ACTIVITY OF ETHANOL EXTRACT OF KETAPANG LEAVES (*Terminalia catappa*) AS AN IMMUNOMODULATOR

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2019
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ABSTRAK

NIKE CHOO LEE-ANN. Aktivitas Ekstrak Etanol Daun Ketapang (Terminalia catappa) sebagai Imunomodulator. Dibimbing oleh ANDRIYANTO dan USAMAH AFIFF.

Terminalia catappa yang dikenal dengan pohon ketapang adalah tanaman obat yang banyak digunakan dalam mengobati berbagai penyakit, termasuk antibakteri. Tujuan penelitian ini adalah mengamati aktivitas ekstrak etanol daun ketapang (Terminalia catappa) sebagai imunomodulator. Penelitian ini menggunakan 20 ekor mencit jantan yang dibagi dalam 4 kelompok, yaitu kelompok A, B, C, dan D dengan masing-masing dosis ekstrak etanol daun ketapang sebesar 0, 1, 3, dan 5 g/kg BB. Pemberian ekstrak etanol daun ketapang dilakukan secara peroral setiap hari selama 7 hari. Pada akhir penelitian mencit percobaan diinduksi Staphylococcus aureus secara intraperitoneal. Satu jam pasca induksi S. aureus, mencit percobaan dinekropsi untuk mengambil cairan peritoneal. Cairan peritoneal kemudian dibuat prepapart ulas dan diwarnai dengan larutan pewarna Giemsa 10% lalu diamati jumlah makrofag di bawah mikroskop. Hasil terbaik ditunjukan pada ekstrak daun ketapang 3 g/kg BB.

Kata kunci: daun ketapang, histopatologi, imunomodulator, makrofag

ABSTRACT

NIKE CHOO LEE-ANN. Activity of Ethanol Extract of Ketapang Leaf (Terminalia catappa) as immunomodulator. Supervised by ANDRIYANTO and USAMAH AFIFF.

Terminalia catappa which is also known as Ketapang tree, is a medicinal plant that is widely used in treating various diseases, including antibacterial. The purpose of this study was to observe the activity of ethanol extract of the leaves of ketapang (Terminalia catappa) as an immunomodulator. This study used 20 male mice which were divided into 4 groups, namely groups A, B, C, and D with dose of ethanol extract of Ketapang leaves 0, 1, 3, and 5 g/kg BW respectively. The administration of ethanol extract of Ketapang leaves was carried out orally every day for 7 days. At the end of the study, the experimental mice were injected with Staphylococcus aureus intraperitoneally. One hour after injection of S. aureus, the mice were necropitized and peritoneal fluid was taken. The peritoneal fluid was then stained with a 10% Giemsa dye solution then observed for the number of macrophages under the microscope. The best results were shown on ketapang leaf extract 3 g/kg BW.

Keywords: histopathology, immunomodulators, ketapang leaf, macrophages
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INTRODUCTION

Background

Disease is a condition that affects a part or the whole structure or function. A disease is often associated with specific symptoms and signs. A disease can be caused by external factors such as pathogens or internal dysfunction. Internal system dysfunction can produce various diseases, including various forms of immunodeficiency, hypersensitivity, allergies, and autoimmune disorders (White 2014).

Herbal medicine is the use of medicinal plants for prevention and treatment of diseases. It is the sum total of the practices based on the theories, beliefs and experiences of different cultures (Firenzuoli and Gori 2007). According to Ahn (2017), medicinal plants, also called herbs, have been found and used in the practice of traditional medicine since prehistoric times. Plants synthesize chemical conflicts for functions including insects, fungi, diseases, and herbivorous mammals. Numerous phytochemicals with potential or established biological activity have been identified. The use of natural remedies for the treatment of disease has a long history and medicinal plants and their derivatives are still used all over the world (Zakhak et al. 2015).

Immunomodulators are an unusual feature for producing antibody responses and cellular responses in human studies (Kensil 2006). Immunomodulatory agents can be used to trigger autoimmune antigens and foreign antigens (Askenasy et al. 2005). Immunotherapy is the treatment of diseases that activates or suppresses the immune system. Immunotherapy that is designed to obtain or strengthen the immune responses is classified as immunotherapy enhancer, while immunization that reduces or supress the immune system is classified as immunotherapy suppressor. Immune effector cells such as lymphocytes, macrophages, dendritic cells, natural killer cells (NK Cell) and cytotoxic T lymphocytes (CTL) works together to protect the body by detecting abnormal antigens expressed on the surface of foreign bodies. The characteristics of plant-based immunomodulatory therapy has attracted the attention of researchers. Innovative technology and further research on immunomodulatory natural products, plants, their extracts, and their active parts with potential immunomodulators, can provide us with valuable entities to be developed as new immunomodulatory agents to support the current chemotherapy (Jantan et al. 2015).
Research purposes

This study aims to determine the activity of ethanol extract of the Ketapang leaves (Terminalia catappa) as an immunomodulator by looking at the number of macrophages and the number of bacteria that are phagocytized by the active macrophages.

Benefits of research

The expected benefit is being able to add information about the usefulness of traditional medicines such as Ketapang leaves (Terminalia catappa) and the activities as an immunomodulator against infections from S. aureus.

Research Hypothesis

The administration of the ethanol extract of Ketapang leaves (Terminalia catappa) can function as an immunomodulator against infections from S. aureus.

LITERATURE REVIEW

Terminalia catappa

Plants of the genus Terminalia are one of the most widely used traditional medicines in the world. Many species of the genus are used for antibacterial, antifungal, antiprotozoa, antiviral, antidiarrheal, analgesic, antimalarial, antioxidant, anti-inflammatory, and anticancer. The leaves and fruit of T. catappa has been used as traditional medicines for antipyretics and hemostatics, as well as the prevention of hepatoma and hepatitis (Ko et al. 2002). According to Cabi (2018), the classification of Ketapang leaves is as followed:

Kingdom : Plantae
Division : Magnoliophyta
Class : Eudicots
Order : Myrtales
Family : Combretaceae
Genus : Terminalia
Species : Terminalia catappa
Common Name : Sea almond tree, Ketapang

Ketapang leaves contain several flavonoids such as kaempferol or quercetin, some tannins such as punicalin, punicalagin or tercatin, saponins, and phytosterols. Based on this phytochemical content, Ketapang leaves and bark are used in herbal medicine with various purposes (Hnawia et al. 2011). The common phytochemical constituents of Terminalia species include flavonoids, tannins, anthocyanins, and gallic acid (Cock 2015). According to Chitmanat et al. (2005), Ketapang leaves have been used by fish farmers for years and prevent certain parasites and pathogenic bacteria. Ketapang leaves are also believed to help prevent mold formation in fish eggs. According to Chansue and Assawawongkasem (2011),
water extract of dried Ketapang leaves can rapidly increase the regeneration of carp tail fins.

According to Cock (2015), Ketapang leaf extract was also used to heal wounds and had cardiovascular effects on several species. Ketapang leaf extract is a popular traditional medicine used in Taiwan to prevent hepatoma and treat hepatitis (Chen et al. 2000). Another study stated that *T. catappa* extract was able to inhibit osmotic-induced hemolysis in human erythrocytes at certain doses. Ketapang leaf extract is also able to prolong the time of blood clotting and shows its potential in treating sickle cell disorders (Mgbemene and Ohiri 1999). According to Babayi et al. (2004), methanol extract of Ketapang leaves can inhibit the growth of *S. aureus* bacteria.

**Immunomodulator**

Immunity is the body's natural defense system against various infectious diseases. Factors that triggers the immunity includes previous microorganism infections, immunization, and various external stimuli. Immunity is able to distinguish between proteins or body cells and foreign particles. As soon as the foreign particle is identified, the collective and coordinated response of specific cells, and mediators against strange substances constitutes the immune response (Baxter 2007). Based on its function, the immune system is categorized into the innate immune system whereby the immune system that is not specific and the adaptive immune system which are special or acquired immune system. Microbiological, chemical and physical defenses are also sometimes included in innate immunity, however, the main mediators of the immune system that provide instant defense include cytokines, acute phase proteins, macrophages, monocytes, complement, and neutrophils (Vesely et al. 2011).

Healthy organisms have an immune system that is able to maintain homeostasis in the body. The function and efficiency of the immune system is influenced by various exogenous and endogenous factors that produce immunosuppression or immunostimulation. Several agents that have activities to normalize or modulate the pathophysiological process are called immunomodulators (Puri et al. 1994). Immunomodulators are generally categorized into immunoadjuvant, immunostimulant, and immunosuppressants in clinical practice. Immunoadjuvant is a specific immune stimulator that increases vaccine success. Agents that activate or induce mediators or components of the immune system are called immunostimulants. Resistance to autoimmunity, cancer, allergies, and infections is enhanced by immunostimulants (Jantan et al. 2015).

On the other hand, immunosuppressants are molecules that inhibit the immune system and can be used to control pathological immune reactions after organ transplants. In addition, these agents can also be used in immunopathological treatment related to infections, hypersensitivity reactions, and autoimmune diseases. A number of monoclonal antibodies and synthesized chemical compounds are also used as immunomodulators (Jantan et al. 2015).
**Staphylococcus aureus**

*Staphylococcus aureus* is a Gram-positive bacterium in the form of cocci and are arranged in groups (Masalha *et al.* 2001). *Staphylococcus aureus* is the main human pathogenic bacterium in humans that causes clinical manifestations. *Staphylococcus aureus* can be found in the environment and is a normal flora located on the skin and mucous membranes of healthy individuals. *Staphylococcus aureus* does not cause infection on healthy skin. However, if it enters the bloodstream or internal tissues, it can cause potentially serious infections. *Staphylococcus aureus* is a causative agent for several human infections, namely bacteremia, infective endocarditis, skin and soft tissue infections such as impetigo, folliculitis, furuncles, carbuncles, cellulitis, scalded skin syndrome, osteomyelitis, septic arthritis, prosthetic device infections, lung infections such as pneumonia and empyema, gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections. These bacteria can cause invasive infections and/or toxin-mediated diseases depending on the strain involved and the location of the infection (Taylor and Unkal 2017).

**Mus musculus**

Mice (*Mus musculus*) are important research materials for modeling the development of human disease and development in the laboratory. Mice have the ability to reproduce and mature quickly making them efficient and economical research materials for scientific studies. Compared to other mammal species, mice are simple and inexpensive to treat in the laboratory. *Mus musculus* may originally be distributed from the Mediterranean region to China, but now it has spread throughout the world. According to Waterston *et al.* (2002), the classification of *Mus musculus* is as follows:

- **Kingdom**: Animalia
- **Phylum**: Chordata
- **Class**: Mammals
- **Order**: Rodentia
- **Family**: Muridae
- **Genus**: Mus
- **Species**: M. musculus

*Mus musculus* has several strains, one of which is DDY. The name strain, DDY, stands for uppercase Deutschland (= German), Denken, and Yoken. This strain shows good reproductive performance, superior growth, and is used in trials for drug efficacy in various fields of research, such as pharmacology, pharmacokinetics, and toxicology studies. This DDY strains originated from mice colonies at the Institute of Infectious Diseases of Tokyo University (Suzuki *et al.* 1999).
METHODOLOGY

Time and Place of Research

The study was conducted from June 2017 to May 2018. The study was conducted in the mouse housing in the Educational Animal Hospital of Bogor Agricultural University (RSHP IPB). The examination of peritoneal fluid was carried out in the Microbiology and Bacteriology Laboratory. Phytochemical testing was carried out in the Laboratorium Pusat Studi Biofarmaka IPB.

Tools and materials

The tools used were a washcloth, blender, 1 mL syringe, scalpel, tissue, scissors, tweezers, Oral gavage feeding tube, mortar and pastle, 100 mL beaker, glass slides, microscope, and a timer. The materials used in this study were male DDY strain mices, Ketapang leaves, S. aureus \(10^8\) CFU/mL, 70% ethanol, 70% alcohol, ketamine, xylazine, sterile distilled water, methanol, 10% Giemsa solution, immersion oil, and peritoneal fluid samples.

Research procedure

Making Ethanol Extract of Ketapang Leaves

A total of 5 kg of fallen Ketapang leaves were collected from around the Bogor Agricultural Institute campus from May to September 2017. The Ketapang leaves were then washed and rinsed with water before drying without direct contact of sunlight and then stored in a black plastic bag. After that, the Ketapang leaves were blended to become a fine powder. Later, 3 kg of the fine powdered leaves were extracted with 10 L of 70% ethanol at room temperature and stirred every 1 hour for 36 hours. The results of ethanol extraction were then filtered using a cloth and evaporated using an evaporator at SEAFAST to obtain the extract in a condensed powder form.

In Vivo Test

The animal ethical approval forms were submitted and was approved with the code number 141/KEH/SKE/V/2019. The mice have a body weight around 25–30 g and are around 2 months old. The mice were healthy at the start of the study. This study used 20 male mice of strain DDY which were divided into 4 groups and each group consisted of 5 mice, namely:

Group A: Mice were not given the ethanol extract of Ketapang leaves as a control.
Group B: Mice were given ethanol extract of Ketapang leaves in a dose of 1 g/kg BW.
Group C: Mice were given ethanol extract of Ketapang leaves with a dose of 3 g/kg BW
Group D: Mice were given ethanol extract of Ketapang leaves with a dose of 5 g/kg BW

The mice were acclimatized for 2 weeks. The treatment was carried out by feeding the ethanol extract of the Ketapang leaves once a day for 7 days using an
oral gavage feeding tube and a 1 mL syringe. On the last day of feeding the ethanol extract, the mice were injected with 1 mL of $10^8$ CFU/mL *S. aureus* intraperitoneally.

**Collection of Peritoneal Fluid Samples**

The experimental mice were injected with 0.2 mL xylazine (10 mg/kg BW) and ketamine (25 mg/kg BW) intraperitoneally one hour after the injection of *S. aureus*. During anesthesia, the mice are terminated by cervical dislocation and then dissected to take intraperitoneal fluid preparations using a 1 mL syringe.

**Peritoneal Fluid Preparations**

The glass slide was cleaned with 70% alcohol then wiped clean, dry, and free of fat. Peritoneal fluid samples were dropped onto one side of the glass slide. Then, another glass slide that is still good and the edges are still flat, is placed on one side of the tip on the first preparation glass by forming an angle of approximately 30°–45°. The second slide glass was pulled until it touches the drops of peritoneal fluid and is allowed to spread along the edge of the second glass slide, then pushed along the glass surface of the first glass slide with sufficient speed so that a thin and even layer of peritoneal fluid is formed and then dried. The glass slides were then marked according to the treatment groups.

**Staining of Peritoneal Fluid Preparations**

The peritoneal fluid preparations were then fixed in a methanol solution and left for 5 minutes. The glass preparations were then soaked in 10% Giemsa solution and left for 30-45 minutes. Distilled water was then used to rinse the glass slide and was dried.

**Observation of Peritoneal Fluid Preparations**

A microscope with 100 x 10 magnification was used with a drop of immersion oil to observe macrophage cells. The macrophages were calculated at 50 different fields of view. The macrophage was then distinguished between active and passive. The number of *S. aureus* bacteria seen in the active macrophages was also recorded.

**Data analysis**

The research data were analyzed by using analysis of variance (ANOVA) method and continued with the Tukey test. The data analysis was performed using Microsoft Excel 2013 software and Minitab 16 program.

**RESULTS AND DISCUSSION**

Testing the activity of ethanol extract of Ketapang leaves as an immunomodulator was carried out to provide additional data in the field as a consideration for the treatment of *S. aureus*. The test was carried out by injecting 1 mL of *S. aureus* $10^8$ CFU / mL intraperitoneally in the experimented mice. One hour after the induction of *S. aureus*, the experimental mice were anesthetized and
terminated by cervical dislocations and then dissected to take intraperitoneal fluid. After that, the identification of the number of macrophages, active macrophages and the number of bacteria in the macrophages was recorded, as can be seen in Figure 1.

Figure 1 Test results for the ethanol extract activity of Ketapang leaves as immunomodulators. A: active macrophages that contain bacteria in the cytoplasm of macrophages; B: passive macrophages that do not contain bacteria in the cytoplasm.

The activity test of ethanol extract of Ketapang leaves (*Terminalia catappa*) as an immunomodulator was carried out using 4 treatment groups, namely, a control, 1 g/kg BW, 3 g/kg BW, and 5 g/kg BW. The test results are shown in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dosage of Ketapang leaf extract (g/kg BB)</th>
<th>Control</th>
<th>1</th>
<th>3</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Macrophage</td>
<td>48.80±36.89b</td>
<td>70.20±13.22b</td>
<td>143.60±27.81a</td>
<td>64.20±17.15b</td>
<td></td>
</tr>
<tr>
<td>Active Macrophage</td>
<td>12.20±12.95b</td>
<td>13.80±3.56b</td>
<td>41.33±16.46a</td>
<td>16.00±9.06b</td>
<td></td>
</tr>
<tr>
<td>Total <em>S. aureus</em> bacteria in macrophage</td>
<td>15.40±13.69b</td>
<td>21.80±4.66b</td>
<td>57.00±19.02a</td>
<td>23.00±13.44b</td>
<td></td>
</tr>
</tbody>
</table>

Description: Different superscript letters on the same line show significant differences (p<0.05)
The test results of the ethanol extract activity of Ketapang leaves as immunomodulators had an effect on \textit{S. aureus} infection. Based on the results obtained, the highest number of macrophages found in 50 fields of view under the microscope was from a dose of 3 g/kg BW with an average value of 143.60 ± 27.81. The lowest number of macrophages found in 50 visual fields under the microscope was the control group that was not given the ethanol extract of the Ketapang leaf. The average value of the number of macrophages in this control group is 48.80 ± 36.89. The second highest number of macrophages was found in leaf doses of ketapang 5 g/kg BW with an average value of 64.20 ± 17.15. Group B with a dose of 5 g/kg BW ethanol extract of the leaves of Ketapang had an average value of 70.20 ± 13.22.

According to Mims (1964), peritoneal macrophages have the opportunity to phagocytosis of microorganisms in intraperitoneal regions and may live up to one week before dividing, dying, or moving to another place. The total normal macrophages found intraperitoneally in mice is around 119.0 ± 16.2. However, there were wide variations in the number and type of cells revealed among the various species tested and between strains of the given species (Padawer and Gordon 1956).

Immunomodulatory agents from Ketapang leaf plants increases an organism's immune response towards pathogens by inactivating the immune system (Jayathirtha and Mishra 2004). According to Abiodun et al. (2016), tannins and flavonoid glycosides are found in \textit{T. catappa} leaves. Many studies show that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilatory actions. However, most interest has been devoted to the antioxidant activity of flavonoids, which is caused by their ability to reduce the formation of free radicals and to scavenge free radicals. Terminalia Asia species contains high antioxidant. (Cock 2015).
Most of the ingested flavonoids are widely degraded to various phenolic acids, some of which still have radical cleansing abilities (Pietta 2000). According to Ajith et al. (2017), studies have shown that some of these antioxidant agents can have potential immune regulation activities. In another study, Saroja (2012) explained that flavonoid fractions effectively stimulated humoral, cell-mediated and non-specific mediated immunity.

Immunostimulative drugs produce an increase in immune reactions which primarily implies stimulation of non-specific immunity such as granulocytes, macrophages, complement, certain T lymphocytes and different effectors (Makare 2001). This can explain the increase in macrophages seen from the group 3 g/kg BW which indicates that the extract can be used as an immunostimulant.

CONCLUSIONS AND SUGGESTIONS

Conclusion

The administration of Ketapang leaf extract can improve and enhance immunity through the parameters of the number of macrophages, the number of active macrophages, and the number of S. aureus in macrophages which according to statistics shows a significant difference. The best results were shown on Ketapang leaf extract 3 g/kg BW.

Suggestion

Further testing is needed regarding the content of the active ingredients found in leaf extract, knowing that the most effective ingredients for enhancing immunity.

REFERENCES


BIOGRAPHY

The author was born in Perak, Malaysia on April 4, 1995 from the parent Mr. Morgan Choo Kee Peng as the father and Ms. Lee Nyok Chun as the mother. The author is the first of three children. In 2012 the author graduated from the SMK(P) Methodist Girl School and in 2014 the author passed the selection to enter the Bogor Agricultural Institute and was accepted at the Faculty of Veterinary Medicine. The author is also active in several types of competitions while being a student. Some of the achievements achieved by the author includes the 1st winner of the OMI swimming competition at IPB University, 1st winner of the women's marathon and OLIVE women's swimming and 3rd place OLIVE long jump.