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ORIGINAL ARTICLE

Cordyceps militaris Polysaccharide Exerted Anticancer Effect via Activating the Endogenous Apoptosis Pathway

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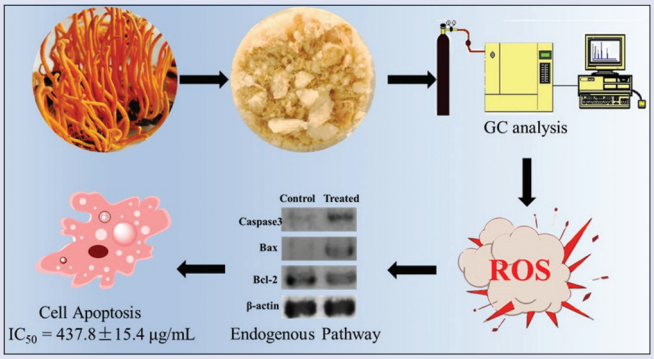
ABSTRACT

Background: Despite tremendous efforts that have been made, cancer is still the leading cause of death all over the world. Chemotherapy, considered a routine method, always faces severe side effects and drug resistance. *Cordyceps militaris (C. militaris)* is a kind of folk tonic food and traditional Chinese medicine and was reported to have anticancer capacity. Since it is difficult to cure cancer via chemotherapy, preventing or inhibiting malignant cells by diet behavior seems useful and attractive. **Objectives:** In this study, we aim to assess the anticancer capacity of *C. militaris* polysaccharide (CMPs) and explore its anticancer mechanism. **Materials and Methods:** Polysaccharide was extracted by water from *C. militaris*. Its total sugar, protein percentage, and monosaccharide composition were measured via the phenol-sulfuric acid method, Bradford kit, and GC assay, respectively. The anti-proliferation effect of the CMPs was screened against several cancer cell lines by CCK8. Its anticancer effect was further studied by cell morphology, live/dead cell staining, and cell apoptosis study. Cellular reactive oxygen species (ROS) evaluation and western blots assay were conducted to explore its anticancer mechanism. **Results:** According to our data, the CMPs could effectively inhibit the proliferation of cancer cells, with IC_{50} values ranging from 437.8 μg/mL to 545.1 μg/mL. Administration of CMPs could cause morphological change among cells and induce cell apoptosis. The study mechanism revealed that the CMPs exerted an anticancer effect via increasing the cellular ROS level and activating the endogenous apoptosis pathway. **Conclusion:** The CMPs can effectively inhibit cancer cells via arousing cellular ROS and activating the endogenous apoptosis pathway.

Key words: Anticancer, apoptosis, *C. militaris polysaccharide*, endogenous, mechanism

SUMMARY

- Polysaccharides extracted from *Cordyceps militaris* inhibited cancer cells
- Polysaccharides extracted from *Cordyceps militaris* could upregulate the cellular ROS level
- Polysaccharides extracted from *Cordyceps militaris* induced cancer cell apoptosis by activating the endogenous pathway



Abbreviations used: *C. militaris*: *Cordyceps militaris*; CMPs: C. militaris polysaccharide; CCK8: cell counting kit 8; ROS: reactive oxygen species; MMP: mitochondrial membrane potential; FBS: fetal bovine serum; O.D.: optical density; BSA: bovine serum albumin; TFA: trifluoroacetic acid; GC: gas chromatography; EDTA: ethylenediamine tetraacetic acid disodium salt; PBS: phosphate buffer solution; FITC: fluorescein isothiocyanate; PI: propidium iodide; DCFH-DA: dichlorodihydrofluorescein-acetoacetate; DNA: deoxyribonucleic acid; S.D.: standard deviation; Bcl-2: Bcell lymphoma 2; p53: tumor protein p53; Bax: Bcl-2 associated X protein.

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INTRODUCTION

As an entomogenous fungus belonging to the class *Ascomycetes*, *Cordyceps militaris* (*C. militaris*) has been widely used in the field of folk tonic food and traditional Chinese medicine.[1,2] With a similar pharmacological function to that of *Cordyceps sinensis*,[3] *C. militaris* possesses a variety of bioactive components, including adenine, adenosine, cordycepic acid, cordycepin, and polysaccharides. Among these, polysaccharides take the most part in the *C. militaris*.[4,5] Previous studies indicated that the CMPs have multiple biological functions, such as antioxidation,[6-8] anticancer,[2,9,10] immunoregulation,[11-13] and anti-inflammation.[14]

Cancer is still an intractable problem all over the world. Chemotherapy, surgery, and radiotherapy are considered routine clinical treatments for cancer. While these strategies always face severe obstacles due to side effects and resistance.[15] To this end, preventing cancer via diet is meaningful.

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Xiao's team has revealed that the temperature could influence the anticancer activity of CMPs.[2] The research results of Tai's group indicated that the CMPs could reduce the side effects of doxorubicin in chemotherapy.[16] Increasing cellular ROS levels is an important mechanism in the battle against cancer.[17,18] Upregulated ROS level exerts an anticancer effect via multiple mechanisms.[17-21] It was reported that exceeding ROS in cancer cells can reduce the mitochondrial membrane potential (MMP),[22] causing the release of cytochrome C from mitochondrial to the cytoplasm,[23] activating the endogenous apoptosis pathway and so inducing cell apoptosis.[22] Since CMPs were reported to have an anticancer effect,[2,24,25] in this work, we conducted a series of studies to assess the anti-proliferation capacity of CMPs toward cancer cells and unfold its anticancer mechanism.

MATERIALS AND METHODS

Materials

The *C. militaris* was purchased from the local market (Strain: CM-jd, China). HCl, $ZnSO_4$, and ethanol were obtained from SinoPharm (China) and were all used without purification. Fetal bovine serum (FBS), RPMI1640, DMEM, trypsin, cell apoptosis kit, live/dead cell staining kit, Bradford protein concentration determination kit, and DCFH-DA kit were purchased from Jiangsu Keygen Co., Ltd (China). MGC803 (human gastric cancer cells), HCT-116 (human colorectal cells), MCF-7 (human breast cancer cells), HepG2 (human hepatocellular carcinomas cells), and RAW264.7 (mouse macrophage leukemia cells) cell lines were obtained from ATCC (USA). The primary antibodies used in the western blot assay were all purchased from Santa Cruz (USA). Monosaccharide composition analysis was conducted on an Agilent 7890B GC system (USA). Electrophoresis separation of proteins was conducted on Bio-Rad vertical electrophoresis tank (USA). The O.D. value was detected by a microplate reader (Tecan, INFINITE200Pro, Switzerland). Cell apoptosis was measured on flow cytometry (BD Accuri, C6, USA). Cell images were obtained on a laser confocal scanning microscope (Olympus, FV3000, Japan).

Extraction of polysaccharide from *C. militaris*

The CMPs were extracted following the reported literature with some modifications.[26] The *C. militaris* was dried in an air-dry oven at 60°C for 2 h and smashed by a grinder. The powder was filtered by a 100-mesh sieve. Then 20 g of the *C. militaris* powder was extracted by water at 70°C for 4 h. The solid-liquid ratio was 30:1, g/mL. After cooling to room temperature, the mixture was centrifuged at 4000 r/min for 20 min. The supernate was collected and concentrated to 14% of the origin volume. Then the 4-time volume of ethanol was added to the aqueous solution and the mixture was stored at 4°C for 8 h. The solid was collected by centrifugation at 4000 r/min for 20 min and dried in an air-dry oven at 60°C for 2 h. After dissolving with water, the solution was added to $ZnSO_4$ (4% weight of the solid). The mixture was further stored at 4°C for 8 h. Then the mixture was centrifuged at 4000 r/min for 20 min to remove the precipitate. Finally, the solution was freeze-dried to afford the polysaccharide as pale yellow solid (3.52 g). The extraction yield was 17.6%.

Sugar and protein determination

The sugar content was determined by the phenol-sulfuric acid method with D-glucose as a standard.[27] The protein content of the extract was analyzed by Bradford kit, in which BSA (bovine serum albumin) was used as a standard sample.[28]

Monosaccharide composition analysis[29]

The extract was sealed in a tube containing 2 mL of TFA (trifluoroacetic acid, 2 mol/L). Then the mixture was hydrolyzed in an oil bath at 120°C for 4 h. The TFA was evaporated under reduced pressure with a bath temperature below 45°C. Then the hydrolysate with myo-inositol as internal standard was acetylated in presence of acetic acid and pyridine, followed by evaporation of reagents under the same condition as that of TFA. Then the sample (final concentration: ~2 mg/mL) dissolved in dichloromethane was taken to be analyzed by Agilent 7890B GC system equipped with an OV-225 capillary column (0.22 mm × 25 m) (WGA, Dusseldorf, FRG). The GC analysis was conducted following the literature.[29] Briefly, the temperature program was set as 50–230°C with a rate of 2°C/min. The helium carrier gas was set as 1.2 mL/min. The flame ionization detector (FID) was set to 270°C. L-rhamnose, D-arabinose, D-xylose, D-mannose, D-glucose, and D-galactose were also converted to their alditol acetates under the same condition as that of the polysaccharide sample and used as a standard sample to assign the GC peak. All the standard samples were with purity above 98%.

Cell culture

Cells were cultured following the manufacturer's instructions. MGC803, HCT-116, and HepG2 cells were cultured with FBS: RPMI1640 = 1: 9 (v/v) at 37°C in 5% CO_2. MCF-7 and RAW264.7 cells were cultured with FBS: DMEM = 1: 9 (v/v) under the same condition. All the cells were dissociated by trypsin-EDTA except for RAW264.7 cells, which were dissociated by the scraper.

CCK-8 assay

The CCK-8 kit was used according to the protocols provided by the manufacturer. All the cells were seeded into a 96-well plate till the density reached 80%. The culture medium was replaced by 100 μL of fresh culture medium containing 0, 100, 200, 400, 600, 800, and 100 μg/mL of polysaccharides. The cells were further cultured for 72 h at 37°C in 5% CO_2. Then all the cells were added with 10 μL of CCK-8 work solution and the culture medium was thoroughly mixed. Then the cells were further cultured for 2 h at 37°C in 5% CO_2. The microplate reader measured the O.D. values at 450 nm and the IC_{50} values were calculated by SPSS 23.0 software (IBM, USA).

Cell morphology[2]

The cells were cultured in the glass-bottom confocal plate in the same condition as that of the above-mentioned experiments for 72 h with or without 800 μg/mL of polysaccharide. Then the cell images were taken by laser confocal scanning microscope under 40× objective lens using 640 nm laser as the excitation light under a bright field.

Cell apoptosis[30]

Cells were cultured in a 6-well plate under the same condition as the CCK-8 experiment. The concentration of the polysaccharide was 800 μg/mL. After being treated with polysaccharides for 24 h, the cells were collected into a 15-mL tube and washed with PBS (10 mmol/L, pH = 7.4). Then the cells were suspended in 1 × binding buffer (containing 0.01 mol/L of Hepes/NaOH pH = 7.4, 1.40 mol/L of NaCl, 25 mmol/L of $CaCl_2$) and incubated with 100 ng/mL of FITC-Annexin V and 2.00 μg/mL of PI at 25°C for 15 min in dark. Then the apoptosis rate was tested on flow cytometry and evaluated by Cell Quest software (BD Biosciences, USA).

Live/dead cell imaging[31]

The cells were cultured in the glass-bottom confocal plate in the same condition as that of the above-mentioned experiments. The concentration of the polysaccharide was 800 μg/mL. After being treated with polysaccharides for 24 h, the cells were washed with PBS (10 mmol/L, pH = 7.4) twice and incubated with 5.00 μmol/L of Calcein AM and PI, respectively, for 30 min in dark. The cell images were obtained by laser confocal scanning microscope under a 20 × objective lens. Green channel: λ_{ex} = 488 nm, λ_{em} = 500–540 nm; Red channel: λ_{ex} = 561 nm, λ_{em} = 600–640 nm.

Cellular ROS level assessment[32]

The RAW264.7 cells were cultured in the same conditions as the live/dead cell imaging experiment. The cells were administrated with vehicle or 800 μg/mL of polysaccharide for 24 h. Then the culture medium was removed and the cells were incubated with 5.00 μmol/L of DCFH-DA work solution for 30 min in dark. After that, the cells were washed with PBS (10 mmol/L, pH = 7.4) three times and were added to 1 mL of PBS (10 mmol/L, pH = 7.4). The ROS signal was detected by laser confocal scanning microscope under a 40× objective lens. Green channel: λ_{ex} = 488 nm, λ_{em} = 500–600 nm.

Western blots[30]

Cells were cultured in a 6-well plate and treated with 800 μg/mL of polysaccharide under the same condition as that of the above-mentioned experiments. Then the cells were dissociated and lysed by RIPA lysis buffer. The concentration of protein was determined by a BCA kit. The protein was loaded on 15% PAGE gel and separated by electrophoresis. Then the protein was transferred onto the PVDF Immobilon-P membrane. The blots were blocked with 5% defatted milk, followed by incubation with caspase3, Bax, Bcl-2, and β-actin primary antibodies at 4°C overnight. Then the membranes were washed with PBST twice and incubated with peroxidase-conjugated goat anti-mouse IgG (H + L) secondary antibody for 1 h and further washed with PBST twice. The dilution ratio for primary antibodies was 1: 1000. The dilution ratio for secondary antibodies was 1: 8000. The protein expression was measured on a multi-functional imaging system. The gray intensity was evaluated by image J software (National Institutes of Health, USA) and analyzed with Excel software (Microsoft, USA).

RESULTS

Extraction and component analysis

The polysaccharide, a pale yellow solid, was extracted from *C. militaris* according to reported literature,[26] with some modifications. The extraction rate was 17.6%. The percentages of total sugar and protein were 86.92% and 0.80%, respectively [Table 1]. The monosaccharide composition of the extract was also assessed by GC assay. As shown in Table 1 and Figure 1, the molar ratio of the CMPs was 9.50:4.48:1.00:2.40:15.29:7.47 (rhamnose: arabinose: xylose: mannose: glucose: galactose).

In vitro cytotoxicity

To access the *in vitro* anticancer effect of the polysaccharide, we conducted the CCK-8 experiment. As shown in Table 2, the polysaccharide exhibited obvious anticancer effect, whose IC_{50} values ranged from 437.8 ± 15.4 μg/mL to 545.1 ± 19.9 μg/mL ($p = 0.002 < 0.01$). Since the polysaccharide possessed the most potent anti-proliferation capacity toward RAW264.7 cells, further biological research was carried out on this cancer cell line.

Cell morphological study

To vividly depict the anticancer effect of CMPs, a cell morphological study was conducted.[2] As shown in Figure 2, compared with the untreated group, cell crushing and shape change were observed, indicating that the cells were inhibited.

Live/dead cell imaging experiments

The cancer cell inhibitory capacity of the polysaccharide was further confirmed by live/dead cell imaging experiments. Calcein AM and PI were used to label living and dead cells, respectively. Calcein AM can enter living cells and emit green fluorescence after being hydrolyzed by hydrolase. PI, which could not enter living cells, can bind to the DNA of dead cells and emit red fluorescence. Figure 3 illustrated that the treatment of CMPs significantly enlarges the portion of dead cells compared with control, hinting at the obvious anticancer effect of the CMPs.

Cell apoptosis

The inhibitory effect of polysaccharides toward RAW264.7 cells was further studied by cell apoptosis assay. As shown in Figure 4, after incubation with CMPs, the apoptosis rate (early apoptosis and late apoptosis) of RAW264.7 cells was upregulated from 8.34% to 27.66%. These results indicated that the CMPs could suppress cancer cell proliferation by inducing cell apoptosis.

Cellular ROS level

To further explain the cancer cell inhibitory effect of the polysaccharide extracted from *C. militaris*, cellular ROS level in CMPs-treated

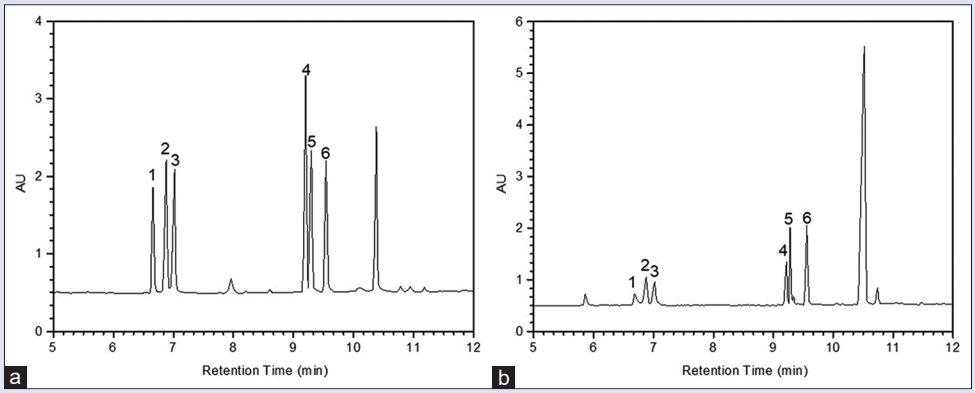

Figure 1: GC spectra of (a) standard monosaccharides and (b) CMPs. Peak assign: 1. Rhamnose, 2. Arabinose, 3. Xylose, 4. Mannose, 5. Glucose, 6. Galactose

Table 1: Total sugar and protein percentage and monosaccharide composition of CMPs

Total Sugar (%)	Protein (%)	Monosaccharide (molar ratios)					
		Rhamnose	Arabinose	Xylose	Mannose	Glucose	Galactose
86.92±2.950	0.80±0.066	9.50	4.48	1.00	2.40	15.29	7.47

Table 2: IC_{50}[a] values of CMPs

Cancer cells	MGC803	HCT-116	MCF-7	HepG2	RAW264.7
IC_{50} values (μg/mL)	459.6±12.90	545.1±19.90	496.7±11.20	521.9±18.60	437.8±15.40

[a]The data were representative of three independent experiments and the mean±S.D. is shown

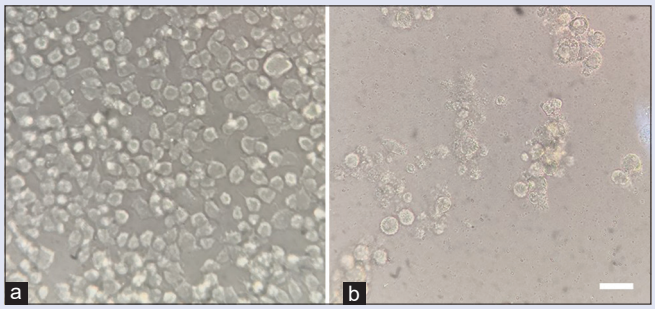

Figure 2: Cell morphology images of RAW264.7 cells. (a) Control. (b) RAW264.7 cells were treated with 800 μg/mL of CMPs. All the cells were cultured for 72 h with or without polysaccharide administration. The pictures were taken with a 40× objective lens. Scale bar: 40 μm

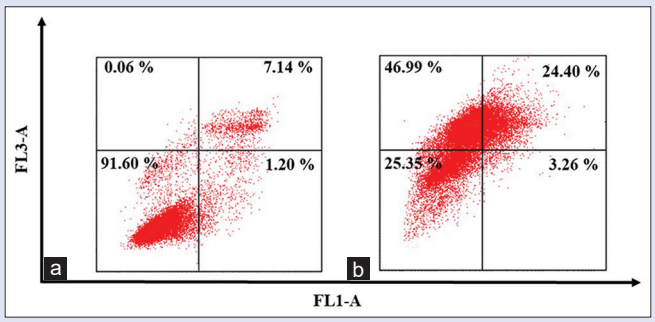

Figure 4: Cell apoptosis results of RAW264.7 cells. (a) Control. (b) RAW264.7 cells were treated with 800 μg/mL of CMPs for 24 h

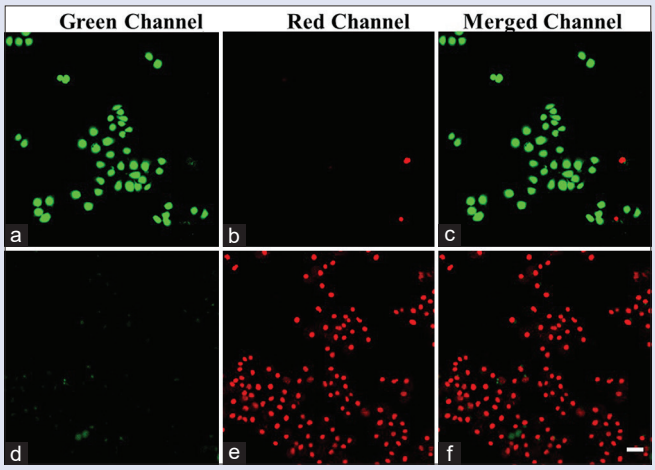

Figure 3: Live/dead cell images of RAW264.7 cells. (a, b, c) Control. (d, e, f) RAW264.7 cells were treated with 800 μg/mL of CMPs. Green channel: λ_{Ex} = 488 nm, λ_{Em} = 500–540 nm; Red channel: λ_{Ex} = 561 nm, λ_{Em} = 600–640 nm. The pictures were taken by 20× objective lens. Scale bar: 40 μm

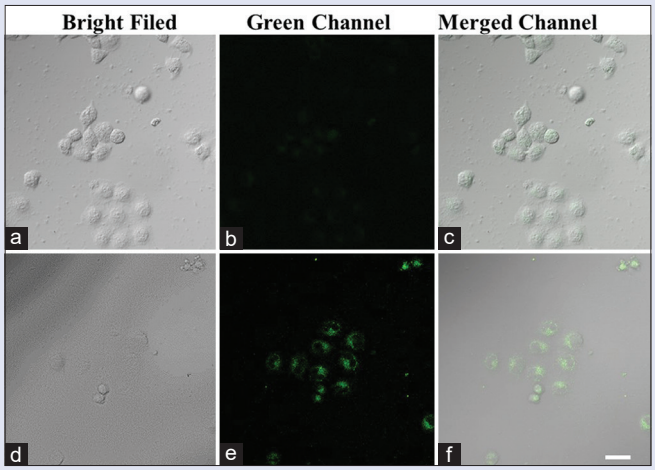

Figure 5: Cellular ROS assessment of RAW264.7 cells. (a, b, c) Control. (d, e, f) RAW264.7 cells were treated with 800 μg/mL of CMPs for 24 h. Green channel: λ_{Ex} = 488 nm, λ_{Em} = 500–600 nm. The pictures were taken with a 40× objective lens. Scale bar: 40 μm

RAW264.7 cells was evaluated by DCFH-DA assay.[32] DCFH-DA is a ROS fluorescent probe that can increase its green fluorescence in cells when the ROS level was upregulated. As shown in Figure 5, after being treated with 800 μg/mL of CMPs, green fluorescence was observed. This phenomenon was in accordance with the previous report.[25] While there was only negligible fluorescence in the untreated group, which could be considered as a background signal. These results demonstrated that the treatment of CMPs could increase the cellular ROS in RAW264.7 cells and so that induces cell apoptosis.

Western blots assay

Western blots study was conducted to explain the mechanism of the anticancer effect of CMPs. In this study, the expression level of endogenous apoptosis pathway-related proteins, including caspase3, Bax, and Bcl-2, were measured.[31,33] As depicted in Figure 6, caspase3 and Bax, a pro-apoptosis protein, were both upregulated and the expression of anti-apoptosis protein Bcl-2 was downregulated, which was in accordance with the previous report.[24] These findings hinted that the administration of CMPs could activate the endogenous apoptosis pathway.

DISCUSSION

Despite tremendous efforts that have been made to fight against cancer, the leading cause of death all over the world, there is still no

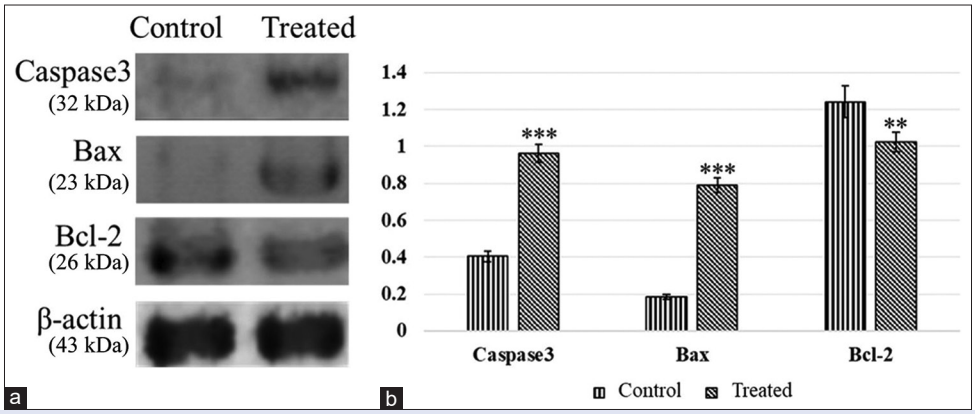

Figure 6: Western blots result of RAW264.7 cells with or without administration of 800 μg/mL of CMPs for 24 h. (a) Blots. (b) Gray intensity analysis of the bolts. The data were representative of three independent experiments and the mean ± S.D. is shown. ***$p < 0.001$, **$p < 0.01$. Two side Student's t-test

effective solution to address this problem.[16] For example, although chemotherapy could kill cancer cells *in vitro*, it faces a variety of embarrassments in clinical usage, including drug resistance, severe side effects, etc.[15,34] Therefore, an alternative that could prevent or treat cancer with safe behavior seems attractive. Daily used natural products are precious candidates for this purpose. Polysaccharides from the natural product might be a potential agent for cancer prevention or treatment.[29,35]

There are several excellent works about the anticancer effect of CMPs.[2,9,24,25] Some of them have revealed that the anticancer behavior of CMPs comes from the upregulation of caspase3 via western blot assay.[24] While its upstream mechanism remains unclear. Much of the studies focus on the antioxidant effect of polysaccharides.[36] Possessing the cell-protective effect, CMPs were studied in anticancer research aiming to reduce the side effects of chemo drugs. Tai and his co-workers demonstrated that the CMPs could attenuate the cytotoxicity of doxorubicin in chemotherapy.[16] Like a double-edged sword, polysaccharides also possess the ability to arouse oxidative stress in cells. Conducting the DCFH-DA staining and flow cytometry assay, Sun[37] and Matsukura[38] demonstrated that polysaccharides could increase the cellular ROS level and so that induces cell apoptosis. While they did not discuss the endogenous apoptosis pathway. To this end, we explored the linkage between the upregulated ROS level aroused by CMPs and the activation of the endogenous apoptosis pathway to explain its underground mechanism of anticancer effect. The anticancer effect of the CMPs was confirmed and the results were similar to that of other reports.[2,9,24] Importantly, the DCFH-DA staining assay indicated that after being treated with CMPs, the ROS signal was observed in RAW264.7 cells. Then the endogenous apoptosis pathway was evaluated to be activated by a western blot experiment. To our knowledge, this work explained the detailed mechanism of the anticancer effect of the CMPs for the first time. The CMPs could upregulate the cellular ROS level, activate the endogenous apoptosis pathway, and finally induce cell apoptosis. This study, in our opinion, gave direct evidence that CMPs could increase cellular ROS levels and activate endogenous apoptosis pathways in cancer cells. The anticancer study is one of the most important parts of the CMPs research. While the usage of CMPs anticancer effect still remains in the laboratory stage. An elaborate study mechanism could point the way to the anticancer research of CMPs. The finding of the ROS-endogenous apoptosis pathway mechanism put a foundation for the further development of CMPs. For instance, CMPs alone exhibit limited anticancer effect, which needs a supplementary element to prevent or cure cancer. The explanation of the mechanism could transparentize the pathway-related proteins to make synergistic therapy possible. Together with other daily used natural products, CMPs might possess unexpected anticancer effects.

CONCLUSION

In this work, we extracted 3.52 g of polysaccharide from 20 g of *C. militaris* by water-extraction and alcohol-precipitation method. The extraction rate was 17.6%. The anticancer effect of the CMPs was tested by CCK-8 assay. The IC_{50} values were around 500 μg/mL, among which the best result was 437.8 ± 15.4 μg/mL toward RAW264.7 cells. The *in vitro* anticancer activity was further studied by cell morphology study and live/dead cell imaging experiment. The results showed that the polysaccharide could effectively cause cancer cell death. A cell apoptosis study revealed that the CMPs could suppress cell proliferation by inducing cell apoptosis. The cellular ROS study indicated that the polysaccharide could increase the ROS level in RAW264.7 cells. Western blot experiment proved that the CMPs could activate the endogenous apoptosis pathway. Taking together, the study revealed that the CMPs could upregulate cellular ROS levels and so arouses the endogenous apoptosis pathway, then finally inducing cell apoptosis. This work also faces some limitations. In this study, the factors of extraction temperature[2] and duration, harvest season of the *C. militaris*, and the maximum tolerated dose of the CMPs were not considered. In future research, we would focus our interest on the above issues. In the end, we assume that the co-administration of CMPs and other natural food-based anticancer components might possess a synergistic effect and exhibit unexpected surprise. So, the research on the combined treatment of CMPs and other effective elements might be meaningful.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

Author contributions

Fenglin Li and Yumiao Ma conducted most of the experiments. Wuyang Hua analyzed the data and wrote the manuscript. Yanxia Liu confirmed the authenticity of all the raw data. Li Li, Zhongkui Lu and Xiaokun Jiang conducted the HPLC study. Chao Liu and Jingxue Liu reviewed the manuscript.

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