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Effects of *Cirsium setidens* extract on gene expression related to apoptosis induction and metastasis inhibition in human breast cancer

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Abstract

Cell apoptosis induction and metastasis suppression in human breast cancer cell, MDA-MB-231, were studied to assess the potential of *Cirsium setidens* extract (CSE) on breast cancer treatment. Cell viability and metastasis analysis using MDA-MB-231 confirmed CSE significantly inhibited the growth and proliferation of cancer cell without suppressing normal cell growth in the range of 0.25 to 0.5 mg/mL of CSE. To analyze the anticancer effect of CSE, the expressions of major genes related to in the apoptosis, including *p53, AMPK-α, Bcl-2*, and *Bax* were analyzed by reverse transcription-polymerase chain reaction. The expressions of *AMPK-α*, *p53*, and *Bax* increased by 45, 43, and 68%, respectively, while the expression of *Bcl-2* decreased by 12%, showing that CSE treatment induced apoptosis of MDA-MB-231. The major compound of CSE was identified as rhoifolin, a type of flavone, through analysis using LC-MS/MS. Our findings confirmed that rhoifolin, known to possess a variety of significant biological activities, could be obtained from *C. setidens* and used as an effective natural compound to suppress breast cancer.

Keywords: *Cirsium setidens*; breast cancer; apoptosis; metastasis; phytochemical; rhoifolin.

Practical Application: *Cirsium setidens* is a perennial herb of the Asteraceae family, which contains various bioactive compounds such as rhoifolin and has been reported to have beneficial effects on hypertension, anti-tumor, antioxidant, and liver protection. Therefore, this study aims to investigate the effect of *Cirsium setidens* extract on breast cancer through the production of phytochemicals.

1. Introduction

Breast cancer is one of the three major cancers in women, accounting for 24.2% of cancers in women worldwide. As of 2018, 2.09 million patients were diagnosed with breast cancer, and in Korea, the incidence rate was 59.8 per 100,000 people, one of the highest among Asian countries (Liu & Luo, 2021). Breast cancer is caused by the abnormal proliferation of mammary lobules, which are located in the distal regions of the mammary gland, and of connective tissue cells around them; this is attributable to the exposure to endocrine disruptors (Kim & Lee, 2020). Owing to the large number of lymph nodes distributed in the breast, the metastasis rate of breast cancer is very high at 38%, and the viability rate in case of breast cancer metastasis decreases by 14%. In addition, because it is classified as an invasive cancer with a recurrence rate of 30% or higher, early diagnosis, prevention, and treatment are necessary to prevent complications such as lymphangitis and ulcers (van der Leij et al., 2012; Lee et al., 2021). Although the viability rate of breast cancer is very high at 93.3% with chemotherapy, currently applied chemotherapy has side effects including venous thrombosis; thus safer, and more effective breast cancer prevention and treatments based on natural compound are necessary (Heo & Heo, 2021; Feng et al., 2022).

Radiation therapy is currently considered the most effective cancer treatment; it is a non-surgical method and is widely used for the treatment of breast cancer and laryngeal cancer. However, irradiation causes side effects including cancerization of normal cell, fatigue, and difficulty in breathing, making it difficult to apply to immunocompromised or weakened elderly patients (Tae et al., 2012). In addition, targeted therapy, a drug treatment method, is a method of controlling and selectively removing cancer cell through an elimination mechanism that targets receptors or proteins related to cancer cell growth (Kwon, 2019). Targeted therapy minimizes damage to normal cell, but as it can develop resistance to the treatment when used in long-term or continuously, interest in developing treatments that can compensate for this shortcoming has been increasing progressively (Yu et al., 2015). Apoptosis, known as an important cell death mechanism in targeted therapy-based chemotherapy, regulates cell growth through extrinsic and intrinsic pathways to selectively eliminate abnormal cell (Ding et al., 2022). According to a recent pathological study revealing the mechanism of apoptosis according to the expression level of the target gene, it is reported that phenolic compounds such as polyphenols have an apoptosis activity through suppression of cancer cell metastasis and growth with fewer side effects (Shafay et al., 2021).

Cancer cells have the same cell cycle as normal cells and are divided into four stages: G0/G1, S, G2, and M phases (Maruszewska & Tarasiuk, 2019). Unlike normal cells, however, cancer cells escape from cell cycle control due to genetic mutations, and abnormal progress in the G1/S phase or G2/M phase occurs, resulting in continuous division and proliferation (Fischer et al.,

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2022). Cell cycle control is controlled by the activation of caspase, and polyphenols increase the activity of caspases, triggered apoptosis via a mitochondrial and a death-receptor pathway (Hafezi et al., 2020) Due to their capacity to control a number of cellular processes involved in carcinogenesis, polyphenols could be able to prevent or delay the development of certain cancers. In instance, it causes apoptosis and suppresses cell proliferation (Wolter et al., 2001; Billard et al., 2002). polyphenols have been shown to induce apoptosis, which has been linked to increased caspase activity, disrupting the cell cycle, decreased *Bcl*-2 and *Bcl-XL* levels, and increased *Bax* levels. It's interesting to note that these pro-apoptotic activities have been reported to frequently be associated to *p53* activation in many cancer cells (D'Archivio et al., 2008; Selvendiran et al., 2006). In addition, caspases contain cysteine and are normally present as proenzymes, but when cleaved by polyphenols, they limit the growth of cancer cells and induce apoptosis (Alaswad et al., 2021).

As dietary habits diversify these days, research on natural products for cancer prevention is becoming more important, so in this study, the anticancer effect of *Cirsium setidens* is to be investigated (Pandey & Tripathi, 2022). *Cirsium setidens* is a perennial herb of the Asteraceae family and has been used as a medicine and food because it is effective for hypertension, antitumor, antioxidant, and hepatoprotective (Shin et al., 2022). *C. setidens* contains bioactive compounds such as silymarin and is known to have an apoptotic effect in neuroblastoma in addition to liver protection, but studies on the induction of apoptosis in breast cancer cell are relatively insufficient (An et al., 2022; Lee et al., 2014). Therefore, in this study, in order to confirm the inhibitory effect on proliferation and metastasis of breast cancer cell from CSE, gene expression inducing breast cancer cell apoptosis was analyzed to confirm the anticancer mechanism, and major compound in *C. setidens* was analyzed to provide basic data for uses such as anticancer-related health functional foods or pharmaceutical compound in the future.

2 Materials and methods

2.1 Materials and reagents

C. setidens was purchased as a powder product from Agricultural Corporation Purunsan (Seoul, Korea). Human-derived breast cancer cell (MDA-MB-231) and kidney cell (HEK-293) for cytotoxicity test and anticancer gene expression were provided by Korea Cell Stock Bank (KCLB, Seoul, Korea). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Thermo Fisher (Waltham, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) for cell viability measure were obtained from Sigma-aldrich (St. Louis, MO, USA). Formic acid and acetonitrile used the LC-MS/MS mobile phase were HPLC grades from Sigma-aldrich.

2.2 Ultrasound-assisted extraction

Ultrasound extraction was used by modifying extraction condition such as Gam et al. (2021a). *C. setidens* powder (1 g) and 50% ethanol (10 mL) were mixed and conducted using an ultrasound device (SD-250H, Mujigae Co., Seoul, Korea) at 60 °C and 40kHz for 30 min. The obtained extract was recovered form supernatant at 10,000 rpm (5,600 g) for 10 min through a centrifuge (1236R, Labogene, Daejeon, Korea) at 4 °C and used as a sample for evaluating breast cancer cell proliferation and metastasis inhibitory effects.

2.3 Cell culture

MDA-MB-231 was used to analyze anticancer effect in order to confirm inhibitory effect on cancer cell proliferation and metastasis through *C. setidens*. HEK-293 was used to compare cell viability in order to confirm non-toxicity in normal cell. When culturing cell, it is used DMEM added 10% FBS and 1% penicillin-streptomycin and used to 0.05% trypsin-EDTA for subculture and cell recovery. The culturing was cultured in a CO_2 culture (Panasonic, Osaka, Japan) while maintaining a wet state at 37 °C and 5% CO_2.

2.4 Cell viability analysis

The MTT assay was performed to analyze the anticancer effect of CSE on the inhibition of breast cancer growth. MDA-MB-231 and HEK-293 were plated 5×10^3 cell/mL of 96-well-plate and cultured for 24 hr at 37 °C in a humidified incubator. After the CSE was added by concentration (0.0 ~ 1.0 mg/mL), cultured for an additional 48 hr, and 0.25 mg/mL MTT reagent (0.1 mL) was dispensed and reacted for 3 hr. The resulting formazan was dissolved in DMSO, and absorbance was measured at 540 nm with a microplate reader (AMR-100, Allsheng, Seoul, Korea), and the cell viability by the expressed as a percentage according to the following Formula 1 Calculated.

$$Cell\,Viability(\%) = \left(1 - \frac{Abs(Experimental)}{Abs(Control)}\right) \times 100 \quad (1)$$

Abs (Experimental): CSE treated sample, Abs (Control): DMEM treated sample.

2.5. Analysis of cell metastasis

To analyze inhibitory effect of cell metastasis on breast cancer cell of CSE, MDA-MB-231 was seeded into 24-well-plates at 2.5×10^5 cell/mL and cultured for 24 hr. A vertical incision of about 0.9 mm was formed with a sterilized pipette tip in the center of each well. After the CSE was treated with 0.0 ~ 1.0 mg/mL, and then observed metastasis according to cell growth in the incision using an inverted microscope (Leitz Fluovert FS, Leica, Wetzlar, Germany) at 12 hr. Metastasis of MDA-MB-231 by extract was measured by the incision area observed each time using image analysis program Image J (US National Institutes of Health, Bethesda, MD, USA). It was calculated as a percentage according to the following Formula 2.

$$Cell\,Metastasis(\%) = \left(1 - \frac{Area(Experimental)}{Area(Control)}\right) \times 100 \quad (2)$$

Area (Experimental): CSE treated sample, Area (Control): DMEM treated sample.

2.6. Apoptosis-related gene expression analysis

To analyze gene expression associated with apoptosis, MDA-MB-231 was cultured at 1×10^6 cell/mL in a 24-well-plate for 24 hr. And then cell were cultured with 0.0 ~ 1.0 mg/mL of CSE for an additional 24 hr. After collecting the cell, the total RNA was extracted from cell using AccuPrep® Universal RNA extraction kit (Bioneer, Daejeon, Korea) and quantified using NanoDrop™ 2000 spectrophotometer (Thermo fisher, Waltham, MA, USA). Reverse transcription was amplified cDNA of genes according to Table 1 using AmfiRivert cDNA synthesis platinum master mix (GenDEPOT, Barker, TX, USA). Each PCR product was electrophoresed on 1.5% agarose gel with Gel red® nucleic acid gel stain (Komabiotech, Seoul, Korea) and visualized by using Gel Doc™ XR+ System and Quantity One software (Bio-Rad, Hercules, CA, USA)

2.7 Quantification of Major compound

To analyze major compound of *C. setidens*, it separated major compound according to molecular weight distribution using ROC C18 column (3.0 mm × 150 mm, Restek Ltd., Bellefonte, PA, USA) put on LC-MS/MS (Finnigan TSQ Quantum, Thermo Fisher Sci., WA, USA). CSE was filtered 0.22 μm syringe filter (Hyundai Micro CO., Ltd., Seoul, Korea) and 0.01 mL injected. The operating condition used an electrospray ionization (ESI) method that ionizes molecules in a sample according to vaporization in a mass spectrometer, and the mass spectrum was analyzed through the full scan mode at 0 to 800 m/z. The flow rate and temperature of column was kept constant throughout the analysis at 0.2 mL/min and 30 °C and the elution mobile phases were mixed (A): 0.1% formic acid and (B): 99.9% acetonitrile. The elution conditions were as follows; (A): 0 ~ 11 min, 95%; (A): 0 ~ 15 min, 0%; (A): 0 ~ 20 min, 95%.

2.8 Statistical analysis

The experiment was repeated three times, and the results were marked with an average ± standard deviation and statistically processed using GraphPad Prism (version 5.0; GraphPad, San Diego, CA, USA). The significance analysis between the two experimental groups was an independent sample t-test and the statistical significance was analyzed based on $p < 0.05$

3 Results and discussion

3.1 Assessment of inhibition of breast cancer cell proliferation

To elucidate the anticancer effects of CSE through the evaluation of breast cancer cell proliferation inhibitory effect and non-toxicity on kidney cell, MDA-MB-231 and HEK-293 were treated with extracts of varying concentrations and cell viability was compared (Figure 1). When the cytotoxicity of CSE was confirmed, the viability rate of HEK-293 was ≥ 98.2% at a concentration of ≤ 0.5 mg/mL, showing no significant difference from the control group, and subsequently confirming the non-toxicity within the treatment range. However, MDA-MB-231 showed a significant decrease in cell proliferation compared to the control group at ≥ 0.25 mg/mL of the extract, and CSE effectively inhibited breast cancer cell growth at 0.25 ~ 0.5 mg/mL without inhibiting cell growth in normal cells and its anticancer effect was confirmed.

Phytochemical of *C. setidens* is thought to inhibit proliferation of cancer cell by blocking the progression from G1 phase to S phase, the stage of DNA replication, through inhibition of signal transduction between the cytoplasm and the membrane by binding to the C1b region of protein kinase Cα (PKCα) in the plasma membrane (Kim et al., 2021; Park et al., 2013). Accordingly, CSE is predicted to inhibit breast cancer cell proliferation by arresting the cell cycle of breast cancer cell and thereby inhibiting cell division. Therefore, CSE is non-toxic to normal cell at a concentration of ≤ 0.5 mg/mL while showing the effect of inducing MDA-MB-231 apoptosis. In the following studies, to exclude cell death effect by suppressing normal cell growth by the extract, the experiment was performed by setting the treatment concentration of CSE to ≤ 0.5 mg/mL.

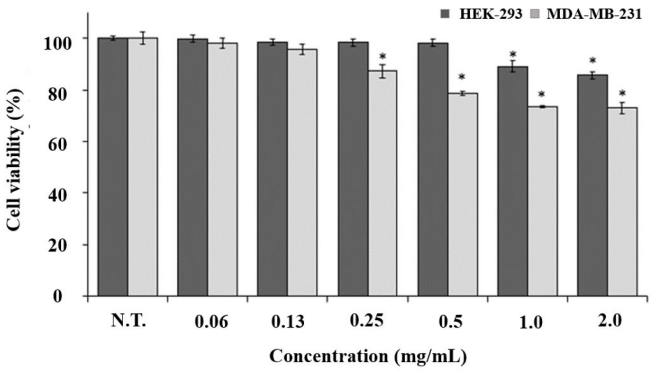

Figure 1. Comparison of cell viabilities of MDA-MB-231 treated with CSE (0.0 ~ 2.0 mg/mL) compared to HEK-293 for the analysis of cytotoxicity and anticancer effect. The cell viability was compared with non-treated cell by performing three repeated experiments and the asterisk (*) expresses significance compared to the control group (*$p < 0.05$).

Table 1. Primer sequences used in RT-PCR for analysis of major genes related to breast cancer suppression in MDA-MD-231.

Primers	Sequences	
	Forward (5' → 3')	Reverse (3' → 5')
p53	CCCATCCTCACCATCATCACAC	GCACAAACACGCACCTCAAAG
AMPK-α[1)]	GACACCAGTTTTGCCTCCAGTA	TCCAGAGGCGGAAGTTCTGT
Bcl-2[2)]	ATTGGGAAGTTTCAAATCACG	TCTATTCCTCTGTGATGTGT
Bax[3)]	GAGCTGCAGAGGATGATGATTCG	AAGTTGCCGTCAGAAAACACG
β-actin	TCACCCACACTGTGCCCATC	CAGCGGAACCGCTCATTGCCA

[1)]*AMPK-α*: AMP-activated protein kinase-α; [2)]*Bcl-2*: B-cell lymphoma-2; [3)]*Bax*: Bcl-2-associated x.

3.2 Assessment of breast cancer cell metastasis inhibition

Invasion and metastasis, the major mechanisms of cancer development, refer to the processes in which cancer cells enter the circulatory system through intravasation and form metastatic cancer in adjacent tissues through extravasation (Su et al., 2017). Therefore, preemptively blocking invasion and metastasis of cancer to inhibit cell metastasis, and in particular, a method of reducing cell mobility is necessary and is considered an effective strategy (Gam et al., 2021b). In this study, the effect of CSE on MDA-MB-231 metastasis was analyzed to confirm the metastasis inhibitory effect of the extract (Figure 2). After 36 hr in the untreated group, 78% of the incision area was restored, and 65% and 39% were restored at concentrations of 0.25 and 0.5 mg/mL, respectively. The restoration area decreased as the concentration of the extract increased, and the cell metastasis ability of MDA-MB-231 was effectively suppressed, proving that there was an effect of inhibiting metastasis.

Cell metastasis is promoted by filopodia in the form of long spindle-shaped structures formed by cross-linking of actin and myosin (Beak et al., 2008). Caspase, a protease activated during apoptosis, inhibits metastasis of cancer cells by breaking down filopodia (Shah et al., 2021). Based on the previous study that plant-derived phytochemical could enhance caspase activation, CSE is believed to induce metastasis suppression through caspase activation, thereby suppressing the viability rate and metastasis of breast cancer cell (Kim et al., 2009). Caspase is activated by *Apaf-1*, which is formed when cytochrome c released into the cytoplasm by phosphorylation of *AMPK-a* in the mitochondria binds to dATP. Therefore, the anticancer effect of *C. setidens* in breast cancer cell is expected to be suppressed by *AMPK-a*, an upstream regulatory protein of caspase, and it is considered necessary to analyze *AMPK-a* gene expression by CSE (Sai et al., 2006).

3.3 Analysis of apoptosis-related gene expression

In the analysis of breast cancer proliferation and metastasis inhibition based on the previous cell culture experiments, the anticancer effect of CSE was confirmed through the inhibition of MDA-MB-231 cell growth and reduced mobility. To confirm the effect on cell cycle inhibition and mitochondrial membrane disruption according to the mechanism of apoptosis, the expression of key carcinogenic genes was analyzed (Figure 3). In intracellular mitochondria, *AMPK-a* promotes a catabolism mode, contributes to the establishment of the glutamine degradation pathway, or induces phosphorylation of the tumor suppressor protein *p53* at Ser15 and accumulation in the mitochondria (Liang & Mills, 2013; Park et al., 2003). When phosphorylated *p53* on the mitochondrial surface increases, the complex which is formed by *Bcl-2*, which promotes cell viability in the endogenous pathway, and *Bax*, a protein that liberates cytochrome c from mitochondria and induces apoptosis, is rearranged and apoptosis occurs through their antagonism (Jo et al., 2019). Therefore, the anticancer mechanism through apoptosis is the accumulation of phosphorylated *p53* by the increase of *AMPK* in cancer cell, reducing *Bcl-2* that inhibits apoptosis and increasing *Bax* that promotes apoptosis, resulting in loss of cell membrane potential and induces apoptosis through the destruction of the mitochondrial membrane and release of cytochrome c (Park et al., 2018; Cho et al., 2016).

To confirm the anticancer effect based on the apoptosis induction mechanism related to cell cycle regulation and mitochondrial membrane destruction, the expression levels of *AMPK-α, p53, Bcl-2*, and *Bax*, which are major genes involved in apoptosis, were measured in breast cancer cell. First, the increase of *AMPK* that encodes the subunits of *AMPK*-α in expression was not significant up to 0.25 mg/mL, but a 67% increase compared to the control group was confirmed in 0.5 mg/mL treatment, suggesting that the extract has an inhibitory effect on cancer cell proliferation according to *AMPK-a* activation. *p53*, which is involved in cell cycle regulation, showed a tendency to increase upon the extract concentration in a concentration-dependent manner, and at the highest treatment concentration of 0.5 mg/mL, it increased by 43% compared to the control group, suggesting that effective breast cancer suppression could be enabled by the interaction between *AMPK-a* and the cancer suppressor gene *p53*. Additionally, *Bcl-2*, which encodes a protein that inhibits apoptosis in mitochondria, showed a concentration-dependent decrease, and it was confirmed that 0.5 mg/mL treatment decreased by 12% compared to the control group. However, *Bax*, a gene that promotes apoptosis by destroying the outer membrane of mitochondria in cancer cell, increased its expression at 0.25 mg/mL treatment concentration, and

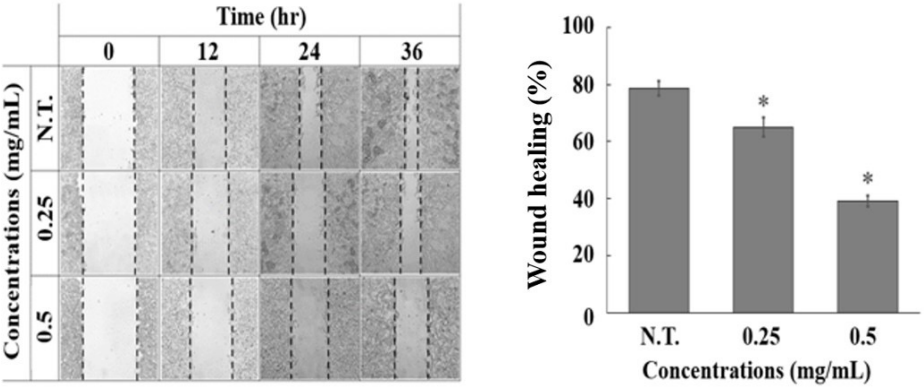

Figure 2. Effect of the treatment of various concentrations of CSE on the invasion and metastasis of MDA-MB-231. Cells were cultured with CSE with 0.25 and 0.5 mg/mL for 36 hr. The metastasis of CSE treated HEK-293 was compared with that of non-treated cell by performing three repeated experiments and the asterisk (*) expresses significance compared to the control group (*$p < 0.05$).

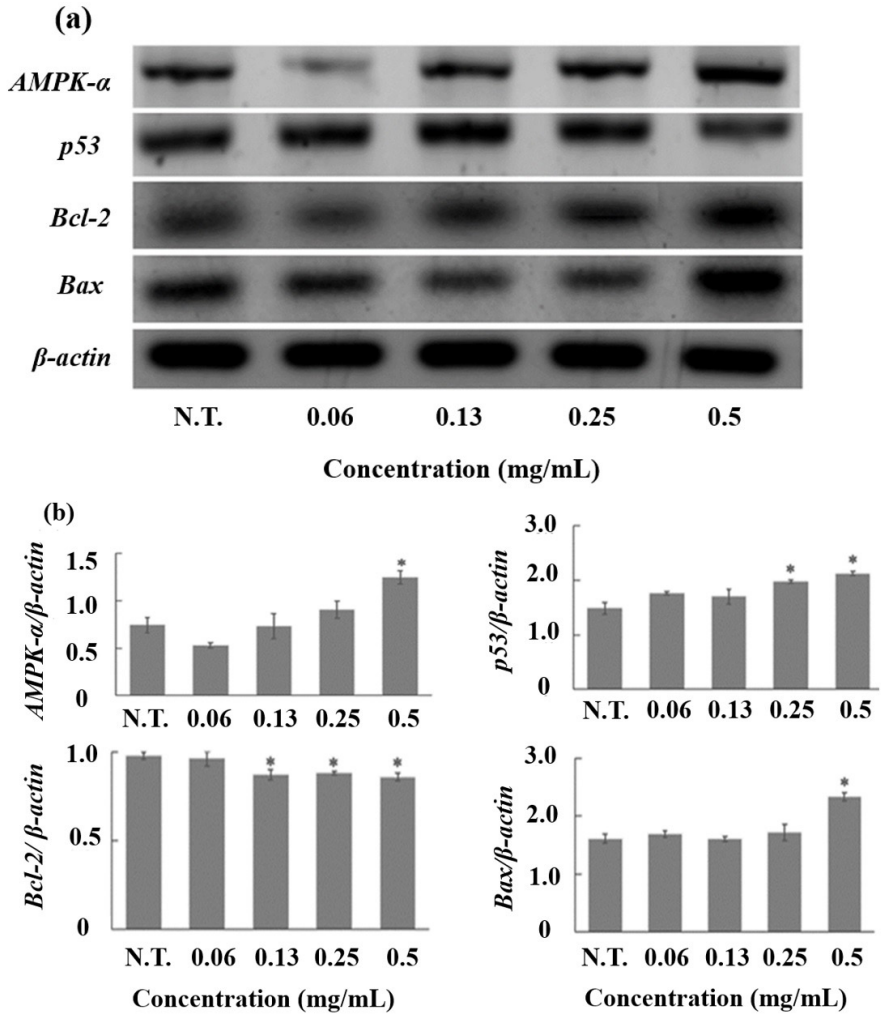

Figure 3. (a) Effect of CSE on the expressions of *p53*, *AMPK-α, Bcl-2*, and *Bax* in MDA-MB-231. (b) Cells were treated with 0.0 ~ 0.5 mg/mL of CSE for 24 hr. The asterisk (*) expresses significance in gene expression of each treatment compared to the control group (*$p < 0.05$).

showed a significant increase of 45% compared to the control group when treated with 0.5 mg/mL. Accordingly, it is judged that *C. setidens* induces apoptosis by increasing *Bax* by inducing an increase of *AMPK-a* in breast cancer cell and by suppressing *Bcl-2* related to apoptosis. Through analysis of the expression of major genes in apoptosis, it has been proven that CSE effectively induces apoptosis and inhibits the proliferation and metastasis of breast cancer cell; thus, it is understood that *C. setidens* can be used as a functional food and pharmaceutical compound that exhibits anticancer effects.

3.4 Quantification of Major compound

CSE was quantitatively analyzed using LC-MS/MS to search for major compound that express apoptosis-related genes in breast cancer cell (Figure 4). When the molecular weight of CSE was measured in negative ion mode in LC-MS/MS, m/z 577, which could be seen in the form of rhoifolin [M-H], was detected at the retention time of 5 min, and thus the major compound of *C. setidens* was predicted to be rhoifolin. Rhoifolin, which was identified as a major compound of *C. setidens*, is a flavonoid-based phytochemical, and flavonoids are known to possess various bioactivities, including antitumor, antioxidant and hepatoprotective effects by controlling mechanisms such as removal of reactive oxygen species, suppression of angiogenesis, and reduction of cell viability and proliferation (Wan & Huang, 2022).

According to a recent study, it was reported that the anticancer effect of flavonoids to hinder the proliferation of breast cancer cell occurs by inducing apoptosis through G1-phase arrest or G2-phase and M-phase arrest in the cell cycle of breast cancer cell. In particular, rhoifolin is known to reduce the metastatic ability of breast cancer cell by inhibiting signal transduction between the plasma membrane and the actin cytoskeleton (Cho et al., 2011; Xiong et al., 2021). Breast cancer cell invades and infiltrates into blood vessels via the epithelial-mesenchymal transition, and rhoifolin inhibits ezrin, a protein that connects the plasma membrane and the actin cytoskeleton and transmits signals, preventing cancer cell invasion and metastasis (Li et al., 2008). It was believed that the anticancer effect of *C. setidens* confirmed

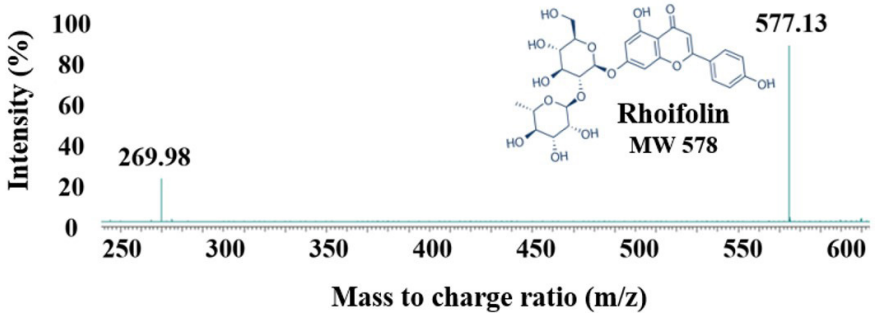

Figure 4. Spectrum of LC-MS/MS fragmentation pattern of major compound, rhoifolin (MW 578) from CSE (full scan in negative ion mode).

through cell mobility and gene expression experiments was caused by rhoifolin, which was identified as a major compound, inducing apoptosis through cell cycle inhibition. In addition, caspase activated by rhoifolin is believed to have inhibited breast cancer invasion and metastasis by degrading filopodia or interfering with breast cancer cell signal transduction in the cytoskeleton, and thus, *C. setidens* is presumed to have anticancer effect through suppression of breast cancer cell proliferation and metastasis.

4 Conclusions

In this study, to confirm the inhibitory effect of CSE on breast cancer cell proliferation and metastasis, the metastatic activity of related cancer cell was analyzed, gene expression was compared, and extract analysis was performed to search for compound with breast cancer inhibitory effect. CSE was confirmed to be non-toxic to normal cell at 0.5 mg/mL or less, and it was confirmed that it had an anticancer effect by effectively inhibiting breast cancer cell proliferation and metastasis by inhibiting cell viability and cell migration in a concentration-dependent manner. When the mRNA expression of *AMPK-α*, a major anticancer gene, was confirmed to identify the apoptosis-related mechanism of CSE in breast cancer cell, the expression of *AMPK-α* and *p53* tended to increase in a concentration-dependent manner. In addition, since *Bcl-2* decreased but *Bax* increased, it is believed that the apoptotic effect of CSE occurred through loss of cell membrane potential, destruction of mitochondrial membrane and release of cytochrome by regulating *Bcl-2* and *Bax*, which are proteins related to cell viability. As a result of analyzing the major compound of *C. setidens*, rhoifolin, known as a flavonoid, was detected, suggesting that rhoifolin could be an effective compound that inhibits invasion and metastasis through apoptosis of breast cancer cell and interfering with cell-cell interactions. Therefore, CSE or rhoifolin was concluded to be effective in suppressing the proliferation and metastasis of breast cancer cell by effectively inducing the apoptotic mechanism of breast cancer cell, and it was considered to have high potential for uses as a functional food and pharmaceutical material for breast cancer treatment in the future.

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