

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION OF THE ETHANOLIC EXTRACT OF KIPAHIT LEAVES ON THE GROWTH OF *Propionibacterium acnes*

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ABSTRACT

Acne is a skin disorder characterized by inflammation in the form of comedones, papules, pustules, and nodules. The disease is caused by the gram-positive bacterium, *Propionibacterium acnes*. Treatment of the disease using antibiotic agents such as erythromycin and clindamycin has been reported to cause some side effects. Therefore, alternative antibacterial agents derived from natural products are required to reduce the occurrence of side effects. Kipahit (*Tithonia diversifolia* A. Gray) is one of the plants with pharmacological activities, such as antibacterial and antiprotozoal activities. The aim of this study was to determine the minimum inhibitory concentration (MIC) of a 96% ethanolic extract of kipahit leaves on the growth of *P. acnes*. Kipahit leaves were extracted through the ultrasound-assisted extraction (UAE) method. Minimum inhibitory concentration (MIC) was determined using the liquid dilution method. A serial dilution was performed to prepare a series of extract concentrations: 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78%, and 0.39%. Whereas, clindamycin and DMSO 1% were used as positive and negative controls, respectively. Phytochemical screening showed that the 96% ethanolic extract of kipahit leaves contains flavonoids, alkaloids, saponins, steroids/triterpenoids, tannins, and phenolic compounds. In addition, the extract showed inhibitory activity against *P. acnes* at concentrations ranging from 25% to 1.56%. In contrast, the extract showed no inhibitory activity at concentrations of 0.78% and 0.39%. Further experiments confirmed that kipahit leaf extract acted as a bacteriostatic agent (inhibiting bacterial growth) at a minimum concentration of 1.56%.

Keywords: Acne, *Propionibacterium acnes*, Kipahit, MIC

INTRODUCTION

Acne is a skin disorder characterized by inflammation such as comedones, papules, pustules, and nodules (Sutaria et al., 2023). The disease is caused by the gram-positive bacterium, *Propionibacterium acnes*. The treatment of the disease using antibiotic agents, such as erythromycin and clindamycin, has been reported to cause some side effects, and the bacteria also demonstrate resistance to some antibiotics (Dessinioti & Katsambas, 2022).

One of the most challenging problems in the treatment of bacterial infections is the increase in antibiotic resistance. Bacterial resistance to commercially available antibiotics has shown an increasing trend every year (Prestinaci et al., 2015). In 2017, the WHO released a list of emerging studies to obtain a new treatment for antibiotic-resistant bacteria. On this list, bacteria are classified into several categories based on their urgency to find a new therapy against this bacterial infection (Fischbach & Walsh, 2009). In addition, in the US, the Centers for Disease Control and Prevention (CDC) estimated the cost of antimicrobial

resistance to be \$ 55 billion annually: \$20 billion in excess of healthcare costs and \$35 billion in society cost for the loss of productivity (Prestinaci et al., 2015).

Following the invention of penicillin by Alexander Fleming in 1928, many antibiotic agents of various classes were invented and used to treat infections (Geisinger & Isberg, 2017). However, the misuse and overuse of antibiotics for decades has triggered an increase in antibiotic resistance (Lister et al., 2009). Therefore, the development of novel therapeutic strategies to treat infections is urgently required. In the past decade, several new strategies have been proposed as alternative therapies against infection.

Indonesia is a tropical country with the second highest plant biodiversity in the world after Brazil. This flora comprises many unique tropical plant varieties. It has a tropical climate and approximately 17,000 islands. Many plants have been reported to exhibit pharmacological activities in in vitro and in vivo studies.

According to recent studies, kipahit (*Tithonia diversifolia* A. Gray) leaves show potential for treating several diseases, for example, antidiabetic, antibacterial, and antiprotozoal effects. In addition, a recent study reported antibacterial activity of kipahit leaves against *P. acnes* (Purwaningsih et al., 2020). Therefore, to obtain better insight into the potency of the leaves, in this study, we evaluated the minimum inhibitory activity (MIC) of kipahit leaves on the growth of *P. acnes* using a macrodilution method.

RESEARCH METHOD

Equipment and Materials

The equipment and materials used in this study are as follows: sonicator (Powersonic® 610), incubator (Mettler®), autoclave (American®), oven (Mettler®), rotary evaporator (Ika®), clindamycin disc (Oxoid®), nutrient agar (Oxoid®), Mueller-Hinton Broth (Oxoid®), standard McFarland No. 0.5, *P. acnes*, 96% ethanol (Brataco®), \pm 1 month-old of kipahit leaves (cultivated from Banjarmasin, Indonesia in March 2023), Dragendorff, Mayer, AlCl_3 , FeCl_3 , H_2SO_4 .

Research Procedure

1. Phytochemical Screening

A standard protocol for phytochemical screening (Farnsworth, 1966) was used to investigate the secondary metabolites of kipahit leaves.

2. Extraction of Kipahit Leaves

Kipahit leaves were extracted following the protocol of Zahari et al. (2020). One kilogram of dry kipahit leaves was powdered to obtain a homogenous mass of leaves. Subsequently, the powder was extracted in a sonicator (40 kHz, 40 °C, 30 minutes) by adding 5 liters of 96% ethanol as solvent. The obtained extract was filtered using Whatman paper to separate the filtrate and the residue. The solvent was removed from the filtrate to obtain a dry extract in a rotary evaporator (50 C, 150 rpm).

3. Determination of The MIC

A standard protocol (macrodilution method) was performed to determine the MIC of the extract of kipahit leaf (CLSI, 2018; EUCAST et al., 2003; Tankeshwar, 2022). Five mL of stock solution of the dry extract (50 % b/v) was prepared in 1% DMSO. Serial dilutions of the extracts (25–0.78 %) were prepared by diluting the stock solution. DMSO 1% and clindamycin were used as the negative and positive controls, respectively. One milliliter of *P. acnes* suspension ($\sim 1 \times 10^8$ cfu/mL) was added to each tube containing the 0.5 mL extracts and 0.5 mL Muller-Hinton broth (MHB). An equal amount of *P. acnes* suspension was added to the control tube (containing only MHB media). The suspensions were mixed thoroughly and the tubes were sealed properly and incubated for 24 hours at 37 °C. All experiments were conducted aseptically in laminar airflow (LAF) and performed in triplicate.

4. Determination of The Bactericidal or Bacteriostatic Activity of The Extract

To further know the effect of the extract on the growth of the bacteria, 100 μ L of bacterial suspension obtained from the no-bacterial-growth tube containing the lowest concentration of the extract was transferred onto a nutrient agar (NA) plate. The plates were then incubated overnight at 37 °C (Collins et al., 1999).

RESULTS AND DISCUSSION

1. Phytochemical Screening

To investigate the secondary metabolites of kipahit leaves, a standard protocol for phytochemical screening was used. The results show that kipahit leaves contain flavonoids, alkaloids, saponins, steroid/triterpenoids, tannins, and phenols. Our result is consistent with a previously reported result (Fauziana, 2021), where the leaves contained all secondary metabolites except saponin (Table I). Although the leaves were cultivated from different islands in Indonesia, they did not affect the secondary metabolites of the leaves. This finding is interesting because another study on different species reported that a plant can produce different secondary metabolites depending on where the plant was cultivated. This is because of the different soil microbiota contributing to the different secondary metabolites of a plant (Schütz et al., 2021).

Table I. Phytochemical Screening of Kipahit Leaves

Secondary Metabolite	Result	Conclusion
Flavonoid	White precipitate	+
Alkaloid		+
- Dragendorff	Orange precipitate	+
- Mayer	White precipitate	+
Saponin	No foam	-
Steroid/Triterpenoid	Green-brown	+
Tannin	Pale yellow	+
Phenol	Dark red	+

2. Extraction of Kipahit Leaves

An ultrasound-assisted extraction (UAE) was performed to obtain an ethanolic extract of kipahit leaves. This method is recommended to increase the yield of plant extracts. In this study, we obtained a relatively high yield (12.14 %) compared to that obtained using the maceration method (8.73 %) (Figure 1) (Purwaningsih et al., 2020). The use of 96% ethanol as a solvent also increased the yield. Ethanol is a universal solvent that can be used to extract non-polar, semipolar, and polar compounds from kipahit leaves (Wendersteyt et al., 2021). On the other hand, UAE provides us with more efficient work where the method only requires 30 minutes to obtain the extract compared to 3 days of maceration. As reported previously, 30 minutes was the optimum extraction duration using the UAE method (Buanasari et al., 2019).

The UAE method is highly effective owing to at least two mechanisms: fragmentation and erosion. Fragmentation causes the particles of the leaf powder to decrease, thereby increasing the surface area of the leaves to interact with the solvent. Erosion is a constant vibration on the surface of the powder particles that breaks the binding between the secondary metabolites and the cell, resulting in a higher amount of

secondary metabolites being released into the solvent during extraction (Suhendar et al., 2020).

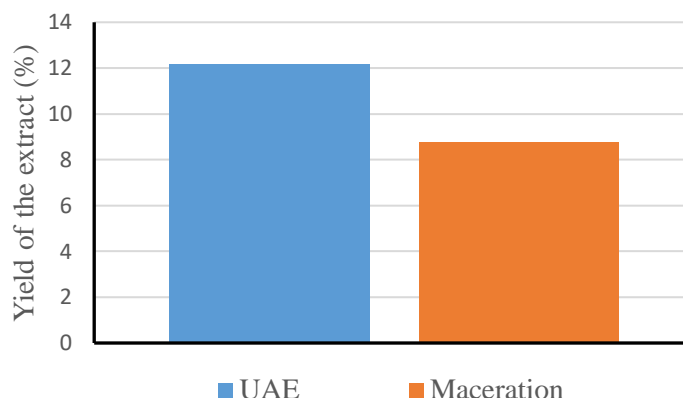


Figure 1. Yield Comparison of The Extract of Kipahit Leaves (%) Between UAE and Maceration Method

3. Determination of The MIC Value of The Extract

Purwaningsih et al., (2020) have reported a study on the activity of kipahit leaves on *P. acnes*. Therefore, as a follow-up study, we further investigated the lowest concentration at which the leaves were able to inhibit the growth of *P. acnes* through a macrodilution method following a standard protocol (CLSI, 2018). As mentioned above, we set up a series of concentrations of kipahit leaves (25–0.39 %) to determine the MIC of the extract.

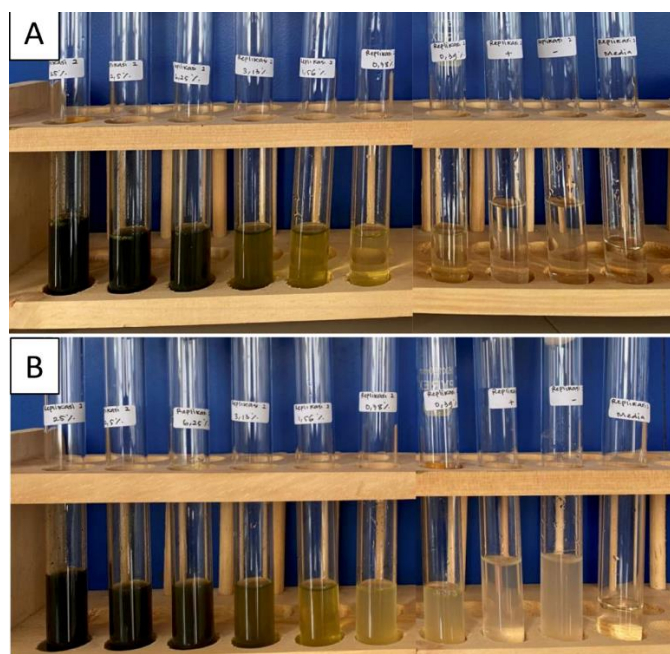


Figure 2. The Macrodilution Result of The Extract of Kipahit Leaves on The Growth of *P. acnes* was Before Incubation at 37°C for 24 h (A), and After Incubation at 37°C for 24 h (B). Clear Suspensions at Concentrations of 25 to 1.56% of The Extract, Positive and Media Control Indicate No Bacterial Growth. Turbid Suspensions at

Concentrations of 0.78 and 0.39% of The Extract and Negative Control Indicate Bacterial Growth After Overnight Incubation at 37°C

The result shows after overnight incubation at 37 °C, in the tubes containing kipahit extract at concentrations of 25 to 1.56% no turbidity was observed with unaided eye compared to the control tube. This indicated that *P. acnes* did not grow in tubes containing the aforementioned concentrations of the extract (CLSI, 2018). This means that the extract effectively inhibited the growth of the bacteria at these concentrations, and the lowest concentration of the series was 1.56%. Meaning concentration of 1.56% is the MIC value of kipahit leaves extract for *P. acnes* (Tankeshwar, 2022). This result is consistent with a previously reported study, in which the extract inhibited the growth of *P. acnes* using an agar diffusion method (Purwaningsih et al., 2020).

The extract was dissolved in DMSO 1% prior to distribution into tubes to determine its MIC value. DMSO 1% was used because of its efficacy in dissolving plant extracts. Moreover, DMSO 1 % has been widely used as a solvent in antibacterial studies because it does not influence the growth of the tested bacteria (Brito et al., 2017). In this study, we used clindamycin as a positive control because it is the first-line drug for the treatment of acne vulgaris, especially that caused by *P. acnes* (Zaenglein et al., 2016). The result showed that the positive control confirmed that the bacteria were still sensitive to the existing antibiotic.

As mentioned above, the extract contained flavonoids, alkaloids, steroids, triterpenoids, tannins, and phenolic compounds. These secondary metabolites apparently contribute to the bacterial inhibitory activity of kipahit leaves.

Flavonoids have been reported to inhibit bacterial growth through at least 3 different mechanisms. Flavonoids are capable of interrupting the production of nucleic acids, blocking the ability of bacteria to produce energy through the inhibition of metabolism, and binding to the protein of the cell membrane, leading to cell lysis (Rahman et al., 2017; Shailla Gharnita et al., 2019).

Phenolic compounds inhibit bacterial growth by denaturing bacterial proteins. The hydroxyl groups of the compound are believed to contribute to this mechanism (Sapara and Waworuntu, 2016). Alkaloids interact with the peptidoglycan layer of bacterial cell membranes, leading to cell lysis. In addition, these compounds can also interact with the DNA of bacteria, resulting in cell death (Ramonah et al., 2020).

Tannins contribute to the inhibition of bacterial growth by inhibiting reverse transcriptase and DNA topoisomerases. These compounds also bind to polypeptides on the cell membrane, causing cell lysis (MARLIANA et al., 2005; Ramonah et al., 2020). The interaction of steroids with transmembrane proteins of the outer cell membrane causes damage to the porins. Damage resulting in a lack of nutrition in the cell leads to cell death (Rahman et al., 2017).

4. Determination of The Bactericidal or bacteriostatic activity of the extract

Even though the extract of kipahit leaves shows inhibition activity on the growth of *P. acnes* but the type of inhibition remains unknown. Therefore, we investigate the activity of the extract in a follow-up experiment. After incubation overnight at 37 °C, the inoculum of bacterial suspension (obtained from the tube containing 1.56 % of extract) grows on an NA plate (Figure 3). This result confirms that the ethanolic extract of kipahit leaves demonstrates a bacteriostatic agent on the growth of *P. acnes*.

Based on the results discussed above, a follow-up study to investigate and isolate active compounds with inhibitory activity against the growth of *P. acnes* is important. By isolating

the active compound, we can further determine its IC_{50} and set up in vivo studies, such as developing an animal model of infection, to evaluate the efficacy of the compound.

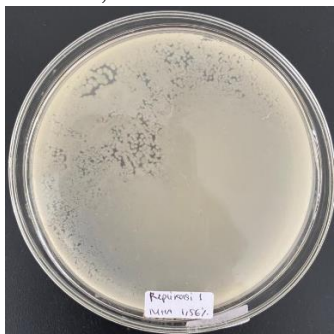


Figure 3. Tube No. 5 (1.56% of The Extract) was Transferred on to an NA Plate and Incubated Overnight at 37°C, The Result Shows The Growth of *P. acnes* Indicating at This Concentration The Extract Does Not Kill The Bacteria But Only Inhibits The Growth of The Bacteria

CONCLUSION

Kipahit (*Tithonia diversifolia* A. Gray) shows inhibition activity on the growth of *P. acnes*, and the minimum inhibitory concentration (MIC) value of the 96% ethanolic extract was 1.56 %.

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