Antiproliferative activity and apoptosis-inducing effects of *Trametes polyzona* polysaccharides against human breast cancer cells

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Abstract. Fungal polysaccharides have garnered interest due to their biological activities in terms of anticancer properties and antioxidant activity. The present study aimed to evaluate the anticancer properties and antioxidant activity of a newly isolated white-rot fungus, *Trametes polyzona* CU07 from Thailand. Crude *T. polyzona* polysaccharides (CTPPs) were extracted from mycelia using hot water. The chemical properties, including total carbohydrates, molecular weight and protein content, and Fourier-transform infrared spectroscopy analysis, were then investigated. The antioxidant activity was determined against the radicals 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The anticancer properties were evaluated in MCF-7 breast cancer (BC) cells, whereas the 293 cell line was used as a control. The inhibitory effects of CTPPs on viability were determined by MTT assay, followed by BrdU incorporation assay to assess cell proliferation. The induction of apoptosis was determined by flow cytometry. CTPPs were considered polysaccharide-protein conjugates, which had molecular weights in the range of 0.3-22,528 kDa. They contained ~50 and 37% carbohydrate and protein, respectively, with glucose as the main monosaccharide component. Notably, CTPPs had high antioxidant activity against ABTS, and had a significant inhibitory effect on the MCF-7 cell line with a half-maximal inhibitory concentration value of 0.58 mg/ml. However, they exhibited little effect on the 293 cell line. The BrdU incorporation assay demonstrated that CTPPs inhibited proliferation by ~20% compared with that in untreated cells. CTPPs also induced early- and late-stage apoptosis of MCF-7 cells. These results indicated that the CTPPs may exhibit potential antiproliferative and antioxidant activity, and apoptosis-inducing effects against human BC cells.

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Introduction

Breast cancer (BC) is considered the most common type of invasive cancer in women worldwide, with the prevalence of cases increasing in recent decades (1). The risk of BC has been worsened by multifactorial lifestyle and environmental factors, which can lead to the initiation of cancer progression (2). Treatments, such as surgery, radiotherapy and hormone therapy, have been applied in patients with BC; however, the side effects cannot be ignored (3,4). Screening of therapeutic agents without extensive side effects that are effective for patients with BC has been performed, and the use of natural products, as single agents or adjuvants, has been suggested for cancer treatment (5,6). In a recent study, natural medicines used as adjuvants with chemotherapeutic drugs have been shown to have clinical use by sensitizing cancer cells to be more responsive to the drug and to reverse chemoresistance (7). The small number of side effects, low toxicity and variety of bioactive compounds are considered advantages of using a natural product for BC treatment.

Polysaccharides, including polysaccharide-protein complexes, are bioactive compounds, which have been highlighted for their potential therapeutic application. Several studies have reported on their antiproliferative, apoptosis-inducing, antioxidative and immune-enhancing effects (8-10). Another study reported that protein-bound polysaccharides can promote the proliferation of peripheral blood mononuclear cells (11). In addition, in *in vitro* and *in vivo* experiments, polysaccharides have been reported to enhance immune responses, with an increasing number of cytokines, such as IL-β, IL-6 and TNF-α, and lymphocyte activation (12,13). In addition, polysaccharides have also been reported to induce B-cell proliferation through the activation of MAPK and NF-κB pathways has been reported (14).

The discovery of the two most notable protein-bound polysaccharides from *Trametes versicolor* (a medicinal mushroom), polysaccharopeptide (PSP) and polysaccharide Krestin (PSK),

have been reported to act as useful adjuvants in cancer treatment (9,15), which has prompted researchers to explore the use of polysaccharides from other *Trametes* fungi as therapeutic compounds, including *T. polyzona*. Based on its physiological properties, *T. polyzona* is a polypore mushroom belonging to white-rot fungi, which is similar to *T. versicolor* (16). This fungus has been reported to secrete various enzymes, such as laccase and manganese peroxidase, which demonstrate the degradation of aromatic compounds in a polluted environment and high tolerance for biodegradation (17,18). In a previous study, the phenolic extract of *T. polyzona* was found to have antimicrobial abilities against various bacterial strains and antioxidant properties against several radicals (19). However, to the best of our knowledge, no evaluation of other bioactive compounds from this fungus, such as polysaccharides, in cancer treatment has been performed. Therefore, this research aimed to isolate polysaccharides from *T. polyzona* CU07, which has been successfully isolated from its natural habitat in Thailand, and to evaluate its bioactivity on BC cells for the development of alternative drugs for cancer and other related diseases.

Materials and methods

Chemicals and reagents. MTT membrane-permeable dye (cat. no. ab146345) and Annexin V-DY-634 PI Apoptosis Staining/Detection Kit (cat. no. ab214484) were purchased from Abcam. Cisplatin was obtained from Glentham Life Sciences Ltd. Cell proliferation ELISA, BrdU (colorimetric) was purchased from Roche Applied Science. Fetal bovine serum (FBS), antibiotic-antimycotic (100X; containing penicillin, streptomycin and amphotericin), 0.25% Trypsin-EDTA (1X) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco; Thermo Fisher Scientific, Inc. Standard monosaccharides (arabinose, glucose and mannose) were purchased from Ajax Finechem Pty Ltd.

Preparation of CTPPs. For the present study, *T. polyzona* CU07 was obtained from the culture collection of Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University (Bangkok, Thailand). After cultivation for 5 days on potato dextrose agar, 10 mycelial discs of CU07 (diameter, 3 mm) were transferred into 150 ml potato dextrose medium and cultivated at room temperature (25±2˚C) under static conditions for 14 days. The mycelial biomass was then harvested by paper filtration, freeze dried at -60˚C, ground and sieved to a fine power (≤1 mm). The crude polysaccharide was obtained by hot water extraction in a reflux refractor with solid-to-liquid ratio of 1:40 (g/ml) at 90˚C for 4 h. The supernatant was collected and freeze dried to obtain crude *T. polyzona* polysaccharides (CTPPs).

Total carbohydrate and protein contents. The total amount of carbohydrate in CTPPs was determined using the anthrone assay (20). The extract (0.1 mg/ml) was mixed with 1.25 ml anthrone reagent (MilliporeSigma) and incubated at 4˚C for 10 min. The mixture was then heated in boiling water for 10 min and the absorbance was observed at 630 nm. The protein content was estimated according to Lowry's method (21) with slight modifications. Briefly, 400 μl sample was added to 2 ml Lowry reagent and incubated at room temperature for 10 min in the dark. Subsequently, Folin reagent (100 μl; MilliporeSigma) was added and incubated for 10 min under the same conditions. The absorbance of mixture was observed at 750 nm. Glucose and BSA (MilliporeSigma) were used as standard solutions for the anthrone assay and Lowry's method, respectively.

Monosaccharide composition. To obtain the monosaccharides, CTTPs (300 mg) were mixed with concentrated sulphuric acid [72% (v/v), 3 ml] and incubated at room temperature for 60 min, followed by autoclaving at 121˚C for 1 h (22). The hydrolysate was neutralized with calcium carbonate and filtered through a microporous membrane (pore size, 0.45 μm). The type and content of monosaccharides were determined by high-performance liquid chromatography (HPLC; Prominence Modular; Shimadzu Corporation), using a refractive index detector and Shodex SUGAR SH-1011 column (8.0x300 mm; Shodex; Resonac Corporation), at 40˚C with 5 mM H_2SO_4 as the mobile phase with a flow rate of 1.0 ml/min. Each sugar was quantified according to the sugar standards (arabinose, glucose, mannose and xylose).

Structural analysis. The primary structure of the sample was characterized by Fourier-transform infrared spectroscopy (FTIR) with wave numbers in the range of 4,000 to 400 cm^{-1} (8). The sample was prepared by mixing with KBr powder (10:1 by weight). The average molecular weight analysis was performed by gel permeation chromatography (GPC; Shimadzu HPLC 10Avp; Shimadzu Corporation) equipped with a refractive index detector and Shodex PLgel column (7.5x300 mm; Shodex; Resonac Corporation) that was operated at 40˚C. The sample (10 μl) was dissolved with ultra-pure water (2 mg/ml, 10 μl) and eluted with the same solution at a flow rate of 0.5 ml/min. Pullulan was used as a polysaccharide standard for calibration and molecular weights were analyzed using Lab Solutions software (Shimadzu Corporation).

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) inhibition assay. The radical cation of $ABTS^+$ was prepared by mixing 7.00 mM ABTS solution (MilliporeSigma) with 2.45 mM $K_2S_2O_8$ at a ratio of 1:1 (v/v) and was incubated at room temperature for 24 h in the dark (23). The $ABTS^+$ solution was then diluted with methanol to obtain the absorbance of 0.700±0.05 at 734 nm. Subsequently, 150 μl $ABTS^+$ solution was mixed with CTPPs dissolved in distilled water (50 μl) at different concentrations (0.4-1.6 mg/ml). Ascorbic acid was used as a positive control. The mixture was incubated at room temperature for 10 min in the dark and the absorbance was measured at 734 nm. The percentage of ABTS inhibition was calculated according to the following equation: Inhibition (%)=(A control-A sample)/A control x100; where A control and A sample refer to the absorbances of $ABTS^+$ solution without and with the extract, respectively. All tests were performed at least in triplicate. The percentage of inhibition at each concentration of the sample was plotted to generate the linear equation for using to calculate the half-maximal inhibitory concentration (IC_{50}) value.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition assay. Samples (CTPPs) dissolved in distilled water (50 μl) at different concentrations (1.5-10.5 mg/ml) were added to 150 μl DPPH reagent (100 μM; MilliporeSigma) and incubated in the dark for 30 min at room temperature. The absorbance was observed at 517 nm, and the inhibition of DPPH and IC_{50} value were calculated as described for the ABTS inhibition assay. Ascorbic acid was used as a positive control (24). All tests were performed in triplicate and results are expressed as the mean ± SD.

Cell lines. The MCF-7 BC cell line (product no. ATCC HTB-22) was obtained from the American Type Culture Collection, whereas the 293 control cell line were obtained from the Center of Excellence in Molecular Genetic and Human Disease, Department of Anatomy, Faculty of Medicine, Chulalongkorn University. The cell lines were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic. The four cell lines were cultured at 37˚C in a humidified atmosphere with 5% CO_2. The cells were passaged before being used in subsequent experiments (25).

Cell treatment. The MCF-7 BC cell line and the 293 control cell line were treated with various concentrations of CTPPs (0.3-1.6 mg/ml) at 37˚C for 72 h. The results were compared with untreated cells, which were considered the negative control group. Cisplatin (10-35 μM) was used as a positive control with the same treatment conditions.

MTT assays. The MTT assay was performed as previously described with minor modifications (25). Briefly, the cells ($5x10^3$ cells/well) were plated in 96-well plates with medium and incubated for 24 h at 37˚C in a 5% CO_2 incubator. The medium was aspirated and fresh complete medium was added containing different concentrations of CTPPs. The plates were incubated for 72 h at 37˚C in a 5% CO_2 incubator, after which, MTT reagent was added. After incubation at 37˚C for 2.5 h, the solution was removed, and DMSO was added and mixed for 5 min. The optical density values were read at 492 and 630 nm. The MTT assay was performed to assess cell viability and to determine the IC_{50} values, which were used to further analyze cell proliferation and apoptosis.

Cell proliferation assay. Cell proliferation was assessed using a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis. The Cell Proliferation ELISA, BrdU (colorimetric) assay was used according to the manufacturer's instructions. The cells ($5x10^3$ cells/well) were treated with CTPPs (IC_{50}) and incubated at 37˚C for 72 h. The results were compared to the untreated cells, which were used as a negative control.

Detection of apoptosis. Cells ($2x10^5$ cells/well) were seeded in a 6-well plate and were incubated for 24 h at 37˚C in a 5% CO_2 incubator. Subsequently, the medium was removed, and the cells were treated with CTPPs (IC_{50}) for 72 h at 37˚C and 5% CO_2. The treated and untreated samples were trypsinized and centrifuged at 250 x g for 5 min, after which the cell pellet was collected. The cells were rinsed with PBS and stained with Annexin V-DY-634 and PI according to the manufacturer's instructions, and flow cytometry (Beckman Coulter Dx Flex Flow Cytometer; Beckman Coulter, Inc.). Graphs showing the percentages of early apoptotic cells, late apoptotic cells and necrotic cells were generated using GraphPad Prism software, version 8 (Dotmatics).

Table I. Chemical properties and monosaccharide composition of crude *Trametes polyzona* polysaccharides.

Parameter	Value
Components, %	
Yield	15.19±1.54
Carbohydrate content	42.59±1.04
Protein content	37.85±1.92
Water solubility	96.11±1.62
Molecular weight, kDa	0.322,528.00
Monosaccharide composition, %	
Glucose	97.34
Arabinose	2.52
Mannose	0.15

Statistical analysis. All of the experiments were conducted in triplicate, and the data are presented as the mean ± SD and were analyzed using SPSS 29.0 (IBM Corp.). The statistical significance of differences among groups was analyzed using one-way analysis of variance, followed by Tukey's post hoc tests for cell proliferation and apoptosis assays. Two-group comparisons were performed using unpaired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics and structural analysis of CTPPs. The yield and chemical properties of CTPPs, including total carbohydrates, protein content, monosaccharide composition and molecular weight, are described in Table I. The obtained weight of CU07 mycelia after cultivation in potato dextrose broth for 14 days was 5.04 g/ml, and the yield of CTPPs extracted by hot water extraction was 0.15±0.02 g/g dry weight of mycelia. The total carbohydrate and protein contents were 42.59±1.04 and 37.85±1.92%, respectively. Glucose (97.34%) was the dominant sugar in the CTPP hydrolysate, followed by trace amounts of arabinose (2.52%) and mannose (0.15%) (Fig. S1). In the GPC analysis, two major peaks at 0.3 and 22,528 kDa were detected (Fig. S2). The FTIR spectrum of CTPPs is shown in Fig. 1, with typical peaks observed within the range of 3,700 to 500 cm^{-1}. The broad peak around 3,288.06 cm^{-1} was caused by the stretching of hydroxyl groups and the small peak in the region of 2,935.07 cm^{-1} was attributed to the stretching vibration of the C-H bond (8,26,27). The spectra peaks of CTPPs at 992.54, 1,025.37, 1,074.63 and 1,148.51 cm^{-1} were considered the-CO and -C-O-C-groups in a pyranose ring, which are the fingerprint of polysaccharides (10,28). A β-configurated peak at 869.41 cm^{-1} was also detected (29,30). In addition, the existence of protein was recognized by the peak at 1,632.83 cm^{-1}, which suggested the vibration of a carboxyl group in the amide I band. The peaks between 1,247.01 and 1,336.57 cm^{-1} were possibly due to the C-N amide III band of the protein structure (31,32).

Table II. IC_{50} (mg/ml) value of the scavenging activities of antioxidants in the ABTS and DPPH assays.

	IC_{50}, mg/ml	
Sample	ABTS	DPPH
CTPPs	1.89±0.06	11.81±0.17
Ascorbic acid	0.0318±0.0003	0.0490±0.0005

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); CTPPs, crude *Trametes polyzona* polysaccharides; DPPH, 2,2-diphenyl-1-picrylhydrazyl; IC_{50}, half-maximal inhibitory concentration.

Table III. Inhibitory effect of CTPPs on the MCF-7 breast cancer cell line and normal human cells (293).

	Cell line IC_{50}	
Sample	MCF-7	293
CTPPs, mg/ml	0.58±0.0260	-
Cisplatin, μM	18.75±0.8956	12.32±1.4460

Results are expressed as the mean ± SD of three independent experiments. CTPPs, crude *Trametes polyzona* polysaccharides; IC_{50}, half-maximal inhibitory concentration.

Antioxidant-scavenging activities. The percentages of inhibition in response to each concentration of CTPPs were plotted on a scatter graph (Fig. S3) to determine the IC_{50} values, which are presented in Table II. The CTPPs were able to inhibit both ABTS and DPPH radicals at IC_{50} values of 1.89 and 11.81 mg/ml, respectively. The results revealed that CTPPs had stronger antioxidant activity against the ABTS radical compared with that against the DPPH radical.

Inhibitory effects of CTPPs on BC cell viability and proliferation. The effect of each concentration of CTPPs on the viability of MCF-7 BC cells was determined by MTT assays (Fig. S4). As shown in Table III, CTPPs decreased the viability of MCF-7 cells compared with the untreated cells, with an IC_{50} value of 0.58 mg/ml. Moreover, the CTPPs did not show much of a cytotoxic effect on the viability of 293 cells; even at the maximum concentration (1.6 mg/ml), the percentage of cell viability was >80% (Fig. S4). By contrast, cisplatin was toxic to 293 cells (Table III; Fig. S4). The IC_{50} values of CTPPs on BC cell lines were used for further analysis of proliferation. The level of cell division was compared to that in the control (untreated) group, as determined using the BrdU incorporation assay. After 72 h of treatment with CTPPs (IC_{50}), the proliferation of MCF-7 cells was significantly reduced by ~22.59% (Fig. 2).

Apoptosis induction in cancer cell lines. Measuring the apoptosis of MCF-7 cells treated with CTPPs (IC_{50}) was performed by staining the cells with Annexin V-FITC and PI for flow cytometry. The representative flow cytometry dot plots of cells

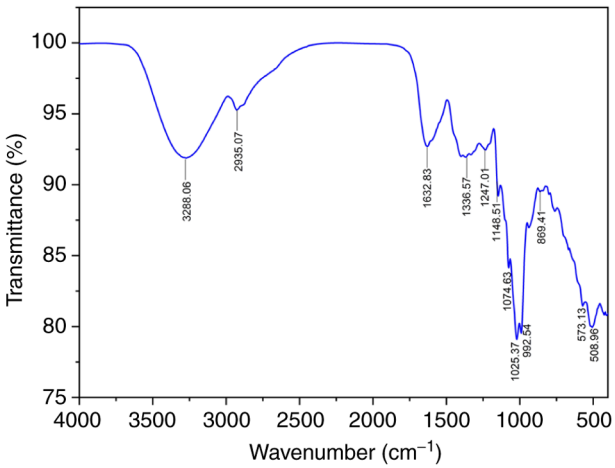

Figure 1. Fourier-transform infrared spectroscopy spectra of crude *Trametes polyzona* polysaccharides.

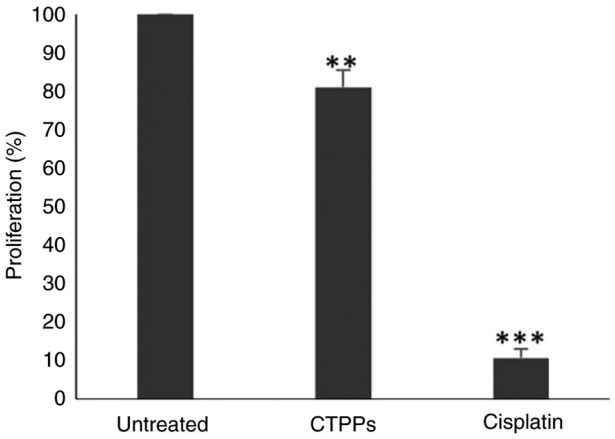

Figure 2. BrdU incorporation assay to determine the proliferation of MCF-7 cells. Cisplatin and CTPPs concentrations were 25 μM and 0.58 mg/ml, respectively. The results are expressed as the mean ± SD of three independent experiments. The differences between the groups were evaluated by one-way ANOVA and Tukey's post-hoc test. **P<0.01 and ***P<0.001 vs. untreated. CTPP, crude *Trametes polyzona* polysaccharides.

showed fluorescence intensities (Fig. 3A). The percentage of cells undergoing early and late apoptosis was analyzed and compared with the untreated control group (Fig. 3B). Treatment with CTPPs significantly accelerated early and late apoptosis in MCF-7 cells, and the levels of induction were 13.21 and 9.21%, respectively. CTPPs also induced late apoptosis at a higher level than cisplatin (5.90%), which was used as a positive control.

Discussion

Based on the FTIR spectrum and sugar analysis, it was revealed that the obtained CTPPs in this study mainly consisted of a β-glucan, since glucose was detected as the major constituent along with the presence of a β-configuration. In addition, CTPPs were revealed to be protein-bound polysaccharides, which consisted of two major components at different molecular weights. The total sugar and protein contents of these CTPPs were similar to those of PSP and PSK, commercial polysaccharides from *T. versicolor*, which are composed of

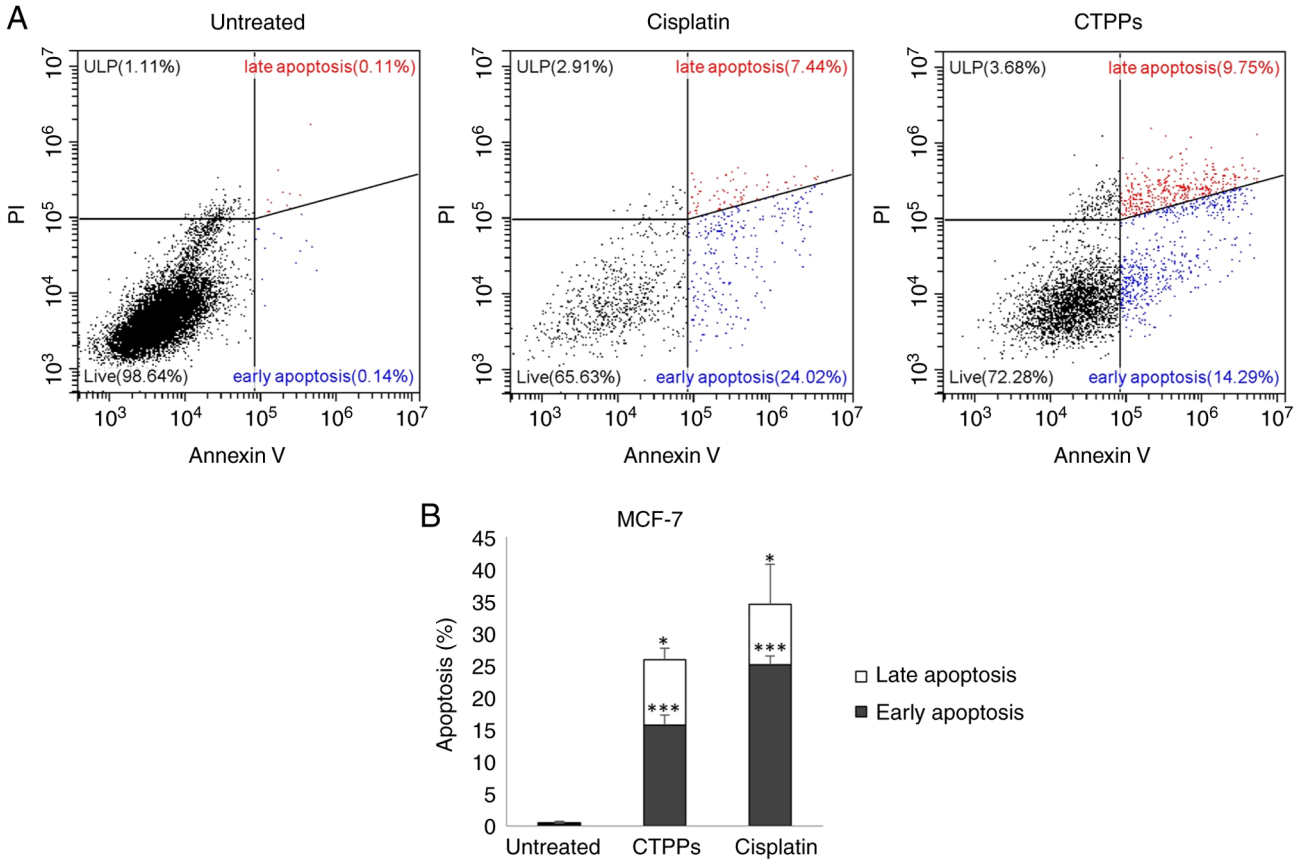

Figure 3. Analysis of apoptosis using the Annexin V FITC-PI analysis in MCF-7 cells. (A) Dot-plots of Annexin V and PI staining. (B) Bar graphs showing the average percentage of early and late apoptotic cells. Cisplatin and CTPPs concentrations were 25 μM and 0.58 mg/ml, respectively. The results are expressed as the mean ± SD of three independent experiments. The differences between the groups were evaluated by one-way ANOVA and Tukey's post-hoc test. *P<0.05 and ***P<0.001 vs. untreated. CTPP, crude *Trametes polyzona* polysaccharides.

soluble carbohydrates and proteins in the ranges of 34-46% (w/w) and 28-35% (w/w), respectively (9,12).

Although fungal polysaccharides are considered biological response modifiers, these polysaccharides possibly support cellular homeostasis by protecting cells from oxidative stress and free radicals through the innate antioxidant system (33,34). Therefore, the radical-scavenging activity of CTPPs was evaluated, and they were revealed to scavenge both radicals assessed, particularly ABTS. According to a previous report, the supply of hydrogen contributes to the bioactivities of polysaccharides (33). Meanwhile, as polysaccharide conjugates, the amino group of proteins and carboxyl group of polysaccharides are associated with radical-scavenging activity, and they are capable of proton-electron donation (35,36).

The IC_{50} value of CTPPs on ABTS was comparable to the antioxidant activity of some polysaccharides from white-rot fungi that are commercially referred to as medicinal mushrooms and is in the average IC_{50} range of CTPPs on ABTS reported in previous studies (23,37,38). Moreover, the IC_{50} value of CTPPs on ABTS was higher compared with that in some other *Trametes* species, for which ethanol was used as a solvent for polysaccharide extraction of >20 mg/ml (39). These findings indicated that using hot water extraction for polysaccharides could lead to an increase in the antioxidant properties of the compounds. The present study revealed that CTTPs may have potential antioxidant activities, particularly against the ABTS radical, although they were shown to be inferior to the ascorbic acid reference.

As determined using the MTT assay, CTPPs were revealed to affect the viability of MCF-7 cells. These results indicated that CTPPs could be considered in the treatment of various types of BC, which is comparable to several polysaccharides obtained from medicinal mushrooms of the *Trametes* species, as described in previous reports (14,40,41). Notably, CTPPs were revealed to be safer for normal cells than cisplatin, which is toxic to both cancerous and normal cells. Cisplatin has commercially been used as an antineoplastic drug with a broad spectrum for various types of cancer, including BC (42). In BC treatment, cisplatin has been shown to have anticancer effects on several types of BC cells. Thus, cisplatin has been chosen as a control in BC research (43-45). Furthermore, the 293 cell line was selected as a normal cell line due to the nephrotoxic evaluation of CTPPs. Recently, nephrotoxicity or renal toxicity of herbal medicines (HM) has been considered as one of the main toxicities in this type of treatment; notably, some HMs have been shown to cause kidney damage in patients (46).

The present study also assessed the effects of CTPPs on cell proliferation. It was shown that CTPPs suppressed the proliferation of MCF-7 cells, which exhibited >20% inhibition compared with untreated cells. Taken together, the present results indicated that CTPPs had cytotoxic activity against the viability of MCF-7 cells. The cytotoxic effects of CTPPs were probably responsible

for suppressing of the proliferating cells, as a recent report demonstrated that cytotoxic drugs could disturb the process of cell division (47). Even though the results on cell proliferation were inferior compared with those in the positive control group, this was a beneficial result in terms of cancer treatment, because cell proliferation is a critical process of cell division. In cancer development, the targeting of cell proliferation is important due to the features of cancer progression and tumorigenesis (48-50).

Another targeted feature of cancer cells is apoptosis, a program cell death process that have therapeutic potential for cancer therapy (51). Notably, CTPPs induced early and late apoptosis in MCF-7 cells. It has been indicated that CTPPs may be involved in the expression of various apoptosis-regulating genes, including pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) genes, or in the activation of caspase-mediated apoptosis (caspase-3, -8 and -9), through intrinsic or extrinsic pathways (52,53). Moreover, CTPPs could be associated with tumor suppressor signaling pathways, such as p53, through which they may induce apoptosis and suppress proliferation (54). A recent report found that a *Trametes* polysaccharide-peptide could promote cell apoptosis through epidermal growth factor receptor (EGFR) pathways by suppressing the expression of EGFRs and the level of programmed cell death-ligand 1 genes (55).

In patients with BC, the treatment is limited, and the current gold standard is chemotherapy. However, these treatments are associated with side effects in patients (56). Therefore, the findings of the present study showed that CTPPs may be helpful as an alternative adjuvant for cancer treatment. In addition, further evaluation of gene expression related to the signalling pathway of apoptosis is strongly suggested in order to reveal the downstream mechanism of CTPPs for further investigation on BC cells.

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Availability of data and materials

The datasets generated and/or analyzed during the study are available from the corresponding author on reasonable request.

Authors' contributions

SP, PY and WB were involved in study conceptualization. BK, SP, PY and WB confirmed the authenticity of all the raw data and designed the methodology. BK performed experiments and wrote the original draft. SP, PY, and WB reviewed and edited the manuscript. SP and PY acquired the funding and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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