Antibacterial activity and characterization of *Annona muricata* Linn leaf extract-nanoparticles against *Escherichia coli* FNCC-0091 and *Salmonella typhimurium* FNCC-0050

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**Abstract.** The objective of this study was to evaluate the characterization and inhibitory effects of *Annona muricata* Linn leaf extract loaded by chitosan nanoparticles on the growth of *Escherichia coli* FNCC-0091 and *Salmonella typhimurium* FNCC-0050. The chitosan-sodium tripolyphosphate-nano particles (CS-TPP-NPs) were prepared by ionic cross-linking of CS-TPP and were characterized physico-chemically (particle size, zeta potential) and morphologically. Antibacterial activity of the CS-TPP-NPs-AmE was performed by well diffusion method. The optimum formula for CS-TPP-NPs was analyzed using centrifugation at 3,500 rpm for 30 min. A wide range, 1:1:0.02 to 1:1:0.01 of CS:AmE:TPP ratios were evaluated with constant CS. Data obtained for bacterial activities were statistically analysed using one-way analysis of variance (ANOVA). The means between groups were separated with Duncan post-hoc test. The result showed that the optimum formula for CS-TPP-NPs of AmE was 1:1:0.01. The optimum formula of CS-TPP-NPs-AmE was characterized by particle size, zeta potential, and morphology. Results showed that the particle size of CS-TPPNPs-AmE was 234.00 ± 21.5 nm with spherical shape and positive charges on their surface with the zeta potentials 34.77 ± 4.97 mV. CS-TPP-NPs of AmE in the ratio 1:1:0.01 has efficient antibacterial activities against *E. coli* FNCC-0091 and *S. typhimurium* FNCC-0050.

1. **Introduction**

Antibiotics had been widely used as an effective tool to improve animal performance, through modifying microflora in the gut, decreasing pathogenic bacterial population that can damage the intestinal cell wall, and suppressing bacterial catabolism. All of these are important to improve health, micronutrient availability, and growth performance [1]. However, the continuous and uncontrolled use of antibiotics can lead to accumulative residue which is harmful to animal and their products for human consumption [2,3]. The European Union countries have long prohibited the use of antibiotics in poultry feed [4]. In Indonesia, the Ministry of Agriculture has banned the use of antibiotics in feed since January 2018. This is in-line with the policy of many developing and developed countries to restrict the use of antibiotics as growth promoters in poultry [5].

In order to spike-off the adverse effects of the banning of in-feed antibiotics, poultry nutritionist are searching for alternatives. One of the potential alternatives for antibiotics is the phytobiotics. Bioactive
compounds contained in the phytobiotics are reported to be beneficial to inhibit the growth and population of pathogenic bacteria in the gut of broiler chickens [6]. *Annona muricata* Linn leaf extract is one of phytobiotics which has some biological properties, including antibacterial function [7]. Phytochemical screening showed that *A. muricata* Linn leaf extracts were rich in secondary class metabolite compounds, such as: flavonoids, alkaloids, terpenoids, saponins, coumarins, lactones, anthraquinones, tannins, cardiac glycosides, phenols, and phytosterols [8,9]. Often the different components in herbs have synergistic activities or buffering toxic effects. Previous studies have shown that ethanolic extract of *A. muricata* Linn leaf had antibacterial activity and inhibited the growth of enteric bacteria, namely *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, and *Salmonella typhimurium* [10–12].

The advantageous strategy for phytobiotics or herbal drugs considered the numerous features that nano-structured systems have to offer with applying nanotechnology to herbs plant extracts has been revealed, including bioavailability, solubility, sustained delivery, pharmacological activity enhancement, protection from toxicity, as well as protection from physical and chemical degradation [13,14]. Technology and polymer are needed to maximize the transport and absorption of bioactive compound from herbs extract, such as in chitosan nanoparticles (CS-NPs) which is cross-linked to sodium tripolyphosphate (TPP) [15]. The mechanism behind the ionic gelation method involves an ionic cross-linking between cations on the backbone of CS and anion such as TPP [16]. The present study evaluated the antibacterial activity and characterization of chitosan-sodium tripolyphosphate nanoparticles (CS-TPP-NPs) charged with *A. muricata* Linn leaf standardized extract (AmE).

2. Material and methods

2.1. Material

Chitosan-sodium (MW, 70 kDa; de acetylation degree, 85%) (Merck, Darmstadt, Germany) and TPP (0.84% w/v) (Bratchem, Indonesia) were used for the preparation of chitosan-TPP nanoparticles. Ethanolic extracts of *Annona muricata* leaves, glacial acetic acid (0.25% v/v), distilled water were used for the formulation of CS-TPP-NPs-AmE. *Escherichia coli* FNCC-0091 and *Salmonella typhimurium* FNCC-0050, provided by the Food and Nutrition Culture Collection (FNCC), Food and Nutrition Development and Research Center (FANDARC), Universitas Gadjah Mada, were used to evaluate the antibacterial activity in vitro. Brain heart infusion (BHI) broth, BHI agar and Muller–Hinton (MH) agar (Difco Laboratories, Detroit, Michigan, USA) were used as growth media of bacteria.

2.2. Methods

2.2.1. Preparation of plant material. Fresh leaf of *A. muricata* were collected from farms in Yogyakarta, Indonesia. The leaves were allowed to dry in a 55°C oven for 3 days. The dried leaves were milled in a Thomas-Willey mill (Arthur H. Thomas Co., Philadelphia, USA) to pass through a 1 mm sieve, stored in a tight bottle and used for analysis.

2.2.2. Ethanol Extraction of plant material. Two hundred grams of *A. muricata* leaf meal were weighed using analytical scales (Ohaus GA200D, Pine Brook, New Jersey, USA) and placed in a beaker glass. One litre of ethanol was poured into the samples using maceration techniques. The set up were left to stand for 72 hours to enable the constituents dissolve thoroughly in ethanol. The filtrates were collected into a beaker and subsequently transferred into water bath in order to concentrate them. The AmE was obtained by dissolving 2% in distilled water.

2.2.3. Preparation of CS-TPP-NPs-AmE. CS-TPP-NPs were prepared according to the ionic gelation method, cross-linking of CS with TPP according to Calvo [15] with slight modifications. The CS 0.2 g was dissolved in 100 ml distilled water with acetic acid 1% (b/v). AmE solution was added into CS
solution with a ratio of 1:1. In addition, 0.04 g of TPP was dissolved in 40 ml distilled water. Solution of TPP was then added into CS-AmE solution with constant magnetic stirring (700 rpm) for 30 min at room temperature. CS-TPP-NPs-AmE was centrifuged at 3500 rpm for 30 min.

2.2.4. Formulation and optimization of CS-TPP-NPs-AmE. In order to use different formulation CS:AmE:TPP ratios (1:1:0.02 to 1:1:0.01), the initial concentration of CS and AmE was kept constant while the volume of TPP added was varied. The CS-TPP-NPs were harvested with centrifugation process (Gemmy PLC-03, Gemmy Industrial Corp., Taipei, Taiwan) in 3500 rpm speed for 30 min. After centrifugation process, sediment and supernatant were separated. The optimum formula was selected for further analysis, i.e. without sediment in solution through visual observation.

2.2.5. Characterization of CS-TPP-NPs-AmE. Particle size of CS-TPP-NPs-AmE were determined using Zetasizer Nano ZS (Horiba Scientific SZ-100, Horiba, Kyoto, Japan). The samples were diluted with a ratio of 1:0.01 (v/v) using distilled water. All measurements were performed in triplicates at 25°C with a detection angle of 90°, following the method proposed by Balakumar [17] and Hussain-Sahudin [18].

Zeta potential of the optimum formulations was determined by dynamic light scattering technique using particle size analyzer (Horiba Scientific SZ-100, Horiba, Kyoto, Japan). The samples were diluted with a ratio of 1:100 (v/v) with distilled water and repeated in triplicate, following the method proposed by Balakumar [17] and Hussain-Sahudin [18].

The CS-TPP-NPs-AmE morphology was examined using high-performance digital imaging transmission electron microscopy (Joel JEM-1400 CX, Hitachi High-Technologies Corp., Tokyo, Japan). For TEM analysis, the CS-TPP-NPs-AmE were diluted with water, a drop of CS-TPP-NPs-AmE dispersion was placed onto the copper micro grid which was natively stained by phosphotungstic acid, and was allowed to evaporate and dry at room temperature (25±2°C). The dried micro grids were then viewed at various resolutions under TEM [18].

2.2.6. Evaluation of antibacterial activity. Formulation of CS-TPP-NPs-AmE were tested for their antibacterial activity by well-diffusion method against two pathogenic organisms, *E. coli* FNCC-0091 and *S. typhimurium* FNCC-0050. The pure cultures of organism were sub cultured on MH broth at 35°C on rotary shaker at 200 rpm. Each strain was swabbed uniformly on the individual plates using sterile cotton swab. Wells of size 6 mm have been made on MH agar plates using gel puncture. 50 μl of the sample of nanoparticles solution, extract and tetracycline standard controls were poured into wells on all plates using micropipette. After incubation at 35°C for 24 h, the different levels of zone of inhibition were measured.

2.3. Statistical analysis
The data were statistically analyzed according to one-way analysis of variance (ANOVA) using Statistical Package for the Social Science (SPSS) software version 2.2 and the means presented as means ± SD. The different means between groups were separated with Duncan post-hoc test.

3. Results and discussion

3.1. Formulation optimization of CS-TPP-NPs-AmE
The CS:TPP ratio is one of the prime parameter to control the particle size and size distribution. A wide range, 1:1:0.02 to 1:1:0.01, of CS-AmE-TPP ratios were evaluated with constant CS concentration. The result showed that CS-AmE-TPP ratio 1:1:0.01 (Table 1) was used as the basis for finding the optimal formula. The wide range of TPP concentrations that form particles is due to the fact that TPP can create as many as five ionic cross-links with amino groups of CS resulting in formation of individual particle or aggregate [19].
Table 1. Formulation of CS-TPP-NPs-AmE

| CS (ml) | AmE (ml) | TPP (ml) | CS concentration (w/v) | TPP concentration (w/v) | Visual observation |
|---------|----------|----------|------------------------|-------------------------|-------------------|
| 1       | 1        | 0.020    | 0.02                   | 0.001                   | Cloudy            |
| 1       | 1        | 0.017    | 0.02                   | 0.001                   | Cloudy            |
| 1       | 1        | 0.014    | 0.02                   | 0.001                   | Cloudy            |
| 1       | 1        | 0.012    | 0.02                   | 0.001                   | Cloudy            |
| 1       | 1        | 0.011    | 0.02                   | 0.001                   | Cloudy            |
| 1       | 1        | 0.010    | 0.02                   | 0.001                   | Clearly           |

3.2. Characterization of CS-TPP-NPs-AmE

3.2.1. Particle size. The mean particle size and polydispersity-index (PI) were calculated from volume, intensity, and bimodal distribution assuming spherical particles. PI was a measure of particle homogeneity [20]. The particle size distribution of the selected formulation was found to be 234.00 ± 22.5 nm, which was highly desirable. The polydispersity-index (PI) was low (0.42 ± 0.20), indicating that the system had narrow size distribution. The particle size was an important factor in CS-TPP-NPs formulation, as this determines the rate and extent of drug release as well as absorption and improve bioavailability [21].

3.2.2. Zeta potential analysis. The zeta potential values were positive for selected formulation, indicating a positive charged surface. Zeta potential values of the selected formulation were found 34.77 ± 4.97 mV. Zeta potential values which were less −30 mV or greater than 30 mV ensuring particle stability [22,23]. Therefore, the results of zeta potential measurement indicated a stable CS-TPP-NPs-AmE formulation.

![Transmission electron micrograph of CS-TPP-NPs-AmE](image)

3.2.3. Morphological examination. The CS-TPP-NPs-AmE appeared a bright spot on a dark background with a spherical shape which homogenous particle size (Figure 1). Different and non-aggregated nanoparticles droplets indicated physically stable nanoparticles [24].

3.3. Antibacterial activity of CS-TPP-NPs-AmE

The antibacterial activity of the CS-TPP-NPs-AmE) was further investigated by bacterial growth time course compared with extract and tetracycline standard control (Table 2).
The antibacterial activity of CS-TPP-NPs-AmE was investigated against pathogenic organisms, namely *E. coli* and *S. typhimurium* using well diffusion method. The diameter of inhibition zones (mm) around each well treated with AmE, CS-TPP-NPs-AmE, and tetracycline standard control (50 μg/ml) were represented in Table 2. The CS-TPP-NPs-AmE was able to significantly inhibit all the bacteria growth i.e. *E. coli* FNCC-0091 and *S. typhimurium* FNCC 0050. The results showed that CS-TPP-NPs-AmE were found to have highest (*p<0.01*) antibacterial activities against *E. coli* FNCC-0091 (16.33 ± 0.57 mm) and *S. typhimurium* FNCC 0050 (17.67 ± 0.57 mm), when compared to AmE and standard antibiotic Tetracycline. The chitosan nanoparticles showed efficient antimicrobial property due to their extremely large surface area, which provides more sensitive contact with microbes. The nanoparticles get attached to the cell membrane and also penetrated inside the bacteria. The chitosan nanoparticles release bioactive compounds of *A. muricata* leaf extract in the bacterial cells, which enhance their bactericidal activity [25–27].

Bioactive compounds such as phenol and flavonoid in *A. muricata* leaf extract have the property as an antibacterial agent which can inhibit the growth of pathogenic bacteria [11]. Flavonoid compounds act on pathogens via three mechanisms: inhibiting nucleic acid synthesis, inhibiting cell membrane function and inhibiting energy metabolism [28]. Previous study identified and isolated new flavonoid triglycosides present in soursop leaves, namely gallic acid, chlorogenic acid, epicatekine, quercetin, catechin, and argentinine [29]. Gallic acid compounds can damage bacterial membranes, including their charge and intra and extracellular permeability. Gallic acid also affects the physicochemical properties of bacterial cell membranes by changing hydrophilic properties to hydrophobicity and disrupting membrane function due to damage to essential intracellular constituents [30].

4. Conclusion

The CS-TPP-NPs of *A. muricata* Linn leaf extract can be formulated in the ratio of CS:AmE:TPP at 1:1:0.01. The formulation had a particle size of 234.00 ± 22.5 nm with PI 0.42 ± 0.20, stable at zeta potential 34.77 ± 4.97 mV, and passed the thermodynamic stability with centrifugation. The formulation improved the CS-TPP solubility and AmE stability. Optimizing formula of *A. muricata* Linn in the form of leaf extract-loaded chitosan-sodium tripolyphosphate nanoparticles may allow its use as an antibacterial and potentially be used as an alternative to the antibiotic in poultry production.

**Table 2. Antibacterial activity of CS-TPP-NPs-AmE**

| Isolate                      | Inhibition Zone Diameter (mm) ± SD | SEM | p-value |
|------------------------------|-----------------------------------|-----|---------|
|                              | AmE | CS-TPP-NPs-AmE | Tetracycline |       |
| *E. coli* FNCC-0091          | 8.50 ± 0.57<sup>a</sup> | 16.33 ± 0.57<sup>b</sup> | 11.67 ± 0.76<sup>b</sup> | 1.15 | <0.01 |
| *S. typhimurium* FNCC 0050   | 7.83 ± 0.28<sup>c</sup> | 17.67 ± 0.57<sup>a</sup> | 15.50 ± 0.50<sup>b</sup> | 1.57 | <0.01 |

<sup>a,b,c</sup>Superscript different within the same row shows a significant difference (*p<0.05*), SD=Standard deviation, SEM=Standard error of the mean.

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