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RESEARCH ARTICLE

# Evaluation of phagocytic index and haematological parameters of *Sida rhombifolia* extracts in mice as immunomodulator

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## Abstract

**Background:** *Sida rhombifolia* was reported to have potential anti-hepatitis C virus activity. Anti-viral activities of plants were also reported to modulate immunity. **Objective:** This research was conducted by testing the immunomodulating activities of *Sida rhombifolia* extract. **Method:** This study evaluated cell viability in HepG2 culture cells to determine the 50% of cell viability. The Immunomodulatory activity was evaluated by examining the phagocytic index value and haematology profile. **Result:** The results showed that the $IC_{50}$ *Sida rhombifolia* was 32.5 µg/mL, while the $IC_{50}$ of $CCl_4$ as negative control was 16.68 µg/mL. Moreover, 100% of cell viability was observed at ½ $IC_{50}$ and ¼ $IC_{50}$ of *Sida rhombifolia*, indicating no toxic effect from the extract. Immunomodulatory assay found that *Sida rhombifolia* extract demonstrated an immunostimulant effect. The phagocytic index value of *Sida rhombifolia* was higher than that of the standard drug (a commercial immunostimulant product). Moreover, the haematology evaluation demonstrated no negative effect on haemoglobin, haematocrit and erythrocyte, which showed no difference in values compared to the normal control and the immunostimulant product. **Conclusion:** *Sida rhombifolia* plant extract has the potential as an immunomodulator candidate for treating hepatitis.

## Introduction

Weak immunity becomes an important factor in infectious diseases, including virus infection (Das, 2022). Strengthening the immune system is a strategy to defend from virus attacks. Several plants medicinal plants were reported to enhance the human immune system through various mechanisms (Zebeaman *et al*., 2023)

Indonesia has a large variety of natural resources, especially the diversity of plants used for medicinal plants. There are 40,000 species of medicinal plants in the world, 30,000 of which are widespread in Indonesia, with 9,600 species of them having known

medicinal properties. Since time immemorial, Indonesian people have used traditional medicinal ingredients for disease prevention, maintenance and health care (Arozal *et al.*, 2020; Silalahi *et al.*, 2015).

*Azadirachta indica* A., *Emblica officinalis*, *Picrorhiza kurroa* Royle, *Tinospora cordifolia*, *Moringa oleifera, Ocimum sanctum* L, and *Withania* are several plants that have been used to prevent the infection of novel viruses (Das, 2022). Many studies also reported the activity of medicinal plants as immunostimulants (Tanti Azizah *et al*., 2021; Zebeaman *et al*., 2023). *Echinacea purpurea* is a medicinal plant that has been used as a component in many commercial immunostimulant products. The plant was reported to possess immunostimulant, anti-inflammatory, anticancer, antimutagenic, antioxidant, antibacterial, antifungal, and antiviral activities. *E. purpurea* stimulates the immune system through phagocytosis activation, strengthening innate immunity and the stimulation of leukocyte mobility and fibroblasts. Interestingly, the plant also shows anti-inflammatory activity by reducing the production of pro-inflammatory cytokines through the inhibition of COX-1 and COX-2. Studies hinted that the biological substances responsible for the immunomodulatory activity of *E. purpurea* were alkamides, ketoalkenes, caffeic acid derivatives, polysaccharides, and glycoproteins. Other studies also reported the antiviral activities of certain *E. purpurea* extracts against *herpes simplex* virus 1 (HSV-1) and *herpes simplex* virus 2 (HSV-2), human immunodeficiency virus type 1 (HIV-1), influenza A2, and vesicular stomatitis virus *in vitro* (Manayi et al., 2015).

In this study, the plant *Sida rhombifolia* L., which belongs to the genus Sida and the family Malvaceae, was used. *S. rhombifolia* L. contains secondary metabolites of alkaloids, flavonoids, phenolic compounds, coumarins, phaeophytins, steroids and ecdysteroids (Abat *et al*., 2017). It was stated that metabolite compounds such as flavonoids, alkaloids, coumarins, and polyphenolic compounds were reported to have activity as an antiviral for hepatitis C (Wahyuni *et al.*, 2016). *S. rhombifolia* has also been reported to have hepatoprotective activity. The ethanolic extract of *S. rhombifolia* was proven to significantly decrease the toxin-induced elevated liver enzymes and repair liver damage (Dhalwal *et al.*, 2007).

This study aimed to investigate the immunomodulatory activities of *S. rhombifolia* in vivo model by examining the phagocytic index value and haematology profile compared to the group given the commercial immunostimulant product containing E. purpurea extract to seek a potential alternative to *E. purpurea*. Moreover, the cytotoxic evaluation of the hepatocyte cells was also measured to determine the doses.

## Methods

### *Preparation of sida rombifolia extract*

*Sida rhombifolia* leaves were obtained from Kediri Regency, East Java, which was determined by Materia Medika Indonesia Batu City. Leaves of *S. rhombifolia* L. were dried at room temperature. After drying, they were milled to obtain sidaguri leaf simplicia powder (*S. rhombifolia*). 600 g of powder was extracted by maceration with 70% of ethanol. After being allowed to stand for 24 hours, the filtrate and residue were separated by filtration using filter paper, a Buchner funnel, and a vacuum pump. The residue was conducted for remaceration with the same solvent twice. The filtrate was collected and then concentrated using a rotary evaporator (with a water bath temperature of 40°C and a rotation speed of 80 rpm) until a thick extract was obtained. Finally, this thick extract was put into the oven at 40°C until a constant weight was reached.

### *Ethical clearance*

The ethical clearance of this research has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, for in vitro research (Ref. No. KE/FK/1044/EC/2022) and in vivo research (Ref. No. KE/FK/1085/EC/2022).

### *HepG2 cell culture*

HepG2 cells were grown in Dulbecco's modified Eagle liquid medium and allowed to be 80-90% confluent. Cells were then harvested and replanted at a density of 5 x 106 in 3 mL of medium in a sterile 3.4 cm diameter petri dish and left overnight.

### *HepG2 cell viability assay*

HepG2 cells were grown on 96-well plates with a density of 5 x $10^6$ and incubated for 24 hours at 37°C. Plant extracts of *S. rhombifolia* were given to HepG2 cultures in the volume of 100 µL of each concentration (50, 10, 1, 0.1, and 0.01 µg/mL) and followed further incubation for 24 hours. 10µl of MTT reagent was added to each well and incubated for two to four hours. Then, the medium was discarded, and 100µl DMSO was added. The absorbance of the sample was measured at a wavelength of 550nm using the GloMax-Multi Microplate Multimode Reader (Promega). Percent cell viability was calculated against control. A growth

inhibition ($IC_{50}$) of 50% was determined using Probit analysis by making a relationship curve between the percent inhibition and the log dose (Wahyuni *et al*., 2018).

***Preparation of experimental animals***

The experimental animals used in this study were eight-week-old white mice weighing between 25 and 35 g. Before the study started, the mice were acclimatised for ten days. The mice were kept in standard cages with a temperature of 24-26°C, humidity of 60-65%, and a 12-hour light-dark cycle. The mice were housed in plastic cages measuring 40 x 20 x 12 $cm^3$ with a cover made of wire and a padded cage covered with rice husks. The cage was equipped with a place to eat and drink. One cage contains two mice. The cage is positioned on a multilevel stainless rack. The mice were given food and water, and libitum was provided in the cage.

***Immunomodulation test of S. rhombifolia extract***

The immunomodulatory effect test of the extract was carried out using the carbon removal method by measuring the absorbance using a UV-visible spectrophotometer. Thirty mice were divided into five treatment groups: 1) Group I (suspension extract at a dose of $IC_{50}$); 2) Group II (suspension extract at a dose of ½ $IC_{50}$); 3) Group III (suspension extract at a dose of ¼ $IC_{50}$); and 4) Group IV (positive control, given a commercial immunostimulant product containing 250 mg of *E.purpurea* extract and 10 mg of Zinc picolinate). Each group was given suspension orally once a day for seven consecutive days. On the eighth day, the tip of the mice's tail was cut. One ml of blood was drawn and put into a tube containing sodium citrate, then 25 µl of blood was taken, and 4 ml of 1% acetic acid was added to lyse red blood cells. This first extraction of blood was used as a blank (0 minutes). Carbon suspension was injected intravenously through a vein in the tail, and at 5, 10, 15, and 20 minutes after carbon injection, blood was collected in a tube containing sodium citrate. Then 25 µl of blood was taken, 4 ml of 1% acetic acid was added to each sample to lyse the red blood cells, then the absorbance was measured using a UV-visible spectrophotometer at a wavelength of 640.5 nm (Sahu *et al*., 2010). The phagocytosis constant was calculated with the following formula:

$$K = \frac{In\ OD1 - In\ OD2}{t2 - t1}$$

K: Phagocytosis constant; OD 1: Absorbance at 0 minutes; OD 2: Absorbance at 15 minutes; t: Time (0 and 15 minutes).

The haematology parameters, including haemoglobin, haematocrit, and erythrocytes, were examined. Twelve hours after blood collection, the mice were euthanised by injecting an overdose of ketamine-xylazine mixture (0.15ml/100gr body weight) into the outer thigh intramuscularly.

***Data analysis***

The data obtained were analysed and tested statistically using SPSS (Statistical Package for The Social Sciences) for Windows software. The data normality test uses either the Shapiro-Wilk test followed by one-way Analysis of Varian (ANOVA) and the *t*-test if the data is normally distributed, and if it is not normally distributed, the Kruskal-Wallis test followed by the Mann-Whitney test. The value of $p < 0.05$ is used to determine the level of significance.

## Results

**HepG2 cells viability assay**

Extract of *Sida rombifolia* showed no toxic effect; the 50% viability cell inhibition ($IC_{50}$) value was 32.5µg/ml. Further evaluation at the dose of ½ $IC_{50}$ and ¼ $IC_{50}$ were obtained, with a percentage of cell viability of 100%. While the positive control of $CCL_4$ treated-HepG2 was obtained, the $IC_{50}$ value was 16.68 µg/ml. It indicates that S. rombifolia did not reveal any toxic effect.

**Phagocytocytic assay**

The phagocytic assay was conducted with the concentration of extracts ½ $IC_{50}$ and ¼ $IC_{50}$. The dose was obtained by converting the in vitro dose to the in vivo dose based on the blood volume of mice.

The phagocytic index after administration of *Sida rhombifolia* extract can be seen in Table I. Administration of an $IC_{50}$ dose of *Sida rhombifolia* extract gave a higher phagocytic index value compared to the positive control group. All of the treated doses revealed a higher phagocytic index compared to the positive control group even though statistically, only the ½ $IC_{50}$ and ¼ $IC_{50}$ doses were significantly higher than the positive control group with *P* values of 0.0144 and 0.0064, respectively. The lowest phagocytosis index was seen in the group given *Sida rhombifolia* extract at a dose of $IC_{50}$ and the highest at a dose of ¼ $IC_{50}$.

**Table I: Phagocytic index value of mice in gradient concentration of *Sida rhombifolia***

| Group | N (number of samples) | Phagocytic index (mean ± standard deviation) |
|---|---|---|
| IC50 | 5 | 0.20 ± 0.04 |
| ½ IC50 | 5 | 0.21 ± 0.07 |
| ¼ IC50 | 5 | 0.22 ± 0.03 |
| Positive control | 5 | 0.04 ± 0.02 |

**Haematological parameter**

The haematological parameters were measured to examine the effect of Sida rhombifolia extract on the profile of haemoglobin, haematocrit and erythrocytes. The results demonstrated that there is no significant difference between the treated groups and the normal control. Moreover, it also revealed a similar profile with standard, commercial immunostimulants used in this study.

The haematological indices of treated and control mice are presented in Table II. Haematocrit, Erythrocytes, and Leukocytes increased in the treated group compared to the control group.

**Table II: Haematology result of mice in gradient concentration of *Sida rhombifolia***

| Haematology | Group | N (number of samples) | Value (mean ± standard deviation) |
|---|---|---|---|
| Haemoglobin (gr/dL) | IC50 | 5 | 15.66 ± 1.21 |
| | ½ IC50 | 5 | 15.88 ± 1.31 |
| | ¼ IC50 | 5 | 15.68 ± 1.85 |
| | Positive control | 5 | 16.08 ± 0.15 |
| Haematocrit (%) | IC50 | 5 | 46.76 ± 3.10 |
| | ½ IC50 | 5 | 46.70 ± 2.98 |
| | ¼ IC50 | 5 | 46.47 ± 4.50 |
| | Positive control | 5 | 49.74 ± 0.40 |
| Erythrocytes (106/µL) | IC50 | 5 | 9.20 ± 0.91 |
| | ½ IC50 | 5 | 9.70 ± 0.56 |
| | ¼ IC50 | 5 | 9.33 ± 1.16 |
| | Positive control | 5 | 9.18 ± 0.43 |

## Discussion

A viability test was carried out to determine the dose of 50% growth inhibition ($IC_{50}$). This test is needed as a preliminary evaluation to determine the safety of the extract to supply basic information for in vivo study. Extracts that have a low $IC_{50}$ value are assumed to have a high degree of toxicity because only a small amount or low concentration is needed to kill 50% of the total cell population. Extracts with low $IC_{50}$ values have the potential to contain highly active compounds that provide strong effects at low concentrations. Nevertheless, the extract still needs to be tested with lower concentration levels than the $IC_{50}$ concentration to find a concentration that is effective and does not cause cell death (Gola, 2019).

The results of the HepG2 cell viability test using ½ and ¼ $IC_{50}$ values in the extracts showed that the *Sida rhombifolia* plant extracts had 100% viability values. This value means that the extract is safe to use in HepG2 cell culture. The $IC_{50}$ values of the *Sida rhombifolia* extracts were also relatively low so that in the future, the immunomodulator ability of the extracts could be tested in vitro using graded concentrations of $IC_{50}$, ½ $IC_{50}$, and ¼ $IC_{50}$. However, to know for sure the safety of a given extract, it is necessary to carry out additional tests.

The systemic ability of the extract to immunomodulate (involving interactions of all body systems and feedback/homeostatic mechanisms) was tested in experimental animals using the carbon clearance assay method. Carbon clearance in the body of the mouse involves phagocytic cells and is reflected in the phagocytic index. The phagocytic index represents the capacity of the extract to modulate phagocytes to provide an immune response in the form of phagocytic activity against antigens/foreign molecules (carbon) that enter the circulation of experimental animals. A high phagocytosis index value indicates that the extract has good potential as an immunomodulator (Sahu *et al*., 2010; Tanti Azizah *et al*., 2021). In this study,

administration of *Sida rhombifolia* at a dose of $IC_{50}$ resulted in a higher phagocytosis index compared to the group given a commercial immunomodulator product. This shows that the *Sida rhombifolia* extract has good immunomodulation potential. However, further research is needed to confirm these results. The administration of the extract also did not change the haematological parameters evaluated in this study, indicating that the given extract was not toxic to the haemopoietic system (Ladokun *et al*., 2015).

Among the prevention mechanisms that are also stated in the traditional healthcare systems of different parts of the world, enhancing the human immune system is one solution to reduce the increasing incidence of diseases and deaths. Immunotherapies using plant-sourced phytochemicals are now receiving attention to combat the spread of cancer, autoimmune disease, and infection (Trinh *et al*., 2020; Zebeaman *et al*., 2023).

Many studies have investigated various pharmacological activities of *Sida rhombifolia*. The plant demonstrated anti-tumour activities by inducing apoptosis in HepG2 cells (Ahmadi *et al*., 2022). The n-hexane extract of *Sida rhombifolia* also showed high anti-inflammatory, cytotoxicity, and anti-cholinesterase activities (Mah *et al*., 2017). The plant has also been used as an herbal medicine against a number of diseases, such as hypertension, diabetes, stomach pain, diarrhoea, swelling, broken bones, cuts, herpes, and ulcers (Debalke *et al*., 2018). Due to its various functions, research on the plant is far-reaching. The plant has the potential to be developed into a natural product or supplement, as shown by this study. However, before targeting product development, extensive research on a certain pharmacological activity of the plant should be conducted first to provide conclusive evidence of the effectiveness of the plant in exerting its specific actions.

## Conclusion

*Sida rhombifolia* plant extract has the potential to act as an immunomodulator and could be a prospective drug candidate for complementary treatment of infection.

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