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ORIGINAL ARTICLE

Mechanisms of crude protein from medicinal mushroom *Ophiocordyceps sobolifera* against human breast MCF-7 cancer cells

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Abstract

The mycelial extract of the entomopathogenic fungus *Ophiocordyceps sobolifera* has recently been reported to have anti-bacterial, anti-fungal and anti-cancer effects; however, there have been no reports on the medicinal value of the crude protein against the Michigan Cancer Foundation-7 (MCF-7) human breast cancer cell line. Therefore, in this study, crude proteins from 11 isolates of *O. sobolifera* were subjected to screening and evaluation for potential anticancer activity against a breast cancer cell line. The cytotoxic and antiproliferative effects of the crude proteins on human breast cancer MCF-7 cell line was investigated using the sulforhodamine B (SRB) assay and colony formation. The gene expression and cell cycle-associated protein levels were assayed by reverse transcription-quantitative polymerase chain reaction and western blotting analysis. The results showed that the crude proteins of all isolates had anti-cancer activity in a time- and dose-dependent manner. Among them, isolates Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 had greater inhibitory effects against cancer cells; therefore, these were chosen for further evaluation of anti-cancer mechanisms. These crude proteins exhibited a potent anticancer effect on the MCF-7 cells by inhibiting colony formation in a dose-dependent manner. Furthermore, the crude proteins significantly decreased the cyclin-dependent kinase 6 (cdk6) and cyclin D1 expression. Additionally, crude proteins significantly enhanced the potent cyclin-dependent kinase inhibitor (p21) levels, and reduced the expression level of the cell cycle protein, cyclin D1. These findings indicate that the crude protein of the entomopathogenic fungus *O. sobolifera* may be useful for developing an anti-cancer drug candidate for breast cancer therapeutic treatment.

Keywords Anti-cancer activity · Breast cancer cell line · Crude protein · Entomopathogenic fungus · *Ophiocordyceps sobolifera*

Introduction

Breast cancer is a major cause of morbidity and mortality. In Asia, breast cancer has caused a significant number of cases and cancer-related deaths in women (Agarwal et al. 2007). The development of novel agents based on the biological activity of many compounds involving antimicrobial proteins (AMPs) could be promising, and has emerged as a new strategy to conquer cancer (Deslouches and Di 2017). Antimicrobial proteins are small proteins that have activity to inhibit or kill microbial pathogens, and AMPs have also been reported to have anti-cancer activity. For example, AMPs from the mushrooms *Calvatia lilacina*, *Pleurotus ostreatus* and *Volvariella volvacea* could decrease cell viability of the human colorectal adenocarcinoma cell line (SW480 cells) and a human monocytic leukemia cell line (THP-1 cells) (Wu et al. 2011).

Some of the possible candidate medicinal fungi, include the entomopathogenic fungi in the genera *Cordyceps* and *Ophiocordyceps*, are known and reported to produce AMPs contain anti-cancer, antifungal and anti-viral activities (Park et al. 2009; Wong et al. 2011; Wang et al. 2012; Bai and Sheu 2018). For example, Wong et al. (2011) demonstrated that the peptide cordymin from the medicinal fungus *Cordyceps militaris* displayed anti-proliferative activity toward the breast cancer MCF-7 cell line and had the ability to inhibit the fungal

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mycelial growth of *Bipolaris maydis*, *Mycosphaerella arachidicola*, *Rhizoctonia solani* and *Candida albicans*. Bai and Sheu (2018) reported that a novel protein from the fruiting bodies of *C. militaris* had the ability to inhibit cell viability in murine primary cells in a time- and dose-dependent manner. Wang et al. (2012) found a novel alkaline serine protease "Cordysobin" isolated from *O. sobolifera* had the HIV-1 reverse transcriptase inhibitory activity. Based on these abilities, the entomopathogenic fungi in the genera *Cordyceps* and *Ophiocordyceps* could be used as sources of novel AMPs possessing a diverse spectrum of anti-cancer activities.

In Thailand, the entomopathogenic fungus *Ophiocordyceps sobolifera* (Ophiocordycipitaceae, Ascomycota) have been found in various locations in the northeastern region. Moreover, the anti-bacterial, anti-fungal and anti-cancer activites (Sangdee et al. 2018a, b) of the mycelial extract from this fungal species have been reported; however, the activities of AMPs have not yet been investigated. Therefore, in this study we screened and investigated the effects of the crude protein from *O. sobolifera* against the human breast cancer MCF-7 cell line, and the mechanism of action responsible was also examined.

Materials and methods

Fungal strains and crude protein preparation

Eleven potential strains of the medicinal fungus identified as *O. sobolifera* based on the internal transcribed spacers of nuclear ribosomal DNA repeats (ITS-DNA) sequencing in previous studies (Jaihan et al. 2016; Sangdee et al. (2018a) (Table 1) were used to preliminary screen anti-breast cancer activity by evaluation of cell viability and the anti-breast cancer mechanisms in this study. The crude protein of these fungal isolates was prepared according to the method previously described by Sangdee et al. (2015) Briefly, the fungal mycelial of all the isolates were cultured in induced medium (Huang et al. 2009) at 28 °C for 20 days. After that, the mycelium was harvested, dried and powdered. Then, 50% ethanol was added to dried mycelium at a final concentration of 100 mg/mL (w/v). Then, the mixture was sonicated, centrifuged and filtered through a 0.2-μm filter. Following this, three milliliters of the 50% (v/v) ethanol extract were pipetted into 15-mL Focal tubes before precipitating the crude protein by adding three volumes of protein precipitation buffer and mixed by inverting 20 times. Then, the mixture was incubated at -20 °C for 2 h before being centrifuged at 9100 × g for 20 min. The total protein pellets were dissolved in 500 μL of 1 × PBS and were then quantized by a Micro-Volume UV-Vis Spectrophotometer (Nanodrop 2000, USA) at an optical density of 280 nm and then adjusted to the final protein concentration was 5 mg/mL before being used to investigate the anti-breast cancer activity.

Sulforhodamine B (SRB) assay

The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC, USA) and maintained according to ATCC's recommendations. SRB assay was used to measure the effect of the crude protein from 11 strains of *O. sobolifera* on the cell viability of MCF-7 cells as previously described Buranrat et al. (2016) Sangdee et al. (2018b) Briefly, breast cancer cells (1×10^4 cells/well) were seeded in 96-well plates and incubated overnight at 37 °C with 5% CO_2. After incubation, the growth medium was discarded and replaced with fresh DMEM medium (Gibco BRL Life Technologies, USA) containing each crude protein extract. The MCF-7 cells were further incubated for 24–72 h prior to the viability being examined by SRB assay. Briefly, cancer cells were fixed with 10% trichloroacetic acid, stained with 0.4% SRB (Sigma-Aldrich, St. Louis, MO, USA) and solubilized with 10 mM Tris base. Next, the optical density was measured by a spectrophotometer at 540 nm. The IC_{50} concentration (50% inhibition of cell viability) was calculated from the dose-response curves.

For the combination treatment between the crude protein of fungal strain Cod-KK1524, Cod-KK1630, Cod-KK1643, Cod-KS1601 and doxorubicin were investigated by SRB as described above. Cancer cells were treated with various concentrations of the crude protein of these isolates (0-250 μg/mL) with 1 μM doxorubicin (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Then the cancer cell viability was dertermined by SRB assay.

Table 1 The effects of 11 isolates of *O. sobolifera* on MCF-7 viability

Fungal isolates	IC_{50} of cell viability (μg/mL)		
	24 h	48 h	72 h
Cod-KK1506	305.13 ± 16.54 [a]	257.20 ± 10.21 [b]	151.90 ± 4.62 [c]
Cod-KK1524	205.27 ± 20.75 [a]	62.21 ± 4.21 [b]	24.64 ± 1.53 [c]
Cod-KK1625	299.30 ± 26.54 [a]	162.87 ± 6.22 [b]	42.11 ± 0.44 [c]
Cod-KK1630	345.80 ± 15.22 [a]	88.22 ± 1.74 [b]	37.48 ± 2.57 [c]
Cod-KK1634	522.99 ± 47.62 [a]	432.77 ± 26.36 [b]	100.94 ± 12.94 [c]
Cod-KK1643	206.60 ± 10.08 [a]	44.51 ± 2.91 [b]	26.10 ± 1.74 [c]
Cod-KS1601	185.27 ± 12.7 [a]	45.45 ± 3.59 [b]	26.80 ± 1.12 [c]
Cod-NB1302	294.69 ± 24.69 [a]	222.50 ± 21.58 [b]	202.47 ± 22.92 [b]
Cod-SN1606	300.23 ± 7.44 [a]	168.47 ± 3.48 [b]	105.26 ± 12.56 [c]
Cod-SN1610	173.87 ± 16.06 [a]	141.53 ± 6.25 [a]	49.77 ± 2.32 [c]
Cod-SN1626	343.10 ± 10.66 [a]	322.70 ± 7.31 [a]	78.68 ± 8.33 [b]

* Different letters indicate statistically significant differences

Colony formation assay

The colony formation assay was performed as described by Zhang et al. (2012) and Buranrat et al. (2016) with some modifcations. Briefly, MCF-7 cells were seeded at the density of 500 cells/well in six-well plates for 24 h. Breast cancer cells were then treated with various concentrations (0-250 μg/mL) of crude protein of strains Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 for 24 h. Then, the breast cancer cells were grown for another 14 days to form colonies. The tissue culture medium was discarded and replaced with fresh complete DMEM medium every 2–3 days. The cancer cells were fixed with 100% cold methanol for 15 min, stained with 0.5% crystal violet (Millipore, Bedford, MA, USA) for 15 min and colonies were observed and counted under the microscope.

Gene expression assay

The effect of the crude protein on cyclin-dependent kinase 6 (cdk6), cyclin D1 and cyclin E gene expressions were determined using real-time polymerase chain reaction (RT-PCR) as described Buranrat et al. (2019) with some modifcations. Briefly, MCF-7 cells were plated in 6-well plates at the density of 2.5×10^5 cells/well for 24 h and then incubated with 250 μg/mL of crude protein of isolates Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 for 24 h. The total RNA was extracted from breast cancer cells using Trizol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions, and the total RNA was then reverse transcribed to single-stranded cDNA using iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA). Real-time PCR was carried out using specific primers for the target gene from PrimerBank (MGH CCIB DNA core) as follows: cdk6 forward 5' GCT-GAC-CAG-CAG-TAC-GAA-TG 3' and cdk6 reverse GCA-CAC-ATC-AAA-CAA-CCT-GAC-C 3'; cyclin D1 forward 5' GCT-GCG-AAG-TGG-AAA-CCA-TC 3' and cyclin D1 reverse 5' CCT-CCT-TCT-GCA-CAC-ATT-TGA-A 3'; cyclin E forward 5' ACT-CAA-CGT-GCA-AGC-CTC-G 3' and cyclin E reverse 5' GCT-CAA-GAA-AGT-GCT-GAT-CCC 3'; and beta-actin forward 5' GTG-ACG-TTG-ACA-TCC-GTA-AAG-A 3' and beta-actin reverse 5' GCC-GGA-CTC-ATC-GTA-CTC-C 3'. The PCR was performed with SsoFast™ EvaGreen Supermix with low Rox (Bio-Rad, Hercules, CA, USA) using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc.) under the following conditions: denaturation at 95 °C for 3 min and amplification by cycling 40 times at 95 °C for 15 s and 60 °C for 31 s. The relative expression ratio of the target gene was calculated compared to the reference beta-actin gene.

Protein expression assay

The effect of the crude protein on protein expression was assessed as described by Wang et al. (2017) and Buranrat et al. (2017a) with some modifcations. Briftly, the MCF-7 cells were seeded at 2.5×10^5 cells/well in 6-well plates for 24 h before treatment with 250 μg/mL of the crude protein of isolates Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 for 24 h. The whole cell lysates were prepared using RIPA cell lysis buffer supplemented with protease cocktail inhibitor according to the manufacturer's instructions. The proteins (20 μg) were resolved with SDS-PAGE using 12% polyacrylamide gel (Bio-Rad, Laboratories, Inc.) and the separated proteins on the gel were electrophoretically transferred to a PVDF membrane (Millipore, USA). The PVDF membranes were blocked for 2 h with 5% (w/v) bovine serum albumin in Tris buffered saline containing 0.1% Tween-20 (TBST). Expression of the cell cycle regulator and protein-related cell cycle were assessed by incubation with primary antibodies (p21, cyclin D1 and beta-actin, Cell Signaling Technology, Beverly, MA, USA) overnight at 4 °C, followed by incubation for 2 h at room temperature with HRP-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA). To determine the expressions of the proteins, the membranes were probed with Clarity™ Western ECL substrate and captured using ChemiDoc™ Touch Imaging System (Bio-Rad, Laboratories, Inc.).

Results

Effects of crude protein on human breast cancer MCF-7 cell line viability

The effects of the crude protein from 11 strains of *O. sobolifera* were investigated against the human breast cancer cell line MCF-7 using the SRB assay. The results clearly indicated that the crude proteins of each isolate had anti-cancer activity against the MCF-7 cells. The crude proteins from seven isolates, Cod-KK1524, Cod-KK1625, Cod-KK1630, Cod-KK1643, Cod-KS1601, Cod-SN1610 and Cod-SN1626, had a high percentage of MCF-7 cell death with quite low IC_{50} values (less than 100 μg/mL) for 72 h (Table 1). Among them, the isolates Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 had the greater activities while showing the lowest IC_{50} values of 24.64 ± 1.53 μg/mL, 37.48 ± 2.57 μg/mL,

26.10 ± 1.74 μg/mL and 26.80 ± 1.12 μg/mL, respectively, for 72 h. The IC_{50} values of the remaining isolates on the cell viability of MCF-7 cells ranged from 42.11 ± 0.44 μg/mL to 202.47 ± 22.92 μg/mL (Table 1). Moreover, increasing the concentrations of the crude proteins, resulted in increasing the percentage of MCF-7 cell death (Fig. 1). This finding indicates that the crude proteins exhibited concentration-dependent cytotoxic activity against the MCF-7 cell line.

Based on the IC_{50} values of the cell viability for 48 and 72 h, isolates Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 were chosen for further investigation of the anti-cancer activity. Next, the combination treatment between the crude proteins of these fungal strains and doxorubicin were also investigated. The data indicated that doxorubicin alone caused induced MCF-7 cells death in a dose-dependent manner with an IC_{50} value of 5.32 ± 0.23 μM at 24 h and for the next study, doxorubicin was chosen at 1 μM in combination as a treatment with increasing cell death by approximately 20%. The results revealed that the combination treatments had the ability to cause MCF-7 cell death more than the individual crude protein treatments. The IC_{50} values of the crude protein and crude protein plus 1 μM doxorubicin on the cell viability of MCF-7 cells are shown in Fig. 2. This result indicates that the anticancer activity may be due to an

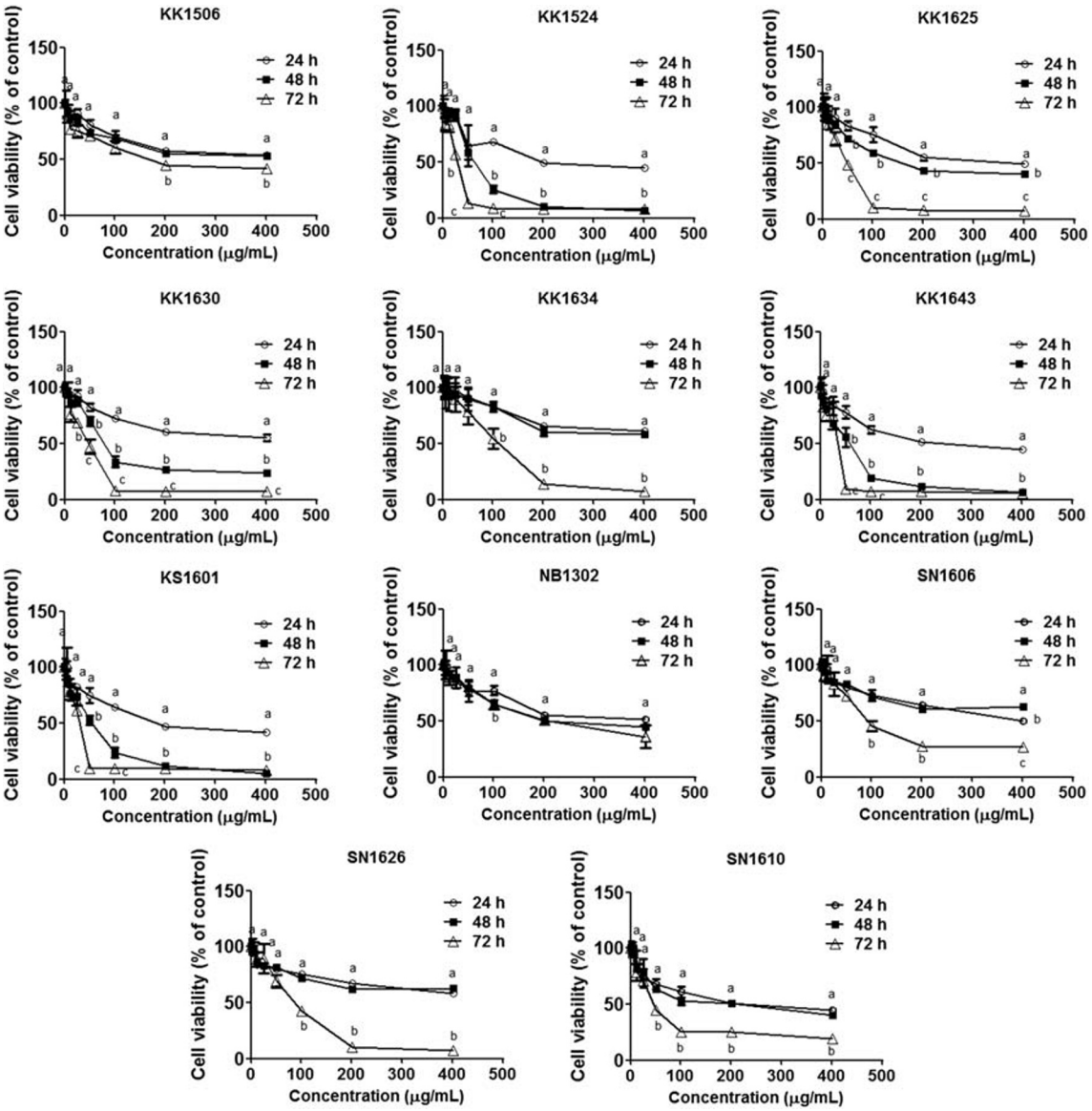

Fig. 1 Effects of crude protein from 11 isolates of *O. sobolifera* on MCF-7 cell viability. Cells were treated with various concentrations of crude proteins for 24–72 h and measured cancer cell death with SRB assay. Results are mean ± SEM of three independent experiments

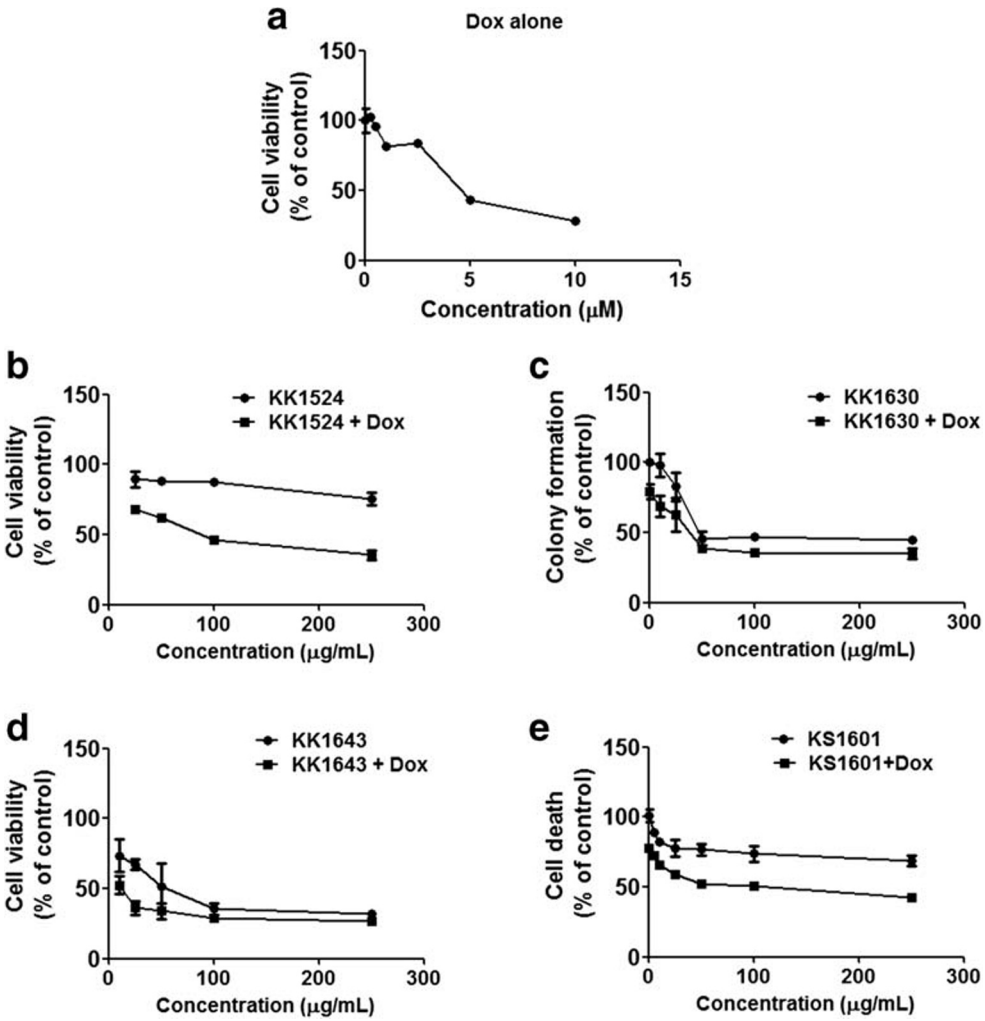

Fig. 2 Effects of crude protein from isolates Cod-KK1524 (**b**), Cod-KK1630 (**c**), Cod-KK1643 (**d**) and Cod-KS1601 (**e**) plus 1 μM doxorubicin on MCF-7 cell viability. Cells were treated with various concentrations of crude protein from each isolate supplemented with 1 μM doxorubicin for 24 h and measured viability of cancer cells with SRB assay. Results are mean ± SEM of three independent experiments. Dox; doxorubicin (**a**)

additive effect from a combination of crude protein plus 1 μM doxorubicin and the combination significantly accelerated the MCF-7 cancer cell death when compared to the crude protein treatment alone (Fig. 2; Table 2).

Effects of crude protein on MCF-7 colony formation

The effects of the crude proteins of isolates Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 on MCF-7 cell growth were investigated using the colony formation assay. The results showed that the crude protein of isolates Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 decreased the colony forming ability (Fig. 3). This finding indicates that the crude protein caused dose-dependent cytotoxic and anti-proliferative effects against the MCF-7 cancer cell type (Fig. 3).

Effects of crude protein of *O. sobolifera* on downstream gene expression in MCF-7 cancer cell

The effects of the crude protein on downstream gene expression of the cell cycle regulator and protein-related cell cycle in

Table 2 IC_{50} of cell viability of the crude protein from 4 isolates of *O. sobolifera* with anticancer drug (Doxorubicin) for 24 h

Treatment groups	IC_{50} of cell viability (μg/mL)
Doxorubicin alone	5.32 ± 0.23 (μM)
Cod-KK1524 alone	693.33 ± 90.04
Cod-KK1524 + Dox	85.95 ± 10.71*
Cod-KK1630 alone	57.65 ± 12.75
Cod-KK1630 + Dox	51.47 ± 6.82*
Cod-KK1643 alone	47.19 ± 9.59
Cod-KK1643 + Dox	20.21 ± 1.48*
Cod-KS1601 alone	353.33 ± 116.42
Cod-KS1601 + Dox	59.61 ± 4.04*

* The difference from crude extracts treatment alone

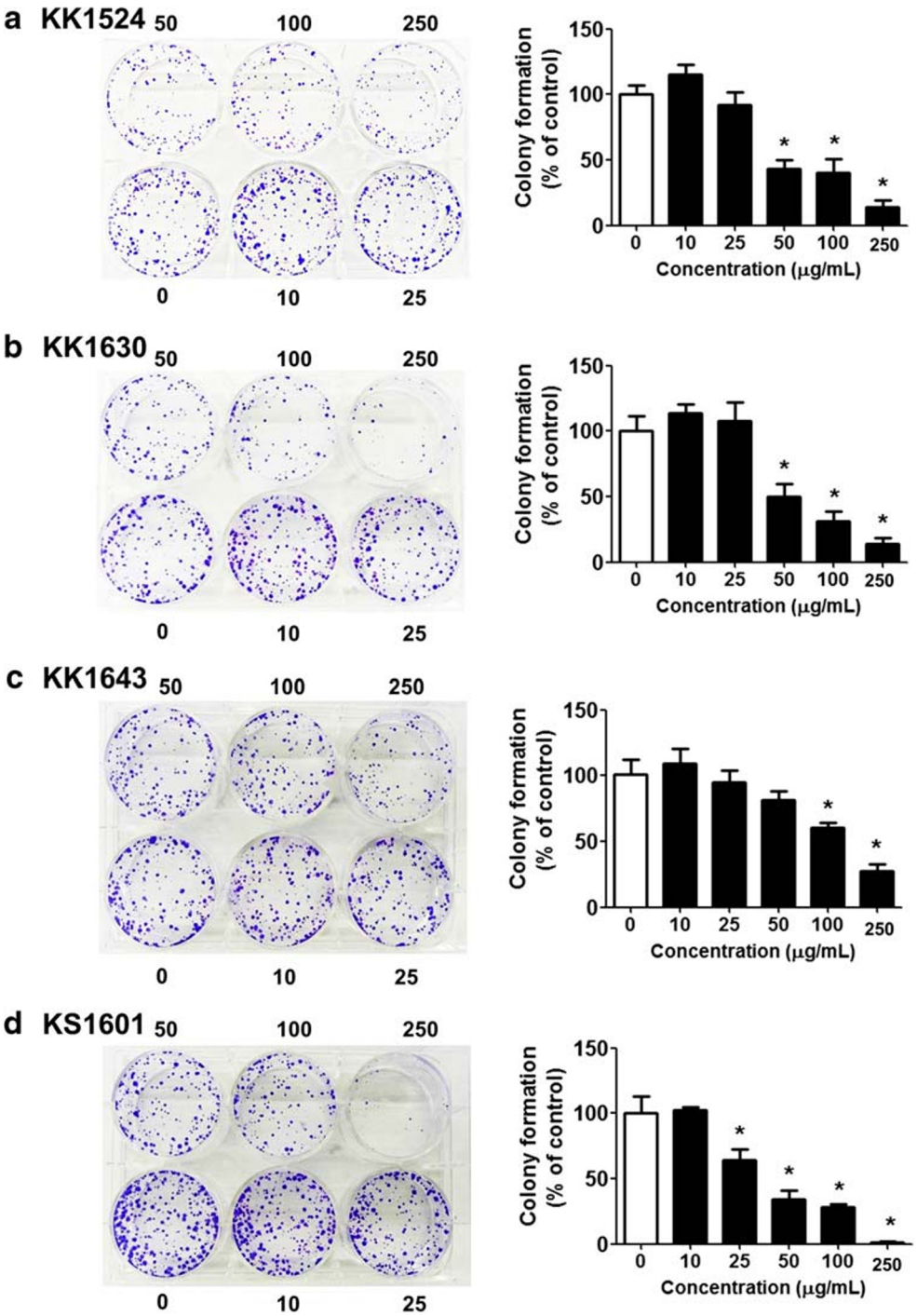

Fig. 3 Effects of crude protein of *O. sobolifera* isolates Cod-KK1524 (**a**), Cod-KK1630 (**b**), Cod-KK1643 (**c**) and Cod-KS1601 (**d**) on MCF-7 cell colony formation. Cells were treated with various concentrations of crude protein of each isolate for 24 h. Cancer cell colony formation was assessed after 14 days of treatment. Results shown are mean ± SEM of three independent experiments. *$p < 0.05$ versus control

MCF-7 cancer cells, mRNA expression of cdk6, cyclin D1 and cyclin E were examined. The results revealed that the treatment of the MCF-7 cells with each crude protein modulated the cdk6, cyclin D1 and cyclin E mRNA expressions in MCF-7 cancer cells (Fig. 4). Interestingly, Cod-KS1601 showed a significant reduction of all mRNA gene expression levels. Moreover, the effects on protein related to cell death, p21 and cyclin D1 were also determined by western blot analysis. The results clearly indicated that the crude proteins significantly induced p21 expression to inhibit the cell cycle. This is correlated with a reduction in the cyclin D1 protein expression in MCF-7 cancer cells (Fig. 5). This evidence confirmed that MCF-7 cancer cell proliferation was inhibited by increasing p21 and reducing cyclin D1 protein expression.

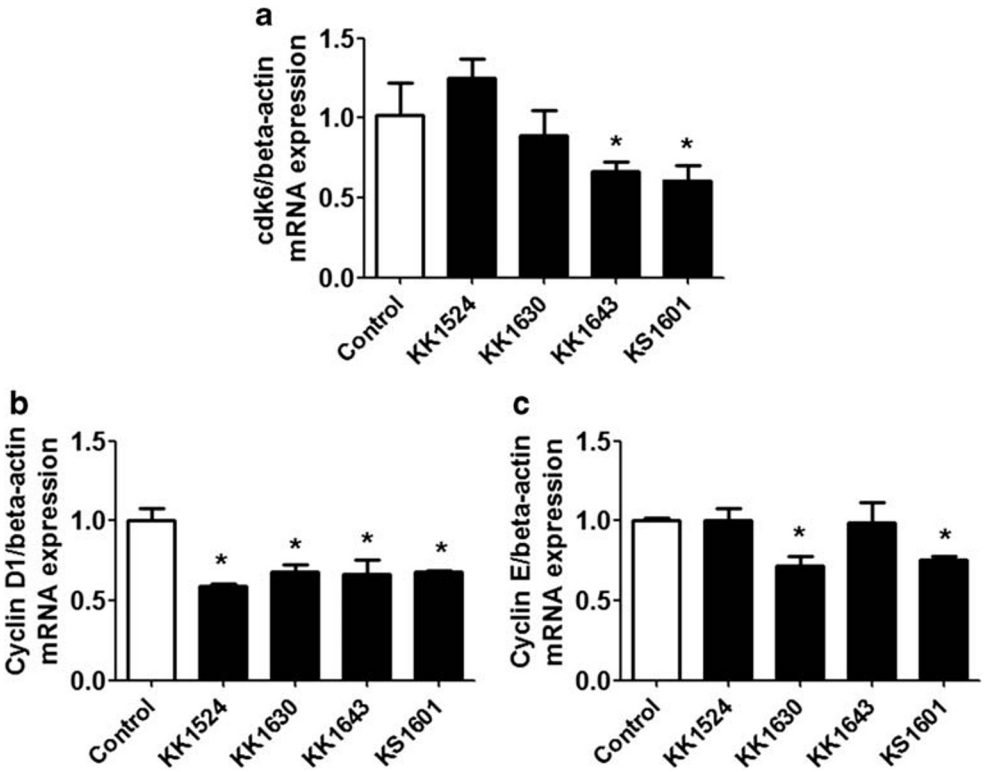

Fig. 4 Effects of crude protein of *O. sobolifera* isolates Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 on downstream gene expression of MCF-7 cell. Cells were treated with crude protein at the concentration of 250 μg/mL for 24 h, then mRNA was extracted and cdk6 (**a**), cyclin D1 (**b**) and cyclin E (**c**) gene expressions were detected by RT-PCR. Results are expressed as mean ± SEM of three independent experiments. *$p < 0.05$ versus control

Discussion

Cancer is one of most common fatal diseases that causes morbidity and mortality worldwide. In Asia, breast cancer caused a significant number of cases and cancer-related deaths in women (Agarwal et al. 2007). It was estimated that 1,671,149 new cases of breast cancer were identified and 521,907 cases of death due to breast cancer occurred in the world in 2012 (Ghoncheh et al. 2016). Resection, chemotherapy and radiotherapy are the most effective strategies used for breast cancer treatment (Pagani et al. 2010). However, the resistance of breast cancer to some anti-cancer drugs has now been reported (O'Driscoll and Clynes 2006); therefore, alternative strategies including new improved drugs or bioactive compounds from natural sources are being identified and screened. The medicinal entomopathogenic fungus *O. sobolifera* is a possible candidate that has been reported to have anti-cancer cell activities, including against breast cancer (Sangdee et al. 2018a) and colon cancer (Yang and Zhang 2016). Based on these activities, we screened and evaluated the potential of strains of *O. sobolifera* against human breast cancer MCF-7 cells using antimicrobial proteins, because AMPs from many medicinal entomopathogenic fungi have been reported to have great potential to inhibit cancer cells (Wong et al. 2011; Bai and Sheu 2018).

In this study we provided evidence for the anticancer activities of crude protein from each tested strain of *O. sobolifera* on breast cancer cells using established assays for measuring the cytotoxic activity of cancer cells. Among the tested isolates, Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 have the best inhibitory effects against cancer cells; therefore, they were chosen for further evaluation of the mechanism of action. Interestingly, the four isolates combined with doxorubicin, a standard anticancer drug for breast cancer, showed additive effects when compared with the extract treatment alone, especially in Cod-KK1524. Likely, as Buranrat et al. indicated, simvastatin plus doxorubicin induced MCF-7 cells death more than simvastatin in treatment alone (Buranrat et al. 2017a). After evaluation, the crude protein of the isolates Cod-KK1524, Cod-KK1630, Cod-KK1643 and Cod-KS1601 exhibited a marked effect on the breast cancer MCF-7 cells by inhibiting colony formation. In addition, we found that 250 μg/mL of crude protein decreased cdk6, cyclin D1 and cyclin E mRNA expression and also reduced the expression level of the cell cycle protein, cyclin D1, which resulted in enhanced breast cancer cell death and inhibition of cell proliferation. This was correlated with Lamb et al. (Lamb et al. 2013), who found that the migration and stem-like cell activity of the four cell lines and primary cells from six breast cancers were decreased when inhibited by cyclin D1 and cdk4/6. As the level of cyclin D1 protein expression may effect breast cancer cell proliferation, such that the overexpression ranged from 25 to 60% of cyclin D1 it plays an important role in invasive breast cancer cells

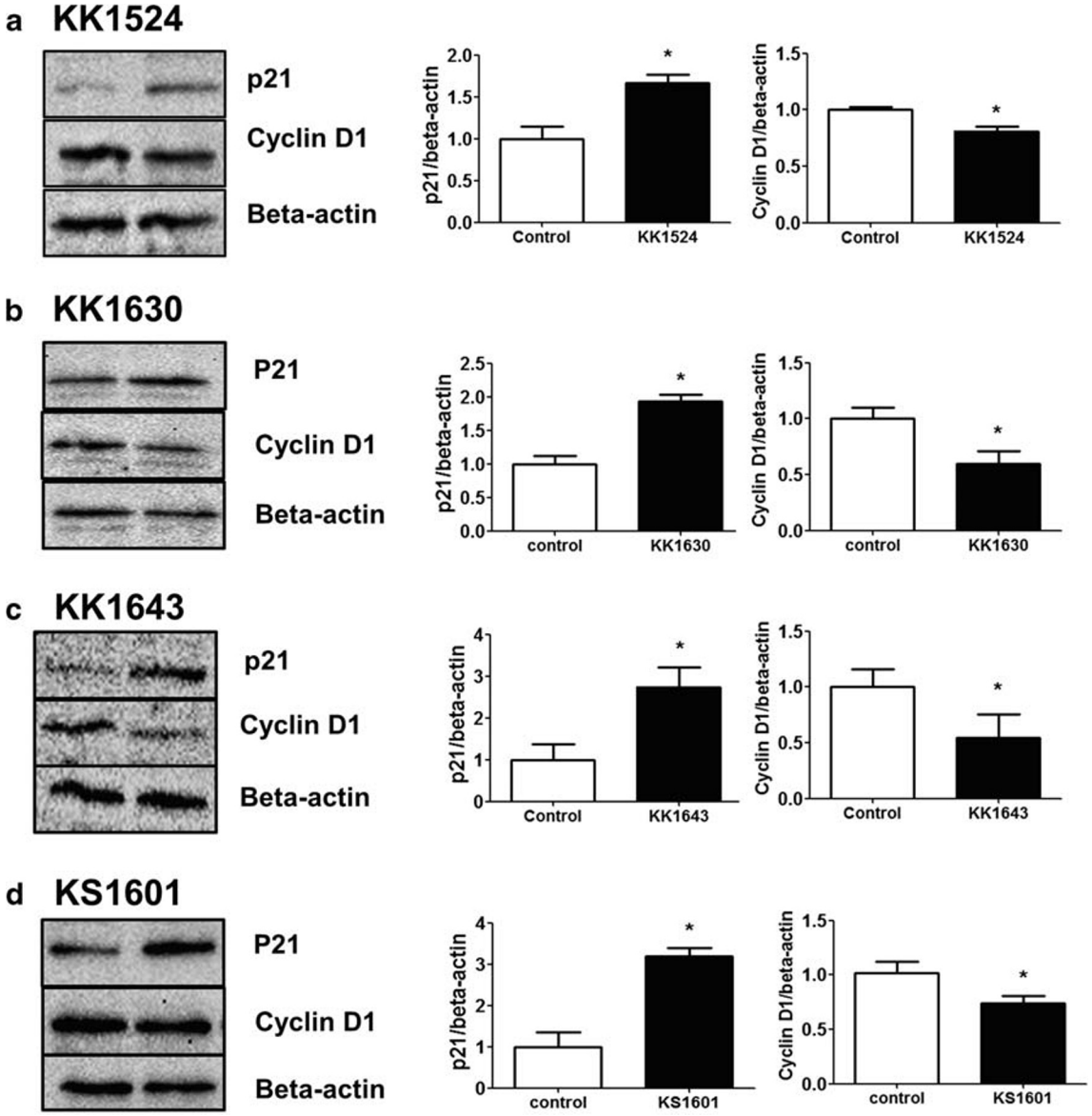

Fig. 5 Effects of crude protein of *O. sobolifera* isolates Cod-KK1524 (**a**), Cod-KK1630 (**b**), Cod-KK1643 (**c**) and Cod-KS1601 (**d**) on the protein expression of MCF-7 cancer cell. Cells were added to crude protein at the concentration of 250 μg/mL for 24 h and protein expression of p21 and cyclin D1 were measured by Western blotting. Results are expressed as mean ± SEM of three independent experiments. *$p < 0.05$ versus control

(McIntosh et al. 1995; Courjal et al. 1996; Gillett et al. 1996; Arnold and Papanikolaou 2005; Akin et al. 2014). Moreover, breast cancer cell activation and cell proliferation occurred when the cyclin D1 bound to the partner cdk6, then eventual modification of the Rb phosphorylation (Lundberg and Weinberg 1998; Ezhevsky et al. 2001) and have been independently implicated in breast cancer (Lamb et al. 2013).

In addition, in this study, crude proteins at a concentration of 250 μg/mL induced an expression level of cyclin-dependent kinase inhibitor protein (p21) that is responsible for inhibiting cellular growth and promoting cell apoptosis (Xiong et al. 1993). The protein p21 may effectively bind cdk4 and cdk6, resulting in preventing cyclin D1 form binding to these protein kinases (Gray-Bablin et al. 1996; Buranrat et al. 2017a). In addition, the protein p21 can bind the cyclin E/cdk2 complex and cause the inactivation of the protein kinase activity, and then eventually causing G1 arrest due to Rb phosphorylation (Zhang et al. 1994; Harper et al. 1995; Sherr and Roberts 1995). This supported our findings and indicated that crude protein enhances breast cancer MCF-7 cell death by reducing cdk6, cyclin D1 and cyclin E mRNA expression as well as by inducing p21 protein expression. Moreover, in this study, the combination treatment between the crude protein of the fungal strains Cod-KK1524, Cod-KK1630, Cod-KK1643, Cod-KS1601 and doxorubicin were also investigated. The results showed that the percentage of cell viability reduced in the combination treatment group (crude protein of the fungal strains plus 1 μM doxorubicin) more than those of breast

cancer cells that were treated with crude protein alone. This finding indicates that the anticancer activity may be due to an additive effect. This was supported by the study of Buranrat et al. (2017b) who found the potentiate effects of *Cratoxy formosum* leaf extract on four anticancer drugs including doxorubicin, cisplatin, 5-fluorouracil (5-FU) and gemcitabine. The percentage of cell viability of MCF-7 cells reduced in the combination treatment group more than in the treatment with *C. formosum* extract or the anticancer drugs alone.

Based on the presented findings, we suggest that the crude protein of *O. sobolifera* enhances breast cancer MCF-7 cell death by reducing cdk6, cyclin D1 and cyclin E mRNA expression as well as by inducing p21 protein expression. This fungus could be used as a novel source of anticancer agents for therapeutic strategies for breast cancer; however, the deep mechanism of this crude protein-induced potentiation needs to be identified in further study.

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Author contributions The author Aphidech Sangdee designed, performed, analyzed the experiments and wrote the manuscript. Benjaporn Buranrat designed, performed and analyzed the experiments. Kusavadee Sangdee and Sutthiwan Thammawat critically proofed the data and manuscript.

Compliance with ethical standards

Disclosure statement The authors have no potential conflict.

References

Agarwal G, Pradeep PV, Aggarwal V, Yip CH, Cheung PS (2007) Spectrum of breast cancer in Asian women. World J Surg 31: 1031–1040. https://doi.org/10.1007/s00268-005-0585-9

Akin S, Babacan T, Sarici F, Altundag K (2014) A novel targeted therapy in breast cancer: cyclin dependent kinase inhibitors. J BUON 19:42–46

Arnold A, Papanikolaou A (2005) Cyclin D1 in breast cancer pathogenesis. J Clin Oncol 23:4215–4224. https://doi.org/10.1200/JCO.2005.05.064

Bai KC, Sheu F (2018) A novel protein from edible fungi *Cordyceps militaris* that induces apoptosis. J Food Drug Anal 26:21–30. https://doi.org/10.1016/j.jfda.2016.10.013

Buranrat B, Senggunprai L, Prawan A, Kukongviriyapan V (2016) Simvastatin and atorvastatin as inhibitors of proliferation and inducers of apoptosis in human cholangiocarcinoma cells. Life Sci 153:41–49. https://doi.org/10.1016/j.lfs.2016.04.018

Buranrat B, Suwannaloet W, Naowaboot J (2017a) Simvastatin potentiates doxorubicin activity against MCF-7 breast cancer cells. Oncol Lett 14:6243–6250. https://doi.org/10.3892/ol.2017.6783

Buranrat B, Mairuae N, Konsue A (2017b) *Cratoxy formosum* leaf extract inhibits proliferation and migration of human breast cancer MCF-7 cell. Biomed Pharmacother 90:77–84. https://doi.org/10.1016/j.biopha.2017.03.032

Buranrat B, Sangdee K, Sangdee A (2019) Comparative study on the effect of aqueous and ethanolic mycelial extracts from *Polycephalomyces nipponicus* (Ascomycetes) against human breast cancer MCF-7 Cells. Int J Med Mushrooms 21(7):671–681. https://doi.org/10.1615/IntJMedMushrooms.2019031140

Courjal F, Louason G, Speiser P, Katsaros D, Zeillinger R, Theillet C (1996) Cyclin gene amplification and overexpression in breast and ovarian cancers: evidence for the selection of cyclin D1 in breast and cyclin E in ovarian tumors. Int J Cancer 69:247–253. https://doi.org/10.1002/(SICI)1097-0215(19960822)69:4<247::AID-IJC1>3.0.CO;2-X

Deslouches B, Di YP (2017) Antimicrobial peptides with selective antitumor mechanisms: prospect for anticancer applications. Oncotarget 8(28):46635–46651. https://doi.org/10.18632/oncotarget.16743

Ezhevsky SA, Ho A, Becker-Hapak M, Davis PK, Dowdy SF (2001) Differential regulation of retinoblastoma tumor suppressor protein by G_1 cyclin-dependent kinase complexes *in vivo*. Mol Cell Biol 21: 4773–4784. https://doi.org/10.1128/MCB.21.14.4773-4784.2001

Ghoncheh M, Momenimovahed Z, Salehiniya H (2016) Epidemiology, incidence and mortality of breast cancer in Asia. Asian Pac J Cancer Prev 17(S3):47–52. https://doi.org/10.7314/apjcp.2016.17.s3.47

Gillett C, Smith P, Gregory W, Richards M, Millis R, Peters G, Barnes D (1996) Cyclin D1 and prognosis in human breast cancer. Int J Cancer 69:92–99. https://doi.org/10.1002/(SICI)1097-0215(19960422)69:2<92::AID-IJC4>3.0.CO;2-Q

Gray-Bablin J, Zalvide J, Fox MP, Knickerbocker CJ, DeCaprio JA, Keyomarsi K (1996) Cyclin E, a redundant cyclin in breast cancer. Proc Natl Acad Sci USA 93:15215–15220. https://doi.org/10.1073/pnas.93.26.15215

Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai LH, Zhang P, Dobrowolski S, Bai C, Connell-Crowley L, Swindell E, Fox MP, Wei N (1995) Inhibition of cyclin-dependent kinases by p21. Mol Biol Cell 6:387–400. https://doi.org/10.1091/mbc.6.4.387

Huang L, Li Q, Chen Y, Wang X, Zhou X (2009) Determination and analysis of cordycepin and adenosine in the products of *Cordyceps* spp. Afr J Microbiol Res 3:957–961. http://www.academicjournals.org/ajmr

Jaihan P, Sangdee K, Sangdee A (2016) Selection of entomopathogenic fungus for biological control of chili anthracnose disease caused by *Colletotrichum* spp. Eur J Plant Pathol 146:551–564. https://doi.org/10.1007/s10658-016-0941-7

Lamb R, Lehn S, Rogerson L, Clarke RB, Landberg G (2013) Cell cycle regulators cyclin D1 and CDK4/6 have estrogen receptor-dependent divergent functions in breast cancer migration and stem cell-like activity. Cell Cycle 12:2384–2394. https://doi.org/10.4161/cc.25403

Lundberg AS, Weinberg RA (1998) Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol 18:753–761. https://doi.org/10.1128/mcb.18.2.753

McIntosh GG, Anderson JJ, Milton I, Steward M, Parr AH, Thomas MD, Henry JA, Angus B, Lennard TW, Horne CH (1995) Determination of the prognostic value of cyclin D1 overexpression in breast cancer. Oncogene 11:885–891. https://www.ncbi.nlm.nih.gov/pubmed/7675447

O'Driscoll L, Clynes M (2006) Biomarkers and multiple drug resistance in breast cancer. Curr Cancer Drug Targets 6:365–384. https://doi.org/10.2174/156800906777723958

Pagani O, Senkus E, Wood W, Colleoni M, Cufer T, Kyriakides S, Costa A, Winer EP, Cardoso F, ESO-MBC Task Force (2010) International guidelines for management of metastatic breast cancer: can metastatic breast cancer be cured? J Natl Cancer Inst 102:456–463. https://doi.org/10.1093/jnci/djq029

Park BT, Na KH, Jung EC, Park JW, Kim HH (2009) Antifungal and anticancer activities of a protein from the mushroom *Cordyceps*

militaris. Korean J Physiol Pharmacol 3:49–54. https://doi.org/10.4196/kjpp.2009.13.1.49

Sangdee K, Nakbanpote W, Sangdee A (2015) Isolation of the entomopathogenic fungal strain Cod-MK1201 from a cicada nymph and assessment of its antibacterial activities. Int J Med Mushrooms 17: 51–63. https://doi.org/10.1615/intjmedmushrooms.v17.i1.60

Sangdee K, Buranrat B, Jaihan P, Thongchai S, Sangdee A (2018a) Evaluation of antibacterial and anticancer activities of the medicinal fungus *Ophiocordyceps sobolifera* (Ascomycetes) from Thailand. Int J Med Mushrooms 20:471–484. https://doi.org/10.1615/IntJMedMushrooms.2018026247

Sangdee A, Sangdee K, Buranrat B, Thammawat S (2018b) Effects of mycelial extract and crude protein of the medicinal mushroom, *Ophiocordyceps sobolifera*, on the pathogenic fungus, *Candida albicans*. Trop J Pharm Res 17(12):2449–2454. https://doi.org/10.4314/tjpr.v17i12.21

Sherr CJ, Roberts JM (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 9:1149–1163. https://doi.org/10.1101/gad.9.10.1149

Wang SX, Liu Y, Zhang GQ, Zhao S, Xu F, Geng XL, Wang HX (2012) Cordysobin, a novel alkaline serine protease with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom *Cordyceps sobolifera*. J Biosci Bioeng 113:42–47. https://doi.org/10.1016/j.jbiosc.2011.09.005

Wang ST, Ho HJ, Lin JT, Shieh JJ, Wu CY (2017) Simvastatin-induced cell cycle arrest through inhibition of STAT3/SKP2 axis and activation of AMPK to promote p27 and p21 accumulation in hepatocellular carcinoma cells. Cell Death Dis 23(2):8. https://doi.org/10.1038/cddis.2016.472

Wong JH, Ng TB, Wang H, Sze SC, Zhang KY, Li Q, Lu X (2011) Cordymin, an antifungal peptide from the medicinal fungus *Cordyceps militaris*. Phytomedicine 18:387–392. https://doi.org/10.1016/j.phymed.2010.07.010

Wu JY, Chen CH, Chang WH, Chung KT, Liu YW, Lu FJ, Chen CH (2011) Anti-cancer effects of protein extracts from *Calvatia lilacina*, *Pleurotus ostreatus* and *Volvariella volvacea*. Evid-Based Complementary Altern Med. https://doi.org/10.1093/ecam/neq057

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach DV (1993) p21 is a universal inhibitor of cyclin kinases. Nature 366:701–704. https://doi.org/10.1038/366701a0

Yang S, Zhang H (2016) Optimization of the fermentation process of *Cordyceps sobolifera* Se-CEPS and its anti-tumor activity *in vivo*. J Biol Eng 10:1–8. https://doi.org/10.1186/s13036-016-0029-0

Zhang H, Hannon J, Beach D (1994) p21-containing cyclin kinases exist in both active and inactive states. Genes Dev 8:1750–1758. https://doi.org/10.1101/gad.8.15.1750

Zhang N, Yang Y, Cheng L, Zhang XM, Zhang S, Wang W, Liu SY, Wang SY, Wang RB, Xu WJ, Dai L, Yan N, Fan P, Dai LX, Tian HW, Liu L, Deng HX (2012) Combination of Caspy2 and IP-10 gene therapy significantly improves therapeutic efficacy against murine malignant neoplasm growth and metastasis. Hum Gene Ther 23:837–846. https://doi.org/10.1089/hum.2011.136