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Antipathogenic effects of emulsion and nanoemulsion of cinnamon essential oil against *Rhizopus* rot and grey mold on strawberry fruits

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Abstract: Application of essential oils in controlling plant pathogens is generally associated with difficulty due to low solubility in water, strong odor, physical and chemical instability. One of the ways to minimise these effects is to use a nanoemulsion system. It also increases the antimicrobial properties. In this research, after preparation of cinnamon (*Cinnamon zeylanicum* L.) essential oil (CEO), nanoemulsion of the essential oil was prepared and its physical and chemical properties were determined. The particle size of nanoemulsion was determined to be 115.33 ± 3.97 nm. Emulsification and nanoemulsion of the essential oil along with thiabendazole as an antifungal agent at various concentrations of active ingredient were studied for control of *Rhizopus stolonifera* and *Botrytis cinerea* fungi, strawberry fruit decay. Results in solid Potato Dextrose Agar (PDA) medium indicated that emulsion and nanoemulsion of CEO have a significant difference in antifungal activity against *B. cinerea* and *R. stolonifera*. The minimum inhibitory concentration was 500 and 1,000 µl fungi per liter of culture medium. According to the results of the research, essential oil nanoemulsion had a significant effect on the reduction of a fungal cartilage of strawberry fruit. In general, nano-emulsions of the essential oil showed more antifungal activity than essential oil. There was no significant difference in decay control between thiabendazole and CEO. The nano-emulsion of cinnamon oil at a concentration of 0.2% proved significant effect in reducing fruit decay and showed the lowest fruit infection (5.43%). Consequently, nano-emulsion of essential oil is recommended for the production of natural fungicides.

Keywords: Cinnamon essential oil, strawberry, control of fungal rot, nanoemulsion, *Botrytis cinerea*

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INTRODUCTION

Strawberries have a very short shelf life because of the high susceptibility to fungal agents. The use of artificial antifungal chemical compounds has been a concern for increasing the shelf life of this fruit. For this reason, it is necessary to use safe methods to control decomposition and maintain the quality of strawberry fruit during storage.

The strawberry belongs to the family Rosaceae and belongs to the genus Fragaria [1]. Due to the presence of ascorbic acid and anthocyanin, it has a high antioxidant effect and has many healing properties, such as enhancing the immune system and reducing the incidence of cancers [2], Its susceptibility to fungal rot, especially *Rhizopus stolonifer*, and grey mold produced by *Botrytis cinerea*, as well as high respiration rate, high water content (about 91%), and high metabolic activity have made strawberry one of the most corrosive fruit with short lifespan [3]. Therefore, the use of chemical pesticides to reduce the damage caused by pathogens after harvest is a matter of course [4–6].

The use of fungicides is the best way to control post-harvest diseases. Spraying fruits with Benomyl before and with Thiabendazole immediately after harvesting is the most commonly used control method for post-harvest disease. Another effective method is washing fruits with sodium ortho-phenyl phenate or Imazalil as soluble or homogeneous treatments in packaging and packing lines of factories [7]. The use of these chemical pesticides results in acne or chronic toxicity to non-target organisms, including humans, which are associated with cumulative properties of living organisms or carcinogenicity [8].

Today, due to the special attention to human health and the environment, incentives to find alternatives to pesticides have become much more advanced. Therefore, it is also essential to strengthen natural methods, using of antifungal herbal products, Gyawali and Ibrahim [9]. Essential oils also provide cropping of agricultural products and food due to their influence on the vapor phase and the possibility of controlling afterbirth [1, 10, 11].

An emulsion is a mixture of two immiscible and suspended liquid phases. Depending on the dispersed phase, the emulsion is divided into two groups: oil-in-water (O/W), where oil droplets are suspended in water, and water-in-oil (W/O), where the water is suspended in oil. The emulsion systems used for conveying compounds are divided into conventional macromolecular emulsions and nanoemulsions [12]. Some of their properties are similar, including composition, structure, and thermodynamic properties, but some of the characteristics are different because of differences in particle size. The size of nanoemulsion particles is 20–50 nm, which is much less than the wavelength of the light, so they do not diffuse the light intensity and thus are transparent or somewhat foggy [13].

Nanoemulsions have used in the manufacture of chemicals, pharmaceuticals, and cosmetics. In cosmetics, rice bran oil is used in the preparation of sunscreens, anti-aging creams, and for skin disease treatment. The size and diffusion of particles can affect the properties of nanoemulsion, such as stability, rheological properties, colour, and texture [14].

Unfortunately, some of the characteristics, including low solubility in water, volatility and strong odor of essential oils, have restricted the use of these natural compounds in foods, especially drinking materials [15, 16]. Moreover, essential oils in foods make unpleasant smells and tastes to consumers, so nowadays we try to reduce the undesirable effects of essential oils.

One of the ways to minimise these adverse effects is to use the nanoemulsion that increases the stability of volatile compounds, protects them against interactions with other compounds, and increases antimicrobial properties by increasing cellular absorption [17, 18].

In this research, we tried to investigate the effect of emulsion and nanoemulsion of cinnamon on controlling the cartilage and grey mold rot of strawberry. The aim of the research was to minimise the strawberry strain losses between the manufacturer and the consumer and the remaining residue of chemical pesticides. If desired, it can be used as an alternative to chemical fungicides to reduce post-harvest lesions of strawberry fruit.

STUDY OBJECTS AND METHODS

Plant material and essential oil extraction. The dried bark of the Cinnamon tree (*Cinnamomum zeylanicum* Blume) was provided from the laboratory sample maintenance unit of RUDN University, Moscow, Russia and then grounded. The essential oil was extracted via distillation by water during three hours using a Clevenger apparatus. Fifty grams of herbal sample was used for essential oil extraction for each treatment. Experiments were carried out in three replications. The obtained essential oil was dewatered by dry sodium sulfate and kept in dark glasses at 4°C until antifungal test [6].

Formulation of nanoemulsion essential oil. Formulation of 10% nanoemulsion essential oil of the Cinnamon was provided from chemistry engineering group of plants and pharmaceutical raw materials from Shahid Beheshti University in Tehran, Iran [18]. In order to obtain nanoparticles of required size, the ultrasonic transducers of a Swiss MTI model (400 W, 220 V, 20.5 kHz, 30% amplitude, and 19 cm probe diameter) was used. Span 80 (Sorbitan monooleate), Tween 80 (Polysorbate 80), and Lecithin were used to make nanoemulsion. Finally, the containers with the nanoemulsion were covered and kept in the fridge.

The particle size of nanoemulsions. The particle size of nanoemulsion was determined with the help of a DLS device. The device obtained the range of particle distribution, as well [19, 20].

Providing the propagule of pathogen. Two most common fungi as the decay factor of strawberry fruits namely *B. cinerea* SBU205 and *R. stolonifera* SBU205 were taken from a collection of microbes of RUDN University. In order to supply the intended isolated propagule, it was cultivated for a week on PDA medium at 25°C. A 12-hour light/darkness period was used. The spore suspension was prepared in sterile distilled water containing 0.05% of Tween 80 and then reached to concentration of 10^4 spores per millimeter of sterile distilled water by a hemocytometer [11].

Antifungal effect of emulsion and nanoemulsion of the Cinnamon essential oil. The antifungal effect of emulsion and nanoemulsion of CEO on two fungi of strawberry fruits, namely grey mold and soft rot was investigated by the method of mixing the essential oil and PDA cultivation medium. For this purpose, a 10% emulsion was prepared from the intended essential oil in a solution of Tween 80 (0.5%). Moreover, the Tween 80 solution (0.05%) was considered as the control treatment. Flasks containing the PDA cultivation medium were kept at room temperature after being put in the autoclave until 42–45°C. Concentrations of 0.125, 0.25, 0.5 and 1.0% of CEO emulsion and nanoemulsion, which equaled 125, 250, 500 and 1,000 μl of essential oil respectively, were transferred separately into flasks with the PDA medium and were mixed to be unified.

The samples obtained were immediately put into Petri dishes with the diameter of 9 m^{-2}. After they got solid, the cork borer provided fungal disks with the diameter of 5 mm from young cultivations of the stated fungus. One fungi disk was then put in the middle of the Petri dishes with cultivation medium which were put in an incubator at 25°C. After 24 hours, the vegetative growth of fungi was measured until the surface of the cultivation medium of control Petri dishes were totally occupied by fungi. In this test, three replications were carried out for each treatment.

Antifungal effects on strawberry fruits. Strawberry fruits of Selva variety (*Fragaria* × *ananassa* cv. Selva) without any physical or chemical problems and equal in size and ripeness index were provided by a greenhouse situated in Moscow, Russia. For superficial disinfection, the fruits were immersed in 0.1% hypochlo-

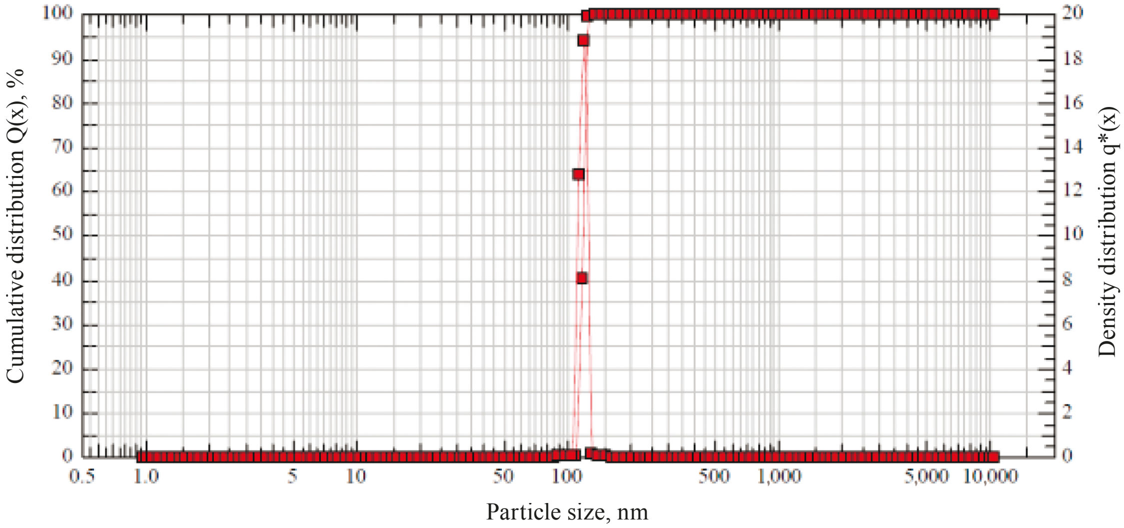

Fig. 1. Particle size and distribution of nanoemulsion obtained by DLS analysis.

rite sodium for 30 s and then they were washed twice by sterile distilled water. After getting dry, in sterile conditions, they were immersed in spore suspensions of *R. stolonifera* and *B. cinerea* for one minute via two separate tests. The concentration was 1×10^4 of spores per a millimeter of sterile distilled water. Further, CEO emulsion and nanoemulsion at concentrations of 0.5, 1 and 2% were added into sterile distilled water and were used for treatment of the inoculated fruits via spraying.

Thiabendazole fungicide (tacto 60) in sterile distilled water (at the concentration of 0.05, 0.1, and 0.2% active ingredient) was used as a control. Other control treatments included infected samples (fruits inoculated by pathogen spore and sprayed by sterile distilled water containing 0.5% of Tween 80) and sound/safe samples (healthy fruits sprayed by sterile distilled water containing 0.5% of Tween 80) [21]. Every five fruits were put as a repetition in one plastic transparent dish with dimensions of 14×11×14 cm [22].

Percentages of *Rhizopus* rottenness and grey decay in strawberry fruits were measured after five and 10 days of storage at 25°C, respectively. The infection of each fruit was based on its mashing, rottenness of fruit's surface; as such that each fruit was divided into eight parts, the symptoms of infection in each part estimated as 12.5% [23, 24]. In other words, the intensity of disease among the infected strawberry fruits ranged between 0 and 8. Here, zero indicates healthy fruit and numbers 1, 2, 3, 4, 5, 6, 7, and 8 shows rottenness to be 12.5%, 12.5–25%, 25–37.5%, 37.5–50%, 50–62.5%, 62.5–75%, 75–87.5%, and 87.5%, respectively. In all treatments, the infection rate of fruit was recorded and rate of control in different treatments was computed by using the proportion of infection in treatment to infection rate in infected samples minus figure of one [23, 24].

Statistical Analysis. A completely randomized design was conducted for testing among treatments with three replications. The normality of data set was determined by Kolmogorov-Smirnov test. The GLM method was used for analysis of variance. After the analysis of variances, the mean comparison of the data was done at significance levels of 1 and 5% with the help of Duncan's multiple range test. All statistical analyses were performed using SPSS software.

RESULTS AND DISCUSSION

Size dispersion and distribution of nanoemulsion. According to Fig. 1, the mean size of the Cinnamon nanoemulsion was equal to 115.33 ± 3.97 nm. The distribution range of particle size of the Cinnamon nanoemulsion was 15.56 ± 3.90 nm.

Antifungal effect of essential oil emulsion and nanoemulsion in laboratory. The results obtained from investigating the antifungal effect of CEO emulsion and nanoemulsion on the growth of fungus showed that generally, an increase in the concentration of essential oil has led to increasing in antifungal activity. The intensity of increase in the deterrence property was observable on *B. cinerea* fungi (Table 1). Regarding the results, emulsion and nanoemulsion of CEO with minimum complete deterrence concentration equaled 500 µl per litre of cultivation medium demonstrated the antifungal activity against *B. cinerea* fungi. It also showed complete deter-

Table 1. Effect of different concentrations of emulsion and nanoemulsion of Cinnamon essential oil on *R. stolonifera* and *B. cinerea* fungus in PDA cultivation medium

Treatment	Active ingredient, µl/l	Deterrence percentage*	
		Rhizopus stolonifer	Botrytis cinerea
Essential oil emulsion	125	3.16 ± 0.33	41.59 ± 0.25
	250	29.42 ± 0.92	65.88 ± 0.85
	500	63.8 ± 0.97	100nd
	1,000	100nd	100nd
Essential oil nanoemulsion	125	11.25 ± 0.19	49.15 ± 0.44
	250	45.68 ± 0.75	68.14 ± 0.82
	500	72.35 ± 1.12	100nd
	1,000	100nd	100nd

Note: nd = not determined (lack of measurable fungal colony)

* The presented number is the mean value of three repetitions

Table 2. Effect of different concentrations of emulsion and nanoemulsion of Cinnamon essential oil on strawberry grey mold after 10 day of storage in darkness at 25°C

Treatment	Fruit infection, %					
	0.05%		0.1%		0.2%	
Essential oil emulsion	41.7 ± 1.79	b	11.84 ± 1.79	e	7.58 ± 1.79	fg
Essential oil nanoemulsion	12.5 ± 1.52	d	7.58 ± 1.52	fg	5.43 ± 0.85	g
Tween 80 (0.05%)	98.43 ± 0.85	a	96.65 ± 0.85	a	97.48 ± 0.85	a
Thiabendazole	23.52 ± 2.65	c	14.13 ± 0.85	e	11.54 ± 1.59	e

Note: Comparing the means has been done in all levels of treatments.
Dissimilar letters indicate significant difference ($p \leq 5\%$)

rence against *R. stolonifera* fungi in the concentration of 1,000 μl per litre of cultivation medium (Table 1). Results of sub-culturing of fungal disks in the treatments where no fungal growth was observed showed that CEO emulsion and nanoemulsion at the concentration of 1,000 μl of active ingredient per one liter of cultivation medium had strong fungicide effect against both fungi.

Reduction of grey mold on strawberry fruits. Amount of strawberry grey mold in infected control samples was computed as 98.33%. There is a significant difference among treatments in terms of grey rottenness amount (Table 2). Generally, an increase at the concentration of the essential oil emulsion and nanoemulsion led to increasing in antifungal activity. Essential oil emulsion at the concentration of 0.2% and essential oil nanoemulsion at concentrations of 0.1 and 0.2% showed the highest amount of deterrence from the grey mold of fruit. There was no significant difference between them, but they had the significant difference with thiabendazole. CEO nanoemulsion at the concentration of 0.2% showed no significant difference with the sound control samples where the infection was zero. Thiabendazole fungicide at the highest concentration (0.2%) with 11.54% infection showed less impact than nanoemulsion.

Reduction of soft rot on strawberry fruits. Emulsion and nanoemulsion of the essential oil had a significant effect on the rate of infection due to the reduced activity of the *R. stolonifera* fungus (Table 3). The results obtained from examining the essential oil showed a decline of rottenness due to *R. stolonifera* on the fifth day of storage. Generally, an increase in the concentration of the essential oil led to increasing in antifungal activity and the essential oil at the concentration of 0.2% demonstrated the highest deterrence from fungi growth. Other concentrations of CEO were not so effective. The antifungal activity of nanoemulsion enhanced with increased concentration, thus Cinnamon nanoemulsion at the concentration of 0.2% displayed the highest rate of control by 13.50% of infection.

The use of medicinal herbs in the pharmaceutical and food industries are expanding due to the active biological compounds in herbs. In addition, extensive research has showed that secondary metabolites of certain medicinal plants are effective in preventing the growth of pathogenic fungi. This makes them an appropriate alternative to chemical pesticides [25].

A number of studies report about the effectiveness of essential oils of some medicinal herbs, such as Sour, Thyme, Cinnamon, Marjoram, Basil, etc., in the control of post-harvest disease in storehouses [10, 26–28]. In Iran, the use of essential oils such as basil, fennel, green substitute, sweet pepper, and peppermint to control of *B. cinerea* and *R. stolonifer* growth is proved to be effective [29–32]. Similarly, the use of herbal essential oils such as marjoram and cinnamon in vegetable washings has had great success in controlling molds and vegetable pathogens [33, 34]. In the study by Saranya *et al.* [7] about the antibacterial properties of a nanoemulsion of eucalyptus oil against *Proteus mirabilis*, the size of particles produced was on average 20 nm. Differences in the size of nanoparticles were due to differences in the types of production methods, while physicochemical properties of the dispersed phase were constant [7, 35].

In our research, CEO also showed significant antifungal activity in controlling grey caries and strawberry fruiting. The preventive effect of essential oils on the growth of the fungus was due to the presence of its active substances. Results of the analysis of essential oils have shown that the main component of cinnamon is cinnamaldehyde which has had antifungal and antimicrobial activity [28, 36].

Unfortunately, essential oils are difficult to use due to low solubility in water, high vapor pressure, and physical and chemical instability. Also, essential oils also

Table 3. Effect of different concentrations of emulsion and nanoemulsion of cinnamon essential oil on strawberry soft rot after five days of storage in darkness at 25°C

Treatments	Fruit infection, %					
	0.05%		0.1%		0.2%	
Essential oil emulsion	92.35 ± 0.85	a	63.33 ± 2.27	d	31.66 ± 1.69	e
Essential oil nanoemulsion	92.35 ± 2.27	a	65.00 ± 1.72	d	13.50 ± 1.72	g
Tween 80 (0.05%)	92.35 ± 2.27	a	92.35 ± 2.27	a	92.35 ± 2.27	a
Thiabendazole	87.50 ± 1.72	b	69.16 ± 0.85	c	20.18 ± 3.17	f

Note: Dissimilar letters indicate significant difference ($p \leq 5\%$)

have an adverse effect on sensory characteristics of the product [37]. One of the ways to minimise this effect is to use nanoemulsion, which allow increasing the stability and half-life of the active substance as well as the ease of reaching the targeted spot on the surface, provided size of the particles is small. In addition, it improves the antimicrobial properties by increasing cellular uptake [12, 17].

On the other hand, the particle size of a nanoemulsion indicates the degree of stability: the larger the particle size, the greater particle size distribution. Thus, the nanoemulsion is more unstable because interconnection of larger particles cause aggregate formation and lead to the instability of the nanoemulsion [38, 39].

In this study, the particle size distribution of the essential oil was about 115 nm, indicating its stability. In fact, the physical and chemical properties of nanoemulsions can be quite different from those of emulsions, as we demonstrate herein. The use of solvents provides the nanoemulsion of essential oils with appropriate viscosity and stability [15]. In addition, the small size of the particles is also very important for increasing the stability and half-life of the active substance and the ease of reaching the workplace [35, 40]. In this research, nanoemulsion of CEO compared to essential oil showed higher antifungal activity against grey caries and soft carcass strawberries.

Similar results were obtained by [18, 20, 41] about the effectiveness of CEO nanoemulsion, savory, and thyme in controlling human pathogenic bacteria. Also, the application of nanoemulsions of *Thymus vulgaris L.* and *Satureja khuzistanica* L. has been effective in controlling strawberry's soft deciduous caries [16]. Nanoemulsions are used as lipophilic transfer systems in the pharmaceutical industry, as flavoring and antibacterial agents in the food industry, as solvents for solving insoluble pesticides in water in the agriculture, as well as carriers of skin care and personal care products in cosmetic products [19]. Particles of nanoemulsions contact the patient's cell wall and begin to destroy them. This non-specialised mechanism does not cause resistance to strains.

On the other hand, regarding the application of essential oils as plant fungicides, the production of a stable, homogeneous, organic aquatic system facilitates its commercial use. The ease of use is possible due to the rapid dilution of emulsions in water by farmers and other consumers [16]. The results of our research allow us to suggest cinnamon nanoemulsion for controlling strawberry storage fungus under post-harvest conditions.

CONCLUSION

Larger particle size is known to lead to unstable nanoemulsion because particles form aggregates. In the current research, essential oil of cinnamon showed considerable antifungal activity against the grey *Rhizopus* rots of strawberry. The preventive effect of essential oil against fungi growth is due to their active ingredients. The nanoemulsion technology may be a potential method to satisfy the demands of commercialization formulations.

In most cases, CEO nanoemulsion in comparison to essential oil indicated greater antifungal activity in controlling the soft rot of strawberry. Generally, increasing the concentration of essential oil raised the antifungal activity and essential oil at the concentration of 0.2% showed the highest rate of deterrence from fungi growth that had a significant difference with other concentrations. In nano essential oil, increasing the concentration led to increasing in antifungal activity, as such that cinnamon nanoemulsion in the concentration of 0.2% with 13.5% infection represented the highest rate of control of antifungal activity against the grey *Rhizopus* rots of strawberry.

According to the results of this research, the cinnamon nanoemulsion is suggested to control the growth of fungus on strawberry in a post-harvest period. In conclusion, improved control activity and maintenance of strawberry quality by cinnamon nanoemulsion indicate that such a formulation is very promising. In addition, further experiments to explain the relationships between antimicrobial activity and chemical composition are underway. After completing the studies about its effects on qualitative characteristics of fruit and obtaining desirable results, it is proposed to produce and use it commercially as an alternative for chemical fungicides in order to reduce the postharvest wastes for strawberry.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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