Food Sci. Biotechnol. Vol. 18, No. 6, pp. 1423 ~ 1429 (2009)

Food Science and Biotechnology

Extracts from *Rhizopus oryzae* KSD-815 of Korean Traditional *Nuruk* Confer the Potential to Inhibit Hypertension, Platelet Aggregation, and Cancer Metastasis *in vitro*

Sang-Jin Lee[1,3], Hyun-Jin Bae[1], Jiyeon Ryu[1], Daeyoung Lee[2], Gye-Won Kim[3], Namin Baek[2], Moosik Kwon[1], and Sungyoul Hong[1]*

[1]*Department of Genetic Engineering, Sungkyunkwan University, Suwon, Gyeonggi 440-746, Korea*
[2]*Natural Products Chemistry Laboratory, Department of Biotechnology, Kyung Hee University, Yongin, Gyeonggi 446-701, Korea*
[3]*Research Laboratories, Kooksoondang Brewery Co., Ltd., Seongnam, Gyeonggi 462-120, Korea*

Abstract *Rhizopus oryzae* KSD-815 was isolated from *nuruk* that has been used to make Korean traditional wines. This study was performed to investigate the effect of cultures of *R. oryzae* KSD-815 on cardiovascular disorders and cancer metastasis. Firstly, these cultures were sequentially fractionationed with *n*-hexane (TAHe), ethylacetate (TAE), *n*-butanol (TAB), and H_2O (TAW). The TAE inhibited the activity of angiotensin-converting enzyme (ACE) and TAB suppressed platelet aggregation *in vitro*. TAE and TAB inhibited cell motility of human breast cancer cells. Furthermore, TAW interrupted the formation of neo-vasculature and tube-like structure, and down-regulated the expression of angiogenic factors, basic fibroblast growth factor (bFGF), tumor necrosis factor-α (TNF-α), and hypoxia-inducible factor-1α (HIF-1α) in breast cancer cells. These results indicated that cultures of *R. oryzae* KSD-815 display the inhibitory activities on hypertension, platelet aggregation, and metastasis, and suggest that these cultures might be further probed for the purposes as therapeutic agents or dietary supplements.

Key words: *Rhizopus oryzae* KSD-815, Korean traditional *nuruk*, anti-hypertension, platelet aggregation, anti-metastatic activity

Introduction

Recent increase in consumption of red wine has been putatively associated with the reports concerned with lowering the risk of developing coronary heart disease (1). Epidemiological studies have shown that the mortality rate from coronary artery disease (CAD) in France is approximately half the rate in other Western European countries and the United States, despite similar intakes of dietary saturated fat and alcohol. This observation is referred to as the 'French paradox' (2,3). Therefore, as wines tend to enhance the quality of life and individual health, interest in the functionalities of red wines, in general, and in Korean traditional alcoholic beverages, in particular, has increased dramatically over the past few years.

The *nuruk* (Korean-style mold bran) consists of unboiled raw barley and various grains. The barley and grains are ground to a paste, moistened, and then naturally inoculated by airborne microorganisms composed mostly of fungi, yeasts, and other bacteria (4,5). Useful fungi with high saccharogenic and dextrinogenic ability have been isolated from the *nuruk,* including *Aspergillus* sp., *Absidia* sp., *Rhizopus* sp., and *Saccharomyces cerevisiae* (4,6). The suppression of the serum cholesterol levels and the decrease in the amount of oxygen free radical in rat were reported (7).

*Corresponding author: Tel: +82-31-290-7862; Fax: +82-31-290-7895
E-mail: syhong@skku.edu
Received June 19, 2009; Revised September 16, 2009;
Accepted September 16, 2009

Korean traditional alcoholic beverages have long been brewed by classical methods using *nuruk*, rice, flour, yeast, and some medicinal herbs that have been used as folk remedies because of their curing effects (8). Recently, many research groups have been involved in studies on the various aspects such as fermentation characteristics, the qualitative improvement, the amelioration in microorganisms, and the standardization of the manufacturing processes (9,10). In addition, angiotensin I converting enzyme (ACE) inhibitory activity, superoxide dismutase (SOD)-like activity, fibrinolytic activity, and electron donating ability (11-13) were reported. Chung *et al*. (14) showed the preventive effects on the circulatory problems and decrease of low density lipoprotein (LDL) cholesterol levels in a high fat diet model *in vivo*.

Previously, *Rhizopus oryzae* KSD-815, which can be brewed with raw rice starch and has high glucoamylase activity, was isolated from *nuruk* (4). Whole extract of alcoholic beverage fermented with this strain suppressed cancer cell proliferation and exerted an anti-metastatic effect on B16BL6 mouse melanoma cells injected into C57/BL6 mice (15,16). In addition, this extract showed a potent gastroprotective effect on acute gastric ulcer *in vivo* (17). However, the physiological characteristics and activities of the strain responsible for these effects remained unclear.

In this study, we prepared the fractions of methanol extracts from *R. oryzae* KSD-815 that was isolated and purified from the *nuruk*, and evaluated the physiological activities of these fractions, particularly focusing on the anti-cardiovascular disorders and anti-metastatic activities.

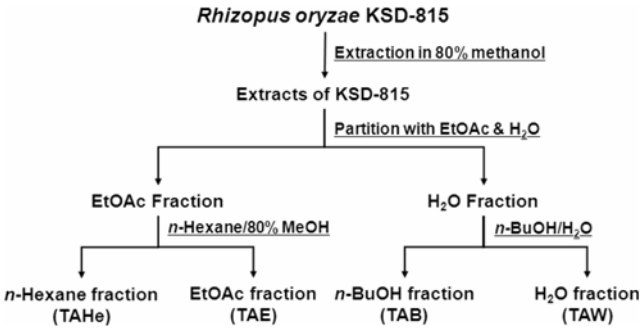

Fig. 1. The procedure for producing *nuruk* and its fractions.

Materials and Methods

Chemicals Recombinant human basic fibroblast growth factor (bFGF) was a kind gift of Dr. Lee, SH (Dong-A Pharmaceutical Co., Ltd., Seoul, Korea). Dulbecco's modified Eagle's minimum essential medium (DMEM), Medium 199 (M199), fetal bovine serum (FBS), heparin, penicillin, and streptomycin were purchased from Gibco-Invitrogen (Grand Island, NY, USA). Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Malony-murine leukemia virus (M-MLV) reverse transcriptase was from Rexgene Biotech (Ochang, Chungbuk, Korea). Polymerase chain reaction (PCR) premix was purchased from Bioneer (Daejeon, Korea). Fertilized eggs for chorioallantoic membrane (CAM) assay were purchased from Yangsung Animal Farm (Yongin, Gyeonggi, Korea). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Preparation of *nuruk* extracts Powdered dry *nuruk* (8 kg) was extracted 3 times with 80% methanol (10 L each) at room temperature. The removal of solvent under vacuum afforded methanol extract (1,227 g). The extracts were partitioned with H_2O (3 L) and ethylacetate (3 L×2), and the latter was evaporated under vacuum. The ethylacetate layer (231 g) was suspended in 80% methanol (2 L) and further extracted with *n*-hexane (2 L×2). Concentrating each layer under vacuum yielded the *n*-hexane fraction (TAHe, 135 g) and the ethylacetate fraction (TAE, 92 g), respectively. The H_2O layer was partitioned with *n*-butanol (2 L×2) and evaporated under vacuum to provide the *n*-butanol (TAB, 114 g) and aqueous (TAW, 870 g) fractions, respectively. The procedure to obtain the extracts and fractions of *nuruk* is depicted in Fig. 1.

Cell culture The human breast cancer cell lines, MDA-MB-231 and MDA-MB-468 were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 units/mL). Human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Lee, SH (Dong-A Pharmaceuticals). HUVECs were cultured in M199 with 20% FBS, 100 units/mL penicillin, 100 units/mL streptomycin, 3 ng/mL bFGF, and 5 units/mL heparin. HUVECs used in this study were from passages 3 to 7. All cell lines were incubated at 37°C in a humidified 5% CO_2.

Assay of ACE activity ACE (EC 3.4.15.1) activity was assayed according to the method of Cushman and Cheung (18). *In vitro* enzyme activity was quantified by means of hippuric acid formation by reacting hippuryl-histidyl-leucine (HHL) with ACE in the presence or absence of fractions. The decrease in absorbance is proportional to the inhibition caused by the fractions. Each 150 mL assay mixture contained ACE solution (20 mU) in 150 mM borate buffer (pH 8.3) with 300 mM NaCl, 50 mL of the sample solution, and 50 mL of 20 mM HHL as substrate, added in that order. The assay mixture was incubated at 37°C for 30 min and the reaction was terminated by adding 1 mL of 1.75 M HCl. The hippuric acid (HA) formed by ACE activity was extracted with 1 mL of ethylacetate. The solvent was then removed by heat evaporation and the HA was then redissolved in water. The amount of HA was measured at 228 nm on an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Sunnyvale, CA, USA). The percent of inhibition was calculated from the following equation:

$$\text{Inhibition } (\%)=[(E_c-E_s)/(E_c-E_b)]\times 100$$

where *E* is the absorbance, *s* is the reaction mixture (sample), *c* is the buffer (control), and *b* is the blank when stop solution was added before the reaction occurred.

Washed platelet preparation and platelet aggregation assay Blood was withdrawn from the artery of male Sprague-Dawley rat (200-250 g) and collected directly into 0.15 (v/v) of anticoagulant citrate dextrose solution that contained 0.8% citric acid, 2.2% trisodium citrate, and 2% dextrose (w/v). Washed platelet-rich plasma was obtained by centrifugation of rat blood at 230×g for 10 min. Platelets were sedimented by centrifugation of the platelet-rich plasma at 800×g for 15 min and then washed with HEPES buffer [pH 6.5, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, and 3.8 mM HEPES] containing 0.35% bovine serum albumin and 0.4 mM ethylene glycol tetraacetic acid (EGTA). The washed platelets were resuspended in HEPES buffer (pH 7.4) and adjusted to 4×10^8 cells/mL. Platelet aggregation was measured with an aggregometer (Chrono-Log Co., Harvertown, PA, USA) according to turbidimetry methods of Born and Cross (19). Briefly, washed platelet suspension was incubated at 37°C in the aggregometer with stirring at 1,000 rpm, and then distilled water or various concentrations of fractions from *R. oryzae* KSD-815 were added. After preincubation for 3 min, platelet aggregation was induced by addition of collagen (5 mg/mL) or adenosine diphosphate (ADP) (5 mM). Aspirin was used as a positive control.

Wound healing assay A confluent monolayer of MDA-MB-231 (2×10^5 cells/well in a 24-well plate) was scratched with a 200-μL tip to create a cell-free zone about 1 mm wide in each well. The medium was then replaced with complete medium containing 50 μg/mL of TAE, TAB, or TAW. After 24 hr, the cells were observed under the inverted light microscope.

***Ex vivo* CAM assay** The CAM assay was carried out by the method of Oikawa *et al.* (20). Fertilized eggs were maintained in a humidified incubator at 37°C. After 3.5 days, small holes were drilled at the narrow end of the eggs

and about 3 mL of albumin were aspirated from the eggs with a 22-gauge hypodermic needle through the holes, allowing the small CAM and the yolk sac to drop away from the shell membrane. The shell covering the air sac was removed with forceps. At the 4.5-day-old chick embryo stage, 1 μL of 10 μL/mL TAE, TAB, or TAW fraction was loaded onto a Thermanox coverslip (Nunc, Naperville, IL, USA) surface and dried. Retinoic acid and dimethyl sulfoxide (DMSO) were used as positive and negative controls at 1 μg and 10 μL, respectively. Two days later, 3-4 mL of Intralipose (Green Cross, Suwon, Gyeonggi, Korea) were injected into the embryo chorioallantois using a 22-gauge needle. The eggs were observed at day 7 under a microscope. Twenty eggs were used at each concentration. The percentage relative activity was calculated as the number of avascular eggs/total number of viable eggs.

HUVEC tube formation assay Solid gels were prepared according to the manufacturer's manual on a 48-well tissue culture plate. HUVEC incubated in M199 with 1% FBS for 6 hr were harvested, seeded onto the surface of Matrigel at a density of 1×10^5 cells/well and then followed by the addition of TAB and TAW. After 24 hr, tube formation was observed under an inverted light microscope at 40× magnification. Microscopic fields were photographed with a digital camera (PowerShot A630; Canon, Tokyo, Japan).

Reverse transcriptase-PCR (RT-PCR) Human breast cancer cells, MDA-MB-468, were treated with TAW. After 24 hr, total cellular RNA was isolated from cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For each sample, RNA was reverse-transcribed into cDNA with M-MLV reverse transcriptase using a thermal cycler GeneAmp (PCR system 2400; Perkin-Elmer, Boston, MA, USA) and CG1-96 palm cycler (Corbett Research, Sydney, AU). The primers used in this work were: vascular endothelial cell growth factor (VEGF) (sense 5'-GCC TCG CCT TGC TGC TCT ACC TC-3'; antisense 5'-GTT CTG TCG ATG GTG ATG GTG TG-3'), bFGF (sense 5'-ACT TCA AGG ACC CCA AGC GG-3'; antisense 5'-GCT CTT AGC AGA CAT TGG AA-3'), tumor necrosis factor-α (TNF-α) (sense 5'-AAG CCT GTA GCC ATG TTG TAG C-3'; antisense 5'-GAA GAC CCC TCC CAG ATA GAT G-3'), hypoxia-inducible factor-1α (HIF-1α) (sense 5'-CTC AAA GTC GGA CAG CCT CA-3'; antisense 5'-CCC TCG AGT AGG TTT CTG CT-3'), and GAPDH (sense 5'-CCT GGC CAA GGT CAT CCA T-3'; antisense 5'-GCC ATG TAG GCC ATG AGG T-3'). PCR was performed in 20 μL of solution containing the primers, PCR Premix, and RT products. After preincubation for 3 min for 94°C, 30 cycles (94°C for 30 sec, 54-56°C for 45 sec, and 72°C for 30 sec) of amplification were performed, followed by 3 min extension at 72°C. GAPDH was used as an internal control. Each PCR reaction was analyzed on 1.2% agarose gel containing 0.6 μL/mL ethidium bromide.

Results and Discussion

Fractionation of cultures of *R. oryzae* KSD-815 The cultures of *R. oryzae* KSD-815 were fractionationed to perform the activity-base assays. The 80% methanol extract of *R. oryzae* KSD-815 was fractionated with various solvents of different polarity such as hexane, ethylacetate, butanol, and water to obtain the most bioactive extract with the final aim to identify the chemical class responsible for the biological activities. In further study, we demonstrated that major active compounds of TAHe and TAE were identified as polyunsaturated fatty acids (PUFAs) and steroids and TAW was composed of more polar compounds than TAHe and TAE fraction such as oligosaccharides (data not shown).

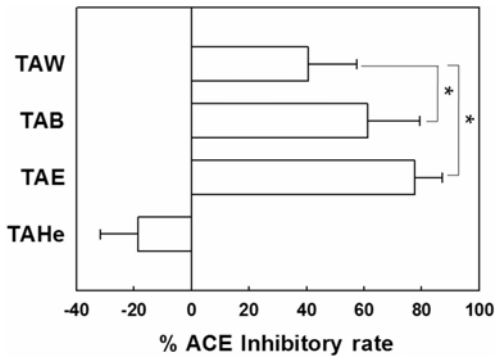

Fig. 2. ACE inhibitory activities of each fraction from *R. oryzae* KSD-815. Fractions of *n*-hexane (TAHe), ethylacetate (TAE), *n*-butanol (TAB), and H_2O (TAW). Data are expressed as % of ACE inhibition at a concentration of 50 mg/mL. The results are the mean±SD of 3 independent experiments (*$p<0.05$; Student's *t*-test).

Inhibitory effect on hypertension and platelet aggregation ACE plays an important role in the regulation of blood pressure as well as cardiovascular function and inhibition of ACE results in a decrease in blood pressure (21). PUFAs as ACE inhibitors are useful to lower blood pressure through modulating rennin formation, ACE activity, and endothelial nitric oxide (eNO) generation. Essential fatty acids (EFAs) and their long-chain metabolites inhibit platelet activation and prevent platelet aggregation (22). The blood pressure lowering mechanism of dietary α-linoleic acid is involved in the reduction of ACE activity and mRNA expression levels in the aorta of spontaneously hypertensive rats (23).

To elucidate the relationship between *R. oryzae* KSD-815 and blood pressure, we performed ACE assays in the presence of each fraction extracted from *R. oryzae* KSD-815. As shown in Fig. 2, TAE fraction showed 77.7% ACE inhibitory activity, which was the highest among the fractions. The inhibitory effects for TAB and TAW were 61.2 and 40.5%, respectively. However, the TAHe fraction, which had strong non-polar properties, did not exhibit the inhibitory activity.

To investigate whether fractions of *R. oryzae* KSD-815 inhibit blood clotting, platelet aggregation assays were performed with ADP-induced (Fig. 3A) and collagen-induced (Fig. 3B) plasma. Fractions of TAHe, TAE, TAB, and TAW inhibited ADP-induced platelet aggregation by 13.51, 88.44, 82.22, and 75.56%, respectively, at the highest concentration (10 mg/mL). Meanwhile, these fractions exhibited 38.6, 68.25, 96.83, and 14.25% inhibition, respectively, in collagen-induced platelet aggregation when

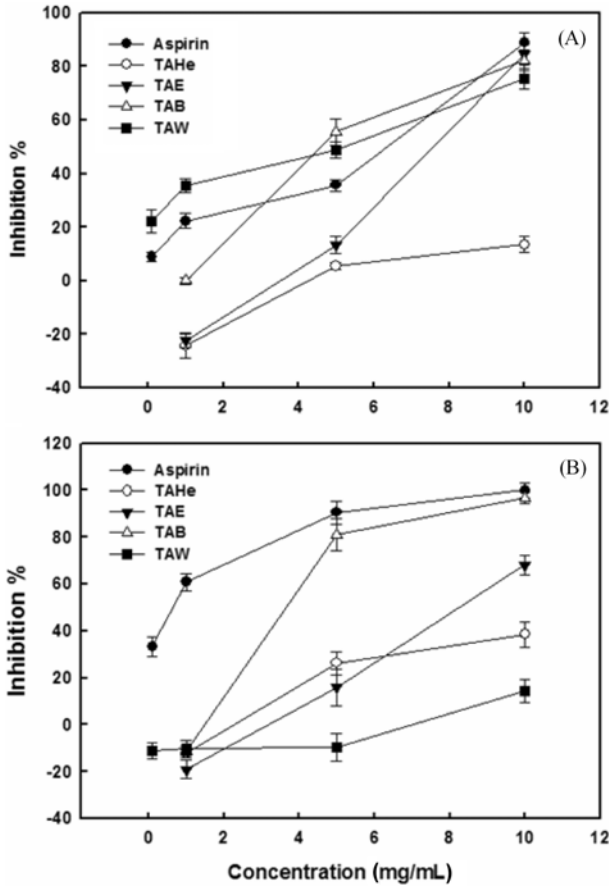

Fig. 3. Effects of fractions from *R. oryzae* KSD-815 on washed rat platelet aggregation induced by ADP (A) and collagen (B) *in vitro*. Aspirin was used as a positive control. Fractions of *n*-hexane (TAHe), ethylacetate (TAE), *n*-butanol (TAB), and H_2O (TAW). The results are the mean±SD of 3 independent experiments.

treated with 10 mg/mL of the fractions. TAB dose-dependently inhibited platelet aggregation induced by both antagonists and had similar potency with positive control, aspirin. Disruption of the endothelium allows platelet to contact with and adhere to subendothelial structures, such as collagen in order to provide an active membrane surface (24,25) and to interact with soluble agonists, such as ADP and thrombin (26). Platelet-derived ADP plays a major role in collagen-induced aggregation of rat platelet (27). Thus, the inhibition of collagen-induced aggregation by TAB is most probably attributed to inhibition of the effects of ADP.

Inhibitory effect on migration and angiogenesis of cancer cells Human breast cancer cells, MDA-MB-231 were used for wound healing assays because of their higher migration potential. Twenty-four hr after making the wound, the 'road' in the center of non-treated cells was recovered in this cell line. As shown in Fig. 4, extracts of *R. oryzae* KSD-815 revealed higher inhibitory action when added to MDA-MB-231 cells. In these cells, whole extracts inhibited wound healing to the same extent. Among the fractions, TAE and TAB had stronger effects than TAW. Despite the difference of intensity among each fraction, all *R. oryzae* KSD-815 extracts, TAE, TAB, and TAW, inhibited the migration of cancer cells tested.

Angiogenesis plays a role in pathologic processes such as the growth and metastasis of tumors. There have been reports that oligosaccharide derivate significantly inhibited an anti-angiogenic activity based on a CAM model and the human microvascular endothelial cells (HMEC) tube formation (28). Oligosaccharides exhibited anti-angiogenic activity in a protein tyrosine kinase (PKT)-dependent manner (29) and represent a novel and exciting therapeutic approach that targets a spectrum of angiogenic molecules that cannot be inhibited through established drug development programmes (30).

Firstly, the anti-angiogenic effect of *R. oryzae* KSD-815 fractions was examined in the CAM assay. After loading each fraction and control samples, 12-16 eggs among 20 had survived. Retinoic acid showed 66.7% relative activity as a positive control. The inhibitory effect of each fraction in neovascularization ranged from 50 to 69.2% (Table 1). The fraction TAW substantially inhibited *in vivo* angiogenesis. As shown in Table 2, TAW inhibited new blood vessel growth in a dose-dependent manner. Outstanding avascular zone formed in the presence of 100 μg/mL of the TAW fraction (Fig. 5d); DMSO, as a negative control, had no effect on formation of new vasculature (Fig. 5a). Based on the results of the wound healing and CAM assays, the *R.*

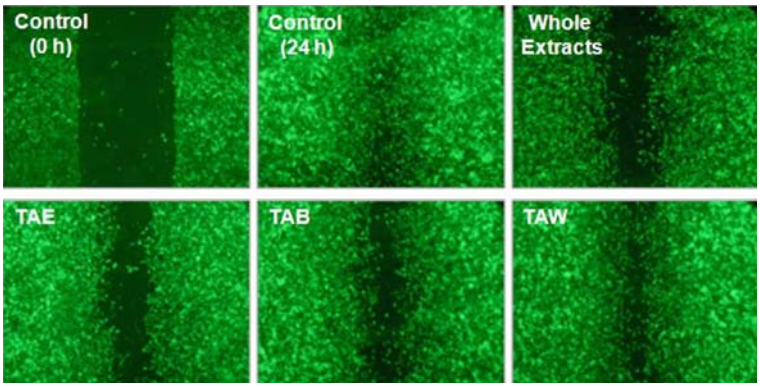

Fig. 4. Effects of fractions on cell motility using wound-healing assay. Wound healing assay was performed with MDA-MB-231 human breast cancer cells. Cells were treated with 50 μg/mL of each *R. oryzae* KSD-815 fraction. Cells were then observed under a microscope. Fractions of *n*-hexane (TAHe), ethylacetate (TAE), *n*-butanol (TAB), and H_2O (TAW).

Table 1. Effects of fractions from *R. oryzae* KSD-815 on angiogenic activity

Fractions	Final concentration (μg/mL)	No. of viable eggs	No. of positive eggs	% Relative activity[1)]
0.1% DMSO (μL)	10	13/20	0	0
Retinoic acid (μg)	1	12/20	8	66.7
Ethylacetate	10	15/20	9	60.0
Butanol	10	16/20	8	50.0
H_2O	10	13/20	9	69.2

[1)]Each assay employed about 20 eggs/sample. Relative activity (%)=(number of positive eggs/number of viable eggs)×100.

Table 2. Effects of H_2O fraction from *R. oryzae* KSD-815 on angiogenic activity

		Final concentration (μg/mL)	No. of viable eggs	No. of positive eggs	% Relative activity[1)]
0.1% DMSO (μL)		10	13/20	1	7.69
Retinoic acid (μg)		1	12/20	8	66.7
	1	10	12/20	6	50.0
H_2O	10	10	13/20	9	69.2
	100	10	15/20	11	73.3

[1)]Each assay employed 20 eggs/sample. Relative activity (%)=(number of positive eggs/number of viable eggs)×100.

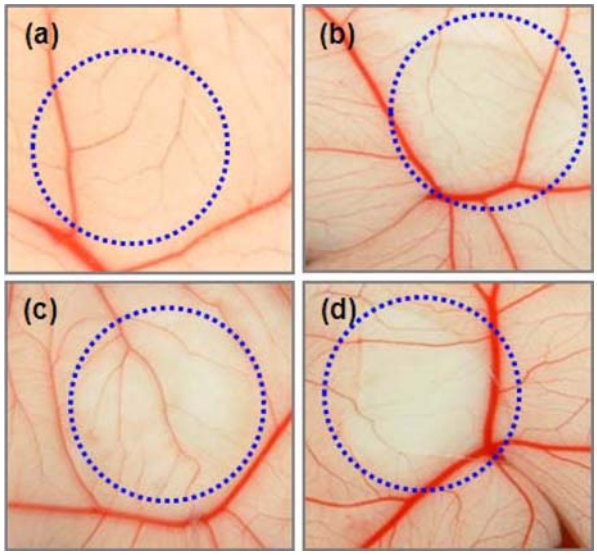

Fig. 5. Inhibitory activities on angiogenesis in chorioallantoic membrane (CAM). Photographs of TAW-treated CAM were selected as represents because of high relative activity. Dotted-lined circle represents the region where the coverslip was loaded. 1% DMSO was used as a negative control (a) and TAW was loaded at (b) 1, (c) 10, or (d) 100 μg/mL.

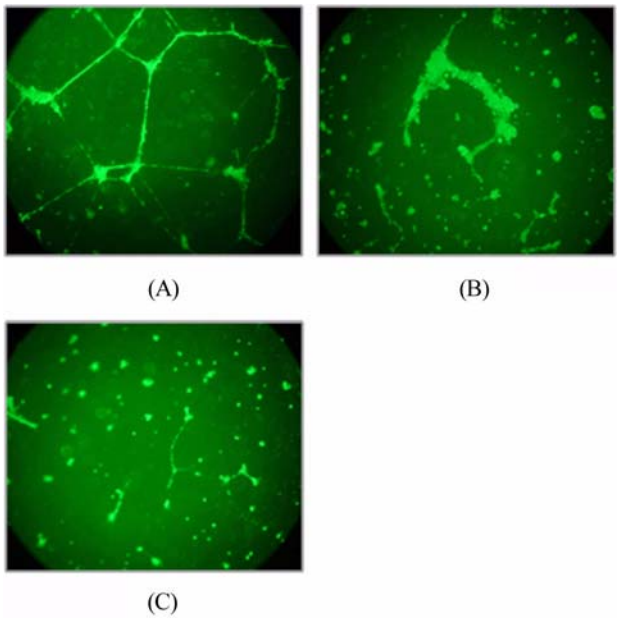

Fig. 6. Effect of fractions on tube formation of HUVEC on reconstituted basement membrane. Cells were plated on reconstituted gel, exposed at the concentration of 50 μg/mL and observed 24 hr later. (A), control; (B), TAB; (C), TAW.

oryzae KSD-815 extract and its fractions appear to have biological activities that inhibit cancer metastasis including cell motility and angiogenesis.

The effect of TAB and TAW from *R. oryzae* KSD-815 was examined in *in vitro* angiogenesis model using HUVECs. As shown in Fig. 6A, HUVECs formed the elongated and robust tube-like structure in the presence of angiogenic factors. TAB formed incomplete and narrow tube-like structure in HUVECs (Fig. 6B). Treatment of HUVECs with TAW led to a complete suppression of HUVEC tube formation (Fig. 6C).

To investigate the changes in mRNA expression with TAW from *R. oryzae* KSD-815, RT-PCR was performed for several angiogenic factors in human breast cancer cells, MDA-MB-468. VEGF is a strong inducer of angiogenesis and is up-regulated in various tumor types and endothelial cells (31). Despite the inhibitory activities of TAW on neovascularization and tube formation, TAW did not affect the VEGF expression level even at high concentrations (Fig. 7). Interestingly, the major regulators of VEGF expression, HIF-1α and TNF-α, were down-regulated in our experiments. In addition, another important regulator of tumor angiogenesis, bFGF was also underexpressed in the presence of TAW (10 μg/mL or more). As previously shown, HIF-1α can be used as a prognostic factor for breast cancer because of its overexpression. Furthermore, according to recent results, bFGF triggered hypoxic

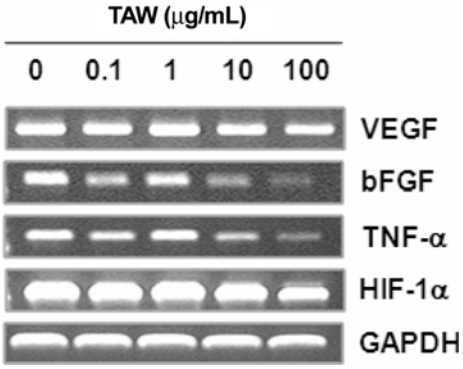

Fig. 7. The transcriptional expression level of representative angiogenic factors. Human breast cancer cell lines, MDA-MB-468 were treated with indicated concentrations of TAW and incubated for 24 hr.

induction of HIF-1α and release of VEGF in T47D breast cancer cells (32). Moreover, TNF-α can induce HIF-1α overexpression (33). Thus, we hypothesized that TAW can suppress tumor angiogenesis by inhibiting the expression of TNF-α and bFGF, and down-regulation of those factors can reduce expression of HIF-1α.

In summary, we evaluated the inhibitory effects of fractions from *R. oryzae* KSD-815 cultures on hypertension, platelet aggregation, migration of cancer cells, and angiogenesis. Ethylacetate fraction showed the *in vitro* anti-hypertensive activity assessed by ACE inhibitory assay. Butanol fraction exhibited a dose-dependent inhibitory effect on ADP- and collagen-induced *in vitro* platelet aggregation. Ethylacetate and butanol fractions caused the attenuation of cell migration in breast cancer cells, MDA-MB-231. In addition, H_2O fraction displayed a potent anti-angiogenic activity in the CAM neovascularization model. Treatment with H_2O fraction resulted in the suppression of tube formation of HUVECs and a significant downregulation of angiogenic factors, TNF-α, bFGF, and HIF-1α in human breast cancer cells, MDA-MB-468.

Acknowledgments

This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea (506008-03-2-CG000).

References

1. Agli MD, Busciala A, Bosisio E. Vascular effects of wine polyphenols. Cardiovasc. Res. 63: 593-602 (2004)
2. Renaud S, de Lorgeril M. Wine, alcohol, platelets, and the French paradox for coronary artery disease. Lancet 339: 1523-1526 (1992)
3. Tunstall-Pedoe H, Kuulasmaa K, Mahonen M, Tolonen H, Ruokokoski E, Amouyel P. Contribution of trends in survival and coronary-event rates to changes in coronary heart disease mortality: 10-years results from 37 WHO MONICA project populations: Monitoring trends and determinants in cardiovascular disease. Lancet 353: 1547-1557 (1999)
4. Shon SK, Rho YH, Kim HJ, Bae SM. *Takju* brewing of uncooked rice starch using *Rhizopus koji*. Korean J. Appl. Microbiol. Biotechnol. 18: 506-510 (1990)
5. Yu TS, Yeo SH, Kim HS. A new species of hypmycetes, *Aspergillus coreanus* sp. nov., isolated from traditional Korean *nuruk*. J. Microbiol. Biotechn. 14: 182-187 (2004)
6. Jeon BY, Kim SJ, Kim DH, Na BK, Park DH, Tran HT, Zhang R, Ahn DH. Development of a serial bioreactor system for direct ethanol production from starch using *Aspergillus niger* and *Saccharomyces cerevisiae*. Biotechnol. Bioprocess Eng. 12: 566-573 (2007)
7. Yoon CG, Chae SN, Huh NE, Kim HS, Yu TS. Effects of *nuruk* or wheat bran supplemented diet on the serum levels of cholesterol and activities of hepatic oxygen free radical metabolizing enzymes rats. J. Korean Soc. Food Sci. Nutr. 28: 212-217 (1999)
8. Jung H, Kang JC. Documental studies on anti-cancer therapy by using medi-alcohol. J. Korean Oncol. 5: 119-136 (1999)
9. Kim HR, Ahn BH. Research trend of Korean traditional alcoholic beverage. Food Ind. Nutr. 6: 5-10 (2001)
10. Lee SJ, Kwon YJ, Kim HR, Ahn BH. Chemical and sensory characterization of Korean commercial rice wines (*yakju*). Food Sci. Biotechnol. 16: 374-380 (2007)
11. Kim JH, Lee DH, Choi SY, Lee JS. Characterization of physiological functionalities in Korean traditional liquors. Korean J. Food Sci. Technol. 34: 118-122 (2002)
12. Kim JH, Lee JH, Kim HJ, Choi SY, Lee JS. Effects of barley *koji* and legumes on the quality and fibrinolytic activity of Korean traditional rice wine. J. Korean Soc. Food Sci. Nutr. 32: 1066-1070 (2003)
13. Kim JH, Jeong SC, Kim NM, Lee JS. Effect of Indian millet *koji* and legumes on the quality and angiotensin I-converting enzyme inhibitor activity of Korean traditional rice wine. Korean J. Food Sci. Technol. 35: 733-737 (2003)
14. Chung HS, Hong SH, Do KR, Rhee HK, Jung SK, Hwang WJ, Kim HM. MooPoong (*Gye Young Jeong*) increases HDL-cholesterol but decreases LDL cholesterol and body weight. Immunopharm. Immunot. 26: 225-232 (2004)
15. Chung KS, Oh WT, Nam SM, Son BS, Park YS. Effect off Korean rice-wine (*yakju*) on *in vitro* and *in vivo* progression of B16BL6 mouse melanoma and HRT18 human colon adenocarcinoma cells. Korean J. Food Sci. Technol. 30: 1470-1475 (1998)
16. Kim SJ, Ko SH, Lee WY, Kim GW. Cytotoxic effects of Korean rice-wine (*yakju*) on cancer cells. Korean J. Food Sci. Technol. 36: 812-817 (2004)
17. Kim SJ, Baek JY, Park CK, Kim GW. Gastroprotective effect of Korean rice-wine (*yakju*). Korean J. Food Sci. Technol. 36: 818-822 (2004)
18. Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol. 20: 1637-1648 (1971)
19. Born GV, Cross MJ. The aggregation of blood platelets. J. Physiol. 168: 178-195 (1963)
20. Oikawa T, Hirotani K, Nakamura O, Shudo K, Hiragun A, Iwaguchi T. A highly potent antiangiogenic activity of retinoids. Cancer Lett. 48: 157-162 (1998)
21. Li GH, Liu H, Shi YH, Le GW. Direct spectrophotometric measurement of angiotensin I-converting enzyme inhibitory activity for screening bioactive peptides. J. Pharmaceut. Biomed. 37: 219-224 (2005)
22. Das UN. Essential fatty acids and their metabolites could function as endogenous HMG-CoA reductase and ACE enzyme inhibitors, anti-arrhythmic, anti-hypertensive, anti-atherosclerotic, and anti-inflammatory, cytoprotective, and cardioprotective molecules. Lipids Health Dis. 15: 7-37 (2008)
23. Oqawa A, Suzuk Y, Aoyama T, Takeuchi H. Dietary alpha-linoleic acid inhibits angiotensin-converting enzyme activity and mRNA expression levels in the aorta of spontaneously hypertensive rats. J. Oleo Sci. 58: 355-360 (2009)
24. Frarndale RW, Sixma JJ, Barnes MJ, De Groot PG. The role of collagen in thrombosis and hemostasis. J. Thromb. Haemost. 2: 561-573 (2004)
25. Sarratt KL, Chen H, Zutter MM, Santoro SA, Hammer DA, Kahn ML. GPVI and α2β1 play independent critical roles during platelet adhesion and aggregate formation to collagen under flow. Blood 106: 1268-1277 (2005)
26. Jackson SP, Nesbitt WS, Kulkarni S. Signaling events underlying

thrombus formation. J. Thromb. Haemost. 1: 1602-1612 (2003)
27. Emms H, Lewis GP. The role of prostaglandin endoperoxides, thromboxane A2, and adenosine diphosphate in collagen-induced aggregation in man and the rat. Brit. J. Pharmacol. 87: 109-115 (1986)
28. Hu K, Liu Q, Wang S, Ding K. New oligosaccharides prepared by acid hydrolysis of the polysaccharides from *Nerium indicum* Mill and their anti-angiogenesis activities. Carbohyd. Res. 26: 198-203 (2009)
29. Ma J, Xin X, Meng L, Tong L, Lin L, Geng M, Ding J. The marine-derived oligosaccharide sulfate (MdOS), a novel multiple tyrosine kinase inhibitor, combats tumor angiogenesis both *in vitro* and *in vivo*. PloS One 3: e3774 (1-11) (2008)
30. Cole CL, Jayson GC. Oligosaccharides as anti-angiogenic agents. Expert Opin. Biol. Th. 8: 351-362 (2008)
31. Jubb AM, Pham TQ, Handy AM, Frantz GD, Peale FV, Wu TD, Koeppen HW, Hillan KJ. Expression of vascular endothelial growth factor, hypoxia inducible factor 1α, and carbonic anhydrase IX in human tumours. J. Clin. Pathol. 57: 504-512 (2004)
32. Shi YH, Bingle L, Gong LH, Wang YX, Corke KP, Fang WG. Basic FGF augments hypoxia induced HIF-1-α expression and VEGF release in T47D breast cancer cells. Pathology 39: 396-400 (2007)
33. Hellwig-Bürgel T, Stiehl DP, Wagner AE, Metzen E, Jelkmann W. Hypoxia-inducible factor-1 (HIF-1): A novel transcription factor in immune reactions. J. Interf. Cytok. Res. 25: 297-310 (2005)