Combinational effects of non *n*-Hexane Fractions of ant-plant (*Myrmecodia tuberosa* Jack) hypocotyl with doxorubicin against lymphocyte and cancer cells

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Abstract: Doxorubicin is widely used as a chemotherapeutic drug despite having many side effects. It may cause the dysfunction of macrophage, decreasing proliferation of lymphocytes, decreasing CD4+/CD8+ ratio and inducing hepatotoxicity. Doxorubicin inhibits the growth of Vero, HeLa, and T47D cell lines, and also induces a resistance of MCF-7 cells. Previous studies showed that ethanolic extract and ethyl acetate fraction of ant-plant (*Myrmecodia tuberosa* Jack*)* hipocotyl could increase macrophage phagocytosis activity and lymphocyte proliferation in vitro. Therefore, ant-plant is a potential immune stimulator. Combinational treatment of non *n*-hexane fraction (NHF) of ant-plant with doxorubicin did not affect the doxorubicin's potency. Nevertheless, increased lymphocyte viability induced by doxorubicin in varied dosages of NHF that lethal to HeLa, MCF-7 and T47D cells. Moreover, on Vero cells, doxorubicin became less toxic when induced together with NHF. Thus, NHF of ant-plant is potential to be proposed as doxorubicin co-chemotherapeutic agent against cancer cells.

Keywords: *Myrmecodia tuberosa* Jack, doxorubicin, lymphocyte, HeLa, MCF-7, T47D

INTRODUCTION

Plant based therapy has started to gain importance as the increasing of the spirit of "back to nature" worldwide. Indonesia is known of its potential natural resources to treat several diseases. Ant-plant (*Myrmecodia tuberosa* Jack) , which belongs to family Rubiaceae can be found in Papua, is used as a herbal remedy with a promising potential against various diseases. The Papuans used its decoction to treat pain due to rheumatic or cancer, to increase body immune system and as energy booster. The present study reported that ethanolic extract and its ethyl acetate fraction of the ant-plant hypocotyls increased the phagocytosis activity of macrophages and lymphocytes proliferation *in vitro* (Hertiani *et al*., 2010) which was supported by results of the *in vivo* assay of its non hexane fraction (Sumardi *et al*., 2013).

Many side effects are still major problems in recent cancer treatments although chemotherapeutic agents are constantly being introduced. Immune suppression in the long period of usage is one of the major adverse effect which can lead to increasing susceptibility to other diseases including severe infections (Patel *et al*., 2006). The condition may influence patient's quality of life and even lead to a life-treathening situation.

Doxorubicin is widely used as chemotherapeutic drug despite having many side effects. It may cause dysfunction of macrophage (Asmis *et al.*, 2006), decreasing proliferation of lymphocytes, decreasing CD4+/CD8+ ratio (Zhang *et al*., 2005), and also inducing hepatotoxicity effect (Chen *et al*., 2011). Doxorubicin has been reported to affect the growth of several type of cell lines including Vero and HeLa (Phonnok *et al*., 2010), T47D (Abdolmohammadi *et al*., 2008), and the resistance of MCF-7 cell (Doublier *et al*., 2012; Sarmoko *et al*., 2014).

In this research we investigated the combination effect of NHF with doxorubicin toward lymphocyte (spleen cell) of Balb/c mice and Vero (as a normal cell); HeLa (cervix adenocarcinoma), MCF-7 (human breast adenocarcinoma) and T47D (human ductal breast epithelial carcinoma) cells by in vitro techniques. Immune modulation property of the NHF is expected to overcome the cytotoxicity effect against normal cells but yet, the possible disturbance in the cytotoxicity of the doxorubicin against cancer cells were also evaluated.

MATERIALS AND METHODS

Plant m aterial

Ant-plant samples were obtained from Bintuni, West Papua, Indonesia. Plant taxonomy determination was done in Pharmacognosy Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia (No: BF/ 14/Ident/Det/I/2012).

Chemicals and reagents

Doxorubicin of 2mg/mL was purchased from Kalbe Farma, Indonesia; ethanol 95% (technical grade) from Brataco company, Indonesia; *n*-hexane and M199

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medium were obtained from Sigma-Aldrich; distilled water from Brataco, PMI 1640 was obtained from Gibco, USA; RPMI complete medium [(RPMI 1640 + fetal bovine serum (FBS) and fungizon (Caisson) + penicillin-streptomycin (Penstrep 1.5%, Gibco)] was prepared in sterile water (Otsuka); MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetra-zolium-bromide] reagent was supplied from Sigma, USA; DMSO was from E-Merck.

Preparation of non n-hexane fractions (NHF) of ant-plant

The ant-plant hypocotyls were sorted and washed with water and sliced 3 mm in thickness, followed by oven-dried (Memmert, German) at 40-60oC and milled. Dried powders were macerated in ethanol 95%, followed by solvent evaporation. Crude extract was then fractionated by liquid-liquid partitioning with *n*-hexane to yield the *n*-hexane fraction and non *n*-hexane fraction (NHF). Further, NHF was evaporated to yield NHF extract and then diluted in 1 % DMSO solution to yield concentration of 12.5µg/mL; 25µg/mL; 50µg/mL and 100µg/mL.

Cell cultures

The cancer cells used (HeLa, MCF-7 and T47D) were the collection of the Research and Assessment Integrated Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia. HeLa, MCF-7 and T47D cell cultures were grown in RPMI 1640 medium complete and Vero cell culture was grown in M199 medium.

Lymphocyte cell isolation and it's proliferation measured by MTT assay

Lymphocyte isolation was prepared according to Sumardi *et al*. (2013). Suspension of the lymphocyte cell (1,5x106 mL-1) in 100 µL medium was distributed into 96 wells microtiter plate (Nunc). Afterwards, 10µL of hepatitis B vaccine as antigen was added into each well and incubated in 5% CO2 incubator at 37°C for 24h. NHF suspensions in varied concentration of 12.5µg/mL; 25µg/mL; 50µg/mL and 100µg/mL, were combined with doxorubicin in three different concentrations of 0.54 µg/mL; 0.22µg/mL and 23µg/mL, which were based on the IC50 values of doxorubicin towards HeLa, MCF-7 and T47D respectively. Incubation was continued for another 48 h. Following addition of 10µL 5mg/mL MTT in phosphate buffer solution, viable cells reacted with MTT to form purple colour of formazan, and then added stopper reagent (10% SDS) in 50µL of HCl 0.01 N. The purple colour resulted from the formation of formazan, was measured by using a microtiter plate reader (Bio-Rad Benchmark, Japan) at 550 nm. Stimulation Index (SI) of lymphocyte cell was calculated as the ratio of the stimulated lymphocyte to an unstimulated control.

Viability cells m easured by MTT assay

Viability cells measured by MTT assay prepared according to Mossman T., 1983.The 48 h cultured of Vero and cancer cells (HeLa, MCF-7 and T47D), were treated with the combination of different concentration of 12.5µg/mL; 25µg/mL; 50µg/mL and 100µg/mL suspensions of NHF extract and doxorubicin in concentration of 0.54µg/mL for HeLa, 0.22µg/mL for MCF-7 and 0.23µg/mL for T47D (based on the IC50 toward respective cancer cells). After 48 h incubation, 10 µl of MTT stock solution was added per well. Following addition of 10µL 5mg/mL MTT in phosphate buffer solution, the viable cells reacted with MTT to form purple colour of formazan. A reagent stopper (10% SDS) in 50µL of HCl 0.01 N was added. The viable cells were measured by using a microtiter plate reader (Bio-Rad Benchmark, Japan) at 550 nm. Percentage cell viability was calculated as, number. of viable Cells Counted / Total Cells Counted (viable and dead) x 100**.**

RESULTS

This study demonstrated the combinational effects of NHF with doxorubicin toward lymphocyte, Vero and cancer cells. The effects were investigated by using three different cancer cells, HeLa, MCF-7 and T47D. The effects of NHF were evaluated at an increasing concentration started from 12.5, 25, 50 and 100µg/mL which were combined with doxorubicin at its IC50 values against the respective cells (a). 0.54µg/mL, (b). 0.22 µg/mL and (c). 0.23µg/mL. The results were gathered as stimulation index for lymphocyte proliferation and % viability for Vero, HeLa, MCF-7 and T47D cells. Vero represents a normal cell, whereas lymphocyte represents a cellular body. The results were analysed using *One Way ANOVA post hoc Tukey* with $p<0.05$ to show the significant differences. The results of this research were shown by figs. 1, 2 and 3.

Fig. 1 showed that doxorubicin decreased lymphocyte proliferation insignificantly ($p>0.05$). NHF seems to play role to modulate the condition. The data proved that increasing concentration of NHF was followed by the increasing of lymphocyte proliferation, quantitatively. The combinational NHF with doxorubicin showed a significant concentration-dependent manner towards HeLa cells in dosage 25, 50 and 100µg/mL ($p<0.05$). Whereas T47D and MCF-7 was significantly increased in all dosage levels ($p<0.05$).

Result of a combinational treatment of NHF and doxorubicin towards normal cell, Vero cell as as a model cell is displayed in fig. 2. Doxorubicin decreased Vero cell viability, except in MCF-7 cells, and the addition of NHF helped to modulate the doxorubicin, which observed to be concentration dependant. The increasing of Vero viability was significantly different towards doxorubicin control in the given of both combination and single dose in all dosage levels except 25µg/mL in HeLa cells and 12.5 µg/mL in T47D cells ($p<0.05$). The NHF 100µg/mL has the best effect to enhance Vero cell viability and reduce

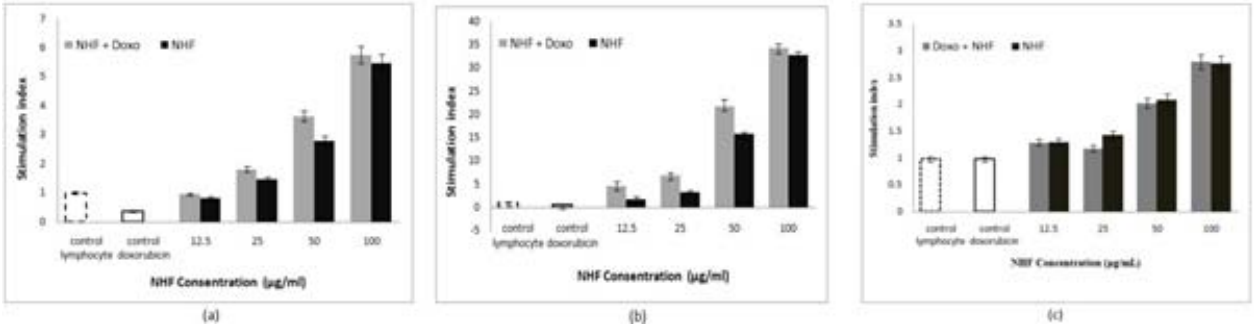

Fig. 1: Stimulation index of lymphocyte proliferation in three different doxorubicin concentrations as mentioned above.

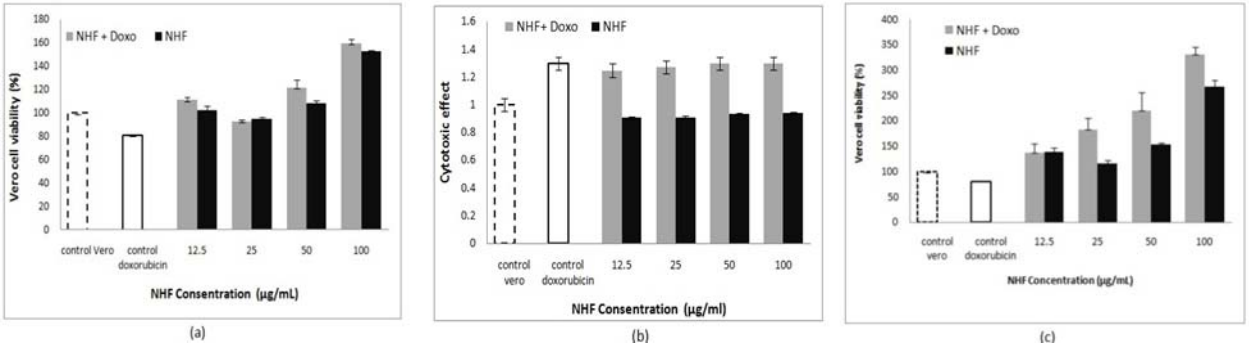

Fig. 2: Viability of Vero cell in three different doxorubicin concentrations as mentioned above.

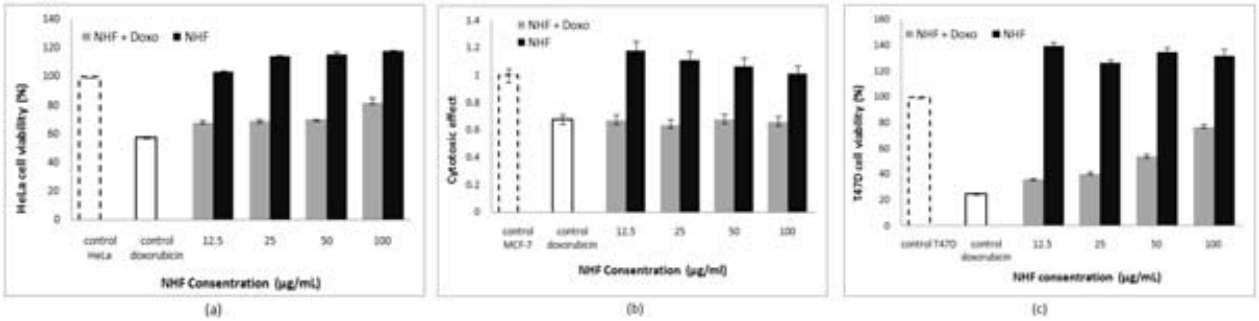

Fig. 3: Single and combinational effect of doxorubicin as mentioned above towards (a) HeLa; (b) MCF-7; and (c) T47D cells

doxorubicin side effect. The same results were observed in a single treatment of NHF.

Fig. 3 indicated that NHF do not have a cytotoxity effect toward HeLa cell on the concentration tested. However, a combinational treatment of NHF and doxorubicin increased cells viability which were observed on HeLa that significantly difference towards doxorubicin control in 25, 50 and 100µg/mL dosage level and T47D cells that significantly different in all dosage level.

DISCUSSION

Increasing lymphocyte proliferation may be due to the phenolics and flavonoids contained in NHF. The NHF was reported by Sumardi *et al*. (2013) to contain 3.548± 0.058% GAE of total phenolics and 0.656±0.026% QE of total flavonoids, beside steroid/terpenoid compounds. Phenolics and flavonoids are widely accepted as effective antioxidant agents which may contribute to their role as immune modulator. Further on their report, Sumardi and collaborators presented that the immunomodulatory effect of *M. tuberosa* showed a strong correlation with the total flavonoid content (Sumardi *et al*., 2013).

Combinational treatment of NHF and doxorubicin increasing cells viability which was observed on HeLa and T47D cells (fig. 3). Possible explanations are either a declining doxorubicin effect due to an interaction with chemical contents of NHF or the presence of antioxidants content in NHF which overcome the drug toxic effect to the cells. As explained by Rossi (2013), doxorubicin property to produce free radical contributes to its

cytotoxicity, beside its main effect to intercalate DNA of target cells and inhibit macromolecule biosynthesis. The addition of NHF together with doxorubicin towards MCF-7 did not affect the cytotoxicity of doxorubicin. Our previous reports on a close related ant plant, *Hydnophytum formicarum* that the ethanolic extract at a concentration up to 100 g/mL showed a inhibition of T47D proliferation in the presence and absence of doxorubicin but no significant effect observed towards Vero cells viability (Darwis *et al*., 2014).

CONCLUSION

Based on these results, the non *n*-hexane fraction of ant-plant (*Myrmecodia tuberosa* Jack) hypocotyls is a potential candidate as immune modulator agent. It increases lymphocyte proliferation, reduces immune suppressant effect of doxorubicin toward Vero cell hence does not disturb doxorubicin's cytotoxicity on MCF-7. However, a further study is needed to confirm the combinational effect of NHF with doxorubicin towards HeLa and T47D cells of which possible restore of cancer cell viability was occured.

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