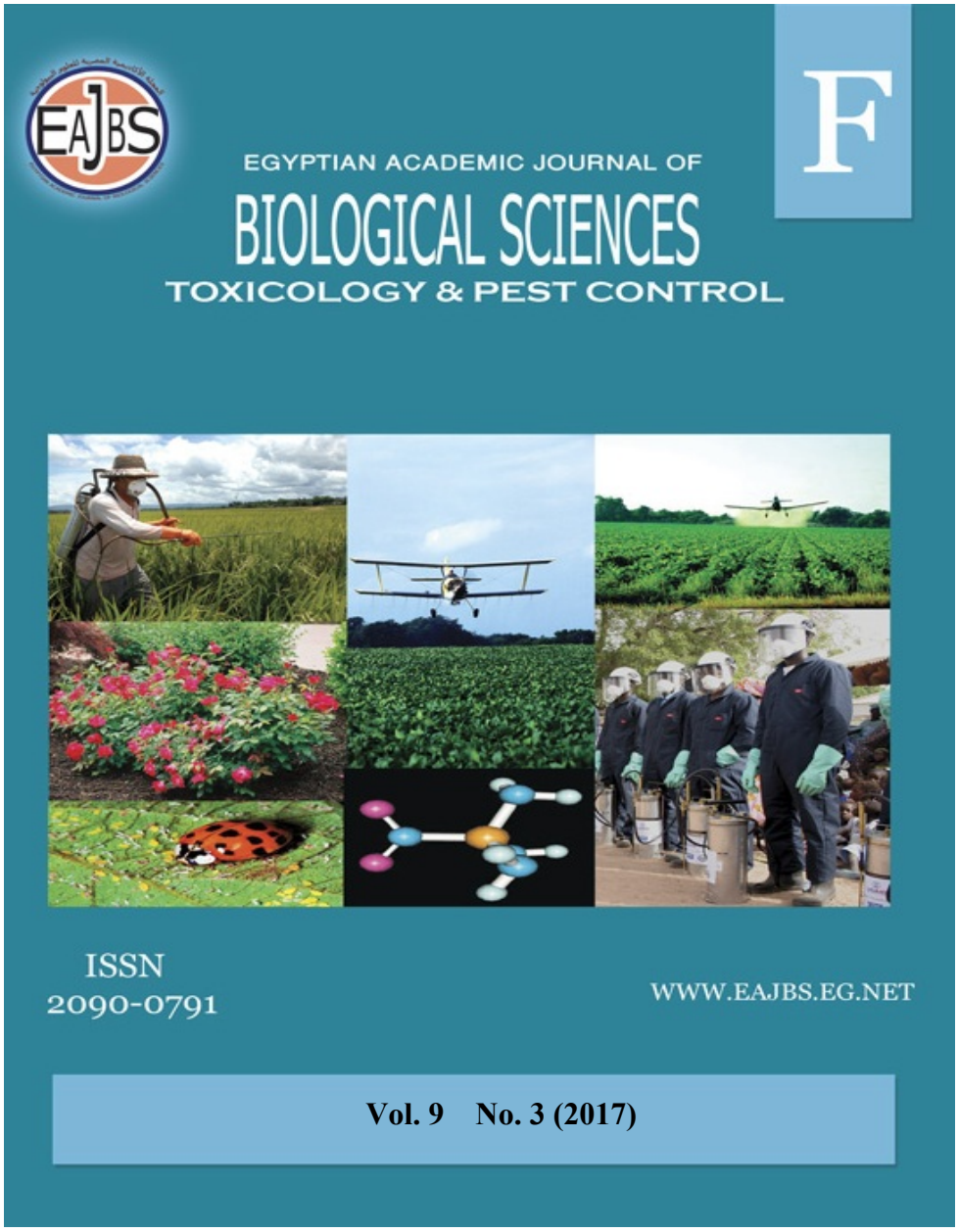


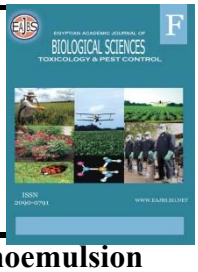
The journal of Toxicology and pest control is one of the series issued twice by the Egyptian Academic Journal of Biological Sciences, and is devoted to publication of original papers related to the interaction between insects and their environment.

The goal of the journal is to advance the scientific understanding of mechanisms of toxicity. Emphasis will be placed on toxic effects observed at relevant exposures, which have direct impact on safety evaluation and risk assessment. The journal therefore welcomes papers on biology ranging from molecular and cell biology, biochemistry and physiology to ecology and environment, also systematics, microbiology, toxicology, hydrobiology, radiobiology and biotechnology.

www.eajbs.eg.net

Egypt. Acad. J. Biolog. Sci., 9(3): 169-182 (2017)

Egyptian Academic Journal of Biological Sciences
F. Toxicology & Pest control
ISSN: 2090 - 0791
www.eajbs.eg.net



Ultrasonic Emulsification and Characterizations of Bio-based Nanoemulsion Formulations Containing Citral with Their Antimicrobial Activity

Gehan I. Kh. Marei[1], Entsar I. Rabea[1], Mohamed E. I. Badawy[2]

1- Department of Plant Protection, Faculty of Agriculture, Damanhour University, Damanhour 22516, Egypt.

2- Department of Pesticide Chemistry and Technology, Faculty of Agriculture, 21545 El -Shatby, Alexandria University, Alexandria, Egypt

ARTICLE INFO

Article History
Received:5/9/2017
Accepted:11/11/2017

Key words:
Citral
Chitosan
Nanoemulsion
Ultrasonication
SEM
DLS
Antimicrobial activity

ABSTRACT

Natural antimicrobial agents, particularly essential oils existing broad-spectrum antimicrobial activity, unique mechanisms of action and low tendency to induce resistance. However, their potential as a viable antimicrobial alternative greatly compromised due to their hydrophobic and volatile nature. The current study deals with the formulation and characterization of bio-based oil in water (O/W) nanoemulsions and their potential antimicrobial activity against some plant pathogens. Nanoemulsion was prepared using citral as the oil phase, chitosan as a biopolymer carrier, tween 80 as a surfactant, and sodium tripolyphosphate (TPP) as a polyanioncrosslinker by ultrasonication method. The success of formulation was confirmed by dynamic light scattering (DLS) and scanning electron microscopy (SEM) techniques. Physical stability and viscosity were investigated in details. The antibacterial activity of formulations were evaluated against *Erwinia carotovora* using ELISA technique by measuring the minimum inhibitory concentration (MIC). The results of DLS and SEM measurements showed that the nanoemulsions had a nearly polydispersity index(PDI) ranged from 0.508 to 0.614 and these values decrease when the concentration of the citral increase. Particle size analysis showed that the mean particle sizes of these formulations ranged from 27 to 1283 nm. Stability studies showed that the formulations were stable under centrifugation test at 5000 rpm for 30 min. Stability under different storage temperature showed that the five formulations were stable with no phase separation for the duration of 1 month at 25°C and 4°C. The antibacterial activity of the essential oil against *E.carotovora* was enhanced considerably when it was converted into a nanoemulsion, which was attributed to easier access of the essential oils to the bacterial cells. The highest antibacterial activity (MIC= 23 mg/L) was observed with the low concentration of citral, which had the lowest Particle size value (27 nm).

INTRODUCTION

Essential oils (EOs) are aromatic and volatile oily liquids obtained from plants. They normally formed in cells or groups of cells, found in leaves and stems, and commonly concentrated in one particular region such as leaves, bark or fruit (Gouvea *et al*. 2017; Oussalah *et al*. 2006; Solórzano-Santos and Miranda-Novales 2012). EOs extracted from plants or spices are rich sources of biologically active compounds such as terpenoids and phenolic acids which having antibacterial, antifungal, antioxidant and antiviral activities (Aumeeruddy-Elalfi *et al*. 2015).

The antibacterial and antifungal activities of EOs have been long recognized (Nielsen *et al*. 2017). However, the EOs are volatile compound which easily evaporates and/or decomposes during preparation of antimicrobial film owing to direct exposure to heat, pressure, light or oxygen. Therefore, in order to overcome the susceptibility and improve the stability of bioactive compounds, there are a number of potential technological challenges associated with incorporating EOs into suitable formulations (Sugumar *et al*. 2013). Encapsulation of functional EOs within nanoparticles has been investigated as a potential strategy for improving their utilization, stability, and efficacy (Donsì *et al*. 2011; McClements and Rao 2011; Qian and McClements 2011). Among the nanoformulations currently be utilized for the delivery of bioactive components, nanoemulsions have been reported to be especially appropriate for utilization in crop protection, due to their ease of preparation and desirable functional attributes (Balaure *et al*. 2017; Kah and Hofmann 2014).

The small particle size may increase interactions between the active compounds with biological membranes, as well as their transfer through them. Moreover, nanoemulsions can be designed to have a good kinetic stability and low turbidity, which is appropriate for a broad range of commercial applications (Solans *et al*. 2005). Sever a techniques have been applied for producing nanoemulsions, including various low-energy and high-energy methods. Ultrasonic emulsification is a high-energy method that is rapidly and efficiently capable of preparing nanoemulsions with small droplet diameters and narrow size distributions (Ghosh *et al*. 2013; Manchun *et al*. 2014). EOnanoemulsions have been reported previously to be effective antibacterial treatments (Liang *et al*. 2012; Zhang *et al*. 2014; Ziani *et al*. 2011); however, knowledge of their mode of action against microorganismsis currently limited.

Citral is an R,β-unsaturated aldehyde with one additional double bond (its two isomers, neral and geranial) (Schieberle and Grosch 1988). Citral is present in the oils of several plants, including lemon myrtle *Backhousia citriodora* (90-98%), *Litsea citrata* (90%), *Litsea cubeba* (70-85%), lemongrass (65-85%), lemon tea-tree (70-80%), *Ocimum gratissimum* (66.5%), *Lindera citriodora* (65%) and *Calypranthes parriculata* (62%). It also has strong antimicrobial qualities and pheromonal effects in insects. Citral showed appreciable antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi.

The nanoemulsion consists of an oil phase dispersed in an aqueous continuous phase, with each oil droplet surrounded by a thin interfacial layer of surfactant molecules, which helps in stabilizing the nanoemulsion system to a more stable formulation (Tadros *et al*. 2004). Nanoemulsions are kinetically stable and are transparent to translucent in appearance. Recently the nanoemulsion formulations using EOs and non-ionic surfactant have been used as natural antimicrobial formulations in the agriculture sector. This has become the point of interest, because of the improvement in the safety. The role of the plant based oil in the emulsion system helps in preventing degradation and improving bioavailability and biocompatibility (Tadros *et al*. 2004). In addition, naturally occurring polymers such as chitosan is widely used in agricultural fields in various forms such as nanoparticles, capsules, and emulsions. This polymer has attractive properties because of their biodegradability, biocompatibility, and nontoxic nature. Ionic gelation technique

based on the electrostatic interaction between the positively charged primary amino groups of chitosan and the negatively charged groups of polyanion, such as sodium tripolyphosphate (TPP) (Yang *et al*. 2011). The aim of the present work was therefore formulation and characterization of bio-based oil in water nanoemulsions containing citraland their potential antimicrobial activity against bacterium *Erwinia carotovora* and fungi *Aspergillus niger* and *Rhizopus stolonifer*. Nanoemulsion preparation method was high-shear stirring, which is extensively used for pesticide formulation preparation. The mixing ratio of different levels of active ingredient citral, sonication, chitosan and TPP were performed to design the experiment. The effect of these factors on the long-term stability of the nanoemulsions, droplet size distribution (PDI) by dynamic light scattering, viscosity and surface morphology by scanning electron microscope were investigated.

MATERIALS AND METHODS

Chemicals and reagents

Citral oil, 3,7-dimethyl-2,6-octadienal(a mixture of geranial (Trans, 55-70%) and neral (cis, 35-45%), low molecular weight chitosan (made from coarse ground crab, 89% degree of deacetylation), Tween 80, dimethyl sulphoxide (DMSO), sodium tripolyphosphate (TPP) and 2,3,5,-triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potato Dextrose Agar (PDA), Nutrient Broth (NB), and Nutrient Agar (NA) media were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK). All other commercially available solvents and reagents were used without further purification.

Tested microorganisms:

Plant pathogenic bacterium *Erwinia carotovora* was obtained from Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt. The culture was maintained on NA medium at 37ºC. Plant pathogenic fungi black mould *Aspergillus niger* (Family: Trichocomaceae, Class: Eurotiomycetes) and black bread mould *Rhizopus stolonifer* (Family: Mucoraceae, Class: Zygomycetes) were provided by Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt, and kept during the experiments on PDA medium at 27 ± 2°C.

Nanoemulsions preparation:

Chitosan solution (1% (w/v)) was prepared by agitating chitosan in an aqueous acetic acid solution (1% (v/v)) overnight. Tween 80 added as a surfactant to the solution and stirred at 45°C for 2 h to obtain a homogeneous mixture. Citral (0.04, 0.08, 0.16 and 0.32 g) was dissolved separately in DMSO (4 mL) and then this oil phase gradually dropped into the aqueous chitosan solution (40 mL)during homogenization at a speed of 13,000 rpm for 10 min under an ice-bath condition to obtain an oil-in-water emulsion. TPP solution (0.4%, w/v) was then added dropwise into the agitated emulsion. Agitation was continuously performed for 40 min. Finally, ultrasonication was performed by a Sonicatior (Ultrasonic Homogenizers HD 2070 with HF generator (GM 2070), ultrasonic converter UW2070, booster horn SH 213 G and probe microtip MS 73, Ø 3 mm) as shown in Figure1.The tip of the horn was symmetrically placed in the coarse emulsion, and the process was carried out at10 min, power 50 kHz and pulses or cycles 5 cycle /sec controlled by the software of the device to produce the nanoemulsions. Weight ratios of chitosan: citral of 1:0.1, 1:0.2, 1:0.4, 1:0.8 and 1:0.0, respectively as F1, F2, F3, F4 and F5, respectively were used for the present study (Hosseini *et al*. 2013).

Characterization of nanoemulsions

Scanningelectron microscopy (SEM):

Scanning electron microscopy (SEM) is a method for high-resolution imaging of surfaces. SEM analysis was done by using a JEOL JSM-5410 (Japan) electron microscope with a W-source and operating at 80 kV. Sample was prepared on a glass slide (1 × 1 cm) after washing it with ethanol.A tiny drop of nanoemulsion was spreaded evenly over glass slide and allowed to air dry. In order to make it conductive, gold coating with Jeol Quick Auto Coater was performed (JFC-1500). The nanoemulsions were then subjected to SEM analysis under ambient conditions.

Particle size and poly dispersity index (PDI) measurement:

Measurement of particle size and poly dispersity index (PDI) of the nanoemulsions were performed using a Dynamic Light Scattering (DLS) method using Zetasizer Nano ZS (Malvern Instruments, UK) at room temperature(Yuan *et al*. 2008). All nanoemulsion samples were diluted before measurements to 10% with deionized water to avoid multiple scattering effects. Emulsion droplet size was estimated by the average of three measurements and presented as mean diameter in nm. The higher the PDI value refers to the lower uniformity of globules size of nanoemulsion.

Viscosity measurement:

The dynamic (absolute) viscosity of the nanoemulsions was determined using a Rotary Myr VR 3000 digital viscometer with L1spindle at 200 rpm at 26.5°C. Viscosity of the nanoemulsion formulations were measured from approximately 50 mL of sample without further dilution at range 30mPa.s. Each reading was taken after the equilibrium of the sample for 2 min. The samples were repeated three times and the data expressed in mPa.s (Badawy *et al*. 2017).

Centrifugation assay:

An accelerated storage testing was carried out to predict the long-term physical stability of the nanoemulsions. This analysis of nanoemulsions was performed by using centrifugation at 5000 rpm for 30 min and then measuring any phase separation. The nanoemulsions should have maximum stability, which is not a phase separation (creaming and cracking). The measurements performed in triplicate (Shafiq and Shakeel 2010).

Freeze thaw cycle nanoemulsions:

This test was carried out for the determination of the accelerated stability of the nanoemulsion formulations. The formulations were subjected to the two different temperatures (-21°C and 21°C.) for each temperature test of at least 24 h.(Kadhim and Abbas 2015).The measurements were performed in triplicate.

Stability at temperature of 25°C:

About 25 mL of freshly prepared nanoemulsions were transferred to a glass tube. The transition from steady state to creaming and coalescence was examined during the storage period of4 week sat temperature of 25°C.

Antimicrobial assay:-

Antibacterial activity :

NB mediumwas used to grow the bacterial strains to a final inoculumsize of 5×10^5cfu/mL calculated as a number of colonies× dilution factor/volume of culture plate using haemocytometer. Nanoemulsion formulations were added to the wells of a sterile 96well microtitre plate, followed by the addition ofNB medium and then 20 μL of bacterial suspension. The final volume in each well was 200 μL and the concentrations of 0.0, 56.25, 62.5, 112.5, 125, 225, 250, 450, 500, 900, and1000 mg/L were tested for each compound. Control wells were prepared with culture medium, bacterial suspensiononly, and solvent. The contents of each well were mixed ona microplate shaker at 200 rpm for 1 min prior to incubation for 24 h at 37°C. To

indicate respiratory activity the presence of color was determined after adding 25μL/well of TTC dissolved in water (0.01%, w/v) as a chromogenic marker and incubated under appropriate cultivation conditions for30 min in the dark (Badawy *et al*. 2016). The absorbance was measuredat 492 nm in an Ultra Microplate Reader (Robonik, PVT, LTD). Positive controls were wells with a medium and the compounds. Negative controls were wells with the growth medium, bacterial suspension, and the TTC reagent. The minimum inhibitory concentration (MIC) was determined as the lowest concentration where no viability was observed after 24 h based on metabolic activity.

Antifungal activity:

The antifungal activity was tested using mycelia radial growth technique (Badawy *et al*. 2014). The compounds were dissolved and serial concentrations ranged from 50 to 3000 mg/L were tested. The aliquots of the stock solutions were added to the PDA medium and then transferred to Petri dishes. After solidification, the mixtures were inoculated with a 5 mm in diameter mycelium fungi at the center of Petri dishes and these were incubated in the dark at 27 ± 2^{o}C. Fungal growth was measured when the control had grown to the edge of the plate. The inhibition of fungal growth was calculated as the percentage of inhibition of radial growth compared to the control. The effective concentration that inhibits 50% of mycelial growth (EC_{50}) for each compound was estimated by probit analysis (Finney 1971) using SPSS 21.0 software.

Statistical analysis:

Statistical analysis was performed using SPSS 21.0 software (Statistical Package for Social Sciences, USA). All experiments were repeated at least 3 times. The data were expressed as the mean ± standard error (SE).The log dose-response curves allowed determination of the EC_{50}values for the fungal bioassay according to the probit analysis (Finney 1971). The 95% confidence limits for the range of EC_{50}values were determined by the least-square regression analysis of the relative growth rate (% control) against the logarithm of the compound concentration.

RESULTS AND DISCUSSION

Preparation of chitosan-citralnan-oemulsions:

When one of two immiscible liquid phases is dispersed as droplets, the resulting mixture is referred to an emulsion (Akoh 2017). Nanoemulsions consist of oil droplets in the nano-ranged size dispersed within an aqueous continuous phase, with each oil droplet surrounded by surfactant molecules (Acosta 2009). Citral is a monoterpene aldehyde, which is the major component of lemon grass oil extracted from *C. citratus* belonging to Gramineae. Citral oil based nanoemulsionwas formulated in two phases (Fig. 1). First, coarse emulsion was prepared by adding chitosan to the oil phase containing citral oil in different ratios (1.0:0.1, 1.0:0.2, 1.0:0.4 and 1.0:0.8, chitosan: citral, respectively) which after subjecting to ultrasonic emulsification resulted into nanoemulsions and then subjected to various characterization techniques to ascertain their size and shape.

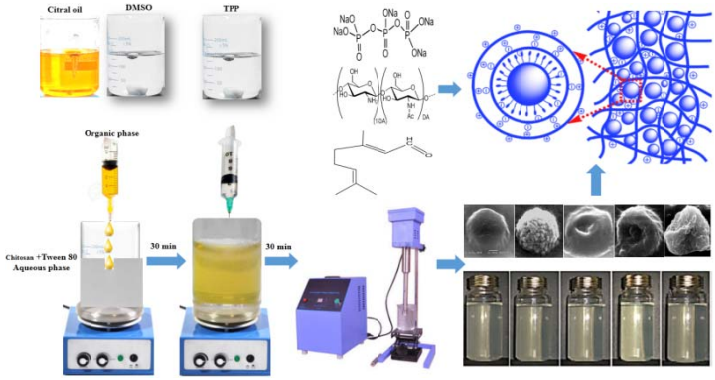


Fig. 1: Schematic representation of formation of chitosan-citralnanoemulsions.

Characterization of chitosan-citralnanoemulsions:

Morphology and shape of the citral-loaded nanoemulsions were studied using scanning electron microscopy (SEM) as shown in Fig. 2. The shape of droplets was found to be spherical for F1-F4. However, the droplet of F5 (chitosan only) was of uniform shape and size. Tween 80 was preferred as a surfactant owing to its high hydrophilic-lipophilic balance (HLB) value, which is favorable for formulating oil-in-water nanoemulsion.

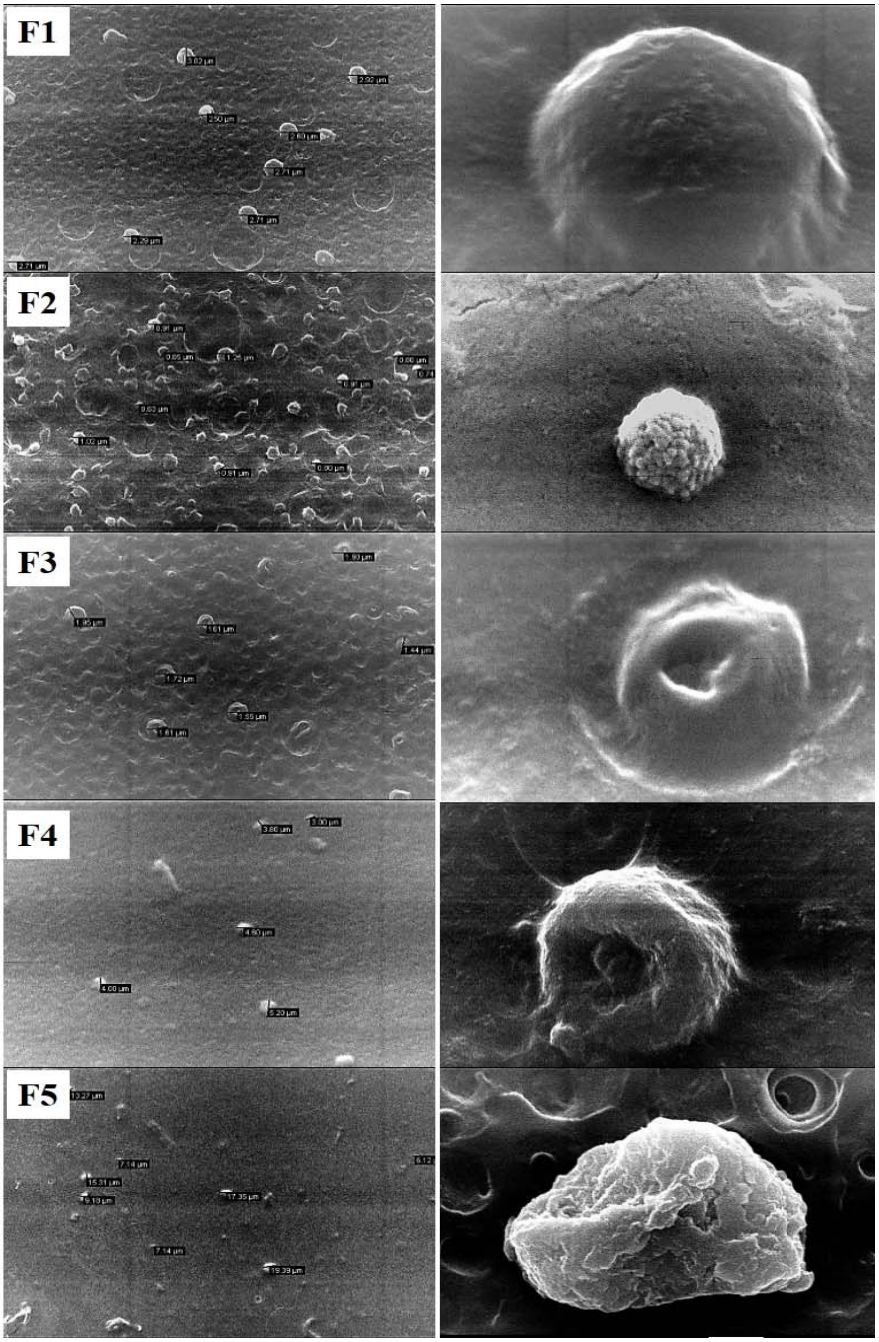


Fig. 2: Scanning electron micrograph of prepared chitosan-citralnanoemulsion formulations, F1 to F5. The SEM was performed on a JEOL JSM-1200EX II scanning electron microscope operating at an acceleration voltage of 80.0 kV with 20 μm aperture.

Tween 80 has an HLB value of 15. In addition, Tween 80 is being a small molecule and more efficient in minimizing droplet diameter. Surfactants acts as emulsifiers and serve the process by lowering the free energy required for the preparation of nanoemulsion by decreasing interfacial tension at oil/water interface (Tadros *et al*. 2004). The polydispersity index (PDI) value represents the particle size distribution of the droplets and the homogeny and stability of the droplet size in the emulsion. A small PDI value indicates a narrow particle size distribution. The results of the average droplet size and PDI of nanoemulsions are shown in Table 1 and their droplet size distribution is presented in Fig. 3. The PDI values were ranging from 0.508 to 0.614, indicating that all of the emulsions had a relatively narrow range of size distribution. PDI decreased significantly ($P< 0.05$) from 0.614 to 0.508 as the mass ratio of chitosan/citral increased from 1:0.1 to 1: 0.8, respectively. The citral nanoemulsion appeared transparent and the particle size is concentration dependent where the low citral concentration (ratio of 1:0.1 and 1:0.2) has the lowest particle size (27.0and 28.5 nm, F1and F2)as compared to the other formulations (from F3 to F5). The droplet size increased with the increase in concentration of oil in the formulations (see Table 1).

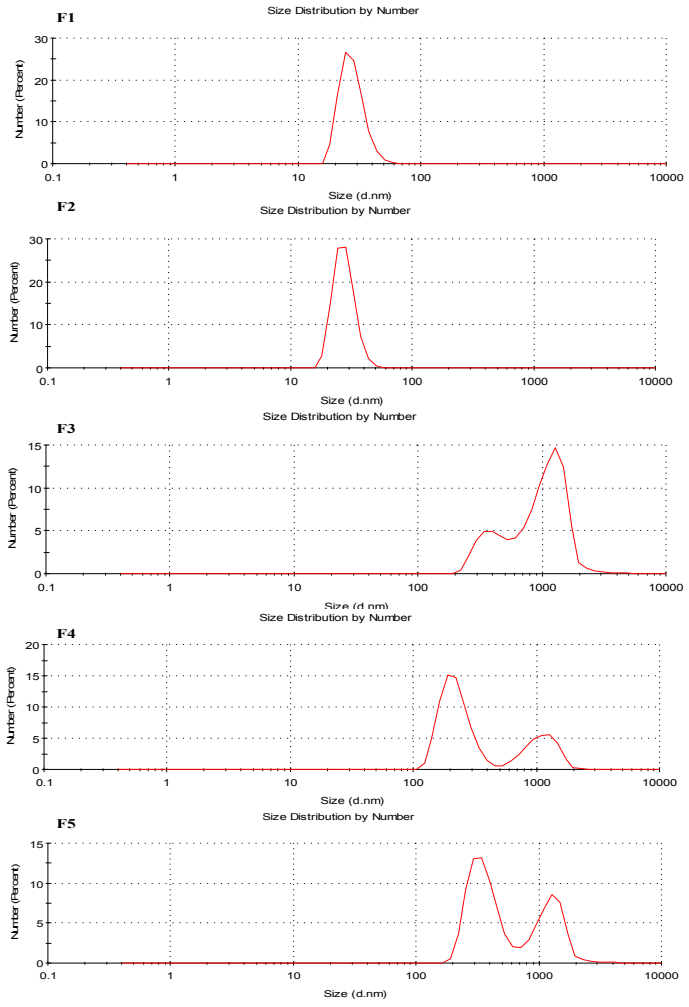


Fig. 3: A typical particle size distribution by a dynamic light scattering of the formulated chitosan-citralnanoemulsion formulations, F1 to F5.

Table 1: Mean droplet size, poly dispersing index and dynamic viscosity of chitosan-citralnanoemulsion formulations

Formulation	Weight ratios (chitosan: citral)	Droplet size (nm)	Poly dispersing index (PDI)	Dynamic viscosity (mPa.s.) ± SE
F1	1.0 :0.1	27.0	0.614	5.1±0.05
F2	1.0 :0.2	28.5	0.594	6.0±0.05
F3	1.0 :0.4	387	0.536	7.0±0.04
F4	1.0 :0.8	1115	0.508	8.0±0.05
F5	1.0 : 0.0	1283	0.571	3.1±0.04

The viscosity of nanoemulsions was found in the range of 5.1 to 8.0 mPa.s. Formulation F5 had the lowest viscosity (3.1 mPa.s.) ascompared to the other formulations (5.1-8.0mPa.s.) as shown in Table 1. The difference in viscosity of the formulationF5 was significant from other formulations but it was observed that the viscosity of all formulations was very low which is expected, as one of the characteristics of the nanoemulsion formulation is lower viscosity (Shakeel *et al*. 2009).It can also noticed that the viscosity is concentration dependent where F1 formulation with the lowest ratio of chitosan/citral (1:0.1, respectively) had the lowest viscosity. There are several factors influence emulsion viscosity such as the disperse phase volume fraction, rheology of component phases, droplet size, colloidal interactions, or droplet charge (McClements 2015; Pal 2011). Palreported that the higher the droplet size the lower the emulsion viscosity, at the same dispersed phase concentration and shear rate (Pal 2011).

Accelerated stability tests of chitosan-citralnanoemulsions

Nanoemulsions are thermodynamically stable systems and formed at a particular composition of oil, surfactant and water, with no phase separation, creaming, cracking, or coalescence. Centrifugation can accelerate the rate of sedimentation or incineration demonstrates that degradation of an emulsion may be related to the action of gravitational force. O/W emulsion system often exhibits creaming rather than sedimentation due to the lower density of the oil droplet compared to the aqueous medium. If nanoemulsions are going to be used as delivery systems for antimicrobial agents, then it is important that they have good physical stabilities during long-term storage. Thus, the selected citralnanoemulsions were subjected to different thermodynamic stability tests using centrifugation, heating-cooling cycle, and freeze-thaw cycle stress tests. The result of the accelerated stability study of formulations is shown in Table 2.

Stability at 25ºC was tested for four weeks. From these tests, it was found that all of the prepared formulations were stable at centrifugation of 5000 rpm, heating cycle, and freeze-thaw cycle.

Table 2: Thermodynamic characterization of chitosan-citralnanoemulsion formulations

Formulation	Weight ratios (chitosan: citral)	Centrifugation at 5000 rpm	Stability at temperature of 25°C				Freeze thaw cycle
			First week	Second week	Third week	Fourth week	
F1	1.0 :0.1	√	√	√	√	√	√
F2	1.0 :0.2	√	√	√	√	√	√
F3	1.0 :0.4	√	√	√	√	√	√
F4	1.0 :0.8	√	√	√	√	√	√
F5	1.0 : 0.0	√	√	√	√	√	√

Note: √, stable (without phase separation); ×, unstable (with phase separation).

No creaming, cracking, coalescence, or phase separation was observed on these formulations (Table 2). The steric effect of the non-ionic surfactant plays a vital rolein the stabilization of nanoemulsion (Tadros *et al*. 2004).

Antibacterial activity of chitosan-citralnanoemulsions against *E. carotovora*

The *in vitro* antibacterial activity of chitosan-citralnanoemulsionsagainst *E. carotovora*is presented in Table 3 as MIC values in mg/L. The measured MICs of the pure and emulsified citral showed significantly different inhibitory effects. The results demonstrated that all formulations showed higher inhibition (MIC ranged from 23to 520 mg/L) than citral (MIC >700 mg/L) against the tested bacterium. The data indicated that the inhibitory effects increased with the concentration of citraldecrease and the most inhibition effect was observed with F1 (1:0.1chitosan/citral, respectively).

Table 3: The *in vitro* antibacterial activity of chitosan-citralnanoemulsion formulations against *E. carotovora*by ELISA technique

Formulations	MIC* (mg/L)
F1	23
F2	40
F3	113
F4	238
F5	520
Chitosan	635
Citral	>700

* Minimum inhibitory concentration (MIC).

Chitosan formulation alone without citral gave MIC of 635 mg/L. The high activity of these nanoemulsions is due to their reduced droplet size and increased droplet surface area available to interact with bacterial cell. The nanoemulsions were found to have an effective bactericidal property whereas; enhanced growth was observed when bacteria were treated with oil alone.

Numerous studies have reported an enhancement of the physical and antimicrobial properties of EO-loaded nanoemulsions as compared to their conventional emulsions (Bilia *et al*. 2014; Buranasuksombat *et al*. 2011; Guerra-Rosas *et al*. 2017; São Pedro *et al*. 2013). A comparison of the antibacterial activity of pure and emulsified essential oils showed that the nanoemulsions were much more effective. Presumably, this is because the small lipid particles within the nanoemulsion were able to bring the essential oil to the cell membrane surface, whereas the pure oil (low water solubility) could not easily interact with the cell membranes. The obtained results are in agreement with other recent studies that showed that conversion of flavor oressential oils into nanoemulsions greatly improved their antibacterial activity, D-limonene (Zhang *et al*. 2014) and oregano oil (Bhargava *et al*. 2015). Lambert and co-authorsreported that the essential oils containingcarvacrol and thymolas monoterpenes, such as thyme oil have a strong bactericidal action (Lambert *et al*. 2001). However, eugenol and citral, the major components of clove and lemongrass or rosewood essential oil, respectively, have been found to inactivate against broad spectrum of microorganisms (Friedman *et al*. 2004). Other aromatic compounds such as linalool, pinene, geraniol and borneol, which can be found in plant essential oils, exhibit a lower inhibitory influence against bacteria (Scortichini and Rossi 1991; Zachariah and Leela 2006). Hamouda and Bakerreported that the soybean oil based nanoemulsion had a

good bactericidal activity against gram-positive bacteria (Hamouda and Baker 2000).

Antifungal activity of chitosan-citralnanoemulsions against *A. niger*and *R. stolonifera*:

The antifungal activity of chitosan-citralnanoemulsions against *A. niger* and *R. stolonifer* using mycelia radial growth technique is presented in Table 4.For the five formulations, F1 formulation exerted significantly potent antifungal activity with EC_{50} of 278 and 221 mg/L against *A. niger* and *R. stolonifer*, respectively. Followed by F2 in the descending order with EC_{50} of 396and 340 mg/L against *A. niger* and *R. stolonifer*, respectively. However, F4 and F5 formulations were the lowest active (EC_{50}= 1887, 1568, 1074 and 1433mg/Lagainst *A. niger* and *R. stolonifer*, respectively). For unformulated compounds, citral only showed good antifingal activity (EC_{50}= 474 and 437mg/L, respectively) compared with chitosan only (EC_{50}= 3016 and 2649mg/L, respectively). When we consider the susceptibility of the microorganisms, another point deserves attention; it can be noticed that fungous of*R. stolonifer* was more susceptible than *A. niger* to all formulations (Table 4). It can also noticed that the antifungal activity is concentration dependent where F1 formulation with the lowest ratio of chitosan/citral (1:0.1, respectively) had the highest antifungal activity. In conclusion, the antifungal activity of citral against *A. niger* and *R. stolonifer* was enhanced considerably when it was converted into a nanoemulsion (see F1 and F2 vs. citral only).

Table 4: The in vitro antifungal activity of chitosan-citralnanoemulsion formulations against A. nigerand R. stolonifer by mycelia radial growth technique

Formulations	EC_{50}[a] (mg/L)	95% confidence limits		Slope[b] ± SE	Intercept[c] ± SE	(χ^2)[d]
		Lower	Upper			
A. niger						
F1	278	150	514	3.45±0.33	-8.44±0.82	7.22
F2	396	337	449	2.54±0.31	-6.60±0.84	1.92
F3	710	626	827	2.40±0.32	-6.85±0.88	2.09
F4	1887	1293	4587	1.57±0.35	-5.16±0.97	1.75
F5	1568	1146	3068	1.66±0.34	-5.31±0.95	0.85
Chitosan	3016	2363	4157	1.19±0.25	-4.13±0.84	0.99
Citral	474	410	538	2.38±0.30	-6.37±0.83	0.62
R. stolonifer						
F1	221	162	309	6.16±0.49	-14.45±1.15	6.72
F2	340	128	514	5.42±0.47	-13.75±1.24	9.10
F3	708	610	856	1.98±0.30	-5.65±0.84	1.92
F4	1074	861	1600	1.70±0.32	-5.15±0.87	0.86
F5	1433	1045	2903	1.47±0.32	-4.63±0.89	1.08
Chitosan	2649	1222	7494	2.1±0.26	-7.05±0.89	4.73
Citral	437	72	785	4.67±0.39	-12.35±1.04	13.04

[a]The concentration causing 50%mycelialgrowth inhibition.
[b]Slope of the concentration-inhibition regression line ± standard error.
[c]Intercept of the regression line ± standard error.
[d]Chi square value.

In the previous literatures, a number of studies investigating the antimicrobial activity using nanoemulsion against fungi and yeast have been published (Joe *et al*. 2012; Ziani *et al*. 2011). Saddiq and Khayyat reported the growth of *P. italicum* and *R. stolonifer* on the solid media was reduced in the presence of citral and its epoxide (Saddiq and Khayyat 2010). Low concentrations (12.50 - 200 mg/L) of citral caused the greatest inhibition of

mycelium growth in both *Magnaporthe grisea* and *Botrytis cinerea* with mycelial inhibition values of 22.84% to 91.34% and 9.76% to 92.12%, respectively (Li *et al*. 2015). Moreover, citral caused a lower inhibition rate of mycelium growth of *Rhizoctonia solani* with EC_{50} values of 193 mg/L. Luoand others suggested a possible mechanism of citral action against *A. flavus* Link.After penetrating cell wall, irreversibly damages plasma membrane and DNA with consequent spore loss germination (Luo *et al*. 2001). Also in *A. niger*, the primary citral site action seems to be cell membrane. Moreover, citral was shown able of forming charge transfer complexes with tryptophan, a good electron donor. Apparently, the antifungal actions of the aldehydes, as citral, are due to their abilities to form charge transfer complexes with electron donors in addition to their reactivity with SH groups. In general, the mechanism of action of essential oils against microorganisms involves the interaction of phenolic compounds with the proteins in the cytoplasmatic membrane that can precipitate and lead to leakage of ions and other cell content causing the cell breakdown (Nychas *et al*. 2003).

CONCLUSION

Nanoemulsions are non-equilibrium colloidal systems formed by forcing two immiscible liquids into homogeneous state, which is kinetically stable. Based on the present study, the conversion of citral into a nanoemulsion greatly enhanced its antibacterial activity against an important plant pathogenic bacterium (*E. carotovora*) and fungi (*A. niger*and *R. stolonifer*). In conclusion, nanoemulsions may be particularly effective delivery systems for essential oils due to their ability to facilitate antimicrobial application and increase antimicrobial efficacy.

REFERENCES

Acosta E (2009). Bioavailability of nanoparticles in nutrient and nutraceutical delivery. Current opinion in colloid & interface Science, 14(1):3-15

Akoh CC (2017). Food lipids: chemistry, nutrition, and biotechnology. CRC press

Aumeeruddy-Elalfi Z, Gurib-Fakim A and Mahomoodally F (2015). Antimicrobial, antibiotic potentiating activity and phytochemical profile of essential oils from exotic and endemic medicinal plants of Mauritius. Industrial Crops and Products 71:197-204

Badawy ME, Rabea EI, AM El-Nouby M, Ismail RIand Taktak NE (2017). Strawberry Shelf Life, Composition, and Enzymes Activity in Response to Edible Chitosan Coatings. International Journal of Fruit Science 17(2):117-136.

Badawy ME, Rabea EI and Taktak NE (2014). Antimicrobial and inhibitory enzyme activity of N-(benzyl) and quaternary N-(benzyl) chitosan derivatives on plant pathogens. Carbohydrate polymers, 111:670-682

Badawy ME, Rabea EI, Taktak NE and El-Nouby MA (2016). The Antibacterial Activity of Chitosan Products Blended with Monoterpenes and Their Biofilms against Plant Pathogenic Bacteria. Scientifica Volume 2016, Article ID 1796256, 10 pages

Balaure PC, Gudovan D and Gudovan I (2017). NANOPESTICIDES: A NEW PARADIGM IN CROP PROTECTION. New Pesticides and Soil Sensors:129

Bhargava K, Conti DS, da Rocha SR and Zhang Y (2015). Application of an oregano oil nanoemulsion to the control of foodborne bacteria on fresh lettuce. Food microbiology, 47:69-73

Bilia AR, Guccione C, Isacchi B, Righeschi C, Firenzuoli Fand Bergonzi MC (2014). Essential oils loaded in nanosystems: a developing strategy for a successful therapeutic approach. Evidence-Based Complementary and Alternative Medicine, 242 (2) 7-12

Buranasuksombat U, Kwon YJ, Turner M and Bhandari B (2011). Influence of emulsion droplet size on antimicrobial properties. Food Science and Biotechnology, 20(3):793-800

Donsì F, Annunziata M, Sessa M and Ferrari G (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. LWT-Food Science and Technology, 44(9):1908-1914

Finney DJ (1971). Probit Analysis: 3d Ed. Cambridge University Press

Friedman M, Henika PR, Levin CEand Mandrell RE (2004). Antibacterial activities of plant essential oils and their components against Escherichia coli O157: H7 and Salmonella enterica in apple juice. Journal of agricultural and food chemistry, 52(19):6042-6048

Ghosh V, Mukherjee A and Chandrasekaran N (2013). Ultrasonic emulsification of food-grade nanoemulsion formulation and evaluation of its bactericidal activity. Ultrasonics Sonochemistry, 20(1):338-344

Gouvea FdS, Rosenthal A and Ferreira EHdR (2017). Plant extract and essential oils added as antimicrobials to cheeses: a review. Ciência Rural, 47(8): 1-9

Guerra-Rosas M, Morales-Castro J, Cubero-Márquez M, Salvia-Trujillo Land Martín-Belloso O (2017). Antimicrobial activity of nanoemulsions containing essential oils and high methoxyl pectin during long-term storage. Food Control, 77:131-138

Hamouda T and Baker J (2000). Antimicrobial mechanism of action of surfactant lipid preparations in enteric Gram-negative bacilli. Journal of applied microbiology, 89(3):397-403

Hosseini SF, Zandi M, Rezaei Mand Farahmandghavi F (2013). Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: preparation, characterization and in vitro release study. Carbohydrate Polymers, 95(1):50-56

Joe MM, Bradeeba K, Parthasarathi R, Sivakumaar PK, Chauhan PS, Tipayno S, Benson A and Sa T (2012). Development of surfactin based nanoemulsion formulation from selected cooking oils: evaluation for antimicrobial activity against selected food associated microorganisms. Journal of the Taiwan Institute of Chemical Engineers, 43(2):172-180

Kadhim DHand Abbas HA (2015). Formulation and characterization of carvedilol nanoemulsion oral liquid dosage form. International Journal of Pharmacy and Pharmaceutical Sciences, 7(12):209-216

Kah Mand Hofmann T (2014). Nanopesticide research: current trends and future priorities. Environment international, 63:224-235

Lambert R, Skandamis PN, Coote PJand Nychas GJ (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of applied microbiology, 91(3):453-462

Li R-Y, Wu X-M, Yin X-H, Long Y-Hand Li M (2015). Naturally produced citral can significantly inhibit normal physiology and induce cytotoxicity on Magnaporthe grisea. Pesticide biochemistry and physiology, 118:19-25

Liang R, Xu S, Shoemaker CF, Li Y, Zhong Fand Huang Q (2012). Physical and antimicrobial properties of peppermint oil nanoemulsions. Journal of agricultural and food chemistry, 60(30):7548-7555

Luo M, Jiang Land Zou G (2001). The mechanism of loss of germination ability of A. flavus spore with citral. Chinese journal of biochemistry and molecular biology, 18(2):227-232

Manchun S, Dass CRandSriamornsak P (2014). Designing nanoemulsion templates for fabrication of dextrin nanoparticles via emulsion cross-linking technique. Carbohydrate polymers, 101:650-655

McClements DJ (2015). Food emulsions: principles, practices, and techniques. CRC press

McClements DJand Rao J (2011). Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. Critical reviews in food science and nutrition, 51(4):285-330

Nielsen CK, Kjems J, Mygind T, Snabe T, Schwarz K, Serfert Yand MeyerRL (2017). Antimicrobial effect of emulsion-encapsulated isoeugenol against biofilms of food pathogens and spoilage bacteria. International journal of food microbiology, 242:7-12

Nychas G, Skandamis P, Tassou Cand Roller S (2003). Antimicrobials from herbs and spices. Natural antimicrobials for the minimal processing of foods,176-200

Oussalah M, Caillet Sand Lacroix M (2006). Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of Escherichia coli O157: H7 and Listeria monocytogenes. Journal of food protection, 69(5):1046-1055

Pal R (2011). Rheology of simple and multiple emulsions. Current Opinion in Colloid & Interface Science, 16(1):41-60

Qian Cand McClements DJ (2011). Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: factors affecting particle size. Food Hydrocolloids, 25(5):1000-1008

Saddiq AAand Khayyat SA (2010). Chemical and antimicrobial studies of monoterpene: Citral. Pesticide Biochemistry and Physiology, 98(1):89-93

São Pedro A, Santo I, Silva C, Detoni Cand Albuquerque E (2013). The use of nanotechnology as an approach for essential oil-based formulations with antimicrobial activity. Microbial Pathogens and Strategies for Combating Them (Méndez-Vilas, A, ed) Formatex Research Center Pulisher, 2:1364-1374

Schieberle Pand Grosch W (1988). Identification of potent flavor compounds formed in an aqueous lemon oil/citric acid emulsion. Journal of Agricultural and Food Chemistry, 36(4):797-800

Scortichini Mand Rossi MP (1991). Preliminary in vitro evaluation of the antimicrobial activity of terpenes and terpenoids towards Erwinia amylovora (Burrill) Winslow *et al.* Journal of Applied Microbiology, 71(2):109-112

Shafiq Sand Shakeel F (2010). Stability and self-nanoemulsification efficiency of ramipril nanoemulsion containing labrasol and plurol oleique. Clinical Research and Regulatory Affairs, 27(1):7-12

Shakeel F, Ramadan Wand Ahmed MA (2009). Investigation of true nanoemulsions for transdermal potential of indomethacin: characterization, rheological characteristics, and ex vivo skin permeation studies. Journal of drug targeting, 17(6):435-441

Solans C, Izquierdo P, Nolla J, Azemar Nand Garcia-Celma M (2005) Nano-emulsions. Current opinion in colloid & interface science 10(3):102-110

Solórzano-Santos Fand Miranda-Novales MG (2012). Essential oils from aromatic herbs as antimicrobial agents. Current opinion in biotechnology, 23(2):136-141

Sugumar S, Nirmala J, Ghosh V, Anjali H, Mukherjee A and Chandrasekaran N (2013). Bio-based nanoemulsion formulation, characterization and antibacterial activity against food-borne pathogens. Journal of basic microbiology, 53(8):677-685

Tadros T, Izquierdo P, Esquena J and Solans C (2004). Formation and stability of nano-emulsions. Advances in colloid and interface science, 108:303-318

Yang S-J, Lin F-H, Tsai H-M, Lin C-F, Chin H-C, Wong J-M, Shieh M-J (2011). Alginate-folic acid-modified chitosan nanoparticles for photodynamic detection of intestinal neoplasms. Biomaterials, 32(8):2174-2182

Yuan Y, Gao Y, Zhao J, Mao L (2008). Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. Food Research International, 41(1):61-68

Zachariah T, Leela N (2006). Volatiles from herbs and spices. Handbook of herbs and spices 1st ed Cambridge, UK: Woodhead Publishing, p:177-211

Zhang Z, Vriesekoop F, Yuan Q, Liang H (2014). Effects of nisin on the antimicrobial activity of D-limonene and its nanoemulsion. Food chemistry, 150:307-312

Ziani K, Chang Y, McLandsborough L, McClements DJ (2011). Influence of surfactant charge on antimicrobial efficacy of surfactant-stabilized thyme oil nanoemulsions. Journal of agricultural and food chemistry, 59(11):6247-6255.

ARABIC SUMMERY

التحلل الضوئى لمبيدات الكاربميت (ميثوميل) باستخدام جزيئات ثانى اكسيد التيتانيوم النانوية ضد حشره دوده ورق القطن

احمد محمود شاكر[١]، ايمن حسن زكى[٢]، الهام فاروق[١] محمود، محمد خضر[٢]
١- مركز البحوث الزراعية .معهد بحوث وقاية النباتات .محطة بحوث سدس
٢- كلية الدراسات العليا للعلوم المتقدمة جامعة بنى سويف
amshaker2003@gmail.com

لقد اصبح التحلل الضوئى وتكسير المبيدات العضوية هو الشغل الشاغل الذى يشغل المجتمع العلمى واصبح السبب الرئيسى للتلوث بالمبيدات هو الاستخدام المفرط لتلك المبيدات كما تقدر الكمية المستخدمة من المبيدات من ١الى ٢.٥ مليون طن سنويا والتى بدورها تسبب تلوثا بيئيا خطيرا. ولذلك تلعب تكنولوجيا النانو دورا هاما لحل هذه المشكلة باستخدام جزيئات ثانى اكسيد التيتانيوم النانوية والتى لها دور كبير فى ازالة الاثر السام الخطير للمبيدات تحت تاثير ضؤ الشمس وفى فتره زمنيه قصيره . فى هذه الدراسة يتم استخدام ثلاث معاملات على العمر الثانى والرابع من دودة ورق القطن وذلك بالتغذية على هذه المعاملات باستخدام ورق نبات الخروع بعد غمسة فى كل معاملة على حده ثم اطعام يرقات العمر الثانى والرابع لحشرة دودة ورق القطن والثلاث معاملات هم خليط من مبيد الميثوميل مع جزيئات ثانى اكسيد التيتانيوم النانوى والمعاملة الثانية مبيد الميثوميل منفرد والمعامله الثالثة ماء مقطر وقد تم تقييم التجربة خلال فترات زمنيه متفاوته ٣، ٥، ٧، ٩، ١٢ يوم وقد لوحظ انه باستخدام الخليط من مبيد ميثوميل مع جزيئات ثانى اكسيد التيتانيوم النانوية خفض فى نسب الموت ليرقات العمر الثانى والرابع لحشرة دودة ورق القطن مقارنة بمبيد الميثوميل منفرد .اما المعاملة الثالثة باستخدام الماء المقطر لايوجد نسب موت وهذا مؤشر قوى على انه بعد خلط مبيد الميثوميل بجزيئات ثانى اكسيد التيتانيوم النانوية تم تحلل ضوئى للمبيد وتقليل معدل سمية المبيد ولذلك يمكن اضافة جزيئات ثانى اكسيد التيتانيوم النانوية لمبيد الميثوميل للتقليل من تاثيره السمى المتبقى.