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ANTIBACTERIAL ACTIVITY OF NUTMEG SEED (*Myristica fragrans* Houtt.) METHANOL EXTRACT AGAINST THE GROWTH OF *Staphylococcus aureus* and *Eschericia coli*

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ABSTRACT

Nutmeg seeds (*Myristica fragran* Hout*t.*) are natural materials containing active compounds with potential antibacterial properties. This study aims to determine the antibacterial activity of nutmeg seed methanol extract and the effective concentration for inhibiting the growth of *S. aureus* and *E. coli*, as well as to compare the inhibition zones produced against both bacteria. The nutmeg seed extract was obtained through maceration extraction using 96% methanol solvent. The antibacterial activity was tested using the disc diffusion method with three different concentrations of the extract: 20%, 30%, and 40%, along with chloramphenicol (positive control) and 10% DMSO (negative control). The inhibition zone data were analyzed using ANOVA with SPSS 26, followed by the DMRT test. Phytochemical testing revealed that the methanol extract of nutmeg seeds positively contains alkaloids, saponins, flavonoids, quinones, and triterpenoids. The results showed that the methanol extract of nutmeg seeds at concentrations of 20%, 30%, and 40% produced inhibition zones against *S. aureus* of 11.83 mm, 12.56 mm, and 15.06 mm, respectively, and against *E. coli*, the inhibition zones measured 11.83 mm, 12.56 mm, and 15.06 mm. The study concludes that the methanol extract of nutmeg seeds exhibits strong antibacterial activity against *S. aureus* and *E. coli*, with the most effective concentration being 40%. Additionally, the nutmeg seed methanol extract tended to have better antibacterial properties against *E. coli* than against *S. aureus*.

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Introduction

The nutmeg plant (*Myristica fragrans* Houtt.) is native to Indonesia, originally flourishing in the Maluku Islands before spreading across North Sulawesi to Aceh. The province of Aceh has become one of the main nutmeg-producing regions, with its largest production in South Aceh District, where the plantations cover an area of 16,898 hectares, producing approximately 5,363.5 tons annually (Sari & Devi, 2022). Nutmeg is known as a high-value spice because every part of this plant can be utilized in various industries, including food and beverages, cosmetics, and medicine. The potential for developing nutmeg as a raw material for pharmaceuticals is significant, as several studies have demonstrated its biological activities, such as anti-inflammatory, antioxidant, antidepressant, anticancer, antifungal, and antibacterial properties (Suloi & Andi, 2021).

Antibacterial agents are substances capable of inhibiting the growth of bacteria and are specifically used to treat infections (Pommweville, 2020). Bacteria that cause infectious diseases in humans include *Escherichia coli* and *Staphylococcus aureus*. *Staphylococcus aureus* is a gram-positive bacterium typically found on the outer layers of the epidermis, in the nose, and throat. This bacterium can cause infections by producing enterotoxins and leading to the formation of pus in wounds, often encountered on the skin, mucous membranes, boils, and wounds. *Escherichia coli* is a gram-negative bacillus that resides in the human colon but can cause infections in the urinary tract, abdominal cramps, and diarrhea (Harti, 2015).

Antibiotics are one of the options for treating infectious diseases caused by bacteria. However, the increasing use of antibiotics has begun to pose new problems, particularly because most of the antibacterial agents used are harmful chemicals and are unsafe for health (Muntasir et al., 2021). To date, the management of diseases caused by bacteria still relies on synthetic antibiotics. This situation raises concerns about the emergence of new bacterial strains resistant to antibiotics (Kurnia et al., 2023), necessitating efforts to avoid such resistance by utilizing natural materials that can inhibit bacterial growth.

Several studies have reported that parts of the nutmeg plant have potential as antibacterials, such as leaves (Widyasfanny & Yusianti, 2003), fruit flesh (Siegrs et al., 2022), fruit skin (Gasareng et al., 2018), seed membranes (Wiguna, 2020), and nutmeg seeds (Syarifah et al., 2018). According to Panggabean et al. (2019), nutmeg seeds contain compounds such as alkaloids, flavonoids, tannins, and phenols, which have antibacterial properties. Previous research by Syarifah et al. (2018) stated that 96% ethanol extract of nutmeg seeds at concentrations of 1%, 10%, and 20% did not exhibit significant inhibitory effects on *E. coli*, where the inhibition zones measured were 9.73 mm, 8.5 mm, and 8 mm, respectively. The inhibitory activity formed was categorized as moderate. Generally, the diameter of the inhibition zone tends to increase with increasing extract concentration. However, in that study, the opposite occurred where the diameter of the inhibition zones decreased with increasing concentrations of nutmeg seed extract. This was caused by the use of Carboxymethyl Cellulose (CMC) as a solvent, which made the extract solution too viscous, thereby preventing it from diffusing effectively in the agar medium.

Based on the above discussion, researchers are interested in conducting further research on nutmeg seeds as an antibacterial agent. However, in this study, the solvent used in the extraction process is different, namely 96% methanol. In addition, two types of test bacteria will be used, *E. coli* and *S. aureus*. If the previous study used CMC as a solvent

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in the treatment concentrations of the extract, this study uses Dimethyl Sulfoxide (DMSO). The extract concentrations used in this study are 20%, 30%, and 40%. In addition to testing for antibacterial activity, testing of active compounds from the nutmeg seeds will also be conducted to determine the secondary metabolite compounds contained in the nutmeg seeds obtained from Tapaktuan. This study aims to determine the antibacterial activity of the methanol extract of nutmeg seeds and to determine the effective concentration in inhibiting the growth of *S. aureus* and *E. coli*, as well as to compare the inhibition zones produced against both bacteria.

Materials and Methods

Materials

The materials used in this study were nutmeg seeds (*Myristica fragrans* Houtt.), bacterial isolates of *Staphylococcus aureus* and *Escherichia coli*, 96% methanol, 70% alcohol, distilled water, Nutrient Agar (NA) medium, Mueller Hinton Agar (MHA) medium, chloramphenicol, physiological NaCl, 1% H_2SO_4, 1% $BaCl_2$, disc paper, aluminum foil, and reagents for the identification of active compounds.

Procedures

Sample Collection

Nutmeg seeds (*Myristica fragrans* Houtt.) were collected from the nutmeg plantation in Gelumbuk Village, South Kluet District, Tapaktuan.

Plant Determination

Plant determination was conducted at the Biosystematics Laboratory of the Faculty of Mathematics and Natural Sciences, Biology, Syiah Kuala University. The aim was to verify the accuracy of the plant's identity that would be used in the research, thus preventing errors in material collection.

Preparation of Simplisia

One kilogram of nutmeg seeds was washed, drained, and then dried under sunlight for three days. The dried seeds were then sorted to remove impurities and foreign objects, and cracked to separate the seeds from the outer shell of the nutmeg fruit. The nutmeg seeds were ground using a blender and sieved through an 18 mesh to obtain a fine powder (Ningsih, 2016).

Extraction Process

Nutmeg seed extraction was performed using the maceration method. A total of 500 grams of simplisia powder was macerated with 5000 mL of 96% methanol (simplisia to solvent ratio of 1:10). It was then covered with aluminum foil to prevent evaporation. Maceration was conducted for 24 hours, with occasional stirring. The maceration product was then filtered to obtain the macerate. The simplisia residue was re-macerated in the same manner. The macerate obtained from both maceration processes was then evaporated using a rotary evaporator at a temperature of 40°C for four hours to produce a thick extract (Depkes, 2017). The yield of the thick extract was calculated using the following formula :

$$\text{yield} = \frac{\text{weight of extract obtained (g)}}{\text{initial weight of simplisia (g)}} \text{ x } 100\%$$

Phytochemical Screening

The methanol extract of nutmeg seeds was then subjected to phytochemical screening to determine the content of secondary metabolites in the seeds. This screening was conducted qualitatively by observing the color reaction using reagents. The secondary metabolites tested included alkaloids, saponins, tannins, flavonoids, quinones, polyphenols, and steroids/triterpenoids (Pangemanan et al., 2020).

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Antibacterial Activity Test

a. Sterilization

Glassware and media were sterilized using an autoclave at a temperature of 121ºC for 15 minutes (Novel et al., 2010).

b. Preparation of 0.5 McFarland Standard Solution

A mixture of 9.95 mL of 1% H_2SO_4 with 0.05 mL of 1% $BaCl_2$ was vortexed until it formed a turbid solution (Dalynn Biological, 2022).

c. Culture Revival

One loop of *Staphylococcus aureus* and *Escherichia coli* cultures was inoculated into slanted NA media and then incubated at 37ºC for 24 hours (Smith & Aferd, 2022).

d. Preparation of Test Bacterial Suspension

A loop of revived bacteria was transferred into a test tube containing 10 mL of sterile 0.9% NaCl solution. It was homogenized using a vortex and compared with the 0.5 McFarland standard solution. The 0.5 McFarland standard is equivalent to a cell suspension with a concentration of 1.5 x 10^8 CFU/mL (Kherid et al., 2020).

e. Preparation of Nutmeg Seed Extract Concentrations

Methanol extracts of nutmeg seeds were prepared in three concentrations: 20%, 30%, and 40%, by adding DMSO to the thick nutmeg seed extract in specific amounts for each concentration.

f. Antibacterial Activity Test

The antibacterial activity was tested using the agar diffusion method (disc diffusion Kirby and Bauer) (Smith & Alferd, 2022; Koeth & Linda, 2022). A sterile cotton swab was used to spread the bacterial suspension, equivalent in turbidity to 0.5 McFarland, evenly over the surface of the MHA medium. Discs containing nutmeg seed methanol extract at concentrations of 20%, 30%, and 40%, a negative control (10% DMSO), and a positive control (chloramphenicol) were each placed on the agar surface. The plates were then incubated at 37°C for 24 hours, and the diameters of the inhibition zones—clear zones formed around the discs—were measured using calipers.

Results and Discussion

This study began with the determination of the plant species at the Biosystematics Laboratory of the Biology Department, Faculty of Mathematics and Natural Sciences, Syiah Kuala University, to ensure the correct identification of the nutmeg species (*Myristica fragrans* Houtt.). Based on the plant determination letter number 378/UN11.1.8.4/TA.00.03/2024, the results confirmed that the plant used in this study was indeed *Myristica fragrans Houtt*. (Rasnovi, 2024).

The sample preparation process for nutmeg seeds included washing, drying, and grinding. Washing aimed to remove impurities adhering to the sample. The drying process was conducted to reduce moisture content and prevent fungal growth in the sample, allowing it to be stored for extended periods without chemical composition changes. Drying reduced the water content and stopped the enzymatic processes occurring in the sample, which also helped preserve the sample and facilitated the solvent's ability to extract active compounds during the extraction process. The grinding process aimed to increase the surface area of the sample, thereby enhancing the contact between the solvent and the sample during extraction. According to Anwar et al. (2017), the greater the contact between the sample and the solvent, the more extensive the damage to the cell walls, facilitating the extraction of active compounds from nutmeg seeds.

The extraction process was performed using the maceration method. This method was

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chosen for its simplicity and ease compared to other methods. Maceration of 500 grams of nutmeg simplisia yielded 131.8 grams of methanol extract, with a yield weight of 26.36%. The use of 96% methanol as the extraction solvent in this study was due to methanol's ability to perfectly extract both polar and non-polar chemical components. Methanol is capable of dissolving active compounds such as alkaloids, flavonoids, saponins, quinones, triterpenoids, and others (Astarina et al., 2013). Furthermore, the use of 96% methanol is more efficient in degrading cell walls, thus extracting more secondary metabolites compared to a 70% concentration. Methanol 96% also minimizes contamination and the growth of other microorganisms in the extract due to its only containing 4% water (Tiwari et al., 2011).

Phytochemical Screening

The identification of active compound groups contained in nutmeg seeds (*Myristica fragrans* Houtt.) was conducted through phytochemical screening. Phytochemical screening is a qualitative test used as a preliminary guess to identify the groups of compounds in nutmeg seeds that exhibit antibacterial activity. The active compound groups tested included alkaloids, saponins, tannins, flavonoids, quinones, polyphenols, steroids, and triterpenoids. Based on the results of the phytochemical tests, the methanol extract of nutmeg seeds was found to contain alkaloids, saponins, flavonoids, quinones, and triterpenoids (Table 1).

Table 1. Phytochemical Screening Results of Nutmeg Seed Methanol Extract

Secondary Metabolite Type		Description	Result
Alkaloid			
A.	Dragendorff	Formation of an orange-brown precipitate	+
B.	Mayer	Formation of yellowish-white precipitate	+
C.	Wagner	Formation of brown precipitate	+
Saponin		Foam formation	-
Tanin		No formation of a cloudy white solution	+
Flavonoid		Formation of purple solution	+
Kuion		Formation of yellow color	-
Polifenol		No formation of blue solution	-
Steroid		No formation of green color	+
Triterpenoid		Formation of red color	+

Antibacterial Activity Test

The antibacterial activity of the nutmeg seed methanol extract against *S. aureus* and *E. coli* was assessed using the disc diffusion method (Kirby and Bauer). The principle of the disc diffusion method involves using disc papers as the medium to carry the test extract at various concentrations. The test extract in the disc papers diffuses into the agar media that has been inoculated with bacteria and forms an inhibition zone after incubation. Figure 1 shows that the higher the concentration of nutmeg seed extract applied, the larger the inhibition zone

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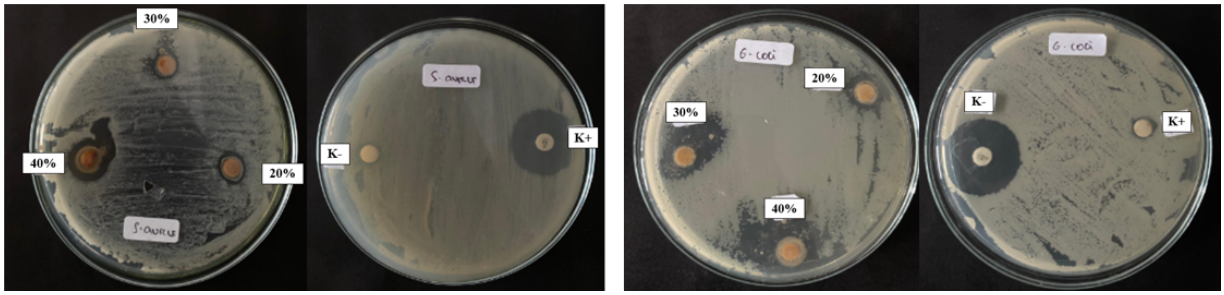
formed. According to Black & Laura (2018), the larger the inhibition zone, the better the antibacterial activity.



(a) (b)

Figure 1. Antibacterial activity of nutmeg seed methanol extract on MHA media after incubation for 24 hours at 37°C: (a) against *S. aureus*, (b) against *E. coli*.

The inhibition zones measured in this study were analyzed using ANOVA in SPSS 26. The results indicated that the treatments with nutmeg seed methanol extract at concentrations of 20%, 30%, and 40%, the negative control (10% DMSO), and the positive control (chloramphenicol) significantly affected the formation of inhibition zones, thus necessitating further analysis with Duncan's Multiple Range Test (DMRT). The measurements of the inhibition zone diameters of the methanol extract of nutmeg seeds against *Staphylococcus aureus* are presented in Table 2, while the results against *Escherichia coli* are shown in Table 3.

Table 2. Results of Inhibition Zone Activity Test against *Staphylococcus aureus*

Treatment	Inhibition Zone Diameter (mm)
Negative control (DMSO 10%)	0,00 ± 0,00[a]
Nutmeg seed methanol extract 20%	11,83 ± 2,00[b]
Nutmeg seed methanol extract 30%	12,56 ± 1,93[b]
Nutmeg seed methanol extract 40%	15,06 ±2,57[b]
Positive control (chloramphenicol)	22,90 ± 0,98[c]

Note : Numbers followed by the same letter are not significantly different, while those followed by different letters indicate significant differences according to DMRT α=0.05; SD (N=3)

Table 3. Results of Inhibition Zone Activity Test against *Escherichia coli*

Treatment	Inhibition Zone Diameter (mm)
Negative control (DMSO 10%)	0,00 ± 0,00[a]
Nutmeg seed methanol extract 20%	13,60 ± 3,32[b]
Nutmeg seed methanol extract 30%	14,40 ± 1,82[b]
Nutmeg seed methanol extract 40%	17,86 ± 4,36[b]
Positive control (chloramphenicol)	24,03 ± 1,82[c]

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Note : Numbers followed by the same letter are not significantly different, while those followed by different letters indicate significant differences according to DMRT α=0.05; SD (N=3)

Tables 2 and 3 demonstrate that higher concentrations of nutmeg seed methanol extract result in larger inhibition zones against *S. aureus* and *E. coli*. According to Harvey et al. (2014), the concentration of an antibacterial test substance can influence the size of the inhibition zone. The difference in inhibition zones at each concentration is due to the varying amounts of active compounds contained within those concentrations. The higher the concentration of nutmeg seed methanol extract, the greater the amount of active components it contains, which in turn increases the size of the inhibition zone. This finding aligns with Madigan et al. (2021), who stated that the concentration of antibacterial compounds is one of the factors affecting the efficiency and effectiveness of the antibacterial agent. Indryani et al. (2020) suggest that an inhibition zone diameter of 20 mm or more indicates very strong inhibitory activity, 11-20 mm is considered strong, 6-10 mm is moderate, and 5 mm or less is categorized as weak.

The measurement results of the inhibition zone diameters of the nutmeg seed methanol extract against *S. aureus* (Table 2) show that the highest inhibition zone was at the 40% concentration with an average diameter of 15.06 mm, while the smallest was at the 20% concentration (11.83 mm), and the 30% concentration exhibited an inhibition zone of 12.56 mm. The inhibition zones formed at all three concentrations are categorized as strong. According to the DMRT, the administration of nutmeg seed methanol extract at concentrations of 20%, 30%, and 40% did not significantly differ in their effect on the diameter of the inhibition zone for *Staphylococcus aureus*, but there was a significant difference compared to the positive control treatment (chloramphenicol).

The measurements of the inhibition zone diameters for the methanol extract of nutmeg seeds against *Escherichia coli* (Table 3) show that concentrations of 20%, 30%, and 40% resulted in inhibition zones of 13.60 mm, 14.40 mm, and 17.86 mm, respectively. The inhibition zones at these concentrations are categorized as strong. According to the DMRT, the concentrations of nutmeg seed methanol extract at 20%, 30%, and 40% did not differ significantly, but they showed a significant difference compared to the positive control (chloramphenicol).

Additional research by Lagha et al. (2020) demonstrated that, in addition to inhibiting *Staphylococcus aureus* and *Escherichia coli*, nutmeg seed extract can also inhibit other pathogenic bacteria such as *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*. The antibacterial activity of nutmeg seeds is due to the presence of secondary metabolites such as alkaloids, saponins, flavonoids, quinones, and triterpenoids. Alkaloids disrupt the components that make up the peptidoglycan of bacterial cells, preventing the formation of a complete cell wall and leading to cell death (Rahmadeni et al., 2019). Saponins, flavonoids, and triterpenoids act by

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damaging the cell wall structure and affecting membrane permeability, causing disruptions in the function and structure of the bacterial cell membrane (Anggrahini, 2013; Diyan et al., 2015; Madduluri et al., 2013). Quinones inhibit bacterial growth by forming irreversible complexes with nucleophilic amino acid residues on plasma membrane proteins, cell wall polypeptides, and enzymes located on the cell membrane surface (Erina et al., 2019). Additionally, nutmeg seeds contain fixed oils (20-40%) composed of myristic acid, trimyristin, glycerides, stearates, and palmitates. Trimiristin and myristic acid are secondary metabolites that can inhibit bacterial growth by disrupting the structure of the cell wall after its formation or altering it post-formation, as well as damaging cell permeability. This leads to nutrient leakage within the cell, resulting in inhibited growth or cell death (Palawi, 2024).

In the negative control, 10% DMSO was used. The use of DMSO as a solvent in the preparation of extract concentrations is due to DMSO's ability to dissolve both polar and nonpolar compounds. The diameter of the inhibition zone for the negative control, both against *S. aureus* and *E. coli*, was not formed. This indicates that the antibacterial activity is not influenced by the solvent (DMSO), confirming that the analyzed antibacterial activity is solely attributable to the potential of the nutmeg seeds. This is consistent with the findings of Utami (2011), who stated that DMSO does not inhibit bacterial growth, thereby not interfering with the results of antibacterial activity tests. Furthermore, according to Amalia et al. (2020), negative controls using DMSO at various concentrations have proven not to have antibacterial activity, ensuring that the antibacterial activity observed is purely from the extract without any influence from the solvent.

In the positive control (chloramphenicol), the diameter of the inhibition zone produced was larger compared to that produced by the methanol extract of nutmeg seeds. The inhibition zones produced by chloramphenicol showed an average value of 22.90 mm against *S. aureus* and reached 24.03 mm against *E. coli*. This is because chloramphenicol is a broad-spectrum antibiotic capable of killing both gram-positive and gram-negative bacteria. Chloramphenicol disrupts protein synthesis by reversibly binding to the ribosomal unit, thereby inhibiting the formation of proteins necessary for bacterial growth and survival (Zhang et al., 2022).

Based on the discussion above, it is evident that the methanol extract of nutmeg seeds forms a larger inhibition zone against *Escherichia coli* compared to *Staphylococcus aureus*. This variation in results is due to the differing antibacterial activity of compounds from nutmeg seeds against each bacterial species, which depends on the thickness and composition of their cell walls. This is in line with Pommerville (2011), who noted that differences in bacterial sensitivity to antibacterial agents are influenced by the structure of the bacterial cell wall. Gram-positive bacteria like *S. aureus* have a thick peptidoglycan layer that contains teichoic acid compounds, whereas gram-negative bacteria like *E. coli* have a thin peptidoglycan layer and do not contain teichoic acids, making them more susceptible to physical disruptions, such as the application of antibiotics or other antibacterial agents (Vaiwala et al., 2022).

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Supporting research conducted by Stery et al. (2015) on the inhibitory effects of methanol extracts from *Selaginella delicatula* and *Diplazium dilatatum* against *S. aureus* and *E. coli* also showed similar results, where the inhibition zones formed on *E. coli* were larger compared to those on *S. aureus*. The methanol extracts of *S. delicatula* and *D. dilatatum* produced inhibition zones of 7.80 mm and 6.70 mm against *S. aureus*, respectively, while against *E. coli*, they were 13 mm and 8 mm. Furthermore, another study by Sudarmi et al. (2017) indicated that leaf extract from Syzygium cumini at concentrations of 5%, 10%, 15%, 20%, and 50% resulted in larger inhibition zones against *E. coli* compared to *S. aureus*.

Conclusions

Based on the results of this study, it can be concluded that the methanol extract of nutmeg seeds (*Myristica fragrans* Houtt.) at concentrations of 20%, 30%, and 40% exhibits antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The inhibition zones for *E. coli* were 13.60 mm, 14.40 mm, and 17.86 mm, respectively, while for *S. aureus*, they were 11.83 mm, 12.56 mm, and 15.06 mm. The inhibition zones formed are categorized as strong, and the most effective concentration for inhibiting bacterial growth was found to be 40%. Additionally, it was observed that the methanol extract of nutmeg seeds tends to have better antibacterial properties against *E. coli* compared to *S. aureus*.

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