BIOLOGY, MEDICINE, & NATURAL PRODUCT CHEMISTRY ISSN 2089-6514 (paper)
Volume 15, Number 2, October 2026 | Pages: 2339-2346 | DOI: 10.14421/biomedich.2026.152.2339-2346 ISSN 2540-9328 (online)

Taxonomic Study and Bioprospecting the Little Giant Mushroom

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Manuscript received: 14 April 2026. Revision accepted: 21 November 2026, Published: 25 November 2026.

Abstract

Termitomyces microcarpus is a small termite mushroom with broad distribution and pose various benefits. To date, there was no comprehensive taxonomical reports of *T microcarpus* in Indonesia, especially the molecular data of this species. In addition, there has been no research on the antibacterial activity of *T. microcarpus* in particular country. This study aimed to provide the taxonomical information and reveal the potential of Indonesian *T. microcarpus* as an antibacterial agent. Samples of *T. microcarpus* were collected from the oil palm plantation of IPB University, Dramaga, Bogor. Identification was carried out based on macroscopic and microscopic observation, then confirmed by molecular analysis using ITS1 and ITS4 primers. Fungal metabolite compounds were extracted using absolute methanol by maceration method. Antibacterial activity was tested against enteropathogenic bacteria *Escherichia coli* by agar disc diffusion assay. The inhibition zone diameter of each concentration was analyzed using one-way Annova and DMRT. Based on microscopic and macroscopic observations, the termite mushroom was identified as *T. microcarpus*. Based on molecular analysis, our specimen has the closest relative species to *Termitomyces microcarpus* voucher HKAS123263. The crude extract of this mushroom has antibacterial activity against enteropathogenic *E. coli*, at a concentration of 25 mg/ml has an inhibition zone diameter of 8.18 mm. The current study provides comprehensive description of *T. microcarpus* for Indonesia and displayed its potency as an alternative agent for combatting pathogenic human bacteria.

Keywords: Exploration; Edible; ITS; Termite mushroom; Wild Mushroom.

INTRODUCTION

Sources of macronutrients for humans can be obtained from foods that contain carbohydrates, fats, proteins, and fiber. It is also important to balance these macronutrients with micronutrients found in foods that contain vitamins and minerals. Incorporating edible mushrooms into the diet is an excellent option for promoting health. Edible mushrooms have the potential to serve as both a source of essential nutrition and functional food (Hamza et al. 2024). Their taste and texture characteristics are like meat, which increases their appeal and versatility for processing (Paloi et al. 2023). Edible mushrooms have been popularly consumed in the past and continue to be chosen by many as part of their daily diet (Singh et al. 20025).

Termitomyces is a mushroom that forms symbiotic with termites and distributes in the pleotropic region (Kirk et al. 2001). *Termitomyces* is an edible wild mushroom that belongs to the order Agaricales (Lyophyllaceae) (Mossebo et al. 2009). This genus was first coined by Heim has morphological characteristics with a pseudo-root structure or pseudorhiza and a tapered structure on the pileus or perforatorium (Heim 1942). More than 330 termite species, mainly belonging to the Odontotermes, Macrotermes, and Microtermes genera, are reported to be associated with the growth of *Termitomyces* fungi (Aanen et al. 2002). The symbiosis was established at least 31 million years ago (Nobre and Aanen 2012). Termites provide habitat for fungal growth and help spread spores, while fungi provide food for termites (Wood and Thomas 1989). *Termitomyces* are spread by alates or winged termites (larvae) (Putra 2020). Currently, based on Index Fungorum (indexfungorum.org), the distribution of *Termitomyces* in the world consists of 58 taxa (Paloi et al. 2023).

One of the unique *Termitomyces* species is *T. microcarpus.* This mushroom is unique and different from other *Termitomyces* because of the absence of pseudorhiza, so it is included in the subgenera Praetermitomyces (Hojo 2019). *Termitomyces microcarpus* (Berk. & Broom) R. Heim is small compared to other *Termitomyces* species with a pileus diameter of less than 3 cm. Pileus creamy white to light gray, with dark beige in the center, smooth texture with flat edges, small spiniform perforatorium, umbo, without papilia, cap split from the margin and resembling a petal. Lamella free, white and dense, thick, turning beige when

mature. Stipe: central, slender, hollow, white, fibrous fleshy, smooth, 4.0 to 10.0 cm long and 0.3 cm to 0.8 cm in diameter, uniformly thick, flat, slightly enlarged underground, lacking annulus and velar remnants (Aryal et al. 2014).

T. microcarpus is utilized for consumption due to its delicious meat-like flavor and has various health benefits. In Nepal this mushroom is used as a tonic to relieve fever (Aryal et al. 2014). *T. microcarpus* contains 0.052 mg/g alkaloids (Atri et al. 2012). In addition, *T. microcarpus* also contains carotenoids, steroids, triterpenoids, fatty acids, emodin, flavonoids, coumarins, saponins, and tannins (Aryal and Budhathoki 2013). These bioactive compounds are useful as antibacterial, antifungal, and antioxidant (Gebreyohannes et al. et al. 2019). The methanol extract of this mushroom has an MIC value of 7 mg/ml against Escherichia coli, 6 mg/ml against Proteus vulgaris, and 5 mg/ml against Staphylococcus aureus (Jonathan et al. 2008). However, research on *T. microcarpus* in Indonesia has so far been limited to macroscopic and ethnomycological studies without proper taxonomical description. This mushroom is believed to be edible and has health effects by the people of Jember, East Java through oral history (Dewi et al. 2022). A comprehensive study on the taxonomy and potential of *T. microcarpus* needs to be conducted. Therefore, this study aimed to reveal the potential of *T. microcarpus* collected from Indonesia as an antibacterial agent and to complete the character information of *T. microcarpus.*

MATERIALS AND METHODS

Study area

Samples of *Termitomyces microcarpus* were collected from Campus Forest of IPB University (Figure 1), Dramaga, Bogor, West Java 6°32'58.6"S 106°43'00.5"E using opportunistic sampling method (Mueller et al. 2011). Samples were collected in January 2024. Some samples were preserved in liquid herbarium using 70% alcohol then deposited to Herbarium Bogoriense, Indonesia with collection number BO24652, other samples were dried at 50°C using dehydrator. The fruiting bodies were documented in situ and the main ecological information was noted.

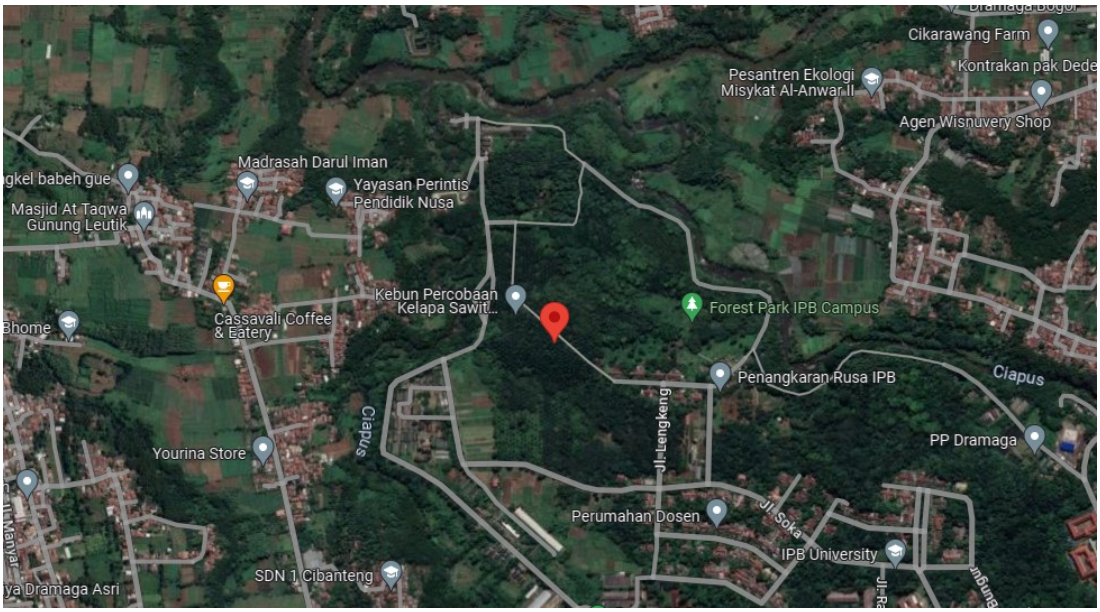

Figure 1. Sampling location of *Termitomyces microcarpus* (red dote).

Morphological Identification

Morphological description was done by macroscopic and microscopic observations. Macroscopic parameters examined following Putra (2021) such as color and size of pileus, lamellae, stipe ornamentation, and pseudorhiza were examined *in situ* and in the Mycology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University. Microscopic observations were made using a Leica light microscope with magnifications of 400X and 1000X. Microscopic data observed were the structure of pileipellis, basidia, cystidia, spores, and hyphae. The description was then used for morphological identification based on related references (Pegler and Vanhaecke 1994).

Molecular Analysis

DNA was extracted from fresh specimens using a Qiagen Dneasy Plant Mini Kit according to the manufacturer's instructions. DNA amplification was done using A Thermo Scientific Arktik Thermal Cycler (ThermoFisher-Scientific). The universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G -3′) and ITS4

(5′-TCC TCC GCT TAT TGA TAT GC-3′) (Wood and Thomas 2007) were used to amplify the ITS1-5.8S-ITS2 region (Luo and Mitchell 2002). The PCR condition was set as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 1 minute. The final extension was set at 72°C for 10 minutes. PCR product was analyzed using 1.5% agarose gel electrophoresis run with TAE buffer (40 mm Trisacetate, pH 8.0 1 mM EDTA), stained with FloroSafe DNA stain and visualized by the Gel Doc EZ Gel Documentation System (Biorad). Amplicons that have been verified based on electrophoresis visualization are then sequenced to determine the nucleotide sequence through a third-party service (https://ptgenetica.com). FASTA files were then edited using Seqtrace software version 0.9.0 to obtain contig sequences. The contig sequences obtained were then analyzed on the website (https://blast.ncbi.nlm.nih.gov). Contig and reference sequences from NCBI were aligned using the ClustalW Multiple Sequence Alignment Program. The analysis and phylogenetic tree were then constructed using the UPGMA method with 1000× bootstrap statistical analysis using MEGA version 11 software (Tamura et al. 2021). The out group used was *Pleurotus ostreatus* strain 2106 (Accession number: HQ286595) (Table 1). The sequence data held will then be submitted to NCBI GenBank following the NCBI sequence deposit procedure.

Table 1. Species, outgroup, collection code, and GenBank accession numbers used in this study

Species	Collection code	ITS accession number
Termitomyces globulus	voucher BUMR03	MK743965
Termitomyces eurrhizus	voucher TMC32	OQ571368
Termitomyces heimii	voucher TMC75	OQ571386
Termitomyces titanicus	voucher BR5020172891379	OQ275443
Termitomyces aurantiacus	voucher BR5020149963016	OQ275437
Termitomyces microcarpus	voucher HKAS123263	OQ275424
Termitomyces microcarpus	**Voucher BO24652**	**PP851013**
Termitomyces striatus	voucher TERM012	MN160266
Termitomyces medius	voucherBUMR07	MK743976
Termitomyces clypeatus	voucher TERM062	MN160316
Termitomyces bulborhizus	voucher HMAS84445	OQ275468
Pleurotus ostreatus	strain 2106	HQ286595

Fruiting bodies Extraction

A total of 15 grams of dry sample was pulverized, then added absolute methanol with the ratio of sample and solvent 1:10. The mixture was shaken for 24 hours at 100 rpm at 30°C. The mixture was filtered using Whatman paper. The filtrate was collected, then the pulp was re-macerated with methanol in the same ratio. Maceration was carried out 3 times. The collected filtrate was evaporated using a rotary evaporator at 50°C until the extract was obtained in paste form. The extract obtained was stored in the refrigerator before being used for the next stage (Sai Latha 2020).

Antibacterial activity test

The antibacterial test was carried out by disc diffusion assay method using the target bacteria enteropathogenic *Escherichia coli* collection belonging to Mr. Wahyu Aji Mahardika, IPB University. A 1% v/v suspension of bacteria concentration 10^8 was put on warm MHA media, then poured into a petri dish. A total of 20 µl of fungal extract that has been diluted using 50% DMSO (extract concentrations of 200, 100, 50, 25, 12.5 mg/ml), DMSO, and positive control were dripped on a 6 mm diameter disc paper, respectively. The test was carried out for 3 replicates. The dishes were incubated at 37°C for 24 hours. The zone of inhibition formed was measured using a ruler (Priyanto et al. 2022).

Antibacterial activity was analyzed through the results of inhibition zone measurements with the following equation:

$$\frac{(\boldsymbol{DH}-\boldsymbol{DC})+(\boldsymbol{DV}-\boldsymbol{DC})}{\mathbf{2}}$$

Notes:
DH: Horizontal diameter
DC: Disc diameter (6mm)
DV: Vertical diameter

Data analysis

Antibacterial data was analyzed using one-way ANOVA with 95% confidence interval using Statistical Product and Service Solutions (SPSS) software version 27.0. Data showing significance $\alpha = 0.05$ were subjected to post-hoc test with Duncan Multiple Range Test (DMRT) 5%.

RESULTS AND DISCUSSION

Taxonomy

Termitomyces microcarpus (Berk. & Broome) R. Heim, Arch. Mus. Natl. Hist. Nat. Sér. 6, 18: 147 (1941).

Basionym:
Agaricus microcarpus Berk. & Broome, Bot. J. Linn. Soc. 11: 537 (1871)
Synonym:
Podabrella microcarpa (Berk. & Broome) Singer, Lloydia 8: 143 (1945)
Gymnopus microcarpus (Berk. & Broome) Overeem, Heyne, De Nuttige Planten van Nederlandsch Indië, ed. 2: 76 (1927)
Entoloma microcarpum (Berk. & Broome) Sacc., Syll. Fung. 5: 687 (1887)
Collybia microcarpa (Berk. & Broome) Höhn.: 993 (1908)
Mycena microcarpa (Berk. & Broome) Pat.: 210 (1913)

Description. Pileus cream to white, whiter at center, smooth texture, margin glabrous and sometimes tear towards the center, small spiniform perforatorium, 1,5 – 2 cm in diameter. Lamellae free, crowded, white, creamy at maturity, 1-2 mm wide. Stipe central, white, fleshy-fibrous, 3-4 cm long, absence of annulus. Pseudorhiza absent. Basidiospores 3,5 x 2 µm, ellipsoid with a pointed tip. Basidia 16-17 µm long, tetra sterigma. Pleurocystidia 7,5 µm long. Cheilocystidia 30 µm long. **Habitat.** Grow grouped on soil in oil palm. **Specimen Examined**. Oil palm plantation forest, IPB University, Dramaga, West Jawa, Indonesia, 6°32'58.6"S 106°43'00.5"E, on soil, Januari 2024, collected by Jati OM.

Termitomyces is reported to be an endemic mushroom in Asia and Africa (Kirk et al. 2001). Currently, there have been some reports on the presence of *Termitomyces microcarpus* in Indonesia. This mushroom was found in Jelbuk, Silo, Patrang, Tempurejo, and Sumberbaru, Jember (Dewi et al. 2022) and in Gunungpati District, Semarang City, Central Java Province (Augustinus and Putra 2021). However, those reports lack taxonomical information. The discovery of *T. microcarpus* in the IPB University campus environment is the first comprehensive data regarding this species in Indonesia. The mushroom found in this sampling location is the same size as that found Gunungpati District (Augustinus and Putra 2021), which has a pileus measuring 1.5-2 cm and a stipe length of 3-4 cm. In addition, this mushroom is unlike other *Termitomyces,* which does not have a pseudorhiza. The development of the fungal fruiting body is influenced by the light intensity at the place of growth, which can affect the presence of mucus on the stipe and optimize development (Tibuhwa et al. 2010). Basidiospores of our specimen was slightly smaller than same species found in Thailand (Jannual et al. 2020). The Basidia in this study have the same dimension as those found in the mushroom, with sizes between 15-30 µm. Cheilocystidia of this mushroom has the same length as that found in Northwest Ethiopia (Sitotaw et al 2015), with a size of 30 µm. However, the fungal pleurocystidia in this study are smaller than those found in the journal, with a size of 15-40 µm. Cystidia is a sterile structure found alongside basidia, which indicates the mushroom is heteromorphous (Atri et al. 2017).



Figure 2. Fresh fruiting body of *Termitomyces microcarpus*. (A) Basidiocarp growing on loose soil. (B) cap. (C) stipe. (D) lamellae.

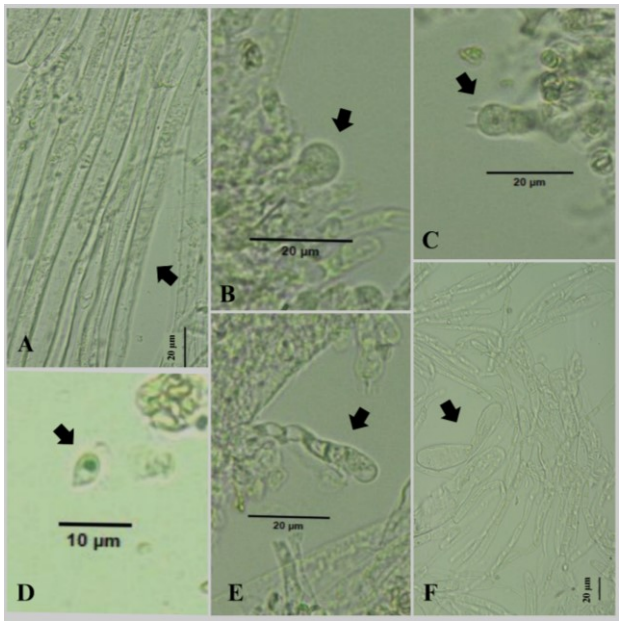

Figure 3. Microscopic features of *Termitomyces microcarpus*. (A) Hifa structure. (B) Pleurocystidia. (C) Basidia. (D) Spore. (E) Cheilocystidia. (F) Pileipelis.

Molecular Analysis

Molecular analysis confirmed that the mushroom found in the oil palm plantation, IPB University was *T.*

microcarpus, as shown from the phylogeny tree that the mushroom was in the same clade as *T. microcarpus* voucher HKAS123263 specimen from Thailand with a BS value of 100% (Figure 4). The ITS sequence of this mushroom has been submitted to the GenBank database and received the accession number PP851013 (Table 1). Molecular analysis needs to be used to determine the accurate taxonomy of wild fungi. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) region is often used as a marker for resolution at the genus or even intra-species level to identify geographic races and is recommended as a marker by the International Fungal Barcoding Consortium (Schoch et al. 2012). ITS1 and ITS4 primers were used because they can selectively amplify fungal sequences. The primer pair amplifies the 5.8S rDNA intervening region and the adjacent ITS2 and ITS3 regions (White et al. 1990).

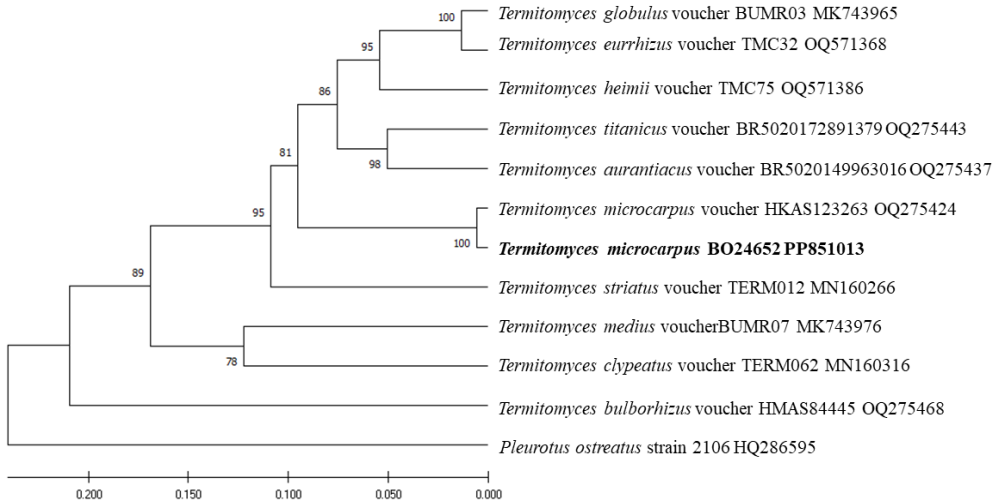

Figure 4. *Termitomyces microcarpus* BO24652 phylogenetic tree based on ITS1-4 region.

Antibacterial Activity

The methanol extract of *T. microcarpus* was able to inhibit the growth of enteropathogenic *E. coli*, characterized by a clear zone around the disc (Figure 5). The concentration that produces the highest inhibition zone diameter is 25 mg/ ml with a value of 8.18 mm and the lowest inhibition was in 200 mg/ml treatment (Table 2). In addition, the inhibition zone produced from mushroom extract was not significantly different compared to each other and was lower than the commercial antibiotic.

Table 2. Antibacterial activities from *Termitomyces microcarpus* crude extract to enteropathogenic *Escherichia coli.*

No.	Treatment	Inhibition zone diameter ± stdev (mm)*
1	200 mg/ml	7.4±0.05^{b}
2	100 mg/ml	7.66±0.18^{b}
3	50 mg/ml	7.73±0.23^{b}
4	25 mg/ml	8.18±0.03^{b}
5	12.5 mg/ml	7.45±0.25^{b}
6	Ciprofloxasin 100 µg/ml (positive control)	21.33±0.58^{a}
7	DMSO (negative control)	0.00±0.00^{c}

*The number followed by a different superscript letter in the same column indicates a significant difference in level ($p < 0.05$) based on Duncan's multiple range tests (DMRT) 5%.

Several previous studies have reported the antibacterial activity of *T. microcarpus* extract against other bacteria. The methanol extract of the mushroom has antibacterial activity against *Bacillus cereus* and *Proteus vulgaris*, but it was not mentioned how large the inhibition diameter was (Giri et al. 2020). On the other hand, Jonathan *et al.* (2008) reported the methanol extract of this mushroom produced an inhibition zone diameter of 13 mm against *E. coli,* 17 mm against *Proteus vulgaris*, and 16 mm against *Staphylococcus aureus*. In addition, the methanol extract of this mushroom at a concentration of 100 µg/ml had an inhibition zone diameter of 22.3 mm against *S. aureus* ATCC 25923, and 15.5 mm against *B. subtilis* ATCC

6633 (Nakalembe and Kabasa 2012). Although the concentration of the mushroom extract used in this study was higher than other reported mushroom extracts, this study revealed the ability of *T. microcarpus* extract to inhibit the growth of enteropathogenic *E. coli,* which has been reported to be the cause of thousands of deaths from diarrhea (WHO 2024).

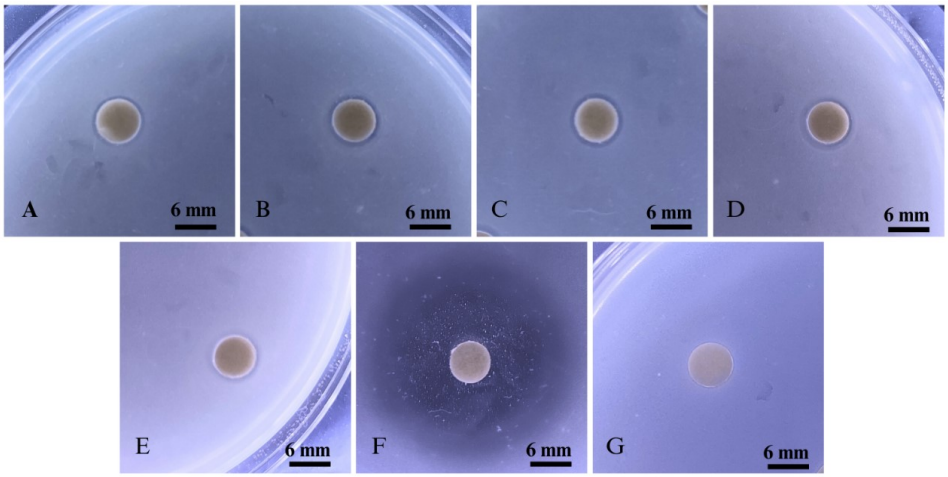

Figure 5. Antibacterial activity of *T. microcarpus* crude extract against enteropathogenic *Escherichia coli*. (A) Concentrations of 200 mg/ml. (B) 100 mg/ml. (C) 50 mg/ml. (D) 25 mg/ml. (E) 12.5 mg/ml. (F) 100 µg/ml ciprofloxacin. (G) DMSO.

The inhibitory activity against pathogenic bacteria is due to metabolites produced by *T. microcarpus*. The solvent used to extract fungal metabolites was methanol. Methanol is a polar solvent that can attract compounds with a high polarity index, such as flavonoids, phenols, and alkaloids (Pandey et al. 2021). Flavonoids can inhibit bacterial growth through various ways, including inhibiting nucleic acid synthesis, energy metabolism, and increasing cell membrane permeability (Biharee et al. 2002; Xie et al. 2014; Gorniak et al. 2019; Donadio et al. 2021). On the other hand, phenols can damage the structure of bacterial cell membranes and cause DNA damage (Martins et al. 2015). Likewise, alkaloids can damage cell membranes, disrupt DNA function, and inhibit bacterial protein synthesis (Kelley et al. 2014; Li et al. 2014; Larghi et al. 2015). Nonetheless, purification of crude extracts and identification of compounds from these fungi need to be done to determine the type of compounds and mechanisms responsible for antibacterial activity.

CONCLUSION

The current study provides the first herbarium, molecular data, and taxonomical description of *Termitomyces microcarpus* for Indonesia. The raw extract from this mushroom has demonstrated antibacterial effects on enteropathogenic *E. coli*, showing an inhibition zone of 8.18 mm at a concentration of 25 mg/ml. This research offers a detailed overview of *T. microcarpus* in Indonesia and highlights its potential as a substitute treatment for harmful human bacteria. Further research needs a warrant to purify the mushroom extract for future mycopharmacological purposes.

Acknowledgements: We are grateful to the Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, and Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Indonesia for the research facilities.

Authors' Contributions: IPP designed the study. OMJ and RFP performed the data collection. IPP performed morphological and molecular identification. All authors wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing Interests: The authors declare that there are no competing interests.

Funding: This research received no funding.

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