Immunomodulatory Effects of Cermai Leaves (Phyllanthus acidus (L.) Skeels) Ethanol Extract on Normal Male Rats and Cyclophosphamide Induction

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Abstract

BACKGROUND: Ethanol extract of Phyllanthus acidus (L.) Skeels leaf contains flavonoids and saponins that were widely used as herbal medicinal plant. The present study focused on exploring the biological potential as well as immunomodulatory effects.

AIM: The present study aimed to investigate the immunomodulatory effects of P. acidus extract on total and differential leukocyte count, antibody titer, delayed-type hypersensitivity (DTH) response in normal male rats, and immunosuppressed male rats.

METHODS: P. acidus extract was obtained by maceration technique by using ethanol as a solvent. Standardization of P. acidus and phytochemical screening includes examination of alkaloids, flavonoids, glycosides, saponins, tannins, and steroids/triterpenoids.

RESULTS: Phytochemical screenings were showed alkaloids, flavonoids, glycosides, saponins, tannins, and steroids/triterpenoids. The trial animals were male rats which were divided into two large groups, namely normal group and immunosuppressed group. All animals were given extract on the 1st day until day 14. The immunomodulatory effect was analyzed by evaluating total leukocytes, leukocyte differential, antibody titer value, and the volume of rat paws.

CONCLUSION: The results indicated that the P. acidus extract had immunomodulatory effects and its potential to be developed as immunomodulator agent.

Introduction

The immune system is the body’s defense mechanism in charge of responding or responding to attacks from outside our body. When an attack occurs, usually antigens in the body will begin to stimulate the immune system. This mechanism will protect the body from attacks by various microorganisms such as bacteria, viruses, fungi, and various germs that cause disease [1].

Antigens are substances that the immune system can recognize and bind to. Antigens can come from organisms (bacteria, viruses, fungi, and parasites) or molecules foreign to the body [2]. The immune system or the body’s defense is related to antibodies. Antibodies or immunoglobulins are a group of proteins formed by plasma cells (B-cell proliferation) due to contact with antigens. In this study, the antigen is 2% sheep red blood cells (SRBC) [3]. T cells with specific receptors on their surface are stimulated by the appropriate antigen and secrete substances called lymphokines. The stimulated lymphocytes undergo transformation into large ones such as lymphoblasts that are able to damage target cells that have receptors on their surface so that tissue damage can occur [4]. Levamisole suspension at a dose of 25 mg/Kg bw was used as a positive control because of the mechanism of action of levamisole which affects defense by regulating cellular immune responses, including the function of leukocytes, macrophages, and T cells [5].

Cyclophosphamide is an alkylating agent antineoplastic drug that is commonly used to treat cancer, especially blood cancers such as lymphoma, multiple myeloma, or leukemia. This drug is generally used in combination therapy with other chemotherapeutic agents, for example, with thalidomide. However, the use of cyclophosphamide has some side effects lymphocyte proliferation and CD4+/CD8+ ratio as well as increasing tumor necrosis factor-a, interleukin (IL)-1a, and IL-10 cytokines [6]. Immune system activity tests can be carried out by various methods, including by looking at phagocytic activity using the carbon clearance method, total leukocyte count and leukocyte differential, delayed-type hypersensitivity (DTH) response, and antibody titer hemagglutination test [7].
A substance that can correct an imbalance in the immune system is called an immunomodulator. This material plays a role in protecting the body from foreign objects that enter so that body functions are not disturbed. Immunostimulant is a substance that acts as an immune enhancer or enhancer that can be obtained by using herbs that are efficacious as immunostimulants [7].

Phyllanthus acidus is one of the Indonesia’s leading medicinal plants; this can be seen from the benefits and effectiveness of these medicinal plants in curing several diseases, including allergic asthma. Based on the chemical research, it is known that P. acidus is a plant that is rich in various chemical constituents, including flavonoids, tannins, and saponins. Flavonoids in P. acidus leaves can provide immunomodulatory effects by increasing IL-12 activity and lymphocyte proliferation. CD4+ cells will affect lymphocyte proliferation and then cause Th-1 cells to be activated. In addition, the study showed that P. acidus extract could inhibit bacterial growth [8]. This study aims to determine the immunomodulatory effects of P. acidus extract on rats induced cyclophosphamide.

**Methods**

**Materials**

The materials used in this study were carboxymethyl cellulose (Na CMC; Sigma, USA), ethanol 96% (Smart Lab, Indonesia), Phosphate buffer saline (PBS; Sigma, USA), cyclophosphamide (Cyclovid®; Novel, Indonesia), hydrochloric acid (Mallinckrodt, USA), Mayer’s reagent (Mitra kimia, Indonesia), Bourchardat (Mitra kimia, Indonesia), Zn powder (Fisons Scientific Equipment, England), concentrated sulfuric acid (Mallinckrodt, USA), Molisch reagent (Rofa Laboratorium Centre, Indonesia), iron (III) chloride reagent (Merck, Germany), Liebermann Burchat reagent (Mitra kimia, Indonesia), distilled water, levamisole (Askamex®; Soho, Indonesia), and aquades.

**Animal**

Male Wistar rats weighing 150–200 g were used and acclimated for 7 days with water ad libitum. The procedure was evaluated by Animal Research Ethics Committees, University of Sumatera Utara (Ethic No. 0511/KEPH-FMIPA/2021).

**Plant**

Sampling was carried out purposely without comparing with the same plants from other areas. The P. acidus leaves were obtained from Tanjung Balai, North Sumatra (2.9659° N, 99.7984° E).

**Preparation of extract**

The leaves were dried, ground, then extracted by maceration method using ethanol as the solvent. Briefly, the dried leaves material (1.2 kg of P. acidus simplicia) was macerated with ethanol (1:10 b/v). Then, the solvent was evaporated using a rotary evaporator to yield ethanol extract of P. acidus (120 g) [9].

**Standardization of Phyllanthus acidus**

Standardization of P. acidus includes the determination of water content, water-soluble extract content, ethanol-soluble extract, total ash content, and acid insoluble ash content [10].

**Phytochemical screening**

Phytochemical screening includes examination of alkaloids, flavonoids, glycosides, saponins, tannins, and steroids/triterpenoids [11].

**Total leukocyte count and leukocyte differential**

The study was performed in normal and cyclophosphamide induced rats. Animals were divided into 6 groups for each condition, including normal control rats without any treatment, Na CMC 0.5% suspension, P. acidus extract 100 mg/kg BW, P. acidus extract 200 mg/kg BW, and 400 mg/Kg BW, levamisole 25 mg/kg BW was used as a positive control. P. acidus extract suspension in Na CMC 0.5% was orally administered to animals once a day until 14 days [12]. Meanwhile, the negative control group received vehicle only (Na CMC 0.5%). On day 4, the animals were injected by intraperitoneal injection 0.1 ml of 2% SRBC in all groups. Cyclophosphamide (70 mg/kg BW) was used to suppress the immune response as reported in a previous study [11]. Cyclophosphamide was administered on days 8, and 13 in immune-suppressed groups. On day 14, the blood leukocyte cell count and leukocyte cell differential were measured.

**Antibody titer test**

P. acidus extract suspension in Na CMC 0.5% and levamisole 25 mg/kg BW used as a positive control were orally administered to animals once a day until 14 days [12]. Meanwhile, the negative control group received vehicle only (Na CMC 0.5%). On day 4, the animals were injected by intraperitoneal injection 0.1 ml of 2% SRBC in all groups. Cyclophosphamide (70 mg/kg BW) was used to suppress the immune
response as reported in a previous study [12]. Cyclophosphamide was administered on days 8, and 13 in immune-suppressed groups. On day 14, blood samples from each rat were taken through a vein in the tail and the serum was transferred. The antibody titer value was determined by hemagglutination technique [13]. The antibody titer value was determined based on the last dilution at which the antibody was still detectable by visually visible hemagglutination. The antibody titer value was then transformed with (2log [titer]+1) [14].

Delayed-type hypersensitivity

P. acidus extract suspension in Na CMC 0.5% and levamisole 25 mg/kg BW used as a positive control were orally administered to animals once a day until 14 days. Meanwhile, the negative control group received vehicle only (Na CMC 0.5%). On day 4, the animals were injected by intraperitoneal injection 0.1 ml of 2% SRBC in all groups. Cyclophosphamide (70 mg/kg BW) was used to suppress the immune response, as reported in a previous study. Cyclophosphamide was administered on days 8, and 13 in immune-suppressed groups. On the 14th day, the volume of the rat's right foot was measured which had previously been marked with a volume measurement limit using a marker, the volume of the rat's paw was measured as the initial volume (V0). Re-injected 2% SRBC intraplantar as much as 0.1 ml into the sole of the right foot. Rat paw volume was measured on day 15 (after 24 h) and expressed by the mean increase of paw volume [15].

Results

Standardization of Phyllanthus acidus

The results of P. acidus standardization include the determination of water content, water-soluble extract content, ethanol-soluble extract content, total ash content, and acid insoluble ash content are shown in Table 1.

Table 1: The results of Phyllanthus acidus standardization

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Parameter</th>
<th>Result (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Determination of water content</td>
<td>6.68</td>
</tr>
<tr>
<td>2</td>
<td>Determination of water-soluble extract content</td>
<td>37.98</td>
</tr>
<tr>
<td>3</td>
<td>Determination of ethanol-soluble extract content</td>
<td>21.99</td>
</tr>
<tr>
<td>4</td>
<td>Determination of total ash content</td>
<td>8.47</td>
</tr>
<tr>
<td>5</td>
<td>Determination of acid insoluble ash content</td>
<td>2.65</td>
</tr>
</tbody>
</table>

Table 1 shows that P. acidus has a water content of 6.68%, water-soluble extract content of 37.98%, ethanol-soluble extract content of 21.99%, total ash content of 8.47%, and insoluble ash content acid 2.65%. The water content is determined to maintain the quality of the leaves because it is associated with fungal growth, if the water content exceeds 10%, it can be a good medium for microbial growth so that the quality of the leaves decreases. The determination of the water-soluble extract content was to determine the levels of polar chemical compounds contained in P. acidus. The determination of the total ash content aims to determine the external and internal mineral content from the initial process until the extract is formed. The presence of low-acid insoluble ash content indicates the presence of sand or other impurities in low levels [15].

Phytochemical screening results of Phyllanthus acidus extract

The results of total phenol and total flavonoid content of the extract are shown in Table 2.

Table 2: Phyllanthus acidus ethanol extract phytochemical screening results

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Secondary metabolic compound</th>
<th>Result (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Glycoside</td>
<td>+</td>
</tr>
</tbody>
</table>

Information: (−): Absent, (+): Contains a group of compounds

Table 2 shows that P. acidus extract contains a class of compounds of alkaloids, flavonoids, saponins, tannins, glycosides, and steroids/triterpenoids.

Total leukocytes

This study showed the immunomodulatory activity of P. acidus extract to increase the number of leukocytes. The effect of P. acidus extract on total leukocytes number from normal and cyclophosphamide-induced rats is shown in Table 3.

Table 3: The effect of Phyllanthus acidus extract on total leukocytes number from normal and cyclophosphamide-induced rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Total leukocytes 10⁹/L (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without cyclophosphamide induction</td>
</tr>
<tr>
<td>Normal control</td>
<td>12.30 ± 0.84*</td>
</tr>
<tr>
<td>Negative control</td>
<td>4.91 ± 0.70</td>
</tr>
<tr>
<td>Levamisole</td>
<td>11.62 ± 0.93*</td>
</tr>
<tr>
<td>P. acidus extract 100 mg/kg BW</td>
<td>9.75 ± 0.81*</td>
</tr>
<tr>
<td>P. acidus extract 200 mg/kg BW</td>
<td>10.49 ± 1.08*</td>
</tr>
<tr>
<td>P. acidus extract 400 mg/kg BW</td>
<td>11.36 ± 0.64*</td>
</tr>
</tbody>
</table>

Information: *p < 0.05 indicates significant differences compared to the negative control group (Na CMC 0.5%). P. acidus: Phyllanthus acidus, SD: Standard deviation, BW: Body Weight, Na-CMC: Carboxymethyl Cellulose Sodium.

Table 3 shows that in the group of rats that were not induced with cyclophosphamide (normal group) P. acidus extract 100 mg/kg bw, 200 mg/kg bw, and 400 mg/kg bw were significantly different from the negative control group (p < 0.05). In the group of rats induced with cyclophosphamide (immunosuppressed group) P. acidus extract dose of 100 mg/kg bw, 200 mg/kg bw, and 400 mg/kg bw were significantly different from the negative control groups (p < 0.05). P. acidus extract doses of 100, 200 mg/kg bw, and 400 mg/kg bw were able to increase total leukocytes in normal rats and immunosuppressed rats [16].
Differential leukocyte

There are five parameters that represent white blood cell differentiation, namely neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The effect of *P. acidus* extract on differential leukocyte counts from normal rats is shown in Table 4.

Table 4: The effect of *Phyllanthus acidus* extract on differential leukocyte counts from normal rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Leukocyte differential (mean ± SD)</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Basophil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>6.74 ± 0.93</td>
<td>2.90 ± 0.61</td>
<td>0.66 ± 0.19</td>
<td>0.33 ± 0.25</td>
<td>1.67 ± 0.56</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>3.42 ± 0.82</td>
<td>0.87 ± 0.49</td>
<td>0.03 ± 0.02</td>
<td>0.18 ± 0.12</td>
<td>0.41 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>6.93 ± 0.94</td>
<td>2.67 ± 1.16</td>
<td>0.59 ± 0.19</td>
<td>0.22 ± 0.05</td>
<td>1.23 ± 0.55</td>
<td></td>
</tr>
<tr>
<td><em>P. acidus</em> extract 100 mg/kg BW</td>
<td>7.21 ± 1.13</td>
<td>2.02 ± 0.72</td>
<td>0.20 ± 0.09</td>
<td>0.16 ± 0.11</td>
<td>0.91 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>200 mg/kg BW</td>
<td>7.21 ± 1.13</td>
<td>2.02 ± 0.72</td>
<td>0.20 ± 0.09</td>
<td>0.16 ± 0.11</td>
<td>0.91 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>400 mg/kg BW</td>
<td>7.21 ± 1.13</td>
<td>2.02 ± 0.72</td>
<td>0.20 ± 0.09</td>
<td>0.16 ± 0.11</td>
<td>0.91 ± 0.42</td>
<td></td>
</tr>
</tbody>
</table>

The effect of *P. acidus* extract on differential leukocyte counts from cyclophosphamide-induced rats is shown in Table 5.

Table 5: The effect of *Phyllanthus acidus* extract on differential leukocyte counts from cyclophosphamide-induced rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Leukocyte differential (mean ± SD)</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Basophil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>6.74 ± 0.93</td>
<td>2.90 ± 0.61</td>
<td>0.66 ± 0.19</td>
<td>0.33 ± 0.25</td>
<td>1.67 ± 0.56</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>3.42 ± 0.82</td>
<td>0.87 ± 0.49</td>
<td>0.03 ± 0.02</td>
<td>0.18 ± 0.12</td>
<td>0.41 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>6.93 ± 0.94</td>
<td>2.67 ± 1.16</td>
<td>0.59 ± 0.19</td>
<td>0.22 ± 0.05</td>
<td>1.23 ± 0.55</td>
<td></td>
</tr>
<tr>
<td><em>P. acidus</em> extract 100 mg/kg BW</td>
<td>7.21 ± 1.13</td>
<td>2.02 ± 0.72</td>
<td>0.20 ± 0.09</td>
<td>0.16 ± 0.11</td>
<td>0.91 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>200 mg/kg BW</td>
<td>7.21 ± 1.13</td>
<td>2.02 ± 0.72</td>
<td>0.20 ± 0.09</td>
<td>0.16 ± 0.11</td>
<td>0.91 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>400 mg/kg BW</td>
<td>7.21 ± 1.13</td>
<td>2.02 ± 0.72</td>
<td>0.20 ± 0.09</td>
<td>0.16 ± 0.11</td>
<td>0.91 ± 0.42</td>
<td></td>
</tr>
</tbody>
</table>

Antibody titer test

The effect of *P. acidus* extract on antibody titer value from normal and cyclophosphamide-induced rats is shown in Table 6.

Table 6: The effect of *Phyllanthus acidus* extract on antibody titer value from normal and cyclophosphamide-induced rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Antibody titer value (mean ± SD)</th>
<th>Without cyclophosphamide</th>
<th>Induced by cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>3.05 ± 0.33*</td>
<td>3.05 ± 0.33*</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>1.84 ± 0.50</td>
<td>1.72 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>5.09 ± 0.33*</td>
<td>5.09 ± 0.50*</td>
<td></td>
</tr>
<tr>
<td><em>P. acidus</em> extract 100 mg/kg BW</td>
<td>3.77 ± 0.34*</td>
<td>3.65 ± 0.33*</td>
<td></td>
</tr>
<tr>
<td>200 mg/kg BW</td>
<td>4.25 ± 0.33*</td>
<td>3.77 ± 0.33*</td>
<td></td>
</tr>
<tr>
<td>400 mg/kg BW</td>
<td>4.73 ± 0.27*</td>
<td>4.37 ± 0.33*</td>
<td></td>
</tr>
</tbody>
</table>

Table 6 shows that *P. acidus* extract doses of 100 mg/kg, 200 mg/kg bw, and 400 mg/kg bw were significantly different from the negative control groups (p < 0.05) on normal and cyclophosphamide-induced rats.

Delayed-type hypersensitivity test

The volume of swelling of the rat's feet was determined based on the difference between the volume of a certain time (Vt) and the initial volume (V0). The effect of *P. acidus* extract on DTH response of normal and cyclophosphamide-induced rats is shown in Table 7.

Table 7: The effect of *Phyllanthus acidus* extract on delayed-type hypersensitivity response of normal and cyclophosphamide induced rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat's paws volume (mean ± SD)</th>
<th>Without cyclophosphamide</th>
<th>Induced by cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>1.90 ± 0.71*</td>
<td>1.90 ± 0.71*</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>0.67 ± 0.27</td>
<td>0.41 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>1.83 ± 0.69*</td>
<td>1.72 ± 0.74*</td>
<td></td>
</tr>
<tr>
<td><em>P. acidus</em> extract 100 mg/kg BW</td>
<td>1.37 ± 0.72*</td>
<td>1.28 ± 1.19*</td>
<td></td>
</tr>
<tr>
<td>200 mg/kg BW</td>
<td>1.67 ± 1.02*</td>
<td>1.47 ± 0.74*</td>
<td></td>
</tr>
<tr>
<td>400 mg/kg BW</td>
<td>1.75 ± 1.05*</td>
<td>1.63 ± 0.86*</td>
<td></td>
</tr>
</tbody>
</table>

Table 7 shows that *P. acidus* extract group at dose 100 mg/kg bw, 200 mg/kg bw, and 400 mg/kg bw of normal and cyclophosphamide induced rats were significantly different with the negative group (p < 0.05).

Discussion

*P. acidus* (L.) plant is one of the Indonesia's leading medicinal plants; this can be seen from the benefits and effectiveness of these medicinal plants in curing several diseases, including allergic asthma. *P. acidus* (L.) is a plant that has rich of various chemical constituents, including flavonoids, tannins, and saponins. Flavonoids in *P. acidus* can provide immunomodulatory effects by increasing IL-12 activity and lymphocyte proliferation. CD4+ cells will affect lymphocyte proliferation and then cause Th-1 cells to be activated. In addition, the study showed that ceremai leaf extract could inhibit bacterial growth [16].

The in vivo studies on immune response were conducted to investigate the immunomodulatory effects of *P. acidus* extract on increasing leukocyte count, antibody titer value, and DTH response. Wistar rats were used and divided into two groups, including normal rats and cyclophosphamide-induced rats. Cyclophosphamide was used to suppress the immune responses of test animals to evaluate the ability of *P. acidus* extract to enhance the host defense in immune-suppressed conditions.
Cyclophosphamide is widely used as an anticancer agent and also used to treat autoimmune diseases. This drug can suppress the immune response by acting on both cyclic and intermitotic cells, thus causes depletion of immune cells. In this study, cyclophosphamide suppressed the immune responses, this was because cyclophosphamide was a compound that can reduce an excessive immune response. Cyclophosphamide is able to inhibit the transcription of cytokines and destroy T cells that stimulate B cells to form antibodies that play a role in the body's immune defense system and decreased leukocyte count, antibody titer value, DTH response [16].

The ethanol extract of P. acidus was able to enhance leukocyte count, antibody titer value, and the paw volume of rats after being injected by 2% SRBC. The enhanced leukocyte count, antibody titer value, DTH response indicates the immunomodulatory effect of ethanol extract of P. acidus in normal and cyclophosphamide-induced.

Leukocytes are the mobile/active unit of the body's defense system. The benefit of these leukocytes is that most of these cells are specifically transported to areas of severe inflammation, so they provide a rapid and powerful defense against possible infectious agents [17]. The increase in the number of leukocytes is because ethanol extract of P. acidus contains flavonoid and saponins compounds that have the ability to increase the immunomodulatory system by increasing the effectiveness of the proliferation of lymphokines produced by T cells so that this situation stimulates phagocytic cells to carry out phagocytic responses and will increase leukocyte production [18].

Hemagglutination is the binding of red blood cells as antigens with antibodies to form a visible clot. The increase in antibody titer values occurs due to increased activation of Th cells that stimulate B cells to produce antibodies and increased activation of B cells in antibody formation [19]. The increase in antibody titer value was because P. acidus extract contain flavonoid compounds. The flavonoids were involved in the differentiation of B cells into antibody-producing plasma cells [20].

The DTH response is a cellular immune response that involves the activation of Th cells, which will release pro-inflammatory cytokines and increase macrophage activity which is characterized by swelling of the animal's legs [21]. In summary, P. acidus extract has immunomodulator activity by increasing total of leukocytes, differential leukocytes, and hypersensitivity delayed-type.

References

17. PMid:19786984
PMid:10421421
PMid:26103628