inflammatory cardinal signs: rubor (redness), calor (heat), tumor (swelling), and dolor (pain), via prostaglandin generation.[20] Induction with LPS alone increased the level of NO, iNOS, and COX-2, but pretreatment with *Z. rhetsa* extracts inhibited the various pro-inflammatory messengers.

NF-κB is a protein complex responsible for the activation of the inflammatory cascade and has been an earnestly pursued target in the study of inflammation due to its multiple roles such as in pro-inflammatory cytokine and leukocyte synthesis as well as in cell survival.[21] In normal state, before the presence of stimuli, NF-κB is located in the cytoplasm in an inactive form as a complex with Iκα protein, an inhibitor. The stabilizing ankyrin repeat domain in IκBα is responsible for its high affinity toward NF-κB in a resting state (Bergqvist *et al*., 2008). In the presence of stimuli, phosphorylation and degradation of IκBα release p65 from the IkB-p65 complex.[17] The phosphorylation of IκBα happens on the serine 32 and 36 residues of the N-terminus,[22] which helps the NF-κB to translocate to nucleus and initiate the transcription of target pro-inflammatory genes. Post-translocation into the nucleus, NF-κB undergoes some modification for its DNA-binding activity, and p65, being one of the best among the Rel proteins, plays a role in accelerating the transcriptional activity for the synthesis of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α and inflammatory genes iNOS and COX-2.[23]

Chloroform fraction of *Z. rhetsa* reduced the pro-inflammatory cytokines by blocking the degradation of IκBα that kept NF-κB inactive. The protein expression of IκBα increased gradually in a dose-dependent manner proving that it was intact without being phosphorylated. Nuclear accumulation of NF-κB p65 was downregulated upon treatment of LPS-stimulated macrophages. These observations indicate that the chloroform fraction of *Z. rhetsa* plays its anti-inflammatory role by blocking the phosphorylation of IκBα in NF-κB signaling pathway.

The anti-inflammatory potential of the bark extract and its fractions may be due to the presence of diverse classes of compounds present in it. Recently, several lignans, alkaloids, flavonoids, and triterpenes were identified in various solvent fractions of *Z. rhetsa* bark extract through gas chromatography–mass spectrometric analysis.[16] Moreover, it was reported that among other fractions, chloroform fraction and compounds such as kobusin and columbamine, present in the fraction, possess toxic effects against B-16 melanoma cells. Compounds such as lupeol, xanthoxyletin, osthol, scopoletin, and rutecarpine were also successfully isolated from this species.[24,25] The major compounds identified and isolated from this species were reported to possess anti-inflammatory activity. Particularly, lupeol was reported to suppress the expression of prostaglandin E2 (PGE2), TNF-α, and IL-1β in an *in vitro* model and also reduced allergic inflammation in airways in a murine model.[26] Osthol, another active compound, reduced the production of TNF-α, NO, and reactive oxygen species as well as inhibiting MAPK signaling pathway in LPS-stimulated macrophages.[27] Besides, a study on hepatic fibrosis using hepatic stellate cells showed that osthol hinders several inflammatory and fibrosis-related cytokines and chemokines.[28] Whereas, scopoletin also reduces malondialdehyde levels by reducing anti-inflammatory cytokines such as TNF-α, NO, PGE2, iNOS, and COX-2 in carrageenan (Carr)-induced paw edema model.[29] Rutecarpine, a bioactive compound, has also shown to suppress pro-inflammatory and inflammatory mediators in LPS-stimulated RAW 264.7 cells.[30] The presence of these above discussed bioactive compounds in this species strongly supports its anti-inflammatory value.

In the present investigation, the chloroform fraction inhibited the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and downregulated the inflammatory mediators in LPS-stimulated macrophages. Therefore, the chloroform fraction of *Z. rhetsa* extract may act as a safer anti-inflammatory agent that can diminish the inflammatory signaling cascade during tissue injury or infections.

## CONCLUSION

The chloroform fraction was the best among all the solvent fractions of *Z. rhetsa* bark extract as it efficiently reduced the level of pro-inflammatory cytokines and mediators in LPS-stimulated RAW 264.7 macrophages. The chloroform fraction of bark extract of *Z. rhetsa* may, therefore, be considered as a potential anti-inflammatory agent against inflammatory diseases. Further animal studies have been planned to confirm the molecular mechanism(s) of this extract in an animal model of inflammation.

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### Conflicts of interest

There are no conflicts of interest.

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