

THE USE OF CURCUMIN-LOADED CHITOSAN NANOPARTICLES TO CONTROL ANTHRACNOSE DISEASE ON PAPAYA

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Abstract

Chitosan (Ch) is a low toxic, biodegradable, biocompatible and safe polymer which is used for various purposes. Antifungal activity of chitosan can be optimized by integration with plant secondary metabolites such as curcumin (Cur). The aim of this study is to obtain the best formulation of curcumin-load chitosan nanoparticles (Cur-ChNP) to control post-harvest anthracnose disease (*Colletotrichum gloeosporioides*) on papaya. The Cur-ChNP was synthesized by an ionotropic gelation method using sodium tripolyphosphate as the crosslinking agent. The effectiveness of Cur-ChNP to *C. gloeosporioides* was evaluated under *in vitro* and *in vivo* assay. Results showed that the size of Cur-ChNP (654.3 ± 5.6 nm) was greater than ChNP alone (167.8 ± 1.7 nm). Cur-ChNP had a spiral shape with the PI value of 0.327 ± 0.041 , and ZP of 47.2 ± 1.0 mV. The Cur alone inhibited the growth of *C. gloeosporioides* at both *in vitro* and *in vivo* assays. However, the Cur-ChNP with formula ratio of 5:5 (w/w), had similar effect with Cur treatment to suppress anthracnose disease severity under *in vivo* assay, with the relative disease inhibition rate of 71.19%. This study, demonstrated that Cur-ChNP may be utilized as a potential biocontrol agent against anthracnose disease on papaya.

Keywords: Antifungal activity, curcumin, chitosan, *Curcuma longa*, nanoparticles, *C. gloeosporioides*, papaya

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Introduction

Papaya (*Carica papaya* L.) is one of important fruit crops that is rich in calcium and vitamin. Papaya production in Indonesia reaches 909 tons/ ha in 2013, but decreased to 840 tons/ha in 2014 (Director General Horticulture, 2015). Anthracnose disease caused by *C. gloeosporioides* is postharvest disease causing low productivity as well as low quality of harvested papaya fruits. The fungal infection causes browning and rotten on fruit that further unsuitable for human consumption (Alberida *et al.*, 2014; Hamdayanti *et al.*, 2017).

Synthetic fungicides are normally applied to control anthracnose; however, this method can cause environmental pollution, hence it is needs to develop alternative control based on the environmentally safe method. Anthracnose disease may also be controlled by a coating application method using natural compounds such as curcumin and chitosan. Curcumin, a natural yellow pigment, as a bioactive agent had been separated from the root of turmeric (*Curcuma longa*) and utilized in various traditional medicines (Hatcher *et al.*, 2008; Chiranjib *et al.*, 2009). The rhizome extract of *Curcuma longa* has antifungal activity containing curcuminoids and essential oils of terpenoids that can inhibit fungal growth and interfere mitochondrial function in the cells (Fresieben and Jager, 2014). Turmeric (*Curcuma longa*) is a medicinal plant belonging to the Zingiberaceae family, which generally has anti-inflammatory, antioxidant, and antimicrobial properties (Cikrikci *et al.*, 2008; De *et al.*, 2009). Of the Zingiberaceae family (ginger, and turmeric), turmeric extract was found as the best antifungal agent to suppress growth of fungal *Colletotrichum fragariae*, *C. acutatum*, and *C. gloeosporioides* (Radwan *et al.*, 2014).

Chitosan is a natural polymer made from the process of deacetylation of chitin. It can be used as a natural fungicide by inhibition of conidial germination, as well as hyphae lysis of fungi (Goy *et al.*, 2009; Suryadi *et al.*, 2016). Chitosan has a different molecular weight (MW) and different antifungal activity. The smaller the chitosan MW has a greater

antifungal activity (Li *et al.*, 2008). The fruit coating assay using hydrolyzed chitosan on the surface of the fruit may inhibit the growth of pathogen as well as prolong fruit shelf life (Hewajulige *et al.*, 2009; Suryadi *et al.*, 2017). The antifungal activity of chitosan may stimulates the papaya defense response to anthracnose disease by increasing the chitinase and β ,1-3 glucanase activities (Hewajulige *et al.*, 2009).

Chitosan could accelerate and enhance the antifungal activity of mango fruits to *Colletotrichum* spp (Suryadi *et al.*, 2017), and tomato fruits to *Aspergillus* sp and *Penicilium* sp. (Da Silva *et al.*, 2018). Moreover, the antifungal properties of chitosan can be enhanced by reducing its size to nanoparticles scale until their dimensions are generally less than 500 nm. Chitosan nanoparticles (ChNP) have advantage i.e., non-toxic, stable during usage, high surface area, and can be used as matrices of various drugs and plant extracts (Anitha *et al.*, 2011; Rismana *et al.*, 2013).

ChNP as a nano-system may increase curcumin delivery in infected tissue; thus it is necessary to obtain the best formula of curcumin extract load with chitosan to inhibit anthracnose disease on papaya fruit as well as to prolong the shelf life of papaya during storage. In the present study, an optimal method for extraction of turmeric rhizome containing curcumin and loaded with ChNP was investigated for antifungal activity to control *C. gloeosporioides* infections. The aim of this work is to obtain the best formula of the Cur- ChNP to inhibit anthracnose disease caused by fungus *C. gloeosporioides* on papaya fruit.

Materials and Methods

Determination of Terpenoid (Curcuminoid) Extraction

Turmeric (*Curcuma longa*) rhizome extract was made by the maceration method using 96% ethanol solvent. Turmeric powder (200 g) was macerated with 1,000 mL of 96%

ethanol for 24 h at room temperature, then filtered and re-macerated twice by the same treatment, and further concentrated to get the raw extract of turmeric rhizomes (Bagchi, 2012). One mg of turmeric rhizome extract was dissolved in 2 mL of ether solvent, then the ether phase was added by Liebermann-Burchard reagent (acetic acid glacial and H_2SO_4). The extract contained terpenoids was indicated by a reddish or purple color (Harborne, 2007).

Quantitative analysis of curcuminoid in the turmeric rhizome extract was performed by spectrophotometric method (Bagchi, 2012). Briefly, 0.1 g of the extract was weighed, then dissolved in glacial acetic acid in a 50 mL flask. The solution was heated at 90°C for 30 min, then cooled and added to 1 g of oxalic acid and 1 g of boric acid. The solution is reheated at the same temperature for 10 min. The solution was cooled and adjusted to a volume of 50 mL with the addition of acetic acid glacial. The absorbance of standard solution and sample were measured using spectrophotometer at 530 nm wavelength (Rahayu *et al.*, 2015).

Determination of Chitosan MW

Low molecular weight (LMW) chitosan was made through acid hydrolysis. A total of 15 g Chitosan powder was put into a cup glass, then a 300 ml of 0.6 M HCl was mixed with the solution; afterwards it was heated to 40°C . The hydrolysis process was carried out for 200 min with stirring and the temperature was kept in constant condition. Furthermore, the hydrolysate was neutralized with NaOH, then the solids filtrate obtained were further filtered using filter paper, washed with aquadest and dried (Adhiatama *et al.*, 2012).

Determination of MW was done by the viscometer method using Ostwald viscometer (Kasaai *et al.*, 2013). Chitosan standard solution of 0.02%; 0.04%; 0.06%; 0.08% and 0.10% (w/v) were prepared in sodium chloride acetate 0.02 M solvent. The relative, specific, reductive, and intrinsic viscosity was determined based upon Huggins and Mark- Houwink equation (Adhiatama *et al.*, 2012) as

follows: $[\eta] = KM\alpha$, where: η = intrinsic viscosity, K = Huggins constant (3.04×10^{-5}), α = constants (1.26), M = MW.

Cur-ChNP Synthesis and Characterization

Preparation of nanoparticles was done by an ionotropic gelation method using 0.1% sodium tripolyphosphate (NaTPP) as a crosslinking agent following the procedure of Nguyen *et al.*, (2014). A stock solution of chitosan and NaTPP was made by dissolving 0.2% (w/v) chitosan in 100 mL 1% acetic acid. The chitosan solution (1 mL) was stirred for 10 min and its volume was adjusted to 1.5 mL with distilled water, then it was added to 20 mg of NaTPP in 0.1 N HCl to a pH of 3.0-3.5, while continuously stirring for 1 h.

To obtain a nano- system with a maximum ratio of metabolite extract loading, different ratios of curcumin/chitosan (w/w) were tested. Curcumin stock (1 mg/mL) was dissolved in ethanol. To the chitosan solution, the curcumin in various concentrations was added. The ratio tested of chitosan to curcumin was 5: 1, 5: 2, 5: 3, 5: 4, and 5: 5 (w/w). Nanoparticles solution was stabilized with 0.25 mL Tween 80 0.1%. The mixture was further stirred for 30 min and centrifuged at $4000 \times g$ for 5 min. Finally, the supernatant was transferred into a new tube and kept in the refrigerator for subsequent analysis (Bhawana *et al.*, 2011).

The samples were directly applied for particle measurements. The particle size and zeta potential (ZP) value of prepared nanoparticles were characterized using Malvern Zetasizer ZS series and Scattering Particle Size Analyzer (Malvern Instruments Ltd. Co, UK). The morphological shape, size, and aggregation of Cur-ChNP were observed by Scanning Electron Microscope (SEM) (Carl Zeiss EVO MA10, Germany). Later on, the Fourier transforms infrared (FTIR) spectra of chitosan and Cur-ChNP were assessed using FTIR Tensor 37 (Bruker Optik GmbH, Karlsruhe, Germany). The sample analysis was performed using wave numbers $4000 - 400 \text{ cm}^{-1}$ (Qi *et al.*, 2004; Nur Laili *et al.*, 2014).

Evaluation of Cur-ChNP to *C. gloeosporioides* under *In vitro* and *In vivo* Assay

Preparation of Fungus *C. gloeosporioides*

C. gloeosporioides was grown for 24 h in potato dextrose agar (PDA) medium. The fungal conidia were collected, washed, and resuspended in sterile phosphate- buffered saline (PBS, pH 7.4). The solution was adjusted to a cell suspension of 10^7 conidia/mL using a UV spectrophotometer (Hitachi U-2010) at 620 nm wavelength.

In vitro Assay

A warm-luke potato dextrose agar (PDA) in 9 cm petri dish (40°C) was mixed with 1 mL of nanoparticle solution, then the medium was allowed to harden (Correa-Pacheco *et al.*, 2017). In the middle of the PDA medium was perforated using cork borer, then the *C. gloeosporioides* isolates were placed into the hole and incubated at 24-26°C until the full growth of control treatment was reached to the edge of petridish. Each treatment was repeated three times. Observation was carried out by measuring diameter colony of fungal growth (cm), then it was converted to radial growth (cm²). The positive control treatment was performed using commercial fungicides (Carbendazim). The effectiveness of nanoparticles to inhibit the pathogen was expressed in the relative degree of inhibition (RDI) which calculated by the formula:

$$RDI (\%) = \frac{(F_c - F_t)}{F_c} \times 100\%$$

where: F_c = fungal growth of control negative (untreated); F_t = fungal growth of treatment.

In Vivo Assay

Papaya fruit cv. "California" was washed with sterile water, and then it was dried and disinfected with 70% alcohol. After that, the fruit was sprayed with a nanoparticles solution and then dried. The fruit was artificially wounded with a sterile needle (about 30-40 puncture), then the fruit was dipped into a *C. gloeosporioides* conidial suspension (10^7 conidia/mL) for 30 min until dried (Suryadi

et al., 2017). The fruit was stored on a sterile tray and wrapped with clear plastic to keep the moisture away. A non-treated fruit was also used as a negative control. The experiment was performed in triplicate. Observations were made at 6 days after inoculation (DAI). The assessment of anthracnose disease in papaya fruit follows the 5 scale basis, i.e: 0 = lesion area 0-1%; 1 = lesion area 1-20%, 2 = lesion area 21-40%, 3 = lesion area 41-60%, 4 = lesion area 61-80% and 5 = lesion area 81-100%. The disease severity (DS) was determined following formula as follow:

$$DS (\%) = \sum \frac{(\text{number of samples per category} \times \text{severity score})}{\text{number of samples observed} \times \text{the highest score}} \times 100\%$$

Then, the effectiveness of nanoparticle to inhibit anthracnose disease is expressed in percent relative degree of inhibition (RDI) by the following formula:

$$RDI (\%) = \frac{(\text{DS of control} - \text{DS of treatment})}{\text{DS of control}} \times 100\%$$

Statistical Analysis

Statistical analyses were performed using Sirichai software ver 6.00. The Duncan multiple range test (DMRT) was used for mean comparison, and different *p* values less than 0.05 were considered statistically significant.

Results and Discussion

Terpenoid Content (Curcuminoid) of Turmeric Rhizome Extract

The color change to reddish purple indicated the presence of terpenoids in turmeric rhizome extract (Figure 1). Terpenoids are a secondary metabolite of nature, having an oxygenated hydrogen- enriched hydrocarbon formula (C₅H₈). Turmeric rhizome contains various types of terpenoid compound. The dominant terpenoid forms in the turmeric rhizome are monoterpenoid and sesquiterpenoid (Afzal *et al.*, 2013). According to Freiesleben and Jager, (2014), terpenoids can inhibit the fungal growth of mycelia and spores by destroying the cell membranes. In this study,

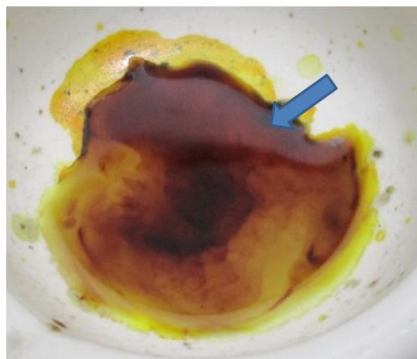


Figure 1. Terpenoid (curcumin) extraction from turmeric rhizome. A reddish purple color (arrow) indicated terpenoid content

the rate of curcuminoid in the turmeric rhizome extract was amounted to $21.71 \pm 0.9\%$ with a concentration of 0.877 ± 0.05 ppm.

Hydrolysis and Chitosan MW

Chitosan is hydrolyzed in order to obtain chitosan with a lower MW as measured by the viscometric method. In this study, the chitosan MW before hydrolysis was obtained by 878.185 kDa. The intrinsic viscosity and chitosan MW value after hydrolysis was decreased to 564.98 kDa. Chitosan hydrolysis is a chitosan chain depolymerisation reaction due to the termination of the glycosidic bond by H_2O molecule, resulting in chitosan with a shorter chain and lower MW. Depolymerisation is influenced by catalyst concentration, temperature, hydrolysis time, and stirring. The higher the concentration of the catalyst will accelerate the higher depolymerisation used temperature. The longer hydrolysis and stirring cause many chains of chitosan to be degraded, therefore the depolymerisation will increase (Adhiatama *et al.*, 2012).

Table 1. Characteristics of ChNP and Cur-ChNP

Treatment ^a	Z-average (nm)	PDI	ZP (mV)
ChNP	167.8 ± 1.7	0.200 ± 0.003	21.5 ± 0.7
Cur-ChNP	654.3 ± 5.6	0.327 ± 0.041	47.2 ± 1.0

^a PDI= polydispersity index; ZP= Zeta potential

In line with this study, Adhiatama *et al.*, (2012) obtained chitosan with a MW of 94.6679 kDa from an initial MW of 1047 kDa, using condition of hydrolysis with a 0.8 M HCl for 200 min in a reactor whose temperature and stirring was kept constant (40°C). The difference in the MW of chitosan molecules is due to differences in catalyst concentration and constant stirring. The higher the concentration of the MW will be the faster catalyst drop. A constant stirring can increase the mass transfer, thus accelerating the MW reduction.

The concentration and type of catalyst (acidifying properties) affect the MW of the hydrolysis results. The addition of acid is usually with a strong acid such as HCl, H_2SO_4 , and HNO_3 , causes a decrease in pH ($pH < 4$) then causes protonation of amine and oxygen groups in chitosan glycosidic bonds, and swelling of the chitosan structure. Swelling structure allows water to break glycosidic bonds, resulting in depolymerisation. Depolymerisation under acidic conditions is random, but the reaction is faster than depolymerisation with enzymes. The random reaction causes the length of the polymer chain is not uniform, so the MW of chitosan molecules is difficult to control (Kasaai *et al.*, 2013).

Characteristics of Cur-ChNP

Curcumin and chitosan formulations are reduced in size to nanoparticles by the ionic gelation method. The synthesis of nanoparticles using the ionic gelation method is based on the interaction between chitosan amine polycation and polyanion tripolyphosphate. Chitosan polycation was obtained from the dissolution of chitosan in 1% acetic acid. The presence of stirring causes polycation and polyanion interactions more evenly, but interactions also become complex and nanoparticles tend to form spherical. The ChNP measurement using a particle size analyzer (PSA) was 167.8 ± 1.7 nm, with PDI value of 0.200 ± 0.003 . After addition of curcumin extract, the nanoparticle size had increased to 654.3 ± 5.6 nm, with the polydispersity index (PDI) value of 0.327 ± 0.041 (Table 1). In this study, the size of Cur-ChNP obtained was in the range of 10-

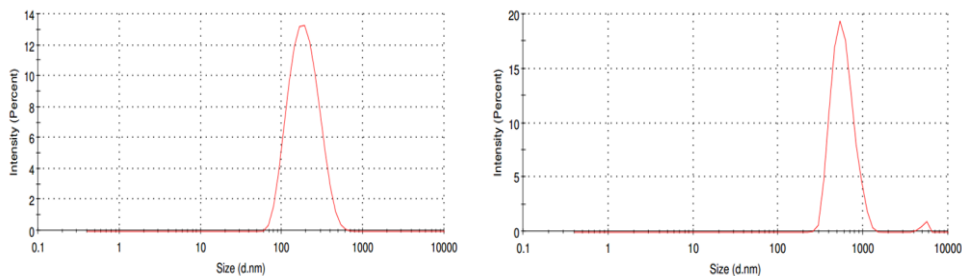


Figure 2. Particle size of ChitosanNP (a) and Cur-ChNP (b)

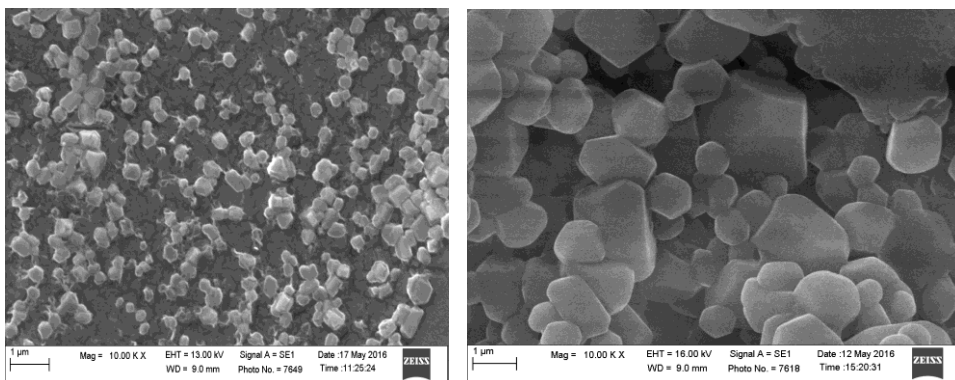


Figure 3. SEM characteristics of ChNP (a) and Cur-ChNP (b)

1,000 nm, therefore the size has reached to nanoparticles size. Moreover, the charge of ChNP becomes lower after curcumin loading.

The size of ChNP after adding the turmeric rhizome extract enlarged more than 4 fold from 167.8 ± 1.7 nm to 654.3 ± 5.6 nm (Figure 2). In line with our study, (Rismana *et al.*, 2013) reported that the size of the ChNP increased 2-5 fold from 95 nm to 200-500 nm after the addition of mangosteen peel skin extracts. The enlargement of particle size may caused by extracts that enter into chitosan, resulting in the encapsulation of extracts by chitosan. According to Haneefa *et al.* (2014), curcumin had a size of 40-70 nm in the form of cubic morphology and long rounded.

The PDI or uniformity of the nanoparticle solution of both sample solutions obtained is less than 0.5, so it can be said that the size of the nanoparticles is uniform. PDI is directly proportional to the particle distribution. The

smaller the PDI shows the narrower particle size distribution, the greater the PDI value of the larger particle size distribution (Nurkhasanah *et al.*, 2015).

The surface morphological structure was further examined by SEM analysis. The diameter average of ChNP was a spherical shape about 160 ± 10 nm (Figure 3), which is close to similar size as observed by PSA analysis (167.8 ± 1.7). On the contrary, Das *et al.* (2010) reported that Cur-ChNP had a particle size of 100 ± 20 nm. This condition might be due to different cross linker applied in the preparation. They used sodium alginate instead of sodium tripolyphosphate (NaTPP) salt as used in this study. The Cur-ChNP with a size of 654.267 ± 5.590 nm obtained in our research was still in the range of nano scale dimension (<1000 nm). However, Akhtar *et al.*, (2012) observed particle size of Cur-ChNP was more than 200 nm.

The ZP value can be used to determine the charge on the surface of the nanoparticles and the stability of the nanoparticles. The ChNP have a ZP of 21.6 mV, after addition of extract the ZP increases to 46.2 mV. The ZP increase can be caused by several factors such as length of stirring and Cur-ChNP composition. The longer the stirring time, the ZP value increases. This result is in accordance with research of Nagaonkar *et al.* (2015) that the ZP value increases with the increment length of stirring condition. ZP is influenced by chitosan-extract composition. If the charge of the extract is positive, then the more extracts added the ZP value increases. The addition of turmeric rhizome extract into the ChNP causes the ZP value to increase. In line with this study, Duan *et al.* (2010) also reported that the ZP value polyethyl (butyl cyanoacrylate) after curcumin addition was increased from 1.46 ± 0.03 mV to 29.11 ± 1.69 mV.

In this study, the surface charge of Cur-ChNP was 46.2 mV, whereas the surface charge of ChNP alone has been reported 21.6 mV. The ZP value can be applied to determine the stability of nanoparticles. Nanoparticles with a ZP greater than (+/-) 30 mV indicate stable nanoparticles since surface charges prevent particle aggregation (Nurkhasanah *et al.*, 2015).

The measurement results show the ZP of ChNP is less than +30 mV, whilst the Cur-ChNP is more than +30 mV. This shows that

the Cur-ChNP are more stable than ChNP, and there is a tendency of ChNP easily aggregated compared with that of Cur-ChNP.

The morphology of nanoparticles that characterized by SEM, implied that the extract can enter or outside the aggregate surface of the ChNP. According to Popat *et al.* (2015), curcumin extract can enter into chitosan because the size of the extract is smaller than that of ChNP, so that curcumin can enter into the chitosan aggregate. The form of ChNP tends to be round, when entered the extract, nanoparticles remain round but tend to elongate. According to Nguyen *et al.* (2014), chitosan can be encapsulated by extracts due to chitosan interactions with turmeric extract on the surface of nanoparticles. The encapsulated chitosan in the extract will be aggregated and becomes irregular shape. The results showed that the morphology of ChNP is round, and irregular. It is also shown that there is an extract encapsulated by chitosan, and there is also chitosan that is encapsulated by the extract. Moffazal Jahromi *et al.* (2015) reported that curcumin can enter into chitosan about $75 \pm 2\%$.

FTIR Spectra of Chitosan, Curcumin and Cur-ChNP

Characterization using FTIR was performed to determine the functional groups present in chitosan, ChNP and Cur-ChNP. Cur-ChNP showed a different spectrum compared with chitosan.

The chitosan absorbance spectra (4-A), ChNP (4-B), and Cur-ChNP (4-C) was shown in Figure 4. Absorbance at $3,500\text{--}3,100\text{ cm}^{-1}$ and $1,640\text{--}1,550\text{ cm}^{-1}$ showed the presence of N-H vibration. Absorbance at wave number $1,657\text{ cm}^{-1}$ indicated the presence of the C=O (amide) group on chitosan. The absorption of NH (amide) chitosan (4-A) undergoes a shifted in ChNP (4-B) from the absorbance at wave numbers $1,657\text{ cm}^{-1}$ to $1,633\text{ cm}^{-1}$, increased absorbance at $1,560\text{ cm}^{-1}$ waves, and the appearance of absorbance P=O ($1,280\text{ cm}^{-1}$) which show the hydrogen bonding interaction in the chitosan NH group with the sodium tripolyphosphate (NaTPP) (Figure 4). The hydrogen bonds cause crosslinking of positively charged

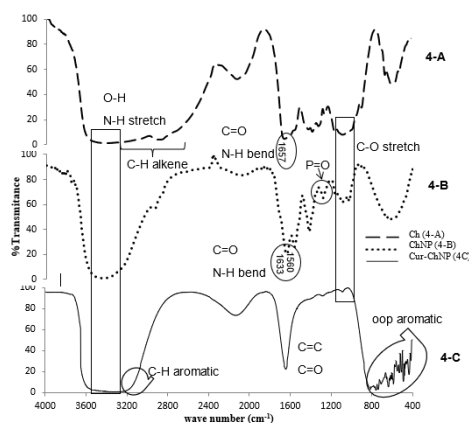


Figure 4. FTIR analysis of Chitosan, ChNP and Cur-ChNP

chitosan with the negatively charged of NaTPP (Käuper and Forrest, 2005).

Absorbance at wave number $1,644\text{ cm}^{-1}$ (4-C) indicates the presence of the C = C (alkenes) group present in curcuminoid and terpenoid. Absorbance at the wave number $3,150\text{--}3,050\text{ cm}^{-1}$ indicated the presence of aromatic C-H (Pavia *et al.*, 2015). The absorbance of the C = O, N-H, and P = O groups in the Cur-ChNP is not visible, this is possible because of the absorbance accumulation by the functional groups of certain wavelengths. The dominant functional group will produce the highest absorbance. Cur-ChNP absorbance (4-C) at the wavelengths of $1,560\text{ cm}^{-1}$ and $1300\text{--}1000\text{ cm}^{-1}$ showed the highest absorbance compared to 4-A and 4-B, and an absorbance at the wavelength of $1,644\text{ cm}^{-1}$ becomes sharper. This indicates the presence of an alkene-functional group in 4-C is dominant, so that alkene absorbance (C = C) is more visible than N-H (bend) and C = O absorbance. The functional groups of chitosan and its combination with Curcumin exhibit significant fungicidal properties that support the importance of these bioactive agent in suppressing fungal infection.

The Effectiveness of Cur- ChNP in Inhibition of *C. gloeosporioides* under *In vitro* and *In vivo* assay

The degree of inhibition of fungal pathogen (*C. gloeosporioides*) was analyzed to explore the effect of curcumin, ChNP and Cur-ChNP on anthracnose infection. The antifungal properties of chitosan are enhanced through hydrolysis, the addition of turmeric rhizome extract, and the reduction of size.

Under *in vitro* assay, the antifungal property of the rhizome extract of turmeric is better than chitosan with the fungal growth of 8.33% or RDI of 85.65%. However, the statistical analyses also showed that Cur-ChNP (5:5) significantly decreased the growth of fungal pathogen ($p < 0.05\%$), with the fungal radial growth of 21.33 cm^2 or RDI value of 64.71% compared with the untreated control (Table 2). This suppression value was similar

with RDI of control positive (Carbendazim treatment).

Under *in vivo* assay, the disease severity (DS) as shown by lesion infection on papaya fruit was ranged from 26.50% to 92% as (Figure 5). It was also shown that Cur alone was effective compare with that of ChiNP alone to reduce anthracnose disease severity. When they combined, the best formula was obtained by Cur-ChNP 5:5 (w/w) with the disease severity of 26.5% or RDI value of 71.19% (Table 3). Although this was not significantly different with Cur alone (RDI = 69.56%).

Chitosan and turmeric have different antifungal mechanisms. Chitosan can inhibit the growth of the fungus *C. gloeosporioides* through the growth of mycelia and spores in the fungus. Mechanism of chitosan inhibition against fungi based on chitosan MW. Chitosan with LMW can enter into fungal cells. Chitosan will make the cell membrane become thicker. The amount of chitosan in the cell will increase the activity of chitinase in fungus cells, so indirectly can disrupt the activity of the fungus (Li *et al.*, 2008), whilst the turmeric rhizome extract acts as antifungal because it

Table 2. The effectiveness of Cur- ChNP to *C. gloeosporioides* (in vitro)

Treatment	Fungal radial growth (cm^2)	RDI (%)
ChNP	40.67 ^b	34.02 ^e
Cur	8.33 ^f	85.65 ^a
ChNP : Cur (5:1)	38.00 ^{bc}	37.69 ^{de}
ChNP : Cur (5:2)	37.00 ^{bc}	39.13 ^{de}
ChNP : Cur (5:3)	35.00 ^c	42.64 ^d
ChNP : Cur (5:4)	27.33 ^d	55.13 ^c
ChNP : Cur (5:5)	21.33 ^e	64.71 ^b
Control negative (untreated)	61.33 ^a	0 ^f
Control positive (Carbendazim)	22.67 ^e	62.62 ^{bc}

$\text{RDI} (\%) = \frac{(\text{Fc}-\text{Ft})}{\text{Fc}} \times 100\%$; RDI= relative degree of inhibition; Fc= fungal growth of control negative (untreated); Ft= fungal growth of treatment. Means not sharing the letter in common differ significantly by Duncan's Multiple Range Test ($P < 0.05$).

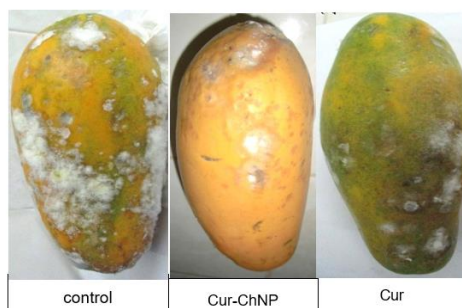


Figure 5. Lesion of anthracnose on papaya with different treatments. Control (a); Cur-ChNP (b); Cur (c)

Table 3. The effectiveness of Cur- ChNP to *C. gloeosporioides* on papaya fruit (in vivo)

Treatment	Disease Severity (%)	RDI (%)
Control (untreated)	92.00 ^a	0 ^a
ChNP	63.50 ^b	30.97 ^b
Cur	28.00 ^{cd}	69.56 ^e
ChNP : Cur (5:1)	63.00 ^b	31.52 ^b
ChNP : Cur (5:2)	65.00 ^b	29.34 ^b
ChNP : Cur (5:3)	51.00 ^{bc}	44.56 ^c
ChNP : Cur (5:4)	43.50 ^{bcd}	52.72 ^d
ChNP : Cur (5:5)	26.50 ^d	71.19 ^e

RDI (%) = $\frac{(DSc - DSt)}{DSc} \times 100\%$. where: RDI= relative degree of inhibition; DSc= disease severity of control (untreated); DSt= disease severity of treatment; Means not sharing the letter in common differ significantly by Duncan's Multiple Range Test (P<0.05).

contains terpenoids and curcuminoid. Terpenoids and curcuminoids are lipophilic that enter the fungal cells through the membrane resulting in impaired membrane stability and causing lysis cells. In addition, terpenoids also interfere with mitochondrial activity by inhibition of transport electrons (inhibition of proton pumps) and ATPase inhibition (Afzal et al., 2013).

The content of curcuminoids in the extract can increase antifungal activity. Curcuminoid is as antifungal by inhibiting the growth of mycelia and spores in the fungus. The addition of turmeric rhizome extract of

nanoparticles is expected to improve the antifungal properties of nanoparticles. According to Radwan *et al.* (2014), the best antifungal activity of curcuminoid is present in desmetoxy- curcumin. Use Curcumin as a potent, biocompatible and bioactive agents with an antimicrobial property has been applied in nanoparticles synthesis.

Biofungicides is environmentally friendly because it does not leave a residue of dangerous chemicals in the soil. Compounds produced by plants can be used as a source of biofungicides because they contain active compounds such as alkaloids, terpenoids, steroids, phenolics, tannins, and others. One of the plants that produce bioactive compounds that can be used as biofungicide is turmeric rhizome (*Curcuma longa* L.). Turmeric rhizome contains a class of curcuminoid compounds and terpenoid essential oils are an important part in biological activities such as anti-inflammatory and anti-oxidant (Afzal *et al.*, 2013), and anti-microbial (De *et al.*, 2009; Anitha *et al.*, 2011).

Chitosan [poly- (2-amino-2-deoxy-β- (1-4) -D-glucopyranose)] is a poly-amino saccharide obtained from the removal of the 2- acetyl (deacetylated) chitin [poly (2-acetamido-2 -Desco-β- (1-4) -D- glucopyranose)], linear biopolymers with 2000-5000 units of monomers interconnected through β- (1-4) glycosidic bonds (Dai *et al.*, 2012). The deacetylation process can be carried out chemically and enzymatically as well as a combination of both. The removal of acetyl groups usually uses alkali which break off the strong hydrogen bond between the nitrogen atoms and the carboxyl groups of the chitin structure.

Chitosan has a different MW that can be divided into low, medium, and high MW. According to Goy *et al.* (2009) chitosan LMW range of 0-50 kDa, medium-MW of 50-100 kDa, and HMW >150 kDa. Chitosan with different MW have different antifungal activity. According to Li *et al.* (2008), the smaller chitosan MW will have the greatest antifungal activity. The antifungal properties of chitosan can be enhanced by the expansion of the chitosan surface by reducing the size of chitosan to become nanoparticles (the size ranges from

10-1,000 nm) with some advantages that are not toxic, stable during use, high surface area, and can be matrices of various types of drugs and plant extracts (Rismana *et al.*, 2013).

This study has shown that the antifungal effect of Cur-ChNP is significantly better than individual curcumin. The Cur-ChNP could significantly suppress the progression of fungal pathogen infection, whereas ChNP by itself could not inhibit pathogen infection. Hence, a combination of ChNP and curcumin produces a synergistic effect on the antifungal activity that is more effective than both of them individually.

Chitosan alone can inhibit fungal growth up to 80% (Gonzalez *et al.*, 2008). The inhibitory effect of chitosan to anthracnose was presumably due to the combination effect of its antifungal property and its ability to stimulate defense response in papaya fruits (Hewajulige *et al.*, 2009). The antifungal effect of Cur-ChNP was observed at ratio (5:5). In contrary to our study, the best formula in the synthesis of ChiNP-extracts under acidic pH was found at the mass ratio Chi: extract of 3:1 (v/v) (Nagaonkar *et al.*, 2015). The smaller the ChiNP, the more ChiNP antifungal activity will increase.

Under *in vivo* assay, this study had also been shown an antifungal effect of curcumin to suppress anthracnose in papaya, as well as displayed a higher potency of Cur-ChNP rather than the individual curcumin.

The property of Cur-ChNP is a good candidate for control fungal infection including *C. gloeosporioides*. The formula can potentially be utilized and applied as a strategy to specifically control anthracnose on papaya fruit. In our future work, optimization formula of Cur-ChNP will be done against different post harvest horticulture crops.

Conclusions

Size of Cur-ChNP was greater than ChNP. The ZP value of Cur-ChNP is higher than that of ChNP alone, so that it assumes to be more stable property than ChNP. The stability of nanoparticles is very important to prevent the

aggregation of nanoparticles, hence the presence of turmeric extract in nanoparticles will improve the properties of better formula. The best antifungal properties of Cur-ChNP was obtained by a ratio of 5: 5 (w/w).

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