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The effect of natural ingredients extracts on the phagocytosis index of the carbon clearance animal models

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ABSTRACT

The immune system has a very important role in fighting foreign substances in the body. Immunostimulants are needed to stimulate the activity of the immune system and immunosuppressants are needed to suppress the immune system. The aims of this study is to evaluate phagocytosis index of several natural ingredient extract using the carbon clearance method. The natural ingredients are the ethanol extract of Ageratum conyzoides leaves 160 mg/kg BW, ethanol extract of Artocarpus heterophyllus leaves 500 mg/kg BW, ethanol extract of Nephelium lappaceum peel 650 mg/kg BW, ethanol extract of Cucurbita moschata seed 342 mg/kg BW and ethanol extract of Citrullus lanatus rind 55 mg/kg BW. Tests were carried out on male mice Balb/C strains using the carbon clearance method, where colloidal carbon ink acts as an antigen. The phagocytic activity of macrophages in eliminating carbon ink was measured based on the stimulation index value and then the immunomodulatory effect was classified according to Wagner's criteria. The stimulation index of A.heterophyllus was 1.423; A. conyzoides was 1.201; N. lappaceum was 1.236; C.lanatus was 0.909 and C. moschata was 1.299. The potential extract as immunostimulant was A.heterophyllus, as the immunosuppressant was C. lanatus. A.conyzoides probably had no effect (act as immunorestorant) and C. moschata 342 mg/kg BW had slight immunostimulant effect.

Keywords: immune system; macrophages; phagocytosis; carbon clearance; phagocytosis index.

INTRODUCTION

The immune system is a mechanism that develops the body's capacity to combat pathogens which would exclude numerous external substances that are taken to prevent disease. The body's defense mechanisms against pathogens are all part of the immune system, which can be divided into two types: the non-specific innate immune system and the specific adaptive immune system. Both have cellular and humoral components. The innate immune system will react quickly and non-specifically if the pathogen breaches the physical barriers. The innate immune system is not able to continuously defend against a given infection (Marshall et al., 2018).

Immunomodulators are substances that can affect the immune system and have the ability to enhance the immune response or protect against pathogens or tumors. Immunomodulators include immuno-stimulants, which can boost the immunological response, immuno-suppressants, which can reduce the immune response, and immunorestorants, which can enhance immune system performance

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(Mahima et al., 2013).

Some plants and natural ingredient have the ability to modify the human immune system, hence enhancing the immune system's ability to combat infections and reducing the occurrence of illness (Swaroop et al., 2022). Immunomodulatory plants typically include secondary metabolites that modulate the immune system by acting as antioxidants during cell metabolism, as enzyme cofactors, promoting the differentiation and proliferation of B and T cells, influencing cytokine synthesis, and decreasing histamine levels (Kumar and Yadav, 2022). This study intends to evaluate the immunomodulatory effects of a number of natural substances, including *Ageratum conyzoides* leaves, *Artocarpus heterophyllus* leaves, *Nephelium lappaceum* peel, *Cucurbita moschata* seed and *Citrullus lanatus* rind.

METHODS

Good-quality material of *Ageratum conyzoides* leaves, *Artocarpus heterophyllus* leaves, *Nephelium lappaceum* peel, *Cucurbita moschata* seed, and *Citrullus lanatus* rind were acquired from the Manoko Medicinal Plants Garden in Lembang. This study utilized 50% ethanol, phytochemical reagents, Pelikan B-17 ink (Pelican-Werke, Hannover, Germany), gelatin, 0.9% NaCl, heparin, 1% acetic acid, Zymosan A (Merck®), and methylprednisolone (Quantum Labs®) as reagents. The instrument utilized is a Visible spectrophotometer (CamSpec). Male BALB/c mice from PT Biofarma's animal research facility served as animal models. The test animals used were 20–30 g in weight.

Production of the Simplicia

The simplicia that have been collected are determined at the Pharmacognosy Laboratory of Padjadjaran University. The test material was well cleaned with running water before being dried at 40°C for 24 hours, then the simplicia was ground into a powder using a blender, and last it was sieved.

Phytochemicals screening of test material

Phytochemical screening was carried out for the content of alkaloids, flavanoids, polyphenols, saponins, tannins, quinones, monoterpenoids, sesquiterpenoids, triterpenoids, steroids, and protein (Depkes RI, 2000).

Extraction

200 grams of the test substance that had been ground into a powder were weighed, placed in a flask with a flat bottom, and then covered with 50% ethanol. Reflux was performed three times for a total of 120 minutes on the filtrated reflux residue. The filtrate was then dried in an oven at 50°C to produce a thick extract.

Testing the immunomodulatory effect of the carbon clearance method

Blood was obtained from the test animals via the tail vein at the start of the experiment, lysed with 1% glacial acetic acid, and then the transmittance at T0 was determined. The test animals were then separated into eight groups: Control, Zymosan A 10 mg/kg BW, methylprednisolone 4 mg/kg BW, ethanol extract of *A.conyzoides* leaves 160 mg/kg BW, ethanol extract of *A.heterophyllus* leaves 500 mg/kg BW, ethanol extract of *N.lappaceum* peel 650 mg/kg BW, ethanol extract of *C.moschata* seed 342 mg/kg BW and ethanol extract of *C.lanatus* rind 55 mg/kg BW. For 15 days, every treatment was administered orally. Every animal fasted on the

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fifteenth day. On the sixteenth day, a suspension of carbon ink 0.025 mL/10g BW was administered to the animals. Following the injection of the carbon ink suspension, blood samples were obtained at 5, 10, 15, and 20 minutes. Blood samples 25 µL were diluted with 4 mL 1% acetic acid solution, and the transmittance was then determined using a visible spectrophotometer at a wavelength 650 nm. Then the stimulation constant (SC) and stimulation index (SI) were determined (Vikasari et al., 2015).

The stimulation constant was calculated from the difference in optical density (OD) at 5 and 20 minutes to the difference in the time of blood sampling. The stimulation index was calculated from the comparison of the KS test to the KS control. Based on the SI value, the classification of the immunomodulatory effect of a substance is no effect (SI = 1.0-1.2), slight (SI=1.3-1.5) and strong (SI ≥ 1.5) (Vikasari et al., 2015).

RESULT AND DISCUSSION

The determination results showed that the plats were *Ageratum conyzoides*, *Artocarpus heterophyllus*, *Nephelium lappaceum*, *Cucurbita moschata* and *Citrullus lanatus*.

Phytochemical screening was carried out to determine the class of secondary metabolite compounds belonging to the plant. The results of phytochemical screening can be seen in Table 1. The results showed that all simplicia contain flavonoids, polyphenols, quinones and monoterpenoids/ sesquiterpenoids.

Table 1. The results of characteristics and phytochemical screening assay

Simplisia	***A.conyzoides* leaves**	***A.heterophyllus* leaves**	***N.lappaceum* peel**	***C.moschata* seed**	***C.lanatus* rind**
Phytochemical screening					
Alkaloids	+	+	+	+	–
Flavonoids	+	+	+	+	+
Polyphenol	+	+	+	+	+
Saponins	–	–	+	–	–
Quinones	+	+	+	+	+
Tannins	–	+	+	–	–
Monoterpenoids/ Seskuiterpenoids	+	+	+	+	+
Triterpenoids/Steroids	–	–	–	–	–

Note: + : contains the tested compound, – does not contain the tested compound

The Carbon Clearance test is designed to measure the capacity of reticuloendothelial cells in the blood to eliminate carbon ink colloidal particles that function as antigens. The activity of macrophages in phagocytosis can be assessed using the stimulation constant, which is the ability of the test preparation to excite the non-specific immune system in phagocytosing carbon ink colloidal particles (Utama, Rosidah and Yuandani, 2020).

The stimulation index indicates the ability of active macrophages to phagocytize colloidal particles of carbon ink based on the optical density value. The optical density and phagocytosis index values increase with the amount of phagocytosed colloidal carbon particles.

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Table 2. The results of the immunomodulatory effect using the carbon clearance test

Groups	Transmittance at the minute:		OD at minute		LnOD20-LnOD5	SC	SI
	5	20	5	20			
Control	42.24	34.48	0.374	0.462	0.212	0.014	-
Methylprednisolone 1.4 mg/kg BW	39.20	34.40	0.407	0.463	0.131	0.009	0.617
Zymosan A 10 mg/kg BW	44.60	33.48	0.351	0.475	0.304	0.020	1.437
A.conyzoides 160 mg/kg BW	41.24	31.92	0.385	0.496	0.254	0.017	1.201
A. heterophyllus 500 mg/kg BW	44.20	33.18	0.355	0.479	0.301	0.020	1.423
N. lappaceum 650 mg/kg BW	46.20	36.68	0.335	0.436	0.261	0.017	1.236
C. lanatus 55 mg/kg BW	39.42	32.36	0.404	0.490	0.192	0.013	0.909
C.moschata 342 mg/kg BW	46.44	36.44	0.333	0.438	0.275	0.018	1.299

Comparative analysis revealed that Methylprednisolone 1.4mg/kg BW had an immunosuppressive effect (SI=0.617), whereas Zymosan A 10 mg/kg BW had an immunostimulating effect (SI = 1.437). Based on data from table 2, it was found that the potential extract as an immunosuppressant was *C. lanatus* 55 mg/kg BW (SI = 0.909), and extracts with potential as a strong immunostimulator were *A. heterophyllus* 500 mg/kg BW (SI = 1.423). The result also showed that *A.conyzoides* 160 mg/kg BW (SI=1.201) and *N. lappaceum* 650 mg/kg BW (SI=1.236) probably had no effect or act as immunorestorant, but it needs further study, and and *C. moschata* 342 mg/kg BW (SI=1.299) had slight immunostimulant effect. Contrary with this result, the study from Iwo et al (2014) showed pumpkins seed extract at doses of 3.8-7.6 g/kg BW, pumpkins seed extract had strong immunostimulant effect (Iwo, Insanu and Dass, 2014). Based on this, the increase in effect is affected by an increase in dose, therefore further research is needed at a minimum dose of *C. moschata* that has an immunostimulating effect.

Methylprednisolone-treated control subjects were used as a benchmark for immunosuppressant comparison. It reduces the amount of lymphocytes in the blood circulation by preventing the production of proinflammatory cytokines (Noack, Ndongo-Thiam and Miossec, 2016). The Zymosan group has immunostimulatory properties. Zymosan can boost immunological response by activating macrophages, leukocytes, and monocytes as well as secreting proinflammatory cytokines (Vikasari, Soemardji and Sutjiatmo, 2015; Abou Elazab et al., 2017). The high phagocytosis index value demonstrates that the amount of carbon ink colloidal particles decreases over time as a result of macrophage activity in phagocytizing antigens in the blood of test animals.

Flavonoids found in *A.conyzoides, A.heterophyllus, N.lappaceum, C.moschata* have potential as immunomodulators. Flavonoids have activity in modulating the immune response, by activating macrophages, triggering the work of NK cells in producing IFN-γ and suppressing the activity of mTOR mediators in producing T lymphocytes (Hosseinzade et al., 2019). Exocarp of *C.lanatus* contains the amino acid L-citrulline which is a precursor of L-arginine as a substrate for the synthesis of nitric oxide, which is an important signal molecule in cell regulation in binding radical

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hydroxyl groups (Aguayo et al., 2021).

It is believed that the Zn concentration in *C.moschata* contributes to RNA synthesis, lymphoid cell proliferation, T cell maturation, and the creation of regulatory cytokines that are important for the immune system, particularly for the removal of antigens (Hojyo and Fukada, 2016; Purnamasari et al., 2022). *C.moschata* seeds contain carotenoid compounds which also have antioxidant activity (Kulczyński, Sidor and Gramza-Michałowska, 2020).

This study was conducted to determine the fundamental properties of a substance for non-specific immunomodulatory activity via the carbon clearance test. Therefore, further study on specific immunomodulatory activity using other animal models or cell lines is required. Specific immunomodulator evaluation may be performed using the humoral antibody response technique, delayed type hypersensitivity response, effect on total leucocyte count, and leucocyte mobilization studies. In vitro testing using a cell line can be performed on the K562 cell line, J 779 macrophage cell, K562 cell line and cutaneous squamous cell carcinoma cell line (Ganeshpurkar and Saluja, 2017).

CONCLUSION

Extracts that have potential as immunostimulants is *A.heterophyllus* and *C. lanatus* had potency as immunosupressant. It is important to conduct additional study on the immunostimulatory effect of these natural substances utilizing other, more specialized testing methods.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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