

Original Research Article

Evaluation of immunomodulatory activity of hydroalcoholic extract of *Carum carvi* seeds in cyclophosphamide-induced Swiss albino mice

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Abstract

Objective: *Carum carvi*, a medicinal plant commonly used in traditional medicine, is the focus of this study, which aims to assess the protective effects of its hydroalcoholic seed extract (HECCS) against immunosuppression caused by cyclophosphamide.

Materials and Methods: Thirty Swiss albino mice were used. The control group received normal saline (oral (p.o.)) for 29 days. The cyclophosphamide (CTX) group received CTX (30 mg/kg, intraperitoneal (i.p.)) on the 8th, 15th, and 21st days. The standard group was given levamisole (50 mg/kg, oral (p.o.)) daily for 29 days, combined with CTX (30 mg/kg, intraperitoneal (i.p.)). The treatment groups received HECCS at doses of 200 and 400 mg/kg (oral (p.o.)) daily for 29 days, together with CTX (30 mg/kg, intraperitoneal (i.p.)). On day 30, mice were sacrificed for functional, hematological, biochemical, and histological analyses of blood and spleen.

Results: HECCS contained flavonoids, tannins, terpenoids, saponins, phenols, and carbohydrates. CTX treatment caused a notable reduction in phagocytic activity, neutrophil adhesion, hematological parameters, and Tumor Necrosis Factor Alpha (TNF- α) levels. Conversely, HECCS (200 and 400 mg/kg) showed a dose-dependent increase in phagocytic activity, neutrophil function, and hematological restoration, with elevated TNF- α levels compared with the CTX group) in splenic tissue. These effects were comparable to those elicited by the standard medication levamisole (50 mg/kg). Histological analysis of spleens showed preservation of structural integrity, especially in the 400 mg/kg HECCS group.

Conclusion: HECCS exhibits significant immunostimulatory and hematoprotective properties in CTX-induced immunosuppressed mice, likely due to the synergistic effects of its bioactive phytoconstituents.

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Introduction

The immune system is a complex, interconnected network of cells, tissues, and organs (Catanzaro et al. 2018). When faced with challenges like infections or tissue damage, resident immune cells in the skin and those migrating from the periphery work together to form a complex defense system. This system aims to reduce injury and restore the tissue to its original state (Nguyen and Soulika 2019). Immunomodulation comprises two primary approaches: immunostimulants enhance the body's resistance to infections by enhancing the baseline response of the immune system, especially in individuals with immune deficiencies (Mandal et al. 2023) and immunosuppressants affect multiple facets of the immune response, notably lymphocyte activity and the regulation of the humoral response, such as altering antibody titers and affinity levels (Strzelec et al. 2023). Numerous chronic illnesses such as inflammatory and autoimmune diseases, are linked to immune system dysfunctions or imbalances, highlighting the importance of immunomodulation (Wan-Nor-Amilah et al. 2021).

Cyclophosphamide (CTX), a prominent alkylating agent, is commonly used in preclinical models to cause immunosuppression by damaging DNA. It destroys immune cells, hampers the growth and development of B and T cells, and reduces both humoral and cellular immune responses (Chen et al. 2019; Liu et al. 2018). Reports indicate that oxidative stress-induced changes in redox balance after CTX exposure result in biochemical and physiological disturbances (Pratheeshkumar and Kuttan 2010).

Modulating the immune system with plant-based products is an effective strategy for treating and preventing immune system disorders because of their various biological activities, good tolerability, and high patient compliance (Di Sotto et al. 2020). Due to their structural diversity and multi-targeted action, natural products remain a rich source of immunomodulators

with good safety profiles (Saklani and Kuttu 2008). Various plants have the potential to modulate the immune system through mechanisms like antioxidant, anti-inflammatory, and anti-cancer activity, which regulate cytokines, and boost the body's immune responses (Alhazmi et al. 2021).

Carum carvi (Caraway) is a member of the Apiaceae family. It has been extensively utilized as a vegetable, aromatic spice, preservative, and in traditional herbal medicine (Liu et al. 2023). This plant has been historically utilized for an extended period for numerous medical conditions including digestive disturbances such as flatulence, anorexia, bloating, heartburn, and minor spasms of the stomach and intestines (Hajlaoui et al. 2021). Caraway fruits contain essential oils, fatty acids such as petroselinic, linoleic, and oleic acids, along with carbohydrates, proteins, phenolic acids like caffeic acid, and flavonoids including quercetin and kaempferol. Additionally, it has monoterpenes like carvone and limonene, which often constitute 95% of the essential oil and are accountable for its antioxidant, antibacterial, anti-inflammatory, diuretic, and antifungal properties (AbdElgawad et al. 2021; Aly et al. 2023; Saremi et al. 2024). Oxidative stress and inflammatory dysregulation significantly contribute to CTX-induced immunosuppression and hematological damage (Ibrahim et al. 2024). Prior research has shown that carvone and limonene can influence inflammatory mediators, diminish oxidative damage, and enhance cellular defense mechanisms (AlSaffar et al. 2022; Sousa et al. 2023). Hence, *C. carvi* may restore immunological competence and hematological parameters in CTX-induced immunosuppression due to its pharmacological qualities.

Many therapeutic plants and natural medicines (Barakat et al. 2023; Rezaei et al. 2025) show immunorestorative properties in CTX-induced immunosuppressed animal

models. Polysaccharides, alkaloids, flavonoids, and phenolic compounds increase leukocyte count, cytokine production, antioxidant levels, and lymphoid organ protection against CTX. These findings suggest that plant-derived bioactive chemicals may treat immunosuppression (El-Yagoubi et al. 2026; Zhang et al. 2025; Zhu et al. 2025). Despite the widespread use of caraway seed in ethnomedicine and its pharmacological potential, there is a lack of preclinical investigations assessing its immunomodulatory and hematoprotective efficacy in a CTX-induced immunosuppressed model (Johri 2011). Although previous studies have focused on polysaccharides and isolated phytochemicals, there has been limited research into whole extracts containing multiple bioactive constituents. Furthermore, the role of caraway seeds in modulating innate immune response, regulating cytokine expression, and splenic architecture remains insufficient.

This work aims to address a significant gap by examining the Hydroalcoholic extract of *Carum carvi* seed (HECCS) in CTX-induced immunosuppressed mice using functional, hematological, biochemical, and histological assessments to scientifically validate its traditional application and explore its potential as a plant-derived immunorestorative agent.

Materials and Methods

Chemicals

Levamisole was procured from Khandewal Pharmaceutical Ltd., Mumbai, India, and CTX was obtained from Getwell Oncology Pvt. Ltd., India. Sodium carbonate (Na_2CO_3) was sourced from MSN Laboratories, Chennai, India. Pelikan 4001 (Brilliant Black) ink was used in the

study. All other chemicals and reagents used were of analytical grade.

Experimental animals

30 Male Swiss albino mice, aged 8-10 weeks and weighing 25-35 g, were employed in the present study. During the acclimation process, rodents that were sick or abnormal were eliminated. The animals were acquired from MASS BIOTECH in Chennai, India. They were transferred and maintained in polypropylene cages under standard laboratory conditions (25–30°C, 35–45% relative humidity, 12-hr light/dark cycle) throughout the course of the investigation. The mice had free access to standard pellets and clean water. The Institutional Animal Ethical Committee (IAEC) has approved all experimental protocols in accordance with ethical standards (protocol approval number: 05/321/PO/Re/S/01/CPCSEA/dated 24/11/23). Additionally, we implemented precautions to mitigate animal discomfort throughout the investigation. We assessed the animals' general condition, behavior, and presence of distress or illness on a daily basis. All investigations were conducted in accordance with the ARRIVE guidelines (<https://arriveguidelines.org>).

Collection and preparation of *C. carvi* seed extract

The fresh seeds of *C. carvi* were procured from Madipakkam, India, and authenticated by Dr. K.N. Sunil Kumar, Research Officer/Sci-II, Head of the Department of Pharmacognosy at the Siddha Central Research Institute, Arumbakkam, Chennai, India, with voucher code: C18122304C. Fresh *C. carvi* seeds were washed and shade-dried at room temperature to eliminate any residual moisture. Seeds were coarsely pulverized using an electrical grinder. For the purpose

of extraction, the powder was stored in a sealed container.

Extraction procedure of *C. carvi* seeds

The pulverized seeds were extracted using the Soxhlet apparatus in the hot continuous extraction system, with a (70:30 v/v) ethanol: water mixture serving as the solvent. *Carum carvi* material, weighing 900 g, was extracted with 2 L of solvent at 55°C for 42 hr. Approximately 168 extraction cycles were performed until the siphoning solvent became almost colourless. The extract was concentrated using a rotary evaporator following the filtration process. The desiccated extract residue was meticulously weighed and stored for future use after a 30-min cooling period in the desiccator (Seddighfar et al. 2020). The percentage yield was calculated using the following formula:

Yield (%) = (Weight of dried extract / Weight of dried plant material) × 100 (Mada et al. 2022)

Experimental design

Thirty healthy male Swiss albino mice were used in this investigation. The total number of mice in each group used for the experiment (n = 6 per group) was determined based on prior studies employing the identical model of CTX-induced immunosuppression (Yan et al. 2021; Zhou et al. 2024). Animals were assigned to treatments using a simple

randomization process. After a one-week acclimatization under standard laboratory conditions, the mice were randomly assigned to five experimental groups (n = 6 per group).

Group I (Normal Control): Received normal saline orally (p.o.) for 29 consecutive days.

Group II (CTX Control): Received CTX (30 mg/kg, i.p.) on days 8, 15, and 21.

Group III (Standard control): Received levamisole (50 mg/kg, p.o.) daily for 29 days along with CTX (30 mg/kg, i.p.) on days 8, 15, and 21.

Group IV (HECCS 200 mg/kg): Administered HECCS at 200 mg/kg (p.o.) daily for 29 days along with CTX (30 mg/kg, i.p.) on days 8, 15, and 21.

Group V (HECCS 400 mg/kg): Administered HECCS at 400 mg/kg (p.o.) daily for 29 days along with CTX (30 mg/kg, i.p.) on days 8, 15, and 21.

On day 29, the animals fasted for 24 hr before being sacrificed. On day 30, the mice were euthanized via a CO₂ chamber. Blood samples were collected through cardiac puncture and tail vein collection, and thereafter kept at −80°C for biochemical and immunological analysis. The spleen was excised as the primary immunological organ for further evaluation (Kumar and Venkatesh 2016). Figure 1 provides a schematic presentation of the experimental design.

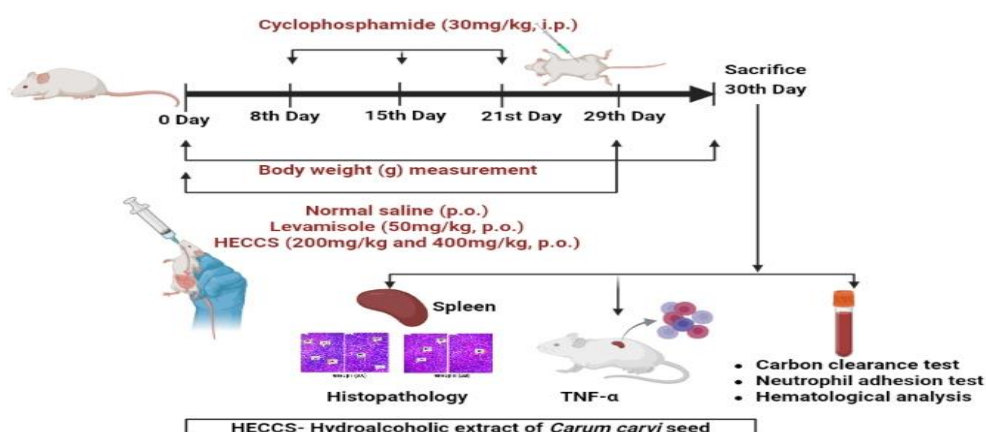


Figure 1. Schematic presentation of the experimental protocol for evaluating the immunomodulatory effects of HECCS in CTX-induced mice.

Preliminary phytochemical screening

The extract was subjected to a qualitative phytochemical analysis to detect the presence of secondary metabolites such as tannins (Braymer's test), flavonoids (NaOH test), carbohydrates (Molisch's test), terpenoids (Salkowski's test), saponins (Froth test), phenols (Folin-Ciocalteu test), glycosides (Bontrager's test) and alkaloids (Hager's test) (Dubale et al. 2023; Pant et al. 2017).

Carbon clearance test

On the 30th day of the study, mice from each group were randomly chosen for the carbon clearance test. A 0.1 mL dosage of Pelikan 4001 (Brilliant Black) ink, employed as the carbon particle suspension, was administered intravenously through the tail vein. Blood samples (25 µL) were obtained at 5 min (t₁) and 15 min (t₂) post-injection. Each sample was promptly diluted in 2 mL of a 0.1% (w/v) sodium carbonate (Na₂CO₃) solution to inhibit further phagocytic activity. The absorbance of the resultant solution was quantified at 650 nm utilizing a UV-Visible spectrophotometer.

The phagocytic index, presented as the carbon clearance rate (K value), was calculated using the following formula:

$$K = (\ln OD_1 - \ln OD_2) / (t_2 - t_1)$$

where OD₁ and OD₂ represent the optical densities at 5 and 15 min, respectively, and (t₂-t₁) denotes the time interval between the two measurements (Xie et al. 2023).

Neutrophil adhesion test

On the 30th day, blood samples were collected from the tail vein of animals in all groups and collected into heparinized vials. The total leukocyte count (TLC) and differential leukocyte count (DLC) were evaluated by preparing blood smears and staining them with Field Stain I and II (Leishman's stain). The samples were then treated with nylon fibers (80 mg/mL) at 37 °C for 15 min. Following incubation, TLC

and DLC were reassessed (Suresh Gupta et al. 2006).

The neutrophil index and neutrophil adhesion percentage were calculated according to the following formulas:

$$\text{Neutrophil Index (NI)} = \text{Total leukocyte count (TLC)} \times \% \text{ Neutrophils}$$

$$\text{Neutrophil Adhesion (\%)} = \text{NI}_{\text{u}} - \text{NI}_{\text{t}} \times 100 \div \text{NI}_{\text{u}}$$

NI_u represents the neutrophil index before the incubation with nylon fibers, whereas NI_t denotes the neutrophil index after the incubation (Obi et al. 2018).

Hematological analysis

For hematological analysis, blood samples were drawn by cardiac puncture after sacrifice into heparinized tubes on the 30th day. An auto-analyzer was utilized for analyzing hematological parameters such as the concentration of red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb) level, and platelet count (Raj and Gothandam 2015).

Determination of Tumor Necrosis Factor Alpha (TNF-α) in the mouse spleen

On the 30th day, the mice were euthanized by CO₂ chamber prior to tissue collection. The spleens were excised, weighed, and immediately kept in Eppendorf tubes at -80°C. Levels of TNF-α in splenic tissues were quantified using an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol. The minimum detectable dose of mouse TNF-α is 18.75pg/mL. Absorbance was quantified at 450 nm utilizing a microplate reader, and TNF-α values were derived from a standard calibration curve (Elabscience®, TNF-α ELISA Kit; Catalogue No. E-EL-M0049) (Yacoub et al. 2018).

Histopathological analysis of the mouse spleen

After euthanasia, the spleens were aseptically harvested on day 30 and stored for 24 hr in 10% neutral-buffered formalin.

Following a standard dehydration procedure, the tissues were preserved in paraffin. Sections were cut with a microtome into 4 µm-thick pieces. The sections were stained with hematoxylin and eosin (H&E) (Zhang et al. 2023) with hematoxylin highlighting the nuclei and eosin highlighting the cytoplasm (Li et al. 2024). Using a light microscope, the stained slides were examined to assess any general histological changes (Hamouda et al. 2022).

Statistical analysis

Statistical analyses were performed utilizing one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. The data are presented as the mean ± standard error of the mean (SEM). All analyses were conducted using GraphPad Prism software (version 10.2.2) on a Windows operating system. A p-value of less than 0.05 was considered statistically significant.

Results

Percentage yield of HECCS

Following Soxhlet extraction of 900 g of coarsely powdered *C. carvi* seeds, a dark green extract with a percentage yield of 22% (w/w) was obtained.

Phytochemical screening of HECCS

The phytochemical analysis of the HECCS revealed the presence of tannins, flavonoids, carbohydrates, terpenoids, saponins, and phenols, while glycosides and alkaloids were not detected. This suggests that the immunomodulatory effects of HECCS may result from the synergistic interactions among its bioactive compounds.

Effect of HECCS on carbon clearance

A carbon clearance test was conducted in mice to investigate the impact of HECCS on the reticuloendothelial system and innate immunity (Table 1). The phagocytic index of the CTX-treated group exhibited a significant reduction in phagocytic activity ($p < 0.0001$) compared to the control. The phagocytic index for the 200 and 400 mg/kg HECCS-treated group, showing significant improvements compared to the CTX group ($p < 0.0001$). A dose-dependent effect was observed, with higher doses restoring phagocytic function. The findings suggest that HECCS significantly enhances reticuloendothelial system function in immunosuppressed animals, possibly due to immunostimulatory mechanisms.

Table 1. Effect of HECCS on carbon clearance and neutrophil adhesion

Groups treated	Phagocytic index	Neutrophil adhesion %
Control	0.1450±0.0006	36.16±0.045
CTX control	0.0084±0.0001 <i>a</i> ****	10.79±0.035 <i>a</i> ****
Standard control	0.0590±0.0001 <i>a</i> **** <i>b</i> ****	36.70±0.20 <i>b</i> ****
HECCS 200 mg/kg	0.0492±0.0002 <i>a</i> **** <i>b</i> ****	17.28±0.025 <i>a</i> **** <i>b</i> ****
HECCS 400 mg/kg	0.0556±0.0002 <i>a</i> **** <i>b</i> ****	28.64±0.43 <i>a</i> **** <i>b</i> ****

Data are presented as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. "a" indicates comparison with the normal control group (Group I), whereas "b" indicates comparison with the cyclophosphamide-treated group (Group II). Statistical significance is expressed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. CTX = cyclophosphamide; HECCS = hydroalcoholic extract of *Carum carvi* seeds.

Effect of HECCS on neutrophil adhesion

The effect of HECCS on neutrophil adhesion was assessed in mice to investigate its immunomodulatory effects on the innate immune system (Table 1).

CTX treatment led to a significant reduction in neutrophil adhesion ($p < 0.0001$) compared to control, indicating immunosuppression. Treatment with 200 and 400 mg/kg HECCS led to a significant

restoration of neutrophil adhesion ($p<0.0001$) compared to the CTX group. An enhancement associated with dose was observed, with the increased dose approaching standard adhesion values. The results demonstrate that HECCS efficiently alleviates the inhibition of neutrophil adhesion induced by CTX.

Effect of HECCS on WBC, hemoglobin, RBC and platelet

CTX-induced myelosuppression was confirmed by significant changes in hematological parameters. Table 2 shows that the CTX group experienced significant reductions in WBC, Hb, RBC, and platelet counts compared with control group, confirming effective induction of myelosuppression ($p<0.0001$) for WBC, RBC, and platelets, and ($p<0.01$) for Hb.

Treatment with 200 mg/kg HECCS resulted in partial recovery of hematological parameters. WBC and Hb levels were significantly reduced. In contrast, RBC levels and platelet count showed significant recovery compared to the CTX group ($p<0.0001$). The 400 mg/kg HECCS exhibited a significant protective effect, indicated by increased levels of WBC, Hb, RBC, and platelets in comparison to the CTX group ($p<0.0001$ for WBC, RBC, and platelets and $p<0.05$ for Hb). The findings demonstrate a dose-dependent recovery of hematological parameters. The administration of HECCS mitigated CTX-induced myelosuppression, with a more pronounced hematoprotective effect observed at 400 mg/kg treated group.

Table 2. Effect of HECCS on WBC, Hemoglobin, RBC and platelet

Groups treated	WBC (cell/mm ³)	Hb (g/dL)	RBC (million/mm ³)	Platelet (lakh/mm ³)
Control	6705±5.00	14.55±0.250	10.49±0.015	8.680±0.032
CTX control	1058±2.50 <i>a</i> ****	10.75±0.550 <i>a</i> **	7.810±0.040 <i>a</i> ****	1.857±0.026 <i>a</i> ****
Standard control	1763±2.50 <i>a</i> **** <i>b</i> ****	13.50±0.300 <i>b</i> *	9.485±0.035 <i>a</i> **** <i>b</i> ****	6.380±0.037 <i>a</i> **** <i>b</i> ****
HECCS 200 mg/kg	1091±1.00 <i>a</i> ****	9.100±0.200 <i>a</i> ***	5.465±0.035 <i>a</i> **** <i>b</i> ****	2.747±0.059 <i>a</i> **** <i>b</i> ****
HECCS 400 mg/kg	1667±16.50 <i>a</i> **** <i>b</i> ****	12.35±0.250 <i>a</i> *	8.385±0.065 <i>a</i> **** <i>b</i> ***	5.017±0.053 <i>a</i> **** <i>b</i> ****

Data are presented as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. "a" indicates comparison with the normal control group (Group I), whereas "b" indicates comparison with the cyclophosphamide-treated group (Group II). Statistical significance is indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. CTX- cyclophosphamide, HECCS- Hydroalcoholic extract of *Carum carvi* seed.

Effect of HECCS on TNF- α in the mouse spleen

The effect of HECCS on TNF- α levels in the spleen was assessed in mice to evaluate its immunomodulatory potential (Table 3). Mice treated with CTX exhibited a significant reduction in TNF- α levels ($p<0.0001$) compared to the control group, indicating immunosuppression. Treatment with HECCS 200 ($p<0.01$) and 400 mg/kg ($p<0.0001$) significantly restored TNF- α levels compared to the CTX group. This restoration demonstrates a dose-dependent immunostimulatory effect of HECCS on

cytokine production in the spleen, effectively alleviating CTX-induced immunosuppression.

Histopathological examination of the spleen

Histopathological examination of spleen tissues using H&E staining is shown in Figure 2A–E. The control group (Group I, Figure 2A) exhibited normal splenic architecture with well-defined white pulp (WP), central arterioles (CA), red pulp (RP), and a limited number of splenic macrophages (SM). In the CTX-treated

group (Group II, Figure 2B), spleen sections exhibited a significant decrease in lymphocytes in the WP and marginal zones. The WP exhibited a reduction in size, characterised by a decreased number of SM and a less defined RP, suggesting immunosuppression and structural damage. Group III (Figure 2C) exhibited a partial restoration of splenic architecture. In the 200 mg/kg HECCS group (Group IV, Figure 2D), the splenic structure remained intact. WP and RP were similar to those in the control group, although SM exhibited

sparse distribution. In the 400 mg/kg HECCS group (Group V, Figure 2E), mild enlargement of the WP was noted, accompanied by a well-defined CA. The RP maintained structural integrity, and there was an increased presence of SM. A partial restoration of normal splenic architecture was observed in the high dose, suggesting a dose-dependent protective effect. The findings demonstrate that HECCS effectively mitigated spleen damage caused by CTX.

Table 3. Effect of HECCS on TNF- α in the mice spleen

Groups treated	TNF- α (pg/ml)
Control	276.7 $\pm$ 2.90
CTX control	117.0 $\pm$ 3.78 <i>a****</i>
Standard control	656.3 $\pm$ 4.09 <i>a**** b****</i>
HECCS 200 mg/kg	310.0 $\pm$ 8.32 <i>a** b****</i>
HECCS 400 mg/kg	378.0 $\pm$ 4.61 <i>a**** b****</i>

Data are presented as mean $\pm$ SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. "a" indicates comparison with the normal control group (Group I), whereas "b" indicates comparison with the cyclophosphamide-treated group (Group II). Statistical significance is indicated as follows: ***p*<0.01, *****p*<0.0001. . CTX- cyclophosphamide, HECCS- Hydroalcoholic extract of *Carum carvi* seed.

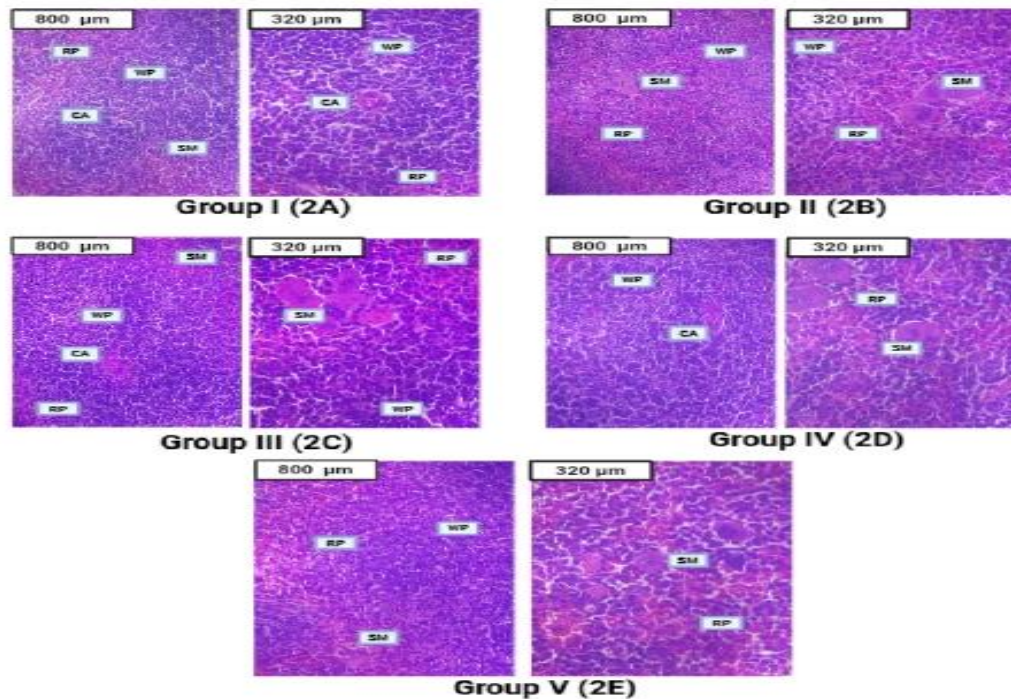


Figure 2. Histopathological analysis of mice spleen tissues stained with hematoxylin and eosin (H&E). (3A) Control group treated with normal saline; (3B) CTX-treated group; (3C) Levamisole-treated group; (3D) Group treated with 200 mg/kg of HECCS; (3E) Group treated with 400 mg/kg of HECCS. Scale bar – 800 μ m and 320 μ m. WP- White pulp, RP- Red pulp, SM- Splenic macrophages and CA- central arterioles.

Discussion

The immune system is a complex network of cells, tissues, and signalling molecules that defends the host against harmful pathogens through innate and adaptive responses (Nam et al. 2022). Its impairment is due to malnutrition, infections, environmental stress, and chemotherapy, which increase susceptibility to infection (Kim et al. 2022; Qu et al. 2022). This study investigates the immunomodulatory potential of HECCS in CTX-induced mice. *Carum carvi*, a medicinal plant, possesses anti-oxidant, anti-inflammatory and anti-cancer properties, suggesting a potential for immunomodulation (Mahboubi 2019). Our findings show that HECCS mitigates the CTX-induced immunosuppression by improving various immune functions, such as phagocytic activity, macrophage function, the adherence of neutrophils, hematopoiesis and upregulating TNF- α expression. This multitargeted approach may offer broader therapeutic potential, especially in chemotherapy-induced immunosuppression.

The preliminary phytochemical analysis of HECCS shows the presence of bioactive components such as flavonoids, tannins, terpenoids, saponins, phenols, and carbohydrates. Among these, flavonoids, terpenoids, and saponins are recognized for their immunomodulatory potential. Flavonoids in HECCS help to reduce oxidative stress caused by CTX by removing harmful free radicals in the bone marrow, which is important for the survival and improves the survival of hematopoietic progenitor cells in cytotoxic environments (Daw and Law 2021). Terpenoids may also enhance antibody production and regulate T-cell responses (Venkatalakshmi et al. 2016). Saponins may have immunomodulatory effects by activating the NF- κ B and JAK/STAT signalling pathways (Zhu et al. 2023); hence, they induce levels of Interleukin (IL)-2, TNF- α , and Interferon-gamma (IFN- γ) (Nalbantsoy et al. 2012). Significantly, HECCS has

been determined to be non-toxic at oral doses of up to 2000 mg/kg, with no indications of toxicity or mortality in rat (Obeid et al. 2020).

CTX is a widely used cytotoxic agent (Fathima et al. 2022) that induces DNA damage and inhibits the proliferation and differentiation of immunocompetent cells (Berköz et al. 2021; Huyan et al. 2011). In addition, CTX induces gastrointestinal toxicity which encompasses anorexia, vertigo, increased caloric intake, and muscle and fat atrophy (Wang et al. 2016).

The reticuloendothelial system (RES) comprises macrophages and other phagocytic cells, playing a crucial role in immunological surveillance against invading pathogens (Yona and Gordon 2015). The functional condition of the RES is often assessed by the clearance of foreign substances such as colloidal carbon, which serves as an indicator of phagocytic activity and immunological response (Labed et al. 2016; Mbaaji et al. 2020). CTX significantly suppressed RES activity by reducing the phagocytic index (Qi et al. 2018). HECCS treatment reversed this inhibition in a dose-dependent manner, restoring activity to its normal level at the optimal dose of 400 mg/kg. HECCS may regenerate reticuloendothelial system function owing to the immunomodulatory and antioxidant bioactive constituents of *C. carvi* such as carvone and limonene. These monoterpenes could enhance macrophage activation and phagocytic function, similarly to the actions of thymoquinone, p-cymene, α -pinene, and carvacrol in *Nigella sativa*, resulting in enhanced carbon clearance and phagocytic index in CTX-immunosuppressed mice.

Neutrophils are key components of the innate immune response, and their adherence to nylon fibers serves as a marker of their activation state (Alberts et al. 2024; Nfambi et al. 2015). This method relies on interactions between integrins on the neutrophil surface and ligands on endothelial cells, such as intracellular adhesion molecules (ICAMs). Integrins are

sequestered in cytoplasmic granules in a resting state, while L-selectins are perpetually expressed on the surface of neutrophils. Upon activation, the integrins are presented on the cell surface, enabling enhanced adherence to the endothelium (Rasheed et al. 2016). CTX significantly inhibited neutrophil adherence, indicative of impaired leukocyte-endothelial interaction (Kurnijasanti et al. 2024). HECCS significantly enhanced neutrophil adherence in a dose-dependent manner. This improvement may be mediated by MAPK and NF- κ B pathways which control the production of adhesion molecules and the activation of neutrophils (Luo et al. 2010). HECCS may thereby enhance neutrophil-endothelial interactions crucial for pathogen clearance. HECCS' phytochemicals such as flavonoids, phenolics, and terpenoids, improve neutrophil adherence. Similar to the findings of (El Fager et al. 2022), these phytochemicals may activate neutrophils and improve leukocyte-endothelial interactions, thereby increasing neutrophil trafficking and boosting innate immune responses in CTX-immunosuppressed animals.

The hematopoietic microenvironment is essential for the production and maintenance of immune cells. It comprises bone marrow stromal cells, hematopoietic stem cells (HSCs), cytokines, metabolic cellular byproducts, and extracellular matrix, with HSCs serving as progenitors of immune cells and facilitating B-lymphocyte development. (Gao et al. 2023; Mbaaji et al. 2020). CTX-induced myelosuppression resulted in a significant decrease in WBC, RBC, platelets, and hemoglobin levels, indicating hematopoietic toxicity (Fei et al. 2022). The mice that were administered HECCS demonstrated a dose-dependent enhancement in hematological profile, with the 400 mg/kg dose resulting in a notable increase. The hematoprotective properties of HECCS may result from its antioxidant capacities and the stimulatory effects of its

phytochemicals on HSCs proliferation (Hajlaoui et al. 2021). The hematological recovery observed after HECCS treatment may be related to its phytochemical components, notably flavonoids, phenolics, and terpenoids. Consistent with the findings of (Tariq et al. 2024), these phytochemicals might protect hematopoietic tissues from oxidative injury and facilitate the proliferation and differentiation of hematopoietic stem cells, thus reinstating WBC, RBC, platelet, and hemoglobin levels in CTX-treated animals.

Cytokines are pivotal in cellular communication within the immune system, and regulating the lymphoid, inflammatory, and HSCs to sustain and restore homeostasis (Wang et al. 2016). CD4⁺ T cells include type 1 T helper (Th1) cells, which mostly release IFN- γ and TNF- α , facilitating cell-mediated immune responses, and type 2 T helper (Th2) cells, which largely secrete IL-4, IL-6, and IL-10, promoting humoral responses (Noh et al. 2019). Among these cytokines, TNF- α serves as a crucial mediator in antibacterial defense by enhancing the bactericidal activity of neutrophils (Giai et al. 2013). Furthermore, treatment with CTX markedly reduced TNF- α levels in the spleens, indicating a molecularly targeted silencing of essential pro-inflammatory pathways in the trigeminal ganglion (Liu et al. 2019). The HECCS at 400 mg/kg reversed this effect by restoring TNF- α to baseline through CD4⁺ T cells activation. The reestablishment of TNF- α expression by HECCS may be primarily due to L-carvone, a principal bioactive component of *C. carvi*. Prior research indicates that L-carvone safeguards bone marrow cells from toxic damage by diminishing oxidative stress and regulating inflammatory pathways. Consequently, L-carvone may facilitate the restoration of cytokine production and immune cell functionality after CTX-induced immunosuppression (Kong et al. 2024).

Histopathological investigation confirmed the biochemical and functional

findings. The spleens of CTX-treated mice exhibited significant architectural disturbance, lymphocyte loss, and disruption of both red and white pulp (Pan et al. 2022). The HECCS treatment conserved splenic architecture in a dose-dependent manner, with the 400 mg/kg group exhibiting superior macrophage infiltration and white pulp integrity, indicative of enhanced immune activity. This histological evidence reinforces the immunorestorative capabilities of HECCS and its subsequent therapeutic efficacy. The semi-quantitative histology rating may enhance the objectivity of architectural preservation assessment in future studies.

Although the results are encouraging, there are some limitations. The molecular mechanisms that govern the immunomodulatory effects of HECCS are not yet elucidated. While NF- κ B, MAPK, and JAK/STAT pathways are proposed as plausible mechanisms according to other publications on phytochemical activity, their direct involvement was not investigated in this work. Future research utilizing molecular and protein-expression analyses is necessary to verify if these pathways are responsible for the noted immunorestorative effects. TNF- α was analyzed as a model pro-inflammatory cytokine; it is necessary to include the measurement of various other cytokines to gain a more comprehensive understanding of the immunoregulatory activities of HECCS. To establish HECCS as a clinically applicable immunotherapy, prospective mechanistic studies and pharmacokinetic characterization are required.

The current study indicates that the HECCS has remarkable immunorestorative properties in an animal model of CTX immunosuppressed mice. HECCS stimulated innate immune responses, such as enhanced phagocytic activity, neutrophil adhesion, and macrophage function, while promoting hematological healing and counteracting CTX-induced decrease in TNF- α . These reactions are likely related to

the synergistic effects of different bioconstituents, like flavonoids, saponins, terpenoids, phenols, and their previously reported antioxidant, anti-inflammatory and anticancer properties, which regulate oxidative stress, inflammatory signalling, and cytokine pathways (Mahboubi 2019). Histopathological alterations further support the immunoprotective capacity of HECCS, suggesting it as a promising choice for mitigating chemotherapy-induced immunosuppression. Further research is essential to identify the biologically active components and clarify their specific mechanisms of action, which will enhance our understanding of the immunopharmacological significance of *C. carvi*.

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Conflicts of interest

No conflicts of interest are declared by the authors.

Ethical Considerations

Experimental animals were acquired from the animal facility of 'Mass Biotech', Chennai, India. The Institutional Animal Ethical Committee at C.L. Baid Metha College of Pharmacy in Chennai, India, authorized this work. Approval number: 05/321/PO/Re/S/01/CPCSEA/dated 24/11/23. Throughout the trial, every measure was made to minimize the animals suffering.

Authors' Contributions

MG was responsible for gathering essential materials, designing and executing the experiments, conducting data analysis,

and drafting the manuscript. AV contributed to the execution of the research work. PA provided conceptual guidance, oversight throughout the study, and critical revisions of the manuscript. All authors reviewed and approved the final version of the manuscript.

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