

Accepted Manuscript

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PII: S0144-8617(16)30721-4
DOI: <http://dx.doi.org/doi:10.1016/j.carbpol.2016.06.046>
Reference: CARP 11231

To appear in:

Received date: 14-3-2016
Revised date: 8-6-2016
Accepted date: 11-6-2016

Please cite this article as: Luo, Zhiyong., Hu, Xiaopeng., Xiong, Hua., Qiu, Hong., Yuan, Xianglin., Zhu, Feng., Wang, Yihua., & Zou, Yanmei., A polysaccharide from Huaier induced apoptosis in MCF-7 breast cancer cells via down-regulation of MTDH protein. *Carbohydrate Polymers* <http://dx.doi.org/10.1016/j.carbpol.2016.06.046>

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**A polysaccharide from Huaier induced apoptosis in MCF-7 breast cancer cells
via down-regulation of MTDH protein**

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HIGHLIGHTS

- A polysaccharide SP1 was purified from Huaier fruiting bodies.
- The chemical properties and sugar linkage of SP1 were evaluated.
- SP1 inhibited the proliferation of MCF-7 cells by inducing apoptosis.
- SP1 treatment led to a rise of Bax/Bcl-2 ratio in MCF-7 cells.
- SP1 treatment repressed MTDH protein expression in MCF-7 cells

ABSTRACT

In this study, one homogeneous polysaccharide (SP1), with a molecular weight of 56 kDa, was isolated from the Huaier fruiting bodies. It had a backbone consisting of 1, 4-linked- β -D-Galp and 1, 3, 6-linked- β -D-Galp residues, which was terminated with 1-linked- α -D-Glcp and 1-linked- α -L-Araf terminal at O-3 position of 1, 3, 6-linked- β -D-Galp unit along the main chain in the ratio of 1.1: 2.0: 1.1: 1.1. MTT assay showed that shMTDH or SP1 (100, 200 and 400 μ g/ml) was able to suppress the proliferation of MCF-7 cells, due to a significant increase in the number of apoptotic cells as determined by flow cytometric analysis. Furthermore, Western blot analysis revealed that SP1 or shMTDH treatment led to a rise of ratio between proapoptotic Bax and antiapoptotic Bcl-2 protein in MCF-7 cells. In addition, carcinogene MTDH protein expression in MCF-7 cells received SP1 (100, 200 and 400 μ g/ml) or shMTDH treatment was also repressed after 48h incubation. Taken together, these findings indicated that SP1 has anticancer potential in the treatment of human breast cancer.

Keywords: Huaier polysaccharide; Breast cancer; MTDH; Apoptosis

1. Introduction

Breast cancer is the most frequent type of malignancy among women in many countries worldwide (DeSantis et al., 2014; Kummalue et al., 2006) and is believed to be the second leading cause of cancer death (Merrill & Weed, 2001). It is reported that breast cancer occurred in women is more than 100-fold more frequent than in men (Cooper, 1992). Despite significant advances in treatment of breast cancer, such as hormonal therapy, chemotherapy, radiotherapy and surgery, the incidence and mortality rates of the disease due to drug resistance and metastases are still on the rise (Clarke et al., 2005; Scully et al., 2012). Unfortunately, breast cancer is highly resistant to chemotherapy, therefore, it is extremely urgent to seek new therapeutic strategies and develop novel drug with low toxicity for the prevention and treatment of breast cancer. In this perspective, the study of natural products extracted from Traditional Chinese Medicine (TCM) remains one of the most challenging fields in cancer research, since TCM has been investigated for the discovery of potential anticancer agents in China in the past few decades (Cameron & Bell, 2004; Hsiao & Liu, 2010; Zhou et al., 2014).

Huaier, a traditional Chinese medicine, has been widely used in China as a supplementary therapy on cancer treatment since the discovery of its anti-tumor effect (Xu et al., 2011). Large amount of evidence had demonstrated that one proteoglycan isolated from Huaier extract was believed to be the main active ingredient responsible for its effectiveness in cancer therapy (Guo et al., 1993; Zhang et al., 2010). In our previous study, we showed that a polysaccharide from mushroom Huaier (SP1)

inhibited the growth of hepatocellular carcinoma and metastasis in mice via down-regulation of HIF-1 α , VEGF, MMP2, bcl-2, Ncadherin, STAT3, and MTDH, together with up-regulation of bax and NE-cadherin (Zou et al., 2015a). More importantly, Huaier aqueous extract has been proved to be a promising therapeutic drug for breast cancer in many documents (Zhang et al., 2010; Wang et al., 2013; Wang et al., 2014; Wang et al., 2015). To the best of our knowledge, the polysaccharides mainly exist in this water extract. Although the antitumor activity of Huaier aqueous extract on breast cancer has been revealed, the principal active ingredients in this extract and the exact underlying mechanisms still remain largely unknown so far. In the present study, we aim to characterize this polysaccharide SP1 and evaluate the effect of this polysaccharide on breast cancer cells, as well as explore the underlying mechanisms.

2. Materials and methods

2.1. Materials and chemicals

The Huaier fruiting bodies were purchased from a local market (Wuhan city, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trifluoroacetic acid (TFA), standard monosaccharides (fucose, arabinose, rhamnose, galactose and mannose, glucose, xylose, glucuronic acid, and galacturonic acid), T-series dextran (T-2000, T-70, T-40, T-20, and T-10), penicillin, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco

Invitrogen Co. (Rockville, IN, USA). DEAE-cellulose-52 and Sepharose CL-6B were from the Pharmacia Co. (Uppsala, Sweden). Antibodies against MTDH, Bcl-2, Bax, β -actin, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin V-FITC Apoptosis Detection Kit was obtained from Pharmingen- Becton Dickinson (San Diego, CA, USA). The enhanced chemiluminescence (ECL) detection kit was purchased from Pierce (Rockford, IL, USA). A BCA protein assay kit was from Pierce (Rockford, IL, USA). All other chemicals were from Merck (Darmstadt, Germany) and Sigma-Aldrich (St Louis, MO, USA).

2.2. Isolation and purification of polysaccharide SPI

The dried materials was first ground into fine powder (20 meshes) and further extracted with 5 volumes of 95% ethanol at 75 °C under reflux to remove some small molecule materials for 24h. After filtration, the left residues were dried and extracted with distilled water at 95 °C for three times and 3h each time, and then the entire aqueous extract was collected by centrifugation at $5000 \times g$ for 15 min, concentrated to the one-fifth of original volume under reduced pressure at 40 °C, and finally precipitated with 4-fold volumes of 95% ethanol at 4 °C overnight. The resulting precipitate collected by centrifugation was resuspended in distilled water and treated with Sevag reagent (1:4 n-butanol:chloroform, v/v, 400 ml) to remove free proteins (Staub, 1965). The final aqueous solution was exhaustive dialyzed against tap water and distilled water for each for 48 h. At last, the retained fraction in cellulose membrane tube was combined, concentrated and lyophilized to yield the crude

polysaccharides, coded as SCP.

The SCP was resuspended in distilled water, filtered through a filter paper (pore size, 0.45 μm), and then subjected to a DEAE-cellulose-52 column (2.6 cm \times 40 cm, Cl^-) eluting successively with distilled water and a 5-step gradient of NaCl aqueous solution (0.05 M, 0.1 M, 0.2 M, and 1.0M) at a flow rate of 0.5 ml/min. Each tube fraction containing polysaccharides were determined for the carbohydrate content by the phenol-sulfuric acid assay (Dubois et al., 1956). The collected fractions eluted by distilled water were combined and further purified by size-exclusion chromatography on a Sepharose CL-6B gel filtration column (2.6 \times 100 cm), eluting with distilled water at a flow rate of 1 mL/min. One major peak monitored by phenol-sulfuric acid assay was collected, dialyzed and lyophilized to achieve one purified polysaccharide fraction (SP1), which was used for subsequent physicochemical and biological analyses.

2.3. Chemical properties and monosaccharide composition analysis

Total carbohydrate content of SP1 was measured according to the phenol-sulfuric acid method (Dubois et al., 1956). Protein content of SP1 was determined by the method of Bradford (1976). Uronic acid content of SP1 was detected by *m*-hydroxydiphenyl colorimetric method (Blumenkrantz & Asboe-Hansen, 1973).

Gas chromatography (GC) was used for monosaccharide composition for the polysaccharide as described by Xie et al. (2010). Briefly, SP1 (10 mg) was hydrolyzed

with 2 M TFA and converted into the alditol acetates by the method of Honda et al. (1981). The resulting alditol-acetates of the standard monosaccharide or the sample were subjected to GC analysis using a Agilent 6280 instrument equipped with HP-5MS capillary column (30 m × 0.32 mm × 0.25 μm) and detected with a flame ionization detector (FID) at temperatures programmed from 160 °C to 260 °C at a rate of 5 °C /min and then hold on 5 min. N₂ was used as the carrier gas and inositol as the internal standard. Quantification of the monosaccharide was performed based on the peak area.

2.4. Homogeneity and molecular weight

The homogeneity and average molecular weight of the polysaccharide was determined by high performance size exclusion chromatography (HPSEC) on a Allances 2695 HPLC system (Waters, USA), coupled with a TSK-GEL G3000 PWXL column (7.8mm×300 mm) and a refractive index detector (RID) detector. The sample (2.0 mg) was dissolved in mobile phase (2 ml) and filtered through a 0.45 μm filter before sample injection. A 20 μl sample was injected in each run and eluted with 0.05 mol/L Na₂SO₄ at a flow rate of 0.5 mL/min. The molecular weight distribution of the polysaccharide was estimated by reference to the standard curve made from standard T-series Dextran of known molecular weight (T-2000, T-70, T-40, T-20, and T-10).

2.5. Methylation analysis

Each SP1 sample (20 mg) was methylated three times according to Needs and Selvendran (1993). Complete methylation was confirmed by the disappearance of the

OH band (3200–3700 cm^{-1}) in the IR spectrum (Zhang et al., 2015). The methylated products were hydrolyzed, then reduced and acetylated with acetic anhydride–pyridine as described by Sweet et al. (1975). The partially methylated alditol acetates were performed on a Shimadzu QP-2010 gas chromatography/mass (GC/MS) spectrometer coupled with a DM-2330 capillary column (30 m \times 0.32 mm, 0.2 mm film thickness) at programmed temperatures as described by Yan et al. (2015).

2.6. Ultraviolet (UV), Fourier-transform infrared spectroscopy (FT-IR) and Nuclear magnetic resonance (NMR) analysis

The ultraviolet absorption spectra were recorded with an ultraviolet spectrophotometer (Shanghai Precision and Scientific Instrument Co. Ltd., Shanghai, China) between 190 and 400 nm. The FT-IR spectra (potassium bromide pellets) were recorded on a Nicolet FT-IR spectrophotometer (Scimitar, America Varian Technology Co. Ltd.) in the frequency range of 400–4000 cm^{-1} . For NMR analysis, the sample (30 mg) was dissolved in 0.6 mL of D_2O (99.9%) and transferred into a 5 mm NMR-tube followed by recording with a Bruker Avance400 MHz spectrometer (Rheinstetten, Germany) at 55 °C.

2.7. Cell culture

Breast cancer cell lines MDA-MB-231, MDA-MB-435, MCF-7 and MCF10A human breast epithelial cells were purchased from Shanghai Institute of Cell Biology in the Chinese Academy of Sciences (Shanghai, China). Three cancer cell lines were routinely grown in DMEM containing 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$

streptomycin. MCF10A cells were cultured in DMEM/F-12 Nutrient Mixture (1:1) containing 5% horse serum, 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin (Sigma), 500 ng/ml hydrocortisone, 10 µg/ml insulin, 100 U/ml penicillin and 100 µg/ml streptomycin. All cell lines were maintained at 37 °C in a humidified incubator with 5% CO₂ gas environment.

2.8. Plasmid construction and transfection

The plasmid constructs carrying shRNA (pSUPER-EGFP-MTDH) against MTDH were performed as previously described (Kock et al., 2007; Yoo et al., 2009). The following MTDH sequences used to generate MTDH short hairpin RNA (shRNA) were cloned into the pSUPER-EGFP plasmid: forward, 5'-ATGAACCAGAATCAGTCAGC-3'; and reverse 5'-GCTGACTGATTCTGGTTCAT-3'. The Breast cancer MCF-7 cells were transfected with pSUPER-EGFP-MTDH using Lipo-fectamine 2000 (Invitrogen) as suggested by the manufacturer's instructions. After 48 hours of transfection, MCF-7 cells were diluted and seeded into the medium containing 500µg/ml G418 for 2-3 weeks. Then, the positive clones were picked up and expanded to establish cell lines after maintaining to select for 4 weeks. The stable transfection cell clones MTDH shRNA was verified by Western blotting analysis and subjected to further experiments.

2.9. MTT assay

Cell viability was determined by the MTT assay as described previously (Zou et al., 2015b). In brief, after 24 h seeding, the transfected MTDH shRNA MCF7 cells or cancer cells were replaced with fresh medium supplemented with different concentrations of SP1 (0, 50, 100, 200, 400, 800 and 1600 μ g/ml) for 24, 48 or 72 h. After incubation at 37 °C for indicated period, 10 μ L MTT solution (5 mg/ml in PBS) was added to the wells for another 4 h at 37 °C in 5% CO₂ incubator, followed by addition of 200 μ L of DMSO to dissolve purple formazan crystals. The color intensity was measured with an ELISA micro-plate reader at a wavelength of 490 nm. The cell viability was calculated with untreated samples as the control. The color intensity was read at 540 nm with an ELISA reader. The cell viability was calculated by comparing the absorbance of treated verses untreated cells.

2.10. Annexin V-FITC/PI analysis

After exposure to SP1, the apoptotic cell death was determined using an Annexin V-FITC/PI (propidium iodide) apoptosis detection kit according to the modified manufacturer's protocol. In brief, transfected MTDH ShRNA MCF7 cells or MCF7 cells were seeded a 12-well plate and left for 24 h in incubator to resume exponential growth followed by the addition of different concentrations of SP1 for another 24h. After the incubation, the cells were harvested and washed with phosphate buffered saline (PBS) twice for determining apoptosis. Then the adherent and floating cells were combined and subjected to Annexin V and PI staining. The samples were

analysed on a FACScan cytometry instrument (Becton Dickinson, San Jose, CA) and the data were then performed using CellQuest software to differentiate apoptotic cells from necrotic cells following the step-by-step protocol provided by the manufacturer.

2.11. Western blot analysis

After incubation, shMTDH, vehicle- or SP1-treated cells were harvested and lysed. For western blot analysis, equal amounts of proteins as quantified using a BCA protein assay kit were subjected to 10% SDS-PAGE, followed by immunoblotting on nitrocellulose membranes, and then blocked in 5% nonfat milk at room temperature for 2 h before being incubated with the indicated primary antibody (1:400 for MTDH, 1:1,000 for β -actin, Bax and Bcl-2) overnight at 4°C in blocking solution. The blots were then reacted with HRP-conjugated secondary antibodies and the signals were quantified using the ECL system using β -actin level as a control.

2.12. Statistical analysis

Data were expressed as mean \pm standard deviation (SD) of three independent determinations and each experiment was done at least three times. Statistical analysis was performed using SAS 8.0 software. The comparisons between treatment and control groups were conducted using the Student's *t*-test and *P*-value (<0.05) was considered to be statistically significant.

3. Results and discussion

3.1. Isolation and purification of the polysaccharide

The water-soluble crude polysaccharide (SCP) was prepared from Huaier fruiting bodies as a brown yellow powder, with a yield of 4.89% of the dried material. The SCP was further fractionated on DEAE-cellulose column and Sepharose CL-6B gel filtration column chromatography to afford different purified polysaccharide with different average molecular weight distribution, of which one water-soluble neutral fraction SP1 (0.35% of dried materials) was collected and lyophilized for further study.

3.2. Chemical properties of the polysaccharide

In **Table 1**, the chemical properties of SP1, including total carbohydrate content, uronic acid content, protein content, average molecular weight and monosaccharide composition were summarized. The total carbohydrate content of SP1 was determined to be 94.35% and no uronic acid and protein content was detected. A single and symmetrical peak on a Waters HPLC system indicated its homogeneity, and its average molecular weight was estimated to be 56 kDa (**Fig. 1**). GC analysis revealed that SP1 consisted of galactose, arabinose, and glucose in a molar ratio of 3.1: 1.1: 1.1.

3.3. Structural identification of the polysaccharide

The methylation analysis is one of the best ways to offer information about glycosidic linkage location of polysaccharides (Vieira et al., 1991). For the purpose of further determining the sugar linkage of SP1, the analysis of the fully methylated product of SP1 was conducted by GC/MS and the polysaccharide showed the presence of four linked types (**Table 2**), namely 1, 4-linked- β -D-Galp (Residue A), 1, 3, 6-linked- β -D-Galp (Residue B), 1-linked- α -D-Glcp (Residue C) and 1-linked- α -L-Araf (Residue D) in molar ratio of 1.1: 2.0: 1.1: 1.1. The proportions of the methylated alditol acetates of SP1 showed a good correlation between terminal and branched residues. Particularly, these molar ratios are in good agreement with the monosaccharide composition of SP1 obtained from GC analysis. It could thus be proposed that the backbone of SP1 contains Residue A and B, with Residue C or Residue D branches attached to O-3 position of Residue B along the backbone.

The data on UV, FT-IR and NMR analysis of SP1 were shown in **Table 3**. The UV-vis spectra of SP1 were recorded on an ultraviolet spectrophotometer. As shown in the UV spectra, SP1 showed no optical absorption at 260 nm and 280 nm, demonstrating the absence of nucleic acid and protein (Sun et al., 2008). The results were consistent with the results of chemical analyses. The characteristic intense broad band at 3434.24 cm^{-1} was assigned to the hydroxyl O-H stretching vibration and the weak signal at 2924.52 cm^{-1} was attributed to C-H stretching vibration (Jiang, et al., 2013). Bands visible at approximately 1428.12 and 1374.47 cm^{-1} were ascribed to the angle vibration of C-H bond (Chen et al., 2008; Shao et al., 2014), whilst a band at

1043.11 cm^{-1} was indicative of the presence of non-symmetric C–O–C stretching vibration (Ding et al., 2012). Furthermore, the characteristic absorption at around 826.75 and 874.45 cm^{-1} confirmed the presence of both α - and β -type glycoside bonds in the purified polysaccharides (Yu et al., 2009). All characteristic absorptions of SP1 in the FT-IR spectrum are common to all the polysaccharide (Barker, et al., 1954)

To further confirm the linkage sites and sequence among residues, the ^1H NMR and ^{13}C NMR spectrum of SP1 were determined. In ^1H NMR spectrum of SP1, one low field and three high field signals in the anomeric region indicated that SP1 has four sugar residues and 4.65, 4.53, 5.51 and 5.10 ppm were assigned to Residue A, B, C and D on the basis of chemical shifts of anomeric proton (Pei et al., 2015; Wang et al., 2014; Yan et al., 2013; Yang et al., 2016; Yu et al., 2015). Chemical shifts of all residues C-1 protons at 5.0–5.4 and 4.4–5.0 ppm confirmed that the existence of α - and β -type glycosidic linkages in SP1 according to the literature (Zhao et al., 2014). These observations agreed with the presence of 826.75 and 874.45 cm^{-1} on FT-IR spectra data (Barker et al., 1954; Yu & Yang, 1999). Other proton signals of H2-H6 were overlay appeared at 3.4–4.5 ppm. Particularly, no chemical shift larger than 6.0 ppm appearing that indicates the absence of uronic acid in SP1 again (Shi et al., 2016). In the ^{13}C NMR spectrum of SP1, the peaks at 107.3, 103.6, 100.1 and 109.4 ppm attributed to the anomeric carbons of Residue A, B, C and D, respectively (Yan et al., 2013; Yang et al., 2016; Zou et al., 2014). The signal of substituted C-4 of Residues A had moved downfield to 80.9 ppm (Zou et al., 2014). Peak at 80.3 ppm and 70.1 in

low magnetic field appeared to come from C-3 and C6 resonance of Residue-B, respectively (Yang et al., 2016). On the contrary, three signals of 64.1, 62.9 and 63.9 at high magnetic field seemed to unsubstituted C-6 of Residue A, C and C-5 of Residue D, respectively (Yan et al., 2013; Zou et al., 2014).

3.4. Effect of SP1 on the cell viability of MCF-7 Cells

To determine whether SP1 has an in vitro anti-tumor activity on breast cancer MDA-MB-231, MDA-MB-435 and MCF-7 cells, we performed the MTT assay to determine the percent of viability of cancer cells when treated with increasing concentration of SP1 (50, 100, 200, 400, 800 and 1600 $\mu\text{g}/\text{mL}$) for indicated period. The results in **Fig. 2A** revealed that SP1 exhibited a time- and dose dependent inhibitory effect on the growth of MCF-7 cells after exposure with SP1 at the concentrations of 50, 100, 200, 400, 800 and 1600 $\mu\text{g}/\text{mL}$ for 24, 48, and 72 h. With the concentration rising to 100 $\mu\text{g}/\text{ml}$, the cell viability declined visibly at all time periods. Beyond 48h exposure, the decrease of cell viability was close to each other, especially between 100 to 400 $\mu\text{g}/\text{ml}$. However, above the concentration of 400 $\mu\text{g}/\text{ml}$, the change of cell death in any situation all became slightly and gently. As seen in **Fig. 2 B** and **C**, breast cancer cell lines MDA-MB-231 and MDA-MB-435 showed weak sensitive response to SP1 treatment compared with MCF-7 cells in the same concentration and treatment time. Thus, we chose MCF-7 cell line as research target using the concentration of SP1 (100, 200 and 400 $\mu\text{g}/\text{ml}$) and the exposure of 48 h as favorite parameters to facilitate the next experiments. More importantly, MTDH shRNA transfection in MCF-7 cells also led to a decrease of the cell viability and SP1

did not show any significant cytotoxic effect on MCF10A human breast epithelial cells (**Fig. 2D**)

3.5. Effect of SP1 on apoptosis in MCF-7 Cells

To ascertain whether the observed inhibitory effect of SP1 on MCF-7 cell viability is associated with programmed cell death, namely apoptosis, we stained the cells simultaneously with AnnexinV and PI and measured the percentage of apoptotic cells in SP1-treated cells on a flow cytometry. After 48 hours of treatment with PBS or shCon (pSUPER-EGFP plasmid), only about 6.5 and 6.4% apoptotic cells appeared in **Fig.3 A** and **B**, respectively. Following exposure of MCF-7 cells with 100, 200, and 400 µg/ml of SP1, amount of apoptotic cell death appearing in entire cells account for 45.3 (**Fig. 3E**), 55.6 (**Fig. 3E**), and 66.8 % (**Fig. 3F**), respectively, which demonstrated that MCF-7 cells undergo apoptosis following SP1 treatment. Interestingly, MTDH shRNA treatment to MCF-7 cells also markedly induced 68.7% apoptotic cell death (**Fig. 3 C**), indicating involvement of MTDH in SP1-triggered apoptosis in MCF-7 cells.

3.6. Effect of SP1 on the expression of Bax, Bcl-2 and MTDH in MCF-7 Cells

It is widely accepted that Bax is a proapoptotic protein and Bcl-2 is an anti-apoptotic protein. The ratio between Bcl-2 and Bax family protein has been demonstrated to play a crucial regulatory role in the regulation of apoptosis (Harris &Thompson, 2000; Rossé et al., 1998; Yang et al., 2006). Therefore, we examined the protein expression of Bax and Bcl-2 in SP1-treated or MTDH-depleted MCF-7 cells.

The results of Western blot presented in **Fig. 4** revealed that there was significant increase in the expression of Bax protein in MCF-7 cells, whereas the level of the anti-apoptotic Bcl-2 protein remained nearly unchanged upon SP1 treatment (100, 200 and 400 $\mu\text{g/ml}$) at any dose, thus leading to a rise of Bax/Bcl-2 ratio. At the same time, MCF-7 cells silenced by shMTDH also appear the same trend.

It has been reported that MTDH expression is frequently up-regulated in various human tumor types, including breast cancer, and plays multiple roles in the process of oncogenesis (Li et al., 2008; Liu et al., 2011). Accumulating published studies have suggested that overexpression of MTDH has been demonstrated to be critically associated with progression of disease and poorer prognosis in breast cancer (Li et al., 2008; Liu et al., 2011; Su et al., 2010). From **Fig. 4**, we observed that the MTDH protein almost successfully vanished in MCF-7 cells after transfecting with MTDH shRNA. In line with these findings, treatment with SP1 (100, 200 and 400 $\mu\text{g/ml}$) for 24 h also induced a marked decrease in the expression of MTDH protein in MCF-7 cells, which was found to be in a dose-dependent manner compared with the control (**Fig. 4**). These observations indicated that SP1-induced apoptosis in MCF-7 cells was involved in the downregulation of Bax/Bcl-2 and MTDH protein expression

4. Conclusions

In the current study, one water-soluble polysaccharide SP1, with a molecular weight of 56 kDa, was isolated and purified from *Huaier* fruiting bodies. SP1 was composed of galactose, arabinose, and glucose in a ratio of 3.1: 1.1: 1.1., as detected by GC and the GC/MS results indicated that SP1 had a backbone of 1, 4-linked- β -D-Galp and 1, 3, 6-linked- β -D-Galp, which was occasionally branched at O-3 position of 1, 3, 6-linked- β -D-Galp with 1-linked- α -D-Glcp and 1-linked- α -L-Araf along the main chain in the ratio of 1.1: 2.0: 1.1: 1.1. We

demonstrated the in vitro antitumor effects of SP1 on human breast cancer MDA-MB-231, MDA-MB-435 and MCF-7 cells for the first time. MTT assay proved that SP1 demonstrated a more effective inhibitory effect on cell growth of MCF-7 cells in a dose-dependent manner than other cancer cell lines, and especially at the dose of 400 $\mu\text{g/mL}$, the inhibitory effect was nearly close to the MCF-7 cells transfected with shTMDH. Simultaneously, the percentage of apoptotic cells was increased in response to SP1 treatment along with concentration increasing after 48 h incubation, indicating its antitumor potential in the cancer therapy. We also observed that shMTDH MCF-7 cells undergo apoptosis, meaning the involvement of MTDH in SP1-induced apoptotic cell death. Particularly, we provided data of the involvement of Bcl-2 family in SP1-induced apoptosis, as evidenced by the upregulation of the ratio of Bax/Bcl-2 protein. Furthermore, the same phenomenon occurred in shMTDH-transfected cells. More importantly, attenuation of carcinogene MTDH in MCF-7 cells was also observed after SP1 treatment. All these findings indicated that SP1 could serve as a novel promising chemopreventive agent for the treatment of breast cancer via downregulation of the ratio Bax/Bcl-2 and MTDH protein expression. However, a deep investigation to elucidate its other pathways in vitro and to carry out the in vivo activity of SP1 toward breast cancer is in progress.

Acknowledgements

This work was supported by Grant No. 81201779 (Hua Xiong) from the National Natural Science Youth Foundation; Grant No. 81502118 (Yanmei Zou) from the National Natural Science Youth Foundation; Grant No. 2014CFB250 (Yanmei Zou) from the Natural Science Foundation of Hubei Province.

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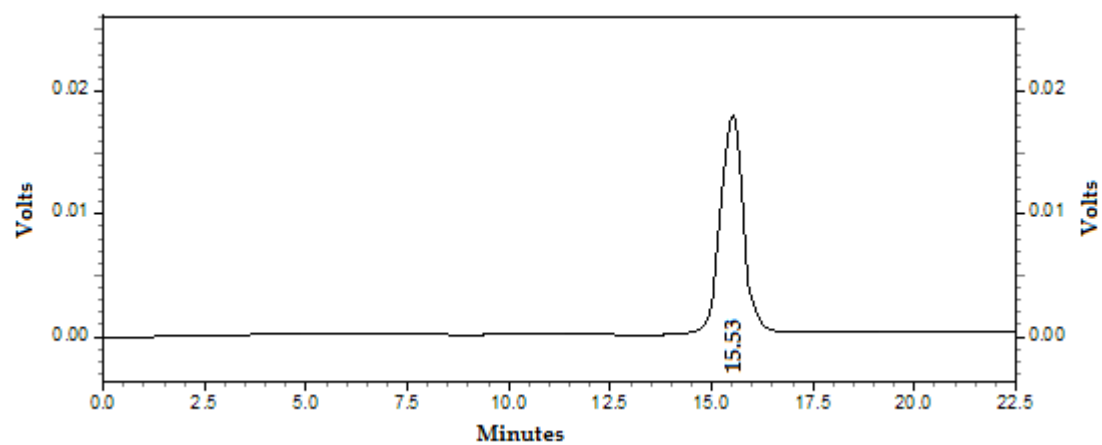


Fig. 1 The profile of SP1 in HPSEC

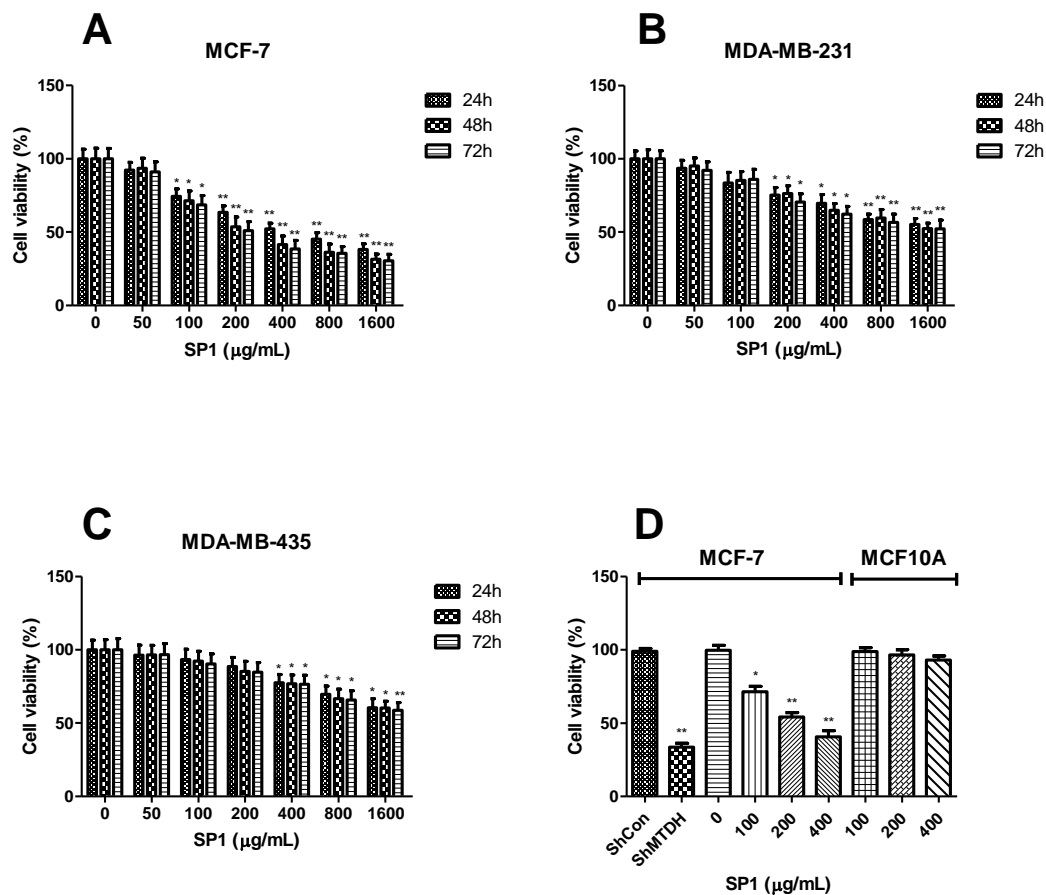


Fig. 2 Inhibitory effects of SP1 on the cell viability of (A) MCF-7, (B) MDA-MB-231 and (C) MDA-MB-435; (D) Inhibitory effects of SP1 on the cell viability of MCF10A and the cell viability of MTDH shRNA (ShMTDH)-infected MCF-7 cells using MTT assay. ShCon represents the cells transfected with pSUPER-EGFP plasmid. The data expressed as mean \pm S.D. from three individual experiments.

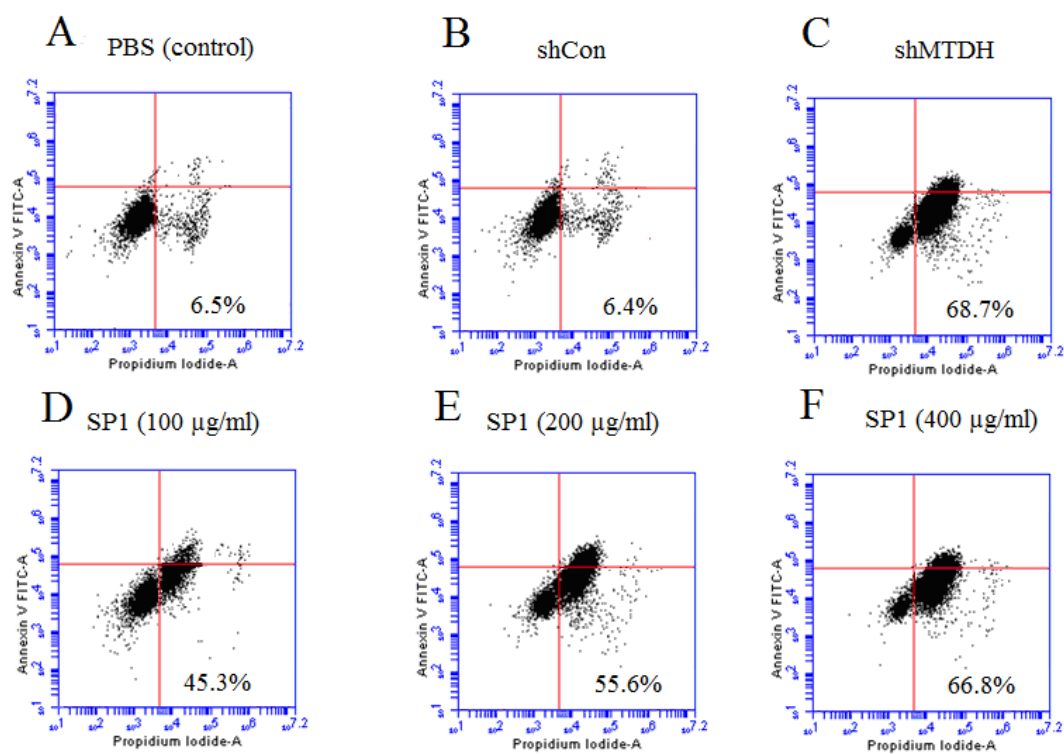


Fig. 3 Quantitative analysis of apoptosis in MCF-7 cells after treatment with (A) PBS (control); (B) shCon (pSUPER-EGFP plasmid); (C) shMTDH (pSUPER-EGFP-MTDH); (D) SP1 (100 $\mu\text{g/ml}$); (E) SP1 (200 $\mu\text{g/ml}$); (F) SP1 (400 $\mu\text{g/ml}$) for 48 h using Annexin V-FITC/PI binding using flow cytometry analysis.

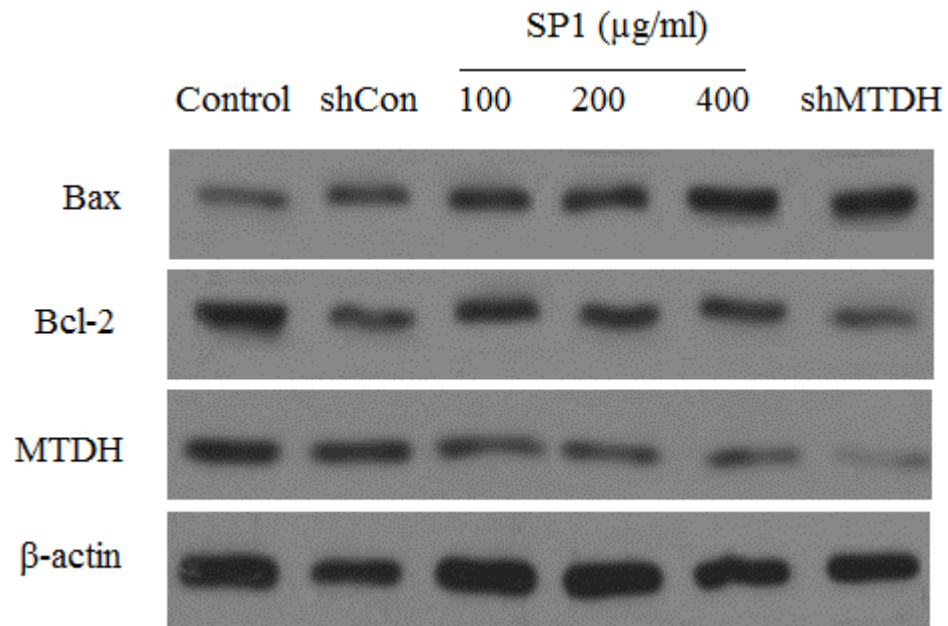


Fig. 4 Effect of SP1 (100, 200, and 400 μ g/mL) or shMTDH (pSUPER-EGFP-MTDH) on the protein expression of Bax, Bcl-2 and MTDH in MCF-7 cells using Western blot assay.

Table 1 Monosaccharide composition and chemical properties of the polysaccharide SP1

Sample	SP1
Carbohydrate (wt%)	94.35
Protein (wt%)	0
Uronic acid (wt%)	0
Sugar components (mol%)	
Galactose (Gal)	3.1
Arabinose (Ara)	1.1
Glucose (Glc)	1.1
Molecular weight (kDa)	56

Table 2 Gas chromatography/mass (GC/MS) analysis of the methylated products of SP1

Peak no.	Methylated sugar	linkage type	Molar ratios
Residue A	2,3,6-Me3-Gal	1, 4-linked- β -D-Galp	1.1
Residue B	2,4-Me3-Gal	1, 3, 6-linked- β -D-Galp	2.0
Residue C	2,3,4,6-Me4-Glc	1-linked- α -D-Glcp	1.1
Residue D	2,3,5-Me3-Ara	1-linked- α -L-Araf	1.1

Table 3 The data of Ultraviolet (UV), Fourier-transform infrared spectroscopy (FT-IR) and Nuclear magnetic resonance (NMR) analysis of SP1

Assay	Peaks or signals at
UV (nm)	260; 280
FT-IR (cm^{-1})	3434.24; 2924.52; 1428.12; 1374.47; 1043.11; 874.45; 826.75
^1H NMR (ppm)	5.51; 5.10; 4.65; 4.53
^{13}C NMR (ppm)	109.4; 107.3, 103.6, 100.1; 80.9; 80.3; 70.1; 64.1; 63.9; 62.9