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Effect of *Piper betle* Leaf Extract-Loaded Chitosan Nano Particle as Fruit-Coating Assay to Control Storage Anthracnose on Mango

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ABSTRACT

Green betel leaf (*Piper betle* L.) is one of the plants being used for traditional herbal medicine. This study aimed to determine betel leaf extract-chitosan nanoparticles to control storage anthracnose of mango. Chitosan nanoparticles (ChiNP) were prepared by ionic gelation method using sodium tripolyphosphate. Characterization of the betel leaf extract was done by pyrolysis Gas Chromatography Mass Spectrophotometriy (GC-MS); while chitosan nanoparticles were characterized using Fourir Transfer Infra Red (FTIR) spectroscopy, Scanning Electron Microscopy (SEM), and Particle Size Analysis (PSA). For in vivo assay, fruit coating assay was carried out by dipping storage mango in a coating formulated ChiNP-betel leaf extract suspension before inoculation with conidial of *C. gloeosporioides*. Non-inoculated fruits were similarly treated as control. The green betel leaf extract contains 71.18 $\pm$ 0.3% of antifungal phenolic compounds. The most phenolic compounds and their derivatives in the betel leaf extract was 1-hydroxy-4-methylbenzotriazole. Chi-hydrolysis reduced the molecular weight (MW) from 754.89 kDa to 245.85 kDa. Based on FTIR analysis, hydrolysis treatment and the addition of extract affected the existence of chitosan functional group, and wave numbers. The absorption of aromatic groups was observed at 1000 - 650 cm^{-1} wave numbers. The sizes of particle were ranged from 101 $\pm$ 6.25 nm to 431.1 $\pm$ 4.32 nm. Chi without hydrolysis had bigger size than that of Chi with hydrolysis. The SEM morphology of the ChiNP-betel leaf extract was rounded/spherical shape. Chi hydrolysis treatment revealed higher antifungal effect than that of Chi without hydrolysis. The mass ratio of ChiNP and betel leaf extracts (3:1; v/v) of both without hydrolysis and with hydrolysis was found as a good formula in suppressing storage anthracnose of mango with the degree of disease inhibition of 85.88% and 98.82%, respectively. The betel leaf extract-loaded ChiNP treatment may offer the fruit shelf life up to 6 days.

Keywords: Anthracnose, *Colletotrichum* spp, chitosan nanoparticle, *Piper betle*

INTRODUCTION

Colletotrichum gloeosporioides the causal agent of anthracnose disease infects mango fruits in almost all mangoes-producing countries. In Indonesia, the decreased of mango production due to anthracnose was up to 7.72% (Ministry of Agriculture, 2014). Anthracnose disease most often attacks horticulture commodities (vegetables and fruits) such as on papaya, mango, chilli, cocoa, avocado, guava, passion fruit, orange, apple, grape, and cashew nuts causing considerable post-harvest losses for farmers (Agrios, 2005; Tasiwal, 2008). *C. gloeosporioides* can infect immature mangoes in trees, as well as disease develops during storage. Spores that germinate appressorium form do not develop until the fruit is harvested and ripe. Symptoms of anthracnose attacks during postharvest are marked by dark brown spots, concave, and round shape found on the surface of the skin. Spots will be more widespread and enter the flesh if the level of attack is getting worse. Problems faced by producers in the development of mango production and quality are the earliest damage caused by fungal pathogen infections from planting to fruit harvesting, so anthracnose is considered as the main problem of postharvest disease on mangoes and still difficult to control (Alemu et al., 2014).

To date, the most commercials synthetic fungicide used are environmentally polluted, toxic, and not easily decomposed; hence natural fungicides that easily prepared, non-toxic and environmentally friendly are required to suppress the development of anthracnose fungi on storage mango. Chitosan produced from crustacean animal shells waste has been widely used as antifungal, medicinal coating, wound healing, and antioxidants (Suryadi et al., 2017; Rejane et al., 2009; Gonzalez et al., 2008). The benefits of Chi, are non-toxic, cheap, biocompatible, degradable, and water-soluble (Rismana et al., 2013). Chi is a polysaccharide consisting of glucosamine and N-acetylglucosamine that serve to induce plant resistance response to pathogen infection (Pamekas et al., 2009). The antimicrobial mechanism of Chi may occur at both extracellular and intracellular levels (Lauzardo et al., 2010). Chi can diffuse into the fungus cells through the cell wall, so that the entire cells become swell and rupture (cell lysis). It is also very effective in inhibiting fungal spore germination, as well as hyphal elongation, and radial growth of the fungus (Rejane et al., 2009). Chi was reported to able to inhibit the growth of *C. musae* by inhibiting the germination of conidia, minimizing the width of hyphae, shortening hyphae segment, and causing hyphae lysis (Pamekas et al., 2009).

The suitability of the Chi to be applied as a biocontrol agent depends upon the molecular weight (MW) (Nguyen et al., 2009). The Chi MW was reported ranged from 50-190 kDa (LMW) to 310-375 kDa (HMW). The Chi LMW contains more antimicrobial activity compared with that of the Chi HMW (Rejane et al., 2009; Suryadi et al., 2016). Therefore, it is necessary to increase the effectiveness of Chi as a fungistatic agent by reducing the MW of Chi molecule. Chi LMW can be obtained using an acid hydrolysis method such as sulphuric acid, hydrochloric acid, and nitric acid by breaking of the Chi glycosidic bond (Dinarsari and Alfiana, 2013; Adhiatama et al., 2012).

In this study the hydrochloric acid catalyst was used due to easily obtain and cheap. The effectiveness of Chi fungistatic can be increased by reducing the particle size into nanoparticle (NP) (submicron colloidal <1 μm) range. The ChiNP have many advantages that are stable during use, high surface area, high adsorption, and high antimicrobial activity (Rismana et al., 2013; Guaix et al., 2008). ChiNP was able to inhibit the growth of fungi by 80% (Rejane et al., 2009).

Another natural fungistatic is green betel leaf (*Piper betle* L.) that is widely used as traditional medicinal properties. The antimicrobial activity of the betel plant extracts (contained saponin, phenol, alkaloids, amino acids, tannins, flavonoid, steroid and other compounds) have been used in healing, wound antiseptic and antifungal ability (Guha, 2006). Moreover, the betel leaf extract has the potential to suppress the development of the fungus *C. capsici* (Sheikh et al., 2012). Alkaloids have antimicrobial activity by inhibiting esterase, DNA, RNA, polymerase, and cell respiration and play an important role in DNA intercalation. It also acts as antifungal by inhibiting the biosynthesis of nucleic acids in the fungus (Kusumaningtyas et al., 2008). Phenols and their derivatives can denature the fungal cell protein. The phenolic compounds may inhibit pathogenic

microorganisms. In addition, they can increase diffusion through the fungal cell membranes, thereby impairing the permeability of the fungal membranes (Nouri et al., 2014).

The incorporation of antimicrobial agents i.e; mineral oils for potential use in coatings, has been demonstrated in different food commodities which inhibit various pathogenic fungi, bacteria, and yeasts (Cagri et al., 2004). So far, lack of study has been reported on used of betel leaf extract to suppress post-harvest fungal pathogen such as *C. gloeosporioides* on mangoes; therefore it is necessary to determine antifungal activity of betel leaf green against *C. gloeosporioides*. Moreover, the inhibitory activity of betel leaf extracts needs also to be optimized with the addition of ChiNP. The betel leaf extract-ChiNP inhibitory activity may influenced by the composition of each compound, hence it is necessary to determine the formula which can inhibit the growth of the fungus optimally.

In this study, we determined the composition of the betel leaf extract-ChiNP which is optimal as an anthracnose disease inhibitor agent on mango. This study aimed to determine the effectiveness of betel leaf extract-ChiNP composition in inhibiting the growth of fungus *C. gloeosporioides* on mango. We hypothesis that of betel leaf extract can be useful in reducing postharvest disease (anthracnose) as antifungal agent and its activity may be enhanced in combination with ChiNP.

MATERIALS AND METHODS

Phytochemical Analysis of Betel Leaf Extract

The green harvested betel leaves (100 g) were milled and extracted using 1000 mL ethanol 80% solvent. The extraction of betel leaves was done using the maceration process of extracting symplicia by using ethanol solvent with 4 stirring times at room temperature. Then, the rough ethanolic extract was concentrated using a rotary evaporator, then alkaloids was determined following procedure of Nouri et al., (2014).

In alkaloid assay, 0.1 g of extract was put into the test tube, then it was added with 2 mL of chloroform and 2 mL of ammonia, whipped and then added with 2N HCl. The positive alkaloid test results were characterized by the formation of green precipitate after the residue was dropped with Dragendorf reagent, yellow precipitate after dyeing with Mayer reagent, and brown sediment after sputtering with Wagner reagent. For phenolic test, green betel leaves were saturated and then rinsed with running water to get rid of dirt, then drained and chopped or cut into small pieces. It was dried at 50-60°C in the dryer cup for 3-5 days then dry sorting. The 10 g betel leaves were dissolved in 100 mL ethanol 80%. The solution was stirred with a magnetic stirrer for 1 h, then stand and filtered for 24 h. The residual plus 80% ethanol is then filtered again. After that it was heated in a water bath at 60°C and stored in the exicator (Ali et al., 2010). Total phenol test was determined using spectrophotometric method, with gallic acid as standard (AOAC, 2006). The sample measurement procedure was done by weighing 100 mg samples, and then it was added with 0.5 mL of methanol, 2.5 mL of distilled water, 2.5 mL of 50% Folin-Ciocalteau reagent. The solution was mixed for 5 min. It was then added with 2 mL of 7.5% Na_2CO_3 and vortexed, and then incubated for 15 min at 45°C. Absorbance was measured at a wavelength of 765 nm using a UV-VIS spectrophotometer (Hitachi U-2800 spectrophotometer).

Phenolic compounds analysis of the betel leaf extract was performed using Pyrolysis Gas Chromatography Mass Spectrophotometric (GC-MS) (Shimadzu type GCMS-QP2010) with the operational condition as follows: Helium gas, mass spectrophotometer detector, Rtx-5MS 0.25 mm phase capillary columns, 50°C column temperature, 100 kPa inlet pressure, water rate of column 0.85 mL/min, split ratio 112.3, SPL temperature 280°C, MS interface 280°C, ion source 200°C, and pyrolysis temperature of 400°C. The sample mixtures were inserted into quartz space in pyrolysis and heated in oxygen free environment at 610°C for 10 sec. The reaction produces heat which was mediated by the division of chemical bonds in the macromolecular structure and produces a LMW indicating a specific type of macromolecule.

Hydrolysis of Chi, Formulation of Betel Leaf Extract-ChiNP, and Determination of MW

Hydrolysis of Chi and *NP Preparation*

The Chi hydrolysis was carried out following the procedure of Adhiatama et al., (2012). Briefly, 3 g of Chi was dissolved into HCL 0.8 N. Chi solution was heated while stirring at 70°C for 2 h and left at room temperature for 24 h. Chi solution was neutralized by adding NaOH. The obtained solutions was then filtered, washed using distilled water to neutral, and dried at room temperature.

The ChiNP preparation was done following procedure of Lifeng et al., (2004), by dissolving Chi in 1% acetic acid. A 0.2% Chi solution was prepared in a cup glass with 100 mL solution volume, then it was added with 20 mL of 0.2% sodium tripolyphosphate (NaTPP) while stirring using magnetic stirrer for 24 h. After that, the solution was added with 0.25 mL of 0.2% Tween 80 by stirring constantly. The addition of NaTPP and Tween 80 may enhance the mechanical properties of Chi that was fragile to form ionic crosslinks between Chi molecules (Nadia et al., 2014). The repeat procedure was prepared to get a ratio between Chi and betel leaf extract (v/v) composition of (1:1), (2:1), (3:1), (4:1), and (5:1) (Laili et al., 2014).

Determination of MW

Determination of MW was done by viscometric method using Ostwald viscometer. Chi was dissolved in a 0.1 M NaCl and 0.02 acetic acid solvent. Chi without hydrolysis dissolved to concentration of 0.01%, 0.02%, 0.04%, 0.05%, and 0.06%; while Chi concentration with hydrolysis, was prepared at concentration of 0.02%; 0.046%; 0.06%; 0.08%; and 0.1%. The flow time of each solution was measured using a capillary Ostwald viscometer with an inner capillary diameter of 0.9 mm. The measurement of Chi solution time was done using 10 replicates. Flow time was determined by calculating the time between initial liquid flows to the second flows index line. After that, the intrinsic viscosity value which was known through the curve was then converted to obtain the Chi MW. The relationship between the reduction viscosity and the concentration yields was determined using formula (Kasaai, 2007) as follows:

$$\eta\,\mathrm{red} = [\eta] + K'[\eta]2\,C \qquad \textbf{Eqn. 1}$$

The MW of the Chi molecule can be determined using Mark–Houwink formula (Hiemenz and Timothy, 2007):

$$[\eta] = K.Mv2 \qquad \textbf{Eqn. 2}$$

where, the values of K and α for Chi with 0.01 M HCl solvent at 30°C were 5.48×10^{-4} and 0.72, respectively.

Fourier Transforms Infrared (FTIR) Particle Size, Zeta Potential (ZP) and SEM Analysis

The FTIR spectra of Chi and extract-loaded ChiNP were assessed using FTIR Tensor 37 (Bruker Optic GmbH, Karlsruhe, Germany). The sample analysis was performed at wave numbers 4000-400 cm^{-1} (Pavia et al., 2013). Particle size and ZP analysis were performed using PSA (Malvern Instruments, UK). A total of 1 mL of a ChiNP with betel leaf extract suspension was put into a PSA devise, then measured with the software. The system was equipped with 4 Mw helium/neon lasers at a 633 nm wavelength, which measures particles with backscattering technology at an angle of 173°. Parameters analysed included means of particle diameter (ZAve) and polydispersity index (PI). The Zeta Potential (ZP) was measured by Laser Droppler Electrophoresis (LDE) method using the same equipment [4]. The ZP value indicates the stability of the NP, whereby the stable ChiNP have a ZP of ± 30 mV. Particle size was done in duplo analysis (mean ± standard deviation). The specimen sample was then analysed using SEM device (Zeiss, Germany) to analyse the size distribution of ChiNPs. Suspension sample of ChiNP was put onto the grid. It was dried for 24 h, and then coated with gold using an ion coating device (Q150R ES, Quorum).

Fruit Coating Assay using Betel Leaf Extract-ChiNP on Storage of Mango

The mango (*Mangifera indica* L) fruit cv. "Manalagi" was used in the fruit-coating assay using ChiNP-betel leaf extract. The homogenous size of mango fruit with similar maturity stage (ripen maturity) was briefly rinsed with tap water and then dried. After that the fruit was soaked in ChiNP solution for 30 min and let it dried. The fruit was inoculated and immersed with a *C. gloeosporioides* conidial suspension (10^7 conidia/mL) using a sterile needle, and then dried (Suryadi et al., 2017). The ChiNP-betel leaf extract of various formulas (i.e., 1: 1, 2: 1, 3: 1, 4: 1, and 5: 1; v/v) were applied to the mango fruit. The control treatment was carried out without immersion with the ChiNP- extract.

The treated and control fruits were placed onto plastic boxes (45 cm height x 40 cm length x 15 cm width) with relative humidy adjusted to >90%. It was wrapped with clear plastic to maintain the moisture away. The treatment was incubated under ambient temperature for 7 days. The experiment was arranged in randomized completely design (CRD) with 4 replications. Each treatments consist of 5 fruits. The experiment was repeated twice. Data obtained was anal;ysis by ANOVA at P(<0.5).

For storage shelf life trial, the mango fruit was washed with sterile water then dried. After that the fruit was soaked and dried in various formulas of ChiNP-betel leaf extract. The control treatment was performed without immersion with ChiNP. The fruit was stored on a sterile plastic box and wrapped with clear plastic to keep the moisture away. The experiment was arranged in CRD, and observations were taken by counting the longevity storage of mango fruit. Similar data obtained (mean of tretaments) was anaysis by ANOVA at P (<0.5).

Under moist and ambient temperature at the lab trial, most mango fruit will rotten for one week, hence the observation on disease development of artificial inoculated fruits was done up to 7 days. The effectiveness of fungal inhibitory by ChiNP was measured by calculating the percentage of disease inhibition (DI), with the following formula:

$$\text{DI}\ (\%) = \frac{\text{CT}}{\text{CC}} x\ 100\% \qquad \textbf{Eqn. 3}$$

where: CT= growth area of fungal colony treatment; CC= growth area of fungal colony control.

Analisis Data

All data were statistically analyzed using one way analysis of variance (ANOVA) and followed by Duncan Multiple Range Test (DMRT) to compare between treatments. Means were compare between treatments at the 5% level based on Sirichai v11.software.

RESULTS AND DISCUSSION

Phytochemical Analysis of Betel Leaf (*Piper betle* L)

The phenolic content of betel leaves was obtained by 71.18 $\pm$ 0.3%. The mechanism of phenolic compounds as antifungal acts as a toxin in destruction of protoplasm, penetrates and deposits the protein of the fungal cells walls. Large molecular phenolic compounds capable of inactivating the essential enzymes in the fungus cells. Phenols can cause damage to the fungus cells, protein denaturation, enzyme activates, and cell leakage (Ji et al., 2013).

The solvents organic solvents (ethanol, ethylacetate and n-hexane) are widely used in the extraction process, which can dissolve flavonoid compounds, saponins, agglomerates flavonoids, steroids and others. The ethanol extract of green betel leaves extract yielded of 3.1% of extracted symplicia and chemical identification showed that the positive extract contained phenolic and alkaloids. This is because the compound is more aromatic in ethyl acetate solvent. The compound in the extract that is thought to act as an antimicrobial activity is a

phenolic compound. In addition to the total phenolic analysis, there is also a qualitative analysis of alkaloids in the betel leaf ethanol extract. Based on alkaloids test, the betel leaf extract shows green colour if dropped by Dragendorf reagent, forming a brown precipitate if dropped by Wagner reagent, and forming a yellow precipitate if dropped by Mayer reagent (Fig. 1).

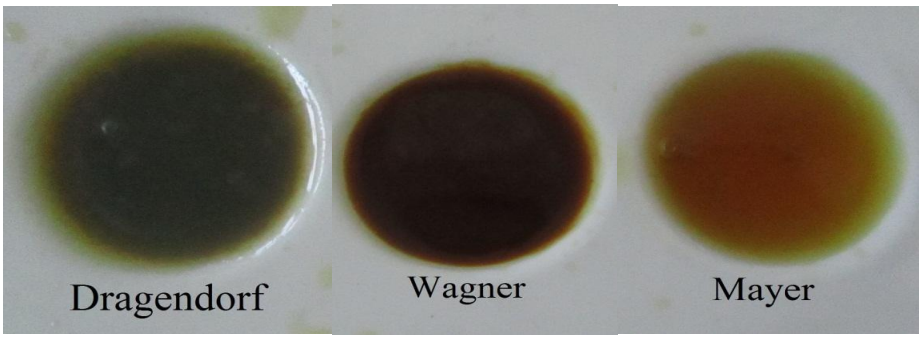


Fig. 1. Alkaloid assays of betel leaf extract (green Dragendorf; brown-Wagner; Yellow-Mayer)

The betel leaf extract contain active alkaloid as antifungal compounds that also can destroy the permeability of cytoplasmic membranes. The destruction of the cytoplasmic membrane, will affect membrane permeability and further disrupted, resulting in leaking of cell contents or the entry of unwanted substances into the cytoplasm that will result in cell death.

According to Rathee et al., (2006), the ethanol extracts of three betel leaf varieties ("Bangla", "Sweet", and "Mysore") possess the best antioxidant activity. In addition, the extract of the Bangla contains chevibetol (CHV), allylpyrocatechol (APC), and their respective glucosides. The results of analysis using pyrolysis GC-MS of phenolic compounds was presented in Table 1. The reaction produces the division of chemical bonds in the macromolecular structure and produces LMW with chemical composition which indicates the specific type of macromolecule. In this study the most phenolic compounds contained in betel leaf extract was found 1-hydroxy-4-metyl-benzotriazole with a concentration of 52%. This compound belong triazole group that effective as an antifungal agent. Triazole has been widely used as an antifungal, anticancer, antitumor, and analgesic drug. This compound can replace lanosterol from cytochrome P450 and block the biosynthesis of ergosterol and other essential components inside the fungus cell membrane (Zabolotnyi et al., 2016).

Table 1. Phenolic compounds analysis of leaf betel extract

Peak	Retention time (s)	Width area	Concentration (%)	Chemical compound
1	2.915	358841017	**32.78**	Methylsulfidtiole
2	17.024	2728455	0.25	Trans-Caryophyllene
3	17.333	25304039	2.31	Beta.-Selinene
4	17.609	569258308	**52.00**	1-Hydroxy-4-Methylbenzotriazole
5	17.818	105609570	**9.65**	2-Methoxy-5-Methyl-Benzaldehyde
6	18.726	5764853	0.53	4-Allyl-1,2-diacetoxybenzene
7	19.941	1893488	0.17	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS) Phytol
8	20.861	16528648	1.51	Hexadecanoic acid (CAS) Palmitic acid

9	21.900	4092340	0.37	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS) Phytol
10	22.575	4653769	0.43	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS) Phytol

Bold letter indicated three main component of phenolic from leaf betel extract based on GC-MS analysis

Chi Hydrolysis, Formulation and Particle Size Analysis

Chi LMW can be obtained by using chemical process by hydrolysis at acidic atmosphere. Chi hydrolysed using HCl acid decreases the drastic MW. The Chi MW before hydrolysed was amounted to 754.887 kDa; while the Chi MW molecule after hydrolysis was 245.85 kDa. This shows the degradation of HMW polymers into LMW polymers.

The mechanism of ChiNP formation based on electrostatic interaction between Chi amine groups (-NH2) and negative charges on polyanion such as tripolyphosphate (TPP). The complexity of these different inter-charge interactions results in Chi undergoing ionic gelation and precipitated to form particles. ChiNP is formed spontaneously due to mechanical stirring at room temperature (Mardliyati et al., 2012). The optimal condition of ChiNP making process is 0.2% Chi concentration with 0.1% NaTPP concentration and Chi volume ratio: TPP of (5:1) v/v (Mardliyati et al., 2012). Using this optimal condition, the size of ChiNP was obtained with high uniformity and good stability.

The parameter used for the uniformity of measurement was the PI value of the particle size distribution, whereas the parameter to determine the stability was the ZP value. Based on the results of the analysis shown in Table 2, Chi with hydrolysis treatment had a smaller mean particle size of 101 nm compared with that of ChiNP without hydrolysis with average particle size of 231.9 nm. Hydrolysis causes the polymer chain to break into a polymer or oligomers that have shorter chains so that the Chi MW is lower (Adhiatama et al., 2012).

Chi LMW that was cross-linked by NaTPP produced particles smaller than that of Chi HMW. The particle size of Chi without hydrolysis based on Hassani et al., (2015) study was reported about 320 nm. The particle size of ChiNP produced in this study is smaller than that of the Hassani et al., (2015) result. Hydrolysed ChiNP has a smaller particle size than ChiNP without hydrolysis. This is due to the fact that hydrolysis which involves the Chi LMW molecules resulting in smaller particles. ChiNP hydrolysis has a smaller particle size (101 ± 6.25 nm) than that of ChiNP without hydrolysis treatment (231.9 ± 2.37 nm). The addition betel leaf extract to ChiNP also has smaller particle size (193.4 ± 1.79 nm), meanwhile the ChiNP without hydrolysis-betel leaf extract has a particle size of 431.1 ± 4.32 nm (Table 2). This might be due to the addition of extract compound which which make a larger size to the ChiNP. The extracts may attched to ChiNP or are inside of ChiNP that causing larger particle size.

Table 2. Results of particle size and ZP analysis of ChiNP and ChiNP-betel leaf extract

Treatment	ZAve (nm)	PI	ZP (mV)
a.ChiNP without hydrolysis	231.9 ± 2.37	0.518 ± 0.09	58.6 ± 4.72
b.ChiNP without hydrolysis + Extract	431.1 ± 4.32	0.545 ± 0.12	41.1 ± 4.07
c.ChiNP with hydrolysis	101 ± 6.25	0.285 ± 0.10	56.0 ± 6.4
d.ChiNP with hydrolysis + Extract	193.4 ± 1.79	0.424 ± 0.08	26.4 ± 12.5

ZAve= particle diameter size; PI = Polydispersity Index; ZP= Zeta Potential

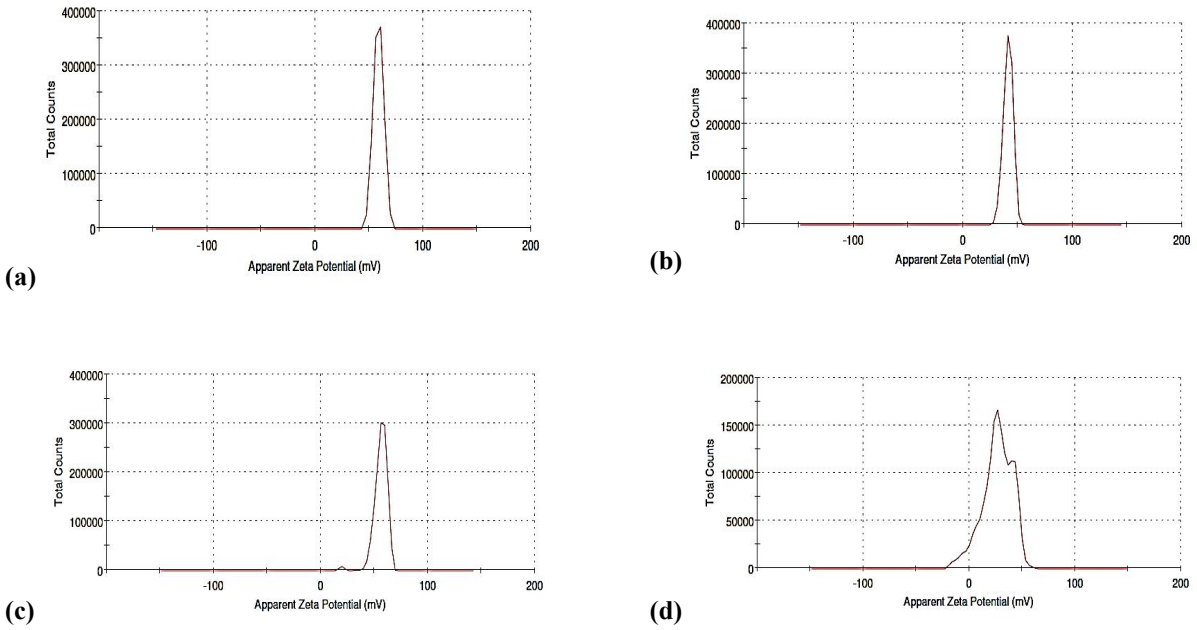


Fig 2. The Zeta Potential distribution curve based on PSA: (a) ChiNP without hydrolysis, (b) ChiNP-betel leaf extracts without hydrolysis, (c) ChiNP with hydrolysis, and (d) ChiNP-betel leaf extracts with hydrolysis

ChiNP without hydrolysis treatment has a greater PI (0.518) than ChiNP with hydrolysis treatment (PI of 0.285). The lower the PI treatment has a higher uniformity of size than ChiNP without hydrolysis. ChiNP without hydrolysis has a fairly high heterogeneity because it has a PI of more than 0.5. The uniformity of particle size is expressed in the PI value. Index value of PI was range of 0 to 1. A PI value near 0 indicates homogenous size dispersion, whilst the PI value of greater than 0.5 indicates high heterogeneity (Avadi et al., 2010). The ChiNP hydrolysis-extract has a PI of 0.424 $\pm$ 0.08 which shows the ChiNP-extract particles are quite uniform; whilst the ChiNP without hydrolysis-extract had also a PI of 0.545 $\pm$ 0.12 indicating the uniform particles (Table. 2).

The ZP of ChiNP without hydrolysis was 58.6 $\pm$ 4.72 mV, whilst the ZP of ChiNP with hydrolysis was 26.4 $\pm$ 12.5 mV (Fig. 2). This shows that particles without hydrolysis are more stable than ChiNP with hydrolysis. NPs with a ZP value of $\pm$ 30 Mv have good suspension stability as surface charge and can prevent the aggregation (Mohanraj and Chen, 2005). The higher the ZP value, the higher the stability of ChiNP the lower the chances of ChiNP being agglomerated. ChiNP is less stable to agglomerate, but the agglomeration formed is small and still nano-sized because the ZP value is close to 30mV. The ZP value shows the charge of the particles in the solution. The ChiNP have positive ZP, this might due to the presence of amine groups in chitosan and OH groups in protonated TPP by H + from acetic acid. ChiNP hydrolysis-extract has a ZP value of 56 $\pm$ 6.4 mV, whereas ChiNP without hydrolysis-extract has a ZP value of more than 30 mV (41.1 $\pm$ 4.07 mV) (Fig. 2). This condition still implied that the ChiNP-extract has possible instability between the lower particles, and the particle tends to agglomeration. Acid catalysed the hydrolysis reaction resulting in breaking bonds between chitosan molecules.

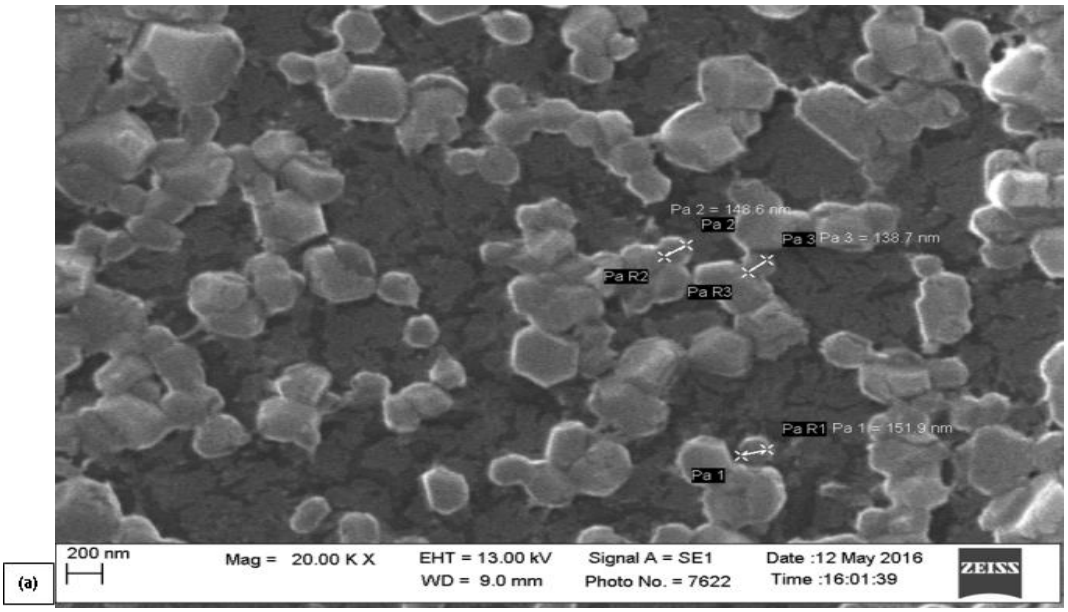
Zeta potential (ZP) values of ChiNP + extract (without hydrolysis) was less positive compared to ChiNP (without hydrolysis), whereas ChiNP + extract (with hydrolysis) was more positive compared to ChiNP without extract. The addition of the extract to ChiNP may causes the ZP value to increase. This might be due to the N group present in the protonated extract by H^+ of acetic acid, increasing the positive charge of the ChiNP.

The ChiNP-extract presented in this study didn't seem have high uniformity and may result in weak physical stability (Fig. 3). The size of NP are highly dispersed, since there were particles with more than 1 micron present in the samples which indicating non-uniform size, although it occurs in less intensity. Hence, to further validate the result, the percent encapsulation study to the efficiency of the extract in ChiNP should be determined to ensure the loading efficiency of the ChiNP.

Scanning Electron Microscopy (SEM) Analysis

Based on morphological analysis, the form of ChiNP was rounded and wrinkled (Fig. 3). The result was in accordance with others study that ChiNP were found in rounded and wrinkled morphology (Jafarinejad et al., 2012; Xu et al., 2007).

The distribution of ChiNP size between SEM and PSA was not significantly different. The distribution of ChiNP size based on PSA result was 193.4 ± 1.79 nm, while the mean ChiNP size distribution based on SEM results, was 146.4 ± 6.86 nm (Fig. 3). The addition of betel leaf extract to ChiNP causes the size of ChiNP to enlarge. This was presumably due to the inclusion of the extract into the ChiNP that has been cross-linked by TPP or the presence of a layer of extract on the surface of ChiNP. Based on SEM analysis the mean size of ChiNP was 305.56 ± 88.2 nm which less differ compared to the result of PSA (431.1 ± 4.32).



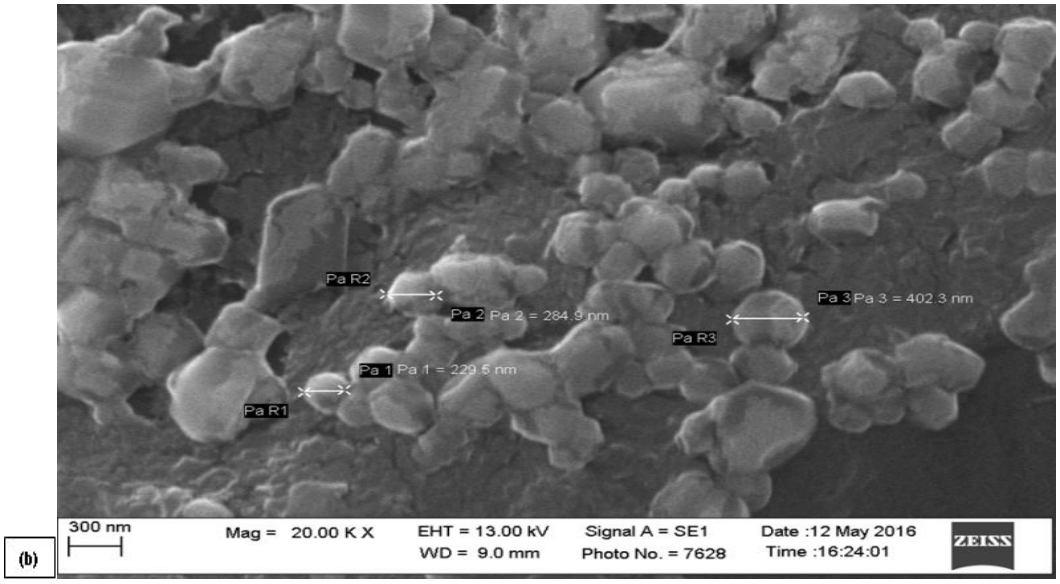


Fig. 3. SEM morphology analysis to hydrolysed ChiNP (a), and morphology analysis to hydrolysed ChiNP-betel leaf extract (b).PaR1, 2, 3= showing size distribution of SEM micrograph

FTIR Analysis of Functional Groups

IR spectra showed that the absorption intensity at wave numbers 3396 cm^{-1}, 3299 cm-1, 3368 cm^{-1}, and 3343.67 cm^{-1} were overlapping N-H and O-H groups (Fig. 4). The wave numbers 3650-3200 cm-1 and N-H uptake occurs at wave numbers 3500-3300 cm^{-1} (Pavia et al., 2013). The absorption spectra of O-H and N-H groups from Chi overlap in the range of 3300 cm^{-1}. The overlap of N-H and O-H causes a small N-H uptake. The absorption peak shifts toward smaller wave numbers due to the H bonds that occur between water and N-H.

At the wave number 3000-2850 cm^{-1} there is absorption of the alkane functional group. At the peak of 2931 cm^{-1} and 2885 cm^{-1} there is an uptake showing the presence of C-H stretching. The absorption peaks at wave numbers 2135 cm^{-1}, 2136 cm^{-1}, 2133 cm-1, and 2133 cm^{-1} indicate the presence of an O-H group. At the wave number 1644 cm^{-1} shows the absorption of C = O stretching. At the wave number 1350-1000 cm^{-1} there is absorption of the amine group, i.e. the C-N bond. In ChiNP after hydrolysis-extract there is no absorption at wavelength of 1088 cm^{-1}. This is due to the presence of hydrolysis treatment and the addition of extracts that cause the C-O bond to interact with other compounds. At the wave number 1153 cm^{-1} there is an absorption which shows the presence of the P = O group of TPP. In the ChiNP there are absorptions in numbers below 1000-650 cm^{-1} indicating out-plane (= CH) aromatic rings originating from extract (Zabolotnyi et al., 2016). The addition of the extract also causes a shift in the absorption intensity and wave number on some functional groups.

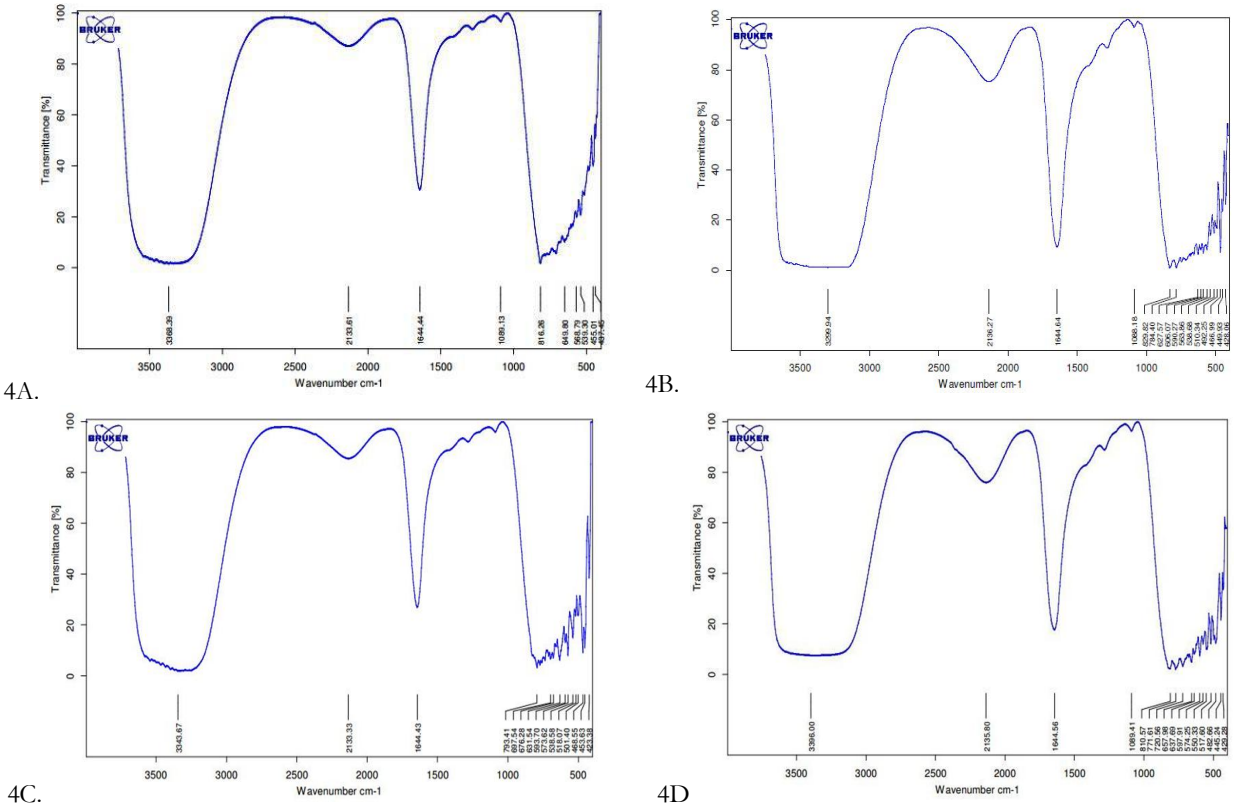


Fig. 4. FTIR analysis to ChiNP-extract. 4A=ChiNP without hydrolysis, 4B=ChiNP with hydrolysis; 4C= ChiNP-extract without hydrolysis, 4D=ChiNP-extract with hydrolysis.

Fruit coating assay using ChiNP to *C. gloeosporioides* on artificially inoculated fruit

A symptom of this disease on fruits is shown by typical spots in the form of rounded concave, with light brown or black colour at the edges. The fungus produces concentrated conidia of spotted, with light orange to pink colour (Agrios, 2005; Tasiwal, 2008). Chi LMW is very effective as antifungal agent compared with Chi HMW (Rejane et al., 2009). There was a significant difference between treatments. The degree inhibition of fungal growth using ChiNP with hydrolysis was higher than ChiNP without hydrolysis (Fig. 5a,5b). This shows that hydrolysis treatment increases Chi antifungal activity. The MW affects the solubility of Chi in acetic acid. The lower the Chi MW, the higher the solubility of Chi. The hydrolysis treatment yields smaller ChiNP size. The smaller the ChiNP that is formed, the more ChiNP antifungal activity increases. The best composition ratio of ChiNP and extract in inhibiting the growth of the fungus *C. gloeosporioides*, was at ratio (3: 1 v/v). This was in accordance with the study of Nagaonkar et al., (2014) that states the best formula in the synthesis of ChiNP-extracts under acidic pH was obtained in the mass ratio Chi: extract of (3:1, v/v). Chi can inhibit an average of 80% against the growth of fungi (Gonzalez et al., 2008). Based on this work, the formula with the mass ratio of ChiNP: extract (3:1; v/v) as prepared without hydrolysis or with hydrolysis could inhibit the growth of the fungus with degree of disease inhibition by 85.88% and 98.82%, respectively. This presumably due to the extract that can increase the Chi antifungal activity. If the concentration of Chi is too high and too low, it will reduce the degree inhibitory of the formula to the growth of fungi.

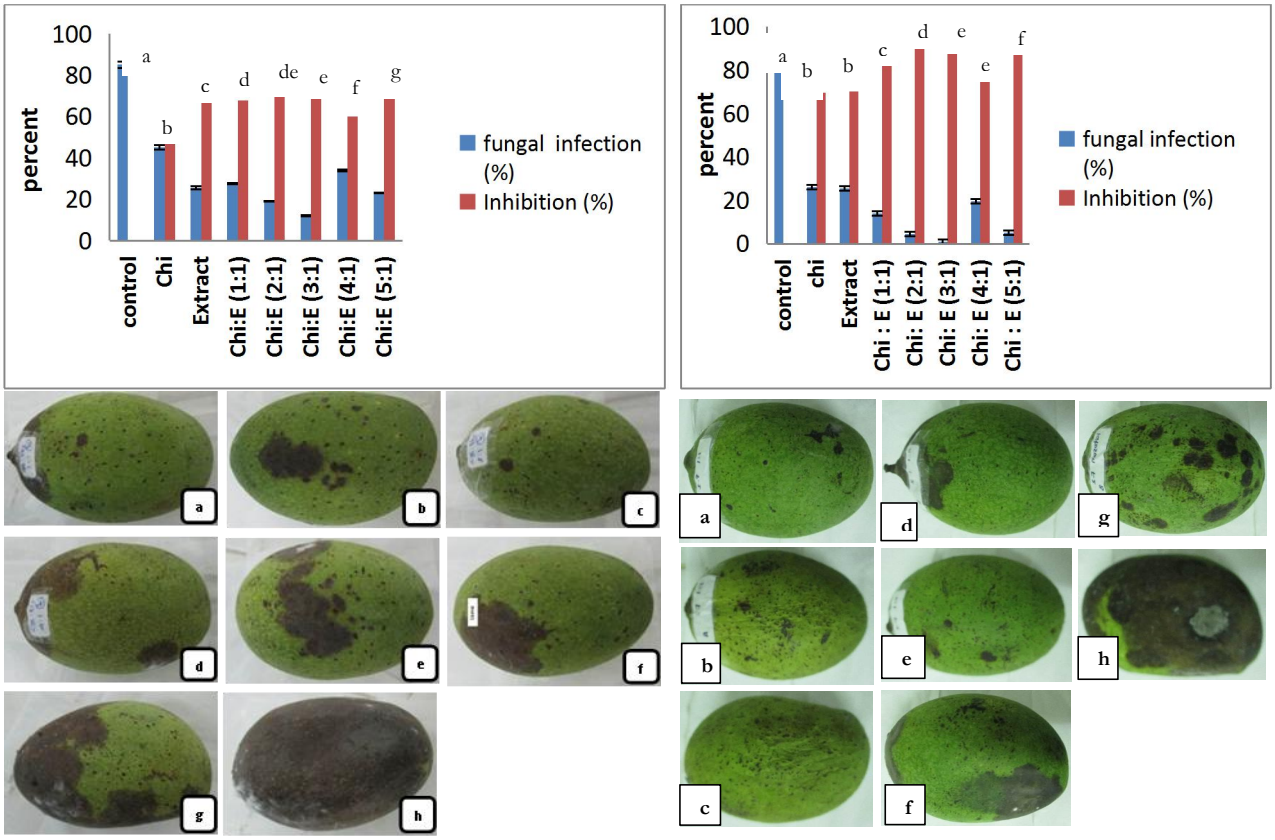


Fig. 5a. Effect of unhydrolyzed ChiNP-betel leaf extract treatment to mangoes anthracnose. v/v. (a) ChiNP: extract (1: 1), (b) ChiNP: extract (2: 1), (c) ChiNP: extract (3: 1) (d) ChiNP: extract (4: 1), (e) ChiNP: extract (5: 1), (f) betel leaf extract (0.2%), (g) ChiNP (0.2%), and (h) control.

Fig. 5b. Effect of hydrolysed ChiNP-betel leaf extract to mangoes anthracnose.v/v. (a) ChiNP: extract (1: 1), (b) ChiNP: extract (2: 1), (c) ChiNP: extract (3: 1), (d) ChiNP: extract (4: 1), (e) ChiNP: extract (5: 1), (f) betel leaf extract (0.2%), (g) ChiNP (0.2%), and (h) control.

* Means followed by the same letter are not significantly different as determined by DMRT test (P≤0.05)

The infection process of *C. gloesporioides* begins with the attachment of spores to the surface of the skin of the fruit, then the spores germinate and form appressoria and hyphae to infect and remain dormant in the cell layer of the skin in latent conditions. Activation of conidia and dormant appressoria is induced by ethylene produced by the fruit during the physiological process of maturation. The older and ripen fruit, the dormant fungus will gradually develop so that it becomes active again and shows symptoms and the fruit becomes rotten. Fungal growth development is faster with over ripen fruit after harvesting. Chi coating on mangoes can prolong the life of fruits because it suppresses the process of respiration, transmission and growth of spoilage microbes, reducing weight loss and moisture content. Low of respiration can result in the breakdown of starch including sugar runs slowly so that the lower the respiration of the fruit the slower process of fruit maturity (Pumchai et al., (2005).

Based on the previous study, ChiNP alone can add a long storage of mango fruit for 4 days (Gonzalez et al.., 2008). In this study, mango that applied with ChiNP-betel leaf extract have not yet decay and still feasible to be consumed after 6 days, while the mango fruit without treatment has been decomposed and is not feasible for consumption. This shows ChiNP-betel leaf extract can inhibit the ethylene content of fruit, thus inhibiting the process of ripening fruit that causes the fruit does not quickly rot and the storage lifetime of fruit increases. Chi is able to inhibit the respiration rate and ethylene content of fruit so that it can prolong the fruit shelf life.

This study has shown the potential of using ChiNP in reducing the important storage disease of mango fruit. In addition, betel leaf extract are considered as excellent antifungal agent. The application of coating assay by

this mixtures may extends the storage life of fruit since they coverage fruit by physical barriers to delay the side effects of respiration. Therefore, this study clearly indicate that the ChiNP-betel leaf extract have a tremendous effect on growth of *C. gloeosporioides* that leads to reduction in disease severity. The reduction might occurs because positive ions are released from the surface of the ChiNP-betel leaf extract by oxidation processes and interact with biological molecules upon entering the fruits during physiological processes. However the actual mechanism of disease reduction is inadequately understood, hence more study are needed in this direction.

Future postharvest uses of ChiNP-betel leaf extract might be facilitated by development of alternative formulations. Less is known about the volatiles produced by the extract in the biocontrol of *C. gloeosporioides*. For the effective protection, the formula of ChiNP must act at the earliest stage of fruit infection (preventive) to delay disease onset of post-harvest pathogens (Suryadi et al*.,* 2017). Moreover, to validate valuable feature to control post-harvest pathogens, further study on the potential of fruit coating assay on mangoes fruits in the field test (*in situ*) needs to be investigated.

CONCLUSION

The betel leaf extract contains alkaloids and phenols. The quantity of phenolic compounds contained in betel leaves was 71.18 $\pm$ 0.3%, with the most phenolic compounds obtained was 1-hydroxy-4-methylbenzotriazole. Chi hydrolysis reduced the Chi MW from 754.89 kDa to 245.85 kDa. Based upon FTIR analysis, hydrolysis treatment and addition of extract affected the existence of Chi functional group, and wave number. The addition of the extract resulted in the absorption of aromatic groups at 1000-650 cm^{-1} wave numbers. The morphological form of ChiNP was spherical shape. The size of NPs was ranged from 101 $\pm$ 6.25 nm to 431.1 $\pm$ 4.32 nm. The sizes of ChiNP without hydrolysis were larger than ChiNP with hydrolysis. The ChiNP and betel leaf extract composition showed better anthracnose disease suppression at ratio (3:1; v/v). The non-hydrolysed ChiNP: betel leaf extract composition with ratio (3:1; v/v) showed degree inhibition of *C. gloeosporioides* growth of 85.88%; while the inhibition of hydrolysed ChiNP-betel leaf extract was higher (98.82%). The ChiNP-betel leaf extract may extend the shelf life of mango fruit for up to 6 days.

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