RESEARCH PAPER

Chitosan Nanocapsules of Tarragon Essential Oil with Low Cytotoxicity and Long-lasting Activity as a Green Nano-larvicide

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ABSTRACT

Frequent use of synthetic larvicides has led to the development of resistance in many species of mosquitoes as well as risk of environmental pollution. Recently, encapsulating essential oils (EOs) in surfactants or polymers is being employed as an approach to control the volatility of EOs as green larvicides. In this research, components of tarragon (Artemisia dracunculus) essential oil were identified by GC-MS analysis. Forty-eight components were identified, with 5 major components including estragole (67.623%), cis-Ocimene (8.691%), beta-Ocimene Y (7.577%), Limonene (4.338%) and 3-Methoxy cinnam aldehyde (1.49%). Tarragon EO was encapsulated in chitosan nanocapsules using ionic gelation method and confirmed by FT-IR analysis. Encapsulation efficiency and size of the chitosan nanocapsules were determined 34.91 ± 2% and 203 ± 16 nm, respectively. For the first time, a long-lasting green larvicide was reported which remained active for 10 days, against Anopheles stephensi. Furthermore, cytotoxicity of the nanoformulation was found to be similar to that of temephos on human skin normal cells (HFFF2). This nanoformulation can be a good alternative for synthetic larvicides due to its long-lasting activity, proper effectiveness and also its green constituents.

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INTRODUCTION

More than half of the world's population lives in areas where several species of mosquitoes are present. Ability of mosquitoes to carry and spread diseases such as encephalitis, dengue fever and malaria to human, has caused around one million deaths every year (1, 2). Continuous efforts to control the mosquitoes are being done to prevent outbreaks from such diseases. All mosquitoes have aquatic immature stages and larviciding is a common method to reduce population of mosquitos and also to prevent the diseases (3, 4). However, in recent years, indiscriminate use

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of synthetic larvicides has caused environmental pollution and development of resistance in many species of mosquitos (5, 6). Also, by affecting non-target organisms, they cause disruption of biological control systems such as *Bacillus* family or *Gambosia* fishes (7, 8). Thus, a lot of attention is being paid to herbal extracts or essential oils (EOs) as alternatives for control of mosquito larvae (9-11).

A main disadvantage of EOs to be used as larvicides is that many components of EOs are volatile (12, 13), thus, they do not remain active for several days. Therefore, many reported studies on EOs as larvicides only have investigated their activity for limited exposure times (e.g. 24h) (14, 15). To resolve this downside, nowadays EOs are being encapsulated in different surfactant or polymer structures to increase their durability (16-18). By formulating EOs in nanoemulsions, due to reducing their particle size, improved permeation into larvae bodies is observed which increases their efficacy. For example, in our previous report, larvicidal activity (LA) of tarragon essential oil (TEO) against *An. stephensi* was evaluated at bulk and nanoemulsion forms. At concentration of 18 ppm, LA increased from 83% to 92% in bulk and nanoemulsion preparations, respectively (19). In another report of our group, nanoemulsion of EO of *Anethum graveolens* with particle size of ~10 nm showed LA of 81%, compared to the bulk form with LA of 73% (18). However, no work so far has reported a long-lasting LA from nanoemulsions. Our own experiences show that nanoemulsions of EOs commonly fail to show a proper duration of action (i.e. several days, data not given).

Comparing with nanoemulsions, polymeric carriers are more stable, thus, may be considered for improving duration of larvicidal actions of EOs. From the literature, by loading EO of *Lippia sidoides* in beads of chitosan/cashew gum (~1.5 mm), LA remained stable for 3 days, against *Aedes aegypti* (20). However, this period is not enough yet, as larval stages of mosquitos are 9-11 days (21) and there is still a need to prolong the duration of action of such preparations.

In this research, for the first time, chitosan nanocapsules containing TEO were prepared as a sustained release larvicide to control volatility of the EO. Furthermore, we aimed to benefit from advantages of reducing the size (improvement of effectiveness of TEO) and providing enough stability (increasing of duration of LA, comparable with larval stages period). Duration of LA of the prepared nanoformulation was evaluated against *An. stephensi.* Then, for the first time, its cytotoxicity on human skin normal cell i.e. HFFF2 (as an important non-target species), was investigated at different concentrations and compared with WHO recommended larvicide (i.e. temephos).

Materials and Methods

Materials

TEO was bought from Zardband Pharmaceuticals Co, Iran. Chitosan (MW: 100 KDa and deacetylation degree: 93%) was purchased from Easter Holding Group, China. Human normal skin fibroblast-like cell line (HFFF2, NCBI Code: C163) was obtained from Pasteur Institute, Iran. Tripolyphosphate (TPP), acetic acid, Tween 20, ethanol, and powder of 4,5-Dimethyl-2-thiazolyl-2,5- diphenyltetrazolium bromide (MTT) were supplied from Merck Chemicals, Germany. Kit of lactate dehydrogenase (LDH) was supplied by Roche Molecular Diagnostics, Germany. DMEM cells medium culture and fetal bovine serum (FBS) were purchased from Biosera, France. Penicillin/streptomycin and Temephos EC (500 g/L) were provided by Gibco and BASF from USA and Italy, respectively.

GC-MS Analysis

GC-MS analyses were performed using a 6890 GC system coupled with a 5973 network mass selective detector (Agilent Technologies, USA). Separation of the essential oil components was carried out on an HP-5MS silica fused column (60 m length; 0.25 mm i.d, and 0.25 µM film thickness *5% Phenyl*-methylpolysiloxane). The GC-MS column temperature was programmed as follows: initial temperature was set at 40°C and fixed for 1 min, then, increased with rate of 3°C/min to the final temperature of 250°C and hold for 60 min. Temperature of the injection port and detector was fixed at 250 and 230°C, respectively. Other instrument parameters were as follows: flow, 25 mL/min, septum purge, 6 mL/min and column flow rate: 1 mL/min. Helium gas with purity of 99.999% was used as carrier gas. Mass spectra were taken at 70 eV ionization energy and full scan mode. The scanned mass range was set at 50–350 m/z.

Components of the TEO were identified by comparison of their retention indices (RIs)

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determined with reference to a homologous series of C_9–C_{24} *n*-alkanes. Firstly, this was confirmed by chromatographic injection of available analytical standard compounds (C_9–C_{24} *n*-alkanes) and comparison of their retention times with those obtained for the essential oil. Wherever standard compounds were not available, identification was carried out by comparison with traditional retention indices. The identification was also confirmed by comparison of their mass spectra with those stored in the Wiley7n.l MS computer library. The linear temperature programmed RIs of all the constituents were calculated from the gas chromatogram by interpolation between bracketing n-alkanes (Equation (1)).

$$RI = 100 \times [(t_{R(i)} - t_{R(z)} / t_{R(z+1)} - t_{R(z)}) + z] \quad (1)$$

Where *z* is the number of carbon atoms in the smaller n-alkane and $t_{R(i)}$, $t_{R(z)}$ and t are retention times of the desired compound, the smaller n-alkane and the larger n-alkane, respectively. In addition, search match factor (SMF), rank number (RN) in the mass library, and five highest peaks in the mass spectra were prepared and used for identification of the components.

Preparation of chitosan nanoparticles containing TEO

Chitosan nanocapsules containing TEO were prepared using ionic gelation technique with some modifications (22). In summary, a solution of chitosan was prepared by dissolving chitosan (1% w/w) in an aqueous solution of acetic acid (1%). The prepared chitosan solution was added dropwise (800 µL/mL) into a solution containing TEO (16 µL/mL), Tween 20 (28 µL/mL) and ethanol (58 µL/mL) at room temperature. The mixture was stirred (1800 rpm) for 30 min. Then, 98 µL/mL of aqueous solution containing TPP (0.04% w/v) was added to the prepared mixture and stirred for another 15 min (1800 rpm, room temperature) to obtain primary formulation of chitosan nanocapsules containing TEO.

Final nanoformulation (named 1F) was obtained by centrifugation of 40 mL of the primary formulation was centrifuged (4°C, 17700 g, 60 min). 35 mL of supernatant was discarded to obtain a sample with a final volume of 5 mL, which was re-dispersed using a probe homogenizer (IKA, Germany, 30 min).

Characterization of the nanoformulation

Particle size and particle size distribution of 1F were evaluated using dynamic light scattering (DLS, scatteroscope-I, K-ONE, Korea). d50 (d: median diameter of particles at 50 cumulative percent) as reported by the DLS instrument was considered as particle size and particle size distribution was also calculated using equation 2. To confirm the size and evaluate morphology of 1F, transmission electron microscope (TEM, LEO 906E Zeiss, Germany), was used, after 200 times dilution.

For investigating encapsulation efficiency (EE) of 1F, the sample was centrifuged (4°C, 17700 g, 60 min) and EE was calculated using Equation 3.

FT-IR (Nicolet iS10 FT-IR spectrometer, USA) was used to confirm TEO loading in the chitosan nanocapsules. The FT-IR spectra of TEO, chitosan, Tween 20, 1F and nanoformulation without TEO (i.e. 1F(-oil)) were recorded in wavenumber range of 500–4000 cm^{-1}, using KBr pellets.

$$\text{Particle size distribution} = \sqrt{d75/d25} \quad (2)$$

$$EE\ (\%) = \text{Initial amount of TEO} - \text{amount of TEO in the supernatant} / \text{Initia Initial amount of TEO} \times 100 \quad (3)$$

Determination of duration of LA of 1F

Larvicidal potency of bulk TEO has been reported in our previous research, its LC_{50} and LC_{90} against *An. stephensi* were determined as 11.36 and 17.54 ppm, respectively (19). In this work, the length of LA of 1F was compared with the similar concentration of bulk TEO (dissolved in ethanol), in line with WHO guideline with some modifications (23). Both 1F and bulk TEO contained 6.04% of EO which equals 302 ppm of the EO, during larvicidal bioassays. The laboratory bioassays were performed at recommended conditions (i.e. relative humidity 65 ± 5 (%), temperature 28 ± 1 (ºC) with 12:12 light and dark photoperiods).

According to the guideline of WHO (23), after evaluation of LA in the lab tests, simulated semi-field (SSF) tests were performed. SSF tests were carried out at specific conditions: relative humidity 25 ± 10 (%) and temperature 32 ± 6 (ºC) with 12:12 light and dark photoperiods, comparable to those of a shaded location, during hot season of Tehran, Iran. For both tests (lab and SSF), laboratory-reared

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of *An. stephensi* (Beech-Lab strain), were obtained from anophelini insectarium, Tehran University of Medical Sciences.

To perform the larvicidal bioassays, briefly, batches of larvae (containing 25 3rd and 4th instar larvae), were added to each test container, having 199 mL no chlorine water. Then, 1 mL bulk TEO or 1F was added so that final concentration of TEO was fixed eventually at 302 ppm in each test container. Containers were isolated from the environment using nets to prevent environmental mosquitoes mixing with the tests. After 24 h exposure, dead larvae were counted, then, all the larvae (i.e. live and dead) were removed by rubber pipette. Subsequently, fresh batches of larvae were added to each container and larvicidal tests continued. The tests were stopped when LA of containers equalled that of containers having 1F(-oil) or when mortality in control groups (1 mL ethanol added only) increased to 5%. All the larvicidal bioassays were repeated 15 times in 3 different replicates.

Comparing cytotoxicity of 1F with temephos

Cell culture

HFFF2 cell line was cultured in DMEM containing FBS (12%) and penicillin/streptomycin (1%). The cell line was incubated with an atmosphere of air (95%) and CO_2 (5%) at 37°C. When the cells reached confluence, they were harvested and seeded in 96-well plates (~7×10^3 cells/well). Cells were then treated with 1F at different concentrations 1/2, 2/3, 1, 2 and 4 times TEO compared to that of 1F (i.e. 1/2F, 2/3F, 2F and 4F, respectively).

For determining cytotoxicity of the ingredients of the nanoformulation, 1F(-oil) was prepared at different concentrations in line with a concentration of tested nanoformulations (i.e. 1/2F(-oil), 2/3F(-oil), 1F(-oil), 2F(-oil) and 4F(-oil)). Temephos (Abate), WHO recommended larvicide, was used as positive control at an advisory concentration of 1 ppm (24). Additionally, bulk TEO was considered containing 25% TEO, comparable with the highest concentrated preparation (i.e. 4F), which was directly added to the culture media.

MTT assay

For determination of cell viability, MTT powder (5 mg/mL) was dissolved in PBS and filtered in light-protected containers. HFFF2 cells were cultured in 96-well plates containing 100 µL complete medium at 37°C for 24 h in the incubator. Subsequently, the mentioned samples were added to each well and incubated for another 4 h. Metabolic activity of each well was determined by MTT assay and compared to those of untreated cells (control). After removal of 100 µL medium, MTT dye solution was added (15 µL/100 µL medium) and the plates were incubated at 37°C for 4 h in 5% CO_2 atmosphere. Then, 100 µL of DMSO was added to each well and mixed thoroughly to dissolve the dye crystals. Absorbance was measured using an ELISA plate reader (ELx800, Bio Tek, USA) at 570 nm with a reference wavelength of 630 nm.

High optical density (OD) corresponded to a high intensity of dye colour, which is related to untreated control cells. The MTT assay was repeated in triplicate. The measured data were expressed as mean ±SD. The cell viability was calculated by equation 4.

$$\text{Cell viability (\%)} = \frac{\text{Mean absorbance of sample test} - \text{Mean absorbance of blank}}{\text{Mean absorbance in control wells} - \text{Mean absorbance of blank}} \times 100 \quad (4)$$

LDH test

LDH assay was carried out according to the Roche's protocol. Briefly, HFFF2 cells seeded in 96-well plates containing 100 µL serum-free medium were incubated for 24 h at 37°C. Subsequently, prepared formulations with/out TEO (i.e. 1F and 1F(-oil)) and different concentrations (1/2, 2/3, 2 and 4)), aqueous solution of temephos (1%), and TEO 25% were added to wells and incubated for another 4 h. As a control for maximum LDH release, cells were treated with lysis solution of the kit. 100 µL of the cell-free culture supernatants were transferred into new 96-well plates. The reaction mixture (100 µL/well) was added to each well for 30 min. The absorbance was determined using plate reader (ELx800, Bio Tek, USA) at 490 nm wavelength and 630 nm as reference wavelength. The LDH assay was repeated in triplicate and the data were expressed as the mean ±SD. Equation 5 was used to determine the percentage LDH release.

$$\text{LDH Release (\%)} = \frac{\text{Mean absorbance of experimental value} - \text{Mean absorbance of low control}}{\text{Mean absorbance of high control} - \text{Mean absorbance of low control}} \times 100 \quad (5)$$

where "low control" corresponds to LDH activity released from the untreated normal cells and "high control" corresponds to maximum releasable LDH activity in the cells**.**

Statistical analysis

For comparing duration of LA of 1F and bulk TEO as well as results of larvicidal bioassays in the lab tests in comparison with those of the SSF tests, independent sample *t*-test with a confidence interval of 95% (CI 95%) was used.

For analyzing and comparing the viability and LDH release of each concentration of the 1F (i.e. 1/2F, 2/3F, 2F and 4F) with temephos, one way ANOVA (CI 95%) was used. Also, to compare different concentrations of 1F with corresponding sample without oil (i.e. 1/2F(-oil), 2/3F(-oil), 1F(-oil), 1F(-oil), 2F(-oil) and 4F(-oil)), independent sample *t*-test (CI 95%) was used. All the mentioned tests were repeated 12 times in 3 different replicates and related statistical analyses were performed using SPSS V22 software (SPSS Inc, USA).

RESULTS AND DISCUSSIONS

Determination of chemical composition of TEO

Components of TEO were identified by GC-MS analysis. Forty-eight components were determined and listed in Table 1. Five major components include estragole (67.623%), cis-Ocimene (8.691%), beta-Ocimene Y (7.577%), Limonene (4.338%) & 3-Methoxy cinnam aldehyde (1.491%).

Table 1. List of components identified in tarragon essential oil using GC-MS analysis.

Physicochemical properties of 1F

From Fig. 1 (Left), particle size of 1F is 222 ± 12 nm and its particle size distribution is 2.43, showing appropriate monodispersity. A TEM image of 1F is depicted in Fig. 1 (Right), indicating spherical particles with a size of 203 ± 16 nm, which agrees with results of DLS. Size of the prepared chitosan nanocapsules is similar to other studies reporting 200 and 400 nm for chitosan nanocapsules containing other EOs (25, 26).

Our previous researches indicated that decreasing size of the nanoparticles leads to increasing its effectiveness. For instance, mortality of larvae when using nanoemulsion of TEO (size of 11 nm) was significantly higher than the corresponding emulsion (size of 9310 nm) at 18 ppm concentration of EO (i.e. 92.71% vs. 81.67%) against *An. stephensi* (19). Also, smaller particle size distribution values are preferred to improve physical stability (27, 28), performance (29, 30) and loading capacity (31) of prepared nanoformulations. Thus, prepared nanoformulation in this study (1F) meets both mentioned properties.

Fig. 2 shows calibration curve and regression equation used for calculating the amounts of TEO in the supernatant after centrifugation of 1F. EE of 1F was calculated as 34.91 ± 2%, similar to other studies which reported encapsulating oil or EO in chitosan nanoparticles, eg, carvacrol (31%) (32), *Carum copticum* (36.2%) (33) and *Zataria multiflora* (45.24%) (34).

Due to presence of a relatively high amount of water in 1F, use of chromatography methods was not possible in this study as carbon columns are corroded by water. Therefore, EO in the supernatant was determined using UV-Vis analysis.

Fig. 3 shows FT-IR spectra of chitosan, Tween 20, TEO, 1F(-oil) and 1F. Chitosan powder shows to have specific peaks at different wavenumbers (cm^{-1}) such as 3400-3500 (O-H and N-H (amine I) stretching) and 1550-1650 (N-H (amid II) bending) (33, 35). Tween 20 has characteristic peaks at 1000 and 2800, attributed to C-O and C-H (in Alkanes groups), respectively (36).

Chitosan nanocapsules (1F(-oil)) were formed after addition of an aqueous solution containing TPP into aqueous solution of chitosan, Tween 20 and ethanol. As a result of electrostatic interactions between phosphoric and ammonium ions of TPP and chitosan, new peak (P=O) appears around 1000-1100 cm^{-1}. Another strong broad peak related to O-H bonding of ethanol also appears at 3200-3400 cm^{-1}. Besides, the encapsulation process leads to fixing some vibration or shifting the peaks. For instance, many peaks of Tween 20 disappeared, also, bending peaks of amide II shifted to lower wavenumber values (1400-1500) (37, 38).

EOs usually consist of many different constituents possessing various functional groups. Here in, to confirm presence of the EO in the nanoformulation, presence of the major component was investigated (22, 33). As was described in section 3.1, estragole (p-allylanisole) is the major component of TEO (67.62% of total EO). Peaks at 1220 and 1550 are related to C-O stretching and C=C in estragole (39, 40).

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Table 1. List of components identified in tarragon essential oil using GC-MS analysis.

No	tR*	Compound	Peak area	%	RI**
1	6.141	Tricyclene	1551424	0.004	
2	29.15	Cinnamyl acetate	4956966	0.014	1066
3	6.35	alpha-Thujene	5230036	0.015	
4	29.456	alpha-Humulene	7004369	0.020	1072
5	22.69	Nerol	8812108	0.025	944
6	29.582	trans-beta-Farnesene	10123459	0.029	1074
7	27.007	beta-Elemene	10571314	0.030	1025
8	28.742	alpha-Bergamotene	14295464	0.041	1058
9	45.615	Nonadecane	15292173	0.044	1411
10	21.698	Carvone	16960150	0.048	925
11	31.617	trans-trans-alpha-Farnesene	18456048	0.053	1114
12	30.095	2(3H)-Furanone, 5-hexyldihydro-	18753954	0.054	1084
13	21.855	p-Allylphenol	19717087	0.056	928
14	30.561	Germacrene D	23644363	0.067	1093
15	9.504	Phellandrene	27173227	0.078	625
16	25.265	Cyclohexylmorpholine	27948634	0.080	992
17	39.807	7-Methoxycoumarin	28349494	0.081	1278
18	26.362	alpha-Copaene	33352538	0.095	1013
19	24.827	alpha-Terpinene	33391272	0.095	984
20	7.171	Camphene	36376876	0.104	
21	8.211	Sabinene	39661740	0.113	
22	31.131	cis-trans-alpha-Farnesene	39918948	0.114	1104
23	22.365	Geranial	41184671	0.118	938
24	32.246	beta-Sesquiphellandrene	43067473	0.123	1126
25	21.947	Anisaldehyde	43431241	0.124	930
26	26.698	Cinnamic acid methyl ester	55253155	0.158	1019
27	28.119	trans-Caryophyllene	55810389	0.159	1047
28	23.132	Benzenemethanol, .alpha.-2-propenyl-	65325726	0.186	952
29	8.979	beta-Myrcene	81623487	0.233	605
30	31.242	peri-Ethylenenaphthalene	83391636	0.238	1106
31	30.314	Acoradiene	89347554	0.255	1088
32	13.36	alpha-Terpinolene	91988314	0.263	749
33	34.537	Spathulenol	99969871	0.285	1172
34	14.214	Linalool	100804203	0.288	770
35	25.711	Eugenol	103221898	0.295	1001
36	10.448	o-Cymene	143777034	0.410	662
37	15.853	Allocimene	153590024	0.438	809
38	22.929	Bornyl acetate	181055212	0.517	948
39	8.341	beta-Pinene	217125236	0.620	
40	27.687	Methyleugenol	269269779	0.768	1038
41	12.166	gamma-Terpinene	338598016	0.966	719
42	21.66	Cuminic aldehyde	389019146	1.110	924
43	6.664	alpha-Pinene	513847405	1.466	
44	34.245	3-Methoxycinnamaldehyde	522580078	1.491	1166
45	10.727	Limonene	1520194907	4.338	673
46	11.899	beta-Ocimene Y	2654890107	7.577	712
47	11.321	cis-Ocimene	3045372978	8.691	696
48	19.176	estragole (p-Allylanisole)	23695362067	67.623	876

*tR: retention times **RI: retention indices

In the spectrum of the final nanoformulation (1F), in comparison with 1F(-oil), addition of TEO leads to an importantly increased intensity of the peak at 1000-1100, and 1550-1650. Also, new peaks related to estragole appear around 1200. Considering this finding and results of

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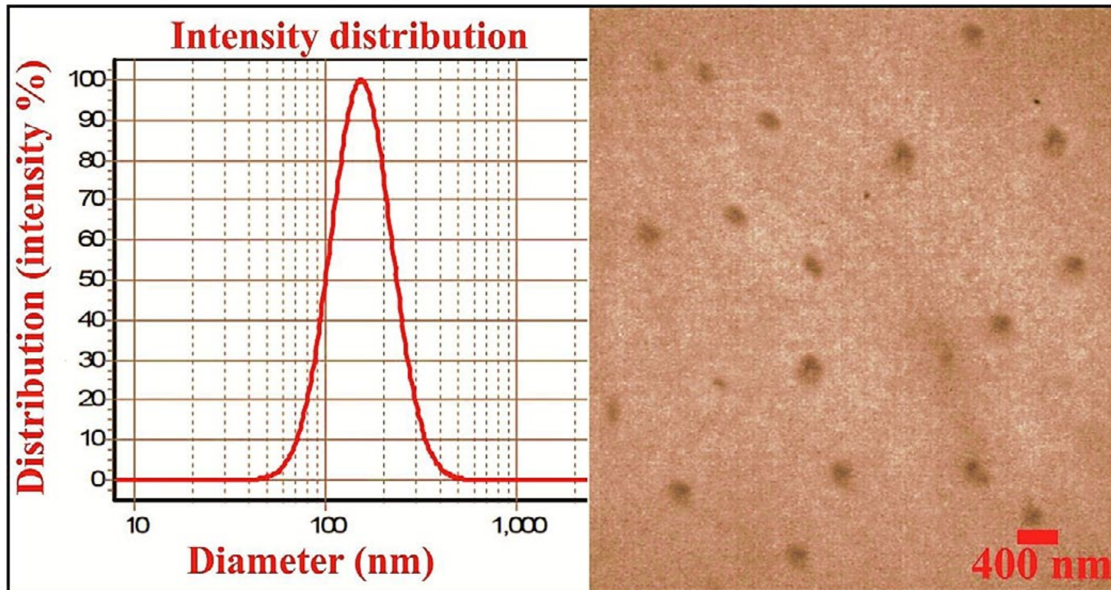

Fig. 1. Results of DLS (left) and TEM (right) analyses on the nanoformulation (1F)

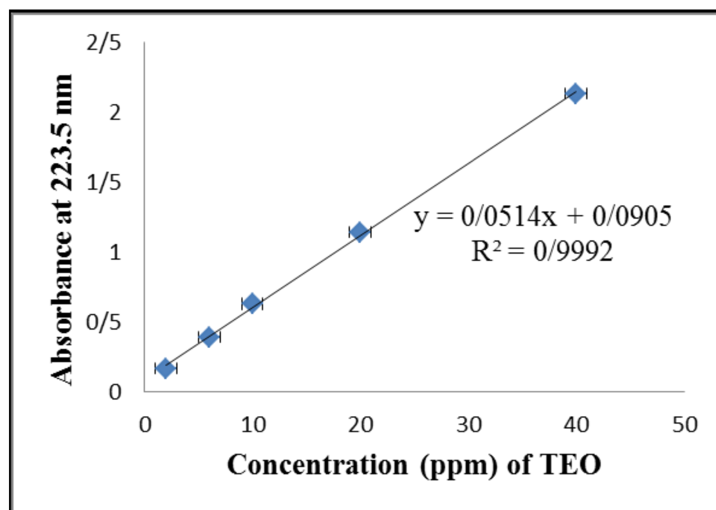

Fig. 2. Calibration curve and regression equation for determining amounts of tarragon essential oil (TEO) in the supernatant of the nanoformulation (1F)

UV-Vis analysis, it could be argued that TEO has been successfully encapsulated in the chitosan nanocapsules.

Comparison of duration of LA of 1F and bulk TEO in the lab and SSF tests

Fig. 4 compares LA of 1F with bulk TEO having similar concentrations of TEO (i.e. 6.04%) in the lab and SSF tests. LA of 1F shows to continue for 10 days in both the lab and SSF tests, while LA of bulk TEO was significantly lower ($p<0.05$), in both the lab and SSF tests (i.e. 5 and 3 days, respectively). LA of 1F(-oil) is negligible and reaches zero at days 5 in the lab and SSF tests, showing that ingredients of the nanoformulation do not have an important effect on larvae. As the concentration of TEO in the containers treated with 1F and bulk TEO was fixed at 302 ppm, it is arguable that longer LA of 1F is due to slow release of TEO for this preparation.

Duration of perfect LA (causing 100% mortality in treated larvae) of bulk TEO in the lab test (4 days) was significantly higher than that of the SSF test

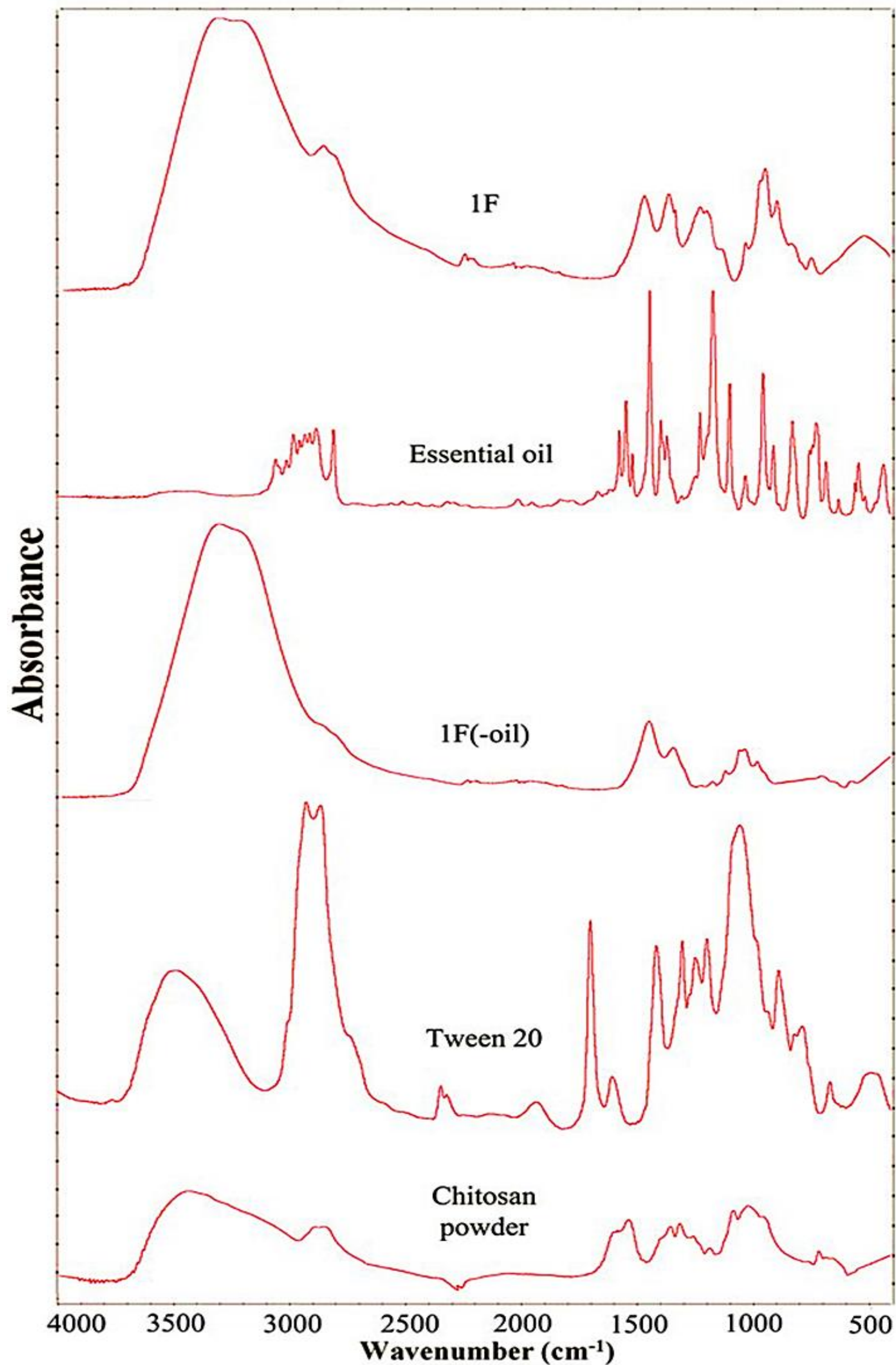

Fig. 3. FT-IR of the nanoformulation (1F), nanoformulation without oil (1F(-oil)) and their constituents

(2 days) ($p<0.05$). This is due to harsh conditions in the SSF, including air circulation and higher evaporation of EO during the test. In general, EOs in their bulk form have a short duration of action due to volatility of ingredients. Reviewing the literature, many reports are focusing only on LA of EOs, not their duration of the effect (41, 42). While, in this study, using nanoparticles, encapsulated TEO was protected environmental factors.

The only reports we could find about larvicide systems with slow release properties contained EO of *Lippia sidoides*. By encapsulating *Lippia sidoides*

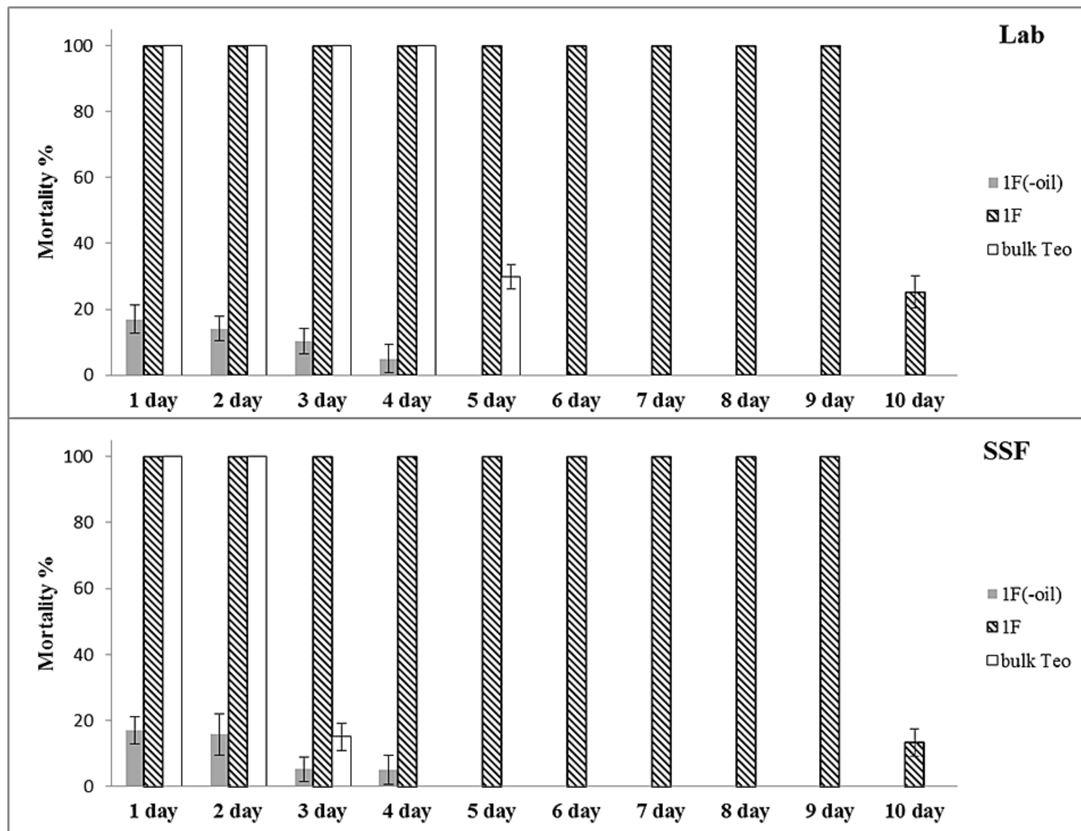

Fig. 4. Comparison of larvicidal activity of the nanoformulation (1F) vs. bulk tarragon essential oil (bulk TEO) both in the lab test (upper) and SSF (simulated semi-field test) (down).

in chitosan/cashew gum beads, non-floating beads with a size of around 1.5 mm were prepared which were able to control larvae of *Ae. aegypti* for up to 3 days (20). In another formulation, *Lippia sidoides* was loaded in Alginate/cashew gum beads in 2 forms (i.e. floating and non-floating beads (~ 1.7 mm)). Floating beads showed very good buoyancy up to 6 days and its LA after 48 h was around 85% against *Ae. aegypti*, while corresponding mortality in non-floating beads was around 30% (43). As larval stages of mosquitos are 9-11 days, a larvicide with duration of action comparable to this period is preferred. While the mentioned reports provide the substantially lower duration of LA, our nanoformulation (1F) remained active for 10 days. Furthermore, chitosan is a biodegradable polymer (44, 45), which was used as carrier of TEO. Both of the active agent (TEO) and the carrier (chitosan nanocapsules) are degradable in the environment. Therefore, long-lasting LA, proper efficiency and environmental safety are achieved simultaneously using this preparation.

Fig. 4. Comparison of larvicidal activity of the nanoformulation (1F) vs. bulk tarragon essential oil (bulk TEO) both in the lab test (upper) and SSF (simulated semi-field test) (down).

Comparing cytotoxicity of 1F and temephos
MTT assay

Results of viability evaluation of HFFF2 cell line, treated with 1F and 1F(-oil) at 5 different concentrations and also temephos (1 ppm) is depicted in Fig. 5. Cell viability of blank group (not treated) in comparison with the bulk TEO group (i.e. cells treated with TEO 25%) shows no significant difference (p>0.05), both having 100% viability, indicating that TEO has no cytotoxicity at concentration of 25%.

Additionally, cell viability in different concentrations of the nanoformulation (i.e. 4F: 48.19%, 2F: 67.34%, 1F: 74.62%, 2/3F: 77.33% and 1/2F: 82.84%) showed no significant changes (p>0.05), compared with their corresponding formulations without oil (i.e. 4F(-oil): 50.55%, 2F(-oil): 72.27%, 1F(-oil): 82.00%, 2/3F(-oil): 87.86% and 1/2F(-oil)): 88.51%). This confirms lack of

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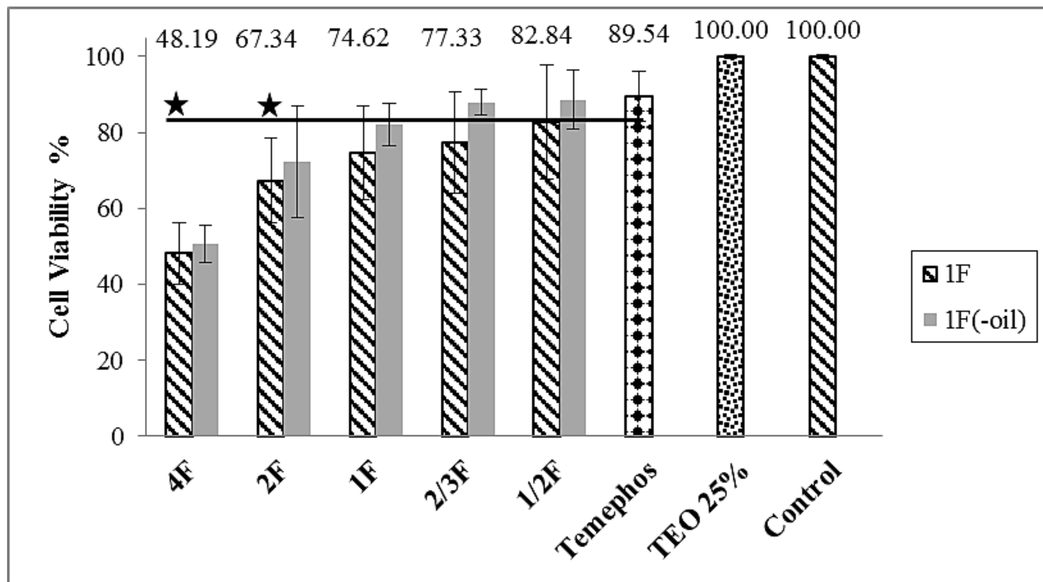

Fig. 5. Comparison of cytotoxicity of different concentrations of the nanoformulation with and without tarragon essential oil (TEO) (4F, 4F(-oil), 2F, 2F(-oil), 1F, 1F(-oil), 2/3F, 2/3F(-oil), 1/2F, 1/2F (-oil)) with temephos (1 ppm) and TEO 25% by MTT assay. *Represents significant changes compared with temephos ($p<0.05$).

cytotoxicity of TEO up to concentration of 25%.

Furthermore, there are no significant differences ($p>0.05$) between cell viability of temephos (89.54%), as standard larvicide (positive control) with the nanoformulation 1F or those having lesser TEO (i.e. 1/2F and 2/3F). As the details show, by increasing the concentration of the nanoformulation to 2F and 4F, cytotoxicity significantly increases ($p<0.05$) compared with temephos.

The MTT assay was carried out to evaluate the mitochondrial activity of succinate dehydrogenase for measuring cell viability. Nowadays, MTT assay is becoming a routine test for evaluating the cytotoxicity of formulated or non-formulated EOs. In a report, toxicity of cardamom oil loaded into chitosan was evaluated just in a single concentration (i.e. 100 ppm); no cytotoxicity was observed on human corneal epithelial cells in MTT assay (46). Having said that, cytotoxicity of some bulk EOs such as *Myrtus nivellei*, *Juniperus communis* and *Zanthoxylum bungeanum* on the human keratinocyte cell line (HaCaT) has already been evaluated. The EOs have no toxic effect up to a concentration of 1.25, 0.64 and 1.075 µL/mL, respectively (47-49).

LDH test

Fig. 6 demonstrates LDH release of cells after treatment with 5 different concentrations of F and F(-oil) as well as treatment with temephos (1%) and TEO 25%. LDH release of cells treated with TEO 25% was 0.05%, showing that TEO has no important effect on this cell line at 25% concentration, a result which agrees well with our MTT findings.

Furthermore, no significant difference ($p>0.05$) was observed between cytotoxicity of different concentrations of nanoformulation with and without TEO up to concentration of 2F (i.e. 2F, 1F, 2/3F and 1/2F, compared with 2F(-oil), 1F(-oil), 2/3F(-oil) and 1/2F(-oil), respectively). LDH release for 4F was however significantly higher than 4F(-oil): (i.e. 78.00% compared with 27.07%, respectively).

Comparing the results of the LDH test on cells treated with 1/2F, 2/3F and 1F, with those treated with temephos also shows no significant change ($p>0.05$). However, with increasing the concentration to 2F and 4F, LDH release increases significantly ($p<0.05$).

LDH is an oxidoreductase which catalyzes interconversion of NADH and NAD^+. When injury or toxic material damage cells, LDH is released into the cell culture. LDH is widely used to evaluate cell toxicity/damage (50). Using LDH test, cytotoxicity of EO of *Salvia officinal* has been evaluated by LDH test. Results showed no cytotoxic effect on freshly isolated rat hepatocytes up to 2 µL/mL (51).

Fig. 6. Comparison of toxicity of different

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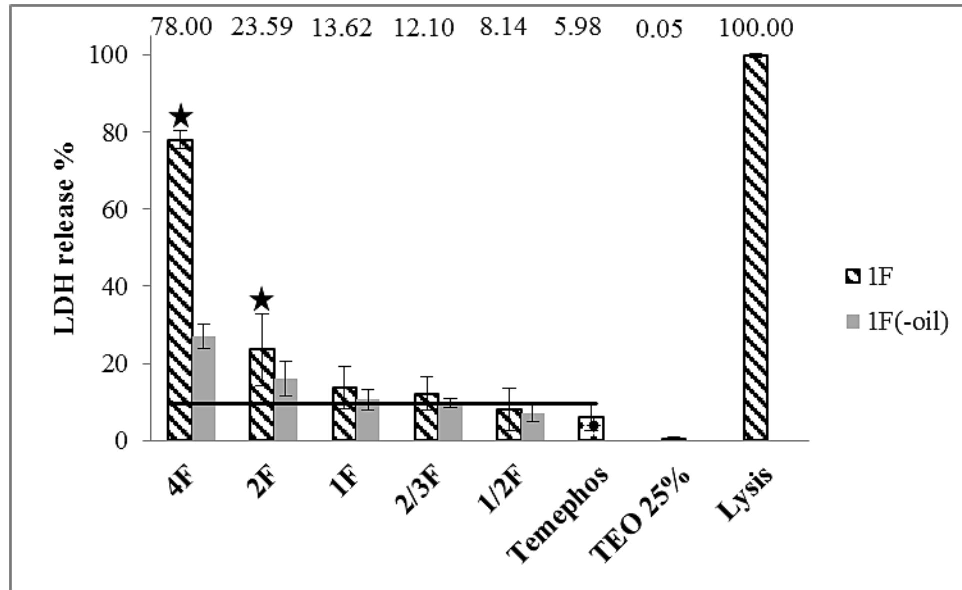

Fig. 6. Comparison of toxicity of different concentrations of the nanoformulation with and without tarragon essential oil (TEO) (4F, 4F(-oil), 2F, 2F(-oil), 1F, 1F(-oil), 2/3F, 2/3F(-oil), 1/2F, 1/2F(-oil)) with temephos (1 ppm) and TEO 25% by LDH test. *Represents significant change compared with temephos (p<0.05)

concentrations of the nanoformulation with and without tarragon essential oil (TEO) (4F, 4F(-oil), 2F, 2F(-oil), 1F, 1F(-oil), 2/3F, 2/3F(-oil), 1/2F, 1/2F(-oil)) with temephos (1 ppm) and TEO 25% by LDH test. *Represents significant change compared with temephos (p<0.05)

CONCLUSION

In this research, chitosan nanocapsules containing TEO (1F) were prepared as green nano-larvicides. Its larvicidal activity in lab and SSF tests showed to last for 10 days, comparable with synthetic larvicides. Cytotoxicity of 1F on human normal skin cells (HFFF2) had no significant difference with temephos which is used as a standard larvicide. This study demonstrates the potential of this herbal nanoformulation as a suitable alternative for synthetic larvicides in terms of long-lasting activity, efficiency and environmental friendliness.

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CONFLICT OF INTEREST

There is no conflict of interest to the authors.

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