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Immunomodulatory Activity of *Combination* of *Phyllanthus niruri* Linn, *Typhonium flagelliforme* (Lodd.) Blume, and *Piper crocatum* on Macrophage Phagocytosis *In Vitro*

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ABSTRACT

Research on the activity of *Phyllanthus niruri* Linn, *Typhonium flagelliforme* (Lodd.) Blume and *Piper crocatum* have been conducted and showed various immunomodulatory activity. This study aims to investigate the immunomodulatory activity of the combination of the ethanolic extracts of *Phyllanthus niruri* Linn, *Typhonium flagelliforme* (Lodd.) Blume, and *Piper crocatum* by determining its macrophages phagocytic index and macrophages phagocytic capacity. Therefore, such a combination could be an alternative drug to increase immune response. In this study, the extraction procedure was carried out through maceration by using an ethanolic solvent. Combinations of herbs ethanol extract were varied in four groups of combination, at three different concentrations of 1 μg/ml, 10 μg/ml, and 100 μg/ml for each group. Macrophages were isolated from the peritoneum cavity of male mice (Mus musculus), and its phagocytic activity was quantified through the Leijh method (1986). The phagocytic index and phagocytic capacity of macrophages were determined by using latex beads as a trigger of phagocytosis and compared with negative controls of media, DMSO, and four groups of ethanolic extract combinations in different concentrations. The results indicate that all of combination group ethanol extract with a concentration of 10 μg/ml was significantly ($p<0.05$) optimum activated phagocytic index. Therefore the combination of *Phyllanthus niruri* Linn, *Piper crocatum*, and *Thyphonium flagelliforme* (Lodd.) Blume ethanolic extract might be prospective to increase nonspecific immune response.

Keywords: *Phyllanthus niruri* Linn; *Thyphonium flagelliforme* (Lodd.) Blume and *Piper crocatum*; macrophages phagocytic index; macrophages phagocytic capacity; immunomodulator activity.

INTRODUCTION

The administration of immunostimulatory products to patients with infectious diseases, especially in the case of a weakened immune system condition, is essential since the immunostimulatory products can be clinically used as treatment and prevention of diseases (Sherwood, 2012). In Indonesia, high biodiversity provides various types of plants that can be used as immunostimulatory agents due to the content of flavonoids and alkaloids (Ukhrowi, 2011).

The pharmacological activity of each of *Phyllanthus niruri* Linn, *Thyphonium flagelliforme* (Lodd.) Blume and *Piper crocatum* have been investigated, and they have been used empirically in daily life as medicinal plants. Ethanolic extract of these plants containing flavonoids and alkaloids that allow these plants to be efficacious as immunomodulators (Nobakht *et al.*, 2010; Nugrahani, 2012; Sianipar, Ariandana and Maarisit, 2015; Optimization *et al.*, 2018). Flavonoids can increase IL-2 activity and lymphocyte proliferation, which increases motility and phagocytic activity quickly and is more efficient in killing bacteria or pathogenic microorganisms, while alkaloids have antibacterial activity by inhibiting microbial protein adherence to host polysaccharide receptors (Ukhrowi, 2011).

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Researchers define mechanisms of immunomodulators as a stimulant, suppressor, or restorative (Baratawidjaja and Rengganis, 2010). Flavonoid compounds have the effects of lowering the immune system; therefore, the combination of these three extracts might inhibit the activity of macrophages or work as suppressor at certain concentrations due to the content of flavonoids (Middleton, Kandaswami and Theoharides, 2000).

Research on immunomodulatory effects of a combination of ethanolic extract of *Phyllanthus niruri* Linn, *Thyphonium flagelliforme* (Lodd.) Blume, and *Piper crocatum* have never been carried out, so this study was conducted to determine the immunomodulatory effect of combinations of these three ethanolic extracts. The parameters to be observed are the phagocytic index and the phagocytic capacity of macrophages.

(a) (b) (c)

Figure 1. Dried Plant Materials (a) *Piper crocatum* (b). *Phyllantus niruri* Linn (c). *Thyphonium flagelliforme* (Lodd.) Blume

Table I. Dosage of Each Extract in Human

Combination	Dosage in Human			
	Piper crocatum (mg)	*Thyphonium flagelliforme* (Lodd.) Blume (mg)	*Phyllantus niruri* Linn (mg)	Total (mg)
1	50	100	100	250
2	50	100	50	200
3	25	100	100	225
4	150	200	150	500

METHODOLOGY

Materials And Method

This study was designed to measure phagocytic index and phagocytic capacity *in vitro* by culturing macrophages from the peritoneum of mice. The cultured macrophages were subjected to a combination of extracts with a specific formula. Phagocytic quantification by Leigh method (Jensch-Junior *et al.*, 2006) and calculated by the following formula:

Phagocytic Index:

$$\frac{Number\ of\ Phagocytosed\ Beads}{\text{Number of Phagocytic Macrophages (100)}}$$

Phagocytic Capacity:

$$\frac{Number\ of\ Phagocytic\ Macrophages}{\text{Number of Counted Macrophages (100)}} \text{ x } 100\%$$

The research plant materials used in this study were collected from the Gama Herbal Indonesia®, provided with a certificate of identification to ensure the conformity of the species used. Dried plant materials were extracted in Pharmaceutical Biology Department, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. These plant materials were *Piper crocatum*, *Phyllanthus niruri* Linn, and *Thyphonium flagelliforme* (Lodd.) Blume (Figure 1).

The animals' test used were male Balb/C strain mice (*Mus musculus*), age 8 weeks with a bodyweight of ± 35 g which were acclimated with the laboratory environment for 2 weeks.

Extraction

Maceration of samples carried out by soaking each powdered plant material in 70% ethanol with solid-liquid ratio of 1:7, for 3x24 hours at room temperature (±27°C) and stirred with a shaker for 6 hours 150 rpm, following the method mentioned in the Indonesian Herbal Pharmacopeia (Departemen Kesehatan Republik Indonesia, 2000). The liquid extract separated from the pulp and then concentrated on a rotary evaporator followed by water bath at a temperature of 50-60°C. Furthermore, the thick ethanolic extract obtained weighed and the yield was expressed in percent against the weight of the plant material the human dose conversion of each extract was shown in Table I.

Characterization of Extracts

The characterization of bioactive compounds was conducted with thin layer chromatography (TLC). The Samples was prepared by dissolving each single extract and their combination in methanol so that the extract is

Table II. Formula of each combination (%) of *Piper crocatum, Thyphonium flagelliforme* Blume, and *Phyllantus niruri* Linn

Combination	***Piper crocatum* (mg)**	***Thyphonium flagelliforme* (Lodd.) Blume (mg)**	***Phyllantus niruri* Linn (mg)**
1	20	40	40
2	25	50	25
3	11	44	44
4	30	40	30

obtained with a concentration of 10 mg/mL. The samples were loaded on to pre-coated TLC (60 F_{254}) approximately 10 µL and it was developed by using the mobile phase in the ratio of ethyl acetate: methanol: water (100:13.5:10 v/v). The TLC plate was exposed under visible light and with fluorescent UV light at 360 nm and 240 nm. Subsequently, the chromatogram was observed under visible light and UV 366 nm by previously treated the TLC plate using anisaldehyde-sulphuric acid spray reagent and heated the plates to 105°C for 5 minutes.

Immunological test

Macrophages were isolated from the peritoneum of Balb/C mice aged 8 weeks. After mice were sacrificed under chloroform anesthesia, 10 ml of cold complete RPMI medium, completed with FBS and Penicillin-streptomycin, was injected into the peritoneal cavity. Furthermore, the peritoneal fluid was aspirated and centrifuged at 1200 rpm at 4°C for 10 minutes. Afterward, the supernatant was discharged, and 3 ml of complete RPMI medium containing 10% v/v FBS was added to the pellets. The cells were calculated with a hemocytometer and resuspended in complete RPMI medium to obtain viability cell of 2.5 x 10^{6}/ml. The cell suspension was cultured in a 24 well plate on the round coverslips, with the volume of cell suspension in each well was 200 µl (5x10^{5} cells), followed by incubation for 30 minutes in 5% CO_2 incubator at 37°C. Eight hundred µl of complete RPMI medium was added into each well, and then the cells were re-incubated for 24 hours. After being cultured, the macrophage cells were washed with complete RPMI twice.

The macrophage phagocytic activity evaluation was conducted in 2 sample groups, negative control (complete RPMI medium and DMSO) groups and treatment groups (Combination 1, Combination 2, Combination 3, and Combination 4 as shown Table II). One ml of each solution was added into the wells containing macrophage cells, with three replication/solution, which was then incubated for 4 hours in a CO_2 incubator at 37°C. The wells were washed with RPMI, then latex beads suspension at a concentration of 2.5 x 10^{7} latex/ml in PBS was added in the volume of 200 µl into each well, and re-incubated for 20 minutes. Samples were washed with PBS twice to remove un-phagocyted latex beads and dried at room temperature. The fixation process was conducted with methanol for 30 seconds, followed by 30 minutes of Giemsa staining, washed with distilled water, and dried at room temperature. The numbers of active macrophages were observed under a microscope. Phagocytic index and phagocytic capacity were calculated by observing the number of phagocytic macrophages and the number of phagocytosed beads by macrophages on the coverslip under a 1000x magnification microscope.

Statistical analysis

The results were expressed as means ± standard error of the mean. Two-way ANOVA with Post Hoc LSD test was applied. The data with a confidence level of 95% ($p<0.05$) was considered statistically significant.

RESULT AND DISCUSSION

Result

Extract Characterization

The fresh plant materials are dried in an oven at 40-60°C for 24 hours. The extraction method was carried out by maceration with 70% ethanol (1:7) for 24 hours. The method and duration of extraction were based on the procedure issued by the Ministry of Health of the Republic of Indonesia, 1986 (Departemen Kesehatan Republik Indonesia, 1986), following the method described in the Indonesian Herbal Pharmacopeia (Departemen Kesehatan Republik Indonesia, 2000). Ethanol was chosen as a solvent because it was able to dissolve many compounds with a wide range of polarities. Macerate was obtained by filtration and evaporated to form a thick extract. The organoleptic descriptions of the extracts were as follows: *Phyllanthus niruri* Linn extract was dark brownish with pungent smell and taste, *Thyphonium flagelliforme* (Lodd.) Blume extract was light brown mass, sweet aroma, and

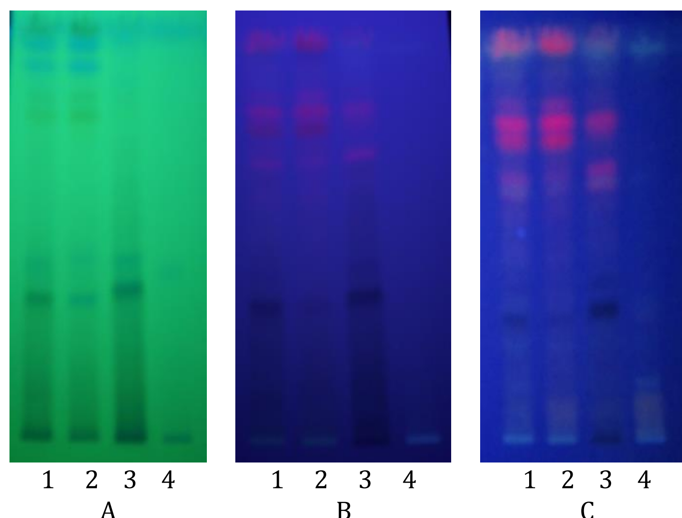


Figure 2. Chromatogram Profile each extract in (A). UV 254 nm, (B). UV 366 nm, (C). UV 366 nm after treated with anisaldehyde-. sulphuric acid spray reagent; Spot: (1) Extract Combinations, (2). *Piper crocatum* ethanolic exttract, (3). *Phyllantus niruri* Linn extract, (4). *Thyphonium flagelliforme* (Lodd.) Blume extract

Table III. Phagocytic index of macrophages treated with combination of *Piper crocatum, Thyphonium flagelliforme* Blume, and *Phyllantus niruri* Linn.

Combination	**1 µg/ml**		**10 µg/ml**		**100 µg/ml**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
1	3.18	0.48	4.24	0.56	3.22	0.28
2	3.14	0.67	4.05	0.33	4.04	0.62
3	3.10	0.38	4.37	0.75	3.69	0.27
4	3.15	0.25	4.05	1.09	3.17	0.20
Control Media	2.25	0.35	2.53	0.09	2.24	0.23
Control DMSO	2.50	0.43	2.50	0.43	2.66	0.25

bitter taste, *Piper crocatum* extract was dark green mass with bitter taste and aroma. The extract yield of *Phyllanthus niruri* Linn, *Thyphonium flagelliforme* (Lodd.) Blume and *Piper crocatum* were 3,35 %, 4,25 % and 9,86 % respectively. The characterization of the extract was carried out with chromatogram profiling. This chromatogram is not intended to identify the presence of certain compounds, but to show the secondary metabolite profile contained in each extract and its combination. From figure 2, it can be seen that there are some compounds that have a chromophore group characterized by quenching spots under UV light at wavelength 254 nm. The results of detection with UV light at wavelength 366 nm after being treated with anisaldehyde-sulphuric acid spray reagent indicate the presence of terpenoid compounds that are adorned with the appearance of red color-purple on TLC plate after being heated 105°C for 5 minutes.

Phagocytic Index and Phagocytic Capacity (%)

Based on the average phagocytic index histogram shows in Graphic 1 and Graphic 2; Figure 9 and Figure 10, in each combination with a concentration of 10 µg/ml there was an increase in phagocytic index and capacity compared to controls, while the phagocytic index and phagocytic capacity of the combination of extracts with different compositions at concentrations of 1 µg/ml, 10 µg/ml, and 100 µg/ml were not significantly different. The results of the LSD test (Table V and Table VI) on phagocytic capacity did not differ significantly compared to inter-combination. However, all combinations were different from controls, which means that there was an effect of increasing the number of active macrophage cells in phagocytosis when compared with controls, whereas between the combination of extracts increased the number of active macrophages cells in phagocytosis were not significantly different.

Table IV. Phagocytic capacity of macrophages treated with combination of *Piper crocatum, Thyphonium flagelliforme* Blume, and *Phyllantus niruri* Linn

Combination	1 µg/ml		10 µg/ml		100 µg/ml	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
1	85	3.00	87.67	4.51	83.33	2.52
2	82	1.00	84.33	3.51	86.00	1.00
3	81.67	3.51	85.33	4.93	84.67	2.87
4	80.33	2.08	88.00	3.00	83.33	3.06
Control Media	77.33	0.58	76.67	1.53	76.00	4.00
Control DMSO	74.67	4.93	73.00	3.61	71.67	3.79

Table V. Significance of the phagocytic index of *Piper crocatum, Thyphonium flagelliforme* Blume, and *Phyllantus niruri* Linn analyzed with LSD Test

Combination	1	2	3	4	K. Media	K. DMSO
1		TB	TB	TB	B	B
2	TB		TB	TB	B	B
3	TB	TB		TB	B	B
4	TB	TB	TB		B	B
Control MK	B	B	B	B		TB
Control DMSO	B	B	B	B	TB	

Note: B = Significantly differences; TB = Differences is not significance

Table VI. Significance of phagocytic capacity (%) of *Piper crocatum, Thyphonium flagelliforme* Blume, and *Phyllantus niruri* Linn analyzed with LSD Test

Combination	1	2	3	4	K. Media	K. DMSO
1		TB	TB	TB	B	B
2	TB		TB	TB	B	B
3	TB	TB		TB	B	B
4	TB	TB	TB		B	B
Control MK	B	B	B	B		TB
Control DMSO	B	B	B	B	TB	

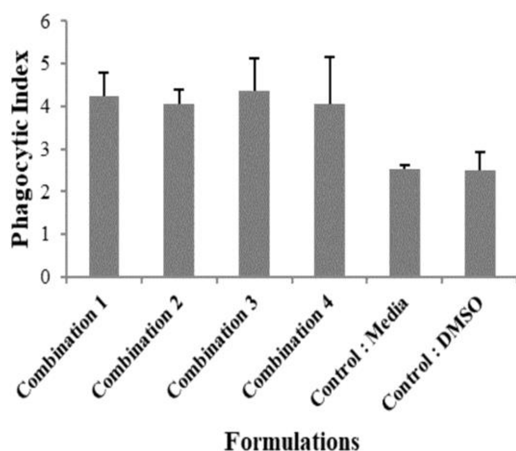
Note: B = Significantly differences; TB = Differences is not significance

Discussion

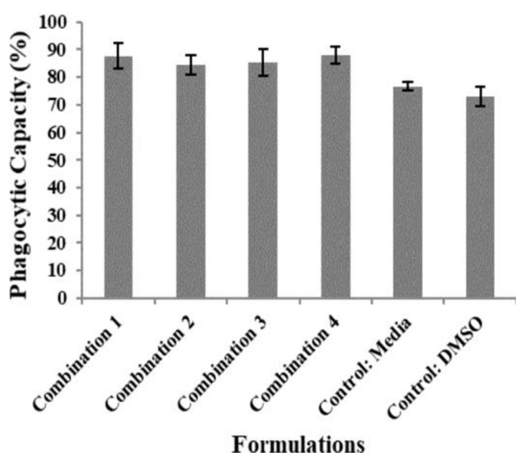
Analysis results (Table III, Table IV, Table V, and Table VI) clearly show that the combination of these ethanol extracts with varied compositions led to increasing phagocytic and capacity index as compared to control. The use of DMSO to improve the solubility of the extract also did not affect the increase in the phagocytic index. Therefore, it can be concluded that the increase in the phagocytic and capacity index was due to a combination of extracts.

The researchers have been developed the activity of each ethanolic extract of *Piper crocatum*, *Phyllanthus niruri* Linn, and *Thyphonium flagelliforme* (Lodd.) Blume. According to (Wiweko, 2010; Apriyanto, 2011), the extract of *Piper crocatum* may lead to enhancement of nonspecific immune system in increasing the macrophage phagocytic index. The activity of ethanolic extract of *Thyphonium flagelliforme* (Lodd.) Blume may be associated with cytokine expression of **IFN**γ that these molecular may impact in activating phagocytosis (Shen and Louie, 2005). Ethanolic extract of *Phyllanthus niruri* Linn is increasingly reported to be associated with an immunomodulator by inducing chemotaxis and phagocytic activity (Ibnul, 2012; Maat, 1996).

In this study, the calculation of the phagocytic index and phagocytic capacity for all combination dosage of ethanolic extracts were determined by using the formula of Jensch-Junior *et al.* (2006). The calculation of the phagocytic index was performed by observing the number of



Graphic 1. Phagocytic index of Macrophages treated with the combination extracts.



Graphic 2. Phagocytic capacity of Macrophages treated with the combination extracts.

latex beads phagocytosed by macrophages, and the calculation of the phagocytic capacity was performed by observing the number of active phagocytic macrophages.

The characterization of each extract was conducted by using thin-layer chromatography. The chromatogram profile of each extract was obtained in UV 254 nm, UV 366 nm, and UV 366 nm after sprayed with sulfuric acid and anisaldehyde, as shown in Figure 2. The chromatogram profile was used as a fingerprint of each extract identity.

The previous studies have been demonstrated that the presence of the phenolic compound was linear related to the immunomodulatory activity (Daulay, 2012). The mechanism of immunomodulatory activity in ethanolic extract of *Thyphonium flagelliforme* (Lodd.) Blume was proposed by the role of IL-10 and TNF-α. The researcher reported the dosage the optimum dosage for obtaining immunomodulatory effect was 250 mg/kg, with its value of IC_{50} was 632 μg/ml, which induces cytotoxic effect toward T47D cell. A phenolic compound consisting of its extracts might be taking a role in increasing the activity of macrophage cells because of stimulating the release of cytokine IL-12 and IFN-γ (Shen and Louie, 2005). Some researchers have reported that the immunomodulatory activity of plants may be attributed to the presence of flavonoids and alkaloids. Ibnul (2012) demonstrated that the extract of *Phyllanthus niruri* Linn could be used in activating macrophage phagocytic activity and to increase the production of oxide nitrite in Balb/C mice. This result is in line with the previous research conducted by Maat, (1996), which showed a similar effect in mice that the extract of *Phyllanthus niruri* Linn increased phagocytic and chemotaxis of macrophage, neutrophil chemotaxis, cytotoxicity NK cell, and hemolysis complement activity. Yuristiyani (2012) previously investigated that the insoluble fraction of n-hexane ethanolic extract of *Piper crocatum* at 0.1 mg/ml, 0.5 mg/ml, and 1 mg/ml could not increase macrophage phagocytic index and decreasing lymphocyte proliferation. Ethanolic extract of *Piper crocatum* does not have activity in the specific immune systems, which are lymphocyte proliferation and antibody titer unless could be taking a role in increasing nonspecific immune system, by using increased macrophage phagocytic (Wiweko, 2010; Apriyanto, 2011). Werdyani, (2012) also reported that a fraction of n-hexane of ethanolic extract of *Piper crocatum* might be able to increase macrophage phagocytic, although it tends to suppress the amount of $TCD4^+$ and $TCD8^+$ cells.

The optimum dosage of combination extracts in immunomodulatory activity was observed in line with the phagocytic index. Therefore, the results of statistical tests are significantly increasing in the phagocytic index at a concentration of 1 μg/ml to a concentration of 10 μg/ml (Graphic 1). Although the phagocytic index at a concentration of 10 μg/ml to a concentration of 100 μg/ml from Graphic 1 is not significantly increasing. This condition might be caused by a decrease of macrophages' ability to phagocytose latex beads at a concentration of 100 μg/ml. Based on the performed orientation, the experiment at a concentration of 1000 μg/ml could induce apoptosis of macrophage cells, as shown in Figure 3 and Figure 4. In addition, the results of this study are in accordance with previous research conducted by Middleton *et al.*, (2000) that flavonoid has the effects of lowering the immune system; therefore there is a possibility of inhibition to the activity of macrophages at optimum

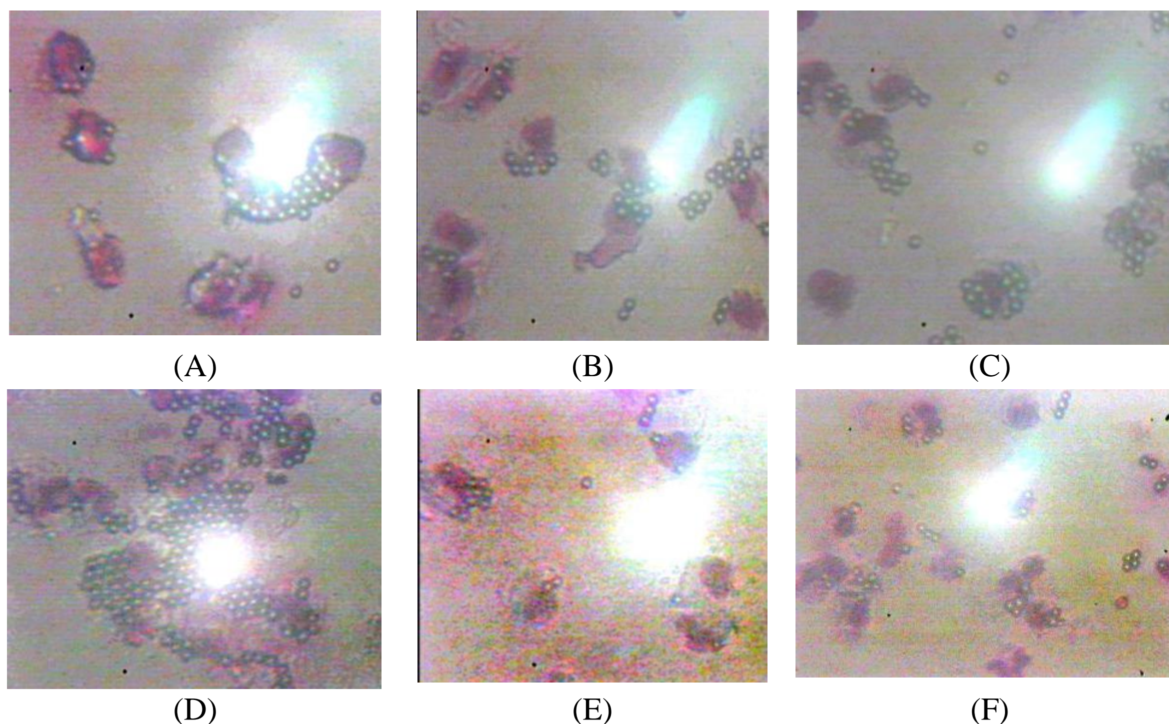


Figure 5. The phagocytocis of macrophages. Concentration 10 µg/ml Combination 1 (A); concentration10 µg/ml combination 2 (B); concentration 10 µg/ml combination 3 (C); concentration 10 µg/ml combination 4 (D); concentration 10 µg/ml Control DMSO (E); macrophages without treatment (F).

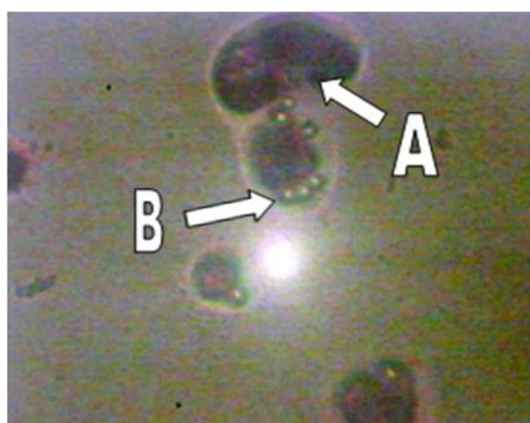


Figure 3. Macrophages was treated with extract combination 2, 1000 µg/ml. (A): Dead macrophage; (B): Latex beads was not opsonized by the macrophage

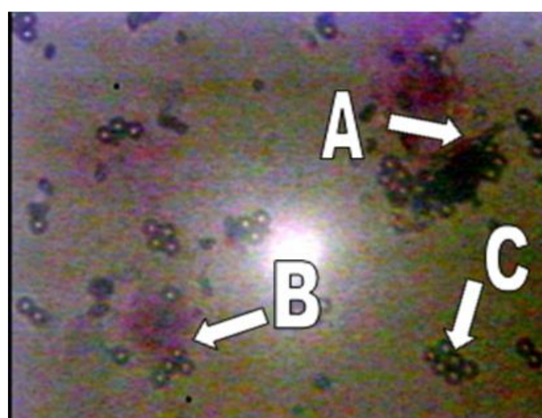


Figure 4. Macrophages were treated with extract combination 3; 1000 µg/ml. (A) Dead macrophage; (B) Macrophage which phagocytes latex beadsLatex beads

concentrations due to the content of flavonoid. Therefore, we might assume that the combination extracts could contain compounds that suppress the immunomodulatory activity at higher concentrations, where these effects do not occur at the optimum concentration.

Overall, it can be concluded that all combinations of extracts were able to increase the number of macrophages that phagocytes latex beads, and the number of latex beads phagocyted by active macrophages at the optimum concentration of 10 µg / ml (Figure 5, Figure 6, Figure 7, and Figure 8). The ability of phagocytic activity of macrophages in almost every combination is possible because the volume of each extract added to the treatment for each combination (Table II) is not significantly different as compared with the dose needed in humans (Table I). *Thyphonium flagelliforme* (Lodd.) Blume extract could increase cytokine expression, one of

them is IFNγ, which function is to activate macrophages(Shen and Louie, 2005), and *Phyllanthus niruri* Linn extract can increase phagocytosis and chemotaxis of macrophages (Galuh, 2008; Maat, 1996).

CONCLUSION

The formulations of combinations ethanol extracts of *Phyllanthus niruri* Linn, *Thyphonium flagelliforme* (Lodd.) Blume and *Piper crocatum* affected the phagocytic index as compared to negative controls with abilities that were significantly different for all combinations with a dose of 10 μg / ml so that the increase in a phagocytic index and phagocytic capacity value might be able to improve the nonspecific immune response.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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