Journal of Environmental Health and Sustainable Development

Zataria Multiflora Essential Oil Loaded with Starch Nanoparticles to Protect Strawberries against Botrytis Cinerea

Maryam Mobininejad [1], Elham Khalili Sadrabad [1], Seyed Hossein Hekmatimoghaddam [2], Ali Jebali [3], Aziz A. Fallah [4], Fateme Akrami Mohajeri [1]*

[1] *Zoonotic Diseases Research Center, Department of Food Hygiene and Safety, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.*
[2] *Department of Advanced Medical Sciences and Technologies, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.*
[3] *Medical Biotechnology Research Center, Ashkezar Branch, Islamic Azad University, Ashkezar , Yazd, Iran.*
[4] *Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran.*

ARTICLE INFO

ORIGINAL ARTICLE

Article History:
Received: 13 June 2021
Accepted: 20 August 2021

***Corresponding Author:**
Fateme Akrami Mohajeri
Email:
Fateme.akrami@gmail.com
Tel:
+9831492274

Keywords:
Botrytis Cinerea,
Antifungal Agents,
Food Packaging,
Nanoparticles,
Zataria Multiflora.

ABSTRACT

Introduction: The post-harvest damage to fruits is estimated to be about 10-30% of the total products, which reaches up to 30-50% in some perishable fruits. About 25 species of fungi and bacteria including *Botrytis* spp. and in particular *Botrytis cinerea* are known to contaminate fruits, vegetables and ornamental greenhouse plants. The aim of this study was to investigate the antifungal activity of different concentrations of *Zataria multiflora* essential oil (ZEO) against *B. cinerea*.

Materials and Methods: The ZEO was extracted through steam distillation and analyzed by gas chromatography-mass spectrometry. The strawberries packages were exposed to ZEO with different concentrations (0, 200, 400, 600, and 800 ppm) and satarch nanoparticles. The exposed fruits were kept for 24 days at two temperatures of 20°C and 4°C.

Results: The ZEO decreased mycelium growth even when only 200 ppm of it was added to each container. The response was dose-dependent, so that the 800 ppm dose of ZEO showed complete inhibitory effect. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values for ZEO against *B. cinerea* were 200 µg/mL and 500 µg/mL, respectively. Additionally, ZEO preserved the sensory characteristics.

Conclusion: The ZEO may be effectively used in packaging of strawberry to increase its shelf life by inhibition of *B. cinerea*.

Citation: Mobininejad M, Khalili Sadrabad E, Hekmatimoghaddam SH, et al. ***Zataria Multiflora Essential Oil Loaded with Starch Nanoparticles to Protect Strawberries Against Botrytis Cinerea*.** J Environ Health Sustain Dev. 2021; 6(3): 1376-87.

Introduction

Strawberry is a member of the Rosacea family and is known as a non-climacteric fruit (i.e., it slowly passes the puberty and development stage on the mother plant). The intensity of breathing is high in this fruit [1]. Iran occupied the 18th ranking in the world in production of strawberries with 42000 to 4080000 tons of strawberry produced in 2011 [2]. The quality of this highly perishable fruit depends on its appearance, texture, aroma, taste, and nutritional value [1]. Due to the presence of ascorbic acid and anthocyanins, this fruit is rich

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[DOI: 10.18502/jehsd.v6i3.7245]

in antioxidants and has many therapeutic properties, such as improving the body's immunity and reducing the risk of cancers [3]. Strawberry has a short shelf-life because of its high levels of breathing, high water content (~ 91%), high metabolic activity, and susceptibility to fungal spoilage [4]. Annually, fungal contamination of fruits and vegetables causes huge losses of the products. The post-harvest damage to fruits is estimated 10–30% of the total products in most studies, which reaches up to 30–50% in some perishable products. *Botrytis cinerea* is a gray mold which contaminates fruits, vegetables and ornamental greenhouse plants [5]. The application of safe and biodegradable fungicides to control the post-harvest fungal decay instead of synthetic chemical substances has drawn public attention [1]. In recent years, some essential oils (EOs) have been used in active films to protect foods from spoilage [6]. *Zataria multiflora Boiss* (Persian name: Avishan-e-Shirazi) is a medicinal plant belonging to *Lamiaceae* family and grown in Iran, Pakistan and Afghanistan. In Iran, *Z. multiflora* is used as a food spice and also as a traditional medicine for treatment of various diseases. The antioxidants, an antibacterial and antifungal activity of Z. multiflora essential oil (ZEO) have been demonstrated and are attributed to phenolic compounds such as carvacrol and thymol [6-8]. The essential oil can be obtained by distilling or pressing the plant's leaves, roots, fruits, seeds, stems or flowers [9].

Antimicrobial compounds can reduce or delay the growth of microorganisms through direct contact with foods or by their presence in packaging polymers [10]. Active packaging of foods could increase the foods' shelf-life using absorbent agents, releasing the agents of various compounds, heating and cooling the food, and determining foods' shelf-life using various sensors and reagents [6, 11]. Some active packages provide continuous migration of antimicrobial compounds to the surface of the food, hence preventing growth of the microorganisms and enhancing the safety and quality of foods [12, 13]. In this research, we aimed to evaluate the effects of starch nanoparticles containing ZEO on the shelf life of strawberries through inhibition of *B. cinerea* growth.

Materials and Methods

Essential oil

The ZEO used in this study was the same used in our previous study [14], which was obtained by steam distillation method and analysed by gas chromatography–mass spectrometry (GC–MS). The major components were thymol (34.44%), carvacrol (33.45%) and ρ-cymene (15.62%) followed by α-terpinolene (1.85%), γ-terpinene (1.80%), and myrcene (1.65%).

Preparation of mixture of starch nanoparticles and ZEO

Starch nanoparticles (particle size 30–60 nm) were obtained from Masiha-Shargh Corporation (Yazd, Iran) and used to maintain the stability and prevent ZEO from fast evaporation. In this way, 10 mL of ZEO was added to 1 g of starch nanoparticle powder, mixed vigorously, and incubated in an acid- and heat-resistant polyethylene container at 37 °C for half an hour. The achieved concentrated colloidal mixture had a relatively thick texture and was used to perform the tests [15, 16].

Preparation of multi-layer micrometer container for nanoparticles/ZEO mixture

At first, a thin piece of sterile cellulose paper (4 × 4 cm, loaded with the prepared nanoparticles/ZEO mixture) was sandwiched between two layers of alcohol-sterilized polypropylene polymer (5 × 5 cm), and all three layers were joined together by heating their outer edges. The top layer already had 20 tiny holes of about 100 µm diameter manually made by a thin pin. This final coating was examined for color, clarity, and content.

In vitro antifungal activity of ZEO

A strain of *B. Cinerea* (ATCC 90870) was purchased from the microbial collection of the Industrial Scientific Research Organization of Iran. For the agar diffusion method, potato dextrose agar (PDA) medium containing 0, 50, 100, 200, 300, 400, 500, 600 and 800 ppm of

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ZEO were prepared. For each concentration, the test was performed in triplicate. A 5 mm disk of Whatman No. 1 filter paper, previously inoculated with 10 µl of suspension of *B. cinerea* containing 10^6 spores/mL, was placed in the center of each plate. The plates were then incubated at 25 °C for 7 days with daily measurement of the colony diameter until the entire control plate (without essential oil) was covered with the fungus. To evaluate the MFC, the discs containing spores that did not show growth in the medium were transferred to dishes with no essential oils, and kept for additional 2 days at 25 °C.

For the vapor method, the antifungal property of the package was examined in a Petri dish containing PDA medium cultivated with 100 µl of suspension comprising 10^6 spores/mL. In this experiment, an active label (containing 0.015 g of starch nanoparticle powder and concentrations of 50, 100, 200, 300, and 400 ppm of ZEO) was glued to the inside of Petri dish cap. In the control culture, the label containing 0.015 g of starch nanoparticle powder was used without adding ZEO. Inhibition of fungal growth was checked by measuring the inhibition zone on the plate surface around the label after 48 and 72 hours as well as on days 7 and 20 of the experiment, which was then compared with the control plate [17].

Antifungal activity of the packaging in strawberries

In each electron beam-sterilized disposable container, a piece of the 3-layered 5×5 cm label was placed. The fruits' surfaces were individually disinfected with 2.5% sodium hypochlorite solution and washed with sterile distilled water. Then, they were immersed in *B. cinerea* suspension containing 10^4 spores/mL for 1 min to become contaminated before being placed inside a sterile foil under the hood for half an hour so that the spores can be settled on strawberries. In the next step, 70 g of these fruits were put in the above-mentioned containers, incubated at either 4 or 20 °C and evaluated daily for 24 days [18].

Sensory properties of strawberries during shelf life

At this stage, some non-sterile strawberries were stored inside two polyethylene containers in refrigerator, and the active label was added to one of them. To investigate the effect of ZEO on sensory properties, 12 trained students were selected to check the properties. The criteria for selection of evaluators were physical health, lack of allergies, the desire to consume the nutrient under investigation, and intact senses of smell and taste. Before the test, the students were given the necessary instructions regarding the fruit's smell, texture and taste. Fresh drinking water was available to the evaluators between the test stages. The sensory judges were asked to evaluate the taste, texture, smell, and overall acceptability of strawberry samples through the five-point hedonic scale (1 = bad, 2 = poor, 3 = moderate, 4 = good, 5 = very good). The sensory evaluation was performed in two stages (one and 10 days after the storage at the refrigerator temperature) and the assessment was carried out under the same light and temperature conditions [19].

Statistical analysis

The data were analyzed by one-way analysis of variance in a completely randomized design with factorial arrangement using SPSS 16. Differences among treatments were expressed in the Duncan test. The p value < 0.05 was considered as significant difference.

Ethical issue

This study was conducted with the approval of Shahid Sadoughi University of Medical Sciences and Health Services, Medical Ethics Committee. Code: IR.SSU.SPH.REC.1395.93.

Results

Disc diffusion method

The effect of different concentrations of ZEO on B. *cinerea* growth is presented in table 1 and figure 1. The growth of fungus was completely prevented at concentrations of 200 µg/mL. The concentration of essential oil and the incubation time had a significant effect on the fungal colony diameter of the *B. cinerea* ($p < 0.05$). The Minimum Inhibitory Concentration (MIC) and Minimum Fatal Concentration (MFC) were 200

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and 500 ppm, respectively. Antifungal activity was assessed by measuring the zone of fungal growth inhibition and MIC determination. The results of disk diffusion assay are summarized in table 1. The results indicated that ZEO at all tested concentrations inhibited the growth of *Botrytis cinerea* significantly ($P < 0.01$).

Uppercase and Lowercase letters indicate a significant ($p < 0.05$) difference among the values of each row and column, respectively (comparison between days). NG: no growth.

Vapor diffusion method

Vapor diffusion method was used to examine the ZEO antifungal activity in the vapor phase. The results are represented in table 2. The growth rate decreased proportionally by the increase in ZEO concentration. Mycelia did not grow at concentrations of 400 ppm or higher.

Table 1: Colony diameter of *B. cinerea* (mm) exposed to various concentrations of *Zataria Multiflora* essential oil (Mean ± SD)

Concentration (µg/mL)	Incubation time (day)		
	Day 2	Day 4	Day 7
0	$47.33^{A,a} \pm 0.58$	$65.27^{B,a} \pm 0.37$	$90.00^{C,a} \pm 0.0$
50	$14.50^{A,b} \pm 0.50$	$27.66^{B,b} \pm 0.41$	$40.61^{C,b} \pm 0.35$
100	$0.0^{A,c} \pm 0.0$	$20.33^{B,c} \pm 0.58$	$35.28^{C,c} \pm 1.52$
200	NG	NG	NG
300	NG	NG	NG
400	NG	NG	NG
500	NG	NG	NG
600	NG	NG	NG
800	NG	NG	NG

Different small letters in each column shows significant differences at $P < 0.05$. Different capital letters in each row shows significant differences at $P < 0.05$.
NG: No Growth

Table 2: Diameter of the growth inhibition zone (mm) under the label containing the essential oil of *Zataria Multiflora* (mean ± SD),

Concentration (µg/mL)	Incubation time (day)			
	Day 2	Day 3	Day 7	Day 20
Control	$0.0^{A,c} \pm 0.0$	$0.0^{A,e} \pm 0.0$	$0.0^{A,e} \pm 0.0$	$0.0^{A,d} \pm 0.0$
50	$54.33^{A,b} \pm 0.58$	$46.33^{B,d} \pm 0.58$	$28.50^{C,d} \pm 0.87$	$0.0^{D,d} \pm 0.0$
100	$55.83^{A,b} \pm 0.76$	$52.33^{B,c} \pm 0.58$	$45.83^{C,c} \pm 0.29$	$35.33^{D,c} \pm 0.58$
200	$90.00^{A,a} \pm 0.00$	$70.67^{B,b} \pm 0.58$	$58.17^{C,b} \pm 0.76$	$43.00^{D,b} \pm 1.00$
300	$90.00^{A,a} \pm 0.00$	$90.00^{A,a} \pm 0.00$	$63.83^{B,a} \pm 0.76$	$45.33^{C,a} \pm 0.58$
400	-	-	-	-

Different small letters in each column shows significant differences at $P < 0.05$. Different capital letters in each row shows significant differences at $P < 0.05$.

Antifungal activity of the new package in strawberries

In figures 2a-d and 3a-d, the changes of the appearance of strawberry samples kept in containers with labels are shown. The fruits were exposed to vapors of ZEO in various volumes during 24 days of storage at two temperatures of 4 °C (refrigeration) and 20 °C (ambient temperature). There was continuous growth of fungus in the next days as demonstrated in table 3. The comparison of strawberry shelf-life percentage at different times is presented in table 4. As the table depicts, no fungal growth was observed in the second day, and all of the strawberries were mold-free.

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Table 3: The percentage of moldy strawberries exposed to different amounts of ZEO in the 24th day.

***Zatara multiflora* essential oil amount (µL)**	**No. (%)**
Refrigerated control samples	9 (100)
Environment control samples	9 (100)
400 Refrigerated	5 (55.56)
400 Environment	8 (88.89)
600 Refrigerated	2 (22.22)
600 Environment	6 (66.67)
800 Refrigerated	0 (100)
800 Environment	4 (44.44)
Total	43(59.72)

Table 4: The percentage of moldy strawberries (N = 72) at different times.

Days after treatment	**No. (%)**
2	0
6	4 (5.56)
10	15 (20.83)
14	28 (38.89)
20	39 (54.17)
24	43 (59.72)

Sensory evaluation of the strawberries

Sensory evaluation of strawberry samples with different concentrations of ZEO as well as control sample was performed triplicate by four panellists at days 1 and 10 of storage. In this study, the parameters of taste, smell and texture were evaluated. The sensory evaluation results of strawberry with different concentrations of essential oil are given in table 5. At day 1, the scores of taste and odor were the highest in the control, 400 and 600 µL of ZEO groups, while increase in the essential oil level to 800 µL caused a significant decrease in taste scores ($p < 0.05$). During the storage period, the taste and odor scores of all samples were significantly reduced ($p < 0.05$) except for odor scores of 800 µL ZEO. At the day 10, the highest scores of odor and taste were reported for samples with 600 and 800 µL ZEO. Comparing 600 and 800 µL ZEO groups for odor and taste scores, the 600 µL ZEO got higher scores.

Table 5: Sensory evaluation of strawberries with different concentrations of essential oil stored at 4 ± 0.5 °C.

Sensory parameter	**Concentrations (ppm)**	**Storage time (days)**	
		1	10
Taste	Control	5^{a}	3.8^{b}
	400	4.7^{ab}	3.8^{b}
	600	4.7^{ab}	4.3^{a}
	800	4.5^{b}	4.1^{a}
Odor	Control	5^{a}	3.7^{b}
	400	5^{a}	3.9^{b}
	600	5^{a}	4.3^{a}
	800	4.3^{b}	4.1^{a}
Texture	Control	5^{a}	3.8^{b}
	400	5^{a}	4.1^{b}
	600	5^{a}	4.7^{a}
	800	5^{a}	4.7^{a}
General acceptance	Control	5^{a}	3.5^{b}
	400	5^{a}	3.7^{b}
	600	4.8^{ab}	4.6^{a}
	800	4.5^{b}	4.4^{a}

Different letters in each column shows significant differences at $P < 0.05$.

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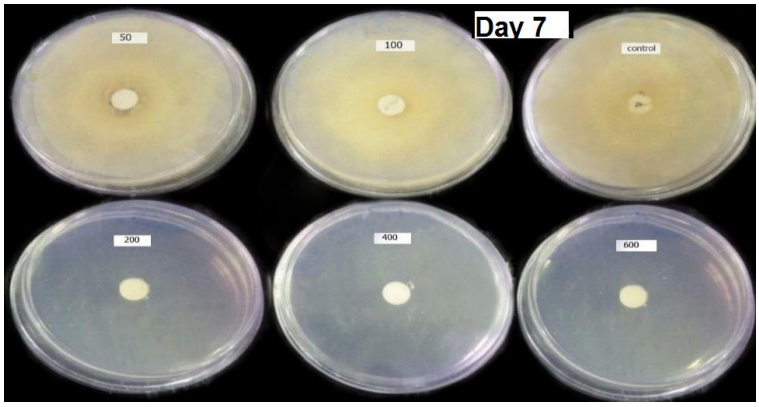

Figure 1: Growth of *B. cinerea* at different concentrations of ZEO on the 7th day of incubation.

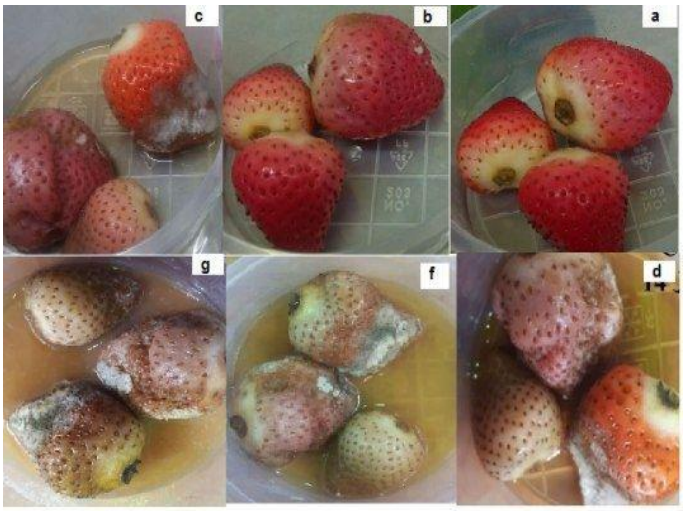

Figure 2a: Appearance changes in the strawberry control samples during storage at 20 °C. a: day 2, b: day 6, c: day 10, d: day 14, f: day 20, g: day 24.

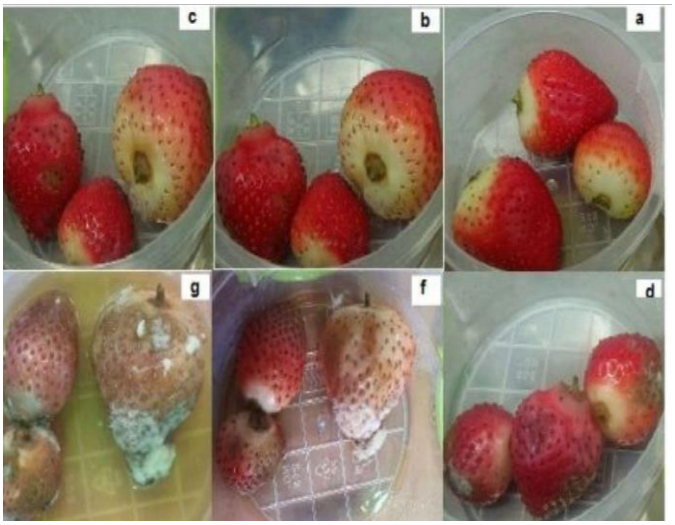

Figure 2b: Appearance changes in the strawberry samples exposed to vapors from 400 µL of ZEO during storage at 20 °C. a: day 2, b: day 6, c: day 10, d: day 14, f: day 20, g: day 24.

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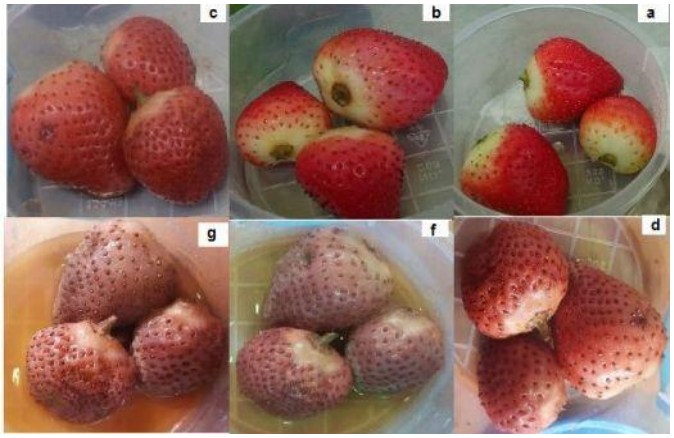

Figure 2c: Appearance changes in the strawberry samples exposed to vapors from 600 µL of ZEO during storage at 20 °C. a: day 2, b: day 6, c: day 10, d: day 14, f: day 20, g: day 24.

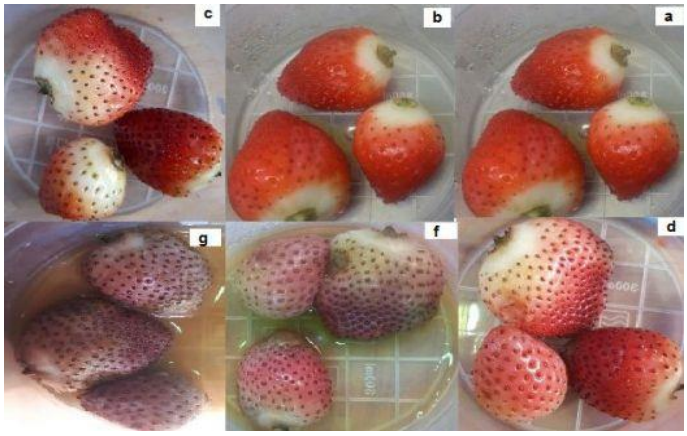

Figure 2d: Apearance changes in the strawberry samples exposed to vapors from 800 µL of ZEO during storage at 20 °C. a: day 2, b: day 6, c: day 10, d: day 14, f: day 20, g: day 24.

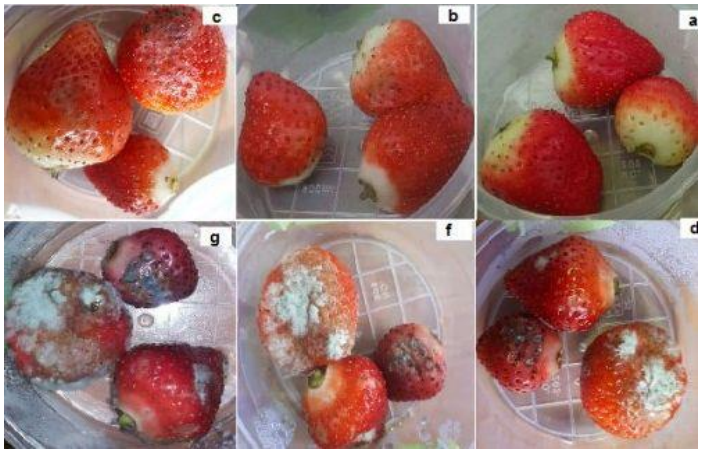

Figure 3a: Apearance changes in the strawberry control samples during storage at the refrigerated temperature. a: day 2, b: day 6, c: day 10, d: day 14, f: day 20, g: day 24.

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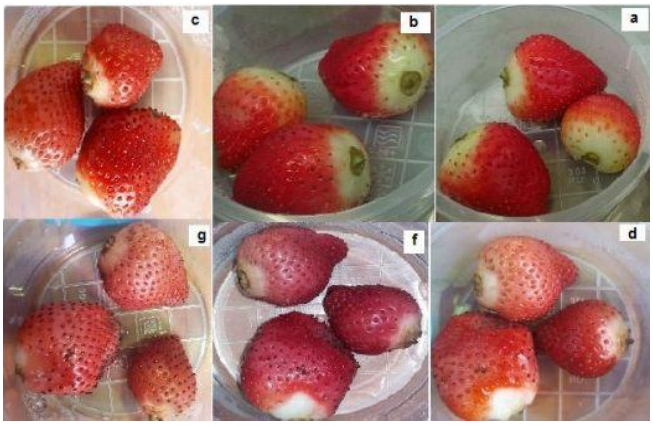

Figure 3b: Appearance changes in the strawberry samples exposed to vapors from 400 µL of thyme ZEO during storage at 4 °C. a: day 2, b: day 6, c: day 10, d: day 14, f: day 20, g: day 24.

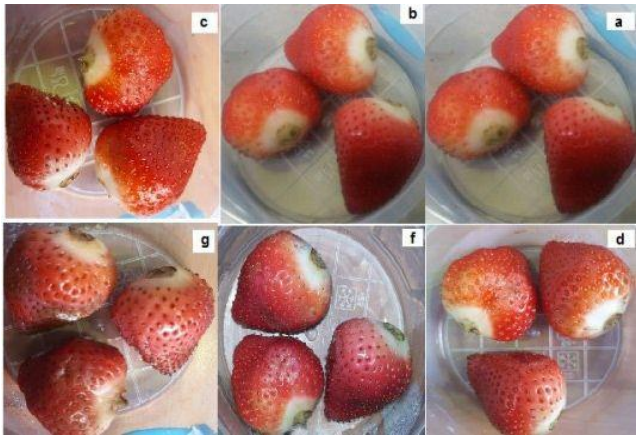

Figure 3d: Appearance changes in the strawberry samples exposed to vapors from 600 µL of ZEO during storage at 4 °C. a: day 2, b: day 6, c: day 10, d: day 14, f: day 20, g: day 24.

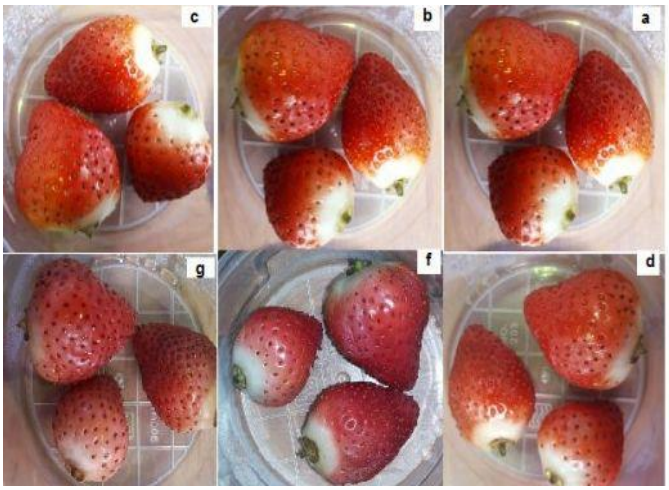

Figure 3e: Appearance changes in the strawberry samples exposed to vapors from 800 µL of ZEO during storage at 4 °C. a: day 2, b: day 6, c: day 10, d: day 14, f: day 20, g: day 24

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Discussion

Findings of the present study indicated that by increasing the concentrations of ZEO, mycelia growth was inhibited. Antifungal activity of ZEO against *B. cinerea* was dose-dependent. Amini et al. stated that the concentration of 500 mg/L of ZEO had complete inhibitory effect on the growth of *P. italicum* [20]. Sabounchi et al revealed that by increasing the concentrations of ZEO, the anti-fungal activity against *Botrytis cinerea* was increased [21]. Findings by Etemadi et al. on the antifungal effect of ZEO on *Botrytis* are consistent with our results [22]. Akrami et al suggested that ZEO can be used as a natural preservative against *Pencillium citrinum* in foods [8, 23].

According to results of the vapor , diffusion method. it can be concluded that the essential oil was consistently active for up to 20 days. In order to investigate the effect of starch nanoparticles on the anti-fungal activity of the label, a label containing only starch nanoparticles was considered. The results were similar to those of the control sample, indicating that nanoparticles alone do not show antifungal activity. The concentration of ZEO and the incubation time had a significant effect on the diameter of the *B. cinerea* growth inhibition zone ($p < 0.05$). In order to evaluate the shelf-life of the essential oil in the produced label, the prepared plates were observed regarding the growth of *B. cinerea* fungus for 20 days. The results of the vapor method showed that by increasing the ZEO concentrations, the growth of mycelia fungus decreased. Furthermore, the production of spores was limited. Akrami et al. investigated the antimicrobial properties of papers containing 2, 4, and 6% ZEO against gram-negative and gram-positive bacteria. They reported that concentrations of 4% and 6% ZEO inhibited the growth of all bacteria, whereas 2% essential oil prevented only the growth of *Staphylococcus aureus* and *Salmonella* [6]. Rodriguez et al. evaluated different essential oils in a new active paper and board coating by paraffin against *in vitro* and *in vivo* growth of *Alternaria alternata*. The concentrations of 3 and 6% (w/w) of bark cinnamon and oregano essential oil, respectively showed the best performance (versus clove and leaf cinnamon essential oils) when incorporated to active paper or board packaging. Total inhibition of the fungus was obtained when 6% concentration of bark cinnamon essential oil was applied to the packaging [24].

With increase in the storage time, the growth of fungus increased gradually in strawberry samples, so that 56.5% of the samples had mold in the sixth day and 59.72% in the last day (day 24). Perdones et al. used films containing 1% chitosan and 3% lemon in strawberry packing, and examined the degree of water vapor permeability and antimicrobial activity [18]. They also investigated the amount of fungal decay, physicochemical properties, and the breathing rate of strawberries at 5 °C. They found that addition of lemon, oil had a significant effect on the destruction of *B. cinerea* and increased antifungal activity of chitosan[18]. Moreover, Rodríguez et al. applied paraffin paper containing cinnamon essential oil as an active packaging against *Rhizopus stolonifer* or black bread mold [25]. It was found that the volatile substances derived from the essential oil of cinnamon that enter the atmospheric gas phase of the package play an antifungal role. Moreover, increasing the amount of essential oil from 1 to 6 % in the paraffin paper cover increased its anti-fungal property. The package containing 6% essential oil could prevent the growth of fungus after 3 days.

In general, the main problem with the use of natural compounds such as essential oils is their intense odor as well as low solubility in water, high vapor pressure, and physical and chemical instability, which is unpleasant to consumers [26]. To solve this problem, it is possible to add these essential oils into the packaging film which renders antimicrobial activity, or to make labels containing these compounds that release over time and prevent growth of microorganisms. The latter method has many advantages. Another way to minimize these undesirable effects is to use their Nano emulsion that increases the stability of volatile compounds, protects them against interactions with other compounds, and increases

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[DOI: 10.18502/jehsd.v6i3.7245]

antimicrobial properties by increasing cellular absorption [27]. We tried to apply a concentration of essential oil that, in addition to high antifungal properties, did not have adverse effects on the sensory properties of strawberries. Then, with designing new packages and using nanoparticles of starch, this important goal was achieved. In line with this strategy, Guerreiro et al. examined the effect of polysaccharide-based edible coatings enriched with citral and eugenol to improve the sensory quality of strawberries. They showed that the overall acceptance scores of all samples significantly decreased during the 14-days storage at refrigerator. They stated that the highest sensory rating was related to the treatment covered with 2% alginate + 0.1% eugenol.

The results of sensory analysis of tissue at the first day showed that all samples had the same scores. On the tenth day, the lowest score of tissue was that of the control and 400 ppm of ZEO. By increasing essential oil content from 400 to 600 µL, tissue scores increased significantly ($p < 0.05$), but the tissue score of 600 and 800 ppm of ZEO was similar on this day. In various scientific texts, the effect of herbal essential oils, either alone or in combination with edible coatings, has been studied as antimicrobial and antioxidant agents. However, in most of these articles the sensory acceptance of the treatments from the consumers' point of view has not been studied. In this research, the effect of ZEO (400, 600 and 800 ppm) and starch nanoparticles on the sensory properties of taste, odor, texture and general acceptance of strawberry were investigated. The results of the evaluation of general acceptance of strawberry samples indicated that on the first day of storage, the highest score was related to the control and 400 ppm of ZEO. On the tenth day of storage, the highest general acceptance rate was for the 600 ppm of ZEO followed by 800 ppm. General acceptance scores of all samples were significantly decreased ($p<0.05$) over time except for samples with 800 ppm of essential oil. Overall, samples with 600 ppm of essential oil had the highest scores of sensory analysis.

The main problem with the use of natural compounds is the presence of intense smell, which sometimes has a harmful effect on the sensory properties of the product [14]. This is the reason for the low sensory score of sample exposed to 800 ppm of essential oil. However, it is possible to use compounds that create less smell. Additionally, using the appropriate methods, we must try to use the lowest concentrations of essential oils to minimize their harmful effects. Nowroozi et al. investigated the effect of thymol and menthol on the sensory properties of strawberries. Thymol significantly contributed to undesirable odor of the fruits in control, and produced the highest level of thymol (15 µL), with the most severe odor though. Menthol essential oil did not induce a significant difference in the odor of the fruit vs. control [28]. Guerreiro et al. examined the effect of polyacetate coatings enriched with citral and eugenol essential oils on the sensory quality of strawberries and showed that during the storage period of 14 days at refrigerator temperature, the overall acceptance scores of all samples were significantly decreased. They stated that the highest sensory rating was related to the treatment covered with 2% alginate + 0.1% eugenol [29]. Rodriguez et al. reported on the effect of chitosan coating containing orange and lemon essential oils on the sensory properties of mangoes [30]. The highest taste scores were related to chitosan-coated samples containing lemon oil [30].

Conclusion

According to the results obtained in this study, it can be concluded that the essential oil of *Zataria multiflora* has the potential to be used as a natural preservative to increase the shelf-life of strawberries. Considering the new packaging design and use of starch nanoparticles, *Zataria multiflora* essential oil can maintain the quality and increase the shelf life of strawberries without causing undesirable effects on sensory properties of products. Application of this essential oil for other herbal products, especially fruits and vegetables seems possible, and needs further studies.

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[DOR: 20.1001.1.24766267.2021.6.3.6.2]
[DOI: 10.18502/jehsd.v6i3.7245]

Acknowledgements

The authors appreciate the supply of instruments by department of food hygiene and safety, international campus of Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Funding

This research was funded by Shahid Sadoughi University of Medical Sciences.

Conflict of Interest

The authors declare no conflict of interest.

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