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ORIGINAL ARTICLE

The therapeutic immunomodulatory benefits of natural medications against hepatic inflammation by modulating the immune system and inhibiting pro-inflammatory cytokines

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ABSTRACT

Background: Phytomedicines act as immunomodulators, enhancing protective immune responses and reducing liver inflammation through immune and anti-inflammatory mechanisms. **Aim:** This study assessed the therapeutic immune modulation effects of herbal treatments from silymarin (Slm), curcumin (Cur), amygdalin (Amg), and ginger (Gng) on liver inflammation induced by carbon tetrachloride (CCL4) in mice. **Methods:** Mice underwent liver inflammation via CCL4 injections (0.5 mg/kg) three times a week for 8 weeks. Twenty-four hours post each CCL4 treatment, mice in group 1 received saline, while mice in groups 2 to 5 were treated with herbal extracts: Slm (112 mg/kg), Cur (522 mg/kg), Amg (64 mg/kg), and Gng (115 mg/kg) thrice/week for 8 weeks. Immune and biochemical responses were evaluated through flow cytometric analysis of $CD4^+$ T, $CD8^+$ T lymphocytes, natural killer (NK) cells, and splenocyte necrosis rates, and ELISA assay for pro-inflammatory molecules C-reactive protein (CRP) and interleukin-6 (IL-6), alongside leukocyte counts and renal and hepatic function analyses. **Results:** The results revealed that in CCL4-treated mice, the expression rate of $CD4^+$ and CD8+ T lymphocytes, as well as NK cells, was significantly higher after treatment with Slm, Cur, Amg, or Gng; while IL-6, CRP levels, and splenocyte necrosis rates were markedly decreased as compared to the control CCL4 group. Biochemical assessments showed nearly normalized ALT and urea levels similar to control mice post-herbal extract treatments. **Conclusion:** Herbal medications from Slm, Cur, Amg, and Gng effectively regulated immunity and inhibited liver inflammation through immune and anti-inflammatory mechanisms. Phytomedicines target immunological mechanisms, potentially impacting hepatic inflammation and demonstrating promise in clinical applications.

Keywords: Cytokines, Herbal therapy, Ehrlich tumor, Immunity, Inflammation, Phytomedicines

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INTRODUCTION

The liver plays a crucial role in sustaining life, which includes the detoxification of harmful agents. Additionally, it performs complex immunological functions that are associated with its interaction with antigens that reach the liver through the bloodstream (Kubes and Jenne, 2018). The liver is essential for immune surveillance and regulatory functions in addressing challenges such as pathogens or tissue damage, while the mechanisms that mitigate inflammation are crucial for maintaining liver homeostasis (Singh et al., 2024) .

The onset of most liver injuries caused by chemicals starts with the metabolic conversion of substances into reactive intermediate species, such as electrophilic compounds or free radicals. The factors contributing to chemical-induced liver injury (CILI) may originate from the direct toxicity of the chemicals themselves or their metabolites, as well as from immune-mediated pathways. While these factors exhibit distinct attributes, they can also be linked. For example, an inflammatory response that occurs subsequently can intensify the initial damage to hepatocytes inflicted by direct chemical toxicity (Lammert et al., 2020).

The use of conventional and synthetic medications for treating liver diseases has sparked a lot of discussion, mainly because these treatments often do not work very well and can cause serious side effects. This situation highlights the importance of looking into traditional and herbal remedies that could offer effective treatment options for liver disorders (Munakarmi et al., 2021). There is growing interest in using phytochemicals, which act as antioxidants to reduce oxidative stress and inflammation. In the field of environmental toxicology, plant-based natural products have been shown to neutralize harmful free radicals by boosting the body's levels of antioxidant enzymes and anti-inflammatory substances (Dwivedi et al., 2022)

Liver tissue damage caused by inflammation in the liver can be influenced by proinflammatory cytokines such as interleukin-6 (IL-6) and C-reactive protein

(CRP) (Koyama and Brenner, 2017). These cytokines can cause Kupffer cells to become active, which in turn leads to the production of different inflammatory substances and free radicals (Decker, 1990). The activation of immune cells, especially Kupffer cells, is an important part of starting liver inflammation. Interleukin-6 (IL-6) and C-reactive protein (CRP) are essential in activating the innate immune system (Pestka and Zhou, 2002). When exposed to external risk factors, tissues trigger the innate immune response and increase the levels of IL-6 and CRP. After they are activated, these factors continue to support and enhance immune responses. Herbal extracts, such as those derived from Silymarin (Slm), curcumin (Cur), Amygdalin (Amg), and Ginger (Gng), have been shown to provide antioxidant and anti-inflammatory benefits. These extracts also help reduce the susceptibility of tissues to damage caused by lipid and protein oxidation. In addition, they improve the function of blood vessel walls, which in turn helps to adjust the body's balance of oxidation and reduction reactions, especially in situations where there is inflammation. This effect is connected to their ability to reduce the levels of harmful inflammatory substances while increasing the production of substances that help reduce inflammation, such as IL-6 (Koyama and Brenner, 2017).

Herbal extracts derived from Slm, Cur, Amg, and Gng are widely recognized for their ability to strengthen the immune system, especially in cases of liver-related illnesses. Extracts made using methanol from these plants are often promoted as natural ways to boost immunity and treat liver conditions caused by chemicals. The effectiveness of these natural substances, including Slm, Cur, Amg, and Gng, has been widely highlighted, with flavonoids showing promise in this regard (Zhang et al., 2023).

The polyphenolic substances found in most herbal extracts have notable anti-inflammatory, antioxidant, and immune-regulating effects. Recent studies have highlighted the unique ability of Slm, Cur, Amg, and Gng to influence immune responses in liver inflammation caused by chemicals, making these compounds important areas of study in immunology (Nemeth et al., 2009). Herbal treatments using Slm, due to their various molecular effects, act as effective regulators of the liver's immune system, which is closely connected to metabolic processes, detoxification, and immune surveillance.

Maintaining this balance is essential for liver health, as an overactive immune response can cause damage to liver cells and speed up the progression of liver diseases (Lin et al., 2011). In addition to their role in regulating the immune system, these herbal treatments are also strong antioxidants, as they actively remove harmful free radicals and reduce oxidative damage that could weaken the structure and function of the liver and its immune system (Janeway et al., 1992) .

The use of herbal treatments such as Slm, Cur, Amg, and Gng in cases of liver inflammation caused by chemicals can help restore the levels of CD4+ T lymphocytes, CD8+ T lymphocytes, B lymphocytes, natural killer (NK) cells, and dendritic cells (DCs). This restoration helps bring back the normal function of Th1-secreted cytokines. Studies have shown that these herbal treatments can prevent the loss of central and memory T cells, which is important for the immune system to properly monitor and respond to threats (Lin et al., 2011).

Many herbal medicines are considered to influence the immune system by reducing the activation of nuclear factor kappa B (NF-κB). This reduction leads to lower production of IL-6, which is a type of cytokine that causes inflammation, and increases the production of IL-10, a cytokine that helps reduce inflammation. This overall effect helps control and balance the immune response (Abenavoli et al., 2011). Herb-based drugs could play a vital role in safeguarding the liver by employing various mechanisms, such as inhibiting the NF-κB pathway, lowering the levels of the proinflammatory cytokine IL-6, adjusting the immune response, and managing the activity of T lymphocytes (Liao et al., 2024). Herbal treatment influences both adaptive and innate immune cells by modifying the secretion of pro-inflammatory and anti-inflammatory cytokines, thereby affecting a range of immune cells, including mast cells, monocytes, macrophages, and neutrophils, as well as CD4+ T lymphocytes, CD8+ T lymphocytes, and NK cells (Nisar et al., 2023; Qiu et al., 2025) .

Phytomedicines can address chemically induced hepatic inflammation by regulating the immune system and offering antioxidant and anti-inflammatory advantages. They accomplish this by inhibiting pro-inflammatory pathways such as NF-κB, reducing the synthesis of cytokines (for instance, CRP, IL-6), promoting the anti-inflammatory pathway, and stimulating antioxidant defenses via mechanisms like nuclear factor erythroid 2-related factor 2 (Nrf2). Notable examples of beneficial plants include Slm and Cur, which play a significant role in the traditional treatment of liver inflammation through their modulation of the immune system (Achary et al., 2025; Laka et al., 2022). Consequently, we aimed to investigate the immunomodulatory and immunotherapeutic properties of herbal treatments

derived from Slm, Cur, Amg, and Gng in experimental mice with chemically induced hepatic inflammation.

Materials and Methods

Reagents and antibodies

CCL4 sourced from Invitrogen (California, USA). Complete RPMI-1640 media were supplied by Invitrogen (California, USA). RBC lysis buffer was provided by Invitrogen (California, USA). Tetrazolium MTT was bought from ThermoFisher Scientific (California, USA). Propidium iodide (PI) cell viability dye was purchased from eBioscience (San Diego, USA). Monoclonal antibodies involving anti-annexin V antibody (clone #: C13), anti-mouse NK antibody (clone #: PK136), anti-mouse CD8 antibody (clone #: YTS169.4), and anti-mouse CD4 antibody (clone #: GK1.5) (Invitrogen, California, USA) were used to determine the phenotypic expression of different immune cells .

Mice

A total of 60 male Swiss albino mice, aged 6-8 weeks and weighing 30±2.0g, were obtained from the animal house at the National Research Centre (Dokki, Giza, Egypt). This study was conducted in full compliance with the guidelines in the Guide for the Use and Care of Laboratory Animals delivered by the National Institutes of Health. The protocols received approval from the ethics committee for Animal Use and Care, Science Faculty, Tanta University (Ethical approval #: Rec-sci-Tu-Q-036) .

Preparation of herb extraction

The herbal materials derived from Slm, Cur, Amg, and Gng were subjected to air drying in the shade for a duration of 4 days, after which they were ground firmly into a fine powder. A total of 100 g of powdered material from each herb was extracted using ethanol (95%) over a period of 72 hours, with continuous shaking at a rate of 200 rpm. The resultant extracts were sieved through Whatman qualitative filter paper (Sigma-Aldrich, MO, USA). The extracted yields were concentrated with a rotary evaporator at a temperature of 50°C, followed by lyophilization to produce the final extracts. The production of individual extract (w/w) was assessed, and the lyophilized extracts were kept at -80°C until required for subsequent experiments.

In vitro MTT cytotoxicity assay

An in vitro cytotoxicity test was conducted on splenocytes using herbal extracts of Slm, Cur, Amg, and Gng through the MTT assay. In summary, cultured splenocytes were placed into 96-well plates with RPMI-1640 medium at a concentration of 2×10^4 cells per well and allowed to incubate overnight at 37°C in a 5% CO2 incubator. Next, the herbal extracts from Slm, Cur, Amg, and Gng were dispensed to the prospective wells (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, and 800 µg/ml). Following an additional incubation period of either 24 or 48 hours, 50 µL of MTT solution (2 mg/mL) was added to each well, and the mixture was allowed to incubate for 4 hours. Subsequently, the medium was discarded, and 150 µL of dimethyl sulfoxide was introduced to each well, followed by a 20-minute incubation. The OD was then measured at 570 nm .

The IC50 values for the herbal extracts against splenocytes were determined using GraphPad Prism software (San Diego, CA, USA), yielding results of 1.18 µg/ml for Slm, 1.87 µg/ml for Cur, 0.27 µg/ml for Amg, and 1.26 µg/ml for Gng, respectively (Figure 1, Table 1). According to the IC_{50} values, the LD_{50} values were calculated at 112 mg/kg, 522 mg/kg, 64 mg/kg, and 115 mg/kg for Slm, Cur, Amg, and Gng, respectively, according to the methods of Halle (2003) (Table 1). Considering that the average weight of the experimental mice utilized in this study is estimated to be 30±2 g, we selected 1/5 of the LD_{50} value as the sublethal dose for each herbal extract. Consequently, the designated intraperitoneal (IP) doses were calculated to be 0.7 mg/mouse, 3.2 mg/mouse, 0.4 mg/mouse, and 0.7 mg/mouse, as well as 1 mg/mouse for Slm, Cur, Amg, and Gng, respectively.

Induction of liver inflammation

To induce liver inflammation, CCL4, dissolved in olive oil in a ratio of 1:3 (v/v), was given by intraperitoneal (IP) injection in a dose of 0.5 mg/kg of mice thrice weekly for eight consecutive weeks (Mahmoodzadeh et al., 2017).

Experimental design and treatment

To study the outcome of herbal treatment on immunological and biochemical changes caused by CCL4-induced hepatic inflammation in male Swiss albino mice, a total of 60 mice were divided into six groups, 10 mice each. Group 1: Mice were IP inoculated with olive oil three times/week for eight weeks (naïve group). Group 2: mice IP inoculated with CCL4 thrice/week for 8 weeks, followed by administration of PBS (CCL4 group). Group 3: Mice were IP administered with CCL4 thrice/week for eight weeks; and 24 h following each CCL4 injection, the mice were IP injected with Slm (112 mg/kg) thrice/week for 8 weeks (Slm group). Group 4: Mice were IP inoculated with CCL4 thrice/week for 8 weeks; and 24 h following each CCL4 injection, the mice were IP administered with Cur (522 mg/kg) thrice/week for 8 weeks (Cur group). Group 5: Mice were IP treated with CCL4 thrice/week for 8 weeks;

and 24 h following each CCL4 injection, the mice were IP injected with Amg (64 mg/kg) thrice/week for 8 weeks (Amg group). Group 6: Mice were IP inoculated with CCL4 thrice/week for 8 weeks; 24 h following each CCL4 injection, the mice were IP administered with Gng (115 mg/kg) thrice/week for 8 weeks (Gng group).

Serum preparation

Before the euthanasia of the mice, blood samples were collected from the retro-orbital plexus using plastic test tubes. These samples were left to stand for three hours to ensure that the blood had fully clotted. Once clotted, the blood samples were centrifuged (10 min, 4°C, 3000 rpm). Following this process, the clear serum was carefully removed and stored at a temperature of -80°C for future immunological and biochemical testing.

Leucocyte analysis

Mice were mildly ether-anesthetized, and blood was drawn from the retro-orbital plexus using heparin-coated microhematocrit tubes. The collected blood samples were analyzed using an automated hematology analyzer (model MEK-6318K, Japan) to evaluate blood parameters, including leucocytes (WBC) and their differential percentages (neutrophils, lymphocytes, basophils, and monocytes).

Splenocyte suspension preparation and counting

Splenocytes were isolated by dissociating spleens and thymuses on 60-µm cell strainers to eradicate clumps and debris. The cell suspension underwent centrifugation (1500 rpm, 5 mins, 4°C), after which the supernatants were removed. Lysing of RBC in splenocytes was carried out with RBC lysing buffer (5 mL RBC lysis buffer/spleen or thymus).

Subsequently, splenocyte suspensions were allowed to incubate at room temperature (RT) for a duration of 5 minutes, with shaking. The lysis was stopped by diluting the buffer with 15 mL of PBS. The cells were spun for 5 min (400 ×g, 4°C). Next, splenocytes were washed and diluted in PBS. The count of splenocytes and thymocytes was assessed using a Trypan blue dye exclusion assay using a hemocytometer.

Flow cytometry

Fresh single-cell suspensions of splenocytes (1.0×10^6) were incubated for 30 min on ice in 2% bovine serum albumin (BSA) and 0.02% sodium azide-containing PBS with the fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs), or with a control irrelevant isotype-matched mAb.The monoclonal antibodies used were anti-mouse CD4 monoclonal antibody, anti-mouse CD8 monoclonal antibody, and anti-mouse NK monoclonal antibody. The cells that were stained underwent washing with PBS and were subsequently fixed using 2% paraformaldehyde (500 ml) in PBS. Next, the cells were maintained on ice, protected from light, until the flow cytometry analysis was performed. The detection and analysis of CD4+ T, CD8+ T, and NK subsets was performed using a Partec flow cytometer (SysmexPartec Company, Germany) and FlowJo software (Treestar, Ashland, OR, USA) .

Assessment of necrosis by flow cytometry

Splenocytes obtained from experimental mice were washed using ice-cold PBS, the cell density was determined, and the cells were resuspended in 1X annexin-binding buffer to achieve a final density of 1 × 106 cells/ml. Cell suspension was transferred into 1.5-ml Eppendorf tubes, after which 5 µL of annexin V-fluorescein isothiocyanate (FITC) and 1 µL of propidium iodide (PI) working solution, at a concentration of 100 µg/ml, were added.

After incubation, 400 µL of 1X annexin-binding buffer was introduced and mixed gently, and the cells were then analyzed using a Partec flow cytometer (SysmexPartec Company, Germany). The phenotypic analysis of the samples obtained was conducted using FlowJo data analysis software (FlowJo, California, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-6 and CRP in the serum of experimental mice were assessed utilizing ELISA. Mouse IL-6 Quantikine ELISA Kit (Cat #: PM6000B) and Mouse C-Reactive Protein/CRP Quantikine ELISA Kit (Cat #: MCRP00) (Biotech, Minneapolis, USA) were used for IL-6 and CRP, respectively, as per the manufacturer's instructions. All steps involving incubation were carried out at room temperature. The OD was specified at 470 nm.

Analysis of liver and kidney functions

The serum levels of alanine aminotransferase (ALT) and urea were calculated calorimetrically via ALT Activity Assay Kit (Cat #: MAK571) and Urea Assay Kit (Cat #: MAK006) (Sigma-Aldrich, USA) following the manufacturer’s instructions .

Statistical analysis

The results were deemed as mean±SE. The statistical analysis was carried out using GraphPad Prism version 7.00 (La Jolla, CA, USA) and the SPSS software package (version 16, IBM, SA). Group comparisons were performed using one-way analysis of variance (ANOVA), with Tukey's post-hoc test, Dunnett's multiple comparison test, and Student's t-test as

appropriate. The difference was deemed statistically significant at $P < 0.05$.

Results

The anti-proliferative effects of herbal medication on splenocyte counts

The current results indicated that the in vitro IC_{50} values of tested herbal extracts were investigated at 1.18 µg/ml, 1.87 µg/ml, 0.27 µg/ml, and 1.26 µg/ml for Slm, Cur, Amg, and Gng, respectively (Figure 1, Table 1). The in vivo LD_{50} values were evaluated from the IC50 values to be 112 mg/kg, 522 mg/kg, 64 mg/kg, and 115 mg/kg for Slm, Cur, Amg, and Gng, respectively (Figure 1, Table 1).

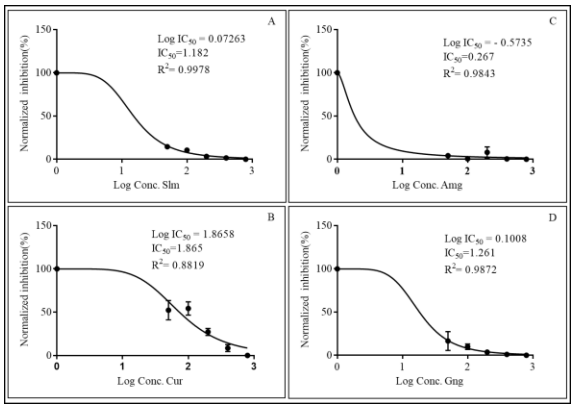

Figure 1. In vitro cytotoxic and anti-proliferative activity of Slm (A), Cur (B), Amg (C), and Gng (D) against splenocytes after 72 h of incubation using MTT assay. Splenocytes were cultured in standard conditions and treated with different concentrations of Slm, Cur, Amg, and Gng.

Table 1. In vitro IC_{50} and in vivo LD_{50} values of Slm, Cur, Amy, and Gng against splenocytes.

IC_{50} and LD_{50} values	Herb extracts			
	Slm	Cur	Amg	Gng
In vitro IC_{50} values (µg/ml)	1.18	1.87	0.27	1.26
In vivo LD_{50} values (mg/kg)	112	522	64	115

Splenocytes were cultured in standard conditions and treated with different concentrations of Slm, Cur, Amg, and Gng (50, 100, 200, and 800 µg/ml). The log (inhibition)-curve fitting model was used to calculate the IC_{50} values.

Potentials of herbal medication on the total counts of splenocytes

The data obtained here revealed a significant decrease in spherocyte counts in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, respectively, as compared to naïve mice that received olive oil (Figure 2). Compared to CCL4-treated mice post-treated with PBS, splenocyte counts showed a significant decrease in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-bearing mice post-treated with Amg, and CCL4-treated mice post-treated with Amg (Figure 2).

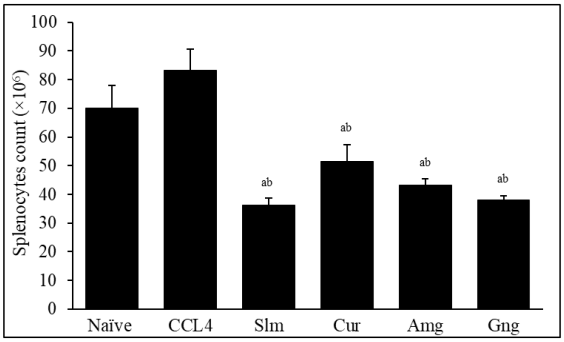

Figure 2. Potentials of extracts of Slm, Cur, Amg, and Gng on the splenocyte count in mice with chemically induced hepatic inflammation. Values were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. [a]: statistically significant vs. negative control naïve mice received olive oil alone; [b]: statistically significant vs. CCL4-treated mice received PBS.

Immunological efficacy of herbal medication on the phenotypic expression of CD4+ T lymphocytes

The current data revealed a remarkable increase in the phenotypic expression percentage of lymphocyte CD4+ T lymphocytes in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to naïve mice that received olive oil and CCL4-treated mice post-treated with PBS (Figure 3A). Conversely, CCL4-treated mice post-treated with PBS showed a decreased phenotypic expression percentage of lymphocyte CD4+ T lymphocytes compared to naïve mice that received olive oil (Figure 3A). Additionally, the present data showed a significant increase in the expression percentage of CD4+ T lymphocytes in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to naïve mice that received olive oil and CCL4-treated mice post-treated with PBS (Figure 3B).

Immunomodulatory effects of phytomedicine on the phenotypic expression of $CD8^+$ T lymphocytes

The present results showed a remarkable increase in the phenotypic expression percentage of CD8+ T lymphocytes in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, and

CCL4-treated mice post-treated with Amg, compared to naïve mice that received olive oil and CCL4-treated mice post-treated with PBS (Figure 4A).

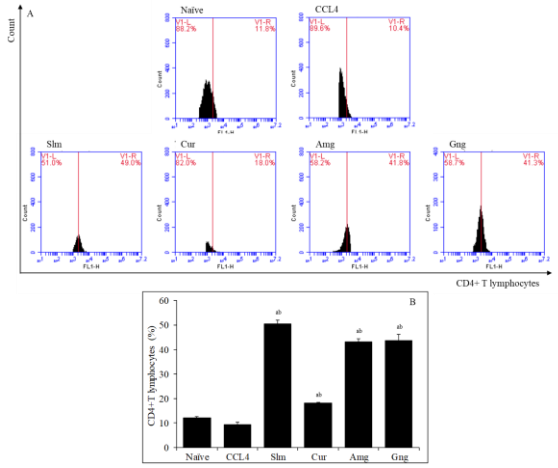

Figure 3. Flow cytometry analysis showing the phenotypic frequency rate (A) and relative number (B) of CD4+ T lymphocytes in mice with chemically induced hepatic inflammation treated with extracts of Slm, Cur, Amg, and Gng. Values were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. [a]: statistically significant vs. negative control naïve mice received olive oil alone; [b]: statistically significant vs. CCL4-treated mice received PBS.

Furthermore, the current data showed a significant increase in the expression percentage of CD8+ T lymphocytes in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to naïve mice that received olive oil and CCL4-treated mice post-treated with PBS (Figure 4B).

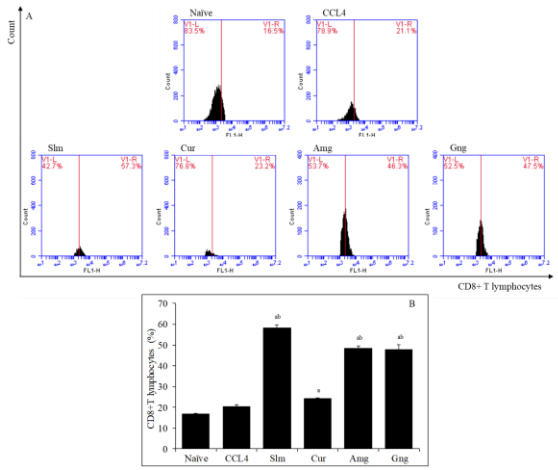

Figure 4. Flow cytometry analysis showing the phenotypic frequency rate (A) and relative number (B) of CD8+ T lymphocytes in mice with chemically induced hepatic inflammation treated with extracts of Slm, Cur, Amg, and Gng. Values are denoted as mean ± standard error (n = 10). Differences among groups were deemed statistically significant at $P < 0.05$. [a]: statistically significant vs. negative control naïve mice received olive oil alone; [b]: statistically significant vs. CCL4-treated mice received PBS.

Immunological efficacy of herbal medication on the phenotypic expression of NK cells

The existing results showed an obvious decrease in the phenotypic expression percentage of NK+ lymphocytes in CCL4-treated mice post-treated with PBS, CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to naïve mice that received olive oil (Figure 4A). Contrarily, compared to their percentages in CCL4-treated mice post-treated with PBS, the phenotypic expression percentage of NK cells recorded remarkable increases in the CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng (Figure 4A). Contrarily, a significant increase in the expression percentage of NK+ lymphocytes was observed in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to CCL4-treated mice post-treated with PBS (Figure 5B).

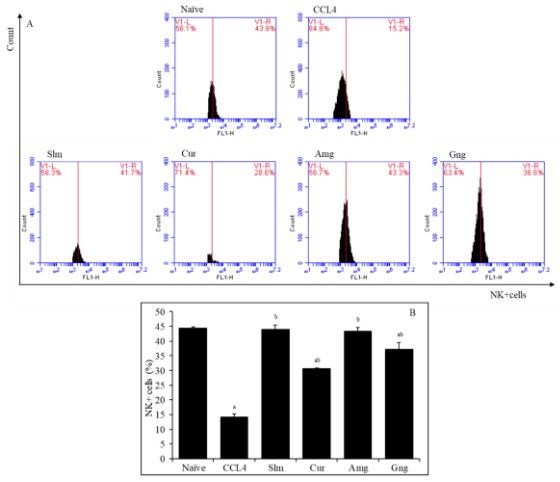

Figure 5. Flow cytometry analysis showing the phenotypic frequency rate (A) and relative number (B) of NK cells in mice with chemically induced hepatic inflammation treated with extracts of Slm, Cur, Amg, and Gng. Values are denoted as mean ± standard error (n = 10). Differences among groups were deemed statistically significant at $P < 0.05$. [a]: statistically significant vs. negative control naïve mice received olive oil alone; [b]: statistically significant vs. CCL4-treated mice received PBS.

Effects of herbal treatments on the splenocyte necrosis

The current data showed an obvious decrease in the necrosis rate of splenic cells in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to CCL4-treated mice post-treated with PBS (Figure 6A). Interestingly, there was a

remarkable decrease in the necrosis rate of splenocytes in CCL4-treated mice post-treated with Cur, compared to CCL4-treated mice post-treated with Slm (Figure 6A). Furthermore, our data showed a significant decrease in the necrosis rate of splenocytes in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to CCL4-treated mice post-treated with PBS (Figure 6B). Interestingly, there was a significant decrease in the necrosis rate of splenocytes in CCL4-treated mice pre-treated with Amg, CCL4-treated mice pre-treated with Cur, and CCL4-treated mice pre-treated with Amg compared to CCL4-treated mice post-treated with Slm (Figure 6B).

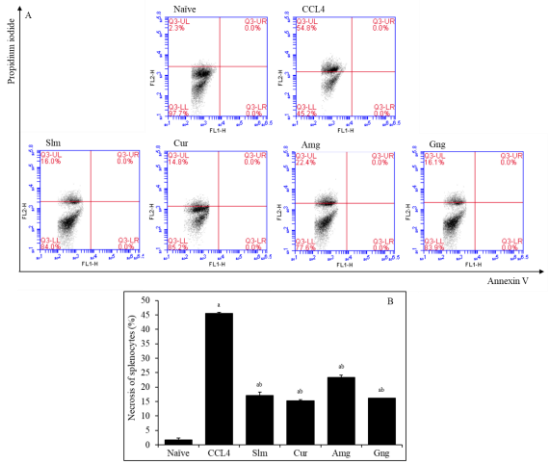

Figure 6. Flow cytometry analysis showing phenotypic frequency rate (A) and expression rate (B) of splenocyte necrosis rate in mice with chemically induced hepatic inflammation treated with extracts of Slm, Cur, Amg, and Gng. Values were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. [a]: statistically significant vs. negative control naïve mice received olive oil alone; [b]: statistically significant vs. CCL4-treated mice received PBS.

Effects of herbal treatments on the serum levels of pro-inflammatory cytokine IL-6

The efficacy of herbal extractions of Slm, Cur, Amg, and Gng on the serum level of pro-inflammatory cytokine IL-6 is shown in Figure 7A. The current data revealed a significant increase in the level of IL-6 in CCL4-treated mice post-treated with PBS, CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to naïve mice that received olive oil (Figure 7A). Contrarily, significant decreases in the level of IL-6 were revealed in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng compared to CCL4-treated mice post-treated with PBS (Figure 7A). Interestingly, the data showed a significant decrease in the level of IL-6 in CCL4-treated mice post-treated with Amg compared to CCL4-treated mice post-treated with Slm (Figure 7A) .

Effects of herbal treatments on the level of pro-inflammatory molecule C-CRP

The potentials of herbal extracts of Slm, Cur, Amg, and Gng on the level of pro-inflammatory molecule CRP are indicated in Figure 7B. The current results displayed a significant reduction in the level of CRP in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to naïve mice receiving olive oil (Figure 7B). Interestingly, the data presented here show a significant elevation in the level of CRP in CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng in comparison to CCL4-treated mice post-treated with Slm (Figure 7B).

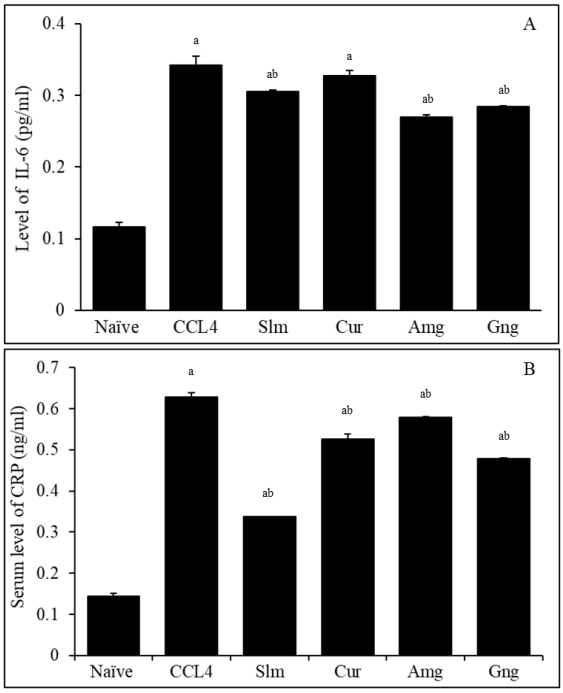

Figure 7. Influences of extracts of Slm, Cur, Amg, and Gng on the serum level of pro-inflammatory cytokine IL-6 (A) and CRP (B) in the mice with chemically induced hepatic inflammation. Values were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. [a]: statistically significant vs. negative control naïve mice received olive oil alone; [b]: statistically significant vs. CCL4-treated mice received PBS.

Impacts of herbal treatments on the total and differential leucocyte counts

The efficacy of herbal extractions of Slm, Cur, Amg, and Gng on the mice leucocyte (WBC) counts and their differentials in a time-dependent manner is

indicated in Table 2. Compared to CCL4-treated mice post-treated with PBS, leucocyte counts recorded an obvious increase in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, and CCL4-bearing mice post-treated with Amg (Table 2). Interestingly, leucocyte counts recorded a significant increase in CCL4-treated mice post-treated with Amg compared to those in naïve mice that received olive oil, and CCL4-treated mice received PBS (Table 2).

Compared to CCL4-treated mice that received PBS, the relative number of lymphocytes recorded a remarkable increase in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-bearing mice post-treated with Gng (Table 2). Compared to CCL4-treated mice receiving PBS, the relative number of monocytes revealed an obvious elevation in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, and CCL4-treated mice post-treated with Amg (Table 2). The relative number (%) of neutrophils revealed an increase in CCL4-treated mice post-treated with Slm, CCL4-treated mice post-treated with Cur, and CCL4-bearing mice post-treated with Amg, compared to those in CCL4-treated mice that received PBS (Table 2). Compared to CCL4-treated mice post-treated with PBS, the eosinophil relative number revealed a remarkable decrease in CCL4-treated mice post-treated with Slm, CCL4-treated mice pre-treated with Amg, and CCL4-bearing mice pre-treated with Gng (Table 2).

Table 2. Effectiveness of extracts of Slm, Cur, Amg, and Gng on the total and differential leucocyte counts in the mice with chemically induced hepatic inflammation.

Groups	WBC count (10^3/µL)	Differential counts (%)			
		Lymphocytes	Monocytes	Neutrophils	Eosinophils
Naive	10.75±0.45	72.50±1.50	7.00±1.00	18.50±1.50	2.00±1.00
CCL4	4.65±0.05	78.50±0.50	5.00±0.00	14.50±0.50	2.00±0.00
Slm	7.60±1.30	76.00±4.00	8.00±0.00	14.50±3.50	1.50±0.50
Cur	6.95±0.05	69.00±1.00	6.00±2.00	22.50±2.50	2.50±050
Amg	27.60±4.80[ab]	70.00±2.00	6.50±0.50	22.00±3.00	1.50±0.50
Gng	3.45±0.55	76.50±5.50	5.00±1.00	17.00±7.00	1.50±0.50

Values were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. [a]: statistically significant vs. negative control naïve mice received olive oil alone; [b]: statistically significant vs. CCL4-treated mice received PBS.

Consequences of herbal treatments on the hepatic and renal functions

The capacities of herbal extracts of Slm, Cur, Amg, and Gng on the level of liver ALT and kidney urea in a time-dependent approach are revealed in Figures 8A and 8B. The current results displayed a significant increase in the level of serum ALT in CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng, compared to naïve mice receiving olive oil and CCL4-treated mice post-treated with PBS (Figure 8A). Furthermore, a significant increase in the level of serum ALT was established in CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice post-treated with Gng in comparison to CCL4-treated mice post-treated with Slm PBS (Figure 8A) .

The recent outcomes showed significant increases in the level of serum urea in CCL4-treated mice post-treated with PBS, CCL4-treated mice post-treated with Cur, CCL4-treated mice post-treated with Amg, and CCL4-treated mice pre-treated with Gng in comparison to naïve mice receiving olive oil alone (Figure 8B). Contrarily, the level of serum urea showed obvious decreases in CCL4-treated mice post-treated with Slm compared to naïve mice receiving olive oil (Table 8B). Additionally, the level of serum urea revealed a remarkable elevation in CCL4-treated mice post-treated with Amg in comparison to control CCL4-treated mice post-treated with PBS (Figure 8B). Rebelliously, the level of serum urea showed a remarkable reduction in CCL4-treated mice post-treated with Slm compared to that in CCL4-treated mice post-treated with PBS (Figure 8B).

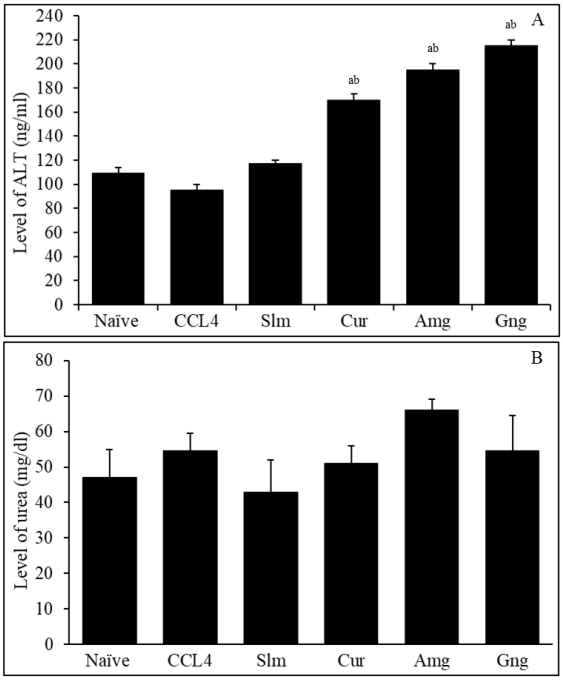

Figure 8. Efficacy of extracts of Slm, Cur, Amg, and Gng on the level of serum ALT (A) and urea (B). Values were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. [a]: statistically significant vs. negative control naïve mice received olive oil alone; [b]: statistically significant vs. CCL4-treated mice received PBS.

Discussion

Herbal-based medicines protect against chemical-induced liver inflammation through immunomodulatory, anti-inflammatory, and antioxidant mechanisms. They primarily function by reducing levels of inflammatory cytokines and free radicals. Furthermore, they enhance innate immune and antioxidant defenses within hepatocytes and reduce inflammatory cell infiltration. The immune protective effects attributed to phytochemicals such as flavonoids and polyphenols found in herbs may involve the regulation of innate immune system components and the restoration of liver architecture (Firdous and Fayed, 2021). Herbal remedies have the potential to alleviate the inflammatory response in the liver, which is known to trigger inflammation and the release of pro-inflammatory cytokines. By integrating these immunological and biochemical mechanisms, herbal medicines present a viable approach to alleviating severe chemically induced hepatic inflammation, thereby offering promising therapeutic strategies for liver diseases (Ugwu and Suru, 2021).

The recent data indicated that, in comparison to CCL4-treated mice subsequently treated with PBS, there was a notable alteration in splenocyte counts in CCL4-treated mice that were subsequently treated with Slm, as well as in those post-treated with Cur, Amg, and Gng. Our findings are corroborated by evidence that oxidative damage caused by chemical administration significantly affects the quantity, structure, and functionality of splenocytes (Ganie et al., 2011).

The harmful impacts of CCL4 on splenocytes are associated with its metabolites, especially the trichloromethyl free radical and the trichloromethyl peroxyl radical, which encourage the secretion of inflammatory cytokines such as IL-6 and CRP (Jiang et al., 2012).Additional studies have shown that herbal extracts of RAMP may stimulate splenocytes through toll-like receptor 4 (TLR4)-independent mechanisms that involve mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB) (Xu et al., 2019).

Our results demonstrate a significant enhancement of immunological defences against chemical-induced hepatitis. Specifically, we observed a marked increase in the percentage of phenotypic expression of CD4+ T lymphocytes, CD8+ T lymphocytes, and NK cells in CCL4-treated mice following post-treatment with Slm, Cur, Amg, or Gng, compared to CCL4-treated mice receiving PBS . In alignment with our research, Hogg et al. (2017) illustrated that the lymphocytes CD4+ T, CD8+ T, and NK+ are essential in the immune response to chemically induced liver inflammation in mice. In instances of chemically induced hepatic inflammation in mice, there was a marked increase in CD4+ T, CD8+ T, and NK lymphocytes. This resulted in a significant rise in the CD4+/CD8+ T lymphocyte ratio when compared to the control group, signifying a considerable change in immune status due to prolonged chemical exposure. These results imply that the herbal remedies may improve immune function in mice suffering from chemically induced inflammation (Huang et al., 2020) .

Moreover, the inclusion of immunomodulatory herbal treatments is essential for the regulation of immune cell functions and cytokine synthesis. These influences enhance immune responses to liver inflammation induced by chemicals, thus preserving immune balance and mitigating immune-related liver damage (Rajanna et al., 2021). A polyherbal formulation exhibits significant hepatoprotective properties against various hepatotoxic agents through multiple mechanisms, such as antioxidant, anti-inflammatory, and immunomodulatory effects, as well as the regeneration of liver cells .

The current findings indicated a significant reduction in the necrosis rate of splenic cells in CCL4-treated mice that were subsequently treated with Slm, as well as in those treated with Cur, Amg, and Gng, when compared to CCL4-treated mice that received PBS. Notably, there was a substantial decrease in the necrosis rate of splenocytes in CCL4-treated mice that were post-treated with Cur and Gng, in contrast to CCL4-treated mice that were post-treated with Slm.

In accordance with our findings, Prakash and Mukherjee (2010) noted a significant splenocyte necrosis in animals treated with CCL4. Importantly, a marked improvement in the necrotic state was noted in CCL4-treated animals that were administered the herbal extract Prak-20. Furthermore, herbal medications from Slm, Cur, Amg, and Gng not only prevent necrosis and degeneration but also encourage considerable cellular regeneration, including that of splenocytes and hepatocytes (Prakash and Mukherjee, 2010).

The herbal treatments involving Gentiana lutea (G. lutea) notably improved the inflammatory cellular infiltration and necrosis in splenocytes and hepatocytes, indicating that it offers protective benefits against liver and spleen damage induced by chemical exposure (Zhang et al., 2023). The use of herbal medicines containing Slm in mice suffering from chemical-induced inflammation significantly diminished the severity of necrosis and inflammatory cell infiltration in the liver and spleen (Hamza et al., 2020). In general, our findings have

uncovered a shared scientific concept indicating that the post-treatment of mice with chemically induced hepatic inflammation using Slm, Cur, Amg, or Gng led to significant reductions in the levels of pro-inflammatory and inflammatory cytokines and molecules, such as interleukin-6 (IL-6) and C-reactive protein (CRP), as opposed to those in CCL4-treated mice that were administered PBS.

It is widely acknowledged that inflammation plays a crucial role in liver damage induced by CCL4. Reactive oxygen species (ROS) not only contribute to liver injury but also instigate inflammation by facilitating the release of inflammatory and proinflammatory cytokines and molecules such as IL-6 and CRP from activated macrophages and other immune cells (Dong et al., 2016) .

In alignment with our data, CCL4 significantly elevated the expression levels of IL-6 and CRP. Conversely, the use of herbal medications appeared to alleviate this effect, likely attributable to their immunomodulatory and anti-inflammatory characteristics. Herbal treatments involving Slm, Cur, Amg, and Gng supplements were found to diminish the production of IL-6 and CRP that was induced by CCL4, by inhibiting the release of pro-inflammatory cytokines (Reyes-Gordillo et al., 2007). The interaction of IL-6 and CRP with their respective receptors (IL-6R, CRPR) triggers the activation of the STAT3 pathway via its association with glycoprotein 130 (gp130) (Reyes-Gordillo et al., 2007).

One of the primary objectives of the present study was to ascertain whether the post-treatment (curative) application of herbal extracts from Slm, Cur, Amg, and Gng in Swiss albino mice that were intoxicated with CCL4 would enhance the potential induced cellular counts, abnormalities, and damages in immune peripheral blood cells: leucocytes (WBCs), lymphocytes, neutrophils, monocytes, and eosinophils of Swiss albino mice. The findings of this study revealed significant alterations in the immunological parameters of mice suffering from chemically induced hepatic inflammation. The treatment of these animals with herbal extracts from Slm, Cur, Amg, and Gng led to an improvement in the diminished counts and abnormalities of WBCs, lymphocytes, neutrophils, monocytes, and eosinophils that were induced by CCL4 administration.

The results indicate that, in comparison to CCL4-treated mice that were post-treated with PBS, the total counts of WBCs and the relative numbers of lymphocytes, monocytes, neutrophils, and eosinophils exhibited a notable increase in CCL4-treated mice that were subsequently post-treated with Slm, as well as in those post-treated with Cur, Amg, and Gng, when compared to CCL4-treated mice that received PBS.

In alignment with our research findings, the treatment of mice suffering from chemically induced hepatic inflammation with herbal therapy derived from Nigella sativa (N. sativa) significantly alleviated the alterations and reductions in total WBC count and the relative proportions of their differentials, which include lymphocytes, neutrophils, monocytes, and eosinophils (Sinha et al., 2006). This study illustrates that herbal medications have the potential to protect hematopoietic cells from the detrimental effects of chemically induced hepatic inflammation, likely due to the immunological, anti-inflammatory, and antioxidant properties of these treatments (Essawy et al., 2010) .

Our research demonstrated that the herbal remedies derived from Slm, Cur, Amg, and Gng significantly improve the harmful effects on liver and kidney functions resulting from chemically induced hepatic inflammation. This is evidenced by a notable decrease in the levels of serum alanine aminotransferase (ALT) and urea in CCL4-treated mice that were subsequently treated with Slm, Cur, Amg, and Gng, when compared to untreated CCL4-treated mice.

The administration of Slm, Cur, Amg, and Gng in CCL4-treated mice has been demonstrated to reverse the elevated serum levels of ALT and urea, indicating a protective effect on the liver against CCL4-related toxicity (Lee et al., 2016). In rats, medicinal herbs such as Slm, Cur, Amg, and Gng offer substantial protection against chemically induced hepatic inflammation by reducing ALT and urea activity, boosting hepatic glutathione levels, and alleviating oxidative stress in the liver, which subsequently lowers lipid peroxidase levels (Lee et al., 2016). Negi et al. (2008) credited this phenomenon to the strong antioxidant characteristics found in herbal treatments. Furthermore, these medicinal therapies serve as a powerful inhibitor of cytochrome P450, which may assist in restoring the equilibrium of antioxidant enzymes and non-enzymatic antioxidants like GSH (Bulku et al., 2012). This positive outcome may be associated with the antioxidant characteristics of Cur, as reactive oxygen species (ROS) have been recognized as contributors to the impairment of the glomerular filtration rate (Farombi and Ekor, 2006).

Herb-based drugs could play a vital role in safeguarding the liver by employing various mechanisms, such as inhibiting the NF-κB pathway, lowering the levels of the proinflammatory cytokine IL-6, adjusting the immune response, and managing the activity of T lymphocytes (Liao et al., 2024).

Herbal treatment influences both adaptive and innate immune cells by modifying the secretion of pro-inflammatory and anti-inflammatory cytokines, thereby affecting a range of immune cells, including mast cells, monocytes, macrophages, and neutrophils, as well as CD4+ T lymphocytes, CD8+ T lymphocytes, and NK cells (Nisar et al., 2023; Qiu et al., 2025). Phytomedicines can address chemically induced hepatic inflammation by regulating the immune system and offering antioxidant and anti-inflammatory advantages. They accomplish this by inhibiting pro-inflammatory pathways such as NF-κB, reducing the synthesis of cytokines (for instance, CRP, IL-6), promoting the anti-inflammatory pathway, and stimulating antioxidant defences via mechanisms like nuclear factor erythroid 2-related factor 2 (Nrf2). Notable examples of beneficial plants include Slm and Cur, which play a significant role in the traditional treatment of liver inflammation through their modulation of the immune system (Achary et al., 2025; Laka et al., 2022).

Conclusion

In brief, our research demonstrated that herbal remedies derived from Slm, Cur, Amg, or Gng significantly reduced hepatic inflammation while also modulating the immune system's reaction to chemical exposure. They enhanced the immune mechanisms to liver inflammation by raising the levels of immune cells, including NK cells, CD8+ T cells, and CD4+ T cells, while concurrently inhibiting inflammatory cytokines such as IL-6 and CRP. As a result, our findings underscore the potential of phytomedicines obtained from Slm, Cur, Amg, and Gng in influencing immune regulatory pathways specific to hepatic inflammation. Additional trials should focus on isolating and identifying effective bioactive compounds from crude phytomedicines of Slm, Cur, Amg, and Gng, enhancing our understanding of how they regulate immune and inflammatory responses, with potential clinical applications in preventing and treating inflammatory diseases.

Availability of data

Data presented in the study and any related data are available upon request.

Conflict of Interest

No conflict of interest is declared.

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