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Formulation, Characterizations and Antibacterial Activity of some Nanoemulsions Incorporating Monoterpenes

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ABSTRACT

In the current study, we formulated and characterized bio-based oil in water nanoemulsions incorporating monoterpenes; (*R*)-carvone, cinnamaldehyde, citral, geraniol and pulegone and tested their antimicrobial activity against two plant pathogenic bacteria, *Pectobacterium carotovorum sub carotovorum* and *Ralstonia solanacearum in vitro* and *in vivo* studies. Nanoemulsions (NEs) were prepared by adding dropwise monoterpenes at concentration of 5% in an aqueous solution containing 10% a surfactant (tween 80) with constant stirring and then ultrasonication. NEs were confirmed by dynamic light scattering and transmission electron microscopy. Physical stability and viscosity were, also, investigated. NEs monoterpenes had a poly dispersity index ranged from 0.130 to 0.630 and droplet size in the range of 56.64-176.00 nm. Results revealed that NE - cinnamaldehyde had great antibacterial activity against *P. carotovorum* and *R. solanacearum* (MIC = 60 and 100 mg/L, respectively). NE cinnamaldehyde was induced effective defense responses *in vivo* in potato plant and tubers against two tested bacteria at a rate 1000 and 3000 mg/kg. Higher activities of polyphenoloxidase (PPO), peroxidase (POD) activities and total phenolic content of tubers and leaves were significantly recorded compared to control at all tested times. This was evident from reduced soft and brown rot diseases symptoms of potato spunta *cv* treated with elicitors subsequently with tested bacteria. These results showed that the PPO, POD and total soluble phenols play a role in instructing resistance to potato soft and brown mold infections. The observed relationship between formulation and activity can lead to the rational design of nanoemulsion based systems for monoterpenes for applications in antimicrobial and agrochemical industries. Amalgamation of such economical treatments might lessen management costs and minimize the environmental pollution.

Keywords: Monoterpenes; Nanoemulsions; Antimicrobial activity; Plant pathogens.

INTRODUCTION

Nowadays rapid advancements in nanoformulations incorporating agrochemical agents have opened up new expectations for several industrial and consumer sectors of agricultural production (Kah, 2015; Badawy *et al.*, 2017; Balaure *et al.*, 2017). This mainly to control the sensitivity and ameliorate the stability of the compounds those have high volatilization and decomposition such as plant essential oils (EOs) and their main constituents such as monoterpenes (Marei *et al.,* 2018).The nanoformulations can, also, promote the uptake, absorption, and bioavailability of the active components in the plants and pests comparing with bulk equivalents (Qian and McClements 2011).

Nanoemulsions defined as emulsions consisting of an oil nano-scale (20 to 200 nm) disseminated in the outer phase of obverse polarity by the effect of surfactants acting on the oil/water interface (Sadurní *et al.*, 2005; Persson *et al.,* 2014). Pesticide companies use NEs, which can either be oil-based or water-based and have standard inhibitors of herbicidal or insecticidal nanoparticles (200-400nm) (Madhuri *et al.,* 2010; Sekhon 2014). Many techniques have been applied to the production of NEs, including low- or high-energy methods(Ultrasonic emulsification). The latter high energy method characterizes as fast and efficient capable of preparing nanoemulsions with diameters of tiny droplets and distributions of small size (Ghosh *et al.*, 2013). Monoterpenes, the main constituents of plant essential oils, have been documented as NEs to be effective as antimicrobial agents (Ghosh *et al.*,2014; Zhang *et al.*,2014; Zahi *et al., 2*015; Ma *et al.*, 2016; Li *et al.*, 2017).

Bacterial soft rot is a dangerous disease in potato, causing considerable reduction of yield and quality. *Pectobacterium carotovorum* subsp. *Carotovorum, Pcc* (Family: Enterobacteriaceae; Class: Gamma Proteobacteria) is soft rot bacteria and favored by low temperature and moist conditions (Chung *et al.*, 2013). The *Pectobacterium* as is facultative anaerobe, no spore-forming, gram-negative enter-bacteria that cause disease in several vegetable crops (Agrios 2005). The virulence of *Pcc* is dependent on the production and secretion of pectinases and cellulases as plant cell wall-degrading enzymes (Verma *et al.*, 2017). Plants that vegetative propagated such as potato was extremely infected, where the bacteria can spread from plant to plant (Pérombelon 2002).

Potato brown rot is caused by the bacterium *Ralstonia solanacearum* (Family: Ralstoniaceae; Class: Betaproteobacteria). This disease represents a serious threat to the potato production in many countries, especially in cosy growing areas and cause marketable yield loss (Kabeil *et al.*,2008; Mansfield *et al.*, 2012). Disease resistance in plants depends upon slowing down ,suppressing or halting infection through the activation of several arrays of defense responses. It like many phytopathogenic bacteria produce multiple extracellular plant cell wall degrading enzymes. Some of these enzymes have been implicated as virulence factors contributing to the pathogen ability to invade and colonize host tissues causing the wilt disease (Liu *et al.,*2005; Leonard *et al.,* 2017).

Therefore, the aim of this article was to prepare and characterize nanoemulsions containing (*R*)-carvone, cinnamaldehyde, citral, geraniol and pulegone in order to enhance their antimicrobial effects against plant pathogenic bacteria *P. carotovora* and *R. solanacearumin vitro* and *in vivo* studies. The interactive effect between the bacterial pathogens and the host plant were studied in

details through determination of the disease index and peroxidase (POD), polyphenol oxidase (PPO) and total soluble phenolic content as defense related enzymes.

MATERIALS AND METHODS

Chemicals and reagents

Five monoterpenes (Figure 1) include cinnamaldehyde (98%), geraniol (98%) and pulegone (92%) were purchased from ACROS Organics Company, New Jersey, USA whereas (*R*)-carvone (98%), and citral (95%) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Zenga fungicide (10% Cu +5% Metalaxyl + 10% Mancozeb) was purchased from Bio Nano Technology Company, Nubaria, El-Behera. All other solvents and reagents were used without further purification.

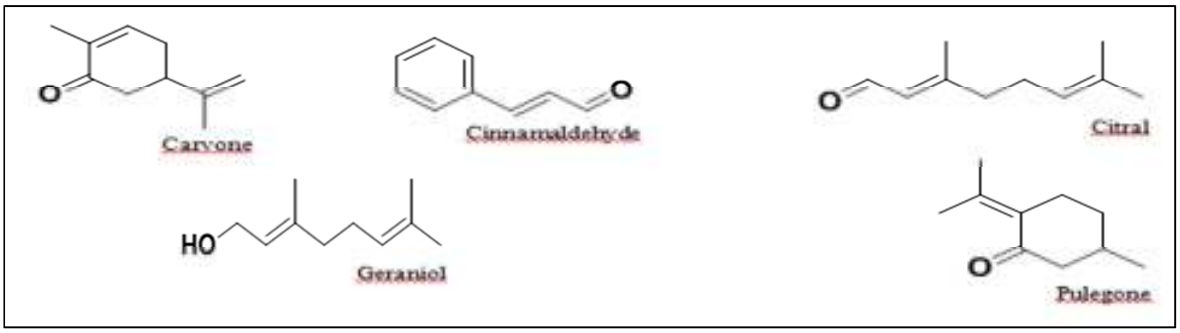

Figure 1. The chemical structure of the tested monoterpenes.

Bacterial cultures

Bacteria of soft rot and brown rot disease were obtained from Plant Pathology Department, Faculty of Agriculture, Damanhour University, Egypt. The bacterium *P. carotovorum* was maintained on the surface of plates containing NA medium (peptone 10, meat extract 5, sodium chloride 2.5 and agar 10 g/L in distilled water) and the plates were incubated for 48 h at 37˚C (Atlas 2005). It was purified through a single colony isolation technique. While, oozes of infected potato tubers with *R. solanacearumin* were placed in sterile water, a loopful of bacterial suspension was streaked on tetrazolium agar medium (Abo-El-Dahab and El-Goorani 1969). Medium was composed of peptone 5 g, beef extract 3 g, glycerol 20 ml, agar 15 g, distilled water 1L and 0.05%tetrazolium agar medium (pH 7.0) and incubated for 48 h at 37ºC. Identification was carried out according to Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2005). All cultural, morphological, physiological and biochemical characteristics of the bacteria were performed (Klement *et al.*, 1990; Cowan and Steel 2004).

Preparation of nanoemulsions

Five oil-in-water (O/W) nanoemulsions containing monoterpenes concentration of 5% , solvent, surfactant and deionized water were prepared using a high-energy ultrasonication method (Badawy *et al.,*2017). The raw emulsions were first prepared by stirring, then emulsification was performed using a high-energy ultrasonic process (Figure 2, Table 1).

Emulsion was formed by dropping monoterpenes which dissolved in DMSO in a water phase containing tween 80 as a surfactant under continuous stirring by a magnetic stirrer at 4000 rpm for 30 min. The emulsions were then subjected to ultrasonic emulsification under optimum conditions (Badawy *et al.,*2017). The optimal conditions for ultrasonic emulsification involved sonication power 75 kHz and pulses or cycles, 9 cycle /sec for 15 min (Ultrasonic Homogenizers HD 2070) (Anjali *et al.*,2012).

Transmission electron microscopy (TEM)

The morphology of the prepared NEs was envisaged by TEM (JEOL JSM-1200EX II, USA). Samples (50 µL) were added to 200-mesh form war-coated copper TEM sample holders and were then stained with phosphotungstic acid (1.5%). Excess liquid was removed and the TEM samples were observed with TEM equipped with 20 µm aperture at 67 kV.

Zeta potential

The electrophoretic properties of nanoemulsion droplets were measured by Zeta sizer nano system (Malvern instrument Ltd., UK). The nanoemulsion was transferred to zeta-potential cell and mustered at 25˚C (Honary and Zahir 2013).

Droplet size and poly dispersity index (PDI)

The droplet size and poly dispersity index (PDI) of NEs were measured by a dynamic light scattering (DLS) at room temperature. Samples prior to measurements were diluted to 10% with deionized water. The size of the droplet(in nanometer) was calculated as outlined by Hamed *et al*., (2016).

Viscosity and pH

The dynamic viscosity (η)was measured by a Rotary Myr VR 3000 viscometer using L4 spindle at 200 rpm at 25˚Cand the data expressed in mPa.s. The pH value was measured by digital pH meter (Mi 151 Martini Instruments, Model Mi 150, UK).

Thermodynamic stability

The nanoemulsions were tested for stability at centrifugation, freeze thaw cycle and also at room temperature. Nanoemulsions were centrifuged at 5000 rpm for 30 min and were observed for phase separation and successful combinations were tested (Kadhim and Abbas 2015). Stability test at freeze thaw cycle was carried out by storage of the nano-formulations at -21˚C for 24 h and then at 21˚C until melt for also 24 h (Kadhim and Abbas 2015), separation or creaming layer was examined. In addition, stability at room temperature was performed. All measurements were performed in triplicate.

Table 1. Preparation conditions of different nanoemulsions containing monoterpenes

Monoterpene	Active ingredient (%)	Solvent DMSO	Surfactant (%)	Water (%)	Sonication time (min)	Sonication power (kHz)	Sonication cycle (cycle/s)
Carvone	5	5	10	80	15	75	9
Cinnamaldehyde	5	5	10	80	15	75	9
Citral	5	5	10	80	15	75	9
Geraniol	5	5	10	80	15	75	9
Pulegone	5	5	10	80	15	75	9

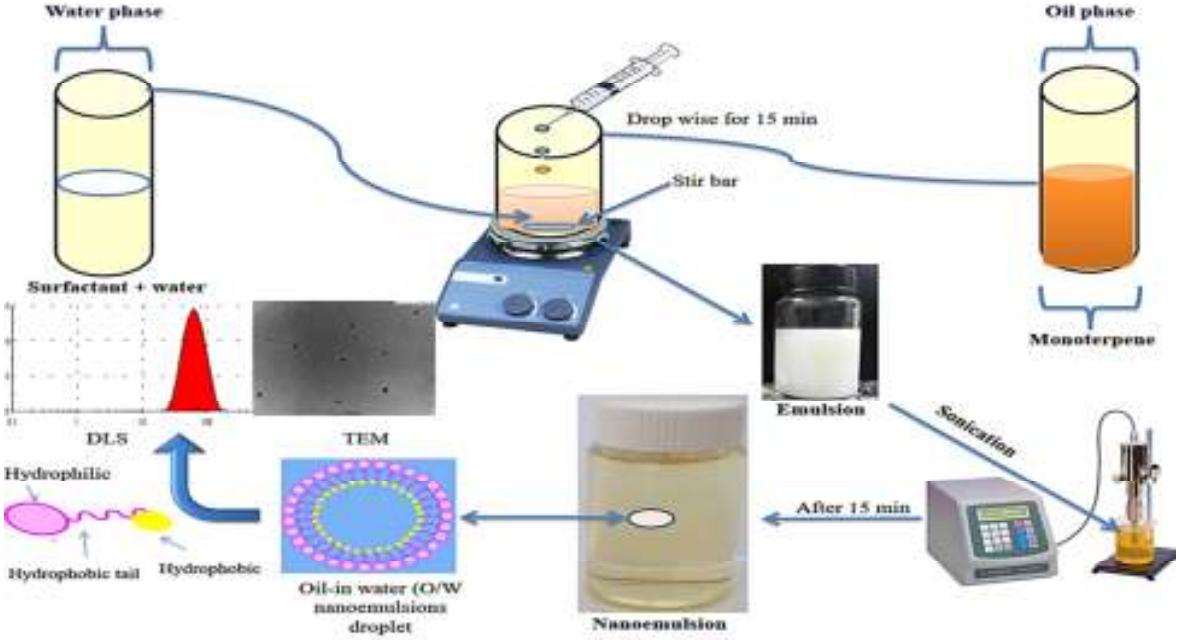

Figure 2. Schematic presentation shows preparation of different nanoemulsions containing monoterpenes.

Antibacterial assay

Minimum inhibitory concentration (MIC) assay

The *in vitro* antibacterial activity of pure and nanoemulsions were assayed using NA dilution method according to the European committee for antimicrobial susceptibility testing (EUCAST 2000) against *P. carotovorum sub carotovorum*, and *R. solanacearum*. The tested monoterpenes were dissolved in tween 20 and Zenga was used as a reference fungicide. Appropriate volumes of the stock solutions were added to molten NA to obtain a range of concentrations (10 to 10000 mg/L) before pouring to petri dishes. After solidifications, bacterial cultures (approximately 10^8 CFU/mL) was spotted (ten spots per each plate) using 2 µL standard loop on the surface of agar. The inoculums spots were allowed to dry before inverting the plates for incubation at 37°C for 24 h. Parallel controls were maintained with distilled water and tween 20 mixed with NA medium. The MIC was determined as lowest concentration of monoterpenes showing no visible bacterial growth in the agar plates.

Activity of NE-cinnamaldehyde on enhancing resistance of potato leaves against *R. solanacearum*

Tubers were obtained from the International Potato Center, Kafr El-Zayat, Gharbiya Governorate, Egypt. *R. solanacearum* isolate was tested for pathogenicity on spunta *cv* potato, which was known to be highly susceptible. Surfaces of the aforementioned cultivar potato tubers were sterilized with 70% ethanol for 5 minutes then washed with sterile water and planted in plastic pots (15 cm diameter) filled with sterile peat moss and clay (one tuber per pot) in a green house at 25±2ºC. After 4-5 weeks, Foliar spraying until draining with NE cinnamaldehyde at rate, 1000 mg/L (NC-1000) and 3000 mg/L(NC-3000) was applied. The treatment was replicated three times. Plants sprayed with water used as a control negative (un- inoculated, UU) and control positive(inoculated,UI) (Montesano et al.,2005). Disease severity of inoculated leaves was assessed after fifteen days of inoculation and severity of wilting was recorded daily on the scale as follows: 1 = no symptoms, 2 = one leaf wilted, 3 = two or three leaves wilted, 4 = four or more leaves wilted, and 5 = plant dead (He, 1983).

Activity of NE-cinnamaldehyde on enhancing resistance of potato tubers against *P. carotovorum*.

Tubers of spunta *cv* potato (about 60 g in weight) were thoroughly washed then immersed in NC cinnamaldehyde at rate, 1000 and 3000 µg/mL for 60 min. After drying, tubers were kept at room temperature for one day before being inoculation (Hajhamed *et al.,*2007). Bacterial suspension (10^8cfu/mL) was prepared from 48 h old culture. Tubers were prepared and sterilized then inoculated by bacterial suspension Inoculated slices were kept for 48 h in an incubator at 37^{o}C. Tubers immersed in water were used as control negative (UU), but inoculated with *Pc* isolate used as control positive (UI). After two days of incubation, the diameters of rotted area (cm) were measured (Hollis and Goss 1950). Three replicates of slices were used.

Effect of NE- cinnamaldehyde on defense related enzymes activities

Spunta *cv* was used to determine the effect of elicitors on defense related enzymes activities such as, PPO and POD and total phenolic content. Tubers and leaves were treated with NE cinnamaldehyde doses as previously mentioned. The samples were taken from around infection site. Enzymes activities were evaluated in samples after 0, 6, 12, 24 and 48 h post inoculations and each treatment was triplicated. One gram of the leaves or tubers was homogenized in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5) in a pre-chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 15 min at 4ºC and the supernatant served as an enzyme source. Polyphenol oxidase (PPO) and peroxidase (POD) activities of leaves and tubers were measured by the method of Mayer *et al.,*(1966) and Hammerschmidt *et al.,*(1982), respectively, and were expressed as change in absorbance (OD) min^{-1} g^{-1} of fresh tissue. While, total phenolic contents of leaves and tubers (catechol equivalent/g of fresh tissue) were determined by the method of Zieslin and Ben Zaken,(1993) .

Statistical analysis

Statistical analysis was performed using SPSS 21.0 software. The data of enzymes activity were analyzed by one-way ANOVA. Duncan's Multiple Range Test (DMRT) was employed to test for significant between the treatments at $P<0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

Characterization of nanoemulsions

Physical and thermodynamic stabilities

The thermodynamic characterizations are presented in Table 2. The nanoemulsions were stable at centrifugation of 5000 rpm, heating cycle, and freeze-thaw cycle for 4 weeks. No creaming or phase separation was observed on these formulations. It is well known that centrifugation can speed the rate of sedimentation indicting that degradation of any emulsion may be referred to the gravitational force action. Oil/Water emulsion often appears compared to deposition of precipitation due to low oil droplet density rather than aquatic medium. It is important to have good physical properties during long-term storage, If NEs are to be used as an antimicrobial delivery systems, (Tadros *et al.*, 2004).

Viscosity and pH

The NEs *R*-carvone, cinnamaldehyde, citral, geraniol and pulegone have viscosity values of 3.33, 7.00, 2.67, 4.33 and 2.80mPa.s, orderly (Table 2). The pH value of the tested five formulations ranged from 6.0 to 6.5. The pH can results in a great effect on the stability of NEs (Badawy and Rabea 2017). Viscosity strongly affected by several factors such as disperse phase volume fraction, colloidal interactions, droplet size , archeology of component phases, and droplet charge (El-Mohamedy *et al.*, 2015; McClements 2015; Pal 2011).

Table 2. Characterizations and thermodynamic stability of different NEs-monoterpenes

Nanoemulsion	Characterizations					Thermodynamic stability			
	μ (mPa.s) ±SE	pH	Droplet diameter (nm) ± SE	PDI ± SE	Zeta potential (mV)	Room temperature (25°C)	Centrifugation (5000 rpm)	Freezing cycle (-21°C)	Heating cycle (21°C)
Carvone	3.33±0.34	6.5	67.23±0.21	0.177±0.01	+0.816	√	√	√	√
Cinnamaldehyde	7.00±0.58	6.1	128.07±1.34	0.322±0.01	-0.052	√	√	√	√
Citral	2.67±0.34	6.0	56.64±0.13	0.130±0.01	-0.216	√	√	√	√
Geraniol	4.33±0.34	6.5	176.00±3.21	0.630±0.08	-1.110	√	√	√	√
Pulegone	2.80±0.20	6.0	58.83±0.15	0.311±0.01	-0.572	√	√	√	√

Droplet size and PDI

Droplet size and PDI are presented in Table 2 and Figure 3. Results reflected that NEs recorded droplet size of 67.23 nm for *R*-carvone, 128.07 nm for cinnamaldehyde, 56.64 nm for citral, 176.00 nm for geraniol and 58.83 nm for pulegone. This result demonstrated that preparation in the nanometric size for all compounds were successful. NEs *R*-carvone, citral and pulegone recorded the lowest value of droplet diameter size (67.23, 56.64 and 58.83 nm, respectively) compared to NEs cinnamaldehyde and geraniol (128.07 and 176.00, respectively). Generally, the range of 10 to 500 nm was the same range which the average droplet size of O/W nanoemulsions falls within it. Li and Chiang (2012), reported that the NEs D-limonene reflected a droplet size smaller than 100 nm .

The PDI values were 0.177, 0.322, 0.130, 0.630 and 0.311 for NEs *R*-carvone, cinnamaldehyde, citral, geraniol and pulegone, respectively. This indicated that all NEs had a relatively narrow range of size distribution. In addition, NEs *R*-carvone and citral are the highest homogeneous compared to NEs cinnamaldehyde, geraniol and pulegone. This finding is proved by Shakeel *et al.,*(2007), who reported that the PDI value remained less than 0.2 exhibits the relative homogeneity of the NEs and PDI > 0.3 indicating system heterogeneity.

Transmission electron microscopy (TEM)

Figure 4 shows a microscopic image of TEM of NEs which shows a spherical shape in NEs that provides the same appearance of oil in water type. It was noticed that the droplet size of TEM analysis seemed to correlate with the range of droplet diameter obtained using Zetasizer. The discrepancies in morphology might be related to the composition of dispersed phase of each formulation (Li and Chiang 2012; Liu and Wu 2010).

Zeta potentials (electrophoretic properties)

The zeta potential values were +0.816, -0.052, -0.216, -1.110 and -0.572 mV for the NEs *R*-carvone, cinnamaldehyde, citral, geraniol and pulegone, respectively (Table 2). Increasing the surface charge improve the emulsions stability because of the increase of repelling forces between droplets against contraction and coalescence (Stachurski and Michalek 1996). Li and Lu

(2016), found that the NE D-limonene (10.0%) at the pH 6.4 recorded approximately -35 mV as a zeta potential.

The differences in the dissociation degree and ability for compound ionization is the reason of the negatively charged zeta potentials of emulsions and NEs formulated with different monoterpenes (Bonilla *et al.*, 2012). However, only short-term stability was noticed for NEs droplets with zeta potentials of ±20 mV, but the droplet tend to come together (Mishra *et al.*, 2009).

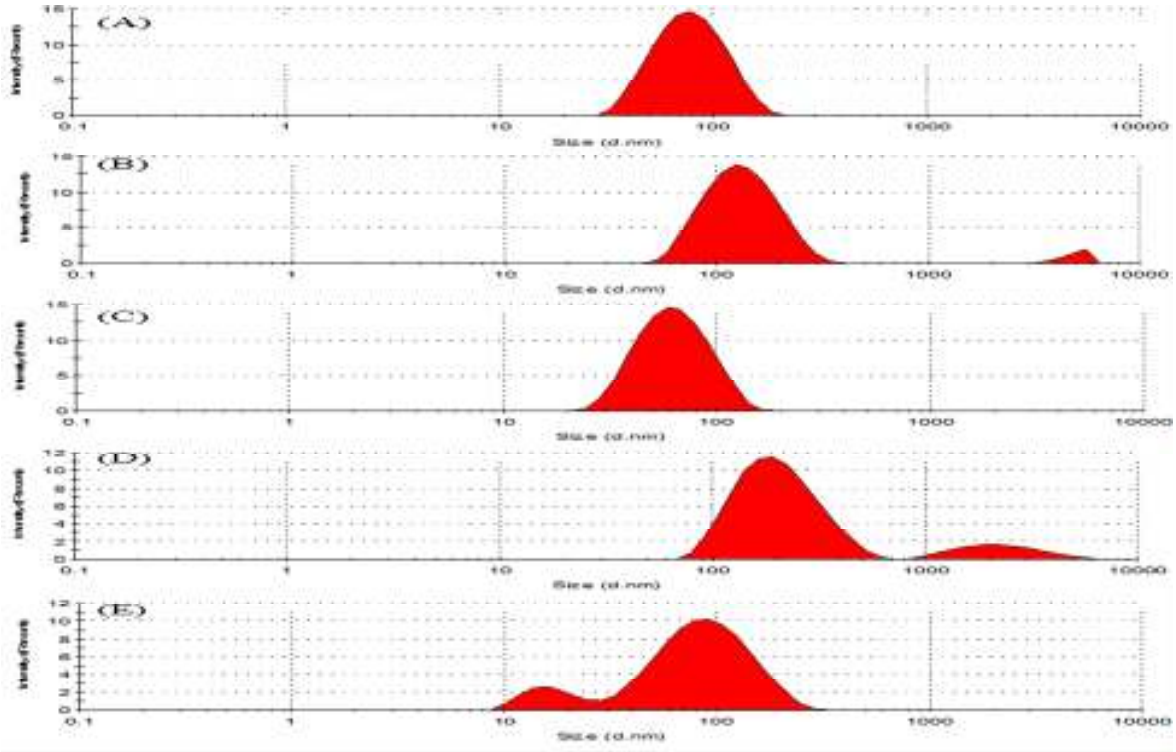

Figure 3. Droplet size distribution measurement by dynamic light scattering (DLS) of NEs monoterpenes. A: (*R*)-carvone, B: cinnamaldehyde, C: citral, D: geraniol and E: pulegone.

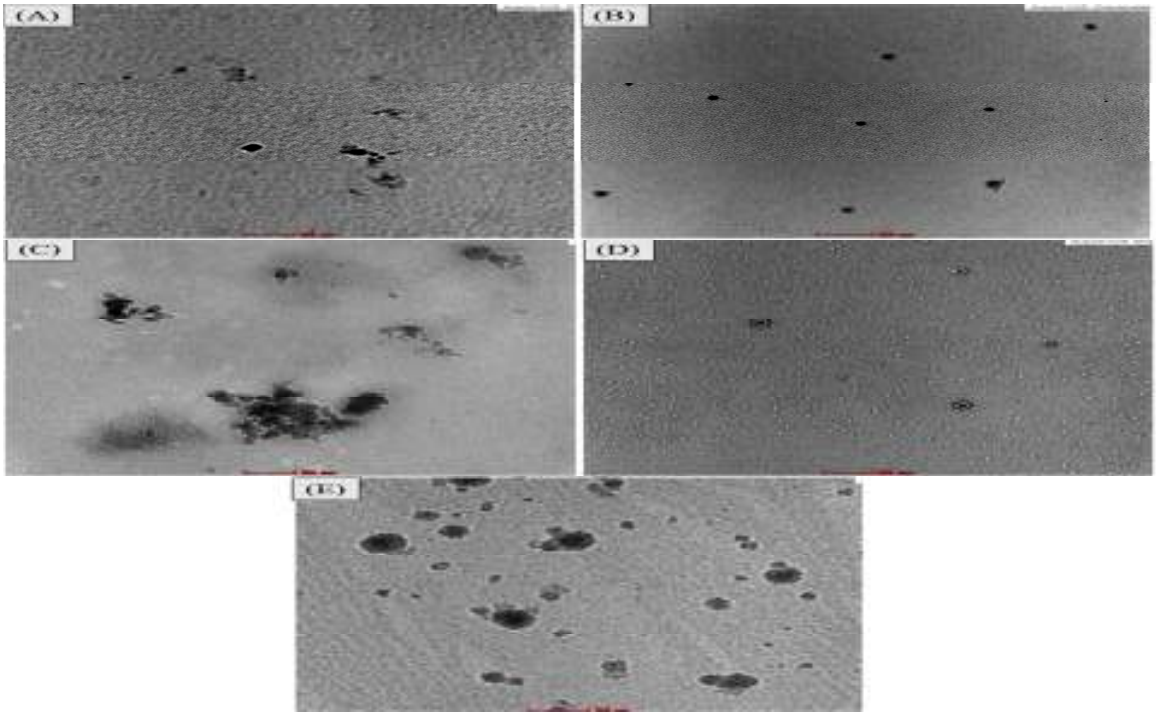

Figure 4. TEM image of NEs monoterpenes. A: (*R*)-carvone, B: cinnamaldehyde, C: citral, D: geraniol and E: pulegone.The TEM was performed on a JEOL JSM-1200EX-electron microscopy operating at an acceleration voltage of 67 kV with 20 µm aperture.

Isolation and identification of bacteria

The two tested bacterial have rods, non spore, gram negative and motile (Table 3). Positive reaction for catalase activity, gelatin liquefaction and acid production from lactose, Arabinose, Mannose, Raffinose and Sorbitol was detected for *P. carotovorum* and 40 ºC for *R. solanacearum*. Furthermore, both two tested bacterial grow at 37ºC However, negative reaction for hydrolysis of starch and inability to produce acids from maltose, adonitol and dextrin were detected.

Table 3. Some morphological, physiological and biochemical activities of *P. carotovorum* and *R. solanacearum*.

Characteristics	Bacterial isolates	
	P. carotovorum	*R. solanacearum*
Cell shape (Rods, single)	+	+
Sporulation	-	-
Motility	+	+
Gram reaction	-	-
Catalase activity	+	+
Gelatin liquefaction	+	+
Hydrolysis of starch	-	-
Sensitivity to erythromycin	-	
Anaerobic growth	+	+
Kovac's oxidase	-	+
Growth on NaCl 6%	+	-
Growth at	37C°	40 C°
Production of acid from		
Arabinose	+	+
Lactose	+	+
Manose	+	+
Maltose	-	-
Adonitol	-	-
Raffinose	+	+
Sorbitol	+	+
Dextrin	-	-
Sucrose		+

+ = positive reaction. - = negative reaction.

Antibacterial activity

The MICs of nanemulsion and normal cinnamaldehyde showed significantly higher inhibition (MIC ranged from 60 and 275 mgL^{-1}) against *P. carotovorum* than *R. solanacearum* (MIC ranged from 100 and 450 mgL^{-1})(Table 4). Several studies have reported enhancing the physical properties and antimicrobials of NEs-essential oils (EOs) compared to conventional emulsions (Buranasuksombat *et al.* 2011; São Pedro *et al.* 2013; Bilia *et al.* 2014; Guerra-Rosas *et al.* 2017). Results displayed that the NE was more effective as an antibacterial activity compared to normal monoterpenes. This is presumably due to the fact that the nanostructures of fat particles are able to bring primary oil to the surface of the cell membrane, while pure oil (low water solubility) failed or cannot easily interact with cell membranes. The results obtained are previously confirmed with (Zhang *et al.*, (2014), who reported n that NEs-EOs markedly enhanced antibacterial activity, D-limonene. Lambert *et al.*,(2001), clarified that the EOs containing carvacrol and thymol as monoterpenes, such as thyme oil exhibited a strong bactericidal action. Furthermore, eugenol and citral, the main components of clove, lemon or rosewood EO, were found, respectively to disrupt against a wide spectrum of microorganisms (Friedman *et al.*, 2004). Other aromatic compounds such as linalool, pinene, geraniol and borneol showed less inhibition effect against bacteria (Zachariah and Leela 2006). Hamouda and Baker (2000) reported the NE soybean oil had a good bactericidal activity against gram-positive bacteria.

Table 4. Minimum inhibitory concentration (MIC) of tested normal monoterpenes and their nanoemulsions against plant pathogenic bacteria *P. carotovorum* and *R. solanacearum*.

Treatment		Minimum inhibitory concentration (mg/L)	
		P. carotovorum	*R. solanacearum*
Carvone	N	1000	1250
	NE	400	500
Cinnamaldehyde	N	275	450
	NE	60	100
Citral	N	300	500
	NE	100	170
Geraniol	N	350	575
	NE	160	300
Pulegone	N	650	1000
	NE	200	600
Zenga		50	90

N: Normal, NE:Nanoemulsion

Effect of NE cinnamaldehyde defense related enzymes activity and total phenolic content of potato tubers and leaves.

The role of PPO, POD and total phenolic content on imparting resistance to soft and brown rot diseases in potato tubers and leaves was considered in this study. Defense reactions were evaluated after 0, 6, 12, 24 and 48h post inoculations by *P. carotovorum* and *R. solanacearum* after the treatment with NE-cinnamaldehyde. This NE was the most significantly effective on reducing the rotted area diameters in tubers inoculated with *P. carotovorum* and wilt disease index in leaves inoculated with *R. solanacearum* compared to positive control (Table 5).

Table 5. Pathological reaction of potato tubers to *P. carotovorum* and wilt disease index developed on potato leaves inoculated with *R. solanacearum*.

Treatment	Diameterrotted area (cm)	Wilt disease index
	P. carotovorum	*R. solanacearum*
UU	0.00 ± 0.00^{a}	1
UI	3.57 ± 0.10^{a}	5
NC-1000	1.22 ± 0.67^{c}	2
NC-1000	2.22 ± 0.11^{b}	4

UU, untreated uninoculated; UI, untreated inoculated; NC-1000, Nanoemulsion cinnamalehyde at 1000mg/L, NC-3000, Nanoemulsion cinnamalehyde at 3000mg/L, *Data with the same letter(s) within a column are not significantly different according to Duncan's a new multiple range test.

Results in Table 6 showed that the PPO and POD activities in infected potato tubers were increased by the infection with *P. carotovorum* compared with the positive control. NE-cinnamaldehyde at rate 1000 mg/L (NC-1000) was the most effective treatment, followed by NE-cinnamaldehyde at rate 3000 mg/L (NC-3000) and they, significantly, raised the values of the total phenolic content as compared to the control. Both applied treatments, significantly, enhanced enzymes activity comparing with the control. Results also showed that the PPO and POD activities in NC-1000 treatment was significantly increased under all the tested time. However, application of NC-3000 exhibited the highest of both PPO and POD activities at 12h are 0.67 and 0.75 (OD min g^{-1} fresh tissue, respectively), then, declined. An increase of total phenolic content in NC-1000 level was detected at 24 h (95.84 µg of catechol g^{-1} fresh tissue), then, declined, whereas; total phenolic increased with the time at rate NC-3000.

Likewise, data in Table (7) revealed that PPO and POD activity and total phenolic content were significantly increased with NE cinnamaldehyde by the infection with *R. solanacearum* in potato leaves compared with control. PPO and POD activities in NC-1000 treatment was showed highest effect at 42 h are 3.89 and 1.37(OD min g^{-1} fresh tissue, respectively), then, declined. However, NE cinnamaldehyde at rate 3000 mg/L exhibited the highest of both PPO and POD activities at 6h are 2.78 and 1.12 (OD min g^{-1} fresh tissue, respectively), then, declined. An increase of total phenolic content in doses NC-1000 and NC-3000 was detected at 24 h (140.84 and 126.39 µg of catechol g^{-1} fresh tissue), then, declined, and increased with the time at rate NC-3000 at 48h.The resistance of the potato was associated with high PPO and POD enzyme activities. In addition, higher activation of total soluble phenols (high concentration values) were detected indicating. These obtained results demonstrate that the PPO, POD, and total phenolic have an important role in inducing resistance to potato soft and brown rot infections. PPO catalyzes oxidation of phenols to quinones causing highly toxic to the microorganisms than the original phenolic compounds (Gandía-Herrero *et al.,*2005).The increase in the activities of oxidant defensive enzymes such as, POD and PPO were associated with resistance to *E. carotovora* (Ngadze *et al.,*2012).

Higher activation of the defense related enzymes; POD, PPO and total soluble phenols were detected due to the interactive effect between the pathogen and the host plant which induces some changes in cell metabolism (Vance *et al.* 1980; Fry 1982; Ngadze *et al.,*2012). Phenols and their oxidative products were shown to inhibit *P. carotovorum* (Lyon and McGill 1989; Weber *et al.,*1996) and the cell wall degrading enzyme activity of the bacteria (Lyon 1989).

Table 6. Polyphenol oxidase (PPO), peroxidase (POD) activity and total phenolic content in potato tubers after treatment with NE cinnamaldehyde followed by inoculation with *P. carotovorum*.

Treatment	Time (h)				
	0	6	12	24	48
PPO activity (ODmin^{-1}g $^{-1}$ fresh tissue)					
UU	0.45±0.01^{d}	0.51±0.01^{c}	0.52±0.01^{d}	0.54±0.00^{d}	0.55±0.00^{c}
UI	0.39±0.00^{e}	0.36±0.00^{d}	0.34±0.00^{e}	0.33±0.00^{e}	0.28±0.02^{d}
NC-1000	0.63±0.00^{a}	0.75±0.00^{a}	0.77±0.00^{a}	0.78±0.00^{a}	0.79±0.02^{a}
NC-3000	0.51±0.00^{c}	0.60±0.01^{b}	0.67±0.01^{c}	0.59±0.00^{c}	0.53±0.01^{c}
POD activity (OD min^{-1}g $^{-1}$ fresh tissue)					
UU	0.90±0.01^{c}	0.96±0.00^{c}	0.98±0.00^{c}	1.03±0.00^{c}	1.06±0.00^{c}
UI	0.51±0.00^{e}	0.50±0.00^{e}	0.45±0.00^{e}	0.41±0.00^{e}	0.33±-.01^{e}
NC-1000	1.00±0.00^{a}	1.16±0.03^{a}	1.25±0.00^{a}	1.34±0.33^{a}	1.48±0.04^{a}
NC-3000	0.73±0.00^{d}	0.88±0.01^{d}	0.75±0.00^{d}	0.61±0.00^{d}	0.59±0.00^{d}
Total Phenolic (µg of catchole g $^{-1}$fresh tissue)					
UU	48.77±0.26^{d}	59.43±0.17^{c}	51.359±0.10^{d}	71.39±0.29^{c}	83.62±-.35^{d}
UI	84.33±0.12^{a}	60.22±0.10^{c}	53.51±-0.66^{c}	78.90±1.02^{b}	93.83±0.20^{b}
NC-1000	76.91±0.28^{b}	84.84±0.31^{ab}	94.15±0.25^{a}	97.29±0.10^{a}	88.26±0.12^{c}
NC-3000	68.21±0.74^{c}	81.47±0.51^{b}	91.34±0.25^{b}	95.84±0.59^{a}	104.14±0.18^{a}

UU, untreated uninoculated; UI, untreated inoculated; NC-1000, Nanoemulsion cinnamaldehyde at 1000mg/L, NC-3000, Nanoemulsion cinnamaldehyde at 3000mg/L, *Data with the same letter(s) within a column are not significantly different according to Duncan's a new multiple range test.

Table 7. Polyphenol oxidase (PPO), peroxidase(POD) activity and total phenolic content in potato leaves after treatment with NE cinnamaldehyde followed by inoculation with *R. solanacearum*.

Treatment	Time (h)				
	0	6	12	24	48
PPO activity (OD min^{-1}g $^{-1}$ fresh tissue)					
UU	1.93±0.012^{c}	2.02±0.10^{c}	2.06±0.00^{c}	2.10±0.01^{c}	2.25±0.04^{b}
UI	2.05±0.01^{c}	1.93±0.03^{c}	1.25±0.00^{d}	1.17±0.01^{d}	0.97±0.01^{d}
NC-1000	3.17±0.12^{a}	3.48±0.09^{a}	3.71±0.03^{a}	3.89±0.01^{a}	3.24±0.05^{a}
NC-3000	2.52±0.06^{b}	2.78±0.07^{b}	2.77±0.02^{b}	2.29±0.09^{b}	1.96±0.04^{c}
POD activity (OD min^{-1}g $^{-1}$of fresh tissue)					
UU	0.82±0.00^{d}	0.84±0.00^{c}	0.86±0.00^{c}	0.88±0.00^{c}	0.91±0.00^{c}
UI	0.83±0.00^{c}	0.81±0.01^{d}	0.77±0.00^{d}	0.73±0.00^{d}	0.70±0.00^{d}
NC-1000	1.05±0.00^{a}	1.16±0.00^{a}	1.23±0.00^{a}	1.37±0.04^{a}	1.09±0.00^{a}
NC-3000	0.97±0.00^{b}	1.12±0.01^{b}	1.08±0.00^{b}	1.03±0.00^{b}	1.00±0.00^{b}
Total Phenolic (µg of catechol g $^{-1}$fresh tissue)					
UU	74.91±0.37^{d}	87.09±0.91^{c}	111.02±0.37^{c}	124.61±0.33^{b}	125.68±0.67^{b}
UI	91.62±1.50^{c}	68.21±0.74^{d}	55.79±0.71^{d}	111.02±0.96^{a}	125.94±0.46^{b}
NC-1000	127.22±0.36^{a}	138.37±0.13^{a}	140.84±0.18^{a}	133.64±0.10^{a}	125.80±0.52^{b}
NC-3000	124.52±0.23^{b}	135.38±0.36^{b}	126.39±0.26^{b}	117.67±0.35^{c}	134.27±0.26^{a}

UU, untreated uninoculated; UI, untreated inoculated; NC-1000, Nanoemulsion cinnamaldehyde at 1000mg/L, NC-3000, Nanoemulsion cinnamaldehyde at 3000mg/L, *Data with the same letter(s) within a column are not significantly different according to Duncan's a new multiple range test.

CONCLUSION

Based on the results of this study, the conversion of monoterpenes to nanoemulsion has significantly improved its antibacterial activity against important plant pathogenic bacteria (*P. carotovorum* and *R. solanacearum*). Nanoemulsion may be specially effective transmission systems for essential oils and their components because of their ability to facilitate the application of antimicrobials and increase the effectiveness of antimicrobials. However, as these tests were conducted *in vitro*, and *in vivo* studies on potato.

Conflict of interest

None.

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تجهيز وتوصيف مستحلبات النانو لبعض التربينات الأحادية ونشاطها البكتيرى

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في الدراسة الحالية، تم تحضير وتوصيف للتربينات الاحادية من الكارفون والسينامالدهيد والسيترال والجيرانيول والبوليجون في صورة مستحلبات نانوية باستخدام الموجات فوق الصوتية وتقييم الفاعلية البيولوجية على ممرضات النبات البكتيرية مثل البكتريا المسببة للعفن البنى والطرى. وتم تحضير المستحلبات النانوية من التربينات الاحادية بتركيز ٥٪ بالتدريج في محلول ماني يحتوي على مستحلب توبين ٨٠ (١٠%) مع التقليب المستمر ثم معاملتها بالموجات فوق الصوتية. وتم تقييم ثبات التجهيزه النانوية ودراسة خواصها عن طريق جهاز تشتيت الضوء الديناميكى (DLS) والمجهر الالكترونى النافذ (TEM). وكذلك معرفة حجم القطرات بالتجهيزه مع دراسة الثبات الحرارى واللزوجة. وتراوح موشر PDI من ٠.١٣٠ إلى ٠.٦٣٠ وحجم القطرات في نطاق ٥٦.٦٤-١٧٦.٠٠ نانوميتر. وأظهرت تجهيزه النانو لزيت السينامالدهيد نشاط بكتيرى عالي على بكتريا *P. carotovorum* و*R. solanacearum* حيث كانت قيم MIC تساوى ٦٠ و ١٠٠ مجم/لتر، على التوالي. وتم دراسة التأثيرالوقائى لمستحلب النانو لمركب السينامالدهيد كمقاومة مستحثة باستخدام جرعات ١٠٠٠و٣٠٠٠ مجم/لتر على فترات زمنية مختلفة. وتم التاكد من ذلك بدراسة العديد من الأنزيمات ذات الصلة بالدفاع النباتي مثل البولي فينول أوكسيديز (PPO) والبيروكسيديز (POD) ومحتوى الفينول الكلى في نبات البطاطس (صنف سبونتا) وذلك بحدوث عدوى للدرنات ببكتريا *P. carotovorum* وللاوراق ببكتريا *R. solanacearum*. واظهرت النتائج انخفاض أعراض مرض العفن البني والطرى للبطاطس على مدى الفترات الزمنية المختبرة. وتظهر هذه النتائج أن انزيمات PPO ، POD، والفينول الكلي القابل للذوبان تلعب دورا هاما في المقاومة المستحثة للعدوى بالعفن البني والطرى لنبات البطاطس. وتظهر الدراسة الحالية انه يمكن تجهيز مستحلبات النانو من الزيوت الطيارة (مثل التربينات الاحادية) كمبيدات نانوية خضراء عالية الفاعلية ضد البكتريا النباتية الممرضة كمبيدات اقل تكلفة وامنة وصديقة للبيئة.