Urtica diocia extract-mediated biosynthesis of nanoemulsion as antimicrobial products effected on *Sander lucioperca* at Refrigerator temperature

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Abstract

The presence of fish in the family food basket is one of the important goals in completing the food regime, but as fish are highly perishable, use of coverage or active packaging could be benefit. In this research, in order to determine the shelf life of the fish, the effect of nettle extract loaded nanoemulsion and storage temperature (4 and 8 ° C) was investigated. To evaluate the effect of temperature and presence of nanoamulsion, various factors such as chemical, microbial and sensory factors were investigated. The samples were divided into 5 groups, which included control treatment, loaded samples with nanoemulsion containing 1, 2, 3 and 4% nettle extract and each of these samples kept the fresh fish separately at temperatures of 4 and 8 ^{o}C. The study of the effect of treatments on chemical properties, peroxide value, total volatile nitrogen (TVN) and microbial characteristics (counting total and microscopic microorganisms) showed that using 4% nanoemulsion of nettle could produce proper properties within 14 days, as well as in the sensory evaluation, which the results also confirmed it. Therefore, the use of nettle nanoemulsion at a level of 4% allowed for 14 days keeping the fish at 4 ° C in optimal quality.

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Keywords: *Urtica diocia*, nanoemulsion, Emulsion Phase Inversion, Shelf life, *Sander lucioperca*

Introduction

Fresh fish or ready to use fish product is one of the most susceptible one to spoilage protein types owing to post-mortem bacteria growth, biochemical repercussion and the rapid

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bacteriological degradation (Reddy, Schreiber, Buzard, Skinner & Armstrong 1994). This procedure returns to deterioration activity of sea food microorganisms such as *Pseudomonas fluorescens* (Gram and Dalgaard 2002) as aerobic fish pathogen, *Listeria monocytogenes* (Min and Oh 2009) and the bacteria, *Aeromonas hydrophila* and *L. monocytogenes* which are able to grow at the refrigerated temperature (Nedoluha & Westhoff 1997). Due to the rising request for consumption of minimally processed fish, obviously natural antimicrobial compositions have been recommended to be utilizing in food industry as additive stabilizers (Holley & Patel 2005). Herbal medicine and other plant extracts have played antimicrobial role against a couple variety of food bacteria and spoilage microorganisms (Tajkarimi, Ibrahim & Cliver 2010). Extractions from plants such as Rosemary, thyme and citrus fruits and etc. have been more considered by scientists for controlling food microorganisms in food packaging or freshly use (Iturriaga, Olabarrieta & de Marañón 2012). *U. dioica* have shown to be used effectively in folk medicine in Turkey and other Asian countries (Erdogrul 2002, Ganie, Ghani & Nissar 2013). Isomeric phenolic reagents such as thymol, eugenol and carvacrol of essential oils (Michiels, Missotten, Fremaut, De Smet and Dierick 2007) and flavonoids (Rattanachaikunsopon and Phumkhachorn 2010) extracted from plant were found to possess antimicrobial properties. Hopefully, results gained from studies encourage food industries to incorporate herb and plant extracts into food packaging and films or as edible emulsioning layers developing shelf-life of perishable food such as fresh or minimally processed fish in inhibiting the microbial growth (Gurib-Fakim 2006). The antimicrobial materials are incorporated into food packaging may gradually migrate to the surface of the food, reacting and inactivating by the ingredients. For this reason, the minimum inhibitory reaction will be needed to prevent some chemical reactions and losing antibacterial properties (Appendini & Hotchkiss 2002). Neetle (*U. dioica*) classified to the Urticaceae, grows and find throughout the world, particularly from Asia, showed high nutritional values with potential antioxidant activity (Alp & Aksu 2010; Rutto, Xu, Ramirez & Brandt 2013). *Sander lucioperca*, which distributed in lakes of Turkey and Iran, is a commercially chief freshwater fish due to its desirable taste with high value compared with others at the same habitat (Özyurt, Özogul, Özyurt, Polat, Özogul, Gökbulut, Ersoy & Küley 2007).

The purpose of this study was to evaluate the properties of *S. lucioperca* fish covered with nanoemulsion of *U. dioica* essential oil at various levels. For this purpose, chemical, microbial and sensory factors were investigated and the duration of storing fish was measured at temperatures of 4 ^{o}C and 8 ^{o}C.

Materials and methods

Chemical materials

Boric Acid, Hydrochloric Acid, Alcohol, Plate Count Agar, NaCl, Magnesium Oxide, Chloroform and Sodium hydroxide were the compounds used in this study (Merck,

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Germany). Nettle extract was obtained from the Kazeroon Agro-industry Company. The extract had an expiration date of 2 years at a temperature of 25 °C and indicated should be far from light.

Preparation of nanoemulsion through the Emulsion Phase Inversion (EPI)

This method involved adding an organic phase to an aqueous phase, which usually contained water and surfactants that was easy to perform. In order to synthesize nanoemulsion, the aforementioned method was used. According to the hydrophilic nature of the extract, a binary emulsion method was used. This means that the aqueous-phase extract at different levels (0, 1, 2, 3 and 4%) was first placed in a surfactants mixture of Spen-80 and Tween-80, with a ratio of 75 to 25, and dichloromethane was used as an outer phase (oil phase). The product was then added to the aqueous phase (containing PVA 1%, Tween-80 10%) to form an emulsion. After 48 hours, the product OF LDPE (Low Density of Polyetylene) was placed under vacuum-oven condition at 40 °C to remove dichloromethane (Khajouei, Peyravi & Jahanshahi 2017).

Determination of nanoemulsion characteristics

In this way, particle size, particle size distribution and particle diffusion index were measured based on the intensity of the transmitted light scattering (caused by the Brownian motion of particles) (Xu 2015).

Sampling method

In order to load the emulsion for the fillets of fish, *S. lucioperca* and to examine required test, each fillet in triplicate was immersed in prepared nanoamulsions at different concentrations for 3 minutes and then folded in polyethylene bags at 4 °C and 8 °C for 14 days (Iturriaga et al. 2012).

Microbiological assays

Total counts of aerobic mesophilic microorganisms

Plants count agar medium (PCA) was used for total counting of aerobic microorganisms. 0.1 milliliter of various dilutions was adding to the surface of the medium and completely spread over the plate surface with a sterilized L-shaped rod. Plates were then incubated at 35 °C for 48 hours (Hozbor, Saiz, Yeannes & Fritz 2006).

Psychrophilic Bacteria count

Similar to the cultivation of mesophilic bacteria, PCA medium was used for culture of psychrophilic bacteria. Next, 0.1 ml of various dilutions were poured onto the medium and distributed on the surface of the medium. it was then incubated at 5-6 °C for 10 days (Mol, Erkan, Uecok & Tosun 2007).

Chemical assay

Peroxide value (PV) determination

One gram of the sample was previously weighed and consequently added in centrifugal tubes and mixed with 11 ml of chloroform solution. Methanol with a ratio of 2 to 1 was added and the compound homogenized for 2 minutes at a centrifuge of 13500 rpm. The homogeneous solution was then straightened using Whatman's No 1 filter paper. Seven ml of the post-filtration

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solution was added to the centrifuge tubes, 2 ml of 0.5% sodium chloride solution added and mildly mixed. Samples were centrifuged at 3000 g for 3 minutes, until the intravenous solution became biphasic. Three milliliter of the lower phase mixed with 2 ml of a cold chlorine-methanol mixture, 25 ml of 30% ammonium thiocyanate and 25 ml of iron chloride and finally the test tubes were kept at room temperature for 30 minutes (Mureşan, Muste, RACOLTA, Semeniuc, Man, Birou & Chircu 2010). Finally, absorbance of the samples was read at 500 nm against a blank that whole compound included except for the sample, using a spectrophotometer (UV-VIS 1700 Shimadzu).

Total Volatile Nitrogen (TVN) measurement

Ten grams of fresh fish samples were mixed with 50 ml of distilled water, the resulting mixture was transferred to 200 ml of distilled water to the round-bottomed flasks and 2 g of magnesium oxide and a silicon droplet (as anti-foam) were added. The 250 ml Erlenmayer flask containing 25 ml of boric acid 3%, 0.04 ml of mixer of methylene blue - methyl red (1:2 v/v) reagents was used as a marker for volatile ammonia titration. The distillation continued until the volume of the distillation phase reached 125 ml. When Boric acid solution distilled with volatile nitrogen, it became alkaline and the color changed to green. This mixture was titrated with 0.1 HCl solution (Goulas & Kontominas 2007). Equation for calculating the amount of TVB-N is given below:

$$\text{TVB-N (mg } 100g^{-1}) = (V \times C \times 14) \times 100/10$$

As V is the amount of chloride acid and C is its concentration

Sensory analysis

Sensory evaluation of color, odor, texture, and general acceptance of *S. lucioperca* on day 14 was carried out by five evaluators who were trained in food industry. Raw fish samples were named with random codes and were evaluated in the same conditions by panels, and a 5-point scale of Hedonik method was employed for sensory evaluation (Saloko, Darmadji, Setiaji & Pranoto 2014).

Statistics analysis

The amounts of the bacteriological index corresponding to microbiological and sensory assay were globally compared by One-Way ANOVA and controlled with bonferroni's multi comparison test, which was a pairwise comparison of the means defined at a 95% confidence level for the treatments. To this purpose, SPSS for Windows 18.0 (SPSS Inc., Chicago, IL, USA) was used.

Results

As shown in Fig. 1, all treatments had a particle size distribution of less than 200 nm, and there were little differences among the treatments ($p>0.05$). The results showed the stability of the nanoemulsion for use in fish *S. lucioperca*. In fact, the stability of the nanomulsion controls the release of the extract on the surface of the fish and improves its effect.

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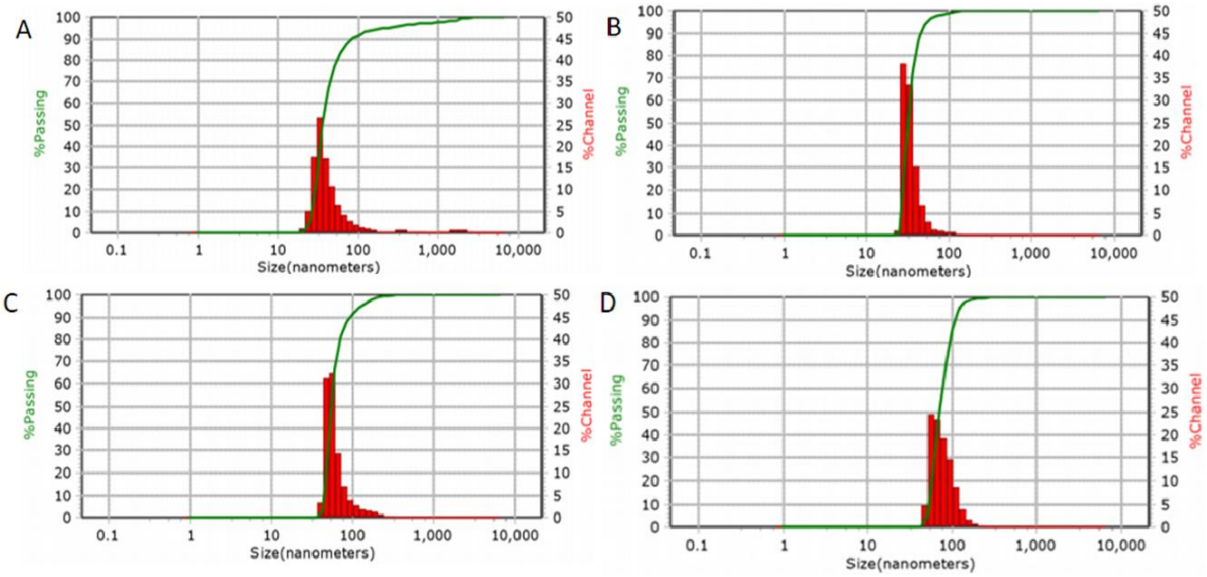


Figure 1. measuring the diameters of nanoemulsion particles in different treatments. A: Nanomulsion containing 1% extracts, B: Nanomulsion containing 2% extracts, C: Nanomulsion containing 3% extracts, D: Nanomulsion containing 4% extracts.

According to Figure 2 and regardless to the concentration of nanoemulsion emulsioning of nettle extract, less temperature (4 ^{O}C) showed more antibactericidal properties ($p<0.05$) compared with those of temperature of 8 ^{O}C for all the treatments. After three days passed the experiment, there was no significant ($p>0.05$) concerning the antimicrobial activity among the treatments nanoemulsion loaded of nettle extract with 2%, 3% and 4%. After 7 days, the 1% and 2% of nettle extract- loaded nanoemulsion presented no significant difference ($p>0.05$) as well as that of 3% and 4% of nanoemulsion. After 14 days, the results showed rather difference among the four aforementioned treatments ($p<0.05$). The least antimicrobial activity occurred after 3 days of the experiment, with considerable difference with control group ($p<0.05$) when the value of treatments 1-3 was about 4.2 log CFU g^{-1}.

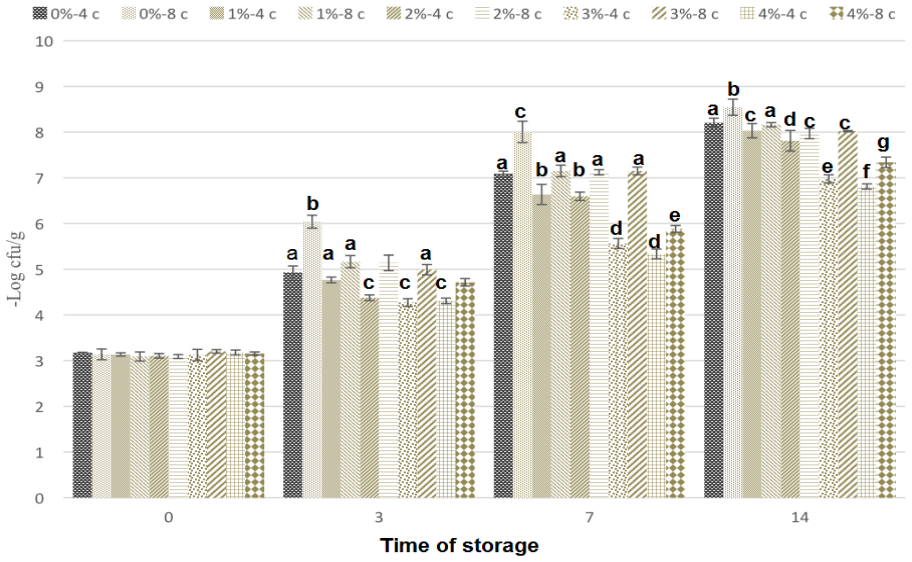


Figure 2. Results of counting aerobic mesophilic bacteria. Different superscripts indicate a significant difference between treatments ($p<0.05$).

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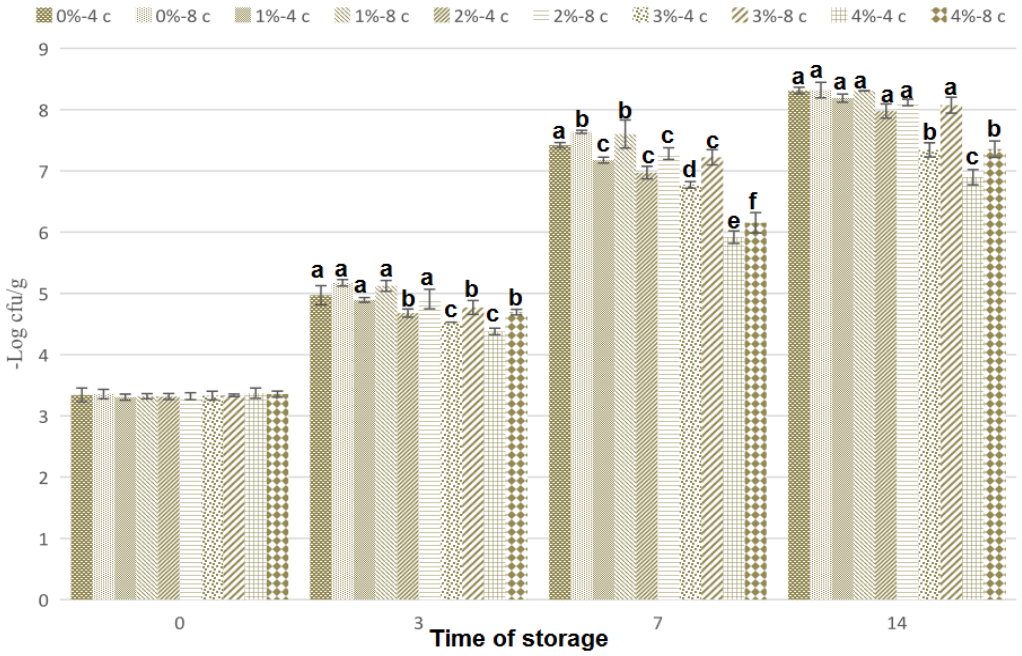


Figure 3. Results of counting aerobic psychrophilic bacteria. Different superscripts indicate a significant difference between treatments ($p<0.05$).

Based on the results of Figure3 and irrespective to the concentration of nettle extract-loaded nanoemulsion, the CFU g^{-1} for the treatments measured at temperature of 4 ^{O}C were more than that of evaluated for 8 ^{O}C ($p<0.05$). For the treatments conducted at 4 ^{O}C, the CFU g^{-1} represented a decrease trend in a concentration-dependent manner with a few exceptions. The minimum CFU g^{-1} was gained on the day 3 was measured about 4.3 log CFU g^{-1} for the treatment of nettle extract 4% with no significant difference against to that of extract 3% ($p>0.05$). This pattern was continued during 4 and 7 days.

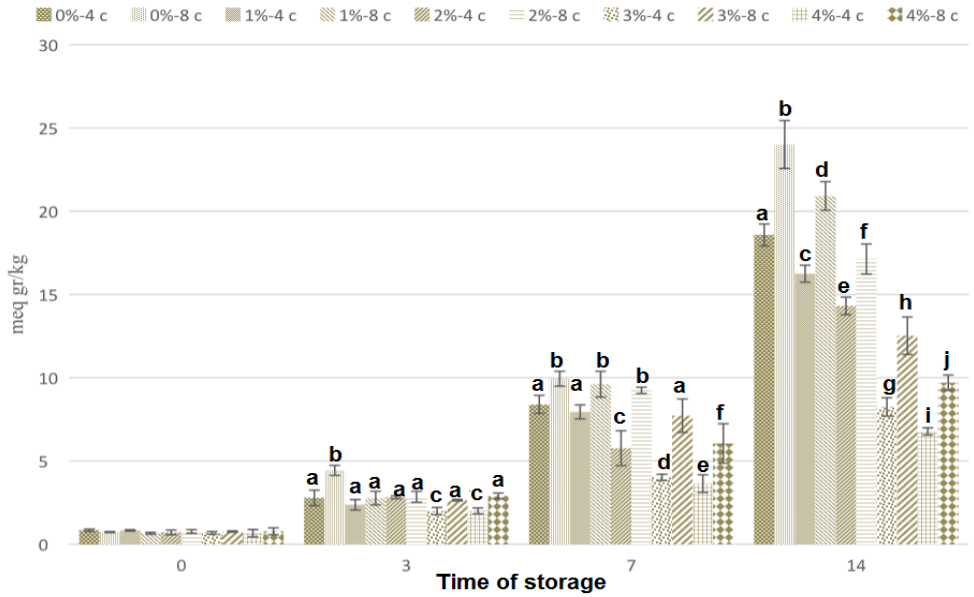


Figure 4. Results of peroxide values at different concentration of nanoemulsion. Different superscripts indicate a significant difference between treatments ($p<0.05$).

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The peroxide values for all treatments decreased in a concentration-dependent manner for 7 and 14 days of the experiment but was not similarly occurred after 3 days of the study (Figure 4). The peroxide value showed no significant difference among the treatments as well as control exception for the treatments of 3% and 4 % of groups. The minimum value of peroxide was measured for the two former groups (about 2.4 meq gr kg^{-1}) with slight difference with other groups.

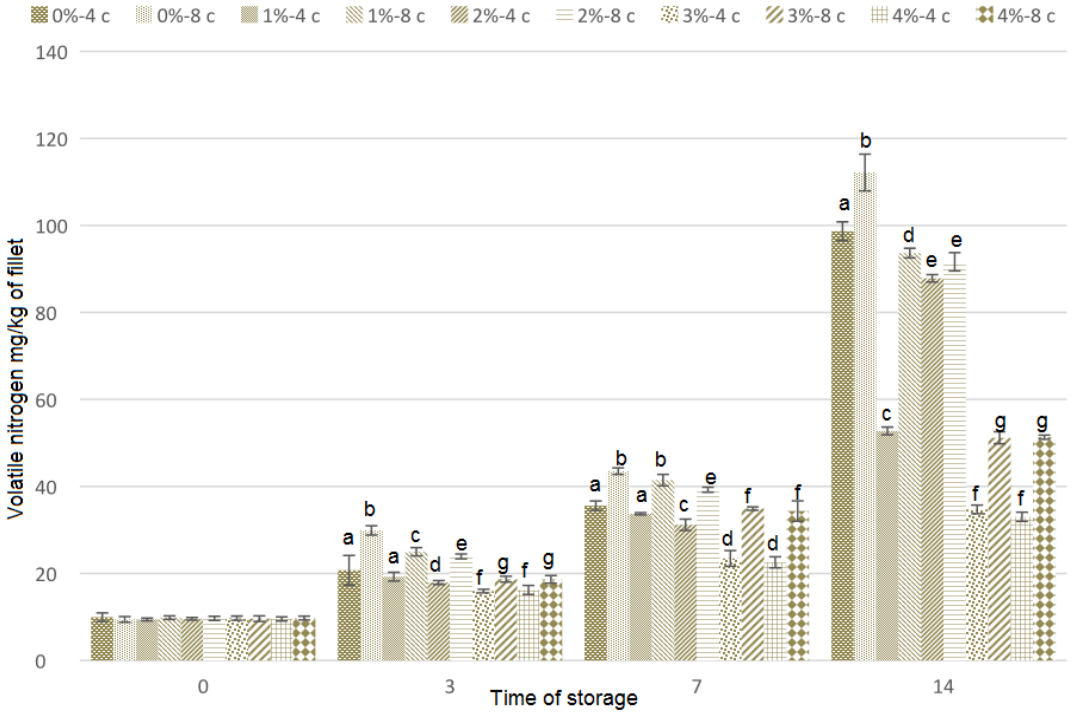


Figure 5. Results of volatile nitrogen at different concentration of nanoemulsion. Different superscripts indicate a significant difference between treatments ($p<0.05$).

The minimum value measured at 3 days post experiment for the 3% nanoemulsion (about 18 mg kg^{-1} of fish fillet) with no significant difference ($p>0.05$) compared with that of 4% nanoemulsion. This pattern was followed at 7 and 14 days for 3% and 4% nanoemulsion.

In order to evaluate the sensitivity of the four factors, the flavor, color, texture and total acceptance of fish were examined (table1). The evaluation of flavor characteristics on day 14 showed that all samples, which were stored at 8 °C had poor quality. But the fish fillets were kept at a temperature of 4 °C and loaded with nanoemulsion containing 3% and 4% of the nettle extract, significantly ($p<0.05$) prevented the production of amine compounds and undesirable odor. On the other hand, in terms of color, these two samples also had good ranks that could be due to prevention of myoglobin oxidation and emittance reaction. However, with respect to fish texture, whole the samples showed texture defects and were not sufficiently acceptable for panelists. But in general, a sample containing 3% and 4% nanoemolsion loaded of nettle extract were acceptable for general acceptance.

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Table 1. Panelist values for the sensory evaluation after 14 days of the experiment

Emulsioning% /Temperature	Odor	Color	Texture	General Acceptance
Control, 4ºC	1.8 ± 0.4a	3.3 ± 1.7[a]	2.2 ± 0.8[a]	2.4 ± 0.4[a]
1%, 4ºC	2.0 ± 0.0a	3.0 ± 0.7[a]	2.8 ± 0.5[b]	3.4 ± 0.5[b]
2%, 4ºC	3.2 ± 0.4[b]	3.4 ± 0.5[a]	3.2 ± 1.4[c]	3.3 ± 0.3[b]
3%, 4ºC	3.6 ± 05[c]	3.6 ± 0.4[a]	2.8 ± 1.8[b]	3.3 ± 0.4[b]
4%, 4ºC	3.8 ± 0.4[c]	3.6 ± 0.5[a]	3.8 ± 1.6[d]	3.8 ± 0.5[c]
Control, 8ºC	1.6 ± 0.5[a]	2.8 ± 0.4[a]	2.2 ± 0.4[a]	1.6 ± 0.5[a]
1%, 8ºC	1.4 ± 0.4[a]	3.4 ± 0.5[b]	2.0 ± 1.0[a]	2.2 ± 0.8[b]
2%, 8ºC	3.0 ± 0.7[b]	3.2 ± 0.4[b]	2.2 ± 0.8[a]	2.8 ± 0.4[c]
3%, 8ºC	3.0 ± 0.0[b]	3.4 ± 0.4[b]	2.4 ± 0.4[a]	2.8 ± 0.4[c]
4%, 8ºC	3.0 ± 1.7[b]	3.2 ± 0.4[b]	2.2 ± 0.5[a]	3.0 ± 0.7[c]

Discussion

This article focus on the effect of nanoemulsion loaded with *U. diocia* extract on fillet of fish *S. lucioperca* through 14 days. Alp Erbay, Dağtekin, Türe, Yeşilsu and Torres-Giner (2017) represented that preserved fresh fish fillets has not properly shown mesophilic and psychrophilic bacteria growth when were emulsion loaded with *U. dioica* water extract.

Li and Peng (2015) showed the use of chitosan nanofiber enhanced the antimicrobial activity when used as layer on the surface food. The explained due to hydrophilicity and positive charge of *E. coli*, the effect of chitosan nanofiber could be enhanced. The findings of this study showed that the temperature of 8 °C encouraged the spoilage microorganisms to rather growth on the surface of foods in contact with the nanoemulsion.

The study of Alp Erbay et al. (2017) concentrated on Rainbow trout showed a 2 log CFU g^{-1} cycle of bacteria on second days of the exposure with an increase of 3.79 log CFU g^{-1} on the 3rd day. the fillets tainted on the 7th day with highest level of 6.27 log CFU g^{-1} while our findings showed 4.2 log CFU g^{-1} and 5.3 log CFU g^{-1}, respectively on 3rd and 7th days, which showed the more effectiveness of 4% *U. dioica* after 7 days (Mol et al. 2007). All these studies confirmed that incorporation of *U. dioica* L. either in the form of water extract or nanoemulsion extended the shelf life of the minimum processed stored fish fillets.

The nettle extract with concentrations of 2-4 % showed effectively diminution the growth (about 4.3 log CFU g^{-1}) of fish spoilage bacteria. From 3 to 7 days of storage at 4 °C, only a cell load increase of about 1 log cycle for the samples obtained that could be due to the ability of nettle loaded nanoemulsion inhibited the growth of the microorgnisms. Iturriaga et al. (2012) showed that biopolymer active films with extract had antimicrobial properties against the bacteria *L. innocua* at 4 °C as a synergic outcome, between temperature depletion and the use of essential oil of herbs, indicating that the use of the extract with fish filet could be useful for controlling the growth of bacteria.

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In fact, samples (treated and untreated) stored at 8 °C after 7 days could not be used and showed high microbial growth. On the other hand, treated samples (nanoemulsion containing 1, 2, 3 and 4% nettle extract) kept at 4 °C properly able to control the microbial growth for 7 days and keep it within range (Respectively, 7.1, 6.9, 6.7, and 5.33 log CFU g^{-1}). of course, the least CFU g^{-1} value at 7 days and 4 °C was gained (about 5.33 log CFU g^{-1}) for the treated fillet with 4% of nettle extract that meant the fillet can be stored for 3 days with 1-4 percent of nettle extract-loaded nanoemulsion but if the strategy would be on increase of shelf life for more storage time of fish fillet, only 4% of *U. diocia* extract can be loaded to nanoemulsion. The findings of peroxide value and violate nitrogen also confirmed the abovementioned result of CFU g^{-1} so that they showed the storage time of *S. lucioperca* fillet can be increased up to 7 days.

The sensorial indicators containing odor, color and texture of the *S. lucioperca* fillet got appropriated scores from panelists after 14 days. Similarly, Gharibzahedi and Mohammadnabi (2017) confirmed that Beluga sturgeon fillets emulsioned with 2% loaded-nanoemolsion containing of nettle extract gained the 'acceptable' scores for overall sensorial assays by the 7th day.

Similar results were obtained in a study conducted by Shadman, Hosseini, Langroudi and Shabani (2017). it was found that after 15 days, the control sample had 11 meq g kg^{-1} of hydro peroxide, while this was 4.2 meq g kg^{-1} for the sample emulsioned with nanoemulsion containing 1 percent of thyme essential oil.

This is concluded that the nanoemulsion loaded with *U. diocia* 4% can be an appropriate compound as antibacterial use to preserve the fish *S. lucioperca* at refrigerator temperature for 7 days.

Conflict of interests

The authors declare that there is no conflict of interest.

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بیوسنتز عصاره گزنه توسط نانوذرات نقره به عنوان محصولی ضد میکروبی مؤثر روی ماهی سوف معمولی در دمای یخچال

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چکیده

حضور ماهی در سبد غذایی خانوار یکی از اهداف مهم در تکمیل رژیم غذایی است، اما بعلت فسادپذیری بالای ماهی‌ها، از پوشش‌ها و بسته بندی فعال برای این محصولات استفاده می شود. از این رو در این پژوهش اثر پوشش نانوامولسیون عصاره گزنه در دمای نگهداری (۴ و ۸ درجه سانتی‌گراد) به منظور تعیین طول مدت نگهداری ماهی سوف معمولی در یخچال مورد بررسی قرار گرفت. برای ارزیابی تأثیر دما و حضور پوشش نانوامولسیون، عوامل مختلفی مانند عوامل شیمیایی، میکروبی و حسی مطالعه شد. نمونه ها به ۵ گروه تقسیم شدند که شامل گروه شاهد و نمونه های پوشش داده شده با نانو امولسیون حاوی ۱، ۲، ۳ و ۴ درصد عصاره گزنه بودند. هر یک از این نمونه ها به صورت جداگانه در دمای ۴ و ۸ درجه سانتی گراد نگهداری شدند. مطالعه اثر تیمارها بر خواص شیمیایی (ارزش پراکسید و مقدار کل نیتروژن) و ویژگی های میکروبی (شمارش کل و میکروارگانیسم های میکروسکوپی) نشان داد که استفاده از نانو امولسیون حاوی ۴٪ عصاره گزنه می تواند خواص مناسب را ظرف ۱۴ روز ایجاد نماید، داده های ارزیابی حسی نیز این نتایج را تأیید کرد. بنابراین، استفاده از نانو امولسیون گزنه در سطح ۴٪، می‌تواند کیفیت مطلوب فیله ماهی را در دمای نگهداری ۴ درجه سانتی‌گراد به مدت ۱۴ حفظ نماید.

کلمات کلیدی: گزنه، نانوذرات نقره، وارونگی فاز امولسیون، طول دوره نگهداری، سوف معمولی

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