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Physicochemical Properties and Antitumor Activities of Polysaccharides Separated from Chinese Chestnut by Membrane Filtration

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Abstract: Chestnuts, characterized by their dual attributes of nutritional and health-promoting properties, are notably rich in polysaccharide constituents. In this study, we employed hot water extraction to extract the water-soluble saccharide components from chestnut kernels. The extracted solution underwent membrane separation and column chromatography for the isolation and purification of polysaccharides, yielding N-CP, a neutral polysaccharide with a purity of 95.20%. N-CP exhibited characteristic infrared absorption features of typical polysaccharide compounds, consisted primarily of arabinose, galactose, and glucose, with a weight-average molecular weight of 482.34 kDa. The anti-proliferative activity of N-CP was evaluated against Hela and A549 cancer cell lines using the CCK-8 assay, with respective IC_{50} values of 35.45 μg/mL and 25.94 μg/mL. The results offer an efficient method for the extraction and purification of functional chestnut polysaccharides, laying a foundation for their application in functional foods and anti-tumor pharmaceuticals.

1. Introduction

Castanea mollissima Blume, commonly known as Chinese chestnut, is one of the ecological and economical tree species indigenous to China, renowned for its rich composition of polysaccharides, proteins, polyphenols, vitamins, and essential minerals[1]. These constituents render the chestnut not only a nutritious food source but also a reservoir of active compounds with potential health benefits[2]. In the realm of Traditional Chinese Medicine (TCM), Chinese chestnut holds a distinguished position as a vital ingredient in medicinal diets, rooted in ancient prescriptions. It is celebrated for its multifaceted health benefits, including spleen and stomach fortification, kidney nourishment, muscle strengthening, blood circulation enhancement, and hemorrhage cessation[3-5]. These therapeutic properties are intricately linked to the functional factors and active ingredients encapsulated within the chestnut.

Carbohydrate compounds, particularly polysaccharides, constitute the primary chemical components of Chinese chestnut kernels. In recent years, there has been a surge in research focusing

on the isolation, structural identification, and physiological activities of functional sugars within the chestnut kernels[6,7]. This burgeoning body of knowledge is propelling the valorization of Chinese chestnut in the domains of functional food development and novel drug discovery.

Membrane separation technology is a sophisticated technique grounded in the principles of mechanical sieving[8]. It employs pressure as the driving force to achieve selective separation at the molecular level. This innovative separation method is characterized by its non-destructive nature to active substances, high separation efficiency, capability for continuous production, cost-effectiveness, and pollution-free operation[9]. These attributes render it particularly promising in various industries, including chemical engineering, pharmaceuticals, and food processing. In the realm of membrane separation, ultrafiltration and nanofiltration technologies are pivotal. They exploit the molecular weight differences among mixed components to accomplish separation objectives[10]. These techniques are adept at effectively segregating biopolysaccharide components from other low-molecular-weight constituents. Moreover, they are capable of facilitating the graded purification of polysaccharides[11]. This is particularly beneficial for polysaccharides that exhibit substantial molecular weight and a broad distribution range, coupled with high solution viscosity and instability[12].

This study employs Yanshan chestnuts as the primary material, utilizing a simplistic hot water extraction method to isolate water-soluble sugars contained within the chestnut kernels. The extracted solution is then processed through a combined membrane separation technique, followed by gel column purification to isolate and purify chestnut polysaccharides. The derived polysaccharides are subjected to a series of comprehensive analyses to determine the infrared absorption characteristics, micro-morphology, thermal stability, molecular weight, and monosaccharide composition. Additionally, the CCK-8 assay is carried out to assess the in *vitro* anti-tumor activity of polysaccharides against HeLa and A549 cells. These evaluations contribute to the theoretical underpinning that supports the expanded utilization and increased value of chestnuts in the formulation of health foods and specialized pharmaceuticals.

2. Materials and Methods

2.1 Experimental Material

The chestnut variety used in this study is "Yanfeng", originating from Qinglong County, Qinhuangdao City, Hebei Province, China. Following the husking and drying processes, the kernels were pulverized through a 40-mesh sieve, and the resulting powder was stored at -20℃ for future utilization. HeLa and A-549 cell lines were procured from Shanghai Institute of Biochemistry and Cell Biology (SIBCB). All other reagents utilized in this study were of analytical grade and were acquired from Shanghai Aladdin Biochemical Technology Co., Ltd.

2.2 Instrument and Equipment

FlowMem-0005-PN40 High-Pressure Plate Membrane, N-1100 Rotary Evaporator, ALPHA 2-4 LD Plus Freeze Dryer, UV-5500 UV-Visible Spectrophotometer, SU8010 Field Emission Scanning Electron Microscope, TENSOR27 Fourier Transform Infrared Spectrometer, ICS-5000 Ion Chromatography System with PAD Pulsed Amperometric Detector, Agilent 1260 Infinity High-Performance Liquid Chromatography System, 3111 CO_2 Cell Culture Incubator, PowerWave XS2 Full-Wavelength Microplate Reader.

2.3 Experimental Method

2.3.1. Extraction and Purification of Chestnut Polysaccharides

50 g of kernel powder were mixed with deionized water at a 1:15 ratio and stirred for 2 hours at 80℃. The resulting extraction was centrifuged at 6000 rpm for 5 minutes and the supernatant was collected. The residue underwent a second extraction under the same conditions, and the supernatants were combined. The combined supernatants were subjected to microfiltration through a 0.1 μm membrane. When the retentate volume reached 500 mL, 500 mL of deionized water was added to the feed tank until the permeate reached a volume of 1500 mL. The permeate was further processed through a 3000 Da ultrafiltration membrane. When the retentate volume reached 500 mL, 500 mL of deionized water was added until the permeate reached a volume of 2000 mL. Next, the permeate was passed through a 300 Da nanofiltration membrane. When the retentate volume reached 500 mL, deionized water was added to the feed tank until the permeate reached a volume of 3500 mL. The retentate was collected, vacuum-concentrated, and then freeze-dried to obtain crude chestnut polysaccharides, labeled as C-CP.

100 mg of the crude polysaccharides were dissolved in 20 mL deionized water, filtered through a 0.45 μm membrane, and loaded onto a DEAE-Sepharose FF cellulose column. The elution process was sequentially conducted using deionized water, 0.1 M NaCl, 0.5 M NaCl, and 1.0 M NaCl solution at a flow rate of 1 mL/min. An automatic collector was employed to gather the eluates in 5 ml per tube increments. The phenol-sulfuric acid method was employed to determine the polysaccharide content in the elution fractions. Fractions containing neutral polysaccharides were concentrated and freeze-dried to obtain white powder, named as N-CP.

2.3.2. Cell Proliferation and Cytotoxicity Assay

HeLa and A-549 cells were routinely cultured in a controlled environment maintained at 37℃, with 5% CO_2 and saturated humidity. The culture medium employed was RPMI 1640, supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells in the logarithmic growth phase were seeded into a 96-well plate at a density of 1×10^4 cells per well, with three replicate wells per condition. The plate was then incubated at 37℃ in an atmosphere containing 5% CO_2. After the cells adhered to the bottom of the wells, they were treated with various concentrations of the sample and incubated for an additional 4 hours under the same conditions. Following this incubation period, 10 μL of CCK-8 reagent was added to each well and gently mixed to ensure uniform distribution of the reagent. The plate was then returned to the 37℃, 5% CO_2 incubator for a further 14 hours. After incubation, the absorbance of each well was measured at 450 nm using a microplate reader to evaluate the cells' viability and proliferation in response to the sample treatments. The percentage of cell viability or cytotoxicity was calculated based on the absorbance readings of treated wells compared to control wells. The dose-response curves for different concentrations of compounds were generated to determine IC_{50} values.

3. Results and Analysis

3.1 Purification of Chestnut Polysaccharides

The polysaccharides C-CP, prepared through membrane separation, were subjected to gradient elution on a DEAE Sepharose FF gel column. An elution curve was plotted with the tube number collected by an automatic fraction collector as the x-axis and the absorbance at 490 nm as the y-axis, as illustrated in Figure 1. A sharp and symmetrical elution peak was observed when deionized water was used as the eluent. In contrast, the use of 0.1 M and 0.5 M NaCl solutions resulted in relatively

shorter elution peaks. No significant elution peak was evident with the 1 M NaCl solution. Compared to the elution peak obtained with water, the area proportions of the peaks obtained with different concentrations of NaCl were all less than 18%, indicating the predominant presence of neutral polysaccharides in the crude extract. Corresponding to the elution curve, the eluates from tubes 6 to 27 were combined, concentrated under reduced pressure, and lyophilized to obtain N-CP. The sugar content of the sample N-CP was determined to be 95.20%, while the protein and uronic acid contents were significantly lower, measured at 1.35% and 0.11% respectively. These findings confirm the high degree of purity and integrity of the extracted polysaccharide, establishing a solid foundation for further analytical studies and potential applications.

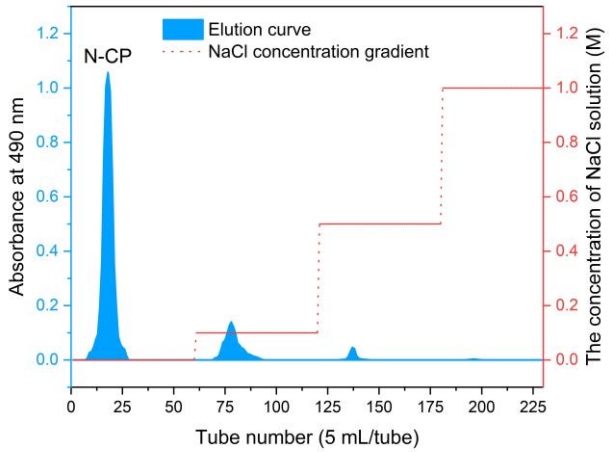

Figure 1: The gradient elution profile of C-CP on DEAE Sepharose FF column.

3.2 Physicochemical Characterization of Chestnut Polysaccharides

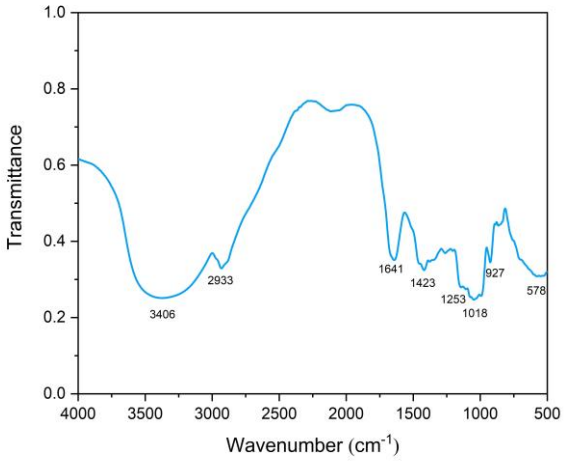

Figure 2: FTIR spectra of N-CP.

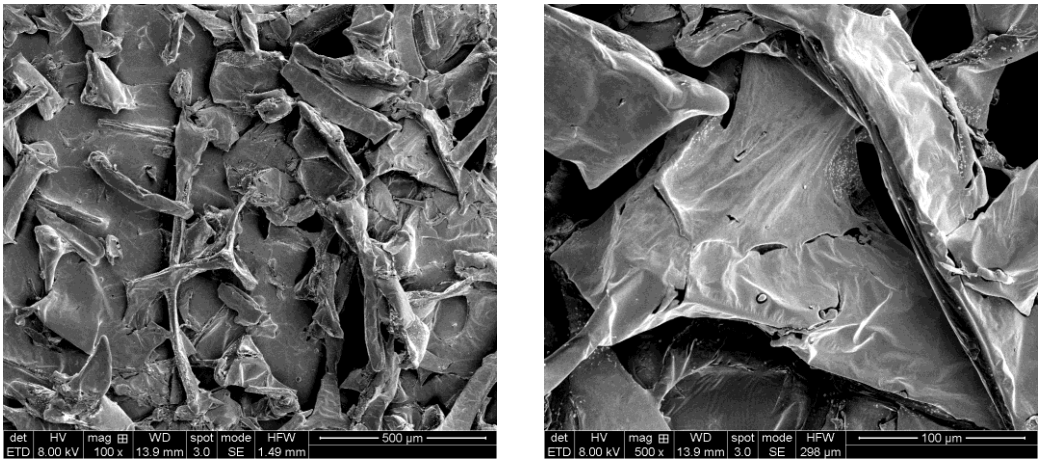

Figure 3: SEM micrographs of N-CP.

Figure 2 presents the FTIR spectrum of N-CP. The sample exhibits characteristic polysaccharide absorption peaks, including a broad and intense O-H stretching vibration absorption peak near 3406 cm^{-1}, a C-H stretching vibration absorption peak at 2933 cm^{-1}, a C=O symmetric stretching vibration absorption peak around 1641 cm^{-1}, a C-H bending vibration absorption peak at 1423 cm^{-1}, characteristic absorption peaks of C-O-H and C-O-C in the pyranose ring at 1018 cm^{-1}, an asymmetric stretching vibration absorption peak of pyranone at 927 cm^{-1}, and a bending vibration absorption peak of C-C-O at 578 cm^{-1}[13-15]. Figure 3 depicts SEM images of polysaccharide N-CP at different magnifications. At low magnification, N-CP particles display an irregular, fragmented morphology with significant aggregation. Higher magnification reveals a surface characterized by folds and protrusions, with pores evident on some fragmentary particles.

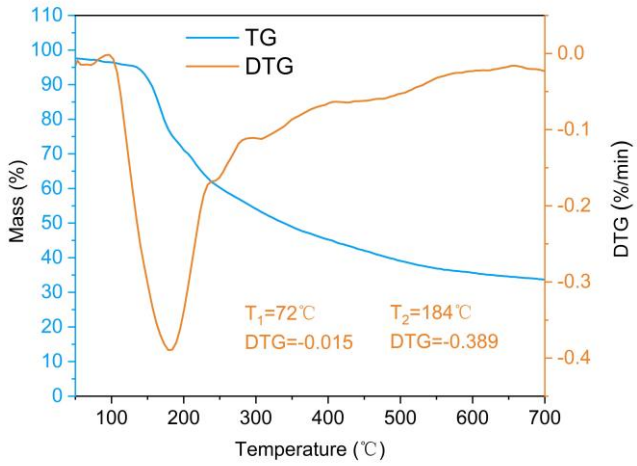

Figure 4: TG and DTG curves of N-CP.

Figure 4 presents the TG and DTG curves of N-CP. The initial weight loss is attributed to the removal of free and bound water in the polysaccharide, with the peak weight loss rate occurring at temperature T_1 of 72 ℃. The second phase of weight loss peaks at T_2 of 184 ℃, with a 50.70% loss rate, resulting from the elimination of functional groups and degradation of molecular chains. At 700 ℃, the weight of polysaccharide remains relatively stable, with a residual mass fraction of 32.85%.

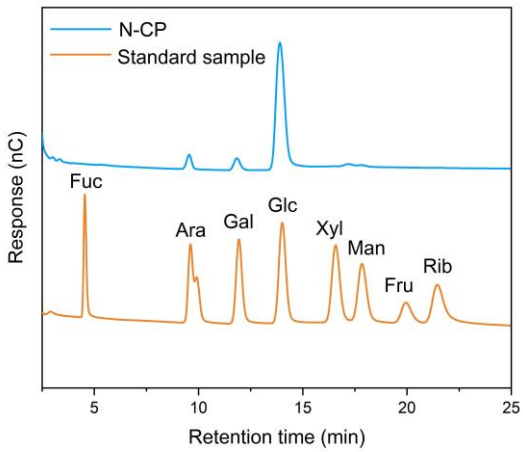

Figure 5: Ion chromatograms of the standard and N-CP after hydrolysis.

Table 1: Molecular weight and distribution of N-CP.

	M_n(kDa)	M_w(kDa)	M_z(kDa)	Polydispersity(M_w/M_n)
N-CP	171.60	482.34	2520.41	2.81

The monosaccharide composition and concentration of N-CP were elucidated post-hydrolysis with TFA, utilizing the HPAEC-PAD technique. The monosaccharides were qualitatively identified based on the retention time of the chromatographic peaks, and quantified using external standard method. The chromatograms of the standard and N-CP are presented in Figure 5. By comparing the retention times with the standard, it was determined that N-CP primarily contains three types of monosaccharides: arabinose, galactose, and glucose, with a trace amount of mannose. Quantitative analysis revealed the molar ratios of these monosaccharides to be 7.72:8.35:83.27:0.66, respectively. Table 1 displays the data obtained using HPSEC for the determination of the relative molecular weight of N-CP. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) were found to be 171.60 kDa and 482.34 kDa, respectively, with a polydispersity index of 2.81, indicating a relatively narrow molecular weight distribution.

3.3 Effects of N-CP on Proliferation of Cancer Cells

The inhibitory activity of N-CP on the proliferation of Hela and A549 cancer cell lines was evaluated using the CCK-8 assay, and the results are depicted in Figure 6, illustrating the inhibition rates at various concentrations. It is evident from the graph that the anti-proliferative effect of N-CP on both cancer cell types is augmented with increasing concentrations, demonstrating a distinct dose-effect relationship. At a higher concentration of 250 μg/mL, N-CP exhibited an inhibition rate exceeding 85% on A549 cells and over 75% on Hela cells. The dose-response curves, derived from plotting the inhibition rates against the concentrations, facilitated the calculation of the IC_{50} values for N-CP against Hela and A549 cancer cells, which were determined to be 35.45 μg/mL and 25.94 μg/mL, respectively. These findings underscore the potent anti-proliferative activity of N-CP, highlighting its potential as a therapeutic agent in cancer treatment.

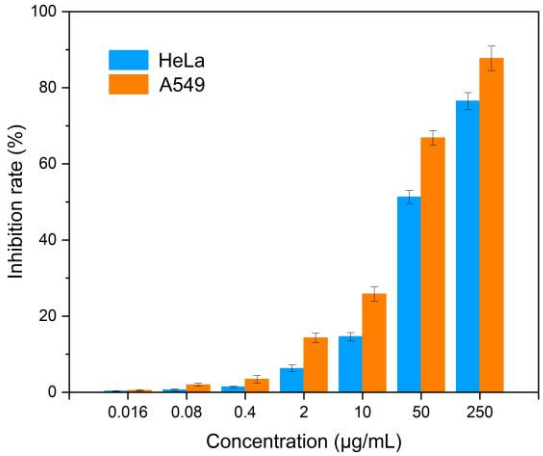

Figure 6: Inhibition effects of N-CP against Hela cells and A549 cells at different doses.

4. Conclusions

This research establishes that N-CP, a polysaccharide isolated via membrane separation and purified through gradient elution, demonstrates pronounced inhibitory effects on the proliferation of Hela and A549 cancer cells. The compositional analysis affirms elevated sugar concentration of N-CP, with negligible traces of protein and uronic acid, attesting to its purity. FTIR spectrum and SEM images further validate the structural and morphological characteristics of N-CP. Thermal analysis reveals its stability, and monosaccharide composition test gives the composition information of the polysaccharide. The potent inhibitory effects of N-CP, evident in the CCK-8 assay, are characterized by a distinct dose-effect relationship, with IC_{50} values of 35.45 μg/mL and 25.94 μg/mL for Hela and A549 cells, respectively. These empirical findings accentuate the prospective application of N-CP in oncological treatments, necessitating further in-depth studies to elucidate its functional mechanisms and therapeutic potential in cancer management.

Acknowledgements

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