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Effect of edible coatings based on zein and chitosan and the use of Roman aniseed oil on the microbial activity of Mazafati dates

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ABSTRACT

Maintaining optimum quality and eliminating dates of contamination, cleanup and packaging to extend the shelf life is one of the country's priorities and policies. Edible coatings with vegetable oils are one way to prevent the growth of microorganisms. In this study, the influence of the coating with natural zein and chitosan polymers along with the essential oil of Roman anise on the microbial behavior of Matsafati dates during one-year storage at 2 and 4 °C and a second year at 10 °C was examined. The results showed that the microbial sample of Bam region with 32% moisture content had a high microbial burden. *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus stolonifer*, and *Alternaria alternata* were growing on it. These fungi, especially *Aspergillus niger* were involved in the rotting and lactic acid bacteria were involved in the rancidity of Mazafat dates. In this study, the number of *Aspergillus niger* fungi using edible coatings of zein and chitosan as well as anise oil in three logarithmic cycles was reduced and the *Penicillium* fungi were eliminated. In general, the lowest microbial growth was observed in zein treatment and the highest in the control sample. The use of anise oil had the effect of reducing the total count of microorganisms. According to microbial results, the use of zein treatment is recommended.

Keywords: Roman anise, Natural polymer, Coating, Mazafati date, Microbial properties

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1. Introduction

Iran is one of the largest date-producing countries, accounting for around 14% of world production. Date fruit production in the country is estimated to be approximately 1.2 million tons per year (Ahmadi et al., 2018). Dates are a valuable product in terms of nutritional value, producing about 3000 kcal per kg and having adequate amounts of vitamins A, B_1, B_2, niacin and various minerals such as potassium, calcium and iron. However, there is also a moderate amount of chlorine, copper, magnesium and sulfur in the dates (Jalili, 2005; Khodabakhshian Kargar & Emadi, 2014). Mazafati dates are one of the most delicious and pleasant varieties in the world and are also used as Rotab. The main birthplace of Mazafat dates is Bam Kerman. This is a variety from the group of soft (moisty) and semi-dried reddish-brown dates with a dark red color (Afshari Jouybari & Farahnaky, 2011). Depending on the harvest time and planting phase, the moisture content is between 15 and 35%. This high humidity is a suitable environment for the growth and activity of fungi and other microorganisms that cause the date fruits to be corrupted frequently in domestic and foreign consumer markets. The amount of waste in the storage process is also high due to the high humidity and in many cases, the damage to product quality is so severe that sometimes the whole product becomes unusable (Salajegheh et al., 2017; Golshan Tafti et al., 2015).

Most palm growers of the region and even packaging stores pack the date product in the simplest way, which reduces exportability and durability and ultimately has a negative impact on export. Many factors such as the type of date, its initial microbial burden, the way the dates are stored before entering the workshop, the method of transport, and the physical operations performed during packing on the dates, washing and drying are effective.

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Little information is available on the microbiology of dates. However, according to Bolin et al. (1972), Ahmed et al. (1997), and Adams and Moss (2000), corruption in date fruit are associated with the growth and activity of yeast and lactic acid bacteria. The activity of these microorganisms causes sour and bad taste. Fungal *Aspergillus* species in different date's fruit ripening stages and species have also reported by Al- Shaickly et al. (1986), and Ahmed et al. (1997). Aflatoxin-producing fungi can grow and become active on damaged dates during the harvest in the field. Therefore, such fruits are not suitable for human consumption. In the investigation of 16 date palm varieties in the UAE, *Aspergillus flavus* and *Aspergillus parasiticus* were isolated in chimeric, Rotab and Tamr stages. The highest number of fungi was in the root stage and the lowest count in the Tamr stage. Shenasi et al. (2002) also reported that the total number of microorganisms in the Rotab stage was high and that lactic acid bacteria in the varieties containing *Aspergillus* and aflatoxins were isolated at this stage. In recent years, the use of antibacterial materials, coatings, and edible films have allowed manufacturers to produce healthier and more satisfying foods (Davidson and Zivanovic, 2003). Food coatings with various edible coatings act as a barrier to the exchange of gases, moisture and microorganisms and maintain the shelf life of food from production to the reach of the consumer (Salajegheh, 2017; Joerger, 2007). Based on research by Xie et al. (2002), Ryu et al. (2009) and Vahedikia et al. (2019) gas exchange (mainly carbon dioxide) and moisture exchange through the surface of dates can be minimized using edible zein and chitosan coatings. Chitosan polysaccharide is a biodegradable, nature-friendly, non-toxic polymer with good functional properties such as antimicrobial, antioxidative and formation of coating and film (Majeti and Kumar, 2000; Shahidi and Abuzaytoun, 2005; No et al., 2007; Tajik et al., 2008). Zein is also a natural polymer with film and coating properties (Ildiko, 2006; Ghanbarzadeh et al., 2007). Anise (*Pimpinella anisum*) is originally a seed of Roman fennel. Perennial fennel (*Foeniculum vulgare*) is one of the most important and most commonly used medicinal plants of the *Apiaceae* family. It contains compounds with microbicidal properties and is therefore used in various pharmaceutical, food and health industries (Gross et al., 2002). Currently, the sterilization process with methyl bromide gas is used to maintain the product quality of the Mazafati variety. This method is problematic due to its toxicity and environmental hazards. As a result, it is necessary to use other appropriate methods (Delkhah et al., 2012). Edible coatings with vegetable oils seem to prevent the growth of microorganisms. Due to the perfect covering effect and the formation of a protective layer on the dates, the product appears neat and shiny. Besides, there is no rotting and rancidity of fruit due to the effect of rinsing fruit. Therefore, in this study, the effect of various edible coatings of zein and chitosan and the use of Roman anise oil on Mazafati dates under different storage conditions (fridge and freezer) was studied for 12 months in two consecutive years. Next, microbial tests and determining the percentage of date fruit contamination during storage, the optimal conditions for reducing the microbial burden of dates and maintaining its quality were studied.

2. Material and Methods

After inspecting palm trees in the Bam region, from Azizabad station 10 bunches of Mazafati date fruits at the Rutab stage were randomly sampled from ten trees. To avoid contamination, fruit bunches were placed in clean baskets and quickly transferred to the laboratory of Kerman Research and Training Center for Agriculture and Natural Resources. To find out the microbial status of date samples and obtain zero-day data, total mold and yeast count tests and coliform tests were carried out immediately. The experiments were conducted over a two-year period from 2014 to 2016 at the Agricultural Research Center and Natural Resources of Kerman.

2.1. Providing coating solutions

Kim et al. (2006) method was used to prepare the chitosan solution. Since chitosan and zein are two protein compounds, proper solvents should be used to dissolve them. Therefore, chitosan was immersed in 2% acetic acid at 55 °C with continuous stirring for two and a half hours to obtain the chitosan solution. The resulting solution was divided into two parts. Half of the anise oil the air-dried seeds of black caraway and anise were supplied from fields of Agricultural Research Center and Natural Resources of Kerman of, Iran. After the plant seeds were authenticated, then 100 g of these medicinal plants was subjected to hydro distillation for 3 h using a Clevenger-type apparatus. The oil was dried over anhydrous $Na_2SO_4$and preserved in a sealed vial at 4 °C for future analysis was added to 1000 µg/ml and the other half without essential oil was used for the *C* treatment (Table 1). 10% zein solution was prepared by 1000 ppm carnation extract and 2% glycerol as plasticizer. Samples were dried as mentioned in previous method. The samples were kept in fridge with 3 °C temperatures and 60% relative humidity for 6 months (Vargas et al., 2006). A solution was prepared by (Janes et al., 2002) method using 95% ethanol. Zein was also divided into two parts. One part was mixed with anise oil and treated to samples, and the other part without essential oil was used for treatment *D* (Table 1).

After applying the above-mentioned treatments, all samples were packed in small boxes. First, small cardboard cartons with dimensions of 5 by 10 cm were selected, after placing the dates inside the low-density polyethylene protector, they were placed inside the carton and stored in the first year at 4 and -18 °C (refrigerator or freezer) and in the second year at 10 °C. Every three months, samples from each treatment were brought to the laboratory without opening in their original packaging and tested for microbial activity. The data were analyzed with the SPSS software and compared with the Duncan method.

2.2. Microbial test

After preparing the culture medium and sterilizing the required dishes and equipment, samples of Mazafat rhubarb were cultured. To do so, the fruits were nucleated using sterile forceps under sterile conditions. Then, 10 g of the nucleated samples were added to 90 ml of quarter-ring diluent to prepare the initial solution with a quarter of dilution. The homogenization of the resulting mixture was carried out using a pulsing machine for 45 s. Test tubes containing 9 ml Ringer's solution were used for lower dilutions.

Overall enumeration of microorganisms was performed according to the Pour Plate method using plate count agar Merck (Germany) plate medium. After culture, the plates were inverted in the incubator at 37 °C for 24 to 48 h and examined at 24 h intervals. The yeast was counted using the Oxytetracycline Glucose Yeast Extract (OTGYE) Merck (Germany) medium in an incubator at 25 °C for 3–5 days.

After the incubation, the colonies on the plate medium were counted. The total enumeration of microorganisms and fungi was carried out according to the Karim (2015). Two plates were

selected from a dilution containing 15 to 300 colonies and all colonies were counted using the colony count. The arithmetic mean of the two counts was multiplied by the dilution factor and the resulting number was reported as the total number of microorganisms and fungi. Only two significant cultivars were reported in the microbial count report. Finally, the number of microorganisms per gram of date fruit sample (Eq. 1) was calculated using the following equation (Salajegheh, 2013).

$$N = \frac{\sum C}{(n_1 + 0.1n_2) \times d} \quad (1)$$

Σ_C :Total colony counts in all selected Petri (with two consecutive dilutions)
n_1 :Petri number of first selected dilution
n_2 :Petri number of Second Selected dilution (Next Dilution)
d: Dilution factor in the first selected dilution

The calculated result was numerically rounded to two significant digits. The result was expressed as a numerical multiplication between (1.0–9.9) at 10^x.

The studied fungi included *Aspergillus niger*, *Aspergillus flavs*, *Alternaria Alternata* and *Rhizopus astolonifer*, which were cultured using PDA culture medium.

Table 1. Treatments used to cover the Mazafati dates.

Row	Treatments	Chitosan	Zein	Chitosan with Essences of Anise	Zein with Essences of Anise	Acetic Acid 1%	Ethanol 95%	Witness
1	A	+	-	-	-	-	-	-
1	B	-	+	-	-	-	-	-
3	C	-	-	+	-	-	-	-
4	D	-	-	-	+	-	-	-
5	E	-	-	-	-	+	-	-
6	F	-	-	-	-	-	+	-
7	G	-	-	-	-	-	-	+

Table 2. Results of analysis of variance of microbial counting in the first year of storage.

Sources of change	Degrees of freedom	Mean squares	
		Overall count	Mold and yeast
Treatment	6	112132720**	3870064**
Temperature	1	70448145**	488444.6**
Shelf time	3	39448892*	2261320.8**
Treatment × Time	18	74658648.8*	219490.5$^{n.s}$
Temperature × Time	6	40189820.8$^{n.s}$	118236$^{n.s}$
Temperature × Treatment	6	28105107.5$^{n.s}$	82651617**
Experimental error	38	230533694	129636.5

n.s, * and **, respectively, without significant difference, the significant difference at 5% and 1% probability level.

Table 3. Average microbial load effect of treatment and storage time on dates during storage.

Storage time	Total count of microorganisms (log cfu/g)	The total count of fungi (log cfu/g)
Three months after storage	4.70 ± 0.29	0.80 ± 0.80
Six months after storage	4.00 ± 0.68	1.00 ± 0.16
Nine months after storage	1.00 ± 0.63	0.17 ± 0.69
Twelve months after storage	1.00 ± 0.29	0.31 ± 0.07

Table 4. Comparison of the average effect of treatments on the total count and number of fungi and date molds in the first year.

Treatments	Total count (log cfu/g)	Number of molds and fungi (log cfu/g)
chitosan	761.25^{a}	348.75^{ab}
Zein	362.50^{a}	136.25^{a}
Chitosan + anise oil	1351.25^{a}	198.75^{ab}
Zein + anise oil	888.75^{a}	562.50^{b}
Acetic acid control	1825^{ab}	440^{ab}
Witness ethanol Ethanol control	437.50^{a}	207.50^{ab}
Control	10750^{a}	2112.50^{c}

Values bearing different lowercase letters in the same row are significantly different ($p < 0.05$).

Table 5. Results of analysis of variance in microbial counting of dates in the second year of storage.

Sources of change	Degrees of freedom	Mean Square	
		Total count	Mold and yeast
Between treatments	4	646755**	81339**
Without treatment	15	153565	94595
Error	19		

** the significant difference at 1% probability.

Table 6. Comparison of the average effect of treatments on total count and number of fungi and date molds in the second year.

Treatments	Total count (log cfu/g)	Number of Mold and yeast (log cfu/g)
Chitosan	625[ab]	175[a]
Zein	150[a]	62[a]
Chitosan + anise oil	140[a]	205[a]
Zein + anise oil	57.5[a]	65[a]
Control treatment	1025[b]	1125[b]

Values bearing different lowercase letters in the same column are significantly different ($p < 0.05$).

3. Results and Discussion

The results of the analysis of variance for the total number of microorganisms are shown in Table 2. There was a significant difference in the number of microbes between treatments. Temperature and shelf time have a greater impact on microbial growth than other factors.

The greatest number of microbes was observed three months after storage. Thereafter, a significant decrease in the microbial count was observed, due to the high moisture content of the crop three months after storage (Table 3).

Among the treatments, the lowest microbial count was zein treatment and the highest was belong to control one (Table 4). This treatment has been able to reduce the number of microbes by 30 times compared to control treatment.

In the second year of storage, there was a significant difference between the treatments in terms of total count and number of molds and yeasts (Table 5).

As it can be seen in the table below (Table 6), the lowest number of microbes is belonging to chitosan + anise oil and zein treatment.

In general, a microbial investigation of Mazafati dates, which were studied at various stages of storage, showed the presence of different species of *Aspergillus*, *Alternaria* and *Rhizopus* fungi. Species of *Aspergillus*, *Rhizopus*, and *Penicillium* have been identified by other researchers in date cultivars (Salajegheh, 2013; Golshan Tafti et al., 2019). The abundance of *Aspergillus niger* and *Aspergillus flavus* in Rotab may be due to the high concentration of sugar in the product. This species of fungus is osmotolerance and can grow in dense sugar environments. Mazafati Rotab has high sugar content and an almost neutral pH value, which is why it is invaded by a variety of microorganisms, especially molds and yeasts. Some researchers attribute the growth and activity of fungi in date cultivars to high levels of reducing sugar and non-reducing sugars (Salajegheh, 2013). However, internal factors in Mazafati Rotab (high humidity, pH close to neutral) along with the increase in relative air humidity during ripening and harvesting allow Rotab Mazafati to rot and acidify. Research has shown the role of *Aspergillus fungi*, especially Niger, in rotting and lactic acid bacteria in Ratab Mazafati acidosis. Golshan-Tafti and Fooladi (2006) stated that the rotting of the Mazafati Rotab begins under the cap and its margins, and after the flesh of the fruit is crushed, it turns black around the core. After three months of storage, high fungal contamination of the Mazafati fruits was reported. However, high microbial and fungal counts at this stage of storage are due to the presence of nutrients and moisture which are suitable for the growth of microorganisms. Edible protein-based coatings such as chitosan and zein effectively increase durability and preserve nutrients and ingredients amount in fruits (Salajegheh et al., 2017; Vahedikia et al., 2019). For example, the use of edible coatings to increase the shelf life of fresh and frozen strawberries and raspberries to 2 °C and 88% relative humidity for three weeks or negative temperatures of 23 °C for six months has been recommended (Han et al., 2004). The results of this study also indicate that the coated date fruit has increased shelf life three times. In addition to the microbial results mentioned above, the samples were also examined in terms of fungi. *Aspergillus niger*, *Aspergillus Flaus*, *Rhizopus stolonifer* and *Alternaria alternata* in samples of dates coated with Zein and Chitosan and their combined treatment with anise oil were observed. However, in the control treatment, in addition to the above fungi, the fungi *Penicillium Italicum*, *Penicillium expansum* and *Aspergillus* were observed with three higher logarithmic cycles. The identification of these fungi was confirmed by the Iranian Research Institute of Plant Protection of the Agricultural Research, Education and Extension Organization (AREEO).

3.1. *Aspergillus niger* (*A. niger*)

Fungal colonies were observed on the CZA culture medium in black with dense conidiophores. The conidia were radially placed on the vesicle. The conidiophore was seen with a smooth, colorless wall, and the vesicle was hemispherical, measuring 100-50 micrometers. The conidia were brown in color, semi-spherical in shape, and 3.5-5.5 mm in diameter (Fig. 1).

3.2. *Aspergillus flavus* (*A. flavus*)

Fungal colonies appeared on a yellowish-green CZA culture medium. The conidia were radial and placed on the vesicle. The

conidial cell was seen as a single-row, double-row conidiophore with a rough, colorless wall and a spherical vesicle with a diameter of 25 to 45 micrometers. The conidia were barbed and semi-spherical with a diameter of 3.5 micrometers (Fig. 2). Sclerotia were also seen in some isolate.

3.3. *Alternaria alternata* (*A. alternata*)

The fungus colonies grow rapidly on the PCA culture medium with a gray to olive-green appearance and in powder form. Conidiophores were often unbranched with one or more condensate sites larger than 50 micrometers and 6-3 micrometers wide. The conidia were stick-shaped to elliptical with a short beak measuring 8-17 × 23-56 micrometers (Fig. 3).

3.4. *Rhizopus stolonifera* (*R. stolonifera*)

The fungus was on a white cotton PPA culture medium that turned dark brown over time. At the nodes, there was a long sporangiosphyroid rhizoid with a length of 1000-2000 micrometers and a width of 13-125 micrometers. Columella with a length of 70-90 micrometers, round and egg-shaped angiospor spores with dimensions of 10-20 × 7-8 micrometers and round and egg-shaped zoospores with a size of 160-220 micrometers were observed. The microscopic image of the mentioned fungus is shown in Fig. 4.

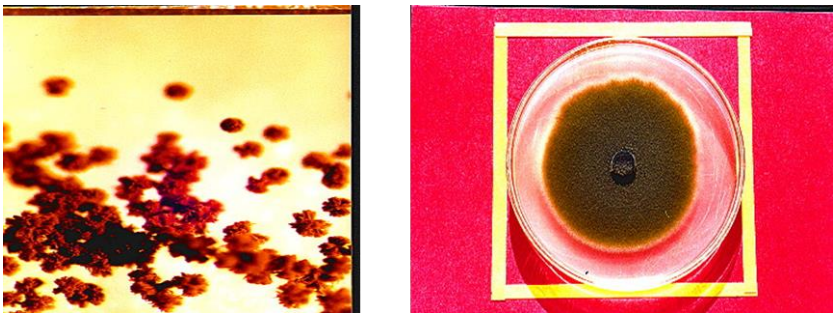

Fig. 1. *Aspergillus niger* fungus.

Fig. 2. *Aspergillus flavus* fungus.

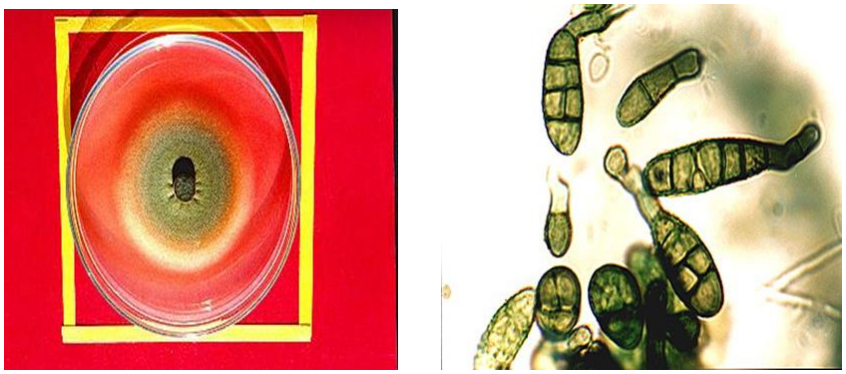

Fig. 3. *Alternaria Alternate* fungus.

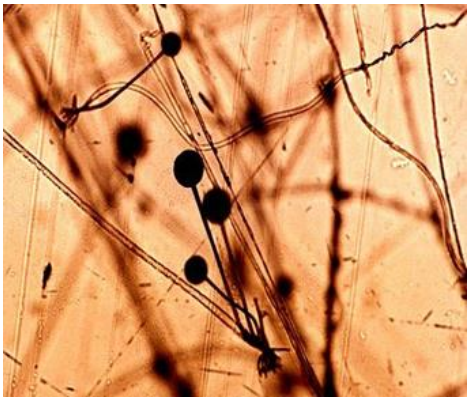

Fig. 4. *Rhizopus stolonifer* fungus.

4. Conclusion

When examining the microbial contamination of Mazafati date fruit in the Rotab stage in terms of a total number of microorganisms and fungi, fungi of *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus stolonifer* and *Alternaria alternate* were observed under different treatments of this study. The highest number was related to *Aspergillus niger*. In other words, different treatments were able to stop the growth of other microbes. But the *Aspergillus niger* fungus grew to a small extent. There was a significant difference between the treatments in terms of the number of microbes. Thus, temperature treatment and storage time had a greater effect on the increase in the number of microbes than other factors. The effect of storage time on the total number of microorganisms at a probability level of one percent was significant. Also, the sampling stage and the interaction of the two were significant at the level of fungal contamination at the probability level of one percent, while the year and its interaction were not significant at the level of fungal contamination. The highest number of microbes was observed in the three months after storage and then there was a significant reduction in the number of microbes, which is due to the high moisture content of the product at this stage. Among the treatments, the lowest microbial count was zein treatment and the highest was control one. This treatment was able to reduce the number of microbes compared to the control treatment in the three logarithmic cycles. The results of the analysis of variance and the mean comparison in the second year also show that the smallest number of microbes grown in zein treatment and the largest number in the control sample. There was a significant difference in the analysis of variance between treatments. The average total number of microorganisms in the Mazafati fruit during storage was in the range of 1.50-4.7 log cfu/g. The highest total number of microorganisms was observed in samples three months after storage. The average total number of fungi and yeasts in Mazafati fruits sampled from dates treated at different periods studied was found in the 0.17-1 log cfu/g range. In general, zein has been recognized as the best treatment either alone or in combination with anise oil in terms of microbial test results.

Internal factors in Mazafati dates and increased relative humidity during ripening and harvesting can lead to rotting and rancidity of the Mazafati dates. Rot and rancidity are among the factors that spoil Mazafati Rotab, leading to a decrease in marketability and an increase in waste. Microorganisms, especially fungi and some bacteria, play a role in rotting and spoiling the crop. Given the importance of Mazafati Rotab and its high production and consumption in the country, comprehensive studies on effective methods for removing fungal contaminants from Mazafati Ratab using physical methods, and studies on the role of lactic acid bacteria in preventing the growth and activity of aflatoxin-producing fungi and aflatoxin toxins in date fruits should be carried out.

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Conflict of 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.

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