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ORIGINAL RESEARCH

Turmeric and *Eucalyptus* Aqueous Extracts Reduced Inflammation in Macrophage Activation Syndrome (MAS) Mouse Model

Mariam M Al-Omari[1], Manar M AL-Yousef[2], Khaled M Al-Qaoud[2], Nesreen M Bataineh[1]

[1]Department of Basic Pathological Sciences, Faculty of Medicine, Yarmouk University, Irbid, 21163, Jordan; [2]Department of Biological Sciences, Faculty of Science, Yarmouk University, Irbid, 21163, Jordan

Correspondence: Mariam M Al-Omari, Department of Basic Pathological Sciences, Faculty of Medicine, Yarmouk University, Irbid, Jordan, Tel +962(2)7211111 Ext.711, Email Maryam.o@yu.edu.jo

Introduction: Macrophage activation syndrome (MAS) is a spectrum of cytokine storm syndrome (CSS) associated with autoimmune conditions. Due to the high number of cytokine storm patient deaths, mainly those that occurred during the COVID-19 pandemic and the lack of effective treatments against CSS spectrums, many people returned to nature and used plant-based remedies at the household level.
Methods: This study investigated the anti-inflammatory activity of commonly used medicinal plant decoctions; Turmeric (Tur) and *Eucalyptus* (Euc) on lipopolysaccharide (LPS) and cytosine guanine dinucleotide (CpG)-induced MAS-BALB/c mouse model, Dexamethasone (Dex) was used as anti-inflammatory control.
Results: A restoration in weight gain was noticed on day 8 by 2.8 ± 1.722 and 1.7 ± 1.976 g, for MAS Tur and MAS Euc, respectively. Levels of TNF-α and CCL2 in mice sera showed a peak after 2 hrs of LPS and CPG treatment in the MAS group. However, a significant reduction of 85.9% and 84.3% in TNF-α, and 77.1% and 54.6% in CCL2 were observed for MAS Tur and MAS Euc, respectively. In contrast, the reduction in IL-6 was less in MAS-Tur and MAS-Euc when compared to MAS-Dex (6.0%, 5.2%, and 17.4%, respectively). Cytokine gene expression in lung tissue at day 10 revealed a significant reduction in the expression of IL-1β, TNF-α, IL-6, and TGF-β in the MAS Tur and MAS Euc groups compared to the MAS group. Histopathological investigation revealed that lung tissue in MAS Tur and MAS Euc groups showed mild to moderate inflammatory areas with no signs of necrosis compared to the MAS group. High MMP-9 expression was noticed in the MAS group with a score of positively stained neutrophils of 3.8 ± 1.6 that has been reduced to 1.9 ± 0.9 and 2.3 ± 0.7 in MAS Tur and MAS Euc groups, respectively. Conclusion, natural plant extracts may help ameliorating MAS in a better way than dexamethasone.

Keywords: cytokine storm syndrome, macrophage activation syndrome, *Eucalyptus*, tumor necrosis factor-alpha, interleukin-6, matrix metalloproteinase-9

Introduction

The recent and rapid spread of the coronavirus disease 2019 (COVID-19) has reminded us of the precise vital role of an effective immune response and the destroying reaction of host immune dysregulation. Macrophage activation syndrome (MAS) is a spectrum of cytokine storm syndrome (CSS) associated with autoimmune conditions, and it can be defined as a dangerous systemic inflammatory response characterized by high levels of circulating cytokines and immune-cell hyperactivation.[1] It can be initiated by different underlying triggers such as pathogens, cancers, viruses, some therapies, and autoimmune disorders,[2] and it has been reported to be associated with severely ill COVID-19 patients recently.

Severe COVID-19 cases display increased production of certain cytokines such as interleukin (IL)-1, IL-6, IL-7, and tumor necrosis factor-alpha (TNF-α) which is a systemic cytokine profile similar to MAS.[3] These pro-inflammatory cytokines are mainly produced by activated macrophages in response to stimuli and cause activation and amplification of a cascade of inflammatory pathways.[4] The severe illness progression in patients does not appear to be strictly related to

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viral load but may include a defect in interferon response.[5] Hyperinflammatory response to the viral infection is expected to be the major reason for disease severity, and it is correlated with increased levels of circulating cytokines and mononuclear cell infiltration into the primary site of infection and other organs such as heart, lymph nodes, and kidneys.[6,7] It is also very important to understand the pathogenesis of MAS to help diagnose it and provide the appropriate therapy to the patient on the right time since delayed treatment results in high morbidity and mortality usually.

Many studies have estimated the potential of several medications and therapeutic protocols for MAS treatment. A recent study has summarized some of these medications in their report and such therapies rely basically on immunosuppression.[8] The aim of using these therapies is to attenuate the hyperinflammatory state of the immune system and target the underlying trigger. Such medications are hampered by the risk of immune suppression or development of autoantibodies and some other serious side effects. Therefore, many people tried to return to nature and use common medicinal plants. This topic has been highly discussed in social media during the COVID-19 pandemic. Curcumin and Eucalyptus decoction took the lead potent anti-inflammatory and cytokine storm damper.

The potential anti-inflammatory activity of curcumin, a natural compound found in the rhizome of the perennial herb *Curcuma longa* L. was demonstrated by in vitro and in vivo experimental studies suggesting that it beneficially induced the development of anti-inflammatory M2 macrophage over an inflammatory M1 subset in a variety of inflammatory diseases.[9,10] Moreover, curcumin suppressed immune cells and inflammatory cytokines in models of rheumatoid arthritis.[11] Furthermore, *Eucalyptus* oil treatment on murine lung alveolar macrophages (MH-S) cell line significantly attenuated IL-1 cytokines, resulting in downstream activation of the signaling cascade (MAPKs and NF-κB) and diminishing of the overall pro-inflammatory response to LPS in lung inflammation.[12] Similar anti-inflammatory activity was also seen in Eucalyptus oil-treated human monocyte-derived macrophages.[13]

The purpose of this study is to investigate the potential anti-inflammatory effects of turmeric and eucalyptus aqueous extracts on Macrophage Activation Syndrome (MAS). Given the limitations and side effects associated with current treatments, we aim to explore alternative therapeutic options that are potentially more accessible, affordable, and have a better safety profile.

This goal was accomplished by using a MAS mouse model that received injections of both LPS and CpG.[14,15] The anti-inflammatory potency of decoctions drinking by MAS mouse groups was assessed by measuring the inflammatory signs, inflammatory cytokines concentration reduction, and cytokine gene expression in lung tissue.

Methodology

Preparation of Plant Extracts

Turmeric rhizomes from *Curcuma longa* plant and *Eucalyptus* leaves from *Eucalyptus Camaldulensis* were obtained from a local market and classified in collaboration with Prof. Jamil Lahham, plant taxonomist in Biology Department, Yarmouk University. A voucher specimen of samples was kept in the herbarium (Turmeric, voucher no 13003, Eucalyptus, voucher no 13004). Then dried Turmeric rhizomes were powdered in a sterile grinding machine, and *Eucalyptus* leaves were grounded into small pieces for further extraction. Plant extracts were freshly prepared as 25 g dry weight in 1 L hot water and boiled for 3 min then filtered and stored at 4°C for further use.

Mice

Wild-type BALB/c mice were bred under specific pathogen-free conditions and kept at the Animal House Facility of Yarmouk University (Irbid, Jordan) with free access to food and water. Six- to eight-week-old male mice with an average weight of 25 ± 4 g were matched within each experiment and kept under steady temperature and 12-hour light–dark cycles. All experiments were approved by the Institutional Animal Care and Use Committee at Yarmouk University (number IACUC/2021/8). According to the National Advisory Committee for Laboratory Animal Research guidelines.

MAS Animal Model

Each BALB/c male mouse was injected with a starting intraperitoneal Lipopolysaccharide (LPS) (from Escherichia coli 0127:B8 Sigma Aldrich, USA) injections (2 µg/g body weight) [50 µg/200 µL phosphate-buffered saline (PBS)] along with intranasal inhalation of CpG ODN 1826 (Integrated DNA Technologies, USA) (400 ng/g body weight) [10 µg/50 µL PBS (25 µL in each nasotril)]. Followed by two repeated LPS boost doses [1 µg/g body weight (25 µg/200 µL PBS)] injected intraperitoneally on days 5 and 9 as described by Behrens et al[16] with minor modifications. Dexamethasone phosphate (1mg/kg/day) (Sigma Aldrich, USA) was provided as a standard anti-inflammatory drug in drinking water throughout the experiment days.[17] Experimental animals were randomly divided into seven groups (n = 10 mice per group). The first three groups were considered as control groups (control, Turmeric extract control, and *Eucalyptus* extract control). Group one (Ctrl) was considered as the control, where mice were kept without any treatments. Groups two- (Ctrl-Tur) and three- (Ctrl-Euc)-mice were given Turmeric and *Eucalyptus* extracts in drinking bottles, respectively, without any other injections. The next four groups were established as the MAS model where MAS was induced as previously mentioned by LPS and CpG injections. Group four mice (MAS) were given only injections and considered as a negative control. Group five (MAS-Dex) was treated with dexamethasone phosphate orally as a standard anti-inflammatory drug and considered a positive control. Groups six and seven were given the plant extracts as mentioned for groups two and three along with LPS and CpG injections and labeled as (MAS-Tur) for the Turmeric extract-treated group and (MAS-Euc) for the *Eucalyptus* extract-treated group. Treatment with Turmeric and *Eucalyptus* extracts was given daily to mice in drinking bottles from the starting day of the experiment to the termination day. Mice body weight was recorded before injections, a day after the first and second injections, and before the termination day (Figure S1).

Blood Collection and Serum Isolation

Blood was collected before and after injections from the retro-orbital sinus. Samples were collected after 2,24 hours (hrs) and 10 days upon the first injection. Sera were collected by allowing blood to clot for 30 minutes (min) after blood withdrawal followed by centrifugation at 2500 rounds per minute (rpm) for 15 min then stored at −20°C for further analysis.

Organs Collection

On the day of termination, mice were euthanized by CO_2 asphyxiation; they were decapitated for whole blood collection and serum isolation purposes. Upon termination, lungs, spleen, and liver tissue were collected from all mice. Part of the lung was stored at −80°C for further RNA extraction, and the other part was fixed in 10% buffered formalin and kept at room temperature (RT) for further histopathological examination. Spleen and liver tissues were collected, weighed, and compared for the presence of splenomegaly and hepatomegaly, respectively.

Mouse IL-6, TNF-α, and CCL2 ELISA

Two-site sandwich ELISA was performed to determine concentrations of IL-6, TNF-α, and CCL2 in serum samples. EliKine™ Mouse IL-6 ELISA, Mouse TNF-α ELISA, and Mouse CCL2 ELISA Kits (Abbkine, California, USA) were used according to the manufacturer's instructions. A hundred µL of diluted sera (1:25) with sample diluent or standards were added to each well of the antibody-pre-coated ELISA microplates. A blue color was developed in proportion to the amount of mouse IL-6, TNF-α, and CCL2 bound in the initial step. Color development was stopped by adding 50 µL stop solution then color intensity was recorded using Thermo Fisher Scientific Multiscan GO (Finland) at 450 nm. Standard curves were established, and concentrations were calculated accordingly.

Ribonucleic Acid (RNA) Extraction and DNase Treatment

Fifty to hundred mg of lung tissue were homogenated well with 1 mL TRIzol Reagent® (Sigma Aldrich, USA) in a homogenator and transferred into an Eppendorf tube. Total mRNA extraction was isolated by phenol-chloroform extraction, and DNase was treated using a Thermo Fisher Scientific DNase kit (Thermo Fisher Scientific, Lithuania) following the standard protocols established in our laboratory.[18] The pellets were resuspended by 20 µL nuclease-free water (NFW). Samples were stored at −80°C for further estimation. The concentration of RNA was measured using

a nano-drop reader at 260 nm using Thermo Fisher Scientific Multiscan GO (Finland). All the extraction steps were performed in cold conditions.

Quantification of Cytokine Expression of Lung Tissue Using Quantitative Real-Time-Polymerase Chain Reaction (RT-PCR).

Quantitative RT-PCR was performed using a TransScript® Green One-Step qRT-PCR SuperMix (TransGen Biotech Co., LTD, China) according to the manufacturer's instructions. For the various genes used in the study, appropriate primers have been designed or previously used. Expression levels of IL-1β (F: 5′-GGTACATCAGCACCTCACAA-3′; R: 5′-TTAGAAACAGTCCAGCCCATAC-3′),[19] TNF-α (F: 5′-CTACCTTGTTGCCTCCTCTTT-3′; R: 5′-GAGCAGAGGTTC AGTGATGTAG-3′),[20] IL-6 (F: 5′-CTTCCATCCAGTTGCCTTCT −3′; R: 5′-CTCCGACTTGTGAAGTGGTATAG-3′), and TGF-β (F: 5′-CCTGCAAGACCATCGACATG-3′; R: 5′-TGTTGTACAAAGCGAGCACC-3′) were normalized to the expression of GAPDH (F: 5′-GGCATGGACTGTGGTCATGA-3′; R: 5′-TTCACCACCATGGAGAAGGC-3′).[21] Relative gene expression was calculated using the following comparative Ct ($2^{-\Delta\Delta Ct}$) analysis method:[22]

$$\Delta Ct = \text{AVG. Ct (gene of interest)-AVG. Ct (housekeeping gene)}$$
$$\Delta\Delta Ct = \Delta Ct \text{ (treated sample)}-\Delta Ct \text{ (control sample)}$$
$$\text{Relative quantification of gene expression} = 2^{-\Delta\Delta Ct}$$

The findings were verified and analyzed using GraphPad Prism software (v8.0.1). The PCR product was run on 1.5% agarose gel for 45 min on 120 V for amplicon validation purposes.

Histology and Immunohistochemistry (IHC)

Formalin-fixed lung tissues were embedded in melted paraffin. Deparaffinized 6 µm sections were stained by Mayer's Hematoxylin solution (UFC Biotechnology, USA) and Eosin Y stain solution (H&E) (GCC, UK) or labeled with biotinylated anti-Matrix Metalloprotease-9 (MMP-9) antibody (MyBioSource, #MBS2005966, CA). Then detected with Mouse and Rabbit Specific HRP/DAB IHC Detection Kit - Micro-polymer (Abcam, Ab236466, UK) using secondary anti-rabbit IgG horseradish peroxidase (HRP) antibody (Promega, USA) after counterstaining with hematoxylin. Stained slides were sent to a collaborator pathologist (Dr. Nesreen Bataineh, Assistant Professor of Histopathology, Department of Medical Basic Science, Yarmouk University) to be microscopically examined for histopathological changes.

Statistical Analysis

The collected data were analyzed and visualized using the GraphPad Prism Software (v8.0.1). The experimental groups (Ctrl-Tur, Ctrl-Euc, MAS, MAS-Dex, MAS-Tur, and MAS-Euc) were compared with the Ctrl group in all tests. Data were expressed as mean values ± standard deviations (SD) for each determination. Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by the multiple *T* test or Welch's *T* test depending on raw data requirements. The mean and SD were calculated with a statistical significance probability level of $p \leq 0.05$. Statistically, significant differences were marked with the symbol (*) between tested groups.

Results

Decoctions Consumption

The average daily consumption of decoctions was between 4.8 and 5.7 mL for all groups. All groups got the recommended concentration of extracts or dexamethasone. The average dose of consumed plant extracts was about 100–130 mg plant dry weight/mouse daily.

Body Weight

The mean body weight of mice was recorded on days 0, 1, 4, and 8 during the experiment. Mean weight decreased among MAS groups, especially after 24 hrs of the first LPS injection compared to the Ctrl group, especially in MAS and

Table 1 MAS-Tur and MAS-Euc Demonstrate a Significant Weight Gain on Day 8. Mean Weight Gain (g) ± SD

Weight Gain (g)	Groups	Ctrl	Ctrl-Tur	Ctrl-Euc	MAS	MAS-Dex	MAS-Tur	MAS-Euc
Day 8	Mean ± SD	1.4 ± 1.35	1.5 ± 1.43	1.0 ± 1.63	−1.62 ± 2.20	−0.36 ± 1.07	2.8 ± 1.72	1.71 ± 1.13
	No	10	10	10	8	10	6	7
	P value		0.476	0.345	0.035	0.150	<0.01	0.026

Notes: Data were analyzed using two-way ANOVA followed by multiple *T*-tests for mean weight compared to Ctrl weight. The p-value shows the statistical difference between each group and the MAS group. Values are significant when $p \leq 0.05$.

MAS-Dex groups with a weight gain of −2.3 and −3.4 g, respectively (data not shown). A normal consistent weight gain was detected between the three Ctrl groups (Ctrl, Ctrl-Tur, and Ctrl-Euc) by 1.4, 1.5, and 1.0 g, respectively, throughout the experiment. Moreover, the MAS-Dex group experienced continuous weight loss during the experiment with a final mean weight of 24.6 g compared to the starting weight of 28.2 g (data not shown). No significant change was detected in all groups after day 4 during the experiment (data not shown). Even though, MAS-Tur and MAS-Euc groups showed a promising weight restore on day 8 by 2.8 and 1.7 g, respectively (Table 1).

Serum Cytokines Analysis (IL-6, TNF-α, and CCL2)

The pro-inflammatory cytokine IL-6 has been altered after 2 hrs of LPS and CpG injections in all MAS groups. IL-6 concentration was significantly increased to 35.0, 29.8, 33.0, and 31.3 ng/mL in MAS, MAS-Dex, MAS-Tur, and MAS-Euc groups, respectively, after the first LPS injection (Figure 1A). The best anti-inflammatory activity was detected in the positive control group who received dexamethasone treatment. The inflammatory parameter exhibited noteworthy reductions throughout the experiment with inhibitory activity of plant extracts on MAS-Tur and MAS-Euc groups a day after the injections to 1.17 and 2.58 ng/mL IL-6 compared to 4.24 and 4.57 ng/mL in MAS and MAS-Dex groups,

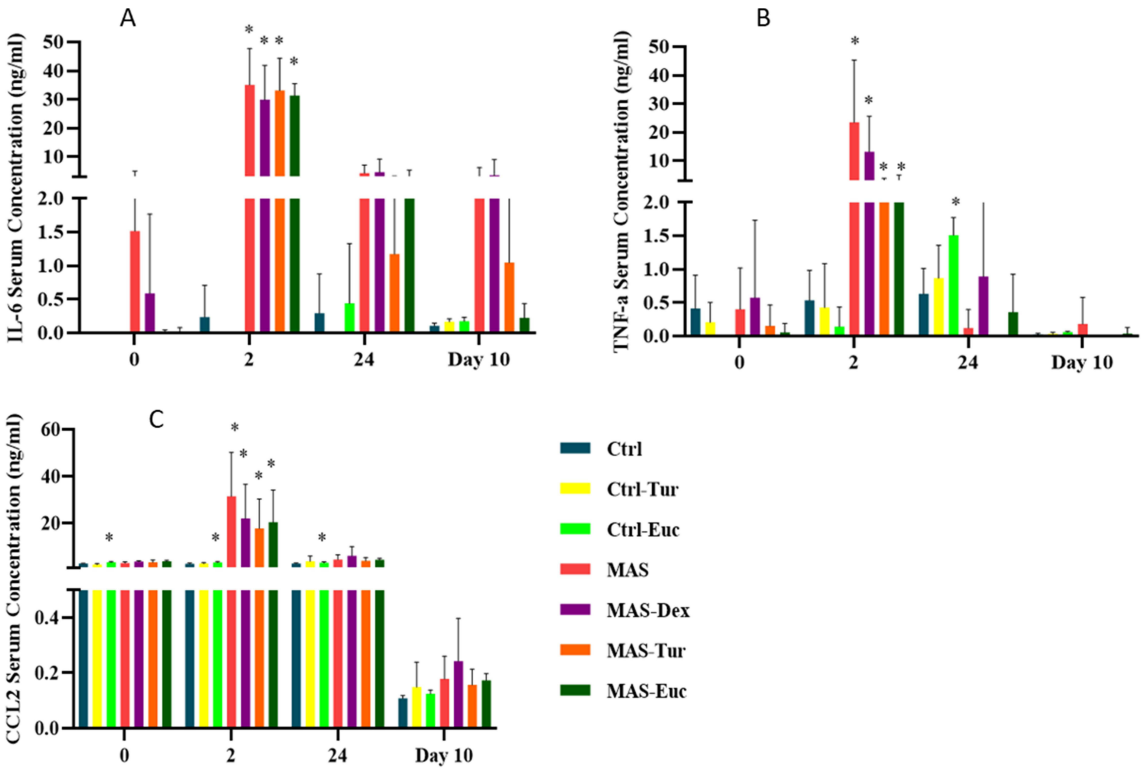

Figure 1 Mean cytokines serum concentrations (ng/mL) ± SD before and after LPS and CpG injections (after 2 and 24 hrs) and at day 10. (**A**) mean IL-6 serum concentration. (**B**) mean TNF-α serum concentration. (**C**) mean CCL2 serum concentration. Data were analyzed using two-way ANOVA followed by multiple *T* test for each group compared to Ctrl group. (*) means values are significant when $p \leq 0.05$ compared to Ctrl.

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respectively (data not shown). The reduction percentage in IL-6 was less in MAS-Tur and MAS-Euc when compared to MAS-Dex (6.0, 5.2, and 17.4%, respectively).

The serum concentration of TNF-α has been raised after 2 hrs of LPS and CpG injections in all MAS groups with a significant increase to 23.4 and 13.1 ng/mL in MAS and MAS-Dex groups and to 2.4 ng/mL in both MAS-Tur and MAS-Euc groups, respectively (Figure 1B). The potent inhibitory activity surprisingly was detected in plant extracts treated groups (MAS Tur and MAS Euc) with below-detection concentrations after 24 hrs and on day 10 (data not shown). A notably high reduction percentages were detected in TNF-α in MAS-Tur group (85.9%) and MAS-Euc group (84.3%) compared to MAS-Dex (79.2%).

CCL2 concentration has been altered after 2 hrs of first LPS and CpG injections in all MAS groups with a significant increase to 31.49, 21.96, 17.78, and 20.37 ng/mL in MAS, MAS-Dex, MAS-Tur, and MAS-Euc groups, respectively (Figure 1C). The inhibitory activity of *Eucalyptus* was similar to dexamethasone and a higher inhibitory effect was detected after 24 hrs for MAS-Euc group as 4.33 ng/mL compared to 6.07 ng/mL for MAS-Dex group (data not shown). Similar effect was detected within MAS-Tur group after 24 hrs of injection with a concentration 3.92 ng/mL (data not shown). CCL2 was also reduced in MAS-Tur and MAS-Euc with 77.1% and 54.6%, respectively. Table 2 represents a summary data for IL-6, TNF-α, and CCL2 concentrations in sera after 2 hrs of the first LPS and CpG treatment and the percentage reduction.

RT-PCR Analysis of Cytokine Genes Expression in Lung Tissues

The cytokine relative gene expression of IL-1β, TNF-α, IL-6, and transforming growth factor-beta (TGF-β) using RT-PCR in mice lungs at day 10 presented variable and significant reduction in decoction-treated mice. Data revealed that the relative gene expression of IL-1β, TNF-α, IL-6, and TGF-β increased in the course of inflammation in MAS groups. The expression of the mentioned cytokines in MAS group was 2.02, 4.61, 3.97, and 4.27 for Il-1β, TNF-α, IL-6, and TGF-β, respectively. The anti-inflammatory activity of plant extracts was seen in the context of expression in MAS-Tur and MAS-Euc groups compared to MAS and MAS-Dex groups. The relative expression of IL-1β was 1.75 and 1.29 in

Table 2 Mean Cytokines Concentration (ng/Ml) ± SD in Serum at 2 hrs After LPS and CpG Injections and the Reduction Percentage (%)

Group	Cytokine	Mean Concentration (ng/mL) ± SD	P value	The Percentage Reduction (%) of MAS level
MAS	IL-6	35.082 ± 12.679		
	TNF-α	23.486 ± 21.786		
	CCL-2	31.495 ± 18.718		
MAS-Dex	IL-6	29.884 ± 11.955	0.278	17.4
	TNF-α	13.113 ± 12.476	0.104	79.17
	CCL-2	21.967 ± 14.644	0.128	43.33
MAS-Tur	IL-6	33.1 ± 11.307	0.629	5.98
	TNF-α	2.452 ± 1.333	0.0007	85.87
	CCL-2	17.786 ± 12.529	0.023	77.1
MAS-Euc	IL-6	31.359 ± 4.087	0.270	5.18
	TNF-α	2.495 ± 2.452	0.0001	84.33
	CCL-2	20.377 ± 13.717	0.068	54.59

Notes: Data were analyzed using two-way ANOVA followed by multiple T tests for each cytokine in each group compared to MAS group. The reduction percentage was calculated with respect to MAS group. Values are significant when $p \leq 0.05$.

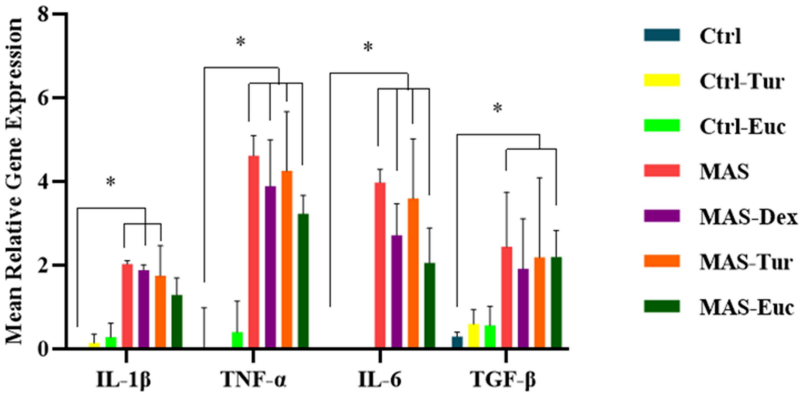

Figure 2 Mean relative gene expression ± SD of selected cytokines in lung tissue for all animal groups. Data of IL-1β, TNF-α, IL-6, and TGF-β were normalized to the expression of GAPDH. Data were analyzed using one-way ANOVA followed by multiple *T* test for all groups compared to Ctrl group. (*) means values are significant when $p \leq 0.05$ compared to Ctrl group.

MAS-Tur and MAS-Euc compared to 2.02 and 1.88 in MAS and MAS-Dex groups, respectively (Figure 2). The expression of the anti-inflammatory cytokine, TGF-β increased in all MAS groups. IL-6 expression was relatively low in all Ctrl groups. Although, the expression of IL-6 was 3.97, 2.71, 3.59, and 2.05 in MAS, MAS-Dex, MAS-Tur, and MAS-Euc groups, respectively. Generally, MAS groups showed higher cytokine relative gene expression compared to Ctrl, however, MAS-Euc group presented with the best inhibition activity on the expression level compared to all MAS groups. MAS-Euc showed the highest reduction in IL-1β, TNF-α, IL-6 (36, 30, and 48%, respectively), whereas, Dex treatment resulted in highest reduction in TGF-β (21.3%). Table 3 represents a summary data for the relative gene expression in lung tissue after 10 experimental days of the LPS and CpG treatment and the reduction percentage.

The $2^{-\Delta\Delta CT}$ equation was used to calculate the relative change values of IL-1β, TNF-α, IL-6, and TGF-β in mouse lung tissue normalized to GAPDH expression. Data were analyzed using one-way ANOVA followed by multiple *T* test for all groups compared to MAS group. Values are significant when $p \leq 0.05$.

Histopathological Analysis

Histopathological investigation revealed that lung tissue of MAS group presented with patchy and prominent areas of inflammation with a possibility of necrosis. Some samples showed signs of alveolar fragmentation and damage with

Table 3 Mean Relative Gene Expression ± SD in Lung Tissue and the Reduction Percentage (%)

Group	Cytokine	Mean Relative Gene Expression ± SD	P value	(%) Reduction of MAS Group
MAS	IL-1β	2.029 ± 0.081	0.03	
	TNF-α	4.614 ± 0.485	<0.01	
	IL-6	3.979 ± 0.3119	<0.01	
	TGF-β	2.442 ± 1.301	0.05	
MAS-Dex	IL-1β	1.885 ± 0.1279	0.04	7.09
	TNF-α	3.887 ± 1.109	0.03	15.75
	IL-6	2.717 ± 0.7516	0.04	31.72
	TGF-β	1.920 ± 1.192	0.07	21.37

(*Continued*)

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Table 3 (Continued).

Group	Cytokine	Mean Relative Gene Expression ± SD	P value	(%) Reduction of MAS Group
MAS-Tur	IL-1β	1.753 ± 0.7144	0.03	13.60
	TNF-α	4.258 ± 1.411	0.02	7.72
	IL-6	3.595 ± 1.422	0.02	9.65
	TGF-β	2.190 ± 1.898	0.15	10.32
MAS-Euc	IL-1β	1.291 ± 0.408	0.07	36.37
	TNF-α	3.228 ± 0.442	<0.01	30.03
	IL-6	2.058 ± 0.834	0.03	48.28
	TGF-β	2.197 ± 0.631	<0.01	10.03

Notes: The $2^{-\Delta\Delta CT}$ equation was used to calculate the relative change values of IL-1β, TNF-α, IL-6, and TGF-β in mouse lung tissue normalized to GAPDH expression. Data were analyzed using one-way ANOVA followed by multiple *T* test for all groups compared to MAS group. Values are significant when $p \leq 0.05$.

inflammatory lymphoplasmacytic infiltrate associated with histiocytes. The same was seen in MAS-Dex group but with preserved overall architecture and hemorrhage areas with no hemosiderin-laden macrophages. All samples of MAS-Tur and MAS-Euc groups showed mild to moderate patchy and some prominent inflammatory areas with no signs of necrosis. The tissue also showed hemorrhage areas with no hemosiderin-laden macrophages and type II pneumocyte hyperplasia. On the other hand, tissue investigation of Ctrl group showed normal tissue architecture with scant to absent signs of inflammation and no signs of necrosis. The same architecture was seen in both Ctrl-Tur and Ctrl-Euc groups with minimal signs of inflammation and areas with hemorrhage with no hemosiderin-laden macrophages (Table 4).

Immunohistochemistry results indicated that MMP-9 secretion by neutrophils was high in MAS groups. The assessment score for MMP-9^+ expression was based on the percentage of neutrophils showing MMP-9^+ positive staining as: 0: 0–5%, 1: 6–25%, 2: 26–50%, 3: 51–75% and 4: 76–100%.[23] Comparing to Ctrl, the highest MMP-9 score was in MAS group with a score of 3.8 ± 1.6 MMP-9^+ neutrophils followed by MAS-Dex with a score of 2.7 ± 0.6. Stained neutrophils score was significantly lower in MAS, groups that received plant extracts MAS-Tur and MAS-Euc (score 0.9 ± 0.09 and 1.3 ± 0.7 respectively) (Table 5).

Table 4 Percentage of Animals with Inflammatory Signs in Lung Tissues Based on Histopathological Investigations

Group	Inflammation			Overall Architecture		Necrosis		
	Absent	Minimal	Patchy and prominent	Preserved	Disrupted	Absent	Local	No definite (possibility)
Ctrl	100%			100%		100%		
Ctrl-Tur		100%		100%		100%		
Ctrl-Euc		100%		100%		100%		
MAS			100%		100%		20%	80%
MAS-Dex			100%	100%			20%	80%
MAS-Tur		60%	40%	100%		60%		40%
MAS-Euc		60%	40%	100%			20%	80%

Notes: *Calculation were done on 5 histopathologicaly analyzed animals per group.

Table 5 Mean Neutrophil Number ± SD and Mean Score of MMP-9$^+$ Neutrophils ± SD Infiltrated Into Lung Tissue

Group	Mean Number of Neutrophils ± SD	P value	Mean Score of MMP-9$^+$ Neutrophils ± SD	P value
Ctrl	0.4 ± 0.285		0.812 ± 0.341	
Ctrl-Tur	3.1 ± 1.645	0.062	1.073 ± 1.1	0.54
Ctrl-Euc	2.1 ± 1.719	0.091	1.250 ± 1.135	0.75
MAS	117.2 ± 40.93	0.0031	3.844 ± 1.603	<0.01
MAS-Dex	63.82 ± 13.45	0.0005	2.729 ± 0.609	<0.01
MAS-Tur	16.48 ± 13.13	0.0086	0.992 ± 0.098	<0.01
MAS-Euc	18.36 ± 8.36	0.052	1.344 ± 0.759	<0.01

Notes: Cells were counted in 4 high-power fields. Data were analyzed using one-way ANOVA followed by multiple *T* test for all groups compared to Ctrl group. Values are significant when $p \leq 0.05$ compared to Ctrl group.

Discussion

Untreated chronic inflammation has been associated with the onset of a cytokine storm, a condition implicated in various inflammatory disorders, including COVID-19. In our study, we aimed to develop an acute and chronic inflammation model resembling MAS in mice, simulating COVID-19-associated cytokine storm, thus lung inflammation was an important parameter to spot the light on as a pivotal parameter. Prior research, such as that by Behrens et al,[16] was conducted to develop a MAS mouse model by repeated toll-like receptor 9 (TLR-9) stimulation driven by five doses of 100 µg LPS or 50 µg CpG over a ten-day period. They concluded that mice suffered from severe weight loss, thrombocytopenia, and hyperferritinemia. This model was used in our study and slightly modified. Our model revealed distinct signs of inflammation within the initial 24 hrs following injections, although detailed data regarding these inflammatory markers are not shown.

Our established MAS model showed a decrease in body weight following LPS injection, with subdued weight gain throughout the experiment. Although specific data on the degree of weight reduction are not shown. Our findings indicate that the range of decrease in body weight in the four MAS groups spanning from 0.7 to 3.4 g compared to their respective starting weight for each group. A recent study concluded that sustained inflammation was found to significantly affects lipid metabolism.[24] Their research involved daily low-dose LPS administration to 9- to 10-day-old mice over seven days, and body weight was recorded daily. They observed a dose-dependent manner in body weight reduction and fat-mass loss. Interestingly, the decrease was most prominent in the initial two days, followed by a gradual increase in body weight until the seventh day, aligning closely with our observed trends. Interestingly, our study noted a weight restoration specifically in MAS-Tur and MAS-Euc groups on day 8 (Table 1), suggesting a potential restorative effect unique to Turmeric and *Eucalyptus* treatments within our experimental timeframe. In this study, we aimed to investigate the anti-inflammatory potential of Turmeric and *Eucalyptus* aqueous extracts on the developed MAS model in a way that resembles human consumption. Our aim was to assess a dosage comparable to the typical preparation method which equals 5 g plant-dry weight in 200 mL boiling water. Therefore, we provided the decoctions in drinking bottles recognizing this as the only way to simulate human use. Prior research has investigated the effect of administration the aquatic extracts via different methods, such as intragastric gavage or oral administration to experimental animals.[25–27] The results of the mentioned studies were encouraging. The substantial reduction percentages in mice sera observed in our study for pro-inflammatory markers IL-6, TNF-α, and CCL2 in MAS-Tur and MAS-Euc groups underscore the promising anti-inflammatory potential of these aqueous extracts. Notably, MAS-Tur group exhibited a significant reduction of 5.98% in IL-6 levels and a remarkable reduction of 85.87% in TNF-α levels. Similarly, MAS-Euc group demonstrated a reduction of 5.18% in IL-6, 84.33% in TNF-α, and 54.59% in CCL2 levels. These findings highlight the

efficacy of both Turmeric and *Eucalyptus* extracts in modulating the levels of key pro-inflammatory cytokines. The substantial reduction in TNF-α, a pivotal mediator of inflammation, alongside notable decreases in IL-6 and CCL2.

A recent study conducted by Nagaraju et al[28] revealed that LPS-induced acute lung injury in rats was ameliorated by pre-treatment with functional food mix composed of Turmeric and other anti-inflammatory plant products. They found that circulating TNF-α peaked after 60 min post-injection, followed by a subsequent decline by 120 min. Conversely, IL-6 levels continued to increase till 120 min, reflecting a sustained presence in the bloodstream. The terminal elimination half-life of IL-6 and TNF-α is 15.5 and a notably shorter duration of 0.5 hrs, respectively according to several studies.[29,30] This contrasting half-life data explains the swift clearance of TNF-α from mice serum within 24 hrs due to its rapid decay, while IL-6 persisted for an extended duration in circulation. Additionally, multiple low-doses of LPS injections may trigger the host's protective mechanism aiding in host resistance to subsequent LPS stimulation again and protect the spleen from further damage according to Zhong et al.[31] This may explain the significant decline in cytokines concentrations within the bloodstream 24 hrs after LPS treatment.

The mechanism of inflammation induced by LPS and subsequent cytokine production is elucidated by many studies.[32,33] It is evident that LPS acts as a potent trigger for nuclear factor kappa B (NF-κB) pathway activation by LPS/TLR4 signaling[34] and pro-inflammatory cytokines like TNF-α, IL-6, and IL-1β trigger the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Furthermore, activation of NF-κB and STAT3 by IL-6 is well-established in previous studies.[35,36] While our study does not specifically delve into identifying the precise mechanism through which the selected decoctions mitigate inflammation, recent research investigating the impact of Eucalyptus essential oils on LPS-activated macrophages revealed a reduction in the inflammatory response by downregulating Mitogen-Activated Protein Kinases (MAPK) and NF-κB pathways.[37] Another recent review highlighted the anti-inflammatory mechanism of curcumin.[38] Curcumin demonstrates the capacity to bind to TLRs and subsequently downregulates NF-κB pathway, MAPK, and other signaling pathways, thereby effectively regulating inflammatory mediators and ameliorating inflammatory diseases. Curcumin also has an anti-inflammatory effect by regulating the JAK/STAT inflammatory signaling pathway.

Histological analysis of lung tissues revealed minimal inflammatory signs and neutrophilic count in MAS-Tur and MAS-Euc groups compared to those in the MAS and MAS-Dex groups. Concurrently, these groups displayed lower MMP-9^{+} neutrophilic score in the corresponding tissue. Neutrophil-mediated inflammation and MMP-9 secretion were evaluated in a study conducted by Hsu et al[39] using intratracheal LPS to cause lung injury. Their findings indicated a continuous increase in MMP-9 activity over 72 hrs, indicating that MMP-9 was upregulated and secreted by primed neutrophils. Our findings are in agreement with their model where lung injury triggers a generalized neutrophil priming with subsequent neutrophils secreting of MMP-9 in the pulmonary vasculature to encourage their migration into the alveolar space. Analysis of neutrophils count in lung tissue showed a distinct variation in our experiment. Specifically, in MAS-Tur and MAS-Euc groups (16.4 and 18.3 cell/HPF, respectively) that exhibited a significant reduced count compared to MAS (117.2 cell/HPF). These findings align with the known role of neutrophils in the course of lung inflammation and tissue damage, however, the significant lower counts in plant-treated groups underscore the potential activity of decoctions in mitigating neutrophil-mediated responses, thus minimizing the inflammatory response.

These natural extracts might exert their inhibitory effects through several pathways. Firstly, anti-inflammatory compounds present in Turmeric and *Eucalyptus*, such as curcumin and flavonoids, are known for their ability to modulate signaling pathways involved in cytokine production,[37,40] potentially mitigating the expression of pro-inflammatory cytokines. Moreover, these compounds may regulate the activity of key transcription factors like NF-κB, integral to the control of genes involved in the inflammatory response, thereby contributing to the downregulation of cytokine expression.[41] Additionally, modulation of critical signaling pathways, including JAK-STAT or MAPK, likely plays a role in dampening the production of these cytokines.[40] Furthermore, immune modulation might be a key factor, influencing the activities of immune cells and their ability to produce pro-inflammatory mediators.

Taken together, our established model provides valuable insights in studying chronic inflammation deeply, offering a window into its intricate dynamics. We suggest that the optimal termination point may be between 2 and 6 hrs post the final boost injection on day 9. Among the experimental groups, MAS group exhibited the most pronounced impact, characterized by significant weight loss and notably heightened levels of key inflammatory parameters (such as IL-6, TNF-α, and CCL2) observed at the two-hour mark post-injection. Administration of Turmeric and *Eucalyptus* decoctions

had an anti-inflammatory effect on both acute and chronic inflammation besides its protective effect on vital organs [lungs, spleen, and liver (data not shown)].

Collectively, this study was conducted with the primary objective of assessing the efficacy of Turmeric and *Eucalyptus* decoctions on MAS mouse model. According to our findings, Turmeric and *Eucalyptus* decoctions have a substantial impact in controlling inflammation progression. These natural extracts exhibited multifaceted roles by pretense of analgesic and reducing pro-inflammatory cytokines as well mitigating pulmonary neutrophil recruitment. At a molecular level, these decoctions significantly impacted gene expression, leading to a reduction in the relative expression of specific cytokines in plant-treated groups. Additionally, our study indicated the safety of these formulations on vital organs, exhibiting no signs of toxicities. However, the precise mechanisms underlying the actions of Turmeric and *Eucalyptus* extracts remain elusive in vivo, presenting an important avenue for future research.

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Disclosure

The authors report no conflicts of interest in this work.

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