

Evaluation of *in-vitro* antifungal activity against *Fusarium incarnatum* of binary and ternary combinations of lemongrass, garlic and mustard oil-encapsulated lipid nanoemulsions

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Summary Encapsulated lipid nanoemulsions (EO-LNs) from garlic oil, lemongrass oil and mustard oil were prepared by a combined method of homogenization and sonication with the aim to generate highly effective formulations against *Fusarium incarnatum* (laboratory bioassays). Their combined binary and ternary formulations (preparations by mixing an equal volume ratio of each EO-LNs) were also tested. The synergistic/additive/antagonistic antifungal effect of the EOs (under nanoform) in their combined formulations was determined using the SynergyFinder software with the Bliss independence model. Results revealed the synergistic effect of the combined binary and ternary formulations of garlic oil-encapsulated LNs (NaG), lemongrass oil-encapsulated LNs (NaL) and mustard oil-encapsulated LNs (NaM). Furthermore, the ternary combination, at the same concentration of each constituent EO, had higher antifungal activity than the binary combinations. Nonetheless, at 600 times dilution the NaLG (binary combination) inhibited 96% the mycelial growth of *F. incarnatum*, which was significantly higher than the efficacy of NaMLG (ternary combination) in the same dilution. This could be possibly attributed to the 1.5-time higher concentration of each constituent EO in the binary combination compared to that in the ternary formulation. In addition, NaLG, even at the high EO concentration of 0.4 g/L, did not show any phytotoxicity symptoms on lettuce plants.

Additional keywords: Antifungal activity, essential oils, synergistic effect

Introduction

Every year, pathogenic microorganisms cause a large loss of crop yields (Jiang *et al.*, 2020). *Fusarium incarnatum* is an important fungal pathogen that causes diseases of the root, stem, leaves, and seed, leading to the reduction of the quality and quantity of the yield of many crops such as rice, banana, sorghum, and maize (Akram *et al.*, 2019). Nowadays, chemical fungicides are commonly used to control this fungal pathogen (Yang *et al.*, 2019). Despite their benefits, their use has been linked to environmental pollution, toxicity issues to humans and other organisms, and resistance development of *Fusarium* strains, thus making it difficult to control the pathogen (Zubrod *et al.*, 2019). There-

fore, the use of biological methods to control plant diseases is an urging alternative solution.

Plant essential oils (EOs) are increasingly used to control plant diseases caused by fungi (Omar *et al.*, 2019). This is because EOs are eco-friendly, biodegradable with negligible or no toxicity to mammals and plants, and highly effective against a wide range of fungal diseases (Omar *et al.*, 2019; Amini *et al.*, 2012). Furthermore, EOs, as other biofungicides, have a multiple mechanism of action, hence they are less prone to the development of resistance of pathogenic fungi (Gressel *et al.*, 2020). Lemongrass oil with geranial (42.2%) and neral (31.5%), garlic oil with diallyl disulfide (27.1–46.8%) and diallyl trisulfide (19.9–34.1%), and mustard oil with allyl isothiocyanate (71.06%), cyclopropyl isothiocyanate (12.16%) and furfural (3.36%) have been previously demonstrated high fungicidal activity (Satyal *et al.*, 2017; Boukhatem *et al.*, 2014; Peng *et al.*, 2014). Other studies showed that isothiocyanate and gerani-

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al could strongly inhibit the growth of fungi such as *Fusarium graminearum*, *Fusarium verticillioides*, *Fusarium sambucinum*, *Alternaria alternata* and *Verticillium dahliae* (Drakopoulos *et al.*, 2020; Roselló *et al.*, 2015). Diallyl disulfide and diallyl trisulfide were found to possess a strong fungicidal activity for a broad spectrum of fungi (Wang *et al.*, 2019). Kamsu *et al.* (2019) reported that lemongrass oil inhibited the mycelial growth and conidia germination of *F. incarnatum* at a high concentration of 500 µL/L and 185 µL/L, respectively. Although the antifungal activity of garlic oil and mustard oil against *F. incarnatum* is still unknown, their effects on other *Fusarium* species at high concentrations have been reported (Dutta *et al.*, 2021; Wang *et al.*, 2020; Hayat *et al.*, 2016).

High antifungal activities of EOs may be due to their ability to destroy the cellular structure of hyphae and cell organelles, leading to the leakage of both cytoplasm and macromolecules. Some EOs can cross the cell membrane, interacting with the enzymes and proteins of the membrane, thereby producing a flux of protons towards the cell exterior which induce changes in the cells and, ultimately, cell death (Omidbeygi *et al.*, 2007). Silva *et al.* (2008) indicated the inhibitory effect of lemongrass oil against *Candida* spp., because the oil is able to form a charge-transfer complex with an electron donor of fungal cells, resulting in fungal death.

Nevertheless, the practical application of EOs in fungal control is challenging as EOs seem not to exhibit a strong antifungal activity at low concentrations due to low water solubility and poor penetration into plants, they are easily degradable under adverse environmental conditions and their price is often high (Nguyen *et al.*, 2020; Kamsu *et al.*, 2019; Rao *et al.*, 2005). To overcome these drawbacks, essential oil-encapsulated lipid nanoemulsions (EO-LNs) have been developed. LNs have a low cost, enhance the water dispersion of EOs by many times, improve the penetration of EOs into plant tissues and control their release, and protect active compounds in EOs from ad-

verse environmental conditions (Nguyen *et al.*, 2020). Bedoya-Serna *et al.* (2018) demonstrated that oregano oil-encapsulated LNs had minimal effective concentration against *Fusarium* sp. at an oregano oil concentration of 0.11 µg/mL, while the native form (pure oil form) exhibited an effect against the fungus at a concentration of 150 µg/mL. Additionally, Hassanin *et al.* (2017) reported that LNs containing thyme oil and basil oil exhibited a significantly higher antifungal activity against *Fusarium oxysporum* f. sp. *cumini* và *Fusarium oxysporum* than the native forms. Therefore, the minimal inhibitory concentration of EOs could be significantly reduced by encapsulating them into LNs, leading to a considerable reduction in the quantity and cost of EOs and enhancing their potential broad application as antifungal agents in green and sustainable agriculture.

Some recent studies have indicated that combinations of EOs result in significantly higher antifungal activities compared to the antifungal activities of single EOs due to the synergistic or additive effects (Bounar *et al.*, 2020; Park *et al.*, 2017; La-Torre *et al.*, 2016; Hossain *et al.*, 2016). For example, Park *et al.* (2017) demonstrated that the synergistic effect of origanum oil and thyme oil (ratio 1:1, v/v) significantly enhanced their inhibitory efficiency against mycelial growth of *Fusarium oxysporum* f. sp. *fragariae* of 40%, 50% and 70% compared to origanum oil, thyme oil and untreated control, respectively. La-Torre *et al.* (2016) reported that combined treatment with rosemary oil, clove oil and thyme oil could reduce tomato *Fusarium* wilt much more efficiently in comparison with single treatment by clove oil, thyme oil, or rosemary oil. Moreover, EOs extracted from *Thymus vulgaris* and *Oregano vulgare* exhibited a synergistic interaction to give a lower minimal inhibitory concentration and a higher antifungal activity against *Fusarium* spp. compared to the single forms (Bounar *et al.*, 2020).

The synergistic effect of EOs in a combined formulation has been demonstrated by using the fractional inhibition concentration index which was manually calculat-

ed (Bounar *et al.*, 2020; Hossain *et al.*, 2016). Meanwhile, the SynergyFinder software with the Bliss independent model was recently developed to demonstrate the synergistic/additive/antagonistic effect of drugs (Ianevski *et al.*, 2017), but it has not been intensively applied to investigate the synergistic/additive/antagonistic effect of EOs for the control of plant fungal pathogens. Only Nguyen *et al.* (2020) have successfully applied this software to demonstrate the synergistic effect of chili oil, cinnamon oil and neem oil (EO-LN forms) in nematode control. The application of the SynergyFinder software with the Bliss independent model to demonstrate the interaction (synergistic/additive/antagonistic effect) of EO components (the combined formulations) in the control of *F. incarnatum* will give the outcome of this study more scientific significance.

In the present research, the *in-vitro* antifungal activities of these three EO-LNs against *F. incarnatum* and of their combined binary and ternary formulations were assessed in laboratory bioassays. In addition, the synergistic/additive/antagonistic effects of these EOs in the combined formulations were determined using the SynergyFinder software with the Bliss independence model. Finally, the effect of the optimal EO-LN formulation on plant growth was evaluated. To our knowledge, the encapsulation of lemongrass (*Cymbopogon citratus*) oil (NaL), garlic (*Allium sativum*) oil (NaG) and mustard (*Sinapis alba*) oil (NaM) into LNs to control *F. incarnatum* has not been studied so far nor the antifungal activities of combined binary and ternary formulations of the EO-LNs.

Materials and Methods

Reagents and Biological Material

Garlic oil, mustard oil, and lemongrass oil were purchased from Dalosa Vietnam Essential – Herbal Co., LTD. (Vietnam). Soybean lecithin (Junsei Chemical, Tokyo, Japan) and Tween 80 (Samchun Pure Chemical, Seoul, Korea) were used as surfactants. A *F. incar-*

natum strain was isolated and cultured at DaLat Nuclear Research Institute (Vietnam). Lettuce plants were purchased from a local market. All other chemicals were of analytical grade.

Preparation of EO-LNs

Three different EO-LNs corresponding to lemongrass oil (NaL), garlic oil (NaG) and mustard oil (NaM) were prepared using a combined method of homogenization and sonication (Nguyen *et al.*, 2020). Briefly, a mixture (10 g) of lecithin and Tween 80 (ratio 1:1, w/w) was added to distilled water (80 g) and stirred at 70°C for 45 min. Each EO (10 g) was then added into the surfactant mixture and homogenized using an Ultra Turax T25 basic homogenizer (IKA, Japan) at 16000 rpm for 4 min. This was followed by sonication using an *Ultrasonic Processor VCX750* (Sonics & Materials, U.S.) at 20% for 3 min to form a nanodispersion of NaL, NaG and NaM.

The combined binary formulations were prepared by mixing two (2) EO-LNs with an equal volume (1:1, v/v), and the combined ternary formulation was prepared by mixing the three (3) EO-LNs at a ratio 1:1:1 (v/v/v).

Physical characterization of the EO-LNs and their binary and ternary combinations

The mean particle size, the polydispersity index (PDI) and the zeta potential of the 3 EO-LNs and of their combined binary and ternary formulations were determined using a Nano-ZS nano-size analyzer (Malvern, UK). Briefly, samples were diluted 100 times with distilled water. The resulting dispersion was then added to a polystyrene latex cell. The measurements were carried out at a temperature of 25°C and a detector angle of 173°C.

In-vitro antifungal activities of the EO-LNs and their binary and ternary combinations

The *in-vitro* antifungal activities against *F. incarnatum* of the EO-LNs and their binary and ternary combinations were evaluated in Petri dishes at laboratory bioassays (Kam-

su *et al.*, 2019). The fungus was grown on a PDA (potato dextrose agar) medium supplemented with the EO-LN formulations (single forms or combined forms) at dilutions of 250, 500, 600 and 750 times (corresponding to total EO-concentration of 0.4 g/L, 0.2 g/L, 0.1667 g/L and 0.1333 g/L). The control was a sample without addition of any EO-LN formulation. After 5 days, the growth of *F. incarnatum* in all treatments was evaluated and the mycelial diameter was measured. The antifungal activity was determined based on the mycelial growth inhibition (GI %) calculated using the following formula (1):

$$GI (\%) = \frac{D_c - D_t}{D_c} \times 100 \quad (1)$$

Where D_c and D_t represent the mycelial growth diameter of the control and treated sample, respectively.

Evaluation of the effect of EO-LN formulation on plant growth

The effect of EO-LN formulation on plant growth (phytotoxicity) was investigated by spraying the optimal EO-LN formulation (determined in the previous section) at a high dilution concentration (250 times) on lettuce plants. Particularly, lettuce plants (112 plants/treatment) were grown in a greenhouse under natural light and at a room temperature of 22–28°C. When the plants had reached the stage of 3–4 leaves, the optimal EO-LN formulation was sprayed on the plants (about 250 mL/treatment). Spraying with water was used as a control. The experiment was repeated two more times. The average height of the plants at 3 and 7 days after treatment was determined, and the percentage of additional growth (AG %) was calculated using the formula (2):

$$AG (\%) = \frac{L_a - L_c}{L_c} \times 100 \quad (2)$$

Where: L_a is the lettuce height after 7 days
 L_c is the lettuce control height

Data analysis

One-way ANOVA with the post-hoc Duncan test on the SPSS software ($P < 0.05$) was used for statistical analysis. The synergistic/additive/antagonistic effects of NaL, NaG and NaM in their binary and ternary combinations were determined using the SynergyFinder software with the Bliss independence model. In this software, scores < -10 indicate that the interaction between two agents is likely antagonistic, scores from -10 to 10 indicate that the interaction between two agents is likely additive, and scores > 10 indicate that the interaction between two or three agents is likely to be synergistic (Ianevski *et al.*, 2017).

Results

Preparation of the EO-LNs

The garlic oil-encapsulated LNs (NaG), lemongrass oil-encapsulated LNs (NaL), and mustard oil-encapsulated LNs (NaM) had a mean particle size of 71.81 nm, 72.06 nm, and 138 nm, respectively. The polydispersity index (PDI) of these EO-LNs was less than 0.245 and their zeta potential was larger than -40 mV. The combined binary and ternary formulations of NaG, NaL and NaM had a small particle size less than 120 nm, a good PDI value of 0.180–0.245, and a high zeta potential more than -40 mV (Table 1).

As shown in Figure 1, the lemongrass oil, the garlic oil and the mustard oil were poorly dispersed in water (Figure 1A), while the dispersion of their corresponding EO-LNs in water was very high (Figure 1B). After the oils were encapsulated into LNs, the colors of the EO-LNs became lighter compared to the colors of the native EOs.

In-vitro antifungal activities of the EO-LNs and their binary and ternary combinations

The *in-vitro* antifungal activities against *F. incarnatum* of the three single formulations (NaM, NaG, NaL) and their combined formulations (NaMG, NaML, NaLG, NaMLG) were determined from their mycelial growth inhi-

bition (GI %) values, using bioassays in Petri dishes (Kamsu *et al.* 2019). The image in Figure 2 shows that the mycelium of *F. incarnatum* in the control sample grew fast with the largest growth diameter after incubation for 5 days, while the supplements of the EO-LN formulations in the PDA medium resulted in smaller mycelial growth diameters, corresponding to smaller GI values. As shown in Table 2, at 250 times dilution, all EO-LN formulations completely inhibited the growth of *F. incarnatum*. At 500 times dilution, the GI values of NaL, NaM, and NaG decreased from 100% to 20.78%, 32.55% and 43.53%, respectively, while the GI values of their binary (NaML, NaLG, NaMG) and ternary combinations (NaMLG) remained 100%. A further increase in the dilution concentration to 600 times resulted in a decrease in the GI values of NaML, NaMG and NaMLG from 100% to 33.33%, 56.08% and 22.35%, respectively. However, the GI value of NaLG

was above 96% at this dilution concentration. At 750 times dilution, the GI values of the combined formulations sharply decreased to below 40% each, except for NaLG whose GI value became 62.35%.

When the synergistic/additive/antagonistic effects of NaL, NaG and NaM in their combined binary and ternary formulations were determined, the Bliss synergy scores (SynergyFinder software with the Bliss independence model) of the binary combinations of NaM and NaL, NaM and NaG, and NaL and NaG were 26.72, 19.05, and 22.37, respectively, while that of the ternary combination (NaMLG) was 100 (Figure 3).

Evaluation of the effects of EO-LN formulations on plant growth

Before being practically applied, the effect of optimal EO-LNs formulation on plant growth should be considered. Based on the *in-vitro* antifungal activities determined

Table 1. Physical characteristics of NaL, NaG and NaM and their combined binary, ternary formulations (n = 3).

EO-LNs formulations	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
NaM	138.00 ± 2.38a	0.174 ± 0.008d	-46.28 ± 1.29c
NaL	72.06 ± 1.45d	0.243 ± 0.012a	-41.37 ± 1.07ab
NaG	71.81 ± 1.51d	0.199 ± 0.013bc	-40.65 ± 1.31ab
NaM + NaL (NaML)	106.15 ± 2.28b	0.182 ± 0.011cd	-42.25 ± 0.93ab
NaM + NaG (NaMG)	107.10 ± 1.15b	0.241 ± 0.009a	-43.00 ± 1.12b
NaL + NaG (NaLG)	72.22 ± 1.21d	0.215 ± 0.012b	-40.10 ± 2.35a
NaM + NaL + NaG (NaMLG)	95.56 ± 1.74c	0.242 ± 0.011a	-41.48 ± 1.14ab

Binary combinations: NaML (NaM + NaL at a ratio 1:1, v/v); NaMG (NaM + NaG at a ratio 1:1, v/v); NaLG (NaL + NaG at a ratio 1:1, v/v). Ternary combination: NaMLG (NaM + NaL + NaG at a ratio 1:1:1, v/v/v).

Any two means followed by the same superscript letters in the same column are not significantly different by One-way Anova with post-hoc Duncan test in the SPSS software ($P < 0.05$).

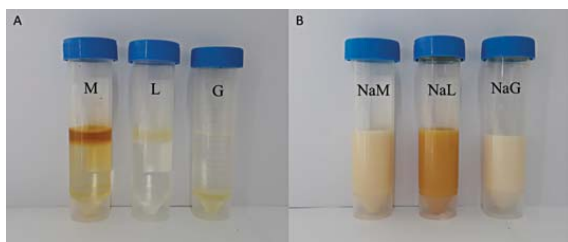


Figure 1. Water dispersion of (A) three Essential Oils (EOs) and (B) their corresponding Lipid Nanoemulsions (EO-LNs); M: mustard, L: lemongrass, G: garlic.

in the previous section, NaLG was chosen. Assessment of its effect on plant growth by spraying NaLG at 250 times dilution on lettuce plants, indicated that after treatment by NaLG for 3 and 7 days, all lettuce plants still developed normally, with a survival rate of 100%. In addition, there was no statistical difference in the percentage of additional growth between the treated and untreated plants (control) (Table 3). The heights of

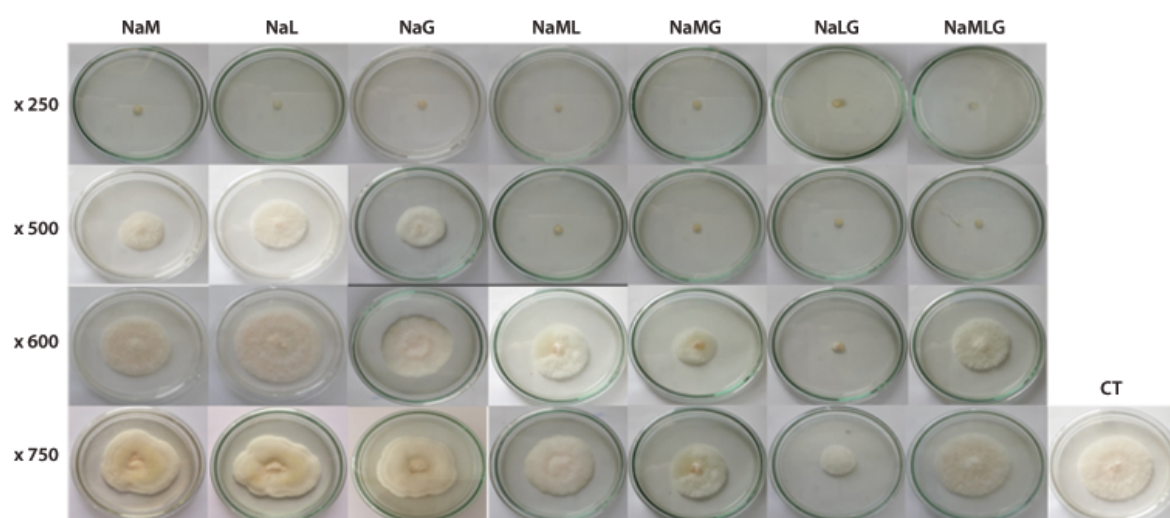


Figure 2. In-vitro antifungal activity against *Fusarium incarnatum* of NaL, NaG, NaM and their binary, ternary combinations. CT: control.

Table 2. In-vitro antifungal activity against *Fusarium incarnatum* of nanoemulsions NaL (lemongrass), NaG (garlic), NaM (mustard) and their combined binary, ternary formulations.

Nanoformulation of essential oils	Growth inhibition (%)			
	250 times dilution	500 times dilution	600 times dilution	750 times dilution
NaM	100 ± 0a	32.55 ± 1.36c	5.88 ± 2.35e	0e
NaL	100 ± 0a	20.78 ± 3.59b	0.39 ± 1.36f	0e
NaG	100 ± 0a	43.53 ± 4.08d	2.75 ± 1.36ef	0e
NaML	100 ± 0a	100 ± 0a	33.33 ± 3.59c	13.73 ± 3.59c
NaMG	100 ± 0a	100 ± 0a	56.08 ± 2.72b	38.82 ± 2.35b
NaLG	100 ± 0a	100 ± 0a	96.08 ± 1.36a	62.35 ± 4.08a
NaMLG	100 ± 0a	100 ± 0a	22.35 ± 2.35d	7.45 ± 2.72d

Binary combinations: NaML (NaM + NaL at a ratio 1:1, v/v); NaMG (NaM + NaG at a ratio 1:1, v/v); NaLG (NaL + NaG at a ratio 1:1, v/v). Ternary combination: NaMLG (NaM + NaL + NaG at a ratio 1:1:1, v/v/v).

Any two means followed by the same superscript letters in the same column are not significantly different by One-way Anova with post-hoc Duncan test in the SPSS software ($P < 0.05$).

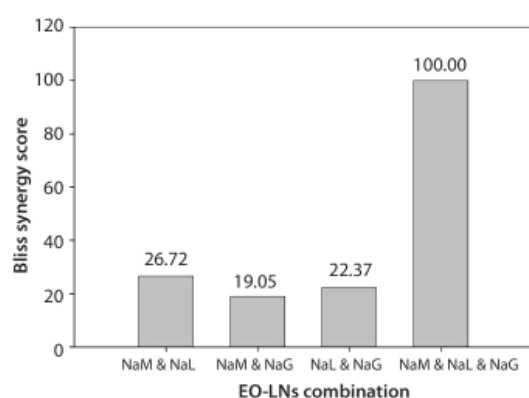


Figure 3. Bliss synergy score of binary and ternary combinations of NaL, NaG and NaM. The synergistic scores were determined by SynergyFinder software based on the results from three independent experiments.

lettuce plants in both cases increased by approximately 48% after 7 days.

Discussion

Based on their small mean particle sizes, good PDIs and high zeta potentials, it is clear that the EO-LNs of lemongrass, garlic and mustard were successfully prepared by a combined method of homogenization and sonication. The difference in the mean particle sizes of NaM, NaG, and NaL could be explained by the correlation between the RHLB

Table 3. Effect of NaLG at 250 times dilution on the growth of lettuce plants (n = 112).

Sample \ Plant height	Before treatment (cm)	After 3 days (cm)	After 7 days (cm)	AG value (%)
Control	3.10 ± 0.21	3.47 ± 0.21	4.59 ± 0.29	47.97 ± 3.36a
Treated by NaLG at 250 times dilution	3.88 ± 0.32	4.53 ± 0.28	5.74 ± 0.38	47.94 ± 2.54a

Any two means followed by the same superscript letters in the column of AG value are not significantly different by One-way Anova with post-hoc Duncan test in the SPSS software ($P < 0.05$).

(required hydrophilic–lipophilic balance) of each EO and the HLB (hydrophilic–lipophilic balance) of the surfactant mixture (Kim *et al.*, 2014). The high zeta potential values (above -40 mV) of the prepared EO-LNs indicate the high stability of these nanoformulations. The high stability can be explained by the fact that the high absolute value of the zeta potential (more than 30 mV) of the nanoparticles in the nanoformulations provided repulsive forces strong enough to prevent the particles from joining together to form larger particles (Mehnert *et al.*, 2001). In addition, the high dispersion of the EO-LNs in water compared to the dispersion of their corresponding oil form (native form) re-affirmed their successful formation. On the other hand, after equal volume ratios of the single EO-LNs were mixed to form combined binary or ternary formulations, the resulting formulations still maintained good physical characteristics nearly similar to those of their “parent” single formulations. In addition, no strange phenomena such as creaming, precipitation or chemical reaction occurred. These indicated that it is possible to prepare combined binary and ternary formulations from NaL, NaG and NaM.

Previous studies have reported high antibacterial and antifungal activities of garlic oil, lemongrass oil and mustard oil (Kamsu *et al.*, 2019; Roselló *et al.*, 2015; Wang *et al.*, 2019). However, as mentioned before, the antifungal activities of these EOs against *Fusarium sp.* are not so strong and usually require a high EO concentration to exhibit an obvious effect (Drakopoulos *et al.*, 2020; Kamsu *et al.*, 2019; Sharif *et al.*, 2017; Hayat *et al.*, 2016). For example, Kamsu *et al.* (2019) showed that lemongrass oil (density of 0.893 g/mL) only

moderately inhibited mycelial growth of *F. incarnatum* at a concentration of more than 500 µL/L (approximately 0.4465 g/L). Hayat *et al.* (2016) reported that an obvious inhibition against *F. oxysporum* of garlic extract was achieved at allicin concentration of approximately 3.9 g/L (10 g garlic homogenized in 100 mL distilled water). In addition, Drakopoulos *et al.* (2020) also showed that mustard seed powders (containing more than 28–32% oil) could inhibit the mycelial growth of *Fusarium graminearum* at a high concentration of 20 g/L, corresponding to an approximately mustard oil concentration of 6 g/L (Drakopoulos *et al.*, 2020; Sharif *et al.*, 2017).

In this study, based on the GI values, it is clear that all three EO-LNs had strong antifungal activities against *F. incarnatum*; each one had a GI value of 100% at 250 times dilution (corresponding to an EO concentration of approximately 0.4 g/L). In addition, the GI values at 500 times dilution indicated that the antifungal activity of NaG against this fungal strain was the highest compared to those of NaL and NaM. Moreover, the results also indicated that the binary and ternary combinations of these single EO-LNs were considerably more effective against *F. incarnatum*, with GI values of 100% each at 500 times dilution (corresponding to concentrations of 0.1 g/L and 0.0667 g/L for each EO in the combined binary formulations and combined ternary formulation, respectively). The antifungal results at 600 times dilution showed that the binary combination of NaL and NaG at a ratio 1:1 (v/v) (NaLG) had the highest antifungal activity of all binary combinations. This nanoformulation nearly completely inhibited the growth of *F. incarnatum* (GI value of 96.08%) at a concentra-

tion of 0.0833 g/L for each constituent EO. This EO concentration was many times lower than that obtained by Kamsu *et al.* (2019). This could be explained by the synergistic effect of NaL (lemongrass oil) and NaG (garlic oil), as indicated by a Bliss synergy score of 22.37 calculated by the SynergyFinder software with the Bliss independence model.

The ternary combination of the EOs had a higher Bliss synergy score than that of the binary combinations, which indicates a higher synergistic effect. More specifically, although the concentration of each constituent EO of the binary formulation at 750 times dilution was 0.0667 g/L and equalled to that of the ternary formulation at 500 times dilution, the GI value of NaMLG reached 100%, compared to 13.73%, 38.82% and 62.35% of NaML, NaMG and NaLG, respectively. This has illustrated that the synergistic effect of ternary combination resulted in higher antifungal activity against *F. incarnatum* than the binary combinations. This is similar to the result reported in our previous study, where the ternary combination of chili oil-encapsulated LNs, garlic oil-encapsulated LNs and cinnamon oil-encapsulated LNs also exhibited the highest *in-vitro* antifungal activity against *Alternaria alternata* due to their synergistic effect (Nguyen *et al.*, 2022). It has also been reported that the ternary combination of chili oil-, cinnamon oil- and neem oil-encapsulated LNs exhibited higher nematocidal activity than their combined binary formulations (Nguyen *et al.*, 2020). Moreover, a study of Jyoti *et al.* (2019) also revealed that the ternary combination of clove oil (*Syzygium aromaticum*), cinnamon oil (*Cinnamomum zeylanicum*), and lemongrass oil (*Cymbopogon citratus*) at a ratio of 1:1:1 (v/v/v) had higher synergistic efficacy than the binary combinations in controlling *Rhizoctonia solani*.

Nevertheless, at 600 times dilution, the binary combination of NaL and NaG (NaLG) exhibited higher antifungal activity compared to the ternary combination (NaMLG) in the same dilution. This could be explained by two reasons. First, the concentration of each constituent EO of the ternary formu-

lation at 600 times dilution was only 0.0556 g/L, while that of the binary formulation was 0.0833 (approximately 1.5 times higher). Secondly, at this dilution, the low concentration (0.0556 g/L) of lemongrass oil, garlic oil and mustard oil contained in the ternary formulation might be lower than the minimal inhibitory concentration (MIC) needed to effectively inhibit the growth of *F. incarnatum* (Dutta *et al.*, 2021; Eke *et al.*, 2020; Wang *et al.*, 2020). Particularly, MIC for controlling *Fusarium solani* of lemongrass oil was 0.5 g/L (Eke *et al.*, 2020), while MIC against *Fusarium oxysporum* f. sp. *lycopersici* of mustard oil was 0.256 g/L (Dutta *et al.*, 2021). In addition, MIC against *F. oxysporum* and *Fusarium solani* of garlic oil was approximately 0.05 g/L (Wang *et al.*, 2020). On the other hand, the combination of different EOs (or EO-LNs) can also limit the resistance of fungal strains compared to the use of single-EO (or EO-LNs) formulations (Hossain *et al.*, 2016). From all above results and discussion, it is possible to conclude that NaLG was the best formulation for controlling *F. incarnatum*.

Furthermore, NaLG did not negatively affect the growth of lettuce plants, even at the high total EO concentration of 0.4 g/L (corresponding to 250 times dilution). None of the symptoms of chlorosis, cell death and reduced growth was observed during the experiment. This might be because after spraying on plants, nano-carriers (NaLG) had deeply penetrated into the plant tissues and slowly released EOs thus maintaining the concentration of EOs in the plant tissue at a level lower than the minimal inhibition concentration (Rehman *et al.*, 2021). Moreover, sulfur compounds in garlic have also been reported to be able to promote plant growth (Cheng *et al.*, 2016).

Conclusion

NaM, NaL, and NaG were successfully prepared by a combined method of homogenization and sonication with good physical characteristics (small mean particle size, small PDI and high zeta potential). Their combined

binary and ternary formulations were also successfully prepared by mixing with the equal volume ratio of each EO-LNs. The *in-vitro* antifungal activity of NaG against *F. incarnatum* was higher than those of NaL and NaM. In addition, the combined binary, ternary formulations exhibited higher antifungal activity than the single formulations due to the synergistic effect as determined by the SynergyFinder software with the Bliss independence mode. The results also demonstrated that, at the same concentration of each constituent EO, the ternary combination had higher antifungal activity compared to the binary combinations. However, at 600 times dilution, the antifungal activity against *F. incarnatum* of NaLG (binary combination) achieved the highest GI value (more than 96%), which was significantly higher than that of NaMLG (ternary combination), possibly due to the 1.5-time higher concentration of each constituent EO compared to that of the ternary formulation. In addition, NaLG at 250 times dilution (corresponding to total EO concentration of 0.4 g/L) did not negatively affect the growth of lettuce plants. Therefore, NaLG has the potential to be widely applied in sustainable agriculture to protect plants from diseases caused by *F. incarnatum*.

This study was partly supported by Institute of Developmental Philosophy (Vietnam) and Nuclear Research Institute (Vietnam). We also would like to thank student Minh-Trong Le for his contribution in this study.

Declarations

Conflicts of interest/Competing interests

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Received: 22 September 2021; Accepted: 28 September 2022

Αξιολόγηση της *in-vitro* αντιμυκητιακής δράσης έναντι του *Fusarium incarnatum*, σκευασμάτων σε μορφή νανογαλακτώματος με ενθυλάκωση δύο ή τριών αιθέριων ελαίων λεμονόχορτου, σκόρδου και λευκού σιναπιού

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Περίληψη Νανογαλακτώματα με ενθυλάκωση αιθέριων ελαίων σκόρδου, λεμονόχορτου και λευκού σιναπιού παρασκευάστηκαν με συνδυασμένη μέθοδο ομογενοποίησης και υπερήχων με στόχο τη δημιουργία αποτελεσματικών σκευασμάτων κατά του *Fusarium incarnatum* (εργαστηριακές βιοδοκιμές). Επίσης δοκιμάστηκαν διμερείς και τριμερείς συνδυασμοί τους (παρασκευάσματα με ανάμειξη ίσης αναλογίας όγκου κάθε αιθέριου ελαίου). Η συνεργιστική/αυξανόμενη/ανταγωνιστική αντιμυκητιακή δράση των νανογαλακτωμάτων στις συνδυασμένες συνθέσεις των αιθέριων ελαίων προσδιορίστηκε με τη χρήση του λογισμικού SynergyFinder και το μοντέλο ανεξαρτησίας Bliss. Τα αποτελέσματα έδειξαν συνεργιστική δράση για τους διμερείς και τριμερείς συνδυασμούς νανο σκευασμάτων των αιθέριων ελαίων σκόρδου, λεμονόχορτου και λευκού σιναπιού. Επιπλέον, ο τριμερής συνδυασμός αιθέριων ελαίων, με συμμετοχή κάθε συστατικού στην ίδια συγκέντρωση, είχε υψηλότερη αντιμυκητιακή δράση από τους διμερείς συνδυασμούς. Ωστόσο, το νανογαλάκτωμα του συνδυασμού αιθέριων ελαίων σκόρδου-λεμονόχορτου, με αραιώση 600 φορές, ανέστειλε κατά 96% τη μυκηλιακή ανάπτυξη του *F. incarnatum*, αποτελεσματικότητα που ήταν σημαντικά υψηλότερη από αυτή του συνδυασμού των τριών αιθέριων ελαίων στην ίδια αραιώση. Αυτό θα μπορούσε πιθανώς να αποδοθεί στην κατά 1,5 φορά υψηλότερη συγκέντρωση κάθε συστατικού αιθέριου ελαίου στον διμερή συνδυασμό σε σύγκριση με εκείνη στον τριμερή. Επιπλέον, το νανογαλάκτωμα του συνδυασμού αιθέριων ελαίων σκόρδου-λεμονόχορτου, ακόμα και στην υψηλή συγκέντρωση αιθέριων ελαίων των 0,4 g/L, δεν προκάλεσε συμπτώματα φυτοτοξικότητας σε φυτά μαρουλιού.

Hellenic Plant Protection Journal **16**: 1-11, 2023