Contact toxicity of ploy lactic acid nanofibers loaded with two essential oils against *Plodia interpunctella* Hub. (Lepidoptera: Pyralidae)

Somaye Allahvaisi[1], Khalil Talebi Jahromi[*2], Sohrab Imani[1] and Mohammad Khanjani[3]

ABSTRACT

In the most recent decade, nanoscale materials have received much attention because of their applications in the field of toxicology and biopesticides. *Mentha piperita* L. and *Salvia officinalis* L., as green pesticides were incorporated into Poly Lactic Acid (PLA) solution about 14%wt which were successfully electrospun into mats with ~58 nm fiber diameters. The contact toxicity of essential oils (EOs) was tested on first instar larvae of *Plodia interpunctella* after 72-h to evaluate the effect of PLA nanofibers loaded with the EOs versus the pure essential oils (PEO)s on the mortality of the larvae over one month. The comparison between LC_{50} of the formulated essential oils (NFO)s and PEOs showed a significant difference ($p< 0.05$). The NFOs showed higher contact toxicity than the PEOs to control this pest for a longer time with slow release efficiency. Moreover, *M. piperita* showed more toxicity than *S. officinalis*. The nanofibers cause surface tension. Therefore, it is evaluated that this formulation increases the contact toxicity efficiency of essential oils as green contact pesticide. The nanofibers cause surface tension. Therefore, it is inferred that this formulation increases the contact toxicity efficiency of essential oils as green contact pesticide.

Keywords: Electrospining, contact toxicity, PLA, Nano-Pesticide.

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INTRODUCTION

Foodstuffs packaging in stores are frequently exposed to the attack of pest insects. Despite the hazardous effects of synthetic pesticides for pest control, there is a general tendency for safe pesticides (Conway and Pretty, 2013). It has been demonstrated that these compounds affect different pest species by insecticidal, antifungal and antimicrobial activity (George *et al.*, 2014). There are several studies using the high percent of EOs in warehouses against stored pest insects (Tapondjou *et al.*, 2002; Sahaf *et al.*, 2007; Sahaf and Moharramipour, 2008; Shojaaddini *et al.*, 2008).

Plodia interpunctella (Hübner) is one of the destructive cosmopolitan pest insects in stores, which extensively causes economic damages on agricultural foodstuffs especially dried fruit, nuts and chocolate (Johnson *et al.*, 1992; Fontenot *et al.*, 2012). Most of these foodstuffs are infected by the secretion of silky webs of larvae of Indian meal moth causing strong reduction of yield and quality of products (Almasi, 1984; Fasulo and Knox, 2008).

The contact toxicity of EOs of *Mentha piperita* and *Salvia officinalis* has been documented (Mondal and Khalequzzaman, 2006; Pavela, 2008; Palacios *et al.*, 2009; Geranmayeh and Hashemi, 2014). Nevertheless, it is reported that the fumigant toxicity of Essential Oils is more effective than the contact toxicity (Mondal and Khalequzzaman, 2006; Koul *et al.*, 2008; Wang *et al.*, 2014). Therefore, increasing the impact of contact toxicity of the EOs is needed.

On the other hand, there are some disadvantages regarding the application of the purified form of the EOs including low persistence, volatility, high concentrations required for effective protection, poor water solubility, and potential for oxidation (Moretti *et al.,* 2002; Korunic *et al.,* 2008; Rajendran and Sriranjini, 2008). Recently, the slow-release formulation of the EOs is applied to stabilize the release of the EOs for a longer time than the purified EOs (Rieger and schiffman, 2014; Mori *et al.,* 2015). Previous studies (Rieger and schiffman, 2014; Claudia *et al.,* 2015) reported that the EOs molecules can sufficiently be distributed in some concentrations through nanofibers via electrospining method and cause uniform structures similar to spider webs.

It has been reported that some advantages of the fibers such as large surface area and many active surface sites can be enhanced by shrinking the diameters of the fibers from micrometer to submicron or nanometer materials (Dabirian *et al.,* 2010).

Electrospinning is a simple and versatile process which uses the electrical forces to produce polymer fibers with nanometer scale diameters ranging from nanometers to micrometers (usually between 50-500 nm). This method is a technique utilizing the electric force to drive polymer fluid for producing polymer nanofibers (Lowe, 2000; Wei *et al.,* 2012; Bonthagarala *et al.,* 2015). The polymeric material is Poly Lactic Acid (PLA) as spun, which is an aliphatic polyester of high weight molecular substance derived from renewable resources such as wheat, corn, sugar-cane or potato corn starch (Gao *et al.,* 2002).

The feasibility of using the nanofibers in water treatment and purification (Feng, 2013; Bagheri *et al.,* 2010), plant protection (Hellmann *et al.,* 2009), and diseases of agricultural products (Rieger and Schiffman, 2014) has been studied. Recently, Angeles *et al.* (2008) have shown a successful electrospining of oil-in-water emulsion featuring an aqueous solution of poly (ethylene oxide) (PEO) as the continuous phase and mineral oil (non-volatile oil) as the drop phase. Some researchers demonstrated the successful incorporating and delivering of EOs from nanofiber mats (Rieger and Schiffman, 2014; Mori *et al.,* 2015). In addition, in 2009, Hellmann and his collogues used polyamide 6 as spun from formic acid and cellulose acetate as spun from acetone as solvent to be carriers of pheromone to release pheromone from nanofibers. The present study was conducted to compare the contact toxicity and persistence of two volatile EOs of *M. piperita* and *S. officinalis* against *P. interpunctella* in both formulated and purified forms.

MATERIALS AND METHODS

Insect collection and maintenance

Tested insects (*P. interpunctella*) were collected from nuts stores in Tehran. The generations of this pest were reared under laboratory conditions, at 29±1°C and 60±5% r.h. in a thermostat chamber (Vukajlovic and Pesic, 2012). These insects had no history of exposure to the insecticides for two years. The standard laboratory diet (S.L.D.) for this moth contains white cornmeal (26%), whole wheat flour (23%), glycerol (16%), honey (14%), ground dog meal (10%), brewers' yeast (5%), rolled oats (4%) and wheat germs (2%) (Silhacek and Miller, 1972). For the tests, 1st larvae (two-days old) were obtained from cultures maintained in the Entomology Laboratory of Research and Science University of Tehran.

Collection and preparation of plant materials

Leaves of *S. officinalis* and *M. piperita* were collected from the field in Medical Plant Center of Shahid Beheshti University, Tehran, Iran in May 2012. The harvested leaves were separated from other parts of the plants, cleaned, and packed. Then, they were dried in the sun for a week, winnowed, and stored at -24 ºC until required for extraction, at the beginning of the experiment on August 2012.

Extraction of EOs

Two medical plants *S. officinalis* and *M. piperita* leaves were hydrodistilled for

extraction of their EO using a modified Clevenger-type apparatus. Leaves (50 g) were grinded and put into a round-bottom flask over water at a temperature around 100 ºC. Volatile oil assembled in the reservoir was collected after 4-hours distillation process. Anhydrous sodium sulfate was used to remove water after extraction. Then, extracted oil was stored in a refrigerator at 4 ºC (Sahaf *et al.,* 2007). Chemical composition of the EO was performed by gas chromatography coupled with Mass Spectrometry (GC-MS). The EO was analyzed on an Agilent 6890 gas mass selective detector (Agilent Technologies, Palo Alto, USA). A vaporization injector operating in the split mode at 250°C and a fused silica capillary column (dimethyl poly dimethyl siloxane, Agilent Technologies) were used. The oven temperature was programmed at 45°C per 1min, raised to 250°C at 5°C min-1 and maintained at 250°C for 5 min. Helium was used as carrier gas at 30 cm s-1 and the injection volume was 1-L. The temperatures of transfer line, ion source, and quadrupole analyzer were maintained at 280°C, 230°C, and 150°C, respectively. A turbo molecular pump (10J5 Torr) was used. A solvent delay of 3 min was selected. The acquisition data and instrument control were performed by the MSD Chem- Station. The identity of each compound was assigned by comparison of their relative retention time relating to a standard mixture of n-alkanes (Adams, 2001), as well as by comparison with the mass spectra characteristic features obtained with the Wiley's library 275 spectral data bank (G1035B; Rev D.02.00; Agilent Technologies, Santa Clara, CA, USA) (Teixeiraa *et al.,* 2013).

EO-nanofibers preparation

A total of 50 g of air-dried sample; 1:10 leaves material/water volume ratio, 4 h distillation. Anhydrous sodium sulphate was used to remove water after extraction. Oil yield (4.16% w/w) was calculated on a dry weight basis. EOs were stored in a refrigerator at 4°C. Poly (lactic acid) (PLA, Mn=90,000 g/mol) was dissolved in chloroform.

Poly (lactic acid) (PLA, M_w=60,000g/mol), Dimethylformamide (DMF), hexan, chloroform, Sodium chloride (NaCl, purity ≥99.0%, CAS 7647-14-5) and Tween 80 (Polysorbate 80, CAS9005-65-6) were purchased from Sigma-Aldrich.

The formulation was prepared by Electrospinning method using the E-SPIN NANO apparatus according to a modification of the method of previous studies (Angeles *et al.,* 2008; Rieger and Schiffman, 2014; Mori *et al.,* 2014) who demonstrated the ability of oil-in-water emulsion to electrospun. PLA was solved in chloroform as a solvent.

Due to high evaporation rate of chloroform, DMF was added as another part of the solvent to control the evaporation rate and create uniform fibers. PLA was dissolved in 9 Chloroform/ 1 DMF (10 wt % PLA) yielding fiber diameters in conventional electrospinning from 50 to 350 nm. The viscosity for electrospinning process was 10.7 Pa sec. Pure NaCl was added to the solution in different percentage (0-4%) because of the electrical properties of polymeric solution. On the other hand, different concentrations of EOs of *M. piperita* and *S. officinalis*, diluted by Tween 80 (Polysorbate 80) as emulsifier, was added to the prepared pre-polymer solution yielding solid nanofibers with up to 14 wt% of EOs (Allahvaisi *et al.,* 2017). The solution was mixed for 24 h at 20 rpm using an Arma-Rotator which indicated that the transparent liquid changed to white color.

Each EO/PLA solution was loaded into a 5 mL Luer-Lock tip syringe capped with Precision Glide 20 gauge needles, secured to an ultra syringe pump. Alligator clips were used to connect the positive anode of a high-voltage supply to the needle and the negative anode to a copper plate wrapped in aluminum foil. A constant feed rate of 60 L/min, an applied voltage of 15 kV (Zong *et al.,* 2002) and a separation distance of 120, 160 and 140 mm were used to electrospin EOs/PLA solutions, respectively. The assembled electrospinning apparatus was housed in an environmental chamber with a desiccant unit to maintain in a temperature of 24°C and a relative humidity of 65 % (Mori *et al.,* 2014).

Bioassay tests

In order to assess the contact toxicity of EOs by topical application, preliminary dose setting experiment was carried out to determine a range of doses that would cause a range of 20–80% mortality. In order to determine the LC_{50} of contact toxicity, the concentrations were ranged as18.97 to 34.7 µL/L air (PEO) and 5.2 to 15.9 µL/L air (NFO) for *S. officinalis*; also as 24.65 to 45.1 µL/L air (PEO) and 3.9 to 14.4 µL/L air (NFO) for *M. piperita*. Then, treated and untreated groups of insects (25 larvae in 1–3 days old for each group) introduced into each plate.

The bottom of the screw plates were covered by filter papers (Whatman No.1) for the experiments of PEOs or formulated NFOs with appropriate concentrations. The caps were screwed tightly and vials placed in incubator set at 27 ± 1ºC and 65 ± 5 % RH in continuous darkness. After 72 h, the plates were opened, mortality for each exposure time was evaluated independently and concentrations were replicated for each exposure time separately (Robertson *et al.*, 2007). The insects were considered to be dead when no movement could be observed over a period of 2-3 minutes, even after gentle prodding with a fine brush.

Statistics

No mortality was observed in the control group, so there was no need to correct the data for natural mortality of control. For percent mortality, square root of Arc Sine transformation was used to stabilize the variance and normalize the data (Osborne, 2010); however non-transformed data are presented in tables. The probit analysis (Finney, 1971) was used to estimate LC_{50} values. The categorical data of mortalities were analyzed using one-way analysis of variances (ANOVA) and the post-ANOVA Tukey's test used to separate means at 0.05 probabilities (SPSS, 2007).

RESULTS

The surface topography of the nanofibers of EOs were observed by a scanning electron microscope (SEM). When 14% of EOs was loaded, the NFOs indicated the most uniform distribution in size (58 nm). SEM images consisted of nanofibers with a relatively uniform thickness as non-woven webs that EO droplets incorporate into these nanofibers (Fig.1).

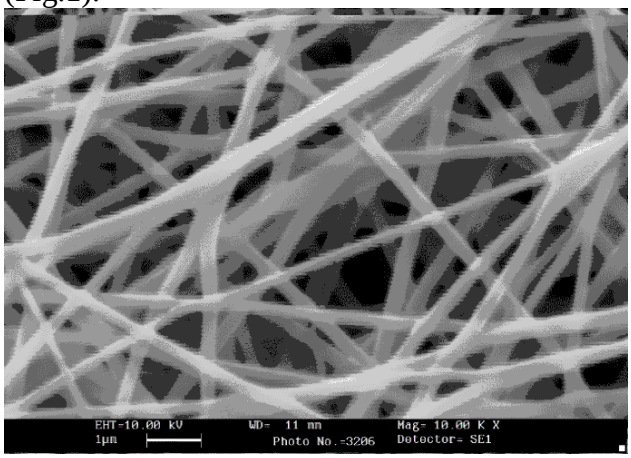

Fig. 1. SEM photographs of the diameter distribution of the electrospun-nanofibers of the different formulations

It is clear that the degradation rate of synthesized PLA was more than that of PLA polymer. Based on the LC_{50} values of the contact toxicity, NFO caused more mortality than PEO. The LC_{50} of the NFO, calculated after 72-hrs of exposure time, was 11.9 and 9.1 µl/L air for *S. officinalis* and *M. piperata*, respectively.

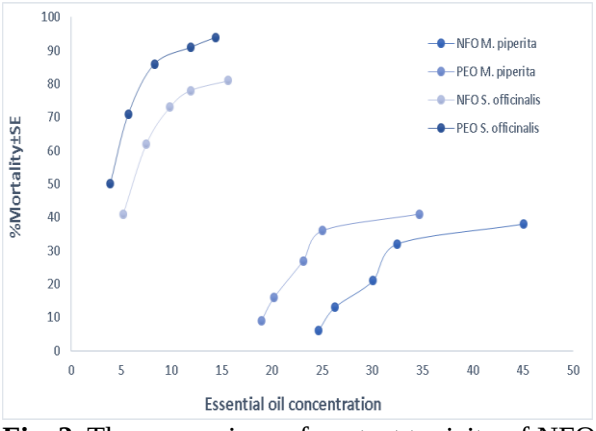

Fig. 2. The comparison of contact toxicity of NFO and PEO against first larvae of *P. interpunctella* for both EOs of *M. piperita* and *S. officinalis.*

Table 1. Average mortality of contact toxicity of NFOs against *P. interpunctella* first larvae after 72 hours exposure at 27°C and 65% r.h.

Plant	PEOs		NFOs	
	Concentration (µl/L air)	Mean	Concentration (µl/L air)	Mean
S. officinalis	18.97	9±2.22c(B)	4.5	41±3.74c(B)
	20.24	16±2.34b(B)	6.8	62±2.45b(B)
	23.13	27±2.34ab(B)	9.75	72±2.45ab(B)
	25.04	36±1.92a(B)	11.9	75±2.45a(B)
	34.7	41±1.89a(B)	15.67	77±2.00a(B)
M. piperita	24.65	6±2.46d(A)	3.9	53±2.66c(A)
	26.3	13±2.00c(A)	5.7	75±2.37b(A)
	30.1	21±2.22b(A)	8.3	85±1.98ab(A)
	32.5	32±2.22a(A)	11.4	87±2.34a(A)
	45.1	38±2.12a(A)	12.9	88±2.12a(A)

Means with same letter are not significantly different (at 5% level of significance)

Table 2. Contact toxicity of NFOs and PEOs against *P. interpunctella* first larvae after 72 hours exposure at 27°C and 65% r.h.

Plant	Type	N	LC_{50} (95% fiducial limits)	Slope±SE	df	P-value	Chi square (χ^2)
S. officinalis	NFO	625	9.1(10.61-7.84)	2.62±0.42	0.35	0.96	3
	PEO	625	30.3(26.75-34.23)	3.98±0.35	1.43	0.88	4
M. piperita	NFO	625	11.7(10.08-13.47)	2.00±0.09	1.75	0.94	3
	PEO	625	39.3(35.65-43.83)	3.44±0.44	0.51	0.94	3

The LC_{50} of the PEO for first instar larvae after 72 hours exposure were 39.3 µl/L air and 30.3 µl/L air for *S. officinalis* and *M. piperita*, respectively. The contact toxicity of *M. piperita* was significantly higher (df=3, F=38.26, p <0.05) than that of *S. officinalis* (Table 2). The relative toxicity obtained from the LC_{50} values showed that NFOs was approximately 3.5-fold lower than those of PEO against *P. interpunctella* first instar larvae (Table 2). However, both PEOs and NFOs caused death in exposed three-day-old larvae after 72-hrs. In the first 24-hrs, there was significantly no mortality for the larvae exposed to the NFO formulation when compared with the control test; while the mortality percentages for the PEO ranged from 0 to 45. On the other hand, according to the results of GC-MS; all constituents of EOs were monoterpenes (Table 3).

A significant increase (p <0.05) in the residual toxicity of the NFOs was observed for the first larvae of *P. interpunctella.* The mortality percentage of the *M. piperita* in the form of PEO at the maximum and minimum concentrations (45.1 and 24.65 µl/L air) was 38±2.12 and 6±2.46%, respectively in the period of 72-hrs (Table 1). It was found that *M. piperita* in the form of NFO at the maximum and minimum concentrations (14.4 and 3.9 µl/L air) have %94 ± 2.12 and %53 ± 2.66 mortalities respectively after 72-hrs (Table 1). Similarly, there was a significant difference between *M. piperita* and *S. officinalis* (p <0.05).

Tukey's test showed that the contact toxicity between different concentrations of EOs was significant (df= 4, F=22.51, p <0.05). In all these cases, the mortality increased with the enhancing of the concentration levels. The toxicity effect of both PEO and NFO was significant (p <0.05) on the *P. interpunctella* larvae. LC_{50} of PEO for *S. officinalis and M. piperita* were respectively 39.3 and 30.3 µl/L

air; moreover, LC_{50} of NFO for *S. officinalis* and *M. piperita* were 9.1 and 11.7 µl/L air. It is clear that the NFO of *M. piperita* was more effective than *S. officinalis* against *P. interpunctella* (Table 2). As can be seen from Fig. 3, the mortality percentage of the larvae was not higher than 45% even after 72-hrs of exposure to PEOs; which, the mortality of NFOs can be about 95%. The mortality percentage of NFOs form was about 3.5-fold lower than PEO form.

Table 3. Chemical constituents of the *S.officinalis* and *M. piperita* PEOs

RI	Compounds	*M. Piperita*	*S. officinalis*
894	Salvene	-	0.5
938	α-Thujene	0.15	2.45
965	α-Pinene	2.4	4.48
979	Camphene	-	3.8
1018	Sabinene	0.4	0.49
1031	β-Pinene	0.9	1.5
1035	α-terpineol	0.33	0.2
1062	Limonene	-	4.7
1087	1,8-Cineole	3.8	12.2
1102	α-Thujone	-	42.5
1119	Camphor	-	8.22
1133	Menthone	52.5	-
1142	β-Thujone	-	5.96
1144	Menthofuran	3.7	-
1145	Borneol	-	4.6
1152	Isoment ol	2.06	-
1160	Menthol	12.64	-
1175	Germacrene D	1.2	-
1193	Myrcene	-	0.5
1216	Bornylacetate	-	0.31
1248	Menthylacetate	8.4	-
1269	Piperitone	0.62	-
1282	Neoisomenthol	3.6	-
1305	β-Farnesene	0.4	-
1341	Guaiol	0.4	-
1367	β-Caryophyllene	2.6	3.24
1409	α-humulene	0.23	0.94

RI :Retention index

DISCUSSION

This study focuses on enhancing the contact toxicity and persistence of EOs without the use of a surfactant. The interaction between EOs units and polymer chain in the nanofiber formulation governed the controlled release of the EOs. SEM images showed the communication of the EOs and PLA in 58 nm, in which there was no knot or phase separation in the structure of the nanofibers (Allahvaisi *et al.*, 2017). It seems that the addition of EOs increases the uniform nanofibers and decreases their thickness. This result is in agreement with the observation of Rieger and Schiffman (2014) who displayed chitosan/CA (5%) /PEO nanofiber mats are less thick in comparison with chitosan and CA at 0% and 0.5% /PEO nanofiber mats.

In the present study, the LC_{50} of contact toxicity of both NFO and PEO was calculated for controlling 1st larvae of *P. interpunctella*. It was found that the LC_{50} value of NFO was significantly higher than PEO after 72-hrs. Moreover, the contact toxicity of the EOs of *S. officinalis* was more than that of *M. piperita*. The results indicated that a unique distribution and the small size of the oil-loaded nanofibers increase their penetrability in the insect body via cuticule in comparison with the pure form of the oil. It is reported that the small diameter of the nanofibers increases the strength of the fibers (Griffth, 1921).

It can be said when EOs are added to the polymer solution, about 14% (w/w) of these compounds could be optimally incorporated through electrospun nanofibers. In nanofiber structures the surface tension is decreased and consequently, the property of contact toxicity of EOs enhances. Therefore, the small fiber diameter and large aspect ratio results in significant high surface to volume ratio and makes the electrospun-nanofibers suitable for different applications (Wei *et al.*, 2012; Mori *et al.*, 2015). It increases the probability of the pesticide touching with the larvae. Thus, it can be concluded that the nanofibers are suitable carriers for the EOs. Despite the low

concentrations of NFO form compared with PEO, the LC_{50} of the NFOs are 3.5-fold higher toxic than PEOs.

Our results showed that the NFOs have the controlled-release effect which acts over a period of 72h. It was observed that pesticides loaded through nanofibers, which are used with the planted seeds, control the pests for several weeks to months (Anonymous, 2009). The use of low concentrations of the EOs in the form of nanofibers for a long time compared with their pure form shows the property of slow release and their persistence. Therefore, the contact toxicity efficiency of this formulation was more than the pure form. Thus, the contact toxicity of *S. officinalis* oil is significantly higher than that of *M. piperita* against 1st larvae of *P. interpunctella.*

GC-mass analyze of EOs indicate the presence of complex mixtures of monoterpenoids and sesquiterpenoids. It was reported that the superior quality of *S. officinallis* oil should contain α-thujone + β-thujone> 50% and camphor < 20% (Guenther, 1949; Putievsky, 1992). The major components of peppermint oil usually include menthol, menthone, and menthofuran. Moreover, it was documented that monoterpenes have insecticidal toxicity including fumigant, contact, and ingestion action against stored product insect pests (Prates *et al.*, 1998; Lee *et al.*, 2003, Rozman *et al.*, 2007; Abdelgaleil *et al.*, 2009; Ziaee *et al.*, 2014). High lipophilicity of monoterpenes causes their rapid penetration in the insect body, where they interfere with the insect's physiological functions (Haouas *et al.*, 2012).

Therefore, the use of non-chemical combinations especially EOs with a short-period of toxicity causes nanofibers to be considered as a new formulation of pesticides in stores. These products increase the persistence of the EOs against the environmental factors. Moreover, Poly Lactic Acid polymer as a biodegradable polymer could load EOs particles and thereby produce a safe formulated pesticide that can control pests for next generations.

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Somaye Allahvaisi[1], Khalil Talebi Jahromi[*2], Sohrab Imani[1] and Mohammad Khanjani[3]

[1]Department of Entomology, Science and Research Branch, Islamic Azad University, Tehran, Iran

2Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

3Department of Plant Protection, College of Agriculture, Bu–Ali Sina University, Hamedan, Iran

***Corresponding author**

Phone No: 09123634001

Fax No: 08735122306

E-mail: *khtalebi@ut.ac.ir*

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