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 1000 μg/mL of Citrus extract, it induced cell proliferation, where high concentrations increased cell viability significantly by up to 116-136% where as high concentration reduced cell viability significantly by up to 10-61%. The value of the 50% inhibitory concentration (IC50) of Citrus extract was more than three times higher than that of hesperidin at 3.1 μM. Additionally, 250 μg/mL of Citrus extract increased 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 antioxidant 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,
flavonoids, 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) (Table I). In addition, hesperidin, as one of the main compounds, has been studied (Table I).

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

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

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**In vitro**, it induces cell proliferation and angiogenesis in MCF-7 breast or WiDr colon cancer (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, 150-600 μM Citrus peel (Citrus extract) significantly 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 (5-200 μM) increases cell proliferation 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 IC₅₀ value varying from 11 to 200 μM; 100; 250; 500; 750; and 1000 μM UV lights (λ=254 and 366 nm) with a maximum final DMSO concentration of 2% (v/v). The effect of Citrus extract on MCF-7/HER2 is contrary to the estrogenic effect seen in ovariecotomized 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) / OçiÔÇÔÇ ÔÇÔ× ÍÔ ÈAOÁÅÁÁ 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, 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 citroboric treatments, positive spots indicating flavonoids were developed 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 citroboric treatments, positive spots indicating flavonoids were developed and evaporated in a rotary vacuum evaporator until a brown viscous extract was obtained (yield was 3.21% (b/b)) (Armandari, 2010). This extract is heretofore referred to as Citrus reticulata.

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 1,000 μM), 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 500 μM).

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 5% CO₂ incubator and grown to confluence in 75cm² tissue culture flasks. After they reached 80% confluence, cells were scraped and used for experiments.
**Cell viability assay**

Cells (5 x 10^4 cells/well) were plated in 96-well plates and cultured in a complete medium for 48 h. The medium was then replaced with a medium containing various concentrations of Citrus extract or hesperidin and incubated for 24 h. 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 595 nm of diluted formazan after addition of 0.5 mg/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 \( A_{\text{t}} \) - absorbance of control \( A_{\text{c}} \))/absorbance of control \( A_{\text{c}} \) x 100. A linear regression analysis was performed to determine the IC_{50} value as a function of the concentration.

Because of the variability, the IC_{50} were calculated as a blank. The percent cell viability and the IC_{50} values were calculated as (absorbance of treated \( A_{\text{t}} \) - absorbance of control \( A_{\text{c}} \))/absorbance of control \( A_{\text{c}} \) x 100. A linear regression analysis was performed to determine the IC_{50} value as a function of the concentration.

**Immunostaining**

Immunocytochemistry with an anti-IL-10 mouse monoclonal antibody (Dako) was carried out by the avidin-biotin complex method as previously described (Armandari et al., 2008). Cells (1 x 10^4 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 15 h. After removal of the medium, cells were washed with cold PBS, and then were fixed in cold methanol for 10 min at -20 °C. The endogenous peroxidase activity was blocked with hydrogen peroxide, and nonspecific sites were blocked with normal goat serum (Novocastra), for 10 min 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 10 min. Following washing, cells were incubated with streptavidin-peroxidase complex reagent (Novocastra) for 10 min and then incubated with 3,3-diaminobenzidine (DAB) substrate solution (Novocastra) for 2-10 min to visualize the bound biotin. Cover slips were washed in distilled water and counterstained with Mayer's hematoxylin (Dako) for 1-3 min. 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 1,000 μg/mL, for 24 h. As expected, lower concentrations of Citrus extract did not affect cell morphology. The cell morphology at lower concentrations (i.e., 10 μg/mL, and 50 μg/mL) appeared the same as that of control cells (Figure 1A). Moreover, the cell density increased after treatment with 50 μg/mL, at higher concentrations of Citrus extract, starting at 500 μg/mL, 1,000 μg/mL, more cells appeared rounder and less flattened out in the well. These morphological observations imply a biphasic effect of Citrus extract.

After 24 h of incubation with Citrus extract, cell viability was assayed by the MTT method. The higher concentrations of Citrus extract (500 μg/mL, 1,000 μg/mL) showed 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 250 μg/mL 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, with 1 μg/mL, and 46% showed 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...
whether this compound would exert a similar biphasic effect to the extract. A series of concentrations varying from 1μM to 500μM (equal to 0.6 μg/mL−305.3 μg/mL) was used (Figure 2).

The lowest tested concentration did not significantly affect cell viability. However, at 5−100 μM, hesperidin increased cell viability significantly (116%−136% compared with 100% in the control without hesperidin treatment). At higher concentrations, hesperidin decreased cell viability (***; p<0.001 ****; p<0.0001) in cell viability compared with the control.

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 MTT assay in pentaplicate. A. Cell morphology and density were observed under a microscope, and representative pictures are presented. Scale bars = 100 μm. 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.
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 μg/mL and 203 μg/mL (332 μM), 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.

Citrus extract can induce IL-10 expression

Treatment with 250 μg/mL 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.

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 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 1 μg/mL to 250 μg/mL (Figure 1B) compared with 10-1,500 μg/mL in cancer cell lines (Yunas et al., 2007; Ardiani et al., 2008; Puspita et al., 2008). Meanwhile, hesperidin at low concentrations 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 25 μg/mL, Citrus extract did not affect IL-10 expression and appeared similar to cells without Citrus extract treatment (0 μg/mL). Further studies be tested further to confirm whether the modulation of IL-10 expression caused by Citrus extract is also ensuing the biphasic trend.
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 immunomodulatory agent should include careful consideration of its biphasic effect on each cell type.

ACKNOWLEDGMENT

4ΕΕΟ ΟΩΔΑ Υ ΑΘΩΔΟ ΑΙ ΔΑΑ ΑΩ * ΑΘΕ ΑΟΑΙ ΓΑ ΑΟΕ Α ΑΩΑΔΟ (ΑΦΕ ΑΘΕ ΑΟΑΙ ΓΑ ΑΟΕ Α ΑΩΑΔΟ) Research Grant) Faculty of Pharmacy UGM 2009 (granted to MI). The authors wish to state their contributions as follows: IA performed experiments for the Citrus extract; AK and YE performed cell viability assays for hesperidin; MI and IA wrote the manuscript and prepared the figures; MI oversaw all aspects of the study. All authors have read and approved the manuscript.

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