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Differential Biochemical Content and Free Radical Scavenging Potency of Three Solvent Extracts of *Porodaedalea pini*

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ABSTRACT

Porodaedalea pini, a non-edible mushroom, is a notable source of bioactive compounds with traditional medicinal uses. Solvent selection is crucial in research and development of natural products. This study examined how solvent polarity affects the biochemical composition and antioxidant properties of *P. pini*. Crude extracts were prepared from whole mushrooms using chloroform, methanol, and ethyl acetate through cold extraction. Total carbohydrate, flavonoid, and phenolic contents were measured, and free-radical scavenging activities against DPPH and superoxide anion were assessed by employing standard protocols. Methanolic extracts yielded significantly more than ethyl acetate ($p<0.05$) and chloroform ($p<0.001$) extracts, showing higher levels of carbohydrates, proteins, flavonoids, and phenolics ($p<0.001$). All extracts displayed notable free-radical scavenging activity, with methanol being the most potent, followed by ethyl acetate and chloroform. Thus, solvent polarity greatly affects the biochemical constituents and antioxidant activity of *P. pini* extracts, highlighting the importance of solvent choice in optimising their bioactive potential.

Keywords: Antioxidant; P. pini; Extraction yield; Flavonoids

NOMENCLATURE

AA	: Ascorbic Acid
CE	: Chloroform Extract
DPPH	: Diphenyl-1-picrylhydrazyl
EAE	: Ethyl Acetate Extract
GAE/g	: Gallic Acid Equivalent
ME	: Methanolic Extract
QE	: Quercetin Equivalent
SOD	: Super Oxide Dismutase
TFD	: Total Flavonoid Content
TPD	: Total Phenolic Content

1. INTRODUCTION

Numerous natural products have gained ethnopharmacological recognition for their medicinal attributes. In many instances, these natural products are utilised to prepare crude extracts using various solvents for the formulation of herbal medicine. Alternatively, bioactive molecules are isolated from such plants for use as therapeutic agents. Regardless of the method used, selecting the right solvent is vital for ensuring the quality and quantity of the final product[2]. Despite the dominance of synthetic agents derived from parent compounds in the modern medicinal landscape, traditional medicinal systems and therapies based on natural products continue to hold significance due to their efficacy and minimal side effects. Moreover, they serve as renewable sources of raw materials for product derivation[1].

Among natural sources, mushrooms, particularly non-edible varieties, occupy a special niche for their medicinal properties[3]. A wealth of literature exists on the medicinal values of various mushroom species. Furthermore, mushrooms are recognised as potential sources of new therapeutic agents for a range of health disorders, including cancers[4,5]. It has been estimated that approximately 130 bioactive molecules with properties such as cardioprotective, radical scavenging,antitumor, antiviral,immunomodulatory effects have been identified from various mushroom species[6]. Several mushroom species have been documented to possess diverse therapeutic properties, encompassing antihyperlipidemic, antidepressant, hypotensive, osteoprotective, anti-inflammatory, immunomodulatory, hepatoprotective, antibacterial, antifungal, antiallergic, antioxidative, antiviral, cytotoxic, antidiabetic, nephroprotectiveand neuroprotectiveactivities[5].

Porodaedaleapini (formerly known as *Phellinus pini* (Brot.) Murrill 1905 belongs to the Hymenochaetaceae family of wood-inhabiting fungi. As a fungal plant

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pathogen, its growth leads to a tree disease called red ring rot, alternatively referred to as white speck. *P. pini* is recognised as a major cause of stem decay in conifers in the northern temperate zone[7]. Despite its pathogenic nature towards certain plants, it has acquired medicinal importance among humans. In Asian countries, *P. pini* has been utilised as a folk medicine for cancer and gastrointestinal diseases[8]. Polysaccharides EP-AV1 and EP-AV2, derived from the hot-water extraction of *P. pini*, show potential as effective agents against HSV-1 infections[9]. Ryang[10], *et al.* isolated an antioxidant molecule from the ethanolic extract of *P. pini*, in an integrated chromatographic techniques and demonstrated its ameliorating effect against H_2O_2-induced toxicityusing rat H9c2 cell line. Im[8], *et al.* reported the butyrylcholinesterase and acetylcholinesterase inhibitory effects of methanolic and hot water extracts of *P. pini* fruiting bodies, indicating their potential as anti-cholinesterase and anti-inflammatory agents. Certain metabolites isolated from *P. pini* have shown antifungal activity against several tree pathogens[11]. *P. pini* contains phenolics, alkaloids, lactones, steroids, and terpenoids, and exhibits potent antioxidant activity[12].

Considering the medicinal and nutraceutical significance of mushrooms, as well as the critical role of solvent selection in extracting bioactive compounds from natural sources, this study was conducted to comparatively assess certain biochemical characteristics of crude extracts of *P. pini* obtained from three solvents: methanol, ethyl acetate, and chloroform.

2. MATERIAL AND METHODS

2.1 Chemicals and Reagents

The reagentsused for the present investigation include: Aluminium chloride (CAS: 7746-70-0); Anthrone (CAS: 90-44-8); Ascorbic acid (CAS: 50-81-7); DMSO (CAS: 67-68-5); Gallic acid (CAS: 5995-86-8); NBT (CAS: 1138321300); DPPH (CAS: 1898-66-4); Quercetin (CAS: 117-39-5) were procured from Sigma Aldrich Chemicals Private Limited, marketed from Bengaluru, Karnataka, India. The other chemicals were purchased from Himedia Laboratories, LLC, Mumbai.

2.2 Mushroom Collection and Extraction

The selected species of the mushroom was collected in the wild areas of Wayanad, a hill station in the Kerala state of India.The collected mushroom was identified using ataxonomic key[13] and the website[14] https://www.mushroomexpert.com/. *P. pini* was identified by scrutinising several key characteristics. The criteria utilised encompassed the following: fruiting body shape and size; color and texture; underside features; flesh color and texture; spore print; habitat and host tree; as well as microscopic examination of spores. The identification was confirmedwith the assistance of a planttaxonomist from the Department of Applied Botany at Mangalore University. Upon collection, the samples were cleaned, sealed, and transported to the laboratory for further analysis. They were photographed, identified, and authenticated. Subsequently, the mushrooms were desiccated at 40 °C in a hot air oven, pulverised, and subjected to cold extraction following a standard method[15] with a slight modification.

For the extraction process, three solvents, namely chloroform, methanol, and ethyl acetate, were employed to prepare the crude extracts. A predetermined quantity of preliminarily processed mushrooms was taken in a flask, one for each solvent, and kept in a water shaking water bath and set to a shaking speed of 120 strokes per minute, maintained at 37 °C for 24 hours. Then, the preparation was centrifuged at 5000 x g for 15 minutes, followed by filtration of the supernatant using Whatman No. 1 filter paper. The residue underwent an additional extraction step with 100 ml of solvent. The filtrates from both extraction cycles were then lyophilised and preserved at 4 °C for later use.

2.3 Yield of the Crude Extracts

After processing a 10 gram mushroom sample, sequential extraction using chloroform, ethyl acetate, and methanol, wascarried out. Subsequently, the resulting crude extracts were individually obtained and weighed using a precision balance.

The percentage yields of the solvent extracts were determined using the following eqn (1)

$$\%\,\text{Yield} = \left(\frac{W1}{W2}\right) \times 100 \qquad \text{Euq (1)}$$

W_1 = Weight of the crude extract

W_2 =Weight ofthe mushroom powder used for extraction

2.4 Phytochemical Screening

The following tests were conducted for phytochemical screening of the crude extracts[16,17].

Phenols (ferric chloride test and leadacetate test); flavonoids (alkaline reagenttest and lead acetate test); terpenoids (Salkowski test); tannins (ferric chloride test); saponins (frothing test and foam test); alkaloids (Mayer's test and Hager's test); carbohydrates (Molisch's test and Benedict's test); steroids (Salkowski test and Liebermann Burchard test); proteins (ninhydrin test and biuret test).

2.5 Estimation of Carbohydrate

The anthrone method[18] was employed to determine the total carbohydrate content in the crude extracts. Initially, 200 µl of the extracts was added to test tubes and diluted to 1 ml with distilled water. Subsequently, 5 ml of anthronereagent was added, and the mixture was incubated at 100 °C for 10 minutes. After reaching to ambient temperature, the O.D. values were measured at 620 nm using a spectrophotometer. A calibration curve was generated for standard glucose solution ranging from 5 to 320 µg/ml following the same procedure.

2.6 Quantification of Protein Content

The total protein content in each of crude extracts was determined by adopting theLowry protein assay[19] with some modifications. The Methanol Extract (ME), Ethyl Acetate Extract (EAE), and Chloroform Extract (CE), each in a volume of 0.5 ml and at a concentration of 1 mg/ml, were added to separate test tubes. To each test tube, 0.5 ml of distilled water and 4.5 ml of reagent 1 were added, followed by incubation for 10 minutes at room temperature. Subsequently, 0.5 ml of reagent 2 was added to each mixture, and the tubes were incubated for 30 minutes at ambient temperature (37 °C). The protein content of the crude extracts was determined by following the same procedure with standard Bovine Serum Albumin (BSA) solutions across a range of concentrations from 5 µg/ml to 320 µg/ml. Optical Density (O.D.) values were measured at 660 nm, and a calibration curve was generated using the BSA standards to quantify the protein content in the extracts.

2.7 Quantification of Total Phenolic Content (TPC)

The crude extracts of *P. pini* were analysed for their TPC using the Folin–Ciocalteu (F–C) assay[20]. Each crude extract (0.5 ml) was mixed with 1.5 ml of 10 % Folin–Ciocalteureagent and 1.5 ml of 5 % Na_2CO_3 at room temperature. The mixture was then incubated for 60 minutes, and O.D. values were measured at 720 nm using a spectrophotometer. Gallic acid in concentrations ranging from 5 to360 µg/ml was used as the standard. A calibration curve was constructed to determine the phenolic contents of the samples.

2.8 Quantification of Total Flavonoid Content (TFC)

The TFC in the extracts was determined using the aluminum chloride colorimetric assay[21]. A calibration curve was constructed using quercetin as a standard. In this assay, 0.5 ml of standard quercetin solution and the extracts were mixed with 1.5 ml of 10 % aluminum chloride solution. Then, 0.1 ml of 1M potassium acetate was added, followed by 2.8 ml of distilled water to the reaction mixture. Then, it was incubated in ambient condition for 30 minutes, and the OD values were taken at 510 nm in aUV-Visible Spectrophotometer.

2.9 Determination of DPPH Radical Scavenging Activity

The free radical scavenging activity of ME, EAE, and CE from *P. pini* was assessed using the DPPH(1,1-diphenyl-2-(2,4,6 trinitro phenyl hydrazine) method as outlined by Brand-Williams[22], *et al.* A 0.1 mM DPPH solution was prepared in methanol. Test samples were dissolved in methanol at concentrations ranging from 10 to 1000 µg/ml. Each sample solution (0.1 ml) or methanol (for the blank) was added to 2.9 ml of freshly prepared DPPH solution in a test tube and mixed thoroughly using a vortex mixer. The reaction mixture was then incubated in the dark at room temperature for 30 minutes to allow the antioxidant compounds to react with DPPH radicals. After incubation, the absorbance was measured at 517 nm using a spectrophotometer. L-ascorbic acid served as the reference standard

Inhibition of DPPH radicals was calculated using the eqn (2)

$$\% \text{ Inhibition} = \left[\frac{\left(A_{\text{Control}} - A_{\text{sample}}\right)}{A_{\text{control}}}\right] \times 100 \qquad \text{Equ (2)}$$

A_{sample} = absorbance of the sample

$A_{Control}$ = absorbance of control

2.10 Superoxide Radical ($O_2^{\bullet-}$) Scavenging Activity

A standard Super Oxide Dismutase (SOD) assay using alkaline DMSO as the superoxide-generating system was conducted following the method of Hyland[23], *et al.* The reaction mixture was formulated by combining 1 ml of 0.20 M potassium phosphate buffer (pH 8.6) containing 10^{-4} M EDTA and 2 x 10^{-5} M cytochrome c in test tubes. To each test tube, 200 µL of each crude extract was added and the tubes were placed in an ice bath for 20 minutes of incubation. Subsequently, 0.5 ml of alkaline DMSO (DMSO containing 1 % water and 5 mM NaOH; pH 9.5) was added to each test tube with continuous stirring. The reduced cytochrome c was then measured by taking the OD values at 550 nm using a spectrophotometer. DMSO without NaOH and L-ascorbic acid were used as the blank and reference standard, respectively. The superoxide radical ($O2^{\bullet-}$) scavenging potency was expressed as percentage inhibition.

2.11 Statistical Analysis

The experiments, conducted in triplicate to ensure result reliability, are presented as mean ± SD. Data analysis was performed using ANOVA, with p-values less than 0.05 considered significant. Graph Pad software was used for statistical analysis and plotting the graphs

3. RESULTS

Table 1. Yield of the crude exteacts of *P. Pini* derived fro the three solvents

Solvent extracts	% Extraction yield (mean± SD)
ME	9.64±1.19[b]
EAE	4.42±0.56[a]
CE	2.21±0.35

[a]$p<0.05$ (ME Vs CE); [b]$p<0.001$ (ME Vs EAE)

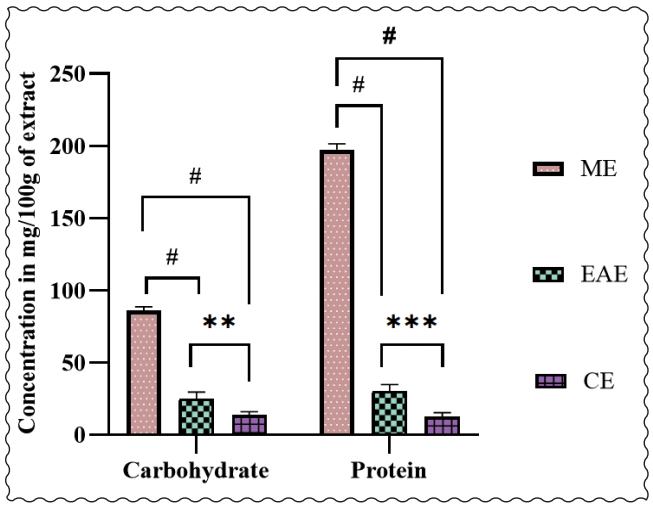

Figure 1. Carbohydrateand protein contents in three solvent extracts of *P.pini.*

**p<0.05; ***p<0.01 and #p<0.001.

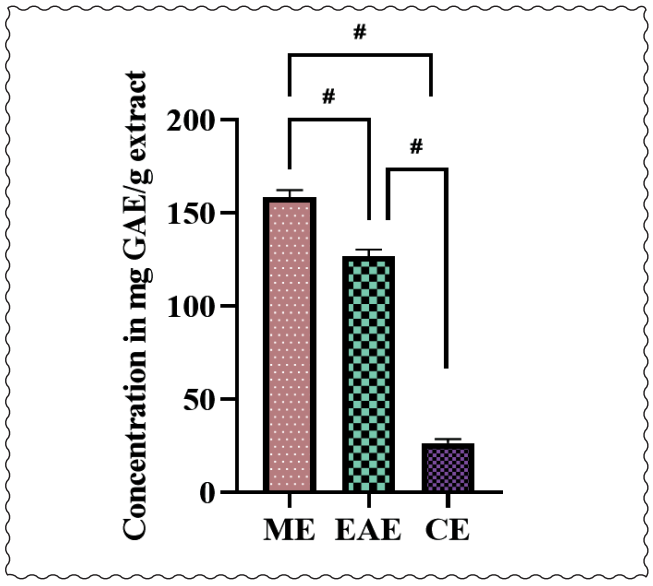

Figure 2. Total phenolic content in the three solvent extract of *P. pini,* #p<0.001.

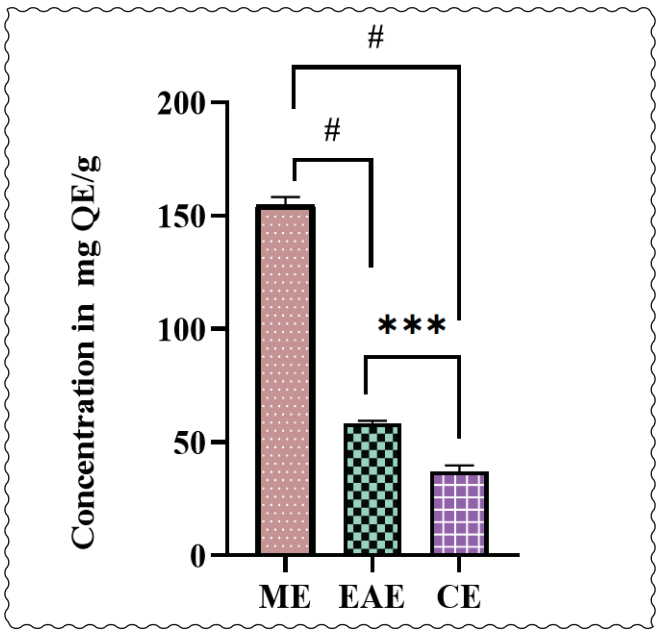

Figure 3. Flavonoid contentin the three solvent extracts of *P. pini.*

***p<0.01 and#p<0.001

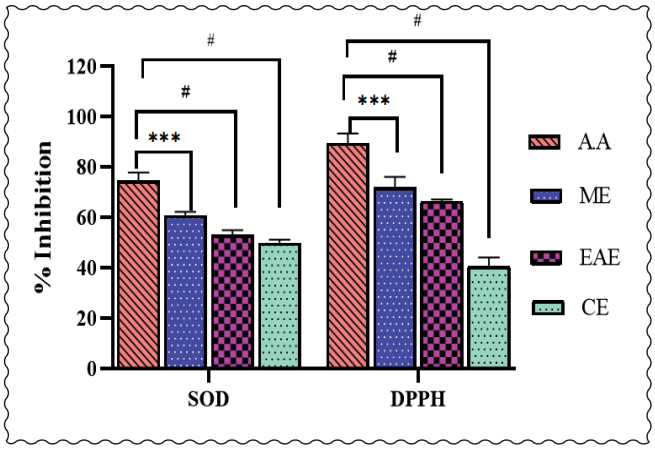

Figure 4. DPPH and superoxide scavenging activity of the three solvent extraxts.

***p<0.05;#p<0.001

4. DISCUSSION

Macrofungi, commonly known as mushrooms, represent a crucial source of natural products for drug discovery[24,25]. The phytochemical processing of raw materials from natural sources is advocated as essential for optimising the concentration of known constituents and preserving their activities[26,27]. Solvent extraction plays a pivotal role in this processing, facilitating the isolation and discovery of bioactive molecules from mushrooms, which are recognised as potential sources of such molecules. Numerous studies have underscored the importance of selecting an appropriate extraction method and solvent for standardising herbal products. This selection is critical for obtaining the desired soluble constituents while excluding unwanted ones[27-29].

In the present study, cold extraction, a conventional method, was utilised for the preparation of three solvent extracts of *P. pini*. Cold extraction offers several advantages compared to traditional extraction methods that involve heat. Many compounds are sensitive to heat and can degrade or lose their beneficial properties when exposed to high temperatures. Cold extraction allows for the extraction of these compounds without subjecting them to heat, preserving their integrity and potency[30]. Further, it permits for finer control over the extraction process, including the rate of extraction and the selectivity of the solvent enabling the extraction conditions to be optimised for specific compounds, resulting in higher yields and better quality extracts[31].

Table 1 presents the yields of the crude extracts derived from three solvents: ME, EAE, and CE. The crude extract yields of *P. pini* were observed to be in the order of ME (9.64±1.19), EAE (4.42±0.56) and CE (2.21±0.35). It is noteworthy that the yield of ME was significantly higher compared to EAE and CE (p<0.001). Furthermore, when comparing EAE and CE, the former yielded a significantly

higher amount compared to the latter ($p<0.05$). These results imply that the selection of solvent, or its polarity, greatly influences the quantity (yield) of the crude extract obtained. This observation aligns with previous reports indicating that solvent selection impacts the quantity of extracts obtained[27-32]. Mushrooms contain various constituents with different solubility in solvents, ranging from polar to nonpolar, depending on the biochemical composition of the samples. Methanol (CH_3OH), for instance, possesses a hydroxyl group, making it highly polar due to the greater electronegativity of oxygen compared to carbon and hydrogen atoms[33]. The higher yield observed in the methanolic extract suggests that P. pini comprises a higher quantity of polar and hydrophilic constituents compared to non-polar ones.

Preliminary phytochemical analysis of the three solvent extracts of *P. pini* identified phenols, flavonoids, terpenes, alkaloids, proteins, and carbohydrates through color and precipitation reactions. These constituents have also been reported in earlier studies on *P. pini*[12]. Interestingly, CE did not exhibit the presence of steroids, unlike ME and EAE, which could be a contributing factor to its lower yield. However, there remains a possibility of water-soluble steroids being present in the mushroom extract, which warrants further investigation. It is worth noting that previous research by Lourenço[34], *et al.* reported the isolation of ceramides, steroids, and ergosterol peroxide from *P. pini* these constituents may indeed be present in the methanolic extract of *P. pini*. This information sheds light on the diverse array of phytochemicals present in *P. pini* extracts and accentuates the need for comprehensive studies to elucidate their composition and potential bioactivities.

The results for the total carbohydrate and protein contents of the three solvent extracts of *P. pini* are depicted in Figure 1. Polysaccharides are crucial components of mushrooms, with some demonstrating antioxidant, immunological, antitumor, anticancer, and anti-HSV-1 infection properties[9,35-36]. Water-soluble polysaccharides have been proposed as promising agents for treating free radical-mediated illnesses[37]. Jiang[38], *et al.* isolated and purified mannose, galactose, and glucose from the mycelium of *P. pini*. Additionally, a previous study by Lee[9], *et al*[9]. showed that *P. pini* contains polysaccharides mainly composed of glucose, with minor sugars including galactose, xylose, and mannose. Many of these sugars possess numerous polar groups, rendering them hydrophilic or sparingly hydrophilic. Therefore, the higher carbohydrate content observed in the methanolic extract of *P. pini* is justifiable. These findings emphasise the potential health benefits associated with polysaccharides found in *P. pini* extracts, and further research in this area may yield valuable insights into their therapeutic applications.

Using Lowry's method Figure 1. the protein content of *P. pini* crude extracts was determined, showing that the methanolic extract had significantly higher total protein levels (197.44±4.03 mg/100 g of extract; $p<0.001$) compared to EAE and CE. Hunt and Ekramoddoullah[39] reported one-dimensional and two-dimensional protein profiles of *P. pini*. Further, Yang[36], *et al.* documented the presence of three water-soluble fractions of crude polysaccharides extracted from the fruiting bodies of *P.pini*, each comprising heteropolysaccharide complexes accompanied by a minor protein content ranging from 1.14 % to 2.55 %. It has been suggested that ability of methanol to disrupt non-covalent interactions can aid in the extraction of proteins and other biomolecules[40]. These observations highlight the effectiveness of methanol as a solvent for extracting proteins and carbohydrates from *P. pini*, suggesting its potential utility in obtaining bioactive compounds from this source.

The highest extractable total phenolics Figure 2. was found in the ME (158.5±3.8 mg GAE/g) of the mushrooms, followed by EAE (126.7±3.6 mg GAE/g), with the lowest amount observed in the CE (26.65±2.122). The significantly higher levels of phenols in ME and EAE compared to CE of *P. pini* indicate that polar solvents are more effective in recovering polyphenols from natural sources such as mushrooms. Similar results were reported by Esmaeili[41], *et al.* where crude extracts derived from polar solvents like methanol exhibited higher phenolic content. Ayer[11], *et al.* documented the presence of phenolics in *P. pini* and their antifungal activity against tree pathogens. Additionally, it has been suggested that chloroform may yield extracts rich in lipids or other nonpolar compounds, which might not significantly contribute to antioxidant activity but could have other biological effects[42]. The presence of phenolics in *P. pini* and their cardioprotective effect have been demonstrated[10]. The methanolic extract of *P. pini* has been shown to contain eleven phenolic compounds through HPLC analysis, exhibiting anticholinesterase and inflammation inhibitory activities[8]. Devi[43], *et al.* utilised Ultra-High Performance Liquid Chromatography (UHPLC) profiling on the methanolic extract of *P. pini*, identifying twelve different standard polyphenolic compounds.

Flavonoids are an important class of biomolecules known for their potent antioxidant and other medicinal properties. Certain mushroom species have been reported to produce flavonoids[44]. The experimental results revealed that all three solvent extracts contained flavonoids. Notably, the methanolic extract had a significantly higher flavonoid content, measuring 154.5±3.6 mg QE/g; $p<0.001$, compared to the EAE and CE Figure 3. EAE exhibited a moderate value of flavonoids (59.48±6.2 mg QE/g), while the least amount was observed in the CE of the mushroom (35.03±2.7 mg QE/g). An earlier study also confirmed the flavonoids in the methanolic extract of *P. pini*[44]. Additionally, a previous report by Xu[45], *et al.* showed high phenol and flavonoid content in polar solvent extracts of a plant. Therefore, based on the present study and earlier reports, it can be inferred that the methanolic extract of *P. pini* is the preferred choice for deriving phenolics with therapeutic properties.

It is widely acknowledged that free radicals are causative factors for biomolecular damage, leading to various health disorders. Antioxidants sourced from natural products play a pivotal role in mitigating health disorders mediated by free radicals, as demonstrated by their effectiveness in attenuating hyperglycemic conditions through the use of a plant extract[46]. This emphasises the importance of identifying potential sources of molecules with free radical scavenging activity. Mushrooms are considered a promising source of antioxidants, and the exploration of new species rich in antioxidants is currently a global research focus. It has been noted that the crude extracts of three solvents derived from P.*pini* exhibited substantial antioxidant properties in terms of DPPH and superoxide scavenging activities, as depicted in Figure 4. Notably, the methanolic extract of the mushroom displayed significantly superior antioxidant activity compared to the other two extracts of the mushroom ($p<0.001$). This finding aligns with previous reports demonstrating the DPPH free radical scavenging activity of *P. pini*[12]. This observation is coherent with earlier studies demonstrating the potential antioxidant activity of *P. pini*[10,43]. It is advocated that effective extraction and appropriate evaluation of antioxidant molecules from natural sources like herbs and mushrooms are crucial in endorsing their usefulness in functional foods, phytotherapy, and food additives[44]. Given that many antioxidant molecules may be polar, the methanolic extract may be considered to derive maximum free radical scavenging activity from this particular mushroom.

The correlations between total phenolics and DPPH radical, as well as SOD radical scavenging activities. The strong positive correlation ($R^2 = 0.995$ for DPPH and $R^2 = 0.740$ for SOD scavenging activity) suggests that the antioxidant activity relies on the concentration of phenolics present in the crude extracts of *P. pini* obtained from the three solvents. Higher phenolic concentrations correspond to greater antioxidant activities. The relationship between total flavonoid content and free radical scavenging property of the extracts is shown ($R^2 = 0.574$ for DPPH and $R^2 = 0.988$ for SOD scavenging activity). Again, a positive correlation was noted between flavonoid concentration and free radical scavenging property. These findings reinforce the understanding that phenolics and flavonoids play a crucial role in free radical scavenging activity, and solvent selection significantly influences the preparation of extracts enriched with antioxidant molecules.

5. CONCLUSION

Thus, methanol, ethyl acetate, and chloroform differ in their polarity and chemical properties, resulting in varying abilities to extract specific classes of compounds from *P. pini*. Methanol, being polar, is likely to yield extracts rich in polar constituents such as phenolics and flavonoids, contributing to antioxidant activity. Ethyl acetate, with moderate polarity, may offer a balanced extraction profile capturing a range of bioactive compounds. Chloroform, being nonpolar, may selectively extract nonpolar compounds with potential biological activities distinct from antioxidant properties. The electro-negativity difference between bonded atoms and the presence of a higher non-zero dipole moment, which are important features of polar solvents, play a significant role in extracting bioactive molecules from plants or other sources for drug discovery purposes[47].

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