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Review

Advance in *Cordyceps militaris* (Linn) Link polysaccharides: Isolation, structure, and bioactivities: A review

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ABSTRACT

Cordyceps militaris is a unique and precious medical fungus in Chinese Cordyceps, which has been widely used as the traditional medicines or as biocontrol agents against pests in China for centuries. Polysaccharides are one of bioactive constituents in *Cordyceps militaris* with a variety of biological activities, including immunomodulation, antioxidant, anti-tumor, and anti-aging activities, among others. However, natural *Cordyceps militaris* are very rare and expensive, most of literatures indicated that the polysaccharides were mostly extracted from artificially cultivated fungal fruiting bodies (intracellular polysaccharides) or mycelia fermentation broths (extracellular polysaccharides). Moreover, separation and purification of polysaccharides was a very complicated and cumbersome process. Nevertheless, a large number of polysaccharides were purified and its characterization was elucidated by structure and biological activities. However, the relationship between structure and activity of polysaccharides has not been well established. Therefore, this review detailed the recent advance in several aspects (i.e., extraction, isolation, structure, and bioactivities) of the polysaccharides from fruiting body of *Cordyceps militaris*. This information could provide theoretical basis for the research on related polysaccharides, and also have important reference value in the field of functional foods and medicine in the future.

Contents

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1. Introduction

Cordyceps militaris (*C. militaris*), a scorpion that infects lepidopteron insects, was consisting of two parts: the stalk (the grass part, also known as the fruit body) and the sclerotium (the corpse part of the insect). Briefly, it is combination of the worm and the grass [1,2]. In China, it is a very precious herbal medicine, which also called "YongChongCao" in Chinese [3–6]. The natural *C. militaris* fruiting body-caterpillar is greatly distributed worldwide from 0 to >2000 m [7–10]. Interestingly, *C. militaris* is also known as orange *Cordyceps sinensis*, which is easily cultivated in liquid and solid media, and with a variety of carbon and nitrogen sources. *C. militaris* have similar chemical composition and medicinal properties with *Cordyceps sinensis*, they are increasingly considered as substitutes for *Cordyceps sinensis* [11–22]. The *C. militaris* fruiting body-caterpillar complexes were showed in Fig. 1.

C. militaris has been used as a tonic for hundreds of years in China [23]. Previous pharmacological studies have shown significant therapeutic effects on a variety of diseases and conditions, including respiratory, renal, hepatic, neurological and cardiovascular diseases, as well as tumors, aging, hyposexuality and hyperlipidemia [16,18,24–31]. It is precisely because of the enormous application potential of natural *C. militaris*, and its limited production has been unable to meet the growing demand. Therefore, fermentation technology has begun to be widely used in the mass production of *C. militaris* fungal mycelium and other useful ingredients [32–36]. Some studies have found that fermented mycelia of some fungal strains have shown similar pharmacological effects with fungal materials and have been widely used in various food health products [14,37].

The various pharmacological effects of *C. militaris* are attributed to the chemical components, mainly including polysaccharides, proteins, cordycepin, adenosine, ergosterol, and myriocin, etc. [33,38–44]. Especially, polysaccharide is one of the most abundant and important components of many biologically active components in fungi, which was extracted and separated from fruiting bodies, mycelium and fermentation broth, which can exhibit a variety of various physicochemical properties. In addition, polysaccharides have been the target of the development and quality control of *C. militaris* health products. To the best of our knowledge, there has been no review about the extraction, isolation, structure, and bioactivities of *C. militaris* polysaccharides. In addition, the relationship between the structure and biological activity of *C. militaris* polysaccharides has not been clarified. Therefore, this review mainly focus on separation techniques, structural features and bioactivities of intracellular polysaccharides (IPSs) and extracellular polysaccharides (EPS) from the natural fruiting body of *C. militaris*, cultured mycelia, and the mycelia fermentation. Herein, this review summarizes the recent research on the extraction, isolation, and purification of *C. militaris* polysaccharides as well as the characterization of their structural features, chain conformations, and biological activities.

2. Extraction, isolation and purification of polysaccharides

C. militaris polysaccharides can be divided into intracellular polysaccharides (IPSs) and extracellular polysaccharides (EPSs) according to their location in fungal cells. In general, pure water, acidic/alkaline solution, heating buffer solution can usually be used to extract from the *C. militaris* fruit body and mycelium [43,45–53]. Extraction of fungal polysaccharides with hot water or boiling water is the most common and convenient method. However, hot water extraction has the disadvantages of high heating temperature, long extraction time, and low extraction yield, etc. [54,55]. Based on above limitation, some novel extraction methods have emerged to improve the extraction efficiency, including subcritical water extraction (SWE) [56,57], ultra-high pressure extraction (UPE) [58], microwave extraction (ME) [59], and ultrasonic extraction (UE) [60]. It was worth noting that ultrasonic assisted extraction (UAE) has attracted more and more attention for extracting polysaccharides from different plant resources [61]. The reason why UAE increases the yield of polysaccharides is mainly due to the mechanical effects of ultrasound, especially the shear forces generated by the effects of ultrasonic cavitation [62,63]. In addition, for the extraction step of EPSs, the fermentation broth of *C. militaris* is centrifuged and concentrated, and then ethanol is added to obtain the precipitate. Finally, the crude extracellular polysaccharides were collected after centrifugation. The steps of extracting polysaccharides from *C. militaris* were summarized in Fig. 2.

The extracts should be further purified by deproteinization using the Sevage method with the mixture of chloroform and 1-butanol (4:1), and then dialyzed and freeze-dried before characterization. After that, the crude fungal polysaccharides were obtained. The next step is to dissolve and decolorize the crude polysaccharides. The solution can be

Fig. 1. *Cordyceps militaris* (Linn) Link fruiting body-caterpillar complexes: morphology and natural habitat.

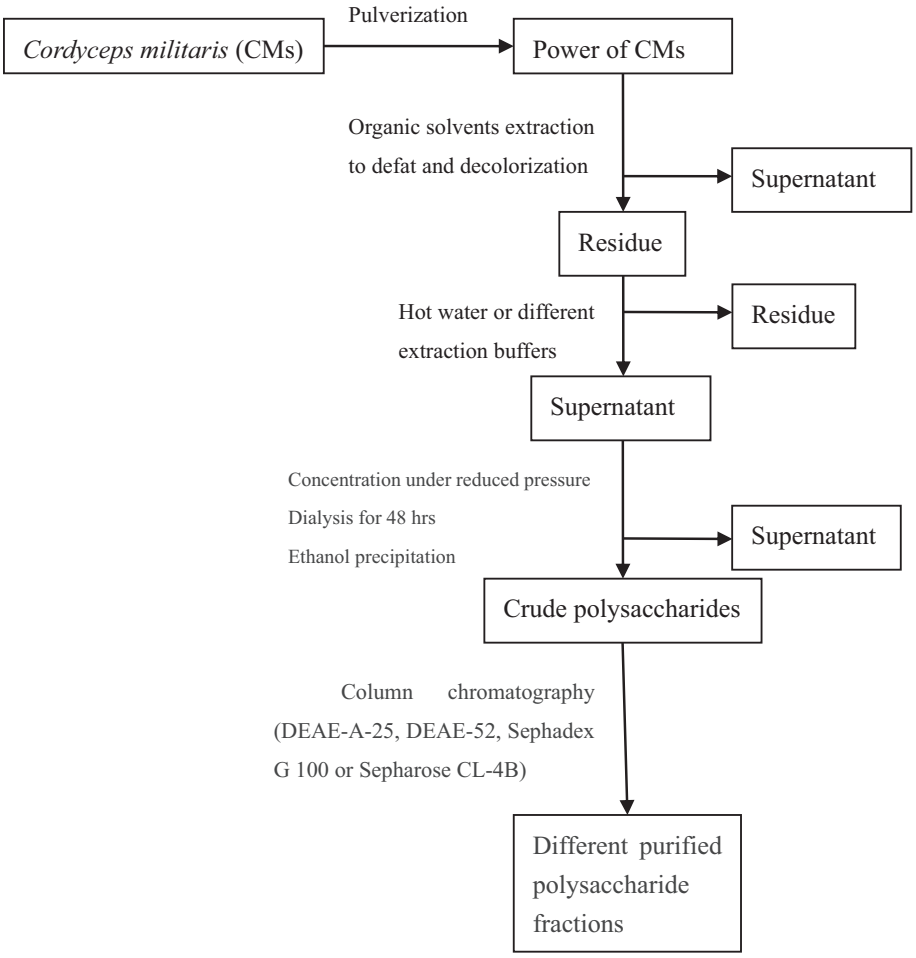

Fig. 2. Flow chart of purifying polysaccharides from *Cordyceps militaris*.

applied to various column chromatography, such as anion exchange chromatography, gel filtration chromatography or affinity chromatography, eluting with an appropriate running buffer, and then concentrating, dialyzing and freeze drying, the pure polysaccharides can be obtained [64–72].

3. Chemical and structural characteristics

The chemical structure of fungal polysaccharides is complex. The polysaccharides extracted from the same raw material also could have different structures and exhibit different biological activities. In general, the chemical structural features of polysaccharides are defined by its monosaccharide composition, configuration of glycosidic linkages, position of glycosidic linkages, sequence of monosaccharide, solubility, and rheological properties, etc. [73–76]. There are significant differences in monosaccharide composition and chemical structure of wild or artificially cultivated *C. militaris* polysaccharides. A large number of previous studies have characterized extracellular polysaccharides (EPSs) or intracellular polysaccharides (IPSs) extracted from *C. militaris*. The main techniques of polysaccharides characterization were mainly used including liquid nuclear magnetic resonance (one and two dimensions), solid-state NMR, methylation analysis, infrared spectroscopy, gas chromatography (GC), gas chromatography mass spectrometry (GC–MS), high performance liquid chromatography (HPLC), complete acid hydrolysis, partial acid hydrolysis, periodic acid oxidation and smith degradation [43,46,47,49,54,77]. The sources, molecular properties, chemical structures, and bioactivities were summarized in Table 1.

3.1. Monosaccharide composition

In general, the monosaccharide composition analysis should firstly break the glycosidic bond by acid hydrolysis, then derivatives it, and finally performs quantitative detection by GC. Besides, high-performance anion exchange chromatography with pulsed amperometric detection has been applied to monosaccharide composition analysis to replace traditional methods, because it doesn't require the derivatization of monosaccharides [95]. Recently, the monosaccharide composition had been also determined by using a 1-phenyl-3-methyl-5-pyrazolone pre-column derivatization method [96–98].

Although a large number of different polysaccharides were extracted from IPSs and EPSs, the monosaccharide composition is mostly composed of mannose (Man), glucose (Glu), and galactose (Gal) in different molar ratios [47,49,52,56,77,82–84,99]. In addition, some monosaccharides such as rhamnose (Rha), xylose (Xyl), and arabinose (Ara) were also found in some extracted polysaccharides [43,45,46,48,58,89]. The different monosaccharide component with various molar ratios of polysaccharides may be related to raw materials, separation method and purification method, etc.

3.2. Average molecular weight

Currently, techniques for determining the average molecular weight and polydispersity index of polymers mainly include osmometry, viscosity measurement, sedimentation, and HPLC [100–104]. It is worth noting that high performance gel permeation

Table 1
Polysaccharides originated from *Cordyceps militaris* fungi: source, chemical structure and bioactivities.

Living strains	Polysaccharides source	Extraction medium	Components	Molecular weight	Types of linkages	Bioactivities	References
Cordyceps militaris	Mycelium	Hot water	Rha:Xyl:Man:Glu:Gla = 1:6.3:25.6:16.0:13.8	23 kDa	1 → 2, 1 → 3, and 1 → 4 linkages	Humoral immunity	[43]
Cordyceps militaris	Mycelium	Hot water	Rha:Glu:Gla = 1:4.62:2.43	13 kDa	–	–	[45]
Cordyceps militaris	Mycelium	Hot water	α-Glu	5 kDa	1 → 4,1 → 6 linkages	–	[45]
Cordyceps militaris	Mycelium	Hot water	Ara:Gal:Man:Glu = 1:2.23:7.06:9.79	8.9 kDa	α-D-Glu, α-D-Man	Antioxidant activities	[46]
Cordyceps militaris	Mycelium	Hot water	Man:Glu:Gal = 1:14.95:1.18	–	1 → 4, 1 → 3,6 linkages	Antioxidant activities	[77]
Cordyceps militaris	Mycelium	Alkaline	Man:Gal:Glu = 3.15:4.34:1	–	1 → 4, 1 → 6 linkages	Antioxidant activities	[47]
Cordyceps militaris	Mycelium	Hot water	–	–	–	Immunomodulatory and antioxidant	[78]
Cordyceps militaris	Mycelium	Subcritical water	Man:Glu:Gal = 2.05:1:1.09	460 kDa	1 → 3, 1 → 4, 1 → 4,6, 1 → 6 linkages	Immunostimulatory activity	[56]
Cordyceps militaris	Mycelium	Hot water	–	1.2 kDa	–	α-Glucosidase inhibitory activity	[79]
Cordyceps militaris	Mycelium	Hot water	–	–	–	Immunostimulatory activity	[80]
Cordyceps militaris	Mycelium	Hot water	–	–	–	Immunomodulatory and antioxidant	[81]
Cordyceps militaris	Mycelium	2 M NaOH solution	Xyl:Glu:Rha = 2.19:6.73:1	28 kDa	1 → 2, 1 → 3,1 → 4, 1 → 6 linkages	α-Glucosidase inhibitory activity	[48]
Cordyceps militaris	Mycelium	Hot water	Man:Gal:Glu = 3.22:1.35:1	42 kDa	1 → 4, 1 → 6, and 1 → 3,6 linkages	Antioxidant activity	[82]
Cordyceps militaris	Cultured mycelia	Hot water	Man:Gal:Glu = 7.87:2.03:1	210 kDa	1 → 4, 1 → 6 linkages	Immunostimulatory activity	[83]
Cordyceps militaris	Culture both	Ethanol	Man:Gal:Glu = 10.64:4.59:1	36 kDa	β-Linked polysaccharide	Immunostimulatory activity	[84]
Cordyceps militaris	Mycelium	Alkaline	Man:Gal:Glu = 6.44:3.92:1	23 kDa	Glucogalactomannan	–	[49]
Cordyceps militaris	Mycelium	Ultrasound, hot water	Glu:Rha = 23.02:1	4.3 kDa	–	Immunity, anti-oxidation, anti-tumor	[60]
Cordyceps militaris	Culture both	Ethanol	–	–	–	Immunostimulatory activity	[50]
Cordyceps militaris	Mycelium	Hot water	Glu:Ara:Man = 62:1.6:1	2.86 kDa	–	Antihyperlipidemic, hepatoprotective, and antioxidant activities	[51]
Cordyceps militaris	Mycelium	Hot water	Man:Glu:Gal = 1:28.63:1.41	1.402 kDa	1 → 3,1 → 4 linkages	–	[52]
Cordyceps militaris	Culture both	Hot water	Man:Glu:Gal = 1:12.41:0.74	1.273 kDa	1 → 3,1 → 4 linkages	–	[52]
Cordyceps militaris	Mycelium Culture both	Ethanol	–	–	–	Anti-inflammatory	[28]
Cordyceps militaris	Mycelium	Hot water	–	–	–	Antitumor activities	[85]
Cordyceps militaris	Mycelium	Hot water	–	–	–	Antitumor activity	[86]
Cordyceps militaris	Mycelium	Ultrahigh pressure	Man:Rha:Gal:Glu = 38.3:1:13.5:15.9	43.6 kDa	–	Antioxidant activity	[58]
Cordyceps militaris	Mycelium	Hot water	–	–	–	Immunostimulating activity	[87]
Cordyceps militaris	Mycelium	Hot water	–	–	–	Immunostimulating activity	[88]
Cordyceps militaris	Cultured mycelia	Hot water	Rha:Ara:Man:Gal = 1.48:11.34:11.62:1	–	1 → 2, 1 → 4, 1 → 6 linkages	Antihypoxic effect	[89]
Cordyceps militaris	Mycelium	Simulated gastric juice	Ara:Man:Gal = 1:2.89:2.03	9.3 kDa	–	Anti-oxidation, anti-tumor	[90]
Cordyceps militaris	Mycelium	Hot water		20.2 kDa	1 → 2, 1 → 4, 1 → 6 linkages	Immunomodulatory and anti-aging activities	[91]
Cordyceps militaris	Mycelium	Boiling water	Gal:Ara:Xyl:Rha = 9.1:4.3:1.2:1		Araf-(1→, →5)-Araf-(1→, →4)-Galp-(1 → and →4)-GalAp-(1 → residues	Immunostimulating activity	[92]
Cordyceps militaris	Mycelium	Microwave	–	–	–	–	[93]
Cordyceps militaris	Mycelium	Hot water	Man:Glu:Gal = 1.52:8.53:1	–	–	Antioxidant activity	[94]

chromatography (HPGPC) is the most widely used method for determining molecular weight distribution and is also applied to the determination of molecular weight in IPSs and EPSs [76]. In addition, high performance size-exclusion chromatograph (HPSEC) which equipped with a MultiAngle Laser Light Scattering detector (MALLs) are also a powerful method for assessing the absolute molecular weight (Mw) of polysaccharides and have higher resolution than traditional gel permeation chromatography (GPC) [105–107]. Through literatures investigation, the molecular weight distribution of *C. militaris* polysaccharides obtained under various source materials and experimental conditions is between ~10^3 Da and ~10^5 Da [45,56,83,84,91].

3.3. Chemical structures

Although different *C. militaris* polysaccharides were obtained in different research groups, only a small amount of structural information was published. The basic structural characteristics of some polysaccharides extracted from *C. militaris* are listed below. The polysaccharides extracted from *C. militaris* with hot water (60–70 °C) might contain mannose bonded by (1 → 2) linkage, xylose bonded by (1 → 4) linkage, and rhamnose bonded with galactose by (1 → 2) or (1 → 3) linkage [43]. A water soluble polysaccharide (CPS-3) isolated from cultured *C. militaris*, which was composed of a α-(1 → 4)-D-glucose and a α-(1 → 6)-D-glucose at 6-O positions once in every eight glucose residues

[45]. A purified *C. militaris* polysaccharide was consisted of (1 → 4)-linked-galactose and (1 → 3, 6)-linked mannose which existed in the branch might be unleashed from the main chain of (1 → 4)-linked-glucose according to the results of FT-IR and ^{13}C NMR [77]. A novel polysaccharide (CBP-1) was isolated from the fruiting body of cultured *C. militaris* by alkaline extraction and its structural features were determined by partial hydrolysis, methylation analysis, GC–MS, ^{13}C NMR, HPAECPAD, FT-IR and HIO_4 oxidation-Smith degradation. The results showed that CBP-1 has a backbone of α-(1 → 4)-D-mannose residues which occasionally branches at O-3 and the branches were mainly composed of α-(1 → 4)-D-glucose residues and β-(1 → 6)-D-galactose residues, and terminated with β-D-galactose residues [47]. Our team also used subcritical water extraction (SWE) to obtain an acidic polysaccharide (CMP-S1) and a neutral polysaccharide (CMP-W1) from cultured *C. militaris*. We found that most sugar residues of CMP-S1 were 1→, 1 → 6, 1 → 2, 1 → 2, 6, 1 → 4, and 1 → 4, 6 linked, and mannose and glucose in CMP-S1 were 1 → 3 linked. However, we may supposed that mannose, as the main chain of the CMP-W1, was connected with 1 → 3, 1 → 2,3, 1 → 2,4, 1 → 3,4, 1 → 3,6, or 1 → 2,3,4 glycosidic bond, and glucose and galactose could be connected with 1→, 1 → 2,6, 1 → 4, or 1 → 4,6 glycosidic linkages in branches chain [56]. One low molecular weight polysaccharide (LCMPs-II) was obtained from the crude *C. militaris* polysaccharides (CMPs). The results showed that LCMPs-II was 1, 3-branched-rhamnoxyloglucan which had a linear backbone of (1 → 4)-linked α-D-glucopyranose (α-d-Glc*p* units) [48]. The water-soluble polysaccharide (P70-1) was purified from crude *C. militaris* polysaccharides by DEAE cellulose-52 and Sephacryl S-100 HR columns. Structural features analysis showed that P70-1 has a backbone of (1 → 6)-linked β-D-mannopyranosyl residues, which occasionally branches at O-3. The branches were mainly composed of (1 → 4)-linked α-D-glucopyranosyl and (1 → 6)-linked β-D-galactopyranosyl residues, and terminated with β-D-galactopyranosyl residues and α-D-glucopyranosyl residues [82]. A high molecular weight polysaccharide (CPMN Fr III) was obtained from cultured mycelia of *C. militaris* (CPM) by hot water extraction, this polysaccharide has a random coil conformation of the β-1,4-branched-β-1,6-galactoglucomannan [83]. The water-soluble polysaccharides (CPSN Fr II) obtained from the liquid culture both of *C. militaris*, the configuration of the β-linkage and random coil conformation of CPSN Fr II were confirmed by using a Fungi-Fluor kit and Congo red reagent, respectively [84]. The polysaccharides were extracted with 5% KOH solution from *C. militaris* dried fruiting bodies, which were purified by freeze-thawing treatment, and dialysis (100 kDa). The homogeneous polysaccharides (Mw 23,000 Da) showed that the main chain was connected with 2,3,4-Me_3-Manp (11.9%) and 3,4,6-Me_3-Manp (28.6%). The branches were (1 → 6)-linked-α-D-Man*p* or (1 → 2)-linked-α-D-Gal*f*, terminating with β-D-Gal*f*, α-D-Gal*f*, α-D-Gal*p*, or α-D-Man*p*. 42.7% of the partially hydrolyzed product consisted of 3,4,6-Me3-Manp, suggesting a (1 → 2)-linked backbone [49]. Two polysaccharides (CMPS-II and CBPS-II) were obtained from the fermented mycelium and cultivated fruiting bodies of the *C. militaris*. Their structural features were investigated by a combination of chemical and instrumental analysis. The results showed that both of CMPS-II and CBPS-II were 1,3-branched-galactomannoglucan that had a linear backbone of (1 → 4)-linked α-D-glucopyranose (Glc*p*) [52]. A purified polysaccharide (CMN1) was obtained from *C. militaris* by a DEAE-52 cellulose anion exchange column and a Sepharose G-100 column. The results showed that the backbone of CMN1 comprised (1 → 2) and (1 → 3) linkages, with branched (1 → 6) and (1 → 4) linkages [89]. In addition, a polysaccharide (CP2-c2-s2) from *C. militaris* (CMP) was investigated. Their results revealed that CP2-c2-s2 is a β-pyran polysaccharide, probably with 1 → 2, 1 → 4, and 1 → 6 glycosyl linkages [91]. Meanwhile, an acidic polysaccharide (APS) was extracted from *C. militaris* grown on germinated soybeans. On the basis of the result of methylation analysis, APS was considered to be mainly composed of Ara*f*-(1→, →5)-Ara*f*-(1→, →4)-Gal*p*-(1 → and →4)-GalA*p*-(1 → residues [92].

3.4. Conformational features

The biological activity of polysaccharides is related to molecular weight, chemical structure, and chain conformation. Generally speaking, polysaccharides may exhibit different chain conformations in solution, such as single helix [108], double helix [109], triple helix [110], aggregates [111], random coil [112], rod-like structures [113], sphere-like structures [114]. However, there are few reports on the solution properties and chain conformation of the *C. militaris* polysaccharides. The literatures about the chain conformation of the polysaccharides from *C. militaris* were listed below. For example, the morphological characteristics of *C. militaris* polysaccharide (CPS) were carried out by SEM and AFM. Results suggested that the surface topography of CPS was smooth with balls on the edge of the chain structure, which indicated that CPS molecules existed crosslinking to form mesh structure [77]. In addition, some polysaccharides were separated and purified from cultured *C. militaris*. The results showed that the heights of spherical structures are higher than a single polysaccharide chain, which revealed that these polysaccharides were branched and formed aggregation [56,90,91]. The solution behavior of polysaccharides (CPS) obtained from *C. militaris* were determined by Congo red assay, circular dichroism spectra and Atomic force microscopy (AFM). The results revealed that CPS can be complexed with Congo red, which indicating that it has a triple helix structure. Interestingly, DMSO can change the intramolecular hydrogen bonds and destroy the triple helix structure of CPS [48,52,79]. Some studies have also obtained some polysaccharides with random coil conformation [83,84]. There is also a report that the ultrastructure of *C. militaris* polysaccharide composed of a sheet-like appearance and randomly distributed ovoid-shape particles [60]. Therefore, the polysaccharides extracted from *C. militaris* have a chain conformation, mainly including agglomeration, triple helix structure, and random coil conformation.

The relationships among solution behavior, chain conformation, chemical structure and biological activity are difficult to explain. Therefore, the chain conformational properties of *C. militaris* polysaccharides need to be further studied by other techniques, such as static and dynamic laser light scattering, viscosity analysis based on dilute polymer solution theory, transmission electron microscopy, AFM-based single-molecule force spectrum, fluorescence spectroscopy, and NMR spectrum [115]. For some new methods, such as dilute solution theory, molecular modeling and computer-aided energy minimization can also be applied to the analysis of polysaccharides chain conformation [116–118].

4. Bioactivities

Previous reviews have demonstrated the pharmacological and biochemical aspects of *C. militaris* from various laboratories [3,17,18,119,120]. Polysaccharide is the most important biological active ingredient in *C. militaris*, and its health effects and pharmacological activities have been confirmed according to a large number of animal and clinical experiments. The various biological activities and health benefits of *C. militaris* polysaccharides are summarized and discussed in detail below.

4.1. Immunomodulatory activity

For natural polysaccharides, immunomodulatory effects are one of its most important biological functions, which are related to its putative role as a biological response modifier [121]. In general, the immunostimulatory and immunosuppressive properties of *C. militaris* polysaccharides are assessed by using natural killer cells, T cells, B cells and macrophage-dependent immune system responses [56,80,81,83]. The phagocytosis of phagocytic cells is the first step in response when a pathogen invades the human body. In addition, macrophages rapidly secrete pro-inflammatory factors (e.g., tumor necrosis

factor (TNF)-α and interleukin (IL)-1) and release cytotoxic and inflammatory molecules to protect against pathogen invasion [e.g., nitric oxide (NO) and reactive oxygen species (ROS)] [122].

Most studies on the immunological activity of *C. militaris* polysaccharides were evaluated by activating macrophages. Previous study demonstrated that the mechanism of macrophage activation induced by a novel polysaccharide (PLCM) from *C. militaris* culture broth. The results showed that PLCM could enhance the immunostimulatory activity of RAW264.7 macrophages, including the release of toxic molecules (NO and SOD), the release of cytokine tumor necrosis factor (TNF)-α, and the phagocytosis of macrophages. In addition, PLCM induces the specificity of mitogenactivated protein kinase (MARK) and nuclear factor kappa B (NF-κB) to inhibit the production of nitric oxide and the uptake of phagocytic cells. Moreover, antibodies specific to the extracellular domain of Toll-like receptor-2, Tolllike receptor-4 or the macrophage receptor Dectin-1 significantly attenuated PLCM-induced secretion of TNF-α [50]. Cordyceps polysaccharide can overcome CY-induced immunosuppression, increase the index of spleen and thymus, and significantly enhance the activity of spleen lymphocytes and the function of macrophages [78]. Two polysaccharides (CMP-W1 and CMP-S1) obtained from *C. militaris* by SWE could significantly promoted lymphatic spleen cell proliferation of mice [56]. The functional polysaccharides (CMP_{40} and CMP_{50}) were extracted from *C. militaris*, which can significantly enhance lymphocyte proliferation, serum antibody titers, rove serum interferon-gamma and interleukin-4 concentrations. *C. militaris* polysaccharides was able to up-regulate the functional events mediated by activated macrophages, such as production of nitric oxide (NO)/reactive oxygen species (ROS) and expression of cytokines (IL-1β, IFN-γ and TNF-α) [81,83,84,87], which also can significantly stimulated the proliferation of T and B lymphocytes [91].

4.2. Antioxidant activity

Oxidation can cause a variety of diseases including diabetes, arteriosclerosis, nephritis, cancer, and so on [123–125]. Previously, a large number of studies have extracted antioxidants from plants, fungi and seaweeds, which can be used as nutraceuticals and functional foods, and have been widely used for health protection and disease prevention [126]. At present, antioxidant activity has been one of the research focuses in the determination methods and activity index of Chinese herbal medicine nutrition and therapeutic mechanism [127–130].

Three polysaccharides (W-CBP50, W-CBP50I and W-CBP50II) were isolated from the fruiting bodies of *C. militaris*, where W-CBP50II exhibited strong stable free radical 1,1-diphenyl-2-picrylhyrazyl (DPPH) scavenging activity, while W-CBP50 and W-CBP50I had strong ability to scavenge DPPH, hydroxyl radicals and superoxide radicals [46]. A novel polysaccharide which named CBP-1 was extracted and purified from the fruiting body of cultured *C. militaris* by alkaline extraction. The results showed that CBP-1 has strong hydroxyl radical scavenging activity with IC_{50} value of 0.638 mg/mL in the in vitro antioxidant assay [47]. Similarly, a polysaccharide (P70–1) obtained from the fruiting bodies of cultured *C. militaris* by hot water extraction was also found to possess hydroxyl radical-scavenging activity with an IC_{50} value of 0.548 mg/mL [82]. There are also some related literatures reporting that polysaccharides extracted from cultured *C. militaris* have a variety of antioxidant activities, including DPPH free radical scavenging, ferrous ions chelating ability, and ferric reducing antioxidant power (FRAP) [58,60,90,99].

In addition, oxidative stress is associated with an abnormal immune response and can cause many diseases. Oxidative stress can occur when the production of free radicals exceeds its ability to defend itself in cells. When oxidative stress occurs, large amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can accumulate and produce deleterious effects such as lipid peroxidation (LPO), protein oxidation, and DNA damage, which ultimately lead to structural and functional changes, and even to apoptosis [131–133]. In studies where oxidative stress causes cell damage, antioxidant enzymes such as SOD, CAT and GSH-Px as well as LPO products such as MDA are often used as potential biomarkers [134–136]. *C. militaris* polysaccharides (CMP) have been studied to prevent reactive oxygen species scavenging activity induced by Cyclophosphamide. The results showed that CMP could significantly increase the SOD activity ($p < 0.01$), CAT activity ($p < 0.01$), GSH-Px activity ($p < 0.01$), and TAOC activity ($p < 0.01$) in the hearts, livers and kidneys. In addition, all CMP doses significantly decreased the MDA levels [78]. In another study, SOD, CAT, GSH-Px and MDA were also used as potential biomarkers to study the role of *C. militaris* polysaccharides in preventing oxidative stress. Their findings indicated that middle-dose and high-dose of CMP significantly inhibited MDA formation in liver kidney and heart, which indicating that high-dose CMP is effective in scavenging various types of oxygen free radicals and their products and protecting against oxidative stress [81]. All these results indicated that *C. militaris* polysaccharides can exert strong antioxidant activity both in vivo and in vitro, and can be used as a potential drug for treating oxidative stress-related diseases.

4.3. Antitumor activity

Mushroom polysaccharides were first reported to have anti-tumor activity in the 1960s [137]. To date, a large number of polysaccharides having antitumor activity have been isolated from plants, animals and fungi. Previous review has shown that mushroom polysaccharides have inhibitory effects on a variety of tumor cells, such as Sarcoma 180 solid tumor, Ehrlich solid tumor, Sarcoma 37, Yoshida sarcoma, and Lewis lung carcinoma [137]. The anti-tumor mechanism of mushroom polysaccharide can be briefly summarized as follows: (1) The effect of preventing tumors is achieved by oral administration of mushroom polysaccharides. (2) Increase the immunity of the human body to the tumor cells carried. (3) Directly inhibit tumors and induce apoptosis. (4) Prevent the spread or migration of tumor cells in the body [76,121,138].

Previous studies have revealed that *C. militaris* polysaccharides showed an anti-tumor effect mainly through the above mechanisms. The cytotoxicity induced by *C. militaris* polysaccharides (CMP-1) was investigated in four human cancer cell lines by using MTT assay. The results indicated that CMP1 could significantly inhibit the growth of HT-29, HeLa, HepG2 and K562 cells [60]. In addition, the polysaccharides obtained from *C. militaris* showed significant antitumor activities against HeLa and HepG2 cells in vitro [85]. In another study, it was found that *C. militaris* polysaccharides also inhibited SMMC-7721, BGC-823 and MCF-7 cells, and showed a concentration-dose effect [86]. Similarly, a novel polysaccharide from cultured *C. militaris* showed inhibitory activity against A549 cells, with the IC_{50} values of 39.08 μg/mL [90]. In addition, the studies also found that *C. militaris* polysaccharides also had a strong inhibitory effect on NCI-H460, colon 205, PC-3 cells [139,140]. In summary, the *C. militaris* polysaccharides can inhibit various tumor cells. Therefore, the research on the inhibition of other tumor cells needs further research.

4.4. Anti-inflammatory activity

The pathogenesis of many diseases is caused by inflammation, including cancer, atherosclerosis, neurodegenerative diseases, obesity, arthritis, etc. [141–143]. In addition, inflammation can cause genetic defects and imbalances of immune regulation, and can lead to damage to the body tissues [144]. A large number of studies have shown that polysaccharides have significant immunological activity, including *C. militaris* polysaccharides. A polysaccharide obtained from medicinal mushroom *C. militaris*, which showed significant immunological activity. Besides, the results showed that the immunological activity is associated with β-D-Glc*p* (1 → 3)-linked [145]. Similarly, CSP1, a polysaccharide obtained from cultured *C. militaris*, showed a significant immunological activity [43]. Different concentrations of *C. militaris*

polysaccharides can significantly reduce the secretion of NO, TNF-α and IL-6 which induced by LPS, and has a dose-dose effect. These results indicated that *C. militaris* polysaccharides have a good inhibitory effect on inflammatory mediators and thus exhibit good anti-inflammatory activity.

4.5. Other bioactivities

As mentioned above, *C. militaris* polysaccharides exhibit a variety of biological activities, including immunological activity, antioxidant activity, antitumor activity, and anti-inflammatory activity. Besides, it was reported that it also exhibited other activities, including inhibitory of α-glucosidase activity [48,79], antihyperlipidemic [51], hepatoprotective activities [51], antinociceptive activity [28], anti-hypoxic effect [89], anti-aging activity [91], anti-influenza virus activity [92], anti-influenza effect [92], among others.

5. Conclusion and future trends

C. militaris is a very valuable medicinal fungus in China because it can be used to treat a variety of diseases in humans, including lung function, kidney function, and immune system diseases, among others. Meanwhile, it also has improved people's quality of life and physical performance. Polysaccharide is considered to be the most important component of *C. militaris* and has a wide range of biological activities, including immunological activity, antioxidant activity, antitumor activity, and anti-inflammatory activity, etc. In recent years, the isolation, purification, structural identification and biological activity of *C. militaris* polysaccharides have been extensively studied. However, the structure of polysaccharide molecules presents complexity and variety, and it is very difficult to establish the relationship among the structure, solution behavior, chain conformation and biological activity. Due to the differences in raw materials, extraction methods, separation and purification methods, there are many differences in the structure and bioactivities of polysaccharides extracted from *C. militaris*, so it is difficult to ensure the consistency, repeatability and reliability of polysaccharides. Therefore, it is necessary to establish a standard method of polysaccharide collection and preparation to solve this problem in the future. This information can provide a reference for determining chemical structure, chain conformation and biological activity, and can be used in foods, medicine and cosmetics.

Regarding the research direction of polysaccharides, the complexation with other components (i.e., protein, polyphenol) has become the focus of research. These polymers exhibit better structural properties and biological activity. However, the mechanisms of biological activity for these compounds are not clear and may be the focus of research in the future.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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