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Antimicrobial Activity of Polylactic Acid Film Incorporated With Marjoram and Clove Essential Oils on Microbial and Chemical Properties of Minced Beef During Refrigerated Storage

Razieh Partovi[1*], Fazeleh Talebi[2], Amir Babaei[3], Aghil Sharifzadeh[4,5]

[1]Department of Food Hygiene, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran
[2]Department of Food Hygiene, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[3]Department of Polymer Engineering, Faculty of Engineering, Golestan University, Gorgan, Iran
[4]Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[5]Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

***Corresponding Author:**
Razieh Partovi, Department of Food Hygiene, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran
Tel: +98 11 44271057,
Fax: +98 1144271054,
Email:dvmr.partovi@ut.ac.ir

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Abstract

Background: Active packaging is one of the new packaging technologies which causes interaction between packaging material and food with the aim of food shelf life extension while maintaining food safety and quality. Biodegradable films like polylactic acid (PLA) can be good alternatives to non-biodegradable plastics because of environmental pollution and concerns about the limitations of petroleum resources.

Objective: This study was conducted to evaluate the efficacy of PLA film incorporated with marjoram and clove essential oils (EOs) (0.5 and 1% v/v) in maintaining the microbial and chemical quality of minced beef during refrigerated storage.

Materials and Methods: Minced beef was packaged with PLA film incorporated with marjoram and clove EOs (0.5 and 1% v/v) alone and in combination and stored at refrigerator temperature for 10 days. Then, microbiological and chemical analyses were done at 0, 2, 4, 7 and 10 days of examination.

Results: A reduction of 1 log CFU/g in total count was observed between groups with simultaneous use of EOs and control group ($P<0.05$) at day 7; however, there was not any significant difference between the mentioned groups at day 10. Active packaging with marjoram and clove EOs decreased the number of psychrotrophs in comparison to the control group and it was more evident at days 7 and 10. The number of Enterobacteriaceae in control and 1% clove EO/1% marjoram EO groups showed a difference of 3 log units at day 10. TVB-N of 1% clove EO/1% marjoram EO and 0.5% clove EO/1% marjoram EO showed significant differences from control at day 10 ($P<0.05$).

Conclusion: The results of the current study have shown that the active PLA films can be a promising approach in order to maintain microbial and chemical quality of minced beef at refrigerator temperature for 10 days.

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Background

Biodegradable films can be good alternatives to non-biodegradable plastics because of environmental pollution and concerns about the limitations of petroleum resources.[1-3] Polylactic acid (PLA) is a biodegradable polymer produced by carbohydrate fermentation and lactic acid polymerization. PLA has a rigid structure with micro or nanospheres and porous scaffolds with considerable tensile strength. Moreover, PLA is resistant to oil and water vapor and has low gas transmittance. PLA is a non-toxic and affordable polymer with good forming ability (plasticity), high transparency and good mechanical and thermal properties.[4,5] Hydrophobic nature of PLA film does not decrease the antimicrobial activity of Essential Oils (EOs) due to hydrophobic properties of EOs.[1]

Active packaging is one of the new packaging technologies which causes interaction between packaging material and food with the aim of food shelf life extension while maintaining food safety and quality.[6,7] Antimicrobial

active packaging as a kind of active packaging inhibits microbial growth, thereby preventing food spoilage and food poisoning.[6,8] Antimicrobial compounds would be more powerful when incorporated in a film than when contacted directly with food. The use of active packaging would be embedded in the antimicrobial compound, which could prevent it from evaporation or diffusion.[9] A number of studies were done on the food packaging with PLA film incorporated with antimicrobial agents.[10-13] The antimicrobial activity of plant EOs have been proved in numerous studies.[14,15] The use of EOs in food packaging can be effective in maintaining the biological activity of EOs for a long time.[16] Using antimicrobial agents in combination can increase antimicrobial activity and decrease unfavorable organoleptic effects.[17]

Caryophyllus aromaticus (clove) belongs to Myrtaceae family which has been used as a traditional dental medicine, antimicrobial additive, and flavoring agent. Eugenol is the most important component of *C. aromaticus* EO with strong biological effects such as antimicrobial, insecticidal, antioxidant, antiseptic, anti-vomiting, anti-carminative, analgesic, antispasmodic, and anti-phlogistic activities.[18,19] Some researchers have reported the antimicrobial activity of clove EO against *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Salmonella enteritidis*, *Bacillus cereus, Staphylococcus epidermidis*, and *Micrococcus luteus*.[20-22] Antimicrobial activity of EOs is attributed to the inhibition of nucleic acid synthesis, disturbance of the cytoplasmic membrane, and energy metabolism.[23] Clove EO, alone or in combination with other EOs and antimicrobial agents, has been used in food packaging technology which extended food shelf life.[24-26]

Origanum majorana (marjoram) belongs to Lamiaceae family. *O. majorana* EO is used in cosmetics and medications and as a flavoring compound in food. Major compounds of marjoram EO are carvacrol and thymol.[14] The antimicrobial effect of *O. majorana* EO against *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, *Brochothrix thermosphacta*, *Pseudomonas fluorescens* and *Listeria innocua* has been reported by some researchers.[27,28] The ability of EOs to increase the cell membrane permeability and induce the leakage of macromolecules and lysis determines their antimicrobial activity.[29] *O. majorana* EO, alone and in combination with other EOs and antimicrobial agents, have been used as food packaging ingredients to improve qualitative properties of food.[30,31]

The authors have assessed the chemical composition of *C. aromaticus* and *O. majorana* EOs and their antimicrobial activity against some food-borne bacteria previously.[14] The aim of this study was to assess microbial and chemical properties of minced beef packaged with PLA films incorporated with *C. aromaticus* and *O. majorana* EOs at refrigerator temperature for 10 days.

Materials and Methods

Preparation of Active PLA Films

PLA films were produced by solvent casting technique. PLA granules (FkuR Kunststoff GmbH, Germany, molecular weight: 197 000 g/mol, density: 1.3 g/cm^3) were solved in chloroform to reach 1% (w/v) solution. The solution was stirred on a magnetic stirrer for 8 hours at room temperature. *C. aromaticus* and *O. majorana* EOs used in the current study were isolated and identified by gas chromatography–mass spectrometry (GC-MS).[14] *C. aromaticus* and *O. majorana* EOs (0.5 and 1% v/v) were added to the solution and homogenized at 8000 rpm for 2 minutes (IKA T 25 digital ULTRA-TURRAX, Germany). Afterwards, 50 mL of the solution was poured into glass petri dishes with 8 cm diameter and dried overnight at room temperature in a biological safety cabinet.[32] Then, the films were removed from the petri dishes and placed in a silica gel desiccator until use.

Preparation of Meat Samples and Treatments

The minced beef from hindquarter muscle was purchased from a local slaughterhouse in Amol, Iran, and transferred to the laboratory at refrigerator temperature within 30 minutes. Then, the minced beef was divided into 25-g samples under aseptic condition and packaged with PLA films containing 0.5 and 1% v/v *C. aromaticus* and *O. majorana* EOs alone and in combination. The samples were then completely placed in permeable polyethylene bags and stored in refrigerated incubators at 4°C for 10 days. The minced beef sample packaged without PLA film was used as a control.

Microbiological Analysis

At 0, 2, 4, 7 and 10 days of examination, 10 g of each sample was homogenized with 90 mL of 0.1% peptone water in a stomacher for 2 minutes (stomacher 400, Interscience, France). A ten-fold serial dilution was made, cultured and then incubated at a certain temperature.[12] Selective media and incubation condition were as follows: plate count agar for total bacterial count (TBC) at 37°C for 48 hours,[33] plate count agar for psychrotrophic bacteria (PSY) at 7°C for 10 days,[34] Man Rogosa Sharpe agar (MRS) for lactic acid bacteria (LAB) at 30°C for 48 hours,[35] Violet Red Bile Glucose (VRBG) agar for Enterobacteriaceae at 30°C for 24 hours, and Baird Parker agar for *Staphylococcus aureus* at 37°C for 48 hours.[35,36] Microbiological results were reported as logarithms of colony-forming units per gram (log CFU/g) of minced beef. All culture media were supplied from Merck (Merck, Germany).

Chemical Analysis

Total volatile base nitrogen (TVB-N) content of meat samples was determined according to the method of AOAC.[37] To this end, 10 g of the samples was used and the

results were expressed as mg nitrogen/100 g minced beef.

Statistical Analysis

All experiments were conducted in triplicate. All data were reported as mean ± standard deviations. Statistical analysis was performed using SPSS version 25.0 (SPSS, Chicago, IL, USA). All data were statistically analyzed by one-way analysis of variance (ANOVA) and multiple comparisons between treatments were done using Bonferroni test. For all analyses, $P<0.05$ was considered statistically significant.

Results

Microbiological Analysis

The microbial changes of minced beef packaged with PLA film incorporated with different concentrations of *C. aromaticus* (0.5% and 1%) and *O. majorana* (0.5% and 1%) EOs during 10 days of storage at refrigerator temperature are shown in Figure 1. Active packaging with PLA film incorporated with *C. aromaticus* and *O. majorana* EOs affected microbial groups in different ways. TBC of the control group was 3.8 log CFU/g at day 0 and reached 8.85 log CFU/g at day 10. TBC of all bacterial groups showed significant differences between days 7 and 4 and also between days 10 and 7 ($P<0.05$). TBC of minced beef samples decreased by adding *C. aromaticus* and *O. majorana* EOs to PLA films in a concentration-dependent way and this was more evident at days 4 and 7. TBC in groups with one EO did not show any significant differences from the control group ($P>0.05$). As shown in Figure 1, a difference of 1 log CFU/g in TBC was observed between groups with simultaneous use of EOs and the control group at day 7 ($P<0.05$); however, there was not any significant difference between the mentioned groups at day 10 ($P>0.05$).

Psychrotrophic bacteria with 9.33 log CFU/g in the control group was the dominant bacterial group in the minced meat at the final day of storage. Active packaging with *C. aromaticus* and *O. majorana* EOs decreased the number of psychrotrophs in comparison to control group, which was more evident at days 7 and 10. The number of psychrotrophs in most of the treatment groups showed a significant difference from control group at day 7 ($P<0.05$), while only groups with simultaneous use of EOs showed a significant difference from control group at day 10 ($P<0.05$).

The PLA films containing *C. aromaticus* and *O. majorana* EOs showed good inhibitory effects against *S. aureus* in treated minced meat at day 7. *S. aureus* count of all treated samples except for 0.5% *C. aromaticus* showed a significant difference from the control group at day 7 ($P<0.05$), while only groups with simultaneous use of EOs showed a significant difference from control group at day 10 ($P<0.05$). *S. aureus* counts in the PLA group and 1% *C. aromaticus*/1% *O. majorana* EOs were 5.32 and 3.34 log CFU/g at day 7, respectively ($P<0.05$), which showed

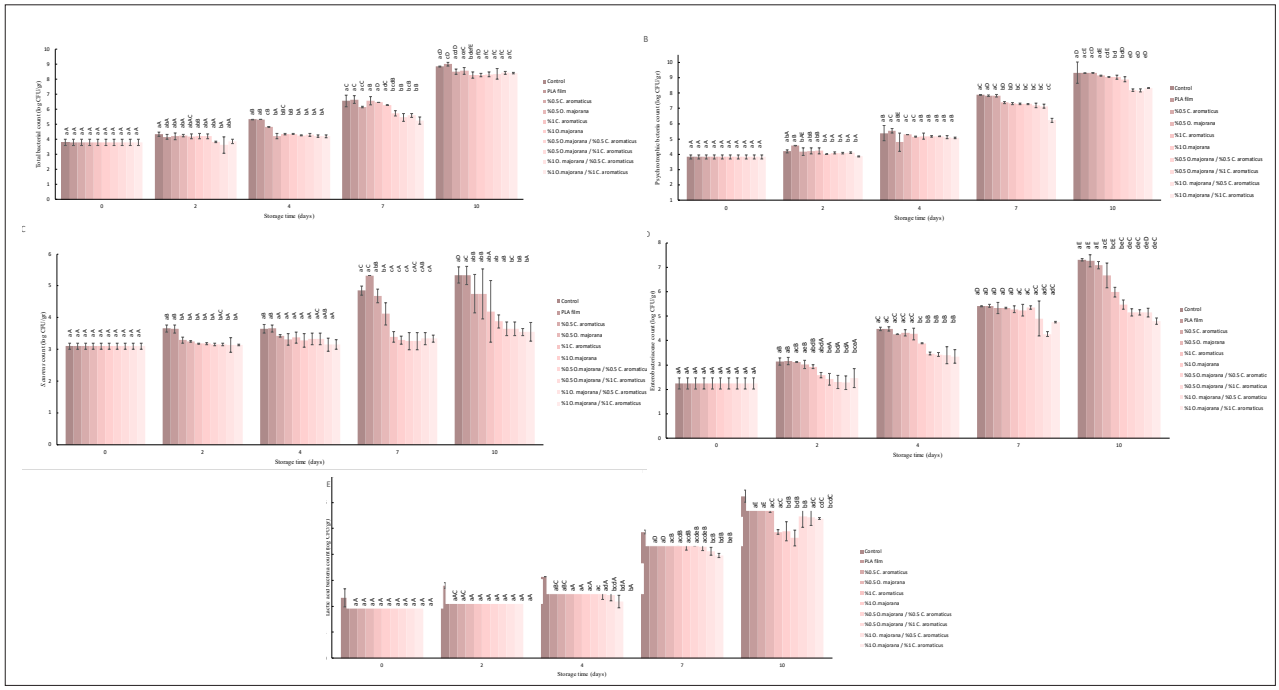

Figure 1. (A) Total Bacterial Count, (B) Psychrotrophic Bacteria Count, (C) S. aureus Count, (D) Enterobacteriaceae Count and (E) Lactic Acid Bacteria Count of Minced Beef with Active Packaging of PLA Film Incorporated with Different Concentrations of C. aromaticus and O. majorana EOs During 10 Days of Storage at Refrigerator Temperature. Bars show the mean ± SD (n = 3). Different lowercase letters indicate significant differences between treatments at the same storage time ($P<0.05$). Different uppercase letters indicate significant differences between storage times at the same treatment ($P<0.05$).

a difference of 2 log units.

The number of Enterobacteriaceae in the control group at day 0 was 2.25 log CFU/g. The number of Enterobacteriaceae showed a significant difference between all treatment groups except for 0.5% *C. aromaticus* and 0.5% *O. majorana* and control group at day 10 ($P<0.05$). The number of Enterobacteriaceae in PLA group and 1% *C. aromaticus*/1% *O. majorana* EOs at day 10 showed a difference of 3 log units ($P<0.05$).

LAB count of minced meat in control group was 2.33 log CFU/g at day 0 and reached 6.24 log CFU/g at day 10. LAB count showed a significant difference between all treatment groups except for 0.5% *C. aromaticus* and 0.5% *O. majorana* groups and control group at day 10 ($P<0.05$). The number of LAB in all groups showed significant differences between days 4 and 7 ($P<0.05$).

The results of the current study showed no significant differences in the in vitro antimicrobial effect of *C. aromaticus* and *O. majorana* EOs against foodborne bacteria.

PLA and control group did not show any significant differences regarding microbial count, indicating that PLA did not have antimicrobial effect.

Chemical Analysis

The effect of packaging with PLA film containing different concentrations of *C. aromaticus* (0.5% and 1%) and *O. majorana* (0.5% and 1%) EOs on TVB-N of minced meat at 10 days of storage at refrigerator temperature has been shown in Table 1. TVB-N values of the control group at day 0 and day 10 were 14.1 and 27.83 mg/100g, respectively ($P<0.05$). According to the results, in both control and treatment groups, TVB-N values increased during storage time while the increase was lower in treatment groups in comparison to the control group ($P<0.05$). TVB-N values of groups with simultaneous use of EOs showed significant differences from control group at day 7 ($P<0.05$), while only 1% *C. aromaticus*/1% *O. majorana* EOs and 0.5% *C. aromaticus*/1% *O. majorana* EOs showed significant differences from the control group at day 10 ($P<0.05$).

Discussion

TBC of minced meat at the first day is in agreement with some other studies.[38,39] The main compounds of *C. aromaticus* EO were eugenol and carvacrol and the major constituents of *O. majorana* EO were carvacrol, thymol, trans-caryophyllene, and cymene.[14] Antimicrobial properties of Eugenol and carvacrol have been proven in food matrix.[14,40-43] Moreover, the antimicrobial effect of *C. aromaticus* and *O. majorana* EOs were investigated as an ingredient of packaging films such as PLA, chitosan, alginate, and hydroxypropyl methylcellulose.[16,31,44,45] The synergistic, partial synergistic and additive interaction of the combination of *C. aromaticus* and *O. majorana* EOs against food-borne bacteria have been proven.[14] It seems that EOs combinations disrupt the membrane integrity, increase permeabilization of microorganisms and cause rupture and lysis of the membranes.[46] Talebi et al reported that PLA film incorporated with *Mentha piperita* and *Bunium percicum* EOs simultaneously decreased TBCs of ground beef in comparison to the control group ($P<0.05$).[12] The effect of polyethylene films containing rosemary and cinnamon EOs on the growth inhibition of microorganisms in Pacific white shrimp was reported previously.[47] In the current study, groups with simultaneous use of EOs showed a reduction of 1 log CFU/g in TBCs in comparison to the control group at day 7. Emiroğlu et al reported that TBC of fresh ground beef patties packaged with soy protein edible films incorporated with *Origanum heracleoticum* and *Thymus vulgaris* EOs was lower compared to the control group at days 3 and 6; however, this difference was not significant.[48] This was attributed to the lower effectiveness of antimicrobial agents in the complicated structure of ground beef patties. In the present study, PLA could provide a suitable environment for EOs to be released into food. Sánchez-González et al reported that antimicrobial

Table 1. TVB-N of Minced Beef with Active Packaging of PLA Film Incorporated with Different Concentrations of C. *aromaticus* and *O. majorana* EOs During 10 Days of Storage at Refrigerator Temperature

Storage Time (days)	Treatment									
	Control	PLA	PLA/ 0.5 CA	PLA/ 0.5 OM	PLA/ 1 CA	PLA/ 1 OM	PLA/ 0.5 OM/ 0.5 CA	PLA/ 0.5 OM/ 1 CA	PLA/ 1 OM/ 0.5 CA	PLA/ 1 OM/ 1 CA
0	14.1 ± 0.1^{aA}	14.1 ± 0.1^{aA}	14.1 ± 0.1^{aA}	14.1 ± 0.1^{aA}	14.1 ± 0.1^{aA}	14.1 ± 0.1^{aA}	14.1 ± 0.1^{aA}	14.1 ± 0.1^{aA}	14.1 ± 0.1^{aA}	14.1 ± 0.1^{aA}
2	15.33 ± 0.20^{aA}	15.26 ± 0.15^{aA}	15.26 ± 0.15^{aA}	15.23 ± 0.15^{aA}	15.20 ± 0.17^{aB}	15.20 ± 0.17^{aA}	14.30 ± 0.26^{bA}	14.26 ± 0.23^{bA}	14.26 ± 0.23^{bA}	14.23 ± 0.20^{bA}
4	24.56 ± 2.28^{aB}	20.93 ± 0.11^{bB}	$20.86\pm0.15^{bd}B$	20.80 ± 0.72^{bdB}	20.86 ± 0.23^{bdCD}	18.40 ± 0.52^{cdB}	17.86 ± 0.23^{cB}	16.96 ± 0.15^{cB}	17.06 ± 0.40^{cB}	16.83 ± 0.28^{cB}
7	26.20 ± 0.34^{aBC}	21.80 ± 0.72^{bBC}	21.36 ± 0.32^{bB}	21.26 ± 0.25^{bdB}	20.16 ± 0.51^{bdC}	20.13 ± 1.20^{bdC}	19.56 ± 0.51^{cdC}	17.26 ± 0.45^{eB}	17.16 ± 0.15^{eB}	16.80 ± 0.30^{eB}
10	27.83 ± 0.15^{aC}	24.83 ± 2.37^{acC}	24.76 ± 4.96^{acB}	21.00 ± 88^{bcB}	21.16 ± 0.20^{bcD}	20.13 ± 0.23^{bcC}	19.93 ± 0.85^{bcC}	19.73 ± 0.47^{bcC}	18.90 ± 0.17^{bC}	18.06 ± 0.11^{bC}

OM, *O. majorana* EO; CA, *C. aromaticus* EO; PLA, polylactic acid.

Data are expressed as mean ± SD (n = 3). The different lowercase letters in the same row indicate significant differences ($P<0.05$). The different uppercase letters in the same column indicate significant differences ($P<0.05$).

effect of bergamot EO in chitosan films on *Penicillium italicum* reduced after day 5 due to the reduction in the availability of the antimicrobial compounds throughout the time.[9] Similar to the results of the current study, Han et al showed that the use of packaging films consisting of polypropylene/polyvinyl alcohol with 0.5% (v/v) rhubarb ethanolic extract and 0.04% (v/v) cinnamon EO lead to slower microbial growth compared to the control group during 10 days of storage.[49] It was shown that the difference between the aforementioned group and control group decreased on the 12th day of storage due to the decrease of antimicrobial concentration.[49] The reduction of antimicrobial activity of the EOs on the 10th day in comparison to the seventh day in the current study can be attributed to this fact.

At refrigerator temperatures, Gram-negative psychrotrophic bacteria are the most important bacteria causing food spoilage and even food poisoning.[5] The results of the current study were consistent with other studies reporting that the antimicrobial activity of PLA film containing *M. piperita* and *B. percicum* EOs on ground beef was less apparent in the first 7 days.[12] Pseudomonas spp. counts in ground beef patties packaged with soy edible film incorporated with *T. vulgaris* plus *O. heracleoticum* EOs were 1.13 and 1.27 log lower compared to the control group, respectively.[48]

Packaging ground beef with PLA film containing *M. piperita* and *B. Percicum* EOs and nanocellulose particle decreased *S. aureus* count by 2 logarithmic units in comparison to the control group on day 12.[12] Soy protein edible films incorporated with *O. heracleoticum* and *T. vulgaris* EOs did not have any significant effect on *S. aureus* count in fresh ground beef patties packaged with soy protein edible films.[48]

Enterobacteriaceae is a family of microorganisms with the ability to shorten food shelf life and also to induce food poisoning.[5,50] The number of Enterobacteriaceae in the control group at day 0 was in accordance with the results obtained by Shavisi et al.[5] The results of the present study were in accordance with previous studies which showed that the final count of Enterobacteriaceae of fresh meat in treatment groups was significantly different from the control group ($P < 0.05$).[5] Results of the current study were in agreement with those reported by Alizadeh Sani et al. on lamb meat.[51]

The results of the current study on LAB are in line with the results reported in a previous study.[51] Similar to the results of the present study, the initial LAB count of minced beef in the control group was 2.72 log CFU/g.[52] Rezaeigolestani et al showed that packaging vacuum-packed cooked sausages with PLA film containing *Zataria multiflora* Boiss. EO and propolis ethanolic extract decreased LAB count in comparison to control group significantly ($P < 0.05$).[4] Contrary to the results of the current study, soy protein edible films incorporated with *O. heracleoticum* and *T. vulgaris* EOs could not decrease LAB count of fresh ground beef patties due to the resistance of LAB to these antimicrobial agents.[48]

The results of the current study proved the previous results which showed no significant differences in the in vitro antimicrobial effect of *C. aromaticus* and *O. majorana* EOs against foodborne bacteria.[14]

Talebi et al showed that PLA film had no antibacterial activity in ground beef assessed.[12] Shavisi et al showed that oxygen permeability and bacterial population of minced meat insignificantly decreased in the treated samples ($P > 0.05$).[5]

TVB-N acts as an important chemical indicator of bacterial spoilage; therefore, it can be used to predict food shelf life.[53] Protein content of meat is destroyed by bacterial growth and changed to some volatile basic nitrogen compounds such as ammonia, trimethylamine, and dimethylamine.[54] Dong et al reported that TVB-N content in shrimps packaged with polyethylene films containing rosemary and cinnamon EOs was significantly lower compared to those packaged in film without the EOs ($P < 0.05$).[47] Similarly, the lowest TVB-N value belonged to the shrimps packed in films incorporated with 1% (w/w) rosemary/1% (w/w) cinnamon EOs.[47] Synergistic effect of the EOs on decreasing TVB-N value has been proven in the current study; however, the difference was more significant at day 7 in comparison to day 10. This difference between days 7 and 10 was due to the decrease of antimicrobial effect of the EOs due to the evaporation of their volatile agents. Packaging fresh beef with polypropylene/polyvinyl alcohol film containing ethanolic extract of rhubarb and cinnamon EO decreased TVB-N value in treatment samples in comparison to the control group.[49]

Conclusion

The most potent and long-lasting antimicrobial activity of PLA films incorporated with *C. aromaticus* and *O. majorana* EOs against TBC, PSY, LAB, Enterobacteriaceae, *S. aureus* and TVB-N in minced meat was observed in films with the highest concentration of both EOs (1%/1%). The results of the current study have shown that the active PLA films can be a promising approach to maintain microbial and chemical quality of minced beef at refrigerator temperature for 10 days. Further studies are needed regarding sensory acceptability and antioxidant activity of the minced beef packaged with the active PLA films.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Ethical Approval

All procedures performed in this study were in accordance with the ethical standards of the National Research Committee.

Authors' Contributions

RP designed the study and wrote the manuscript. FT drafted the manuscript. AB prepared the PLA films. AS obtained the samples.

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