SUPPLEMENTARY MATERIAL

Chemical composition and potentiation of insecticidal and fungicidal activities of *Citrus trifoliata* L. fruits essential oil against *Spodoptera littoralis, Fusarium oxysporum* and *Fusarium solani via* nano-cubosomes

Mostafa A. Abdel-Kawy[a], Camilia G. Michel[a], Farid N. Kirollos[a], Rania A. A. Hussien[b], Abdulaziz M. Al-mahallawi[c,d] and Mohamed S. Sedeek[a]*

[a] *Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Postal Code 11562, Cairo, Egypt*

[b] *Fungicide, Bactericide and Nematicide Dept., Central Agricultural Pesticides Lab (CAPL), Agriculture Research Center (ARC), Dokki, Giza, Egypt.*

[c] *Department of pharmaceutics and industrial pharmacy, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Postal Code 11562, Cairo, Egypt*

[d] *Department of pharmaceutics, Faculty of pharmacy, October University for Modern Sciences and Arts (MSA), Giza, Egypt*

* Corresponding author: Mohamed S. Sedeek

Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Postal Code 11562, Cairo, Egypt

E-mail address: drmohamedsedeek@live.com, Tel. 01282534822

Chemical composition and potentiation of insecticidal and fungicidal activities of *Citrus trifoliata* L. fruits essential oil against *Spodoptera littoralis*, *Fusarium oxysporum* and *Fusarium.solani* via nano-cubosomes

Abstract

Development of natural nano-based plant-protection formulations represents an emerging phenomenon that has been widely improved for crops protection and for enhancing the efficiency and safety of pesticides. In the present study we isolated the essential oil from the fruits of *Citrus trifoliata* L. and investigated it using gas chromatography-mass spectrometry analysis. Limonene (78.46%) was the major component followed by β-Myrcene (7.94%) and Caryophyllene (4.20%). *Citrus trifoliata* essential oil (CTEO) loaded nano-cubosomes were successfully prepared by the emulsification technique. The insecticidal and fungicidal activities of formulated CTEO nano-cubosomes and unformulated CTEO were tested. While both of them exhibited substantial activities, CTEO nano-cubosomes were more effective than unformulated oil. It is the first time to formulate CTEO in nano-cubosomes and examine their insecticidal and fungicidal activities. In light of the current study, CTEO as it is or as nano-cubosomes is recommended as a promising candidate for pest and fungal pathogens control.

Keywords:

Citrus trifoliata L. fruits; essential oil; nano-cubosomes; insecticidal; fungicidal.

Experimental section

Plant material

Fruits of *Citrus trifoliata* L. were collected from Horticulture Research Institute, Giza, Egypt. The identity of the plant was kindly confirmed by Dr. Mohamed El-Gebaly, Plant taxonomy department, National Research Centre, Giza, Egypt. A voucher specimen 9.12.2018 (1) of the plant were registered and deposited in the herbarium of Faculty of Pharmacy, Cairo University. *Citrus trifoliata* L., the plant list (http://www.theplantlist.org), last access on 7 May 2019.

Preparation of the essential oil from fruits of C. trifoliata L

Peels of the fruits of *C. trifoliata* L. were washed, dried and powdered mechanically. Their essential oils were extracted by hydro distillation in a Clevenger's apparatus for 5 hrs. According to the procedure described in the Egyptian Pharmacopeia (E.P.2005). The essential oils were dried with anhydrous sodium sulphate and stored in amber glass vials at 4 °C for use in further chemical and biological studies.

GC–MS analysis and quantification (Table S1)

Mass spectra were recorded using Shimadzu GCMS-QP2010 (Tokyo, Japan) equipped with Rtx-5MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, USA) equipped with a split–splitless injector. The capillary column was directly coupled to a quadrupole mass spectrometer (SSQ 7000; Thermo-Finnigan, Bremen, Germany). The initial column temperature was kept at 45 °C for 2 min (isothermal) and programmed to 300 °C at a rate of 5 °C/min, and kept constant at300 °C for 5 min (isothermal). Injector temperature was 250 °C. Helium carrier gas flow rate was 1.41 ml/min. All the mass spectra were recorded applying the following condition: (equipment current) filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 200°C. Diluted samples (1% v/v) were injected with split mode (split ratio 1: 15). The sample (1 µL) was injected automatically to the chromatograph using AOC-20i auto sampler. Volatile components were deconvoluted using AMDIS software (www.amdis.net) and identified by its mass spectrum matching to EPA/NIH database and with authentic standards (when available). Peaks were quantified by selected abundant fragments (m/z) to overcome the problem of co-eluted compounds

and relative volatile abundances were calculated using accustom PEARL script to extract peak areas of individual ions characteristic of each component (Halket et al. 1999).

Preparation of oil loaded cubosomes

Material

Glyceryl monooleate (GMO), polyvinyl alcohol (PVA) and Pluronic F127 were obtained from Sigma Chemical Co. (St. Louis, USA).

Preparation of nano-cubosomal dispersions

It is well known that GMO is able to incorporate water-soluble, oil-soluble, and amphiphilic substances within its aqueous and lipid domains(Nasr et al. 2015; Karami & Hamidi 2016; Rao et al. 2018). Being a water insoluble essential oil, the CTEO is incorporated within the lipid domain of the nano-cubosomes. Cubosomal dispersions were prepared by emulsification technique described by (Morsi et al. 2014) with slight modifications. In brief, GMO (900 mg) and Pluronic F127 (100 mg) were melted at 70^{o}C and the oil (1000 mg) was added to this molten mixture. The obtained oily solution was added drop-wise to 8 mL distilled water (70°C) under mechanical stirring at 1500 rpm (final volume 10 ml). Essential oil represented only 10% of the prepared nano cubosomes which allow us to economically prepare nano pesticide using small amounts of essential oil. Dispersion was maintained under stirring and cooled to room temperature to achieve homogenous state followed by bath sonication (Crest Ultrasonic Corp., NJ, USA) for 10 min to ensure the development of fine-tuned dispersion (Abdelbary et al. 2016; Saber et al. 2018). The average particle size and poly dispersity index as measured by dynamic light-scattering technique (Abdelbary et al. 2015) using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) were found to be 220 nm and 0.17, respectively (Figure S1). Figure S2 demonstrated that the prepared nano-formulation exhibited a zeta potential of -37 mV indicating that they have sufficient charges that would inhibit their aggregation (Abdelbary et al. 2016). Transmission electron microscope (TEM) revealed that the prepared cubosomes are in the nano-size, which confirms the results of particle size measurement, showed nanoparticles of cubical nanostructure and red arrows indicate more or less cubic nano-particles (Figure S3). The appearance of spherical globules in the transmission electron micrographs is

attributed to the existence of emulsified oil globules together with the nano-cubosomes (the oil was emulsified by the added Pluronic F127, which is a hydrophilic surfactant).

Insecticidal activity of CTEO and its nano cubosomes against S.littoralis

Mass rearing of S. littoralis

A laboratory strain of the cotton leaf worm , *Spodoptera littoralis* (Lepidoptera:noctuidae) was obtained and reared in the cotton leaf worm department , plant protection research institute , Dokki , Giza under constant laboratory conditions as described by (Hamouda et al. 2016) at 26◦C, 60% RH and 12:12 h light:dark photoperiod. They were reared on modified artificial diet contained 20 g of agar, 5 g of ascorbic acid, 150 g of chickpea flour, 40 g of yeast, 1 g of benzoic acid and 1 g of nipagin in a total water volume of 800 ml.

Assay (Table S2&S3)

Artificial diet bioassay , similar to that described by (Hamouda et al. 2016), was used to determine the toxicity of *C. trifoliata* L. fruits essential oil (CTEO) as described by previous studies of essential oils toxicity (Knaak et al. 2014) and its nano cubosomes against 2nd instar larvae using different volumes of 25, 50, 75 and 100 µl from the oil and 50, 100, 150, 200 µl from nano cubosomes form.

Newly emerged 2nd instar larvae were placed individually in small covered cups. Then, they were fed with 5g of treated artificial diet for 24 h. Three replicates of 10 larvae were used for each treatment. Three replicates of 10 larvae were fed on artificial diet for 24 h to serve as control. Larval mortality was recorded after 24h.

Statistical analysis

Lethal Concentrations (LC_{50}) were calculated based on the obtained results of larvicidal effect. LC_{50} values were calculated using probit analysis as described by Finney 1971 (Okumu et al. 2007) , using "L d p line" software.

Antifungal assay of CTEO and its nano cubosomes against F. oxysporum and F.solani

Fungal strains

Pure cultures of *F. solani* and *F. oxysporum* were obtained from the department of fungicides, bactericides and nematicides, central agricultural pesticides laboratory.

Assay (Figure S4) and (Table S4&S5)

In vitro antifungal activity of CTEO and its nano cubosomes was determined by measuring the growth inhibition through agar well diffusion assay as described (Khokhlov 2015; Al-Zahrani & Al-Garni 2019).

Potato dextrose agar (PDA) medium (containing 20gm dextrose, 200gm starch and 15 gm agar) was poured into sterilized Petridishes (20mm diameter) and kept for solidification. After solidification, well of 6mm diameter were punctured in the culture medium using sterile CDRK borer. Different volume (50, 100, 150, 200 µl) of *Citrus trifoliata* L. volatile oil and its nano-cubosomes were loaded in the well using sterilized micropipettes. The tested fungi were inoculated with 6 mm mycelia plugs after 7 days incubation at 25±2 ºC. Other plates containing free media (without volatile oil) were inoculated with the pathogenic fungi and were used as a check treatment. Four replicates were used for each treatment. The percentage inhibition of the radial growth by different volumes of volatile oil or its nano cubosomes Compared to control was calculated using the following formula:

Percentage mycelia inhibition = dc – dt/dc X 100

where dc is the mean colony diameter for the control sets and dt is the mean colony diameter for the treatment sets as described by (Rai et al. 2014).

Statistical analysis

EC_{50} and EC_{90} values were determined by the linear regression (LPD line computer program) of the probit of the tested fungus. Percentage inhibition versus logs volumes of volatile oil and its nano cubosomes. The EC_{50} notation used to indicate the effective concentration (volumes) of the tested oil that causes 50 % inhibition of the mycelial growth.

			Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	220.1	**Peak 1:**	265.7	100.0	124.6
PdI:	0.171	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.966	**Peak 3:**	0.000	0.0	0.000
Result quality	Good				

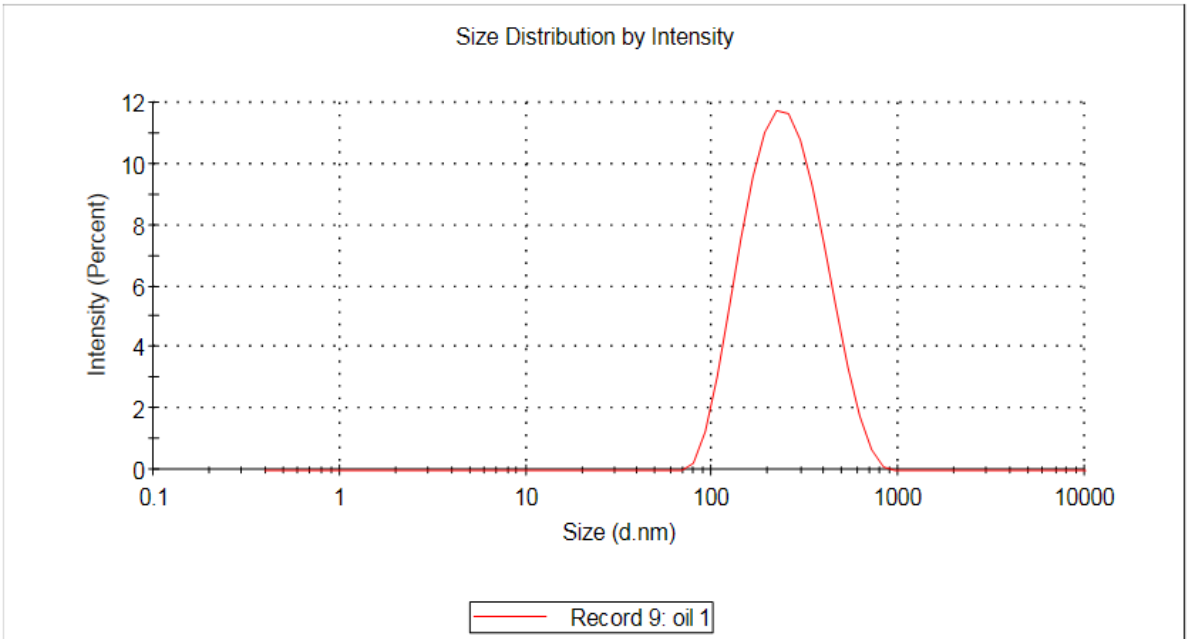

Figure S1: Particle size distribution of *C. trifoliata* L essential oil nano-cubosomes

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-37.6	**Peak 1:**	-37.6	100.0	5.01
Zeta Deviation (mV):	5.01	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0147	**Peak 3:**	0.00	0.0	0.00
Result quality	Good				

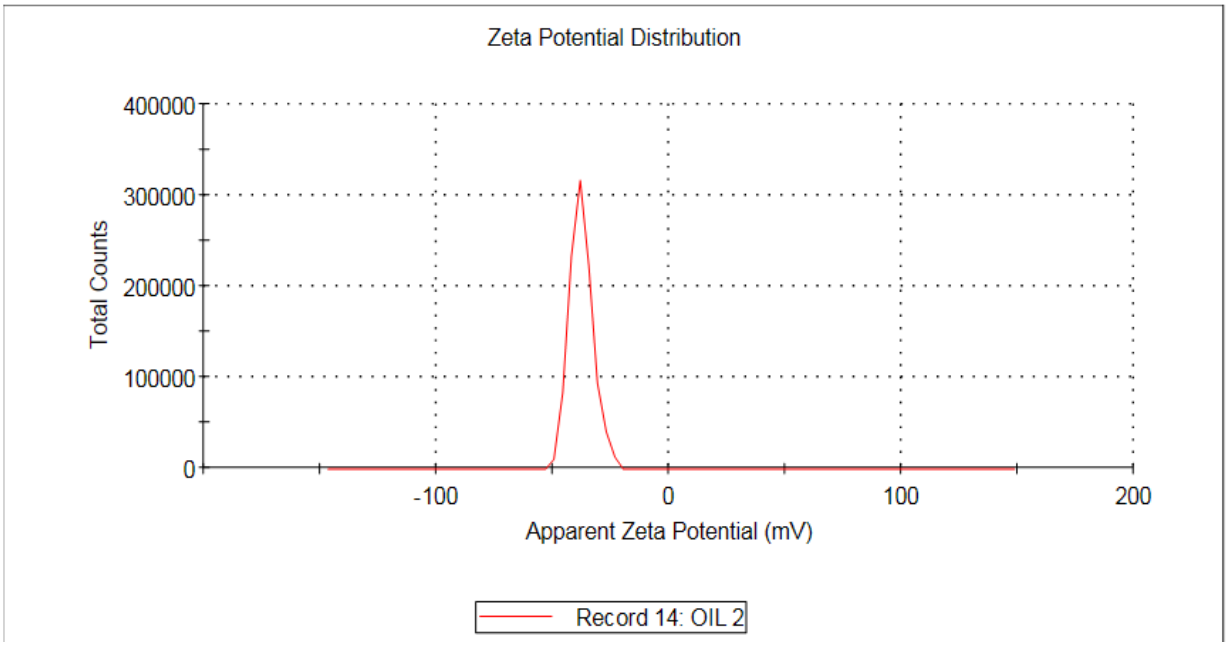

Figure S2: Zeta potential of *C. trifoliata* L essential oil nano-cubosomes

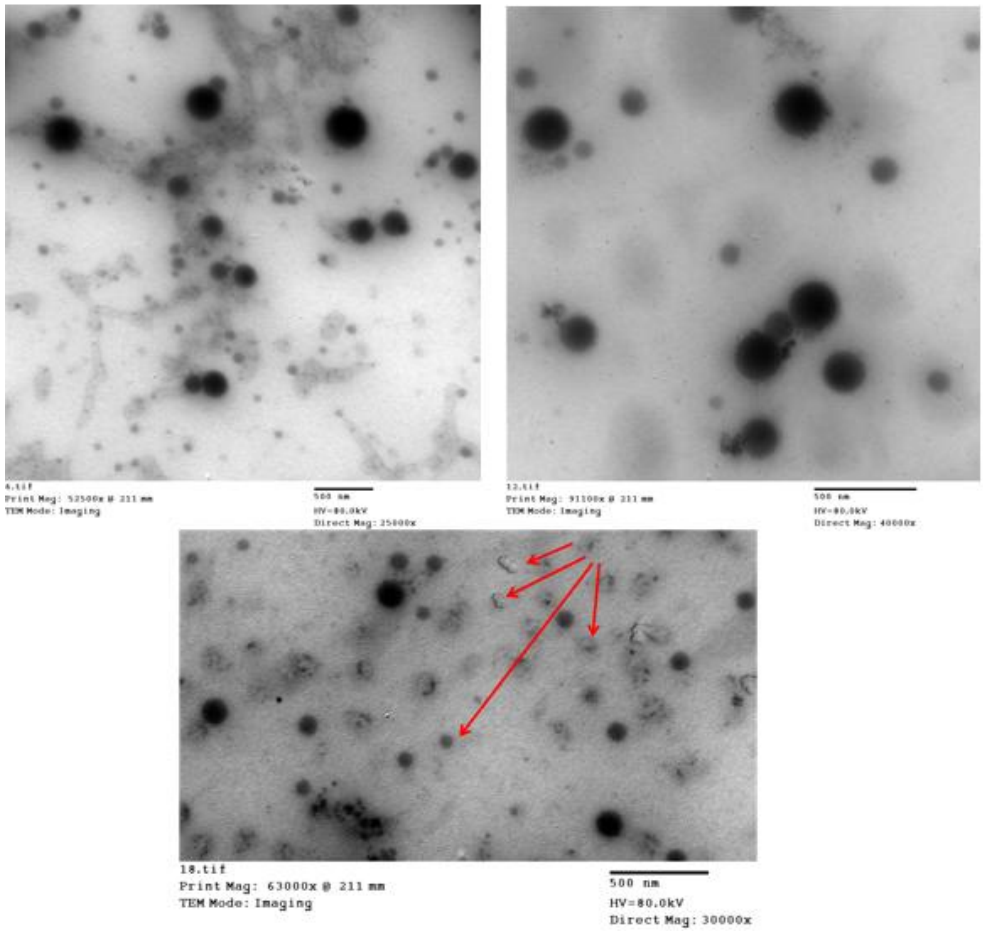

Figure S3: Transmission electron micrographs of different nano-cubosomal dispersions of *C. trifoliata* L essential oil nano-cubosomes, red arrows indicate more or less cubic nano-particles.

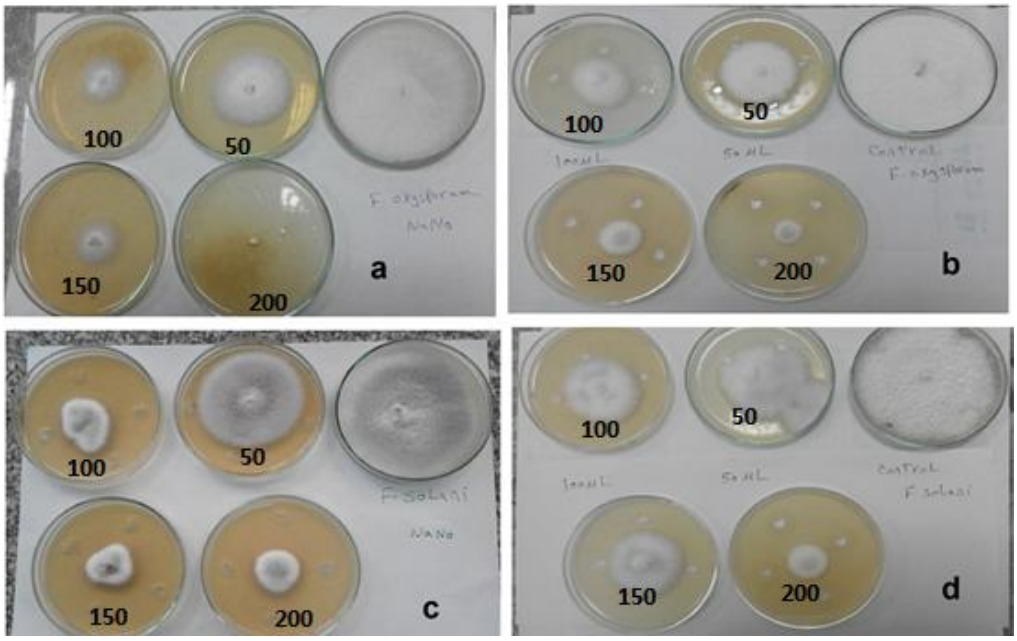

Figure S4: Antifungal activity of CTEO and its nano-cubosomes against *F.solani* and *F.oxysporum* at different volumes using agar well diffusion assay. Numbers on the photos correspond to the tested concentrations (µl/ml).

a. Nano-cubosomes against *F. oxysporum*

b. CTEO against *F. oxysporum*

c. Nano-cubosomes against *F. solani*

d. CTEO against *F. solani*

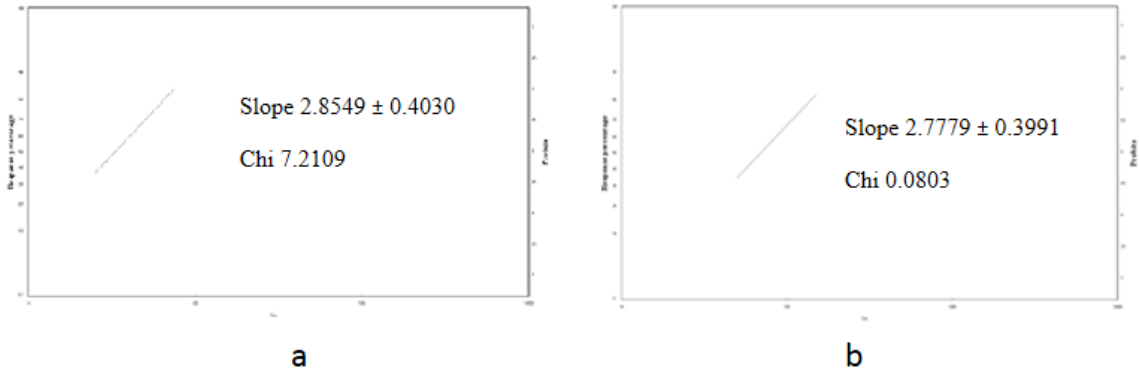

Figure S5: probit analysis of mortality of *S. Litteralis* larvae after 24h of exposure to different concentrations of a) CTEO b) its nano-cubosomes

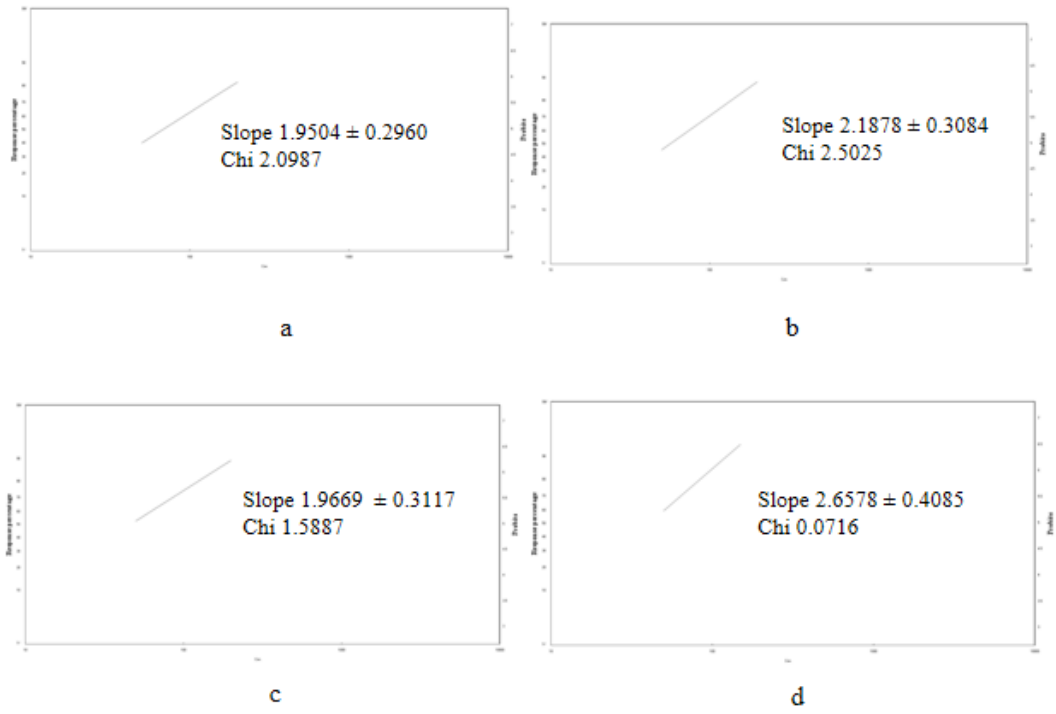

Figure S6: probit analysis of Antifungal activity of CTEO against a) *F. solani* b) *F. oxysporum* and antifungal activity of its cubosomes against c) *F. solani* d) *F. oxysporum*

Table S1: Composition of the fruits essential oil of *Citrus trifoliata* L.

No.	RT	Name	Area%
1	7.58	α-Pinene	0.20
2	8.79	Sabinene	0.03
3	8.88	β-Pinene	0.53
4	9.36	β-Myrcene	7.94
5	9.74	α-Phellandrene	1.05
6	10.13	α-Terpinene	0.07
7	10.66	Limonene	78.46
8	10.82	β-*cis*-Ocimene	0.03
9	11.13	β-*trans*-Ocimene	0.58
10	11.46	γ-Terpinene	0.24
11	11.92	*cis*-Sabinene hydrate	2.13
12	12.40	*cis*-Linalool oxide	1.17
13	12.75	Linalool	0.89
15	13.44	Carveol	0.04
16	15.18	*trans*-Sabinene hydrate	0.67
17	15.60	α-Terpineol	0.77
18	21.41	γ-Elemene	0.11
19	22.23	Caryophyllene	4.20
20	23.06	Humulene	0.17
21	23.11	β-Farnesene	0.24
22	23.67	α-Selinene	0.02
23	24.40	Germacrene	0.03
24	24.48	α-Farnesene	0.03
		Total identified	99.6
		Total monoterpene hydrocarbons	94.8
		Total oxygenated monoterpene hydrocarbons	3.54
		Total sesquiterpene hydrocarbons	4.80

Table S2: Mortality of S. litteralis larvae after 24h of exposure to different concentrations (µl / 5g diet) of *C. trifoliata* oil and its nano-cubosomes

	LC_{50}	Upper[a] Lower	LC_{90}	Upper[a] Lower
CTEO	41.6 ± 0.9	42.62	75 ± 1.3	76.47
		40.58		73.53
Nano-cubosomes	77.3[*] ± 1.2	78.66	165.8 ± 1.1	167.04
		75.94		164.56

[a] 95% lower and upper confidence limits

[*] Significantly different from CTEO at $p<0.05$. Statistical analysis was performed by t-test.

Table S3: Antifungal activity of *C. trifoliata* oil and its nano-cubosomes against *F. solani*

	EC_{50}	Upper[a] Lower	EC_{90}	Upper[a] Lower
CTEO	65.5 ± 0.8	66.4	220 ± 1.2	221.36
		64.6		218.64
Nano-cubosomes	41.5[*] ± 0.6	42.18	190 ± 0.9	191
		40.82		189

[a] 95% lower and upper confidence limits

[*] Significantly different from CTEO at $p<0.05$. Statistical analysis was performed by t-test.

Table S4: Antifungal activity of *C. trifoliata* oil and its nano-cubosomes against *F. oxysporum*

	EC_{50}	Upper[a] Lower	**EC_{90}**	Upper[a] Lower
CTEO	55.1 ± 0.7	55.89	185.7 ± 1.3	187.17
		54.31		184.23
Nano-cubosomes	35[*] ± 0.5	35.566	130 ± 0.8	130.9
		34.434		129.1

[a] 95% lower and upper confidence limits

[*] Significantly different from CTEO at $p<0.05$. Statistical analysis was performed by t-test.

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