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Research article

Potential water-based extracts from fresh and dried *auricularia auricula-judae*on the inhibitory effect of human papillomavirus type 16 and cervical cancer cell line in caski

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ABSTRACT

Auricularia auricula-judae, also called the wood ear mushroom, is a well-known edible mushroom in various countries. Notably, the extracts of this mushroom inhibited the growth and expression of human papillomavirus (HPV) oncogene in the HPV-positive cervical cancer cell line. However, there has been no reported comparison of the effect of the extracts from fresh and dried *A. auricula-judae* on anti-HPV16 in CaSki cell lines. Thus, this study investigated the effect of the water-based extracts from fresh and dried *A. auricula-judae* at 100ºC on anti-HPV16 pseudovirus infection and anti-cancer in CaSki as well as the expression of viral oncogenes and cellular genes. HPV16 pseudovirus was produced and used for anti-viral infection assay in pre-attachment and adsorption steps in treated 293FT cells. Cell apoptosis, cell proliferation, cell migration and colony-forming assay were investigated in treated CaSki cells. In addition, mRNA expression levels of HPV16E6/E7, *BCL2*, *BAK* and *CASP3* were determined using quantitative real time polymerase chain reaction. The results indicated that the extract from dried mushroom caused higher cytotoxicity than fresh mushroom. Notably, the extracts from the dried mushroom more effectively inhibited the infection of HPV16 pseudovirus in 293FT cells than did fresh mushroom. Furthermore, it significantly decreased HPV16E6/E7 and *BCL2* and increased *BAK* and *CASP3* expression, in contrast to the untreated and water-treated CaSki cells, but not in the extract derived from fresh mushroom. The main conclusion was that the extract from dried mushroom more effectively inhibited growth of CaSki and HPV16-pseudovirus infection than from fresh mushroom.

Keywords: Auricularia auricula-judae, Human papillomavirus, HPV16 pseudovirus, Cervical cancer, Water-based extract.

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INTRODUCTION

Cervical cancer is a malignancy that is fourth in the world and the second most common in Thailand [1, 2]. The main risk factor for this cancer is human papillomavirus (HPV), particular HPV16, which is most commonly detected in cervical cancer patients worldwide [3]. Even though there are effective prophylactic vaccines against HPV16 infection, the incidence of HPV16-related cervical cancer is still high, particularly in low income countries [4, 5]. Furthermore, a therapeutic vaccine to cure HPV-infected patients is still under development. The screening of potential drugs from natural materials is a promising option, especially in foods having medical properties, including edible mushrooms, to inhibit pre- and post-infection of HPV [6-8].

Auricularia auricula-judae belong to the family *Auriculariaceae*, also called the wood ear mushroom that is widely consumed and commercially cultivated in Thailand. There are many reports of important *A. auricula-judae* substances including dietary fiber (51–56%), polysaccharides (13–28%), proteins (10–16%), melanin (1–3%), polyphenol/flavonoid (1%), and fat (0.3–0.7%) [9]. Polysaccharides of this mushroom isolated using water-based extraction comprise β-glucan which is water-soluble [9]. Monosaccharides also found using this extraction method include mannose, galactose, glucose, glucuronic acid, and xylose [9, 10]. In addition, total phenol, flavonoid and antioxidant agents have been found in *A. auricula-judae* water-based extract [11]. *A. auricula-judae* extracts using various extraction methods inhibited the growth of breast cancer, lung cancer, colon cancer, gastric cancer, sarcoma, and hepatocellular carcinoma cell lines [12-14]. The mechanism of these extracts suppressed BCL2, induced BAX protein expression and caspase 3/9 activation to promote apoptosis, and downregulated the expression levels of VEGF and CD31 tumor angiogenesis factors [9, 15]. As expected, *A. auricula-judae* extract caused cell death in the CaSki and HeLa cell lines and suppressed E6 protein expression [16-18]. However, the effects have never been studied of *A. auricula-judae* extracts on the inhibition of HPV16 pseudovirus.

This study investigated the effects of water-based extracts from both fresh and dried *A. auricula-judae* to evaluate the inhibition

of HPV16 pseudovirus infection and the expression of HPV16E6/E7, *BCL2*, *BAX*, and *CASP3* genes, as well as the inhibitory effect on cell proliferation, cell migration, colony-forming and apoptosis in CaSki cell lines. This study first demonstrated the ability of *A. auricula-judae* extracts on anti-HPV16 pseudovirus infection and its effects on gene-related cancer, as well as cellular biology in CaSki.

MATERIALS AND METHODS

Sample Preparation and Mushroom Extract

The mushroom sample was purchased from the Talat Thai market in Pathum Thani province, Thailand during December 2020. The mushroom was washed with sterile distilled water, then cut into small pieces, and divided into 2 groups (100 gram/group). One group was immediately extracted with hot water (100 ml) at 100ºC for 1 hour. The other part was dried at 50ºC for 96 hours, and then was boiled in 100 ml sterile water at 100ºC for 1 hour. The mushroom in water was crushed in a sterile plastic bag. The supernatant was collected based on centrifugation at 5,000 rpm for 30 minutes, and an aliquot was added into the tube. The extracts were stored at -20ºC.

Cell Culture

The human embryonic kidney (HEK) 293FT cell line was purchased from the Invitrogen Company (Carlsbad, CA, USA) and the CaSki cell line was kindly provided by Prof. Dr. Chamsai Pientong, Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY, USA) medium containing 10% fetal bovine serum (FBS) and antibiotics (40 µg/ml gentamicin, 2.5 µg/ml amphotericin B, 100 µg/ml streptomycin, and 100 unit/ml penicillin G). The cell culture was incubated in a humidified atmosphere containing 5% CO_2 at 37ºC.

Cytotoxicity Assay

Either the 293FT or CaSki cell line at a density of 10,000 cells per well was set up in 96-well plates and incubated for 24 hours. The extracts were treated into the cells at various concentrations and incubated for 48 hours. Ten microliters of MTT reagent (5 mg/ml) were added into the cells per well and continuously incubated for 4 hours. A formazan pellet was dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at OD540 using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Vantaa, Finland).

Production of Human Papillomavirus Type 16 Pseudovirus

293FT cells were used to produce HPV16 pseudovirus using Lipofectamine 2000® (Invitrogen Corp., Carlsbad, CA, USA) to co-transfect 2 plasmids including p16SheLL (HPV16 L1/L2 expression vector) and pfwB (green fluorescence/reporter vector). After 48 hours post-transfection, pseudovirus was isolated using lysis buffer (0.05% BriJ 58, 0.01% RNase, 9.5 mM $MgCl_2$ in PBS). The viral titer was measured by 10-fold dilution and counted as the percentage of infections under a fluorescent microscope (Olympus BX51, Olympus Co., Ltd., Tokyo, Japan) to estimate the multiplicity of infection (MOI).

Anti-HPV16 Pseudovirus Infection Assay in Pre-attachment and Adsorption Steps

For pre-attachment step assay, HPV16 pseudovirus at MOI 0.5 was mixed with the extracts at the 20% and 50% cytotoxic concentrations (CC20 and CC50, respectively) and then incubated for 1 hour at 37ºC. The mixture was added to 293FT cells and continuously incubated for 4 hours at 37ºC. After removing the mixture, the cells were maintained in complete medium for 48 hours. For adsorption step assay, 293FT cells were adsorbed by HPV16 pseudovirus at MOI 0.5 for 2 hours at 20ºC. After removing viral mixture, the extracts at CC20 and CC50 were added to the cells for 48 hours. Total cells and fluorescent cells were counted under a microscope (Olympus BX51, Olympus Co., Ltd., Tokyo, Japan). The percentage of inhibition was calculated by subtracting the percentage of infection from 100.

RNA Extraction and cDNA Synthesis

CaSki cells were seeded into a 12-well plate (120,000 cells/well) and incubated for 24 hours. The cells were treated with the extracts at the 20% inhibitory concentration (IC20) in complete medium and incubated for 48 hours and then harvested and subjected to TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) for RNA isolation. Two hundred nanograms of total RNA was processed using a RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA) with Oligo dT primers according to the manufacturer's instructions. The cDNA was stored at -20ºC.

Determination of Gene Expression using Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Two microliters of diluted cDNA at 1:10 was subjected to Sso-Advanced SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), and each primer specific to the HPV16E6, HPV6E7, *BCL2*, *BAK* and *CASP3* genes. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control and for normalizing the target gene expression. The conditions were: pre-naturing at 95ºC for 2 minutes followed by 45 cycles of 95ºC for 15 seconds and 60ºC for 45 seconds, run on an Eco™ Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). Expression analysis was analyzed using the Eco™ Software v4.0.7.0. Relative quantification (RQ) was calculated using $2^{-\Delta\Delta CT}$.

Cell Proliferation Assay

CaSki cells were seeded into a 96-well plate (5,000 cells/well) and maintained in DEME containing 2.5% FBS and antibiotics (40 µg/ml gentamicin, 2.5 µg/ml amphotericin B, 100 µg/ml streptomycin, and 100 unit/ml penicillin G) for 24 hours. The extracts were prepared at IC0 and IC20 in the same medium as

mentioned above, and incubated for 24, 48 and 72 hours. Ten microliters of MTT reagent (5 mg/ml) were added to each well and then incubated for 4 hours. Formazan was dissolved in DMSO. The absorbance was detected at OD540 using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Vantaa, Finland).

Cell Migration by Wound Healing Assay

CaSki cells were seeded into a 24-well plate at a density of 60,000 cells/well and maintained in DMEM supplemented with 2.5% FBS and antibiotics for 24 hours at 37ºC. The wound was scraped using a sterile 200 µl tip. Cell culture medium was removed and replaced with the extracts at IC0 and IC20 in DMEM supplemented with 2.5% FBS and antibiotics. The wound distance was photographed at 0, 24 and 48 hours under an inverted phase-contrast microscope (Nikon, Eclipse TS100, Nikon Co., Shinjuku, Tokyo, Japan) using a 4x objective lens. The wound distance was analyzed using the ImageJ software (version 1.53k, Wayne Rasband NIH, USA) to calculate the percentage of wound closure according to the formula: Percentage of wound healing = [(wound length at 0 hour) − (wound length at 24 or 48 hours)]/(wound length at 0 hour) x 100.

Colony-forming Assay

CaSki cells were seeded into a 6-well plate (500 cells/well) and incubated at 37ºC for 24 hours. The extracts at IC0 and IC20 were prepared in complete medium and incubated for 48 hours. The medium was removed and replaced with fresh complete medium and continuously incubated for 14 days, changing the medium every 4 days. The colonies were counted under an inverted phase-contrast microscope (Nikon, Eclipse TS100, Nikon Co., Shinjuku, Tokyo, Japan).

Apoptosis Assay Based on Dual Acridine Orange/Ethidium Bromide Staining

CaSki cells were seeded into a 12-well plate (120,000 cell/well) and incubated for 24 hours. The cells were treated with the extracts at IC20 and IC50 and continuously incubated for 48 hours. Then, the cells were harvested and washed using trypsin and PBS, respectively. The cell pellet was resuspended in 20 µl complete medium. One microliter of acridine orange/ethidium bromide (AO/EB) solution was added into the cell suspension; subsequently, cells were counted in a hemocytometer under a fluorescent microscope (Olympus BX51, Olympus Co., Ltd., Tokyo, Japan).

Statistical Analysis

Statistical differences in the data between 2 groups were analyzed using an unpaired, two-tailed Student's t test with a Microsoft Excel 2019 application (Intel® Software; Microsoft Corporation, Redmond, WA, USA).

RESULTS & DISCUSSION

Cytotoxic Effect of Extracts on CaSki and 293FT Cell Lines

The CC of the extracts from dried mushroom were higher than from the fresh mushroom for 293FT, whereas the IC of the extract from dried mushrooms was lower than for fresh in CaSki, as shown in Table 1. The CC20 and CC50 of the extracts from fresh and dried mushroom in 293FT are 25.77 ± 2.44 and 73.26 ± 3.66 mg/ml, and 48.74 ± 1.39 and 119.38 ± 11.78 mg/ml, respectively. Simultaneously, the IC0, IC20 and IC50 of the extracts from fresh and dried mushroom in CaSki are 3.17 ± 0.01, 73.69 ± 13.84 and 187.71 ± 31.91 mg/ml, and **2.55** ± **0.08**, **55.01** ± **13.77** and **155.66** ± **52.71** mg/ml, respectively. The IC50 of cycloheximide (CHX) in CaSki is 0.00097 mg/ml. These values were used in further experiments.

Table 1: Cytotoxic and inhibitory concentrations of 293FT and CaSki

Cell type	Extract	Cytotoxic concentration (CC), mg/ml (wet weight)		
		CC20	CC50	
293FT	Fresh, 100ºC	25.77 ± 2.44	73.26 ± 3.66	
	Dried, 100ºC	48.74 ± 1.39	119.38 ± 11.78	
Cell	Extract	Inhibitory concentration (IC), mg/ml (wet weight)		
		IC0	IC20	IC50
CaSki	Fresh, 100ºC	3.17 ± 0.01	73.69 ± 13.84	187.71 ± 31.91
	Dried, 100ºC	**2.55** ± **0.08**	**55.01** ± **13.77**	**155.66** ± **52.71**
	CHX	< 0.0000016	0.0000091 ± 0.00	0.00097 ± 0.00

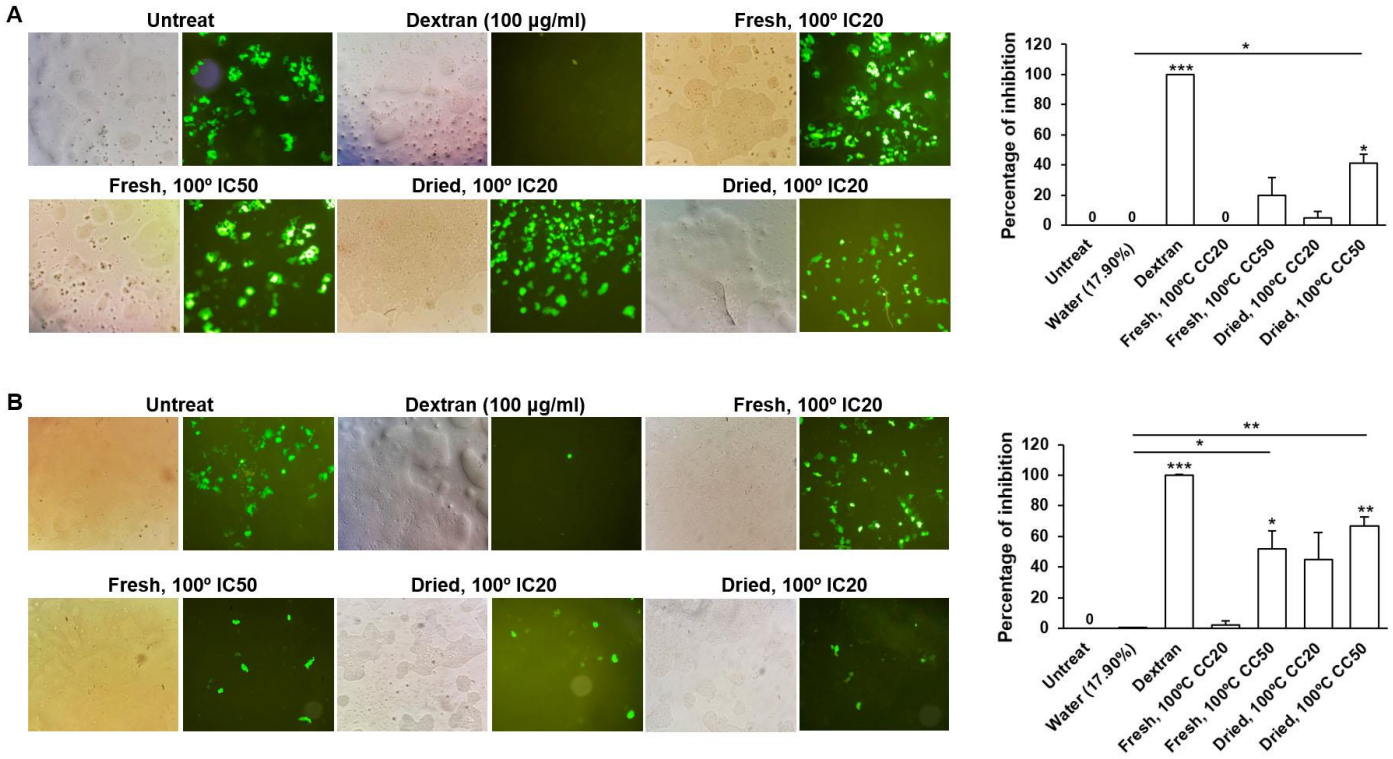
Note: Data expressed as mean ± standard deviation. IC value was used in cancer cell line, whereas CC was used in non-cancerous cell line.

Effect of *A. auricula-judae* Extracts on HPV16 Pseudovirus Infection in Pre-attachment and Adsorption Steps

The extract from dried mushroom significantly inhibited HPV16 pseudo virus infection in both the pre-attachment and adsorption steps, whereas the extract from fresh mushroom significantly inhibited the infection only in the adsorption step (Figure 1). It seemed that the extracts acted on anti-HPV16-pseudovirus infection in a dose-dependent manner. Dextran is a polysaccharide sulphate that is highly effective at inhibiting HPV16 pseudo virus infection [19, 20]. Thus, in the current study, polysaccharide in the extracts was investigated using the sulfuric-phenol method, and amounts of 1.08 ± 0.09 mg/ml and 43.06 ± 0.29 mg/ml were found in the fresh and dried mushroom, respectively. Therefore, this high level of *A. auricula-judae* polysaccharide in the extracts, particularly in the dried mushroom, might have interfered with HPV16 pseudovirus infection. In addition, the extract from the fresh mushroom was a light-yellow turbid solution with low viscosity, whereas the extract from the dried was dark brown and more viscous. This might be implied that both extraction methods yielded different bioactive compounds because fresh mushroom's cell wall network was firmer and may have been more difficult to be broken and penetrated by water compared to dried mushroom that

contained larger pores and less network contributing to a different amount of bioactive compounds dissolved in water [21].

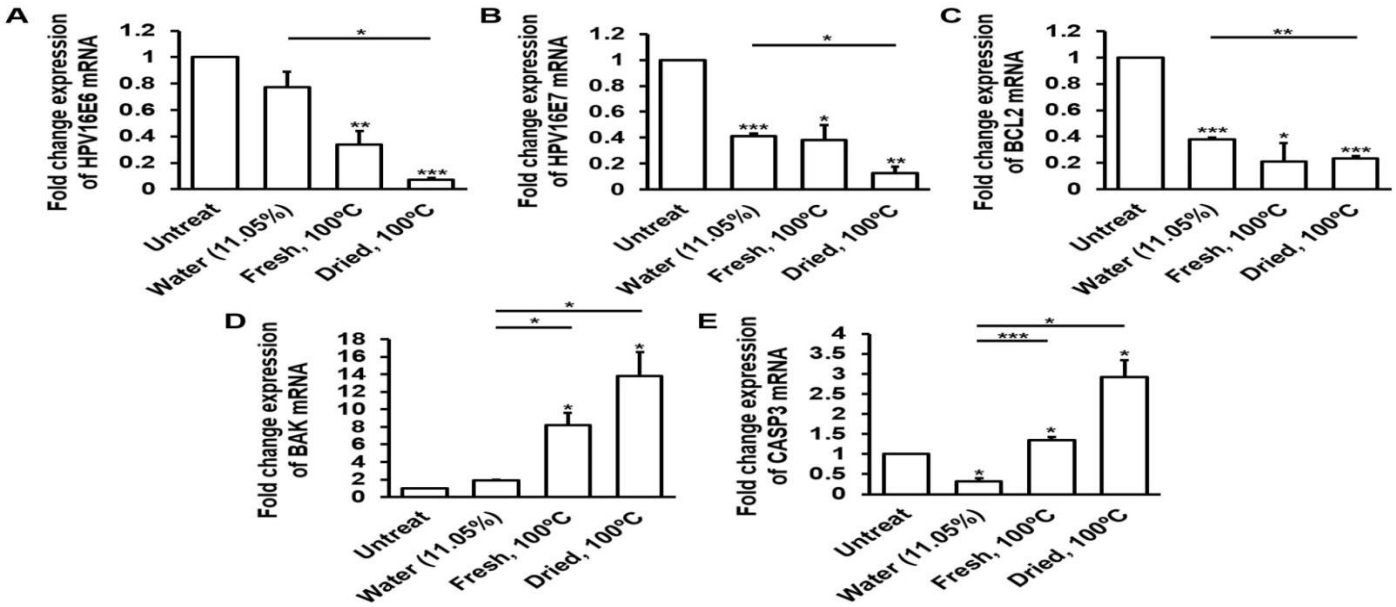
Figure 1: Effect of *A. auricula-judae* extracts on anti-HPV16 pseudovirus infection. Pre-attachment (a) and adsorption (b) steps were performed to determine the effect of the extracts on anti-HPV16 pseudovirus infection.



Effect of *A. auricula-judae* Extracts on mRNA Expression of HPV16E6/E7, *BCL2*, *BAK* and *CASP3*

The HPV16E6 and HPV16E7 proteins play an important role in the development of cervical cancer trough suppression in several tumor suppressor genes, including BAK and caspase 3 (CASP3), and in the trough induction of anti-apoptotic BCL2.

Figure2: Expression of HPV16E6 (a), E7 (b), *BCL2* (c), *BAK* (d) and *CASP3* (e) genes in *A. auricula-judae* extract-treated CaSki cells, respectively.

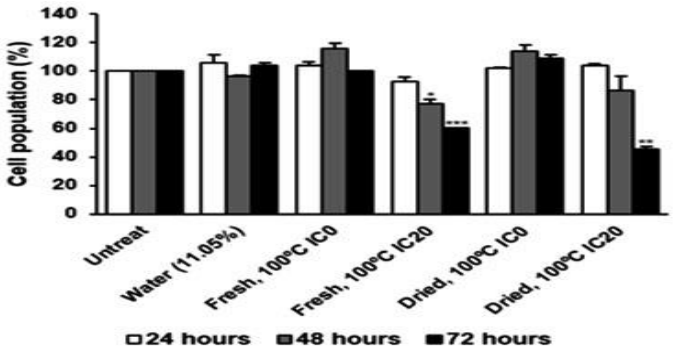


Interestingly, the extract at IC20 from the dried mushroom significantly reduced HPV16E6/E7 and *BCL2* and elevated *BAK* and *CASP3* mRNA expression levels in contrast to the untreated and water-treated CaSki cells (Figure 2). The extract from fresh mushroom significantly upregulated the expression of *BAK* and *CASP3* compared with untreated cells (Figure 2d and e). In previous study, Nagarathinam et al., (2007) used immunohistochemistry to demonstrate that *A. auricula-judae* extracted in ethanol, methanol, dichloromethane and water suppressed the expression of HPVE6 in CaSki cells. Simultaneously, we found that *A. auricula-judae* extract

reduced both HPV16E6 and E7 based on qRT-PCR analysis. In addition, there are reports the effects of *A. auricula-judae* extracts that downregulate BCL2, upregulate BAX, and activate caspase 3 in hepatoma, Sarcoma and Acinar cell carcinoma but no report in cervical cancer [15, 25, 26]. Therefore, this study firstly reports the effects of *A. auricula-judae* extracts on *BCL2* downregulation, *BAX* upregulation and caspase 3 activation in CaSki cells [22].

A. auricula-judae Extracts Suppressed Cell Proliferation in CaSki Cells

Figure 3: Effect of *A. auricula-judae* extracts on cell proliferation in CaSki cells

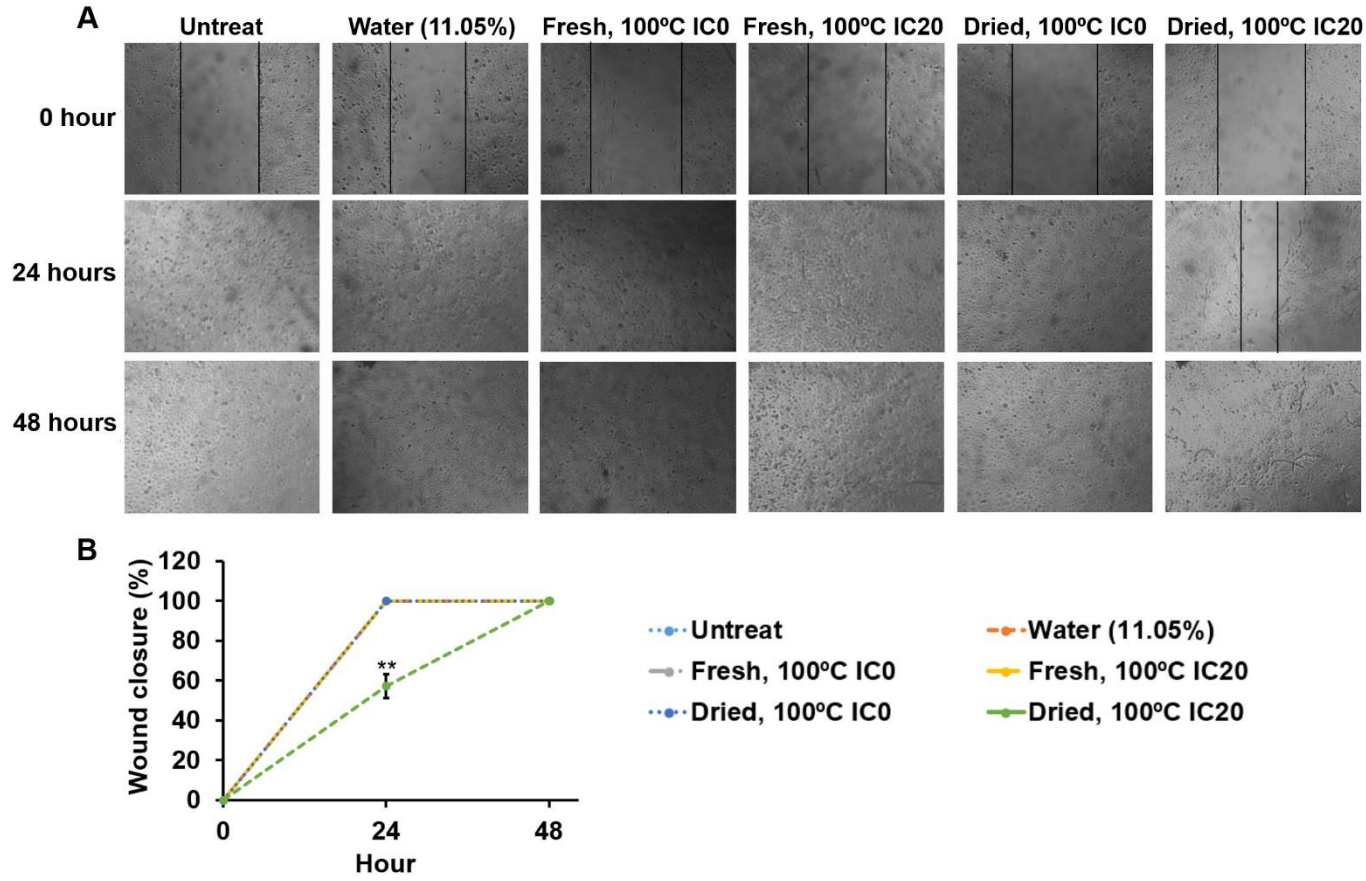


To study the effect of the extracts of cell proliferation in CaSki cells, the extracts from fresh and dried mushrooms at IC0 and IC20 were used to treat CaSki cells for 24, 48 and 72 hours. From the results, both extracts at IC20 reduced the cell population percentage, particularly after 72 hours (Figure 3), with the percentage reduction of the extract from the dried mushroom being higher. These results demonstrated that *A. auricula-judae* extracts inhibited cell proliferation of CaSki cells in a dose- and time-dependent manner

A. auricula-judae Extracts Decreased Migration in CaSki Cells

To determine the effect of *A. auricula-judae* on the inhibition of distant metastasis in CaSki cells, the extracts at IC0 and IC20 were prepared in DMEM containing 2.5% FBS and added into the cell for 0, 24 and 48 hours. Wound distance was measured at each time point. The results indicated that the extract from dried mushroom at IC20 significantly suppressed wound closure (57.18 % ± 6.08) compared with the controls (100% ± 0.00), as shown in Figure 4. From this experiment, it firstly suggested that *A. auricula-judae* extracts from dried mushrooms inhibited cell migration in CaSki cells [23].

Figure 4: Effect of *A. auricula-judae* extracts on cell migration in CaSki

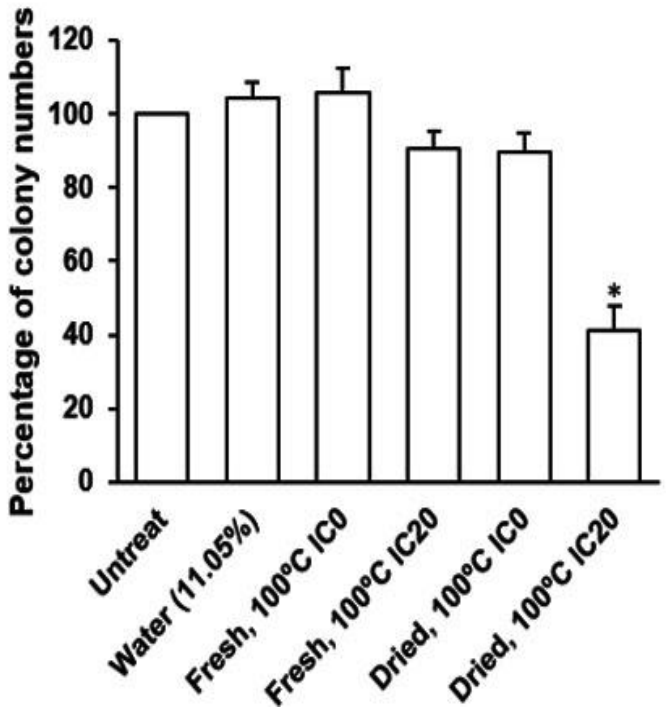


A. auricula-judae Extracts Decreased Colony Forming in CaSki Cells

In order to investigate the effect of *A. auricula-judae* on the inhibition of a single cell grown into a colony, the extracts at IC0 and IC20 were tested in completed medium and treated CaSki cells for 48 hours. After 10 days post-treatment, colonies were counted. From the results, the extract from dried mushroom at IC20 significantly reduced colony forming (41.06% ± 6.70), as shown in Figure 5. This information indicated that *A. auricula-judae* extract inhibited the

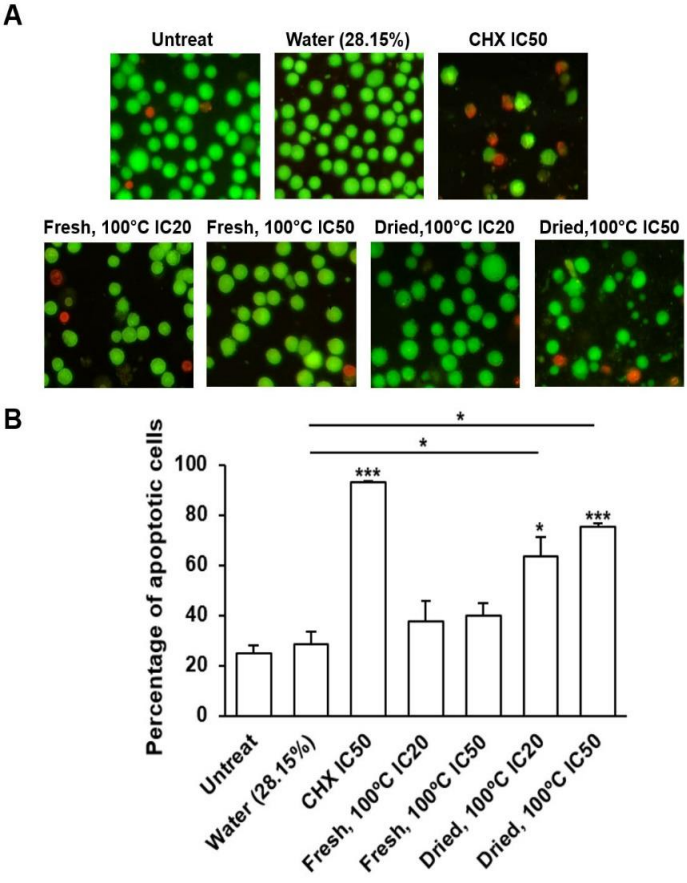
growth of CaSki cells.

Figure 5: Colony numbers in *A. auricula-judae* treated CaSki cells



Effect of *A. auricula-judae* Extracts on CaSki Apoptosis Based on AO/EB Staining

Figure 6: Effect of *A. auricula-judae* extracts on apoptosis in CaSki cells



Each extract at IC20 and IC50 was added into CaSki cells for 48 hours. The cells were treated with AO/EB solution to distinguish any apoptotic cells that were subsequently counted. The percentage of apoptotic cells was determined based on the total cells counted. Cycloheximide (CHX) was used as a positive control. The criteria to differentiate apoptotic cells was 1) normal live cell: a normal nucleus with green fluorescence; 2) early apoptosis: plasma membrane blebbing with yellow-green fluorescence chromosomal condensation and concentration into a crescent or granular; 3) late apoptosis: chromatins condensed with orange-red fluorescence; 4) Necrosis, a uniform orange-red color and cell volume increased. Both extracts induced higher early apoptosis than late apoptosis (Figure 6a). In addition, the extracts at IC50 significantly increased apoptotic cells, particularly from the dried mushroom more than from the fresh mushroom compared to untreated and water-treated cells, as shown in Figure 6b. This information suggested that the extracts from *A. auricula-judae* promoted early apoptosis more than late apoptosis in CaSki cells.

CONCLUSION

This study demonstrated that the hot water-based extract from dried *A. auricula-judae* inhibited HPV16 infection and growth of CaSki cells.

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Conflict of interest

The author declare that there are no conflicts of interest.

Ethics approval

Not applicable.

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