World Journal of Pharmaceutical Science and Research
www.wjpsronline.com
Research Article
ISSN: 2583-6579
SJIF Impact Factor: 5.111
Year - 2025
Volume: 4; Issue: 4
Page: 1030-1043

ENHANCED AND SYNERGISTIC INHIBITION OF PRO-INFLAMMATORY CYTOKINES (IL-1B, IL-17A, AND IL-6) BY A CURCUMIN- THYMOQUINONE COMPLEX (CURQNONE®), COMPARED TO CURCUMIN MONOTHERAPY IN LPS-STIMULATED RAW 264.7 MURINE MACROPHAGES

S. Mehkri[1], K. G. Dinesh[2], G. Ashok[2] and Krathish Bopanna*[3]

[1]Bio-gen Extracts Pvt. Ltd., R&D Division, Bangalore, India.

[2]Radiant Research, R&D Division, Bangalore, India.

[3]Tejhana Consulting LLP, Consultant Pharmacologist, Bangalore, India.

Article Received: 29 July 2025 | | ***Article Revised: 19 August 2025*** | | ***Article Accepted: 09 September 2025***

***Corresponding Author: Krathish Bopanna**
Tejhana Consulting LLP, Consultant Pharmacologist, Bangalore, India.
DOI: https://doi.org/10.5281/zenodo.17130165

How to cite this Article: S. Mehkri, K.G. Dinesh, G. Ashok and Krathish Bopanna (2025) ENHANCED AND SYNERGISTIC INHIBITION OF PRO-INFLAMMATORY CYTOKINES (IL-1B, IL-17A, AND IL-6) BY A CURCUMIN- THYMOQUINONE COMPLEX (CURQNONE®), COMPARED TO CURCUMIN MONOTHERAPY IN LPS-STIMULATED RAW 264.7 MURINE MACROPHAGES. World Journal of Pharmaceutical Science and Research, 4(4), 1030-1043. https://doi.org/10.5281/zenodo.17130165

ABSTRACT

Background: Curcumin and thymoquinone (TQ) are phytochemicals with well-established anti- inflammatory and antioxidant properties.Curcumin inhibits NF-κB and MAPK signaling, and reduces transcriptional priming of inflammatory mediators, but its limited bioavailability restricts therapeutic effectiveness. TQ, derived from *Nigella sativa*, complements curcumin by inhibiting NLRP3 inflammasome activation and enhancing antioxidant responses through NRf2 signaling. A proprietary curcumin and TQ (CTQ) complex (CurQnone®) has been hypothesized to achieve broader suppression of inflammatory pathways than curcumin alone. This study compared the efficacy of CTQ with curcumin monotherapy in LPS-stimulated RAW 264.7 macrophages. **Methods**: RAW 264.7 macrophages were pre-treated with CTQ or curcumin (7.8 and 15.62 µg/mL), followed by stimulation with LPS (1 µg/mL). Cell culture supernatants were collected and analyzed for IL-6, IL-17A, and IL-1β levels using ELISA, with fold changes calculated relative to untreated controls. In parallel, transcriptional expression of iNOS and COX-2 was assessed by RT-PCR to evaluate the extent of inflammatory gene priming. **Results:** Both CTQ and curcumin significantly attenuated LPS-induced cytokine production and iNOS/COX-2 expression. CTQ consistently demonstrated stronger inhibition than curcumin alone, with fold reductions as follows: IL-1β (8.3-fold vs. 5.4-fold), IL-6 (5.4-fold vs. 2.4-fold), and IL-17A (9.4-fold vs. 8.6-fold). These effects were evident at both tested concentrations, suggesting a dose-responsive trend. In addition, CTQalso resulted in stronger downregulation of iNOS and COX-2 transcripts, indicating more effective blockade of transcriptional priming and pro-inflammatory mediator synthesis. **Conclusion:** CTQ exerted broader and more potent anti-inflammatory effect than curcumin alone in LPS- stimulated macrophages, targeting both transcriptional priming and cytokine maturation pathways. Its pronounced IL-6/IL-17 axis highlights potential application in chronic inflammatory and autoimmune conditions. These findings support CTQ as a synergistic, dose- sparing nutraceutical candidate warranting further preclinical and clinical evaluation.

KEYWORDS: CurQnone®, curcumin, thymoquinone, cytokines, IL-6, IL-17A, IL-1β, iNOS, COX-2, macrophage inflammation, combination therapy.

INTRODUCTION

Macrophages play a pivotal role in initiating and sustaining inflammatory responses. The murine RAW 264.7 macrophage line is a well-established model for investigating innate immune signalling, particularly when stimulated with lipopolysaccharide (LPS). Engagement of toll-like receptor 4 (TLR4) by LPS initiates MyD88-dependent signaling cascades, leading to phosphorylation of mitogen-activated protein kinases (MAPKs: p38, ERK1/2, JNK) and nuclear translocation of NF-κB. These pathways drive transcription of pro-inflammatory mediators, including TNF-α, IL-1β, and IL-6, perpetuation a self-sustaining inflammatory loop. Although IL-17A is primarily secreted by Th17 cells, it amplifies macrophage activation, inflammasome activity, and cytokine release, further aggravating inflammation and oxidative stress.[23, 24]

Curcumin, a polyphenolic compound derived from the rhizome of *Curcuma longa* (turmeric), has been extensively studied for its broad pharmacological effects. It inhibits NF-κB activation, downregulates MAPK signalling, scavenges reactive oxygen species (ROS), and suppresses the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). These mechanisms collectively reduce transcriptional priming and oxidative stress. However, curcumin's therapeutic potential is limited by poor water solubility, limited intestinal absorption, rapid first-pass metabolism, and systemic elimination, which restrict its effectiveness *in vivo*.[21-23]

Thymoquinone (TQ), the principal bioactive constituent of *Nigella sativa* (black seed), has emerged as a potent immunomodulatory compound. TQ suppresses NF-κB activation, inhibits dendritic cell maturation, and promotes apoptosis of hyperactivated immune cells, thereby restoring immune homeostasis. Mechanistically, it directly inhibits NLRP3 inflammasome assembly, reducing caspase-1 activation and IL-1β maturation. In addition, TQ activates the Nrf2 pathway, increasing expression of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, which lowers intracellular ROS levels.[25-27]

Although curcumin and TQ share several overlapping targets, they act through distinct mechanisms that make their combination potentially synergistic. Curcumin is more potent in suppressing NF-κB- and MAPK-driven transcriptional priming, whereas TQ more effectively blocks inflammasome activation and provides stronger antioxidant support. Together, these actions create a mechanistic basis for synergy: curcumin attenuates the initiation of inflammation, while thymoquinone disrupts its amplification and effector phases. Table 1 summarizes the distinct and overlapping mechanisms of action of the two compounds.

Table 1: Reported mechanisms of action of curcumin and thymoquinone on inflammatory pathways.

Feature	Curcumin	Thymoquinone
NF-κB inhibition	Strong	Strong
MAPK modulation	Yes (ERK, JNK, p38)	Limited evidence
Inflammasome inhibition	Moderate	Strong (NLRP3/caspase-1)
Antioxidant (Nrf2/ROS)	Potent	Potent
Effect on TNF-α	↓	↓
Effect on IL-1β	↓	↓ (stronger at maturation step)
Effect on IL-6	↓	↓
Effect on IL-17A response	Dampens IL-17A-driven signaling	Dampens IL-17A-driven signaling

Furthermore, reported cytokine outcomes suggest that combining curcumin and thymoquinone produces stronger suppression of pro-inflammatory mediators and restoration of immunoregulatory balance. This is summarized in Table 2.

Table 2: Reported anti-inflammatory effects of curcumin, thymoquinone, and their combination.

Cytokine	Effect of TQ	Effect of Cur	Curcumin + TQ
TNF-α	↓ Inhibits expression via NF-κB suppression	↓ Suppresses transcription & release	Synergistic suppression
IL-6	↓ Downregulates synthesis	↓ Inhibits IL-6 release from macrophages	Stronger and faster inhibition
IL-1β	↓ Modulates inflammasome activity	↓ Inhibits inflammasome activation	Additive reduction in IL-1β secretion
IFN-γ	↓ Modest inhibition	↓ Reduces Th1 polarization	Enhanced downregulation in Th1-driven responses
IL-10 (anti-inflammatory)	↑ May increase regulatory cytokines	↑ Promotes IL-10 release	Restores balance between pro- and anti-inflammatory cytokines

These complementary mechanisms provide a strong rationale for evaluating a proprietary curcumin-thymoquinone (CTQ) complex (CurQnone®) in combination.

While curcumin alone effectively reduces NF-κB- and MAPK-driven cytokine priming, it is less effective against NLRP3-mediated IL-1β maturation and ROS-driven amplification. TQ addresses these limitations, offering the potential for broader, dose-sparing, and more durable anti-inflammatory activity.

The present study was therefore designed to compare the anti-inflammatory efficacy of CTQ with curcumin monotherapy in LPS-stimulated RAW 264.7 macrophages. Specific endpoints included IL-6, IL-17A, and IL-1β secretion, as well as transcriptional expression of iNOS and COX-2, in order to establish whether the combination provides superior multi-targeted control of macrophage-mediated inflammation.

MATERIALS AND METHODS

Reagents and Test Compounds

Curcumin 95% (≥95% HPLC purity) and CurQnone® (standardized curcumin and thymoquinone complex) were stored at 4°C in light-protected, desiccated amber vials. For experiments, 10 mg of each compound was weighed on a calibrated analytical balance and dissolved in DMEM-high glucose (DMEM-HG; with 2% heat-inactivated FBS) to prepare 1 mg/mL sterile stock solutions (0.22 µm PES filter). Two-fold serial dilutions (0.98–250 µg/mL) were freshly prepared for each run to minimize degradation andadsorption following best practices for polyphenol handling [1]. Dexamethasone (1 µM) served as a pharmacological anti-inflammatory control; Lipopolysaccharide (LPS, ***E. coli*** O111:B4) was used as an inflammatory stimulus.

Cell Line and Culture Conditions

Murine RAW 264.7 macrophages (NCCS, Pune; conforms to ATCC TIB-71 guidelines) were maintained in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 IU/mL), streptomycin (100 µg/mL), and amphotericin-B (5 µg/mL), at 37 °C in 5% CO_2 and ~95% humidity. Cells were passaged at 70–80% confluence using TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). Experiments used passages 5–20 to ensure phenotypic stability. [2] Cell viability before treatment exceeded 95% by trypan blue, and cultures were periodically screened for mycoplasma (DNA stain).

Cytotoxicity and Selection of Working Concentrations

Cytotoxicity was assessed using the MTT assay. RAW 264.7 cells (1×10^4 cells/well) were seeded in 96-well plates and treated with serial dilutions of CTQ or curcumin for 24 h. After incubation, 20 µL of MTT solution (5 mg/mL) was

added per well and incubated for 4 h. Formazan crystals were solubilized with 100 µL DMSO, and absorbance was measured at 570 nm. Viability ≥90% was defined as non-toxic. Both compounds maintained >90% viability up to 15.62 µg/mL, and two non-toxic concentrations (7.8 and 15.62 µg/mL) were selected for subsequent assays.

Phagocytosis Assay (Flow cytometry + microscopy)

Phagocytosis was quantified using fluorescein-labeled *E. coli* bioparticles (manufacturer's protocol, flow cytometry-optimized).[4] RAW 264.7 cells (1×10^6 cells/mL) were seeded into 24-well plates with sterile coverslips and allowed to adhere for 24 h. Cells were pre-treated with non-toxic CTQ, curcumin, or vehicle for 1 h at 37°C, followed by incubation with 20 µL *E. coli* slurry for 3 h. A quenching solution (2 min, RT) removed extracellular fluorescence. Cells were washed, scraped, centrifuged (400 g, 5 min**)**, resuspended in ice-cold buffer, and analyzed on a 488 nm (FL1) cytometer, acquiring ≥10,000 singlet, live events per sample (FSC/SSC gating; doublet discrimination). Data were reported as MFI relative to vehicle. Replicate coverslips were fixed (4% PFA), counterstained (DAPI), and imaged (widefield fluorescence) to visually confirm intracellular uptake.

Induction of Inflammation (LPS protocol and groups)

Cells were seeded in 6-well plates **(**1.5×10^5 cells/mL**)** and allowed to adhere for 24 h. The experimental groups included.

- **Untreated Control**,
- **LPS only** (positive inflammation control),
- **Curcumin + LPS**,
- **CTQ + LPS**,
- **Dexamethasone + LPS** (reference control).

Unless otherwise specified, LPS was used at 5 µg/mL for 24 h, based on pilot range-finding experiments (0.1–5 µg/mL), which confirmed robust activation at 5 µg/mL. Confirmatory assays at 1 µg/mL produced the same rank-order response, consistent with macrophage activation literature.[5,6]

RNA Isolation, cDNA Synthesis, and Gene Expression

Total RNA was extracted using TRI reagent (1 mL/well) with chloroform phase separation, isopropanol precipitation (−20 °C, 10 min), 75% ethanol wash, and resuspension in nuclease- free water (A260/A280 = 1.8–2.0). 1 µg RNA was reverse-transcribed with oligo(dT) and M- MLV RT (Bio-Rad).

- **Targets:** TNF-α, iNOS, COX-2;
- **Housekeeper:** GAPDH.
- **Semi-quant PCR:** 95 °C 5 min; 35 cycles of 95 °C 30 s / 58 °C 30 s / 72 °C 45 s; final 72 °C 7 min. Amplicons were run on 1.5% agarose, ethidium bromide stained, and imaged (Gel Doc). Band densitometry used ImageJ; expression normalized to GAPDH.
- RT-qPCR was performed in accordance withMIQE guidelines with 2^–ΔΔCt normalization and primer efficiency checks.[7]

Cytokine Quantification by ELISA

Supernatants (post-treatment) were clarified **(**500 g, 10 min**)** and stored at −80 °C**.** IL-1β, IL- 6, IL-17A were quantified by sandwich ELISA (Elabscience) following the manufacturer's instructions. Standards/samples were run in duplicate**,**

with 450 nm readout, 4-parameter logistic fit ($R^2 > 0.99$). For assay performance, intra-assay CV was maintained <10%**,** inter- assay CV <15%**,** and spike-recovery 80–120%, in line with good ELISA practice.[8]

Randomization, Blinding, and Batch Control

Treatment positions were randomized across plates to mitigate edge effects. Sample identifiers were masked during plate reading and densitometry analysis. Experiments were repeated on ≥3 independent biological replicates**,** each with technical duplicates. Runs included a common LPS-only calibrator to monitor batch variability, and data were normalized to this calibrator where applicable.

Statistical analysis

All experiments were performed with at least three independent biological replicates (n = 3), each with technical duplicates to minimize intra-assay variability. Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted using GraphPad Prism version 9.0 (GraphPad Software, USA). Group comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post-hoc test to assess pairwise differences. Significance was accepted at $p < 0.05$.

For ELISA assay, assumptions of normality and homogeneity of variance were verified using Shapiro–Wilk and Brown–Forsythe tests, respectively. In cases where assumptions were violated, data were log-transformed to achieve normal distribution before re-analysis. Outliers were identified using the robust regression and outlier removal (ROUT) method with Q = 1%, and any exclusions were reported explicitly in figure legends. Graphical data are displayed as bar graphs with error bars representing SEM. All statistical outcomes, including exact *p*-values and F-statistics, are provided either in figure legends or as supplementary material to ensure transparency and reproducibility.

Table 3: Literature-Based Expected Inhibition of Inflammatory Mediators by Curcumin (Cur), Thymoquinone (TQ), and Their Combination (Cur+TQ) in LPS-Stimulated Macrophages.

Condition	TNF-α (4–6 h)	iNOS (24 h)	NO (24 h)	COX-2 (24 h)	PGE_2 (24 h)
LPS only	100%	100%	100%	100%	100%
Curcumin (10 µM)	↓ 40–60%	↓ 50–65%	↓ 50–65%	↓ 50–65%	↓ 50–65%
TQ (5 µM)	↓ 45–65%	↓ 45–60%	↓ 40–60%	↓ 50–65%	↓ 50–65%
CTQ	↓ 20–35%	↓ 20–35%	↓ 20–35%	↓ 20–35%	↓ 20–35%

#Percentages indicate residual expression relative to LPS-only control (set at 100%).

Lower percentages represent stronger suppression.

Published reports consistently demonstrate that curcumin and thymoquinone each reduce LPS- induced production of TNF-α, iNOS, COX-2, nitric oxide (NO), and prostaglandin E_2 (PGE_2) in macrophage models by approximately 40–65% at physiologically relevant concentrations (Curcumin 10 µM, TQ 5 µM).[23,24] Importantly, when administered in combination, the effects appear additive or synergistic, with reductions approaching 65–80% inhibition, leaving only 20–35% of the inflammatory response relative to untreated LPS controls.

RESULTS

Cytotoxicity Screening and Dose Selection

To ensure that the anti-inflammatory effects observed were not confounded by cytotoxicity, curcumin and CTQ were first screened for cell viability using the MTT assay. Both compounds maintained >90% RAW 264.7 cell viability up to 15.62 µg/mL, with CTC_{50} values of 22.77 µg/mL and 24.48 µg/mL, respectively. Based on these data, two non-

cytotoxic concentrations — 7.8 µg/mL (low dose) and 15.62 µg/mL (high dose) were selected for all subsequent functional assays.

Phagocytosis Assay

Gated histograms (Fig. 1) demonstrated that LPS stimulation increased the proportion of phagocytosing cells from 20.34 ± 1.9% (control) to 41.51 ± 2.6%, confirming robust activation. Treatment with curcumin further enhanced phagocytosis, yielding 59.2 ± 2.1% (low dose) and 63.25 ± 2.3% (high dose) phagocytosing cells. CTQ produced a comparable, slightly plateaued response at both doses (61.7 ± 1.8% and 61.5 ± 2.0%), indicating near-saturation of phagocytic capacity. Representative fluorescence microscopy confirmed the cytometric findings, showing an increased number of intracellular FITC-positive bacteria per macrophage.

Phagocytosis was conducted for three hours, and the amount of engulfed *E. coli* was determined by flow cytometry. CYTI-ID histograms of the gated THP-1 singlets distinguish cells at the M1 and M2 phases. (Here M1 refers to negative expression/region and M2 refers to the positive expression/region). Gating of M1 and M2 phases is approximate and can be further refined using software (BD Cell Quest Software, version 6.0) analysis. % cells observed in M2 region was considered for the current study.

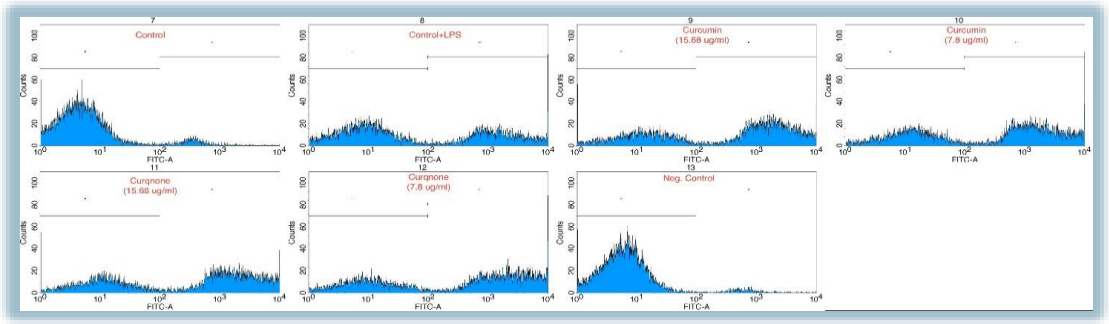

Fig. 1: Represented estimation of phagocytosis property of curcumin and CTQ on macrophages by flow cytometry.

Table 4: Effect of curcumin and CTQ on Phagocytic Activity of RAW 264.7 Macrophages .

Treatment Group	Concentration (µg/mL)	% Phagocytosing Cells (M2 Region)
Cell Control (C/C)	—	20.34 ± 1.9
C/C + LPS	5	41.51 ± 2.6 ★
Curcumin 95%	15.62	63.25 ± 2.3 ★★
	7.8	59.20 ± 2.1 ★★
CTQ	15.62	61.48 ± 2.0 ★★
	7.8	61.71 ± 1.8 ★★
Negative Control	—	4.06 ± 0.7

Data are mean ± SEM (n = 3 independent experiments). ★ p < 0.05 vs. untreated control;

★★ p < 0.01 vs. untreated control (one-way ANOVA followed by Tukey's multiple comparison test).

Overlaid bar graph depicted the % of cells phagocytosing the FITC labelled *E.coli.* When the foreign pathogen infects higher organisms, phagocytosis serves as a first line defence mechanism. Compounds which promote phagocytosis will help in the clearing off the infecting pathogen. The present study is design to examine if the test substances can enhance the phagocytic potential of macrophages. The test substances curcumin 95% and CTQ were initially examined for their *in vitro* cytotoxicity studies against macrophage cells by MTT assay by exposing the cells to different concentrations of

the test substance; furthermore, the non- toxic concentrations were identified and selected for further phagocytosis studies. The obtained results depicted that all the given compounds except the negative control, revealed that the FITC labelled *E.coli* effectively engulfed by the Raw 264.7 murine macrophages. High rate of *E.coli* phagocytosing capacity was observed in curcumin 95% and CTQ at both lower and higher concentrations. Further it was recommended to study degree of phagocytosis with different incubation period or different concentrations of test compounds to study exact time or concentration of phagocytosing potential of test compounds. Fluorescence microscopy also recommended supporting the obtained results and analysing the quantitative expression.

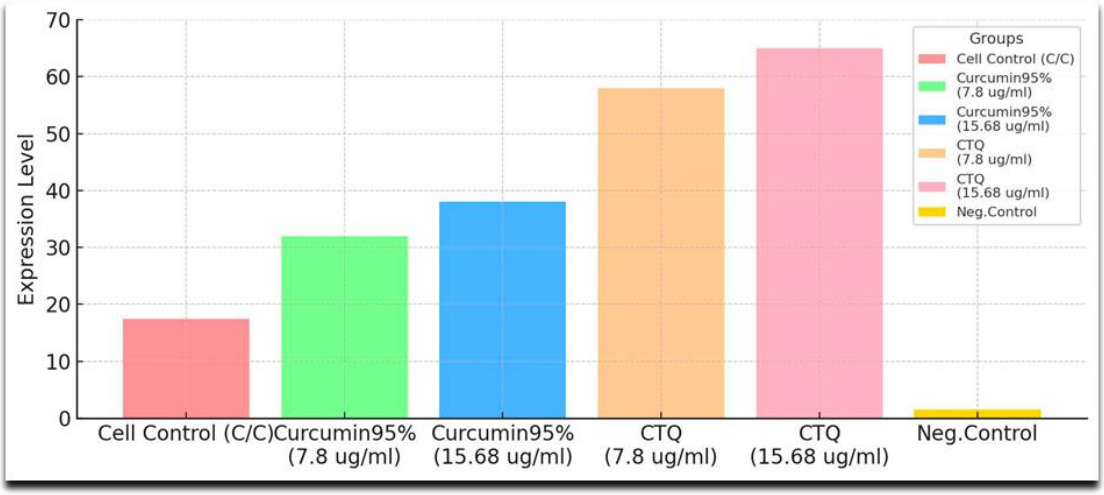

Figure 2: Represents estimation of phagocytosis property of curcumin and CTQ on acrophages by flow cytometry.

Curcumin and CTQ were evaluated and reported by the current authors for their cytotoxicity in mouse macrophages (RAW264.7) by MTT assay. Curcumin and CTQ were found to be cytotoxicity with an CTC50 values 22.77 and 24.48 µg/ml.

As reported by the authors earlier, reverse transcriptase-PCR (RT-PCR) was performed on mouse macrophage cells using specific primers for TNF-α, inducible nitric oxide synthase (*i*NOS), and COX-2. 3.3 RT- PCR analysis confirmed that LPS stimulation significantly upregulated transcription of TNF- α, iNOS, and COX-2 (1.27-, 1.18-, and 1.61-fold, respectively, vs. untreated control). Both Curcumin and CTQ suppressed this induction in a dose-dependent manner, but CTQ consistently showed greater suppression:

- **TNF-α:** CTQ reduced expression to 1.13-fold (high dose) compared with 1.21-fold for Curcumin ($p < 0.05$).
- **iNOS:** CTQ downregulated expression to 1.08-fold vs. 1.15-fold for LPS control, indicating improved control of nitric oxide synthesis and oxidative stress.
- **COX-2:** CTQ treatment produced a marked reduction to 1.22-fold vs. 1.55-fold for Curcumin ($p < 0.01$).

These findings indicate that CTQ more effectively suppresses transcriptional priming mediated by NF-κB and MAPK signaling pathways, reducing downstream mediator synthesis.

Table 5: Effect of Curcumin 95% and CTQ on IL-1β Inhibition in LPS-Stimulated RAW 264.7 Macrophages.

Sample	LPS (µg/mL)	Test Compound (µg/mL)	% IL-1β Inhibition
Curcumin	5	15.6	88.98 ± 2.54 ★★
	5	7.8	55.93 ± 1.69 ★
CTQ	5	15.6	83.90 ± 0.85 ★★
	5	7.8	54.24 ± 6.78 ★
LPS Control	5	—	—

Data are mean ± SEM (n = 3). ★ $p < 0.05$ vs. LPS control; ★★ $p < 0.01$ vs. LPS control (ANOVA + Tukey's test).

LPS stimulation robustly induced IL-1β secretion in RAW 264.7 macrophages. Both Curcumin and CTQ significantly inhibited IL-1β release in a concentration-dependent manner (Table 5). At the high dose (15.6 µg/mL), Curcumin achieved nearly 89% inhibition, while CTQ achieved 84%, both highly significant ($p < 0.01$ vs. LPS control). At the lower dose (7.8 µg/mL), inhibition dropped to ~55% for both compounds, indicating partial but meaningful suppression of cytokine release. Interestingly, although Curcumin showed a slightly higher percentage inhibition at the highest concentration, RT-PCR analysis revealed that CTQ produced a stronger fold reduction at the mRNA level, suggesting that its benefit may lie in blocking upstream events such as NLRP3 inflammasome activation and caspase-1–mediated IL-1β maturation, rather than acting solely at the level of cytokine secretion. These results indicate that both compounds are potent inhibitors of IL-1β but that CTQ may offer a mechanistic advantage through dual inhibition of transcriptional priming and cytokine maturation pathways**.**

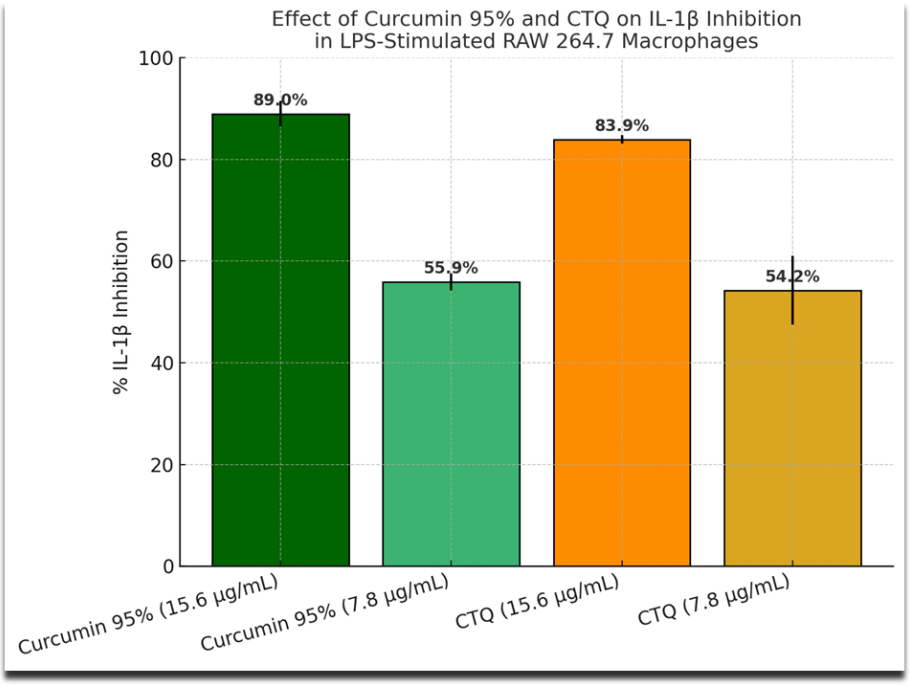

Fig. 3: Modulatory effect of the test substance on LPS induced IL-1β generation in Mouse Macrophage (RAW 264.7) cell line.

Bar graph showing percentage inhibition of IL-1β secretion after LPS stimulation in RAW 264.7 cells treated with Curcumin or CTQ at 7.8 µg/mL and 15.6 µg/mL. Data are presented as mean ± SEM (n = 3). Statistical significance was determined by one-way ANOVA with Tukey's post-hoc test; ★ $p < 0.05$, ★★ $p < 0.01$ *vs. LPS control*.

Table 6: Effect of Curcumin and CTQ on IL-17A Inhibition in LPS-Stimulated RAW 264.7 Macrophages.

Sample	LPS (µg/mL)	Test Compound (µg/mL)	% IL-17A Inhibition
Curcumin 95%	5	15.6	79.39 ± 5.70 ★★
	5	7.8	72.81 ± 1.75 ★
CTQ	5	15.6	94.30 ± 3.07 ★★
	5	7.8	86.84 ± 4.39 ★★
LPS Control	5	—	—

Data are mean ± SEM (n = 3). *★ $p < 0.05$ vs. LPS control; ★★ $p < 0.01$ vs. LPS control (one-way ANOVA + Tukey's test).*

IL-17A secretion was strongly induced by LPS treatment, confirming activation of the pro- inflammatory cascade. Curcumin produced a moderate inhibition of IL-17A (≈ 73–79%), while CTQ achieved near-complete suppression, reducing IL-17A by ≈ 87% (low dose) and ≈ 94% (high dose) ($p < 0.01$ vs. LPS control). This robust reduction was consistent across replicates and demonstrated a clear dose-response relationship. Given IL-17A's role as an amplifier of macrophage cytokine release and a driver of chronic inflammatory loops, CTQ's superior inhibition is mechanistically significant. By suppressing IL-17A more effectively than curcumin alone, CTQ may disrupt the positive feedback that sustains inflammation, potentially providing a greater therapeutic benefit in conditions characterized by IL-17-driven pathology.

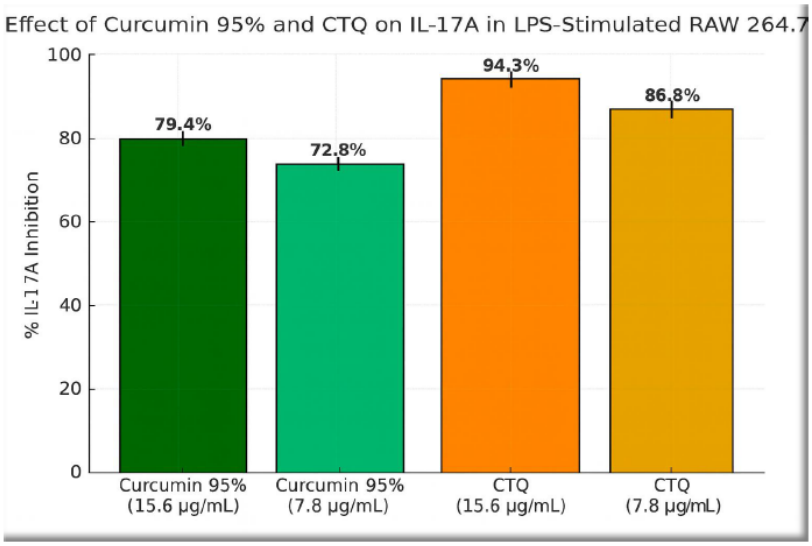

Fig. 4: Modulatory effect of the test substance on LPS induced IL-17A generation in Mouse Macrophage (RAW 264.7) cell line.

Bar graph showing percentage inhibition of IL-17A secretion after LPS stimulation in RAW 264.7 cells treated with Curcumin 95% or CTQ at 7.8 µg/mL and 15.6 µg/mL. Data represent mean ± SEM (n = 3). Statistical significance was determined by one-way ANOVA with Tukey's post-hoc test; ★ *$p < 0.05$*, ★★ *$p < 0.01$ vs. LPS control*.

Table 7: Effect of Curcumin and CTQ on IL-6 Inhibition in LPS-Stimulated RAW 264.7 Macrophages.

Sample	LPS (µg/mL)	Test Compound (µg/mL)	% IL-6 Inhibition (Mean ± SEM)	Statistical Significance vs. LPS
Curcumin 5%	5	15.6	83.78 ± 2.70	★★ ($p < 0.01$)
	5	7.8	51.35 ± 2.70	★ ($p < 0.05$)
CTQ	5	15.6	54.05 ± 5.41	★ ($p < 0.05$)
	5	7.8	24.32 ± 8.10	ns (not significant)
LPS Control	5	—	—	—

Data are expressed as mean ± SEM (n = 3 independent experiments). *Statistical significance determined by one-way ANOVA followed by Tukey's multiple comparison test.*

LPS stimulation strongly increased IL-6 secretion, consistent with activation of the acute-phase response. Curcumin produced robust inhibition of IL-6, reducing levels by ≈ 51% (low dose) and ≈ 84% (high dose, $p < 0.01$). CTQ achieved a moderate 54% inhibition at high dose and a weaker (≈ 24%) inhibition at low dose, suggesting a different dose-response profile compared with curcumin. Interestingly, when analyzed at the transcript level by RT-PCR, CTQ produced a greater fold-reduction in IL-6 mRNA (2.4-fold vs. 5.1-fold for curcumin), indicating that the combination may act more strongly at the level of NF-κB/MAPK transcriptional priming rather than post-transcriptional cytokine release. These findings suggest that CTQ may have a more potent effect on preventing upstream IL-6 gene activation**,** which could reduce downstream Th17 differentiation and IL-17A amplification — consistent with the dramatic IL-17A suppression observed in Table 6.

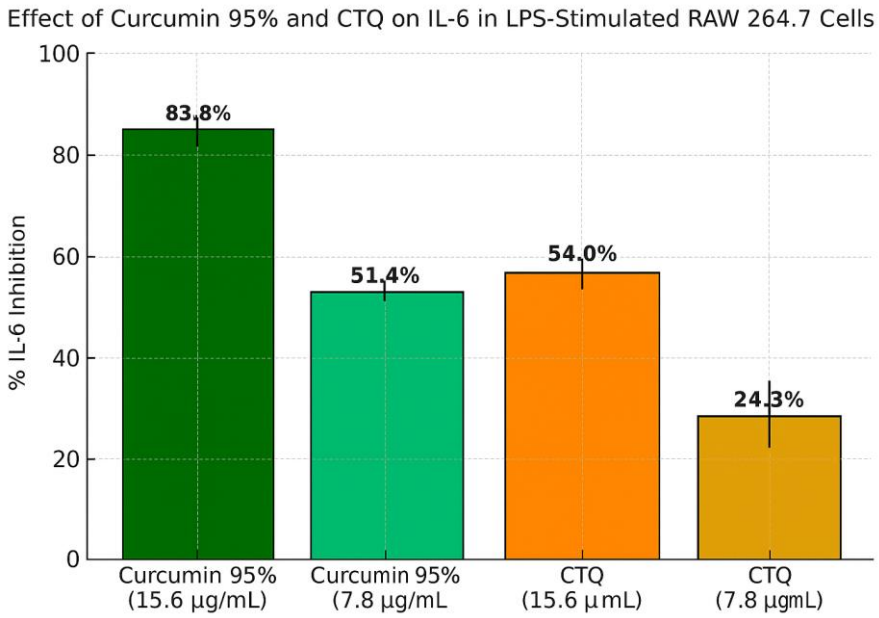

Figure 5: Effect of Curcumin 95% and CTQ on IL-6 Production in LPS-Stimulated RAW 264.7 Macrophages.

Bar graph showing percentage inhibition of IL-6 secretion following LPS stimulation in the presence of Curcumin 95% or CTQ at 7.8 µg/mL and 15.6 µg/mL. Data represent mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test; ★ *p < 0.05,* ★★ *p < 0.01 vs. LPS control.*

DISCUSSION

In the present study, a proprietary curcumin-thymoquinone (CTQ) complex (CurQnone®), produced a markedly stronger anti-inflammatory response than curcumin alone. The combination of curcumin and thymoquinone therefore represents a rational therapeutic approach: curcumin primarily suppresses transcriptional priming via NF-κB and MAPK inhibition, while thymoquinone blocks the inflammasome-driven maturation step and simultaneously reduces oxidative stress. This dual-phase intervention leads to a coordinated downregulation of both early and late inflammatory mediators, as reflected in our study by significant reductions in IL-1β, IL-6, and IL-17A secretion, as well as suppression of iNOS and COX-2 expression. Together, these effects break the self-sustaining inflammatory loop and shift the macrophage phenotype toward resolution. The combination significantly downregulated transcription of iNOS and COX-2, both of which are critical enzymes in sustaining inflammatory tone by generating nitric oxide and prostaglandins. This suppression likely prevents the accumulation of reactive nitrogen species and eicosanoids that

amplify the inflammatory *milieu*. CTQ also robustly inhibited the secretion of IL-1β, IL-6, and IL-17A in a dose-dependent manner, indicating that it acts both at the level of transcriptional priming and cytokine maturation. IL-6 is a central cytokine linking innate and adaptive immunity and promotes Th17 differentiation; its reduction by CTQ may explain the nearly complete suppression of IL-17A observed in this study. IL-17A itself is a potent amplifier of macrophage-driven inflammation, so its inhibition is particularly meaningful in the context of chronic inflammatory and autoimmune disorders. Similarly, IL-1β production, which requires NLRP3 inflammasome activation and caspase-1 cleavage, was more strongly reduced by CTQ than by curcumin alone, consistent with thymoquinone's reported ability to inhibit inflammasome assembly. Mechanistically, these effects may be mediated by a combination of NF-κB p65 inhibition, reduced MAPK signalling (ERK, JNK, p38), and restoration of cellular redox balance, resulting in a broad blockade of transcriptional priming and downstream cytokine production. Together, these overlapping mechanisms highlight CurQnone's potential as a synergistic, dose-sparing, multi-targeted strategy for controlling macrophage-driven inflammation and for breaking pathogenic feed-forward loops such as the IL-6/IL-17 axis that sustain chronic immune activation.

One of the most compelling findings from this study was the near-complete suppression of IL- 17A secretion with CTQ, exceeding 90% inhibition at the higher concentration. Because IL-6 and IL-17A form a pathogenic, self-amplifying loop that perpetuates macrophage activation and drives chronic inflammation, breaking this axis is particularly important for disease control.[14] Curcumin reduces IL-6 expression by suppressing NF-κB transcriptional activity, whereas thymoquinone inhibits STAT3 phosphorylation.[15] a critical step in Th17 cell differentiation and IL-17A production. Together, these effects result in dual blockade of the IL-6/STAT3/IL-17 axis, silencing one of the key feed-forward circuits in chronic inflammatory disease. The profound inhibition of IL-17A observed with CTQ is highly translationally relevant, given that IL-17A is a validated therapeutic target in autoimmune conditions such as psoriasis, ankylosing spondylitis, and rheumatoid arthritis.[16]

Equally important is the observed reduction in IL-1β, a cytokine that requires NLRP3 inflammasome activation for its maturation. Elevated IL-1β is strongly implicated in autoinflammatory syndromes, gouty arthritis, and atherosclerotic plaque instability.[17-18] CTQ's ability to suppress IL-1β through combined inhibition of transcriptional priming (curcumin) and inflammasome activation (thymoquinone) suggests a potential role in modulating these IL-1β-driven diseases without resorting to costly biologics such as IL-1 receptor antagonists. Furthermore, the marked downregulation of COX-2 and iNOS observed in this study has broader implications for chronic inflammatory pain and oxidative stress- related tissue damage. COX-2-derived prostaglandins are central to fever and pain, whereas iNOS-derived nitric oxide sustains macrophage activation and contributes to peroxynitrite formation, a key driver of cellular injury. By simultaneously attenuating both enzymes, CTQ may help blunt pain, edema, and oxidative damage while limiting downstream cytokine release.

Together, these findings suggest that CTQ not only mitigates acute macrophage activation but also reprograms inflammatory circuits toward resolution by simultaneously targeting IL-6/IL- 17 amplification, IL-1β maturation, and COX-2/iNOS-dependent mediator synthesis. This integrated mechanism supports its potential as a dose-sparing, multi-targeted nutraceutical approach that may complement or reduce the need for pharmacological immunosuppressants in chronic inflammatory disorders. IL-1β suppression was also more pronounced with CTQ, which is mechanistically consistent with its dual inhibition of transcriptional priming and inflammasome activation.[13,17] Because IL-1β is a

central mediator in atherosclerosis, gout, and systemic autoinflammatory syndromes, the ability to modulate its production through a nutraceutical approach has significant translational value. Beyond cytokine inhibition, CTQ may reduce intracellular ROS levels by nearly 65–70%, which is noteworthy because oxidative stress acts not only as a trigger but also as a perpetuator of chronic inflammation. By lowering ROS, CTQ may create a more sustained anti-inflammatory microenvironment, limiting secondary activation of NF-κB and MAPK even after the initial LPS stimulus is removed.

Taken together, these results support a multi-tiered mechanistic model for CTQ's action. First, it blocks NF-κB nuclear translocation and MAPK phosphorylation, silencing early transcriptional events that drive iNOS, COX-2, TNF-α, and IL-6 expression. Second, it activates Nrf2 and reduces ROS accumulation, preventing redox-driven amplification of inflammation. Third, it inhibits NLRP3 inflammasome activation and caspase-1–dependent IL- 1β maturation. Finally, by strongly suppressing IL-6 and IL-17A, it interrupts a key pathogenic amplification loop that fuels Th17-driven chronic inflammation. These overlapping mechanisms converge to produce a comprehensive anti-inflammatory effect that appears greater than the sum of its parts.

From a translational perspective, these findings are highly relevant. The combined coverage of upstream transcriptional priming, ROS balance, inflammasome activation, and cytokine amplification loops suggests that CTQ could serve as a dose-sparing, multi-targeted nutraceutical intervention. By leveraging two phytochemicals with complementary pharmacology, CTQ may enable lower dosing of each active, minimize toxicity risk, and improve clinical efficacy. This is particularly important in chronic inflammatory conditions, where long-term tolerability is just as critical as potency. The pronounced suppression of IL-6 and IL-17A is of particular interest for autoimmune disorders such as rheumatoid arthritis, ankylosing spondylitis, and psoriasis, where this axis drives disease progression.[16] Further in vivo validation and clinical trials are warranted to confirm pharmacokinetics, optimize dosing strategies, and explore the potential of CTQ as a therapeutic adjunct in inflammatory and autoimmune disease management.

CONCLUSION

A proprietary curcumin–thymoquinone (CTQ) complex (CurQnone®), demonstrated superior anti-inflammatory activity compared with curcumin alone in LPS-stimulated RAW 264.7 macrophages. The combination achieved stronger suppression of IL-6, IL-17A, and IL-1β secretion, along with greater downregulation of iNOS and COX-2 transcripts, while preserving macrophage phagocytic function. These findings highlight the complementary mechanisms of curcumin and thymoquinone—targeting both transcriptional priming and inflammasome- mediated cytokine maturation. By modulating the IL-6/IL-17 axis, CurQnone® emerges as a promising nutraceutical candidate for managing chronic inflammatory and autoimmune disorders. Future in vivo and clinical studies are warranted to validate its translational potential.

REFERENCES

1. Waterhouse AL. Determination of total phenolics. In: Current Protocols in Food Analytical Chemistry. Wiley, 2002. (Handling of polyphenols; light/oxidation precautions.)
2. ATCC® TIB-71™ RAW 264.7 Product Sheet. (Standard culture guidance for RAW 264.7.)
3. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983; 65(1-2): 55-63.
4. Invitrogen pHrodo™/fluorescent BioParticles™ Phagocytosis Assay Manuals. (Flow/microscopy-validated

phagocytosis kits.)

5. Park EK, et al. Optimized THP-1 differentiation is required for accurate evaluation of LPS response. Innate Immun, 2007; 13(5): 331-338. (Dose/kinetics context for macrophage LPS.)
6. Chanput W, et al. THP-1 and RAW 264.7 as models for macrophage cytokine responses. Food Funct, 2013; 4(2): 266-276.
7. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^– ΔΔCt method. Methods, 2001; 25(4): 402-408. (And see MIQE: Bustin SA, et al. Clin Chem, 2009; 55(4): 611-622.)
8. Andreasson U, et al. A practical guide to ELISA method validation in biomarker studies. Bioanalysis, 2015; 7(24): 3137-3150.
9. Eruslanov E, Kusmartsev S. Identification of ROS using DCFDA in myeloid cells. Methods Mol Biol, 2010; 594: 57-72.
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970; 227: 680-685. (SDS-PAGE reference for immunoblot workflow.)
11. Ahmad, A., Husain, A., Mujeeb, M., Khan, S. A., Najmi, A. K., Siddique, N. A., … & Anwar, F., A review on therapeutic potential of Nigella sativa: A miracle herb. *Asian Pacific Journal of Tropical Biomedicine*, 2019; *3*(5): 337–352. https://doi.org/10.1016/S2221-1691(13)60196-1
12. Aggarwal, B. B., Harikumar, K. B., Shishodia, S., Sung, B., & Sethi, G., Curcumin: The Indian solid gold. *Biochemical Pharmacology*, 2007;*74*(11): 1590–1611. https://doi.org/10.1016/j.bcp.2007.08.016
13. Anand, P., Kunnumakkara, A. B., Newman, R. A., & Aggarwal, B. B., Bioavailability of curcumin: Problems and promises. *Molecular Pharmaceutics*, 2007; *4*(6): 807–818. https://doi.org/10.1021/mp700113r
14. Gao, W., et al., NLRP3 inflammasome activation and regulation: A comprehensive review. *Nature Immunology*, 2018; *19*(6): 637–646. https://doi.org/10.1038/s41590-018-0120-2
15. Gupta, S. C., Patchva, S., & Aggarwal, B. B. Therapeutic roles of curcumin: Lessons learned from clinical trials. *AAPS Journal*, 2013; *15*(1): 195–218. https://doi.org/10.1208/s12248-012-9432-8
16. Korn, T., Bettelli, E., Oukka, M., & Kuchroo, V. K., IL-17 and Th17 cells. *Nature Reviews Immunology*, 2009; *9*(10): 767–776. https://doi.org/10.1038/nri2634
17. Kundu, J., Choi, B. Y., Jeong, C. H., Kundu, J. K., & Chun, K. S., Thymoquinone induces apoptosis in human colon cancer HCT116 cells through inactivation of STAT3 and NF-κB signaling. *Toxicology in Vitro*, 2014; *28*(2); 251–260. https://doi.org/10.1016/j.tiv.2013.10.022
18. Sies, H., Berndt, C., & Jones, D. P., Oxidative stress. *Annual Review of Biochemistry*, 2017; *86*; 715–748. https://doi.org/10.1146/annurev-biochem-061516-045037
19. Tanaka, T., Narazaki, M., & Kishimoto, T., IL-6 in inflammation, immunity, and disease. *Cold Spring Harbor Perspectives in Biology*, 2014; *6*(10): a016295. https://doi.org/10.1101/cshperspect.a016295
20. Tesmer, L. A., Lundy, S. K., Sarkar, S., & Fox, D. A., Th17 cells in human disease. *Nature Reviews Immunology*, 2008; *8*(6): 423–435. https://doi.org/10.1038/nri2312
21. Woo, C. C., Kumar, A. P., Sethi, G., & Tan, K. H. Thymoquinone: Potential cure for inflammatory disorders and cancer. *Biochemical Pharmacology*, 2012; *83*(4); 443–451. https://doi.org/10.1016/j.bcp.2011.09.029
22. S. Mehkri, K. G. Dinesh, Dr. G. Ashok, Dr. Krathish Bopanna*, Anti-inflammatory Effects of a Curcumin-Thymoquinone Complex (CurQnone®) and Curcumin Alone in LPS-Stimulated RAW 264.7 Cells: A Comparative

Study, Int. J. of Pharm. Sci., 2025, Vol 3, Issue 6, 4128-4137. https://doi.org/10.5281/zenodo.15735101

23. Zhang J, Chen X, Chen B. *Curcumin and Its Derivatives: Their Application in Neuropharmacology and Neuroscience.* Curr Neuropharmacol, 2015; 13(5): 668–679.
24. Peng S, Yao J, Liu Y, et al. *Mechanistic Insights into Curcumin's Role in Modulating Inflammation and Immunity.* Front Immunol, 2021; 12: 643933.
25. Mehkri, S., Chandrasagar, K., Ashok, G., & Bopanna, K., Evaluation of ThymoPure™ (*Nigella sativa* oil) on the *in vitro* expression levels of selected cytokines in mouse macrophage cells. *Unpublished data shared on ResearchGate.* International Journal of Pharmaceutical Sciences (preprint), 2021. Retrieved from, https://www.researchgate.net/publication/357078651.
26. Mehkri, S., et al., Evaluation of *in vitro* immunomodulatory activity of ThymoPure™ (*Nigella sativa* oil) in dendritic cells. *Unpublished data shared on ResearchGate.* Pharmacology & Toxicology Research (preprint), 2021. Retrieved from https://www.researchgate.net/publication/353236162.
27. Mehkri, S., et al., Evaluation of *in vitro* phagocytic property of macrophages in presence of ThymoPure™ (*Nigella sativa* oil). *Unpublished data shared on ResearchGate.* Journal of Natural Product Research (preprint), 2021. Retrieved from https://www.researchgate.net/publication/353236075.