

The effects of *Hydnophytum formicarum* ethanolic extract towards lymphocyte, vero and T47d cells proliferation *in vitro*

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ABSTRACT

This research aims were to explore the effects of *Hydnophytum formicarum* Jack (Rubiaceae) known as the Ant plant on proliferations of mouse lymphocyte, Vero and T47D's cell lines. Plant hypocotyls were collected from West Papua. Some parts were dried to gain plant with ant residue (A), while other parts were separated from the ant residue to gain plant without ant residue (B) and ant residue (C). After being macerated in ethanol and evaporated to dryness, each extract was tested on Balb/c mice lymphocytes and Vero cell lines by MTT assay in the presence and absence of Doxorubicin (Dox). Effect on T47D was observed by using Dox 25 µg/mL as control. Data obtained were analyzed by ANOVA and paired sample T-test. Thin layer chromatography profiles were evaluated to identify the difference in chemical contents. The extracts A and B had the ability to increase lymphocyte proliferation by increasing concentration. Both extracts could not significantly enhanced the proliferation of Vero cells, neither in the presence nor absence of Dox. The extract B in 100 µg/mL showed the highest inhibition against T47D cells by 53.45%. There was no chemical content difference observed on extracts A and B.

INTRODUCTION

Doxorubicin is a drug of choice for certain cancer drug due to its high efficacy in killing cancer cell. However, Dox treatment can cause suppression of body immune system, i.e., natural killer cells (NK cell), lymphocyte proliferation and ratio CD4+ and CD 8+ (Zhang *et al.*, 2005) and leucocytes cell (Todorova *et al.*, 2005).

Immunomodulator is an agent which modulates immune system in form of stimulation or suppressive or restorative actions (Baratawidjaja, 2002). Flavonoids, monoterpenes, iridoid glycosides have been reported by Chiang *et al.* (2003) to have ability to increase lymphocyte proliferation and secretion stimulation of INF-γ, while Zhang *et al.* (2005) reported that proanthocyanidine from wine seed could repair immune system function in Dox treatment. Therefore a complementary treatment in cancer chemotherapy with an immune stimulator is expected to overcome Dox side effects. Ant plants (*Myrmecodia tuberosa* and *M. pendens*) extracts have been reported in previous research to

have capability in increasing lymphocyte proliferation (Hertiani *et al.*, 2010) but *M. pendens* was reported to be cytotoxic to cancer cell lines (Soeksmanto *et al.*, 2010). It was expected that the plant extracts can be used to overcome Doxorubicin's immunosuppressive effect. Another ant plant species, *Hydnophytum formicarum* is expected to also possess similar activity. It is interesting to find out whether its ant residue plays a role in the activity.

This research aims were to determine the potency of *H. formicarum* ethanolic extract on mice Balb/c lymphocyte proliferation by *in vitro* method and to evaluate whether the ant residue plays a role in the activity; to determine the extract cytotoxic activity to Vero and T47D cell lines; and to evaluate the TLC profile of extract.

MATERIALS AND METHODS

Materials

H. formicarum; ethanol 95% (technical grade, Brataco); distilled water; HCl, ethyl acetate, n-hexane, NaOH, AlCl₃, FeCl₃, toluene, cerium sulphate, CHCl₃, H₂SO₄ (pro analyse grades, Merck); Silica F254 (Merck); Balb/c mice (male, 2 months old)

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Vero and T47D cell lines (*LPPT-UGM*); medium RPMI (Gibco, USA); MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium-bromide) (Sigma, USA); Doxorubicin; Giemsa; Tripa blue (Merck, USA); tween 80 (Merck, USA); Fetal Bovine Serum (FBS) (Caisson, USA); PBS (Gibco, USA); SDS (Merck, USA); Phytohemagglutinin = PHA (Gibco, USA); hepatitis B (Engerix®, UK).

Equipments

Microplate reader (Bio-Rad, Japan), Haemocytometer (Neubauer, Germany), CO₂ incubator (Heraeus®, Germany), inverted mikroskop (Olympus, Japan).

Sample preparation

Plant samples were obtained from Bintuni, West Papua on November 2011. Plant taxonomy determination was done in Pharmacognosy Laboratory, Faculty of Pharmacy, UGM under registration number: BF/131/Ident/Det/XII/2011.

After the hypocotyls were being sliced, some parts were dried directly to gain plant with ant residue (A), while other parts were separated from the ant residue to gain plant without ant residue (B) and ant residue (C). Oven dried samples were macerated in 96% ethanol, followed by vacuum evaporation to yield ethanol extract of A,B, and C.

Lymphocytes proliferation assay *in vitro*

Each extract was tested for the lymphocyte proliferation activity in various concentration (10, 20, 50, 100, and 200 µg/ml) and in presence of Dox (1 µg/mL). Lymphocyte preparation was performed according to Sano *et al.* (2007). Spleen tissue was collected from mice aseptically and subsequently transferred to a sterile centrifuge tube containing 5 mL of RPMI. Cell suspension was centrifuged for 10 min. Clumps were suspended in 2 mL Tris ammonium chloride buffer and left in room temperature for 2 min. Afterwards, 1 mL complete medium was added and was followed by centrifugation at 1200 rpm 4°C for 5 min. After being rinsed twice with RPMI, clumps were suspended in complete medium. Cells were counted by haemocytometer and inverted microscope. The lymphocytes were ready for assay and were cultured in CO₂ incubator at 37°C.

Lymphocytes cells 100 µL (1.5×10⁶ mL⁻¹) were distributed into 96-wells micro plates. Into each well was added 10 µL of hepatitis B vaccine and incubated for 24 h in 5% CO₂ at 37°C. After incubation, samples were added and incubated again for another 48 jam. Furthermore, each well was given 10 µL of MTT 5 mg mL⁻¹, followed by 4 h incubation at 37°C. Viable cells will convert the MTT to form purple color. Reagent stopper (10% SDS) in 50 µL HCl 0.01 N was added into each wells. Cells density were determined by using micro titer plate reader at 550 nm Data of cells densities were used to calculate the Proliferation Stimulatory Index.

$$SI_{proliferation} = \frac{(Abs\ sample - medium)}{(Abs\ normal - medium)} \dots\dots\dots(1) \text{ (Sano } et al., 2007).$$

T47D and Vero cells proliferation assay *in vitro*

Each extract was tested for the lymphocyte proliferation activity in various concentrations (10, 20, 50, 100, and 200 µg/ml). DMSO was used as sovent. Cytotoxicity assays on Vero and T47D cell lines were done according to protocol method by *Cancer Chemoprevention Research Center (CCRC) UGM* (2010).

Cells with confluency of 80% were harvested from the CO₂ incubator and transferred to wells (100 µL) and incubated overnight. Afterwards, media were discarded and 100 µL PBS was added into each well for rinsing. Samples were added into respective wells and incubated in a CO₂ incubator (incubation period was 24-48 h). MTT reagent (0,5 mg/mL) was prepared by diluting the MTT stock (in PBS, 5 mg/mL) in complete medium to achieve a total 10 mL suspension (FBS, fungizone and medium M199). Afterwards, media were discarded and MTT was added into each wells (100µL), followed by incubation for 2-4 h in the CO₂ incubator. Reagent stopper (100 µL SDS 10% in 0.1 N HCl) were added into each wells. Cells density were determined by using micro titer plate reader at 550 nm. Percentage of viable cells was calculated formula as follows:

$$\text{Percentage of viable cells} = \frac{(Abs\ sample - medium)}{(Abs\ normal - medium)} \times 100\% \dots\dots\dots(2)$$

Thin layer chromatography (TLC) profiling

Thin layer chromatography profile of each extract was evaluated to identify the difference in chemical contents according to methods recommended by Jork *et al.* (1990).

Data Analyses

Data obtained were analyzed by ANOVA and paired sample T-test with Confidence level 95%.

RESULTS AND DISCUSSION

The plant sample is consisted of ant residue inside the hypocotyls hollows (Fig 1). An Ant plant lives as an epyphyte in big trees and conjugates itself in a tight, almost obligatory mutualistic simbiosis with ants. The ants are benefiting by having a warm and safe home inside the plant's hypocotyl, while the plants' needs for soil resource are provided by the ants. It was reported by Solano and Dejean (2004) and Defosse *et al.* (2011), the ants provide nitrogen to their host plant. Moreover, translocation of ant residues to the plants does occurred (Beattie, 1989). Iridoids, which is a monoterpenoid plant secondary metabolite was also reported to be found in *Iridomyrmex* sp. (ant species).

This group of compounds was detected in *M. tuberosa* extract, a taxonomy-close-related Ant plant species with *H. formicarum* (Efendi and Hertiani, 2013). Aucubin, which is an iridoid glycoside was reported by Chiang *et al.* (2003) to play a role in immunomodulatory activity of *Plantago* species. Therefore it is worth to know whether this ant residue is also responsible for many therapeutic effect of the plant.



Fig. 1: *Hydrophytum formicarum* Jack, the whole plant (left) and the slices showing the hollows containing ant residues (right)

In this research we separated the ant residue and compare its activity and phytochemical profile with the plant itself.

Extract samples recoveries compared to dried powder were as follows: A, 4.93%; B, 3.38%; C, 1.18%. There was no chemical content difference observed on extracts A and B, suggesting flavonoid, phenolic, aldehyde / ketone, terpenoids and tannin contents. There was no iridoids detected in the samples, which may be due to instability of this group of compound. Furthermore, phytochemistry profile suggested no group of plant secondary metabolites detected in the ant residue (C) by TLC method.

An example of terpenoids having a potential immunostimulatory agent was aucubin (Chiang *et al.*, 2003) and saponins (Lacaille-Dubois, 2005). Iridoid was not detected in the extract which may be due to its nature being easily decomposed. Other important plant substances are phenolics and flavonoids (Chiang *et al.*, 2003).

Polyphenols such as flavonoids, and tannin are potential antioxidant substances which may explain its immunostimulatory effect. Antioxidative compounds promote changes in redox-sensitive signalling pathways involved in the expression of many genes, and further in several cell functions including immune response (Ramiro-Puig and Castell, 2009). Propolis is an example of extract rich in antioxidant phenolics compounds, which is also a potential immunomodulatory agent (Syamsudin *et al.*, 2009). *H. formicarum* itself is a potential antioxidant sources. Bustanussalam *et al.* (2009) reported the prominent activity of the ethyl acetate fraction which was comparable with Vitamin C, while Prachayasittikul and collaborators reported the plant's antioxidative potency as well as its chemical constituents, i.e flavonoid and phenolic acid, beside stigmaterol (Prachayasittikul *et al.*, 2008). The result showed that extracts A and B had the ability to increase lymphocyte proliferation by increasing concentration in the presence and absence of Doxorubicin 1 µg/mL (Figure 2 - 3).

The activity was similar, suggested that Dox did not affect the stimulation activity. On the other hand extract C did not show significant activity in comparison to normal control, which

might be correlated to the result of the phytochemical assay previously described. Therefore it can be suggested that the ant residue did not play a role in the enhancement of lymphocyte proliferation. Nevertheless, extract A which was prepared without eliminating the ant residue showed a slightly higher activity in comparison with extract B (derived from plant without the ant residue).

In order to explore the possibility of extract A or B as complementary to Dox treatment, cytotoxicity assays on cell lines T47D and Vero cells were performed. T47D cell is a Human Mammary gland Ductal carcinoma which has mutation in p53, while Vero and HeLa cells are epithelium-like (Goodwin and DiMaio, 2000). Another close related Ant plant, *M. pendens* has been reported to be toxic to Vero and HeLa cell lines (Soeksmanto *et al.*, 2010).

Therefore we were eagered to explore the activity of this plant extract as well, in the presence and absence of Dox. The extract would not be beneficial as an immunomodulator agent for Dox therapy if the application can somehow increase the cancer cells proliferation as well. On the other hand, cytotoxicity assay on the normal cells, in this case represented by Vero cells, was also assayed to find out about the extract potential to intoxicate the healthy cells. Vero cells proliferation was inhibited by Doxorubicin (Fig. 4). This is due to metabolic conversion of this compound into Doxorubicinol by certain enzymes such as carbonyl reductase. Major mechanism of doxorubicinol toxicity was occurred due to its interaction with iron and the formation of the Reactive Oxygen Species which further destroys the cell macromolecules (Minotti *et al.*, 2004). Both extract A and B were unable to increase the cell proliferation in the presence of Doxorubicin (Fig 5). It can be observed that the extract slightly decreased the Vero cells proliferation in absence of Doxorubicin but not statistically significant (Fig. 6).

The extracts A and B affected the T47D proliferation in a concentration-activity relationship except for result of extracts A and B 200 µg/mL of and at 12.5 µg/mL of extract A. (Fig. 7). Extract B showed the highest inhibition at concentration 100 µg/mL (53.45%).

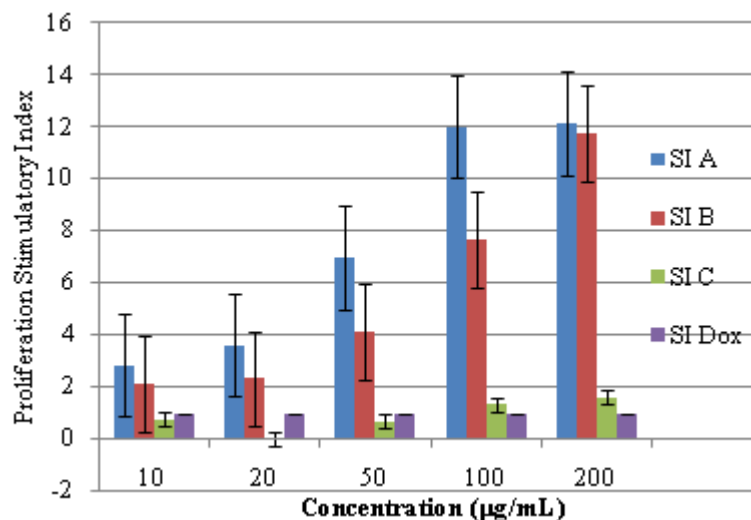


Fig. 2: Graphic of Proliferation Stimulatory Index ($SI_{proliferation}$) of extracts A, B, C in the presence of Doxorubicin (1 µg/mL)

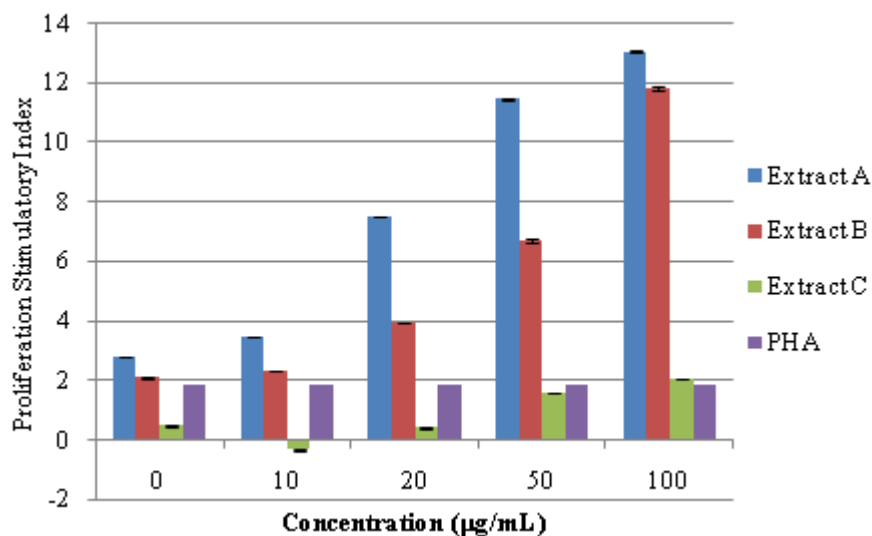


Fig. 3: Graphic of Proliferation Stimulatory Index ($SI_{proliferation}$) of extracts A, B, C without Doxorubicin (in a presence of Hepatitis B vaccine 1 µg/mL); PHA was used as a positive control (n=3).

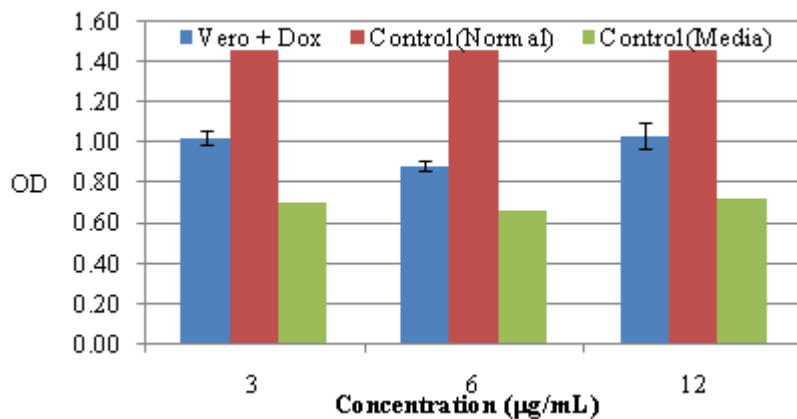


Fig. 4: Optical densities of Vero cells treated with Doxorubicin in various concentrations.

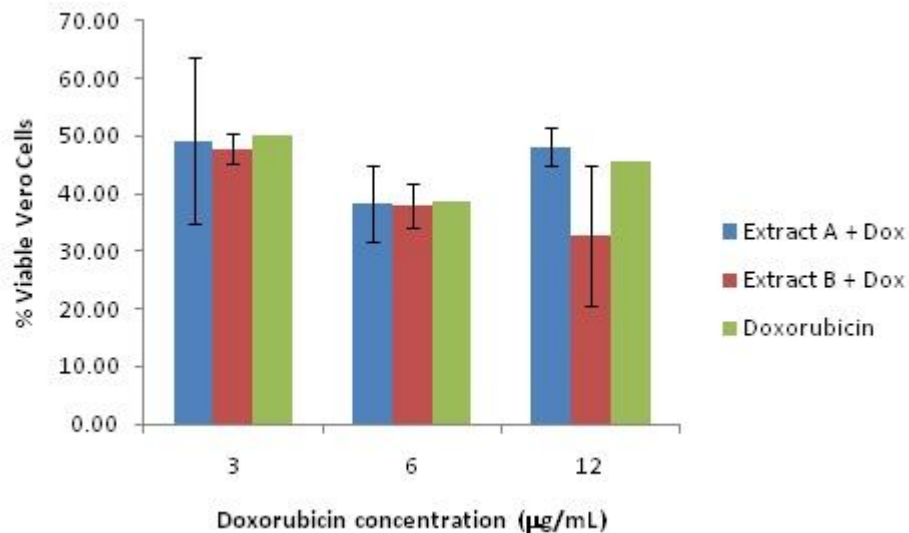


Fig. 5: Percentage of viable Vero cells treated with Doxorubicin in various concentrations and extract in 100 µg/mL (n = 3).

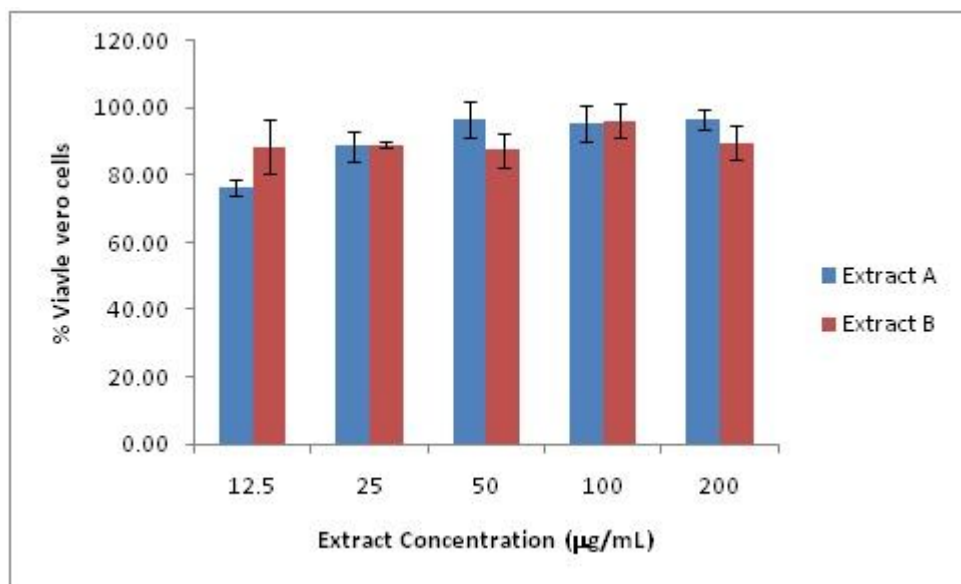


Fig. 6: Percentage of viable Vero cells treated with extracts in various concentrations (n=3)

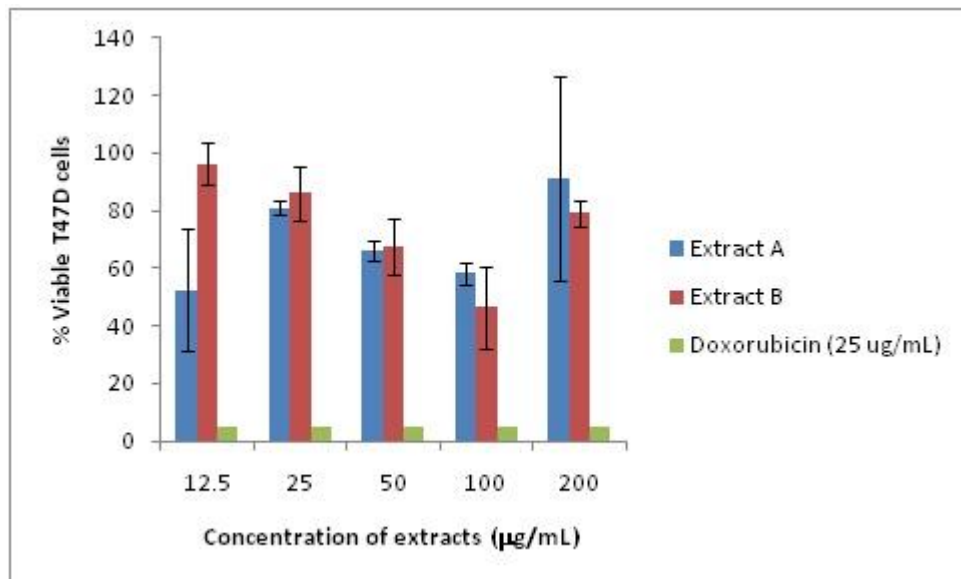


Fig. 7: Percentage of viable T47D cells treated with extracts in comparison with Doxorubicin 25 µg/mL as a control (n = 3).

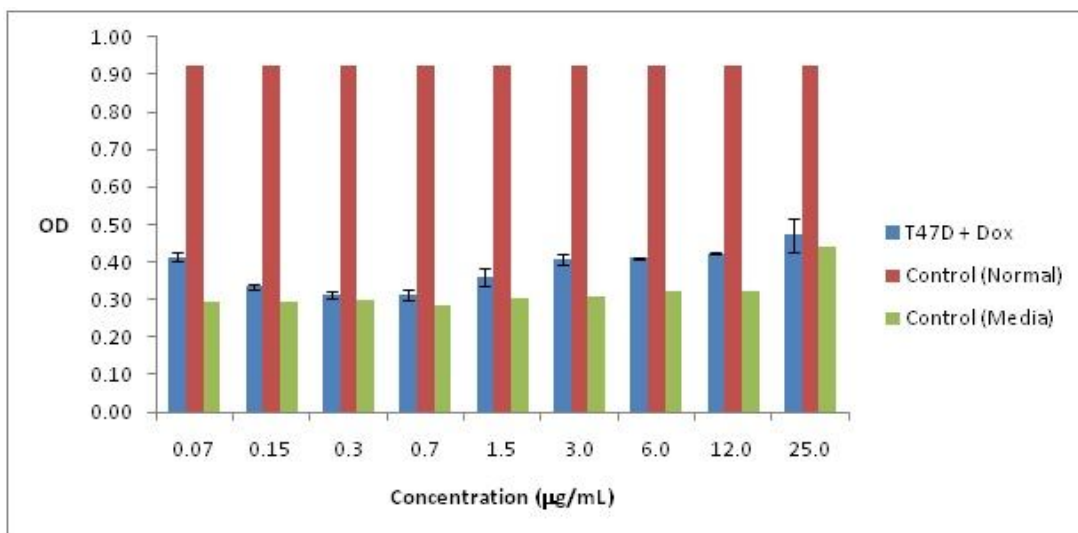


Fig. 8: Optical Densities of T47D cells in the presence of Doxorubicin (n=3).

Nevertheless this inhibition was still incomparable to the lowest concentration of Dox tested (0.07 µg/mL) which was 99.81% (Fig. 8). Considering that the inhibition of extracts, both A and B were decreased at 200 µg/mL, *H. formicarum* extracts should be used in caution. Further experiment to explore the extracts activity towards cell lines in the presence of Dox should be performed in order to evaluate its potency as a complementary treatment in Dox therapy.

CONCLUSION

The result showed that extracts A and B had the ability to increase lymphocyte proliferation by increasing concentration. Both extracts could not significantly enhanced the proliferation of Vero cells, neither in the presence nor absence of Dox. The extract B in 100 µg/mL showed the highest inhibition against T47D cells by 53.45%. The inhibition of extracts, both A and B were decreased at 200 µg/mL, recommends further analyses on the effect of *H. formicarum* extracts towards T47D proliferation in the presence and absence of Dox. Extract C showed no activity on lymphocyte proliferation which can be explained by no compounds detected by TLC. There was no chemical content difference observed on extracts A and B. These extracts contained flavonoid, phenolic, aldehyde / ketone, terpenoids and tannin.

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