

# Mycochemical screening, antioxidant, and cytotoxic properties of *Panaeolus cyanescens*

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**Abstract.** Galay KV, Plimaco SMY. 2025. Mycochemical screening, antioxidant, and cytotoxic properties of *Panaeolus cyanescens*. *Nusantara Bioscience* 17: 137-154. This study focuses on exploring the subtleties of the hallucinogenic mushroom *Panaeolus cyanescens* Sacc., known for its psychoactive component psilocybin. The study focuses on exploring the phytochemical, antioxidant, and cytotoxic properties of ethanolic extracts through rigorous procedures while considering factors such as chemical composition, physiological characteristics, possible therapeutic applications, and the broader implications of its psychoactive nature. The methods used are mycochemical screening, Gas Chromatography-Mass Spectrometry (GC-MS) analysis, DPPH free radical scavenging assay, and brine shrimp lethality test. The goal of the study is to determine the bioactive components in the mushroom and investigate their potential therapeutic applications, with an emphasis on their anticancer capabilities. The process entails gathering, confirming, and producing the extracts. A wide spectrum of secondary metabolites, including flavonoids, steroids, alkaloids, and fatty acids, are shown in the results, suggesting several potential therapeutic advantages. 25 unique compounds with antibacterial, antioxidant, anticancer, and anti-inflammatory properties have been identified by GC-MS analysis, offering a comprehensive understanding of their bioactivity. With an IC<sub>50</sub> value of 91.19 ppm, the DPPH assay exhibits strong antioxidant qualities and notable concentration-dependent antioxidant activity. With an LC<sub>50</sub> value of 26.63 ppm, cytotoxicity studies show substantial toxicity and strong anticancer effects. The study highlights *P. cyanescens*'s varied bioactive properties, antioxidant capacity, and intriguing anticancer potential while offering crucial insights into the proper dosage and responsible usage of the plant. The findings of the present assessment provide initial scientific validation for the mushroom's historic use in long-standing cultural customs. This study catalyzes additional research and confirmation of its therapeutic value.

**Keywords:** Antioxidant, brine shrimp lethality test, DPPH free radical scavenging assay, Gas Chromatography-Mass Spectrometry (GC-MS) analysis, mycochemical screening, *Panaeolus cyanescens*, psilocybin

## INTRODUCTION

Mushrooms, with their diverse mycochemical compounds, offer a wide range of physiologically active substances that might be exploited as functional components in pharmaceutical formulations to treat various ailments (Kostić et al. 2020). They include different mycochemical compounds such as alkaloids, steroids, flavonoids, terpenoids, tannins, phenols, glycosides, and more (Ogidi and Oyetayo 2016). Mushrooms stand out for their immune-modulating and antitumor properties, which make them particularly promising in the treatment of serious diseases. They also exhibit other therapeutic attributes such as antioxidant, antihypertensive, cholesterol-lowering, liver protection, anti-obesity, anti-inflammatory, antidiabetic, and antimicrobial effects (Shamtsyan 2016). Mushrooms also produce secondary metabolites and antibiotics with various chemical groups, which are well-suited for natural products (Shao et al. 2020; Chugh et al. 2022).

The prospective use of this crucial knowledge in the field of medicine has been largely overlooked. There have been limited ethnomycological research projects conducted

in the Philippines. There is a need for scientific validation of such empirical studies (Tantengco and Ragraio 2018; Brown 2019; Zeleke et al. 2020; Živković et al. 2021; Ríos-García et al. 2023). Half of the drugs on the market are thought to be derived from natural sources such as medicinal plants, herbal drugs, animals, fungi, and marine organisms. Only a small fraction of mushrooms with medicinal properties have been studied and evaluated for their therapeutic benefits (Ahmed et al. 2021). As a result, it is believed that many compounds found in plants and mushrooms have antioxidant characteristics.

Psychedelic mushrooms, sometimes referred to as magic mushrooms, contain chemicals that can induce hallucinations; psilocybin may have potential benefits for both physical and mental well-being in addition to producing euphoric effects (Wieczorek 2015; Barrett 2020). *Panaeolus cyanescens* Sacc., from the Galeropsidaceae family, is known as the blue meanie due to its blue staining reaction when bruised. It is a coprophilous fungus found growing on dung in tropical and subtropical regions worldwide. *Panaeolus cyanescens* is well-known for its psychedelic effects due to the presence of the hallucinogenic chemical psilocybin (Awan et al. 2018).

Research has focused on the anti-inflammatory properties, safety profile, and potential therapeutic uses of *P. cyanescens* (Nkadimeng et al. 2020; Nkadimeng et al. 2021). Caution should be exercised when considering the consumption of *P. cyanescens*, as it bears a resemblance to other poisonous species, emphasizing the need for accurate identification and expert supervision (Kaur et al. 2014).

Bustillos et al. (2014) found that *P. cyanescens* have several pharmacological characteristics. It has hallucinogenic effects due to the psychoactive compounds, antioxidant activity to combat free radicals, anti-inflammatory potential, and can suppress the formation of cancer cells. Significant bioactive substances such as psilocybin, psilocin, and baeocystin have been extracted from *P. cyanescens*. Psilocybin and psilocin are the main psychoactive compounds that produce hallucinogenic effects by interacting with serotonin receptors. They hold the potential to assist individuals in overcoming challenging-to-treat or treatment-resistant conditions such as psychogenic diseases and mental health disorders (Dawood Hristova and Pérez-Jover 2023). Psychedelic mushrooms are known for their ability to alter consciousness and induce mystical experiences, which has resulted in their incorporation into many spiritual and medicinal practices (Guzmán 2008; Johnson et al. 2014).

The study extensively examines the ethanolic extracts of *P. cyanescens*, investigating secondary metabolites and bioactive substances using mycochemical screening and GC-MS analysis (Brands et al. 2021). The evaluation involves determining the antioxidant capabilities of the mushroom by the DPPH free radical scavenging assay, which demonstrates its ability to counteract oxidative stress (Gulcin and Alwasel 2023). Brine shrimp lethality assay is used in cytotoxicity studies to evaluate the effectiveness of mushrooms in treating disorders associated with uncontrolled cell development, providing valuable information on their health benefits (Wahab and Hussain 2021). Although *P. cyanescens* has been traditionally used for treating several health disorders, little scientific data is confirming its safety and effectiveness. Hence, further investigation is required to study the pharmacological and

toxicological characteristics of these fungi, along with their medication interactions and adverse consequences. The study examined the biological characteristics and antioxidant and cytotoxic activities of *P. cyanescens*. Further research is required to explore their potential in other health-related activities, including anticancer qualities and treatment of mental health issues.

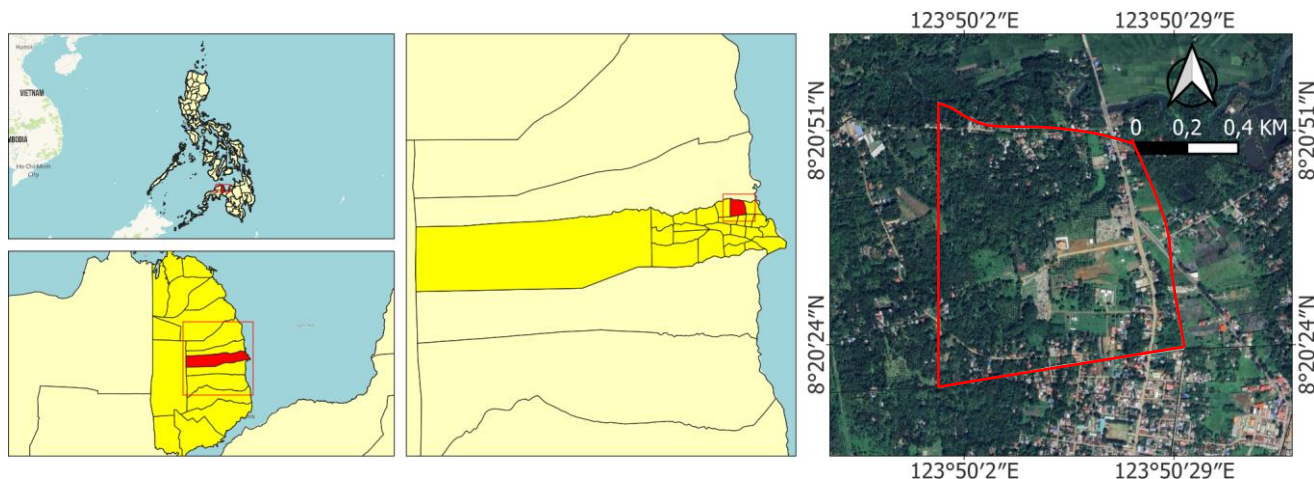
## MATERIALS AND METHODS

### Study area

Mushrooms of *P. cyanescens* were collected at Dicoloc, Jimenez, Misamis Occidental (Figure 1). To ensure authenticity, the mushroom samples were meticulously documented and subsequently sent to Jennifer M. Niem, the highly experienced Curator of the Mycological Herbarium at the Museum of Natural History, University of the Philippines-Los Banos, for further verification. To prevent deterioration, 230 fresh-weight grams of mushroom samples were submerged in 1000 mL of ethanol subsequent to the harvesting process. The sample underwent filtration using Whatman Number 1. It was subsequently concentrated in a rotary evaporator at a temperature range of 40-50°C while maintaining reduced pressure, resulting in the formation of a semisolid material. The extracted samples were then carefully stored in vials specifically designated for subsequent analysis, ensuring their integrity and reliability.

### Mycochemical screening

A qualitative assessment of different components in ethanolic extracts, such as flavonoids, steroids, saponins, alkaloids, tannins, cyanogenic, anthraquinones, and glycosides, was carried out at the Chemistry Laboratory of Mindanao State University's - Iligan Institute of Technology (MSU-IIT). The Handbook of the Philippine Medicinal Plants was used as the foundation for a three-point rating system (+turbid, ++moderate, and +++heavy) (De Padua et al. 2012; Dela Peña et al. 2019).



**Figure 1.** Geographical location of the sampling site. The map of the Philippines is raised in the upper left portion. The map of Dicoloc, Jimenez, and Misamis Occidental, Philippines is inset in the lower left section

### Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The analysis of the *P. cyanescens* mushroom sample was conducted using Gas Chromatography-Mass Spectrometry (GC-MS) in accordance with the methodology outlined by Chipiti et al. (2015). This approach has previously been employed to detect chemical compounds in mushroom fruiting bodies. The identification of substances was accomplished by the comparison of the mass spectrum of the analyte at a specific retention time with a reference standard in the collection of the National Institute of Standards and Technology (NIST). The qualitative investigation of the ethanolic extracts from *P. cyanescens* mushrooms was undertaken by the Chemistry Analytical and Research Laboratory at Ateneo de Davao University in Davao City.

### DPPH – Free radical scavenging assay

The antioxidant properties of *P. cyanescens* mushroom extracts were assessed by measuring their ability to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The evaluation technique was adapted from Jacinto et al. (2011) with a modification of the protocol. Multiple samples with varying concentrations (1,000, 500, 300, 200, 100, 50, 30, 20, and 10 µg/mL) were combined with 3 mL of an ethanolic DPPH solution (0.1 mM). The mixture was vigorously shaken for 10 seconds using a vortex mixer and then left to stand in a dark area at room temperature for one hour. Subsequently, the absorbance was quantified at a wavelength of 517 nm. Control samples were tested using DPPH solution in ethanol, with each sample analyzed three times. The radical scavenging activity was directly related to the DPPH quenching intensity, and the percentage of DPPH discoloration in the sample was determined. The results were quantified as the percentage of inhibition using the formula: % Inhibition =  $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$ . Here,  $A_{\text{control}}$  refers to the absorbance values of the control, while  $A_{\text{sample}}$  indicates the absorbance values of the sample. The extract concentration or  $IC_{50}$  needed to inhibit DPPH radicals by 50% was used to indicate the antioxidant activity of the extracts.

### Cytotoxicity test using brine shrimp lethality test

The cytotoxic effects of *P. cyanescens* mushroom extracts were evaluated by the brine shrimp lethality test, following the techniques outlined by Lumogdang et al. (2021) with certain modifications. The Chemistry Department at MSU-IIT evaluated the fatal effects on brine prawns (*Artemia salina* (Linnaeus, 1758)) to determine the possible bioactivity of ethanolic extracts. The brine shrimp larvae were used for the cytotoxicity test two days after hatching. Using ethanol as the extraction solvent, an ethanolic extract was prepared from *P. cyanescens* at three concentrations: 10, 100, and 1000 µg/mL. A stock solution was prepared by dissolving 36.5, 25.18, and 35.4 mg of the extract in

ethanol to make a 10,000-ppm stock solution for each concentration. The ethanol extraction solvent was allowed to stand for two days to facilitate evaporation. Additionally, specific quantities of Dimethylsulfoxide (DMSO) were added; next, diluting the prepared stock solution created concentrations of 1,000, 100, and 10 ppm. Three duplicates of the ethanolic extract were made, and 5 mL of sterile filtered seawater was used as the control setup for comparison. Each prepared extract received ten nauplii, and the control setup (sterile seawater) had an additional ten nauplii added. Afterward, the test tubes were examined, and the quantity of died (non-motile) nauplii in each tube was recorded at 6 and 24 hours.

### Data analysis

The network analysis conducted in this study utilized Gephi software (0.10.1 202301172018) under the dual license CDDL and GNU General Public License v3, (©2008-2023). The data was imported in the form of an edges table, with commas as the separator and the graph type set to directed. Various statistical analyses were performed using Gephi, including network overview, node overview, edge overview, and community detection. Each dataset was analyzed separately by Gephi due to the unique communities and network graphs associated with each dataset. In this context, bioactive compounds and their respective biological functions were represented as nodes, while the connections between them were depicted as edges. The layout of the resulting graph was adjusted using the Fruchterman-Reingold algorithm. Furthermore, Gephi facilitated the analysis of network centrality and modularity. The final network was examined for its bioactive compounds and functions based on the visual outcomes generated from the network analysis.

## RESULTS AND DISCUSSION

### Mycochemical screening

Mycochemical screening of the ethanolic extracts of *P. cyanescens* using ethanol as the solvent confirms the potential advantages associated with exposure to secondary metabolites. *Panaeolus cyanescens* extracts were found to contain a range of bioactive compounds, including significant concentrations of antioxidants such as steroids, fatty acids, and alkaloids, believed to contribute to its therapeutic properties. Minimal quantities of flavonoids and saponins were detected, although tannins and anthraquinone were entirely absent in the extracts (Table 1). Previous research has identified comparable chemicals in several species of the Galeropsidaceae family but with minor variations (Bustillos et al. 2014; Nkadameng et al. 2020a). The chemicals are thought to be accountable for the fungi's remarkable therapeutic characteristics.

**Table 1.** Mycochemical profile of the ethanolic extracts of *Panaeolus cyanescens*

Phytoconstituents	Interference
Alkaloids	+++
Anthraquinone	-
Cyanogenic-Glycosides	-
Flavonoids	+
Fatty Acids	+++
Saponins	+
Steroids	+++
Tannins	-

Note: + mean signifies present: + = turbid, ++ = moderate, +++ = heavy; - mean signifies absent

Alkaloids, emphasized by Willems et al. (2020), are potent bioactive compounds that are essential for therapeutic purposes and are commonly present in mushrooms. Alkaloids, whether in pure form or synthesized, are used as medicinal agents due to their pain-relieving, muscle-relaxing, and antimicrobial properties (Ogidi et al. 2021). This finding is consistent with previous research by Dulay et al. (2015) that examined *Panaeolus antillarum* (Fr.) Dennis. Alkaloids are effective antioxidants that fight free radicals and show promise for the future development of cancer therapeutic drugs (Nkadimeng et al. 2021). These substances have various pharmacological effects such as pain relief, antibacterial properties, cell division inhibition, malaria treatment, anti-inflammatory effects, antiviral activity, psychotropic effects, and potential anticancer properties (Wink 2016; Ding et al. 2017). Particular alkaloids, such as quetiapine, are essential in psychiatric medications used to treat diseases, including schizophrenia and bipolar disorder (He et al. 2020).

Similarly, flavonoids, another class of bioactive compounds, contribute to the medical properties of *P. cyanescens*. These phenolic compounds are present in a turbid state. Phenolics are recognized for their antioxidant and antibacterial characteristics. Research has shown that flavonoids from ten Malaysian wild mushrooms have significant beneficial effects on human health, demonstrating anti-inflammatory, antimutagenic, and anticarcinogenic properties, free radical scavenging, and acting as reducing agents (Azieana et al. 2017; Ang et al. 2019; Chávez-González et al. 2020; Ekalu and Habila 2020). Flavonoids have been found to offer various medicinal benefits, such as anticancer effects, antidepressant, anticonvulsant, anti-proliferative, antihypertensive, antiallergenic, antiviral, vasodilating actions, antifungal, hepatoprotective, and cytotoxic activity (Dias et al. 2021). The major characteristic of flavonoids is that they exert antidiabetic effects (Panche et al. 2016). Alternatively, phytochemicals like flavonoids have a broad variety of biological roles and can be used as medicines directly (Badshah et al. 2021).

Beyond flavonoids, mushrooms are also rich in natural sources of fatty acids that are essential components in human nutrition (Çayan et al. 2016). Unsaturated fatty acids can counteract diet-induced inflammation and reduce body fat by directly affecting the hypothalamus (Cintra et al. 2012). Analysis of fifteen types of edible mushrooms in

Korea shows that fat quality indicators, including the ratio of hypocholesterolemic/hypercholesterolemic fatty acids, the atherogenic index, and the thrombogenic index, demonstrate the health benefits of fats derived from mushrooms (Saini et al. 2021). Unsaturated fats, essential fatty acids obtained from the diet, are necessary for basic bodily functions such as cell growth, organ function, and nerve function. They may also have protective effects against heart disease and diabetes. Arachidonic acid plays a role in blood vessel constriction and blood clotting, while oleic acid has a relatively lower negative impact on carcinogenicity (Kayode et al. 2015). According to Rathore et al. (2017) reported that linoleic acid exhibits anticarcinogenic characteristics in animal models of breast, prostate, and colon cancers, inhibiting tumor cell proliferation at different stages of carcinogenesis.

Saponins are also compounds present in a turbid state in the ethanolic extracts of *P. cyanescens*, which are considered potential anticarcinogens. Saponins are believed to have anticarcinogenic qualities through mechanisms such as direct cytotoxicity, immune-modulatory actions, bile acid binding, and normalization of carcinogen-induced cell proliferation (Podolak et al. 2010). Saponins have various pharmacological effects such as antimicrobial, anti-tumor, hemolytic, anti-inflammatory, antidiabetic, antifungal, expectorant, vasoprotective, hypocholesterolemic, hypoglycaemic, molluscicidal, and antispasmodic properties. The substance has been discovered to possess antioxidant, anticancer, and antiviral characteristics (Sharma et al. 2021). It is also utilized as an adjuvant in addition to being employed in the food and cosmetic sectors as an emulsifier or sweetener (Majinda 2012). Saponins have anticancer capabilities by disrupting cellular DNA replication and inhibiting the growth of cancer cells (Yıldırım and Kutlu 2015). The research on saponins in various forms as a treatment for cancer has generated a lot of potential (Thakur et al. 2011). Several saponins have demonstrated significant anticancer properties and have also shown promise in combating neurological illnesses, cardiovascular disease, and renal disease (Zhong et al. 2022).

Furthermore, steroids are a vital phytochemical detected in high levels in the ethanolic extracts being studied. In vitro and in vivo studies show that certain triterpene and steroid compounds, particularly those with unsaturated side chain bonds, have enhanced antitumor activity. Triterpene and steroid compounds derived from mushrooms have a wide range of therapeutic benefits, such as hepatoprotective, hypoglycemic, antifungal, antioxidant, anti-inflammatory, and cholesterol biosynthesis regulation (Nikitina et al. 2016). Additionally, Ogidi et al. (2021) confirmed that steroids present in *Chromolaena* DC. plant species have antibacterial and antiviral properties against *Streptococcus mutans* Clarke, 1924 and *Streptococcus sobrinus* strains. A novel steroid derived from *Ganoderma sinense* J.D.Zhao, L.W.Hsu & X.Q.Zhang has a strong binding affinity to HK2 and significantly binding free energy. It has been identified as an HK2 inhibitor and may be utilized to target HK2 in cancer treatment (Badalyan et al. 2019). Phytochemical analysis of *P. cyanescens* extracts using various solvents resulted in the following findings.

Analysis of aqueous extracts from both the mycelia and fruiting body of *P. cyanescens* detected several mycochemicals such as saponins, alkaloids, and tannins. Flavonoids were solely present in the fruiting body. No phlobatannins or cardiac glycosides were detected in the mycelium or fruiting bodies of *P. cyanescens* (Bustillos et al. 2014). The results are consistent with the study carried out by Nkadimeng et al. (2023) on the cardiovascular safety of extracts from *P. cyanescens*, *Psilocybe natalensis* Gartz, D.A.Reid, M.T.Sm. & Eicker, *Psilocybe cubensis* (Earle) Singer, and *P. cubensis* leucistic A+ strain mushrooms. The ethanol and water extracts from the four psilocybin-containing mushrooms exhibited cardio-protective effects against angiotensin II-induced oxidative stress. The antioxidant and anti-inflammatory qualities of the water and ethanol extracts from the four psilocybin-containing mushrooms were validated by the phytochemical analysis of the extracts. The selection of solvents and extraction methods had a notable influence on the extraction of bioactive chemicals, producing different outcomes in each medicinal species examined (Lezoul et al. 2020). Various solvents extracted varied amounts and types of phytochemicals from various parts of the species, indicating that a single solvent may not be able to extract all the known beneficial components (Madike et al. 2017). The results showed variations in the different extracts, highlighting the importance of choosing the precise solvent to extract the optimal amounts of bioactive compounds (Toiu et al. 2018). Analysis of mycochemical screening in *P. cyanescens* has unveiled a diverse array of mycochemical compounds inherent to this mushroom species. A wide range of mycochemical groups, including flavonoids, saponins, alkaloids, steroids, and tannins, have been found in this investigation. It is important to understand that these secondary metabolites are bioactive substances that exhibit a wide range of physiological effects that significantly increase the overall biological activity of this mushroom. They are more than just their chemical makeup.

These mycochemicals specific and focused impacts on the human body make them fascinating subjects for further scientific study. Research on these chemical compounds' potential for creating innovative treatments is fascinating. These drugs are expected to have minimal adverse effects on human health and provide therapeutic advantages for a broad range of medical disorders. This research aligns with the broader goal of advancing knowledge about mycochemicals, which will result in the development of novel pharmacological treatments. The potential use of these mycochemicals as therapeutic agents heralds a new era in natural product-based medicine and their possible integration into pharmaceutical research, opening a world of possibilities for developing more effective and healthier cures for various illnesses.

#### Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analysis was crucial for pinpointing the precise bioactive ingredients that give the ethanolic extract from *P. cyanescens* its medicinal qualities. Table 2 provides an intricate detail of the complex characteristics of these bioactive compounds and the documented biological effects associated with each. The analysis revealed 25

compounds in the mushroom extract, enhancing our knowledge of its biological makeup.

Figure 2 depicts the interconnectedness of the scientific properties of the twenty-five distinct bioactive compounds identified in the ethanolic extracts of *P. cyanescens*. Fifty distinct nodes signify unique biological attributes or individual bioactive compounds within a complex network uncovered by the Gephi analysis. Besides that, there were 112 edges, and this represents an illustration of how complex the interdependencies are among every bioactive component and its biological feature. It shows interdependence among many components in the biological landscape. To further understand the structure of this network, we estimated the mean degree, which is determined to be 2.24. This metric indicates that, on average, each node is connected to approximately two other nodes, suggesting a relatively sparse yet interconnected network. Such a degree of connectivity can provide insights into the potential interactions and collaborative functions of the bioactive compounds and their biological roles.

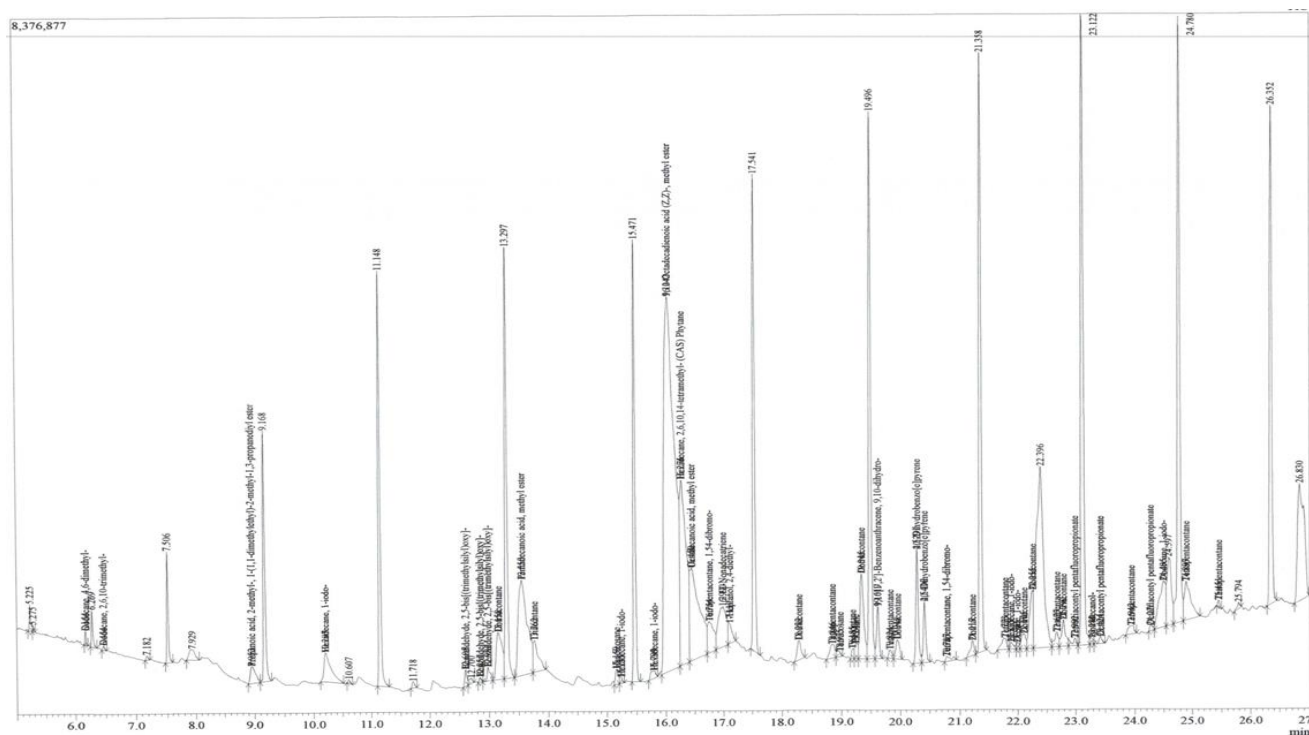
As depicted in Figure 2, antimicrobials emerge as a predominantly significant therapeutic category within this network, highlighting their key role in biological connections and potential applications in health and disease management. Furthermore, the analysis reveals that the core node demonstrates a remarkable level of centrality, signifying its vast interconnections with multiple bioactive substances. This central node may represent a key player in the network, potentially influencing various biological processes and interactions, thereby warranting further investigation into its functional implications and therapeutic potential. Overall, the findings from the Gephi analysis provide a valuable framework for understanding the complex interplay between bioactive compounds and their biological attributes, paving the way for future research and applications in the field of biomedicine. The mass peak of the bioactive compounds against its retention time is depicted in Figure 3.

Fatty Acid Methyl Esters (FAMES) offer a more environmentally friendly substitute for regular fatty acids. They have improved qualities that make them appropriate for use as lubricants, biofuel, and sustainable applications by substituting a methyl ester for carboxylic acid (Röttig et al. 2009). A notable example that highlights the adaptability and importance of FAMES is the molecule 9,12-Octadecadienoic acid (Z, Z)-, methyl ester. This chemical has strong binding affinity and potential for modulating target proteins, indicating its relevance in drug development. The potential effectiveness of targeting proteins for therapeutic purposes is shown by the docking score of -1.284 kcal/mol seen against the EGFR kinase domain, an essential part of the Epidermal Growth Factor Receptor (EGFR) protein, functioning as a molecular switch that helps transmit signals for cell growth and division, making it a target in drug development as documented by (Reza et al. 2021). Another significant compound known as octadecanoic acid, methyl ester, has been acknowledged for its capacity to inhibit tumor growth, demonstrate cytotoxic properties in the context of cancer treatment, and have anti-inflammatory properties (Shahin et al. 2022).

**Table 2.** Bioactive substances were identified qualitatively by analyzing the ethanolic extracts of *Panaeolus cyanescens* using GC-MS

Compound name	Retention time	Peak area (%)	Molecular formula	Reported biological properties with reference
Dodecane, 4,6-dimethyl-	6.165	93	C <sub>14</sub> H <sub>30</sub>	Antibacterial activity (Geethalakshmi and Sarada 2013; Zou et al. 2021); antioxidant (Zou et al. 2021); antimicrobial (Zou et al. 2021); inhibitory potential (Abdel-Ghaffar and Youssef 2013).
Dodecane, 2,6,10-trimethyl-	6.464	90	C <sub>15</sub> H <sub>32</sub>	Cytotoxic and antimicrobial activity (Jaradat et al. 2021); antifungal (Padma et al. 2019); antibacterial (Faridha Begum et al. 2016; Padma et al. 2019).
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	8.953	92	C <sub>10</sub> H <sub>20</sub> O <sub>4</sub>	No known activity.
Hexadecane, 1-iodo-	10.207	92	C <sub>16</sub> H <sub>33</sub> I	Antioxidant (Kim et al. 2022; Manzano et al. 2023); antiallergic, inhibitory, antimicrobial, and anticancer (Kim et al. 2022); antibacterial (Yogeswari et al. 2012).
Benzaldehyde, 2,5-bis[(trimethylsilyl)oxy]-	12.851	73	C <sub>16</sub> H <sub>26</sub> O <sub>2</sub> Si <sub>2</sub>	Inhibitory (Alijani et al. 2020).
Dotriacontane	13.158	82	C <sub>32</sub> H <sub>66</sub>	Antioxidant activity (Soosairaj and Dons 2016; Ekambaram et al. 2020; Qanash et al. 2022; Gazwi et al. 2023); antiviral (Qanash et al. 2022; Gazwi et al. 2023); antimicrobial and antibacterial (Asong et al. 2019; Kawuri and Darmayasa 2019; Değirmenci and Erkurt 2020; Ekambaram et al. 2020; Qanash et al. 2022; Gazwi et al. 2023); antispasmodic (Jeyaraman et al. 2018; Ekambaram et al. 2020; Teleb et al. 2022; Gazwi et al. 2023); cytotoxicity (Soosairaj and Dons 2016; El-Fayoumy et al. 2021; Qanash et al. 2022); anticancer (Dhanraj et al. 2021; El-Fayoumy et al. 2021); antifungal (El-Fayoumy et al. 2021); anti-inflammatory (El-Fayoumy et al. 2021); hypercholesterolemic (Ekambaram et al. 2020).
Pentadecanoic acid, methyl ester	13.550	94	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	Antioxidant (Gheda and Ismail 2020; Reza et al. 2021); antimicrobial (Nisar et al. 2013; Gheda and Ismail 2020; Reza et al. 2021); antibacterial (Salem et al. 2018); antifungal (Belakhdar et al. 2015).
Triacotane	13.762	88	C <sub>30</sub> H <sub>62</sub>	Cytotoxic (Mellado et al. 2019; Paudel et al. 2019; Saud 2023); antioxidant (Paudel et al. 2019; Sadiq et al. 2020; Sharma and Panwar 2022; Mohammed et al. 2021); antihyperlipidemic (Mohammed et al. 2021); antibacterial, antidiabetic, and antitumor (Paudel et al. 2019); antimicrobial (Cunha et al. 2018); anticancer (Nidugala et al. 2015).
Heneicosane	15.159	90	C <sub>21</sub> H <sub>44</sub>	Antimicrobial (Añides et al. 2019; Jihene et al. 2020; Kaplan 2021; Riaz et al. 2022); antioxidant (Vanitha et al. 2020); antineoplastic (Subramanian et al. 2020); antibacterial (Ekambaram et al. 2020); inhibitory potential (Okechukwu 2020); anticancer (Yusuf et al. 2020; Kaplan 2021); anti-proliferative (Yusuf et al. 2020).
9,12-Octadecadienoic acid (Z, Z)-, methyl ester	16.042	94	C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>	Anti-inflammatory (Hadi et al. 2016; Iroaganachi et al. 2023); analgesic and ulcerogenic properties (El-Sayed et al. 2023); antioxidant (Chakraborty et al. 2021; Reza et al. 2021; Shahin et al. 2022); antiarthritic (Muflihunna et al. 2021); anticancer, antihistaminic, and hepato-protective (Gheda and Ismail 2020); hypocholesterolemic, antiacne, and antieczemic (Ahmad et al. 2020); antimicrobial (Rahman et al. 2014).
Hexadecane, 2,6,10,14-tetramethyl- (CAS) Phytane	16.276	91	C <sub>20</sub> H <sub>42</sub>	No known activity.
Octadecanoic acid, methyl ester	16.450	90	C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>	Antioxidant, antitumor, cytotoxic, antimicrobial, and anti-inflammatory (Shahin et al. 2022); antimycobacterial (Ahmad et al. 2020).
Tetrapentacontane, 1,54-dibromo-	16.754	86	C <sub>54</sub> H <sub>110</sub> Br <sub>2</sub>	Antioxidant (Enema et al. 2019; Tanod et al. 2019; Bensaad et al. 2022); antimicrobial (Bensaad et al. 2022; Oviya et al. 2022); antimutagenic (Bensaad et al. 2022); antifungal (Oviya et al. 2022); antibacterial (Selim et al. 2013; Oviya et al. 2022).
1,3,12-Nonadecatriene	16.990	85	C <sub>19</sub> H <sub>34</sub>	Antioxidant (Moustafa et al. 2015; Mangawang et al. 2020; Ogundajo et al. 2022; Truong et al. 2023); hepatoprotective (Moustafa et al. 2015); anti-inflammatory (Truong et al. 2023); antimicrobial (Khan et al. 2019; Lykholat et al. 2021); antifungal (Parveen et al. 2017; Khan et al. 2019; Lykholat et al. 2021).
1-Heptanol, 2,4-diethyl-	17.110	79	C <sub>11</sub> H <sub>24</sub> O	Antimicrobial (Ruixia et al. 2017; Ferdosi et al. 2023).





**Figure 3.** The mass peak of the bioactive compounds against its retention time

Another significant compound, by Abdel-Ghaffar and Youssef (2023), revealed that dodecane, 4,6-dimethyl- exhibits potent effects on glycogen synthase kinase 3- $\beta$  (GSK-3 $\beta$ ), an essential enzyme involved in metabolic and signaling pathways. GSK-3 $\beta$  plays an important role in regulating kinds of cellular processes, including cell survival, gene expression, and metabolism, making it a key target in therapeutic research for various health conditions like diabetes and also brain diseases such as Alzheimer's and Parkinson's. The study highlights a strong interaction between dodecane, 4,6-dimethyl- and the enzyme's active site, as indicated by a free binding energy of -28.49 kcal/mol. The compound forms significant alkyl and  $\mu$ -alkyl bonds with specific amino acid residues (Tyr134, Ala83, Cys199, Leu188, and Leu132), suggesting a suppression mechanism through targeted binding interactions. These findings highlight the potential of dodecane, 4,6-dimethyl- for the development of GSK-3 $\beta$ -related therapies. Comparably, hexadecane, 1-iodo-, is an aliphatic alkane chemical that shares its classification with dodecane, 2,6,10-trimethyl- (Padma et al. 2019). A study conducted by Kim et al. (2022) revealed that hexadecane, 1-iodo-, a naturally occurring molecule present in the essential oil of *Chrysanthemum boreale*, has potential as a therapeutic intervention for atopic dermatitis, also known as eczema. Their study showed that this chemical can alleviate skin symptoms like those of AD in mice primarily through its anti-inflammatory and skin barrier-enhancing properties. By preventing mast cells from releasing histamine and other important inflammatory proteins and encouraging keratinocytes to produce essential skin barrier proteins, the chemical hexadecane, 1-iodo-, had two distinct effects.

According to the results, hexadecane, 1-iodo may prove useful in creating new strategies for the management of AD. Similarly, Heneicosane showed strong antibacterial activity against *Aspergillus fumigatus* Fresen. and *Streptococcus pneumoniae* A, highlighting its potential application in antimicrobial or therapeutic settings (Vanitha et al. 2020). Additionally, in a rat model, Heneicosane, along with other n-alkanes, exhibited analgesic effects via potentially suppressing inflammation. These chemicals have the potential to create analgesic or anti-inflammatory medications (Okechukwu 2020). Similarly, the stem extract of *Dendrobium crepidatum* Lindl. & Paxton contains triacontane, a compound associated with cytotoxic activity. The cytotoxic effects on HeLa and U251 cancer cell lines were assessed in the study. The study revealed that the chloroform extract had the highest level of efficacy in decreasing the proliferation of HeLa cells. In comparison, the hexane extract demonstrated the greatest effectiveness in suppressing the growth of U251 cells. The cytotoxic activity found against cancer cell lines is attributed to the presence of tetracosane, tetracosane, and various phenol derivatives (Paudel et al. 2019).

Moreover, the methanolic extract of the kei apple (*Dovyalis cafra* (Hook.fil. & Harv.) Warb.) revealed that Dotriacontane plays a crucial role in its numerous therapeutic advantages. Its detrimental effects on HepG2 cells demonstrated the anticancer capabilities of Dotriacontane. Additionally, it demonstrated promising antibacterial properties when utilized in conjunction with other constituents of the extract, particularly against *Escherichia coli* E and *Proteus vulgaris* B. The extract's modest antiviral action against human coronavirus 229E

was also attributed to the presence of Dotriacontane. The therapeutic significance of Dotriacontane in the kei apple extract was further substantiated by molecular docking research, as evidenced by the findings of Qanash et al. (2022). In another study, Mishra et al. (2019) highlight the presence of Dotriacontane, a high constituent found in the acetone extract of *Curcuma raktakanta*, a relatively obscure species of curcuma originating from Kerala, India. The objective of this study was to evaluate the efficacy of several fractions (acetone, hexane, and ethyl acetate) derived from *C. raktakanta* on cell lines associated with glioma, cervical, and breast cancer. Dotriacontane in the acetone extract reduced the vitality of cancer cells in a concentration-dependent manner, with a particularly strong effect on C-6 glioma cells. Dotriacontane was identified as a prominent constituent in the acetone extract of *C. raktakanta* using GC-MS analysis, suggesting its potential involvement in the plant's anticancer activities. Further research by Kawuri and Darmayasa (2019) emphasizes that *Streptomyces* sp. 9, specifically *Streptomyces capoamus* Gonçalves de Lima et al., 1964, had noteworthy biocontrol capabilities in combating banana bacterial wilt disease. In antagonistic tests, Dotriacontane showed a notable inhibition of *Ralstonia solanacearum* (Smith, 1896) Yabuuchi et al., 1996, and in greenhouse-scale trials, it was able to suppress the disease completely.

The study conducted by Alijani et al. (2020) highlights the importance of Benzaldehyde, 2,5-bis[trimethylsilyl]oxy]- as a key component in the volatile chemicals emitted by *Stenotrophomonas maltophilia* B strain UN1512. The GC-MS study of this molecule suggests that it is likely involved in the antifungal actions that have been documented against the pathogen that causes strawberry anthracnose, *Colletotrichum nymphaeae* (Pass.) Aa. Furthermore, it has been shown that volatile compounds, such as Benzaldehyde and 2,5-bis[trimethylsilyl]oxy]-, play a crucial role in promoting the growth of tomato plants by influencing the development of both roots and shoots. This observation underscores the notable contribution of Benzaldehyde, 2,5-bis[trimethylsilyl]oxy]- in augmenting the antifungal properties of the bacteria against strawberry anthracnose and facilitating the growth and maturation of tomato plants.

Alkanes are hydrocarbon compounds consisting of carbon and hydrogen atoms connected by single bonds (Clark et al. 2009). These compounds are used as solvents and lubricants in medicines, cosmetics, and industrial applications because of their non-reactive nature and stability (Van and Funhoff 2007). The ethanolic extracts of *P. cyanescens* included alkane chemicals such as tetrapentacontane, 1,54-dibromotetrapentacontane, tetracosane, and eicosane. The synthesized compounds were tested for their antibacterial, antifungal, and antioxidant activities that conferred resistance to free radicals in yeast (Holla et al. 2001). Alkanes found in *P. cyanescens* contribute to its antioxidant properties, including dodecane, tetradecane, and hexadecane (Bustillos et al. 2014). Prior studies have shown that tetrapentacontane, namely 1,54-dibromo-, an alkane compound, had antioxidant and antibacterial properties when administered to the bacterial species under investigation.

In addition to their role in *P. cyanescens*, tetrapentacontane and its derivatives have been shown to possess potent antimicrobial, antifungal, and bactericidal properties (Oviya et al. 2022). According to Sri et al. (2021), the tetrapentacontane molecule has shown significant efficacy in promoting hair growth, reducing uric acid production, and inhibiting arachidonic acid. The anticancer effects of tetracosane have been shown against many cell lines, including MDA-MB-231, HT-2918, AGS, and NIH 3T3. The chemicals demonstrate antioxidant, anticancer, anti-inflammatory, and antibacterial properties due to their structural attributes, such as the presence of phenolic compounds in antioxidants or specific alkaloids in antimicrobial medications (Özçelik et al. 2011). According to Oviya et al. (2022) and Okechukwu (2020), eicosane, an alkane molecule present in the fungus *P. cyanescens*, has significant anti-inflammatory, antibacterial, analgesic, and antipyretic characteristics.

1,3,12-Nonadecatriene is classified as an alkene, a kind of compound that contains carbon-carbon double bonds (Smith 2016). Alkenes are very desirable initial substances for producing epoxides, which serve as crucial and adaptable intermediate components for the pharmaceutical, flavoring, and polymer sectors. Previous studies have concentrated on using various oxidizing agents and enzymes, such as cytochrome P450 monooxygenases, for alkene epoxidation. Additionally, microbial whole-cell catalysts have been explored despite their limitations (Özçelik et al. 2011; Truong et al. 2013; Babot et al. 2022). This improved the antioxidant capacity of cells by blocking the generation of reactive oxygen species and decreased the inflammatory response by lowering the release of nitric oxide.

Esters are another group of compounds found in mushrooms that exhibit distinct physicochemical features and functionalities (Xu et al. 2022). Ester compounds showed potent antibacterial effects against several oral infections (Huang and Ebersole 2010). Tetraatriacontyl pentafluoropropionate and Octatriacontyl pentafluoropropionate are Esters known for their strong antimicrobial, antibacterial, anti-inflammatory, antioxidant, and anticarcinogenic properties that are also found in the ethanol and water extracts of four psilocybin mushrooms (Zulhendri et al. 2022; Mohammed and Al-Katib 2023; Nkadimeng et al. 2023). The ethanolic extracts of *P. cyanescens* also contain halogenated alkanes such as decane, 1-iodo, and dodecane, 1-iodo, which are known for their antibacterial, antifouling, and repellent activities (Gribble 1994). Saltwater contains certain halogenated alkanes that are not directly affected by human activities (Dembitsky and Tolstikov 2003). According to Dutta and Hyder (2019), the inclusion of halogen substituents has the potential to augment the toxic, mutagenic, and other deleterious characteristics of compounds.

The concentration of alcohol in *P. cyanescens* was evaluated by the utilization of gas chromatography, identifying 1-Heptanol, 2,4-diethyl. The antibacterial activity of the compounds has been well-documented (Ferdosi et al. 2023). According to Mohamed and Farghaly (2014), alcohols have a variety of functions, including

flavoring, antifreeze, antiseptic, fuel, preservative, solvent, antioxidant, and antibacterial capabilities. Isotridecanol is a fatty alcohol derived from the isotridecanol hydrocarbon and is present in *P. cyanescens*. According to Lakhera et al. (2022), fatty alcohol molecules are composed of nitro groups that possess the ability to firmly remove electrons and hydroxyl groups that have the ability to donate electrons. Fatty alcohol molecules are widely utilized in medicinal science, insecticides, dyes, cosmetics, and other fields (Federle and Itrich 2006). Isotridecanol, a kind of fatty alcohol, has been categorized as possessing anticancer, antibacterial, antimicrobial, antifungal, phototoxic, and other chemical and therapeutic activities (Kuklev et al. 2003).

Polycyclic Aromatic Hydrocarbons (PAHs) are a type of persistent organic chemical present in *P. cyanescens* mushrooms, known for their significant cancer-causing properties and widespread presence in the environment (Zhou et al. 2020). According to MacLeod et al. (1979), PAH compounds have the potential to create cancers in many species and tissues. Additionally, they may induce mutagenesis and convert cells into malignant cells when grown in a laboratory setting. 9-dihydroanthracene (D-A-D) Polycyclic aromatic hydrocarbons (PAHs) encompass 9,10-dihydrobenzo[e]pyrene and 4,5-dihydrobenzo[e]pyrene. The chemicals possess potential antimutagenic, anticarcinogenic, and cytotoxic properties (MacLeod et al. 1979). Numerous studies have confirmed that Polycyclic Aromatic Hydrocarbons (PAHs) having one, two, or three rings exhibit acute toxicity, whereas those encompassing more than three rings exhibit genotoxicity (Li et al. 2010).

#### DPPH – Free radical scavenging assay

The DPPH radical scavenging technique is a crucial experiment used to assess the antioxidant ability of natural substances by inhibiting lipid oxidation. This methodology assesses the efficacy of the analyzed substances in counteracting free radicals, providing crucial information on their antioxidant properties. The results of the DPPH radical scavenging activity of ethanolic extracts of *P. cyanescens* are presented in Table 4. The findings of the study indicate that the scavenging activity of the extracts exhibited a concentration-dependent pattern, whereby higher concentrations of the extract resulted in a more pronounced scavenging activity.

Free radicals are chemical entities that possess one or more unpaired electrons in their atomic or molecular orbitals (Qazi and Molvi 2018). Cellular damage caused by

unstable atoms can result in illness and the process of aging (De Leon et al. 2020; Adetuyi et al. 2022). Oxygen-derived radicals are the predominant type of radical species generated within biological entities. Oxygen molecule acts as a free radical, causing the production of reactive oxygen species that can harm cells (Sánchez 2017). According to Husain and Kumar (2012), an excessive presence of Reactive Oxygen Species (ROS) can disrupt the equilibrium between antioxidants and oxidants, hence causing oxidative harm to many cellular components such as cell structures, DNA nucleic acid bases, lipids, and proteins. These cells serve as the body's primary defense against damage caused by free radicals (Abd 2012; Khatua et al. 2013).

Bustillos et al. (2014) assessed the antioxidant properties of *P. cyanescens* by varying culture medium and pH levels. The antioxidant activity of the coconut water with a pH of 5 was found to be the highest, as evidenced by its total phenolic content of 25.19 mg AAE/g sample and a radical scavenging activity of 14.40%. The findings underscore the significance of culture medium and pH profiles in influencing the antioxidant characteristics of *P. cyanescens*. The DPPH radical scavenging activity of *P. antillarum* was assessed by Dulay et al. (2015) across several culture media and pH conditions. The antioxidant potential and DPPH radical scavenging activity of potato broth were found to be the greatest, whereas coconut water exhibited the lowest levels. The study determined that the ideal pH level for effective DPPH radical scavenging in *P. antillarum* was 7.0. The findings suggest that the culture media and pH strongly influence the mushroom's capacity to scavenge DPPH radicals. The antioxidant capacity of potato broth was shown to be enhanced by a pH of 7.0. *Marasmius oreades* (Bolton) Fr., in the same taxonomic order as *P. cyanescens* in Agaricales, has notable DPPH free radical scavenging activities, suggesting it might be a valuable natural antioxidant. The findings suggest that the DPPH free radical scavenging activity of *M. oreades* varies depending on the concentration of the extract. The antioxidant capabilities of the ethanol extract derived from *M. oreades* were found to be substantial, as evidenced by scavenging activities ranging from 38.98 to 72.58% across several dosages (0.25, 0.5, 1, and 2). The maximal concentration (2) yielded the highest reported scavenging activity, leading to the neutralization of about 72.58% of the DPPH free radicals. The findings suggest that *M. oreades* might serve as a beneficial natural source of antioxidants with prospective uses (Karalti et al. 2022).

**Table 4.** DPPH radical scavenging activity of the ethanolic extracts of *Panaeolus cyanescens*

Concentration (µg/mL)	% Inhibition
20	3.00
50	48.21
100	63.82
200	70.98
300	77.23
500	83.58
1000	87.85
IC50	91.19

**Table 5.** Brine Shrimp Lethality Test of the ethanolic extract of *Panaeolus cyanescens*

Concentration (µg/mL)	Mortality rate (%) 24 hours exposure	LC50 (ppm)
10	1.33%	26.63
100	33%	
1000	33%	

Several investigations have demonstrated that mushrooms contain a diverse range of secondary metabolites, including phenolic chemicals. Phenolic chemicals play a major role in the antioxidant effect of some types of mushrooms (Edeoga et al. 2005). The mycochemical analysis of *P. cyanescens* in this study showed significant levels of antioxidants, secondary metabolites such as fatty acids, alkaloids, and steroids, as well as flavonoids and saponins. The bioactive compounds were thought to be responsible for the mushroom's antioxidant effects. The phenolic content and antioxidant activity of this specific mushroom, which are commonly used to assess antioxidant properties, may offer new insights into the creation of treatment strategies for oxidative diseases (Cheung et al. 2003). Previous studies on *P. cyanescens* have largely examined its medicinal qualities, but information regarding its antioxidant qualities is limited (Bustillos et al. 2014). According to Rajan et al. (2020), the solvent extraction processes have the potential to induce alterations in polarity, phytoconstituents, antioxidant activity, and other biological activities, hence impacting the effectiveness of free radical scavenging. The utilization of ethanol as the solvent in the study of *P. cyanescens* underscores the potential advantages associated with exposure to secondary metabolites. The evaluation of antioxidant activity is commonly conducted by the utilization of 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable free radical, as described by Dulay et al. (2016). The DPPH radical is an artificial nitrogen radical that possesses extraordinary characteristics. The experimental procedure is predicated upon the phenomenon of electron transfer, wherein an antioxidant compound reduces the oxidant by transferring an electron, resulting in a change in color and subsequent modification in absorbance. The generation of DPPH results in the removal of violet hue, exhibiting a peak absorption at the wavelength range of 517-520 nm (Dwivedi and Paul 2020). The concentration of extracts necessary to inhibit 50% of radicals is determined using the IC<sub>50</sub> value. The IC<sub>50</sub> value is a widely used metric for quantifying antioxidant activity since it measures the concentration of antioxidants required to lower the initial DPPH concentration by 50% (Molole et al. 2022).

The ethanolic extract exhibited substantial inhibition of DPPH, displaying a highly potent antioxidant activity of 83.58% at a concentration of 500 µg/mL. Peña et al. (2019) classify substances as potent antioxidants if their inhibition rate exceeds 80%, moderate antioxidants if their inhibition rate ranges from 50 to 80%, and weak antioxidants if their inhibition rate is below 50%. The IC<sub>50</sub>, which stands for half-maximal inhibitory concentration, is a vital metric used in pharmacological research to assess the strength of antagonist drugs. The term refers to the concentration of a pharmaceutical compound required to impede a biological process by 50%. This parameter is frequently assessed using whole-cell assays (Aykul and Martinez-Hackert 2016). The determination of an antioxidant's effectiveness heavily relies on the IC<sub>50</sub> value. If a chemical's IC<sub>50</sub> value is less than 50 ppm, it is considered highly potent; if it is between 50 and 100 ppm, it is considered strong; and if it is between 101 and 150 ppm, it is considered moderately

potent. Sukweenadhi et al. (2020) define weak antioxidants as compounds with an IC<sub>50</sub> value greater than 150 ppm. A lower IC<sub>50</sub> value indicates higher antioxidant activity of the extracts, suggesting more efficiency in scavenging DPPH (Wright et al. 2017). The findings of this study provide clear evidence that the extract possesses significant antioxidant properties, as seen by its IC<sub>50</sub> value of 91.19 ppm. According to the study's findings, *P. cyanescens* may have a biochemical basis that supports its traditional medical use, such as its ability to treat infections and its potential as an anticancer and antioxidant.

#### **Cytotoxicity test using brine shrimp lethality test**

The Brine Shrimp Lethality Test (BSLT) is a crucial instrument utilized for evaluating the lethal effects of various chemical substances. The purpose of this test is to assess the potential toxicity of chemicals by quantifying the mortality rates of brine shrimp larvae within a designated timeframe. The results of the BSLT provide crucial insights into the difficult characteristics of the chemicals under examination, where higher fatality rates suggest higher toxicity levels. Table 5 demonstrates that the mortality rates of brine shrimp larvae exposed to ethanolic extracts of *P. cyanescens* exhibited a concentration-dependent manner within 24 hours. The results demonstrated that increased concentrations of the extract had a more pronounced impact on prawn mortality, hence underscoring the hazardous characteristics of the extracts.

The Brine Shrimp Lethality Test (BSLT) is a concise, economical, and simple technique for evaluating the lethality of mushroom extracts. It often has a strong correlation with cytotoxic and antitumor properties (Baravalia et al. 2012). This test is used to predict drugs or extracts that may have anticancer properties, as demonstrated by Moshi et al. (2004). Furthermore, Krishnaraju et al. (2005) have established the efficacy of this method in the identification of fungal toxins and the assessment of mushroom extract toxicity. BSLT's quickness is a significant advantage, along with the resemblances between *Artemia* larvae and mammalian cells, the quick, uncomplicated, and cost-effective testing procedure, the minimal amount of material needed for testing, and the outcomes that closely mimic real-world conditions due to the large number of larvae utilized (Mirzaei et al. 2013).

The toxicity of the ethanolic extract of *P. cyanescens* was assessed using the BSLT. Throughout 24 hours, there was an apparent increase in fatality rates among brine shrimp larvae as the concentration of the ethanolic extract increased, hence underscoring its significant toxicity. The findings exhibited a progressive rise in lethality, with rates of 1.33, 33, and 33% seen at doses of 10, 100, and 1000 µg/mL, correspondingly. The LC<sub>50</sub> value is the concentration at which 50% of the brine shrimp population dies after 24 hours of exposure, serving as a crucial measure of the extract's toxicity (Tawaha 2006). The concentration of the *P. cyanescens* extract was determined to be 26.63 parts per million (ppm).

Extracts are classified according to their LC<sub>50</sub> values: categorizing them as extremely hazardous if they fall within the range of 0 to 100 µg/mL, medium toxic if they

fall within the range of 100 to 500  $\mu\text{g}/\text{mL}$ , and low toxic if they fall within the range of 500 to 1000  $\mu\text{g}/\text{mL}$ . Substances having  $\text{LC}_{50}$  values greater than 1000  $\mu\text{g}/\text{mL}$  are classified as non-toxic (Yurasbe et al. 2023). Lestari et al. (2015) and Wanyoike et al. (2004) emphasized that a smaller  $\text{LC}_{50}$  value indicates more toxicity, whereas a bigger  $\text{LC}_{50}$  value indicates lesser toxicity. Based on its  $\text{LC}_{50}$  value of 26.63 ppm, *P. cyanescens* extract is categorized as very hazardous within 24 hours. This result highlights the importance of doing more investigations to evaluate its potential impact. The possible hazards and ethical implications concerning the toxic effects of the ethanolic extracts from *P. cyanescens* are noteworthy and mostly due to their high-hazard classification. There are considerations on its potential impact on human health, the need to obtain consent in a study, and ethical responsibility regarding safety while investigating the curative potential of these substances.

Previous studies have demonstrated that *P. cyanescens* have teratogenic and toxic properties at higher doses when employed as a model organism in zebrafish embryos. The present study proposes that mushrooms of *P. cyanescens* possess teratogens that have promising potential for the development of anticancer pharmaceuticals. The *P. cyanescens* species includes psilocin (0.48%), psilocybin (0.11%), serotonin (0.072%), urea (1.8%), and baeocystin (0.02%), which might potentially have harmful and teratogenic effects on embryos (Bustillos et al. 2016). This finding aligns with the research conducted by Meyer (2017), which suggests that extracts derived from psychedelic mushrooms, specifically *P. cubensis* (which belongs to the same order as *P. cyanescens* within Agaricales), exhibit notable cytotoxic properties on brine shrimp. These effects are observed at high levels of toxicity, regardless of the concentration used. The observed mortality rate of brine prawns upon exposure to extracts derived from *P. cubensis* serves as evidence for the extract's toxic properties in comparison to the control groups. According to the research, all brine prawns in the treatment vials died within 24 hours. The toxicity of *P. cubensis* towards brine prawns and soil bacteria is noteworthy, suggesting that the presence of psilocybin and related compounds may have evolved as a defensive mechanism.

Psychoactive compounds found in *P. cyanescens* are mainly psilocybin and psilocin. These cause adverse effects like hallucinations, anxiety, and possibly neurotoxicity. The process of ethanolic extraction may increase the concentration of such compounds and the risk of toxicity. Acute exposure may result in intense psychological distress, but chronic use might cause severe impairment of cognitive functioning or a severe psychological disorder. Special consideration is required for the vulnerable population, who may already be diagnosed with some mental health condition, since they may be at higher risk of adverse reactions. Therefore, understanding the full spectrum of these effects is crucial for assessing the safety of these extracts.

According to Lumogdang et al. (2021), the cytotoxic effects of ethanolic extracts can be attributed to their

elevated levels of mycochemicals, antioxidants, and antibacterial properties. The first evaluation of the bioactive substances found in *P. cyanescens* benefits from the application of the brine shrimp lethality assay. The remarkable efficacy of *P. cyanescens* extract at dangerous concentrations suggests that further investigation is necessary to see whether it could be a good substitute for anticancer treatments. To create new anticancer treatments, more research is required to utilize the extract's cytotoxic properties fully. This emphasizes the necessity of investigating the dual properties of some dangerous substances for possible therapeutic uses. Hence, this underscores the significance of ongoing research in elucidating the cytotoxic characteristics of *P. cyanescens*, thus facilitating the development of sophisticated and efficacious therapeutic interventions for cancer.

The current study has shown promise for developing anticancer drugs from ethanolic extracts of *P. cyanescens* due to its observed cytotoxicity. However, such findings must be translated into clinical applications through a holistic approach involving phytochemical characterization, mechanistic studies, in vivo assessments, formulation development, and rigorous clinical testing. Through this, future researchers will be able to utilize the therapeutic benefits of this bioactive compound, hence furthering efforts toward successful treatments for cancer. Traditional knowledge, in combination with the application of scientific techniques, will bring forth groundbreaking therapeutic interventions, bettering patients' conditions with regard to cancer diseases.

In conclusion, a thorough analysis of ethanolic extracts has revealed important information about the mycochemical, antioxidant, and cytotoxic properties of the hallucinogenic mushroom *P. cyanescens*. The ethanolic extract showed strong antioxidant properties at 1,000  $\mu\text{g}/\text{mL}$ , which led to an 87.85% suppression of DPPH. This inhibition's  $\text{IC}_{50}$  value was found to be 91.19 ppm. The GC-MS analysis conducted in this study revealed the presence of 25 bioactive compounds, including fatty acid methyl esters. These compounds have been well acknowledged for their numerous health benefits, such as their anticancer, anti-inflammatory, and antibacterial properties. As demonstrated by the high toxicity of *P. cyanescens* with an  $\text{LC}_{50}$  value of 26.63 ppm, indicating significant anticancer properties, the brine shrimp lethality test validated the extract's potential as an anticancer agent. This illustrates only the initial phases of exploring the wide range of possibilities offered by *P. cyanescens*. More research is required to understand the complex mechanisms underlying these impacts. The need for further research is not just a necessity but an exciting opportunity to delve deeper into the potential of *P. cyanescens*. Furthermore, it is imperative to conduct comprehensive clinical trials in order to validate the efficacy and safety of the intervention within a medical framework. To fully realize the potential of this intriguing opportunity, ethical and accurate scientific investigation is required.

Considering the ethical implications of promoting the medicinal use of hallucinogenic mushrooms is vital. Public health and safety must always be a priority. It's important

to advocate for responsible use, including proper dosage, setting, and patient education. Clear communication about potential risks and benefits is crucial in fostering an informed public discourse. Collaborating with healthcare professionals, researchers, and policymakers will ensure that any advancement in using these substances is done ethically, prioritizing safety and informed consent. When discussing the variability of natural compounds in clinical applications, it's crucial to advocate for standardized extraction methods and dosing. It's true that individual responses to compounds can differ significantly due to genetic, environmental, and psychological factors. However, this variability also points to the need for personalized medicine approaches, which consider individual differences in treatment plans. Emphasizing ongoing research and the importance of controlled studies can help address concerns regarding inconsistency.

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