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Original Article

The Evaluation of Antibacterial Activity of Silver Nanoparticle in Combination with *Lavandula angusifolia* Extract through Response Surface Methodology

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ABSTRACT

Background: Many research studies have investigated the antimicrobial activity of nanoparticles and herbal extracts on pathogenic bacteria. The aim of this study was to evaluate the antibacterial activity of silver nanoparticles in combination with *L. angusifolia* leaf extract against *Escherichia coli* and *Staphylococcus aureus* using response surface methodology.

Methods: To evaluate the antibacterial activity of silver nanoparticles and *L. angusifolia* extract at different pH values against *E.coli* and *S. aureus*, the response surface methodology was used along with a central composite design. Agar well diffusion method was used to determine the antibacterial activity.

Results: The results showed that the antibacterial activity of the combination of silver nanoparticles and *L. angusifolia* extract on *E.coli* (15.4 - 23.6 mm) was greater than that on *S. aureus* (11.7 – 21.6 mm). In addition, the antibacterial activity of the silver nanoparticles against *E. coli* and *S. aureus* was higher than that of *L. angusifolia* extract. The pH values had no effect on the antibacterial activity of the silver nanoparticles and *L. angusifolia* extract.

Conclusion: The findings of this study showed that the combination of silver nanoparticles and *L. angusifolia* extract could be used as a possible source of effective antibacterial agent in infections.

Keywords: Silver Nanoparticle, *L.angusifolia*, Antibacterial, Response Surface Methodology

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Introduction

Bacterial infections are one of the leading causes of death today. Antibiotics are increasing day by day due to their cost-effectiveness and powerful results and are a desirable treatment for bacterial infections. These days the bacterial resistance to antibiotics has become a global problem (1). The increased use of antibiotics has increased the bacterial resistance and therefore, antibiotic resistance has led researchers to look for suitable alternatives. One of the suitable candidates is nanoparticles (2). Nanoparticles are materials that having dimensions in the order of 1–100 nm (3). Nanoparticles, such as nano-metal materials, have appeared to be promising candidates, since they have wide cytotoxic activity against the broad- spectrum of microorganisms such as bacteria, fungi and even viruses (4-6). Many investigations have performed on the antimicrobial activity of nanoparticles (7-9), and recent research achievements revealed that it is possible to produce new types of nanoparticles for achieving more antibacterial activity (10). The studies have proven that some changes on nanoparticles such as surface modifications with biomolecules and polymers are effective strategies to make the nanoparticles more effective (11). Recently, the combination of different drugs has been used for the treatment of many infectious diseases to reduce toxicity and increase medicinal effect against resistant bacteria (12). One approach that has been considered recently is the combination of nanoparticles with other antimicrobial agents such as herbal extracts (13, 14).

The herbs are traditionally used around the world to treat many diseases, especially infectious diseases. The plants can be considered as a source of potentially useful chemicals. These chemicals can be used not only in the form of drugs, but also as a unique model and starting point for manufacturing drug analogues. *Lavendula angustifolia* has been considered as the most popular of all essential oils used in aromatherapy (15). *L.angustifolia* belongs to the *Labiatae* family, which is native to the Mediterranean region. There are more than 30 species in genus of *Lavandula*. The extract of *Lavandula* leaves and flowers has a lethal effect on a wide range of bacteria, so that its antimicrobial effect has been identified in most studies (15- 17).

The Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables (18). One of the main objectives of RSM is to optimize response variable. Optimization of several parameters by the method of one factor at a time is time consuming, therefore, RSM can be replaced it particularly when the interactions among factors are important (19, 20).

Despite the proven antibacterial activity of silver nanoparticles, no studies could be found on the antimicrobial ability of silver nanoparticles in combination with *L. angustifolia* extract. Due to this issue, the present study was carried out to evaluate the antibacterial activity of the silver nanoparticles in combination with *L. angusifolia* leaf extract using the response surface methodology against *E. coli* and *S. aureus*.

Materials and Methods

Chemicals

The silver (Ag) nanoparticle with dimensions of 20 nm was purchased from US Research Nanomaterials Co (USA). All other chemicals were of analytical grade from standard suppliers.

Microorganisms and media

Antibacterial activities of silver nanoparticle and *L.angusifolia* leaf extract were tested for two bacteria obtained from clinical specimens including *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive). These strains were maintained on Nutrient Agar at 4°C for subsequent studies.

Extraction

For extraction, 60 g of dried leaves of *L.angusifolia* powder was mixed with 300 ml of methanol as the solvent in the flask. The flask was placed in Soxhlet extractor and was boiled for 8 hours. This solvent was then slowly evaporated at 40 °C using a rotary apparatus to get dried methanol free extract of *L.angusifolia*.

Determination of minimum inhibitory concentration of silver nanoparticle and L.angusifolia leaf extract

To obtain the approximate values of the activity of silver nanoparticle and *L.angusifolia* leaf extract against *E.coli* and *S.aureus* for central composite design, the minimum inhibitory concentrations (MIC) of silver nanoparticle and *L.angusifolia* leaf extract were determined. For this purpose, the microtiter plate

assay was used. The silver nanoparticle was used with concentrations of 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/ml for each bacterial strain. The *L.angusifolia* leaf extract was also used with concentrations of 5, 10, 15, 20, 25, 30, 35 and 40 mg/ml for each bacterial strain. The bacterial strain suspensions were prepared in the sterile normal saline to a concentration of 0.5 McFarland standard solutions.

Agar well diffusion method

Antibacterial activity of the silver nanoparticle and *L.angusifolia* leaf extract against *E.coli* and *S.aureus* was studied using agar well diffusion method. The petri dishes including semi solid Mueller Hinton agar (MHA) were cultured with 1.5×10^8 CFU/mL suspensions of test bacteria. Different concentration of silver nanoparticle and *L.angusifolia* leaf extract (the values designed by central composite design) were inoculated into the wells (6 mm diameter). The plates were incubated for 24 hours at 37°C. Antimicrobial activity was measured based on the diameter of inhibition zone in mm. The pH adjusted by addition of 1 M NaOH and 1 M HCl.

Statistical analysis

In order to optimize the antimicrobial activity of the silver nanoparticle and *L.angusifolia* extract, the RSM with central composite design (CCD) was applied containing three factors. The factors include silver nanoparticle (AgNPs), *L.angusifolia* extract (Extract), and pH, which were selected as independent variables. In addition, the inhibition zone diameters of *E.coli* and *S.aureus* were chosen as the response variables. Minitab statistical software (version 18.1) was used for experimental design and data analysis (21). The levels of independent variables are shown in Table 1. A total of 20 experiments designated using Minitab software and statistical analysis was performed to evaluate the analysis of variance (ANOVA). It should be noted that 95% level of confidence was used in this research. Table 4 reports all data of 20 runs that were used to estimate the coefficients for the evaluation of antibacterial activity of the silver nanoparticle in combination with *L.angusifolia* extract.

Table 1. Levels of three independent variables used for designing central composition design

	Levels				
Factors	-1.68	-1	0	1	1.68
AgNPs (mg/mL)	0.132	0.2	0.3	0.4	0.468
***L.angustifolia* extract (mg/mL)**	16.59	20	25	30	33.41
pH	5.32	6	7	8	8.68

Results

Minimum Inhibitory concentration of the silver nanoparticle and L.angusifolia extract

The MIC results of silver nanoparticles and *L. angusifolia* leaf extract against *E. coli* and *S. aureus* are shown in Table 2. The maximum MIC values of silver nanoparticles and *L. angusifolia* leaf extract belonged to *S. aureus* (0.3 and 25 mg/mL, respectively). Therefore, 0.2 and 0.4 mg/mL of silver nanoparticles were selected as levels of -1 and +1 and 20 and 30 mg/mL of *L. angusifolia* extract as levels of -1 and +1, respectively.

Table 2. The Minimum inhibitory concentration of silver nanoparticles and L. angusifolia extract against E. coli and S. aureus

	AgNPs(mg/ml)	L.angusifolia extract(mg/ml)
S.aureus	0.3	25
E.coli	0.2	20

Antibacterial activity of the silver nanoparticle and L.angusifolia extract against E.coli and S.aureus

In this study, the antimicrobial activities of the silver nanoparticle and *L.angusifolia* leaf extract were investigated by growing *S.aureus* and *E.coli* colonies on MHA plates, supplemented with different concentrations of silver nanoparticle and *L.angusifolia* leaf extract at different pH which designed by CCD. According to the designed experiments, 20 experiments were carried out and the results have been shown in Table 3.

Table 3. Central composite design and responses for *E.coli* and *S.aureus*

Run number	AgNPs (mg/m)	L.angusifolia (mg/mL)	pH	*E.coli* (mm)	*S.aureus* (mm)
1	0.300	16.59	7.00	15.5	0.25
2	0.300	25.00	7.00	20.2	0.75
3	0.132	25.00	7.00	13.9	0.25
4	0.300	25.00	7.00	23.6	0.5
5	0.400	20.00	8.00	19.4	1
6	0.300	25.00	7.00	18.5	0.25
7	0.200	30.00	8.00	18.2	0.75
8	0.468	25.00	7.00	22.3	0.5
9	0.400	30.00	6.00	23.2	0.25
10	0.200	20.00	6.00	16.5	0.75
11	0.200	20.00	8.00	15.4	0.25
12	0.400	20.00	6.00	19.2	0
13	0.300	25.00	8.68	20.1	0.75
14	0.400	30.00	8.00	25.2	0.5
15	0.300	25.00	5.32	17.9	0.75
16	0.300	25.00	7.00	22.7	0.75
17	0.300	25.00	7.00	19.7	0.75
18	0.200	30.00	6.00	16.3	0.75
19	0.300	33.41	7.00	23.6	0.5
20	0.300	25.00	7.00	20.9	0.5

The regression models for inhibition zone diameter of the silver nanoparticle for *E.coli* and *S.aureus* as response variables were found in the linear regression equations as follows:

E.coli = 19.615 + 2.543 AgNPs + 1.905 Extract (1)

S.aureus = 17.17 + 2.439 AgNPs + 1.431 Extrac (2)

The adequacy of regression models was evaluated using analysis of variance (ANOVA). The ANOVA results for *E.coli* and *S.aureus* based on Equations (1) and (2) have been shown in Tables 4 and 5, respectively and it can be concluded that all model terms are significant (P-value< 0.05). Additionally, the F-value of 22.83 and P-value<0.05 indicate that the response surface linear model (1) is significant. In addition, the F-value of 26.17 and P-value<0.05 reveal that the response surface linear model (2) is significant as well. Tables 6 and 7 show the test of significance for regression coefficients of Equations (1) and (2), respectively. The P-vales of these tables also confirm that all model terms are significant (P-value< 0.05).

Table 4. Regression analysis by ANOVA for *E.coli*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	2	137.89	68.945	22.83	0.000
Linear	2	137.89	68.945	22.83	0.000
AgNPs	1	88.31	88.305	29.24	0.000
Extract	1	49.58	49.585	16.42	0.001
Error	17	51.34	3.020		
Lack-of-Fit	12	33.12	2.760	0.76	0.680
Pure Error	5	18.21	3.643		
Total	19	189.23			

Table 5. Regression analysis by ANOVA for *S.aureus*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	2	109.21	54.604	26.17	0.000
Linear	2	109.21	54.604	26.17	0.000
AgNPs	1	81.26	81.262	38.94	0.000
Extract	1	27.95	27.947	13.39	0.002
Error	17	35.47	2.087		
Lack-of-Fit	12	18.09	1.508	0.43	0.890
Pure Error	5	17.38	3.476		
Total	19	144.68			

Table 6. Testing of the significance of the regression coefficients associated with *E.coli*

Term	Coef	SE Coef	T-Value	P-Value
Constant	19.615	0.389	50.48	0.000
AgNPs	2.543	0.470	5.41	0.000
Extract	1.905	0.470	4.05	0.001

Table 7. Testing of the significance of the regression coefficients associated with *S.aureus*

Term	Coef	SE Coef	T-Value	P-Value
Constant	17.170	0.323	53.16	0.000
AgNPs	2.439	0.391	6.24	0.000
Extract	1.431	0.391	3.66	0.002

Figures 1 and 2 show the surface plots of *E.coli* and *S.aureus*, respectively, versus the factors AgNPs and Extract. It can be easily seen from the figures that the inhibition zone diameter tends to grow for *E.coli* and *S.aureus*, when the amount of silver nanoparticle and *L.angusifolia* leaf extract increases. This is in complete agreement with the previous results obtained from the linear regression models (1) and (2).

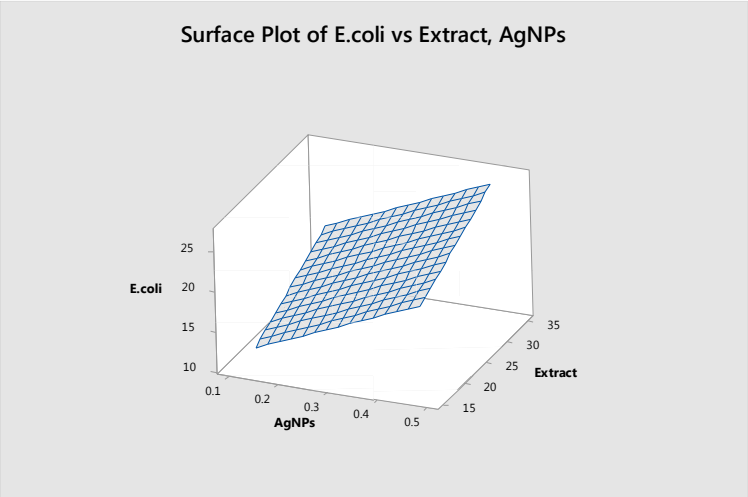

Figure 1. Surface plot of *E.coli* versus AgNPs and Extract

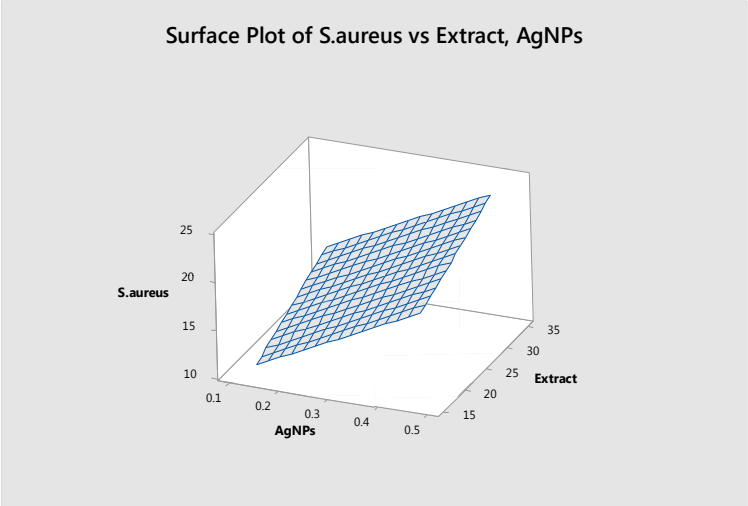

Figure 2. Surface plot of *S.aureus* versus AgNPs and Extract

Discussion

There have been a number of investigations on the antimicrobial activities of nanoparticles and herbal extracts individually (7, 22-24). However, some studies have focused on the combination of nanoparticles or plant extract together or with other antimicrobial agents such as antibiotics (13, 25-27). Rapper *et al.* studied the antimicrobial activity of *Lavandula angustifolia* essential oil in combination with antimicrobial agents against *S. aureus*, *P. aeruginosa* and *Candida albicans* and the finding showed that *L. angustifolia* essential oil had synergy in combination with the mentioned antibiotics (15). A study conducted in 2014 aimed to identify the antimicrobial properties of 35 essential oil antimicrobial compounds such as *L. angustifolia*. The oils were tested in combination with beta-lactam antibiotics and the results showed that *L. angustifolia* essential oil in combination with piperacillin had the highest synergy (17). Regarding the combination of nanoparticles and herbal extract, it was reported that a mixture of the nettle extract with silver nanoparticles and a mixture of the shallot extract with silver nanoparticles had a synergistic effect and an additive effect on *Acinetobacter baumannii* isolates, respectively (12). Jafari *et al.* showed that the effect of the combination of silver nanoparticles and *Calendula officinalis* extract was much greater than the effect of each of them against *S. aureus*, *B. cereus* and *E. coli* (28). In another study, the combination of the silver nanoparticles with *Drosera binata* extract were studied against resistant *S. aureus* and the results showed that their combination had more antibacterial activity than the use of each of them alone (14).

In this study, the antibacterial activity of silver nanoparticles in combination with the *L.*

angusifolia leaf extract against *E. coli* and *S. aureus* was evaluated using RSM. The results were consistent with the above results. The results of this study showed that the effect of the combination of silver nanoparticle and *L. angusifolia* extract on *E. coli* and *S. aureus* was much greater than the effect of each of them alone. The effect of silver nanoparticles and *L. angusifolia* extract on the diameter of the inhibition zone was greater for *E. coli* than for *S. aureus*. The results also showed that as the amount of silver nanoparticles and *L. angusifolia* extract increased, the diameter of the inhibition zone increased for *E. coli* and *S. aureus.* Another result of this study was that the silver nanoparticles and *L. angusifolia* extract did not show any interaction. This means that in this experiment, silver nanoparticles had no effect on *L. angusifolia* extract and vice versa.

The quality of fitted models was evaluated based on the adjusted coefficient of determination denoted by R^2_{adj} . It is well known that $0 \leq R^2_{adj} \leq 1$ and values close to 1 imply that the associated regression model can be used as an appropriate predictor of response variable. The R^2_{adj} for Equations (1) and (2) was 0.6968 and 0.726, respectively. These values of R^2_{adj} are relatively high indicating that the proposed regression models have the ability to predict inhibition zone diameter of silver nanoparticle for *E.coli* and *S.aureus* with the help of two factors. The F-values of models were significant (P-value <0.05), which confirmed the suitability of the proposed models. In addition, the P-values showed that all model terms in equations (1) and (2) were significant (P-value <0.05). The linear regression models proposed in equations (1) and (2) are highly informative.

In this study, it was found that the antimicrobial activity of the silver nanoparticles and leaf extract of *L. angustifolia* against *E. coli* and *S. aureus* remained relatively unaffected at different pH values (pH 5.0, 6.0, 7.0, and 8.0). Similar to our study, Mahfuzul *et al.* reported that altering pH values showed no significant effect on the antibacterial activities of Guava and Neem extracts against *S. aureus* and *Listeria monocytogenes* (29). Other similar results showed no significant changes in the antibacterial activities of *Ficus sycomorus* and *Ficus platyphylla* extracts at different pH values (30). However, some studies have shown that the antibacterial activity of some herbal extracts was significantly altered with increasing or decreasing the pH values (31).

Conclusion

It is concluded that the silver nanoparticle and leaf extract of *L.angustifolia* could be a possible source of an effective antibacterial agent. However, comprehensive studies and clinical trials are required to justify and appraise the potential of the mixture of silver nanoparticle and *L.angustifolia* extract as a useful antimicrobial agent in medical applications.

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Author's contributions

FM designed the study, carried out the experiments and wrote the manuscript. MK carried out the statistical analysis and wrote the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Ethical approval

This article does not contain any test on human participants or animals.

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