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Anti-cancer Activities of the Ethyl Acetate Fraction from *Opuntia humifusa* in PANC-1 Human Pancreatic Cancer Cells

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Abstract With the development of health technology, effective treatments for many types of cancer have been developed and contributed to increasing the survival rate of cancer patients, but pancreatic cancer still has a very low survival rate. In this study, the effect of ethyl acetate (EtOAc) fraction from *Opuntia humifusa* on PANC-1 human pancreatic cancer cells was investigated. The extract was obtained by treating the fruit powder of *O. humifusa* with 50% EtOH, and then the EtOAc fraction was selected to conduct this study. The PANC-1 cells cultured in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum and penicillin were seeded on a 96 well plate, cultured for 24 h, and treated with EtOAc fraction for 12 to 72 h. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay confirmed that cancer cells were reduced by more than 50% in the experimental group treated with EtOAc fraction for 48 h compared to the control group. In order to explore the signal transduction pathway in anti-cancer activity, western blotting was performed. ERK1/2 and Akt, enzymes involved in the upstream signaling pathway directing cell proliferation and invasiveness were reduced by the EtOAc fraction compared to the control. Cleaved caspase-3, a representative effector caspase involved in apoptosis was increased compared to the control by the EtOAc fraction. The EtOAc fraction extensively induced apoptosis through extrinsic, intrinsic, and ER-stress signaling pathways. Effective anti-pancreatic cancer activity was achieved by treating PANC-1 cells with EtOAc fraction through reduction of poly (ADP-ribose) polymerase (PARP), a necessary factor for cell repair, lamin A/C, a major component of nuclear membrane structures, and CDK4, which is required to start the G1/S phase in the cell cycle. In conclusion, it was found that the EtOAc fraction from *O. humifusa* inhibited the proliferation, invasiveness, cell cycle progression, and cell repair ability of PANC-1 pancreatic cancer cells, while effectively inducing apoptosis of them through extrinsic, intrinsic, and ER-stress signaling pathways.

Keywords: *Opuntia humifusa, PANC-1, apoptosis, cell cycle arrest, signaling pathways*

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1. Introduction

Indian fig (*Opuntia humifusa*) is often called devil's tongue, Eastern prickly pear, and in Korea it is called Cheonnyeoncho. It bears red or purple fruits, and has a strong characteristic of maintaining vitality without freezing to death by reducing its own moisture to less than half even in extreme cold of –20°C. Therefore, development as a crop for regionally specialized industries is promising, but its pharmacological properties have not been clearly identified systematically and scientifically. In order to improve the usability as a health functional food and health care products, in-depth research on its ingredients and functionality is required [1,2,3].

Malignant tumors, that is, cancers, generally lose the specificity of cell division that normal cells have, and grow excessively without control. Cancer cells rapidly proliferate and metastasize to other surrounding tissues, ultimately leading to death of the patient. These cancers are one of the main causes that threaten the health and life of modern people, and the reason why pancreatic cancer was selected in this study is that pancreatic cancer is a cancer that is very difficult to diagnose early, although early diagnosis is more important than any other cancer. Like other cancers, the occurrence of pancreatic cancer is influenced by genetic factors and environmental factors such as smoking, drinking, stress, and obesity. Among them, as a genetic factor, the mutation of a gene called K-Ras is found in more than 90% of pancreatic cancer, but it is difficult to know statistically clear factors [4].

Accordingly, pancreatic cancer is a carcinoma with a very low survival rate, and the 5-year relative survival rate remains in the range of 2-9%. This is very low compared to other cancer types [5].

The reason why pancreatic cancer has a higher mortality rate than other carcinomas is that it is very likely that it has already reached its final stage when symptoms are manifested and diagnosed. Pancreatic cancer shows few symptoms in the early stages of its occurrence, so early detection is very difficult, and treatment is delayed. Excisional surgery is the only way to expect a cure, but only 20% of patients can be operated at the time of diagnosis. Thus, the prognosis from diagnosis to treatment is still very poor. In addition, due to pancreatectomy, digestive juices and insulin are not sufficiently secreted, so there is a high risk of problems such as diarrhea and diabetes, and recurrence is frequent within 1 to 2 years after surgery [6,7,8,9]. Consequently, it is more important to prevent pancreatic cancer than any other cancer. In particular, active treatment for chronic pancreatitis is important, and correct eating habits along with steady exercise are important [10]. It is also considered important to consistently consume anti-oxidant, anti-inflammatory, and anti-cancer foods.

In this study, in order to find out whether *O. humifusa* contains components effective for anti-cancer activity, the extraction with 50% ethanol (EtOH) and then fractionation with ethyl acetate (EtOAc) was executed to obtain the experimental materials. Subsequently, the western blot analysis was mainly performed to identify the signal transduction pathways related to anti-cancer activity [11,12,13,14]. To identify the mechanism of specific anti-cancer activity, apoptosis was first focused and key caspase markers were identified. The cleaved caspase-3 is a final executioner of extrinsic, intrinsic, and ER-stress pathways in apoptosis, and an increase in cleaved caspase-3 is a clear clue that apoptosis must occur [15].

The purpose of this study is to investigate the signaling mechanisms of anti-pancreatic cancer activity induced by the EtOAc fraction from *O. humifusa* against PANC-1 human pancreatic cancer cells.

2. Materials and Methods

2.1. Plant Material and Preparation of the EtOAc Fraction

The cold dry powder of *O. humifusa* was provided by Upo Wetland (Goodday Farming Corporation, Changnyeong, South Korea). To prepare the EtOAc fraction, 500 g of *O. humifusa* powder was boiled in 50% EtOH for 6 h, repeating the boiling process five times to ensure thorough extraction. The EtOH extract was then concentrated using rotary evaporation at a controlled temperature of 55°C. The resulting concentrate was split into small portions and further separated into components using a 1:1 ratio of EtOAc to water (H_2O). The organic EtOAc fraction, containing the target compounds, was obtained and subsequently concentrated under reduced pressure using rotary evaporation at 45°C and stored at -20°C until use. The extraction and partitioning process was carried out following established protocols with minor modifications [12,13].

2.2. Cell Line and Reagents

PANC-1 human pancreatic cancer cells were bought from the Korea cell line bank (KCLB, Seoul, South Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin were purchased from Hyclone (Logan, UT, USA). Phosphate Buffered Saline (PBS, pH 7.4) was purchased from Welgene (Gyeongbuk, South Korea). Antibodies against cleaved caspase-3, pro-caspase-8, pro-caspase-9, pro-caspase-12, ERK1/2, Akt, lamin A/C, PARP, and CDK4 were purchased from Cell Signaling Technology (Beverly, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibody was, also, bought from Enzo Life Sciences (Farmingdale, NY, USA). All other reagents used were of the highest grade available.

2.3. *In vitro* Experiment

2.3.1. Culture of PANC-1 Cells

PANC-1 cells were incubated at 37°C with 5% CO_2 in DMEM including 100 units/mL penicillin and 10% FBS. The cells were subcultured every 3-5 days. The culture medium was changed every day. The cells were cultured for 24 h and treated with various concentrations of *O. humifusa* EtOAc fraction or 0.1% dimethyl sulfoxide (DMSO).

2.3.2. Cell Viability Assay

Cell viability was examined by a CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manual. PANC-1 cells were seeded on 96-well plates and incubated at 37°C for 24 h. After then cell culture media were replaced with serum-free media (SFM) and incubated for an additional 12 h. Next, the cells were treated with various concentrations of *O. humifusa* EtOAc fractions for 12, 24, 48, 72 h. Subsequently, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was added and incubated for 3 h at 37°C in a humidified atmosphere with 5% CO_2 Using a microplate reader, absorbance was measured at 490 nm. The cell viability assay was repeated at least 3 times.

2.3.3. Western Blot Analysis

PANC-1 cells were treated with 500 μg/mL of *O. humifusa* EtOAc fractions, washed twice with PBS, and harvested using a cell scraper. The pellets of PANC-1 cells were resuspended in a lysis buffer and kept on ice for 1 h and cell debris was removed using centrifugation at 10,000 x g for 10 min. Protein concentrations were measured by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein were mixed with 2 × Laemmli sample buffer and heated at 95°C for 5 min. Samples were then loaded onto 10-12% SDS-polyacrylamide gels and transferred onto a polyvinylidene fluoride (PVDF) membrane for 1 h 30 min

with a wet transfer system (Hoefer, MA, USA). Membranes were blocked with 5% non-fat milk dissolved in PBST for 1 h at room temperature and then incubated overnight with primary antibodies at a 1:1,000 dilution on Open Air Rockers (Thermo Fisher Scientific, MA, USA) in the refrigerator. After then, membranes were washed for 10 min with PBST 3 times. Subsequently, the membranes were incubated on Open Air Rockers for 1 h at room temperature with 1:5,000 dilution of the secondary antibody (HRP conjugated goat anti-rabbit IgG) and washed three times with PBST for 10 min. Final detection was performed using ECL detection kits (Santa Cruz, CA, USA).

3. Results

3.1. Preparation of EtOAc Fraction from *O. humifusa*

EtOH extracts and EtOAc fractions of *O. humifusa* fruit powder were successfully prepared. The fruit powder of the *O. humifusa* was extracted with 50% EtOH, and then fractionated with EtOAc. The EtOAc fraction was concentrated under reduced pressure to be the form of mucus, not the form of powder. Using a total of 500 g of *O. humifusa* fruit powder, 4.9 g of EtOAc fraction was obtained (Figure 1).

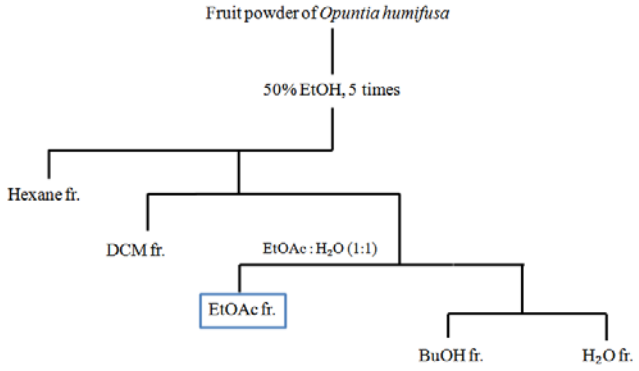
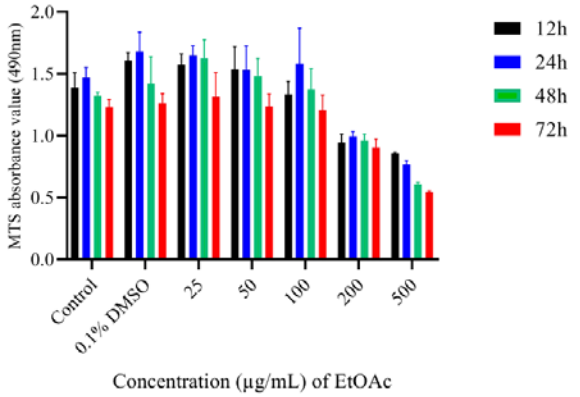
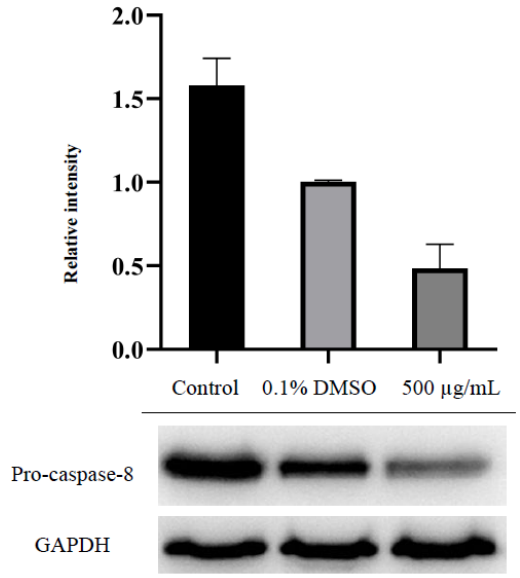


Figure 1. Procedures of extraction and fractionation of experimental materials from *Opuntia humifusa*



PANC-1 cells ($1x10^4$ cells/well) were treated with the indicated concentration (control, 0.1% DMSO, 25, 50, 100, 200, and 500 μg/ml) of *O. humifusa*. Cell viability was measured after 12, 24, 48, and 72 h by MTS assay. The results were expressed as means ± SD (n = 3).

Figure 2. Inhibitory effect of *O. humifusa* EtOAc fractions on proliferation of PANC-1 cells



PANC-1 cells ($1.5×10^6$ cells/100 mm plate) were incubated with *O. humifusa* EtOAc fraction (500 μg/mL). The expression levels of pro-caspase-8 were determined by western blot analysis. The results were expressed as means ± SD (n = 3).

Figure 3. Effect of *O. humifusa* EtOAc fraction on protein levels of pro-caspase-8 in PANC-1 cells

3.2. Cell Viability Test

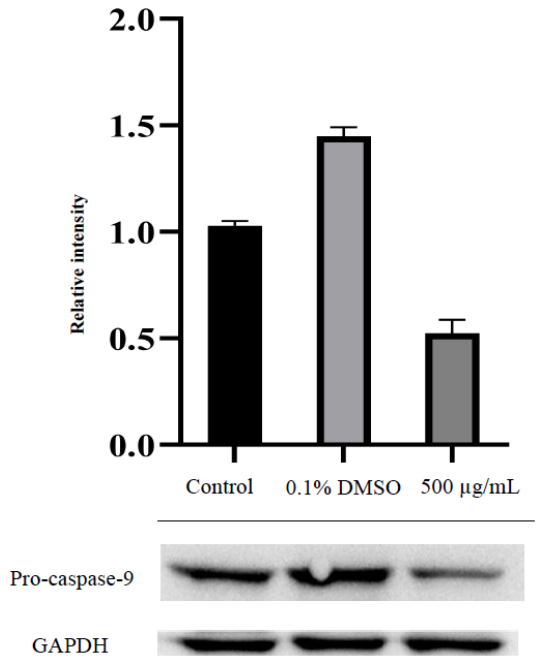
The viability of PANC-1 cells was effectively decreased by adding *O. humifusa* EtOAc fraction. When the *O. humifusa* EtOAc fractions of various concentrations were treated to PANC-1 cells for 12-72 h, more than 50% of the cells were reduced at a concentration of 500 μg/mL (Figure 2).

3.3. Induction of Apoptosis

PANC-1 cells ($1.5×10^6$ cells/100 mm plate) were incubated with *O. humifusa* (500 μg/mL) for 48 h. The expression levels of pro-caspase-8, pro-caspase-9, and pro-caspase-12 were reduced, while those of cleaved caspase-3 were elevated (Figure 3, Figure 4, Figure 5, Figure 6). Pro-caspase-8 is an enzyme involved in the extrinsic signaling pathway, pro-caspase-9 is a mediator involved in the intrinsic signaling pathway, and pro-caspase-12 is an agent involved in the ER-stress signaling pathway. And cleaved-caspase-3 is an effector caspase that acts as the final executioner in these three apoptosis inducing signaling pathways. Therefore, it was confirmed that the *O. humifusa* EtOAc fraction extensively induces apoptosis through extrinsic, intrinsic, and ER-stress signaling pathways.

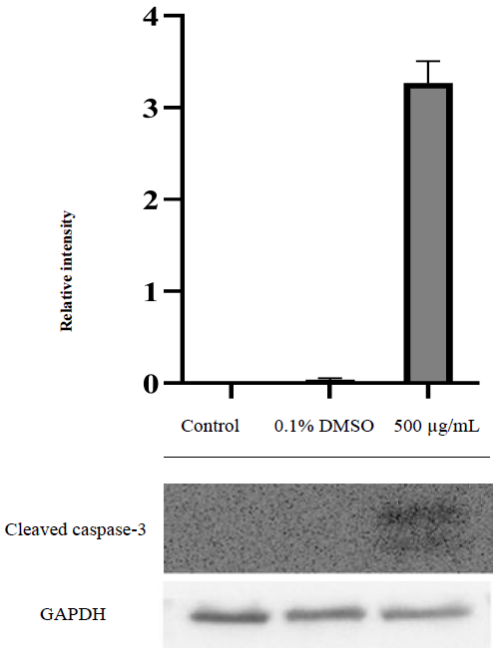
3.4. Inhibition of Cell Proliferation

PANC-1 cells ($1.5×10^6$ cells/100 mm plate) were incubated with *O. humifusa* (500 μg/mL) for 48 h. ERK1/2 and Akt, kinases involved in upstream signaling pathways of apoptosis, proliferation, and invasiveness were reduced (Figure 7, Figure 8). Therefore, it was assumed that upstream signaling pathways are affected by *O. humifusa* EtOAc fraction to activate the main signaling pathways of apoptosis or cell cycle arrest.



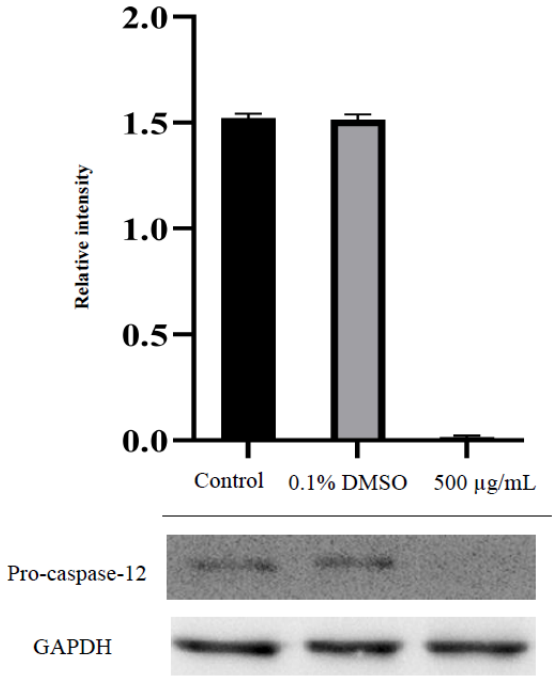
PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* EtOAc fraction (500 μg/mL). The expression levels of pro-caspase-9 were determined by western blot analysis. The results were expressed as means ± SD (n = 3).

Figure 4. Effect of *O. humifusa* EtOAc fraction on protein levels of pro-caspase-9 in PANC-1 cells



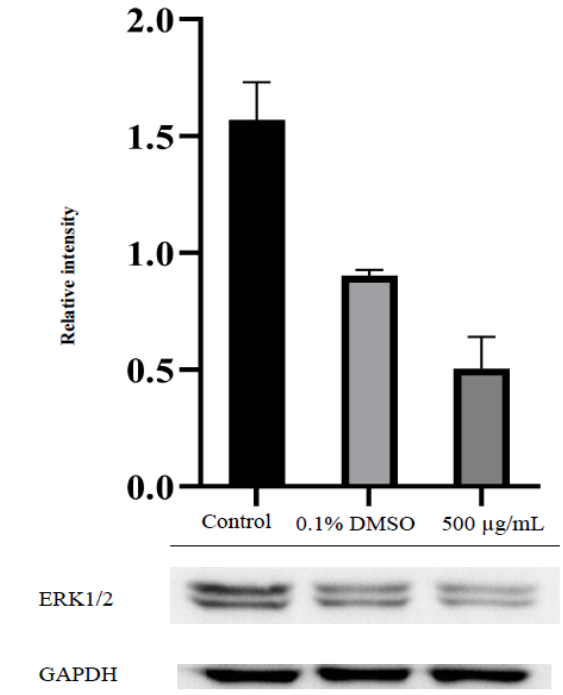
PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* EtOAc fraction (500 μg/mL). The expression levels of cleaved caspase-3 were determined by western blot analysis. The results were expressed as means ± SD (n = 3).

Figure 6. Effect of *O. humifusa* EtOAc fraction on protein levels of cleaved caspase-3 in PANC-1 cells



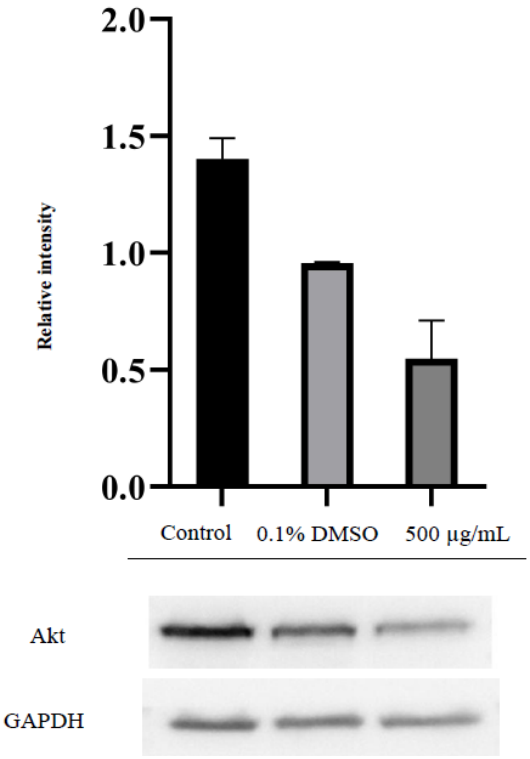
PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* EtOAc fraction (500 μg/mL). The expression levels of pro-caspase-12 were determined by western blot analysis. The results were expressed as means ± SD (n = 3).

Figure 5. Effect of *O. humifusa* EtOAc fraction on protein levels of pro-caspase-12 in PANC-1 cells



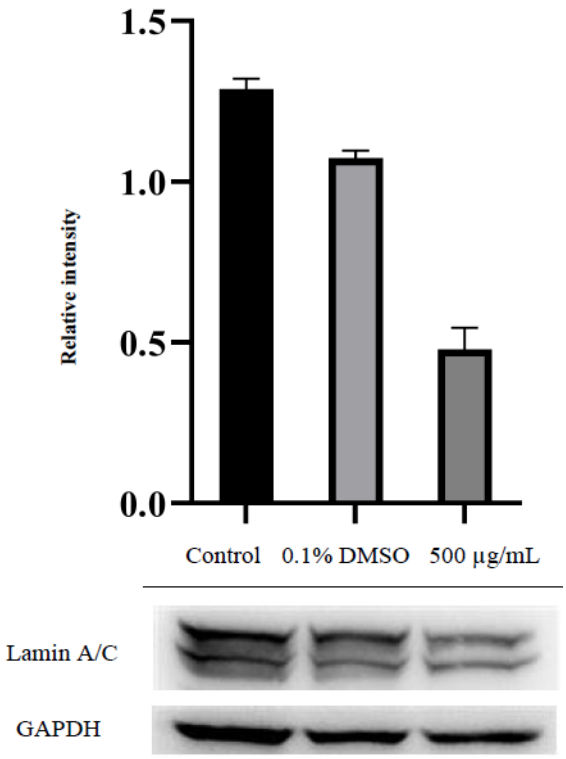
PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* EtOAc fraction (500 μg/mL). The expression levels of ERK1/2 were determined by western blot analysis. The results were expressed as means ± SD (n = 3).

Figure 7. Effect of *O. humifusa* EtOAc fraction on protein levels of ERK1/2 in PANC-1 cells



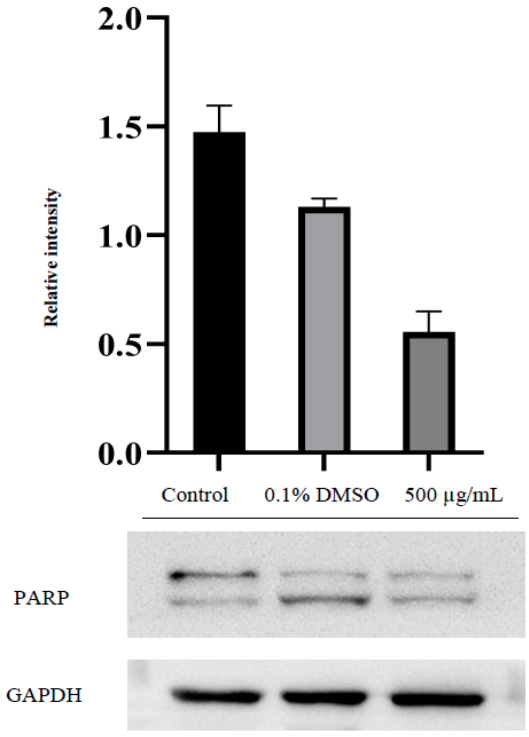
PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* EtOAc fraction (500 μg/mL). The expression levels of Akt were determined by western blot analysis. The results were expressed as means ± SD (n = 3).

Figure 8. Effect of *O. humifusa* EtOAc fraction on protein levels of Akt in PANC-1 cells



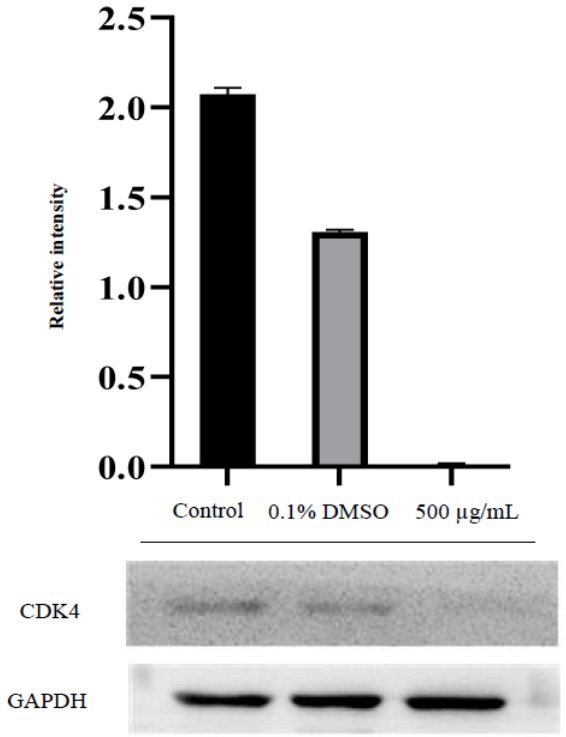
PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* EtOAc fraction (500 μg/mL). The expression levels of lamin A/C were determined by western blot analysis. The results were expressed as means ± SD (n = 3).

Figure 10. Effect of *O. humifusa* EtOAc fraction on protein levels of lamin A/C in PANC-1 cells



PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* EtOAc fraction (500 μg/mL). The expression levels of PARP were determined by western blot analysis. The results were expressed as means ± SD (n = 3).

Figure 9. Effect of *O. humifusa* EtOAc fraction on protein levels of PARP in PANC-1 cells



PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* EtOAc fraction (500 μg/mL). The expression levels of CDK4 were determined by western blot analysis. The results were expressed as means ± SD (n = 3).

Figure 11. Effect of *O. humifusa* EtOAc fraction on protein levels of CDK4 in PANC-1 cells

3.5. Inhibition of Cell Repair

PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* (500 μg/mL) for 48 h. Poly (ADP-ribose) polymerase (PARP), a protein involved in DNA repair was reduced (Figure 9). Therefore, it was confirmed that *O. humifusa* EtOAc fraction activates caspase-3 through extrinsic, intrinsic, and ER-stress mediated signaling pathways, and caspase-3 in turn inactivates PARP, directly involved in DNA repair, and finally causes apoptotic bodies.

3.6. Deformation of Nuclear Membrane

PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* (500 μg/mL) for 48 h. The expression levels of lamin A/C were reduced. Lamin A/C is a major component of the nuclear membrane, and its decline means that the instability of the nuclear membrane increases, leading to apoptotic bodies (Figure 10).

3.7. Cell Cycle Arrest

PANC-1 cells (1.5×10^6 cells/100 mm plate) were incubated with *O. humifusa* (500 μg/mL) for 48 h. The expression levels of CDK4 were dramatically reduced. CDK4 binds with Cyclin D1 to initiate the cell cycle progression at G1/S phase, and the decrease of CDK4 indicates that the cell cycle is arrested at G1/S stage, eventually causing PANC-1 cells to lose their ability to proliferate (Figure 11).

4. Discussion

This study aimed to investigate the anti-cancer effects of the EtOAc fraction derived from *O. humifusa* on PANC-1 human pancreatic cancer cells. The results unveiled a multifaceted mechanism underlying the anti-cancer activity of the *O. humifusa* EtOAc fraction. Downregulation of ERK1/2 and Akt, key components of the upstream signaling pathway governing proliferation and invasiveness, suggests the potential to alleviate the aggressive behavior of cancer cells. Additionally, observed reductions in pro-caspase-8, pro-caspase-9, and pro-caspase-12 levels underscore activation of multiple apoptotic pathways, including extrinsic, intrinsic, and ER-stress mediated pathways. Inactivation of PARP and reduction of lamin A/C, key mediators of downstream signaling pathways that complete the apoptotic bodies, provide the ability to accelerate apoptosis in cancer cells. Additionally, this study identified the role of CDK4 in cell cycle progression. Downregulation of CDK4 levels by the *O. humifusa* EtOAc fraction indicates that the *O. humifusa* EtOAc fraction may be a mediator causing cell cycle arrest in the G1/S phase. These findings are in good agreement with the characteristics of signaling pathways reported in previous studies [16-25]. These results suggest that the *O. humifusa* EtOAc fraction effectively and cooperatively inhibits proliferation and invasiveness, induces apoptosis, and arrests the cycle of PANC-1 cells.

Looking at previous related studies, the EtOAc fraction extracted from *O. humifusa* contained the flavonoids, trans-taxifolin (dihydroquercetin) and aromadendrin (dihydrokaempferol), and induced cell cycle arrest in the G1 phase of HeLa human cervical cancer cells. It has been found to exhibit anti-cancer activity [26]. Several organic solvent fractions, including the EtOAc fraction from *O. humifusa*, have been shown to exert anti-oxidant and anti-inflammatory activities [27,28,29]. Previous studies on the anti-cancer activity of *O. humifusa* were limited to HeLa cervical cancer, HT-29 colon cancer, and MCF-7 breast cancer cells [25,30,31]. Therefore, this study, which confirmed the anti-cancer effect on PANC-1 pancreatic cancer cells using the *O. humifusa* EtOAc fraction, is a completely new research result and presents a promising vision for the development of pancreatic cancer treatment.

In summary, this study provides insight into the anti-cancer effect of *O. humifusa* EtOAc fractions on PANC-1 human pancreatic cancer cells, which can be valuable information for research and development of various cancer therapies.

5. Conclusion

The *O. humifusa* EtOAc fraction shows significant potential as an anti-cancer agent against PANC-1 human pancreatic cancer cells. Its effects include induction of apoptosis via extrinsic, intrinsic, and ER-stress-related pathways, attenuation of proliferation and invasiveness, and cell cycle arrest in the G1 phase. Inactivation of PARP and reduction of lamin A/C, main mediators of the downstream signal transduction completing the apoptotic bodies, offer the capacity to facilitate apoptosis of cancer cells. As these results suggest the possibility as a new candidate for various cancer treatments, additional research on actual application is needed.

Conflict of Interest Statement

Authors declare no conflict of interest.

Authors' Contributions

MCC was involved in designing the work, data collection, data analysis, and data interpretation. DSL was involved in data interpretation and drafting the article.

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