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Characterization and Antioxidant Activities of Crude Polysaccharide from Mushroom *Hypsizygus ulmarius*

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Abstract

In this study, crude polysaccharide extracted from *Hypsizygus ulmarius* (HUCP) by hot water extraction method at 55˚C and subsequently precipitated with cold ethanol. And then, deproteinized, dialysed, lyophilized. The antioxidant activities of HUCP were evaluated by established *in vitro* systems, including scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, hydroxyl radicals, reducing power and ferrous ion chelating ability. HUCP structure characterized by FTIR, UV spectral analysis, X-ray diffraction and conformation analysis by congored analysis. Physiochemical properties of HUCP were evaluated. The moisture, pH, carbohydrate, protein, uronic acid, reducing sugar and sulfate content of HUCP were 77.33%, 7.00%, 6.4%, 5.30% and 5.77%, respectively. Morphological analysis by scanning electron microscopy (SEM). HUCP had moderate antioxidant potential (51.55% DPPH radical scavenging, 51.73% Fe^{2+} chelation and 0.544 reducing power at 20 mg/ml, 62.33% hydroxyl radical scavenging activity at 10 mg/ml). HUCP can be used an antioxidant supplement in the pharmaceutical and food sectors.

***Key words*:** *Hypsizygus ulmarius,* Mushroom, Polysaccharide, Antioxidant, Characterization

Mushrooms, a well-known delicacy, contain a wide range of biomolecules with nutritious properties [1]. Polysaccharides are polymers generated by the polycondensation of ten or more monosaccharides [2]. Polysaccharides, a structurally diverse family of natural polymers found in living beings, exhibit a wide range of possible biological actions, including anticoagulant, antioxidant, immune-stimulating, and anticancer properties [1]. Polysaccharides are developing as a viable health dietary additive that can protect the human body from a variety of ailments based on their biological activities [3]. In a recent year, polysaccharides have been reported antioxidants and characterization in *Catathelasma ventricosum* [1], *Poria cocos sclerotium* [4], *Pleurotus eous* [5], *Pleurotus eous* [6]. Extraction is the first and most important step in the characterization and application of bioactive polysaccharides. Extraction methods and conditions not only alter polysaccharide yield, but also vary the composition and structure of polysaccharides, resulting in variations in bioactivity [7]. *Hypsizygus ulmarius*, commonly known as the Elm Oyster Mushroom large and fleshy with excellent taste. It is an edible mushroom which can be easily grown either for commercial purpose of home consumption. The purpose of this study was to first characterize the structure of HUCP, and then to evaluate its physicochemical qualities. Furthermore, the *in vitro* antioxidant activity of HUCP was investigated using *in vitro* chemical studies (DPPH, hydroxyl radical, reducing power, and chelating ability experiments).

MATERIALS AND METHODS

Chemicals

Hypsizygus ulmarius fresh mushroom was procured from IIHR, Bangalore. Ethanol, Chloroform, Methanol, Butanol, Ascorbic acid, Ferric chloride, Dialysis membrane was purchased from Himedia in Mumbai, India and Merck, India. 2,2-diphenyl-1-picrylhydrazyl (DPPH), Salicylic acid was purchased from Sigma Aldrich. All other reagents used in the experiments were of analytical grade.

Extraction of crude polysaccharide

For 24 hours at room temperature, dried mushroom powder was soaked in petroleum ether. Centrifugation was used to collect the solid residue, and the technique was done twice to remove the lipids and pigments [8-9]. The residue was air dried and extracted three times with hot water (1:30 (w/v), 55°C, 2 h)., filtrate collected by centrifugation. After filtered, the filtrate was concentrated by rotary evaporator under the reduced pressure. Concentrated sample was dialysed against distilled water for 48 hr. After dialysis, centrifuged, concentrated and precipitated with 3 volumes of ice-cold ethanol. Polysaccharide precipitate was collected by centrifugation and lyophilized to obtain crude *Hypsizygus ulmarius* polysaccharide (HUCP).

The polysaccharide yield (%) was calculated using the following formula:

Polysaccharide yield (%, w/w) = [weight of dried polysaccharide (g)/weight of raw material (g)] × 100%

Chemical composition analysis

The total carbohydrate content was determined using the phenol-sulfuric acid method with D-glucose as a standard [10].

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Protein content was measured using the Bradford technique using bovine serum albumin as the reference [11]. The sulphate content was determined using the Tehro and Haritiala method, with potassium per sulphate serving as the standard [12] (Tehro and Haritiala, 1971). DNS (Dinitro salicylic acid) was used to assess sugar reduction [13]. The uronic content was determined using the sulfuric acid and carbazole technique [14]. For quantification, the carbazole reaction was used, which is the most reliable method for measuring uronic acid. Galacturonic acid was used as the standard. The results are expressed as % for HUCP.

Determination of moisture content

The moisture content of the HUCP was determined by following the method of Sudarsan *et al*. [15]. Moisture content was estimated by subtracting the dry weight of sample from the known wet weight of the sample dried in the hot air oven.

Determination of pH

A 1% w/v dispersion was prepared by dissolving the polysaccharide in distilled water and the pH determined.

Characterization of HUCP

UV and FTIR spectrometric analysis

2mg of sample dissolved in 1ml of distilled water, used for UV spectral analysis. Fourier transform infrared spectroscopy (FTIR) is commonly used to study molecular vibration and atom interactions. The structure of polysaccharides (monosaccharide configuration, glycosidic bond type, and functional groups) can be determined via an FT-IR scan. The FTIR spectra of HUCP were acquired using a BRUKAR 657, Tensor II spectrometer. The data was gathered in the 4000-400cm^{-1} range using the ATR technique.

X-ray diffraction analysis

A DMAX-2000 diffractometer was used to obtain polysaccharide X-ray diffraction patterns (Rigaku, Tokyo, Japan). The diffractometer was set up with nickel filtered Cu K radiation, 36 kV and 26 mA, a scan speed of 0.05 min1, and an angular range of 5-70°(2θ) [16].

Congo red test

The Ogawa procedure, with minor modifications, was used to determine the triple helical structure of HUCP. Monitoring the maximum absorption wavelength (max) of Congo-red polysaccharides at varied sodium hydroxide concentrations revealed a transition from a triple-helix structure to a single-stranded conformation. In a gradient of sodium hydroxide solution, the sample (6 mg/2 mL) was diluted in distilled water and reacted with Congo red (2.0 mL, 100 M) (0.1–0.5 M). The absorbance was measured at each sodium hydroxide concentration. Distilled water served as control.

Scanning electron microscopy (SEM) analysis

SEM (S-4800, FE-SEM, Hitachi High-Technologies, Japan) was used to examine the morphological characteristics of HUCP. Images were captured at a magnification of 2000X and 10 X accelerating voltage.

Antioxidant activity

DPPH radical scavenging activity

DPPH radical scavenging activity of HUCP was determined using the previously described method [17-18]. 1ml of the sample (HUCP) was combined with 1mL of 0.2mM DPPH in methanol solution at various doses (4-20 mg/mL). For 30 minutes, the mixture was stored in the dark at room temperature. At 517nm, the decrease of the DPPH radical was measured. The reaction mixture's lower absorbance suggested more free radical scavenging activity. For comparisons, ascorbic acid was employed as a positive control. The EC_{50} value (mg/mL) is the effective concentration at which the scavenging ability is calculated using linear regression interpolation. Scavenging activity (%) = (Ac-As) /Ac × 100 was used to calculate DPPH radical scavenging activity. Where Ac is the absorbance control (1mL methanol and 1mL DPPH solution), and As is the absorbance of the sample mixture (1mL sample and 1mL DPPH solution).

Scavenging of hydroxyl radicals

For this activity, 1 mL of polysaccharide solution was mixed with 1.0 mL of 9 mmol/L $FeSO_4$, 1.0 mL of 9 mmol/L salicylic acid, and 0.5 mL of 0.1% H_2O_2 and kept at 37°C for 30 minutes Li *et al*. [19]. The resultant suspensions were measured for absorbance (A_1) at 510 nm with deionized water as a reference. To determine the absorbance of the solution that replaces the polysaccharide solution with deionized water, the same treatment was used (A_0). To determine absorbance, several concentrations of 1.0 mL polysaccharide solutions were added to 2.5 mL of deionized water (A_2). All of these assays used Vc as a positive control.

Scavenging activity = $[1-(A_1-A_2) / A_0] \times 100$, where A_0 is the absorbance of the control (without extract), A_1 is the absorbance in the presence of the extract, and A_2 is the absorbance without sodium salicylate.

Reducing power

The reducing power of HUCP was determined by according to the method of Liu *et al*. [20] with some modifications. In brief, ascorbic acid served as positive control, whereas the absence of sample served as a negative control. The reaction mixture was incubated at 50°C for 20 minutes with various concentrations (4-20 mg/mL) of sample solution (1mL), 1mL of 0.2M sodium phosphate buffer (pH 6.6), and 1ml of 1% potassium ferricyanide [K3 Fe(CN6)] (w/v). The reaction was then terminated by adding 1mL of 10% TCA (w/v). The mixture was then centrifuged for 10 minutes at 3000rpm. 1mL of supernatant was pipetted and mixed with 1mL of deionized water and 0.2 ml of 0.1% ferric chloride ($FeCl_3$) solution. At 700nm, absorbance was measured against a blank. The greater the absorbance, the greater the reducing power. The EC_{50} values were calculated as the effective concentrations at which the absorbance exceeded 0.500.

Chelating ability

Using the ferrozine assay was used to examine ion chelating behaviour. Tang *et al*. [21]. 0.1 mL 2 mM ferrous chloride and 3.7 mL water were mixed with HUCP (1-5 mg/mL). Starting the reaction, 0.2mL of 5mM ferrozine was added and allowed to react for 10 minutes at room temperature. 562nm was calculated as the absorbance of the reaction mixture. The ratio of inhibition of the ferrozine-Fe2+ complex development was calculated as $A_{control}$ - A_{sample} / $A_{control}$ x 100, where $A_{control}$ is the absorbance of only $FeCl_2$ and ferrozine and A_{Sample} is the absorbance of HUCP. Ethylenediaminetetraacetic acid (EDTA) was used as a control in this experiment.

Statistical analysis

The results were reported as mean ± standard deviation (SD) of three replicates. Statistical analyses were performed using Analysis of Variance (ANOVA) and the significances of the differences between samples were determined using

Tukey's multiple range test. Statistical significance was set at a level of $P < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of HUCP

The yield of HUCP was 3.87%. Bioactivity of polysaccharides inevitably depended on the chemical components [22]. As shown in (Table 1), HUCP exhibited content of carbohydrate was 77.33%, reducing sugar (5.27%), uronic acid (6.4%), sulphate (5.77%) and protein (7.07%). The moisture content and pH of crude polysaccharide was found to be 7.2% and 7.4.

In previous study Miao *et al*. [23] reported, total sugar content ranged between 536-95.4%, uronic acid content ranged between 11.46-26.3%, protein content ranged between 0.2 - 20.35% of four different polysaccharides from the fruiting bodies of *Lepista sordida.*

Table 1 Physicochemical properties of HUCP

Yield (%)	Carbohydrate (%)	Reducing sugar (%)	Protein (%)	Uronic acid (%)	Sulphate (%)	Moisture content (%)	pH
3.87±0.058	77.33±2.08	5.27±0.23	7.07±0.058	6.40±0.10	5.77±0.09	7.2±0.252	7.4

Characterization analysis

UV spectra analysis

The UV spectra of HUCP was shown in (Fig 1). Absorption at 260 nm was observed for HUCP, revealing the presence of protein.

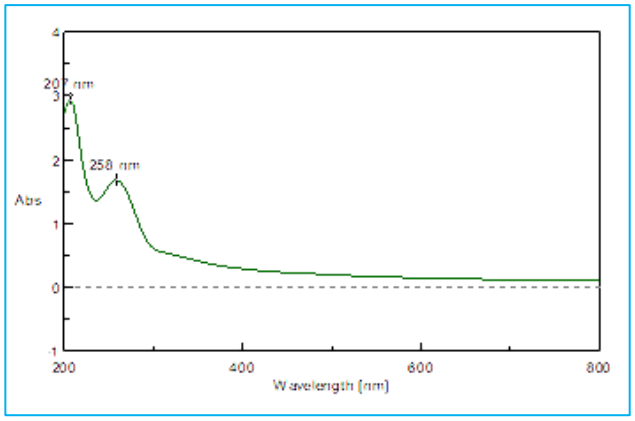


Fig 1 UV Spectra of HUCP in the range of 200–400 nm

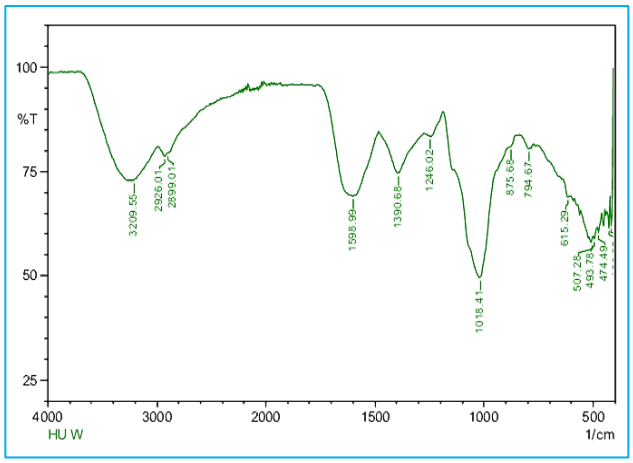


Fig 2 FTIR Spectral analysis of HUCP

FTIR spectral analysis

The FT-IR spectroscopic analysis was used to investigate molecular vibrations and polar bonding between distinct atoms [24]. FT-IR spectra of HUCP, which is common for carbohydrate polysaccharides (Fig 2). A characteristic main broad stretching peak between 3209 cm^{-1} corresponded to the hydroxyl group, while a faint band between 2926 cm^{-1} occurred, indicating the C-H stretching vibration [25]. The absorbance at 1598 cm^{-1} was ascribed to C—O stretching vibration. The bands at 1018 cm^{-1} were due to the stretching vibrations of C-O-C and C-O-H [26]. In addition, the peak at 857 cm^{-1} was also observed, which was ascribed to α-type glycosidic linkage.

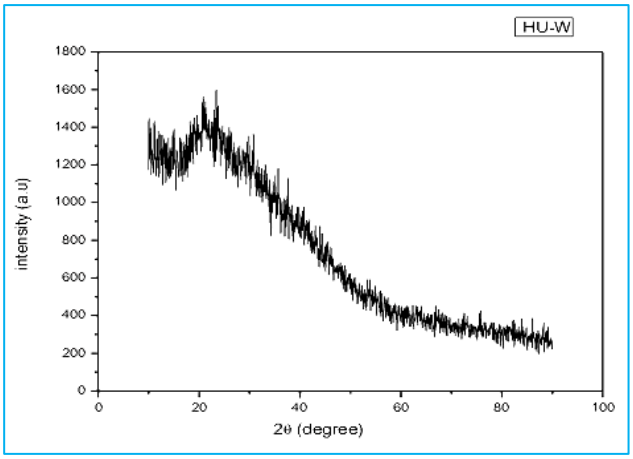


Fig 3 XRD diffraction of HUCP

X Ray diffraction analysis

XRD has been frequently utilized to determine the structure of many compounds [27-28]. The crystalline zone was seen at the angles (2Θ) 20° and 26° in (Fig 2D), indicating that the HUCP was a semi-crystalline polymer with low crystallinity (Fig 3).

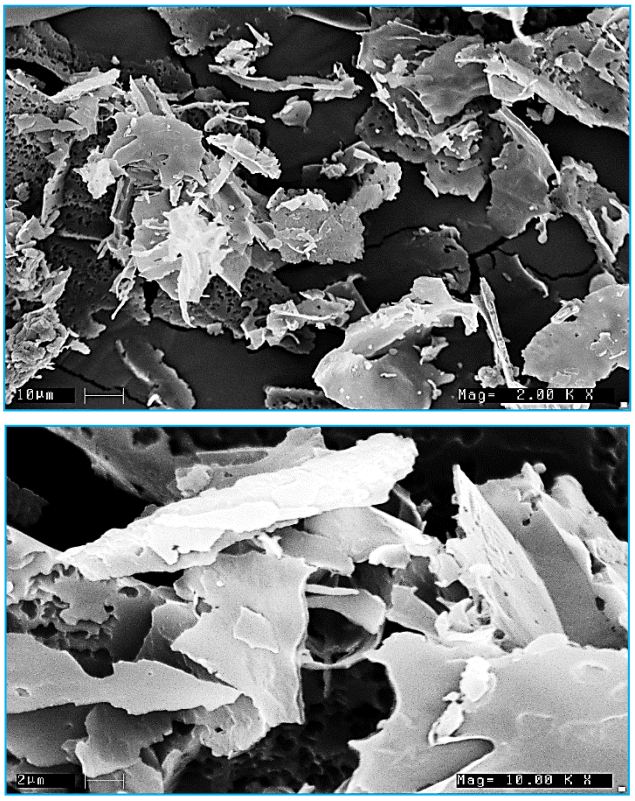


Fig 4 SEM images of HUCP

Surface morphology

SEM revealed a considerable change in the surface morphology of HUCP has a smooth surface with an inconsistent pattern (Fig 4).

Congo red analysis

In general, Congo red could attach to polysaccharides having a triple-helix structure, resulting in a bathochromic shift of the complex's maximum absorption wavelength (λmax). Furthermore, as the NaOH concentration increased, the complex would be destroyed, resulting in a rapid decrease in λmax. The Congo red-HUCP group's λmax change significantly at varied NaOH concentrations, as illustrated in (Fig 5). As a result, HUCP had a triple-helix structure. This finding was supported by triple-helix polysaccharides (*Millettia Speciosa Champ*) [29].

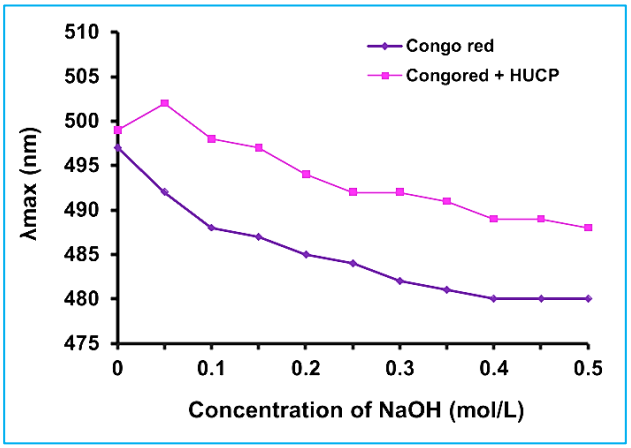
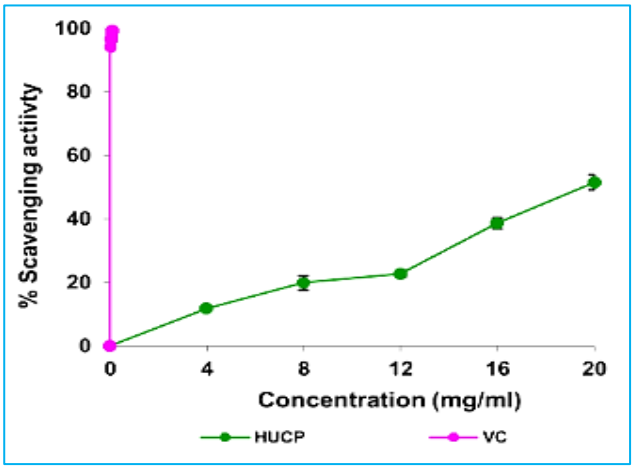
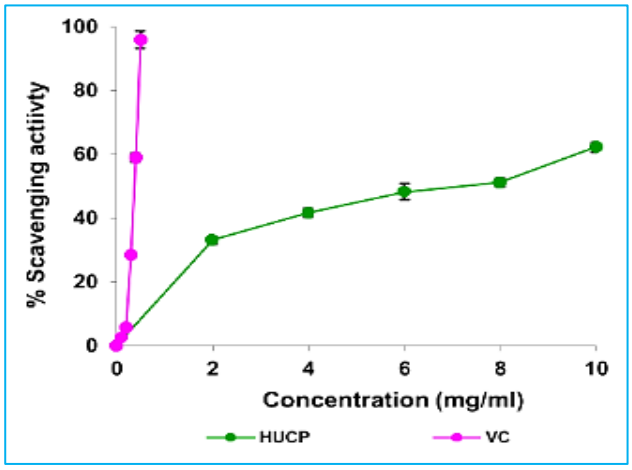


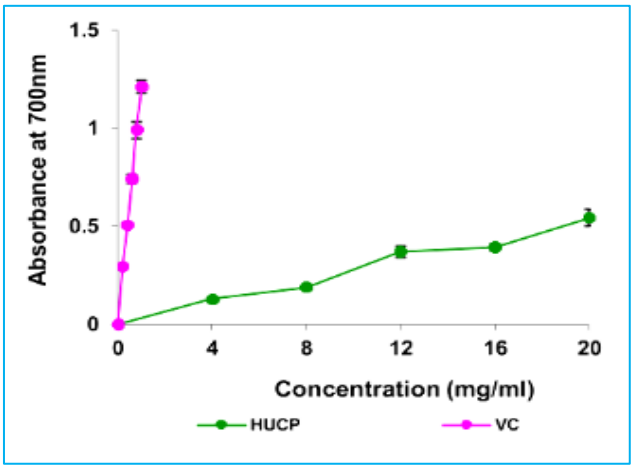
Fig 5 Congored analysis



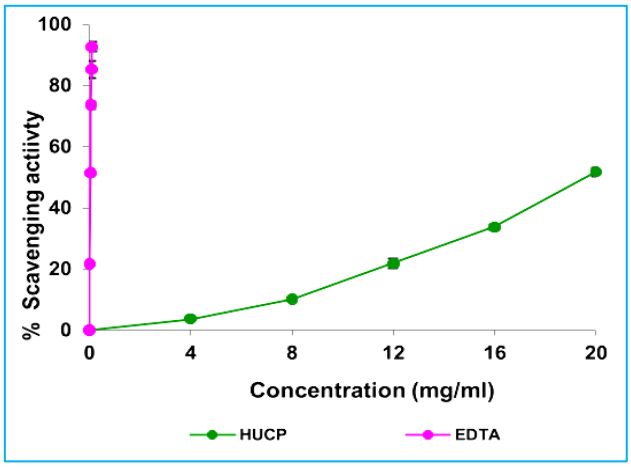
(a) DPPH radical scavenging activity



(b) Hydroxyl radical scavenging activity



(c) Reducing power



(d) chelating ability

Fig 6 Antioxidant activities of HUCP

Antioxidant activities

DPPH radical scavenging activity

DPPH radical has been extensively used for screening antioxidant activity, because it can become a stable molecule after acceptance of an electron or hydrogen atom that shows a strong absorption at 517nm [30]. It could be seen from (Fig 6a) that the scavenging abilities increased with increase of the concentration from 4-20 mg/ml. The scavenging ability of HUCP from *Hypsizygus ulmarius* showed 51.50% at 20 mg/ml when compared with ascorbic acid which showed 99.21% at 0.1 mg/ml, respectively. A significant difference ($p<0.05$) in DPPH radical scavenging activity was observed with different sample concentrations. The half maximal effective concentration (EC_{50}) is defined as the concentration of sample at which the scavenging rate reaches 50%. Practically, a lower EC_{50} value corresponds to stronger antioxidant activity of tested sample [31]. The EC_{50} value of HUCP was 19.40mg/ml within the concentration of test range. However, the scavenging activity of HUPS was lower than that of Vc (0.005mg/ml). This might be attributable to its strong hydrogen-donating ability caused by activating the hydrogen atom of the anomeric carbon [32]. Antioxidant activities of polysaccharides are usually influenced by many factors, such as their molecular weight, water solubility, uronic acid content and monosaccharide composition, glycoside bond type [33]. CVPS extracted from *Chuanminshen violaceum* has been reported to have a DPPH radical scavenging activity of 71.34% at a concentration of 1mg/ml [34].

Reducing power

In reducing power assay, the presence of antioxidants in the samples would result in the reduction of $Fe^{3+/}$ferricyanide complex to Fe^{2+} by donating the hydrogen atoms to break chain reactions. Fe^{2+} can be monitored by measuring the absorbtion of the Prussian blue at 700 nm. It had been reported that the reducing power positively related to the antioxidant activities [35], and that the absorbance values can directly indicate the reducing power, a higher absorbance indicates a higher reducing power [36]. (Fig 6b) shows the reducing power activity of HUCP. At the concentration of 20mg/ml, the reducing power of HUCP was 0544. Vc was used as the positive control, had a reducing power was 0.909 at 0.05mg/ml. A significance difference ($p<0.05$) was observed in the EC_{50} value between HUCP (19.51mg/ml) and Vc (0.027mg/ml). It showed that the reducing power of HUCP was significantly lower than that of Vc.

Chelating ability

Metal chelating activity is widely recognized as an antioxidant mechanism because it reduces the concentration of the catalytic transition metal in lipid peroxidation. Ferrozine is a sensitive reagent that can react with ferrous ions to create colourful species (iron (II)-ferrozine complex). When antioxidants are added to the system, they compete with ferrozine for ferrous ions, lowering the solution's absorbance. The metal chelating rate increased from 3.63% to 51.73% when the concentration of HUCP was increased from 4.0 to 20.0 mg/mL, as shown in (Fig 6c). EDTA, on the other hand, may exhibit a 92.73% scavenging effect at a low dosage of 0.1 mg/mL. This suggests that HUCP has a moderate and stable scavenging ability.

Scavenging of hydroxyl radicals

Hydroxyl radicals are a reactive oxygen species with a high oxidation capacity that can destroy red blood cells, break DNA and cell membranes, and cause tissue damage or cell apoptosis. Li *et al*. [37]. However, the action of hydroxyl free radical scavengers can dramatically restore these negative effects to normal health conditions. The experimental results (Fig 6d) demonstrated that HUCPs had a concentration-dependent action for scavenging hydroxyl radicals, which was enhanced as polysaccharide concentration. Hydroxyl radical clearance actions of HUCP ranged from 33.17% at 2 mg/mL 62.33% at 10 mg/mL. Li *et al*. [37] reported *Polygonatum cyrtonema* Hua had moderate hydroxyl radical scavenging activity.

CONCLUSION

In this study, we evaluated the physicochemical properties and antioxidant activity of the primary fraction (HUCP). The physicochemical properties of the polysaccharide showed a degree of purity. HUCP had low level moisture content. Morphological studies exhibited that the particles had smooth surface with an inconsistent pattern. HUCP showed high antioxidant potential and could inhibit the radicals' chain reaction propagation, either by the donation hydrogen/electron to free radicals, or by chelating ion involved in the Fenton reaction. So as to combat the oxidative stress and treat humanoid ailments.

Abbreviations

HUCP - *Hypsizygus ulmarius* crude Polysaccharide
DPPH - 1,1-Diphenyl 2-picrylhydrazyl
VC - Vitamin C
O$\mathring{H}$ - Hydroxyl radical

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Conflict of interest

The authors declare that there are no conflicts of interest.

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