Polyphenol mixtures of *Euphorbia supina* the inhibit invasion and metastasis of highly metastatic breast cancer MDA-MB-231 cells

YOUNG SHIN KO¹, WON SUP LEE², YOUNG NAK JOO¹, YUNG HYUN CHOI³, GON SUP KIM⁵, JIN-MYUNG JUNG³, CHUNG HO RYU⁷, SUNG CHUL SHIN⁶ and HYE JUNG KIM¹

Departments of ¹Pharmacology, ²Internal Medicine and ³Neurosurgery, School of Medicine, Institute of Health Sciences, Gyeongsang National University, Jinju 660-702; ⁴Department of Biochemistry, College of Oriental Medicine, Dongeui University and Department of Biomaterial Control (BK21 Program), Dongeui University Graduate School, Busan 614-052; ⁵School of Veterinary Medicine and ⁶Department of Chemistry, Research Institute of Life Science, Gyeongsang National University, Jinju 660-701; ⁷Division of Applied Life Science (BK21 Program), Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea

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Abstract. The Korean prostrate spurge *Euphorbia supina* is abundant in polyphenols and has been used as a folk medicine in Korea against a variety of diseases. Thus, we aimed to investigate the effect of polyphenol mixtures of Korean *Euphorbia supina* (PES) on the invasion and metastasis of highly metastatic breast cancer MDA-MB-231 cells. Firstly, PES showed no cytotoxicity on cancer cells and endothelial cells (ECs) at the doses of 0.1-10 µg/ml, but showed significant cytotoxicity from 50 µg/ml. Thus, we performed subsequent experiments with PES at doses up to 5 µg/ml. PES dose-dependently suppressed epithelial-mesenchymal transition by downregulating the mesenchymal markers, Snail1 and N-cadherin, showing significant inhibition from 1 and 5 µg/ml, respectively. In addition, PES significantly inhibited MMP-9 activity and LOX release induced by TNF-α at 5 µg/ml. Then, we determined the effect of PES on the expression of adhesion molecules and VE-cadherin phosphorylation. The results showed that PES effectively reduced TNF-α-mediated VCAM-1 expression but not ICAM expression both in the MDA-MB-231 cells and ECs, resulting in the reduced adhesion of MDA-MB-231 to ECs. Finally, PES effectively inhibited MDA-MB-231 cell invasion through ECs, suggesting that PES may serve as a therapeutic agent against cancer metastasis with minimal cytotoxicity to normal cells.

Introduction

Natural compounds exhibit a wide range of anticancer effects, including cell cycle arrest, apoptosis, anti-angiogenesis, and anticancer invasion and migration. Natural phytochemicals containing phenolic compounds have been widely documented to have the capability to prevent cancer metastasis (1). The Korean prostrate spurge *Euphorbia supina* is a weed that belongs to the Euphorbiaceae family. The plant has been used in folk medicine in Korea against a variety of conditions such as bronchitis, jaundice, hemorrhage and gastrointestinal diseases including gastritis, peptic ulcer, diarrhea and hemorrhoid (2,3). It has been reported that this plant contains a number of biologically important organic substances (4-6). Among these, polyphenols have attracted a great deal of interest due to their beneficial effects on human health. Epidemiological studies have shown that polyphenols reduce the risk of chronic diseases (7,8), and have anti-oxidant, anti-aging, and anti-microbial properties (9). According to Song *et al* (10), nine polyphenols have been isolated and identified from Korean *E. supina* and quercetin and kaempferol derivatives account for 84.8% of the total polyphenols. Therefore, the inhibitory effects of *E. supina* on cancer growth and metastasis could be expected; however, few studies have been performed to demonstrate this effect.

Breast cancer is the most common cancer diagnosed in Western European and North American women. Asian
populations are generally at the lowest risk, but the incidence has been steadily increasing. Particularly, in Korea, the incidence of breast cancer has increased by more than four times from 1996 to 2010, showing the highest growth of breast cancer in the OECD countries (11). Most breast cancer patients virtually die of metastasis. Cancer metastasis is the spread of cancer cells from the primary neoplasm to distant sites, where secondary tumors are formed. Its process involves several steps: the entrance of cancer cells from the primary tumor into the vasculature, migration to distant organs, adhesion to endothelial cells lining the blood vessels, extravasation from the blood vessels, and the final proliferation of secondary tumors (12). Thus, to enhance the survival of cancer patients as well as the quality of life, the blockade of the metastatic cascade with natural compounds has gained research interest. In the present study, we investigated the effects of polyphenol mixtures of Korean Euphorbia supina on the invasion and metastasis of highly metastatic breast cancer MDA-MB-231 cells.

Materials and methods

Preparation of polyphenol mixtures of Euphorbia supina (E. supina). Polyphenols from E. supina (PES) were extracted and purified by Professor S.C. Shin as reported in Song et al (10). Briefly, the lyophilized E. supina tissue (10 g) was ground into powder and extracted in ethyl acetate (300 ml) at 80°C for 20 h, and eluted using a mixture of methanol:dichloromethane (1:5, 25 ml). The isolated polyphenol mixtures were identified by HPLC-MS/MS according to a previous method (13). The nine polyphenols in the Korean E. supina were as follows: gallic acid, protocatechuic acid, nodakenin, quercetin-3-O-hexoside, quercetin-3-O-pentoside, kaempferol 3-O-hexoside, kaempferol 3-O-pentoside, quercetin and kaempferol. Quercetin and kaempferol derivatives formed 84.8% of the total polyphenols (10).

Materials. Anti-VCAM-1, anti-ICAM-1, anti-Snail, anti-N-cadherin, anti-β-catenin, anti-E-cadherin, anti-VE-cadherin and anti-LOX antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-VE-cadherin (phospho-Y658) antibody was purchased from Abcam (Cambridge, MA, USA). Matrigel™ basement membrane matrix supplied was purchased from BD Biosciences (San Diego, CA, USA). Enhanced chemiluminescence (ECL) western blotting detection reagent was obtained from Amersham (Buckinghamshire, UK). All other chemicals, including β-actin, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. Human breast cancer cell line, MDA-MB-231, was obtained from the Korean Cell Line Bank (Seoul, Korea) and grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM N2-hydroxyethylpiperazine-N2-2-ethanesulfonic acid, 25 mM NaHCO3, 100 IU/ml penicillin and 10 µg/ml streptomycin. Human umbilical vein endothelial cell line (EA.hy 926 cell) was obtained from the American Type Culture Collection (ATCC) and grown in medium 199 supplemented with 20% FBS, 2 mM L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, 10 µg/ml streptomycin and 50 µg/ml EC growth supplements. Cells were cultured in 100-mm dishes at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Cell viability assay. Cells were seeded at 104 cells/well in 24-well plates. Cells were treated with PES at the indicated doses for 24 h. After treatments, 50 µl of 5 mg/ml MTT solution was added to each well and incubated for 4 h. The supernatants were aspirated, and the formazan crystals were dissolved with 200 µl of 4 N HCl-isopropanol in each well. The optical density of the colored product was measured at 570 nm, as suggested by the manufacturer, using an Infinite 200 microplate reader (Tecan Austria GmbH, Grödig, Austria).

Western blot analysis. Western blot analysis was performed as described previously (14), with minor modifications. Briefly, cells were lysed using PRO-PREP protein extraction solution (iNtRON Biotechnology, Seoul, Korea), and proteins in conditioned media (CM) were concentrated 20-fold with Pierce concentrator 7 ml/9K, MWCO devices (Thermo Pierce, Rockford, IL, USA). The protein concentration was determined by the Bradford method. Aliquots of 50 µg of protein were subjected to 7.5-12.5% sodium dodeyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences UK Ltd.). The membranes were incubated with the indicated primary antibodies. The bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and an ECL western blotting detection reagent (Bionote, Gyeonggi-do, Korea). β-actin was used as a loading control.

Adhesion assay. ECs and MDA-MB-231 cells were treated with PES for 24 h and subsequently stimulated with TNF-α for 6 h. Thereafter, MDA-MB-231 cells (7.5x105 cells/ml) were added to the ECs. After 30 min at 37°C, cell suspensions were withdrawn, and the ECs were gently washed with PBS three times. The cells were then counted under a light microscope, and images were taken using an Olympus microscope (CKX41) equipped with a camera (DS-U3; Nikon).

Matrigel invasion assay. The Matrigel invasion assays were performed using EC coated-Matrigel. ECs were pretreated with PES for 24 h and then washed with PBS three times. After ECs were stimulated with TNF-α for 6 h, MDA-MB-231 cells were added to EC-Matrigel-coated wells and incubated for 24 h. The non-invasive cells that remained on the upper side of the insert were removed. The cells on the lower part of the insert membranes were stained with 4,6-diamino-2-phenylindole (DAPI) and counted under a light microscope.

Gelatin zymography. Media were prepared from MDA-MB-231 cells and concentrated 20-fold using protein concentrators (9K MWCO). Proteins in the media were precipitated with 80% cold acetone. Precipitated proteins were mixed with sample buffer (0.03% bromophenol blue, 0.4 M Tris-HCl pH 7.4, 20% glycerol, 5% SDS) and separated on 8% SDS-polyacrylamide gels containing gelatin (1 mg/ml). Thereafter, the gels were washed with renaturing buffer (2.5% Triton X-100) for 1 h and subsequently incubated for 24 h at 37°C in developing
buffer (50 mM Tris, 20 mM NaCl, 5 mM CaCl₂, 0.02% Brij35, pH 7.5). Gels were stained with 0.05% Coomassie Brilliant Blue R-250 and destained with 50% methanol and 10% acetic acid. Within the blue background, clear zones indicated MMP proteolytic activity.

Statistical analysis. Scanning densitometry was performed using Image Master® VDS (Pharmacia Biotech Inc., San Francisco, CA, USA). The treatment groups were compared using one-way analysis of variance and the post hoc test by Scheffe. All data are expressed as the mean ± standard error of the mean (SEM). P<0.05 was considered to indicate a statistically significant result.

Results

Effect of PES on the cell viability of MDA-MB-231 breast cancer cells and ECs. First, we examined the cell viability of MDA-MB-231 breast cancer cells and ECs in response to PES treatment in a dose-dependent manner (1, 10, 50 and 100 µg/ml). When MDA-MB-231 cells and ECs were treated with the indicated doses of PES for 24 h, the results revealed that the doses of 1 and 10 µg/ml of PES did not decrease the cell viability of both MDA-MB-231 cells and ECs, but the doses of 50 and 100 µg/ml significantly decreased the cell viability of both types of cells (Fig. 1).

PES downregulates the levels of mesenchymal markers and inhibits MMPs and lysyl oxidase (LOX) secretion in TNF-α-treated MDA-MB-231 cells. Next, we observed changes in MDA-MB-231 cell morphology after PES treatment. Fig. 2A showed that PES induced morphologic changes in the MDA-MB-231 cells from a mesenchymal form to an epithelial form in a dose-dependent manner. Furthermore, PES significantly inhibited TNF-α-induced VCAM-1 expression, but not ICAM-1 expression in both the MDA-MB-231 cells and ECs (Fig. 3). Then, we investigated the effect of PES on the adhesion of MDA-MB-231 cells to ECs. The adhesion of the MDA-MB-231 cells to ECs was markedly increased by TNF-α (10 ng/ml, 6 h), compared to unactivated ECs. In contrast, the treatment of PES (0.1-5 µg/ml) to ECs for 1 h before TNF-α stimulation resulted in a significant reduction in the adhesion of the MDA-MB-231 cells to ECs (Fig. 4).

PES inhibits the phosphorylation of VE-cadherin mediated by TNF-α in ECs. Tyrosine phosphorylation of VE-cadherin is known to be associated with weak junctions and impaired barrier function. Therefore, we investigated the effect of PES on the phosphorylation of VE-cadherin at tyrosine residue 658 (Y658) by western blotting. PES treatment 1 h prior to TNF-α decreased TNF-α-induced phospho-VE-cadherin from 1 µg/ml of PES, showing a significant inhibition at 5 µg/ml (Fig. 5).

PES downregulates MDA-MB-231 cell invasion induced by TNF-α. Finally, we examined the effect of PES on MDA-MB-231 cell invasion through ECs. ECs were pretreated with PES for 1 h and stimulated with TNF-α for an
additional 6 h. Then, MDA-MB-231 cells were added to the EC-Matrigel-coated wells and incubated for 24 h. As shown in Fig. 6, PES dose-dependently inhibited MDA-MB-231 cell invasion through TNF-α-stimulated ECs.
Discussion

Great advances in medical science have been achieved. Yet, the population of individuals diagnosed with cancer is steadily increasing, and cancer patients virtually die due to metastasis. Therefore, it is important to develop a strategy to inhibit cancer metastasis with minimal toxicity to normal cells to enhance the quality of life of patients. In this regard, much research has focused on natural compounds with anticancer effects. Particularly, natural phytochemicals containing phenolic compounds are known to prevent cancer metastasis (1). The Korean prostrate spurge Euphorbia supina is reported to contain a number of biologically significant organic substances such as polyphenols and has been used as a folk medicine in Korea against a variety of inflammatory conditions. Therefore, we aimed to ascertain whether the polyphenols in Euphorbia supina (PES) have a suppressive effect on the invasion and metastasis of breast cancer cells.

Cancer metastasis involves several steps in which cellular responses between cancer cells and normal cells are coordinately involved. These include the entrance of cancer cells from the primary tumor into the vasculature, migration to distant organs, adhesion to endothelial cells lining the blood vessels, extravasation from the blood vessels and proliferation of secondary tumors. EMT is a process that converts an epithelial cell to a mesenchymal cell by promoting the loss of cell-cell adhesion, leading to the release of cells from the surrounding tissue, and finally enables cells to acquire the migratory capability to invade. Therefore, EMT can be regarded as an initial process of metastasis, and EMT occurring during tumor progression is considered to be the major mechanism that is responsible for the invasion and metastasis of cancer cells (22-24). Within the tumor microenvironment, tumors release several factors which can promote cancer metastasis; MMPs are involved in the proteolytic digestion of the ECM as well...
KO et al.: INHIBITORY EFFECT OF POLYPHENOL MIXTURES OF Euphorbia supina ON CANCER INVASION

as angiogenesis, which are major steps in cancer invasion. In addition, adhesion molecules such as ICAM-1 and VCAM-1 are involved in cell-cell and cell-ECM interactions and are mechanistically important for the extravasation of cancer cells during metastasis (20,21). In particular, VCAM-1 is expressed preferentially or highly on breast cancer endothelium compared to normal endothelium (25,26). Another important factor is endothelial cell membrane permeability regulated by transmembrane endothelial adherens junctions (AJs). In endothelial cells, AJs are largely composed of VE-cadherin. The phosphorylation, cleavage and internalization of VE-cadherin are thought to affect endothelial permeability (27). Thus, in the present study, we determined the effect of PES on the invasion and metastasis of highly metastatic breast cancer MDA-MB-231 cells.

As expected, PES significantly suppressed EMT by downregulating the mesenchymal markers, Snail1 and N-cadherin. In addition, PES significantly inhibited MMP-9 activity induced by TNF-α at 5 µg/ml. Moreover, the release of LOX, an enzyme that crosslinks ECM proteins such as collagen and promotes breast cancer metastasis, was induced by TNF-α, which was inhibited by PES treatment (mainly at 5 µg/ml). Then, we determined the effect of PES on the expression of adhesion molecules and the phosphorylation of VE-cadherin. The results showed that PES effectively reduced TNF-α-induced VCAM-1 but not ICAM expression in both the MDA-MB-231 cells and ECs, resulting in the decreased adhesion of MDA-MB-231 cells to ECs. Furthermore, PES suppressed LOX secretion by TNF-α, suggesting that PES efficiently inhibited the invasion of MDA-MB-231 cells. Finally, when we assessed whether PES inhibits the invasion of MDA-MB-231 cells through ECs, the results showed that PES effectively inhibited MDA-MB-231 cell invasion through ECs at a very low concentration (0.1 µg/ml) where it showed no cytotoxicity on cancer cells and ECs.

The most abundant flavonoids in the diet, flavonols, exhibit numerous biological and pharmacological effects including anticancer-related properties (28), and quercetin and kaempferol derivatives are the major components of total polyphenols isolated and identified from Korean E. supina. Quercetin (3,5,7,3’,4’-pentahydroxyflavone) is an active component of flavonoids that abundantly exists in many fruits and vegetables, and dietary food sources. Quercetin exhibits various beneficial biological activities, such as anti-oxidant, anti-inflammatory, anti-atherosclerotic, and anti-tumorigenic activities (29-31). Quercetin was found to inhibit hL-60 leukemia cell proliferation in association with the inhibition of cytosolic protein kinase C and membrane tyrosine protein kinase in vitro (32). Furthermore, quercetin exerted anti-proliferative effects against glioma and breast cancer cells (33-35). It has been suggested that quercetin may be a potential anti-invasive compound in breast cancer cells (36,37). In addition, kaempferol and its derivatives also exhibit a wide range of pharmacological activities including anti-oxidant, anti-inflammatory, anticancer, anti-microbial, cardioprotective, neuroprotective, and anti-diabetic activities (38). Kaempferol induced apoptosis in HL-60 leukemia cells which was accom-
panied by significant DNA condensation and increased ATP levels. It also altered the expression of caspase-3 and apoptosis-inducing factor (39). The regular consumption of foods containing kaempferol has been positively correlated to a reduction in the risk for developing several disorders including cancer. In breast cancer, similar to the activity of quercetin, kaempferol exhibited inhibitory activity on the invasiveness and MMP-3 levels in MDA-MB-231 cells (37). Based on our results and these studies, PES containing quercetin and kaempferol may suppress the process of metastasis of highly metastatic breast cancer cells by regulating the adhesion of MDA-MB-231 cells to ECs by inhibiting VCAM-1 expression in ECs, and by reducing EC permeability and inhibiting EMT in MDA-MB-231 cells. Finally, PES may serve as a therapeutic agent against cancer metastasis with minimal cytotoxicity to normal cells.

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References


