

Preparation and potential of nanoparticles containing curcuminoids to control fungal diseases in tropical fruits

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Abstract. *Colletotrichum* species cause anthracnose in various tropical crops in both cultivation and postharvest periods. The current synthetic fungicides to treat anthracnose sometime show toxic effects for farmers, residues on foods, and environmental impacts. In this work, turmeric extract containing curcuminoids was used as an active ingredient and formulated into a nanoemulsion to control anthracnose in red pepper and tomato fruits. The nanoparticles containing turmeric extract (25 %) were successfully prepared by encapsulation using Tween-80 and PEG 400 as emulsifiers. Out of the three main curcuminoids in the turmeric extract, BDMC was determined as the most abundant constituent via HPLC analysis. Turmeric nanoparticles showed an average particle size of 203 nm, polydispersity index (PI) of 0.322, and zeta potential of -92.7 mV. In the *in vitro* antifungal bioassay, this nanoformulation significantly inhibited the mycelial growth of *Colletotrichum gloeosporioides* and *Colletotrichum orbiculare* in a dose-dependent manner. Red pepper anthracnose was also consistently suppressed by turmeric nanoparticles but the tomato anthracnose was resistant to this formulation at 10 mg/mL *in vivo*. The study results proved the effectiveness of turmeric nanoparticles to control red pepper anthracnose in postharvest preservation and suggested developing the innovative nanoformulation as a green fungicide.

Keywords: anthracnose, *Colletotrichum gloeosporioides*, *Colletotrichum orbiculare*, curcuminoids, turmeric extract.

Classification numbers: 1.3.3, 1.4.5.

1. INTRODUCTION

Filamentous fungus *Colletotrichum* sp. causes a serious plant disease named anthracnose in various tropical crops and fruits in both cultivation and postharvest periods. The disease has been treated currently with synthetic chemical fungicides that harm human health and cause environmental pollution [1, 2]. To replace the harmful fungicides, botanical materials and nanomaterials have been investigated and developed into green and innovative fungicides to control plant fungal diseases including anthracnose [3]. Nanopesticides have been considered as a new approach at controlling plant diseases and pest insects during cultivation and postharvest [3 - 6].

Curcuminoids such as curcumin (CUR), bis(4-hydroxy-3-methoxyphenyl)-1,6-diene-3,5-dione, together with dimethoxy curcumin (DMC) and bisdemethoxycurcumin (BDMC) are the main yellow constituents in the turmeric rhizome of *Curcuma longa*. The curcuminoids in turmeric rhizomes are safe and can be used in high doses due to they possess various pharmacological properties such as anticancer, antioxidant, and antimicrobial effects [7]. Curcuminoids and other constituents of turmeric rhizome are antimicrobial compounds against plant pathogenic microorganisms [7, 8]. However, there are no turmeric nanoformulations that have been applied in the protection of red pepper and tomato fruits from infection by anthracnoses.

Over the last five years, more and more studies have reported that turmeric extracts helped control plant diseases and preserve vegetables. The antifungal activity of five species of turmeric (*Curcuma* spp.) against *Fusarium solani* has also been discussed, where the turmeric species *Curcuma longa* was shown to have a high content of curcuminoids and showed excellent antifungal activity [9]. The methanol extracts of turmeric (*Curcuma longa*) inhibited seed germination and seedling growth [10]. *Curcuma longa* extracts recorded a maximum inhibition of 89 % against *Alternaria solani* [11]. The chitosan-based coating containing turmeric and green tea extracts was developed and evaluated for the preservation of postharvest strawberries [12]. Antimicrobial effect of rhizome and turmeric extract in controlling postharvest anthracnose of dragon fruit [13]. Curcumin nanoparticles including nanoemulsion have been investigated and applied in various fields such as the pharmaceutical and food industries [7, 8, 14, 17, 18]. In the food industry, Sari *et al.* [18] suggested that curcumin from the nanoemulsion is supposed to increase bioavailability. The authors made curcumin nanoemulsions by ultrasonication using whey protein concentrate-70 and Tween-80 as emulsifiers to yield nanoparticles with average sizes of 141.6 nm [18]. Curcumin-loaded zein/ carboxymethyl dextrin nanoparticles (220 nm) showed an increase in antioxidant, thermal, and photochemical stability of Cur as well as a postponed release of Cur in simulated gastrointestinal fluids. In particular, our recent study published that curcumin-removed turmeric oleoresin nanoemulsion as a fungicide for the control of anthracnose in litchi [14].

In this study, the turmeric extract was prepared by solvent extraction of the turmeric rhizome with ethyl acetate. A nanoemulsion formulation was prepared by encapsulating the turmeric extract and its particle size, zeta potential, and PI were determined. Moreover, the antifungal efficacies of this nanoformulation were evaluated against *Colletotrichum* sp. causing anthracnose of tropical fruits *in vivo* and *in vitro*. Our work demonstrated for the first time the preparation of an innovative nanoemulsion containing turmeric extract and suggested using the formulation to effectively control anthracnose of tropical fruits during postharvest preservation.

2. MATERIALS AND METHODS

2.1. Materials

The dried turmeric rhizomes were purchased in Hung Yen province in 2021. Ethyl acetate (99 %, Singapore) was provided by Vietchem Co. (Viet Nam). Curcumin (95 %) was provided by Vietnam Institute of Industrial Chemistry. Polysorbate 80 (Tween 80®) was purchased from Anhui BBKA Pharmaceutical Co. (China). Ethanol 96 %, propylene glycol (PG, 99.5 %, Sigma-Aldrich), and PEG 400 (Kanto, Japan) were used in the experiment of nanoparticle preparation.

2.2. Preparation and characterization of the nanoparticles containing curcuminoids

The dried powders of turmeric rhizomes (*Curcuma longa*) (2.5 kg) were reflux extracted with ethyl acetate twice to obtain ethyl acetate extract. The extract was evaporated in a rotary evaporator under reduced pressure to a volume of 30% of the beginning volume. The extract was allowed to cool at room temperature for 3 days and the upper layer was collected from the turmeric precipitation. The solvent was evaporated from the upper layer to yield a turmeric extract (186 g) that contains the un-precipitated curcuminoids and oils. The turmeric extract was used as an active ingredient for turmeric nanoemulsion preparation. The presence of curcuminoid constituents in this turmeric extract was analyzed based on HPLC method.

The preparation of turmeric nanoformulation was modified based on the previously published protocols [7, 8, 14]. In brief, the turmeric extract (25 g) was dissolved in ethanol (25 mL), mixed with 10 mL water, 1 g of PEG 400, 3 g of Tween 80 and 2 mL of propylene glycol and stirred at 450 rpm for 30 min to form an organic phase. The aqueous phase was composed of Tween 80 (5 g), water (25 mL) and propylene glycol (4 mL). The organic phase was dropped at the rate of one drop per 10 s to the aqueous phase during ultrasonication with a power of 700 W using an ultrasonic bath and stirring at 450 rpm and the stirring state was kept for 30 min after the organic phase was completely added. The particle diameter of turmeric nanoemulsion was determined by dynamic light scattering (DLS) (Zetasizer Ver. 6.20, Malvern Instruments, UK). Transmission electron microscope (TEM, JEOL JEM-2100, Japan) was applied to study the nanoparticle's actual size.

2.3. HPLC analysis of curcuminoids in turmeric extract

The HPLC analysis of turmeric extract (1.2 mg/mL) was carried out with Agilent 1260 HPLC system and used an Agilent Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of acetonitrile (A) and H₂O containing 0.1 % formic acid (B). The gradient elution was performed with 42 - 47 % A in B running from 0 - 40 min, at a flow rate of 0.8 mL/min and column temperature of 40 °C. The detector wavelength was set at 420 nm. Curcumin 95 % (0.6 mg/mL) was used as an authentic compound to identify curcumin, DMC and BDMC in the sample.

2.4. *In vitro* bioassay of turmeric nanoemulsion against *Colletotrichum species*

To achieve the desired concentrations, the tested extracts were dissolved in DMSO and then added to a sterilized PDA medium in Petri dishes with diameters of 6 cm. The DMSO concentration in PDA was below 2 % and the medium was allowed to cool at room temperature. *Colletotrichum gloeosporioides* and *C. orbiculare* were stored in our laboratory and used

consistently in various experiments [15, 16]. The antifungal treatments were processed at concentrations ranging from 625 to 10000 µg/mL. A mycelial plug was placed in the center of each Petri dish as an inoculant before being incubated at 20 - 25 °C for 2 - 7 days. As negative controls, Petri dishes that had been exposed to 2 % DMSO were used. The inhibition efficacy of the test materials calculated based on the following formula (1) was used to determine the percentage inhibition of mycelial growth (%) for the tested samples.

$$\% \text{ inhibition} = 100 \times \frac{A - B}{A - 4} \quad (\text{Formula 1})$$

where A is the diameter of the fungus's mycelial growth in the dishes used for the negative control, B is the diameter of the fungus's growth in the dishes used for the treatment, and 4 is the diameter of the PDA plug used to hold the fungal inoculum (mm).

2.5. *In vivo* bioassay for controlling anthracnose on red pepper and tomato fruits

Fungal strains *Colletotrichum* sp. a causal agent of anthracnose were isolated from the infected tissues of red pepper and tomato and the fungal collection was stored at Faculty of Agriculture, Can Tho University. *Colletotrichum* sp. was cultured in PDA agar, in the dark, at 25 - 30 °C. After 7 days, the fungal spores were collected and determined in the range of 10⁴ - 10⁵ CFU/mL.

Tomatoes (or red peppers) in a uniform size were selected to test in the *in vivo* bioassay. The fruits are disinfected with water, ethanol 70°, dried, then soaked with 1 % NaOCl solution for 5 minutes, finally the fruits were washed with sterilized deionized water, dried and stored in a plastic box (or clean plastic bag).

A small circular wound approximately 3 - 5 mm (2 - 3 mm on the pepper) in diameter was inflicted on the surface of each fruit with a sterilized col tip. A 5 µL of *Colletotrichum* spores was piped into each wound, then a 5 mm diameter round (PDA-free) agar plug was placed over the wound. Inoculated fruits were stored in plastic bags at room temperature for 3 days. After 3 days, diseased fruits with diameters of 6 - 7 mm (tomato), and 4 - 5 mm (red pepper) were selected and treated with the nanoemulsion. Each treatment was replicated 5 times and the experiment was repeated twice. The test nanoformulation was diluted with sterilized water to get the concentrations from 1.25 to 10 mg/mL. The test solutions were sprayed evenly on the site of the disease infection and the whole fruit. The treated fruits were dried naturally and stored in a plastic bag at room temperature. The lesion image was taken and the mean wound diameter was determined from the length of the two diagonals of the wound after 7 - 10 days. Distilled water was used for the negative control and a fungicide Score 250EC (at 100 µg/mL) was used for the positive control.

2.6. Statistical analysis

All bioassays were conducted in at least triplicate. The data were analyzed using variance analysis (ANOVA) and presented as means ± standard deviation (SD). *In vivo* lesion inhibitions of the test materials against *Colletotrichum* sp. were calculated and presented using GraphPad Prism 8 software.

3. RESULTS AND DISCUSSION

3.1. Characterization of the nanoemulsion containing curcuminoids

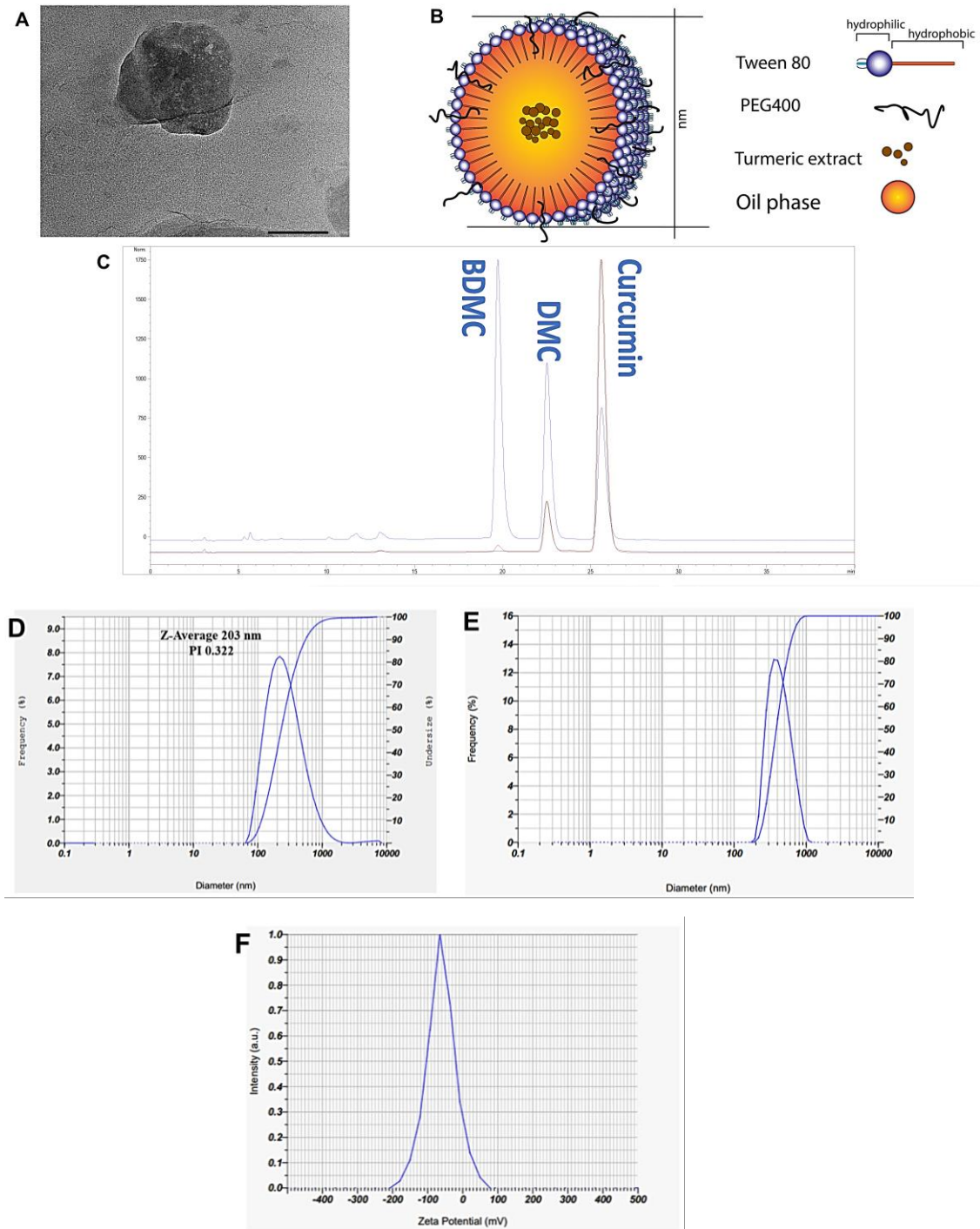


Figure 1. The main constituents and morphology characteristics of nanoparticles containing curcuminoids. **A.** TEM image of nanoparticles (the scale bar is at 100 nm). **B.** Diagram of the nanoemulsion. **C.** the HPLC chromatogram of turmeric extract containing curcumin, DMC and BDMC; blue line: turmeric extract; and red line: curcumin 95%. **D.** Particle size and distribution of nanoemulsions measured by DLS. The particle size (**E**) and zeta potential over (**F**) of nanoemulsion measured after 7 days.

The turmeric extract fabricated at the rate of 25 % (see section 2.1) using Tween 80 and PEG 400 as emulsifiers gave the nanodroplets with an average size of 203 nm (Figure 1A, B, and D), zeta potential of -92.7 mV and PI of 0.322. The nanoparticles containing curcuminoids displayed stability after 7 days (average size of 327.7 nm and zeta potential of -61.7 mV) (Figure 1E, F). The turmeric extract obtained in our study was found to have three main curcuminoids curcumin, DMC and BDMC, in which the content of BDMC was determined to be the highest compared with the others (Figure 1C). The study on the antifungal activity of *C. longa* grown in Thailand reported that ethanol extracts containing curcumin (11.6 %), DMC (10.32 %) and BDMC (10.77 %) exhibited antifungal activity against 29 strains of dermatophytes using the agar disc diffusion method [19]. In a recent study by Choi et al., the methanol extract of turmeric rhizomes (*C. longa*) effectively controlled anthracnose (*C. coccodes*) in red pepper [20]. The ingredients in the extract included curcumin, DMC, and BDMC, at which BDMC strongly inhibited spore germination of *C. coccodes* and was effective in the greenhouse test. This showed a significant difference from curcumin-removed turmeric oleoresin (CRTO), of which curcumin was the most abundant constituent in a previous study by Bui *et al.* [14]. Moreover, CRTO was prepared at a ratio of about 40 % (with the highest content of curcumin) in CRTO-nanoemulsion [14], while turmeric extract was 25 % in nanoparticles obtained by the present study.

3.2. *In vitro* inhibitivity of turmeric nanoparticles against *Colletotrichum* species

The *in vitro* bioassay of turmeric nanoparticles in nanoemulsion type was performed by poisoned-food technique to observe the mycelial growth inhibition of *Colletotrichum* species. As shown in Table 1, nanoparticles significantly inhibited the mycelial growth of both *C. gloeosporioides* and *C. orbiculare* in the concentration ranging from 625 to 10000 µg/mL (equivalent to the range of turmeric extract content from 156.25 to 2500 µg/mL). The nanoemulsion did mycelial growth inhibitions from 24.8 to 70.3 % over a range of test concentrations and displayed *in vitro* antifungal activity in a dose-dependent manner (Table 1). At the highest concentration test, turmeric nanoparticles inhibited an inhibition by 70 % (Table 1 and Figure 2). According to the *in vitro* results, the formulation was selected to test by *in vivo* bioassay on the postharvested red peppers and tomatoes. The test concentrations were selected from 1.25 to 10 mg/mL. The concentration of 625 µg/mL was not included in the *in vivo* bioassay due to its weak inhibition observed (Table 1 and Figure 2).

Table 1. *In vitro* antifungal activity of turmeric nanoparticles containing curcuminoids against *Colletotrichum gloeosporioides* and *Colletotrichum orbiculare*.

Concentration (µg/mL)		Mycelial growth inhibition (%)	
Turmeric extract	Nanoformulation	CG	CO
156.25	625	27.7 ± 1.1	24.8 ± 1.2
312.5	1250	36.4 ± 1.9	34.5 ± 1.9
625	2500	43.7 ± 2.4	43.9 ± 0.7
1250	5000	51.4 ± 2.3	66.1 ± 0.4
2500	10000	70.3 ± 1.4	70.0 ± 1.0

The mycelial growth was observed 4 days after treatment and the fungi were incubated in an incubator at 25 °C. CG: *Colletotrichum gloeosporioides* and CO: *Colletotrichum orbiculare*.

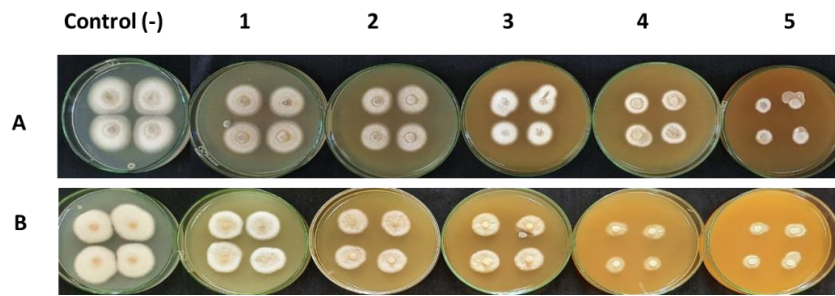


Figure 2. *In vitro* inhibition of turmeric nanoparticles containing curcuminoids against *Colletotrichum gloeosporioides* and *Colletotrichum orbiculare*. The fungi were incubated in an incubator at 25 °C for 4 days. A: *C. gloeosporioides* and B: *C. orbiculare*. Lanes 1 = 156.25 µg/mL; 2 = 312.5 µg/mL; 3 = 625 µg/mL; 4 = 1250 µg/mL; 5 = 2500 µg/mL.

3.3. *In vivo* efficacy of turmeric nanoparticles against anthracnose in red pepper and tomato

As the results of anthracnose suppression in red pepper bioassay shown in Figure 3, the disease lesion diameters on the anthracnose-infected fruits were reduced when treated with turmeric nanoparticles in the range of test concentrations. In the observation at 3 days after treatment, the reduction of lesion diameters was dependent on the increase of test concentrations (Figure 3B).

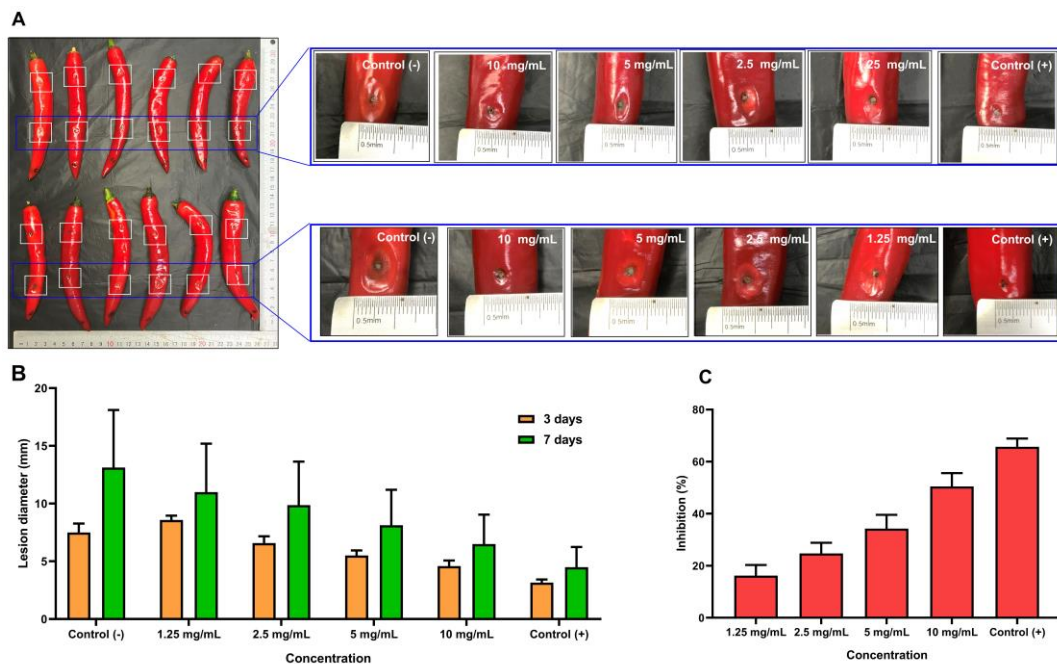


Figure 3. *In vivo* suppression of turmeric nanoparticles against anthracnose caused by *Colletotrichum* sp. on the fruits of red pepper. A: The disease lesion on red pepper fruits 7 days after treatment. B: The disease lesion diameters were measured after 3 and 7 days. C: Lesion inhibitions (%) were calculated by relative comparison with negative control 7 days after treatment. Control (-): negative control using water alone. Control (+): positive control using Score 250EC.

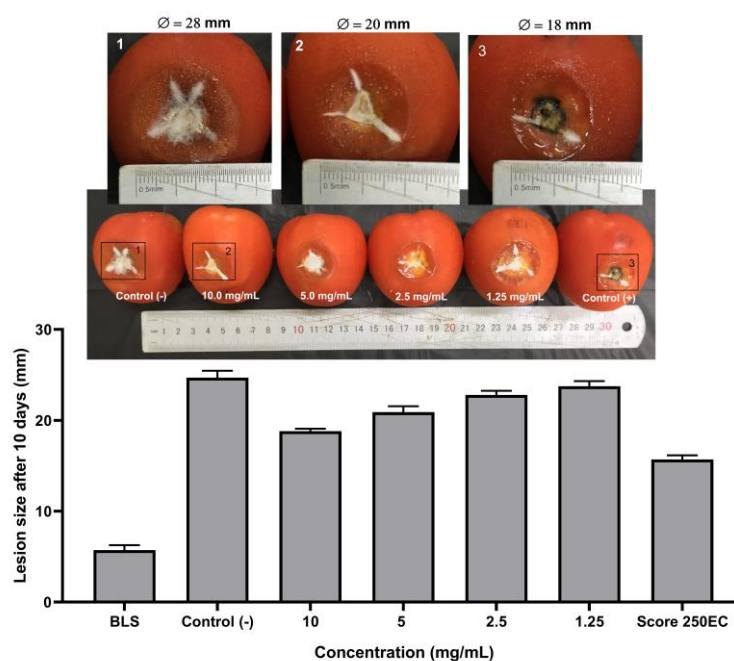


Figure 4. *In vivo* suppression of turmeric nanoparticles against anthracnose caused by *Colletotrichum* spp on tomato fruits. The diameters of the disease lesion were recorded 10 days after treatment. BLS: baseline lesion size of treated tomato fruits. Control (-): negative control using water alone. Control (+): positive control using Score 250EC.

Similarly, at 7 days after treatment, the lesion diameters and control efficacy were also shown in a dose-dependent manner (Figures 3B and C). Interestingly, at 10 mg/mL, the control efficacy of turmeric nanoparticles was 50.5 % at 7 days after treatment equivalent to 76.9 % of the positive control which used Score 250 EC and caused suppression of 65.7 % for red pepper anthracnose (Figure 3C). The results demonstrated that the turmeric nanoparticles possess effective protection for red pepper from the infection of *Colletotrichum* sp. during 10 days postharvest.

In the *in vivo* bioassay on tomato fruits, the treatments were also tested at the same concentrations for red pepper. However, turmeric nanoparticles exhibited a weak suppression for anthracnose in tomatoes *in vivo*. At 10 mg/mL, this formulation caused a suppression of about 20 % against tomato anthracnose (Figure 4). At the concentrations of 1.25 and 2.5 mg/mL, the control efficacies of nanoemulsion were very low; the lesion diameters in the samples treated with nanoemulsion were insignificantly different from those of the negative control (Figure 4). The positive control also showed weak effectiveness against *Colletotrichum* sp. when treated on tomato. The strain of *Colletotrichum* sp. was resistant to the test nanoformulation and positive control. Therefore, the species needs to be carefully identified by molecular tools and determined for its pathogenicity in further studies. In a previous study, CRTO-nanoemulsion was reported to have good efficacy to control anthracnose in litchi [14]. In the present study, a new nanoemulsion containing 25 % of turmeric extract with BDMC as the most abundant constituent was investigated and showed effective control against anthracnose in both *in vitro* and *in vivo* bioassays. Currently, curcumin nanoparticles caused the attraction for scientists because of their bioavailability and permeability enhancement, controlled release of active ingredients, and high dispersion in water [7, 8, 14, 17, 18]. According to Bhawana et al., a wet-

milling method was used to formulate curcumin nanoparticles with a specific particle size range between 2 and 40 nm. The curcumin nanoparticles exhibited a good dispersion in water and were much more effective than curcumin against bacteria *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and fungi *Penicillium notatum*, and *Aspergillus niger* [8].

Concerning antifungal and protection properties of nanoformulations of phytochemicals, eugenol (4-allyl-2-methoxyphenol) and limonin were formulated into a nanoemulsion and showed high inhibition against blue mold caused by *P. italicum* in citrus fruits [6]. Cinnamaldehyde, eugenol and carvacrol nanoemulsions strongly inhibited *P. digitatum* and were applied to the postharvest preservation of citrus fruit [21]. Our study opens a new application of turmeric nanoparticles in the protection of red pepper fruits from *Colletotrichum* sp. infection during postharvest. However, the stability of this nanoformulation should be tested in future research.

4. CONCLUSIONS

In our study, turmeric nanoparticles containing 25 % turmeric extract were successfully fabricated by encapsulation using Tween 80 and PEG400 as emulsifiers. By HPLC analysis, the turmeric extract was proved to contain curcumin, DMC and BDMC as main constituents, in which BDCM has the highest content. The turmeric nanoparticles with an average size of 203 nm were tested for *in vitro* and *in vivo* antifungal bioassays against *Colletotrichum* species. The nanoformulation effectively inhibited *C. gloeosporioides* and *C. orbiculare*. In the *in vivo* bioassay, red pepper anthracnose was also consistently suppressed by turmeric nanoparticles at 10 mg/mL. The study results demonstrated that turmeric nanoparticles effectively control red pepper anthracnose and suggested developing the innovative nanoformulation as a green fungicide.

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CRedit authorship contribution statement. Nguyen Tuan Anh, Le Dang Quang, Tran Quang De and Nguyen Thi Thu Trang: Methodology, Investigation, Funding acquisition. Le Dang Quang, Nguyen Cuong Quoc, Vo Thi Kieu Anh, and Bui Van Cuong: Investigation, Formal analysis. Tran Thanh Men, Do Tan Khang, and Nguyen Trong Tuan: Formal analysis. Le Dang Quang, Nguyen Tuan Anh, Vu Xuan Minh, Tran Dai Lam, and Tran Quang De: Supervision.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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