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Potency of Endophytic fungi Isolated from *Muntingia calabura* as Antifungal Substances against *Candida parapsilosis*

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Abstract

Alternative is needed to overcome the problem of dandruff caused by C. parapsilosis. Endophytic fungi isolat-ed from plants are capable of synthesizing compounds to produce various secondary metabolites. This study aimed to obtain the most active endophytic fungal isolates, determine the class of compounds, and determine the antifungal activity of the endophytic fungi (M. calabura) against C. parapsilosis. Fifteen isolates of endo-phytic fungi isolated from M. calabura were coded FDK1-FDK15. There was a color change in the media after 30 days of cultivation isolates of fungi. FDK4 and FDK13 have potential as antifungals. FDK13 fungal extract had the highest inhibition zone diameter of 20.00 mm. 20 eluates were obtained from the isolation of com-pounds (Thin Layer Chromatograhy), FDK 131, 1314, 1317, and 1320 eluates are compounds of the terpenoid group and FDK1316 eluates are phenol group compounds that are active in inhibiting yeast growth. FDK1317 was the most active compound isolate in inhibiting the growth of C. parapsilosis with an inhibition zone diameter of 12.5 mm.

Keywords : M. calabura; C. parapsilosis; Isolate; Antifungal

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1. Introduction

Candida parapsilosis is a yeast that is a species of the Genus *Candida* that often infects humans and causes several diseases [1]. It generally infects the skin and is one of the species that causes dandruff on the human head. *C. parapsilosis* from human dandruff capable of spreading through human hands [2]. *C. parapsilosis* that infects the scalp can cause dandruff whose severity ranges from a mild scale similar to dry skin to Seborrhoeic Dermatitis (SD). The major difference between dandruff and SD is that SD is an inflammation of the scalp that arises from dandruff formation [3]. The growth and pathogenesis of *C. parapsilosis* begins with colonization and infection, which depend on its ability to adhere to host cells and tissues, particularly mucosal surfaces [4].

Endophytic fungi are microorganisms that colonize intercellular or intracellular host plants without causing any disease, play an important physiological and ecological role [5], [6]. Endophytic fungi isolated from plants are capable of synthesizing compounds to produce various secondary metabolites. Its antifungal activity causes endophytic fungi to attract attention and are considered as promising biological control agents [7].

M. calabura is known worldwide as the Jamaican cherry and has a different name for each country. *M. calabura* is known to be a wild plant in Indonesia. The fruit is generally eaten directly due to its sweet taste, but as a traditional medicine, it is not well known in Indonesia [8]. Research has been carried out to test the phytochemical content present in the leaves. The extract contains secondary metabolites, including flavonoids, tannins, and saponins [9]. Buhian et al. [10] stated that *M. calabura* has very promising potential as an antifungal agent.

The research on endophytic microorganisms in *M. calabura* so far has been carried out by Leiwakabessy and Latupeirissa [11] only to explore and test the antibiosis of endophytic bacteria against the fungal pathogen *Rhizoctonia solani*, The test results stated that there was an endophytic bacterial isolate on *M. calabura* (BK6 Isolate) which had high inhibition against *R. solani*. There has been no research on the isolation and testing of antifungal activity of

endophytic fungi isolated from *M. calabura* leaves, especially as antifungal against *C. parapsilosis*.

This research was conducted to obtain the most active endophytic fungal isolates, determine the class of compounds, and determine the antifungal activity of the endophytic fungi (*M. calabura*) against *C. parapsilosis.*

2. Materials and Methods

2.1 Isolation and Purification of Endophytic Fungi and *C. parapsilosis*

All the isolation and purification of endophytic fungi were carried out aseptically [7]. The leaf was sterilized with 70% alcohol for 30 seconds, then immersed in 5% sodium hypochlorite solution for 1 minute, rinsed with distilled water and dried. A sterile knife was used for cutting the dried samples, each sample was cut to a size of 20 x 10 mm. Incubation at room temperature (± 25^{o}C). Different colonies were separated and transferred to a new Potato Dextrose Agar (PDA) dish until the pure cultures were obtained.

2.2 Cultivation and Extraction of Endophytic Fungi

Potato Dextrose Broth (PDB) as a medium for fungi and controls were prepared in each 1 liter bottle of 300 ml. Pure fungal strains were cut into 10 pieces using the agar plug method using a 0.5 cm cork borer and inoculated into bottles. Incubation at room temperature for 30 days until a color change occurs on the media. Fungi and secondary metabolites are then segregated through filtration. Extraction was carried out twice with the addition of ethyl acetate to PDB with a ratio of 1:1, the second metabolites was evaporated using a rotary evaporator at a temperature of 40^{o}C. The thick extract obtained was then used in the activity test [12].

2.3. Antifungal Activity Test

The inhibitory activity test of *C. parapsilosis* was carried out using the paper disk method [6], [13]. *C. parapsilosis* was obtained from the isolation of dandruff. A petri dish containing sterile Saboraud Dextrose Agar (SDA) was prepared. The suspension was made using *C. parapsilosis* Culture as much as 1 ose into 3 ml of 0.9% NaCl and homogenized with a vortex until the turbidity is equivalent to McFarland 0.5 with the number of microorganism colonies 10^6 CFU/ml. *C. parapsilosis* was inoculated from the suspension using the spread plate method. Each petri dish is placed a maximum of 5 paper disks, then 20 µl of endophytic fungi extract is dripped onto 4 paper disks with an extract concentration of 10% using DMSO (Dimethyl Sulfoxide) solvent, 1 DMSO paper disk as a control (-) and 1 paper disc contained ketoconazole (+). All cultures were incubated in an incubator at 37^{o}C for 7 days and observed the diameter of the inhibition zone (clear area that was not overgrown with yeast) on each agar in a dish. Measurements were made on the diameter of the inhibition area formed.

2.4 The Isolation of Secondary Metabolites

The secondary metabolites in the most active extracts were isolated by column chromatography. Silica gel powder was inserted into the column reaching three quarters of the column height, then endophytic fungi extract was added and eluted with a gradient solvent system, n-Hexane-ethyl acetate in a ratio of 9:1; 8:2 ; 7:3 ; 6:4 ; 5:5 ; 4:5 ; 3:7 ; 2:8 ; 1:9 as much as 50 mL and poured into the column gradually, with the column tap opened. The compound was then accommodated in a 20 mL vial and evaporated using a hair dryer and a concentrated eluate was obtained. All vials were retested for antifungal activity [14], [15].

2.5 Thin Layer Chromatography (TLC)

The most active eluate was diluted 1% using ethyl acetate as solvent. The plate is eluted with the eluent (a mixture of organic solvents). The chromatogram is made of dots where the eluate is spotted. The eluate is spotted on the chromatogram and expanded with the mobile phase. The chromatogram was then sprayed with 10% H_2SO_4 and heated on a hot plate, color spots appeared and then observed in UV light (254nm). The value of the retention factor (Rf) is determined using the formula:

$$\mathbf{Rf} = \frac{\textbf{Distance travelled by component}}{\textbf{Distance travelled by solvent}}$$

2.6 Bioautography Test

The chromatogram of the TLC test results was then placed in a petri dish containing the *C. parapsilosis*. The chromatogram were stick to the agar medium for 1 hour so that the active compounds diffused into the agar medium, then carefully removed and incubated for 48 hours. The clear zone formed is the area where the active compound is [16]

2.7 Identification of Endophytic Fungi

Determination of endophytic fungi species was carried out on fungi that produced the most active compounds as antifungals by identification based on macroscopic and microscopic morphological characteristics. Macroscopic views of the colony structure, colony color, zoning, radial and concentric lines. Microscopy using a microscope with the observed characteristics are hyphal structure (insulated or not,

branched or not), hyphae color (transparent or dark), presence or absence of conidia, and conidia shape (round, oval, chained, or irregular) [12].

3. Results and Discussion

The results of isolation and purification of endophytic fungi have been obtained from the *M. calabura* with 15 isolates from the leaves. There was a difference in the fungal colonies that grew for 7 days after inoculation of *M. calabura* leaves on Potato Dextrose Agar (PDA) media. The appearance of colonies originating from leaves was based on the sequence of fungal numbers which were then coded FDK 1-15. Hyphae that appeared on FDK 1-4 appeared on day 1-2, FDK 5-10 appeared on day 3-5, and FDK 11-15 appeared on day 6-7. 15 Isolates of the endophytic fungi *M. calabura* are shown in Figure 1.

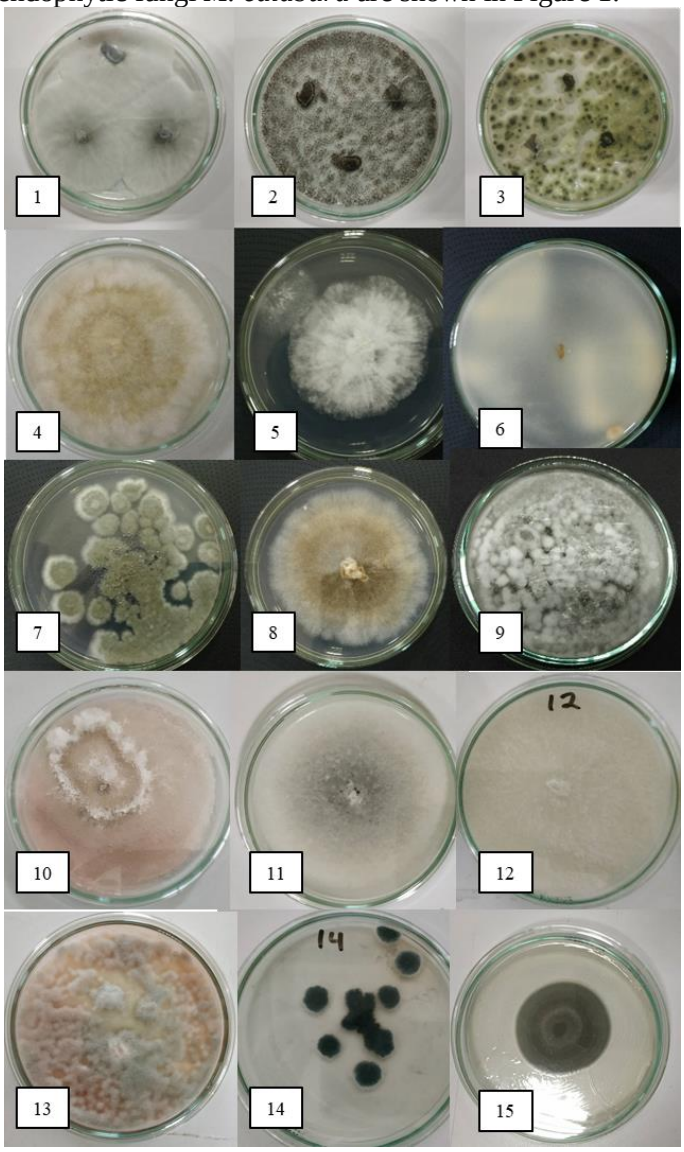

Figure 1. 15 Isolates of the endophytic fungi *M. calabura*, 1-15 shows the order based on fungal isolates (FDK 1-FDK 15).

The endophytic fungi obtained underwent a growth process which was marked by the wider diameter of the colony since it was inoculated on a plate filled with media. Fungal growth occurs when there is an increase in biomass volume and overall cell number. This is inseparable from the nutritional factors that come from PDA media. PDA is a medium made up of natural ingredients such as potatoes and synthetic materials, such as agar and dextrose. This medium contains carbohydrates, vitamins, micronutrients, and dextrose as simple carbohydrates and energy sources that can be immediately used and utilized by fungi [17], [18].

The initial PDB volume of 300 ml has been cultivated by endophytic fungi, after 30 days There was a color change in the media after 30 days of cultivation which indicated that the medium was rich in secondary metabolites formed by endophytic fungi. The results of cultivation of endophytic fungi isolates are presented in Figure 2.

Figure 2. PDB media as control (A), PDB media after 30 days of cultivation (B).

Based on Figure 2 it is known that PDB media changed color for 30 days after being inoculated by the endophytic fungus *M. calabura*. Transparent pale yellow will change to different colors such as deep yellow, brownish, black, and orange. depending on the species of the fungus.

PDB media made of dextrose and potato infusion are often used in the growth of fungi. Fungi can break down starch in potatoes into soluble sugars which then become a source of carbon and energy for growth. Fungi in PDB medium for 30 days will grow and produce secondary metabolites. The color change of PDB is caused by fungi that produce secondary metabolites from metabolic

processes. Secondary metabolites are produced by fungi at the end of the stationary phase and secondary metabolite synthesis occurs when nutrients in the fungal growth medium have been limited [19].

The inhibition zone was indicated by the absence of *C. parapsilosis* growth around the paper disc, the formation of a clear zone or inhibition zone. The inhibition zone around the paper disc formed indicated the presence of antifungal compounds in the extract. The diameter of the inhibition zone of the endophytic fungi extract *M. calabura* is summarized in table 1 and figure 3.

Table 1. Activity Test Results of the endophytic fungus *M. calabura*

Isolate	Inhibition zone diameter (mm)
FDK1	0
FDK2	0
FDK3	0
FDK4	10.5
FDK5	0
FDK6	0
FDK7	0
FDK8	0
FDK9	0
FDK10	0
FDK11	0
FDK12	0
FDK13	20.00
FDK14	0
FDK15	0
Ketoconazole(+)	32.00
DMSO(-)	0

Based on Table 1 and figure 3, it can be seen that the FDK13 isolate was the extract with the higher effect compared to FDK4 in inhibiting the growth of *C. parapsilosis* with a value of 20 mm. This indicates that there is an endophytic fungus *M. calabura* which is capable of producing antifungal compounds, as evidenced by the inhibition zone produced. Extract from endophytic fungal isolate code FDK13 has high antifungal activity.

Endophytic fungi extract inhibited the growth of *C. parapsilosis* because endophytic fungi were able to synthesize compounds producing various metabolites produced and isolated from higher plants, while *M. calabura* L contained antifungal substances derived from secondary metabolites, namely flavonoids, tannins, terpenoids, lignin, alkaloids, glycosides, etc [10], [20], [21].

Compound isolation was carried out on FDK13 which was the most active extract and produced 20 eluates. The eluate activity test of the FDK13 fungi isolate was carried out with the data obtained in the form of an inhibition zone formed on a paper disc. The results of the antifungal activity of the eluate isolate FDK13 are presented in Table 2 and figure 4.

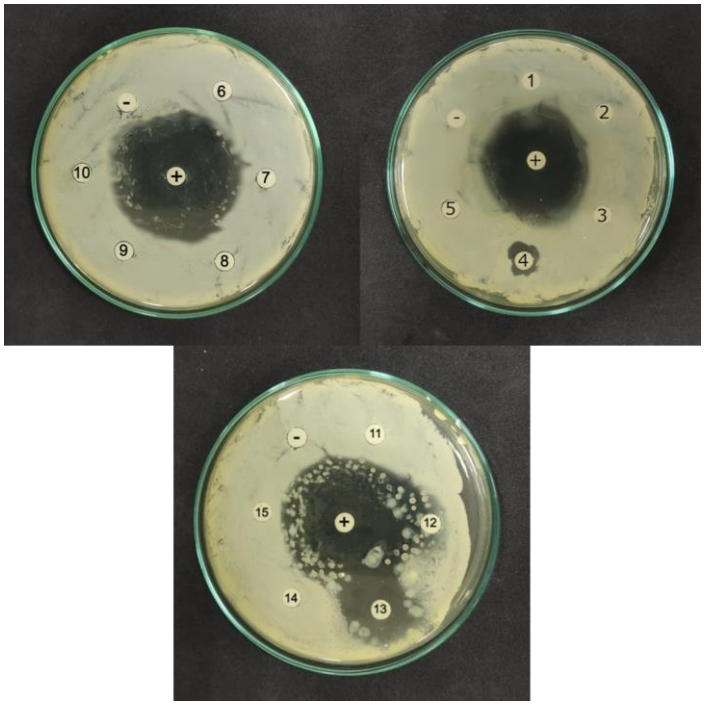

Figure 3. The antifungal test results of endophytic fungi isolates against *C. parapsilosis*, (numbers 1-15) isolates extract code FDK 1-15, (-) negative control of dimethyl sulfoxide (DMSO), (+) positive control of 10% Ketoconazole

Table 2. FDK13 extract eluate activity test results

Eluates	Inhibition Zone Form (mm)
$FDK13_1$	**10.50**
$FDK13_2$	0
$FDK13_3$	0
$FDK13_4$	0
$FDK13_5$	0
$FDK13_6$	0
$FDK13_7$	0
$FDK13_8$	0
$FDK13_9$	0
$FDK13_{10}$	0
$FDK13_{11}$	0
$FDK13_{12}$	0
$FDK13_{13}$	0
$FDK13_{14}$	**8.5**
$FDK13_{15}$	0
$FDK13_{16}$	**10.33**
$FDK13_{17}$	**12.5**
$FDK13_{18}$	0
$FDK13_{19}$	0
$FDK13_{20}$	**11.00**
DMSO (-)	**0**
Ketoconazole(+)	**33.00**

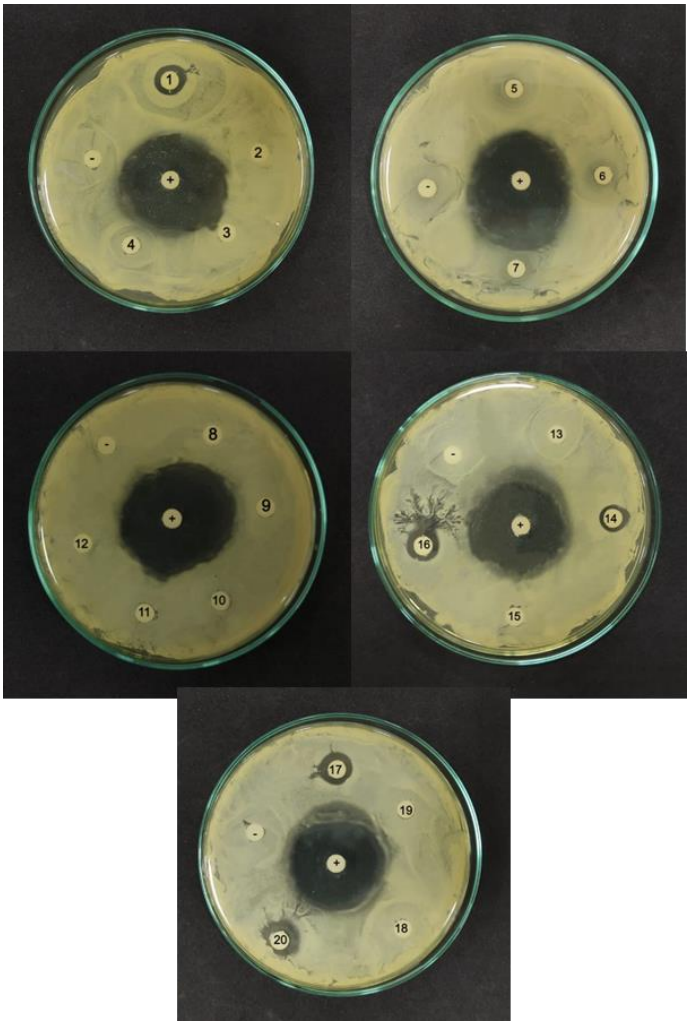

Figure 4. Eluate Antifungal Activity Test Results with Control

Based on table 2 and figure 4, it can be seen that there are several eluates that are active in inhibiting the growth of *C. parapsilosis*. The eluates of FDK 13_1, 13_{14}, 13_{16}, 13_{17}, and 13_{20} contained antifungal compounds as evidenced by the presence of an inhibitory zone formed. The most active eluate in inhibiting the growth of *C. parapsilosis* was FDK13_{17} eluate with an inhibition zone diameter of 12.5 mm.

All eluates that have been tested for activity have a smaller diameter of inhibition than the FDK13 extract because the compounds are separated by column chromatography. Based on this, there was a reduction in the concentration of the extract containing many antifungal compounds to an eluate containing only isolates of antifungal compounds.

Identification of secondary metabolite isolates and bioautographic tests contained in the active eluate seen from the resulting spots and stains produced from FDK 13_1, 13_{14}, 13_{16}, 13_{17}, and 13_{20}. Identification of compounds and calculated Rf values are presented in Table 3 and figure 5.

Table 3. Identification of compound groups and Rf values

Eluate	Color	Rf	Compounnd
FDK13_1	Purple	0.57	Terpenoids
FDK13_{14}	Purple	0.70	Terpenoids
FDK13_{16}	Yellow	0.46	Phenol
FDK13_{17}	Purple	0.80	Terpenoids
FDK13_{20}	Purple	0.75	Terpenoids

Figure 5. The result of identification eluates A)FDK13_1 (purple) B) FDK13_{14} (purple) C) FDK13_{16} (yellow) D) FDK13_{17} (purple) E) FDK13_{20} (purple)

The color spots produced by the four eluates (FDK13_1, FDK13_{14}, FDK13_{17} and FDK13_{20}) are purple. Purple spots were seen after the plate was sprayed with 10% H_2SO_4 reagent and heated on a hot plate. The appearance of blue, purplish blue or purple on the TLC plate with H_2SO_4 reagent indicates the presence of terpenoid compounds. The appearance of yellow color (FDK 13_{16}) with H_2SO_4 reagent indicates the presence of phenolic compounds in the eluate.

The single spot on the TLC plate indicates that the isolated compound being tested is a single compound or also called a pure compound. this is evidenced by the five eluates, each of which shows one spot. The secondary metabolites contained in the extract have been separated into each eluate. The ratio of the amount of solvent (n-hexane; ethyl acetate) makes the compounds contained in the extract move down and separate between one compound and another, according to the polarity of the compound to each eluent. Isolation of compounds by column chromatography

method aims to separate compounds based on their level of polarity, namely through the stationary phase and the mobile phase [22].

The results of the bioautography test have been carried out by looking at the similarity of the position of the spots on the plate and the clear zone formed on the agar. It showed that the clear zone formed on the plate had the same position as the spots on the TLC plate. In addition, the clear zone only saw 1 spot in each cup. This indicates that the spots on each plate have pure antifungal compounds. The results of the bioautography of the whole eluate are presented in Figure 6.

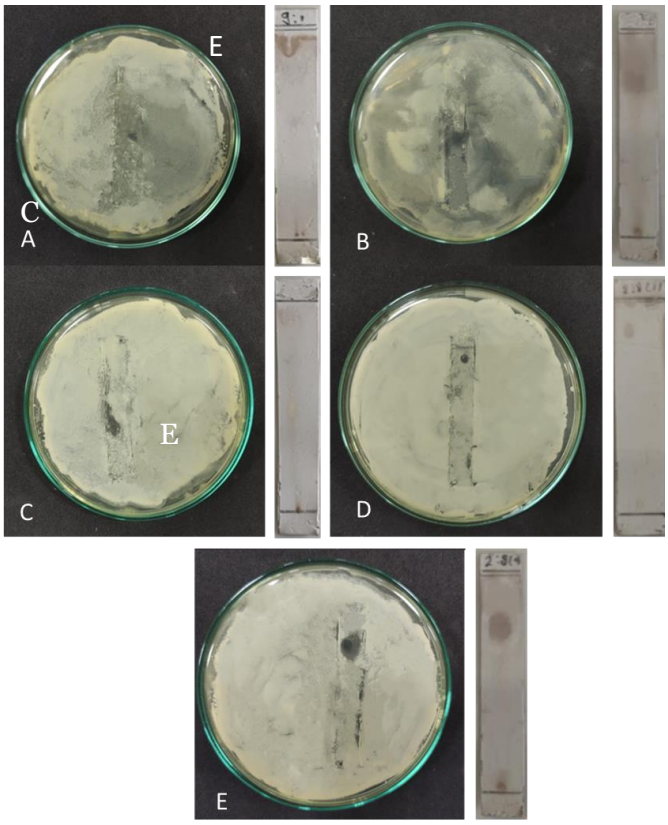

Figure 6. The results of the eluate bioautography test A) $FDK13_1$ B) $FDK13_{14}$ C) $FDK13_{16}$ D) $FDK13_{17}$ E) $FDK13_{20}$

The appearance of the clear zone cannot be separated from the ability of each active compound to inhibit microbial growth. The active compounds consist of two types of compounds, namely terpenoids and phenols which have been shown to be active in inhibiting the growth of *C. parapsilosis*. Terpenoids and phenols have different mechanisms in interacting with yeast cells, terpenoids cause cells to die due to lack of nutrients while phenols cause cells to die due to denaturation of their cell walls [16].

Fungal isolate FDK_{13} was the most active fungus inhibiting the growth of *C. parapsilosis*, then identified to determine the species of the fungus. Fungi were identified based on morphological characteristics (macroscopic and microscopic) and the species was identified as *Trichophyton mentagrophytes*. The morphological structure of *Trichophyton mentagrophytes* is shown in Figure 7.

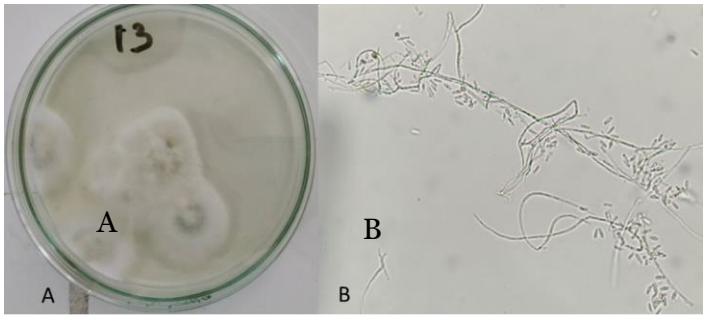

Figure 7. The morphological structure of *Trichophyton mentagrophytes*, (A) Macroscopic, (B) Microscopic

The macroscopic characteristics of the colony started from the initial growth which showed a shape like fuzz, flat and white-yellowish color, then turned into fluffy-powdery and brownish-cream color, the growth showed the nature of concentric rings. Microscopic characteristics showed that the hyphae were septate and branched, some of the tips of the hyphae were spiral-shaped. Macroconidia are multiseptate cigar-like shapes, consist of 3 to 4 cells, have thin and smooth walls, attached to hyphae with short stalks. Microconidia are cylindrical, and spherical in shape seen in clusters arranged on both sides along the hyphae. In addition, chlamydospores can also be found.

Determination of the genus *Trichophyton* has a characteristic based on macroconidia that distinguishes it from another Genus in the same family. Trichophyton are smooth-walled, mostly cylindrical in shape, 3-4 septa, and borne singly [23]. Species that distinguishes it from other *Trichophyton* species is based on key characteristics that are reinforced by the results of Campbell, et al [24] research. The species contains abundant cylindrical microconidia and spiral-shaped hyphae.

Trichophyton mentagrophytes is a pathogenic fungus but is capable of producing antifungal metabolites. This is because this fungus was isolated from muntingia calabura which has antifungal compounds as well. Endophytic fungi isolated from plants are able to synthesize compounds to produce various secondary metabolites. Endophytic fungi have many sources of new chemical compounds that have potential as antifungals, their active metabolites as excellent antifungals not only against pathogenic fungi in humans but also in plant pathogenic fungi [20].

4. Conclusion

There were 15 isolates of endophytic fungi obtained from *M. calabura*. The fungal extract which actively inhibited the growth of yeast was FDK13 which was identified as *Trichophyton mentagrophytes* with a 20 mm

inhibition area. 20 eluates were obtained from the isolation of compounds, FDK 13_1, 13_{14}, 13_{17}, and 13_{20} eluates are compounds of the terpenoid group and $FDK13_{16}$ eluates are phenol group compounds that are active in inhibiting yeast growth. $FDK13_{17}$ was the most active compound isolate in inhibiting the growth of *C. parapsilosis* with an inhibition zone diameter of 12.5 mm.

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