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Acute toxicity evaluation and immunomodulatory potential of hydrodynamic cavitation extract of citrus peels

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ABSTRACT

Citrus peels have been explored vastly due to their high methoxy flavonoid contents that show several biological activities, including immunomodulation, anti-inflammation, and chemoprevention. A water-based extraction method, hydrodynamic cavitation (HC), has recently been adopted to obtain a high methoxy flavonoid extract from *Citrus reticulata* peels in order to increase its acceptance and utilization. Aiming to verify the safety and immunomodulatory potency of this HC extract of citrus peels (HCE-CP), an acute toxicity test and immunomodulatory assay in rats were carried out. The single oral administration of HCE-CP did not show signs of either toxicity or death up to the 15th day even at the highest dosage, resulting in an lethal dose 50 of more than 5,000 mg/kg body weight (BW). In immunomodulatory evaluation, HCE-CP was given orally daily for 11 weeks, induced with lipopolysaccharide (LPS), and the blood was sampled 5 hours after LPS induction. HCE-CP treatments increased the relative weight of the spleen. The dosage of 2,000 mg/kg BW reduced CD4+ T lymphocytes significantly, but not CD8+. Bioinformatics analysis predicted that hesperidin and hesperetin, two major methoxy flavonoids in HCE-CP, target several proteins that are involved in inflammation in LPS-induced lymphocytes. Taking these data together, HCE-CP is not toxic and shows immunomodulatory potency, especially anti-inflammation.

INTRODUCTION

Citrus fruits, especially the peel, are rich in methoxy flavonoids, including hesperidin and diosmin (Barreca *et al.*, 2017; Mahato *et al.*, 2018; Meiyanto *et al.*, 2012). The extract from *Citrus reticulata* peels and hesperidin/diosmin have been reported to show antiviral properties *in silico* (Chen *et al.*, 2020; Utomo *et al.*, 2020) while *in vitro* and *in vivo* they display immunomodulatory activities (Cheng *et al.*, 2020; Ikawati *et al.*, 2019), anti-inflammation (Tejada *et al.*, 2018), and chemoprevention and anticancer activities (Gilang *et al.*, 2012; Kusharyanti *et al.*, 2011; Meiyanto *et al.*, 2011). Hesperidin and diosmin have good pharmacodynamic and pharmacokinetic properties in the human body (Manach *et al.*, 2003) and do not show significant toxic effects (Cheng *et al.*, 2020). The lethal dose 50 (LD_{50}) of hesperidin/diosmin is 2 g/kg body weight (BW) (Zanwar *et al.*, 2014), and thus it is safe for therapeutic use. Hesperidin and diosmin are currently used as therapeutic agents to improve blood circulation (Nagasako-Akazome, 2014; Zanwar *et al.*, 2014), so, for other therapeutic purposes, phase 1 and 2 clinical trials can be omitted.

Despite the vast proven benefits of its methoxy flavonoid contents, the utilization of the extract from citrus peels

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is still limited by the acceptance, toxicity, and environmental issues since most extractions use organic solvents (Lachos-Perez *et al.*, 2018). To overcome this, a water-based extraction system for *C. reticulata* peels, as adopted from Meneguzzo *et al.* (2020), has been developed. With a goal to provide a safe and highly potential immunomodulatory agent from natural ingredients that are produced locally and independently, a further validation on the safety and efficacy of this hydrodynamic cavitation extract of citrus peels (HCE-CP) is needed. In this study, an acute toxicity test and immunomodulatory assay in rats were carried out. Considering the safety and the immunomodulatory properties of hesperidin/diosmin as main compounds in HCE-CP as described above, it can be predicted that HCE-CP would show no acute oral toxicity in rats and possibly display immunomodulatory activities in lipopolysaccharide (LPS)-induced rats. A follow-up bioinformatics analysis would provide more information on the HCE-CP target proteins that are involved in the immunomodulatory activity.

MATERIALS AND METHODS

Preparation of citrus peels extract with HC

Citrus reticulata peels were collected from West Java, Indonesia, in July 2020 and were authenticated in the Department of Biological Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada (UGM), with a reference number of 2.02.06/UN1/FFA/BF/PT/2021. The peels were air-dried and covered with black plastic. Dried peels were then powdered and prepared for extraction using the HC technique with water as solvent (Meneguzzo *et al.*, 2020) and modified by the Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, UGM. The liquid extract was then evaporated in a vacuum evaporator until a brownish crude extract was obtained. The extract was detected for its phytochemical content by thin-layer chromatography to detect flavonoid components as previously described (Anggoro *et al.*, 2021; Harborne, 1984). The obtained extract, heretofore referred to as HCE-CP, was dissolved in sterile water to obtain a designated dosage with a final volume of 3 ml for the oral administration to animals.

Animals

Adult (8-week-old) male Sprague-Dawley rats (*Rattus norvegicus*), weighing 80–120 g, were used in this study. Animals were acclimatized for around 2 weeks before the experiments and were randomized into control and experimental groups. Rats were housed per group in polypropylene cages with paddy husks as bedding and maintained in the animal facility of our institution accordingly to a standard procedure at room temperature with a 12-hour light/dark cycle (Lestari *et al.*, 2019). Free access to a standard pellet diet and water *ad libitum* was available. All experimental procedures were approved by the Animal Welfare Committee of UGM (Approval no. 00053/04/LPPT/XI/2020).

Acute oral toxicity study

The acute oral toxicity study was carried out referring to Organization of Economic Cooperation and Development (OECD) guideline 420 for acute oral toxicity fixed dose procedure (OECD, 2001) and the guideline for *in vivo* nonclinical toxicity testing by the National Agency of Drug and Food Control (NADFC) of Indonesia (NADFC, 2014). Single administration of HCE-CP at doses of 5, 50, 300, 2,000, and 5,000 mg/kg BW was given orally to the treatment groups (n = 5 per group). As HCE-CP is water based, the control group was kept untreated.

Clinical observation

Rats were observed for 24 hours, with an intensive observation for the first 30 minutes and 4 hours, and this was then continued once daily up to 14 days after treatment (NADFC, 2014; OECD, 2001). Any signs of acute toxicity including various changes in physical appearances, behavior, and mortality were visually assessed (NADFC, 2014; OECD, 2001).

BW measurement

The BW of each rat was measured before the test and every 3 days during the study (NADFC, 2014; OECD, 2001).

Histopathological analysis

At the end of observation, 14 days after oral administration, the animals were sacrificed by intraperitoneal ketamine-xylazine euthanasia (0.1 ml/100 g containing 4−10 mg ketamine and 0.5−1.3 mg xylazine) and the vital organs including the heart, liver, lungs, stomach, and kidneys were isolated. The organs were observed for the presence of nodules, abnormality in shape and consistency, and discoloration. Following a macroscopic observation, the organs were processed by the formalin-fixed paraffin-embedded method. Sections were made and stained with hematoxylin and eosin (HE) (Feldman and Wolfe, 2014). The examination was carried out microscopically at ×400 in a blind manner by a pathologist. Five slides from each tissue of four animals per group were included. The HE-stained sections were observed for the presence of inflammation, congestion, and hydropic degeneration.

Immunomodulatory study

Animal treatment and LPS induction

The immunomodulatory study was conducted by using the acute induction of LPS in rats (Singh and Jiang, 2003). A daily dose of HCE-CP at 200, 500, 1,000, and 2,000 mg/kg BW was administered orally to the treatment groups (n = 5 per group) for 11 weeks (Elelaimy *et al.*, 2012). The following day after the last treatment, 1 µg/g BW of LPS (from *Escherichia coli* O111:B4, Catalog no. L4391, Sigma) was injected intravenously (Singh and Jiang, 2003). Five hours after LPS induction, the blood was sampled, and the animals were sacrificed for organ isolation. Two control groups without HCE-CP treatment, one for untreated and one for LPS control, were also included.

BW and relative organ weight measurement

Before and during the treatment, the BW was monitored every 3 days. The liver, kidney, spleen, heart, lungs, and stomach that were isolated at the end of the study were cleaned in phosphate-buffered saline and dry-blotted by using a paper towel, and the wet organ was weighed. The ratio of organ weight to BW at the end of the treatment was calculated (Porwal *et al.*, 2017; Soufane *et al.*, 2013).

Hematology analysis

The blood collected in ethylenediamine tetraacetic acid-containing tubes was evaluated for various hematological parameters as listed previously (Porwal *et al.*, 2017; Soufane *et al.*, 2013) at the Department of Clinical Pathology and Laboratory Medicine, Faculty of Medicine, Nursing, and Public Health, UGM.

CD4+ and CD8+ flow cytometry

The flow cytometry analysis was carried out to observe CD4+ and CD8+ T cell lymphocyte population as described previously (Alif *et al.*, 2021; Kasianningsih *et al.*, 2011). Briefly, the collected whole blood from each animal was stained by anti-rat CD4 or CD8 fluorescein isothiocyanate antibodies from Invitrogen (Catalog nos. 11-0040-82 and 11-0084-82, respectively) and then was subjected to flow cytometry (BD FACS Aria III). The population of CD4+ and CD8+ cells was calculated as a percentage to total reads (Alif *et al.*, 2021; Anggriani *et al.*, 2019).

Statistical analysis

All values were presented as mean ± standard deviation (SD). The results were analyzed statistically by Student's *t*-test (two-tailed, type 2) (Excel 16.45, 2019) at a confidence level of 95% ($p < 0.05$).

Bioinformatics analysis

To gain more information on the effect of HCE-CP in LPS-induced lymphocytes, we predicted target proteins of methoxy flavonoids. The Simplified Molecular Input Line Entry System structure of hesperidin, as the major methoxy flavonoid in HCE-CP (Meneguzzo *et al.*, 2020; Utomo *et al.*, 2020), and its aglycon form, hesperetin, was pasted in SwissTargetPrediction (http://www.swisstargetprediction.ch) (Daina *et al.*, 2019) by choosing *Homo sapiens* as the species (Hasbiyani *et al.*, 2021; Musyayyadah *et al.*, 2021). Meanwhile, the protein expressed in LPS-induced lymphocytes was acquired from GeneCards (https://www.genecards.org) by using "LPS-induced lymphocytes" as the keywords. Proteins expressed in LPS-induced lymphocytes were then sliced with target proteins of hesperidin and hesperetin in a Venn diagram (http://www.interactivenn.net) (Heberle *et al.*, 2015).

RESULTS

Acute oral toxicity study of HCE-CP in rats

The acute toxicity potential based on the value of LD_{50} and the toxic effects that appeared within 24 hours after the single oral administration of HCE-CP and 14 days after was observed in rats. Clinical observation of possible toxic symptoms was carried out after 30 minutes, 4 hours, and 24 hours after treatment, followed up by daily observation up to day 15 to monitor possible delayed effects. The parameter observation included changes in somatomotor activities and behavior patterns that consisted of tremors, paralysis, and compulsion to move. The fur, skin, and eyes and secretions were also monitored. All treatment groups did not show any toxic symptoms or changes in behavior and other observed parameters (Table 1).

Until the 15th day of observation, there was no mortality in all groups (Table 1). The single oral administration of HCE-CP in male rats up to a maximum dose of 5,000 mg/kg BW did not induce death. Thus, the LD_{50} of HCE-CP was more than 5,000 mg/kg BW and can be considered practically nontoxic according to the Hodge and Sterner toxicity scale (NADFC, 2014).

The observation of general condition was also carried out on the average BW and changes in BW. The BW was measured every 3 for 15 days. As seen in Table 1, there was no decrease in BW throughout the study. The weekly average BW of the tested animals in all groups increased normally (Table 2). Before the treatment (week 0), the average BW of the HCE-CP group at the dosages of 5 and 5,000 mg/kg BW was higher than that of the control group. Meanwhile, at the end of the study (week 2), the 50 mg/kg BW group showed a lower average BW. However, these differences can be considered normal due to the biological variation among tested animals, taking into account that differences occurred before the treatment.

There were no deaths and no signs of toxicity for clinical signs including changes in fur, skin, and eyes, the consistency of feces, changes in somatomotor activity and behavior pattern, and BW decrease in all the tested animals. Hence, a confirmation was followed up by the examination of the rats' organs. Vital organs including the stomach, lungs, heart, liver, and kidneys were isolated at the end of the study. No particular conclusion could be drawn from the macroscopic observation (Fig. 1). Thus, a histopathological analysis was carried out to further confirm possible toxic effects.

The liver and kidneys from four rats from each group were processed for histopathological examination. Based on the qualitative analysis, hydropic degeneration was found in the livers from all groups, including the control group, and inflammation was found in some of the treated groups (Fig. 2 and Table 3). Meanwhile, abnormality changes including inflammation and congestion were found only in the kidneys of group administered with high dosages of HCE-CP (2,000 and 5,000 mg/kg BW) (Fig. 2 and Table 3). Thus, based on this qualitative analysis, it can be concluded that in general the single oral administration of HCE-CP until 300 mg/kg BW did not cause particular histopathological abnormalities.

Immunomodulatory potency of HCE-CP in LPS-induced rats

After confirming its safety, we then further evaluated the immunomodulatory potency of HCE-CP. Dosages of 200–2,000 mg/kg BW were chosen based on the acute toxicity test, and the treatment was given orally daily for 11 weeks. To induce immune responses and to mimic acute inflammation, LPS induction was applied as modified from a previous study (Singh and Jiang, 2003).

First, we measured the ratio of organ weight to BW at the end of the treatment. From six organs that were isolated, only the spleen and stomach showed differences (Table 4). In general, the average of the relative weights of the spleen and stomach in the untreated control was lower; meanwhile, the relative stomach weight in the LPS control group was higher. All HCE-CP treatment groups had significantly higher relative spleen weight compared to the untreated control ($p < 0.01$), while the LPS control also showed higher weight but at a lower significant level ($p < 0.05$) (Table 4). We concluded that the HCE-CP possibly causes increases in the spleen's weight. However, on the other hand, the effect of HCE-CP on the relative stomach weight was difficult to conclude, taking

Table 1. Clinical observation and mortality of rats in the acute toxicity study.

Treatment[a]	Observation[b]	Parameter				
		Fur, skin, and eyes	Feces consistency	Somatomotor activity and behavior pattern	Decrease in BW	Mortality
Untreated control	Intensive	n	n	n	nf	nf
	24 hours	n	n	n	nf	nf
	Up to day 15	n	n	n	nf	nf
HCE-CP 5	Intensive	n	n	n	nf	nf
	24 hours	n	n	n	nf	nf
	Up to day 15	n	n	n	nf	nf
HCE-CP 50	Intensive	n	n	n	nf	nf
	24 hours	n	n	n	nf	nf
	Up to day 15	n	n	n	nf	nf
HCE-CP 300	Intensive	n	n	n	nf	nf
	24 hours	n	n	n	nf	nf
	Up to day 15	n	n	n	nf	nf
HCE-CP 2,000	Intensive	n	n	n	nf	nf
	24 hours	n	n	n	nf	nf
	Up to day 15	n	n	n	nf	nf
HCE-CP 5,000	Intensive	n	n	n	nf	nf
	24 hours	n	n	n	nf	nf
	Up to day 15	n	n	n	nf	nf

[a] HCE-CP was given in single oral administration. The number indicates the dosage in mg per kg BW.

[b] Intensive observation was carried out after 30 minutes and 4 hours, followed by 24 hours and daily observation up to day 15 of all rats of each group ($n = 5$).

n, normal; nf, not found.

Table 2. BW of rats in the acute toxicity study.

Treatment[a]	BW (g)[b]		
	Week 0	Week 1	Week 2
Untreated control	189.78 ± 26.44	253.31 ± 20.88	287.74 ± 19.54
HCE-CP 5	226.86 ± 17.96*	257.33 ± 19.32	284.48 ± 20.09
HCE-CP 50	201.48 ± 12.83	234.80 ± 14.10	259.72 ± 13.13*
HCE-CP 300	207.52 ± 15.98	244.96 ± 15.65	278.84 ± 18.61
HCE-CP 2,000	208.22 ± 12.30	238.11 ± 8.58	271.53 ± 13.61
HCE-CP 5,000	220.74 ± 13.82*	248.45 ± 16.68	277.25 ± 16.85

[a] HCE-CP was given in single oral administration. The number indicates the dosage as mg per kg BW.

[b] Data are expressed as mean ± SD ($n = 5$).

Statistical significance was determined by Student's *t*-Test. Asterisks indicate a significant difference compared to the untreated control (*$p < 0.05$).

into account that a similar significant increase was also observed between the untreated and LPS control ($p < 0.01$).

At the end of treatment, the hematological profile was measured and presented in Table 5. Overall, the oral administration of HCE-CP for 11 weeks did not cause changes except for decrease in platelet distribution width (PDW) and increase in mean corpuscular volume (MCV) at the dosage of 1,000 and 2,000 mg/kg BW, respectively ($p < 0.05$). LPS induction alone also did not affect all the observed hematological parameters.

Acute LPS induction did not induce changes in all hematological profiles compared to the untreated control (Table 5). To further evaluate specific populations on T cell lymphocytes as immune responses, the population of CD4+ and CD8+ T cell lymphocytes was measured. Likewise, acute induction of LPS in this design (5 hours after intravenous injection) did not affect CD4+ and CD8+ cell population. Conversely, the treatment group at the highest dose (2,000 mg/kg BW) showed a significant reduction in CD4+ count compared to the untreated

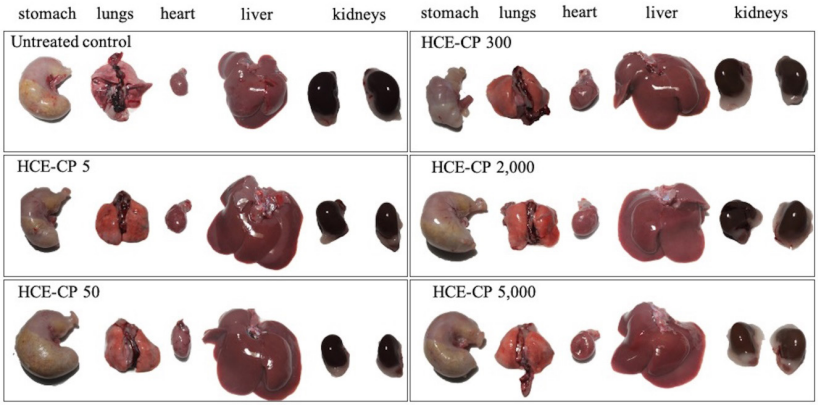


Figure 1. Macroscopic observation of rats' organs at 15 days after single oral administration of HCE-CP. HCE-CP was given in single oral administration with dose as indicated in mg per kg BW. Fifteen days after oral administration, the animals were sacrificed, and the organs were isolated. Representative images of the stomach, lungs, heart, liver, and kidneys from each group are shown.

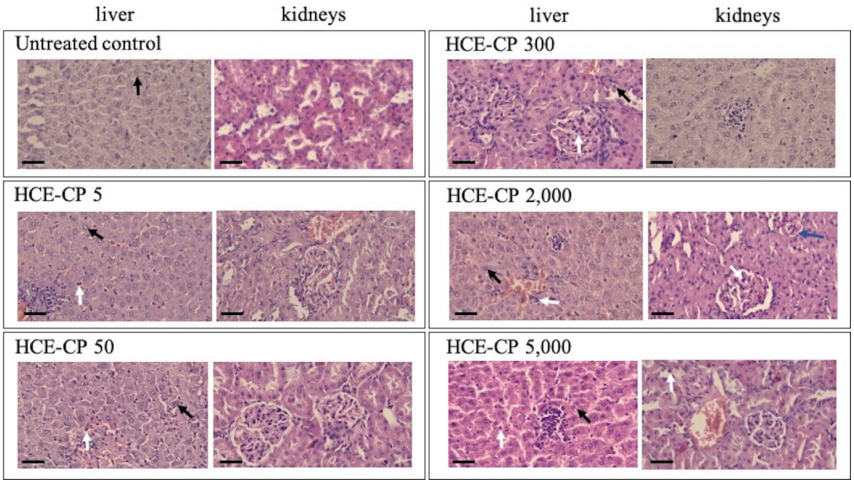


Figure 2. Histopathological examination of rats' livers and kidneys at 15 days after single oral administration of HCE-CP. Organs were isolated and processed for HE staining, and examination was carried out microscopically at ×400. Representative figures of each group at the same magnification were shown, and the abnormalities were pointed by arrows. The black arrow indicates hydropic degeneration, the white arrow indicates inflammation, and the blue arrow indicates congestion.

and LPS control groups (Fig. 3a). Although not significant, HCE-CP 2,000 mg/kg BW also showed a tendency toward decrease in CD8+ count (Fig. 3b). It is possible that HCE-CP may inhibit the proliferation and differentiation of lymphocytes.

Predictive target proteins of methoxy flavonoids of citrus peels in LPS-induced lymphocytes

Our acute LPS induction was not able to show distinctive effects, but HCE-CP did affect T cell lymphocytes as shown above. We hypothesized that HCE-CP inhibits lymphocyte proliferation and differentiation to encounter inflammation induced by LPS. Thus, a bioinformatics study was executed to approach our hypothesis. One hundred protein targets of hesperidin (major citrus flavonoid in HCE-CP) and hesperetin (aglycon form of hesperidin) were sliced with 1,557 proteins expressed in LPS-induced lymphocytes, resulting in 14 overlapped proteins (Fig. 4a). Among those 14 proteins, the proteins involved in inflammation and inflammatory cell migration were identified, including prostaglandin-endoperoxide synthase 1 (PTGS1), also known as cyclooxygenase-1 (COX-1), matrix metalloproteinases (MMPs), and signal transducer and activator of transcription 1 (STAT1) (Fig. 4b).

DISCUSSION

Citrus sp. is one of the commodities which is highly consumed and possesses significant health benefits (Meiyanto *et al.*, 2012). Not only the edible part but also the peels that consist of flavedo and albedo, which are considered waste, provide high value due to their high phytochemical contents and can be used as nutra-pharmaceuticals (Mahato *et al.*, 2018). Common extraction involving an organic solvent to extract methoxy flavonoids limits the usage of citrus peel extract (Lachos-Perez *et al.*, 2018).

Table 3. Histopathological analysis of rats' livers and kidneys at 15 days after single oral administration of HCE-CP.

Treament[a]	Animal	Abnormality	
		Liver	Kidney
Untreated control	1	HD	nf
	2	HD	nf
	3	HD	nf
	4	HD	nf
HCE-CP 5	1	HD, I	nf
	2	HD, I	nf
	3	HD, I	nf
	4	HD	nf
HCE-CP 50	1	HD, I	nf
	2	HD, I	nf
	3	I	nf
	4	HD, I	nf
HCE-CP 300	1	HD, I	nf
	2	HD, I	nf
	3	HD, I	nf
	4	HD, I	nf
HCE-CP 2,000	1	HD, I	C
	2	HD, I	I
	3	HD	nf
	4	HD	nf
HCE-CP 5,000	1	HD, I	nf
	2	HD, I	nf
	3	HD, I	I
	4	HD	nf

[a] HCE-CP was given in single oral administration with dose as indicated in mg per kg BW. Histopathological analysis of rats' livers and kidneys was carried out (HE, ×400) for each group ($n = 4$).

HD, hydropic degeneration; I, inflammation; C, congestion; nf, not found.

Table 4. The relative organ weight in LPS-induced rats treated with HCE-CP for 11 weeks.

Treament[a]	Relative organ weight (×100)					
	Liver	Kidneys	Spleen	Heart	Lungs	Stomach
Untreated control	4.67 ± 0.33	1.21 ± 0.09	0.29 ± 0.04$^{\#}$	0.46 ± 0.05	0.92 ± 0.22	0.77 ± 0.01$^{\#\#}$
LPS control	4.94 ± 0.41	1.16 ± 0.07	0.38 ± 0.07*	0.47 ± 0.08	0.97 ± 0.10	2.45 ± 0.77**
LPS + HCE-CP 200	4.75 ± 0.48	1.13 ± 0.09	0.39 ± 0.05**	0.41 ± 0.04	0.95 ± 0.24	2.04 ± 0.52**
LPS + HCE-CP 500	4.82 ± 0.47	1.24 ± 0.30	0.41 ± 0.08**	0.45 ± 0.04	0.96 ± 0.25	2.25 ± 0.53**
LPS + HCE-CP 1,000	4.94 ± 0.51	1.17 ± 0.16	0.45 ± 0.04**	0.51 ± 0.10	0.92 ± 0.12	2.16 ± 0.57**
LPS + HCE-CP 2,000	5.21 ± 0.85	1.22 ± 0.08	0.51 ± 0.04**	0.48 ± 0.07	0.85 ± 0.16	2.33 ± 0.22**

[a] HCE-CP was given orally daily, and the dosage in mg per kg BW is indicated. At the end of week 11, an intravenous injection of LPS was given, followed up by termination and organ isolation on the following day. Data are expressed as means ± SD ($n = 5$).

Statistical significance was determined by Student's *t*-test. Asterisks and hashtags indicate a significant difference compared to the untreated control ($^{*}p < 0.05$; $^{**}p < 0.01$) and to the LPS control ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$), respectively.

Table 5. Hematology profile of LPS-induced rats treated with HCE-CP for 11 weeks.

Parameter	Unit	Treament[a]					
		Untreated control	LPS				
			Control	HCE-CP 200	HCE-CP 500	HCE-CP 1,000	HCE-CP 2,000
White blood cell	10^9/l	5.26 ± 0.74	6.06 ± 2.09	5.34 ± 0.59	5.82 ± 0.75	4.88 ± 0.68	5.96 ± 1.75
Red blood cell	10^{12}/l	6.44 ± 0.25	6.84 ± 0.33	6.62 ± 0.36	6.46 ± 0.24	6.68 ± 0.24	6.42 ± 0.37
Hemoglobin	g/dl	12.68 ± 0.33	13.08 ± 0.27	13.08 ± 0.34	12.78 ± 0.52	13.02 ± 0.33	12.80 ± 0.81
Hematocrit	%	39.34 ± 1.24	40.52 ± 1.37	40.16 ± 1.63	39.62 ± 1.63	40.60 ± 1.20	39.66 ± 2.32
MCV	fl	60.92 ± 0.52	59.28 ± 1.70	60.74 ± 1.60	61.38 ± 1.15	61.04 ± 0.80	61.78 ± 0.52*#
Mean corpuscular hemoglobin	pg	19.62 ± 0.25	17.14 ± 4.44	19.80 ± 0.76	19.82 ± 0.59	19.58 ± 0.48	19.92 ± 0.24
Mean corpuscular hemoglobin concentration	g/dl	32.20 ± 0.45	32.20 ± 0.45	32.40 ± 0.89	32.40 ± 0.89	32.20 ± 0.45	32.40 ± 0.55
Platelet count	10^9/l	1,142 ± 133	990 ± 208	1,115 ± 104	1,032 ± 94	1,132 ± 78	1,105 ± 190
PDW	fl	8.88 ± 0.22	9.14 ± 0.56	8.66 ± 0.47	8.72 ± 0.55	8.36 ± 0.37*#	8.58 ± 0.58
Mean platelet volume	fl	7.54 ± 0.11	7.62 ± 0.41	7.32 ± 0.41	7.38 ± 0.40	7.22 ± 0.40	7.32 ± 0.39
Platelet larger cell ratio	%	8.62 ± 0.75	8.70 ± 2.53	7.52 ± 0.31	7.68 ± 2.03	6.92 ± 1.95	7.72 ± 2.00
Red blood cell distribution width	%	12.72 ± 1.23	12.06 ± 0.70	12.60 ± 0.49	12.48 ± 0.54	12.16 ± 0.42	12.64 ± 0.99
Lymphocyte	%	75.88 ± 5.91	75.96 ± 5.76	74.98 ± 7.37	73.22 ± 4.39	74.92 ± 6.63	77.30 ± 2.07
Monocytes-basophils-eosinophils mixed	%	3.60 ± 0.89	2.88 ± 0.67	3.30 ± 0.88	3.58 ± 1.12	2.84 ± 0.64	3.48 ± 1.25
Neutrophil	%	20.52 ± 5.08	18.38 ± 9.35	21.72 ± 6.52	23.20 ± 3.88	22.20 ± 6.17	19.98 ± 2.33

[a] HCE-CP was given orally daily, and the dosage in mg per kg BW is indicated. At the end of week 11, intravenous injection of LPS was given and 5 hours after that the blood was sampled for analysis. Data are expressed as means ± SD (n = 5).

Statistical significance was determined by Student's t-test. Asterisks and hashtags indicate a significant difference compared to the untreated control ($^*p < 0.05$) and to the LPS control ($^\#p < 0.05$), respectively.

Instead, a water-based extraction would provide flexibility in the development of nutra-pharmaceuticals from citrus peel extract. Thus, our group has adopted a HC method (Meneguzzo *et al.*, 2020) for *C. reticulata* peels and resulted in the HCE-CP with high hesperidin content. Even though citrus is a food commodity that is generally recognized as safe (NADFC, 2014) and hesperidin has also been proved to be safe for therapeutic use (Zanwar *et al.*, 2014), we have to ensure the safety of HCE-CP. Here, we proved that oral administration of HCE-CP in the acute toxicity test prolonged until day 15 did not cause lethality in rats, even at the highest tested dose of 5,000 mg/kg BW (Table 1). Thus, HCE-CP is practically not toxic and safe and potentially can be utilized as a nutra-pharmaceutical.

In addition to lethality, other clinical signs including BW profiles and histopathological analysis of vital organs are equally important and extensively used to elucidate the possible effect of tested materials in toxicity studies (Sutrisni *et al.*, 2019). Indeed, HCE-CP did not cause alteration in clinical signs in all the treated groups up to the highest tested dose (Table 1) nor histopathological abnormality up to 300 mg/kg BW compared to the control (Table 3). The BW was also increased normally throughout the 2 weeks observation after single oral administration of HCE-CP (Table 2). Moreover, daily intake of HCE-CP for 4 weeks had no particular effect on the hematological profiles of rats (Table 5). Taken together, these data supported our conclusion that HCE-CP is safe and has no negative effects.

Since our goal is to utilize HCE-CP as a nutra-pharmaceutical that can be taken daily safely and is beneficial for health, especially for the immune system, we set a design study over the course of 11 weeks with daily oral administration of HCE-CP. The spleens in the HCE-CP treatments had a higher relative organ weight than the untreated control (Table 4), indicating possible immune system activation. Considering the spleen as a secondary lymphoid organ in which lymphocytes reside (Li *et al.*, 2008), a further evaluation on specific populations of lymphocytes revealed the significant reduction in CD4+ T cell lymphocytes (Fig. 3). Although not significant, acute LPS induction increased CD4+ count compared to the untreated control. HCE-CP probably encounters an immune reaction activated by LPS induction that mimics inflammation caused by infections. Hence, HCE-CP possibly has anti-inflammatory activity. Further confirmation in an *in vitro* model (Shetty *et al.*, 2013) using LPS-induced cells (Al-Rikabi *et al.*, 2020; Fuior *et al.*, 2020) would provide clearer evidence.

To confirm the possible mechanism of HCE-CP in encountering inflammation, a bioinformatics analysis was carried out by using the SwissTargetPrediction and GeneCards databases. The major methoxy flavonoid in HCE-CP, hesperidin, and its aglicon, hesperetin, target 14 proteins in LPS-induced lymphocytes (Fig. 4). STAT1 is important since it affects lymphocyte survival and proliferation, including CD4+ (Lee *et al.*, 2000). A proinflammatory cytokine, interleukin-27 (IL-27), is induced by LPS via STAT1 (Shimizu *et al.*, 2013). Moreover, hesperidin and hesperetin also target inflammatory proteins such as PTGS1, MMP12 (macrophage metalloelastase), MMP13 (collagenase), and MMP8 (neutrophil collagenase) that are involved in inflammation.

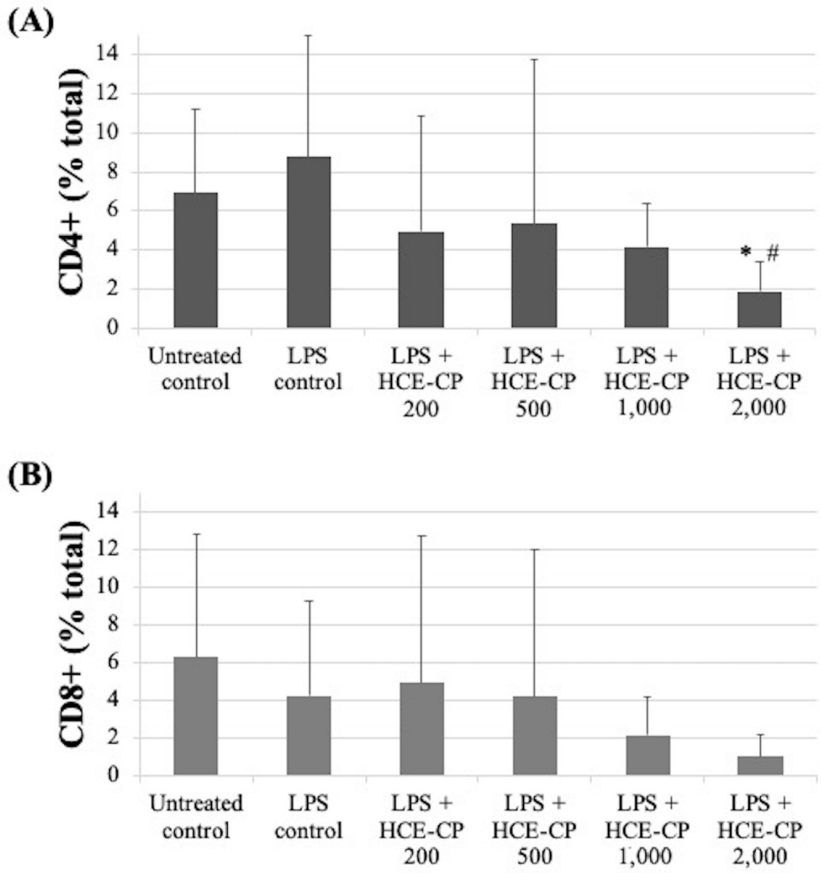
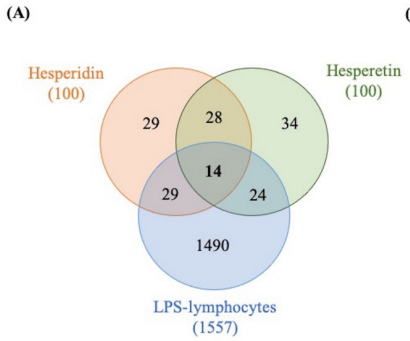


Figure 3. CD4+ and CD8+ count in LPS-induced rats treated with HCE-CP for 11 weeks. HCE-CP was given orally daily, and the dosage in mg per kg BW is indicated. At the end of week 11, intravenous injection of LPS was given, and 5 hours after that the blood was sampled. Blood samples were processed, stained with antibody anti-CD4 or CD8, and analyzed with flow cytometry. Percentages of CD4+ (A) and CD8+ (B) cells from total reads were calculated and presented, respectively. Data are expressed as means + SD ($n = 5$). Statistical significance was determined by Student's *t*-test. Asterisks and hashtags indicate a significant difference compared to the untreated control ($^{*}p < 0.05$) and to the LPS control ($^{\#}p < 0.05$), respectively.



(B)

Target	Common name
Sodium/glucose cotransporter 2	SLC5A2
Cyclooxygenase-1	PTGS1
Matrix metalloproteinase 13	MMP13
Matrix metalloproteinase 12	MMP12
Matrix metalloproteinase 8	MMP8
Telomerase reverse transcriptase	TERT
Adenosine A1 receptor	ADORA1
Adenosine A2 receptor	ADORA3
Testis-specific androgen-binding protein	SHBG
Estrogen receptor alpha	ESR1
Dual specificity protein phosphatase 3	DUSP3
DNA (cytosine-5)-methyltransferase 1	DNMT1
Apoptosis regulator Bcl-2	BCL2
Signal transducer and activator of transcription 1-alpha/beta	STAT1

Figure 4. Predictive target proteins of methoxy flavonoids in LPS-induced lymphocytes. Prediction of target proteins for hesperidin and hesperetin was obtained from SwissTargetPrediction (http://www.swisstargetprediction.ch). The protein expressed in LPS-induced lymphocytes was acquired from GeneCards (https://www.genecards.org). (A) Venn diagram of protein targets for hesperidin and hesperetin, and LPS-induced lymphocytes was generated by InteractiVenn (http://www.interactivenn.net). (B) Fourteen overlapping proteins as targets of hesperidin and hesperetin in LPS-induced lymphocytes are displayed.

PTGS1 plays a pivotal role in prostanoid synthesis in basal conditions but also may be upregulated in various pathological conditions including inflammation (Zidar *et al.*, 2009). MMP12, MMP13, and MMP8 are functioning in catalytic activity degrading extracellular matrices, thus allowing cell movements or cell recruitment in inflammation reaction, and are also involved in chemokine/cytokine activation (Hardy and Fernandez-Patron, 2021; Manicone and McGuire, 2008). Targeting these proteins by methoxy flavonoids from citrus peels may block the inflammation. Measuring the level expression of these proteins as well as the cell migration under methoxy flavonoids or CHE-CP treatment *in vitro* would prove this theory.

Taken together, our data provide evidence that HCE-CP yielded by a water-based extraction and high in methoxy flavonoids is safe. Daily intake of HCE-CP is also considered safe with a benefit in the immune system. We concluded that HCE-CP is promising to be developed as a nutra-pharmaceutical.

CONCLUSION

The oral administration of HCE-CP up to 5,000 mg/kg BW in rats practically does not give a lethal effect. Furthermore, the single oral administration of HCE-CP does not change clinical signs. HCE-CP reduces CD4+ T lymphocyte cells. On the other hand, hesperidin and hesperetin as the major methoxy flavonoids in citrus peels target proteins that are involved in LPS-induced lymphocytes. To sum up, HCE-CP is safe and has potential to be developed as an immunomodulatory agent and an anti-inflammatory agent caused by infection.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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ETHICAL APPROVAL

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Universitas Gadjah Mada (Approval no. 00053/04/LPPT/XI/2020, November 2, 2020).

AUTHORS' CONTRIBUTIONS

EM and MI contributed to the conceptualization of the study. DDPP, GGM, EM, and MI contributed to the methodology. YK contributed to extract preparation. DDPP, GGM, EM, and MI contributed to formal analysis. DDPP, GGM, EM, and MI contributed to the investigations. EM and MI curated the data. MI contributed to writing and original draft preparation. YK, RAS, EM, and MI contributed to writing and reviewing and editing of the manuscript. GGM and MI contributed to the visualization of the study. RAS and EM supervised the study. EM and MI acquired the funding. EM gave final approval of the version of the manuscript to be published. All authors have read and agreed to the published version of the manuscript.

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