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Microbial Decontamination in Saffron (*Crocus sativus* L.) by Cold Plasma Treatment: An *In vitro* study

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ABSTRACT

Background & Objective: One of the problems associated with exporting traditional bulk saffron during the harvest season is the uncontrolled microbial load. Hence, it is crucial to discover a suitable method for microbial decontamination. Cold plasma technology is a safe, non-thermal, and efficient approach to deactivating different pathogenic microorganisms in different food or agricultural products. Considering the nutritional and medicinal importance of saffron (*Crocus sativus* L.), the present research aimed to study the efficacy of cold plasma for decontaminating saffron.

Materials & Methods: The cold plasma method was used to treat samples of saffron under various conditions (two different gas types, two different pressures, and two different frequencies, but one power, one exposure time, one gas injection, and one temperature). Subsequently, the treatments' microbial load was measured at a probability level of 1% compared to the control.

Results: Our results indicated that the microbial load in the treated samples was reduced by cold plasma technology. The highly effective in reducing microbial load was shown in conditions with cold-vacuumed plasma systems, radio frequency energy (RF) with a 13.56 MHz, pressure at 10^{-2} Torr, exposure time of 5 min, injected gas with air and argon (50 L min^{-1}), the temperature at 25ºC, and power of 30 W. This treatment was highly effective in decreasing microbial load by 46.6%.

Conclusion: Hence, the cold plasma technology can be a risk-free approach to reduce the microbial load in spices, particularly saffron. Ultimately, our *in vitro* results introduce an optimized conditions and primary information for future research on the industrial decontamination of medicinal plants.

Keywords: Plasma Technology, Microbial Load, Decontamination, Non-thermal Treatment, Saffron

1. Introduction

Currently, spices are an important part of the human diet worldwide. Herbs are found in the green parts of plants, and spices are found in the bark, seeds, and underground stems. They enhance flavor and color, impart fragrance, and preserve foods through their protective components, like antioxidants and antimicrobial properties (1, 2).

The costliest spice worldwide is saffron, often referred to as red gold. It consists of dried red stigmas from an autumnally grown flower that belongs to the *Iridaceae* family, *Crocus* genus, and *C. sativus* species (3). Saffron has been widely grown in Spain, Morocco, Afghanistan, India, Greece, and particularly Iran due to its adaptability to arid and semiarid climates (2, 4). Iran is one of the important countries for producing and exporting this expensive spice, producing over 430 tons and exporting 154,318,000 USD (5). Today, saffron is utilized and traded for its medical, coloring, cosmetic, and flavoring benefits. Using saffron can benefit neurological disorders, antioxidant, anticancer, antimicrobial, and anti-inflammatory properties (2, 5, 6).

Due to their growing environment, plant products tolerate a high level of microbial contamination (7). Plant and medicinal products are deactivated using essential disinfectants, such as methyl bromide, thermal treatment, ethylene oxide, gamma rays or high-energy electrons, UV radiation, and ozone (5, 8). Despite their effectiveness in reducing the microbial load, these treatment methods have limitations and drawbacks (release of sustained toxic compounds and toxic by-products, change in the color, nutritional and effective substance of plant products, and environmental issues) (9, 10). One of the problems associated with exporting traditional bulk saffron during the harvest season is the uncontrolled microbial load. Hence, it is crucial to discover a novel and suitable approach for microbial decontamination.

Cold plasma is becoming an environmentally friendly technology with auspicious decontamination features in many industries, which can be utilized to make healthy vegetables and food and enhance the plant material quality (11-13). Reactive compounds produced by cold plasma result in the destruction of cell membranes. Through UV radiation and ion bombardment, reactive compounds can denature proteins and DNA, which in turn inactivates a broad range of bacteria, fungi, and viruses (14, 15). Cold plasma technology is a non-thermal food processing effectively used for food preservation (8, 16, 17). Recent investigations into plant products have demonstrated that pretreatment by cold plasma can be used before drying to preserve desired quality traits and improve drying properties (18).

In Iran, saffron is important in nutrition, medicine, and economics. Therefore, reducing the microbial load of this valuable plant is crucial. Since insufficient studies have concentrated on the impact of the cold plasma on spices, the present research seeks to study the impact of cold plasma method on the saffron microbial load. A new solution for saffron microbial decontamination is expected to emerge through the results of this research.

2. Materials and Methods

2.1 Saffron Powder Preparation

Saffron (*Crocus sativus* L.) powder was purchased from Rezwan Company (Bisotun, Kermanshah, Iran). Experts from Razi University's Faculty of Agriculture verified the purchased plant.

2.2 Plasma Treatment

Cold plasma (CUTE Model) was utilized to treat saffron under various conditions (Table 1). Three experiments were designed for cold plasma treatment, and operational variables were optimized. The control was included in the sample that was not treated. The results were compared to those from the control experiment by comparing different treatment conditions. Five minutes was considered for exposure time. The used frequencies were 13.56 and 20 MHz, and the reactive power was 30 w. The sampling process was initiated immediately upon exposure to vacuum plasma, and preserved at 4°C for the following analysis.

Table 1. The used levels of variables for optimizing cold plasma treatment.

Treatments	Cold plasma form	Energy generator	Power (W)	Time of exposure (min)	Gas form	Pressure (Torr)	Temperature (ºC)	Gas injection (L min^{-1})	Frequency (MHz)
Treatment A	Under vacuum	LF	30	5	Oxygen	10^{-1}	25	50	20
Treatment B	Under vacuum	RF	30	5	Air (50%) + Argon (50%)	10^{-1}	25	50	13.56
Treatment C	Under vacuum	RF	30	5	Air (50%) + Argon (50%)	10^{-2}	25	50	13.56

2.3 Antimicrobial Assay

To determine cold plasma antimicrobial activity, we compared the microbial load of non-treated (control) samples to that of treated samples. After the treatment of the samples, an initial sample suspension was prepared under sterile conditions by mixing 1 g of the treated sample with 9 ml of peptone water (0.1%) and then homogenized at room temperature. The dilution was carried out and 100 µL of each dilution sample was cultured on the plate count agar (PCA) at 37°C for 24 h. Each experiment was subjected to three replications (19).

Colonies were counted using a magnifying glass after 24 hours at 37°C incubation. The logarithmic cycle was used to express the reduction in microbial load, and formula 1 was used to calculate it. In this formula, MLR (%) refers to microbial load decrease (%), N_0: the number of colonies in the un-treated sample, N1: the number of colonies in samples that were treated with cold plasma (20).

$$\text{MLR (\%)}=\frac{\text{Log } N_0 - \text{Log } N_t}{\text{Log } N_0}\times 100 \qquad (1)$$

2.4 Bacterial Identification with API 20E System

Gram-negative rods, *Enterobacteriaceae,* and other non-fastidious were identified using the API 20 E Kit. The experiments were conducted twice, and the average values were assessed. An oxidase kit from Padtan Teb Co. was used to evaluate the oxidase activity of all isolates.

2.5 Statistical Analysis

Each treatment was subjected to three repetitions in a completely random design of all tests. The tests resulted in data analysis via the ANOVA (Analysis of Variance) in SAS (version 9.3). Duncan's multiple range test was used to perform the mean comparison with a probability level of 1%. The graphs were drawn using GraphPad Prism 8 software.

3. Results

The microbial load of saffron was analyzed to study the impact of treatment with cold plasma. The results showed that all treatments significantly decreased bacterial contamination compared to the control. Our results indicated that the highest microbial decontamination was related to sample C (pressure at 10^{-2} Torr, injected gas 50 L min^{-1}, exposure time of 5 min, RF-13.56 MHz, 25ºC, and 30 w power) with 46.06% (Figure 1). When plasma treatment was applied to Saffron samples, the untreated control had the highest microbial load. Still, the treatment *C*, *B,* and *A* had the lowest microbial load (Figure 1). The ANOVA analysis is given in Table 2. This indicates that the *P*-value is statistically very highly significant at the probability level 0.0001.

According to the results of the API 20 E Kit on saffron samples, *Pseudomonas luteola* was the Gram-negative bacteria present in both treated and control samples of saffron. Gram-positive cocci were *Enterococcus faecium* and *Enterococcus durans* (Table 3).

Table 2. The two-way analysis of variance (ANOVA)

Variation source	DF	Sum of Squares	Mean Squares	F-Value	*P*	R-Square
Treatments	3	3.7167	1.2389	291.51	<0.0001****	0.990935
Error	8	0.034	0.00425			
Total	11	3.7507				

****= Statistically very highly significant

Table 3. API 20 E Kit and Gram-staining methods for diagnosis of bacteria in the treated samples of Saffron

Saffron Samples	Stained Samples
Control (Untreated)	Gram-negative bacilli, Gram-positive cocci, Gram-negative cocci, Diplococcus, Gram-positive bacilli, Gram-positive coccobacilli
Treatment A	Gram-negative bacilli, Gram-positive cocci
Treatment B	Gram-positive cocci, Gran-negative cocci
Treatment C	Gram-negative bacilli, Gram-positive cocci

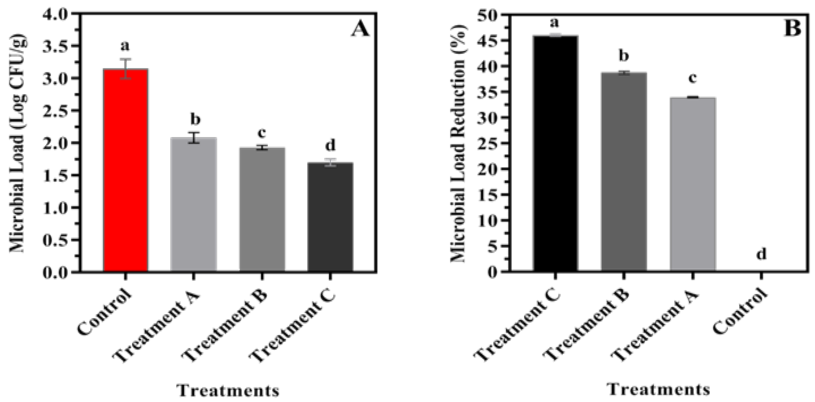

Figure 1. The impact of cold plasma method on microbial load of Saffron samples. Duncan's multiple range test was used to compare the means with a probability of 1%. There is no significant difference between means with common letters. A) Logarithmic microbial load. B) Microbial load reduction.

4. Discussion

The health status of consumers can be affected by microbial contaminants found in plant and herbal products, making them a prevalent health difficulty globally. Therefore, to ensure product quality and safety, it is crucial to choose applicable and non-thermal approaches for microbial decontamination of plants or their obtained extracts (21). Cold atmospheric plasma is widely suggested due to its non-thermal, fast, and environmentally friendly properties. It has effectively inactivated various microorganisms (22, 23). In light of the significance of saffron in terms of nutrition, medicine, and economics in Iran and the importance of reducing the microbial load of this valuable plant, the purpose of this research was to study the effect of cold plasma technology on saffron microbial load. According to our findings, cold plasma decreased the bacterial load in the treated samples of saffron. Compared to the control treatments at a 1% probability level, all treatments significantly decreased the microbial load. The highly effective in reducing microbial load was shown in conditions with cold-vacuumed plasma systems, radio frequency energy (RF) with a 13.56 MHz, pressure at 10^{-2} Torr, exposure time of 5 min, injected gas with air and argon (50 L min^{-1}), the temperature at 25ºC, and power of 30 w. This treatment was highly effective in decreasing microbial load by 46.6%.

According to previous studies, cold plasma is a promising non-thermal technology for decontaminating spices without significant variations in other quality parameters (2, 5, 24). In a study, saffron was decontaminated using a cold plasma method. Nitrogen and normal air were used to produce cold plasma in that research. The findings show that nitrogen plasma has less disinfection power than air plasma. The reduction of microbes was significantly affected by increasing plasma exposure time. The microbial reduction reached its maximum at 12 minutes (25). Darvish et al (2) used low pressure cold plasma treatment to microbial decontaminate saffron. Their findings revealed that increasing the low-pressure of cold plasma power and exposure time decreased the microbial decontamination of saffron stigmas. At 110 w for 30 minutes, the highest microbial log reduction was observed (2). In another study, the effect of Radiofrequency Low-Pressure Cold Plasma treatment on the decontamination of saffron was investigated. Their results showed that the best set was at an RF power of 76 W for 26 min using the gas mixture (Air 65%, Oxygen 5%, and Argon 30%), which decreased the total bacteria, *Escherichia coli*, molds, coliforms, and yeast. According to their findings, applying cold plasma treatment can decontaminate saffron without causing significant harm to its appearance or metabolites (5).

Microbial decontamination by cold plasma technique is a process that involves interacting with produced reactive species, reactive molecules, and radicals with cellular functions and microbial cell membranes (26). It has been proposed that cold plasma damages the cell membrane, DNA, among other internal structures (13). Reactive compounds produced by cold plasma result in the destruction of cell membranes. Through UV radiation and ion bombardment, reactive compounds can denature proteins and DNA, which in turn inactivates a broad range of microorganisms (14, 15). Various reactive species are formed together with plasma discharge, have microbial decontamination properties (13). According to previous literature, cold plasma is a successful non-thermal method for achieving microbial decontamination. Nonetheless, many unexplored areas remain, particularly regarding the quality of food products after cold plasma treatment.

5. Conclusion

Our research was conducted to optimize the effects of cold plasma method on Saffron (*Crocus sativus* L.)

microbial load. Our findings suggested that conditions with cold-vacuumed plasma systems, radio frequency energy (RF) with a 13.56 MHz, pressure at 10^{-2} Torr, exposure time of 5 min, injected gas with air and argon (50 L min^{-1}), the temperature at 25ºC, and power of 30 W were the most efficient set for microbial deactivation. Thus, the cold plasma technology can be a risk-free approach to reduce the microbial load in spices, particularly saffron. Eventually, our laboratory-based results introduce optimized conditions and primary data for future research on the industrial decontamination of medicinal plants.

6. Declarations

6.1 Acknowledgments

None.

6.2 Ethical Considerations

The medical ethics committee of Kermanshah University of Medical Sciences (IR.KUMS.REC.1397.291) approved the study protocol.

6.3 Authors' Contributions

P.Y: Data curation, methodology, writing-original draft; K.Y and M.K: Funding, methodology, administration of project, & manuscript editing. A.T: Data curation, methodology; S.H: Data curation, Formal analysis, Software, Writing-original draft and review.

6.4 Conflict of Interest

The authors declare that there are no conflicts of interest.

6.5 Fund or Financial Support

This research received no external funding.

6.6 Using Artificial Intelligence Tools (AI Tools)

The authors were not utilized AI Tools.

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