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The Effect of Aqueous Extract of Saffron (Crocus sativus L. Stigma) on the Behavior of Salmonella Typhimurium in A Food Model during Storage at Different Temperatures

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ABSTRACT

Background: Given the concerns about the use of chemical preservatives in food, the consumers and producers have been interested in natural alternatives, such as plant essential oils and extracts. Since there are limited studies about the effect of saffron (*Crocus sativus* L.) on the behavior of foodborne pathogens in food models, this study aimed to determine the inhibitory effect of aqueous extract of saffron stigma on the growth behavior of *Salmonella* Typhimurium (*S.* Typhimurium) in commercial barley soup (as a food model) during storage at different temperatures. **Methods:** The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the extract were determined against *S.* Typhimurium using broth microdilution method. The growth of *S.* Typhimurium was investigated in the presence of this extract in commercial barley soup during 12 days of storage at 10, 20, and 30 °C. **Results:** The MIC and MBC values for saffron extract against *S.* Typhimurium were 100 and >200 mg/ml, respectively. Also, the saffron extract at a concentration of 200 mg/ml and temperature of 10 °C had the highest inhibitory effect on the growth of bacteria in commercial barley soup during storage. **Conclusion:** According to the results of this study, the antimicrobial effect of this extract increased in a dose-dependent manner against this bacterium. Therefore, the use of proper concentrations of this extract together with appropriate storage temperature can have an appropriate inhibitory effect on the growth of this bacterium, improving food safety shelf life.

Keywords: *Saffron; Crocus Sativus* L; *.Antimicrobial; Salmonella*

Introduction

Various microorganisms, including gram-positive and gram-negative bacteria, as well as fungi, cause a variety of infections in humans. Over the years, effective antimicrobial substances have been developed to overcome pathogenic microorganisms. However, in recent years,

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microorganisms resistance to common antimicrobial drugs has increased, leading to an urgent need for novel antimicrobials (Kalaivani *et al.*, 2012, Khatibi *et al.*, 2020a, b). According to the reports of the centers for disease control and prevention (CDC), 76 million people in the United States are infected with foodborne pathogens annually, leading to 225,000 cases of hospitalizations and 5,000 cases of death (Oussalah *et al.*, 2007).

From ancient times, plant extracts have been used for various purposes, such as improving the flavor of foods and beverages, and the treatment of various diseases. Since public concerns have increased about the side effects of chemical preservatives in recent years, the use of plant extracts has been known as a promising way to increase the shelf life of food, due to their natural origin and more safety compared to chemical preservatives (Khatibi *et al.*, 2015, Khatibi *et al.*, 2017, Khatibi *et al.*, 2018, Moosavy *et al.*, 2017, Moosavy *et al.*, 2018, Pandey *et al.*, 2017, Santos-Sánchez *et al.*, 2017).

Saffron (*Crocus Sativus* L.), belonging to the Iridaceae family, is a perennial plant that is traditionally used to improve the taste and flavor of food (Raj *et al.*, 2015). The dried stigma of this plant is usually used in the food industry as an aromatic spice and coloring agent (Hill, 2004, Mzabri *et al.*, 2019). According to the findings of previous studies, the extract of this plant has antimicrobial, antioxidant, and anticancer properties (Amoozadeh *et al.*, 2016, Karimi *et al.*, 2010, Milajerdi *et al.*, 2016, Parray *et al.*, 2015). Saffron contains over one hundred and fifty different volatile compounds. The main constituents of this plant are Crocin, Picrocrocin, and Safranal, which are effective in color, taste, and smell of saffron, respectively. Each of these compounds plays an important role in the antioxidant and antimicrobial properties of saffron (Ökmen *et al.*, 2016).

Different types of soup have been reported as a source of salmonellosis outbreak in Germany (Geiss *et al.*, 1993), Vietnam (Vo *et al.*, 2014), and USA (Hedican *et al.*, 2009). In Europe, the consumption rate of soup per capita is estimated at about 0.8 kg/person/year (Dionisi and Oldring, 2002). Commercial/homemade barley soup is one of the most popular types of soup throughout the world. Due to having special and different compounds, such as meat, onion, carrot, parsley, and barley, it is a rich source of high-quality protein, vitamins, and minerals. It provides an appropriate medium for the growth of food-borne pathogens and spoilage microorganisms (Pajohi *et al.*, 2010, Shahbazi *et al.*, 2017). In recent years, barley soup has been used by many researchers as a food model to study the effect of antimicrobials on the growth of pathogenic foodborne microorganisms (Ahmadi *et al.*, 2017, Moradi and Sadeghi, 2017, Pajohi *et al.*, 2010, Shahbazi *et al.*, 2017, Sharafati Chaleshtori and Fallah, 2019). Therefore, this study aimed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the aqueous extract of saffron stigma on *Salmonella* Typhimurium, as the most important foodborne pathogen. Also, the commercial barley soup was used as a food model to investigate the antimicrobial effect of the extract on the behavior of this bacterium at different temperatures of storage.

Materials and Methods

Bacteria: Salmonella enterica subspecies *enterica* serovar Typhimurium (PTCC: 1709) was used for this study. The lyophilized bacteria were prepared from the collection center of industrial microorganisms (Iranian Research Organization for Science and Technology, Iran). The bacterium was cultured consequently in the nutrient broth (Merck, Germany) at 37 °C for 24 hours. The second culture was then mixed with sterile glycerin in a ratio of 5: 1 and stored at -20 °C to be used during the study (Pintado, 2011).

Preparation of bacterial inoculums: Briefly, 100 µl of the bacterial suspension was transferred to 10 mL of the nutrient broth and incubated at 37 °C for 24 h. This culture was repeated under the same condition. Using 0.1% sterile peptone water, serial dilutions of the culture were prepared, and the

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bacteria were cultured on the surface of nutrient agar (Merck, Germany). After incubation of plates at 37 °C for 24 h, colonies were counted. Using the surface culture method, the number of bacteria was calculated as colony-forming unit (CFU) per mL of the culture medium.

Extraction of aqueous extract: The saffron was collected from a field in Gonabad city. The stigmas of the plant have been fully dried in the shade and under a gentle current of air. To prepare the aqueous extract, 13.8 g of clean and crushed saffron stigmas were mixed with 50 ml of distilled water and boiled for 20 minutes. The resulting mixture was passed three times through filters with large to small porosity degrees. The filtered solution was placed in a water bath at 50 °C until the water evaporated completely. The residual dry matter was distributed in sterile microtubes and stored in a refrigerator for the next experiments (Pitsikas and Sakellaridis, 2006).

Determination of minimum inhibitory concentration of the extract by microdilution broth method: To determine the MIC of the extract, 96-round well microplates with a volume of 300 µl were used. Different extract concentrations of 12.5, 25, 50, 100, 200 mg/ml were prepared using distilled water. Firstly, 200 mg of dried saffron extract was dissolved in 1 ml of distilled water. The resulting solution was passed through a 0.45 µm filter and used for the preparation of lower dilutions. Then, 20 µl of the extract with the desired concentration and 160 µl of broth medium were poured into each well. Twenty (20) µl of bacterial suspension was added to each well. The final bacterial concentration was 10^5 CFU/ml (the exact number of bacteria was determined by surface culture and colony counting). A well containing 180 µl of nutrient broth medium and 20 µl of bacteria was considered as the positive control. Also, a well containing the nutrient broth was used as the negative control. To control possible contamination of the extract, a well containing 20 µl of the extract and 180 µl of nutrient broth medium was also used. The contents of the microplate were mixed for 2 minutes using a microplate shaker. After incubating the microplates at 37 °C for 24 h, the wells were visually monitored for the presence of turbidity. The minimum concentration of the extract that inhibited bacterial growth was considered as the MIC. The experiments for detecting the MIC value were performed in triplicates (Khatibi *et al.*, 2018, Wadhwani *et al.*, 2009).

Determination of minimum bactericidal concentration of the extract: For this purpose, wells in which bacterial growth was inhibited were used for this experiment. A sterile swab was impregnated with the content of each well and cultured on the surface of nutrient agar. The culture was incubated for 24-48 h at 37 °C, and the count of bacteria was counted. The minimum concentration of extract that inhibited the growth of 99.9% of bacteria was considered as the MBC. These experiments were performed in triplicates (Khatibi *et al.*, 2017).

Preparation of substrate: Each package (85 g) of commercial barley soup (Nestle, Iran) was added to 1 liter of distilled water according to the manufacturer's guidelines, and were heated for 15 min. The mixture was passed through a strainer and distributed in microtubes. Finally, it was sterilized at 121 °C for 20 min (Moosavy *et al.*, 2017).

Addition of the extract to soup and storage at different temperatures: After sterilization and cooling of the barley soup, 100 and 200 µl of aqueous extract was added to 900 and 800 µl of soup, respectively. Then, 100 µl of bacterial suspension was added to the mixture with a final concentration of 10^5 CFU/ml. A control sample was also prepared. The samples were incubated at 10, 20 and, 30 °C for 12 days. To evaluate the antibacterial effect of extract against *S.* Typhimurium, the bacterial colonies were counted after 0, 1, 2, 3, 6, 9, and 12 days. For colony count, serial dilutions of the samples were prepared using 0.1% peptone water and were cultured on the surface of nutrient agar plates. They were incubated at 37 °C, and the count of bacteria was enumerated after 24 h. This experiment was performed in triplicates (Moosavy *et al.*, 2017).

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Data analysis: The SPSS software version 19 (IBM Corporation, Armonk, NY, USA)" was used for statistical analysis of data, and p-value less than 0.05 was considered statistically significant.

Results

Minimum inhibitory and bactericidal concentrations of the extract: After adding the desired amounts of extract and bacteria to each well and incubating microplate at 37 °C, it was found that a concentration of 100 mg/ml of the extract can inhibit the growth of S. Typhimurium. Therefore, this concentration was considered as the MIC against this bacterium. After the culture of the contents of the clear wells, it was found that none of the studied concentrations could kill this bacterium. Therefore, the concentrations above 200 mg/ml were determined as the MBC.

The effect of the extract on the behavior of Salmonella Typhimurium in commercial barley soup at different temperatures: To investigate the inhibitory effect of aqueous extract of saffron stigma on the growth of the bacteria in barley soup during storage, the bacterial count was enumerated at the selected days and temperatures. The results of the bacterial colony count are shown in **Figures 1-3**. Bacterial count during the storage period of barley soup was affected by the extract in a dose-dependent manner. By increasing the concentration of the extract from 100 to 200 mg/ml, the bacterial count significantly ($P < 0.05$) decreased after 12 days. Storage temperature also had a significant effect ($P < 0.05$) on the bacterial count. After 12 days of storage at 10 °C, the concentration of 200 mg/ml of the extract decreased the bacterial count by 3.1±0.15 log compared to the control sample, while this difference was 2.32 ± 0.23 and 2.37 ± 0.18 log at 20 °C and 30 °C, respectively.

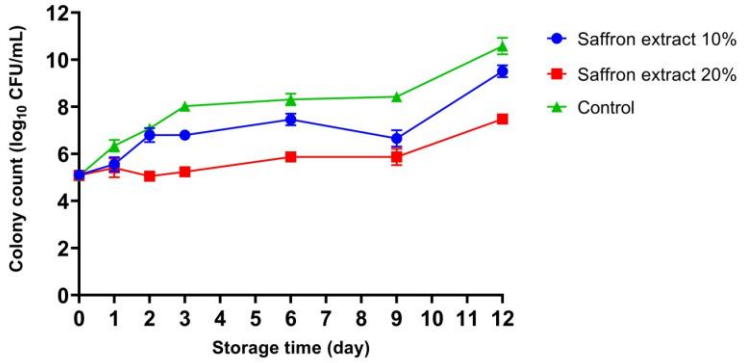

Figure 1. Behavior of *Salmonella* Typhimurium in commercial barley soup using different concentrations of aqueous extract of saffron stigma during 12 days of storage at 10 °C

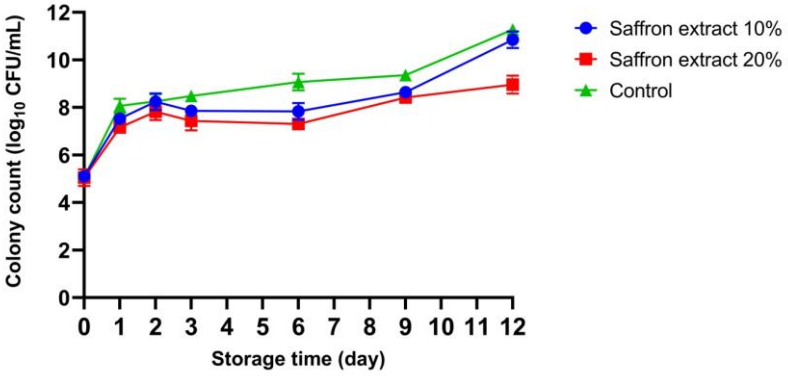

Figure 2. Behavior of Salmonella Typhimurium in commercial barley soup using different concentrations of aqueous extract of saffron stigma during 12 days of storage at 20 °C

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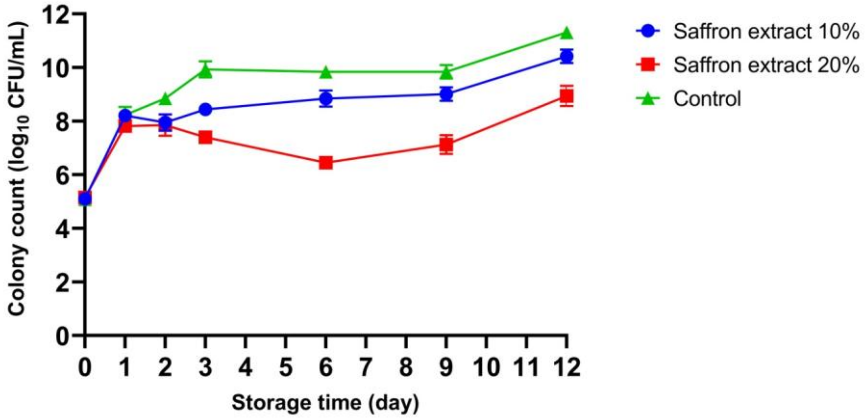

Figure 3. Behavior of Salmonella Typhimurium in commercial barley soup using different concentrations of aqueous extract of saffron stigma during 12 days of storage at 30 °C

Discussion

In this study, the inhibitory effect of the aqueous extract of saffron was evaluated on the growth of *S.* Typhimurium. It was found that extracts at concentration of 100 mg/ml could inhibit the growth of *S.* Typhimurium and this concentration was considered as MIC against this bacterium. It has been reported that ingredients, such as Crocin and Safranal are involved in this property. Probably due to the solubility in water, these compounds can bind to food-borne bacteria - and kill them (Pintado, 2011).

In line with the present study, Hashemi *et al*. evaluated the effect of aqueous extract of saffron stigmas on some pathogenic microorganisms. The MIC of aqueous extract in their study against all the studied bacteria was 40 mg/ml (Hashemi *et al.*, 2017). According to the results of the present study, the aqueous extract of saffron at a concentration of 100 mg/ml had an inhibitory effect against *S.* Typhimurium. In other similar studies, the antimicrobial effect of this extract on food-borne pathogens has also been proven (Cenci-Goga *et al.*, 2018, Jomehpour *et al.*, 2019, Pintado, 2011, Raj *et al.*, 2015, Razzaghi *et al.*, 2003). The results of a study performed on the aqueous extract of saffron stigmas collected from Torbat-e Heydarieh, Gonabad, and Khorasan (Iran) showed modest and obvious antibacterial activities against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Enterococcus faecalis* (Cenci-Goga *et al.*, 2018). Antibacterial activity of aqueous and methanolic extracts of *Crocus Sativus* stigma has also been reported against clinical isolates of some gram-positive and gram-negative pathogenic bacteria (Jomehpour *et al.*, 2019). Razzaghi *et al*. isolated the main components of aqueous extract and found that Safranal (an organic compound isolated from saffron) can inhibit the growth of *E. coli* and *S. aureus*. Other compounds of saffron had no inhibitory effects against the studied bacteria (Razzaghi *et al.*, 2003). In a study on the ethyl acetate, ethanol, petroleum ether extracts of stigma, cream, and saffron leaves on the *S. aureus*, *Staphylococcus epidermidis*, *E. coli*, *Micrococcus luteus*, *Candida albicans*, Cladosporium, and *Aspergillus niger*, it was shown that ethyl acetate extract of the saffron leaf did not affect the above microorganisms. The antifungal effect of the ethyl acetate extract of saffron stigma against the above microorganisms was more than style. The antibacterial activity of ethyl acetate extract of saffron style was more than other parts of the plant (Vahidi *et al.*, 2010). Muzaffar *et al*. also reported strong activity of the petroleum ether and methanolic extracts of saffron stigmas against various bacterial strains (*S. aureus, E. coli, Pseudomonas aeruginosa, Proteus vulgaris,* and *Klebsiella pneumonia*) and fungi (*Aspergillus*

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fumigates, *Aspergillus niger*, and *Candida albicans*) (Muzaffar *et al.*, 2016). Antibacterial activity of the methanolic extract of stigma and petal of *Crocus spp.* (*Crocus caspius*, *Crocus speciosus* and *Crocus sativus*) has been reported against *S. aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *E. coli*. The authors found that *Bacillus subtilis*, and *E. coli* were the most sensitive and resistant bacteria to the extract, respectively (Afshar Mohammadian *et al.*, 2016).

It has been reported that the aqueous extract of saffron has a great antimicrobial effect on coagulase-negative *staphylococci* (CoNs) (Ökmen *et al.*, 2016). Also, Fazeli Nasab *et al*. studied the antibacterial effects of hydroalcoholic extract of saffron petals on some gram-positive and gram-negative bacterial pathogens. They reported that the antimicrobial activity of this extract was more effective against gram-positive than gram-negative bacteria. The differences in the resistance of gram-negative bacteria to antimicrobial substances of the extract may be related to differences between bacteria in the cell wall structures. Generally, the cell wall of gram-positive bacteria consists of a single layer, whereas the gram-negative cell wall has a multilayered structure surrounded by an outer cell membrane (Fazeli Nasab, 2019, Gao *et al.*, 1999, Kim *et al.*, 2013).

In recent years, similar studies have been conducted to investigate the effect of plant essential oils and extracts against *S.* Typhimurium in food models. Moosavy *et al*. conducted a study about the effect of *Zataria multiflora* Bioss essential oil against *S.* Typhimurium and *S. aureus* in the commercial barley soup at 8 °C and 25 °C. They found that the increase of incubation temperature has a significant effect on the growth rate of this bacterium (Moosavy *et al.*, 2010). The results of the present study also indicated that the bacterial growth in commercial barley soup stored at 30 °C was more than those stored at 10 °C and 20 °C. Similar behavior has also been reported by other authors (McAuley *et al.*, 2015, Prudêncio *et al.*, 2015, Shakeri *et al.*, 2017, Tassou *et al.*, 2000). It may be related to the lower metabolic activity of *S.* Typhimurium at low temperatures (Shakeri *et al.*, 2017, Tassou *et al.*, 2000). The temperature range for the growth of *S.* Typhimurium is from 6.2 °C to 45 °C. This microorganism can grow well at room temperature, but the optimum temperature for its growth is about 37 °C (Albrecht, 2021, Moosavy *et al.*, 2010). The low temperature had a bacteriostatic effect on *Salmonella* spp. allowing the antimicrobial to put more stress on the organism, resulting in the reduction of their count in food (Porter *et al.*, 2020). Moosavy *et al*. found that the count of *S.* Typhimurium in barley soup decreased by increasing the concentration of essential oil (Moosavy *et al.*, 2010). The results of the present study also showed that higher concentrations of saffron aqueous extract (200 mg/ml) increased the antibacterial effect of the saffron extract. Also, the results of this study are in agreement with the findings of other researchers. Moradi *et al*. studied the effect of *Cuminum cyminum* essential oil on *Bacillus cereus* in commercial barley soup, which was stored at 10 °C and 25 °C. They found that the count of bacteria at 10 °C was significantly lower than 25 °C (Moradi *et al.*, 2012). The results of Pajoohi *et al*. on the antimicrobial activity of *Origanum vulgare* and *Cuminum cyminum* essential oils in the barley soup showed that the use of these essential oils at low temperatures (8 °C) significantly inhibits the growth of the vegetative form of *Bacillus cereus* and *Bacillus subtilis* in commercial barley soup at the lowest concentration of each essential oil (Pajouhi *et al.*, 2012).

Generally, plant extracts and essential oils have been shown to prolong the delayed phase of bacterial growth while reducing the growth rate in the logarithm phase. Their performance follows a mechanism related to their accumulation in the lipid bilayer of the cell membrane and the destruction of its structure (Tassou and Nychas, 2000, Valero and Giner, 2006).

Conclusion

The results of this study showed that the concentration of aqueous extract of saffron stigma and incubation temperature had a significant effect

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on the behavior of *S.* Typhimurium. Therefore, high concentrations of this extract together with appropriate storage temperature can have an acceptable inhibitory effect on the growth of this bacterium, thereby increasing shelf life and improving food safety.

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Authors' Contribution

Valizadeh S: Data gathering and analysis, Project administration, Writing-original draft. Moosavy M: Conceptualization, Methodology, Supervision, Validation, Writing-original draft, Writing-review & editing. Ebrahimi A: Data analysis, Validation, Writing-original draft. Akhondzadeh Basti A: Conceptualization, Methodology, Supervision and, Validation. Mahmoudi R: Conceptualization, Data analysis, Methodology, Supervision, Validation. Khatibi SA: Software, Validation, Writing-original draft, Writing-review & editing.

Conflicts of interest

The authors declare that there is no conflict of interest.

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