The Effect of *Ziziphora clinopodioides* Essential Oil and Nisin on Chemical and Microbial Characteristics of Fish Burger During Refrigerated Storage

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ABSTRACT: *Ziziphora clinopodioides is from the genus of Ziziphora and family of Lamiaceae, which grows in Iran and Turkey. This study was conducted to preserve the chemical and microbial quality of trout fish burger during storage using Ziziphora clinopodioides essential oil (ZEO) individually and in combination with nisin. Firstly, the chemical composition of ZEO was determined using GC-MS analysis. Different treatments of trout fish burger were formulated using ZEO and nisin as natural preservatives, stored in refrigerator for 20 days and were analyzed for chemical (pH and Total Volatiles Base-Nitrogen (TVB-N)) and microbial (total viable count, psychotropic counts, Enterobacteriaceae count and Pseudomonas spp count) characteristics.at 5-day intervals. The Results indicated a yield of 1% (w/w) for ZEO isolation and Pulegone (40.09%), Menthone (13.76%) and Isomenthone (12.31%) were identified as the major components of phytochemicals of ZEO. According to the obtained results combination of ZEO and nisin had the strongest effect on chemical and microbial quality of fish burger; however, their individual use had significant effects on preserving the chemical and microbial quality of fish burger as well. based on results of this study, formulation of ZEO and Nisin in fish burger especially in combinations can prolong its shelf life and control chemical and microbial changes during storage at 4˚ C.*

KEYWORDS: C*hemical composition; Chemical quality; Fish burger; Total volatile base nitrogen; Ziziphora clinopodioides.*

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INTRODUCTION

Fishery products can form a very nutritious part of man's diet and contribute to children's proper growth and humans heart health [1]. Microbial spoilage, as one of the main causes of spoilage, declines the shelf life of fish meat and other fishery products [2, 3]. The changing factors of the composition and the spoilage microflora of fish burger are including 1) the natural microflora of fish and another component of fish burger, 2) cooking methods 3) microbial contamination during production, and 4) kind of using preservatives, storage conditions and temperature. In recent years, researchers have focused on controlling foods from chemical and microbial spoilage using new techniques to increase the shelf life of food products. Increasing the concentration of these essential oils decreased the logarithm of the number of microorganisms. Essential oils showed inhibitory effect on *Salmonella spp[4]*. The essential oils of Mentha spicata and Mentha pulegium in low concentrations have an antimicrobial effect on spoilage yeast and can improve the sensory properties of Doogh[5, 6]. Various plants extract and essential oils, are used as preservatives in different products, due to being healthy and natural; because these components are accepted by consumers and have antimicrobial and antioxidant activities to preserve food products [7]. A safe and acceptable approach to increase the safety and shelflife of tropical foods is the use of essential oils [8].

Ziziphora clinopodioides is known by the local name of mountain *kakoty*, it is a genus of *Ziziphora*, a family of the Lamiaceae, which grows in Iran and Turkey. The genus of *Ziziphora* consists of four species, including Z. *clinopodioides Lam, Z. capitata L, Z. tenuior L and Z. persica*. *Ziziphora clinopodioides* is a perennial plant with stemmy bushes, short stem (5-16 cm) and thin, sharp leaves [8]. Previous studies have identified its major phenolic compounds such as *pulegone*, *1,8-cineole*, *thymol*, *carvacrol* and *limonene* [9]. Generally, in the Iranian folk medicine, healing properties of this plant have been used for flu disease, wound heal, carminative effects, treatment of stomach tonic and its other effects such as antibacterial, anthelmintic, antifungal and antiviral properties [8]. Traditionally, this plant has been powdered and was used in meat and dairy products especially yogurt to enhance their flavor and aroma in different areas of Iran for a long time [10].

Nisin is a small, cationic, hydrophobic antimicrobial peptide (AMPs) and well-known bacteriocin with 34 amino acids. It can inhibit the growth of some Gram-negative bacteria [11] and has inhibitory effect to growing a broad spectrum of Gram-positive bacteria; therefore, it has been applied industrially in various food products [12]. Commercial *Nisin* is produced by *Lactococcus lactis* subspecies *lactis* [13] and is declared as a GRAS (generally recognized as safe) substance by the Food and Drug Administration (FDA) [14].

There are few studies about the application of different Essential Oils(EOs) and *Nisin* as natural preservatives in fishery products especially fish burgers[15, 16]. It is important to evaluate the effect of natural preservatives in fish product in order to extend their shelf life; however, *ZEO* has never been used as the preservative in the component of fish burger and it is the first time that it is used for shelf life extension of the fish burger. Thus, the present study aimed to investigate 1) the chemical composition of ZEO 2) the ability of ZEO and nisin to control chemical and microbial changes of fish burger and extend its shelf life.

EXPERIMENTAL SECTION

Essential Oil Extraction and Analysis:

Isolation and analysis of ZEO were performed by the Hydro distillation method. The Clevenger type apparatus was utilized to extract essential oil of dried aerial parts, collected during the flowering stage from the Mountains of *Bojnourd* County in North Khorasan province of Iran, identified by the Herbarium of Iranian Institute of Medicinal Plant in Alborz province. Plant material was distilled with water for 4 hours according to technique was used by *A. Ehsani at al.* 2016 [18]. The obtained ZEO was dehydrated by sodium sulfate, filtrated and stored at 4 °C until further analysis. The composition of the essential oil was determined by Gas Chromatography and coupled with Mass Spectrometry (GC-MS) described previously according to the method [18]. Nisin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA) and dissolved (50 mg) in 5 ml hydrochloric acid (0.02 mol/L) to provide standard solutions 200 IU/g. All culture media such as Plate Count Agar (PCA), Pseudomonas Agar Base supplemented with CFC, Violet Red Bile Glucose Agar **(**VRBG agar**)** and chemical material such as Butanol, Methanol, Chloroform, were purchased from Merck Company (Merck, Darmstadt, Germany).

Table 1: List of combinations and treatments.

	Treatment	Description
A	Control	Without Nisin and EO
B	Addition of ZEO	0.25% (w/w)
C	Addition of ZEO	0.50% (w/w)
D	Addition of ZEO	1% (w/w)
E	Addition of Nisin	200 IU/g
F	Addition of ZEO	200 IU/g plus ZEO 0.25% (w/w)
G	Addition of ZEO	200 IU/g plus ZEO 0.50% (w/w)
H	Addition of ZEO	200 IU/g plus ZEO 1% (w/w)

Sample Preparations

Fresh rainbow trout's (average weight and length: 300-400 g and 250 mm) were purchased from an aquaculture farm of *Bojnourd* County, Iran and transferred in ice boxes to the laboratory at North Khorasan Branch of Academic Center for Education, Culture and Research (NK-ACECR). At the first, all fresh fish were beheaded, gutted and washed. Then, they were disbanded, filleted and minced by a meat mincer with a pore size of 1.0 mm. Fish burgers were formulated regarding the following method [19]: 6% corn flour, 0.2% garlic powder, 4% wheat flour, 0.6% sugar, 1.2% salt and 0.2% onion powder were added with 87.8% fresh minced fish flesh and mixed well. The formulated fish burger was divided into eight equal sample portions as treatments which are shown in Table 1. Then, a sample of each group was separately mixed until a homogeneous distribution was obtained.

50 g of each mixture was placed on the plastic, pressed by hamburger press machine, frozen at -18 °C for two hours and was stored in the refrigerator (4˚C) for 20 days. Fish burgers were formed to 11 cm diameter and 0.8 cm thickness. Then the samples were analyzed periodically on days 0, 5, 10, 15 and 20 for Total Viable Count (TVC), *psychotropic* count, total Enterobacteriaceae count and *Pseudomonas* spp count as well as chemical parameters such as TVB-N and pH values.

Chemical Analysis

Determination of Total Volatiles Base Nitrogen materials (TVB-N) was performed by *Kjeldahl* apparatus according to the method previously was described by *Pikul* 1989 [20] (Gerhardt company-Germany). In this method after MgO addition into the homogenized samples flask containing the aqueous solution of boric acid of 3% concentration as well as indicator (produced by dissolving 0.1 g methyl red and 0.1 g methylene blue in 100 ml absolute ethanol) was utilized to collect the distillate. To determine TVB-N values, the boric acid solution was titrated using 0.05 M sulphuric acid solution. Results have been expressed in milligram of nitrogen per 100 g of sample. Measurement of pH value was performed by a pH meter (model 780; Metrohm, Switzerland) as well, according to the Iran national standard No 1028-1386 [19, 18].

Microbiological Analysis

An amount of 10 g of samples from each treatment was weighted and aseptically transferred to sterile tubes containing 90 mL sterile peptone water (0.1% w/w) and homogenized for 3 min. For microbial enumeration, 10 µl of serial dilutions of homogenates was spread on specific agar plates using drop plate technique [21, 22]. Total Viable Counts (TVC) were determined by Plate Count Agar after incubation at 30 °C for 48 h [23]). To enumerate the *Pseudomonas* spp., plates containing Pseudomonas Agar Base with Cetrimid, Fucidin, Cephalosporin (CFC) supplement were incubated at 25°C for 72 h [23]. Total *enterobacteriaceae* counts were enumerated by violet red bile agar and the plates were incubated at 30 °C for 24 h [23]. In order to enumerate psychotrophic bacteria, Nutrient Agar plates were incubated at 6.5 °C for 10 days [23]. The counts were expressed as log10 Cfu/g.

Statistical analysis

All experiments in this study were performed in quintuplicate. The average of the logarithm of the bacterial count and chemical values of this study were compared between treatments using repeated measure ANOVA followed by Bonferroni post-hoc test for pairwise comparison. All statistical analysis performed by SPSS version 21 and $P<0.05$ considered as significant.

RESULTS AND DISCUSSION

***Characteristics of raw fish*:**

The proximate composition of fish burgers was performed as follows: protein 16%, fat 7.2%, ash 2%, carbohydrate 7.8%, and moisture 67%, it was approximately similar to *Ehsani* findings [24].

The chemical composition of ZEO

The essential oil content of the aerial parts of *Ziziphora Clinopodioides* was obtained in a yield of 1 % (w/w), which was in accordance to *Amiri et al* (2014) and *Morteza-Semnanis* (2005) [25] reports (1.1% and 0.98% respectively), but different results were reported by another researcher [9]. In this study *pulegone* (40.09%), *Menthone* (13.76%) and *Isomenthone* (12.31%), were the most representative components of ZEO (Table 2). Similar result was reported by a former study *as* well [26]; Although other researchers reported some other compounds (*pulegon* 46.8% , P-menth-3-en-8-ol14%) [27] and (pulegone 61.67%, cis-caran-trans-2-ol 12.66%) [9], as the most representative components of ZEO. This differences among chemical composition of ZEO in the different study could be attributed to the differences in climates, seasons, regions and geology of the plants have grown [27, 28].

Chemical characteristics

TVB-N and pH were used as chemical parameters related to spoilage and quality reduction of fish burgers these changes occur during deterioration by spoilage bacteria and endogenous enzymes which are present in fish tissue [29].

TVB-N changes

Total Volatile Basic Nitrogen (TVB-N) is known as a product of bacterial spoilage and endogenous enzymes action and its content is often used as an index to assess the keeping quality and shelf-life of products [30].

Results showed no statistically significant differences between the initial value of pH (range: 5.35-5.7) and TVB-N (range: 6.8-7.2 mg/100 g) among treatments at the beginning of example and at the end of 20th day. *Jokiet, Meynel, and Ojagh* have reported similar results [7, 29, 31]. TVB-N levels increased in all groups during storage, but this increase was progressively at the highest level (15 mg N/ 100g) in the control group (A) throughout the storage period ($p<0.001$). But in other groups (B, C, D, E, F, G, and H), increases were very lower than the control group. The Lowest TVB-N levels were observed in group H with ZEO 1% plus Nisin (v/v) treatment (9 mg N/100g) on the last day of storage (Fig. 1) ($p<0.001$). These results were obtained based on *Gimenez, Roncales, and Beltran* (2002)[32]. Inhibitory effect of component sample might be mainly due to the inhibitory effect of *Nisin* and ZEO on the growth of spoilage bacteria, resulting in the reduction in bacterial growth and/or a decrease in the capacity of bacteria for oxidative deamination of proteins [33-35], and eventually resulted in a significant extension in shelf-life of fish burgers[36].

pH change

In this study, pH value of fresh fish burger was reported between 6.35-6.7(Fig. 2) similar to the result of *Kyrana, V.R et al.*, 1997 (determined between 6.0 - 6.5) [37-40]. *Varlik et al.* explained The decrease in the pH value of fish balls could be caused by the fermentation of the potato and bread ingredients of the burgers [41].

Regarded the pH values of 6.8-7.0 as the limit of acceptability for fish. This shows that pH is not a suitable quality indicator for trout burgers[42]. It decreased during storage (20th day) in control (A) and ZEO treated samples (B, C, D) to 5.5. This pH decrease is due to bacterial spoilage and fermentation of the flour and other ingredients of fish burgers [43]. Different results were reported about decrease or increase of pH value in various fish species as well [44].

pH values in control (A) group and (H) group were at the lowest and the highest level respectively. pH value was more stable in samples with *Nisin* and *Nisin* plus ZEO, that might be attributed to amino acids decomposition, inhibitory effects of ZEO and *Nisin* against bacterial growth, the glycogenolysis, and dissolution of CO_2 and its conversion to carbonic acid

Table 2: Chemical composition of essential oil from Ziziphora clinopodioides Lam identified by GC-MS.

Chemical Tests				
No	RT	%	Components	KI
1	11.33	0.21	α-Thujene	927
2	11.72	1.13	α-Pinene	934
3	12.62	0.14	CampHene	958
4	13.80	0.89	Sabinene	975
5	14.06	1.60	β-Pinene	981
6	14.32	0.19	1-Octen-3-ol	986
7	14.63	0.55	Myrcene	992
8	15.18	0.45	3-Octanol	1003
9	16.62	0.67	p-Cymene	1030
10	16.78	0.99	Limonene	1033
11	16.97	6.79	1,8-Cineole	1037
12	18.31	0.65	γ-Terpinene	1063
13	19.03	0.16	p-Mentha-3,8-diene	1077
14	23.16	2.04	p-Mentha-3-en-8-ol	1159
15	23.53	1.15	Menthone	1166
16	23.99	9.91	iso-Menthone	1175
17	24.17	0.63	neo-Menthol	1179
18	24.37	0.30	Borneol	1183
19	24.56	0.82	neoiso-Isopulegol	1187
20	24.71	0.19	Terpinen-4-ol	1190
21	25.27	3.33	iso-Menthol	1201
22	27.46	0.27	Thymol methyl ether	1248
23	27.70	58.78	Pulegone	1253
24	28.39	1.08	Piperitone	1267
25	29.74	0.23	Methyl acetate	1296
26	30.05	1.43	Carvacrol	1303
27	32.31	2.32	Piperitenone	1354
28	38.10	0.60	Germacrene D	1489
29	40.39	0.11	Z-β-Bisabolene	1547
		97.61	Total Identified	

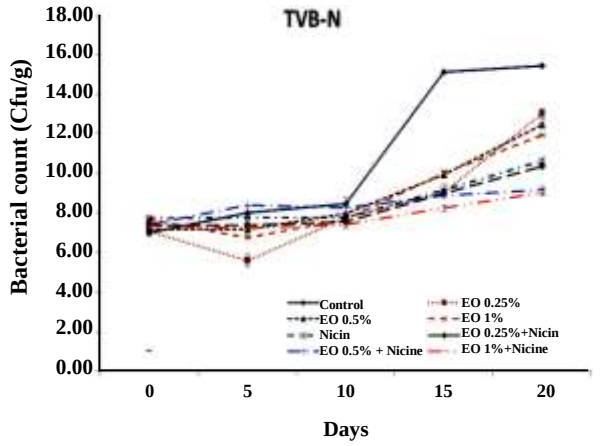

Fig. 1: TVB-N Changes in different treatment groups (Control, EO, NISIN) of fish burger kept during storage at 4 °C.

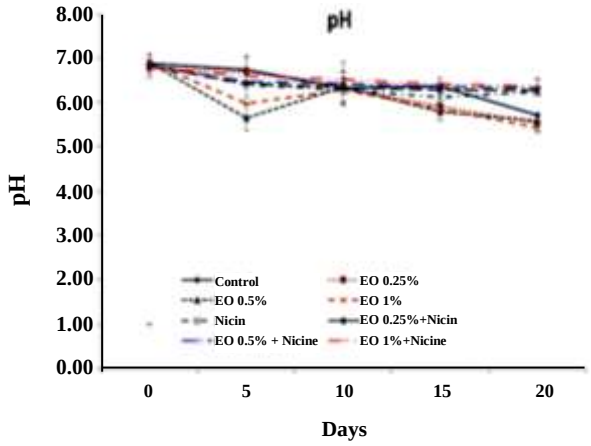

Fig. 2: pH Changes in different treatment groups (control, ZEO, Nisin) of Fish burger kept during storage at 4°C.

in the fish burgers during storage period [34, 45, 46]. pH and Total Volatile Basic Nitrogen (TVB-N) (including ammonia and other volatile basic nitrogenous compounds [47]) [48] are used as an indicator of fish freshness or deterioration [49]. In agreement with our findings, the same results related to the effect of *Nisin* in increasing the shelf life of fish products have been reported by another researcher [36].

Microbiological characteristics

The values of (TVC), (PBC), (TEC) and *Pseudomonas* spp (PS) are presented in Figs. 3-6 respectively. In this study, there were low amounts of the initial value of bacterial counts with no significant difference among the groups ($P > 0.05$) indicating a high hygienic quality of raw materials which was consistent with pH and TVB-N results [24]. The initial counts of TVC, TEC, PBC, and Ps were obtained 4.1±0.2 l, 3.2±4, 3±2 [15, 16] and Ps count <2 log10 cfu/g respectively. Some of these values were relatively higher than fresh fish's meat counts since the ingredients were not sterilized, these results could be connected to contamination of ingredients that had been used for the production of fish burgers such as corn flour, wheat flour and eTEC [15, 50]. In all samples, bacterial counts were seen significant differences between the control and there was no similarity between rates of increasing control and treated samples with ZEO, Nisin and their corporation groups $p<0.001$. By International Commission on Microbiological Specification for Food, (ICMSF 1978), the level of TVC, TEC, PBC, and Ps was proposed as $\log^7$-$\log^8$ CFU/g in fish products [1, 51].

Total Viable Count (TVC)

In this regard, the average of TVC count between the different group was shown during the storage period ($p<0.001$). The average of the bacteria's count in control (A) group (8.4 Log CFU/g) was higher than fish burger experimental groups (B, C, D, E, F, G and H) during the storage period and showed over the range of the acceptable maximum level until the 10th day and the higher level of essential oil was more effective [52]. The microbial (TVC) loads rising were decreased respectively in (B, C, D, E, F, G, and H) groups during the examination and the perishable day for fish burger (B, C, and D) groups were the 15th day [52]. In the other experimental groups (E, F, G, and H) obtained nonacceptable value by the 20th day. The TVC loads rising in the sample treated with ZEO 1 % plus Nisin (H) was lowest and the ZEO 0.5% plus Nisin (G) was lower than sample treated with Nisin E. The results of TVC changes are shown in Fig. 3 according to *Shahbazi et al.* 2016 reported [53]. The essential oil of aerial parts of *Z.clinopodioides* has a broad spectrum of antimicrobial activity against all tested microorganisms [53]. The results of TVC counted shown inhibition synergistic effect of *Nisin* and *ZEO* (E, F, G, and H) against bacterial growth in fish burgers. The researchers suggested bacteriostatic or bactericidal effectiveness of *Nisin plus ZEO* complex, against both gram negative and positive bacteria, is due to, (i)

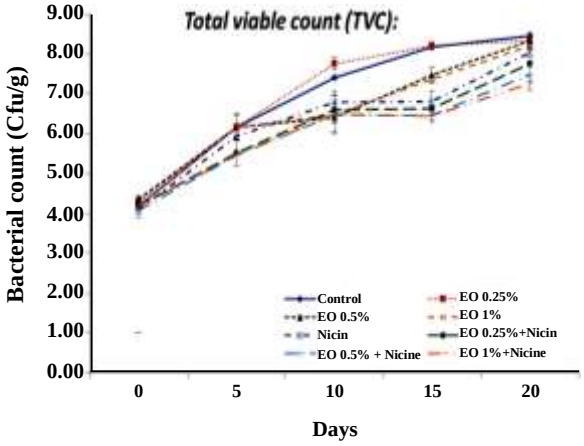

Fig. 3: TVC Changes in different treatment groups (control, ZEO, Nisin) of fish burger kept during storage at 4°C.

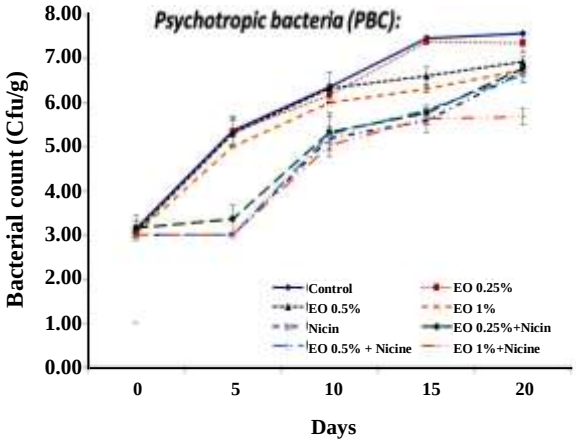

Fig. 4: Psychotropic bacteria (PBC): in different treatment groups (control, ZEO, Nisin) of fish burger kept during storage at 4°C.

Synergistic inhibitory effect between them (iii) changing the membrane of the cell and rising the penetrating capabilities of *Nisin*, this inhibition occurs by binding metal ions in the lipopolysaccharide (LPS) layer of the bacteria [54]. In other study was reported, the effectiveness of *Nisin* usually enhanced at a lower pH and incorporation with other preservatives such as essential oils [54].

Regarding to the results obtained from former studies, the antimicrobial activity of ZEO is mostly due to the presence of phenolic compounds mainly, *pulegone* and *isomenthone*. Inactivation of cellular enzymes and destruction of the permeability of cell membranes were known causes of antimicrobial action of phenolic compounds [55]. It has been reported that *pulegone* and other peptides in ZZEO composition are cause of these antimicrobial activities [56, 57] that it is respectively against both gram positive and gram negative bacteria [52, 56, 58].

Psychotropic bacteria (PBC)

Aerobic and anaerobic conditions, level of existent salt and other preservatives, materials and temperature are several factors that are relative with viability of psychotropic bacteria [59]. In this study psychotropic bacterial count in control (A) group, was shown highest amounts (7 Log CFU/g) (Fig. 4) during storage time ($p<0.001$). Lawria.R.A and Ledward.D.A (2016) got out of acceptable range at 15th day and the sample treated with ZEO 0.25 % (B) Was spoiled in 20th day and in other treatment nonacceptable values were not obtained. The average bacterial count was statistically significant between the groups during the storage period ($p<0.001$). value of the PBC loads was raised in H group, the result of this sample was the lowest deal in all treatments groups and amount of the ZEO 0.5% plus Nisin (G) was shown lower than sample treated with Nisin alone. It was proposed that appropriate limit of psychotropic bacteria in foods is 10^3 to 10^4 cfu/g [16] and non-acceptable value is10^7 cfu/g because this amount of bacteria able to produce 30 mg(N-TMA) 100 g and is found in spoiled fish [60].

Total Enterobacteriaceae Count (TEC)

In the present study, the level of non-acceptable value of TEC in control (A) and B, C and D groups were obtained until 15th day. *Guran et al.* 2015 reported 5.84 log10 cfu/g at the end of the 14th day of storage period [50]. For other groups(E, F, G and H)), spoilage day was at the 20th day, those were consistent with results of Kassem et al., 2011and *Erkan*, 2012 [24] studied, during used of thyme essential oil and sodium acetate in decreased the microbial load in fish burgers, in G group, was found statistically significant with other treatment. The lowest enterobacteriaceae bacteria count was observed in the H group (6.6 log10 cfu/g). Logarithmic changing of the bacterial count wasn't same in the different groups during the period of study ($p<0.001$). The average of TEC count in (H) group was lower than the other and the (A) group had the highest amount. The results are shown in Fig. 4.

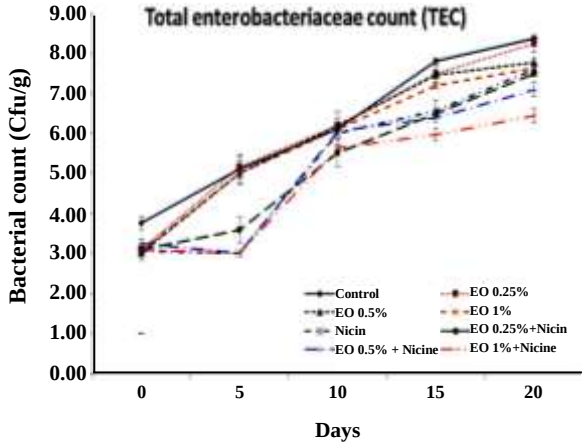

Fig. 5: Total Enterobacteriaceae count (TEC) changes in different treatment groups (control, ZEO, Nisin) of fish burger kept during storage at 4ºC.

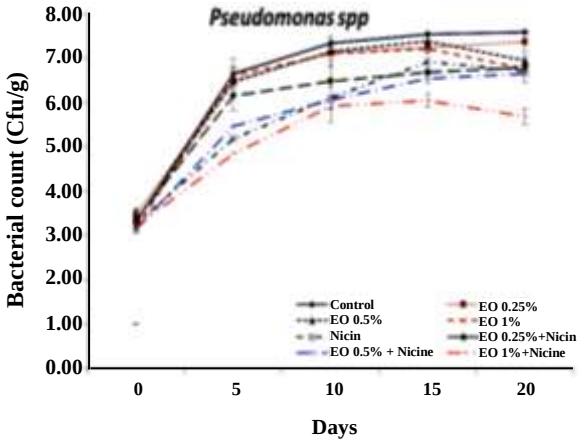

Fig. 6: Pseudomonas spp changes in different treatment groups (control, ZEO, Nisin) of fish burger kept during storage at 4ºC.

Pseudomonas spp

The initial Pseudomonas spp counts of samples were <2 log10 cfu/g in all fish burgers (fig. 6). Load of *Pseudomonas spp* gradually increased with an increase in storage time, The result of PS was approximately the same as result of PCC changes. Logarithmic changing of the bacterial count wasn't same in the different groups during the period of study ($p<0.001$). However, the increase rate in A, B, C and D were and higher than E, F, and G and their value were higher than the H treatments sample ($P< 0.05$). *Anzabi et al.* 2013 reported the minimum inhibitory concentration of ZEO was 250 µg/mL for most of the gram-negative bacteria unless for *Pseudomonas aeruginosa* [61]. These findings were also parallel to the results of (PBC) changes during in this study. This fact confirms the low effects of ZEO on inhibition PS growing and presents synergistic antimicrobial effect between ZEO and nisin. According to bacterial result of Another study, *Soltanian et al.* 2011 reported that Nisin could be used to extend the shelf-life of filleted burger (rainbow trout) [36] and *Mirshekari* found that using the Nisin incorporation of other preservative compounds decreased the bacterial loading at the end of stage(16th day) [62], that these results are consistent with the present study.

CONCLUSIONS

As a conclusion, this research reported that the application of ZEO incorporated with Nisin resulted in extension of shelf life of rainbow trout fish burger and can be usefully applied in food industries. The results of this study found that microbiological and chemical characteristics of these treated fish burger with NISIN plus ZEO were better than those of control samples during refrigerated storage. The higher concentrations of ZEO cooperated with Nisin resulted in stronger antimicrobial and antioxidant activities. These natural components can be used for extending the shelf life of fish burgers during refrigerated storage.

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