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Antibacterial Impact of Methanol Extract (*Eryngium Caeruleum*) on Escherichia Coli and Staphylococcus Aureus in a Food Model at 4 °C

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ABSTRACT— In this study, which was an interventional research, impact of Eryngium Caeruleum in 5% and 10% concentrations on bacteria Escherichia Coli and Staphylococcus Aureus in soya cheese (tofu), to each gram of which 106 bacterium cell was added, was studied at 4°C in a 15-day period. Eryngium Caeruleum was collected from Javaherdeh Heights, Ramsar Town. Extract of Eryngium Caeruleum was prepared from drenching dried Eryngium Caeruleum in 85% methanol, and soya cheese was prepared from conclusion of soya milk by calcium sulfate. Results of this evaluation were analyzed by one-way variance method. In 4°C, Staphylococcus Aureus confronted a 2 and 4 logarithmic reduction in both concentrations after 15 days. Antibacterial impact of 10% Eryngium Caeruleum on Staphylococcus Aureus in 4°C was higher than the 5% concentration. Escherichia Coli had a 1 logarithmic reduction after 15 days in both concentrations. Statistically, it had no significant difference (P>0.05). Generally, results are indicative of the fact that Eryngium Caeruleum has antibacterial effects on both Staphylococcus Aureus and Escherichia Coli. Moreover, its impact on gram-positive bacteria was by large higher than that on gram-negative bacteria.
Key words: Staphylococcus Aureus; Escherichia Coli; Eryngium Caeruleum; methanol extract; soya cheese

Introduction

The 21st century has been named as the century of returning to the nature and consuming natural and plant materials in treatment. This is made possible as a consequence of acknowledgement of side effects of many artificial materials manufactured by humankind (Safaei, 2004). In the recent years, resistance against chemical materials in both human beings and plant pathogens due to excessive use of commercial antibacterial medications and chemical materials applied in treatment of infectious diseases has been evident (Baratta et al., 1998; Bax et al., 2000; Gulluce et al., 2007; Sadeghi-nejad, 2010; Yadegarinia, 2006). On the other hand, diseases ensuing from food materials have a serious problem in all countries of the world even such developed countries as the US. Corruption in food materials ensuing from all types of microorganisms is a major concern borne by well-known food companies. Pollution from raw and/or processed materials with microflora might happen at different processing phases including manufacturing to distribution. Thus, food industry currently makes use of chemical preservatives to prevent development of factors causing food spoilage (Gulluce et al., 2007). Since bacterial resistance against chemical materials is on the rise, measures should be taken to reduce bacterial resistance, which is one of the ways to discover new drugs from natural resources (Baratta et al., 1998). *Eryngium caeruleum* belongs to parsley family that has a relatively wide range of dispersion in northern Iran (Khoshbakht et al., 2007; Morteza Semnani et al., 2003). In some regions of Iran, leaves of some species are used in salads, foods, and traditional medication. Some species are known as diuretic factors (Khoshbakht et al., 2007; Saeeidi and Morteza-Semnani, 2008). This plant is a thorny one whose thorns are colored in light blue. Species of this plant are appetizer, laxative, agents of menstruation, agents of flatulence, anti-inflammatory, healers of skin and hepatic irritations affecting on renal dysfunction and sexual organs (Morteza-Semnani et al., 2002). This plant has pain killing impacts due to its limonene compounds. Having a high food value, soya cheese (tofu) is a plant product that is generated in Southeast Asian countries since long ago (Ghanbar Zadeh, 2010). Soya cheese includes mineral materials like sodium, potassium, iron, and phosphorus and has vitamins E and B. Soya contains isoflavones, amino acids, fiber, and unsaturated fatty acids. Application of soya reduces possibility of cardiovascular diseases. This product has relatively lower degrees of calorie compared to the cheese coming from cow milk (Ghanbar Zadeh et al., 2010; Lim et al., 1990). Soya cheese does not include cholesterol and lactose. Hence, this is a good alternative for people affected by lactose intolerance and those with high cholesterol (Spern et al., 2011; Lim et al., 1990).

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Escherichia Coli is a member of enterobacteriaceae family. Most *Escherichias* are able to ferment lactose and produce gas and acid. Enterobacteriaceae is an extended family of gram-negative bacilli that is largely found in human and animal colons as a member of natural flora (Safar Zadeh and Modarres M., 2000). Most *Escherichia Coli* strains are innocuous saprophytes, but other strains cause disease in humans and animals. Diseases ensuing from *Escherichia Coli* include intestinal infections, septicemia, urinary tract infections, and mastitis. On the other hand, *Staphylococcus Aureus*, followed by *Escherichia Coli*, is the most important origin of human food pollutions (Afraz et al., 2008). Range of *Escherichia Coli* infections includes pimples and boils to toxic shock syndrome and infection, which mainly depends on several virulence factors (Tasci et al., 2011). On the other hand, some infections such as *Escherichia Coli* food poisoning rely on one type of virulence factors like staphylococcal enterotoxin (Tasci et al., 2011). According to general food health and safety experts, millions of diseases are annually caused by pathogen agents transmitted from food materials in the world, and *Staphylococcus Aureus* is regarded as one major cause of diarrhea and vomiting ensuing from consumption of food materials (Khudor et al., 2012). Since plant extracts are presently taken into consideration as a preserver of food materials from pathogens. Presently, many consumers have paid attention to flavoring properties of some extracts. Impact of this plant extract on these two bacteria in a food material like soya cheese is addressed.

Materials and Methods

To conduct this interventional study, extract of *Eryngium Caeruleum* was used. *Eryngium Caeruleum* was collected from Javaherdeh Heights, Ramsar Town, and was affirmed by the Department of Botany, Faculty of Science, and University of Shahrekord. The plant was cleansed and dried outside of the sunlight. Then, *Eryngium Caeruleum* was dried and drenched in methanol 85% for 24 hours during which it was constantly shaken. After this time, extract was filtered by filter paper (Whatman filter paper, 0.2 micron) and was placed in the temperature of 36°C. Methanol was permitted to be removed. Finally, dried extract was collected and preserved in refrigerator up to the examination. To prepare soya cheese from soya milk, drenching and grinding method was applied. To do so, 139 grams of soybean were drenched in water with the amount six times its volume for nine hours in the temperature of 20-22°C. Drenched soybeans were pulverized in 625 ml water for 4 minutes. Finally, the watery solution was filtered by chintz fabrics in order to obtain filtered soy milk. Required soya cheese was prepared from conclusion of boiled soya milk and mixing it with 2% of soya dried weight from calcium sulfate (Cai et al., 1997). It was pasteurized in the temperature of 70°C for 10 minutes. Twenty cc of homogenized cheese was poured in steel beaker. Then, 10^7 CFU/g from *Escherichia Coli*, standard strain RTCC2310, and *Staphylococcus Aureus*, standard strain ATCC6538, procured by Razi Vaccine and Serum Manufacturing Institute were prepared. Each was added to one beaker containing soya cheese to get the bacteria number to10^6 CFU/g. Methanol extract of *Eryngium Caeruleum* was added to beakers in two concentrations 5% and 10%. To prepare concentrations of extract, distilled water was added to the extract and sterilized by 0.45 micrometer syringe filter. For each group of concentration, two controls were regarded. The first control included soya cheese, the intended bacterium without extract, and the second control was composed of distilled water, bacterium, and *Eryngium Caeruleum* extract. Beakers under study were subjected to an atmosphere of 4°C. Bacteria were counted in 0^{th}, 3^{rd}, 6^{th}, 9^{th}, 12^{th}, and 15^{th} days on plate count agar medium. Three repetitions were considered for each group. Average of results obtained from three repetitions was analyzed using the software Sigma Stat 2.1, one-way variance, and two Dunnett and Tukey methods ($P<0.05$).

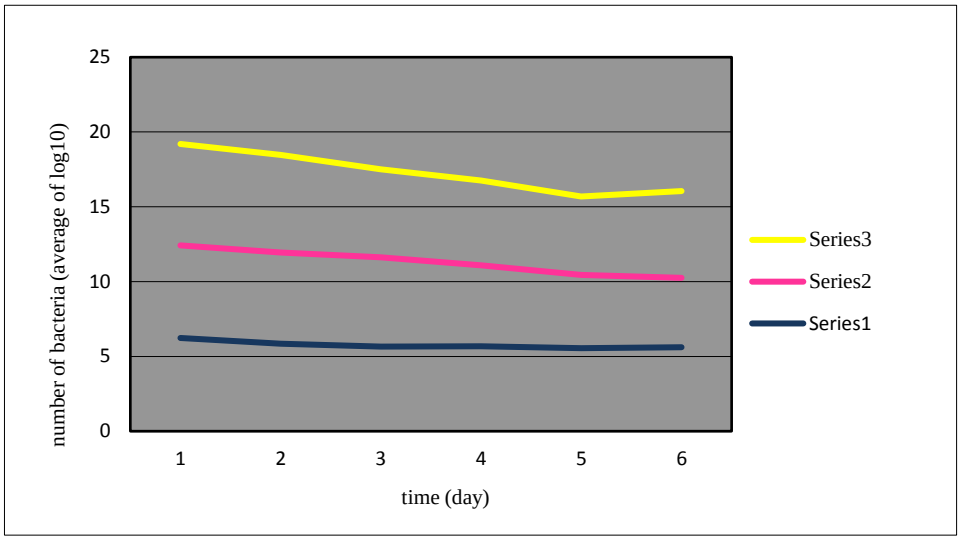
Results

Impact of *Eryngium Caeruleum* extract in 5% and 10% concentrations on *Escherichia Coli* in the temperature of 4°C is displayed in Table 1 and Graphs 1, 2, and 3. Number of *Escherichia Coli* bacteria in presence of 5% *Eryngium Caeruleum* extract decreased from 0^{th} to 3^{rd} day in a 1 logarithmic reduction. Then, however, this number remained almost fixed. In control sample 1, which included water, 5% *Eryngium Caeruleum* extract, and *Escherichia Coli* bacteria, number of bacteria was almost permanent from 0^{th} to 3^{rd} day. Afterwards, there was a 1 logarithmic reduction until the 6^{th} day, the amount which remained fixed until the 9^{th} day. Then, number of bacteria experienced a 1 logarithmic reduction from 9^{th} to 12^{th} day, the amount which remained fixed until the15^{th} day. According to the Table 1, there was no significant statistical difference between two groups ($P>0.05$). In the sample under investigation of 10% *Eryngium Caeruleum* extract, number of bacteria was fixed from 0^{th} to 3^{rd} day, after which it faced a 1 logarithmic reduction. Then, from 6^{th} to 9^{th} day, there was a 1 logarithmic increase in the number of bacteria, the amount which remained permanent until the 12^{th} day. There was a 1 logarithmic reduction, then, until the 15^{th} day. In control sample 2, there was a 1 logarithmic reduction from the 0^{th} to 3^{rd} day, the amount which remained fixed until the 9^{th} day. Afterwards, from the 9^{th} to 12^{th} day, bacteria were not segregated. In the sample 3, number of bacteria was fixed from 0^{th} to 3^{rd} day, after which it faced a 1 logarithmic reduction until the 6^{th} day. It remained fixed until the 15^{th} day. According to the Graph 3, there was no significant statistical difference between two groups ($P>0.05$). In Graph 3, a comparison is made between impact by 5% and 10% *Eryngium Caeruleum* extracts on *Escherichia Coli.* According to this graph, there was no significant statistical difference between two groups ($P>0.05$).

Table1: impact by 5% and 10% *Eryngium Caeruleum* extracts on *Escherichia Coli* in the temperature of 4°C (number of bacteria in terms of CFU/g and time in terms of days)

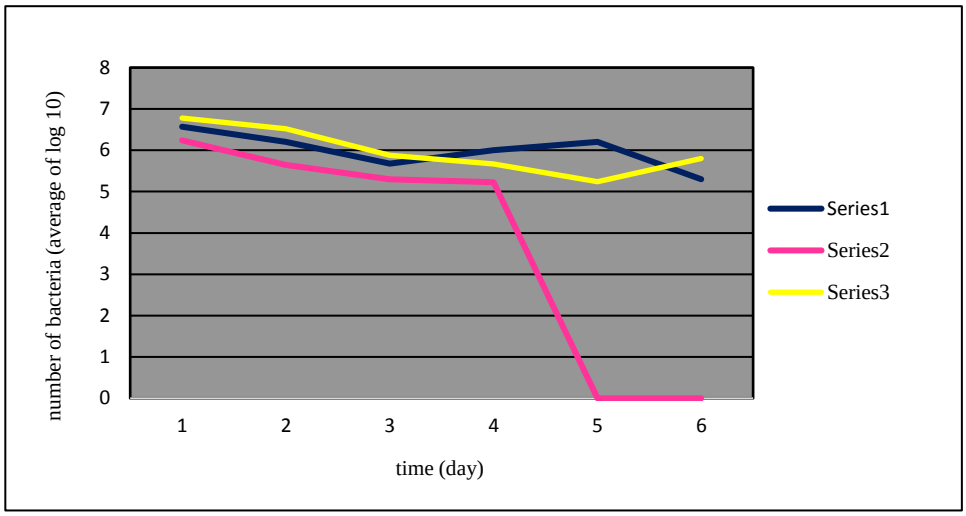
Sample	**Cheese+5% *Eryngium Caeruleum*+ *Escherichia Coli***	**Control 1**	**Cheese+10% *Eryngium Caeruleum*+ *Escherichia Coli***	**Control 2**	**Control 3**
Days					
0	1.69×10^{6}	1.56×10^{6}	3.746×10^{6}	1.73×10^{6}	6.03×10^{6}
3	7.16×10^{5}	1.14×10^{6}	1.583×10^{6}	4.43×10^{5}	3.30×10^{6}
6	4.53×10^{5}	9.10×10^{5}	4.65×10^{5}	0.016×10^{5}	7.56×10^{5}
9	4.66×10^{5}	2.59×10^{5}	1.01×10^{6}	1.70×10^{5}	4.70×10^{5}
12	3.586×10^{5}	7.853×10^{4}	1.62×10^{6}	0	1.73×10^{5}
15	4.16×10^{5}	4.26×10^{4}	1.99×10^{5}	0	6.20×10^{5}

Control 1 → water + 5% *Eryngium Caeruleum* + *Escherichia Coli*
Control 2 → water + 10% *Eryngium Caeruleum*+ *Escherichia Coli*
Control 3 → cheese+ *Escherichia Coli*



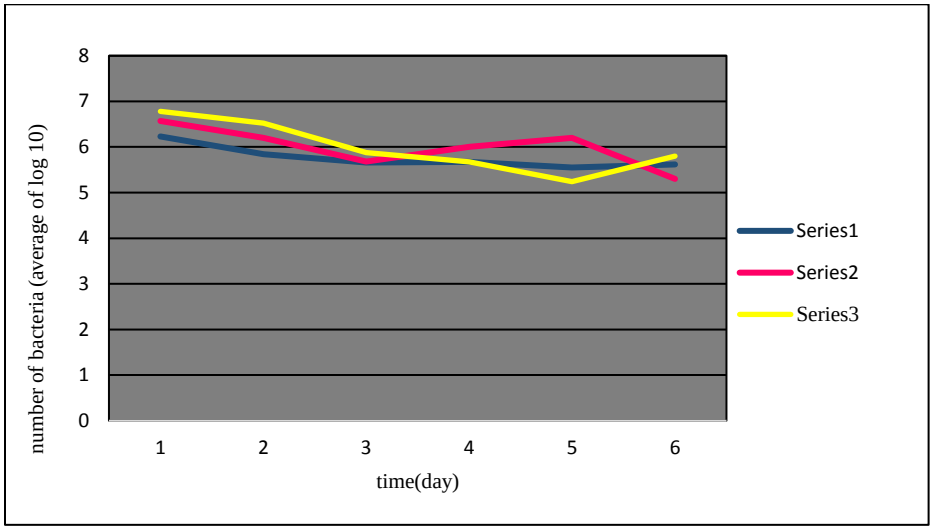
Graph 1: impact by 5% Eryngium Caeruleum methanol extract on Escherichia Coli in soya cheese in the temperature of 4°C

Control 1 → water + 5% *Eryngium Caeruleum+ Escherichia Coli*
Control 2 → cheese+ *Escherichia Coli*



Graph 2: *impact by 10% Eryngium Caeruleum methanol extract on Escherichia Coli in soya cheese in the temperature of 4°C*

Control 1 → water + 10% *Eryngium Caeruleum+ Escherichia Coli*
Control 2 → cheese+ *Escherichia Coli*



Graph 3: *impacts by 5% and 10% Eryngium Caeruleum methanol extracts on Escherichia Coli in soya cheese in the temperature of 4°C*

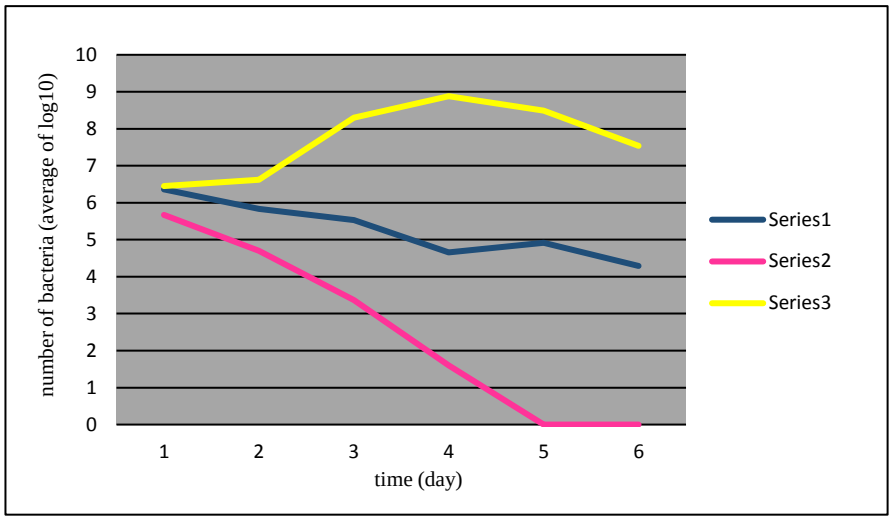
Control 2 → cheese + *Escherichia Coli*

In table 2 and graphs 4, 5, and 6, impacts by 5% and 10% *Eryngium Caeruleum* methanol extracts on *Escherichia Coli* in soya cheese in the temperature of 4°C are observed. With respect to the following table, 5% concentration of *Eryngium Caeruleum* causes a 1 logarithmic reduction in the number of bacteria from the 0^{th} to the 3^{rd} day. This trend remained almost fixed until the 6^{th} day, since then, from the 6^{th} to 9^{th} day, the number of bacteria experienced a 1 logarithmic reduction and remained almost permanent until the 15^{th} day. In the control sample 2, the number of bacteria reduced from the 0^{th} to 3^{rd} day in a 2 logarithmic reduction. After that, there was a decrease in the number of bacteria from the 3^{rd} to 6^{th} day. A 2 logarithmic reduction happened for the number of *Staphylococcus Aureus* from the 6^{th} to 9^{th} day, after which bacteria did not segregate. According to the Graph 4, there was a significant difference between the group with 5% concentration extract and control group 3 (cheese and bacterium) ($P<0.05$). In addition, there was a significant difference between control groups 1 and 3 ($P<0.05$). 10%-concentration *Eryngium Caeruleum* extract caused a 1 logarithmic reduction in the number of bacteria from 0^{th} to 3^{rd} day, the amount which remained almost permanent until the 6^{th} day. There was a 3 logarithmic reduction in the number of bacteria from the 6^{th} to 15^{th} day. In control sample 2, no bacterium was segregated from the 0^{th} to 3^{rd} day. In control sample 3, the number of bacteria remained almost fixed from the 0^{th} to 3^{rd} day; while, there was a 3 logarithmic increase until the 6^{th} day, the amount which remained almost permanent until the 12^{th} day. A 1 logarithmic reduction occurred afterwards until the 15^{th} day, which is justifiable according to the bacteria growth curve. According to the Graph 5, there was a significant difference in the group with 10% concentration and control group 3 ($P<0.05$). Additionally, the comparison between control 2 and control 3 had a significant difference ($P<0.05$).
Graph 6 shows a comparison between impact of 5% and 10% *Eryngium Caeruleum* extracts with control 3. According to this graph, there was a significant difference between 5% concentration extract and control 3 ($P<0.05$). And, the comparison between 10% concentration extract and control 3 had a significant difference ($P<0.05$). Statistically, there was no significant difference between 5% and 10% concentrations ($P>0.05$).

Table 2: impact by 5% and 10% *Eryngium Caeruleum* extracts on *Staphylococcus Aureus* in the temperature of 4°C (number of bacteria in terms of CFU/g and time in terms of days)

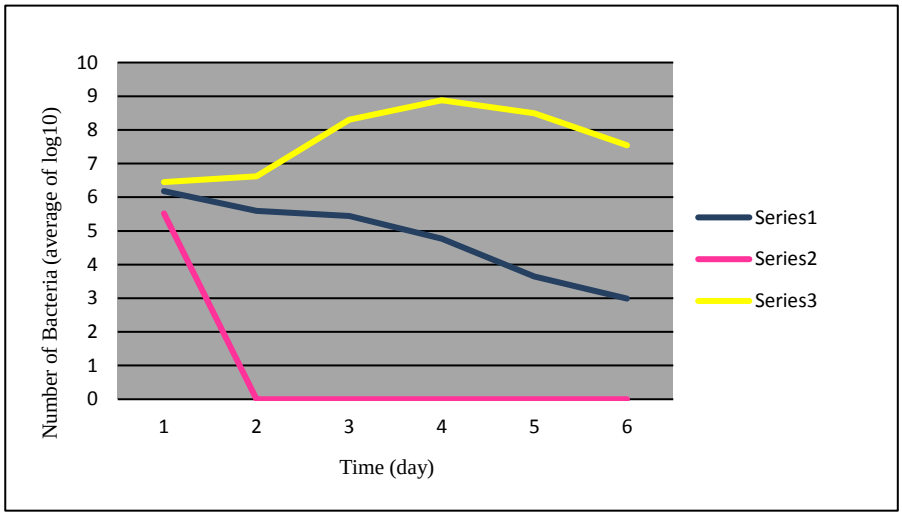
Sample	**Cheese+5% *Eryngium Caeruleum*+ *Staphylococcus Aureus***	**Control 1**	**Cheese+10% *Eryngium Caeruleum*+ *Staphylococcus Aureus***	**Control 2**	**Control 3**
Days					
0	2.316×10^{6}	4.63×10^{6}	1.51×10^{6}	3.32×10^{5}	2.793×10^{6}
3	6.79×10^{5}	5×10^{4}	3.86×10^{5}	0	4.208×10^{6}
6	3.40×10^{5}	2.33×10^{3}	2.75×10^{5}	0	2.002×10^{8}
9	4.61×10^{4}	4×10	5.85×10^{4}	0	7.60×10^{8}
12	8.313×10^{4}	0	4.46×10^{3}	0	3.06×10^{8}
15	1.97×10^{4}	0	9.80×10^{2}	0	3.50×10^{7}

Control 1 → water + 5% *Eryngium Caeruleum*+ *Staphylococcus Aureus*
Control 2 → water + 10% *Eryngium Caeruleum*+ *Staphylococcus Aureus*
Control 3 → cheese+ *Staphylococcus Aureus*



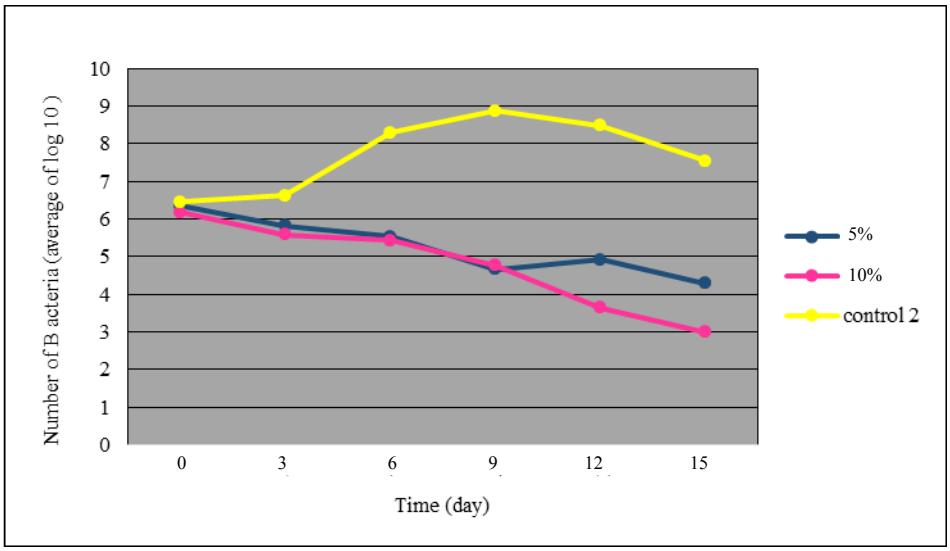
Graph 4: *impact by 5% Eryngium Caeruleum methanol extract on Staphylococcus Aureus in soya cheese in the temperature of 4°C*

Control 1 → water + 5% *Eryngium Caeruleum+ Staphylococcus Aureus*
Control 2 → cheese+ *Staphylococcus Aureus*



Graph 5: *impact by 10% Eryngium Caeruleum methanol extract on Staphylococcus Aureus in soya cheese in the temperature of 4°C*

Control 1 → water + 10% *Eryngium Caeruleum+ Staphylococcus Aureus*
Control 2 → cheese+ *Staphylococcus Aureus*



Graph 6: *impacts by 5% and 10% Eryngium Caeruleum methanol extracts on Staphylococcus Aureus in soya cheese in the temperature of 4°C*

Control 2 → cheese+ *Staphylococcus Aureus*

Discussion and Conclusion

Presently, diversified methods have been applied to control pathogen food-caused factors. One of these methods is application of additives. While, with regard to present-age genetic diversity in microbial pathogenic factors, appearance of resistant strains, and side effect ensuing from consumption of many of such additives, their replacement by other materials and methods has gained special importance. This has given rise to several studies on finding herbal additives that cause no side effects. Few studies, however, have been conducted on *Eryngium Caeruleum* as this is an indigenous plant. Most studies on different species of *Eryngium* include analysis of components in essences of these plants. Semnani et al. (2003), in their study, analyzed *Eryngium Caeruleum* compounds, and could thereby identify 12 compounds in this plant. Among these compounds, limonene (52.1%), beta-sesquiterpene phellandrene (8.1%), alpha-pinene (5.5%), and delta-2-Karen (5.3%) constitute chief *Eryngium Caeruleum* compounds. Furthermore, studies by a group of researchers have shown anti-inflammatory benefits gained by *Eryngium* species. According to these studies, extracts obtained from both root and aerial parts of eight *Eryngium* species in Turkey, including *E. Campestre*, *E. Creticum*, *E. Davisii*, *E. Falcatum*, *E. Isauricum*, *E. Kotschyi*, *E. Maritimum*, and *E. Trisectum* demonstrate anti-inflammatory and analgesic activities (Kupeli et al., 2000). Another study affirmed anti-inflammatory and analgesic properties in leaves of *Eryngium Foetidum L. (*Saenz et al., 1997*).* Safari et al. (2005) examined antibacterial effects of some species of the country's native plants against *S. iniae*. This study was performed on essences of Shirazi thyme, caraway, anaricheh, and *Eryngium campestre.* The last-mentioned plant is a species of *Eryngium.* Results of measuring size of the zone of inhibition showed that diameter of inhibition zone in streptomycin was 16.9±0.4 millimeters and that of essence of *Eryngium campestre* was 18.5±0.7 millimeters. In fact, size of non-growth haloes in this bacterium was close to streptomycin under the effect of essence of *Eryngium campestre.* No significant difference was observed among them ($p>0.05$). In this study, MIC values of essence of *Eryngium campestre* were calculated to be 0.5 microgram/liter for *S. iniae* (Safari et al., 2005). According to the results obtained from evaluation of antibacterial effect of *Eryngium Caeruleum's* alcoholic extract on *Staphylococcus Aureus* and *Escherichia Coli* in soya cheese, it was concluded that this alcoholic extract had antibacterial effect on both examined bacteria. The effect on gram-positive bacterium of the test, i.e., *Staphylococcus Aureus*, was calculated to be higher than on the gram-negative bacterium, i.e., *Escherichia Coli*. In addition, this effect was higher in 10%-concentration extract than that on the 5%-concentration one. There was no significant statistical difference between 5%- and 10%-concentration extracts on *Escherichia Coli*. Essences and extracts have natural antibacterial activities on a large number of pathogenic bacteria. Most of these compounds have a common presence of active phenolic groups in their structures. In reality, a special attention is paid to them due to having large amounts of aromatic volatile compounds that some of them make changes in food tastes (Tabatabaei Yazdi, et al., 2016; Settineri and Krassner, 2003). Impact of plant extracts on gram-negative bacteria is higher than gram-positive ones. This is possibly because of protein-lipopolysaccharide layers in peptidoglycan cell wall of gram-negative bacteria than gram-positive ones that cause a higher resistance against antibacterial compounds existing in extracts (Sahraeiyan et al., 2012). In analyzing *Eryngium Caeruleum* compounds by Semnani et al. (2003), it was revealed that around 71% of *Eryngium Caeruleum*

compounds is composed of monoterpenoids, 12.6% of sesquiterpene terpenoids, and 0.6% of de-terpenoids. Accordingly, this is possible that antibacterial impacts of this plant are caused by existence of special sesquiterpene terpenoids compounds therein. Consequently, this is proposed to conduct further studies in relation to antibacterial effects of this plant in order to either reject or affirm the outcomes gained herein. With regard to impact of this extract on organoleptic properties of soya cheese, this is safe to indicate that although taste of the cheese produced by 10%-concentration *Eryngium Caeruleum* might not be liked by all, it is consumed by the people in northern Iran who have a good acquaintance with this plant.

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