Research Article

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Immunomodulatory activity of *Ananas comosus* (L.) Merr. flesh and stem fruit juice on mice with carbon clearance and *Staphylococcus aures*-induced methods

Dicki Bakhtiar PURKON [1*], Zahwa Ai Nunisa NURAISYAH[1], Dieta Relany NAMINA[1], M.H. ROSENO [1], Ayu Nala El Muna HAERUSSANA [1], Faizah Min FADHLILLAH [2], Yogi Khoirul ABROR [3]

1 Department of Pharmacy, Poltekkes Kemenkes Bandung, Jl. Prof. Eyckman No. 24, Pasteur, Kecamatan Sukajadi, Kota Bandung, Jawa Barat, Indonesia, 40161.

2 Department of Pharmacy (Pharmaceutical Biology), Faculty of Mathematics and Science, Universitas Garut, Jl. Jati No. 42 B, Tarogong, Kabupaten Garut, Jawa Barat, Indonesia.

3 Department of Medical Laboratory Technology, Poltekkes Kemenkes Bandung, Jl. Babakan Loa No. 10 A – Gunung Batu, Pasir Kaliki, Kecamatan Cimahi Utara, Kota Cimahi, Jawa Barat, Indonesia, 40154.

* Corresponding Author E-mail: dickibakhtiar_farmasi@staff.poltekkesbandung.ac.id (D.B.P); Tel. +62-85321482650.

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ABSTRACT: The body's immune system plays a very important role in defending the body from antigens and infectors that enter the body and strive to eliminate them. Along with increasing public awareness and knowledge, the development of medicines from herbal/natural ingredients is being intensively carried out. *Ananas comosus* is a type of plant that is widely spread in tropical countries including Indonesia. The fruit is rich in nutrients, vitamins, essential minerals, and the content of the enzyme bromelain which has a wide range of pharmacological activities, including its alleged efficacy as an immunomodulator.This study aims to determine the effective dose of squeezing the flesh and fruit stems of *Ananas comosus*as an immunomodulator against its phagocytic activity based on the carbon clearance method and the application of its immunomodulator in test animal models induced by *Staphylococcus aureus* bacteria. The experimental animals used were male mice of the *BALB/c* strain, which was divided into several groups, namely: normal control group, negative control, immunostimulant positive control(Stimuno Forte®), immunosuppressant positive control (methylprednisolone), *Ananas comosus* fruit flesh and stem squeezing test(ACFSF) dosis 1 (125 mg/kg bw), dose 2 (250 mg/kg bw), and dose 3 (500 mg/kg bw).Examination of these immunomodulatory activity tests, including: therate of carbon elimination velocity, the phagocytosis index, the organ index, and the percentage of phagocytic activity (%).The results of this study have shown that the ACFSF group with the most effective dose as an immunostimulant based on the carbon clearance method and the application of immunomodulators in test animal models induced by *S. aureus* bacteria is ACFSF dose 3 (500 mg/kg bw) with an index value phagocytos is 1,40 and the largest percentage of phagocytic activity (%) compared to the negative control group and close to the immunostimulant comparison group Stimuno Forte®.

KEYWORDS: Immunomodulatory; *Ananas comosus*; carbon clearance method; phagocytic index; *Staphylococcus aureus*-induced.

1. INTRODUCTION

The body's immune system plays a very important role in protecting the body from antigens and infection events to remain in a homeostasis and healthy condition. When the functional immune system is not optimal, the body cannot protect itself against various exposures to infectors (such as: bacteria, viruses, parasites, worms, and other pathogens), antigens/foreign bodies, or abnormal cells that risk threatening the body's normal organism. So that in the end there are several infectious events, tumors/cancers, or other immune system disorders that can cause death [1–4].

Invasion of various pathogens originating from the surrounding environment such as bacteria, viruses, worms, fungi, parasites, or protozoa can cause infections in the body[1]. One of them is the Covid-19 virus which can cause a significant increase in mortality rates around the world at some time to have caused a death rate of more than 539 million cases worldwide [5,6]. One of the causes of death of around 70% of people with Covid-19 infection is due to the occurrence of acute respiratory distress syndrome or ARDS (Accute

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Respiratory Distress Syndrome) which results in severe respiratory distress due to the accumulation of fluid in the alveoli of the lungs with the main symptoms of severe shortness of breath due to the high number of cytokines and other immune cells in the blood, causing hyperinflammation and organ damage [5,7,8].

The importance of the role of immunomodulatory agents that can modulate the immune system, one of which works as an immunostimulant to prevent infection or the severity of infection symptoms (preventive) by stimulating the expression of immune agents of the body, but also acts as an immunosuppressant by lowering the level of immune cells when too much is produced due to infection in the body (curative) depending on the dose given [9,10].Along with the increasing public knowledge and awareness of health and the importance of utilizing various herbal ingredients that have the effect of modulating/improving the immune system. So, many people are trying to use various products from natural ingredients that are increasingly in demand because they are considered safer and more profitable [11]. Herbal medicine has a wide range of phytochemical constituents and active substances that are thought to have a synergistic role to produce therapeutic effects and cover each other's side effects that can be caused [12–14].

Various types of plants that have been used for generations in traditional medicine, especially in Indonesia, to improve/modulate the body's immune system but are still not optimally related to scientific data on various parts of the plant as immunomodulatory products. One of the plants that are widely found in Indonesia and are in demand by the public and have good benefits for the health of the human body is *Ananas comosus* (L) Merr. Several previous studies have stated that the bromelain content in the plant *part of Ananas comosus* on the skin and crown of the fruit has antioxidant and immunomodulatory activity [11,15,16].The enzyme bromelain is also thought to be a natural immunomodulatory agent because it can activate various pro-inflammatory mediators. But also vice versa, bromelain can reduce the secretion of such proinflammatory mediators when it is already produced too much through inhibition of transcription factors NF-κB and cyclooxygenase 2 (COX-2) [8,11].The content of the enzyme bromelain in the fruit stem of *Ananas comosus* has a higher concentration compared to the pulp [17]. However, in other studies it has been mentioned that bromelain in the fruit of *Ananas comosus* has a higher proteolytic activity and greater specificity compared to bromelain on the stem [18].However, scientific data related to the potential immunomodulatory activity and application of immunomodulators in test animal models induced by *Staphylococcus aureus* bacteria on the combined part of the flesh and fruit stem of *Ananas comosus* have no scientific data preclinically (in-vivo).

Research on pharmacological activity in preclinical (in-vivo) on *Ananas comosus* plants that has been carried out previously, namely related to the potential as an immunomodulator with the *carbon clearance* method on the skin of the fruit at a dose of 250 mg/kg bw as an immunostimulant [15], also on the crown section at a dose of 3.12 mg/20 g bw as an immunostimulant with a phagocytic index obtained of 1.71 compared to the control group [16]. Meanwhile, the study of immunomodulatory activity and the application of immunomodulators in test animal models induced by *Staphylococcus aureus* bacteria on the flesh and fruit stems of *Ananas comosus* have no scientifically recorded immunomodulatory activity. Based on the description above, it is necessary to conduct further research to examine the immunomodulatory activity of squeezing the flesh and fruit stems of *Ananas comosus* (L.) Merr. to non-specific immune responses and their immunomodulatory applications in test animal models induced by *Staphylococcus aureus* bacteria in vivo in male white mice of *the BALB/c* strain because the immune cells in the strain mice are more easily activated/sensitized, so they can provide a good response to immunological testing [19,20]. Testing of immunomodulatory activity by the carbon clearance method to see non-specific immune responses was then carried out to see the parameters of carbon elimination velocity, phagocytosis constant, organ index, and phagocytic index values of the entire test group during 7 days of preclinical testing using carbon ink inducers as foreign substances (antigens) [15]. This test was divided into 6 test groups which included a negative control group, immunostimulant positive control/comparator (Stimuno Forte®), positive control/immunosuppressant comparator (methylprednisolone), *Ananas comosus* fruit flesh and stem squeeze group (ACFSF) dose 1 (125 mg/kg bw), dose 2 (250 mg/kg bw), and dose 3 (500 mg/kg bw). In addition, the next activity testing was the application of immunomodulators from ACFSF on a *BALB/c* test animal model induced by *Staphylococcus aureus* bacteria using 7 test groups, namely the normal control group, immunostimulant positive control (Stimuno Forte®), immunosuppressant positive control (methylprednisolone), and 3 differentACFSF dose groups on immunomodulatory activity testing with the carbon clearance method (n=5). On day 8, each test group (except normal control) was then injected with *Staphyloccus aureus* bacteria intraperitonially. The percentage of phagocytosis activity/macrophage cell activity was calculated from the smear of the peritoneal fluid of the test animal mice. The data obtained is then processed statistically using the SPSS software application version 25 with the One Way ANOVA method for normal and homogeneous distributed data [20–23].

2. RESULTS AND DISCUSSSION

2.1. Plant determination results

The results of the determination of test plants have been carried out at the Plant Taxonomy Laboratory, Department of Biology, FMIPA, Padjadjaran University with Certificate No. 24/HB/01/2022 which is proven that the plant to be used in this study is *Ananas comosus* (L.) Merr species.This test plant was obtained from plantations in the Subang Regency, West Java, Indonesia, which is one of the largest *Ananas comosus* fruit producing areas in Indonesia. In 2018, Subang Regency produced at least around 1,778,360 quintals of *Ananas comosus* fruit which is at least 98.36% of all *Ananas comosus* fruit production in West Java [24].

2.2. Squeezed flesh and stalks fruit *Ananas comosus* (L.) Merr. result

The raw material for the test sample used in this study was the flesh and stem of *Ananas comosus*fruit, which was about 12 kg. The flesh and stem of the *Ananas comosus* fruitthat has been peeled off the crown and the skin are then cut into smaller sizes to be squeezed using a juicer (Phillips®) so that it is separated between the water and the pulp. Then 2 liters (L) of flesh and stem fruit juice of*Ananas comosus* were obtained. The squeeze is then dried using the lyophilization method (freeze-drying) to remove the water content in it but for the content of the bromelain enzyme in it is not denatured or does not suffer damage due to drying. Dry product from *freeze-drying*results on ACFSF as much as 12.0116 grams (percentage of rendement of 0.6% w/v).

This lyophilized methods uses a low temperature at -60°C and is dried in a *freeze-dryer* [25]. The freeze drying method on *Ananas comosus* fruit juice has been carried out in previous studies that were able to produce a dry product of *Ananas comosus* fruit juice with a bromelain content of 96% [26], and is considered to have advantages in maintaining the quality of the product, both from sensory characteristics, nutritional value, physical and chemical compared to ordinary drying using thermals [27,28]. Thus, the use of the *freeze-drying* process in the ACFSF test sample in this studycan obtain the content of the enzyme bromelain and the content that has other immunomodulatory efficacies from ACFSF with a high concentration so that it can provide pharmacological effects on test animals.

2.3. Screening of phytochemicals squeezed flesh and fruit stems of *Ananas comosus* (L.) Merr.

Phytochemical screening has been carried out to see the big picture of various compounds contained from the squeezing of the flesh and stems fruit of *Ananas comosus* as therapeutic agents/have inseparable pharmacological activity [18,29]. The results of phytochemical screening showed that the squeezing of the flesh and stalks of the fruit of *Ananas comosus* (L.) Merr. contains compounds of the saponin and terpenoid compounds shown in Table 1.

Some saponin compounds have antitumor efficacy in many cancer cells by inhibiting the cell cycle and apoptosis mechanisms in those tumor cells. Saponins are also part of the essential compounds of triterpenoids, in addition to steroids, and glycosides. Some terpenoid compounds have efficacy as antibiotics and have the ability to modulate immunological processes (immune system), such as: increasing the production of antibodies and suppressing the response of T lymphocyte cells [1].

2.4. Carbon clearance test results in *BALB/c* strain male mice

This study aims to determine the activity of squeezing the flesh and fruit stems of *Ananas comosus* (L.) Merr. as a preclinical immunomodulator (in-vivo) in a test animal model of male white mice strain *BALB/c* using the carbon clearance method [9,30,31]. The test animal used in this study was a *BALB/c* strain male mice aged ± 10 weeks which is an adult age so that the organ system of the test animal has been well formed and can respond well as well. The mice test animal was chosen because it has similarities with humans in terms of absorption, distribution, metabolism, and excretion (ADME), so it is hoped that this study can be used as a preclinical handle (in-vivo) to be clinically applied to humans with similar treatments. The selection of *BALB/c* strain mice is because this strain provides a good and representative level of immunity response sensitivity, both for testing and observing specific and non-specific immune responses. The use of male sex mice is based on this mice having fewer reproductive hormones compared to female mice, so it is hoped that there will be fewer variations that can affect the results of the study [15,32,33]. Various studies on animal models of *BALB/c* strain mice test for testing immunomodulatory activity have also been widely carried out [9,20,34].

Table 1. The result of phytochemical screening of the flesh and fruit stems of *Ananas comosus* (L.) Merr.

Compound Group	Screening Method	Screening Results	Conclusion
Flavonoid	Wilstater Test: 5 drops of concentrated HCl+ Mg powder tape	No discoloration	-
Alkaloid	Mayer Reagent	No white precipitate forms	-
	Reagen Wagner	No red/orange deposits form	-
Saponin	Froth test: Shaking + a few drops of HCl	Stable foam formed	+
Steroid	Test of Lieberman Burchard:	No discoloration	-
Terpenoid	1 mL of anhydrous acetic acid + 1 mL of H2SO4	Red rings are formed	+

Information: + = Present, - = Absent

Data from immunomodulatory activity testing using the carbon clearance method obtained in the form of absorbance to be then processed into a phagocytosis constant value that describes the speed of elimination of carbon particles in each time period of blood sampling. The carbon elimination velocity curve can be seen in Figure 1 below.

Based on the carbon particle elimination velocity curve in Figure 1, the ACFSF test group data dose 3 (500 mg/kg bw) had almost the same carbon elimination velocity as the Stimuno Forte® immunostimulant comparison group. The decrease in absorbance value is proportional to the speed of elimination of carbon ink, so it can be said that the Stimuno Forte® comparison group and the ACFSF test group dose 3 (500 mg/kg bw) had a greater decrease in the amount of carbon ink compared to other groups at each interval of blood serum collection. The value of the phagocytosis constant in each test group is shown in Table 2. The absorbance value of each time interval of blood draw in each group of test animals is then entered into the calculation formula to obtain the value of the phagocytosis constant. The value of the phagocytosis constant describes the speed of elimination of carbon ink particles in the blood, so that the value of the high phagocytosis constant against the negative control group and immunosuppressants indicates the activity of the compound as an immunostimulant, and vice versa [9,35,36].

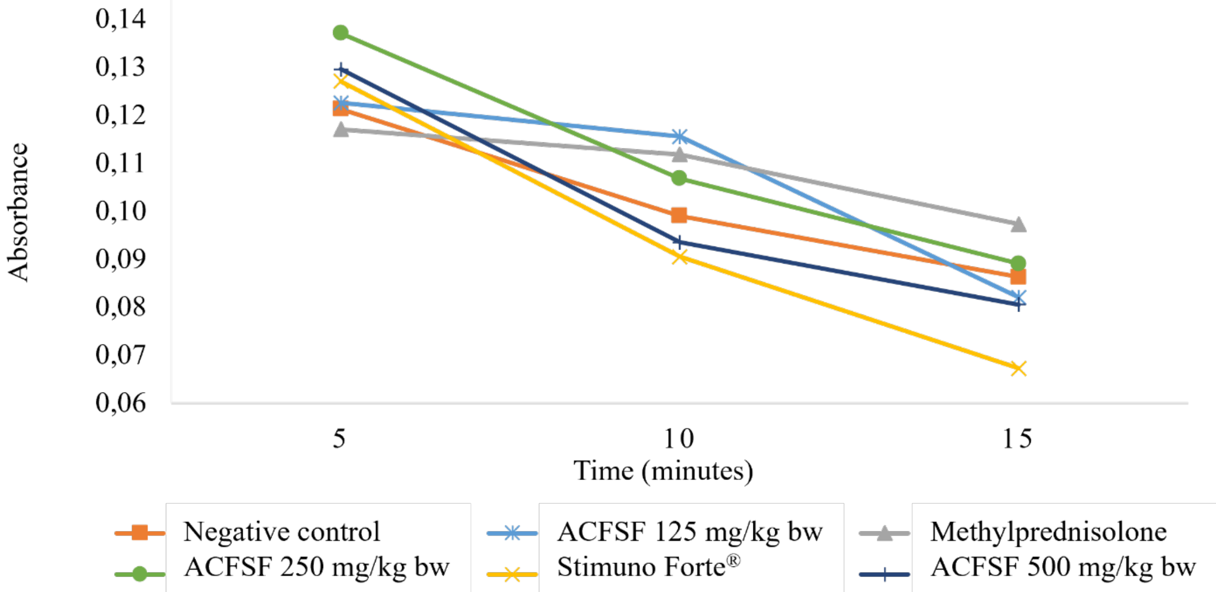

Figure 1. The carbon elimination rate in each test group on day 8 after colloidal induced carbon ink (in a 0.9% NaCl physiological solution) of 0.1 mL/10 g bw intravenously

The enzyme bromelain according to research conducted by Rathnavelu, V., et al. (2016), Chakraborty, A. J., et al. (2021), and Serkan, S., et al. (2021) has immunomodulatory activity, which can act as an

immunosuppressant and immunostimulant agent through several mechanisms of action [5,11,18]. The content of this bromelain enzyme is known to be found in the flesh and stem of the relatively large fruit of *Ananas comosus*[18,37]. The mechanism of action of the enzyme bromelain as an immunomodulator, namely: lowering the regulation of pro-inflammatory prostaglandin E-2 (PGE-2) through inhibition of NF-κB and cyclooxygenase-2 (COX-2), increased regulation of PGE-1 (anti-inflammatory agent), lowering T cell activation by blocking the T cell signal transduction pathway when inflammatory mediators are already excreted too much in the body.However, it is also known that bromelain can increase phagocytic activity by activating inflammatory mediators such as (IL)-1β, IL-6, INF-γ, and TNF-α as an acute immune response to oxidative stress in cellular, improve the antibody response of specific B cells and T cells, and modulate the T cell response in vitro and in vivo [11,17,38].

The value of the phagocytic constant is obtained by calculating the absorbance logs of the 5th, 10th, and 15th minutes, then compared with the time difference in minutes for each retrieval period. The value of the phagocytic constant describes the speed of carbon elimination at each time interval of each group. The greater the value of the constant (K) the more the compound tends to be immunostimulant [30,31,34]. Based on Table 2, the Stimuno Forte® immunostimulant comparison group had the largest K value among other groups and also for the entire test extract group had a K value greater than the negative control group. Thus, the entire group of test extracts has a tendency to be immunostimulants based on the value of their phagocytosis constant.

Table 2. The value of phagocytosis constant (K) and phagocytosis index (α) on the results of the carbon clearance method of the entire test group after treatment

Test Animal Group	The Value of the Phagocytosis Constant (K)	Phagocytosis Index (α)	Comparison of Phagocytosis Index	Classification of Immunomodulatory Effects (Wagner, H., 1999) [39]
Negative Control[a]	0.013 ± 0.019	0.08	1.00	-
Positive Group of Immunosuppressant[b]	0.032 ± 0.091	0.16	2.00	Immunostimulant
Positive Group of Immunostimulants[c]	0.008 ± 0.011	0.05	0.59	Immunosupressant
ACFSF Dose[d]	0.017 ± 0.022	0.09	1.20	Immunostimulant
ACFSF Dose 2[e]	0.019 ± 0.074	0.10	1.32	Immunostimulant
ACFSF Dose 3[f]	0.021 ± 0.064	0.11	1.40	Immunostimulant

Information:
[a]Group given 0.5% Na-CMC and induced carbon ink
[b]Methylprednisolone suspension and carbon ink-induced groups
[c]Stimuno Forte® suspension and carbon ink-induced groups
[d]The group was squeezed with flesh and stems fruit of *Ananas comosus* at a dose of 125 mg/kg bw and induced carbon ink
[e]The group was squeezed with flesh and stems fruit of *Ananas comosus* at a dose of 250 mg/kg bw and induced carbon ink
[f]The group was squeezed with flesh and stems fruit of *Ananas comosus* at a dose of 500 mg/kg bw and induced carbon ink
*The data used was in the form of an average standard deviation ± (SD) using 5 mice test animals per group (n = 5).

Based on Wagner's criteria (1999) and other related references that a compound is said to have moderate immunostimulant activity (active) if the value of the phagocytic index ranges from 1.0-1.5 and a strong immunostimulating effect (very active) if its value is more than 1.5. Immunosuppressive obtained if the value of the phagocytic index is less than one (<1) [9,21,39,40]. From the data in Table 2 above, it can be seen that the ACFSF test group of doses 1, 2, and 3 has moderate (active) immunostimulating activity and the Stimuno Forte® comparison control group has strong immunostimulating activity (very active). Meanwhile, the methylprednisolone comparison control group had activity as immunosuppressants with a phagocytic index value of less than one. The immunomodulatory activity of squeezing the flesh and fruit stems of *Ananas comosus* was tested using the carbon clearance method and accompanied by organ index values to determine their effect on immune organs in the body, especially on non-specific immune systems [16,41]. The carbon clearance method is a method of testing immunomodulatory activity in a non-specific immune system, namely by measuring the speed of immune cells in phagocytosis of carbon ink injected intravenously in test animals. The purpose of this method is to evaluate the effect of squeezing the flesh and fruit stems of *Ananas comosus* on the reticuloendothelial system (RES) [9,41].

The carbon clearance method uses colloidal carbon particles injected into the systemic circulation directly, the speed at which macrophages eliminate carbon from the blood is determined based on the equation of each time interval. The speed of such carbon elimination correlates with an increase in phagocytic activity observed in each treatment group. The decrease in the amount of carbon ink at intervals is an indicator of an increase in carbon clearance speed, an increase in the ability of phagocytic activity in vivo, and the competence of the granulopoietic system in eliminating foreign particles, which is an indicator of an increase in the immunological response to antigens [34,42,43].Absorbance describes the amount of concentration of carbon ink present in the blood and passed by monochromatic light from a UV-clinical photometer instrument. Carbon ink is considered an antigen or foreign body that will be eliminated from the body by means of phagocytosis by macrophages or non-specific immune cells. Thus, if there is immunostimulating activity, there will be a greater decrease in carbon ink absorbance at each time interval. The absorbance value will then be entered into an equation to find out the phagocytic constant, the greater the K value, the better the activity in stimulating the body's immune system to eliminate carbon that has been injected [15,42,43].

The value of the phagocytosis constant or the speed of carbon clearance in the blood of each time period of taking indicates that the Stimuno Forte® comparator has the best immunostimulant activity, because it has the largest K value among other groups which means that the immunostimulant activity possessed by the Stimuno Forte® comparator can provide an excellent immune response. Furthermore, it was followed by the ACFSF test group dose 3 (500 mg/kg bw) which showed an increase in the ability of phagocytic cells to phagocytosis carbon so that the amount of carbon is reduced in the blood. It is seen in the carbon elimination velocity curve shown in Figure 1 that the lines of decrease (carbon elimination velocity) of the dose 3 test group are very close together and almost the same as the comparison in the 5th to 10th minutes. As for the test group of ACFSF dose 2 (250 mg/kg bw) and ACFSF dose 1 (125 mg/kg bw) did not differ significantly in constant values, both showed immunostimulating activity seen from a greater K value of the negative control group although still below the ACFSF test group dose 3 and the Stimuno Forte® comparison control.

Stimuno Forte® was chosen because it contains *Phyllanthus niruri* extract which is proven to be an immunomodulatory/immunostimulant agent with a mechanism of action to provide stimulation to immune cell receptors and send intracellular signals at cell receptors so as to improve the work of immune cells better [21,22,44].Stimuno Forte® medicine is a "Fitofarmaka" class herbal medicine that has gone through clinical trials with standardized ingredients marketed in Indonesia and several other countries globally with its content, namely extracts from the herbaceous *Phyllanthus niruri* which has efficacy in improving the immune system [21,22,44].While the use of methylprednisolone as a comparison group of immunosuppressants is based on its pharmacological activity which is efficacious as an anti-inflammatory or immunosuppressant. Methylprednisolone is a glucocorticoid group compound that can diffuse passively across cell membranes and bind to intracellular glucocorticoid receptors. This methylprednisolone-receptor glucocorticoid complex can bind and block pro-inflammatory agents, increase the expression of anti-inflammatory agents, and inhibit the synthesis of inflammatory cytokines, especially by blocking the function of transcription factors, such as nuclear factor-kappa-B (NF-κB) [9,45,46]. Methylprednisolone includes compounds from corticosteroids that have been commonly used to lower the immune system and are anti-inflammatory. This compound is also known to lower immunoglobulin levels (including Ig G) in rodensia test animals such as mice [46,47].

Along with the value of the phagocytosis constant, measurements of the weight of the lymphoid organs in the liver and spleen organs have also been carried out to obtain a phagocytic index describing the number of macrophages that phagocytize carbon ink. Naturally, immune cells in certain organs also eliminate carbon particles because they are considered harmful antigens to the body [42,43].Carbon particles contained in the injected ink will be phagocytosed by macrophage cells/Kupffer cells contained in the liver in high amounts and also by phagocytic cells in the spleen and thymus gland as a form of non-specific immune response. The spleen as a secondary lymphoid organ containing B lymphocyte cells and T lymphocytes plays a role in specific immune responses. But in addition, in the spleen organ there are also dendritic cells and macrophages that act as APC (Antigen Presenting Cells) which function to present antigens to T cells [34–36].

2.5. Liver and spleen index test results

The non-specific cellular immunity response involves the lymphoid system, in particular the liver and spleen organs that protect the body from exposure to infectors and foreign substances [48]. The two organs from the results of treatment by induction of carbon ink were then carried out a process of isolation, washing, weighing, and comparison process between the weight of the liver and spleen organs with the body weight of each test animal from the entire test group. The value of the organ index is indicated in percentage form in Table 3.

In this test, the test animal was sacrificed and then surgically performed and isolated lymphoid organs. The organs are weighed and then the weight is compared with the body weight of each test animal, until the organ index percentage value is obtained. The greater the value of the organ index, the indicated immunomodulatory activity leads to immunostimulants. This is because when the organ is exposed to antigens (in this case carbon) then naturally the immune agents in the body will "exchange information" with each other and try to eliminate the foreign substance. The more immune agents that are stimulated, the more weight of the lymphoid organ will increase because that is where non-specific immunostimulant agents work, namely by phagocytosis foreign substances [30,48].The greater the value of the organ index, the greater the weight of each organ, meaning that there is an increase in the number of immune cells in the area of these organs [30,48]. Based on the organ index values shown in Table 3, the test group (ACFSF) dose 2 (250 mg/kg bw)and dose 3 (500 mg/kg bw) had a greater organ index value than the negative control and normal control, which means that the test group has immunostimulant activity based on the organ index value.

Table 3. Indices of lymphoid organs (liver and spleen) in each test group of the carbon clearance method testing

Test Animal Group	Organ Index Value (%)	
	Liver	Spleen
Kontrol Normal	5.50 ± 1.01**	0.69 ± 0.25
Kontrol Negatif	4.65 ± 1.38	0.74 ± 0.33
Methylprednisolone	4.98 ± 0.55	0.57 ± 0.16
Stimuno Forte®	6.13 ± 0.33**	0.78 ± 0.07
ACFSF Dose 1	5.20 ± 0.51	0.66 ± 0.15
ACFSF Dose 2	6.48 ± 0.13**	0.98 ± 0.09**
ACFSF Dose 3	6.60 ± 0.43**	0.50 ± 0.22*

*Information:
The data is displayed as an average ± standard deviation (SD). Mean data values written with superscript symbols have meaningful differences against negative control groups (* = P<0.05 and ** = P<0.01).

The organ index data of each test animal was further processed using SPSS software to determine whether there were significant differences between one treatment group and another. First, a normality and homogeneity test is carried out, which is the initial requirement in statistical analysis [49]. Based on the results of the normality test and the data obtained are distributed normally because the value of P (Sig.) > 0.05 in the Saphiro-Wilk column (with the number of data <50). Based on statistical analysis using the One Way ANOVA test, it was later found that the P (Sig.) values for the liver and spleen organ indices were 0.000 and 0.003 (<0.05) respectively, so it can be said that there were significant differences between groups. The analysis process continued with Post Hoc-LSD testing to determine meaningful differences between treatment groups. As seen in Table 3, the values of the liver organ index (%) in the ACSF Dose 2 and 3 groups, Stimuno Forte®, and normal controls differed significantly compared to the negative control groups (P<0.05 and P<0.01).

Staphylococcus aures bacteria as shown in Figure 2 and Table 4 act as infectors/antigens which are gram-positive bacteria that are able to bind Giemsa dyes clearly so as to facilitate calculations under a microscope. In addition, this bacterium also does not contain protein A (a protein that is antiphagocytic), so *Staphylococcus aureus* cannot avoid phagocytosis of macrophages of the peritoneum [22]. The induction of such bacteria is administered intraperitonially and then left for an hour (on day 8) because it can make non-specific immune responses work. Where, the non-specific immune response can work about 0-12 hours after infection occurs[50]. In the event of an infection process, T lymphocyte cells will produce a number of lymphokines that pull macrophage cells to where they need them and activate them. Active macrophages will secrete several important substances, namely: enzymes, lysozymes, elastase, collagenases, complements, and cytokines. Cytokines to be secreted by macrophage cells, namely: IL-1, IL-6, IL-8, IL-12, IL-15, and TNF-α [23,42,50].In Table 4, the percentage of phagocytic activity in the optimal ACFSF dose group as an immunostimulant was ACFSF dose 3 (500 mg/kg bw) because it could increase significantly compared to the negative control group (P<0.01) and approach the Stimuno Forte® immunostimulant comparison group.

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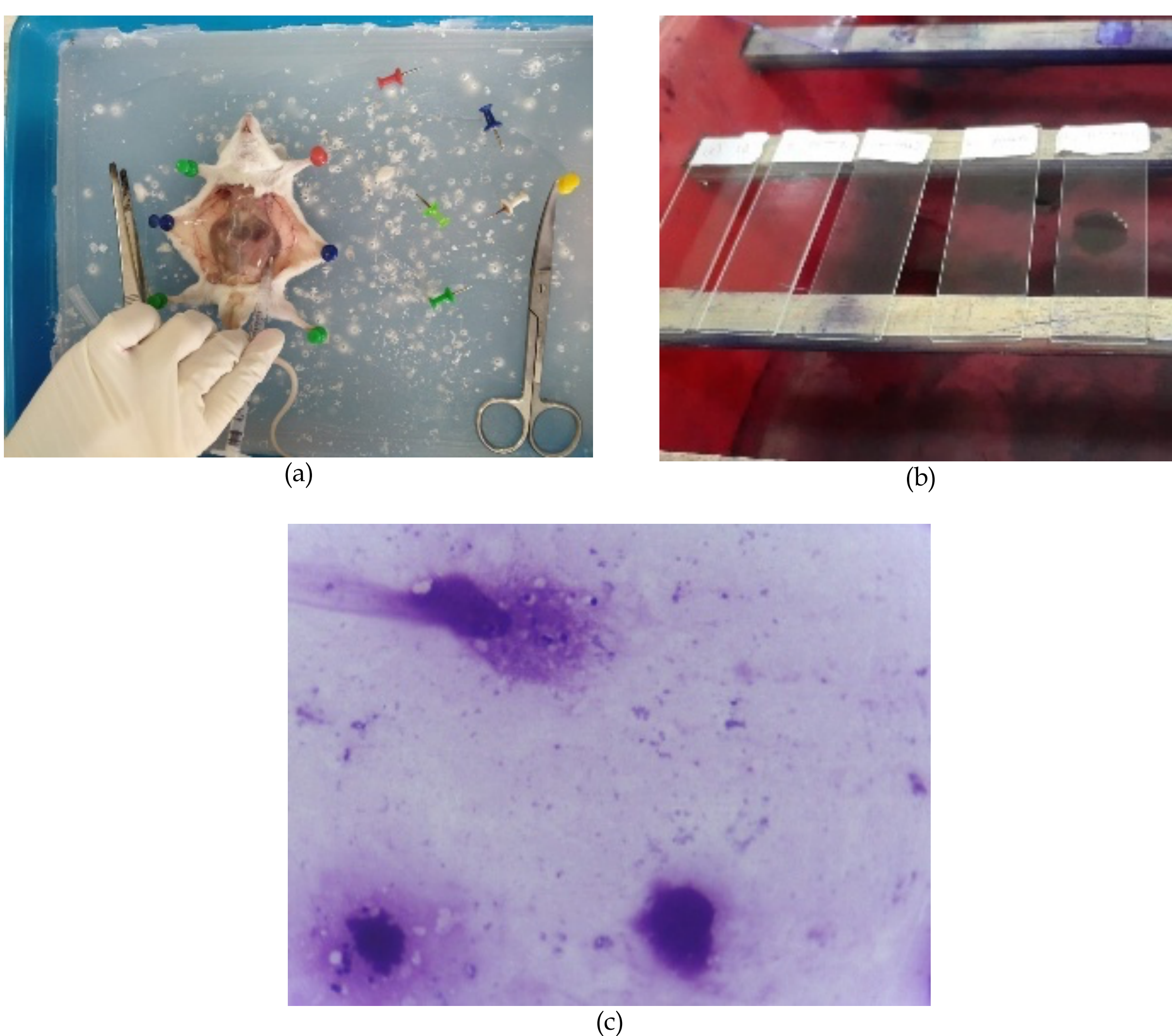
3. CONCLUSION

Squeeze the flesh and fruit stem of *Ananas comosus* (L.) Merr. has immunomodulatory activity, namely as an immunostimulant in male mice strain *BALB/c* with the carbon clearance method and its application in test animal models induced by *Staphylococcus aureus* bacteria. The best dose of *Ananas comosus* fruit flesh and stem squeezing extract group (ACFSF) that provides immunomodulatory activity as an immunostimulant is ACFSF dose 3 (500 mg/kg bw) with the greatest carbon ink antigen elimination rate and a phagocytic index value of 1.40.In the ACFSF group dose 3 also had the largest percentage of phagocytic activity (%) among other test groups which increased significantly compared to the negative control group, and approached the immunostimulant comparison control group Stimuno Forte®.



(a) (b)

(c)

Figure 2. Photo documentation of peritonial fluid specimen collection in all test animals from each group (a), the process of making peritonial fluid (b), and observations on a microscope device from the test specimen with a magnification of 10-100 times (c)

4. MATERIALS AND METHODS

4.1. Materials

The various ingredients used in this study were the flesh and stem of the fruit of *Ananas comosus* (L.) Merr. obtained from the Experimental Garden of the Subang Tropical Fruit Plant Research Center (the results of the determination of the *Ananas comosus* plant have been carried out at the Plant Taxonomy Laboratory, Department of Biology FMIPA UNPAD with the certificate number, namely: 24/HB/01/2022), aquadest, NaCl 0.9% physiological, Na-CMC, glacial acetic acid, 1% acetic acid, methylprednisolone (PT. Gracia Pharmindo), Stimuno Forte® (PT. Dexa Medica), carbon ink (Pelican China Ink B-17®), Giemsa dyes, methanol, and agar nutrients. While the tools used in this study, namely: UV-Vis spectrophotometer (Pharo 100

Spectroquant®), clinical UV photometer (Biolabo®), analytical scales (Metler Toledo®), rotary vacuum evaporator (PT. Buchi®), waterbath (CRWB-30®), refrigerator, parchment paper, evaporative cup, spatel, mortar and stamper, cuvette, micropipette, vacutainer, animal scales, test animal surgical equipment, mice cages, microscopes, incubators, drip pipettes, ose wire, syringes (Terumo®), and a variety of other glassware.

Table 4. Percentage of phagocytic activity (%) of the entire test group from the results of immunomodulatory testing applications in test animal models induced by *Staphyloccus aureus* bacteria

Treatment Group	Percentage of Phagocytic Activity (%)
Normal Control	18.00±1.63**
Negative Control (*S. aureus*)	41.00±3.46
Positive Control of Immunostimulants (Stimuno Forte®)	56.50±2.52**
Positive Control of Immunosuppressants (Methylprednisolone)	38.00±1.63
ACFSF Dosis I(125 mg/kg bw)	42.00±1.63
ACFSF Dosis II (250 mg/kg bw)	43.50±3.00
ACFSF Dosis III (500 mg/kg bw)	50.50±1.91**

Information:
* There was a significant difference in the negative control group (P<0.05).
** There were significant differences in the negative control group (P<0.01).
The data is displayed as the average ± standard deviation (SD).

4.2. Experimental animals

A total of thirty-five *BALB/c* strain mice each aged approximately 8-12 weeks with an average body weight of 25-30 g were selected in immunomodulatory activity testing by the carbon clearance method and preclinical application of immunomodulators in test animal models induced with *Staphylococcus aureus* bacteria. The test animal was acclimatized for 1 week before testing. All test animals are normally given standard laboratory feed and clean drinking water. These test animals were divided into 7 test groups consisting of normal control (Na-CMC 0.5%), negative control (Na-CMC 0.5% and carbon ink inducing compound on the clearance carbon test; or 0.5% Na-CMC and *Staphylococcus aureus* inducing bacteria), immunostimulant positive control group/comparator (Stimuno Forte® 0.13 mg/20 g bw), positive control/immunosuppressant comparator (methylprednisolone 0.0208 mg/20 g bw), ACFSF test group dose 1 (125 mg/kg bw), dose 2 (250 mg/kg bw), and dose 3 (500 mg/kg bw). The administration of Na-CMC 0.5% in the entire test group because Na-CMC is a suspension agent in the test preparations made (normal control group, negative control, immunostimulant positive control, immunosuppressant positive control, and test extract preparations), and has inert properties and can produce stable suspensions, meaning that Na-CMC does not contain active substances that can provide any pharmacological effects on test animals [51].This research has gone through the test of the Animal Use Ethics Committee test by the Health Research Ethics Committee Team (KEPK) of Poltekkes Kemenkes Bandung with an approval ethics number, namely: No. 74/KEPK/E/III/2022.

4.3. Methods

4.3.1. Making of the flesh and stem of ananas comosus fruit (L.) Merr.

The flesh and stems of the still intact *Ananas comosus* fruit were first weighed. The flesh of the fruit that has been peeled from the peel and cut into small pieces and the stem of the fruit is determined by weight. The flesh and stem of the fruit are put into a juicer to get the squeezed water. The squeezed water is then measured in volume using a measuring cup.

The results of squeezing the flesh and stems of *Ananas comosus* fruit are then thickened using a rotary vacuum evaporator at a temperature of 50°C so that a thick squeeze of flesh and fruit stems of *Ananas comosus* is obtained. It aims to extend the shelf life of the preparation and obtain its more active substance content. The thickening process is carried out until the difference from each weighing (at least two times the weighing) in a row is not more than 0.05 mg (or a constant weight has been obtained) [52–54].

4.3.2.Phytochemical screening

Test extracts from the flesh and stems of *Ananas comosus* fruit (ACFSF) carried out a qualitative evaluation process for phytochemical screening using various reagents for each group of secondary metabolites common to plants. Phytochemical screening carried out includes: tests for the content of flavonoids, alkaloids, phenolics, tannins, saponins, and steroids/triterpenoids. Positive results show changes in color, turbidity with certain colors, or the formation of a precipitate in accordance with guidelines from WHO, the Ministry of Health of the Republic of Indonesia, and various other scientific articles [9,55–58].

4.3.3.Testing immunomodulatory activity by carbon clearance method

1. Preparation of carbon ink colloidal as antigen

A total of 1.6 grams of carbon ink was suspended into 8.4 mL of 1% w/v gelatin (in 0.9% physiological NaCl solution) [9,10,41].

2. Testing immunomodulatory activities with carbon clearance method

Test animals that had been grouped into 7 groups were then given treatment according to their respective groups for 7 days. On day 8, animal blood samples were taken as much as 25 µL through the tail vein at the 0th minute (T0) then put into a plastic tube (vacutainer) (without anticoagulants) with a cover to be lysis with the addition of 4 mL of 1% acetic acid. A blood sample at the 0th minute (T0) is used as a preliminary blank before the induction process is carried out. The test animals were then injected intravenously with colloidal carbon ink in 1% w/v gelatin (in a 0.9% NaCl physiological solution) of 0.1 mL/10 g bw. Blood samples are then taken back in the 5th minute (T5), 10 (T10), and 15 (T15) with the same amount through the tail vein. The blood samples were also inserted into a vacutainer tube (without anticoagulants) containing 4 mL of 1% acetic acid solution which was then measured the percentage of transmitting at a wavelength of 675 nm using a UV-clinical photometer [9,34,41]. The percentage of transmittan or absorbance data is then entered into the equation below to obtain the elimination rate (constant) of the carbon clearance and the phagocytic index as carried out by Yuandani, et al. (2022) and Purkon, et al. (2021) [9,42].

$$\text{Rate of carbon clearance (K)} = \frac{\text{Log OD5} - \text{Log OD 15}}{\text{T2} - \text{T1}}$$

$$\text{Phagocytic index } (\alpha) = \frac{\frac{K}{3} \text{ x Mice weight (g)}}{\text{Liver weight (g)} + \text{Spleen weight (g)}}$$

Exposure: Log OD5 = absorbance value of blood sample log at 5th minute; Log OD15 = absorbance value of the blood sample log at the 15th minute; T1 = initial pick-up point (5th minute); T2 = final pick-up point (15th minute).

3.Determination of liver and spleen organ index (%)

In determining the immunomodulatory activity, the indices of lymphoid organs, such as: liver and spleen organs become one of the parameters of the test. After being given a test preparation for 7 days, right on the 8th day, all test animals were sacrificed/euthanized by putting them in a chamber container containing CO_2 gas. Furthermore, the surgical process is performed and the liver and spleen organs are taken on each test animal to measure the weight of the organ. The organ indices of the two organs are calculated and expressed against the body weight of each test animal [9,30,48].

4.3.4.Application of immunomodulatory activity in animal models of Staphylococcus aureus bacteria-induced mice

The test animals were randomly grouped into 7 groups with each group consisting of 5 test animals. The entire test group was given test extracts and comparison compounds orally every day for 1 week (7 days). The division of the test animal groups in this test method can be seen in Table 5.

In the next day (on the 8th day), the entire group of test animal mice (except the normal control group) was induced intraperitonially (i.p.) with a 0.5 mL suspension of *Staphylococcus aureus* bacteria and left for one hour. The mice test animal was then euthanized with CO_2 gas and surgically performed on its abdomen using surgical equipment and tweezers. The peritoneal fluid is then taken using a 1 mL syringe and inserted into the vial. In the case of taking peritoneal fluid in mice test animals, PBS (Phosphate Buffered-Saline) liquid with a pH of 7.4 is first given into the entire peritoneum of *BALB/c* mice. This aims to help dissolve macrophage cells attached to the mice organ and can also make it easier to take peritoneal fluid. Peritoneal fluid is taken as much as 10 µL which is then dripped on the preparation glass and fixed with methanol solvent for 5 minutes, then stained with Giemsa staining. After drying, the sample on the preparation glass can be seen under a

microscope using emersion oil at magnification (10x – 100x) and then calculated the phagocytic activity of the macrophages according to the formula below [21–23,35,59].

$$\% \text{ Phagocytic activity} = \frac{\text{Number of active macrophages}}{\text{Overall number of macrophages}} \times 100\%$$

Table 5. Division of test animal groups on the application of immunomodulatory activity in a test animal model of mice induced by *Staphyloccus aureus*bacteria

Group	Treatment
Normal Control	Na-CMC 0.5%
Negative Control (*S. aureus*)	Na-CMC 0.5% + induced *S. aureus*
Positive Control of Immunostimulants (Stimuno Forte®)	Stimuno Forte® 0,13 mg/20g bw + induced*S.aureus*
Positive Control of Immunosuppressants (Methylprednisolone)	Metilprednisolon 0,13mg/20g bw + induced*S.aureus*
ACFSF Dosis I(125 mg/kg bw)	Squeeze the flesh and fruit stem of *Ananas comosus* 125 mg/kg bw + induced*S.aureus*
ACFSF Dosis II (250 mg/kg bw)	Squeeze the flesh and fruit stem of *Ananas comosus* 250 mg/kg bw + induced*S.aureus*
ACFSF Dosis III (500 mg/kg bw)	Squeeze the flesh and fruit stem of *Ananas comosus* 500 mg/kg bw + induced*S.aureus*

4.4 Data analysis

The observation/examination data that has been obtained is then analyzed statistically using the SPSS (Statistical Product and Service Solution) version 25 application. Data analysis was carried out using the One Way ANOVA test method because more than two test groups were then continued with the Post Hoc test, namely using LSD (Least Significant Differences) analysis to find out the differences between treatment groups and see which groups showed significant differences in activity. This One Way ANOVA test is carried out if the data obtained are normal and homogeneous, so that it can show a difference with the value of $P<0.05$ (95% confidence level). If one of the conditions is not met, then the data analysis process uses alternative tests, namely: the Kruskal-Wallis test and to see the differences between treatment groups, an analysis is carried out with the Mann-Whitney test [22,33,60,61].

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