Natural Product Sciences
22(4) : 287-292 (2016)
https://doi.org/10.20307/nps.2016.22.4.287

Maximization of Extracted Condition of Pro-angiogenic Components in *Citrus unshiu* Peels using Dimethyl Sulfoxide

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Abstract – Aqueous extraction of *Citrus unshiu* peels (AECUP) is mainly comprised with pro-angiogenichesperidin and narirutin. In this study, we report approaches to increasing the yields of extracted hesperidin and narirutinfrom *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.
Keywords – Angiogenesis, *Citrus unshiu* peels, Hesperidin, Narirutin, DMSO, MeOH

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 *Citrusunshiu* 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, wound healing, 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*. 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.

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Experimental

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).

Cell culture and reagents – HUVECs were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in EGM-2 Bullet kit 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).

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).

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].

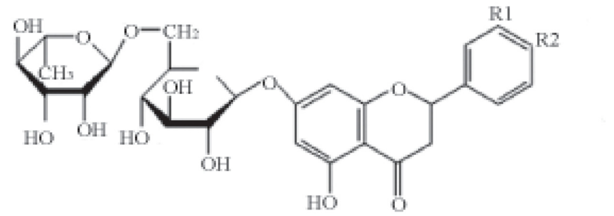
Tube formation – Tube formation assays were performed as previously described with some modifications[9]. In brief, 250 μL growth factor-reduced matrigel (BD Biosciences, San Diego, CA) was used to coat 24 well plates (SPL Life Sciences, Pocheon, Republic of Korea) and allowed to polymerize at 37 °C 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.

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 (5 μm particle size, 4.6 mm × 250 nm; 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]

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 mL 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. According 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).

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 top-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 for1h at room temperature. Bands were measured by densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA).

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.



	R1	R2
Hesperidin	OH	OCH_3
Narirutin	H	OH

Fig. 1. Chemical structures of hesperidin and narirutin.

Result 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. 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. 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

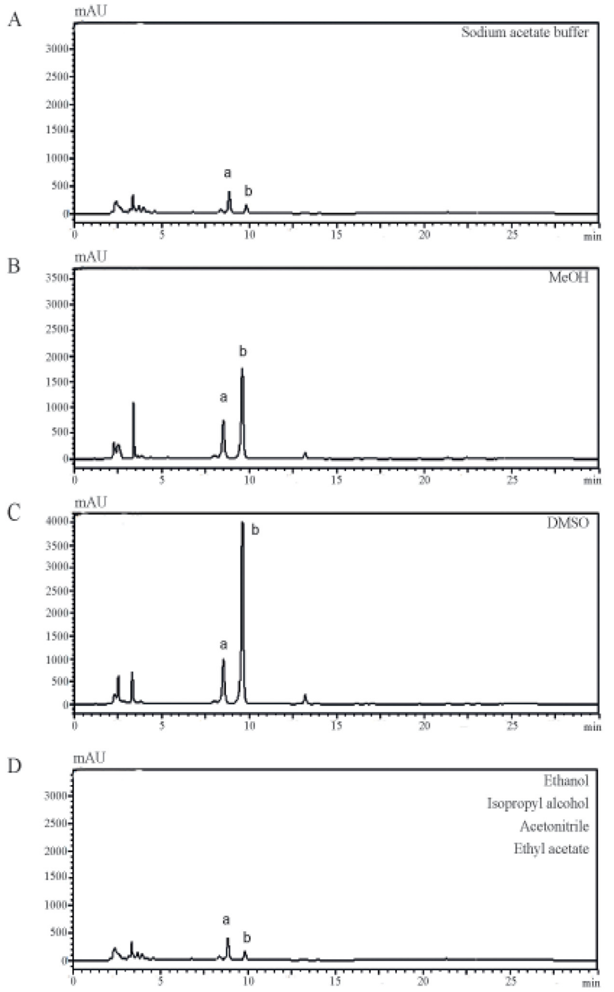


Fig. 2. Characterization of constituent elements using different solvents. *Citrus Unshiu* peel was extracted with sodium acetate buffer (A), MeOH (B), or DMSO (C). The extracts were dissolved in methanol and were injected into a HPLC system at a column flow rate of 1 mL/min and analyzed (two major peaks of narirutin and hesperidin were designated a and b, respectively).

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. 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 a 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

A

	LOD	LOQ
Hesperidin	12.2749	40.9164
Narirutin	23.8208	79.4029

B

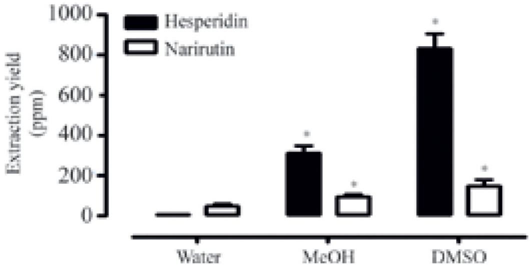


Fig. 3. Quantification of extracted narirutin and hesperidin.(A) LOD and LOQ values of standard hesperidin and narirutin were calculated using HPLC chromatograms, respectively. (B) Extracted hesperidin and narirutin were quantified using HPLC analysis (data represent the percentage ± SD and are representative of three individual experiments, *$p < 0.05$).

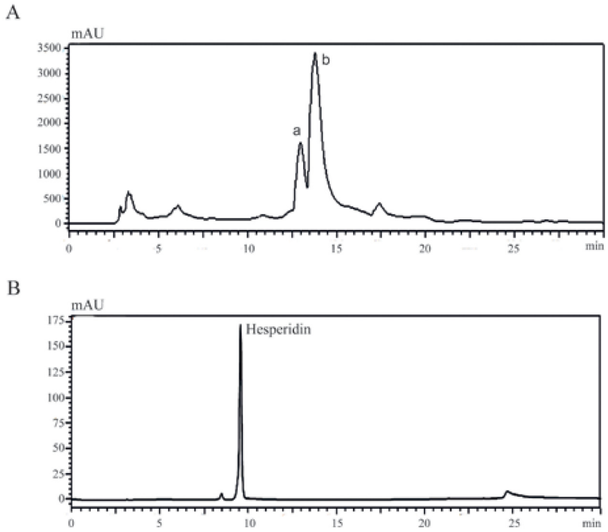


Fig. 4. Purification of DMSO extracted hesperidin from *Citrus Unshiu* peel. (A) Extracted hesperidin was purified using a prep-LC system (two major peaks of narirutin and hesperidin were designated a and b, respectively). (B) Extracted hesperidin and standard hesperidin were dissolved in methanol and injected into the HPLC system at a column flow rate of 1 mL/min.

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 *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.

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. 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-inducedERK1/2 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 develop-

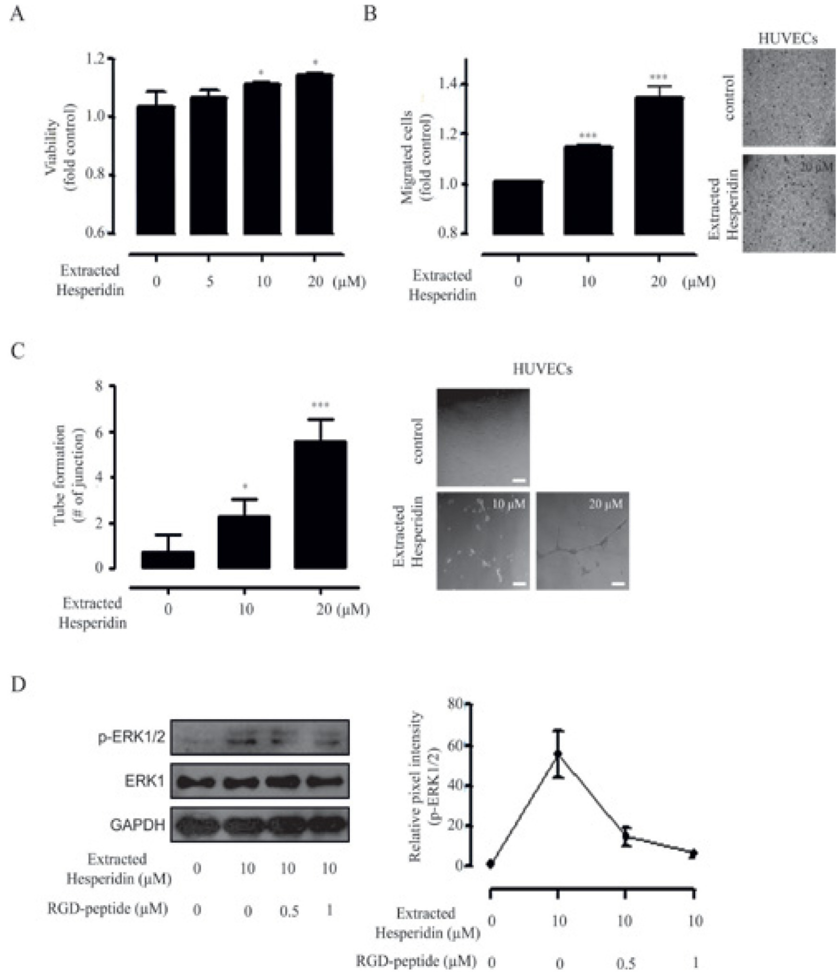


Fig. 5. Pro-angiogenic effects of DMSO extracted hesperidin.(A) HUVECs were incubated with varying concentrations (0, 5, 10, and 20 μM) of DMSO extracted hesperidin for 72 h (data represent the percentage ± SD and are representative of three individual experiments, **p* < 0.05, ***p* < 0.01). (B) HUVECs 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.

ment 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.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1A6A1A03012862) and (2016R1A6A3A01010676).

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Received August 19, 2016
Revised September 26, 2016
Accepted September 27, 2016