**Pinus maritima** Extract Induces Apoptosis in Human Malignant Melanoma Cells via ROS/Caspase-3 Signaling

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**Abstract**

Melanoma is the most aggressive type of skin cancer due to its rapid metastasis with a high recurrence rate following conventional therapy. Pine bark extract (PBE) from *Pinus maritima* contains numerous phenolic compounds and functions as a potent antioxidant. The present study aimed to analyze the potential anticancer properties of PBE on human malignant melanoma A375 cells. The chemical composition of PBE was determined by high-performance liquid chromatography/photodiode array detector. The effects of PBE on cell death, migration, and invasion were determined using xCELLigence Technology real-time cell analysis. Annexin/propidium iodide flow cytometry and Hoechst 33342 staining were conducted to detect cell apoptosis. PBE induced apoptosis and inhibited cell migration and invasion. Cleaved caspase-3 expression and activity were significantly increased (*P* < 0.01) in cells treated with PBE compared with control cells. PBE ameliorated hydrogen peroxide (H₂O₂)-induced reactive oxygen species (ROS) formation. Treatment of the cells with PBE in the presence of H₂O₂ led to significant (*P* < 0.001) reduction of matrix metalloproteinase-9, which is a mediator responsible for advanced melanoma. PBE induces A375 programmed cell death and suppresses cellular invasion by attenuating the ROS-dependent pathway associated with MMP-9 reduction.

**Keywords**
malignant melanoma, pine bark extract, *Pinus maritima*, phenolic compound, catechin, migration

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**Introduction**

Malignant melanoma, a disease in which malignant cancer cells arise from melanocytes, is known for its rapid progression, metastasis, and high mortality rates. Malignant melanoma accounts for 75% of all skin cancer mortality and its 5-year survival rate is lower than 15% for all metastatic cases.¹ The incidence of malignant melanoma continues to increase significantly worldwide over the last several decades. Exposure to intense and intermittent ultraviolet (UV) irradiation can affect the risk of melanoma.² Despite a wide variety of therapies available to treat melanoma, the prognosis remains very poor.³ Therefore, there is an urgent need to identify and develop novel and effective treatments.

Apoptosis is a programmed cell death pathway that balances cell death with cell survival.⁴ The 2 commonly described initiation pathways of apoptosis are the intrinsic (mitochondrial) and extrinsic (death receptor) pathways.⁵ Many proteins play critical roles in regulating apoptosis in human melanoma including caspases and their upstream regulators, as well as Bcl-2, Bcl-X(L), Mcl-1, and nuclear factor kappa-light-chain-enhancer of activated B cells.⁶ Some molecules such as matrix metalloproteinase (MMP) family members (MMP-1, MMP-2, MMP-9, and MMP-13) are present on the cell surface of invasive tumor cells⁷ and can induce malignant melanoma cell invasion, tumor growth, and metastasis formation.⁸

The pine bark extract (PBE) from *Pinus maritima* Lam. (or *Pinus pinaster* Ait.) has been reported to have major functions as an antioxidant because of its strong free radical-scavenging activity against reactive oxygen species (ROS) and nitrogen...
species\textsuperscript{9} and its anti-inflammatory activity.\textsuperscript{10,11} PBE contains numerous phenolic compounds with a wide variability of chemical structures that differ in polarity and size, from simple phenolic compounds to oligomers such as polyphenolic monomers, proanthocyanidins, and phenolic acids (derivatives of benzoic and cinnamic acids). Some of these components have received considerable attention because of their anti-inflammatory, antimutagenic, antitumorigenic, and anticarcinogenic activities.\textsuperscript{9} Several studies have demonstrated the antimicrobial and antiviral activities of PBE.\textsuperscript{12} Furthermore, PBE may protect against sarcopenia\textsuperscript{13} and may help improve conditions relating to poor circulation, high blood pressure,\textsuperscript{14} osteoarthritis,\textsuperscript{15} and diabetes.\textsuperscript{16} PBE has been reported to have cardiovascular benefits and aid in problems with circulation.\textsuperscript{9} In vivo results showed that PBE can inhibit lipid peroxidation\textsuperscript{17} and platelet aggregation.\textsuperscript{18} In addition, PBE has antitumor effects via activation of caspase 3/9 and can suppress migration and invasion of ovarian cancer cells.\textsuperscript{19} One clinical trial evaluated the proapoptotic activity of the PBE pycnogenol (PYC) on human fibrosarcoma cells.\textsuperscript{20} However, the effects and molecular mechanisms of PBE in malignant melanoma remain unknown. Here we determined the effects of PBE on human melanoma A375 cell proliferation, migration and invasion, as well as caspase activation in the intrinsic pathway. We also examined the effects of PBE on MMP-9 secretion through a ROS-dependent pathway.

## Results

### Catechin Content in PBE Analyzed by HPLC

Procyanidins consist mainly of flavan-3-ol units of (+)-catechin.\textsuperscript{21} PBE was analyzed by high-performance liquid chromatography (HPLC) with detection at 230 nm using HPLC/photodiode array. A peak of catechin was detected eluting at 3.82 minutes. For quercitin was not detected. UV–visible spectroscopy (Vis) wavelength spectral quantification showed that PBE contained ~61.26 mg/g of catechin.

### PBE Reduces A375 Cell Viability and Proliferation

Treatment of A375 cells with PBE resulted in a reduction in cell viability in a concentration-dependent manner (Figure 1(a)). Cell cytotoxicity was significantly observed ($P < 0.05$) following treatment with 5 µg/mL PBE (83.3% ± 1.9%) along with a significant decrease ($P < 0.01$) in cell viability (72.2% ± 1.1%) observed at 50 µg/mL PBE; however, no significant difference ($P > 0.05$) was observed between cells treated with 50 µg/mL PBE and 100 µg/mL PBE, with comparable half-maximal inhibitory concentration values of 12.5 µmol for 24-hour stimulation. Similar results were obtained using xCELLigence real-time cell analysis (RTCA) over 72 hours (Figure 1(b)).

![Figure 1](image-url)

**Figure 1.** Pine bark extract (PBE) suppressed A375 cell viability and proliferation. (a) Dose-dependent inhibitory effects of PBE (1-100 µg/mL) on cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium assay. (b) Effects of PBE (1-50 µg/mL) on cell proliferation evaluated by xCELLigence real-time cell analysis. Data are shown as mean values ± standard deviation from 3 independent experiments. *$P < 0.05$ and **$P < 0.01$ vs control.
cell proliferation reduced in a dose-dependent manner when cells were grown in the presence of PBE from 5 to 50 µg/mL.

PBE Induces Apoptotic Cell Death in A375 Cells

We examined whether the inhibitory effects of PBE on cell viability and proliferation are the result of apoptotic cell death. Morphological analysis with Hoechst staining revealed nuclei with chromatin condensation and apoptotic bodies in cells cultured with PBE (Figure 2(a)), and these effects significantly increased (P < 0.01) in a dose-dependent manner (Figure 2(b)). In contrast, only minimal morphological changes were observed in the control culture. We further examined PBE-induced cell death by flow cytometric analysis of apoptotic subpopulations using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining (Figure 2(c)). PBE induced A375 cell apoptosis in a dose-dependent manner at 5, 25, and 50 µg/mL. Next, the levels of apoptosis-related proteins were determined by Western blotting. Cleaved (ie, activated) caspase-3 in the cells as protein bands with a molecular mass of 17 kDa were detected. PBE dose-dependently induced caspase-3 cleavage (Figure 2(d)), which peaked at 24 hours (Figure 2(e)). In order to identify the enzymatic activity of caspase-3 enzyme during PBE-induced apoptosis, caspase-3 activity was measured following treatment with PBE using specific fluorogenic peptide substrates. Next, Z-DEVD-FMK caspase-3 inhibitor was used to show that its activation is a key step in the PBE-induced apoptosis. Pretreatment with Z-DEVD-FMK dose-dependently prevented PBE-induced chromatin condensation and nuclear blebbing of apoptotic cells (Figure 2(f)). Furthermore, the activity of caspase-3 was significantly (P < 0.01) increased in a concentration-dependent fashion, as compared with untreated control cells (Figure 2(g)). These results demonstrate an association between the cytotoxic effects observed in response to PBE extract and the induction of apoptosis in A375 cells via caspase-3 activity.

PBE Suppresses Cell Migratory and Invasive Properties

The effects of PBE on migratory properties of A375 cells were investigated using uncoated cell invasion and migration (CIM)-16 xCELLigence plates. The rate of cell migration through uncoated membranes was decreased following 32 hours of exposure to 1, 5, 25, or 50 µg/mL PBE compared with control cells grown for the same period without PBE (Figure 3(a)). The results showed a significant decrease (P < 0.05) in cell migration following stimulation from 1 to 50 µg/mL PBE (Figure 3(b)) compared with control (5% fetal bovine serum [FBS]). The effects of PBE exposure were investigated in invasive properties of the cells using CIM-16 xCELLigence plates that had been coated with Matrigel (Figure 3(c)). The rate of cell invasion through Matrigel was decreased in cells following 45 hours of exposure to 25 and 50 µg/mL PBE as compared with cells grown for the same period without PBE as a control (0% and 5% FBS). We found that the concentration of PBE used in this study induced a significant decrease in cell invasion (P < 0.05) compared with data from a control group (Figure 4(d)).

PBE Inhibits H$_2$O$_2$-Mediated ROS and MMP-9 Formation

Oxidative stress refers to elevated intracellular levels of ROS that cause damage to lipids, proteins, and deoxyribonucleic acid (DNA). Pinus maritima PYC exerted strong scavenging activities against ROS generated by hydrogen peroxide (H$_2$O$_2$) and phorbol 12-myristate 13-acetate in RAW 264.7 cells. We next determined the effect of PBE on ROS formation in the presence of H$_2$O$_2$ by CM-H2DCFDA fluorescence staining (Figure 4a). In H$_2$O$_2$-treated A375 cells, ROS level was significantly increased compared with controls (P < 0.001), while PBE from 10 to 50 µg/mL significantly reduced the ROS level in a dose-dependent manner (P < 0.001). MMP-9 induces melanoma cell invasion, tumor growth, and metastasis formation. Cryptotanshinone, a quinoid diterpene isolated from the root of Salvia miltiorrhiza Bunge, has a variety of anticancer actions, including the activation of apoptosis. Recent studies have revealed that cryptotanshinone blocked A375 cell migration and invasion in vitro that was associated with the downregulation of MMP-9. We observed a dose-dependent increase in MMP-9 levels in the supernatant of H$_2$O$_2$-treated A375 cells (Figure 4b). PBE at 5 µg/mL (P < 0.01), 25 µg/mL, and 50 µg/mL (P < 0.001) significantly inhibited MMP-9 release following H$_2$O$_2$ exposure (Figure 4c).

Discussion

Here we report that PBE from P. maritima (or P. pinaster) reduced cell proliferation and cell viability of A375 malignant melanoma cells. We demonstrated that PBE is effective in inducing apoptosis through the activation of caspase-3 activity. Our results also showed that PBE induced dose-dependent reductions of cell migration and invasion that was associated with MMP-9 downregulation following ROS or H$_2$O$_2$ exposure.

Proanthocyanidins are oligomeric proanthocyanidins or condensed tannins. The most widely studied proanthocyanidins are based on flavan-3-ol(-), such as procyanidins, prodelphinidins, and propelargonidins. PYC is a mixture of phenols and polyphenols that is broadly divided into monomers such as (+)-catechin, epicatechin, and taxifolin and condensed flavonoids such as procyanidin B1, B3, B7, and others. PBE from P. maritima is a multicomponent mixture with pharmacologically active substances. The antiproliferative activity of PBE in our study may be attributed to a number of proanthocyanidins and PYC found in the plants.

Maritime pine bark has been reported to have many health benefits including cardiovascular protection, vasorelaxant activity, and the ability to enhance the microcirculation by increasing capillary permeability and also demonstrates a
Figure 2. Pine bark extract (PBE) induced apoptotic cell death and increased caspase-3 activation and activity. (a) PBE induced apoptotic bodies as determined by Hoechst 33342 staining. Cells were treated with 25 or 50 µg/mL of PBE for 24 hours. Photomicrographs of nuclei with chromatin condensation and apoptotic bodies stained cells are shown (arrows). Images were captured digitally at 10× (left panels) and 20× (right panels) magnification. (b) The numbers of nuclei with morphological changes were averaged from four 40× field-of-view images and values were calculated as the percentage of apoptotic cells. (c) Cells were treated with PBE (1-50 µg/mL). PBE-induced cell death was determined by flow cytometric analysis of apoptosis with propidium iodide (PI) and Annexin V double staining. Triplicate experiments were performed. (d) Effect of PBE (5-50 µg/mL) on cleaved caspase-3 protein levels by Western blot. (e) Effect of PBE for different periods (0, 12, 24, 48 hours) on caspase-3 protein expression. A representative blot is shown above each plot of quantified band intensities (normalized to β-actin signal). (f) Cells were grown under the same conditions as (a and b) except that cells were pretreated with Z-DEVD-FMK, caspase-3 inhibitor 15 or 50 µM, before incubated with PBE. The numbers of nuclei with morphological changes were averaged from four 40× field-of-view images and values were calculated as the percentage of apoptotic cells. (g) Caspase-3 activity was determined by a colorimetric assay kit and the enzyme-catalyzed release of p-nitroaniline was monitored at 405 nm. Data are the means ± standard deviation. The results presented are from 3 independent experiments; *P < 0.01 vs unstimulated cells; **P < 0.01 vs PBE treatment.
strong free radical-scavenging activity against ROS\(^9\) and anti-inflammatory.\(^{10}\) In addition, Kamran et al demonstrated proapoptotic activity of PYC on human fibrosarcoma cells.\(^{20}\) *Pinus pinaster* PYC shows anticancer effects in breast cancer cell culture.\(^{26}\) However, little is known about the anticancer properties of PYC from this plant in skin cancer. Our study shows for the first time the potential antiproliferative activity and induction of an apoptosis effect of PBE on malignant melanoma skin cancer cells. A previous study showed that PBE from *P. pinaster* at 10-40 µg/mL reduced human oral mucoepidermoid carcinoma (MC-3) cell viability and induced chromatin condensation.\(^{27}\) In contrast with our results, the previous study reported that 40 µg/mL PBE reduced the number of MC-3 cells more than 80% and increased the numbers of apoptotic cells by 6-fold. In our study, 50 µg/mL PBE induced cell viability and apoptosis at lower levels compared with results in the previous study. The differences in effects may be related to the differences among pine species and cultivars as well as differences in the extraction, separation, and purification protocols and cell types.

UV radiation is the major carcinogenic factor for all types of skin cancers via oxidative mechanisms generated by ROS. ROS can oxidize lipids, proteins, and DNA, leading to the formation of oxidized products such as lipid hydroperoxides, protein carbonyls, and 8-oxo-guanosine.\(^{28}\) If these alterations occur in genes involved in normal homeostatic mechanisms that control proliferation and cell death, significant abnormalities can be observed in the cell cycle, leading to the first stage.

**Figure 3.** Dose-dependent effect of pine bark extract (PBE) on cell migratory and invasive activities in A375 cells. (a) Cells were grown in cell invasion and migration (CIM) plates and stimulated with 5% fetal bovine serum (FBS) or 1-50 µg/mL PBE for 30 hours. Cell index (CI) was determined. (b) The rate of cell migration was determined by calculating the slope of the line between 2 time points. (c) The effect of PBE on invasion. Cells were grown on CIM plates coated with Matrigel and exposed to 0%, 5% FBS, or 25 and 50 µg/mL of PBE. CI is shown. (d) The rate of cell invasion was determined by calculating the slope of the line between 2 time points. Cells exposed to 5% FBS were used as a positive control. Data are shown as mean values ± standard deviation from 3 independent experiments; \(*P < 0.05\) vs PBE treatment.
of cancer.\textsuperscript{29} In the present study, we examined the effect of PBE which induced apoptosis on intracellular ROS generated by H\textsubscript{2}O\textsubscript{2} in A375 malignant melanoma cells. The results showed that PBE significantly increased (\(P < 0.01\)) apoptosis and reduced (\(P < 0.001\)) ROS formation, suggesting that the reduction of A375 cell viability may be at least in part due to the inhibition of cellular ROS production during PBE treatment. Consistent with our results, Kyriazi et al.\textsuperscript{30} reported the chemopreventive effects of oral and topical administration of PBE in hairless mice exposed to UV radiation and showed that PBE inhibited the appearance of skin papillomas, skin carcinogenesis, and tumor multiplicity.

A number of MMP family members (MMP-1, MMP-2, MMP-9, and MMP-13) play functions in invasive tumor cells and can induce malignant melanoma cell invasion, tumor growth, and metastasis formation.\textsuperscript{7} MMP-9 is expressed by or can be induced in cell lines derived from advanced primary melanomas and is absent in cell lines derived from early stage primary lesions.\textsuperscript{7} Our results demonstrated the dose-dependent PBE inhibitory effects on MMP-9 release in the malignant melanoma cell supernatant following incubation with H\textsubscript{2}O\textsubscript{2} and suppression of cell migration and invasion, implying that PBE may have a beneficial effect on advanced-stage melanoma.

Polymeric tannins and monomeric flavonoids, such as catechin and epicatechin, in PBE could be responsible for the high antioxidant activity and anticancer effect.\textsuperscript{31} In the present study, we found that PBE contained ~61.26 mg catechin/g, so the maximum concentration of PBE used in our study (50 µg/mL) contained ~3.11 µg/mL catechin. In a previous study, quercetin in an ethanolic extract of \textit{P. pinaster} Aiton subsp. \textit{atlantica} was identified at a detection wavelength of 280 nm.\textsuperscript{32} Increased levels of quercetin can regulate the expression of the Bcl-2 family of proteins and induce apoptosis.\textsuperscript{33} However, we could not identify quercetin in the PBE used in our study at a detection wavelength of 370 nm (retention time 2.16 minutes) following a validated HPLC method in a previous report.\textsuperscript{34} Therefore, our study implies that the catechin content of PBE is linked to the anticancer actions in A375 cells.\textsuperscript{35} Clarification of the quercetin content in PBE used in this study should be examined in more detail.

\textbf{Conclusion}

This study aimed to determine the constituents of PBE from \textit{P. maritima}. Our study indicated that PBE exhibited cytotoxicity against A375 cells and inhibited cell migration and invasion \textit{in vitro}, indicating that the crude extract may be a potential candidate for the treatment of malignant melanoma. Further screening \textit{in vivo} will help to understand the pharmacological activity of the compounds against this skin cancer.
**Experimental**

**Chemicals, Reagents, and Antibodies**

(+) -Catechin and quercetin (purity ≥ 99.0% standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Annexin V-FITC and PI from Miltenyl Biotech (Bergisch Gladbach, Germany), Hoechst dye 33342 from AnaSpec (Fremont, CA, USA), modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Thermo Fisher (Rochester, NY, USA), Matrigel Anti-caspase-3, and horseradish peroxidase-conjugated secondary (goat anti rabbit) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-β-actin antibody from Cell Signaling Technology (Beverly, MA). Other chemicals were obtained as listed in the methods.

**Plant Material and Identification**

PBE from *P. maritima* was extracted following a previous study and stored as a powder at −20°C. All chemicals were of analytical grade purity. Dried powder was dissolved in dimethyl sulfoxide (DMSO) before use. All PBE fractions were analyzed using HPLC with an external standard following a previous study on a Nexera series HPLC system (Shimadzu Corporation, Japan) with TSKgel ODS-100V HPLC Column phase C18 (Tosoh Bioscience GmbH, Griesheim, Germany) (15 cm length ×4.6 mm I.D., 5 μm particle size for catechin and 25 cm length ×4.6 mm I.D., 5 μm particle size for quercetin) with SPD-M20A photodiode array detectors. Methanol:acetonitrile with 0.1% acetic acid and methanol:0.1% orthophosphoric acid were used as the mobile phase for catechin and quercetin, respectively, at a flow rate of 1.0 mL/minute. The injection volume for all standards and samples was 10 μL. The chromatograms were recorded at 230 and 370 nm for peaks detected compared with those of standards.

**Cell Culture and Treatment**

The A375 malignant melanoma cell line (American Type Culture Collection, ATCC, Manassas, VA, USA) was obtained from the Department of Dermatology, Kagoshima University Graduate School of Medical and Dental Sciences, Japan. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL) containing 7% FBS, penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin), and 2 mmol/L glutamine (Hyclone Logan) at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Cells were cultured in serum-free DMEM before stimulation with PBE extract.

**MTT Assay**

Cells were cultured into 96-well plates in either the absence or presence of PBE (1 100 μg/mL) for 24 hours. Cell viability was assessed using MTT assay, as previously described. Absorbance was measured at 570 nm using an automatic microplate reader (ImmunoMini NJ-2300; InterMed, Tokyo, Japan). The cell survival percentage was calculated using DMSO-treated cells as a standard.

**Detection of Apoptotic Cells**

Cells were plated in 8-well Lab-Tek chamber slides (Nagle Nunc International, Rochester, NY, USA) at 1 × 10^4 cells/well. Cells were subsequently treated with PBE for 24 hours, fixed, washed, and stained with Hoechst 33342. Nuclei were examined and photographed using a fluorescence microscope (BX51; OLYMPUS, Tokyo, Japan). Images were captured. Four images per group/time point were obtained to evaluate morphological changes in the nuclear chromatin of apoptotic cells, and values were calculated as a percentage of apoptotic cells. For the inhibition assays, cells were pretreated with 15 μM or 50 μM Z-DEVD-FMK, caspase-3 inhibitor for 1 hour before 50 μg/mL PBE treatment.

**Flow Cytometric Analysis of Apoptosis with Annexin V-FITC/PI Staining**

The induction of apoptosis was determined using Annexin V-FITC/PI double staining following the manufacturer’s instruction. Briefly, A375 cells were treated either with or without PBE (1, 25, and 50 μg/mL) for 24 hours. The collected cells were fixed with PBS and 70% ethanol (v/v) at 4°C. The cell pellet was suspended in Binding Buffer and mixed with Annexin V-FITC. After washing, cells were centrifuged at 300×g for 10 minutes and suspended in binding buffer before adding PI solution in the dark at room temperature. Samples were analyzed using a NovoCyte flow cytometer with NovoExpress software (ACEA Bioscience, Inc., San Diego, CA, USA).

**Preparation of Cell Lysates and Western Blotting**

Apoptosis-protein profiling by Western blot was performed according to a previous study. Cells were treated with PBE at 0-50 μg/mL for 0, 12, 24, and 48 hours and were lysed in radi-immunoprecipitation assay (RIPA) buffer (1% [w/v] NP40, 1% [w/v] sodium deoxycholate, 0.1% [w/v] sodium dodecyl sulfate, 0.15 M sodium chloride, 0.01 M sodium phosphate buffer, pH 7.2, 2 mM ethylenediaminetetraacetic acid, and 50 mM phosphatase inhibitor cocktail). Protein concentrations were determined by Bradford protein assay using bovine serum albumin as standard (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (30 μg) were subjected to 14% sodium dodecyl sulfate-polyacrylamide gel, electrophoretic transfer, and Western blotting. Band intensities were quantified using National Institutes of Health Image 1.63 software and were normalized to β-actin signals.
Caspase Activity Assay

Enzymatic activity of caspases was assayed using a colorimetric assay kit according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA). Briefly, cells were lysed in lysis buffer. After the samples were centrifuged, 100 µg of the protein was incubated with reaction buffer and colorimetric tetrapeptide Asp-Glu-Val-Asp (DEVD)-p-nitroaniline for 2 hours at 37°C. Optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

xCELLigence Technology RTCA

Cell proliferation was monitored in real-time using the xCELLigence system E-Plate (ACEA Biosciences Inc., San Diego, CA, USA). A total of $1 \times 10^4$ cells were seeded in the E-plate. Cells were stimulated with PBE at 1-50 µg/mL. The impedance value of each well was automatically monitored by the xCELLigence system for a duration of 72 hours and expressed as a cell index (CI) value. Data were normalized at 24 hours. The normalized cell index (NCI) was calculated as CI at a given time point divided by the CI at the normalization time point. Automatically, the CI was calculated by the RTCA Software Package 2.1.0., based on the following formula:

$$CI = \frac{(Z_i - Z_0)}{15}$$

CI calculation used to measure the relative change in electrical impedance, representing the cell status. The unit of impedance is ohm (Ω). $Z_i$ is the impedance at an individual point of time during the experiment and $Z_0$ is the impedance at the start of the experiment.

Monitoring Cell Migration and Invasion by RTCA

The rate of cell migration was observed in real-time using CIM plates (ACEA Biosciences Inc., San Diego, CA, USA) by the xCELLigence system as previously described.38 Several experimental tests were recorded for Video clips to determine the optimal seeding density of the cells (Supplementary Movie 1). The optimal cell density at each time point for these migration profiles was $2 \times 10^4$ cells/well, and this number of cells was seeded in each well. Cells were serum starved for approximately 24 hours. Cells were seeded in the upper chamber of each well of a CIM plate in serum-free medium. Next, fresh medium was added to the lower chamber and cells were stimulated with 1-50 µg/mL PBE. The impedance value of each well was monitored automatically by the xCELLigence system for 30 hours and represented as CI value. The CI value was calculated by the RTCA Software, as described above. The slope of the line between 2 given time points was calculated for the rate of cell migration. For the cell invasion analysis, a similar protocol was performed as in a previous study,39 except that the upper chamber of the CIM plates was coated with 1:40 solution of Matrigel (BD Biosciences, NY, USA) before seeding the cells ($4 \times 10^4$ cells/well).

Detection of Intracellular ROS

Levels of ROS were measured using a dichlorodihydrofluorescein diacetate (DCFH-DA) detection kit (Sigma, St. Louis, MO, USA), according to the manufacturer’s instructions. A375 cells ($8 \times 10^3$ cells/well) were seeded in 96-well plates and treated with PBE (10, 25, 50 µg/mL) before incubation with or without H$_2$O$_2$ (50 µM) for 1 hour. Cells were incubated with 10 µM of DCFH-DA plus Hank’s buffered salt solution for 30 minutes at 37°C. The levels of ROS were measured using a spectrophuorometer (PerkinElmer, Waltham, MA, USA; Ex: 490 nm, Em: 570 nm).

Quantification of MMP-9 Levels

Cells were treated with PBE (5, 25, or 50 µg/mL) before incubation with or without H$_2$O$_2$. The levels of MMP-9 in the A375 cell supernatant were determined by the MMP-9 human enzyme-linked immunosorbent assay Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions.

Statistical Analysis

Data were analyzed using SPSS software (version 20.0; SPSS Inc., Chicago, IL, USA). Data are presented as mean values ± SD. Significant differences between 2 groups were assessed using Student’s t-test, and differences among multiple groups were assessed by one-way analysis of variance, followed by Scheffe’s multiple range testing. P < 0.05 was considered statistically significant.

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Supplemental Material

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