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Antimetastatic activity of polyphenol-rich extract of *Ecklonia cava* through the inhibition of the Akt pathway in A549 human lung cancer cells

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ABSTRACT

An ethyl acetate extract (ECE) of a brown alga, *Ecklonia cava*, was examined for its anti-metastatic effect, using A549 human lung carcinoma cells. ECE treatment significantly suppressed the migration and invasion of A549 cells in a concentration-dependent manner. It also strongly down-regulated the matrix metalloproteinase (MMP)-2 activity of the cancer cells by gelatin zymography assay. For elucidating its mechanism of action in cancer cell metastasis, ECE was further investigated for various cell signalling pathways, including JNK, ERK, p38, and Akt. In this, ECE showed an anti-metastatic effect in a concentration- and time-dependent manner by the mechanism of suppression of Akt and p38, but not JNK and ERK. These results, for the first time, suggest that ECE (a polyphenol-enriched, highly anti-oxidative fraction of brown alga, *E. cava*) may have therapeutic potential in metastatic lung cancer, based on its strong inhibitory effects on the migration and invasiveness of A549 human lung adenocarcinoma cells.

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1. Introduction

Lung cancer is now the most common cause of cancer-related deaths, which accounts for more than one million worldwide annual deaths (Jemal et al., 2008). It is one of the most malignant types of human cancer and about 70% of patients die as a result of cancer metastasis. Approximately, 80% of people diagnosed with lung cancer have non-small cell lung cancer (NSCLC), such as adenocarcinoma, which remains an aggressive cancer with poor prognosis. Therefore, our knowledge on the invasion and migration of lung cancer cells is crucial for designing a new therapeutic strategy against the malignant lung tumour.

In cancer cell metastasis, the degradation of extracellular matrix (ECM) is essential; this is associated with the overexpression of proteolytic enzymes, such as matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA). MMP-2 and MMP-9 can degrade most of the ECM components of basal membrane and the strong expression of MMP-2 has been well characterised in highly metastatic human lung cancer cells, such as A549 cell (Chu, Chiou, Chen, Yang, & Hsieh, 2004). Up to now, the search for MMP-2 inhibitors has been a focus of interest for the development of anti-metastatic agents. However, currently available inhibitors have often exhibited severe side effects at their therapeutic doses, including inflammation, musculoskeletal pain and joint stiffness (Whittaker, Floyd, Brown, & Gearing, 1999). Therefore, it is important to develop a novel MMP inhibitor with fewer side effects and a high therapeutic efficacy. It has been achieved by library screenings of various origins such as natural products. For example, curcumin (turmeric) has been observed to block invasion and migration of malignant tumour cells (Duvoix et al., 2005) and it inhibits metastasis in animal models (Menon, Kuttan, & Kuttan, 1999). In murine melanoma cells, the treatment with curcumin reduced the activities of MMP-2 and MT1-MMP (Banerji, Chakrabarti, Mitra, & Chatterjee, 2004).

In general, seaweeds are a rich source of natural antioxidants and some of them are known as pigments (e.g. fucoxanthin, astaxanthin, and carotenoids) and polyphenols (phenolic acid, flavonoid, and tannins) (Heo, Park, Lee, & Jeon, 2005). Among these components, phlorotannins, a class of compounds with polymerised phloroglucinol units found in brown algae, especially in *Ecklonia cava* (*E. cava*), have strong antioxidant activities. *E. cava* is a member of the family of Laminariaceae, which belongs to the order Laminariales, as a perennial brown alga. It is distributed prolifically along the coasts of South Korea and Japan. It has long been used in Korea, in food ingredients, cosmetics and folk medicine in gynecopathy (Ahn et al., 2007). There are several phloroglucinol derivatives in *E. cava*, and its crude methanol extract, and single phlorotannins have proved to have various biological activities, including radical-scavenging (Kang et al., 2004), and anti-allergic (Kim et al., 2008), bactericidal and protease inhibition effects (Ahn et al., 2004). However, the activity of *E. cava* on cancer cell

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metastasis remains poorly understood. In the present study, we have elucidated the anti-metastatic potential of the ethyl acetate fraction of *E. cava* (ECE) and its regulatory mechanism, using A549 human lung carcinoma cells.

2. Materials and methods

2.1. Chemicals and reagents

RPMI 1640 medium without phenol red, Trypsin–EDTA, penicillin–streptomycin–amphotericin B solution, fetal bovine serum (FBS), and phosphate buffer solution (PBS) were from Gibco BRL, Life Technologies (USA). MTT reagent, gelatin (G8150), Folin–Ciocalteu's phenol reagent, and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). LY294002 (a PI3K inhibitor) was from Tocris Cookson Ltd. (Bristol, UK). For ERK1/2, p38, JNK, and Akt, their total and/or phosphorylated protein-specific antibodies were purchased from Cell Signaling Technology (Beverly, MA). Invasion assay chambers ware obtained from BD Biosciences (San Jose, CA, USA).

2.2. Plant material and extraction

E. cava, a brown seaweed, was collected along the coast of Jeju island, South Korea, during the month of March. Fresh *E. cava* was washed three times with tap water to remove salt and impurities, then dried at room temperature for 3 days and stored at −20 °C. The dried samples were homogenised, using a grinder, before extraction. The methanol extracts of *E. cava* were obtained by using previously described methods (Kang et al., 2010) with minor modifications. Briefly, dried *E. cava* powder (10 g) was dissolved in 70% MeOH (200 ml) and shaken for 24 h. After the extraction, the supernatant was recovered by centrifugation (7000 rpm) at 4 °C for 30 min and the methanol was evaporated using a rotary vacuum evaporator (Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The extract powder was suspended in distilled water and then partitioned with *n*-hexane, methylene chloride, ethyl acetate (EtOAc) in sequence. Each of these fractions was evaporated and kept on −20 °C until used. The total polyphenol contents and DPPH radical-scavenging activities of the fractions were determined according to methods previously described (Kang et al., 2010).

2.3. Measurement of total polyphenol content

Total polyphenol content was determined by using the method Zhang et al. (2006) with a minor modification (Zhang et al., 2006). For polyphenol standard, a stock solution (1 mg/ml) of gallic acid (in distilled water) was prepared and the stock was diluted to give working standards of 0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/ml concentrations, respectively. For samples, an aliquot (5 mg) of several seaweeds methanol extract was dissolved in 1 ml of distilled water. To measure total polyphenol content, an aliquot (10 μl) of each sample or standard solution was mixed with 50 μl of Folin–Ciocalteu's reagent in a 96-well microplate format and incubated for 5 min. The reactant was mixed with 40 μl of 7.5% sodium carbonate solution and incubated in a dark place for 2 h. Absorbance was measured at 750 nm with a spectrophotometric microplate reader (BioTek Instruments Inc., Winooski, USA).

2.4. DPPH radical-scavenging assay

The antioxidant activity of *E. cava* methanol extract was determined using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method with a minor modification (Kang et al., 2010). For this, each fraction of *E. cava* extract was prepared as described above. A working solution (6×10^{-5} M) of DPPH· was also freshly prepared by dissolving it in DMSO and was vortex-mixed on the day of experiment. In brief, 900 μl of DPPH· solution was mixed with 100 μl of distilled water (control), or 100 μl of each fraction of *E. cava* extract (sample). For the calibration of sample background, 100 μl of each fraction of *E. cava* extract was mixed with 900 μl of DMSO alone (sample background) instead of DPPH· solution. Butylhydroxytoluene (BHT) and L-ascorbic acid were used as positive controls. An aliquot of control, sample solution, or sample background was transferred to a 96-well microplate and incubated for 1 h in a dark place. The absorbance at 517 nm was measured using a spectrophotometric microplate reader (BioTek Instruments Inc., Winooski, USA) and DPPH radical-scavenging activity of a sample was analysed using the equation as below.

$$\text{DPPH radical-scavenging activity } (\%) = [1(A_1 - (A_2 - A_3))/A_1] \times 100$$

where, A_1 is the absorbance of control; A_2 is the absorbance of sample; A_3 is the absorbance of sample background.

2.5. Cell culture

A549 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 supplemented with 10% FBS, 100 μg/ml of penicillin–streptomycin-amphotericin B solution at 37 °C in a 5% CO_2-humidified incubator. Cells were passaged three times a week by treating with trypsin–EDTA and used for experiments after five passages.

2.6. MTT assay for cell viability

Cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay, as described previously (Kang & Kim, 2010). Briefly, A549 cells were incubated at a density of 4×10^4 cells/well in 24-well plates for 24 h in 500 μl of RPMI 1640 with 10% FBS. The cells were treated with ECE (0–200 μg/ml) for 24, or 48 h. Then, MTT dye (5 mg/ml) was added to the cells and they were incubated for an additional 3 h. After the medium was removed, DMSO was added to the cells for the solubilisation of generated formazan salts. The amount of formazan salt was determined by measuring the optical density (OD) at 540 nm, using a GENios® microplate spectrophotometer (PowerWave™XS, BioTek Instruments Inc., Winooski, USA). Relative cell viability of treatment was calculated as a percentage of vehicle-treated control ([OD of treated cells − OD of blank/OD of control − OD of blank] × 100).

2.7. Wound migration assay

Cell migration assay was performed using 6-well plates, as previously described (Lee et al., 2006). A549 cells were seeded into 6-well plates (1×10^5 cells/ml) and grown to 80–90% confluence for the experiment. After aspirating the medium, cells were scraped with a sterile micropipette tip to create wound. They were washed twice with PBS to remove cellular debris and then replaced with complete RPMI 1640. A549 cells were treated with ECE (0, 12.5, 25, and 50 μg/ml) and incubated for 48 h. Cell migration into the wound area was photographed at the stages of 0 and 48 h, respectively, for the image analysis of each treatment. The level of cell migration was determined using a Hewlett–Packard scanner and NIH Image software (Image J), and then it was expressed as a percentage of each control for the mean of wound closure area.

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