



Journal of Medical Bacteriology



Evaluation of Antibacterial Activities of Hydroalcoholic Extract of Saffron Petals on Some Bacterial Pathogens

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ARTICLE INFO

Article type:

Research Article

Article history:

Received: 10 Jun 2019

Revised: 09 Jul 2019

Accepted: 02 Aug 2019

Published: 05 Sep 2019

Keywords:

Crocus sativus, *E. coli*, *L. monocytogenes*, *S. aureus*, *S. typhimurium*.

ABSTRACT

Background: Saffron is an interesting minor spice with a food coloring history and its active components have shown several useful pharmacological effects such as antidepressant, anti-inflammatory, antitumor, radical scavenger effects, learning and memory-improving effects, anti-Alzheimer, antipruritic, etc. Because of negative consumer perception of chemical preservatives, attention is shifting towards natural alternatives. Particular interest has been focused on the potential application of plant essential oils. The aim of the present study was undertaken to evaluate the interaction of hydroalcoholic extract of saffron petals and different concentrations of it against different Gram-positive and Gram-negative bacteria.

Methods: The hydroalcoholic extract of saffron petals and different concentrations of extract (60, 90, 120 mg/ml) by a blank sterilized disc diffusion test was assessed against four different gram-positive and negative bacterial pathogens. Also, a full randomized factorial design experiment was conducted with three replications. The statistical analysis was performed using two-way ANOVA followed by LSD test for multiple comparisons.

Results: The results of ANOVA showed that saffron petals hydroalcoholic extract and antibiotics were an effective on all tested bacterial strains and the most effective antibacterial properties has been observed on *Listeria monocytogenes* followed by *Escherichia coli*. The antibacterial activities of concentration of saffron petals hydroalcoholic extract was not significant on *E. coli*, but was significant on *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella typhimurium*. Also, it was shown that the increases of the concentration of the extract caused higher antibacterial effects. The highest antibacterial effect of saffron petals hydroalcoholic extract was 120 mg which was observed against the *L. monocytogenes*. Saffron petals hydroalcoholic extract showed more antibacterial activity against the Gram-positive bacterium.

Conclusion: The results of this research showed that saffron petals extract has considerable potential as a herbal-base antimicrobial compound.

- **Please cite this paper as:** Fazeli-Nasab B. Evaluation of Antibacterial Activities of Hydroalcoholic Extract of Saffron Petals on Some Bacterial Pathogens. *J Med Bacteriol.* 2019; **8** (5, 6): pp.8-20.

Introduction

Crocus sativus Linn (family: Iridaceae) is a flowering and bulbous perennial stem-less plant in the Iridaceae family and is commonly known as saffron. Saffron, widely cultivated in Iran, is well adapted to arid and semi-arid lands and produces stigmas, annually. It is also adaptable to temperate and sub-tropical climates. Saffron has been used in folk medicine for more than 3000 years (1). It is native to Iran and widely used as a spice and as a coloring and flavoring agent in the preparation of various foods and either cosmetics. Also the stigmas of the plant are mainly used for therapeutic purposes (2).

Most of the plant polyphenols act as antioxidants, but recent evidences support the idea that these compounds primarily activate mild oxidative stress to elicit a positive, beneficial response from cells (3, 4). Phenolic compounds like flavonoids and anthocyanins are the biologically active components of the saffron petal that are thought to have medicinal effects in some human and animals diseases (5, 6).

Important constituents of saffron, which are pharmacologically active, are bitter (e. g. picrocrocin), volatile agents (e. g. safranal), and are coloring (e. g. crocetin and its glycoside crocin) (7). Saffron has been used as a food additive for several centuries and this supports its safety for human and medicine purposes (8). The antioxidant and antimicrobial properties of saffron have been noticed in recent years (3). The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity (9-11). The application of these compounds for detoxification and scavenging of ROS is still a matter of debate but it can be explained that their role is even more complicated. It is assumed that they might be signaling molecules in some pathways involved in the energetic balance (3). It is also mentioned that preventive effects by modulation of lipid peroxidation, antioxidants, and detoxification systems which is caused by saffron may be used in some diseases (12).

In general, saffron petal is not applied as a herbal tea or food component and also is a by-product that is usually discarded as waste (13). But, in some areas, the rest by-products of saffron are provided with food to flocks of domestic animals. A number of studies indicate that ethanol extract of saffron petals possesses antidepressant (14), anti-inflammatory (15), ant nociceptive (16), antihypertensive (17), anti-cancer and antitumor activities (18, 19), and also has been shown that saffron can promote the diffusivity of oxygen in different tissues (8). Intra-peritoneal LD50 values of saffron stigma and petal are reported to be 1.6 and 6 gr/kg in mice, respectively (20). Nevertheless, it is not toxic when administrated orally with LD50 value being above 5000 mg/kg (21). Although ingestion of less than 1.5g of saffron is nontoxic for human (22), it is considered toxic when ingested with doses more than 5 gr and could be lethal if taken about 20gr/day (22). However, the common effective doses applied in clinical trials are considerably lower than this level (30-50 mg/day) (7).

It is mentioned that the stigmas and petals of saffron ethanolic and aqueous extracts have some effects like anti-nociceptive and anti-inflammatory activities. Hence, this supports its traditional use as an anti-edematogenic remedy by the effects in the writhing test, xylene-induced ear edema in mice and formalin-induced edema in the rat paw (15).

The aqueous and ethanolic extracts of *Crocus sativus* petals causes a decrease in blood pressure in a dose-dependent manner in various models including: anesthetized rats, in isolated rat vas deferens, guinea pig ileum etc. where responses were initiated by electrical stimulation. This decrease in blood pressure was proposed to be mediated post-synoptically (13, 23, 24).

Crocus sativus petals and hydroalcoholic extracts of the stigmas have shown to possess anti-depressant activity in a 6-week double-blind, randomized and placebo-controlled trial and in animal-based pre-clinical studies. This antidepressant activity was similar to the activity

of standard drugs imipramine and fluoxetine (14, 17, 25).

The aim of the present study was undertaken to investigate the antibacterial activities status of the hydroalcoholic extract of the saffron petal.

Materials and Methods

Plant Materials and Preparation of saffron petals extract

The saffron were collected in 2017 from Qaen (Ghayen) County, South Khorasan Province, Iran and then identified by the Department of Plant Biology, University of Zabol.

The saffron petals were washed several times with deionized water and dried at room temperature. Then, 10 gr of the petals was homogenized in 100 ml of hydroalcoholic (30 mL double distilled water and 70 mL ethanol) and stirred (SKIR-601L model, UniEquip company, Germany) and kept on a rotator shaker (RO02 model, ParsAzma, Iran) at 190-220 rpm for 24h at room temperature in an airtight container till further use with the help of electric grinder (A11 basic model, IKA company, Germany), glass dish and filtered (26). After centrifugation at 10,000 rpm for 15 mins, the supernatant was collected and stored at 4 °C.

Antibacterial Activities Test

The antibacterial activity of saffron petals hydroalcoholic extract was assessed against *Escherichia coli* (*E. coli*) (ATCC 25922), *Staphylococcus aureus* (*S. aureus*) (ATCC 25923), *Salmonella typhimurium* (*S. typhimurium*) (PTTC 1609) and *Listeria monocytogenes* (*L. monocytogenes*) (ATCC 19118) and also was carried out based on propagation method in Mueller-Hinton agar medium (manufactured by Merck Germany) using paper blank sterilized disc (6 mm, Padtan-Teb Company, Iran) diffusion test (27, 28) and microbial susceptibility determination was performed by Bauer and Kirby (29).

In order to evaluate the interaction of hydroalcoholic extract of saffron petals and different concentrations of the extract (60, 90, 120 mg/ml) on four different Gram-positive and Gram-negative bacteria, a factorial design experiment was conducted based on completely randomized design with three replications and also in this research some antibiotics including Amikacin (AN), Ceftriaxone (CRO), Gentamycin (Gm), Ampicillin (Am), Penicillin (P), Sulphamethoxazole/trimethoprim (SXT), Cefoperazone (CP) and Azithromycin (AZM) were used as positive control.

Paper disc diffusion test method (28) has been widely used to assess the antimicrobial influences of various compounds. This method was described by Bauer, Kirby, Sherris, and Turck (generally known as the Kirby- Bauer test) (29). The paper disc diffusion technique is commonly used widely in assessing the antimicrobial activity of an impaired inhibitor (30). In this method, 6 mm sterilized filter paper disks are saturated with an antimicrobial agent at the desired concentration (31). Then, saturated discs are placed on the surface of suitable solid agar media such as Mueller-Hinton, tryptic soy agar or Nutrient agar, which are already inoculated by inorganic organisms (30).

The standard amount of bacteria inoculated is 10^8 CFU/mL (32) and this is equal to the turbidity standard of 0.5% McFarland. The drying time of the inoculated paper disc differs among the researchers from 2 hours to one full night under the laminar hood (32). Insert the inoculated plates into bacteria for 24 hours at 37 °C (31). After incubation, we reported the diameter of the inhibition zones in millimeters and the nearest point where 80% growth decreased (33).

After bacterial turbidity reached the half-MacFarland turbidity, the plates were inoculated with sterile swabs inoculated with the microbial suspension, and the disks were stacked with sterile pins and flame along with discs with plate walls of at least 5 mm and at least 25. The millimeter was determined and after complete contact with medium, 10 µl of herbal extract was

poured onto the discs (Nichipet EXII, Japan). After the above steps, the plates were kept at 37 °C for 18 to 24 hours in an incubator (RS232 model, Memmert, Germany). After the required time has elapsed, the inhibition zones (including the diameter of disc) were measured accurately 0.02 mm using the digital caliper (Mitutoyo, Japan) and also values < 10 mm were considered as nonnative extracts against bacteria (34, 35).

Statistical analysis of data

Data analysis was performed using Statistix 10 software (36). Due to the small amount of data, the normal distribution frequency was determined by Kolmogorov-Smirnov test ($P > 0.05$) then the statistical analysis was performed using two-way ANOVA followed by LSD (Least Significant Difference) test for multiple comparisons. Statistical significance was set at $P < 0.05$.

Result

The results of ANOVA showed that different concentration of saffron petals hydroalcoholic extract affected ($P < 0.01$) all the bacterial strains studied in this research (table 1) and also the highest antibacterial influence was detected on *Listeria monocytogenes* followed by *Escherichia coli* (Figure 1). The most effective concentration of saffron petals hydroalcoholic extract was 120 mg/ml and then by increasing the concentration of the extract, the antibacterial effect increased (Figure 2). Saffron petals hydroalcoholic extract showed more antibacterial activity against the gram-positive bacterium.

The results of ANOVA indicated that saffron petals hydroalcoholic extract and all antibiotics were inhibitory ($P < 0.01$) on *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella typhimurium* (table 2).

The most inhibiting material on *E. coli* were CP and then AN (Figure 3), but SXT and then CP were most active against *L. monocytogenes*, (Figure 4), SXT and then CP on *S. aureus*

(Figure 4), CP and then SXT against *S. typhimurium* (Figure 5). The antibacterial activities on *E. coli* of saffron petals hydroalcoholic extract was more effective than P and AM antibiotics (Figure 3), On *L. monocytogenes* was more effective than AM and CRO antibiotics (Figure 4), On *S. aureus* was more effective than AM antibiotics (Figure 5), On *S. typhimurium* was more effective than AM antibiotics (Figure 6).

The antibacterial activities of concentration of saffron petals hydroalcoholic extract was not significant on *E. coli*, but was significant on *L. monocytogenes*, *S. aureus* and *S. typhimurium* (table 3) that by increasing the concentration of the extract, antibacterial effect has increased. So, the highest antibacterial effect of saffron petals hydroalcoholic extract was 120 mg on the *L. monocytogenes* bacteria.

Discussion

The results of this study showed that saffron petals hydroalcoholic extract has an antibacterial effect on all bacterial strains studied, especially on *L. monocytogenes* and *E. coli* and also had a higher effect than AM antibiotics. Based on the fact that many herbal extracts have been reported to have insignificant inhibitory effect on *E. coli* as the result of the positive effect of saffron petals hydroalcoholic extract on *E. coli* it can be a good result and then the high efficiency of saffron petals hydroalcoholic extract on *L. monocytogenes* at 120 mg/ml concentration it can be an another good result but saffron petals hydroalcoholic extract has not the high antibacterial effect base on the majority of antibiotics studied. The current data showed that the petal extract of saffron contains antibacterial compounds.

Table 1. Variance Analysis of different concentration of saffron petals hydroalcoholic extract against bacteria.

S.O.V	df	SS	MS	F
Bacteria (B)	3	4.75	1.5833	844.44**
Saffron Extract Concern(SEC)	2	21.875	10.9375	5833.33**
B * SEC	6	10.625	1.7708	944.44**
Error	24	0.045	0.0019	
Total	33	37.295		
CV			3.5	

** Significant at 1 % probability

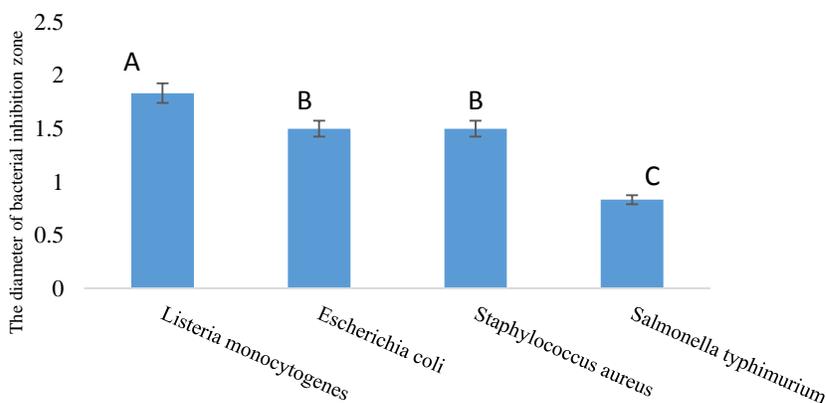


Figure 1. Mean comparison of the diameter of the bacterial inhabitation zone of saffron petals hydroalcoholic extract. The same letters indicate a significant difference.

Table 2. Variance analysis of the different concentration of saffron petals hydroalcoholic extract and antibiotics against bacterial strains.

S.O.V	df	MS			
		<i>E. coli</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>S. typhimurium</i>
Treatment of Saffron extract and Antibiotics (T)	8	6.59724**	10.2875**	3.00033**	8.73808**
Concern(C)	2	0.01661ns	1.0278**	0.58333**	0.19444**
T * C	16	0.00034ns	1.0278**	0.58333**	0.19444**
Error	54	0.00958	0.003	0.00305	0.00304**
CV		4.97	3.8	4.5	5.6

** Significant at 1 % probability

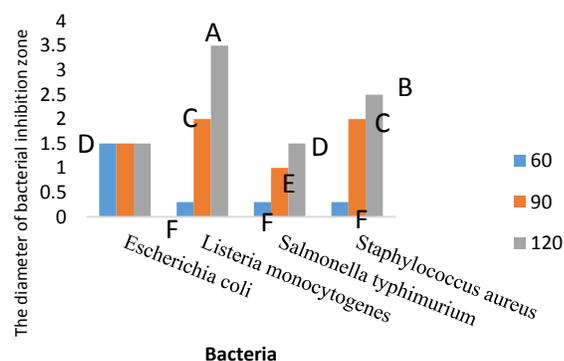


Figure 2. Mean comparison of the diameter of bacterial inhibitor zone of different concentrations of saffron petals hydroalcoholic extract. The same letters indicate a significant difference.

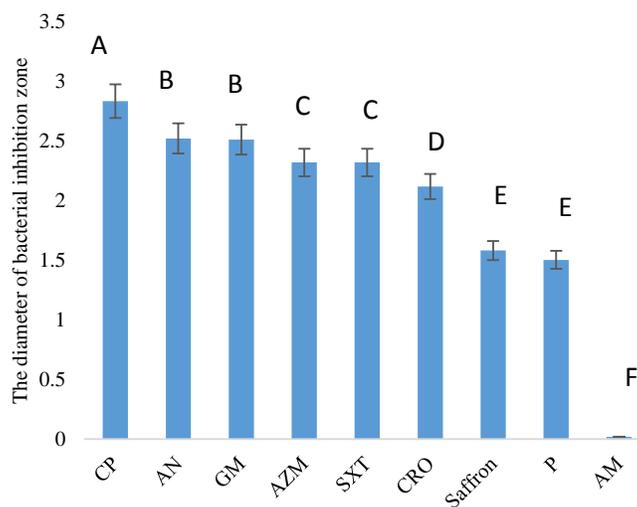


Figure 3. Evaluation of antibacterial activities of saffron petals hydroalcoholic extract and some antibiotics against *E. coli*. The same letters indicate a significant difference.

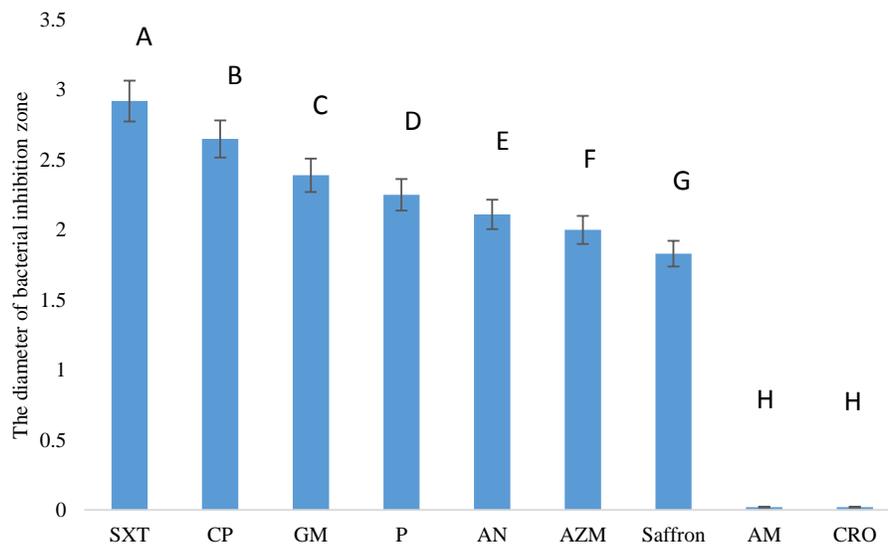


Figure 4. Evaluation of antibacterial activities of saffron petals hydroalcoholic extract and some antibiotics against *L. monocytogenes*. The same letters indicate a significant difference.

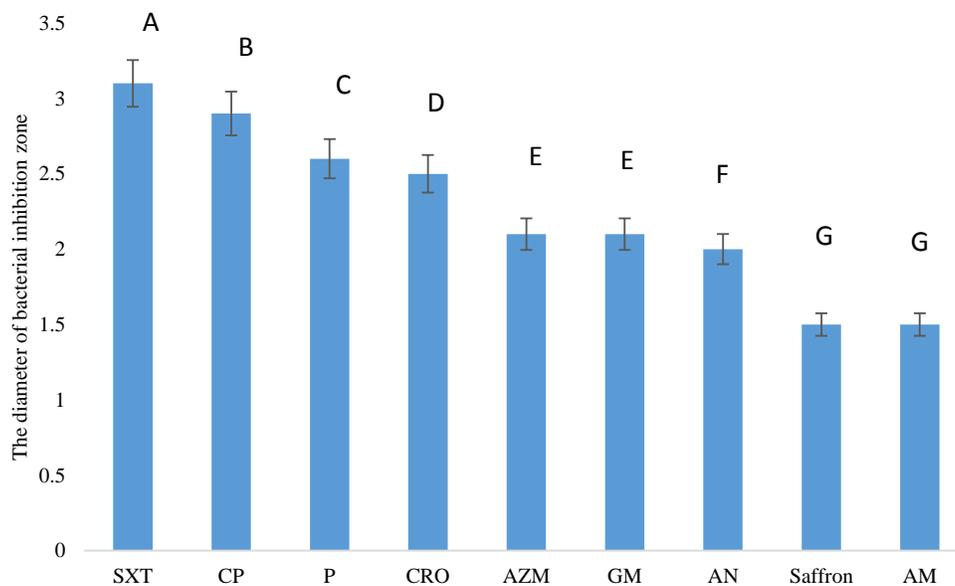


Figure 5. Evaluation of antibacterial activities of saffron petals hydroalcoholic extract and some antibiotics against *S. aureus*. The same letters indicate a significant difference.

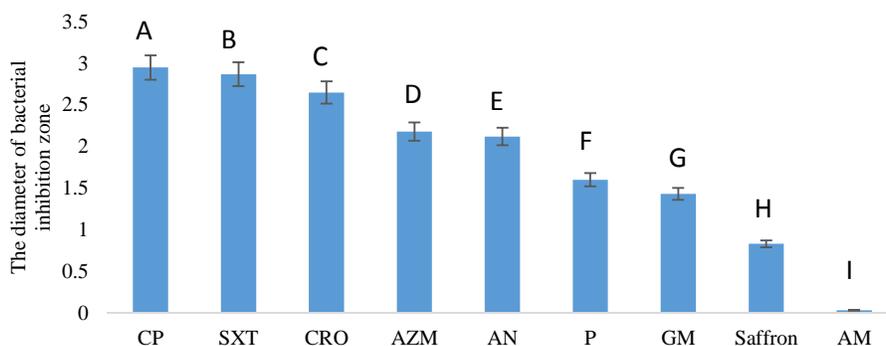


Figure 6. Evaluation of antibacterial activities of saffron petals hydroalcoholic extract and some antibiotics against *S. typhimurium*. The same letters indicate a significant difference.

Table 3. Evaluation of concentration of saffron petals hydroalcoholic extract and some antibiotics against bacterial strains .

S.O.V		<i>L. monocytogenes</i>	<i>S. Aureus</i>	<i>S. typhimurium</i>
AM	60	0.0 h	1.5 g	0 g
AM	90	0.0 h	1.5 g	0 g
AM	120	0.0 h	1.5 g	0 g
AN	60	2.11 f	2 f	2.12 c
AN	90	2.11 f	2 f	2.12 c
AN	120	2.11 f	2 f	2.12 c
AZM	60	2.00 g	2.13 e	2.18 c
AZM	90	2.00 g	2.13 e	2.18 c
AZM	120	2.00g	2.13 e	2.18 c
CP	60	2.65 c	2.98 b	2.95 a
CP	90	2.65 c	2.98 b	2.95 a
CP	120	2.65 c	2.98 b	2.95 a
CRO	60	0.00 h	2.51 d	2.65 b
CRO	90	0.00 h	2.51 d	2.65 b
CRO	120	0.00 h	2.51 d	2.65 b
GM	60	2.39 d	2.12 e	1.43 e
GM	90	2.39 d	2.12 e	1.43 e
GM	120	2.39 d	2.12 e	1.43 e
P	60	2.25 e	2.61 c	1.6 d
P	90	2.25 e	2.61 c	1.6 d
P	120	2.25 e	2.61 c	1.6 d
Saffron	120	3.50 a	0 h	1.5 e
Saffron	90	2.00 g	2.5 d	1 f
Saffron	60	0.00 h	2 f	0 g
SXT	60	2.92 b	3.1 a	2.87 a
SXT	90	2.92 b	3.1 a	2.87 a
SXT	120	2.92 b	3.1 a	2.87 a

In a research, buffered methanol extracts of *A. indica* and *R. chalepensis* showed inhibition zones against *B. cereus* at lower doses (400 Ag per disc) and also their acetone extracts were only active at doubled concentration (38). Of course, in another research (39) related that the ethanolic extracts (4000 Ag per disc) of the two plants were inactive against both Gram-positive and Gram-negative bacteria while in the present study the high efficiency of saffron petals hydroalcoholic extract was at 120 mg/ml concentration on *L. monocytogenes*.

A study has indicated that experiments with presence of activity at concentration of 100 µg/disc for extracts demonstrated a potential activity for antibacterial (37).

In some researches related that ethanol extract is the preferred solvent to extract phenol, flavonoids and other antioxidant material of herbs with antibacterial activities (11, 27, 40), but in some others researches, it has been shown that the ethanol extract of plants like *Vitex negundo* has no antibacterial activity against the bacterial strains studied. This is probably due to the preparation of the extract in ethanol. It is reported that ethanol extract of some medicinal plants lack antibacterial activities (41) in this research we found that ethanol solvent is suitable to extract antioxidant material with more antibacterial activities.

Some researchers have inked the paper blank sterilized discs before placing on the inoculated plates into the antimicrobial agent, while others preferred to immerse them in the antimicrobial agent after placing the paper disks on the inoculated plates (32, 42).

In a research (43) the antimicrobial activity of some plant extracts such as *Achillea millifolium*, *Caryophyllus aromaticus*, *Melissa officinalis*, *Ocimum basilicum*, *Psidium guajava*, *Punica granatum*, *Rosmarinus officinalis*, *Salvia officinalis*, *Syzygium joabolanum* and *Thymus vulgaris* against *Staphylococcus aureus*, *Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans*, *Proteus* spp., *Klebsiella pneumonia*, *Shigella* spp. *Enterobacter aerogenes* and *Escherichia coli* were

evaluated with antibiotic susceptible and resistant microorganisms. Their results showed that the highest antimicrobial potentials for the extracts of *Caryophyllus aromaticus* and *Syzygium joabolanum*, which inhibited 64.2 and 57.1% of the tested microorganisms, respectively, with higher activity against antibiotic-resistant bacteria (83.3%) and then they related that *Salvia officinalis* and *Achillea millifolium* extracts did not present any antimicrobial activity, but in this research the antibacterial activities of saffron petals hydroalcoholic extract were effective on all the bacteria studied.

It has been also shown that the crude extracts of *Ephedra gerardiana* (root and stem) harbors different antimicrobial properties against *B. atrophaeus*, *K. pneumonia*, *P. aeruginosa* and *B. subtilis*. Also, it has been indicated that the highest inhabitation zone (13.3mm) belong to the methanolic crude extract of stem against *B. atrophaeus*. A similar research (45) highlighted the fact that the medicinal plants such as *Ziziphus vulgaris*, *Malva sylvestris*, *Onosma bracteatum*, *Hyssopus officinalis*, *Ephedra gerardiana*, *Cordia latifolia*, *Althaea officinalis*, *Mentha piperita*, *Glycyrrhiza glabra*, *Justica adhatoda* seem to be inhibitory against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The order of antibacterial activity was *S. aureus*>*P. aeruginosa*>*E. coli*. Maximum zones of inhibition were seen in *Hyssopus officinalis* (3.37 cm) against *S. aureus*, *Glycyrrhiza glabra* (3.6 cm) against *E. coli* and *Justica adhatoda* (2.67 cm) against *P. aeruginosa*. But, in the present study, the highest inhabitation zone (35.5 mm) belongs to saffron petals hydroalcoholic extract against *L. monocytogenes*.

In a research (46) the antimicrobial activities of the methanol, ethyl acetate and hexane extracts of *Crocus biflorus*, *C. baytopiorum* and *Crocus flavus* subsp. *Dissectus* were investigated and it has been shown that the methanol extract of *Crocus flavus* subsp. *dissectus* had maximum activities on *Yersinia enterocolitica* and also the minimum inhibition concentrations of plant extracts have been found on *Staphylococcus aureus*, *Bacillus*

subtilis and *Bacillus cereus*. In comparison, another study (47) showed that crude extracts from *Inula aucherana*, *Fumaria officinalis*, *Crocus sativus*, *Vicum album*, *Tribulus terrestris*, *Polygonatum multiflorum*, *Alkanna tinctoria*, and *Taraxacum officinale* were screened for their *in vitro* antioxidant and antimicrobial properties. They showed that the plants are different based on antioxidant activity and total phenolic contents. *Viscum album* and *Crocus sativus* had the highest antioxidant (82.23%) and total phenolic content (42.29 mgGAE/g DW), respectively. they showed that the highest inhibition zone (15 mm) was seen in both *Inula aucherana* and *Fumaria officinalis* against *Staphylococcus aureus*.

In a research (48) the antimicrobial activity of different parts of *Croccus sativus* L. (saffron) including stigma, stamen, leaves, and colora, extracted by various solvents, were tested against different bacteria (*Micrococcus luteus*, *Staphylococcus epidermitis*, *Staphylococcus aureus* and *E. coli*) and fungi (*Candida albicans*, *Aspergillus niger* and *Cladosporium* sp.) by cup plate diffusion method and they showed that the ethyl acetate extract of stigma, stamen, and colora exhibit activity against the majority of the fungi and bacteria tested. However, no activity was observed when the ethyl acetate extract of leaves was used against test organisms at the concentration of 100 mg/ml. they said that the relative antifungal activity of ethyl acetate of stigma was higher than stamen. In contrast, the relative antibacterial activity of ethyl acetate extract of stamen was higher than the other parts of *Croccus sativus*. In the present study, the saffron petals hydroalcoholic extract has shown the effect on 60 mg/ml and also by increasing the concentration of the extract from 60 mg/ml to 120 mg/ml, the antibacterial effect has increased too.

In this research the antibacterial activity of hydroalcoholic extract of saffron was more against Gram-positive than Gram-negative bacteria. In accordance with previous findings, Gram-negative bacteria were not susceptible to plant extracts when compared to Gram-positive bacteria (42). The resistance of Gram-negative bacteria towards

antibacterial substances is related to lipopolysaccharides in their outer membrane (49).

The use of plants to heal diseases, including infectious one, has been extensively applied by people. Data from the literature, as well as our results revealed the significant potential of saffron for therapeutic treatment, in spite of the fact that they have not been completely investigated. Therefore, more studies need to be conducted to search for new compounds. Once extracted, and before being used in new therapeutic treatments, they should have their toxicity tested *in vivo*. Bioassays (51, 52) have demonstrated the toxicity of extracts from different plants.

Conclusion

Saffron petals hydroalcoholic extract has great potential as antimicrobial compounds against microorganisms. Thus, it can be used in the treatment of infectious diseases caused by resistant microbes and also the synergistic effect from the association of antibiotic with saffron extract against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment.

Acknowledgment

This work was supported by the Vice-Chancellor for Research grant (no. 23159) of Tehran University of Medical Sciences (Tehran, Iran) and Alborz University of Medical Sciences, Karaj. The authors are thankful to Karimi aviculture for providing the chickens for this research.

Funding information

This research was supported by the Vice Chancellor: Research and technology, University of Zabol.

Ethics approval and consent to participate

Authors confirm the progress of the study, anything occurring in the course of the study, any revision in the protocol. Consent to publish were obtained from all the participants.

Conflict of interest

The authors declare that they have no conflicts of interest.

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