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Phytochemical Analysis and Nanoparticle Formulations of Extracts *Myristica fragrans* Houtt Leaves as Antibacterial

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Abstract

Myristica fragrans Houtt leaves contain secondary metabolites such as flavonoids, terpenoids and alkaloids, which have antibacterial properties. At the nanoscale, the particles have a bigger surface contact area, resulting in increased amounts and solubility of the active chemical. This leads to a stronger antibacterial activity. This study aims to examine the influence of several chitosan variations on particle size, antibacterial properties and the form of nanoparticles produced from *Myristica fragrans* leaves ethanol extract. Ethanol extract was analyzed using the UV-Vis spectrophotometric method to quantify total flavonoid content. Nanoparticles were synthesized using the ionic gelation technique with several ratios of chitosan and sodium tripolyphosphate, specifically 8:1, 10:1 and 12:1. Nanoparticles were analyzed with a Particle Size Analyzer (PSA) and Scanning Electron Microscopy (SEM). Agar diffusion test for bacteria germs using a paper backer. *Myristica fragrans* leaves ethanol extract was 50, 40 and 30 %, while nanoparticle extract was 5, 4 and 3 %. The overall flavonoid concentration in the QE/ethanol extract was 39.7252 ± 1.9596 mg. Antibacterial test results against Staphylococcus aureus and Escherichia coli bacteria and *Myristica fragrans* leaves extract limit inhibition area at 30 % (13.35 mm), 40 % (13.68 mm) and 50 % (13.93 mm) for Staphylococcus aureus and 30 (13.28), 40 (13.45) and 50 % (13.81 mm) for Escherichia coli. *Myristica fragrans* leaves extract chitosan nanoparticles included 3 (14.70), 4 (15.30) and 5 % (15.56 mm) for Staphylococcus aureus and 3 (14.60), 4 (14.65) and 5 % (15.05 mm) for Escherichia coli. Ionic gelation can create nanoparticles of *Myristica fragrans* leaves ethanol extract with chitosan and sodium tripolyphosphate. *Myristica fragrans* leaves nanoparticle ethanol extract exhibits better antibacterial activity than ethanol extract. Increasing chitosan concentration affects particle size. The ethanol extract of *Myristica fragrans* nanoparticle leaves showed slightly better antibacterial activity than the ethanol extract.
Keywords: Antibacterial, Chitosan, *Myristica fragrans*, Nanoparticle

Introduction

Nanotechnology is ancient. Using nanotechnology, Nature has continuously synthesized biological molecules, including enzymes, proteins, carbohydrates and lipids [1,2]. The physical, chemical and biological properties of 1 - 100 nm nanoparticles differ from bulk materials and small molecules [3,4]. Due to their unique material properties, nanoparticles are employed in industrial catalytic processes, energy conversion and storage, image display technologies, cosmetics, medical devices, therapies, antimicrobial, antibacterial and diagnostic. Particle shape and size affect medication solubility, absorption and distribution, which affects therapeutic [5-7].

Nanoparticles can be made from co-conservation complexes and ionic gelation. 2 oppositely charged hydrophilic colloid dispersions can form a co-conservation complex or ionic gelation. Negative charges cancel positive charges, making separation difficult [8,9]. Nanoparticles of chitosan can transport medicines. Chitosan breaks down naturally, is nontoxic and biocompatible. Chitosan micro/nanoparticles are stable, have a wide surface area and can be employed as a matrix for medicines and plant products [10-15].

Using palate leaves in nanoparticle compositions was inspired by Indonesia's ongoing development of nanoparticle technology for herbs. This herbal plant inspired researchers to employ palate plants in nanoparticles [16,17]. The leaves of M. fragrans plants are essential in microbiology. Antibacterial flavonoids, terpenoids and alkaloids occur in M. fragrans leaves. 3 substances disrupt bacterial microorganisms to stop bacteria growth [18,19]. The nutmeg plant is a typical Indonesian plant that is often used by people as a cooking spice and sweet, especially the flesh of the fruit. However, as technology advances, nutmeg has been developed as an herbal medicine that has the potential to act as an antibacterial against Escherichia coli [20].

Myristica fragrans is commonly known as "nutmeg" and belongs to the family Myristiceae in Magnoliales, comprising around 150 genera and many species. Myristica species are natives of Eastern Indonesia, Sri Lanka, India and they are now cultivated in many countries worldwide. It is a common spice in many food items such as sauces, meats, soups and confectionery stuff M. fragrans is the chief source of 3 commercial goods such as nutmeg, mace (fleshy aril) and essential oil, which are broadly used for flavoring purposes in many dishes and offer valuable health effects. *Myristica fragrans* leaves contain several secondary metabolites. This includes alkaloids, flavonoids, saponins, tannins and steroids. M. fragrans leaves can treat coughs, digestion, hunger, blood flow, stroke, nausea and vomiting, angina infections and vomiting, menstruation discomfort and rheumatism due to its compounds [21-24]. This research aims to create a nanoparticle preparation formulation, determine the antibacterial activity of ethanol extract and nutmeg leaf extract nanoparticles against Staphylococcus aureus and Escherichia coli and determine flavonoid levels using UV-vis spectrophotometry.

Materials and methods

Rotary evaporator, filter paper, analytical balance, cup, porcelain level, set of water content determination tools, laboratory glassware, magnetic bar, magnetic stirrer, centrifuge (Hitachi), Particle Size Analyzer (Vasco) and Scanning Electron Microscope (Hitachi TM3000), drop pipette, test tube, petri dish, tube needle, micropipette, spirit lamp, incubator (Memmert), oven (Memmert), autoclave (Fison) and UV-Vis spectrophotometry.

Sample collection

Myristica fragrans was collected from Medan, North Sumatra Province, Indonesia. Other materials used are 70 % ethanol, toluene, 2N hydrochloric acid, chloroform, distilled water, sodium tripolyphosphate (NaTPP), chitosan, acetic acid, test bacteria: *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8938 (stock culture collection from the Microbiology Laboratory, Faculty of Pharmacy, University of North Sumatra), nutrient agar (NA) media, nutrient media broth (NB) and dimethyl sulfoxide (DMSO), acetic acid anhydride, nitric acid, sulfuric acid, amyl alcohol, iron (III) chloride, bismuth (III) nitrate, iodine, potassium iodide, magnesium powder, mercury (II) chloride, alpha-naphthol, lead (II) acetate, toluene, chloroform, n-hexane, distilled water, hydrochloric acid, quercetin, aluminum chloride and sodium acetate.

Preparation of ethanolic leaves extract of *Myristica fragrans*

The leaves of M.fragrans are extracted by maceration. 500 g of simple powdered leaves are put into a glass container and mixed with 3.75 liters of 96 % ethanol. The container is sealed and left out of the light for 5 days. The pulp is husked, squeezed and washed with enough water to get 4 liters. Put it in a container with a lid and seal it up. Keep it in a cool place away from light for 2 days. Used a filter or a pour. A rotary evaporator boiled down the results until most of the liquid was gone. The process was then repeated in a water bath until a thick extract was made [26,27].

Examination of extract characterization

As part of characterizing an extract, the amount of water present, the amount of water-soluble juice present, the amount of ethanol-soluble juice present, the amount of total ash present and the amount of acid-insoluble ash present are all measured [27].

Determination of total flavonoid content of ethanol extract of *Myristica fragrans* leaves

The 25 mL volumetric flask was filled with ethanol extract of 25 mg of M. fragrans leaves and methanol up to the limit mark (C = 1000 µg/mL). 1 mL was pipetted into a 10-mL volumetric flask and filled with 1.5 mL methanol, 0.1 mL 10 % aluminum chloride, 0.1 mL 1 M sodium acetate, 2.8 mL distilled water and enough methanol to achieve the limit. The mixture was mixed well and left to sit for 6 to 9 min. The absorption was found at 437 nm, which is the longest wavelength. 6 copies of the sample were made for each test and the average absorption value was found [28,29].

Preparation of chitosan solution 0.08, 0.10 and 0.12 %

Chitosan solution was made by adding 100 mL 1 % acetic acid into three 250 mL beakers. Put 0.08, 0.10 and 0.12 g of chitosan into each beaker glass, stirring with a magnetic stirrer until the chitosan was dissolved [30].

Preparation of 0.01 % NaTPP solution

NaTPP solution was produced by adding 0.035 g NaTPP to 350 mL distilled water in a 500 mL beaker. The solution was magnetically stirred until dissolved.

Preparation of nanoparticles of ethanol extract of *Myristica fragrans* leaves

Weighting 1 g of *M. fragrans* leaves extract produced *Myristica fragrans* nanoparticles. After dissolving *Myristica fragrans* leaves extract in 35 mL 96 % ethanol and 15 mL distilled water in a 1000 mL beaker, 100 mL chitosan solution in 1 % acetic acid was added. While stirring for 2 h, 350 mL of NaTPP was slowly added to the mixture. After mixing the contents, a magnetic stirrer stirred for 2 h at a steady pace. Next, centrifugation separated chitosan and NaTPP-*Myristica fragrans* leaves nanoparticle colloids. The ethanol extract nanoparticle solids were refrigerated at ± 3 °C until dry [31,32]. Chitosan and NaTPP concentrations in (%) are compared in **Table 1**.

Table 1 Concentration ratio of chitosan and NaTPP.

Formula	Chitosan (%)	NaTPP (%)	Ratio
A	0,08	0,01	8:1
B	0,10	0,01	10:1
C	0,12	0,01	12:1

Preparation of *Myristica fragrans* leaves ethanol extract assay solution

A total of 2 g of M. fragrans leaves ethanol extract was weighed, then 96 % ethanol was added to a total volume of 4 mL and stirred until dissolved and obtained a concentration of 500 mg/mL or 50 %. Dilutions were made with concentrations of 40 and 30 % [33].

Preparation of nanoparticle test solution of ethanol extract of *Myristica fragrans* leaves

A total of 0.1 g of *M. fragrans* leaves ethanol extract nanoparticles was weighed, then dimethyl sulfoxide (DMSO) was added to a total volume of 2 mL and stirred until dissolved and obtained a concentration of 50 mg/mL or 5 %, then dilutions with concentrations of 4 and 3 % were made [34].

Antibacterial activity assay

The antibacterial activity of *M. fragrans* leaves extract in ethanol and nanoparticles in various concentrations was investigated. This test used paper plate agar diffusion [35].

Staphylococcus aureus and Escherichia coli bacteria assays

After adding 0.1 mL of inoculum to a clean Petri dish, 9 mL of nutritional agar media was added. Keep the temperature between 45 and 50 °C. A table surface was used to shake the cup so that the media and bacterial suspensions were mixed evenly. On the densely packed media, several paper plates were soaked (+15 min) in a test solution of ethanol extracts of M. fragrans leaves and nanoparticles of *Myristica fragrans* leaves extracts with different concentrations. After incubating plates at 35 ± 2 °C for 18 - 24 h, the clear zone diameter was measured with a caliper [36].

Results and discussion

Extraction of *Myristica fragrans* leaves

The maceration method was used to extract 500 g of M. fragrans leaves simplicia powder M. fragrans in 96 % ethanol. A rotary evaporator concentrated the filtrate until most of the solvent evaporated and then the procedure was repeated over the water bath to create a thick extract. The thick macerated extract weighed 42.77 g, yielding 8.5 %.

Characterization of *Myristica fragrans* leaves simplicia powder

Characterization of M.fragrans leaves extract yielded 7.3 % water, 13.46 % water-soluble essence, 13.17 % ethanol-soluble extract, 1.42 % total ash and 0.795 % acid-insoluble ash. The results of the characterization of simplicia and ethanol extract of M. fragrans can be seen in **Table 2**.

Table 2 Characterization of Simplicia and Ethanol Extract of *Myristica fragrans* leaves.

No.	Parameter	Characterization results (%)	MMI (%)
1	Water content	7.3	< 10
2	Water soluble essence content	13.46	> 5.5
3	Ethanol soluble essence content	13.17	> 8
4	Total ash content	1.42	< 3
5	Acid-insoluble ash content	0.795	< 1

The results of determining the characterization of the simplicia show the results meet the requirements and are guaranteed quality based on the Indonesian Materia Medika (MMI).

Phytochemical screening

Alkaloids, flavonoids, glycosides, tannins, saponins and triterpenoids are found in simplicial powder and ethanol extract of M. fragrans leaves, as shown in **Table 3**. Researchers have found M. fragrans leaves have alkaloids, flavonoids, glycosides, steroids and flavonoids [37].

Table 3 Phytochemical screening results of simplisia and *Myristica fragrans* leaves extracts.

No.	Group of compounds	Simplicia	Extracts
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Tanins	+	+
4	Glycosides	+	+
5	Saponins	+	+
6	Steroids/Triterpenoids	+	+

Based on the results, M.fragrans leaf extract and simplicial contains flavonoids, alkaloids, saponins, tannins and steroids metabolites. Phytochemical screening is required to obtain information on the classes of secondary metabolites found in M.fragrans ethanol extract.

Determination of total flavonoid levels in *Myristica fragrans* Leaves

Determination of total flavonoid content from *Myristica fragrans* leaf ethanol extract was carried out using 6 replications. The calibration curve line equation may calculate total flavonoid content by comparing *M. fragrans* leaves ethanol and ethyl acetate extract absorption values, as shown in **Table 4** [38].

Table 4 Determination of Total Flavonoid Content of *Myristica fragrans* Leaves.

Test solution	No.	Sample Weight (g)	Absorbance	Concentration (μg/mL)	Total flavonoid content of extract (mgQE/g)	Actual level (mgQE/g Extract)
Ethanol Extract of *Myristica fragrans* Leaves	1	0.025	0.429	3.9725	39.725	39.7252 ± 1.9596 mgQE/g extract
	2	0.025	0.428	3.9631	39.631	
	3	0.025	0.435	4.0293	40.293	
	4	0.025	0.427	3.9536	39.536	
	5	0.025	0.428	3.9631	39.631	
	6	0.025	0.431	3.9914	39.914	

The study found that the *M. fragrans* leaves ethanol extract contained 39.7525 ± 1.9596 mgQE/g of total flavonoids. Flavonoids form a yellow complex with 10 % aluminium chloride, determining their quantity.

Particle size distribution

The liquid nanoparticles were made by mixing 3 chitosan and linking them with NaTPP. There were 3 different ratios of chitosan to NaTPP: formula A (8:1), formula B (10:1) and formula C (12:1). PSA measured particle size distribution for each recipe, which can be seen in **Figures 1 - 3** and **Table 5**.

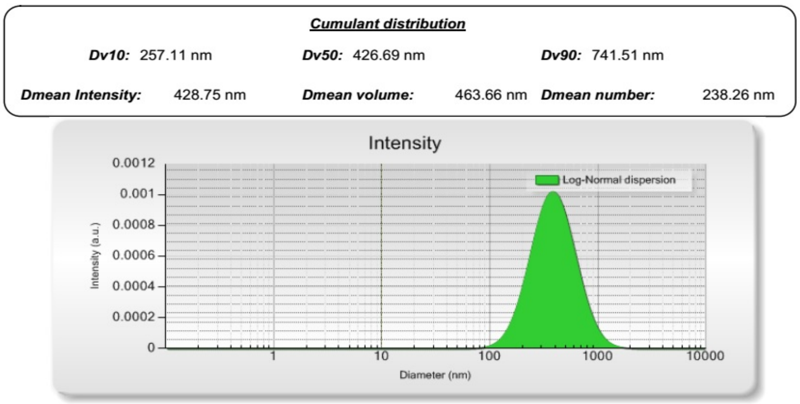


Figure 1 Formula A (8:1).

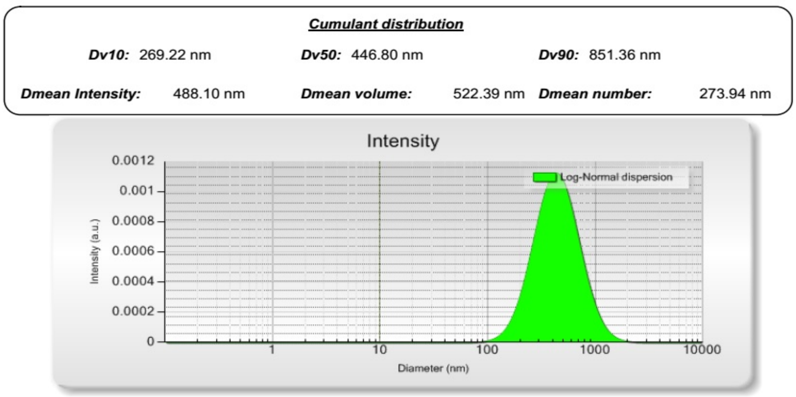


Figure 2 Formula B (10:1).

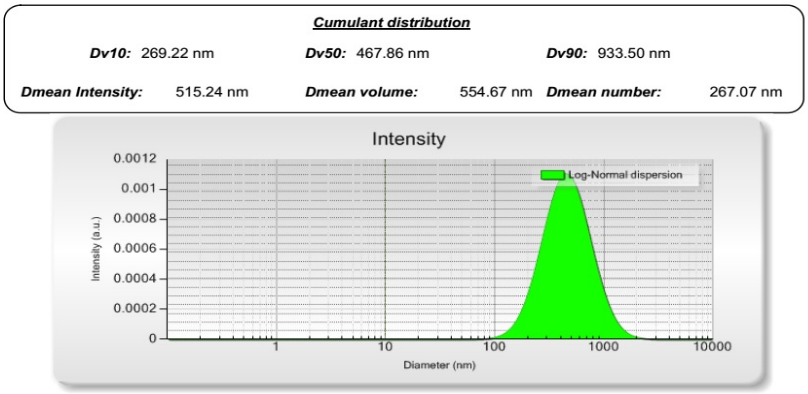


Figure 3 Formula C (12:1).

Table 5 Particle size analyzer measurement results.

No.	Chitosan (%)	NaTPP (%)	Ratio chitosan and NaTPP	Particle size distribution (nm)	Average particle size (nm)
1	0.08	0.01	8 : 1	147.95 - 977.95	428.75
2	0.10	0.01	10 : 1	169.87 - 1,122.32	488.10
3	0.012	0.01	12 : 1	169.87 - 1,230.59	515.24

The chitosan-NaTPP makeup ratio (8:1) **Figure 1**, which has 0.08 % chitosan and 0.01 % NaTPP, made the particles with the smallest average size. The largest particle size that was made was 428.75 nm and the smallest particle size that was made was 147.95 nm. In formula B, the average particle size was 488.10 nm and the range of particle sizes was 169.87 to 1,122.32 nm. In formula C, the average particle size was 515.24 nm and the range of particle sizes was 169.87 to 1,230.59 nm [39].

The measurements show that the particle size increases as the chitosan concentration rises. Adding more chitosan to a mixture with the same amount of NaTPP makes it more likely for particles to stick together. Because of this, the higher the chitosan concentration, the more particles of bigger sizes are formed [40].

Zeta potential

Zeta potential is an assessment of the efficient electrical charge on the surface of a nanoparticle by measuring its charge. When a nanoparticle has a surface charge, the charge is filtered by the concentration of oppositely charged ions close to the nanoparticle's surface. These layers of oppositely charged ions move with the nanoparticle and collectively with the layer. The magnitude of the zeta potential provides an idea of the stability of the particle [41]. The result of zeta potential as shown in **Table 6**.

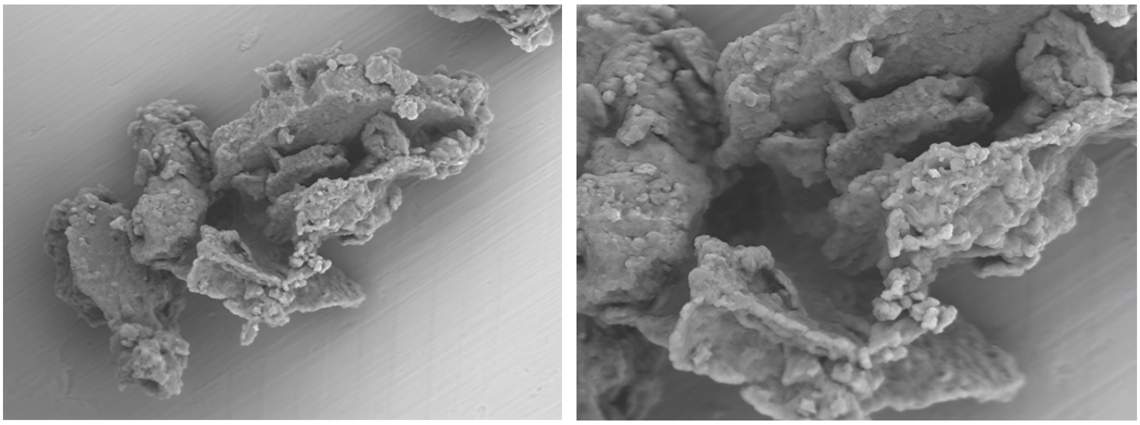
Table 6 Zeta Potential Results

Replication	Zeta potential (mV)		
	Formula A	Formula B	Formula C
1	−28.9	−29.3	−29.5
2	−29.1	−29.2	−29.7
3	−29.1	−29.2	−29.6
Mean	−29.03	−29.23	−29.6
SD	0.11	0.05	0.10

The zeta potential value shows the charge on the surface of a particle. The charge of the particles causes particles that experience a tendency to aggregate or repel. The zeta potential results obtained by formulas A, B and C are −29.03, 29.23 and 29.6 mV, respectively, provided that the zeta potential value is smaller than −30 mV and greater than +30 mV and has higher stability. The results obtained do not meet the requirements; it can be concluded that the zeta potential value of African leaf Simplicia nanoparticles has a zeta potential value that is less stable and shows that the strength of the particles to repel is getting weaker so that the particles tend to experience aggregation and dispersion [42].

Particle morphology

The dried solid particles produced from *Myristica fragrans* leaves ethanol extract were tested for form using SEM. We looked at the shape and size of particles in formula A (8:1) under 500 times magnification and 1000 times magnification, as shown in **Figure 4**.



(a) (b)

Figure 4 SEM results of formula A with magnification of (a) 500 times magnification and (b) 1000 times magnification.

The surface morphology of the *Myristica fragrans* leaves ethanol extract nanoparticles, which shows irregular surface conditions and aggregates forming. This condition also illustrates that *Myristica fragrans* leaves ethanol extract in the form of nanoparticles, which is not uniform and has an uneven surface [43,44].

Antibacterial activity test of ethanol extract and nanoparticles of ethanol extract of *Myristica fragrans* leaves against *Staphylococcus aureus* and *Escherichia coli*

The inhibition of the ethanol extract from *M. fragrans* leaves as shown in (**Table 6** and **Figure 5**) and the nanoparticles made from that extract (**Table 7** and **Figure 6**). The test for antibacterial action showed that nanoparticles made from ethanol extract of *M. fragrans* leaves and Staphylococcus aureus and Escherichia coli bacteria can stop their growth.

Table 7 Antibacterial activity test results of ethanol extract of *Myristica fragrans* leaves against *Staphylococcus aureus* and *Escherichia coli.*

Concentration of ethanol extract of *Myristica fragrans* leaves (mg/mL)	**Diameter of the area of resistance *(mm)**	
	Staphylococcus aureus	***Escherichia coli***
30	13.35 ± 0.15	13.28 ± 0.16
40	13.68 ± 0.10	13.45 ± 0.15
50	13.93 ± 0.12	13.81 ± 0.15
Blank	-	-

Information: * = average result of 3 measurements
- = no barriers

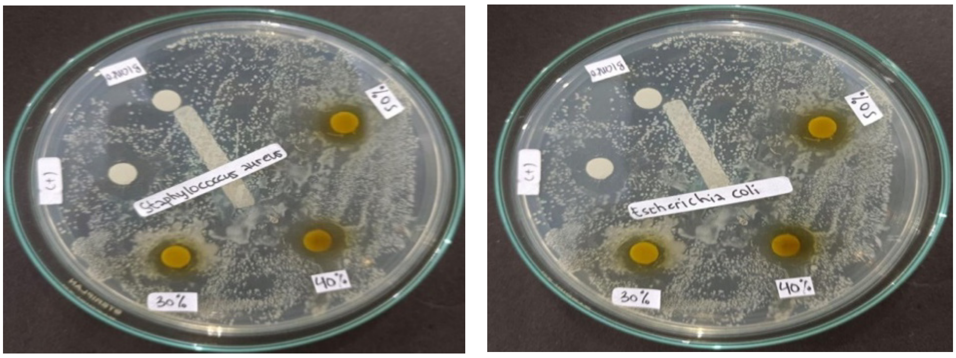


Figure 5 Ethanol extract against *Staphylococcus aureus* and *Escherichia coli.*

Table 8 Antibacterial activity test results of *Myristica fragrans* leaves ethanol extract nanoparticles against *Staphylococcus aureus* and *Escherichia coli.*

Concentration of ethanol extract of *Myristica fragrans* leaves (mg/mL)	**Diameter of the area of resistance *(mm)**	
	Staphylococcus aureus	***Escherichia coli***
3	14.70 ± 0.15	14.20 ± 0.13
4	15.30 ± 0.13	14.65 ± 0.15
5	15.56 ± 0.11	15.05 ± 0.11
Blank	-	-

Informations: * = average result of 3 measurements
- = no barriers

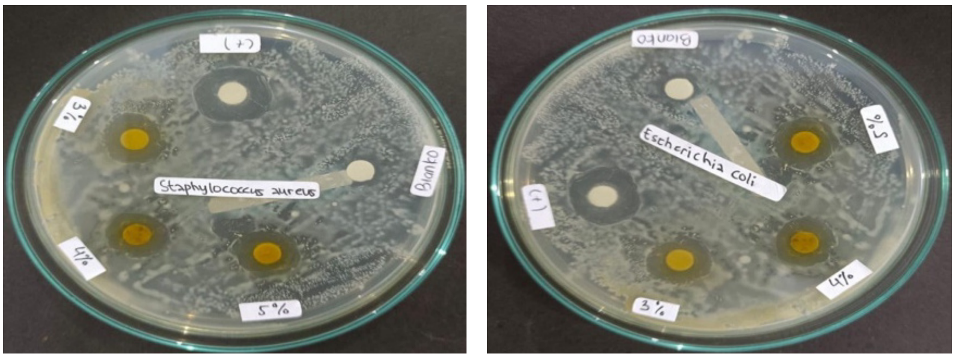


Figure 6 Ethanol extract nanoparticles against *Staphylococcus aureus* and *Escherichia coli.*

Paper plates were used with the agar diffusion method, no colony growth of germs in the clear area formed around the treatment. There are clear areas in the medium for bacterial growth where the diameter of the inhibition zone grows as the concentration does. There is a positive relationship between the concentration of the extract and the growth inhibition zone diameter of Escherichia coli bacteria [45,46]. Antibacterial test results against Staphylococcus aureus and Escherichia coli bacteria and *Myristica fragrans* leaves extract limit inhibition area at 30 (13.35), 40 (13.68) and 50 % (13.93 mm) for Staphylococcus aureus and 30 (13.28), 40 (13.45) and 50 % (13.81 mm) for Escherichia coli. *Myristica fragrans* leaves extract chitosan nanoparticles included 3 (14.70), 4 (15.30) and 5 % (15.56 mm) for Staphylococcus aureus and 3 (14.60), 4 (14.65) and 5 % (15.05 mm) for Escherichia coli. M. fragrans leaves can suppress Staphylococcus aureus and Escherichia coli growth in viscous extract and nanoparticle formulations. The blank, however, does not affect either of the bacteria.

Conclusions

There is total flavonoid activity in the ethanol extract of *Myristica fragrans* leaves, which means they can kill germs. The growth of Staphylococcus aureus and Escherichia coli bacteria can also be stopped by ethanol extract and nanoparticles of the leaves of this plant. The best antibacterial test results for *Myristica fragrans* leaf extract against Staphylococcus aureus and Escherichia coli were found at a concentration of 50 % with respective inhibition zones of 13.93 and 13.81 mm. In the antibacterial test of *Myristica fragrans* leaf nanoparticles against staphylococcus aureus and Escherichia coli, the best concentration was found at 5 % with respective inhibition zones 15.56 and 15.05 mm.

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