In Vitro antibacterial activity of Mindi (Melia azedarach Linn.) leaf extract with nanoencapsulation technology

N A S Masjid1, R Martien2, Zuprizal1 and N D Dono1

1Department of Animal Nutrition and Feed Science, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia.
2Department of Pharmaceutics, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Corresponding author: nanungdd@ugm.ac.id

Abstract. Purpose of this study was to examine the effect of nanoencapsulation technology in protecting the bioactive components of mindi (Melia azedarach Linn.) leaf extract. The first step was formulation of the ratio of mindi leaf extract:chitosan:sodium tripolyphosphate (STPP) for nanoencapsulation by ionic cross-linking. The results showed that the optimum formulation ratio of mindi leaf extract:chitosan:STPP was 1:7:1/175. Nano-capsulation characteristics of the fitobiotic was measured (particle size (PSA), zeta potential value, morphology of nanoencapsulation (TEM), and bacterial growth inhibition). The second step was measuring the bacterial growth inhibition using the well diffusion technique with six treatments and three replications. The treatments were: aquadest (T1), aquadest with 100 ppm antibiotic Tetracycline addition (T2), aquadest with 0,2% chitosan (T3), aquadest with 0,04% STPP (T4), aquadest with 2% mindi leaf extract (T5); and aquadest with nanoencapsulated mindi leaf extract (T6). The results showed that particle size of nanoencapsulation mindi leaf extract was 535.2±12.83 nm with Polydispersity Index (PI) 0.436±0,21 and zeta potential value 59.73±0.35 mV. The formulation of mindi leaf extract was found to possess inhibitory activity against Escherichia coli, Lactobacillus acidophilus, and Salmonella typhimurium. The research concluded that formulation of mindi leaf extract was clear, stable and had antibacterial activity.

1. Introduction

Feed additives including antibiotics or drugs are commonly used in poultry feed to maximize the efficiency of production, product quality, and control diseases [1]. The continuous and uncontrolled use of antibiotics can lead to accumulative residue which is harmful for animals and their products for human health [2,3]. In the European Union, the use of antibiotic growth promoters as feed additives is now prohibited. In Indonesia, authorization of the use of antibiotics as growth promoter in animal feed has been withdrawn since January 2018. This is in accordance with the policies of several developing and developed countries to limit the use of antibiotics as growth promoters to maximize meat quality products [4].

The use of biological products, including: probiotics, prebiotics, synbiotics, enzymes, organic acids, and plant extracts (phytobiotics) as alternatives to in-feed antibiotic for poultry animals has recently increased [1,5]. Many of these additives have been reported to maximize the gut function in poultry. One of the potential alternative for antibiotics is phytobiotics, a plant–derived natural bioactive compounds that can be incorporated in the diets to enhance growth performance of animals.
Besides stimulating appetite, bioactive compound in phytobiotics were also reported to stimulate endogenous secretions (such as: enzymes) and has some beneficial properties, such as: anti-pathogens (antibacterial, anti virus, etc.), anthelmintic, and antioxidant [5,7-9].

Mindi (Melia azedarach Linn) leaf extract was reported to contain various bioactive compounds, largely as phenols and flavonoids, but also tannins, alkaloids, phytosterols, and saponins. All of these have various functions, including: antioxidants, antimicrobials, coccidiostatic, anti-inflammatory, analgesic, and anti cancer [10,11]. Mindi leaves extract was reported to limit the growth or to kill Staphylococcus aureus, Escherichia coli, and Bacillus thuringiensis [12]. On the other hand, mindi leaves extract were moderately effective against Eimeria tenella infection in broilers. However, the bioactive compound in mindi leaves extract have negative characters, such as: lipophilic, low solubility in water, rapidly degraded, low bioavailability, quickly damaged by environmental influence of digestive tract, and limited absorption in digestive tract [13].

One of strategy that can be used to solve this problem is nanotechnology. Nanotechnology maximizes the use of bioactive compound from herb plant extracts in their transportation for effective absorption, as in chitosan nanoparticles which is cross-linked with STPP [14]. Among the various methods to create chitosan nanoparticles, ionic gelation method is the most popular method as the process is simple and relatively easy to control. This study reported the antimicrobial effect and characterization of mindi leaves extract with nanoencapsulation technology.

2. Material and methods

2.1. Material

Materials which used for preparation of nanoparticles are chitosan (Merck, Darmstadt, Germany) and STPP, distilled water, ethanol 70% for extraction of mindi leaves, mindi leaves, Escherichia coli and Salmonella typhimurium provided by Food and Nutrition Development and Research Center (FANDARC), Universitas Gadjah Mada, was used for evaluate the antimicrobial activity by in vitro. Muller-Hinton (MH) agar and MH broth (Difco, USA) were used as growth media of microbial or bacteria.

2.2. Methods

2.2.1. Preparation of plant material (mindi leaves). The fresh leaves sample of plant mindi was collected from Sleman, Yogyakarta, Indonesia. The sample were dried with oven 55°C for 2 days. They were milled with Thomas-Willey mill (Arthur H. Thomas Co., Philadelphia, USA) to pass through a 1 mm sieve and stored in room temperature.

2.2.2. Extraction of plant material (mindi leaves). An amount of 250 grams of mindi leaf powder were placed in a glass beaker and added with 750 ml ethanol 70%. It was macerated for 3 days and stirred regularly. After 3 days the macerate was filtered and the resulting filtrate was evaporated using a waterbath until it produces thick extract of mindi leaves and diluted with distilled water to 2%.

2.2.3. Preparation of nanoencapsulation mindi leaves extract: chitosan: STPP. The nanoencapsulation process was an ionic gelation method by mixing mindi leaf extract: chitosan: STPP (1: 7: 1/175%) w/v. Two (2) grams chitosan were added into 100 ml in acetic acid (1% b/v) and dissolve 0.04 ml into distilled water and are stirred for 1 hour. Mindi leaf extract were was then added to the mixture (chitosan dissolved in acetic acid pH 4 solution), and stirred using a magnetic stirrer for 30 minutes. Then STPP was added and stirred for 30 another minutes. Then the solution and sediment are separated. The precipitate was removed and the solution in the form of nano encapsulation was used in the antibacterial in vitro test.
2.2.4. Characterization of Nanoencapsulation mindi leaves extract. **Particle size.** Particle size of nanoencapsulation mindi leaves extract was determined using Zetasizer Nano ZS (Horiba Scientific SZ-100, Horiba, Kyoto, Japan) following the method of by Balakumar[15] and Hussain-Sahudin[16]. The samples were diluted with a ratio of 1:0.01 (v/v) using distilled water and the samples were performed in triplicates at 25°C with a detection angle of 90°.

**Zeta potential analysis.** Zeta potential of the optimum formulations was determined by dynamic light scattering technique using particle size analyzer (Horiba Scientific SZ-100, Horiba, Kyoto, Japan) with method of Balakumar[15] and Hussain-Sahudin[16] The samples were diluted with a ratio of 1:100 (v/v) with distilled water and the samples were repeated in triplicate.

**Morphological analysis.** Morphology was examined using high-performance digital imaging Transmission Electron Microscopy (Joel JEM-1400 CX, Hitachi High-Technologies Corp., Tokyo, Japan). TEM analysis, the nanoencapsulation mindi leaves extract were diluted with distilled water, a drop of it was placed into the copper micro grid which was earlier stained by phosphotungstic acid, and was allowed to evaporate to dry at room temperature (25±2°C). The dried micro grids were then viewed at various resolutions under TEM [16].

2.2.5. Antimicrobial activity of Nanoencapsulation mindi leaves extract. The nanoencapsulation mindi leaves extract were tested for antimicrobial activity by well diffusion method against two pathogenic organism namely, *Escherichia coli* and *Salmonella typhimurium*. The pure cultures of these were sub cultured on MH broth (35°C) on rotary shaker with 200 rpm. Each strain was swabbed uniformly on the individual plates using sterile cotton swab and wells (6 mm) made using gel puncture. Sample of distilled water (negative control), chitosan, STPP, mindi leaves extract, nanoencapsulation mindi leaves extract, and tetracycline (positive control) were pour into wells using micropipette. The mixture were stored in 35 °C room incubator for 1 day.

2.2.6 Statistical analysis. Result were analyzed using Completely Randomized Randomized Design using Statistical Package for the Social Science version 22. The data with significant differences were further separated using Duncan's new Multiple Range Test. All indications of significant differences in this study were based on probability of less than 5%.

3. Results and discussion

3.1 Optimum formulation of mindi leaves extract: chitosan: STPP

One of the factors that influence the size of nanoparticles is the ratio of chitosan and STPP [17] in the formulation. Comparison of formulations from 1: 1/100 to 1: 7/ 1:175 of mindi leaf extract: chitosan: STPP was evaluated one by one to get the optimum formulation. The results show that a ratio of 1: 7/ 1:175 (Table 1) was selected to be the optimum formulation based on visual observations that there was no sediment after centrifuging for 30 minutes.

| Ratio of Extract : Chitosan: STPP | Visual Observation      |
|----------------------------------|-------------------------|
| 1:1:1/100                        | brown precipitate       |
| 1:2:1/100                        | brown precipitate       |
| 1:3:1/100                        | brown precipitate       |
| 1:4:1/100                        | brown precipitate       |
| 1:5:1/100                        | brown precipitate       |
| 1:6:1/100                        | brown precipitate       |
| 1:7:1/100                        | white precipitate       |
| 1:7:1/175                        | Clear (no precipitate)  |
3.2 Characterization of mindi leaves extract: chitosan: STPP

3.2.1. Particle size. The average particle size and polydispersity index (IP) were calculated from the volume, intensity, and bimodal distribution with PI accumulation as a measure of particle homogeneity [18][19]. Table 2., shows that the average particle size distribution of the optimum formulation was 535.2±12.83 nm and the average polydispersity index (IP) was 0.436 ± 0.21. The IP value was low, which indicated that nanoencapsulation systems had an equivalent size distribution. Danaei et al. [19] clarify that the numerical value of PI ranges from 0.0 (for a perfectly uniform sample with respect of particle size) to 1.0 (for a highly polydisperse sample with multiple particle size populations).

3.2.2. Zeta potential analysis. The zeta potential value of the optimum formulation of mindi leaves extract: chitosan: STPP was 59.73±0.35 mV. The positive potential zeta value for the optimum formulation was selected, showing a positively charged surface. The combination of chitosan and STPP by ionic gelation method produces a positive potential zeta. On the other hand, the ionization process in the amino group on chitosan causes cationic process which can produce potential zeta with a positive value [20]. Potential zeta values of less than 30 mV or more than 30 mV indicate particle stability [21].

3.2.3. Morphological analysis. Nanoencapsulation formulation of mindi leaves extract: chitosan: STPP appears small dots or circles on a dark background with a homogeneous round shape (Figure 1). The morphology of the nanoencapsulation form of this nanoencapsulated extract was spherical in shape, like a ball. Self-aggregation and different nanoparticles droplets indicated homogeneously distributed in size and stable [22].

![Figure 1. Transmission electron micrograph of mindi leaves extract: chitosan: STPP](image)

3.3 Antibacterial activity of mindi leaves extract: chitosan: STPP

Table 2 showed that STPP have inhibition activity only on Salmonella typhimurium bacteria. Whereas, chitosan, mindi leaves extract, nanoencapsulation of mindi leaves extract, and tetracycline have the ability as antibacterial activity for Escherichia coli, Lactobacillus acidophilus, and Salmonella typhimurium. The highest diameter of inhibitory zone against Salmonella typhimurium bacteria was in tetracycline 100 ppm which was 20.58±0.38 (P<0.05). While the diameter of the chitosan inhibition zone was 15.00±0.50 and STPP has a diameter of inhibitory zone of 8.00±0.50 (P<0.05). The diameter of the inhibitory zone nanoencapsulation of mindi leaf extract was 12.50 ± 0.50, which was higher (P<0.05) when compared to the diameter of the inhibitory zone by mindi leaf extract with a value of 8.50±0.50.

The highest inhibition zone diameter of Escherichia coli was 2% chitosan (P<0.05). The diameter of the inhibition zone by nanoencapsulation of mindi leaf extract was 9.91±0.52 while tetracycline was 9.66±0.28. No significant differences between both treatments suggests that nano-encapsulation of
mindi leaf extract is considered capable of replacing tetracycline as an antibacterial agent against *Escherichia coli* bacteria. The highest diameter of inhibitory zone against *Lactobacillus acidophilus* bacteria was on tetracycline 100 ppm which was equal to 14.5±0.86 (P<0.05). The diameter of the chitosan inhibition zone, mindi leaf extract, and nanoencapsulation of mindi leaf extract were 8.50±0.50, 7.75±0.25, and 7.91±0.14 respectively.

**Table 2.** Antibacterial activity of nanoencapsulated extract (mindi leaves extract: chitosan: STPP)

| Isolate           | Inhibit Zone Diameter (mm) ± SD |
|-------------------|---------------------------------|
|                   | T1   | T2   | T3   | T4   | T5   | T6   |
| S. typhimurium    | 0e   | 15.00±0.50b | 8.00±0.50d | 8.50 ± 0.50d | 12.50±0.50c | 20.58±0.38a |
| E. coli           | 0d   | 11.16±0.28c | 0e   | 7.83±0.28c | 9.91±0.52b | 9.66±0.28b |
| L. acidophilus    | 0e   | 8.50±0.50d | 0e   | 7.75±0.25b | 7.91±0.14b | 14.5±0.86a |

T1: Aquadest (negative control); T2: chitosan 2.0%; T3: STPP 0.04%; T4: mindi leaves extract (MLE) 2%; T5: Nanoencapsulation MLE; T6: Tetracycline 100 ppm (positive control).

The secondary metabolites from mindi leaves also have functions as an antimicrobial by reducing the activity of pathogenic bacteria, both in gram positive bacteria (*Bacillus thuringiensis* and *Staphylococcus aureus*) and gram negative (*Escherichia coli*) [12]. Flavonoids as antimicrobials have three mechanisms, which inhibit nucleic acid synthesis, inhibit cell membrane function and inhibit energy metabolism [23], and Phenol compounds have antimicrobial mechanisms, namely denaturing cell proteins and Chitosan and STPP (in the manufacture of nano encapsulation formulations) also have antibacterial activity against *E. coli, S. aureus, Pseudomonas aeruginosa* and *Salmonella typhimurium*.

### 4. Conclusion

From the results of this study it can be concluded that for improved mindi leaf extract solubility and stability the optimum formulation for nanoencapsulation (mindi leaf extract: chitosan: STPP) was 1:7:1/175 with particle size 535,2±12,83, IP value 0,436±0,21 and potential zeta 59,73±0,35. Nanoencapsulation of mindi leaf extract and chitosan have antibacterial activity against *Escherichia coli, Lactobacillus acidophilus*, and *Salmonella typhimurium*.

### References

[1] Bedford M 2000 *Worlds. Poultr. Sci. J.* 56 347–65
[2] Timbermont L, Haesebrouck F, Ducatelle R and Van Immerseel F 2011 *Avian Pathol.* 40 341–7
[3] Wachira W M, Shitandi A and Ngure R 2011 *Int. Food Res. J.* 18 1203–8
[4] Verstegen M W A and Williams B A 2002 *Anim. Biotechnol.* 13 113–27
[5] Wenk C 2003 *Herbs and Botanicals for Monogastric Animals Asian Aust. J. Anim. Sci.* 16 282–90
[6] Vidanarachchi J K, Mikkelsen L L, Sims I, Iji P A and Choct M 2005 *Recent Adv. Anim. Nutr. Aust.* 15 131–44
[7] Guo F C, Kwakkel R P, Williams B A, Li W K, Li H S, Luo J Y, Li X P, Wei Y X, Yan Z T and Verstegen M W A 2004 *Br. Poult. Sci.* 45 684–94
[8] Goyena R 2019 *No Title No Title J. Chem. Inf. Model.* 53 1689–99
[9] Windisch W, Schedle K, Pitzner C and Kroismayr A 2008 *J. Anim. Sci.* 86 E140–8
[10] Singh R, Singh S, Kumar S and Arora S 2007 *Food Chem. Toxicol.* 45 1216–23
[11] Vijayanand S and Wesely E G 2011 *Int. J. Pharm. Sci. Res.* 2 1298–302
[12] Munir T, Mohyuddin A, Khan Z and Haq R 2017 *Scientific Inquiry and Review* ( SIR ) 1 19–26
[13] Silva H D, Cerqueira M À and Vicente A A 2012 *Food Bioprocess Technol.* 5 854–67
[14] Calvo P, Remuñán-López C, Vila-Jato J L and Alonso M J 1997 *J. Appl. Polym. Sci.* 63 125–
32

[15] Balakumar K, Raghavan C V, selvan N T, prasad R H and Abdu S 2013 *Colloids Surfaces B Biointerfaces* 112 337–43

[16] Hussain Z and Sahudin S 2016 *Int. J. Pharm. Pharm. Sci.* 8 297–308

[17] Sreekumar S, Goycoolea F M, Moerschbacher B M and Rivera-Rodriguez G R 2018 *Sci. Rep.* 8 1–11

[18] Goyal U, Arora R and Aggarwal G 2012 *Acta Pharm.* 62 357–70

[19] Danaei M, Dehghankhod M, Ataei S, Hasanzadeh Davarani F, Javanmard R, Dokhani A, Khorasani S and Mozafari M R 2018 *Pharmaceutics* 10 1–17

[20] Motiei M, Kashanian S, Lucia L A and Khazaei M 2017 *J. Control. Release* 260 213–25

[21] Servat-Medina L, González-Gómez A, Reyes-Ortega F, Sousa I M O, Queiroz N de C A, Zago P M W, Jorge M P, Monteiro K M, de Carvalho J E, San Román J and Foglio M A 2015 *Int. J. Nanomedicine* 10 3897–909

[22] Masarudin M J, Cutts S M, Evison B J, Phillips D R and Pigram P J 2015 *Nanotechnol. Sci. Appl.* 8 67–80

[23] Cushnie T P T and Lamb A J 2006 Erratum: Antimicrobial activity of flavonoids (International Journal of Antimicrobial Agents (2005) 26 (343-356) DOI: 10.1016/j.ijantimicag.2005.09.002) *Int. J. Antimicrob. Agents* 27 181

[24] Fernandes B C S, Martins M R F B, Mendes A A, Milbradt E L, Sanfelice C, Martins B B, Aguiar E F and Bresne C 2014 *Rev. Bras. Cienc. Avic.* 16 417–24

[25] Anes U C, Nettey H and Jen J V 2016 *Int. J. Res. Stud. Microbiol. Biotechnol.* 2 15–21