Indonesian Journal of Cancer Chemoprevention, October 2021
ISSN: 2088–0197
e-ISSN: 2355-8989

Citrus Flavonoids from *Citrus reticulata* Peels Potentially Target an Autophagy Modulator, MAP1LC3A, in Breast Cancer

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Abstract

Citrus flavonoids have been known for their vast biological activities including chemoprevention activities. However, the organic solvent extraction system limits its potential utilization. We recently adopted a hydrodynamic-cavitation method to extract citrus flavonoids from citrus peels. In this study we verified the high flavonoid content of the hydrodynamic-cavitation extract from *Citrus reticulata* peels and explore the potency of its citrus flavonoid contents as targeted chemoprevention agent for breast cancer by using bioinformatics. Based on a thin layer chromatography, the extract positively yielded high content of citrus flavonoids represented by hesperidin. The toxicity analysis by Protox II Online Tool revealed that hesperidin as the major citrus flavonoid in the extract was considered safe with a predicted LD_{50} of 12,000 mg/kg. We then further exploring citrus flavonoids' capacity in targeting MAP1LC3A, a key protein in autophagy. UALCAN analysis validated that low expression of MAP1LC3A is associated with low survival rates in breast cancer patients. Limonin, hesperidin, narirutin, neohesperidine, and naringin are flavonoids from citrus peels that predicted to have inhibitory activity against Protein Kinase A (PKA), a negative upstream of MAP1LC3A, calculated by KNIME. Citrus flavonoids scoparone, cirsimaritin, 4',5,7-trimethoxyflavone, eupatorine, and hesperidin were also exhibit similar structure to an agonist of ATG4B, a protein that plays a role in MAP1LC3A activation. Furthermore, eupatorine, hesperidin, and cirsimaritin displayed a high affinity to ATG4B based on a molecular docking. We concluded that citrus flavonoids from citrus peels are safe to normal cells, and the citrus flavonoids potentially targets MAP1LC3A by inhibiting PKA and acting as ATG4B agonists. Thus, this extract-contained flavonoids from citrus peels is potential to be investigated further as a chemoprevention agent by inducing autophagy, especially for breast cancer.

Keywords: *Citrus reticulata, citrus flavonoid, autophagy, MAP1LC3A, breast cancer.*

INTRODUCTION

Breast cancer has a high prevalence of cases in Indonesia (World Health Organization International Agency for Research on Cancer [IARC], 2020). The government and health workers organize and support the "Breast Self-Examination

Submitted: October 22, 2021
Revised: December 3, 2021
Accepted: December 7, 2021

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(SADARI)" program for early detection and prevention of malignancy of breast cancer cells. However, breast cancer that is detected early needs to be supported by appropriate therapy to increase the patient's chances of healing. Many cancer therapies are targeted at inhibiting metastasis, angiogenesis, induction of cell death, and other mechanisms, each of which has advantages and disadvantages. One of the ways to increase the chances of survival of patients in early-stage cancer is to target the early stages of tumorigenesis. One of the body's physiological mechanisms that play a role in this stage is the autophagy. The mechanism of autophagy causes degradation of damaged organelles, reduces inflammation, and protects cells from damage in the early stages of tumor growth (Li, *et al*., 2020).

Treatment for breast cancer therapy can be obtained from natural ingredients. One of the great healing potentials possessed by orange peel which is still considered as waste. Citrus peel has bioactive properties that have been shown to modulate various cancer progression pathways through cell cycle arrest, inhibition of cell proliferation, induction of apoptosis, and inhibition of angiogenesis and metastasis (Goh, *et al*., 2019; Koolaji, *et al*., 2020; Nair, *et al*., 2018). Most of the utilization of citrus peels to extract its phytochemical compound by using organic solvents leads to several issues, such as the acceptance and environment issue. A water-based extraction called hydrodynamic-cavitation method adopted from Meneguzzo, *et al*. (2020) has been developed recently to extract flavonoids from citrus peels (Utomo, *et al*., 2020).

This study was conducted to identify the flavonoid content of the hydrodynamic-cavitation extract from *Citrus reticulata* peels and to explore the potential of citrus flavonoids from citrus peel extract as autophagy modulators in breast cancer. The modulation of citrus flavonoids was targeted specifically at the MAP1LC3A that plays a role in autophagy. The MAP1LC3A or microtubule-associated protein 1 light chain 3A is one of the proteins that play a role in the autophagy (Costa, *et al*., 2016). The MAP1LC3A is involved in the autophagosome formation stage (He, *et al*., 2003). The MAP1LC3A part was explored with a proteomic study based on UALCAN, KEGG Pathway, and STITCH data, which was then used as the basis for molecular interaction analysis using KNIME and Molecular Operating Environment (MOE). The study was strengthened by a verification of the extract's safety by Protox II Online Tool to predict the LD_{50} value of citrus flavonoids from the peel extract. The results of this study support the development of citrus peels' extract as autophagy modulators for breast cancer.

MATERIALS AND METHODS

Identification of Phytochemical Profile of the Citrus Peel Extract

The hydrodynamic-cavitation extract of *Citrus reticulata* peels was prepared by Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada as modified from previous method (Utomo, *et al*., 2020; Meneguzzo, *et al*., 2020). A thin layer chromatography (TLC) was utilized to qualitatively analysis the citrus flavonoid contents. The extract was dissolved in methanol and spotted on the bottom of the silica gel F254 TLC plate, which were then eluted with butanol, acetic acid, and water (4:1:5 v/v) as the mobile phase. The plate was detected by the UV light at 254 and 356 nm. Hesperidin (H5254, Sigma-Aldrich, St. Louis, Missouri, USA) (1 mg in 5 mL methanol) was included as the citrus flavonoid standard (Ikawati, *et al*., 2019).

Prediction of Compound Toxicity with Protox II Online Tool

Selected citrus flavonoids from peel extract in SMILES format were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/), then processed using the Protox II Online Tool (http://tox.charite.de/tox/) to predict the LD_{50} and toxicity class (Banerjee, *et al*., 2018).

Protein Expression Profile with UALCAN

TCGA analysis was selected in the UALCAN main menu (http://ualcan.path.uab.edu/) (Chandrashekar, *et al*., 2017). The data exploration was started by entering the MAP1LC3A into the search field and selecting breast invasive carcinoma in the TCGA dataset option. The next analysis chosen was survival. The results obtained were the effect of MAP1LC3A expression on the survival of invasive breast cancer patients. Next, the page was returned to the main menu, and then CPTAC analysis was selected. MAP1LC3A was typed into the search field with the breast cancer option. The results obtained are MAP1LC3A expression in breast cancer. The data obtained is then stored for the data analysis process.

Protein Interactions with The KEGG Pathway

Signal transduction pathway analysis was obtained from the KEGG Pathway bioinformatic system (www.genome.jp/kegg/pathway.html). Access database entry number for autophagy was hsa04140.

Protein Interactions with STITCH

The protein common name (MAP1LC3A) was entered into the item by name search field. The organism selected was Homo sapiens. In the settings menu, the interaction lines were set in molecular action mode with a minimum interaction score of 0.400 and a maximum number of interactions: ten in the 1st shell and five in the 2nd shell. Other settings were left in default mode. All settings were applied by pressing the update command. Direct target proteins (DTPs) and indirect target proteins (ITPs) can be obtained from STITCH (http://stitch.embl.de/) (Hermawan, *et al*., 2020).

Molecular Interactions with KNIME

Based on the analysis of protein interactions and signaling pathways from KEGG and STITCH, citrus flavonoids in citrus peels were predicted for their inhibitory activity against negative upstream of MAP1LC3A or were analyzed for their structural similarities to agonist of related proteins using the Teach Open CADD workflow. The result was a sequence of citrus flavonoids in citrus peels accompanied by a predictive inhibitory activity score and structural similarity.

Molecular Docking with Molecular Operating Environment (MOE)

The receptors on the prepared compounds were then docked using the MOE software (licensed by the Faculty of Pharmacy, Universitas Gadjah Mada). Selected citrus flavonoids from citrus peels in 3D form as well as flubendazole as a standard were included in the ligand database and docked on the ATGB4B protein (PDB ID: 2D1I). At this step, data were obtained in the ligand compound's docking conformation and the bond score. The resulting output was displayed in the form of _.mdb with docking options for site ligand: pocket atom and ligand: MDB file. The docking score was shown in the form of a database viewer that can be exported in _.xls. The results of the interaction were displayed in 2D or 3D (Utomo, *et al*., 2020).

RESULTS

Citrus Flavonoid Content in the Citrus Peel Extract

The qualitative analysis by TLC revealed that the extract was rich in flavonoids, represented by hesperidin as the standard at hRf around 4 (Figure 1). We verified that this water-based extraction is able to extract high flavonoid contents from citrus peels.

Citrus Flavonoids from Citrus Peel Extract are Considered not Toxic

An ideal chemoprevention agent not only cytotoxic to cancer cells, but equally importantly is safe to normal toxic, thus a selectivity index should be considered (Artun, *et al*., 2015). The safety of a compound seen from its toxicity to organisms

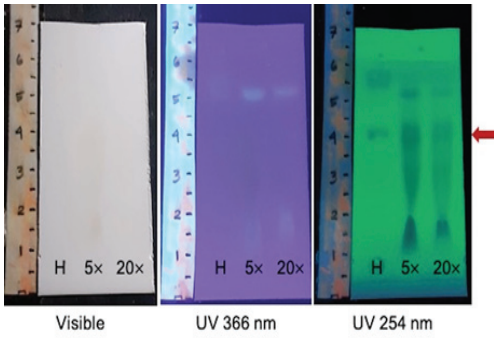

Figure 1. Identification of citrus flavonoids in the hydrodynamic-cavitation extract of *Citrus reticulata* peels. A thin layer chromatography was carried out and representative images were observed at visible light (left), UV 254 nm (middle), and 365 nm (right) is shown. The arrow indicates the clear presence of hesperidin spots. H: hesperidin; 5×: extract at 5× dilution; 20×: extract at 20× dilution.

is an absolute requirement for a compound to be consumed. In this study, the safety of the extract target toxicity and LD_{50} values of citrus flavonoids from citrus peels was predicted using the Protox II Online Tool platform. The toxicity class is in the range of 1 to 6: the higher the toxicity class, the safer a compound is. As hesperidin appeared as the major citrus flavonoids in the citrus peel extract, we focused on hesperidin. The prediction tools with accuracy level of 73% revealed that hesperidin is in class 6 with an LD_{50} of 12,000 mg/kg. Based on this result, we confirmed that the extract is not toxic.

Expression of MAP1LC3A Positively Correlated with Survival Rate of Breast Cancer Patients

UALCAN was used to investigate the effect of MAP1LC3A expression on survival rate in invasive breast cancer patients. Breast cancer patients with low MAP1LC3A expression had a lower survival rate when compared to those with high MAP1LC3A protein expression (Figure 2). Thus, it is evident that the associated MAP1LC3A plays an important role in the pathophysiology of breast cancer.

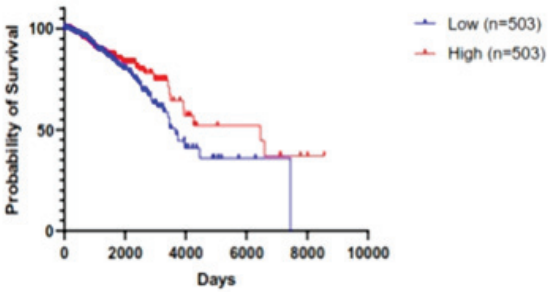

Figure 2. The association of MAP1LC3A expression levels with survival in breast cancer patients. The red and blue lines indicate high and low expression levels, respectively. The high expression level of MAP1LC3A was proportional to survival rates in invasive breast cancer patients. Analyzes were performed with UALCAN (http://ualcan.path.uab.edu/).

Indonesian Journal of Cancer Chemoprevention, October 2021
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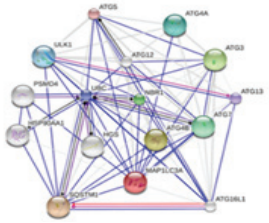
e-ISSN: 2355-8989

A.



B.

Protein	Score
SQSTM1	0,999
ATG4B	0,993
ATG3	0,992

Figure 3. Protein-protein interactions. (A) Analysis of MAP1LC3A-induced expression pathways using STITCH. (B) Prediction of interacting protein scores.

Protein Interactions

Tracing the MAP1LC3A expression pathway was carried out to see the associated proteins. PKA was obtained based on the KEGG Pathway (www.genome.jp/kegg/pathway.html), which is a negative upstream protein that interacts directly with MAP1LC3A (Bonam, *et al*., 2020; Cherra, *et al*., 2010). PKA inhibits the MAP1LC3A protein so that the MAP1LC3A protein decreases (Bonam, *et al*., 2020). PKA is expected to be inhibited by the compounds contained in citrus peels. Further analysis with STITCH showed that proteins SQSTM1, ATG4B, and ATG3 were the highest-scoring MAP1LC3A-interacting proteins (Figure 3). ATG4B is a protein that plays a role in MAP1LC3 cleavage, which plays an important role in the autophagy process (Costa, *et al*., 2016). Reducing PKA expression and increasing ATG4B can increase MAP1LC3A expression, thus inducing autophagy.

Interaction of Target Proteins with Flavonoids from Citrus Peels

As previously reported, PKA is directly targeting MAP1LC3A by phosphorylation (Cherra, *et al*., 2010). Based on the KEGG, PKA is a potential target protein, which can increase MAP1LC3A expression when inhibited. KNIME was used to predict the inhibitory activity of compounds in orange peel against the upstream negative MAP1LC3A protein, and the results can be seen in Table 1.

The flavonoids from citrus peels that were predicted to have inhibitory activity against PKA were limonin, hesperidin, narirutin, neohespiridine, and naringin (Table 1). In addition, hes-

Table 1. The inhibitory activity of flavonoids from citrus peels against PKA as predicted by KNIME.

Compound	Activity	Score
Limonin	1.0	0.71
Hesperidin	1.0	0.58
Narirutin	1.0	0.56
Neohesperidin	1.0	0.55
Naringin	1.0	0.54
Sinensetin	0.0	0.89
Salvigenin	0.0	0.89
Cirsimaritin	0.0	0.88
Tangeretin	0.0	0.86
Ponkanetin	0.0	0.86
Tangeritin	0.0	0.86

Table 2. Similarities in chemical structure of flavonoids from citrus peels with ATG4B agonists analyzed by KNIME.

Compound	Score
Scoparone	0.486486
Cirsimaritin	0.473684
4',5,7-trimetoksiflavon	0.472222
Eupatorin	0.467532
Hesperidin	0.465116
Neohesperidin	0.45977
Nobiletin	0.453333
Tangeretin	0.453333
Isosinensetin	0.453333

peridin also has a similar chemical structure with ATG4B agonists (Table 2). Scoparone, cirsimaritin, 4',5,7-trimethoxyflavone, and eupatorine also have greater structural similarities than the ATG4B agonists.

The Predicted Interaction Between ATGB4B and Flavonoids from Citrus Peels

MOE was used to predict the potential of flavonoids from citrus peels as protein agonists that had a positive role on MAP1LC3A through molecular docking. All compounds had high-affinity values with (RMSD) <2, which indicated the validity of the docking results (Figure 4). The flavonoids from citrus peels have high-affinity values, except for scoparone. High affinity is indicated by a lower docking score than the agonist compound (flubendazole). The highest compound affinity order is as follow: eupatorin>hesperidin>-cirsimaritin>4',5,7-trimethoxyflavone>scoparone. All compounds interacted with the amino acid residue Thr 22 (Figure 4B).

DISCUSSION

To obtain large amounts of hesperidin compounds from orange peels, extraction using the hydrodynamic-cavitation technique has been developed (CCRC, unpublished data). The hydrodynamic-cavitation method produces large yields and extract large quantities of hesperidin (Meneguzzo, *et al*., 2020). We confirmed that the water-based extract from *Citrus reticulata* peels contains hesperidin as one of the major citrus flavonoids (Figure 1). The quantification as well as the characterization of hesperidin or other flavonoids in the extract should be described further in a more detail. Nevertheless, we still be able to conclude that citrus flavonoids from peels can be extracted by using the hydrodynamic-cavitation method.

We hypothesize that the flavonoids in citrus peels are targeting MAP1LC3A, so that autophagy are optimal, and eventually suppress the development of breast cancer. To proof our hypothesis, an investigation was carried out through bioinformatic studies. The bionformatic analysis was carried out by using several databases (UALCAN, OncoLnc, KEGG, STITCH, Protox II Online Tool, KNIME) and MOE software.

Both MAP1LC3A and MAP1LC3B are expressed in normal breast tissues and breast carcinoma tissues, but we focus more in MAP1LC3A since is more immunoreactive in carcinoma (Othman, *et al*., 2009). Breast cancer patients with low MAP1LC3A expression had a lower probability of survival when compared to patients with high MAP1LC3A protein expression based on data obtained from the UALCAN and OncoLnc websites (Figure 2). MAP1LC3A protein plays a role in the autophagy process (He, *et al*., 2003). Autophagy is necessary to suppress tumor growth at an early stage by degrading damaged organelles, reducing inflammation, and protecting cells from damage (Li, *et al*., 2020). The low expression of MAP1LC3A inhibits autophagy in breast cancer, resulting the lower probability of survival

Indonesian Journal of Cancer Chemoprevention, October 2021
ISSN: 2088–0197
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A.

Compound	Score Docking (kkal/mol)	RMSD (Å)
Flubendazole (agonis)	-12.3389	1.1071
Scoparone	-11.4574	0.8008
Cirsimaritin	-17.3660	1.4017
4',5,7-Trimetoksiflavon	-14.2738	1.3077
Eupatorin	-20.1086	1.3117
Hesperidin	-16.6338	1.8615

B.

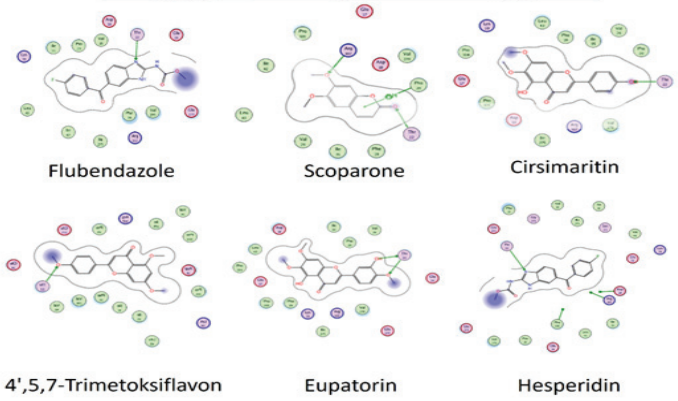

Figure 4. Results of molecular docking between flavonoids from citrus peels and ATG4B (PDB ID: 2D1I). (A) Docking score results. (B) Visualization of compound interaction with ATG4B protein.

in cancer patients. This relationship underlies the importance of targeting MAP1LC3A in modulating the autophagy process to increase the probability of survival in breast cancer patients.

We took two approaches to keep the autophagy process optimal by targeting MAP1LC3A. First is to increase MAP1LC3A expression by inhibiting its negative upstream, and second is by inducing MAP1LC3A activation. These approaches resulted in two proteins closely related to MAP1LC3A, namely PKA and ATG4B. Flavonoids from citrus peels were targeted to inhibit PKA protein activity and, on the other hand, induce ATG4B protein activity. Based on the analysis results via KEGG, PKA inhibits the activity of MAP1LC3A through a phosphorylation process so that it is targeted to be inhibited and the interaction is a direct one (Cherra, *et al*., 2010). ATG4B is required to activate MAP1LC3A protein so that its activity is targeted to be induced. The STITCH database confirmed the close interaction between citrus flavonoids and ATG4B. Proteins in the ATG family play a role in the posttranslational modification of the MAP1LC3A protein to produce a functional MAP1LC3A variant that will become a component of the autophagosome membrane (He, *et al*., 2003). In addition to the close interaction with MAP1LC3A, the selection of ATG4B protein as a target in this study was based on a known ATG4B agonist compound, namely flubendazole can target ATG4B and induce autophagy (Zhang, *et al*., 2015). The protein structure of ATG4B is also available in the PDB database so that the interaction between ATG4B and citrus flavonoids can be further confirmed by the molecular docking.

The ability of flavonoids from citrus peels to inhibit PKA protein activity and induce ATG4B protein activity was predicted through KNIME and MOE software in this study. The flavonoids in citrus peels that were predicted to have inhibitory activity against PKA were limonin, hesperidin, narirutin, neohesperidin, and naringin (Table 1), while the compounds predicted to induce ATG4B protein activity were hesperidin, scoparone, cirsimaritin, 4',5,7- trimetoxyflavones, and eupatorine with high binding affinity to ATG4B (Figure 4). Among these compounds, only hesperidin could inhibit PKA activity and, at the same time, induce ATG4B activity. Analysis using the Protox II Online Tool showed the diversity of toxicity levels of

these compounds on normal cells (data not shown). Hesperidin has the lowest toxicity with a predicted LD_{50} of 12,000 mg/kg. Thus, we hypothesized that hesperidin is a lead compound in the hydrodynamic-cavitation extract of citrus peels that can provide the expected activity against PKA and ATG4B proteins and is safe against normal cells.

CONCLUSION

The hydrodynamic-cavitation extract from *Citrus reticulata* peels with flavonoid contents represented by hesperidin is considerably not toxic to normal cells. Flavonoids from citrus peels, including hesperidin, are possibly modulate autophagy via MAP1LC3a by PKA inhibition and ATG4B induction. Thus, hydrodynamic-cavitation extract from citrus peels with rich flavonoids is potential to be developed as supporting agents for breast cancer through autophagy modulation.

ACKNOWLEDGMENT

The authors thank the Directorate General of Learning and Student Affairs, Ministry of Education and Culture, Research and Technology, Republic of Indonesia for their funding by "Program Kreativitas Mahasiswa-Riset Eksakta" (PKM-RE) 2021. We also grateful for supports from Prof. Edy Meiyanto and all members of Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada in this research. The authors wish to state their contribution as follow: BA, DK, DA methodology, analysis, visualization, original draft preparation. BA–extraction, bioinformatic, and docking investigation; DK–*in vitro* assay; DA–phytochemical investigation; MI–conceptualization, methodology, supervision, visualization, manuscript review and editing.

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