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# Immunoregulatory effects of *Choerospondias axillaris* (Roxb.) B.L.Burtt & A.W.Hill fruit extract in mice with insights on in vitro mechanism

Ravi Gautam[1,6], Anju Maharjan[1,7], JaeHee Lee[1], SuJeong Yang[1], JiHun Jo[1], Manju Acharya[1,8], DaEun Lee[1], Narayan Prasad Ghimire[2], Saroj Lamichhane[3], ByungSun Min[4], ChangYul Kim[1], HyoungAh Kim[5] and Yong Heo[1*]

## Abstract

**Background** Lapsi (*Choerospondias axillaris*), a plant native to Nepal, has been traditionally used in Asian countries to treat cardiovascular conditions. However, its effects on immune regulatory function remain largely unexplored. This study aimed to in vivo evaluate the immunoregulatory properties of Lapsi fruit extract in mice on immunotoxic responses with analysis on in vitro mechanism for immune suppression, oxidative stress, and inflammatory response. Male Balb/c mice were intragastrically administered various doses of the extract for 21 days. In some mice, immune suppression was induced with cyclophosphamide, and subsequent immune recovery was assessed. In addition, RAW264.7 cells and THP-1-derived macrophages were treated in vitro with lipopolysaccharide and different concentrations of the extract.

**Results** Administration of extract increased the IgG2a/IgG1 ratio while reducing serum IgE and IgG1 level compared with control mice. Tumor necrosis factor (TNF)-α and interleukin (IL)-17 levels were lower in splenic culture supernatants of mice administered extract. Lapsi extract also effectively reversed cyclophosphamide (CP)-induced immunosuppression by enhancing serum levels of IgA and IgG2a, of interferon-γ and interleukin (IL)-4 secreted by splenic T cells, and of IgG1 and IgG2a secreted by B cells, as well as by increasing immune cell counts. In cell cultures, the extract decreased the levels of inflammation markers, including nitric oxide, reactive oxygen species, prostaglandin E2, and pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β). Mechanistic analysis showed that Lapsi extract modulated the NF-κB p65, MAPK, and inflammasome pathways.

**Conclusions** Lapsi extract may act as both an immunostimulatory and anti-inflammatory agent, indicating its potential as a candidate immunomodulatory activity under polyclonal and CP-suppressed conditions; however, further disease-specific studies, along with isolation and characterization of active phytochemicals, are warranted to evaluate its therapeutic applicability.

**Keywords** Lapsi (*Choerospondias axillaris*), Immunomodulation, Immunostimulation, Immunoglobulins, Cytokines, Anti-inflammatory, In vivo

*Correspondence:
Yong Heo
yongheo@cu.ac.kr

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## Background

Functional foods, which have the potential ability to promote health and prevent disease, have garnered significant interest, particularly regarding their roles in reducing the risks of cardiovascular diseases, aging-related problems, cancer, and inflammation [1, 2]. *Choerospondias axillaris* (Roxb.) B.L.Burtt & A.W.Hill, also called Lapsi, is a large, edible fruit tree belonging to the family Anacardiaceae. Although indigenous to Nepal, Lapsi is also found in other Asian countries, including India, China, Japan, Vietnam, and Thailand. Lapsi fruits, which weigh 8–18 g, are oval in shape, with a thin yellow peel (< 1 mm thickness) and a large seed [3]. In Nepal, Lapsi fruits are processed into various edible products, including candies, sweets, and pickles, which are marketed both locally and internationally. Additionally, dried ripe Lapsi fruit has been traditionally used as an ethnic medicine in China, Mongolia, and Nepal for therapeutic application on cardiovascular diseases or neurological problems such as insomnia or restlessness [4–7]. Various investigations on pharmacological activities of Lapsi fruit have been carried to reveal its anti-myocardial pathogenesis, antioxidant activity, anti-inflammation, or antitumor activity [5, 6, 8, 9].

The pharmacological and anti-oxidant effects of Lapsi are mainly attributed to polyphenols. The levels of these compounds are higher in the peel than in the flesh, with the peel having greater anti-oxidant, anti-bacterial, and anti-proliferative effects [8, 10]. Lapsi fruits contain various nutritive components, including amino acids, vitamin C, saccharides, pectin, organic acids, and trace elements. Chemical analysis has shown that Lapsi fruits contain dihydroquercetin, quercetin, protocatechuic acid, gallic acid, 3,3-di-o-methylellagic acid, B-sitosterol, daucosterol, stearic acid, triacontanoic acid, octacosanol, syringaldehyde, vanillic acid and citric acid [8, 11]. Total flavones from Lapsi fruit have been shown to protect rats against adriamycin-mediated myocardial peroxidation injury and to exert an anti-arrhythmic action in rats with aconitine-induced arrhythmia [12].

Although crude drugs in medicinal plant research have often been dismissed as complex mixtures lacking a singular concentrated compound, the therapeutic efficacy of medicinal plants may be due to synergistic interactions among multiple compounds [13–15]. Most previous studies of Lapsi fruit have assessed the effects of isolated components or total flavones on various health-related factors, including cardiac-related disorders, cancer, and oxidative stress [16–18]. Total flavones from Lapsi were also used for evaluating immunological effects in mice [19, 20], where total flavones enhanced thymic development or phagocytic function of mononuclear macrophages. Most recently immunomodulatory effects of Lapsi have been evaluated using isolated polysaccharides or fractionated extracts in several models. It was shown that purified polysaccharide improved immune and intestinal barrier function in cyclophosphamide (CP) treated mice [21]. A study by Labh et al. demonstrated upregulation of few cytokine genes in fish fed with Lapsi extract and a study by Mann et al. reported anti-inflammatory effects of methanolic extract in synoviocytes and collagen-induced arthritis rat model [22, 23].

Considering approaches for evidence-based immunotherapeutic effects of herbal total extract on humoral and cellular immunities [24, 25], the present study, to the best of our knowledge, is the first to demonstrate the humoral and cell-mediated immunomodulatory effects of an ethanolic extract of Lapsi fruit on normal or immunosuppressed mice, and its anti-inflammatory or anti-oxidative stress effects on macrophage cell lines derived from mice and human. This targeted investigation may enhance understanding of the immunomodulatory potential of Lapsi fruit extract.

## Methods

### Preparation of *Choerospondias axillaris* extracts

*Choerospondias axillaris* fruits were collected in Kathmandu, Nepal, and identified by the Department of Plant Resources, Ministry of Forest & Environment, Nepal. After washing the fruits with distilled water, their peels, flesh, and seeds were separated. The dried flesh and peel were ground into powder. Ten grams of powder were extracted twice with 300 ml of 70% ethanol by sonication for 30 min each at room temperature. The combined extracts were filtered through Whatman filter paper, concentrated using a rotary evaporator at 45 °C under vacuum and the extract was freeze-dried in one run, aliquoted and stored at -20 °C in the dark to minimize batch to batch variability. Endotoxin level in the extract (0.01 mg/mL) was measured using the Kinetic-QCL™ LAL assay kit (Lonza, USA) on an Epoch microplate spectrophotometer (BioTek, USA) and resulted in below the detection limit.

### Animals and study design

All animal procedures were approved by the IACUC of Daegu Catholic University (IACUC-2018-015). Specific pathogen-free, 4-week-old male Balb/c mice were obtained from DBL, Korea, and housed in sterile laminar airflow cages under pathogen-free conditions at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 5%, with a 12-hour light-dark cycle and *ad libitum* access to standard rodent food and autoclaved filtered water. Experimental treatments were initiated after one week of acclimatization.

To assess the immunomodulatory activity of Lapsi fruit extract, 24 mice were randomly divided into four groups of six mice each, with one group each intragastrically

administered 5, 50, or 500 mg/kg/day/100 µl of extract, and the fourth group administered vehicle for 21 days (Fig. 1A). To assess the immune-boosting activity of the extract, mice were divided into five groups of 6 ($N = 6$ per group), with one group each intragastrically administered extract, as above; one administered vehicle (2% DMSO and 2% Tween 80 in saline, concentrations deemed non-toxic and well-tolerated by mice) [26]; and one cyclophosphamide (CP) model control group. Vehicle control groups were injected intraperitoneally with 100 µl of saline, whereas the other groups were injected with 100 µl of 100 mg/kg/day CP in saline, on days 1, 7, and 14 to induce immune suppression (Fig. 1B). Body weights were measured weekly, and mice were sacrificed on day 21 using CO2 asphyxiation.

#### Hematology

Blood samples were collected via cardiac puncture into $K_2$EDTA vacutainer tubes (BD Biosciences, USA) and peripheral blood counts were measured using an AVIDA 2120 automatic analyzer (Siemens, Germany).

#### Ex vivo production of cytokines and immunoglobulins by splenic lymphocytes

To evaluate ex vivo cytokine and immunoglobulin production by splenic lymphocytes, spleens were aseptically collected and maintained on ice in 3 ml of RPMI 1640 (Hyclone, USA). Single-cell suspensions were prepared as described [27]. Spleen cells were cultured in RPMI 1640 supplemented with 1 mM sodium pyruvate (Lonza), 1 mM non-essential amino acids (Lonza), 0.075% sodium bicarbonate (Sigma Aldrich, USA), 1% penicillin-streptomycin (Gibco, USA), 50 µM 2-mercaptoethanol (Sigma Aldrich), and 10% heat-inactivated fetal bovine serum (FBS, Hyclone). Splenic T cells were stimulated in vitro with immobilized anti-CD3e monoclonal antibody (5 µg/5 × $10^5$ cells, BD Biosciences) and 5 units of recombinant human IL-2 (Peprotech, USA) for 48 h at 37 °C in a 5% $CO_2$ incubator. Simultaneously, $10^6$ splenic B cells were cultured with lipopolysaccharide (LPS, 1 µg; Sigma), recombinant mouse interleukin (IL)-4 (50 ng; R&D Systems, USA), and recombinant human a proliferation-inducing ligand (APRIL; 10 ng; R&D Systems) for 96 h at 37 °C in a 5% $CO_2$ incubator [28, 29]. Culture supernatants were collected and stored at -80 °C for subsequent analysis.

#### Assays of cytokine and immunoglobulin concentrations in culture supernatants and sera

Concentrations in culture supernatants of the cytokines Interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-4 were measured using OptEIA ELISA kits (BD Biosciences), and IL-17 was measured using DuoSet (R&D Systems, USA). Concentrations of immunoglobulins, including IgE, IgA, IgG1 and IgG2a, were determined by sandwich ELISA. Serum IgE and IgA levels were assessed using anti-mouse IgE and IgA monoclonal antibodies, respectively, as capture antibodies, with biotinylated anti-mouse IgE and IgA, respectively, as detection antibodies (BD Biosciences). IgG1 and IgG2a concentrations in plasma and B-cell culture supernatants were assessed using goat anti-mouse IgG1 and IgG2a (Serotec, UK), respectively, as capture antibodies, and goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma) as detection antibodies [30, 31].

#### Flow cytometry

Splenocyte preparations were subjected to flow cytometry to identify $CD3^+CD4^+$ helper T cells, $CD3^+CD8^+$ cytotoxic T cells, $CD3^-B220^+$ B cells and $CD3^-CD335^+CD49b^+$ NK cells, following established protocols [28, 30]. Briefly, $10^6$ cells were washed with staining buffer and incubated with 1 µg of FcBlock (BD Biosciences) for 30 min to prevent nonspecific binding. Cells were stained with anti-CD3 PE, anti-CD3 FITC, anti-CD4 FITC, anti-CD8 PE, anti-CD45RB/B220 PE, anti-CD335 (NKp46) PE, and anti-CD49b APC (BD Biosciences) antibodies, followed by flow cytometry using a BD Accuri plus flow cytometer (BD Biosciences). Non-specific binding of fluorescent antibodies was assessed by incubating these cells with FITC-, PE-, and APC-conjugated isotype controls.

#### Cell cultures

RAW 264.7 cells, obtained from the Korean Cell Line Bank (Seoul, Korea), were cultured in DMEM (Hyclone) with 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C in a humidified 5% CO2 incubator. THP-1 cells were obtained from the American Type Culture Collection (ATCC, #TIB-202, Manassas, VA, USA) and cultured in RPMI-1640 (Gibco) with 10% heat-inactivated FBS, 0.05 mM 2-mercaptoethanol, and 1% penicillin-streptomycin. Macrophage differentiation was induced in THP-1 cells by treatment with 100 ng/ml phorbol 12-myristate-13-acetate (PMA) for 48 h, yielding THP-1 derived macrophages (TDM). These cells were allowed to rest in PMA-free media for an additional 48 h before extraction and Western blotting analysis.

#### Cell viability assay

RAW 264.7 ($1 \times 10^4$ cells/well) and TDM ($4 \times 10^4$ cells/well) were plated in a 96-well plate (Corning, USA) and incubated for 24 h. The cells were incubated with various concentrations of Lapsi fruit extract for 2 h, followed by stimulation with 1 µg/ml LPS for 24 h. A 10 µl aliquot of CCK-8 reagent (Dojindo Molecular Technology Inc., Japan) was added to each well, and the cells were

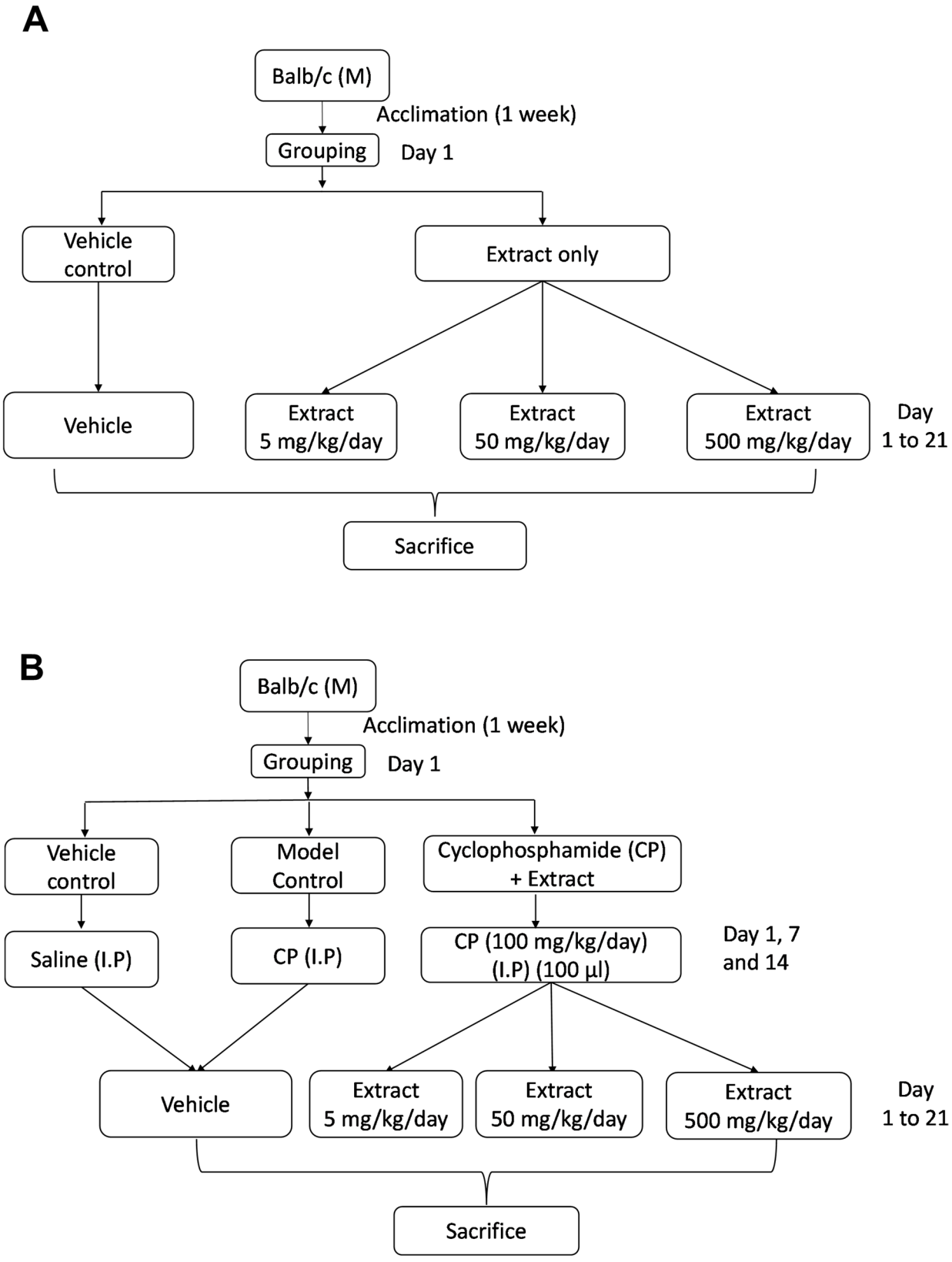



**Fig. 1** Diagrammatic illustration of in vivo study design. (**A**) Study design for immunomodulation study of Lapsi extract in normal mice. (**B**) Study design for immunostimulant study in immunosuppressed mice

incubated for 3 h. Absorbance was measured at 450 nm with a reference wavelength of 650 nm.

#### Nitric oxide (NO) and reactive oxygen species (ROS) assays

RAW 264.7 ($5 \times 10^4$ cells/well) were seeded in 24-well culture plates and incubated for 24 h. The cells were incubated with various concentrations of Lapsi fruit extract for 2 h, followed by incubation with 1 μg/ml LPS for 24 h, and collection of the supernatant. NO concentrations were measured by mixing a 50 μl aliquot of each supernatant with 50 μl of Griess reagent in each well of a 96-well plate, followed by incubation for 15 min at room temperature. NO levels were quantified by measuring the absorbance at 540 nm and comparing it with a standard curve. Intracellular ROS concentrations were determined by treating cells in 5 ml round-bottom polystyrene tubes with 10 μM $H_2$DCF-DA for 20 min at 37 °C and measuring mean fluorescence intensity on a BD Accuri C6 plus flow cytometer with FlowJo v. 10 software (BD Life Sciences).

#### Inflammatory markers in culture supernatants

RAW 264.7 cells and TDM were seeded in a 24-well plate, cultured for 24 h, and treated with various concentrations of extract for 24 h in the presence or absence of LPS (1 μg/ml). The supernatants were collected and the concentrations of inflammatory markers determined. In RAW 264.7 cell supernatants, prostaglandin E2 (PGE2) levels were analyzed using a ParameterTM prostaglandin E2 assay kit (R&D Systems), whereas IL-6, IL-1β, and TNF-α levels were measured using BD OptEIA mouse ELISA kits (BD Biosciences). In TDM supernatants, TNF-α levels were measured using BD OptEIA Human ELISA kits (BD Biosciences), and IL-6 and IL-1β concentrations were measured using human DuoSet ELISA kits (R&D Systems). To measure IL-1β, 3 mM ATP was added to the cell cultures 45 min before supernatant collection.

#### Western blot analysis

RAW 264.7 cells and TDM were treated with various concentrations of extract for 3 h, followed by treatment with LPS. RAW 264.7 cells were harvested at 15 min, 30 min, and 24 h intervals for protein analysis; TDM were collected 3 h after LPS treatment, with ATP added 45 min before collection. Cells were lysed with RIPA buffer (Cell Signaling Technology, USA) containing 1% protease inhibitor cocktail (Sigma), 1 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride (Sigma). Nuclear proteins were extracted using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, USA), according to the manufacturer's protocol. Protein concentrations were measured using BCA protein assay kits (Thermo Scientific). Equal aliquots of proteins were loaded onto 10–12% SDS gels, electrophoresed and transferred to PVDF membranes (Millipore Corporation, USA). After blocking, the membranes were incubated with specific antibodies against β-actin, phospho-p38 (p-p38), phospho-Jun N-terminal kinase (p-JNK) (Santa Cruz Biotechnology Inc., USA), p38, JNK, phospho-extracellular signal-regulated protein kinases (p-ERK)1/2, nuclear NF-κB p65, and phospho-IκBα (p-IκBα, Cell Signaling Technology, USA). Antibody binding was detected using Immobilon Western Chemiluminescent HRP substrate (Millipore, USA) and visualized on a UVITEC Cambridge Alliance Mini 2 M imaging system.

#### Statistical analyses

Data were expressed as mean ± SE or mean ± SD. Depending on data normality, differences among groups were determined by one-way analysis of variance (ANOVA) or the Kruskal-Wallis test by ranks, followed by the Student-Newman-Keuls post hoc method. Differences between two groups were analyzed by Student's t-tests or distribution-free non-parametric tests, depending on data distribution. All statistical analyses were performed using Sigma Plot ver 14 (Systat Software, USA), with p-values $< 0.05$ considered statistically significant.

## Results

#### Effects of Lapsi extract on mouse body weight and splenic and thymic indices

Mouse body weights were not significantly affected by the various concentrations of Lapsi extract, or by the injection of CP. Injection of CP was found to significantly increase the splenic index (spleen weight, mg/body weight, g) compared with control mice, but this index in CP-treated mice was reduced following injection of Lapsi extract. Conversely, CP significantly reduced the thymic index (thymus weight mg/body weight, g) compared with control mice. However, Lapsi extract tended to dose-dependently increase this index, reaching significance at the maximum concentration (500 mg/kg/day; Fig. 2).

#### Effects of the extract on immunoglobulin levels

Administration of Lapsi extract to normal mice resulted in a dose-dependent reduction in IgG1 levels, with the highest concentration of the extract (500 mg/kg/day) significantly reducing both IgG1 and IgE levels, while having little effect on IgG2a levels. At all concentrations, this extract increased the IgG2a/IgG1 ratio. Additionally, the extract tended to dose-dependently increase the concentrations of IgA (Fig. 3).

CP injection was found to induce significant reductions in IgG1, IgE, and IgA levels, while having little effect on IgG2a levels. The extract did not affect IgE and IgG1 levels in CP-treated mice, and their levels remained markedly lower than those in control mice. Conversely, the levels of IgG2a and IgA were significantly higher in mice

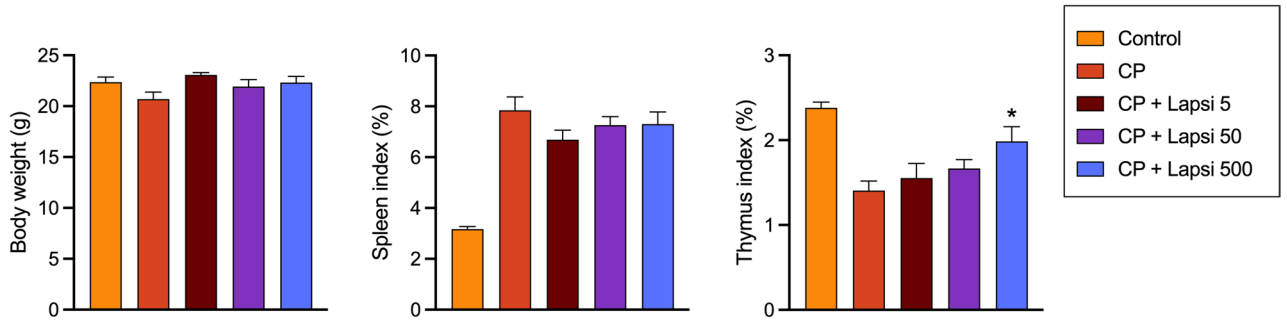



**Fig. 2** Effect of Lapsi extract and cyclophosphamide (CP) on splenic and thymic index and body weight of mice. Data are expressed as mean ± SE ($N = 6$). $^{*}p < 0.05$, $^{***}p < 0.001$ vs. CP group. The indices were calculated by dividing the weight of the spleen and thymus (mg) by total body weight (g)

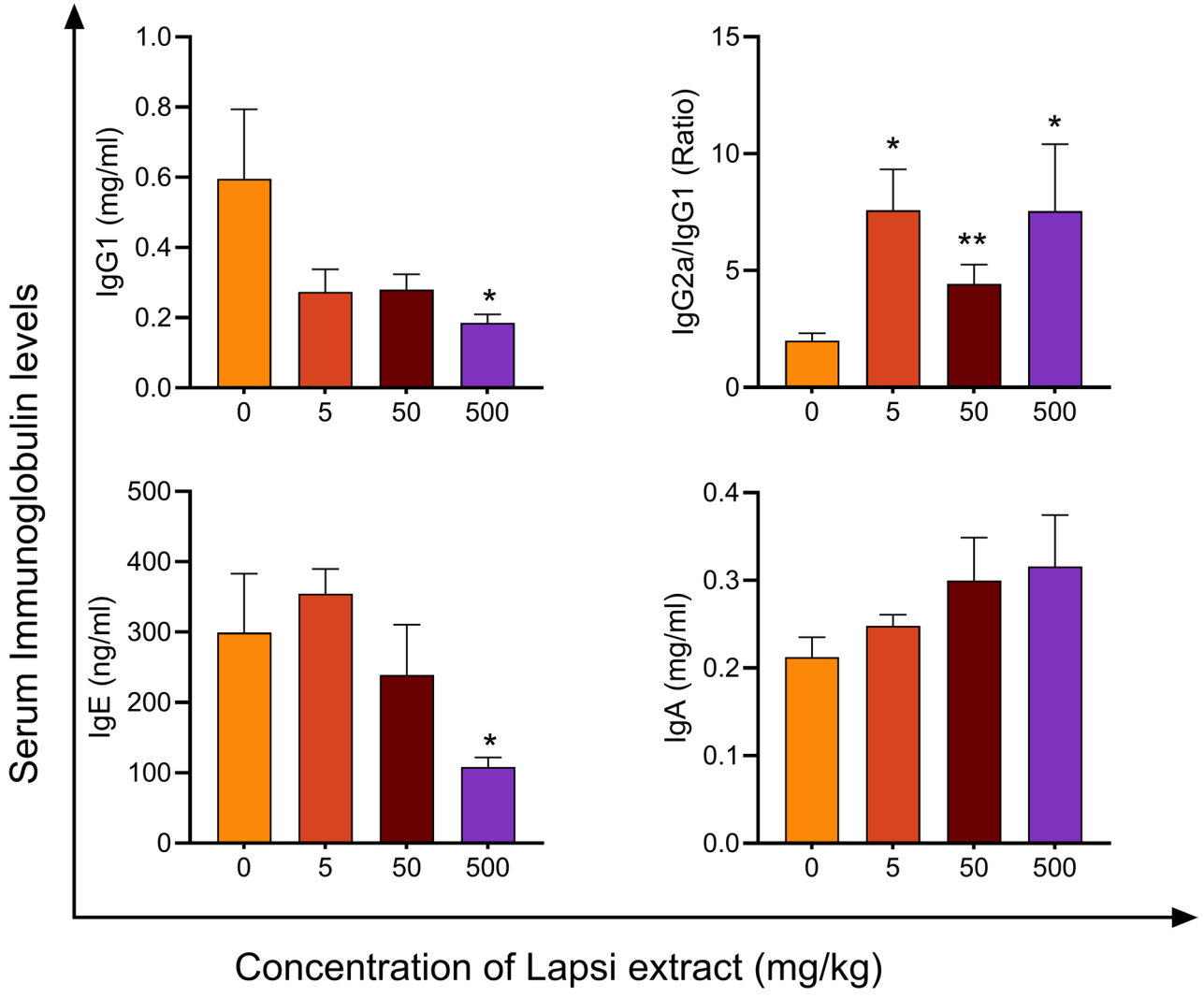



**Fig. 3** Effect of Lapsi extract on serum levels of IgG subtypes (IgG1 and IgG2a), IgA and IgE. Immunoglobulins were analyzed in serum collected from the heart blood of mice. Data are expressed as mean ± SE ($N = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. non-treated group

treated with the extract than in mice treated with CP. The elevated IgG2a/IgG1 ratio observed in CP-injected mice was further increased by treatment with the extract, particularly at the lowest extract dose (5 mg/kg/day), whereas the higher dose showed no additional effect (Fig. 4A). Similarly, B-cell cultures derived from CP-injected mice exhibited diminished production of both IgG1 and IgG2, indicating that CP has an immunosuppressive effect on

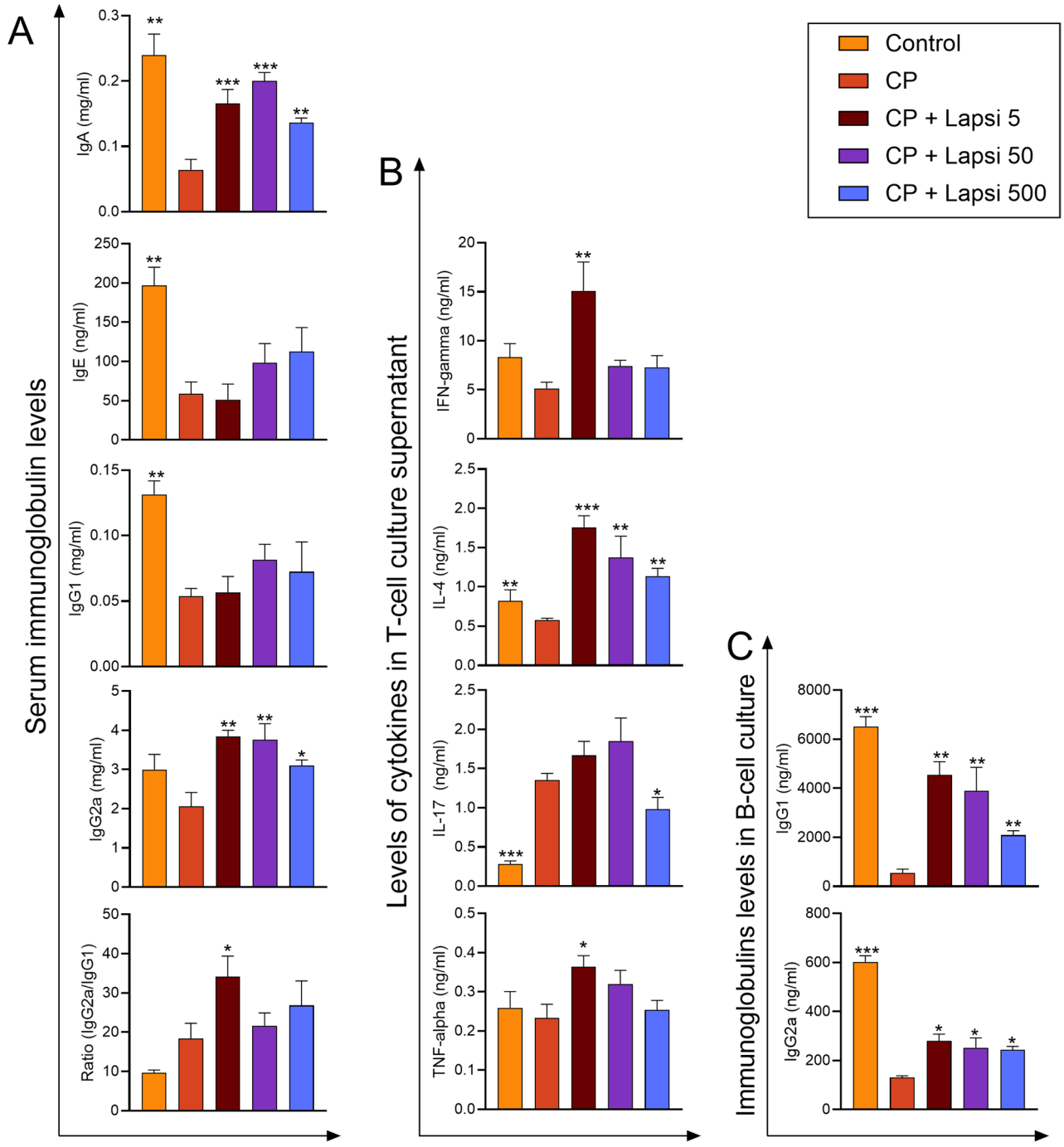



**Fig. 4** Lapsi reverses the immunosuppressive effect of cyclophosphamide. (**A**) serum immunoglobulins, (**B**) cytokine levels in splenic T-cell culture supernatant, and (**C**) immunoglobulins in B-cell culture of splenocytes. Data are expressed as mean ± SE ($N = 6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. immunosuppressed and nontreated mice (CP)

B cells as well. However, administration of the extract to CP-injected mice resulted in an upregulation of both IgG1 and IgG2a with maximal enhancement at the lowest extract dose (Fig. 4C).

### Effect of the extract on cytokine production by splenic T cells

In normal mice, low and intermediate doses of the extract slightly reduced IFN-γ, IL-17 levels, and the IFN-γ/IL-4 ratio. These doses also significantly decreased TNF-α levels. Additionally, the highest extract concentration (500 mg/kg/day) markedly reduced IL-17 levels (Table 1).

**Table 1** Concentrations of cytokines produced by splenic T-cell cultures and proportions of immune cells in the spleens of mice following treatment with Lapsi fruit extract

| Variables | Concentrations of Lapsi extract (mg/kg/day) | | | |
|---|---|---|---|---|
| | 0 | 5 | 50 | 500 |
| **Cytokines** | | | | |
| IFN-γ (pg/ml) | 4238 ± 1580 | 2763 ± 1690 | 3771 ± 2657 | 7702 ± 4412 |
| IL-4 (pg/ml) | 1138 ± 252 | 1043 ± 475 | 1265 ± 335 | 826 ± 265 |
| Ratio (IFN-γ/IL-4) | 3.8 ± 1.5 | 2.6 ± 0.9 | 3.0 ± 2.2 | 10.1 ± 7.2 |
| TNF-α (pg/ml) | 202 ± 28 | 100 ± 14** | 97 ± 37* | 186 ± 91 |
| IL-17 (pg/ml) | 1019 ± 507 | 886 ± 179 | 889 ± 382 | 686 ± 355* |
| **Immune cells** | | | | |
| $CD3^+$cells (%) | 60.6 ± 10.1 | 53.4 ± 3.0 | 52.9 ± 2.3 | 57.5 ± 10.8 |
| $CD4^+$ T cells (%) | 30.8 ± 7.1 | 30.7 ± 2.5 | 29.1 ± 3.6 | 27.0 ± 3.6 |
| $CD8^+$ T cells (%) | 20.3 ± 6.6 | 16.4 ± 3.0 | 15.1 ± 2.0 | 20.0 ± 5.2 |
| B cells (%) | 22.8 ± 8.3 | 30.0 ± 4.0 | 29.7 ± 2.0 | 27.5 ± 8.7 |
| NK cells (%) | 5.4 ± 0.5 | 4.4 ± 1.5 | 4.6 ± 2.4 | 4.8 ± 0.8 |

Data are expressed as mean ± SD ($N = 6$). *$p < 0.05$, ** $p < 0.01$ significant difference compared to non- treated group. Supernatant collected from splenic T-cells culture with anti-CD3e mAb and Human IL-2 for 48 h was used for cytokine estimation and splenocytes were stained with different Abs to obtained percentage of immune cells in lymphocytes

**Table 2** Hematological parameters and flow cytometric determinations of splenic cells of mice treated with cyclophosphamide (CP) and Lapsi fruit extract

| Immune cell phenotype | Concentrations of Lapsi extract (mg/kg/day) | | | | |
|---|---|---|---|---|---|
| | 0 | CP + 0 | CP + 5 | CP + 50 | CP + 500 |
| RBC ($10^6$/ µL) | 9.7 ± 0.64* | 7.8 ± 1.2 | 9.0 ± 0.4 | 9.1 ± 0.6 | 9.0 ± 0.4 |
| HGB (g/dL) | 15.5 ± 1.3 | 11.5 ± 3.9 | 11.7 ± 6.0 | 14.0 ± 1.0 | 13.9 ± 0.6 |
| Neutrophils (%) | 20.2 ± 2.9*** | 71.6 ± 5.0 | 56.7 ± 4.9*** | 61.9 ± 10.6*** | 60.1 ± 13.0** |
| Lymphocytes (%) | 76.6 ± 10.2*** | 19.6 ± 4.1 | 32.8 ± 4.1*** | 29.9 ± 9.5*** | 31.3 ± 10.2*** |
| $CD3^+$ cells (%) | 77.7 ± 5.3*** | 29.4 ± 4.7 | 43.6 ± 7.5*** | 45.8 ± 5.6*** | 44.8 ± 6.1*** |
| $CD4^+$ cells (%) | 42.9 ± 4.1*** | 17.3 ± 3.1 | 25.4 ± 4.6** | 25.6 ± 3.9** | 25.4 ± 3.2** |
| $CD8^+$ cells (%) | 22.4 ± 6.0*** | 6.94 ± 1.7 | 11.7 ± 2.2*** | 12.6 ± 1.6** | 13.1 ± 3.6*** |
| B cells (%) | 13.7 ± 3.0*** | 6.3 ± 1.5 | 11.9 ± 2.0*** | 10.0 ± 2.2** | 11.3 ± 1.0*** |
| NK cells (%) | 4.7 ± 1.4* | 3.1 ± 0.9 | 6.3 ± 0.5** | 5.7 ± 1.8** | 6.7 ± 1.7*** |

Data are expressed as mean ± SD ($N = 6$). * $p < 0.05$, ** $p < 0.01$, ** $p < 0.001$ significant difference compared to CP injected and non-treated group (CP + 0). RBC, red blood cells; HGB, hemoglobin. RBC, HGB, neutrophils and lymphocytes were estimated in blood, and $CD3^+$ cells, $CD4^+$ cells, $CD8^+$ cells, B-cells and NK cells were estimated from splenocytes after staining with Abs

CP significantly reduced IFN-γ and IL-4 levels, increased IL-17 levels, but had little effect on TNF-α levels. At concentrations of 5 and 50 mg/kg/day, the extract significantly increased IFN-γ and IL-4 levels compared with the control group, with all concentrations of extract maintaining higher cytokine levels than CP (Fig. 4B). The ratios between IFN-γ and IL-4 were not significantly different among all groups (Supplementary Fig. 1). The heightened IL-17 levels were significantly mitigated by the highest extract concentration (500 mg/kg/day) (Fig. 4B). Furthermore, at its lowest dose (5 mg/kg/day), the extract significantly increased TNF-α levels compared with the CP group, whereas other concentrations of extract had little or no effect on TNF-α levels (Fig. 4B). Considering the maximal production of IFN-γ, IL-4, and TNF-α from activated T cells at the lowest extract dose group, the horrmetic effect of Lapsi extract administration to CP-immunosuppressed mice seems apparent since the most upregulation of these cytokines was observed at its lowest concentration.

### Effects of the extract on hematological parameters and immune cells

Administration of Lapsi extract to normal mice did not significantly affect their hematological indices (data not shown). By contrast, CP administration led to significant reductions in the levels of red blood cells, hemoglobin, and lymphocytes, accompanied by an increase in the percentage of neutrophils. These effects were significantly reversed by the administration of Lapsi extract (Table 2).

Similarly, in normal control mice, Lapsi did not have pronounced effects on splenic immune cells, whereas CP reduced the levels of $CD3^+$, $CD4^+$, $CD8^+$, and B cells. These effects were reversed by the administration of Lapsi extract. Additionally, CP reduced the level of NK cells, whereas the Lapsi extract increased NK cells significantly higher compared to CP treated (Table 2). These

findings suggest that Lapsi extract may have a potential immunostimulatory effect, mitigating the CP-induced alterations in hematological parameters and immune cells.

### Viability of LPS-stimulated macrophages

At concentrations of 250 µg/ml and 500 µg/ml, the extract exhibited cytotoxic effects against Raw 264.7 cells and TDM. At a lower concentration of 125 µg/ml, the extract maintained the viability of both Raw 264.7 cells (almost 100%) and TDM (approximately 75%) (Fig. 5A). The extract concentration that consistently maintained ≥ 75% viability in both cell lines was selected for the assessment of soluble pro-inflammatory markers in culture supernatants.

### Extract-mediated inhibition of NO, PGE2, and intracellular ROS production

To investigate the effects of the extract on LPS-induced inflammatory responses, its ability to regulate NO production in response to LPS stimulation was examined. LPS-stimulated cells produced significantly higher levels of NO, and the extract reduced the NO levels in a dose-dependent manner (Fig. 5B). Additionally, LPS-induced elevation of PGE2, a crucial eicosanoid lipid mediator implicated in the pathogenesis of inflammatory diseases [32], was significantly and dose-dependently attenuated by the extract (Fig. 5B). LPS also markedly increased intracellular ROS generation and this increase was effectively attenuated by the extract (Fig. 5B).

### Extract induced suppression of pro-inflammatory cytokines

LPS treatment of RAW 264.7 and TDM cells resulted in notable increases in the production of pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β. The extract dose-dependently suppressed the production of IL-6 and IL-1β by LPS-treated macrophages. Although the extract did not dose-dependently inhibit TNF-α production, the extract significantly reduced the levels of TNF-α in LPS-treated cells (Fig. 6).

### Mechanism associated with the anti-inflammatory activity of Lapsi extract

Mitogen-activated protein kinases (MAPKs), including c-JNK, ERK1/2 and p38 MAPK, are thought to play

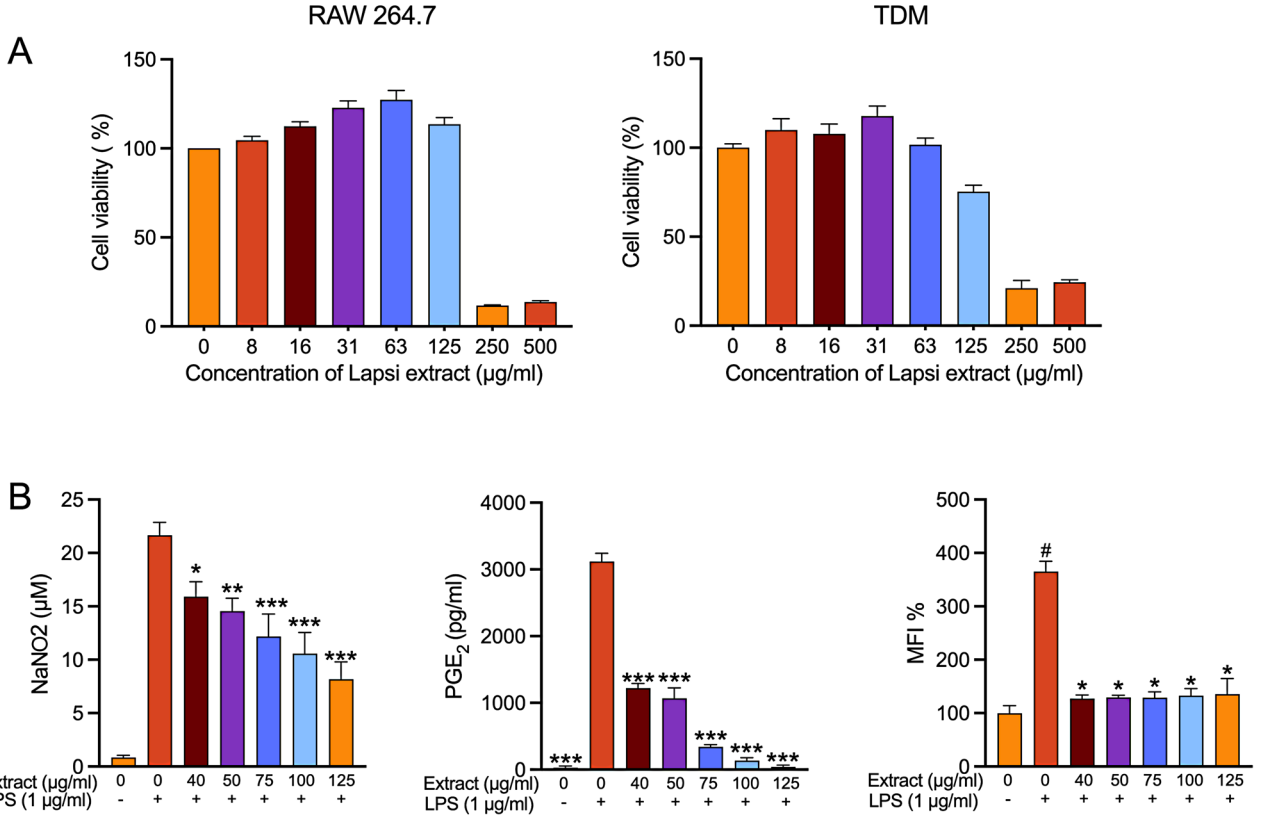



**Fig. 5** Effect of Lapsi extract on the cell viability and LPS-induced production of nitric oxide (NO), prostaglandin $E_2$ ($PGE_2$) and intracellular reactive oxygen species (ROS). (**A**) RAW 264.7 and THP-1 derived macrophage (TDM) were incubated with extract and LPS for 24 h and viability was determined. (**B**) Level of NO, $PGE_2$ and intracellular ROS were quantified in RAW 264.7 cells following Lapsi extract and LPS treatment. Data are represented as mean ± SD of three independent experiments. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ vs. LPS control (no extract but LPS treated)

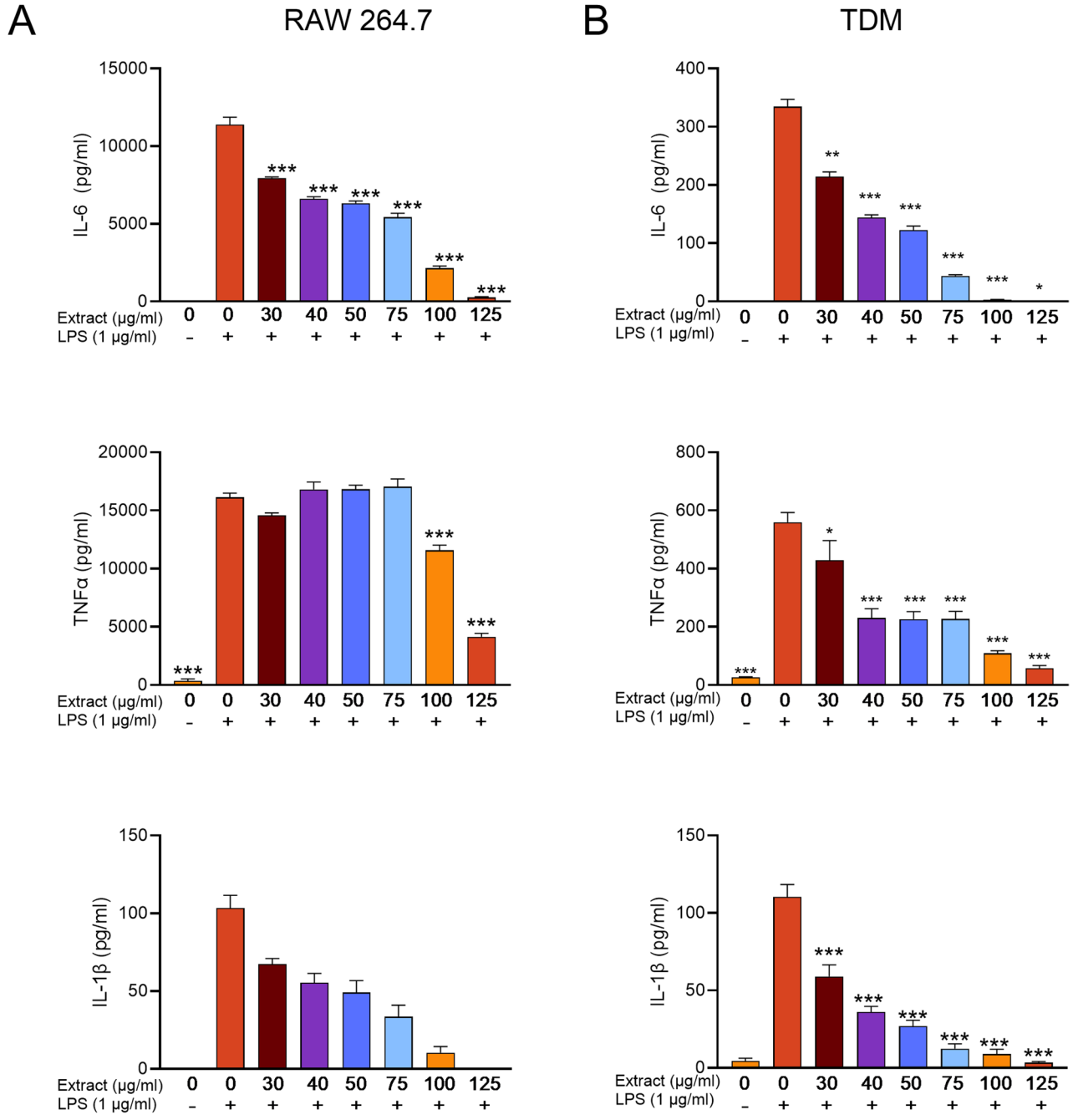



**Fig. 6** Effect of Lapsi extract on LPS induced pro-inflammatory cytokines IL-6, TNFα and IL-1β in (**A**) RAW 264.7 and (**B**) TDM. The values are presented as mean ± SE of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. $^{***}p < 0.001$ vs. LPS control (no extract but LPS treated)

a major role in regulating inflammatory mediators. LPS stimulation activated phosphorylation of p38 (2.4 fold), ERK (4.4 fold) and JNK (2.2 fold), while extract treatment suppressed their phosphorylation to 1.6, 3.3 and 1.3 fold (Fig. 7A). Similarly, the ability of the extract to modulate the NF-κB pathway was assessed by examining the level of NF-κB p65. LPS stimulation of RAW 264.7 cells induced approximately a 2.1-fold increase in NF-κB nuclear translocation compared with control cells, whereas the extract markedly reduced this translocation to 0.2 fold (Fig. 7C). This reduced nuclear translocation of NF-κB may result from the ability of the extract to decrease the phosphorylation of IκBα, which increased 3.1 fold after LPS stimulation but was lowered to 2.2 fold with extract treatment (Fig. 7B). Moreover, stimulation of TDM with LPS and ATP resulted in NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) upregulation to 1.2 fold, which was inhibited by the

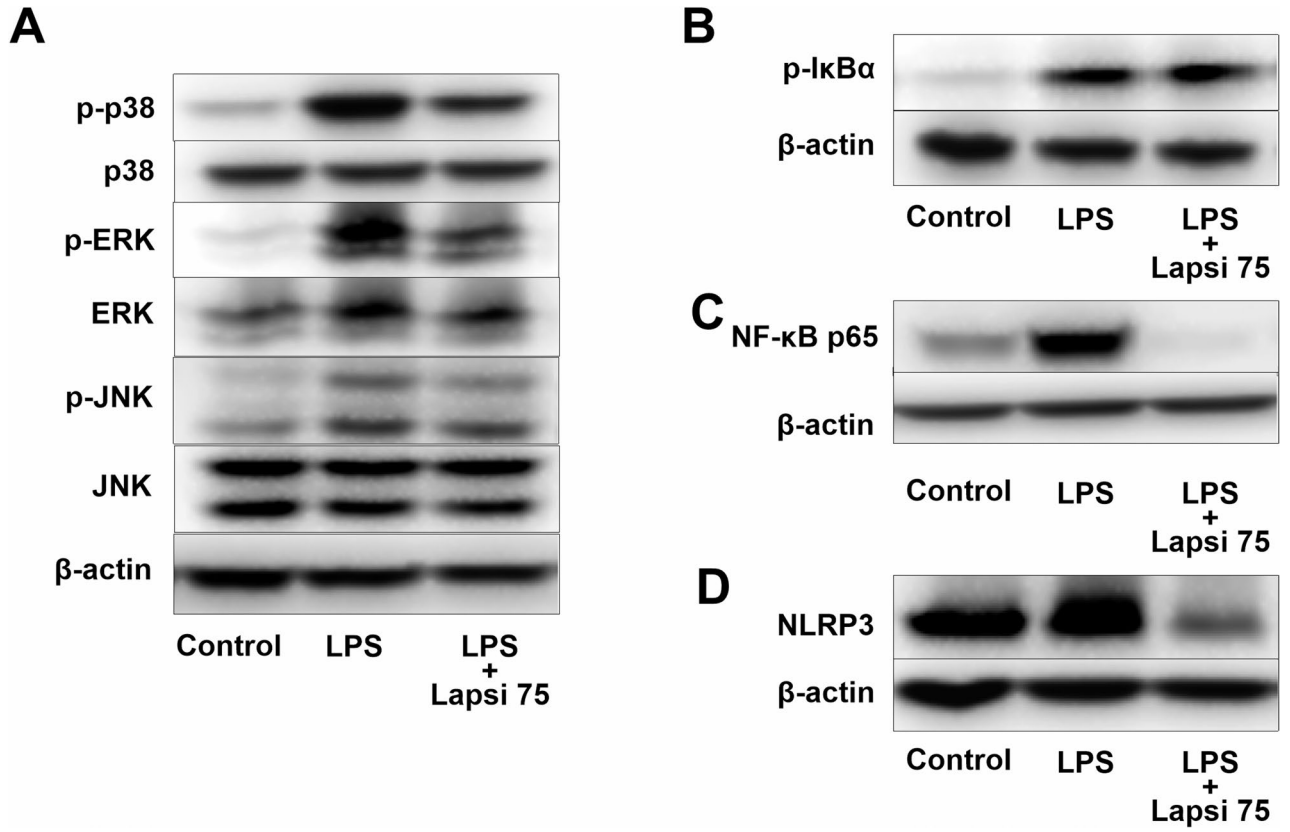



**Fig. 7** Effect of extract on the expression of (**A**) MAPK phosphorylation, (**B**) p-IκBα, and (**C**) nuclear NF-κB (P65) in LPS stimulated RAW 264.7 cells and on the NLRP3 inflammasome in LPS and ATP activated TDM (**D**). Protein expression was analyzed by western blot assay, with results normalized using β-actin or non-phosphorylated counter proteins of MAPK. The blots are representative of two independent experiments

extract to 0.7 fold by extract (Fig. 7D). Taken together, these results suggest that the anti-inflammatory activity of Lapsi extract is mediated through the inhibition of NF-κB, MAPKs, and the inflammasome-related events.

## Discussion

Medicinal plants with immunoregulatory properties may substitute for conventional drugs in the treatment of many diseases, particularly when host defense mechanisms have to be activated under immunosuppressed conditions or when selective immunosuppression is required in situations such as autoimmune disorders. Although medicinal plants have been regarded as "crude drugs", as they are dilute mixtures of hundreds of compounds without a single concentrated entity, their efficacy may be due to the synergistic effects of several compounds [13–15]. In this study, we evaluated the immunomodulatory and immunostimulant effects of ethanolic extract of *Choerosopsondias axillaris* fruit in vivo using normal and immunosuppressed mice models and screened for potential anti-inflammatory activities in vitro macrophage cell lines.

The immunostimulatory properties of plant-derived products are often evaluated using CP-induced immunosuppression models in vivo [33]. CP typically induces a decrease in thymus weight and an increase in spleen weight, serving as a biomarker of its immunotoxic effects. Consistent with these findings, CP treatment in our study elevated the splenic index and decreased the thymic index. However, co-administration of Lapsi extract notably counteracted these changes, particularly at the highest tested dose (500 mg/kg/day), which significantly restored the thymic index. These findings align with previous reports demonstrating that total flavones from *Choerospondias axillaris* leaves can enhance thymic development in mice [20].

Hematological assessments showed that CP-induced reductions in RBC, HGB, and lymphocyte counts, along with an increase in neutrophils, were effectively restored by Lapsi extract treatment. CP was found to induce similar alterations in Wistar rats, including increased neutrophil counts in bronchoalveolar lavage fluid and decreased RBC, HGB, and lymphocytes [34, 35]. The ability of Lapsi extract to upregulate RBC and HGB levels may be attributed to the anti-hypoxic properties of flavonoids found in *C. axillaris* [36], and the ability of the extract to elevate blood lymphocyte counts suggests that this extract may act as an immunostimulant in peripheral immune cells.

T cells are divided into CD8 + cytotoxic T cells, which target cancerous and virus-infected cells, and CD4 + helper T cells, which regulate immune responses via cytokine secretion [27, 30]. NK cells, key players in innate immunity, also target tumor and infected cells. In the present study, Lapsi extract alone did not significantly alter the proportions of immune cell subsets in the spleen or thymus under normal conditions. In contrast, CP-treated mice showed significant reductions in B cells, T cells and NK cell populations, consistent with prior findings on CP-induced immunosuppression [37]. Co-administration Lapsi extract effectively restored these immune cell population, suggesting a protective effect against CP-induced immune cell depletion.

Proliferating T cells differentiate into type-1 (Th1) and type-2 (Th2) helper T cell subtypes, with Th1 responses protecting against cancer, viral, and intracellular infections, and Th2 responses guarding against autoimmune diseases and extracellular pathogens. Th2 activity is also linked to allergic conditions such as asthma and respiratory allergies [38, 39]. The IFN-γ/IL-4 ratio is widely used as an indicator of Th1 versus Th2 dominance, with a higher ratio suggesting Th1 polarization. In this study, administration of the highest dose of Lapsi extract increased the IFN-γ/IL-4 ratio, likely due to a selective reduction in IL-4 levels, indicating a shift toward Th1-type immunity. In contrast, lower and intermediate doses reduced the ratio due to reduction in level of IFN-γ. CP treatment reduced both IFN-γ and IL-4 levels, consistent with previous findings [40], and increased IL-17, reflecting activation of Th17 cells, a phenomenon also observed in cancer-associated inflammation [41]. In CP treated mice, Lapsi extract lower dose upregulated both IFN-γ and IL-4 levels relative to CP group whereas higher doses had minimal effect on IFN-γ, but IL-4 level was upregulated compared to CP. Despite the elevated IL-4, there was a dose dependent reduction in IL-4 levels when compared to the lower dose group. This trend suggests a complex concentration-dependent immunoregulatory effect of the extract. Notably, the IFN-γ/IL-4 ratio remained largely unchanged across doses in CP-treated mice, suggesting overall Th1/Th2 balance in all groups of treatment. Lapsi extract also attenuated the CP-induced rise in IL-17, particularly at highest dose, suggesting potential protective effects against Th-17 mediated immune dysregulation or inflammatory responses. Regarding TNF-α, the extract decreased its level in normal mice at low and intermediate dose, indicating potential anti-inflammatory activity. In CP-treated mice, TNF-α levels were not altered by CP injection alone; however, the lowest concentration of the extract significantly upregulated TNF-α levels, while the intermediate and highest concentrations showed no such effect. In a previous study, polysaccharides extracted from *Choerospondias axillaris* administered at 300 mg/kg/day for 12 days in CP-treated mice led to upregulation of IFN-γ and TNF-α, indicating an immunostimulatory effect [21]. Together, these findings suggest that Lapsi exerts the immunoregulatory effects implicated with restoration of suppressed immune parameters while restraining excessive inflammation, thereby underscoring its homeostatic immunomodulating potential.

Administration of the extract to mice also had immunomodulating effects on immunoglobulins. Lapsi extract promoted a Th1 immune response, as shown by decreases in IgG1 levels and resulting increases in IgG2a/IgG1 ratios. Skewness towards Th1 or Th2 responses is commonly assessed by comparing the relative levels of IgG1 and IgG2a [42, 43]. In addition, the extract gradually reduced IgE concentrations, an allergic marker linked to IL-4-mediated isotype switching. This anti-allergic effect may be attributed to the presence of polyphenols and anthocyanidins in the extract, in agreement with previous findings that a mixture of these compounds decreased IgE and IgG1 levels in mice with DNCB-induced atopic dermatitis [44]. The extract also dose-dependently increased levels of IgA, demonstrating its effect on a broader range of immunoglobulins. CP reduced serum immunoglobulin levels, whereas the extract had a minimal effect on serum IgG1 and IgE. Interestingly, in CP treated mice, IgG2a/IgG1 ratio was most elevated at the lowest dose of extract and other concentrations did not significantly regulate the ratio compared to CP and control. This pattern is similar to that observed for IFN-γ in T-cell culture supernatants. The extract also restored the CP-induced decrease in IgA levels. CP also reduced the release of IgG1 and IgG2a by splenic B cells, an effect restored by Lapsi extract. Notably, the lowest dose of Lapsi extract induced the highest upregulation of IgG1, whereas the highest dose resulted in the least upregulation. This pattern may indicate that Lapsi extract stimulates the immune system but also has mechanisms to limit excessive activation, helping to maintain immune balance. In this way, it might prevent overstimulation of Th2-mediated responses, such as allergies.

Lapsi extract did not always exhibit a linear or dose-dependent response, with maximal effects sometimes observed at the lowest dose of 5 mg/kg/day in CP-immunosuppressed mice, especially for serum IgG2/IgG1 ratio, levels of IgG1 and IgG2a in B cell culture supernatants, and production of IFN-γ, IL-4, and TNF-α from activated T cells. This likely reflects the complex composition of the extract, where multiple bioactive constituents may act synergistically at lower concentrations, but antagonistic interactions or receptor saturation at higher doses could diminish efficacy. Pharmacokinetic factors, including absorption, distribution, metabolism, and clearance,

as well as in vivo regulatory mechanisms, feedback loops, and immune modulation pathways, may further contribute to these non-linear effects. Such biphasic or hormetic responses have been documented for other plant extracts or natural bioactive compounds such as curcumin, resveratol, or linoleic acid [45, 46]. These findings highlight the complexity of interpreting dose–response relationships in multi-component botanical preparations and underscore the need for careful dose optimization in future studies.

We observed that the extract downregulated IFN-γ, TNF-α, and IL-17 levels in vivo in normal mice, while in CP-treated mice, higher extract concentrations either had no effect or showed downregulating trends on these cytokines. Based on these in vivo cytokine effects and previous reports of the extract's anti-inflammatory properties, we were interested in further exploring its effects on macrophages using two different models: RAW 264.7 cells derived from mice and THP-1-derived macrophages from humans. Macrophages are key effector cells of the innate immune system that rapidly respond to pathogens by recognizing molecules such as LPS through Toll-like receptor 4 (TLR4). LPS treatment activates macrophages, triggering inflammatory signaling pathways and the release of pro-inflammatory cytokines and mediators essential for the innate immune response [47, 48]. Prolonged dysregulation of these mediators, including ROS, NO, and iNOS, can lead to chronic inflammation, which may contribute to the development of various diseases, including cancers [49, 50]. ROS acts as signaling molecules, influencing cellular activities such as cytokine secretion and cell proliferation. At higher concentrations, however, ROS can cause cellular injury and death. Additionally, iNOS contribute to ROS induction [50, 51]. Elevated production of NO due to increased iNOS levels has been associated with the pathogenesis of inflammation, septic shock, and carcinogenesis [52]. In the present study, Lapsi extract reduced the levels of LPS-induced NO, PGE2, and intracellular ROS.

The LPS-stimulated activation of critical signaling components in the present study, including IκBα, NF-κB, p-38, JNK, and ERK, was in agreement with established pathways associated with inflammatory responses [53]. Lapsi extract was able to affect these pathways, as shown by its ability to diminish IκBα phosphorylation, impede NF-κB translocation to the nucleus, and modulate the phosphorylation levels of pivotal molecules, such as p38, JNK, and ERK. This multi-faceted intervention by Lapsi extract resulted in concentration-dependent reductions in IL-6 and IL-1β levels induced by LPS in RAW 264.7 cells, along with the attenuation of TNF-α, particularly at higher extract concentrations. Even at lower concentrations, Lapsi extract reduced the levels of LPS-induced cytokines, such as IL-6, TNF-α, and IL-1β, in TDM. This dual impact of Lapsi extract on pro-inflammatory mediators across both murine and human macrophages underscores its promising potential as a botanical anti-inflammatory agent.

The present study also evaluated the effects of Lapsi extract on the activation of NLRP3 inflammasomes in macrophages. Inflammasomes in macrophages can be activated by acute LPS stimulation, followed by the addition of ATP [54], resulting in the cleavage of pro-IL-1β by caspase-1 and the release of mature IL-1β [55]. The present study found that THP-1 derived macrophages treated with LPS followed by ATP exhibited elevated levels of NLRP3 protein. However, administration of Lapsi extract markedly reduced the levels of NLRP3 in cells exposed to both LPS and ATP. This finding provides further evidence for the potential ability of Lapsi extract to inhibit the NLRP3 inflammasome complex, suggesting that this extract can counteract inflammasome-mediated inflammation.

The anti-inflammatory properties of Lapsi extract may be due to its constituents, as individual compounds were shown to have distinct anti-inflammatory activities. For example, quercetin was found to inhibit NLRP3 interaction, dihydroquercetin to suppress ROS and NLRP3 assembly, procyanidins to inhibit ROS, and catechin to hinder NLRP3 interaction, thus reversing the inflammation process [56–59]. Similarly, several flavonoids were found to reduce the expression of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, in RAW 264.7 cells. Flavonoid activity was also associated with polyphenol-mediated inhibition of NF-κB, AP-1, and MAPK activity [60, 61]. The total flavones of Lapsi extract were also found to prevent chronic myocardial injury in rats by reducing p-IκBα expression and blood TNF-α and IL-6 levels [62]. Similarly, the total flavones of *C. axillaris* were found to counteract ischemia/reperfusion-induced myocardium impairment through anti-oxidative and anti-apoptotic activities, modulating effects through the MAPK signaling pathway [18]. These results from our in vitro assays support and complement previous studies reporting the anti-inflammatory effects of Lapsi extract. While these findings reinforce the extract's potential, further investigations using disease-relevant experimental models are necessary to comprehensively evaluate its therapeutic applicability in inflammation-mediated conditions. Moreover, it is essential to isolate and characterize the active phytoconstituents responsible for the observed bioactivity in order to enable targeted application and provide mechanistic insight into their role in immunomodulation and anti-inflammatory effects.

## Conclusions

The ethanolic extract of *Choerospondias axillaris* (Lapsi) exhibited broad immunomodulatory, immunostimulatory, and anti-inflammatory effects. In vivo and ex vivo studies revealed that this extract promoted Th1 immune responses as evidenced by decreased IgG1 and IgE linked to Th2 response and increased IgG2a/IgG1 ratio. It also reduced inflammatory cytokines such as TNF-α and IL-17, suggesting a potential to limit basal inflammation and allergens associated response. In cyclophosphamide induced immunosuppressed mice, Lapsi restored suppressed cytokines and immunoglobulin level without increasing TNF-α, IgE and IgG1, this selective restoration underscores its role as an immune homeostatic agent, stimulating suppressed immunity without provoking excessive inflammation. These in vivo results reflect the interplay between innate and adaptive responses in a systemic setting whereas our in vitro assay using LPS stimulated macrophages cover primarily the acute innate immune response, where Lapsi suppressed key molecules of inflammatory pathways such as NF-κB, MAPKs, IκBα phosphorylation, NLRP3 and reduced other inflammatory mediators and proinflammatory cytokines.

Lapsi extract exhibited immunostimulatory effects in CP-treated mice and anti-inflammatory effects in LPS-activated macrophages, highlighting its potential as a candidate immunomodulatory activity under both polyclonal activation and CP-induced immunosuppressed conditions. This dual activity may benefit conditions involving immune dysregulation; however, its broad action could limit specificity in therapeutic applications. Therefore, prior to therapeutic applications of Lapsi extract, isolation and detailed characterization of the active phytoconstituents are essential for developing targeted therapeutic applications with improved specificity and translational promise. Further investigation utilizing disease-specific and inflammation-specific animal models such as chronic or persistent inflammation animal model related with tissue or organ damage, e.g. rheumatoid arthritis mouse model or murine model for immunosuppression in sepsis [63–65] is warranted to more precisely evaluate the therapeutic potential of Lapsi extract.

### Abbreviations

| | |
|---|---|
| ANOVA | One-way analysis of variance |
| CP | cyclophosphamide |
| ERK | Extracellular signal-regulated protein |
| HGB | Hemoglobin |
| IFN-γ | Interferon-gamma |
| IL- | Interleukin- |
| JNK | Jun N-terminal kinase |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| NK | Natural killer |
| NO | Nitric oxide |
| NLRP3 | NOD-like receptor family pyrin domain-containing protein 3 |
| PGE2 | Prostaglandin E2 |
| PMA | Phorbol 12-myristate-13-acetate |
| RBC | Red blood cell |
| ROS | Reactive oxygen species |
| TDM | THP-1 derived macrophage |
| Th1 | Type-1 helper T cell |
| Th2 | Type-2 helper T cell |
| TNF-α | Tumor necrosis factor-alpha |
| TLR | Toll-like receptor |

## Supplementary Information

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Supplementary Material 1

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Not applicable.

### Author contributions

Conceptualization: Gautam R, Kim CY, Kim HA, Heo Y; Investigation: Gautam R, Maharjan A, Lee JH, Yang SJ, Jo JH, Acharya M, Lee DE; Methodology: Gautam R, Min BS, Kim HA; Resources: Gautam R, Ghimire N, Lamichhane S; Writing-original draft: Gautam R, Maharjan A, Heo Y; Writing-review and editing: Gautam R, Kim CY, Kim HA, Heo Y; Data curation: Maharjan A; Project administration: Ghimire N, Kim CY, Heo Y; Supervision: Lamichhane S, Kim HA, Heo Y; Funding acquisition: Kim HA, Heo Y.

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### Data availability

The data that supports the findings of this study are available from corresponding author, Dr. Yong Heo, upon reasonable request under permission of the DragonImmuno Inc., and the Industry Academic Cooperation Foundation, Daegu Catholic University, Republic of Korea.

## Declarations

### Competing interests

All authors declared no potential conflicts of interest to this article's research, authorship, and/or publication.

### Author details

[1]Department of Health and Safety and GLP Center, Daegu Catholic University, Gyeongsan-Si, Gyeongbuk Province 38430, Republic of Korea
[2]Central Department of Botany, Tribhuvan University, Kathmandu, Nepal
[3]Prakritik Herbaceuticals Pvt Ltd., Thapathali, Kathmandu, Nepal
[4]College of Pharmacy, Daegu Catholic University, Gyeongsan-Si, Gyeongbuk Province 38430, Republic of Korea
[5]Department of Preventive Medicine, College of Medicine, The Catholic University of Korea, Seoul 06591, Republic of Korea
[6]Present address: Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA
[7]Present address: Department of Microbiology & Immunology, Thomas Jefferson University, Philadelphia, PA 19107, USA
[8]Present address: Gene Regulation Section, Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD 20892-4264, USA

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