약학석사학위논문

칸타리딘에 의한 MDA-MB-231 유방암 세포의 항암활성연구

Cantharidin induces apoptosis and suppresses cell migration and invasion of MDA-MB-231 human breast cancer cells through inhibition of EGFR-STAT3 signaling

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약학과 천연물과학 전공

박 민 경

Abstract

Cantharidin induces apoptosis and suppresses cell migration and invasion of MDA-MB-231 human breast cancer cells through inhibition of EGFR-STAT3 signaling

Min Kyoung Park

Natural Products Science Major

College of Pharmacy

Master Course in the Graduate School

Seoul National University

Breast cancer is the most frequently diagnosed life-threatening cancer in women. Common chemotherapeutic agents target three receptors: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Breast cancer cells that

lack ER, PR and HER2 are referred to as triple negative breast cancer (TNBC). TNBC occurs in about 15~20 % of diagnosed breast cancer. TNBC is known to increase recurrence and mortality rate within 5 years of cancer detection and is thus considered to have poorer prognosis. Recently, the signal transducer and activator of transcription 3 (STAT3) is reported as a key factor in TNBC treatment, due to high levels of STAT3 expression in TNBC.

Bioassay-guided fractionation and purification were performed to isolate the cytotoxic compound from cantharides. The dried cantharides were crushed and extracted with acetonitrile and then separated into methylene chloride, acetonitrile, n-hexane and water layers. Methylene chloride and acetonitrile layer which had strong activity were further separated and purificated using various chromatographic techniques. The extract was fractionated into 4 fractions by Prep-LC, and the third fraction showing cytotoxic activity was separated using CCC (Counter-Current Chromatography). Separated fraction was structurally determined and identified as cantharidin by comparing NMR and HRMS data with literature value.

Cantharidin is an active constituent of the blister beetles, belonging to Meloidae family, which is traditionally used to treat wart and relieve

blood stasis. Moreover, numerous studies have revealed that cantharidin has a cytotoxic effect on cancer cells.

However, there have been no reports on cantharidin's effect on inhibiting cell growth of TNBC. Herein, we demonstrated that cantharidin induced cell death in one of TNBC cells, MDA-MB-231, by suppressing STAT3 activation. The result showed that cantharidin reduced STAT3 tyrosine-705 in MDA-MB-231 cells. Cantharidin significantly inhibited activation of EGFR, Src, and STAT3, not affecting JAK-STAT3 signaling. Moreover, cantharidin inhibits cell proliferation and induces apoptosis by regulating a transcription of STAT3 target genes such as cox-2, cyclin D1, bcl-2, caspase-3 and parp1.

Taken together, this study provides that cantharidin may be used as a potential therapeutic agent against TNBC, especially MDA-MB-231 cells, by reducing EGFR-STAT3 signaling.

Keywords: Triple negative breast cancer, MDA-MB-231, EGFR, STAT3, Cantharidin, Apoptosis, Bioassay-guided, HPCCC

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I. INTRODUCTION

1. Triple negative breast cancer (TNBC)

Breast cancer remains the most common malignant and clinically heterogeneous disease[1]. Currently, one in eight women in the US will develop breast cancer in her lifetime, and breast cancer is the second leading cause of cancer death among women[2]. The primary molecular subtypes of breast cancer have been identified by molecular markers: Luminal A, Luminal B, Triple negative/basal-like and HER2 type. Triple negative breast cancer (TNBC), characterized by tumors that exhibit little or no estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2) expression, can be a particularly aggressive form and high-grade metastasis[3]. These tumors are unlikely to respond to anti-estrogen therapy or HER2 antagonists. Because TNBCs lack ER, PR and HER2 receptors, they don’t respond to hormone therapy[4]. TNBC occurs in about 15~20 % of diagnosed breast cancer. TNBC is known to increase recurrence and mortality rate within 5 years of cancer detection and is thus considered to have poorer prognosis[5]. Thus, the development of alternative therapy for TNBC treatment is essential.

2. Signal Transducer and Activator of Transcription 3 (STAT3)

STAT (signal transducers and activators of transcription) proteins are latent transcription factors that become activated by phosphorylation on a single tyrosine, typically in response to various extracellular polypeptide ligands[6]. STAT phosphorylation is caused by growth factors and cytoplasmic cytokines[7]. STAT phosphorylation occurs at the cytoplasm, which induces STAT homo and heterodimer formation between two monomers via their Src homology 2 (SH2) domain interactions[8]. From the cytoplasm, activated STAT dimers translocate and accumulate in the nucleus, where they initiate and mediate gene transcription by binding to DNA response elements. The dimers accumulate in the nucleus, recognize specific DNA elements and activate transcription[9]. The STAT proteins are subsequently inactivated by tyrosine dephosphorylation and return to the cytoplasm[10]. STAT3 is one of seven members of the STAT family of transcription factors, namely, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT7. STAT activation is critical for a number of biological processes, including cell proliferation, survival, differentiation, and inflammation[11]. This results in either

upregulation or downregulation of the biological effectors and subsequent cellular processes that are critical for cellular homeostasis[12].

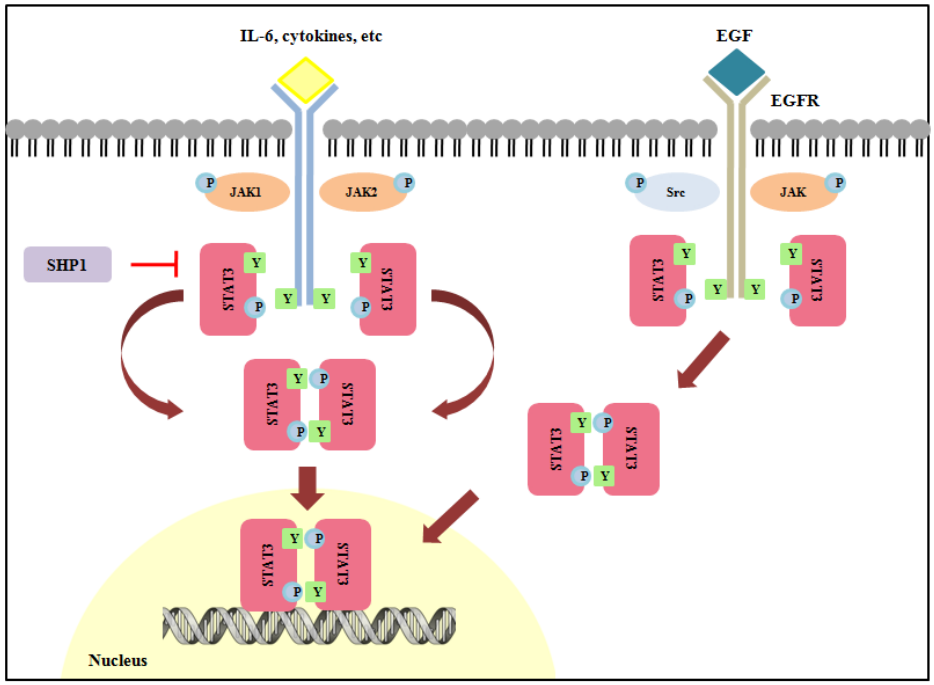

Figure 1. STAT3 signaling pathway

3. EGFR (Epidermal growth factor receptor)

EGFR is a member of the growth factor receptor tyrosine kinase family consisting of EGFR (ErbB1), HER2 (ErbB2, Neu), HER3 (ErbB3), and HER4 (ErbB4). Each receptor has a unique extracellular region, a transmembrane domain, and an intracellular kinase domain that is highly conserved within the family[13]. EGFR is activated by receptor overexpression, ligand-independent, and most commonly ligand-dependent mechanisms[14]. EGFR is involved in diverse cellular processes including growth, differentiation and survival[15].

EGFR has six known ligands, including EGF, transforming growth factor alpha (TGFa), amphiregulin, betacellulin, heregulin, and heparin-binding EGF[16]. Activation of the receptor through various ligands binding to the extracellular domain leads to receptor dimerization and autophosphorylation. Autocrine signaling occurs when a cell produces both a ligand and its receptor, as is the case in cells that overexpress both EGFR and TGFa leading to uncontrolled cell growth[17].

4. EGFR-STAT3 pathway

The epidermal growth factor receptor (EGFR) and signal transducers and activators of transcription (STATs) are commonly expressed and activated in many malignancies[18]. EGFR is an upstream activator of several pathways involved in tumor progression, and STATs activate selected genes involved in oncogenesis[19]. There are several different mechanisms by which STAT proteins can mediate intracellular EGFR signaling, including direct activation of STATs by EGFR binding and indirect activation of STATs through Src-mediated EGFR signaling[20]. EGFR likely activates STAT in a manner distinctive from other mechanisms of STAT activation[19]. Ligand binding to EGFR has been shown to activate STAT1, STAT3, and STAT5 via Src, not JAK in a breast cancer cell line[21]. The role for JAKs in ligand-induced EGFR activation of STAT3 is cell-type dependent. JAKs provide maximal activation of STAT proteins in an EGF-dependent signaling scenario[19]. Inhibiting JAKs in breast cancer cell lines, however, only partially blocks EGF-dependent STAT protein activation, further supporting the role of Src in STAT-mediated EGFR signaling[22]. In the presence of EGF stimulation, STAT3 is activated via EGFR and Src kinase activity is cooperative but not required[23].

4. Cantharides

Cantharides are the dried body of the blister beetle. The species used in medicine are *Mylabris phalerata* and *M. cichorii* (Meloidae). The use of cantharides as a traditional medicine in China can be traced back more than 2000 years, and it, is still used as a folk medicine today[24]. In recent studies, it has been found that cantharides possess antitumor properties and increase the number of leucocytes[25]. The active constituent of cantharides is cantharidin[26]. Cantharidin is an inhibitor of protein phosphatases 1 and 2a leading to multiple cellular effects such as DNA damage, cell cycle arrest, and apoptosis[27].

II. MATERIALS AND METHODS

1. MATERIALS

1.1 Insect materials

Mylabris sidae FABR. (cantharides) were provided from Professor Kyoung Jin Lee of Kyung Hee University.

1.2 Chemicals and reagents

All organic solvents used for extraction, column chromatography and High Performance Countercurrent Chromatography (HPCCC) were of analytical grade and were purchased from Seoduk Chemical Co. in South Korea. Acetonitrile (HPLC grade) and hexane, methylene chloride and methanol were supplied by J.T. Baker (Phillipsburg, NJ) and Fisher Scientific (Pittsburg, PA). Distilled water (NANO pure Diamond, Barnstead, USA) was used for all solutions and dilutions. Cantharidin was dissolved in DMSO and the final concentration of DMSO in the cell culture was kept below 0.05%. Dulbecco's phosphate buffered saline (DPBS), a protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other cell culture reagents were purchased from Sigma Aldrich (St.

Louis, MO). Bovine serum albumin (BSA) was obtained from Pierce (Rockford, IL, USA). All other chemicals were obtained from Sigma-Aldrich Co. (St Louis, Mo, USA) unless otherwise indicated.

1.3 Instruments

HPLC analyses were carried out on Hitachi L-6200 instrument equipped with UV system and SIL-9A auto injector (Shimadzu, Japan) and Agilent 1100 equipped with PDA UV detector. Columns used for analysis are iNNO C18 column (150 mm x 4.6 mm id, 5 µm particle size) from Young Jin biochrom Co. Ltd (Seongnam, South Korea).

Preparative HPLC separation was performed using Hitachi JP/L-7100 equipped with Hitachi L-4000 UV detector. Column used for separation was iNNO C18 column (100 mm x 250 mm, 10 µm particle size).

Spectrum HPCCC from Dynamic Extractions Ltd (Slough, UK) with tubing id 1.6 mm, total volume 135.5 mL, and sample loop 6 mL was used for CCC separation. The revolution speed was adjusted with a controller to an optimum speed of 1,600 rpm. Hitachi L-6200 intelligent Pump (Tokyo, Japan) was used to fill the CCC apparatus with the stationary phase and elute the mobile phase. The effluent was continuously monitored by Dynamax UV Absorbance Detector from

Rainin Instruments, LLC (California, USA).

The high resolution electrospray ionization source (HR-ESI/MS) was performed using a Finniga LCQ ion trap mass spectrometer from Thermo Finnigan (San Jose, CA) equipped with an electrospray (ESI) probe. The NMR analyses were recorded on Bruker Avance 500 and 600 spectrometers. ^{1}H and ^{13}C NMR spectra were measured in a DMSO-d_6 solution at 500 and 125 MHz or 600 and 150 MHz.

1.4 Antibodies

The primary antibodies for p-STAT3 (Tyr705), p-STAT3 (Ser727), STAT3, p-JAK1, and p-JAK2 were from Abcam (Cambridge, MA). The primary antibodies for PARP1, p-JNK, JNK, p-ERK, ERK, COX-2, SHP-1, SHP-2, bcl-2, Caspase-8, Caspase-3, p-p38, p38, and β-actin and all secondary antibodies were from Santa Cruz Biotechnology (SantaCruz, CA). The primary antibodies for p-Akt, Akt, p-EGFR, EGFR, and cyclin D_1 were from Epitomics (Burlingame, CA). The primary antibodies for JAK1 and JAK2 were from Cell Signaling Technology (Beverly, MA). Penicillin, streptomycin, DMEM (high glucose), and fetal bovine serum (FBS) were obtained from GenDepot (Barker, TX).

2. METHODS

2.1 Cell culture

MDA-MB-231 human breast cancer cells were obtained from the Korea Cell Bank (Seoul, Korea). MDA-MB-231 were maintained in DMEM supplemented with 10% FBS and antibiotics (penicillin 100 μg/mL and streptomycin 100 μg/mL). Cultures were maintained in an incubator at 37℃ in 5% CO_2

2.2 Cell viability assay

Cell viability was measured by MTT colorimetric assay. Briefly, MDA-MB-231 cells were seeded in 24-well plate at density of 1 x 10^5 cells/well, and they were incubated at 37℃ for 24 h. The cells were then treated with various concentration of cantharidin (0, 5, 10, 15 μM) for 24h. After the incubation for 24 h, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) was added to each well and incubation was continued for an additional 2h under the same conditions. The medium was removed and DMSO was added to each well to dissolve the formazan crystals, and then plate was shaken. The amount of formazan derivative was measured at 595 nm using an ELISA micro plate reader (Molecular

Devices, Sunnyvale, USA)

2.3 Preparation of nuclear and cytosolic fraction

Whole cell lysates were prepared using a lysis buffer (20 mM HEPES, pH 7.6, 350 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 50 mM NaF, 0.1mM DTT, 0.1mM PMSF, and protease inhibitor cocktail) for 30min on ice. The lysates were centrifuged at 15,000rpm for 10min. Nuclear extracts were prepared using a lysis buffer (10mM HEPES, pH7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mMDTT,1mM PMSF, and protease inhibitor cocktail) for 15 min on ice.Then, 10% NP-40 was added and the mixtures were centrifuged for 5min. The nuclear pellets were resuspended in nuclear extraction buffer (20mM HEPES, pH7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF, and protease inhibitor cocktail) and centrifuged at 15,000 rpm for 10 min. The protein concentration was determined by the Bradford reagent (Bio-Rad, Hercules, CA).

2.4 Luciferase reporter assay

MDA-MB-231 cells were plated in 24-well plates at a density of 1×10^4 cells/well. After 24 h, the cells were transiently transfected with

pSTAT3-Luc reporter vector in the presence of pCMV-Luc vector (Firefly) using transfection reagent (Intron Biotechnology, Seoul, Korea). At 24 h post-transfection, the cells were treated with cantharidin for 24 h. Luciferase assay were performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The luminescence signal was measured using a luminometer (MicroLumat Plus, Berthold Technologies, Dortmund, Germany).

2.5 Western blot analysis

The cells were seeded at a density of 2×10^5 cells per well in 6-well plates and attached for 24 h. After incubation, the adherent and floating cells were collected, washed with DPBS and centrifuged. The pellet were extracted in lysis buffer (20 mM HEPES [pH 7.6], 350 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 50 mM NaF, 0.1 mM DTT, 0.1 mM PMSF, and protease inhibitor cocktail) for 30 min on ice. The extract was centrifuged at 15,000 rpm for 10 min at 4℃. The protein concentration was estimated using a Bradford reagent (Bio-Rad Laboratories Inc., CA). An equal amount (20-30 μg) of protein was loaded on 8-12% SDS-polyacrylamide gels and transferred

using a semi-dry method to a nitrocellulose membrane. After being blocked with 5% skim milk, the membrane was incubated at 4℃ overnight with specific primary antibodies. The membrane was washed and incubated at room temperature for 1 h with secondary antibodies conjugated with horseradish peroxidase (HRP). Finally, the blot was developed using a chemiluminescence kit (AbFrontier) and the immunoreactive bands on the blot were visualized using a LAS-1000image analyzer (Fujifilm)

2.6 Wound-healing assay

To determine cell motility, the cells were seeded into 24-well plates and grown to 80–90% confluence. A monolayer of cells was scratched with a sterile micropipette tip, followed by washing with DPBS to remove cellular debris. The cells were exposed to cantharidin, and cell migration was observed and counted under a CKX41 microscope (Olympus, Tokyo, Japan) at a magnification of 100×. The cells that migrated across the yellow lines were counted in five randomly chosen fields from each triplicate treatment. The percentage of inhibition was expressed using untreated wells at 100%.

2.7 Annexin V & PI analysis

Apoptotic cells were differentiated from viable or necrotic cells using a combined staining of Annexin V-FITC and PI. To examine the apoptosis-inducing potential of compounds, flow cytometry-based Annexin V staining was performed to detect the externalization of phosphatidylserine. After treatment with compounds, adherent and floating cells were collected, washed with DPBS, and subsequently centrifuged. The cells were suspended in 1 X binding buffer and stained with Annexin V-FITC and PI according to the manufacturer's directions (BD Biosciences). After incubation for 15 min at room temperature in the dark, the cells were analyzed with flow cytometry using Becton Dickinson FACS Calibur (BD Biosciences) to estimate the population of apoptotic cells.

2.8 Invasion assay

Cell invasion assay was conducted using Transwell chambers (SPL,Seoul,Korea). Transwell inserts with 8 μm pore size were coated with matrigel (1mg/mL) and dried for 30min. The cells treated with cantharidin were suspended in serum-free DMEM. Approximately 5×10^4 cells per insert were added to the upper chambers, and DMEM

was added to the lower chambers. After incubating for 24h, the non-invasive cells that remained on the upper side of the insert were removed using cotton swabs. The cells that invaded on the lower side of the insert were stained with 2% crystal violet and observed under a CKX41 microscope at a magnification of 200×. To quantify the invasiveness of the cells, methanol was added to dissolve the stain and the absorbance was determined at a wavelength of 595nm.

2.9 Extraction

Dried cantharides was provided from professor Kyoung Jin Lee of Kyung Hee University. The dried cantharides (50 g) were ground and were extracted three times with 80% acetonitrile (250mL) by sonication at room temperature for 1 h. This process was repeated for three times. The extract was dried with rotary evaporator under 50℃. Subsequently, dried extract was suspended in 80% acetonitrile and solvent partition was performed with hexane, methylene chloride (MC), acetonitrile and water. The obtained fractions were evaporated to dryness under vacuum and stored in desiccators until further use. Each fraction was tested for its cytotoxicity against MDA-MB-231 human breast cells. Since methylene chloride and acetonitrile fraction showed

higher cytotoxicity than other fractions, further separation was performed on this fraction.

2.10 Preparative HPLC Separation

Methylene chloride and acetonitrile fraction was carried out with preparative HPLC technique. The mobile phase used was distilled water (solvent A) and HPLC-grade acetonitrile (solvent B). The HPLC condition of experiment was as follows: gradient elution, 0 – 40 min (25% - 45% B), 40 - 60 min (45% - 100% B). The flow rate was 3.0 mL/min for 60 minutes and monitored with UV detector at 230 nm. Four fractions were collected, evaporated and stored in refrigerator until further use. Each fraction was tested for its inhibition activity against MDA-MB-231 cells. Since the third fraction showed higher cytotoxicity than other fractions, further isolation was performed on the third fraction.

2.11 Measurement of the partition coeffient (*K*)

The solvent system was selected based on the *K* values of the target compounds. The values were measured based on the peak area shown in HPLC chromatogram. The two-phase solvent system was prepared in a

separating funnel flask, shaken to allow the solvents mix and settle it for 2 hours to allow the solvent system separate and equilibrate. The lower phase and upper phase were collected in separated tubes. The extract was dissolved in equal volumes of lower and upper phases of the equilibrated two-phase solvent system in test tube. The tube was shaken to equilibrate the sample between the two phases. The phases were separated and evaporated to dryness under nitrogen (N_2) gas. The residue was dissolved in acetonitrile and analyzed with HPLC. The *K* value was indicated as the peak area of the target compound in the upper phase divided by that in the lower phase. The settling time, which is highly correlated to the retention of the stationary phase, was expressed as the time to form a clear layer between the two phases after mixing.

2.12 HPCCC Separation

In each separation run, the multi-layered coiled column was filled with the upper phase to form the stationary phase. The first step was performed in reverse phase mode at 25℃. The preparative column (total 135 mL) was filled with upper phase as the stationary phase. The mobile phase (lower phase) was pumped to the system at a flow rate of

2 mL/min while the columns were rotating at a speed of 1300 rpm. Sample was dissolved in same ratio of upper and lower phase with total volume of 6 mL and injected into the HPCCC system.

2.13 Identification of isolated compound

Isolated compound was analyzed with HPLC coupled with high resolution electrospray ionization source (HR-ESI/MS) for molecular weight determination and their structure were identified by comparing the ^{1}H and ^{13}C NMR spectra in DMSO-d_6 with references

2.14 Statistical analysis

All the data are presented as the mean ± SD from at least three independent experiments. An analysis of variance (ANOVA) with the Dunnett's t-test was used for the statistical analysis of multiple comparisons. A value of $p<0.05$ was chosen as the criterion for statistical significance.

III. RESULTS

1. Extraction and solvent partition

Powdered cantharides (50 g) was extracted three times with 80% acetonitrile by sonication at room temperature for 1 h. This process was repeated for three times. The extract was dried with rotary evaporator under 50℃. Dried extract was partitioned with three layer solvent system (water, methylene chloride, acetonitrile, and hexane)

Obtained fractions were tested with MTT assay to screen cytotoxicity in MDA-MB-231 cells. All fractions were tested with 25, 50, 100, and 200 μg/mL, and compared with control treating only DMSO.

The cell viability decreased at the highest concentration tested but it is still in the acceptance range (≥50%), except for methylene chloride and acetonitrile fraction which its IC_{50} value is 35.75 μg/mL

Therefore, a further separation was performed on methylene chloride and acetonitrile fraction (2.1g).

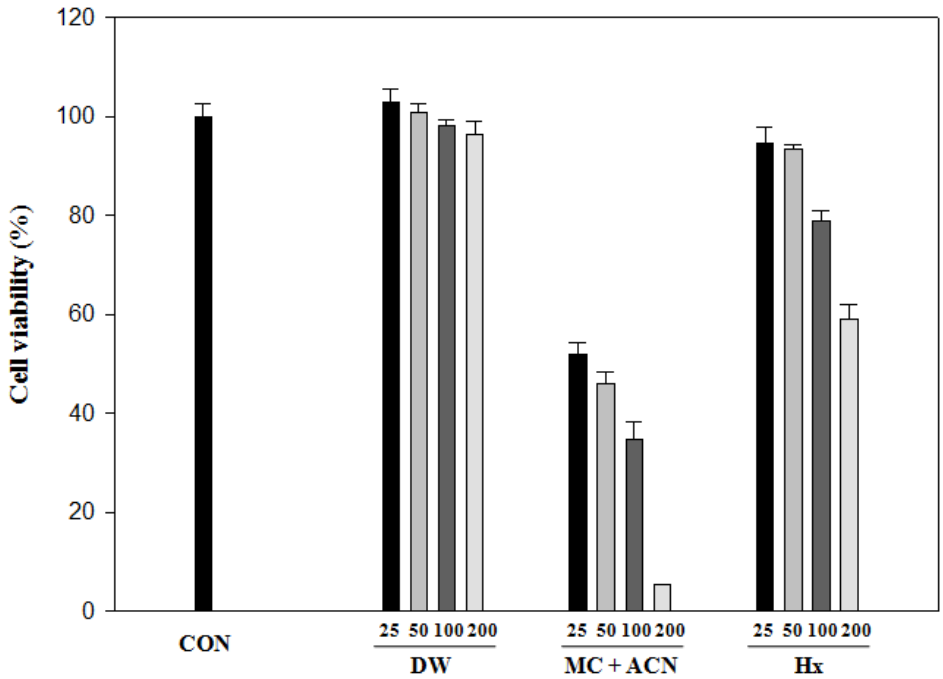

Figure 2. The cell viability of fractions obtained from solvent partition

Cell (1 x 10^5 cells/well) were seeded in 24-well plates and were treated with different dose of obtained fractions (25, 50, 100, 200 μM) for 24 h. Cell viability was determined by MTT assay. Data were derived from three independent experiments and are expressed as mean ± standard deviation. (S.D). Control (vehicle), DW (distilled water fraction), MC + ACN (methylene chloride and acetonitrile fraction), Hx (hexane fraction)

2. Separation of methylene chloride and acetonitrile fraction by preparative HPLC and the cell viability of obtained four fractions

The separation of methylene chloride and acetonitrile fraction was employed by preparative HPLC. Preparative HPLC condition was described in methods section. Part 1, part 2, part 3, and part 4 were obtained with yield 59.9 mg, 61.1 mg, 98 mg, and 9.8 mg, respectively. The representative HPLC chromatogram is shown in Figure. 3.

Four fractions obtained from preparative HPLC were tested with MTT assay to screen cell viability in MDA-MB-231 cells. All fractions were tested with 1, 2.5, 5, and 10 μg/mL, and compared with control (vehicle).

As shown in Figure. 4, MA1 and MA2 were showed no cytotoxicity at maximum concentration (≥10 μg/mL). MA3 showed the strongest inhibition of cell viability with IC50 value 3.15 ± 0.13 μg/mL, followed by MA4 with IC_{50} value 7.18 ± 0.35 μg/mL. Hence, a further separation was performed on MA3 fraction showing the strongest cytotoxicity (2.1g).

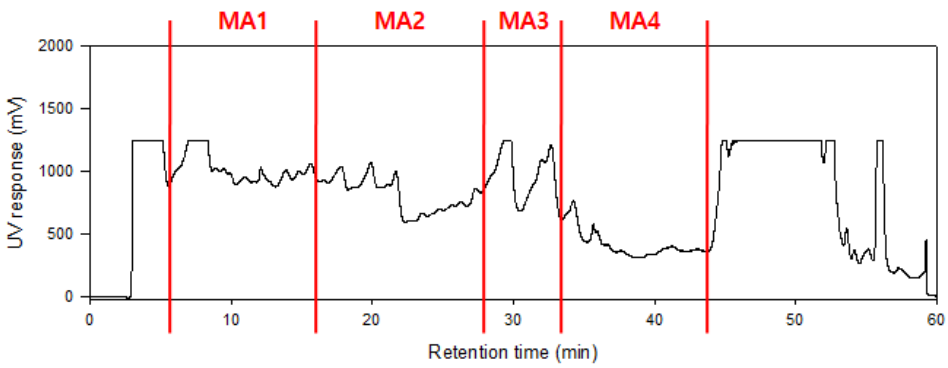

Figure 3. Preparative HPLC chromatogram of methylene chloride and acetonitrile fraction

Methylene chloride and acetonitrile fraction were isolated using preparative HPLC method. The mobile phase used was distilled water (solvent A) and HPLC-grade acetonitrile (solvent B). The HPLC condition of experiment was as follows: gradient elution, 0 – 40 min (25% - 45% B), 40 - 60 min (45% - 100% B). The flow rate was 3.0 mL/min for 60 minutes and monitored with UV detector at 230 nm.

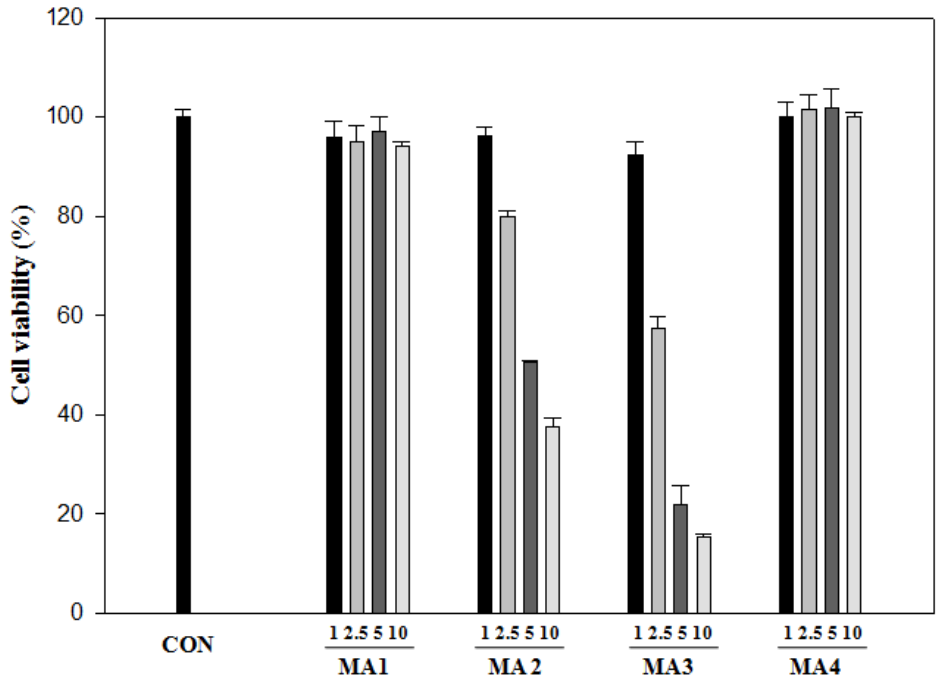

Figure 4. The cell viability of four fractions obtained preparative HPLC

MDA-MB-231 cells were seeded and treated with obtained fractions (1, 2.5, 5, 10 μM) for 24 h. MTT assay was performed to screen cell viability of fractions. Data were derived from three independent experiments and are expressed as mean ± standard deviation. (S.D)

3. Separation of MA3 fraction by HPCCC and the cell viability of separated fractions.

HPCCC was performed to separate MA3 fraction. Several solvent system were tested to investigate the optimum partition coefficient (*K*) and selected solvent system was Hexane, methylene chloride, acetonitrile, methanol, water (2 : 4 : 1 : 4 : 3, v/v/v/v/v). The HPCCC operation was conducted as described in methods section and the HPCCC chromatogram is shown in Figure. 5.

Separated MA3A, MA3B, MA3C from HPCCC were tested at 1, 2.5, 5, and 10 μg/mL. As shown in figure 6, MA3B fraction showed the highest inhibition of cell viability among all tested fractions with IC_{50} value 2.67 μg/mL(2.1g). Based on this result, further identification was performed on MC3B.

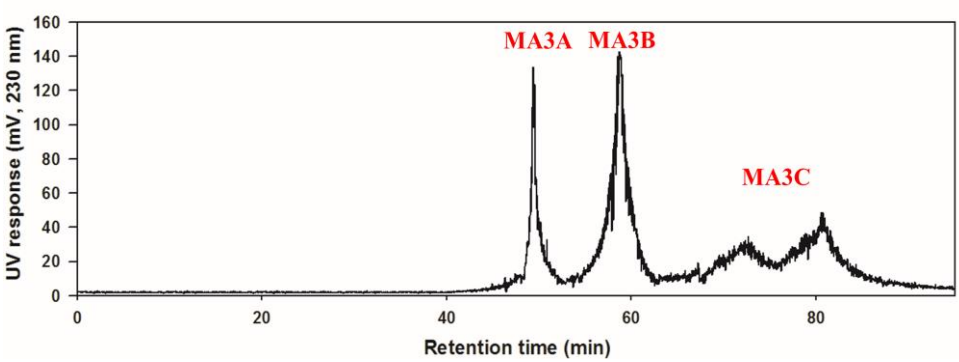

Figure 5. HPCCC chromatogram of MA3 fraction

HPCCC chromatogram of MA3 fraction is shown in above figure. The mobile phase was pumped to the system at a flow rate of 2 mL/min while the columns were rotating at a speed of 1300 rpm. Sample was dissolved in same ratio of upper and lower phase with total volume of 6 mL and injected into the HPCCC system.

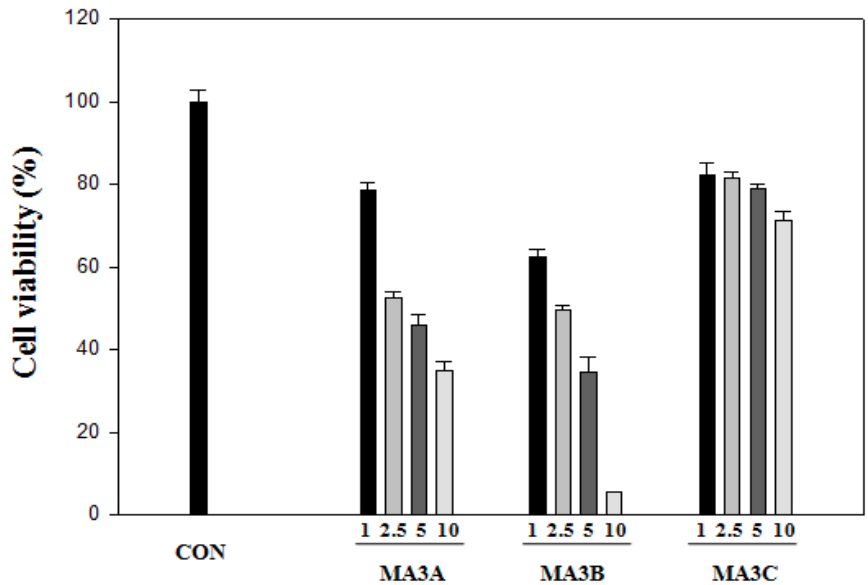

Figure 6. The cell viability of fractions separated from HPCCC

Cell (1 x 10^5 cells/well) were seeded in 24-well plates and were treated with MA3A, MA3B, MA3C fractions (1, 2.5, 5, 10 μM) for 24 h. Cell viability was determined by MTT assay. Data were derived from three independent experiments and are expressed as mean ± standard deviation. (S.D)

4. Identification of compound

Compound was isolated as white amorphous powder from separation of fraction. The UV detection of compound showed maximum absorption at 230 nm. The ESI-MS positive-ion mode spectra showed molecular ion peak $[M+H]^+$ at m/z 197.08 (Fig. 7)

1H NMR spectrum of compound showed signals at δ 4.73 (1H, d), 4.72 (1H, d), 1.81 (2H, m), 1.76 (2H, m), and 1.24 (6H, s) ppm. The 13C NMR spectrum of compound showed signal at δ 176.1 (C-1 and C-3), 55.5 (C-3a and C-7a), 23.6 (C-5 and C-6), 84.9 (C-4 and C-7), 12.9 (C-8 and C-9) (Fig. 8 and Table. 1). Comparing with another literature[28], it is proposed that this compound is cantharidin. The structure is shown in Fig. 9.

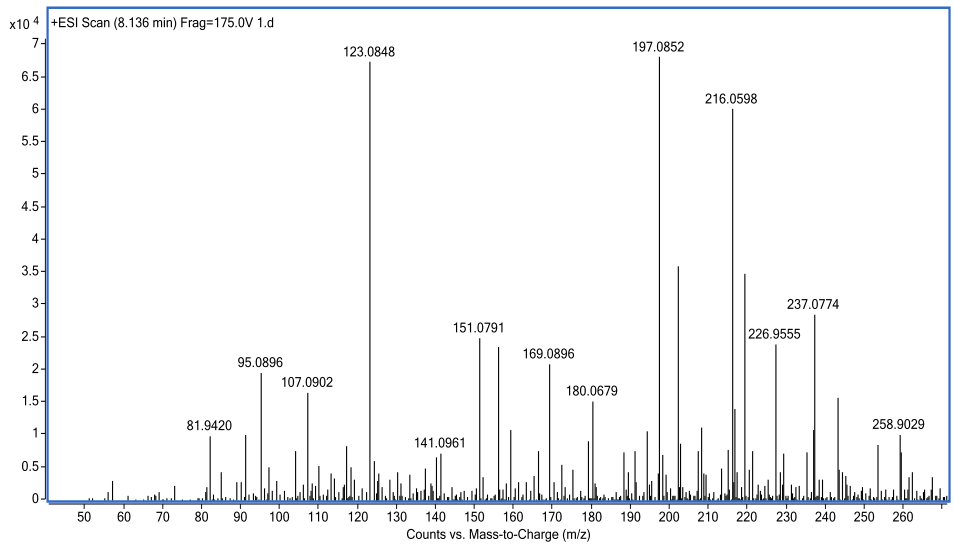

Figure 7. High resolution mass spectrometry analysis of compound.

ESI-MS positive-ion mode

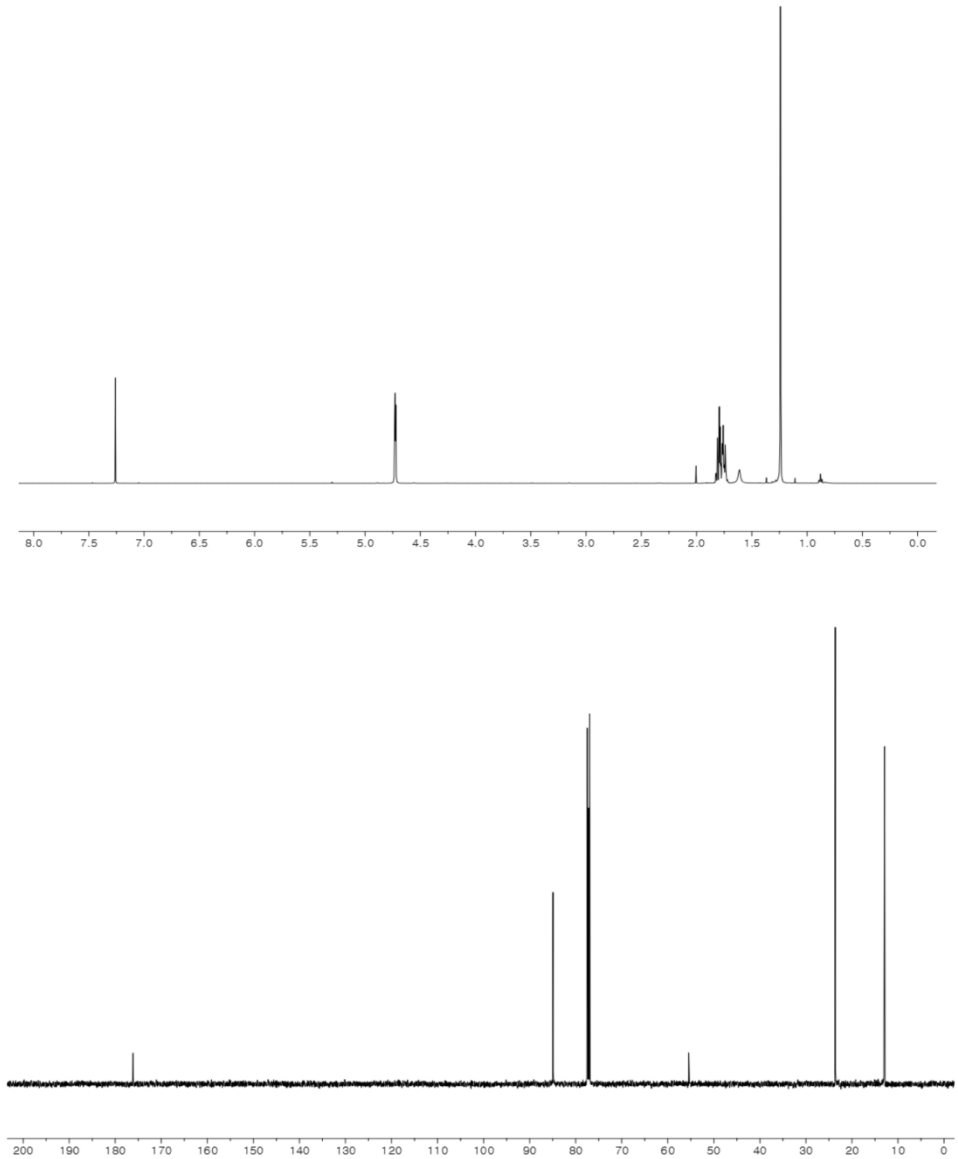

Figure 8. 1H and 13C NMR spectrum (500 and 125 MHz, respectively) of compound in $CDCl_3$

Table 1. ^{13}C and ^{1}H NMR Assignment for compound in $CDCl_3$

Position	δ_C	δ_H
1	176.1, C	
3	176.1, C	
3a	55.5, C	
4	84.9, CH	4.72, d (2.0)
5	23.6, CH_2	1.81, m
6	23.6, CH_2	1.76, m
7	84.9, CH	4.73, d (2.0)
7a	55.5, C	
8	12.9, CH_3	1.24, s
9	12.9, CH_3	1.24, s

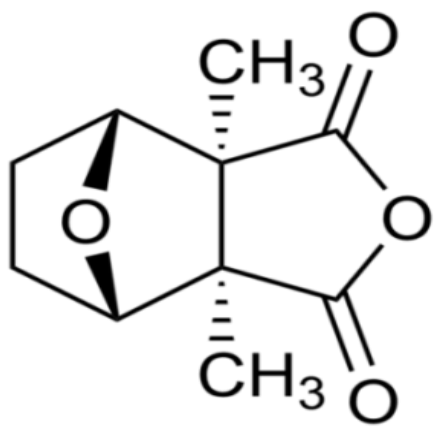

Figure 9. Chemical structure of cantharidin

5. Effect of cantharidin on the cell viability in MDA-MB-231 cells

First, we examined the cytotoxic effects of cantharidin on the proliferation of MDA-MB-231 cells. The cells were exposed to cantharidin at different concentrations for 6, 12 and 24 hours, and cell viability was measured by MTT assay. The dose- dependent inhibition of cantharidin on the proliferation of cells was observed (Fig. 10).

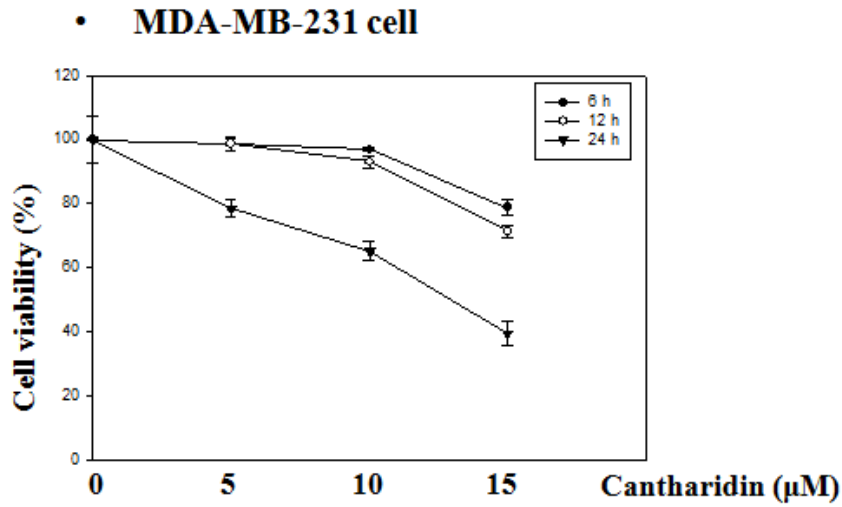

Figure 10. Cytotoxicity effect of cantharidin on MDA-MB-231 human breast cancer cells

MDA-MB-231 cells (1 x 10^5 cells/well) were seeded in 24-well plates and treated cantharidin (0, 5, 10, 15 μM) for different times. MTT assay was performed to determine cell viability.

6. Inhibitory effect of cantharidin on STAT3 activity

Next, we determined the effect of cantharidin on STAT3 phosphorylation in MDA-MB-231 cells. Western blot analyses demonstrated that cantharidin significantly inhibited protein expression of STAT3 phosphorylation at tyrosine 705 in a dose- and time dependent manner. However, this treatment did not reduce the protein levels of STAT3 phosphorylation at serine 727(Fig. 11A). Because STAT3 phosphorylation at tyrosine 705 leads to STAT3 dimerization and then nuclear translocation, we determined whether cantharidin suppresses the nuclear translocation of STAT3. Consistent with the inhibition of STAT3 phosphorylation, cantharidin inhibited STAT3 translocation to the nucleus (Fig. 11B). Although cantharidin inhibits STAT3 translocation, it is not known whether cantharidin regulate Stat3 transcriptional activity. Luciferase reporter gene assays were employed to detect STAT3 transcriptional activity. We found that cantharidin suppressed STAT3 transcription activity in MDA-MB-231 cells (Fig. 11C).

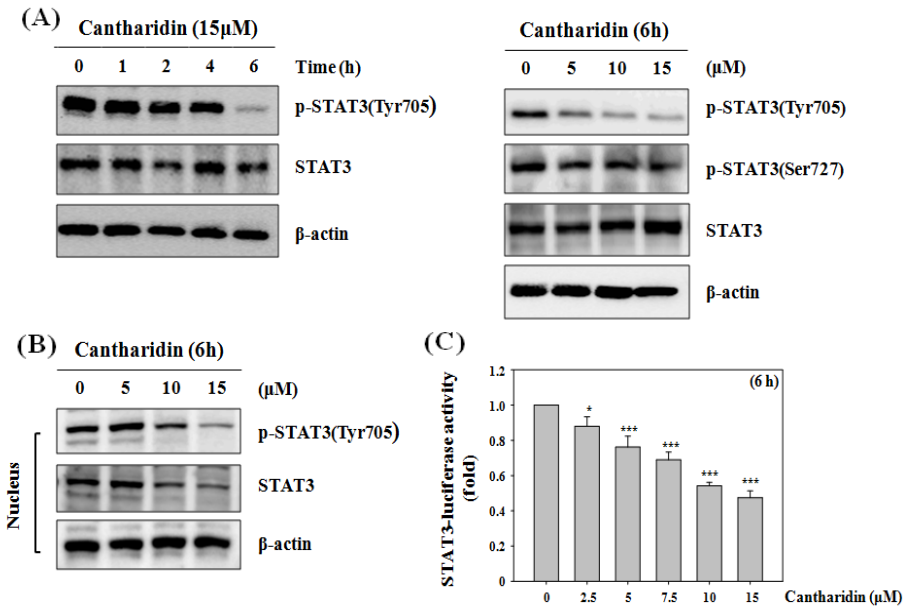

Figure 11. Effects of cantharidin on constitutively active STAT3 tyrosine phosphorylation in MDA-MB-231 cells

(A) The cells were treated with the indicated concentrations of cantharidin for 6 h and with 15 μM cantharidin for the indicated times. Cell lysates were prepared and subjected to SDS-PAGE and Western blotting to determine the p-STAT3 (Tyr705), p-STAT3 (Ser727), STAT protein levels. (B) The cells were treated with 0, 5, 10, 15 μM of cantharidin for 6 h. Nuclear extracts were prepared and Western blotting to determine nuclear translocation of STAT3. (C) MDA-MB-231 cells were transfected with the indicated plasmid, and then treated with each drug for 24 h. MDA-MB-231 cells were then treated with 0,

2.5, 5, 7.5, 10, 15 μM of cantharidin. Cell lysates were analyzed using luciferase reporter assay. Each value represents the mean ± SD. All data are p\0.0001 by Student t-test.

7. Effect of cantharidin on STAT3 upstream signaling pathways

To determine whether the inhibitory effect of cantharidin on STAT3 phosphorylation is associated with the suppression of upstream signaling pathways, we examined the effect of cantharidin on the activation of JAKs, PTPs, EGFR, and Src in MDA-MB-231 cells. When MDA-MB-231 cells were treated with cantharidin at the indicated concentrations for 6h, cantharidin did not reduce the protein levels of phosphorylated JAK1, JAK2, and PTPs, suggesting that cantharidin inhibits STAT3 phosphorylation independent of the upstream kinases JAK1, JAK2, and PTPs (Fig. 12A).

Activation of EGFR is found in many human cancers[29]. Inhibition of EGFR and its intracellular signal transduction pathways such as STAT3, AKT, and ERK could be a promising strategy for novel and selective anti-cancer therapies. There are several intracellular EGFR signaling by which STAT3 protein is phosphorylated, including direct activation of STAT3 by EGFR binding and indirect activation of STAT3 through Src-mediated EGFR signaling[19].

This result shows that cantharidin suppressed the activation of EGFR

and Src in a dose-dependent manner (Fig. 12B). MDA-MB-231 had sensitive to the expression of EGFR and Src after cantharidin treatment. To confirm whether STAT3 mediated EGFR signaling and cantharidin suppressed EGF stimulated STAT3 phosphorylation, cantharidin was treated on cells with EGF (100 ng/mL). As shown in Fig. 12C, cantharidin significantly inhibited activation of EGF-induced EGFR and STAT3.

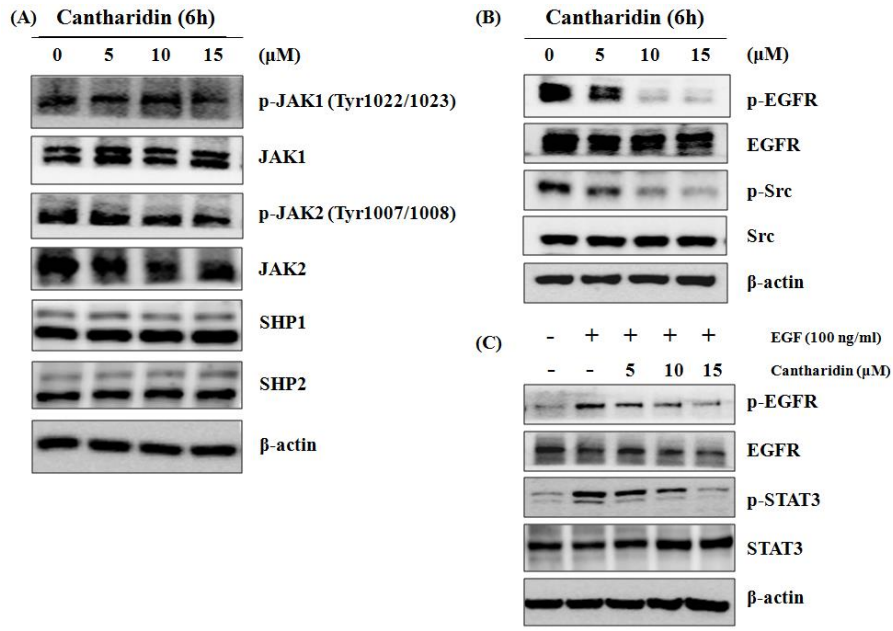

Figure 12. The effects of cantharidin on STAT3 upstream signaling including JAKs, PTPs, EGFR, and Src in MDA-MB-231 cells

Cells were seeded in 6-well plate and treated with 0, 5, 10, 15 μM of cantharidin for 6 h. (A) Effect of cantharidin on phosphorylation of JAKs and PTPs. (B) Inhibitory effect of cantharidin on activation of EGFR and Src. (C) Effect of cantharidin on EGF-induced activation of EGFR and STAT3. Cells were stimulated with EGF (100 ng/ml) for 10 minutes and treated with different doses of cantharidin. Cell lysates were prepared and subjected to SDS-PAGE and Western blotting with antibodies against p-JAK1, JAK1, p-JAK2, JAK2, SHP1, SHP2, p-EGFR, EGFR, p-Src, and Src.

8. Effect of cantharidin on STAT3 associated proteins

STAT3 associated proteins, including Akt, ERK, p38, and JNK play important role in signal transduction and regulating apoptosis[30]. To elucidate the mechanisms responsible for the effect of cantharidin on expression of proteins, we examined the phosphorylation of Akt, ERK, p38 and JNK in MDA-MB-231 cells. The phophorylation levels of Akt were decreased and the phophorylation levels of p38, JNK and, ERK were increased in a time-dependent manner at 24 h after cantharidin treatment. These results suggest that cantharidin suppressed cell proliferation through inhibition of phosphorylated Akt and potently induced the apoptotic activity of MDA-MB-231 cells by activating MAPK signaling pathways (Fig. 13).

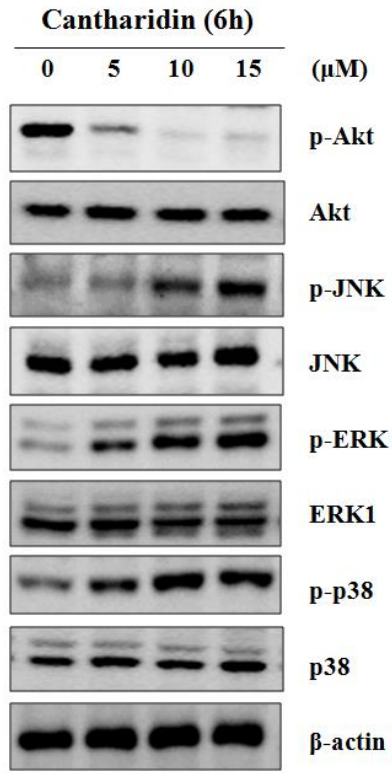

Figure 13. Effects of cantharidin on STAT3 associated proteins in MDA-MB-231 cells

Cantharidin induced the activation of Akt, ERK, p38, and JNK in a dose-dependent manner. MDA-MB-231 cells were treated with the indicated concentrations of cantharidin for 6 h. Cell lysates were subjected to SDS-PAGE and Western blot analysis with antibodies against p-Akt, Akt, p-p38, p38, p-JNK, JNK, p-ERK, and ERK.

9. Effect of cantharidin on expression of STAT3 down-stream genes

STAT3 activation has been reported to regulate the expression of various genes involved in cell proliferation, survival, angiogenesis, cell cycle progression, and programmed cell death[31]. Therefore, we assessed the effect of cantharidin on STAT3 target genes in MDA-MB-231 cells. The results showed that expression of STAT-3 target genes products that regulate proliferation (cyclin D_1), metastasis (COX-2), and anti-apoptotic protein (Bcl-2), was modulated by treatment of cantharidin (Fig. 14).

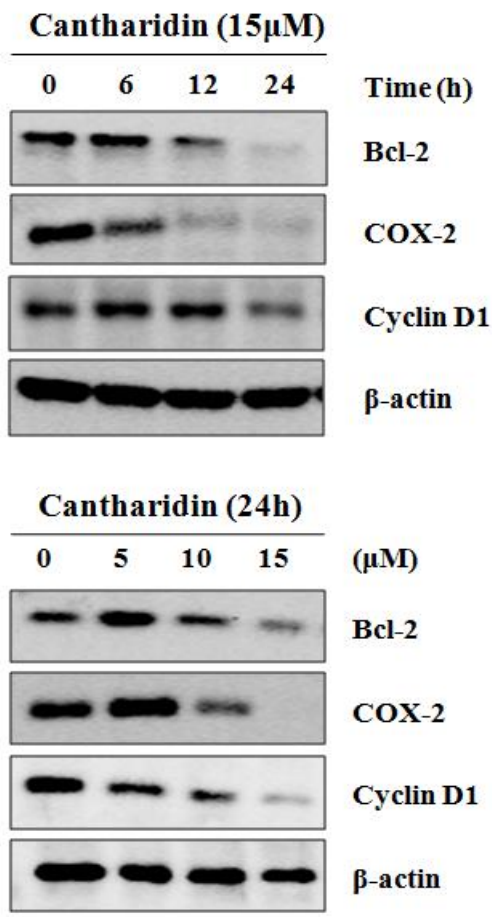

Figure 14. Effects of cantharidin on STAT3-regulated gene products

Cantharidin suppresses STAT3-regulated proliferative gene products. MDA-MB-231 cells were treated with 5, 10, and 15 μM of cantharidin for 6, 12 and, 24 h. Whole cell lysates were prepared and subjected to Western blotting for Bcl-2, COX-2, and cyclin D_1..

10. Induction of caspase-mediated apoptosis by cantharidin

It was hypothesized that cantharidin induces apoptosis through the activation of caspase-3, caspase-8 and PARP1. To prove that the apoptotic effect of cantharidin was due to caspase-dependent apoptosis, the levels of proteins implicated in apoptosis were evaluated using Western blotting. The results showed that cantharidin treatment of MDA-MB-231 cells increased apoptosis through both the extrinsic pathway, mediated by caspase-8 activation, thus ultimately activating the common downstream apoptosis effectors caspase-3 and PARP1(Fig. 15). These results suggested that cantharidin induce caspase-dependent apoptosis in MDA-MB-231 cells through both the extrinsic and intrinsic pathways.

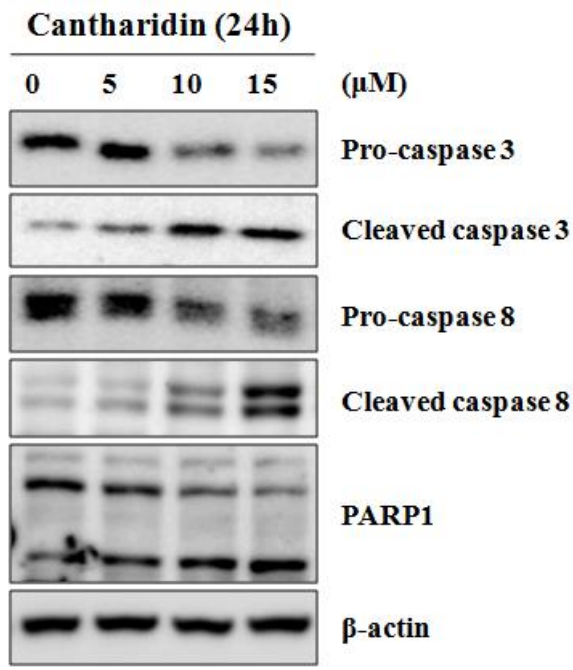

Figure 15. Induction of caspase-mediated apoptosis by cantharidin

Cantharidin induced apoptosis through a caspase-dependent pathway. Western blotting was performed using PARP1, caspase-3, and caspase-8antibodies. The results shown are representative of three independent experiments.

11. Effect of cantharidin on changes in cell morphology and apoptosis

Along with cytotoxicity, it was observed by microscope that cell morphology by cantharidin treatment was changed. When apoptosis occurs in cells, they undergo cell rounding, membreane blebbing, cytoplasmic condensation and fragmentation and finally chromatin condensation/fragmentation[32]. As MDA-MB-231 cells were incubated in a dose-dependent (0, 5, 10, 15), the shapes of cells were changed rounder and the formed to membrane blebbing (Fig. 16A).

In order to investigate whether cantharidin induces apoptosis, it was measured by flow cytometry using Annexin V-FITC and propidium iodide (PI) staining. The cells undergoing apoptosis expose phosphatidylserine (PS) on the external surface of the cell membrane and AnnexinV prefetentially binds to negatively charged phospholipids like PS in the presence of Ca2+[33, 34]. The quantification of AnnexinV binding to the cell membrane is a useful tool in detecting apoptotic cells. As PI is a fluorescence dye to bind to DNA by intercalcting between the bases, PI is membrane impermeant and generally excluded from viable cells[35]. Therefore, PI is commonly

used for identifying dying or dead cells and it can also evaluate the cell death and apoptosis.

Cantharidin was treated to MDA-MB-231 cells at the concentrations of 5, 10, and 15 μM for 24h. In MDA-MB-231 cells, the rates of early and late apoptosis increased markedly. The values of dead cell were 10%, 13%, and 24%, respectively (Figure. 16B).

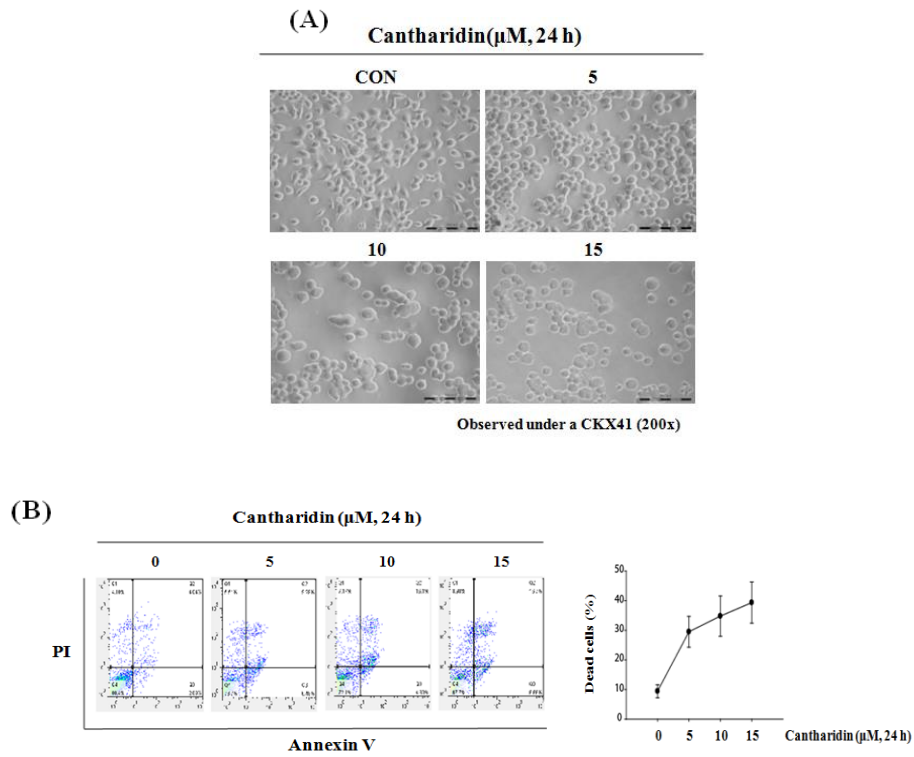

Figure 16. Effect of cantharidin on changes in cell morphology and apoptosis

(A) For the migration assay, cell monolayers were scratched with a micropipette tip, and the cells were exposed to cantharidin for 24 h. The cells were observed under a microscope at a magnification of 200×. (B) The lower right quadrant represents early apoptosis, and the upper right quadrant represents late apoptosis. The results were determined using a flow cytometry in which 15,000 events were counted per

sample. MDA-MB-231 cells were treated with 0, 5, 10, 15 μM of cantharidin for 24 h and subsequently analyzed with flow cytometry. The results shown are representative of three independent experiments.

12. Effect of cantharidin on cell migration

To further investigate the pharmacological activity of cantharidin against cancer metastasis, we examined the effect of cantharidin on cell migration in MDA-MB-231 cells. The effect of cantharidin on cell migration was examined using the wound-healing assay. As shown in Fig. 17, cantharidin significantly decreased cell motility in a dose-dependent manner for 24h. The values of cell migration were 55%, 30%, and 8%, respectively.

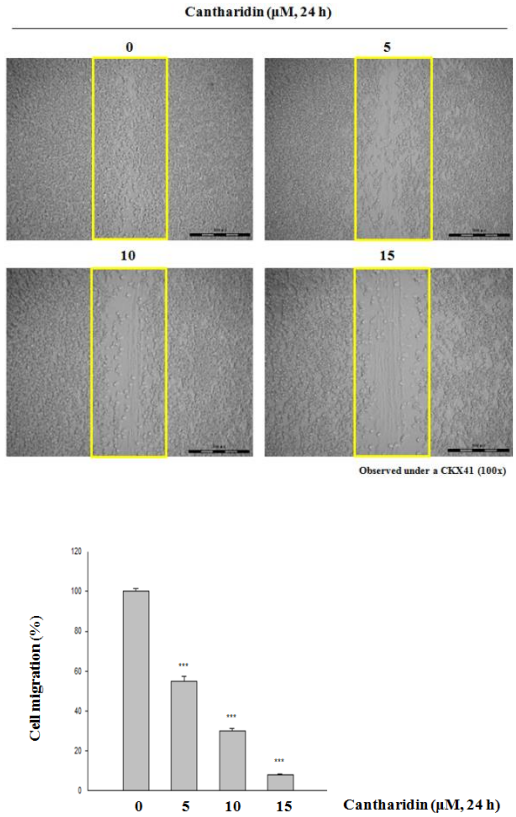

Figure 17. Effects of cantharidin on the migration of MDA-MB-231 cells

Cell monolayers were scratched with a micropipette tip, and the cells were exposed to cantharidin for 24 h. The cells were observed under a microscope at a magnification of 100×. Migrated cells across the yellow lines were calculated as a percentage of migration. The data shown are the means ±SD of three experiments. Significant difference from control group, $^{**}p<0.01$ and $^{***}p<0.001$.

13. Effect of cantharidin on cell invasion

The effect of cantharidin on cell invasion was examined using the Transwell chamber assay. As shown in Fig. 18, cantharidin significantly decreased the level of cell invasion through the matrigel in a dose-dependent manner. The values of cell invasion were 60%, 30%, and 13%, respectively.

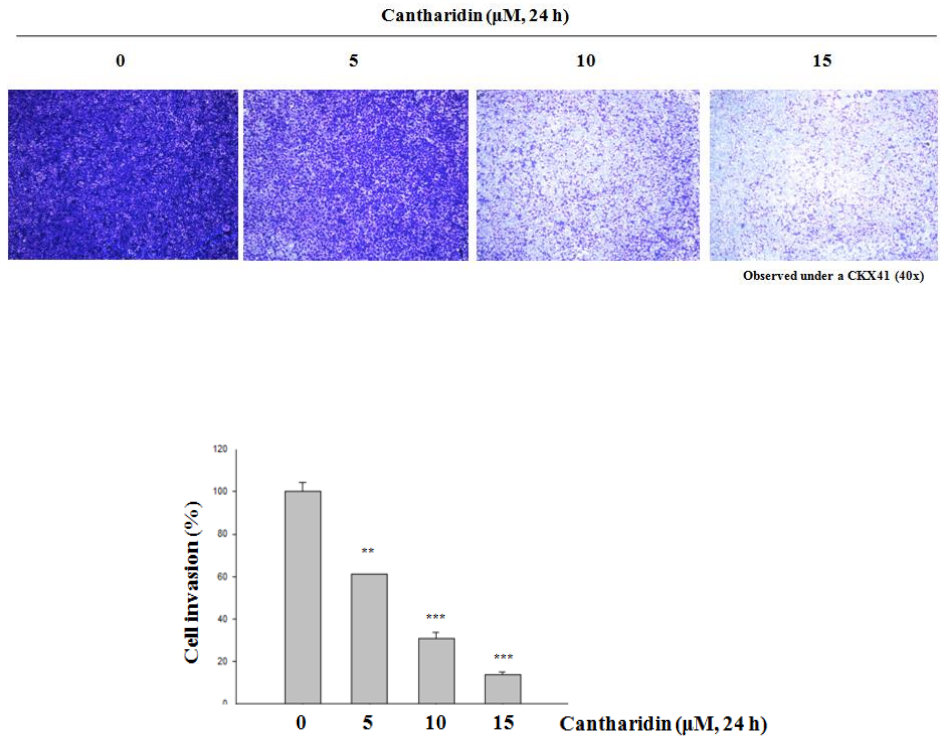

Figure 18. Effects of cantharidin on the invasion of MDA-MB-231 cells

Cells treated with cantharidin for 24 h were seeded into matrigel-coated transwell chambers and incubated for 24 h. The invading cells on the lower insert membranes were stained with crystal violet and observed under a microscope at a magnification of 40×. The number of invading cells was calculated as a percentage of invasion. The data shown are the means ±SD of three experiments. Significant difference from control group, $^{**}p<0.01$ and $^{***}p<0.001$.

IV. DISCUSSION

Triple negative breast cancer (TNBC) is characterized by the absence of the therapeutically targetable hormone receptors and HER2 protein[36]. For this reason, TNBC is an aggressive cancer for which prognosis remains poor and limited to chemotherapy[37]. STAT3 which is highly expressed in TNBC plays an important role in cell proliferation, survival, chemoresistance and metastasis[12]. Thus, Inhibition of STAT3 has potential for the treatment and prevention of TNBC. Cantharides are the dried body of the blister beetle. They used in traditional medicine for antitumor properties and wart treatment[24]. The aim of our study was to find potential compound from cantharides and to investigate the effects of compound on the STAT3 signaling pathway.

Activity-directed fractionation and purification processes were employed to identify the anticancer active compound from cantharides. Powdered cantharides were extracted with 80% acetonitrile and then separated into methylene chloride, acetonitrile, n-hexane and water layers. Among them, only methylene chloride and acetonitrile layer showed strong activity and therefore, subjected to separation and

purification using various chromatographic techniques. The extract was fractionated into 1-4 fractions by Prep-LC, and the third fraction showing potent activity was separated using CCC (Counter-Current Chromatography). Isolated fraction was identified by comparing spectral data (UV, NMR, and HRMS) with literature values to be cantharidin.

Cantharidin is an active constituent of cantharides[26]. It has been reported that cantharidin induces cell-cycle arrest and apoptosis in many human cancer cells, including hepatoma[38], colon cancer[39], bladder cancer[40], oral cancer[41], and pancreatic cancer[42]. Although numerous studies have shown that cantharidin induced cell death and apoptosis in many human cancer cells, there are no reports that cantharidin affected TNBC, especially MDA-MB-231. Thus, herein, we investigated the effects of cantharidin on cell death of MDA-MB-231 human breast cancer cells in vitro and the results indicated that cantharidin decreased the percentage of cell viability (Fig. 10). We demonstrated that cantharidin can inhibit the activation of STAT3. Cantharidin suppressed STAT3 phosphorylation at Tyr 705 (Fig. 11A). In addition, the result show that cantharidin inhibited nuclear translocation of STAT3 and STAT3-DNA binding activities (Fig. 11B).

Tyrosine residues on STAT3 are regulated by STAT3 upstream signaling cascades such as JAK1, JAK2, SHP1 and SHP2[43]. To determine whether the inhibitory effect of cantharidin on STAT3 phosphorylation is associated with the suppression of upstream signaling pathways, we evaluated the effects of cantharidin on phosphorylation of JAKs and PTPs. However, cantharidin did not affect the protein levels of constitutively phosphorylated JAK1, JAK2, and PTPs (Fig. 12A).

STAT3 has been reported to be activated by EGFR which is an upstream activator of several signaling pathways involved in progression of tumors[19]. We examined whether cantharidin inhibits the protein level of EGFR and Src in MDA-MB-231 cells. As shown in (Fig. 12B), cantharidin significantly inhibited Src and EGFR phosphorylation and EGF-induced activation of EGFR and STAT3 (Fig. 12C). EGFR is one of the main kinases involved in activation of a number of downstream pathways such as Akt, ERK, JNK and p38, which are also associated with STAT3[20]. The result shows that cantharidin suppressed the phosphorylation of Akt and increased the phosphorylation of ERK, p38, and JNK in a dose-dependent manner without affecting their total protein levels (Fig. 13).

STAT3 downregulates the expression of STAT3 target genes associated with survival, proliferation, and apoptosis[44]. The results showed that treatment of cantharidin resulted in inhibition of cyclin D_1, COX-2 and Bcl-2. In addition, the significant increase in the levels of the cleaved forms of caspase-3, caspase-8, and PARP1 in cantharidin-treated cells were also shown in Fig. 15. This result indicated that cantharidin induces apoptosis on MDA-MB-231 through extrinsic and intrinsic apoptosis pathway. To confirm whether cantharidin activates apoptosis, cell morphology observation and FACS analysis were conducted. From morphology observation optical microscope, we observed that the shapes of cells were changed rounder and the formed to membrane blebbing dose-dependently. In order to determine the apoptosis effect of cantharidin, FACS analysis has been employed. After treatment of cantharidin on MDA-MB-231 cells, the rates of early and late apoptosis significantly increased. To demonstrate the pharmacological activity of cantharidin against cancer metastasis, we examined the effect of cantharidin on cell migration and invasion. The results indicated that cantharidin has inhibitory effect on the migration and invasion in MDA-MB-231 cells.

In conclusion, this study provides that cantharidin induces apoptosis

and suppresses migration and invasion via inhibiting EGFR and STAT3 activation in MDA-MB-231 cells. Therefore, cantharidin may be a novel and potential anticancer agent against TNBC, especially MDA-MB-231 cells, by reducing phosphorylation of EGFR and STAT3.

V. CONCLUSION

These results indicated that cantharidin, an active constituent of cantharides, significantly inhibited EGFR-STAT3 signaling and blocked the nuclear translocation of STAT3 and its DNA binding in MDA-MB-231 cells. Cantharidin results in the inhibition of migration and invasion by regulating expression of STAT3 target genes. Moreover, cantharidin induces apoptosis through both the intrinsic pathway mediated by activation of caspase-3 and PARP1 and the extrinsic pathway mediated by caspase-8 activation. These results provide that cantharidin is proposed to serve as a novel therapeutic agent against Triple negative breast cancer.

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ABSTARCT IN KOREAN

유방암은 여성에게 있어서 가장 흔히 생명을 위협하는 암으로 진단된다. 일반적인 화학 치료제는 에스트로겐 수용체 (ER), 프로게스테론 수용체 (PR) 및 인간 상피 성장 인자 수용체 2 (HER2)의 3가지 수용체를 표적으로 합니다. ER, PR 및 HER2가 결핍 된 유방암 세포를 삼중음성유방암 (TNBC)이라고 합니다. TNBC는 전체 유방암의 약 15~20%를 차지합니다. TNBC는 암 검진 후 5년 이내에 재발 및 사망률을 증가시키는 것으로 알려져 있으므로 예후가 좋지 않은 것으로 간주됩니다. 최근 TNBC의 신호겸 전사활성화인자-3 (STAT3) 발현 수준이 높기 때문에 STAT3가 TNBC 치료의 주요 인자로 보고된다.

반묘로부터 세포 독성 화합물을 분리하기 위해 생물학적 검정 (bioassay-guided) 분별 및 정제가 수행되었다. 건조된 반묘를 분쇄하고 아세토니트릴로 추출한 다음, 염화메틸렌, 아세토니트릴, n-헥산 및 물층으로 분리하였다. 강한 활성을 갖는 염화메틸렌 및 아세토니트릴 층을 추가로 분리하고 다양한 크로마토그래피 기술을 사용하여 정제하였다. 추출물을 Prep-LC로 4개의 분획으로 나누어 가장 높은 세포독성을 나타내는 세번째

분획을 향류분배크로마토그래피 (CCC)로 분리하였다. 분리된 분획은 NMR 및 HRMS 데이터를 문헌 값과 비교함으로써 구조적으로 결정하고 최종적으로 칸타리딘으로 확인되었다.

칸타리딘은 가뢰과에 속하는 물집딱정벌레의 구성성분으로 전통적으로 사마귀를 치료하고 울혈을 완화시키는 데 사용된다. 또한, 많은 연구에 의하면 칸타리딘은 암세포에 세포 독성 효과가 있음이 밝혀졌다.

그러나 칸타리딘이 TNBC의 세포 성장을 억제하는 효과에 대한 보고는 아직까지 없었다. 본 연구자들은 칸타리딘이 STAT3 활성화를 억제함으로써 TNBC 세포 중 하나인 MDA-MB-231에서 세포 사멸을 유도함을 입증 하였다. 실험 결과 칸다리딘은 MDA-MB-231 세포에서 STAT3의 Tyr-705 잔기의 인산화를 감소시켰다. 칸타리딘은 EGFR, Src 및 STAT3의 활성화를 유의하게 억제하였으며, JAK-STAT3 신호 전달에는 영향을 미치지 않았다. 또한 칸타리딘은 cox-2, cyclin D1, bcl-2, caspase-3 및 parp1과 같은 STAT3 표적 유전자의 전사를 조절함으로써 세포증식을 억제하고 세포사멸을 유도하였다.

결론적으로, 이러한 연구결과를 토대로 칸타리딘이 EGFR-STAT3 신호전달을 억제함으로써 TNBC, 특히 MDA - MB - 231 세포에 대한 잠재적인 치료제로 사용될 수 있을 것이라고

기대된다.

주요어: 삼중음성유방암, MDA-MB-231, 표피성장인자수용체, 신호전달 겸 전사활성화인자 3, 칸타리딘, 세포사멸, 생물학적 검정, 향류분배크로마토그래피

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