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Immunostimulatory effects of ethyl acetate extract of galing plants on breast cancer model mouse: Analysis of Interferon Gamma and CD14 expression

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ABSTRACT: Breast cancer continues to be a significant global health concern, resulting in considerable morbidity and mortality. This is primarily due to the fact that patients frequently encounter compromised cellular immune function, which is the primary means by which cancer cells are eliminated; therefore, effective treatment strategies that increase the patient's immune system's workload are required. For enhancing immune system functionality in cancer, the galing plant (*Cayratia trifolia* L. Domin) may present a viable alternative due to the presence of active compounds such as delphinidin, malvidin, resveratrol, and quercetin. These compounds have been empirically validated for their ability to regulate both non-specific and specific cellular immune responses. The objective of this research endeavor is to investigate the potential of galing ethyl acetate extract to modulate the cellular immune system in white mouse models of breast cancer by upregulating the expression of IFN-γ and CD14. Inducing the carcinogen DMBA transformed a cohort of 22 female white mouse into a model of breast cancer. These mice were subsequently divided into three groups: normal mouse (K0), cancer model mouse (K1), and those that received a 300 mg/kgBW galing ethyl acetate extract. after administering for a duration of 28 days. Following this, a monoclonal antibody immunohistochemical examination was conducted using anti-rat CD14 and anti-rat IFN-γ. The findings of the study demonstrated that the administration of galing ethyl acetate extract significantly increased the levels of IFN-γ and CD14 expression in comparison to the negative and normal control groups ($p<0.05$). Furthermore, a robust correlation was observed between the increase in IFN-γ expression and CD14 expression ($p<0.01$). Consequently, this research sheds light on the potential therapeutic applications of galing plants as immunostimulatory agents.

KEYWORDS: *Cayratia trifolia* L (Domin); breast cancer; IFN-γ; CD14; immunostimulatory

1. INTRODUCTION

Cancer is the second leading cause of mortality on a global scale; according to projections, the disease will be responsible for 12.3 million fatalities in 2022. Based on data provided by Globocan, the International Agency for Research on Cancer (IARC) in 2022, breast cancer is the leading cause of morbidity and mortality on a global scale, affecting approximately 2.201 million individuals. Mortality ranks fifth, affecting 714.09 million individuals. Indonesia will have the highest breast cancer morbidity and mortality rates in 2022, with 65.42 million and 22.4 million cases, respectively [1].

Cancer treatment is currently carried out in various ways, including surgery, radiation, chemotherapy, endocrinopathy, immunotherapy and hormone therapy [2]. The anticancer drugs currently available are still limited by the problem of less selective effects, by killing normal cells, so that the therapeutic effect is not optimal [3], such as treatment with chemotherapy can cause depression of the bone marrow which produces blood cells which results in infection, bleeding, fatigue, hair loss, nausea, vomiting diarrhea and serious immunodeficiency [4]. An approach to surmounting cancer involves the administration of substances that regulate the cellular immune system, with a particular emphasis on enhancing the

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functionality of T helper lymphocytes (CD4), macrophages and natural killer (NK) cells as immunocompetent cells that generate the cytokine interferon-gamma (IFN-γ), which functions as a cytokine activator of NK cells. in addition to cytotoxic/cytotoxic T-lymphocyte (CTL/CD8) T lymphocyte cells, which eliminate cancer cells efficiently while avoiding adverse effects in patients [5-7].

In order to enhance the cellular immune system, one can achieve this by providing an immunomodulator molecule, such as delphinidin/malvidin, resveratrol, and quercetin, resveratrol dan quercetin [8]. The chemical substance is present in the galing plant (*Cayratia trifolia* L (Domin)) [9]. he anthocyanin chemicals (delphinidin, malvidin) found in galing plants have the ability to regulate macrophages, stimulating the production of interferon (IFN) and tumor necrosis factor-alpha (TNF-α) [10]. cientific evidence has demonstrated that the quercetin chemical effectively enhances the activity of T helper lymphocytes (CD4) in the cellular immune system and triggers the production of TNF-α and iNOS (inducible nitric oxide synthase) by macrophages [11]. Resveratrol, a molecule known as stilbenes, has the ability to modulate the immune response by affecting helper T cells (CD4) and cytotoxic T lymphocytes/CTL (CD8). It can also boost the activity of cytotoxic T lymphocytes (CD8) and helper T lymphocytes (CD4), as well as promote the secretion of IFN-γ in tumors. Resveratrol has been demonstrated to regulate the immune system in laboratory settings by stimulating the production of the cytokines IFN-γ, IL-2, and IL-4, and activating CD4 T lymphocytes, CD8/CTL, and NK cells [12,13].

The existing scientific knowledge regarding the process by which galing plants prevent the growth of cancer cells through cellular immune system mechanisms is lacking. Previous studies have found that the ethanol extract of galing plants contains compounds that can potentially affect the cellular immune system. Specifically, the flavonoid compounds in the ethyl acetate extract are believed to have a more significant impact on enhancing the function of macrophage cells and T helper lymphocytes (CD4) in expressing IFN-γ in cancer. Uncovered via this research. The upregulation of IFN-γ expression is of significant importance as this primary cytokine stimulates the activation of NK cells (natural killer cells) and CTL (cytotoxic T-Lymphocytes) to selectively eliminate cancer cells while sparing normal cells. The current understanding of the impact of galing plant ethyl acetate extract on immunocompetent cells producing IFN-γ and CD14 expression in cancer is lacking. Therefore, this study aims to uncover the potential role of galing plant ethyl acetate extract as an immunostimulator candidate in breast cancer cells. The study used the use of white mouse to simulate breast cancer. This was done by administering the carcinogen DMBA (7,12-Dimethylbenz(a)anthracene) intragastrically twice a week for a duration of 5 weeks [14,15].

2. RESULT AND DISCUSSION

2.1 The impact of Galing leaf extract on the expression of IFN-γ

Immunohistochemical technique using monoclonal anti-rat antibody IFN-γ catalog no. 507802 (Biolegend) was used to determine the amount of IFN-γ expression in spleen tissue, counted throughout the number of follicles in the spleen tissue which were stained brown in cytoplasm with blue nuclei (positive for IFN-γ) at 400 times magnification. The results showed that administration of ethyl acetate extract from the Cayratia trifolia (KP) plant could significantly increase the amount of IFN-γ expression in the K0 and K1 groups ($p<0.05$). The number of expression IFN-γ in the group given ethyl acetate (KP) extract was significantly higher compared to the group without extract (K1) and the normal group (K0), where the mean value and standard deviation were obtained for the KP group ($73.18^{b} \pm 8.49$), group K1 ($20.17^{a} \pm 3.26$) and group K0 ($19.43^{a} \pm 4.41$) (table 1). The results of this study prove that there is an effect of giving ethyl acetate extract of the galing plant to white mouse with breast cancer on significantly increasing the number of IFN-γ expression. The research results can be seen in table 1 and Figures 1-3.

Table 1. Mean and standard deviation of the number of IFN-γ expression in mouse spleen tissue follicles

Group (n=8)	IFN-γ (Mean ± SD)	p-value
K0= Normal cells	$19.43^{a} \pm 4.41$	
K1= DMBA	$20.17^{a} \pm 3.26$	0.000
KP= DMBA+ Ethyl acetate extract	$73.18^{b} \pm 8.49$	

Note: Superscripts with different letters in the same column are meaningful ($p<0.05$)

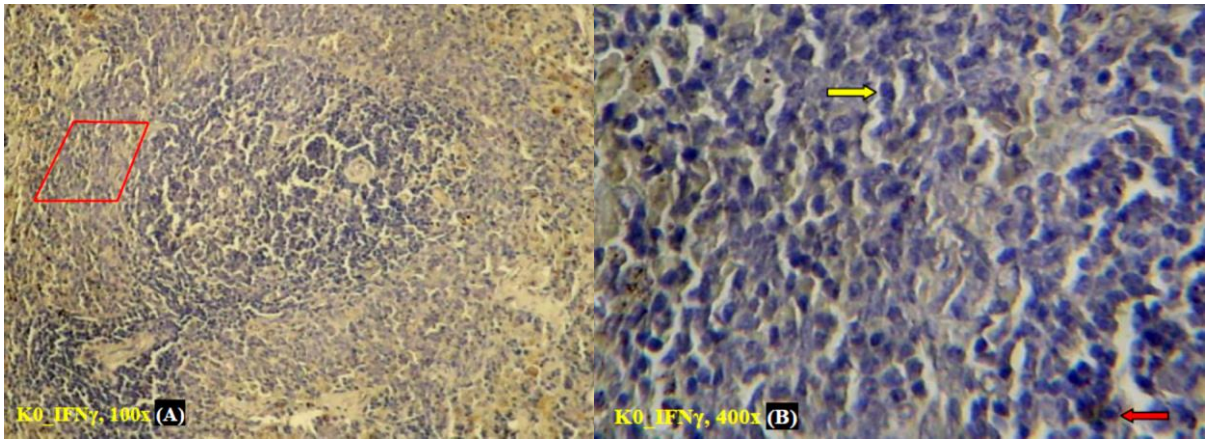

Figure 1. (A). Microscopy of spleen tissue incisions from mouse in the K0 group. Follicles with white pulp areas are visible, there is a proliferation process (⟶) reading area. **(B).** Expression of IFN-γ producing cells in the spleen of white mouse, brown IFN-γ producing cells appear (➡) and cells that do not produce IFN-γ are colored blue (⇨), IHC staining.

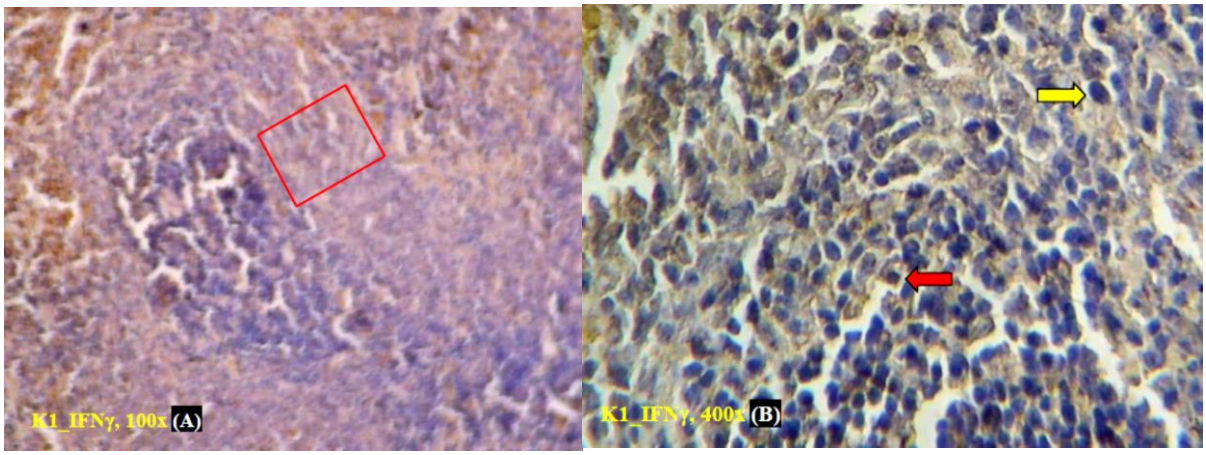

Figure 2. (A). Microscopy of spleen tissue incisions from mouse in group K1. Follicles with a white pulp area are visible, there is a proliferation process (⟶) in the area that is read. **(B)**. Expression of IFN-γ producing cells in the spleens of K1 group white mouse. Cells producing IFN-γ are seen in brown (➡) and cells that do not produce IFN-γ in blue (⇨), IHC staining.

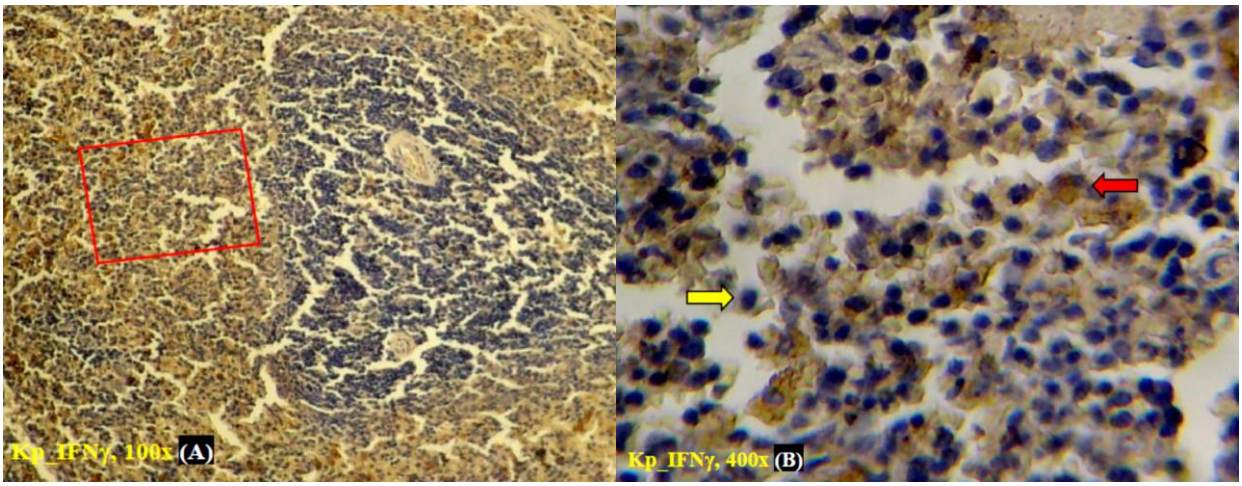

Figure 3. (A). Microscopy of spleen tissue incisions from mouse in the KP group. Follicles with a white pulp area are visible, there is a proliferation process (⟶) in the area that is read. (B). IFN-γ producing cells in the spleens of KP white mouse. Cells producing IFN-γ are seen in brown (➡) and cells that do not produce IFN-γ in blue (⇨), IHC staining.

Figure 3 shows that the amount of IFN-γ expression in the spleen tissue of white mice from the KP group was significantly higher than the K0 and K1 groups ($p<0.05$).

The research results obtained are in accordance with previous research by Kumar et al (2011) where the group of anthocyanin compounds (delphinidin, malvidin) and quercetin are compounds contained in the ethyl acetate extract of galing plants [16], which have the effect of modulating macrophages to produce IFN-γ, and stimulating the cellular immune system of T helper lymphocytes as IFN-γ producing cells [13,17]. According to previous research, the resveratrol compound in galing plants has an immunomodulatory effect on T helper lymphocytes and modulates cytotoxic T lymphocytes (CD8), T helper lymphocytes (CD4) and IFN-γ secretion in tumors, as well as activating NK cells [18,19].

IFN-γ, which is secreted by immune cells such as Th1 lymphocytes and macrophages, plays a crucial role in enhancing the body's cancer defense mechanisms. It can exert an antiproliferative effect on cancer cells and directly impede their growth by upregulating gene expression. Tumor suppressor genes are genes that inhibit the development of tumors [19]. IFN-γ is the primary cytokine responsible for activating macrophages and is involved in both non-specific and specific cellular immunity. Th1 lymphocytes secrete IFN-γ, which stimulates macrophages to perform non-specific lysis of cancer cells. This is achieved through the secretion of the cytokine tumor necrosis factor-alpha (TNF-α), which activates phagocytic activity in macrophages and induces the release of cytotoxic enzymes (perforin and granzyme). In addition to activating the caspase cascade, IFN-γ is required to enhance the activity of macrophages as antigen presenting cells (APCs). This allows them to be more efficient and responsive in presenting foreign objects on the cell surface through MHC class I or MHC class II molecules. Additionally, macrophages secrete IL-12, which stimulates the proliferation and differentiation of helper lymphocytes into Th1 and Th2 lymphocytes [6,17].

The effect of IFN-γ secreted by Th1 lymphocytes and macrophage cells can be an autocrine cytokine which can activate its expressing cells and a paracrine cytokine which can trigger the activation of cytotoxic/CTL lymphocyte cells and NK cells, which is a cellular immune system that is more protective than the humoral immune system. in dealing with cancer cells through the Fas-Ligand pathway which can trigger FADD (Fas associated protein death domain) activity and through cytotoxin enzymes released by CTL and NK cells, so that cancer cells can be lysed or undergo apoptosis [5,20].

2.2 The effect of the ethyl acetate extract from Galing leaves on CD14 expression

Examination of the amount of CD14 expression was carried out using immunohistochemical techniques using an anti-rat CD14 monoclonal antibody that is reactive to mouse tissue, catalog no. HM1060 (Hycult Biotech). In this study, the results showed that administration of galing plant ethyl acetate extract (KP) could significantly increase the amount of CD14 expression in the normal group (K0) and without K1 extract ($p<0.05$). The amount of CD14 expression in the extract group was significantly higher than the group without extract administration and the normal group, where the mean value and standard deviation were obtained for the KP group ($113.55^{c} \pm 19.58$), K1 group ($51.08^{b} \pm 4.33$) and K0 group ($38.38^{a} \pm 5.68$) as in Table 2. The results of this study prove that there is an effect of giving ethyl acetate extract of galing plants to white mice with breast cancer on significantly increasing the number of CD14-expressing cells. The research results can be seen in table 2 and Figures 4-6.

Table 2. Mean and standard deviation of the number of CD14 expressing cells in mouse spleen tissue follicles

Group (n=8)	CD14 (Mean ± SD)	p-value
K0= Normal cells	$38.38^{a} \pm 5.68$	
K1= DMBA	$51.08^{b} \pm 4.33$	0.000
KP= DMBA+ Ethyl acetate extract	$113.55^{c} \pm 19.58$	

Note: Superscripts with different letters in the same column are meaningful ($p<0.05$)

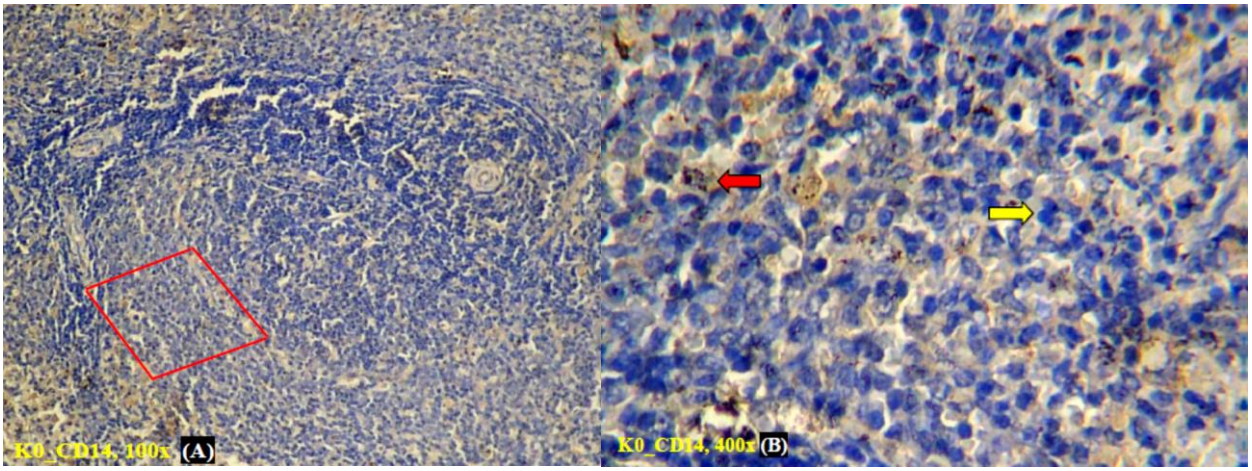

Figure 4. **(A).** Microscopy of spleen tissue incisions from mouse in the K0 group. Follicles with a white pulp area are visible, there is a proliferation process (⟶) in the area that is read. **(B).** CD14 expressing cells in the spleen tissue of K0 group white mouse. CD14-expressing cells are seen in brown (➡) and non-CD14-expressing cells in blue (⇨), IHC staining.

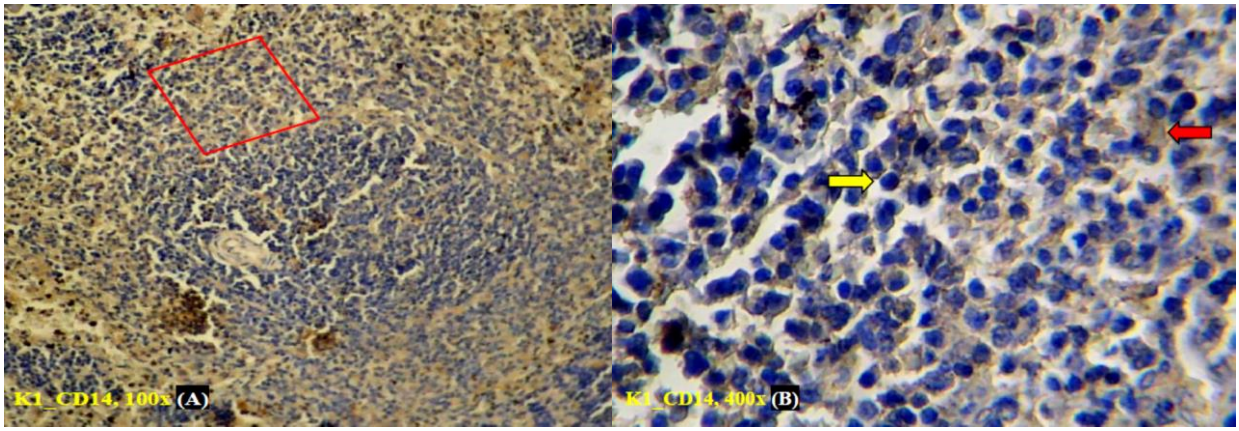

Figure 5. **(A).** Microscopy of spleen tissue incisions from mouse in group K1. Follicles with a white pulp area are visible, there is a proliferation process (⟶) in the area that is read. **(B).** CD14 expressing cells in the spleen tissue of K1 group white mouse. CD14-expressing cells are seen in brown (➡) and non-CD14-expressing cells in blue (⇨), IHC staining.

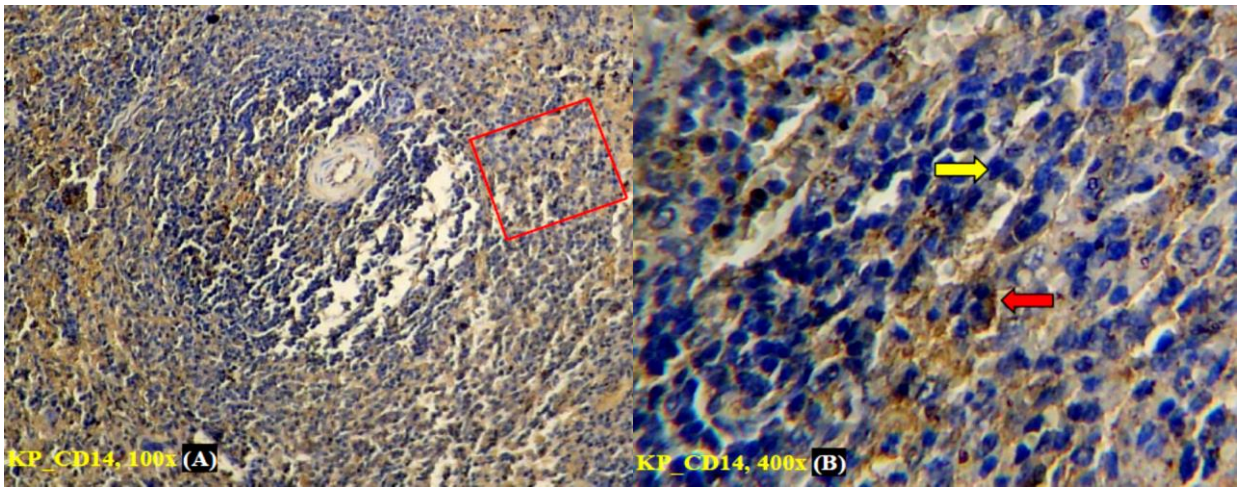

Figure 6. **(A).** Microscopy of spleen tissue incisions from mouse in the KP group. Follicles with a white pulp area are visible, there is a proliferation process (⟶) in the area that is read. **(B).** CD14 expressing cells in the spleen tissue of KP group white mouse. CD14-expressing cells are seen in brown (➡) and non-CD14-expressing cells in blue (⇨), IHC staining.

The results of this study are consistent with previous research that explains the presence of anthocyanin compounds (delphinidin, malvidin) in galing plants, which have been proven to modulate macrophages to produce IFN-γ and TNF-α [21,22]. Additionally, these plants contain quercetin compounds that have been shown to stimulate the cellular immune system of T helper lymphocytes (CD4) and activate macrophages to produce TNF-α and iNOS (inducible nitric oxide synthase) [5,23], he research conducted by Falchetti in vitro shown that the compound resveratrol effectively modulates the immune system, particularly macrophage cells, by inducing the cytokines IFN-γ, IL-2, and IL-4, thereby activating CD4 T lymphocytes, CD8/CTL cells, and NK cells [19,21].

Enhancing CD14 expression on macrophage cells is highly beneficial for eradicating cancer cells. This is because the cytokines released by macrophages, such as IFN-γ, can stimulate the activation of natural killer (NK) cells and cytotoxic T lymphocytes (CTL). These immune cells then eliminate cancer cells by utilizing the FADD (Fas Associated Protein Death Domain) activity pathway. The activation occurs when ligands on the surface of NK cells and CTL cells link to Fas receptors on the surface of cancer cells. The interaction between Fas-Ligand triggers signal transduction within the cytosol of cancer cells, leading to the activation of the FADD protein. FADD activity initiates caspase cascades, leading to the activation of caspases. These active caspases subsequently activate DNA-se, an enzyme that breaks and destroys the DNA of cancer cells. As a result, cancer cells undergo apoptosis (cell death) and their population decreases. Furthermore, the secretion of IFN-γ by CD4 T helper lymphocytes will stimulate macrophages to enhance their ability to eliminate cancer cells. This is achieved through the secretion of TNF-α, which can induce phagocytic activity in macrophages. Additionally, the release of cytotoxic enzymes (perforin and granzyme) by macrophages activates the caspase cascade, leading to the death or lysis of cancer cells [20,24].

2.3 Analysis of corelation the amount of IFN-γ expression and the amount of CD14 expression

The statistical analysis revealed a robust and statistically significant connection ($r=0.891$, $p<0.01$) between the levels of IFN-γ expression and CD14 expression. This demonstrates a positive correlation between the levels of IFN-γ expression and CD14 expression. In other words, as the amount of IFN-γ expression increases, so does the expression of CD14, and vice versa. IFN-γ has several functions, such as stimulating macrophage and CTL cells to fight against tumors, enhancing the activity of NK cells as powerful cells that destroy cancer cells, and regulating the function of B lymphocytes in the immune response [7,21].

When macrophages are activated by IFN-γ, they release a cytokine called IFN-γ. This cytokine then activates CTL cells and NK cells. These cells, in turn, eliminate cancer cells by using the Fas-Ligand pathway. The activation of FADD by these cells triggers a series of caspase cascades. The active caspases then activate an enzyme called DNA-se, which breaks down the DNA of cancer cells. As a result, the cancer cells undergo apoptosis (cell death) and their numbers decrease [20,25].

Furthermore, the secretion of IFN-γ by T helper lymphocytes stimulates macrophages to enhance their ability to eliminate cancer cells. This is achieved through the release of TNF-α, which induces phagocytic activity in macrophages. Additionally, the secretion of cytotoxic enzymes (perforin and granzyme) by macrophages activates the caspase cascade, leading to the death or lysis of cancer cells [11,12]. Furthermore, the production of IFN-γ by Th1 lymphocytes and macrophages can stimulate the activation of NK cells and CTL cells. As a result, these activated NK cells and CTL cells will eliminate cancer cells (abnormal cells) and modulate the cellular and humoral immune system. This includes the activation of Th2 lymphocytes to release the cytokines interleukin-4 (IL-4), IL-5, IL-6, IL-10, and IL-13. IL-4, IL-5, and IL-6 stimulate B lymphocytes to differentiate into plasma cells, which subsequently produce and release antibodies (IgG) along with memory cells. Antibodies, particularly IgG, attach to the Fc receptor of NK cells, triggering ADCC (antibody dependent cell-mediated cytotoxicity). This activation leads to the destruction of cancer cells by the action of cytotoxin enzymes, namely perforin and granzyme [6,25].

3. CONCLUSION

The administration of the ethyl acetate extract of the galing plant (*Cayratia trifolia*) has been demonstrated to enhance the expression of IFN-γ and CD14 in mice with a breast cancer model. This research highlights the plant's role as an immunostimulatory agent with significant potential for development. It offers a potential solution for treating breast cancer by improving the immune system in cancer patients.

4. MATERIALS AND METHODS

4.1. Materials

The substances used in the experiment were ethyl acetate, 95% ethanol, maize oil (CV M&H Farm Bogor), DMBA (Sigma), and poly-L-lysine coated glass objects (muto pure chemical). The following substances are included in the list: 10% formalin buffer solution, liquid paraffin, block paraffin, glycerin, absolute ethanol, alcohol at concentrations of 96%, 90%, 80%, and 70%, xylol, distilled water, Mayer's Hematoxylin solution, eosin 2-3% solution, phosphate buffered saline (PBS), xylol, ethanol, hydrogen peroxide (H_2O_2), fetal bovine serum (FBS), 3,3-diaminobenzidine (DAB) solution, primary antibodies: monoclonal antibodies anti-Rat CD14 (Hycult Biotech) and anti-Rat IFN-γ (Biolegend), and secondary antibodies: ultravision monoclonal Rabbit anti mouse (Thermo Scientific).

4.2. Procedure for the Preparation of Ethyl Acetate Extract from Galing Leaf

Galing leaf samples were collected in Kendari City. The leaves were washed, cut into pieces, and dried in an oven at a temperature of 50^{o}C. After drying, the leaves were blended and sieved using a 4/18 mesh size. Measure 500 grams of the sample and transfer it into a macerator. Add ethyl acetate solvent until the entire sample is fully immersed, or use three times the weight of the sample. Allow it to sit for 24 hours, stirring regularly. Afterward, filter the mixture to obtain the filtrate. The process of extraction, soaking, and filtration is repeated three times or until the sample no longer exhibits any coloration. The liquid that passed through the filter was gathered in a container that does not allow light to enter and was securely sealed. Subsequently, the gathered filtrate is subjected to evaporation using a rotating vacuum evaporator until a concentrated extract is achieved. The concentrated filtrate is dried in an oven at a temperature of 50-60 oC until a raw extract is achieved in the form of powder. It is then stored in a clean opaque glass bottle, properly labeled, and prepared for use as research material [26-28].

4.3. Preparation of the DMBA Carcinogen

The powdered form of DMBA is thereafter measured based on the dosage required for the experimental animal and combined with corn oil. The dosage of DMBA (7,12-Dimethylbenz(a)anthracene) is 20 mg/kg BW and is administered twice a week, for a total of 10 administrations over a period of 5 weeks. The administration is done through the use of a nasogastric tube for intra-gastric delivery [29].

4.4. Creating Breast Cancer Models in Animal Experiments

The white mouse cancer model was developed at the Integrated Research and Testing Laboratory (LPPT) UGM Yogyakarta. The research has undergone testing and received approval from the Halu Oleo University LPPM Research Ethics Committee, with the reference number 889-KE_2023. Twenty female SD strain white mouse, aged 30-40 days and weighing 100-150 grams, were used to create breast cancer models. This was achieved by administering the carcinogen DMBA at a concentration of 20 mg/kg BW dissolved in corn oil, which was given to the mouse through intra-gastric administration. Donations are made twice a week, totaling 10 times over a period of 5 weeks. The experimental animal's breast is examined weekly through palpation until a lump is detected. Breast cancer development begins at the 10th week and continues for 25 weeks [30,31]. The mouse cancer model was acquired 11 weeks after the administration of the carcinogen DMBA, resulting in a 40.90% incidence rate. Subsequently, between weeks 13 and 20, 18 white mouse developed breast cancer.

4.5. Management of Mouse Experimental Subjects

The treatment group of mouse that had previously developed cancer was administered the ethyl acetate extract at the Experimental Animal Laboratory Unit, Faculty of Medicine, Halu Oleo University. The ethyl acetate extract was administered for a duration of 4 weeks at a dosage of 300 mg/kg BW per day. The administration was done using a 0.5% Na.CMC solution through an intragastric probe. The positive control group received a daily dose of 0.5% Na-CMC placebo for a duration of 4 weeks, whereas the negative control group (normal) received a daily dose of 0.5% Na-CMC for the same duration. On the initial day of the fifth week, all mice in groups K0, K1, and KP were euthanized, and their spleen tissue was collected. Subsequently, all the tissue samples were preserved using a 10% formalin solution and underwent tissue processing. Paraffin blocks were then created and the tissue was sliced using a microtome positioned on a poly L-lysine glass substrate [15,32].

4.6. Haematoxylin Eosin (HE) Staining

The process involves paraffin from tissue that has been cut with a microtome and placed on a glass object. The tissue incision should be inserted into xylol three times, each time for 3-5 minutes. Then, it shouldbe successively inserted into ethanol with decreasing concentrations: 100% ethanol three times for 1-3 minutes, 95% ethanol twice for 1-3 minutes, and 90%, 80%, and 70% ethanol each for 1-2 minutes. After that, the tissue should be washed with tap water for approximately 5 minutes. Next, it should be placed in Haris Haematoxylin for 6 minutes, followed by rinsing with water and dipping it in 1% acidic alcohol for 3-5 times. First, rinse it again with water. Then, immerse it in ammonia water until it changes color to blue. Next, submerge it in eosin solution. After that, sequentially immerse it in 95% ethanol two times for one minute each, followed by immersion in xylol three times for two minutes each. Finally, mount it with entelan and observe it under a light microscope at 400x magnification [30,31].

4.7. Immunohistochemical Examination Procedure

The immunohistochemical staining used in this study was to determine or examine CD14 and IFN-γ. Immunohistochemical staining of IFN-γ and CD14 : Tissue that has been cut with a microtome that has been placed on a glass object is then deparaffinized, namely pulling/removing the paraffin that is in the tissue. then washed with PBS 3 times (a': 2 minutes), then added to primary monoclonal antibody (mouse anti Rat IFN-γ) catalog no. 507802 (Biolegend) and (mouse anti Rat CD14) catalog no. HM1060 (Hycult Biotech) for 30 minutes, then wash with PBS 3 times (a': 2 minutes), then add secondary monoclonal antibody, namely Rabbit anti-mouse biotinylated label, for 30 minutes. Then washed with PBS 2 times (a': 2 minutes), then successively put into labeled streptavidin HRP: 30 minutes, washed with PBS 3 times (a': 2 minutes), put into chromogen substrate: 5 minutes (DAB solution), washed with PBS 3 times (a': 2 minutes), then rinsed with distilled water, placed in Mayer Hematoxylin: 6 minutes, washed with running water, and finally mounted and observed/read the results on a light microscope at 400x magnification. 10 fields of view [30,31].

4.8. Data Analysis

Observational data is the result of examining IFN-γ expression and CD14 expression by counting the number of spleen tissue follicles, then statistically analyzed using; Data normality test, Wilk's Lamda test in Multivariate Analysis of Variance (Manova), Post hoc Games-Howell test, Pearson correlation test to see the correlation between IFN-γ and CD14. The data that has been collected is processed quantitatively manually and using the SPSS 18.00 program.

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REFERENCES

[1] The Global Cancer Observatory. Cancer incident in Indonesia. International Agency for Research on Cancer. 2022; 858: 1–2. https://gco.iarc.fr/today/data/factsheets/populations/360-indonesia-fact-sheets.pdf

[2] Wahyuni W, Diantini A, Ghozali M, Subarnas A, Julaeha E, Amalia R, Sahidin I. Cytotoxic and antimigration activity of *Etlingera alba* (A.D.) Poulsen Rhizome. Adv Pharmacol Pharm Sci. 2021; 2021: 6597402. https://doi.org/10.1155/2021/6597402

[3] Cejalvo JM, Pascual T, Fernández-Martínez A, Braso-Maristany F, Gomis RR, Perou CM, Muñoz M, Prat A. Clinical implications of the non-luminal intrinsic subtypes in hormone receptor-positive breast cancer. Cancer Treat Rev. 2018; 67(3): 63–70. https://doi.org/10.1016/j.ctrv.2018.04.015

[4] American Cancer Society. Facts & Figures 2022. American Cancer Society. 2022: 1–76.

[5] Ilyas MY, Diantini A, Ghozali M, Sahidin I, Fristiohady A. Immunomodulatory potency *Etlingera rubroloba* A.D. poulsen fruit ethanol extract against macrophage phagocytic activity and CD4 levels in wistar male rats. Res J Pharm Technol. 2022; 15(9): 4067–4072. https://doi.org/10.52711/0974-360X.2022.00682

[6] Ilyas MY, Sahidin I, Jabbar A, Yodha AWM, Diantini A, Pradipta IS, Amalia R, Febrianti RM, Hadisaputri YE, Ghozali M, Julaeha E. Effect of immunomodulating extract and some isolates from *Etlingera rubroloba* A.D. Poulsen

fruits on diabetic patients with tuberculosis. Molecules. 2023; 28: 2401. https://doi.org/10.3390/molecules28052401

[7] Ilyas MY, Diantini A, Halimah E, Amalia R, Ghozali M, Julaeha E. Phytochemical analysis and immunomodulatory potential on diabetic-infected tuberculosis by fruit *Etlingera rubroloba* A.D. Poulsen. Pakistan J Biol Sci. 2022; 25(7): 669–675. https://doi.org/10.3923/pjbs.2022.669.675

[8] Hong J, Fristiohady A, Nguyen CH, Milovanovic D, Huttary N, Krieger S, Hong J, Geleff S, Birner P, Jager W, Ozmen A, Krenn L, Krupitza G. Apigenin and luteolin attenuate the breaching of MDA-MB231 breast cancer spheroids through the lymph endothelial barrier in vitro. Front Pharmacol. 2018; 14(9): 220. https://doi.org/10.3389/fphar.2018.00220

[9] Yusuf MI, Wahyuni, Susanty S, Ruslan, Fawwaz M. Antioxidant and antidiabetic potential of galing stem extract (*Cayratia trifolia* Domin). Pharmacogn J. 2018; 10(4): 686-689. https://doi.org/10.5530/pj.2018.4.113

[10] Holzner S, Brenner S, Georgiev A, Senfter D, Stadler S, Huu C, Fristiohady A, Milovanovic D, Huttary N, Sigurd Krieger S, Bago-Horvath Z, Wever O, Tentes I, Ozmen A, Jager W, Dolznig H, Dirsch VM, Mader RM, Krenn L, Krupitza G. Intravasation of SW620 colon cancer cell spheroids through the blood endothelial barrier is inhibited by clinical drugs and flavonoids in vitro. Food Chem Toxicol. 2018; 111(11): 114–124. https://doi.org/10.1016/j.fct.2017.11.015

[11] Ilyas MY, Diantini A, Halimah E, Amalia RM, Ghozali M, Julaeha E, Sahidin I. Potential immunomodulator fraction fruit of *Etlingera rubroloba* A.D Poulsen against macrophage phagocytosis and interleukin-12 levels in BCG-stimulated Balb/C mice. Int J Pharm Res. 2021; 13(1): 3262–3269. https://doi.org/10.31838/ijpr/2021.13.01.478

[12] Decendit A, Mamani-Matsuda MV, Amount P, Waffo-Tugeo D, Moynet K, Boniface E, Richard S, Krisa J, Rambert JM, Merillon MD, Mosallayi. Malvidin-3-O-β glucoside, major grape anthocyanin, inhibits human macrhophage-derived inflamatory mediators and decreases clinical scores in arthritic rats. J Biochem Pharmacol. 2013; 86(10): 1461–1467. https://doi.org/10.1016/j.bcp.2013.06.010

[13] Jang JY. Mechanism of resveratrol-induced programmed cell death and new drug discovery against cancer : A Review. Int J Mol Sci. 2022; 23(22): 13689. https://doi.org/10.3390/ijms232213689

[14] Brockmueller A, Sameri S, Liskova A, Zhai K, Varghese E, Samuel SM., Büsselberg D, Kubatka P, Shakibaei M. Resveratrols anti-cancer effects through the modulation of tumor glucose metabolism. Cancers (Basel). 2021; 13(2): 188. https://doi.org/10.3390/cancers13020188

[15] Rojas-Armas JP, Arroyo-Acevedo JL, Palomino-Pacheco M, Herrera-Calderón O, Ortiz-Sánchez JM, Rojas-Armas A, Calva J, Castro-Luna A, Hilario-Vargas J. The essential oil of *Cymbopogon citratus* Stapt and carvacrol: An approach of the antitumor effect on 7,12-Dimethylbenz-[α]-anthracene (DMBA)-induced breast cancer in female rats. Molecules. 2020;25(14):3284. https://doi.org/10.3390/molecules25143284

[16] Kumar D, Kumar S, Gupta J, Arya R, Gupta A. A review on chemical and biological properties of *Cayratia trifolia* Linn. (Vitaceae). Pharmacogn Rev. 2011; 5(10): 184–188. https://doi.org/10.4103/0973-7847.91117

[17] Amini P, Moazamiyanfar R, Dakkali MS, Khani A, Jafarzadeh E, Mouludi K, Khodamoradi E, Johari R, Taeb S, Najafi M. Resveratrol in cancer therapy: From stimulation of genomic stability to adjuvant cancer therapy: A comprehensive review. Curr Top Med Chem. 2023;23(8):629-648. https://dx.doi.org/10.2174/1568026623666221014152759

[18] Lalani AR, Fakhari F, Radgoudarzi S, Rastegar-Pouyani N, Moloudi K, Khodamoradi E, Taeb S, Najafi M. Immunoregulation by resveratrol; implications for normal tissue protection and tumour suppression. Clin Exp Pharmacol Physiol. 2023; 50(5): 353-368. https://doi.org/10.1111/1440-1681.13760

[19] Jang JY, Im E, Kim ND. Mechanism of resveratrol-induced programmed cell death and new drug discovery against cancer : A Review. Int J Mol Sci. 2022; 23(22): 13689. https://doi.org/10.3390/ijms232213689

[20] Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. Apoptosis and molecular targeting therapy in cancer. Biomed Res Int. 2014; 2014: 150845. https://doi.org/10.1155/2014/150845

[21] Wadsworth TL, Koop DR. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. Biochem Pharmacol. 1999; 57(8): 941-949. https://doi.org/10.1016/s0006-2952(99)00002-7

[22] Kumar D, Arya V, Kaur R, Bhat ZA, Gupta VK, Kumar V. A review of immunomodulators in the Indian traditional health care system. J Microbiol Immunol Infect. 2012; 45(3): 165–184. http://dx.doi.org/10.1016/j.jmii.2011.09.030

[23] Jabbar A, Sahidin I, Malik F, Ilyas MY, Rusli N, Apriyanto, Reymon, Qadar J. Evaluation of xanthine oxidase inhibitory activity of sinapyl alcohol diacetate and stigmasterol compounds and phytochemical screening stem of Etlingera. Food Res. 2023; 7(4): 101–105. https://doi.org/10.26656/fr.2017.7(4).009

[24] Imran, Wahyuni, Fristiohady A, Leorita M, Malaka MH, Ilyas MY, Musadar, Rahmatika NS, Darmawan A, Fajriah S, Yodha AWM, Sahidin I. Radical scavenger and anti-diabetic potencies of *Etlingera elatior* fruits growing in south east Sulawesi-Indonesia. Res J Pharm Technol. 2022; 15(5): 2141-2146. https://doi.org/10.52711/0974-360X.2022.00355

[25] Yahfoufi N, Alsadi N, Jambi M, Matar C. The immunomodulatory and anti-inflammatory role of polyphenols. Nutrients. 2018; 10(11): 1618. https://doi.org/10.3390/nu10111618

[26] Jabbar A, Sahidin I, Monstavevi SA, Malaka MH, Malik F, Ilyas MY. Antioxidant and anti-inflammatory activity of ethanol extract stem of *Etlingera rubroloba* A.D. Poulsen. Pakistan J Biol Sci. 2022; 25(10): 885–891. https://doi.org/10.31838/ecb/2022.11.05.009

[27] Fristiohady A, Leorita M, Malik F, Thamrin ASW, Ilyas MY, Wahyuni, Purnama LOMJ, Sahidin I. Pancreatic histological profile on the efficacy of extract of *Etlingera calophrys* (K.Schum) A.D. Poulsen stem against streptozotocin-induced diabetes in diabetic model rats. Biointerface Res Appl Chem. 2021; 11(2): 9209–9217. https://doi.org/10.33263/BRIAC112.92099217

[28] Irma, Tee SA, Fithria, Ilyas MY. The antibacterial inhibition test of gonad sea urchin (*Diadema setosum*) against the growth of *Staphylococcus aureus*. J Phys Conf Ser. 2021; 1899(1). https://doi.org/10.1088/1742-6596/1899/1/012053

[29] Barros ACSD, Muranaka ENK, Mori LJ, Pelizon CHT, Iriya K, Giocondo G, Pinotti JA. induction of experimental mammary carcinogenesis in rats with 7,12-dimethyl-benz(a)antrhacene. Rev Hosp Clin Fac Med S Paulo. 2004; 59(5): 257-261. https://doi.org/10.1590/s0041-87812004000500006

[30] Koivunen ME, Krogsrud RL. Principles of immunochemical techniques used in clinical laboratories. Lab Med. 2006; 37(8): 490–497. https://doi.org/10.1309/MV9RM1FDLWAUWQ3F

[31] Miller JL, Bartlett AP, Harman RM, Majhi PD, Jerry DJ, Van de Walle GR. Induced mammary cancer in rat models: Pathogenesis, genetics, and relevance to female breast cancer. J Mammary Gland Biol Neoplasia. 2022; 27(2): 185-210. https://doi.org/10.1007/s10911-022-09522-w

[32] Duderstadt EL, McQuaide SA, Sanders MA, Samuelson DJ. Chemical carcinogen-induced rat mammary carcinogenesis is a potential model of p21-activated kinase positive female breast cancer. Physiol Genomics. 2021; 53(2): 61-68. https://doi.org/10.1152/physiolgenomics.00112.2020