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Preparation, characterization and antioxidant activity of polysaccharides copper from Qingzhuan dark tea

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Abstract

Tea polysaccharides was one of the active ingredients of Qingzhuan dark tea, which had antioxidant, anti-tumor, and immune-enhancing effects. The complexation of metal ions of tea polysaccharides was one of the current research hotspots. It can combine the advantages of tea polysaccharides and their complexed trace metal elements, and had great potential development. In this study, the trace element-copper required by the human body was used for complexation with Qingzhuan dark tea polysaccharides, and the structure was characterized by various chemical methods. The copper complexes of Qingzhuan dark tea polysaccharides were characterized by UV spectroscopy, FT-IR, thermogravimetric analysis, X-ray diffraction (XRD), SEM, AFM and X-ray photoelectron spectroscopy (XPS). The tests results show that it was successfully complexed with tea polysaccharides. Meanwhile, the Qingzhuan dark tea polysaccharides copper complex compound (termed TPS-Cu) showed higher scavenging activity of hydroxyl radical and ABTS free radical than the tea polysaccharides (termed TPS). Therefore, TPS-Cu can be used as a potentially effective and stable copper supplement and preventive agent.

Keywords: Qingzhuan dark tea; polysaccharides; copper complex compound; antioxidant activity.

Practical Application: Develop Qingzhuan dark tea for pharmaceutical industry.

1 Introduction

In recent years, TPS had been very active in international scientific community, especially in the fields of nutrition, pharmacy and food science, among which the development of metal ion complexation of TPS is one of the research hotspots (Liu et al., 2018; Mei et al., 2017; Huang et al., 2017). Compared with TPS, its complexation with iron ions and selenium ions had enhanced the antioxidant properties (Zhou et al., 2022a, 2022b; Almanaa et al., 2022; León et al., 2022), and its complexation with scandium was used in various tumors such as MNNG/HOS osteosarcoma, A375 melanoma, A549 Lung adenocarcinoma, U251 glioma, etc. with antiproliferative properties and as a nuclear medicine theragnostic agent (Mazza et al., 2021; Muñoz-Garcia et al., 2021). Due to the properties exhibited by chemically modified TPS, it was suitable for many industries such as pharmaceuticals and health food.

Qingzhuan dark tea was a deep-fermented dark tea unique to Xianning City, Hubei Province, China (Hui, 2014). The application significance of TPS-Cu was that make full use of copper's functions in the human body. Copper was an essential micronutrient for organisms, which as a cofactor of superoxide dismutase participated in the oxidation of cytochrome C and participated in cognitive impairment. Meanwhile, copper was considered safe for topical application to human skin with a very low risk of adverse effects (Zeng, 2016; Vincent et al., 2018).

These research results had provided a theoretical basis for study of TPS-Cu. which had the potential development in the field of new materials.

Thus, TPS-Cu as a type of Cu supplement was worthy for further research and development. The presents study aims to perform the structural characterization and investigate the antioxidant activity of TPS-Cu. In this study, the structure and physical properties of TPS-Cu were characterized by high performance gel filtration chromatography, atomic absorption spectroscopy, ultraviolet spectroscopy (UV), Fourier-transform infrared spectroscopy (FT-IR), thermogravimetric analysis (TGA), X-ray diffraction (XRD) analysis, scanning electron microscopy (SEM), atomic mechanics microscopy (AFM) and X-ray photoelectron spectroscopy (XPS) (Wang et al., 2021). Additionally, a series of in vitro experiments was measured to the antioxidant activity of the TPS-Cu complex (Kaska et al., 2021).

2 Materials and methods

2.1 Experimental materials

The following reagents were used: The Qingzhuan dark tea was obtained from Chibi city, Hubei Province, China. cupric chloride dihydrate (AR), 2,2-diphenyl-1-picrylhydrazyl and

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2,2'-azobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were procured from McLean (China). All reactants were used, unless otherwise specified, in this study. Deionized water was used for all experiments.

2.2 Preparation of TPS and TPS-Cu

TPS Production: Polysaccharides obtained from the Qingzhuan Dark tea was grown in Xianning (Hubei Province, China) and the polysaccharides were performed as previously described. Briefly, 20 g of the Qingzhuan Dark Tea sample was placed and heating in a constant temperature at 60 °C oil-bath pans with 200 mL of petroleum ether for 3 h, and then was dried at 60 °C to a constant weight. Subsequently, the residue was placed and heating in a constant temperature at 80 °C oil-bath pans with the solid-liquid ratio of 1:10 of 80% ethanol for 2 h, and then was dried at 80 °C to a constant weight. Subsequently, the residue was extracted with ultra-pure water under the same conditions of 60 °C for 4 h (the solid-liquid ratio of 1:16 of ultra-pure water was added to the sample). And then, the sample was rotary evaporated and concentrated in a vacuum extractor/concentrator (Sample Concentrator). Subsequently, the extracts were evaporated, concentrated and lyophilized for the complete evaporation of the solvent to obtain the dry extract (A polyamide column), after which it was left for 12 h in the condition of 4 °C, followed by adding some anhydrous ethanol. The eluent was extracted with lyophilized, followed by collected, concentrated, and dialyzed for 48 h under conditions of polyamide column and rotary evaporators.

TPS-Cu Preparation: The TPS-Cu complex was prepared by directly adding CuCl2 to 160 mL of polysaccharide solution. The polysaccharide-Cu complex was stirred gently with a magnetic stirrer for 2 h at room temperature. 80 mg TPS was added to 40 mL ultrapure water and stirred until fully dissolved. Add 40 mL of cupric chloride dihydrate solution into TPS solution and stir for 2 h at temperature of 30 °C (Figure 1).

2.3 Characterization of TPS and TPS-Cu

Molecular weight and determination of Cu content: The relative molecular masses and homogeneity of TPS and TPS-Cu were determined by high performance gel filtration chromatography. Then, 2 mg of TPS and TPS-Cu were weighed and dissolved in 400 μL of 0.1 mol/L sodium nitrate solution, respectively, and the homogeneity and molecular weight distribution of TPS and TPS-Cu were determined. The mobile phase was 0.1 mol/L sodium nitrate solution at a flow rate of 0.9 mL/min and the experimental column temperature was 45 °C. The cobalt content in TPS-Cu was determined by ContraA700 atomic absorption spectrometer (flame method).

UV spectroscopic analysis: TPS and TPS-Cu samples were prepared into a 1 mg/mL solution and added to a quartz cuvette to measure the UV absorbance in the range of 200-400 nm using a UV-visible spectrophotometer (UV-1800, Shimadzu, Japan).

Fourier transform infrared spectroscopy analysis: TPS and TPS-Cu samples were weighed and pressed into sheets with KBr powder, respectively. Each sample within 4000 ~ 500 cm^{-1} used in the three regions of Nicolet 6700 Fourier Transform infrared spectrophotometer record spectrum. FT-IR data were collected using software and using automatic baseline correction.

Thermal performance analysis: Samples of TPS and TPS-Cu were subjected to thermogradient-analysis (TGA) using a synchronous thermal analyzer (STA449F3, NETZSCH, Germany). Thermogravimetric analysis (TGA) was carried out under the protection of 50 mL/min dry nitrogen at a heating rate of 10 °C/min from 30 °C to 1000 °C, and the thermogravimetric curve was made and the thermal stability of the material was analyzed.

SEM/EDC analysis: The morphological characteristics of TPS and TPS-Cu were analyzed and observed with an X-MAX N80 energy spectrometer (JSM-7500F, Japan) under accelerating voltage. At the same time, the surface element composition between TPS and TPS-Cu was compared by EDS. The TPS and TPS-Cu crystal structures of the samples were characterized using an Empyrean X-ray diffractometer (Panko, the Netherlands).

Atomic Force Microscopy analysis: Atomic force microscopy (AFM) (Nasoscope, VEECO, USA) was used to study the morphology of TPS and TPS-Cu.

X-ray photoelectron spectroscopy (ESCALAB 250Xi Thermo Fisher Technology Co., LTD.) was used to determine copper valence and binding energy in TPS-Cu.

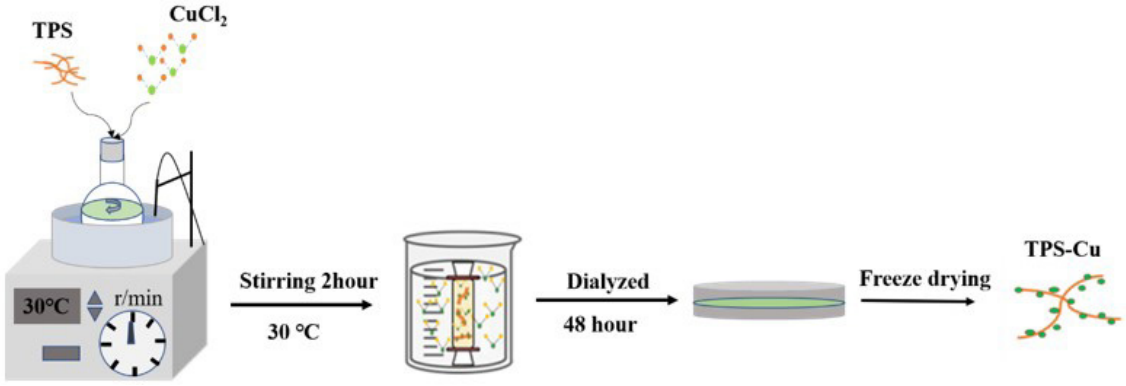

Figure 1. Schematic illustration for the preparation of TPS-Cu.

2.4 Antioxidant test

The hydroxyl radical scavenging activity was determined through spectrophotometry. The method was used with slight modifications. To the six test tubes, 1.5 mL of 7.5 mol/L ferrous sulfate solution and 3 mL of 8 mmol/L salicylic acid-ethanol solution were added. Further, 1.5 mL of TPS and TPS-Cu solutions with different concentrations (0.0, 0.2, 0.4, 0.8, 1.6, 3.2 mg/mL) were added. Finally, 3 mL of 7.5 mmol/L hydrogen peroxide solution was added, which was fully mixed and incubated for 45 min at 37 °C. The absorbance was determined at 510 nm (A_1). According to aforementioned operation, the solution without sample was taken as A_0, and the clearance rate was calculated as (Equation 1) (Hang et al., 2022).

$$Hydroxyl\ radical\ scavenging\ rate\ (\%) = (A0 - A1)/A0 \times 100\% \quad (1)$$

According to Li et al. (2022) and Pereira et al. (2020) research method with a slightly modified, 7.4 mmol/L ABTS solution was mixed with 2.6 mmol/L potassium persulfate, and the reaction was carried out at room temperature and protected from light for 12 h. The solution was diluted 40-50 times with pH 7.4 phosphate buffer that the absorbance at wavelength 734 nm was about 0.7, and ABTS+ working solution was obtained. Then, 0.2 mL of samples with different concentrations were taken and 0.8 mL of diluted ABTS+ working solution. After the reaction at room temperature for 6 min, the absorbance was measured at 734 nm using an enzymatic standard. The ability of TPS and TPS-Co to scavenge ABTS+ radical was calculated as (Equation 2) (Li et al., 2022; Pereira et al., 2020).

$$ABTS+\ scavenging\ ability\ (\%) = \left[1 - (A_1 - A_2)/A_0\right] * 100 \quad (2)$$

Where A_1 denotes the sample mixed with ABTS+ solution, A_2 denotes sample without ABTS+ solution, and A_0 is the absorbance of ABTS+ solution without the sample as a blank control.

2.5 Statistical analysis

All results are expressed as mean ± standard deviation of six repetitions. Statistical analysis was performed using Origin 2019 software (Origin Lab Corporation, Northampton, MA, USA). One-way analysis of variance (ANOVA) was performed using SPSS (version 18.0, IBM, Armonk, New York, USA). A P value < 0.05 was considered to be statistically significant.

3 Results and discussion chemical characterization of the polysaccharide-Cu complex

3.1 Molecular weight and determination of Cu content

The weight average molecular weight (Mw), number average molecular weight (Mn) and polydispersity (Mw/Mn) of TPS and TPS-Cu were determined to evaluate the relative molecular weight of the combination of copper and polysaccharide. Table 1 showed that the Mn of TPS and TPS-Cu were 7033 g/mol and 4222 g/mol, respectively. The Mw of TPS was 110121 g/mol, while the TPS-Cu was 51274 g/mol. The Mw of TPS were decreased when it binds to Cu. The complexation of tea polysaccharides with copper may lead to the degradation of TPS due to its quaternary structure of macromolecules. The PDI index decreased from 15.66 to 12.14, which also indicated that the complexation of copper reduced the dispersion of TPS.

Table 1. Molecular weight and determination of coper content.

Index	TPS	TPS-Cu
M_n	7033	4222
M_w	110121	51274
PDI	15.66	12.14
Coper content (%)	-	4.54

3.2 Ultraviolet spectrum analysis

Qingzhuan Dark TPS and TPS - Cu of ultraviolet spectrum was showed in Figure 2A. As shown in the figure, TPS had unique small absorption peaks at 280 nm, indicating that they may contain small amounts of protein (Zhang et al., 2016). However, the absorption peak disappeared after complexation of copper and tea polysaccharide, which may be due to the changes in the structure of tea polysaccharide after complexation of tea polysaccharides and copper. The results showed that TPS was successfully complexed with Cu.

3.3 Infrared spectral analysis

The FT-IR spectrum analysis was shown in Figure 2B. According to the vibration frequency table of organic compound groups, the stretching vibration frequency of O-H was between 3570 and 3050 cm^{-1}, the vibration frequency table of benzene ring skeleton was between 1650 and 1450 cm^{-1}, and the bending vibration frequency of O-H was between 1100-1000 cm^{-1}. The C-H stretching vibration was between 3000-2500 cm^{-1} (Lu et al., 2016). As shown in the figure, the O-H stretching frequency of TPS was 3384.74 cm-1, the C-H stretching vibration was 2935.47 cm^{-1}, the O-H bending vibration frequency was 1021.08 cm^{-1}, and the C=O stretching vibration frequency was 1617.10 cm^{-1}. The O-H stretching frequency was 3399.11 cm^{-1}, the C-H stretching vibration was 2935.47 cm^{-1}, the O-H bending vibration frequency was 1021.08 cm^{-1}, and the C=O stretching vibration frequency was 1636.34 cm^{-1}. After complexation, the absorption peak at 3384.74 cm^{-1} shifts to 3399.11 cm^{-1} and the peak shape becomes wider, which indicated that the participation of O-H leads to the enhancement of vibration peak. The absorption peak from 1617.10 cm^{-1} to 1636.34 cm^{-1}, and C=O may also be involved in the complexation of copper. These results had indicated that the complex reaction between polysaccharides and copper may occurred and the TPS-Cu retains the basic structure of polysaccharides.

3.4 Thermal property analysis

TGA analysis was performed to evaluate the thermal stability of TPS and TPS-Cu at different temperature ranges. As can be seen in Figure 2C, the thermal decomposition curve of TPS and TPS-Cu showed that the mass loss of TPS-Cu was 67.87% and that of TPS was 70.03%, which may be due to the complexation of copper. The TPS-Cu was more stable than TPS.

3.5 XRD crystal structure of the TPS-Cu complex

XRD analysis was used for the structural analysis of the internal distribution of atoms. X-rays was pass through a crystalline material of a particular wavelength that can be dispersed because the atoms or ions were arranged regularly within the crystal. The phase of the scattered X-rays was improved in some directions. Hence, it had specific diffraction phenomena correspond to the crystal structure (Figure 3). Both samples exhibited less crystallinity and lacked sharp crystallization peaks. After the coordination reaction, the crystal morphology had changed. The XRD results of TPS-Cu exhibited different peak positions, which signified that the crystallinity of polysaccharides changed after incorporation of Cu. Collectively, Cu was introduced into TPS, and the TPS-Cu was successfully formed, which confirmed through XRD characterization.

3.6 SEM/EDS analysis

The surface morphology of TPS and TPS-Cu was studied by SEM and EDS (Energy Dispersive Spectroscopy) (Figure 4). The structure of TPS was fibrous and appeared to be dispersed. Meanwhile, TPS-Cu was in a relatively aggregated state. Differences in the tightness and pore size between TPS and TPS-Cu were observed. The results showed that the surface of Cu complexed with polysaccharides changed from fibrous to sheet, which might be the aggregation state after complexed with Cu, which could increase the stability of –TPS-Cu complex.

3.7 AFM analysis

The morphology of TPS was studied by atomic mechanics microscope. Chemical modification could change the spatial structure of polysaccharides and affect their structure-activity relationship. The spatial structure or conformation of polysaccharides had played a significant role in biological activities. Figure 5 shows the 2 μm AFM image of TPS and TPS-Cu. It could be seen from the obtained image that the TPS sample as a whole exhibit's universal roughness, with a surface roughness of 2.7 nm. In contrast, the surface of TPS-Cu complex was flatter than TPS in the comparison structure, with a surface roughness of only 1.2 nm. These results indicated that the stability of the polysaccharides was improved due

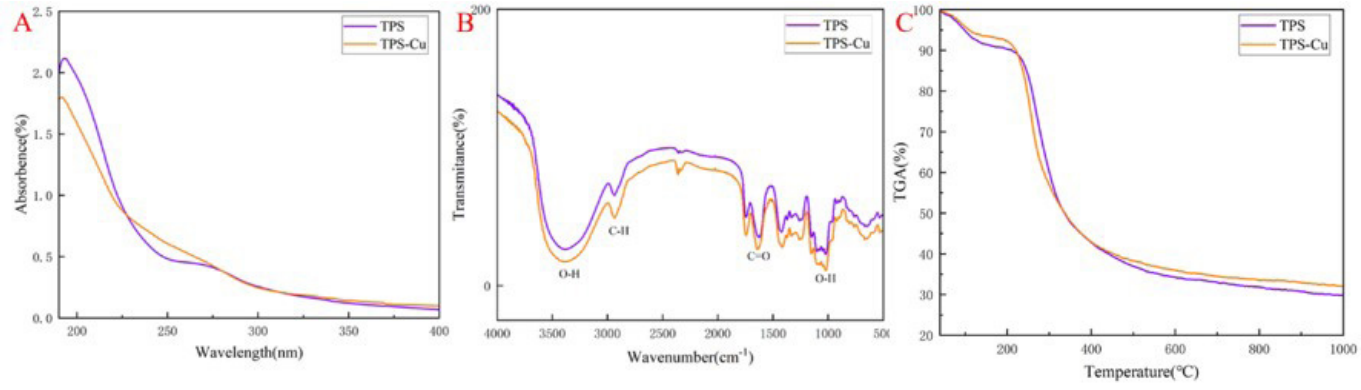

Figure 2. The ultraviolet spectrum of the TPS and the TPS-Cu (A), FT-IR transmission spectra of the TPS and the TPS-Cu (B), thermal property analysis of TPS and TPS-Cu (C).

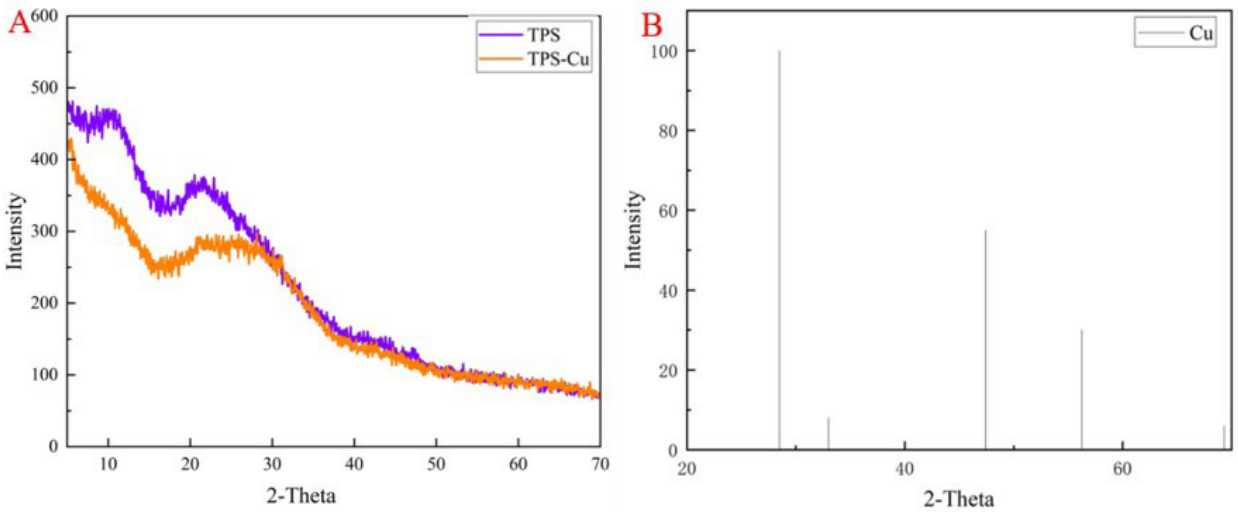

Figure 3. The XRD analysis of TPS and TPS-Cu.

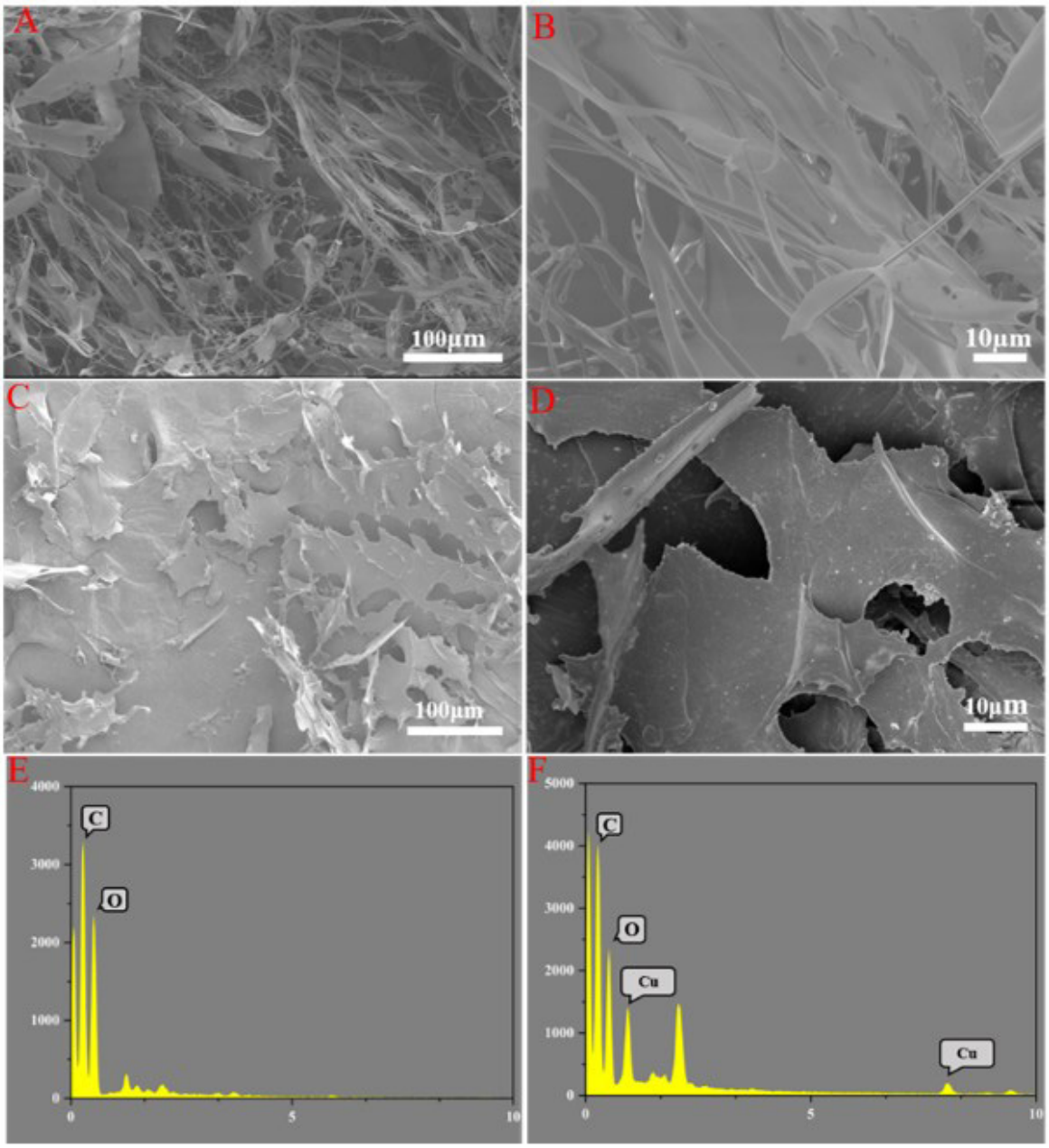

Figure 4. The image of SEM of TPS (A), the image of SEM of TPS (B), the image of SEM of TPS-Cu (C), the image of SEM of TPS-Cu (D), EDS (elemental analysis) for TPS (E) and TPS-Cu (F).

to the introduction of copper. It indicated that the polysaccharides were successful complexation of copper.

3.8 X-ray photoelectron spectroscopy

XPS provided information on chemical bonding and molecular composition of the material located on the surface of the sample or in the first layer below it. The full-spectrum XPS results of TPS and TPS-Cu were shown in Figure 6A, and the Cu2p scanning spectra of TPS and TPS-Cu were shown in Figure 6B. It could be concluded that there was no characteristic peak of copper in the full-scan spectrum and Cu2p of sugar samples, indicated that the polysaccharides samples do not contain copper element. The characteristic peaks of Cu2P1 and Cu2P1 of copper appeared at the binding energies of 937 eV and 952 eV of TPS-Cu, indicated that copper complexed with polysaccharide successfully.

4 Antioxidant activity assays

4.1 Hydroxyl radical scavenging activity

Hydroxyl radical was an active substance of reactive oxygen that has a very strong oxidation ability. It could destroy red blood cells, degrade DNA, cell membrane, macromolecules, and cause great harm to human health. Thus, the removal of hydroxyl free

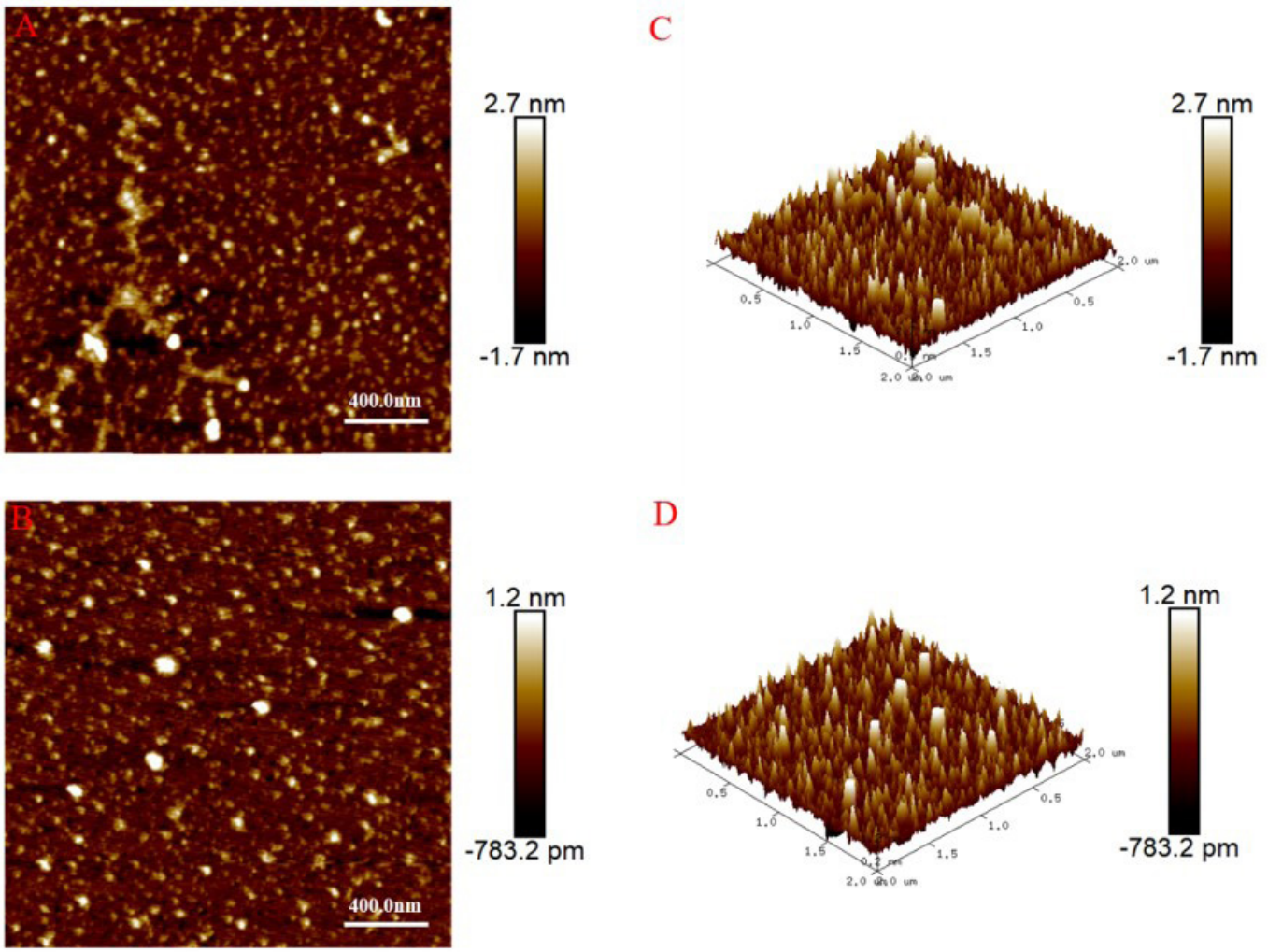

Figure 5. AFM 2D images of TPS (A) and TPS-Cu (B), AFM 3D images of TPS (C) and TPS-Cu (D).

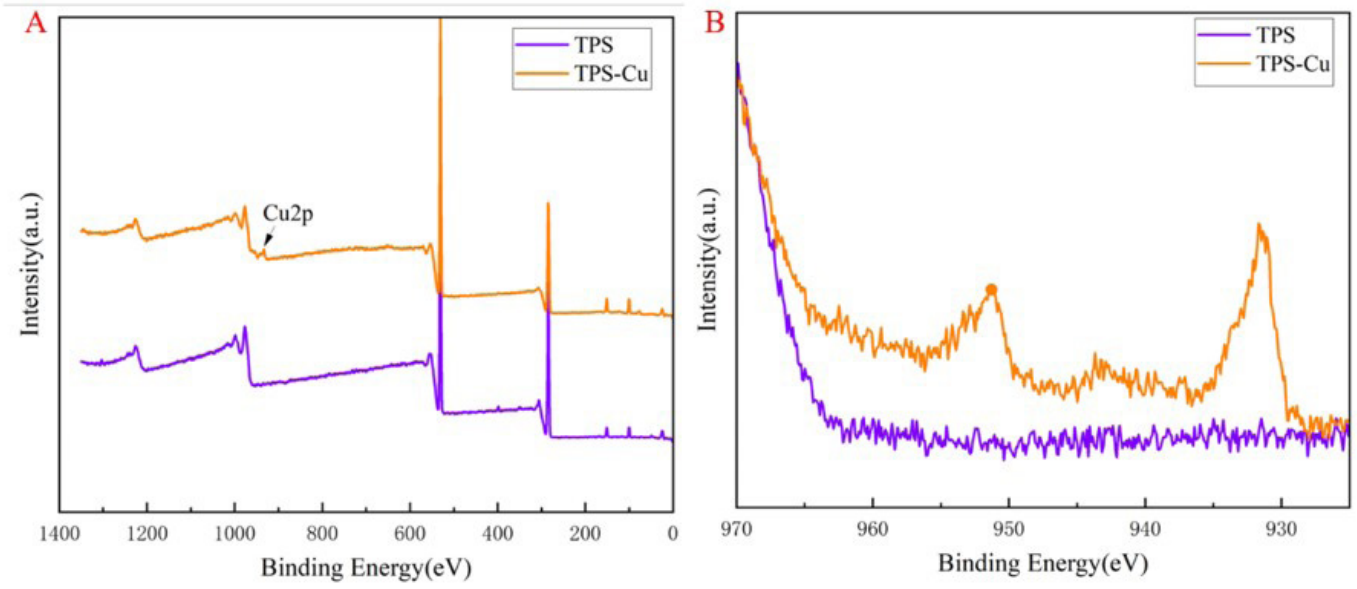

Figure 6. XPS of the TPS and the TPS-Cu.

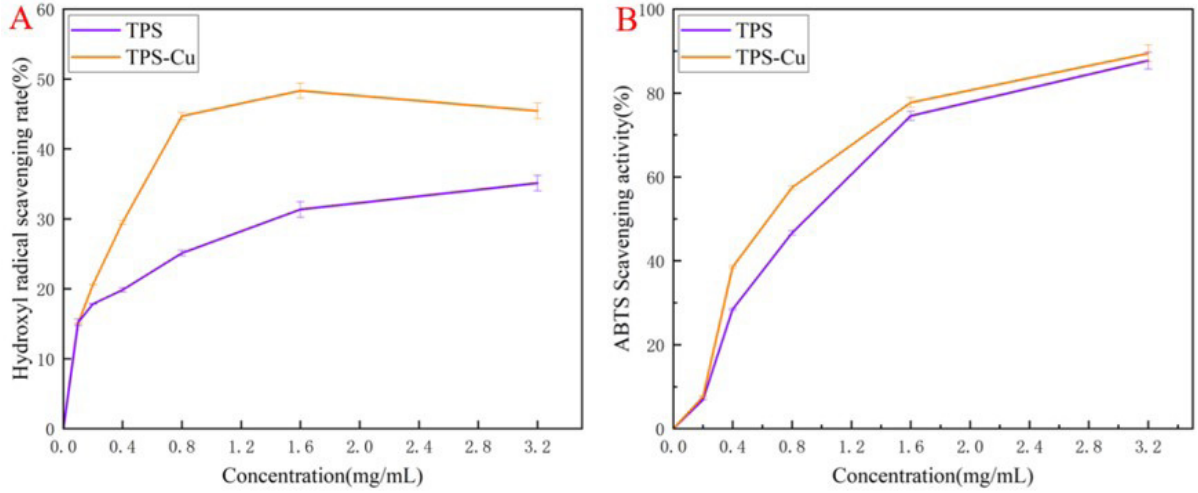

Figure 7. Hydroxyl radical scavenging of TPS and TPS-Cu (A), ABTS radical scavenging of TPS and TPS-Cu (B).

radicals was one of the effective measures to prevent diseases. Here, we adopted the Fenton method to establish a reaction model. When the sample had the antioxidant ability to scavenge hydroxyl radical, it could compete with salicylic acid and react with hydroxyl radical to absorb 2, 3-dihydroxybenzoic acid and decrease the absorbance at 510 nm. The hydroxyl radical scavenging activity of TPS and the TPS-Cu was delineated in Figure 7A. In the experiment, the concentration of the samples ranged from 0.0 mg/mL to 3.2 mg/mL. The hydroxyl radical scavenging activity of the samples increased with the increase in concentration and exhibited a certain degree of concentration dependence. In general, the TPS-Cu complex had a higher hydroxyl radical scavenging ability than TPS. The scavenging activity of TPS and the TPS-Cu complex was 35.11% and 45.45%, respectively, at 3.2 mg/mL concentration.

4.2 ABTS radical scavenging activity

In the form of 2,2-diazo-bis (3-ethyl-benzothiazole-6-sulfonic acid) diammonium salt, ABTS could react with $K_2S_2O_8$ to generate a stable, blue cationic radical and exhibits a clear absorption peak at 734 nm (Wu et al., 2018). Its concentration could be determined by detecting the absorbance at 734 nm. According to the aforementioned principles, the ABTS scavenging activity of TPS and the TPS-Cu at different concentrations was estimated, the results were displayed in Figure 7B. The TPS-Cu displayed a clear ABTS radical scavenging activity of 89.45% at the concentration of 3.2 mg/mL. Though the ABTS free radical scavenging activity of TPS was lower than the TPS-Cu that it still showed certain activity. The maximum ABTS free radical scavenging activity of TPS reached 87.72% at 3.2 mg/mL. The ABTS radical scavenging activity of TPS and the TPS-Cu complex exhibited some degree of concentration dependence.

5 Conclusion

In this study, we characterized the structure of TPS-Cu. TPS-Cu contains 4.5% copper that was more thermal stable than TPS. It was shown that Cu ions bind to polysaccharides. TPS showed different UV spectroscopic, FT-IR, SEM, XRD, AFM and XPS from TPS-Cu, indicated that TPS-Cu has a different structure from TPS. The test results had showed that copper has been successfully complexed into TPS. TPS was used as a substrate for copper incorporation. The surface of TPS-Cu had changed significantly, and a new surface morphology was produced. The antioxidant activities of TPS and TPS-Cu were determined by the scavenging activity of hydroxyl radical and ABTS radical. TPS-Cu showed stronger free radical scavenging ability than TPS, and both of them were concentration dependent. The results showed that TPS-Cu could be used to form organic copper supplements with excellent antioxidant activity. However, the complex relationship between the structural characteristics and antioxidant properties of TPS-Cu may be affected by different combinations of factors, and the mechanism of antioxidant activity of TPS-Cu needs to be further studied. These results had accelerated the further development of unique TPS-Cu and other metal-polysaccharides combinations, and provided a scientific basis for the further development and utilization of TPS and its complex compound.

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