

# Immunosuppressive and Ameliorative Effects of Dietary Combined Herbs Extract of *Curcuma zedoaria* (Christm.) Roscoe and *Phyllanthus niruri* L. in DMBA-induced Breast Cancer Mouse Model

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*Immunosuppressive and Ameliorative Effects of Dietary Combined Herbs Extract of Curcuma zedoaria (Christm.) Roscoe and Phyllanthus niruri L. in DMBA-induced Breast Cancer Mouse Model*

*DMBA ile indüklenen Meme Kanseri Fare Modelinde Curcuma zedoaria (Christm.) Roscoe ve Phyllanthus niruri L. Kombine Bitki Ekstrelerinin İmmünsüpresif ve İyileştirici Etkileri*

## SUMMARY

Cancer continues to be the most prevalent health issue on a worldwide basis. Each year, there is an increased number of new incidence and mortality. Numerous approaches, such as medicinal plant-based treatments, have been put out as therapies. In this study, we aimed to evaluate the immunomodulation effect of dietary combined herbs extract of *Curcuma zedoaria* and *Phyllanthus niruri* (cheral) in 7,12-Dimethylbenz[a]anthracene (DMBA)-induced breast cancer mouse model. The experimental mice were divided into roughly six treatment groups, including administering cheral extract with a series of doses including 1.233 mg/kg BW, 2.466 mg/kg BW, and 4.932 mg/kg BW. Flow cytometry analysis was performed to evaluate immune parameters including NK cells, TNF- $\alpha$  and IFN- $\gamma$ -expressing NK cells, CD4<sup>+</sup> TNF- $\alpha$  IFN- $\gamma$ <sup>+</sup> type 1 helper T cells, and CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells population. In this present study, we demonstrated that cheral extract exerts immunosuppressive activity by attenuating the properties of the immune system, such as NK cells, Th1 cells, and regulatory T cells in DMBA-inducing mice to the normal levels. Thus, we suggested that cheral extract has ameliorative potency as alternative or complementary therapy against cancer incidence.

**Key Words:** Cheral, *Curcuma zedoaria*, DMBA, Immunosuppressive, *Phyllanthus niruri*

## ÖZ

Kanser dünya çapında en yaygın sağlık sorunu olmaya devam etmekte, her yıl yeni vaka ve ölüm oranlarında artış yaşanmaktadır. Tıbbi bitki bazlı tedaviler gibi çok sayıda yaklaşım tedavi seçeneği olarak konmuştur. Bu çalışmada, 7,12-Dimetilbenz[a]antrasen (DMBA) ile indüklenen meme kanseri fare modelinde *Curcuma zedoaria* ve *Phyllanthus niruri* (cheral) kombine bitki ekstrelerinin immünomodülatör etkisini değerlendirmeyi amaçladık. Deney hayvanları, 1.233 mg/kg VA, 2.466 mg/kg VA ve 4.932 mg/kg VA dahil olmak üzere bir dizi dozla kombine ekstrelerin (cheral) uygulandığı altı tedavi grubuna ayrıldı. NK hücreleri, TNF- $\alpha$  ve IFN- $\gamma$  ekspres eden NK hücreleri, CD4<sup>+</sup> TNF- $\alpha$  IFN- $\gamma$ <sup>+</sup> tip 1 yardımcı T hücreleri ve CD4<sup>+</sup> CD25<sup>+</sup> düzenleyici T hücreleri popülasyonu dahil olmak üzere bağışıklık parametrelerini değerlendirmek için akış sitometri analizi yapıldı. Bu çalışmada, kombine ekstrelerin, DMBA'yı indükleyen farelerde NK hücreleri, Th1 hücreleri ve düzenleyici T hücreleri gibi bağışıklık sisteminin özelliklerini normal seviyelere azaltarak immün baskılayıcı aktivite gösterdiği saptandı. Bu nedenle, kombine ekstrelerin kanser vakalarına karşı alternatif veya tamamlayıcı tedavi olarak iyileştirici güce sahip olduğunu düşünmekteyiz.

**Anahtar Kelimeler:** Cheral, *Curcuma zedoaria*, DMBA, İmmünosüpresif, *Phyllanthus niruri*.

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## INTRODUCTION

Cancer is a broad term for a chronic condition caused by uncontrolled cell proliferation. It is a common disease primarily because of prolonged unhealthy lifestyles like extreme high-calorie diets and high levels of stress, exposure to carcinogens, genetic factors, or a combination of those risk factors (Rock et al. 2020). With more than 19 million newly diagnosed cases and 10 million fatalities annually, it is regarded as one of the leading causes of mortality and one of the most important burdens on healthcare systems and communities globally (Sung et al. 2021). However, the death rate in America has decreased, although this phenomenon may occur only in developed countries with modern and competent healthcare systems and greater ability for earlier diagnosis (Siegel et al. 2021). Nonetheless, some reports indicate that the burden of cancer incidence and mortality, particularly breast cancer, is increasing significantly because breast cancer has surpassed lung cancer as the leading cancer diagnosed regardless of gender, with more than 2.3 million new cases diagnosed in 2020 (Lei et al. 2021, Sung et al. 2021). It accounts for more than 30 percent of the projected new cases in the United States alone during the previous two years among women (Siegel et al. 2022, 2021) the American Cancer Society estimates the numbers of new cancer cases and deaths in the United States and compiles the most recent data on population-based cancer occurrence and outcomes. Incidence data (through 2018, while it accounts for roughly 12% of cases globally, with over 7% of recent fatalities recorded (Sung et al. 2021). One in every eight women in the United States is at risk of developing invasive breast cancer, which can spread to other organs, and one in every 36 women dies from the disease (Britt et al. 2020). Breast cancer patients must be treated as soon as they are diagnosed. Delaying therapy for just three months after the first diagnosis may result in a worse prognosis because breast cancer can progress swiftly into more severe stages. However, in low-to-middle-income nations, most cases are identified later, either locally advanced or metastatic

the chances of survival are very high (Ginsburg et al., 2020). However, women in many settings face complex barriers to early detection, including social, economic, geographic, and other interrelated factors, which can limit their access to timely, affordable, and effective breast health care services. Previously, the Breast Health Global Initiative (BHGI). Because of its complexity, there is currently no universal cure for every type and subtype of cancer.

The immune system plays a vital role in developing and eliminating cancer. It is frequently associated with a persistent inflammatory response. Both innate and adaptive immune components carry out this reaction. It is considered the primary component of the tumor microenvironment, together with the tumor itself, surrounding capillaries, fibroblasts, and extracellular matrix (Henke et al. 2020). Cancer and its surrounding microenvironment are inextricably linked and continually interact. Tumors can impact the microenvironment by releasing extracellular signals, boosting tumor angiogenesis, and establishing peripheral immunological tolerance, whereas immune cells in the microenvironment can influence malignant cell proliferation and development (Korneev et al. 2017, Ghoshdastider et al. 2021). Cancer immunosurveillance is the process by which immune cells continually monitor the tissue to detect and eradicate cancer or pre-cancer cells that exhibit or lose specific surface markers. This method involves several immune cells, including natural killer (NK) cells, macrophages, dendritic cells (DC), helper T cells, and cytotoxic T cells. Furthermore, the immune cells and tumor cells involved produce cytokines, chemicals that carry out intercellular communication in the immune system (Henke et al. 2020, Ghoshdastider et al. 2021).

Because they influence the evolution of chronic inflammatory disorders such as cancer, NK cells are critical components of the inflammatory response. It is an important part of tumor immunosurveillance (Tosello-Tramont et al. 2017, Parisi et al. 2017, Zhang et al. 2021). It is the CD8 T cell equivalent of

innate immunity, which means it may kill tumor cells in the absence of antigen-presenting cells (APCs) by acting directly as cytolytic effector lymphocytes. Resting NK cells are activated to attack cancer by a multi-step signaling mechanism that needs numerous ligations of molecules expressed on their surface. Multiple apoptosis-inducing pathways are triggered by the contacts, including the release of granules containing perforin and granzymes, stimulation of apoptosis via Fas-FasL or TRAIL-TRAIL ligand, and different cytokine and chemokine releases (Zhang et al. 2021, Fang et al. 2018). After activation, NK cells secrete cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines such as CCL1, CCL2, CCL3, CCL4, CCL5, and CXCL8, which can modulate and activate adaptive immune response effector cells to eliminate cancer cells (Paul & Lal 2017, Marischen et al. 2018). TNF- $\alpha$  and IFN- $\gamma$ , whose primary role is activating NK cells' lytic capabilities in response to aberrant type I IFNs and other inflammatory cytokines. When NK cells detect cancerous cells, they release cytotoxic granules containing perforin and granzyme, causing cancer cells to undergo apoptosis.

Tumor-infiltrating lymphocytes (TILs) have been identified as a biomarker of anti-tumor response in breast cancer. TIL subsets such as CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes can identify tumor antigens and kill tumor cells. CD4<sup>+</sup> helper T cells are primarily involved in tumor immunology and are functionally categorized into Th1, Th2, and Th17 cells based on secretory cytokines and immunologic activities (Zhao et al. 2019, Lee et al. 2019). Th1 helper cells boost macrophage and cytotoxic T-cell responses. The IL-12 generated by antigen-presenting cells in response to antigenic stimulation promotes the polarization of helper T cells into the Th1 subset, which activates the signal transducer and activator of the transcription 4 (STAT4) pathway. They primarily generate pro-inflammatory cytokines, including IL-1, IL-2, IL-12, TNF- $\alpha$ , and IFN- $\gamma$ , which are linked to a better prognosis in cancer patients because they mediate the

anti-tumor action. Th1 cells' IFN- $\gamma$  may attract and activate additional NK cells at the tumor site (Wculek et al. 2020, Powell et al. 2019). Another important component in anti-cancer inflammatory responses is regulatory T cells, a subpopulation of T cells identified by the expression of CD4, CD25, and FOXP3, expressing highly immunosuppressive activity against anti-tumor immune responses, explicitly downregulating induction, and proliferation of effector T cells. Because of these qualities, tumor cells secrete CXCL1, CCL2, and CCL20 to attract additional regulatory T cells to the tumor location. They then block the anti-tumor responses from cytotoxic cells by secreting immunomodulatory cytokines such as IL-10, IL-35, and TGF- $\beta$  (Jean Baptiste et al. 2022, Togashi et al. 2019, Ohue & Nishikawa 2019).

This delicate balance of immune cells in TME has become an attractive area of research, as has the effect of giving bioactive components from diverse herbal and culinary plants. The majority of these studies advocate for a shift in the immune cell balance toward pro-inflammatory cells by promoting helper T cell polarization to the Th1 phenotype, recruiting and promoting the expansion of NK cells as well as their activities, as well as inhibiting T cell polarization to the Th2 phenotype and reducing the activity of regulatory T cells. As a result of the administration of numerous bioactive chemicals, the immune system has been altered by decreasing cancer-promoting factors. Those findings are supported by research on *Withania somnifera*, *Panax genus*, *Rhodiola rosea*, *Coriolus versicolor*, and *Lentinula edodes* (Venturella et al. 2021, Li et al. 2017, Jean Baptiste et al. 2022, Dubey et al. 2021). However, a study focused on Korean red ginseng suggests that it suppresses the Th1 responses by inhibiting Th1 polarization promoted by IL-12 produced by DC (Cho et al. 2019).

Cheral, a combination of *Zedoaria rhizome* (*Curcuma zedoaria* (Christm.) Roscoe) and chamberbitter (*Phyllanthus niruri* L.), is our major focus in this study. Indigenous societies frequently claim that they have anti-cancer effects. However, research on that combination and its impact on

immune cells during cancer is limited. Previous research on *C. zedoaria* and *Phyllanthus* extracts suggests that the extracts' bioactive compounds could remodel the immunosuppressive TME by decreasing Treg infiltration and differentiation and increasing NK cell activity, particularly cytotoxicity, inhibiting IL-2/IL-2R signal-dependent Treg cell differentiation, and preventing metastasis and EMT in the TGF-1-induced signaling pathway. However, the majority of their processes remain unknown (Hou & Fang 2021, Li et al. 2021, Oh et al. 2018, Subramaniam et al. 2019, Tendean & Riwanto 2021, Tjandrawinata et al. 2017). Meanwhile, research reveals that a bioactive ingredient in *Phyllanthus* suppresses B and T cell proliferation and the down-regulation of Th1 (IL-2 and IFN- $\gamma$ ) and Th2 (IL-4) cytokines and CD4<sup>+</sup> and CD8<sup>+</sup> cells (Nisar et al. 2018). Therefore, we studied cheral for its potential use as a curative agent for breast cancer using immunity parameters based on the relative quantity of NK cells, CD4TNF- $\alpha$ , CD4IFN- $\gamma$ , and T-regs in the spleen of the mice model represents the immune response against cancer.

## MATERIAL AND METHODS

### Experimental design and sample preparation

Combined herbs extract of *C. zedoaria* and *P. niruri* which is called cheral was obtained from Makassar. Pathogen-free female mice were ordered from LPPM, Gadjah Mada University, Yogyakarta. About 24 female mice weighing approximately 20 grams and aged 7-8 weeks were used in this study. The experimental mice underwent an acclimatization procedure for a week. The cages were cleaned daily, and food and water were provided daily, *ad libitum* (Putra & Rifa'i 2020).

A completely randomized design was performed in this study. The treatment was divided into six groups with four replications. Except for the negative control, all of the mice were injected with DMBA (Tokyo Chemical Industry, Japan) for six weeks to induce breast cancer formation. The DMBA group as a positive control was left untreated after the cancer induction (Putra & Rifa'i 2019). The first treatment

group was treated with cisplatin (Dankos Farma-Kalbe Medika, Jakarta) 1 mg/kg BW by subcutaneous injection as a control treatment. The second, third, and fourth treatment group was treated orally with cheral with 1.233 mg/kg BW (CE1); 2.466 mg/kg BW (CE2); and 4.932 mg/kg BW dosage (CE3), respectively for 14 days. The Research Ethics Commission of Brawijaya University issued a declaration of ethical conduct for this study, with reference number 925-KEP-UB.

### Immunostaining and flow cytometry analysis

This study's immunostaining and flow cytometry analysis were based on our previous protocols (Putra et al. 2016; Putra et al. 2015). After the mice were sacrificed, the isolated samples from the spleen were mixed with 50  $\mu$ l of extracellular antibody, such as anti-NK.1.1, anti-CD4, or anti-CD25 (BioLegend, USA) then incubated in the dark at room temperature for 20 minutes for extracellular antibody labeling. The solution was then incubated at room temperature for 20 minutes with 50  $\mu$ l of cytofix (BioLegend, USA). Afterward, 500  $\mu$ l of washperm (BioLegend, USA) was applied and incubated at room temperature for 20 minutes before centrifugation. The solution was centrifuged, and the separated pellet was treated with 50  $\mu$ l of intracellular antibodies such as anti-TNF- $\alpha$  or anti-IFN- $\gamma$  (BioLegend, USA) for 20 minutes at room temperature. All solutions were then diluted with 300  $\mu$ l of BSA-supplemented PBS before being transferred to a cuvette for flow cytometry analysis (FACS Calibur<sup>TM</sup>). Further, an *in silico* study related to the evaluation of chemical interaction with specific proteins was performed through the STITCH (<http://stitch.embl.de/>) database (Putra et al. 2023, Putra et al. 2017).

### Data and statistical analysis

The data on the relative numbers of NK cells, TH1 cells, and regulatory T cells were obtained via flow cytometry analysis and statistically analyzed using SPSS software version 22.0 for Windows. The gathered data will be evaluated using a normality test to see whether it is regularly distributed and a homogeneity test to see if it is homogenous. The data variance was

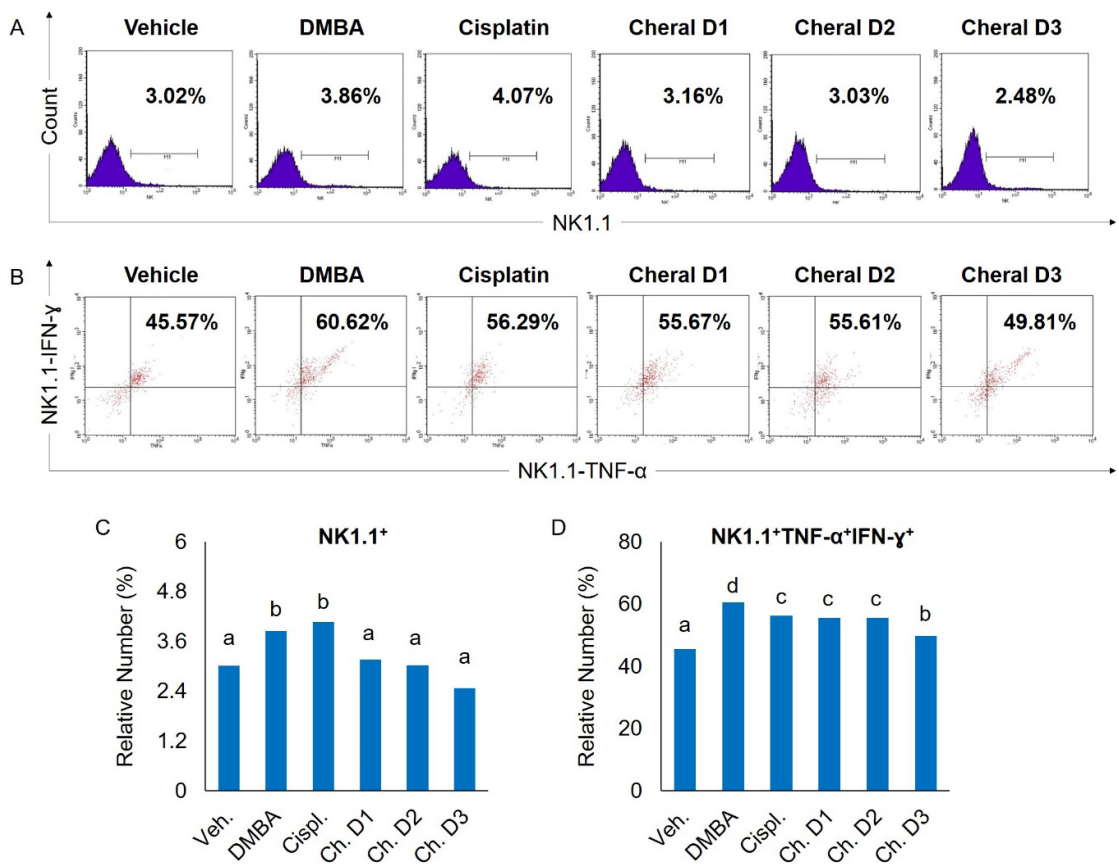
then determined using a one-way ANOVA followed by Tukey's Honestly Significant Difference test with a 95% confidence.

**RESULTS and DISCUSSION**

**Immunosuppressive effect of cheral extract toward NK cells expression**

According to the flow cytometry analysis, each treatment group's proportion of NK cells produced different outcomes (Figure 1A and 1C). The average relative number of NK cells in negative controls was 3.02%, lower than the DMBA treatment, with a mean relative number of NK cells as high as 3.86%. Furthermore, the cisplatin treatment group had an average relative number of NK cells of 4.07%, higher

than the positive control group. The increase in the relative number of NK cells is suggested due to the response of immunocompetent cells against cancer cells, which need a higher number of NK cells to eliminate the cancer cells. Conversely, cisplatin used in this treatment can suppress cancer-causing mutations by stopping DNA replication, specifically in the DNA adducts segments. Meanwhile, the cheral treatment groups showed continuously lower NK cells' relative number as the treatment dose increased. The CE1 group has an average of 3.16%, the CE2 group has an average of 3.03%, and the CE3 group has 2.48%. Among the cheral treatments, it did not result in a significant difference as the dose progressed. However, this cheral group's result is insignificant compared to negative control groups.



**Figure 1.** Immunomodulatory effects of cheral extract toward NK1.1<sup>+</sup> natural killer cells (A and C) and NK1.1<sup>+</sup>TNF-α<sup>+</sup>IFN-γ<sup>+</sup> natural killer cells (B and D) in DMBA-induced mice. The results were shown as the mean ± SD. Results were considered significant at p < 0.05. Different alphabetic symbols on the graph indicate a significant difference.

### **Cheral extract attenuates the relative number of NK Cells expressing TNF- $\alpha$ and IFN- $\gamma$**

TNF- $\alpha$  and IFN- $\gamma$  are the two major pro-inflammatory cytokines expressed by NK cells. The proportional levels of TNF- $\alpha$  and IFN- $\gamma$  expressed by NK cells yielded varied results in each treatment group (Figure 1B and 1D). The increasing inflammatory response resulting from DMBA induction in the positive control group resulted in a rising relative number of activated pro-inflammatory cytokines, the TNF- $\alpha$  and IFN- $\gamma$ , with an average of 60.62%, higher than the negative control, which has an average number of 45.57%. The average relative levels of TNF- $\alpha$  and IFN- $\gamma$  in the cheral treatment group also decreased compared to the positive control group. Moreover, the CE1 group has an average relative amount of TNF- $\alpha$  and IFN- $\gamma$ , around 55.67%, whereas the CE2 group has 55.61%. Those two groups had insignificantly different average numbers of TNF- $\alpha$  and IFN- $\gamma$  compared to each other. Thus, we proposed that those two groups have similar treatment effects. The CE3 group had an average relative level of TNF- $\alpha$  and IFN- $\gamma$  as low as 49.81%, significantly lower than the two lower dosages. Compared to other treatments, the CE3 group has a lower number of NK cells and their expression of TNF- $\alpha$  and IFN- $\gamma$ .

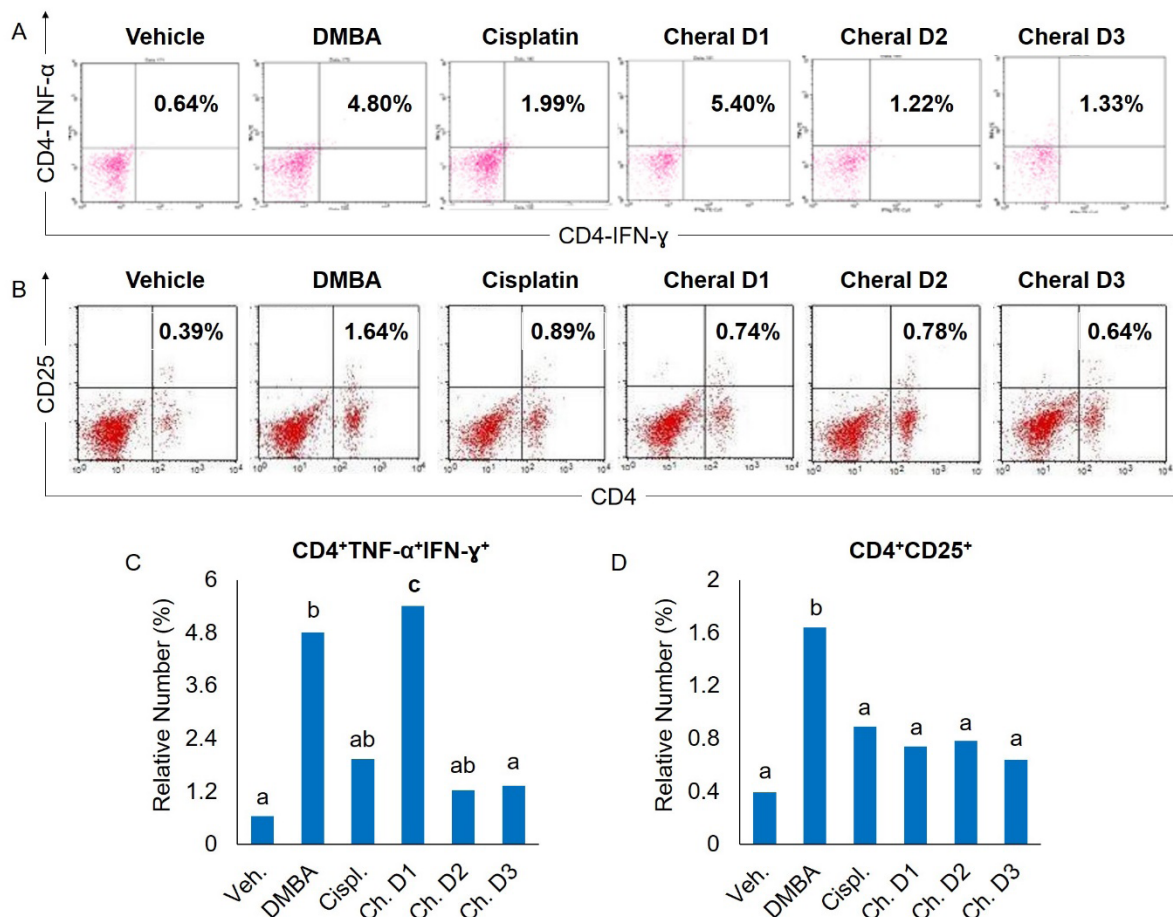
### **Cheral extract constricts the relative number of CD4<sup>+</sup>TNF- $\alpha$ <sup>+</sup>IFN- $\gamma$ <sup>+</sup> type 1 helper T cells**

The cisplatin and cheral treatment groups reduced

the relative number of CD4<sup>+</sup> T cells expressing the pro-inflammatory cytokine TNF- $\alpha$ <sup>+</sup>IFN- $\gamma$ <sup>+</sup>, also known as Th1, except for the CE1. According to the statistical results, the positive control group had a higher proportion of Th1 cells than the untreated group. There was no significant difference in the number of Th1 cells between the cherals and cisplatin treatment groups ( $p>0.05$ ). The most significant decrease in the relative number of CD4<sup>+</sup>TNF- $\alpha$ <sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells occurred in the CE2 and CE3 groups, which demonstrated close results to the negative control (Figure 2A and 2C).

### **Cheral extract reduces the relative number of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cell population**

The results showed that the relative number of regulatory T cell populations in breast cancer model mice increased significantly ( $p<0.05$ ) compared to the normal group (Figure 2B and 2D). The increase in the relative number of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the cancer model may be attributed to the inflammation in cancer patients. Subsequently, a large quantity of tumor antigens presented by MHC II on the surface of the APC cells, causing an increase in population and activation of CD4<sup>+</sup>CD25<sup>+</sup> T cells, promoting the further proliferation of immunocompetent cells. Compared to the cancer group, cheral treatment group also showed a significantly lower relative number of regulatory T cell populations ( $p<0.05$ ). The highest cheral dose treatment outperformed cisplatin or other cheral doses.



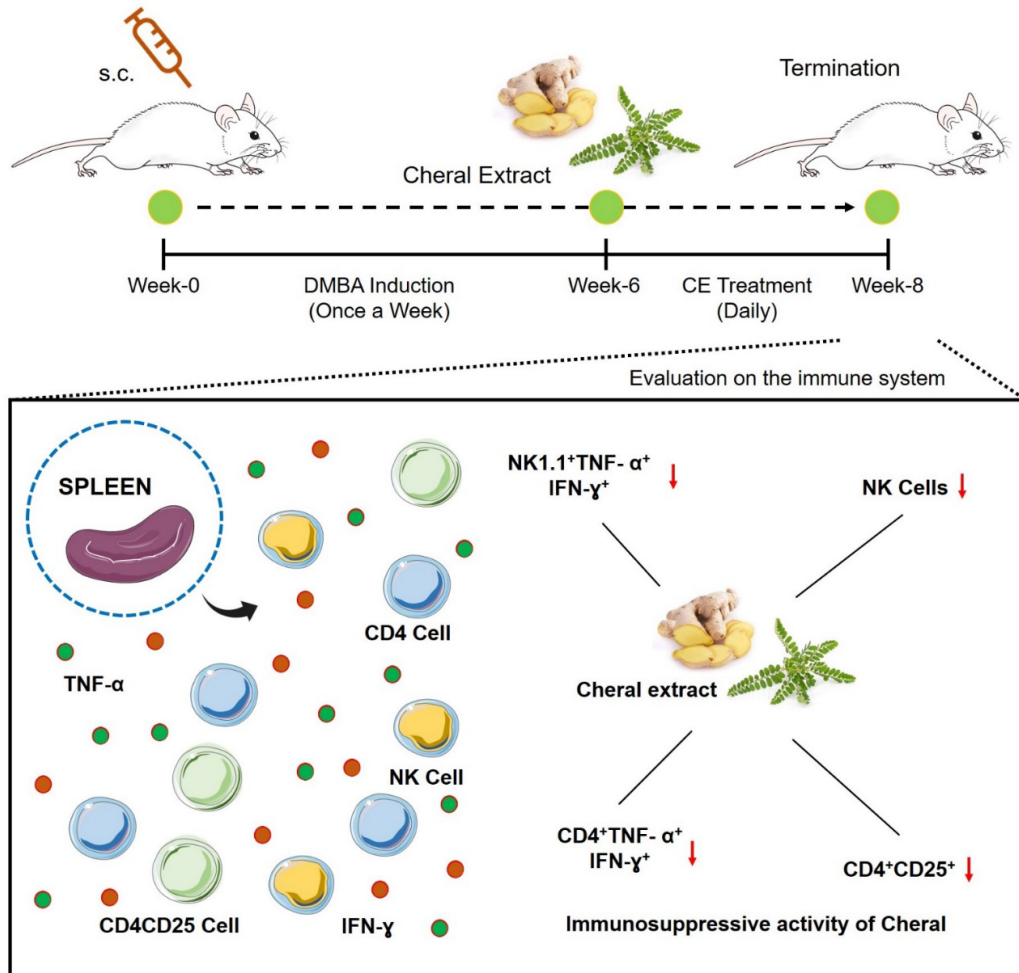
**Figure 2.** Immunomodulatory effects of cheral extract toward CD4<sup>+</sup>TNF- $\alpha$ <sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells (A and C) and CD4<sup>+</sup>CD25<sup>+</sup> effector T cells (B and D) in DMBA-induced mice. The results were shown as the mean  $\pm$  SD. Results were considered significant at  $p < 0.05$ . Different alphabetic symbols on the graph indicate a significant difference.

The rise in the DMBA-induced group’s NK cell population might be due to the early phases of immunosurveillance mediated by NK cells (Buque et al., 2020). This happens because NK cells constantly monitor the tumor site and recognize growing malignant cells lacking certain self-antigens, such as MHC class I. Tumor cell activation of surface receptors such as CD16, NKG2D, NKp46, DNAM-1, 2B4, NTB-A, and CRACC is also followed by the recognition of missing MHC class I, which triggers the cytotoxic mechanism as well as cytokine production. TNF- $\alpha$ , IFN- $\gamma$ , and IL-2 generated by CD4<sup>+</sup> cells, particularly Th1, attract more NK cells

and encourage limited proliferation, increasing their population size as the immune system tries to limit tumor progression (Liu et al. 2021, Meza Guzman et al. 2020). The interaction between the carcinogenic DMBA and cytochrome P-450, on the other hand, promotes DNA adduction and activates a detoxifying process, boosting ROS generation since it transforms DMBA into DMBA-3,4-diol, indicating the existence of free radicals (Mazambani et al. 2019) individual differences in the susceptibility to xenobiotic-induced breast cancer remain unclear. Since epigenetic modifications could control the expression of metabolic enzymes, our goal was to determine whether

epigenome modulated metabolic networks determine susceptibility to xenobiotic-induced breast cancer. The effect of epigenetic background on predisposition to carcinogen 7,12-dimethylbenz(a. Because NK cells are extremely vulnerable to ROS, an increased

concentration of ROS in the TME might result in cell dysfunction as well as triggering apoptosis, favoring an immunological escape mechanism (Kotsafti et al. 2020, Wang et al. 2021).



**Figure 3.** The immunomodulatory role of cheral extract in DMBA-induced breast cancer mouse model

According to certain research, providing bioactive substances may boost the cytotoxic activity of NK cells against tumor cells. Most studies, however, imply that administering bioactive compounds tends to increase the number of NK cells because the bioactive compounds encourage hematopoietic stem cells that develop into NK cells to mature quicker while promoting NK cell proliferation (Huijskens et al. 2015). The findings might imply that administering cheral, which includes many

antioxidants, could reduce oxidative stress generated mainly by ROS, hence improving conditions in the tumor microenvironment (Figure 3). Meanwhile, lower ROS content in the tumor microenvironment may improve NK cells' cytotoxicity and ability to eliminate cancerous cells because the nature of NK cells is susceptible to oxidative stress, particularly in the tumor microenvironment, resulting in a lower number of NK cells required to eliminate cancerous cells effectively (Whiteside 2020, Wang et al. 2021).



The significant rise in both cytokines appears to be unrelated to the number of NK cells. Even though we cannot identify the subset of NK cells from the mice, based on the results obtained, the increase of both cytokines in DMBA-induced mice is likely due to the more dominant subset of NK cells being cytokine-producing NK cells rather than cytotoxic NK cells, which are thought to have lower cytotoxicity but actively express TNF- $\alpha$  and IFN- $\gamma$ . Furthermore, the NK cell subgroup appears to have not yet reached the fatigued phase. TNF- $\alpha$  continues to cause cancer cell death by activating caspase 8. At the same time, IFN- $\gamma$  urges DCs to trigger robust CTL responses and further sensitize the cancer cells to NK cytotoxicity by cytotoxic NK cells (Zheng et al. 2019, Wang et al. 2012). In the cisplatin treatment group, the average relative quantity of TNF- $\alpha$  and IFN- $\gamma$  was lower than the positive control group, at 56.29%. We cannot pinpoint the cause of the decrease. However, it is believed to relate to cisplatin's antineoplastic properties, which interferes with DNA repair mechanisms, primarily in the adducted segment, causing DNA damage and subsequently lowering the number of cancer cells via apoptosis the immune responses by the NK cells (Ghosh 2019).

Typically, the lower number of NK cells and their expression of TNF- $\alpha$  and IFN- $\gamma$  indicate higher tumor incidence and further tumor progression because it reflects that the NK's function has been impaired. These results contradict other research, favoring a higher number of NK cells and their pro-inflammatory cytokine expression (Tjandrawinata et al. 2017, Hou & Fang 2021, Lee & Cho 2018, Sabry et al. 2019). However, another study stated that curcumin, a dominant bioactive compound in *C. zedoaria*, inhibits the IFN- $\gamma$  synthesis in NK cells by blocking STAT1 signaling in human pancreatic cancer cells and upregulating the STAT4 and STAT5, which promotes cytotoxicity of the NK cells (Lee & Cho 2018, Fiala 2015, Grudzien & Rapak 2018). The research focuses on an ethanol extract of *Phyllanthus* and found that it significantly reduced the release of IFN- $\gamma$ , TNF- $\alpha$ ,

IL-1, IL-2, and IL-6. It is claimed that corilagin in the extract significantly inhibited the production of pro-inflammatory cytokines and mediators such as TNF- $\alpha$ , IL-1, IL-6, NO (iNOS), and COX-2 at the protein and gene levels via NF- $\kappa$ B pathway inhibition. However, this mechanism occurred in cultured cancer cells rather than NK cells (Yuandani et al. 2013, Jantan et al. 2019). It is suggested that the administration of cheral could lower the synthesis of both inflammatory cytokines while enhancing the cytotoxicity of the NK cells, which means shifting the population of NK cells towards the cytotoxic NK cells rather than its cytokines-producing counterparts and directly killing the cancer cells.

The higher number of Th1 in the cancer group is driven by a higher concentration of T-cell stimulating factors, particularly the IL-12 secreted by APCs such as DC. The DCs acquire process and present tumor-associated antigens (TAAs) on MHC molecules in the TME and provide co-stimulation and soluble factors to shape T cell responses. These soluble factors, including IL-12 and IFN- $\gamma$ , promote T helper polarization towards the Th1 phenotype and increase its relative number (Wculek et al. 2020). The high relative number of Th1 could induce a high degree of anti-tumor responses mediated by the release of pro-inflammatory cytokines and higher recruitment of NK cells to the TME, as mentioned in the previous chapter. Meanwhile, a high concentration of TNF- $\alpha$  in the tumor microenvironment triggers a signaling cascade that activates NF- $\kappa$ B, initiating the expression of anti-apoptotic genes such as TRAF1 and TRAF2 that could promote tumor survival (Tang et al. 2017). Excessive pro-inflammatory cytokines can promote tumor growth through angiogenesis and cell migration (Esquivel-Velázquez et al. 2015).

The exact reason why the cisplatin treatment group exhibits a significantly lower number of Th1 cells remains unclear. A study suggests that cisplatin downregulates the expression of PD-L2 in DC cells, subsequently enhancing cytokine

synthesis that promotes Th1 polarization, as well as dephosphorylation of STAT6 required for Th2 polarization (Galluzzi et al. 2015, Boustani et al. 2021) accumulating evidence indicates that the efficacy of conventional and targeted anticancer agents does not only involve direct cytostatic/cytotoxic effects, but also relies on the (re, which seems contrary to our results. However, another study suggests that cisplatin induces IL-10 synthesis by activating DC's p38 MAPK and NF- $\kappa$ B signaling pathways, promoting Th2 cell polarization and inducing DC to adopt a tolerogenic phenotype. Balance shifting towards Th2 promotion hinders the Th1 cell proliferation and polarization (Kim et al. 2016). Because of cisplatin's initial deployment as a cytotoxic drug directly targeting the cancer cell is thought to induce cell death by forming crosslinks in DNA in fast-proliferating cells and initiating apoptosis (Raudenska et al. 2019). It also kills the cancer cell by increasing the expression of the Fas receptor, which recruits a variety of proapoptotic proteins, including caspase-8, to create the death-inducing signaling complex upon stimulation by FasL (Peter et al. 2015, Raudenska et al. 2019). These effects by cisplatin could lower the number of cancer cells, which also reduces the response of DC to promote helper T cell polarization towards the Th1 phenotype trying to eliminate the cancer cells.

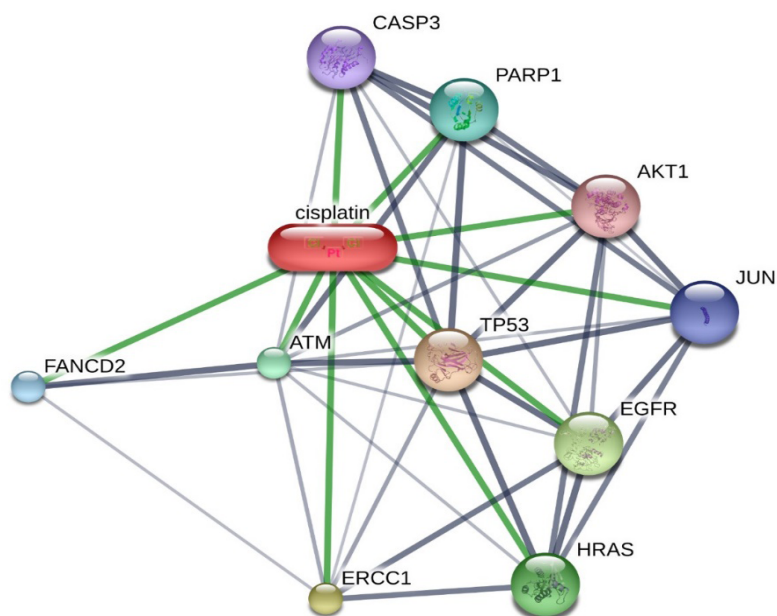
The information about the effects of administering *C. zedoaria*, *P. niruri*, or a combination of both towards the population and activity of Th1 cells could possess a similar effect as cisplatin, acting as a cytotoxic agent against cancer cells. A study suggested that  $\alpha$ -curcumene contained in *C. zedoaria* extract could activate the caspase-3 and caspase-9 pathways by releasing cytochrome c, inducing apoptosis (Shehna et al. 2022). Similarly, a study showed the alkaloid content of *Phyllanthus* plant called securinine and allosecurinine exerts anti-proliferation activity in several cancer cells. Further, the securinine induces apoptosis through cell cycle arrest activation in HeLa cells (Stefanowicz-Hajduk et al. 2016). Other studies also suggest that *C. zedoaria* and *Phyllanthus* extract

could induce apoptosis in cancer cell through down-regulation of Bcl-2, which act as an inhibitor of Bax and Bak, leading to cytochrome c-induced apoptosis (Lourembam et al. 2019). These mechanisms directly reduced the cancer cell count, simultaneously lowering the immune cell responses involved in TME, such as APCs, CTL, helper T cells, and NK cells. It could be predicted that a lower number of TAA recognized by the DCs could lead to the lower synthesis of the Th1 polarization agent, decreasing the number of Th1 cells in the TME (Wculek et al. 2020, Kim et al. 2016).

TNF- $\alpha$  is also released by macrophage-induced phagocytosis in human malignant mesothelioma. It can increase cell viability, reduce abscess-induced cytotoxicity, and may increase the assemblage of abscess-damaged mesothelium cells prone to malignant transformation. It also contributes to tumor initiation by increasing the production of genotoxic molecules, such as NO and ROS, which can cause DNA damage and mutations. Its expression has also been linked to an increased risk of other types of cancer, including bladder cancer, hepatocellular carcinoma, gastric cancer, and a poor prognosis in many hematological cancers. TNF- $\alpha$  promotes tumor progression rather than tumor initiation by promoting angiogenesis and metastasis and impairing immune surveillance by strongly suppressing many macrophage-activated cytotoxic T-cell activity responses (Cai et al. 2017, Kawanishi et al. 2017). TNF- $\alpha$  expression increases CXCL1/2 production by tumor cells via the NF- $\kappa$ B activation cascade by releasing an inflammatory modulator protein that can activate the p70S6K protein and the ERK1/2 signaling pathway, providing an advantage for primary tumor cell survival and cell metastasis (Ni et al. 2021). The ILs, TNF- $\alpha$ , and IFN- $\gamma$  regulate immune function, inflammatory response, and other physiological and pathological activities. These cytokines regulate cell survival, proliferation, and differentiation by signaling through the Jak/STAT and NF- $\kappa$ B pathways. When ILs and IFN bind to their cognate receptors, Jak Kinase activates and phosphorylates the STAT

protein, allowing STAT dimerization and nuclear translocation. The NF- $\kappa$ B proteins, including p50, p52, RelA/p65, and RelB, function as dimers and are typically inactively maintained by I $\kappa$ B proteins. Extracellular stimuli, such as TNF- $\alpha$  secretion, cause I $\kappa$ B degradation, releasing the NF- $\kappa$ B complex. The active NF- $\kappa$ B protein enters the nucleus and interacts with STAT, a transcription factor that regulates a wide range of target genes involved in this process. The TGF- $\beta$ , with its numerous and critical functions in the immune system, is involved in many ILs, TNF- $\alpha$ , and IFN- $\gamma$  signaling cascades by regulating the bioavailability and signal transduction of these cytokines. These factors, in turn, influence TGF- $\beta$  activity in various ways (Esquivel-Velázquez et al. 2015). The TNF- $\alpha$  binds to two receptors, TNFR1

and TNFR2, to initiate a signaling cascade that results in transcriptional regulation of mediators important in tumor cell survival, invasion, angiogenesis, and impaired immune system surveillance. However, the primary receptor mediator of TNF- $\alpha$  in tumor promotion is TNFR1 (Tang et al. 2017). On the other hand, the cheral treatment group reduced the relative number of macrophage cells that secrete both pro-inflammatory cytokines because it contains polyphenol and flavonoid antioxidants, reducing free radical concentration and activity of anti-inflammation and anti-malignancy properties. These bioproperties are not cytotoxic to normal cells, while the anti-cancer effects are limited to tumor cells.



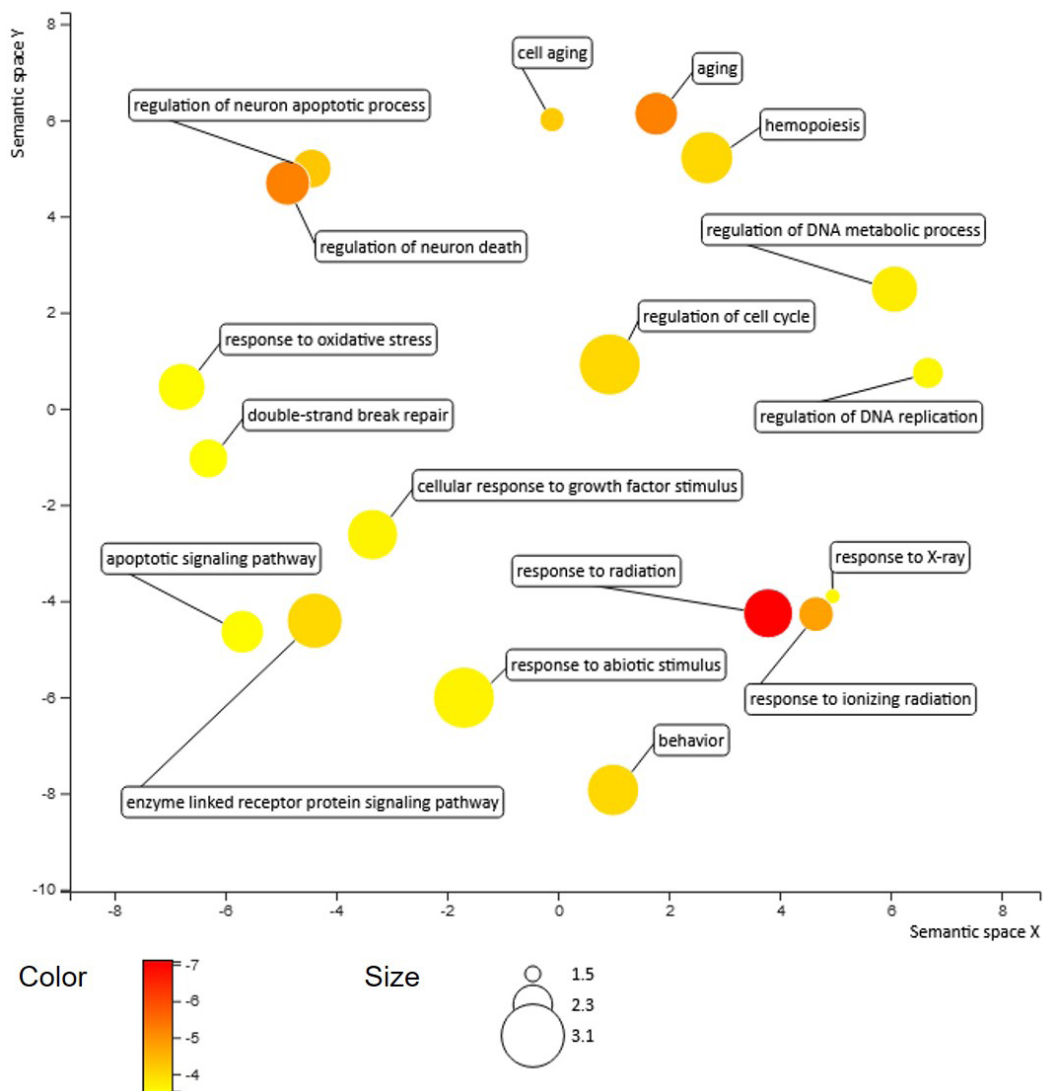
**Figure 4.** Cisplatin-proteins interaction network analysis. Several proteins showed in the network analysis have responsibility for cancer development and progression.

The CD4<sup>+</sup>CD25<sup>+</sup> T cell population can modulate the immune system's response to infection, autoimmunity, inflammation, and malignancies. This regulatory T cell typically helps the immune system maintain homeostasis (Okeke & Uzonna 2019). Cancer cells will exploit the Treg population, which

has immunosuppressive properties by inhibiting the activity of dendritic, CTL, and NK cells, resulting in continuous immunological invasion and hastening the development of breast cancer (Jean Baptiste et al. 2022, Togashi et al. 2019, Ohue & Nishikawa 2019). Another study discovered that the cancer mouse

model had more regulatory T lymphocytes in the spleen and lymph nodes than normal mice, which is commonly associated with poor prognosis (Riaz et al. 2017). This phenomenon is expected in the escape phase because tumor cells secrete chemokines such as CXCL1, CCL2, CCL5, CCR6, and CCL20 to recruit more Treg cells into the TME while also promoting Treg cell proliferation (Togashi et al. 2019, Ozga et al. 2021, O'Donnell et al. 2019). Recirculating Treg cells express a high number of IL2R (CD25), which is highly important for its development because it

would actively bind to IL-2, hence inhibiting the immunological response to malignant cells or the environment by lowering the production of cytokines, which also play a role in tumor growth suppression (Chinen et al. 2016). Increased CD25 expression in the CD4<sup>+</sup> T cell population is primarily due to increased ROS synthesis in the TEM by cancer cells and TAMs during oxidative stress, dampening the immunological response to the cancer cells (Chen, Han, et al. 2015).



**Figure 5.** Map visualization of Cisplatin’s biological response in the body. The map showed multiple biological processes were affected by Cisplatin.

Cisplatin groups show a lower number of Treg cells in the spleen compared to the DMBA-induced group. Although the exact molecular mechanism is unclear, it is suggested that a lower number of Treg cells in this group is correlated to cisplatin's lymphopenic characteristic which has a direct negative impact on actively proliferating cells, including the Treg cells (Heeren et al. 2019). The fact that the number of Treg cells remains higher than the negative control suggests that the surviving cancer may be feeding the mechanism driving the elevated Treg levels (Chen, et al. 2015).

Cisplatin is an organometallic platinum complex with two chlorine and amine atoms next to one another (Brown et al. 2019). Commonly, patients who have been diagnosed with lymphomas, breast, testicular, ovarian, head and neck, cervical, and sarcomas are still given cisplatin and other platinum-based drugs such as oxaliplatin and carboplatin as first-line medication (Mortensen et al. 2020). Cisplatin has been shown to affect many oncogenes involved in cancer development and remission directly (Figure 4). In addition, various biological processes, including the apoptotic signaling pathway, double-strand break repair, and oxidative stress response, are involved in the impact of cisplatin (Figure 5).

Generally, cisplatin's cytotoxic mechanism is triggered by its interaction with DNA to produce adducts, which induces apoptosis or programmed cell death (Siddik 2003). After entering the cell, cisplatin exerts its lethal effect by losing one chloride ligand, binding to DNA to generate intra-strand DNA adducts, and limiting DNA synthesis and cell growth. DNA lesions resulting from cisplatin-induced DNA damage stimulate DNA repair via NER (nuclear excision repair system) by inhibiting cisplatin-induced cell death via the ATM (ataxia telangiectasia mutated) pathway. Since cisplatin-induced DNA damage activates many signal transduction pathways that can facilitate or inhibit apoptosis, investigations have demonstrated that gene p53 is also related to

DNA damage and repair (De Laurenzi & Melino 2000, Lin & Howell 2006).

These similar outcomes suggested an effect of curcumin that reduced the relative amount of regulatory T cells and its activity by down-regulating CTLA-4, a protein on their cell surface, and interacting with CD80 molecules, causing signal transduction from Treg cells, and FOXP3 which important for T cells differentiation (Paul & Sa 2021, Bose et al. 2015). A study also suggests that curcumin could transform Treg cells into Th1 cells through downregulating FOXP3 and upregulating IFN- $\gamma$  synthesis (Shafabakhsh et al. 2019). Curcumin could also inhibit the nuclear translocation of p65 and cRel in the regulatory T cells population (Burge et al. 2019). The chamber bitter contains phytochemicals such as Phyllantin and flavonoids, which have the potential to modify and activate the immune system. Previous research has demonstrated that flavonoids can lower the expression of CD25 and IL-2 molecules, scavenging free radicals, lowering ROS levels, and inhibiting NF- $\kappa$ B activation, resulting in reduced pro-inflammatory cytokines such as IL-1 $\beta$ , IL-2, IL-6, and TNF- $\alpha$  (Leyva-López et al. 2016).

## CONCLUSION

In this present study, we demonstrated that cheral extract exerts immunosuppressive activity by attenuating the properties of the immune system, such as NK cells, Th1 cells, and regulatory T cells in DMBA-inducing mice into normal levels. Further research needs to be carried out, especially to determine the broader effects on the immune system and the specific mechanism of action from cheral extract in treating cancer.

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### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

### AUTHOR CONTRIBUTION STATEMENT

Concept and Design (M.R.); Supervision (M.R.); Funding Acquisition (M.R.); Materials (W.E.P., A.M.L.S.M., D.R., D.C., A.H.); Data Collection and Processing (W.E.P., A.M.L.S.M., D.R., D.C.); Analysis and Interpretation (W.E.P., A.H., M.R.); In Silico Study (W.E.P., A.H.); Writing Manuscript (W.E.P., A.H., M.R.); Review and Editing (W.E.P., A.M.L.S.M., D.R., D.C., A.H., M.R.).

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