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Research Article
Effect of Papaya Leaves (*Carica papaya* L.) Extract on Immune Response (TLR-7, TLR-9) and Inflammation (COX-2) in Rats Induces DMBA (7,12-Dimethylbenz[a]antrasen)

[1]Fatma Zuhrotun Nisa, [2]Mary Astuti, [1]Sofia Mubarika Haryana and [2]Agnes Murdiati

[1]University of Gadjah Mada, Faculty of Medicine, Jl. Farmako Sekip Utara Yogyakarta, 55281, Indonesia
[2]University of Gadjah Mada, Faculty of Agriculture Technology, Jl Flora No 1 Yogyakarta, 55281, Indonesia

Abstract

Background and Objective: TLR is known to regulate the immune system in cancer. TLR-7 and TLR-9 can enhance the antitumor immune system in many types of solid tumors. Cyclooxygenase-2 (COX-2) is a biomarker of inflammation. This study aimed to investigate the effect of papaya leaves extract on immune response (TLR 7, TLR 9) and inflammation (COX-2) in rats induced DMBA. **Materials and Methods:** This experimental study used *Sprague dawley* female rats of age more less 50 days. Rats were divided into 4 groups: Negative Control (NC), Positive Control (PC), Cancer Drug Doxorubicin (DOXO) and Papaya Leaves Extract (PLE). The study was conducted for 13 weeks. DMBA induction performed for 5 weeks with administration of 2 times per week. **Results:** the expression of TLR-7 of PLE and DOXO was higher than PC groups significantly different ($p<0.05$). The expression of TLR-9 of PLE was higher than NC, PC and DOXO groups but not significantly different ($p>0.05$) while the expression of COX-2 of PLE and DOXO groups was lower than NC and PC groups but not significantly different ($p>0.05$). **Conclusion:** It can be concluded that papaya leaves extract can improve the immune system and reduce inflammation. It shows that papaya leaves extract has potent as anti-cancer.

Key words: Papaya leaves, tool like receptor (TLR), cyclooxygenase (COX-2), immune response, inflammation

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Corresponding Author: Fatma Zuhrotun Nisa, University of Gadjah Mada, Faculty of Medicine, Jl. Farmako Sekip Utara Yogyakarta, 55281, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Toll-like receptors (TLRs), as the most important pattern recognition receptors in innate immunity, play a pivotal role in inducing immune response through recognition of microbial invaders or specific agonists. Accumulating evidence indicated that the expression of TLRs on tumor cells, which is known to mediate innate immune response, influences the proliferation and migration of tumor cells. Toll-like receptor itself also has the potential to be a therapeutic target against cancer[1]. Several TLR agonists have been demonstrated to produce antitumor effects. TLR-7 and TLR-9 agonists, nucleic acid analogues improve antitumor immunity in many solid tumor types and are currently in clinical trials[2].

One mechanism of modern cancer treatment is based on the theory of the hallmark of cancer is to improve the immune system. The immune system is a defense system that protects the body against invading organisms and cancer cells[3]. The body's defense system consists of a humoral and cellular immune system. Humoral immune system mediated by antibody molecules that secreted by plasma cells[4]. TLR associated with natural and adaptive immune responses and have a significant role in the immune system in cancer[5]. Battacharya and Yusuf[6] reviewing that TLR is known to regulate the immune system in cancer by controlling the suppressive function of Treg cells (regulatory T) and through the natural immune responses mediated by other immune cells. TLR-7 and TLR-9 can enhance the antitumor immune system in many types of solid tumors[2].

Dynamic interaction between the tumor and the immune system is important for the survival of tumor growth and metastasis[7]. The development of invasive cancer is the result of genetic and epigenetic changes in the body[8]. The adaptive immune system is very important to eliminate pathogens and tumor cells in the final phase of the host immune response and produce more tumor-specific immunity[9]. TLR is known to regulate the immune system of cancer by controlling the suppressive function of Treg cells and through the mediation of the natural immune response by immune cells[9,10,11]. TLR activates dendritic cells and macrophages to secrete IL-12 that is an adaptive immune response cytokine produced by Th1 cells[12].

Inflammation has been associated with the development of cancer. Cyclooxygenase-2 (COX-2) is one of the biomarkers of inflammation. Increased levels of mRNA and cyclooxygenase-2 (COX-2) protein are found in breast cancer cells[13]. The tumor tissue induced by COX-2 appears to have decreased levels of proapoptotic proteins (Bax and Bcl-xL) and increased Bcl-2 anti-apoptotic proteins. This suggests that decreased apoptosis of breast epithelial cells contributes to tumorigenesis[14]. The previous study showed that papaya leaves extract can inhibit proliferative of breast cancer cell MCF-7 *in vitro*[15]. There is no study about the effect of papaya leaves extract on immune response and inflammation *in vivo*. Furthermore, This study investigated the effect of papaya leaves extract on immune response (TLR-7, TLR-9) and inflammation (COX-2) in rats induced DMBA.

MATERIALS AND METHODS

Papaya leaves extract: Papaya leaves dried in oven 60°C for 3 hrs 3 times then milled. One gram of dried papaya leaves milled into 20 mL of solvent and stirred. The solvents used in this study were methanol, 70% ethanol and water. The microwave extraction procedure is to set heat percentage of 50% then the sample solution is placed into the microwave and then extracted by setting 4 sec on and 60 sec off three times. After that filtering is done using filter paper then stored in the refrigerator for further testing.

***In vivo* study:** This study was conducted in PAU laboratory Gadjah Mada University from January to May 2017. This experimental study used *Sprague Dawley* (SD) female rats. The age of rats used ±1 month and administered DMBA done at the age of rats reached ±50 days. Twelve female rats were divided into 4 groups consist of Negative Control (NC), Positive Control (PC), Doxorubicin (DOXO) and Papaya Leaves Extract (PLE) groups. The NC group was given standard feeding and drinking water, PC group was given standard feeding and DMBA (7.12-dimethylbenz (a) anthracene), DOXO group was given standard feeding, DMBA and breast cancer drug that is doxorubicin, PLE group was given standard, DMBA and papaya leaf extract at doses 1.319 μg mL^{-1}. The dose of papaya leaf extract based on IC_{50} dose at previous study[16]. The study was conducted for 13 weeks and before research rats were adapted for 3 days using standard AIN-93 feed. DMBA induction is administered for 10 times, twice a week. DMBA 20 mg kg^{-1} weight gain was administered in a solution in corn oil with a concentration of 4 mg mL^{-1}. The analysis was performed at the end of week 13. Parameters are TLR-7, TLR-9 and COX-2 in plasma. The treatment was given by force-feeding. Blood sampling has taken from retro-orbital plexus.

Preparation of blood sample: The preparation of blood samples was done by centrifuging blood at a rate of 3000 rpm for 15 min. The serum was taken and transferred into an Eppendorf tube and stored at -40°C until analysis.

Analysis of TLR-7, TLR-9 and COX-2: The expression of TLR-7, TLR-9 and COX-2 of rat serum were measured using ELISA with a kit from the fine test. The procedure of analysis was explained in the kit document. Before analysis, the kit standard was checked to know is the kit is good or not.

Statistical analysis: Data were analyzed using ANOVA with Duncan's test to analyze the significant difference between treatments.

RESULTS AND DISCUSSION

The results showed that papaya leaf extract could enhance the adaptive immune response indicated by the expression of TLR-7 and TLR-9. The expression of TLR-7 and TLR-9 may be due to flavonoids contained in papaya leaf extract. The results showed that the expression of TLR-7 of PLE and DOXO was higher and significantly different ($p<0.05$) compared with PC group. The TLR-7 expression of PLE, NC and DOXO were not significantly different ($p>0.05$) Fig. 1. Papaya leaf extract flavonoids may activate TLR-7 and TLR-9 because flavonoids have been reported as immunomodulator[15]. Edewor[16] reviewed that the immunological potential of bioactive compounds flavonoids and flavonoid content in plants. Otsuki *et al.*[18] reported that papaya leaf extract has the potential to modulate the immune system by mediating changes Th1 in the human immune system, it indicates that papaya leaf extract has the potential for treatment and prevention of several diseases such as cancer, allergies and can also serve as immunoadjuvant for therapy vaccine. Immunomodulator potential of the flavonoid extract of *S. ocymastrum* on macrophage phagocytosis showed that the extracts have immunostimulator effects at low concentrations and has immunosuppressive effects at higher concentrations[19]. Flavonoids of *Oldenlandia diffusa* can prevent acute ulcerative colitis in rat through inhibition mechanism of activation of NF-kB p65, a decrease in IL-8, TNF-α expression and increased anti-inflammatory factor IL-10[20].

It was observed that the expression of TLR-9 of papaya leaf extract was higher than NC, PC and DOXO groups but not significantly different ($p>0.05$) Fig. 2.

All TLR were synthesized in endoplasmic reticulum then into the golgi apparatus and outputted to the cell surface or intracellular compartments such as endosomes. Localization of intracellular TLR is very important for the acceptance of the ligand and to prevent TLR entry and contact with their nucleic acids can cause autoimmune[21-24]. Multi-pass trans-membrane protein or UNC93B1 control intracellular TLR journey from the endosome to endoplasmic reticulum. Interestingly, UNC93B1 regulates excess activation of TLR-7 with the use of TLR-9 against TLR7. This is shown by experiments on mice that store amino acid substitution (D34A) in UNC93B1, which shows hyperactive TLR-7 and hypoactive TLR-9. Thus, optimizing the balance of TLR-7 and TLR-9 is a potential mechanism to

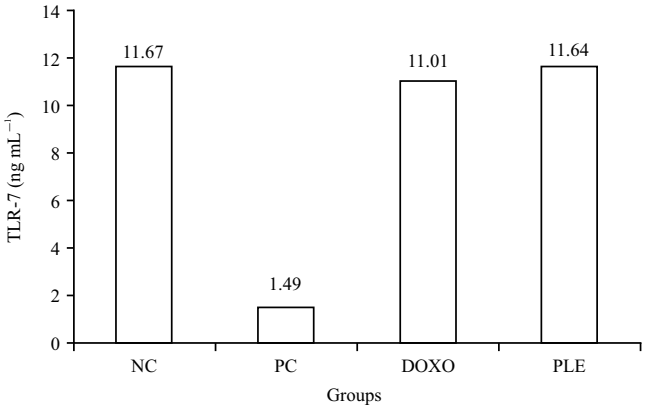

Fig. 1: Expression of Toll Like Receptor-7 (TLR-7) rat plasma-induced DMBA

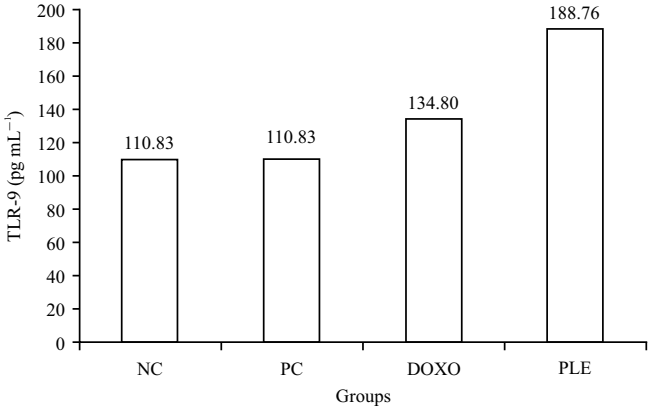

Fig. 2: Expression of Toll Like Receptor-9 (TLR-9) rat plasma-induced DMBA

regulate the autoimmune[25]. TLR trip is also controlled by PRAT4A protein that regulates the release of TLR-1, TLR-2, TLR-4, TLR-7 and TLR-9 from endoplasmic reticulum and trips to the plasma membrane and endosome[26].

Some TLR can induce antitumor activity by regulating the function of immune cells that infiltrated the tumor microenvironment. Has conducted clinical trials of new anticancer therapies based on the administration of TLR ligands that are agonists[27]. Example of TLR ligands is imiquimod. Provision of imiquimod 5% cream in 90 patients with basal cell carcinoma resulted in a cure rate of 96% and only two have relapsed during the study and no systemic side effects[28]. TLR-7 is activated by several low molecular weight compounds including imiquimod, which have anti-tumor properties that can induce cytokines IFN α, IFN-γ and IL-12, activate tumor associated antigen (TAA), specific cytotoxic T lymphocytes (CTL) and activate myeloid dendritic cells[29,30,31].

Activation of TLR-9 by using synthetic agonists is considered a useful mechanism to against cancer[32]. CpG-ODN has been developed and explored for the treatment of hematological and solid tumors. CpG-ODN is a TLR-9 agonist can stimulate the activation and maturation of cells dendritic, increasing the differentiation of B cells into antibody plasma cells and promote the development of anti-tumor T cells[33]. In a murine model of human ovarian cancer, intraperitoneal administration of CpG-ODN resulted in anti-tumor effects of intravenous administration[34]. Early clinical trials are being carried out research on the safety and efficacy of TLR-9 agonists for the treatment of breast cancer, colorectal cancer, lung cancer, melanoma, glioblastoma and some lymphomas and leukemia[35]. Activation of TLR signaling plays an important role against inflammation and immunity. TLR has three functions of the immune response that are identify pathogens, initiate innate response directly and stimulate the adaptive immune response is in accordance with the pathogen[36]. Activation of TLR-9 signaling in dendritic cells can induce the production of IFN-α[37,38] and tumor necrosis factor α (TNF-α)[39], promoting migration leukocytes from the bloodstream to the site of infection. IFN-α and TNF-α also perform effector functions of innate immunity by stimulating the synthesis of antimicrobial peptides and cytokines and activate phagocytosis in macrophages[40].

The mechanism of increased expression of TLR-7 and TLR-9 by flavonoids papaya leaf extract may be through several approaches: (1) The flavonoid compound papaya leaf extract acts as an agitated TLR ligand as an antitumor by inducing IFN-α, IFN-γ and IL-12 cytokines and activates tumor associated antigen (TAA) -specific cytotoxic T lymphocytes (CTL) (2) flavonoid of papaya leaf extract as TLR ligand agonist induce of activation and maturation dendritic cells, increase cell differentiation B into plasma cell antibodies and promote anti-tumor development; (3) flavonoid compounds can activate TLR signaling. Activation of TLR signaling plays an important role in inflammation and immunity to identify pathogens, stimulate innate immune responses and stimulate adaptive immune responses compatible with pathogens.

Figure 3 showed that the expression of COX-2 papaya leaf extract and DOXO group was lower than NC and PC. However, there was no significant difference ($p>0.05$) between treatment groups.

The results of this study indicate that papaya leaf extract can decrease inflammation as indicated by low COX-2 expression. The ability of papaya leaf extract in reducing inflammation is probably caused by flavonoid compounds of quercetin contained in papaya leaf extract. Previous research

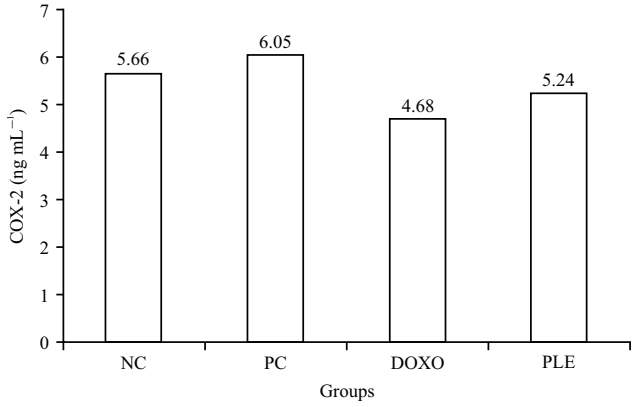

Fig. 3: Expression of Cyclooxygenase-2 (COX-2) rat plasma-induced DMBA

has proved that quercetin is an antiproliferative compound against cell line MCF-7 through activation of Adenosine monophosphate-activated protein kinase (AMPK) resulting in COX-2 expression barrier[41]. The results of Herwiyati[42] showed that 1,2-Epoxy-3 (3- (3,4-dimethoxyphenyl) -4H-1-benzopyran-4-one) propane induced on cell line MCF-7 and T47D had expression of COX-2 lower than control. Tocopherols may inhibit COX-2 activity whereas quercetin and some quercetin conjugates affect transcription and COX-2 activity[43].

CONCLUSION

Based on the results of this study show that papaya leaves extract can increase the expression of TLR-7 and TLR-9 while the expression of COX-2 of papaya leaves extract decrease in rats induced-DMBA. It means that the papaya leaves extract can increase immune response and decrease inflammation. This research give solution for people that papaya leaf extract have potent as anti-cancer by an increased immune response and decreased inflammation. This extract good to prevent cancer but still need the research to prove that papaya leaves extract can prevent cancer in human.

SIGNIFICANCE STATEMENT

This study discovered the effect of papaya leaves extract on immune response and inflammation in rat induced DMBA that can be beneficial for the development of knowledge about cancer therapy by functional food. This study will help the researchers to uncover the critical areas of the mechanism of the increased TLR-7, TLR-9 and the decreased COX-2 by functional food that many researchers were not able to

explore. Thus a new theory on papaya leaves extract can increase immune response and decrease inflammation may be arrived at.

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