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Cytoprotective And Anti-Inflammatory Effects Of Manilkara Zapota Ethanolic Flower Extract Against H_2O_2-Induced Oxidative Stress In Raw 264.7 Macrophages

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Abstract

Background:

Oxidative stress plays a crucial role in the initiation of inflammation and cellular damage. Hydrogen peroxide (H_2O_2) is commonly used to induce oxidative stress in vitro. The present study evaluates the cytoprotective, antioxidant, and anti-inflammatory properties of the ethanolic extract of Manilkara zapota *flowers (SFE) on H_2O_2-induced RAW 264.7 macrophage cells.*

Methods:

Sapota flowers were collected, shade-dried, and ground into powder. Ethanolic extracts were prepared and tested for cytoprotective and anti-inflammatory activities using MTT assay, nitric oxide (NO) and prostaglandin E2 (PGE2) quantification, DCFDA staining for reactive oxygen species (ROS) assessment, and gene expression analysis via real-time PCR. Morphological changes were observed using phase contrast microscopy.

Results:

SFE was non-toxic up to 200 µg/ml and significantly improved cell viability in H_2O_2-treated cells. SFE effectively reduced NO, PGE2, and ROS levels in a dose-dependent manner. Morphological analysis confirmed the protective effect of SFE against oxidative damage. Gene expression studies showed that SFE downregulated pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, Cox-2a) and moderated IL-10 levels, indicating its anti-inflammatory potential.

Conclusion:

The ethanolic extract of Manilkara zapota *flowers exhibits strong cytoprotective, antioxidant, and anti-inflammatory activities, suggesting its potential as a therapeutic agent against oxidative stress-related inflammatory conditions.*

Keywords: *Oxidative stress,Anti-inflammatory,RAW 264.7 macrophages,Hydrogen peroxide (H_2O_2),Reactive oxygen species (ROS),Nitric oxide (NO),Prostaglandin E2 (PGE2)*

INTRODUCTION

Inflammatory activity refers to the body's response to harmful stimuli, which can lead to various diseases if dysregulated. Recent studies have explored both natural and synthetic anti-inflammatory agents, highlighting their mechanisms and potential applications. The following sections detail the sources, mechanisms, and implications of inflammatory activityPlant Extracts: Several medicinal plants, such as Dichrostachys cinerea and Ehretia rigida, exhibit significant anti-inflammatory properties by inhibiting key enzymes like 15-lipoxygenase and reducing nitric oxide release in macrophages[1].Adipose Tissue: Adipose tissue acts as an endocrine organ, releasing pro-inflammatory cytokines that contribute to chronic inflammation, particularly in obesity[2].Natural Compounds: Omega-3 fatty acids (EPA and DHA) from fish oil have been shown to inhibit inflammation by competing with arachidonic acid in the COX pathway, leading to reduced prostaglandin synthesis[3].Synthetic Agents: New synthetic compounds, such as quinazolinone derivatives, have demonstrated significant anti-inflammatory activity, suggesting potential for drug development[4].Chronic Inflammation: Persistent inflammation can lead to diseases like arthritis, cancer, and metabolic syndrome, emphasizing the need for effective anti-inflammatory strategies[5].Therapeutic Safety: Natural anti-inflammatory agents are often preferred due to their lower side effects compared to synthetic drugs, making them suitable for long-term use[6].This study explores the anti-inflammatory activity of sapota flower ethanolic extract .

MATERIAL AND METHODS

Collection of sapota flower

Fresh flowers were carefully plucked from healthy sapota trees, selected for their optimal quality, then subjected to shade drying. The dried flowers were subsequently ground into a fine powder for further analysis.

Chemicals and reagents

Hydrogen Peroxide (H2O2), Dulbecco's modified Eagle medium (DMEM), MTT, Dimethyl sulphoxide (DMSO), phosphate buffer saline (PBS), and antibiotic-antimycotic solution (100U penicillin, 100µg streptomycin, and 0.25µg amphotericin B per ml) were purchased from Sigma-Aldrich. Fetal bovine serum was purchased from GIBCO/BRL Invitrogen. Isorhamnetin was purchased from TCI chemicals, India. All other chemicals, reagents and solvents used were of analytical grade and purchased from SRL chemicals, India.

Cell line maintenance

RAW macrophage cell lines (RAW 264.7) were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37◦C in a humidified atmosphere containing 5% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

Cell viability (MTT) assay

The cell viability of Sample A treated with RAW 264.7 cells was assessed by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. RAW 264.7 cells were plated in 96 well plates at a concentration of 5x103 cells/well 24 hours after plating, cells were washed twice with 100µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37◦C. After starvation, cells were treated with different concentrations of Sample A or in combination with H2O2 (100 µg/mL) for 24 h. At the end of treatment, the medium from control and Sample A or in combination with H2O2 treated cells were discarded and 100µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4h at 37◦C in the CO2incubator. The MTT containing medium was then discarded and the cells were washed with 1x PBS. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100µl) and incubated in the dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as percentage of control cells cultured in serum-free medium. Cell viability in control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

Morphology study

Based on MTT assay we selected the optimal doses (200µg/ml of Sample A) for further studies. Analysis of cell morphology changes by a phase contrast microscope. 2×105 cells were seeded in 6 well plates and treated with Sample A or in combination with H2O2 (100µM/ml) for RAW 264.7 cells) for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope.

NO level measurement

RAW 264.7 cells (1x105 cells/ml) were pretreated with various concentrations of Sample A in 100 µM/ml H2O2 at 37℃ an incubator with 5% CO2. The NO level in the culture supernatants was measured using Griess reagents by adding 50 µl 1% sulphanilamide and 50 µl 0.1% NED in 5% phosphoric acid to 100 µl of culture supernatant in each well and incubating at room temperature for 15 min in the dark. Subsequently, absorbance at 540 nm was measured with a ELISA plate reader. A standard curve was prepared using NaNO2 as a standard solution in the same manner, and was used to calculate the concentration of NO.

PGE2 level measurement

RAW 264.7 cells were pretreated H2O2 with various concentrations of Sample A for 24h at 37 ℃ in an incubator with 5% CO2. PGE2 levels were assessed by a commercially available PGE2 enzyme immunoassay kit (cayman chemical co., Ann Arbor, MI, USA) in accordance with the instructions of the manufacturer. The PGE2 concentration was analyzed in accordance with the formula obtained from the standard curve generated using the PGE2 standard solution provided in the kit.

DCFDA staining

The intracellular ROS level in treated RAW 264.7 cells was analyzed by DCFDA staining. The cells were grown in 24 well plates and treated with Sample A or in combination with H2O2 (100µM/ml) for 24h time point After the treatment period, the cells were incubated with 200µl of DCFDA (10µM) working solution at 37°C for 20 min. After incubation, DCFH-DA working solution was removed and cells were washed with PBS and the intracellular ROS level was examined under a fluorescence microscope.

Real Time PCR

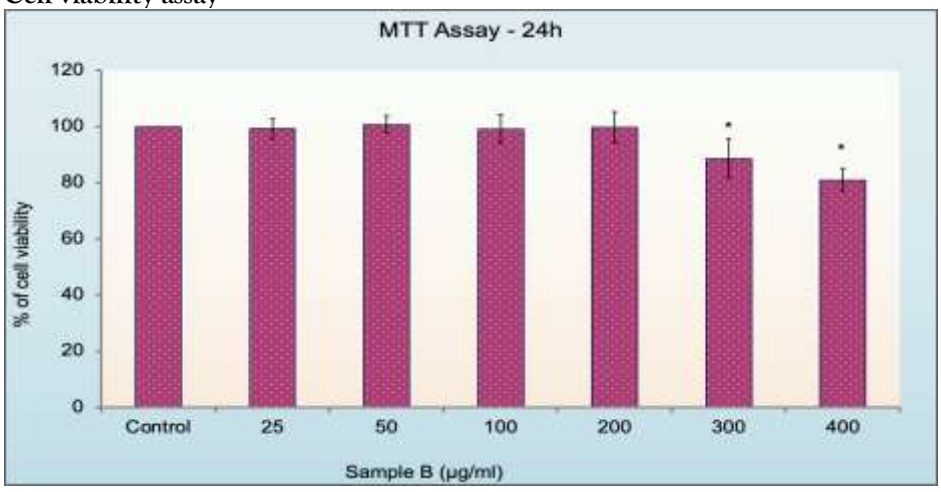
The gene expression of pro-inflammatory cytokines levels was analysed using real-time PCR. RAW macrophages were treated with Sample A with 100µM/ml of H2O2 and incubated for 24h. The total RNA was isolated by the standardized protocol using Trizol Reagent (Sigma). 2µg of RNA used for cDNA synthesis using reverse transcription using a PrimeScript, 1st strand cDNA synthesis kit (TakaRa, Japan). The targeted genes were amplified using specific primers. PCR reaction was performed with GoTaq® qPCR Master Mix (Promega), it contains SYBR green dye and all the PCR components. Real time-PCR was performed in a CFX96 PCR system (Biorad). The results were analyzed by comparative CT method and 2−ΔΔCT method was used for fold change calculation described by Schmittgen and Livak, 2008

Statistical analysis

All data obtained were analyzed by One way ANOVA flowed by Students-t-test using SPSS, represented as mean ± SD for triplicates. The level of statistical significance was set at $p<0.05$.

RESULTS

Cell viability assay

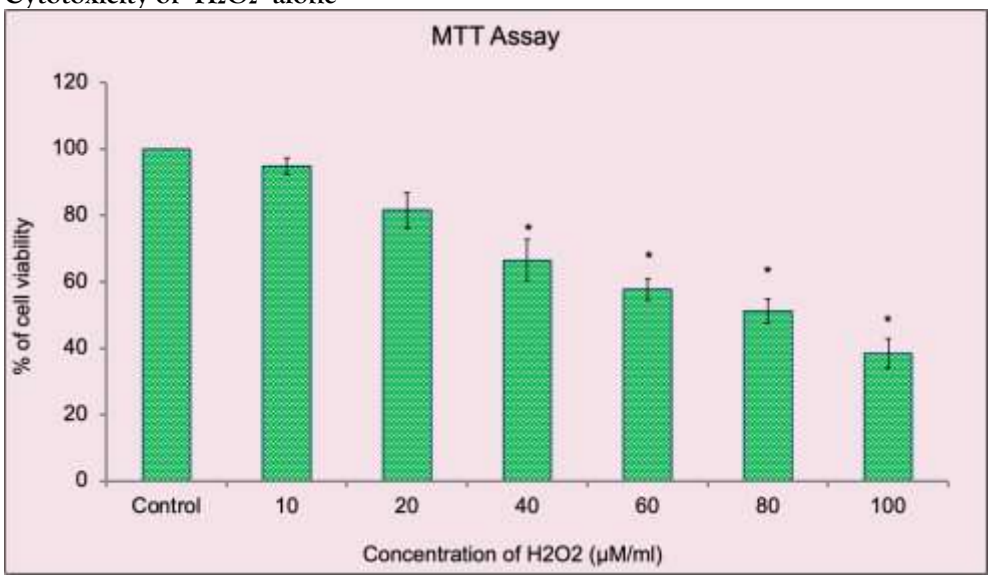


Effect of SFE on the viability of RAW 264.7 cells. The cells were treated with the indicated concentrations of H2O2 alone for 24 h. Cell viability was assessed using the MTT assay, and the results are expressed as the percentage of surviving cells over control cells. Each value is presented as the mean ± SD and is

representative of the results obtained from three independent experiments. The significance was determined by the Student's t-test (*p<0.05, compared with control group).

SFE is non-toxic up to 200 µg/ml, with cell viability close to or at 100%.At higher concentrations (300 and 400 µg/ml), a statistically significant reduction in cell viability ($p < 0.05$) is observed, suggesting mild to moderate cytotoxicity.This confirms that SFE is biocompatible at lower doses and can be safely used up to 200 µg/ml for therapeutic applications without harming macrophage cells.

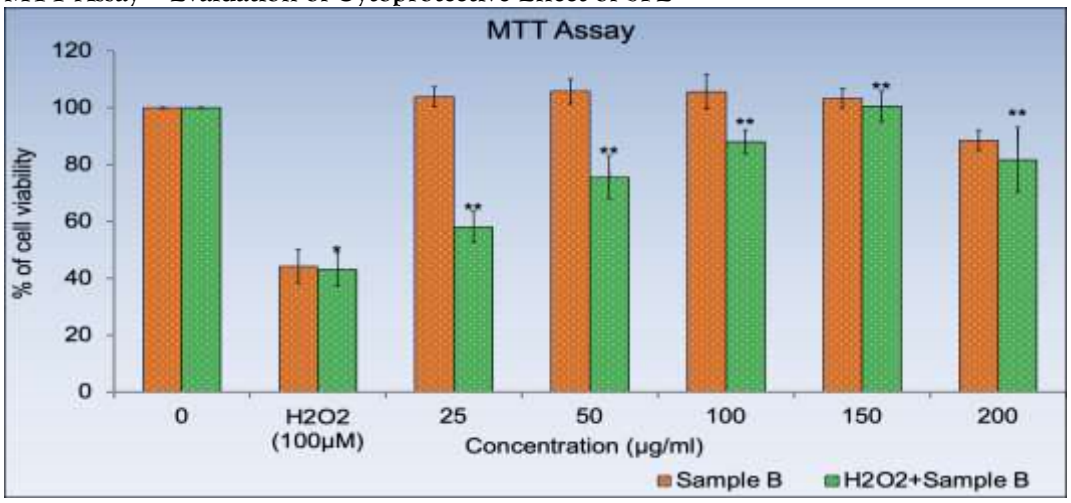
Cytotoxicity of H_2O_2 alone



Effect of H2O2 on the viability of RAW 264.7 cells. The cells were treated with the indicated concentrations of H2O2 alone for 24 h. Cell viability was assessed using the MTT assay, and the results are expressed as the percentage of surviving cells over control cells. Each value is presented as the mean ± SD and is representative of the results obtained from three independent experiments. The significance was determined by the Student's t-test (*p<0.05, compared with control group).

H_2O_2 reduces cell viability in a dose-dependent manner.At 100 µM, viability drops to ~38%, indicating severe oxidative damage.This justifies the use of 100 µM H_2O_2 in the previous experiment (SFE + H_2O_2), as it creates a standard oxidative stress condition.The MTT assay confirms the model of oxidative damage and provides a baseline to evaluate antioxidant/cytoprotective interventions.

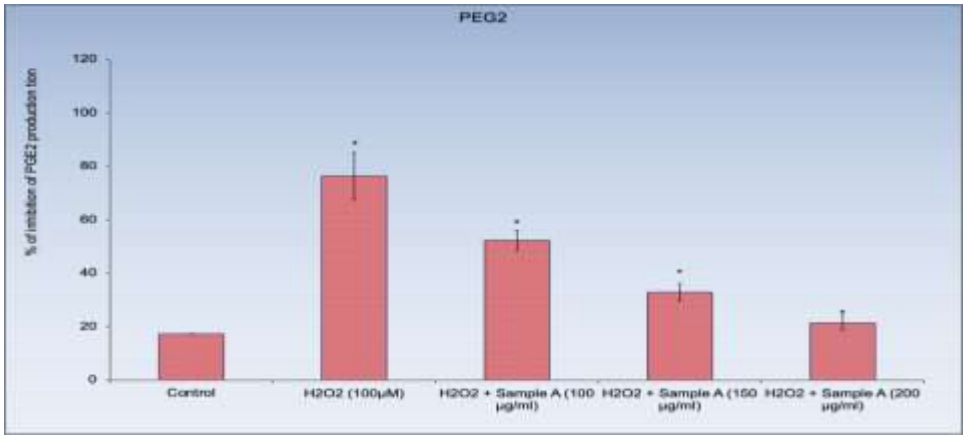
MTT Assay – Evaluation of Cytoprotective Effect of SFE



Effect of SFE on the viability of H2O2 induced RAW 264.7 cells. The cells were treated with the indicated concentrations of SFE or in combination with H2O2 (100 uM/ml) for 24 h. Cell viability was assessed using the MTT assay, and the results are expressed as the percentage of surviving cells over control cells. Each value is presented as the mean ± SD and is representative of the results obtained from three

independent experiments. The significance was determined by the Student's t-test (*p<0.05, compared with control group, ** p<0.05, compared with H2O2 vs H2O2+SFE treated). SFE is not cytotoxic up to 200 µg/mL.H_2O_2 reduces viability by more than half, confirming its cytotoxicity.SFE significantly reverses H_2O_2-induced damage in a dose-dependent manner.Maximum cytoprotection is observed between 100–150 µg/mL, where cell viability is restored close to normal.

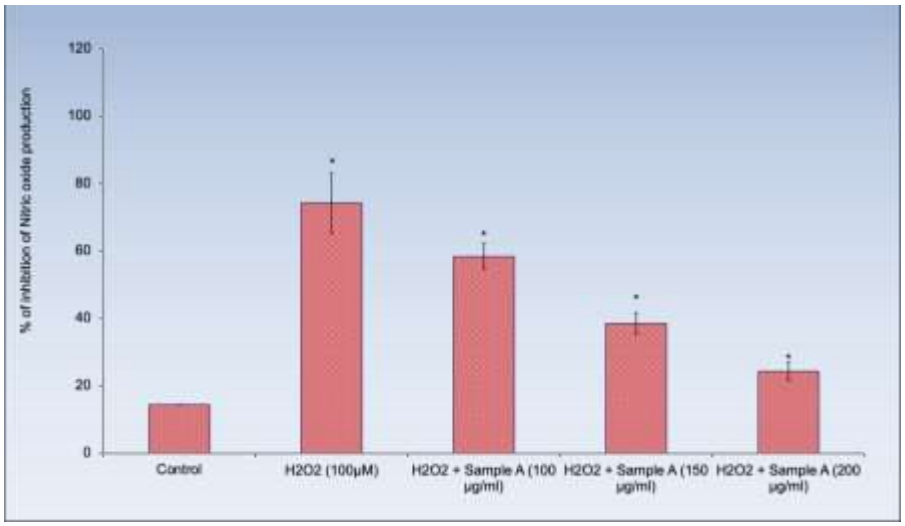
Inhibition of PGE2 Production in H_2O_2-Induced RAW 264.7 Cells



Effects of SFE on PEG2 production in H2O2-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of SFE or in combination with H2O2 (100 µM/mL) for 24 h. NO production was analyzed by ELISA kit. The data are presented as mean ± SD of at least three independent experiments. * indicates Control vs H2O2. ** indicates H2O2 vs Other groups $p < 0.01$.

The H_2O_2-treated group showed a significant increase in PGE2 production due to oxidative stress-induced inflammation. However, co-treatment with Sample A reduced PGE2 levels in a dose-dependent manner, suggesting that Sample A may inhibit COX-2 expression or reduce free radical-induced PGE2 synthesis.The highest dose of Sample A (200 µg/mL) most effectively inhibited PGE2 production, implying potential for anti-inflammatory therapeutic use.This outcome aligns with the nitric oxide (NO) inhibition results, where Sample A also reduced NO levels under similar conditions, further confirming its anti-inflammatory capacity.

Nitric Oxide Production in H_2O_2-Induced RAW 264.7 Cells

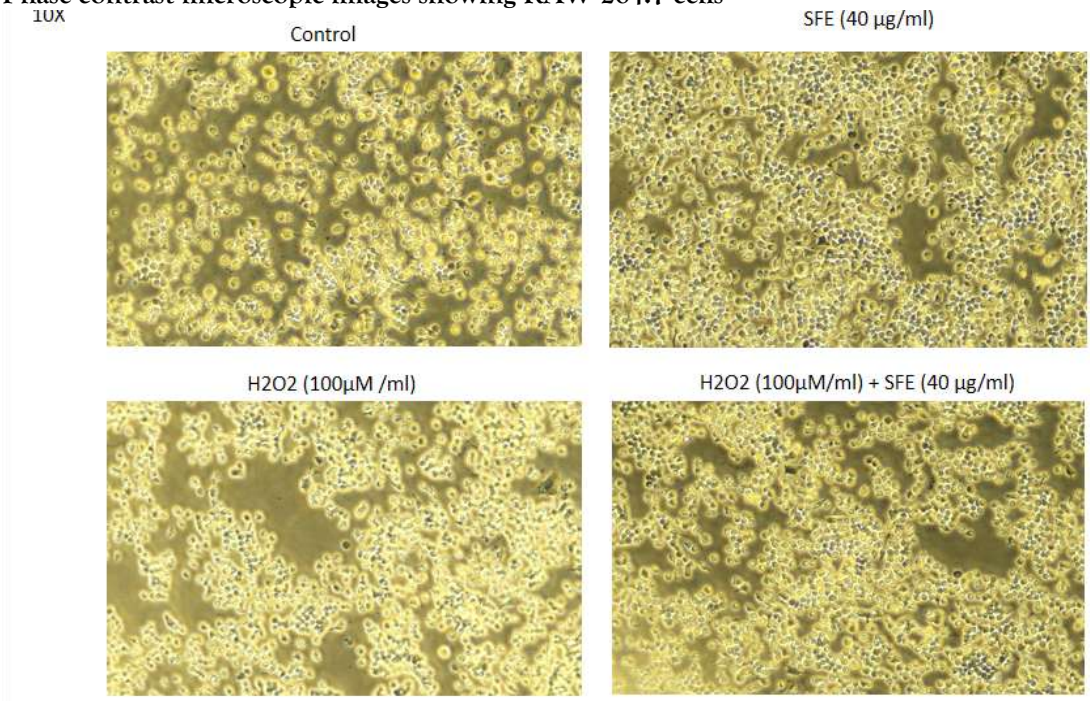


Effects of SFE on NO production in H2O2-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of SFE or in combination with H2O2 (100 µM/mL) for 24 h. NO production was analyzed by ELISA kit. The data are presented as mean ± SD of at least three independent experiments. * indicates Control vs H2O2. ** indicates H2O2 vs Other groups $p < 0.01$.

Hydrogen peroxide (H_2O_2) is known to generate reactive oxygen species (ROS), leading to oxidative stress, which can result in cellular damage, morphological distortion, and apoptosis in macrophages.

In this study, H_2O_2 treatment caused significant morphological alterations, such as cell shrinkage and reduced adherence.In contrast, treatment with SFE (40 µg/mL) alone showed no harmful effects on RAW 264.7 cells, demonstrating its non-toxic nature. Importantly, co-treatment with SFE and H_2O_2 mitigated the oxidative damage and helped restore normal cell morphology, suggesting that SFE exhibits protective, possibly antioxidant properties.

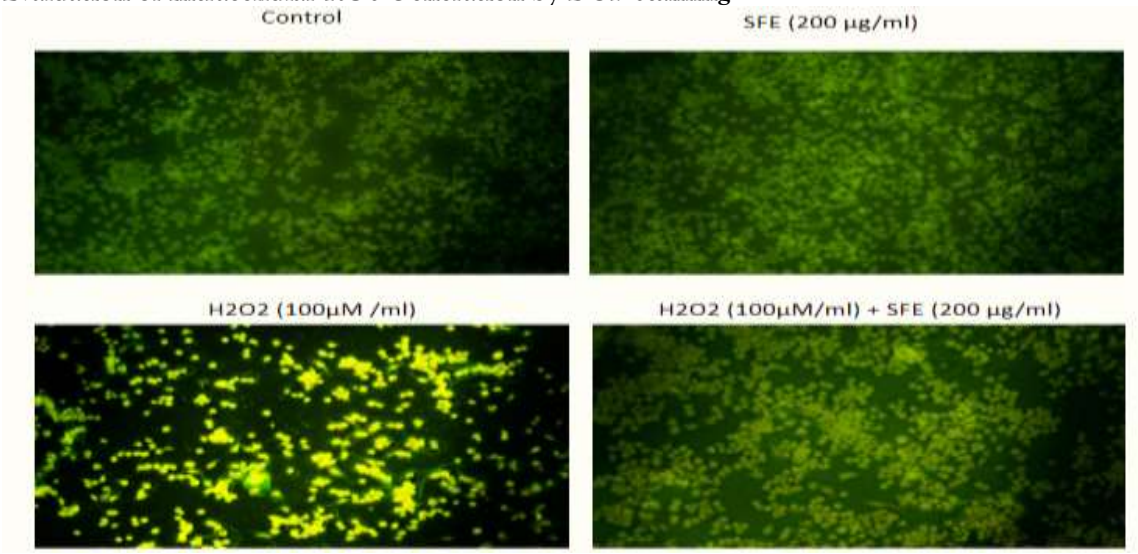
Phase contrast microscopic images showing RAW 264.7 cells



Effect of SFE on cell morphology of RAW 264.7 cells. Cells were treated with indicated concentrations of SFE or in combination with H2O2 (100 µM /mL) for 24 h and cells were observed under an inverted phase contrast microscope. Hydrogen peroxide (H_2O_2) is known to generate reactive oxygen species (ROS), leading to oxidative stress, which can result in cellular damage, morphological distortion, and apoptosis in macrophages. In this study, H_2O_2 treatment caused significant morphological alterations, such as cell shrinkage and reduced adherence.

In contrast, treatment with SFE (40 µg/mL) alone showed no harmful effects on RAW 264.7 cells, demonstrating its non-toxic nature. Importantly, co-treatment with SFE and H_2O_2 mitigated the oxidative damage and helped restore normal cell morphology, suggesting that SFE exhibits protective, possibly antioxidant properties.

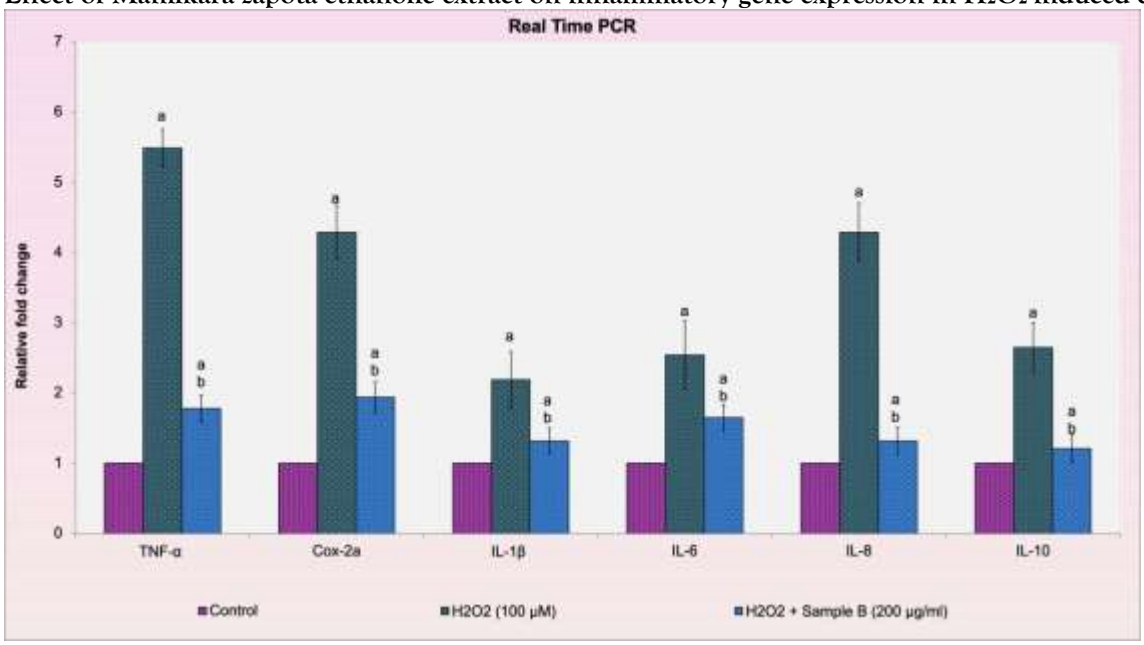
Evaluation of Intracellular ROS Generation by DCF Staining



Assessment of ROS level in SFE treated H2O2 induced RAW 264.7 cells. Cells were treated with indicated concentrations of SFE or in combination with H2O2 (100µM /ml) for 24 h and cell Images were obtained using an inverted fluorescence microscope. Oxidative stress is important in inflammation and cellular injury, mainly through the generation of ROS. H_2O_2 exposure in this research caused high levels of intracellular ROS accumulation, as shown by enhanced green fluorescence.

Treatment with Manilkara zapota ethanolic extract (SFE) drastically prevented ROS production in H_2O_2-treated RAW 264.7 cells. This was delineated as less fluorescence intensity in the co-treated group, which reveals the antioxidant and cytoprotective nature of the extract.DCF fluorescence analysis confirmed that the ethanolic extract of Manilkara zapota effectively reduces intracellular ROS in oxidative stress-induced RAW 264.7 cells.This supports the extract's potential as an anti-inflammatory and antioxidant agent, making it a promising candidate for therapeutic applications in oxidative stress-related disorders.

Effect of Manilkara zapota ethanolic extract on inflammatory gene expression in H_2O_2-induced cells.



Effect of SFE in pro-inflammatory cytokines expression in H2O2-stimulated RAW 264.7 cells. Total RNA was prepared for reverse transcriptase PCR (RT-PCR) analysis of pro-inflammatory cytokines gene expression in H2O2-stimulated RAW 264.7 cells. The experiment was repeated three times. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls for the RT-PCR analyses.Hydrogen peroxide significantly upregulated pro-inflammatory cytokines and enzymes, including TNF-α, Cox-2a, IL-1β, IL-6, and IL-8, indicating an acute oxidative stress and inflammatory response. Interestingly, IL-10 — an anti-inflammatory cytokine — also showed elevated expression in the H_2O_2 group, likely as a feedback mechanism.Co-treatment with the ethanolic extract of Manilkara zapota notably suppressed the expression of these inflammatory genes, bringing them closer to basal (control) levels. This indicates that the extract exhibits significant anti-inflammatory potential, possibly due to the presence of bioactive compounds such as flavonoids, tannins, and phenolic acids.The reduction in IL-10 expression in the sapota-treated group could be attributed to the overall reduction in pro-inflammatory stimuli, negating the need for compensatory IL-10 upregulation.Other studies often report downregulation of **NFκB1/2, TNFα, IL-1β, COX-2, NOS2** and upregulation of **NFE2L2 (NRF2)** as part of the antioxidant response.[7]

SUMMARY

This study aimed to evaluate the protective effects of *Manilkara zapota* flower extract (SFE) against hydrogen peroxide (H_2O_2)-induced oxidative stress in RAW 264.7 macrophage cells. Other studies also use **H_2O_2-induced oxidative stress in RAW 264.7 macrophages**. For example, research evaluated plant extracts (e.g., ginseng, bilberry) showing reduced expression of TNFα, COX-2, IL-1β, NOS2, and NFκB

genes in H_2O_2-stimulated RAW 264.7 cells[8].The extract was found to be non-toxic up to a concentration of 200 µg/ml and significantly improved cell viability in cells exposed to H_2O_2.The H_2O_2 dose typically used is **200 µM** (non-toxic) for inducing stress in such macrophage models[9] SFE effectively reduced oxidative stress markers, including nitric oxide (NO), prostaglandin E2 (PGE2), and intracellular reactive oxygen species (ROS).Downregulation of **NO**, **ROS**, **iNOS/NOS2**, and **COX-2** is a recurring finding. Bilberry, Pseuderanthemum, and many other flavonoid-rich extracts show this trend [10] Morphological observations confirmed that SFE mitigated H_2O_2-induced structural damage in macrophages. Furthermore, gene expression analysis revealed that SFE suppressed the expression of key pro-inflammatory cytokines and enzymes. Other studies often report downregulation of **NFκB1/2, TNFα, IL-1β, COX-2, NOS2** and upregulation of **NFE2L2 (NRF2)** as part of the antioxidant response[11]These results indicate that SFE possesses potent antioxidant and anti-inflammatory properties and may serve as a promising natural therapeutic agent for managing oxidative stress-related inflammatory disorders.Many comparative studies focus on leaves e.g., M. zapota leaves show anti-inflammatory effects in vivo or other parts like fruit, seeds[12].But this study is among the few investigating *flower extract* (Sapotaceae) in oxidative-inflammatory macrophage models, making it **novel** within the M. zapota literature.

CONCLUSION

The *Manilkara zapota* ethanolic flower extract provides substantial cytoprotection and anti-inflammatory benefits in macrophages subjected to oxidative stress. It restores cell viability, reduces inflammatory mediators, and limits intracellular ROS production. The downregulation of inflammatory gene expression further supports the therapeutic potential of SFE in oxidative stress and inflammation-associated diseases. This study highlights SFE as a promising natural agent for future exploration in the management of inflammatory and oxidative stress-related health conditions.

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