Master's Thesis

Characterizations of a novel glucosyltransferase and pro-angiogenic components in *Citrus.*

Myung-Seung, KIM

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

February, 2017

감귤에서의 새로운 당전이효소와

혈관형성촉진 물질의 특성분석

지도교수 김 재 훈

김 명 승

이 논문을 이학 석사학위 논문으로 제출함

2016 년 12 월

김명승의 이학 석사학위 논문을 인준함

심사위원장   류 기 중   ㊞

위   원 운노타쯔야   ㊞

위   원 김 재 훈   ㊞

제주대학교 대학원

2016 년 12 월

Characterizations of a novel glucosyltransferase and pro-angiogenic components in *Citrus.*

Myung Seung, KIM

(Supervised by Professor Jae Hoon Kim)

A thesis submitted in partial fulfillment of the requirement

For the degree of Master of Science

December, 2016

This thesis has been examined and approved.

Chairperson of the supervising committee

Professor Key-Zung Riu, Ph.D., College of Applied Life Sciences, Jeju National University

---

Professor Tatsya Unno, Ph.D., College of Applied Life Sciences, Jeju National University

---

Professor Jae Hoon Kim, Ph.D., College of Applied Life Sciences, Jeju National University

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

PART 1

Characterization of a novel glucosyltransferase from

Citrus platymamma Hort. ex Tanaka

CONTENTS

LIST OF FIGURES

ABSTRACT

A flavonoid glucosyltransferase, bGT612, was cloned from Byungkyool (*Citrus platymamma* Hort.et Tanaka) that is a Korean native citrus species. Using a genomic sequence data, specific primers for bGT612 were designed and PCR was performed with total RNA prepared from leaves. The open reading frame of bGT612 cDNA consists of 1446 base pairs and contains PSPG (Plant Secondary Product Glucosyltransferase) box. The cloned gene was expressed in *E.coli* and gene product was analyzed by chromatography methods. Six kinds of flavonoids such as apigenin, eriodictyol, kaempferol, luteolin, naringenin, and qucertin were used to confirm the specific activity of bGT612. Gene product of bGT612 can transfer glucose to all of tested substrates. Interestingly, bGT612 shows specific activity for Kaempferol, producing kaempferol-5-O-glucoside (K-5). These results help understanding specificity of GTs for various substrates and K-5 characterization.

1. INTRODUCTION

Citrus platymamma Hort.et Tanaka(CHT) belongs to the Rutaceae family and its fruits widely used in healthy effect such as anti-cancer, anti-oxidant, anti-inflammatory [1]. And its fruits shape looks like gourd-bottle, so called by "byeong-kyul" in korea and fruits was not sweet, also it is not commercial plant. But this plant more stronger against diseases. This is why we studied this plant.

We found a glucosyltransferase(GTs) in CHT. GTs transfer glucose to various flavonoids. They were consist of PSPG motif that related with GT activity [2]. This essential enzyme has been reporting multilateral studies, for example, a potential drug target, catalyst for the vaccines, and bio-pharmaceuticals. Understanding GT function, mechanism, and structure will contribute   to increase the potential drug target [3].

Some kinds of flavonoids are ubiquitous substances in various plants. Flavonoids are phytochemical and included in polyphenols. Their various activities were anti-inflammatory, anti-microbial, anti-viral infections and modulate growth by interaction with hormone level in various plants. [4] With in vivo (oral) treatment, flavonoid glycosides demonstrated more effects on antidiabetes, antiinflammatory, antidegranulating, antistress and antiallergic activity than their flavonoid aglycone [5].

Glucosylated product was studied in various reports about common characterization [6]. An unusual product, quercetin-5-O-glucoside has a UV protection effect and fluorescent in the silkworm [7].

From previous studies, there has been increasing interest in the bio-catalytic synthesis of glycosides with potential medicinal properties using regio-specific GTs [8], [9], [10], [11]. We studied the GTs that its product is a

2

rare kaempferol-5-O-glucoside. The GTs can offer a potential solution to overcome the problems of rare chemical synthesis. Significant activity toward 5-O position of flavonols, quercetin has been observed by other enzymes [12], but not in kaempferol. Similarly, studies of regio-specific of glucosylation of kaempferol demonstrated that effects of block to UV. Therefore, the inert glucosylation activity of OH- group at C-5 could explain why 5-O-glcosylated flavnoes and flavonols are important compounds as reported by Iwashina et al [13]. We consider that this unique *Citrus platymamma* Hort.et Tanaka glucosyltransferase with a regio-specific toward the glucosylation of the 5-O position of kaempferol would be useful as a novel biocatalyst for the production of potential pharmaceuticals.

In conclusion, a novel GT that could induce the formation of K-5 glucosylation was found and characterized. We investigated the regio-specific glucosyltransferase and its product functional effects. Further study need to obtain information about the role of 5-O-glucosylated flavonol effects.

2. MATERIALS AND METHODS

2.1 Cloning and expression of bGT 612 at protein level

Total RNA was extracted from leaves of *Citrus platymamma*, Hort. ex. Tanaka using Trizol reagent method (ThermoFisherScientific). The cDNA was amplified using ReverseTranscription-PCR kit (Takara). We used following primers to PCR, Forward primer : GCGC G GATCC ATGGCATCCGAAGCCAGCAGGTT, Reverse pimer : CGCCGCCTCGAGTTAAATCCCT TCTGA G CTTGGC. PCR condition began an initial denaturation at 94 ℃ for 2min, and followed by 34 cycles that is denaturation at 94 ℃ for 30s, annealing at 55 ℃ for 30s, and extention at 72 ℃ for 1min 30s with final 10 min extension at 72 ℃. PCR product was digested with BamHⅠ and XhoⅠ and then ligated into pGEX4T-1 vector, finally cloned plasmid DNA. Transformed *E.coli* BL21 (DE3) cells were pre-cultured at 37 ℃ over night in Luria-Bertani broth medium volume was 5 ml containing ampicillin concentration was 100 μg/ml. These cells sub-cultured at LB medium volume was 20ml at 37℃ until the cell density reached to absorbance at 600 nm optical density was 0.6 ~ 0.7, and then IPTG (isopropyl-1-thio-β-D-galactopyranoside) was added at 0.1 mM, followed by growing for additional 20 hours at 18 ℃.

2.2 Purification of bGT612

bGT612 protein cultured in 1L and induced in same condition, previously. GTs induced glucosyltransferase in *E.coli* cells were harvested by

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

centrifugation. And then added lysis buffer to harvested cell. Sonicated to cell. We purified the protein by GST affinity column.

2.3 Alignment of glucosyltransferase

glucosyltransferase alignment was determined using a BLASTW solution (NCBI, Mannheim, USA). We selected three things with high homology, used BioEdit software for alignment.

2.4 Phylogenetic tree

bGT612 amino acid sequence and GT amino acid sequence of arabidopsis database (http://www.p450.kvl.dk/UGT.shtml) were used for phylogenetic tree analysis. Amino acids were aligned using the Multiful sequence alignment by CLUSTALW. Phylogenetic tree was constructed using MEGA7 software.

2.5 Enzyme assay that using bio-transformation

To analysis enzyme activity, glucosyltransferase induced in *E.coli* cells were harvested by centrifugation, then added the reacting mixture (300 μl) consist of 50 mM potassium phosphate (pH 7.0), 1% glucose and 50μM flavonoid (sigma aldrich) to cells. It was incubated for 24 hr at 28 ℃ and extraction using Ethyl acetate. Ethyl acetate was evaporated and then reaction product was dissolved in methanol of 300 μl for HPLC sample.

2.6. Purified bGT612 protein activity

Purifiied protein activation condition was incubated with 10 μg purified enzyme, 5 mM MgCl$_2$, 5 mM UDP-glucose, 50 μM substrate, 50 mM potassium phosphate buffer for 3 hours then extracted by ethylacetate, finally dissolved at methanol.

2.7 HPLC analysis

GT activity product were analyzed with a Shimadzu HPLC e2695 Alliance separations module (Shimadzu, Milford, MA) and photo didode array (PDA) detector. YMC C18 column (5.05 μm particle size, 250x4.6 mm) was used for all analysis. HPLC grade Acetonitrile and Water (Fisher Scientific, Fair Lawn, NJ) were used as a mobile phases. The products were separated with a gradient elution codition : 20 % acetonitrile at initial condition, 40 % acetonitrile at 20 min, 70 % acetonitrile at 25 min, 20% acetonitrile during run time, 30 min.

2.8 Production of kaempferol-5-O-glucoside

Production of kaempferol-5-O-glucoside condition was cultured in LB broth volume was 200ml, induction condition (O/D value 0.6 to 0.8, IPTG concentration 0.1 mM, 18℃, 20 hr). glucosyltransferase induced in *E.coli* were harvested by centrifugation, then added the reacting mixture 40 ml consist of 50 mM potassium phosphate (pH 7.0), 1% glucose and 50 μM flavonoid to cells. It was incubated for 24 hr at 28 ℃ and extraction using Ethyl acetate, dissolved in methanol.

2.9 Preparative-HPLC analysis

Kaempferol-5-O-glucoside(K-5) was isolated by prep-HPLC in kaempferol product. K-5 were isolated by a Shimadzu HPLC e2695 Alliance separations module (Shimadzu, Milford, MA) and photo didode array (PDA) detector. YMC C18 column (5 um particle size, 250 x 10 mm) was used for analysis. HPLC grade Acetonitrile and Water (Fisher Scientific, Fair Lawn, NJ) were used as a mobile phases. The products were separated with a same HPLC gradient elution conditon, previously.

2.10 LC-MS analysis

Samples were analyzed with a Waters UPLC(SCIEX Q-TRAP 5500). Q1 MS scan, Product ion scan, MRM scan, Hypersil GOLD column(100 x 2.1 mm, 1.9 μm, Thermo) was used for analysis. Injection amount was 10ng, injection volume was 1ul and flow rate : 0.3 μl/min. 0.1 % FA in water and 0.1 % FA in Acetonitrile were used as a mobile phases. The products were separated with a gradient elution : 95 % water at 0 min, 40 % water at 20 min, end run time.

2.11 Measurement of Fluorescent

Fluorescence spectra were recorded using a multi-microreader machine(spectramax i3, USA). We dissolved in dimethyl sulfoxide(DMSO) each compound at 1 mM, that is kaempferol-3-O-glucoside, kaempferol-4'-O-glucoside, kaempferol-5-O-glucoside, kaempferol-7-O-glucoside. Transfer the sample to the 96 well plate, then measured by multi-microreader machine(spectramax i3, USA)

3. RESULTS

3.1 Phylogenetic tree and Alignment

Phylogenetic tree showed that bGT612 belonged to group D. bGT612 has a 97 % homology with *Citrus Sinensis*, 68% homology in *Theobroma cacao* and 61 % homology in *Astragalus membranaceus*. The GTs were consists of 44 aminoacid sequence, PSPG motif for glucosylation with various substrates. Group D from Arabidopsis that UGT73B subfamily was known to formation of glucosidic metabolite with an important fuction in plant defense and induced by abiotic stress. For example UV light, wounding, oxidative stress[14].
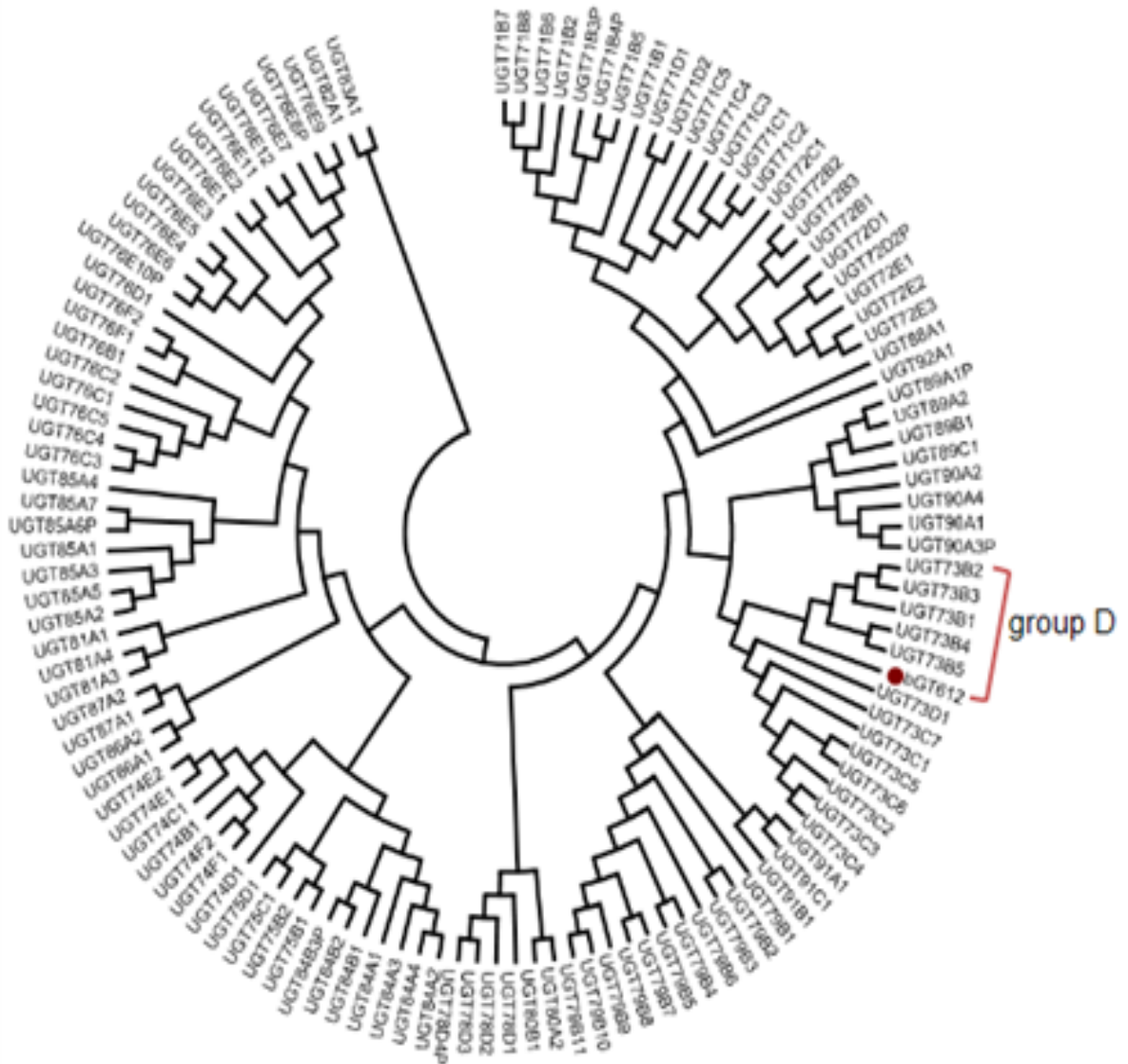
Figure 1. Phylogenetic analysis of bGT612 and arabidopsis. GTs database Phylogenetic tree based on the 122 amino acid sequences from arabidopsis GTs databse. Evolutionary analyses were conducted in MEGA7.
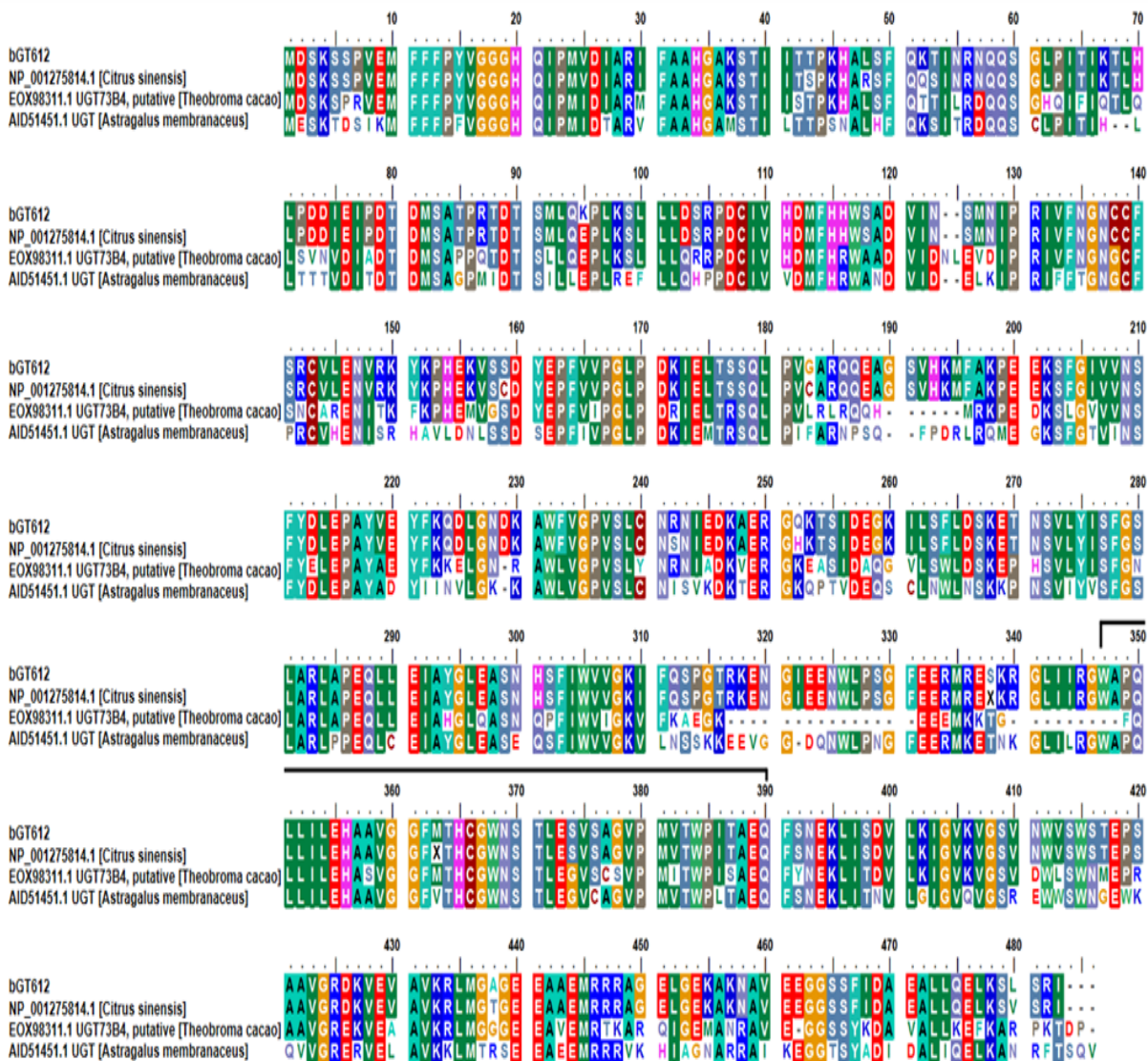
Figure 2. Alignment of bGT612 aminoacid sequence. Alignment operated by BLAST with 3 different genes that is *Citrus sinesis*, *Theobroma cacao* and *Astragalus membranaceus* each has a each 97 %, 68 %, 61 % homology. Black line indicated PSPX box sequence.

3.2 Cloning and gene expression at protein level

Glucosyltransferase gene was gained from genomic sequence of *Citrus platymamma* Hort.ex. Tanaka. A part of glucosyltransferase were identified from *Citrus platymamma* hort. Ex Tanaka. These genes recombinanted to *E.coli*, then investigated enzyme activity through bio-transformation[15]. bGT612 showed another glucosyltransferase activity with kaempferol. bGT612 (1446 bp) contains an open reading frame (ORF) that encodes a protein with 481 amino acid. bGT612 gene was expressed using glutathione-S-transferase fusion protein system. bGT612 was induced soluble protein, then we purified protein. The molecular weight of the recombinant bGT612 was approximately 79.3 kDa with a fused GST tag (26.3 kDa). Purified protein one band (GST-fused bGT612) was detected by SDS-PAGE (Fig 3.).
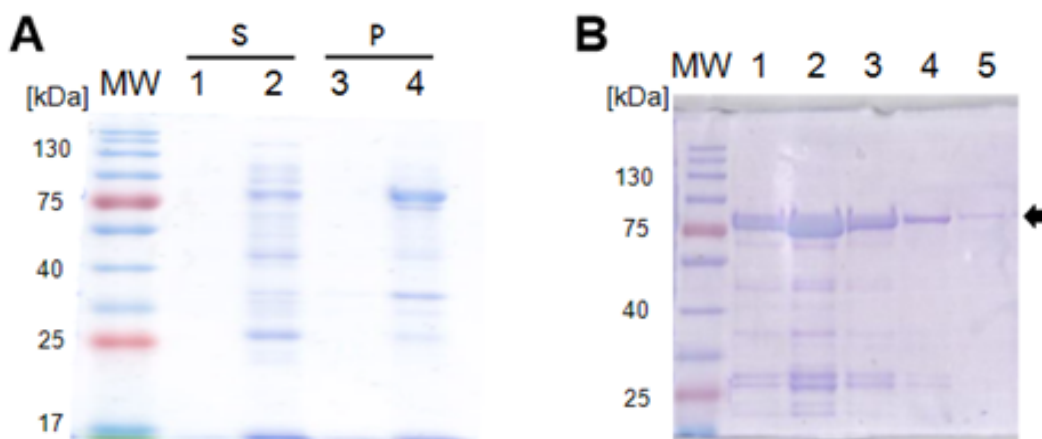

Figure 3. SDS-PAGE of recombinant protein expression and purified protein..Figure 3A, 1 = uninduced superantant; 2 = induced supernatant, 3 = uninduced pellet, 4 = induced pellet. Figure 3B, 1 = purified protein 1, 2 = purified protein 2, 3 = purified protein. 4 = purified protein, 5 = purified protein.   .

3.3 Characterization of glucosyltransferase

We examined the enzyme assay for glucosyltransferase activity to using purified protein and bio-transformation with apigenin, eriodictyol, kaempferol, luteolin, naringenin, quercetin. We detected standard flavonoids in each HPLC figure A, B and we compared with products peak, as a result apigenin converted to 7-O-glucoside, eriodictyol converted to glucoside, kaempferol converted to 5-O-glucoside and 7-O-glucside, luteolin converted to 4'-O-glucoside, naringenin didn't converted and quercetin converted to 4'-O-glucoside. And same results from purified protein activity.
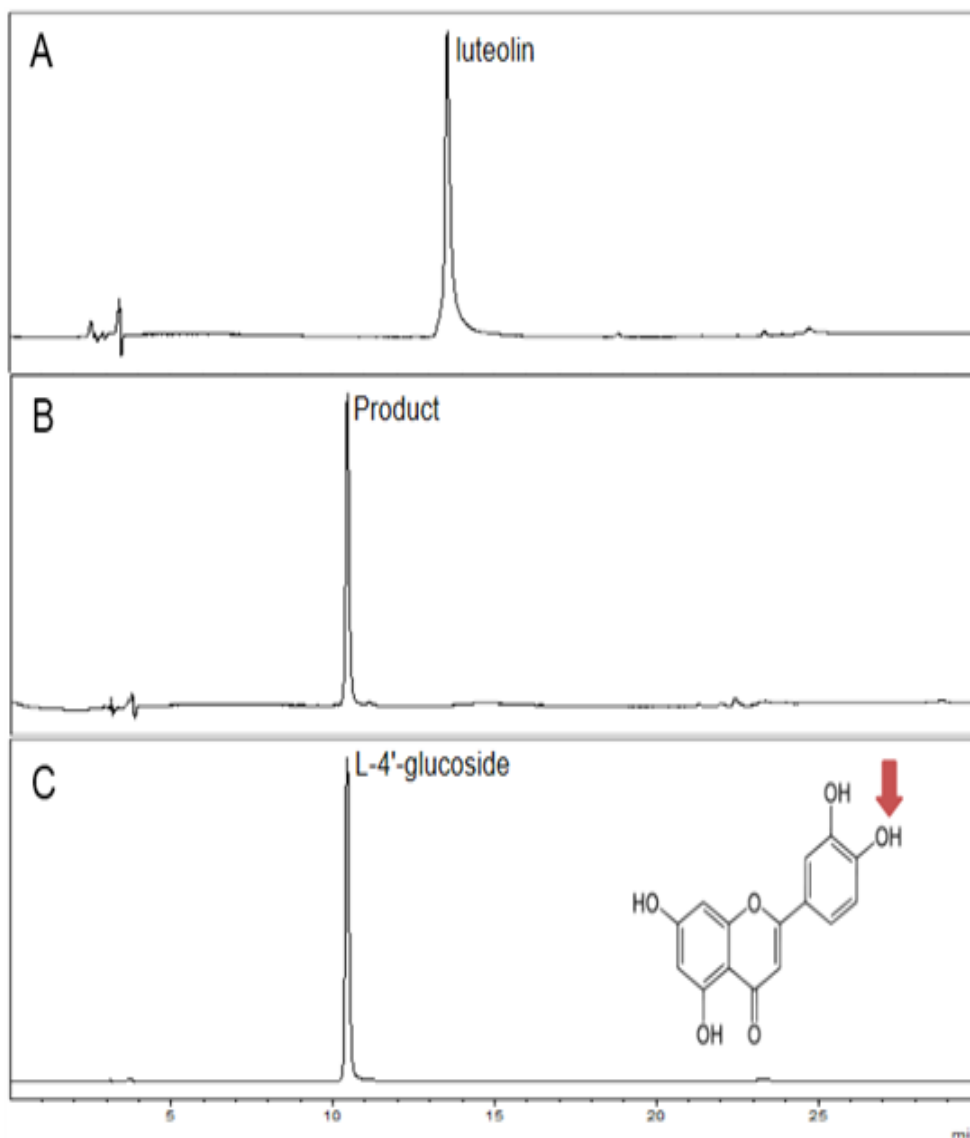
Figure 4. HPLC analysis of purified bGT612 protein reaction with luteolin. Fig 4A was standard analysis, luteolin, Fig 4B was product analysis, Fig 4C was standard analysis, luteolin-4'-glucoside.
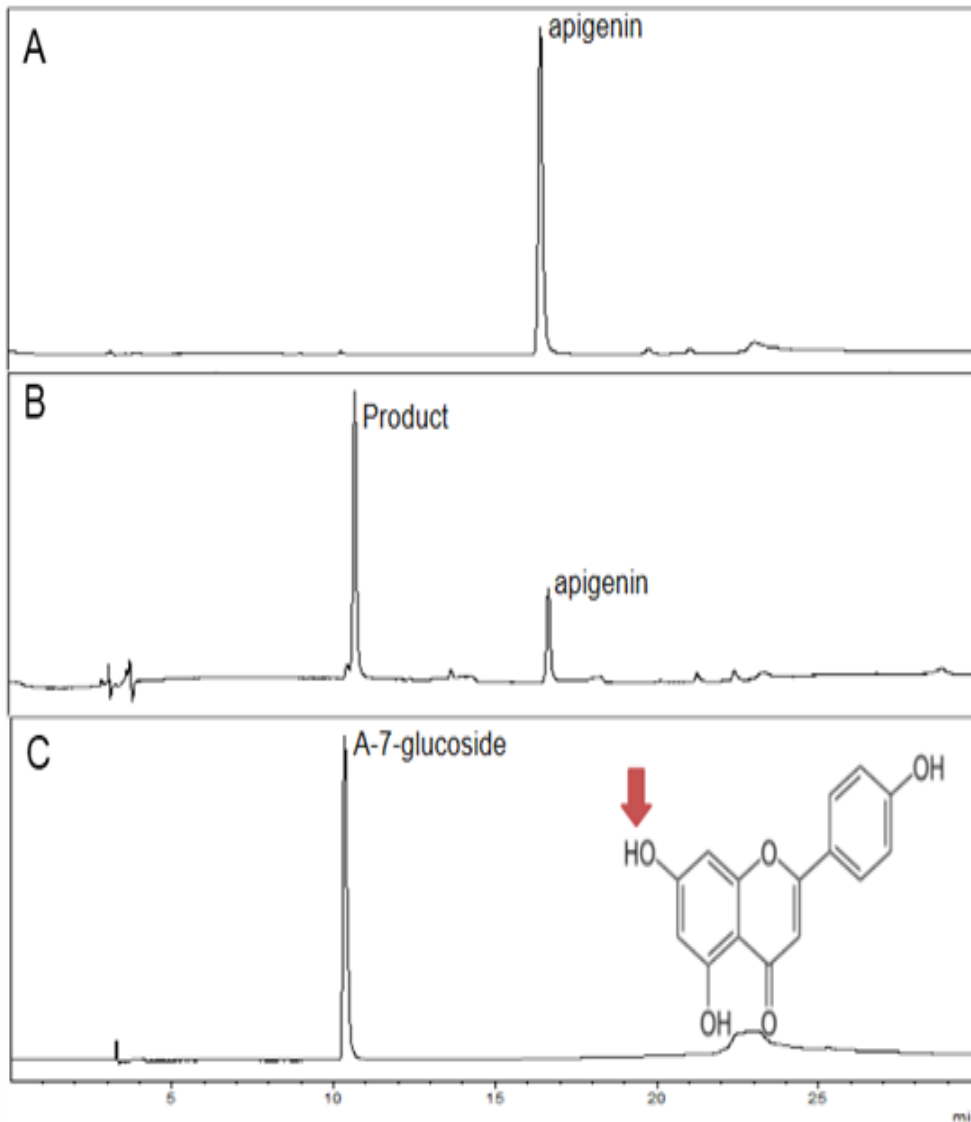
Figure 5. HPLC analysis of purified bGT612 protein reaction with apigenin. Fig 5A was standard analysis, apigenin, Fig 5B was product analysis, Fig 5C was standard analysis, apigeinin-7-glucoside.
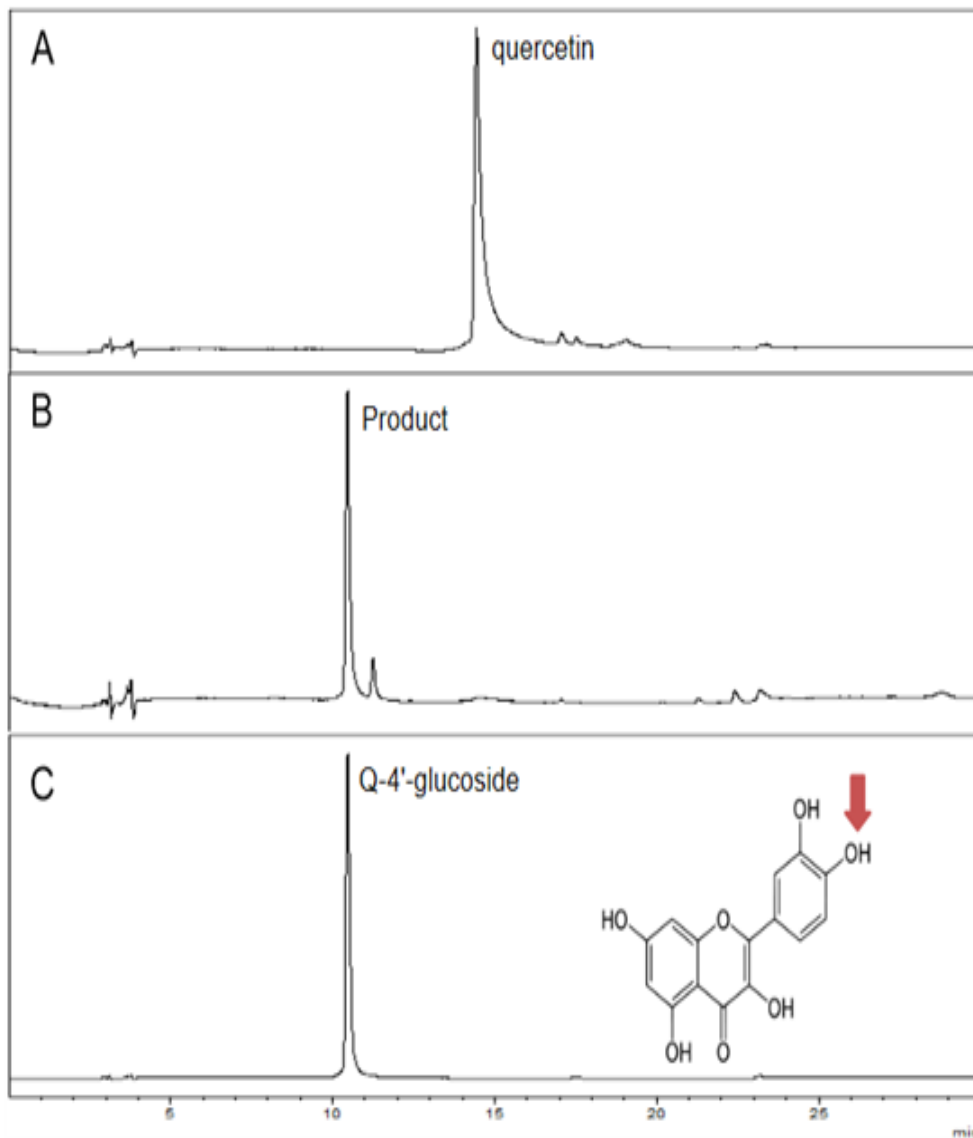
Figure 6. HPLC analysis of purified bGT612 protein reaction with quercetin.
Fig 6A was standard analysis, quercetin, Fig 6B was product analysis, Fig 6C
was standard analysis, quercetin-4'-glucoside.
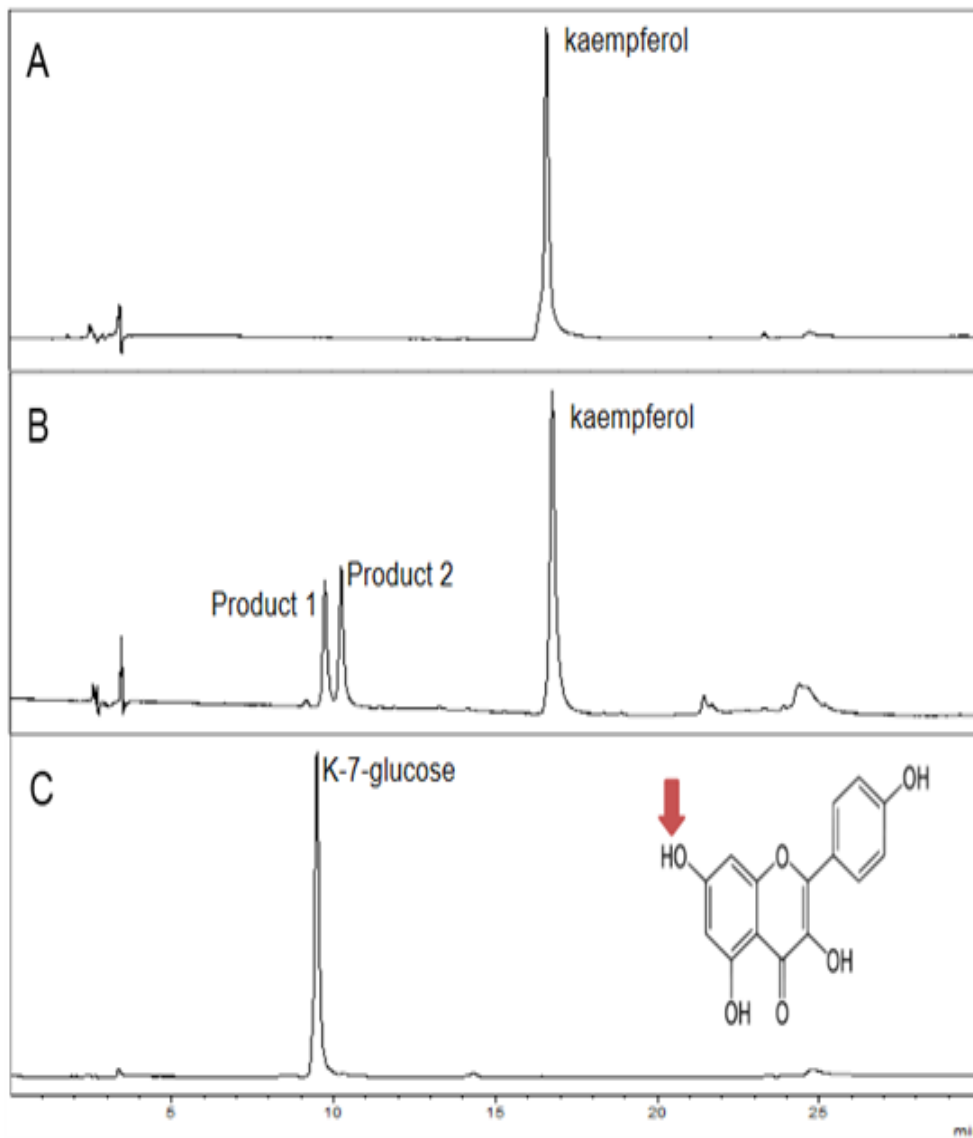
Figure 7. HPLC analysis of purified bGT612 protein reaction with kaempferol. Fig 6A was standard analysis, kaempferol, Fig 7B was product analysis, Fig 7C was standard analysis, kaempferol-7-glucoside.
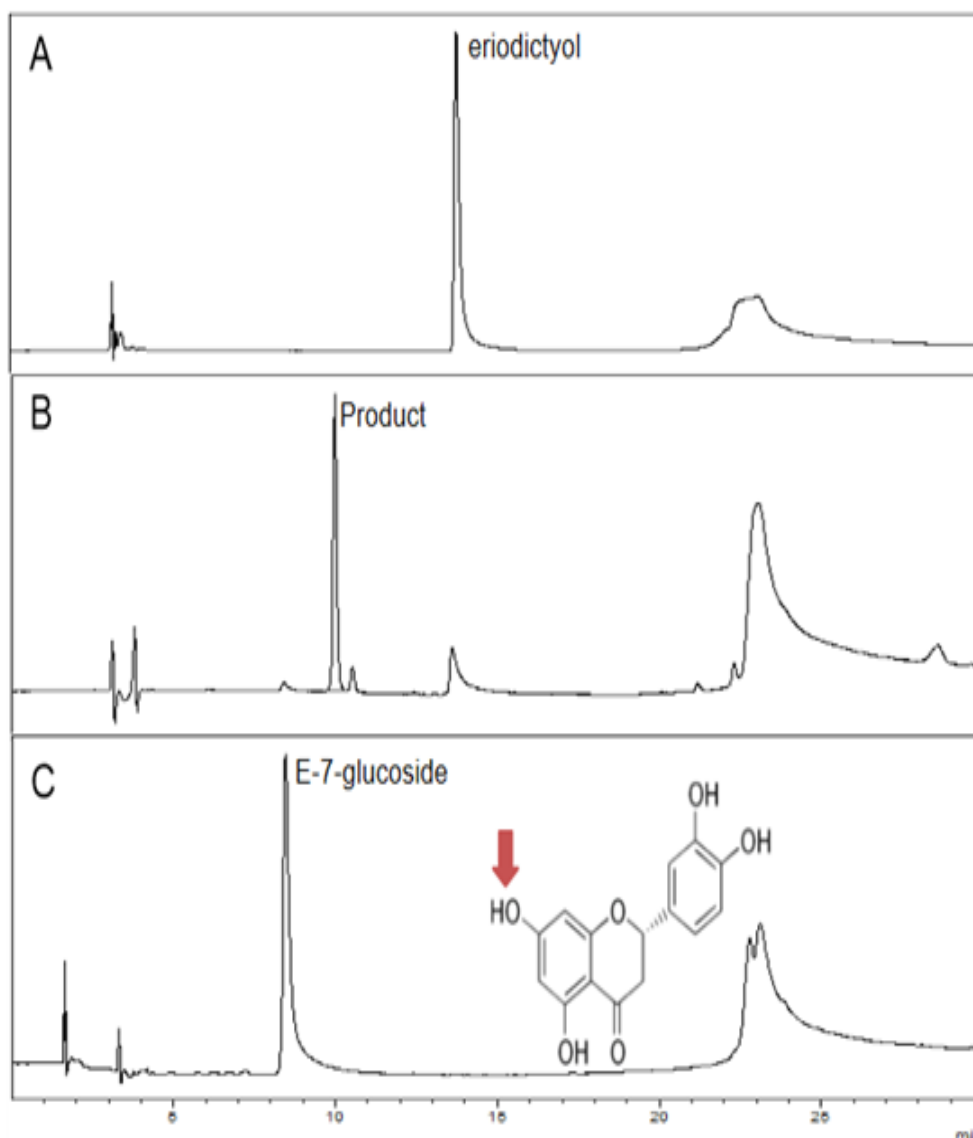
Figure 8. HPLC analysis of purified bGT612 protein reaction with eriodictyol. Fig 8A was standard analysis, eriodictyol, Fig 8B was product analysis, Fig 8C was standard analysis, eriodictyol-7-glucoside.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY
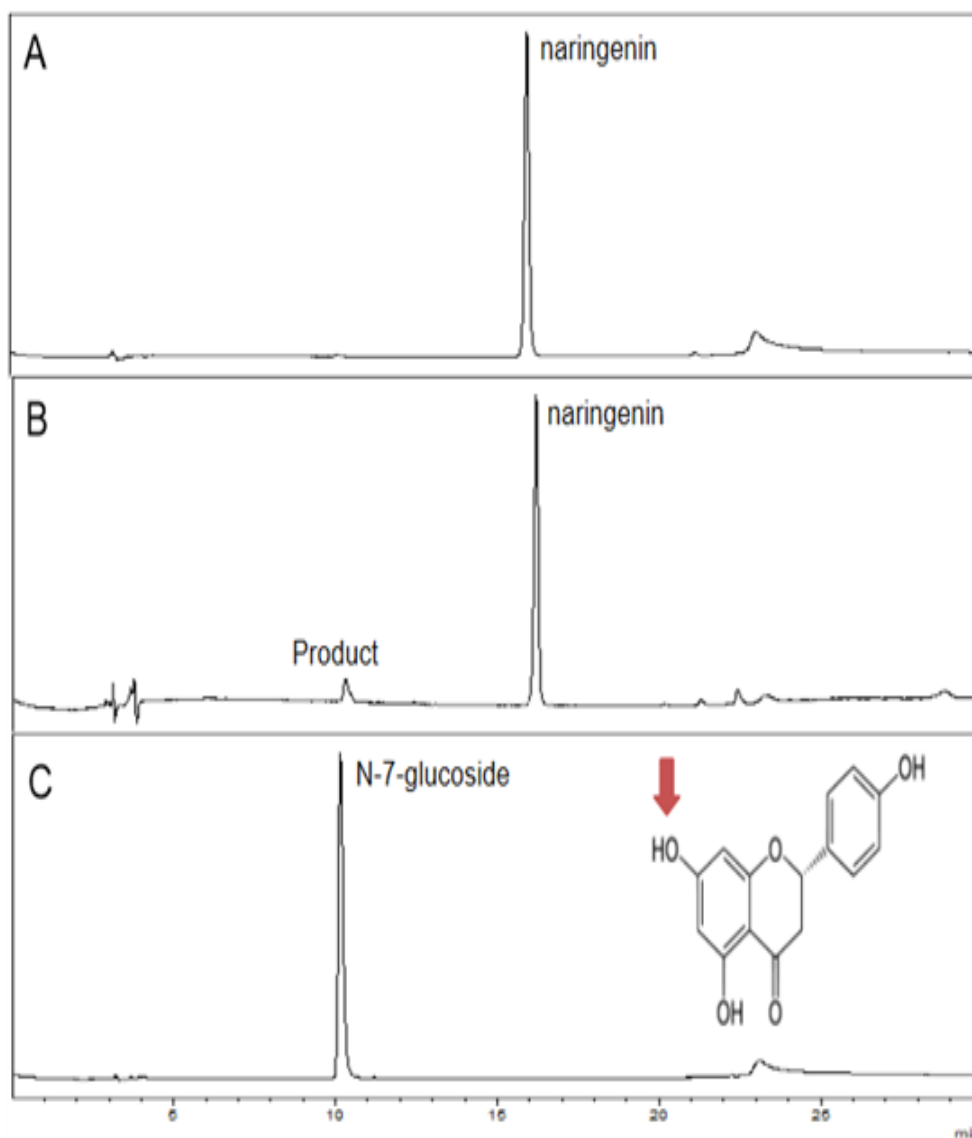
Figure 9. HPLC analysis of purified bGT612 protein reaction with naringenin. Fig 9A was standard analysis, naringenin, Fig 9B was product analysis, Fig 9C was standard analysis, naringenin-7-glucoside.

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

3.4 Preparative-HPLC analysis

We separated to unknown peak by prep-HPLC(Fig 10). Isolated peak was confirmed by HPLC with retention time.
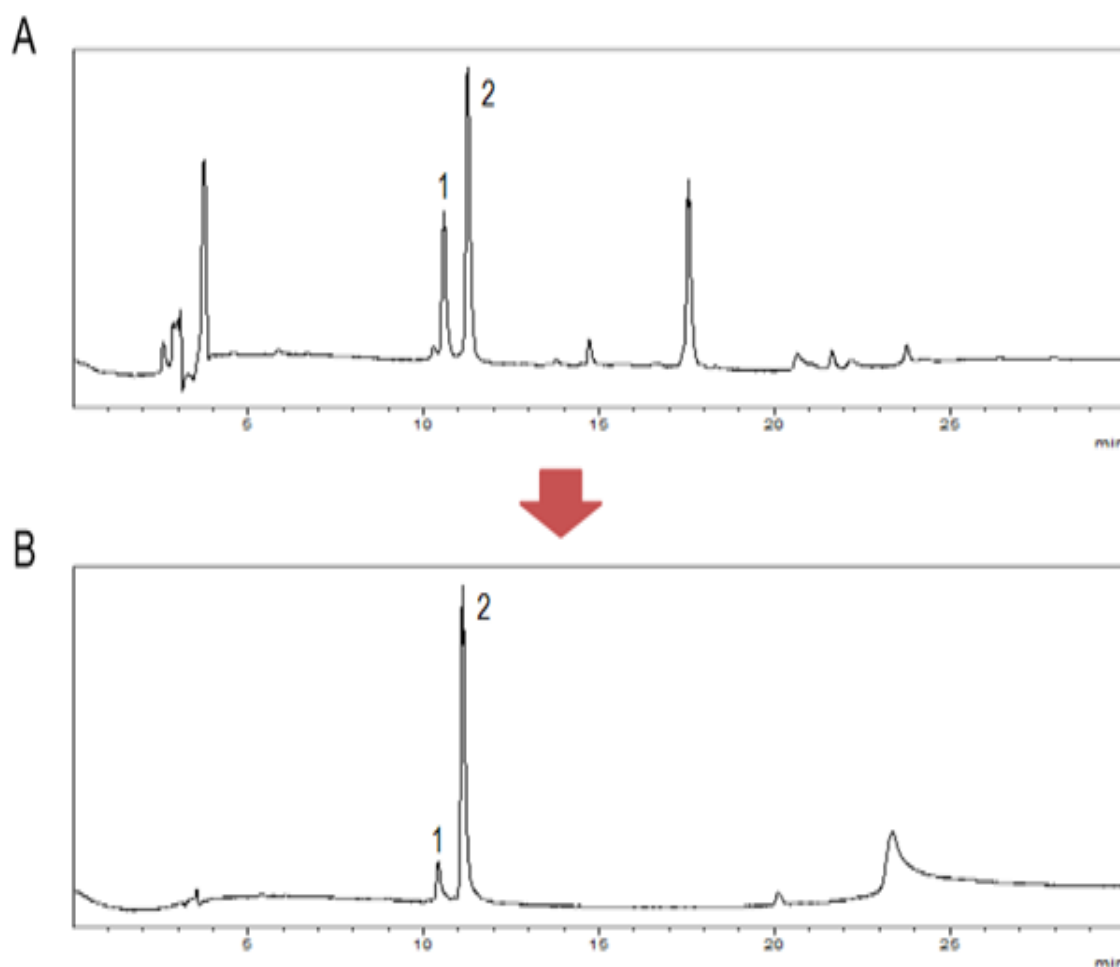
Figure 10. Purified product that bGT612 reaction with kaempferol. Fig 10A, before prep-HPLC of product.1 = kaempferol-7-O-glucoside, 2 = unknown product. Fig 10B, HPLC of isolated 2 = unknown product.

3.5 LC-MS analysis

We compared with other kaempferol aglycone retention time, we suggested that unknown peak was kaempferol-5-O-glucoside. Fig 11A shown kaempferol-3-O-glucoside, kaempferol-4'-O-glucoside, kaempferol-7-O-glucoside, kaempferol-5-O-glucoside in order of number. As a result, Fig 11B shown isolated kaempferol-5-O-glucoside peak that indicated by 4.
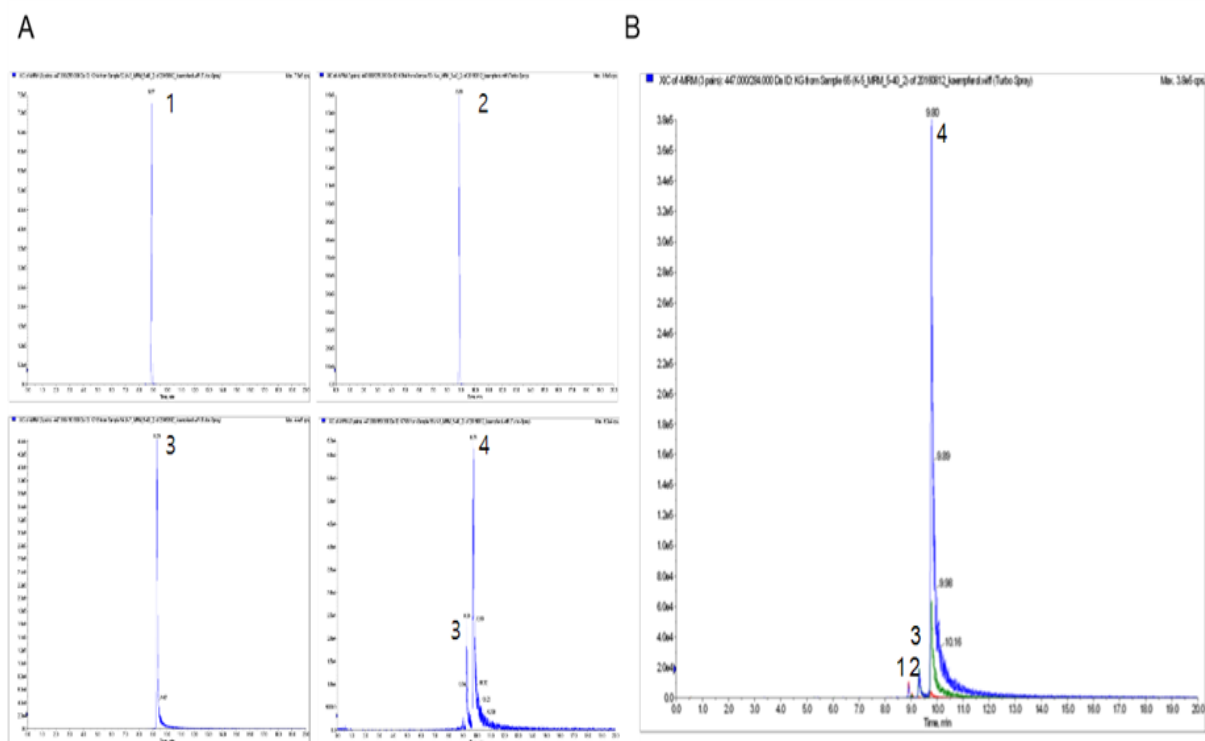
Figure 11. LC-MS analysis of purified product that bGT612 reaction with kaempferol. Fig 11A was standard analysis, 1 = kaemferol-3-O-glucoside, 2 = kaemferol-4'-O-glucoside, 3 = kaemferol-7-O-glucoside, 3 and 4 = product, bGT612 reaction with kaempferol. Fig 11B was analysis of product that bGT612 reaction with kaempferol.

3.6 Fluorescent of Kaempferol-5-O-glucoside

When compare with other kaempferol glycoside, Kaempferol-5-O-glucoside had a strong fluorescent effect at every excitation length (250 to 390 nm) and emission length. (350 to 750 nm) in Fig 12, Kaempferol-5-O-glucoside has a high absorbance at excitation 390 nm wave length and at emission 350 nm wave length. Fluorescent in various wave length of excitation (250, 270, 290, 310, 330, 350, 370 and 390 nm) and emission (350, 370, 390, 410, 430, 450, 470, 490, 510, 530, 550, 570, 590, 610, 630, 650, 670, 690 nm).
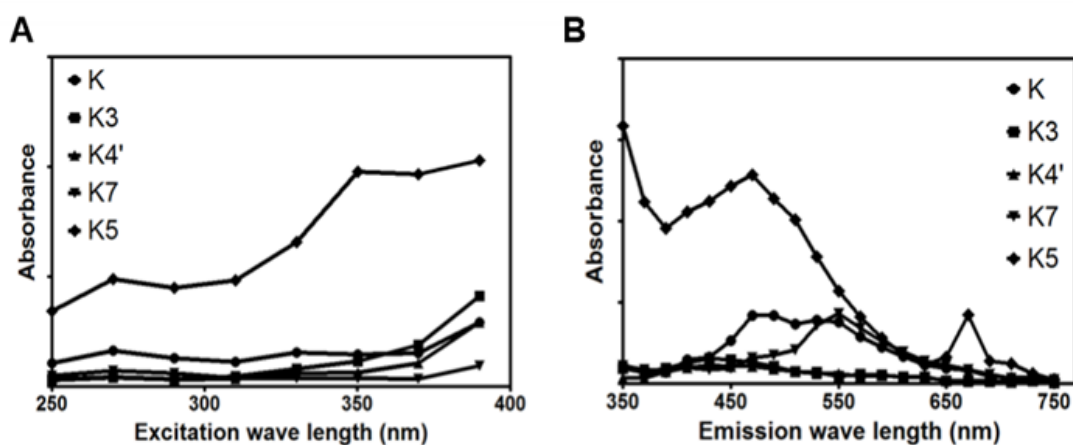
Figure 12. The absorbance spectra of kaempferol and kaempferol glucoside that dissolved in DMSO at 1 mM. Fig 12A, absorbance analytical values at emission wavelength was 490 nm and excitation wavelength were 250 to 390 nm, Fig 12B, absorbance analytical values at excitation wavelength was 280 nm and emission wavelength were 350 to 750 nm,

4. DISCUSSION

GT gene is present in many plants and its function is known. However, there was no study of substrate specificity and no study on the functional change by substrate activity. To investigate the substrate specificity of novel GT gene in citrus fruit, we analyzed the glucose transfer site using flavonoid.

In flavone activity, bGT612 glucosylated at the 4 'position of luteolin. but in apigenin which is without OH at the 3' position did not glucosylated at the 4 'position and glucosylated at the 7 position howerver, the activity toward 7-OH was low.. The presence or absence of 3'-OH in the substrate affects the specificity of the enzyme.

In flavonol activity, bGT612 glucosylated at 4 'position of quercetin but in kaempferol which is without OH at the 3' position didn't glucosylated at 4 'position and glucosylated at position 7. Also, The activity was relatively low. Unlike Flavone, kaempferol was found to be able to glucosylated at the position 5. This is can be thought that the effect of presence position 3-OH, unlike flavone.

In flavanone activity, bGT612 weakly glucosylated at 7 position of naringenin, without OH at the 3 'position, similar to activity of flavone and flavonol in the flavanone structure. In eriodictyols which is presence -OH at 3' posotion, 7 position was not glucosylated and 4' position was expected to glucosylation, as in the case of flavone and flavonol.

We didn't compare with eriodictyol glucosylated at 4' position, becasuse the substance is not sold, further study need to produce a large amount of

eriodictyol-7-glucoside and it is necessary to determine the structure using NMR. As a result, we demondstrated the specificity of the glucose transfer site and the production of K-5 and the UV shield effect.

5. CONCLUSION

bGT612 was belonged to group D that was classified by arabidopsis glucosyltransferase. bGT612 has a 97% homology with *Citrus Sinensis*, 68% homology in *Theobroma cacao* and 61% homology in *Astragalus membranaceus.* bGT612 (1446 bp) contains an open reading frame (ORF) that encodes a protein with 481 amino acids.

In flavonoid activity, when the 3'-OH was present in the flavonoid substrate, bGT612 glucosylated at 4 '. but when the of 3'-OH was absent in the flavonoids substrate, bGT612 glycosylated at 7-OH. bGT612 additionally received the effect of 3-OH of flavonol, showed activity for 5-OH as well as 7-OH in the case of kaemferol.

We found a kaempferol-5-O-glucoside from products in bGT612 reaction with kaempferol. When compare with other kaempferol glycoside, kaempferol-5-O-glucoside had a strong fluorescent effect at every excitation length (250 to 390 nm) and emission length   (350 to 750 nm).

Up to date, no gene has been reported to produce kaemferol-5-O-glucoside.   Also, we domonstrated kaempferol-5-O-glucoside has a very good ability to absorb UV, indicating that kaemferol-5-O-glucoside is highly applicable.

6. REFERENCE

1   Arulkumar Nagappan, Ho Jeong Lee, Venu Venkatarame Gowda Saralamma, Hyeon Soo Park, Gyeong Eun Hong, Silvia Yumnam, Suchismita Raha, Shobana Nancy Charles, Sung Chul Shin, Eun Hee Kim, Won Sup Lee, Gon Sup Kim: Flavonoids isolated from *Citrus platymamma* induced G2/M cell cycle arrest and apoptosis in A549 human lung cancer cell, CA: oncology letters 2016;1394-1402.

2   Ritesh Kumar, Rajender S. Sangwan, Smrati Mishra Farzana Sabir, Neelam S. Sangwan: In silico motif diversity analysis of the glycon preferentiality of plnat secondary metabolic glucosyltransferase, CA: plant omics journal 2012;200-210.

3   Aram Chang, Shanteri Singh, George N. Phillips Jr, Jon S. Thorson: Glycosyltransferase structural biology and its role in the design of catalysts for glycosylation, CA: a cancer journal for clinicians 2011;22:800-808.

4   Harald P. Hoensch, Reinhard Oertel: The value of flavonoids for the human nutrition: Short review and perspectives, CA: clinical nutrition experimental 2015:8-14.

5   Jianbo Xiao, Tingting Chen Hui Cao: Flaovnoid glycosylation and biological benefits, CA: biotechnology advances 2014;21

6   Chikara Hirayama, Hiroshi Ono, Yasumori Tamura, Kotaro Konno, Masatoshi Nakamura: Regioselective formation of quercetin 5-O-glucoside from orally administered quercetin in the silkworm, *Bombyx mori*, CA: phytochemistry 69 2008:1141-1149.

7   Takaaki Daimon, Chikara Hirayama, Masatoshi Kanai, Yoshinao Ruike, Yan Meng, Eiichi Kosegawa, Masatoshi Nakamura, Gozoh Tsujimoto, Susumu Katsuma, Toru Shimada: The silkworm *Green b* locus encodes a

quercetin 5-O-glucosyltransferase that produces green cocoons with UV-shielding properties, CA: PNAS 2010: 11471-11476

8   Kramer, C.M., Prata, R.T.N., Willits, M.G., De Luca, V., Steffens, J.C., Graser, G., 2003. Cloning and regiospecificity studies of two flavonoid glucosyltransferases from Allium cepa. Phytochemistry 64, 1069-1076.

9   Lim, E. K., 2005. Plant glycosyltransferases - their potential as novel biocatalysts. chem.-Eur. J. 11, 5486-5494.

10   Weis, M., Lim, E.K., Bruce, N., Bowles, D., 2006. Regioselective glucosylation of aromatic compounds: screening of a recombinant glycosyltransferae library to identify biocatalysts. Angew. Chem.-Int. Ed., 45, 3534-3538.

11   Willits, M.G., Giovanni, M., Prata, R.T., Kramer, C.M., De Luca, V., Steffens, J.C., Graser. G., 2004. Bio-fermentation of modified flavo-noids: an example of in vivo diversification of secondary metabolites. Phytochemistry 65, 31-41.

12   Hirayamo, C., Konno, K,. Shinbo, H., 1997. The pathway of ammonia assimilation in the silkworm, *Bombyx mori*. J. Insect Physiol. 43, 959-964.

13   Iwashina, T., Matsumoto, S., Nishida, M., Nakaike, T., 1995. New and rare flavonol glycosides from Asplenium trichomanes ramosum as stable chemotaxonomic markers. Biochem. Syst. Ecol. 23, 283-290.

14   Mar Morant, Claus Ekstrom, Peter Ulvskov, Charlotte Kristensen, Mats Rudemo, Carl Erik Olsen, Jorgen Hansen, Kirsten Jorgensen, Bodil Jorgensen, Birger Lindberg Moller, Soren Bak: Metabolomic, Transcriptional, Hormonal, and Signaling Cross-Talk in Superroot2. Molecular Plant. volume 3, number 1 2010:192-211.

15   Kim JH, Kim BG, Ko JH, Lee Y, Hur H-G, Lim Y, Ahn J-H,: Molecular cloning, expression, and characterization of a flavonoid glycosyltransferase from Arabidopsis thaliana. Plant Science 2006, 170(4):897-903.

요약문

bGT612 당전이효소는 한국 재래감귤인 병귤에서 클로닝하였다. 병귤의 genomic DNA 서열을 통해 bGT612 서열의 특이적인 프라이머를 합성하고 병귤잎에서 뽑은 total RNA 에서 PCR 수행하였다. bGT612 cDNA 의 전사해석틀은 1446 개의 염기쌍으로 이루어졌고 PSPG BOX 를 포함하였다. 복제한 유전자를 대장균에서 발현하였고 당전이효소 생성물은 크로마토그래피 방법으로 분석하였다. apigenin, eriodictyol, kaempferol, luteolin, naringenin, quercetin 의 6 가지 flavonoid 로 bGT612 의 활성을 검정하였다. bGT612 당전이효소 생성물은 5 가지 기질에서 당전달하였고 또한 특이적으로 kaempferol-5-O-glucoside 생성하였다. 이러한 결과는 다양한 기질과 kaempferol-5-O-glucoside 에 특이적인 당전이효소를 이해하는데 도움이 될 수 있다

PART 2

Effective extraction of pro-angiogenic components

in *Citrus unshiu Peels*

CONTENTS

LIST OF FIGURES

ABSTRACT

Aqueous extraction of *Citrus unshiu* peels (AECUP) is mainly comprised with pro-angiogenic hesperidin and narirutin. In this study, we report approaches to increasing the yields of extracted hesperidin and narirutin from *Citrus unshiu* peels using proper solvents. Significantly improved yields of both compounds were obtained using methanol and dimethyl sulfoxide (DMSO) compared to acetonitrile, ethyl acetate, ethanol, and isopropyl alcohol. Especially, effect of DMSO was by far the better of the two solvents in extraction of hesperidin. In addition, the DMSO extracted hesperidin significantly induced the pro-angiogenic effects of human umbilical vein endothelial cells (HUVECs) and markedly up-regulated phosphorylation of the ERK1/2 signaling pathway. These results demonstrate that pro-angiogenic inducer; hesperidin and narirutin can be simply, easily, and effectively extracted from *Citrus unshiu* peels.

1. INTRODUCTION

Satsuma mandarin (*Citrus unshiu* Marc.; number SKC. 111022) peel has been used for traditional medicine to improve the chronic diseases such as bronchial asthma and blood circulation [1]. *Citrus unshiu* peel also has been reported to relieve allergic reactions, inflammation, oxidative stress, and tumor progression. The beneficial effect of *Citrus unshiu* peel in human body is originated by various bioactive-compounds such as phenolic acids and flavonoids [2-5].

Recently, *Citrus* fruits are used as source of juice and processed foodstuffs in the food industry. However, more than half of the *Citrus* fruit weight is discarded as by-products including peel, pulp, and seeds. These by-products have been used for animal feed, fiber production, and fuel production [6-8]. *Citrus* wastes, especially *Citrus* peel ingredients, may inhibit allergic reactions, skin inflammation, oxidative stress, and tumor promotion [2-5]. Therefore, effective extraction of functional components from by-products is an economical and environmental imperative.

We have previously shown that narirutin and especially hesperidin in aqueous extracted *Citrus unshiu* peel induce pro-angiogenic effects via the activation of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK)1/2 signaling pathway in HUVECs [9]. Angiogenesis is the formation of new blood vessels from existing endothelium; this process is detrimental in various diseases including cancer, rheumatoid arthritis, and ocular disorders [10]. However, angiogenesis is beneficial in treating burns, woundhealing, stroke, cardiac disorders, and various diabetes-related diseases [11]. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are the most potent angiogenic inducers *in vitro* and *in vivo*.

2

Unfortunately, their use in clinical applications has been restricted due to their high cost and other factors [12, 13].

A variety of constituents of *Citrus* wastes can be extracted with organic solvents. However, some of these solvents are toxic to humans. Still, the high value of natural products from *Citrus* wastes has spurred the examination of the potential of food grade solvents [14].

In the present study, we report that functional components from *Citrus Unshiu* peel can be effectively extracted without specialized equipment and technique.

2. MATERIALS AND METHODS

2.1 Preparation of *Citrus Unshiu* peel extraction

Citrus unshiu peels was prepared from citrus juice processing wastes obtained from a facility on Jeju island as previously described [9]. Briefly, citrus peel waste was lyophilized and dissolved 50 mM sodium acetate buffer (pH 4.8), acetonitrile, ethanol, ethyl acetate, methanol, isopropyl alcohol, and dimethyl sulfoxide (DMSO).

2.2 Cell culture and reagents

HUVECs were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in EGM-2 Bulletkit medium (Lonza Biologics, Hopkinton, MA, USA) containing 1×10^5 unit/L Penicillin-100 mg/L Streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO_2 as previously described [15]. All experiments were performed using HUVECs within 3-7 passages. Antibodies forphospho-ERK1/2 (Thr202/204), ERK, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Beverly, MA, USA). Inhibitor of integrins (RGD-peptide) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Qualified sodium acetate, acetonitrile, ethanol, ethyl acetate, methanol, isopropyl alcohol, DMSO, and hesperidin were obtained from Sigma-Aldrich (St. Louis, MO, USA).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2.3 Measurement of cell viability

To evaluate cell viability with treatment of extracted hesperidin, WST-1 reagent (Nalgene, Rochester, NY) was used as described previously [15]. After 30 min incubation at room temperature, the absorbance was measured at 490 nm by using a microplate reader (Bio-Rad, Richmond, CA, USA).

2.4 Migration assay

Migration assay were performed using a 24-Transwell apparatus (Corning, Corning, NY, USA) according to the supplier′s protocols as previously described [16].

2.5 Tube formation

Tube formation assays were performed as previously described with some modifications [9]. In brief, 250 μL growth factor-reduced matrigel (BD Biosciences, San Diego, CA) was used to coat 24 well plates (SPL Life Sciences, Pocheon, Republic of Korea) and allowed to polymerize at 37°C for 30 min. HUVECs (3×10^4 cells/well) were suspended in 500 μL serum-free EBM medium containing different dosages of extracted hesperidin. After incubation for 16 h at 37°C, photographs of four representative fields per well were taken using phase contrast microscopy. Endothelial tubes were quantified by counting the number of junctions defined as the origin of two or more branch protrusions.

2.6 High-performance liquid chromatography (HPLC) analysis

A commercial HPLC system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a photo diode array (PDA) detector and a Luna C18(2) column (5μm particle size, 4.6mm X 250nm;Phenomenex, Torrance, CA, USA) was used using 50 mg/mL extracted samples as previously described [9]. Quantification of extracted hesperidin and narirutin was calculated using limit of detection (LOD) and limit of quantification (LOQ) values using HPLC analysis as previously described [17].

2.7 Preparative-HPLC

Preparative HPLC (prep-HPLC) was used to highly purify the hesperidin from DMSO extracted *Citrus Unshiu* peel. The prep-HPLC system (Shimadzu Scientific Instruments) consisted of a model LC-20AP pump, model SPD-M20A photodiode array detector, model SIL-10AP autosampler, and model CBM-20A system controller. Qualitative analysis was performed with step gradient mode using various ratios of acetonitrile and water (2:8, 4:6, 7:3, and 2:8) for different times at a flow-rate of 4 mL/min. The samples (50 mg) were dissolved in 1 mL of eluent and 250 ul of solution was injected. The total running time was 30 min. Detection was performed by monitoring the absorbance signals at 270 nm. The chromatographic analysis was also performed by comparing retention times of each peak with reference HPLC data. Accoding to this peak, extacted hesperidin was collected by DMSO-free condition using s supplier's manual. After freezing-dry process, hesperidin powder was dissolved in sodium acetate buffer (pH 4.8).

2.8 Western blot analysis

To evaluate the phosphorylation levels of ERK1/2 in hesperidin treated HUVECs, Western blot analysis was performed as described previously [16]. Briefly, HUVECs were stimulated with 10 μM of DMSO extracted hesperidin with different dosages of RGD-peptide. Cells were lysed in M-PER lysis buffer (Thermo Scientific, Carlsbad, CA, USA) with protease and phosphatase inhibitors to prepare the cell lysates. Antibodies specific to p-ERK (Thr202/204), ERK, and GAPDH were 1:1000 and incubated overnight at 4°C. Secondary antibodies included HRP-conjugated donkey anti-rabbit or donkey anti-mouse (Santa Cruz Biotechnology) were diluted 1:4000 and incubated for 1 h at room temperature. Bands were measured by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.9 Statistical analysis

Data are presented as the mean ± standard deviation (SD). Levels of significance for comparisons between two independent samples were determined using the Student's t-test.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

3. RESULTS AND DISCUSSIONS

We previously reported that hesperidin and narirutin in aqueous extracted *Citrus unshiu* peel have pro-angiogenic effects in HUVECs and hesperidin is the more prominent pro-angiogenic inducer [9]. To further demonstrate whether application of different solvents can improve the extraction yield of functional components from *Citrus unshiu* peel, lyophilized *Citrus unshiu* peel was dissolved with sodium acetate buffer (pH 4.8), ethanol, methanol, isopropyl alcohol, DMSO, acetonitrile, and ethyl acetate. We first performed HPLC analysis using extracted narirutin and hesperidin (Fig. 1) from sodium acetate buffer (pH 4.8), ethanol, methanol, isopropyl alcohol, DMSO, acetonitrile, and ethyl acetate, respectively.
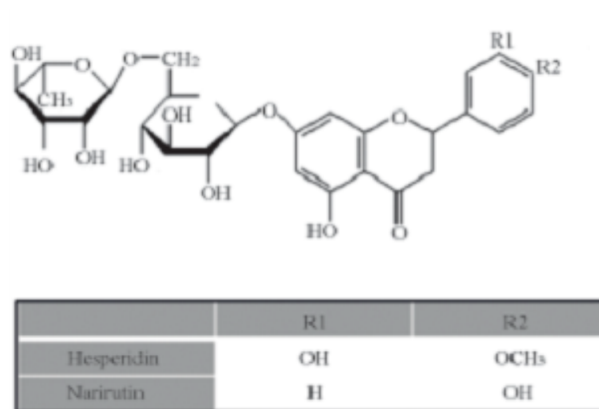
	R1	R2
Hesperidin	OH	OCH₃
Narirutin	H	OH

Figure 1. Chemical structures of hesperidin and narirutin.

As shown Fig. 2a-c, methanol and DMSO improved the extraction yield of narirutin and hesperidin compared with sodium acetate buffer extraction. However, there were no increases in extraction efficiency from *Citrus unshiu* peel using ethanol, isopropyl alcohol, acetonitrile, and ethyl acetate (Fig. 2d). In particular, DMSO maximized the hesperidin content compared with applications of sodium acetate buffer and methanol. We next quantified the extracted narirutin and hesperidin in sodium acetate buffer, methanol, and DMSO using HPLC chromatograms.
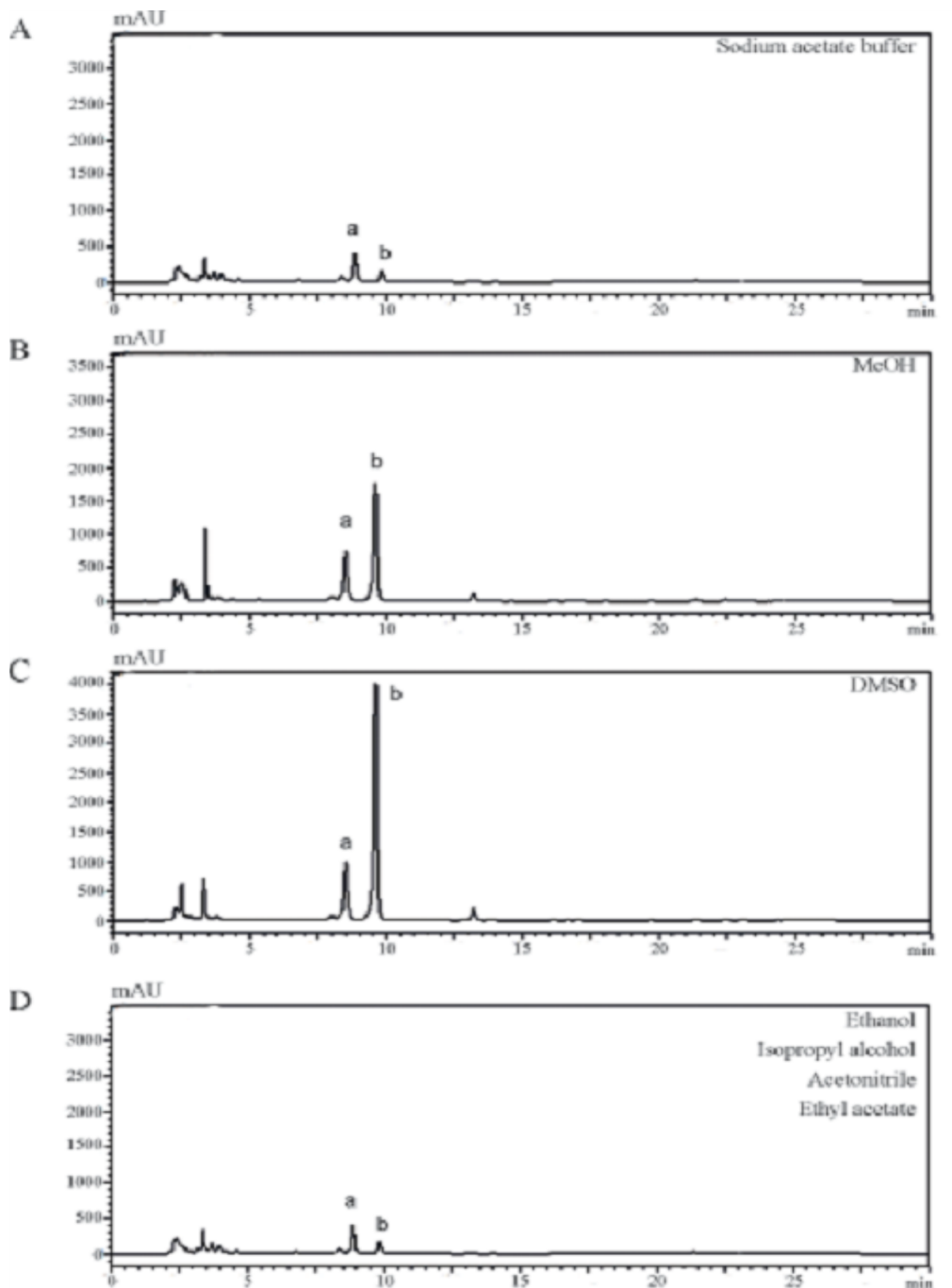
Figure 2. Characterization of constituent elements using different solvents. *Citrus Unshiu* peel was extracted with sodium acetate buffer (A), MeOH (B), or DMSO (C). The extracts were dissolved in methanol and were injected into a HPLC system at a column flow rate of 1 mL/min and analyzed (two major peaks of narirutin and hesperidin were designated a and b, respectively).

To quantify the extracted hesperidin and narirutin, we firstly measured LOD and LOQ values of standard hesperidin and narirutin, respectively, using HPLC analysis (Fig. 3a). Next, we calculated extracted hesperidin and narirutin in sodium acetate buffer, MeOH and DMSO, respectively. The yield of narirutin using methanol and DMSO was significantly increased, respectively, compared to using sodium acetate buffer (water: 41.10 ± 15.12 ppm, MeOH: 95.21 ± 15.11 ppm, DMSO: 148.71 ±38.59 ppm). Especially, the yield of hesperidin using methanol and DMSO as the solvent was more particular, respectively, compared to yield obtained using sodium acetate buffer (water: 1.95 ± 1.04 ppm, MeOH: 308.72 ± 50.27 ppm, DMSO: 828.11 ± 106.84 ppm). Improvement of natural product extraction yield can be obtained by optional choice of extraction method.
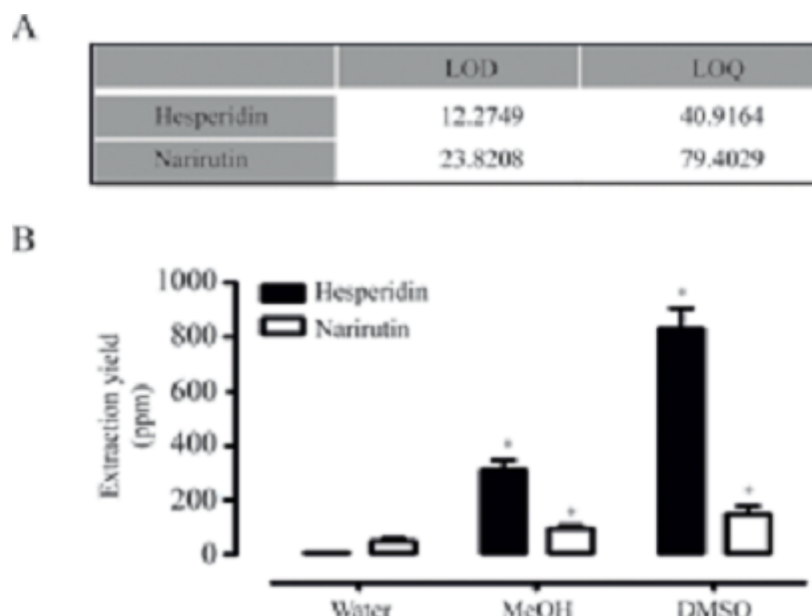
Figure 3. Quantification of extracted narirutin and hesperidin. (A) LOD and LOQ values of standard hesperidin and narirutin were calculated using HPLC chromatograms, respectively. (B) Extracted hesperidin and narirutin were quantified using HPLC analysis (data represent the percentage ± SD and are representative of three individual experiments, *$p < 0.05$).

We show for the first time the effective extraction of functional pro-angiogenic stimulator without specialized equipment and technique. Organic solvents, such as ethanol and methanol, are often used in conventional extraction processes. However, their use is limited by long extraction time, toxicity, and strict legal statutes [18]. In contrast, DMSO has been recognized as an uniquely non-toxic organic solvent in drug synthesis and delivery studies involving humans for over 50 years [19-22]. In agreement with previous reports, increased yields of narirutin and hesperidin were obtained using methanol and DMSO as extraction solvents. Especially, DMSO optimally extracted functional hesperidin from *Citrus Unshiu* peel. Further research will be needed to evaluate the safety of methanol and DMSO extraction method from

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Citrus Unshiu peel. In addition, temperature, pH, and pressure will be considered to prepare the most suitable conditions for optimal extraction yield. Moreover, optimal recovery-strategy of narirutin or hesperidin from DMSO or MeOH extracted mixture should be designed more effectively.
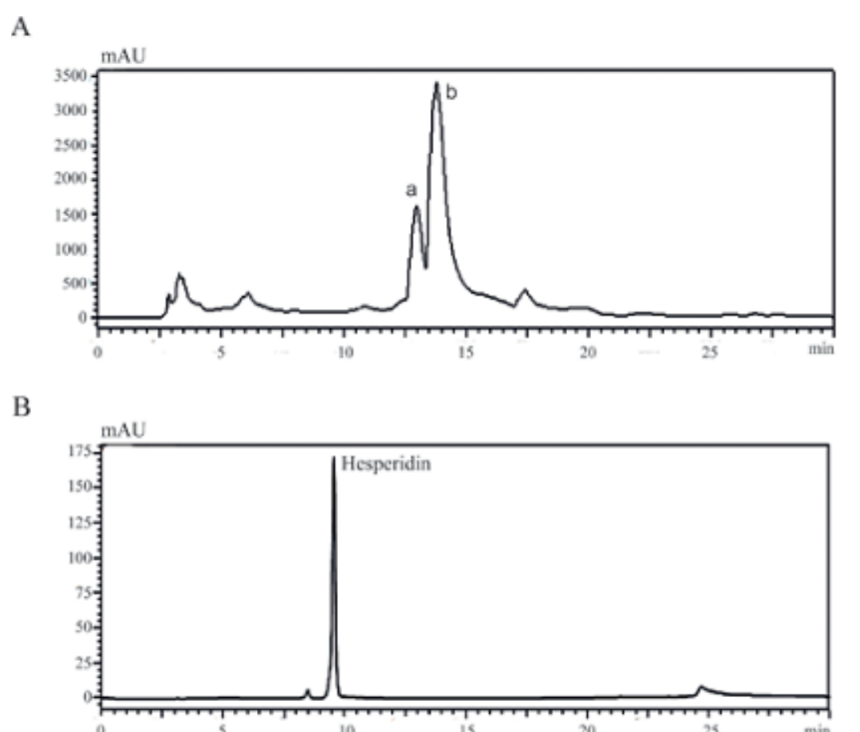
Figure 4. Purification of DMSO extracted hesperidin from *Citrus Unshiu* peel. (A) Extracted hesperidin was purified using a prep-LC system (two major peaks of narirutin and hesperidin were designated a and b, respectively). (B) Extracted hesperidin and standard hesperidin were dissolved in methanol and injected into the HPLC system at a column flow rate of 1 mL/min.

Additionally, we further verified that DMSO-extracted components have pro-angiogenic effects in HUVECs. To assess the functional effects of extracted components, we separated dominant hesperidin from the DMSO extraction mixture containing narirutin and hesperidin using prep-LC (Fig. 4a). The DMSO extracted hesperidin was isolated and identified using HPLC retention time analysis (Fig. 4b). To investigate the functional effects of DMSO extracted hesperidin in HUVECs, we examined the HUVECs proliferation, migration, and tube formation under extracted hesperidin.
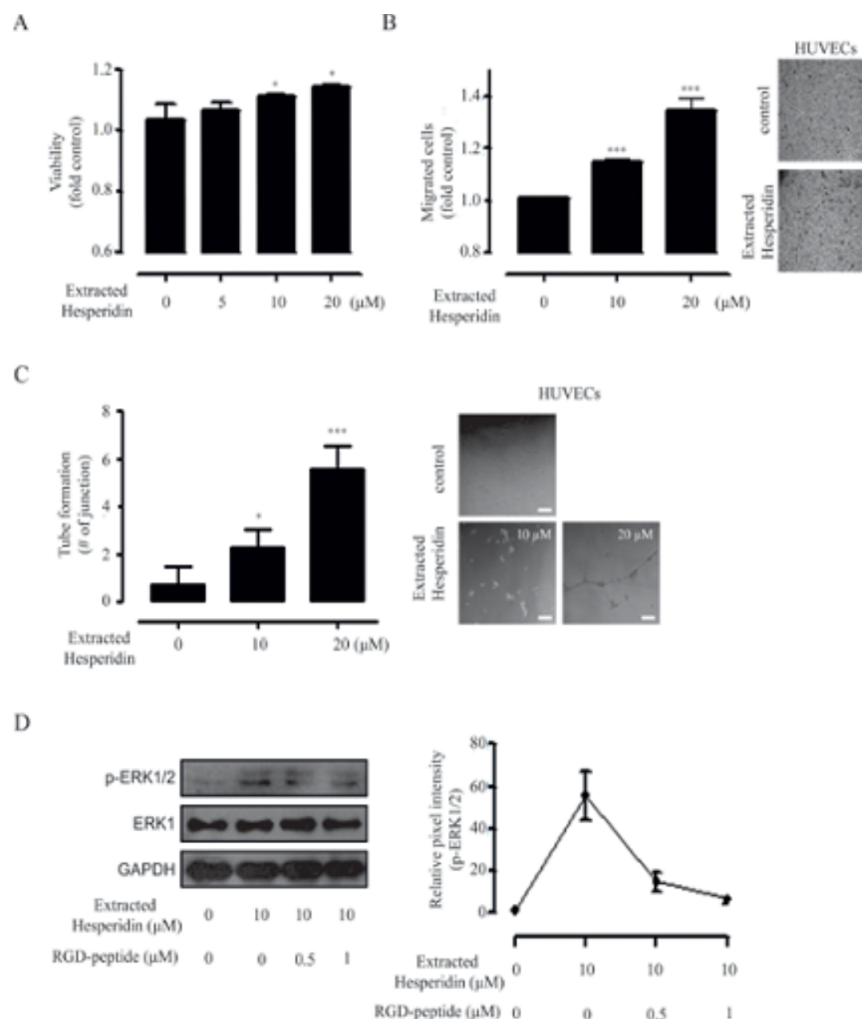
제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 5. Pro-angiogenic effects of DMSO extracted hesperidin. (A) HUVECs were incubated with varying concentrations (0, 5, 10, and 20 μM) of DMSO extracted hesperidin for 72 h (data represent the percentage ± SD and are representative of three individual experiments, *$p < 0.05$, **$p < 0.01$). (B) HUVECs were incubated in the presence or absence of DMSO extracted hesperidin for 6 h using a Transwell migration assay (data represent the percentage ± SD and are representative of three individual experiments, **$p < 0.01$, ***$p < 0.001$). Representative image of a Transwell migration assay (scale bar=50 μm). (C) Left, Capillary like structure (CLS) formation of HUVECs assayed after 16 h of incubation of cells in the presence or absence of DMSO extracted hesperidin (data represent the percentage ±

SD and are representative of two individual experiments, *p* < 0.05, ***p* < 0.001).
Right, Representative image of tube-formation assay (scale bar=50 μm). (D) Left,
HUVECs were pre-incubated with different dose of RGD-peptide (0, 0.5, and 1 μM)
for 30 min before DMSO extracted hesperidin (10 μM) were added for 60 min and
cell lysates were subjected to immunoblot analysis using antibodies for p-ERK1/2
and ERK1. GAPDH was used as a loading control. Right, relative pixel intensities
for p-ERK1/2 were measured using p-ERK1/2/GAPDH.

As shown in Fig. 5a, the treatment produced weak, but statistically significant,
increase in the proliferation of HUVECs as the treatment dosages increased from 10
μM (11±0.007 %) to 20 μM (14±0.0087 %). We also examined the effects of
extracted hesperidin on HUVEC migration using a Transwell-assay. The different
dosages of hesperidin markedly affected migration of HUVECs (10 μM: 14±0.015 %
and 20 μM: 34±0.043 %) compared with the control treatment (Fig. 5b, left and right
panels). In addition, treatment with different dosages of extracted hesperidin induced
tube formation of HUVECs. Quantitative evaluation of tube formation by counting
the junctions of branches revealed that exposure to different dosages of extracted
hesperidin (10 and 20 μM) significantly increased the number of junctions of the
tubular structure compared with control (Fig. 5c, left and right panels). To further
analyze the involvement of hesperidin as the pro-angiogenic effector, we examined
the phosphorylated levels of ERK1/2 signaling under the arginine-glycine-asparate
(RGD)-peptide pre-treatment as previously described [9]. Exogenous treatment with
extracted hesperidin increased the phosphorylation of ERK1/2 and pre-treatment
with RGD-peptide dose-dependently inhibited hesperidin-induced ERK1/2

phosphorylation (Fig. 5d, left and right panels). Various pro-angiogenic inducers, *i.e.* recombinant fibroblast growth factor, hepatocyte growth factor, placental growth factor, and vascular endothelial growth factor have been suggested as reagents for angiogenic-related therapy [23, 24]. However, their use in clinical applications is restricted due to the high cost and short half-life. In this respect, bio-active components from various natural substances including hesperidin seem to be attractive substitutes. Bio-active components from natural products or the wastes after processing of foodstuffs are attractive options, because they can be obtained in huge quantity and at a low price, and produce fewer side effects. Various reports support the rationales of bio-active component development from various natural substances by demonstrating their marked therapeutic effects [2-5]. In the present study, we examined functional pro-angiogenic effects of DMSO extracted hesperidin in HUVECs. We also demonstrated that DMSO extracted hesperidin induced the activation of the ERK1/2 signaling pathway and that was involved in integrins and their ligand interactions.

Taken together, our findings provide the effective, low-priced, and easy method to obtain the natural materials for strong pro-angiogenic inducer.

4. REFERENCE

(1) Choi, I.Y.; Kim, S.; Jeong, H.; Park, S.; Song, Y.; Lee, J.; Kang, T.; Park, J.; Hwang, G.; Lee, E.; Hong, S.; Kim, H.; Um, J. *Mol. Cell. Biochem.* **2007**, *305*, 153-161.

(2) Jeong, S.M.; Kim, S.Y.; Kim, D.R.; Jo, S. C.; Nam, K.C.; Ahn, D.U.; Lee, S.C. *Agric. Food Chem.* **2004**, *52*, 3389-3393.

(3) Murakami, A.; Nakamura, Y.; Torikai, K.; Tanaka, T.; Koshiba, T.; Koshimizu, K.; Kuwahara, S.; Takahashi, Y.; Ogawa, K.; Yano, M.; Tokuda, H.; Nishino, H.; Mimaki, Y.; Sashida, Y.; Kitanaka, S.; Ohigashi, H. *Cancer Res.* **2000**, *15*, 5059-5066.

(4) Kim, D.K.; Lee, K.T.; Eun, J.S.; Zee, O.P.; Lim, J.P.; Eum, S.S.; Kim, S.H.; Shin, T.Y. *Arch. Pharm. Res.* **1999**, *22*, 642-645.

(5) Higashi-Okai, K.; Kamimoto, K.; Yoshioka, A.; Okai, Y. *PTR.* **2002**, *16*, 781-784.

(6) Manthey, J.A.; Grohmann, K. *J. Agric. Food Chem.* **2001**, *49*, 3268-3273.

(7) Chau, C.F.; Huang, Y.L. *J. Agric. Food Chem.* **2003**, *51*, 2615-2618.

(8) Llorach, R.; Espin, J.C.; Tomas-Barberan, F.A.; Ferreres, F. *J. Agric. Food Chem.* **2003**, *51*, 2181-2187.

(9) Lee, J.; Yang, D.S.; Han, S.I.; Yun, J.H.; Kim, I.W.; Kim, S.J.; Kim, J.H. *J. Med. Food.* **2016**, *19*, 569-577.

(10) Khurana, R.; Simons, M.; Martin, J.F.; Zachary, I.C. *Circulation.* **2005**, *112*, 1813-1824.

(11) Folkman, J. *Nat. Med.* **1995**, *1*, 27-31.

(12) Redlitz, A.; Daum, G.; Sage, E.H. *J. Vasc. Res.* **1999**, *36*, 28-34.

(13) Teruyama, K.; Abe, M.; Nakano, T.; Iwasaka-Yagi, C.; Takahasi, S.; Yamada, S.;

Sato, S. *J. Cell. Physio*. **2001**, *188*, 243-252.

(14) Bucar, F.; Wube, A.; Schmid, M. *Nat. Prod. Rep.* **2013**, *30*, 525-545.

(15) Lee, J.; Han, S.I.; Yun, J.H.; Kim, J.H. *Tumour biol.* **2015**, *36*, 9385-9393.

(16) Lee, J.; Kim, J.H. *PloS one*. **2016**, *11*, e0155264.

(17) Sanagi, M.M.; Ling, S.L.; Nasir, Z.; Hermawan, D.; Ibrahim, W.A.; Abu-Namin,

A. *J. AOAC Int.* **2009**, *92*, 1833-1838.

(18) Anwar, F.; Przybylski, R. *Acta. Sci. Pol. Technol. Aliment.* **2012**, *11*, 293-301.

(19) Jacob, S.W.; Rosenbaum, E.E. *Headache*. **1966**, *6*, 127-136.

(20) Jacob, S.W.; Wood, D.C. *Am. J. Surg*. **1967**,*114*, 414-426.

(21) Jacob, S.W. *Am. Surg*. **1969**, *35*, 564-573.

(22) Wood, D.C.; Weber, F.S.; Palmquist, M.A. *J. Pharmacol Exp. Ther.* **1971**, *177*,

520-527.

(23) Carmeliet, P. *Nature*. **2005**, *438*, 932-936.

(24) Dor, Y.; Djonov, V.; Abramovitch, R.; Itin, A.; Fishman, G.I.; Carmeliet, P.;

Goelman, G.; Keshet, E. *EMBO J.* **2002**, *21*, 1939-1947.

요약문

감귤껍질의 수용성 추출액은 신생혈관형성 효과물질의 헤스페리딘과 나리루틴으로 구성되었고 이번 연구는 감귤껍질에서 헤스페리딘과 나리루틴의 추출수율을 높이기 위해 적절한 용매를 찾는 것이었다. Methanol 과 dimethyl sulfoxide 를 사용했을 때 아세토니트릴과 에틸아세테이트, 에탄올, 아이소프로판올 비교했을 때 훨씬 높아진 추출율을 얻을 수 있었고 특히, DMSO 의 헤스페리딘 추출율이 메탄올보다도 훨씬 높았다. 또한 DMSO 로 추출한 헤스페리딘은 인간 제대정맥혈관내피세포의 신생혈관형성 유도를 하였고 ERK1/2 신호전달의 인산화를 up-regulate 하였다. 이러한 결과는 추출된 헤스페리딘의 신생혈관형성 효과와 감귤껍질에서 헤스페리딘과 나리루틴을 간단하고 쉽게 그리고 효과적으로 추출하는 방법을 보여준다.

ACKNOWLEDGEMENTS