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Original Article

In-Vitro Evaluation of Crocus Sativus L. Petals and Stamens as Natural Antibacterial Agents Against Food-Borne Bacterial Strains

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Abstract

Growing interest to use natural preservatives and spices with antimicrobial effects and large amounts of floral bio-residues (92.6 g per 100 g) generated and wasted in the production of saffron spice guided this study to evaluate the opportunity to expand the uses of *C. sativus* flowers (petals and stamens), beyond the spice (dried stigmas). The antibacterial potential of total extracts and different sub-fractions of floral bio-residues of saffron production (petals and stamens) were primarily evaluated against five bacterial strains potentially causing food-borne disease (*Bacillus cereus*, *Staphylococcus aureus*, *Salmonella enterica*, *Escherichia coli* and *Shigella dysenteriae*) using well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined by macrodilution method. Methanol extract of petals had shown more antibacterial activity against *S. aureus, S. enteric,* and *S. dysenteriae* compared to stigma. Methanol extract and ethyl acetate sub-fraction of stamens showed more antimicrobial effect against *B. cereus* and *E. coli*. The petals total extract showed the most antibacterial activity against *Shigella dysenteriae* (MIC 15.6mg/ml) while the ethyl acetate and chloroform sub fractions showed the maximum effect against *Bacillus cereus*(MIC 62.5mg/ml). Stamen methanol total extract and aqueous sub fraction have the maximum effect against *Staphylococcus aureus* and *Bacillus cereus* (MIC 62.5mg/ml) while the ethyl acetate sub fraction has the best effect against *Shigella dysenteriae* (MIC 15.6mg/ml). Results showed that both petals and stamens could act as new and natural sources of antibacterial agents with food industrial applications.

Keywords: antibacterial, Crocus sativus , food-borne diseases , petals, stamens.

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1. Introduction

Aromatic plants have been used for their preservative and medicinal attributes, as well as to improve food taste. There has been considerable interest in essential oils and extracts of medicinal and edible plant and spices for the development of alternative food additives, in order to prevent the growth of food-borne pathogens or to interrupt the onset of food spoilage. [1-2]. Food poisoning is usually caused by bacterial factors assumed as an acute disease followed by eating contaminated food or beverages [3]. Factors which can cause this disease are eating parasites, toxic plants, fungi or animal materials. Annually about 76 million people worldwide are affected and most of the cases occur in summer. Generally, children and old people are at higher risk of food poisoning. World Health Organization has estimated that in 2005, 1.8 million people had died due to diarrheal diseases and a considerable number of these cases were affected by contaminated food or water [4].

Crocus sativus L. commonly known as "Saffron" has a long history of spicy food uses in many countries. It belongs to Iridaceae family in the order of Asparagales that contains about 66 genera and more than 2000 species [5]. Saffron spice is the dried stigma of the flower that only represents 7.4% of *C. sativus* flowers. It is known for its aroma, color and medicinal properties [6]. Although the source of saffron is obscure, it is apparently originated from Asia Minor and Iran. Since one dry stigma in saffron plant weighs about 2 mg and each flower contains three of them, approximately 150,000 saffron flowers must be carefully picked for the production of 1 kg of the spice and harvesting the flowers and separating the stigmas is very time consuming procedure. Consequently, saffron is still the world's most expensive spice [7-8]. Mechanization of flower collection, stigma separation, and dehydration process, increases the product capacity and extend the uses of *C. sativus* flowers different parts, beyond the production of saffron spice. Large amounts of floral bio-residues (92.6 g per

100 g of flowers) are generated and wasted in the production of saffron spice [9]. Saffron petal is the main by-product of saffron harvesting which prorduced more than 1000 tons yearly [10]. The petals and stamens are excessive parts of *C. sativus* which are locally used as food additives with low prices. Nowadays, saffron petals are only used for dye extraction, which is not flourished yet [11]. Due to the mass production of this plant, evaluating some other uses for the other parts of saffron could be so useful [12].

Antimicrobial agent, including food preservatives have been used to inhibit food-borne bacteria and extend the shelf life of the processed food. Many natural products such as essential oils, herbs and spices have been proven to possess antimicrobial functions and could serve as a source of antimicrobial agent against food spoilage and pathogens [13]. The purpose of the present study was to evaluate comparative antibacterial activities of total extracts and different sub-fractions of *C. sativus* petals and stamens separately against selected microorganisms causing food-borne diseases.

2. Materials and Methods

2.1. Plant material

The fresh *C. sativus* flowers were collected in October 2012 from the saffron farms of Ferdows, Southern Khorasan Province, Iran. The plant sample was identified by Dr. Asgarpanah in Department of Pharmacognosy, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran. A sample was deposited in the Herbarium of the University with voucher specimen No. 639. The petals and stamens were separated and shade dried.

2.2. Extract and sub-fraction preparation

The dried petals and stamens (500 g) were separately extracted by maceration with methanol. The extract was dried by rotary evaporator (Heidolph laborota 4000). 15 g of the each crude extract was kept separately in sterile sample tubes and stored at 4 °C. The crude extracts were then partitioned by chloroform, ethyl acetate, methanol and water. Solvents of the extracts and sub-fractions were removed under reduced pressure in a rotary evaporator until they became completely dry. The obtained extracts and different sub-

fractions were dissolved in Tween 20 (10% V/V) to give stock solutions; filter sterilized and kept in dark bottles at 4° C.

2.3. Bacterial strains

Bacterial strains including *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* PTCC 1247, *Escherichia coli* ATCC 25922, *Shigella dysantriae* PTCC 1188, and *Salmonella typhi* ATCC 19430 were obtained from Iranian Research Organization for Science and Technology, Tehran, Iran.

2.4. Primary antibacterial assay

Antibacterial activities of the methanol total extracts and different sub-fractions of petals and stamens were primarily investigated by the Cup plate method [14]. The Muller-Hinton Agar medium was purchased from Merck Company, Germany. The cups each of 6mm diameter were made by scooping out medium with a sterilized cork borer in a petri dish which was streaked with the microorganism's saline suspension from overnight bacterial agar culture with a turbidity equivalent to a 0.5 Mc Farland standard. Concentrations of 1000, 500, 250, 125, 62.5 and 31.2 mg/ml of each total extract and sub-fraction were prepared in Tween 20 (10% V/V). 100 µl of each dilution was added in cups and petri dishes were subsequently incubated at 37 °C for 24 h. Tween 20 (10% V/V) was also added to one cup as the negative control which did not reveal any inhibition. Zone of inhibition produced by each dilution of total extracts and sub-fractions was measured in mm after 24h. The test was repeated three times and the means of the results were reported [14-15].

2.5. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

After confirming the antibacterial activity of total extracts and sub-fractions, MIC and MBC of the extracts and sub-fractions were determined by broth macro dilution method using Muller-Hinton Broth medium(Merck, Germany) against the test organisms by macro dilution method[15-16].

The total extracts and sub-fractions were properly prepared in Tween 20 (10% V/V) and transferred to tubes in order to obtain a twofold serial dilution of the original extract/sub-fraction (from 1:2 to 1:512 starting from the concentration of 500 mg/ml). Each tube contains approximately 5 x 10^5

CFU/ml of microorganism after inoculation. 2 tubes were reserved for sterility control (no inoculum added), inoculum viability (no extract added) and the Tween 20 (10% V/V) inhibitory effect. Tubes were incubated at 35- 37 °C for 24 hours. The MIC was determined as the lowest concentration of the extract/sub-fraction which completely inhibits visible growth of the microorganisms [15-16].

To confirm MICs and to establish MBC, 50 µl of each tube with no visible growth was removed and inoculated in MHA plates. After 24 h of aerobic incubation at 37 °C, the numbers of surviving microorganisms were determined. MBC was then recorded as the lowest concentration that killed at least 99.99% of the initial number of bacteria. Each experiment was repeated at least three times [15-16].

2.6. Total phenolic content

The concentration of phenolic compound in the extract and fractions was determined using methanolic solutions of the extract and fractions in the concentration of 1 mg/ml. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of the extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The absorbance was determined using spectrophotometer at λmax = 765 nm after 45 minute. The samples were prepared in triplicate and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of Gallic Acid and the calibration curve was construed. Based on the measured absorbance, the concentration of the phenolic compounds was determined (mg/ml) from the calibration curve; then the content of phenolics in extract and fractions was expressed in terms of Gallic Acid equivalent (mg of GA/g of extract)[15-17].

2.7. Total flavonoid content

The content of flavonoids in the extracts and sub fractions were determined using spectrophotometric method. The samples were dissolved in 80% methanol to obtain a final concentration of 1 mg/ml. The calibration curve was prepared using 0.1-1 ml aliquots of Rutin solution, 500 µL of the acetic acid solution, 2 ml

Table1. The mean inhibition zone diameter (mm) of total extract and sub-fractions of *C. sativus* petals *(n=3).*

concentration (mg/ml) / microorganism		1000	500	250	125	62.5	31.2
Staphylococcus aureus ATCC 25923	Methanol	20	19	14	12	10	-
	Chloroform	14	12	-	-	-	-
	Ethyl acetate	15	-	-	-	-	-
	water	13	10	-	-	-	-
Bacillus cereus PTCC 1247	Methanol	14	12	10	10	-	-
	Chloroform	15	12	10	-	-	-
	Ethyl acetate	15	14	10	-	-	-
	water	12	-	-	-	-	-
Salmonella typhi ATCC 19430	Methanol	17	13	10	-	-	
	Chloroform	10	-	-	-	-	
	Ethyl acetate	15	13	11	-	-	
	water	14	12	-	-	-	
Escherichia coli ATCC 25922	Methanol	13	10	-	-	-	
	Chloroform	13	10	-	-	-	
	Ethyl acetate	11	-	-	-	-	
	water	11	-	-	-	-	
Shigella dysantriae PTCC 1188	Methanol	22	19	17	15	14	11
	Chloroform	16	15	12	-	-	-
	Ethyl acetate	17	15	12	-	-	-
	water	20	17	15	13	12	-

of the pyridine solution and 1 ml of aluminium chloride solution. The final volume was adjusted to 10 ml with 80% methanol and the final Rutin concentration was 1-10 µg/ml. To quantify the flavonoids, 0.5 ml of total extract and/or each fraction was transferred to a test tube and 0.5 ml of the acetic acid solution, 2 ml of the pyridine solution, 1 ml of the reagent aluminium chloride solution and 6 ml of 80% methanol were added. The samples remained at room temperature for 30 minutes. The spectrophotometer was adjusted to a wavelength of 420 nm. The test was performed in triplicate and the flavonoid content is expressed as milligrams of Rutin equivalents (RE) per gram of sample of extracts (mg RE/g) [15-18].

3. Results and Discussion

The antimicrobial activities of the total extracts and different sub-fractions of *C. sativus* petals and stamens were evaluated in different concentrations against bacterial strains causing food-borne disease by cup plate method. The diameter of inhibition zones have been presented in tables 1 and 2.

As shown in table 1, the methanol extract of *C. sativus* petals with concentration equal

Table2. The mean inhibition zone diameter(mm) of total extract and sub-fractions of *C. sativus* stamen *(n=3).*

Concentration (mg/ml)		**1000**	**500**	**250**	**125**	**62.5**	**31.2**
microorganism							
Staphylococcus aureus ATCC 25923	Methanol	17	14	-	-	-	-
	Chloroform	12	10	-	-	-	-
	Ethyl acetate	15	12	-	-	-	-
	water	17	13	11	-	-	-
Bacillus cereus PTCC 1247	Methanol	16	13	10	-	-	-
	Chloroform	12	11	-	-	-	-
	Ethyl acetate	15	13	11	10	-	-
	water	16	13	10	-	-	-
Salmonella typhi ATCC 19430	Methanol	14	12	10	-	-	
	Chloroform	14	12	10	-	-	
	Ethyl acetate	17	13	11	-	-	
	water	16	13	11	10	-	
Escherichia coli ATCC 25922	Methanol	11	10	-	-	-	
	Chloroform	12	11	-	-	-	
	Ethyl acetate	14	11	-	-	-	
	water	11	10	-	-	-	
Shigella dysantriae PTCC 1188	Methanol	18	15	12	-	-	-
	Chloroform	16	14	11	-	-	-
	Ethyl acetate	21	15	13	11	-	-
	water	17	13	15	11	10	-

to1000mg/ml indicated significant antibacterial activity against *S. aureus, B.cereus, S. typhi, , E. coli* and *S. dysenteriae* with inhibition zone diameters ranging from 13 to 22 mm while the ethyl acetate sub-fraction showed the best antibacterial activity against *B. cereus* with inhibition zone diameter of 15 mm. Aqueous and Chloroform sub-fractions of *C. sativus* petals were less effective against the studied strains. Table 2 shows that Ethyl acetate sub-fraction of *C. sativus* stamens has the best antibacterial activity on *S. typhi*, *E. coli* and *S. dysenteriae* with inhibition zone diameters ranging from 14 to 21 mm while the most active extract against *B. cereus* and *S. aureus* is total methanol extract with inhibition zone diameters ranging from 16 to 17 mm. The best activity against *S. dysenteriae* was seen by petals methanol extract.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts and sub-fractions of *C. sativus* petals and stamens were determined by macro

Table3. Minimum inhibitory concentration (MIC) of total extract and sub-fractions of *C. sativus* petals and stamens[a].

Extracts	Petal(mg/ml)				Stamen(mg/ml)			
Microorganism	M	C	EA	W	M	C	EA	W
Staphylococcus aureus ATCC 25923	31.2	125	62.5	125	62.5	125	125	62.5
Bacillus cereus PTCC 1247	125	62.5	62.5	125	62.5	125	125	62.5
Salmonella typhi ATCC 19430	62.5	125	62.5	125	125	125	31.2	62.5
Escherichia coli ATCC 25922	125	125	125	125	125	125	62.5	125
Shigella dysantriae PTCC 1188	15.6	62.5	31.2	31.2	62.5	62.5	15.6	31.2

M,C,EA and W represents Methanol, Chloroform, Ethyl Acetate and Water respectively.

dilution method and the results are shown in Tables 3 and 4.

The total methanol extract, ethyl acetate and aqueous sub-fractions of *C. sativus* petals had the maximum antimicrobial effects against *S. dysenteriae* respectively with MIC values equal to 15.6, 31.2 and 31.2 mg/ml. The inhibitory effects of petals chloroform and ethyl acetate sub-fractions were equal against *B. cereus* with the MIC value of 62.5 mg/ml. All the extract and sub-fractions of petals had equal activities on *E. coli* with the MIC value of 125 mg/ml. The inhibitory activities of ethyl acetate and aqueous sub-fractions were also equal against *S. dysenteriae* with the MIC value of 31.2 mg/ml.

The stamen Methanol extract and the aqueous sub-fraction showed equal inhibitory activities against *S.aureus* with the MIC value of 62.5 mg/ml. The stamens ethyl acetate sub-fraction had the maximum antibacterial effect against *S. typhi*, and *S. dysenteriae* with the lowest MIC values of 31.25and 15.6 mg/ml respectively. The aqueous sub-fraction of the stamens showed significant inhibitory effect on *B. cereus* growth with MIC value of 62.5 mg/ml.

Table4. Minimum Bactericidal concentration (MBC) of total extract and sub-fractions of *C. sativus* petals and stamens[a]

Extracts	Petal(mg/ml)				Stamen(mg/ml)			
microorganism	M	C	EA	A	M	C	EA	W
Staphylococcus aureus ATCC 25923	125	500	125	500	125	250	125	250
Bacillus cereus PTCC 1247	250	500	250	500	125	500	500	125
Salmonella typhi ATCC 19430	125	250	250	250	125	250	125	125
Escherichia coli ATCC 25922	250	250	250	250	250	125	125	250
Shigella dysantriae PTCC 1188	125	125	125	125	250	250	125	125

M,C,EA and W represents Methanol, Chloroform, Ethyl Acetate and Water respectively.

Methanol total extract and ethyl acetate sub-fractions of *C. sativus* petals and stamens had the maximum bactericidal effects against *S. aureus* with the lowest MBC of 125 mg/ ml. The stamen methanol extract and the aqueous sub-fraction showed a good activity against *B. cereus* with the MBC value of 125 mg/ ml. Chloroform and ethyl acetate sub-fractions of stamens had the bactericidal activities with the MBC values of 125 mg/ml against *E.coli*. All the extract and the sub-fractions of the petals and also the stamen ethyl acetate and aqueous sub-fractions showed equal bactericidal effect on *S. dysenteriae* with the MBC values of 125 mg/ml while the solvent as negative control presented no inhibitory or cidal effect against the tested strains.

Total phenolic content was measured after making a standard calibration curve by Gallic Acid ($y=1.48x - 0.05$, $r^2 =0.99$). The content was ranged from 4.09 to 17.34 mg GAE/g of dry powder of petal. Phenolic content of stamen also was ranged from 3.07 to 15.37 mg GAE/g . As it has been indicated in Table 5 the lowest total phenolic content was found in the chloroform fraction (C) and the highest was in the aqueous fraction (W) for both petal and stamen.

Standard calibration curve of Rutin was used to evaluate the content of flavonoid in the extract and its fractions ($y=0.02x – 0.05$, $r^2=0.97$). Results are

shown in Table 5. The lowest flavonoid content was measured in chloroform fraction (1.8 mg RE/g of dry powder for petal and 0.6 mg RE/g of dry powder for stamen) and the highest was in aqueous fraction (3.8 mg RE/g of dry powder for petal and 2.5 mg RE/g of dry powder for stamen). In fact the polar sub-fractions generally indicated more antibacterial activity which can be related to the total phenolic content of fractions. Polyphenols are hydrophilic phytochemicals and thus more hydrophilic extractants are better solvents for their recovery from plants [15-16].

4. Conclusion

The most common cause of diarrhea in developing countries is enterohemorrhagic *Escherichia coli* [19]. Another pathogen that usually spreads through contaminated food and water *is Shigella* which is the third most common causes of bacterial food-borne disease in the United States [20]. Other bacterial agents such as *Staphylococcus aureus, Bacillus cereus* are involved in the incidence of food poisoning [21-22]. Food-borne diseases may have long-term and variable symptoms such as watery or bloody diarrhea, meningitis, chronic renal disorders, and respiratory, immunologic and cardiovascular complications [23-24]. In Iran the most common causative agents were *Escherichia coli* (type unspecified), *Salmonella, Shigella* and *Staphylococcus aureus* [25]. *Salmonella* is the second a most common cause of bacterial food-borne disease in the United States [20]. Reports from Japan and Korea showed that food poisoning of bacterial origin in Korea was 58.6% of the total case, including *Salmonela* spp. (23.1%), *Staphylococcus* spp. (14.9%), *E. coli* (6.8%). In Japan, the majority of bacterial causes were *Staphylococcus* spp. (24.6%), *Salmonella* spp. (14.8%), *E. coli* (3.5%) [26]. A study of major pathogens that caused food-borne disease in the United States, mostly from 2000-2008, estimated that approximately 131,000 episodes of acquired food-borne infection with *Shigella* occurred each year, with 20 percent of these patients requiring hospitalization. The prevention and treatment of disease caused by these organisms are complicated by the increase in multidrug-resistant strains and the lack of an effective vaccine and preservatives.

From the present study, it was concluded that methanol extract of petals had more antibacterial

activity against *S. typhi* compared to stamens. Methanol extract and aqueous sub-fraction of stamens showed more antimicrobial effect against *B. cereus*. Results showed that all extract and fractions from both petals and stamens have the MIC value equal or less than 125 mg /ml so they could act as new and natural sources of antibacterial agents with industrial applications. Employing of these floral materials as functional ingredients with the subsequent added value also suggested. Since *C. sativus* petal and stamen as the main by-products of saffron production possessed considerable phenolic compounds which showed high antioxidant power comparable with TBHQ (as a strong synthetic antioxidant) [10] and regarding the results of the present study, floral bio-residues of *C. sativus* especially the petals which are discarding more than thousands tons each year could be employed as natural antibacterial and antioxidant food preservative. Also based on the results of this study, further in vivo and ex vivo confirmatory tests for effective extracts and sub-fractions of *C. sativus* petals and stamens are recommended.

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