

Phytochemical and Bioinformatic Studies of Citrus Flavonoids as Chemopreventive Agents Targeting GGPS1 for Liver Cancer

Ratih Kurnia Wardani^{1,2} I Made Rhamandana^{1,2}, Christiana Mutiara Putri Gono^{1,2},
Muthi' Ikawati^{2,3*}

¹Undergraduate Program, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia

²Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia

³Macromolecular Engineering Laboratory, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia

Abstract

Overexpression of geranylgeranyl diphosphate synthase 1 (GGPS1) is an unfavorable prognosis in liver cancer development. The side effects of therapeutic standards encourage the development of therapeutic agents from herbal materials. Citrus peels are rich of phytochemical compounds, especially citrus flavonoids, that possess cytotoxic activities. This study aimed to determine the potential of citrus flavonoids as chemopreventive agents targeting GGPS1 protein by phytochemical and bioinformatic studies. Dried peels of *Citrus reticulata* were extracted by hydrodynamic-cavitation method followed by identification of compounds using thin layer chromatography (TLC). The expression level of GGPS1 was obtained from UALCAN, while its correlation with survival rate was obtained from the GEPIA. Prediction models regarding the potential inhibitors of citrus peel compounds against GGPS1 were obtained through KNIME and ChEMBL, followed by literature studies on chemopreventive activity of citrus flavonoids. The molecular docking was used to predict the molecular interaction followed by tracking of target genes that were positively correlated with GGPS1 by SwissTargetPrediction. Yielded 75% (v/v), the extract positively contained citrus flavonoid with hesperidin as comparison. Overexpression of GGPS1 significantly reduced the survival rate of liver cancer patients (p value=0.019). Four citrus flavonoid compounds, namely tangeretin, nobiletin, hesperidin, and naringenin showed potential inhibition to GGPS1. The molecular docking showed that tangeretin had a strong affinity compared to the native ligand and zoledronic acid, as positive control. PARP1, CSNK2A1, TNKS2, and GSK3B were clarified as targeted genes for tangeretin and nobiletin that positively correlated with GGPS1. *In vitro* and *in vivo* studies will validate our findings and support the development of citrus peel extract with rich flavonoid contents as a chemopreventive agent.

Keywords: *geranylgeranyl diphosphate synthase 1 (GGPS1), liver cancer, hydrodynamic-cavitation, citrus flavonoid, bioinformatic.*

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*Corresponding author: muthi_ikawati@ugm.ac.id

INTRODUCTION

Damaged regulation in cancer allows cells to grow abnormally and uncontrollably exceed the threshold and invade other tissues. Liver cancer is one of cancer that needs to be addressed immediately. Based on GLOBOCAN 2020, liver cancer in men ranks as the second leading cause of cancer-related death (577,522 cases) and the most frequently diagnosed cancer (632,230 cases) worldwide (Sung, *et al.*, 2021). Hepatocellular carcinoma (HCC) is the main histological type of primer liver neoplasm and accounts for 85-90% of total liver cancer cases (Lafaro, *et al.*, 2015). Geranylgeranyl diphosphate synthase 1 (GGPS1), also known as GGDPS, is a 20-carbon isoprenoid phospholipid whose lipid moiety can be incorporated post-translationally into proteins to enhance membrane association (Agabiti, *et al.*, 2016). This protein is an unfavorable prognosis in the development of liver cancer. In liver hepatocellular carcinoma (LIHC) patients, GGPS1 mRNA expression is increased by 85.29% of tumor tissue compared to surrounding cells (Yu, *et al.*, 2014). Therefore, GGPS1 can serve as a potential therapeutic target in liver cancer.

To date, most of the treatment uses chemical therapy with chemotherapeutic agents. However, existing anticancer agents can induce chemoresistance that can lead to cell death. Therefore, the development of liver cancer chemotherapy needs to be directed at the development of chemopreventive agents from herbal ingredients. Citrus peels are commonly considered as waste. However, the ethanolic extract from the peels of *Citrus reticulata*, one of most consumed citrus commonly known as mandarin oranges, showed anticarcinogenic, antiproliferative, co-chemotherapy, and estrogenic effects (reviewed in Meiyanto, *et al.*, 2012). In addition, it is also a cheap and abundant source of anti-oxidants and potentially bioactive phenolic compounds (Ferreira, *et al.*, 2018).

Our group currently developed the extraction method (Utomo, *et al.*, 2020) for *Citrus*

reticulata peels by adapting a hydrodynamic-cavitation method (Meneguzzo, *et al.*, 2020), resulting high flavonoid contents. This study was conducted to determine the ability of citrus flavonoid compounds from citrus peel extract as potential chemopreventive agents targeting GGPS1 in liver cancer. The research carried out based on bioinformatics that involved computational performance and utilized several databases. In addition, phytochemical profile analysis and molecular docking approach were carried out to observe molecular interactions between citrus flavonoids and GGPS1. The collected data support the potency of citrus flavonoids from citrus peels as chemopreventive agent for liver cancer.

METHODS

Material Preparation and Extraction

The dried citrus peels were purchased from a commercial marketplace. A determination carried out by the Department of Biological Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada (UGM) confirmed that the material was *Citrus reticulata*. The extraction was prepared by Cancer Chemoprevention Research Center (CCRC) Faculty of Pharmacy UGM by using the hydrodynamic-cavitation method as modified from Meneguzzo, *et al.* (2020) (Utomo, *et al.*, 2020;) and the yield was calculated.

Identification of Phytochemical Profile

The hydrodynamic-cavitation citrus peel extract (EHC) was then analyzed qualitatively by thin layer chromatography (TLC) and compared with hesperidin (1 mg/5 mL methanol), a citrus flavonoid as the standard, as previously described (Ikawati, *et al.*, 2019). The extract sample was dissolved with methanol as solvent and spotted on the bottom of the silica gel F254 TLC plate. The samples were diluted 5 and 20 times, which were then eluted with butanol, aquadest and glacial acetic acid (4:5:1 v/v) as the mobile phase. The plate was detected by the UV light at 254 and 356 nm.

GGPS1 Expression Profile Analysis and Patient Survival Rate

The analysis was carried out through web-based tools UALCAN (<http://ualcan.path.uab.edu/index.html>) and GEPIA (<http://gepia.cancer-pku.cn/index.html>). The UALCAN database was used to examine the expression of the GGPS1 gene in the liver hepatocellular carcinoma (LIHC) and normal tissues. The GEPIA database was used to analyze the survival rate of liver cancer patients which correlated with GGPS1 overexpression. All statistical data is taken directly from the appropriate database. The data is then downloaded and presented in graphical form.

Identification of Potential Compounds of *Citrus reticulata* Peels

Data on secondary metabolites contained in *Citrus reticulata* peels were obtained from Dr. Duke's Phytochemical online database (<https://phytochem.nal.usda.gov/phytochem/search>). The search was conducted using the keywords "*Citrus reticulata*" and "on the pericarp (skin)". Furthermore, a literature search on chemoprevention activity against liver cancer was carried out on the compounds obtained from the database. The literature search was carried out using the keywords "citrus flavonoid", "liver cancer", "liver hepatocellular carcinoma", and "hepatocellular carcinoma" on the PubMed platform (<https://pubmed.ncbi.nlm.nih.gov/>) and Google Scholar. (<https://scholar.google.com/>). The potential compounds of *Citrus reticulata* peels are referred as potential compounds of citrus peels or citrus flavonoid thereafter.

Compound Prediction with KNIME Machine Learning

KNIME is a data processing platform with one of the methods in the form of opensource computer aided drug design. The inhibitory activity of compounds from citrus peels against GGPS1 was analyzed using machine learning KNIME. The data entered into the KNIME software are

activity datasets obtained from the ChEMBL database (<https://www.ebi.ac.uk/chembl/>) and compound SMILES data obtained through PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The analysis was carried out using artificial intelligence Random Forest algorithm with the dependent variable used was pIC_{50} . Prediction model of potential compound activity in orange peel is then presented with a prediction value with a value close to one, meaning that the compound is most closely related to the biological activity being sought.

Analysis of the Interaction of Potential Compounds of citrus peel with GGPS1

Molecular docking was carried out to determine the molecular interaction between potential compounds of citrus peels and GGPS1 using MOE 2010.10 software licensed by the Faculty of Pharmacy UGM. Protein data was downloaded through the Protein Data Bank (<https://www.rcsb.org/>) and compound data was downloaded through PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). A computational study is presented to simulate molecular binding, calculate root mean deviation square (RMSD), docking score (S) and visualize protein-ligand interactions using the Chimera platform (Utomo, *et al.*, 2020).

Analysis of Target Genes that are Positively Correlated with GGPS1

Genes correlated with GGPS1 were obtained using the UALCAN database (<http://ualcan.path.uab.edu/analysis.html>). The search was carried out using the keywords "GGPS1" in the gene column and "liver hepatocellular carcinoma" in the cancer column. Data on genes that were positively correlated with GGPS1 were then sliced with data on target genes of potential compounds in citrus peels obtained from the SwissTargetPrediction database (<http://www.swisstargetprediction.ch/>) by using InteractiVenn (<http://www.interactivenn.net>) (Heberle, *et al.*, 2015; Musyayyadah, *et al.*, 2021).

RESULTS

Phytochemical Profile of Hydrodynamic-cavitation Citrus Peel Extract (EHC)

The hydrodynamic-cavitation extraction yielded 75.44% (v/v) of liquid extract, referred as EHC (hydro-cavitated citrus peel extract) thereafter. Citrus peels contain methoxy flavonoids, such as hesperitin, hesperidin, and diosmin, an enantiomer structure of hesperidin (Ikawati, *et al.*, 2019; Meneguzzo, *et al.*, 2020). Qualitative analysis by TLC revealed that EHC positively contains hesperidin, the major methoxy flavonoid in *Citrus reticulata* (Barreca, *et al.*, 2017) as it also shown the same spot as hesperidin as the standard at hRf 4, indicating that EHC possibly rich in flavonoids (Figure 1). Thus, this water-based extraction was able to produce extract with remarkable flavonoid contents.

GGPS1 Expression Profile and Its Correlation with Survival Rate in Liver Cancer Patients

Based on data obtained from UALCAN, GGPS1 was significantly higher in liver cancer than in normal cells (Figure 2A) with *p* value of 1×10^{-12} , indicating that a high level of GGPS1 expression is associated with the incidence of liver

cancer hepatocellular carcinoma (LIHC). Thus, it is further validating the GGPS1 protein as one of the undesirable prognoses in liver cancer. High GGPS1 expression also correlated with significantly decreased survival rate in liver cancer patients ($p < 0.05$) when compared with low expression of GGPS1 (Figure 2B). Therefore, inhibiting GGPS1 could be a potential strategy to overcome liver cancer.

The Chemopreventive Activity of Compounds in Citrus Peels

A total of 627 compounds contained in *Citrus reticulata* peels were obtained from Dr. Duke's Phytochemicals online database. A literature search on chemoprevention activity against cancer was carried out on citrus flavonoid compounds obtained from the database. Flavonoid compounds are compounds with the highest abundance in citrus and are known to have potent cytotoxic activity in previous studies. The chemopreventive activity of citrus flavonoid compounds from *Citrus reticulata* peels is shown in Table 1. Hesperidin, naringin, nobiletin, and tangeretin are known to have cytotoxic activities including inducing apoptosis, antiproliferation, anti-metastasis, and inducing cell cycle arrest. This shows that citrus flavonoids could be used as chemopreventive agents.

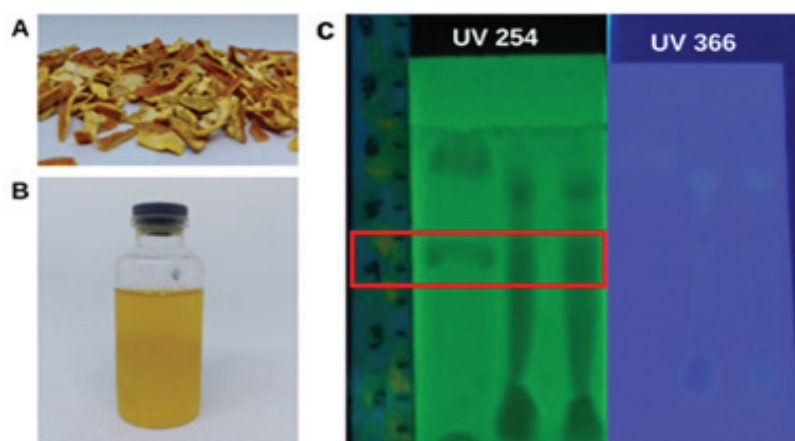


Figure 1. The hydrodynamic-cavitation extract of citrus peels and the phytochemical profile. (A) Dried citrus peels. (B) Hydrodynamic-cavitation citrus peel extract (EHC). (C) Phytochemical profile of EHC. A thin layer chromatography was carried out as described in the Methods and a representative image of observations at UV 254 nm (left) and 365 nm (right) is shown. The red box indicates the presence of hesperidin spots. Left lane: hesperidin; middle lane: EHC at 5 \times dilution; right lane: EHC at 20 \times dilution.

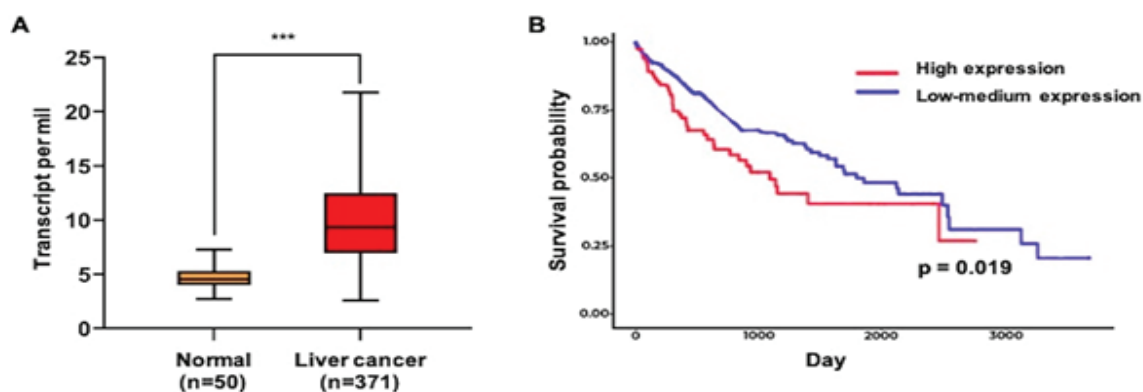


Figure 2. GGPS1 expression and liver cancer. (A) Expression of GGPS1 in normal cells and liver cancer cells analyzed by UALCAN (<http://ualcan.path.uab.edu/analysis.html>). ***: $p < 0.001$. (B) The effect of GGPS1 expression level on survival probability of liver cancer patients analyzed by GEPIA (<http://gepia.cancer-pku.cn/index.html>).

GGPS1 Inhibition by Citrus Flavonoids using KNIME

A total of 627 compounds were obtained from the Dr. Duke's Phytochemical database was then analyzed for its inhibitory activity against the GGPS1 protein. The prediction model was made using machine learning KNIME using the Random Forest algorithm. The dataset of ChEMBL used is a dataset with code ChEMBL319144 and cut off pIC_{50} of 4.5. The parameters of the validity of the algorithm prediction model produced are overall accuracy and Receiver Operating Characteristic (ROC) with each of 85.13% with an ROC value of 0.939. The ROC indicates whether discrimination threshold of binary classifier such as the algorithm is varied while the overall accuracy indicate the overall result of the created algorithm to the submitted data itself. Both the ROC value and overall accuracy determine the validity of the prediction model. The results indicated that the prediction model used is valid because the ROC and overall accuracy values are close to 1 and 100%, respectively. Based on the results of the prediction model, we found that 303 of the 627 compounds in citrus peels were predicted to inhibit GGPS1 protein expression. The prediction then focused on citrus flavonoid compounds (Figure 3) which are the compounds with the highest abundance in citrus peels and have cytotoxic activity, thus potential as

chemopreventive agents (Koolaji, *et al.*, 2020). The predicted value of the inhibitory activity of GGPS1 protein by citrus flavonoid compounds is shown in Table 2. Tangeretin, nobiletin, and naringenin has activity prediction of 1.0, meaning that the algorithm predict these compounds could potentially bind to GGPS1. Whereas hesperidin showed activity prediction of 0.0, meaning that this compound could not bind to GGPS1. Prediction value as seen in Table 2 indicates the score of the activity.

Molecular Interactions between Citrus Flavonoid and GGPS1

Citrus flavonoid compounds predicted to have activity to inhibit GGPS1 namely tangeretin and nobiletin were then analyzed further using molecular docking. Molecular docking was carried out to the protein structure of GGPS1 when it binds to a bisphosphonate inhibitor named FV0109 (PDB ID 6C57). The result of the interaction is indicated by the docking score (S) with a lower docking score indicating that it has a stronger bond with less energy to inhibit the GGPS1 protein. The parameter validity of the method is indicated by the RMSD value which is said to be valid if the RMSD value is < 2 . Molecular docking was carried out on GGPS1 substrates, namely dimethylallyl diphosphate and isopentenyl diphosphate; zoledronic acid, an approved therapeutic for HCC (Nguyen, *et al.*,

Table 1. Citrus flavonoids in *Citrus reticulata* peels and their pharmacological activities.

Compound	Activity	Mechanism	Reference
Hesperidin	apoptosis induction	Increases caspases-9, 8, 3; Bax, Bak Decreases Bcl-xl	Banjerdpongchai, <i>et al.</i> , 2016
	anti-proliferation	CAMKIV inhibitor	Aggarwal, <i>et al.</i> , 2020
	anti-metastasis	Decreases MMP-9 via AP-1, JNK, and NF- κ B signaling pathway	Aggarwal, <i>et al.</i> , 2020
Naringin	anti-metastasis	Inhibits MMP-2, MMP-9, and ERK-P38-JNK signaling pathway	Aroui, <i>et al.</i> , 2016
	anti-proliferation	Inhibits Zeb-1 expression	Ming, <i>et al.</i> , 2018
	apoptosis induction	Inhibits Bcl-2 expression Induces NF- κ B, p53, Bax	Ming, <i>et al.</i> , 2018
Nobiletin	cell cycle arrest at G0/G1	Inhibits ERK1/2, cyclin D1 activity Induces p21	Chen, <i>et al.</i> , 2014
	apoptosis induction	Inhibits Bcl-xl expression	Ming, <i>et al.</i> , 2018
Tangeretin	anti-proliferation	Inhibits COX-2 expression	Arivazhagan & Sorimuthu, 2014
	cell cycle arrest at G1/S	Induces p53/p21	Arivazhagan & Sorimuthu, 2014
	anti-metastasis	Inhibits expression of MMP-2, MMP-9, VEGF	Arivazhagan and Sorimuthu, 2014

2018) as comparison; and on tangeretin, nobiletin, naringenin, and hesperidin as citrus flavonoid compounds that predicted potent in inhibiting GGPS1 expression based on machine learning KNIME.

Based on the results of the docking that has been done, the value of RMSD<2 is obtained so that the analysis carried out is valid (Table 3). Tangeretin, nobiletin, hesperidin, and naringenin

had lower docking scores than the GGPS1 substrate and zoledronic acid (Table 3). This indicated that the four citrus flavonoid compounds are more strongly bound to GGPS1 protein than their substrates, so they have the potential to become potent competitive inhibitors of GGPS1. The visualization of molecular docking is shown in Figure 4.

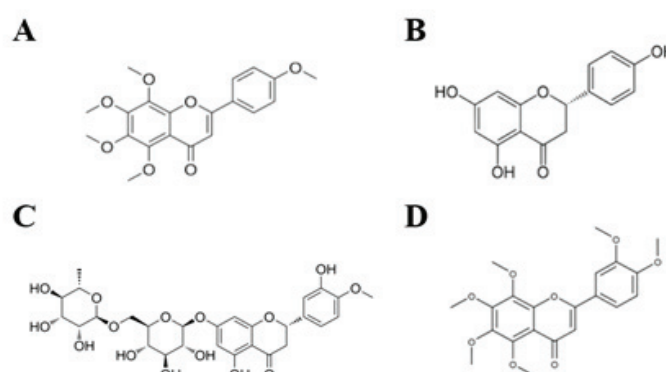


Figure 3. Citrus flavonoids. (A) Tangeretin (B) Naringenin (C) Hesperidin (D) Nobiletin.

Table 2. Prediction results of citrus flavonoid compounds that inhibit GGPS1 based on random forest algorithm.

Compound	Activity Prediction	Prediction Value
Tangeretin	1.0	0.71
Nobiletin	1.0	0.60
Naringenin	1.0	0.51
Hesperidin	0.0	0.63

Target Gene of GGPS1 and Citrus Flavonoids

A total of 1,074 genes that were positively correlated with GGPS1 in LIHC were obtained from the UALCAN database with Pearson-CC data taken, namely Pearson-CC >0.5. The positive correlation indicates that when GGPS1 expression in liver cancer is high, the positively correlated genes are also highly expressed in liver cancer. Therefore, these genes can be used as targets other than GGPS1 in the development of chemopreventive agents in liver cancer. Tangeretin and nobiletin were predicted to be potent inhibitors of GGPS1 based on previous molecular docking results. Each of the 100 target genes of tangeretin and nobiletin obtained from the SwissTargetPrediction database were then sliced with genes that were positively correlated with GGPS1 using InteractiVenn.

A total of 4 genes were obtained from the slices including: PARP1, CSNK2A1, TNKS2, and GSK3B are shown in Figure 5. These four genes can then be used as targets for the development of chemopreventive agents from citrus flavonoids, especially tangeretin and nobiletin in liver cancer.

DISCUSSION

Resistance to existing anticancer agents and lack of biomarkers that can detect recently surgically resected nodules can lead to liver cell death (Yu, *et al.*, 2014). To date, most treatments have been chemically treated with chemotherapeutic agents such as zoledronic acid (ZOL) which are used to reduce cancer-induced osteolysis. ZOL can slow the growth and progression of bone metastatic

Table 3. Molecular docking between GGPS1 and citrus flavonoids.

Protein	Compound R	MSD (Å)	S (kcal/mol)
GGPS1	Isopentenyl diphosphate*	1.25	-12.3269
GGPS1	Dimethylallyl diphosphate*	1.81	-14.9444
GGPS1	Zoledronic acid**	1.21	-11.9640
GGPS1	Tangeretin	1.96	-22.1638
GGPS1	Nobiletin	1.55	-22.1386
GGPS1	Naringenin	1.69	-16.6916
GGPS1	Hesperidin	1.44	-21.5638

*substrate of GGPS1; **approved drug (inhibitor) as comparison.

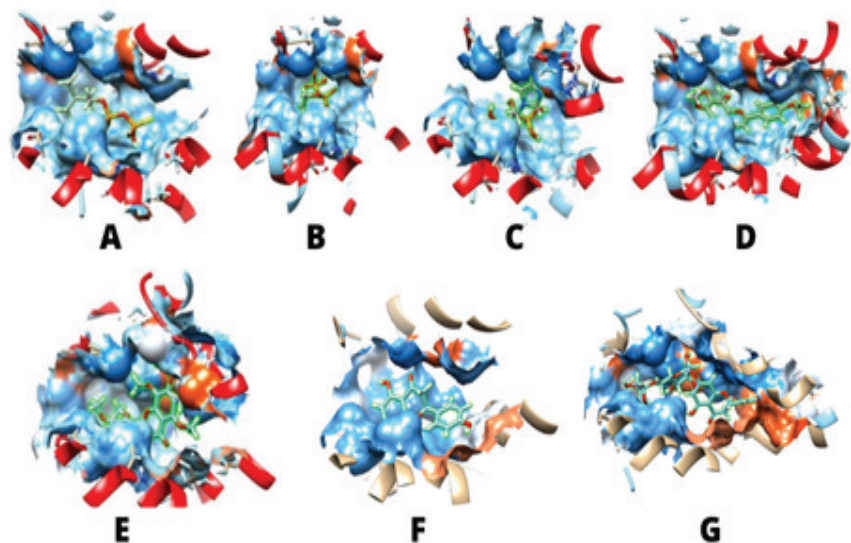


Figure 4. Visualization of the interaction of GGPS1 protein with tested compounds. Substrates: (A) isopentenyl diphosphate and (B) dimethylallyl diphosphate. Inhibitor (approved drug): (C) zoledronic acid. Citrus flavonoids: (D) tangeretin, (E) nobiletin, (F) naringenin, and (G) hesperidin.

pain from hepatocellular carcinoma (HCC) (Honda, *et al.*, 2015). However, existing anticancer agents can induce chemoresistance that can lead to cell death. The many limitations possessed by existing chemotherapeutic agents such as limited effectiveness, the presence of side effects to cause resistance cause the urgency of the need for the development of liver cancer chemotherapy that needs to be directed at the development of chemopreventive agents from herbal ingredients.

Citrus peels, which are often used as waste, are known to contain compounds such as flavonoids, vitamins, minerals, and carotenoids which are commonly found in the colored part of the orange peel (flavedo), giving rise to the distinctive aroma of citrus peels. Flavonoids in citrus peel are known to have anticancer activity, cancer chemopreventive, antioxidant, antiproliferative, and estrogenic effects (Meiyanto, *et al.*, 2012; Parhiz, 2014). Important and distinctive citrus flavonoids are classified as methoxy flavonoids including hesperidin, tangeretin, naringenin, and nobiletin (Fast, 2019). Although the contents can be found in every part of citrus, but generally the greatest abundance

is in the peel with hesperidin showing the highest content in *Citrus reticulata* (Meneguzzo, *et al.*, 2020).

The results of the phytochemical test using thin layer chromatography showed that the hydro-cavitated extract of citrus peels (EHC) contains hesperidin compounds. Hesperidin belongs to the flavonoid group, more precisely the flavanones found in citrus fruits, especially in the peel. Hesperidin is known to inhibit cancer cell proliferation by inducing apoptosis and cell cycle arrest. Hesperidin also has the potential to inhibit tumor cell metastasis, angiogenesis, and chemoresistance (Aggarwal, *et al.*, 2020). Although previous studies have revealed the potential of citrus flavonoids as anticancer, no studies have reported their suppressive role in liver cancer targeted at GGPS1. Geranylgeranyl diphosphate (GGPS1) also known as GGDPS is a 20-carbon isoprenoid phospholipid whose lipid moiety can be incorporated post-translationally into proteins to enhance membrane association. The geranylgeranylation process has been implicated in the antiproliferative effect of clinical agents that inhibit me-

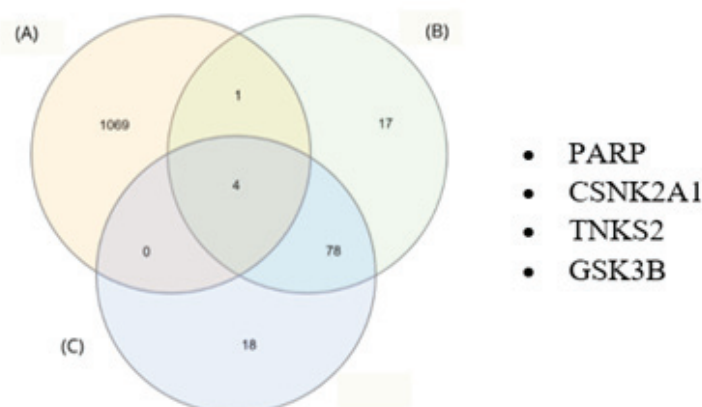


Figure 5. Venn diagram of predictive target proteins of citrus flavonoids and GGPS1-correlated genes in liver cancer. (A) Genes that are positively correlated to GGPS1. (B) Targets of tangeretin. (C) Targets of nobiletin. The four-intersection-genes are shown on the right panel.

valonate pathway enzymes (*i.e.* statins and nitrogen bisphosphonates) (Agabiti, *et al.*, 2016). In addition, GGPS1 showed an important relationship between soprenoids and cell survival and proliferation. Up-regulation and/or dysregulation of isoprenoid biosynthetic pathway enzymes including GGPS1 has been indicated in oncogenic development (Pandya, *et al.*, 2015). GGPS1 is an unfavorable prognosis in the development of liver cancer, especially during neoplastic progression. It was found that GGPS1 protein expression was increased in tumor tissue compared to adjacent tissue in 11 of 15 patients due to the mutation. The overexpression of GGPS1 in liver cancer with its correlation with decreased patient survival rate shows the important role of GGPS1 as a potential target in the treatment of liver cancer.

A total of 627 secondary metabolites of orange peel were obtained from the database of Dr. Duke's Phytochemical and was then analyzed for its inhibitory activity against the GGPS1 protein. In previous studies it was known that citrus flavonoid compounds such as tangeretin, naringenin, nobiletin, and hesperidin, have chemopreventive activity with various mechanisms such as inducing apoptosis, inhibiting cell proliferation, anti-metastasis, and inducing cell cycle arrest (Banjerdpongchai, *et al.*, 2016; Aggarwal, *et al.*, 2016; Aggarwal,

et al., 2020; Aroui, *et al.*, 2016; Ming, *et al.*, 2018; Chen, *et al.*, 2014; Arivazhagan and Sorimuthu, 2014).

Based on the KNIME machine learning prediction model using the Random forest algorithm, it was found that citrus flavonoids, namely tangeretin, nobiletin, and naringenin were predicted to have inhibitory activity on GGPS1 based on the fingerprint inhibitor pattern of GGPS1 against GGPS1. In addition, the results of the molecular docking test to determine the molecular interactions that occur indicate that hesperidin, which was confirmed to be contained in the hydrocavitation extract of orange peel by thin layer chromatography test has a strong bond with GGPS1 when compared to substrates and inhibitors of GGPS1. Not only that, the test results showed that tangeretin had the strongest bond with GGPS1 followed by nobiletin, hesperidin, and naringenin. The lower the docking score, the more potent its binding affinity to the ligand (Hermawan, *et al.*, 2021), implying that GGPS1 tend to bend and interact with tangeretin. Tangeretin and nobiletin have similar docking scores due to their similarity in the chemical structure which differ only in 1 OCH3 group. Many studies have shown that polymethoxylated flavonoids such as tangeretin and nobiletin are more potent inhibitors of tumor cell growth than free hydroxylated flavonoids

(Dong, *et al.*, 2014). The higher docking score of tangeretin on GGPS1 indicated lower binding affinity compared with native ligands. All of the result indicates citrus flavonoid compounds have the potential to be developed as chemopreventive agents targeted in GGPS1 inhibition. A total of 4 genes were obtained from the slices of 1074 genes that were positively correlated with GGPS1 in LIHC: PARP1, CSNK2A1, TNKS2, and GSK3B (Figure 5). These four genes can then be used as targets for the development of chemopreventive agents from citrus flavonoids, especially tangeretin and nobiletin in liver cancer. The following research can be used as the basis for the development of further research that focuses on the chemopreventive activity of citrus flavonoid compounds targeted at GGPS1 both *in vitro* and *in vivo* tests, as well as research on formulations to increase the stability and bioavailability of citrus peel extract as a chemopreventive agent targeting GGPS1 for liver cancer.

CONCLUSION

The water-based extraction with hydro-cavitation method was successfully yielded citrus peel extract with high flavonoid contents, including hesperidin. Bioinformatically, citrus flavonoid contents showed a good inhibitory activity to GGPS1 which its high expression leads to low survival rate in liver cancer. In addition to direct molecular interaction, citrus flavonoids also targeted genes that are positively correlated to GGPS1. Overall, our findings supported the potential of citrus peel extract for further development as a co-chemotherapeutic agent targeting GGPS1 for liver cancer.

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REFERENCES

- Agabiti, S.S., Liang, Y., and Wiemer, A.J., 2016, Molecular mechanisms linking geranylgeranyl diphosphate synthase to cell survival and proliferation, *Molecular Membrane Biology*, **33**(1-2), 1-11.
- Aggarwal, V., Tuli, H.S., Thakral, F., Singhal, P., Aggarwal, D., Srivastava, S., *et al.*, 2020, Molecular mechanisms of action of hesperidin in cancer: Recent trends and advancements, *Experimental Biology and Medicine*, **245**(5), 486-497.
- Arivazhagan, L., and Pillai, S., 2014, Tangeretin, a citrus pentamethoxyflavone, exerts cytostatic effect via p53/p21 up-regulation and suppresses metastasis in 7,12-dimethylbenz(α) anthracene-induced rat mammary carcinoma, *The Journal of Nutritional Biochemistry*, **25**(11), 1140-1153.
- Aroui, S., Najlaoui, F., Chtourou, Y., Meunier, A.-C., Laajimi, A., Kenani, A., and Fetoui, H., 2016, Naringin inhibits the invasion and migration of human glioblastoma cell via downregulation of MMP-2 and MMP-9 expression and inactivation of p38 signaling pathway, *Tumor Biology*, **37**, 3831-3839.
- Banjerdpongchai, R., Wudtiwai, B., and Khaw-on, P., 2016, Hesperidin from Citrus seed induces human hepatocellular carcinoma HepG2 cell apoptosis via both mitochondrial and death receptor pathways, *Tumor Biology*, **37**, 227-237.
- Barreca D., Gattuso G., Bellocco E., Calderaro A., Trombetta D., *et al.*, 2017, Flavanones: Citrus phytochemical with health-promoting properties, *BioFactors*, **43**(4), 495-506.

- Dong, Y., Cao, A., Shi, J., Yin, P., Wang, L., Ji, G., Xie, J., and Wu, D., 2014, Tangeretin, a citrus polymethoxyflavonoid, induces apoptosis of human gastric cancer AGS cells through extrinsic and intrinsic signaling pathways, *Oncology Reports*, **31**(4), 1788-1794.
- Fast, D.J., Stern, N.P., Chuang, J., Li, Y., Scholten, J.D., and Hu, C., 2019, Flavanones common to citrus fruits activate the interferon-stimulated response element by stimulating expression of IRF7, *Journal of Food Bioactives*, **8**.
- Ferreira, S.S., Silva, A.M., and Nunes, F.M., 2018, Citrus *reticulata* Blanco peels as a source of antioxidant and anti-proliferative phenolic compounds, *Industrial Crops and Products*, **111**, 141-148.
- Hermawan, A., Putri, H., and Utomo, R.Y. 2021, Exploration of targets and molecular mechanisms of cinnamaldehyde in overcoming fulvestrant-resistant breast cancer: a bioinformatics study, *Network Modeling Analysis in Health Informatics and Bioinformatics*, **10**(1), 1-14.
- Honda, Y., Takahashi, S., Zhang, Y., Ono, A., Murakami, E., Shi, N., et al., 2015, The effects of bisphosphonate zoledronic acid in hepatocellular carcinoma, depending on mevalonate pathway: Anticancer effects of zoledronic acid, *Journal of Gastroenterology and Hepatology*, **30**, 619-627.
- Ikawati, M., Armandari, I., Khumaira, A., and Ertanto, Y., 2019, Effect of peel extract from *Citrus reticulata* and hesperidin, a citrus flavonoid, on macrophage cell line, *Indonesian Journal of Pharmacy*, **30**(4), 260-268.
- Koolaji, N., Shammugasamy, B., Schindeler, A., Dong, Q., Dehghani, F., and Valtchev, P., 2020, Citrus peel flavonoids as potential cancer prevention agents, *Current Developments in Nutrition*, **4**(5), nzaa025.
- Lafaro, K.J., Demirjian, A.N., and Pawlik, T.M., 2015, Epidemiology of Hepatocellular Carcinoma, *Surgical Oncology Clinics of North America*, **24**(1), 1-17.
- Meiyanto, E., Hermawan, A., and Anindyajati, A., 2012, Natural Products for Cancer-Targeted Therapy: Citrus Flavonoids as Potent Chemopreventive Agents, *Asian Pacific Journal of Cancer Prevention*, **13**(2), 427-436.
- Meneguzzo, F., Ciriminna, R., Zabini, F., and Pagliaro, M., 2020, Review of Evidence Available on Hesperidin-Rich Products as Potential Tools against COVID-19 and Hydrodynamic Cavitation-Based Extraction as a Method of Increasing Their Production, *Processes*, **8**(5), 2227-9717.
- Ming, H., Chuang, Q., Jiashi, W., Bin, L., Guangbin, W., and Xianglu, J., 2018, Naringin targets Zeb1 to suppress osteosarcoma cell proliferation and metastasis, *Aging*, **10**(12), 4141-4151.
- Nguyen, A.L., Cao, H., and Thinn, M.M., 2018, Metastatic Hepatocellular Carcinoma Treated with Zoledronic Acid, *Journal of Oncology Practice*, **24**(1), 52-54.
- Pandya, A.A., Mullen, P.J., Goard, C.A., Ericson, E., Sharma, P., Kalkat, M., et al., 2015, Genome-wide RNAi analysis reveals that simultaneous inhibition of specific mevalonate pathway genes potentiates tumor cell death, *Oncotarget*, **6**, 26909-26921.
- Parhiz, H., Roohbakhsh, A., Soltani, F., Rezaee, R., and Iranshahi, M., 2014, Antioxidant and Anti-Inflammatory Properties of the Citrus Flavonoids Hesperidin and Hesperetin: An Updated Review of their Molecular Mechanisms and Experimental Models, *Phytotherapy Research*, **29**(3), 323-331.
- Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., and Bray, F., 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, *CA: A Cancer Journal for Clinicians*, **71**, 209-249.
- Yu, D., Liu, J., Chen, J., Shao, J., Shen, X., Xia, H., et al., 2014, GGPPS1 predicts the biological character of hepatocellular carcinoma in patients with cirrhosis, *BMC Cancer*, **14**, 248.