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# The Immunostimulant Effects of Alang-Alang (*Imperata cylindrica*) Roots Extract on BALB/c Male Mice (*Mus musculus*)

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**ABSTRACT**
Alang-alang (*Imperata cylindrica* L.) is an annual rhizomatous grass and traditionally well known for its therapeutic values, especially in treating fever, muscle soreness, nosebleed and respiratory asphyxia. It has been reported to contain different classes of secondary metabolites and attracted scientists' attention to assess its biological and pharmacological activities. Previous studies have shown that the roots extract of *I. cylindrica* possess antibacterial, anti-inflammatory, antihypertensive, anti-helminthic, antidiuretic, and antioxidant activities. While the objective of this research was to explore the immunostimulant potential of *I. cylindrica* roots extract by evaluating its effects on the BALB/c male mice immune system. A completely randomized design was used in this research with 5 treatments and 4 replications for each treatment. P0 was the negative or no-treatment control group; P1—P4 consisted of mice given *I. cylindrica* roots extract 0 mg/25 g BW (P1), 6.25 mg/25 g BW (P2), 12.5 mg/25 g BW (P3), and 18.75 mg/25 g BW (P4) daily for two-weeks and intraperitoneally injected with 1 % bovine red blood cell (BRBC) antigen suspension on day-15. The effects of the extract on total leukocyte count, differential leukocyte counts, and relative spleen weight measurement were studied in the mice, while activity on humoral antibody titer was evaluated in vitro. The results showed that all treated groups to which *I. cylindrica* was administrated had a greater count of leukocytes and P3 and P4 were demonstrated statistically significant differences ($p<0.05$) as compared to control groups. The effect of the extract in the number of lymphocytes was similar, whereas the number of monocyte and neutrophil of treatment groups was numerically increased but not statistically significant. It also substantially increased the humoral antibody titer value and relative spleen weight of the treatment groups. Overall, these investigations revealed the potential of the roots extract of *I. cylindrica* as an immunostimulant.

***Keywords:*** *Humoral antibody, Immunostimulant, Imperata cylindrical, Leukocytes.*

## 1. INTRODUCTION

Indonesia possesses a large fraction of the world's biological diversity, second only to Brazil. There are more than 30,000 species of plants grown in the country and around 9,600 among them are traditionally well known to have medicinal benefits. However, out of these only 200 medicinal plant species are utilized for drug production [1]. Therefore, it is highly important to explore and assess these superabundant medicinal resources. Moreover, although modern medicine is highly available in Indonesia, the medicinal plant has been utilized as an immunostimulant by Indonesians for a long time and still maintain their popularity for historical and cultural reasons. Up to 59 percent of Indonesia's population rely heavily on this traditional medicine to prevent, treat and cure their diseases [2].

A great number of medicinal plants can be used as immunostimulants—Chemical compounds that nourish our immune system to prevent, treat and cure diseases. It works by increasing the stimulation and proliferation of leukocytes from hematopoietic stem cells, enhancing the activity of phagocytes cells as well as the

components of humoral immunity particularly by increasing the B cells production that is responsible for antibodies secretion [3, 4, 5]. The exposure of animals to immunostimulant consumption before infection will bolster their immunity against harmful or lethal infectious diseases and therefore, some compounds with immunostimulatory effects are also utilized as adjuvants to increase vaccine effectiveness [6].

Alang-alang (*Imperata cylindrica* L.) is a perennial rhizomatous herb identic as a weed in the agricultural fields and spreads widely in Indonesia. However, the plant's roots contain various beneficial secondary metabolites and are widely used as traditional medicine particularly in treating fever, muscle soreness, nosebleed, and respiratory asphyxia making a large number of researchers attracted to scientifically explore its medicinal benefits [7]. The roots have been reported to contain different classes of phytochemicals including alkaloid, polysaccharide, flavonoid, glycoside, protein, saponin, steroid, and tannin. Previous studies have been reported its biological and pharmacological activities such as antibacterial, anti-inflammatory, antihypertensive, anti-helminthic, antidiuretic, and antioxidant [8-13]. While this research aims to investigate the immunostimulant potential of *I. Cylindrica* roots extract by evaluating its effects on the BALB/c male mice's immune system.

## 2. MATERIAL AND METHOD

This study used 20 white male mice (9 weeks old) of BALB/c strain weighing 26.5±0.21 g which were supplied by the Veterinary Medicine Faculty of USK. The mice were housed in groups of four per plastic cage (48 x 36 x 14 cm) equipped with a wire cover at the top and filled with chaff at the bottom. They were fed on standard commercial pellets and water ad libitum. Upon arriving at the laboratory, mice were acclimated to the standard animal facility, Microtechnique Laboratory, Biology Department, Mathematics and Natural Science Faculty, USK for seven days [14]. All animal care and experimental procedures were supervised by experts from the Biology Department.

A completely randomized design was used in this research with 5 treatments and 4 replications for each treatment. The treatments were, P1 or positive control (0 mg/25 g body weight), P2 (6.25 mg/25 g BW), P3 (12.5 mg/25 g BW), P4 (18.75 mg/25 g BW), and P0 or negative control. The mice in the negative or no-treatment control group were treated identically except the experimental manipulation (were not given *I. cylindrica* roots crude extract and were not injected with BRBC antigen). The mice in the positive control group were not given *I. cylindrica* roots crude extract but injected with 1 % BRBC antigen suspension. The mice in the P2 group were given 6.25 mg/25g BW extract + 1 % BRBC injection. The mice in the P3 group were given 12.5 mg/25g BW extract + 1 % BRBC injection. The mice in the P4 group were given 18.75 mg/25g BW extract + 1 % BRBC injection. The crude extract was administered orally by gavage once per day for two weeks while the 1% BRBC antigen suspension was administrated intraperitoneally on day 15 [17].

The fresh plant material (the roots of *I. cylindrica*) was collected from Matangkuli, Aceh Utara, properly washed in running tap water and followed by a final rinse in distilled water, chopped and subjected to three days of sun drying and then pulverized into a powder with a sterile electric blender. The powder (200 g) was then extracted in boiling water ($dH_2O$) for eight hours on a small flame. The resulting mixture (crude extract) was then filtered and the collected filtrate was stored [15, 16].

BRBC was used as an antigenic material and the making of it was started by collecting 500 ml of bovine's blood. Fresh bovine's blood, withdrawn immediately after slaughter in a local slaughterhouse, was collected in a thermos containing 100 ml sodium citrate (anticoagulant) and ice. Erythrocytes were washed in NaCl saline solution by the same amount (1:1) and weighed to obtain a balanced position and followed by high-speed centrifugation at 2,000 rpm speed for 15 minutes. The resulting supernatant was then discarded and these processes were repeated three times to obtain the erythrocytes. The collected antigenic material was then diluted in physiological NaCl solution (1:99) to make one percent BRBC suspension and finally stored in the fridge for antibody titer test [17].

The effects of the extract on total leukocytes count, differential leukocyte counts, and relative spleen weight measurement was studied in the mice, while activity on humoral antibody titer was evaluated in vitro. The total and differential WBC count (lymphocyte, monocyte, and neutrophil) were visually verified by microscopic evaluation of the stained blood films at a magnification of 400X (oil immersion). In generating an adequate approximation of the white blood cell count, five areas were scanned and the average number of leukocytes seen per high-power field was multiplied by 2,000/$mm^3$. These peripheral film preparations and microscopic evaluations were following the procedures used in the examination of the peripheral blood film and correlation with the complete blood count by Maedel and Doig [18] and were done before treatment and 14 days post-treatment [4]. Determination of the activity on humoral antibody titer was begun by injecting mice with 1 % BRBC suspension intraperitoneally on day 15. The next

day, blood samples (0.5 ml) were collected from the tail vein of each mouse of all groups. Afterward, the blood samples were centrifuged and the serum was separated. The levels of antibodies were then monitored using the hemagglutination (HA) method [19]. Spleen organ relative weight was the last parameter that was assessed in this immunostimulant study. On day 16, this parameter was done firstly by sacrificing the mice. They were killed by cervical dislocation. The spleen was cleaned from connective tissue and blood. Then, it was put into the container containing PBS (pH 7.4). Finally, the weight of the organ was measured by analytical weighing [20].

The results of these investigations were statistically analyzed with SPSS 20.0. After the distribution and homogeneity results were checked, ANOVA was run to detect whether at least one significant difference exists between the treatments. An LSD (Least Significant Difference)-post hoc test was conducted afterward to determine where the significant differences were [21].

## 3. RESULTS AND DISCUSSION

**Table 1.** Effect of *I. cylindrica* on total leukocytes.

| | Total Leukocytes Counts ($10^3$ cell/mm$^3$) | | |
|---|---|---|---|
| | Day-0 | Day-14 | $\Delta_{Day-14 - Day-0}$ |
| P0 | 5.00±0.26 | 6.30±0.50 | 1.30$^{a}$±0.41 |
| P1 | 5.20±0.63 | 6.60±0.38 | 1.40$^{a}$±0.26 |
| P2 | 5.70±0.25 | 9.90±0.62 | 4.20$^{a,b}$±0.68 |
| P3 | 6.00±0.63 | 12.50±0.47 | 6.50$^{b}$±0.53 |
| P4 | 5.80±0.26 | 12.40±0.43 | 6.60$^{b}$±0.53 |

Note: Values are expressed as mean±SD from 4 mice in each group. Group bearing different superscript letters are significantly different according to the LSD test ($p<0.05$).

**Table 2.** Effect of *I. cylindrica* on differential leukocytes.

| | Differential Leukocytes Counts ($10^3$ cell/mm$^3$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day-0 | | | Day-14 | | | $\Delta_{Day-14 - Day-0}$ | | |
| | M | N | L | M | N | L | M | N | L |
| P0 | 0.7±0.10 | 1.5±0.19 | 2.8±0.28 | 1.1±0.10 | 1.9±0.19 | 3.3±0.30 | 0.4±0.16 | 0.4±0.16 | 0.5$^{b}$±0.25 |
| P1 | 0.7±0.19 | 1.6±0.16 | 2.9±0.38 | 1.1±0.10 | 2.0±0.23 | 3.5±0.19 | 0.4±0.16 | 0.4±0.16 | 0.6$^{a}$±0.26 |
| P2 | 0.7±0.10 | 1.6±0.16 | 3.4±0.26 | 1.2±0.16 | 2.1±0.25 | 6.6±0.26 | 0.5±0.19 | 0.5±0.19 | 3.2$^{b}$±0.43 |
| P3 | 0.8±0.16 | 1.5±0.19 | 3.7±0.44 | 1.5±0.19 | 2.2±0.25 | 8.8±0.58 | 0.7±0.25 | 0.7±0.25 | 5.1$^{b}$±0.30 |
| P4 | 0.7±0.19 | 1.5±0.19 | 3.6±0.16 | 1.5±0.25 | 2.5±0.19 | 8.4±0.40 | 0.8±0.36 | 1.0±0.34 | 4.8$^{b}$±0.54 |

Notes: Values are expressed as mean±SD from 4 mice in each group. Group bearing different superscript letters are significantly different according to the LSD test ($p<0.05$). M = monocyte; N = neutrophil; and L = lymphocyte.

The effects of *I. cylindrica* on total leukocytes and differential leukocytes are presented in Table 1 and 2 respectively. The average count of total leukocytes in the blood of negative and positive control groups of mice after 14 days were 1.30±0.41 and 1.40±0.26 x $10^3$ cell/mm$^3$ respectively while the average value of total leukocytes in the blood of treatment groups (P2, P3, and P4) of mice after 14 days were 4.20±0.68, 6.50±0.53 and 6.60±0.53 x $10^3$ cell/mm$^3$ respectively. All treated groups to which *I. cylindrica* roots extract was orally administrated had a greater count of leukocytes after 14 days and two groups (P3 and P4) were demonstrated to have a statistically significant differences ($p<0.05$) with control groups. In differential leukocytes studies, the effect of the extract in the number of lymphocytes was similar, whereas the number of monocyte and neutrophil in the blood of treatment groups of mice after 14 days were numerically increased by the administration of *I. cylindrica* but not statistically significant as compared with the control groups.

Moreover, it is important to note that the count of lymphocyte, monocyte, and neutrophil in this study were in the range of normal limits reported by Arrington [22] for healthy mice.

These results indicate that the crude extract of roots of *I. cylindrica* displays stimulatory effects on the immune system by enhancing white blood cells proliferation, particularly for lymphocyte. The rise in lymphocyte number during the trial probably resulted from the flavonoid content of *I. cylindrica*. This biologically active compound can act as a highly potent mitogen in stimulating the antigen-independent lymphocyte proliferation in healthy animals [5, 19, 23]. No statistically significant increase of monocyte and neutrophil number during the trial was positive because the rise of these types of white blood cells occurs in response to infection and tissue damage [24, 25]. The results of these animal clinical studies could also explain the blood-related functions of the plant extract, determine the ability of mice's immune system to prevent, treat and cure diseases, and offer a higher predictive value for clinical studies on humans [4, 5]. Therefore, the rise in white blood cells counts in these studies revealed the potential of the roots extract of *I. cylindrica* as an immunostimulant. The administration of an immunostimulant will enhance white blood cells proliferation and the rise in leukocytes number indicate that the immune system of the test animals had been stimulated [4,5]. The stimulatory effects of *I. cylindrica* were then further determined by measuring the concentration of antibodies generated from the activation, proliferation, and differentiation of B lymphocytes in response to the antigenic material injection and recording the changes of spleen-one of the main organs responsible for immunological defense mechanism [26].

HA titer was measured in individual mice after the immunization of them with 1 % BRBC suspension, and the level of it is expressed as the reciprocal of the highest dilution of BRBC antigen showing distinct and persistent agglutination [27]. The effect of *I. cylindrica* and BRBC suspension on mice's humoral immune response is shown in Table 3. The average antibody titer of mice in the negative and positive control groups were 1.00±0.00 and 4.75±0.96 respectively while the antibody titer value of mice in the treatment groups were 6.75±1.50, 8.25±2.36, and 10.00±1.41 respectively.

**Table 3.** Effect of *I. cylindrica* on antibody titer.

| | Antibody Titer [2(log titer)+1] |
|---|---|
| P0 | $1.00^{a}$±0.00 |
| P1 | $4.75^{b}$±0.96 |
| P2 | $6.75^{c}$±1.50 |
| P3 | $8.25^{d}$±2.36 |
| P4 | $10.00^{d}$±1.41 |

Notes: Values are expressed as mean±SD from 4 mice in each group. Group bearing different superscript letters are significantly different according to the LSD test ($p<0.05$).

This study revealed that the oral administration of *I. cylindrica* roots extract to mice generated a substantial increase in the production of HA titer in response against the BRBC antigen suspension as compared with the control groups, suggesting that the greater administration of the dosage of *I. cylindrica*, the higher the immunostimulatory potency is and indicated an enhanced antibody synthesis and macrophage and T cell activation [19]. The antibody formed in response to antigenic challenge and the cells highly responsible for agglutination or antibody formation is B lymphocytes [19, 26].

In response to the injection of BRBC antigen, these cells were activated, proliferated, and differentiated into plasma cells and secrete specific antibodies therefore strengthened the role of *I. cylindrica* in the humoral immune competence of mice. As presented in the table 3, the titer antibody in the blood of treatment groups of mice was 5 to 10 times greater in correlation with the titer measured in the blood of control groups of mice. These findings are supported by Owen *et al*. [19]. According to the authors, the humoral immune response against the antigenic material injection causes an interaction between B lymphocytes and the antigens. This B cells-foreign antigens interaction will be activated, proliferated B lymphocytes into plasma cells which generate specific protective antibodies against the injected antigen. These specific antibodies will then eliminate the BRBC antigen through agglutination. This antibody-mediated immune response will also generate various types of T lymphocytes including cytokines producing T lymphocyte cells. When released, these proteins will orchestrate the recruitment and activation of other immune cells including monocytes and macrophages to eliminate the antigen. A diagram explaining these humoral and cell-mediated immune responses can be found in Owen *et al*. [19].

The last assessments of the stimulatory effect of *I. cylindrica* on mice immune system were done by measuring the changes in spleen weight relative to the body. Spleen, the largest mass of lymphoid tissue in the body, plays a highly important role in defense mechanisms and is responsible for producing lymphocytes and antibodies [4,28]. The average spleen relative weight of mice in the negative and positive

control groups were 0.499±0.010 and 0.501±0.010 mg respectively while the relative weight of mice in the treatment groups were 0.559±0.001, 0.604±0.003, and 0.613±0.011 mg respectively (Table 4).

As presented in the Table 4, all treated groups to which *I. cylindrica* roots extract was administrated had a

**Table 4.** Effect of I. cylindrica on spleen

| | Body weight (mg) | Spleen weight (mg) | Spleen relative weight (%) |
|---|---|---|---|
| P0 | 28.05±0.192 | 0.140±0.002 | 0.499$^{a}$±0.010 |
| P1 | 28.11±0.245 | 0.141±0.002 | 0.501$^{a}$±0.010 |
| P2 | 28.23±0.443 | 0.158±0.003 | 0.559$^{b}$±0.001 |
| P3 | 31.08±0.457 | 0.188±0.003 | 0.604$^{c}$±0.003 |
| P4 | 31.10±0.770 | 0.191±0.003 | 0.613$^{c}$±0.011 |

Notes: Values are expressed as mean±SD from 4 mice in each group. Group bearing different superscript letters are significantly different according to the LSD test ($p<0.05$).

greater relative weight of spleen after 14 days as compared to control groups. The differences were also statistically significant ($p<0.05$) and were directly proportional with the significant increase of lymphocyte number and antibody level of treatment groups in the leukocytes and HA titer studies. It is also important to note that the weight of the spleen organ in all mice was within the physiological limit of normal BALB/c mice (≤0.2 g) suggested by Gay [29].

Spleen is an important organ of the immune system and studies on immunostimulants show that a close relationship exists between the increase in spleen weight and the immune competence of the tested animals. In human and animal clinical studies, the changes in organ weight relative to body weight compared to controls are important indicators of test-article effect and are widely utilized parameters in reflecting the organ's developmental status [4, 28, 30]. These findings also agree with the results of the two previous studies and demonstrated that a close relationship exists among the increase in the number of lymphocytes, antibody concentration, and spleen relative weight. According to Young *et al*. [26], as part of the surveillance process of the immune system, B and T lymphocyte cells continuously circulate and transit between various lymphoid and nonlymphoid tissues of the body including the spleen through the circulatory and lymphatic vascular systems, and B lymphocytes may settle down and develop into long-lived antibody-producing plasma cells. The enhancing activity of these reactive cells may increase the weight of the spleen as observed in this study.

Overall, it can be concluded from these studies that the stimulatory influence of *I. cylindrica* roots crude extract on mice immune system was understood by at least four means, by significantly increasing the total leukocytes and the lymphocyte numbers, by significantly increasing humoral antibody titer value and by substantially increased relative spleen weight of mice. This study also revealed that the greater administration of the dosage of *I. cylindrica*, the higher the immunostimulatory potency is and indicated an enhanced antibody synthesis and macrophage and T cell activation. Moreover, because of the immune system complexity, further immunostimulant investigation by in vitro lymphocyte proliferation and other macrophage phagocytosis assays are essential to be investigated.

## AUTHORS' CONTRIBUTIONS

Conceptualization, all authors.; methodology, all authors.; Investigation, F.M.; Resources, all authors.; Formal analysis, F.M.; writing—original draft preparation, F.M.; Supervision, I.M.R., R.R. and K.E.; Visualization, F.M. All authors have read and agreed to the published version of the manuscript.

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