Research Article

The effect of *Phyllanthus niruri* and *Catharanthus roseus* on Macrophage Polarization in Breast Cancer Mice Model

Sefihara Paramitha Sakti, Fikriya Novita Sari, Farida Rachmawati, Sri Widyarti, Sri Rahayu, Aris Soewondo, Yoga Dwi Jatmiko, Muhaimin Rifa'i *

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang 65145, Indonesia

ABSTRACT

Cancer death cases have increased yearly, and there are estimated to be 21.6 million cancer cases in 2030. Studies of herbal compounds for cancer treatment alternatives are essential because cancer treatment is relatively expensive and has adverse effects. *Phyllanthus niruri* (Pn) and *Catharanthus roseus* (Cr) are plants that are known as herbal medicines. Combining the two plants is expected to prevent and enhance the immune system in breast cancer cases. This study aims to analyze the anti-cancer and immunomodulatory effects of *P. niruri* and *C. roseus* extract (PCE) in modulating macrophage polarization in breast cancer mice. Experimental animals are divided into six groups and there is healthy control (normal mice), cancer (DMBA-induced mice), cancer mice with cisplatin administration, cancer mice with PCE administration with three different doses, including dose 1 (500 mg/kg Pn + 15 mg/kg Cr), dose 2 (1000 mg/kg Pn + 75 mg/kg Cr), and dose 3 (2000 mg/kg Pn + 375 mg/kg Cr). The mice were injected with DMBA once a week for six weeks to induce cancer in mice. The breast cancer mice model was administered with PCE orally for 14 days. The expression of CD11b+IL-10+ and CD11b+IFN-γ+ demonstrated macrophage polarization. The results showed that breast cancer induction using DMBA increased the level of IL-10 and decreased the level of IFN-γ significantly compared to the normal group (p < 0.05). In specific doses, administration of PCE could reduce IL-10 levels and increase the level of IFN-γ significantly (p < 0.05). PCE can modulate the polarization of macrophages by suppressing the M2-like macrophage and increasing the M1-like macrophage. The ability of PCE to modulate macrophage polarization indicates that the combination of *P. niruri* and *C. roseus* has activity as an anti-cancer.

Keywords: Cancer, Catharanthus roseus, IFN-γ, IL-10, Phyllanthus niruri

Introduction

According to reports, the number of deaths caused by cancer is increasing. There were about 19.3 million cancer cases in 2020, with a global mortality rate of around 10 million [1], and its prevalence was predicted to reach up to 21.6 million cases in 2030 [2]. Breast cancer is a condition caused by uncontrolled cell growth in breast tissue that is the most frequent malignancy in Indonesia, with 65,858 cases reported [1]. Immune cells help fight breast cancer by discovering and eliminating cancer cells. Macrophages are immune cells that play an essential role in fighting breast cancer by secreting several cytokines and activating the complement system that can trigger inflammation. Macrophages develop into two main groups with different immune defense and surveillance functions. The two main groups are classically activated macrophages (M1-like macrophages) and alternatively activated macrophages (M2-like macrophages) [3].

M1-like macrophages are tumor-suppressing cells interacting in the tumor microenvironment to inhibit tumor cell proliferation. Cytokines such as IFN-γ, GM-CSF, TNF-α, and IL-1 act as

activators of the M1-like macrophage phenotype during the onset and maintenance of inflammation [4]. IFN-γ is a pleiotropic cytokine that is an anti-viral, anti-tumor, and immunomodulator. IFN-γ in inflammatory conditions triggers the activation of the innate immune response and stimulates the elimination of pathogens. IFN-γ plays a role in the innate immune response by activating macrophage polarization to an M1 proinflammatory phenotype [5]. IFN-γ is produced in the tumor microenvironment by cytotoxic immune cells and synergizes with toll-like receptors (TLR) to induce the tumoricidal activity of macrophages and increase the production of NO and proinflammatory cytokines. IFN-γ was known to increase the number of M1-like macrophages and reduce tumor growth [6]. M1-like macrophages secrete molecules such as TNF-α, IL-6, and IL-12 [4], while M2-type macrophages secrete molecules such as IL-10 and TGF-β [3]. M2-like macrophages are dominated by tumor-associated macrophages (TAM). TAM is associated with cancer cell proliferation, metastasis, and latency by releasing cytokines, chemokines, and growth factors. In the microtumor environment, TAM secretes cytokines such as IL-10, TGF-b, and several inflammatory mediators such as PGE2 and MMP-7, which can inhibit the antigen-presenting process so that T cells lose their ability to identify and kill tumor cells [7]. IL-10 is an anti-inflammatory cytokine that plays a role in supporting the proliferation and metastasis of tumor cells. Several studies have shown that IL-10 concentrations in breast cancer patients are higher than in healthy individuals and are associated with poor clinical conditions [8]. M2-like macrophages are known to help the cancer cells in metastasis, angiogenesis, and proliferation, but M1-like macrophages are associated with higher survival. They can be considered potential indicators for improved therapeutic outcomes in various malignancies, including breast cancer [3].

Cancer treatment is relatively expensive and has side effects that make some people use herbal plants for alternative therapies. Exploration of herbal plants to treat cancer continues to develop to get the best herbal medicine and minimize the side effects of chemotherapy. Indonesians have used many herbal plants to treat diseases, including *Phyllanthus niruri* and *Catharanthus roseus*. *P. niruri* and *C. roseus* have several secondary metabolites that act as anti-cancer with se-veral mechanisms, such as inducing M1 macro-phage

polarization. *P. niruri*, used as an ingredient in herbal medicine from Indonesia, is known to have the ability to treat several diseases [9]. *P. niruri* contains bioactive compounds such as alkaloids, anthocyanins, chlorogenic acids, coumarins, flavonoids, lignin, phenolic acids, saponins, tannins, and terpenoids that have the biological activity to treat disease [10]. The compounds in *C. roseus* widely known for treating cancer are vinblastine and vincristine. Vinblastine and vincristine inhibit mitosis by stopping cell division and causing cells to undergo apoptosis [11]. Therefore, the current study evaluated the potential of *P. niruri* and *C. roseus* combination extract on macrophage polarization in breast cancer mice models.

Material and Methods
Plant Material and Extraction
Simplicia of *P. niruri* and *C. roseus* leaves with specimen numbers 074/454/102.7-A/2021 and 074/455/102.7-A/2021 were collected and determined from UPT. Laboratorium Herbal Materia Medica, Batu, Indonesia. Simplicia was dissolved in aquadest at a 1 : 10 ratio (simplicial : solvent, w/v). The sample was mixed with a magnetic stirrer for 24 hours and then filtered with Whatman filter paper. Extraction was done with freeze-dry at -20ºC to get extract in powder form.

Induction of Breast Cancer Model
The experimental animals used were 6-7 weeks old female Balb/C mice from the Pharmacy Laboratory, Faculty of Pharmacy, Gadjah Mada University (n = 24). Mice acclimatized for a week before the experiment in a free-pathogen environment in the Animal Physiology Laboratory, Biology Department, Brawijaya University. DMBA (7,12-Dimethylbenz(a)anthracene) was injected into mouse breasts subcutaneously to make the breast cancer model. The dose of DMBA given is 1.5 mg/g BW and dissolved in 0.1 ml of corn oil. The injection was given once every week for six weeks. Breast cancer was detected after one week through a morphological transformation in mice by palpation methods. All experimental animal procedures in this study were approved by Brawijaya University Ethics Committee No. 125-KEP-UB-2021.

Treatment
P. niruri (Pn) and *C. roseus* (Cr) extracts were weighed according to the dose and dissolved in

distilled water. The combination of *P. niruri* and *C. roseus* (PCE) was given to mice orally for two weeks. There are three dose combinations given to breast cancer model mice; there are dose 1 (500 mg/kg Pn+15 mg/kg Cr), dose 2 (1000 mg/kg Pn+75 mg/kg Cr), and dose 3 (2000 mg/kg Pn+375 mg/kg Cr). The dose of cisplatin given to mice was 5 mg/kg BW and dissolved in PBS. Cisplatin was injected into mice intraperitoneally once every two days for two weeks. The dosage was determined based on previous studies, which showed that 75 mg/kg body weight of *C. roseus* given to mice with breast cancer models showed no toxicity to the body [12]. The *P. niruri* extract at 5000 mg/kg body weight also showed no side effects or organ damage [13].

Antibody Staining and Flow Cytometry Analysis

The spleen was isolated and homogenized with PBS to analyze cytokine-secreting macrophage profile. Homogenate was centrifuged at 2500 rpm and 10°C for 10 minutes. The pellet was resuspended in PBS for antibody staining. 50 µl of the sample was added to the microtube and added with PBS. Extracellular antibodies used to stain the cells are FITC-conjugated rat anti-mouse CD11b. The sample was added with Cytofix and incubated in an ice box for 20 minutes. Washperm was added to the sample and incubated in an ice box for 20 minutes. Samples were centrifuged at 2500 rpm, 10ºC for 5 minutes. Intracellular antibodies (PE-anti-mouse IFN-γ and PE/Cy7-anti-mouse IL-10) were added to the pellet and incubated on an icebox for 20 minutes. The sample was added with 400 µl of PBS then transferred to flow cytometry cuvette for analysis by flow cytometry.

Data analysis

Samples were analyzed using BD CellQuest ProTM software. Statistical analysis was accomplished by SPSS 21.0 software, which included a normality test, homogeneity test, and one-way ANOVA parametric analysis. The significance between treatments was known by performing Duncan's Multiple Range test. The p-value of 0.05 was regarded as significant between the two groups. All data were shown in mean ± standard deviation (SD).

Results and Discussion

The flow cytometry results show that the relative amount of IL-10 in breast cancer mice is 0.78%, higher than in normal mice (Figure 1). The relative amount of IFN-γ in normal treatment is 2.16% and decreases to 1,26% in the breast cancer mice model (Figure 2). According to several studies, IL-10 levels are higher in cancerous conditions and associated with bad prognosis, whereas normal tissue samples did not detect IL-10 expression [14]. This research showed that administration of DMBA can inhibit M1-like macrophage polarization by decreasing the level of IFN-γ but increasing the polarization of M2 macrophages by increasing IL-10 secretion from M2 macrophages. DMBA (7, 12-Dimethylbenz(α)anthracene) is a carcinogenic compound from the PAH group used to make models of breast cancer in experimental animals. CYP1B1 metabolizes the DMBA compound and produces DMBA-3,4-epoxide, followed by a change from mEH to DMBA-3,4-dihydro diol, then changes to DMBA-3,4-dihydro-diol-1,2-epoxide (DMBA-DE) which has immunosuppressive properties [15]. The carcinogenicity of DMBA is known to be due to its mechanisms
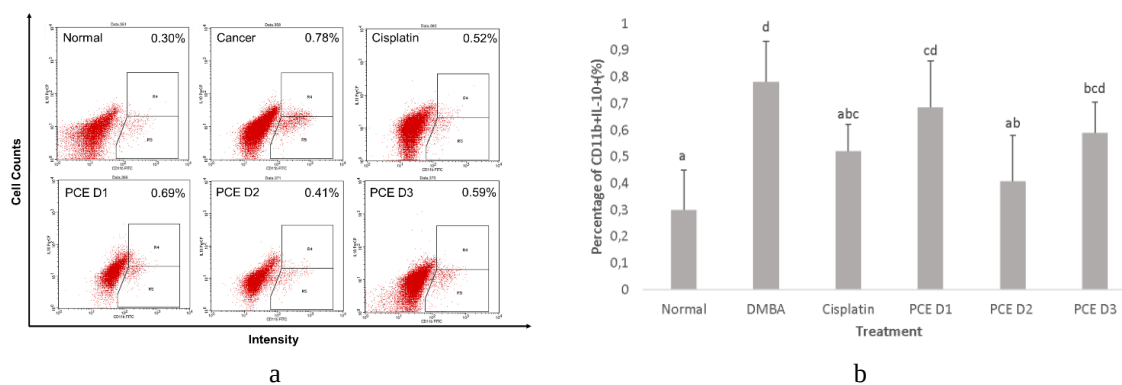

a   b

Figure 1. The relative number of IL-10 expressed by M2-macrophage in each mice group. (a) A plot of flowcytometry analysis and (b) Results of statistical analysis. The data are mean value ± standard deviation of four mice in each group with a significant value p < 0.05 (n=24).
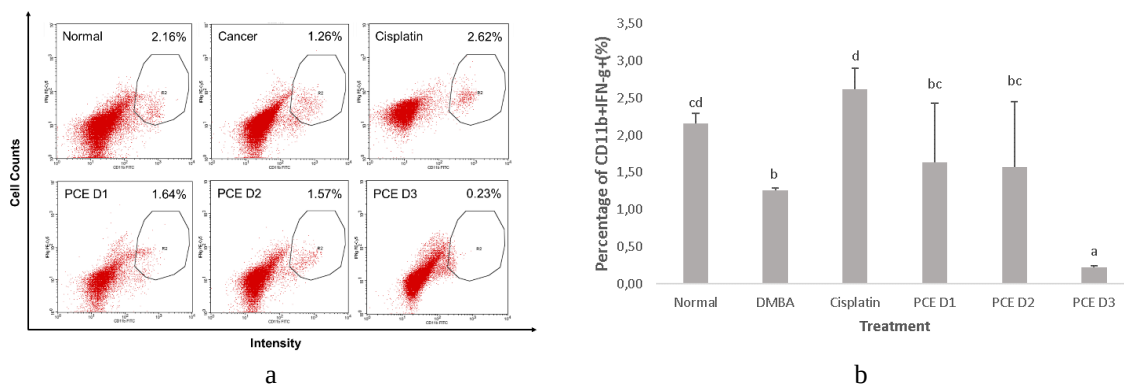
Figure 2. The relative number of IFN-γ expressed by M1-macrophage in each mice group. (a) A plot of flowcytometry analysis and (b) Results of statistical analysis. The data are mean value ± standard deviation of four mice in each group with a significant value $p < 0.05$ (n=24).

involved in DNA damage. Genes that play an essential role in cell growth and survival are damaged, causing mutations and an imbalance between cellular oxidants and antioxidants, which play an essential role in breast cancer development and progression [16].

In tumor conditions, immunocompetent cells will form a tumor microenvironment and interact with tumor cells, affecting initiation, growth, and metastasis in tumors [17]. The tumor microenvironment is a group of cells that support tumor cells during the transition to malignancy, and macrophages are most commonly found in the tumor microenvironment among innate and adaptive immune cells [18]. Macrophages play a role in initiating, maintaining, and resolving inflammation. Inflammation is a defense mechanism of the body against damaged tissue or invading pathogens. The activation and deactivation of macrophages are typically associated with an inflammatory response [3]. The activated macrophages are divided into two main classes; there are M1-type macrophages and M2-type macrophages. Classically activated M1 macrophages exhibit pro-inflammatory behavior by migrating to inflamed tissues, targeting pathogens with reactive oxygen species (ROS) production, and having high antigen-expressing potential. These macrophages can be potent effector cells that kill tumor cells and can recruit cytotoxic T lymphocytes (CTLs) to activate adaptive immune responses. M1-type macrophages are activated by IFN-γ and act as an anti-tumor by producing immunostimulatory cytokines such as IL-12 and IL-23 [19]. IFN-γ plays an essential role in inhibiting the proliferation of cancer cells by regulating p21 expression through STAT1 activation in tumor cells. Moreover, IFN-γ is essential in eliminating tumor cells, promoting tumor cell apoptosis, inducing tumoricidal effects, and inhibiting angiogenesis [20]. IFN-γ activates the JAK-STAT pathway, which triggers the expression of several genes, increasing immunogenicity and stimulating immunity [21]. On the opposite side of macrophage polarization, alternatively, activated M2 macrophages secrete anti-inflammatory cytokines to induce immune tolerance. M2 macrophages facilitate canonical tissue repair functions and, in cancer, are regarded as pro-tumor, where they promote tissue remodeling and repair, stimulate angiogenesis with VEGF, and promote tissue growth with TGF-β. IL-4 and IL-13 activate M2-like macrophages, which play a role in tumor initiation, proliferation, and metastasis and secrete anti-inflammatory cytokines such as IL-10 and TGF-β [3]. IL-10 is a cytokine that acts as an anti-inflammatory and has a role in the proliferation and metastasis of tumor cells. IL-10 secretion by macrophages inhibits the inflammatory response and causes immune system deregulation, allowing the tumor to escape [22].

IL-10 expression in breast cancer mice models decreased by up to 0.52% after cisplatin administration (Figure 1). This result differs from IFN-γ expression, which increases by up to 2.62% (Figure 2). This study showed that cisplatin injection significantly decreases anti-inflammatory cytokines and increases pro-inflammatory cytokines in breast cancer mouse models. The results of this study are related to previous studies, which have proved that cisplatin could decrease the level of IL-10 and increase pro-inflammatory cytokine [23]. Cisplatin is an anti-tumor agent that has long been used in cancer therapy. Cisplatin or cis-diamminedichloroplatinum (II) is an anti-cancer agent

known for treating various types of cancer. The interaction between cisplatin and DNA forms DNA adducts which cause DNA damage and induce apoptosis in cancer cells [24].

Administration of PCE extract increased IFN-γ activity for macrophage polarization to become M1 macrophages. IFN-γ expression in breast cancer model mice increased after PCE doses 1 and 2 administrations to 1.64% and 1.53%, respectively (Figure 2). IL-10 expression decreased after PCE doses 1, 2, and 3 administrations to 0.69%, 0.41%, and 0.59%, respectively. PCE dose 2 was the optimal dose for reducing IL-10 expression. The ability of PCE to reduce IL-10 expression and increase IFN-γ expression is due to secondary metabolites in both extracts. *P. niruri* is a medicinal plant that can treat several diseases, including breast cancer [9]. Previous studies have shown that *P. niruri* extract increases the activity of the cytokine IFN-γ release by Th1, causing activation of M1 macrophages and indicating inflammatory conditions. In addition, previous research also shows that *P. niruri* can increase the production of IFN-γ, IL-6, and TNF-α by increasing NO release [26]. The anti-cancer ability of *P. niruri* is due to secondary metabolites such as gallic acid, caffeoylquinic acid, quercetin, and phyllanthin. Previous research showed that *P. niruri* has anti-cancer activity by inhibiting angiogenesis and reducing the expression of IL-6, Il-17, and CXCL12. *P. niruri* inhibits angiogenesis by suppressing HIF-1α and VEGF expression [25]. *C. roseus* is a plant widely used for breast cancer therapy. Compounds in *C. roseus* that are known to have anti-cancer abilities are vincristine and vinblastine. Vincristine and vinblastine inhibit mitosis cell division and cause cell death or apoptosis [11]. The increase in IFN-γ in the PCE group could be due to the secondary metabolites in *P. niruri* and *C. roseus* that have good functions for the body. The increase in IFN-γ in the PCE group could be due to the secondary metabolites in *P. niruri* and *C. roseus* that have good functions for the body. Quercetin, one of the compounds found in *P. niruri* and *C. roseus*, has the ability to inhibit cancer cell growth and trigger apoptosis. [27]. *C. roseus* contains alkaloid compounds that play a role in cancer cell apoptosis through the NF-kB pathway [28]. Alkaloid compounds in the periwinkle plant can inhibit the proliferation of cancer cells by binding to tubulin in the mitotic spindle. In addition, alkaloids in *C. roseus* can also induce apoptosis in cancer cells [29].

Conclusion

DMBA injection can increase IL-10 expression and decrease IFN-γ expression in macrophages. The reduced expression of IL-10 and increased IFN-γ after administration of the *P. niruri* extract combination indicated that the combination of *P. niruri* and *C. roseus* extracts could suppress macrophage polarization to M2-type macrophages which acts as an anti-inflammatory and increase macrophage polarization to M1-type macrophages which has pro-inflammatory activity. The study concludes that *P. niruri* and *C. roseus* extracts have anti-cancer characteristics and can be used as an alternative for breast cancer treatment.

Acknowledgments

References

1. Sung H, Ferlay J, Siegel RL et al. (2021) Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians 71 (3): 209–249. doi: 10.3322/caac.21660.
2. Gelband H, Jha P, Sankaranarayanan R et al. (2015) Cancer. Washington, DC, The World Bank.
3. Boutilier AJ, Elsawa SF (2021) Macrophage Polarization States in the Tumor Microenvironment. International Journal of Molecular Sciences 22 (13): 6995. doi: 10.3390/ijms22136995.
4. Wynn TA, Chawla A, Pollard JW (2013) Macrophage biology in development, homeostasis and disease. Nature 496 (7446): 445–455. doi: 10.1038/nature12034.
5. Jorgovanovic D, Song M, Wang L, Zhang Y (2020) Roles of IFN-γ in tumor progression and regression: a review. Biomarker Research 8 (1): 49. doi: 10.1186/s40364-020-00228-x.
6. Müller E, Christopoulos PF, Halder S et al. (2017) Toll-Like Receptor Ligands and Interferon-γ Synergize for Induction of Antitumor M1 Macrophages. Frontiers in Immunology 8 (1383): 1–16. doi: 10.3389/fimmu.2017.01383.
7. Zhou J, Tang Z, Gao S et al. (2020) Tumor-Associated Macrophages: Recent Insights and Therapies. Frontiers in Oncology 10 (188): 1–13. doi: 10.3389/fonc.2020.00188.
8. Sheikhpour E, Noorbakhsh P, Foroughi E et al. (2018) A Survey on the Role of Interleukin-10 in Breast Cancer: A Narrative. Reports of Biochemistry & Molecular Biology 7 (1): 30–37.
9. Bagalkotkar G, Sagineedu SR, Saad MS, Stanslas J (2010) Phytochemicals from *Phyllanthus niruri* Linn. and their pharmacological properties: a review. Journal of

Pharmacy and Pharmacology 58 (12): 1559–1570. doi: 10.1211/jpp.58.12.0001.

10. Kaur N, Kaur B, Sirhindi G (2017) Phytochemistry and Pharmacology of *Phyllanthus niruri* L.: A Review: *Phyllanthus niruri* L. Phytotherapy Research 31 (7): 980–1004. doi: 10.1002/ptr.5825.

11. Kumar S, Singh B, Singh R (2022) Catharanthus roseus (L.) G. Don: A review of its ethnobotany, phytochemistry, ethnopharmacology and toxicities. Journal of Ethnopharmacology 284 (2022): 1–44. doi: 10.1016/j.jep.2021.114647.

12. Aslam J, Khan SH, Siddiqui ZH et al. (2010) Catharanthus roseus (L.) G. Don. An Important Drug: It's Applications and Production. Pharmacie Global 01 (4): 1–16.

13. Asare G, Addo P, Bugyei K et al. (2011) Acute toxicity studies of aqueous leaf extract of Phyllanthus niruri. Interdisciplinary Toxicology 4 (4): 206–210. doi: 10.2478/v10102-011-0031-9.

14. Bhattacharjee HK, Bansal VK, Nepal B et al. (2016) Is Interleukin 10 (IL10) Expression in Breast Cancer a Marker of Poor Prognosis? Indian Journal of Surgical Oncology 7 (3): 320–325. doi: 10.1007/s13193-016-0512-6.

15. Gao J, Mitchell LA, Lauer FT, Burchiel SW (2008) p53 and ATM/ATR Regulate 7,12-Dimethylbenz[*a*]anthracene-Induced Immunosuppression. Molecular Pharmacology 73 (1): 137–146. doi: 10.1124/mol.107.039230.

16. Youssef SSM, Ibrahim NK, El-Sonbaty SM, El-Din Ezz MK (2022) Rutin Suppresses DMBA Carcinogenesis in the Breast Through Modulating IL-6/NF-κB, SRC1/HSP90 and ER-α. Natural Product Communications 17 (9): 1–11. doi: 10.1177/1934578X221118213.

17. Teng F, Tian W-Y, Wang Y-M et al. (2016) Cancer-associated fibroblasts promote the progression of endometrial cancer via the SDF-1/CXCR4 axis. Journal of Hematology & Oncology 9 (1): 8. doi: 10.1186/s13045-015-0231-4.

18. Noy R, Pollard JW (2014) Tumor-Associated Macrophages: From Mechanisms to Therapy. Immunity 41 (1): 49–61. doi: 10.1016/j.immuni.2014.06.010.

19. Yang L, Zhang Y (2017) Tumor-associated macrophages: from basic research to clinical application. Journal of Hematology & Oncology 10 (1): 58. doi: 10.1186/s13045-017-0430-2.

20. Castro F, Cardoso AP, Gonçalves RM et al. (2018) Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. Frontiers in Immunology 9 (847): 1–19. doi: 10.3389/fimmu.2018.00847.

21. Mandai M, Hamanishi J, Abiko K et al. (2016) Dual Faces of IFNγ in Cancer Progression: A Role of PD-L1 Induction in the Determination of Pro- and Antitumor Immunity. Clinical Cancer Research 22 (10): 2329–2334. doi: 10.1158/1078-0432.CCR-16-0224.

22. Teresa Gonzalez-Garza M, Elva Cruz-Vega D, Maldonado-Bernal C (2021) IL10 as Cancer Biomarker. Transl Res Cancer. doi: 10.5772/intechopen.90806

23. Kumar P, Barua CC, Sulakhiya K, Sharma RK (2017) Curcumin Ameliorates Cisplatin-Induced Nephrotoxicity and Potentiates Its Anticancer Activity in SD Rats: Potential Role of Curcumin in Breast Cancer Chemotherapy. Front Pharmacol 8 (132): 1–12. doi: 10.3389/fphar.2017.00132

24. Aldossary SA (2019) Review on Pharmacology of Cisplatin: Clinical Use, Toxicity and Mechanism of Resistance of Cisplatin. Biomedical and Pharmacology Journal 12 (1): 07–15. doi: 10.13005/bpj/1608.

25. Ramadhani A, Ahkam A, Suharto A et al. (2021) Suppression of hypoxia and inflammatory pathways by Phyllanthus niruri extract inhibits angiogenesis in DMBA-induced breast cancer mice. Research in Pharmaceutical Sciences 16 (2): 217. doi: 10.4103/1735-5362.310528.

26. Jantan I, Haque MdA, Ilangkovan M, Arshad L (2019) An Insight Into the Modulatory Effects and Mechanisms of Action of Phyllanthus Species and Their Bioactive Metabolites on the Immune System. Frontiers in Pharmacology 10 (878): 1–19. doi: 10.3389/fphar.2019.00878.

27. Rauf A, Imran M, Khan IA et al. (2018) Anticancer potential of quercetin: A comprehensive review: Quercetin as an anticancer agent. Phytotherapy Research 32 (11): 2109–2130. doi: 10.1002/ptr.6155.

28. Widowati W, Mozef T, Risdian C et al. (2010) Apoptosis and Antioxidant Activities of Catharanthus rosues [L] G.Don Extract on Breast Cancer Cell Line. Indonesian Journal of Cancer Chemoprevention 1 (2): 99. doi: 10.14499/indonesianjcanchemoprev1iss2pp99-107.

29. Arora R (2010) Herbal Medicine: A Cancer Chemopreventive and Therapeutic Perspective. doi: 10.5005/jp/books/11166.