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Characterization of Endophytic Fungi in Robusta Coffee (*Coffea canephora* L.) Beans Through 18S rRNA Gene Sequencing and Evaluation of Antioxidant Activity and Chlorogenic Acid Content

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Abstract: Utilizing endophytic fungi to discover metabolites by characterizing the fungi produced from robusta coffee beans can be highly beneficial without causing harm to the plants. Additionally, this approach aids in identifying raw materials for medical drug development, specifically compounds like chlorogenic acid, which hold promise as potential treatments for diabetes mellitus. This study aimed at characterizing the endophytic fungi present in Robusta coffee (*Coffea canephora* L.) beans through 18S rRNA gene sequencing and evaluation of antioxidant activity and chlorogenic acid content found in these endophytic fungi. Green, unripe Robusta coffee beans were harvested from a coffee plantation in Tana Toraja and isolated using Potato Dextrose Agar medium. The isolation process yielded three isolates, designated RF1, RF2, and RF3. Subsequently, the isolates were subjected to testing for endophytic fungi activity, and the isolate that exhibited activity was further characterized using 18S rRNA gene sequencing. Following that, the isolates were fermented using a Potato Dextrose Broth medium, resulting in two phases: filtrate and biomass. The filtrate was then extracted using ethyl acetate as the solvent. The extracted compound was subsequently evaluated for antioxidant activity using ABTS and DPPH methods, and the concentration of chlorogenic acid was measured. From the three isolates of endophytic fungi obtained from green Robusta coffee beans with the codes RF1, RF2, and RF3, all of them exhibited antibacterial activity against Escherichia coli and Staphylococcus aureus. The 18S rRNA gene sequencing analysis revealed that isolate RF1 was identified as *Fusarium sp*. For the evaluation of antioxidant activity using the ABTS method, an IC_{50} value of 53.49 µg/mL was obtained, while the DPPH method resulted in an IC_{50} value of 54.03. Furthermore, the measurement of chlorogenic acid content in the extract of endophytic fungi from Robusta coffee beans indicated a concentration of 11.58%. The characterization result of the endophytic fungi isolates from green Robusta coffee beans revealed the presence of *Fusarium sp*. It exhibited high antioxidant activity and a chlorogenic acid content of 11.58%.

Keywords: Antioxidant Activity; Chlorogenic Acid; Endophytic Fungi; Robusta Coffee

Introduction

Coffee, a beloved beverage among the public, possesses beneficial properties as an antioxidant. It contains many compounds, such as phenolics, chlorogenic acid, and caffeine, which have been used in medicinal applications (Choi & Kim, 2017; Xu et al., 2018). The antioxidant activity of chlorogenic acid makes it a potential candidate for the treatment of diabetes mellitus, as well as for its antibacterial and antihypertensive properties. Additionally, chlorogenic acid has been found to activate glucose transporter 4, making it useful in managing diabetes mellitus (Hasanuddin et al., 2021; Jasmiadi et al., 2020; Rusman &

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Irfiyanti, 2022; Supriana et al., 2020). Robusta coffee exhibits broad-spectrum antibacterial activity, inhibiting both gram-positive and gram-negative bacteria by increasing membrane permeability and disrupting plasma membrane function, resulting in nucleotide leakage. It shows inhibitory effects against the growth of bacteria, such as Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus. As an antifungal agent, chlorogenic acid can impede the growth of Candida albicans by disrupting cell membrane structures (Farah & dePaula Lima, 2019). Although numerous studies have been conducted on the isolation of chlorogenic acid from Robusta coffee, the exploration of microorganism isolation using endophytic fungi to extract compounds from these fungi remains relatively rare.

Endophytic fungi, as microorganisms living in association with host plants, produce secondary metabolites similar to the compounds present in their host plants (Bezerra et al., 2021; Hasanuddin et al., 2022). Isolated endophytic fungi from medicinal plants can yield alkaloids or secondary metabolites identical to those found in the original plant, sometimes in even greater quantities. This eliminates the need to harm the original plant for sampling purposes, a process that would otherwise take several decades to cultivate and grow (Lou et al., 2011). Endophytic fungi also protect plants against various pathogens, including bacteria, fungi, and insects. These antimicrobial properties are commonly found in several fungal genera, such as Aspergillus, Alternaria, Colletotrichum, Fusarium, Penicillium, and Pestalotiopsis (Casella et al., 2013; Gupta et al., 2020; Martín-Rodríguez et al., 2015; Saleem & Ebrahim, 2014). The selection of appropriate host plants for isolating endophytic fungi is crucial when considering their use as a source of biologically active secondary metabolites.

The primary group of phenolic compounds is hydroxycinnamic acid, which is commonly found in almost every plant. Hydroxycinnamic acid can undergo esterification with quinic acid, resulting in the formation of chlorogenic acid (Kesuma, 2015; Meiliana & Wijaya, 2013; Hounsome et al., 2008; Rice-Evans et al., 1996). Based on the information aforementioned, research is necessary to characterize and evaluate the antioxidant activity of endophytic fungi isolates from Robusta coffee beans using 18S rRNA gene sequencing.

Method

This study was conducted in January 2022 at the Microbiology Laboratory of Mega Rezky University and the Biochemistry Laboratory of the Department of Chemistry, Faculty of Mathematics and Natural Sciences, Hasanuddin University. The sequencing of the 18S rRNA gene was performed at the HUMRC Laboratory, Hasanuddin University Teaching Hospital.

Instruments and Materials

The instruments used included an autoclave, Petri dishes, Erlenmeyer flasks (Pyrex iwaki ST), beakers (Infors HT), incubator, hypodermic needles, glass slides, L-glass deg glass, laminar airflow (LAF), alcohol burner, digital microscope, centrifuge, UV-Vis spectrophotometer, analytical balance (ACIS), test tubes (Pyrex), centrifuge tubes (Pyrex), vials, and oven (B-one).

The materials used in this study included distilled water (Aquades; H_2O), aluminum foil, ascorbic acid ($C_6H_8O_6$), chlorogenic acid (Sciencewerke), Robusta coffee beans (*Coffea canephora* L.), 70% ethanol (C_2H_5OH), emergency oil, chloramphenicol, potassium persulfate ($K_2S_2O_8$), methanol (CH_3OH), methylene blue, sodium hypochlorite (NaOCl), nutrient agar (Himedia), potato dextrose agar (Himedia), potato dextrose broth (Himedia), and 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS).

Research Procedures

Sample Collection and Processing

Samples of Robusta coffee (*Coffea canephora* L.) beans were collected from a coffee plantation in the Rano area of Tana Toraja Regency, South Sulawesi, Indonesia. The coordinates of the plantation are -3°432.2332 latitude and 11°4515.0696 longitude. The coffee beans were transported to the Microbiology Laboratory at Mega Rezky University. The samples were thoroughly washed and cleaned with running water, followed by surface sterilization using 70% ethanol to eliminate any potential contaminants or other microorganisms.

Equipment Sterilization

All equipment used in this study was washed with distilled water. Furthermore, glassware was dried and wrapped in paper before being placed in an oven for sterilization. Furthermore, heat-resistant equipment was sterilized at 160°C for 2 hours, while non-heat-resistant equipment was sterilized at 121°C for 15 minutes. Hypodermic needles were sterilized by flaming them with an alcohol burner. After sterilization, the instruments were handled using sterile forceps.

Endophytic Fungi Isolation

Fresh samples of Robusta coffee (*Coffea canephora* L.) beans were collected and treated under aseptic conditions. The samples were soaked in 70% ethanol for 30 seconds, followed by immersion in sodium hypochlorite for 3-5 minutes. They were then rinsed three times with sterile distilled water, each rinse lasting

for 1 minute. The samples were aseptically cut into small pieces of approximately ±1-2 cm. The pieces were directly planted onto Petri dishes containing Potato Dextrose Agar with Chloramphenicol (PDAC) medium using sterile forceps. The Petri dishes were then incubated at 27°C for 5-7 days. After that, fungal colonies that grew around the planted samples were observed.

Subsequently, the fungi were transferred to new Petri dishes containing Potato Dextrose Agar (PDA) medium using aseptic techniques and a hypodermic needle. This process was repeated three times to obtain pure cultures. The obtained fungal cultures were streaked onto a slanted PDA medium for stock purposes. Finally, the fungal cultures were incubated at 27°C and were ready for further testing.

Phylogenetic Analysis of Endophytic Fungal Isolates

The DNA sequencing of PCR products from samples that exhibited positive electrophoresis results was performed by 1stBase through PT. Genetika Indonesia. The DNA sequencing process followed the Sanger dideoxy method.

DNA Sequence Analysis

The sequencing results were analyzed by conducting a BLAST search of the 18S rRNA sequencing data against the available database at www.ncbi.nlm.nih.gov. The BLAST search aimed to identify the similarity between the query sequence (nucleotide or protein sequence of interest) and the subject sequence in the database. Sequence alignment was carried out using the Clustal W program. Subsequently, the phylogenetic relationship was visualized using the MEGA6 program.

Fermentation of Endophytic Fungal Isolates

Pure colony cultures were aseptically obtained using a hypodermic needle and transferred into 100 mL Erlenmeyer flasks containing Potato Dextrose Broth. The flasks were then incubated at 28°C for 14 days. After that, the cultures were shaken using an incubator shaker at a speed of 200 rpm for 7 consecutive days, 24 hours each day. After completion, each medium was centrifuged at 3800 rpm for 15 minutes at 4°C, resulting in two phases: filtrate and biomass (supernatant).

Extraction of Fermentation Products from Endophytic Fungal Isolates

The filtered and separated fermentation results, comprising the filtrate and biomass, were subjected to liquid extraction using ethyl acetate. A volume of 100 mL (v/v) of ethyl acetate was added to the filtrate in a separating funnel and vigorously shaken. The mixture was then allowed to settle until two distinct phases formed. The upper phase, known as the organic layer, contained the ethyl acetate extract, while the lower phase consisted of the aqueous fraction. The two phases were carefully separated, and ethyl acetate was added again to the aqueous fraction. The process was repeated three times, ensuring thorough mixing and separation. Only the upper layer was collected. The ethyl acetate fractions obtained were combined and dried using a rotary evaporator at a temperature of 40°C until a dry extract was obtained. Meanwhile, the biomass obtained from the filtration process was extracted with 100 mL (v/v) of methanol. The biomass was finely ground using a mortar, followed by maceration and shaking for 24 hours. The mixture was then filtered to obtain the filtrate, while the solid residue was subjected to three additional rounds of maceration with fresh methanol. The resulting extracts were concentrated using a rotary evaporator at 40°C to obtain a concentrated extract for antioxidant testing.

Antioxidant Activity Test

The antioxidant activity was assessed using the ABTS method with the following steps:

Measurement of ABTS Blank Solution Absorbance

A 1 mL volume of the ABTS solution was pipetted and diluted to 5 mL with absolute ethanol in a measuring flask. The solution was then measured using UV-Vis spectrophotometer at a wavelength of 750 nm.

Measurement of Free Radical Scavenging Activity with Samples

The stock solution of the extract obtained from the fermentation of the endophytic fungi isolates at 1000 ppm was pipetted with volumes of 0.05 mL, 0.1 mL, 0.15 mL, 0.2 mL, and 0.25 mL. Then, 1 mL of the ABTS solution was added, and the volume was adjusted with absolute methanol to a total of 5 mL, resulting in concentrations of 10 ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm. The mixture was homogenized, and the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 750 nm.

Measurement of Free Radical Scavenging Activity with Pure Ascorbic Acid

The test was performed by pipetting 0.15 mL, 0.20 mL, 0.25 mL, 0.30 mL, and 0.35 mL of the stock solution of ascorbic acid at 1000 ppm. Then, 1 mL of the ABTS solution was added, and the volume was adjusted with absolute ethanol to a total of 5 mL, resulting in concentrations of 3 ppm, 4 ppm, 5 ppm, 6 ppm, and 7 ppm. The mixture was homogenized, and the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 750 nm.

Measurement of Antioxidant Activity of the Fermented Extract from Endophytic Fungi Isolate of Robusta Coffee (Coffea canephora L.) Beans at 500 ppm

The stock solution of 500 ppm was pipetted at volumes of 100 µL, 200 µL, 400 µL, 800 µL, and 1000 µL, and then transferred into 5 mL volumetric flasks. Next, 1 mL of 0.4 mM DPPH solution was added, and the volume was completed with ethanol p.a up to the mark, resulting in concentrations of 10 ppm, 20 ppm, 40 ppm, 80 ppm, and 100 ppm. The mixture was homogenized, covered with aluminium foil, and allowed to stand for 30 minutes. Subsequently, the absorbance was measured using a visible spectrophotometer at a wavelength of 515 nm.

Result and Discussion

Result

Characterization of Endophytic Fungi in Robusta Coffee as seen in Figure 1.

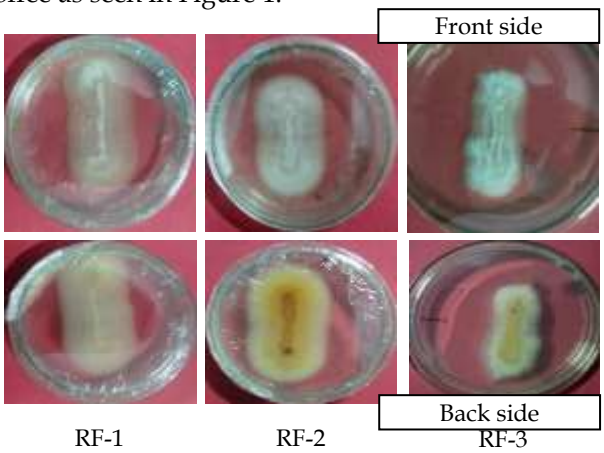


Figure 1. Results of Endophytic Fungi Isolation from Robusta Coffee Beans

Table 1. Results of Macroscopic Observation of Endophytic Fungi Isolates

Isolates	Macroscopic Characteristics			
	Colony Surface Color	Colony Pigmentation	Colony Texture	Colony Shape
RF-1	White	Yellowish White	Fine, Cotton-like	Oval, Elongated
RF-2	White	Yellowish White	Fine, Cotton-like	Oval, Elongated
RF-3	White	Yellowish White	Fine, Cotton-like	Oval, Elongated

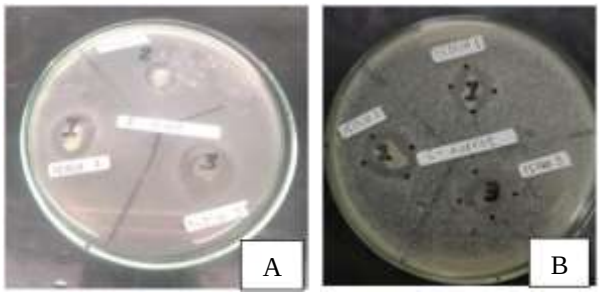
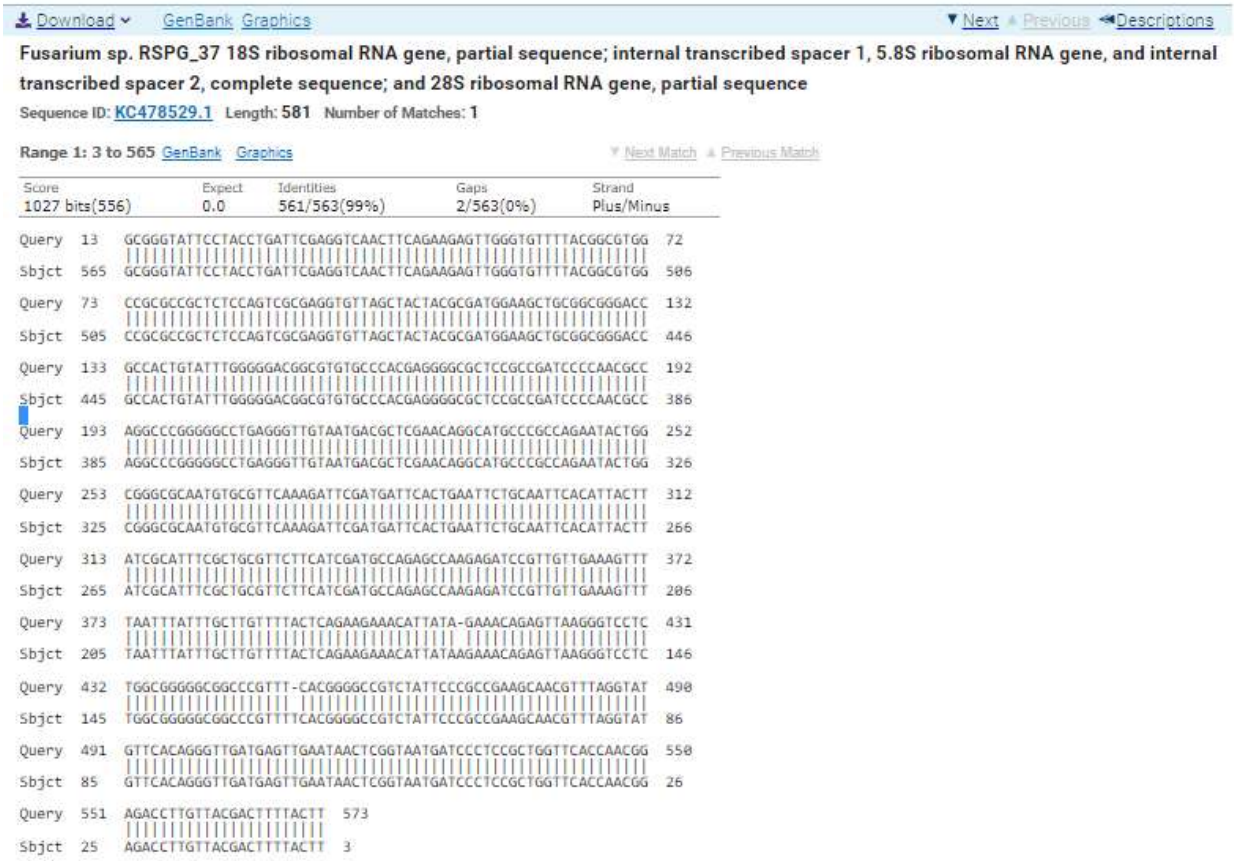


Figure 2. Results of Antimicrobial Activity Test of Endophytic Fungal Isolates from Robusta Coffee (*Coffea canephora* L.) Beans

Note: A. Escherichia coli Bacteria
B. Staphylococcus Bacteria



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Fusarium sp. RSPG_37 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: KC478529.1 Length: 581 Number of Matches: 1

Range 1: 3 to 565 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1027 bits(556)	0.0	561/563(99%)	2/563(0%)	Plus/Minus

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Figure 3. BLAST Results from Images on NCBI Application

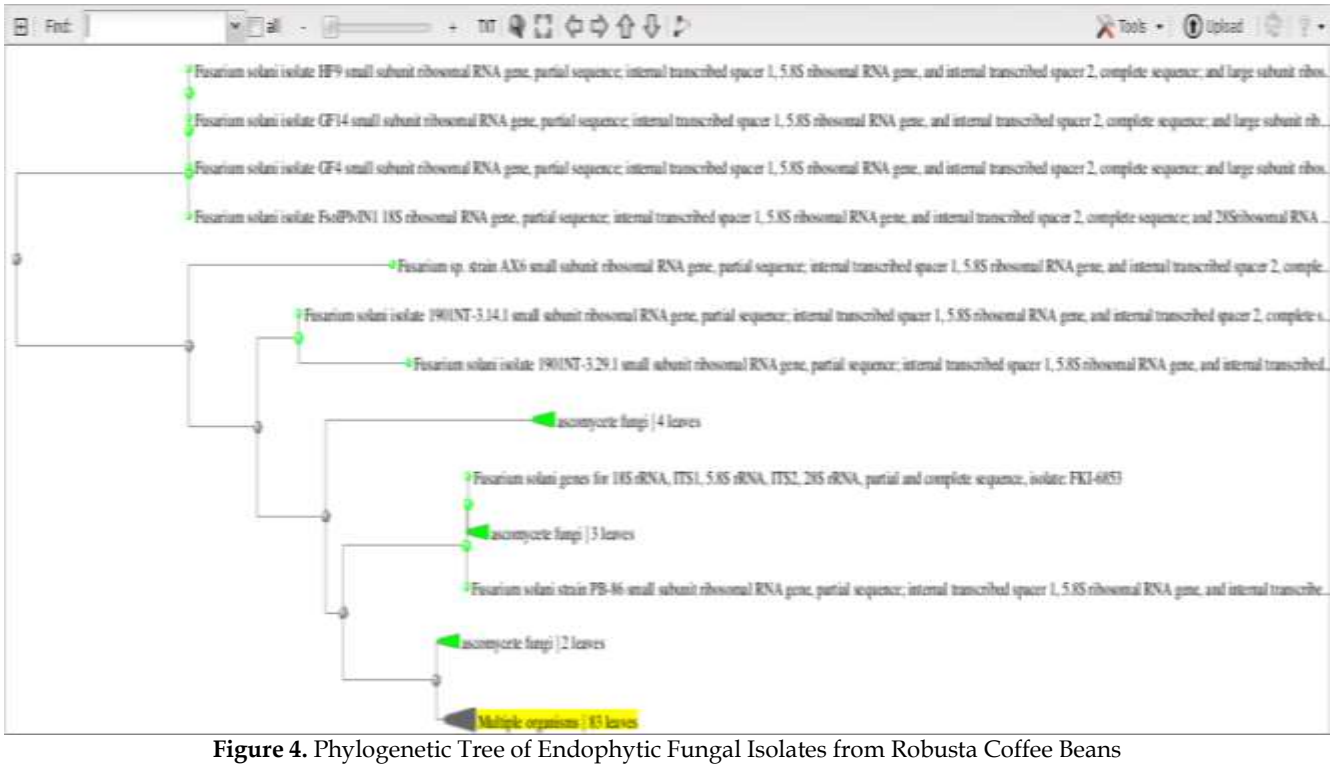

Figure 4. Phylogenetic Tree of Endophytic Fungal Isolates from Robusta Coffee Beans

Table 2. Results of Antioxidant Activity Measurement of Endophytic Fungal Isolates from Robusta Coffee Beans Using the ABTS Method

Concentration (µg/mL)	Absorbance (A) at λ = 515 nm	Antioxidant Activity (%)	IC_{50} Value (µg/mL)
20	0.434	30.67	
40	0.333	46.81	
60	0.277	55.75	53.4975
80	0.239	61.82	
100	0.186	70.29	
Control	0.626		

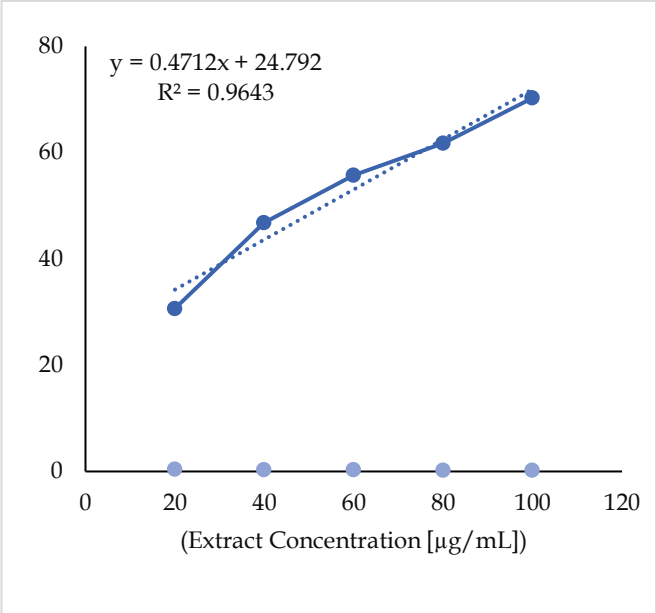

Figure 5. Antioxidant Activity Curve of Endophytic Fungal Isolates from Robusta Coffee Beans Using the ABTS Method

Table 3. Results of Antioxidant Activity Measurement of Endophytic Fungal Isolates from Robusta Coffee Beans Using the DPPH Method

Samples	Concentration (µg/mL)	Antioxidant Activity (%)	IC_{50} Value (µg/mL)
Robusta Coffee Endophytic Fungi	10	36.70	54.03
	20	41.20	
	40	49.81	
	80	54.31	
	100	62.73	

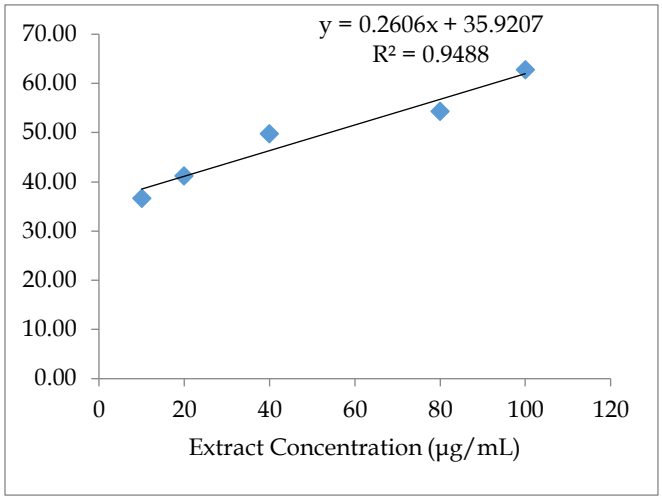

Figure 6. Antioxidant Activity Curve of Endophytic Fungal Isolates from Robusta Coffee Beans Using the DPPH Method

Table 4. Results of Chlorogenic Acid Content Measurement in the Fermented Extract of Endophytic Fungal Isolates from Robusta Coffee (*Coffea canephora* L.) Beans

Samples of Robusta Coffee Bean Ethanol Extract	Absorbance (nm)	Chlorogenic Acid Content (%)	Average (%)
Replication I	5.9677	11.75	11.58
Replication II	5.6491	11.48	
Replication III	5.6871	11.51	

Discussion

Robusta coffee, as a plant rich in antioxidants, particularly polyphenols and chlorogenic acid, has been shown in various studies to have therapeutic potential in managing metabolic syndromes like diabetes mellitus (Rusman & Irfiyanti, 2022). Chlorogenic acid, specifically, is an antioxidant compound that helps reduce cellular damage caused by free radicals and regulates metabolism by minimizing the excessive release of glucose from the liver into the bloodstream (Ma et al., 2015; Yan et al., 2017). Utilizing microorganisms, including endophytic bacteria and fungi found in plants, presents a viable approach to extracting chemical compounds from plants without causing harm to the plant itself (Radji, 2005).

Research on the antibacterial and antioxidant activity testing of Robusta coffee bean extracts has shown their activity against various pathogenic microorganisms, such as Escherichia coli, Staphylococcus aureus, Candida albicans, Streptococcus aureus, and others (Arfadilla, 2021; Bharath et al., 2015; Suryanti et al., 2023). The isolation of endophytic fungi from Robusta coffee beans has resulted in the discovery

of several endophytic fungal isolates that possess the ability to inhibit the growth of microorganisms. Endophytic fungi can produce bioactive compounds or molecules that are beneficial to their host plants, and these compounds can be utilized in medical treatments (Yan et al., 2019). The population of endophytic fungi is highly influenced by taxonomy, genetic background, age, and tissue of the host plant, as well as environmental factors. These aspects greatly aid in the identification of bioactive compounds produced by specific medicinal plants under certain environmental conditions. Furthermore, other endophytic fungi can stimulate the formation and accumulation of secondary metabolites that are exclusively produced by the host plant. Such compounds commonly exhibit antitumor, antipyretic, antimalarial, analgesic, or anti-inflammatory properties, making them valuable for medicinal purposes. Interestingly, there seems to be a specific correlation between native medicinal materials with the highest quality and efficacy for specific diseases and endophytic fungi. Certain types of endophytic fungi associated with medicinal plants are linked to the production of specific bioactive compounds that are sought after by humans (Bezerra et al., 2021).

Endophytic fungi from Robusta coffee beans have been found to possess three isolates that show potential as antibacterial agents. This is evidenced by their antibacterial activity against Escherichia coli and Staphylococcus aureus, representing gram-positive and gram-negative bacteria, respectively. The three isolates were labeled as RF-1, RF-2, and RF-3, with inhibitory zone diameters of 10.46 mm, 13 mm, and 15.39 mm, respectively, against Escherichia coli, and 11.2 mm, 17.06 mm, and 19.06 mm, against Staphylococcus aureus. Isolates exhibiting antibacterial activity were then subjected to fermentation and 18S rRNA gene sequencing. The sequencing results for the isolate RF-1 revealed it to be *Fusarium sp*. Fusarium is a genus of hyphomycete fungi that can be easily identified by its characteristic phragmosporous conidia, which usually have foot-shaped basal cells (Samuels et al., 2009; Sholihah et al., 2019). Fusarium fungi encompass numerous species with diverse roles in ecosystems. Some are pathogens, while others are saprophytic, aiding in the decomposition of organic matter. Certain species produce enzymes that are used in livestock feed biotechnology, food production, and the pharmaceutical industry. Moreover, several Fusarium species produce secondary metabolites, including alkaloids, polyphenols, and antibiotics, which possess pharmacological effects and are utilized in medical treatments (Akhtar & Swamy, 2018; Ek-Ramos et al., 2013; Podgórska-Kryszczuk et al., 2022).

Conclusion

The fermentation product of endophytic fungi isolates from Robusta coffee beans was subsequently extracted using ethyl acetate solvent. The antioxidant activity test of the ethyl acetate extract from endophytic fungi of Robusta coffee beans exhibited antioxidant activity with an IC_{50} value of 53.497 µg/mL. The antioxidant activity test in this research employed the ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) method to determine the concentration of antioxidants capable of inhibiting free radicals by 50% (IC_{50}) ABTS, a radical with a nitrogen center with a distinct blue-green color. The findings of this study were comparable to the antioxidant activity test using ethanol extract from green Robusta coffee beans. Moreover, the analysis of chlorogenic acid content in the ethyl acetate extract from endophytic fungi of Robusta coffee beans yielded a chlorogenic acid content of 11.58%.

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Author Contributions
The authors' contributions are as follows: Rusman Hasanuddin was responsible for conducting and managing the research, obtaining ethical research clearance, analyzing & interpreting the data, ensuring data accuracy, drafting the initial manuscript, and revising & editing the final manuscript. Nur Alim supervised the research, contributed to the discussion of research findings, and provided critical feedback on the initial and final manuscripts. Agus Sangka supervised the research, improved the writing, contributed to the discussion of research findings, and provided critical feedback on the initial and final manuscript. Nur Riska Rahma and Jasmiadi supervised the research, guided the sample isolation procedures, contributed to the discussion of research findings, and revised & edited the final manuscript.

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Conflicts of Interest
The authors declare no conflict of interest.

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