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ORIGINAL PAPER

Bioactivity Potentials of Biodegradable Chitosan/Gelatin Film Forming Solutions Combined with Monoterpenoid Compounds

Tuba Baygar[1]

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Abstract

Novel food packaging systems including biodegradable/edible films have been introduced to the market for consumers who desire natural products for their nutrition. Biochemically active plant compounds are added to the biopolymer-based films to improve their functionality. Within the present study, chitosan (1%) and gelatin (4%) biopolymer-based film forming solutions (FFSs) combined with 1, 2, 5 and 10% (v/v) eugenol, pulegone and carvacrol, monoterpenoid compounds, were evaluated for their antimicrobial and antioxidative potential. Antioxidant activities and total phenolic contents (TPC) of the FFSs were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity and Folin–Ciocalteau assays, respectively. Screening the antimicrobial activity of FFSs were performed against food spoilage microorganisms including *Bacillus cereus*, *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes* and a fungi, *Candida albicans* by using agar well diffusion method. The chitosan FFS containing 10% carvacrol had greater TPC (3857.3 ± 0.07 mg gallic acid equivalent/L). The highest antioxidative capacity was observed for the chitosan FFS containing 10% eugenol as 97.92 ± 0.01%. FFSs with monoterpenoids showed promising antimicrobial activities against tested microorganisms. Based on antioxidative and antimicrobial potentials of the FFSs, it can be envisaged to use monoterpenoid incorporation to biopolymer films for food packaging applications.

Keywords Biodegradable · Film forming solution · Monoterpenoid · Antimicrobial · Antioxidant

Introduction

Packaging materials derived from petrochemical based plastics such as polyolefins, polyesters, polyamides have widespread usage for many applications; due to their availability, low cost and functional properties. On the other hand, there is a growing awareness on environmental problems which are trying to be solved by biodegradability [1]. Based on this global concern, researchers focus on developing new materials which are produced from renewable resources and eco-friendly bio-based polymeric materials [2]. Biodegradable packaging materials from natural polymers can be obtained from several sources (polysaccharide, lipid, protein) [1]. Gelatin is an appropriate biopolymer for its film-forming properties and good barrier functionality to protect food against drying, light, and oxygen [3]. Chitosan, the deacetylated form of chitin, is one of the most abundant natural biopolymers such as cellulose and have antimicrobial and antioxidant characteristics with its excellent oxygen barrier function when used as film [4, 5]. Natural antioxidative and antimicrobial agents are good candidates to be used as alternatives to synthetic preservatives [6]. Such compounds can be added to the formulation of polymeric packaging films to enhance their antioxidant and antimicrobial properties, extend the shelf-life and inhibit or reduce food borne spoilage microorganisms [7–9]. Combination of antimicrobial and antioxidant agents into polymeric packaging films to prolong the shelf-life of packaged foods has remarkable developments in recent years [7].

Terpenes, mostly hydrocarbons with general formula of $(C_5H_8)n$, are the largest and chemically diverse groups of natural products [10]. Monoterpenes, a class of terpenes, are the minor or major constituents of essential oils. Monoterpenoid compounds are widely used as fragrances for cosmetic industry, in the formula of household products (such as detergents, insect repellents, soaps, and others), for the synthesis of perfume chemicals, as flavouring agent for

✉ Tuba Baygar
tubaygar@mu.edu.tr

[1] Research Laboratories Center, Mugla Sitki Kocman University, 48000 Mugla, Turkey

food industry and for the production of alcoholic and non-alcoholic beverages [11, 12]. They are found in edible and medicinal plants, in spices and in the content of drugs. Carvacrol, eugenol and pulegone are naturally occurring phenolic monoterpenoid compounds obtained from the essential oils of a variety of plants (oregano, clove and pennyroyal) and have strong biological activities [13–15].

The incorporation of biologically active substances in edible and/or biodegradable films is an alternative application to provide and improve the properties of the film [16, 17]. Monoterpenoid compounds themselves are known to have enriching potential biological effects on the characteristics of the consumable products such as food, drug and cosmetics. The present study was carried out to compare the antioxidative and antimicrobial effects of monoterpenoids-carvacrol, eugenol and pulegone- when combined with gelatin and chitosan polymers as biodegradable film forming solutions. The purpose of the comparison was to detect the most effective monoterpenoid concentration for antioxidant and antimicrobial edible films that are especially active against common food pathogens. To the best of our knowledge, this is the first study that compares the antioxidant and antimicrobial activities of biodegradable FFSs combined with biochemically active monoterpenoid compounds.

Materials and Methods

Preparation of Film Forming Solutions (FFSs)

Preparation of the FFSs was adopted from Alparslan [18] with slight modifications. To obtain the polymer concentration of 4% (w/v), gelatin powder (Merck, Darmstadt, Germany) and distilled water were mixed at room temperature. Glycerol (0.15 mL/g gelatin) (Merck, Darmstadt, Germany) and D-sorbitol (0.15 mL/g gelatin) (Merck, Darmstadt, Germany) were then added to the gelatin FFS, and the solution was kept at 45 °C to avoid solidifying. Chitosan FFS at a concentration of 1% was prepared by dissolving 1 g chitosan with 100 mL 1% acetic acid solution (Merck, Darmstadt, Germany), the mixture was heated to 45 °C and stirred until the chitosan dissolved. Similar to the gelatin FFS, glycerol (0.15 mL/g gelatin) and D-sorbitol (0.15 mL/g gelatin) were also added to the chitosan FFS. 1, 2, 5 and 10% (v/v) of carvacrol, eugenol and pulegone (Sigma-Aldrich, USA) were added to both gelatin and chitosan FFSs before the analysis. Tween-80 was used to stabilize the emulsions, with a ratio of 0.2% of the monoterpenoid compound. 20 mL of gelatin and chitosan FFSs with different monoterpenoid contents were poured onto a 90 mm petri dishes and dried under ambient conditions to prove the film forming capacities. After drying, it was observed that all FFSs formed clear films.

Total Phenolic Content

Total phenolic contents (TPC) of the FFSs were measured by the Folin–Ciocalteu colorimetric method [19]. Gallic acid standard (50–500 mg/L) and a 20-µL sample aliquot of FFSs were mixed with 1580 µL water and 100 µL Folin–Ciocalteau's reagent was added. After vortexing, samples were incubated at room temperature for 10 min and 300 µL sodium carbonate solution (20%) were added. Then, samples were vortexed and incubated at room temperature for 2 h. Absorbances were recorded at 765 nm on a UV–Vis spectrophotometer (Multiskan GO UV/Vis Microplate Spectrophotometer, Thermo-Fisher Scientific, USA). The concentration of the total phenolic content was calculated as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. Studies were performed in triplicate.

Antioxidant Activity of Gelatin/Chitosan Film Forming Solutions

Antioxidative potentials of the FFSs incorporated with different concentrations of monoterpenoid compounds were assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity [20]. 0.5 mL test sample, 3 mL ethanol and 0.3 mL DPPH radical solution (0.5 mM, prepared in ethanol) were mixed. After 100 min incubation in dark, color changes (from deep violet to light yellow) were read at 517 nm using a UV–Vis spectrophotometer (Multiskan GO UV/Vis Microplate Spectrophotometer, Thermo-Fisher Scientific, USA). Samples without DPPH solution (mixture of ethanol and samples) were served as blanks for each monoterpenoid group. The mixture of ethanol and DPPH solution was used as control group (0.3 mL). Studies were performed in triplicate. The scavenging activity was determined according to the following formula [21]:

$$\%\text{DPPH scavenging activity} = \left[\left(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}\right)/\text{OD}_{\text{control}}\right] \times 100$$

Antimicrobial Activity of Gelatin/Chitosan Film Forming Solutions

The antimicrobial activities of FFSs incorporated with 1, 2, 5 and 10% of monoterpenoid compounds were tested against food spoilage/pathogen microorganisms; *Candida albicans* ATCC 10239, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644, *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923, using agar well diffusion assay [22]. *C. albicans* was grown in Saboraud Dextrose Broth (SDB) at 30 °C; *E. coli* and *S. aureus* were grown in Nutrient

Borth (NB) at 30 °C; and *B. cereus*, *L. monocytogenes* and *S. typhimurium* were grown in Brain Heart Infusion Broth (BHIB) at 37 °C. Inoculums were prepared by adjusting the turbidity of the medium to match the 0.5 McFarland standard dilutions. 20 mL of Saboraud Dextrose Agar (SDA), Nutrient Agar (NA) and Brain Heart Infusion Agar (BHIA) were sterilized in separated flasks and cooled to 45–50 °C. After injecting the microorganism cultures to sterile plates (1000 µL), appropriate media was distributed and mixed homogenously. When the inoculated media solidified, wells of 6 mm diameter were made on agar plates using a cork borer and 20 µL of gelatin/chitosan FFSs combined with monoterpenoid compounds were injected to the wells. Plates inoculated with *C. albicans* were incubated at 30 ± 0.1 °C for 24–48 h; *B. cereus*, *E. coli, L. monocytogenes, S. typhimurium* and *S. aureus* strains were incubated at 37 ± 0.1 °C for 18-24 h. After the incubation periods ended, antimicrobial activities were evaluated by measuring the zones of inhibition against the tested microorganisms. Studies were performed in triplicate.

Statistical Analysis

The data from the experiments were statistically analyzed using the SPSS® software program (SPSS Statistical Software, Inc., Chicago, IL, USA). Statistical comparisons were done by one-way analyses of variance (ANOVA), differences between pairs of means being assessed on the basis of confidence intervals using the Tukey-b test with a level of significance of $P \leq 0.05$.

Results and Discussion

Total phenolic contents of the FFSs are given in Table 1 as mg gallic acid equivalent/L. Chitosan FFSs with eugenol and carvacrol had the highest total phenolic contents while lower results were obtained for FFSs with pulegone. FCR total phenolic contents analysis results revealed out that there is a statistically significant difference among the gelatin and chitosan FFSs combined with monoterpenes at the same concentrations ($P < 0.05$). Carvacrol incorporation caused a statistically significant difference at all concentrations ($P < 0.05$). As an expected result, phenolic content of the FFSs increased paralelly to the monoterpenoid concentration. Carvacrol and eugenol incorporation resulted in higher phenolic contents while FFSs with pulegone had lower phenolic contents ($P > 0.05$).

Free radical scavenging activity results of the gelatin and chitosan FFSs (Table 2) were found to have statistically significant differences for the same concentration rates ($P < 0.05$). For gelatin and chitosan FFSs, the statistical difference between the 1% and 2% eugenol incorporation was not significant while it was found to be significant related to the concentration rate ($P < 0.05$). Antioxidant activity results were similar to the total phenolic contents of the FFSs. In the present study, chitosan FFSs with eugenol

Table 1 Total phenolic contents of the FFSs combined with monoterpenoid compounds

Total phenolic content (mg gallic acid equivalent/L)						
%	Gelatin			Chitosan		
	Eugenol	Pulegone	Carvacrol	Eugenol	Pulegone	Carvacrol
1	2222.63 ± 0.10^{Bc}	983.83 ± 0.09^{Bd}	1876.97 ± 0.09^{Bd}	2920.63 ± 0.10^{Ac}	1196.97 ± 0.16^{Ab}	2354.63 ± 0.21^{Ad}
2	2782.97 ± 0.01^{Bb}	1108.20 ± 0.04^{Bc}	2363.97 ± 0.04^{Bc}	3006.30 ± 0.11^{Abc}	1326.97 ± 0.07^{Ab}	2753.30 ± 0.05^{Ac}
5	2882.97 ± 0.08^{Bb}	1564.97 ± 0.04^{Bb}	2954.63 ± 0.06^{Bb}	3137.63 ± 0.05^{Aab}	2216.63 ± 0.10^{Aa}	3179.97 ± 0.06^{Ab}
10	3491.97 ± 0.12^{Aa}	1722.30 ± 0.06^{Ba}	3635.30 ± 0.08^{Ba}	3580.97 ± 0.09^{Aa}	2368.63 ± 0.04^{Aa}	3857.30 ± 0.07^{Aa}

Different small letters indicate significant difference among means in the same column ($P < 0.05$)

Table 2 DPPH radical scavenging activities of the FFSs combined with monoterpenoid compounds

Antioxidant activity (% inhibition)[a]						
%	Gelatin			Chitosan		
	Eugenol	Pulegone	Carvacrol	Eugenol	Pulegone	Carvacrol
1	40.91 ± 0.02^{Bc}	39.75 ± 0.01^{Bc}	23.21 ± 0.16^{Bd}	89.59 ± 0.02^{Ab}	68.12 ± 0.08^{Ab}	74.47 ± 0.05^{Ad}
2	45.20 ± 0.04^{Bc}	41.99 ± 0.02^{Bbc}	28.81 ± 0.03^{Bc}	92.61 ± 0.03^{Ab}	68.22 ± 0.03^{Ab}	83.33 ± 0.03^{Ac}
5	56.25 ± 0.11^{Bb}	44.93 ± 0.02^{Bb}	45.15 ± 0.04^{Bb}	97.70 ± 0.03^{Aa}	67.90 ± 0.04^{Ab}	93.38 ± 0.03^{Ab}
10	71.08 ± 0.05^{Ba}	50.95 ± 0.03^{Ba}	61.16 ± 0.03^{Ba}	97.52 ± 0.04^{Aa}	70.26 ± 0.05^{Aa}	97.02 ± 0.03^{Aa}

[a]Different small letters indicate significant difference among means in the same column ($P < 0.05$)

showed promising antioxidant capacity. Chitosan has been known to have intrinsic antioxidant properties [23–26], but the most accepted practical way to improve antioxidant property of the biodegradable chitosan films is to incorporate with antioxidant agents [27]. The antioxidant potential of eugenol has been figured out by Gülçin [28] and resulted that eugenol had the most powerful antioxidant activity and radical-scavenging activity among the tested control groups. Similarly, carvacrol incorporation also resulted in higher radical scavenging capacities for chitosan FFSs ($P < 0.05$). The antioxidative potential of carvacrol has been reported [29, 30]. Researchers resulted in a linear correlation between the content of total phenolic compounds and their antioxidant capacity [31–35]. The present study demonstrated that biodegradable FFSs with monoterpenoid compounds scavenged the DPPH radical in a dose-dependent manner directly related to monoterpenoid concentrations. There was a positive correlation between the total phenolic content and antioxidant activity for FFSs, suggesting that total phenolics in the FFSs provided a substantial antioxidant activity.

The measurements of the inhibition zones against all microorganisms are listed in Table 3. The highest inhibition zone was measured against *E. coli* by chitosan FFS with 10% carvacrol (36.00 ± 1.00) while the lowest inhibition zone was measured against *B. cereus* by chitosan FFS with 1% pulegone (7.33 ± 1.15). Both gelatin and chitosan FFSs combined with monoterpenoids were found to be effective against *C. albicans* ($P < 0.05$). The statistical differences of the inhibition zones against *B. cereus* were not found to be significant for the gelatin and chitosan FFSs combined with

Table 3 Antimicrobial activities of the FFSs combined with monoterpenoid compounds

	Zone of inhibition (mm)[a]					
	Gelatin			Chitosan		
	Eugenol	Pulegone	Carvacrol	Eugenol	Pulegone	Carvacrol
C. albicans						
1%	17.33 ± 1.15^{Ac}	16.67 ± 1.53^{Ad}	22.33 ± 1.15^{Bc}	13.67 ± 0.58^{Bc}	16.33 ± 0.58^{Ac}	27.33 ± 1.15^{Ac}
2%	21.33 ± 1.53^{Abc}	20.67 ± 0.58^{Ac}	26.33 ± 1.53^{Bb}	21.33 ± 1.15^{Ab}	19.00 ± 1.00^{Bb}	29.67 ± 1.53^{Ab}
5%	23.67 ± 1.15^{Ab}	22.33 ± 1.53^{Ab}	27.67 ± 0.58^{Bb}	19.33 ± 0.58^{Bb}	20.67 ± 0.58^{Bb}	34.00 ± 1.00^{Aa}
10%	28.33 ± 1.53^{Ba}	24.67 ± 1.15^{Aa}	33.67 ± 1.15^{Aa}	30.00 ± 1.00^{Aa}	24.67 ± 0.58^{Aa}	34.33 ± 1.15^{Aa}
B. cereus						
1%	–	–	13.33 ± 0.58^{Ac}	13.00 ± 1.00^{Ab}	7.33 ± 1.15^{Ab}	11.67 ± 1.53^{Bd}
2%	11.67 ± 0.58^{Bc}	–	16.67 ± 1.15^{Ab}	13.67 ± 1.53^{Ab}	11.67 ± 0.58^{Aa}	16.67 ± 0.58^{Ac}
5%	16.67 ± 2.08^{Ab}	9.33 ± 0.58^{Bb}	30.00 ± 1.00^{Aa}	16.33 ± 0.58^{Aa}	11.67 ± 1.53^{Aa}	23.00 ± 1.00^{Bb}
10%	18.00 ± 2.00^{Aa}	13.33 ± 1.15^{Aa}	30.33 ± 1.53^{Aa}	17.67 ± 0.58^{Aa}	12.33 ± 0.58^{Aa}	26.00 ± 1.00^{Ba}
E. coli						
1%	$12.67 \pm 1{,}53^{Bc}$	15.00 ± 2.00^{Ab}	14.00 ± 1.00^{Bd}	20.00 ± 1.00^{Ac}	–	22.00 ± 1.00^{Ac}
2%	12.67 ± 0.58^{Bc}	11.67 ± 0.58^{Ac}	20.67 ± 0.58^{Ac}	24.67 ± 0.58^{Ab}	10.67 ± 0.58^{Bc}	20.33 ± 0.58^{Ad}
5%	16.67 ± 1.53^{Bb}	15.33 ± 1.53^{Ab}	25.33 ± 0.58^{Bb}	24.00 ± 1.00^{Ab}	13.67 ± 2.52^{Bb}	28.00 ± 0.00^{Ab}
10%	22.33 ± 2.52^{Ba}	18.67 ± 0.58^{Ba}	$28{,}33 \pm 1.53^{Ba}$	27.67 ± 0.58^{Aa}	20.33 ± 1.15^{Aa}	36.00 ± 1.00^{Aa}
L. monocytogenes						
1%	11.33 ± 0.58^{Ac}	–	11.33 ± 1.53^{Ad}	9.33 ± 0.58^{Bc}	–	12.00 ± 1.00^{Ad}
2%	12.67 ± 0.58^{Ac}	–	15.33 ± 1.53^{Ac}	$12.67 \pm 0{,}58^{Ab}$	–	15.67 ± 1.15^{Ac}
5%	14.67 ± 1.15^{Ab}	9.00 ± 1.00^{Ab}	20.67 ± 1.15^{Ab}	12.67 ± 1.53^{Bb}	9.67 ± 1.53^{Aa}	21.00 ± 1.73^{Ab}
10%	$17{,}33 \pm 1.53^{Aa}$	11.33 ± 0.58^{Aa}	$22.00 \pm 1{,}00^{Ba}$	15.33 ± 0.58^{Ba}	10.33 ± 1.53^{Aa}	25.33 ± 1.15^{Aa}
S. aurues						
1%	10.67 ± 1.53^{Bd}	10.67 ± 0.58^{Bc}	13.67 ± 1.53^{Bd}	14.00 ± 1.00^{Ad}	15.00 ± 1.00^{Ac}	24.33 ± 1.15^{Ad}
2%	$13.33 \pm 0{,}58^{Bc}$	11.00 ± 1.00^{Bc}	20.00 ± 1.00^{Bc}	18.67 ± 1.15^{Ac}	16.33 ± 1.15^{Abc}	26.67 ± 1.53^{Ac}
5%	15.33 ± 1.15^{Bb}	17.33 ± 0.58^{Ab}	21.67 ± 1.53^{Bb}	25.00 ± 1.00^{Ab}	17.33 ± 0.58^{Ab}	30.33 ± 0.58^{Ab}
10%	18.33 ± 1.53^{Ba}	21.67 ± 1.53^{Ba}	27.67 ± 0.58^{Ba}	31.67 ± 0.58^{Aa}	24.00 ± 1.00^{Aa}	32.67 ± 0.58^{Aa}
S. typhimurium						
1%	9.67 ± 0.58^{Ac}	10.67 ± 0.58^{Ab}	12.33 ± 1.53^{Ac}	10.33 ± 0.58^{Ad}	9.00 ± 0.00^{Ac}	12.33 ± 0.58^{Ac}
2%	12.33 ± 0.58^{Bb}	10.33 ± 0.58^{Ab}	13.67 ± 0.58^{Bc}	15.67 ± 0.58^{Ac}	10.33 ± 1.53^{Ab}	15.33 ± 1.53^{Ab}
5%	15.33 ± 1.53^{Ba}	12.33 ± 0.58^{Aa}	19.33 ± 0.58^{Ab}	17.33 ± 0.58^{Ab}	10.33 ± 0.58^{Bb}	19.67 ± 0.58^{Aa}
10%	15.67 ± 0.58^{Ba}	13.67 ± 1.15^{Aa}	22.67 ± 1.15^{Aa}	20.00 ± 1.00^{Aa}	12.33 ± 0.58^{Ba}	20.00 ± 1.00^{Ba}

[a]Different small letters indicate significant difference among means in the same column ($P < 0.05$)

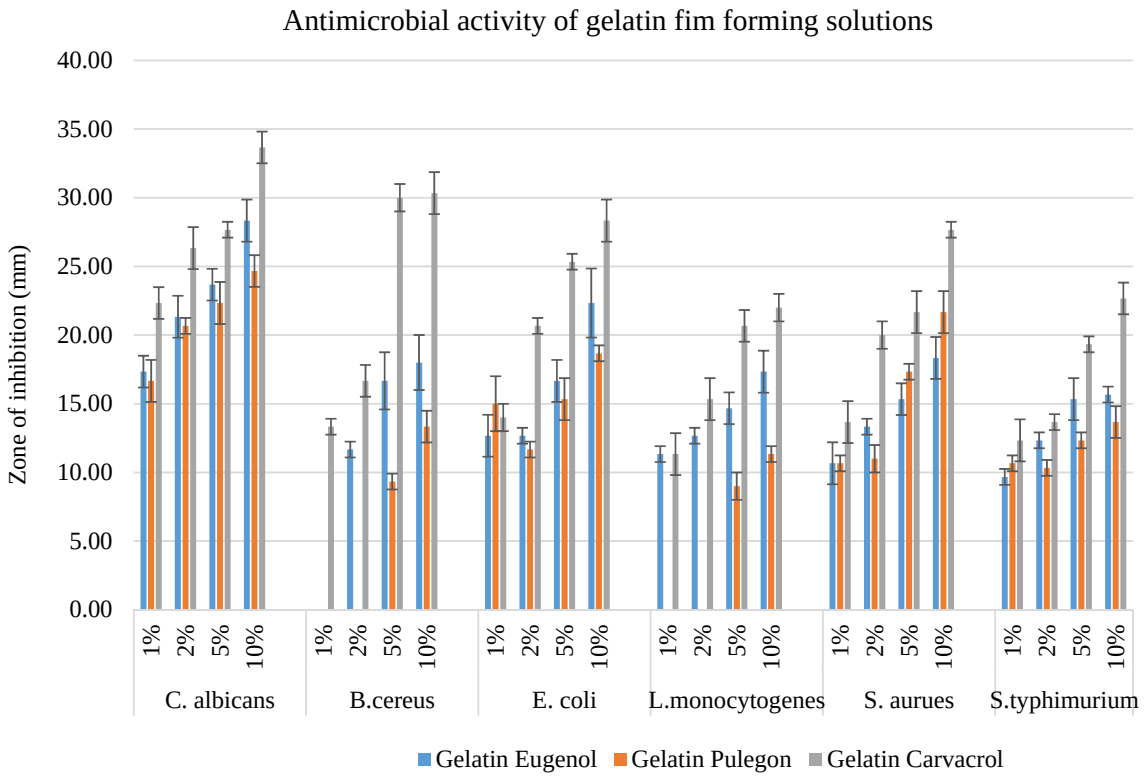

Fig. 1 Graphical results of the antimicrobial activity of the gelatin FFSs combined with monoterpenoid compounds

pulegone ($P > 0.05$). Chitosan FFSs with eugenol and carvacrol were more effective against *E. coli* than other combinations ($P < 0.05$). *L. monocytogenes* and *S. typhimurium* were found to be sensitive against carvacrol incorporation to FFSs ($P < 0.05$). The statistical differences were found to be significant among the inhibition zones of *S. aureus* by eugenol and carvacrol incorporation to FFSs ($P < 0.05$) while this was not significant for pulegone incorporation ($P > 0.05$). The antimicrobial activity of FFSs against *S. typhimurium* was found to be statistically significant for high concentrations ($P < 0.05$) while it was not significant for low concentration ($P > 0.05$).

The results showed that gelatin and chitosan edible films incorporated with monoterpenoid compounds could be used as active films due to their great in vitro antimicrobial activity. Gómez-Estaca et al. [36] reported that neither gelatin nor gelatin-chitosan edible films had antimicrobial activity against *Pseudomonas fluorescens*, *Shewanella putrafaciens*, *Photobacterium phosphoreum*, *Listeria innocua*, *E. coli* and *Lactobacillus acidophilus*. Chitosan has a widespread usage for food applications due to its antimicrobial and antioxidant activities, biodegradability, biocompatibility, non-toxicity and film-forming capacity [37]. However, researchers reported that chitosan films were not effective against *Listeria innocua*, *Serratia marcenscens*, *Aeromonas hydrophila*, *Achromobacter denitrificans* and *Alcaligenes faecalis* [38], *E. coli* and *S. aureus* [5, 39]. FFSs incorporated with monoterpenoid compounds showed promising antimicrobial capacities against tested microorganisms (Figs. 1 and 2). Incorporating natural agents into the biopolymer-based edible films may enhance the protective feature of the foods and prolong the shelf-life. The results of the present study revealed out that as the amount of monoterpenoid compound incorporated to the gelatin and chitosan films increased, the antimicrobial effects on all microorganisms were also increased.

Conclusion

Combination of biopolymers with monoterpenoid compounds resulted in potentially active film forming solutions that are utilizable for food applications. This study demonstrated that the amount of the monoterpenoids directly affect the inhibition strength of the film forming solution against food spoilage/pathogen microorganisms. Antioxidative potential of the FFSs was directly related to the total phenolic content of the FFSs. Carvacrol and eugenol were the most effective monoterpenoid compounds that increased the antimicrobial capacity of both gelatin and chitosan FFSs. On the other hand, pulegone had less potential to enhance the activities of the FFSs. Incorporation of monoterpenoid compounds to gelatin and chitosan

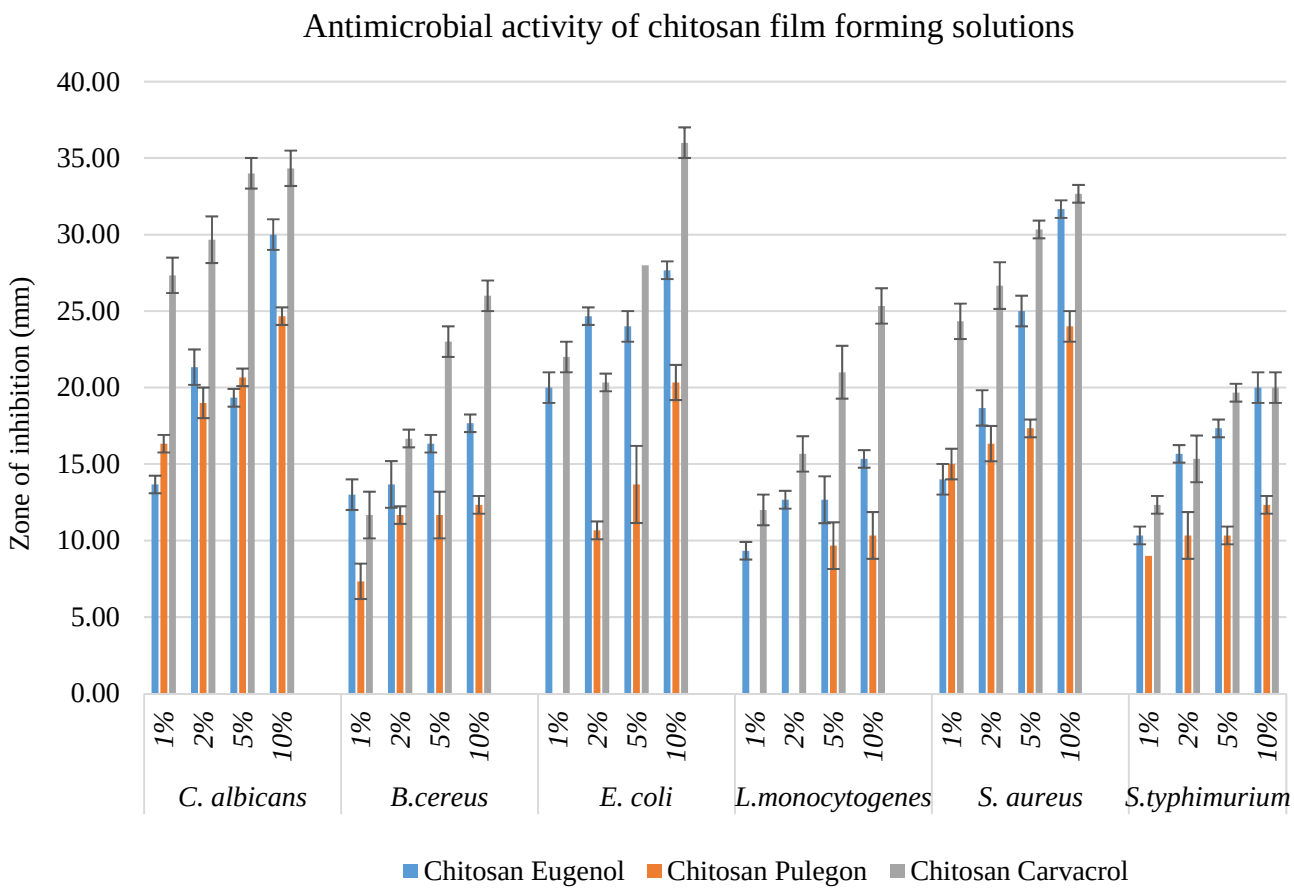

Fig. 2 Graphical results of the antimicrobial activity of the chitosan FFSs combined with monoterpenoid compounds

FFSs had noticeable antioxidant and antimicrobial effects. The results of the present study are important for the further development of biodegradable antioxidant and antimicrobial films with improved biological properties.

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Compliance with Ethical Standards

Conflict of interest The author declare no conflict of interest.

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