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ORIGINAL ARTICLE

The prophylactic immunomodulatory potentials of herbal extract-based treatment on *CD4+/CD8+ T* lymphocytes and the related pro-inflammatory cytokines in chemically related liver inflammation

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ABSTRACT

Background: Herb-based drugs regulate immunity by inhibiting cytokines and chemokines, impacting hepatic inflammation and immune cell recruitment after chemical injury. **Aim:** This study aimed to evaluate the prophylactic immunomodulatory and anti-inflammatory potentials of silymarin (Sylm), curcumin (Curc), amygdalin (Amyg), and ginger (Ging) on carbon tetrachloride (CCL4)-induced liver inflammation. **Methods:** Mice were divided into six groups: group 1 received saline; group 2 received CCL4 (0.5 mg/kg) thrice/week for 9 weeks; and groups 3 to 6 received herbal extracts: Sylm (112 mg/kg), Curc (522 mg/kg), Amyg (64 mg/kg), and Ging (115 mg/kg), respectively, thrice/week for 3 weeks. Then, mice were given CCL4 (0.5 mg/kg) thrice/week for 6 weeks; 24 h following each CCL4 injection, they were given again herbal extracts at the same doses twice a week for 6 weeks. The expression of natural killer (NK) cells, CD4+ and CD8+ T lymphocytes, splenocyte necrosis rates, pro-inflammatory markers, tumor necrosis factor-α (TNF-α), and alpha fetoprotein (AFP), and total and differential leukocyte counts, along with liver and kidney functions, were assessed. **Results:** Mice treated with herbal extracts from Sylm, Curc, Amyg, or Ging post-CCL4 injection exhibited significant increases in the expression rate of NK, CD4+, and CD8+ T lymphocytes, while TNF-α, AFP levels, and splenocyte necrosis rates were significantly reduced as compared to CCL4-intoxicated controls. Moreover, Amyg showed the most effective extract for regulating immune responses in response to liver inflammation. **Conclusion:** Treatment with the herbs Sylm, Curc, Amyg, and Ging shows promise in regulating immune responses to chemical damage, highlighting their potential in treating liver inflammation. Further pre-clinical studies are needed to prove their immune regulation and therapeutic effectiveness in liver inflammation.

Keywords: Cytokines, Herbal therapy, Immune, Inflammation, Liver, NK cells, T cells

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INTRODUCTION

The liver is vital for maintaining life, particularly through the detoxification of deleterious mediators. It serves as a key component in both systemic and local innate immunity, making significant contributions to immune regulation during liver inflammation induced by toxins (Dienes and Drebber, 2010). The liver is essential in bolstering the immune system, safeguarding mammals against harmful pathogens. It is important to acknowledge that, despite the liver encountering a considerable influx of "non-self" substances through the portal blood, it typically maintains the immune status of the surrounding environment in an anti-inflammatory or immunotolerant state (Barouki et al., 2023). As a result, the liver is adept at rapidly and effectively initiating an immune response when specific conditions arise to combat undesirable threats. The delicate balance between triggering a strong immune response when necessary and promoting tolerance at other times is crucial for the liver to function optimally as a primary immune organ (Qin et al., 2024). The mechanisms of hepatic inflammation play a vital role in maintaining the homeostasis of tissues and organs, and any disruption in these processes can lead to liver damage. Consequently, it is essential that the dynamic interactions between these immune cell populations are effectively coordinated, and any circumstances that could result in the disruption of their functional relationships are carefully avoided to promote overall health and well-being (Nemeth et al., 2009).

The mechanisms that lead to chemical-induced liver inflammation (CILI) can arise from the chemical's exposure or its metabolites, in addition to immune-mediated pathways. For instance, an inflammatory reaction that arises later can exacerbate the initial harm to hepatocytes resulting from the chemical exposure (Lammert et al., 2010). The management of liver diseases using both synthetic and conventional medications has generated significant debate, mainly because of inadequate treatment outcomes and prominent side effects. This scenario

highlights the pressing need to investigate herbal and traditional therapies that could offer therapeutic benefits for liver diseases (Munakarmi et al., 2021). The natural products derived from plants effectively neutralize free radicals by enhancing the levels of anti-inflammatory cytokines and cellular antioxidant enzymes (Dwivedi et al., 2022).

Hepatic tissue damage associated with liver inflammation may be promoted by pro-inflammatory cytokines such as C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), alpha fetoprotein (AFP), and interleukin-6 (IL-6) (Reyes-Gordillo et al., 2007). These cytokines can activate Kupffer cells, which results in the release of various inflammatory mediators (Sundari et al., 2018). The enhancement of inflammatory cells, particularly Kupffer cells, represents a crucial step in the onset of hepatic inflammation. The pro-inflammatory markers TNF-α, AFP, IL-6, and CRP have been recognized as significant contributors to the activation of innate immunity (Zhang et al., 2023). In reaction to exogenous risk factors, tissues commence the innate immunity and elevate the expression of these pro-inflammatory factors. Once these factors are activated, they further promote immune responses (Sundari et al., 2018).

Botanical remedies not only exhibit antioxidant and anti-inflammatory characteristics but also reduce tissue susceptibility to protein and lipid oxidation. Additionally, they improve endothelial function, thus altering the cellular redox balance towards oxidative stress in inflammatory states (Surai, 2015). This effect may be due to the capacity of herbal remedies to inhibit the release and synthesis of pro-inflammatory cytokines while facilitating the elevation of anti-inflammatory cytokine mediators, including IL-6 (Surai, 2015).

Herbal substances sourced from amygdalin (Amyg), silymarin (Sylm), ginger (Ging), and curcumin (Curc) are acknowledged for their properties that enhance immunity, particularly concerning liver ailments. Methanolic extracts of these herbs are frequently enhanced as natural immune boosters in the management of chemically induced liver conditions. The therapeutic potential of a range of natural products, including Amyg, Sylm, Ging, and Curc, has been significantly emphasized, with flavonoids showing remarkable effectiveness (Zhang et al., 2023). The polyphenolic compounds in herbal medications exhibit strong anti-inflammatory, antioxidant, and immunomodulatory characteristics. A study by Balaban et al. (2017) has revealed the extraordinary capability of herbal treatment to affect immune responses in chemically induced liver inflammation, positioning them as significant subjects in immunity. Herbal components derived from Amyg, Sylm, Ging, and Curc, with their diverse molecular activities, emerge as efficient regulators of the immune system, which is vital in immune surveillance, metabolic pathways, and detoxification (Dara et al., 2016). This regulation is crucial for preserving the health of the liver, as an intense immune response can lead to hepatocellular damage and exacerbate the development of liver illness (Lin et al., 2011). Besides their immune-regulating functions, the antioxidant capabilities of these herbal remedies are highlighted, as they actively reduce oxidative damage and neutralize free radicals that could otherwise threaten structural integrity and immune function (Muscolo et al., 2024).

The administration of herbal therapies such as Amyg, Sylm, Ging, and Curc in the event of chemically induced hepatic inflammation may facilitate the recovery of B lymphocytes, dendritic cells (DC), CD8+ and CD4+ T lymphocytes, and natural killer (NK) cell populations, thereby restoring the predominance of Th1-secreted cytokines (Lin et al., 2011; Trivadila et al., 2025). Numerous herbal medications are regarded as immune response modifiers, inhibiting the enhancement of nuclear factor kappa B (NF-κB), which in turn reduces the subsequent production of pro-inflammatory cytokine TNF-α, while promoting the synthesis of anti-inflammatory cytokine IL-10, resulting in regulation of immunity (Kandilarov et al., 2023). Accordingly, this study aimed to assess how herbal treatments derived from Sylm, Curc, Amyg, and Ging preventively affect the body's immune response concerning liver inflammation caused by carbon tetrachloride (CCL4) in mice.

Materials and methods

Reagents

CCL4 was sourced from Sigma-Aldrich (Missouri, USA). RPMI-1640 complete medium was bought from Sigma-Aldrich (Missouri, USA). RBC lysing buffer was sourced from G-Biosciences (California, USA). Propidium iodide (PI) dye for cell viability was supplied by ThermoFisher (California, USA). Kits for Enzyme-Linked Immunosorbent Assay (ELISA): Mouse TNF-alpha Quantikine ELISA Kit (Cat #: PMTA00B) and mouse Alpha-Fetoprotein/AFP ELISA Kit – Quantikine (Cat #: MAFP00) were bought from Biotech (Minneapolis, USA).

Antibodies

Flowcytometric monoclonal antibodies: anti-mouse CD4 antibody (Cat # 14-9766-82), anti-mouse CD8 antibody (Cat # MA1-82375), anti-mouse NK antibody (Cat# A14739), and anti-annexin V antibody (Cat # MA5-41552) were purchased from ThermoFisher (California, USA).

Mice

Eight-week-old Swiss albino mice (male), weighing 25-30 g. The mice were acquired from the National Research Center animal facility (Giza, Egypt). Mice were housed at constant laboratory conditions (temperature 25°C, relative humidity 55±10% with a 12 h light/12 h dark cycle). Before the experiments began, the mice were grouped and allowed to acclimatize to their new environment for one week. Ethical clearance was approved by the ethics committee, Science Faculty, University of Tanta (Approval code: Rec-sci-Tu-Q-036) .

Sample preparation

The herbs Sylm, Curc, Amyg, and Ging were shade-dried for a period of 3 days and subsequently ground into a powder. Powdered materials (100 g) from each herb were extracted with 95% ethanol (3×100 mL) for 48 h with constant shaking at 120 rpm. The liquid extracts obtained were filtered through Whatman qualitative filter paper. The liquid extracts obtained were filtered through Whatman qualitative filter paper. The yield of each extract (w/w) was evaluated, and the extracts were stored at -20°C until further experiments.

Mouse model of liver inflammation

To provoke inflammation, 8-week-old Swiss albino mice, weighing 25-30 g, were administered an intraperitoneal (i.p.) injection of CCL4, which was diluted in olive oil at a 1:9 (v/v) ratio, at a dosage of 0.5 mg/kg three times a week for 8 weeks .

Laboratory design and dosage timeline

To investigate the effects of herbal therapy on the biochemical and immunological alterations induced by CCL4-induced liver inflammation and fibrosis, sixty Swiss albino mice were allocated into 6 distinct groups, with 10 mice in each group. Cont group: mice i.p. received olive oil. CCL4 group: mice i.p. injected with CCL4 (0.5 mg/kg) thrice/week for 9 weeks. Groups CCL4/Sylm, CCL4/Curc, CCL4/Amyg, and CCL4/Ging: mice were i.p. administered with herbal treatments—Sylm (112 mg/kg), Curc (522 mg/kg), Amyg (64 mg/kg), or Ging (115 mg/kg)—respectively, thrice/week for 3 weeks. 24 h after the last herbal dose, mice were i.p. inoculated with CCL4 (0.5 mg/kg) thrice/week for 6 weeks, 24 h following each CCL4 injection, mice were i.p. administered with herbal extracts—Sylm (112 mg/kg), Curc (522 mg/kg), Amyg (64 mg/kg), or Ging (115 mg/kg)—respectively, thrice/week for 6 weeks. The reference for the dosages of herbal extracts used in the current study depended on our published data (Nassef et al., 2025) .

Sera collection

Before the euthanasia of the mice, blood was gathered in plastic containers. Blood was allowed to sit for 3 h to ensure complete clotting. After clotting, the blood samples underwent centrifugation (1500 ×g rpm, 15 min, 4°C). Subsequently, the sera were aspirated and preserved at -80°C for subsequent biochemical and immunological analyses.

Hematological assessment

Blood samples intended for the assessment of hematological parameters were gathered in EDTA containers and subsequently analyzed with a hematology analyzer (Mindray Auto Hematology Analyzer, BC-5200, USA) in accordance with the manufacturer's guidelines. The parameters evaluated included the total count of white blood cells (WBCs) and the differential count of lymphocytes, neutrophils, monocytes, and eosinophils.

Preparation of the thymocytes single-cell suspension

Mice were euthanized by cervical dislocation. The thymus was washed with phosphate buffer saline (PBS) and then compressed between two glass slides to form a single-cell suspension. The thymocyte suspension was washed twice with PBS by centrifugation (500 ×g, 5 min, 4°C). To eliminate erythrocytes, the pellet was diluted in RBC lysing buffer, incubated for 5 minutes, and subsequently aliquoted with PBS. Next, spinning of the thymocyte suspension was carried out at 500 ×g for 5 minutes. Next, the pellet was diluted in a suitable buffer and passed through a 45 µm cell strainer. The thymocyte count was done using a hemocytometer with a trypan blue dye assay.

Flowcytometric analyses

A suspension of splenocytes was prepared and calculated using a hemocytometer along with trypan blue dye to check for live cells, as described earlier by Diaz-Montero et al. (2009). In short, a cell suspension of splenocytes (1.0×10^6 cells) was prepared, and their surface molecules were identified using anti-mouse CD4 antibody, anti-mouse CD8 antibody, and anti-mouse NK antibody.

The splenocytes were stained with the specified conjugated monoclonal anti-mouse CD4, monoclonal anti-mouse CD8, or monoclonal anti-mouse NK (10 µl/10^6 cells) and incubated for 30 minutes in the dark, followed by a 1-minute chill on ice.

The cells underwent two washes with PBS and were subsequently aliquoted in 0.3 ml of PBS, which was

enriched with 0.02% sodium azide and 0.5% BSA. Later, the cells were washed and examined by a Cytpix Flow Cytometer (ThermoFisher, California, USA). The analyses of the CD4+ cells, CD8+ cells, and NK cell subsets were phenotypically assessed utilizing FlowJo™ v11 Software (BDBiosciences, California, USA) .

Evaluation of splenocyte necrosis

Splenocytes harvested from mice were washed with ice-cold PBS and then diluted in 1X annexin-binding buffer. Next, 200 µl of splenocyte suspension was placed into Eppendorf tubes. Then, 5 µl of annexin V-fluorescein isothiocyanate (FITC) and 1 µl of PI, both prepared at a concentration of 100 µg/ml, were added. The mixture was allowed to rest, followed by the addition of 400 µl of 1X annexin-binding buffer, and the contents were gently mixed. The cells were subsequently examined with a Cytpix Flow Cytometer (ThermoFisher, California, USA). The data from the phenotypic analysis were processed using FlowJo™ v11 Software (BDBiosciences, California, USA).

ELISA

The concentrations of TNF-α and AFP in the serum of experimental mice were assessed by ELISA. Mouse TNF-alpha Quantikine ELISA Kit and mouse Alpha-Fetoprotein/AFP ELISA Kit – Quantikine (Biotech, Minneapolis, USA) were managed for TNF-α and AFP, respectively, in accordance with the manufacturer's instructions. The optical density was measured at 450 nm.

Biochemical assessments

The concentrations of aspartate transferase (AST), alanine aminotransferase (ALT), urea, and creatinine were calorimetrically measured using the AST Assay Kit (Cat #: MAK467), the ALT Activity Assay Kit (Cat #: MAK571), the Urea Assay Kit (Cat #: MAK006), and the Creatinine Assay Kit (Cat #: MAK475) (Sigma-Aldrich, USA). The instructions provided by the manufacturer for each biochemical test were carefully followed throughout the study.

Statistical analysis

Experimental outcomes were reported as means of standard errors. Statistical comparisons among groups were performed using one-way ANOVA in SPSS version 16 (SPSS, Inc., Chicago, IL). Significance was determined with post-hoc tests and Dunnett's comparisons of treatment means to controls, deeming p-values under 0.05 as significant.

RESULTS

Effects of herbal therapy on the body weight gain of mice with inflamed liver

The effects of extracts from Sylm, Curc, Amyg, and Ging on the weight gain of mice are illustrated in Table 1. The data obtained indicates that, compared to CCL4-intoxicated mice pre-treated with PBS, there were significant rises in body weight gain observed in CCL4-intoxicated mice pre-treated with Sylm, Curc, Amyg, and Ging by 19.10±2.00%, 13.90±1.70%, 10.70±1.20%, and 7.30±1.80%, respectively (Table 1). Additionally, compared to CCL4-intoxicated mice pre-treated with Sylm, there were significant drops in body weight gain in CCL4-intoxicated mice pre-treated with Curc, Amyg, and Ging at 13.90±1.70% and 10.70±1.20%, respectively (Table 1).

Table 1. Efficiency of extracts of Sylm, Curc, Amyg, and Ging on the body weight gain in the mice with CCL4-induced liver inflammation.

Groups	Body weight gain (%)
Cont	22.00±1.00
CCL4	$4.00\pm1.60^{\#}$
CCL4/Sylm	$19.13\pm1.93^{\$}$
CCL4/Curc	$13.91\pm1.75^{\$}$
CCL4/Amyg	$10.67\pm1.18^{\#\$}$
CCL4/Ging	$7.28\pm1.82^{\#}$

Data were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. $^{\#}$: statistically significant vs. negative control mice received olive oil alone; $^{\$}$: statistically significant vs. CCL4-intoxicated mice received PBS.

Effects of herbal therapy on the thymocyte count

The effectiveness of herbal therapy from Sylm, Curc, Amyg, and Ging on the mice splenocyte counts following CCL4-induced liver injury is illustrated in Table 2. The findings revealed an outstanding increase in thymocyte counts in CCL4-intoxicated mice pre-treated with Sylm, Curc, Amyg, or Ging, with values of $95.50\pm2.70\times10^6$, $86.25\pm5.50\times10^6$, $90.00\pm3.50\times10^6$, and $101.25\pm8.26\times10^6$, respectively, when compared to those in CCL4-intoxicated mice receiving PBS (Table 2).

Table 2. Effects of extracts of Sylm, Curc, Amyg, and Ging on the thymocyte counts in the mice with CCL4-induced liver inflammation.

Groups	Thymocytes count ($\times10^6$)
Cont	93.00±3.49
CCL4	89.00±5.54
CCL4/Sylm	95.50±2.72
CCL4/Curc	86.25±5.54
CCL4/Amyg	90.00±3.53
CCL4/Ging	101.25±8.26

Data were evaluated as mean ± standard error (n=10).

Effects of herbal therapy on the total and differential leucocyte counts

The usefulness of herbal treatment sourced from Sylm, Curc, Amyg, and Ging on the total and differential of white blood cell (WBC) counts in the mice with CCL4-induced liver damage is depicted in Figures 1A, 1B, 1C, 1D, and 1E. Importantly, the total WBC counts exhibited a significant rise in CCL4-intoxicated mice pre-treated with Ging at $26.80\pm3.50\times10^3$, in comparison to those in the negative control group. Similarly, leucocyte total counts exhibited a significant increase in CCL4-intoxicated mice pre-treated with Ging at $26.80\pm3.50\times10^3$, compared to those in CCL4-intoxicated mice that did not receive any treatment (Figure 1A). Similarly, when compared to the negative control mice and CCL4-intoxicated mice receiving PBS, those pre-treated with Sylm, Amyg, or Ging showed significant drops in relative values of lymphocytes at 52.50±7.50%, 47.50±2.50%, and 50.00±0.00%, respectively (Figure 1B). When compared to the negative control group, which did not receive any treatment, as well as CCL4-intoxicated mice that were administered PBS, the relative counts of monocytes were increased in CCL4-intoxicated mice pre-treated with Sylm, Amyg, and Ging, with values of 8.50±0.50%, 8.50±1.50%, and 8.50±0.50%, respectively (Figure 1C). In contrast to the negative control mice and CCL4-intoxicated mice receiving PBS, those pre-treated with Sylm, Amyg, or Ging showed significant increases in relative values of neutrophils at 32.50±7.50%, 39.00±1.00%, and 35.00±0.00%, respectively (Figure 1D). Additionally, when compared to the negative control group, which received no treatment, and CCL4-intoxicated mice receiving PBS, the relative counts of eosinophils indicated a significant increase in CCL4-intoxicated mice pre-treated with Sylm, Curc, Amyg, and Ging, at 4.50±0.50%, 4.50±0.50%, 4.00±1.00%, and 4.50±0.50% (Figure 1E).

Effects of herbal therapy on the numbers of CD4+ T cells, CD8+ T cells, and NK cells.

Our data indicated a significant rise in the phenotypic frequency rate of $CD4^+$ T lymphocytes in CCL4-intoxicated mice that were pre-treated with Sylm, Curc, Amyg, and Ging, by 50.60±1.80%, 21.10±1.20%, 48.00±2.30%, and 42.70±1.00%, respectively, when compared to negative control mice that did not receive any treatment (Table 3). Similarly, the recent data demonstrated a significant elevation in the phenotypic frequency rates of CD+ T lymphocytes in CCL4-intoxicated mice pre-treated with Sylm, Curc, Amyg, and Ging at values of 50.60±1.80%, 21.10±1.20%, 48.00±2.30%, and 42.70±1.00%, respectively, when compared to CCL4-intoxicated mice pre-treated with PBS (Table 3).

Table 3. Flow cytometry analysis indicating the phenotypic expression rate of CD4+ T cells in mice with CCL4-induced liver inflammation treated with extracts of Sylm, Curc, Amyg, and Ging.

Groups	CD4+T cells (%)
Cont	12.00±0.35
CCL4	9.40±1.00
CCL4/Sylm	$50.60\pm1.80^{\#\$}$
CCL4/Curc	$21.10\pm1.20^{\#\$}$
CCL4/Amyg	$48.00\pm2.30^{\#\$}$
CCL4/Ging	$42.70\pm1.00^{\#\$}$

Data were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. $^{\#}$: statistically significant vs. negative control mice received olive oil alone; $^{\$}$: statistically significant vs. CCL4-intoxicated mice received PBS.

The current results revealed marked rises in the phenotypic frequency rate of CD8+ T lymphocytes in CCL4-intoxicated mice that were pre-treated with Sylm, Curc, Amyg, and Ging with values at 45.40±1.90%, 36.15±1.55%, 42.45±2.05%, and 42.20±0.30%, respectively, when compared to the negative control mice receiving no treatment (Table 4). Likewise, the phenotypic frequency rate of CD8+ T cells showed a significant increase in CCL4-intoxicated mice pre-treated with Sylm, Curc, Amyg, and Ging by 45.40±1.90%, 36.15±1.55%, 42.45±2.05%, and 42.20±0.30%, respectively, in comparison to CCL4-intoxicated mice receiving PBS (Table 4).

Table 4. Flow cytometry analysis indicating the phenotypic expression rate of CD8+ T cells in mice with CCL4-induced liver inflammation treated with extracts of Sylm, Curc, Amyg, and Ging.

Groups	CD8+T cells (%)
Cont	17.00±0.30
CCL4	20.00±0.85
CCL4/Sylm	$45.40\pm1.90^{\#\$}$
CCL4/Curc	$36.15\pm1.55^{\#\$}$
CCL4/Amyg	$42.45\pm2.05^{\#\$}$
CCL4/Ging	$42.20\pm0.30^{\#\$}$

Data were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. $^{\#}$: statistically significant vs. negative control mice received olive oil alone; $^{\$}$: statistically significant vs. CCL4-intoxicated mice received PBS.

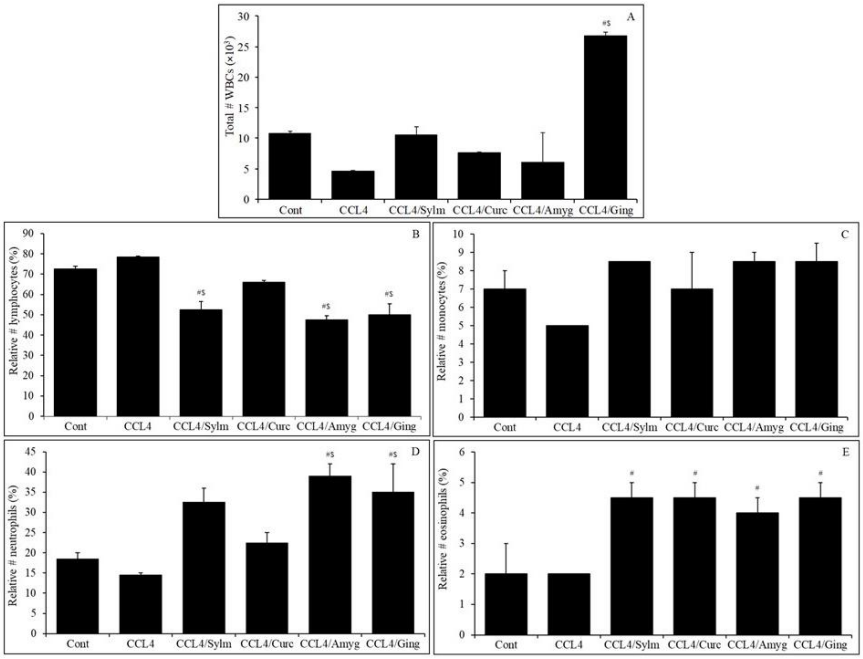

Figure 1. Potentials of extracts of Sylm, Curc, Amyg, and Ging on the total count of leucocytes (A), relative numbers of lymphocytes (B), monocytes (C), neutrophils (D), and eosinophils (E). Data were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at P less than 0.05. [#]: statistically significant vs. negative control mice received olive oil alone; [$]: statistically significant vs. CCL4-intoxicated mice received PBS.

Additionally, the current findings indicated significant reductions in the phenotypic frequency rate of NK cells in CCL4-intoxicated mice that were pre-treated with PBS, as well as in CCL4-intoxicated mice pre-treated with Curc, showing rates of 14.15±1.0% and 29.50±0.70%, respectively, when compared to the negative control mice receiving no treatment (Table 5). Contrarily, the phenotypic frequency rate of NK cells demonstrated a significant elevation in CCL4-intoxicated mice pre-treated with Sylm, Curc, Amyg, and Ging, with respective increases of 48.60±3.40%, 29.50±0.70%, 50.15±1.15%, and 39.70±0.70%, when compared to CCL4-intoxicated mice receiving PBS (Table 5).

Table 5. Flow cytometry analysis indicating the phenotypic expression rate of NK+ cells in mice with CCL4-induced liver inflammation treated with extracts of Sylm, Curc, Amyg, and Ging.

Groups	NK + cells (%)
Cont	44.00±0.55
CCL4	14.00±1.05[#]
CCL4/Sylm	48.60±3.40[$]
CCL4/Curc	29.50±0.70[#$]
CCL4/Amyg	50.15±1.15[$]
CCL4/Ging	39.70±0.70[$]

Data were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. #: statistically significant vs. negative control mice received olive oil alone, $: statistically significant vs. CCL4-intoxicated mice received PBS.

The anti-necrotic effects of herbal therapy on splenocytes

The usefulness of herbal treatment sourced from Sylm, Curc, Amyg, and Ging on the splenocyte necrosis rate in mice suffering from CCL4-induced hepatic damage is illustrated in Table 6. The current findings demonstrated significant reductions in the splenocyte necrosis rate in CCL4-intoxicated mice that were pre-treated with Sylm, as well as in CCL4-intoxicated mice pre-treated with Curc, CCL4-intoxicated mice pre-treated with Amyg, and CCL4-intoxicated mice pre-treated with Ging, with values at 6.70±0.00%, 10.85±0.05%, 8.00±0.40%, and 6.95±0.05%, respectively, comparable to CCL4-intoxicated mice receiving PBS (Table 6). Additionally, the splenocyte necrosis rate showed notable increases in CCL4-intoxicated mice pre-treated with Curc or Amyg, with increases of 10.85±0.05% and 8.00±0.40, respectively, when compared to CCL4-intoxicated mice pre-treated with Sylm (Table 6).

Immunological approaches of herbal therapy on the pro-inflammatory cytokine TNF-α .

The immune capacities of herbal treatments of Sylm, Curc, Amyg, and Ging regarding the levels of TNF-α in mice suffering from CCL4-induced hepatic injury are illustrated in Table 7. The present findings indicate significant increases in TNF-α levels in CCL4-intoxicated mice that were pre-treated with PBS, as well as in those pre-treated with Sylm, Curc, Amyg, and Ging, showing values of 0.375±0.012 pg/ml,

0.269±0.006 pg/ml, 0.320±0.003 pg/ml, 0.220±0.004 pg/ml, and 0.315±0.002 pg/ml, respectively, when compared to the negative control mice receiving normal saline (Table 7). In contrast, significant reductions in TNF-α levels were observed in CCL4-intoxicated mice pre-treated with Sylm, Curc, Amyg, and Ging, with values of 0.269±0.006 pg/ml, 0.320±0.003 pg/ml, 0.220±0.004 pg/ml, and 0.315±0.002 pg/ml, respectively, in comparison to CCL4-intoxicated mice receiving PBS (Table 7).

Table 6. Flow cytometry analysis of phenotypic expression of splenocytes showing a necrosis rate in the mice with CCL4-induced liver inflammation treated with phytomedicine of Sylm, Curc, Amyg, and Ging.

Groups	Splenocyte necrosis (%)
Cont	2.00±0.60
CCL4	46.00±0.35$^{\#}$
CCL4/Sylm	6.70±0.00$^{\#\$}$
CCL4/Curc	10.85±0.05$^{\#\$}$
CCL4/Amyg	8.00±0.40$^{\#\$}$
CCL4/Ging	6.95±0.05$^{\#\$}$

Data were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. $^{\#}$: statistically significant vs. negative control mice received olive oil alone; $^{\$}$: statistically significant vs. CCL4-intoxicated mice received PBS.

Table 7. Potentials of phytomedicine of Sylm, Curc, Amyg, and Ging on the level of pro-inflammatory cytokine TNF-α in the mice with CCL4-induced liver inflammation.

Groups	TNF-α (pg/ml)
Cont	0.140±0.007
CCL4	0.380±0.011$^{\#}$
CCL4/Sylm	0.269±0.006$^{\#\$}$
CCL4/Curc	0.320±0.003$^{\#\$}$
CCL4/Amyg	0.220±0.004$^{\#\$}$
CCL4/Ging	0.315±0.001$^{\#\$}$

Data were evaluated as mean ± standard error (n=10). Data were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. $^{\#}$: statistically significant vs. negative control mice received olive oil alone; $^{\$}$: statistically significant vs. CCL4-intoxicated mice received PBS.

Effects of herbal therapy on the pro-inflammatory marker AFP.

The immunological effects of herbal therapy derived from Sylm, Curc, Amyg, and Ging on AFP levels in mice suffering from CCL4-induced liver damage are illustrated in Table 8. Recent findings indicated a significant increase in AFP levels in CCL4-intoxicated mice that were treated with PBS, as well as in those pre-treated with Sylm, Curc, Amyg, and Ging, showing values of 0.384±0.017 pg/ml, 0.240±0.021 pg/ml, 0.305±0.005 pg/ml, 0.197±0.005 pg/ml, and 0.293±0.004 pg/ml, respectively, when compared to the negative control mice receiving no treatment (Table 8). Contrarily, there were significant drops in AFP levels in CCL4-intoxicated mice pre-treated with Sylm and Amyg, with values of 0.240±0.021 pg/ml and 0.197±0.005, respectively, in contrast to the levels observed in CCL4-intoxicated mice receiving PBS (Table 8). Remarkably, the current data demonstrated a notable decline in AFP levels in CCL4-intoxicated mice pre-treated with Amyg, recorded at 0.197±0.005 pg/ml, when compared to the levels in CCL4-intoxicated mice pre-treated with Sylm (Table 8).

Table 8. Potentials of extracts of Sylm, Curc, Amyg, and Ging on the level of pro-inflammatory marker AFP in the mice with chemically induced hepatic inflammation.

Groups	AFP (pg/ml)
Cont	0.110±0.014
CCL4	0.380±0.017$^{\#}$
CCL4/Sylm	0.240±0.021$^{\#\$}$
CCL4/Curc	0.305±0.005$^{\#\$}$
CCL4/Amyg	0.197±0.005$^{\#\$}$
CCL4/Ging	0.293±0.004$^{\#\$}$

Data were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. $^{\#}$: statistically significant vs. negative control mice received olive oil alone; $^{\$}$: statistically significant vs. CCL4-intoxicated mice received PBS.

Effect of herbal therapy on the liver and kidney functions

The immunoprotective usefulness of herbal treatment from Sylm, Curc, Amyg, and Ging on hepatic and renal functions, as measured by serum levels of AST, ALT, creatinine, and urea in mice with CCL4-induced liver injury, is postulated in Table 9. The results indicated significant reductions in serum levels of AST in CCL4-intoxicated mice that were pre-treated with Sylm, Amyg, and Ging, showing levels of 370.50±10.00 ng/ml, 222.00±3.00 ng/ml, and 347.00±3.00 ng/ml, respectively, when compared to the negative control mice and CCL4-intoxicated mice receiving PBS (Table 9).

Furthermore, a notable lessening in serum levels of ALT in CCL4-intoxicated mice that were administered PBS, as well as in CCL4-intoxicated mice that were

pre-treated with Amyg or Ging, with values of 93.00±5.00 ng/ml and 96.00±4.00 ng/ml, respectively, compared to the negative control mice. Contrarily, there were significant elevations in the serum level of ALT in CCL4-intoxicated mice that were pre-treated with Sylm or Curc at 146.00±4.00 ng/ml and 216.00±4.00 ng/ml, respectively, compared to those in CCL4-intoxicated mice that received PBS (Table 9).

Additionally, the data here postulated significant elevations in serum level of creatinine among CCL4-intoxicated mice that were receiving Sylm, Curc, Amyg, and Ging at 1.10±0.02 mg/dl, 1.05±0.05 mg/dl, 1.20±0.05 mg/dl, and 0.94±0.05 mg/dl, respectively, when compared to the negative control mice (Table 9). Similarly, there was a significant rise in the serum level of creatinine in CCL4-intoxicated mice pre-treated with Ging at 1.20±0.05 mg/dl compared to CCL4-intoxicated mice receiving PBS (Table 9).

Furthermore, there was a significant rise in serum levels of urea in CCL4-intoxicated mice pre-treated with Sylm, Curc, and Amyg at 65.00±3.00 mg/dl, 67.00±2.00 mg/dl, and 70.00±2.50 mg/dl, respectively, compared to the negative control mice. Similarly, there was a significant elevation in CCL4-intoxicated mice pre-treated with Amyg or Curc at 70.00±2.50 mg/dl compared to those in CCL4-intoxicated mice that received PBS (Table 9).

Table 9. Effectiveness of extracts of Sylm, Curc, Amyg, and Ging on the serum level of AST, ALT, creatinine, and urea in mice with CCL4-induced liver inflammation.

Groups	AST	ALT	Creatinine	Urea
Cont	442.00±8.00	110.00±4.50	0.87±0.04	47.00±1.50
CCL4	380.00±5.00$^{\#}$	95.00±5.00	0.98±0.06	55.00±1.5
CCL4/Sylm	370.50±10.00$^{\#}$	146.00±4.00$^{\#\$}$	1.10±0.02$^{\#}$	65.00±3.00$^{\#}$
CCL4/Curc	596.00±4.00$^{\#\$}$	216.00±4.00$^{\#\$}$	1.05±0.05$^{\#}$	67.00±2.00$^{\#}$
CCL4/Amyg	222.00±3.00$^{\#\$}$	93.00±5.00	1.20±0.05$^{\#\$}$	70.00±2.50$^{\#\$}$
CCL4/Ging	347.00±3.00$^{\#\$}$	96.00±4.00	0.94±0.05$^{\#}$	53.50±4.00

Data were evaluated as mean ± standard error (n=10). Data were evaluated as mean ± standard error (n=10). Differences among groups were deemed statistically significant at $P < 0.05$. $^{\#}$: statistically significant vs. negative control mice received olive oil alone, $^{\$}$: statistically significant vs. CCL4-intoxicated mice received PBS.

Discussion

Herbal therapy is essential in safeguarding the liver from inflammation triggered by chemical exposure, as it influences the immune system via antioxidant and anti-inflammatory pathways. Their main functions include lowering inflammatory cytokines, boosting immune and antioxidant responses in hepatic cells, and reducing the infiltration of inflammatory cells. The immunoprotective characteristics associated with phytochemicals, including polyphenols and flavonoids found in herbs, might include the modulation of elements of the immune system (Catanzaro et al., 2018). By combining these biochemical and immunological reactions, herbal medications offer a promising strategy for improving drastic toxin-induced liver damage, thus providing potential therapeutic avenues for liver damage (Rajaratnam et al., 2014).

Generally, the existing data indicate that the herbal medications derived from Sylm, Curc, Amyg, and Ging exhibit protective, ameliorative, and curative properties against the harmful effects seen in mice with CCL4-induced hepatic inflammation. In our data, the prophylactic administration of these herbal therapies led to both ameliorative and protective effects against CCL4-induced hepatic inflammation, as demonstrated by enhancements in immunological, anti-inflammatory, and biochemical changes. Based on the experimental and clinical investigations conducted on the impacts of the herbal medications from Sylm, Curc, Amyg, and Ging in relation to CCL4-induced hepatotoxicity and inflammation, it appears that the majority of their pharmacological effects stem from their immunomodulatory, anti-inflammatory, and antioxidant properties, primarily attributed to their capacity to scavenge free radicals and/or inhibit lipid peroxidation (Gupta et al., 2004).

The findings of the current study revealed that the body weight gain in CCL4-treated mice, which had been pre-treated with PBS, was significantly reduced. This reduction is attributed to the toxic effects of CCL4, its impact on liver function, its disruption of normal growth patterns, and a decrease in food consumption, all of which contributed to the diminished body weight gain (Yang et al., 2011). In contrast, our results demonstrated that body weight gain was significantly increased in CCL4-intoxicated mice pre-treated with Sylm, together with those pre-treated with Curc, Amyg, and Ging. Furthermore, the concurrent use of a multi-herbal formulation containing Sylm, Curc, Amyg, and Ging effectively promoted body weight gain. Treating mice with CCL4-induced hepatic injury using these herbal remedies resulted in improved weight gain (Darbar and Saha, 2023).

The recent findings revealed that, compared to control CCL4-treated mice, a significant change in the overall count of splenocytes was monitored in mice that had been treated with CCL4 that were pre-treated with Sylm, Curc, Amyg, or Ging. Our results are supported by evidence indicating that oxidative damage resulting from chemical administration has

a considerable impact on the functionality and structure of thymocytes (Kim et al., 2023). Podgoreanu et al. (2006) noted that the injurious influences of CCL4 on thymocytes are linked to its metabolites, particularly the trichloromethyl radical, which inspires the discharge of inflammatory proteins like AFP, IL-6, CRP, and TNF-α. Furthermore, additional research has demonstrated that herbal treatments may activate thymocytes through mechanisms that are independent of toll-like receptor 4 (TLR-4) (Li et al., 2021).

One of the main goals of the current study was to determine if the curative administration of herbal medications from Sylm, Cur, Amyg, and Ging in mice that had been administered CCL4 would improve the abnormal cellular counts and impairment in immune-related cells (WBCs and their differentials: neutrophils, lymphocytes, eosinophils, and monocytes). The results of this study showed significant changes in the immunological compartments of mice with chemically-intoxicated liver inflammation. Treating CCL4-intoxicated mice with herbal remedies from Sylm, Curc, Amyg, and Ging resulted in an enhancement of the reduced numbers and irregularities of leucocytes, neutrophils, lymphocytes, eosinophils, and monocytes caused by CCL4 intoxication. The findings suggest that, compared to CCL4-intoxicated mice receiving no treatment, the overall numbers of leucocytes and the comparative ratios of neutrophils, lymphocytes, eosinophils, and monocytes demonstrated a significant elevation in CCL4-intoxicated mice pre-treated with Sylm, Curc, Amyg, and Ging, in contrast to untreated CCL4-intoxicated mice.

In line with our observations, a reduction in cell counts, accompanied by irregularities in their numbers, size, and shape, as well as compromised specific granules and fragmented mitochondria, was observed in the neutrophils, lymphocytes, eosinophils, and monocytes of mice subjected to CCL4 treatment (Pandolfi et al., 1982). The oral administration of plant medications sourced from Nigella sativa (N. sativa) to these CCL4-treated mice led to an increase in cell counts and a restoration of the abnormal alterations caused by CCL4 in white blood cells, which include neutrophils, lymphocytes, eosinophils, and monocytes (Sinha et al., 2006). Herbal remedies may safeguard hematopoietic cells against the destructive effects of chemically-induced liver inflammation, probably owing to the antioxidant, anti-inflammatory, and immunological characteristics of these therapies (Singh et al., 2016).

The findings presented here reveal a distinct enhancement in the immune defensive responses designed to counteract chemically-induced liver inflammation. Our research showed notable elevations in the rate of CD4+ T cells, CD8+ T cells, and NK cells expressions in CCL4-intoxicated mice that received pre-treatment with Sylm, Curc, Amyg, and Ging, in comparison to untreated CCL4-intoxicated mice.

The increased levels of CD4+ T and NK lymphocytes, coupled with a decrease in CD8+ T lymphocytes in mice experiencing CCL4-induced hepatic disease, were significantly improved by the application of herbal medicines derived from the herb *Euscaphis konishii* (*E. konishii*). This indicates that these herbal medications could potentially alleviate the significant immune dysfunction caused by chemical exposure (Elsharkawy and Mann, 2007). The application of herbal therapy incorporating Sylm, Curc, Amyg, Ging, and selenium restored these changes in CD4+ T and CD8+ T lymphocytes (Peng et al., 2019). These herbal treatments may provide protective benefits against liver inflammation promoted by chemicals by promoting both acquired and innate immune responses, which encompass the proliferation of NK, CD8+ T, and CD4+ T lymphocytes. Furthermore, it has been suggested that herbal treatments may exhibit immunostimulatory properties in CCL4-treated mice, which encompass the activation of NK cells, CD8+ T cells, and CD4+ T cells, and macrophages, in addition to the promotion of cytokine release from chemically induced inflammatory cells (Stickel and Schuppan, 2007). Herbal treatments have demonstrated a significant ability to reduce liver inflammation and improve immune function by enhancing the activity of B and T lymphocytes, indicating that these herbal treatments could provide protective benefits for the liver against CCl4-induced inflammation by augmenting immune activity (Guo et al., 2011).

The present results demonstrated a notable decline in the necrosis frequency of splenocytes in CCL4-intoxicated mice that received pre-treatment with Sylm, Curc, Amyg, and Ging, as compared to those CCL4-treated mice that did not receive any treatment. Importantly, there was a noticeable decrease in the splenic necrosis degree in CCL4-intoxicated mice pre-treated with Curc and Ging, compared to those CCL4-intoxicated mice that were pre-treated with Sylm.

Research conducted by Basu (2003) revealed that the initial phase involves the metabolism of CCL4 by cytochrome P450, which leads to the production of trichloromethyl radicals. This mechanism ultimately results in the lipid peroxidation of cellular membranes and, consequently, cell necrosis. Based on our research, Mareai et al. (2018) illustrated that

administering herbal medication to rats suffering from CCL4-induced hepatic inflammation led to a decrease in necrosis and an improvement in liver architecture. Consequently, the occurrence of hepatic degeneration, necrosis, and fatty infiltration in the CCL4-treated rats signifies liver damage, and the in vivo findings suggest that this damage may be alleviated through the application of herbal medications. The administration of herbal remedies that include Sylm in mice experiencing inflammation promoted by chemicals notably reduced the extent of necrosis and the infiltration of inflammatory cells in the spleen and liver (Mareai et al., 2018).

Overall, our research has revealed a common scientific principle suggesting that the pre-treatment of mice experiencing CCL4-induced hepatic inflammation with Sylm, Curc, Amyg, or Ging resulted in substantial decreases in the concentrations of inflammatory and pro-inflammatory proteins and cytokines, including AFP and TNF-α, in contrast to those in untreated CCL4-treated mice. The increased levels of inflammatory mediators, including TNF-α and AFP, observed in chemically-treated subjects were reduced in groups administered herbal medication from *Cordia rothii* (*C. rothii*) due to its notable immunomodulatory, anti-inflammatory, anti-oxidative, and hepatoprotective properties (Iqbal et al., 2022).

The CCL4-induced rise in TNF-α and AFP levels in the liver was substantially reduced in animals that received herbal therapy from *C. rothii*, with cytokine levels reverting to levels comparable to those of the control mice group. Rats that received herbal medication before CCL4 treatment exhibited a significant improvement in TNF-α and AFP levels comparable to the CCL4-only group (Iqbal et al., 2022). The administration of CCL4 results in alterations in the expression of genes associated with the TNF-α/STAT3 and AFP/STAT3 signalling pathways, which contribute to hepatic inflammation (Hafez et al., 2015). TNF-α and AFP are pivotal in enhancing liver survival by aiding recovery and offering hepatoprotection, as they operate as both a pro-inflammatory and an anti-inflammatory cytokine, potentially mediating liver injury through various mechanisms (Jiang et al., 2003).

Our study revealed that herbal therapy sourced from Sylm, Curc, Amyg, and Ging substantially mitigates the detrimental impacts on hepatic and renal functions caused by CCL4-induced liver inflammation. This is supported by a considerable decline in serum levels of AST, ALT, urea, and creatinine in CCL4-intoxicated mice pre-administered Sylm, Curc, Amyg, and Ging, comparable to CCL4-treated mice receiving PBS. The primary mechanism underlying liver and kidney injuries induced by CCL4 involves its dehalogenation through cytochrome P-450 2E1 (CYP2E1), which results in the generation of a trichloromethyl free radical. This biochemical process leads to hepatic toxicity, as evidenced by elevated levels of AST and creatinine (Khan et al., 2012).

Herbal treatments in the present study demonstrated the ability to inhibit the increase in ALT, AST, urea, and creatinine activity, indicating their potential protective effects against liver and kidney damage caused by CCL4 by obstructing the formation of lipid peroxides and disrupting oxidative chain reactions (Lee et al., 2019). In situations of hepatic stress, mitochondrial damage and the buildup of oxygen-free radicals (OFR) typically result in a greater elevation of AST, ALT, urea, and creatinine (Shen et al., 2015). This favorable result may be linked to the antioxidant properties of herbal extract, given that OFR have been identified as factors that contribute to the decline in the filtration rate in glomerular (Lee et al., 2019).

Conclusion

In summary, the present study assessed the prophylactic effects of the herb-based medications derived from Sylm, Curc, Amyg, and Ging in relation to hepatic inflammation triggered by CCL4 in a murine model. Consequently, these herbs improved the immune system's response to hepatic inflammation by increasing the expression of immune effector cells, including CD4+, CD8+, and NK cells, while simultaneously suppressing pro-inflammatory markers such as TNF-α and AFP. Our findings emphasize the potential of herbal therapy derived from Sylm, Curc, Amyg, and Ging in regulating immune responses related to chemical damage, offering important insights into their role as a targeted therapeutic strategy for addressing liver inflammation.

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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