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Antifungal Activity Test of Ethanol Extract of *Manukan* Leaves (*Rhinacanthus nasutus* (L.) Kurz) Against *Malassezia furfur* Growth

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Abstract

Medicinal plants are increasingly being explored as potential sources of natural antifungal agents to address the rising incidence of skin infections in tropical regions. *Manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz) contain various secondary metabolites with potential antifungal, antibacterial, anti-inflammatory, antiviral, and anticancer activities. Although previous research has reported the antifungal activity of *manukan* leaves against *Candida albicans*, their activity against *Malassezia furfur*, a causative agent of tinea versicolor, has not yet been specifically investigated. In Waeperang Village, Buru Island, Maluku, manukan leaves have traditionally been used to treat skin infections; however, this empirical use has not been adequately supported by scientific evidence. This study aimed to evaluate the antifungal activity of the ethanol extract of *manukan* leaves against *Malassezia furfur* and to identify the groups of secondary metabolites potentially associated with this activity. *Manukan* leaves were extracted by maceration using ethanol. Antifungal activity was evaluated using the agar diffusion method, while the active compound groups were identified using thin-layer chromatography (TLC) combined with bioautography. The novelty of this study lies in the evaluation of *manukan* leaf extract against *Malassezia furfur* and the use of TLC-bioautography to identify compound groups potentially associated with its antifungal activity. The ethanol extract contained flavonoids, alkaloids, tannins, steroids, and terpenoids. The extract exhibited antifungal activity against *Malassezia furfur* at concentrations of 5%, 15%, 30%, and 60%, with the highest inhibition zone observed at 60% concentration (14.4 mm). TLC-bioautography indicated that tannins (Rf 0.63) and steroids (Rf 0.68) were suspected to contribute to the antifungal activity against *Malassezia furfur*.

Keywords: Activity; Antifungal; Leaves; *Rhinacanthus*; Malassezia.

INTRODUCTION

Plants are important sources of secondary metabolites and have played a significant role in both traditional and modern medicine because of their bioactive compounds, which possess various biological activities, including antimicrobial effects (Sheikh, 2020). One plant known for these properties is *manukan* (*Rhinacanthus nasutus* (L.) Kurz). *Manukan* leaves possess diverse pharmacological properties, including antifungal, antibacterial, anti-inflammatory, antioxidant, and anticancer activities (Fitriyasti et al., 2024). Several Asian countries have traditionally used manukan leaves in herbal medicine to treat various health conditions (Brimson et al., 2020).

The empirical use of *manukan* leaves as a medicinal plant has been reported in several regions. In East Lombok, the leaves are used as a natural insecticide to control various agricultural pests (Fadli, 2018). In Tulang Bawang Regency, Lampung, local communities also use manukan leaves as traditional medicine for various health conditions (Irawan, 2024). In Waeperang Village, Buru Island, Maluku, manukan leaves are traditionally used to treat skin infections, including tinea versicolor, ringworm, and scabies. The leaves are prepared by pounding them into a smooth paste and applying it directly to the affected skin. Tinea versicolor is a superficial fungal skin infection associated with the overgrowth of *Malassezia* species, including *Malassezia furfur* (Tambunan, 2022). It is commonly characterized by the appearance of hypopigmented or hyperpigmented patches, particularly in areas with a high density of sebaceous glands. Warm and humid environmental conditions, excessive sweating, and sebum production may promote the growth of *Malassezia furfur* (Visiadina, 2023). Although generally not a serious infection, tinea versicolor can cause itching and alterations in skin pigmentation, resulting in discomfort and potentially affecting the patient's self-confidenc (Wakano et al, 2022).

Antifungal activity assays are used to evaluate the capacity of a substance to inhibit fungal growth by interfering with cellular functions and metabolic processes (Sun, 2024). However, studies investigating

the antifungal activity of manukan leaves (*Rhinacanthus nasutus* (L.) Kurz) against *Malassezia furfur* remain limited. Narti (2021) reported that manukan leaf extract exhibited antifungal activity against *Candida albicans*, producing an inhibition zone of 18.92 mm, which was classified as a strong inhibitory effect. This finding suggests that *manukan* leaves have considerable potential as a natural source of antifungal agents. (Wirandanu, 2022).

Based on this background, the research problem can be formulated as follows:

1. Does the ethanol extract of *manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz) have antifungal activity against *Malassezia furfur*?
2. What is the inhibitory activity of the ethanol extract of *manukan* leaves against the growth of *Malassezia furfur*??
3. What groups of secondary metabolites exhibit antifungal activity against *Malassezia furfur*?

This study aimed to evaluate the antifungal activity of the ethanol extract of *manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz) against *Malassezia furfur* using the agar diffusion method and to identify the groups of secondary metabolites potentially associated with antifungal activity through thin-layer chromatography (TLC) coupled with bioautography.

MATERIALS AND METHODS

Material

The materials used in this study consisted of *manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz), ethanol, *Malassezia furfur*, fungal growth medium, distilled water, 2% ketoconazole, paper discs, TLC plates, n-hexane, ethyl acetate, and reagents for secondary metabolite identification, including $AlCl_3$ or ammonia, Dragendorff's reagent, $FeCl_3$, Liebermann–Burchard reagent, and anisaldehyde–sulfuric acid reagent.

Tool

The tools used included maceration equipment, a drying oven, a blender or simplex grinder, an analytical balance, a rotary evaporator or vaporizer, an autoclave, an incubator, Petri dishes, Erlenmeyer flasks, test tubes, pipettes, capillary tubes, TLC chambers, 254 nm and 366 nm UV lamps, calipers, and other supporting laboratory equipment.

Method

Manukan leaves were identified at the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Pattimura University, Ambon. The samples were then sorted, washed, dried, dry-sorted, and ground into simplicia. Extraction was carried out by maceration using ethanol for five days, after which the filtrate was evaporated to obtain a thick extract.

Ways of working

The identified *manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz) were subjected to wet sorting to remove impurities and unwanted plant materials. The leaves were subsequently dried and subjected to dry sorting to obtain materials suitable for the preparation of simplicia. The dried material was then ground and sieved to obtain powdered simplicia prior to extraction. Extraction was performed using the maceration method with ethanol as the solvent for five days. The resulting macerate was then concentrated by evaporation to obtain a thick extract (Maitulung et al., 2022)

Antifungal activity against *Malassezia furfur* was evaluated using the agar diffusion method. The fungal suspension was adjusted to a 0.5 McFarland standard and inoculated onto the agar medium. The ethanol extract was prepared at concentrations of 5%, 15%, 30%, and 60%, with 2% ketoconazole serving as the positive control and distilled water as the negative control. Paper discs impregnated with each test preparation were placed onto the inoculated agar medium and incubated under appropriate conditions. Antifungal activity was evaluated by measuring the diameter of the inhibition zone formed around each disc. All treatments were tested in triplicate (Narti, 2021)

Secondary metabolite profiles were analyzed by thin-layer chromatography (TLC) using a mixture of n-hexane and ethyl acetate (1:2) as the mobile phase. Following elution, the chromatographic spots were examined under UV light at wavelengths of 254 and 366 nm and further characterized using specific detection reagents. The retention factor (Rf) values of the detected spots were subsequently calculated. TLC-bioautography was then conducted to determine the compound groups potentially responsible for the antifungal activity against *Malassezia furfur* (Abdullah, 2021)

Data were analyzed qualitatively based on the diameter of the inhibition zones as an indicator of the antifungal activity of the extract. The analysis also considered the identification of secondary metabolite groups on the TLC plates, their respective Rf (retardation factor) values, and the inhibition zone diameters observed in the TLC-bioautography assay.

RESULTS AND DISCUSSION

Identification of Secondary Metabolite Compound Content

Table 1. Results of the Identification Test for Secondary Metabolite Compounds in *Manukan* Leaves (*Rhinacanthus nasutus* (L.) Kurz).

No	Compound	Nila Rf
1	Flavonoid	0.93
2	Alkaloid	0.71 and 0.87
2	Tannin	0.63
3	Steroid	0.68
4	Terpenoid	0.85

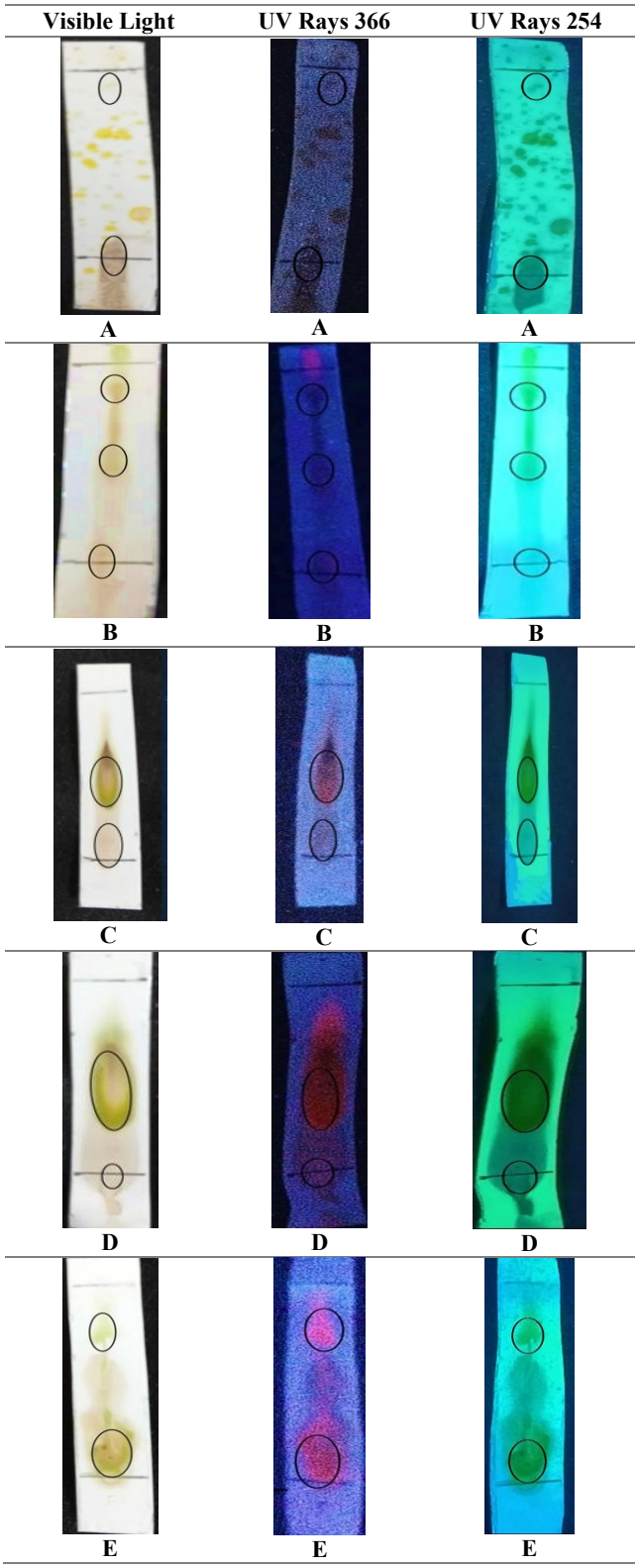


Figure 1. Results of secondary metabolite identification using thin-layer chromatography (TLC) with n-hexane and ethyl acetate (1:2, v/v) as the eluent system.

Information:
A. :Flavonoid Compound Identification Test Results
B. :Results of Alkaloid Compound Identification Test
C. :Tannin Compound Identification Test Results
D. :Steroid Compound Identification Test Results
E. :Terpenoid Compound Identification Test Results

As shown in Table 1, TLC analysis revealed the presence of several secondary metabolite groups in the ethanol extract of *manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz), including flavonoids, alkaloids, tannins, steroids, and terpenoids. The compounds were separated using n-hexane and ethyl acetate as the mobile phase. Flavonoids produced an Rf value of 0.93, while alkaloids yielded two spots with Rf values of 0.87 and 0.77. Tannins showed an Rf value of 0.63, steroids showed an Rf value of 0.68, and terpenoids showed an Rf value of 0.85. These findings demonstrate that the ethanol extract contains various groups of secondary metabolites that may contribute to its biological activities, including potential antifungal activity.

Antifungal Activity Test Using the Disc Diffusion Method

Table 2. Antifungal Activity of Ethanol Extract of *Manukan* Leaves (*Rhinacanthus nasutus* (L.) Kurz) Against *Malassezia furfur.*

Control	Inhibition zone diameter				Information
	PI	P II	P III	Average (mm)	
Aquadest (-)	0	0	0	0	Weak
Ketoconazole (+)	28	29	29	28.7	Very strong
5%	7	9	8	8	Currently
15%	10	13	13	12	Strong
30%	10	13	14	12.4	Strong
60%	14	14	15	14.4	Strong

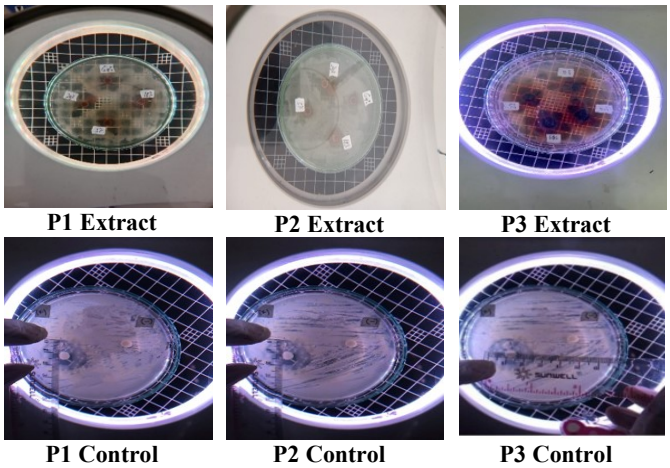


Figure 2. Antifungal Activity of Ethanol Extract of *Manukan* Leaves (*Rhinacanthus nasutus* (L.) Kurz) Against *Malassezia furfur*

As presented in Table 2, the ethanol extract of *manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz) demonstrated inhibitory activity against *Malassezia furfur* at concentrations of 5%, 15%, 30%, and 60%, producing mean inhibition zone diameters of 8.0, 12.0, 12.4, and 14.4 mm, respectively. The inhibition zone

generally increased with increasing extract concentration, indicating a concentration-dependent trend in antifungal activity. The negative control, distilled water, produced no inhibition zone, whereas the positive control, 2% ketoconazole, produced a 28.7 mm inhibition zone, which was classified as very strong. Overall, the findings demonstrate that the ethanol extract of *manukan* leaves possesses antifungal activity against *Malassezia furfur*, although its inhibitory effect was lower than that of ketoconazole.

Antifungal Activity Test Using the Bioautography Method

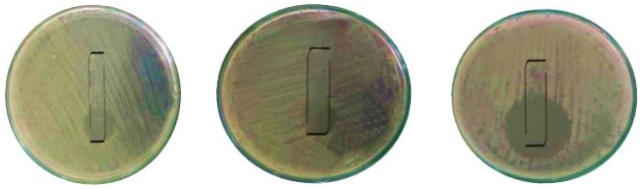


Figure 3. Antifungal Activity Test Using The Bioautography Method.

Based on Figure 3, TLC-bioautography was performed to evaluate the potential antifungal activity of the ethanol extract of *manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz) and to determine the compound groups potentially associated with the observed activity. The direct-contact method was applied by placing the developed TLC plate directly onto agar medium previously inoculated with *Malassezia furfur*. Compounds present in the chromatographic spots with antifungal properties were able to diffuse into the agar medium and inhibit fungal growth. Antifungal activity was indicated by the formation of clear inhibition zones on the agar surface at positions corresponding to the active spots on the TLC plate.

Discussion

During the TLC identification process, several chromatographic spots exhibited tailing, characterized by elongated spots rather than well-defined, circular shapes. This phenomenon may have been caused by several factors, including sample overloading, which can interfere with the separation of compounds during the development process. In addition, tailing may result from an inappropriate mobile-phase composition that does not provide optimal separation, uneven application of the sample, insufficient drying of the spots before development, or inadequate saturation of the developing chamber. These factors may reduce the quality of chromatographic separation and affect the clarity of the resulting spots.

During the TLC identification process, several chromatographic spots exhibited tailing, characterized by elongated rather than well-defined, circular spots. This phenomenon was presumed to be associated with several factors, particularly sample overloading, which may have hindered the optimal separation of compounds during the development process. Other contributing factors may include an inappropriate mobile-phase composition, uneven sample application, insufficient drying of the spots before development, and inadequate saturation of the developing chamber. These conditions may have affected the quality of chromatographic separation, resulting in less distinct spots and reduced separation efficiency on the TLC plate (Fitriyasti et al, 2024).

The variation in inhibition zone diameters among the tested concentrations suggests a general trend toward increased antifungal activity with increasing extract concentration. The 60% concentration produced the largest inhibition zone, which may indicate a higher availability of bioactive compounds capable of inhibiting the growth of *Malassezia furfur* compared with the lower concentrations. However, the increase in inhibition zone diameter was not directly proportional to the increase in extract concentration, as only a slight difference was observed between the 15% and 30% concentrations. This finding is consistent with Sunarti (2020), which reported that increasing concentrations of *manukan* leaf extract were associated with larger inhibition zones against *Malassezia furfur*, with the greatest inhibitory effect observed at a concentration of 60%.

The results presented in Figure 3 indicate that the tested samples exhibited antifungal activity, as demonstrated by the formation of clear inhibition zones on the agar medium. In the thick extract sample, an inhibition zone was observed at the spot with an Rf value of 0.00, indicating antifungal activity against *Malassezia furfur*. In addition, inhibition was observed in the region extending from Rf 0.00 to Rf 0.68. Based on the results of the TLC spot identification, spots with Rf values of 0.63 and 0.68 on the thick extract plate were identified as tannin and steroid compounds, respectively. The presence of inhibition zones at these Rf positions suggests that tannins and steroids may be associated with the antifungal activity of the *manukan* leaf extract against *Malassezia furfur*.

Overall, the findings suggest that steroid and tannin compounds present in *manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz) may contribute to the antifungal activity observed against *Malassezia furfur*. Steroids are a class of secondary metabolites associated with various biological activities. One steroid compound commonly found in plants is β-sitosterol, which has been reported to possess biological activity and potential antifungal effects. Tannins, in addition to steroids, are also known to exhibit antifungal activity through interactions with proteins and other components of microbial cells. Therefore, the presence of steroids and tannins in the ethanol extract of *manukan* leaves may be associated with its inhibitory activity against the growth of *Malassezia furfur* (Brimson, 2020).

CONCLUSION

The ethanol extract of *manukan* leaves (*Rhinacanthus nasutus* (L.) Kurz) exhibited antifungal activity against *Malassezia furfur*, with the highest inhibition zone observed at a concentration of 60%, measuring 14.4 mm. Phytochemical analysis using TLC revealed the presence of flavonoids, alkaloids, tannins, steroids, and terpenoids in the extract. TLC-bioautography indicated that tannins and steroids may contribute to the observed antifungal activity against *Malassezia furfur*.

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

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