SUPPLEMENTARY MATERIAL

Apoptotic Effect of jaceosidin on MCF-7 Human Breast Cancer Cells through modulation of ERK and p38 MAPK pathways

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Abstract

Jaceosidin a flavone abundant in Artemisia species has been used for its beneficial effects. This study investigated the apoptotic effect of jaceosidin treatment on MCF-7 human breast cancer cells at varying concentrations of (0, 10, 20 and 40 μM) for 24 and 48 h treatment times. Jaceosidin treatment induced a significant ($p<0.05$) dose-dependent increase in apoptosis of MCF-7 cells. Jaceosidin similarly modulated the expressions of apoptosis-associated proteins, and revealing a coaction between Bax and Bcl-2, striking a balance between cell survival/cell death. Besides, a significant increase in pro-apoptotic expression of cleaved PARP which is a key executioner in apoptosis was observed. Apoptosis was confirmed in the cells by flow cytometry which indicated an early apoptosis (7%, 17%), as well as late apoptosis (36%, 40%) of the cells in varying percentages as treatment concentration increased. Thus, this study demonstrates that jaceosidin could be used as a potential treatment for breast cancer.

Keywords; MCF-7, jaceosidin, anticancer activity, human breast cancer cell, apoptosis

Experimental

Chemicals and antibodies

Jaceosidin (Lot No. CFS201602) isolated from *Artemisia artica* herbs (4, 5, 7-trihydroxy-3, 6-dimethoxyflavone) was purchased from ChemFaces Biochemical Co., Ltd, (Wuhan, China), fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY, USA), Roswell Park Memorial Institute medium-1640 (RPMI-1640), high glucose- Dulbecco's modified Eagle's medium (DMEM-H). MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was purchased from Amresco (Solon, OH, USA). Primary Antibodies BCL-2, BAX, p-AKT, p38, and p-p38, were acquired from Cell Signaling Technology Beverly, MA, USA), AKT, ERK, p-ERK, PARP and β-actin purchased from (Santa Cruz Biotechnology, Santa Cruz, CA, USA) while Anti-mouse and Anti-rabbit IgG, HRP-Linked secondary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RIPA lysis buffer from iNtRON Biotechnology (Seongnam, South Korea), Bradford reagent (Bio-Rad Laboratories) was used for protein assay.

Cell culture, treatment and morphological assessment

Human breast cancer cells (MCF-7) and human endothelial cells (EA.hy926) were purchased from the Korean Cell Line Bank (KCLB). MCF-7 cells were grown in RPMI-1640 medium containing antibiotics and 10% FBS. EA.hy926 cells were cultured and maintained in DMEM supplemented with 10% FBS. MCF-7 cells and EA.hy926 cells were incubated at 37 °C in a humidified 5% CO_2 incubator until 80% confluence. Following cell growth to suitable confluence, cancer cells were treated with jaceosidin concentrations of 0, 10, 20 and 40 μM dissolved in DMSO at treatment times of 24 and 48 h. The changes in cells morphology were observed and captured using an optical microscope (Leica, MC120 HD, Wetzlar, Germany).

Cell Viability (MTT Assay)

A total number of $2x10^4$ MCF-7 cells and EA.hy926 cells were seeded in a 96-well plate containing 100 μl of their respective medium and maintained overnight. Afterward, cells were treated as mentioned in section 2.2. After the incubation period (24 and 48 h), 0.5 mg/ml MTT solution in PBS was added to each well and incubated for 3 h at 37 °C. After incubation, the

precipitated formazan was dissolved with isopropyl alcohol for 1h and absorbance was measured at 595 nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA, USA). Results were expressed as percentage (%) of control.

Western blot analysis

Total protein in the cultured cells were extracted with radio immunoprecipitation assay(RIPA) lysis buffer containing phosphatase inhibitors (GenDEPOT, TX, USA) and a protease inhibitor cocktail (GenDEPOT, TX, USA) after harvesting. Cell lysates were kept on ice for 15 min, centrifuged at 13000 rpm; 4 °C for 15 min and clear liquid above the sediment was collected after centrifugation. Bradford assay was used to determine the protein concentration and protein samples were separated by SDS-PAGE, with equal amounts of total protein (30 μg) loaded in 7.5–15% SDS polyacrylamide gel, electrophoresed and transferred to Nitrocellulose membranes, a solution containing 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) was used to block membranes for 1 h. Respective primary antibodies such as BCL-2 (1:1000), BAX(1:1000), p38(1:1000), p-p38(1:1000), p-AKT(1:1000) were added and were incubated at temperature of 4°C, overnight. Each membrane was washed five times for 5 mins using TBST and was then incubated with appropriate secondary antibodies anti-mouse for PARP and β-actin while anti-rabbit was used for BCL-2, BAX, p38, p-p38, p-AKT, AKT, ERK, p-ERK at a dilution of 1:1000 for 1 h on a shaker at room temperature. Protein signals were detected using an Enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences, NJ, USA) and measured by Fusion Solo Detector (Vilber Lourmat, Marne La Vallee, France). The densitometry of immunoblots was quantified with Image J software NIH.

Flow cytometry analysis

FITC annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ USA), was used to perform flow cytometry analysis according to manufacturer's instructions. Briefly, Treated and untreated cells were re-suspended in 500 µl phosphate-buffered saline (1x PBS) containing 1% FBS, and centrifuged at 3000 rpm for 5 min thrice. The cells were then stained with 5ul FITC Annexin and PI then incubated for 15mins at RT (25^{0}C) in the dark. 400ul 1x binding buffer was added and cells were analyzed using life technologies Attune NxT acoustic focusing cytometer (SN 2AFC215660517, Thermo Fisher Scientific).

Statistical analysis

In appropriation, all data are shown as mean ± standard deviation (SD) values. Data evaluation was carried out via factorial analysis of variance (ANOVA) and a Fisher's least significant difference test ($p < 0.05$) were used for post hoc analysis.

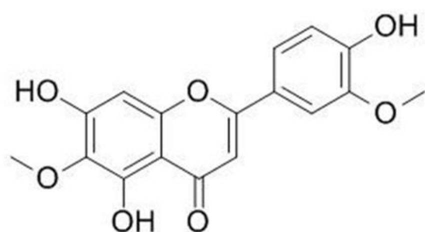


Figure S1. Structure of jaceosidin (4’, 5, 7-Trihydroxy-3,6-dimethoxyflavone)

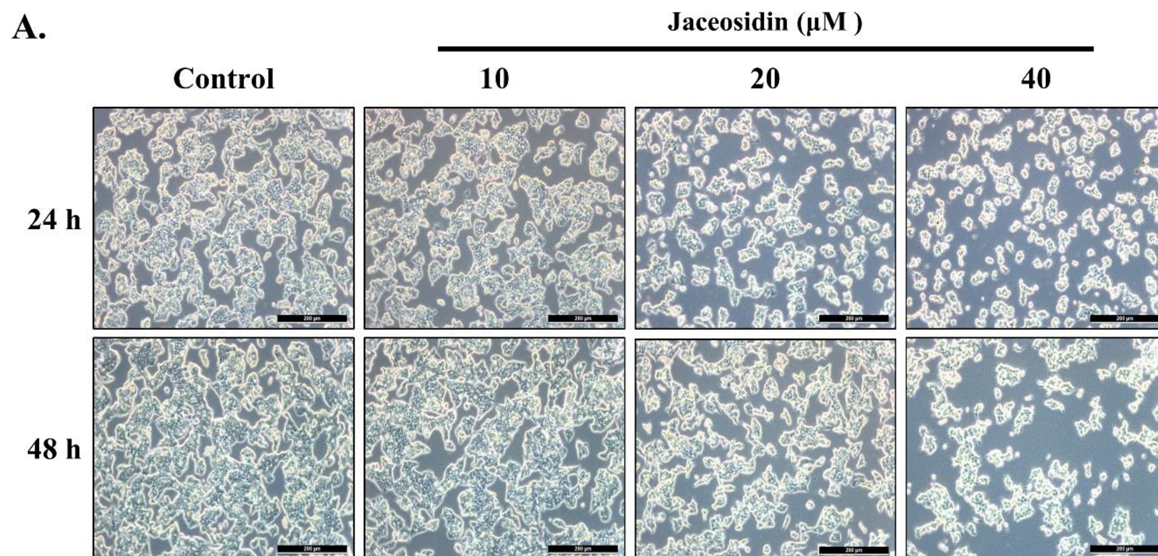


Figure S2a. Apoptotic effect of jaceosidin on MCF-7 cells. (A) MCF-7 cells were treated with 0,10,20,40 μM jaceosidin for 24 h and 48 h. The cell morphology was examined using a microscope (Scale bar represents 200 μM).

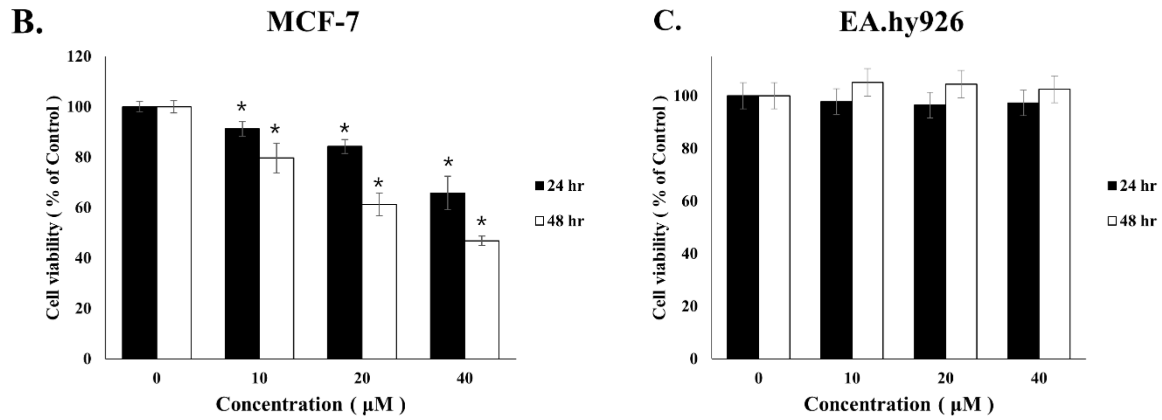


Figure S2b. Jaceosidin inhibited cell proliferation and induced cell death. (B) Mcf-7 Cells were treated with indicated doses of jaceosidin for 24 and 48 hours and cell viability were measured by MTT assay. (C) EA.hy 926 Cells were treated with indicated doses of jaceosidin for 24 and 48 hours and its cytotoxicity was measured by MTT assay. Results were expressed as percentage (%) of control values of three independent experiments. Statistical significance was considered at $p < 0.05$ compared to control

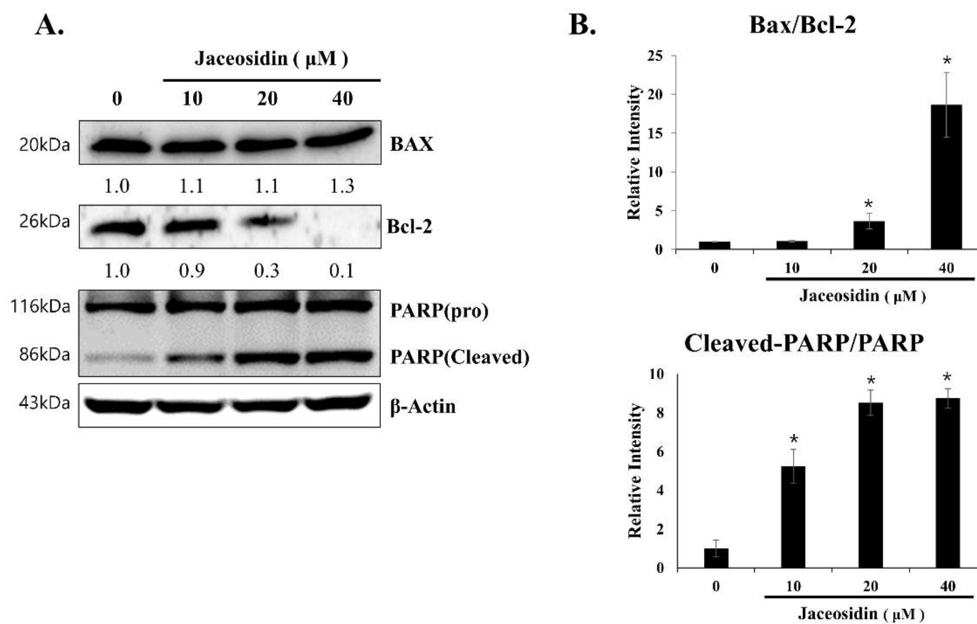


Figure S3. Effect of jaceosidin on the expression of the apoptosis regulators in MCF-7 cells **(A)** MCF-7 cells were treated with indicated doses of jaceosidin (0, 10, 20, or 40 μM) for 48 h. The expression BAX, BCL-2, Pro PARP, cleaved PARP were measured by Western blotting, β-Actin was used as loading control. **(B)** Ratio of Bax/Bcl-2 expression and total/cleaved form of PARP. Representative blots are shown from results of three independent experiments. Statistical significance was considered at $p < 0.05$.

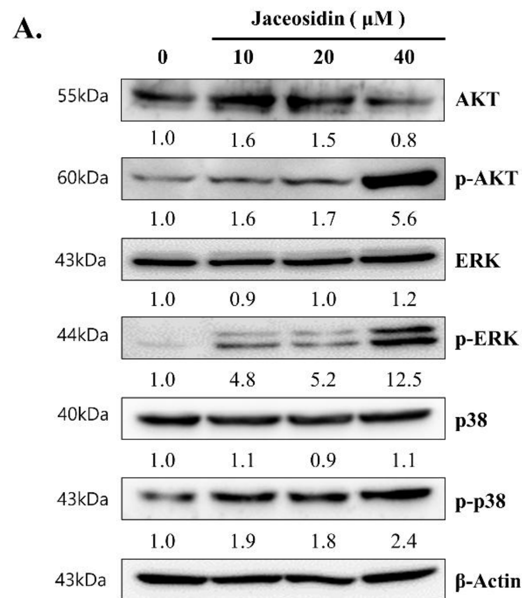


Figure S4. Effects of jaceosidin on the expression of MAPK pathway related proteins in MCF-7 cells. **(A)** MCF-7 cells were treated with indicated doses of jaceosidin (0, 10, 20, or 40 μM) for 48 h. The expression AKT, p-AKT, ERK, p-ERK, p38, p-p38 were measured by western blotting, β-Actin was used as loading control. Statistical significance was considered at $p < 0.05$.

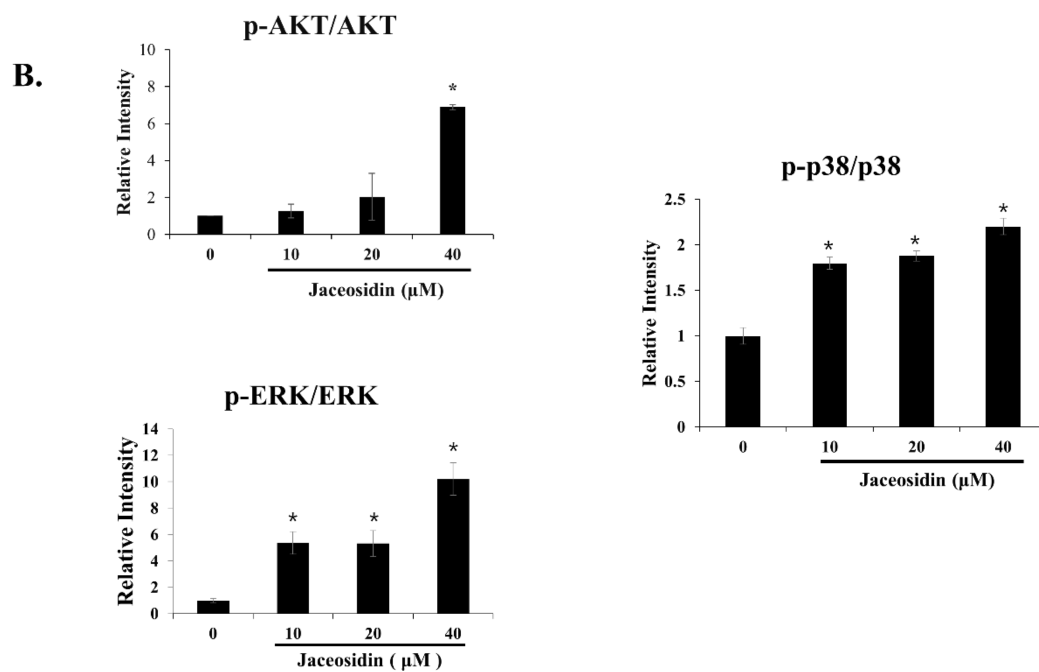
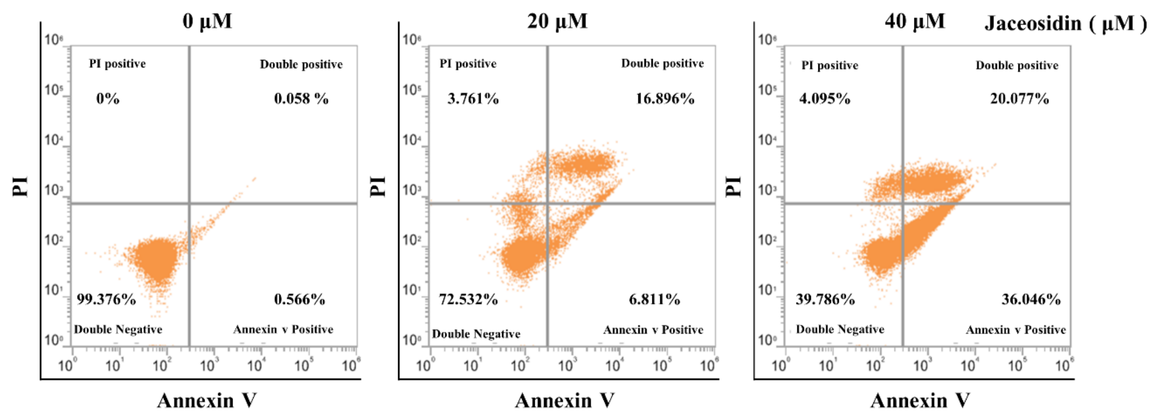


Figure S4. Effects of jaceosidin on the expression of MAPK pathway related proteins in MCF-7 cells.(**B**) Plot ratio of total and phosphorylated forms of Erk and p38 normalizing β-Actin. Representative blots are shown from results of three independent experiments. Statistical significance was considered at $p < 0.05$.



Treatment/Dose	Double Negative	PI Positive	Annexin v Positive	Double Positive
Jaceosidin 0 µM	99.376	0	0.566	0.058
Jaceosidin 20 µM	72.532	3.761	6.811	16.896
Jaceosidin 40 µM	39.783	4.095	36.046	20.077

Figure S5. Jaceosidin induces early and late apoptosis in Mcf-7 cells. To evaluate jaceosidin-mediated apoptosis, jaceosidin-treated Mcf-7 cells were treated with indicated doses of jaceosidin (0, 20, 40 µM) for 48 h and labelled with Annexin V, apoptotic staining profiles were analyzed using flow cytometry.