Immunomodulator Effect of Polyherbal Extract Turmeric-Meniran on Macrophages Profile of Mice Cancer Model by DMBA Injection

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Abstract

The immune system has responsibility for various infections, including cancer. Macrophage cells are divided into Macrophage 1 (M1) and Macrophage 2 (M2) based on polarization against cancer cells. Alternative medicine, such as herbal medicine, have potential to modulate the immune system. This study was conducted to evaluate the potential of the combination of Meniran and Turmeric extracts for regulating immune system cells, especially macrophage cells profile against cancer. This experiment was used Mus musculus cancer model that were injected subcutaneously with 7,12-dimethylbenz[a]anthracene (DMBA) 45 mg/kg body weight. Animal models were treated with Meniran and Turmeric extract combination for 2 weeks. The immunocompetent cell parameters were analyzed using flow cytometry, with markers CD11b cells and cytokines secreted in macrophage [i.e., interleukin (IL)-6, IL-10, also tumor necrosis factor α (TNF-α)]. The study showed that DMBA injection on mice increased the cell level of CD11b+ and CD11b+IL10 (M2), at the same time decreased the cell level of M1, i.e., CD11b+IL6+ and CD11b+TNF-α compared with the Normal group. The Meniran and Turmeric extract combination treatment on DMBA-injected mice decreased the level of the cells of CD11b+ and CD11b+IL10+, meanwhile it increased the level of the cells of CD11b+IL6+ and CD11b+TNF-α. The result indicated that the Meniran and Turmeric combination had the potential to modulate the immune system, especially macrophage cells profile against cancer.

Keywords: Anticancer, cancer, herbal combination, herbal medicine, macrophage, immunomodulator.

Introduction

Cancer has become a concern at this time because the appropriate treatment is still unclear. Breast cancer is the tumor case that most commonly affects women. Breast cancer treatment that has been commonly used so far, in fact, still causes various kinds of side effects. The most reliable and commonly used animal model of carcinogenesis to evaluate the therapeutic potential of a cancer drug candidate is the chemically injected animal model. 7,12-Dimethylbenz[a]anthracene (DMBA) is one of the most frequently used carcinogenic chemicals to model cancer in animals. Furthermore, DMBA injection on mice causes immunosuppressants, such as reduced macrophage profile 1 (M1) cytokine levels.

Eliminating malignant cells and other diseases and all kinds of diseases invading the body is the responsibility of the immune system, such as macrophage cells. However, cancer cells have a way...
of surviving and evading immunocompetent cells. Thus, efforts are needed to help stimulate the immune system to work optimally in eliminating cancer cells. Alternative medicine that can modulate the immune system is currently being used very intensively, for example, phytochemical herbal medicines rich in bioactive compounds [18].

Macrophages are immune system cells that are found in almost all tissues. In cancer cases, macrophages are immune cells that are found in the tumor microenvironment [5]. Macrophages are divided into two subtype populations, namely, Macrophage 1 (M1) or Macrophage 2 (M2) macrophages. M1 macrophages are called antitumors because they play roles for defense and kill tumor cells by secreting proinflammatory cytokines [e.g., interleukin (IL)-6 and tumor necrosis factor α (TNF-α)]. In addition to that, M2 macrophages play roles in promoting tumor development by secreting antiinflammatory cytokines [e.g., IL-10 and tumor growth factor β (TGF-β)] [15].

Traditional herbal medicine, such as Meniran (Phyllantaceae) and Turmeric L. (Zingiberaceae), has been practiced in every country for many centuries, especially in Indonesia. These herbal medicines have pharmacological effects, such as antibacterial, hepatoprotective, antihypertensive, antiinflammatory, and anticancer [6, 7]. Although the pharmacological effects of these herbal medicines have been widely proven, there are no studies that have tested the combination of these herbals for their ability to regulate immune system cells, especially on macrophages cells profile. Thus, this research was conducted to study the potential of the combination of Meniran and Turmeric extracts for regulating immune system cells, especially macrophage cells profile for anticancer.

Materials and Methods

Protein Preparation
Dry powder of Turmeric rhizome and Meniran herb was obtained from UPT Materia Medica Batu, located in Batu City, Indonesia. The powder extraction was performed by dissolving in boiled demineralized water in a 1:10 ratio (herb:solvent, w/v) for 4 h. The extracts were filtered using a fine cloth and then centrifuged at 2500 rpm for 20 min. Next, the solution was filtered using a 2.5 µm Whatman filter. The water was removed using a freezer and frozen until usage. The combination extract was used with 1:1 ratio (Turmeric and Meniran). Thus, this combination in this article is called polyherbal.

Experimental Design
The study protocol was approved by the Universitas Brawijaya’s ethics committee (Ethics No. 1126-KEP-UB). This study was used mice (Mus musculus) female, BALB/c strain, 6-week-old. The animals were acclimatized for 1 week, also had free access to food and water. The cancer animal model was performed in female BALB/c mice (M. musculus) that were injected subcutaneously in the mammary glands with DMBA (Sigma Aldrich) 45 mg/kg body weight (BW) for 8 weeks. After 8 weeks, the animals were randomly divided into seven groups, followed treatment for 2 weeks. After 2 weeks, these groups were sacrificed, and the immunocompetent cells were isolated from the spleen for flow cytometry analysis and mammae organ for histology preparation (Figure 1). The groups detailed as follows:

1. Normal (N) control group, mice were injected with corn oil only without DMBA or polyherbal treatment.
2. DMBA (DMBA) control group, DMBA animal model without any given polyherbal treatment.
3. DMBA + Cisplatin group as drug control, DMBA animal model was injected intraperitoneally with cisplatin (Kalbe Farma) 15 mg/kg BW, once every week, for 2 weeks.
4. DMBA + D1 group, DMBA animal model was treated with polyherbal 100 mg/kg BW daily for 2 weeks.
5. DMBA + D2 group, DMBA animal model was treated with polyherbal 300 mg/kg BW every day for 2 weeks.
6. DMBA + D3 group, DMBA animal model was treated with polyherbal 900 mg/kg BW every day for 2 weeks.
7. DMBA + D4 group, DMBA animal model was treated with polyherbal 1800 mg/kg BW every day for 2 weeks.
Tissue Preparation and Hematoxylin and Eosin Staining

The tissue preparation and staining procedures are similar as in a previous study [18]. The process step includes dehydrating, clearing, embedding, sectioning deparaffinizing, staining, cleaning, dehydrating, and mounting. The mammae tissue section was stained with hematoxylin and eosin. The samples were then viewed under an Olympus BX51 binocular microscope integrated with Optilab Viewer 3.0 software and Optilab Advanced Plus camera.

Flow Cytometry Analysis

The antibodies used included anti-CD11b, anti-TNF-α, anti-IL-6, and anti-IL10 (BioLegend). The spleen was used in this study because spleen a secondary lymphoid organ, which could describe the condition of the circulating immune system. The spleen of animal model was isolated, then homogenized with phosphate-buffered saline (PBS) (Gibco). The spleen’s cells were suspended with PBS and centrifuged at 2500 rpm at 10°C for 5 min. The pellet was resuspended with 1 mL of PBS. As much as 50 μL of suspension cells was transferred into a microtube. The sample was stained with 50 μL extracellular antibodies with a final concentration of 0.005 mg/100 μL (anti-CD11b) and then incubated for 30 min in a darkroom. Intracellular staining was performed by applying 50 μL Cytofix™ fixation buffer (BD Biosciences) and incubated for 30 min. The fixative solution was added to 500 μL of staining perm wash solution (BioLegend) and centrifuged at 2500 rpm at 10°C for 10 min. The cells were stained with 50 μL intracellular antibodies with a final concentration of 0.005 mg/100 μL (anti-IL-6, anti-IL-10, and anti-TNF-α) and then incubated for 30 min. The sample was added with 400 μL of PBS and then analyzed using a flow cytometer (BD FACSCalibur) with BD Cellquest Pro™ software (BD Biosciences). The percentage of IL-6, IL-10, and TNF-α expression by CD11b (Macrophages) from all CD11b populations was calculated using the formula 1. Upper right quadrant showed cells population which have interleukin positive (y-axis) and CD11b positive (x-axis). Lower right quadrant showed cells population which have interleukin negative and CD11b positive.
\[ UR \times 100 = LR + UR \] (1)

where:
\[ UR = \text{cells in the upper right quadrant} \]
\[ LR = \text{cells in the lower right quadrant} \]

**Statistical Analysis**
Statistics for flow cytometry were analyzed using analysis of variance test continued post hoc Tukey HSD test using SPSS 25.0 software. The significant difference between the two groups was obtained using a \( P \)-value score of <0.05. Data are presented as mean ± standard deviation (SD) [18].

**Results and Discussion**

**Histological Analysis**
A histological analysis was conducted to confirm the tissue’s form of mammae organ. This study found that DMBA injection in experimental animals could lead to infiltration of stromal cells in the adipose tissue of mammary organs (Figure 2B). Breast cancer is a disease characterized by irregular ductal and lobular hyperplasia [15]. Irregular proliferation of breast lobular or ductal cells can cause cancer that invades the surrounding tissues, such as adipose tissue [2]. The development of breast cancer is facilitated by stromal cells by a number of processes, such as tumor angiogenesis, inflammation, and fibrosis.

However, these results need to be further verified using specific cancer marker parameters, such as immunohistochemistry using Ki-67. After administration of polyherbal in animal models, the density of stromal cells in adipose tissue in treatment groups is less than DMBA group (Figure 2D–F). The results of this study indicated that the polyherbal could reduce the infiltration of stromal cells in adipose tissue in DMBA-injected mice. This ability is thought to be related to the active compound content in the extract combination that functions as anticancer agent, such as inhibiting cell proliferation and inducing apoptosis.

![Figure 2. Histology of mammary glands from DMBA-injected mice with/without administration of polyherbal (100× magnification). A: Normal group; B: DMBA group; C: DMBA + Cisplatin group; D: DMBA + D1 group; E: DMBA + D2 group; F: DMBA + D3 group; G: DMBA + D4 group. SA: adipose cells; SS: stromal cell](image)

DMBA is a carcinogenic substance most used to induce cancer in experimental animals, including breast cancer. The DMBA mechanisms to induce cancer development involve gene mutation and cellular oxidative stress and may cause alteration to the spleen [3, 8, 14]. Thus, this study used DMBA for creating a cancer model of mice. However, the degree of success of cancer cell formation is influenced by various factors, including various routes of administration, doses, exposure duration, and differences
in the level of immunity in experimental animals [15,16]. The ability of a chemotherapy drug can be seen based on its ability to inhibit the proliferation of cancer cells. This study provides the combination of Meniran and Turmeric extracts can be a potential chemotherapeutic agent. Huang et al. [12] stated that the content of flavonoids and tannins in Meniran causes this plant to have a potential chemotherapeutic agent in suppressing tumor growth in C57BL/6 mice implanted using Lewis lung carcinoma. Previous study showed that the combination of Meniran and Turmeric extracts contains bioactive compounds such as quercetin, kaempferol, curcumin, vanillin, 4-coumaric acid, ar-turmerone, and ferulic acid. These compounds are predicted to have anticancer functions, namely triggering apoptosis (such as stimulants of Caspase-3 and P53 proteins) and inhibiting metastasis (i.e. inhibiting the expression of MMP9 and JAK2 proteins). In addition, these compounds have interactions with cancer-related proteins, such as CASP3, EGFR, and TP53 [20].

Effect of Polyherbal Treatment on Macrophage Cells Profile
Immunocompetent cells that play a role in eliminating cancer cells, including macrophage cells. The macrophage cells were analyzed after 2 weeks of polyherbal treatment in the experimental group. The population of CD11b+ cells from a total of spleen cells in the mice breast cancer group was significantly higher (7.11% ± 0.46%) than that in the Normal group (5.14% ± 0.67%) (P < 0.05). Administration of cisplatin significantly decreased CD11b+ cells (4.58% ± 0.42%) compared to the DMBA group. After polyherbal treatment reduced the levels of CD11b+ cells (3.98% ± 0.52%–5.61% ± 0.78%) compared DMBA group (Figure 3A, B). The results showed that administration of polyherbal in DMBA-injected mice could reduce the population of CD11b+ cells compared to the DMBA group.

![Figure 3. The relative number of macrophage cells (CD11b+) from DMBA-injected mice with/without administration of extracts combination. Meniran and Turmeric combination extracts reduced the relative number of CD11b+ cells compared to the DMBA group. The bars were represented as mean ± SD](image)

Macrophages are abundant in tissues and are essential to the function of the immune system. Immune cells called macrophages are present in the tumor microenvironment in cancer instances [5]. Similar to this study, the macrophage population in the DMBA group was higher than that in the Normal group. Their presence correlates with reduced survival in most cancers, often called tumor-associated macrophages (TAMs) [5]. This study showed that the combination of Meniran and Turmeric extracts treatment might reduce the number of macrophages. Immune system modulation is essential to maintain the homeostasis and physiological stability of an organism. Immunomodulators from natural ingredients are safer because they have fewer side effects [16]. The content of active compounds in herbal plants is reported to have an immunomodulatory function in the immune system. The group of polyphenolic compounds is reported to have the ability to change the composition of immunoglobulins and inflammation and the population of immune cells [11]. Based on this, it can be indicated that the ability of the combination of Meniran and Turmeric extracts in modulating immunocompetent cells is due to the active compounds contained in them, especially in the polyphenol group.

Effect of Polyherbal Treatment on Cytokines Expressed on Macrophage Cells Profile
Macrophages play a dual role in the process of eliminating cancer cells. Polarization of macrophages into type 1 (M1) and type 2 (M2) macrophages can be seen from the cytokines contained in macrophages. M1 secrete proinflammatory cytokines (i.e., TNF-α and IL6), while M2 secrete antiinflammatory cytokines (i.e., IL10). This study has analyzed the cytokines expressed in macrophage cells. The level of CD11b+IL6+ cells in the DMBA group was significantly lower (36.01% ± 0.11%) than
that in the N group (44.75% ± 1.19%) \( (P < 0.05) \). Cisplatin treatment has significantly increased the levels of CD11b^+IL6^+ cells (54.9% ± 5.85%) than the DMBA group. After polyherbal treatment, the levels of CD11b^+IL6^+ cells were increased (37.83% ± 1.0.5%–56.87% ± 4.21%) than the DMBA group (Figure. 4A, B).

The population of CD11b^+TNF-α^+ cells in the DMBA group was lower (31.36% ± 1.15%) than that in the Normal group (32.17% ± 2.23%), although there is no significant difference. Administration of cisplatin increased the levels of CD11b^+ TNF-α^+ cells (44% ± 0.45%) than the DMBA group. The administration of polyherbal increased CD11b^+ TNF-α^+ cells (33.19% ± 1.13%–40.96% ± 0.30%) compared to the DMBA group (Figure 4C, D).

**Figure 4** The relative number of CD11b^+IL-6^+ and CD11b^+TNF-α^+ from DMBA-injected mice with/without administration of extracts combination. Polyherbal increased the relative number of CD11b^+IL-6^+ (A, B) and CD11b^+TNF-α^+ (C, D) cells compared to the DMBA group. The bars were represented as mean ± SD

Macrophages are classified into two cell subtype populations, called M1 or M2 macrophages, based on the polarization process. M1 macrophages are called antitumors because they play roles for defense and kill tumor cells by secreting proinflammatory cytokines (e.g., IL-6 and TNF-α). In addition to that, M2 macrophages play roles in promoting tumor development by secreting antiinflammatory cytokines (e.g., IL-10 and TGF-β). They are considered protumor by immunosuppression mechanism as they are exposed to IL-10, TGF-β, and matrix metalloproteases (MMPs) [23]. Therefore, M2 preferentially will perform suppression rather than immune effector in the tumor microenvironment [9].

The level of CD11b^+IL10^+ cells in the DMBA group was higher (23.66% ± 1.73%) than that in the N group (23.36% ± 1.28%), although there is no significant difference. Unfortunately, cisplatin administration increased the levels of CD11b^+IL10^+ cells (34.04% ± 0.53%) than the DMBA group. The polyherbal treatment decreased the levels of CD11b^+IL10^+ cells (17.53% ± 1.13%–19.79% ± 1.51%) compared DMBA group (Figure 5A, B).
The results indicate that administration of polyherbal in DMBA-injected mice could modulate macrophages that secrete cytokines. These herbal combination might increase the population of macrophage type 1 (i.e., CD11b+IL6+ and CD11b+TNF-α+), also decrease the population of macrophage type 2 (i.e., CD11b*IL10*).

Macrophage activation is influenced by helper T cells (Th). Th cells will be activated in the immune response into two, namely, Th1 cells and Th2 cells. Th1 activation will secrete IL-12 and IFN-γ, causing activation of M1 macrophages as an anticancer response, although the mechanism of M1 macrophages as anticancer is unknown. On the other hand, IL-4 and IL-10 will be secreted by activated Th2 cells, causing M2 macrophage activation as a procancer response [17]. M2 macrophages function to remove dead cells, repair damaged tissue, encourage the development of new blood vessels, and support tumor development [10]. To suppress the antigen-presenting process, TAMs release cytokines such as transforming growth factor-β (TGF-β), IL-10, and inflammatory mediators like prostaglandin E2 (PGE2) and matrix metalloproteinase-7 (MMP-7). The cytokine IL-10 plays a crucial role during infection and inflammation to maintain tissue homeostasis through boosting innate immunity, reducing excessive inflammatory responses, and encouraging tissue repair processes [25]. The breast tumor displays paracrine feedback between M2 and cancer cells. M2 cells secrete monocyte colony-stimulating factor receptor (M-CSFR) that binds to M-CSF expressed by the cancer cell. In addition to that, M2 cells produce epidermal growth factor, which will activate the epidermal growth factor receptor on the cancer cells. This interaction might support the migration cancer cells through enhancing cell motility and enabling intravasation [24]. Thus, blocking these interactions can potentially prevent metastasis or even potential novel cancer therapy. This study found that DMBA injection reduced M1 macrophage (CD11b+IL6+ and CD11b+TNF-α+) and increased M2 macrophage (CD11b+IL10+). This result is similar to a previous study [1] that M1 macrophages was increased, while M2 macrophages was decreased in mice tumor model. The polyherbal treatment on DMBA-injected mice indicates regulating M1 (i.e., increasing CD11b+IL6+ and CD11b+TNF-α+) and M2 (i.e., increasing the population of CD11b+IL10+).

Based on the result, polyherbal Meniran and Turmeric had the potential to maintain the immune cells, especially macrophage cells against cancer cells. The content of active compounds in the polyherbal is believed to have pharmacological functions. Meniran shows potential as an immunomodulator, inhibiting T cells proliferation and proinflammatory cytokines [13]. Curcuminoids are the main active compound constituents of Turmeric. Chandrasekaran et al. found that Turmeric has a potential immune-stimulatory activity on splenocyte cells, including activated macrophages, which secreted inflammatory mediators, such as interleukin 6 and 12, also TNF-α [6]. Although polyherbal Meniran and Turmeric had potential as anticancer agents, future study needs to be conducted to study another cytokine expression and analyze immunocompetent cells in the tumor microenvironment. Furthermore, gene expression in immune cells might determine how polyherbal Meniran and Turmeric can modulate the immune system in cancer conditions.

Conclusions

This study concludes that the Meniran and Turmeric treatment on DMBA-injected mice decreased the level of CD11b+ and CD11b+IL10+, meanwhile it increased the level of CD11b+IL6+ and CD11b+TNF-α+ cells. The result indicated that the Meniran and Turmeric combination had the potential to modulate the
immune system, especially macrophage cells against cancer cells under the experimental conditions described in this work.

Conflicts of Interest

No competing interests were disclosed.

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