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ORIGINAL RESEARCH

Evaluation of Antituberculosis Potential of n-hexane Extract and Ethanol Extract of Soursop Leaves (*Annona Muricata L.*) in Vitro

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ABSTRACT

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis*, primarily affecting the lungs and transmitted through droplets. Indonesia ranks second in the world for pulmonary TB cases. Due to increasing resistance to synthetic TB drugs, alternative treatments are needed, such as soursop leaves, which are known to treat symptoms like coughing and bleeding. This study aims to evaluate the potential of soursop leaf extract against TB. The method used in this study is the Lowenstein-Jensen method with varying concentrations. Sputum samples from TB-positive patients were identified using Zheil-Nelsen staining, while soursop leaf extract was obtained through percolation using n-hexane and ethanol fractions. The results showed that the moisture content of soursop leaf powder was 5%, meeting the simplisia standard. The antituberculosis test revealed resistance to synthetic drugs starting from the second week, while the n-hexane extract showed resistance from the first week. The ethanol fraction inhibited bacterial growth until the third week, but positive growth occurred in the fourth week. Therefore, soursop leaf extract, particularly the ethanol fraction, shows potential as an alternative TB treatment, which warrants further investigation.

Keywords: Antituberculasis, n-hexane extract, ethanol extract, soursop leaves

Introduction

Pulmonary tuberculosis (TB) is an infectious disease caused by the rod-shaped bacteria Mycobacterium tuberculosis. Although the lungs are the most commonly infected organ, these bacteria can also attack other organs. This disease is transmitted from one person to another through droplets from people with pulmonary TB (Sari et al., 2022). Tuberculosis is one of the direct infectious diseases caused by the bacteria Mycobacterium tuberculosis, and the main transmission comes from infected patients. Pulmonary TB is a serious threat to global public health, especially in developing countries. This disease ranks third as the leading cause of death in all age groups and is the number one infectious disease (Nurjannah et al., 2022). According to the Indonesian Ministry of Health report in 2020, the number of Tuberculosis cases in Indonesia reached 351,936. North Sumatra Province is one of the areas with the highest cases of Tuberculosis, with 33,779 cases reported by the North Sumatra Provincial Health Office in the same year. One preventive measure that can be taken is to provide information on the factors that influence the spread of Tuberculosis in North Sumatra (Santi et al., 2022).

According to the 2020 Global Tuberculosis Report from the World Health Organization (WHO), Indonesia ranks second after India in the number of TB cases globally. In 2014, 176,677 new cases of Tuberculosis were found confirmed through sputum tests, a decrease compared to 2013 which recorded 196,310 new cases. Provinces with large populations, such as West Java, East Java, and Central Java, reported the highest number of cases, with these three provinces contributing 40% of the total new TB cases in Indonesia (Depkes, 2013). The increase in TB cases is influenced by various individual factors, such as low patient compliance in undergoing treatment. This is due to the long duration of treatment, which takes at least six months (Suratmini dan Berliana Togatorop, 2023). As a result, many patients experience double

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resistance, reduced drug effectiveness in killing bacteria, and become patients who stop treatment, which ultimately complicates treatment and has the potential to cause double drug resistance (Maelani dan Cahyati, 2019). TB is also more easily spread in densely populated residential areas, with houses with minimal sunlight exposure. Traditionally, some plants can treat coughs with phlegm and blood which are used by drinking the results of soaking or boiling the plant material. Coughing with phlegm and blood is one of the symptoms of tuberculosis (Ummah dan Akbar, 2019). The plant material may be effective as an anti-tuberculosis, one of which is soursop leaves (*Annona muricata* L), but the way to use it is considered less popular because it is less practical and tastes sour and its volume is large (Kusmiyani et al., 2024).

The samples to be tested in this study consisted of sputum specimens from Tuberculosis patients. The sputum specimens were taken containing *Mycobacterium tuberculosis* from 3 patients who tested positive for Tuberculosis. Meanwhile, soursop leaf samples were taken based on purposive techniques without comparing them with similar plants from other areas. The chemical content of soursop leaves to be studied includes alkaloids, glycosides, flavonoids, saponins, tannins, and steroids/triterpenoids. These compounds can function as disinfectants and antiseptics, so it is possible that plants containing these compounds can be used as antibacterials, especially for Mycobacterium tuberculosis (Rizky et al., 2024).

A study conducted by Irmawati (2019) stated that chemical compounds in the form of alkaloids in soursop leaves have antibacterial activity by damaging cell walls through the components of peptidoglycan in bacterial cells. In addition, the tannin content in soursop leaves has antibacterial activity by precipitating proteins, inactivating enzymes, and destroying or inactivating genetic material. This shows that the higher the concentration of tannin given, the better the antibacterial activity (Irmawati, 2019).

Materials and Methods

Materials and Tools

The tools used in this study include: laboratory glassware, aluminum foil, a bunsen burner, wool thread, a petri dish, a porcelain dish, a funnel, cotton bud, an incubator, ose needle, gauze, parchment paper, a stove, an oven, water bath, dropper, tweezers, gas stamper and mortar, spatula, scales, rotary vacuum.

The plants used in this study were soursop leaves that were selected intentionally (purposive sampling). In addition to soursop leaves, other ingredients used include distilled water, eggs, and proanalytical quality chemicals from E'Merck, namely 80% ethanol, toluene, chloroform, Lowenstein-Jensen (LJ) media, glacial acetic acid, hydrochloric acid, nitric acid, sulfuric acid, benzene, bismuth nitrate, ethyl acetate, isopropanol, iodine, potassium iodide, iron(III) chloride, potassium hydrogen phosphate, malachite green, methylene blue, fuchsin, magnesium citrate, sodium glutamate, glycerin, toluene, rifampicin, ethambutol, and isoniazid.

Methods

Determination of Test Materials

Identification or determination of plant types was carried out at the Plant Systematics Laboratory of the Medanense Herbarium (MEDA) University of North Sumatra.

Preparation of Test Materials

Soursop leaves that have been collected go through a wet sorting process and are cleaned. After that, the leaves are sliced and spread on paper, then allowed to dry either in an open or closed room for about 10 days. After that, the leaves are put into a simplicia drying cabinet with a temperature of around 60°C-70°C for approximately 3 days, until the leaves become dry which is indicated by a brittle condition when broken. Furthermore, the simplicia obtained is ground and tested for the characteristics of the simplicia.

Preparation of Soursop Leaf Extract

A total of 1 kg of soursop leaf simplicia powder is put into a closed dark-colored container, then n-hexane solvent is added until all the simplicia powder is well submerged. The container is tightly closed and left for 3 hours, protected from light. After that, the simplicia powder is slowly transferred into a percolator by pressing it carefully. The n-hexane solvent was added sufficiently until the liquid began to drip and there was still a layer of n-hexane above the simplex. The percolator was closed and left for 24 hours.The simplex was squeezed using gauze to separate the liquid. The remaining simplex was then percolated with ethanol solvent, ensuring that all the simplex was submerged. The percolation container was closed and left for 3 hours, protected from light. After that, the liquid drop rate was adjusted to 1 ml per minute, with ethanol added through the reservoir tube sufficiently so that there was still a layer of ethanol above the simplex. The percolation process was continued until the percolate liquid that came out was no longer colored, producing ethanol extract.The results of each extraction obtained were concentrated with a rotary evaporator to obtain a thick extract, then dried by cold drying using a freeze dryer (temperature ± 40 ℃) for 24 hours, so that a thick n-hexane extract and a thick ethanol extract were obtained.

Phytochemical Screening

Phytochemical screening was conducted to identify secondary metabolite compounds contained in the powdered simplicia and ethanol extract of soursop leaves including alkaloids, glycosides, flavonoids, saponins, tannins, steroids/triterpenoids.

Preparation of Drug Materials (Comparator) and Test Materials

In this antimicrobial activity test, the test material and reference drug were mixed in Lowenstein-Jensen (LJ) media with various concentrations to evaluate their effectiveness against microorganisms. Each drug material was dissolved and diluted to the desired concentration. The goal was to determine the minimum threshold of the effective concentration of the test material against the target bacteria and compare it with the effectiveness of the standard drug (Japan International Cooperation Agency).

Table 1. *Mycobacterium tuberculosis* test material

No	Drug substance (control)/test substance	Drug substance/test substance concentration	Amount of substance taken (ml)	Amount of LJ media	Concentration of substance in LJ media
1.	Rifampicin	4000 µg/ml	1	100	40 µg/ml
2.	Ethambutol	1000 µg/ml	1	100	10 µg/ml
3.	Isoniazid	20 µg/ml	1	100	0,2 µg/ml
4.	Ethanol extract of soursop leaves	250 mg/ml	5	50	25 mg/ml
5.	Ethanol extract of soursop leaves	200 mg/ml	5	50	20 mg/ml
6.	Ethanol extract of soursop leaves	100 mg/ml	5	50	10 mg/ml
7.	Ethanol extract of soursop leaves	50 mg/ml	5	50	5 mg/ml
8.	Soursop leaf n-hexane extract	250 mg/ml	5	50	25 mg/ml
9.	Soursop leaf n-hexane extract	200 mg/ml	5	50	20 mg/ml
10.	Soursop leaf n-hexane extract	100 mg/ml	5	50	20 mg/ml
11.	Soursop leaf n-hexane extract	50 mg/ml	5	50	5 mg/ml

Data Analysis

This study used experimental tests and descriptive analysis which aimed to describe the characteristics of the simplicia and the results of phytochemical screening.

Result and Discussion

Extraction Results

The extraction process was carried out using 1000 grams of soursop leaf simplicia with the percolation method. Two solvents were used in this study: n-hexane and 80% ethanol. The extraction with n-hexane produced a thick blackish-green extract weighing 33 grams, while the extraction with 80% ethanol yielded a thick blackish-green extract weighing 157 grams. The percentage of yield was calculated to determine the efficiency of the extraction process for each solvent.

Based on the extraction results, it was found that the percentage of n-hexane extract yield in this study was 3,3%, while the percentage of ethanol extract yield was 15,7%. Based on the requirements of the herbal pharmacopeia, the requirement for the yield of the thick extract is not less than 10% (Kementerian Kesehatan Republik Indonesia, 2017). This shows that the percentage of n-hexane extract yield is less than the requirements of the herbal pharmacopeia, while the percentage of ethanol extract yield meets the requirements of the Indonesian herbal pharmacopeia.

Phytochemical Screening Results

From the results of phytochemical screening that has been carried out on fresh soursop leaves, soursop simplicia, n-hexane extract of soursop leaves, and ethanol extract of soursop leaves. Based on the results of phytochemical screening of ethanol extracts, fresh leaves and soursop leaf simplicia contain alkaloids, flavonoids, saponins, tannins, steroids/triterpenoids, and glycosides. Ethanol extract contains alkaloids, flavonoids, tannins, saponins, steroids, and glycosides. While the n-hexane extract only contains alkaloids, and steroids/triterpenoids. This is likely to occur because the ethanol extract can attract secondary metabolites present in whereas the n-hexane extract cannot attract secondary metabolites.

Table 2. Results of phytochemical screening of soursop leaves, soursop simplex, n-hexane extract, and ethanol extract

No	Phytochemical Screening Examination	Results			
		Fresh soursop leaves	Soursop simplicia	Soursop n-hexane extract	Soursop ethanol extract
1.	Alkaloids	+	+	+	+
2.	Flavonoids	+	+	-	+
3.	Saponin	+	+	-	+
4.	Tannin	+	+	-	+
5.	Triterpenoids/Steroids	+	+	+	+
6.	Glycosides	+	+	-	+

Results of Identification of *Mycobacterium Tuberculosis* Bacteria

Identification of bacteria was carried out using the Zeihl-Nelsen staining method, this staining was carried out to see whether or not there were Mycobacterium tuberculosis bacteria in the patient's sputum to be used. The results of the bacterial staining were declared positive, by checking using a microscope using x100 magnification there were red bacteria in the form of bacilli/rods.

Results of Anti-Tuberculosis Activity Test

The test results showed that ethanol extract was more effective in inhibiting the growth of Mycobacterium tuberculosis than n-hexane extract, possibly because active compounds such as alkaloids, flavonoids, saponins, and tannins were extracted more by ethanol. Before the addition of the test material, the Lowenstein-Jensen medium was green due to 2% malachite green as an indicator. After the addition of

the test material, there was no change in color in the media, which remained green as before the test material was given.

Table 3. Anti-Tuberculosis activity test results

Test material	Concentration	Colony growth in specimen A week to				Colony growth in specimen B week to				Colony growth in specimen C week to			
		1	2	3	4	1	2	3	4	1	2	3	4
Control	0 µg/ml	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Rymphamphycin	40 µg/ml	1+	3+	4+	4+	1+	3+	4+	4+	1+	3+	4+	4+
Ethambutol	10 µg/ml	1+	3+	4+	4+	1+	3+	4+	4+	1+	3+	4+	4+
Isoniazid	0,2 µg/ml	1+	3+	4+	4+	1+	3+	4+	4+	1+	3+	4+	4+
N-hexane extract	25 mg/ml	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	20 mg/ml	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	10 mg/ml	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	5 mg/ml	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Ethanol extract	25 mg/ml	-	-	-	-	-	-	-	-	-	-	-	-
	20 mg/ml	-	-	-	-	-	-	-	+1	-	-	-	-
	10 mg/ml	-	-	-	+1	-	-	-	+2	-	-	-	+2
	5 mg/ml	-	-	-	+2	-	-	-	+2	-	-	-	+2

The scientific investigation explored the complex interactions between *Mycobacterium tuberculosis* and various anti-tuberculosis interventions through a meticulously designed experimental protocol. Researchers collected bacterial specimens from tuberculosis-infected volunteers and cultivated them on specialized Ogawa media, employing a comprehensive approach to understanding bacterial resistance mechanisms. The experimental design incorporated synthetic anti-tuberculosis drugs like rifampicin, ethambutol, and isoniazid as positive control measures, with additional negative control media to establish baseline bacterial behavior. Throughout the four-week observation period, the research team documented a fascinating progression of bacterial growth and resistance patterns.

Initial observations revealed a consistent and concerning trend across all three bacterial specimens. The bacterial growth demonstrated a systematic escalation, starting with minimal positive growth in the first week (+1), dramatically increasing to significant positive growth in the second week (+3), and ultimately reaching maximum proliferation in the third and fourth weeks (+4). Researchers critically analyzed potential factors contributing to this drug resistance. Their findings suggested multiple interconnected reasons, including patient-related issues such as medication non-compliance, irregular treatment schedules, and inconsistent drug administration. This observation highlighted the complex human factors that can significantly impact tuberculosis treatment effectiveness.

The extract analysis provided particularly intriguing insights. The n-hexane extract showed consistent resistance throughout the experimental period, effectively demonstrating its limitations as an antibacterial agent. In stark contrast, the ethanol extract exhibited a more nuanced response, with no bacterial growth observed during the initial three weeks and minimal growth emerging only in the fourth week (Vicente-Zurdo et al., 2024). The superior activity of the ethanol extract can be attributed to its ability to extract a broader range of active phytochemical compounds (Kusuma dan Nur, 2019). Compounds such as alkaloids, flavonoids, saponins, and tannins, which are known for their antibacterial properties, were likely responsible for the observed inhibitory effects on Mycobacterium tuberculosis. This highlights the role of ethanol as an effective solvent for isolating bioactive compounds from soursop leaves (Wijayanti et al., 2023).

Dose-dependent experiments with the ethanol extract unveiled a complex relationship between concentration and bacterial inhibition. At lower concentrations (5 and 10 mg/ml), bacterial growth remained relatively active, with variations across different specimens. However, a critical breakthrough occurred at 25 mg/ml, where the extract successfully inhibited Mycobacterium tuberculosis growth. The findings emphasize the importance of phytochemical profiling in understanding the efficacy of natural extracts.

Furthermore, the absence of color change in the Lowenstein-Jensen medium, which remained green throughout the study, confirmed the inhibitory activity of the ethanol extract rather than bactericidal effects, pointing toward its potential role as a growth inhibitor (Warnis et al., 2023).

The research's most significant contribution lies in its demonstration of soursop leaf ethanol extract's potential as an alternative antibacterial intervention. By achieving optimal bacterial growth inhibition at specific concentrations, the study opens promising avenues for exploring natural compounds in tuberculosis treatment. Beyond the immediate findings, the research underscores the importance of comprehensive, multi-faceted approaches to understanding bacterial resistance. It emphasizes not only the scientific methodology but also the critical role of patient behavior and treatment adherence in managing complex infectious diseases like tuberculosis. This investigation represents a valuable contribution to microbiological research, offering insights that could potentially inform future therapeutic strategies, drug development, and our understanding of bacterial resistance mechanisms.

Conclusion

The research on the anti-tuberculosis potential of ethanol and n-hexane extracts from soursop leaves (*Annona muricata L.*) against *Mycobacterium tuberculosis* revealed that the ethanol extract, rich in alkaloids, flavonoids, tannins, saponins, steroids, and glycosides, demonstrated significant antimicrobial activity and robust bacterial growth inhibition, highlighting its potential as an alternative treatment. In contrast, the n-hexane extract, containing only alkaloids and steroids, showed minimal to no antibacterial efficacy. These findings emphasize the role of solvent selection in phytochemical research and underscore the promise of plant-based therapies for infectious diseases like tuberculosis.

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