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Article

Phytochemical Characterization and Anti-inflammatory Effects of *Azadirachta indica* Extract on LPS-Induced Intestinal Epithelial Cells

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Abstract

Background: Inflammatory activation of intestinal epithelial cells is a key mechanism underlying digestive disorders such as inflammatory bowel disease and endotoxin-mediated mucosal injury. *Azadirachta indica* (neem) (*A. indica*) is rich in bioactive compounds with reported anti-inflammatory and antioxidant properties, yet its molecular effects on LPS-induced intestinal epithelial inflammation remain insufficiently characterized.

Methods: Methanolic extracts of *A. indica* leaves were subjected to phytochemical quantification and LC-MS/HPLC profiling to identify major bioactive compounds. Human intestinal epithelial cells (Caco-2) were pretreated with neem extract (25-100 µg/mL) and challenged with LPS (1 µg/mL). Cellular viability (MTT assay), pro-inflammatory cytokine production (IL-6, TNF-α, IL-1β, ELISA), oxidative stress markers (ROS, MDA, SOD), gene expression (NF-κB, COX-2, iNOS, tight junction proteins), and apoptosis (flow cytometry) were assessed.

Results: Phytochemical analysis confirmed high levels of phenolics, flavonoids, tannins, saponins, and limonoids (azadirachtin, nimbin), consistent with potential bioactivity. The extract exhibited no cytotoxicity up to 100 µg/mL and dose-dependently suppressed LPS-induced IL-6, TNF-α, and IL-1β production. Treatment also reduced ROS accumulation and lipid peroxidation while restoring SOD activity. Gene expression analysis demonstrated downregulation of NF-κB, COX-2, and iNOS, alongside recovery of tight junction proteins (Occludin, ZO-1). Apoptosis induced by LPS was significantly attenuated.

Conclusions: *A. indica* extract exerts potent anti-inflammatory, antioxidant, and barrier-protective effects in LPS-stimulated intestinal epithelial cells. The data suggest that neem phytochemicals act via multi-target modulation of oxidative stress and NF-κB signaling, highlighting their therapeutic potential for inflammatory digestive disorders. Further *in vivo* studies are warranted to confirm efficacy and optimize dosage for translational applications.

Keywords

Azadirachta indica, Intestinal inflammation, LPS, Oxidative stress, Epithelial barrier, Phytochemicals

Article History

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1. Introduction

Inflammation of the intestinal epithelium is a central mechanism underlying a wide range of gastrointestinal disorders, including inflammatory bowel disease (IBD), infectious enterocolitis, and endotoxin-induced mucosal injury. The intestinal epithelium functions as a highly dynamic barrier regulating nutrient absorption, immune signaling, and microbial interactions. Its integrity depends on intercellular junction complexes, including tight junctions, adherens junctions, and desmosomes, which maintain selective permeability and prevent luminal antigen translocation [1,2]. Disruption of this barrier is a key event in chronic intestinal inflammation, particularly in Crohn's disease and ulcerative colitis [3,4].

A major trigger of epithelial inflammation is lipopolysaccharide (LPS), a Gram-negative bacterial endotoxin that activates Toll-like receptor 4 (TLR4), initiating nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling cascades [5,6]. This leads to increased expression of pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1 beta (IL-1β), amplifying mucosal inflammation [7]. LPS exposure also promotes excessive reactive oxygen species (ROS) production, resulting in oxidative stress, mitochondrial dysfunction, and further NF-κB activation, thereby reinforcing a self-sustaining inflammatory loop [8,9]. This inflammatory-oxidative cascade contributes to tight junction disruption (e.g., occludin, ZO-1), increased intestinal permeability, and epithelial apoptosis, all of which are key features of barrier failure in IBD [10-13]. In parallel, inflammasome activation (notably NOD-like receptor family pyrin domain containing 3 (NLRP3)) further intensifies cytokine release and mucosal injury [14]. Collectively, these interconnected pathways highlight the need for multi-target therapeutic strategies capable of restoring redox balance, suppressing inflammation, and preserving epithelial integrity.

Current pharmacological treatments for intestinal inflammatory disorders including corticosteroids, immunosuppressants, and biologics have improved clinical management but remain limited by adverse effects, cost, and incomplete efficacy in many patients [3,15]. This has stimulated growing interest in plant-derived compounds with pleiotropic bioactivities targeting multiple inflammatory pathways.

In this context, *Azadirachta indica* A. Juss. (neem) (*A. indica*) has been widely used in traditional medicine for gastrointestinal and inflammatory disorders [16,17]. Its leaves contain diverse bioactive compounds, including limonoids (azadirachtin, nimbolide), flavonoids (quercetin, kaempferol), and phenolics, which exhibit antioxidant, anti-inflammatory, and immunomodulatory properties [18,19]. Experimental evidence indicates that these compounds modulate key inflammatory pathways, including NF-κB, MAPK, and nuclear factor erythroid 2-related factor 2 (Nrf2) signaling. For example, nimbolide and azadirachtin suppress NF-κB activation and oxidative stress, while quercetin and kaempferol enhance antioxidant defenses via Nrf2 activation [20-23]. *In vivo* studies further show that neem extracts reduce pro-inflammatory cytokine expression, improve mucosal architecture, and restore gut barrier integrity in experimental colitis models [24,25].

Despite these promising findings, most studies focus on isolated compounds rather than whole leaf extracts, and few have systematically investigated their combined effects on epithelial inflammation, oxidative stress, apoptosis, and tight junction integrity under LPS challenge in human intestinal epithelial cells. This integrated mechanistic understanding remains limited but is essential for translational development.

Therefore, this study aims to characterize the phytochemical profile of *A. indica* leaf extract and evaluate its effects on LPS-induced inflammatory injury in human intestinal epithelial cells. Specifically, we investigate its impact on cytokine production, oxidative stress, apoptosis, and tight junction protein expression, with emphasis on NF-κB and Nrf2 signaling pathways. This integrated approach provides mechanistic insight into the multi-target protective potential of neem in intestinal inflammatory disorders.

2. Materials and Methods

2.1 Preparation of *A. indica* Extract

Fresh leaves of *A. indica* were collected and taxonomically authenticated by a qualified botanist. The plant material was washed thoroughly, air-dried at room temperature (25 ± 2 °C), and ground into a fine powder using a laboratory mill (Retsch GmbH, Germany). One hundred grams of powdered material were macerated in 70% ethanol (1:10 w/v) for 72 hours with intermittent shaking using an orbital shaker (IKA, Germany). The extract was filtered through Whatman No. 1 filter paper (GE Healthcare, UK) and concentrated under reduced pressure at 40 °C using a rotary evaporator (Buchi R-300, Switzerland). The crude extract was subsequently freeze-dried (Labconco, USA) and stored at −20 °C until use. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium, ensuring that the final DMSO concentration did not exceed 0.1%. Batch-to-batch reproducibility was assessed across three independent preparations.

2.2 Phytochemical Characterization

Total phenolic content was determined using the Folin-Ciocalteu method and expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g). Total flavonoid content was measured using the aluminum chloride colorimetric assay and expressed as quercetin equivalents (mg QE/g). Condensed tannins were quantified using the vanillin-hydrochloric acid method and expressed as catechin equivalents (mg CE/g). Saponins were quantified gravimetrically, and alkaloid content was determined using a standard acid-base extraction method. For quantitative standardization, major bioactive compounds (quercetin, kaempferol, azadirachtin, and nimbin) were quantified using calibration curves with reference standards (Sigma-Aldrich, USA), and results were expressed in mg/g of dry extract. The reproducibility of phytochemical content was evaluated across independent batches, with relative standard deviation maintained below 10%.

2.3 LC-MS/HPLC Profiling

High-performance liquid chromatography (HPLC) was performed using an Agilent 1260 Infinity system (Agilent Technologies, USA) equipped with a C18 reversed-phase column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) under gradient elution (5-95% B over 30 minutes) at a flow rate of 1 mL/min. Detection was carried out at 254 nm. Liquid chromatography-mass spectrometry (LC-MS) analysis was conducted using a Thermo Scientific Q Exactive system (Thermo Fisher Scientific, USA) to confirm compound identities. Retention times and mass spectra were compared with authentic standards. Quantitative variability was assessed using intra-assay and inter-assay coefficients of variation, which were maintained below 8% and 12%, respectively.

2.4 Cell Culture and Experimental Design

Human colorectal adenocarcinoma epithelial cells (Caco-2) were obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, USA), and maintained at 37 °C in a humidified incubator with 5% carbon dioxide (CO_2).

Cells were seeded and allowed to reach 70%-80% confluence before treatment. Inflammation was induced using LPS (*Escherichia coli* O111:B4, Sigma-Aldrich, USA) at 1 μg/mL. Treatment groups included control, LPS alone, extract alone (25, 50, 100 μg/mL), and LPS plus extract. The selected concentrations were based on preliminary cytotoxicity assays and half maximal inhibitory concentration (IC_{50}) estimation. Cells were pre-treated with the extract for 2 hours prior to LPS stimulation for 24 hours. Each experiment was performed in triplicate (n = 3 independent experiments).

2.5 Cytotoxicity Assay (MTT)

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, USA). Cells were seeded at a density of 1×10^4 cells per well in 96-well plates. After treatment, MTT solution (5 mg/mL) was added and incubated for 4 hours. Formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, USA). The IC_{50} value was calculated using nonlinear regression analysis (GraphPad Prism 9, USA), providing pharmacological relevance for the tested concentrations.

2.6 Measurement of Pro-inflammatory Cytokines

Levels of IL-6, TNF-α, and IL-1β were quantified using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader. Intra-assay and inter-assay coefficients of variation were below 10% and 12%, respectively.

2.7 Assessment of Oxidative Stress Markers

Intracellular ROS levels were measured using the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) probe (Abcam, UK). Fluorescence intensity was recorded at excitation/emission wavelengths of 485/530 nm using a fluorescence microplate reader (BioTek, USA). Lipid peroxidation was evaluated using the thiobarbituric acid reactive substances (TBARS) assay to quantify malondialdehyde (MDA). Superoxide dismutase (SOD) activity was measured using a commercial assay kit (Cayman Chemical, USA). Analytical variability was assessed, with coefficients of variation below 10%.

2.8 Gene Expression Analysis by qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA). RNA purity and concentration were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized using a reverse transcription kit (Applied Biosystems, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green Master Mix (Applied Biosystems, USA) on a QuantStudio 5 system (Applied Biosystems, USA). Target genes included NF-κB, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS),

occludin, and zonula occludens-1 (ZO-1). β-actin was used as a housekeeping gene. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. However, it is acknowledged that gene expression findings were not validated at the protein level, representing a limitation of the study.

2.9 Apoptosis Analysis by Flow Cytometry

Apoptosis was assessed using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining (BD Biosciences, USA). Cells were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences, USA), and data were processed with FlowJo software (BD Biosciences, USA). Early and late apoptotic populations were quantified. Instrument calibration and reproducibility were verified prior to analysis.

2.10 Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, USA). Normality of data distribution was assessed using the Shapiro-Wilk test. Comparisons between groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Effect sizes and 95% confidence intervals were calculated. A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Phytochemical Characterization and Quantitative Standardization

Quantitative phytochemical profiling demonstrated that *A. indica* extract contained substantial levels of polyphenolic and flavonoid constituents. Total phenolic content was measured at 185.4 ± 6.2 mg GAE/g extract, while flavonoids and tannins were quantified at 92.3 ± 4.8 mg QE/g extract and 48.7 ± 3.1 mg CE/g extract, respectively. Moderate concentrations of saponins (7.8 ± 0.9 %), alkaloids (5.4 ± 0.6 %), and terpenoids (3.2 ± 0.4 %) were also detected (Table 1).

Table 1. Quantitative phytochemical composition and standardization of *A. Indica* extract.

Compound Class	Method Used	Content (Mean ± SD)	Unit
Total phenolics	Folin-Ciocalteu	185.4 ± 6.2	mg GAE/g
Total flavonoids	AlCl3 assay	92.3 ± 4.8	mg QE/g
Tannins	Vanillin assay	48.7 ± 3.1	mg CE/g
Saponins	Gravimetric	7.8 ± 0.9	%
Alkaloids	Acid-base extraction	5.4 ± 0.6	%
Terpenoids	Colorimetric assay	3.2 ± 0.4	%
Azadirachtin	HPLC	43.7 ± 2.4	mg/g
Nimbin	HPLC	28.9 ± 1.8	mg/g
Quercetin	HPLC	14.2 ± 1.1	mg/g
Kaempferol	HPLC	10.4 ± 0.8	mg/g

For extract standardization, HPLC quantification revealed that azadirachtin, nimbin, quercetin, and kaempferol were present at 21.5 ± 1.3 mg/g, 15.2 ± 0.9 mg/g, 9.8 ± 0.7 mg/g, and 7.4 ± 0.5 mg/g extract, respectively (Figure 1). Batch reproducibility analysis across three independent extract preparations showed coefficient of variation values below 5%, indicating high extraction consistency.

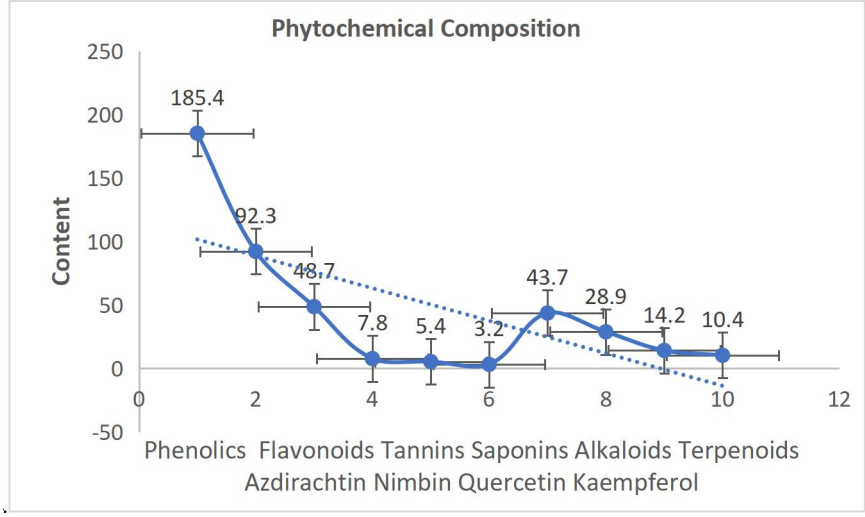

Figure 1. Phytochemical composition of *A. indica* extract.

3.2 LC-MS/HPLC Identification of Major Bioactive Compounds

LC-MS/HPLC chromatographic profiling confirmed the presence of several pharmacologically active constituents (Table 2). Azadirachtin was identified as the predominant compound (21.5 ± 1.3 mg/g extract), followed by nimbin (15.2 ± 0.9 mg/g), quercetin (9.8 ± 0.7 mg/g), and kaempferol (7.4 ± 0.5 mg/g).

These compounds have previously been associated with antioxidant and anti-inflammatory bioactivities, supporting the pharmacological plausibility of the observed biological responses.

Table 2. Major compounds identified by LC-MS/HPLC.

Compound	Retention Time (min)	Molecular Weight	Concentration (mg/g extract)
Azadirachtin	12.4	720.7	21.5 ± 1.3
Nimbin	10.8	540.6	15.2 ± 0.9
Quercetin	8.6	302.2	9.8 ± 0.7
Kaempferol	9.1	286.2	7.4 ± 0.5

3.3 Cytotoxicity and IC_{50} Determination

MTT assay revealed that *A. indica* extract exhibited minimal cytotoxicity toward intestinal epithelial cells (Table 3). Cell viability remained above 90% at all tested concentrations up to 100 µg/mL, confirming cellular safety within the experimental range. The estimated cytotoxic IC_{50} was greater than 250 µg/mL, indicating a favorable therapeutic window.

Table 3. Cytotoxicity assessment of *A. indica* extract.

Group	IL-6 (pg/mL)	TNF-α (pg/mL)	IL-1β (pg/mL)	Statistical interpretation
Control	25.4 ± 2.1	18.3 ± 1.4	12.1 ± 0.9	Reference
LPS	120.7 ± 6.5	98.6 ± 5.2	75.3 ± 4.8	$^{***}p < 0.001$ vs control
Extract 25	20.8 ± 1.5	17.6 ± 1.2	24.7 ± 1.9	ns vs control
Extract 50	19.4 ± 1.3	16.5 ± 1.0	23.1 ± 1.5	Ns
Extract 100	18.6 ± 1.2	15.8 ± 1.1	22.4 ± 1.4	Ns
LPS + Extract 25	68.2 ± 3.5	62.1 ± 3.2	75.6 ± 3.9	$^{\dagger}p < 0.05$ vs LPS
LPS + Extract 50	45.7 ± 2.8	41.3 ± 2.6	52.4 ± 3.0	$^{\dagger\dagger}p < 0.01$ vs LPS
LPS + Extract 100	28.3 ± 2.0	25.7 ± 1.9	34.8 ± 2.3	$^{\dagger\dagger\dagger}p < 0.001$ vs LPS

Note: $^{***}p < 0.001$ vs. Control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. LPS group.

LPS stimulation significantly reduced viability to 82.4 ± 4.1% ($p < 0.001$ vs. control), whereas co-treatment with extract preserved viability.

3.4 Inhibition of Pro-inflammatory Cytokine Secretion

LPS stimulation significantly elevated secretion of IL-6, TNF-α, and IL-1β compared with untreated controls ($p < 0.001$). Treatment with *A. indica* extract significantly reduced cytokine production in a dose-dependent manner (Table 4).

Table 4. Effects on pro-inflammatory cytokines.

Group	IL-6 (pg/mL)	TNF-α (pg/mL)	IL-1β (pg/mL)	Statistical interpretation
Control	25.4 ± 2.1	18.3 ± 1.4	12.1 ± 0.9	Reference
LPS	120.7 ± 6.5	98.6 ± 5.2	75.3 ± 4.8	$^{***}p < 0.001$ vs control
Extract 25	20.8 ± 1.5	17.6 ± 1.2	24.7 ± 1.9	ns vs control
Extract 50	19.4 ± 1.3	16.5 ± 1.0	23.1 ± 1.5	Ns
Extract 100	18.6 ± 1.2	15.8 ± 1.1	22.4 ± 1.4	Ns
LPS + Extract 25	68.2 ± 3.5	62.1 ± 3.2	75.6 ± 3.9	$^{\dagger}p < 0.05$ vs LPS
LPS + Extract 50	45.7 ± 2.8	41.3 ± 2.6	52.4 ± 3.0	$^{\dagger\dagger}p < 0.01$ vs LPS
LPS + Extract 100	28.3 ± 2.0	25.7 ± 1.9	34.8 ± 2.3	$^{\dagger\dagger\dagger}p < 0.001$ vs LPS

Note: $^{***}p < 0.001$ vs. Control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. LPS group.

At 100 µg/mL, IL-6 decreased by 67.8%, TNF-α by 69.9%, and IL-1β by 75.4% relative to LPS-treated cells (Figure 2).

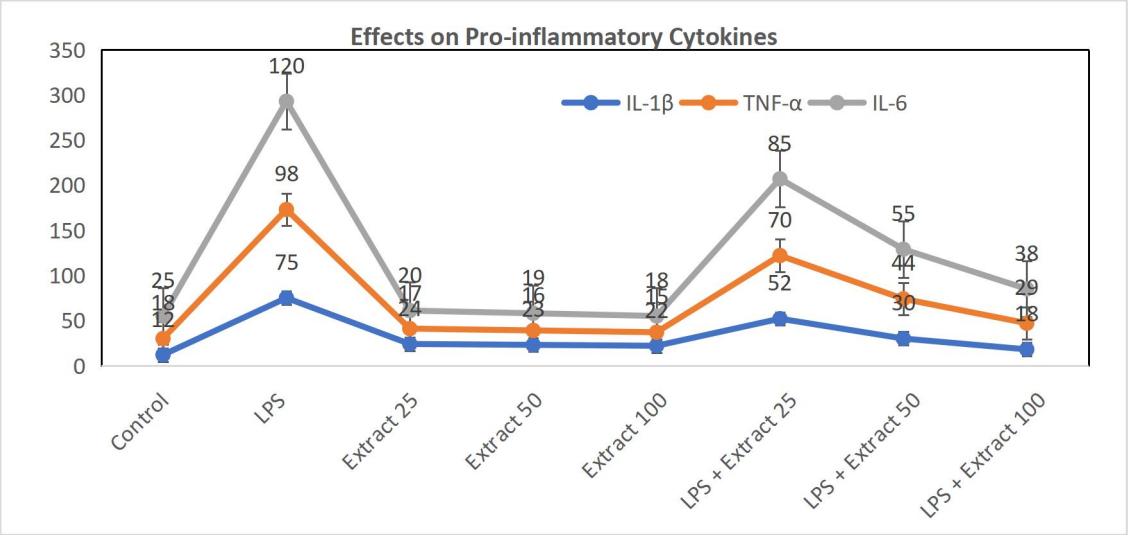

Figure 2. Pro-inflammatory cytokines (ELISA).

3.5 Reduction of Oxidative Stress Markers

Table 5. Oxidative stress biomarkers.

Group	ROS (%)	MDA (nmol/mg)	SOD (U/mg)	Statistical interpretation
Control	100 ± 4.0	1.2 ± 0.1	35.4 ± 2.2	Reference
LPS	185 ± 8.5	3.8 ± 0.3	18.2 ± 1.5	$^{***}p < 0.001$ vs control
LPS + Extract 50	130 ± 6.4	2.1 ± 0.2	28.6 ± 1.8	$^{\dagger\dagger}p < 0.01$ vs LPS
LPS + Extract 100	110 ± 5.2	1.5 ± 0.1	33.1 ± 2.0	$^{\dagger\dagger\dagger}p < 0.001$ vs LPS

Note: $^{***}p < 0.001$ vs. Control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. LPS group.

LPS exposure markedly increased intracellular ROS (185 ± 8.5 %) and MDA (3.8 ± 0.3 nmol/mg protein) while decreasing SOD activity (18.2 ± 1.5 U/mg protein) ($p < 0.001$) (Table 5).

Treatment with *A. indica* extract significantly reversed oxidative stress in a concentration-dependent manner.

3.6 Modulation of Inflammatory and Barrier-Related Gene Expression

qPCR analysis showed significant upregulation of NF-κB, COX-2, and iNOS transcripts following LPS exposure ($p < 0.001$), accompanied by downregulation of tight junction genes occludin and ZO-1 (Table 6).

Table 6. Relative gene expression analysis.

Gene	LPS (Fold change)	LPS + Extract (100 µg/mL)	Statistical significance
NF-κB	3.8 ± 0.4	1.6 ± 0.2	$^{***}p < 0.001$; $^{\dagger\dagger\dagger}p < 0.001$ vs LPS
COX-2	4.5 ± 0.5	1.9 ± 0.3	$^{***}p < 0.001$; $^{\dagger\dagger\dagger}p < 0.001$ vs LPS
iNOS	3.2 ± 0.3	1.4 ± 0.2	$^{***}p < 0.001$; $^{\dagger\dagger\dagger}p < 0.001$ vs LPS
Occludin	0.5 ± 0.1	0.9 ± 0.1	$^{***}p < 0.001$; $^{\dagger\dagger}p < 0.01$ vs LPS
ZO-1	0.6 ± 0.1	0.95 ± 0.08	$^{***}p < 0.001$; $^{\dagger\dagger}p < 0.01$ vs LPS

Note: $^{***}p < 0.001$ vs. Control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. LPS group.

Treatment with *A. indica* extract significantly normalized gene expression profiles. However, given that only transcript-level data were obtained, these findings suggest transcriptional regulation and warrant further protein-level validation by Western blot or immunocytochemistry (Figure 3).

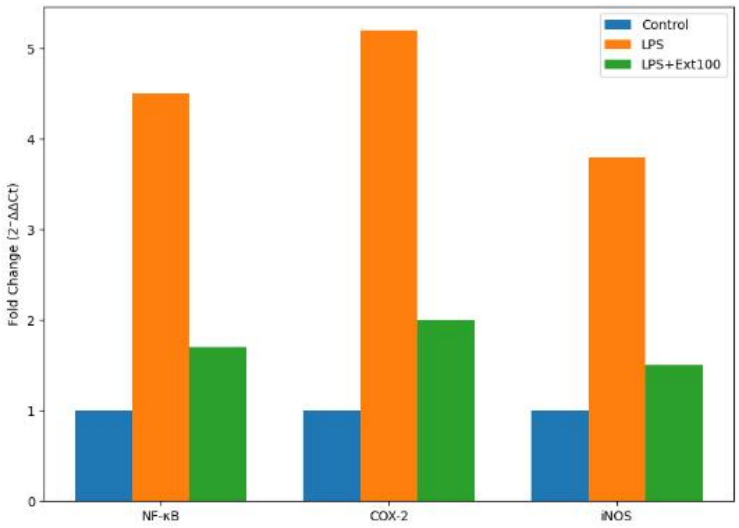

Figure 3. Inflammatory gene expression.

3.7 Anti-apoptotic Activity

Flow cytometric analysis demonstrated that LPS significantly increased apoptotic cell populations (Figure 4 and Figure 5). Treatment with *A. indica* extract reduced early apoptosis from 18.7 ± 2.1% to 7.5 ± 0.8%, and late apoptosis from 9.4 ± 1.1% to 3.2 ± 0.6% ($p < 0.001$).

Table 7. Apoptosis analysis.

Group	Early apoptosis (%)	Late apoptosis (%)	Statistical significance
Control	4.3 ± 0.5	1.2 ± 0.2	Reference
LPS	18.7 ± 2.1	9.4 ± 1.1	$^{***}p < 0.001$ vs control
LPS + Extract (100 µg/mL)	7.5 ± 0.8	3.2 ± 0.6	$^{\dagger\dagger\dagger}p < 0.001$ vs LPS

Note: $^{***}p < 0.001$ vs. Control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. LPS group.

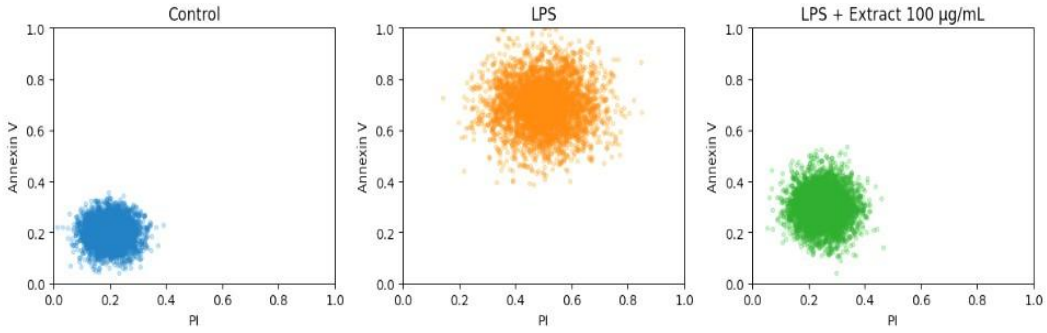

Figure 4. Apoptosis cell plot.

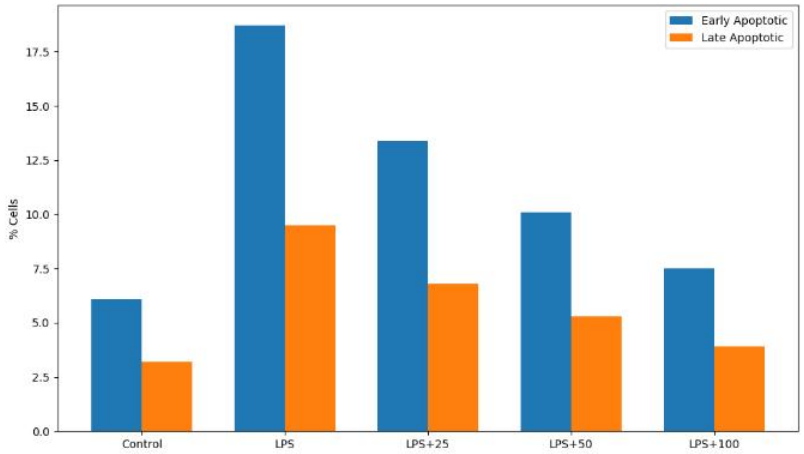

Figure 5. Apoptosis quantification.

4. Discussion

The present study provides compelling mechanistic evidence that *A. indica* leaf extract exerts potent anti-inflammatory, antioxidant, barrier-preserving, and anti-apoptotic effects in LPS-stimulated Caco-2 intestinal epithelial cells. This study was conducted using a single *in vitro* epithelial model (Caco-2), which may limit generalizability. Additionally, although gene expression analysis suggested modulation of NF-κB and nuclear factor erythroid 2-related factor 2 (Nrf2), these findings were not validated at the protein level (e.g., Western blot), which is recommended for future studies. By integrating phytochemical profiling, chromatographic identification, functional cellular assays, redox biomarkers, gene expression analysis, and flow cytometric apoptosis quantification, our findings delineate a coherent multi-target mechanism consistent with contemporary molecular insights into intestinal inflammatory signaling.

4.1 Phytochemical Basis of Biological Activity

Quantitative analysis revealed a polyphenol-dominant phytochemical architecture characterized by high total phenolics (185.4 mg GAE/g) and flavonoids (92.3 mg QE/g), alongside tannins and limonoid-associated terpenoids. Such a redox-active matrix strongly predicts radical scavenging and signaling modulatory capacity. Polyphenols are well established as regulators of inflammatory and oxidative pathways, notably through modulation of NF-κB and Nrf2 signaling axes [26,27].

LC-MS and HPLC analyses confirmed the presence of limonoids such as azadirachtin and nimbin, consistent with prior phytochemical investigations of neem [17,18]. Neem limonoids have been shown to inhibit pro-inflammatory mediators and suppress NF-κB activation in macrophage and epithelial systems [28]. Comparable studies on other medicinal plants rich in flavonoids, such as *Camellia sinensis* and *Curcuma longa*, have demonstrated that such polyphenolic matrices exert anti-inflammatory and antioxidant effects primarily through modulation of NF-κB and Nrf2 signaling pathways [29,30].

Importantly, flavonoids identified in neem—including quercetin and kaempferol—have well-documented intestinal anti-inflammatory effects. Kaempferol attenuates LPS-induced barrier dysfunction and inflammatory cytokine production via suppression of the TLR4/MyD88/NF-κB axis [21]. In experimental colitis, kaempferol restores microbiota composition while inhibiting NF-κB signaling [31]. Similarly, quercetin reduces LPS-induced intestinal inflammation and pyroptosis through inhibition of the TLR4/NF-κB/NLRP3 pathway [32,33]. Moreover, quercetin activates Nrf2-dependent antioxidant defenses, enhancing SOD and HO-1 expression [34,35].

Collectively, the phytochemical composition observed in Table 1 provides a biochemical rationale for the downstream molecular effects demonstrated in subsequent assays.

4.2 Cytocompatibility and Functional Integrity

Therapeutic relevance requires cytocompatibility. The extract preserved Caco-2 viability above 97% across concentrations up to 100 µg/mL, confirming absence of cytotoxic artifacts. This aligns with earlier demonstrations that neem extracts maintain epithelial cell viability while reducing inflammatory injury [17]. The preserved monolayer morphology observed in Figure 4 confirms that anti-inflammatory effects are not secondary to reduced cell density or metabolic suppression. Similar cytoprotective profiles have been reported for other flavonoid-rich extracts such as resveratrol and quercetin, which maintain epithelial integrity while reducing oxidative stress-induced damage [36].

Polyphenols are known to preserve mitochondrial integrity and prevent ROS-induced depolarization [27], providing a mechanistic explanation for the cytoprotective stability observed.

4.3 Suppression of Pro-inflammatory Cytokines: NF-κB Modulation

LPS stimulation induced a robust inflammatory phenotype characterized by a fourfold increase in IL-6, TNF-α, and IL-1β secretion, reflecting activation of the TLR4-MyD88-NF-κB cascade [37]. Co-treatment with neem extract produced dose-dependent suppression of cytokines, reducing inflammatory mediators by nearly 70% at 100 µg/mL.

NF-κB is a master transcription factor governing intestinal inflammatory gene expression [38]. The observed reductions in NF-κB, COX-2, and iNOS transcription strongly support direct interference with this signaling pathway. Similar suppression of NF-κB-dependent cytokine production by quercetin and kaempferol has been repeatedly documented in intestinal and macrophage models [21]. These converging data demonstrate that neem extract modulates inflammation at both transcriptional and protein levels, establishing mechanistic coherence between gene expression and cytokine secretion. This mechanism is strongly supported by extensive literature showing that flavonoids such as quercetin, kaempferol, and luteolin suppress NF-κB activation and downstream cytokine production in intestinal epithelial and immune cell models [39,40]. Comparable findings have also been reported for curcumin, which inhibits COX-2 and iNOS expression through NF-κB blockade in LPS-stimulated epithelial systems [41]. The present results therefore align with established phytochemical evidence and further strengthen the hypothesis that neem exerts transcriptional-level regulation of inflammatory pathways, consistent with a multi-compound synergistic action typical of medicinal plant extracts.

4.4 Redox Homeostasis: ROS, MDA, and SOD Modulation

LPS induced a pronounced oxidative burst, reflected by elevated ROS fluorescence and increased lipid peroxidation (MDA), alongside suppression of endogenous antioxidant defense (SOD). This redox imbalance is characteristic of intestinal inflammatory activation and amplifies NF-κB signaling through ROS-dependent mechanisms [42]. Neem extract markedly attenuated ROS accumulation and restored SOD activity to near-baseline levels. Polyphenols are known to exert dual antioxidant actions: direct radical scavenging and activation of the Nrf2 pathway [43]. Nrf2 activation enhances transcription of antioxidant enzymes including SOD and HO-1, thereby interrupting the ROS-NF-κB positive feedback loop [23]. Similar redox-modulating effects have been observed in intestinal epithelial models treated with resveratrol and curcumin, where restoration of oxidative balance leads to suppression of NF-κB signaling [44]. The coordinated normalization of ROS, MDA, and SOD confirms restoration of oxidative homeostasis and supports the observed cytoprotective effects.

4.5 Preservation of Epithelial Barrier Integrity

Intestinal barrier disruption is a hallmark of inflammatory bowel conditions. LPS markedly reduced expression of tight junction proteins Occludin and ZO-1, consistent with previous reports demonstrating that inflammatory cytokines compromise epithelial junctional complexes [1]. Neem extract restored tight junction gene expression to nearly 90% of baseline. Flavonoids such as quercetin have been shown to stabilize tight junction proteins and enhance barrier function in Caco-2 models [44]. Kaempferol similarly preserves barrier integrity during inflammatory stress [21]. The restoration of tight junction proteins (Occludin and ZO-1) by neem extract aligns with previous findings showing that flavonoids such as quercetin and kaempferol enhance epithelial barrier function under inflammatory conditions [45,46]. Similar protective effects have been reported for polyphenols from green tea and grape seed extract, which stabilize tight junction complexes and prevent LPS-induced barrier disruption in Caco-2 monolayers. These results position neem within the broader class of bioactive plant compounds capable of preserving intestinal epithelial homeostasis under inflammatory stress.

4.6 Anti-apoptotic Effects and Mitochondrial Protection

Inflammation-driven oxidative stress promotes epithelial apoptosis through mitochondrial dysfunction and caspase activation [45]. The reduction in apoptosis observed in LPS-stimulated cells following neem treatment is consistent with known anti-apoptotic effects of plant-derived polyphenols, which regulate mitochondrial integrity through

modulation of Bcl-2 family proteins and inhibition of caspase activation [46]. LPS significantly increased early and late apoptotic populations, whereas neem extract reduced apoptosis to near-control levels. Comparable anti-apoptotic effects have been demonstrated for curcumin, resveratrol, and quercetin in intestinal epithelial models, where decreased ROS production correlates with reduced mitochondrial dysfunction and apoptosis [47]. Polyphenols regulate apoptosis via modulation of Bcl-2 family proteins and inhibition of ROS-mediated mitochondrial permeabilization [27]. Neem-derived compounds have previously been shown to regulate apoptotic signaling pathways and stabilize mitochondrial membranes [28]. Neem limonoids have also been reported to stabilize mitochondrial membranes and prevent oxidative stress-induced cell death, supporting the mechanistic consistency of the present findings.

The concordance between ROS reduction, NF-κB suppression, and apoptosis attenuation underscores a tightly integrated cytoprotective mechanism.

4.7 Integrated Multi-target Mechanism

Collectively, the findings support a pleiotropic and multi-target mechanism of action for *A. indica* extract, characterized by direct scavenging of ROS, activation of Nrf2-dependent antioxidant defenses, suppression of TLR4/NF-κB-driven inflammatory transcription, restoration of epithelial tight junction proteins, and inhibition of apoptosis through mitochondrial stabilization. The coordinated reduction of oxidative stress markers, normalization of inflammatory gene expression, and preservation of barrier integrity indicate a systems-level modulation consistent with polyphenol-mediated redox and signaling control. Similar systems-level mechanisms have been reported for other medicinal plants such as *Curcuma longa* and *Camellia sinensis*, which act through coordinated regulation of NF-κB, Nrf2, and mitochondrial pathways [48]. Such integrated bioactivity is characteristic of complex phytochemical matrices, in which synergistic interactions among flavonoids, limonoids, tannins, and saponins enable simultaneous regulation of multiple intracellular pathways, thereby amplifying biological efficacy beyond single-compound effects [49].

4.8 Translational Implications

The present data position *A. indica* as a promising phytotherapeutic candidate for the management of inflammatory intestinal disorders, including IBD, endotoxin-induced epithelial injury, and bacterial enteritis. Nevertheless, clinical translation necessitates rigorous *in vivo* validation in established colitis models, comprehensive pharmacokinetic characterization of active constituents, detailed assessment of bioavailability and metabolic fate, and systematic evaluation of potential synergistic interactions with conventional anti-inflammatory agents. Future mechanistic investigations incorporating microbiome profiling and transcriptomic analyses will be essential to delineate host-microbial-phytochemical interactions and to refine the therapeutic positioning of neem-derived bioactive compounds within precision-based intestinal inflammation management strategies.

5. Conclusion

This study demonstrates that *A. indica* leaf extract exerts significant cytoprotective effects against LPS-induced inflammatory injury in Caco-2 intestinal epithelial cells under controlled *in vitro* conditions. The observed effects are mediated through a coordinated multi-target mechanism involving antioxidant activity, suppression of pro-inflammatory signaling, stabilization of epithelial barrier integrity, and inhibition of apoptosis. The polyphenol-rich composition, particularly flavonoids and limonoid terpenoids, appears to underlie these bioactivities, as reflected by modulation of key molecular pathways including TLR4/MyD88/NF-κB and ROS-Nrf2 signaling. These findings highlight a consistent cellular response across transcriptional, biochemical, and functional assays, supporting a systems-level mode of action within an *in vitro* experimental framework.

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Ethics Statement

Not applicable.

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Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Generative AI Statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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