

The effect of gelatin films containing *Pimpinella anisum* essential oil on the microbial and chemical properties of minced beef

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ABSTRACT

The use of films with natural origins and natural antimicrobial agents has become one of the novel methods of food packaging. In this study, the antibacterial activity of active films against two microorganisms, *Staphylococcus aureus* (ATCC 65138) and *Escherichia coli* O157: H7 (ATCC 25922) were evaluated by disk diffusion method. Also, the antimicrobial properties of gelatin film containing different concentrations of *Pimpinella anisum* essential oil (0, 0.3, and 0.6 and 0.9%) were studied on minced beef at refrigerator temperature. The treatments were stored for 8 days after packaging and the microbial (total count of aerobic bacteria, psychrophilic bacteria, *Pseudomonas* spp., *Enterobacteriaceae* and mold/yeast), chemical (TVB-N and peroxide value) and sensory (color, odor, and taste) properties were examined. The main components of the essential oil were trans-anethole (87.39%), (Carvone 8.79%), and Hexadecanoic (2.26%), Heptadecane 1.56%). Results showed that all microbial and chemical properties related to treatments containing antimicrobial agents were significantly ($p < 0.05$) less than the control treatment. Considering the acceptable microbial limit for minced beef at refrigerator temperature, the shelf-life of treatments wrapped with films containing essential oils increased by at least 3 days without any adverse without any unfavorable organoleptic properties.

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1. Introduction

Meat is very sensitive to microbial corruption because of its specific chemical and physical properties (aw, pH, and nutrients). The most common way of meat storage is keeping in the refrigerator. In this situation, the growth of different microorganisms, especially all types of psychrophilic and also pathogenic microorganisms is possible (1-3). Therefore, active packaging systems are now designed not only to protect food from environmental factors but also to prevent and delay microbial and chemical degradation and increase the shelf-life of the product (4). Edible films and coatings are a thin layer of food stuffs that may be based on biopolymers such as proteins, polysaccharides, lipids, or a combination of them. Over the past two decades, it has attracted a lot of attention due to its widespread uses, low cost, high bio-availability, and biodegradability properties (5). In fresh or processed products,

microbial contamination with the highest density is found on the surface and requires a system that controls the growth of microorganisms. The direct addition of antimicrobial agents to foods can limit its antimicrobial effect, as different food contents may reduce the effect of these antimicrobial agents. The use of films as an active packaging is more beneficial than the use of adding antimicrobial agents directly in foods because in this case, antimicrobial agents can slowly and selectively migrate from the active compounds of the film to the surface of the food (6). Anise by the scientific name of *Pimpinella anisum* is from the *Umbelliferae* family. Anise is a herbaceous, annual plant, with white flowers, and its main consumed part are seeds, which is a green, small and pear-shaped seed which grows in Turkey, Iran, India, Egypt, as well as European countries such as Spain, Italy and Germany, and also South America (7, 8). Researchers have reported that the main components of the essential oil of this plant include

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anethole, eugenol, methylchavicol, anisaldehyde, and estragole (9). There are also various reports about the antimicrobial and anti-spoilage activity of this essential oil against pathogenic bacteria. The researchers reported that the essential oil has antimicrobial properties against some microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Micrococcus luteus* and *Salmonella Typhi* (6, 10). The aim of this study was to investigate the effect of gelatin films containing anise essential oil on the microbial and chemical characteristics of minced beef in refrigerator temperature.

2. Materials and methods

2.1. Preparation of the plant and extraction of essential oils

Anise fruit was collected from the countryside of Bandar Abbas (Hormozgan province) at the end of spring and after identification by experts of the Faculty of Agriculture, University of Tehran. The essential oil was extracted by the Steam distillation method using a Clevenger apparatus, and dewatered by centrifugation at 2500 rpm for 5 minutes. Anhydrous sodium sulfate (MERCK, GERMANY) was added and re-prepared in the centrifuge (Optima, BHG 500, Germany) under the same conditions, then the essential oil analysis was performed by (GC/MS) (Hewlett Packard (HP) 6890).

2.2. Preparation of film based on gelatin

A 4% gelatin solution was prepared with sterilized distilled water on a magnetic stirrer at 50°C for 30 minutes. The solution was dissolved for 15 minutes in Ben-Marie at 80°C. The solution was then cooled to 40 °C and then anise essential oils (0, 0.3, 0.6, and 0.9%) with 0.25% tween 80 and Glycerol (5% v/v) were added to the solution. The film solution was homogenized using ULTRA-TURRAX homogenizer for 2 minutes at 12,000 rpm. A film-forming solution was degassed using a vacuum pump. After that, 50 ml of these solutions were poured into glass plates of 10 cm in diameter and dried in the ambient temperature under the safety cabinet (11).

2.3. Preparation of samples and packaging

The beef was purchased from the slaughterhouse and was immediately transferred to the laboratory in the vicinity of the ice. The meat was minced by a Grinder machine. The amount of 100 g samples were packed in films. They were kept in sterile stomacher bags at refrigerator temperature (4°C) for subsequent tests. A blank control sample without film was also provided. The minced meat was kept refrigerated (4°C) for 8 days. On days 0, 1, 3, 5, and 8, the microbial and chemical evaluation was performed on minced meat.

2.4. Microbial analysis

2.4.1. In vitro antibacterial activity of active films

In this study, the antibacterial activity of active films against two microorganisms, *Staphylococcus aureus* (ATCC 65138) and *Escherichia coli* O157: H7 (ATCC 25922) were evaluated by disk diffusion method. The amount 1×10^7 CFU/ml of each bacteria was cultured on Muller-Hinton agar. Then, circular discs (1cm diameter) were placed in the center of inoculated plates. Then, plates were placed at 37°C. The diameter of the inhibition zone was measured with a digital micrometer and also measured by Image 1.47 software for digital photo analysis (12).

2.4.2. Total counts of aerobic bacteria

In this method, 10 g of specimens were mixed with 90 cc solution of 0.1% peptone water in a stomacher. Different dilutions were made and cultured in PCA plates. Then plates were incubated at 30°C for 72 hours.

2.4.3. Psychrotrophic bacteria count

It was applied like a total count. Only incubation was carried out at 7°C for 10 days.

2.4.4. Enterobacteriaceae count

To determine the number of *Enterobacteriaceae*, the culture method was used on the VRBGA medium. The plates were incubated for 24 hours at 37°C incubator.

2.4.5. Pseudomonas spp. count

Pseudomonas isolation agar was used to count *pseudomonas* species. For this purpose, after preparing the dilution series, they were cultured on a surface of *Pseudomonas* isolation agar and incubated at 37°C for 24 hours.

2.4.6. Mold and yeast count

In this method, after preparing the appropriate dilution series, and incubate the DRBC medium for 5 days at 22°C.

2.5. Chemical analysis

2.5.1. pH measurement

10 g of the sample was mixed in 50 ml distilled water and homogenized and measured its pH using a digital pH meter.

2.5.2. Lipid oxidation evaluation

10 g of specimens were weighed in a glass Erlenmeyer flask. They were heated to 60 °C for 3 minutes in a water bath to allow its fat to melt. 30 ml of acetic acid and chloroform (v/v 3:2) solution was added to this mixture. The solution was stirred for 3 minutes to dissolve the fat. This solution was

filtered and 0.5 ml of a saturated solution of potassium iodide was added. Then titrated with 25 ml standard sodium thiosulphate solution. The peroxide number was calculated using the following formula (13).

2.6. Sensory evaluation

The sensory evaluation of samples was done by 6 trained referees. To evaluate the samples, the treatments were cooked to 60 ° C and then cooled. The samples were 3 digits coded and provided to the referees along with the 0 to 5. (14).

2.7. Statistical analysis

In this study, SPSS 22.0 software was used to analyze the data. For analyzing and comparing the groups, one-way ANOVA and Duncan tests were used to compare means.

3. Results and discussion

3.1. Chemical components of Anise seeds

After identification of the essential oil compounds, using gas chromatography, mass spectrometry, four different organic compounds were identified that they were 98.64% of them. Among the compounds identified, trans-anethole (87.39%), (Carvone 8.79%) and Hexadecanoic (2.26%), Heptadecane 1.56%) formed the main components of the essential oil (Table 1). In the study of Ozcan and Chalchat (15), the major components of anise essential oil were trans-anethole (9.93%) and estragole (4.2%), whereas other compounds with a concentration of 0.06% included E-methyleugenol, α -cuparene α -himachalene β -bisabolene p -anisaldehyde and cis-anethol. In the study of Orav et al. (16), this essential oil in addition to the main compounds had trans-anethole (76.9-7.73%) and γ -himachalene (8/2-0/4%) trans-pseudoisoeugenyl 2-methylbutyrate p -anisaldehyde methylchavicol. Also, Ullah et al. (17), reported trans-anethole (82.1%), and γ -hemiactalene, trans-pseudoisoeugenyl, 2-methylbutyrate, p -anisaldehyde, and methylchavicol (2.8-4 %) as essential components of the essential oil of this plant, and Abdel-Reheem (10) reported The main components of the anise essential oil as follows: Trans-anethole (82.1%), cis-anethole (5.8%), estragole (methylchavicol) (2.5%), linalool (2.3%), aterpineol (1.5%) and methyl eugenol (1.3%). This difference in the chemical composition of the essential oils may be due to differences in factors such as plant species, plant age, used part of the plant for extraction of essential oil, soil type, weather conditions, harvest time, plant growth zone, and extraction method, etc. (18).

3.2. Antimicrobial activity of films

As shown in Table 1, the inhibition zone was observed for *Staphylococcus aureus*, and with the increase in the essential oil content, the diameter of the halo was also increased, there was no halo formed in the control treatment, while in the

concentration of 0.9% of anise essential oil, the highest diameter in non-growth halo has been observed and had a significant difference with lower concentrations of essential oil. Several studies have been carried out on the antimicrobial activity of this essential oil. For example, Al-Bayati (7) evaluated the antimicrobial activity of methanolic extract and essential oil of anise seeds against *Salmonella typhi*, *Salmonella typhimurium*, *Proteus mirabilis*, *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus cereus* microorganisms by determination method of Minimum Inhibitory Concentration (MIC) and reported the range from 62.5 to 15.56 μ g/ml. Also, Abdel-Reheem (10), evaluated the antimicrobial activity of Anise seeds essential oil against gram-positive and gram-negative microorganisms including *Staphylococcus aureus* (15 mm), *Escherichia coli* (15 mm), *Pseudomonas aeruginosa* (10 mm), *Candida albicans* (10 mm) *Streptococcus pyogenes* (12 mm), *Enterococcus faecalis* (16 mm), *Micrococcus Luteose* (14 mm), *Salmonella typhi* (17 mm) by diffusion disc method and minimum inhibitory concentration of growth of 4-2 μ g/ml have been reported. The reasons for these slight differences in the amount of antimicrobial activity of essential oil were discussed in the previous section. The antimicrobial results of anise essential oil showed high inhibitory potency on gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*). It was also observed that this essential oil has a relatively weak inhibitory effect on the gram-negative bacteria O157: H7. One of the reasons for this fact is the difference in the structure of the cell wall of gram-positive and gram-negative bacteria (19). In this study, inhibition of growth in *Staphylococcus aureus* was observed. By increasing the concentration of essential oil, the diameter of the halo also increases. In the control treatment, no halo was formed, while in the concentration of 0.9% of the anise essential oil, the highest diameter of the inhibition zone was observed and there was a significant difference with the lower concentrations of the essential oil. Also, in *E. coli* cultivation, there was no incidence of growth that was consistent with the results of (12).

3.3. Microbial results of samples

3.3.1. Total aerobic bacteria

The increase of growth of bacteria in the control sample on day 8 was 5.15 log CFU/g when the total bacterial count in the treatment wrapped by the film at a concentration of 0.9% essential oil was 1.9 log CFU/g and less than the total count of aerobic bacteria in the control treatment on the final day of the test. Although the lowest count of aerobic microorganisms after 8 days of storage is related to the mentioned treatment, the difference with the number of treatments packed by gelatin film containing 0.3% essential oil is not significant. But in the treatment containing 0.9% essential oil, there was a significant difference from the earliest days and throughout the maintenance period, with other concentrations of the essential oil. The results showed that the count of aerobic bacteria in the

processed meats treated with gelatin films containing 0.3, 0.6, and 0.9% Anise essential oil was significantly ($p < 0.05$) less than the count of aerobic bacteria in the control treatment during all days of the experiment (Fig. 1).

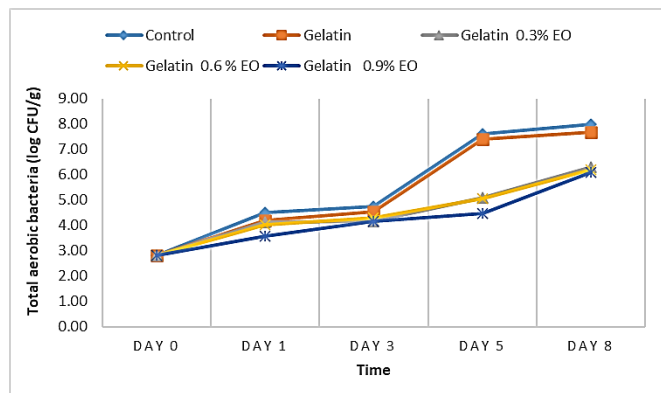


Fig 1. Results of total count of aerobic bacteria (log CFU/g) in minced meat wrapped with gelatin films containing different concentrations of Anise essential oil (EO) stored at 4°C.

3.3.2. Psychrotrophic bacteria

The results showed that the growth rate of psychrotrophic bacteria from day zero to the last day has increased in all conditions (Fig. 2). The growth rate of bacteria in the control sample on day 8 is 4.96 log CFU/g which has increased compared to day zero. Also, the count of psychrotrophic bacteria in treatments packed with gelatin films containing 0.9% of anise essential oil had the lowest microbial population on the final day of the test, which was 2.04 log CFU/g less than the count of psychrotrophic bacteria in the control treatment on the final day. The use of gelatin film in the package did not significantly reduce the growth of the psychrotrophic bacteria compared to the control sample at all days of the study, but adding of the essential oil makes a significant difference. But the difference in growth rate indicates that the increase of essential oil concentration from 0.3% to 0.9%, makes no significant decrease in psychrotrophic growth. With increasing concentrations of essential oil from 0.6% to 0.9%, growth reduction was observed compared to the treatment packed in gelatin film containing 0.6% essential oil, but this difference was not significant. The total count of aerobic bacteria (Fig. 1), as well as the counting of psychrophilic bacteria (Fig. 2), is considered as an indicator of the corruption of meat and seafood products (20, 21). The result of this study shows that the total count of aerobic bacteria during the use of gelatin film with essential oil has a significant reduction compared to the control treatment. These results are consistent with studies by Ahmad et al. (22) and Bao et al. (23). The results show that the total count of aerobic bacteria in the treatment containing gelatin films was similar to the control treatment. The use of gelatin did not make a significant difference. But adding essential oils can slow down the growth rate. By increasing the concentration of essential oil up to 0.6% not much difference was observed. However, with increasing concentrations up to

0.9% on the third day, the difference in growth was significant.

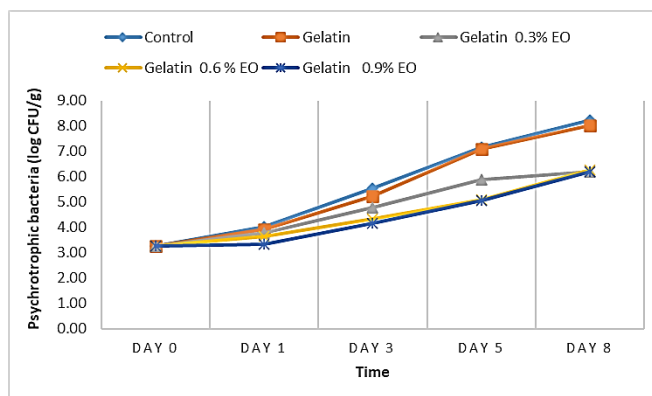


Fig. 2. The results of psychrotrophic bacteria count (log CFU/g) in minced meat wrapped with gelatin film containing different concentrations of Anise essential oil (EO) stored at 4°C.

3.3.3. Enterobacteriaceae

The results showed that the growth rate of *Enterobacteriaceae* family increased in all conditions from day zero to last day (Fig. 3).

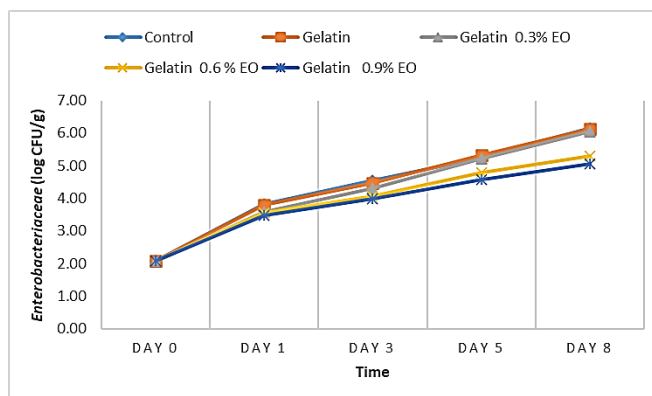


Fig. 3. The results of *Enterobacteriaceae* (log CFU/g) counting in minced meat wrapped with gelatin film containing different concentrations of Anise essential oil (EO) stored at 4°C.

The growth rate of the bacteria in the control sample on day 8 was 0.47 log CFU/g compared to day zero. The results showed that counting of *Enterobacteriaceae* family in minced meat treatment with gelatin films containing 0.3, 0.6, and 0.9% Anise essential oil was significantly ($p < 0.05$) lower than the count of *Enterobacteriaceae* family in the control treatment. The results of *Enterobacteriaceae* family counting in treatment packed in the film containing 0.9% essential oil showed that increasing concentration from 0.6% to 0.9% caused a significant decrease in the growth only on the eighth day. The count of *Enterobacteriaceae* in treatments packed with gelatin films containing 0.9% anise essential oil had the lowest microbial population on the final day of the test and was 0.91 log CFU/g less than the count of *Enterobacteriaceae* family in the control treatment on the final day of the test.

3.3.4. *Pseudomonas* spp.

The results showed that the count of the initial count of *Pseudomonas* spp. was 2.86 log CFU/g and reached to 6.53 log CFU/g on the 8th day of storage. But the *Pseudomonas* spp. count in the samples wrapped with gelatin film containing 0.6 and 0.9% essential oil was 4.95 and 4.77 log CFU/g at the end of storage, respectively. In the first and third day of the study, the treatments with gelatin films had a significant decrease in pseudomonas count in comparison with control treatment. On other days, this reduction was not significant. The use of Anise essential oil (0.9%) in gelatin film did not make a significant decrease in the growth of pseudomonas in minced meat compared to 0.6% essential oil (Fig. 4).

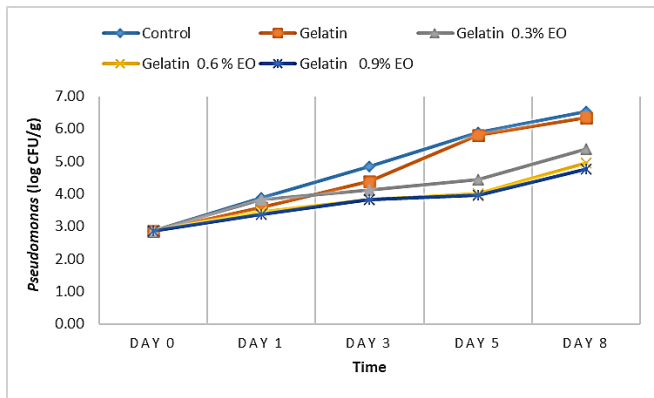


Fig. 4. *Pseudomonas* bacteria count (log CFU/g) in minced meat wrapped with gelatin film containing different concentrations of Anise essential oil (EO) stored at 4°C.

3.3.5. Mold and yeast

The count of mold and yeast in the control sample at day 0 was 2.46 and reached to 6.78 log CFU/g on day 8. The results showed that the growth rate of mold and yeasts have increased in all samples from day zero to the last day. The use of gelatin film in minced meat packaging did not decrease the growth of mold and yeast in comparison with the control sample. Adding 0.3% of the essential oil to the film did not significantly affect the growth of mold and yeast until the third day of the film. But from the fifth day, there was a significant difference in the number of counts compared to the gelatin film sample and the control sample. The addition of 0.9% essential oil in the final days of the study (day 5 and day 8) caused a significant decrease in the growth compared to the treatment containing 0.6% essential oil so that the lowest number of mold and yeast was related to this treatment on the fifth and eighth day (Fig. 5). Kosalec et al. (24) examined the anise essential oil activity on 7 yeast species and 4 species of dermatophytes. They showed that the anise extract can prevent the activity of *Candida* family fungi. Also, in other studies, the inhibitory effect of this essential oil on *Aternaria alternata*, *Aspergillus niger*, and many other fungi is consistent with the results of this study. All microbial tests were modified with the same approach. Antimicrobial effects of gelatin films containing

Anise essential oil were well observed in all microbial factors studied. The same trend was observed for psychrophilic bacteria count. In the treatments containing antimicrobial agents were below the microbial limit until the last day of the test.

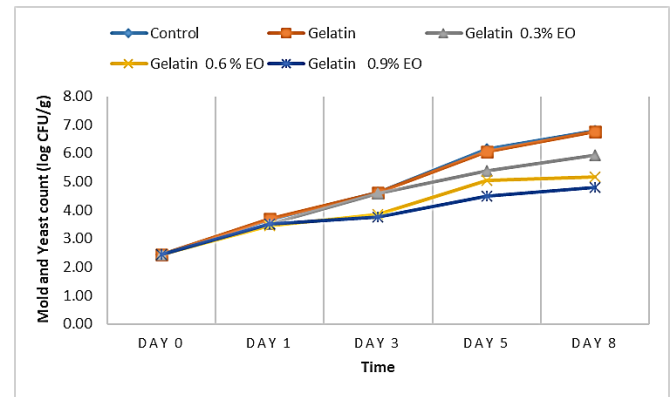


Fig. 5. Results of mold and yeast count (log CFU/g) in minced meat with gelatin film containing different concentrations of Anise essential oil (EO) stored at 4°C.

3.4. Evaluation of chemical factors

An increase in the amount of pH in all treatments is observed. The highest increase was observed in the control sample, ranging from about 6.01 to 7.12. The increase in pH in the control sample was significant in all test days. In the treatments containing gelatin film and gelatin film with 0.3% essential oil, the pH was also significantly increased in most days. Only in the fifth and third day of the study, the increase in pH was not significant. But in the gelatin films and samples wrapped with films containing anise essential oil at concentrations of 0.6% and 0.9%, the increase in pH was not significant during the study days. In the concentration of 0.6% and 0.9%, the increase in pH from day zero to the eighth day was 0.21 and 0.15, respectively (Fig. 6).

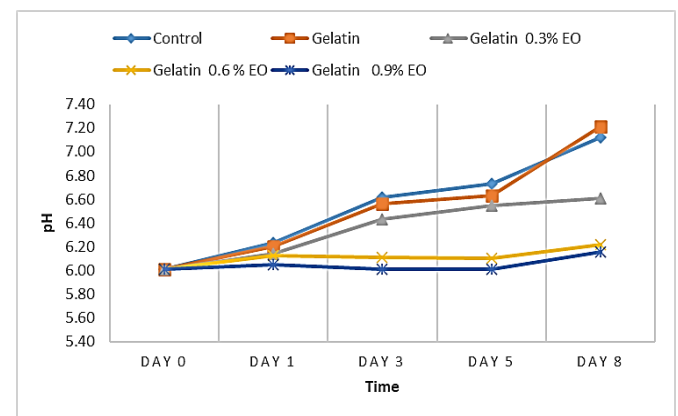


Fig. 6. Results of pH of minced beef wrapped with gelatin film containing different concentrations of anise essential oil (EO) stored at 4°C.

The amount of TVN in the control sample showed an increase of 16.73 mg/100g of zero to the last day. In the package of gelatin film containing 0.9%, the essential oil was 8.39 mg/100g. Adding 0.3% essential oil to gelatin film on day 3 and fifth showed a significant difference compared to the treatments containing only gelatin film. Increasing the essential oil concentration to 0.6% resulted in a significant difference only on the eighth day. There was no significant difference in 0.09% essential oil in with 0.6% essential oil gelatin films in any of the study days (Fig. 7).

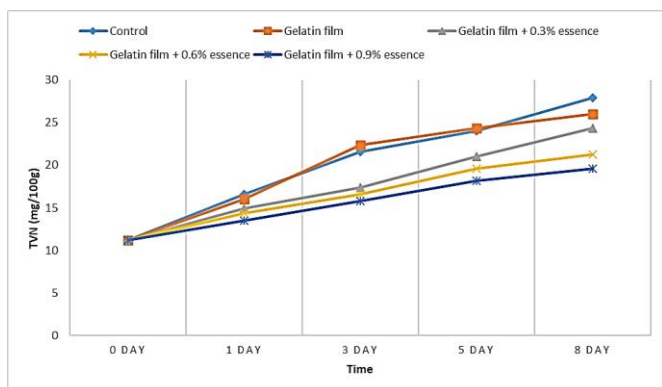


Fig. 7. TVN (mg/100g) results of gelatin film containing different concentrations of anise essential oil (EO) stored at 4°C.

3.5. Lipid oxidation assay

Based on the results, peroxide increased in all treatments during all study days. The amount of peroxide increased in the control sample from the beginning to the end of the study was 4.29 meq/1000g fat. The treatments packed with gelatin film containing 0.9% essential oil had the lowest amount of peroxide (3.3 meq/1000g fat) on the final day. On the first day of study, the use of gelatin and essential oil (0.3%, 6.0%) did not significantly affect the amount of peroxide. But the gelatin film treatment containing 0.9% essential oil was effective. On the third day, using gelatin film and 0.3% essential oil did not lead to significant differences in the amount of peroxide in these treatments. Adding 0.6% of essential oil made a significant difference with the control sample. On the fifth day, the same result was also visible. On the eighth day, in the treatment with gelatin film containing 0.6% essential oil and higher peroxide value, showed a significant difference with the previous treatments and the control sample (Fig. 8). Hydroperoxides are the primary products of oxidation of polyunsaturated fatty acids. They are broken in the second stage of oxidation of fatty acids to Secondary products such as aldehydes. Hydro-peroxides react with pigments and other products, causing color loss and unpleasant smells. The second stage of oxidation begins with the advent of carbonyl compounds (25). Malondialdehyde is produced from hydroperoxides. Its amount is measured by Thiobarbituric acid. Lipid oxidation in meat products is one of the reasons for the degradation of meat quality during storage. The presence of free radicals in meat leads to the formation of aldehydes.

These compounds are responsible for the taste of lipid degradation and changes in the color of the meat. Oxidation of lipids in meat has a complex mechanism. During this process, in addition to undesirable effects on taste and color, protein solubility also decreases. Finally, the product is dropping for nutritional value. For this purpose, compounds such as vitamins (alpha-tocopherol) are often added as antioxidants to the formulation of meat products (25). In the evaluation of taste and odor, the results showed that there was no significant difference in all samples. However, when they were stored with a gelatin film containing 0.6% and 0.9% essential oil, there was a significant difference with the control sample.

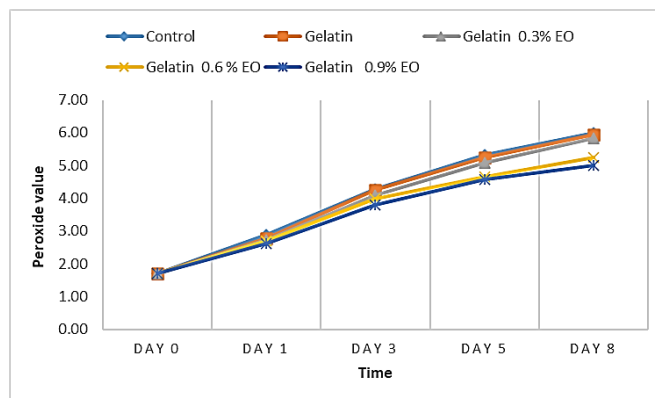


Fig. 8. Results of peroxide test with gelatin film containing different concentrations of anise essential oil (EO) stored at 4°C.

3.6. Organoleptic evaluation

Organoleptic results on the second day show that in all conditions, except for gelatin with 0.6% essential oil, there is no significant difference in flavor and odor compared to the control. There was a significant difference between the taste and taste of the samples in all of the samples compared to the control sample. When the essential oil was added to the gelatin film, there was no significant difference in taste and flavor compared to the treatment containing only gelatin film. Increasing the essential oil concentration to 0.6% resulted in increasing acceptance of flavor. The overall acceptance of films containing pure gelatin films, or films with essential oils was not significantly different from the control sample (Table 2).

4. Conclusion

The results of this study showed that the treatments with essential oil had an antimicrobial effect with a significant ($p < 0.05$) reduction compared to the control treatment after 8 days of storage and reduced the microbial count. Due to the acceptable microbial limit for beef minced meat at refrigerator temperature, the shelf-life of these treatments increased by at least 3 days. The study also showed that the use of anise essential oil and gelatin-coating had a synergistic effect on the shelf-life of beef minced meat. The results showed that concentrations of 0.6% and 0.9% did not differ in their effects.

It seems that the film containing Anise Essential oil can be used as an applicable coating in the meat industry.

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