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Antioxidant Activities of Crude Polysaccharide Isolated from *Pleurotus eous* and its Preliminary Structural Properties

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Abstract

The aim of this research work was to isolate polysaccharide from edible mushroom *Pleurotus eous* and to find out its preliminary structural characterization and *in vitro* antioxidant activities. The crude polysaccharides from *Pleurotus eous* were isolated by using hot water extraction. The chemical composition analysis, and FT-IR analysis were then used to determine the physicochemical parameters and the functional groups of *Pleurotus eous* polysaccharide (PEP) respectively. The antioxidant activities were also investigated using several methods, such as the DPPH assay, ABTS assay, FRAP assay and β-bleaching assay. Surface morphology and crystallinity of PEP was detected using Scanning Electron Microscopy and XRD respectively. Furthermore, the triple helical structure of PEP was determined using Congored Assay. Chemical composition of PEP such as carbohydrate content (76.23%), reducing sugar (6.13%), uronic acid and sulphate content were 6.7% and 4.1% respectively. Protein content of PEP was 6.43%. PEP showed strong antioxidant activity in a dose-dependent manner according to the antioxidant experiments. DPPH scavenging activity (50.73%). ABTS (51.55%), FRAP (1.91 mg\mL) *B*-carotene Assay (54.76%). The finding suggested that hot water extraction could be useful method for producing polysaccharides with greater antioxidant properties, potentially enhancing application in functional foods and medicine industry.

***Key words*:** Polysaccharides, Edible mushroom, *Pleurotus eous*, Antioxidant, Hot water extraction

Polysaccharides are abundant in nature and essential cell building blocks. They play a significant role in the growth, development, and functional regulation of organisms [1]. Natural polysaccharide is receiving more and more attention as ingredients in functional foods, medicine, and dietary supplements [2]. Mushrooms have been consumed as food for generations. Because of their bioactive elements, such as proteins, polysaccharides, phenolic compounds, terpenes, etc. This is due to both their distinctive flavour as well as their significant nutritional and therapeutic significance [3]. Due to their numerous biological properties, including as anti-oxidant, anti-viral, anti-tumor, anti-inflammatory, immunological and hypoglycaemic effects, polysaccharides have attracted a lot of attention in recent years [4]. According to reports, natural polysaccharides were crucial in protecting against oxidative stress by lowering the reactive oxygen species. (ROS). Polysaccharide physiological activities are greatly influenced by their unique chemical compositions and origin-dependent features [5]. Therefore, it is crucial to investigate the separation, isolation and characterization of various polysaccharides.

To prevent or treat the rising number of cancer cases around the world, it is vital to find new, highly safe, and reasonably priced anti-cancer drugs. Natural anti-tumor polysaccharide, in contrast to current pharmaceuticals used in cancer chemotherapy, have negligible or no negative side effects [6]. One of the most widely cultivated mushroom species is the *Pleurotus* species, also known as oyster mushrooms. They are farmed in South East Asia, India, Europe, and Africa, particularly Nigeria, and are edible fungi [7]. *Pleurotus eous* is often referred to as the pink oyster mushroom, *Pleurotus eous* (Berk.) is a valuable and nutrient rich edible mushroom [8]. In order to lay the groundwork for future research on the connection between pharmacological activities and polysaccharide structures, this study set out to demonstrate the preliminary structural properties and antioxidant activities of crude polysaccharides from *Pleurotus eous*.

MATERIALS AND METHODS

Sample collection

Pleurotus eous mushroom was purchased from IIHR Bangalore. All the chemicals used in this experiment were of analytical grade.

Extraction of crude polysaccharide

Dried mushroom powder was de-fattened using petroleum ether (24 hours). To collect the solid residue, it was centrifuged twice to remove the lipids and pigments [9]. The collected residue was air dried and extracted thrice using hot water (1:30(w/v), 55°C, 2 h), followed by centrifugation to collect the filtrate. The filtrate was condensed using rotary evaporator under reduced pressure. Condensed sample was dialyzed against distilled water for 48 hr. The dialyzed sample then centrifuged, condensed and precipitated with 3 volumes of

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ice-cold ethanol. Ethanol precipitated polysaccharide was centrifuged and lyophilized to obtain the crude *Pleurotus eous* polysaccharide (PEP).

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

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

Chemical compositions analysis

The carbohydrate content was determined using phenol sulphuric acid method with D- glucose as a standard [10], Barford method used for the determination of protein content and bovine serum albumin as the standard [11]. Sulphate content was determined according to the Tehro and Haritiala method, potassium per sulphate acting as a reference [12]. Using DNS (Dinitro salicylic acid), the sugar reduction was evaluated [13]. The sulphuric acid and carbazole method were used to determine the amount of uronic acid content [14]. The carbazole reacton, which is the most accurate way t measure uronic acid, was utilized for quantification. Galacturonic acid was used as the standard. For PEP, the results are expressed as %.

Structural characteristics

UV-vis spectrum analysis

The UV spectra of aqueous solution of PEP (1mg/ml) in the 190-400 nm wavelength were obtained by the UV-visible spectrophotometer (UV-2550, Shimadzu. Tokyo, Japan).

Fourier transform infrared (FT-IR) spectroscopic analysis

FT-IR Spectrophotometer was used to obtain the infrared spectra of the polysaccharide sample. PEP polysaccharide were crushed with KBr powder and then pressed into a polymer film for FT-IR measurement between 4000-400 cm^{-1}.

SEM

Using a scanning electron microscope, the shape and surface characteristics were examined and pictures were recorded with a magnification of 500-10,000x. Gold powder was applied by sputtering to polysaccharide samples that had been fixed to the specimen holder.

XRD

X-ray diffraction patterns of the polysaccharide were obtained using a DMAX-2000 diffractomer (Rigaku, Tokyo, Japan). The operating conditions of the diffreactometer were: nickel filtered Cu kα radiation, 36 kv and 26 mA scan Speed 0.05 min^{-1}, angular range of 5-70° (2θ).

Triple helix structure of PEP

Triple helical structure of PEP was determined by Congo red assay, in which a red shift in the light absorption maximum λ_{max} is attributed to the triple helices of polysaccharide chains [15]. 6 mg of polysaccharide was dissolved in 2.0 ml of distilled water and reacted with 2.0 ml µmo;/1 of Congo red in a gradient of sodium hydroxide solution (0.0, 0.1,0.15,0.2,0.3,0.4,0.5, and 1.0 mol/1). The absorbance was measured in the range of 200-800 nm, and max at different concentrations of sodium hydroxide was plotted. Distilled water without adding polysaccharide was served as the control.

Antioxidant activity

DPPH

The capacity of PEAP to scavenge DPPH was assessed using [16]. Mix 4.0 mL PEAP (1-5mg/mL) and Vitamin C as a positive control with 1.0 mL methonolic DPPH solution (final concentration of DPPH is 0.2mM) for 30 minute in the dark, with absorbance measured at 517 nm. The scavenging ability of DPPH free radical is calculated as:

$$(A_0 - A_1) / A_0] \times 100$$

Where; A_0 is DPPH + water absorbance and A1 is DPPH + PEAP Absorbance

ABTS

The radical scavenging ability of ABTS was measured using [17]. In this assay ABTS is oxidized with ammonium persulphate to produce the ABTS+ chromophore. The ABTS radical solution was created by overnight rection of 1.0 ml ABTS stock solution (7.0 mM) with 1.0 mL potassium persulphate (4.95 mM) at 25 °C in the dark. With ethanol, the ABTS radical solution was diluted to an absorbance of 0.7 ± 0.05 at 734 nm. To begin the reaction, 1.0 mL of diluted ABTS+ was added to 10µL of PEAP (0.2 to 1.0 mg/mL or 10 µl of methanol as a control. After 6 minutes of reaction absorbance was measured at 734 nm. The ability of the ABTS to scavenge radicals was calculated using the equation below:

ABTS radical scavenging ability (%) = $[1 - (A_1 - A_2) / A_0] \times 100$

Where; A_0 represents the Abs of control (deionized water instead of sample)

A_1 represents the Abs of sample, and

A_2 is the Abs of the sample with 0.2 M PBS instead of ABTS

Ferric Reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) was evaluated using [18] 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 mmol/L). Mix 900 µL of FRAP reagent, 90 µL of water and 30 µL of PEAP (0.5- 2.5 mg/mL). At 37 °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.

β-carotene bleaching assay

The anti-lipid peroxidation properties of the samples were assessed using a linoleic acid/-carotene combination---- prepare a β carotene solution (2 mg/10 mL chloroform) and pour 2 mL into a flask to mix with 40 mg lioleic acid and tween 40.(400mg). Following the removal of the chloroform, 100 mL of oxidized ultra-pure water was added to the emulsion, which was vigorously shaken. Pipette 2.4 mL of the emulsion into a separate test tube with 0.1 mL PEP (1-5 mg/ml). The positive control was butyl hydroxyl anisole (BHA). PEP was replaced with water in the control group. As soon as the sample was added to each tube, the zero-time absorbance was measured at 470 nm with a spectrophotometer. In a hot water bath, the tubes were then incubated at 50 °C. After 2 hours, the absorbance value was measured again. BHA was used as a positive control. A beta carotene- free blank was created for background subtraction.

Bleaching inhibition (%) = β-carotene content after 2 h of assay / Initial β-carotene content) × 100

Statistical analysis

The results were expressed as mean ± standard deviation (SD) of three replicates. Statistical analyses were carried out

using Analysis of variance (ANOVA) and the significance of the difference 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 PEP

The yield of the PEP was 2.3%. As shown in the (Table 1), carbohydrate content of PEP was 76.23%, reducing sugar was found to be 6.13%, uronic acid and sulphate content were 6.7% and 4.1% respectively. Protein content of PEP was 6.43%.

Table 1 Chemical composition of PEP

Yield (%)	Carbohydrate (%)	Reducing sugar (%)	Protein (%)	Uronic acid (%)	Sulphate (%)
2.3±0.1	76.23±1.078	6.13±0.15	6.43±0.208	6.7±0.3	4.1±0.40

UV-vis spectrum analysis

The UV spectrum of Polysaccharide in the range of 190-400 nm is frequently used to confirm the purity of polysaccharide samples. As presented in (Fig 1) PEP polysaccharide had a peak at 257 indicating the presence of protein.

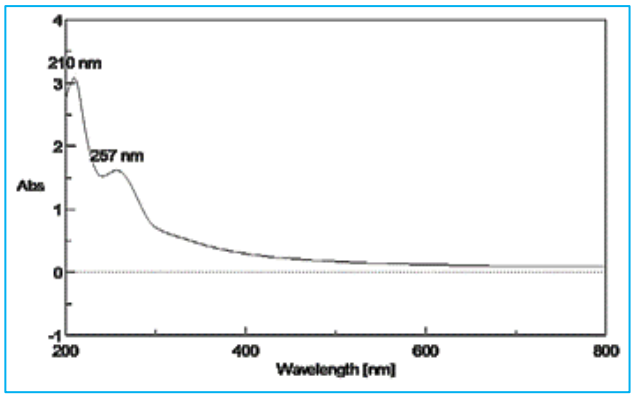

Fig 1 UV spectra of PEP in the range of 200-400 nm

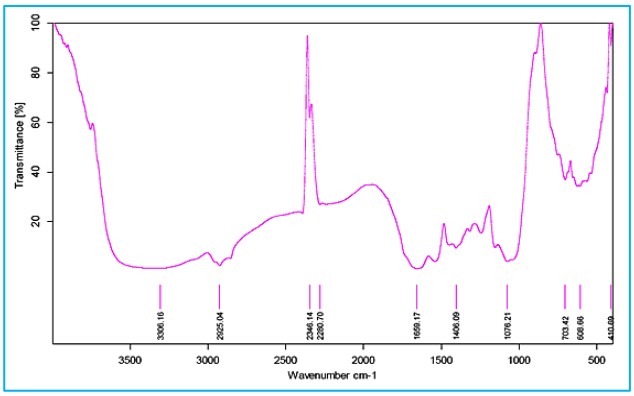

Fig 2 FTIR spectra of PEP

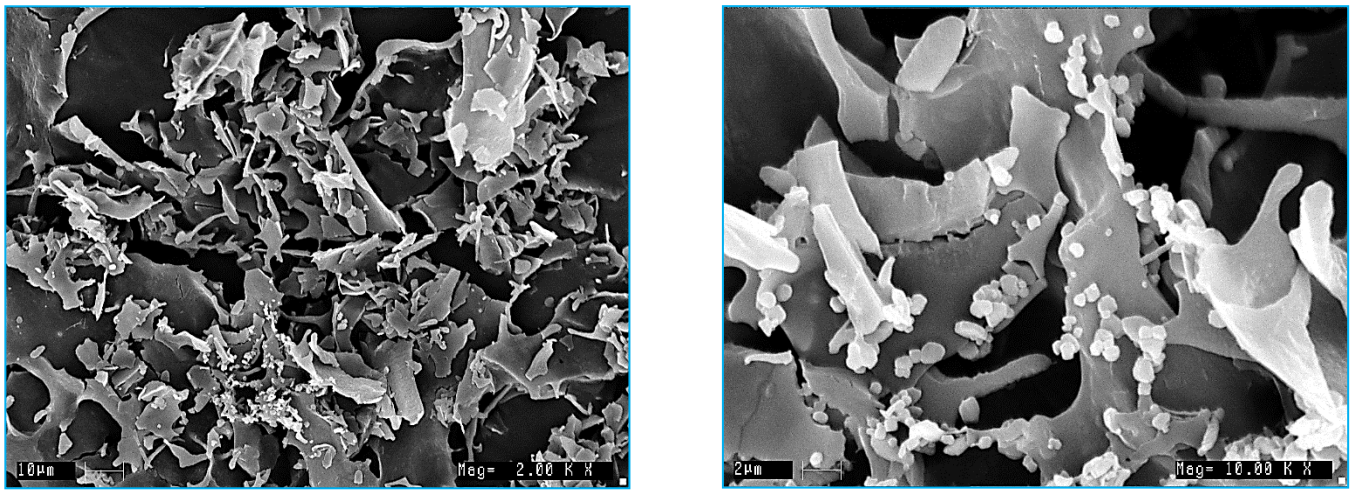

Fig 3 SEM images of PEP

FTIR (Fourier transform infrared)

PEP showed a few typical polysaccharide absorption peaks in the range of 4000-400 cm^{-1}. The bands in the region of 3306.16cm^{-1} was due to the hydroxyl stretching (O-H) vibration in carbohydrates, indicating the presence of inter-molecular hydrogen bonds. (Fig 2) represent the FTIT spectra of PEP. The band in the region of 2925.04cm^{-1} as the characteristic's absorption of C-H stretching vibration. The absorption peaks within the range of 1100-1010 cm^{-1} indicated the possible presence of pyranose ring and furanose ring. The absorbance at 1659.17 cm^{-1} indicated the presence of carbonyl group (C=O). An absorption peak was also observed near 1406.09 suggesting the probable presence of uronic acid in the PEP.

Scanning electron microscopy (SEM)

One way to characterize the apparent morphology of polysaccharide is by scanning electron microscopy (SEM), which can detect the stereoscopic morphology of polysaccharides [19]. PEP appeared as irregular particles with a flat surface and nodule like appearance towards the edge. changes in physiochemical properties and the chosen kind of extraction procedure most likely contributed to the polysaccharides varied surface topography [20]. (Fig 3) shows the SEM images of PEP.

XRD

The XRD pattern was evaluated to determine the crystallinity of *Pleurotus eous* polysaccharide (PEP) (from 10-80°). PEP displayed the norma; XRD pattern for semi crystalline polymers as seen in the (Fig 4). Overall poor crystallinity was seen in *Pleurotus eous* polysaccharide (PEP), and the crystalline region was found at an angle of 20°. The results were comparable to the polysaccharides found in chickpea hulls [21]. Other peaks, on the other hands, are either shoulders of more intense peaks or are relatively weak and unresolved. The solubility of molecules is also influenced by their crystallinity. For instance, more crystallinity in some rugs indicates less water solubility [22].

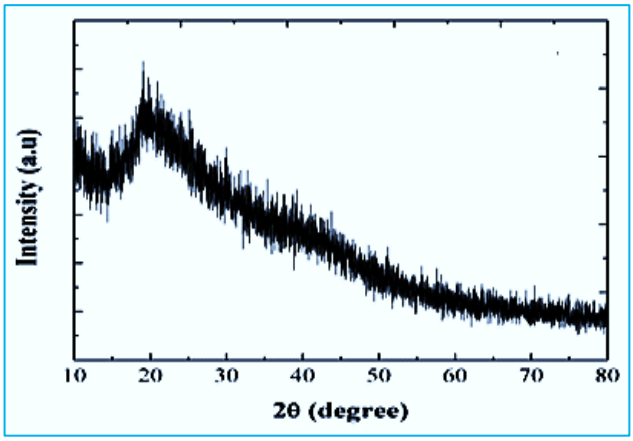

Fig 4 XRD images of PEP

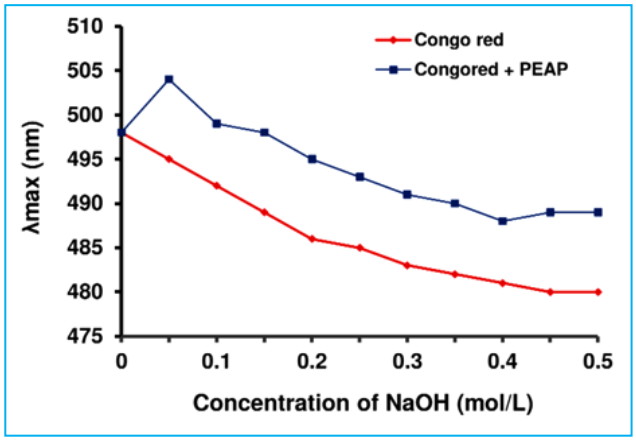

Fig 5 Congored analysis

Triple helical structure

In order to understand function and biological activities, it is important to identify the triple helix conformation of PEP. Polysaccharide with Triple-helical structures may combine to generate a Congo red-polysaccharide complex, which will exhibit λ_{max} red shift in comparison to the Congo red control [23]. (Fig 5) shows the changes in the λ_{max} concentration of the polysaccharide with Congo red complex. Thus, the result indicated that the *Pleurotus eous* polysaccharide (PEP) had triple-helical structures as evidenced by the strong red shifts in λ_{max}, the result was in accordance with the results reported by [24].

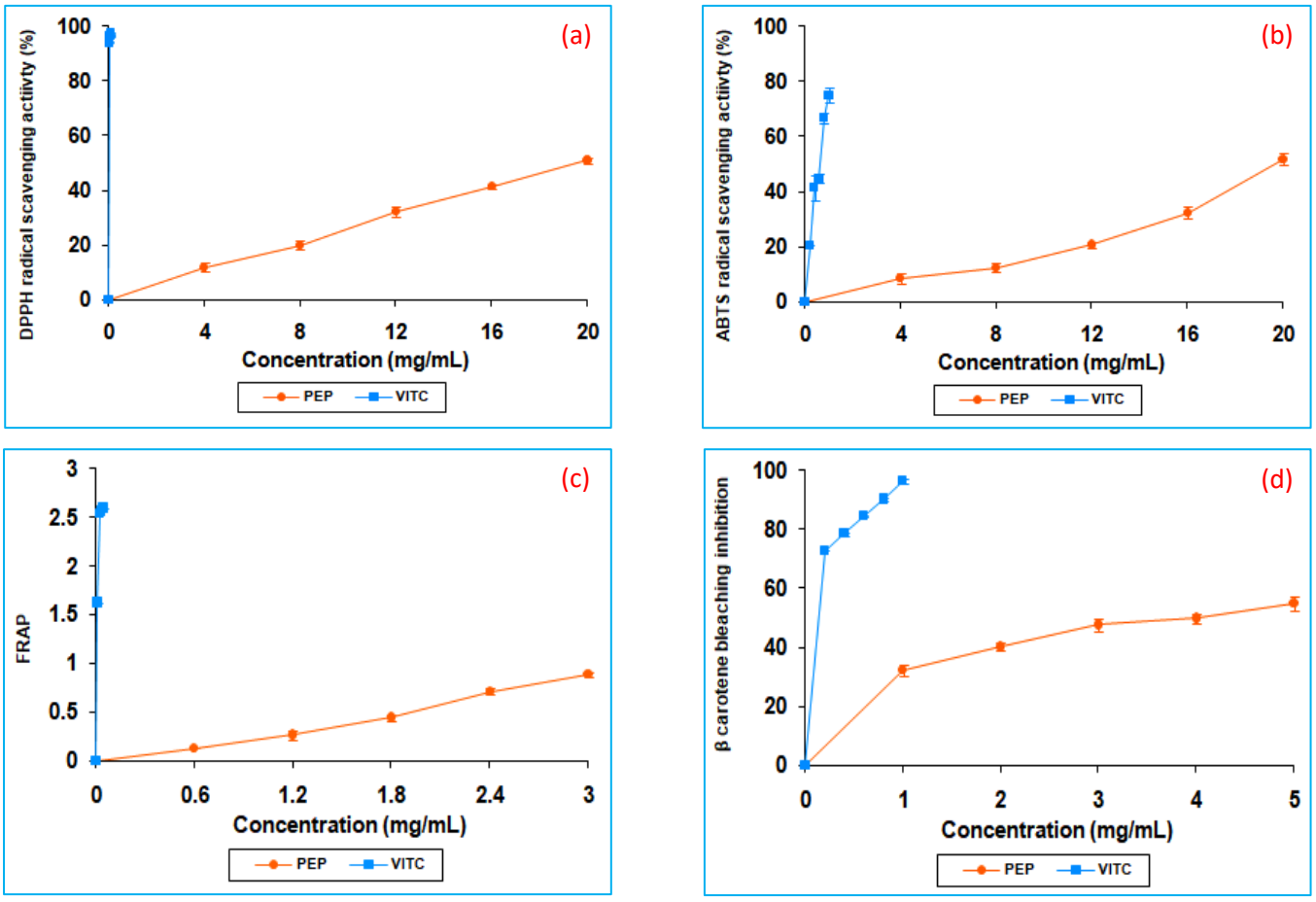

Fig 6 Antioxidant activities of *Pleurotus eous* polysaccharide (PEP)
(a) DPPH radical scavenging activity (b) ABTS radical scavenging activity (c) FRAP (d) β-carotene bleaching assay

Antioxidant activity analysis in vitro

DPPH

The DPPH Radical Assay is one of the most widely used assays for determining polysaccharide antioxidant capacity, because it delivers repeatable results at room temperature [25]. The ability of antioxidants to scavenge stable radicals was determined by measuring the decrease in absorbance caused by antioxidants [26]. The DPPH radical assay has become a standard tool for assessing antioxidant activity in natural products in vitro. In this study, therefore DPPH radical scavenging activities of PEP were determined (Fig 6a). With an EC50 value of 19.8 mg/ml, PEP showed strong DPPH scavenging activity. Although PEP's scavenging activity was lower than that of the positive control VC, it was nonetheless significant.VC has an EC50 of 0.052 mg/ml. At concentration ranging from 4 to 20 mg/ml, PEP demonstrated scavenging activity in a concentration-dependent manner. PEP had a scavenging activity of 11.83±30 at a low dosage of 4 mg/ml. At

the high concentration of 20mg/ml, the scavenging activity were 50.73±2.08. Many factors influence polysaccharide antioxidant activity, including water solubility, uronic acid content, polysaccharide molecular weight and monosaccharide composition, etc. The result suggested that the crude polysaccharide from *Pleurotus eous* acted as a DPPH radical scavenger. A similar result was found in the scavenging effect of crude extract from *M. crassa* [27] (Fig 6a).

ABTS

The ABTS assay is a commonly used method for determining a potential antioxidant's total antioxidant activity [28]. The spectrophotometric techniques used to assess the overall antioxidant activity of pure substance solutions, and beverages were based on the inhibition of the formation of the ABTS+ a radical cation [29]. As the concentration of the PEP increased, so did its ability to scavenge ABTS radicals (Fig 6 b). At a concentration of 4 mg/ml, PEP had a scavenging ratio of 8.53%, while at a concentration of 20mg/ml it had a scavenging ratio of 51.55%. However, the EC50 value of PEP was 19.4 mg/ml which is much less than that of the positive control VC (0.66 mg/ml).

FRAP

A quick and easy way to evaluate antioxidant power is with the FRAP assay. The reducing power may be measured by detecting Prussian blue production at 700 nm. The antioxidant ability to convert $Fe^{3+}(CN^-)_6$ into $Fe^{2+(}CN^-)_6$ [18]. The samples antioxidant abilities showed a strong correlation with concentration (Fig 6c). With increasing sample concentration, a significant difference in ferric reduction antioxidant power was seen. The reducing power of the PEP and ascorbic acid were 0.881 (3mg/ml) and 2.591 (0.03 mg/ml) respectively. Additionally, EC50 value of PEP were 1.91 mg/ml, which is less than that of a vitamin c 0.025mg/mL.

B-carotene Assay

When subjected to thermally induced oxidation, linoleic acid works as a source of free radical that generate peroxyl radicals. The sudden loss of colour is caused by the reaction of β-carotene with linoleic acid hydroperoxides [29]. Since the antioxidant prevents lipid peroxidation by breaking the chain and scavenging the linoleic free radical produced, the presence of antioxidant in linoleic acid emulsion system prevents-carotene bleaching [30]. The ability of PEP to prevent or minimize reduction of β carotene is illustrated in (Fig 6d). The antioxidant capacity of PEP increased with increasing PEP concentration. It was lower than that of the Vc used as a positive control. At a concentration of 1 mg/ml the inhibition of extracted PEP was 32.12±1.77, and at a highest concentration of 5 mg/ml inhibition of PEP were 54.76±2.39 respectively. EC50 value of PEP was 4.32 mg/ml.

CONCLUSION

The crude polysaccharide from PEP used in this investigation were extracted using hot water. The carbohydrate content was significant. FT-IR spectroscopy of PEP showed typical characteristic absorption peaks of polysaccharides. In addition, *P. eous* polysaccharide shown strong free radical scavenging properties in a dose-dependent manner along with a triple helical structure that was confirmed by Congo red analysis. XRD revealed PEP had a semi crystalline structure. Future research will concentrate on the antitumor activity of PEP in vitro and understand its underlying mechanisms. Additional research is also required to determine the monosaccharide composition and molecular weight of PEP polysaccharide.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Abbreviations

PEP - *Pleurotus eous* Polysaccharide
DPPH - 1,1-Diphenyl 2-picrylhydrazyl
ABTS - 2,2'-Azino-bis [3-ethylbenzthiazoline sulphonate]
FRAP - Ferric reducing ability of plasma
VC - Vitamin C

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