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Nanoencapsulation of astaxanthin from *Haematococcus pluvialis* using maltodextrin-sodium caseinate coating and evaluation of antioxidant and antibacterial activities of the carrier nanocapsules

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ABSTRACT

The aim of present research in the first stage was to extract astaxanthin from *Haematococcus pluvialis* using acid-acetone method and then nanoencapsulation of the pigment using maltodextrin-sodium caseinate coating. In the next step, antioxidant and antibacterial activities of nanocapsules carrying astaxanthin and the free form of the pigment was evaluated. In order to evaluate antibacterial activity of the samples, *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus iniae*, *Bacillus subtilis* (Gram positive), *Yersinia ruckeri*, *Escherichia coli* and *Enterobacter aerogenes* (Gram negative) were used. The results showed that the antioxidant activity of nanocapsules carrying astaxanthin is significantly higher than the free form of pigment ($p<0.05$); In addition, this activity was improved by increasing the concentration of samples from 100 to 200 µg/ml ($p<0.05$). By astaxanthin nanoencapsulation, the diameter of non-growth zone of the studied bacteria increased ($p<0.05$), but minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the pigment and its carrier nanocapsules decreased ($p<0.05$). According to the results of zone of inhibition, Gram positive (except *Listeria monocytogenes*) and Gram negative bacteria were resistant up to concentrations of 60 and 80 µg/ml of samples, respectively. In the following, the MIC and MBC of the pigment (free and nanoencapsulated forms) for the seven bacteria ranged from 50 to 400 and 100 to 500 µg/ml, respectively. The results of evaluation the antioxidant and antibacterial activities of nanocapsules carrying astaxanthin during storage period (30 days at 4ºC) indicated stability and no significant change of these properties ($p>0.05$). According to the values of diameter of non-growth zone, MIC and MBC, *Listeria monocytogenes* was the most sensitive bacteria against astaxanthin and its carrier nanocapsules. Based on the findings, astaxanthin extracted from *Haematococcus pluvialis* has antioxidant and antibacterial activities, and these properties are improved by the pigment nanoencapsulation using maltodextrin-sodium caseinate coating.

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1- Introduction

The use of synthetic preservatives (antimicrobials and antioxidants) in the food industry has caused concern due to the threat to the health of consumers. For this reason, food industry experts always looking for remove or reduce the use of these preservatives in food products and replace those using natural compounds. Unlike synthetic preservatives which are very harmful, there are also natural compounds (with antioxidants and antimicrobial properties) that are not only not harmful to health, but also have many benefits in maintaining health, preventing and treating various diseases. Active compounds that extracted from aquatics such as protein hydrolysate, chitosan [1], pigments [2 and 3] and etc. are this type of substances. Phycocyanin [4] and astaxanthin [5] are two pigments extracted from aquatics (microalgae) that are widely used in the food industry [6].

Astaxanthin ($C_{40}H_{52}O_4$ - 596.8 g/mol) is a red to pink pigment that is often extracted from various aquatics such as microalgae [2], fishes and sea crabs [7]. In order to extract this pigment, various solvents, acids, edible oils, microwave and enzymatic methods are used [2, 8 and 9]. Astaxanthin has eleven conjugated double bonds and two hydroxyl groups, that these bonds and ring structures are effective in determining the chemical properties and absorption of the pigment light [10]. This pigment has hydrophilic and hydrophobic characteristics and has the most biological activity compared to other carotenoids [11]. The red to pink color of astaxanthin is due to the presence of conjugated double bonds in the center of the pigment. Also, these double bonds act as very strong antioxidants; in such a way that, they react with free radicals and deactivate those [12]. According to research results, the antioxidant activity of astaxanthin is 10 times higher than zeaxanthin, lutein, canthaxanthin, beta-carotene and 100 times higher than alpha tocopherol [13]. The favorable antioxidant activity of astaxanthin has been proven in many studies [2, 5 and 14]. This pigment also has antimicrobial properties [15 and 16]. In addition to the antioxidant, antibacterial and coloring properties, astaxanthin has many benefits in maintaining health and preventing various diseases. This pigment has anti-cancer [17], anti-inflammatory [18] and anti-diabetic activity [19]. Also, this pigment helps to maintain eye health [20] and prevent cardiovascular diseases [21]. According to the properties mentioned for astaxanthin, its use in the food industry with the two goals of increasing shelf life and enriching food is justified. In the European Union, the use of astaxanthin as a raw material in the production of food supplements has been approved and since 1997, European countries use astaxanthin in 5 types of food, including dairy products, drinks, jellies, sweets and chocolates [22].

The use of astaxanthin in various foods as a coloring or preservative, requires the use of a suitable method for protection from its structure. Because during the production and storage of food, the use of heat, acid, and enzyme treatments, as well as the use of substances that can react with astaxanthin and reduce its properties, probably cause a decrease in the efficiency and performance of this pigment. Also, if the purpose of using astaxanthin in food formulation is enrichment and its positive effects on health, must be protected from the structure of this pigment in the digestive system environment so that it does not decompose under the influence of enzymes and hydrochloric acid [23]. Today, in the food and pharmaceutical industries, the nanoencapsulation technique is one of the effective method to protect active compounds such as essential oils, bioactive peptides [23], aromas, flavors, and natural pigments [3 and 6]. During this process, a capsule is created around the active compounds (core), which protects them from possible changes in

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adverse conditions. This technique increases the durability and survival of active compounds as well as the lack of influence of environmental factors such as pH, light and oxygen on them. Also, the nanoencapsulation method helps to control the release of substances enclosed in the wall, that this increases the effect of active compounds on the quality of food [24 and 25]. In order to active compounds nanoencapsulation, different coatings are used to form the wall. These coatings usually have a protein, carbohydrate, cellulose and lipid structure and are prepared from plant, marine, animal and microbial sources [3]. Gluten, casein, gelatin, albumin, peptides, starch, chitosan, maltodextrin, carboxymethyl cellulose, methyl cellulose, ethyl cellulose, nitrocellulose, cellulose acetate, liposome, wax, paraffin, beeswax, diglycerides, monoglycerides and oils are among these coatings. In choosing the wall, attention should be paid to the properties of active compounds (core) such as chemical composition and structure, molecular weight, solubility, rheological behavior, film forming ability, surface activity, durability and degradability, melting point and boiling point [26].

Maltodextrin is a carbohydrate coating that is prepared from various starch sources such as potato, corn and wheat. This substance is considered one of the most important polysaccharide substances for encapsulation of active compounds due to its high solubility in water and lack of characteristic odor and color [3]. Sodium caseinate is a protein coating and unlike casein, it is soluble in water. This matter has a favorable surface activity and emulsifying properties and the active compounds covered by this substance have low permeability to environmental parameters such as water vapor and oxygen [24 and 25].

The aim of the present research in the first stage is to extract astaxanthin pigment from *Haematococcus pluvialis* microalgae using the acid-acetone method. In the next step, this pigment is nanoencapsulated using maltodextrin-sodium caseinate coating. Finally, the antioxidant activity and antibacterial property of nanocapsules carrying astaxanthin and free form of the pigment will be evaluated.

2- Materials and methods

Materials

The pure powder of *Haematococcus pluvialis* microalgae was prepared from Partovghaza Mazandaran Company that its chemical composition is presented in Table 1. ABTS and hydroxyl radicals, maltodextrin, sodium caseinate, EDTA and deoxyribose were obtained from Sigma Company and the bacteria used in this research (*Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus iniae*, *Bacillus subtilis*, *Yersinia ruckeri*, *Escherichia coli* and *Enterobacter aerogenes*) were prepared in frozen form from Iranian Research Organization for Science and Technology.

Table 1- Qualitative analysis of *Haematococcus pluvialis* pure powder used in this study

Cell composition	Amount (%)
Protein	23
Carbohydrate	37.5
Total fat	35.5
Astaxanthin	3
Beta-carotene	0.037
Lutein	0.018
Canthaxanthin	0.18

Methods

Extraction, saponification and purification of astaxanthin

To extract astaxanthin pigment from *Haematococcus pluvialis* microalgae combined acid-acetone method (initial pretreatment with hydrochloric acid and then extraction with pure acetone) was used [5 and 27]. After extracting the mentioned pigment, in order to hydrolyze or saponify of astaxanthin and convert its monoester and diester forms into free form, the mixture containing 0.02 N sodium hydroxide and extract containing

the pigment were placed in a dark place for 3 hours at 22°C [28]. Purification of astaxanthin also were done according to the methods provided by Kang and Sim [8] and Sun et al [29].

Nanoencapsulation of extracted astaxanthin

In order to astaxanthin nanoencapsulation, maltodextrin and sodium caseinate coating was used with a ratio of 1:1 and the ratio of coatings to the core was considered to be 4:1 [3 and 6]. First, a homogeneous suspension of maltodextrin was prepared in distilled water (4 g/50 ml). To prepare the second coating, a suspension of sodium caseinate was prepared in distilled water (4g/50 ml) and in order to homogenize, placed on a magnetic stirrer at 45 °C for 30 minutes. Then, the solution containing sodium caseinate (after lowering the temperature to 25 °C) was added to the maltodextrin solution and kept for 24 hours at 4 °C to increasing water absorption. In the final stage, 2 grams of astaxanthin was added to the solution containing the coatings, and after its dissolution, to produce nanocapsules was used an ultrasound machine (Hilscher, UP200, Germany) with 40 kHz wavelength for 15 minutes and 6 cycles (the time of each cycle was 30 seconds and the rest time was 15 seconds between cycles). In order to further reducing the particle size and increasing the efficiency of nanocapsule particles, a homogenizer (Ultrasonic, IKa, Italy) with speed of 10000 rpm was used for 15 minutes. The solution obtained from the process was frozen at -18 °C and dried using freeze dryer (Vaco 2 Zirbus, Germany) at 0.051 millibar pressure and -50 °C [24 amd 25].

Evaluation of physical properties of nanocapsules carrying astaxanthin

Average particle size and particle dispersity index of nanocapsules after diluting the samples up to 10 times using buffer (phosphate-buffered saline), were measured by dynamic light scattering method and Zetasizer machine (Malven, Nano ZS, 3000, angle of 90 degree and special tube with width of 0.01 meter, made in England). In order to evaluate zeta potential, the nanocapsules were diluted 10-fold using 50 mM phosphate buffer (pH=7.4). Then zeta potential was measured at scattering angle of 173 degree and helium-tungsten wavelength of 633 nm at 25 °C [30]. The efficiency of the nanoencapsulation process was also determined using the method of Yan et al [24] and below equation (FA= amount of uncoated or free astaxanthin, IA= amount of astaxanthin added at the beginning of the experiment).

Nanoencapsulation efficiency = [(FA-IA)/IA] ×100

Evaluation of antioxidant activity of astaxanthin and its carrier nanocapsules

The antioxidant activity of astaxanthin in free and nanoencapsulated forms (100 and 200 µg/ml) was evaluated using two tests of ABTS and hydroxyl radicals scavenging activity at 0, 15 and 30 days (stored at refrigerated temperature or 4±1°C). The results of antioxidant activity of the samples were compared with butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) at 200 µg/ml.

ABTS free radicals scavenging activity

7 mM ABTS solution was prepared in 2.45 mM potassium persulfate and kept at room temperature (dark environment) for 16 hours. After this period, the solution was diluted with distilled water until reach the absorbance of 0.7±0.02 at 734 nm. In the next step, 20 microliters of the sample was mixed with 980 microliters of diluted ABTS solution and placed in a dark place at 30°C for 10 minutes. The ABTS free radical scavenging activity was determined based on the following equation and reported as percentage [31].

ABTS free radicals scavenging activity= $(Abs_{control}- Abs_{sample}/ Abs_{control}) \times 100$

Hydroxyl radical scavenging activity

In order to evaluate the hydroxyl radical scavenging power of samples, at first, 0.9

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ml of phosphate-buffered saline or PBS (0.2 M, pH 7.4), 0.5 ml of α-deoxyribose (10 mM), 0.2 ml of hydrogen peroxide (10 mM), 0.2 ml of samle solution and 0.2 ml of FeSO4-EDTA (10 mM) were mixed and then incubated at 37 ºC for 1 hour. To stop the reaction, 1 ml of 1% thiobarbituric acid and 3% trichloroacetic acid was added to the mixture. The mixture was heated in boiling water for 15 min, then cooled in ice and the absorbance was monitored at 532 nm. Finally the hydroxyl radical scavenging activity was calculated with below equation [32].

Hydroxyl radical scavenging activity (%) = $(1- Abs_s/ Abs_c) \times 100$

Evaluation the antibacterial property of astaxanthin and its carrier nanocapsules

The antibacterial property of the pigment in two free and nanoencapsulated forms (on days 0 and 30 of storage at refrigerator temperature) was evaluated using two methods of diffusion in agar culture medium and microdilution test.

Cultivation conditions of the bacteria

The culture medium used to prepare the bacteria was the infusion broth of cow brain and heart. After the primary culture of bacteria, the samples were incubated for 18 hours at 35 and 30 °C for Gram positive (*Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus iniae* and *Bacillus subtilis*) and Gram negative (*Yersinia ruckeri*, *Escherichia coli* and *Enterobacter aerogenes*) bacteria, respectively. In the next step, in order to achieve bacterial precipitation, the culture media containing the bacterial suspension were centrifuged at 5000 rpm for 15 minutes and after removing the supernatant and adding phosphate buffer to the remaining sediment, the centrifugation process was repeated two more times. In each step, phosphate buffer was used to remove the effects of the initial broth environment. Then, some phosphate buffer was added to the remaining sediment and compared with the McFarland 0.5 standard tube (equivalent 1.5×10^8 cfu/ml). Finally, by diluting the samples, dilution 1×10^6 was used for the next tests [33].

Diffusion in agar medium (zone of inhibition)

In this method, first, the suspension prepared from bacteria was cultured on Mueller Hinton agar culture medium. In the next step, wells with a diameter of 6 mm were placed on the surface of the embedding environment and 50 microliters of concentrations of 20, 40, 60, 80, and 100 µg/mg of the pigment and its carrier nanocapsules were transferred to the wells. Then, the samples were incubated at two temperatures of 35 ºC and 30 ºC for 24 hours. After incubation, the diameter of the non-growth zone around the well (zone of inhibition) was measured using a ruler and reported in millimeters. Antibiotic doxycycline 30 mg was used as a positive control [3 and 34].

Microdilution test

In microdilution test, the dilutions used were 50, 100, 200, 400, and 500 µg/mg, which were transferred to the test tube along with 1 ml of the infusion medium of the bovine heart and brain. Then, 100 microliters of the 24-hour culture of the studied bacteria were added to the tubes and incubated at 30 °C and 37 ºC. In order to check the resulting changes, the optical absorption of the samples was read at 600 nm [3 and 34]. In this method, a concentration of the microbial suspension that showed the lowest light absorption was determined as the minimum inhibitory concentration (MIC), and a concentration of the suspension that showed no colony growth in the culture medium after 24 hours was also determined as minimum bactericidal concentration (MBC).

Statistical analysis

All tests were performed in three replications and the results were reported as average ± standard deviation. The normality and homogeneity of the data were assessed using the kolmogorov smirnov and Levene test. The data were analyzed by one-way analysis of variance

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(One-Way ANOVA in $SPSS_{21}$ software) and the difference between the means was evaluated by Duncan's test at 95% confidence level ($p<0.05$).

3-Results

Physical properties of nanocapsules carrying astaxanthin

In Table 2, the physical properties of nanocapsules carrying astaxanthin are presented. According to this Table, the average particle size is less than 270 nm, the particle dispersity index is about 0.42, the zeta potential of the surface of nanocapsules is positive and about 47 mV and the nanoencapsulation efficiency of the process is around 85%.

Table 2- Physical properties of nanocapsules carrying astaxanthin coated by maltodextrin-sodium caseinate

Physical properties	Level
Average particle size (nm)	269.1±3.95
particle dispersity index	0.423±1.28
Zeta potential (mv)	+46.71±2.88
Encapsulation efficiency (%)	85.19±4.09

Antioxidant activity of astaxanthin and its carrier nanocapsules

In Tables 3 and 4, the ABTS and hydroxyl radicals scavenging activity of the pigment and its carrier nanocapsules are presented, respectively. As can be seen in these Tables, the antioxidant activity of astaxanthin in both forms (free and nanoencapsulated) and three times has increased significantly by increasing the concentration from 100 to 200 µg/ml ($p<0.05$). Also, by astaxanthin nanoencapsulation using maltodextrin-sodium caseinate coating, the antioxidant activity of the pigment increased significantly and the carrier nanocapsules were more powerful in inhibiting radicals (ABTS and hydroxyl) compared to the free form of astaxanthin ($p<0.05$). In the following, it was found that the antioxidant activity (in both tests) of free form of the pigment decreased significantly during the storage time ($p<0.05$). In contrast, this activity in carrier nanocapsules did not change and remained constant after 30 days ($p>0.05$).

Comparison the antioxidant activity of the samples with synthetic antioxidants (BHA and BHT) at concentration of 200 µg/ml showed that the power of nanocapsules carrying astaxanthin in inhibiting radicals is not significantly different from BHT ($p>0.05$).

Table 3- ABTS free radical scavenging activity of astaxanthin and its carrier nanocapsules on days 0 and 30 of storage at refrigerator temperature (%)

Time (day)	Concentration (µg/ml)	Form of astaxanthin		Positive control	
		Free	Nanoencapsulated	BHA	BHT
0	100	58.15 ± 0.36^{Bc}	67.18 ± 0.64^{Ab}	89.54 ± 1.72^{A}	80.07 ± 1.83^{B}
	200	69.11 ± 0.29^{Ca}	78.86 ± 2.05^{Ba}		
15	100	50.88 ± 1.23^{Bd}	67.21 ± 0.79^{Ab}		
	200	63.34 ± 0.11^{Bb}	78.81 ± 1.06^{Aa}		
30	100	44.89 ± 1.02^{Be}	66.98 ± 1.25^{Ab}		
	200	51.12 ± 0.74^{Bd}	78.72 ± 0.89^{Aa}		

- The different lowercase and uppercase letters in the column and row indicate significant differences between the data, respectively ($p<0.05$).

Table 4- Hydroxyl radical scavenging activity of astaxanthin and its carrier nanocapsules on days 0 and 30 of storage at refrigerator temperature (%)

Time (day)	Concentration (µg/ml)	Form of astaxanthin		Positive control	
		Free	Nanoencapsulated	BHA	BHT
0	100	57.87 ± 0.75^{Bc}	69.98 ± 0.92^{Ab}	91.36 ± 1.85^{A}	82.11 ± 1.91^{B}
	200	72.28 ± 0.12^{Ca}	81.95 ± 1.15^{Ba}		
15	100	51.95 ± 1.1^{Bd}	70.09 ± 0.78^{Ab}		
	200	65.01 ± 0.81^{Bb}	82.03 ± 1.26^{Aa}		
30	100	43.88 ± 0.64^{Be}	70.21 ± 1.05^{Ab}		
	200	52.29 ± 1.23^{Bd}	82.14 ± 0.94^{Aa}		

- The different lowercase and uppercase letters in the column and row indicate significant differences between the data, respectively ($p<0.05$).

Antibacterial activity of astaxanthin and its carrier nanocapsules

Zone of inhibition

In Tables 5 and 6, antibacterial activity of astaxanthin and its carrier nanocapsules (in five concentrations and two times) against Gram positive and Gram negative bacteria by diffusion method in the culture medium containing agar are presented. According to these Tables, by astaxanthin nanoencapsulation using maltodextrin-sodium caseinate coating, diameter of non-growth zone of all seven bacteria has increased significantly ($p<0.05$). Also, after 30 days storage of astaxanthin and nanocapsules at 4 °C, antibacterial activity (zone of inhibition of bacteria) of carrier nanocapsules did not change and remained constant ($p>0.05$). But this activity in free form of the pigment decreased significantly ($p<0.05$).

As seen in Tables 5 and 6, by increasing the concentration of astaxanthin and its carrier nanocapsules, the diameter of non-growth zone (zone of inhibition) of bacteria increased significantly ($p<0.05$). Of course, Gram positive (except *Listeria monocytogenes*) and Gram negative bacteria were resistant up to concentrations of 60 and 80 µg/ml of samples, respectively. In the following, comparison of the antibacterial activity of positive control and the samples showed that the ability of astaxanthin and its carrier nanocapsules to inhibit the growth and proliferation of the studied bacteria is lower than Doxycycline.

According to Tables 5, *Listeria monocytogenes* was only resistant to concentration of 20 µg/ml of astaxanthin and its nanocapsules. But three other bacteria, in addition to the concentration of 20 µg/ml, were also resistant to concentrations of 40 and 60 µg/ml. Based on the results of the inhibition zone diameter, it can be claimed that among the four Gram positive bacteria studied, *Listeria monocytogenes* is the most sensitive and *Streptococcus iniae* is the most resistant bacteria against the free form of astaxanthin and its carrier nanocapsules. The maximum diameter of inhibition zone for *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus iniae* and *Bacillus subtilis* was about 29, 16.1, 11.4 and 19 mm, respectively; that these values (diameter of inhibition zone) are related to the nanoencapsulated form of astaxanthin at concentration of 100 µg/ml.

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Table 5- Diameter of non-growth zone of Gram positive bacteria against astaxanthin and its carrier nanocapsules on days 0 and 30 of storage at refrigerator temperature (mm)

Strain	Time (day)	T	Concentrations (µg/ml)					Positive control
			20	40	60	80	100	Doxycycline
L. monocytogenes	0	F	R	9.96±0.15^{Db}	14.28±0.37$^{C}_{b}$	18.16±0.11$^{B}_{b}$	25.14±0.19$^{A}_{b}$	44.26±1.35
		N	R	12.71±0.84^{Da}	18.98±1.35$^{C}_{a}$	23.12±1.28$^{B}_{a}$	28.94±0.62$^{A}_{a}$	
	30	F	R	7.79±0.46^{Dc}	11.12±0.02$^{C}_{c}$	14.11±0.15$^{B}_{d}$	21.73±0.11$^{A}_{c}$	
		N	R	12.68±0.73^{Da}	19.1±0.97^{Ca}	23.15±1.36$^{B}_{a}$	28.87±0.74$^{A}_{a}$	
Staph. aureus	0	F	R	R	R	11.1±0.32^{Bf}	14.12±0.09$^{A}_{f}$	36.14±1.08
		N	R	R	R	12.97±0.05$^{B}_{e}$	16.19±0.28$^{A}_{e}$	
	30	F	R	R	R	8.18±0.06^{Bh}	11.38±0.37$^{A}_{h}$	
		N	R	R	R	12.98±0.08$^{B}_{e}$	16.1±0.32^{Ae}	
Strep. iniae	0	F	R	R	R	7.19±0.02^{Bi}	9.12±0.1^{Ai}	33.66±1.19
		N	R	R	R	9.26±0.1^{Bg}	11.35±0.43$^{A}_{h}$	
	30	F	R	R	R	6.12±0.03^{Bj}	7.44±0.16^{Aj}	
		N	R	R	R	9.35±0.24^{Bg}	11.41±0.18$^{A}_{h}$	
B. subtilis	0	F	R	R	R	14.11±0.07$^{B}_{d}$	16.15±0.63$^{A}_{e}$	37.98±0.84
		N	R	R	R	16.11±0.23$^{B}_{c}$	18.91±0.38$^{A}_{d}$	
	30	F	R	R	R	11.24±0.1^{Bf}	12.99±0.05$^{A}_{g}$	
		N	R	R	R	16.14±0.12$^{B}_{c}$	18.88±0.24$^{A}_{d}$	

- T: Treatments, F: Free astaxanthin, N: Nanoencapsules carrying astaxanthin, R: Resistant
- The different lowercase and uppercase letters in each column and row indicate significant difference between the data, respectively ($p<0.05$).

According to Table 6, all three Gram negative bacteria were resistant up to concentrations of 80 µg/ml of astaxanthin and its nanocapsules. Also, in concentration of 100 µg/ml, the free form of astaxanthin was not able to inhibit the growth and proliferation of *Yersinia ruckeri* and *Escherichia coli*, but the nanocapsules carrying astaxanthin were able to inhibit these two bacteria. The values of zone of inhibition showed that the sensitivity of *Enterobacter aerogenes* against astaxanthin and its carrier nanocapsules is higher than *Yersinia ruckeri* and *Escherichia coli* ($p<0.05$). The maximum diameter of inhibition zone for *Enterobacter aerogenes* was about 12 mm but this value for *Yersinia ruckeri* and *Escherichia coli* was recorded about 8 mm.

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Table 6- Diameter of non-growth zone of Gram negative bacteria against astaxanthin and its carrier nanocapsules on days 0 and 30 of storage at refrigerator temperature (mm)

Strain	Time (day)	Form of astaxanthin	Concentrations (µg/ml)					Positive control
			20	40	60	80	100	Doxycycline
Y. ruckeri	0	Free	R	R	R	R	R	25.74±0.78
		Nanoencapsulated	R	R	R	R	8.12±0.65[b]	
	30	Free	R	R	R	R	R	
		Nanoencapsulated	R	R	R	R	7.96±0.88[b]	
E.coli	0	Free	R	R	R	R	R	26.56±1.11
		Nanoencapsulated	R	R	R	R	7.98±0.59[b]	
	30	Free	R	R	R	R	R	
		Nanoencapsulated	R	R	R	R	8.11±0.62[b]	
E. aerogenes	0	Free	R	R	R	R	8.16±0.33[b]	29.56±1.11
		Nanoencapsulated	R	R	R	R	11.85±0.43[a]	
	30	Free	R	R	R	R	6.43±0.24[c]	
		Nanoencapsulated	R	R	R	R	11.78±0.52[a]	

- R: Resistant
- The different letters indicate significant difference between the data ($p<0.05$).

MIC and MBC of astaxanthin and its carrier nanocapsules

In Table 7, MIC and MBC of astaxanthin and its carrier nanocapsules for Gram positive and negative bacteria are shown. As can be seen in this Table, the MIC of the pigment (free and nanoencapsulated forms) for the seven bacteria ranges from 50 to 400 µg/ml. This value was recorded for the MBC from 100 to 500 µg/ml. Also, by astaxanthin nanoencapsulation, the MIC and MBC of the pigment were reduced for the studied bacteria. After 30 days (storage of the samples at refrigerator temperature), the MIC and MBC of nanocapsules carrying astaxanthin for the bacteria did not change and remained constant but the MIC and MBC of free form of the pigment increased for most of bacteria.

According to the results, the lowest MIC and MBC were related to *Listeria monocytogenes*; therefore, this species is the most sensitive of bacteria to astaxanthin and its carrier nanocapsules (similar to the results of inhibition zone diameter). Also, based on the findings of inhibition zone diameter, MIC and MBC, it can be claimed that the sensitivity of Gram positive bacteria against astaxanthin and its carrier nanocapsules is higher than Gram negative bacteria. Of course, *Streptococcus iniae* is an exception in this field and has shown a certain resistance against samples.

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Table 7- MIC and MBC of astaxanthin and its carrier nanocapsules against five strain of bacteria on days 0 and 30 of storage at refrigerator temperature (µg/ml)

Strain	Time (day)	Form of astaxanthin	MIC	MBC
L. monocytogenes	0	Free	100	200
		Nanoencapsulated	50	100
	30	Free	200	400
		Nanoencapsulated	50	100
Staph. aureus	0	Free	200	400
		Nanoencapsulated	100	200
	30	Free	400	500
		Nanoencapsulated	100	200
Strep. iniae	0	Free	400	500
		Nanoencapsulated	200	400
	30	Free	400	500
		Nanoencapsulated	200	400
B. subtilis	0	Free	200	400
		Nanoencapsulated	100	200
	30	Free	400	500
		Nanoencapsulated	100	200
Y. ruckeri	0	Free	400	500
		Nanoencapsulated	200	400
	30	Free	400	500
		Nanoencapsulated	200	400
E.coli	0	Free	400	500
		Nanoencapsulated	200	400
	30	Free	400	500
		Nanoencapsulated	200	400
E. aerogenes	0	Free	200	400
		Nanoencapsulated	100	200
	30	Free	400	500
		Nanoencapsulated	100	200

4-Discussion

According to the findings of the present research, astaxanthin and its carrier nanocapsules have shown potential antioxidant activity, that this property makes it possible to use these compounds in various food products as preservatives. In various researches like the present study, the potential antioxidant activity of astaxanthin has been confirmed [2, 5,10,14,16 and 28]. Comparison the antioxidant activity of astaxanthin and its carrier nanocapsules in the current research showed that the nanoencapsulation technique not only helps to improve the antioxidant activity of this pigment, but also prevents the reduction of this activity during the period of pigment storage at refrigerator temperature. This result shows that the maltodextrin-sodium caseinate coating has a high ability to protect the properties and structure of astaxanthin [3]. In a research by Safari et al [3], nanoencapsulation of phycocyanin using maltodextrin-sodium caseinate coating increased antioxidant activity of the pigment and stability of this property during 60 days of storage at -18°C, that this finding is consistent with the present study. In another study, it was reported that microencapsulation of phycocyanin using maltodextrin-caraginan coating, increases its antioxidant activity and protects from it against the oxidation process [35], that this finding confirms the present research. In a study with a similar topic, it was proven that by nanoencapsulation of phycocyanin using 3% chitosan coating, its antioxidant activity increases significantly [4], which is consistent with the results of the present study. All mentioned researches confirm the high efficiency of encapsulation technique in improving the antioxidant activity of various pigments. In the current research, by increasing concentration of

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both forms of astaxanthin (free and nanoencapsulated forms) from 100 to 200 µg/ml, the antioxidant activity of the samples (in both tests) increased significantly. Unlike the present study, in some researches, it has been proven that by increasing the concentration of astaxanthin up to 100 µg/ml, its antioxidant activity increases and at concentrations higher than 100 µg/ml, the trend of this activity is stabled. In other words, due to the high power of the antioxidant activity of astaxanthin, high concentrations of this pigment are not needed to show maximum antioxidant properties [2 and 14].

In the present study, it was found that astaxanthin has a favorable antibacterial activity and can inhibit the growth and proliferation of various Gram positive and Gram negative bacteria. In various researches, this property has been confirmed in astaxanthin extracted from different organisms [36-40]. In a research by Ahmed et al [41], the antibacterial activity of astaxanthin extracted from *Haematococcus pluvialis* microalgae against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* was confirmed. The research findings of Irna et al [16] on the antibacterial activity (zone of inhibition) of astaxanthin extracted from shrimp (using two chemical and high pressure methods) showed that this pigment is able to inhibit the growth and proliferation of *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus* and *Bacillus subtilis* bacteria. In another research, astaxanthin was extracted from three species of crabs (*Portunus sanguinolentus*, *Callinectes sapidus*, *Paralithodes brevipes*) using acetone solvent method and its antibacterial activity was investigated against *Escherichia coli* bacteria isolated from milk and spoiled meat. The findings indicated the desired ability of the pigment in inhibition the mentioned bacteria; So that the zone of inhibition in concentrations of 30 and 50 µg/ml was reported to vary from about 5 to 12 mm [15]. The results of the three mentioned studies are consistent with the findings of the present research.

In the following, the current research showed that the antibacterial activity of astaxanthin can be improved significantly using nanoencapsulation technique. In addition, this method can help to stabilize this property during the pigment storage period at 4°C. Because according to the results, the antibacterial property (zone of inhibition, MIC and MBC) of carrier nanocapsules remained constant and unchanged during the storage period. But in the free form of astaxanthin, there was no such stability and the antibacterial activity decreased significantly (reduction diameter of non-growth zone and also increasing MIC and MBC). According to the results, the free form of astaxanthin was not able to inhibit the growth and proliferation of *Y. ruckeri* and *E.coli*, but the nanocapsules carrying pigment was able inhibit these two bacteria. This result indicates high efficiency of nanoencapsulation technique and maltodextrin-sodium caseinate coating in improving of antibacterial activity of astaxanthin. A research that evaluated the antibacterial activity of phycocyanin in two free and nanoencapsulated forms using maltodextrin-sodium caseinate coating showed that nanoencapsulation technique leads to an increase in antibacterial activity of the pigment and stability this property during 60 days of storage at -18°C [3] that these results are consistent with the present research.

In the present research, it was found that by increase in the concentration of astaxanthin and its carrier nanocapsules (from 20 to 100 µg/ml), the antibacterial activity (zone of inhibition) of samples increases significantly that this finding is consistent with the research of Muthulakshmi et al [42], Sitohy et al [34] and Mohite et al [43]. Of course, to inhibit the growth of most of the studied bacteria, concentrations higher than 60 and 80

µg/ml were needed. In a research which phycocyanin and its carrier nanocapsules were used to inhibit the growth of the bacteria studied in the present research, concentrations of 2.5 to 25 µg/ml of the samples in agar culture medium were able to create inhibition zone [3]. Comparison results of the mentioned study with the current research shows that the antibacterial activity of phycocyanin is much higher than astaxanthin. Because in order to inhibit same bacteria, higher concentrations of astaxanthin are needed compared to phycocyanin.

The sensitivity of bacteria to an antimicrobial substance depends on various factors, including the composition of the cell wall, the ability to produce enzymes, and virulence [3].The values of zone of inhibition, MIC and MBC showed that among the seven studied bacteria, the most sensitive bacteria against astaxanthin and its carrier nanocapsules is *Listeria monocytogenes*. Also, *Y. ruckeri* and *E.coli* were the most resistant bacteria. Therefore, if in a food product the goal is to eliminate *Listeria monocytogenes*, astaxanthin and its carrier nanocapsules can be a suitable option for preservation and increasing shelf life. In general, in the current research, the resistance of Gram negative bacteria against astaxanthin and its carrier nanocapsules was higher than Gram positive bacteria. The reason for this resistance is probably related to the stronger structure (composition) of the cell wall of Gram negative bacteria [3].

The results of present research showed that astaxanthin has favorable antioxidant and antibacterial activity, and these properties can be improved by the pigment nanoencapsulation using maltodextrin-sodium caseinate coating. Also, the mentioned technique and coating is effective in order to preserve and stabilize the inherent properties of astaxanthin during 30 days of storage at 4 °C. Therefore, astaxanthin and its carrier nanocapsules can be used in food formulations as preservatives.

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