



Comparative evaluation of anti-inflammatory properties of thymoquinone and curcumin using an asthmatic murine model

El-Sayed M. Ammar, Nariman M. Gameil, Noha M. Shawky, Manar A. Nader *

Department of Pharmacology and Toxicology, Faculty of pharmacy, Mansoura University, Egypt

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ABSTRACT

This study was designed to compare the inhibitory effects of thymoquinone (TQ) and curcumin (CMN) on the biological changes associating asthma. TQ appeared to exhibit greater inhibitory effects on the aggregation of inflammatory cells in bronchoalveolar lavage (BAL) fluid and in lung tissues. We also measured the effects of the two agents on serum IgE and the changes in the mRNA levels of inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α) and transforming growth factor- β 1 (TGF- β 1). Serum IgE was significantly decreased by TQ and CMN with TQ being more potent. Also, TQ showed superior inhibitory effects on iNOS and TGF- β 1. Meanwhile, CMN was more potent in inhibiting mRNA expression of TNF- α . These results suggest that TQ is more potent in inhibiting the inflammatory changes associating asthma. On the other hand, CMN was a less potent inhibitor of all measured parameters, despite its superior inhibitory effect on TNF- α mRNA levels.

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1. Introduction

Allergic asthma is a complex chronic airway disorder that is characterized by airway inflammation, lung eosinophilia, mucus hypersecretion by goblet cells, elevated serum IgE level, and airway hyperresponsiveness (AHR) [1]. The leukocyte infiltrates are composed of a variety of different cells from the immune system. The most common of which are eosinophils and lymphocytes [2]. Their accumulation in the lung requires that they travel from the peripheral circulation, through the vascular endothelium, through the parenchyma of the lung, and ultimately, to the bronchial and bronchiolar spaces. This process involves a complex interplay of a series of molecules, including adhesion molecules and chemokines [3]. Constriction of airway smooth muscle and the development of AHR, are also hallmarks of bronchial asthma [4]. IgE, bound to high affinity IgE receptors (Fc ϵ RI), triggers the activation of mast cells after cross-linking with specific antigen, resulting in the synthesis and release of a variety of proinflammatory mediators and cytokines [5].

Agents derived from natural sources have attracted great attention in the recent years. In this study, we were concerned with two of those agents; thymoquinone (TQ) and curcumin (CMN). TQ is one of the most active ingredients of *Nigella sativa* or black cumin seeds. The most prominent activity of TQ is its antioxidant and anti-inflammatory effects. TQ was also found to exhibit chemopreventive, anti-proliferative, cell cycle regulatory and apoptosis induction activities [6]. CMN is a phenolic compound, the main ingredient of *Curcuma longa* or

turmeric. CMN exhibited antiparasitic, antispasmodic, antiinflammatory and gastrointestinal effects. It also inhibited carcinogenesis and cancer growth [7]. CMN was also found to possess antioxidant, antimicrobial and antiallergic activities when examined in animal models of allergy [8].

In this study, we used murine model of asthma in mice to compare the abilities of TQ and CMN to inhibit asthma associated changes in mice.

2. Material and methods

2.1. Animals

Adult male Swiss albino mice (4 weeks age and 25–35 g weight) were used in this study. They were purchased from “Urology and Nephrology Center”, Mansoura University, Egypt. All protocols described in this study were approved by the University of Mansoura, Department of Pharmacology Committee for Animal Experimentation. All animals in this study were maintained under standard conditions of temperature, about 25 °C, with regular 12 h light/12 h dark cycle and allowed free access to standard laboratory food and water *ad libitum*.

2.2. Chemicals

CMN was purchased from Nanjing Tianshu Biological Eng. Co., Ltd., China. TQ, ovalbumin (OVA) grade IV, alum, carboxy methyl cellulose (CMC) and urethane were purchased from Sigma Chemicals Co., St. Louis, MO., USA.

* Corresponding author at: Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.

E-mail address: manarahna@yahoo.com (M.A. Nader).

2.3. Sensitization and airway challenge

Mice (4 groups (each comprised of 10 mice); control, OVA, OVA-TQ and OVA-CMN treated groups). Mice were sensitized by subcutaneous injections with 25 µg of OVA adsorbed on 1 mg of alum in 200 µL of normal saline per mouse on days 0, 7, 14 and 21 (only drug free normal saline in control group). Intranasal challenges with OVA (20 ng/50 µL saline) were carried out on days 31, 33, 35 and 37 (only drug free normal saline in control-group). Mice in the treated groups were treated with oral administration of 10 and 15 mg/kg/day of TQ and CMN, respectively, starting from day 30 to day 38 (1 h before challenge on days of challenges) while control group receive drug vehicle (0.5% CMC). The selected doses of the two agents were found to be the most potent doses based on preliminary experiment (data not shown).

2.4. Inflammatory cell counts in bronchoalveolar lavage (BAL)

Twenty-four hours after the last OVA or saline challenge, mice were anesthetized with urethane (2.5 g/kg, I.P.). Tracheae were exposed and cannulated with polyethylene cannula for BAL that was performed by instillation of 0.5 mL of phosphate-buffered saline (PBS). The thorax was gently massaged then the BAL fluid was withdrawn. The process was repeated 4 times. About 1 mL of the instilled fluid was retrieved from each mouse. The retrieved BAL fluid was centrifuged at 500 g for 10 min at 4 °C. The cell free supernatant was removed and the cell pellet was resuspended in 200 µL PBS and used for total and differential leukocyte counts. Total leukocytes in BAL fluid were counted using a hemocytometer. Different cell types were identified by differential staining microscopy with Diff-Quick (International reagents Corp., Kobe, Japan). The cell counts of lymphocytes, eosinophils and monocytes in BAL fluid were obtained.

2.5. Lung tissue histopathology

Lungs were harvested after performing BAL, the right lung was fixed with formalin, cut into sections, stained with hematoxylin and eosin and examined under microscope to evaluate the severity of inflammation. Inflammatory infiltrates were further characterized according to cell type on a morphologic basis. Inflammatory changes were expressed as scores of eosinophils, this semiquantitative scale was from 0 to 3 (0 = no, 1 = mild, 2 = moderate, 3 = marked) for each cell type. The total inflammation score for each animal was calculated as the mean of the scores for 6 lungs [9]. Also plasma cells in lung tissue were assessed by giving a score using the same semiquantitative scale.

2.6. Measurement of IgE in serum

Levels of IgE in sera were determined by enzyme immunoassays kits (Costar, Cambridge, MA) according to the manufacturer's protocol.

2.7. Isolation, purification and reverse transcription (RT) of RNA

The lung was isolated and flash-frozen in liquid nitrogen and stored at –80 °C. Lung samples (about 20–30 mg of tissue for each sample) were mechanically homogenized using a variable speed homogenizer (model 125, OMNI international). Total RNA was isolated from the homogenized lungs and purified using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. To remove the contaminating genomic DNA (gDNA), RNA samples (1 µg each) was added to 2 µL genomic DNA (gDNA) wipeout buffer at 42 °C for 2 min. RNA samples were reverse transcribed at 42 °C for 25 min in 20 µL containing 1 µL quantiscript reverse transcriptase, 4 µL quantiscript RT buffer and 1 µL RT primer mix. The reaction was terminated by heating at 95 °C for 3 min. Real-time polymerase chain reaction (RT-PCR) was carried out in a 23 µL final volume in duplicates using SYBR Green I as a fluorescent detection dye. The reactions contained 2 µL cDNA, 12.5 µL Rotor-Gene

SYBR Green PCR Master Mix, 8.5 µL RNase-free water and forward and reverse primers in final concentration 1 µM each. Primer sequences are described in Table 1. PCR amplification was performed in the thermocycler RotorGene Q (Qiagen, Hilden, Germany). After an initial activation step at 95 °C for 5 min (hot start DNA polymerase activation), 40 cycles with the following thermocycling conditions were carried out: denaturation at 95 °C for 5 s, combined annealing/extension at 60 °C for 10 s at which the fluorescence was acquired. Amplification specificity was checked by generation of a melting curve by heating the PCR product slowly at a rate of 1 °C s^{–1} from 60 °C to 95 °C which causes melting of the double-stranded DNA and a corresponding decrease in SYBR Green fluorescence. The relative quantification values for calibrator-normalized target gene expression were normalized to β-actin. PCR efficiency of both the target (iNOS, TGF-β1 and TNF-α) and reference (β-actin) genes was calculated from the derived slopes of the standard curves.

2.8. Statistical analysis

The results were presented as mean ± standard error of mean (S.E.M.). Statistical significance was considered when $P < 0.05$. Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test.

3. Results

3.1. Effects of TQ and CMN on total and differential leukocyte count in BAL fluid

Total leukocyte count in BAL fluid of the OVA group was significantly increased ($P < 0.01$) when compared with the control group. TQ and CMN treated groups significantly decreased the total leukocyte count ($P < 0.001$ and $P < 0.01$), respectively when compared with the OVA group (Fig. 1). Diff-Quick staining of the cells revealed massive increase in cell count of eosinophils, monocytes ($P < 0.001$) and lymphocytes ($P < 0.01$) when compared with the control group. On comparing the 2 treated groups with the OVA group, we found that both decreased the number of different leukocyte types with varying degrees. TQ treatment significantly decreased the number of the three measured cell types ($P < 0.001$), while CMN treatment significantly decreased the number of eosinophils ($P < 0.05$), monocytes ($P < 0.01$) and lymphocytes ($P < 0.001$) when compared with the OVA group. The number of eosinophils was significantly higher in CMN treated group when compared with control and TQ treated groups ($P < 0.01$ and $P < 0.05$, respectively). It was also found that the number of monocytes was significantly higher in CMN treated group when compared with control and TQ treated groups ($P < 0.05$).

Table 1
Primers used in real-time polymerase chain reaction.

Primer		Sequence	Melting temperature (T _m)	Product size (bp)
Reference gene:				
β-actin	Forward	TGGGTATGGAATCCTGTGG	55.78	96
	Reverse	GCACTGTGTTGGCATAGAGG	56.18	
Target genes:				
iNOS	Forward	GGAGCCTTTAGACCTCAACAGA	58.03	104
	Reverse	GGCTGGACTTTTCACTCTGC	57.17	
TNF-α	Forward	CCACCACGCTCTTCTGTG	57.79	114
	Reverse	ATCTGAGTGTGAGGGTCTGG	57.62	
TGF-β1	Forward	GCTAATGGTGGACCGCAAC	58.57	100
	Reverse	CACCTGCTCCCGAATGTCTG	58.72	

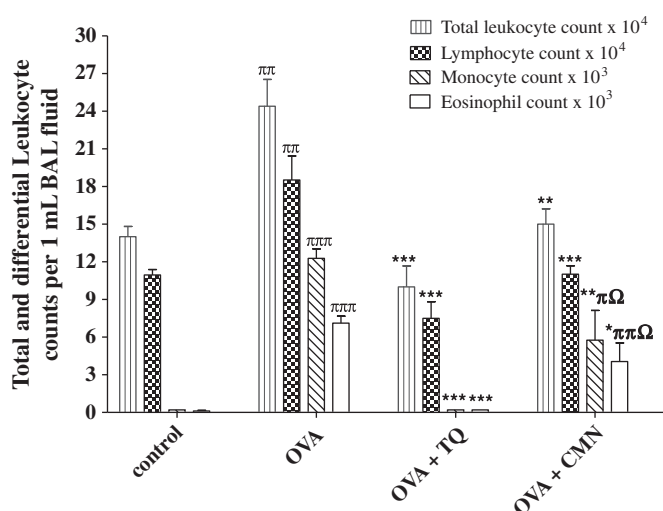


Fig. 1. Effects of TQ and CMN on total and differential leukocyte counts in BAL fluid of asthmatic mice. Values represent mean $\pm$ S.E. TQ; thymoquinone, CMN; curcumin, BAL; bronchoalveolar lavage. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as compared to OVA group. π $P<0.05$, $\pi\pi$ $P<0.01$, $\pi\pi\pi$ $P<0.001$ as compared to control group. Ω $P<0.05$ as compared to TQ treated group (one way ANOVA followed by Tukey–Kramer multiple comparisons test).

3.2. Effects of TQ and CMN on eosinophilic accumulation in lungs of mice

Light microscopic examination of sections of normal lungs of mice revealed normal histology of the lung. Lung from OVA group revealed marked infiltration of eosinophils when compared with normal lung ($P<0.001$). The eosinophils were generally more accumulated towards the bronchioles rather than the bronchi. TQ and CMN significantly reduced the score of eosinophils when compared with the

OVA group ($P<0.001$) with TQ being more potent than CMN (Fig. 2, Table 2).

3.3. Effects of TQ and CMN on the accumulation of plasma cells in lungs of mice

OVA treated group revealed marked accumulation of plasma cells when compared with normal lung ($P<0.01$). TQ significantly reduced the score plasma cells when compared with the OVA group ($P<0.01$). On the other hand, CMN produced an insignificant decrease in the score of plasma cells when compared with the OVA group (Fig. 2, Table 2).

3.4. Effects of TQ and CMN on total IgE in serum

Because Th2 cytokines promote airway inflammation in asthma through increased IgE levels, we investigated the expression of IgE in serum. As shown in Fig. 3, control treated mice exhibit minor detectable OVA-specific IgE in sera. The levels of sera IgE were found to be significantly increased in OVA group compared with control group ($P<0.001$). However, administration of TQ and CMN significantly decreased the levels of serum IgE compared with OVA group ($P<0.001$) but in CMN treated group there is still significant higher level of serum IgE than control and TQ treated groups ($P<0.001$ and $P<0.05$, respectively).

3.5. Effects of TQ and CMN on mRNA-iNOS, TGF- β 1 and TNF- α expression levels

The mRNA levels of iNOS, TGF- β 1 and TNF- α were significantly higher ($P<0.001$, $P<0.01$ and $P<0.001$) in asthmatic mice compared to control treated group. A significant decrease of iNOS mRNA expression was observed with TQ and CMN treatment ($P<0.05$) when compared with OVA group. CMN treated group also showed a significantly

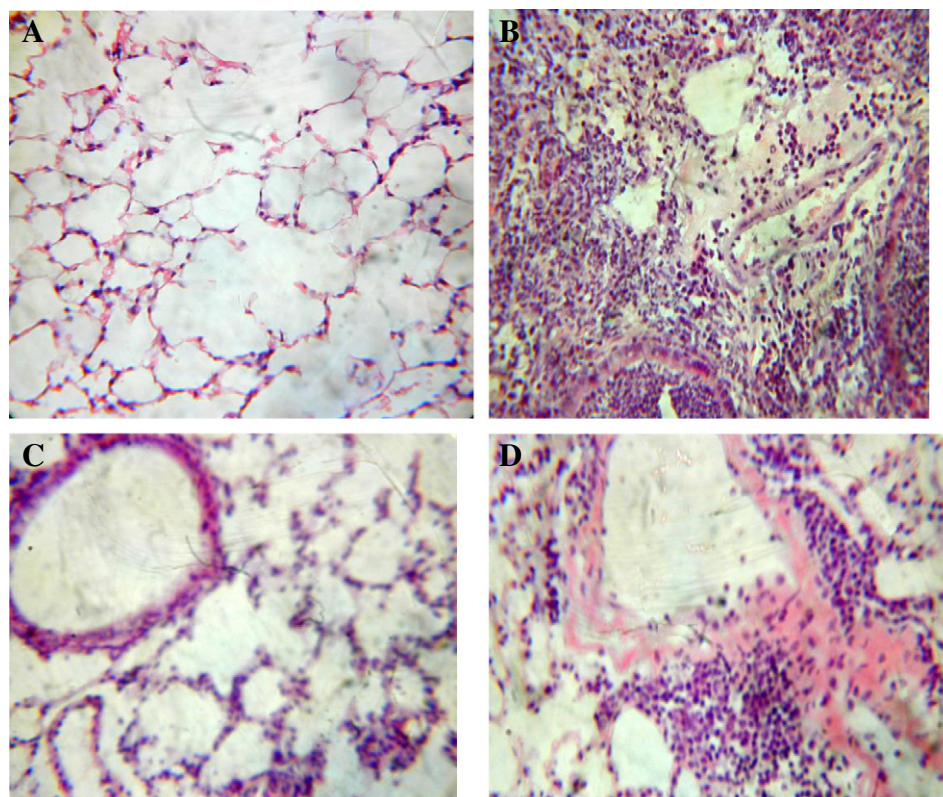


Fig. 2. Representative lung tissue histology showing (A) control group showing an absence of inflammatory cells, (B) OVA group showing marked accumulation of inflammatory cells, (C and D) TQ and CMN treated groups, respectively, showing some inflammatory cells (H&E stain, 40 $\times$). TQ; thymoquinone, CMN; curcumin.

Table 2

Histopathologic scores of eosinophils and plasma cells in lung tissues in a murine model of asthma.

Treatment groups	Score of eosinophils	Score of plasma cells
Control	0 ± 0	0.8 ± 0.2
OVA	2.8 ± 0.2 πππ	2.33 ± 0.21 ππ
OVA + TQ	0.5 ± 0.22 ***	0.75 ± 0.19 **
OVA + CMN	0.75 ± 0.11 ***	1.75 ± 0.58

OVA; ovalbumin, TQ; thymoquinone, CMN; curcumin.

Values represent mean ± S.E.

P<0.01, *P<0.001 as compared to OVA group.

ππ P<0.01, πππ P<0.001 as compared to control group (one way ANOVA followed by Tukey–Kramer multiple comparisons test).

higher iNOS gene levels ($P<0.05$) when compared with the control group. A significant decrease in TGF- β 1 gene expression level was produced upon treatment with TQ ($P<0.05$) in comparison with OVA group. CMN treatment produced an insignificant decrease in the TGF- β 1 mRNA levels when compared with the OVA group. A significant decrease in the mRNA levels of TNF- α was found to associate treatment with TQ ($P<0.05$) and CMN ($P<0.001$) when compared with the OVA group (Fig. 4).

4. Discussion

Asthma is a disease that results from a variety of environmental factors acting on a background of genetic factors. Using a rodent model of allergic airway disease, this study explores the application of emerging toxicogenomic tools in conjunction with bronchoalveolar lavage and lung pathology to investigate variable degrees of inhibitory effects TQ and CMN on the different pathologic changes associating asthma. Both agents effectively suppressed allergic inflammation in rodent model of airway allergic inflammation in variable degrees through decreasing total leukocytes, eosinophils and lymphocytes count, decreased IgE serum level and the magnitude of genes expression of iNOS, TGF- β 1 and TNF- α in lung that was enhanced during OVA sensitization and challenge of mice.

The results of this study revealed that TQ and CMN may be used as adjuvant therapy for patients with allergic airway inflammation. Mice sensitized and challenged with OVA significantly increased an influx of total leukocytes, eosinophils, and lymphocytes, and the secretion of inflammatory mediators. Histological examination of lung tissues,

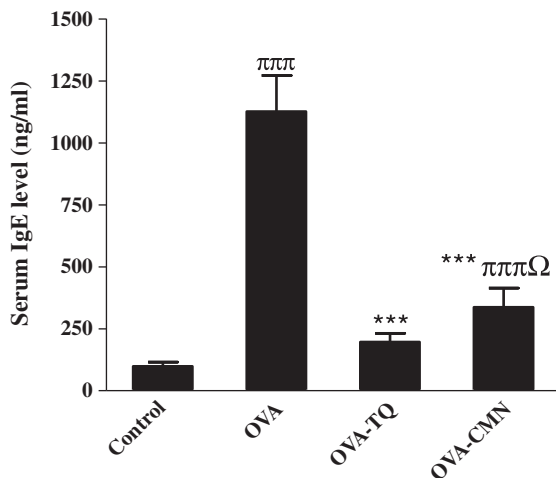


Fig. 3. Effects of TQ and CMN on serum IgE in asthmatic mice. Values represent mean ± S.E. TQ; thymoquinone, CMN; curcumin, IgE; immunoglobulin E. ***P<0.001 as compared to OVA group. πππ P<0.001 as compared to control group. Ω P<0.05 as compared to TQ treated group (one way ANOVA followed by Tukey–Kramer multiple comparisons test).

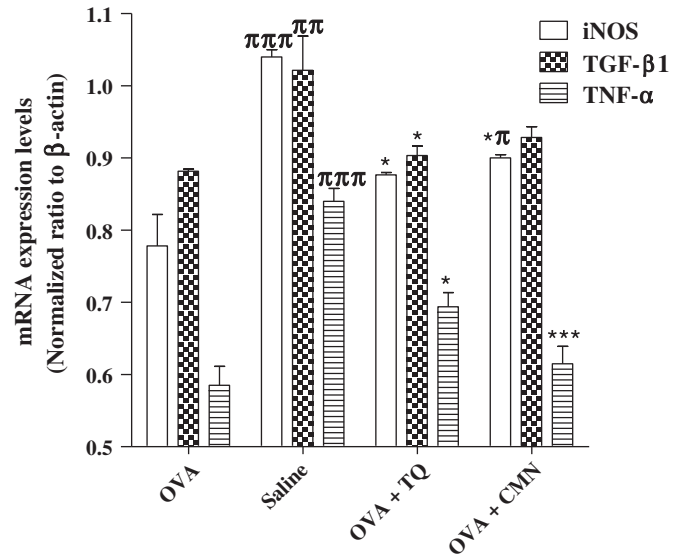


Fig. 4. Effects of TQ and CMN on iNOS, TGF- β 1 and TNF- α expression in lungs of asthmatic mice. Values represent mean ± S.E. TQ; thymoquinone, CMN; curcumin, iNOS; inducible nitric oxide synthase, TGF- β 1; transforming growth factor- β 1, TNF- α ; tumor necrosis factor- α . *P<0.05, ***P<0.001 as compared to OVA group. π P<0.05, ππ P<0.01, πππ P<0.001 as compared to control group (one way ANOVA followed by Tukey–Kramer multiple comparisons test).

paralleled the results of analysis of the cell count in the BAL fluid which showed marked influxes of eosinophils and plasma cells in OVA group. TQ and CMN supplementation can reverse established airway inflammation.

Total infiltrating leukocytes, eosinophils, and lymphocytes in BAL fluid and influxes of eosinophils and plasma cells in lung tissue were reduced by TQ and CMN supplementation. However, TQ was found to be more potent in decreasing total leukocyte, lymphocyte, eosinophil and monocyte counts. TQ was also a more potent inhibitor on the recruitment of eosinophils and on plasma cell aggregation in lung tissue, such effects were more potent than those shown by CMN.

We also observed in this study that oral administration TQ and CMN significantly reduced the release of IgE in serum compared to OVA sensitized mice. Elevated serum IgE level has been reported to be important in the development of asthmatic responses [10,11]. T-cells bear specific variable regions of the β -chain of the T-cell receptor that regulate production of IgE and development of airway inflammation in mice sensitized by OVA [12]. Activation of T-cells with allergen-specific IgE plus airway challenge enhances its function leading to increased Th2-cytokine (IL-4, IL-5 and IL-13) production and eosinophilic airway inflammation [10]. These results suggest that TQ and CMN have an effect on allergic asthma that developed in an IgE-dependent manner. TQ was found to be more potent than CMN in inhibiting the rise of serum IgE, where CMN showed significantly higher levels of serum IgE when compared with control and TQ treated groups.

TNF- α is a potent proinflammatory cytokine with immunoregulatory activities. It is produced by many cell types including monocytes, macrophages, lymphocytes, neutrophils, eosinophils and mast cells [13–15]. In the lung, TNF- α is synthesized and stored mainly in mast cells and alveolar macrophages [16]. It functions as a chemoattractant for neutrophils and monocytes. It can also increase microvascular permeability and activate T cells, eosinophils and mast cells. Furthermore, there is increased expression of TNF- α in the airways of asthmatic patients compared to normal subjects [16,17]. In our study, the upregulated TNF- α mRNA levels in the lungs of asthmatic mice were significantly decreased by TQ and CMN with variable degrees. CMN was found to be more potent inhibitor of TNF- α mRNA expression than TQ.

TGF- β 1 is an intercellular signaling molecule that demonstrates a wide range of biological effects [18]. It has been shown that TGF- β 1 expression increases in asthmatic patients [19–23]. TGF- β 1 was also found to be a proinflammatory and powerful chemotactic agent for neutrophils, eosinophils, macrophages and mast cells [24,25]. Upon measuring the mRNA levels of TGF- β 1 in our two treated groups, TQ and CMN, they significantly decreased the mRNA levels when compared with OVA group, with TQ treatment being more potent than CMN treatment.

The role of iNOS expression leading to the subsequent generation of NO during allergic lung responses is unclear. The involvement of iNOS in modulating interactions between Th1 and Th2 cells was studied. First, NO may exert a self regulatory effect on Th1 cells that are implicated in immunopathological situations. Thus, high concentrations of NO can inhibit the secretion of IFN- γ and IL-2 by Th1 cells, while having no effect on IL-4 production by Th2 cells [26]. This reduction in IFN- γ production would result in increased antigen-driven proliferation of Th2 cells [27]. Thus, iNOS may be involved in the complex balance between Th1 and Th2 cells in immune and inflammatory states, which ultimately favors a Th2 cell outcome [28]. Thymoquinone and CMN were able to significantly decrease the levels of iNOS gene transcripts when compared with the OVA group. On comparing their effects, TQ was a more potent inhibitor than CMN.

This study suggested that TQ is a more potent inhibitor of inflammatory cell aggregation in BAL fluid and lung tissues and TGF- β 1 and iNOS mRNA expression which indicate its potent effects in antagonizing airway inflammation. Curcumin may be suggested to exert weaker inhibitory effects on the different explored parameters. However, it was found to be a more potent inhibitor of the expression of mRNA of TNF- α . This may provide evidence for its superior activities on the late phase allergic reactions mediated by the release of TNF- α from mast cells.

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