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Development and Characterization of Limeberry (*Triphasia* trifolia) Fruit Extract Nanoparticles Prepared by Ionic Gelation and Their Antibacterial Activity Against *Staphylococcus aureus* and *Salmonella sp.*

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ABSTRACT. Limeberry *(Triphasia trifolia)* is a plant with the potential for a traditional medicine. It has an antibacterial agent. Limeberry extract made on a nanoscale can enlarge the surface area of particles which allows isolation of more active ingredients for stronger antibacterial action. The ionic g elation method was used to form nanoparticles with chitosan polymer and sodium tripolyphosphate (NaTPP) as polyanion. The purpose of the current study was to determine the characteristics and inhibition of the antibacterial activity of nanoparticles of ethanol extract of limeberry fruit *(T. trifolia)* against *Staphylococcus aureus* and *Salmonella sp*. The results showed that the best formula of limeberry *(T. trifolia*) was the concentration of 0.75% chitosan and 0.01% NaTPP. The characteristics of nanoparticles obtained a particle size of 365.81 nm with a polydispersity index of 0.718. The shape of the particles is imperfectly round and the FTIR analysis shows cross-linking between chitosan, sodium tripolyphosphate (NaTPP), and the limeberry fruit extract. Antibacterial testing against *S. aureus* and *Salmonella sp.* bacteria obtained an average inhibition zone of 28.56 mm and 23.57 mm with a very strong inhibition activity.

Keywords: Antibacterial activity, limeberry, nanoparticles, *Salmonella sp., Staphylococcus aureus*

INTRODUCTION

The limeberry plant with the Latin name *Triphasia trifolia* (family: Rutaceae) is derived from the Greek where "tripha" means three, referring to the arrangement of its leaves (Theanphong & Mingvanish, 2023). The limeberry plant (*T. trifolia*) is a plant with considerable potential for pharmacological activity. The limeberry plant has properties, such as ethnomedicine for the treatment of dandruff, cough, diarrhea, dysentery, influenza, and lung disorders. Limeberry fruit has benefits as an anti-microbial life. Both the stem and leaves of limeberry have antibacterial activities against *Bacillus subtilis, S. aureus, Escherichia coli,* and *Proteus vulgaris* with inhibition zone values formed, namely 25 ± 1.36mm; 26 ± 1.8mm; 24 ± 1.21mm; and 22 ± 1.68 mm (Theanphong & Mingvanish, 2023). The inhibition can occur due to antibacterial activities in limeberry extract in the forms of flavonoids, tannins, terpenoids, and alkaloids. Limeberry plants contain essential oils which are also useful as antimicrobial compounds. The most essential oil was obtained from the fruit (0.92%, leaves (0.20%), and stems (0.03%), respectively. The oil is rich in sabine (leaves, 31.1%, stem, 21.1%, fruit, 23.9%), and β-pinene (leaves, 40.8%, stem, 36.2%, fruit, 32.4%) (Zoghbi & Andrade, 2009). The essential oil of limeberry fruit can inhibit *B. subtilis* and *Chromobacterium violaceum* (Santos et al., 2008).

The use of active compounds alone is considered ineffective because herbal plant extracts have weaknesses, namely their low effectiveness and solubility in water, thus reducing their bioavailability (Alam et al., 2012). Particle shape and size are some of the factors that affect the effectiveness of active compounds. Smaller particle sizes can increase the surface area. The limeberry fruit extract made on nanoscale can enlarge the surface area of particles, allowing the isolation of more active compounds for stronger antibacterial activity (Kammona & Kiparissides, 2012).

Nanotechnology refers to a technology that involves molecules with a size of 10 – 1,000 nm (Aloys et al., 2016). In recent years, nanotechnology applications for food and medicine have been very interesting and it has showed an increasing trend. It has developed in many industries, such as food manufacturing, agriculture, cosmetics, and construction (Ye et al., 2017). Nanoparticle

technology has many benefits as a delivery agent for active substances in food and drug products. It can control the release rate of active substances, increase the solubility of poorly soluble active substances, the bioavailability of active substances, and absorption in the body (Ma et al., 2023).

The ionic gelation method constitutes the method of making nanoparticles, involving a mixture of two phases, namely chitosan as a polymer and sodium tripolyphosphate as a polyanion (Kafshgari et al., 2011). Chitosan also has the benefit of increasing the antimicrobial properties found in limeberry compounds, and it takes the form of a porous membrane that can absorb water and antimicrobial substances. The preparation of nanoparticles by ionic gelation method involves the interaction between the negative ions of

The compounds contained in the extract nanoparticles and the protonated amine groups of chitosan in an acidic atmosphere. In addition to interacting with negatively charged groups from the ethanol extract of limeberry fruits, chitosan amine groups can also interact with the negative charge of NaTPP (Wang et al., 2023). Although several studies have reported the antimicrobial properties of *T. trifolia*, limited research has explored its formulation in nanoparticle systems to enhance its antibacterial effectiveness. The backgrounds have motivated the researchers to explore limeberry fruit extract nanoparticles using the ionic gelation method and to evaluate their physicochemical characteristics and antibacterial activity against *S.aureus* and *Salmonella sp*.

EXPERIMENTAL SECTION

Material

The materials used in the current study include the limeberry fruits obtained from farmers in Krui, West Coast. Analytical materials consist of distilled water, 96% ethanol, 2% acetic acid, chitosan and sodium tripolyphosphate (NaTPP), Mg powder, HCl, reagents (Mayer, Wagner, Dragendrof), Lieberman-Bouchard reagent, FeCl3, NA (Nutrient agar) brand Merck®, NB (Nutrient Broth) brand Merck®, aquades, 96% ethanol, culture of *S. aureus* and *Salmonella sp.*

A number of the tools were employed in this study, such as an oven, a baking sheet, a chopper, a grinder, a jar, a filter paper, aluminum foil, a Whatman paper, a vacuum rotary evaporator, a magnetic stirrer, an analytical balance,an erlenmeyer, a test tube, a dropper pipette, a sonicator, a particle size analyzer (PSA), a scanning electron microscope (SEM) and fourier transform infrared (FTIR) (Agilent Cary 630), stirring rod, autoclave, petri dish, ose needle, incubator, paper disc, caliper/ruler.

Extraction Process

Preparation of ethanol extract of limeberry fruit used a maceration method. In the maceration process, 450 g of limeberry fruit samples were first mashed and they were then weighed and macerated using 96% ethanol as much as 750 mL. The maceration process spent for 1 x 24 hours with three repetitions and the limbery fuit was filtered. Once finished, the filtrate obtained was concentrated using a vacuum rotary evaporator at 30 °C until a thick extract is obtained (Fitri et al., 2021).

Phytochemical Screening

Phytochemical screening of the limeberry fruit ethanol extract and its nanoparticle formulation was carried out to identify the presence of secondary metabolites, including saponins, steroids, terpenoids, tannins, alkaloids, and flavonoids. The analysis was performed following the procedure described by Kumawardani et al. (2021).

Preparation of Limeberry Extract Nanoparticles by Ionic Gelation Method Preparation of Chitosan Solution

The preparation of chitosan with concentrations of 0.25%, 0.50%, 0.75%, and 1%, respectively was carried out by respectively dissolving 0.25g, 0.5g, 0.75g, and 1.00g of chitosan powder in 100 mL of 2% acetic acid solution using a magnetic stirrer at 1,000 rpm for 30 minutes.

Preparation of Sodium Tripolyphosphate (NaTPP) Solution

Sodium tripolyphosphate solution 0.01% was obtained by dissolving 0.01 g sodium tripolyphosphate powder in 100 mL. Sodium tripolyphosphate itself serves as a stabilizing agent in the preparation of limeberry fruit extract nanoparticles.

Preparation of Limeberry Fruit Ethanol Extract Nanoparticles

Preparation of nanoparticles of limeberry fruit ethanol extract by the ionic gelation method adhered the basis of previous research by Nurviana, (2020) which modified the ratio of the chitosan extract, NaTPP, 10, 5, and 1 in 100 mL. The formulation of the use of chitosan, limeberry extract and NaTPP can be seen in **Table 1.**

The extract was mixed with chitosan solution and homogenized with a magnetic stirrer at 1000 rpm for 30 minutes. NaTPP solution was added dropwise into the chitosan extract solution at room temperature with a magnetic stirrer rotation at 1000 rpm for 4 hours until colloidal nanoparticles were formed. After this, the nanoparticle preparation was sonicated for 30 minutes. The resulting colloidal nanoparticles were characterized using PSA, SEM, and FTIR.

Nanoparticle Characterization of Limeberry Fruit Ethanol Extract

Particle size and particle distribution

Measurement of particle size and size distribution of nanoparticles is carried out using a PSA (Particle Size Analyzer) tool. The working principle of this tool is dynamic light scattering (DLS) (Li et al., 2010).

Table 1. Yield characteristics of limeberry fruit powder simplisia extracts

Description	Extract of Limeberry Fruit
Color	Brown
Scent	Characteristic odor
Texture	Thick

Nanoparticle morphology

Morphology measurements were measured using a Scanning Electron Microscopy (SEM) tool. The sample was dissolved into a copper container coated with carbon. The copper container was then put into a freeze- drying device. The dried samples were then observed using SEM (Sun et al., 2014).

Fourier transform infrared (FTIR)

Sample observation with FTIR (Agilent Cary 630) began with the sample placed between the beam splitter and detector. The beam that falls on the beam splitter transmitted part of the wave to the fixed mirror and partly reflected to the movable mirror. Both beams were recombined with the beam splitter and they were emitted to the sample and then were received by the detector. The results received by the detector were then forwarded to the computer to obtain a display of the spectrum of the sample tested. The analysis was carried out at room temperature in the range of 4,000-400 cm $^{-1}$ (Yuliantini, 2020).

Media Creation

Nutrient Agar (NA) powder (6 grams) was dissolved with 300 mL of distilled water in an erlenmeyer and it was covered with aluminum foil. The solution was then heated while stirring using a stirring rod to boil, after which it was sterilized using an autoclave at 121 °C for 15 minutes. Then the solution was poured into a Petri dish (Manus et al., 2016).

Bacterial Culture

The proliferation of bacteria to be tested was carried out by taking isolates of *Staphylococcus aureus* (gram- positive) and *Salmonella sp.* (gram-negative) from the available preparations. The bacteria were aseptically taken using an ose needle and then scratched on the available Nutrient Agar media and incubated in an incubator at 37 °C for 24 hours.

Antimicrobial Activity Testing of Limeberry Fruit Nanoparticles

The test of antimicrobial activity was carried out by the inhibition method. testing of bacterial activity begins with preparing each concentration of limeberry fruit extract nanoparticles that will be used with 5 different treatments, then a bacterial suspension is taken as much as 0.3 mL and placed in a test tube. Bacterial cultures that will be used as test bacteria are refreshed first by scraping one ose of bacterial culture from the slanted agar medium. Next, inoculation was carried out into 10 mL of appropriate sterile liquid media, followed by incubation at 37 °C for 15 minutes. Then as much as 0.1 mL of bacterial culture was inoculated into 20 mL of NA media and then homogenized and poured into a sterile petri dish until it froze. Then the disc paper was placed on a Petri dish containing media that had previously been soaked with limeberry fruit extract nanoparticles for 1 hour according to the treatment and incubated for 24 hours at 37 °C. Antibacterial activity is declared positive if an inhibition zone is formed in the form of a clear zone around the disc paper. Chloramphenicol was used as a positive control, while sterile distilled water served as a negative control. Antibacterial activity was indicated by the formation of a clear inhibition zone around the disc.

Measurement of Zone Inhibition

Observation of bacterial activity on limeberry nanoparticles after incubation was carried out by measuring the diameter of the inhibition area formed in the clear zone around the disc paper, the diameter of the inhibition zone formed around the disc was observed using a caliper/ruler. The antibacterial activity of the limeberry fruit extract nanoparticles was compared with the positive control (F0). The results showed that treatment C3 (0.75% chitosan) produced the lagerst inhibition zone among the nanoparticle formulations and demonstrated strong antibacterial activity against both tested bacteria.

RESULT AND DISCUSSION

Ethanol Extract of Limeberry Fruit *(T. trifolia)*

The maceration process using 96% ethanol produced a dark-brown extract with a thick consistency and a characteristic odor. The extraction yield of this study was 22.5%, indicating that ethanol was effective to extract bioactive compounds from limeberry fruit. Ethanol is widely used in plant extraction because of its ability to dissolve a wide range of polar and moderately non-polar secondary metabolites (Wendersteyt et al., 2021). It is a solvent capable of extracting flavonoid, saponin, tannin, terpenoid and alkaloid compounds (Gagliardi et al., 2023).

Limeberry fruit weighing 2.0 kg produces 450 g of simplisia. Fresh fruit contains a moisture content of 79.14%, while the simplisia or dry powder of limeberry fruit contains 7.35%. The ratio of powder and solvent in this extraction by soaking 450 g of powder in 750 mL of 96% ethanol solvent obtained 22.5% extract. The maceration process lasts for 24 hours with 3 times remaceration or replacement of new solvents which aims to extract the compounds contained in the sample thoroughly. Every 24 hours,

the liquid extract was filtered and then it was replaced with a new solvent. The characteristics of the resulting limeberry fruit extract are dark brown in color with a thick consistency and distinctive odor.

Phytochemical Screening of Limeberry Fruit Extract Nanoparticles

The secondary metabolite component test aims to determine the content of active compounds contained in a plant. The results of the phytochemical screening nanoparticles of ethanol extract of limeberry fruit can be seen in **Table 2.**

Based on the phytochemical screening results, the nanoparticles of limeberry fruit ethanol extract were found to have contained terpenoids, tannins, alkaloids, flavonoids, and phenolic compounds. These findings are consistent with previous studies reporting that *T. trifolia* contains various secondary metabolites, such as flavonoids, terpenoids, and polyphenolic compounds that contribute to its biological activity. Santos et al. (2008) reported that extracts of *T. trifolia* contain bioactive compounds with antimicrobial properties. Similar phytochemical profiles have also been reported in other plant-based nanoparticle formulations where flavonoids and phenolic compounds play an important role in antibacterial activity. These bioactive compounds originate from the limeberry fruit extract, which is known to contain several secondary metabolites such as alkaloids, terpenoids, polyphenols, tannins, and flavonoids.

Particle Size and Distribution

The diameter size of the limeberry fruit extract nanoparticles was determined using a particle size analyzer. The diameter and polydispersity index values for samples A (0.25%), B (0.50%), C (0.75%), and D (1%) respectively are presented in **Table 3**.

The results of testing the particle size diameter of the four types of formulations showed that only two types were indicated, namely using chitosan 0.75% and 0.5%, with particle size diameter values of 365.81 nm and 943 nm, respectively. However, of the two indicated formulations, only the use of 0.75% chitosan had the nanoparticle size range of 10-1000nm. This is in line with the research by Irawan et al. (2019) that the ionic gelation method using chitosan-NaTPP can produce nanoparticles with the size range of 200-500 nm. This statement is also supported by the results of the research report by Pichla et al., (2021), that the use of chitosan which tends to increase can produce smaller nanoparticle sizes.

The use of chitosan concentrations that does not meet the threshold limit of use cannot form a perfect nanoparticle structure. The use of excess chitosan can result in the formation of cross-links that tend to be unstable and irregular (Elkomy et al., 2022). This is because the interaction of positive charges on chitosan together with secondary metabolites against negative ions is blocked (Wang et al., 2023). Another factor that contributes to the size of limeberry fruit nanoparticles is the amount of protonation of polycations in chitosan in the form of amine groups against polyanions contained in NaTPP. The higher the amount of protonation of polymer cations against anions is, the lower the pH value will be. The pH values within the range of 3.5-5.5 have the potential for aggregate formation that tends to be low along with Van der Waals forces in cross-linking bond interactions, so that electrostatic interactions between the two polymers tend to decrease, especially in particle size distribution (Picos-Corrales et al., 2023).

Table 2. Phytochemical screening results of nanoparticles of ethanol extract of limeberry extract type of qualitative phytochemical test

Phytochemical Test Result	Description	
Saponins	+	Positive
Steroids	–	Negative
Terpenoids	+	Positive
Tannins	+	Positive
Alkaloids	+	Positive
Flavonoids	+	Positive
Phenolic	+	Positive

Table 3. Particle size and distribution of limeberry fruit extract

Treatment	Particle Size (nm)	Polydispersity index (PDI)
A	-	-
C	943.81	0.758
C	365.81	0.718
D	-	-

Description:
(-): Not indicated; A: Use of 0.25% chitosan; B: Use of 0.50% chitosan; C: Use of 0.75% chitosan; D: Use of 1% chitosan

Table 4. Results of inhibition zone diameter of limeberry fruit extract nanoparticles against *S. aureus* bacteria

Treatment	Average (mm)	Sd	Category
B1	24.75[c]	± 0.21	Very strong
B2	25.48[b]	± 0.15	Very strong
B3	28.56[a]	± 0.40	Very strong
B4	23.34[d]	± 0.37	Very strong
E0	29.50	-	Very strong
A0	-	-	No inhibition

Description: Values followed by the same letter notation indicate no significant difference. B1: Use of 0.25% chitosan in nanoparticle preparation; B2: Use of 0.5% chitosan in nanoparticle preparation; B3: Use of 0.75% chitosan in nanoparticle preparation; B4: Use of 1% chitosan in nanoparticle preparation E0(+): Ethanol 96% (Positive control); A0(-): Distilled water (Negative control)

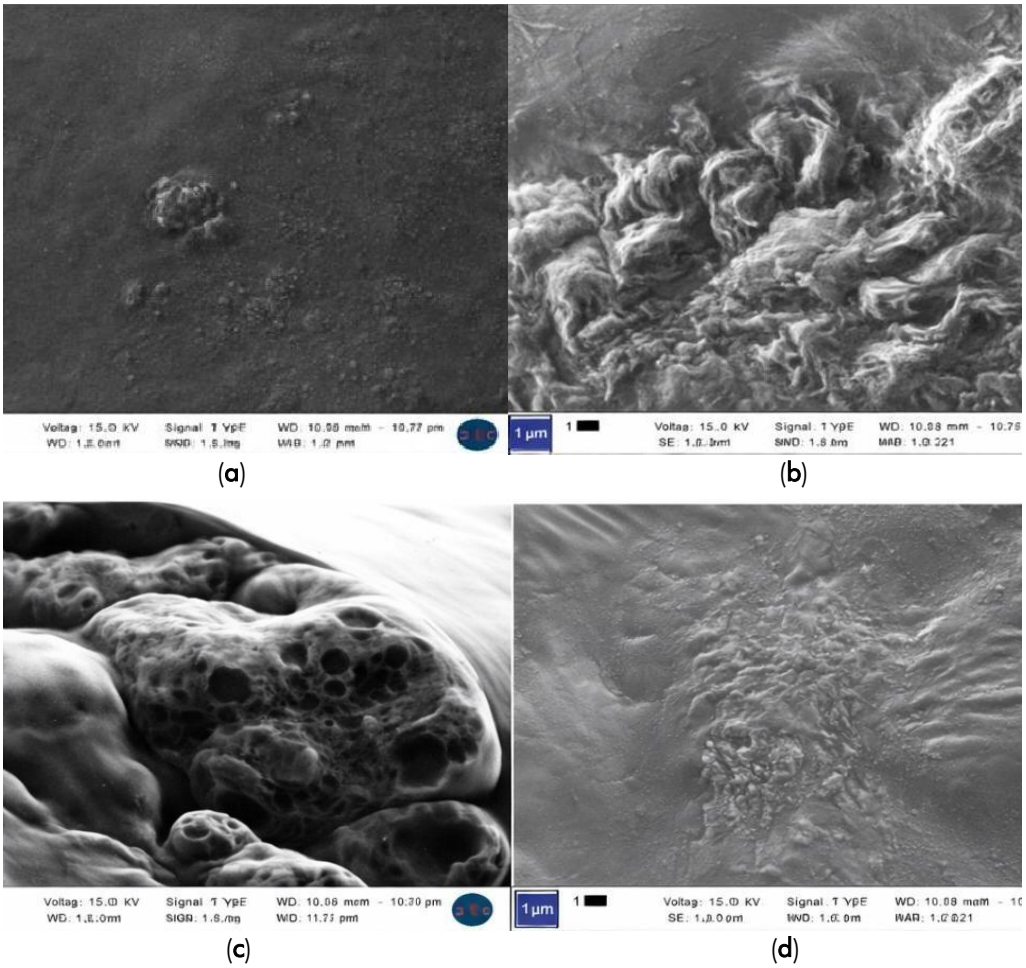

Figure 1. SEM results; (**a**) sample A (using 0.25% chitosan concentration); (**b**) sample B (use of 0.50% chitosan concentration); (**c**) sample C (use of 0.75% chitosan concentration); and (**d**) sample D (use of 1% chitosan concentration)

Particle size distribution is expressed in terms of polydispersity index (PI). The results of the polydispersity index based in **Table 4** showed that only two were indicated of the four types of formulations, namely the use of 0.75% and 0.50% chitosan, with PI values of 0.718 and 0.758, respectively. PI value <1, indicating that the particle size tends to be homogeneous. This is in line with the statement by Abere et al., (2022) that a PI value of <1 indicates that the dispersion between polymer bonds is narrow and evenly distributed, while a PI value of >1 suggests that the dispersion of molecules tends to be wide and uneven. This can occur because the particles formed tend to be heterogeneous, so that the nanoparticle-forming formulation experiences flocculation and caolescense (Karimirad et al., 2019).

Particle Morphology

The shape and surface of nanoparticles can be observed using scanning electron microscopy (SEM). Characterization using SEM aims to see the surface morphology of particles or the 3-dimensional shape of particles and the size of these particles through an image (Fitri et al., 2021). The morphological structure of limeberry fruit extract nanoparticles formed through SEM testing with a magnification of 10,000 times on the use of 4 different chitosan concentrations is presented in **Figure 1.**

The test results showed that during the nanoparticle manufacturing process, particles were formed perfectly at a chitosan concentration of 0.75%. This concentration produced nanoparticle morphology in the form of imperfect spheres that had a fairly uniform size. Meanwhile, the use of chitosan concentrations of 0.25%, 0.50%, and 1% did not produce morphologically perfect molecular particles. This indicates that chitosan concentrations that are either higher or lower than the threshold limit of use cannot form nanoparticle structures perfectly. According to Deshmukh et al. (2022), the use of excess chitosan results in the formation of cross- links that tend to be unstable and irregular. This is because the interaction of positive charges on chitosan together with secondary metabolites against negative ions is blocked.

Fourier Transform Infrared (FTIR)

FTIR analysis testing to determine the functional groups contained in chitosan and limeberry fruit extract nanoparticles on **Figure 2.** The FTIR spectrum of pure chitosan shows a broad absorption band around 3,200-3,400 cm^{-1}, which corresponds to the stretching vibration of hydroxyl (O-H) and amine (N-H) groups. Similar absorption bands have also been reported for chitosan in previous studies (Tomaz et al., 2018), indicating the characteristic functional groups of chitosan. This characterization identifies the interaction between these functional groups (Putri et al., 2019). Some Functional groups such as C-H stretching vibrations were not clearly observed in the spectrum, possibly due to overlapping peaks or low-intensity absorption bands. The specific functional group wavenumbers of chitosan and limeberry fruit extract nanoparticles can be seen in **Table 5.**

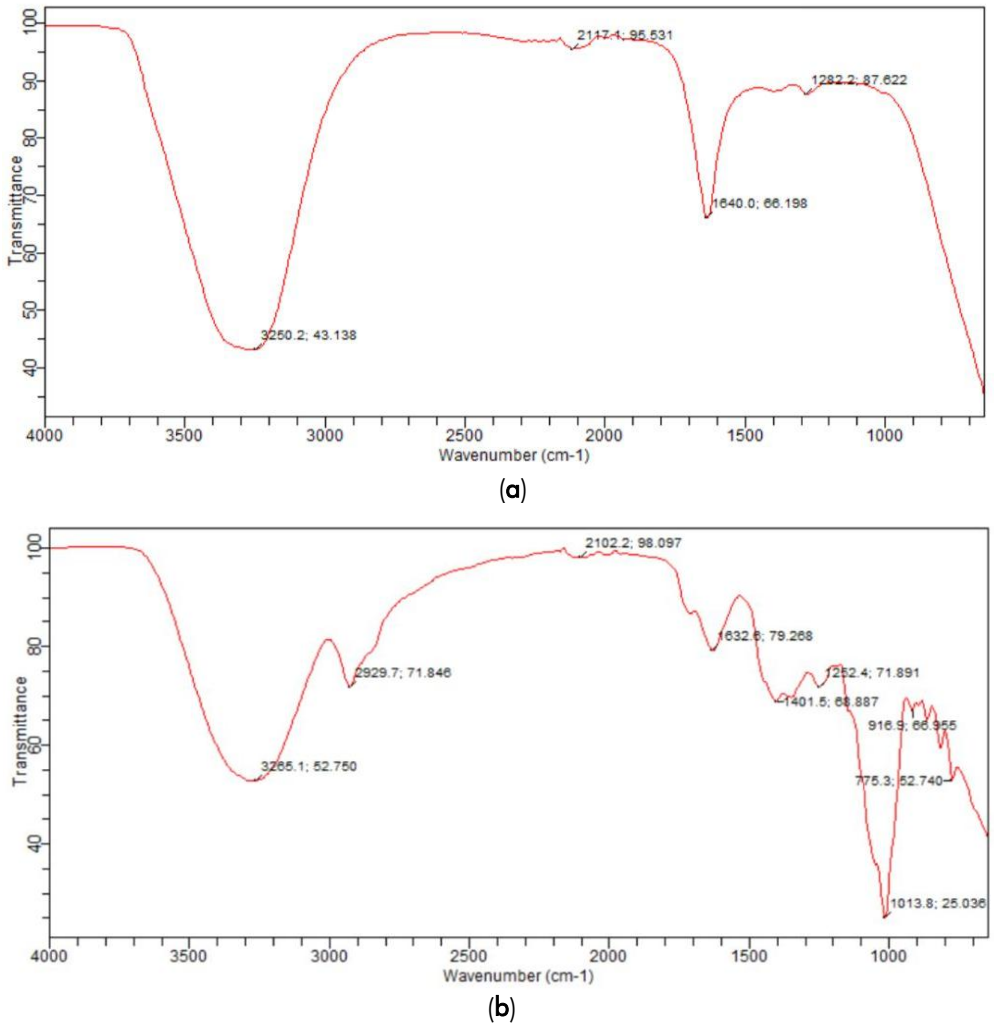

Figure 2. FTIR results; (**a**) FTIR spectra of pure chitosan; (**b**) FTIR spectra of limeberry fruit extract nanoparticles

The graph of FTIR results showed a shift in the wavenumber of the -OH group from 3,250.2 in chitosan to 3,257.7 cm^{-1} on limeberry fruit extract nanoparticles. The shift in the -OH stretching vibration from 3,250.2 cm^{-1} to 3,257.7 cm^{-1} indicates the presence of intermolecular interactions between chitosan and bioactive compounds from limeberry extract (Dwitarani *et al.*, 2021). These interactions may occur through hydrogen bonding between hydroxyl groups of chitosan and phenolic compounds present in the extract, which can alter the vibrational energy of functional groups and cause slight shifts in the FTIR spectrum. Furthermore, in the limeberry fruit extract nanoparticles and chitosan, absorption in the 1,640.0 cm^{-1} refers to a bending vibration of the N-H group which is a chitosan functional group. In the spectra of limeberry fruit extract nanoparticles, a new absorption peak at a wavenumber of 1,013.8 cm^{-1} shows the absorption spectra of phosphate groups (PO3) derived from the use of stabilizers, namely sodium tripolyphosphate (NaTPP). This peak corresponds to the P-O stretching vibration of phosphate groups and confirms the successful ionic interaction between chitosan and NaTPP during nanoparticle formation. The functional groups contained in sodium tripolyphosphate (NaTPP) are P=O groups, PO2, PO3 groups and P-O-P groups (Tomaz et al., 2018). The same findings were also reported by Amilatussholihah (2020) that a phosphate peak (PO3) was reached at wavenumber 1,045. cm^{-1}. The stretching of the PO3 group indicates the formation of a cross-link between the amine group (NH) of chitosan and the anionic group (PO3) on NaTPP.

FTIR spectra of limeberry fruit extract nanoparticles showed the presence of hydroxyl group -OH and C-O group at wavenumber 1,259.8. cm^{-1} indicating secondary metabolite compounds in limeberry fruit extract, namely flavonoid compounds playing roles to form crosslinks with chitosan. The shift occurred in the C-O functional group at wavenumber 1,252.4 of the cm^{-1} chitosan, wavenumber 1,282.2 cm^{-1} limeberry fruit extract and wavenumber 1,259.8 cm^{-1} limeberry fruit extract nanoparticles. The shift in spectra of limeberry fruit extract after the formation of nanoparticles in the -OH and C-O groups indicates that these groups play a role in the formation of cross-links between amine groups in chitosan and phosphate groups from sodium tripolyphosphate (NaTPP). Under acidic conditions, the amino groups of chitosan become protonated ($–NH^{3+}$), enabling electrostatic interactions with negatively charged groups such as phosphate ions from NaTPP or phenolic compounds from plant extracts. These interactions contribute to the formation of stable nanoparticle structures through ionic gelation.

Table 5. Spesific functional group wavenumbers of chitosan and limeberry fruit extract nanoparticles

Function Group Chitosan	Fruit Extract Nano-particles	Literature	Wavenumber (cm^{-1})
O-H	3,250.2	3,257.7	3,700-3,100
C-H	Not Observed	Not Observed	3,000-2,700
C=O	Not Observed	Not Observed	1,900-1,550
N-H	1,640.0	1,640.0	1,660-1,500
PO_3	Not Observed	1,013.8	1,088-920
C-O	128.2	1,259.8	1,300-1,000

Table 6. Results of inhibition zone diameter of limeberry fruit extract nanoparticles against *Salmonella Sp.*

Treatment	Average	Sd	Category
C1	18.22^{c}	± 0.01	Strong
C2	19.00^{b}	± 0.05	Strong
C3	23.57^{a}	± 0.06	Very strong
C4	17.28^{d}	± 0.03	Strong
Positive control	20.81	-	Very strong
Negative Control	-	-	-

Description:
C1: Use of 0.25% chitosan in nanoparticle preparation C2: Use of 0.5% chitosan in nanoparticle preparation C3: Use of 0.75% chitosan in nanoparticle preparation C4: Use of 1% chitosan in nanoparticle preparation positive control: ethanol 96%
negative control: distilled water

Values represent the mean inhibition zone diameter (mm) ± standard deviation from three replicates. Chloramphenicol was used as the positive control, while sterile distilled water was used as the negative control. The antibacterial activity was classified based on the inhibition zone diameter as follows: weak (≤5 mm), moderate (6-10 mm), strong (11-20 mm), and very strong (≥21 mm) (Surjowardojo et al., 2016). The largest inhibition zone diameter was observed in the 0.75% chitosan treatment against *Staphylococcus aureus* and *Salmonella* sp., producing inhibition zones of 28.56 mm and 23.57 mm, respectively. In contrast, the smallest inhibition zone was observed in the 1% chitosan treatment with inhibition zones of 23.35 mm and 17.28 mm. The reduction in antibacterial activity at higher chitosan concentrations may be associated with the formation of denser polymer networks during nanoparticle formation. Excessive chitosan can increase the viscosity of the system and produce tighter cross-linked structures, which may reduce the diffusion of active compounds from nanoparticles into the surrounding medium. As a result, the release of antibacterial compounds becomes less effective, leading to smaller inhibition zones. According to Surjowardojo et al. (2016), the diameter of the inhibition zone of ≤ 5 mm, 6-10 mm, 11-20, and ≥ 21 mm inhibitory strength against a bacterium is categorized as weak, moderate, strong, and very strong respectively. This illustrates that limeberry fruit nanoparticles have good antibacterial effectiveness against gram-positive bacteria (*S. aureus*) and gram-negative bacteria (*Salmonella sp.*).

The ability to inhibit the antibacterial activity of limeberry ethanol extract nanoparticles comes from the content of antibacterial compounds in limeberry fruit. According to Egra et al. (2019), antibacterial compounds generally inhibit bacterial growth starting with damaging the bacterial cell wall. The mechanism of flavonoid compounds in inhibiting bacterial growth is by damaging the permeability of bacterial cell walls, microsomes, and lysosomes. Flavonoid compounds have a group, namely in the form of a hydroxyl group with the capability of changing organic components and nutrient transportation in bacteria which in turn result in the onset of toxic effects on bacteria. The content of antibacterial compounds tends to increase in effectiveness due to the reduction of particle size through a nano-formation system with the ionic gelation method. According to Vargas et al., (2020), the reduction of particle size will lead to an increase in surface area and to increase particle breakdown which causes the benefits of active compounds in limeberry to increase so that bioavailability tends to spread evenly. Smaller nanoparticle sizes are very easy to penetrate deeper bacterial cell walls and interact with membranes that cause cell membrane damage (Dalir et al., 2020). Nanoparticles of limeberry fruit extract are formed due to the cross-linking of ammonium ions contained in chitosan, phosphate ions in NaTPP and limeberry fruit extract which form electrostatic interactions (Uzair et al., 2020).

CONCLUSIONS

The findings of the current study concludes that limeberry fruit extract *(T. trifolia)* can be made into nanoparticle preparations. Nanoparticles of limeberry fruit extract with the use of chitosan concentration of 0.75% (b/v) produce characteristics with a particle size of 365.81 nm, polydispersity index of 0.718 which indicates homogeneous particle size and imperfect spherical particle morphology. The FTIR analysis showed that there was cross- linking between chitosan, sodium tripolyphosphate (NaTPP), and limeberry fruit extract. Nanoparticles of limeberry fruit extract at a concentration of 0.75% produced the best antibacterial effectiveness with the formation of an inhibition zone value of 23.57 nm on *Salmonella* sp. bacteria and 28.56 nm on *S. aureus* bacteria.

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