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Preliminary Study on Immunomodulatory Effect of Sarang-Semut Tubers *Myrmecodia tuberosa* and *Myrmecodia pendens*

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Abstract: Problem Statement: Sarang-Semut tubers, *Myrmecodia tuberosa* and *M. pendens* (Rubiaceae) are both medicinal plants originating from Papua which have broad range of therapeutic values including those to improve vitality. Nevertheless, no scientific proof is available so far on their immunomodulatory effect. The purpose of this study was to reveal the potency of Sarang-Semut tubers as immunomodulatory agent by evaluating their effects on Balb/c mice lymphocytes proliferation and macrophage phagocytosis by *in vitro* techniques. **Approach:** Bioactivity assays were performed on the raw extracts and fractions. Extracts were obtained by macerating the pulverized samples in ethanol 95%, followed by successive fractionation to yield n-hexane, ethyl acetate and water fractions. Series of extracts and fractions were made to concentration 10, 20, 50 and 100 µg mL^{-1} by adding 0.5% Tween 80. Lymphocytes proliferation was observed by the MTT reduction method and analyzed using microplate reader at 550 nm. Macrophage phagocytosis activity was determined based on the number of latex beads uptake by the macrophage cells. **Results:** All extracts and fractions significantly increased the lymphocytes proliferation and macrophage phagocytosis activity in comparison to negative control. The ethyl acetate fraction of *M. pendens* (50 µg mL^{-1}) showed the highest activity in lymphocytes proliferation assay, but the highest macrophage phagocytic index was shown by *M. tuberosa* ethanol extract (50 µg mL^{-1}). **Conclusion:** Our study demonstrated that Sarang-Semut tubers are potential to be developed as immunomodulatory agents.

Key words: Immunomodulatory, *Myrmecodia tuberosa Myrmecodia pendens*, lymphocytes proliferation assay, macrophage phagocytosis

INTRODUCTION

Ant plant is widely used in West Papua as herb with broad range of therapeutic values. Local people categorize the plants into red and white "Sarang-Semut" (Indonesian name for Ant plants) of which the taxonomy were determined further as *M. pendens* and *M. tuberosa* (syn. *M. armata*), respectively. Both are used as part of herbal remedies for many medicinal purposes, starting from mild diseases such as nausea, to severe diseases such as breast cancer. They are used by boiling the dried cut tubers in water and consumed as tea.

According to Soeksmanto *et al*. (2010), Sarang-Semut plant lives as ephyphyte on plants such as cajuput (Melalueca), "Cemara gunung" (Casuarina), Kaha (Castanopsis) and Beech (Nothophagus). This plant, as other myrmecophyte lives in tropical forest is known to be engaged tightly with ants. It has specialized hollow structures, called domatia or nesting cavities to host ant colonies (Blatrix *et al*., 2009).

In mutualistic interactions, ants contribute to the host plant defense mechanism against herbivores (Dejean *et al*., 2009; González-Teuber and Heil, 2010), fungal pathogens (González-Teuber and Heil, 2010) and other plants to compete for living space (Heil *et al*., 2010).

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These plants are potential to be developed in modern herbal medicines because they can grow well as ephyphyte, therefore the exploitation will not endanger the environment.

Unfortunately, scientific proofs reported on this plant, both *M. tuberosa* and *M. pendens* are still lacking. Soeksmanto and collaborators (2010) has reported a potential cytotoxic activity of *M. pendens* against cancer cell lines, HeLa dan MCM-B2. The active fraction contained saponin, alkaloid, tannin and flavonoids (Soeksmanto *et al*., 2010). Another report on the bioactivity came from a related species *Hydnophytum formicarum* Jack (Prachayasittikul *et al*., 2008) which revealed the plant's antioxidant and antimicrobial activities.

Considering its wide range of therapeutic usage in traditional remedy including those to vitality, it is hypothesized the Sarang-Semut tubers effects as immunomodulatory agent. This research aims to reveal the ethanol extracts and fractions effects on Balb/c mice macrophage phagocytic activity and lymphocyte proliferation by *in vitro* methods and to do the phytochemical analyses of the extracts. It is interesting to find out whether there are differences in these two plants' immunomodulatory activities and chemical constituents.

MATERIALS AND METHODS

Samples preparation: Ant plants were collected from Babo, Bintuni, West Papua, Indonesia, on Februari-March 2010. Plants' species identification was performed by Mr. Heri Sujadmiko and Mrs. Ludmilla Fitri Untari (Laboratory of Plant Taxonomy, Faculty of Biology, Gadjah Mada University, Indonesia) with voucher specimen number 0209/T/Tb/VIII/2010.

Ant plant tubers were washed, cut and dried in oven (Memmert, Germany) at 40°-60°C, then ground to obtain dried pulverized samples. After immersing in ethanol 95% (technical grade, Brataco, Indonesia), the supernatant were evaporated by vacuum rotary evaporator (Heidolph® WE 2000, Germany) to obtain ethanol extracts.

The ethanol extracts were fractionated by using liquid-liquid partition with polarity gradient solvents as follows, n-hexane, ethyl acetate and water (technical grades, Brataco, Indonesia) to yield hexane, ethyl acetate and water fractions, respectively. Series of samples were made to concentration 10, 20, 50 and 100 µg mL^{-1} by using 0.5% Tween 80 (Merck, Germany).

Lymphocytes isolation and proliferation assay (Ediati *et al*., 2006): Spleen tissue was isolated aseptically from Balb/c mice (Centre for Integrated Research and Assay, Gadjah Mada University, Indonesia) and subsequently transferred to a 50 mm petri dish containing 10 mL of RPMI 1640 (Sigma-Aldrich, Germany) to yield lymphocytes suspension in medium. The suspension in 10 mL centrifuge tube was then centrifuged at 3,200 rpm 4°C for 4 min. Clumps were suspended in 5 mL Tris ammonium chloride buffer and left in room temperature for 15 minute. RPMI was added to reach 10 mL suspension and centrifugation was taken place at 3,200 rpm 4°C for 4 min. Clumps were separated from the supernatant, washed twice with RPMI and then diluted with complete medium. Lymphocytes cells were counted by hemocytometer (Neubaeur). The cells were then ready to be tested and were cultured in 37°C CO_2 incubator. Suspensions of lymphocytes cells in 100 µL medium (1.5×10^6 cells mL^{-1}) were distributed into 96-wells micro plates (Nunc). Into each well were added 10 µL of hepatitis B vaccine (Engerix®, GlaxoSmithKline), and incubation was taken place at 37°C for 24 h with 5% CO_2 flow (Heraeus®, Germany). After incubation, 100 µL of samples suspensions were added and incubation was continued for another 48h. Into each wells were added 10 µL of MTT 5 mg mL^{-1} [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (Merck, Germany), followed by 4h of incubation at 37°C. Viable cells will react with MTT to form purple color. Reagent stopper (10% SDS) in 50 µL of HCl 0.01 N was added into each wells. Optical densities were determined by using microplate reader (Bio-Rad Benchmark, Japan) at 550 nm. Positive control used was 10 µL of PHA 5 µg/µL (Phytohemagglutinin, Merck, Germany).

Macrophage isolation and phagocytosis activity with latex beads: Macrophages isolation technique was modified from Spilsbury *et al*. (1995). Macrophages were isolated from mice peritoneal fluid with ±10 mL of cold RPMI. Aliquot was centrifuged at 1,200 rpm 4°C for 10 min. About 3 mL of RPMI 1640 complete media (contains FBS 10% (v/v) was added to sediment clumps. Cells were counted by hemocytometer (Neubaeur) and then resuspended in complete medium to obtain cell suspension with 1.38×10^6 cells mL^{-1} density. Cell suspension was then inoculated on microtiter plate 24 wells (Nunc) which covered by round cover slips. Each well contains 200 µL suspension (2.8×10^5 cells). Cells were incubated in CO_2 5% incubator at 37°C for 30 min. Afterwards each well was washed with 250 µL complete medium three times,

followed by incubation for 2 h. Cells was then washed with RPMI 1640 twice and then 1 ml complete medium was added, followed by 24h incubation. Non specific phagocytic activity was performed *in vitro* according to Leijh *et al*. (1986) by using 3 µm *latex beads* (Sigma-Aldrich, Germany). Latex beads were resuspended in PBS to get concentration of 2.5×10^6 particles mL^{-1}. 24h-Cultured of peritoneal macrophages, were washed twice after washing with RPMI 1640. Latex suspension (200 µL/wells) and samples (200 µL/wells) were added into each wells and incubated in incubator CO_2 5% 37°C for 60 min. Cells were then washed with PBS three times to eliminate excess latex beads. After left to dry in room temperature, fixation with methanol was done for 30 sec. Afterwards methanol was aspired and cover slips were left to dry, followed by 2% Giemsa (v/v) staining (Merck, Germany) for 20 min. After being washed with distilled water, supernatant were aspired and wells were left to dry. The amount of macrophages which phagocyted the latex beads and latex beads consumed by the macrophages were counted under inverted microscope (Olympus, Germany) to calculate the macrophage phagocytic index. Positive control used was 10 µL LPS (Lipopolysaccharide, Sigma-Aldrich, Germany) 1 µg/µL.

Phytochemical analyses: Several TLC systems were evaluated to get the best separation (stationary phase used was silica 60 F254 precoated plates, Merck, Germany). UV 254 and 366 nm lamps were used to detect compounds with chromophores. Spraying reagents to detect the compounds were chosen based on functional groups (Spangenberg, 2008).

RESULTS

Result of lymphocytes proliferation assay of *M. tuberosa* and *M. pendens* showed dose dependant manner (Table 1-2). Statistical analyses ($p < 0.05$, n = 3) by non parametric Kruskal Wallis followed by Mann-Whitney methods showed significant differences. In general, *M. pendens* showed higher activity in comparison to *M. tuberosa*. The highest activities were observed in ethyl acetate fractions of each plant. Hexane fractions showed low activity but still significantly higher than negative control suggesting low stimulation of lymphocyte proliferation.

Macrophage phagocytosis assay of both plants showed similar pattern (Table 3-4). One way Anova followed by Tukey test showed significant differences among results ($p < 0.05$, n = 3). The highest activity was shown by the ethanol extracts at concentration 50 $\mu g\ mL^{-1}$.

Phytochemical analyses of the *M. pendens* tuber detected alkaloid, phenolics, and terpenoids compounds, while TLC profile of *M. tuberosa* indicated the absences of alkaloid, polihydroxy flavonoid and compounds with carbonyl groups, but gave positive results for terpenoids and phenolics. After comparing the TLC profile of both plant extracts, it was revealed that no similar spots were detected. This suggests different in chemical contents of both plants.

DISCUSSION

The need for control and balance in the stimulation and suppression of the immune system has urged the search of new substances with immunomodulatory activity from nature (Philippi *et al*., 2010). Modulation of the immune system can be shown by any changes involve induction, expression, amplification or inhibition of any part of phase in immune response (Alamgir and Uddin, 2010). Effect of a substance on immune response itself can be suppressive, tolerogenic or stimulatory (Krensky *et al*., 2006).

Many herbal medicines have already been reported to possess immunomodulatory effect. Compound such as aucubin (an iridoid), phenolics and flavonoids have been reported as potential immunostimulatory agents (Chiang *et al*., 2003), so does saponin (Lacaille-Dubois, 2005). As already been widely accepted, polyphenols such as flavonoids, and tannin are potential antioxidant substances. Anti oxidative compounds might contribute to the immunomodulatory effect by promoting changes in redox-sensitive signaling pathways involved in certain genes expression. These kind of changes will further influence several cell functions including the immune response (Ramiro-Puig and Castle, 2009). Propolis is an example of extract rich in antioxidant phenolics compounds, which is also a potential immunomodulatory agent (Syamsudin *et al*., 2009).

Study on immunomodulatory effect of Sarang-Semut tubers showed that the ethanol extracts were potential to stimulate non specific immune response (phagocytosis) but the effect on specific immunity was not as good as the ethyl acetate fraction. This was expressed by the results of lymphocyte proliferation assay. On the other hand, the ethyl acetate fractions can induce the lymphocyte proliferation but the activity on the macrophage phagocytosis was lower than that shown by the ethanol extracts.

In general, macrophages phagocytic activities in the presence of extracts and fractions were significantly stimulated in comparison to the negative control. Based on the fact that the macrophage plays an important role in generating the immune response, this result supports Sarang-Semut tubers as potential immunostimulatory agents. Nevertheless, since no dose dependant manner observed, mechanism of modulation might occurred, resulting reduced effects by higher dose exposure.

Phytochemical analyses of the extracts showed differences in TLC profiles. The presence of alkaloids, terpenoids, and phenolics of *M. pendens* were consistent to the result of phytochemical study reported by Soeksmanto and collaborators (2010). Since no polyhydroxy flavonoid and carbonyl groups detected in the extract of *M. tuberosa*, a presence of compounds such as polyhydroxy flavons or flavonols in this plant can be neglected. Flavonoid type of compound lacking of carbonyl groups in its structure i.e. deoxyanthocyanidin might occured.

Chiang *et al*., (2003) reported that triterpenoids such as oleanolic acid and ursolic acid were not a potent modulator for lymphocyte proliferation. These compounds are lipophilics, therefore they would be present in hexane fractions. This might explain lower activity of the hexane fractions of both plants in lymphocyte proliferation assay.

Considering that Iridomyrmex ants are reported to be involved in myrmecotrophy of Sarang-Semut plants (Beattie, 1989) a possibility of iridoid compound presence in the extracts cannot be put aside. Iridoids are monoterpenoids which have been reported as part of Iridomyrmex ant defense mechanism. This group of compounds plays an important role in plant defense against herbivores (Gálvez *et al*., 2005). As decribed by Beattie (1989), ant waste materials translocation to host plant do exist. In other reports, Solano and Dejean (2004) and Defossez *et al*. (2010) revealed that ants provide their host plant with nitrogen, resource which is relatively low in ephyhyte like Sarang-Semut plant. These phenomena suggest the possibility of finding ant's metabolites enrich the plant extract.

What is also interesting to note, that symbiosis in myrmecophytes is not only involving host plant and ants, but also include fungi living in domatia (nesting cavities of myrmecophytes) (Defossez *et al*., 2009; 2010). Mutualist ants play a role in the occurrence of this fungus. Therefore it is expected that the fungi are tightly related to ant-plants mutualism (Defossez *et al*., 2009). This kind of symbiotic relationship suggests the fungi contribution in providing useful chemical for plant host defense. As described by Medina and collaborators (2010), some fungi have capabilities to produce enzyme to degrade tannin into antioxidant phenolics. Furthermore, some fungi endophytes also contributed in plant host defense against pathogenic fungi (Ting *et al*., 2010). Further investigation is of course needed to assure the involvement of either fungi or ants or even other organisms in Sarang-Semut chemical contents enrichment.

Table 1: Effect of *M. tuberosa* extract and fractions on mice lymphocytes proliferation. Proliferation activity was expressed by the optical density at 550 nm.Negative control showed value of 0.02±0.00; positive control (PHA 5µg/µL, 10 µL): 0.01±0.01. Values are mean ± standard deviation; n = 3, $p < 0.05$

Samples	Optical density			
	10 µg mL^{-1}	20 µg mL^{-1}	50 µg mL^{-1}	100 µg mL^{-1}
Ethyl acetate fraction	0.04±0.01	0.06±0.01	0.14±0.02	0.31±0.02
n-Hexane fraction	0.05±0.01	0.04±0.01	0.04±0.01	0.12±0.01
Water fraction	0.03±0.00	0.03±0.01	0.05±0.00	0.09±0.01
Ethanol extract	0.04±0.00	0.04±0.01	0.06±0.02	0.15±0.01

Table 2: Effect of *M. pendens* extract and fractions on mice lymphocytes proliferation. Proliferation activity was expressed by the optical density at 550 nm. Negative control showed value of 0.02±0.00; positive control (PHA 5µg/µL, 10 µL): 0.01±0.01. Values are mean ± standard deviation; n = 3, $p < 0.05$

Samples	Optical density			
	10 µg mL^{-1}	20 µg mL^{-1}	50 µg mL^{-1}	100 µg mL^{-1}
Ethyl acetate fraction	0.10±0.01	0.13±0.01	0.24±0.01	0.45±0.02
n-Hexane fraction	0.06±0.01	0.09±0.02	0.11±0.04	0.21±0.04
Water fraction	0.10±0.03	0.09±0.01	0.18±0.02	0.35±0.06
Ethanol extract	0.09±0.02	0.12±0.02	0.16±0.01	0.31±0.06

Table 3: Effect of *M. tuberosa* extract and fractions in macrophage phagocytosis. Negative control showed value of 0.08±0.02; positive control (LPS 1µg/µL, 10 µL): 2.23±0.60. Values are mean ± standard deviation; n = 3, $p < 0.05$.

	Index			
Samples	10 µg mL^{-1}	20 µg mL^{-1}	50 µg mL^{-1}	100 µg mL^{-1}
Ethyl acetate fraction	4.64±0.78	2.76±0.28	3.30±0.53	3.61±0.39
n-Hexane fraction	2.30±0.03	4.17±0.59	4.73±0.11	2.39±0.14
Water fraction	3.78±0.15	3.06±0.24	2.29±0.08	1.99±0.13
Ethanol extract	4.48±0.18	2.24±0.02	5.61±0.39	2.64±0.07

Table 4: Effect of *M. tuberosa* extract and fractions in macrophage phagocytosis. Values are mean ± standard deviation; n = 3, $p < 0.05$. Negative control showed value of 0.08±0.02; positive control (LPS 1µg/µL, 10 µL): 2.23±0.60

	Index			
Samples	10 µg mL^{-1}	20 µg mL^{-1}	50 µg mL^{-1}	100 µg mL^{-1}
Ethyl acetate fraction	3.79±0.43	3.99±0.35	3.83±0.56	2.56±0.31
n-Hexane fraction	2.65±0.53	3.83±0.20	4.16±0.10	3.11±0.16
Water fraction	3.67±0.57	3.02±0.25	2.77±0.59	2.23±0.16
Ethanol extract	3.49±0.22	4.41±0.09	5.52±0.29	3.72±0.57

CONCLUSION

Sarang-Semut tubers significantly increased the Balb/c mice lymphocytes proliferation and macrophage phagocytosis activity. The ethyl acetate fraction of *M. pendens* (50 µg mL^{-1}) showed the highest activity in lymphocytes proliferation assay, but the highest macrophage phagocytic index was shown by *M. tuberosa* ethanol extract (50 µg mL^{-1}).

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