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Evaluation of Reciprocal Pharmaceutical Effects and Antibacterial Activity of Silver Nanoparticles and Methanolic Extract of *Crocus sativus* L. (Saffron) on Some Bacterial Strains

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Abstract

Background: Silver nanoparticles (Ag-NPs) are the most prominent nanoparticles which are recognized for their high antimicrobial efficacy.

Objectives: The aim of this study was to evaluate the reciprocal pharmaceutical effects and antibacterial activity of Ag-NPs and methanolic extract of *Crocus sativus* L. (saffron) on some bacterial strains.

Materials and Methods: For evaluation of antibacterial activity of Ag-NPs and methanolic extract of *C. sativus* L. (saffron) on some bacteria, agar well diffusion method was used. Minimal inhibition concentration (MIC) and minimum bactericidal concentration (MBC) were determined for saffron extract, Ag-NPs, and their combination on methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. pyogenes* and *S. epidermidis*.

Results: The combination of medium concentrations of Ag-NPs (500 µg/mL) and saffron extract (50 mg/mL) was in the optimum mode to eliminate *S. epidermidis* and *S. pyogenes*. The results showed that saffron extract, Ag-NPs, and their combined form had antibacterial effects on these bacteria.

Conclusion: It is suggested to evaluate the synergistic effects of active components of the extract and antimicrobial preservatives used in food, health, pharmaceutical, and cosmetic industries.

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Introduction

Crocus sativus L., commonly known as saffron, belongs to the family of Iridaceae and is widely cultivated in Iran, India, and Greece.[1,2] Saffron is globally the most valuable industrial medical product and Iran is considered as the greatest producer of this plant.[3] Different parts of saffron including stigma, leaves, stamen and colora represent antimicrobial activity. Traditionally, among these parts stigma was mainly used for its medicinal properties. It also contains a variety of chemical constituents such as crocetin, crocin and other flavonoids, utilized for therapeutic purposes.[4-6] Although antimicrobial activities of saffron extract against various types of microorganisms have already been demonstrated, this research aimed at enhancing this activity. Skin as the largest organ is in direct contact with the environment and is the exposed suitable location for microorganisms. *Staphylococcus*, *Streptococcus* and *Pseudomonas* are the most important bacteria involved in skin diseases.

It is well known that among metal nanoparticles, silver nanoparticles (Ag-NPs) are the most dominant ones. During the past decades, due to unique biological properties of Ag-NPs such as antimicrobial activities, they have been of immense importance.[7-9] Materials at the nanoscale behave different from the same mass of material. Because of higher specific surface area to volume ratio of Ag-NPs compared to bulk silver metal, the resultant antimicrobial effects of Ag-NPs are higher.[10-12] Recently, considering the side effects of antibiotics and occurrence of antibiotic resistance in *Pseudomonas aeruginosa* and *Staphylococcus aureus*, usage of natural agents have attracted the attention of scientists.[13,14] *P.*

aeruginosa results from low permeability, antibiotic leakage system (efflux system), acquiring resistance genes via plasmids, transposons, and biofilm formation.[15,16] *P. aeruginosa* is the causative agent of infections in patients with immune deficiency, cystic fibrosis and burns.[17,18] *S. aureus* is the most important opportunistic pathogen in hospital infections and is the cause of endocarditis, toxic shock syndrome, furuncle and abscess.[19-21] Currently, green process is known as an efficient and powerful tool that inspires researchers to avoid usage of toxic chemicals and consequently to protect environment. Specifically, for this purpose herbal extracts can be invaluable resources to synthesize Ag-NPs.[22-24] In green synthesis protocol, there are no signs of the toxic chemicals, so this process offers numerous benefits including ecofriendliness and compatibility to pharmaceutical applications and also provides advancement due to cost-effectiveness.[25-28] In this study we reported the enhanced combinatory effects of Ag-NPs and saffron extract against *S. epidermidis*, *S. aureus*, *Streptococcus pyogenes*, *P. aeruginosa,* and methicillin-resistant *S. aureus* (MRSA).

Materials and Methods

Preparation of Saffron Extract

Saffron stigmas that were used in this research were purchased from Novin Saffron Company (Mashhad, Iran). For preparation of methanolic extract of *C. sativus*, the stigmas of plant were dried at room temperature and powdered by electric blender. About 30 g of dry powdered plant material was extracted on the basis of maceration method with methanol (MeOH). In this method, the powdered plant was soaked in methanol for 3 days. The clear solution obtained with the filtering was collected in a glass container and this procedure was repeated 3 times. Finally, the yield from 30 g dry plant was 10 g extraction. Dried extracts were stored in a dark, cool place away from light and moisture.[14]

Preparation of Different Concentrations of Extract

The prepared methanolic extract was weighted by analytical scale (with accuracy of 0.0001) in the range of 6.25, 12.5, 25, 50, 100 mg. For preparation of the concentrations of 6.25, 12.5, 25, 50, 100 mg/mL, 1 mL of DMSO 10% was added to each of the weighted extracts.

Silver Nanoparticles

Ag-NPs were purchased from Lotus Pasargad Company (Tehran, Iran) in the form of colloidal solution and with the brand of colloidal silver (LNP-CS). This material is completely water soluble and its carrier is distilled water. The concentration of nanoparticles used in this research was 8000 µg/mL and the size of nanoparticles in this product was approximately 40 nm. According to the manufacturers recommendations, monoetilenglicol and distilled water are its solvent and in this study, distilled water was applied as solvent. The concentrations of Ag-NPs colloid were prepared by serial dilution using distilled water.[15]

Preparation of Different Concentrations of Silver Nanoparticles Solution

To obtain the desired concentrations of Ag-NPs colloid with distilled water, the concentrations of 8000, 4000, 2000, 1000, 500, 250, 125 and 62.5 µg/mL from Ag-NPs in sterile deionized water were prepared with serial dilution.

Preparation of Silver Nanoparticles Solution Combined With Saffron Extract

Minimum, medium and maximum concentrations of saffron extract and nanoparticles, combined16 (16) according to the ratio of 1:1, were determined as below (Table 1).

Bacterial Strains

The following 5 strains of bacteria were used for testing, respectively: *S. epidermidis* ATCC 12228, *S. aureus* ATCC 25923, *S. pyogenes* ATCC 27853, *P. aeruginosa* ATCC 19615 and MRSA ATCC 33591. It is noteworthy that Microbiology Laboratory of Tarbiat Modares University, Tehran, Iran provided us with these microorganisms.

Antimicrobial Assay

Agar well diffusion method, minimal inhibition concentration (MIC) and minimum bactericidal

Table 1. Combination of Different Concentrations of Saffron Extract and Silver Nanoparticles

Silver Nanoparticles	Saffron Extract	Number
Minimum concentration (62.5 µg/mL)	Minimum concentration (12.5 mg/mL)	1
Minimum concentration (62.5 µg/mL)	Medium concentration (50 mg/mL)	2
Minimum concentration (62.5 µg/mL)	Maximum concentration (100 mg/mL)	3
Medium concentration (500 µg/mL)	Minimum concentration (12.5 mg/mL)	4
Medium concentration (500 µg/mL)	Medium concentration (50 mg/mL)	5
Medium concentration (500 µg/mL)	Maximum concentration (100 mg/mL)	6
Maximum concentration (8000 µg/mL)	Minimum concentration (12.5 mg/mL)	7
Maximum concentration (8000 µg/mL)	Medium concentration (50 mg/mL)	8
Maximum concentration (8000 µg/mL)	Maximum concentration (100 mg/mL)	9

concentration (MBC) analyses were the applied three methods to determine the *C. sativus* extract, Ag-NPs solution, and the combinatory antimicrobial activities.

Evaluation of Early Antibacterial Effects of Saffron Extract and Silver Nanoparticles Solution

Antimicrobial activity of the extract was assayed using agar well diffusion method. All the bacteria were incubated at 37°C for 24 hours by inoculation in Mueller-Hinton broth. Each colony was separately suspended in a normal saline solution, so the cultured suspensions were adjusted using 0.5 McFarland turbidity standard tubes. Mueller-Hinton agar was poured into each sterilized Petri dish. The wells each of 6 mm diameter, were made on Petri dishes which were streaked with the microorganism's saline suspension. Then, 100, 50, 25, 12.5, 6.25 mg/mL of extract and 8000, 4000, 2000, 1000, 500, 250, 125, 62.5 µg/mL of Ag-NPs were prepared and 100 µL of each extract solution was added in wells along with positive control (antibiotic) and negative control (solvent) and Petri dishes were incubated at 37°C for 24 hours. After 24 hours the zone of inhibition produced by each dilution of extract was measured. In this study, 20 mg/mL of Gentamicin was used as positive control for Gram-negative bacteria and 25 mg/mL of vancomycin was used as positive control for gram-positive bacteria. DMSO 10% was used as negative control for methanolic extract and distilled water was used for nanoparticles. In this research the experiments were performed three times and the results were proposed as mean.

Evaluation of Early Antibacterial Effects of Saffron Extract and Silver Nanoparticles Solution Combination

Each colony was separately suspended in a normal saline solution and the cultured suspensions were adjusted using 0.5 MacFarland turbidity standard tubes. Afterwards,100 µL of each extract solution was added in wells which contained the minimum (12/5 mg/mL), medium (50 mg/mL) and maximum (100 mg/mL) concentrations of methanolic extract and minimum (6.25 µg/mL), medium (500 µg/mL) and maximum (8000 µg/mL) concentrations of nanoparticles solution along with positive control (antibiotic) and negative control (solvent) and Petri dishes that were incubated at 37°C for 24 hours. This procedure also was repeated three times and the average diameter of inhibitory zone of methanolic extract and nanoparticles solution were measured.

Determination of Minimal Inhibition Concentration and Minimum Bactericidal Concentration

In this study, for determination of MIC value, the broth micro dilution method in micro plate was utilized. Based on the evaluation of antibacterial effects of saffron extract, the presence of diameter of inhibitory zone was shown in 2 bacteria of all and we performed MBC value measurement just for these 2 bacteria. But all of the bacteria in this research showed the diameter of inhibitory zone in nanoparticles; so MIC value was measured for all of the bacteria. One hundred microliter of different concentrations of extract, nanoparticles and the combination of extract-nanoparticles were used for each well of micro plate. Then microbial suspension of each strain was prepared according to 0.5 MacFarland in MHB medium and 100 µL of these suspensions were added to each well. The final volume of microbial suspension and final concentrations were calculated by serial dilution method. Each well of micro plate contained medium, bacteria, and extract or nanoparticles or the combination of extract and nanoparticles. Comparing the wells with positive control and negative control can detect the growth or inhibition of bacteria and MIC value. Due to the turbidity in all of the wells, 20 µL of contents of each well was cultured on MHA plates and incubated at 37°C for 24 hours. This procedure was repeated for three times and the colony count was evaluated. The concentration, at which no colonies of bacteria were grown, was reported as MBC. Each test was repeated three times and the results were recorded.

Statistical Analysis

Statistical analysis was performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). For multiple comparisons, data were analyzed by one-way analysis of variance (ANOVA) and followed by LSD test. The *P* value less than 0.05 was considered significant. The results are expressed by mean ± standard deviation (SD).

Results

The antibacterial activity of methanolic extract of saffron was assayed in different concentrations against five species of bacteria by agar well diffusion method. As displayed in Figure 1 and Tables 1 and 2, the saffron extract showed diameters of inhibition zone against *S. epidermidis* and *S. pyogenes* and the mean diameter of inhibition zone regularly increased with higher concentrations of saffron extract. Ag-NPs used in this research had influence on the average diameter of the inhibition zone of *S. aureus*, MRSA, *S. epidermidis*, *S. pyogenes* and *P. aeruginosa*

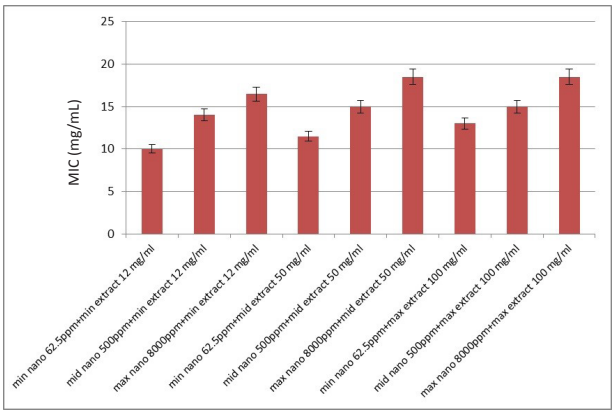

Figure 1. Average Inhibition Zone Diameter (mm) of *Streptococcus pyogenes* in Different Concentrations of Saffron Extract and Ag-NPs by Agar Well Diffusion Method.

Table 2. Comparison of Different Concentrations of Saffron Extract in *Staphylococcus epidermidis*[a]

Method	Bacteria	Concentration mg/mL	Extract	Mean (mm)	Standard deviation (mm)
Agar well diffusion	*Staphylococcus epidermidis*	25.0 Van	Saffron	34.5	0.58
		6.25	Saffron	-	-
		12.5	Saffron	9	0
		25	Saffron	9.25	0.5
		50	Saffron	11	0.82
		100	Saffron	13	0.82

[a] $P=0.001$.

and the mean diameter of inhibition zone increased with higher concentrations of saffron extract. Moreover, combining 2 extracts revealed that the combination cannot influence the mean diameter of inhibition zone but each individually can show this effect. Since higher concentrations of saffron extract and Ag-NPs individually increased, the diameter of inhibition zone when saffron extract and Ag-NPs were in their maximum concentration values, had the highest mean diameter of inhibition zone and vice versa. It means, when saffron extract and Ag-NPs were in their minimum concentration, they had the lowest mean diameter of inhibition zone. According to Tables 1 and 2, various concentrations of saffron extract against *S. aureus* and *S. pyogenes* lead to different diameters of inhibition zone and this difference is significant. Moreover, as observed in Tables 1 and 2, an increase in the concentration of saffron extract is directly related with the increase in diameter of inhibition zone.

The most effective MIC values were methanol extracts of *C. sativus L.* (MIC/MBC=50 mg/mL) for *S. epidermidis* and (MIC/MBC=50 mg/mL) for *S. pyogenes*. Remarkably, the methanolic extract containing Ag-NPs exhibited low MIC values in the range of used medium concentration (500 µg/mL) of methanol extract (50 mg/mL), which is very significant in both *S. epidermidis* and *S. pyogenes* as expected.

According to the results, effect of different concentrations of Ag-NPs on *S. aureus*, *S. epidermidis*, *S. pyogenes,* MRSA and *P. aeruginosa* led to different diameters of inhibition zone and this difference was significant. Moreover, as shown in Figures 2-5, with increasing concentration of saffron extract and Ag-NPs, diameter of inhibition zone in abovementioned bacteria raised.

Discussion

The current study investigated the influence of different concentrations of *C. sativus* L. (saffron) against 4 species of gram-positive bacteria and one species of gram-negative bacteria. The inhibitory effects of methanolic extracts of

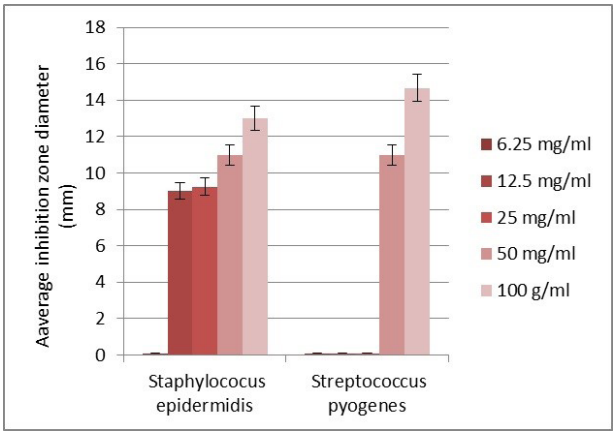

Figure 3. Compared Average Inhibition Zone Diameter (mm) of *Staphylococcus epidermidis* and *Streptococcus pyogenes* in Different Concentrations of Saffron Extract By Agar Well Diffusion Method. This diagram indicates that higher concentrations of saffron extract against *Staphylococcus epidermidis* and *Streptococcus pyogenes* lead to higher average inhibition zone diameter.

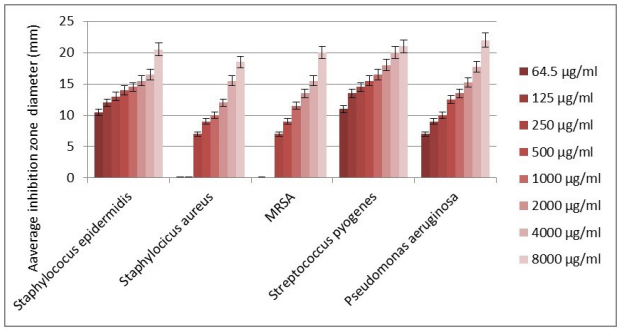

Figure 2. Average Inhibition Zone Diameter (mm) of Bacteria in Different Concentrations of Ag-NPs by Agar Well Diffusion Method. This diagram shows that higher concentrations of Ag-NPs against all of bacteria that were studied in this research lead to increasing average of inhibition zone diameter.

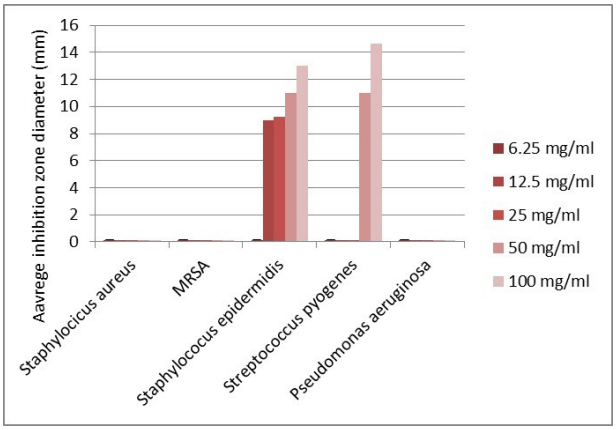

Figure 4. Average Inhibition Zone Diameter of Bacteria in Different Concentrations of Saffron Extract by Agar Well Diffusion Method.

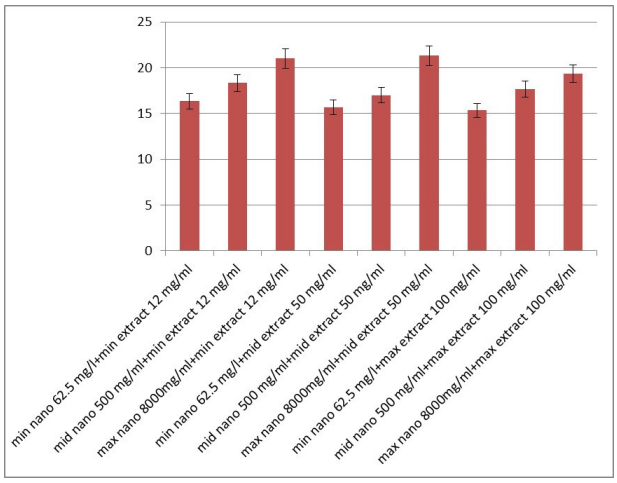

Figure 5. Average Inhibition Zone Diameter (mm) of Staphylococcus epidermidis in Different Concentrations of Saffron Extract and Ag-NPs by Agar Well Diffusion Method.

C. sativus L. were examined on the growth of all species of the tested bacteria. Results showed that *S. epidermidis* had a greater diameter of inhibition zone in low concentrations and was more sensitive than *S. pyogenes*. In this research, Ag-NPs showed antibacterial effect on all five species and according to the chart, it seems that *S. pyogenes* and *S. epidermidis* were more sensitive than *P. aeruginosa*. In the study of Im et al,[22] it was reported that the antibacterial activity of the Leonuri herbal extract against *P. aeruginosa*, *Escherichia coli* and *Enterobacter cloacae* can be enhanced through the combination with Ag-NPs. Bindhu MR, Umadevi in their study deduced that the prepared Ag-NPs had pronounced antimicrobial activity against *Escherichia coli*, *Proteus mirabilis* and *Shigella flexneri*.[29] Veerasamy et al[30] suggested that the biosynthesized Ag-NPs using mangosteen leaves extract proved excellent antimicrobial activity. While various theories were proposed to explain the mechanism of antimicrobial activity of Ag-NPs, it is but widely believed that Ag-NPs by penetrating to cell membrane of bacteria and modulating cellular signaling by dephosphorylating putative key peptide substrates on trypsin residues and tending to bind to sulphur and phosphorous containing compounds like DNA that leads to the condensation of DNA and the loss of its ability to replicate and leakage of intracellular materials can cause the cell death.[13,14] The other way is explained in terms of the interaction of Ag-NPs with bacterial peptidoglycan cell wall, thus silver ion released from Ag-NPs destroys the peptidoglycan. Moreover, based on recent studies the mechanism mediated by the generation of reactive oxygen species by Ag-NPs may result in the significant antibacterial activity of Ag-NPs.[14] These studies indicate a simple, reliable, rapid and economical method to synthesize Ag-NPs and furthermore these biologically synthesized nanoparticles are highly effective against multi-drug resistant pathogens.[29] Dipankar C, Murugan proposed that the biosynthesized Ag-NPs using *Iresine herbstii* leaf aqueous extract represented excellent antimicrobial activity.[31]

For investigation of the antibacterial activities of methanolic extract-mediated Ag-NPs, the incubation method was performed. Ag-NPs exhibited antibacterial activities against five strains, and *P. aeruginosa* possessed slightly higher MIC/MBC values among the strains. The methanol extract did not show any significant antibacterial activity against *S. aureus*, MRSA and *P. aeruginosa*. It had antibacterial activities only against *S. epidermidis* and *S. pyogenes*. The saffron extract was effective on the average diameter of inhibition zone and this effect was significant. However, the effect of nanoparticles despite their relatively high effectiveness was not significant. Additionally, as expected, the reciprocal effects of combination were not significant. But according to our results, the highest effect on the diameter of inhibitory zone of *S. epidermidis* and *S. pyogenes* was occurred in the highest concentrations of saffron extract. And in *S. pyogenes*, the saffron extract and nanoparticles solution were individually effective on the average diameter of inhibitory zone but the combined form was not effective. It is well known that the high surface per volume ratio and crystallographic surface structure of Ag-NPs result in significant antibacterial activity.[17] It seems that the methanol extract containing Ag-NPs showed better antibacterial activities against *S. epidermidis* and *S. pyogenes*. However, *S. pyogenes* is more sensitive than *S. epidermidis*.

To conclude, we used low concentrations of nanoparticles for MIC value. The way in which plant extracts and Ag-NPs formulations were used simultaneously to increase the antibacterial activity is very efficient, and can be used for other plants and nanoparticles in the near future. The isolation of different components of saffron extract and evaluation of their antibacterial effects and furthermore evaluation of antiviral and antifungal effects of extract and Ag-NPs are also recommended. Furthermore, it is suggested to evaluate the synergistic effects of active components of the extract and antimicrobial preservatives used in food, health, pharmaceutical and cosmetic industries.

Authors' Contributions

Study design: AS. Experiment design, experiment conduct, data interpretation and manuscript edition: AS, HRA, NS. Experiment performance: HRA, AS. Data collection, data interpretation, grant application, and manuscript preparation: AS, MR, NS, RH, SHH.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Ethical Approval

Not applicable.

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