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Antibacterial activity of guava *(Psidium guajava* L.*)* leaf ethanolic extract nanosuspension against *Escherichia coli* bacteria

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ABSTRACT

Diarrhea is a condition where a person has bowel movements three or more times a day, with consistent stools. One of the common bacteria that causes diarrhea is *Escherichia coli.* Empirical and preclinical studies have demonstrated the effectiveness of guava leaves *(Psidium guajava* L.*)* in treating diarrhea due to their tannin content. Nanosuspension formulations can be created to simplify the use of guava leaves for medicinal purposes. This study aims to investigate the efficacy of guava leaf extract, both in its natural form and as a nanosuspension preparation, against *Escherichia coli*. Additionally, the study aims to characterize the guava leaf extract nanosuspension used in the experiment. The technique used to make nanosuspension involves ionic gelation methods by using chitosan as a polymer, and subsequent characterization of the resulting product includes organoleptic testing, specific weight, pH, sedimentation volume, and viscosity. After the characterization of the guava leaf nanosuspension, it was found that the optimal formula had a particle size of 245.7 nm at a concentration of 0.01%, a polydispersion index of 0.406, and a zeta potential of +26.9 mV. Guava leaf ethanol extract 1% has a diameter of the inhibitory zone of 4.05±0.45 mm. However, the nanosuspension form of *P. guajava* L at a concentration of 0.01% has an inhibitory zone diameter of 11.45±0.64 mm. The nanosuspension formulation using *P. guajava* L has met the evaluation requirements and has antibacterial activity against *E. coli* bacteria.

Keywords: *Escherichia coli*, guava leaf ethanolic extract, ionic gelation method.

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INTRODUCTION

Diarrhea poses a considerable health challenge and stands as a prominent cause of illness and death among children across numerous nations, including Indonesia. It is characterized by the occurrence of three or more liquid bowel movements within a day or night. Children are particularly susceptible to diarrhea due to their less robust immune systems (Friedel & Cappell, 2023).

Escherichia coli is a bacterium known to trigger diarrhea, often spread through direct contact, and is particularly prevalent in regions with inadequate sanitation. Guava leaves, rich in compounds such as alkaloids, flavonoids, tannins, phenolics, quinones, and saponins, offer the potential to manage diarrhea. Then, guava ethanol extract with an extract solution concentration of 250 mg/mL has a MIC value of 4.3944 mg/mL against *E. coli* bacteria (Maysarah & Apriani, 2016). Furthermore, a 50% guava extract concentration showed antibacterial properties, effectively inhibiting *E. coli* with an inhibition zone diameter of 10.17±0.24 mm (Farhana et al., 2017).

Nanosuspension preparations offer a convenient means of utilizing guava leaves for medicinal purposes. This technology presents various benefits, including the alteration of surface properties and particle size of herbal remedies, facilitating precise delivery to targeted organs like the brain, lungs, kidneys, and digestive tract with enhanced selectivity, effectiveness, and safety. Furthermore, it enables the controlled release of active compounds, thus reducing potential side effects (Al-Kassas et al., 2017; Alaei et al., 2016; Du et al., 2015; Jahan et al., 2016; Wang et al., 2013). Nanosuspension is widely recommended for herbal medicines because it can reduce drug side effects by using smaller doses compared to conventional doses and improve the physical and chemical stability of drugs (Ansari et al., 2012; Narasaiah et al., 2010). Moreover, augmented particle surface area and solubility can result in heightened bioavailability and absorption of active components. Additionally, the size of the nanoparticles tends to prolong residence times in the GI tract (Ahmad et al., 2015).

The method used to prepare the guava leaf nanosuspension is the ionic gelation method. It uses bottom-up technology to create nanoparticles from a molecular solution by controlling their characteristics, such as size and morphology, for example, using solvent evaporation (Du et al., 2016). Chitosan is used as a polymer that has properties bioactive, biocompatible, chelating, antibacterial, and biodegradable properties. Nonetheless, chitosan's rapid water absorption and significant swelling propensity in aqueous environments limit its suitability for biological and medical applications as a drug delivery and release system. Hence, incorporating sodium tripolyphosphate becomes essential to generate chitosan derivatives with heightened biocompatibility and reduced swelling characteristics (Karimirad et al., 2020). Employing sodium tripolyphosphate as a cross-linking agent at a minimal dosage prevents excessive bonding between the TPP polyanion and chitosan's amino groups. This technique, known as the ionic gelation method, combines chitosan polymer and sodium tripolyphosphate. The formation mechanism of chitosan nanoparticles hinges on the electrostatic interaction between chitosan's amino groups and the negative groups of a polyanion, such as tripolyphosphate (Diniatik et al., 2019).

MATERIALS AND METHOD

Equipment

The experiments utilized an analytical balance (Mettler Toledo), macerator, incubator (Memmert IN 55), autoclave (Biobase), rotary evaporator (Ika), vortex (MixMat), magnetic stirrer with heater (model 79-1), oven (B-one), micropipette (Socorex), DelsaTM Nano C particle Analyzer (Beckman Coulter), and standard laboratory glassware.

Materials

Guava leaves (*P. guajava* L.) comes from the Wado Sumedang area with evidence of plant identification No.189/HB/11/2018 from the Jatinangor Herbarium of the Plant Taxonomy Laboratory (Department of Biology, FMIPA, Padjadjaran University), Ethanol 96% (PT. DPH), Muller Hinton Agar (MHA) media (PT. DPH), *E. coli* bacteria (ATCC 25922), NaCl 0,9% (Otsuka), Acetic acid 1% (Merck),

Chitosan (Sigma Aldrich), Sodium tripolyphosphate (Merck), PGS (Pulvis Gummosus) (PT. DPH), Methylparaben (PT. DPH), Simple syrup, Mint flavor (PT. DPH), Aquadest (PT. DPH), Aqua deionization (PT. Jayamas Medica Industri) and reagents required for screening are Ammonia, Chloroform (Merck), H2SO4 (Merck), Dragendorff reagent (PT. DPH), Mayer reagent (PT. DPH), Wagner reagent (PT. DPH), Magnesium metal (Merck), Zinc, HCl 5N, Amyl alcohol (Merck), Ether, Liebermann burchard reagent, FeCl3 (Merck) and Gelatin 1% (Brataco).

Methods

Sample preparation

Initially, guava leaves underwent a powdering process. They were cleansed with water and then dried in an oven at 40°C for 5 days. After drying, the guava leaves were finely ground into powder and filtered through an 80-mesh sieve.

Extraction preparations

The 500 g of the powdered guava leaves were placed in a maceration container and then soaked in 1.5 liters of 96% ethanol for 3 days while stirring several times. The resulting maceration was then filtered to separate the ethanol liquid from the residue. The liquid extract was concentrated using a rotary evaporator.

Characterization of the simplicia and extract

The simplicia and extract were characterized using organoleptic tests, assessing color, odor, and taste. Phytochemical screening was conducted, involving tests for alkaloid, flavonoid, saponin, tannin, and polyphenol content and the determination of total ash content. Additionally, the ethanol-soluble extract content, water-soluble extract content, and water content were determined using the azeotropic distillation method (Harborne, 1996; Departemen Kesehatan RI, 2000).

Antibacterial activity testing methods

The antibacterial testing was carried out using the diffusion method. 20 mL of Muller Hinton Agar (MHA) media was placed in a sterilized petri dish, and 200 µl of bacterial suspension was added. The dish was then swirled to distribute the media and bacteria and allowed to solidify evenly. Four holes were then made with equal spacing between them. In addition, *E. coli* bacteria from the stock were taken and mixed with 0.9% NaCl, then homogenized. The bacterial suspension was then standardized using the McFarland standard solution, and 50 µl of the extract was poured into the prepared wells. The petri dish was then incubated for 16 to 18 hours at 35±2℃; ambient air (CLSI, 2012).

Antibacterial activity of guava leaf extract

Testing for the antibacterial activity of guava leaf extract involves diluting the extract to assess its inhibitory zone activity against bacteria. The concentrations used in this test were 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, and 10%. The antibacterial testing was carried out using the diffusion method. 20 mL of Muller Hinton Agar (MHA) media was placed in a sterilized petri dish, and 200 µl of bacterial suspension was added. The dish was then swirled to distribute the media and bacteria and allowed to solidify evenly. Four holes were made with equal spacing, and different extract concentrations were placed in each well. The petri dish was then incubated for 24 hours at 37°C. After that, bacterial growth was observed, and the inhibition zone diameter was measured.

Nanoparticle preparation (ionic gelation method)

Approximately 4 mL of a 0.01% guava leaf extract solution in a 1% acetic acid solvent is placed into a vial. Subsequently, 1 mL of a 0.25% chitosan solution is added to the vial using a 1% acetic acid solvent. The mixture is stirred using a magnetic stirrer for 10 minutes until all components of the guava leaf extract are entirely dissolved. Then, 4 mL of a 0.1% sodium tripolyphosphate solution, prepared with deionized water, is added dropwise at a rate of 1 drop per second. This addition of sodium

tripolyphosphate is carried out on the magnetic stirrer with a speed of 500 rpm for 1 hour. Subsequently, particle size and zeta potential are measured using a DelsaTM Nano C Particle Analyzer (Beckman Coulter (Woranuch & Yoksan, 2013). The composition of guava leaf extract nanoparticles is detailed in Table 1.

Table 1. Guava leaf extract nanoparticle composition

Formula	The ethanolic extract of guava leaf	Chitosan (0.25%)	Sodium tripolyphosphate (0.1%)
F I			
The ethanolic extract of guava leaf 0.05%	4 mL	1 mL	5 mL
F II			
The ethanolic extract of guava leaf 0.01%	4 mL	1 mL	5 mL

Preparation of nanosuspension formulation of guava leaf extract

PGS (*Pulvis Gummosus*) and water are combined in a mortar and crushed until a homogeneous mixture is obtained. Meanwhile, methylparaben is dissolved in hot water in a glass beaker while stirring until completely dissolved. Subsequently, the dissolved methylparaben, simple syrup, mint flavoring, and guava leaf extract nanoparticles are added into the mortar and crushed until homogeneous. Then, distilled water is added to reach a total volume of 100 mL. The formulation details of the guava leaf extract nanosuspension are provided in Table 2.

Table 2. Nanosuspension formulation of guava leaf extract

Composition	Formula I	Formula II
Guava Leaf Extract	8.3%	-
Guava Leaf Extract Nanosuspension	-	8.3%
PGS (Pulvis Gummosus)	1%	1%
Methylparaben	0.1%	0.1%
Simple syrup	25%	25%
Mint Flavour	qs	qs
Aquadest ad	60 mL	60 mL

Evaluation of Guava Leaf Extract Nanosuspension

Guava leaf extract nanosuspension evaluation includes organoleptic, specific gravity, pH, sedimentation volume, and viscosity testing.

Organoleptic test

Organoleptic test including color, odor, and taste.

Specific gravity test

A pycnometer is weighed and recorded as A grams. It is then filled with water up to the brim and weighed again, recording the weight as A1 grams. The nanosuspension is put into an empty pycnometer, weighed, and recorded as A2 grams. The specific gravity of the nanosuspension is then calculated (Emilia et al., 2013).

pH test

The nanosuspension is put into a beaker, and the pH meter is inserted to measure the pH. The results are recorded.

Sedimentation volume test

The nanosuspension is put into a measuring glass of up to 50 mL and left for approximately 1 hour until two phases form. The sedimentation volume is observed and recorded.

Viscosity test

The viscosity test involves transferring the nanosuspension into a beaker and configuring a viscometer to a predetermined speed for viscosity measurement using a Brookfield Viscometer. Subsequently, the recorded results are documented.

Antibacterial Activity of Guava Leaf Extract Nanosuspension

The method is the same as the antibacterial activity testing methods.

RESULT AND DISCUSSION

The phytochemical screening and characterization of simplicia and ethanolic extract of guava leaf

The outcomes of the phytochemical screening conducted on both the simplicial form and the extract yielded identical results concerning the secondary metabolite content yang, as seen in Table 3. This implies that the extraction method did not eliminate the available secondary metabolites in their original form within the simplicia. The non-specific characteristic testing conducted on both the simplicia form and the extract complied with the standards outlined in the Indonesian Herbal Pharmacopoeia (Kementerian Kesehatan RI, 2017). Furthermore, the extract formulation exhibited a decrease in total ash content, indicating a reduction in impurities. Moreover, there was an elevation in the concentration of water-soluble and ethanol-soluble compounds, signifying an enhanced concentration of compounds in the extract formulation compared to the simplicia form. The results of testing non-specific characteristics of simplisia and guava leaf ethanol extract are presented in Table 4.

Table 3. Results of phytochemical screening of guava leaves

Secondary Metabolite	Simplicia	Extract
Flavonoid	+	+
Tannin/Polyphenol	+	+
Saponin	+	+
Quinon	+	+
Steroid/Triterpenoid	+	+
Alkaloid	+	+

Information:
(+) Positive Results; (-) Negative Results

Table 4. Non-specific characteristics of simplicia & guava leaf ethanol extract

Characteristic	Simplicia (%)	FHI Parameters	Extract (%)	FHI Parameters
Water Content	4.00% ± 0.00	<10%	8.00 ± 0.00	<10%
Ash Content	8.84% ± 0.21	<9%	1.64 ± 0.82	<6.1%
Water Soluble Extract Content	24.48% ± 0.01	>18.2%	49.46 ± 0.39	>18.2%
Ethanol Soluble Extract Content	22.60% ± 0.24	>15%	72.45 ±0.86	>15%
Yields			9.506%	

The antibacterial activity of the guava leaf ethanolic extract

At a concentration of 10%, the ethanolic extract from guava leaves maintains its antibacterial effectiveness, confirming the presence of antibacterial properties within the extract. Identifying the

active extract involves establishing its Minimum Inhibitory Concentration (MIC), which gauges the extract's potency against the test bacteria until reaching the lowest effective concentration. Therefore, the MIC is determined at concentrations ranging from 0% to 10% with a concentration variation interval of 1. Several factors can affect the inhibition of test bacteria by antibacterial compounds, including the thickness of the medium bacteria (Maysarah & Apriani, 2016). The results of the antibacterial activity test of guava leaf ethanolic extract are presented in Table 5.

Table 5. The results of the antibacterial activity of the guava leaf ethanolic extract

Concentration (%)	Inhibition zone (mm) ± SD
0	-
1	4.05 ± 0.45
2	4.30 ± 0.30
3	4.72 ± 0.92
4	5.20 ± 0.50
5	6.35 ± 0.55
6	6.45 ± 0.35
7	6.50 ± 0.50
8	6.55 ± 0.15
9	6.65 ± 0.55
10	6.75 ± 0.15

Optimization of nanoparticle preparation

The optimization of nanoparticle production aims to determine an appropriate formula to obtain an optimal result. The optimization includes the amount of chitosan polymer, cross-linking agent sodium tripolyphosphate, stirring time, rotation speed, and dripping speed. Chitosan is a polymer that includes cationic polymers with a high electric charge at pH <6.5 so that it adhesives to the negative surface and chelate metal ions. The highly reactive hydroxyl and amino groups in chitosan play a role in chemical reactions and salt formation. Amino groups allow chitosan interaction with anionic systems, resulting in changes in the physicochemical characteristics of such combinations (Gomes et al., 2017). Tripolyphosphates in multi-ion crosslinked nanoparticles are used as ion pairs of chitosan. The reason for using tripolyphosphate is its nature as a multivalent anion that can form cross-linked bonds with chitosan to produce nanoparticles that are more stable and have better membrane penetration characteristics (Shi et al., 2011). The formation mechanism of chitosan nanoparticles arises from the electrostatic interaction between the amine group of chitosan and the negative groups of polyanions like tripolyphosphate (Matalqah et al., 2020). The results of the nanoparticles of guava leaf extract can be seen in Figure 1.

Figure 1. Nanoparticles of guava leaf extract

The determination of particle size, polydispersity index, and zeta potential

The polydispersity index is a numeric gauge of nanoparticle size distribution within a formulation. A lower polydispersity index implies greater long-term stability of the dispersion system. When the

index approaches 0, it signifies a uniformly dispersed formulation, while a value surpassing 0.5 indicates notable heterogeneity (Desmiaty et al., 2017). Both formulations yielded particle sizes within the nanoparticle range, typically spanning 10-1000 nm. Nanoparticle size can be influenced by factors such as polymer concentration, crosslinker drip rate, and rotational speed during manufacturing.

The polydispersity index findings reveal favorable values of 0.489 and 0.406 for both formulations, respectively. These values fall below 0.5, indicating that the formulations belong to the monodispersed category. In this category, the narrow distribution of nanoparticle particles promotes even dispersion and greater stability compared to polydisperse formulations. Apart from particle size, zeta potential is a crucial characteristic of nanoparticles. Zeta potential testing is conducted to anticipate the stability of colloidal solutions, where particle interactions significantly influence colloidal stability. Zeta potential quantifies the repulsive forces between particles (Ubgade et al., 2021).

Zeta potential testing was only conducted on formulation II because it had the smallest particle size. The zeta potential measurement result for formulation II yielded a value of +26.9 mV, indicating that the nanoparticles in the formulated colloid suspension are approaching stability as they are close to the value of 30 mV. This result can be seen in Table 6.

Table 6. The characterization of guava leaf nanoparticle preparations *(P. guajava* L.*)*

Formula	Size Particle (nm)	Polydispersity Index	Zeta Potential (mV)
I	451.1	0.489	-
II	245.7	0.406	+ 26.9

The evaluation of nanoparticle guava leaf ethanolic extract preparations

The assessment findings of the nanosuspension formulation revealed a milky white appearance, a mint aroma, and a bitter taste. The specific gravity test aimed to compare the final specific gravity of the formulation with the theoretical specific gravity of the suspension. This test, commonly employed for liquids, relies on comparing the weight of a substance in air at 25°C with the weight of water at the same volume and temperature. In suspensions, when water is used as the carrier, the resulting specific gravity typically exceeds that of the carrier (Wang et al., 2013). The specific gravity of the nanosuspension formulation was determined using a pycnometer, yielding results of 1.263 g/mL, 1.0334 g/mL, and 1.0343 g/mL, as illustrated in Table 7.

Table 7. Evaluation of nanosuspension guava ethanolic extract preparations

Parameters	Formula		
	Blank	I	II
Color	White	White	White
Odor	Mint	Mint	Mint
Flavor	Bitter	Bitter	Bitter
Specific gravity	1.2630 g/mL	1.0334 g/mL	1.0343 g/mL
pH	5.6	6.2	5.4
Viscosity	246.7 cP	214.0 cP	241.3 cP

The pH analysis was carried out to assess the acidity level of the completed formulation. Findings revealed that the formula blank exhibited a pH of 5.6, while formula I displayed a pH of 6.2, and formula II had a pH of 5.4. Viscosity testing was conducted to evaluate the consistency and flow characteristics of the final suspension. Per the national standard (SNI), suspension viscosity typically ranges from 37 to 396 cP. The examination outcomes indicated that the suspension's viscosity fell within the established range documented in the literature. These findings are summarized in Table 8.

Table 8. The sedimentation test of nanosuspension guava leaf ethanolic extract

Evaluation Time		Formula		
		Blank	**I**	**II**
	Vo	50 mL	50 mL	50 mL
15,30,40,60 minutes	**Vu**	50 mL	50 mL	50 mL
1 – 5 days	**F**	**1**	**1**	**1**

Information:
Vo = Initial volume; Vu = Final volume of the precipitate; F= Sedimentation volume

The sedimentation volume assessment aimed to ascertain the sedimentation volume of the completed formulation by comparing it with the expected value. Sedimentation volume is determined as the ratio of the final sedimentation volume (Vu) to the initial suspension volume (Vo) (Syamsuni, 2006). When F = 1 signifies flocculation equilibrium, the suspension is deemed satisfactory. If F>1 indicates a very loose and fine floc, resulting in Vu>Vo. Evaluation findings for the three suspension formulations revealed an F value of 1, suggesting these formulations are well-prepared suspensions.

The activity of nanosuspension guava leaf extract preparations

The activity test results conducted on Formula II, which is the nanosuspension, showed the largest inhibition zone of 11.45±0.64 mm, while Formula I showed 8.40±0.35 mm. This indicates that nanosuspension is more effective, in line with the advantages of nanosuspension, which can deliver drugs more effectively to small units in the body and increase drug delivery efficiency by improving solubility in water, can be targeted to reduce toxicity and increase drug distribution efficiency, improve the delivery of bioengineered drugs through various extreme body anatomy such as the brain barrier. Then, particle size testing can show that particle size influences the antibacterial activity of the preparation. Nanosuspensions with smaller particle sizes and large surface areas are able to increase the rate of dissolution and absorption of drugs (Patel & Agrawal, 2011). The results can be seen in Table 9.

Table 9. The activity of nanosuspension guava leaf ethanolic extract preparations against *Escherichia coli*

Formula	Inhibition Activity (mm)
I	8.40 ± 0.35
II	11.45 ± 0.64

CONCLUSION

According to the research findings, the ethanolic extract of guava leaf demonstrated an inhibition zone of 4.05±0.45mm at a concentration of 1%. Conversely, the nanosuspension of guava leaf extract, at a much lower concentration of 0.01%, exhibited a notably larger inhibition zone value of 11.45±0.64mm. The characterization results for the optimized formula of guava leaf nanosuspension at the 0.01% concentration revealed a particle size of 245.7 nm, a polydispersity index of 0.406, and a zeta potential of +26.9 mV.

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