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Original Article

Antrodia salmonea Extract Inhibits Cell Proliferation through Regulating Cell Cycle Arrest and Apoptosis in Prostate Cancer Cell Lines

Pang-Ting Cheng[1], Yu-Chiao Cheng[1], Muhammet Oner[1], Yu-Hsuan Li[1,2], Mei-Chih Chen[2], Jyh-Horng Wu[3], Ting-Chieh Chang[1], Ayse Celik[1], Fang-Ling Liu[1], Hsin-Yi Wang[4], Chih-Ho Lai[5], Jer-Tsong Hsieh[6], Chieh-Yin Chen[7]*, Ho Lin[1,8,9]*

[1]Department of Life Sciences, National Chung Hsing University, Taichung City, Taiwan, [2]Translational Cell Therapy Center, Department of Medical Research, China Medical University Hospital, Taichung City, Taiwan, [3]Department of Forestry, National Chung Hsing University, Taichung City, Taiwan, [4]Department of Nuclear Medicine, Taichung Veterans General Hospital, Taichung City, Taiwan, [5]Department of Microbiology and Immunology, Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan City, Taiwan, [6]Department of Urology, University of Texas Southwestern Medical Center, Dallas, TX, USA, [7]Experimental Forest, College of Bio-Resources and Agriculture, National Taiwan University, Taichung City, Taiwan, [8]Ph.D. Program in Translational Medicine, National Chung Hsing University, Taichung City, Taiwan, [9]Rong Hsing Research Center for Translational Medicine, National Chung Hsing University, Taichung City, Taiwan

Abstract

Antrodia salmonea (AS) is a fungus, which belongs to a fungal family of Taiwanofungus salmoneus with the features of anti-oxidant, anti-inflammatory, and anticancer. Recent studies have shown that AS has anti-cancer functions in ovarian and breast cancer. However, the effects of AS on prostate cancer (PCa) proliferation remain unknown. Therefore, we investigated the role of AS in PCa proliferation through apoptosis, and cell cycle regulation in PCa cell lines. Our results showed that *Antrodia salmonea* extract (ASE) inhibited PCa cells growth with a dose-dependent manner. In addition, ASE decreased the anchorage-independent growth formation ability in PC3 cells. Moreover, ASE-induced cell growth inhibition in PCa cells (DU145, PC3) was correlated to decreased cell cycle-related proteins such as cyclin A/B and cyclin-dependent kinase CDK1/2/4, and increased cell cycle inhibitor proteins p21. Besides, ASE decreased the total protein level of epidermal growth factor receptor and its downstream signaling pathways Akt and Erk in both PCa cells. We found that apoptotic markers such as cleaved-PARP protein levels increased significantly in DU145 cells indicating ASE might induce apoptosis. In conclusion, our results suggest that ASE may have the ability to induce PCa cell death through regulating cell cycle arrest and apoptosis pathways.

Keywords: Anchorage-independent growth, *Antrodia salmonea*, apoptosis, cell cycle, proliferation, prostate cancer

Introduction

Prostate cancer (PCa) is caused by abnormal cell proliferation in the prostate. PCa is a highly malignant tumor and the second leading cause of cancer death.[1] The PCa occurrence is initiated with older age in men.[2-5] It is also related to each person's living habits, eating habits, and family genetic history.[6] It has been known that there are many treatment methods for PCa, such as surgical resection, radiation therapy, chemotherapy, and hormone therapy. In the early stage, androgens and androgen receptor (AR) are required to maintain the proliferation and migration of PCa cells. Therefore, androgen deprivation therapy (ADT) is one of the effective strategies and is usually used as a treatment for early PCa or accompanied by surgical removal of tumor tissue.[1,4,7-9] However, after ADT, most patients may develop castration-resistant PCa. In this case, the tumor cells appear to be transformed into androgen-independent tumor cells, which are more malignant

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***Address for correspondence:** Dr. Chieh-Yin Chen, Experimental Forest, College of Bio-Resources and Agriculture, National Taiwan University, Taichung City, Taiwan.
E-mail: cyinchen@ntu.edu.tw
Prof. Ho Lin,
Department of Life Sciences, National Chung Hsing University, Taichung City, Taiwan.
E-mail: hlin@dragon.nchu.edu.tw

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and aggressive.[10-13] For these reasons, finding a new drug for PCa treatment becomes a crucial emerging issue.

Antrodia salmonea (AS) is a local fungus belonging to fungal family of Taiwanofungus salmoneus with the features of anti-oxidant, anti-inflammatory, and anticancer. AS has been used to improve the condition of diseases-caused adverse effects, including diarrhea, abdominal pain, hypertension, and itchy skin.[14,15] It has been reported that AS has a similar biofunction to *Antrodia cinnamomea* in Taiwan.[16] It has been reported that AS can induce oxidative stress or suppresses tumor growth in ovarian and breast cancer.[15] However, the effect of AS extracts in PCa remains unknown. In this study, we hypothesize that AS can potentially inhibit PCa cell growth through the regulation of cell cycle arrest and apoptosis.

Therefore, we investigated the safe dosage of AS in two PCa cells PC3 and DU145. Next, we evaluated PCa cell growth with the presence of AS treatment. We found that AS extracts inhibited PCa cell growth considering the outcomes from growth rate and anchorage-independent growth. Moreover, we further evaluated cell-cycle-related proteins, apoptotic pathway proteins, and cell growth-related signaling pathways in PCa cell lines. Taken together, our findings suggest that AS is an effective treatment for the inhibition of PCa growth through regulating cell-cycle arrest and apoptosis.

Materials and Methods

Antrodia salmonea extraction

Depends on the method described previously,[17] the AS was lyophilized by vacuum freeze dryer at −40°C for 72 h. The powder then would be placed in a conical flask with 95% ethanol and sonicated in an ultrasonicator (Branson 5510, Branson Ultrasonic, Ontario, Canada) for 60 min. The extract would then filter with Whatman #1 paper and concentrated in a rotary evaporator and lyophilized.

Reagents and antibodies

Antibodies against to PARP (cat. no. sc-8007, 1:1000), CDK1 (cat. no. sc-54, 1:1500), CDK2 (cat. no. sc-163, 1:1500), cyclin A (cat. no. sc-751, 1:1000), p-Akt (cat. no. sc-7985-R, 1:500), Akt (cat. no. sc-5298, 1:500) were purchased from Santa Cruz Biotechnology. Antibodies against cleaved-PARP (cat. no. AB3565, 1:1000), actin (cat. no. MAB1501, 1:10,000), tubulin (cat. no. #D5-829, 1:15,000) were purchased from Millipore. Antibodies against cyclin B (cat. no. #4138, 1:1000), caspase-8 (cat. No. #9496, 1:1000), p21 (cat. No. 2947s, 1:500), epidermal growth factor receptor (EGFR) (cat. no. #2232, 1:1000), and p-EGFR (cat. no. #2236, 1:1000) were purchased from cell signaling. Antibodies against p27 (cat. no. 610242, 1:500), Erk1 (cat. no. 610031, 1:5000), and p-Erk1/2 (cat. no. 612358, 1:1000) were purchased from BD.

Cell lines and culture

PCa cell lines DU145 and PC3 were purchased from the Center for Bioresource Conservation and Research. The main component of DU145 cell is MEM including 10% fetal bovine serum (FBS, GIBCO), 1% penicillin/streptomycin (P/S, GIBCO), 1.5 g/L sodium bicarbonate (Sigma). PC3 cells were grown in F-12 medium and supplemented with 7% FBS (FBS, GIBCO), 1% penicillin/streptomycin (P/S, GIBCO), and 1.5 g/L sodium bicarbonate (Sigma). The culture environment was incubated at 37°C, 5% CO_2, and 95% air, and is subcultured about every 3–4 days.[18]

Trypan blue assay

PC3 and DU145 cells were seeded in 24-wells plate, the seeding number is 8×10^3 cells/well. After 24 h, cells were collected by trypsin, then stained with trypan blue (T8154; Sigma), and counted by hemocytometer.[18]

Anchorage-independent assay

In 6-well plates, 3 mL of 0.5% agar-growth medium solution with different dosages of *Antrodia salmonea* extract (ASE) was added to each well as base layers. PC3 cells (8×10^3 cells) were seeded in 1 mL of 0.3% agar-growth medium solution with different dosages of ASE on the top of base layers. The cells were cultured in 37°C for 21 days. Images of colonies were captured at × 4 magnification and quantified by using ImageJ.

Cell protein extraction

The cells were placed on ice to reduce the action of intracellular enzymes, and then collected cells and added lysis buffer, and the cells were mixed for 45 min (15 min/time). Centrifuge at × 15,400 g for 20 min, add Bradford to measure the protein concentration, finally, add the sample buffer, then denature the protein in a dry bath at 100°C for 10 min to perform SDS-PAGE or store at −20°C.[18]

Cell nuclear/cytosolic protein fractionation

The cells were collected in PBS and divided into two groups of input and fraction in a ratio of 2:8. The input group was the same as the method of cell protein extraction. The fraction was added to the hypotonic buffer for 12 min, and then centrifuged for 1500 g for 5 min, and the supernatant was collected for cytoplasmic protein. Moreover, the pellet was further lysed through nuclear extract buffer following by ×15,400 g centrifugation for 20 min to get nuclear protein.

Western blot analysis

Depends on the method described previously,[19] treated cells were collected and lysed by lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 10% 2 mM PMSF, and 1% Na_3VO_4/H_2O). The lysates were quantified by Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) and separated by SDS-PAGE (30 µg/lane). The samples were then transferred onto polyvinylidene difluoride membranes (PerkinElmer Life Sciences, Shelton, CT, USA). The membranes were probed with primary antibodies and incubated with horseradish peroxidase-conjugated secondary antibodies. The protein band on the membrane was revealed by using the Enhanced Chemiluminescence (PerkinElmer Life Sciences, Shelton, CT, USA) reaction and exposed to X-ray films (Fujifilm,

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Tokyo, Japan) or presented by Luminescence Imaging System LAS-4000 (GE HealthcareLife Sciences, Pittsburgh, PA, USA).

Statistical analysis

If the magnitude of the experimental results involved different treatment groups, the data of each group were expressed as mean ± standard error and the difference between the two groups was analyzed by *t*-test two-tailed, with $P < 0.05$ as a significant difference, and * as a label; $P < 0.01$ is very different and marked with **; $P < 0.001$ is a very significant difference and is indicated by ***.[18]

Results

Antrodia salmonea extract treatment inhibits cell viability of prostate cancer cells

In this study, we first used ASE (6.25–400 μg/mL) to treat PCa PC3 and DU145 cells to examine the cell viability. After 24 h of treatment with ASE, the cell number was assessed by counting under microscopy with trypan blue staining. Our results showed that ASE decreased the cell viability in a dose-dependent manner [Figure 1a and b]. These data showed that the treatment with ASE in PC3 and DU145 cells decreased the cell proliferation rate.

Antrodia salmonea extract decreases the anchorage-independent growth formation ability in PC3 cells

To further analyze the effects of ASE on the characteristics of cell colony formation, the soft-agar colony assay was performed. After 21 days of treatment with various concentrations of ASE (6.25–100 μg/mL), the colony size of PC3 cells was decreased compared to the control group [Figure 2a]. The quantified results also showed the colony average size decreased with dose-dependent manner compared to the control group [Figure 2b].

Antrodia salmonea extract stimulates apoptosis in prostate cancer cells

To understand the mechanism of ASE-inhibited cell viability, PC3 and DU145 cells were treated with ASE (6.25–100 μg/mL) for 24 h to observe the expression level of apoptosis-related proteins. Our results showed that the protein level of cleaved-PARP was increased and total PARP was decreased when PC3 and DU145 were treated with 100 μg/mL ASE [Figure 3a and b]. These results showed that ASE treatment induced apoptosis in PCa cell.

Antrodia salmonea extract decreases cell cycle-related proteins in PC3 and DU145 cells

Next, the cell cycle-related proteins were detected in PC3 and DU145 cells. Here, we used tubulin and PARP as the marker of

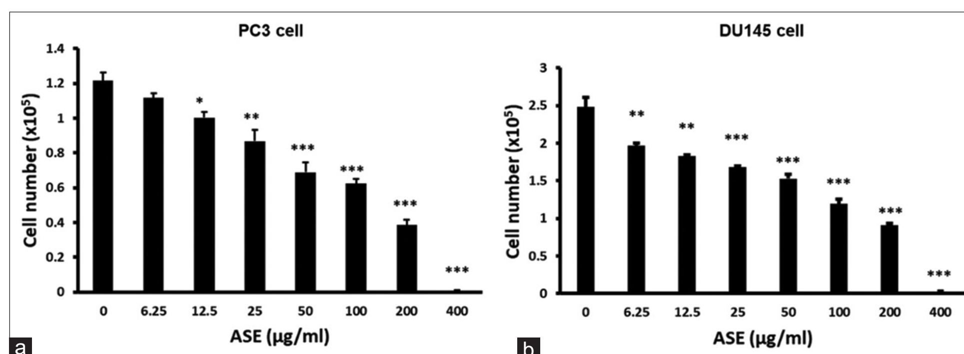

Figure 1: ASE inhibited cell viability of prostate cancer cells. (a) PC3 cells were treated with various concentrations of ASE (0, 6.25, 12.5, 25, 50, 100, 200, 400 μg/ml) for 24 h. (b) DU145 cells were treated with various concentrations of ASE (0, 6.25, 12.5, 25, 50, 100, 200, 400 μg/ml) for 24 h. Cell viability was determined by counting with trypan blue staining. Each value is expressed as the mean ± SD ($n = 3$). Significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control. ASE: *Antrodia salmonea* extract, SD: Standard deviation.

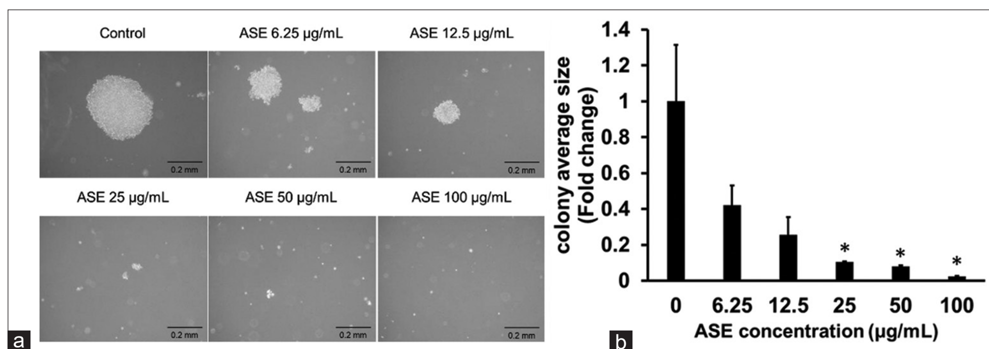

Figure 2: ASE decreased the anchorage-independent growth formation ability in PC3 cells. (a) PC3 cells were treated with ASE (6.25–100 μg/mL). Colonies were allowed to form for 21 days, then observed by microscope. (b) The colony average size was measured by ImageJ. ASE: *Antrodia salmonea* extract.

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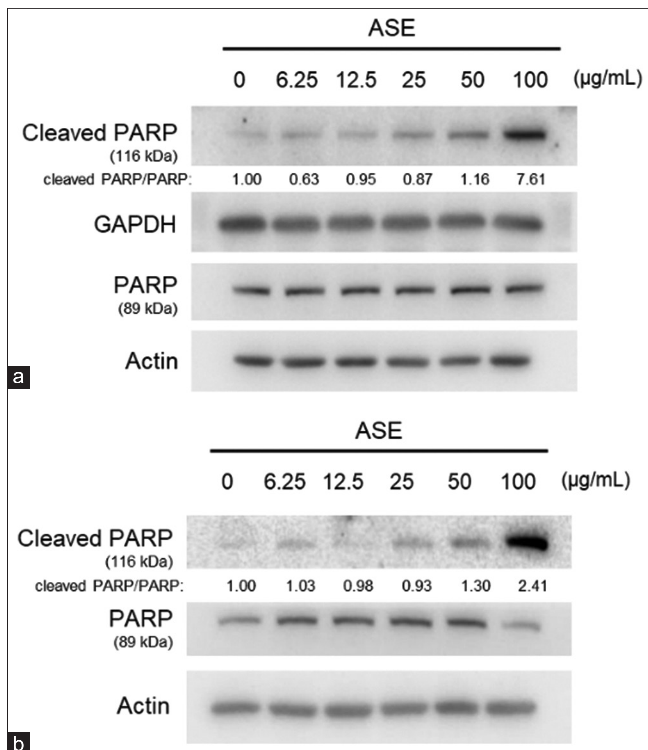

Figure 3: ASE induced apoptosis in PC3 and DU145 cells. In (a) PC3 and (b) DU145 cells, protein levels of cleaved-PARP and PARP were analyzed by western blot analysis with different concentrations of ASE treatment (6.25–100 μg/mL). ASE: *Antrodia salmonea* extract.

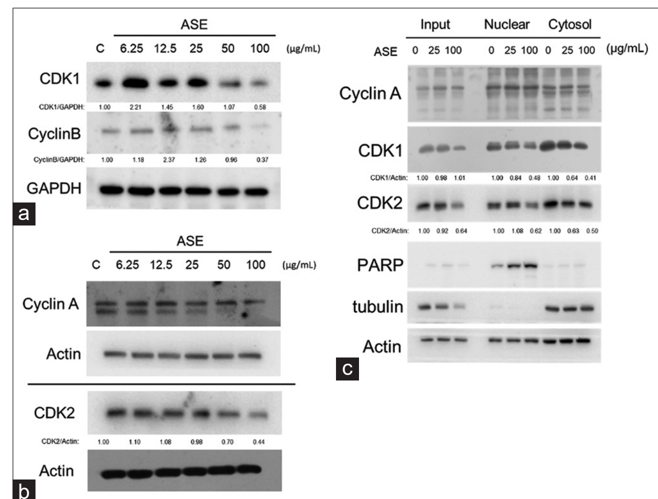

Figure 4: ASE decreased cell cycle-related proteins in PC3 and DU145 cells. (a) In PC3 cells, CDK1 and cyclin B were monitored using western blot analysis. (b) CDK2 and cyclin A have monitored the location in DU145 cells. (c) In PC3 cells, protein levels of cyclin A, CDK1 and CDK2 were analyzed by western blot analysis. ASE: *Antrodia salmonea* extract.

cytoplasm and nuclear fraction individually. After the treatment with ASE (6.25–100 μg/mL), cyclin B and CDK1, which are important for cell cycle M-phase progression, were decreased in PC3 cells [Figure 4a]. In DU145 cells, ASE treatment decreased cyclin A and CDK2 level, which are responsible for helping cell entry into the S-phase [Figure 4b]. Moreover, the result from cell nuclear/cytosolic protein fractionation in PC3 cell showed that the protein level of CDK1, CDK2 in nuclear also decreased [Figure 4c]. These results indicated that ASE treatment inhibited cell cycle progression through decreased protein levels of cell cycle-related proteins.

Antrodia salmonea extract increases cell cycle inhibitor protein p21 or p27 in PC3 and DU145 cells

Previously, we found that increases of the CDK inhibitor proteins, p21 and p27 in PCa reduced its proliferation ability.[5,20] Therefore, we investigated how ASE inhibits the cell cycle by evaluating the protein level of cell cycle inhibitor protein p21 and p27. In PC3 cells, AS increased the protein level of p21. In addition, our result from cell nuclear/cytosolic protein fractionation also showed that AS increased the protein stability and protein expression level of p21 but not p27 in nucleus [Figure 5a]. Besides, we also found an increased level of p21 in DU145 cells with the presence of ASE treatment, especially the highest dose of ASE (100 μg/mL) [Figure 5b]. These results indicated that ASE inhibited the cell-cycle by inducing p21 protein expression levels.

Antrodia salmonea extract decreases the total protein level of epidermal growth factor receptor and its downstream signaling pathways in PC3 and DU145 cells

Further, the protein level of EGFR and its downstream protein extracellular signal-regulated kinase (Erk) or protein kinase B (Akt) were measured with ASE treatment. Our results showed that ASE decreased the total protein level of phospho-EGFR (Tyr1068), Akt and phospho-Akt in PC3 cells [Figure 6a]. In DU145 cells, ASE decreased the protein level of EGFR, phospho-EGFR, Akt, and phospho-Akt. The protein level of Erk and its phospho-form were also decreased [Figure 6b]. Our data suggested that ASE inhibited the EGFR pathway and further affected Akt or Erk activation in PC3 and DU145 cells.

Discussion

Over the last few decades, medicinal mushrooms have been found pharmacologically active with biological properties. Some findings have demonstrated that medicinal mushrooms and their bioactive compounds can be potential treatment against various type of human cancers.[21] AS grows with the indigenous coniferous tree Cunninghamia konishii Hayata (Cupressaceae). *A. salmonea* has recently been reported to have anti-cancer effects as *A. cinnamomea* does. It has been shown that *A. salmonea* can inhibit cancer cell growth and enhance cell apoptosis in human triple-negative breast cancer, ovarian carcinoma cells, and human promyelocytic leukemia.[15,22,23] Since *A. salmonea* showed beneficial effects against multiple diseases, including the inhibition of cancer cell growth. *A. salmonea* can be considered as the alternative to *A. cinnamomea* in the marketplace, because of the rarity and cost in the market.

Therefore, we investigated the anti-cancer effect of the extract of AS on PCa cells. Our current data identified that

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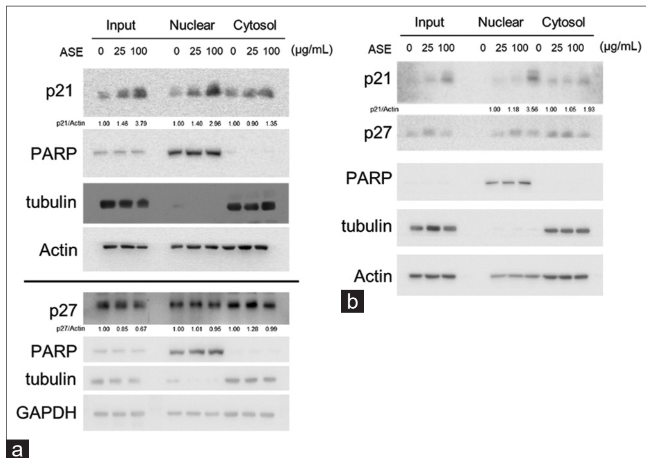

Figure 5: ASE increased cell cycle inhibitor protein p21 in PC3 and DU145 cells In (a) PC3 and (b) DU145 cells, p21 and p27 was monitored for the location using western blot analysis. ASE: *Antrodia salmonea* extract.

ASE treatment significantly inhibited the cell viability, and stimulated apoptosis in human PCa cell lines. Apoptosis not only occurs during development and aging, when cells are damaged by noxious agents or stress, but also occurs as a defense mechanism.[24] The occurrence of apoptosis causes cell death. In this study, we found ASE can induce pro-apoptotic protein PARP transfer to cleaved-PARP form to induce apoptosis in human PCa cell line PC3 and DU145.

Cell cycle progression plays an important role in regulating cell proliferation, growth, and cell division. Cancer cell cycle disruption becomes a goal of treatment in the development of new anticancer drugs.[25] CDK2 is activated by cyclin A to drive the transition from S-phase to G2-phase.[26] The combination of cyclin B1 and CDK1 will induce cell cycle progression from the G2 phase to the M phase. Inversely, inhibiting the expression of CDK2, cyclin A or cyclin B1, CDK1 can inhibit cell cycle progression.[27] Since it has been reported that AS induces G2 phase cell cycle arrest and inhibits the cell cycle-related proteins, Cyclin A, Cyclin B1, Cyclin D, Cyclin E, and CDK2 in human triple-negative breast cancer.[15] Therefore, we investigated the role of AS in the regulation of the cell cycle in PCa cells. Our results implied that ASE downregulated the cyclin B, cyclin A, and CDK1, CDK2 expression levels in PCa cells. This finding suggested that AS might be potential inhibitor of S phase or M phase for the treatment of PCa.

The function of p21 in the nucleus is acting as a universal cyclin-dependent kinase inhibitor. It has been found to inhibit all cyclin-CDK complexes. Further, its overexpression inhibits cancer cell proliferation. Besides the essential role of p21 in cell-cycle regulation, p27 can also inhibit cyclin-CDK complexes at G0 to G1 phase.[28] Our current study demonstrated that the protein level of p21 increased in the nuclear in PC3 and DU145 cells. This data suggested ASE may inhibit the cell cycle by increasing p21 but not p27, then further inhibiting cell proliferation. EGFR inhibition suppressed PC3 and DU145 cells growth and motility.[29] Its downstream, Erk and Akt signaling pathways are the molecular mechanisms that are mainly involved in cell survival, proliferation, motility, differentiation, and metabolism.[29] Activation of EGFR promotes its downstream signaling, including MEK/Erk1/2 or PI3K/Akt.[30] Therefore, we monitored EGFR-related signaling pathways in human PCa cell lines. In our study, we found that AS treatment decreased the protein expression level of p-EGFR, p-Akt, and Akt in PC3 cells. Besides, AS also decreased p-EGFR, EGFR, p-Akt, Akt, and p-Erk in DU145 cells. Therefore, we suggest that ASE may decrease PCa cells proliferation through inhibiting EGFR and its downstream proteins.

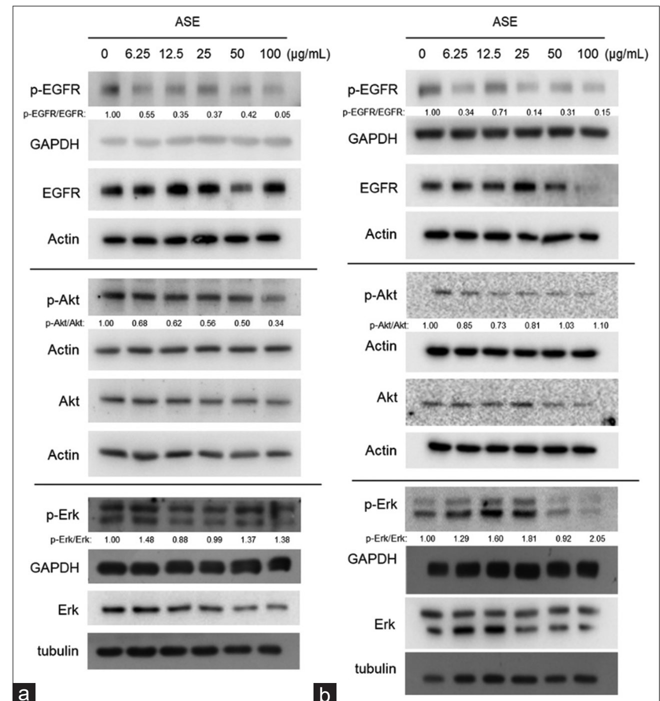

Figure 6: ASE decreased the total protein level of EGFR and its downstream signaling pathways in PC3 and DU145 cells Western blotting results showed the effects of ASE on the protein of EGFR and its downstream protein Akt, p-Akt, Erk, p-Erk in (a) PC3 cells and (b) DU145. ASE: *Antrodia salmonea* extract, EGFR: Epidermal growth factor receptor.

Conclusion

Overall, our data suggested that the inhibitory effects of ASE on PCa cells are associated with its ability to inhibit the expressions of cell cycle-related proteins such as CDK1, cyclin B, CDK2, and cyclin A. Moreover, ASE also induced cell cycle inhibitor protein p21 expression level increase. ASE also induces apoptosis by regulating the expressions of PARP and cleaved-PARP. Taken together, AS extract seems to become an effective treatment, and AS can be utilized as a potential drug against human PCa proliferation in the near future.

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Conflicts of interest

There are no conflicts of interest.

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