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Immunomodulatory Effects of *Citrus* Species Ethanol Extracts on Cellular Immune Responses in Wistar Rats and Mice

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Abstract

Currenty, *Citrus reticulata* has been found to modulate RAW 264.7 macrophage cell line. In the present study, the immunomodulatory effects of *Citrus* species peel extracts, including *Citrus microcarpa*, C. *limon* L., C. *sinensis* L. and C. *hystrix* were investigated on cellular immune responses in Wistar rats and mice. Mice were treated with ethanol extracts of *Citrus* species orally at dosages of 25, 50 and 100 mg/kg bw for 7 days. The phagocytosis activity of the extracts was examined using the carbon clearance method. Meanwhile, oral administration of *Citrus* species ethanol extracts at dosages of 17.5, 35 and 70 mg/kg bw was administered to Wistar rats to assess the effect of extracts on the delayed-type hypersensitivity (DTH) response using paw edema-method. Thin Layer Chromatography (TLC)-densitometry analysis was also performed. In both animal models, all extracts enhanced the cellular immune responses. Amongst the samples, C. *sinensis*, at the dose of 100 mg/kg bw in mice and 70 mg/kb bw in rats, demonstrated the highest stimulation on phagocytosis activity and DTH response, respectively. Except *C. microcarpa*, rutin was found in all *Citrus* species. The results indicate that *Citrus* species, particularly *C. sinensis*, have the potential to be developed into a potent immunostimulatory agent.

Keywords: *Citrus* species, Cellular immune response, Immunomodulator, Phagocytosis, Delayed-type hypersensitivity response, Thin layer chromatography-densitometry, Rutin

Introduction

The human defense system is a complicated system, but each of its parts works together to perform a highly specialized and coordinated function [1]. This function is mediated by various of cells and molecules which are able to recognize and destroy various pathogen and undesirable materials [2]. Phagocytes play a necessary role in the non-specific immune system [3]. The professional phagocytes include many types of white blood cells such as neutrophils, monocytes and macrophages [4]. Phagocytosis which facilitated by

recognizing directly by bacterial structures and by opsonized bacteria is performed using pseudopodia which is extended to surround an organism or particle, finally meet and fuse to form a closed vacuole (phagosome) [5]. Phagocytosis of microorganism triggers generation of superoxide radical (O2•-) and other secondarily derived reactive oxygen species (ROS) such as hypochlorous acid (HOCl), hydoxy radical and chloramines through the activity of myeloperoxidase (MPO). Besides, macrophages produce nitric oxide (NO.), a major radical nitrogen species (RNS), which reacts with superoxide to form peroxynitrite (ONOO.), a potent microbicidal agent, by the enzymatic of inducible nitric oxide synthase (iNOS) [6].

Macrophages process antigen and presented antigen to T cells. T cell Receptor (TCR) recognize a complex on the surface of Antigen-presenting cells (APC's) which consist of both the peptide fragments of antigen and a class II MHC protein through direct binding of CD4+ regions to the APC's class II MHC molecule. Moreover, this binding activates helper T cells. Whereas the activation of cytotoxic T cells become activated if the APC itself is infected with a virus, then viral proteins are synthesized and presented on the surface in association with class I MHC proteins [7]. The regulatory function is mediated mainly by helper CD4+ T cells. Th cells help B cells develop into plasma cells which can produce antibody. However, dysfunction of immune response may cause various diseases, such as ulcerative colitis (UC), psoriasis, rheumatoid arthritis (RA) and immunodeficiency disorders. Thus, modulation of immune responses is required for the treatment of those diseases [8].

Traditional herbs have been used as alternative medicine to treat various diseases including immune related diseases since many years ago. The extracts of many medicinal plants such as *Citrus reticulata, Gynura segetum, Curcuma mangga* and *Picria fel-terrae* and their bioactive secondary metabolites have been investigated for their pharmacological activities [9-12]. *Citrus* species is an economically beneficial plant for body, particularly for preventing infectious organisms. *Citrus* species contains rutin (quercetin-3-O-rutinoside) and diosmin (diosmetin 7-orutinoside) as the major bioactive compound [13]. Hesperidin and naringenin were also reported as the potent bioactive compounds in *Citrus* species [14,15]. The esssential oils of *Citrus* species has been reported for their pharmacological activities, such as antioxidant, antibacterial, and antifungal activities. Moreover, other studies revealed *Citrus* species activity against foodborne pathogens; *Escherichia coli, Campylobacter jejuni, Listeria monocytogenes, Bacillus cereus*, and *Staphylococcus aureus*. Moreover, *Citrus reticulata* has been reported to modulate RAW 264.7 macrophage cell line [9]. However, a lack of study reported the immunomodulatory effect of various *Citrus* species. In this paper, we report on the immunomodulatory effects of *Citrus microcarpa*, *C. limon* L., *C. sinensis* L. and *C. hystrix* on phagocytosis activity in mice as well as DTH response of *Staphylococcus aureus*-infected Wistar rats.

Materials and methods

Plant materials

The peels of *Citrus* species were obtained from North Sumatera province, Indonesia. A botanist verified the authenticity of the plant samples, and voucher specimens bearing the voucher numbers of 1397/MEDA/2023; 1398/MEDA/2023; 1399/MEDA/2023; 1400/MEDA/2023 for *Citrus microcarpa*, *C. limon* L., *C. sinensis* L. and *C. hystrix*, respectively.

Plant extraction

In brief, the powdered dry sample material was soaked at room temperature in absolute ethanol (1:10 (w/v)). The solvent was filtered through Whatman No. 1 filter paper (Whatman, England) and the mixture was constantly agitated. After that, the solvent was extracted under low pressure, yielding a crude extract

for *C. sinensis*, *C. limon*, *C. hystrix*, and *C. microcarpa*, respectively, with yields of 15.76, 15.14, 15.72, and 15.70 %.

Quantitative analysis of *Citrus* species extracts using thin layer chromatography (TLC)-densitometry analysis

The presence of rutin in *Citrus* species extracts was determined using TLC-densitometry method. Sample solutions were prepared by dissolving 100 mg *Citrus* species extract in 10 mL of ethanol. Then, the samples were spotted (20 µL) on a precoated silica gel glass plate 60F245 (20×20 cm^2) (E. Merck, Darmstad, Germany) with a micro liter syringe. The mobile phase was made up of ethyl acetate: Formic acid: Water (100:15:17). A TLC scanner was used to do densitometric analysis at 350 nm. An amount of 10 mg of standard compound (rutin) was dissolved in a standard solution in 10 mL of ethanol. Calibration standards were prepared at concentrations of 10, 20, 30, 40, 50 ppm of rutin. The levels of rutin were determined by the substitution peak area of samples in the regression equation of rutin.

Antigen preparation

A nutrient broth was used to dilute 1 mL aliquot of *Staphylococcus aureus* (ATCC 25923) that was obtained from the American Type Culture Collection (ATCC, USA). This resulted in a cell concentration of 1×108 cells/mL^2, which was determined using spectrophotometry. The mixture was centrifuged for 10 min at 10,000 rpm at 25 °C. After the cells were divided, 1 mL of phosphate buffer saline (PBS) (Sigma, USA) was used to wash them [10].

Animals

There were 25 mice (20 - 30 g) and 50 male Wistar rats (120 - 200 g) used in this study. The test animals were acclimated by housing mice and rats in plastic cages with regular circumstances for 7 to 14 days at room temperature and with enough ventilation. Water was available to the animals at all times, along with a regular pellet meal. Animal Research Ethics Committees (AREC), Faculty of Mathematics and Natural Science (FMIPA), Universitas Sumatera Utara approved all protocols for using animals in this study (approval number: 0687/KEPH-FMIPA/2023).

Phagocytosis assay

The effect of ethanol extracts of *Citrus* species on phagocytosis ability was assessed using a carbon clearance approach [16]. In brief, 25 mice received doses of 25, 50 and 100 mg/kg bw of citrus species extracts every day for 7 days. Imboost® (32.5 mg/kg bw) and the vehicle, 0.5 % sodium carboxymethylcellulose (Na CMC), served as the positive and negative controls, respectively. On day 8, an intravenous injection of a dispersion of China ink (0.1 mL per 10 g) was administered to each animal through the tail vein. Afterwards, blood samples (25 µL) were drawn from each animal at intervals of 5, 10, 15 and 20 min. In order to lyse the erythrocytes, 4 milliliters of 1 % acetic acid were added to the blood samples. The absorbance of the supernatants at 640.5 nm was measured using a Thermo Scientific Microplate Reader (Thermo Fisher Scientific, USA). The animals were finally sacrificed 12 h after the blood was drawn, and their livers and spleens were thrown away. The phagocytic index and rate of carbon clearance was subsequently calculated using the following formulas.

$$\text{Rate of carbon clearance (K)} = \frac{\text{Log OD5} - \text{log OD20}}{\text{t2} - \text{t1}} \quad (1)$$

$$\text{Phagocytic index } (\alpha) = \frac{\text{K1/3 x animal body wt}}{\text{Liver wt + spleen wt}} \quad (2)$$

where t2 is the final time point of blood collection and t1 is the first time point; OD5 and OD20 are the log absorbance of blood at 5 and 20 min, respectively.

Delayed-type hypersensitivity response

The alteration of paw volume was determined to assess the effect of *Citrus* species ethanol extracts on the Delayed-Type Hypersensitivity (DTH) response, as previously reported [10]. In brief, the rats received an intraperitoneal injection sensitizing them to *S. aureus* (1×10^8 cells/mL^2). 72 h later, the rats received the combined extracts (1:1) at doses of 17.5, 35 and 70 mg/kg bw (the doses are comparable to the doses tested in mice for phagocytosis effect) and this process continued for 14 days. Imboost® (32.5 mg/kg bw) was the positive control, while 0.5 % Na CMC was the only substance contained in the negative control. On day 14, the volume of the mice's hind paws was measured using a pletismometer (Ugo Basile, Italy). Following a subcutaneous injection of S. aureus into the animals' hind paws, the mean increase (ΔV) in paw volume was observed 24 h later (ΔV: Vt-Vo; Vt: Final time paw volume; Vo: First time paw volume).

Statistical analysis

The data were statistically evaluated using a one-way analysis of variance (ANOVA) and the post hoc Tukey test. The data were given as mean and standard error mean, or SEM. Differences were considered significant when *p*-values were less than 0.05.

Results and discussion

Quantitative analysis of *citrus* species extracts using thin layer chromatography (TLC)-densitometry analysis

TLC-densitometry analysis was performed to determine the rutin content in several Citrus species extracts. Rutin was eluted with an Rf value of 0.30, whereas 0.27, 0.29 and 0.29 were found in *C. sinensis, C. limon* and *C. hystrix* ethanol extracts, respectively. Calibration curves plotted were linear with a correlation coefficient ($r2$) of 0.9769 (**Figure 1**). Amongst the samples, *C. limon* contained the highest amount of rutin (187.0437 µg/mL) (**Table 1**). Previous studies also reported the presence of rutin in *C.limon* and *C.sinensis* [17-18].

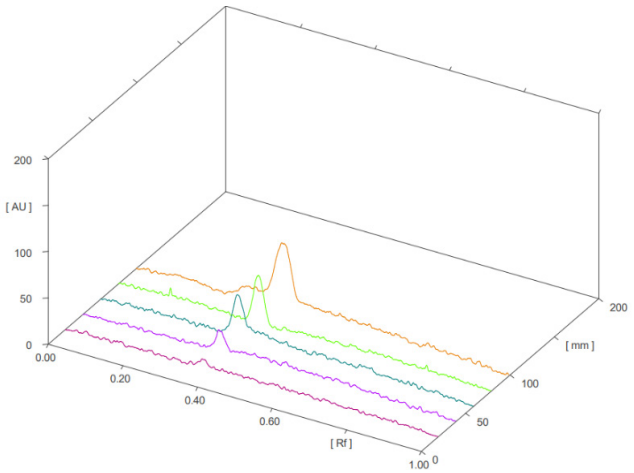

Figure 1 Calibration curve of rutin at Rf 0.34, detected at wavelength 350 nm.

Table 1 Rutin content in *Citrus* species extracts obtained by TLC-densitometry analysis.

Samples	Rf value	Concentration (µg/mL)
Citrus sinensis extract	0.27	126.5241
Citrus hystrix extract	0.29	173.6340
Citrus limon extract	0.29	187.0437
Citrus microcarpa extract	NI	NI

NI: Not Identified.

Phagocytosis assay

The immunomodulatory effects of the ethanol extracts of *Citrus* species on phagocytosis ability was investigated using mice as the animal model. **Table 2** demonstrates that the *Citrus* extracts at the doses of 25, 50 and 100 mg/kg bw increased the rate of carbon elimination which was higher than those of negative control ($p < 0.05$), suggesting that they were increasing the rate of carbon engulfment and therefore promoting the phagocytic cells. The primary cellular innate immune reaction to eliminate infections is phagocytosis. When a phagocyte recognizes the bacterial structures, it begins the process of phagocytosis and forms pseudopodia to enclose the pathogen [5]. Phagocytosis involves ingestion of particulate ligands which are generally big and numerous particles larger than 1 µm. Phagocytosis carries out 2 essential immune roles, i.e. as an innate immune effector as well as a bridge between the innate and adaptive immune responses. The body's defense against invasive organisms is significantly strengthened by the improvement of phagocytosis's capacity to eradicate pathogens [19]. The higher rate at which carbon particles were cleared from the bloodstream indicated that phagocytosis activity of mice leukocytes was enhanced. *Citrus* species ethanol extracts increased carbon intake in a dose-dependent manner. Amongst the extracts, *C. sinensis* extract at the dose of 100 mg/kg showed the highest stimulation at a dose of 100 mg/kg bw, exhibiting a phagocytic index of 3.090, followed by *C. limon, C. hystrix* and *C. macrocarpa* with the phagocytic index of 3.022; 2.845 and 2.726, respectively (**Figure 2**). The result was supported by a previous study which reported the ability of *Citrus limetta* peel to enhance phagocytic index [20].

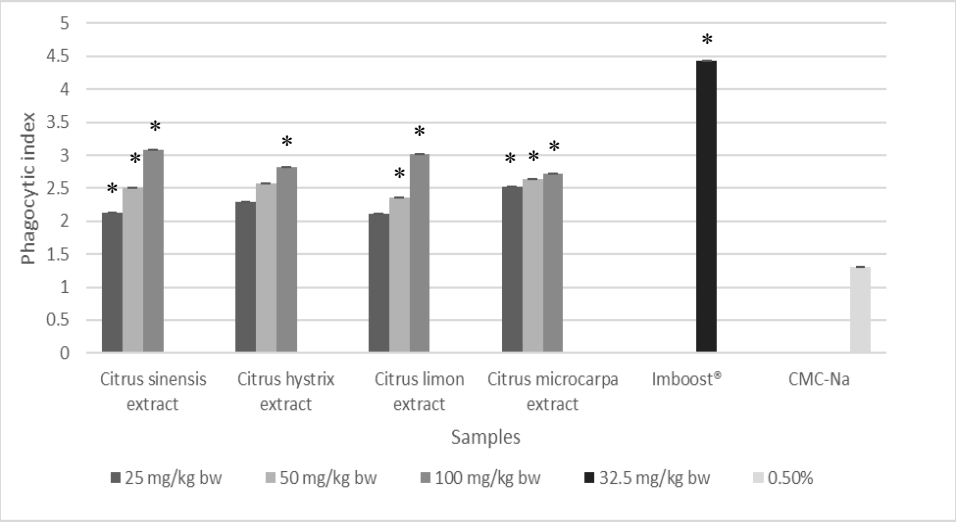

Figure 2 Effect of ethanol extracts of *Citrus* species on phagocytic index (Mean ± SD, $^{*}p < 0.05$ significant to respective control).

Table 2 Effect of ethanol extracts of *Citrus* species on carbon clearance (Mean ± SD).

Samples	Rate of carbon clearance				
	25 mg/kg bw	**50 mg/kg bw**	**100 mg/kg bw**	**32.5 mg/kg bw**	**0.5 %**
Citrus sinensis extract	0.007 ± 0.001*	0.008 ± 0.001*	0.011 ± 0.001*		
Citrus hystrix extract	0.012 ± 0.004*	0.012 ± 0.006*	0.013 ± 0.004*		
Citrus limon extract	0.006 ± 0.002	0.009 ± 0.003*	0.014 ± 0.005*		
Citrusb microcarpa extract	0.011 ± 0.001*	0.012 ± 0.002*	0.012 ± 0.002*		
Imboost®				0.0183 ± 0.005*	
CMC-Na					0.002 ± 0.001

*$p < 0.05$ significant to respective control.

Delayed-type hypersensitivity (DTH) response

The ability of the samples to induce the Delayed-Type Hypersensitivity (DTH) response was demonstrated by the fact that all of the ethanol extracts of *Citrus* species increased DTH response of Wistar rats when compared to those of the negative control ($p < 0.05$). **Table 3** illustrates how extracts of *Citrus* species increased the paw volume of Wistar rats in a dose-dependent manner. DTH response is initiated by the activation of the innate arm of the immune system by environmental or microbial antigen. Thereafter, innate immune recognition triggers off a series of inflammatory cascades that include leukocyte chemotaxis toward the infection site, antimicrobial mechanism activation, and the stimulation of adaptive immune response by the interaction between MHC class II antigen on the Antigen Processing Cells (APCs) with T-cell receptor (TCR) on the surface of T-helper. Th-1 subset produce interferon-γ (IFN γ) that activates macrophage, thus paw edema development is a sign that cytokines have stimulated macrophages to start an inflammatory response as a defense mechanism [21-23]. The DTH response was most stimulated by the ethanol extract of *C. sinensis* at a dose of 70 mg/kg bw (paw volume of 2.848), which was comparable to that of the positive control (paw volume of 1.688). The result was in accordance with phagocytosis ability evaluation which revealed that *C. sinensis* has the strongest phagocytosis stimulation. A previous study also reported that *Citrus limetta* peel enhanced DTH response [20]. The immunostimulatory effect of the *C. sinensis* extract might be due to the presence of rutin, although other constituents may also contribute. Rutin has been found to have immunostimulant effect on phagocytosis and delayed-type hypersensitivity response [24].

Table 3 Effect of ethanol extracts of *Citrus* species on delayed type hypersensitivity (DTH) response in *Staphylococcus aureus*-infected rats (Mean ± SD).

Samples	Paw volume (mL)				
	17.5 mg/kg bw	**35 mg/kg bw**	**70 mg/kg bw**	**25 mg/kg bw**	**0.50 %**
Citrus sinensis extract	2.220 ± 0.229*	2.486 ± 0.089*	2.848 ± 0.162*		
Citrus hystrix extract	2.204 ± 1.229*	2.372 ± 0.452*	2.498 ± 0.509*		
Citrus limon extract	1.924 ± 0.438*	2.296 ± 0.464*	2.071 ± 0.458*		
Citrus microcarpa extract	1.246 ± 0.303*	1.690 ± 0.099*	2.390 ± 0.223*		
Levamisole				1.688 ± 0.545*	
CMC-Na					0.292 ± 0.106

*$p < 0.05$ significant to respective control.

Conclusions

The ethanol extracts of *Citrus sinensis*, C. *hystrix, C. limon* and *C. microcarpa* showed immunostimulatory effects on cellular immune response. The extracts strongly enhanced phagocytic ability in mice and stimulated DTH response in *S. aureus*-infected rats. Amongst the *Citrus* species extract, *C. sinensis* demonstrated the highest immunostimulatory effects on both phagocytosis and DTH response. Rutin was found in all extracts, except in *C. microcarpa.* Rutin might contribute to the immunostimulatory effects of *Citrus* species extract, although other constituents may also contribute. Further elucidaton is required to investigate the mechanisms of action of *Citrus species* and their compounds in different lineages of immune responses.

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Ethical approval

Animal Research Ethics Committees (AREC), Faculty of Mathematics and Natural Science (FMIPA), Universitas Sumatera Utara approved all protocols for using animals in this study (approval number: 0687/KEPH-FMIPA/2023).

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