

Formulation and Characterization of Ethanol Extract Nanoparticles of Gayam *Inocarpus fagifer* (Parkinson) Fosberg Seeds for Anti-Atherosclerotic Applications

I Made Sukadana^{1*}, Sri Rahayu Santi¹, Ni Nyoman Rupiasih²,
Luh Gede Sri Surya Heryani³, Lilik Parwati¹
and Nadya Pamesthi Annisa Ramadhani¹

¹Chemistry Department, Faculty Mathematics and Natural Sciences
Udayana University, Badung, Indonesia.

²Physic Department, Faculty Mathematics and Natural Sciences
Udayana University, Badung, Indonesia.

³Faculty of Veterinary Medicine Udayana University, Badung, Indonesia.

*Corresponding Author E-mail: sr_santi@unud.ac.id

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Preclinical studies indicate that gayam seeds (*Inocarpus fagiferus* Fosb) at a dose of 50 mg/kg body weight exhibit anti- atherosclerotic effects, as evidenced by changes in biomarkers including SOD activity, lipid profile, blood MDA, SOD-3, SOD-2, ICAM-1, IL-6, and TNF- α in aortic endothelial cells. While optimized solid and granule formulations have been developed, the formation of nanoparticles (1–100 nanometers in size) may further enhance the bioactivity of the extract. Nanoparticles provide improved solubility, bioavailability, stability, targeted delivery, increased extract efficacy, reduced dosages, and minimized side effects. This study aimed to prepare and characterize ethanol extract nanoparticles of gayam seeds for anti-atherosclerotic drug development. Nanoparticles were synthesized using the quality by design approach (Fractional Factorial Design, FFD), with the optimal formulation comprising 1.5 mL of 0.1% extract, 10 mL of 1.754% chitosan, and 5 mL of 0.1% NaTPP, stirred at 500 rpm for 15 minutes. The resulting nanoparticles demonstrated 100% encapsulation efficiency, 44.89% transmittance, a polydispersity index (PDI) of 0.3489, a zeta potential of 34.07 mV, and a particle size of 250.63 nm, meeting established nanoparticle criteria.

Keywords: Antioxidant; Atherosclerosis; Drug formulation; *Inocarpus fagiferus* Fosb; Nanoparticles.

Inocarpus fagifer (Parkinson) Fosberg seeds, also known as gayam or gatep in Bali,^{1,2} demonstrate potential for atherosclerosis prevention, as supported by preclinical studies. The ethanol extract exhibits DPPH radical inhibition with an IC₅₀ of 280 ppm, enhances SOD activity in Wistar rat plasma, and improves lipid profiles. These antioxidant effects are attributed to the presence of linoleic acid, ethyl linoleate, ethyl oleate, and homopterocarpine.³ The extract

upregulates SOD-3 expression in aortic endothelial cells and reduces MDA levels in rat plasma,⁴ supporting its role in atherosclerosis prevention. It may also stimulate endogenous antioxidants, particularly SOD-2, which serves as a biomarker for anti-atherosclerotic activity.^{5,6,7,8,9,10} SOD-2 expression influences ICAM-1 levels, positioning ICAM-1 as an early biomarker for atherosclerosis. Reduced plasma MDA is associated with decreased inflammation, as indicated by lower TNF- α and

IL-6 expression in aortic endothelial cells.¹¹ Preclinical biomarker and toxicity assessments have identified 50 mg/kg body weight as an effective anti-atherosclerotic dose. Standardizing this dose in nanoparticle form may further enhance its therapeutic efficacy.¹²

Nanoparticles are 1-100 nanometers in size (one nanometer is one-billionth of a meter) and offer advantages such as improving dissolution of difficult substances, enhancing bioavailability,^{13,14} modifying drug delivery, increasing stability, and boosting absorption.^{15,16} These features make nanoparticle-based extracts more efficient for developing anti-atherosclerosis drugs.¹⁷ Nanoparticles also penetrate spaces between cells that can be accessed by colloidal particles. The formation of nanoparticles is influenced by the characteristics of both the polymer and the drug. The aim of this research is to formulate and determine the characteristics of nanoparticles including organoleptic properties, particle size and distribution, morphology, zeta potential, and percent transmittance.

MATERIAL AND METHODS

Material

Inocarpus fagifer (Parkinson) Fosberg seeds sourced from Tabanan, Bali, served

as the plant material. The chemicals utilized included 96% ethanol (EMSURE), sodium carboxymethylcellulose, chitosan, 1% glacial acetic acid, sodium tripolyphosphate (NaTPP), and demineralized water.

The equipment comprised standard laboratory glassware, a blender, sieve, extractor, rotary vacuum evaporator, centrifuge, analytical balance, pipettes, flasks, freezer, air cooler, oven, mortar, particle size analyzer (PSA), transmission electron microscope (TEM), water bath, plethysmometer, timer, filter paper, aluminum foil, and gloves.

Methods

Preparation of ethanol extract of gayam (*Inocarpus fagifer* (Parkinson) Fosberg) seeds and preparation of nanoparticles

Six kilograms of dried gayam (*Inocarpus fagifer* (Parkinson) Fosberg) seed powder were macerated with ethanol in a dark extractor at room temperature for 24 hours. The extract was filtered, and the remaining solids were re-macerated with ethanol until complete extraction was achieved. The combined filtrates were concentrated using a rotary vacuum evaporator and subsequently weighed. Nanoparticle optimization was conducted using the quality by design approach (Fractional Factorial Design, FFD), as outlined in Table 1.

Table 1. Fractional Factorial Design method

Level	Factors				
	1	2	3	4	5
	Extract Concentration 1.5 mL (%)	Chitosan Concentration 10 mL (%)	Concentration of Na TPP 5 mL (%)	Mixing Speed (RPM)	Mixing Time (Minutes)
Lower level (-1)	0.1	1.75	0.1	500	15
Upper level (+1)	0.2	2	0.2	1000	60

Table 2. Results of total flavonoid content test of gayam extract

Sample	Replication	Flavonoid content (quercetin equivalent) in 1 g extract (ug)	Average (ug)	Flavonoid Content in Gayam Seeds (%)
extract test solution	1	307.778	307.778	0.03 %
	2	309.259		
	3	306.296		

Table 3. The results of the optimization of nanoparticle production with the response of % Entrapment Encapsulation (EE) and % Transmittance

Std	Run	Factor 1 A: Extract Concentration 1.5 mL %	Factor 2 B: Chitosan Concentration 10 mL %	Factor 3 C: Concentration of Na TPP 5 mL %	Factor 4 D: Stirring speed RPM	Factor 5 E: Stirring Time Menit	Response 1 EE %	Response 2 Transmittance %
6	1	0.1	1.75	0.2	1000	60	100	32.43
15	2	0.2	1.75	0.1	1000	60	100	12.06
1	3	0.1	1.75	0.1	500	60	100	41.31
11	4	0.2	2	0.1	500	60	100	18.08
7	5	0.2	2	0.2	500	15	100	22.01
14	6	0.2	1.75	0.1	500	15	100	22.73
16	7	0.2	1.75	0.2	1000	15	100	14.81
5	8	0.1	1.75	0.1	500	15	100	44.81
8	9	0.2	1.75	0.2	500	60	100	19.21
12	10	0.1	1.75	0.2	500	15	100	36.42
4	11	0.2	2	0.2	1000	60	100	11.1
13	12	0.2	2	0.1	1000	15	100	16.26
3	13	0.1	2	0.2	1000	15	100	32.25
10	14	0.1	2	0.2	500	60	100	36.45
9	15	0.1	2	0.1	1000	60	100	26.12
2	16	0.1	2	0.1	500	15	100	42.98

Table 4. Results of optimal formula verification for F1, F2 and F3

Formula	Test Type	Target	Formula Repetition	Observation results	Average observation results	Deviation (%)	Condition	Results
1	% Entrapment Encapsulation	100	F1	100	100	0	Maximize	Qualify
			F2	100				
			F3	100				
2	% Transmittance	44.579	F1	45.35	44.89	0.698 %	Maximize	Qualify
			F2	44.98				
			F3	44.35				
3	PDI	-	F1	0.3450	0.3489	-	< 0.500	Qualify
			F2	0.3511				
			F3	0.3506				
4	Zeta Potential (mV)	-	F1	34.29	34.07	-	> ± 30 mV	Qualify
			F2	33.84				
			F3	34.09				
5	Particle size (nm)	-	F1	296.9	250.63	-	<1000 nm	Qualify
			F2	248.4				
			F3	206.6				

According to Table 1, the extract was dissolved in 96% ethanol, chitosan in 1% acetic acid, and NaTPP in water. The extract and chitosan solutions were combined in a beaker and stirred at 500 rpm for 5 minutes. NaTPP was then added while maintaining the specified stirring speed and duration until the mixture became cloudy. The percentage of entrapment encapsulation (EE) and transmittance for each formulation were measured. The resulting nanoparticle precipitate

was stored in a freezer at 3°C for two days, dried using air cooling, and ground with a mortar prior to characterization.

Characterization of nanoparticles

Following identification of the optimal formula using FFD, formulations F1, F2, and F3 were prepared in triplicate to assess their characteristics. Nanoparticles were evaluated for particle size and distribution using a particle size analyzer (PSA), morphology via transmission

Table 5. The results of the analysis of variance (ANOVA)

Respon	F value	P value	R2
% Entrapment Encapsulation (EE)	0	0	0
% Transmittance	28.44	< 0.0001 (significant)	0.9614

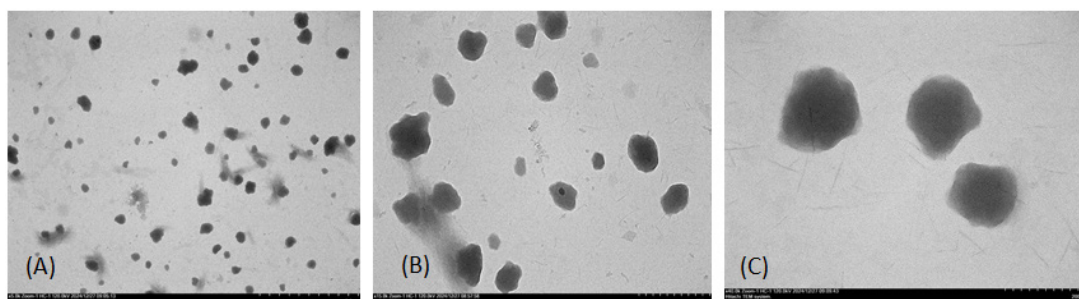


Fig. 1. The results of Hitachi TEM System analysis for optimal formula nanoparticles (one of the formulas is F1): (A) zoom 5x, HC-1 120V; (B) zoom 15x, HC-1 120V; (C) zoom 40x, HC-1 120V

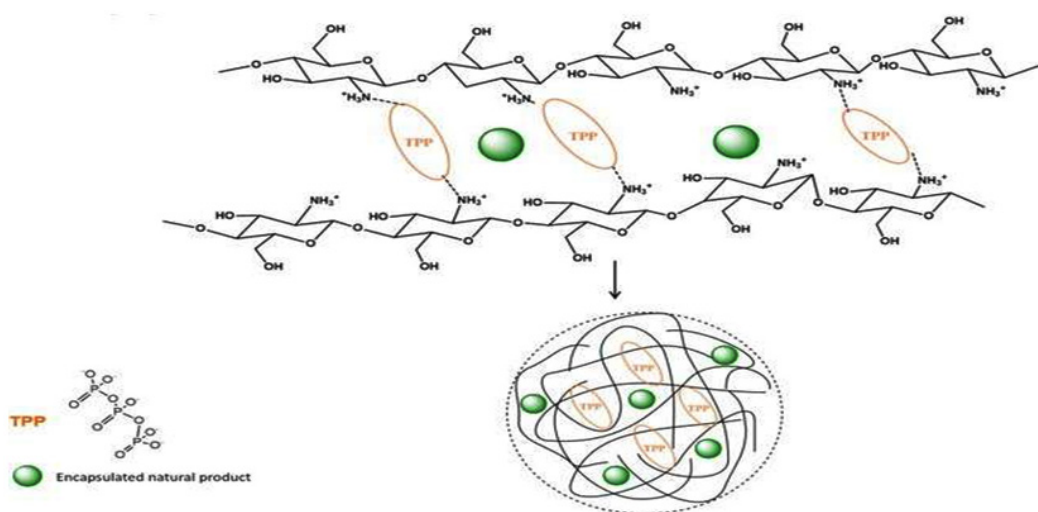


Fig. 2. Scheme of the formation of nano chitosan-tripolyphosphate cross-links that encapsulate active substances from extract³³

Table 6. Criteria of Independent and Dependent Variables

Independent and Dependent Variables	Criteria
Extract Concentration 1.5 mL (A)	<i>In range</i>
Chitosan Concentration 10 mL (B)	<i>In range</i>
Concentration of Na TPP 5 mL (C)	<i>In range</i>
Stirring speed (D)	<i>In range</i>
Stirring Time (E)	<i>In range</i>
% EE (Y1)	<i>Maximize</i>
% Transmittance (Y2)	<i>Maximize</i>

electron microscopy (TEM), as well as zeta potential and percent transmittance.

RESULTS

All data presented in the Results section were generated from research conducted in the Natural Product Chemistry Laboratory, Chemistry and Physical Sciences Departments, Udayana University.

Preparation of ethanol extract of gayam (*Inocarpus fagifer* (Parkinson) Fosberg) seeds and preparation optimum of nanoparticles

Maceration of 6 kg of dry *Inocarpus fagifer* (Parkinson) Fosberg seed powder produced approximately 30 g of concentrated extract. The total flavonoid content of the extract is presented in Table 2.

Following determination of flavonoid content, optimization of gayam (*Inocarpus fagifer* (Parkinson) Fosberg) seed extract nanoparticles was performed using the quality by design approach (Fractional Factorial Design, FFD), with results summarized in Table 3.

Characterization of Optimal Formula Nanoparticles

The characterization results for the optimal nanoparticle formulations, specifically F1, F2, and F3, are presented in Table 4.

TEM analysis revealed that the synthesized nanoparticles exhibited semi-spherical or oval morphologies, as illustrated in Figure 1.

DISCUSSION

Table 2 indicates that gayam (*Inocarpus fagifer* (Parkinson) Fosberg) seed extract contains

Table 7. Optimal formula

Factor 1 A: Extract Concentration 1.5 mL %	Factor 2 B: Chitosan Concentration 10 mL %	Factor 3 C: Concentration of Na TPP 5 mL %	Factor 4 D: Stirring speed RPM	Factor 5 E: Stirring Time Minute	Response 1 EE %	Response 2 Transmittance %	Desirability
0.100	1.754	0.100	500	15	100	44.579	0.993

0.03% flavonoids (quercetin equivalent), confirming the presence of bioactive flavonoid compounds in relatively low concentrations. Despite this, these flavonoids contribute to the pharmacological potential of gayam seeds, including antioxidant, anti-inflammatory, and other biological activities characteristic of flavonoids.

Given the presence of flavonoids (0.03%) in gayam seeds, nanoparticle development was pursued to enhance the stability and bioavailability of these active compounds. The Fractional Factorial

Design (FFD) within the Quality by Design framework was employed to efficiently evaluate and optimize key factors affecting nanoparticle characteristics: extract concentration, chitosan concentration, NaTPP (crosslinker) concentration, stirring speed, and stirring time. The primary responses measured were entrapment efficiency (EE), representing the percentage of flavonoids encapsulated within the nanoparticle matrix, and transmittance, reflecting the clarity and homogeneity of the nanoparticle solution. Higher

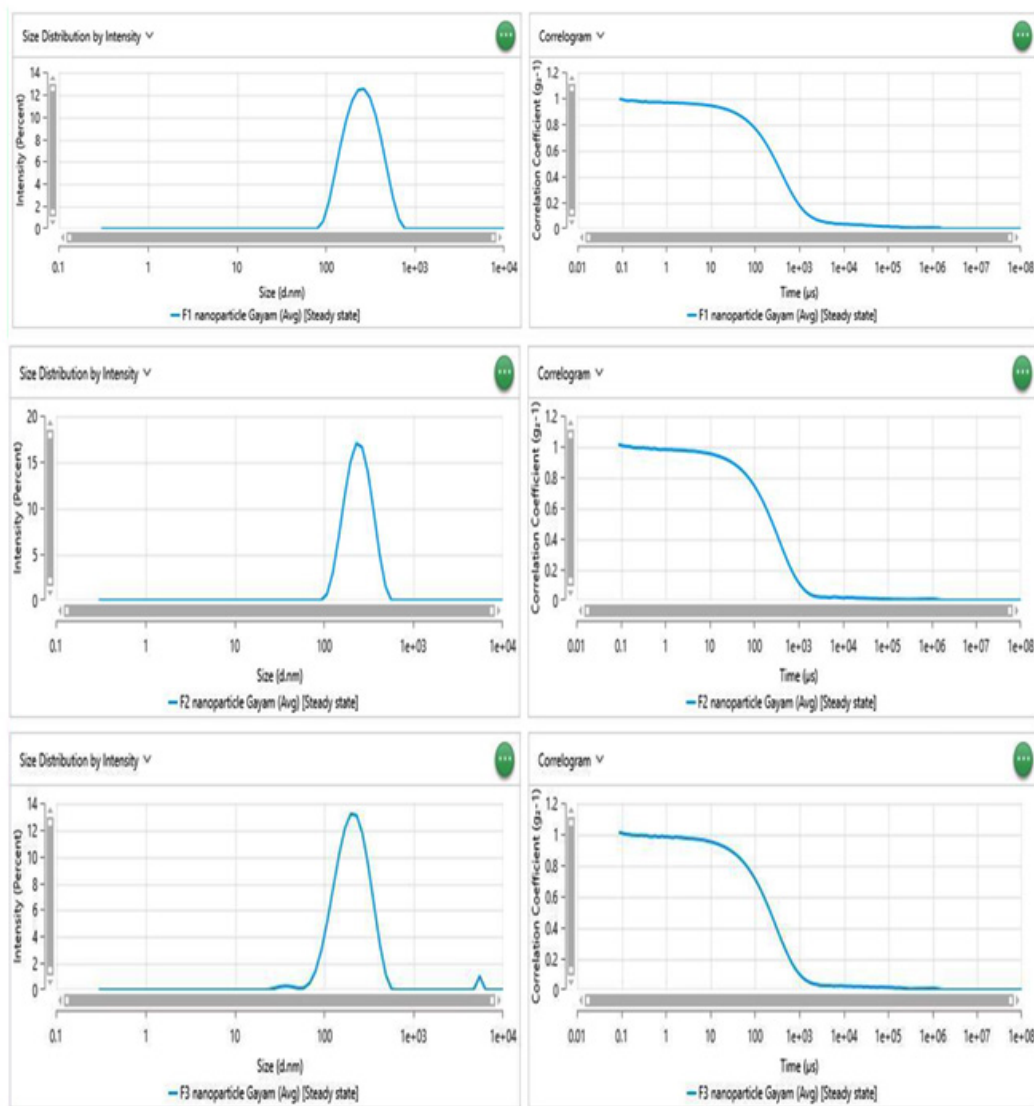


Fig. 3. The results of particle size measurements for optimal formula nanoparticles (F1, F2, and F3)

transmittance values indicate well- formed, uniformly dispersed nanoparticles. Entrapment efficiency was most strongly influenced by chitosan and NaTPP concentrations via the ionotropic gelation mechanism. Excessive extract concentration reduced encapsulation efficiency, while appropriate stirring speed and duration were critical for achieving homogeneous particles with high transmittance. The optimal combination of these parameters produced stable, clear, and effective nanoparticles.

Preparation of gayam extract nanoparticles according to the FFD design (Table 3) resulted in

100% entrapment efficiency across all formulations, with transmittance values ranging from 11.1% to 44.81%. The optimal formula in Table 3 is highlighted prior to conducting analysis of variance (ANOVA) and establishing the criteria for independent and dependent variables. The optimal formula, as determined by ANOVA and subsequent software analysis, was finalized based on the criteria presented in Tables 5 and 6.

The results for the optimal formula, determined through the FFD design (Table 3), ANOVA analysis (Table 5), and established criteria (Table 6), are presented in Table 7.

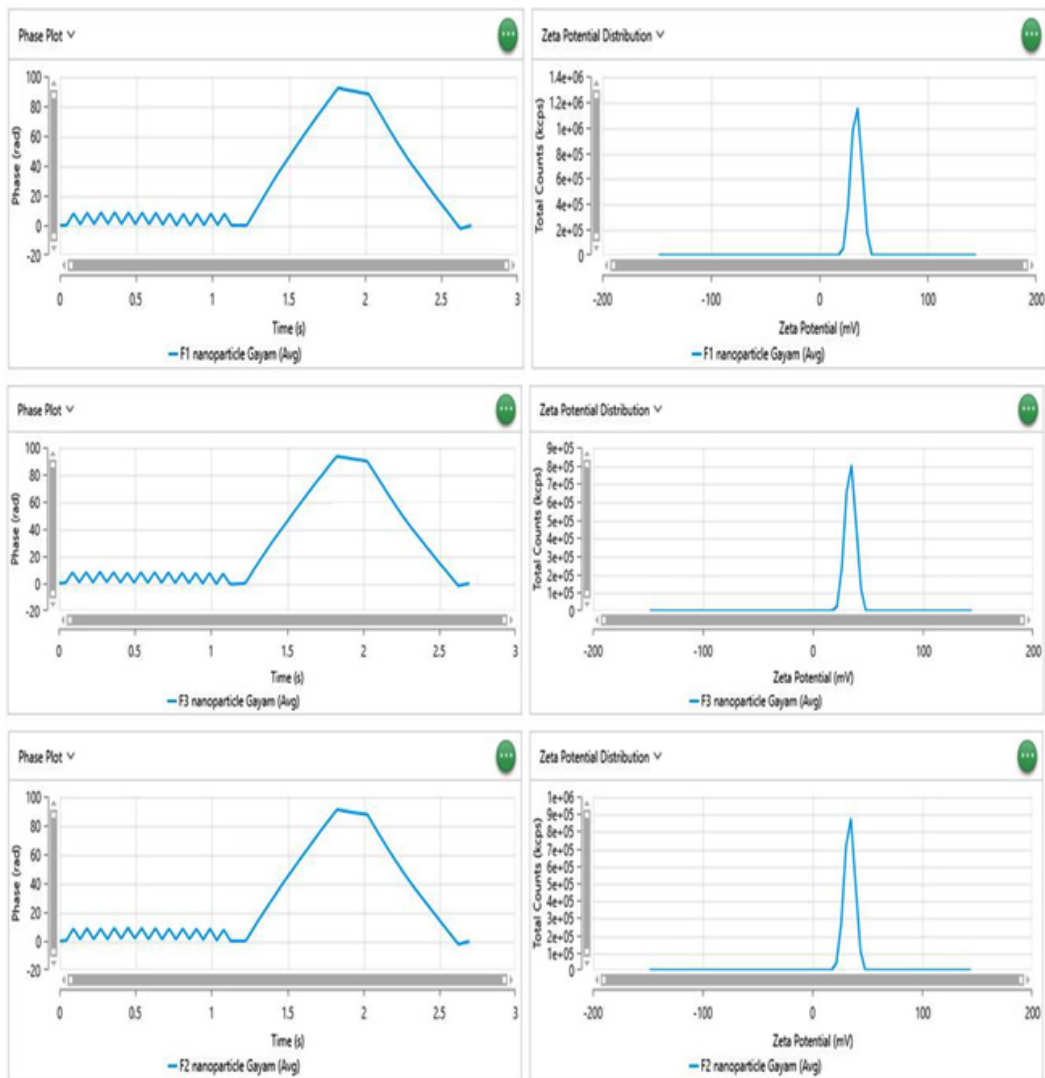


Fig. 4. The results of zeta potential measurements for optimal formula nanoparticles (F1, F2, and F3)

Three formulations (F1, F2, and F3) were prepared in replicate, as determined by the optimal formula in Table 7, to characterize the nanoparticle formulation. The results are provided in Table 4. Gayam seed extract nanoparticles were synthesized using the ionic gelation method, which involves the interaction of positively charged chitosan with negatively charged sodium tripolyphosphate (NaTPP). This approach is straightforward, avoids the use of high temperatures, and allows for precise control.^{18,19} Chitosan, a non-toxic cationic polymer, serves as an effective drug carrier due to its high biodegradability and biocompatibility.^{20,21} Furthermore, chitosan can enhance drug efficacy without inducing side effects.²² During ionic gelation, chitosan dissolves in dilute acid, generating cationic amine groups that cross-link with NaTPP anions to form stable chitosan nanoparticles.^{23,24} NaTPP dissociates in water into sodium and tripolyphosphate ions, which interact with chitosan NH₂ groups and function as a non-toxic crosslinker.^{25,26}

The preparation of nanochitosan-tripolyphosphate-gayam seed extract involves the interaction between the positively charged amine groups in chitosan and the negatively charged tripolyphosphate. The resulting bond facilitates the absorption of the extract.²⁷ Bioactive components from gayam seed extract can associate with the surface of nanochitosan-tripolyphosphate.²⁸ This interaction enables the storage of bioactive substances within the nanoparticle system. The polymer and matrix system encapsulates these substances in the core, embedding the molecules within the polymer matrix.²⁹ Nanoparticles are fine solids with nanometer-scale dimensions formed through these interactions.³⁰ The dropwise addition of NaTPP under constant magnetic stirring prevents solidification, reduces aggregation, and stabilizes nanoparticle formation.^{31,32} Stirring speed influences both the stability of the emulsion and the size of the nanoparticles. Higher stirring speeds decrease interfacial tension and enhance emulsion stability. The formation scheme for nanochitosan-tripolyphosphate-gayam seed extract, which encapsulates active substances from gayam seed extract via the ionic gelation method,³³ is illustrated in Figure 2.

Particle size measurements for 1.754% chitosan combined with 0.1% extract yielded an

average size of 250.63 nm. The mean polydispersity index (PI) was 0.3489, and the zeta potential (ZP) measured 34.07 mV, as detailed in Table 3. Figure 3 and Figure 4 present the particle size and zeta potential data for the optimal nanoparticle formulation.

According to the data in Table 4, nanoparticle formulations are considered stable when the zeta potential exceeds ± 30 mV.³⁴ Nanoparticles are defined as materials with sizes ranging from 1 to 1000 nm, and the results confirm that the particles produced fall within this range.³⁵ The polydispersity index (PI) indicates the distribution width of particle sizes. A low PI value (less than 0.5) corresponds to a narrow size distribution and stable suspension, whereas a high PI value (greater than 0.7) indicates a broad distribution, increased non-uniformity, faster agglomeration, and reduced stability. High non-uniformity accelerates flocculation and coalescence, while uniform particle size supports suspension stability by minimizing size deviation.^{36,37} Therefore, a low PI value demonstrates the effectiveness of the stabilizer in preventing particle agglomeration,^{38,39} while a high PI value reflects a broad size distribution. In this study, the polydispersity index was 0.3489 and the particle size was 250.63 nm, indicating that the nanoparticles are monodisperse and meet the criteria for nanoparticle classification.

Zeta potential reflects the surface charge of particles and determines their tendency to aggregate or remain dispersed.⁴⁰ A zeta potential above +30 mV or below -30 mV indicates a stable suspension, as high values promote electrostatic repulsion and prevent particle aggregation.^{41,42} The ethanol extract nanoparticles of gayam seeds exhibited an average zeta potential of +34.07 mV, suggesting good stability, although some aggregation may still occur over time. Zeta potential also represents the overall charge in the suspension, which is a key predictor of nanoparticle stability.⁴³ The observed value of +34.07 mV indicates favorable storage stability. Further enhancement of stability can be achieved by incorporating surfactants, which create a protective barrier around the particles and inhibit aggregation.^{44,45}

Transmission electron microscopy (TEM) analysis (Figure 1) revealed that the ethanol extract nanoparticles of gayam seeds are predominantly

spherical with smooth surfaces. The observed particle size ranged from 100 to 200 nm, consistent with the average size of 206 nm measured by dynamic light scattering (DLS), which accounts for the hydration layer. The moderate variation in size distribution aligns with the polydispersity index (PDI) value of 0.3489. Although some aggregation was observed, the majority of nanoparticles remained discrete and uniform in shape, indicating a stable system as further supported by the zeta potential of +34.07 mV.

CONCLUSION

The optimal nanoparticles of anti-atherosclerosis gayam seed ethanol extract, prepared by the Fractional Factorial Design method (0.1% extract, 1.754% chitosan, 0.1% NaTPP, 500 rpm, 15 min), demonstrated characteristics as follows 100% encapsulation efficiency, 44.89% transmittance, a PDI of 0.3489, zeta potential of +34.07 mV, and particle size of 250.63 nm. TEM analysis confirmed the spherical morphology and uniform distribution, indicating their suitability as a stable nanoparticle formulation for potential anti-atherosclerosis therapy.

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Conflict of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials

Permission to reproduce material from other sources

Not Applicable.

Authors contributions

I Made Sukadana: conceived the research idea and grant winner; Sri Rahayu Santi: wrote the draft research methodology and is the corresponding author; Ni Nyoman Rupiasih: formulated the nanoparticles; Luh Gede Sri Surya Heryani: prepared the materials for characterization at BRIN; Lilik Parwati: assisted with the research in the laboratory; Nadya Pamesthi Annisa Ramadhani: assisted in editing the manuscript.

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