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Immunomodulatory Activity of Kasumba Turate (*Carthamus tinctorius* L.) Extract Against Non-Specific and Specific Immune Responses in Mice

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Abstract

Immunomodulators are compounds that can regulate the immune system with the objective of normalizing or helping to optimize the immune system. People tend to back to nature by utilizing various plants to be used as medicine, one of which is kasumba turate (Carthamus tinctorius L.) which is thought to be efficacious as an immunomodulator in increasing the activity and capacity of macrophages. This study aims to determine the immunomodulatory activity of kasumba turate ethanol (CTEE) extract against non-specific and specific immune responses in mice. Non-specific immune response tests using the carbon clearance method were carried out on mice with 3 dose ratings. Specific immune response tests were carried out by the hemagglutination test method to determine antibody titers. The results of the carbon clearance test showed that the CTEE dose 50 mg/KgBW was a moderate immunostimulant with a phagocytic index of 1.41, while doses of 100 and 200 mg/KgBW were strong immunostimulants with phagocytic index of 1.57 and 1.82 respectively. While the results of the hemagglutination test method, the antibody titers value is determined from the highest serum dilution value that still indicates hemagglutination. The results show an increase in humoral immune response with the highest primary and secondary antibody titer values of 1:256 and 1:1024 for doses of 100 mg/KgBW, and 1:512 and 1:1024 for doses of 200 mg/KgBW. Phytochemical screening results of CTEE proved to contain alkaloid group compounds, flavonoids, saponins, tannins and triterpenoids. Based on the results obtained, it was concluded that the administration of CTEE showed immunostimulant activity.
Keywords: *Carthamus tinctorius* L., *immunomodulators, phagocytic index, antibody titer*

INTRODUCTION

Immunomodulators are compounds that can regulate the immune system with the objective of normalizing or helping to optimize the immune system. Immunomodulators are divided into two, namely immunostimulators and immunosuppressors (Khadijah, 2017). Immunomodulators assist the body in optimizing the function of the immune system, where the immune system is the main component that plays a role in the body's defense (Suhirman and Winarti, 2010).

Immunity is a reaction in the body to foreign materials that infiltrate the body molecularly or cellularly (Sukmayadi *et al.*, 2014). The immune system if exposed to substances that are considered foreign, then there are two types of immune responses that will occur, namely non-specific and specific immune responses. Non-specific immune response is an innate immune system that has a response to foreign substances that can occur even though the body has not previously been subjected to these substances and provide an early response to pathogens. The adaptive (specific) immune system generates antibodies and employs them to fight specific pathogens with which the body has had previous contact (Puspitaningrum *et al.*, 2017).

People tend to back to nature by utilizing various plants to be used as medicine, one of which is kasumba turate (*Carthamus tinctorius* L.) which is thought to be efficacious as an immunomodulator in increasing the activity and capacity of macrophages (Ismail *et al.*, 2015). According to Lee *et al.* (2008) kasumba turate which is known as safflower also contains immunostimulant and antitumor components that have potential as immunomodulators.

The character and active compounds of kasumba turate are quinochalcones and flavonoids. Kasumba turate has many biological functions including dilating coronary arteries, modulating the immune system, anticoagulation, antithrombosis, antioxidant, anti-inflammatory, antitumor, analgesia (Lee *et al.*, 2008).

Based on this description, the purpose of this study was to determine the immunomodulatory activity of kasumba turate (*Carthamus tinctorius* L.) ethanol extract (CTEE) against non-specific immune responses by carbon clearance method and specific immune response by hemagglutination method in mice.

RESEARCH METHODS

Phytochemical Screening

To ascertain the class of compounds contained in *Carthamus tinctorius* L. ethanol extract (CTEE), a phytochemical analysis was performed. The phytochemical screening carried out included testing for the presence of alkaloids, flavonoids, saponins, tannins, steroids, and triterpenoids based on the Harborne (1987) method.

Ethical Clearance

This research method has met the declaration of Helsinki 1975, Council for International Organizations of Medical Sciences (CIOMS) and World Health Organization (WHO) 2006, and ethically approved by Health Research Ethics Committee Faculty of Medicine of Universitas Muhammadiyah Surakarta No.3737/A.1/KEPK-FKUMS/X/2021.

Non-Specific Immune Response Test by Carbon Clearance Method

Six groups comprise thirty (30) Swiss Webster strain mice with a body weight between 20-35 grams. Group I was administered Carboxymethyl Cellulose Sodium (CMC Na) 0.5% as a negative control; group II was administered levamisole 2.5 mg/KgBW as a positive control immunostimulant; group III was administered methylprednisolone 40 mg/KgBW as a positive control immunosuppressant; group IV was administered extract 50 mg/KgBW; group V was administered extract 100 mg/KgBW; and group VI was administered extract 200 mg/KgBW. Animals were administered test preparations orally once per day for seven days. On day 8, each animal from each group had 25 µL of blood drawn through the tail vein (0 minutes), and mice were injected with 0.1 mL/10 gBW of carbon suspension (the carbon suspension is made by suspending 1.6 mL of Chinese ink B-17 in 8.4 mL gelatin 1% w/v in a 0.9% pyrogen-free NaCl solution (Faradilla and Iwo, 2014)) through the tail vein. Mice blood was taken as much as 25 µL at minutes 5, 15 and 20 calculated from the time the carbon suspension was administered. The obtained blood was mixed with 3 mL of 1% acetic acid in a test tube, and the transmittance value was measured using a visible spectrophotometer at 675 nm.

The carbon clearance rate or phagocytic index (k) measured from the concentration regression coefficients each time (at minutes 5, 15 and 20) was drawn by plotting 100 – % transmittance per mice (X) to time (Y) (Iwo *et al.*, 2000) treatment group and the regression coefficient of the negative control. Phagocytic index <1 indicates immunosuppressant effect, phagocytic index <1.2 indicates no immunostimulant effect, phagocytic index 1.3-1.5 indicates moderate immunostimulant, and phagocytic index >1.5 indicates that the test substance has a strong immunostimulant effect (Wagner *et al.*, 1999)**.** The magnitude of the phagocytosis index can be calculated by the formula: slope of the mice line x / slope of the negative group line.

Specific Immune Response Test by Hemagglutination Method

The antigen used to test the specific immune response is sheep red blood cells (SRBC). SRBC is stored in an EDTA vaculab tube, then centrifugated at a speed of 3000 rpm for 10 minutes. Superfoods were removed with a pipette, while pellets were taken and

washed 3 times using 0.9% NaCl (Faradilla and Iwo, 2014).

The specific immune response test was carried out on thirty (30) Swiss Webster mice weighing 20-35 grams divided into 6 groups. Group I was given CMC Na 0.5% as a negative control, group II was given levamisole 2.5 mg/KgBW as a positive immunostimulant control, group III was given methylprednisolone 40 mg/KgBW as a positive control immunosuppressant, group IV was given extract 50 mg/KgBW, group V was given extract dose 100 mg/KgBW and group VI was given extract 200 mg/KgBW. Mice were given test extracts orally with a frequency of once a day for 13 days. All treatment groups were immunized with 0.1 mL/10 gBW SRBC (Sheep Red Blood Cells) 10% intravenously on the third day after administration of the test extract. On the 8^{th} day after the test extract, mice had their blood drawn in the tail vein and centrifuged to obtain serum. Primary antibody titer tests were performed in each treatment group. Hemagglutination was seen on plate 96 micro bottom V wells. Each well was given 50 µL of NaCl. The stock solution was prepared by diluting the test serum 1:2. The first line is filled with test serum and diluted 1:2 to the 12^{th} line. Wells that have been filled with stock solution are added SRBC suspension as much as 25 µL and incubated 24 hours in an incubator with a temperature setting of 37°C. The primary antibody titer is expressed with the highest dilution value causing hemagglutination. On the same day as the determination of the primary antibody titer, mice are re-immunized with SRBC. Five days after the second immunization, mice have their blood drawn to determine secondary antibody titers by observing the hemagglutination that occurs (Faradilla and Iwo, 2014).

The results of the antibody titer test are determined by looking at the pattern in the deposition of red blood cells at the center of the microplate well. If at the center of the well there is a red dot and clear in the surrounding fluid, then the test is negative (no hemagglutination occurs). The antibody titer value is seen from the highest dilution which still causes hemagglutination (Utami *et al.*, 2016).

RESULTS AND DISCUSSION

The study began with qualitative phytochemical tests using the tube method to ascertain the composition of the group of immunostimulant-suspected compounds found in CTEE. The phytochemical analysis reveals that the CTEE contains flavonoid compounds, alkaloids, saponins, polyphenols, tannins, and triterpenoids (**Table 1**). Ethanol is utilized as a solvent because it is a universal solvent capable of extracting active compounds including tannins, polyphenols, polyacetylenes, flavonoids, terpenoids, sterols, and alkaloids (Pandey and Tripathi, 2014).

Non-specific immune response was tested using the carbon clearance method. The carbon clearance method is used to evaluate the reticuloendothelial system (RES) effects of pharmaceuticals and phytoconstituents. The RES is a diffuse system that generates phagocytic cells. Colloidal carbon particles are injected directly into the blood circulation for phagocytosis-based removal by RES (Ganeshpurkar and Saluja, 2017). The first step in immunomodulatory testing against nonspecific immune responses is the administration of carbon suspension as an antigen. Phagocytic index <1 indicates immunosuppressant effect, phagocytic index <1.2 indicates no immunostimulant effect, phagocytic index 1.3-1.5 indicates moderate immunostimulant effect, and phagocytic index > 1.5 indicates the test substance has a potent immunostimulant effect (Wagner *et al.*, 1999). CMC-Na is used as a negative control because it is devoid of active ingredients and therefore cannot produce pharmacological effects in test animals. In this study, the negative control was administered a suspension of CMC-Na 0.5%, which serves as a suspending agent but has no pharmacological activity (Putri *et al.*, 2014).

Table 1. **Phytochemical screening results of kasumba turate (*Chartamus tinctorius* L.) ethanol extract**

Examination	Theoretical Results	Test Results	Information
Alkaloids	Dragendroff: brick-red, red, orange deposits Mayer: white or yellowish precipitate	Dragendroff: orange deposits Mayer: yellowish precipitation	+
Flavonoids	The color formed on the amyl alcohol layer indicates the presence of flavonoid compounds.	Formed color on the layer of amyl alcohol	+
Saponins	The foam does not disappear for 10 minutes	The foam does not disappear for 10 minutes	+
Tannins	Tannins: dark blue or greenish-black color	Formed greenish-black	+
Steroids and Triterpenoids	Red or purple color indicates the presence of triterpenoids and green or blue color indicates the presence of steroids.	Triterpenoids: Formed purple color	+

Information:
Positive (+) = contains a group of compounds
Negative (-) = contains no class of compounds

Carboxymethyl Cellulose Sodium (CMC-Na) is a cellulose derivative used as a thickener, stabilizer, gelling agent, binder, and emulsifier (Rahmania and Husni, 2017). Positive control immunostimulants administered levamisole 2.5 mg/KgBW. Levamisole is used as an immunostimulant control because it is an immunomodulator that is useful for potentiating immunity by stimulating phagocytosis by monocytes (Rao *et al.*, 2017). Meanwhile, methylprednisolone was used as a positive control for immunosuppressants, because it is a corticosteroid with anti-inflammatory and immunosuppressive properties (Masruri and Harijanti, 2017).

Figure 1 display the 100-% transmittance values from minute 0 to minute 20. The steeper the graph (the greater the slope of the line) indicates the faster it eliminates carbon particles, and vice versa. The slope of the treatment group divided by the slope of the control group produces a phagocytic index

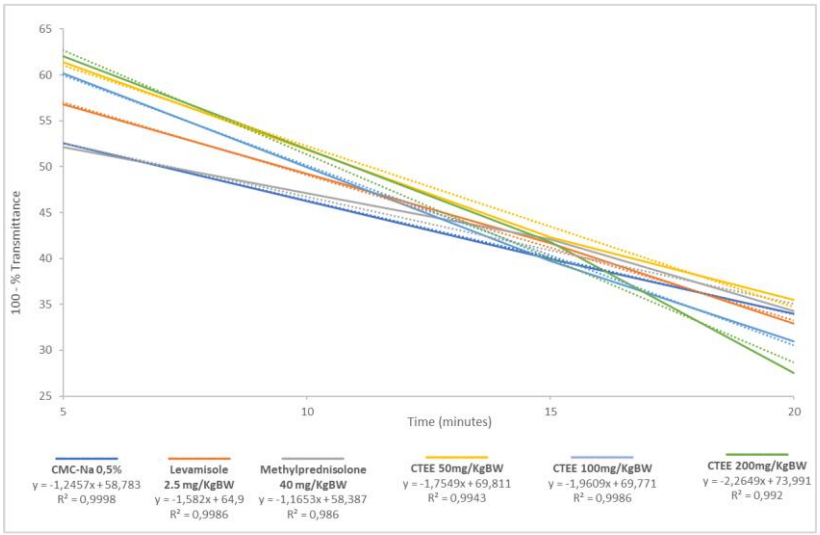

Figure 1. Graph of 100-% transmittance (Y) versus time (X). The graph shows the difference in the rate of carbon clearance from the mice's body which is affected by the difference in the administration of the preparations.

Table 2. Phagocytic index of each treatment group

Group	Phagocytic index	Activity Category
CMC-Na 0.5%	1.00	-
Levamisole 2.5 mg/KgBW	1.27	Weak immunostimulants
Methylprednisolone 40 mg/KgBW	0.94	Immunosuppressants
CTEE 50 mg/KgBW	1.41	Moderate immunostimulants
CTEE 100 mg/KgBW	1.57	Strong immunostimulant
CTEE 200 mg/KgBW	1.82	Strong immunostimulant

Notes: CTEE (*Carthamus tinctorius* ethanol extracts); N=5 per groups

value. The average value of the phagocytic index demonstrates the effect of CTEE on the phagocytosis of carbon particles. **Table** 2 demonstrates that the CTEE has immunostimulant properties. The immunostimulant effect of CTEE increases with increasing dose.

This study demonstrates that CTEE increases non-specific immune responses in Swiss Webster mice. Increased concentrations of 50, 100, and 200 mg/KgBW of CTEE increased immunomodulatory activity from moderate to strong immunostimulant criteria. The next test is testing the specific immune response, which is seen from the value of the antibody titer. Antibody titers are measured using the SRBC hemagglutination method as a component of humoral immunity measurement. Antibody titer is a measurement of variations in the number of antibodies in the immune response of an organism. The group administered the extract will have higher antibody titers than the control group. SRBC functions as an antigen that stimulates the proliferation and differentiation of B cells into antibodies or immunoglobulins by interacting with B cells. Higher levels of immunostimulant substances will result in increased antibody titers (Restuati, 2015). Antibody titer testing against mice serum involves introducing the same antigen (1% SRBC) to a 96-well plate so that antigen-antibody interactions can occur. If there is a reaction between the antigen and the antibodies in the serum, a visually discernible precipitate will form. The fluid in the well will disseminate agglutinated red blood cells. Antibody titers were determined by observing the highest dilution in wells that still showed SRBC hemagglutination (Effendi and Widiastuti, 2014). Conversely, hemagglutination did not occur as can be seen from the SRBC that settled at the bottom of the well. **Table 3** shows the results of the antibody titer test. Based on **Table 3**, CTEE dosages of 50, 100, and 200 mg/KgBW resulted in greater antibody dilution than negative controls. The antibody titer value of CTEE dose 50 mg/KgBW is lower than doses 100 and 200 mg/KgBW, indicating that the higher the dose, the greater the number of antigens that bind to the antibodies. The concentrations of 100 and

Table 3. Results of mice primary and secondary antibody titer

Test group	Primary antibody titer	Secondary antibody titer
CMC-Na 0.5%	1:256	1:256
Levamisole 2.5 mg/KgBW	1:256	1:1024
Methylprednisolone 40 mg/KgBW	1:32	1:128
CTEE 50 mg/KgBW	1:128	1:512
CTEE 100 mg/KgBW	1:256	1:1024
CTEE 200 mg/KgBW	1:512	1:1024

Notes: CTEE (*Carthamus tinctorius* ethanol extracts); N=5 per groups

200 mg/KgBW of CTEE produced the greatest dilution. This demonstrated that concentrations of 100 and 200 mg/KgBW of CTEE had a greater capacity to induce a humoral response than negative controls. It can be concluded that concentrations of 100 and 200 mg/KgBW of CTEE stimulate the production of a greater number of antibodies, causing more antibodies to bind to the antigen than in other test groups. In methylprednisolone group, there is the least amount of dilution compared to the other groups, as methylprednisolone decreases the immune response when administered to normal mice. The titers of secondary antibodies are greater than those of the primary immune response. This is due to the fact that memory cells generated during the primary immune response endure rapid transformation and differentiation into antibody-producing cells (Suwartini *et al.*, 2018).

CONCLUSIONS

Based on the findings of this study, it was determined that CTEE increases the specific humoral immune response or shows immunostimulant activity. CTEE at dose of 50, 100, and 200 mg/KgBW, the phagocytic index are 1.41 (medium immunostimulant), 1.57 (strong immunostimulant), and 1.82 (strong immunostimulant), respectively. CTEE 100 and 200 mg/KgBW can also enhance the humoral response exhibited by the same antibody titer value as the immunostimulant positive control. The CTEE contains alkaloid compounds, saponins, tannins, flavonoids, and triterpenoids.

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