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Assessment of Anticoagulant and Antioxidant Properties of Polysaccharide Extracted from Pink Oyster Mushroom (*Pleurotus eous*)

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Abstract

This study investigates the anticoagulant and antioxidant potential of polysaccharide extracted from *Pleurotus eous*, obtained through alkali extraction. The antioxidant activity of polysaccharide was examined using different in vitro antioxidant assays like FRAP, DMPD radical assay, Cu (II) reduction capacity assay and Lipid peroxidation inhibition assay. The potential anticoagulant activity of PEAP was evaluated using various assays, which targeted the intrinsic and/or common pathways (activated partial thromboplastin time), the extrinsic pathway (prothrombin time), and the common pathway (thrombin time). In vitro antioxidant studies showed that the PEAP exhibited DMPD radical scavenging activity (EC_{50} = 14.66 mg/mL), FRAP (EC_{50} = 1.374 mg/mL), Cu (II) reduction capacity assay (EC_{50}=1.282 mg/mL), and Lipid peroxidation inhibition assay (EC_{50}= 1.323 mg/mL). PEAP significantly prolonged the activated partial thromboplastin time (APTT), thrombin tie (TT) and prothrombin time (PT) in a dose-dependent manner compared to the positive control. These finding suggested that *Pleurotus eous* alkaline polysaccharide (PEAP) might be an explored as a potential natural antioxidant and anticoagulant agent.

***Key words*:** Mushroom polysaccharides, alkali extraction, *Pleurotus eous*, Anticoagulant activity, antioxidant activity

Polysaccharides are type of natural polymer joined by aldose or ketose-containing glycosidic linkage and are abundant in living things [1]. Natural polysaccharides have gained more recognition in the biomedical field in recent years due to their significant anticancer and immunomodulatory activity as well as their low toxicity [2]. Mushrooms are exceptionally nutrient-dense edible fungi with a variety of therapeutic benefits and bioactivities. The primary active ingredients in edible mushrooms are polysaccharide [3]. In recent decades, native polysaccharides, particularly those with triple helical structure, have been thoroughly researched because to their notable bioactivities. They have also drawn increased interest due to their exceptional activities as anti-tumor, anticoagulant and immune-supporting compounds [4] Polysaccharides physical characteristics and biological activities, including their molecular weight distribution, solubility, chemical composition, etc., will be impacted by the extraction procedure [5]. Thrombotic diseases are a major contributor to illness and death worldwide. The primary approach for preventing and treating this condition involves the use of anti-coagulant drugs [6]. Given the rising prevalence of thrombotic diseases, there is a pressing need for highly effective medications. Heparin, an anticoagulant drug widely used in treating and preventing thrombosis, is associated with adverse effects. Due to the risk and substantial expenses associated with current treatments, there is a strong demand for further research to uncover new source of anticoagulants that prevent excessive clotting without increasing the risk of bleeding or causing other side effects. Free radicals are hazardous molecules generated during the body's oxidation reaction. They can destroy the body's tissues and cells, lead to chronic diseases, and accelerate ageing. They have potent oxidizing properties. Numerous polysaccharides increase the activity of antioxidant enzymes and remove free radicals from the body [7]. Therefore, it is important to find such natural antioxidant rich polysaccharide that can reduce the formation of free radicals. *Pleurotus eous* is a nutrient-rich edible fungus belongs to the basidiomycete from the pleurotaceae and classified within the *pleurotus* genus. Previously, we have reported the structural characterization and immunomodulatory effects of PEAP [8]. Thus, the present study aims to determine the, anticoagulant and antioxidant activity of alkali extracted polysaccharide from *Pleurotus eous*.

MATERIALS AND METHODS

Chemicals

Shade dried fruiting bodies procured from the IIHR Bangalore. Chemicals including TPTZ, HCL, $FeCl_3$, Ethanol, Ferric chloride, L ascorbic acid, BHT, DMPD, Acetic acid and PT kit, APTT and TT kits and other reagents, sourced from Sigma-Aldrich and HiMeida respectively, were of analytical grade with purity exceeding 98%.

Extraction of polysaccharide

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The defatted *Pleurotus eous* mushroom powder was combined with a 1 M NaOH and 1% NaBH4 solution (1:30 v/v) and allowed to stand overnight at room temperature [8]. After centrifugation at 5000 rpm for 10 minutes, the supernatant was reduced to one- tenth of its volume using a rotary evaporator. This was followed by dialysis against sodium acetate buffer (pH 5.0) for 24 hours, and then against distilled water for another 24 hours. The supernatant was neutralized with 50% glacial acetic acid, and the resulting precipitate was removed by filtration. The supernatant was further concentrated with a rotary evaporator, precipitated with a fourfold volume of 95% ethanol at 4°C for 24 hours, and deproteinized using the Sevag method. Finally, the solution was freeze-dried to obtain the crude polysaccharide (PEAP).

Antioxidant activities

DMPD radicals scavenging activity

This assay is based on the reduction of the purple-coloured radical DMPD+ and is carried out according to the approach of Apak *et al* (2006). 20.9 mg of DMPD was dissolved in 1 ml deionised water to create freshly prepared DMPD+ (100 mM), 500 µL of this solution was added to 50 mL of acetate buffer (0.1 M, pH 5.25) and the coloured radical cation was generated by adding 0.1mL of a 0.05 M ferric chloride solution, and the reagent was equilibrated to an absorbance of 0.900 0.100 at 505 nm that remined stable for 12 hours. The test tubes were filled with varying concentration of PEAP (0.5-2.5 mg/ml) and standard antioxidant (quercetin) and the total volume was adjusted to 0.5 mL using distilled water. 1mL of DMPD+ solution was added to solution after 30 minutes of incubation, and the absorbance reading were measured at 505 nm. A buffer solution was used as a blank for this experiment. The same formula used in the DPPH radical scavenging assay was used to calculate the results.

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) was evaluated using Benzie and Strain (1996) method. To make the FRAP working reagent, mix 25 mL acetate buffer (0.3 mol/L, pH 3.6), 2.5 ml TPTZ (10mmol/L in 40 mM HCL), and 2.5 ml $FeCl_3$ 6 H_2 0 (20 m mol/L). Mix 900 µL of FRAP reagent, 90 µL of water and 30 µL of PEAP (0.5- 2.5 mg/mL). At 37^0 C for half an hour, the reaction mixture was incubated. Increased reaction absorbance (595nm) indicated improved reducing capacity when compared to vitamin c as a control.

Cu (ll) reduction capacity assay

In the presence of the chelating agent neocuproine, the CUPRAC test assesses the samples ability to reduce Cu 2+ to Cu 1+. In a test tube, different concentration of PEAP (1.0-5.0 mg/mL) were mixed with 0.25 mL ethanolic neocuprine solution (7.5 × 10-3M) and 0.2 mL acetate buffer solution (1 M). After 30 minutes, the total volume was adjusted to 2.0 mL with distilled water, and the absorbance was measured at 450 nm against a reagent blank.

Lipid peroxidation inhibition assay

A modified thiobarbituric acid reactive species (TBARS) assay was used using egg yolk homogenates as lipid rich media to determine the amount of lipid peroxide generated. 0.5 ml of 10% egg homogenate and 0.1 ml of different concentration of (2.0-10 mg/ml) were mixed in a test tube, and the volume was increased to 1.0 mL by adding distilled water. 0.05 ml FeSO4 (0.07 M) was added to the above mixture and incubated for 30 minutes to cause lipid peroxidation. After that, 1.5 mL 20% acetic acid (pH 3.5 adjusted with NAOH), 1.5 mL 0.8 % TBA (made in 1.1% sodium dodecyl sulphate), and 0.05 mL 20% TCA were added, vortexed and heated in a boiling water bath for 60 minutes. Each volume was prepared with 5 mL of n-butanol and centrifuged for 10 minutes at 3000 rpm after cooling. The absorbance of the organic top layer was estimated at 532 nm. BHT was used as a positive control.

Blood coagulation assays

The anticoagulant activity of the polysaccharide was assessed *in vitro* using activated partial thromboplastin time APTT, thrombin time (TT), and prothrombin time (PT) tests. Human blood samples were collected using venipuncture method was used for the study. The collected samples was mixed with a sodium citrate solution (3.8%, w/w) at a ratio of 9:1 (v/v), followed by centrifugation at 3000 r/ for 10 minutes to isolate plasma for further analysis [9]. The APTT assay involved incubating 90 µL of citrated normal human plasma with 10 µL of PEAP (1 and 5 mg/ mL) at 37℃ for 60 seconds. Subsequently, 100µL of prewarmed APTT assay reagent was added and allowed to react at 37℃ for 2 minutes. Following this, 100 µL of prewarmed 0.25 mol/L calcium chloride was added, and the time taken for clot formation was recorded as APTT. For the TT assay, 90 µL of citrated normal human plasma was mixed with 10 µL PEAP (1 and 5 µg/mL) and incubated at 37℃ for 60 seconds. Then 200 µL of prewarmed TT assay reagent (37℃) was added, and the clotting time was recorded. In the PT clotting assay, 90 µL of citrated normal human plasma was mixed with 10 µL of PEAP (1 and 5 mg/mL) and incubated at 37℃ for 1 minute. 200 µL of pre-incubated PT assay reagent (37℃, 10 minutes) was added, and the clotting time was recorded [10]. Each clotting assay was conducted in triplicate, and the results are presented as mean values ± standard deviations (SD). Heparin was used as a positive control to assess the anticoagulant activity.

Statistical analysis

The data are represented as means and standard deviations (SD). Statistical differences were evaluated using one-factor analysis of variance (ANOVA) and Duncan's Multiple Range test. Results were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

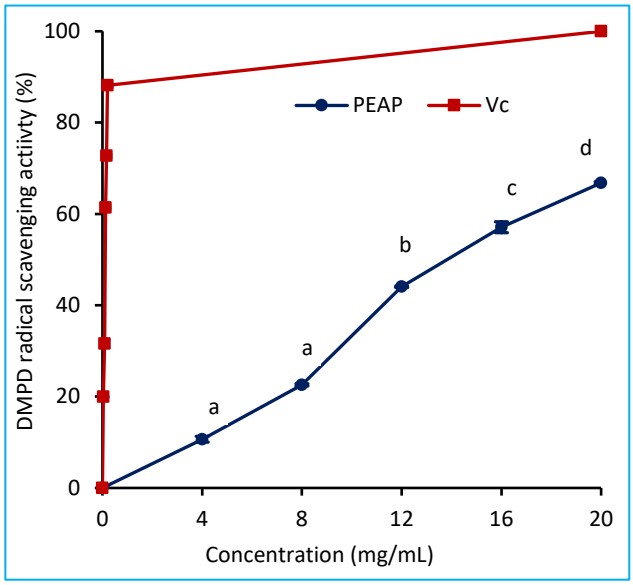
Antioxidant activities

DMPD radical scavenging activity

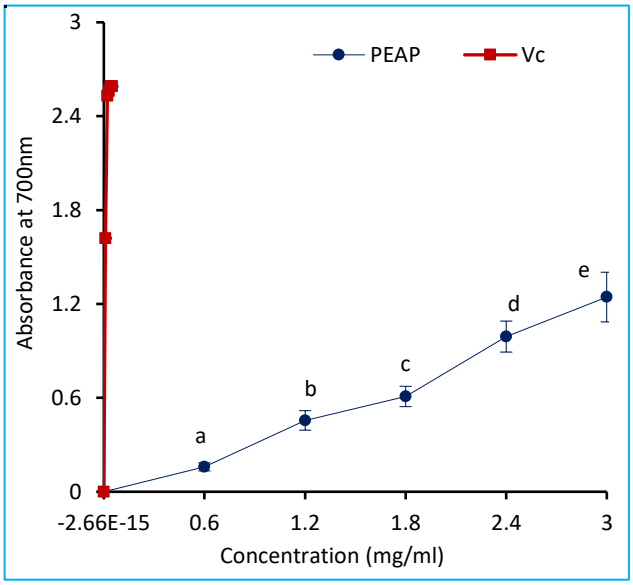
The DMPD assay is a technique to assess antioxidants capacity to donate hydrogen [11]. Antioxidant substances lighten the solution by removing the existing colour by transferring a H atom to $DMPD^+$ [12]. Over the whole range of concentrations from 4 to 20 mg/mL, PEAP demonstrated strong scavenging capacities with scavenging rates of 10.67%, 22.58%, 44.05%, 57.09%, and 66.78 % respectively. These values were lower than the vitamin C (19.96% - 88.19 at 0.04 to 0.2 mg/mL). The EC_{50} of PEAP was 14.66 mg/mL.

Ferric reducing antioxidant power assay

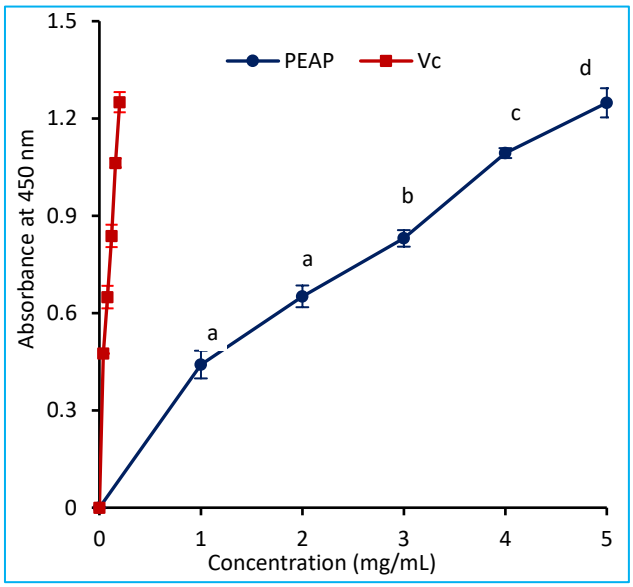
Antioxidants capacity to decrease Fe^{3+} - Fe^{2+} can be assessed using the FRAP assay, and the blue Fe^{2+} tripyridyltriazine complex can be detected at 593nm [13]. The scavenging ability of FRAP increased with the PEAP concentration. At the concentration of 0.6 mg/mL the FRAP value of PEAP were 0.159 and reaches about 1.244 when the concentration was 3 mg/mL. The EC_{50} value of PEAP was 1.374 mg/mL.



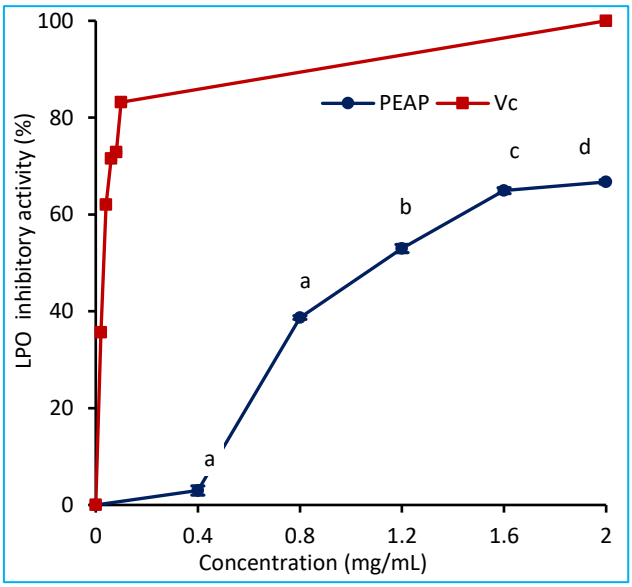
a. DMPD radical scavenging activity



b. Ferric reducing antioxidant power (FRAP)



c. Cu (ll) reduction capacity Assay



d. Lipid peroxidation inhibition

Fig 1 (a-d) Antioxidant activities of PEAP

Cu (ll) reduction capacity assay

The reduction of Cu (ll) to Cu (l) by antioxidants in the presence of neocuprine is the basis of the cupric ion reducing antioxidant power assay, and increase in absorbance implies higher cupric ion reducing power [14]. The concentration ranging from 1-5 mg/mL the cupric ion (cu^{2+}) reducing abilities of PEAP were between 0.441-1.248 respectively and it was lower than the positive control vitamin C value ranging from 0.475-1.250 at 0.04-0.20 mg/ml. EC50 value of PEAP is 1.282 mg/mL.

Lipid peroxidation inhibition assay

Since the polysunsatured fatty acids PUFAs in cell membrane reacts with ROS to cause lipid peroxidation, which modifies the permeability and fluidity of the lipid bilayer and compromises cellular integrity, it is important to quantify the inhibition of lipid peroxidation as a sign of strong antioxidant capabilities [14]. The lipid peroxidation inhibition effects of PEAP demonstrated a dose dependent manner. When the of PEAP was increased from 0.4-2.0 mg/mL respectively, LPO inhibition drastically increased from 2.99% to 66.72%, and it was lower than that of vitamin C values of 35.63%-83.19% at 5-25 mg/mL. The Ec_{50} value of PEAP is 1.323 mg/mL.

Anticoagulant activity

Anticoagulant activity *in vitro* of PEAP was assessed by activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT). In this study, heparin was used as a positive control. As shown in the (Table 1) PEAP significantly prolonged the APTT, TT and PT in a concentration dependent manner. The APTT prolongation suggests inhibition of the intrinsic and/or common coagulation pathways [15]. The PT test, evaluating the impact of PEAP on the extrinsic pathway, also demonstrated a notable effect. Moreover, PEAP was found to significantly enhance its anticoagulant activity, as indicated by the TT assay, which assesses anticoagulant effects on the common pathway of coagulation [16]. The conversion of fibrinogen into fibrin is recognized as the final step in coagulation, with thrombin time (TT) serving as a crucial indicator of this process [9]. Additionally, at a concentration of 1 mg/ mL, PEAP clotting time was about 41.18 seconds in the APTT assay, 15.48 seconds in the PT assay, and 21.78 in the TT assay. At 5 mg/mL, the clotting times were 44.41 seconds for APTT, 16.11 seconds for PT, and 26.6 seconds for TT. These findings indicated that PEAP exhibited strong anticoagulant activity *in vitro*, effectively inhibiting both the intrinsic and/or common pathways of coagulation.

Table 1 Anticoagulant activities of PEAP

Sample	Concentration	Clotting time (s)		
		APTT (Activated partial thromboplastin time)	TT (Thrombin time)	PT (Prothrombin time)
PEAP	1 mg/mL	41.18	21.78	15.48
	5 mg/mL	44.41	26.60	16.11
Heparin	0.01 µg/mL	65.12	30.77	19.83

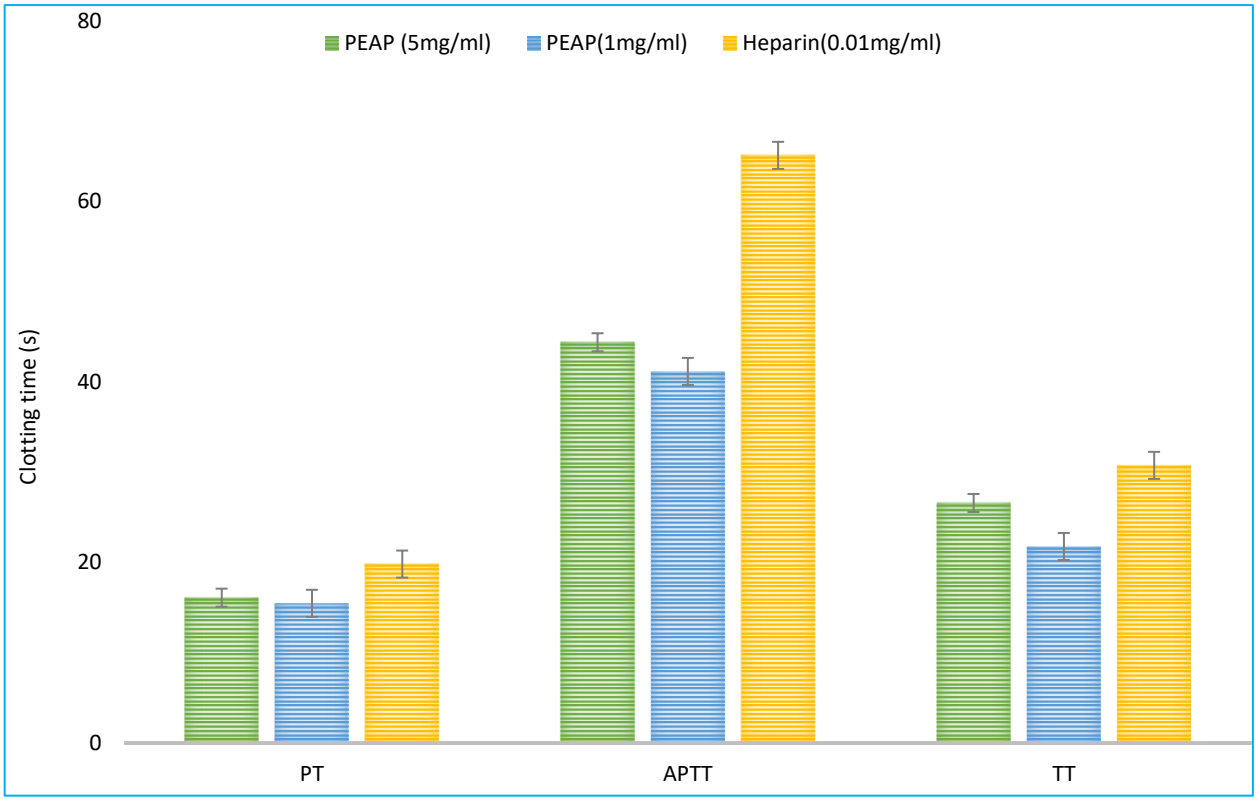


Fig 2 Anticoagulant activities of PEAP

CONCLUSION

A water-soluble, polysaccharide, PEAP was extracted from the edible mushroom *Pleurotus eous*. PEAP was found out to be an antioxidant-rich polysaccharide, as demonstrated by antioxidant assays such as DMPD radical assay, FRAP, Cu (ll) reduction capacity assay and Lipid peroxidation inhibition assay. PEAP were shown to have strong anticoagulant activity especially PEAP exhibited significant anticoagulant activity in the APTT assay and TT assay indicating their primary effectiveness on the intrinsic and/or common pathways of coagulation cascade. These finding suggest that PEAP could be a promising candidate for further development as an anticoagulant with potentially fewer side effects and broader applications.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Abbreviation

PEAP	- *Pleurotus eous* alkaline polysaccharide
APTT	- Activated Partial Thromboplastin Time
PT	- Prothrombin Time
TT	- Thrombin time
VitC	- Vitamin C
DMPD	- N,N-dimethyl-ρ-phenylenediamine
CUPRAC	- Cu (ll) reduction capacity Assay

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