GTLabs

Research Article

Immunostimulating effect of jackbean flour on non-specific immunity of mice in vitro and in silico

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ABSTRACT

Background: Jackbean (*Canavalia ensiformis*) contains protein and secondary metabolite compounds, such as phenolics and flavonoids that can have potential as antioxidants, antibacterial, antimicrobial, and antiviral.

Objectives: This study aims to determine the effect of giving jackbean flour as an immunostimulant on non-specific immunity of mice in-vitro and in-silico.

Methods: This type of research is double methods research with a comparative method and uses an experimental design in the form of a Randomized Group Design and in-silico testing with the NCBI website. Mice with a total of 16 heads were divided into 4 treatment groups, namely the Negative Control group (P0), Dose 1 (P1) with a solution of jackbean flour as much as 2.3 g, Dose 2 (P2) as much as 4.6 g, Dose 3 (P3) as much as 9.2 g. The hemagglutination test was carried out on mice with a total of 16 mice. The hemagglutination test was carried out on the 15th day after giving the flour solution, then tested the content of active compounds in koro pedang in silico according to the literature review of previous research from the website which contains active compounds, namely canavanine, concanavalin A and B.

Results: The results of in-vitro research show that the most influential dose for hemagglutination is P1 with a dose of 2.3 grams, characterized by almost all wells on the microplate there is concentrated clotting, and there is significance ($p < 0.05$) which means there is a difference in the effectiveness of jackbean flour solution as an immunostimulant from each dose.

Conclusion: This study concluded that jack bean flour, especially at a dose of 2.3 grams, effectively enhanced non-specific immunity in mice, as evidenced by significant hemagglutination results. Therefore, jack bean flour has the potential to be a natural immunostimulant in improving immune health and fighting infections.

Keywords: Antibodies, *Canavalia ensiformis*, immunostimulants, proteins

SDGs Relevance: This research supports SDG 3: Good Health and Well-Being, by exploring natural immunostimulants that contribute to improving immunity. By reporting the potential of low-cost natural ingredients, the findings of this study can support global health goals in a sustainable manner.

Laboratory Affiliation: In vitro research was conducted at the Medical Laboratory, Campus 2, University of Muhammadiyah Malang, where the hemagglutination test was conducted. In silico analysis was conducted using a dry lab approach.

INTRODUCTION

The environment contains various infectious agents such as viruses, bacteria, fungi, and parasites. The presence of infectious agents in the environment will cause infectious diseases and even death if their spread is not properly inhibited. Living things (mammals) need an immune system to protect the body from infections caused by microorganisms. Immunity can be enhanced by increasing the function of the immune system using ingredients that can stimulate the immune system or immunostimulants. Immunostimulants can strengthen the body's natural resistance to infections caused by microorganisms.

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The mechanism of action of immunostimulants is by stimulating the body's immune system through phagocytosis, complement system, antibody secretion, interferon release, antibody synthesis by B cells and through cytokinin production by immune cells (Petrunov, et al. in Inayati et al., 2020). In general, the cells involved in the immune system are T cells and B cells, which are produced by the thymus and spinal cord, respectively (Listiani & Susilawati, 2019). In the process of developing these cells, stimulation can be done with immunostimulants (Sukmayadi et al., 2014). It is known that there are various kinds of plants that have the ability as immunostimulants, one of which is from beans. Nuts contain high protein and omega 3 fatty acids, which are common characteristics of foods that can boost immunity (Firdausiyah & Syafah, 2017).

One type of legume that is thought to have the potential to increase immunity is jackbean (*Canavalia ensiformis*). So far, research on the benefits of jackbean (*Canavalia ensiformis*) as a source of functional food has been widely disclosed, but research that reveals the ability of koro pedang to increase the immune response, especially the increase in antibody titer, has never been disclosed. Based on previous research conducted by Istiani (2010), it was explained that in white jackbean there are active flavonoid components of 29.3 mg/100 grams of seed flour and phenol of 245.5 mg/100 grams of seed flour, and has antioxidant activity in whole koro pedang of 47.13%. Giving koro pedang flour that contains protein and secondary metabolite compounds such as phenolics and flavonoids is expected to affect the increase in antibodies. A good immune system will give a good response if there is antigen exposure/induction. Therefore, to prove that immunostimulants have worked effectively, pre-clinical trials are needed.

The in vitro test conducted in this study uses a hemagglutination inhibition test, which is expected to solve the problems raised, namely related to the benefits of jackbean as an immunostimulant against non-specific immunity in test animals in the form of mice (*Mus musculus*). Hemagglutination inhibition test is determined based on resistance at the highest dilution that is still able to bind antigens and perform hemagglutination (at a concentration of 4 HA units) and inhibit red blood cell agglutination (Janovie et al., 2014). In this study, researchers used *Staphylococcus aureus* bacteria as antigens, because these bacteria have hemagglutinin molecules. Hemagglutinin molecules function in mediating the attachment of bacterial cells to red blood cells, namely lectins, both in gram-negative and gram-positive bacteria (Erina et al., 2021). S. aureus bacteria itself is an infectious bacterium that is pathogenic and easily transmitted, because this bacteria spreads in the air so that it can contaminate the environment, and can be the main cause of nosocominal infectious diseases and food poisoning (Puspitasari & Farizal, 2021). In addition, in-silico testing is needed to determine the active compounds contained in jackbean that play a role in increasing immunity.

Research on antibodies in mice has previously been conducted by Rahayu (2015) using sambiloto herb extraction and the results showed an increase in the number of igG antibodies. The content of bioactive compounds produced from jackbean (*Canavalia ensiformis*) can be developed into an alternative in increasing body immunity (Listiani & Susilawati, 2019). The novelty in this research is the utilization of jackbean (*Canavalia ensiformis*) which has the potential as an immunostimulant and has never been tested before. The data results from this study are expected to be a source of information for the community regarding functional food ingredients that have an effect on health.

METHODS

The approach and type of research used in this research is a qualitative-quantitative mixed method. The research method in this study is Comparative Research or comparative research. This research focuses on the effect of different doses of jackbean (*anavalia ensiformis*) flour given to test animals as immunostimulants on the non-specific immune system of the test animals. This research was conducted at the Laboratory of Medicine Campus 2, Universitas Muhammadiyah Malang (UMM) which is located at Jl. Bendungan Sutami No.188, Sumbersari, Lowokwaru, Malang City, East Java, 65145. This research was conducted from May to June 2024.

The samples used in this study were mice with the criteria of male sex, 2 - 3 months old, weighing 20 - 30 grams/tail, having a healthy physique with normal appetite, hair does not fall out, clear red eyes, and normal feces or not mushy or watery. The tools used in the study include test tubes, centrifuge, test tube racks, micropipettes, yellow microtips, microtubes, blood tubes, microplates, mice oral sonde, syringes, digital

scales, 80 mess sieves, spatulas, analytical scales, beakers, measuring cups, sample bottles, surgical scissors, tweezers, table lamps, colony counters, and microscopes.

The materials used in the study included 16 male mice (*Mus musculus*), jackbean flour (*Canavalia ensiformis*), distilled water, Phospate Buffer Saline (PBS), yellow microtip, microtube, and label paper.

The independent variable in this study is the administration of a solution of jackbean flour (*Canavalia ensiformis*) given to mice (*Mus musculus*) at a dose of 2.3 grams, 4.6 grams, and 9.2 grams. The dependent variable in this study is the antibody of mice (*Mus musculus*) that can hemagglutinate Staphylococcus aureus bacteria. The control variables in this study were antibody titer test; age of the test animals; weight of the test animals; cage used; care of the test animals; amount of feed and drink; and sex of the animals.

Test animals in the form of male mice (*Mus musculus*) with a total of 16 animals aged 2 - 3 months and weighing 20 - 30 grams. Mice before being given treatment, acclimatization is carried out for approximately 3 days, to give test animals the opportunity to adapt to their new environment. Mice were placed in plastic cages with a size of 30 cm x 45 cm x 15 cm, equipped with a lid in the form of hollow woven wire, and a base of husk which was replaced every 3 - 4 days. The cages were equipped with feeders, drinkers, and labels indicating care and control procedures. During the adaptation process, the animals were fed with pellets and drinking water.

Before dissolving the flour, it was sieved first with an 80-mesh sieve, then the flour was weighed using analytical scales according to the needs per dose, namely 2.3 grams (P1), 4.6 grams (P2), and 9.2 grams (P3), then the flour was dissolved using distilled water as much as 16.8 ml for P1, 22.4 ml for P2 and 44.8 ml for P3. The jackbean flour solution was then stored in bottles and labeled according to the dose.

Mice (*Mus musculus*) that have gone through the acclimatization process for 3 days, then divided into 4 treatment groups. Group P0 became a control group that was not given the test material, then for treatment group 1 was given a dose of flour solution of 2.3 grams, group 2 of 4.6 grams, and group 3 of 9.3 grams. All dosing is done by oral sonde every day.

On the fifteenth day after administration of the test material solution, mice blood was taken intracardially. The blood that came out was immediately sucked using a syringe and put into a vacutainer tube until a minimum of 0.1 ml was collected, then placed at room temperature until the blood clotted, then the blood sample was centrifuged at 3000 rpm for 15 minutes, and the blood serum was taken.

Erythrocyte sediment at the bottom of the blood tube after centrifuge, taken as much as 1 drop using a pipette and put into a test tube. Aquades as much as 99 drops was added to the test tube containing erythrocytes, and then centrifuged at 3000 rpm for 15 minutes. The test tube was then taken to remove the water and only left the erythrocyte sediment, then added again with 99 drops of distilled water, followed by another centrifuge process at 3000 rpm for 15 minutes.

The serum obtained was then diluted in double dilution process with PBS (Phosphate Buffered Saline) in the ratio of ¼; 1/8; 1/16; 1/32; 1/64; 1/128; 1/256; and 1/512. Based on each predetermined ratio, the dilution results were taken as much as 50 µL, and then placed in 8 microplate holes. 50 µL of serum from well A was taken, then added to well B, then homogenized. 50 µL of serum from well B was taken, then added to well C, then homogenized. So, on until well H, so that 8 dilution series were obtained with a multiple of two. All holes were then added with 50 µL of Staphylococcus aureus bacteria and 50 µL of 1% erythrocyte suspension, the microplate was then allowed to stand for 2.5 hours. The results of the hemagglutination test or antibody titer test can be analyzed.

In-silico test of bioactive compounds contained in jackbean (*Canavalia ensiformis*) based on the results of previous studies and NCBI research.

This study was conducted by taking and examining samples in the laboratory. The data obtained was collected based on the highest dilution that could still agglutinate the antigen and was carried out by observation and documentation. The results of the data obtained from the examination of mice blood serum (*Mus musculus*) were then observed for clotting in the microplate wells after 2.5 hours, then continued with data analysis using the Kruskal-Wallis Test using IBM SPSS to determine whether there was a significant difference between each control group and the dose treatment group.

This study has received ethical approval from the Health Research Ethics Committee (KEPK), Faculty of Medicine, University of Muhammadiyah Malang with an ethical certificate letter number E.5.a/095/KEPK-UMM/V/2024.

RESULTS

Data from the results of the hemagglutination test after administering a solution of jackbean flour (*Canavalia ensiformis*) at doses of 2.3 grams; 4.6 grams; and 9.2 grams based on the value of antibody titers given to test animals in the form of mice (*Mus musculus*) for 14 days and given antigens in the form of Staphylococcus aureus bacteria are presented in Figure 1.

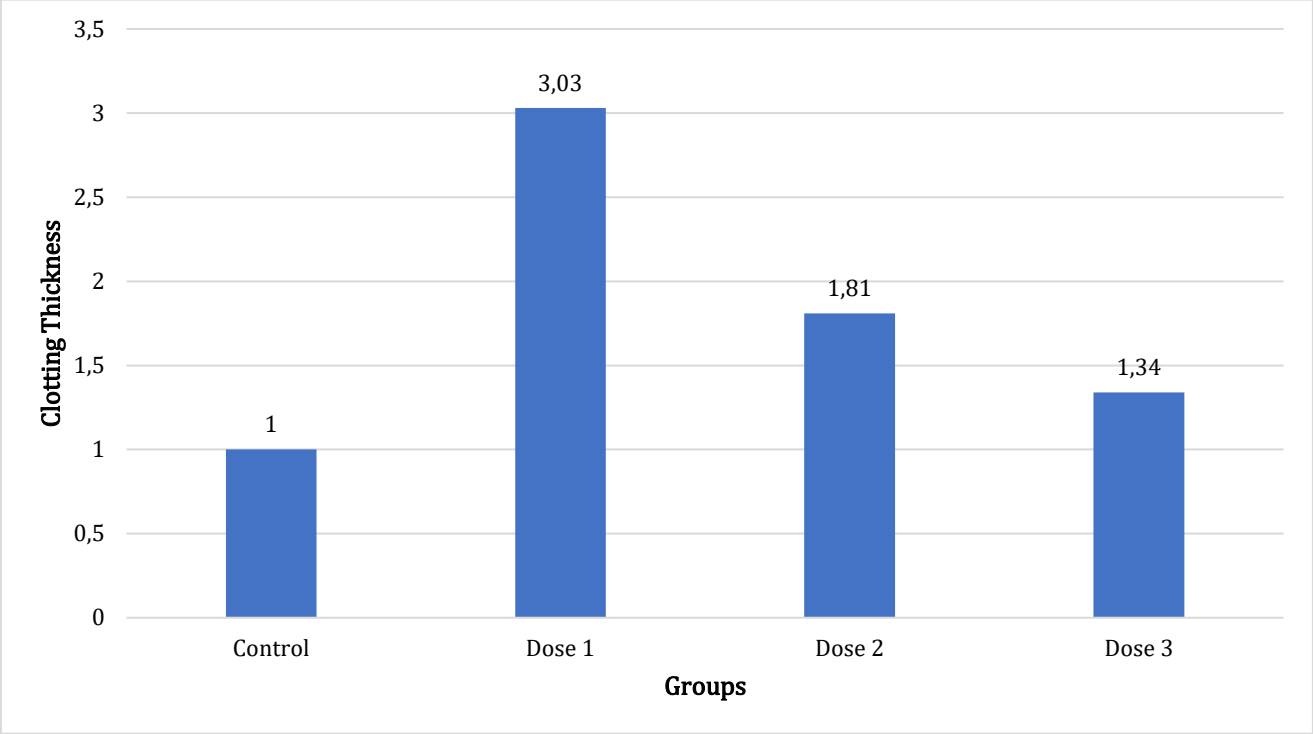
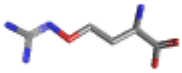
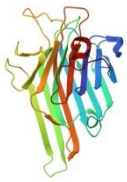
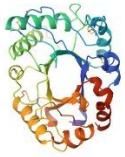


Figure 1. Hemagglutination test result chart

Table 1. In-silico characteristic

Active Compounds	Picture	Structure	Characteristics
Canavanine	 (NCBI, 2024).	$C_5H_{12}N_4O_3$	A non-proteinogenic amino acid similar to arginine.
Concanavalin A	 (Chung, et al., 2017).	$C_{23}H_{32}N_6O_8S$	A group of proteins referred to as lectins, functioning as antiviral molecules, mitogenicity, immunoglubulin isolation, immunomodulators for cancer therapy.
Concanavalin B	 (Hennig, et al., 1995)		A group of proteins referred to as lectins, serve as antiviral, immunomodulatory molecules for cancer therapy.

Based on Figure 1, it can be seen that the dose 1 treatment group has the highest average value compared to other treatments, followed by dose 2 which is 3.03, then dose 3, and the lowest average is in the negative control treatment group. Based on the data that has been obtained, it can be continued by using the Kruskal-Wallis statistical test. Furthermore, based on Table 1, it can be seen that jackbean (*Canavalia ensiformis*) contains active compounds such as canavanine, concanavalin A and concanavalin B which are protein groups that are influential in increasing immunity.

DISCUSSION

Research on the immunostimulant effect of jackbean flour (*Canavalia ensiformis*) on the non-specific immune system in the blood of mice (*Mus musculus*) exposed to *Staphylococcus aureus* bacteria was carried out using the hemagglutination inhibition test. In Erina et al. (2021) explained that the principle of the hemagglutination inhibition test is the inhibition of RBC agglutination due to the binding of the virus (antigen) with specific antibodies characterized by the presence of erythrocyte deposits in the microplate wells and the determination of the antibody titer value. The results of the examination in four treatment groups of mice blood samples (*Mus musculus*) showed the presence of sediment in the microplate wells, especially in the P1, P2, P3, and P0 dose treatment groups. However, in the P0 treatment, the resulting clotting is only a faint line, not too concentrated as seen in the dose treatment group. This may occur presumably due to the influence of the smaller amount of antibodies compared to the treatment groups.

The interaction between antigen and antibody causes a secondary reaction in the form of agglutination, because antigens are small insoluble particles, when specific antigens and anti-serum come together, precipitation will occur as large clumps. Generally, antibodies have several antigen-binding receptors that will react with other antigen molecules to bind to one of the antibody molecules (Suriani, 2019; Sebayang & Hasibuan, 2021). An increase in the antibody titer value is evidenced by an increase in the antibody titer of mice, which indicates the sensitization of T cells and B cells related to antibody production (Sebayang & Hasibuan, 2021). If the ratio between antigen and antibody is balanced, a new agglutination reaction can occur, so that an equivalent zone is formed which is supported by a high temperature (37 - 56°C) and movement that causes contact between antigen and antibody, and the gathering of clots requires salt from the PBS used (Suriani, 2019).

Based on the observations in the table of hemagglutination test results, it is known that the dose that has the most effect on hemagglutination is P1, it can be seen from all microplate wells at dose 1 that clotting occurs, this can be seen from the gradation of the most concentrated clots to the form of thin clots. Whereas in the treatment of higher dose concentrations, namely P2 and P3, not too many very concentrated clots were found, on average only slightly concentrated clots were seen, while the clots produced from P0 were only faint lines. Statistical analysis used to analyze the effect of jackbean flour (*Canavalia ensiformis*) as an immunostimulant presented in the *Kruskal-Wallis* test results table shows that there is significance ($p < 0.05$) which means there are differences in the effectiveness of jackbean flour solution as an immunostimulant at each dose against exposure to Staphylococcus aureus bacteria. This difference in effectiveness is thought to be caused by the influence of each dose of the jackbean (*Canavalia ensiformis*) flour solution.

Excessive antibody conditions can result in the antigen-antibody complex remaining in solution without forming agglutination, while excessive antigen will result in the dissolution of the antigen-antibody complex that has been formed. The flavonoid content in legumes, such as those found in jackbean (*Canavalia ensiformis*) can be developed into an alternative immunostimulant that can increase the body's immunity (Listiani & Susilawati, 2019). In general, beans have a lot of nutritional content that is very good for health, as is the case with koro pedang. Jackbean (*Canavalia ensiformis*) not only contains bioactive components that have antioxidant activity as an antidote to free radicals, but also contains anti-nutritional compounds. Anti-nutritional substances contained in jackbean (*Canavalia ensiformis*) seeds include saponins, tannins, cyanogenic glycosides, kanatoxins, concanavalin A and B, and HCN (Nursalma et al., 2021).

Based on in-silico tests that have been conducted, it is known that jackbean seeds contain the active compound Concanavalin A. Concanavalin A is an anti-nutritional substance, but it also has functional benefits. Concanavalin A is known to function as an antiviral and immunomodulatory molecule for cancer

therapy. Concanavalin A also has positive effects in boosting the immune system. It has been reported that, Con A can stimulate innate immunity and TLR expression in macrophages and induce peripheral blood mononuclear cell proliferation (Alimahana et al., 2023). The content of various compounds in jackbean flour (*Canavalia ensiformis*) is proven to be able to improve the immune system, this is evidenced by an increase in non-specific immunity in mice (*Mus musculus*) after giving a solution of jackbean flour for fourteen days.

CONCLUSION

The administration of *Canavalia ensiformis* flour affects the antibody titer seen from blood clots or hemagglutination that occurs at the bottom of the microplate, the most effective treatment is at dose 1 (2.3 g), and contains active compounds, namely canavanine, concanavalin A and B which have antioxidants to be an immunostimulant, antiviral, and immunomodulators for cancer therapy.

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