Journal of Plant Protection Research ISSN 1427-4345

ORIGINAL ARTICLE

Rosemary essential oil nanoemulsion, formulation, characterization and acaricidal activity against the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae)

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Vol. 59, No. 1: 102–112, 2019

DOI: 10.24425/jppr.2019.126039

Received: October, 2018
Accepted: March 5, 2019

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Abstract

The adverse effects of synthetic acaricides on humans, animals, non-target organisms and the ecosystem are serious problems. Thus, there is a new trend to use nanotechnology for developing new, natural, bio and safe acaricides for mite control in green-pest management. This is the first work for preparing a nanoformulation of rosemary essential oil (EO) and evaluating its effect against the two-spotted spider mite *Tetranychus urticae* Koch. GC/MS analysis of rosemary EO showed that 1,8 cineole (31.45%), borneol (11.07%), α-pinene (10.91%), D-limonene (9.19%), L-linalool (8.86%), D-camphor (7.32%), γ-terpinene (3.92%), linalyl acetate (3.37%), α-terpineol (3.32%), and p-cymene (1.82%) were the major components. After 6 min of sonication, a nanoemulsion of rosemary EO was formulated with a droplet size of 139.9 nm. The balance between oil (lyophilic) and surfactant (hydrophilic) was correlated with the droplet size and the stability of the nanoemulsion. Spray application of rosemary nanoemulsion showed high acaricidal activity against immature and adult two-spotted spider mites *T. urticae* with LC_{50} 723.71 and 865.68 μg · ml^{-1} and the toxicity increased by 54.15 and 52.69% for immature and adult mites, respectively. There were no toxic effects or mortality of rats treated with rosemary nanoemulsion. High acaricidal activity, stability, and safety of rosemary nanoemulsion make this nanoformulation a possible green and nano-acaricidal product. Further studies under field conditions are necessary to study the acaricidal efficiency of rosemary nanoemulsion against two-spotted spider mites and the toxic effect on predacious mites.

Keywords: acaricidal activity, essential oil, nanoemulsion, rosemary, the two-spotted spider mite, toxicity

Introduction

Synthetic pesticides play a significant role in pest control both in agriculture and in public health sectors. Throughout application of pesticides, a mole amount (<5%) of pesticide formulations "such as acaricides" reach the target pests and another amount (>95%) extends to non-target species (Miller and Spoolman 2014). Therefore, pesticide residues were found in fruits, vegetables and other foods, as well as in environmental components. Due to the lipophilicity of pesticides, it can accumulate in fatty tissues, plants, and induce adverse effects in humans, non-target species, and the ecosystem. It can induce genotoxicity, hepato, renal and reproductive toxicity in experimental animals (Mansour and Mossa 2010; Mossa and Abbassy 2012; Marzouk *et al.* 2012) and agricultural workers (Abbassy *et al.* 2014). It can also, induce oxidative stress, lipid

peroxidation, and organ dysfunction in both mothers and their offspring (Mossa *et al.* 2017). Moreover, pest resistance and tolerance have increased due to the increasing application of pesticides which in turn leads to increased costs, contamination and adverse health effects (Damalas and Eleftherohorinos 2011).

There is a new trend for using natural, bio and safe pesticides for pest control in green-pest management (Mossa 2016). Essential oils (EOs) are important and safe compounds to control pests such as phytophagous mites, fungi, insects, nematodes, and weeds (Rossi and Palacios 2015; Pavela 2015; Fatemeh and Moharramipour 2017; Mossa *et al.* 2018). Essential oils are produced by many plants, which contain many active compounds such as second metabolites and play important roles in plant defences (Rattan 2010). Plant extracts and EOs are considered to be green pesticides with low to no toxicity to mammals and are ecofriendly (Mossa *et al.* 2017; Mossa *et al.* 2018).

The two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is one of the most important pests in agriculture that causes damage to many vegetable crops either in open fields or in protected greenhouses. Spider mites are found worldwide under different environmental conditions, to which they can adapt and induce damage by sucking the contents of cell sap. Their natural enemies play an important role in reducing the damage caused by spider mites. When insecticides are used extensively against insect pests, the damage of spider mites increases due to the destruction of natural enemies. Populations of spider mites have been rapidly increasing their resistance to several acaricides and recently have experienced a new aspect of cross resistance (Pree *et al.* 2002; Kimbaris *et al.* 2006).

Previous studies have reported acaricidal activity of many EOs such as garlic, basil, bergamot, black pepper, caraway seed, cedarwood, clove, citronella, eucalyptus, lavender, lemon, hot pepper, ginger and rosemary (Nechita *et al.* 2015; Zandi-Sohani and Ramezani 2015; Tak and Isman 2017; Kim *et al.* 2018; Lee *et al.* 2018). Rosemary EO has been investigated for biological activities by several workers (Elamrani *et al.* 2000; Tak and Isman 2016). Some active compounds were found in rosemary EO e.g., 1,8 cineole, camphor and (+)-α-pinene (Tak and Isman 2016), which have high activity against many insects and acarine species (Lee *et al.* 2001; Yang *et al.* 2004; Waliwitiya and Lowenberger 2009). Although EOs have high activity and are effective for pest control, there are many technical issues that limit EOs application such as insolubility, volatility, degradation and short half-life time (Raut and Karuppayil 2014; Mossa *et al.* 2018). Currently, there is a new trend to use nanotechnology to resolve these problems and to develop many nanoformulations. These nanotechnologies or nanoformulations are important issues for using EOs in modern agriculture and green pest management. This study, thus, was designed to formulate nanoemulsion of rosemary EO by using ultrasonic emulsification method and to study the acaricidal effect against the two-spotted spider mite *T. urticae*.

Materials and Methods

All experiments in this study were carried out in the laboratories of the Pesticide Chemistry Department and Pests and Plant Protection Department, National Research Centre (NRC), Egypt.

Extraction of rosemary EO

Rosemary plants used in the current study were obtained from the market in Giza, Egypt. The dried leaves (at room temperature) were ground and subjected to hydrodistillation for essential oil extraction using a Clevenger apparatus. The obtained oil was dried by anhydrous sodium sulfate (Na_2SO_4) and storage undercooling in amber glass. In this study, the yield of rosemary EO was 1.45 g · 100 g^{-1} dry weight (1.45%).

Gas chromatography-mass spectrometry (GC/MS) analysis

GC/MS (Agilent Technologies) equipped with GC (7890B) and MS detector (5977A) was used for rosemary EO analysis (Central Laboratories Network, National Research Centre, Giza, Egypt). Essential oil was mixed with hexane at a ratio of 1 : 19 (v/v). A GC fused silica capillary column was used with helium as the carrier gas (1 ml · min^{-1}). The following conditions were used 1 µl (injector volume); 1 : 30 (split ratio), 280°C, and 220°C were the injector and detector temperatures. The conditions of MS were as follows: solvent delay time, 3 min; ionization voltage, 70 eV; and m/z range, 50–550 and the GC column was introduced directly into the source of the MS. The MS operating conditions were 3 min (solvent delay time), 70 eV (ionization voltage), and 50–550 m/z range. All obtained results of GC/MS analysis were compared with the data in NIST and Wiley Mass Spectral Library.

Fabrication of rosemary EO nanoemulsion

Rosemary nanoemulsion (oil-in-water) at a concentration of 5% was prepared by using Tween 20 (Polysorbate 20, VWR International 201, Rue Carnot F-94126 Fontenay/Bois, France) as a non-ionic surfactant and deionized water. For nanoemulsion fabrication, the EO and surfactant (Tween 20) were used as an organic

phase at different ratios (1 : 1, 1 : 1.5 and 1 : 2 w/w). Deionized water (D.W.) was used as an aqueous phase, added to the organic phase and subjected to sonication for 2, 4 and 6 min using ultrasound (Sonics & Materials, INC. 53 Church Hill Rd. Newtown, CT, USA) with a probe diameter of 13 mm at a high frequency of 20 kHz and power output of 750 W. A sonicator probe was used to give the energy which was reduced by using ice. In contrast, the normal emulsion of rosemary EO (5%) and Tween 20 was prepared by using 5-seconds as sonication time. This emulsion was stable for 2 days.

Rosemary EO nanoemulsion characterization

Rosemary EO nanoemulsion was studied for physicochemical properties. The stability of nanoemulsion was tested with different stress factors such as heating, cooling, and freezing, as cited by Mossa *et al.* (2018). The stable formulations were centrifuged at 10,000 rpm for 30 min at 25°C (Heraeus Labofuge 400R, Kendro Laboratory Products GmbH, Germany). Finally, the stable nanoemulsions were stored for 4 weeks at room temperature for more observations such as separation or creaming. In this study, two samples after a sonication time of 6 min and at a ratio of EO to Tween 1 : 1.5 (sample 6) and 1 : 2 (sample 9) were stable after the physicochemical studies. Therefore, the droplet size of these samples was evaluated. Using a Adwa (AD8000) pH meter the pH value was measured and the stable formulation nanoemulsions were measured at 25 ± 0.1°C.

Analysis of nanoemulsion droplet size

The size and size distribution of nanoemulsions were determined by a dynamic light scattering (DLS) instrument (PSS, Santa Barbara, CA, USA) at 23°C, using the 632 nm line of a HeNe laser as the incident light with an angle of 90°. The nanoemulsion with a 1 : 2 (w/w) ratio of rosemary EO (5%) and Tween 20 with 6 min sonication time was found to be stable with the lowest droplet size diameter (139.9 nm). So, nanoemulsion (sample 9) was used for further characterization and acaricidal evaluations.

Transmission electron microscopy (TEM)

TEM (JEM-1230, Jeol, Tokyo, Japan) was used for morphology studies of rosemary nanoemulsion. Phosphotungstic acid solution (2%, pH = 6.7) was used for staining nanoemulsion on a carbon-coated copper grid for 1 min. After drying at room temperature, the image was pictured with TEM at 80 KV accelerating voltages.

Acaricidal activity of rosemary EO nanoemulsion

Acaricidal activity of rosemary EO nanoemulsion was evaluated against the two-spotted spider mite (TSSM) *Tetranychus urticae* Koch. Adult females and immature stages of spider mites were collected from an abandoned castor oil plant, *Ricinus communis* (L.) that was found in the Faculty of Agriculture, Cairo University, Cairo, Egypt. Both adult females and immature stages of TSSM were placed on clean acalypha (*Acalypha marginata*) leaves under standard conditions [27 ± 0.5°C, 75 ± 0.5% relative humidity (RH) and 16 h light : 8 h dark]. In our study, the toxic effect of rosemary EO nanoemulsion was evaluated by using spray and residual effect techniques. In addition, the normal emulsion was evaluated on the immature stages and adult females of TSSM.

Spraying technique

For spraying, clean acalypha discs (3 cm in diameter) were placed lower surface downwards on water-saturated cotton in large uncovered Petri dishes (15 cm in diameter), ten discs per Petri dish, as replicates for each treatment where ten adult females or immature stages of TSSM were confined on the upper surface of each acalypha leaf disc. A preliminary concentration (2,500 μg · ml^{-1}) was selected to estimate the range of concentrations of nano and normal emulsions. In this experiment, different concentrations of nanoemulsion or normal emulsion (312.5, 625, 1,250 and 2,500 μg · ml^{-1}) were used both on the adult female and immature stages of spider mites. The controls of both adult females and immature stages were sprayed with distilled water. A glass atomizer was used to spray the spraying solution. Calculations were based on Petri dish area compared to Fadden area (4,200 m^2) and 100 l were considered to be a volume of spray solution to one Fadden (100 l/4,200 m^2).

Residual technique

The residual effect technique was used for the evaluation of rosemary EO nanoemulsion. Clean acalypha discs (3 cm in diameter) were dipped for 30 s in nano and normal emulsions and left to dry at room temperature then arranged and supplied with the adult female and immature stage of *T. urticae*. The same concentration used for the spray technique was used in the residual effect study.

In all experiments, the mortality of mites with treatments and control was recorded after 12, 24 and 48 h of treatment. High mortality was found after 48 h of treatment therefore it was used in this study. Acaricidal activity was repeated three times under the same conditions. No mortality was found in control mites.

Toxicological studies on experimental animals

Acute oral toxicity study

The experiment was conducted in the Animal Breeding House of the National Research Centre (NRC), Dokki, Giza, Egypt. Rats weighing 100 ± 5 g, were placed in cages (five each), with free access to water and food, 12 h light/dark cycle, 24 ± 3°C and 46% RH. All animals were kept according to animal protection and welfare guidelines and in accordance with the "Guide for the Care and Use of Laboratory Animals" (NRC 2011).

Rats were divided into three groups. Group I (control group) received orally distilled water (1 ml/rat). Group II (rosemary EO nanoemulsion group) received orally a single dose of rosemary nanoemulsion (1 ml/rat). This volume corresponded to 0.5 g rosemary EO/kg body weight (average b.wt. 100 g/rat). This dose, corresponding to 30 g of rosemary EO in nanoemulsion/person, was based on average human weights (60 kg), which were equivalent to 600 ml of nanoemulsion/person. Group III (normal-emulsion group) received 1 ml/rat of normal-emulsion (5% rosemary EO, 7.5% Tween 20 and 87.5% water, w/w) (Samojlik *et al.* 2010; Mossa *et al.* 2018). Signs of toxicity and mortality were recorded according to Mossa *et al.* 2018.

Data analysis

Results were expressed as a mean ± standard error (SE). The results of the bioassay were statistically analyzed according to the method of Finney (Finney 1971) by using the log-probit software program Ldp Line® model "Ehabsoft"(Bakr 2000). Moreover, the toxicity index (TI) was calculated as cited in our previous study (Mossa *et al.* 2018) by the following equation.

Toxicity index (TI) = [(LC_{50} or LC_{90} of nano or normal emulsion)/(LC_{50} or LC_{90} of normal emulsion)]. When the TI value is less than one (TI < 1), this means that the nanoemulsion is had high toxicity compared to the normal emulsion.

Results and Discussion

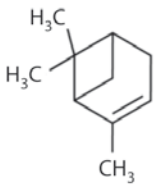
In this study, rosemary EO was obtained by hydrodistillation and subjected to GC/MS analysis. Chemical compounds of rosemary EO were identified by using Wiley Mass Spectral Library data, listed in Table 1. The chemical structures of the major components in rosemary EO are shown in Figure 1. The major compounds were 1,8 cineole (31.45%), borneol (11.07%), α-pinene (10.91%), D-limonene (9.19%), L-linalool (8.86%), D-camphor (7.32%), γ-terpinene (3.92%), linalyl acetate (3.37%), α-terpineol (3.32%), and p-cymene (1.82%).

Table 1. GC/MS analysis of rosemary essential oil

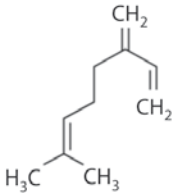
No.	Retention time [min]	Area [%]	Compounds
1	4.71	10.91	α-Pinene
2	5.26	0.01	a-Thujenol
3	5.73	1.01	Sabinene
4	6.16	0.98	α-Myrcene
5	6.74	0.22	α-Phellandrene
6	6.99	0.2	Isocineole
7	7.06	0.10	α-Terpinene
8	7.37	1.82	p-Cymene
9	7.46	9.19	D-Limonene
10	7.61	31.45	1,8 Cineole
11	8.46	3.92	γ-Terpinene
12	9.42	0.46	α-Terpinolene
13	9.72	0.03	Myrtenol
14	10.05	8.86	L-Linalool
15	11.51	0.16	Terpinene-1-ol
16	12.08	7.32	D-Camphor
17	13.06	11.07	Borneol
18	13.36	0.16	4-Terpineol
19	14.04	3.32	α-Terpineol
20	14.19	0.88	γ-Terpineol
21	15.22	0.02	Isopulegol acetate
22	16.06	3.37	Linalyl acetate
23	16.36	0.39	Geraniol
24	22.99	1.03	Caryophyllene
		96.88	

In addition, other components were recorded such as a-thujenol, sabinene, α-myrcene, α-phellandrene, isocineole, α-terpinene, α-terpinolene, myrtenol, terpinene-1-ol, 4-terpineol, γ-terpineol, isopulegol acetate, geraniol, and caryophyllene. Other studies reported a similar chemical composition of rosemary EO. For example, El-Ghorab 2003 found that the major components of rosemary EO are 52.31% 1,8-cineole, 6.54% α-pinene and 5.48% caryophyllene (z), 3.0% α-terpineol, 2.38% camphene, 0.72% eugenol and 0.22% methyl eugenol. Tucker and Maciarello (1986) determined the chemical compositions of 23 cultivars *Rosmarinus officinalis* L. They found a wide range of components in EOs such as α-pinene (from 0.06 to 57.45%), 1,8 cineole (from 3.55 to 42.69%), camphor (from 0.20 to 56.45%), bornyl acetate (from 0.66 to 21.03%), and borneol (from 0.40 to14.69%). Our results of rosemary EO analysis by GC/MS are in accordance with those reported by previous studies (Tucker and Maciarello 1986; Reverchon and Senatore 1992; El-Ghorab 2003).

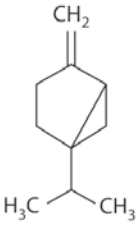
Currently, application of nanotechnology in crop protection is increasing and one of the most



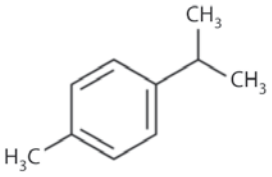
α-Pinene



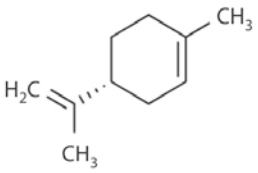
α-Myrcene



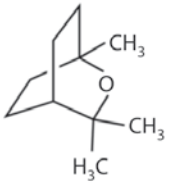
Sabinene



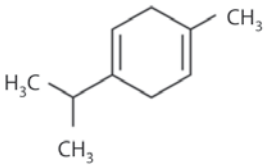
p-Cymene



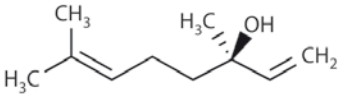
D-Limonene



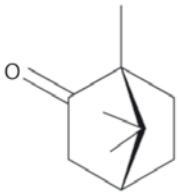
1,8 Cineole



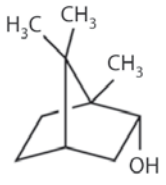
γ-Terpinene



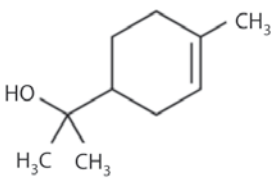
L-Linalool



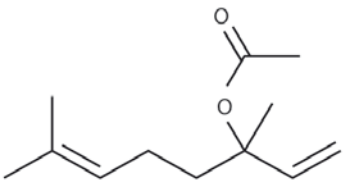
D-Camphor



Borneol



α-Terpineol



Linalyl acetate

Fig. 1. Chemical structure of major chemical compounds in rosemary essential oil based on GC/MS analysis

important uses of this technology is the preparation of eco-friendly green nanopesticides for pest control in green-pest management (Mossa 2016). Nanoformulations of plant extracts or essential oils are considered to be safe for humans and their ecosystem (Mossa *et al.* 2018). There is also a new trend to prepare green nanoformulations by using EOs for controlling mites. Essential oils contain many active components such as second metabolites which play an important role in plant defences (Rattan 2010). These active components are some of the most important sources of new, active and eco-friendly acaricides.

Tween 20 (polysorbate 20) a hydrophilic surfactant and deionized water were used for rosemary EO nanoformulation. Different ratios (1 : 1, 1 : 1.5 and 1.2 w/w) of rosemary EO and the surfactant were used to find the best ratio with low droplet sizes. The organic phase (oil) was used at 5% and the eques phase was a different ratio of Tween 20 and deionized water (Table 2). The samples were subjected to different sonication times (2, 4, and 6 min) by using ultrasound (Table 3). All samples of emulsions (samples 1–9) were studied for stability with thermodynamics and centrifugation tests (Table 3). Results indicated that two samples (samples 6 and 9) were stable after thermodynamics and centrifugation tests while other samples (samples 1–5, 7 and 8) were unstable and showed separation phases with different degrees of creaming. Thus, samples 6 and 9 were subject to analysis of droplet size distribution.

In the present work, samples 6 and 9 had mean droplet size diameters of 150.1 and 139.9 nm, respectively (Fig. 2). Sample 9 of rosemary EO nanoemulsion had the lowest droplet size diameter (139.9 nm). The ratio of EO and Tween 20 (1 : 2) with sonication time (6 min) played an essential role in nanodroplet formations in nanoemulsion which affected the stability of nanoemulsion. Therefore, sample 9 was subjected to morphology study by TEM and acaricidal activity studies. The droplet of rosemary EO nanoemulsion of sample 9 was spherical in shape and transmission electron microscopy showed that there was good dispersion (Fig. 3). The pH was 6.8. It has been reported that when the Hydrophile-Lipophile Balance (HLB) value of an EO couple coincides with that of surfactant (Diaz and Brignole 2005; Yang et al. 2009; Mossa *et al.* 2018) small sized nanoemulsion droplets are formed. Several researchers formulated some essential oils in nanoemulsions such as garlic, eucalyptus, cinnamon, camphor, basil and rosemary (Ghosh *et al.* 2013; Sugumar *et al.* 2014; Duarte *et al.* 2015; Mossa *et al.* 2017, 2018) and established their biological activities.

Nanoemulsion of rosemary EO sample 9 (EO 5% and Tween 20 at a ratio of 1 : 2 with sonication time of 6 min) and normal emulsion were also evaluated as acaricides against the two-spotted spider mite (TSSM) *T. urticae* at two stages (immature and adult females) by using spraying and residual techniques. Results of mortality were recorded after 12, 24 and 48 h of exposure. The greatest effect was recorded after 48 h of exposure and parallel results were found after 72 h. Therefore, 48 h of exposure was selected as the endpoint, and the results were used since both normal, and nanoemulsions have high toxic effects at this time.

Our study revealed that immature stages of *T. urticae* were more sensitive to both normal emulsions and nanoemulsions of rosemary EO after spray and residual techniques (Tables 4, 5). The lethal concentration (LC_{50}) of spray application of normal emulsion (EO) was 1,578.50 and 1,829.94 $\mu g \cdot ml^{-1}$ for immature and adult females of spider mites, respectively. However, nanoemulsion of rosemary EO had a greater toxic effect against two stages of *T. urticae* with LC_{50}

Table 2. Ratio of components used for formulation of rosemary essential oil (EO) nanoemulsion

Sample number	Ratio of EO and Tween 20 (w/w)	Formulation [%]		
		EO	Tween 20	D.W.*
1	1 : 1	5	5	90
2	1 : 1.5	5	7.5	87.5
3	1 : 2	5	10	85

*Deionized Water

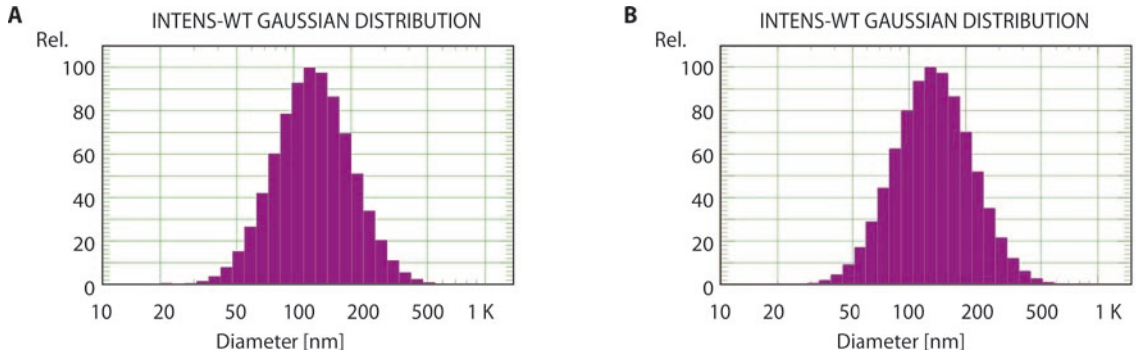


Fig. 2. Particle size of rosemary essential oil nanoemulsion: (A) sample 6 with size 150.1 nm and (B) sample 9 with size 139.9 nm

Table 3. Stability of rosemary essential oil (EO) nanoemulsions and physicochemical studies

Sample code	EO : tween (w : w)	Sonication time [min]	Heating–Cooling cycle	Freeze–Thaw cycle	Centrifugation	Result
1	1 : 1	2	–	–	–	x
2	1 : 1.5	4	+	–	–	x
3	1 : 2	6	+	+	–	x
4	1 : 1	2	–	–	–	x
5	1 : 1.5	4	+	–	–	x
6	1 : 2	6	+	+	+	√
7	1 : 1	2	–	–	–	x
8	1 : 1.5	4	+	–	–	x
9	1 : 2	6	+	+	+	√

X = failed; √ = passed

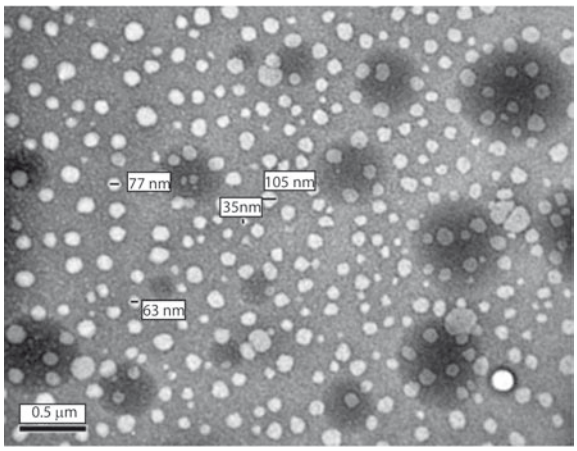


Fig. 3. Transmission electron microscopy (TEM) image of rosemary oil nanoemulsion (sample 9)

723.71 and 865.68 µg · ml^{-1} of the aforementioned stages, respectively. The LC_{50} of residual effect of normal emulsion (EO) accounted for 1,930.13 and 2,363.48 µg · ml^{-1} of immature and adult females of spider mite, respectively. However, nanoemulsions of rosemary EO have higher toxic effects against two stages of *T. urticae* with LC_{50} 1,136.96 and 1,415.26 µg · · ml^{-1} of the aforementioned stages, respectively.

In addition, nanoemulsion of rosemary EO has greater toxicity against both stages of *T. urticae* than the normal emulsion. After spraying, the toxicity index (TI) of nanoemulsion at LC_{50} account 0.458 µg · ml^{-1} with a toxicity increase of 54.15% of immature and 0.473 µg · ml^{-1} with a toxicity increase of 52.69% of adult *T. urticae*, respectively (Table 4 and Fig. 4). In the residual effect study, the toxicity index (TI) of nanoemulsion at LC_{50} account 0.489 µg · ml^{-1} with a toxicity increase of 41.09% of immature and 0.5988 µg · ml^{-1} with a toxicity increase of 40.11% of adult *T. urticae*, respectively (Table 5 and Fig. 5). The application of nanoemulsion by spraying had more toxic effects than the residual effects in both immature and female adults. Moreover, the immature stages of mites were more sensitive than adult females, due to their low development. The high toxicity and mortality induced by nanoemulsion of rosemary EO compared to the normal ones could be due to the small droplet size (139.0 nm) of nanoemulsion which led to increased surface area and acaricidal activity compared to the normal emulsion. Other studies reported high biological activity of some EO nanoformulations. This effect could be due to the physical and chemical properties of nanodroplets (Rodrigues *et al.* 2014; Sugumar *et al.* 2014; Mossa *et al.* 2018). However, the acaricidal activity of rosemary EO may be due to the presence of major and minor active components such as 1,8 cineole, borneol, α-pinene, D-limonene, L-linalool, D-camphor, γ-terpinene, α-terpineol, p-cymene, a-thujenol, sabinene, α-myrcene, α-phellandrene, isocineole, α-terpinene, α-terpinolene, myrtenol, terpinene-1-ol, 4-terpineol, γ-terpineol, isopulegol acetate and geraniol. Previous studies have also reported acaricidal activity of rosemary against several phytophagous mites (Ebadollahi *et al.* 2014; Ismail *et al.* 2015).

To the best of our knowledge, the preparation of rosemary EO nanoemulsion was carried out by using safe material and water. In synthetic acaricides, in addition to active ingredients some inert materials and solvents are used. These materials and solvents have toxic effects, therefore, the World Health Organization highlighted the requirement of assessing the toxic hazards of formulated pesticides (WHO 1992). The adverse toxic effects of synthetic acaricides on non-target organisms are important problems for the ecosystem and human health.

In this study, acute oral toxicity of rosemary EO nanoemulsion was also investigated in male rats. Results

Table 4. Toxicity and toxicity index (TI) of nano and normal emulsions of rosemary essential oil on the movable stages of *Tetranychus urticae* (immature and adult females) using spray technique

Treatment	LC_{50} [µg · ml^{-1}]	LC_{50} [µg · ml^{-1}]				Slope	LC_{90} [µg · ml^{-1}]	LC_{90} [µg · ml^{-1}]			
		lower limit	upper limit	toxicity index (TI)	% of toxicity increase			lower limit	upper limit	toxicity index (TI)	% of toxicity increase
Immatures											
Nanoemulsion	723.71	614.22	841.77	0.458	54.15	2.04	3,079.35	2,361.19	4,528.59	0.233	76.70
Normal emulsion	1,578.52	1,256.03	2,169.65	1	–	1.39	13,216.45	7,127.89	39,582.08	1	–
Adult females											
Nanoemulsion	865.68	728.81	1,026.16	0.473	52.69	1.81	4,415.71	3,160.35	7,353.27	0.289	71.07
Normal emulsion	1,829.94	1,434.65	2,620.06	1	–	1.39	15,265.51	7,973.68	48,925.89	1	–

LC_{50} – concentration induced 50% mortality; TI – toxicity index, TI = [(LC_{50} or LC_{90} of nano or normal emulsions)/(LC_{50} or LC_{90} of normal emulsion)]. High toxicity when TI was less than 1. Toxicity increase [%] = (TI of normal emulsion – TI of nanoemulsion) × 100

Table 5. Toxicity and toxicity index (TI) of nano and normal emulsions of rosemary essential oil on the movable stages of *Tetranychus urticae* (immature and adult females) using residual effects

Treatment	LC_{50} [µg · ml^{-1}]	LC_{50} [µg · ml^{-1}]				Slope	LC_{90} [µg · ml^{-1}]	LC_{90} [µg · ml^{-1}]			
		lower limit	upper limit	toxicity index (TI)	% of toxicity increase			lower limit	upper limit	toxicity index (TI)	% of toxicity increase
Immatures											
Nanoemulsion	1,136.96	942.81	1,408.89	0.5890	41.09	1.5957	12,206.10	7,071.36	29,956.88	0.423	57.60
Normal emulsion	1,930.13	1,505.73	2,800.17	1	–	1.4015	28,789.35	13,120.61	118,959.49	1	–
Adult females											
Nanoemulsion	1,415.26	1,163.69	1,848.64	0.5988	40.119	1.4752	18,444.48	9,564.49	56,242.38	0.4200	58.0
Normal emulsion	2,363.48	1,758.59	3,851.02	1	–	1.2962	43,914.04	17,230.56	263,294.45	1	–

LC_{50} – concentration induced 50% mortality; TI – toxicity index, TI = [(LC_{50} or LC_{90} of nano or normal emulsions)/(LC_{50} or LC_{90} of normal emulsion)]. High toxicity when TI was less than 1. Toxicity increase [%] = (TI of normal emulsion – TI of nanoemulsion) × 100

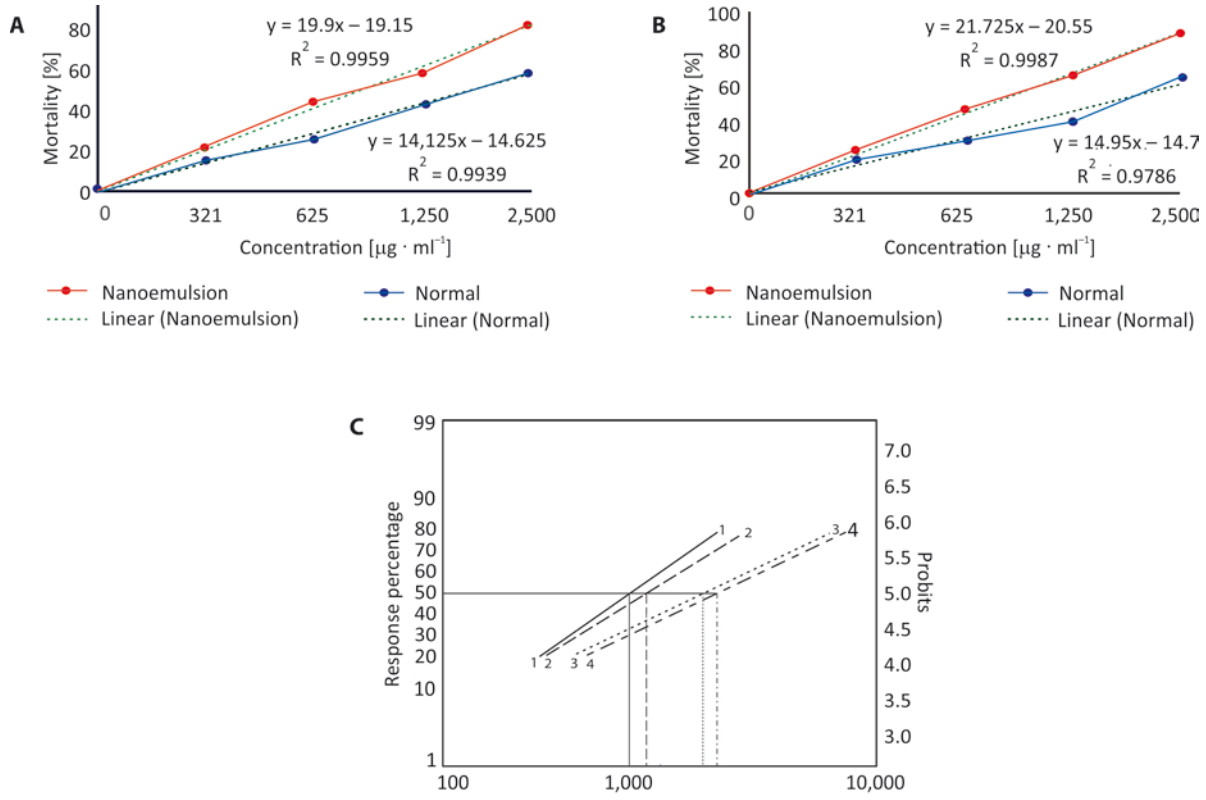


Fig. 4. Concentration-mortality response lines (adults – A; immature – B) and Log-Probit-Line (C) for *Tetranychus urticae* exposed to different concentrations of nano and normal emulsion of rosemary essential oils using spray technique. Toxicity Lines, nanoemulsion on immature (1), nanoemulsion on adults (2), normal emulsion on immature (3) and normal emulsion on adults (4)

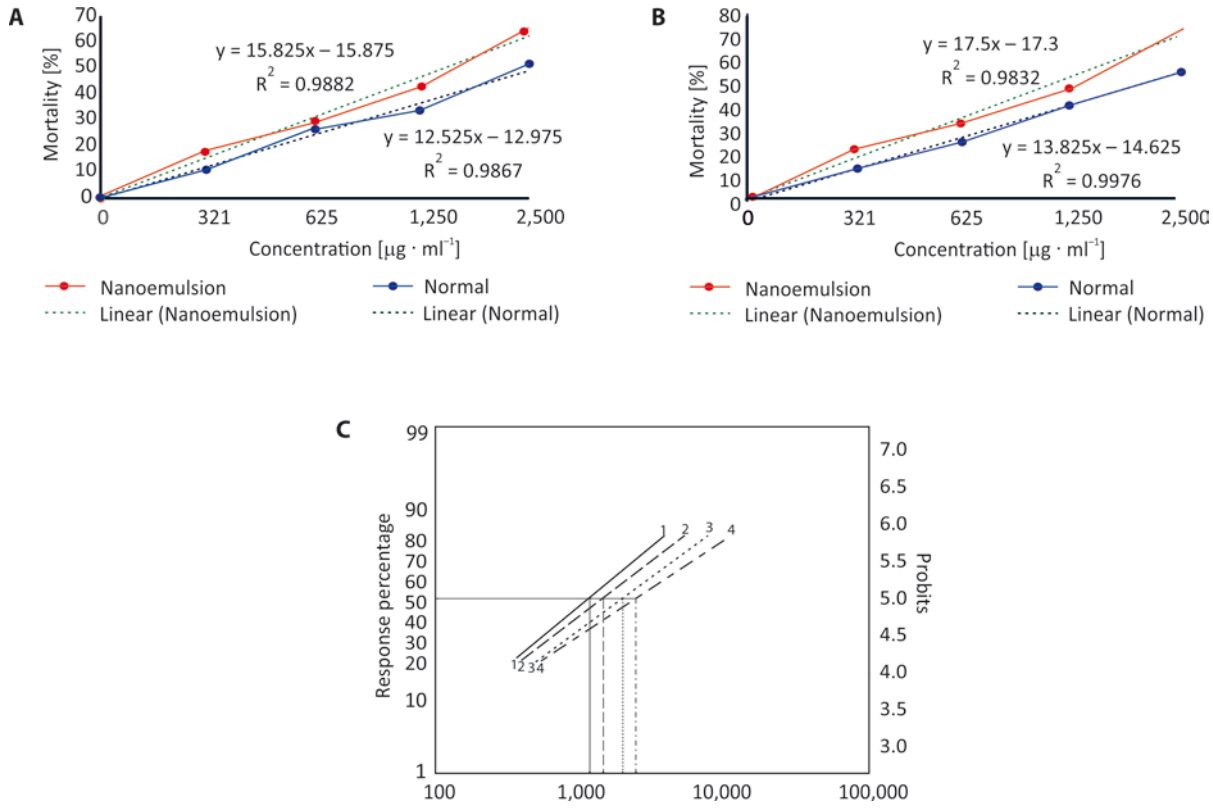


Fig. 5. Concentration-mortality response lines (adults – A; immature – B) and Log-Probit-Line (C) for *Tetranychus urticae* exposed to different concentrations of nano and normal emulsion of rosemary essential oils using residual technique. Toxicity Lines, nanoemulsion on immature (1), nanoemulsion on adults (2), normal emulsion on immature (3) and normal emulsion on adults (4)

indicated that when rats were given a single dose of either rosemary EO normal emulsion or nanoemulsion there were no signs of toxicity or mortality in rats. The dose given in this study equaled 0.5 g · kg^{-1} body weight, which was parallel to 30 g of rosemary EO nanoemulsion/person and equivalent to 600 ml of nanoemulsion/person. Moreover, there were no different effects of all treatments on body weight, water and food consumption in the treated animals. These criteria were extensively used in toxicological studies (Mossa *et al*. 2017, 2018).

Conclusions

Nanoemulsion of rosemary essential oil with a droplet size of 139.9 nm was formulated by using ultrasound for 6 min. The droplet size and stability of nanoemulsion was dependent on HLB values of oil and surfactant and the correlation with emulsification time. Spray application of rosemary nanoemulsion showed high acaricidal activity against immature and adult two-spotted spider mites *T. urticae* with LC_{50} 723.71 and 865.68 µg · ml^{-1}. The toxicity increased by 54.15 and 52.69% for immature and adult mites, respectively compared to the residual effect. No signs of toxicity or mortality were recorded in rats treated by rosemary nanoemulsion. Therefore it may be considered nontoxic for mammals. The high acaricidal activity, stability, and safety of rosemary nanoemulsion makes this nanoformulation a possible green and nano-acaricidal product. Further studies under field conditions are necessary to study the acaricidal efficiency of rosemary nanoemulsion against two-spotted spider mites and the toxic effect on predacious mites.

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