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Effects of Peel Extract from *Citrus reticulata* and Hesperidin, A Citrus Flavonoid, on Macrophage Cell Line

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ABSTRACT

The extract of *Citrus reticulata* has been studied for its biological activities, due to its citrus flavonoid content. The extract and its flavonoid compounds exhibit growth inhibition property in several cancer cell lines and *in vivo* models. Conversely, the extract can also induce cell proliferation and angiogenesis, and shows estrogenic effects by *in vitro* and *in vivo*. Because of the contrasting effects that depend on the concentration or dosage, the precise action of the extract and its flavonoids need to be elucidated in various cell types. The objective of this study is to evaluate the effect of *Citrus reticulata* peel extract (Citrus extract) and hesperidin, a citrus flavonoid, on the modulation of cell proliferation in the RAW 264.7 macrophage cell line. Cell viability under Citrus extract or hesperidin treatment was assessed by using the MTT assay. The expression of interleukin-10 (IL-10), an anti-inflammatory cytokine, modulated by Citrus extract was also examined by immunostaining. Low concentrations of Citrus extract at 1 and 100µg/mL were able to induce cell proliferation, although in significant, as shown by cell viability of 138 and 114%, respectively. At higher concentrations of 500, 750, and 1000µg/mL, Citrus extract decreased cell viability significantly by up to 64, 46, and 36%, respectively. Accordingly, hesperidin at low (3.1µg/mL –61.1µg/mL) concentration increased cell viability significantly by up to 116-136% where as high (152.6203µg/mL–305.3203µg/mL) concentration reduced cell viability significantly by up to 10-61%. The value of the 50% inhibitory concentration (IC_{50}) of Citrus extract was more than three times higher (756µg/mL) than that of hesperidin (203µg/mL = 332µM). Additionally, 250µg/mL of Citrus extract was able to induce IL-10 expression compared to control. These results demonstrated that Citrus extract and hesperidin exerted a biphasic effect on macrophage cells. The future development of Citrus extract as a co-chemotherapeutic, anticancer, or immunomodulatory agent should include careful consideration of its biphasic effect on each cell type.

Keywords: *Citrus reticulata*, hesperidin, macrophage RAW 264.7 cell, proliferation, interleukin-10 (IL-10)

INTRODUCTION

Citrus plants (family: Rutaceae) have attracted researchers' attention due to their biological activities since the early 18th century (Manthey *et al*., 2001). Their phytochemical health-promoting properties are mainly based on the anti-oxidant activities of flavonoid compounds that contribute to the cardiovascular disease and cancer prevention, anti-inflammatory, antiviral, and antimicrobial properties of citrus (Barreca *et al*., 2017; Benavente-Garcia *et al*., 1997). *Citrus* species contain a variety of flavonoids, namely flavonones,

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Table I. Biological activities of *Citrus reticulata* peel extract (Citrus extract) and hesperidin

Material	Experimental design	Biological activity	Concentration /dosage	Reference
Citrus extract	*In vitro* assay in MCF-7 breast cancer cell line	Increases selectivity for doxorubicin		(Yunas *et al.,* 2007)
		Single treatment induces cell proliferation	50-400µg/mL	
	bFGF-induced chicken embryo chorio-allantoic membrane	Anti-angiogenesis	150-600µg	(Chrisnanto *et al*., 2008)
	In vitro assay in WiDr colon cancer cell line	Induces cell proliferation and COX-2 expression	10-500µg/mL	(Ardiani *et al*., 2008)
		Induces VEGF expression	1.500-µg/mL	(Puspita *et al*., 2008)
	Ovariectomized rats	Estrogenic (modulates blood cholesterol profile and increases bone density)	500-1.000 mg/kg BW	(Adelina *et al*., 2015)
	DMBA-induced rats	Inhibits breast epithelial cell proliferation (reduces c-Myc expression)	750-1.500mg/kg BW	(Supriyati *et al*., 2008)
		Inhibits hepatic cell proliferation (reduces c-Myc expression)	750-1.500mg/kg BW	(Meiyanto *et al*., 2012)
Hesperidin	*In vitro* assay in MCF-7 breast cancer cell line	Increases selectivity for doxorubicin		(Hermawan *et al*., 2010)
		Single treatment induces cell proliferation	5-100µM	
	In vitro assay in T47D breast cancer cell line	Cytotoxic	IC_{50} 200µM	(Setiawati *et al*., 2011)
	In vitro assay in HeLa cervical cancer cell line	Cytotoxic	IC_{50} 48µM	(Kusharyanti *et al*., 2011)
	In vitro assay in WiDr colon cancer cell line	Increases selectivity for doxorubicin		(Gilang *et al*., 2012)
		Single treatment is not cytotoxic	5–200 µM	
	In vitro assay in MCF-7/HER2 breast cancer cell line	Increases selectivity for doxorubicin	IC_{50} 11 µM	(Febriansah *et al*., 2014)

Acronyms: bFGF, basic fibroblast growth factor; DMBA = 7,12-dimethylbenz(a)anthracene

flavons, and flavonols (Benavente-García *et al*., 1997). Hesperidin, a flavonone glycoside, is a major flavonoid compound in *Citrus reticulata* (reviewed in Barreca *et al*., 2017). The first description of hesperidin by Lebreton in 1828 marked the beginning of researchers' vast interest in citrus flavonoids (reviewed in Manthey *et al*., 2001). As a fruit crop that is abundantly cultivated and consumed across the globe, citrus fruits generate a large amount of waste every year (Sharma *et al*., 2017). Citrus peels are one of the solid citrus waste products that need to be managed. Since 2007, the Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada (UGM), Indonesia has been exploring the extract of citrus peels in order to reveal its chemopreventive properties and, at the same time, to increase the utility of citrus waste (reviewed in Meiyanto *et al*., 2012). One of the studies was focused on the ethanolic extract of *Citrus reticulata*, commonly known as tangerine or mandarin, which has been shown to contain flavonoids (Armandari, 2010; Meiyanto *et al*., 2011) (Table I). In addition, hesperidin, as one of the main compounds, has been studied (Table I).

In vitro, it induces cell proliferation and angiogenesis in MCF-7 breast or WiDr colon cancer cells at concentrations of 10−1500µg/mL (Ardiani *et al*., 2008; Puspita *et al*., 2008; Yunas *et al*., 2007) but can suppress MCF-7 cell

proliferation when combined with doxorubicin, a cytostatic agent (Yunas *et al*., 2007) (Table I). *In vivo*, 150-600µg Citrus extract shows anti-angiogenic activities (Chrisnanto *et al*., 2008) and suppresses epithelial breast and hepatic cell proliferation in rats with chemically induced cancer at 750-1500mg/kg BW (Supriyati *et al*., 2008; Meiyanto *et al*., 2011). In the same manner as Citrus extract, hesperidin also displays differing properties depending on its concentration and the cell type. Hesperidin (5-200µM) increases cell viability in MCF-7 and WiDr cells but decreases cell viability more effectively in combination with an anticancer agent than the anticancer agent alone (Gilang *et al*., 2012; Hermawan *et al*., 2010). In the other cancer cell lines, T47D breast, HeLa cervix, and MCF-7 with HER2 over-expression (MCF-7 /HER2), hesperidin exhibits an IC_{50} value varying from 11 to 200µM (Febriansah *et al*., 2014; Kusharyanti *et al*., 2011; Setiawati *et al*., 2011). The effect of Citrus extract on MCF-7/HER2 is contrary to the estrogenic effect seen in ovariectomized mice (Adelina *et al*., 2015). In summary, Citrus extract and hesperidin may act differently depending on the concentration and the cell type. The dual or biphasic effect of these compounds on cell proliferation will influence whether the expected effect on cells is antiproliferative or proliferative. Therefore, the precise action of Citrus extract and citrus flavonoids need to be elucidated in various cell types. Besides the exploration of its anticancer potency, citrus and citrus flavonoids have also been studied for their anti-inflammatory activities (Manthey *et al*., 2001). Our group's work has been focused mainly on cancer cells, not on normal or inflammation-related cells (Table I). To our knowledge, no study has yet addressed the dual effect of citrus extract or citrus flavonoids on cell proliferation. Hence, an investigation of Citrus extract and citrus flavonoids on normal or inflammatory-related cells, i.e., macrophages, is important, especially considering the probability of their contradictory concentration-dependent effect. In this study, we evaluate the effect of ethanolic extract from *Citrus reticulata* peel (Citrus extract) and hesperidin at various concentrations on the modulation of cell proliferation in the RAW 264.7 macrophage cell line. We reveal that both Citrus extract and hesperidin show a biphasic effect on RAW 264.7 macrophage cell viability. Additionally, Citrus extract at a moderate tested concentration can induce the expression of interleukin-10 (IL-10), an anti-inflammatory cytokine.

MATERIAL AND METHODS

Preparation of Citrus extract

Citrus reticulata fruits were obtained from Kalisoro, Tawangmangu, Central Java, Indonesia in September and were identified in the Laboratory of Pharmacognosy, Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada (UGM). Healthy, green, mature but unripe fruits were washed, and the peels were collected and air dried without direct sunlight. Dried peels were powdered and macerated in 70% ethanol (Merck, Darmstadt, Germany) (10L for 1kg powder) for 5 days as previously described (Adelina *et al*., 2008). The ethanolic fraction was separated and evaporated in a rotary vacuum evaporator until a brown viscous extract was obtained (yield was 3.21% (b/b)) (Armandari, 2010). The obtained extract was then identified by thin layer chromatography to detect flavonoid components as previously described (Meiyanto *et al*., 2011). After citrobroric treatments, positive spots indicating flavonoids were developed under 245 and 366nm UV lights (Armandari, 2010). This extract is heretofore referred to as "Citrus extract."

Preparation of tested materials

Citrus extract was first diluted in dimethyl sulfoxide (DMSO) (99.5% pro GC, Sigma Aldrich, Germany) as a stock solution of 50mg/mL and then serially diluted in culture medium to reach the designated concentrations (1; 5; 10; 25; 50; 75; 100; 250; 500; 750; and 1,000µg/mL) with a maximum final DMSO concentration of 2% (v/v). The same preparation procedure was also carried out for hesperidin (Sigma Aldrich, Germany), a citrus flavonoid, as a comparison (1, 5, 10, 25, 50, 100, 250, and 500µM).

Cell culture

The murine monocyte macrophage RAW 264.7 cell line was a gift from Prof. Tatsuo Takeya (Nara Institute of Science and Technology, Japan). Cells were cultured in Minimum Essential Media (Gibco, USA) supplemented with 10% (v/v) Newborn Calf Serum (NBCS) (Gibco, USA) and 2% (v/v) penicillin-streptomycin (Gibco, USA) in a 37°C, 5% CO_2 incubator and grown to confluence in 75cm^3 tissue culture flasks. After they reached 80% confluence, cells were scraped and used for experiments.

Cell viability assay

Cells (5×10^3 cells/well) were plated in 96-well plates and cultured in a complete medium for 48h. The medium was then replaced with a medium containing various concentrations of Citrus extract or hesperidin and incubated for 24h. Cell viability was assessed by the MTT method as previously described (Ikawati *et al*., 2018) and carried out in at least triplicate for each experiment. Briefly, the absorbance at 595nm of diluted formazan after addition of 0.5mg/mL 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, Germany) in phosphate-buffered saline (PBS) followed by stopper solutions (10% SDS (Merck, Germany) in 0.1NHCl (Merck, Germany)) were measured in a microplate reader (Bio-Rad, Japan). The detailed step-by-step protocol is described in Armandari (2010). Untreated cells served as a control, while wells without cells served as a blank. The percent cell viability and the IC_{50} value were calculated as (absorbance of treated cells − absorbance of blank)/(absorbance of control − absorbance of blank) × 100% and by linear regression analysis as follows: cell viability (%, y axis) vs log concentration (µg/mL or µM, x axis) (Ikawati *et al*., 2018), including all tested dosage of the concentration series). Data are presented as the mean of two or three measurements per condition per experiment.

Immunostaining

Immunocytochemistry with an anti-IL-10 mouse monoclonal antibody (Dako) was carried out by the avidin-biotin complex method as previously described (Ardiani *et al*., 2008). Cells (1×10^5 cells/well) were plated on coverslips in a 24-well plate until they reached 80% confluence. Cells were then incubated with Citrus extract at the designated concentrations for 15h. After removal of the medium, cells were washed with cold PBS, and then were fixed in cold methanol for 10min at -20°C. The endogenous peroxidase activity was blocked with hydrogen peroxide, and nonspecific sites were blocked with normal goat serum (Novocastra), for 10min each at room temperature, followed by incubation in the primary antibody, anti-IL-10 (1:50), overnight at 4°C. After washing with PBS, cells were incubated in IgG biotinylated universal secondary antibody (Novocastra) for 10min. Following washing, cells were incubated with streptavidin-peroxidase complex reagent (Novocastra) for 10min and then incubated with 3,3-diaminobenzinidine (DAB) substrate solution (Novocastra) for 2-10min to visualize the bound biotin. Cover slips were washed in distilled water and counterstained with Mayer's hematoxylin (Dako) for 1-3min. Cells were dehydrated in ethanol, cleared in xylene, and cover slips were mounted with a mounting medium. Protein expression was observed qualitatively with a light microscope (Olympus). A stained cover slip without primary antibodies served as a control.

Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analyzed for significance using the Student's *t*-test. Values of $p < 0.05$ were considered to indicate significance.

RESULTS AND DISCUSSION

Higher, but not lower, concentrations of Citrus extract decrease RAW 264.7 cell viability

To examine the inhibition or induction of cell proliferation, RAW 264.7 cells were treated with a series of concentrations of Citrus extract, in the range from 1 to 1,000µg/mL, for 24h. As expected, lower concentrations of Citrus extract did not affect cell morphology. The cell morphology at lower concentrations (i.e., 10µg/mL and 50µg/mL) appeared the same as that of control cells (Figure 1A). Moreover, the cell density increased after treatment with 50µg/mL extract. In contrast, at higher concentrations, starting at 500µg/mL, more cells appeared rounder and less flattened out in the well. These morphological observations imply a biphasic effect of Citrus extract.

After 24h of incubation with Citrus extract, cell viability was assayed by the MTT method. The higher concentrations of Citrus extract (500µg/mL, 750µg/mL, and 1000µg/mL) were able to decrease cell viability significantly (64%, 46%, and 36%, respectively, compared with 100% in the control without Citrus extract treatment) (Figure 1B). The cell viability in the presence of 250µg/mL Citrus extract was 96%, while lower concentrations increased the cell viability, though not significantly, ranging from 108% to 138%. The highest cell viability was given by the lowest tested concentration, 1µg/mL. Thus, Citrus extract demonstrated a biphasic effect on RAW 264.6 macrophage cell proliferation.

Biphasic effects of hesperidin on RAW 264.7 cell viability

Hesperidin is one of the citrus flavonoids, a major compound in Citrus extract or citrus in general. Therefore, a similar cell viability assay was also carried out for hesperidin to determine

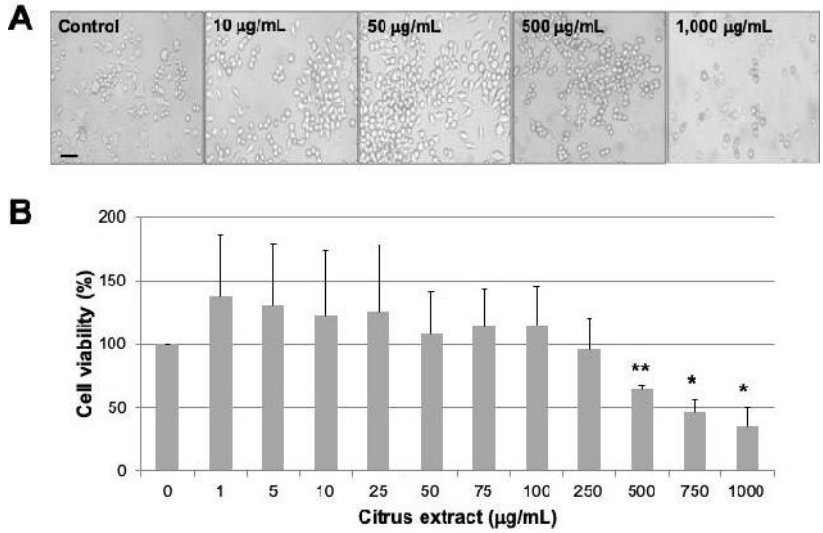

Figure 1. Effects of Citrus extract on cell viability in RAW 264.7 macrophage cells. Cells were treated with a concentration series of Citrus extract, incubated for 24h, and then assayed by MTTassay in pentaplicate. A. Cell morphology and density were observed under a microscope, and representative pictures are presented. Scale bars = 100µm. B. The graph of Citrus extract treatment demonstrated a biphasic effect on cell viability. The experiments were carried out twice, and the graph represents means ±=SD (n=2). Statistical significance was determined by means of the Student's *t*-test. Asterisks indicate a significant decrease (*; p<0.05. **; p<0.01) in cell viability compared with the control.

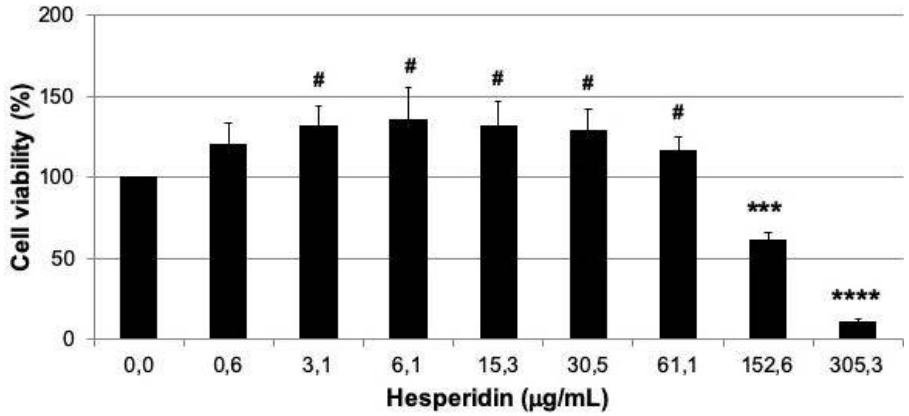

Figure 2. Effects of hesperidin on cell viability in RAW 264.7 macrophage cells. Cells were treated with a concentration series of hesperidin, incubated for 24h, and then assayed by MTT assay in triplicate. The graph of hesperidin treatment demonstrated a biphasic effect on cell viability. The experiments were carried out three times, and the graph represents means ± SD (n=3). Statistical significance was determined by means of the Student's *t*-test. Hash tags indicate significant increase (#; p<0.05) on cell viability compared with the control. Asterisks indicate a significant decrease (***; p<0.001 ****; p<0.0001) in cell viability compared with the control.

whether this compound would exert a similar biphasic effect to the extract. A series of concentrations varying from 1µM to 500µM (equal to 0.6µg/mL−305.3µg/mL) was used (Figure 2). The lowest tested concentration did not significantly affect cell viability. However, at 5−100µM, hesperidin increased cell viability significantly (116%−136% compared with 100% in the control without hesperidin treatment). At higher concentrations, hesperidin decreased cell

viability by as much as 61% and 10% at 250µM and 500µM, respectively. These data confirmed the biphasic effect of Citrus extract on cell proliferation, and that effect was likely caused by its citrus flavonoid content.

Citrus extract has a lower IC_{50} value compared with hesperidin in RAW 264.7 cells

IC_{50} values were calculated based on linear regression equations derived from the graph of concentration versus cell viability (Figure 3). The IC_{50} of Citrus extract and hesperidin was 756 g/mL and 203µg/mL (332µM), respectively, as expected ranging at the high tested concentration. The IC_{50} of Citrus extract was 3.7 times higher than that of hesperidin. This is plausible because Citrus extract may contain other compounds. Nevertheless, at the given IC_{50}, studies on the utilization of Citrus extract rather than hesperidin may yield additional advantages, especially if the accessibility of a pure compound is limited.

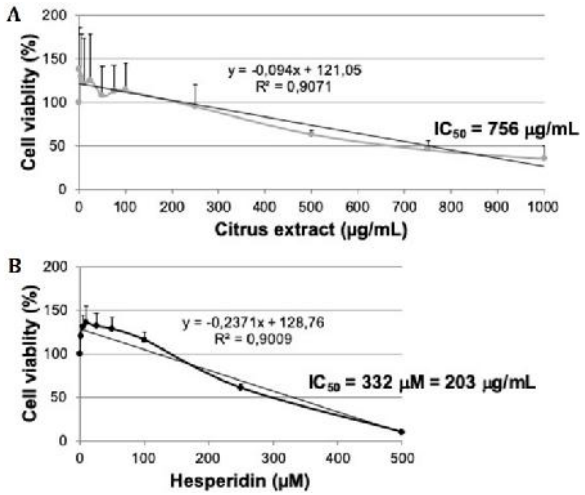

Figure 3. The IC_{50} value of Citrus extract or hesperidin in RAW264.7 macrophage cells. Cells were treated with a concentration series of Citrus extract (A) or hesperidin (B) for 24h and then assayed by the MTT method. Graphs of concentration versus percentage cell viability are presented as indicated. Points in A and B are presented as the mean of two or three experiments, respectively. The IC_{50} value was calculated by linear regression analysis as stated on each graph.

Citrus extract can induce IL-10 expression

Treatment with 250µg/mL Citrus extract induced expression of IL-10, an anti-inflammatory cytokine, indicated by a brownish color in the cytoplasmic area compared with control IgG (Figure 4, rightmost panel versus leftmost panel). However, at a concentration of 25µg/mL, Citrus extract did not affect IL-10 expression and appeared similar to cells without Citrus extract treatment (0µg/mL). Higher concentrations should be tested further to confirm whether the modulation of IL-10 expression caused by Citrus extract is also ensuing the biphasic trend.

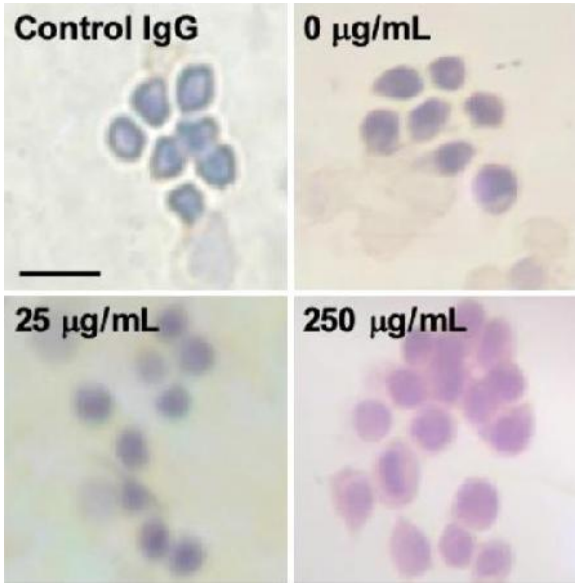

Figure 4. Effects of Citrus extract treatment on the expression of interleukin-10 (IL-10). Cells were incubated with Citrus extract at the indicated concentrations for 15h. Cells were immunostained for IL-10 (brown) and counterstained with Mayer's hematoxylin to visualize nuclei (blue). Staining without primary antibody served as a control (leftmost panel). Scale bar = 50µm.

Our findings exhibit that the ethanolic extract of *Citrus reticulata* (Citrus extract) and hesperidin, a citrus flavonoid, display a biphasic effect on the proliferation of a monocyte macrophage cell line, indicated by the cell viability parameter (Figure 1B and 2). More importantly, the concentration of Citrus extract required to increase RAW 26.7 cell viability varied from 1µg/mL to 250µg/mL (Figure 1B) compared with 10-1,500µg/mL in cancer cell lines (Yunas *et al*., 2007; Ardiani *et al*., 2008; Puspita *et al*., 2008). Meanwhile, hesperidin at low concentrations (5–100µM) can increase cell viability (Figure 2), similarly in cancer cell lines (5–100 or 200µM) (Hermawan *et al*., 2010; Gilang *et al*., 2012). The IC_{50} value of hesperidin in the monocyte

macrophage cell line is 1.7–30 times higher than in cancer cell lines (Figure 3B) (Kusharyanti *et al*., 2011; Setiawati *et al*., 2011; Febriansah *et al*., 2014). Taken together, the use of Citrus extract or hesperidin should carefully deliberate the concentration or dosage and the cell type.

Macrophages express interleukin-10 (IL-10) and usually triggered by inflammatory stimuli (Chung *et al*., 2007). Since citrus flavonoids has been studied extensively for their anti-inflammatory activity (Manthey *et al*., 2001), it is plausible to observe IL-10 expression under Citrus extract or hesperidin treatments. Due to our limitations (Figure 4). At 250µg/mL, Citrus extract can induce IL-10 expression, but not at lower concentrations. In the future study, higher concentrations of Citrus extract should be tested to confirm whether the modulation of IL-10 expression caused by Citrus extract also follows the pattern of a dual effect. Furthermore, the expression of cyclooxygenase-2 (COX-2), one of the key enzymes in inflammation, was also tested. However, there was no COX-2 detected (data not shown). An inflammatory stimulus such as lipopolysaccharide (LPS) should be administered to the cells, similarly to the ones that have been reported previously (Kang *et al*., 2011; Sakata *et al*., 2003).

At lower concentrations, Citrus extract was able to maintain cell viability independently of the expression of the anti-inflammatory cytokine IL-10. Meanwhile, hesperidin at a lower concentration was able to promote cell proliferation. Therefore, a low concentration of Citrus extract and hesperidin is possibly useful for treating inflammatory diseases. On the other hand, application of a low concentration of Citrus extract or hesperidin should be avoided for cancer cells because it induces cell proliferation as previously reported in a colon cancer cell line by Ardiani *et al*. (2008). Alternatively, at higher concentrations, Citrus extract or hesperidin is beneficial to cancer cells due to their cytotoxic effects. Recently, genistein, another flavonoid found in soybean, a widely known potential chemopreventive agent, has been reported to demonstrate a biphasic mechanism on CHO-K1 cells: at low concentrations, it induces senescence and apoptosis in combination with estrogen, and at high concentrations, it modulates the cell cycle (Jenie *et al*., 2019). Hence, an investigation of the dose-dependent mechanism of Citrus extract and hesperidin should be carried out further.

CONCLUSION

Citrus extract and hesperidin exerted a biphasic effect on a macrophage cell line. The future development of Citrus extract as a co-chemotherapeutic, anticancer, or immunemodulatory agent should include careful consideration of its biphasic effect on each cell type.

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