

IN VITRO ANTIFUNGAL ACTIVITY OF PAPAYA LEAF EXTRACT (*Carica papaya* L.) AGAINST *Trichophyton rubrum*

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Abstract: *In Vitro* Antifungal Activity Of Papaya Leaf Extract (*Carica papaya* L.) AGAINST *Trichophyton rubrum*. *Trichophyton rubrum* is the species that causes tinea pedis and dermatophytic infections of the nails. Meanwhile, long-term use of antifungal drugs for the therapy can cause side effects. Therefore, it is necessary to find drugs from natural products such as papaya leaves (*Carica papaya* L.), which are known to have pharmacological activity due to the presence of active compounds such as alkaloids, triterpenoids, steroids, flavonoids, saponins, and tannins. This study aims to prove the *in vitro* effectiveness of the ethanol extract of papaya leaves (*C. papaya* L.) in inhibiting the growth and killing of *T. rubrum*. The antifungal activity test was carried out using the two-fold microdilution method and resazurin staining, with the papaya leaves extract concentration range of 4000 – 31.25 µg/mL. To test the bactericidal effect of papaya leaf extract, assessment of the number colonies of *T. rubrum* in potato dextrose agar was carried out. The relationship between the concentrations of papaya leaf ethanol extract with the number of *T. rubrum* colonies was analyzed using linear regression test. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of papaya leaf ethanol extract against *T. rubrum* was 2000 µg/mL. From the results of linear regression analysis, obtained the equation $y = -0.001x + 4.221$, R^2 value of 0.569, and the correlation coefficient is 0.755. Papaya leaf ethanol extract was able to inhibit and kill *T. rubrum*. Furthermore, there is a strong relationship between the increases in the concentration of papaya leaf extract with its bactericidal activity against *T. rubrum*.

Keywords: Antifungal agents, Plant extract, Resazurin Microplate Assay, Tinea pedis

Abstrak: Aktivitas Antifungal *In Vitro* Ekstrak Daun Pepaya (*Carica papaya* L.) Melawan *Trichophyton rubrum*. *Trichophyton rubrum* merupakan spesies penyebab tinea pedis dan infeksi dermatofitosis pada kuku. Sementara itu, penggunaan obat antijamur untuk terapi dalam jangka panjang dapat menyebabkan efek samping. Oleh sebab itu, dapat dikembangkan obat dari bahan alam seperti daun pepaya (*Carica papaya* L.), yang diketahui memiliki aktivitas farmakologi karena adanya kandungan senyawa aktif seperti alkaloid, triterpenoid, steroid, flavonoid, saponin, dan tanin. Penelitian ini bertujuan membuktikan efektivitas ekstrak etanol daun pepaya (*C. papaya* L.) dalam menghambat pertumbuhan dan membunuh *T. rubrum* secara *in vitro*. Uji aktivitas daya hambat antijamur ekstrak etanol daun pepaya dilakukan menggunakan metode *two-fold* mikrodilusi pewarnaan resazurin dengan rentang konsentrasi 4000 – 31,25 µg/mL. Untuk uji daya bunuh ekstrak etanol daun pepaya dilakukan menggunakan penilaian jumlah koloni *T. rubrum* di media Potato Dextrose Agar. Hubungan antara konsentrasi ekstrak etanol dan pepaya dengan jumlah koloni *T. rubrum* dianalisis menggunakan uji regresi

linier. Konsentrasi minimal ekstrak etanol daun pepaya untuk menghambat dan membunuh *T. rubrum* adalah 2000 µg/mL. Dari hasil analisis regresi linear, didapatkan persamaan $y = -0,001x + 4,221$, R^2 0,569 dan koefisien korelasi 0,755. Ekstrak etanol daun pepaya (*Carica papaya* L.) dapat membunuh dan menghambat pertumbuhan *T. rubrum*. Selain itu, terdapat hubungan yang kuat antara peningkatan konsentrasi ekstrak daun pepaya dengan aktivitasnya untuk membunuh *T. rubrum*.

Kata Kunci: Agen Antijamur, Ekstrak Tanaman, Uji Resazurin, Tinea Pedis

INTRODUCTION

Fungi thrive in Indonesia, which has a tropical climate with high temperature and humidity. Therefore, fungal skin diseases, such as dermatophytosis easy to infect Indonesian people (Nurwulan *et al.*, 2019). Dermatophytosis itself is a superficial mycosis disease that affected the keratinized tissue such as hair, skin, and nails. The diseases can be carried by *Trichophyton*, *Microsporum*, and *Epidermophyton* (Devy and Ervianti, 2016). The most common dermatophytic fungus is *Trichophyton rubrum*, the main cause (78.7%) of tinea pedis (Athlete's foot). *Trichophyton rubrum* was also the most widely isolated microbe from nail fungus (66.7%) (Colosi *et al.*, 2020).

The spread of primary fungal infections and opportunistic infections, accompanied by limited antifungal therapy options, especially in systemic infections can increase antifungal resistance in the future. Long-term and inappropriate treatment of fungal infections with synthetic drugs also often causes side effects. To minimize this, the use of natural products from plants can be used as a strategy for the discovery and development of antifungal drugs (Vandeputte *et al.*, 2012; Apsari and Adiguna, 2013).

Tropical plant such as papaya (*Carica papaya* L.) is a lush plant either in the lowlands or the highlands of Indonesia. Almost all part of papaya, including leaf, sap, seed, fruit, peel, and roots, can contain compounds that are useful for medicine (Mahendra C and Nikhil D, 2016). For example, compounds such as alkaloids, triterpenoids, steroids, flavonoids, saponins, and tannins can be found in the papaya leaves (A'yun and Laily, 2015). These compounds have the potential as antifungal. One study suggested that the

ethanolic extract of papaya leaf which contains alkaloids, saponins, and flavonoids can inhibit the growth of *Candida albicans in vitro* (Rosari *et al.*, 2018; Noorie and Chenthamarai, 2020). In addition, papaya leaf simplicia is known to have an inhibitory effect on several species of saprophytes, dermatophytes, and yeasts (Sherwani *et al.*, 2013). However, until now, the research regarding the efficacy of papaya (*Carica papaya* L.) leaf extract on *Trichophyton rubrum* has not been found.

The concentration of papaya leaf extract which is effective as an antifungal varies depending on the type of fungus tested. To inhibit the growth of *C. albicans*, concentration of 100% is needed to produce an inhibition zone of 23.61 mm (Rosari *et al.*, 2018). While other study stated that the concentration of papaya leaf extract 500 – 1000 µg/mL effectively decrease the growth of *C. albicans*, and produced inhibitory zone with the range of 8.73 – 11.97 mm. If using microdilution method, minimum inhibitory concentration (MIC) of ethanolic papaya leaf extract against *C. albicans* has also been found at a concentration of 350 µg/ml (Noorie and Chenthamarai, 2020).

This study will test the effectiveness of papaya leaf extract (*Carica papaya* L.) in inhibiting the growth of *T. rubrum in vitro*. Based on the description above, the concentration range of papaya leaves extract to be used in this study was set between 31.25 to 4000 µg/mL. Qualitative phytochemical tests were also carried out to analysis the content of compounds in papaya leaf ethanol extract.

METHODS

This research is laboratory experimental with post-test only control group design. *T. rubrum* used in this research was obtained from the Department of Microbiology, Faculty of Medicine Universitas Gadjah Mada. The Papaya leaves (*C. papaya L.*) is derived from the Sumberrejo Village, Donorojo Jepara, Indonesia. This research was approved by the Health Research Ethics Commission (HREC) of the Faculty of Medicine, Universitas Muhammadiyah Semarang with the number 155/EC/FK/2021.

Tools and materials

This research required tools including microplate 96 well (Iwaki), incubator (Mettler), sterile test tube, sterile round loop, oven (Mettler), erlenmeyer tube, laminar airflow (Biobase), autoclave (Hirayama), sterile petri dish, micropipette, colony counter (Funke Gerber), and rotary evaporator (Ika® RV10). The materials used are Potato Dextrose Broth (PDB) (Himedia), Potato Dextrose Agar (PDA) (Merck), papaya leaf extract, Mc Farland 0.5 standard solution, sterile water, Mg

$$\% \text{ extract concentration} = \frac{\text{Weight of extract obtained}}{\text{Weight of extracted simplicia}} \times 100\%$$

Susceptibility testing of antifungal papaya (*C. papaya L.*) leaf extract against *T. rubrum*

Trichophyton rubrum assay suspension was prepared from culture on PDA media aged 2-5 days. *Trichophyton rubrum* suspension was prepared using PDB media with the inoculum concentration adjusted according to the Mc Farland standard of 0.5 (1.5×10^8 CFU/mL). Then the suspension was diluted to the final concentration of *T. rubrum* for the test 1×10^6 CFU/mL (CLSI, 2008).

The minimum inhibitory concentration (MIC) test was carried out using the two-fold microdilution method with resazurin staining (Ohikhen, Wintola and Afolayan, 2017). Papaya leaf extract was dissolved in sterile water with a concentration range of 4000 – 31.25 µg/mL. To ensure the validity of the test results, a positive control group was prepared using ketoconazole with a

powder (Merck), HCl (Merck), gelatines salt (Himedia), FeCl₃ solution (Merck), H₂SO₄ (Merck), ketoconazole (Phapros Tbk), and resazurin (Sigma Aldrich).

Papaya (*C. papaya L.*) leaf extraction

Papaya (*C. papaya L.*) leaves that are still green, not dry, and without pests are taken as much as 3 kg, then washed with running water and cut into pieces. Furthermore, papaya leaves are dried at room temperature for approximately 5 days. The dried papaya leaves are then mashed with a blender until dry simplicia is obtained.

The papaya leaf extraction process was carried out using the maceration method. Papaya leaf simplicia was put into a 5 liter glass bottle, then 96% ethanol (1:4) was added for the soaking process. This soaking process was carried out for 5 days, with 10 minutes of stirring every day. After 5 days, the maceration results were filtered using filter paper to obtain the supernatant. The process of concentrating the ethanol extract of papaya leaves was carried out using a vacuum rotary evaporator. The yield of the extract was calculated by the following formula: (Cahyani, 2020)

concentration range of 16 – 0.125 µg/mL. Furthermore, negative control (sterile water), sterility control of PDB media and papaya leaf extract were also used. Growth controls were prepared from PDB and *T. rubrum*, and each treatment group was replicated four times. The microplate was incubated at 37 °C for 96 hours, and after the incubation, as much as 30 µL of 0.025% resazurin was added to each test well. The reading of the MIC value was done by observing the colour change of the resazurin indicator. The lowest concentration of papaya leaf extract that remained blue was determined as the MIC value.

Furthermore, the MBC determination test was determined by taking 20 µL of solution from each test well. The solution was then grown on Potato Dextrose Agar (Merck) media with an incubation time of 96 hours at 37°C. After incubation, readings of the growth

results of *T. rubrum* colonies were carried out using a colony counter.

Phytochemical analysis of papaya (*C. papaya L.*) leaf extract

Phytochemical test for identification of alkaloid compounds was carried out on 2 ml of papaya (*C. papaya L.*) leaf extract which dissolved using 5 mL of 2 N HCl. The solution was divided into five test tubes, the first tube became a control solution of papaya leaf extract in 2N HCl, the second tube was given Dragendorff's reagent, the third tube was given Mayer's reagent, the fourth tube was given Hager's reagent, and the fifth tube was given Wagner's reagent. Alkaloids are indicated if at least 2 test tubes form a precipitate along with orange precipitate (second tube), white precipitate (third tube), yellow precipitate (fourth tube), and red precipitate (fifth tube).

To identify flavonoids, 1 ml of papaya leaf extract was added with acetone, boric acid, and oxalic acid. The mixed solution was then heated with a water bath, and as much as 10 ml of ether was added to the solution and observed under UV light at 366 nm. The presence of intense yellow fluorescence in the solution indicates the presence of flavonoids. Identification of saponins compounds was carried out by shaking vertically for 10 seconds in a tube containing a solution of papaya leaf extract. Furthermore, the tube is allowed to stand for 10 seconds. If in less than 10 minutes, a stable foam with a size of 1 to 10 cm is formed, then the solution be avowed positive contains saponins.

The Liebermann-Burchard reaction was used to test the content of steroids and triterpenoids in papaya leaf extract. A total of 2 ml of papaya leaf extract solution was mixed with 0.5 ml of chloroform and 0.5 ml of anhydrous acetic acid. Then, through the tube wall,

2 mL of concentrated sulphuric acid was added to the test tube. The formation of a purple ring indicates the presence of triterpenoids, but if the blue-green ring formed the solution avowed positive contains sterols. For testing the tannin content, as much as 1 mL 10% solution of iron (III) chloride was added to papaya leaf extract. The formation of a dark blue or greenish black colour in the test solution indicates the presence of tannins (Mahatrinny *et al.*, 2014).

Data analysis

The MIC value was determined as the lowest concentration of papaya (*C. papaya L.*) leaf extract that inhibits the growth of *T. rubrum*. The MBC value was determined as the lowest concentration of papaya leaf extract that caused no growth of the *T. rubrum* in PDA. To determine the relationship pattern between papaya leaf extract concentrations in killing of *T. rubrum*, the linear regression test was used.

RESULTS

The amount of simplicia from the dried papaya leaves was 581 grams. After the papaya leaf simplicia was extracted using the maceration process, the extract yield was 8.59%.

Susceptibility testing of papaya (*Carica papaya L.*) leaf extract and ketoconazole against *T. rubrum*

In this study, the MIC value was determined by the resazurin staining test. Wells in the microplate that appear blue at the lowest concentration are considered as the MIC values. From the susceptibility testing, papaya (*C. papaya L.*) leaf extract can inhibit the growth of *T. rubrum* with the MIC value of 2000 µg/ml. Meanwhile, ketoconazole was able to inhibit the growth of *T. rubrum* at a concentration of 8 µg/ml (Figure 1).

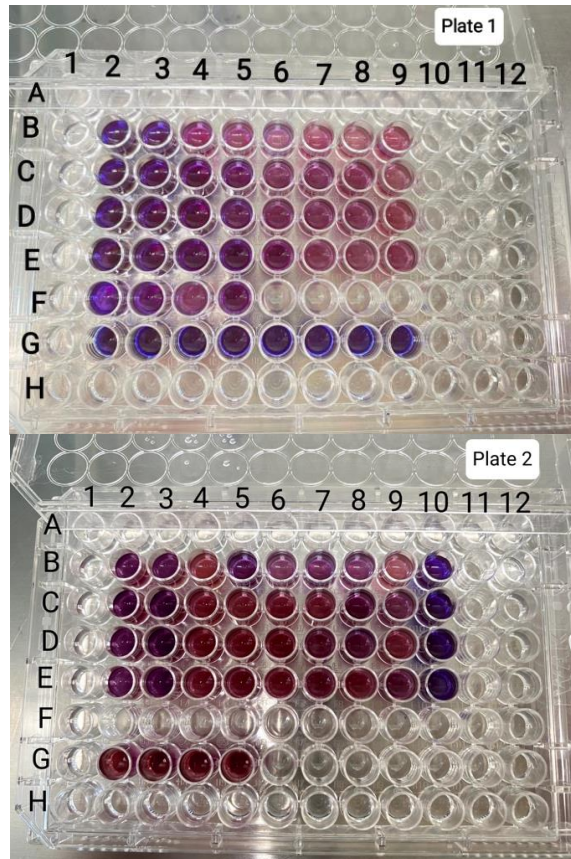


Figure 1. Plate 1 is a microplate for susceptibility testing of papaya (*C. papaya L.*) leaf extract against *T. rubrum* plates (Note: B-E2 to B-E9 are papaya leaf extract with concentration of 4000-31.25 µg/mL; F2-F5 are *T. rubrum* growth control; and G2-G9 are sterility control). Plate 2 is a microplate for susceptibility testing of ketoconazole against *T. rubrum* (Note: B-E2 to B-E9 are ketoconazole with concentration of 16-0.125 µg/mL; B10-E10 is drug sterility control; and plates G2-G5 are negative control)

Based on the results of the determination of the MBC value, papaya (*C. papaya L.*) leaf extract can kill the *T. rubrum* at a concentration of 2000 µg/mL (Table 1). Linear regression analysis was

performed to assess the relationship between papaya leaf extract concentration and the number of *T. rubrum* colonies.

Table 1. The mean ± standard deviation (SD) log of the *T. rubrum* colonies after the administration of various concentrations of papaya (*Carica papaya L.*) leaf extract

No.	Papaya (<i>Carica papaya L.</i>) Leaf Extract Concentration (µg/mL)	Mean ± SD Log Colony of <i>T. rubrum</i>
1.	31.25	4.75 ± 0.32
2.	62.5	4.54 ± 0.47
3.	125	4.51 ± 0.37
4.	250	3.35 ± 2.23
5.	500	3.33 ± 2.22
6.	1000	3.27 ± 2.18
7.	2000	0 ± 0
8.	4000	0 ± 0

Based on the results of linear regression analysis, it can be concluded that there was a decrease in the average number of colonies of the *T. rubrum* due to an increase of the papaya leaf extract concentration ($y = -0.001x + 4.221$ and

R^2 0.569). The correlation coefficient value obtained is 0.755, which means there is a strong correlation between the increase of papaya leaf extract concentration and its bactericidal activity (Figure 2).

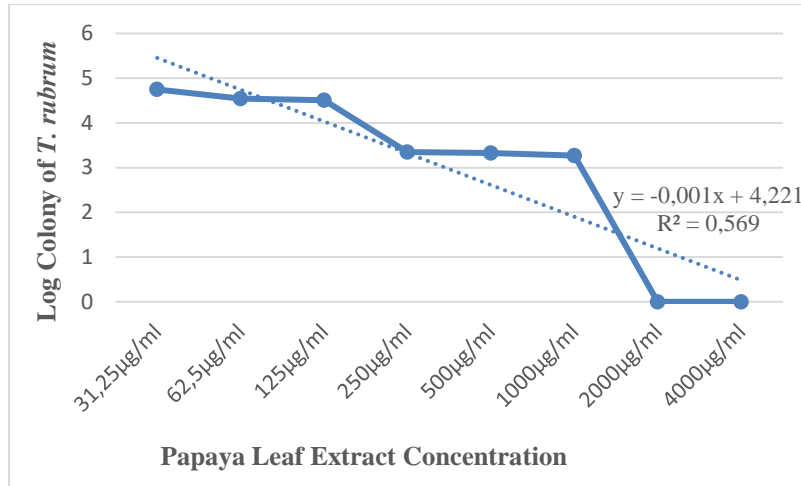


Figure 2. Linear regression analysis between the concentrations of papaya (*C. papaya L.*) leaf extract with *T. rubrum* log colonies

Meanwhile, for the results of the bactericidal activity of ketoconazole against *T. rubrum*, the MBC value of ketoconazole was 16 g/ml (Table 2). This MBC value is twice the MIC value of ketoconazole against *T. rubrum*.

Table 2. The mean ± standard deviation (SD) log of the *T. rubrum* colonies after the administration of various concentrations of ketoconazole

No.	Ketoconazole Concentration (µg/mL)	Mean ± SD Log Colony <i>T. rubrum</i>
1.	0.125	4.73 ± 0.09
2.	0.25	4.59 ± 0.09
3.	0.5	4.40 ± 0.32
4.	1.0	4.40 ± 0.32
5.	2.0	4.45 ± 0.19
6.	4.0	2.00 ± 2.31
7.	8.0	2.00 ± 2.31
8.	16.0	0 ± 0

Based on the results of linear regression analysis, it was seen that there was a decrease in the average number of *T. rubrum* colonies along with the increase in the concentration of ketoconazole. The regression equation obtained was $y = -0.302x + 4.524$, with

the value of R^2 is 0.652 (Figure 3). The correlation coefficient obtained is 0.802, which means that there is a strong relationship between the increase in the concentration of ketoconazole and the bactericidal activity against *T. rubrum*.

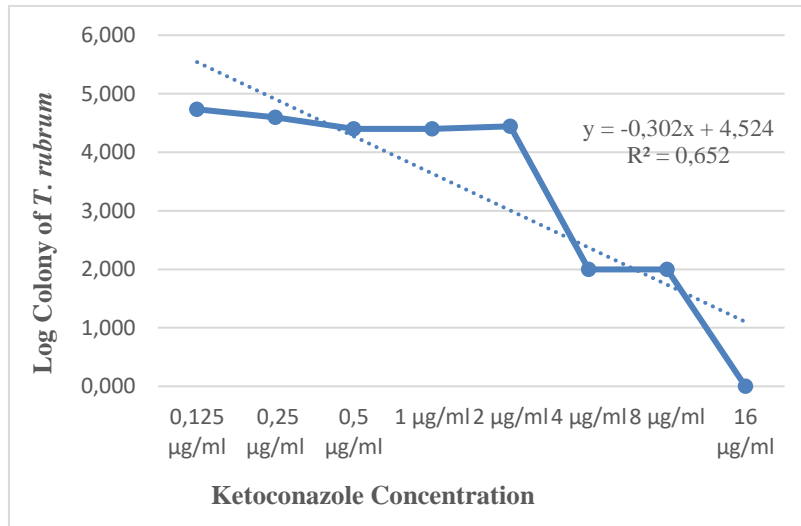


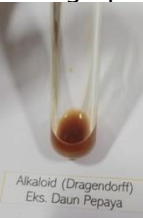


Figure 3. Linear regression analysis between the concentrations of ketoconazole leaf extract with *T. rubrum* log colonies




Phytochemical analysis of papaya (*Carica papaya L.*) leaf extract

Phytochemical tests were carried out to determine the content of compounds in the ethanol extract of

papaya (*C. papaya L.*) leaves. Based on the test results, steroid and terpenoids compounds were not found in the ethanol extract of papaya leaves (Table 3).

Table 3. The results of papaya (*C. papaya L.*) leaf extract phytochemical test

No.	Compound Tested	Description of Phytochemical Analysis Test Results	Conclusion
1.	Alkaloid	Orange precipitate 	(+)
2.	Phenolic Group	Deep turquoise color 	(+)
3.	Flavonoids	Formed pink color 	(+)

No.	Compound Tested	Description of Phytochemical Analysis Test Results	Conclusion
4.	Tannins	Bluish-green solution 	(+)
5.	Saponins Group	Stable foam 	(+)
6.	Steroids Terpenoids	No green or blue color is formed No reddish color is formed 	(-) (-)

DISCUSSIONS

The purpose of this study was to determine the effectiveness of papaya leaf extract (*C. papaya L.*) in inhibiting growth or killing *T. rubrum*. The activity was determined from the value of MIC and MBC of papaya leaf ethanol extract. The susceptibility testing was used resazurin staining (Resazurin Microplate Assay) to facilitate the interpretation of the results. When the test results show the presence of microbial growth, the blue color of resazurin can be reduced to a pink color that fluoresces in the form of resorufin. The microdilution method is also used in this study, because it has several advantages which are relatively short test time, efficient in the use of samples, and can test several different samples at once test (Trimedona *et al.*, 2017).

Based on the result of antifungal susceptibility testing, the MIC value of

papaya leaf ethanol extract to inhibit the growth of *T. rubrum* was 2000 µg/ml. This is in line with other studies which state that papaya extract from boiling the leaf in water can inhibit the growth of several species of dermatophytes like *Trichophyton mentagrophytes* and *Microsporum cannis*, with an average inhibition zone of 16-20 mm using an extract concentration of 180-320 mg/ml. In addition to dermatophytes, papaya leaf extract also has activity to inhibit the growth of several saprophytic species (*Penicillium sp.*, *Aspergillus niger*, *Aspergillus flavus*, and *Rhizopus sp.*) at an extract concentration of 120-160 mg/ml and with an average inhibition zone of 15-20 mm. The concentration required by papaya leaf to inhibit the growth of dermatophytes and saprophytic species is higher (in mg/mL) compared to the concentration of papaya leaf ethanol extract to inhibit the growth of *T. rubrum* in this study. This could be

due to differences in the solvent used for extraction (Sherwani *et al.*, 2013). Papaya leaf ethanol extract was also effective in inhibiting the growth of *Candida albicans* with a MIC value of 350 µg/ml (Noorie and Chenthamarai, 2020). Species from other *Candida* genera (*Candida galbrata*, *Candida tropicalis*, and *Candida kruzei*) can also be inhibited by papaya leaf water boiled extract at a concentration of 80-200 mg/ml to form an average inhibition zone of 12-20 mm (Sherwani *et al.*, 2013).

Meanwhile, the results of the sensitivity test of *T. rubrum* to the control drug resulted in the MIC value of ketoconazole at a concentration of 8 µg/ml, and the MBC value at a concentration of 16 µg/ml. *T. rubrum* can be declared resistant if the MIC value of ketoconazole *in vitro* is more than 0.8 µg/ml (Therese *et al.*, 2006). However, there are other studies which state that ketoconazole resistant in dermatophytes species *in vitro* is expressed when the MIC value is ≥ 1 µg/ml (Anggarini *et al.*, 2015; Shen *et al.*, 2022). Therefore, it can be concluded that the *T. rubrum* used in this study was resistant to ketoconazole. In general, drug resistance can be classified into two, namely intrinsic resistance and acquired resistance. Intrinsic resistance occurs when microorganisms evolve and undergo genetic changes (mutations) that can confer resistance to drugs. Acquired resistance occurs as a failure of clinical response to exposure to antifungal drugs during the treatment process. In *T. rubrum* used in this study, the mechanism of resistance could be due to intrinsic or extrinsic factors, because the isolates used were not wild type isolates (Vandeputte *et al.*, 2012). In general, fungal resistance to azole antibiotics can be caused by fungal activity to degrade drugs, changes in drug targets caused by mutations, and the presence of an efflux pump that pumps drugs out of cells (Apsari and Adiguna, 2013).

Based on the results of phytochemical analysis, the ethanol extract of papaya leaves used in this study contained alkaloids, flavonoids,

phenolic, saponins, and tannins. This is in line with the research conducted by A'yun and Laily (2015). All of these compounds known to have antifungal activity. For example, alkaloids can kill fungi by inhibiting esterase enzymes, DNA and RNA polymerases, cellular respiration, and nucleic acid production. In addition to alkaloids, flavonoids can also destroy fungal cells and increase cell membrane permeability by forming complexes of hydroxyl groups with phospholipids from fungal cell membranes. The activity of these flavonoids can cause denaturation of fungal cell walls. For polar surfactants called saponins, they can damage the lipid layer that covers cell membranes, causing membrane permeability abnormalities, disrupting the diffusion of substances needed by fungi, unstable cell membranes and ultimately causing lysis cell. The last compound, tannins are also able to inhibit the formation of chitin and damage fungal cell membranes (Dewi *et al.*, 2019; Mala, 2020).

Based on the results of this study, the ability of papaya (*C. papaya* L.) leaf ethanol extract to inhibit growth and kill *T. rubrum* can trigger further research. Papaya leaf ethanol extract potential to be developed as antifungal, for example can be made in the form of topical drug for the treatment of tinea pedis infection.

CONCLUSIONS

Ethanol extract of papaya leaves (*C. papaya* L.) has effectiveness in inhibiting the growth and killing of *T. rubrum in vitro*, with MIC and MBC values of 2000 µg/ml. Based on the results of the phytochemical test analysis, the ethanolic extract of papaya leaves contains compounds that have the potential as antifungals such as alkaloids, flavonoids, phenolic, saponins, and tannins.

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