Indian Journal of Biochemistry & Biophysics
Vol. 63, July 2026, pp. 782-795
DOI: 10.56042/ijbb.v63i7.15290

Modulation of inflammatory cytokine expression due to *in vitro* exposure of *Aegle marmelos* in chicken splenocytes

Sonu Ambwani[1]*, Rigzin Dolma[1], Lata Paliwal[1], Amandip Kaur[1] & Tanuj Kumar Ambwani[2]

[1]Department of Molecular Biology and Genetic Engineering, College of Basic Sciences and Humanities; &
[2]Department of Veterinary Physiology and Biochemistry, College of Veterinary and Animal Sciences,
G.B. Pant University of Agriculture & Technology, Pantnagar-263 145, Uttarakhand, India

Received 25 November 2025; *revised 27 May 2026*

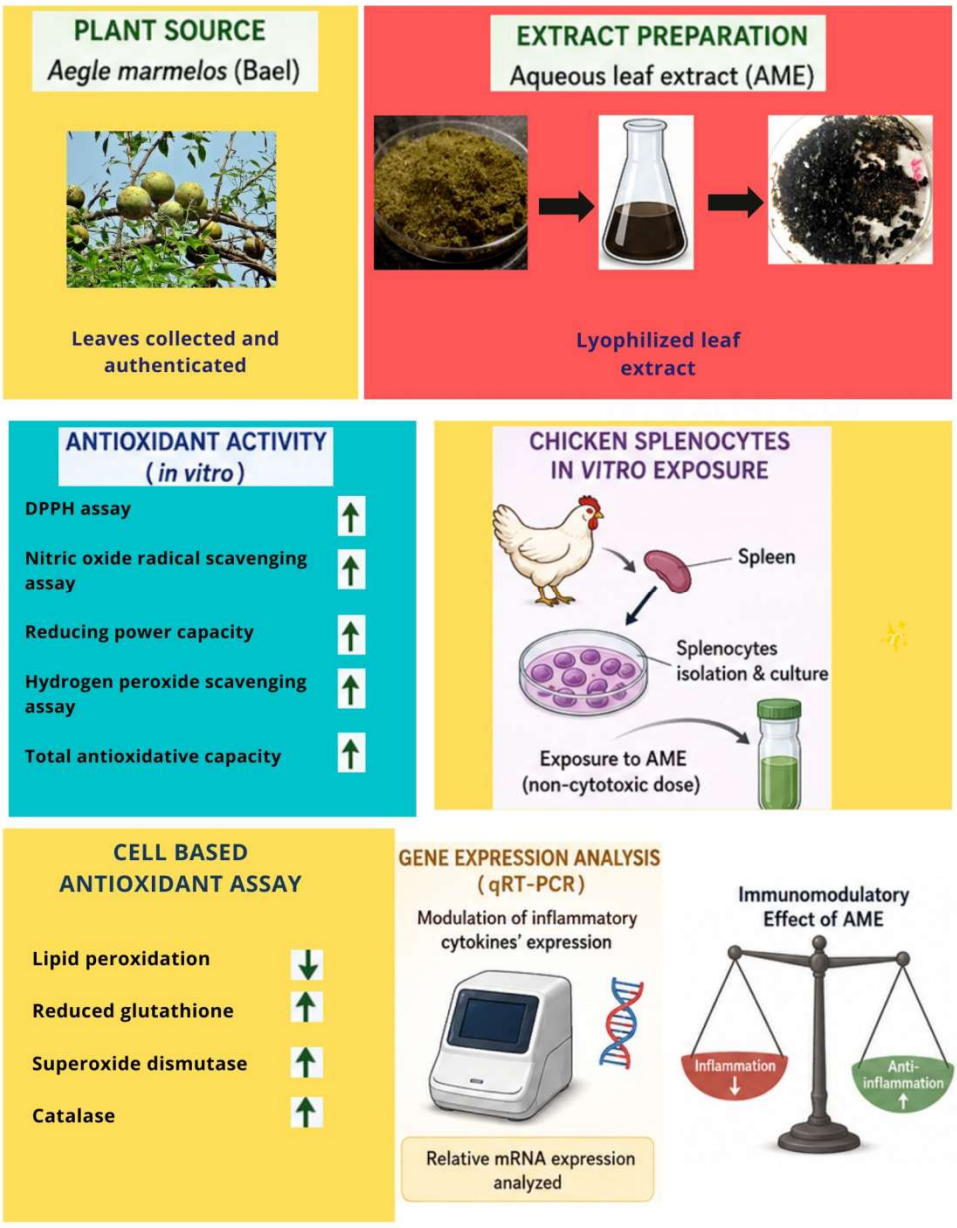
The global popularity of traditional herbal medicine is on the rise, despite facing disparagement for lack of scientific validation. Many traditional herbal preparations are endorsed for their anti-inflammatory properties. Cytokines, as key regulators of the immune system, can significantly impact immune status. Given the limited understanding of molecular targets and mechanisms, there is a crucial need to scientifically validate the potential of anti-inflammatory herbs at the molecular level. In view of this, *Aegle marmelos* (L.) Correa, commonly known as 'Bael', was investigated for its antioxidative and anti-inflammatory properties using chicken lymphocytes culture system. The study utilized aqueous extract of *Aegle marmelos* leaves (AME) to assess antioxidative potential through various assays. The calculated maximum non-cytotoxic dose of AME was used for treatment in chicken lymphocytes *in vitro*, allowing for the examination of transcriptional modulation of selected genes *via* qRT-PCR. Results showed significant *in vitro* antioxidant properties of AME across multiple assays. Gene expression analysis revealed notable alterations due to AME exposure, with pro-inflammatory genes being down-regulated and anti-inflammatory genes up-regulated. The study concluded that AME exhibits potent anti-oxidative and anti-inflammatory potential, warranting further exploration through appropriate *in vivo* experiments due to its strong anti-inflammatory activity.

Keywords: *Aegle marmelos*, Chicken lymphocytes, Cytokines expression, Dexamethasone, Immunomodulation

Over the past two decades, poultry meat has become more and more popular with wider acceptability as economic and easily available source of animal protein. It is currently the most preferred meat from any livestock species, making up about 45% of the overall meat consumed. A critical and fast-growing global concern is use of antibiotics in livestock and poultry industry. Reducing the use of antibiotics in livestock feed necessitates significant improvements in herd health problems, but prudent and expert management of antimicrobial medications is also required to avoid the dangers of antibiotic resistance in humans. Antimicrobial medications can be used for prophylactic and therapeutic purposes that are effective. However, because of the effects on intestinal microbiota and the harmonic function of the gastrointestinal barrier, any use of antibiotics in-feed has been seriously questioned, in addition to public health concerns[1]. Due to disease resistance and antibiotic remnants in the food chain, there is a global push to reduce the usage of antibiotics in poultry. The situation necessitates to investigate feasible alternatives that may be advantageous and economical. Probiotics, gut acidifiers, immunomodulators, eubiotics, organic acids, and other similar items are widely accessible on the market, but further relevant research is required[2].

Organic healthcare systems are utilized as supplemental medicine in many regions of the world[3]. They have also contributed significantly to the global rise in demand for food dietary supplements, herbal remedies, nutraceuticals, phytopharmaceuticals, and other products[4]. Over the past several decades, there has been a massive growth in interest in medicinal plants, their extracts, and multi-component medications that contain natural sources of active ingredients. The generation of safe and effective herbal medicinal substances with few or no undesirable side effects is the primary rationale and objective. As a result, interest in the immunomodulating properties of many commonly used herbs and spices has surged subsequently[5]. Immunomodulation is the practice of boosting or restraining the immune system to treat a variety of

*Correspondence:
E-mail: sonuambwani@yahoo.co.in



Graphical abstract

ailments and diseases. Immune-stimulating and immune-suppressive drugs are medicines that can boost or reduce specific immune system cells or components. To improve chicken health and productivity, a variety of medicinal plants can be utilized as substitutes for antibiotics, serving as sources of novel chemicals with therapeutic potential[6].

Aegle marmelos (L.) is a worthy plant with a range of therapeutic applications. It is a member of the Rutaceae family and is frequently referred to as bael (Bel, Bilwa, Indian bael, Stone Apple). The plant has a variety of beneficial compounds, including fibres, carotenoids, polyphenols, terpenoids, flavonoids, alkaloids, and coumarins. The presence of these compounds in plants provides antibacterial, antioxidant, antidiarrheal, antidiabetic, anti-ulcerative, cardioprotective, anticancer, and gastroprotective properties[7]. The compounds that scavenge free radicals are found in medicinal plants and fruits as phenolics and flavonoids. The antioxidant-active components in the bael tree shield the immune system

from oxidative stress, which promotes B-cell differentiation and proliferation into plasma cells[8]. Numerous phytochemicals, such as aegelin, lupeol, cineole, citral, eugenol, skimmianine, marmesinin, *etc*., are found in *Aegle marmelos* leaves. Two strong cardioactive substances with anti-inflammatory properties are aegelin and lupeol[9]. Strong antioxidant and free-radical scavenging properties are additionally linked to the fruit. Some studies highlighted the significant presence of total phenolic compounds and antioxidant properties in *Aegle marmelos* fruit drink, suggesting its potential as an antiscorbutic and stomach-soothing agent. Additionally, extracts from *Aegle marmelos* were observed to enhance the body's immune system, potentially aiding in anti-cancer activities[10]. Compounds such as d-limonene, eugenol, and citral found in *Aegle marmelos* extract exhibited anti-neoplastic effects[11]. Furthermore, active metabolites from *Aegle marmelos* were found to possess immunosuppressive properties in experimental models, affecting cytokines involved in immune responses, including antibody-mediated and cell-mediated processes[12]. The burgeoning interest in the beneficial effects of cytokines, variations in their expression, and the possibility of targeting their receptors may provide a new avenue for utilizing them in medical treatment. This study aimed to assess the immunomodulatory and antioxidant properties of *Aegle marmelos* leaf extract (AME) on chicken splenocytes by analyzing the expression of various cytokines using qRT-PCR. Because the immune systems of chickens and mammals are comparable, using chickens as an experimental model system has the potential to provide insight into the human immune system.

Materials and Methods

Plant material and extracts

Aegle marmelos (Bael) leaves were obtained from the Agroforestry Research Centre at the Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India (Voucher specimen no.1628). The green leaves were initially dried in the shade to reduce moisture content. Once dried, the leaves were ground into a fine powder and sifted to create a Bael leaf powder meal. This meal was then stored in airtight, moisture-resistant containers until needed. Using the maceration method, an aqueous extract was prepared from the *Aegle marmelos* leaf powder[13].

Phytochemical screening

Various qualitative phytochemical analyses of AME, for the presence of multiple classes of active chemical constituents such as tannins, saponins, glycosides, flavonoids, alkaloids, terpenes, and steroids, *etc*., using standard procedures were conducted[14.]

Test for total phenolic

AME's overall phenolic content was estimated using the Folin-Ciocalteu reagent in accordance with Mansour *et al.* methodology[15]. The total amount of phenolic compounds was calculated using a standard curve made using gallic acid and its linear equation. The expression mg/g gallic acid equivalent (GAE) represents the overall phenolic component concentration.

Test for total flavonoids

A colorimetric assay with aluminium chloride was used to measure the total flavonoid content of AME, as reported by Atanassova *et al*[16]. The amount of flavonoid content obtained in the extract was expressed as milligrams of rutin equivalent (RE) per gram.

Determining the antioxidative potential of AME

DPPH assay

The 2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed using Gulcin *et al.* protocol[17]. The assay's foundation is the evaluation of antioxidants' scavenging ability. Antioxidants found in the plant extract provide a hydrogen atom that reduces the odd electron of a nitrogen atom in DPPH to generate corresponding hydrazine. Because of its odd electron, DPPH exhibits a prominent absorption band at 517 nm, giving the solution a deep violet appearance. As a result, the DPPH test calculates the total amount of reducing agents in the plant extract. The positive control used was ascorbic acid.

The radical scavenging activity (RSA) was calculated using the following equation:

RSA (%) = [(absorbance of control - absorbance of sample)/ absorbance of control] ×100

Nitric oxide (NO) radical scavenging assay

The assay employs sodium nitroprusside to produce free radicals. Subsequently, the reaction is monitored through colorimetric detection of nitrite as an azo dye product of the Griess reaction. The assay was carried out by the method given by Jagetia *et al*[18]. Ascorbic acid was used as a positive control.

The percent NO radical scavenging activity was calculated according to the absorbance at 546 nm.

% NO scavenging = [(absorbance of control-absorbance of sample)/ absorbance of control] ×100

Reducing power capacity

In the presence of a reducing agent, the ferric ion gets reduced to the ferrous ion. An increase in absorbance indicates that the plant extract has a higher reducing power capacity. This method detects an antioxidant chemical at 700 nm by utilizing potassium ferricyanide, trichloroacetic acid, and ferric chloride to form a coloured complex. The procedure described by Alam *et al*. was used to conduct the assay[19]. Ascorbic acid used as standard.

Hydrogen peroxide scavenging assay

Certain enzymes are directly turned inactive by hydrogen peroxide, typically through the oxidation of crucial thiol (-SH) groups. It can quickly traverse cell membranes and is most likely to create hydroxyl radicals while reacting with Fe^{2+} and potentially Cu^{2+} ions. The assay was determined according to the method Ruch *et al.* described using ascorbic acid as standard[20].

Total antioxidative capacity

By using the phosphomolybednum method[21], which relies on the sample analyte's reduction of Mo (vi) to Mo (v) and the subsequent creation of a green phosphate/Mo (v) complex that is detected at 695 nm and compared with ascorbic acid, the total antioxidant capacity of AME was determined. The total antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Cell culture and treatment

Chicken spleens were obtained from healthy broiler chickens aged 3 to 5 weeks at local slaughterhouse. The spleens were collected in sterile Dulbecco's phosphate buffer saline (DPBS) and transported to the laboratory for processing. Lymphocytes were then isolated from these spleens following the method outlined by Ambwani *et al*[22]. The isolated lymphocytes were subsequently cultured in 96-well flat bottom plates using RPMI-1640 medium (Hi-media, India). This medium was enriched with 10% foetal bovine serum (FBS) (Hi-media, India) and supplemented with both antibiotic and antimycotic solutions to prevent contamination. The cells were incubated for 24 h at 40°C in a CO_2 incubator, providing an optimal environment for cell growth and preparation for further experimental procedures.

***In vitro* exposure of AME in chicken splenocytes**

The cytotoxic potential of the AME was evaluated using the MTT (3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide) assay, which measures cell viability. This method relies on the ability of mitochondrial dehydrogenases in living cells to convert the pale yellow MTT into dark blue formazan crystals. These crystals accumulate within healthy cells due to their impermeability to cell membranes, with the amount of formazan produced directly correlating to the number of surviving cells. To determine the maximum non-cytotoxic dose (MNCD), cells were exposed to various concentrations of AME in triplicate and incubated for 24 h at 40°C in a CO_2 incubator. After this period, the media was replaced with MTT dye and incubated for an additional 4 h in darkness under the same conditions. The resulting formazan crystals were then dissolved using dimethyl sulfoxide (DMSO). Following an incubation of 15-minute, the absorbance was measured at 570 nm using a computerized micro-scan ELISA reader, allowing for quantification of cell viability[23].

The rate of growth inhibition was calculated as per the following formula:

Percent growth rate = [absorbance of AME treated cells/ absorbance of control] x 100

The values of percent cytotoxicity are expressed with respect to the control cells (untreated cells) *i.e*., Percent cytotoxicity = (100 - percent growth rate) %

Lymphocyte proliferation assay

The lymphocyte proliferation assay (LPA) evaluates the capacity of lymphocytes to multiply when exposed to stimulants in a laboratory setting. This technique involves placing lymphocytes in a short-term tissue culture and exposing them to various stimuli such as foreign molecules, antigens, or mitogens. In this particular setup, three cell culture-tested stimulants: concanavalin A (Con A), phytohaemagglutinin M form (PHA-M) (Hi-media, India), and lipopolysaccharide (LPS) (Sigma Aldrich, USA) derived from *Escherichia coli* (serotype 0111:B4) were used. These stimulants were applied to splenocytes at a concentration of 5 µg/mL in RPMI-1640 medium. The assay followed the mitogenic stimulation method outlined by Creed *et al*[24]. To establish positive controls, LPS-stimulated cells

without AME treatment for B lymphocytes, while Con A and PHA-stimulated cells without AME treatment served as positive controls for T lymphocytes.

Cell-based antioxidative assay

Chicken lymphocytes were treated with MNCD of AME and incubated overnight in a CO_2 incubator. After incubation, control and treated cells were harvested and cell lysates were prepared and stored at -80°C for determining the antioxidant status.

Lipid peroxidation (LPO)

LPO is measured by quantifying malondialdehyde (MDA), a byproduct of fatty acid oxidation, which forms a coloured complex with thiobarbituric acid (TBA). This complex is detected spectrophotometrically at 532 nm and expressed as nmol MDA per mg protein as described by Li *et al*[25].

Superoxide dismutase (SOD)

The method evaluates SOD activity by utilizing pyrogallol's spontaneous oxidation to produce superoxide. This assay determines how effectively SOD can prevent superoxide from reducing MTT to formazan. After halting the reaction with DMSO, the resulting formazan is measured spectrophotometrically at 570 nm, providing a quantitative measure of SOD activity[26].

Reduced glutathione (GSH)

The concentration of GSH is measured using a method given by Ellman[27]. This approach involves combining GSH with a compound known as DTNB (5,5′-dithio-bis (2-nitrobenzoic acid)). The resulting chemical reaction produces a yellow-coloured substance. The intensity of this yellow colour can be quantified by measuring its light absorption at a wavelength of 412 nm.

Catalase

The UV spectrophotometric method is widely regarded as the primary technique for assessing catalase activity. This approach relies on tracking changes in absorbance at 240 nm when using concentrated hydrogen peroxide solutions, typically 30 mM or higher. However, these elevated H_2O_2 concentrations can rapidly inhibit the catalase enzyme by modifying its active site structure. The degree of this inhibition can vary, but it remains a significant consideration when employing this method for catalase activity measurement[28].

RNA extraction, cDNA synthesis, and quantitative PCR

In this experiment, different conditions were shown to affect the expression of genes in chicken splenocytes. Cells were cultured in six-well plates and either left unstimulated or stimulated with LPS (5 µg/mL), then treated with MNCD of AME (75 µg/mL) as well as dexamethasone (30 ng/mL) as determined earlier by Ambwani *et al*[22]. Using RNA-xpress reagent (Hi-media, India), total RNA was extracted following a 24-h incubation period at 40°C in a CO_2 incubator. Using a cDNA synthesis kit (Thermo Fisher Scientific, USA), 2 µg of RNA was reverse transcribed to cDNA after the concentration of RNA was determined using nanodrop spectrophotometry. Until it was required, the cDNA was kept at -80°C Ultra low temperature freezer. The expression of 13 immune response-related genes, including several cytokines and transcription factors, was then examined using real-time PCR (Eppendorf, Germany) using SYBR green qPCR master mix (Applied Biosystems, USA). The relative expression levels of genes were calculated using $2^{-\Delta\Delta(Ct)}$ method, and GAPDH was used as internal reference gene. The details of the primers used for the study are described in (Table 1).

Statistical analysis

All experiments were conducted in triplicate, with the mean and standard deviation calculated for each set of observations. The data is analyzed using one way analysis of variance (ANOVA), followed by Tukey's test. The critical difference was calculated at a 5% significance level to determine the statistical significance of differences between treatment means**.** Statistical Package for the Social Sciences (SPSS) software is utilized for all the statistical analysis.

IC_{50} value

The IC_{50} value is a crucial measure that quantifies the potency of an inhibitor. It represents the concentration of an inhibitory compound needed to reduce a specific biological or biochemical activity by 50%. This metric provides a standardized way to compare the effectiveness of different inhibitors. A lower IC_{50} value indicates that less of the inhibitor is required to achieve the same level of inhibition, suggesting a more potent compound[29].

Results

From the 100 g of dried leaf powder of Bael (*Aegle marmelos*), 14.01 grams of extract (AME) was obtained, resulting in an extraction yield of 14.01% (Fig. 1).

Table 1 — PCR primers along with their melting temperature

Primer Name	Accession number	Sequence	Annealing temperature
IL-1β	NM_204524.1	CTGACCCGCTTCATCTTCTAC CTTAGCTTGTAGGTGGCGATG	56°C
IL-2	NM_204153.1	AACTGAGACCCAGGAGTGCA CCGGTGTGATTTAGACCCGTA	56°C
IL-4	NM_001007079.1	TCCTGCGTCAAGATGAACGTG GCATTCAGGAGCTGACGCAT	52°C
IL-6	NM_204628.1	CGCCTTTCAGACCTACCTGG CTTCAGATTGGCGAGGAGGG	56°C
IL-10	NM_001004414.2	CAATCCAGGGACGATGAACT ATCTGTGTAGAAGCGCAGCA	53°C
iNOS-2	NM_204961.1	CGTGTTCCACCAGGAGATGT CGTGTTCCACCAGGAGATGT	53°C
IFNγ	NM_205149.1	ACAACCTTCCTGATGGCGTGA AGTTCATTCGCGGCTTTGCG	59°C
LITAF	NM_204267.1	AGATGGGAAGGGAATGAACC GGAAGGGCAACTCATCTGAA	52°C
TGF-β1	NM_001318456.1	ATGGACCCGATGAGTATTGG GGACACGTTGAACACGAAGA	54°C
Caspase-3	NM_204725.1	TGGTGGAGGTGGAGGAGC ATGTCTGTCATCATGGCTCTTG	60°C
Caspase-9	XM_424580.6	TCCATCCCAGTCCAACCTGA GGTACACCAGTCTGTGGTCG	60°C
Nrf2	NM_205117.1	CGATGACCACTCAGGAACTG CCCAGGAGAACTGTCCATGT	54°C
NF-κB1	NM_205134.1	GACCGCCAATAGCTTGTCCT TATGTAGTGCTGTCCGCGTC	56°C
GAPDH	NM_204305.1	GGAAAGTCATCCCTGAGCTG CTTGGCTGGTTTCTCCAGAC	54°C

IL-interleukin, iNOS- inducible nitric oxide synthase, IFN-γ=interferon gamma, LITAF=Lipopolysaccharide-Induced Tumor Necrosis Factor-Alpha factor, TGF-β1= Transforming growth factor beta 1, Nrf2= Nuclear factor erythroid 2-related factor 2, NF-κB1= Nuclear Factor Kappa B Subunit 1, GAPDH= glyceraldehyde 3-phosphate dehydrogenase

Fig. 1 — *Aegle marmelos* (a) *Aegle marmelos* leaf; (b) *Aegle marmelos* dried leaf powder; and (c) Aqueous leaf extract of *Aegle marmelos* (AME)

Phytochemical screening

Qualitative assays revealed presence of various phytochemicals in the AME as presented in (Table 2).

Total phenolic content

Total phenolic content was reported as mg GAE per gm of the extract as presented in (Table 3). The total phenolic content of the AME was estimated to be 480 mg/g.

Total flavonoid content

The total flavonoid content was reported as mg RE per g of the extract as presented in (Table 4). The total

flavonoids content of the AME was found to be 342.70 mg/g.

Antioxidative potential of AME

DPPH assay

A dose-dependent relationship was observed in the DPPH assay *i.e.* scavenging activity was increased as the concentration of the extract was increased (Fig. 2a). The IC_{50} value for AME was found to be 233.11 µg/mL.

Table 2 — Phytochemical screening through qualitative tests of AME

S. No.	Phytochemical Analysis	AME
1	Phenolics	+
2	Flavonoids	+
3	Tannins	+
4	Alkaloids	+
5	Saponins	+
6	Reducing sugars	-
7	Protein	+
8	Amino acids	-
9	Phytosterols	+

Table 3 — Total phenolic content in AME

S. No.	Plant extract	Concentration of extract	Total phenolic content (µg/mg extract)	Total phenolic content (mg GAE/g extract)
1	AME	5 µg/mL	0.48	480

Table 4 — Total flavonoid content in AME

S. No.	Plant extract	Concentration of extract	Total flavonoid content (µg/mg extract)	Total flavonoid content (mg RE/g extract)
1	AME	5 µg/mL	0.342	342.7

NO radical scavenging assay

NO is a reactive free radical generated by phagocytes and endothelial cells. It gives rise to more reactive species, such as peroxynitrite, which can further decompose to produce the hydroxyl radical. Dose-dependent relationship was observed in no radical scavenging assay (Fig. 2b). The AME was found to have IC_{50} value of 131.93 µg/mL, whereas the standard showed an IC_{50} value of 113.80 µg/mL.

Reducing power capacity

The reducing power capacity of AME was found to be increased in a dose-dependent manner. Increased absorbance of the reaction mixture indicated increased reducing power of the extract (Fig. 2c). In this test, the yellow colour of the sample solution changes to different shades of green and blue based on the reducing power of each compound.

Hydrogen peroxide scavenging assay

The plant was assessed for its scavenging potential by hydrogen peroxide scavenging assay. The IC_{50} of the standard was found to be 27.52µg/mL. It was 69.32µg/mL for AME. The aqueous extract of *Aegle marmelos* was reported to possess significant

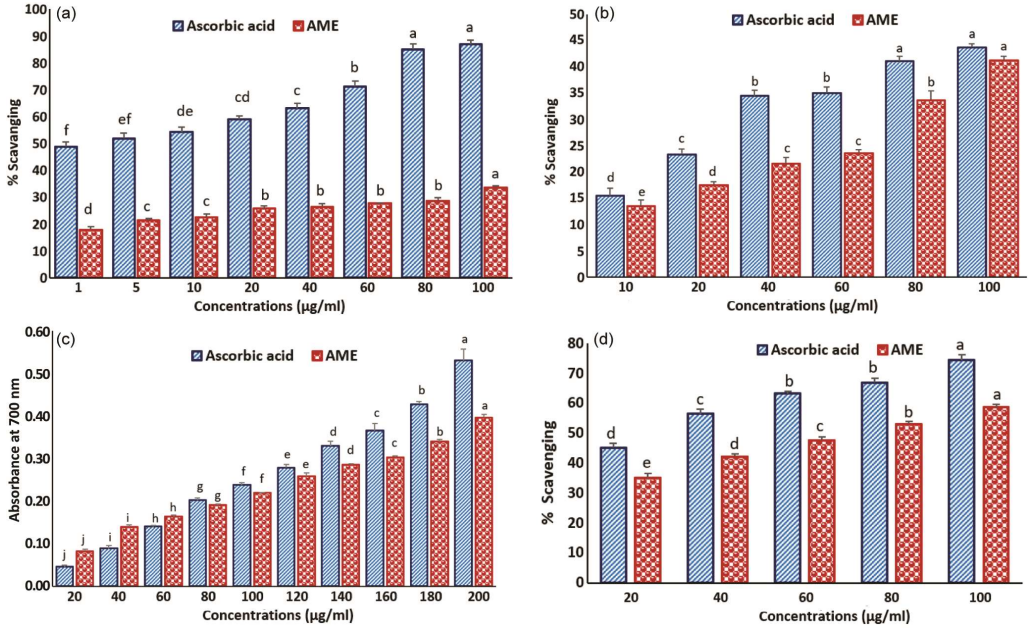


Fig. 2 — Antioxidant activity of AME with reference to Ascorbic acid as standard. (a) DPPH radical scavenging; (b) Nitric oxide radical scavenging; (c) reducing power capacity; and (d) hydrogen peroxide scavenging. Each bar represents the mean value of three determinations ± S.D

antioxidative capacity as displayed through hydrogen peroxide radical scavenging assay (Fig. 2d).

Total antioxidative capacity

The phosphomolybdate method is considered as quantitative because it measures the total antioxidant capacity and expresses it as the equivalent amount of ascorbic acid, allowing for accurate quantification of antioxidant levels. AME was found to possess total antioxidative capacity with 102.3 mg/g of dry matter.

MNCD of AME in chicken lymphocytes

Various concentrations of AME were used for *in vitro* exposure to chicken lymphocytes for MNCD determination and percent viability was observed after AME treatment (Fig. 3). As at dose level of 75µg/mL, cells showed a hundred percent viability and thus this dose was considered as MNCD for further analyses. At higher dose levels of AME exposure revealed dose dependent cytotoxicity that was confirmed through microscopic examination also (Fig. 4).

Lymphocyte proliferation assay

The proliferation of mitogen stimulated B and T lymphocytes was observed after AME treatment at MNCD. For B lymphocytes, LPS stimulated cells without AME treatment were used as positive control. LPS stimulated AME treated cells showed a 9.32% decrease in B lymphocyte proliferation. For T lymphocytes, Con A and PHA stimulated cells without AME treatment were used as positive control. In Con A stimulated cells AME treatment displayed a significant decrease of 6.20% in T lymphocyte proliferation. There was a significant decrease in lymphocyte proliferation in AME treated cells as compared to control untreated cells. The percent inhibition in the case of PHA was found to be 15.53% (Fig. 5).

Cell based antioxidant assay

In different biochemical assays, the antioxidative potential of AME was explored. The enzymatic assays *i.e.* LPO, GSH, SOD and catalase were estimated. Ascorbic acid was used as positive control in each assay (Fig. 6).

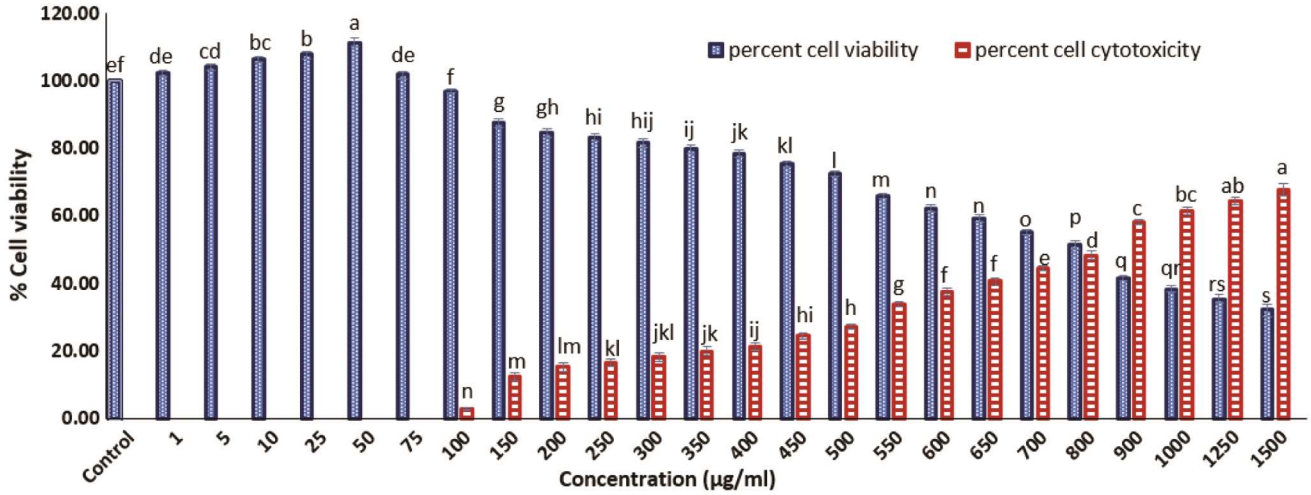


Fig. 3 — Percent viability of chicken lymphocytes in the presence of different concentrations of AME. Data are presented as mean ± S.D of three independent experiments

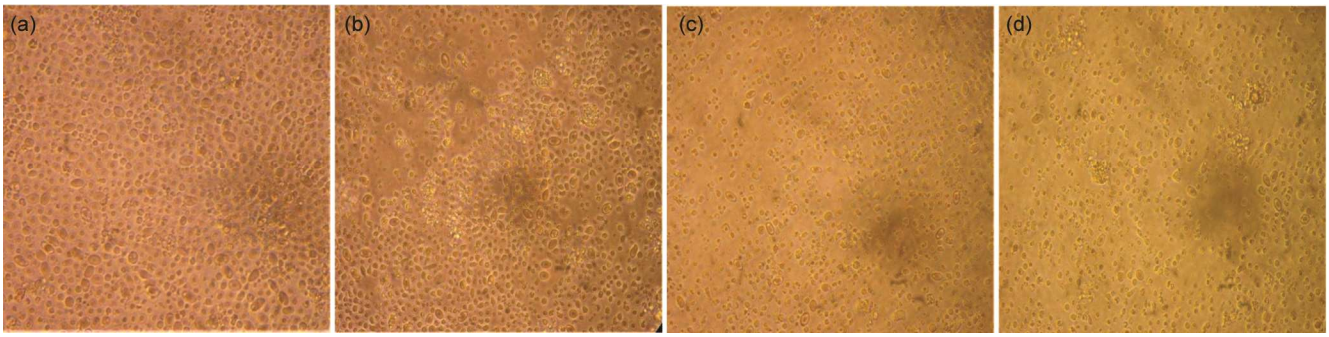


Fig. 4 — Influence on chicken lymphocytes due to *in vitro* exposure of AME after 48 h. (a) Control; (b) 75 µg/mL; (c) 150 µg/mL; and (d) 700 µg/mL) at 40x

Lipid Peroxidation

In membrane lipid peroxidation assay, the decrease in rate of peroxidation was observed after AME exposure. AME treated cells showed significant decrease in LPO as compared to control and ascorbic acid treated cells (Fig. 6a).

Superoxide Dismutase

The content of superoxide dismutase was found to be increased after AME exposure as compared to control cells. Ascorbic acid showed more antioxidative potential (Fig. 6b).

Reduced Glutathione

The reduced glutathione content was estimated which signifies the antioxidative potential of AME in chicken lymphocytes. AME treated lymphocytes showed slight increase in reduced glutathione content as compared to control (Fig. 6c).

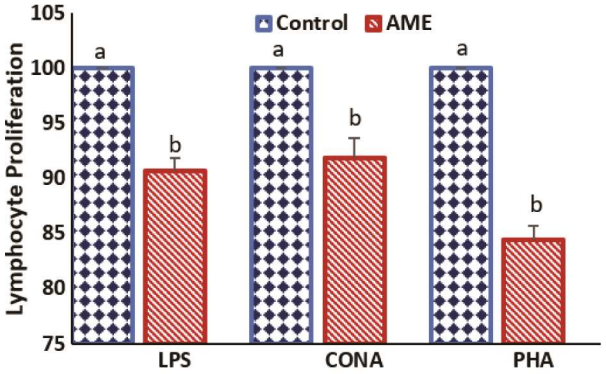


Fig. 5 — Effect of AME on lymphocyte proliferation upon mitogenic stimulation. Data are presented as mean ± S.D of three experiments

Catalase

The catalase content in AME treated cells was estimated which was increased after AME exposure. AME treated cells showed slight increase in content of catalase as compared to control (Fig. 6d).

Expression analysis by real-time quantitative RT-PCR in spleen following LPS treatment

To assess the effect of AME on pro-inflammatory and anti-inflammatory mediators, the chicken splenocytes were treated with MNCD of AME and dexamethasone in LPS unstimulated and stimulated cells where dexamethasone treated cells were kept as positive control (pro-inflammatory) while untreated cells were taken as control. The expression of IL-1β, IL-2, IL-6, iNOS, IFN-γ, LITAF, Caspase-3, Caspase-9 and NF-κB1 was found to be substantially reduced (and has an expression changes that were statistically significant at $P< 0.05$) in AME treated chicken splenocytes as compared to control in LPS unstimulated with mean fold expression of 0.59, 0.42, 0.11, 0.14, 0.69, 0.48, 0.65, 0.84, 0.48 and as well as in LPS stimulated cells with 0.73, 0.69, 0.31, 0.21, 0.75, 0.88, 0.74, 0.83, 0.65, respectively. This could

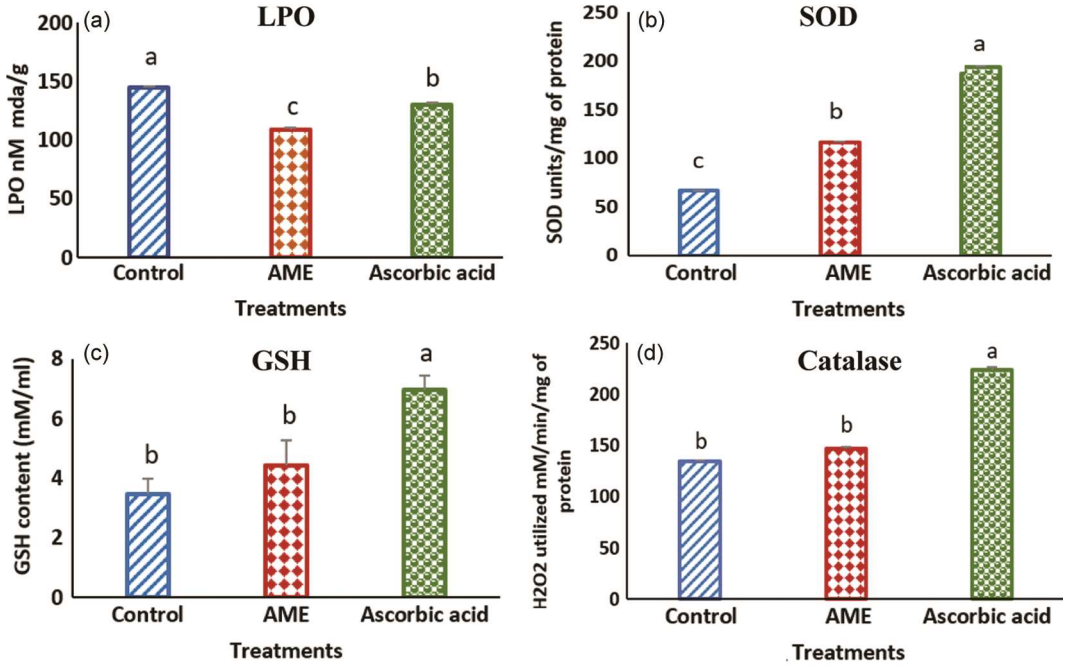


Fig. 6 — Effect of AME on antioxidative status of chicken lymphocytes on (a) lipid peroxidation, (b) SOD; (c) GSH; and (d) Catalase activities of *in vitro* enzyme assays. Vertical bars indicate standard deviation. Mean values in columns with different letters show significant difference at the 5% significance level

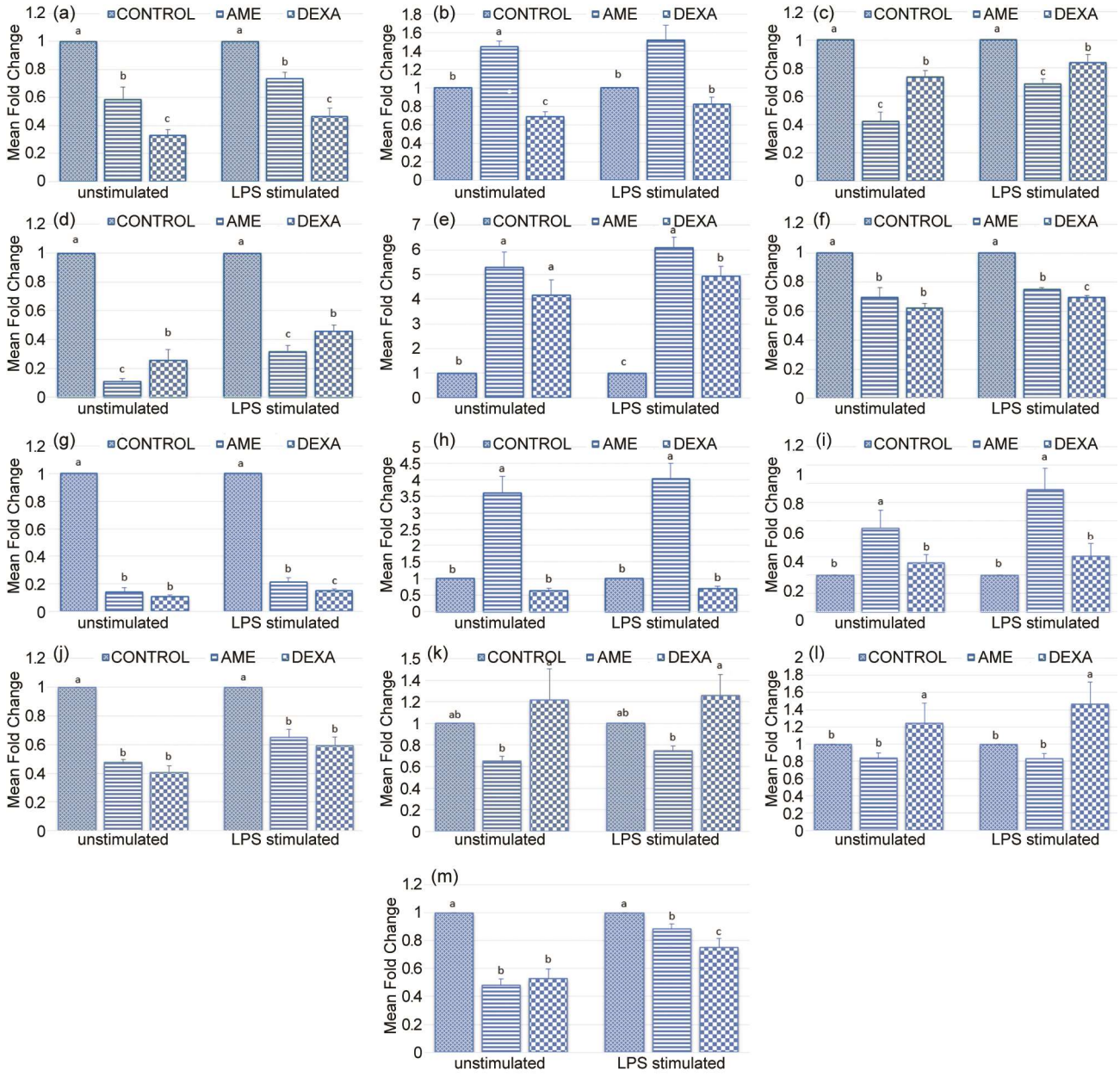


Fig. 7 — Determination of AME and dexamethasone effect on mRNA expression of different cytokine/ transcription factor genes in the chicken splenocytes. (a) IL-1β; (b) IL-4; (c) IL-2; (d) IL-6; (e) IL-10; (f) IFN-γ; (g) iNOS-2; (h) TGF- β; (i) Nrf2; (j) NF-κB; (k) Caspase-3; (l) Caspase-9; and (m) LITAF. Data is presented as mean fold change ± S.D of three independent experiments. Mean values in columns with different letters show significant difference at the 5% significance level

be linked to the anti-inflammatory potential of AME. The expression level of IL-4, IL-10, TGF-β1 and Nrf2 in AME treated chicken lymphocytes was found to be increased significantly in both LPS unstimulated and stimulated chicken lymphocytes with mean fold expression of 1.45, 5.30, 3.59, 2.29 and 1.52, 6.08, 4.04, 3.34, respectively. Although significant reduction in the mRNA expression of IL-1β, IL-2, IL-6, IL-4, iNOS, IFN-γ, LITAF, TGF-β1 and NF-κB1 with substantial increase in the expression of Nrf2, Caspase-3, Caspase-9, IL-10 was observed in dexamethasone treated cells in both LPS unstimulated and stimulated as compared to control as shown in (Fig. 7).

Discussion

Aegle Marmelos has long been recognized as a valuable ayurvedic bio-resource, with its leaves traditionally utilized to address a variety of ailments

such as fever, abdominal pain, urinary issues, heart palpitations, dysentery, dyspepsia, stomach pain, seminal weakness, vomiting, and swellings[30]. Recent scientific research has revealed that *Aegle marmelos* contains a rich array of phytochemicals, with aegeline, aegelenine, aegelinosides, marmelin, marmelosin, anhydromarmeline, marmelide, umbelliferone β-d-galactopyranoside, lupeol, halfordinol, butyl p-tolyl sulfide, 6-methyl-4-chromanone, butylated hydroxyanisole, imperatoin, xanthorrhizol, xanthoarnol, 1-hydroxy-5,7-dimethoxy-2-naphthalene-carboxaldehyde, 1-methyl 2-(3'-methyl-but-2'-enyloxy)-anthraquinone, and other compounds showing significant bioactivity[31-33]. The body's response to oxidative stress involves a disruption in the balance between the generation of reactive oxygen species (ROS) and the capacity of antioxidant enzymes to counteract them, leading to heightened levels of ROS within cells. Antioxidants play a crucial role in averting oxidative damage caused by ROS through inhibiting ROS formation, interrupting ROS attacks, and scavenging reactive metabolites. The present findings demonstrate that the natural antioxidants in AME significantly lower the ROS levels in the splenocytes of chickens. According to a study examining the antioxidants in AME and their potential as a therapeutic for Alzheimer's disease treatment, the ethyl acetate fraction significantly reduced brain lipid peroxidation[34]. Upadhya *et al*. demonstrated that the aqueous extract of *Aegle marmelos* leaves has an anti-diabetic effect. This is confirmed by analysis based on glucose, glutathione-s-transferase, and plasma urea levels, as well as MDA and GSH levels, and checks for erythrocytes in diabetic rats stimulated with alloxan[35]. The significant antioxidant action of flavonoids on human fitness has been recognized. The scavenging or chelating processes demonstrated how the flavonoids work. The large number of hydroxyl groups that phenolics contain may be the cause of their great capacity to scavenge free radicals. Higher polyphenol content in the *Aegle marmelos* aqueous extracts was associated with a higher electron-donating capacity; literature reports on similar findings have also been made. The order in which the reducing power demonstrated was comparable to that of free radical scavenging by DPPH[36]. The ability to scavenge free radicals and antioxidants is demonstrated by the DPPH, reducing power, superoxide anion, hydrogen peroxide anion and LPO assays. According to a study by Lampronti *et al.* extracts from *Aegle marmelos* L. Correa can stop the growth of K562, T-lymphoid Jurkat, B-lymphoid Raji, erythroleukemic Hel, melanoma Colo38, MCF7 breast cancer, and MDA-MB-231 *in vitro*. Additionally, in human cell lines it was discovered that the presence of three derivatives- butyl p-tolyl sulfide, 6-methyl-4-chromanone, and butylated hydroxyanisole strongly inhibited the proliferation of human K562 cells *in vitro*[37].

In light of the above-mentioned context, the present study carried out an *in vitro* investigation to assess the immunomodulatory potential of *Aegle marmelos* on a chicken splenocyte culture system. On exposed cells, AME had a immunosuppressive and antioxidant impact. Using RT-PCR, the underlying molecular mechanism was examined by investigating expression of certain transcription factors and cytokines. Synthetic glucocorticoids like dexamethasone suppress the immune system by preventing the growth and development of lymphocytes and the release of cytokines[38]. Furthermore, Kunicka *et al*. observed that dexamethasone decreases the proliferation of lymphocytes in a dose-dependent way[39]. In the present study, dexamethasone with its MNCD was also utilized in this investigation to assess the expression of cytokines. Inflammation is defined by immune cell infiltration (monocytes, lymphocytes, macrophages, PMN, and plasma cells) into the vascular wall, immune cell invasion into the tissue, and the generation of reactive oxygen species by these cells, which causes tissue damage. Numerous investigations have demonstrated the involvement of several distinct cytokines in inflammatory reactions[40]. During inflammation, macrophages and lymphocytes are the primary producers of cytokines, which are low molecular weight secretory proteins. Activated macrophages produce cytokines, which affect the macrophage's microenvironment and facilitate an efficient immune response that connects innate and adaptive immunity. These proteins regulate many different tasks, such as chemotaxis, tissue healing, and localized to systemic inflammation. Therefore, using dexamethasone and AME to treat LPS-stimulated chicken splenocytes, we assessed pro- and anti-inflammatory cytokines in the current analysis. LPS causes macrophages to secrete a large number of cytokines in significant amounts. The main LPS surface receptor is CD14, which binds to LPS with a high affinity when LPS-binding protein is present.

LPS stimulation of macrophages also triggers a widely reported signalling cascade that involves the activation of the pleiotropic transcription factor NF-κB. According to earlier research, both acute and long-term stress in patients raises the expression of the inflammatory mediators, TNF-α, IL-1β and IL-6, which disrupts neurogenesis in hippocampus cells and may contribute to depression[41]. When the transcriptional factor NF-κB is blocked, pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are reduced, which in turn causes the patients' depressed behaviour to change. According to this mechanism of action, in an acute inflammatory cell culture model system (LPS stimulation) of chicken splenocytes, cytokines generated by genes containing NF-κB responsive elements (TNF-α, IL-1β, and IL-6) were also seen along with a significant AME-mediated suppression of NF-κB. Nitric oxide is produced by the enzyme called inducible nitric oxide synthase (iNOS). Another important inflammatory mediator that sets off the immune system and inflammation is nitric oxide. The generation of proinflammatory cytokines including IL-6 and IL-8 has been linked to a significant release of nitric oxide[42]. In the intrinsic pathway, caspase-9 initiates the caspase cascade, while in the extrinsic pathway, caspase-8 serves a similar role and both paths lead to caspase-3, an irreversible executioner caspase that continues the pre-committed apoptosis cascade[43]. Both of these showed enhanced expression levels in dexamethasone-treated splenocytes and suppressed expression levels in AME-treated splenocytes. An increase in the production of several pro-inflammatory cytokines occurs when oxidative stress activates NF-κB. Moreover, pro-inflammatory oxidative stress results in increased NF-κB activation and cytokine surplus production. Overall, treatment with AME markedly improved the antioxidant status of chicken splenocytes, as evidenced by reduced LPO along with enhanced levels of endogenous antioxidant markers including GSH, SOD, and catalase activity. These biochemical findings were further supported by molecular evidence showing upregulation of Nrf-2, a key regulator of cellular antioxidant defence, and downregulation of iNOS, which is associated with oxidative and nitrosative stress. Collectively, these observations suggest that AME may strengthen cellular antioxidant capacity, maintain redox balance, and protect splenocytes against inflammation-induced oxidative damage.

The Nrf2/ARE system's activation is crucial for breaking this loop. Reports indicate that pro-inflammatory cytokines such as IL-6 and IL-1β do not undergo transcriptional upregulation in the presence of LPS when Nrf2 is activated[44]. According to a recent study using mouse macrophage chromatin immunoprecipitation (ChIP)-seq and ChIP-qPCR data, Nrf2 binds to the promoter regions of pro-inflammatory cytokines including IL-6 and IL-1β and prevents RNA Pol II activation. Consequently, the transcriptional activation of IL-6 and IL-1β, which eventually results in the suppression of gene expression, cannot be processed by RNA Pol II. Ahmed *et al*.[44] demonstrated a novel mechanism through which Nrf2 inhibits the transcriptional activation of certain genes, regardless of the presence of an ARE, by obstructing the recruitment of RNA Pol II, while also transactivating downstream genes *via* AREs[45]. In the current investigation, exposure to dexamethasone and AME significantly increased the levels of mRNA expression of Nrf2 and IL-10 in both LPS-stimulated and unstimulated chicken lymphocytes, suggesting that these agents may have anti-inflammatory properties. The production of cytokines, chemokine-releasing factors, MMPs and other inflammatory agents, such as COX-2 and iNOS, is thus negatively regulated by the Nrf2 signalling pathway. This impacts the NF-κB and MAPK pathways and additional pathways that govern inflammation, either directly or indirectly[44].

Conclusion

Critically analysing the results, it is concluded that AME treatment led to reduced lymphocytes in all three mitogen-stimulated cells as observed through lymphocyte proliferation assay in the present *in vitro* investigation. Furthermore, cells treated with AME had an enhanced antioxidant status as observed through cell-based assays, which may be explained by the increased expression of Nrf2. The down-regulation of NF-κB1 and other pro-inflammatory mediators like LITAF, IL-1β, IL-2, and IL-6 was also a consequence of AME and dexamethasone exposure. The substantial anti-inflammatory properties of *Aegle marmelos* are backed by both conventional usage and contemporary scientific studies. Its bioactive ingredients present encouraging opportunities for the creation of fresh anti-inflammatory therapies. Comprehensive clinical research is necessary, therefore, to verify its safety and usefulness in poultry

and to successfully apply it to the relief of a variety of stressful situations.

Acknowledgement

The authors thank DES and Dean CBSH, G.B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India for support and institutional facilities. Department of Biotechnology, Government of India, for providing Master's thesis research grant to Rigzin Dolma and Amandip Kaur is duly acknowledged that partially supported this study. All the experimental procedures were conducted following approval from the Institutional Animal Ethics Committee, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India (Approval No. IAEC/CBSH/MBGE/406).

Conflict of interest

All authors declare no conflict of interest.

References

1 Hafez HM & Attia YA, Challenges to the poultry industry: current perspectives and strategic future after the COVID-19 outbreak. *Front Vet Sci*, 7 (2020) 516.
2 Chatterjee RN & Rajkumar U, An overview of poultry production in India. *Indian J Anim Health*, 54 (2015) 89.
3 Shroff FM, What is ayurvedic health care and how is it applicable to the modern day. *J Nutr Hum Health*, 1 (2017) 17.
4 Shakya AK, Medicinal plants: Future source of new drugs. *Int J Herb Med*, 4 (2016) 59.
5 Kadiyska T, Tourtourikov I, Dabchev K, Zlatarova A, Stoynev N, Hadjiolova R, Spandidos DA, Adamaki M & Zoumpourlis V, Herbs and plants in immunomodulation. *Int J Funct Nutr*, 4 (2023) 1.
6 Mahanta JD, Borgohain B, Sarma M, Sapcota D & Hussain J, Effect of dietary supplementation of herbal growth promoter on performance of commercial broiler chicken. *Indian J Anim Res*, 51 (2017) 1097.
7 Sekar DK, Kumar G, Karthik L & Rao KB, A review on pharmacological and phytochemical properties of *Aegle marmelos* (L.) Corr. Serr (Rutaceae). *Asian J Plant Sci Res,* 1 (2011) 8.
8 Khan A, Ali NH, Santercole V, Paglietti B, Rubino S, Kazmi SU & Farooqui A, *Camellia sinensis* mediated enhancement of humoral immunity to particulate and non-particulate antigens. *Phytother Res*, 30 (2016) 41.
9 Maity P, Hansda D, Bandyopadhyay U & Mishra DK, Biological activities of crude extracts and chemical constituents of Bael, *Aegle marmelos* (L.) Corr. *Indian J Exp Biol,* 47 (2009) 849.
10 Patel P & Asdaq SM, Immunomodulatory activity of methanolic fruit extract of *Aegle marmelos* in experimental animals. *Saudi Pharm J*, 18 (2010) 161.
11 Baliga MS, Thilakchand KR, Rai MP, Rao S & Venkatesh P, *Aegle marmelos* (L.) Correa (Bael) and its phytochemicals in the treatment and prevention of cancer. *Integr Cancer Ther*, 12 (2013) 187.
12 Asif M, Jenkins KA, Hilton LS, Kimpton WG, Bean AG & Lowenthal JW, Cytokines as adjuvants for avian vaccines. *Immunol Cell Biol*, 82 (2004) 638.
13 Stéphane FF, Jules BK, Batiha GE, Ali I & Bruno LN, Extraction of bioactive compounds from medicinal plants and herbs, in *Natural Medicinal Plants* (Intech Open, London) 2021, 39.
14 Ambwani S, Dolma R, Chand P, Kaur A & Ambwani TK, Phytochemical analysis of *Andrographis paniculata* (Burm.f.) nees leaf extract and *in vitro* assessment of its antioxidant, anti-inflammatory and immunomodulating properties. *Pharmacol Res Nat Prod*, 11 (2026) 100612.
15 Mansour RB, Gargouri B, Bouaziz M, Elloumi N, Belhadj Jilani I, Ghrabi Z & Lassoued S, Antioxidant activity of ethanolic extract of inflorescence of *Ormenis africana in vitro* and in cell cultures. *Lipids Health Dis*, 10 (2011) 1.
16 Atanassova M, Georgieva S & Ivancheva K, Total phenolic and total flavonoid contents, antioxidant capacity and biological contaminants in medicinal herbs. *J Chem Technol Metall*, 46 (2011).
17 Gulcin İ & Alwasel SH, DPPH radical scavenging assay. *Processes*, 11 (2023) 2248.
18 Jagetia GC, Rao SK, Baliga MS & Babu K, The evaluation of nitric oxide scavenging activity of certain herbal formulations *in vitro*: a preliminary study. *Phytother Res*, 18 (2004) 561.
19 Alam MN, Bristi NJ & Rafiquzzaman M, Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharm J*, 21 (2013) 143.
20 Ruch RJ, Cheng SJ & Klaunig JE, Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, 10 (1989) 1003.
21 Prieto P, Pineda M & Aguilar M, Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal Biochem*, 269 (1999) 337.
22 Ambwani S, Dolma R, Sharma R, Kaur A, Singh H, Ruj A & Ambwani TK, Modulation of inflammatory and oxidative stress biomarkers due to dexamethasone exposure in chicken splenocytes. *Vet Immunol Immunopathol*, 262 (2023) 110632.
23 Ambwani S, Kandpal D, Arora S & Ambwani TK, Cytotoxic effects of gold nanoparticles exposure employing *in vitro* animal cell culture system as part of nanobiosafety. *AIP Conf Proc*, 1724 (2016).
24 Creed TJ, Lee RW, Newcomb PV, Di Mambro AJ, Raju M & Dayan CM, The effects of cytokines on suppression of lymphocyte proliferation by dexamethasone. *J Immunol*, 183 (2009) 164.
25 Li Y, Ma QG, Zhao LH, Wei H, Duan GX, Zhang JY & Ji C, Effects of lipoic acid on immune function, the antioxidant defense system, and inflammation-related genes expression of broiler chickens fed aflatoxin contaminated diets. *Int J Mol Sci*, 15 (2014) 5649.
26 Paseban M, Mohebbati R, Niazmand S, Sathyapalan T & Sahebkar A, Comparison of the neuroprotective effects of aspirin, atorvastatin, captopril and metformin in diabetes mellitus. *Biomolecules,* 9 (2019) 118.
27 Ellman GL, Tissue sulfhydryl groups. *Arch Biochem Biophys*, 82 (1959) 70.
28 Aebi HE, Catalase, in *Methods of enzymatic analysis*, (1974) 673.

29 Neubig RR, Spedding M, Kenakin T & Christopoulos A, International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol Rev*, 55 (2004) 597.

30 Manandhar B, Paudel KR, Sharma B & Karki R, Phytochemical profile and pharmacological activity of *Aegle marmelos* Linn. *J Integr Med*, 16 (2018) 153.

31 Baliga MS, Bhat HP, Joseph N & Fazal F, Phytochemistry and medicinal uses of the bael fruit (*Aegle marmelos* Correa): a concise review. *Food Res Int*, 44 (2011) 1768.

32 Dhankhar S, Ruhil S, Balhara M, Dhankhar S & Chhillar A, *Aegle marmelos* (Linn.) Correa: a potential source of phytomedicine. *J Med Plant Res*, 5 (2011) 1497.

33 Rahman S & Parvin R, Therapeutic potential of *Aegle marmelos* (L.) an overview. *Asian Pac J Trop Dis*, 4 (2014) 71.

34 Asaduzzaman M, Uddin MJ, Kader MA, Alam AH, Rahman AA, Rashid M, Kato K, Tanaka T, Takeda M & Sadik G, *In vitro* acetylcholinesterase inhibitory activity and the antioxidant properties of *Aegle marmelos* leaf extract: implications for the treatment of Alzheimer's disease. *Psychogeriatrics*, 14 (2014) 1.

35 Upadhya S, Shanbhag KK, Suneetha G, Naidu BM & Upadhya S, A study of hypoglycemic and antioxidant activity of *Aegle marmelos* in alloxan induced diabetic rats. *Indian J Physiol Pharmacol*, 48 (2004) 476.

36 Raja WW & Khan SH, Estimation of some phytoconstituents and evaluation of antioxidant activity in *Aegle marmelos* leaves extract. *J Pharmacogn Phytochem*, 6 (2017) 37.

37 Lampronti I, Martello D, Bianchi N, Borgatti M, Lambertini E, Piva R, Jabbar S, Choudhuri MS, Khan MT & Gambari R, *In vitro* antiproliferative effects on human tumor cell lines of extracts from the Bangladeshi medicinal plant *Aegle marmelos* Correa. *Phytomedicine*, 10 (2003) 300.

38 Schmidt J, Fleiβner S, Heimann-Weitschat I, Lindstaedt R, Pomberg B, Werner U & Szelenyi I, Effect of corticosteroids, cyclosporin A, and methotrexate on cytokine release from monocytes and T-cell subsets. *Immunopharmacology*, 27 (1994) 173.

39 Kunicka JE, Talle MA, Denhardt GH, Brown M, Prince LA & Goldstein G, Immunosuppression by glucocorticoids: inhibition of production of multiple lymphokines by *in vivo* administration of dexamethasone. *Cell immunol*, 149 (1993) 39.

40 Chuang TY, Cheng AJ, Chen IT, Lan TY, Huang IH, Shiau CW, Hsu CL, Liu YW, Chang ZF, Tseng PH & Kuo JC, Suppression of LPS-induced inflammatory responses by the hydroxyl groups of dexamethasone. *Oncotarget*, 8 (2017) 49735.

41 Liu CH, Zhang GZ, Li B, Li M, Woelfer M, Walter M & Wang L, Role of inflammation in depression relapse. *J Neuroinflammation*, 16 (2019) 1.

42 Singh AP, Singh L, Singh P & Bhatti R, Biological Evaluation of *Aegle marmelos* Fruit Extract and Isolated Aegeline in Alleviating Pain–Depression Dyad: *In Silico* Analysis of Aegeline on MAO-A and iNOS. *ACS Omega*, 6 (2021) 2034.

43 Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S & Reed JC, Regulation of cell death protease caspase-9 by phosphorylation. *Science*, 282 (1998) 1318.

44 Ahmed SM, Luo L, Namani A, Wang XJ & Tang X, Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochim Biophys Acta*, 1863 (2017) 585.

45 Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Hayashi M, Sekine H, Tanaka N, Moriguchi T, Motohashi H, Nakayama K & Yamamoto M, Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat Commun*, 7 (2016) 1.