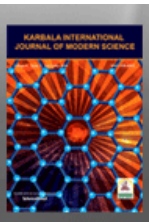

Karbala International Journal of Modern Science

Volume 9 | Issue 2 Article 10

Immunomodulatory and Ameliorative Effect of Citrus limon Extract on DMBA-induced Breast Cancer in Mouse

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Recommended Citation

Putra, Wira Eka; Agusinta, Astrid Karindra; Ali Ashar, Muhammad Sultonun Arifin; Manullang, Vetti Adriani; and Rifa'i, Muhaimin (2023) "Immunomodulatory and Ameliorative Effect of Citrus limon Extract on DMBA-induced Breast Cancer in Mouse," *Karbala International Journal of Modern Science*: Vol. 9 : Iss. 2 , Article 10.
Available at: https://doi.org/10.33640/2405-609X.3273

Immunomodulatory and Ameliorative Effect of Citrus limon Extract on DMBA-induced Breast Cancer in Mouse

Abstract

Breast cancer is the most prevalent malignancy in the world and the leading cause of female cancer-related mortality. Several ways for combating this entity have been proposed, but a significant and effective cure has yet to be established. Natural compounds originating from plants, including Citrus limon, are employed as alternative cancer treatments. In this study, we aimed to evaluate the immunomodulation activity of Citrus limon's extract against breast cancer inci-dence in the mouse model. An in vivo study using female BALB/c mice served as the basis for this investigation. Four groups of eight-week-old mice, each weighing 20 grams, were randomized. The carcinogenic substance called 7-12 Dimethylbenz(a)anthracene (DMBA) was used to generate cancer in the experimental animal model. Several antibodies combination were used for the extracellular and intracellular staining experiments, including FITC-conjugated rat an-ti-mouse CD11b, PE/Cy5-conjugated rat anti-mouse IL-6, FITC-conjugated rat-anti mouse CD4, PE-conjugated rat anti-mouse CD8, PE-conjugated rat anti-mouse CD62L, PE-conjugated rat-anti-mouse TNF-α, and PE Cy5-conjugated rat-anti mouse IFN-ɣ. A one-way analysis of variance test was used to examine the relationship between flow cytometry data and the relative cell number. In this present study, we found that the induction of DMBA decreased the relative number of both naïve CD4 and CD8 T cells. On the other hand, the induction of DMBA increased pro-inflammatory cytokines such as TNF-α, IFN-ɣ, and IL-6. Interestingly, Citrus limon's extract administration can directly change the immune system condition into the normal level in carcinogenic mouse model. Thus, this study suggested that Citrus limon's extract ameliorative activity against breast cancer incidence warrants further investigation.

Keywords

Breast cancer; DMBA; immunomodulation; lemon; therapeutic

RESEARCH PAPER

Immunomodulatory and Ameliorative Effect of *Citrus limon* Extract on DMBA-induced Breast Cancer in Mouse

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Abstract

Breast cancer is the most prevalent malignancy in the world and the leading cause of female cancer-related mortality. Several ways for combating this entity have been proposed, but a significant and effective cure has yet to be established. Natural compounds originating from plants, including *Citrus limon*, are employed as alternative cancer treatments. In this study, we aimed to evaluate the immunomodulation activity of *C. limon's* extract against breast cancer incidence in the mouse model. An in vivo study using female BALB/c mice served as the basis for this investigation. Four groups of eight-week-old mice, each weighing 20 g, were randomized. The carcinogenic substance called 7−12 Dimethylbenz(a) anthracene (DMBA) was used to generate cancer in the experimental animal model. Several antibodies combination were used for the extracellular and intracellular staining experiments, including FITC-conjugated rat anti-mouse CD11b, PE/Cy5-conjugated rat anti-mouse IL-6, FITC-conjugated rat-anti mouse CD4, PE-conjugated rat anti-mouse CD8, PE-conjugated rat anti-mouse CD62L, PE-conjugated rat-anti-mouse TNF-α, and PE Cy5-conjugated rat-anti mouse IFN-ɣ. A one-way analysis of variance test was used to examine the relationship between flow cytometry data and the relative cell number. In this present study, we found that the induction of DMBA decreased the relative number of both naïve CD4 and CD8 T cells. On the other hand, the induction of DMBA increased pro-inflammatory cytokines such as TNF-α, IFN-ɣ, and IL-6. Interestingly, *C. limon*'s extract administration can directly change the immune system condition into the normal level in carcinogenic mouse model. Thus, this study suggested that *C. limon*'s extract ameliorative activity against breast cancer incidence warrants further investigation.

Keywords: Breast cancer, DMBA, Immunomodulation, Lemon, Therapeutic

1. Introduction

Breast cancer is the most common cancer in the world and the leading cause of death from cancer in women [1]. Intriguingly, the number of women diagnosed with breast cancer in the United States has reached over 200 million and is projected to increase over the next several years [2,3]. Breast cancer risk factors include gender, age, genetics, lifestyle, and the presence of carcinogens in the environment [4]. Carcinogens are chemicals that promote cancer incidence, and one of them is DMBA. Carcinogens can affect the immune response, including the elevation of inflammatory conditions. Inflammation is a type of immune response characterized by the secretion of pro-inflammatory cytokines such as TNF-α and IFN-ɣ by various innate or adaptive immune cells. Pro-inflammatory cytokines, in normal conditions, promote the activation of different immune cells such as natural killer cells, macrophages, T cells,

Received 25 July 2022; revised 29 October 2022; accepted 2 November 2022.
Available online 1 May 2023

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https://doi.org/10.33640/2405-609X.3273

and B cells for elimination or defense system purposes [5]. However, long-term and uncontrolled inflammation can elevate the reactive oxygen species (ROS), which can damage DNA, cause genomic instability, and promote carcinogenesis [6].

Anti-cancer drugs and chemotherapy treatments are widely used to cure various cancers, including breast cancer [7]. Although these treatments have great potency in reducing cancer growth in patients, it also leads to other adverse effects. Inappropriate cancer therapy can also increase the inflammatory response by traumatizing necrosis and injuring the tissue, stimulating cancer recurrence and resistance to cancer therapy [6]. According to Luo et al., natural ingredients derived from plants and their extracts are used as alternative cancer therapies due to their low toxicity [8]. On the other hand, Saroja et al. noted that one of the causes of cancer progression is the involvement of ROS, so there must be a compound activity that can reduce high ROS activity to prevent cancer. Antioxidants have been shown to reduce oxidative damage caused by ROS and may even help prevent cancer. Fruits with antioxidants, such as flavonoids, can provide biological effects such as anti-inflammatory and anti-cancer properties [9].

A natural ingredient in the form of *Citrus limon* extract (CLE) was tested in this study to evaluate immune system activity against cancer. Lemon is widely used as a traditional medicine in developing countries because it is thought to have scavenger and anti-inflammatory properties for all types of cancer [10]. Lemon is known to contain anti-cancer compounds. Several previous studies have shown that the flavonoid content in lemons can inhibit the growth of cancer cells and have an antiproliferative effect *in vitro* [11]. Cancer cells, in particular, have a high potential for being eliminated by the immune system. The immune system will recognize cancer as an antigen. Numerous studies have demonstrated that immunogenic signals, such as pro-inflammatory cytokines, which trigger innate and adaptive immunity, can help the immune system identify and destroy cancer cells [12,13]. The immune system is essential to investigate in cancer research because it has a prominent potency to reduce cancer incidence without causing excessive toxicity to normal tissue and induce memory cells to prevent cancer recurrence [12]. According to the above explanation, this study aimed to assess the immunological profile of breast cancer mouse models after they were treated with lemon fruit extract.

2. Materials and methods

2.1. Experimental design

This study was based on an in vivo experiment on female BALB/c mice. A total of sixteen female mice aged eight weeks and weighing 20 g were divided into four treatment groups: vehicle control (Veh.), DMBA group as a positive control (D), CLE 50 group with DMBA induction and treated with 50 mg/kg BW lemon extract, and CLE 200 group with DMBA induction and treated with 200 mg/kg BW lemon extract for 14 days. Except for the vehicle group, all mice were induced with the carcinogenic compound 7–12 Dimethylbenz(a)anthracene (DMBA) at a dose of 0.015 mg/g BW. DMBA was dissolved with 0.1 mL of corn oil. For about six weeks before CLE treatment, DMBA induction was performed subcutaneously in the mammary gland of mice (Fig. 1). The animal (scientific procedures) act of 1986, which restricts the use of designated animals in any research that may cause pain, suffering, distress, or enduring harm to the animal, has been adhered to in this study. Furthermore, the Research Ethics Commission of Brawijaya University has granted this study a certificate of ethical clearance with the following number 779-KEP-UB.

2.2. Splenocytes isolation

Every treatment group of mice was terminated and followed by spleen isolation. Phosphate buffer saline (PBS) was used to wash these organs. The spleen was smoothed by the base of the syringe clockwise until homogeneous. The crushed organ was then stored in a 15 mL propylene tube. A propylene tube is used to hold the cell suspension. The suspension from each organ was centrifuged for 5 min in a propylene tube at 2500 rpm at 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 mL of PBS.

2.3. Antibody staining and flow cytometry analysis

The cell isolation resuspension was taken up to 60 μL and then transferred to a microtube. A total of 300 μL PBS was added to the microtube containing the cells, which was then centrifuged for 5 min at 2500 rpm in 10 °C. The pellets were separated from the supernatant and stained with 50 μL of extracellular antibody, which included FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD8, and PE-conjugated rat anti-mouse CD62L. The

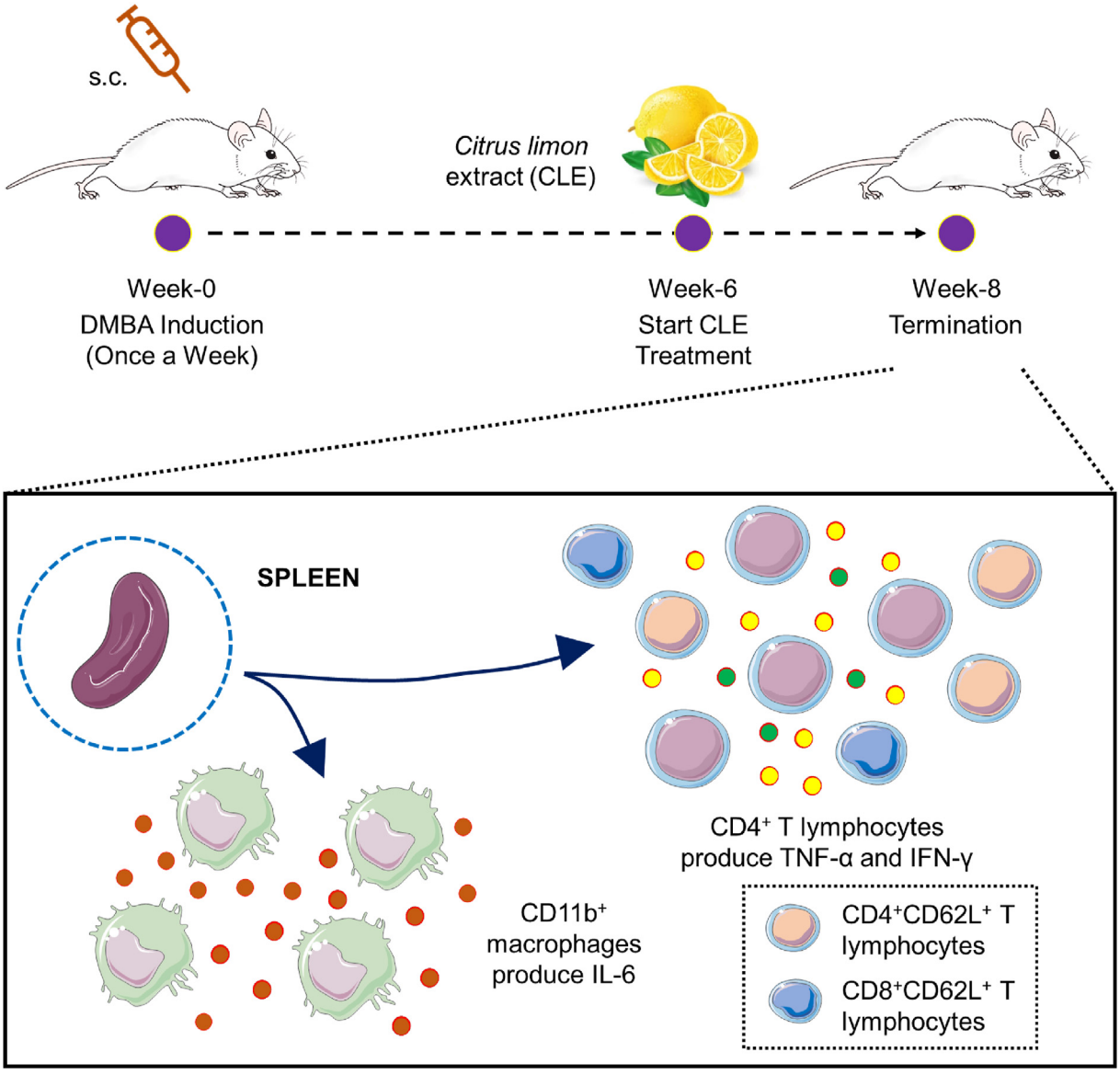

Fig. 1. Schematic picture showed how Citrus limon's *extract interfere the immune system, including the innate and adaptive immunity and the production of cytokines on DMBA-induced breast cancer mice model.*

mixture was then incubated in an ice box for 20 min. After extracellular antibody staining, cells were incubated in 400 μL of PBS before being transferred to flow cytometry cuvettes and analyzed using flow cytometry on a computer with BD Cellquest Pro™ software. Several antibody combinations were used in the extracellular and intracellular staining experiments, including FITC-conjugated rat anti-mouse CD11b, PE/Cy5-conjugated rat anti-mouse IL-6, FITC-conjugated rat-anti mouse CD 4, PE-conjugated rat-anti-mouse TNF-α, and PE Cy5-conjugated rat-anti mouse IFN-ɣ.

Similarly to the previous treatment, 300 μL of PBS was added to the microtube containing 60 μL of samples from each treatment. At 10 °C, samples were centrifuged at 2500 rpm for 5 min. Separately, 50 μL of CD4 and CD11b antibodies were added to the centrifuged pellets. The resuspended pellets were incubated in a dark room for 20 min at ice temperature. The intracellular staining was initiated in each incubated microtube by adding 50 μL of cytofix solution to the microtube and incubating in the ice box at 40 °C for 20 min. After incubation, the fixative was washed with washperm solution as much as 300 μL, then centrifuged at 2500 rpm for 5 min at 10 °C. The supernatant was removed and the pellets from each microtube were added with intracellular antibodies IFN-ɣ, TNF-α, and IL-6 then

incubated in an ice box at 4 °C for 20 min. The PBS was added to the microtube as much as 400 μL, then put it in a flow cytometry cuvette and analyzed flow cytometry. Techniques and data analysis using FACS Calibur™ and BD CellQuest Pro™ software.

2.4. *Protein target of* C. limon's *bioactive compound and network analysis*

We conducted the prediction study to investigate the target protein of *C. limon*'s bioactive main component. After that, the protein target's interaction was examined using the STRING protein–protein interaction database (https://string-db.org/). From database, we analyse further information related to gene ontology, cellular compounds, molecular function, and biological process.

2.5. *Data analysis*

Data on the relative number of cells resulting from flow cytometry were analyzed and followed by a one-way analysis of variance (ANOVA) test with a 95% confidence interval using SPSS version 16 for Windows, then the Tukey test was carried out as a follow-up test to determine the real difference between the treatment groups.

3. Results and discussion

3.1. *CLE increases the relative number of* $CD4^+CD62L^+$ *naïve T cells*

MHC class II-expressing APCs will phagocytically ingest and present antigens generated from deceased cancer cells. The macrophages subsequently activate the CD4 T cells by forming a complex with them through the TCR. These macrophages will generate IL-1, prompting CD4 T cells to generate IL-2. After that, IL-2 promotes CD4 T cell growth through autocrine. Through secreted lymphokines, activated CD4 T cells will also influence other cells such as B cells, macrophages, cytotoxic T cell precursors, and endothelial cells [14]. When CD4 T cells are activated, they lose CD62L molecules, which serve as a marker for T cell homing. The $CD4^+CD62L^+$ T cell analysis data revealed a significant decrease in DMBA treated mice (Fig. 2). Expressly, in breast cancer model mice, a decreased number of naive T cells indicated a cellular immune response that activates effector cells. The immune response that occurs is a result from CD4 T cell activation in the secretion of several types of lymphokines. This is supported by Huang et al. study, which found a significant increase in the number of activated CD4 T cells in mice that were subcutaneously injected with breast tumor cell lines 4T1 and E0771 in the breast fat tissue section [15]. Th1 cells are the dominant CD4 T cell population in the early stages of breast tumor development, and they may play an important role in immunosurveillance. In the late stages of breast cancer, however, the dominant population of $CD4^+$ T cells changes to regulatory T cells and Th17 cells as tumor cells develop. After DMBA induction, administration of lemon extract significantly increased the relative number of naive CD4 T cells (Fig. 2). The increased number of naïve CD4 T cells indicates that the compounds in lemon extract such as flavonoids have immunostimulation properties.

3.2. *CLE increases the relative number of* $CD8^+CD62L^+$ *naïve T cells to a normal level*

Most tumor antigens in cancer cases are presented by the major histocompatibility complex (MHC) class I, which forms a complex with CD8 cytotoxic T cells via the T-cell receptor (TCR). This condition will trigger and activate cytotoxic T cells, destroying the tumor cells. CD8 T cells can directly destroy tumors via effectors such as granzyme B and perforin [16]. The analysis of the number of $CD8^+CD62L^+$ T cells using flow cytometry revealed a significant decrease in the relative number of $CD8^+CD62L^+$ T cells or naive CD8 T cells in DMBA treated compared to the vehicle group (Fig. 2). The decline of naïve CD8 T cells in DMBA-treated mice indicated a cellular immune response in the breast cancer mice model. The immune response occurs due to the activation of CD8 T cells that form a complex through TCR with MHC class I on the cellular surface of the tumor antigen, then CD8 T cells will destroy the tumor cells. This is also consistent with research conducted by Huang et al. (2015) stated that the number of activated CD8 T cells also significantly increased in mice injected subcutaneously with breast tumor cell lines 4T1 and E0771 in the breast fat tissue section [15]. CD8 T cells are mostly found at the tumor site as lymphocytes that infiltrate the tumor tissue, known as tumor-infiltrating lymphocytes.

CD8 T cells play a significant role in the immune response to breast cancer. In the early stages of breast tumor development, CD8 T cells are one of the dominant populations in tumor-infiltrating lymphocytes that can perform immunosurveillance. CD8 T cells are the main effector cells that have an important role in controlling anti-tumor immunity. The number of CD8 T cells is more dominant than CD4 T cells in the early stages of tumor

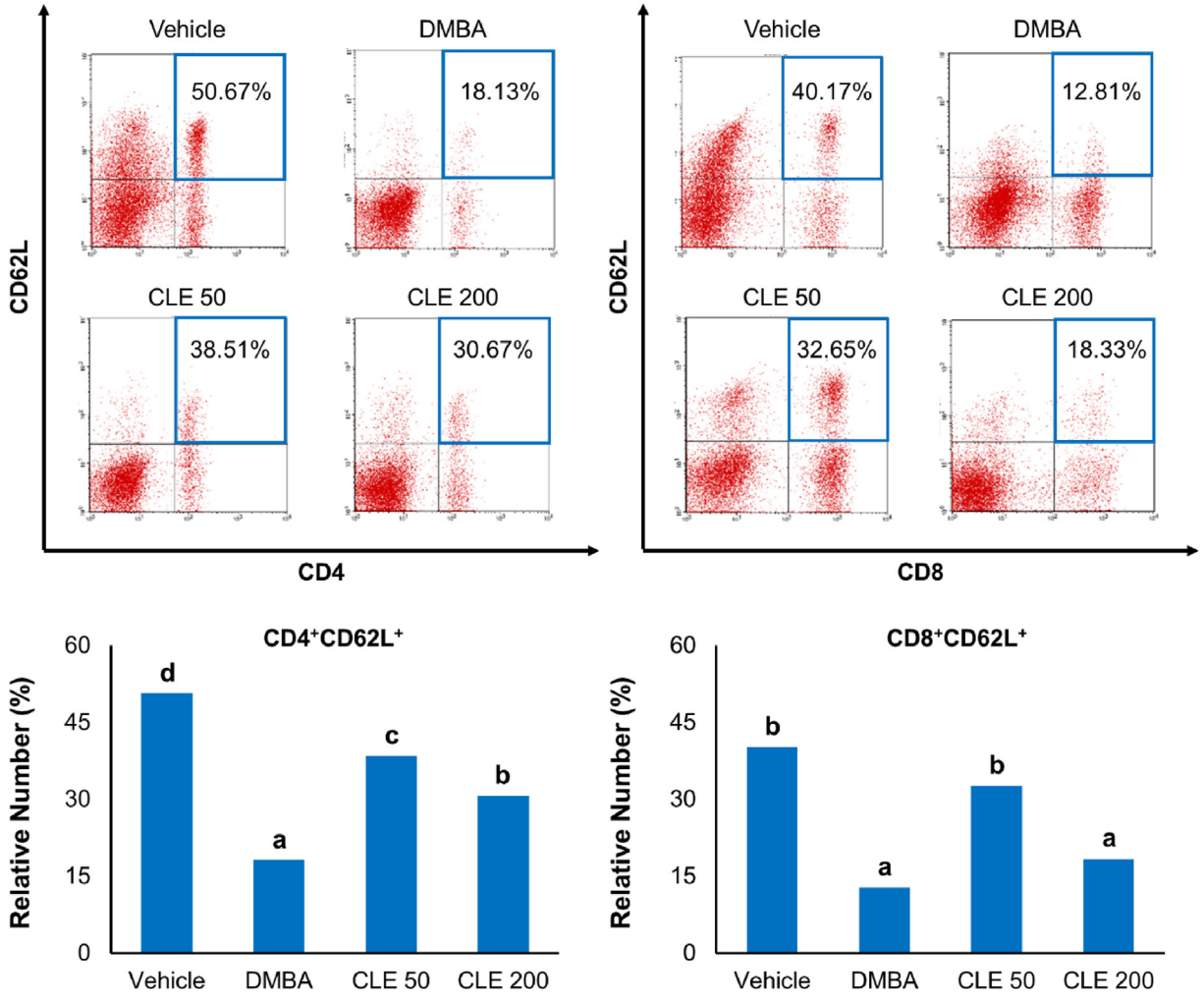

Fig. 2. Relative number of naïve CD4 and CD8 T cells. Both $CD4^+CD62L^+$ and $CD8^+CD62L^+$ were decreased on DMBA induction groups. However, the relative number returned to the normal condition after being treated by Citrus limon's *extract. The table shows a significant difference among the experimental group ($p < 0.05$).*

development. However, CD4 T cells infiltrate the tumor site more rapidly than CD8 T cells in tumor development. Therefore, CD4 T cells become the dominant population in the late stages of tumor development [15,17]. The relative number of naïve CD8 T cells in CLE 50 group was 32.65% higher compared to CLE 200 group (Fig. 2). An experimental mouse model in CLE 50 group was able to significantly increase the relative number of naïve CD8 T cells while the number of naïve CD8 T cells in CLE 200 group showed insignificant change.

Flavonoids in *C. limon* exert anti-inflammatory effect which induce the activation of cells involved in the immune response, including T- and B cells [18]. Apart from flavonoids, hesperidin and tangeretin in *C. limon* are also known to have anti-neuroinflammatory abilities which can suppress the secretion of cytokines TNF-α, IL-1β, IL-6 [19]. Similarly, TNF-α and IL-6 induced by IL-7 will strengthen inflammation and support cancer growth [20]. Furthermore, Arivazhagan & Pillai, (2014) explained that the tangeretin could significantly suppress matrix metalloproteinase (MMP)-2 in animal models induced by DMBA. MMP secretion is one of the hallmark of cancer invading [21]. According to Lee et al., tangeretin also inhibits metastasis in human MCF-7/6 carcinoma cells by suppressing the secretion of MMP-3 and MMP-9 [22].

3.3. The effect of CLE on pro-inflammatory cytokines

Cytokines are proteins in the immune system that regulate interactions among the cells and can trigger immune reactivity, both the adaptive and specific

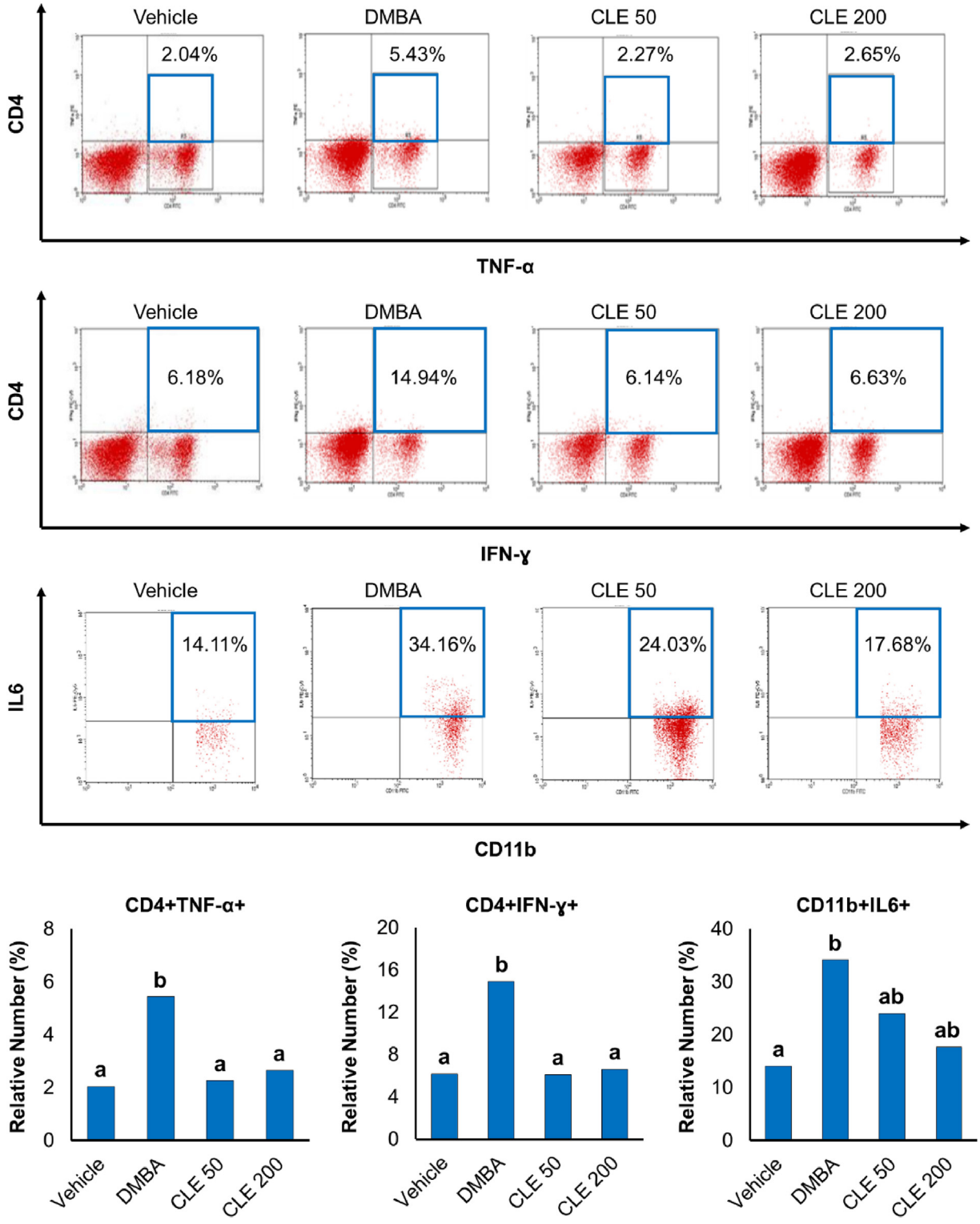

Fig. 3. Relative number of cytokines-producing T cell and cytokines-producing macrophage. Data showed $CD4^{+}TNF\text{-}\alpha^{+}$, $CD4^{+}IFN\text{-}\gamma^{+}$, and $CD11b^{+}IL\text{-}6^{+}$ were significantly increased on DMBA induction group. However, the relative number returned to the normal condition after being treated by Citrus limon's *extract. The table shows a significant difference among experimental group ($p < 0.05$).*

immune systems. Cytokines can carry chemical messages, mediate cell communication, and influence cellular response. Cytokines that play a role in immunity such as IL-6, TNF-α and IFN-γ are generally produced by several cells that act against different target cells [23].

The results showed that the number of $CD4^+$ T cell cytokines that secreted TNF-α significantly differed between the vehicle group and breast cancer mouse model ($p \leq 0.05$). The relative number of $CD4^+TNF\text{-}\alpha^+$ T cells expressed in CLE 50 group was about 2.27% and 2.65% in CLE 200 (Fig. 3). These results indicate that after administration of lemon extract either at a dose of 50 mg/kg BW or 200 mg/kg BW the expression of $CD4^+TNF\text{-}\alpha^+$ cytokines decreased to the normal level. A similar trend was found in the expression of $CD4^+IFN\text{-}\gamma^+$ The data showed the relative number of $CD4^+IFN\text{-}\gamma^+$ cells in the average breast cancer model was higher than vehicle group ($p \leq 0.05$). The relative amount of IFN-γ cytokines expressed in normal mice was 6.18%, whereas in cancer model mice was 14.94%. The graph shows the difference in the average relative cell count of $CD4^+IFN\text{-}\gamma^+$ cells in the group of mice given lemon extract at a dose of 50 mg/kg BW and 200 mg/kg BW (Fig. 3).

In vehicle group, the secretion of pro-inflammatory cytokines tends to be low compared to mice in breast cancer models. In this study, the relative number of pro-inflammatory cytokines in breast

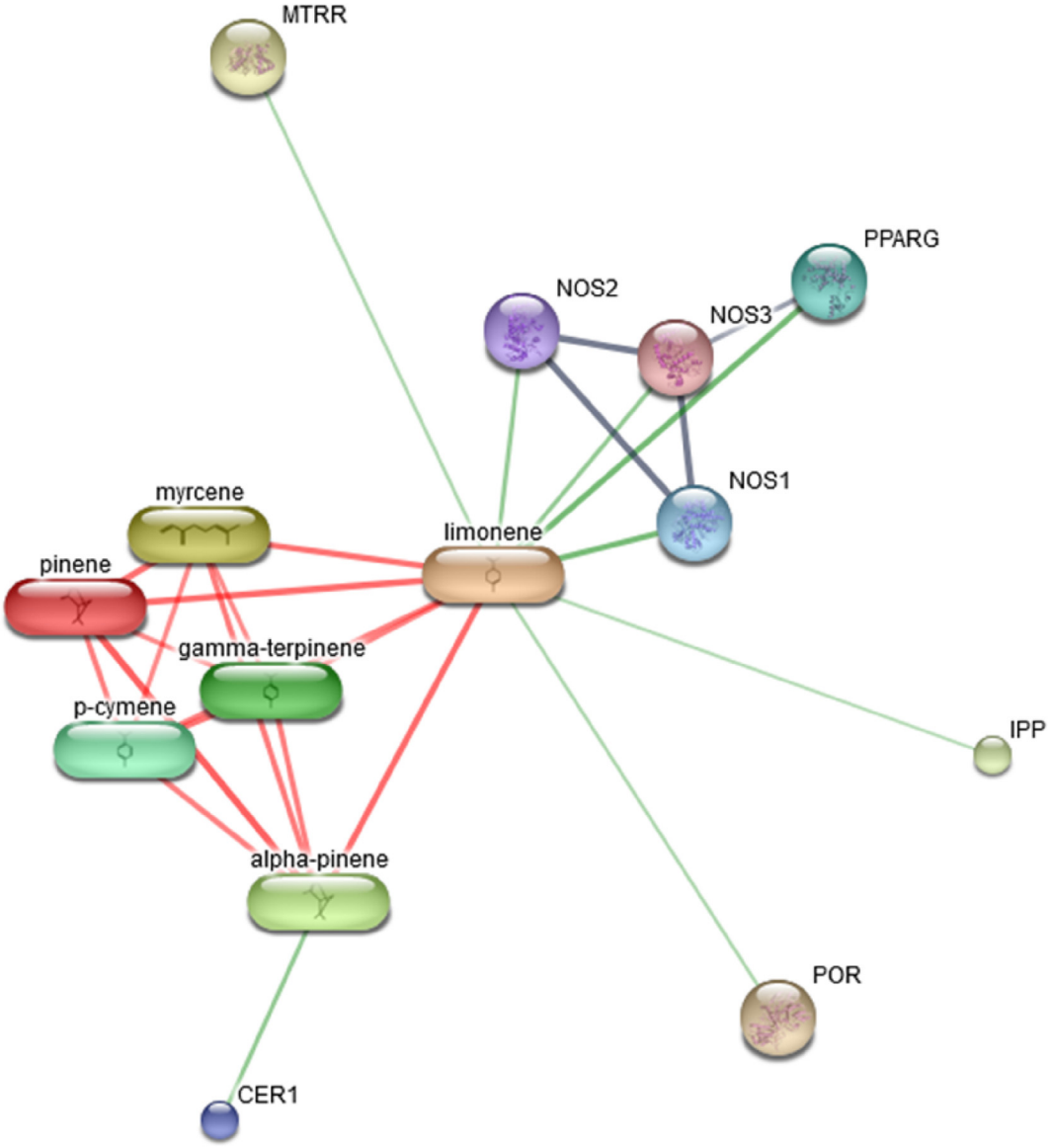

Fig. 4. The interaction among Citrus limon's *bioactive compounds and possible targeted protein that include or affected by those compounds.*

cancer model mice has increased, this is thought to be due to an increase in immune response associated with increased activity of immune cells against breast cancer. Cancer cells express different antigens from cells in general. The antigen is a product of mutated genes or it can be overexpression of normal proteins. The expression of these antigens serves as recognition by the immune system to produce an immune response against antigens [24]. TNF-α and IFN-ɣ are thought to induce apoptosis and function as Th1 subset regulation and are responsible for increasing the proliferation of B cells to produce Ig-G as humoral immunity, which binds to antigens and activates antibody-dependent cellular cytotoxicity function.

Macrophages are also thought to be able to bind to tumor antigens via IgG binding to tumor antigens, then macrophages can phagocytose, break down into peptides and present them to CD4 cells. CD4 that is activated by MHC type 2 will differentiate into Th 1 cells and start secreting cytokines related to immune responses to tumor antigens, including IFN-ɣ and TNF-α [5,25]. Based on research conducted by Amorim et al., (2016) explained that *C. limon*'s extract was able to reduce pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ in mice injected with carcinogenic compounds [26]. According to Devaraj et al. (2011) Several clinical trials that have been conducted have also shown a positive effect of flavonoids in *C. limon* in reducing pro-inflammatory cytokines in humans. In addition, flavonoid compounds are also mitogenic, which can induce IL-2 synthesis, which can induce and stimulate T cell proliferation [27].

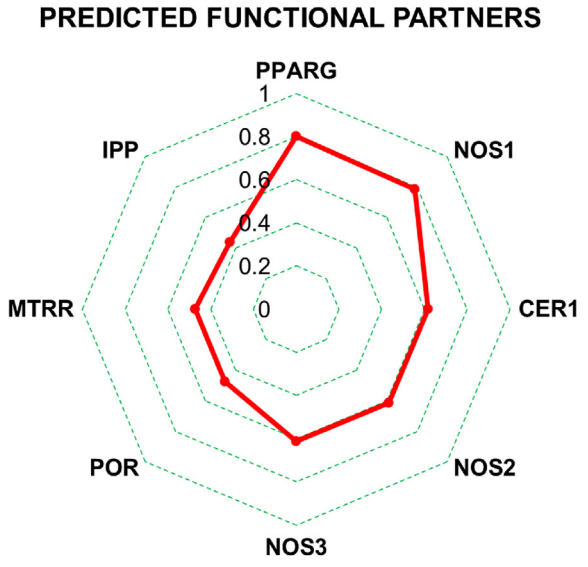

Fig. 5. The predicted functional partners involved within the interaction with Citrus limon's *bioactive compounds.*

Based on the results of flowcytometry analysis on IL-6 producing by macrophage, showing the relative number of cells, it is known that there is a difference in the relative number of $CD11b^{+}IL\text{-}6^{+}$ from each treatment. Mice group treated by DMBA only increased significantly ($p < 0.05$) compared to other treatments. However, the relative number of $CD11b^{+}IL\text{-}6^{+}$ were back to the normal level after treated by lemon extract (Fig. 3). This pattern of results is supported by Park et al. and Heikkilä et al., statement that, under normal or healthy conditions, IL-6 concentrations are low, while the amount of IL-6 increased during tumor developing stage [28,29]. The initial entry of an antigen into the body will be recognized by innate immunity, including macrophages. Macrophages are the main source of pro-inflammatory cytokines which also promote tumor immunosurveillance and cancer cytotoxicity [5].

During chronic inflammatory conditions, pro-inflammatory cytokines including IL-6 promote tumor initiation, cell proliferation, and reduce apoptosis [30]. Increasing amount of IL-6 may become the hallmark of cancer progression, which induce the not only cell proliferation, but also promoting angiogenesis [31]. Another study showed angiogenesis occurs due to chronic inflammation and causes the increasing number of tumor infiltrated lymphocytes which secrete excessively pro-inflammatory cytokines such as IL-6, IL-1α, IL-1β, TNF-α that affect COX-2 and VEGF expression [32]. Investigation conducted by Xu et al. (2016) showed the expression number of CD11b was lower in healthy mice compared to the tumor mice model [33]. This is become the strong evidence that CD11b in macrophage or dendritic cells was significantly increased in the spleen and bone marrow of mice affected by tumors and in conditions associated with impaired immune system reactivity [34].

When mice model positive to breast cancer caused by DMBA, it means that there are lots of ROS or free radicals in the body which make the mechanism in the body to change the cell cycle by disrupting cell proliferation due to DNA damage. The role of flavonoids in *C. limon* act as the ROS scavenger by inhibiting lipid peroxidation reactions, preventing other oxidative damage, and preventing chain reactions of ROS [35]. Therefore, the lemon extract therapy containing flavonoid and other bioactive compounds may possibly inhibit cancer cell proliferation. Flavonoids in *C. limon* can reduce chronic inflammation caused by the production of cytokine IL-6 from excessive macrophage secretion and this can be seen from the treatment of cancer mice given lemon extract. This

is consistent with the statement of Wang et al., that flavonoids can play a major role in the main prevention of chronic disease [36]. When flavonoids react and suppress IL-6 production from macrophage secretion, there is no binding between IL-6 and its receptor, namely IL-6R. This is because when there is a decrease in IL-6R activity, it significantly inhibits the activity of seeding or growth of cancer cells [37]. When there is a cancer condition, the cytokine IL-6 plays a role in inflammation and stimulation of cancer cell proliferation will play an active role, depending on the type of cell and the presence or absence of IL-6R [38].

3.4. The role of lemon against cancer cells

After treated by *C. limon's* extract every day for two weeks it has caused a decrease in pro-inflammatory cytokines concentration in both dose groups, CLE 50 and CLE 200. The decrease in pro-inflammatory cytokines after lemon extract administration is thought to be due to improvements in the body that can reduce the growth of cancer cells. The reduced growth of cancer cells leads to decreased overactivity of immune cells. Lemon has various bioactive components which are thought to have the ability to reduce the growth of cancer cells. The

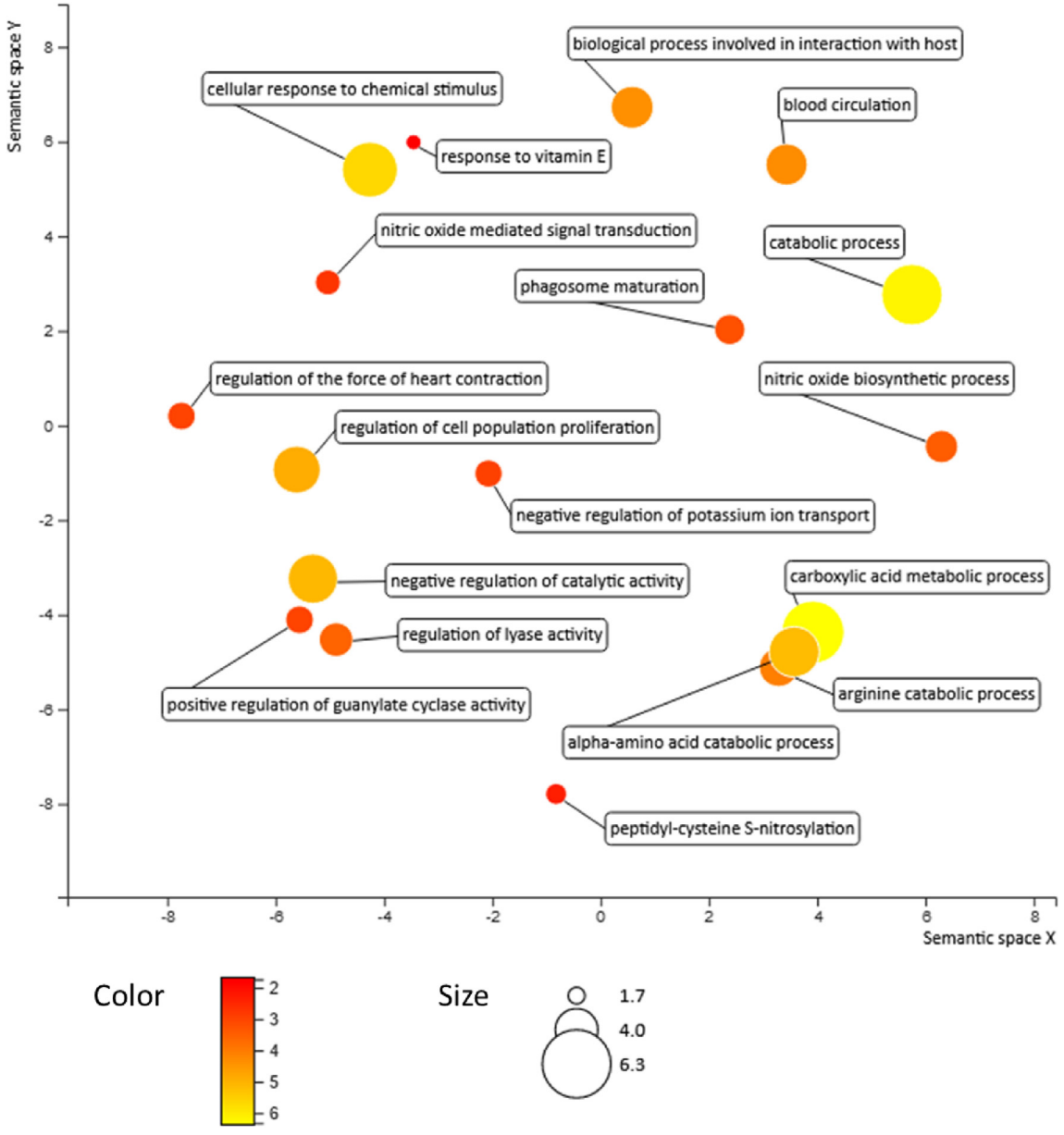

Fig. 6. Possible biological process related to the target proteins which involved in the interaction with Citrus limon's *bioactive compounds.*

bioactive ingredients contained in lemon include flavonoids, flavonoids are secondary plant metabolites that have a mechanism to fight biotic and abiotic stress conditions [39]. In this present study, we found the prediction on how lemon's bioactive compound interact with other proteins which might possible in regulating cancer (Figs. 4 and 5). To a greater extent, we also predict that targeted protein are involved in several biological pathways related to the cancer progression (Fig. 6 and Table 1). Therefore, this prediction suggested that the bioactive compounds of lemon have prominent potency as therapeutic drug-like molecule to ameliorate cancer incidence.

ROS can cause DNA damage that can initiate cancer formation, including strand breakbase modification and DNA crosslinking which can cause mutations and transformations in some cancers. However, the presence of antioxidants can reduce oxidative stress, leading to carcinogenesis by scavenging against ROS. Antioxidants have two mechanisms: the chain breaking mechanism by donating electrons and the second mechanism to remove ROS under catalysis. Antioxidants are divided into enzymatic and non-enzymatic. Enzymatic antioxidants work by interacting with antioxidant enzymes. The non-enzymatic antioxidants found in lemons include vitamin C and flavonoids [40,41]. Vitamin C is thought to reduce ROS directly by inhibiting the formation of nitrosamines, increasing the immune response and accelerating the detoxification of liver enzymes.

Flavonoids are thought to have antioxidant effects, the modulator of enzyme activity, and work as antimutagenic or cytotoxic and protection against oxidative stress. Notably, the flavonoids can be used as a scavenger of peroxyl radicals, an effective inhibitor of lipid peroxidase [41]. Flavonoids have other mechanisms in cancer inhibition including increasing apoptosis, inhibiting cell proliferation, inhibiting lipid peroxidase, inhibiting angiogenesis, and inhibiting DNA oxidation. Flavonoids are thought to induce cell cycle distortion during G1 or G2/M by inhibiting CDK. Apoptosis is a cell death program by eliminating desired cells or damaged cells. Cancer has its own mechanism in inhibiting the cell death program. Flavonoids are thought to induce apoptosis in various ways including inhibition of DNA topoisomerase I and II activity, reducing ROS, modulating signaling pathways, downregulation of NF-κB, activation of endonuclease and decreasing Mcl-1. Flavonoids also have a role to inhibit the activation of metabolic carcinogens by interacting with metabolic enzymes such as P450, these enzymes can activate procarcinogens to become reactive. Flavonoids inhibit the activity of P450 isoenzymes such as CYP1A1 and CYP1A2 so they are expected to inhibit cellular damage or induce metabolic enzymes such as GST or quinine

Table 1. The list of possible biological processes related to the target proteins involved in the interaction with Citrus limon's *bioactive compounds.*

No	Description	False Discovery Rate	Genes Involved
1.	Arginine Catabolic Process	3.26E-05	NOS1,NOS2,NOS3
2.	Nitric Oxide Metabolic Process	3.26E-05	NOS1,NOS2,NOS3,POR
3.	Positive Regulation of Guanylate Cyclase Activity	0.000145	NOS1,NOS2,NOS3
4.	Nitric Oxide Biosynthetic Process	0.000182	NOS1,NOS2,NOS3
5.	Nitric Oxide Mediated Signal Transduction	0.000332	NOS1,NOS2,NOS3
6.	Regulation of Lyase Activity	0.000332	MTRR,NOS1,NOS2,NOS3
7.	Alpha-Amino Acid Catabolic Process	0.000332	MTRR,NOS1,NOS2,NOS3
8.	Positive Regulation of Vasodilation	0.000566	NOS1,NOS2,NOS3
9.	Regulation of Blood Pressure	0.00163	NOS1,NOS2,NOS3,PPARG
10.	Negative Regulation of Blood Pressure	0.00194	NOS1,NOS2,NOS3
11.	Phagosome Maturation	0.00228	NOS1,NOS2,NOS3
12.	Carboxylic Acid Metabolic Process	0.00286	MTRR,NOS1,NOS2, NOS3,POR,PPARG
13.	Peptidyl-Cysteine S-Nitrosylation	0.00321	NOS1,NOS2
14.	Blood Circulation	0.0096	NOS1,NOS2,NOS3,PPARG
15.	Response to Vitamin E	0.0142	NOS1,PPARG
16.	Interaction with Host	0.0202	NOS1,NOS2,NOS3
17.	Negative Regulation of Catalytic Activity	0.0279	MTRR,NOS1,NOS3,POR,PPARG
18.	Single-Organism Catabolic Process	0.0354	MTRR,NOS1,NOS2,NOS3,POR
19.	Regulation of Cell Proliferation	0.0359	CER1,NOS1,NOS2, NOS3,POR,PPARG
20.	Regulation of the Force of Heart Contraction	0.0408	NOS1,NOS3
21.	Negative Regulation of Potassium Ion Transport	0.043	NOS1,NOS3
22.	Cellular Response to Chemical Stimulus	0.0445	CER1,MTRR,NOS1, NOS2,NOS3, POR, PPARG

reductase so that carcinogens are detoxified and eliminated from the body [42].

4. Conclusion

In this present study we found that the induction of DMBA decreased the relative number of both naïve CD4 and CD8 T cells. On the other hand, the induction of DMBA increased pro-inflammatory cytokines such as TNF-α, IFN-ɣ, and IL-6. Interestingly, the treatment of *C. limon*'s extract can directly change the immune system condition into the normal stage. Thus, this study suggested that *C. limon*'s extract have ameliorative activity against breast cancer incidence through the immune system activation that warrant further investigation.

Author's contribution

W.E.P. collected the data, contributed data or analysis tools, performed the analysis, and wrote the paper. A.K.A., M.S.A.A.A., and V.A.M. collected the data, contributed data or analysis tools, and performed the analysis. M.R. conceived and designed the analysis, performed the analysis, and wrote the paper.

Acknowledgement

Authors thank Brawijaya University, Malang for support this study.

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