

RESEARCH ARTICLE

Immunomodulatory Effects of Hexane Insoluble Fraction of *Ficus septica* Burm. F. in Doxorubicin-treated Rats

Agung Endro Nugroho^{1,3*}, Adam Hermawan^{1,2}, Kunti Nastiti³, Suven³, Pritha Elisa³, Tony Hadibarata⁴, Edy Meiyanto^{1,2}

Abstract

The use of chemotherapeutics induces cardiotoxicity and affects immune functions, therefore development of combinatorial agents against cardiotoxicity and immunosuppression needs to be explored. Previous studies of the hexane insoluble fraction (HIF) of an ethanolic extract of *Ficus septica* leaves showed anticancer effects singly and in combination with doxorubicin on T47D breast cancer cells. In this present study, it was evaluated for its immunomodulatory activities in doxorubicin-treated rats. Thirty male Sprague Dawley rats were divided into five groups consisting of six rats each as follows: Group 1, receiving oral saline 10 ml/kg BW (control group); Group 2, receiving HIF dose 750 mg/kg BW orally, once daily; Group 3, receiving HIF dose 1.500 mg/kg BW orally, once daily; Group 4, given oral saline 10 ml/kg BW (normal group); Group 5, receiving HIF dose 1.500 mg/kg BW orally, once daily. The rats of group 1-3 were intramuscularly administered with doxorubicin at a dose of 4.67 mg/kg BW at the days 1 and 4 to suppress immune functions. Concomitantly, the rats were treated with saline or HIF for seven consecutive days (1 to 7). Treatment of HIF succeeded in reducing side effects of doxorubicin based on increasing lymphocyte density and phagocytosis activity and capacity of macrophages, as well as increasing the CD8+ blood level and decreasing spleen IL-10 expression. Hexane insoluble fraction of ethanolic extract of *Ficus septica* leaves has potential as a protective agent combined with doxorubicin.

Keywords: *Ficus septica* Burm. F. - hexane insoluble fraction - doxorubicin - immunomodulator - in vivo

Asian Pacific J Cancer Prev, **13** (11), 5785-5790

Introduction

Cancer is a public health problem in the world and a major cause of death in the world. Around 13% of all deaths due cancer occurred in 2008, and more than 70% of all death due to cancer occurred in developing countries (WHO, 2011). The disease is associated with uncontrolled rapid growth of abnormal cells in the body. They have the capacity to invade the healthy surrounding tissue and spread to other organs, known as metastasis (Hanahan and Weinberg, 2011).

There are several approaches used for treating cancer including chemotherapy. One of the most popular chemotherapeutics is doxorubicin (Rang et al., 2003; Tan et al., 2009). However, its clinical use is limited due to its side effects in high- and repeated-doses. The use of the drug induced cardiotoxicity and affected the immune functions (Santos et al., 2010; Bowles et al., 2012).

Reportedly, doxorubicin suppressed the production of IL-2 and INF-gamma, NK cell cytotoxicity, lymphocyte proliferation and CD4+/CD8+ ratio in tumour-bearing mice (Zhang et al., 2005a). Administration of doxorubicin on rats also increase production of pro inflammatory

cytokines (Santos et al., 2010). Immunological study on advanced breast cancer patients treated with doxorubicin showed decreasing plasma level of IL-1, IL-10, and TNF-alpha (Panis et al., 2012). The use of chemotherapy is often combined with an immunostimulatory agent in order to protect and enhance the patients immune functions during chemotherapeutics administration. The agent combined with chemotherapeutic agents to produce better effects is named co-chemotherapeutic agent.

Our research group named Cancer Chemoprevention Research Center (CCRC) has selected several Indonesian plants for their anticancer activity, one of them is *Ficus septica* Burm. F.. In the previous study, ethanolic extract of *F. septica*. showed a cytotoxic effect on MCF-7 and T47D cells with IC₅₀ value of 13 and 6 µg/mL, respectively (Mubarak et al., 2008; Pratama et al., 2011). The extract not only showed a synergistic effect in combination with doxorubicin (3.75 nM), but also induced apoptosis and downregulated the expression of Bcl-2 protein in breast cancer cells MCF-7 (Seki et al., 2010). In 7,12-dimethylbenz[a]anthracene-induced rat, the ethanolic extract at 750 mg/kg BW succeeded to induce apoptosis through p53-independent pathway in liver

¹Cancer Chemoprevention Research Center, ²Department of Pharmaceutical Chemistry, ³Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Jogjakarta, Indonesia, ⁴Institute of Environmental and Water Resource Management, Universiti Teknologi, Skudai, Johor, Malaysia *For correspondence: agungendronugroho@yahoo.com, nugroho_ae@ugm.ac.id

cancer (Septhea et al., 2011).

Ethanollic extract of *F. septica* leaves was gradually fractionated to provide several fractions. Among them, hexane insoluble fraction has showed most potent cytotoxic effect on T47D cell lines (IC₅₀ value : 9 µg/mL) and considered to be the active fraction (Nugroho et al., 2011). The fraction synergically increases the cytotoxic effect of doxorubicin through apoptosis, decrease of PARP expression and cell cyce arrest (Nugroho et al., 2012). Therefore, the fraction is potential to be developed as co-chemotherapeutic agent for doxorubicin in breast cancer therapy. In the present study, the fraction was evaluated for its immunomodulatory activities in doxorubicin-treated rats.

Materials and Methods

Plant material

Ficus septica Burm. f. leaves were collected from area around Sumber Arum Moyudan, Sleman, Yogyakarta, Indonesia. The plant was authenticated by a taxonomist at Department of Pharmaceutical Biology, Universitas Gadjah Mada, Indonesia. The voucher specimen was deposited in herbarium of the department.

Preparation of hexane insoluble fraction (HIF)

The fraction was prepared according to the previous study (Nugroho et al., 2011). Fresh leaves were washed thoroughly with distilled water. The leaves were then air dried followed by oven drying at 60 °C. The dried leaves were ground and weighed. Subsequently, the dried ground powder was extracted using ethanol 70% with a ratio of 1:5 for 72 hours. The filtrate was collected, and the sediment was re-extracted twice. All filtrates were collected and then evaporated under reduced pressure to provide a viscous ethanolic extract. The extract was diluted with 100 mL aquadest to provide a liquid form of that extract. The extract was fractionated with *n*-hexane at a ratio of 1:3 (extract:*n*-hexane) yielding *n*-hexane soluble fraction and insoluble fraction of *n*-hexane. The insoluble fraction was then concentrated by rotary vacuum evaporator at 50 °C (Heidolph Instrument GmbH&Co., Germany) to obtain a viscous extract. The fraction was dried using a Freeze dryer ALPHA 1-2 LD plus (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) in order to eliminate the existence of the remaining traces of water.

Animals

Wistar rats (weighing 100-150 g) were housed at a constant temperature (25±2°C) with a constant relative humidity (60±10%) on the an automatically controlled 12:12 h light-dark cycle (light on at 7:00 a.m.). Rats were fed with standard rat chow and water as libitum. The rats were acclimatized and quarantined for at least one week prior to the experiment.

The animal handling protocols of this study were in accordance with the guidelines of the animal care of the Department of Pharmacology and Clinical Pharmacy, the Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia.

Experimental design

Thirty male Sprague Dawley rats were divided into five groups consisting of six rats each as follows: Group 1. the rats received oral saline 10 ml/kg BW (control group). Group 2. the rats received HIF dose 750 mg/kg BW orally, once daily. Group 3. the rats received HIF dose 1.500 mg/kg BW orally, once daily. The rats of group 1-3 were intramuscularly administered with doxorubicin (Ebewe, obtained from P.T. Ferron Par Pharmaceutical Cikarang, Indonesia) dose 4.67 mg/kg BW at the day 1 and 4 to suppress the immune functions. Concomitantly, the rats were treated saline or HIF for seven consecutive days (day 1 to day 7). Group 4. the rats received oral saline 10 ml/kg BW (normal group). Group 5. the rats received HIF dose 1.500 mg/kg BW orally, once daily. The rats of group 4-5 were not treated with doxorubicin. The treatment of saline or HIF were administered for seven consecutive days. Body weight was monitored regularly.

Lymphocyte proliferation assay

Rats were sacrificed by decapitation at the day 7. Afterwards, the peritoneal cavity was opened carefully, and the spleen quickly removed and placed in a tube containing RPMI medium to obtain a single cell suspension. The cell was then centrifuged at 1500 rpm, 4°C for 10 min. The pellets were collected and suspended in 2 ml of Tris Buffered Ammonium Chloride (8.3 g/L ammonium chloride in 0.01 M Tris-HCl buffer pH 7.65) to lyse erythrocytes. The cells were mixed gently using a pipette and allowed to stand at room temperature for 5 minutes. Subsequently, the cells were added with 1 ml of Fetal Bovine Serum (FBS) (Gibco[®] BRL, USA). The suspension was then centrifuged at 1500 rpm, 4°C for 5 min, and the supernatant was discarded. The collected-pellets were washed with RPMI twice, and centrifuged. The cell (pellet) was then resuspended with complete medium. Cell density was calculated using hemocytometer, and its viability was determined by trypan blue. The cells were seeded into 24-well culture plates at a density of 10⁶ cells /0.4 ml per each well. The cells were incubated overnight at 37 °C for 24, 48, and 72 hours. Proliferative activity was measured by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (Sigma Chemical, St Louis, MO) test splenocyte assay. MTT were added to each well and then incubated for 4 h and then added stopper and λ Elisa read at 550 nm.

Macrophage phagocytosis

At the day 7, rats were sacrificed by decapitation. Macrophages were isolated by injection of cold RPMI-1640 (Sigma Chem., MO, USA) BSA into the peritoneal cavity and the abdomen was massaged for about 3 min. The peritoneal cavity was opened carefully, and the fluid containing macrophages was collected. The fluid was then centrifuged at 1,200 rpm, 40°C for 10 minutes. The supernatant was discarded and then resuspended with 3 ml of complete RPMI medium containing FBS. The non-specific phagocytosis activity of macrophages was evaluated based on the method of Pratten and Lloyd (1984) with a diameter of 3.0 µm latex beads (Sigma Chem., MO., USA). Macrophage cells were observed under light

microscope for counting the number of phagocytic activity of macrophages to latex bead particles and the number of latex beads phagocytosed by the macrophages.

Determination of CD8+ lymphocytes

At day 0 (basal value) and 7, the blood samples were collected from retro-orbital plexus of the rats. The rats were anaesthetized using sodium pentobarbital (75 mg/kg) during blood collections. Sample preparation was performed by mixing 5 µl blood sample, 10 µL reagent rat antigen CD3 fluorescein isothiocyanate (FITC) (Invitrogen) and phycoerythrin (PE) anti-rat CD8+ (eBioscience) incubated at 5°C for 1 h. After shaking gently, the solution was stored in a dark room for 15 minutes. Subsequently, the mixture was added with 4,500 µl lysing solution (BP FACS) for dilution, and vortex, then allowed to stand in a dark room for 15 minutes. The cells were analyzed with a flow cytometer (FACS Calibur, BD) equipped with an argon laser (488 nm excitation) and appropriate filter settings for observation of FITC fluorescence. The data was the percentage of each type of fluorescent cells representing the relative to a number of CD8+ T cell lymphocyte.

Immunohistochemical study of IL10

The spleens were fixed with 4% formaldehyde in phosphate-buffered saline (PBS), and gradually dehydrated using a series of increasing alcohol concentrations. Subsequently, the tissues were cleared using clearing agents (xylol), and then embedded in paraffine wax prior to subsequent sectioning. Tissue sections 2-3 µm in thickness were mounted on glass slides. After washing in phosphate-buffered saline (PBS), the activity of endogenous peroxidase was blocked using 3% H₂O₂ in methanol for 15 min, and then washed with aquadest. Subsequently, the sections were incubated with 20% horse serum for at least 10 min to prevent non-specific binding. The sections were then incubated with Biotinylated rat anti-mouse monoclonal antibodies to IL-10 (primary antibodies) at a 1:250 dilution for one hour at room temperature. The sections were then incubated with peroxidase-conjugated secondary antibody at a 1:500 dilution for one hour at room temperature. Expression of IL-10 would be visualized after incubation with substrate 3,3'-diaminobenzidine (DAB, Novo Castra) dye for 15 min. The sections were counterstained with hematoxylin, and then mounted in a mounting medium (Sigma). Expression of IL-10 was observed under light microscope.

Statistical analysis

All data were presented as mean±the standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test was used for statistical analysis to compare more than two groups. P-values of less than 0.05 were considered significant.

Results

Effect of HIF on lymphocyte proliferation

In the study, the administration of doxorubicin dose

4.67 mg/kg BW at the day 1 and 4 succeeded to inhibit the proliferation of lymphocytes by 25.77±4.56% in comparison to that of control group. Treatment of HIF for seven consecutive days in absence of doxorubicin did not alter the lymphocytes proliferation significantly. Treatment of HIF for seven consecutive days concomitantly with doxorubicin at the day 1 and 4 could restore the decrease of lymphocytes proliferation due to doxorubicin administration in concentration-dependent manner (Table 1). Treatment of HIF doses 750 and 1,500 mg/kg BW succeeded to restore the lymphocytes densities of doxorubicin single treatment group by 25.59% and 52.26%, respectively.

Effect of HIF on macrophage phagocytosis

The effect of HIF on the phagocytosis of macrophage was evaluated using non-specific phagocytosis activity on latex beads (Pratten and Lloyd, 1984). In addition, the macrophage capacity was also observed. Macrophage functions in non-specific defence (innate immunity) and specific defence (adaptive immunity) in vertebrate animals (Rang et al., 2003; Shibata and Glass, 2009; Shalhoub et al., 2011). Those cells have a main role in immun system as phagocytic cells. In the study, doxorubicin (4.67 mg/kg BW) administration suppressed phagocytosis activity and capacity of macrophages by 27.27±4.21% and 35.67±3.89%, respectively (Table 2). Single treatment of HIF (without doxorubicin administration) did not influence the phagocytosis activity and capacity of macrophages. Treatment of HIF 1,500 mg/kg BW for seven consecutive days concomitantly with doxorubicin

Table 1. Effect of *n*-hexane Insoluble Fraction (HIF) of *F. septica* on Lymphocyte Proliferation in Doxorubicin-induced Rats

Group	Treatment	Lymphocytes density (% control) (Mean±SEM)
I.	Doxorubicin	74.23± 3.32#
II.	HIF 750 mg/kg+doxorubicin	93.00±13.47
III.	HIF 1,500 mg/kg+doxorubicin	113.02±10.21*
IV.	HIF 1,500 mg/kg	104.51± 5.57
V.	Saline (control)	100.00± 7.88

*Significant difference (P<0.05) compared to the doxorubicin group value. #Significant difference (P<0.05) compared to the control (saline) value. Data were expressed as Mean±SD (n=4-6 rats)

Table 2. Effect of *n*-hexane Insoluble Fraction (HIF) of *F. septica* on Phagocytic of Latex Beads by Rat Peritoneal Macrophages in Doxorubicin-induced Rats

Group	Treatment	Percentage of macrophage that phagocytose the beads (= macrophages capacity) (Mean±SEM)	Number of latex beads phagocytosed by 100 macrophage (= phagocytosis activity) (Mean±SEM)
I.	Doxorubicin	46.93±4.96#	84.71±6.03#
II.	HIF 750 mg/kg + doxorubicin	40.56±4.18	70.85±12.87
III.	HIF 1,500 mg/kg + doxorubicin	64.35±2.95*	136.71±4.56*
IV.	HIF 1,500 mg/kg	62.36±4.56*	148.07±6.56*
V.	Saline (control)	64.53±5.20	131.66±10.74

*Significant difference (P<0.05) compared to the doxorubicin group value. #Significant difference (P<0.05) compared to the control (saline) value. Data were expressed as Mean±SD (n=4-6 rats)

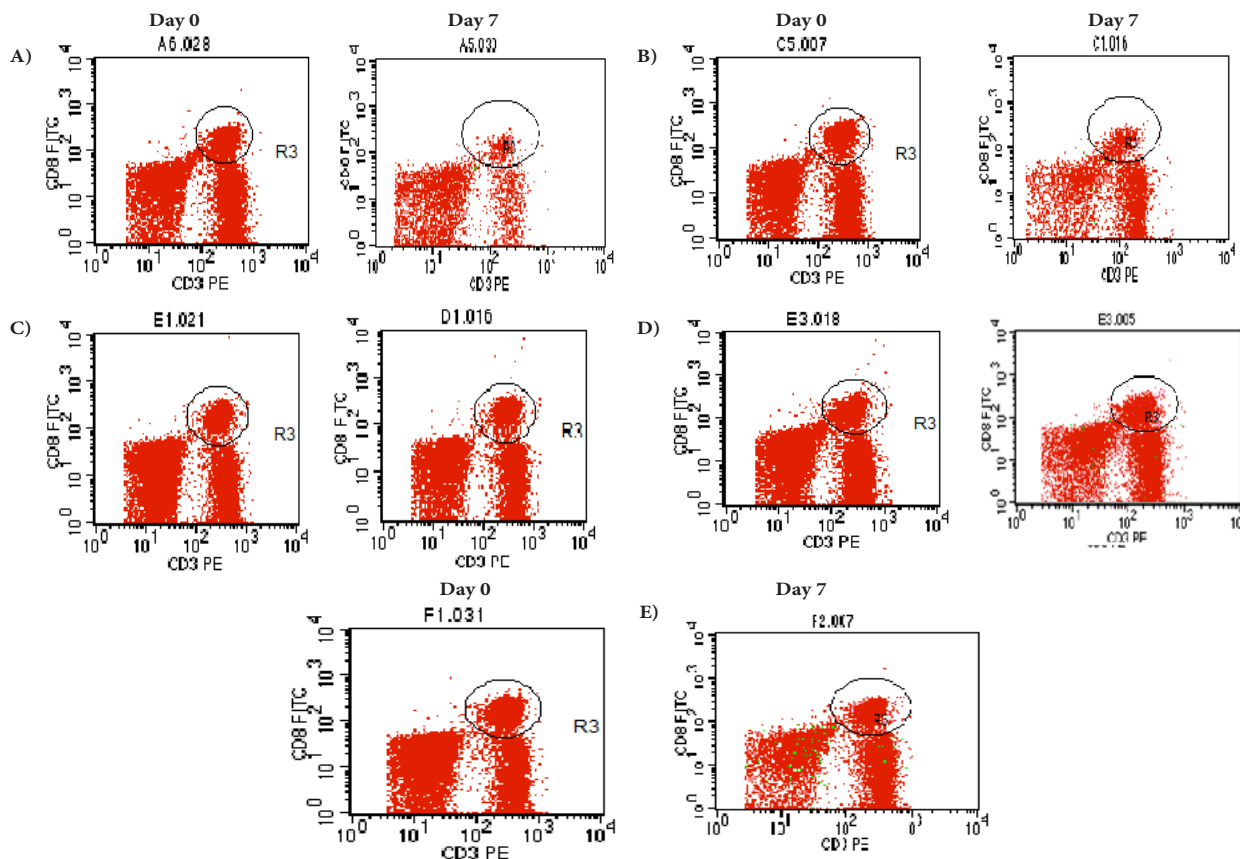


Figure 1. CD8+ Analysis using Flow Cytometry. The analysis was performed at day 0 (pre-treatment) and the day-7 (post-treatment) on doxorubicin-treated rats with administration of saline (A), HIF 750 mg/kg BW (B), or HIF 1,500 mg/kg BW (C), or on untreated rats with administration of HIF 1500 mg/kg BW (D) or saline (E)

succeeded to restore the decrease of phagocytosis activity and capacity of macrophages to be the same value as control group.

Effect of HIF on CD8+ lymphocytes

CD8+ lymphocytes is a T lymphocyte that attack cancer cells, infected cells and damaged cells (DeNardo et al., 2010). In the study, HIF was evaluated for its effect on CD8+ profile using a FACS flowcytometer. Doxorubicin (4.67 mg/kg BW) administration at the day 1 and 4 markedly suppressed CD8+ by 38.9±4.23 % in comparison to that of control group (Table 3 and Figure 1). Single treatment of HIF for seven consecutive days could increase CD8+ by 16.81±2.79% of control group. Treatment of seven consecutive days HIF concomitantly with doxorubicin could improve the decrease of CD8+ due to doxorubicin administration (Table 3).

Effect of HIF on spleen IL-10 expression

Cytokine IL-10, an antiinflammatory cytokine, produced mainly by the spleen. In chemotherapy-treated subjects, there was a significant upregulation of cytokines level mainly IL-10 (Zhang et al., 2005a; Gotoh et al., 2012). The IL-10 expression in spleen was detected by immunohistochemistry staining method (Doster et al., 2010). In the study, the suppression of immune functions due to doxorubicin treatment were accompanied with stimulation of IL-10 expression (Figure 2a). That stimulation occurred when the immune system is suppressed in order for defense mechanism in the body.

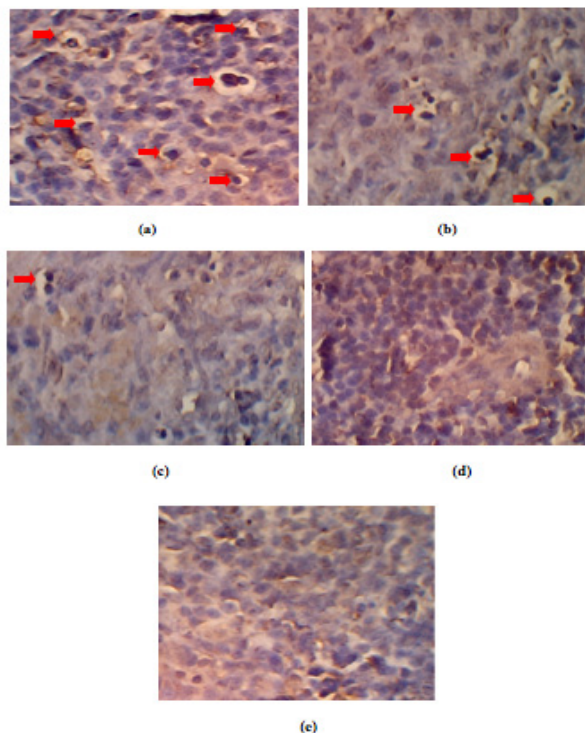


Figure 2. Immunohistochemical Study of IL-10 Expression in Spleen. The analysis was performed on doxorubicin-treated rats with administration of saline (a), HIF 750 mg/kg BW (b), or HIF 1,500 mg/kg BW (c); or on untreated rats with administration of HIF 1500 mg/kg BW (d) or saline (e). IL-10 expression was showed as a red-brown stained mononuclear cells with white (empty) area surrounding the cells (red arrow). Magnification of all figures is x 1600

Table 3. Effect of *n*-hexane Insoluble Fraction (HIF) of *F. septica* on CD8+ in Doxorubicin-induced Rats. Data of CD8+ were Measured at Day 0 (pre-treatment) and Day 7 (Post-treatment)

Group	Treatment	CD8+ (%) Day-0 (Mean±SEM)	CD8+ (%) Day-7 (Mean±SEM)
I.	Doxorubicin	17.62±1.66	10.29±1.45#
II.	HIF 750 mg/kg + doxorubicin	18.37±2.10	13.77±2.50*
III.	HIF 1,500 mg/kg + doxorubicin	16.40±1.78	15.34±2.81*
IV.	HIF 1,500 mg/kg	17.41±2.35	19.66±1.00*
V.	Saline (control)	16.55±0.57	16.83±1.74

*Significant difference (P<0.05) compared to the doxorubicin group value. #Significant difference (P<0.05) compared to the control (saline) value. Data were expressed as Mean±SD (n=3-4 rats)

Single treatment of HIF for seven consecutive days did not influence the IL-10 expression in comparison to that of control group. Treatment of HIF for seven consecutive days concomitantly with doxorubicin mildly decreased the stimulation of IL-10 expression due to doxorubicin (Figure 2b and 2c).

Discussion

In the present study, HIF could restore the immune functions that previously suppressed by doxorubicin treatment. HIF succeeded to stimulate the lymphocytes proliferation and macrophage activation on doxorubicin treated rats. In addition, treatment of HIF reduced the decreasing of T cell subsets on observation with FACS flowcytometry. Subsequently, HIF was also evaluated for its effect on IL-10 that was considered to be responses secondary to the increase of T cells. HIF decreased the stimulation of IL-10 expression in spleen due to doxorubicin treatment.

Doxorubicin suppressed immune system on doxorubicin treated rats, shown by inhibition of lymphocyte proliferation, suppression of phagocytosis activity and capacity of macrophages, suppression of CD8+ cytotoxic T cells and downregulation of IL-10. Macrophages play role in non specific immune response by phagocytosis, while T cells and B cells are responsible for specific immune response. Doxorubicin caused DNA damage on bone marrow cells (Uspenskaya et al., 2002), and also induced apoptosis on lymphocyte cycle by decreasing T cells and B cells in spleen, lymph nodes and thymus (Ferraro et al., 2000). Doxorubicin induced ROS generation that leads to protein and lipid peroxidation, DNA damage, and mitochondrial dysfunction (Ryter and Choi, 2012) and increased anti inflammatory cytokine e.g IL-10 (Santos et al., 2010). Doxorubicin treatment inhibit lymphocyte proliferation on cancer mouse model (Zhang et al., 2005b).

Expression of IL-10 occurred in many cells of adaptive immune system such as CD8+ T cells, B cells, and cells of the innate immune system such as dendritic cells, macrophage, mast cells and natural killer (NK) cells (Saraiva and O'Garra, 2010). Upregulation of IL-10 increased activation and differentiation of CD8+ cytotoxic

cells (Emmerich et al., 2012). Expression of IL-10 in blood is equal to its expression on spleen, the higher IL-10 expression on spleen, the higher IL-10 expression in blood circulation (Min et al., 2004).

Phytochemical study showed that active compounds in the extract of *F. septica* were compounds of the flavonoid and alkaloid, while major compounds of HIF are predicted flavonoids. Wu et al. (2002) found out the active compounds from *Ficus septica* leaves i.e. phenanthroindolizidine alkaloids, including ficuseptine, (+)-tylophorine, and a mixture of (+)-tylocrebrine and (+)-isotylocrebrine, that showed potent cytotoxic effect on two human cancer cell lines. Some flavonoids including genistin, kaempferitrin and coumarin has been also identified from leaves of *F. septica* (Wu et al., 2002). Yang et al. (2005) performed anti inflammatory effect of *F. septica* leaves extract through inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). However, the exact compounds on HIF need to be explored.

Some flavonoids showed anticancer and immunomodulatory activity. Previous in vivo anticancer combinatorial study of doxorubicin showed that the combination of doxorubicin and proanthocyanidin strongly improved the anti-tumour effect of doxorubicin and immune responses, and eliminated myocardial oxidative stress on rats induced by doxorubicin (Zhang et al., 2005a). In vivo study of apigenin on in vitro and in vivo genotoxicity showed decreasing doxorubicin induced genotoxicity on in vitro model, and decreasing genotoxicity on rats induced by cyclophosphamide, possibly due to inhibition of chemotherapeutics metabolic activation (Bokulić et al., 2011). Another in vivo study of flavonoid, showed that single treatment of quercetin is able to induce lymphocyte proliferation and regulate imbalance of Th1/Th2, while its combination with doxorubicin induces persistent T-cell tumor-specific responses in breast cancer mouse model (Du et al., 2010). Therefore probably flavonoids on HIF also act in the same mechanism, but we need to clarified further.

Based on the results, we concluded that combination of *n*-hexane insoluble fraction (HIF) of *Ficus septica* Burm. F. leaves and doxorubicin provided beneficial effects. HIF restored the doxorubicin-induced immunosuppressive activities by stimulate the lymphocytes proliferation, macrophage activation and T cell subsets, and accompanied with mild decrease of IL-10.

Acknowledgements

We gratefully thank to DP2M DIKTI (Directorate of Higher Education) Ministry of Education, Indonesia through "Hibah Bersaing" Research Grant 2012 for financial support in the study. Conflict of interest statement, we declare that we have no conflict of interest.

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