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Nanoparticle Synthesis of Secang Wood Extract (*Caesalpinia sappan* L.) as Antibacterial Agent

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Abstract. Indonesia is a country rich in various types of plants that can be used as medicinal ingredients, including traditional medicine, herbs, and jamu. The sappanwood plant is one of the widely used plants in traditional medicine. The sappanwood plant contains chemicals such as flavonoids, brazilin, alkaloids, saponins, tannins, phenylpropane, and terpenoids. The need for metabolite compounds has recently increased significantly to overcome resistance to bacteria. Infectious diseases in Indonesia are still among the ten highest diseases, so in 2050 deaths due to antibiotic resistance will reach 10 million per year and become the highest cause of death among other causes. The methodology of this study began with sample preparation, making sappanwood extract, isolating secondary metabolite compounds using preliminary TLC, synthesizing nanoparticles using the ionic gelation method, and testing antibacterial activity using *P. acnes* and *P. aeruginosa* bacteria. The results of the study revealed that the synthesis of nanoparticles showed moderate antibacterial activity with an inhibition zone of 8.15 mm against *P. acnes* and 9.1 mm against *P. aeruginosa*. In contrast, ethyl acetate extract showed a stronger antimicrobial effect, reaching inhibition zones of 13.02 mm and 19.54 mm, respectively. This indicates that the activity shown is relatively strong due to the difference in concentration used during the study. It is expected that further research can produce a larger antibacterial inhibition zone than ethyl acetate extract.

Key words: Sappanwood, synthesis, and antibacterial activity

INTRODUCTION

Indonesia is a country with a rich variety of flora that can be utilized as medicinal materials, including traditional medicines, herbs, and herbal medicine. One notable example is the secang plant (*Caesalpinia sappan* L.), which is frequently employed as a traditional medicinal ingredient. The specific part of the ecang plant that is utilized as a medicinal ingredient is the wood, either in pieces or as wood shavings [1].

The following classification system is employed for secang plants:

Kingdom : *Plantae*
Division : *Tracheophyta*
Class : *Magnoliopsida*
Ordo : *Fabales*
Familia : *Caesalpiniaceae*
Genus : *Caesalpinia*
Species : *Caesalpinia sappan L.*

Sappan wood plants have been found to contain a variety of chemical constituents, including flavonoids, brazilin, alkaloids, saponins, tannins, phenyl propane, and terpenoids [2]. A multitude of researchers have documented the biological activities of various secang plants, including antioxidant, anticancer, anti-implantation, hypoglycemic, hepatoprotective, and antibacterial properties [3].

The demand for metabolite compounds has recently increased significantly in order to overcome bacterial resistance. The excessive use of antibiotics, in conjunction with their improper usage, has been demonstrated to expedite the proliferation of antibiotic-resistant bacteria and the genes that confer resistance to them [4].

Antibiotic resistance in Indonesia is a multifaceted problem with the potential to worsen annually. The uncontrolled utilization of antibiotics has led to the proliferation of antibiotic resistance in developing countries. Infectious diseases are a major cause of mortality, accounting for over 13 million deaths annually in these regions. Indonesia is no exception, with infectious diseases ranking among the top ten causes of death. Projections indicate that by 2050, deaths attributable to antibiotic resistance will reach 10 million per year, becoming the leading cause of death among all other causes [5].

Nanotechnology is a technology that increases the penetration, absorption, and bioavailability of active substances by reducing their particle size. This technology has had a profound impact on the pharmaceutical and cosmetic industries, particularly in terms of delivery mechanisms for active ingredients. The use of nanoparticles has been shown to enhance the absorption of active ingredients into the skin by increasing the rate of diffusion from the carrier system towards its surface. One notable application of nanotechnology involves the use of nanoparticles [6].

Nanoparticles are particles measuring between 1 and 100 nanometers in size. The advantage of nanoparticles is that they can penetrate intercellular spaces that colloidal particles cannot. Another advantage is the increase in system affinity due to an increase in contact surface area at the same amount [7]. There are many types of nanoparticle use in drug delivery systems, one of which is chitosan nanoparticles. Chitosan, a cationic polymer, reacts with multivalent anions,

such as tripolyphosphate, and possesses unique polyelectrolyte properties, including the capacity to form gels, biodegradability, biocompatibility, and non-toxicity to living tissues. These properties render it a suitable agent for use in drug delivery systems. A substantial body of research and development has been dedicated to investigating the potential of chitosan as a high-potential antibacterial material, with the ability to modify its structure to achieve desired properties and functions [8].

According to the aforementioned description, the authors are interested in conducting research related to the nanoparticle synthesis of secang wood extract (*Caesalpinia sappan* L.) as an antibacterial agent. The primary objective of this research is to develop nano-sized active ingredients derived from natural materials, particularly secang wood extract, and to assess the antibacterial activity of flavonoid compounds.

METHODS

The instruments employed in this study include an analytical balance, a beaker glass (Pyrex), a measuring cup (Pyrex), a volumetric flask (Pyrex), a glass stirring rod, a KLT chamber, a thermometer, a Buchner funnel, a vacuum pump, a Buchner flask, a wooden stirrer, a capillary pipe, separatory funnel, stative, ring clamp, erlenmeyer flask (pyrex), magnetic stirrer, hotplate (Thunder PS 10A), laminar air flow (B-ONE V 915 S), incubator (Memmert), vacuum rotary evaporator (Heidolph), and Whatman filter paper no. 1, vial bottles, glass vials. 1, vial bottle, glass maceration bottle, micro pipette, autoclave, microtube, microplate, refrigerator, digital vernier, spreader, cotton bud, tweezers, sudip, watch glass, test tube (pyrex), suction ball, volume pipette (pyrex), drop pipette, ose needle, vortex mixer, UV lamp, PSA (particle size analyzer) (Horiba SZ-100) and Zeta Sizer/potential.

The materials utilized in this study encompassed Secang wood powder, Ethyl Acetate extract, aluminum foil, 96% Ethanol, chitosan, Ethyl Acetate, N-Hexane, Silica gel 60 F254 plate, Mueller Hinton Agar (MHA) (Oxoid CM 0337), Mueller Hinton Broth (MHB) (Himedia), distilled water, 0.9% physiological NaCl, 90 mm petridish dish (OneLab), 100% Dimethylsulfoxide (DMSO), and 0.5 Mc. The Farland Standard, composed of 70% ethanol, 1% NaTPP, aquabidest, Tween-80, glacial acetic acid (p.a.), Oxidoid blank disk, plastic cling wrap, chloramphenicol, and *Propionibacterium acnes* and *Pseudomonas aeruginosa* bacteria, was utilized in the experiment.

Sample Preparation

A quantity of 1 kilogram of sappan wood powder was obtained from a reputable establishment specializing in jamu and herbal products, situated in the vicinity of Medan Mall in Medan City, North Sumatra.

Preparation of Secang Wood Extract

Sappan wood powder, with a mass of 1 kilogram, was extracted by maceration method with 96% ethanol in a glass maceration jar. The extraction process was continued until a clear solution was obtained, at which point it was deemed sufficient. This is defined as the point at which all extracts are considered to have been dissolved by the solvent. The solvent was changed every 1x24 hours. It is imperative to stir the mixture occasionally to enhance the interaction between the sample and the solvent, thereby facilitating the release and dissolution of the active compounds contained within the sample into the solvent. In this extraction process, the solvent replacement was conducted for 3x24 hours for a solvent that had already become clear. The three macerates were subsequently amalgamated and concentrated in a rotary evaporator at 50°C. The concentrated extract was weighed and

Separation of Secondary Metabolite Compounds

The thick extract of secang wood was separated by thin-layer chromatography (TLC).A total of 20 grams of ethanol extract of secang wood was fractionated and then separated by KLT by bottling the extract on silica gel 60 F_{254}, with several eluent comparisons of N-hexane and ethyl acetate.The compounds formed from the KLT process were then examined.

Nanoparticle Synthesis

Synthesis of nanoparticles using the ionic gelation method. These nanoparticles were then characterized using a PSA test and a Zeta Sizer/potential.

Antibacterial Activity Test

The experiment commenced with the weighing of 100 milligrams of the sample, followed by its dissolution in 1000 µg/mL of DMSO (dimethyl sulfoxide) at a concentration of 100%. The sample was then vortexed, and 100 µg/mL of the vortexed sample was taken and subsequently diluted with 900 µg/mL of DMSO to obtain a 1% sample solution in 10% DMSO3. In this study, Chitosan-Flavonoid nanoparticle samples and a thick Ethyl Acetate extract were utilized. Subsequently, bacterial rejuvenation was initiated, and bacterial inoculum was prepared and measured. The inhibition zone formed was determined using the disc diffusion method.

RESULT AND DISCUSSION

Sample Extraction

The extraction process of secang wood (*Caesalpinia sappan* L.) samples was executed through the utilization of the maceration method, a technique that was selected due to its simplicity and the absence of the necessity for thermal treatment. This method was selected to preserve the stability of active compounds that are heat-labile. Moreover, this method is regarded as more economical and pragmatic. The extraction process in this study involved the use of ethanol, a polar solvent, as the extraction agent.

The maceration process on secang wood is carried out by soaking approximately 1 kg of powdered samples in an ethanol solvent for a period of 24 hours, with three repetitions. The macerated sample is then stored in a closed container, with the utmost care taken to ensure it is protected from direct sunlight. The container is covered with aluminum foil to prevent the evaporation of the solvent. To enhance the interaction between the sample and the solvent, occasional stirring is necessary to facilitate the release and dissolution of the active compounds contained within the sample into the solvent. During the soaking process, plant samples undergo a disruption in cell walls and membranes due to the pressure differential between the interior and exterior of the cells, resulting in the dissolution of secondary metabolites present in the cytoplasm into organic solvents. During this maceration process, ethanol enters the cell through the cell wall and dissolves the secondary metabolite compounds contained within. Subsequent filtration of the solution yields a filtrate volume of up to 4.5 liters, which exhibits a reddish-brown coloration.

Figure 1: Filtrate of Secang Wood

The filtrate obtained is then subjected to concentration using a rotary evaporator, thereby yielding the concentrated extract. This evaporator process is carried out with the objective of removing the solvent.

Liquid-Liquid Fractionation

The subsequent stage involves the application of organic solvents exhibiting varying degrees of solubility, specifically n-hexane and ethyl acetate.The utilization of a separatory funnel in liquid-liquid extraction aims to differentiate between compounds with disparate polarities present within the extract. The employment of ethanol as a solvent in the maceration process is predicated on its ability to dissolve the vast majority of secondary metabolite compounds. However, the presence of diverse secondary metabolite compounds within the ethanol extracts necessitates the implementation of a fractionation process to purify them. The fractionation process involves the use of solvents that vary in their degree of polarity, as fractionation is essentially the extraction of compounds using two solvents with different polarity properties.

A total of 20 grams of a thick extract of secang wood (Caesalpinia sappan L.) was dissolved in distilled water (100 ml) and then added to a non-polar solvent (n-hexane) (100 ml). The mixture was then placed into a separating funnel and agitated to form two layers. The upper layer, which contains the n-hexane, is collected, and the lower layer, which contains the water and ethanol, is added to a semi-polar solution (ethyl acetate) containing 100 ml. The mixture is shaken and allowed to stand, resulting in the formation of two upper layers of ethyl acetate and a lower layer of water ethanol. The water ethanol layer and the ethyl acetate layer are then separated.

This shaking and separation process is repeated up to four times to ensure the complete extraction of all semi-polar compounds. Ethyl acetate has been shown to have a high affinity for the dissolution of compounds such as flavonoids and polyphenols, which are frequently found in sappan wood extract. Following the completion of the separation process, the n-hexane, ethyl acetate, and ethanol-water fractions were subjected to a rotary evaporator to remove the solvent, resulting in the formation of a thick fraction containing the active compounds. This solvent evaporation process is intended to yield compounds in a more concentrated form, thereby preparing them for subsequent analysis.

Table 1. The weight of the condensed fraction of Secang wood is as follows:

Secang Wood Viscous Extract (g)	Ethyl Acetate Viscous Fraction (g)	Viscous Fraction n-hexane (g)	Ethanol:water Viscous Fraction (g)
20	7,425	0,2	1,3012

The results of the fractionation process indicated that the ethyl acetate fraction (7.425 grams) was the most substantial, suggesting that secang wood is abundant in semi-polar compounds, such as flavonoids. Conversely, the distilled water fraction (1.3012 grams) exhibited a comparatively lower content, attributable to its comparatively lower concentration of polar compounds relative to semi-polar compounds. The n-hexane fraction (0.2 grams) was minimal, suggesting a low abundance of non-polar compounds. This disparity in amounts indicates that secang wood contains a greater proportion of semi-polar compounds compared to other compounds.

Compound Isolation

The initial process of isolation and purification of compounds in the thick fraction of secang wood (*Caesalpinia sappan* L.) was executed through preliminary thin layer chromatography (PTLC). The purpose of PTLC is to ascertain the most suitable solvent for flavonoid

compounds. In PTLC, the components in the mixture are separated based on their affinity for the stationary and mobile phases. The stationary phase employed in this study was a silica gel thin layer plate, while the mobile phase was a non-polar solvent. The separation process focused on the spots formed on the thin layer plate. In addition, preliminary KLT facilitates the identification of components in the sample through detection methods, such as spot highlighting using ultraviolet (UV) light.

In this study, two types of eluents with different properties were utilized: n-hexane (nonpolar) and ethyl acetate (semi-polar). The selection of these three distinct eluents was made with the objective of identifying components in the sample based on their polarity properties. The KLT plate, which employs a silica gel 60 F254 plate measuring 7 cm x 6 cm with upper and lower limits of approximately 0.5 cm, is utilized. The KLT process commences with the transfer of the sample to the prepared plate using a capillary pipette. The samples utilized in this study are pure compounds of secang wood, ethyl acetate fraction, n-hexane fraction, and water. The pure compound is utilized as a reference point to facilitate the analysis of the results obtained from the three fractions that are to be identified.

Subsequently, the plate that has been administered a specimen is eluted with 5 milliliters of eluent within a chamber. In this study, elution was carried out with various eluent comparisons, such as n-hexane:ethyl acetate (1:9; 2:8; 3:7; 4:6; 5:5).

Prior to the initiation of elution, the eluent is subjected to saturation with the objective of eliminating air entrapped within the chamber, thereby ensuring the stability of eluent movement during elution and facilitating optimal sample separation. The elution process was halted when the eluent reached the top of the KLT plate. Subsequent to this, the elution results on preliminary KLT with various eluent comparisons were viewed using a UV lamp at a wavelength of 245-366 nm. The utilization of UV lamps is instrumental in the detection of spots or bands formed on the KLT plate post-eluition, thereby facilitating the more precise identification of the components present in the sample.

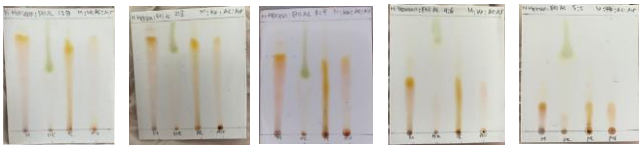


Figure 2. KLT results of Secang Wood Fraction (*Caesalpinia sappan* L.) with Comparison of N-Hexane: Ethyl Acetate (1:9; 2:8; 3:7; 4:6; and 5:5).

Nanoparticle Synthesis of Ethyl Acetate Extract of Secang Wood (Caesalpinia sappan L.)

A solution of 40 mL of chitosan-flavonoid and 1 mL of 1% sodium tripolyphosphate (NaTPP) was prepared, along with a few drops of Tween-80, at room temperature (280-300°C). The solution was then subjected to magnetic stirring at a speed of 1600 rpm for a duration of 2 hours. This process resulted in the formation of a suspension of nanoparticles. The ionic gelation method is employed in this study to prepare the nanoparticles, with the use of NaTPP and Tween-80 aiming to prevent the formation of aggregates and to stabilize the nanoparticles that are formed.The formation of brazilin nanoparticles isolated from chitosan can be seen qualitatively by observing a change in the color of the solution to a greater turbidity and the production of colloids [9].

Figure 3: Nanoparticle synthesis results of sappan wood extract

Nanoparticle Characterization of Secang Wood Ethyl Acetate Extract

In addition, the PSA (Particle Size Analyzer) test was conducted to ascertain the size of the particles formed, and the Zeta Sizer was utilized to assess the stability of the nanoparticles formed. This process was executed with three replicates. The mean value obtained from the PSA test was 1289.1 nm.

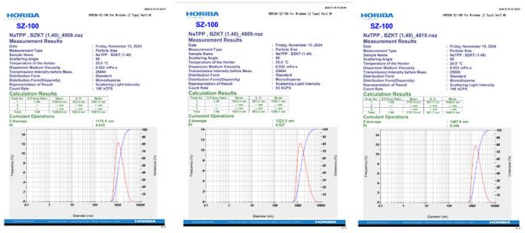


Figure 4. PSA test results (a); (b); (c) replicate repetitions 1, 2, and 3

In accordance with the findings of this study, it has been determined that the results obtained thus far have not yielded nanoparticles, and instead remain in the micro-size range. To further characterize the samples, the Zeta potential/Zeta Sizer instrument has been utilized.

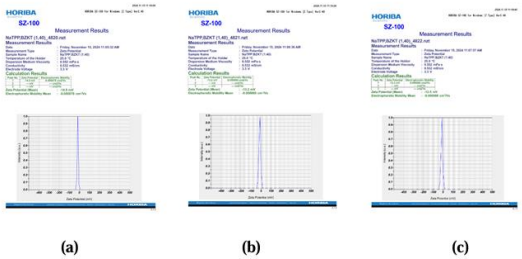


Figure 5. Zeta Potential Characterization Results

Zeta potential characterization was conducted to assess the stability of the formed nanoparticles. The analysis

yielded an average value of -13.53 mV, indicative of stability.

Antibacterial Activity Test

Antibacterial compounds are defined as chemical or biological compounds, both natural and synthetic, that are capable of impeding the growth and activity of bacteria. The classification of antibacterials can be based on the cellular components or systems affected, with agents that cause cell death (i.e., bactericidal agents) and those that merely inhibit cell growth (i.e., bacteriostatic agents) being two examples of this classification. The study of antibacterial activity can be approached through various methodologies, including the dilution method, agar diffusion method, and diffusion-dilution method. The diffusion method is a frequently used technique for analyzing antibacterial activity. The diffusion method can be executed in three different ways: the well method, the disc method, and the cylinder method. The observation results obtained are in the form of the presence or absence of clear areas formed around the disc paper, which shows the inhibition zone on bacterial growth [10].

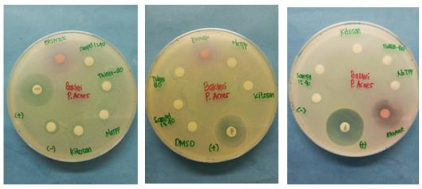
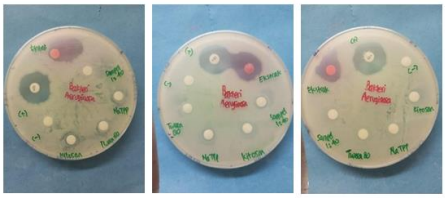


Figure 6. ZOI results on P.acnes bacteria

Table 2. ZOI values for P. acnes bacteria

Sample Name	Replica 1 (mm)	Replica 2 (mm)	Replica 3 (mm)	ZOI Average (mm)
DMSO (-)	0	0	0	0
Chloramphenicol (+)	21,8	18,9	24,95	21,88
Synthesis result (1:40)	8,6	7,1	8,75	8,15
Ethyl acetate extract	10,83	11,1	17,13	13,02
Chitosan	8,4	7,8	8,15	8,11
Tween-80	10,4	6,45	7,8	8,21
NaTPP	6,45	9	8,2	7,88

Description: 2-5 mm (weak); 5-10 mm (medium); 10-20 mm (strong); and >20 mm (very strong).



Gambar 7. Hasil ZOI pada bakteri *P. aeruginosa*

Table 3. ZOI values for *P. aeruginosa* bacteria

Sample Name	Replica 1 (mm)	Replica 2 (mm)	Replica 3 (mm)	ZOI Average (mm)
DMSO (-)	0	0	0	0
Chloramphenicol (+)	20,45	20,6	19,1	20,05
Synthesis result (1:40)	8,5	9,35	9,45	9,1
Ethyl acetate extract	21,16	19,43	18,03	19,54
Chitosan	11	10,15	8,7	9,95
Tween-80	9,2	10	9,35	9,51
NaTPP	9,15	8,9	10,1	9,38

Keterangan: 2-5 mm (lemah); 5-10 mm (sedang); 10-20 mm (kuat); dan >20 mm (sangat kuat)

According to Gisvold (1982) in Sabir (2005), flavonoids have been shown to cause damage to the permeability of bacterial cell walls, microsomes, and lysosomes as a result of the interaction between flavonoids and bacterial DNA.

Naim (2004) further asserts that flavonoids possess lipophilic properties, enabling them to compromise bacterial cell membranes. The extant research demonstrates that microparticles and flavonoid extracts from secang wood have antibacterial activity against P. acnes bacteria by 8.15 mm and 13.02 mm, respectively, and against P. aeruginosa bacteria by 9.1 mm and 19.54 mm, respectively. This finding indicates that the ethyl acetate extract exhibits a more pronounced antibacterial effect compared to the synthesized microparticles (1:40). The observed variation in the diameter of the inhibition zone can be attributed to the varying concentrations of microparticles and extracts utilized in the study, resulting in disparate mean diameters of the inhibition zone.

CONCLUSIONS

Based on the research that has been done, it can be concluded that the antibacterial activity of flavonoid nanoparticles from secang wood isolates (Caesalpinia sappan L.) against Propionibacterium acnes bacteria is 8.15 mm and Pseudomonas aeruginosa is 9.1 mm. This indicates that the activity shown is classified as moderate. While the diameter of the inhibition zone for ethyl acetate extract produced on P. acnes and P. aeruginosa bacteria was 13.02 mm and 19.54 mm, respectively. This is due to the different concentrations used during the study.

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