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The Potential of Wild Mango Leaves from Sumatera as the Immunostimulant Agent

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Abstract

Wild Mango from Sumatera is potentially new immunostimulant. It contains mangiferin, which is potentially become the immunostimulant candidate. This study aimed to prove the immunostimulatory effect of wild mango leaves extract on white rat peritoneum fluid induced by *Staphylococcus aureus* bacteria. The immunostimulatory effect was determined based on the activity and capacity of macrophage phagocytosis. This study used 54 male white rats about 130 g - 290 g. Based on the results,the highest phagocytosis activity of macrophage cells was found in ethanol extract of *Mangga Hutan* leaves dose 2 with a percentage of phagocytosis activity was 84%. The highest phagocytosis capacity of the macrophage cells was also found in ethanol extract of *Mangga Hutan* leaves dose 2 with an average phagocytic capacity of 171.67 from 50 active macrophages. Results of this study indicated that wild mango leaves from Sumatera has the potential immunostimulant activity. This indicates that the wild mango have the potential for therapeutic efficacy for the prevention of degenerative diseases caused by immune deficiencies.

How to Cite

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INTRODUCTION

The increase in the prevalence of degenerative diseases caused by immune deficiencies such as lupus, HIV, diabetes, obesity and hypertension is increasing every day due to nutritional imbalances and lifestyle of society nowadays. This condition leads to the requirement to find the sources of natural ingredients to be developed as new potential immunostimulants. Forest that provides plants as important immunostimulants in the pharmaceutical world is Sumatera's lowland rainforest. One of the potential plants is from genus of *Mangifera* (wild mango) which contains mangiferin as the immunostimulant candidate.

Immunostimulants act to strengthen weak immune systems and to moderate overactive immune systems (Patil *et al*., 2012). De and Chattopadhyay (2008) stated that mangiferin has the modulatory mechanisms for the inhibition to induced the death of T-cell that stimulated macrophage. El-Hawary and Raibeh (2014) investigation proved the possibility of using *M. indica* peels waste fornatural immunostimulant. Immunostimulant-related research on mango species is currently only focused on the type of cultivated mango, such as *M. indica* (Wei *et al*., 2011; Grace *et al*., 2013; Savant *et al*., 2014). However, there is no specific study for wild mango from Sumatera.

Wild mangoes are less valuable fruits and can not be found in traditional market. Though, this type of mango has a high antioxidant ability and is capable of increasing the body's immune system because of its distinctive sour taste with a strong aroma of turpentine. Based on the basic study of antioxidant activity (Fitmawati *et al*., 2018), the result showed that the antioxidant content of wild mango is very strong, especially on *M. magnifica, Mangifera* sp1. (*mangga bukit suligi*) and *Mangifera* sp2. (*mangga hutan*). This study aimed to prove the immunostimulatory effect of wild mango leaves extract on white rats peritoneal fluid induced by *Staphylococcus aureus* based on the activity and capacity of macrophage phagocytosis. This study could also become the pioneer research for wild mangoes species. This study of immunostimulants from wild mango to be interesting and is expected to contribute the information on new sources of immunostimulants.

METHODS

Materials used in this study were white rats (*Rattus norvergicus*), *Mangifera magnifica*, *Mangifera* sp1 (*mangga hutan*), *Mangifera* sp2 (*mangga bukit suligi*) and chemical materials such as ethanol, chloroform, isolates of *Staphylococcus aureus*, NaCl solution 0.8% and Giemsa dye.

In vivo Assay and Doses Determination for Animal Modelling

This study was an experimental study using 54 male white rats aged 3 months with a weighed about 130 g - 290 g. This study used a Randomized Block Design with 18 treatments and 3 replications, consisting of 3 control(control 0 with distilled water, positive control with immunomodulatory drugs (stimuno) and negative control with 1% Carboxymethylcelluose Natricum (CMC Na)

Determine the dose scale of wild mango leaves powder extract based on converting a human consumption dose of 110 ml and ethanol extract of wild mango leaves of 50 ml. This conversion dose was relevant to the weight of all samples multiplied to maximal volume or 200. Normal doses for animal modelling obtained frombody weigh (200g) divided with standart weigh (200g) multiplied with conversion doses to extract of leaves wild mango powder (1.98ml) and ethanol exttract of wild mango leaves (0.99ml). Serial doses given were0.5 (Dose 1), 1 (Dose 2), and 1.5 (Dose 3) that were adapted to the weight of the animal.

Bacteria Test

The tested bacteria were *Staphylococcus aureus* (SA) No. ATCC 12600. SA was embedded on Nutrient Mueller Hinton Broth (MHB) gel, then it was diluted on sterile peptone broth suspension using sterile syringe and subsequently incubated inside an incubator on 37^0C (Fitmawati *et al*., 2017).

Phagocytosis Test

On the 8th days, SA was injected to every treated rat intraperitoneally using the dose formula as follow: $\frac{BW}{SW} \times MD$

Note: BW: Body Weight (grams), SW: Standard Weight (200 grams), MD: Maximum Dose (0.5 mL).

The treated rats were subsequently anesthetized by chloroform and then abdominally dissected by using surgical instruments. The peritoneum fluid of rats was taken out by pipette and dropped on object glass to make smear preparation of samples. Before they were stained with Giemsa, they were fixed on object glass by using methanol, dried for 20 minutes and rinsedby flowing water. The smear of samples were observed under microscope using immersion oil

on 10x-100x magnification. The phagocytosis activity was determined according to the number of active cells on the phagocytosis process out of100 of phagocyte cells. The phagocytic capacity was established from the number of bacteria that has been ingested by 50 active-phagocytic- (Fitmawati *et al.*, 2017).

Data Analysis

The data obtained were analyzed using ANOVA (analysis of variance), with further testing with the Duncan Multiple Range Test (DMRT).The damage percentage was calculated using the formula based on Dey, (1991) and Wagner (1999).

RESULT AND DISCUSSION

The Ability of Peritoneal Fluid Macrophage-Phagocytises by Mango Leaf Extract

The term of phagocytosis in immunology known as the main mechanism for vanishing the pathogen from cells residues. In the test of immunostimulant effect of mango leaves extract, observations on the activity and capacity of phagocytic cells were carried out through an overview of peritoneal fluid smear. In the immunostimulant effectiveness test of mango leaves extract, the activity and capacity of macrophages (white blood cells) from peritoneal fluid was calculated. Macrophages in the animal's body play a role in phagocytosis of pathogens in the body. *S. aureus* was the pathogen that infected to the intraperitoneal route of white rats.

All treatments of mango leaves extract both powder and ethanol extract showed the macrophage phagocytic activity and capacity. In the control group, there was a significant

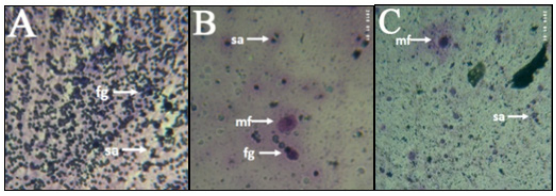

Figure 1. The overview of peritoneal fluid smear (A) Control + (stimuno), (B) Control 0 (distilled water), (C) Negative control (CMC Na 1%). Fg: Phagocytosis, mf: macrophage, sa: *S. aureus* bacteria

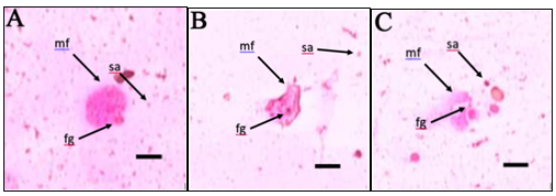

Figure 2. The overview of peritoneal fluid smear with the administration of *M.magnifica* leaves extract. (A) Dose 1, (B) Dose 2, (C) Dose 3. Fg: Phagocytosis, mf: macrophage, sa: *S. aureus* bacteria

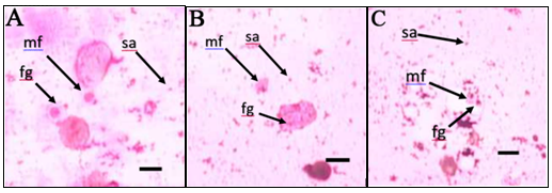

Figure 3. The overview of Peritoneal fluid smear with the administration of *Mangga Hutan* leaves extract. (A) Dose 1, (B) Dose 2, (C) Dose 3. Fg: Phagocytosis, mf: macrophage, sa: *S. aureus* bacteria.

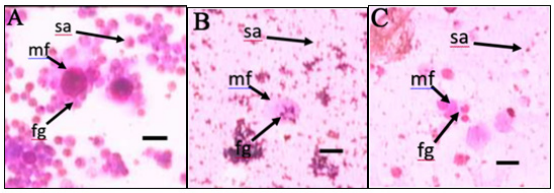

Figure 4. The overview of peritoneal fluid smear with the administration of *Mangga Bukit Suligi* leaves extract. (A) Dose 1, (B) Dose 2, (C) Dose 3. Fg: Phagocytosis, mf: macrophage, sa: *S. aureus* bacteria.

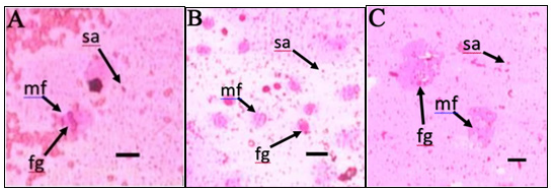

Figure 5. The overview of peritoneal fluid smear with the administration of ethanol extract of *M.magnifica* leaves. (A) Dose 1, (B) Dose 2, (C) Dose 3. Fg: Phagocytosis, mf: macrophage, sa: *S. aureus* bacteria.

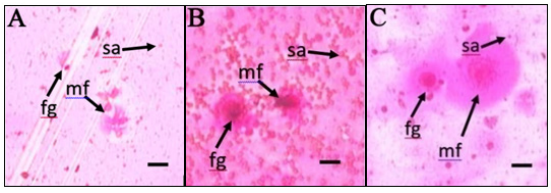

Figure 6. The overview of peritoneal fluid smear with the administration of ethanol extract of *Mangga Hutan* leaves. (A) Dose 1, (B) Dose 2, (C) Dose 3.Fg: Phagocytosis, mf: macrophage, sa: *S. aureus* bacteria

difference between the positive, normal and negative control.The percentage of macrophages that ingested tested bacterial cells was calculated per 100 cells of macrophages in each treatment group (Chairul & Praptiwi, 2007) this condition expressed as active macrophage cells. While, the macrophage phagocytic capacity is the mean value of the number of tested bacteria that were phagocytized by each active macrophage. The quantity of macrophage cells activity and capacity in phagocytosis was analyzed by calculating active phagocytic cells and phagocytized bacterial cells.

Macrophage Phagocytic Activity with the Administration of Mango Leaves Extract

The macrophage phagocytic activity can be seen through the number of phagocytic cells that phagocyte the other cells or particles such as bacteria. Quantity of macrophages activity as a result of mango leaves extract administration can be determined by analyzing the calculation of active macrophages and the tested bacterial cells phagocytized by macrophages.

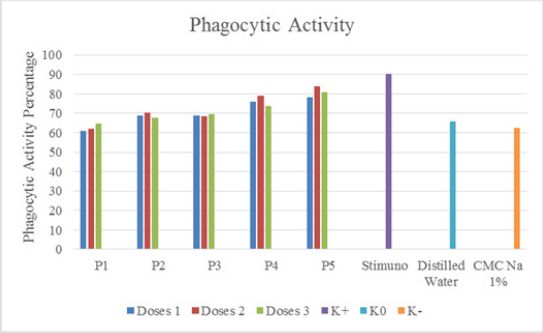

Figure 7. Macrophages phagocytic activity after treatment. P1: Leaves powder extract of *M. magnifica*, P2:Leaves powder extract of *Mangga Hutan*, P3: Leaves powder extract of *Mangga Bukit Suligi*, P4: Leaves ethanol extract of *M. magnifica*, P5: Leaves ethanol extract of *Mangga Hutan*. K+: Positive Control, K0: Control 0, K-: Negative Control.

The macrophage phagocytic activity values of all treatments after the addition of mango leaves both powder and ethanol extract show the difference numbers that arelower than positive controls (Figure 7). Positive control shows a higher phagocytic activity compared to the other treatments. However, the treatment of the ethanol extract of *Mangga Hutan* leaves at dose 2 shows a result that approaches the ability of phagocytic activity of macrophages inpositive control. In order to see the differences of study results between treatment groups, one-way ANOVA statistical test was carried out followed by DMRT test. The percentage of macrophage phagocytosis activity with the administration of mango leaves extract is presented on Table 1.

Table 1. The Average Percentage of Macrophage Phagocytic Activity after the Provision of Mango Leaves Extract

Treatments		Macrophage Phagocytic Activity (%)
Control	Distilled water(K0)	65.67±2.08^{ab}
	Stimuno (K+)	90.33±3.21^{h}
	CMC Na 1% (K-)	62.33±1.53^{a}
P1	Dose1	61±6.00^{a}
	Dose 2	62±4.36^{a}
	Dose 3	64.67±6.43^{ab}
P2	Dose 1	69±6.93^{abcd}
	Dose 2	70.33±4.62^{abcde}
	Dose 3	67.67±2.89^{abc}
P3	Dose 1	69±3.61^{abcd}
	Dose 2	68.33±8.08^{abc}
	Dose3	69.67±5.51^{abcde}
P4	Dose 1	76±4.58^{cdefg}
	Dose 2	79±0.00^{efg}
	Dose 3	73.67±8.08^{bcdef}
P5	Dose 1	78.33±1.15^{defg}
	Dose 2	84±6.56^{gh}
	Dose 3	81±5.57^{fg}

Notes: P1: Leaves powder extract of *M. magnifica,* P2: Leaves powder extract of *Mangga Hutan,* P3: Leaves powder extract of *Mangga Bukit Suligi*, P4: Leaves ethanol extract of *M. magnifica*, P5: Leaves ethanol extract of *Mangga Hutan*. The numbers which followed by different letters on the same column are significantly different on the level of confidence 5 %

The macrophage phagocytic activity with the addition of Mango leaves extract showed P value of 0.000. P value <0.05 means that there is a significant difference in the percentage of macrophage phagocytic activity with the treatment of the addition of mango leaves extract powder extract and ethanol extract in each treatment.

Table 1 shows that the highest macrophage phagocytic activity was seen from treatment with the addition of Stimuno (positive control)

by 90.33%. While the negative control treatment (CMC Na 1%) showed the lower phagocytic activity by 62.33%.The high phagocytic activity was also seen in the treatment with ethanol extract of *Mangga Hutan* dose 2 which were not significantly different from the addition of Stimuno (84%). This shows that the treatment with ethanol extract of *Mangga Hutan* dose 2 has a good ability in phagocytic activity and has the potential as an immunostimulant drug. Research by Kurnianingtyas *et al*. (2013) about the immunostimulant activity of *Polyscias obtuse* on Bone Marrow Broiler immunity after administration of *Salmonella typhimurium* with 3 serial doses showed that treatment at dose 2 (84%) had a higher immunomodulator activity in Bone Marrow Broiler than dose 1 (78,33%) and dose 3 (81%), so that the best dose used is a normal dose which is dose 2.

A high macrophage phagocytic activity is also seen from treatment with the addition of ethanol extract of *Mangga* Hutan leaves dose 2 (84%), ethanol extract of *Mangga Hutan* leaves dose 1 (79%) and 3 (81%) and respectively. The ability percentage of phagocytic activity given by the addition of ethanol extract of *Mangga Hutan* leaves dose 2, ethanol extract of *Mangga Hutan* leaves dose 2 and 3 was still lower compared to the stimuno as positive control. Phagocytosis activity that is too high is feared to cause undesirable side effects and causes damage to other cells.

The treatment with the addition of *M. magnifica* leaves powder extract showed less phagocytic activity compared to control 0 (distilled water). Dose 1 and 2 showed fewer bacterial cell phagocytosis compared to negative controls (giving CMC Na 1%) which were 61% and 62% respectively. Whereas dose 3 showed phagocytosis activity which was slightly higher than negative control, 64.67%. The addtion of the powder extract of *M. magnifica* leaves cannot be used as an immunomodulator because its phagocytic activity is lower than the positive control treatment (distilled water) and negative control (CMC Na 1%).

The treatment with the addition of powder extract of *Mangga Hutan* leaves showed that dose 2 (70,33%) had a higher percentage of phagocytic activity compared to dose 1 (69%) and 3 (67,67%). These three doses have a higher percentage of phagocytic activity than control 0 (Distiled water) and negative control (CMC Na 1%).

In the treatment by adding the powder extract of of *Mangga Bukit Suligi* leaves, it was seen that at dose 3 (69.67%) the percentage of phagocytosis activity was higher than dose 1 (69%) and dose 2 (68.33%). These three doses have a higher percentage of phagocytic activity than 0 (distilled water) and negative control (CMC Na 1%).

The utilization of mango leaves extract with a certain dose provides different advantages or indications on the ability of phagocytic activity. The use of mango leaves extract as a medicinal plant in all treatments with the correct dose could increase the phagocytic activity of macrophage. In addition, the use of appropriate doses of mango leaves extract in all treatments can be use as a new potential of immunomodulator source. According to Rifatul (2009), the negative impact of using traditional medicines can be avoided by using them in a correct way as well as by initially conduct both preclinically and clinically test as is done in chemical medicine.

The Capacity of Macrophage Cells Phagocitosis after the Provision of Mango Leaves Extract

In the observation of immunomodulator effect test of mango leaves extract, the capacity of phagocytic cells to affect tested bacteria will be seen through an overview of white rats peritoneal fluid smear preparations. Phagocytosis capacity was determined based on the number of *S. aureus* bacteria that were phagocytize ed by 50 active phagocytic cells.

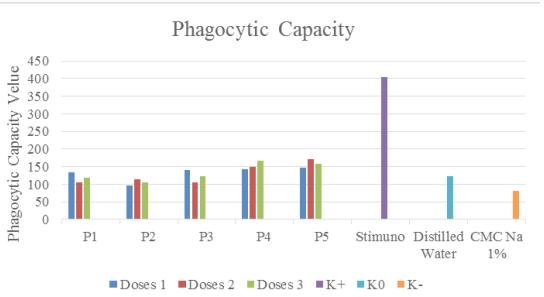

Figure 8. Macrophage phagocytic capacity. P1: Leaves powder extract of *M. magnifica*, P2: Leaves powder extract of *Mangga Hutan*, P3: Leaves powder extract of *Mangga Bukit Suligi*, P4: Leaves ethanol extract of *M. magnifica*, P5: Leaves ethanol extract of *Mangga Hutan*. K+: Positive Control, K0: Control 0, K-: Negative Control.

The macrophage phagocytic capacity of all treatments with Mangoesextractshowed lower numbers than positive control (Figure 8). The mean value of macrophage phagocyticcapacity is presented in Table 2.

Table 2. Mean Value of Macrophage Phagocytic

Capacity

Treatments		Phagocytosis Capacity
Control	Distilled water(K0)	122.33±4.04^{abcde}
	Stimuno (K+)	404.33±3.21^{g}
	CMC Na 1% (K-)	81.67±2.89^{a}
P1	Dose 1	134.33±68.08^{bcdef}
	Dose 2	104.33±29.37^{abc}
	Dose 3	119±1.00^{abcd}
P2	Dose 1	96.67±21.36^{ab}
	Dose 2	114.33±4.62^{abcd}
	Dose 3	105±8.66^{abc}
P3	Dose 1	141.33±27.75^{bcdef}
	Dose 2	104.67±31.39^{abc}
	Dose 3	122±9.85^{abcde}
P4	Dose 1	143±6.24^{bcdef}
	Dose 2	150±5.57^{cdef}
	Dose 3	167.67±39.26^{ef}
P5	Dose 1	147.67±16.17^{cdef}
	Dose 2	171.67±29.50^{f}
	Dose 3	159±6.59^{def}

Notes: P1: Leaves powder extract of *M. magnifica,* P2: Leaves powder extract of *Mangga Hutan*, P3: Leaves powder extract of *Mangga Bukit Suligi*, P4: Leaves ethanol extract of *M. magnifica*, P5: Leaves ethanol extract of *Mangga Hutan*. The numbers followed by different letters in each same column are significantly different at 5 % level of confidence

The phagocytic capacity of macrophage cells with the addition of Mango leaves extract obtained P value of 0.000. The value of P <0.05means that there is a significant difference in the mean value ofmacrophage phagocytic capacity of with the addition of Mango leaves extract in each treatment.

The positive control treatment (Stimuno) has a higher value of phagocytic capacity of macrophage compared to other treatments (Table 2). The value of phagocytic capacity of macrophage positive control treatment (stimuno) is 404.33 from 50 active macrophage cells. The highest macrophage phagocytic capacity with the addition ofmango leaves extract can be seen from the treatment with *Mangga Hutan* ethanol extract dose 2 (171.67 from 50 active macrophage cells). Therefore, the treatment is potential to be an immunomodulator because in the macrophage phagocytic capacity of the treatment is is the highest among all the treatment with mango extracts.

All treatments with the addition of Mango leaves in form ofpowder or ethanol extract shows the better macrophage phagocytic capacity score than the negative control (CMC Na 1%). Some of them also have a better macrophage phagocytic capacity score than the control 0 (distilled water) i.e. P1 dose 1, P3 dose 1, P4 dose 1, 2 and 3, and P5 dose 1, 2 and 3.

Comparison of Activity and Capacity of Macrophage Cell Phagocytosis

Immunostimulant effectiveness is influenced by the ability of macrophagephagocytic activity and capacity in body fluids of living things. If the value of phagocytic capacity is higher than the value of phagocytic activity, then the treatment with mango leavesextract is said to have the ability as an immunomodulator. Comparison macrophage cells phagocytic activity and capacity with the provision of several kinds of mango leaves extracts is presented in Figure 9.

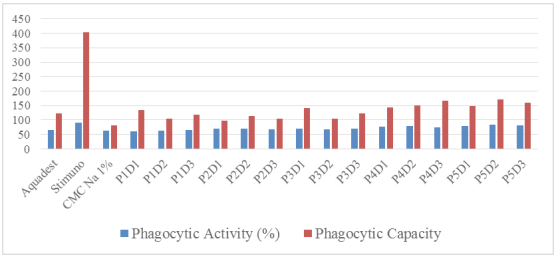

Figure 9. Comparison of Activity and Capacity of Macrophage Cell Phagocytosis. P1: Leaves powder extract of *M. magnifica*, P2:Leaves powder extract of *Mangga Hutan*, P3: Leaves powder extract of *Mangga Bukit Suligi*, P4: Leaves ethanol extract *M. magnifica*, P5: Leaves ethanol extract *Mangga Hutan*. D1: Dose 1, D2: Dose 2, D3: Dose 3.

The phagocytosis capacity of macrophage cells is higher than their phagocytosis activity. It means thatthatwild mangoes leaves are potential to be the new immunostimulant agent (Figure 9). Ethanol extract from *M. magnifica* and *Mangga Hutan* had a higher phagocytosis activity and capacity of compared to other wild mango leaf powder extract treatments. Based on a study by Shailajan *et al.* (2016) about immunostimulant agent from the hexane extract of cultivated mango leaves (*Mangifera indica)* in India, mango stimulates T-cells, macrophages, monocytes, neutrophils etc, which may increase the infiltration of macrophages to the inflammatory site.

This study is a first study about the possibility to produce immunostimulant agent from wild mangoes leaves extract. The wild mangoes are less of a public concern, but they have extraordinary potential abilities in the pharmaceutical industry similar to the cultivated mangoes.

This study provides the information that the extracts of wild mangoes leaves are non-toxic to the human immune cellsand able to modulate the cellular immune response. This finding generates its possible role in killing the microbes by inducing the innate immunity against them and significantly increasing themacrophages phagocyticactivity and capacity. Wild mangoes are rich sources of phytochemical constituents, which can induce the non-specific immune cells such as macrophages, natural killer cells and complementary functions. From this study, it isindicated that wild mangoes leaves from Sumatera have potential immunostimulant activity as well as therapeutic efficacy for the prevention of degenerative diseases caused by immune deficiencies.

CONCLUSION

The highest phagocytosis activity and capacity of macrophage cells were found in ethanol extract of *Mangga Hutan* leaves dose 2 with a percentage phagocytosis activity was 84 and an average phagocytic capacity of 171.67 from 50 active macrophages. The phagocytosis capacity of macrophage cells is higher than their phagocytosis activity which means that wild mango leaves are potential to be used as the new immunostimulant agent.

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