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Anticoagulant and Antioxidant Activities of Water-soluble Polysaccharide from Fruiting Bodies of *Hypsizygus ulmarius*

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Abstract

This study was carried out to elucidate the anticoagulant and antioxidant activities of water-soluble alkali polysaccharide (HUAP) from *Hypsizygus ulmarius* mushroom. Antioxidant activities of HUAP was evaluated by DMPD, Hydroxyl radical scavenging activity, FRAP, Lipid peroxidation inhibition assay. Moreover, there *in vitro* anti-coagulant activities was evaluated by activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) assays. In additionally HUAP having notable scavenging action against DMPD (EC_{50} = 13.29 mg/mL), Hydroxyl radicals (EC_{50} = 3.48 mg/mL), Lipid peroxidation inhibition (EC_{50} = 0.541 mg/mL) and also exhibited Ferric reducing power (FRAP) (EC_{50} = 1.97 mg/mL). At 5mg/ml, HUAP's anticoagulant activity using APTT, PT and TT was calculated to be 42.15s, 14.2s and 25.25s, respectively. Antioxidant assay showed that HUAP exhibited strong radical scavenging activity, reducing power and LPO inhibition. HUAP had remarkably superior antioxidant activity and might be developed as a natural antioxidant. HUAP exhibited potent anticoagulant activity and would be expected to be a potential source of anticoagulant.

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

Polysaccharides, a class of essential biomacromolecules found in plants, animals, and microorganisms, have attracted worldwide attention due to their low toxicity [1]. Natural polysaccharides derived from mushrooms have recently gained academic attention due to their bioactive features such as anti-inflammatory, anti-oxidative, anticancer, hypoglycemic, immune-stimulation, and anti-aging benefits, among others [2]. Mushrooms are regarded as a valuable food in some countries around the world. Asian countries have the oldest heritage of using mushrooms as both a meal and a source of health-promoting substances. However, Poland and other Slavic countries have a long history of harvesting and eating wild mushrooms.

Mushrooms growing in the wild are usually picked for their taste and flavour, but they can also be a natural source of physiologically active substances used in traditional medicine [3]. In a dose-dependent way, *Stropharia rugosoannulata* [4], *Auricularia polytricha* [5] and *Ganoderma lingzhi* [6] shown reducing power, scavenging action against ABTS, hydroxyl, superoxide and chelating capacity. Organisms often maintain a dynamic equilibrium between their coagulation and anticoagulation systems. Inhibiting blood coagulation is effective in preventing thrombosis. The human body's coagulation process involves transforming fibrinogen into fibrin, which is catalyzed by thrombin [7]. In addition to thrombosis, the excess free radicals created during cell metabolism can cause heart disease, cancer, and ageing. Antioxidants are commonly employed to protect the human body against dangerous reactive oxygen species. Nonetheless, the extensively used synthetic antioxidants over the last few decades are considered to have possible damage to the liver, stomach, and potentially cause cancer [8]. Fungal fruiting bodies, mycelium, and culture medium have all been evaluated as possible sources of bioactive substances [9]. *Hypsizygus ulmarius*, also known as the elm oyster mushroom, which belongs to *Lyophyllaceae*. The majority of current studies on *H. ulmarius's* potential medical applications have been conducted in India. Compounds identified in both the mycelium and the fruiting body have been reported to be anti-inflammatory, antioxidant, antitumor and antidiabetic. In China, it is regarded as a medicinal fungus [10]. The current study has been aimed to isolate and identify the polysaccharides from *H. ulmarius.* In this work, the antioxidant activities of HUAP were studied, using *in vitro* assays of DMPD, Hydroxyl radical scavenging activity, FRAP assay and Lipid peroxidation inhibition assays. To evaluate anticoagulant activity of HUAP by APTT, PT, TT clotting assays.

MATERIALS AND METHODS

Chemicals

The fruiting bodies of *Hypsizygus ulmarius* was provided by Narmadha Mushroom, Pune. DMPD, TPTZ were purchased from Sigma Chemicals Company (St. Louis, USA). Ascorbic acid, Ferric chloride Hydrogen peroxide, Sodium salicylate, HCl, $FeCl_3$, TBA, PT kit, TT kit and APTT kit were obtained from Himedia. All other chemicals used in present work were analytical grade.

Polysaccharide extraction

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Hypsizygus ulmarius mushroom powder was defatted by petroleum ether at room temperature for 24hr. The defatted powder was dissolved in a solution of 1 M NaOH and 1% NaBH4 (1:30 v/v) and stored at room temperature overnight [11]. After centrifugation (5000 rpm, 10 min), the supernatant was concentrated to a tenth volume using a rotary flash evaporator. It was then dialyzed against sodium acetate buffer (pH 5.0) for 24 hours, followed by distilled water for another 24 hours. Filtering removes the precipitate after neutralizing the supernatant with 50% glacial acetic acid. The supernatant was concentrated with a rotary evaporator, precipitated with a fourfold volume of 95% ethanol at 4°C for 24 hours, and then deproteinized using the Sevag method. After vacuum freezing, crude polysaccharide (HUAP) was found.

Antioxidant activities

Hydroxyl radical scavenging activity

The reaction mixture (3.0 ml) contains 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate, and varied doses (2.0-10 mg/ml) of HUAP [12]. After 1 hour of incubation at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562nm. Vitamin C (0.02-0.10mg/mL) served as positive control. The percentage scavenging effect was calculated as follows: scavenging activity = [1-(A1-A2) / A0] x 100, where A0 is the absorbance of the control (without extract), A1 is the absorbance in the presence of the extract, and A2 is the absorbance without sodium salicylate.

Scavenging effect on DMPD radical

Different doses of HUAP (4-20 mg/ml) were added to test tubes, and the total volume was diluted with distilled water to 0.5 mL. 10 minutes later, the absorbance was measured at 505 nm [13]. 1.0 ml of $DMPD^{\bullet+}$ solution was added immediately to the reaction mixture, and the absorbance at 505nm was measured. Ascorbic acid (0.04-0.20 mg/mL) was employed as a positive control. The buffer solution served as a blank sample. The $DMPD^{\bullet+}$ scavenging activity was computed using the following equation:

$$\text{Scavenging activity (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where, A_0 is the absorbance of the first concentration of $DMPD^{\bullet+}$ and A1 is the absorbance of the remaining concentration of $DMPD^{\bullet+}$ in the presence of the extract. EC_{50} value (mg extract/ml) was the effective concentration at which DMPD radicals were scavenged by 50% and were obtained by interpolation from linear regression analysis.

Ferric reducing antioxidant power assay (FRAP)

The ferric reducing antioxidant power (FRAP) was estimated using Wang *et al*. [14] method. Mix 25 mL acetate buffer (0.3 mol/L, pH 3.6), 2.5 mL TPTZ (10 mmol/L in 40 mM HCl), and 2.5 mL $FeCl_3.6H_2O$ (20 mmol/L) to make the FRAP working reagent. Combine 900µl of FRAP reagent, 90µl of water, and 30µl of HUAP (0.6-3.0 mg/mL). At 37°C, the reaction mixture was incubated for 30 minutes. When compared to Vitamin C as a comparison, increased reaction absorbance (595nm) meant increased reducing power.

Lipid peroxidation inhibition assay

A modified thiobarbituric acid reactive species (TBARS) assay was used with egg yolk homogenates as lipid rich media to determine the amount of lipid peroxide released [15]. 0.5 mL of 10% egg homogenate and 0.1 mL of different concentrations of HUAP (0.4-2.0 mg/mL) were mixed in a test tube, and the volume was made upto 1.0 mL with distilled water. 0.05 mL $FeSO_4$ (0.07 M) was added to the above mixture and incubated for 30 minutes to induce lipid peroxidation. After that, 1.5 mL 20% acetic acid (pH 3.5 with NaOH), 1.5 mL 0.8% TBA (prepared in 1.1 percent sodium dodecyl sulphate), and 0.05 mL 20% TCA were added, shaked, and heated in a boiling water bath for 60 minutes. Every solution was added with 5.0 mL n-butanol and centrifuged for 10 minutes at 3000 rpm after cooling. The absorbance of the organic upper layer was determined to be 532 nm. Vitamin C was used as a positive control.

Anticoagulant activity

Normal human plasma with no history of bleeding or thrombosis was tested in vitro for Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT), and Thrombin Time (TT), with heparin sulphate as a standard. Human blood obtained through venipuncture was mixed with 3.8% sodium citrate aqueous solution. To complete the experiment, blood was centrifuged for 15 minutes at 3600 r/min. Plasma was obtained within 2-3 hours [16].

Activated partial thromoplastin time (APTT)

To perform the APTT clotting assay, mix 90 µL of citrated human plasma samples with 10 µL of HUAP (1 and 5 mg/mL). Add 100 µL of APTT reagent and incubate for 3 minutes at 37 °C. The clotting time was measured after adding 100 µL of 0.025 mol/L $CaCl_2$ solution, which had been pre-incubated at 37°C for 5 minutes [17].

Prothrombin time (PT)

For PT clotting test, mix 90 µL of citrated human plasma with 10 µL of sample solution (1 and 5 mg/mL) and incubate at 37 °C for 3 minutes. Then, add 200 µL of pre-incubated PT reagent at 37 °C for 10 minutes and record clotting time [17].

Thrombin time (TT)

In TT clotting assay, 90 µL of citrated human plasma was mixed with 10 µL of HUAP (1 and 5 mg/mL) and incubated at 37°C for 3 minutes. Then, add 100 µL of TT reagent at room temperature and record clotting time. All assays were performed three times and mean values were determined [17].

Statistical analysis

All experiments were carried out in triplicate and data were expressed as mean ± standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Antioxidant activities

Antioxidant activity has also been linked to a variety of processes and mechanisms, including radical scavenging, reductive capacity, chain initiation prevention, and transition metal ion catalyst binding [18]. In this investigation, antioxidant assessment methods such as hydroxyl and DPPH radical scavenging assays and reducing power analyses were used to determine the antioxidant *in vitro* of polysaccharides (HUAP).

Hydroxyl radical scavenging activity

Hydroxyl radicals were the most common reactive oxygen free radicals in living organisms, and they were a major contributor to the ageing and tissue damage processes, as well as the progression of many degenerative diseases [19]. In (Fig

1a), the ability of HUAP to scavenge hydroxyl radicals was demonstrated at doses ranging from 2 to 10 mg/mL. HUAP displayed concentration-dependent behavior and demonstrated strong hydroxyl radical scavenging abilities, according to the results. At a dosage of 10mg/mL, HUAP had a scavenging rate of 88.09%. Furthermore, the EC_{50} values of HUAP hydroxyl radical scavenging ability was 3.48mg/mL ($p<0.05$), which was lower than that of Vitamin C (EC_{50} = 0.058 mg/mL).

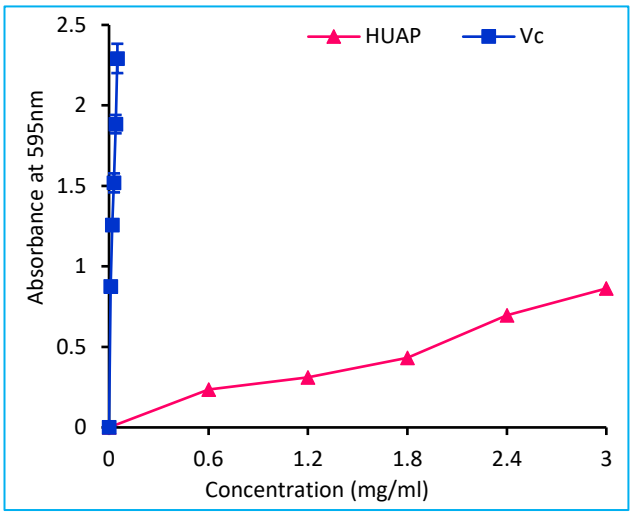

Fig 1(a) Hydroxyl radical scavenging activity

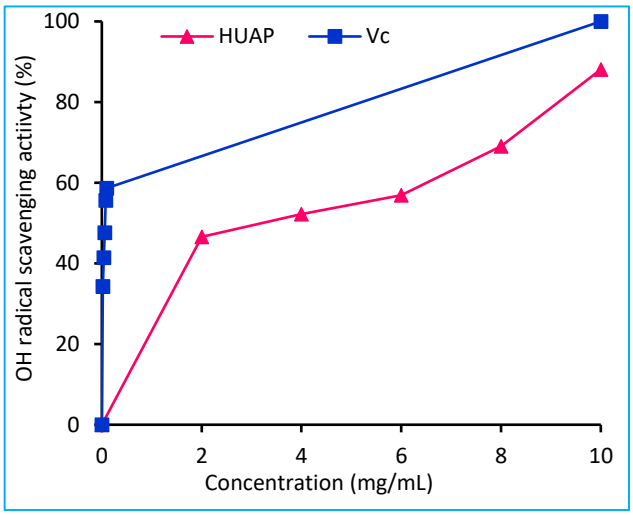

Fig 2(b) DMPD radical scavenging activity

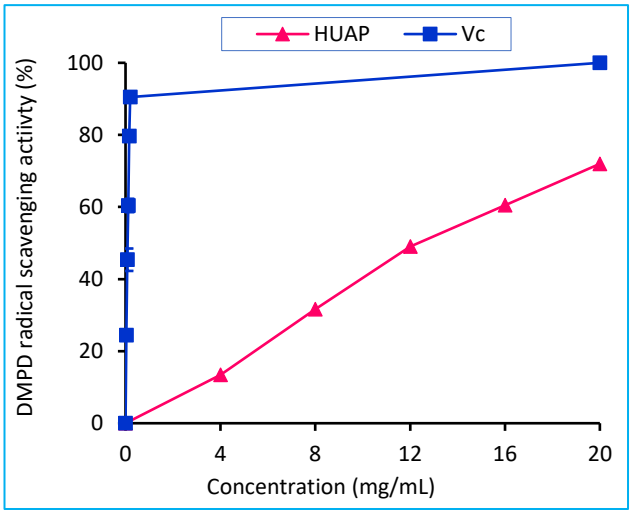

Fig 1(c) Ferric reducing antioxidant power (FRAP)

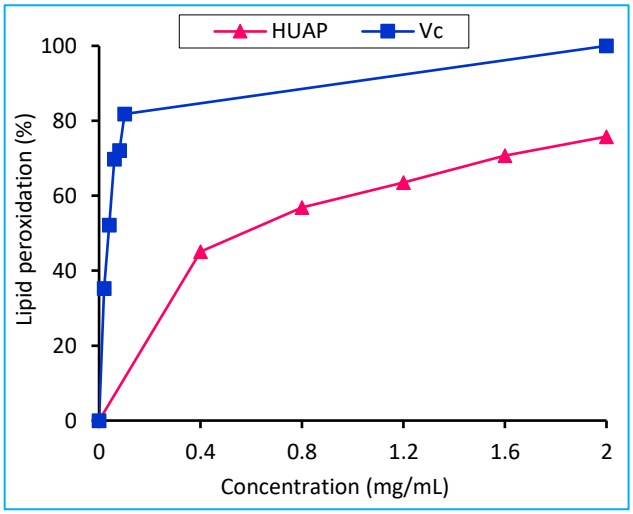

Fig 1(d) Lipid peroxidation inhibition

Fig 1(a-d) Antioxidant activities of HUAP

$DMPD^{\bullet+}$ radical scavenging activity

DMPD can form a stable and coloured radical cation ($DMPD^{\bullet+}$) at acidic pH and in the presence of a suitable oxidant solution. The UV visible spectrum of $DMPD^{\bullet+}$ shows a maximum absorbance at 505 nm. Antioxidant compounds, which are able to transfer hydrogen atom to $DMPD^{\bullet+}$, quench the colour and produce a decolouration of the solution. As shown, the DMPD radical-scavenging capacity of HUAP was dose- dependent (Fig 1b). At the concentration of 4-20 mg/ml, the scavenging effects of HUAP on DMPD radical were 13.44% - 71.97% but lower than Vitamin C (90.54%) at 0.2 mg/mL. A significant difference ($p<0.05$) in DMPD radical scavenging activity was observed with different sample concentrations. The EC_{50} values of HUAP and Vitamin C on DMPD radicals were 13.29 and 0.096 mg/mL respectively.

FRAP exercise

The FRAP assay measures a HUAP's antioxidant capacity to decrease Fe^{3+} to Fe^{2+}. When this happens in the presence of 2,4,6-trypyridyl-s-triazine, the reduction is accompanied by the creation of a coloured complex with Fe^{2+} which offers the total antioxidant or reductant information [14]. At 0.6 to 3.0 mg/ml, ferric reducing power of HUAP was 0.236 to 0.863 (Fig 1c). Moderate reducing power was detected in HUAP extract (EC_{50} = 1.97 mg/mL) despite that lower than that of Vitamin C (0.006 mg/mL).

Inhibition of lipid peroxidation

Food deterioration is mostly caused by lipid peroxidation, which affects colour, flavour, texture, and nutritional content. Low-density lipoprotein (LDL) oxidative modification may have a role in the development of atherosclerosis. The peroxidation of polyunsaturated fatty acid components in LDLs is a frequent beginning step in oxidative modification [16]. In the presence of ferrous sulphate, lipids in egg yolk undergo fast nonenzymatic peroxidation. HUAP and Vitamin C inhibited lipid peroxidation in a dose-dependent manner (Fig 1d). At 0.4- 2.0 mg/mL, HUAP inhibited lipid peroxidation by 45.08-75.78% with varying sample concentrations, there was a significant difference ($p<0.05$) in

lipid peroxidation inhibition. At 0.02-0.10 mg/mL, the prevention of lipid peroxidation by positive control Vitamin C was 35.27-81.79%.

Table 1 Anticoagulant activities of HUAP

Sample (Conc)	Clotting time (sec)		
HUAP	APTT	PT	TT
1mg/mL	39.31	13.89	23.1
5mg/mL	42.15	14.2	25.25
Heparin (0.01mg/mL)	65.33	15.29	30.92

Anticoagulant activity

The samples' *in vitro* anticoagulant activity was assessed using APTT, TT, and PT. Heparin, a commercially available anticoagulant, was employed as a positive control. APTT corresponds to the intrinsic coagulation phase, PT to the extrinsic phase, and TT to the third coagulation phase in plasma. Liu *et al*. [20] refer to these examinations as functional tests, which monitor clot formation.

Data in (Table 1) shows that APTT, PT and TT were prolonged in a concentration-dependent manner within the experimental range. HUAP at 5mg/mL significantly differed from saline and heparin in APTT, PT and TT assays ($P < 0.05$). The prolonged APTT and PT suggest suppression of the intrinsic and extrinsic coagulation cascade, respectively. The lengthening of TT indicates suppression of thrombin-mediated fibrin production. Furthermore, the blood clotting time of HUAP at 1 mg/mL in APTT, PT and TT assays were 39.31s, 13.89s and 23.1s respectively (Fig 2). At 5mg/mL, clotting time of HUAP in APTT, PT and TT were 42.15s, 14.2s, 25.25s which was greater than that of the saline. Moreover, HUAP demonstrating superior anticoagulant activity [16].

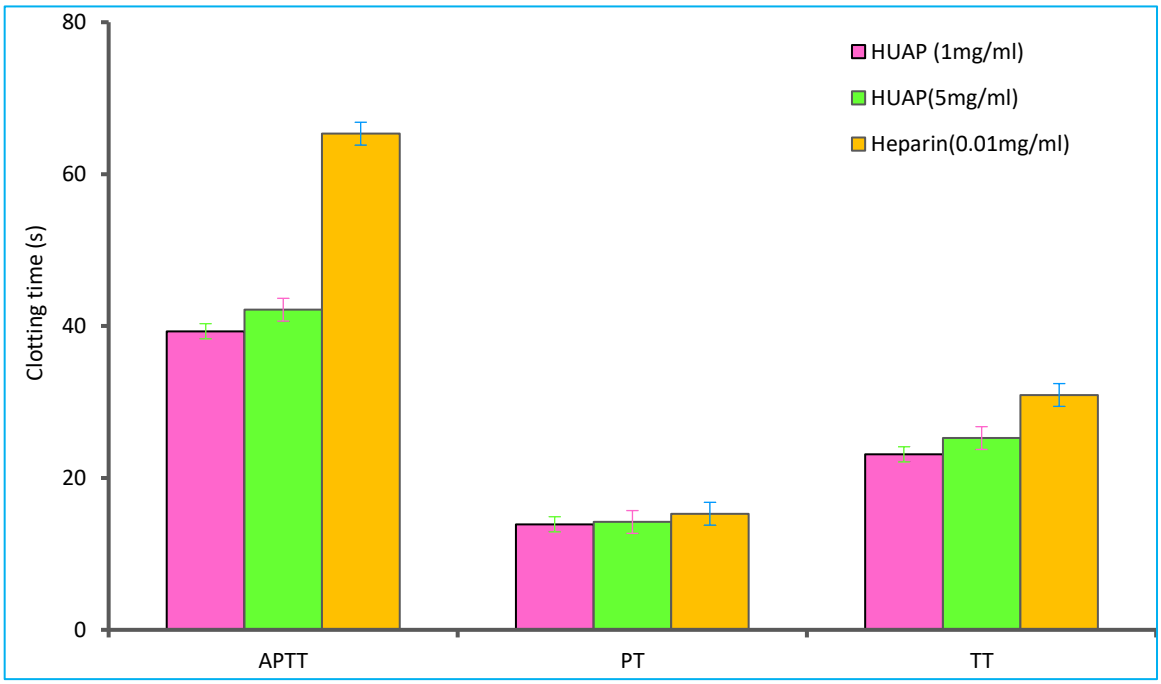

Fig 2 Anticoagulant activities of HUCP

Abbreviations

HUAP	:	*Hypsizygus ulmarius* Alkali Polysaccharide	DMPD	:	N,N-dimethyl-ρ-phenylenediamine
VitC	:	Vitamin C	TBA	:	Thiobarbituric acid
APTT	:	Activated Partial Thromboplastin Time	TPTZ	:	2,4,6 –Tripyridyl-s-triazine
PT	:	Prothrombin Time	FRAP	:	Ferric reducing antioxidant power
TT	:	Thrombin time			

CONCLUSION

In this study, we evaluated the *in vitro* anticoagulant and antioxidant activity of water-soluble alkali polysaccharide from *Hypsizygus ulmarius* mushroom. Compared with heparin, the anticoagulant activity of Heparin-binding urinary angiogenesis factor (HUAP) can prolong activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT). The results showed that HUAP exhibited strong radical scavenging activity, reducing power and lipid peroxidation inhibition. However, further studies are required to improve our understandings on the structure and anticoagulant mechanism of HUAP.

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Conflict of interest

The authors state no conflict of interest.

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