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Suppressive effect of lemon (*Citrus limon* L. Burm. f.) extract toward pro-tumor immune response in DMBA-induced carcinogenesis mouse

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Abstract

Objective: Cancer is a worldwide health issue which recognized as a chronic condition resulting from the unregulated growth of abnormal cells. Breast cancer has overtaken lung cancer as the most often diagnosed malignancy among all genders, with a projected three million new cases by 2040. Many treatment options are available, however, latest therapies are associated with undesirable side effects. *Citrus limon* is among the medicinal plants recognized to have immunomodulatory properties. This research aimed to examine the immunomodulatory effect of *C. limon* extract (CLE) in 7,12-Dimethylbenz[a]anthracene (DMBA)-induced carcinogenesis mice.

Materials and Methods: The treatment group consisted of four subgroups: a vehicle control group, a DMBA induction group, a CLE 50 group, and a CLE 200 group. Flow cytometry was used to assess the proportions of the following cell populations: Gr-1$^+$, CD68$^+$IL-17$^+$, CD68$^+$TNF-α$^+$, NK1.1$^+$, CD4$^+$CD25$^+$, and CD4$^+$CD25$^+$CD62L$^+$.

Results: In this study, we discovered that CLE reduces the number of immune system profiles to normal levels, including granulocytes, macrophages, natural killer cells, and effector T cells, while increasing the population of regulator T cells to normal levels. Moreover, the absorption, distribution, metabolism, excretion, and toxicity (ADMET) evaluation showed that several significant CLE compounds meet the drug-like requirements.

Conclusion: This research revealed that CLE might be developed as a supplemental or additional cancer treatment.

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Introduction

The intricate interplay between cancer and inflammatory responses has been a comprehensive scientific investigation focal point. Chronic inflammation, marked by sustained and dysregulated immune activation, is increasingly acknowledged as a pivotal factor in cancer development, including initiation, promotion, and progression (Zhao et al. 2021). In this dynamic landscape, regulatory immune cells emerge as central orchestrators in modulating the complex milieu of cancer progression. These cells exert immunosuppressive effects within the tumor microenvironment, delicately balancing the need to control the inflammatory responses exerted by pro-inflammatory immune cells and components (Labani-Motlagh et al. 2020). Immune cells like natural killer (NK) cells and granulocytes can either help stop tumors from forming or help them grow depending on the type of tumor and which immune cells are involved (Qu et al. 2018; Ogura et al. 2018).

Recognizing and eliminating aberrant cells, including cancer-associated ones, is one of the critical roles that the immune system has. Harnessing and controlling the immune system's power for cancer therapy has emerged as a revolutionary approach in oncology (Kumar et al. 2021). Immunotherapy, a burgeoning field, targets cancer cells through specific biomarkers, offering a promising alternative to conventional treatments with potentially severe side effects (Dobosz and Dzieciątkowski 2019). However, the underlying molecular pathways contributing to immune escape and responses, particularly in breast cancer which ranks among the most prevalent cancers, must be fully understood (Arnold et al. 2022; Steven and Seliger 2018). Within this complex milieu, bioactive substances, notably flavonoids, stand out for their well-documented immunomodulatory characteristics. These compounds are believed to exert their influence by activating innate immune responses. Specifically, they have been associated with activating NK cells, suppressing M2-type macrophage activation, and facilitating the conversion from M2 to M1, thereby orchestrating a nuanced interplay within the immune landscape. These compounds exhibit a dual regulatory role by activating innate immune responses while concurrently influencing adaptive immune responses by regulating Treg cell proliferation and enhancing Cytotoxic T cell (CTL) activity. Despite their recognized potential, the precise mechanisms underlying flavonoids' actions remain subjects of debate, necessitating further investigation (Han et al. 2022).

Research exploring the immunostimulatory potential of lemon extract (*Citrus limon*), an Asian native plant, adds a layer of intrigue to this narrative. Studies have indicated that the flavonoid content ethanol extract in the crude lemon may not only dose-dependently reduce cell survival against human leukemia HL-60 cells but also exhibit immunostimulatory properties by augmenting mouse splenocyte proliferation (Diab 2016). Previous studies have demonstrated that aqueous extract of lemon could promote immunomodulation to counter the unwanted effect of carcinogenesis in breast cancer models (Putra et al. 2023).

However, there are minimal reports related to the immunosuppressive activity of lemon extract toward the immune cells, especially in the cancer condition. We suggest that lemon extract might contribute to modulating the immune system, whether it increases or suppresses the immune cells which then determine the cancer progression level.

Therefore, in the present study, we aim to evaluate the immunomodulatory effects of lemon extract on pro-tumor immune responses using a 7,12-Dimethylbenz(a)anthracene (DMBA)-induced breast cancer mouse model. Finally, the findings from this work might

provide insights, especially on the benefit of lemon extract as immunomodulation that helps ameliorate cancer progression.

Materials and Methods

Chemicals and reagents

DMBA was purchased from Tokyo Chemical Industry and was administered to the mice after being diluted in corn oil. All antibodies used in this study including Gr-1, CD68, CD4, CD25, CD62L, and NK.1.1 were purchased from BioLegend.

Plant collection and extraction

Lemons were collected from the East Java region of Indonesia. To prepare the crude lemon extract (CLE), the fruit flesh was chopped into small pieces, blended until smooth, and then filtered to remove solid residues. The resulting liquid was freeze-dried to obtain a powdered form of the extract. Prior to administration to mice, the lemon powder was dissolved in distilled water.

Animal model description

A group of female BALB/c mice (*Mus musculus*) aged seven to eight weeks were used. The mice were kept in the animal room for one week before the treatment began to ensure proper acclimation. Each mouse weighed approximately 20 g, was in good health, and had an active body without impairment. The mice were kept clean to prevent any infections during the experiment, and their weight was recorded every time they received treatment to ensure the correct doses were administered (Putra et al. 2015).

The study adhered to a completely randomized design, using 16 mice divided into four treatment groups with four replications each. These groups consisted of the vehicle group, DMBA induction group, CLE 50 group (treated with DMBA and *C. limon* aqueous extract dosage 50 mg/kg BW), and CLE 200 group (treated with DMBA and *C. limon* aqueous extract dosage 200 mg/kg BW). The research was conducted in strict adherence to the Animal Scientific Procedures Act of 1986, which prohibits the use of animals that may experience pain, suffering, distress, or lasting harm during the study.

DMBA and CLE administration

To induce tumor formation, DMBA was administered via subcutaneous injection once a week from week 1 to week 6 in the breast region (Putra et al. 2020). The DMBA dose was 0.015 mg/g BW and diluted in 0.1 ml of corn oil. The amount of DMBA solution injected into each mouse corresponded to its body weight. Then in the week 7 and week 8, the mice were orally given extracts containing 50 and 200 mg/kg BW of CLE daily for 14 days, with distilled water as the solvent. The amount of CLE given to each mouse was calculated based on its weight.

Extracellular and intracellular immunostaining

After completing the necessary animal testing, the mice were euthanized using cervical dislocation, followed by sanitization of the abdominal area with 70% alcohol. Dissection was then performed from the left dorsal to the ventral. To ensure the spleen was free of any fat, it was removed and cleaned twice with phosphate-buffered saline (PBS). The organs were then crushed in a cup with 1 ml of PBS using a blunt-tipped syringe in a clockwise motion. The crushed spleen was dissolved in 15 ml of propylene, followed by centrifugation at 2500 rpm for 5 min at 10°C. After the pellet was mixed with 1 mL of PBS, homogenization was achieved by pipetting.

Antibody staining was performed according to our previous protocols (Rahayu et al. 2022; Putra and Rifa'i. 2020; Putra et al. 2015). Extracellular antibody staining was performed using pellets in 1 ml of PBS, then sample were transferred to 60 µl and we added to 300 µl of PBS. The sample was then centrifuged at 2500 rpm for 5 min at 10°C. The supernatant was

discarded, and the pellet was resuspended in 50 µl of antibody and incubated for 20 min. PE-Cy5-conjugated rat-anti-mouse Gr-1, FITC-conjugated rat-anti-mouse CD68, FITC-conjugated rat-anti-mouse CD4, PE-conjugated rat-anti-mouse CD25, PE-Cy5-conjugated rat-anti-mouse CD62L, and FITC-conjugated rat-anti-mouse NK.1.1 (Biolegend) were the monoclonal antibodies utilized. Meanwhile, intracellular antibody staining was performed in a microtube with pellets, and 100 µl of PBS supplemented with 50 µl of Cytofix™ as a fixative and incubated for 20 min. The cell suspension was then resuspended in 500 µl of washperm to allow the wash over the leftover Cytofix™. The suspension was then centrifuged for 5 min at a speed of 2500 rpm at a temperature of 10°C. We used 50 µl of PE-Cy5-conjugated rat-anti-mouse IL-17 and PE-conjugated rat-anti-mouse TNF-α for intracellular immunostaining. It was then incubated for around 20 min. After separating the pellet from the supernatant, 50 µl of intracellular antibody was added to the pellet, and each cell suspension was placed in a cuvette with 400 µl of PBS. The FACS Calibur™ (BD Bioscience) read the cell solution in the cuvette. Backgating was performed to confirm gating strategies which could distinguish the lymphocyte population or granulocyte, including the monocyte population. After that, the specific cell expression which was determined according to antibody staining then analyzed based on their lineage population.

Small molecule pharmacokinetics prediction analysis

The small molecule pharmacokinetics prediction was assessed through the pkCSM webserver (http://biosig.unimelb.edu.au/pkcsm/). Several main bioactive compounds of CLE, such as limonene (CID: 22311), γ-terpinene (CID: 7461), β-pinene (CID: 14896), p-cymene (CID: 7463), α-pinene (CID: 6654); myrcene (CID: 1253) were used as a subject to observe (Lücker et al. 2002). Furthermore, the specific toxicity evaluation was performed through ProTox 3.0 - Prediction of Toxicity of Chemicals webserver (https://tox.charite.de/protox3/) to evaluate the toxicity class, LD50 prediction, and the possibility of inducing toxicity.

Statistical analysis

The data were statistically analyzed using one-way ANOVA, specifically a one-way ANOVA with a significance threshold of 5%. The Tukey HSD test was then employed to determine the difference between the two groups. These statistical analyses were conducted using SPSS 22.

Results

CLE attenuates the expression of granulocytes, Gr-1$^+$ cells and macrophages, CD68$^+$IL-17$^+$ cells, and CD68$^+$TNF-α$^+$ to the normal level

The analysis results of the average percentage of the relative number of Gr-1$^+$ cells from the spleen are shown in Figures 1A and 1D. The DMBA-treated group exhibited the highest average percentage of Gr-1$^+$ cells (23.82%), which was significantly higher than the other treatment groups ($p<0.05$). However, treatment with CLE 200 resulted in Gr-1$^+$ cell levels returning to near-normal conditions.

Results from Figures 1B and 1E show the average proportion of CD68$^+$ macrophages in the spleen of mice that express the pro-inflammatory cytokine IL-17 (CD68$^+$IL17$^+$). The group of mice treated with DMBA had the highest average relative number of macrophage cells that release IL-17 cytokines, at around 11.91%, which is statistically significant ($p<0.05$). However, the CLE200 treatment demonstrated suppressive activity that promotes CD68$^+$IL-17$^+$ cells to the normal level.

Figures 1C and 1F show the flow cytometry and the analysis of the relative number of TNF-α-expressing macrophages in the spleen. The cancer group had the

highest number of this type of macrophage, followed by the CLE50 group. The average relative number of TNF-α-expressing macrophages in the vehicle group was substantially lower than in all treatments. The CLE200 group had reduced the relative number of TNF-α-expressing macrophages by 18.57%.

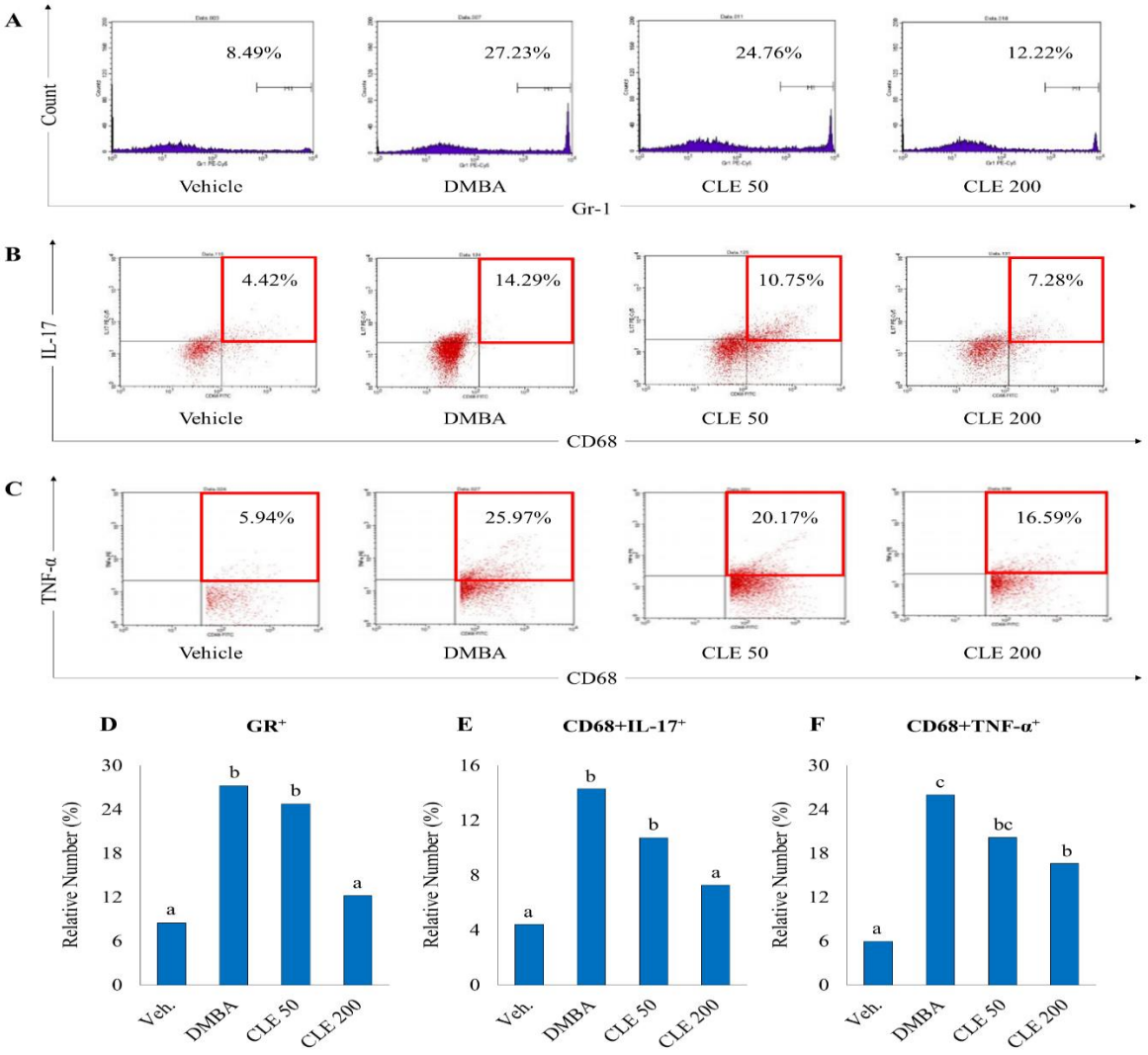

Figure 1. Immunomodulatory effects of CLE toward $Gr1^+$ granulocytes (A and D), $CD68^+IL\text{-}17^+$ (B and E), and $CD68^+TNF\text{-}\alpha^+$ (C and F) macrophages in DMBA-induced mice. The results are shown as the mean ± SD. Results were considered significant at $p<0.05$. Different alphabetic symbols on the table indicate a considerable difference.

CLE attenuates the expression of Natural Killer and effector T cells, but upregulates regulatory T cells

The results in Figures 2A and 2D reveal that mice induced with DMBA had a considerably larger number of NK cells than the vehicle group. Interestingly, CLE200 treatment reduces the number of NK cells into the normal condition. Overall, the administration of CLE improved the profile of NK cells by decreasing their production in the spleens of DMBA-induced cancer mice. When given the CLE at a 200 mg/kg BW dose, the number of NK cells decreased significantly but not considerably more than the standard treatment. The NK cells number in CLE50 group did not differ significantly from the DMBA treatment group. However, it indicates that when CLE is administered, there is an improvement in the immune system of DMBA-induced cancer mice, further lowering the NK cell synthesis.

The flow cytometry study revealed that the relative number of $CD4^+CD25^+$ T cells rose in the spleens of mice with breast cancer, particularly in the DMBA-induced treatment group. However, this number was reduced in DMBA-induced mice treated with CLE (Figure 2B and 2E). The analysis shows that the average change in the relative number of $CD4^+CD25^+CD62L^+$ T cells in the spleen has decreased in the treatment group induced by DMBA. However, the results were the opposite in CLE treatment groups, revealing differences in the average relative number of $CD4^+CD25^+CD62L^+$ T cells between each treatment group (Figure 2C and 2F). The findings showed a significant reduction ($p \leq 0.05$) in the relative number of regulatory T cells in breast cancer conditions compared to the control group. The vehicle group had an average relative number of 44.15%, whereas the DMBA-induced group had 13.25%. However, in the CLE treatment groups, the average relative number of Tregs rose proportionally with the dosage. The relative number of Tregs rose by 22.28% when CLE was administered at 50 mg/kg BW, and by 23.13% when CLE was administered at 200 mg/kg BW. It may be concluded that as the amount of CLE increased, so did the proportional number of Tregs.

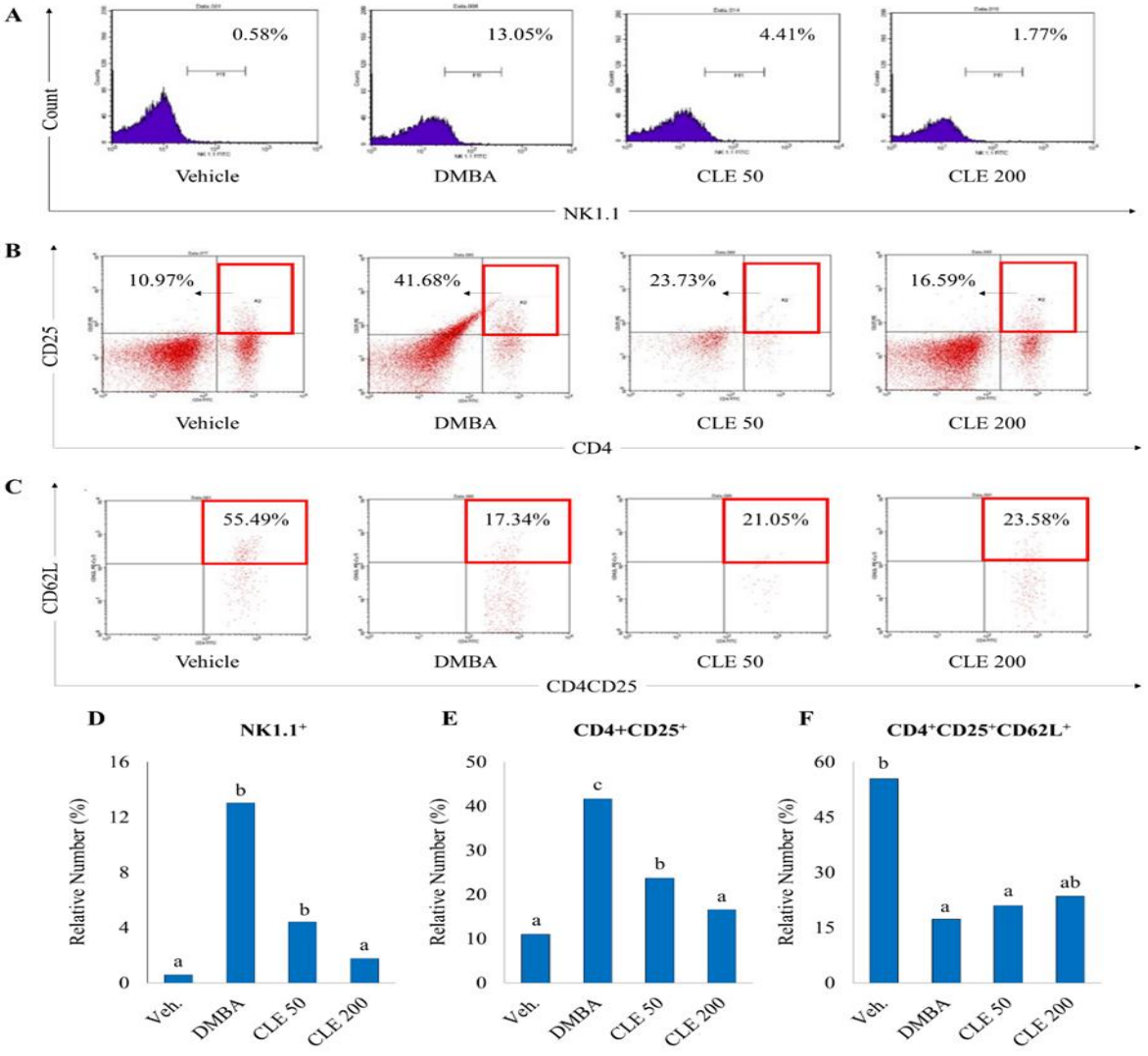

Figure 2. Immunomodulatory effects of CLE toward $NK1.1^+$ natural killer cells (A and D), $CD4^+CD25^+$ effector T cells (B and E), and $CD4^+CD25^+CD62L^+$ regulator T cells (C and F) macrophages in DMBA-induced mouse. The results are shown as the mean ± SD. Results were considered significant at $p < 0.05$. Different alphabetic symbols on the table indicate a considerable difference.

The immunomodulation potency of *Citrus limon* as cancer treatment

The absorption, distribution, metabolism, excretion, and toxicity (ADMET) of the main components of *C. limon* compounds, including limonene, terpinene, pinene, p-cymene, pinene, and myrcene (Figure 3), were evaluated in this study using the pkCSM webserver. The results revealed that *C. limon* compounds outperformed control medications regarding intestinal absorption, Caco-2 Permeability, and water solubility. Compounds with solid absorption recorded an absorption value of at least 80%, while those with poor absorption had a value of at least 30%. A compound with a prediction value of more than 0.90 was determined to have significant Caco-2 permeability. Water solubility indicates how soluble a molecule is in water at 25°C, and lipid-soluble medications are not as readily absorbed as water-soluble ones, especially when administered enterally. The distribution characteristics of *C. limon* components were impressive, with some substances crossing the blood–brain barrier, as indicated by a Log BB value greater than 0.3, and the VDss was in the high range. Furthermore, the chemicals from *C. limon* had no discernible impact on metabolism or the non-contraindication mechanism. *C. limon* compound values were less than 2 ml/minute/kg according to the clearance property, or total clearance, indicating a lengthy half-life before being excreted by the kidney, liver, and gall bladder. *C. limon* compounds were categorized as non-hepatotoxic, making them fit for development, given that drug-induced liver injury is a key safety concern and a substantial cause of drug attrition (Pires et al. 2015). To a greater extent, the toxicity evaluation via ProTox 3.0 webserver indicates most of *C limon* compounds have low toxicity effects on other systems or organs (Figure 4).

Property	Model Name	limonene	γ-terpinene	β-pinene	p-cymene	α-pinene	myrcene
Absorption (log mol/L)	Water solubility	-3.57	-3.94	-4.19	-4.08	-3.73	-4.50
Absorption (log Papp in 10^{-6} cm/s)	Caco2 permeability	1.40	1.41	1.39	1.53	1.38	1.40
Absorption (% Absorbed)	Intestinal absorption (human)	95.90	96.22	95.53	93.54	96.04	94.70
Distribution (log L/kg)	VDss (human)	0.40	0.41	0.69	0.70	0.67	0.36
Distribution (log BB)	BBB permeability	0.73	0.75	0.82	0.48	0.79	0.78
Distribution (log PS)	CNS permeability	-2.37	-2.05	-1.86	-1.40	-2.20	-1.90
Metabolism	CYP2D6 substrate	No	No	No	No	No	No
Metabolism	CYP3A4 substrate	No	No	No	No	No	No
Metabolism	CYP2D6 inhibitor	No	No	No	No	No	No
Metabolism	CYP3A4 inhibitor	No	No	No	No	No	No
Excretion (log ml/min/kg)	Total Clearance	0.21	0.22	0.03	0.24	0.04	0.44
Excretion	Renal OCT2 substrate	No	No	No	No	No	No
Toxicity (log mg/kg/day)	Max. tolerated dose (human)	0.78	0.76	0.37	0.90	0.48	0.62
Toxicity (mol/kg)	Oral Rat Acute Toxicity (LD50)	1.88	1.77	1.67	1.83	1.77	1.64
Toxicity	Hepatotoxicity	No	No	No	No	No	No

Figure 3. ADMET properties of lemons' bioactive compounds as consideration for developing drug candidates for cancer treatment.

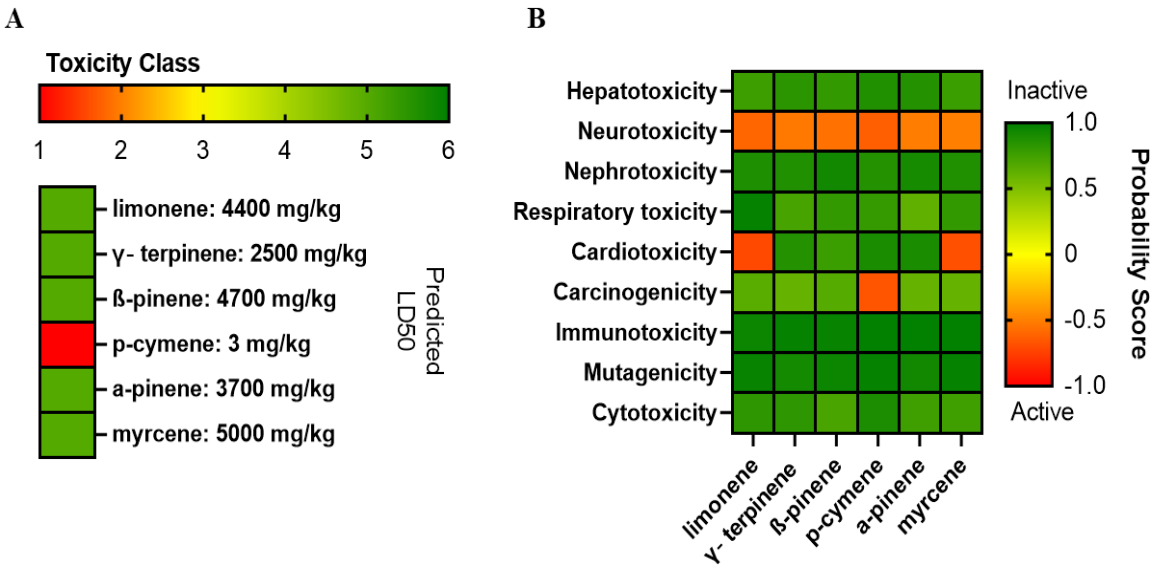

Figure 4. Toxicity evaluation of lemon bioactive compounds. (A). Toxicity class and predicted LD50. (B). The possibility of compounds causing the toxicity in some parameters.

Discussion

Research suggests that the concentration of flavonoids in CLE can potentially affect the oxidative activity of neutrophils, including the release of NADPH-oxidase or myeloperoxidase. This, in turn, may lead to a decrease in the number and activity of neutrophils in cancer patients. Additionally, a separate study found that quercetin, genistein, and flavones can reduce reactive oxidative species (ROS) formation and induce apoptosis in human neutrophils. It is also theorized that these flavonoids could significantly reduce polymorphonuclear leukocyte migration to the site of cancer. Overall, flavonoids may have an immunomodulatory effect that reduces neutrophil damage in cases of chronic or uncontrolled inflammatory responses (Han et al. 2022).

Studies show that the administration of CLE may reduce the number of $CD68^{+}IL17^{+}$ macrophages by inhibiting macrophage polarization towards the M2 phenotype. This is achieved by inhibiting downstream signaling of IL-4, resulting in STAT6 phosphorylation and downregulation of M2-related genes such as CD206 and Arg-1. Alternatively, CLE may inactivate the STAT3 phosphorylation mechanism and PGE2-PPARδ signaling cascades, leading to decreased IL-4Rα and Arg-1 synthesis. Research suggests that flavonoids, including luteolin and isoliquiritigenin, possess these characteristics, but more exploration is necessary to identify other possible flavonoids (Saeedifar et al. 2021). Additionally, CLE's antioxidants, such as flavonoids and their derivatives, may act as anti-inflammatory agents by reducing ROS production and inhibiting NF-κB activity, resulting in the suppression of PD-L1 and immunosuppressive chemokines' expression. This impedes the polarization mechanism favoring the M2-phenotype, further decreasing the population of M2-macrophages in the TME.

On the other hand, the bioactive content found in CLE may play a role in directly increasing M1 polarization. In vitro studies utilizing flavonoid and phenolic compounds, such as curcumin, epicatechin gallate, and chlorogenic acid, have indicated that these compounds may promote the phosphorylation of STAT4 and the expression of IL-12, as well as increase the phosphorylation of STAT1 and NF-κB,

and the expression of IL-12 and iNOS. Additionally, the bioactive substance has been shown to increase the expression of MHC II and CD11c while decreasing M2-Macrophage indicators induced by IL4, such as CD206 and Arg-1 (Saeedifar et al. 2021). However, further research is necessary to confirm if the dominant bioactive compounds in CLE promote M1 polarization through a similar mechanism.

Studies have suggested that administering bioactive compounds may enhance the NK cells's cytotoxic activity against tumor cells (Boichuk et al. 2025; Chaicharoenaudomrung et al. 2023). However, it is commonly observed that administering bioactive compounds increases the population of NK cells. It is unclear whether the observed increase in NK cell population was compared to standard or untreated control. While two studies on genistein suggest that the compound could increase NK cell activity (Guo et al. 2002; Guo et al. 2001), another study indicates that it lowers splenic NK cells in all treatment groups without increasing NK cell activity, with the exact mechanism remaining unclear (Grudzien and Rapak 2018). Additionally, a study on ascorbic acid found that it could drive hematopoietic stem cells to mature faster, resulting in a higher proliferation rate of mature NK cells in its presence (Huijskens et al. 2015).

According to our findings, treatment with CLE increased the relative number of regulatory T cells, indicating that the particular substances in CLE may act as an anti-inflammatory agent, lowering inflammation in the cancer environment. *C. limon* includes flavonoids with anti-inflammatory and antioxidant effects. These flavonoids suppress ROS activity, reduce interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) expression by Th1, and reduce oxidative stress in tissues. Flavonoids can trigger genes that attract and activate regulatory T cells, such as transforming growth factor-beta (TGF-β) which stimulates their proliferation (Yahfoufi et al. 2018).

We examined the impact of CLE on the immune system of mice with carcinogenic-induced tumors. Our results indicate that CLE has an immunomodulatory effect, bringing several immune system profiles back to normal levels. Specifically, CLE reduced levels of $Gr1^+$ granulocytes, $CD68^+IL\text{-}17^+$ and $CD68^+TNF\text{-}\alpha^+$ macrophages, $NK1.1^+$ natural killer cells, and $CD4^+CD25^+$ effector T cells while increasing the population of $CD4^+CD25^+CD62L^+$ regulator T cells (Figure 5).

C. limon is a member of the Rutaceae family, known for its biologically beneficial compounds that have been shown to protect against various cancer types. Previous studies have demonstrated the anticancer potential of citrus peels, particularly lemon peels (Koolaji et al. 2020; Alshatwi et al. 2011). In addition, daily eating of citrus fruits is associated with a reduced risk of stomach cancer (Bae and Kim, 2016). Prior research has also found that *C. limon* ethyl acetate and petroleum ether extracts have antitumor activity against multiple human cancer cell lines (Sawyerr 2018).

In a separate study, a blend of lemon and ginger water extract was found to increase caspase-3 activity, resulting in the induction of apoptosis and a reduction in the expression of vascular endothelial growth factor in MDA-MB231 Cells. The combination also stimulated lymphocyte proliferation in the presence of Concanavalin A (Con A) and lipopolysaccharide (LPS), while peritoneal macrophages showed an increase in pinocytic activity but no phagocytic activity. The study also observed a decrease in tumor percentage with no fatalities (Al-ataby and Talib 2022). Lemon, in particular, demonstrated anticancer activity by suppressing cell proliferation in cancer, inducing apoptosis through TRAIL activation, inhibiting tumor development in chronic myelogenous leukemia, and

exhibiting antioxidant and apoptotic induction in MCF-7 cells (Kim et al. 2012; Raimondo et al. 2015). It also enhanced antioxidative cellular defenses through the ERK/Nrf2 signaling pathway and reduced inflammation by decreasing the NF-κB factor, nitric oxide (II) synthase (iNOS), promoting induced cyclooxygenase (COX-2), and reducing the TLR-signaling pathway (Parhiz et al. 2015).

Finally, CLE can potentially restore various immune system profiles to normal levels. These include $Gr1^+$ granulocytes, $CD68^+IL\text{-}17^+$ and $CD68^+TNF\text{-}\alpha^+$ macrophages, $NK1.1^+$ natural killer cells, and $CD4^+CD25^+$ effector T cells. Moreover, CLE can elevate the population of $CD4^+CD25^+CD62L^+$ regulator T cells to the standard level. Furthermore, our ADMET evaluation revealed that several essential compounds present in CLE meet the drug-like standards. This implies that CLE may be developed as a supplementary or complementary medicine in the fight against cancer. In the present study, we have limitations on determining which compound of lemon extracts provides the immunomodulation activity. Therefore, future research on evaluating specific compounds and their effects including toxicity, pharmacokinetics, immunomodulation, and anti-cancer activities needs to be explored.

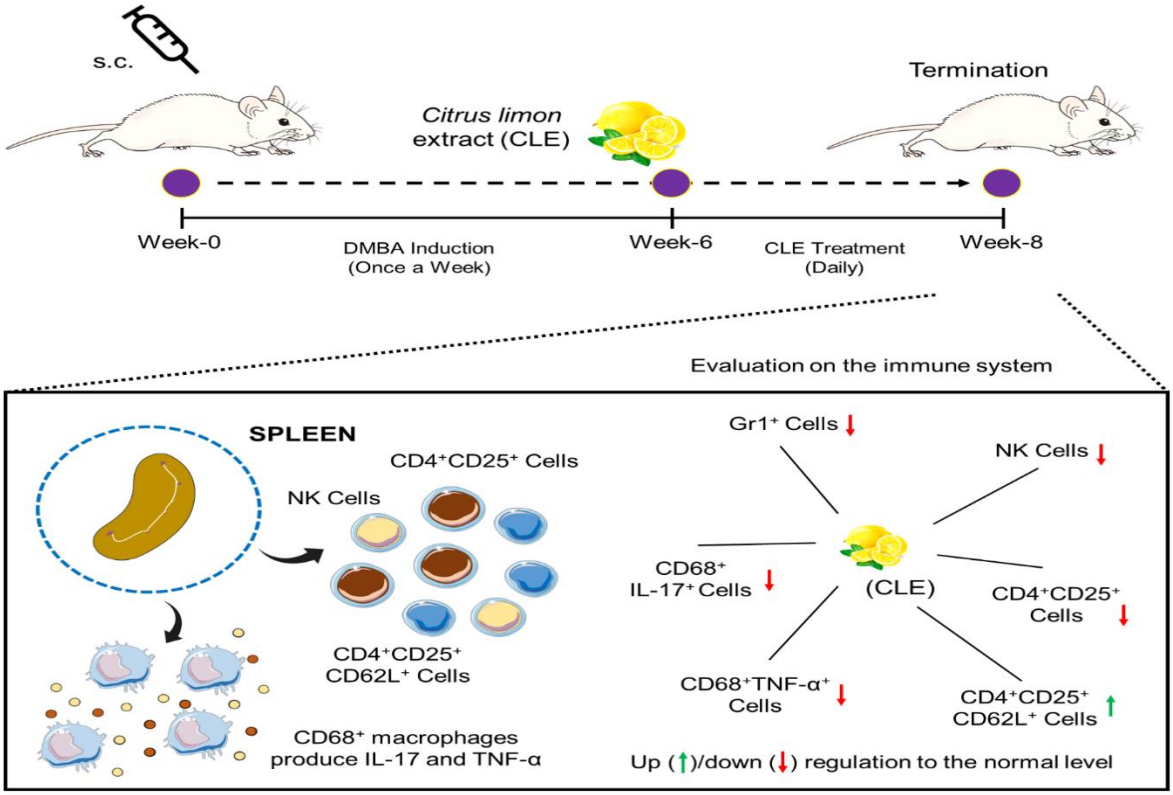

Figure 5. The schematic figure illustrates the immunomodulatory activity of CLE in DMBA-induced mice. CLE attenuates the granulocyte, macrophage, NK cells, and effector T cells and regulates the regulatory T cells to the normal level.

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Conflicts of interest

The authors declare there is no conflict of interest.

Ethical Considerations

All experiments were approved by the Research Ethics Committee of Brawijaya University.

Code of Ethics

The ethical clearance code for this study is 779-KEP-UB.

Authors' Contributions

W.E.P, C.N, D.E.M, R.F.I.N, and A.H contributed to the experimental work, data analysis, computational analysis, data visualization, and writing of the original draft. M.R contributed to the study design, supervision, and manuscript editing. All authors have read and approved the final version of this manuscript.

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