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Isolation and Antioxidant Activity Test of Symbiotic Fungi in Pletok Beer UTA'45 Jakarta

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ABSTRACT

Bir pletok is a traditional Betawi herbal beverage composed of various spices known to contain bioactive compounds. In addition to the host plants, bioactive metabolites may also be produced by symbiotic fungi associated with these natural materials. This study aimed to isolate symbiotic fungi from bir pletok, identify their macroscopic and microscopic characteristics, determine their secondary metabolite content, and evaluate the antioxidant activity of ethyl acetate extracts using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Fungal isolation was carried out on Potato Dextrose Agar (PDA), followed by cultivation on rice medium for one month. Secondary metabolites were extracted by maceration using distilled ethyl acetate with three extraction cycles, and the solvent was removed using a rotary evaporator to obtain crude extracts. Phytochemical screening and antioxidant activity assays were then performed using UV–Visible spectrophotometry. The results showed that two fungal isolates were successfully obtained and designated as J.H and J.P . Based on their macroscopic and microscopic characteristics, the J.H was presumed to belong to the genus Aspergillus, whereas the J.P was presumed to belong to the genus Mucor. Phytochemical screening revealed that both isolates contained alkaloids, flavonoids, and tannins. The J.H extract was positive for steroids, while the J.P extract was positive for triterpenoids. The maximum absorption wavelength of DPPH was observed at 516 nm with an optimum reaction time of 30 minutes. The IC_{50} value of ascorbic acid was 6.91 ppm. The IC_{50} values of the J.H and J.P extracts were 122.79 ppm and 123.84 ppm, respectively, indicating moderate antioxidant activity. In conclusion, symbiotic fungi isolated from bir pletok have the potential to serve as natural sources of antioxidant compounds due to the presence of secondary metabolites capable of scavenging free radicals.

Keyword: *Bir pletok; fungal isolates; antioxidant activity; secondary metabolites*

Introduction

Oxidative stress results from an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense system of living organisms. Excessive ROS production promotes oxidative damage to lipids, proteins, and nucleic acids, contributing to the pathogenesis of numerous chronic disorders, including cardiovascular diseases, diabetes mellitus, neurodegenerative diseases, and cancer. Consequently, the identification of safe and effective natural antioxidants has become an important research priority in pharmaceutical and natural product sciences, particularly those derived from microorganisms capable of producing structurally diverse secondary metabolites. Recent studies have highlighted that fungal metabolism can be manipulated through cultivation

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conditions to enhance the production of phenolic compounds and radical-scavenging metabolites, emphasizing fungi as an emerging source of natural antioxidants (Zaichenko et al., 2026).

Traditional herbal beverages have long been consumed not only for their sensory characteristics but also for their health-promoting properties. Their biological activities are largely attributed to the presence of phenolic compounds, flavonoids, tannins, terpenoids, and other phytochemicals that exhibit antioxidant, anti-inflammatory, and antimicrobial activities. Recent investigations on fermented herbal beverages further demonstrate that fermentation may enhance antioxidant capacity through the formation or biotransformation of bioactive metabolites. These findings indicate that traditional herbal beverages represent complex biological systems in which both plant constituents and associated microorganisms may contribute to the overall bioactivity (Cho & Che Zain, 2026).

Bir pletok is a traditional Betawi herbal beverage prepared from a combination of medicinal spices, including ginger (*Zingiber officinale*), sappan wood (*Caesalpinia sappan*), cinnamon (*Cinnamomum burmannii*), cloves (*Syzygium aromaticum*), lemongrass (*Cymbopogon citratus*), and several other aromatic ingredients. These spices are well recognized as rich sources of polyphenols and flavonoids with pronounced antioxidant activity. Previous investigations have primarily focused on the phytochemical composition and antioxidant potential of the herbal ingredients or the beverage itself. However, little attention has been directed toward fungal communities naturally associated with bir pletok, despite their potential to synthesize bioactive secondary metabolites during colonization or cultivation. Filamentous fungi are recognized as prolific producers of secondary metabolites with diverse chemical structures and biological activities. Numerous fungal metabolites exhibit antioxidant, antimicrobial, anticancer, immunomodulatory, and anti-inflammatory properties, making fungi an important resource for pharmaceutical discovery. The biosynthesis of these compounds is strongly influenced by the cultivation substrate and environmental conditions, allowing fungi to produce metabolites that differ substantially from those synthesized by their plant hosts. Consequently, fungal isolates recovered from herbal materials represent a promising yet underexplored source of naturally occurring antioxidant compounds (Zaichenko et al., 2026).

Despite increasing interest in natural antioxidants, studies investigating fungal isolates associated with traditional Indonesian herbal beverages remain scarce. Most published reports evaluate only the antioxidant activity of herbal extracts without considering the contribution of associated fungi as independent producers of bioactive metabolites. This knowledge gap limits our understanding of fungal biodiversity associated with traditional herbal preparations and restricts

opportunities to identify novel microbial sources of antioxidant compounds for pharmaceutical applications.

The novelty of the present study lies in the isolation of fungal strains from traditional bir pletok, followed by the evaluation of their phytochemical characteristics and antioxidant potential using ethyl acetate extracts. Unlike previous studies that primarily investigated the phytochemical constituents of the beverage, the present work focuses on fungal-derived secondary metabolites as potential contributors to antioxidant activity. This microbial perspective provides new insight into the bioprospecting potential of fungi associated with traditional Indonesian herbal beverages.

Therefore, this study aimed to isolate fungi from traditional bir pletok, characterize the obtained isolates based on their macroscopic and microscopic morphology, identify the major classes of secondary metabolites in their ethyl acetate extracts, and evaluate their antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The findings are expected to provide a scientific basis for the utilization of fungal isolates associated with traditional herbal beverages as prospective sources of natural antioxidant compounds for future pharmaceutical development.

Method

This experimental laboratory study was conducted to isolate fungi associated with traditional bir pletok, evaluate the phytochemical constituents of their ethyl acetate extracts, and determine their antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay(Prima et al., 2022) .

Materials

The bir pletok sample was obtained from the Herbal Laboratory of Universitas 17 Agustus 1945 Jakarta. Potato Dextrose Agar (PDA), rice, methanol, 70% ethanol, distilled ethyl acetate, chloramphenicol, distilled water, DPPH (2,2-diphenyl-1-picrylhydrazyl), ascorbic acid, magnesium powder, concentrated hydrochloric acid, 2 N hydrochloric acid, ferric chloride ($FeCl_3$), ammonia solution, Mayer reagent, Wagner reagent, Dragendorff reagent, lactophenol cotton blue (LPCB), and other analytical-grade reagents were used throughout the study. The instruments included an autoclave, laminar airflow cabinet (LAF), incubator, rotary evaporator, analytical balance (OHAUS), UV–Visible spectrophotometer (Thermo Scientific Genesys 150 and Thermo Spectronic Genesys 2), microscope, hot plate, and standard laboratory glassware (Prima et al., 2022) .

Isolation and Morphological Identification of Fungal Isolates

Contaminated bir pletok was inoculated onto Potato Dextrose Agar (PDA) supplemented with 0.1% chloramphenicol to inhibit bacterial growth. The cultures were incubated at 25°C for approximately 14 days until fungal colonies developed.

Colonies with different morphological characteristics were purified by repeated subculturing on fresh PDA to obtain stable pure isolates. Macroscopic observations included colony color, texture, margin, colony shape, and growth characteristics after 6 days of incubation. Microscopic characterization was performed by placing a small portion of fungal mycelia on a glass slide containing lactophenol cotton blue (LPCB), followed by observation under a light microscope at 100× magnification. Hyphal morphology, septation, conidiophores, vesicles, phialides, and conidia were examined for preliminary genus identification based on standard fungal identification references (Prima et al., 2022).

Solid-State Cultivation and Extraction of Secondary Metabolites

Each purified fungal isolate was cultivated on sterilized rice medium prepared by mixing 75 g of rice with 75 mL of distilled water in 500 mL Erlenmeyer flasks. Following sterilization, the medium was inoculated with fungal cultures and incubated under static conditions for 30 days at room temperature to promote secondary metabolite production. The fermented rice cultures containing fungal mycelia were extracted by repeated maceration using distilled ethyl acetate. Combined filtrates were dried over anhydrous sodium sulfate before solvent removal under reduced pressure using a rotary evaporator. The concentrated crude extracts were weighed to determine extraction yield and subsequently stored at 4°C until analysis. Fermented rice cultures containing fungal mycelia were extracted by maceration using distilled ethyl acetate. Each extraction was carried out for 24 h and repeated three consecutive times. The combined filtrates were concentrated under reduced pressure using a rotary evaporator to obtain crude ethyl acetate extracts (Kjer et al., 2010) .

Phytochemical Screening

Qualitative phytochemical analysis was performed to detect alkaloids, flavonoids, tannins, saponins, terpenoids, steroids, and triterpenoids using standard colorimetric methods. Alkaloids were detected using Mayer and Wagner reagents after acid extraction with 2 N hydrochloric acid. Tannins were identified using 10% ferric chloride solution. Flavonoids were detected using the magnesium–hydrochloric acid reduction method. Terpenoids and steroids/triterpenoids were evaluated using the Liebermann–Burchard reaction, while saponins were determined based on the formation of stable foam after vigorous shaking (Sukmawaty et al., 2020) .

Antioxidant Activity Assay

A total of 30 mg of DPPH was dissolved in 1000 mL of methanol to obtain a 30 µg/mL (30 ppm) DPPH solution. The solution was stored in a dark bottle and protected from light throughout the experiment. The maximum absorption wavelength (λmax) was determined by scanning the DPPH solution between 400 and 800 nm using methanol as the blank. The negative control consisted of 2 mL

methanol mixed with 2 mL DPPH solution. For the positive control, ascorbic acid was dissolved in methanol to obtain a 100 µg/mL stock solution, which was subsequently diluted to concentrations of 1, 2, 3, 4, 5, 6, 7, 8, and 9 µg/mL. All solutions were protected from light by covering the volumetric flasks with aluminum foil. Each fungal ethyl acetate extract was dissolved in methanol to prepare a 1000 µg/mL stock solution. Working solutions were prepared by serial dilution to final concentrations of 1.5, 3.0, 12.5, 50, 100, and 150 µg/mL. For each concentration, 2 mL of sample solution was mixed with 2 mL of DPPH solution (30 ppm) and homogenized. The mixtures were incubated for 30 min at room temperature in the dark, after which absorbance was measured using a UV–Visible spectrophotometer at the predetermined maximum wavelength. The same procedure was applied to the positive control. All measurements were performed in triplicate (Prima et al., 2022) .

Results and Discussion

Isolation and Macroscopic Characteristics of Fungal Isolates

Isolation of fungi associated with bir pletok successfully yielded two morphologically distinct fungal isolates, designated as J.H and J.P. Macroscopic observation revealed clear differences in colony color, texture, and growth pattern on PDA medium, suggesting that the isolates belonged to different fungal taxa. The J.H isolate developed white colonies that gradually turned black during sporulation, whereas the J.P isolate maintained a white, cotton-like colony throughout the incubation period. These differences indicate variations in fungal morphology and sporulation characteristics that are commonly used as preliminary criteria for fungal identification (Erdiansyah & Zaini, 2023) .

Microscopic examination further supported the differentiation of both isolates. J.H exhibited septate hyphae, vesicle-bearing conidiophores, and chains of dark conidia, which are characteristic of the genus *Aspergillus*, particularly *A. niger* (Erdiansyah & Zaini, 2023). In contrast, J.P produced rapidly growing white colonies with abundant aerial mycelia, consistent with the typical morphology of the genus *Mucor* (Fathoni et al., 2016*)*. Although the present identification was based on morphological characteristics, these observations provide an important preliminary basis for distinguishing fungal isolates prior to molecular characterization.

Microscopic Characteristics of Fungal Isolates

Microscopic examination confirmed the morphological differences between the two fungal isolates. Isolate J.P exhibited broad aseptate hyphae, upright sporangiophores, spherical sporangia, and prominent columellae, which are characteristic features of the genus *Mucor* (Fathoni et al., 2016) . The occurrence of *Mucor* in bir pletok may be associated with contamination during raw material

handling, processing, or storage, as this fungus readily proliferates under humid conditions and in carbohydrate-rich substrates.

In contrast, isolate J.H showed septate hyphae, vesicle-bearing conidiophores, and chains of dark conidia produced from metulae and phialides. These morphological characteristics are consistent with those reported for *Aspergillus niger* (Erdiansyah & Zaini, 2023). The presence of *A. niger* is likely related to fungal spores originating from spices or the surrounding production environment, reflecting the widespread distribution and ecological adaptability of this species. Overall, microscopic observations supported the preliminary identification of the fungal isolates and complemented the macroscopic characteristics observed on PDA medium.

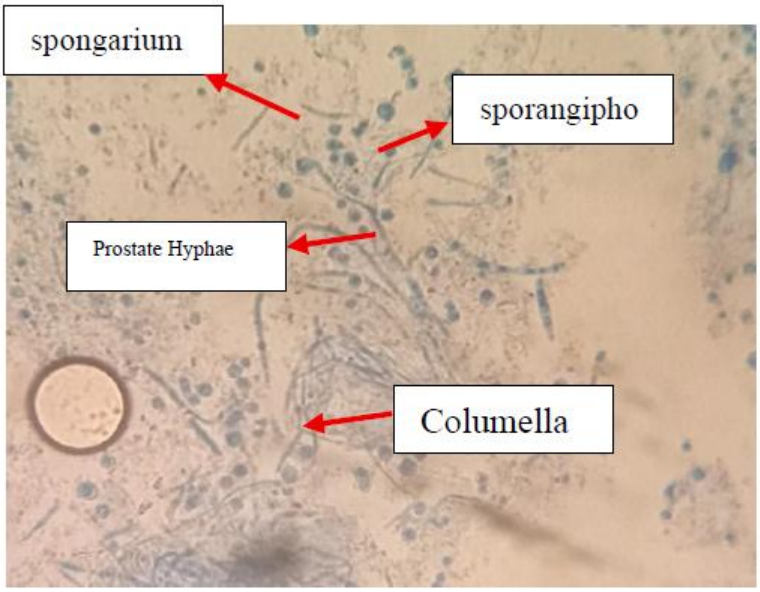


Figure 1 (Microscopic J.P)

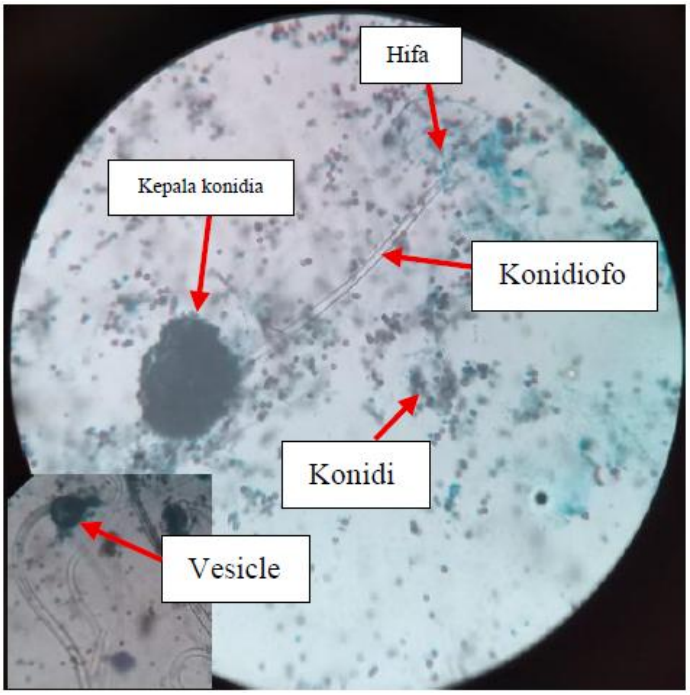


Figure 2(Microscopic J.H)

Cultivation and Extraction Yield of Fungal Metabolites

After the incubation period, the average weight of fermented culture obtained from the J.H and J.P fungal isolates was 151.39 g and 159.789 g, respectively. Extraction using ethyl acetate produced 5.24 g of concentrated extract from the J.H isolate and 4.52 g from the J.P isolate. The extraction yield of the J.H extract was 3.46%, while the J.P extract was 2.83%.

The difference in extraction yield between fungal isolates indicates variations in secondary metabolite production during the fermentation process. Although both isolates were cultivated under similar conditions, differences in fungal metabolism and biosynthetic capacity may affect the amount of metabolites produced. Rice substrate used in solid-state fermentation provides nutrients, particularly carbohydrates, that support fungal growth and stimulate the production of secondary metabolites.

Ethyl acetate extraction was selected because it is effective for extracting semi-polar fungal metabolites, including phenolic compounds, flavonoids, terpenoids, and alkaloid derivatives. The higher yield obtained from the J.H isolate suggests a greater production of ethyl acetate-soluble metabolites. However, extraction yield does not directly determine biological activity, as antioxidant activity depends on the type and concentration of active compounds present in the extract.

Table 1(Yield of crude extracts obtained from fungal isolates cultivated on rice substrate)

Fungal isolate	Weight of fermented culture (g)	Weight of crude extract (g)	Extraction yield (%)
J.H	151.39	5.24	3.46
J.P	159.789	4.52	2.83

Phytochemical Screening

Phytochemical screening revealed the presence of several secondary metabolites in fungal extracts, including alkaloids, flavonoids, tannins, steroids, and triterpenoids. The J.H extract showed positive results for alkaloids, flavonoids, tannins, and steroids, while the J.P extract contained alkaloids, flavonoids, tannins, and triterpenoids.

The detection of these secondary metabolites indicates that fungi associated with bir pletok are capable of producing bioactive compounds. Differences in metabolite profiles between fungal isolates may be related to variations in their metabolic pathways and adaptation mechanisms during growth on the fermentation substrate.

Flavonoids and tannins are considered important antioxidant compounds because their hydroxyl groups can donate hydrogen atoms or electrons to stabilize free radicals. In addition, alkaloids and terpenoid derivatives have also been reported to contribute to antioxidant activity through radical scavenging mechanisms. The presence of these metabolites may explain the antioxidant potential observed in fungal extracts through the DPPH assay.

Table 2(Phytochemical screening results of J-H and J-P fungal extracts)

Secondary metabolite compounds	J.H isolate	J.P isolate
Alkaloid (Mayer reagent)	+	+
Alkaloid (Wagner reagent)	+	+
Alkaloid (Dragendorff reagent)	+	+
Phenolic compounds/Tannins	+	+
Flavonoids	+	+
Steroids	+	-
Triterpenoids	-	+

Antioxidant Activity

The antioxidant activity of fungal extracts was evaluated using the DPPH radical scavenging method with ascorbic acid as the standard antioxidant. Prior to sample analysis, the maximum wavelength and operating time were determined to obtain optimal measurement conditions.

The maximum absorption wavelength of DPPH solution was obtained at 516 nm. This result is within the characteristic absorption range of DPPH radicals,

which generally exhibits maximum absorbance around 515–520 nm. The purple color of DPPH radicals is generated by the presence of unpaired electrons, and the decrease in absorbance occurs due to electron or hydrogen atom transfer from antioxidant compounds to stabilize the radical form.

The operating time determination showed that the reaction reached a stable condition after 30 minutes. This indicates that the interaction between DPPH radicals and antioxidant compounds reached an optimal reaction period, allowing accurate measurement of radical scavenging activity.

The calibration curve of ascorbic acid was established at concentrations of 1–9 ppm. The absorbance decreased with increasing concentration of ascorbic acid, indicating an increase in DPPH radical reduction. The IC_{50} value obtained for ascorbic acid was 6.91 ppm, indicating very strong antioxidant activity. Ascorbic acid was used as a reference because it has a high ability to donate electrons and neutralize free radicals.

The antioxidant activity of fungal extracts was further evaluated using the same DPPH method. The J.H and J.P extracts showed a concentration-dependent decrease in absorbance, where higher extract concentrations resulted in lower DPPH absorbance values. The J.P extract showed an IC_{50} value of 123.84 ppm, while the J.H extract showed an IC_{50} value of 122.79 ppm.

The IC_{50} values of both fungal extracts indicate moderate antioxidant activity based on the DPPH antioxidant activity classification. The slightly higher antioxidant activity of the J.H extract compared with the J.P extract may be associated with differences in the composition and concentration of secondary metabolites produced by each fungal isolate. Based on phytochemical screening, both extracts contained flavonoids and tannins, which are known to contribute to antioxidant activity through hydrogen atom donation and free radical stabilization. The antioxidant activity observed in fungal extracts suggests that fungi associated with bir pletok have the ability to produce bioactive secondary metabolites with potential antioxidant properties. However, the antioxidant capacity of crude extracts is influenced not only by the presence of metabolites but also by their concentration, synergistic interactions, and chemical structures. Further studies using chromatographic separation and compound identification are required to determine the specific metabolites responsible for antioxidant activity.

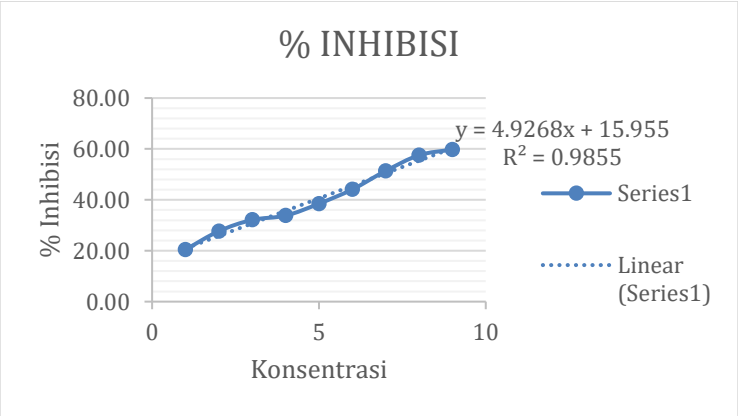


Table 3(Percentage Inhibition Curve of Ascorbic Acid)

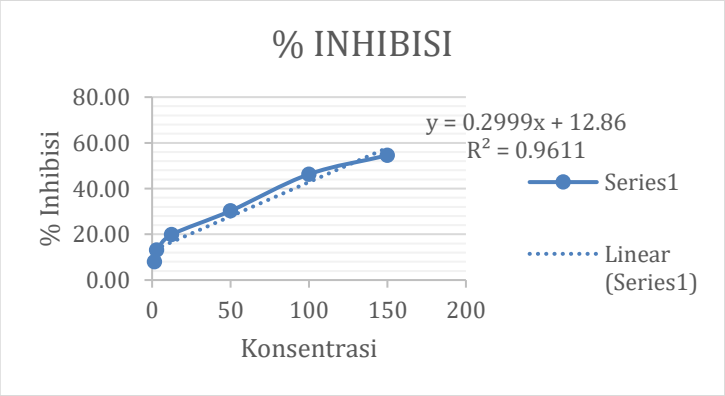


Table 4(Percentage Inhibition Curve of J-P Fungal Extract)

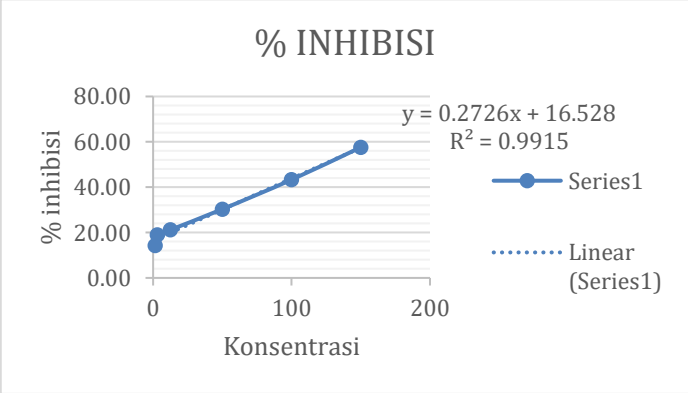


Table 5(Percentage Inhibition Curve of J-H Fungal Extract)

Relationship Between Secondary Metabolites and Antioxidant Activity

The antioxidant activity of fungal extracts may be associated with the presence of secondary metabolites identified through phytochemical screening. Both J.H and J.P extracts showed positive results for flavonoids, tannins, and alkaloids, J.H and J.P extract contained steroids and the extract contained triterpenoids. The presence of these compounds indicates that fungi associated with bir pletok are capable of producing bioactive metabolites with potential antioxidant properties.

The DPPH assay showed that both fungal extracts exhibited moderate antioxidant activity, with IC_{50} values of 122.79 ppm for the J.H extract and 123.84 ppm for the J.P extract. The slightly higher antioxidant activity of the J.H extract may be related to its higher extraction yield (3.46%) compared with the J.P extract (2.83%), indicating a greater amount of ethyl acetate-soluble metabolites obtained from the fermentation culture.

Flavonoids and tannins detected in both extracts are considered important contributors to antioxidant activity due to their ability to donate hydrogen atoms or electrons to neutralize free radicals. In addition, alkaloids, steroids, and triterpenoids may contribute through synergistic antioxidant mechanisms. However, antioxidant activity is not solely determined by the presence of metabolite groups, but also depends on the concentration, chemical structure, and interaction between individual compounds present in the extract.

These findings indicate that fungi isolated from bir pletok have potential as sources of antioxidant secondary metabolites. Further studies using chromatographic separation and structural identification are required to determine the specific compounds responsible for the observed antioxidant activity.

Conclusion

This study demonstrated that fungi associated with bir pletok were capable of producing bioactive secondary metabolites with antioxidant potential. The fungal isolates produced ethyl acetate extracts with different yields, where the J.H extract showed a higher yield (3.46%) compared with the J.P extract (2.83%). Phytochemical screening revealed the presence of several secondary metabolites, including alkaloids, flavonoids, tannins, steroids, and triterpenoids, which may contribute to their biological activity.

The antioxidant evaluation using the DPPH radical scavenging method showed that both fungal extracts exhibited moderate antioxidant activity. The J.H extract showed an IC_{50} value of 122.79 ppm, while the J.P extract showed an IC_{50} value of 123.84 ppm. The antioxidant activity observed in fungal extracts was associated with the presence of secondary metabolites, particularly flavonoids and tannins, which are known to contribute to free radical scavenging activity.

These findings indicate that fungi isolated from bir pletok have potential as sources of natural antioxidant compounds. Further studies involving molecular identification and characterization of active compounds are required to determine the specific metabolites responsible for the antioxidant activity.

Suggest

Molecular identification of the fungi using the ITS rDNA PCR method and sequencing analysis is necessary to obtain a more accurate identification of the

fungal species, particularly to confirm the suspected genus Aspergillus sp. in J.H. and the Mucorales group in J.P. It is necessary to purify and identify active compounds using chromatography and analytical instruments such as TLC, TLC-bioautography, LC-MS, or GC-MS to determine the specific compounds responsible for antioxidant activity.

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