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Original Article

Inhibitory Mechanisms of Soybean Extract on the Development of Breast Cancer Through Modulation of Cellular Immune Response

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ABSTRACT

Background: Breast cancer is the most frequently diagnosed cancer in women worldwide. Consumption of soy products has been reported to reduce the incidence of and mortality rate for some cancers, including breast cancer. However, there are limited *in vivo* studies on the inhibitory effect of soybean extracts on breast cancer. **Objectives:** To examine the effect of soybean extracts on breast cancer cellular immunity and to determine the role of $CD4^+$ and $CD8^+$ T cells in the development and outcome of breast cancer. **Material and Methods:** Rat were induced with DMBA 11 times to get a breast cancer model. A soybean extract was given at different doses starting one week before DMBA induction and continued until the end of the study. At the end of the study, peripheral blood was collected, and the lymphocytes were examined using flow cytometry. **Results:** The phytochemical screening of soybean extract, using the Q-TOF LC/MS method, detected four bioactive components from the isoflavone and saponin groups. The incidence of tumor formation in the NeC, SE-D250, SE-D500, and SE-D1000 groups was 100%, 83%, 33%, and 33%, respectively. The highest proportion of $CD4^+$ T cells was found in the NeC (69.35%), while the lowest was in the SE-D1000 (63.75%). The highest and lowest proportions of $CD8^+$ T cells were found in the SE-D1000 and NeC groups, at 35.95% and 31.15%, respectively. **Conclusions:** The soybean extract was able to reduce the incidence of breast tumor formation in DMBA-induced rat in a dose-dependent manner. The soy extract group's $CD4^+/CD8^+$ ratio was close to that of healthy rats compared to the DMBA-induced group without soy extract. A lowered $CD4^+/CD8^+$ ratio is followed by a lower risk of tumor formation.
Key words: Breast cancer, Cellular immune response, $CD4^+$, $CD8^+$, Soybean extract.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women worldwide.[1] The global burden of cancer study estimates 2.3 million (11.7%) new cases and 685 thousand (6.9%) breast cancer deaths of 36 cancers and all cancers combined in women worldwide. In Indonesia, there were about 66 thousand (16.6%) new cases and 22.5 thousand (9.6%) breast cancer deaths in women, therefore breast cancer ranks first in incidence and second in mortality compared to all types of cancer recorded in Indonesia.[2,3] Several treatment options are available to cancer patients, e.g., surgery, radiation, and chemotherapy. Each course of treatment has the potential to affect patient's quality of life in several different ways.[4] Long-term physical alterations, such as anatomical abnormalities, chronic discomfort, phantom breast pain, axillary web syndrome, and lymphedema, are among the side effects that are extremely likely to manifest in women following breast cancer surgery. Psychological and emotional changes, such as despair, anxiety, exhaustion, worries about one's appearance, and issues with sexuality are additional potential side effects for women.[5] Some people may have telangiectasias developed months to years after breast cancer radiation treatment. Meanwhile, chemotherapy has not always provided satisfactory results, mostly due to serious side effects, including bone marrow suppression. In addition, treatment with chemotherapy is relatively expensive.[6] This has motivated many researchers to look for new cancer drugs that are more effective and/or selective. Many studies have been carried out to look for cancer drugs from natural sources that may have less side effects. Thus, it is necessary to develop and find new medicines derived from natural ingredients, especially plant-based functional food, so that the availability of these drugs is abundant, and the cost is also relatively cheaper.[7]

Soybean is one of the cultivated plants that has been studied for its various beneficial effects including its potential as a cancer chemo-preventive agent.[8] Soy products, such as soy milk, contain abundant functional ingredients, e.g., soy proteins and isoflavones, and consumption of soy products has been reported to reduce breast cancer risk and improve prognosis of breast cancer patients.[9] Natural compounds that may play a role in its biological activities include isoflavones and soyasaponins. Isoflavones are flavonoid compounds that are known to be strong antioxidants. Therefore, many of the health benefits of soy products may be obtained from its isoflavone contents. Among them are daidzein and genistein, which are considered phytoestrogens found in soybeans and some legumes. Genistein and daidzein played important roles such as cancer prevention agents.[10] Daidzein's chemical profile is similar to that of mammalian estrogens, and it has the possibility of acting in both directions by either replacing or inhibiting the estrogen and estrogen receptor complex. Hence, daidzein could be a therapeutic strategy for estrogen-dependent health problems, such as breast cancer.[11]

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Additionally, genistein may inhibit the growth of breast cancer cells due to its structural resemblance to estrogen and its complementary and antagonistic effects on the estrogen itself.[12] Although research has been done to understand the potential anticancer effects of isoflavones, not all the anticancer effects associated with soy consumption are due to isoflavones. There are several studies that showed that other natural components such as soyasaponins also have potential as anti-cancer, including breast malignancy. Soyasaponins, which are also commonly found in legumes such as soybean, are composed of soyasapogenol (an aglycone) and oligosaccharide moieties.[13] Soyasaponins are oleanane-type triterpenoids, which are based on the structures of their aglycones, have been divided into four groups: group A, group B, group E, and DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) soyasaponins. Members of the soyasaponin group A are known to exhibit various biological activities such as promoting bone health, anti-obesity, and antioxidant, whereas compounds from the soyasaponin group B are known to exhibit anti-cancer, anti-inflammatory, hepatoprotective and renin inhibitory activities.[14]

Several studies documented that immune response plays an essential role in controlling cancer progression. These immune responses are predominantly mediated by cell-mediated immunity. Helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) are the main types of lymphocytes in cell-mediated immunity and play a central role in the induction of efficient immune responses against tumors. $CD8^+$ T cells can recognize tumor antigens bound to class I MHC molecules on the tumor cells and directly kill them. On the other hand, the generation of tumor-specific cytotoxic T lymphocyte responses is believed to depend on the help of activated $CD4^+$ T cells, which recognize tumoral antigens presented with class II MHC molecules on antigen presenting cells. Some experiments have shown that in the absence of $CD8^+$ T cells, $CD4^+$ T cells can still eliminate tumor cells to some extent; however, effective tumor elimination needs both $CD4^+$ and $CD8^+$ T cells.[15] $CD4^+$ T cells are critical for priming of tumor specific $CD8^+$ T cells and for the secondary expansion and memory of $CD8^+$ T cells as well.[16,17]

While the benefits of soy products in preventing the development of breast cancer and the roles of $CD4^+$ and $CD8^+$ T cells in anti-tumor immunity have been extensively studied in both animal models and clinical cancer patients, little is known about the potential of bioactive components of soy extracts in modulating breast cancer cellular immunity. This study was conducted due to the limited *in vivo* studies on DMBA-induced rats to explore the anticancer effects of soy extract, especially the effects of cellular immune modulation. To better understand the role of $CD4^+$ and $CD8^+$ T cells in the breast cancer animal models that were given soybean extract, we used a DMBA-induced rat model of breast cancer. One week before and during induction, rats were given standardized soybean extract. At the end of the study, the proportion and ratio of $CD4^+$ T cells and $CD8^+$ T cells in peripheral blood were examined using flow cytometry.

MATERIAL AND METHODS

Soybean extraction and screening of bioactive compounds

Soybean seeds (*Glycine max* (L.) Merr) of Grobogan variety were obtained at the Research Institute for Various Nuts and Tubers, Malang, East Java, Indonesia (https://maps.app.goo.gl/sFDjtE6WZQYMCBg3A). Soybean seeds undergo the process of defatting in several stages. These seeds were washed with water, and ground using a 3 mm grinder (AEG type AMEB 80 FX). The powder was then wrapped in gauze and pressed at 100-150 atm for 30 minutes at 120°F, to create plates. The plates are turned into powder by using a mortar and pestle, as well as a blender. The powder obtained was sieved using a mesh sieve (Size 40) and placed into a plastic container and stored at 4°C until the maceration process. Extraction was carried out by the maceration process with the use of Phosphate Buffer Saline solvent (PBS) at pH 7.4 (Oxoid, cat no. BR0014G). Soybean powder was added with PBS, stirred until homogeneous, and macerated for 60 min with occasional stirring. The maceration product was filtered using a Whatman 54 filter paper. The filtrate was collected and stored at 4°C. In this study screening and identification of bioactive components were also carried out which included tests for alkaloids, flavonoids, saponins, steroids, polyphenols, tannins, peptides, and terpenoids. Phytochemical screening was carried out using the Q-TOF LC/MS method referring to the protocol of Qiaou et al.[18-20]

Study design and breast cancer modelling

This research is a true-experimental *in vivo* laboratory study, which was carried out at the Anatomical Pathology Experimental Laboratory, Faculty of Medicine, Universitas Indonesia - Dr. Cipto Mangunkusumo National Central Public Hospital, Jakarta. This study used female white rats (Sprague-Dawley) as animal models for breast cancer, obtained from the Food and Drug Administration of the Republic of Indonesia, aged 6-7 weeks, with body weight ranging from 100-150 grams. Thirty rats were divided into five groups consisting of six rats each, namely normal control (NoC), negative control (NeC), soybean extract-dose (SE-D250, SE-D500, SE-D1000). Soybean extracts are administrated one week before and continued during DMBA induction, until the end of the treatment, with doses of 250 mg/kg BW, 500 mg/kg BW, 1000 mg/kg BW respectively. Rats were induced with 7,12-dimethylbenz[a] anthracene (DMBA) (Sigma-Aldrich, cat no. D3254-1G) 11 times, twice a week at a dose 20 mg/kg BW. The cancer model was declared successful if all rats in the negative control group (NeC) succeeded in producing breast tumor nodules. During the experiment and analysis, no rats were excluded due to illness or other reasons. The timeline of this study is illustrated in Figure 1.

Ethics approval

The experimental protocols were approved by the Ethics Committee of the Faculty of Medicine Universitas Indonesia - Dr. Cipto Mangunkusumo National General Hospital, with protocol number: KET-1201/UN2.F1/ETIK/PPM.00.02/2021. The treatment and maintenance of the animals are following the guide for the care and use of laboratory animals by the Animal Care and Use Committee, namely by monitoring the temperature of 25°C, 12 h of light-dark cycle, 55% humidity, as well as standard food and drink. Anesthesia and euthanasia procedures are performed according to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals. Anesthesia was performed with ketamine (KTM-100, PT Guardian Pharmatama) at 75-100 mg/kg BW and xylazine (Xyla, Interchemie Holland) at a dose of 10 mg/kg BW intramuscular.

Quantification of $CD4^+$ and $CD8^+$ lymphocytes

The expression markers on T cells were determined by flow cytometry analyses after surface or intracellular staining with anti-rat specific antibodies conjugated with FITC, PE, and APC. These rat antibodies included: anti-CD3 (cat no. E-AB-F1228C), anti-CD4 (cat no. E-AB-F1105D), and anti-CD8 (cat no. E-AB-F1098E), which were purchased from Elabscience. Peripheral blood (100 µL with EDTA) from rats are sacrificed was put into 3 different tubes, 2 µL of antibodies was added to each tube, incubated for 30 minutes. Then add 1 mL of lysing buffer, was vortex and incubate for 15 minutes. Add 1 mL of stain buffer, vortex, and centrifuge at 2100 rpm for 5 minutes, remove the supernatant and add 300 µL of stain buffer to each tube. All stained cells were analyzed on a BD FACSCantoTM II cytometer (BD Bioscience) and data analyzed with FlowJo software (Tree Star Inc., CA, USA).

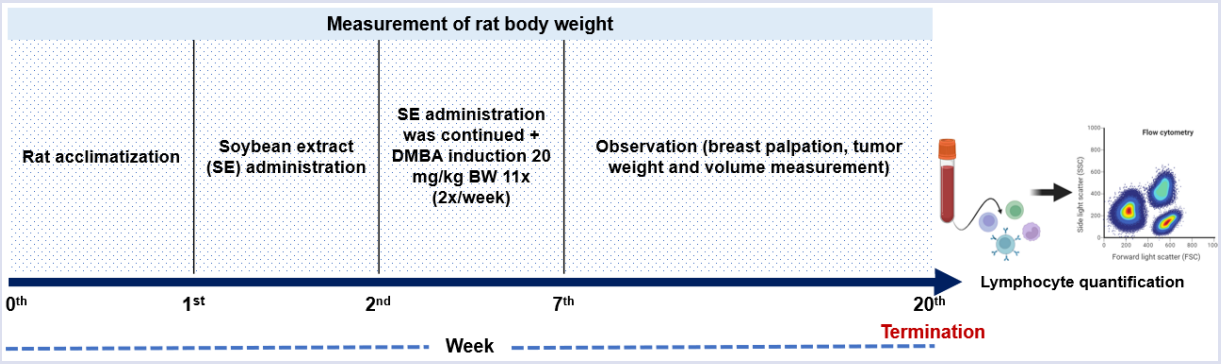

Figure 1. The timeline of study.

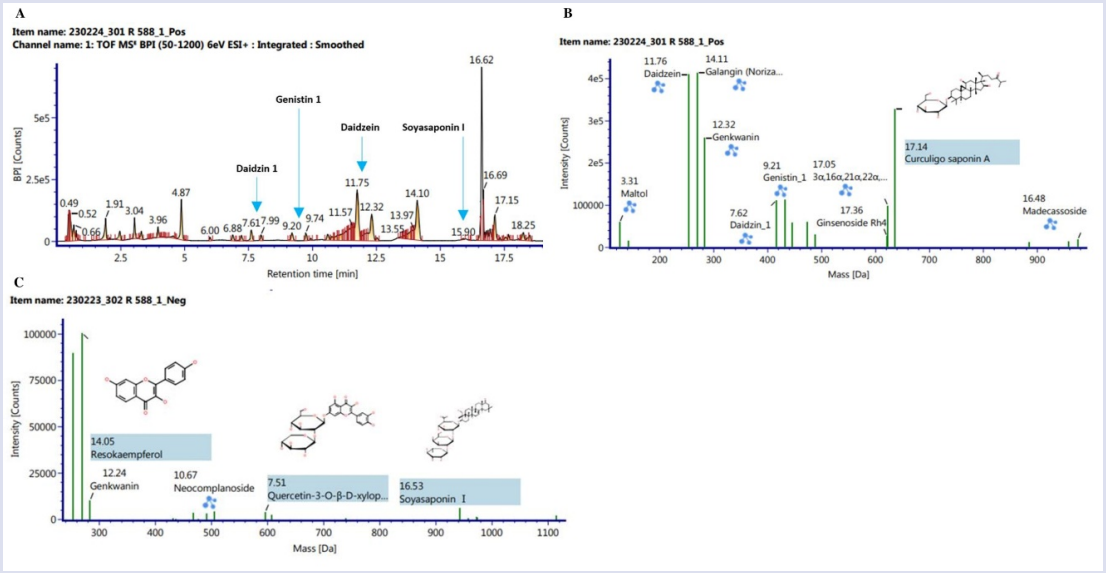

Figure 2. The Q-TOF LC/MS chromatograms of soybean extracts.
(A) The base peak intensity chromatogram of soybean extract; (B) Mass spectrum of soybean extract in a positive ion mode, showing the presence of daidzein, daidzein 1, genestin 1, and others; (C) Mass spectrum of soybean extract in a negative ion mode, showing the presence of soyasaponin I and flavonoids.

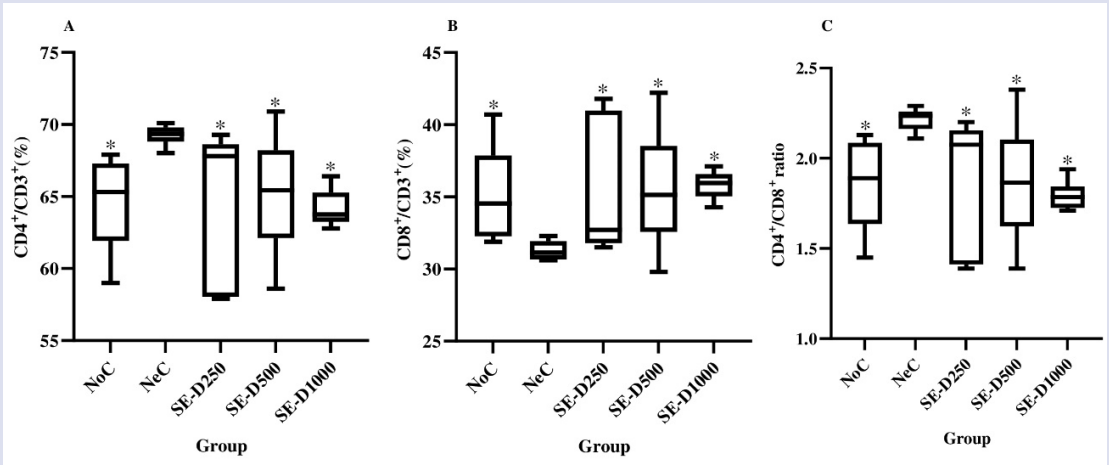

Figure 3. The proportion and ratio of $CD4^+$ and $CD8^+$ lymphocytes in the peripheral blood.
(A) The proportion of $CD4^+$ T cells; (B) The proportion of $CD8^+$ T cells; (C) The ratio of $CD4^+/CD8^+$. NoC, normal control: feed corn oil. NeC, negative control: DMBA induced 20 mg/kg BW, without soybean extract. SE-D250: feed soybean extract 250 mg/kg BW. SE-D500: feed soybean PBS extract 500 mg/kg BW. SE-D1000: feed soybean extract 1000 mg/kg BW. Data represent median (min-max) (n=6). Differences in asterisk indicate the statistical significance at $p<0.05$ based on Dunn's post hoc.

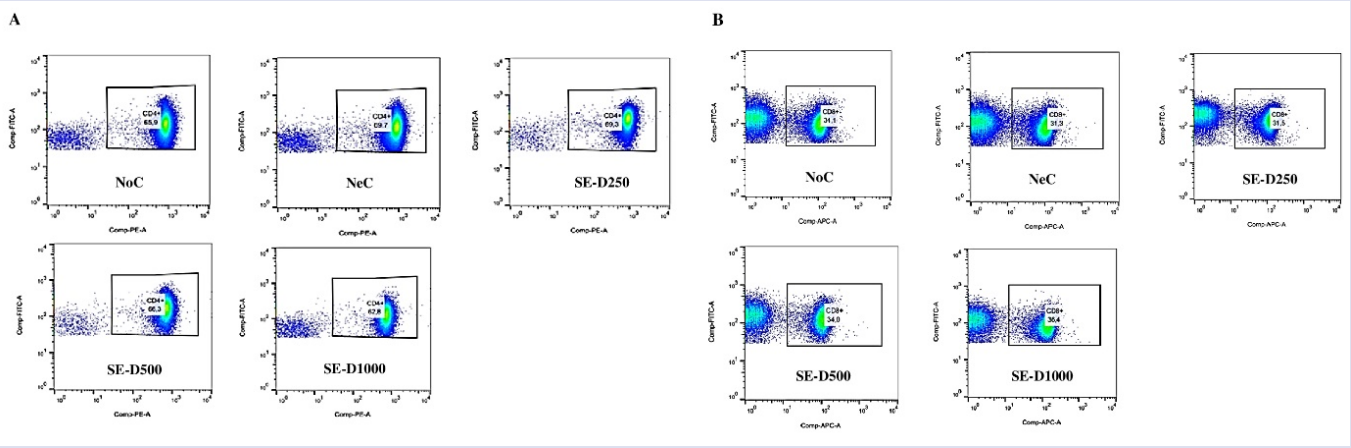

Figure 4. A representative depiction of (A) $CD4^+$ and (B) $CD8^+$ proportions using flow cytometry.
NoC, normal control: feed corn oil. (b) NeC, negative control: DMBA induced 20 mg/kg BW, without soybean extract. (c) SE-D250: feed soybean extract 250 mg/kg BW. (d) SE-D500: feed soybean extract 500 mg/kg BW. (e) SE-D1000: feed soybean extract 1000 mg/kg BW.

Statistical analysis

Data was analyzed using the GraphPad Prism 9. Data analysis was with the use of Kruskal-Wallis, followed by Dunn's test to compare the differences. Differences of $p<0.05$ are considered statistically significant.

RESULTS

Characterization of the chemical constituents of the soybean extract using Q-TOF LC/MS analysis

Soybean extracts have been reported to contain many bioactive compounds such as flavonoids and saponins. However, depending on the method of extraction, the solvent used, and the condition under which the extracts are prepared, the chemical constituents may vary. To investigate the chemical constituents of our soybean extract, which was prepared by maceration with Phosphate Buffer Saline (PBS) solution pH 7.4, the sample was analyzed by Q-TOF LC/MS using a well-established method for analyzing isoflavones and other secondary metabolites from soybean.[18,21,22] The results showed that the soybean PBS extract contains flavonoids (i.e., resokaempferol, quercetin, etc), isoflavones (i.e., daidzein, genistein, etc), soyasaponin, and other known constituents (Figure 2A–C). These constituents are consistent with those previously reported in soybean extracts, confirming the presence of bioactive compounds in the extract.

The effect of soybean extract on rat mammary gland carcinogenesis

To test the beneficial effect of the soybean extract on rat mammary gland carcinogenesis, a total of 24 female SD rats were induced with 7,12-dimethylbenz[a] anthracene (DMBA) 20 mg/kg, administered intragastrical twice a week for a total of 11 times, whereas six rats were used as non-induced controls. Of the 24 rats induced, 6 rats (100%) in the negative control group (NeC) developed tumors, indicating that the 5 rats developed tumors in the extract group SE-D250 (83.33%), while in the SE-D500 and SE-D1000 each obtained 2 rats that gave rise to tumors (33.33%). Observations were carried out for 17 weeks, 5 weeks during induction and extract administration, and observations continued 12 weeks after that. The first tumor appeared at the end of 6^{th} week after the last DMBA induction, which was in the third NeC rat and the last tumor appeared at 10^{th} week after the last DMBA induction which was found in the second NeC rats (Table 1).

Table 1: The number of rats that appear tumor nodules.

Group (n=6)	The number of rats with tumors	Number of tumors (min-max)	Incidence (%)
NoC	0	0	0
NeC	6	10 (1-3)	100
SE-D250	5	8 (0-2)	83.33
SE-D500	2	3 (0-2)	33.33
SE-D1000	2	2 (0-1)	33.33

Abbreviations: NoC: normal control; NeC: negative control; SE-D: soybean extract-dosage.

Proportion and ratio of $CD4^+$ and $CD8^+$ lymphocytes in the peripheral blood

The main types of lymphocytes in cell mediated immunity are $CD4^+$ and $CD8^+$ T cells, which play a main role in the induction of efficient immune responses against tumors. To investigate if the soybean extract affects the proportion and ratio of $CD4^+$ and $CD8^+$ T lymphocytes in the DMBA-induced rats, the blood samples were collected and the number of $CD4^+$ and $CD8^+$ T were counted by flow cytometry after surface or intracellular staining with anti-rat specific antibodies (anti-CD3, anti-CD4, and anti-CD8) conjugated with FITC, PE, or APC. The results showed significant differences in the proportion of $CD4^+$ T cells in the blood between groups ($p=0.021$). These differences included NoC (65.30%), SE-D250 (67.80%), SE-D500 (65.45%), and SE-D1000 (63.75%) compared to NeC (69.35%). The highest proportion of $CD4^+$ T cells was found in the NeC group, while the lowest was in the SE-D1000 group (Figure 3(A) & Figure 4(A)). The $CD8^+$ T cells proportion also showed significant differences between groups ($p=0.024$). There is a significant difference in NoC (34.55%), SE-D250 (32.70%), SE-D500 (35.15%), and SE-D1000 (35.95%) compared NeC (31.15%). The highest proportion of $CD8^+$ T cells was found at SE-D1000 group and the lowest at NeC group (Figure 3(B) & Figure 4(B)).

In addition to the proportions of each lymphocyte subset, we identified significant differences in the $CD4^+/CD8^+$ lymphocyte ratio between groups. Dunn's test revealed a significant difference in the ratio of $CD4^+/CD8^+$ lymphocytes in NoC (1.89), SE-D250 (2.07), SE-D500 (1.86), and SE-D1000 (1.78) compared to NeC (2.23). The highest ratio was observed in the NeC group, while the lowest ratio was found in the SE-D1000 group (Figure 3(C)).

DISCUSSION

The relationship between dietary intake of soybean and breast cancer has been studied for more than two decades. The identification of active components of soybeans, e.g., isoflavones and soyasaponins, has opened new opportunities for the investigation and discovery of anticancer therapeutic agents, especially for breast cancer.[9] Although there were many reports on the presence of isoflavones, saponins, and other metabolites in soybeans.[21,22] The chemical constituents varied depending on the extraction methods and other factors such as time, solvent system, and temperature. Among them, the solvent used for extraction is a very important factor for obtaining secondary metabolites.

Many health benefits, including protection against breast cancer, are associated with consumption of soy foods, mainly due to soy isoflavones (genistein, daidzein, glycitein). Isoflavones are thought to be selective estrogen receptor modulators.[23] In the meantime, soyasaponin I can alter the sialylation pathways to promote the adherence of tumor cells to the cell matrix, which ultimately hinders the dissemination of tumor cells. Soyasaponin I is a good candidate in tumor metastasis prevention strategies.[24] To study the role of isoflavones and soyasaponins in relation to anti-breast cancer through the cellular immune response pathway, the proportion and ratio of $CD4^+$ and $CD8^+$ T cells from peripheral blood was examined in DMBA-induced breast cancer rat models.

The immune system plays an important role in the pathogenesis and progression of breast cancer. $CD4^+$ and $CD8^+$ T cells are the main types of lymphocytes in cell-mediated immunity and play a central role in the anti-tumor immune responses.[15] Several studies have reported that there are changes in the population and ratio of $CD4^+$ and $CD8^+$ T cells in the peripheral blood of patients in various types of cancer. A reduction in the percentage of $CD4^+$ T cells has been reported in patients with colon, gastric, and esophageal cancers.[25]

In this study, we found that the proportions and ratios of $CD4^+$ and $CD8^+$ T cells differed significantly between groups. At the end of the study, the proportion and lymphocyte ratio were examined from the peripheral blood. The percentage of $CD4^+$ T cells was very high in the NeC group and significantly different compared to SE-D250, SE-D500, and SE-D1000. And in contrast to the $CD4^+$ T cell proportion, the percentage of $CD8^+$ T cells was lower in the NeC group and significantly different compared to NoC, SE-D250, SE-D500, and SE-D1000. The highest $CD4^+/CD8^+$ ratio was in the DMBA (NeC) group and the lowest was in SE-D1000. Associated with tumor development, T lymphocyte cells play an important role as anticancer immunity. A high $CD4^+/CD8^+$ ratio due to a high percentage of $CD4^+$ cells in the peripheral blood appears to be associated with the development of breast tumors in the rat in each group. The higher the percentage of $CD4^+$ T cells and the $CD4^+/CD8^+$ ratio in the peripheral blood, followed by the greater the number of tumors formed.

Different from previous studies, which reported that there was a negative correlation between the percentage of $CD4^+$ and $CD8^+$ T cells on tumor formation and tumor size, the higher the T cells, the lower the tumor size.[15,26] However, other findings reported the number of $CD4^+$ T cells increases significantly with the development of breast cancer, suggesting active involvement of the tumor-induced immune response. Importantly, in addition to increasing numbers, the subsets of $CD4^+$ T cells also change dynamically, indicating different functions of $CD4^+$ T cells in different stages of tumor development. In late tumor stages, $CD4^+$ T cells may become more important for promoting tumor growth. This finding was further confirmed in a retrospective study of breast cancer patients, showed that intra-tumoral $CD4^+$ T-cell count positively correlated with advanced tumor stage, large tumor size, and positive tumor metastases. Furthermore, results from clinical cancer patients showed that the $CD4^+/CD8^+$ ratios were strongly correlated with the advanced tumor stage, large tumor sizes and positive lymph node status.[16]

In this study, soybean PBS extract was used which had been confirmed to contain several bioactive components from the isoflavone group (daidzein, daidzin, and genistin) and saponins (soyasaponin I). Soybean extract is used to prevent the development of DMBA-induced breast cancer in rats. The higher the dose of soybean extract given to the rats, the smaller the chance of developing breast tumors. As shown in Table 1, the number of rats with tumors in the SE-D1000 group was the least compared to the tumors formed in NeC, SE-D250, and SE-D500. The decrease in the number of tumors is dose-dependent, suspected due to the activity of the bioactive components of isoflavones and soyasaponins which act as anti-cancer through the cellular immune response pathway. Research conducted by Hsu, et al.[24] showed that soyasaponin I can reduce the migration of metastatic breast cancer cell lines MDA-MB-231. Soyasaponin I works by increasing the adhesion ability of cancer cells. The ability of soyasaponins to suppress expression intercellular adhesion molecule-1 (ICAM-1) has been investigated for a long time and is associated with the inflammatory response and its inhibitory abilities.[27,28]

The mechanism of soy isoflavones in breast cancer is related to ER modulation, especially ERβ. However, current *in vitro* studies show that soy isoflavones interfere with other signaling pathways that control cell development, such as NF-κB, PI3K/Akt or MAPK/ERK. In addition, isoflavones can inhibit the angiogenesis signaling pathway and initiate apoptotic events. Genistein reduces the numbers of peripheral blood $CD4^+$ and $CD8^+$ T-cell, and is associated with thymic atrophy.[29,30] Previous studies suggested that soybean extract lowered EGFR expression and decreased tumor volume in DMBA-induced breast cancer rat models.[31]

CONCLUSIONS

In vivo studies revealed that rats administered soybean extract had less tumor nodules in their clinical outcomes. Additionally, the soy extract group's $CD4^+/CD8^+$ ratio was close to that of healthy rats compared to the DMBA-induced group without soy extract. A lowered $CD4^+/CD8^+$ ratio is followed by a lower risk of tumor formation. Soybean extract appears to exert its effects by influencing the functioning of T cell subsets, ultimately contributing to the suppression of breast cancer progression.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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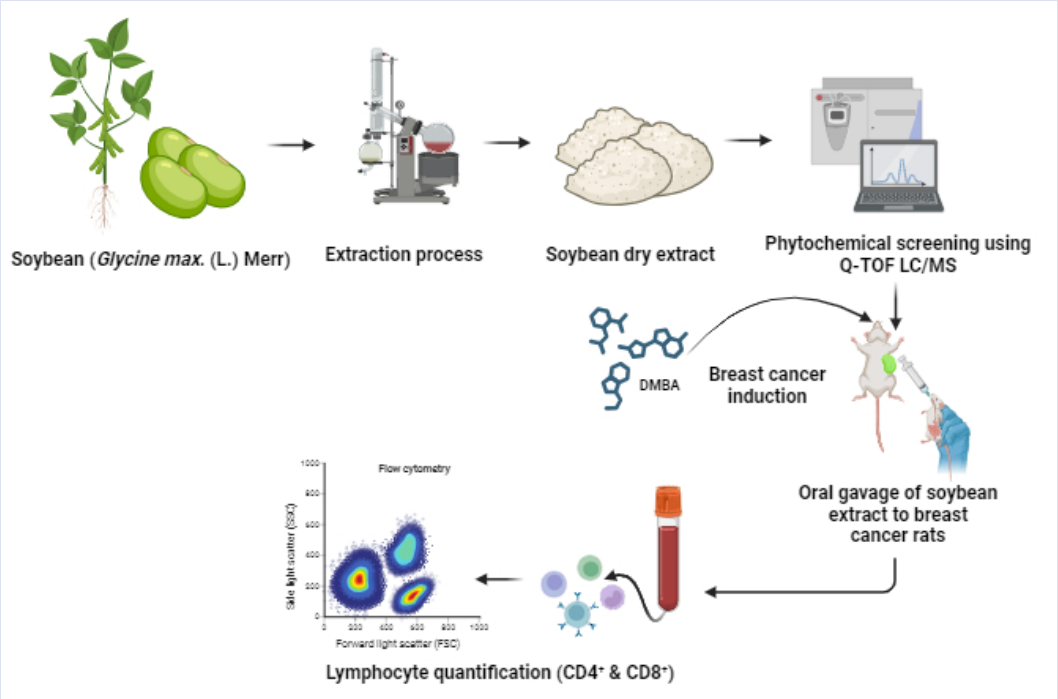
GRAPHICAL ABSTRACT



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