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Polysaccharides from Korean *Citrus* hallabong peels inhibit angiogenesis and breast cancer cell migration

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ABSTRACT

Although the peel of the hallabong (*Citrus sphaerocarpa*) fruit is rich in polysaccharides, which are valuable dietary ingredients for human health, it is normally wasted. The present study aimed to utilize the peel waste and identify properties it may have against breast cancer metastasis. Hallabong peel extract containing crude polysaccharides was fractionated by gel permeation chromatography to produce four different polysaccharide fractions (HBE-I, -II, -III, and -IV). The HBE polysaccharides significantly blocked tube formation of human umbilical vein vascular endothelial cells (HUVECs), at a concentration of 12.5 or 25 μg/mL. Tube formation appeared to be more sensitive to HBE-II than to other HBE polysaccharides. HBE-II also inhibited breast cancer cell migration, through downregulation of matrix metalloproteinase-9 (MMP-9) in MDA-MB-231 triple-negative breast cancer cells. Therefore, inhibition of tube formation and MMP-9-mediated migration observed in HUVEC and MDA-MB-231 cells, respectively, are likely to be important therapeutic targets in triple-negative breast cancer metastasis.

1. Introduction

The hallabong is a new hybrid of mandarin and orange in South Korea. It was named after the Halla Mountain on Jeju Island, where it is mainly grown [1]. Hallabong fruits are primarily used for juice manufacture, with the peel of this *Citrus* species being used in traditional medicine to treat stomach upsets, coughs, skin inflammation, muscle pain, and ringworm infections, as well as to lower blood pressure [2].

The peel of the hallabong is thicker and more bulky than mandarin or orange, and the weight of the peel is almost 30% of the total weight of the hallabong fruit. Although the peel of the hallabong is rich in pectin, flavonoids, carotenoids, limonoids, and coumarins that are valuable and functional dietary ingredients for human health [3–5], it is normally wasted. Therefore, research on the utilization of this peel waste has received much attention in the recent literature. Previous studies showed that a sufficient supply of orange-peel extract might have chemoprotective functions and possibly reduce the risk of cancers [6]. In addition, polymethoxyflavones, which are particularly found in the peel of the *Citrus* genus, inhibited growth of human lung cancer cells, through downregulation of oncogenic proteins and induction of apoptosis [7]. However, little is known about the active constituents and health benefits of hallabong peel.

Breast cancer is one of the most common malignant diseases and the second major cause of cancer death in women [8,9]. In addition, mortality due to breast cancer remains very high, although approaches to its diagnosis and treatment have advanced [10]. The main reason for breast cancer patient death is more commonly distant metastasis than the primary tumor [11]. Tumor metastasis, which is the predominant cause of patient death in breast cancer, is a major obstacle to conventional chemotherapeutic strategies [12].

The aim of the present study was to utilize hallabong peel waste and identify properties it may have against triple-negative breast cancer metastasis. Triple-negative breast cancer is a type of malignant breast cancer that does not have any of the three receptors, the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2, that are the primary targets of chemotherapy drugs for this cancer [13,14]. Moreover, treatment-resistant

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triple-negative breast cancers have a poor prognosis and rapidly metastasize, rendering it difficult to cure [15].

2. Materials and methods

2.1. Chemicals and reagents

Pectinase isolated from *Aspergillus niger* was purchased from Sigma (St. Louis, MO). Sephadex G-75 gel permeation chromatography medium was obtained from GE Healthcare Life Sciences (Piscataway, NJ). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY). The EZ-Cytox enhanced cell viability assay kit was purchased from DoGenBio (Seoul, Korea). Primary antibodies against vascular endothelial growth factor (VEGF), phospho-AKT, extracellular signal-regulated kinase, p53, and cleaved caspase-3, and all secondary antibodies, were obtained from Cell Signaling Technology, Inc. (Danvers, MA).

2.2. Extraction and purification of polysaccharides from hallabong peel

Hallabong fruit was harvested in Gashi-ri, South Jeju-gun, Jeju-do, Korea. The hallabong peel was dried at 60 °C for 3 days using a convection oven, and then pulverized with a mechanical grinder, as previously reported [16]. Four purified polysaccharide fractions (HBE-I, -II, -III, and IV) from hallabong peel were used in this study. The fractions were prepared following the method of Lee et al. In brief, pulverized peel was extracted with water containing pectinase, at 50 °C and pH 5.0, and then boiled to inactivate the pectinase. To obtain crude polysaccharide, the supernatant was then collected, and four volumes of ethanol were added to it. The resulting precipitate was dissolved in water and dialyzed (molecular weight cut-off: 12,000–14,000), and finally lyophilized to yield the crude polysaccharide of the hallabong peel (HBE-0). HBE-0 was fractionated by gel filtration through Sephadex G-75 (GE Healthcare Life Sciences); details of the fractions obtained and their elution profiles have been described in our previous publication [16]. The procedures for obtaining pure polysaccharides (HBE-I, -II, III, and -IV) are as shown in Fig. 1A.

2.3. Determination of carbohydrate composition and content

The total neutral sugar content was calculated using galactose as the standard, by the method of Dubois et al. [17]. Uronic acid content was determined by the *m*-hydroxybiphenyl colorimetric procedure of Blumenkrantz and Asboe-Hansen, with galacturonic acid as the standard [18]. The thiobarbituric acid assay was performed in order to determine the presence of 2-keto-3-deoxy-D-*manno*-2-octulosonic acid (Kdo) and 2-keto-3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha) [19]. The protein content of the polysaccharide fractions was determined by the Bradford assay [20]. The monosaccharide composition of the polysaccharide fractions was determined by gas chromatography (GC; ACME-6100, Yong-Lin Co., Anyang, Korea) of their alditol acetates [21]. The neutral sugars were determined as alditol acetates by GC after 2 M trifluoroacetic acid (TFA) hydrolysis for 90 min at 121 °C using the procedure of Zhao et al. [22]. The composition of Kdo, Dha, and uronic acid were determined via GC as alditol acetates, according to the modified methods of York et al. [23] and Stevenson et al. [24]. The resulting alditol acetates were analyzed via GC, using a SP-2380 capillary column (0.2 μm film thickness, 0.25 mm internal diameter × 30 m; Supelco, Bellefonte, PA) and a flame-ionization detector (FID; Young-Lin Co.). The carrier gas was N_2, with a flow rate of 1.0 mL/min. The oven temperature was initially set at 60 °C for 1 min, increased at 30 °C/min to 220 °C and kept at this temperature for 12 min, then decreased by 8 °C/min to 250 °C and kept at this temperature for 15 min. The molar amount of each component sugar was calculated from the peak area of each derivative, their molecular weight, and the FID molecular response factor.

2.4. Determination of molecular weight of the polysaccharides

The weight average molecular weight of the polysaccharides HBE-I, -II, -III, and -IV were determined by gel permeation chromatography (GPC; 1260 Infinity LC system, Agilent Technologies, Santa Clara, CA) with a Superdex 75 prepacked size exclusion chromatography column (GE Healthcare Life Sciences). The eluent was a 50 mM ammonium formate buffer solution (pH 5.5). The flow rate was maintained at 0.5 mL/min, and the temperature of the column was maintained at room temperature. The eluent was monitored with a refractive index detector (1200 series, Agilent Technologies). Pullulan (Showa Denko Co., Ltd., Tokyo, Japan) was used as a standard to calibrate the column. All data generated by the GPC system were collected and analyzed using the Workstation software package.

2.5. Cell culture

Human umbilical vein vascular endothelial cells (HUVECs) (ATCC, Manassas, VA) were maintained using the Clonetics EGM-2 MV BulletKit (Takara Bio Inc., Shiga, Japan) in a humidified atmosphere (5% CO_2, 95% air). MDA-MB-231 human breast adenocarcinoma cells (ATCC HTB-26) were cultured in DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 unit/mL penicillin at 37 °C in a humidified atmosphere (5% CO_2, 95% air). The cells were fed every 2–3 days and subcultured once they reached 70–80% confluence. For experiments, cells were plated at an appropriate density, according to each experimental design.

2.6. Viability measurements of HUVEC and MDA-MB-231 cells

The cytotoxicity of HBE polysaccharides to HUVEC and MDA-MB-231 cells was tested using the MTT assay [25]. Cells were seeded at 2×10 cells μL^{-1}/well on a 96-well plate. The cells were then treated with various amounts of HBE or with the DMSO vehicle (control), and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. Subsequently, 10 μL of MTT was added to each well and the plates were incubated for an additional 1 h at 37 °C. The absorbance of samples at 450 nm was measured using a microplate reader.

2.7. Tube-formation assay of HUVEC

Plates (96-well) were coated with 60 μL of 10 mg/mL Matrigel, which was allowed to polymerize at 37 °C. Cells were harvested and seeded (3×10^2 cells μL^{-1}/well) onto the Matrigel-coated plate, medium with or without HBE polysaccharide was added, and the plates were then incubated at 37 °C. After 24 h, cells were fixed with 4% paraformaldehyde fixative and stained with Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan). Cell morphology changes and tubular-structure formation were observed with a light microscope. The images were captured, and the degree of tube formation was quantified by measuring the lengths of the tubes using the ImageJ program.

2.8. Cell migration assay

The migration of MDA-MB-231 cells was determined using cell culture polyethylene terephthalate inserts with a pore size of 8 μm

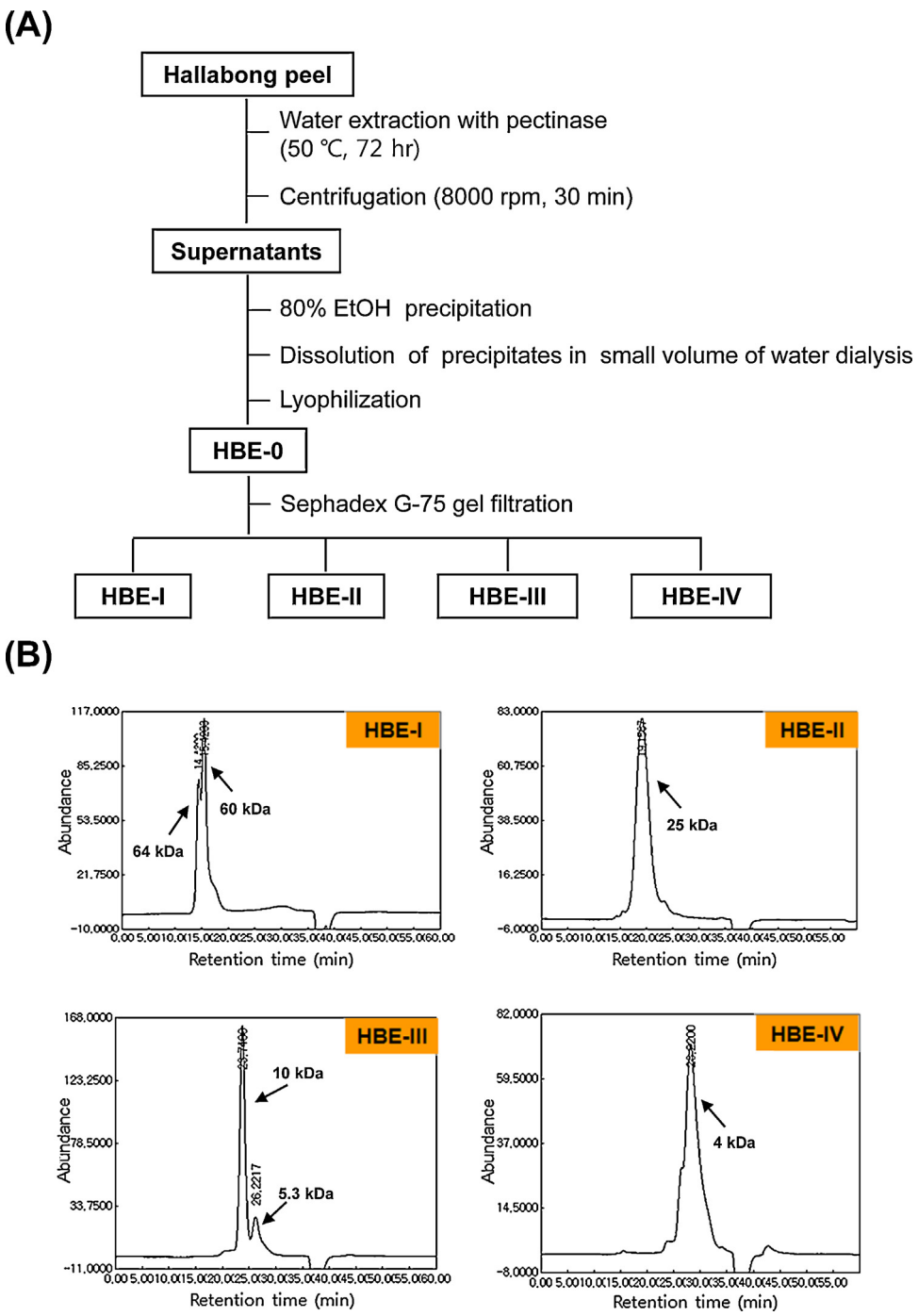


Fig. 1. Isolation and characterization of polysaccharide fractions from hallabong peel. (A) Schematic of the extraction of polysaccharides from the peel of the hallabong *Citrus* fruit, and their purification by size-exclusion chromatography. (B) High performance size-exclusion chromatogram of the polysaccharide fractions, HBE-I, -II, -III, and -IV, isolated from hallabong peel.

and a 24-well format (No. 353097; Falcon, Franklin Lakes, NJ). The lower surface of the filters was coated with 5 μg/mL Matrigel. Cell suspension (2×10^3 cells μL^{-1}/well) was added to the upper compartment of the chamber, and 600 μL of culture medium with or without HBE polysaccharide was added to the lower compartment. The chambers were incubated at 37 °C. Cells that had not migrated were removed with cotton swabs. The migrated cells on the filter were fixed with methanol, and stained with Mayer's hematoxylin and 1% Eosin Y solution (Muto Pure Chemicals). Cells were photographed, counted, and the percentage of migrated cells was calculated.

2.9. Western blotting analysis

MDA-MB-231 cells (8×10^5) grown in 60-mm dishes were treated with HBE polysaccharide samples at various concentrations for 24 h. Whole-cell extracts were then prepared using RIPA buffer, according to the manufacturer's instructions (Cell Signaling Technologies), which was supplemented with 1× protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride. Proteins (whole-cell extracts, 30 μg/lane) were separated by electrophoresis in a precast 4–15% Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA), blotted onto polyvinylidene difluoride membranes, and analyzed with

epitope-specific primary and secondary antibodies. Bound antibodies were visualized using ECL Advance Western Blotting Detection Reagents (GE Healthcare Life Sciences) and a LAS 4000 imaging system (Fujifilm, Tokyo, Japan).

2.10. Reverse transcription-PCR analysis

Total RNA was isolated from MDA-MB-231 cells by using the RNeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer's protocol, and then treated with RNase free DNase (Qiagen) to remove any potential DNA contamination. For reverse transcription-PCR (RT-PCR), 1 μg of total RNA was converted into cDNA by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). To amplify the cDNAs, the reverse-transcribed cDNA was subjected to 40 PCR cycles using the Ex Taq polymerase kit (Takara Bio Inc.) in the presence of 10 pmol of sense and antisense oligonucleotide primers (for the matrix metalloproteinase-9 [MMP-9] cDNA, 5′-TCCAACCACCACCACACCGC-3′ and 5′-CAGAGAATCGCCAGTACTT-3′; for the β-actin cDNA, 5′-GGCTCCGGCATGTGCAAG-3′ and 5′-CCTCGGTCAGCAGCACGG-3′). Each PCR cycle included a denaturation step at 95 °C for 1 min, a primer-annealing step at 58 °C for 1 min, and an extension step at 72 °C for 40 s. Reactions were performed using a Biometra T gradient Thermocycler (Göttingen, Germany). The PCR products were analyzed by electrophoresis in a 2% agarose gel and visualized by UV after ethidium bromide staining.

2.11. Statistical analysis

Statistical significance was determined through analysis of variance, followed by a multiple comparison test with a Bonferroni adjustment. *P* values of less than 0.05 were considered statistically significant.

3. Results and discussion

The four polysaccharide fractions from hallabong peel were extracted from the peel with water at 50 °C containing pectinase, as previously described [16]. Crude polysaccharide (HBE-0) was precipitated from hallabong peel extracts with 80% ethanol, precipitates were dialyzed to remove salt, and finally lyophilized. HBE-0 was further fractionated by GPC and generated four fractions of different molecular weight (HBE-I, -II, -III, and -IV) (Fig. 1A). The molecular weights of the four fractions were determined by HPLC on a system equipped with a Superdex 75 column. As shown in Fig. 1B, the molecular weights of each fractions were estimated to be 64 kDa and 60 kDa (HBE-I), 25 kDa (HBE-II), 10 kDa and 5.3 kDa (HBE-III), and 4 kDa (HBE-IV), according to the calibration curve. A single and symmetrical peak of HBE-II indicated a highly purified polysaccharide fraction.

Analytical results for sugar composition of the polysaccharide fractions are shown in Table 1. HBE-I primarily consisted of neutral sugar (96.3%) containing glucose (51.2% of neutral sugar), mannose (35.9% of neutral sugar), arabinose, rhamnose, galactose, xylose, and a minor amount of uronic acid (3.7%). On the other hand, HBE-II had a high uronic acid content (34.8%), including galacturonic acid and glucuronic acid. The neutral sugar composition of HBE-II was 65.2%, mainly composed of rhamnose (22.9% of neutral sugar), arabinose (12.4% of neutral sugar), and small amounts of fucose, xylose, mannose, galactose, and glucose. HBE-III showed the most varied sugar composition of neutral sugar (52.8%), uronic acid (43.0%), and Kdo-like material (4.2%). The sugars 2-methylfucose, 2-methylxylose, apiose, and aceric acid, and Kdo-like material containing Dha and Kdo, were reported as components of the side chains of the pectic polysaccharide, rhamnogalacturonan II (RG II) [26]. Recently, we reported that the HBE-III polysaccharide fraction (identified as RG II) inhibited tumor metastasis via activation of natural killer cells, using colon 26-M3.1 carcinoma cells in a murine model [16]. The HBE-IV fraction was not fully purified for analyzed further.

Table 1
Chemical properties of the polysaccharide fractions HBE-I, -II, and -III purified from pectinase digest of hallabong peel.

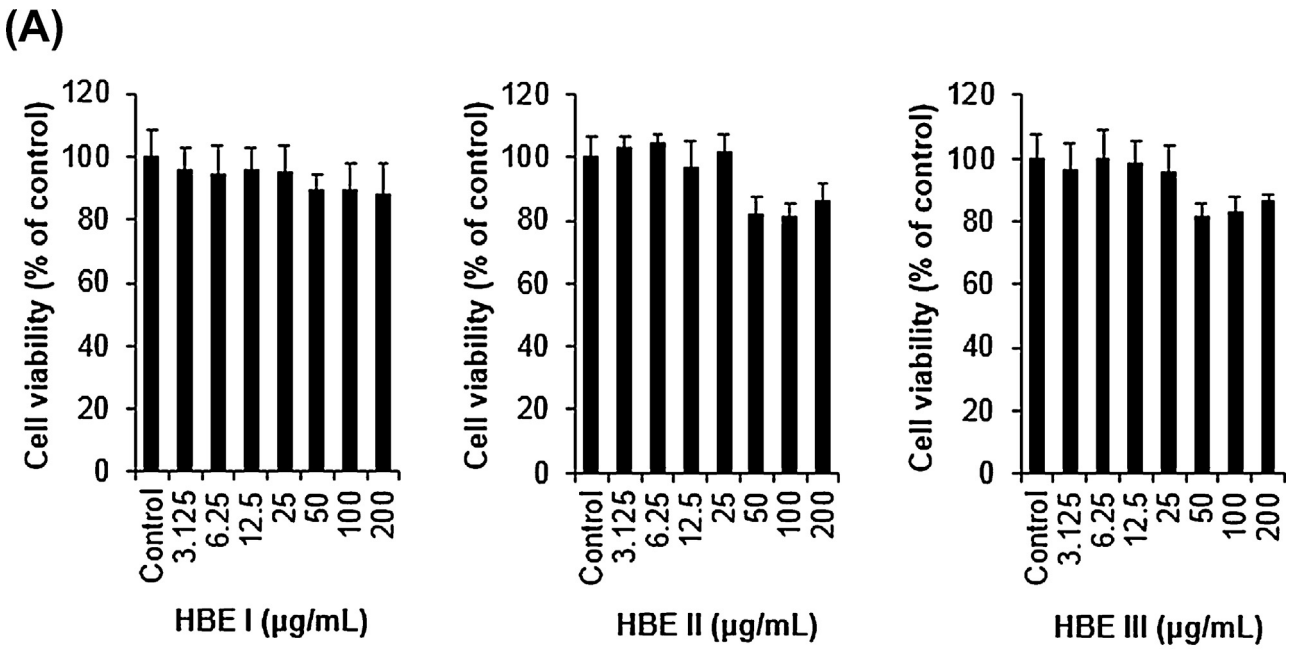
Chemical composition[a]	HBE-I (%)	HBE-II (%)	HBE-III (%)
Neutral sugar	96.3	65.2	52.8
Uronic acid	3.7	34.8	43.0
Kdo-like material	n.d.[b]	n.d.	4.2
Protein	n.d.	n.d.	n.d.
Sugar component[c]	**(mol%)[d]**	**(mol%)**	**(mol%)**
2-Methylfucose	n.d.	n.d.	3.8
Rhamnose	2.6	22.9	10.2
Fucose	0.3	5.7	3.2
2-Methylxylose	n.d.	n.d.	4.6
Arabinose	3.3	12.4	12.4
Xylose	0.4	3.1	n.d.
Apiose	n.d.	n.d.	2.2
Aceric acid	n.d.	n.d.	2.5
Mannose	35.9	7.6	0.8
Galactose	2.6	5.1	9.8
Glucose	51.2	8.4	3.3
Galacturonic acid	3.7	34.8	37.6
Glucuronic acid	n.d	n.d	5.4
Dha	n.d.	n.d.	1.8
Kdo	n.d.	n.d.	2.4

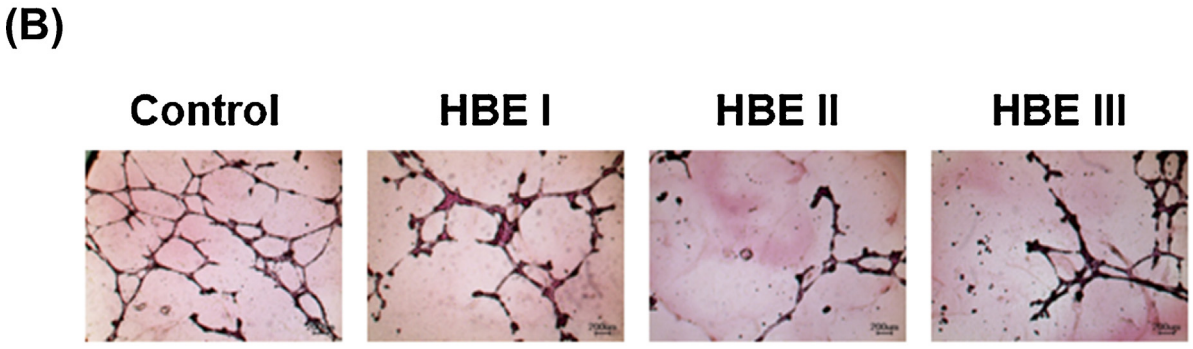
[a] Based on dry material.
[b] Not detected.
[c] Sugar components were analyzed by gas chromatography using alditol acetates.
[d] mol% was calculated from the detected total carbohydrate.

Angiogenesis is defined as the growth of blood vessels from the existing vasculature. These vessels provide the route by which tumor cells exit the primary tumor and enter the circulation. Therefore, inhibition of angiogenesis may be a valuable new approach to cancer therapy [27,28]. The antiangiogenic potential of each HBE fraction was assessed using HUVECs. Endothelial cell proliferation is one of the complex multistep processes involved in angiogenesis. Therefore, the ability of the polysaccharide fractions (HBE I, II, and III) to inhibit the proliferation of endothelial cells was examined. As shown in Fig. 2A, each HBE fraction inhibited HUVEC proliferation in a dose-dependent manner. Treatments with up to 25 μg/mL of each HBE fraction had no effect on the HUVECs, whereas treatment with 50–200 μg/mL decreased cell viability. Consequently, we propose that the HBE polysaccharides exert growth-inhibitory effects on HUVECs, but without causing significant cytotoxicity.

To verify any morphogenic potential of HBE polysaccharides, a tube formation assay was performed; this is one of the most commonly used in vitro angiogenesis tests. The effect of a nontoxic dose (12.5 and 25 μg/mL) of HBE polysaccharides on HUVEC tube formation is shown in Fig. 2B and C. Representative tube images are shown (Fig. 2B); HBE polysaccharides at a concentration of 12.5 or 25 μg/mL significantly blocked tube formation (Fig. 2C). Tube formation appeared to be more sensitive to HBE-II than to the other HBE fractions. Compared with the control, significant inhibition of tube formation of 46.9% and 49.6% at 12.5 and 25 μg/mL, respectively, was observed for HBE-II.

The antimetastatic effect of HBE-II was further assessed using MDA-MB-231 cells. MDA-MB-231 cells are extremely aggressive, invasive triple-negative breast cancer cells, which are resistant to several anticancer agents [29]. Therefore, MDA-MB-231 cells provide an ideal in vitro model to analyze the effects of antimetastatic agents. The effects of HBE-II on the viability of MDA-MB-231 cells,





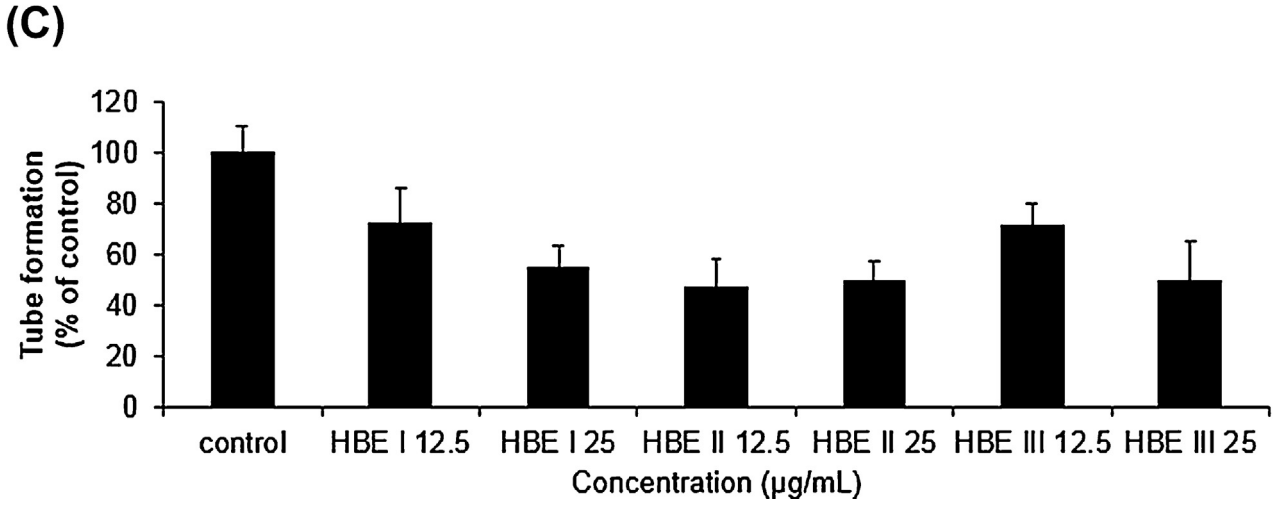


Fig. 2. Antiangiogenic effectiveness of HBE polysaccharide fractions on HUVECs. (A) Comparison of the effects of HBE polysaccharides on HUVEC proliferation, and assessment of cytotoxicity. HUVECs were treated with HBE polysaccharides at a series of concentrations (3.125–200 μg/mL) or the DMSO vehicle (control) for 24 h, and then cell viability was evaluated by the MTT assay. (B) and (C) Comparison of the effects of HBE polysaccharides on tube formation. Tube formation of HUVECs on Matrigel is shown after incubation with HBE polysaccharides (12.5 and 25 μg/mL) or DMSO (control) at 24 h. The relative length of capillaries was measured using the ImageJ program.

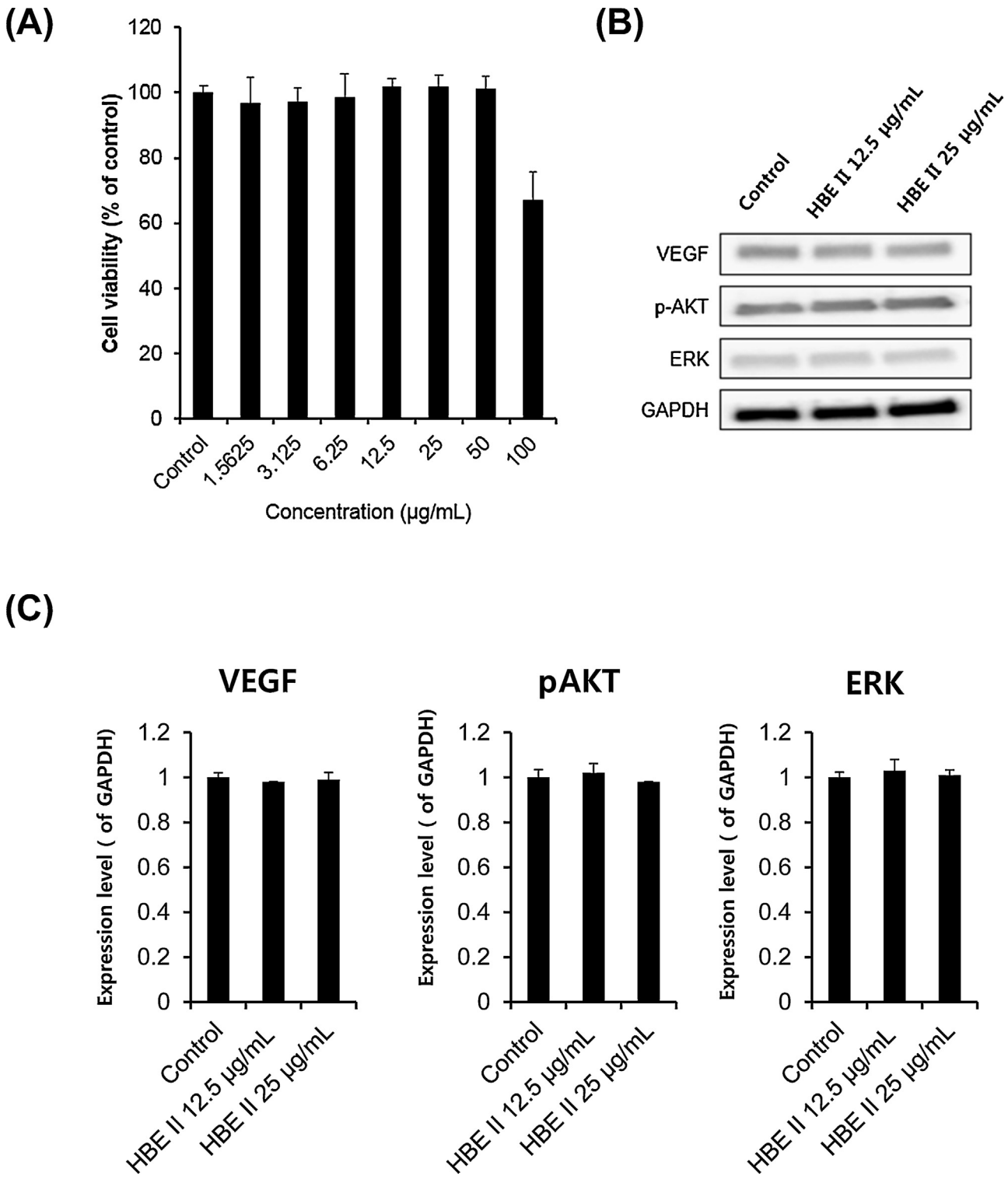


Fig. 3. Effects of HBE-II on protein expressions of angiogenic molecules in MDA-MB-231 cells. (A) Effect of HBE-II on MDA-MB-231 cell proliferation and assessment of cytotoxic effects of HBE-II. MDA-MB-231 cells were treated with HBE II at a series of concentrations (1.56–100 μg/mL) or with the DMSO vehicle (control) for 24 h, and then cell viability was evaluated by the MTT assay. (B) Effect of HBE-II on the protein expression levels of VEGF, phospho-AKT, and ERK in MDA-MB-231 cells. (C) Bar graphs showing quantification of the expression level of each protein compared with the expression of GAPDH. Data are expressed as means ± s.e.m. Western blots are representative of three independent experiments.

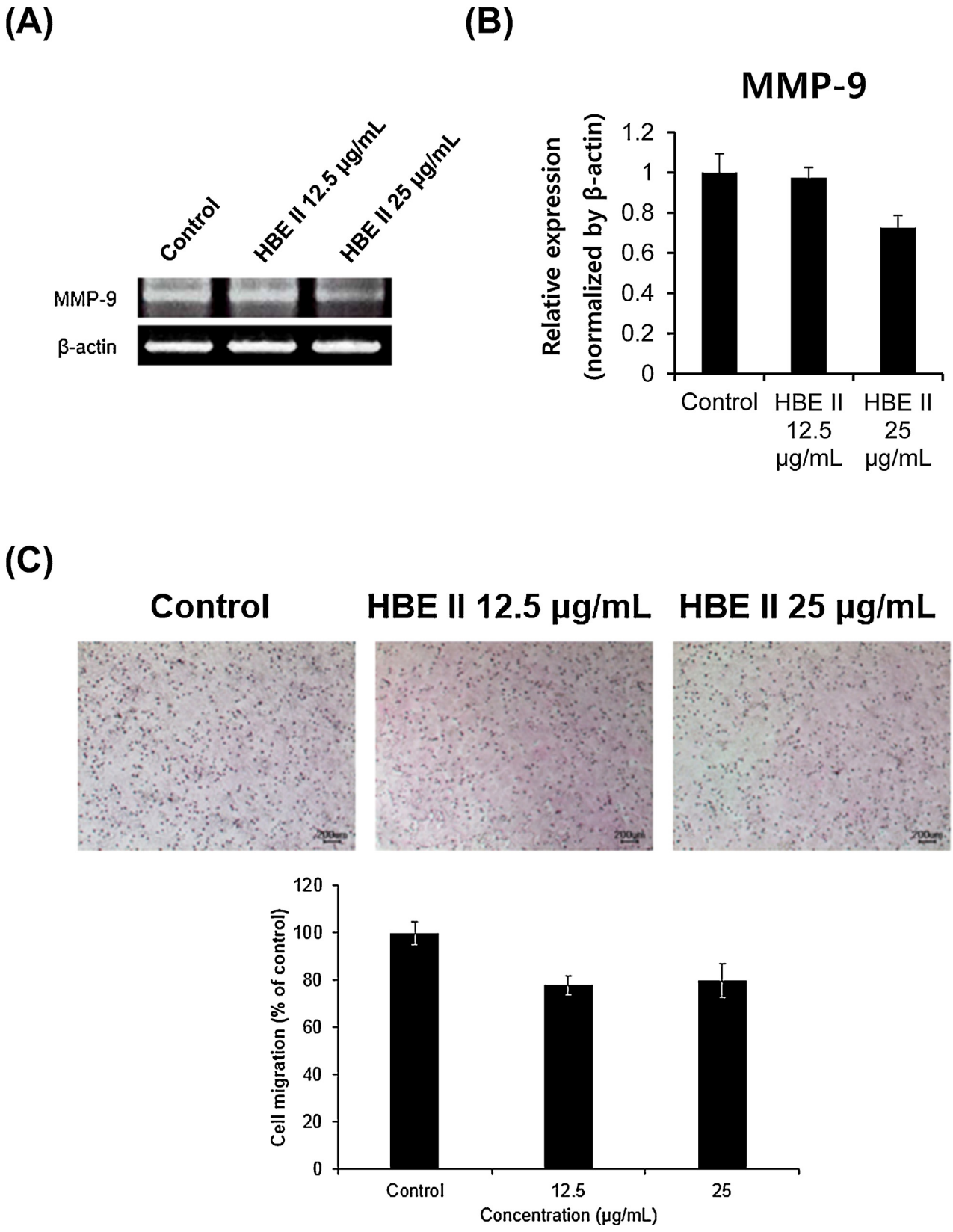


Fig. 4. Antimetastatic effectiveness of HBE-II on MDA-MB-231 cells. (A) Expression of the *MMP9* mRNA in HBE-II-treated MDA-MB-231 cells. MDA-MB-231 cells (2.0×10^6 cells/6-cm dish) were treated with HBE-II at the indicated concentrations (0, 12.5, and 25 μg/mL) for 24 h. Total RNA was extracted from HBE-II-treated MDA-MB-231 cells, and *MMP9* expression was analyzed by reverse transcription-PCR (RT-PCR). RT-PCR reactions were performed as described in Section 2. The size of the product is 272 bp. β-actin was used as an internal control. (B) Bar graphs showing quantification of the expression level of *MMP9* compared with the expression of β-actin. (C) Effect of HBE-II on MDA-MB-231 cell migration. MDA-MB-231 cells were seeded into the insert of each well of a 24-well plate. HBE-II (12.5 and 25 μg/mL) or DMSO (control) was added to the lower compartment of the chamber, which held 0.6 mL of medium. After 24 h of incubation, the cells that migrated through to the lower side of the membrane were fixed, stained with hematoxylin and eosin, and counted.

investigated by treating the cells with increasing concentrations of HBE-II (1.56–100 μg/mL) for 24 h, are shown in Fig. 3A. Treatment with up to 50 μg/mL HBE-II had no effect on MDA-MB-231 cells, whereas treatment with 100 μg/mL HBE-II decreased cell viability.

Angiogenesis starts with the release of growth-related molecules from cancerous tumor cells that send signals to vascular endothelial cells [30]. We measured the expression of angiogenic molecules, including VEGF, in MDA-MB-231 cells by using western blotting. However, no changes in these angiogenic molecules were noted (Fig. 3B and C) and we switched our attention to other molecules that may account for the antimetastatic potential of HBE-II. Matrix metalloproteinases (MMPs) are the main group of proteolytic enzymes that are critically involved in tumor invasion, metastasis, and angiogenesis in cancer [31–33]. MMP-2 and MMP-9 both play an important role in angiogenesis, as well as tumor invasion and metastasis [34,35]. RT-PCR analysis was used to evaluate the expression of MMP-9 in MDA-MB-231 cells. As shown in Fig. 4A and B, the levels of the *MMP9* mRNA were decreased, compared with control untreated cells, after 24 h of incubation with HBE-II. In contrast, there were no changes in *MMP2* mRNA expression following the treatment with HBE-II (data not shown). Cancer progression is associated with abrogation of the normal controls that limit cell migration. Cell migration is a critical process in tumor metastasis [36]. The inhibitory effect of HBE-II on the migration of breast cancer cells was examined using Matrigel-coated filters. In the absence of HBE-II, MDA-MB-231 cells displayed the capability to migrate, as indicated by their being able to completely penetrate through the Matrigel-coated filters (Fig. 4C). The migration activity of the cancer cells was suppressed by exposure to HBE-II. At concentrations of 12.5 and 25 μg/mL of HBE-II, the number of cells able to penetrate through the Matrigel-coated filters was decreased compared with control cells, suggesting a role of HBE-II in suppressing breast cancer metastasis.

In summary, polysaccharide fractions of hallabong peel extract (HBE-I, -II, and -III) significantly inhibited angiogenesis in a dose-dependent manner. Tube formation of HUVECs appeared to be more sensitive to HBE-II than other HBEs. HBE-II also inhibited breast cancer cell migration through downregulation of MMP-9 in triple-negative breast cancer MDA-MB-231 cells. Therefore, the inhibitions of tube formation and MMP-9-mediated migration are likely to be important therapeutic targets in triple-negative breast cancer metastasis.

Acknowledgements

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