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In vitro antioxidant and anticancer activities of *Smilax corbularia* extract combined with *Phellinus linteus* extract against breast cancer cell lines

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Abstract. Treatment with extracts from whole herbs has been reported to synergistically enhance the anticancer activities of therapeutic agents in recent studies. The present study evaluated the antioxidant and anticancer activities of *Smilax corbularia Kunth* (*S. corbularia*) and *Phellinus linteus* (*P. linteus*) crude extracts individually and in combination. *S. corbularia* was extracted using ethanol, whereas *P. linteus* was extracted using hot water. Both crude extracts underwent physiochemical characterization. Subsequently, the possible antioxidant activities of both crude extracts, individually and in combination, were evaluated using 2,2-Diphenyl-1-picrylhydrazyl and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assays. Their effects on breast cancer cell cytotoxicity, proliferation and apoptosis were then assessed. The crude *S. corbularia* extract obtained was found to have a high level of total phenolic content, whilst the crude *P. linteus* extract had high levels of total polysaccharide content. The total phenolic content and total polysaccharide content results of the combinations depended on the respective ratios of the individual extracts. *S. corbularia* alone and combination 3 (which contained 75% *S. corbularia*: 25% *P. linteus*) demonstrated the greatest radical scavenging activity, followed by combination 1 (50% *S. corbularia*: 50% *P. linteus*), combination 2 (25% *S. corbularia*: 75% *P. linteus*) and *P. linteus*. The toxicity results of the extract samples on the cancer cells corresponded with their antioxidant activity. In particular, certain combinations demonstrated clearer inhibitory effects on cell proliferation against three types of breast cancer cells compared with those exerted by the two individual extracts. However, induction of apoptosis was limited, with the degree of apoptosis observed to be <5%. These findings suggested that treatment with combinations of these two extracts could confer enhanced antioxidant and antiproliferative effects on breast cancer cells. Therefore, the potential of these two extracts in combination as anticancer agents warrants further investigation.

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Introduction

In addition to genetic risk factors, unhealthy lifestyles have been implicated in the development of cancer. The adverse effects of environmental conditions, such as free radical exposure, can disrupt biological homeostasis in normal cells and induce oxidative stress (1). This type of oxidative stress has been reported to be involved in the pathogenesis of numerous diseases, including cancer. Cancer cell proliferation and development can be triggered by cellular stress (1,2). Cancer is a complex disease that can be associated with other illnesses such as cardiovascular disease and type II diabetic, which increases mortality rates worldwide. Among females, breast cancer is a particularly common diagnosed cancer with ~19.3 million new cases in 2020, with an increased prevalence worldwide (3,4).

Breast cancer is a heterogeneous and complex disease. Classifications of breast cancer have been developed according to the histological type, tumor grade, lymph node status and the expression profiles of certain proteins, such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (5). MCF-7 is a breast cancer cell line that has been previously used as an *in vitro* model for hormonal evaluation of estrogen-dependent breast cancer, in which PR expression is progesterone-dependent and which is responsive to therapeutic processes. By contrast, MDA-MB-231 and MDA-MB-468 cells are typically used as *in vitro* metastatic breast cancer models, which are characterized by the absence of ER, PR and HER2 expression, which result in higher degrees of proliferation and resistance to therapy (5-7). In addition,

MDA-MB-231 expresses a mutant p53, which makes it a particularly aggressive cancer cell line (8).

Current therapeutic approaches for breast cancer, such as radiotherapy and chemotherapy, also induce severe adverse side effects due to non-selective cell death and drug resistance. Herbal medicine has been assessed as a potential therapeutic treatment and intensive investigations of the reported efficacy, safety and cost-effectiveness is ongoing (9,10). Previous studies have reported characterization of the profiles of bioactive compounds that can provide antioxidant and anticancer potential contained within naturally-occurring plants and fungi (9,10).

S. corbularia is a medicinal plant in Thailand, from the *Smilacaceae* family. The rhizomes of this plant are used in traditional medicine and it has been previously reported to contain several recognized compounds (11-13). Specifically, *S. Glabra* and *S. corbularia* are reported to be enriched with phenolic and flavonoid chemicals, such as catecin, astilbin, isoastilbin, taxifolin and smiglasides. They are bioactive constituents that contribute antioxidant, anti-inflammatory, antibacterial and anticancer properties (11-13). Moreover, *P. linteus* is a well-known medicinal mushroom that contains a number of bioactive compounds, including polyphenols, terpenoids, furans and polysaccharides. Previous studies have reported that extracts from fruiting bodies or cultured mycelium can exhibit numerous bioactivities, such as anticancer, anti-inflammation, immunomodulatory, antioxidant and antidiabetic effects (14-18). Therefore, *Smilax corbularia* and *Phellinus linteus* were the plant and fungi of interest in this study.

The consumption of whole foods and dietary variety has been proposed to lower the risk of developing chronic diseases due to the numerous compounds reported to be contained within the foods and their corresponding associated health benefits. There have been reports that consuming whole herbs (from plants or fungi, either in their natural or extracted forms) may exert greater effects compared to consuming any active single compound alone (19,20). Therefore, natural polysaccharides in combination with phenolic compounds are expected to confer more potent biological activities via physical or chemical approaches due to their proposed cooperative therapeutic effects. A previous study reported that the combination of polysaccharides and phenol compounds from different jujube plant polysaccharides with 6-gingerol demonstrated considerable synergy in antioxidant and antitumor activities (21).

Although the bioactivities of the individual crude extracts of *S. corbularia* and *P. linteus* have been previously reported, the effects of them in combination remain poorly understood. To assess the bioactivities of their co-administration, the present study investigated their possible antioxidant activities using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays after extraction. For extraction, ethanol extracts of *S. corbularia* were obtained and hot water extracts were obtained from *P. linteus*. Both of the extracts were tested either individually or in combination at three different ratios, namely combination 1 (50% *S. corbularia*: 50% *P. linteus*), combination 2 (25% *S. corbularia*: 75% *P. linteus*) and combination 3 (75% *S. corbularia*: 25% *P. linteus*). Subsequently, their effects on three breast cancer cell lines, MCF-7, MDA-MB-231 and MDA-MB-468, were evaluated using the MTT assays and BrdU incorporation for cell proliferation and flow cytometry for cell apoptosis.

Materials and methods

Material and chemicals. S. corbularia (rhizome) and *P. linteus* (fruiting body) powder were purchased from Nature Herb International Holding Co., Ltd. and Nature Herbs International Holding Co., Ltd. The breast cancer MCF-7 (cat. no. ATC. HTB-22) and MDA-MB-231 (cat. no. ATC.HTB-26) cell lines were purchased from The American Type Culture Collection. The breast cancer MDA-MB-468 cell line and the 293 cell line were donated by the Center of Excellence in Molecular Genetics of Cancer and Human Disease (Chulalongkorn University, Thailand). Ethanol, methanol, DMSO, and other chemicals used were of analytical grade.

Extraction procedure. The powdered samples of *S. corbularia* and *P. linteus* were extracted by reflux method using a Soxhlet cellulose filter (Cytiva). The powdered samples were refluxed with 95% ethanol for *S. corbularia* and distilled water for *P. linteus* for 6 h, with a solid-to-liquid ratio of 1:60 (w/v) (22,23). The extraction products were evaporated in a rotary evaporator for ethanol extraction or using the lyophilizing method for water extraction. The yield of each crude extract was calculated and the extracts were stored in the refrigerator at -20°C for further use.

Extracted sample preparations. The determination of total polysaccharide content, total phenolic content, antioxidant and anticancer activities was performed using five extracted samples including three combinations. These were as follows: Crude extract of *S. corbularia*, crude extract of *P. linteus*, combination 1 (50% *S. corbularia*: 50% *P. linteus*), combination 2 (25% *S. corbularia*: 75% *P. linteus*) and combination 3 (75% *S. corbularia:* 25% *P. linteus*). Initially, the dried *S. corbularia* crude extract was dissolved in DMSO (Sigma-Aldrich; Merck KGaA) and *P. linteus* was dissolved in distilled water to generate the stock solutions. Each stock was prepared at a concentration of 500 mg/ml. The extracted samples were then mixed to generate the aforementioned solutions. For generation of the combinations, the individual extracts were prepared from the stock extracts at the same concentration. The combinations were then mixed and shaken vigorously to obtain the required combinations at their respective percentages.

For cell treatments (cytotoxic, cell proliferation and apoptosis assays), extracted sample stocks were freshly diluted in serial concentrations with complete DMEM [DMEM mixed with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic-antimycotic 100X (which contained penicillin, streptomycin and Amphotericin B; Gibco; Thermo Fisher Scientific, Inc.) before use. The final treatment medium contained ~0.1% DMSO. The negative control for anticancer treatment was 0.1% DMSO (untreated cells) and the positive control was cells treated with 25 μM cisplatin (Glentham Life Sciences Ltd.). All assays were performed in triplicate.

Determination of total polysaccharide content. The sample extracts were dissolved in distilled water to the appropriate concentrations. In the present study, the concentrations used were as follows: 0.2 mg/ml *S. corbularia*, 0.1 mg/ml *P. linteus* and 0.2 mg/ml for each when in combination. In total, 250 μl of each sample was put into a reaction tube containing 1.25 ml of 1 mg/ml cold anthrone (Sigma-Aldrich; Merck KGaA)

solution in concentrated sulphuric acid, which was then incubated at 4°C for 10 min. Afterwards, the mixture was heated in boiling water for 10 min. The absorbance of this solution was then measured using a spectrophotometer (Thermo Fisher Scientific, Inc.) at 630 nm after cooling to room temperature. The total polysaccharide content was calculated by comparison with a standard curve for glucose (24).

Determination of total phenolic content. The total phenolic content was determined using the Follin-Ciocalteu (FC) reagent (Loba Chemie Pvt. Ltd., India) method. In total, 25 μl sample extracts were dissolved in distilled water to the appropriate concentrations (0.4 mg/ml S. corbularia, 10 mg/ml P. linteus, 0.4 mg/ml combination 1, 0.8 mg/ml combination 2 and 0.4 combination 3; the combinations contained different ratios of the two extracts from stock solutions and were used at different concentration) and were mixed with 25 μl diluted FC solution [ratio of FC-water was 1:3 (v/v)], which was added to 200 μl distilled water in 96-well plates. This mixture was then incubated for 5 min at room temperature. Subsequently, 25 μl 10% sodium carbonate (w/v) was added and incubated for 60 min in the dark. The measurement of absorbance was performed at 765 nm using a spectrophotometer (Thermo Fisher Scientific, Inc.). Total phenolic content was expressed as mg gallic acid equivalent (GAE) per g defatted crude extract (mg GAE/g dried crude extract) (25).

Preliminary structural characterization of P. linteus extract. The crude polysaccharide of *P. linteus* was hydrolyzed using H_2SO_4 to break it down into individual monosaccharides. Then, the type and content of sugar in the hydrolyzed sample (10 μl) were assessed using High-Performance Liquid Chromatography (HPLC; Prominence Modular; Shimadzu Corporation) with a refractive index detector and sugar SH1011 column (8.0x300 mm; Shodex; Resonac Corporation). The analysis condition was performed at 40°C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min. The sugar content was quantified for the standard sugars (1, 5 and 10% w/v; xylose, glucose, and arabinose; Millipore Sigma) and the data were analyzed using Lab Solutions software (Shimadzu Corporation) (26). The structure of *P. linteus* extract was analyzed by Fourier Transform Infrared (FTIR) Spectrophotometer (Nicolet 6700; Thermo Nicolet Corp.) in the range of 4000-500 cm^{-1} using KBr that was mixed with the sample at a ratio of 10:1 (by weight). The spectral resolution was set at 4 cm^{-1} with the number of scans at 50. The molecular weights (M_ws) of crude polysaccharide (10 μl) was determined by Gel Permeation Chromatography (GPC; LC-10ADVP; Shimadzu Corporation) using a Shodex PLgel column (7.5x300 mm; Shodex; Resonac Corporation) and a refractive index detector that was operated at 40°C and eluted with distilled water at a flow rate of 0.5 ml/min. The sample was dissolved in distilled water at a concentration of 0.1% (w/v) and pullulans (MilliporeSigma) were used as polysaccharide standards for calibration (27). The data were analyzed using Lab Solutions software (Shimadzu Corporation).

DPPH radical scavenging activity. Serial dilutions of sample extracts were mixed with 0.1 mM DPPH (Sigma-Aldrich; Merck KGaA) in methanol solution and incubated in the dark for 30 min at room temperature. The decrease in the absorbance of the samples compared with 0.1 mM DPPH in methanol was then measured at 517 nm using a spectrophotometer (28). The half-maximal inhibitory concentration (IC_{50}) was calculated as the amount of antioxidant required to decrease the initial DPPH concentration to 50%. The blank was designated as sample solution without 0.1 mM DPPH, whereas the negative control was the 0.1 mM DPPH solution and the positive control was ascorbic acid in 0.1 mM DPPH solution.

ABTS radical scavenging activity. The radical cation of $ABTS^+$ was prepared by mixing 7 mM ABTS (Sigma-Aldrich; Merck KGaA) solution with 2.45 mM potassium persulfate at a 1:1 ratio (v/v) before leaving the mixture overnight until stable absorbance was obtained. The $ABTS^+$ solution was diluted with distilled water to an absorbance of 0.700±0.05 at 734 nm (29). The photometric assay was performed by mixing $ABTS^+$ solution with serially diluted aqueous solution sample extracts and incubating for 10 min in the dark at 27°C. The absorbance of the mixture was then measured at 734 nm using a spectrophotometer. The blank solvent was the sample solution without $ABTS^+$, negative control was the $ABTS^+$ solution after dilution, whereas the positive control was ascorbic acid with the $ABTS^+$ solution.

Cell lines culture and preparation. In the present study, three breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-468 and the normal 293 cell line were utilized. All cell lines were cultured in high glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic-antimycotic 100X (which contained penicillin, streptomycin and Amphotericin B; Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured and incubated at 37°C with 5% CO_2. Cells were used when the confluence of the culture reached 70-80% (30).

Cytotoxicity assay. The cytotoxicity of the extracted samples was quantified using the MTT assay (Abcam). In total, $5x10^3$ cells/well were cultured in 96-well plates for 24 h at 37°C with 5% CO_2, before fresh medium containing serial concentrations of the sample extracts were added. After 72 h of incubation at 37°C with 5% CO_2, the extract medium was removed and MTT 5 mg/ml dissolved in fresh media was added into each well, which was then incubated for 3 h at 37°C. This solution was then removed and DMSO was added for incubation for 5 min at 27°C. The absorbance was measured using a spectrophotometer at wavelengths of 492 with a reference wavelength of 630 nm use for all samples (30). The absorbance data were calculated by subtracting the 492 nm result from the 630 nm result, which was subsequently provided as % viability compared with the untreated control. The IC_{50} was reported as the concentration of the extracts that inhibited cell proliferation by 50%. IC_{50} was determined using curves constructed by plotting cell survival (%) against the concentration of extracted samples (31,32). The results of MTT assay (IC_{50}-dependent manner) were used for further assays for cell proliferation and apoptosis.

Cell proliferation assay. A BrdU (5-bromo-2'-deoxyuridine) cell proliferation ELISA kit was used for measuring the proliferation

Table I. Yield and chemical properties of *Smilax corbularia Kunth*, *Phellinus linteus* and combinations.

Sample	Yield, g/g	Total phenolic content, mg GAE/g dry weight	Total polysaccharide content, mg/mg glucose standard
S. corbularia	0.18±0.02	184.41±5.92***	0.25±0.02***
P. linteus	0.53±0.05	4.80±0.40	0.89±0.01
Combination 1		97.55±7.21***	0.60±0.04***
Combination 2		55.60±3.36***	0.82±0.06
Combination 3		134.64±2.06***	0.42±0.02***

All data were presented as the mean ± standard deviation of three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. ***P<0.001 vs. *P. linteus*. GAE, gallic acid equivalent.

rate according to the manufacturer's protocol (Roche Diagnostics GmbH). In a 96-well culture plate, cells were seeded at a density of $5x10^3$ cells/well for 24 h at 37˚C with 5% CO_2. A sample extracts were then added and incubated for 72 h at 37˚C with 5% CO_2, before the cells were cultured for another 24 h with a BrdU working solution at 37˚C with 5% CO_2. The supernatant was removed and the Fixative/Denaturing Solution from the kit was added to fix and denature the cells for 30 min at room temperature. An anti-BrdU antibody solution was added and incubated for 90 min at 25˚C. After washing the unbound primary antibody with PBS, the secondary antibody solution from the BrdU kit was added and incubated for 30 min at 25˚C. Finally, a substrate solution was added and incubated in the dark for 20 min. The amount of incorporated BrdU was measured using an ELISA plate reader at 370 nm (Thermo Fisher Scientific, Inc.).

Apoptosis assay. The apoptosis assay was performed using an Annexin V-DY-634/PI staining/detection protocol kit (Abcam). The cell lines at $2x10^5$ cells/well were cultured and incubated in six-well plates for 24 h at 37˚C with 5% CO_2. Cells were then treated with sample extracts for 72 h at 37˚C with 5% CO_2. The treated and untreated cells were then trypsinized (0.25% Trypsin-EDTA 1X; Gibco; Thermo Fisher Scientific, Inc.), washed once in PBS and centrifuged at 605 x g for 3 min at room temperature (27˚C). To evaluate the extent of apoptosis, the collected cells were re-suspended in the Annexin V binding buffer, before Annexin V-DY-634 and propidium iodide were added and incubated at room temperature (27˚C) for 15 min in the dark. The apoptotic cells were analyzed using flow cytometry (Beckman Coulter Dx Flex Flow Cytometer; Beckman Coulter, Inc.).

Statistical analysis. All treatments were performed in triplicate and the data were presented as the mean ± standard deviation. The statistical significance of differences among groups was analyzed using one-way analysis of variance using SPSS 28.0 (IBM Corp.), post-hoc tests of significant differences were performed using Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Physiochemical characterization. The yield of the crude extract from the raw materials was higher (~53%) for *P. linteus* compared with *S. corbularia* (~18%) (Table I). Analysis of the chemical properties of the extracted samples using spectroscopy demonstrated that the total phenolic content was significantly different P<0.001 other samples vs. *P. linteus*. Specifically, *S. corbularia* had the highest total phenolic content (~18.44% of dry weight), followed by combinations 3 (~13.46% of dry weight), combination 1 (~9.76% of dry weight), combination 2 (~5.56% of dry weight) and *P. linteus* which had a small total phenolic content (~0.48% of dry weight). Total polysaccharide content also demonstrated significant differences for several samples such as *S. corbularia*, combination 1 and combination 3 compared with *P. linteus* (P<0.001). There was no significant difference between combination 2 and *P. linteus*. *P. linteus* had the highest value of ~89% (w/w), followed by combination 2 (~82% w/w), combination 1 (~60% w/w), combination 3 (~42% w/w) and *S. corbularia* (~25% w/w).

Table II. Monosaccharide composition of crude polysaccharide extracted from *Phellinus linteus*.

	Monosaccharide composition (% w/v)		
P. linteus	Glucose	Arabinose	Other monosaccharides
	96.42	1.68	1.89

w/v, mg/ml.

Preliminary structural characterization of P. linteus. The results of the monosaccharide composition analysis were presented in Table II. Based on the monosaccharide standards, it was demonstrated that the crude polysaccharide of *P. linteus* is a heteropolysaccharide that is comprised of glucose, arabinose and other monosaccharides. The FTIR spectra for the *P. linteus* crude polysaccharide at wavenumbers between 500 and 4000 cm^{-1} were presented in Fig. 1. The profile of this crude polysaccharide demonstrated a wide stretching peak at ~3314.35 cm^{-1} for the O-H stretching vibration and a weak stretching peak at ~2914.00 cm^{-1}, for the C-H stretching of CH_2 groups (33,34). Absorption at 1637.01 cm^{-1} indicated the presence of the COO^- deprotonated carboxylic group, suggesting the presence of uronic acid. Furthermore, a peak at ~1411.15 cm^{-1} indicated the -OH group of phenol (35,36).

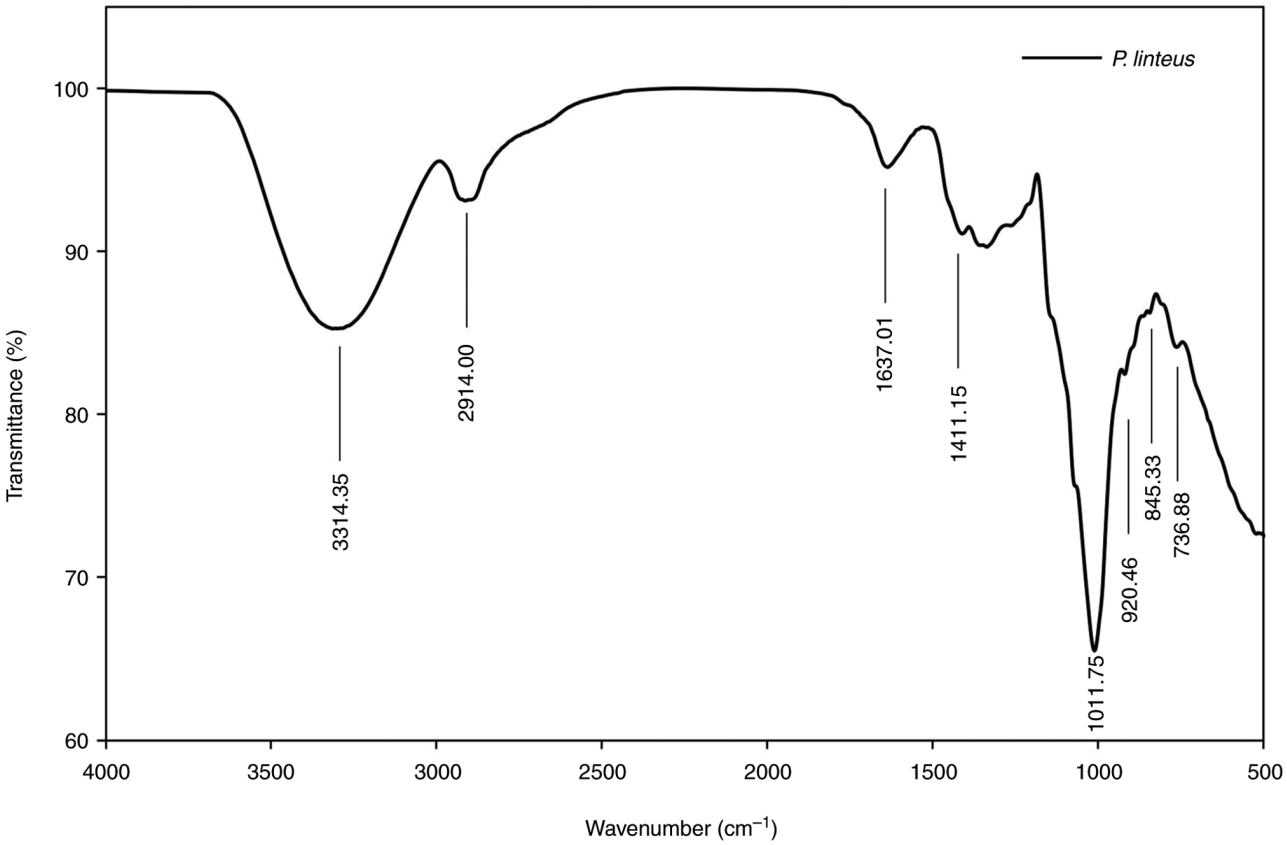

Figure 1. Fourier-transform infrared spectroscopy spectra of crude polysaccharide of *Phellinus linteus*.

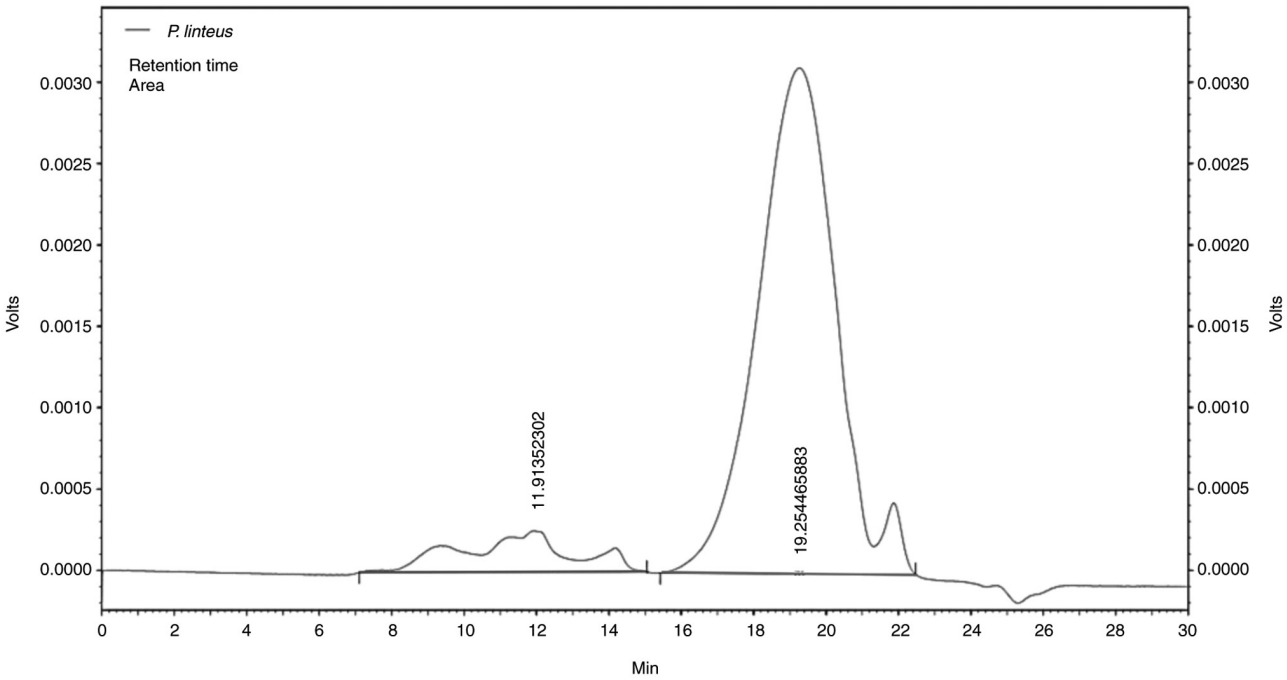

Figure 2. Gel permeation chromatography chromatogram of crude polysaccharide of *Phellinus linteus*.

A potent band at ~1011.75 cm^{-1} indicated stretching vibration of the pyranose ring. A peak at 900 cm^{-1} (920.46 cm^{-1}) indicated β-configuration of the sugar unit. Weak bands at ~845.33 and ~763.88 cm^{-1} indicated β-D-glucosidic linkages (33,35,36). GPC was used to determine the M_ws of the crude polysaccharide extracted from *P. linteus*. This crude polysaccharide demonstrated two main peaks with retention times of 11.913 and 19.254 min (Fig. 2), with average M_ws of

Table III. Antioxidant activities of *Smilax corbularia Kunth*, *Phellinus linteus* and combinations scavenging ability assessed using DPPH and ABTS assays.

Sample	IC_{50} concentration (μg/ml)	
	DPPH	$ABTS^+$
S. corbularia	54.63±0.70	55.37±1.90
P. linteus	2469.00±91.72***	1939.46±49.95***
Combination 1	104.50±2.82	105.46±1.60
Combination 2	258.60±6.99***	179.67±17.81***
Combination 3	73.55±1.63	66.11±4.05
Ascorbic acid (positive control)	35.59±0.55	25.61±0.32

All data were presented as the mean ± standard deviation of three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. ***P<0.001 vs. *S. corbularia*.

2,625.41 and 2.416 kDa, respectively. The second peak was dominant according to its height, which suggested that the majority of the *P. linteus* polysaccharide was at the lower molecular weight.

Antioxidant activity of the extracted samples on free radical reagents. The scavenging activity of all samples on DPPH and ABTS are presented in Table III. DPPH results demonstrated no significant difference among *S. corbularia*, combination 3 and combination 1. However, these three extracted samples appeared to possess a significantly higher radical scavenging activity compared with combination 2 or *P. linteus* (P<0.001). Among all the extracted samples, *S. corbularia*, combination 3 and combination 1 also demonstrated significantly higher ABTS radical scavenging activity compared with that of combination 2 or *P. linteus* in terms of the IC_{50} results (P<0.001). According to the DPPH and ABTS IC_{50} values, the scavenging activities of the samples could be ranked as strong to weak as follows: *S. corbularia*, combination 3, combination 1, combination 2 and *P. linteus*.

Cytotoxic effect of the extracted samples on breast cancer cell lines. *S. corbularia* and combination 3 demonstrated the highest inhibition of cell viability and showed significant differences (*S. corbularia* or combination 3 vs. *P. linteus*, P<0.001) in IC_{50} values for MCF-7 after 72 h of treatment (Table IV). *S. corbularia* also demonstrated a significant difference when compared with combination 1 (P<0.01 combination 1 vs. *S. corbularia*) and combination 2 (P<0.001 combination 2 vs. *S. corbularia*). Similar to MCF-7, *S. corbularia* and combination 3 inhibited the viability of MDA-MB-468 cells at lower concentrations compared with those of other extracted samples. All extracted samples conferred significantly different cell viability results (P<0.001) on the MDA-MB-231 cell line, where *S. corbularia* exerted the highest inhibitory effects compared with other samples. The IC_{50} values against all cancer cell lines demonstrated the same trend when ranked from strongest to weakest inhibition, as follows: *S. corbularia*, combination 3, combination 1, combination 2 and *P. linteus*. Moreover, all extracted samples demonstrated strong cytotoxic effects on MCF-7, but the cytotoxic effects on MDA-MB-231 were weaker. However, 293 cells demonstrated reduced cytotoxicity compared with MCF-7 cells based on IC_{50} data. Apart from *P. linteus*, the majority of samples also demonstrated less cytotoxicity 293 cells compared with MDA-MB-468 cells. However, the toxicity of all samples in 293 cells increased as the concentration increased, as did the results of *P. linteus* on MDA-MB-468 cells and all combinations against MDA-MB-231 cells.

Antiproliferative effect of the extracted samples on breast cancer cell lines. In the present study, BrdU incorporation reagent was added after the treatment of each cell line with samples for 72 h. The results demonstrated the cell proliferation percentage after 24 h of incubation with BrdU. Individual extracts of *S. corbularia* and *P. linteus*, as well as their combinations, significantly inhibited cell proliferation in the MCF-7 cell line (combination 1 vs. untreated, P<0.01; *S. corbularia*, *P. linteus*, combination 2 or combination 3 vs. untreated, P<0.001). The proportion of proliferative cells decreased when treated with the IC_{50} value of each sample extract. MCF-7 (70.96±1.65%) and MDA-MB-231 (23.17±2.52%) results demonstrated that treatment with combination 1 resulted in the lowest extent of cell proliferation compared with that demonstrated by other samples (Fig. 3). However, only combination 1 was significantly different when compared with the untreated group in MDA-MB-231 cells among all of the extracted samples (combination 1 vs. untreated, P<0.001). *S. corbularia* and combination 2 demonstrated the greatest inhibitory effects on cell proliferation by MDA-MB-468 cells. In addition, all extracted samples conferred clear antiproliferative activity on MDA-MB-468 cells as demonstrated by the proliferation of each extract treated sample being <70% (*S. corbularia*, 42.00±1.31%; *P. linteus*, 65.29±3.12%; combination 1, 51.91±1.50%; combination 2, 41.63±1.40%; combination 3, 55.36±1.95%) compared with the untreated control (P<0.001 all extracted samples vs. untreated).

Induction of apoptosis by the extracted samples in breast cancer cell lines. The percentage of late apoptotic cells was increased in MCF-7 cells after treatment with IC_{50} values of *P. linteus* (3.64±0.21%, *P. linteus* vs. untreated, P<0.01) and combination 1 (2.99±1.97%, combination 1 vs. untreated, P<0.05) after 72 h of incubation compared with that in untreated cells (0.20±0.22%). For early apoptosis, *P. linteus* (4.17±0.34%) and combination 1 (1.64±2.28%) demonstrated higher percentage values; however, only *P. linteus* treatment resulted in a significant difference (*P. linteus* vs. untreated, P<0.01) (Fig. 4A and B). The late apoptosis results in MDA-MB-231 cells demonstrated that combination 1 (0.96±0.10%, combination 1 vs. untreated, P<0.001) and combination 3 (0.39±0.13%, combination 3 vs. untreated, P<0.01) had the highest values compared with those in untreated cells (0.03±0.03%; Fig. 4A and C). Combination 1 and 2 presented significantly different results for early apoptosis (combination 1 or combination 2 vs. untreated, P<0.05), with values of 0.58±0.07% (combination 1) and 0.61±0.13% (combination 2), compared to 0.16±0.10% in untreated cells. None of the

Table IV. Inhibitory effects of *Smilax corbularia Kunth*, *Phellinus linteus* and combinations on the viability of certain cell lines.

	IC_{50} concentration (μg/ml)			
Sample	MCF-7	MDA-MB-231	MDA-MB-468	293
S. corbularia	71.02±3.04	218.81±5.25	170.36±7.43	230.03±71.54
P. linteus	405.96±35.77***	1388.59±29.14***	2022.51±67.24***	2029.84±570.04
Combination 1	137.18±4.20**	713.61±5.83***	329.24±13.25**	802.69±20.89
Combination 2	166.04±9.79***	852.97±21.68***	698.51±35.11***	825.74±113.28
Combination 3	96.81±11.72	342.48±17.91***	200.14±1.18	347.40±46.65
Cisplatin, μM (positive control)	16.92±0.61	23.54±2.96	21.00±2.62	13.26±0.71

All data were presented as the mean ± standard deviation of three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. **P<0.01 and ***P<0.001 vs. *S. corbularia*.

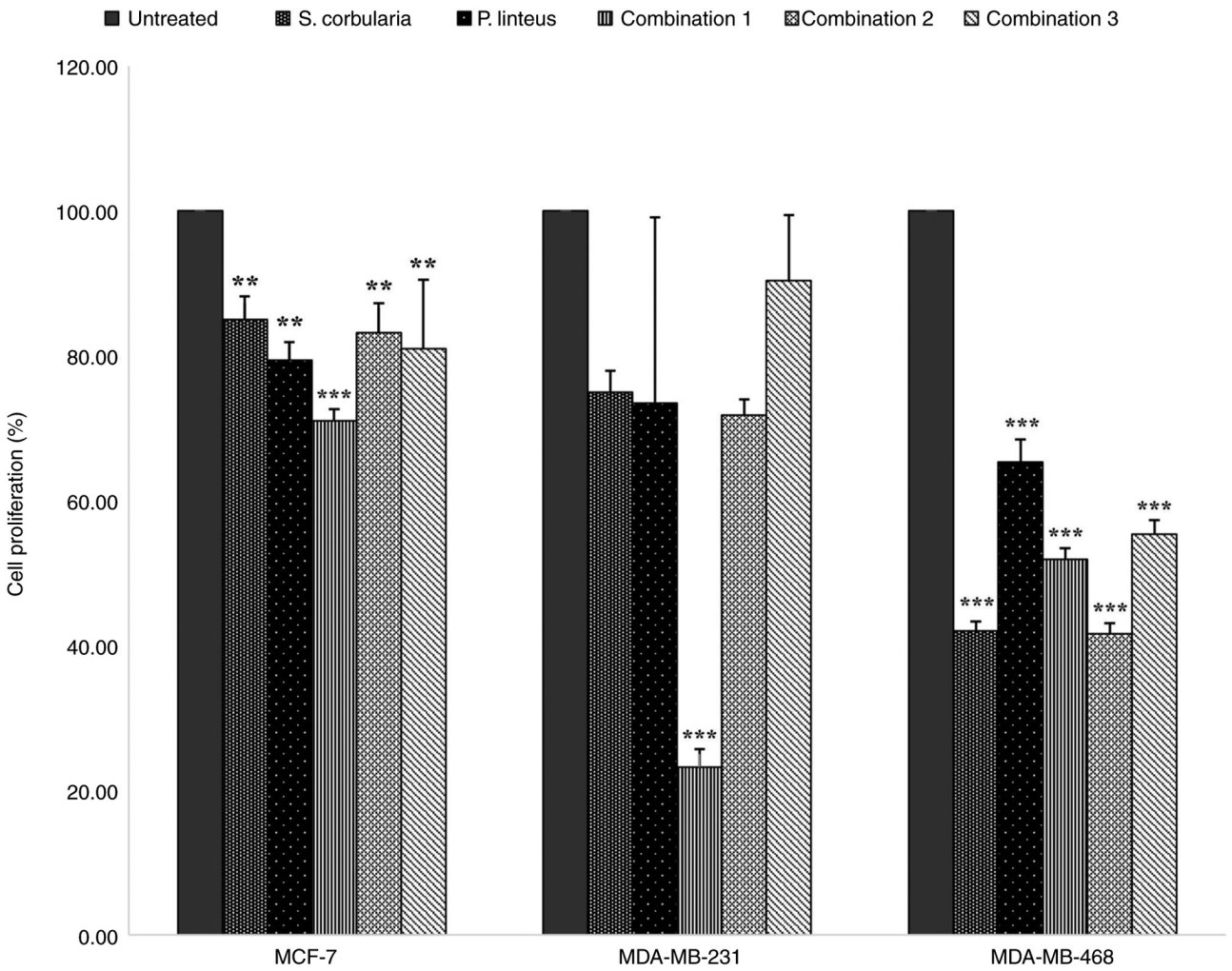

Figure 3. The antiproliferative effects of *Smilax corbularia Kunth* extract, *Phellinus linteus* extract and combinations on three distinct breast cancer cell lines. Percentage cell proliferation was presented as the mean ± standard deviation of three replicates for each sample of IC_{50}-treated cells (one-way ANOVA with Tukey's post-hoc test). **P<0.01 and ***P<0.001 vs. the untreated group.

extracted samples were particularly effective at inducing apoptosis in MDA-MB-468 cells, as demonstrated by the lack of significant difference in either late apoptosis (P=0.127) or early apoptosis (P=0.224) between any of the extracted samples compared with untreated cells (Fig. 4A and D).

Discussion

In the present study, the results demonstrated that the percentage yield of the extracts in the two samples varied. It has been previously reported that the overall yield of extraction,

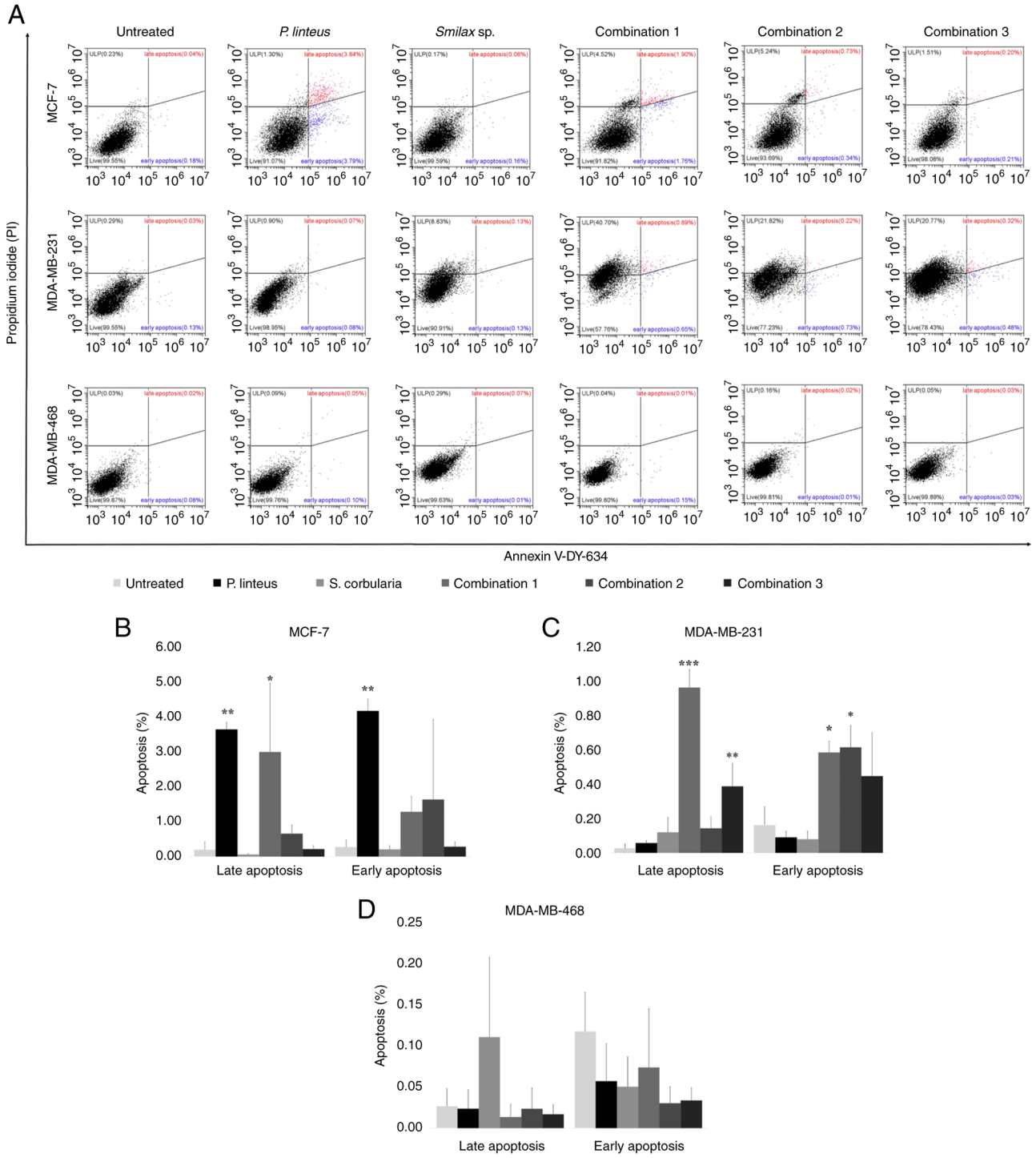

Figure 4. The apoptotic effect of *Smilax corbularia Kunth*, *Phellinus linteus*, and combinations on three distinct breast cancer cell lines. (A) Representative flow cytometry dot plots of the three replicates of 72 h treatment with *S. corbularia*, *P. linteus* and combinations on three distinct breast cancer cell lines using co-stained Annexin V-DY-634 and propidium iodide, the percentage in each dot plot quadrant was generated by determination of the single cell population. Proportion of apoptotic cells for (B) MCF-7, (C) MDA-MB-231 and (D) MDA-MB-468. The proportion of cells in late apoptosis and early apoptosis was presented as mean ± standard deviation of three replicates for each sample of IC_{50}-treated cells (one-way ANOVA with Tukey's post-hoc test). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. the untreated group. ULP, unlabeled part.

chemical characteristics and bioactive compounds can be effected by a variety of parameters, such as solvent type, extraction procedure, sample type and extraction process time (22,37,38). The present study demonstrated that ethanol-extracted *S. corbularia* had a higher total phenolic content compared with that in the *P. linteus* extract using the hot water method. A previous study on the total phenolic content in *S. corbularia* extracted with ethanol using the maceration process reported a value of ~0.006% dry weight (0.006 mg GAE/g), which indicated that the modified extraction procedure using the reflux extractor, used in the present study, provided improved efficiency for total phenolic content extraction (11).

The present study assessed the total polysaccharide content of *P. linteus* crude extract, which was the highest compared

with *S. corbularia* and the combinations. Preliminary structural characterization demonstrated that the polysaccharides extracted from *P. linteus* fruiting bodies to be mainly heteropolysaccharides, which contained certain monosaccharides, such as glucose, arabinose, and other monosaccharides. Certain previous studies had reported that *P. linteus* heteropolysaccharides contained mainly glucose with a minor monosaccharide proportion of galactose, xylose, mannose, fructose and glucosamine (39,40). The majority of bioactive compounds reported amongst the fungal polysaccharides are glucans which contain the α-, β-, or both, configurations (39,40). In addition, the structure of the crude polysaccharide *P. linteus*, in accordance with the FTIR data, appeared to be comprised of β-linked pyranose sugars and contain the presence of a trace amount of phenol, which was consistent with the spectroscopy data. In GPC results, the average M_ws of the crude polysaccharide *P. linteus* was of lower molecular weights (<3 kDa). A previous investigation of degraded polysaccharides which utilized ultrasonic treatment without changing the primary structure of *P. linteus* polysaccharide (original PL-N) reported that the low molecular weight PL-N3 fraction of polysaccharides significantly improved antioxidant activity. Furthermore, polysaccharides with low molecular weights can be more easily absorbed and therefore be more readily applied for therapeutic applications (41). However, the extraction process, molecular weight, monosaccharide compositions and chemical structure of polysaccharides can all have effects on their subsequent biological activities (14,35).

Three distinct combinations of *S. corbularia* and *P. linteus* were used in the present study. Combination 3, which contained 75% *S. corbularia*, had a higher total phenolic content compared with the other combinations. By contrast, combination 2 contained a higher total polysaccharide content, likely due to the major constituent being *P. linteus* crude extract. Results from the present study suggested that the total phenolic content and total polysaccharide content results for all combinations depended on the respective ratios of the substances in each combination.

A recent study of the *S. corbularia* extract alone reported marked DPPH and ABTS radical scavenging activity, which was greater compared with that reported by a previous study on *S. corbularia* obtained by ethanol extraction, using a different extraction method (11). In addition, *P. linteus* alone demonstrated a higher antioxidant activity in the present study compared with that reported by a previous study on the extract of the fruiting body of *P. linteus* obtained by hot water extraction, against the DPPH free radical reagent (39). In the present study, combination 3 demonstrated the strongest antioxidant abilities compared with other combinations. The antioxidant activities demonstrated from high to low were *S. corbularia*, combination 3, combination 1, combination 2 and *P. linteus*. Although the capabilities of the combinations did not exceed those of *S. corbularia* extract alone, combination 2 (which contained 75% *P. linteus* extract) exerted >8 fold greater scavenging activity compared with that of *P. linteus* alone. Which suggested that the combinations can mediate synergistic effects compared with either individual extract alone. Furthermore, a previous study reported a strong correlation between total phenolic content and antioxidant activity (42). This was consistent with the present findings, which demonstrated that extracted samples with the greatest total phenol content, such as individual *S. corbularia* and combination 3, exerted the greatest radical scavenging ability.

MTT assay principal provides a colorimetric based determination of intracellular formazan production by metabolically active cell and common tool to measure drug cytotoxicity. Loss of the ability to convert the tetrazolium salts into colored formazan products, demonstrated the cytotoxicity of the drug to the metabolic processes in the cell and was taken as a positive result (43). The results in the present study demonstrated that the toxicity of all the extracted samples and combinations on the three different breast cancer cell lines had the same trend when ranked from strong to weak activity, with *S. corbularia* as being the most potent, followed by combination 3, combination 1, combination 2 and *P. linteus*. This suggested that the toxicity of the extracted samples on cancer cells was associated with antioxidant activity.

One of the possible mechanisms by which phytochemical antioxidants act as potential cytotoxic agents to cancer cells is by the reduction of reactive oxygen species-induced stresses. This may be by either scavenging free radicals or inducing the production of endogenous antioxidants. Endogenous antioxidants can be elevated by targeting non-enzymatic antioxidant systems, such as nuclear factor erythroid 2-related factor 2 activators, vitamins, N-acetylcysteine and glutathione (GSH) esters, and enzymatic antioxidants, including superoxide dismutase, GSH peroxidases, peroxiredoxins (PRDXs) and catalase (44). A previous study reported that managing H_2O_2-mediated oxidative stress can significantly reduce the survival of MCF-7 cells by downregulating the expression PRDX1 (45). Another previous study, of the medicinal plant Parijoto (*Medinilla speciosa* Blume), reported a positive correlation between the antioxidant and cytotoxic activity in the 4T1 breast cancer cell line. Which indicated that greater antioxidant activities also increase the cytotoxicity on the cancer cell line (46). The previous reports were consistent with the findings of the present study, that *S. corbularia* alone and combination 3, which exerted higher antioxidant activities compared with *P. linteus*, demonstrated greater degrees of cytotoxicity on breast cancer cells at lower concentrations.

The extracts assessed in the present study exerted a potent cytotoxic effect on MCF-7 cells, moderate cytotoxic effects on MDA-MB-468 cells, but low cytotoxic effects on MDA-MB-231 cells. It could be hypothesized that each cell line subtype would respond differently to the extract, including breast cancer cell lines. All extracts required higher concentrations to treat MDA-MB-231 cells compared with the concentration required MCF-7 and MDA-MB-468, which also affected the viability of the 293 cell line. In the present study, the combinations were able to reduce the viability of 293 cells by ~50% at the same concentrations as the IC_{50}-dependent administrations on MDA-MB-231 cells. These results indicated that the crude extracts caused greater damage to certain breast cancer cell lines whilst having less effect on normal kidney cells. According to these findings, this herbal treatment exhibited low nephrotoxicity.

In the present study, cell viability was measured after 72 h treatment with the extracted samples. The cytotoxic agent in the extracted samples may affect the molecular cell mechanisms and disturb cell division (47). Therefore, it could

be hypothesized that not all surviving cells after treatment could proliferate under those conditions. Compared with the cytotoxic assay results which demonstrated that combination 3 had the highest activity, the proliferation assay demonstrated that combination 1 had the greatest anti-proliferative effect on MCF-7 and MDA-MB-231 cells. In addition, combination 2 had the highest inhibitory effect on MDA-MB-468 cell proliferation. This suggested that the interaction of compounds could exert different effects on the cell mechanisms. A polyphenol called hispolon, which is typically found in the genus *Phelinus*, was previously reported to exert certain effects against cancer cells, such as inhibiting cell proliferation (9). Furthermore, compounds such as flavonol rhamnoside, astilbin, cathecin, epicathecin and resveratrol, from *S. corbularia* extract have been reported to possess abilities as antiproliferative agents (48). Therefore, the combination of these bioactive compounds at certain ratios from both individual extracts in the present study may have exerted a synergistic effect to inhibit breast cancer cell proliferation. Previous studies have reported that the total contents of a whole herb extract exerted substantially greater impact compared with that of a single isolated active component, which suggested that the potential anticancer benefits of herb combinations may be more effective (21,49).

According to the apoptosis results, *P. linteus* and combination treatments resulted in higher rates of late and/or early apoptosis. However, the low values (>5%) indicated that the capability the extracted samples to induce apoptosis in breast cancer cell lines was low. It has been previously reported that all cells, including cancer cells, will undergo cell death if the mechanism required for survival is disrupted. Certain biochemical agents such as polyphenolics, flavonoids and alkaloids have been reported to induce programmed cell death. Apoptosis was previously considered to be the only form of programmed cell death that serves a significant role in the optimization of cancer therapy (50,51). However, cancer cells develop strategies to prevent programmed cell death by developing genetic mutations or epigenetic modifications in key modulator pathways, such as mutation of p53 or upregulation of Bcl-2 expression. Therefore, cancer cells are frequently resistant to apoptosis and may evolve apoptotic tolerance. In addition, other forms of programmed cell death, different to apoptosis have been reported, such as autophagy and necroptosis (50-52). The present findings demonstrated that the extracted sample had cytotoxic and antiproliferative effects on breast cancer cell lines. Otherwise, the percentage of apoptosis in all extracted samples-treated cells was low, <5%. Thus, the extracted samples did not demonstrate a potent effect on apoptosis; however, these extracts may induce other forms of programmed cell death, apart from apoptosis.

The present study evaluated the synergistic effect of two herbs, *P. linteus* and *S. corbularia*, in certain proportions. This study differs from our previous research, which examined the effects of three herbs in a single ratio of 3:1:1 (*P. linteus*, *S. corbularia*, and *S. glabra*) (53). Determining the optimal ratio for each herb's mechanism of action provides a basis for the future development of herbal anti-cancer medications. Moreover, since understanding of the specific compounds within the extracts are critical for obtaining strong predictive understanding of the herb's mechanism of action on cancer cell lines. Furthermore, a comprehensive structural compound analysis is needed in future studies to elucidate the details of the characteristics of the compound extracts. The lack of these data, was a limitation of the present study.

In conclusion, the present study demonstrated that the increase in total phenolic content is associated with an increase in antioxidant activity and cytotoxic effects. Certain combinations had a significant antiproliferative effect on three distinct breast cancer cell lines. In addition, other mechanisms of programmed cell death besides apoptosis should be investigated in a future study. The results of the present study suggested that the combination of individual extracts from *S. corbularia* and *P. linteus* may have potential as antioxidant and anticancer agents.

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Availability of data and materials

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

Authors' contributions

AJS, WB, SP and PY designed the study. AJS conducted the experiments, analyzed the data and drafted the original manuscript. WB, SP and PY confirm the authenticity of all raw data. WB, SP and PY reviewed and edited the manuscript. SP and PY supervised the study. PY wrote the proposal for a grant. All authors have 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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