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Research Article

Evaluation of Eucalyptus Oil Nanoemulsion to Control Root-Knot Nematode *Meloidogyne Javanica* in Laboratory Conditions

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ABSTRACT

The effectiveness of Eucalyptus nanoemulsion as an alternative for chemical control and suppression of destructive pests *Meloidogyne javanica* was investigated in laboratory conditions. The droplet size of the Eucalyptus nanoemulsion was 23.88 nm and recorded the best results in laboratory research. The uniform structure of eucalyptus nanoemulsion in the liquid produced by this process has improved the reactivity of the particles. The duration of making nanoemulsion was done in the time interval of 0 to 30 minutes in order to investigate the effect of time of nanoemulsion formation and the physical stability of the formulation with this method, the transparency and stability of nanoemulsion will increase with time. The effect of eucalyptus nanoemulsion on the percentage of mortality of the second instar larvae of the root-knot nematode *Meloidogyne javanica* was investigated in laboratory conditions. The results showed that eucalyptus nanoemulsion can penetrate the cuticle surface and destroy the inner tissue after 24 hours. Also, the results showed that Eucalyptus nanoemulsion has a significant effect on the mortality rate of larvae and the percentage of larval deaths is significantly different in different concentrations of nanoemulsion. In laboratory conditions, using a concentration of 2.76 ppm resulted in the highest loss of second-instar larvae (90%), and the loss rate was 50%, 62%, and 75%, respectively, for concentrations of 2.14, 1.48, and 0.77 ppm it was. Based on the results, a low concentration of nanoemulsion has significant effects on larval mortality, which indicates the nematicidal potential of eucalyptus nanoemulsion.

Keywords: Root-knot nematode; Eucalyptus oil; Ultrasonication; Cuticle; Nanoemulsion

INTRODUCTION

Parasitic nematodes are indeterminate worms found in soil, aquatic environments, plants, or animal bodies. Nematodes can be useful and pathogenic and have a great impact on environmental balance, human and animal health, and agricultural production [1]. Meloidogen root-knot nematodes are the most important plant parasitic nematodes that damage agricultural products in the world [2]. Meanwhile, *M. incognita* and *M. Javanica* species are more important than others in terms of their host range and the amount of damage caused to agricultural products, which has led to the complete destruction of agricultural products [3,4]. In addition, the plant parasitic nematode is considered the most important harmful species [5]. The effect of root-knot nematodes on the subsurface organs causes changes in the size and structure of plant roots, which in certain conditions prevent the formation of secondary roots or prevent the proliferation of capillary roots around the damaged area [6,7]. The natural absorption of water and nutrients by the roots and causing problems for these materials to reach other tissues and organs of the plant, which causes a lack of minerals in some parts of the plant [8]. For this reason, the use of plant essential oils is stronger and safer to use as an antibacterial compared to chemical products. Due to the bioactive components present in plant-derived oils, plant essential oils have insecticidal, antifungal, and antibacterial properties [9]. For example, lemon grass and thyme oils showed complete inhibition against *Fusarium oxysporum* [10]. Nanotechnology can be used in the production of pesticides, insecticides, and the use of new insect repellants. One of the reasons for the production of nano-pesticides is that they cause easier and faster absorption of the pesticide in the plant and pest tissue [11,12]. Nanoparticles prepared from biological extracts have many advantages such as tolerance, variety, and reproducibility. In synthesis, they are complex and biocompatible [13]. The difference between emulsion essential oil and nanoemulsion essential oil is in the size of the oil particles, the stability of the emulsion improves significantly with the size of the oil and the particles become smaller,

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and surfactants are added to the mixture. Oil and water to increase the kinetic stability of such a system, the surfactant is an amphiphilic molecule that has a hydrophilic head group (polar region) that has a high affinity for water and a lipophilic tail group (nonpolar region) that has a high affinity for oil [14]. Essential oils in nanoemulsions seem to have a faster permeability in microbial membranes due to the increase in surface area per unit weight and decrease in concentration to reach a balance or even a greater microbial effect than conventional emulsions [15]. Also, previous studies showed that silver nanoparticles reduce the level of glycogen, lipids, and proteins in the nematode body. Contents of *H. contortus*. Parasites generate energy from stored carbohydrate energy (glycogen) to carry out basic metabolic processes. One of the other ways to control and dispose of pests, especially types resistant to common poisons, is to prepare and use extracts extracted from plants. Nanoemulsions of plant extracts such as Indian hyacinth, hairy basil, and vetiver with medium particle size (150-220 nm) were prepared and tested against *Aedes aegypti*. For example, a concentration of 5% of basil and vetiver and 10% of Indian hyacinth killed *Aedes aegypti* for 4.7 hours (18) [16]. For this purpose, the effect of Eucalyptus essential oil in the form of Eucalyptus nanoemulsion on the larvae of the second instar root-knot nematode was investigated, because the nanoemulsion can easily penetrate into the nematode's body and be absorbed. Because in this experiment, second instar root-knot nematode larvae were destroyed and controlled by nanoemulsion with dimensions of 23.88 nm and 2.68 ppm concentrations that led to a 90% reduction. For this reason, the development of nano pesticides may be seen as a significant advance in the fight against agricultural pests and other disease vectors. Since the nanoemulsion can be used with the lowest concentration and the highest percentage of pest losses can be observed for 200 seconds instar larvae of the root-knot nematode on a laboratory scale and in terms of the natural nature of the eucalyptus nanoemulsion. Based on this, the effects of nanoparticles cause the complete destruction of the cuticle and the shrinking of the nematode's body from a morphological point of view, and from a physiological point of view, the level of reactive oxygen and nitrogen species increases, which causes oxidative stress and physical damage to the nematode. Eucalyptus nanoemulsion does not cause any harmful damage to agricultural products, soil nutrients, and biological ecosystems.

Nematode *meloidogyne javanica*

Root-knot nematode also makes the root system vulnerable to infections by disease-causing fungi and bacteria. *Meloidogyne incognita* and *M. arenaria* have been shown to induce root-knot disease in *C. forskohlii* [17]. They are distributed worldwide and are obligate root parasites of thousands of plant species. However, more than 90 species of *Meloidogyne* have been described, of which *Meloidogyne incognita* (Kofoid and White) *Chitwood, Meloidogyne javanica* (Treub) *Chitwood,* and *Meloidogyne arenaria* (Neal) *Chitwood* are highly harmful and endangered apomictic species. These three species are found worldwide, typically in tropical and subtropical regions, but also occur in more temperate regions, especially in protected cultivation [18]. In modern agriculture, chemical control is one of the main methods of fighting nematodes, and for this reason, the synthetic nematicides are widely used today. Wide use of these substances can reduce their effectiveness. Therefore, it is necessary to use new and effective methods to control plant parasitic nematodes. Based on this, discovery, and development of nematicide plant products or nematode inhibitors has significantly increased [19-22].

MATERIALS AND METHODS

Materials

Extraction, preparation, and analysis of essential oil: In this method, first, 400 kg of *Eucalyptus Globus* leaves, together with 1,200 litres of drinking water, were poured into the distillation pot, which is inside a closed chamber with steam tubes embedded in its bottom, and it started to boil at a temperature of 90°C for 3 hours. Boiling, hence, distillation with steam, essential oil, and aromatic water are separated from the leaves. The water vapour entering the chamber from the bottom passes through the leaves and the heat of the water vapour destroys the glands containing the essential oil and causes the essential oil to leak from these glands and be carried upwards by the water vapour. Water vapour along with essential oil exits from the top of the chamber and enters the condenser column, cools down, and enters the separator. Finally, the essential oil is separated from the aromatic water. During the process, no impurities are added during the essential oil extraction stage (Figure 1).

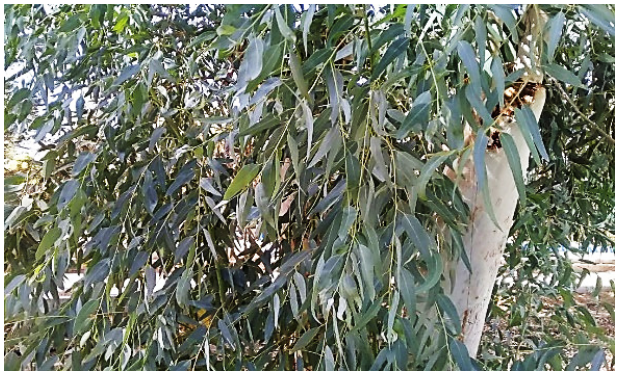

Figure 1: *Eucalyptus globus* tree.

Preparation of nanoemulsion using ultrasonic waves

The essential oil obtained from eucalyptus leaves was poured in an amount of 30 mg in one liter of double distilled (deionized) water and placed in an ultrasonic bath (cleaner brand) with an output power of 50-100 watts, a frequency of 40 kHz and a temperature range of 0°C-80°C. Leave it for 30 minutes until the essential oil in the distilled water turns into a eucalyptus nanoemulsion. The advantage that this method has over the methods that have been used to prepare nanoemulsion is that in this method the nanoemulsion obtained is stable for 6 months and does not lose its nanoemulsion properties because in this process water is distilled twice. which causes the stability of nanoemulsion has been used and this is the reason that there is no need for the impurity of a substance such as a surfactant, this method can increase the stability of nanoemulsion and this process for different times of nano emulsification in time intervals (0.5, 10, 15, 20, 25, 30 minutes) has been used. The desired nanoemulsion has smaller dimensions in the 30-minute period compared to other times.

Eucalyptus nanoemulsion indicators

Particle size distribution and polydispersity index: Droplet size distribution (analysis by volume) and dispersity index of nanoemulsion formulation of eucalyptus oil were determined using a 90-plus particle. Particle size was analyzed using Dynamic

Light Scattering (DLS) technique. Also, photodynamic scattering was measured at different time intervals (0, 5, 10, 15, 20, 25 and 30 minutes). In each water phase, droplet sizes in nanometers are described to minimize multiple scattering effects prior to testing. Using the DLS device, the diameter of the produced particles was measured and their size was 23.88 nm. The uniform structure of eucalyptus nanoparticles in the liquid produced by this process improves the reactivity of these particles. Since the nanoparticles in the prepared suspension have the necessary stability and do not settle quickly, it shows the stability of the nanoemulsion in this method (Figure 2) [23].

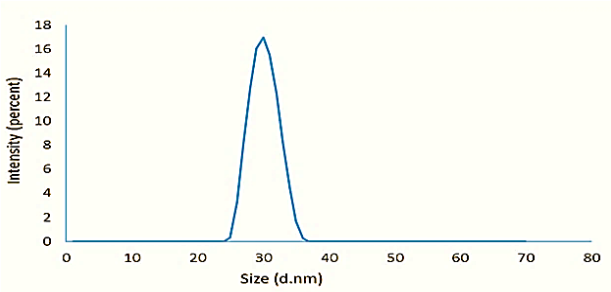

Figure 2: Dimensions of eucalyptus nanoemulsion in 30 minutes.

pH measurement of nanoemulsion: The pH values of the were determined. At specific time intervals for every 5 minutes, ultrasound was measured at room temperature of 24°C under the same conditions with a pH meter. The measurement of different pHs in different time intervals showed the effective effect of eucalyptus nanoemulsion on root-knot nematode because the closer the nanoemulsion is too acidic pH, it makes the root-knot nematode is unable to survive in these conditions and hence the preparation method of nanoemulsion By using ultrasonic waves, reduced the pH within 30 minutes, according to the experiments conducted in the laboratory, in addition to the dimensions of the nanoemulsion, low pH is very effective for the removal and control of the root-knot nematode in the soil.

Identification and morphological investigation of root-knot nematode

First, the roots of the American eggplant, (Figures 3A and 3B) which has shiny purple skin and a soft, and sweet texture that is a good host for root-knot nematode, were taken out of the soil from eggplants cultivated in a greenhouse. The roots are washed and cut into equal sizes (2 cm length), and sterilized by 1% hypochlorite solution for 30 minutes [24]. Then the roots were washed for removing the hypochlorite from the chopped roots preventing to the destruction of nematode eggs. The roots were then put into a blender, pureed, and combined for 40 seconds, and then placed onto a 325 and 500 mesh sieve [25]. The nematode eggs in the 500 mesh sieve were collected in an Erlenmeyer flask after sifting, and the solution was then thinned down with water [26]. Before producing larvae from eggs, some of the produced suspension was put on filter paper and kept in an incubator for 72 hours at a temperature of 28°C. The second instar nematodes were placed in a petri dish containing water for 72 hours and then passed *via* a 500 mesh sieve [27]. The density of the nematode population was checked under binoculars (Figure 3) [28].

Determination of nematode type: Gelatine sacs containing root-knot nematode eggs were isolated from American eggplant roots using Hussey and Barker's method [29]. The eggs were placed on filter paper and kept in an incubator at 28°C for 72 hours. According to Coyne, after 72 hours the eggs were hatched in the water and the active chicks of *M. javanica* were collected [30]. To determine the type of nematode, a female nematode (Figure 4A) separated from the infected roots by fine needle and a sterilized razor under binoculars and placed on the slide along with a drop of glycerin and a drop of 45% lactic acid (Figure 4B). Then, with a thin blade, a slice is taken from the end of the nematode's body and placed in an osmotic environment, the contents of the nematode's body removed, and the layers were completely separated from each other, and using microscope with magnification of 1000 types of nematodes were observed and analyzed. Based on the observations the separated root knot nematode was *M. javanica* (Figure 4) [31,32].

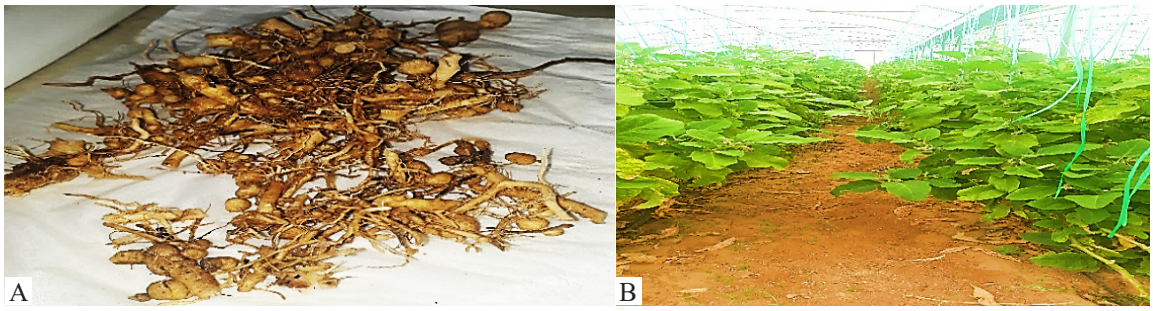

Figure 3: (A) Eggplant roots infected with nematodes; (B) Eggplant greenhouse infected with nematodes.

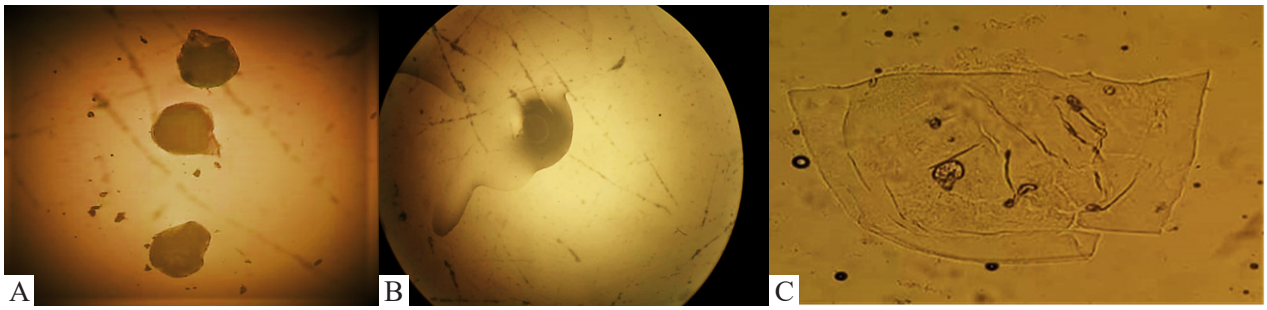

Figure 4: (A) Pear-shaped female nematode; (B) Female nematode in osmotic environment; (C) End section of female nematode body.

Investigating the effect of eucalyptus nanoemulsion on root-knot nematode (*M. javanica*) in laboratory conditions

In order to investigate the effect of Eucalyptus nanoemulsion on the mortality rate of second instar larvae of root-knot nematode in laboratory conditions, an experiment was conducted to compare 4 concentrations of 0.77, 1.48, 2.14 and 2.76 ppm of Eucalyptus nematode along with furalar nanoparticles. Mortality in a completely randomized design with four replications. Each replicate consisted of a 12 cm diameter Petri dish containing 200-second instar larvae. To distinguish active worms from inactive worms, the nematode suspension was placed on filter paper and placed in an incubator at a temperature of 28°C. Petri plates were examined under a microscope and 72 hours after leaving the incubator, the number of active nematodes was measured [33]. 50 ml of drinking water with pH 7.4 and 200-second instar larvae were poured into each petri dish, and then four concentrations of Eucalyptus nanoemulsion were added to each petri dish along with control of 200 larvae by adding nanoemulsion with concentrations of 2, 4, 6, and 8 ml. became. The nanoemulsion solution with an initial concentration of 20 ppm, all treatments were placed in a dark laboratory at a temperature of 29°C. In this experiment, sterile distilled water with a pH of 4.89 was used as a control [34]. All the steps performed in 48 hours have been examined with the mentioned concentrations, and the duration of 48 hours for the concentration of 2.67 ppm of eucalyptus nanoemulsion, all the larvae of the second instar of the root knot nematode have been destroyed and the mortality rate it was 100%.

Statistical analysis

Each measurement was performed in four replicates data analysis was done by SPSS software and graphs were drawn using Microsoft Excel, and the data were statistically analyzed in a one-way way, and the comparison test was performed when p-values <0.05 were considered for significant differences.

RESULTS AND DISCUSSION

Effect of Eucalyptus nanoemulsion on mortality of second instar larvae of root-knot nematode (*M. javanica*) in laboratory conditions.

Data analysis

Each measurement was performed in four replicates. Data analysis was done by SPSS software and graphs were drawn using Microsoft Excel, and the data were statistically analyzed in a one-way way, and the comparison test was performed when p-values <0.05 were considered for significant differences. The results of the analysis of variance showed that there is a significant difference between different concentrations of nanoemulsion for the percentage of larval losses at the probability level of 1% (Table 1). The mean comparison results showed a significant effect of Eucalyptus nanoemulsion on larval mortality compared to the control treatment (Figure 5). Larval mortality of 50%-90% was achieved using 0.77 to 2.76 ppm nanoparticle concentrations as treatments. Also, there was a significant difference between the nanoemulsion treatments, and the highest mortality rate of 90% was observed at the concentration of 76.2 ppm. Regression analysis of nanoemulsion on larval mortality showed a linear relationship (Figure 6) (Table 2). The percentage of larval mortality showed a linear increase in one ppm of 20% increase in nanoemulsion concentration (Table 2). After 24 hours, the microscopic examination of the eucalyptus nanoemulsion on the root-knot nematode skin showed that the worm was flattened and floated in the nanoemulsion as dry wood on the surface of the nanoemulsion, which also pierced the epidermis and destroyed the lipids and internal tissue of the nematode. The advantage of using eucalyptus nanoemulsion over eucalyptus extract and essential oil is that you can use eucalyptus nanoemulsion at the lowest concentration, which has a great effect on nematodes while using eucalyptus extract and essential oil at the lowest concentration does not have a high effect. On nematodes in laboratory conditions, the use of a concentration of 2.76 ppm resulted in 90% of the highest losses of second-instar larvae. In the conducted research, it was shown that the presence of chitosan in peppermint essential oil can be effective on the size, nucleation and nematicidal activity of nanoemulsion, as well as essential oil emulsion and nanoemulsion components may have a negative effect on the nervous system of nematodes, and another possibility is that the essential oils disrupt the nematode cell membrane and can change its permeability [35]. The extract has a more inhibitory effect against nematode second-stage larvae and nematode egg hatching, which causes the production of root-knot nematode [36]. All the steps have been investigated over a period of 48 hours with the mentioned concentrations that period of 48 hours for the concentration of 2.67 ppm of eucalyptus nanoemulsion has destroyed all the larvae of the second instar of the root-knot nematode and the mortality rate, it was 100% (Table 1), (Figures 5 and 6), (Table 2).

Table 1: Result of analysis of variance for mortality percentage of nematodes.

S.O.V	D.F	Mean square	Pr>F	C.V (%)	R^2 (%)
Treatment	3	1182.33	0.003	17.14	77.22
Error	12	141			

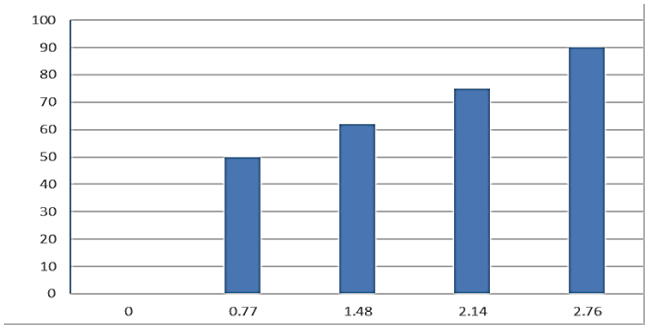

Figure 5: Comparison of the average concentration of nanoemulsion for the percentage of larval death. The mean of having at least the same letters in the test does not have a significant difference. **Note:** Eucalyptus nanoemulsion concentration.

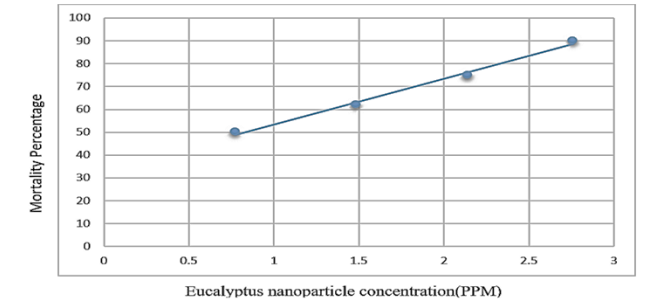

Figure 6: Regression relationship between mortality percentage and nanoemulsion concentration.

Table 2: Regression analysis of variance of nanoemulsion concentration on nematode mortality.

S.O.V	D.F	Mean square	F	Pr>F
Regression	1	880.95	303.79	0.0033
Error	2	5.89		
R^2=0.9935	Linear equation: Mortality percentage=33.48+20.01 Concentration			

Microscopic analysis and skin scan of root-knot nematode *M. javanica*

In this section, the interpretation and discussion of the images taken by the light microscope and the scanning of the nematode skin with the AFM (Atomic Force Microscope) are discussed.

Based on Figure 7A taken by light microscopy, shows that a healthy root nematode has healthy internal tissue, and the dark lines inside the nematode body indicate lipid accumulation under the nematode cuticle. Figure 7B shows a root-knot nematode exposed to 2.76 ppm of Eucalyptus nanoemulsion in a laboratory environment for 24 hours. This worm is shown dead and motionless on the surface of the water. Figure 7C, which was prepared with a light microscope with a magnification of 1000, shows the root-knot nematode that was treated with eucalyptus nanoemulsion for 24 hours, and it was observed that the internal body tissue and fats were destroyed (Figures 7C-7E). Essential oil can penetrate the fungal cell wall and cytoplasmic membranes and disrupt them, make them permeable and finally damage the mitochondrial membrane. On the other hand, essential oils as nanoemulsions or natural nematicides have different mechanisms and functions [37,38]. The penetration of chitin into the cell wall of the cytoplasmic membrane destroys the lipoprotein and enables it to be released from the cytoplasm, thereby producing an antifungal effect suggested and the antipictide activity of nanoemulsion increased while the cytotoxicity decreased [39]. Aldehydes in essential oil components may cause irreversible changes in protein structures, especially those on the nematode surface, such as formaldehyde, etc. Aldehydes are interesting to note that benzaldehyde and furfural (D2-furaldehyde) attract C. elegans at low concentrations [40]. In this study, nanoemulsion containing eucalyptus essential oil extract has nematicidal activity against root knot nematodes with toxicity, the cell is low (Figure 7).

Scanning nematode skin using AFM device: According to the photos taken by the AFM device, it shows (Figure 8A) that the skin of the control that was placed in distilled water was only wrinkled and the skin of the nematode was not destroyed. The surface has been cuticled, and eucalyptus nanoemulsion has destroyed the cell wall and lipids (Figure 8B) (Figure 8).

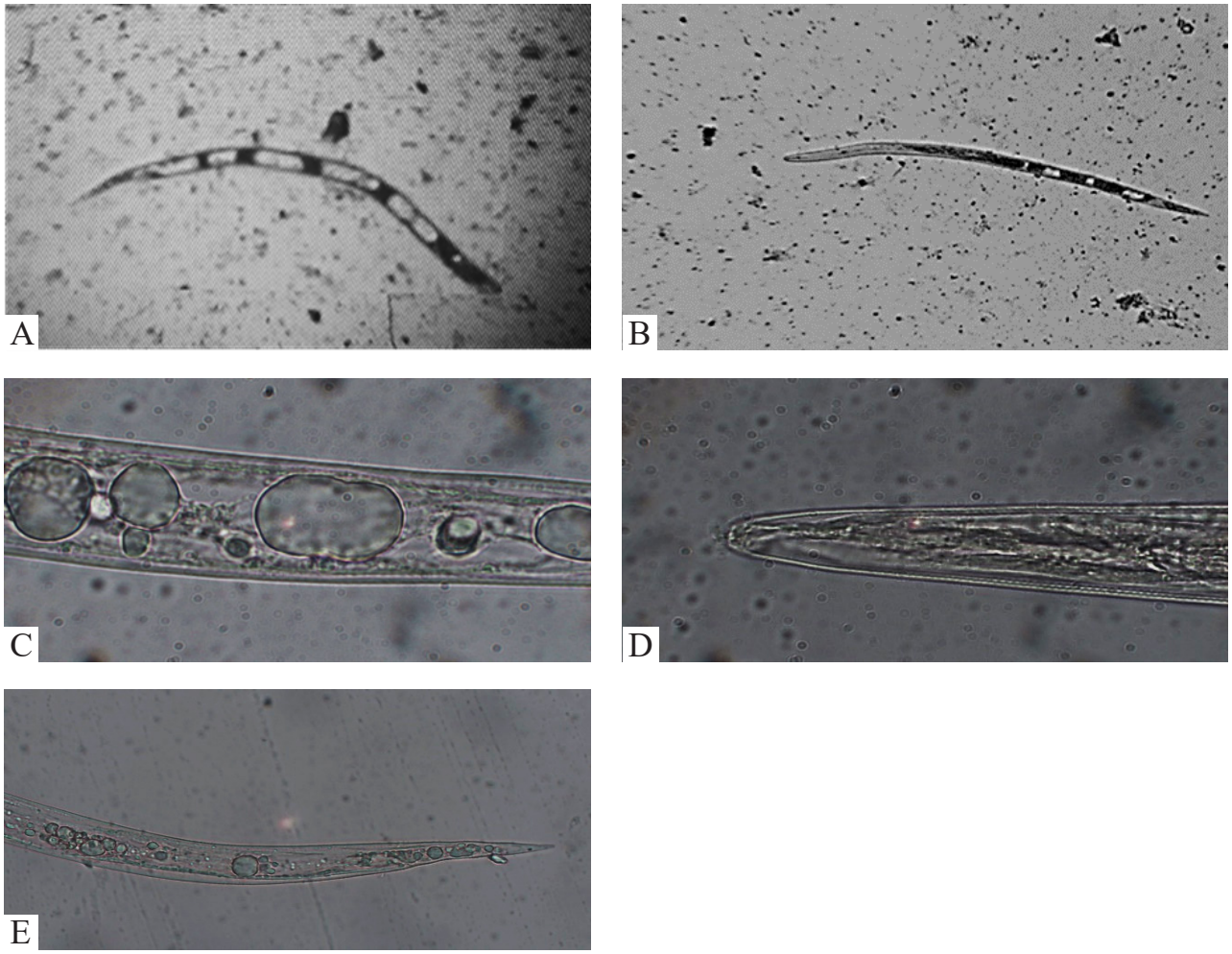

Figure 7: (A) Photo of a healthy root-knot nematode; (B) Photo of a root-knot nematode inside eucalyptus nanoemulsion; (C) Microscopic photo of a root-knot nematode with 1000 magnification; (D) The head part of root-knot nematode in eucalyptus nanoemulsion; (E) The terminal part of root-knot nematode in eucalyptus nanoemulsion.

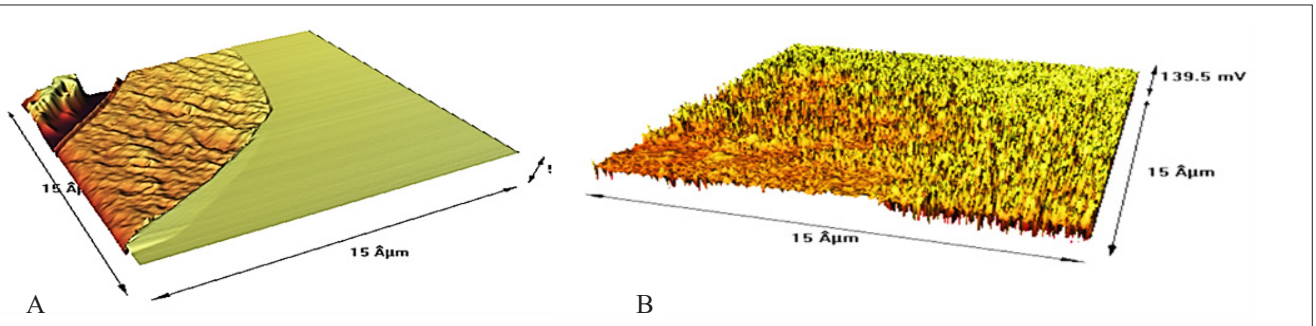

Figure 8: (A) Root-knot nematode healthy skin (3D); (B) Skin of root-knot nematode in eucalyptus nanoemulsion after 24 hours (3D).

The effects of the eucalyptus nanoemulsion mechanism on the cuticle surface when the nanoemulsion enters the larval cuticle are clearly seen in the results observed from the scanned images using an AFM device and a microscope with 1000 magnification data analysis in laboratory conditions. The nanoemulsion has penetrated the cuticle of the second instar larva and destroyed it. The internal tissue of the nematode body is composed of lipids and proteins, and the nanoemulsion is easily absorbed by the cuticle because the dimensions of the eucalyptus nanoemulsion are much smaller than the dimensions of its extract and essential oil, which are in liquid form. Cuticle has the ability to absorb nanoemulsion. Dead nematode skin is smooth, elongated and resembles the dried wood of a eucalyptus tree. It floats motionless on the surface of the liquid. This effect is observed in laboratory conditions and in a dark environment for 24 hours with a concentration of 2.75 ppm and the nano size used in this experiment is 23.88 nm. This effectiveness may be due to the presence of cineole (eucalyptol), which is well known and increases penetration in the drug delivery system through the skin and topical application studies [41-43]. In the studies, it was shown that the effect of essential oils against Nematodes are not well defined [44]. The involvement of plant compounds in essential oil on the nervous system of nematodes and the process of transmission of nerve messages is not completely clear. Some researchers believe that essential oils cause the death of nematodes by destroying the skin of nematodes or by disrupting the cell membrane of nematodes and changing its permeability [45]. Due to the variety of antimicrobial compounds of plant extracts and essential oils, there are different mechanisms for their set of activities. Due to the combined effect and overlapping of different compounds, the chemicals in the extract and essential oil break the cell wall and membrane of pests and pathogens and increase the permeability and ionic destruction of cells. Following the breakdown of cell wall lipids, mitochondria and membrane proteins, as well as cytoplasm coagulation, it causes the death of damaged cells [46]. According to the report of Tapondjou et al., the lethality of plant essential oils is caused by the biological effect of their constituent compounds on insects In the tests conducted using eucalyptus nanoemulsion and eucalyptus emulsion on mosquitoes, it has been shown that in general, synthetic pesticides have disadvantages such as health problems, harmful to the environment, and pests may become more resistant to some chemicals over time [47]. Tolerate and pollute water and soil resources [48-50]. However, the indiscriminate use of pesticides has led to the widespread development of resistance among pests as well as vector insects [51]. However, their use is often limited due to their instability and rapid degradation. The use of plant pesticides related to nanotechnology offers a significant potential to increase the efficiency of plant pesticides [52-54]. Eucalyptus oil is an oil distilled from eucalyptus leaves, and the repellent activity of Eucalyptus oil against *Cx. quinquefasciatus* was demonstrated [55,56]. The seed and leaf extracts of eucalyptus oil contain compounds that are toxic to mosquito larvae [57,58]. Since they are not harmful to health, their effects are used as insect repellants and safe and environmentally friendly alternatives to synthetic pesticides [59]. In general, botanical pesticides have a low effective duration. So researchers used nanotechnology to overcome this problem. For this purpose, the use of plant essential oils in terms of constituent compounds, mainly monoterpenes, have significant toxicity for many pests, including nematodes. The presence of some chemicals in plant extracts causes the death of nematode larvae. In some cases, these chemicals directly penetrate the nematode body and inhibit the activity of acetylcholinesterase and cholinesterase-like esterases. Acetylcholinesterase inhibitors inhibit activity and motility and delay the shedding process. In the study of Santhoshkumar et al, the larvicidal effects of silver nanoparticle formulation of plant extract on malaria vectors and *Nelumbo Nucifera* filaria were evaluated. In the research conducted, the formula of nano silver cut from the mentioned plant is almost 10 times more effective than its standard extract. This silver nanoparticle is a larvicide prepared by reduction. According to the obtained results, it can be concluded that eucalyptus oil nanoemulsion can be used as an effective and non-toxic solution to control root-knot nematodes in laboratory conditions, but for use in agricultural fields, these results should be used to organize programs. Appropriate research was used to investigate its effect in real direct conditions [60].

CONCLUSION

As a result, the nanoemulsion obtained from eucalyptus emulsion showed that it can have larvicidal properties for root knot nematode. The size of the particles may be a reason for the rapid penetration into the nematode body, and also the low pH of eucalyptus nanoemulsion is also an effective insult for to increase the amount of larvicide, eucalyptus nanoemulsion can be used in agriculture. In laboratory conditions, Eucalyptus oil nanoemulsion was evaluated as a possible control agent against the root-knot nematode *Meloidogyne javanica*.

CONFLICT OF INTEREST

Authors declare that no conflict of interest.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Data will be made available on request.

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