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Optimization Studies on Green Synthesis of Silver Nanoparticles from different Plant Extracts Using Taguchi Design

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Abstract

Background/Objectives: The main objective of the present study is to screen an efficient plant extract sources for the green synthesis of silver nanoparticles (AgNPs) and optimization of various conditions for the synthesis using a statistical methodology like Taguchi design. The antimicrobial activity of the synthesized AgNPs against common wound causing dermal pathogens was also checked. **Methods**: The conditions for green synthesis, such as the concentration of silver nitrate, amount of plant extract, synthesis reaction time, temperature, and pH of the plant extract, were optimized by using Taguchi design (L_{27}type). Ultraviolet-visible spectroscopy, scanning electron microscopy (SEM), and Transmission electron microscopy (TEM) were used for the characterization of produced green AgNPs. The antibacterial activity of the obtained green AgNPs was tested against most common wound forming dermal pathogens like S_1(*Staphylococcus aureus*-Multi drug-resistant, hospital strain), S_2 (*Staphylococcus aureus*-pimple source), S_3 (*Staphylococcus aureus*-wound sample), S_4 (*Pseudomonas sp.*-hospital sample), and S_5 (*Pseudomonas sp.*-wound sample) using Agar well diffusion method. Findings: Thirty different aqueous leaf extracts were screened for AgNPs using a flask level unidimensional study. Among all, the aqueous leaf extract of *Aloe vera* was found to be the most suitable for the green synthesis of AgNPs. The optimum conditions for the green synthesis of AgNPs were observed as pH- 7.0, temperature 35°C, 10mM silver nitrate ($AgNO_3$), 50% (v/v) plant extract for a time period of 30 min. **Novelty/Applications**: The Taguchi statistical method was used for the optimization of the green synthesis of AgNPs with suitable experiential tests. Finally, this rapid and easy process of green synthesis of AgNPs using aqueous leaf extract of *Aloe vera* demonstrated broad spectrum activity as an antibacterial agent against the wound-causing dermal pathogens in the medical field by exhibiting the promising minimum inhibitory concentration (MIC) of AgNPs as $\leq$ 0.0535mg/L.

1 Introduction

Metallic nanoparticles have huge consideration deductively and innovatively because of their extraordinary and strange physico-chemical properties when contrasted with mass materials[1,2]. The green synthesis of nanoparticles using both microbial sources like bacteria[3], fungi[4], yeast[5], and plant materials[6,7] have gained prominence as they are clean, nonhazardous, and eco-friendly when compared to physical and chemical methods[8,9]. Among these green methods, microorganisms intervened synthesis of nanoparticles isn't industrially reasonable as maintenance of culture and fermentative process require profoundly sterile conditions[10].Additionally, the reduction of metallic ions by microbes requires longer incubation time as compared to plant extracts[11]. The utilization of plant extracts is additionally gainful over microorganisms because of the easiness to scale-up[12]. The plant extracts can be used both intracellularly and extracellularly for the synthesis of different nanoparticles[13–15].The intracellular synthesis includes growing plants in metal-rich media or soil[16,17], whereas extracellular methods include boiling the plant material in sterile water or any organic solvent[18,19].

Many researchers have focused on AgNPs due to their significant applications in color filters, optical switching, optical sensors, surface-enhanced Raman scattering, etc.[20–22] and also in clothing, respirators, household water filters, antibacterial sprays, cosmetics, detergents, dietary supplements, cutting boards, shoes, cell phones, laptop keyboards, and children toys are typical products currently in the market that exploit the antimicrobial properties of silver nanoparticles[23–25].Taking their multiple applications into consideration, the annual synthesis of silver nanoparticles has been estimated to be 55 tons globally, and this is increasing according to the demand[26,27].

The present work focused on the application of statistical experimental designing tools for green synthesis of AgNPs using different plant extracts. The synthesis process of these nanoparticles involves the reduction of silver ions, nucleation, and growth of the particles[28] that is influenced by several factors like the amount of both silver nitrate and plant extract, pH, incubation period, temperature, etc.[29,30]. By implementing statistical tools for the synthesis of nanoparticles specifically for regulating the size is resulted in enhancing bioactive attributes of the synthesized AgNPs. A statistical method Taguchi was applied to minimize the number of experiential trails during optimizing different parameters for efficient green synthesis of AgNPs. Taguchi is a basic and powerful measurable strategy that arranges orderly experimentation to decide the close to ideal settings of plan parameters for execution, quality, and cost. Right now, a huge number of factors are concentrated with few tests utilizing symmetrical exhibits, and the fluctuation of parameters is communicated by signal-to-noise(S/N) proportion, which represents the ratio of desirable results (signal) to undesirable results (noise). Thus, the optimized parameters would help in the bulk synthesis of AgNPs by the green method.

2 Materials and Methods

2.1 Preparation of Plant Extract

Fresh leaves of different plants (Table 1) were collected from different areas like the college campus, local garden, and nursery. The leaves were washed thoroughly, cut, and dried. Approximately 20g of the dried powder of respective plant sp. were boiled in 100ml of double-distilled water for 10-15min.[31]. The aqueous solution was filtered

and stored at 40^0 C until further use.

Table 1. Different plant species used for green synthesis of silver nanoparticles

S.No	Plant species Scientific name	Common name
1	*Amaranthus*	Pigweed
2	*Aloe vera*	First aid plant
3	*Annona reticulata*	Custard apple
4	*Calotropis*	Jilledu
5	*Carica papaya*	Papaya
6	*Chrysanthemum*	Chamanthi
7	*Cinnamomum tamala*	Bay leaf
8	*Citrus limon* (L.)	Lemon
9	*Coriandrum sativum*	Coriander
10	*Curcuma longa*	Turmeric
11	*Eucalyptus globulus*	Southern blue gum
12	*Lawsonia inermis*	Henna
13	*Mentha*	Pepper mint
14	*Millettia pinnata*	Indian beech
15	*Mirabilis jalapa*	4'o clock plant
16	*Momordica charantia* Linn.	Karela
17	*Moringa oleifera*	Drum stick
18	*Murraya koenigii*	Curry leaf
19	*Nerium oleander*	Nerium
20	*Ocimum basilicum*	Basil
21	*Phyllanthus emblica*	Amla
22	*Piper betle*	Tamalapaku
23	*Psidium guajava*	Guava
24	*Punica granatum*	Pomegranate
25	*Ricinus communis*	Castor bean
26	*Sapindus mukorossi*	Soap berry
27	*Syzygium cumini*	Malabar plum
28	*Tagetes*	Marigold
29	*Trigonella foenum-graecum*	Fenugreek
30	*Zingiber officinale*	Ginger

2.2 Green synthesis of silver nanoparticles and optimization experiments

The silver nanoparticles were synthesized by adding both silver nitrate solution and aqueous plant extract at various concentrations. These reaction mixtures were incubated at different time intervals and different temperatures and pH (Table 2). The experiment was performed in duplicates (set A and set B).

Table 2. Parameters with their levels used for green synthesis of silver nanoparticles

Factor		Levels		
		1st	2nd	3rd
A	Concentration of AgNO$_3$ (mM/ml)	2	5	10
B	Concentration of plant extract (ml)	25	50	75
C	pH	5	7	9
D	Incubation time period (min)	10	30	60
E	Incubation temperature (^{0}C)	25	35	50

The design of an experiment is a powerful tool to conduct experiments with minimum resources. Therefore, the experimental parameters were studied using a statistical design called the Taguchi method. The parameters and their levels were set up based on the selected Taguchi design, as indicated in Table 2. The variables like the amount of silver nitrate and plant extract, incubation period, incubation temperature, and pH were studied at levels 1, 2, and 3. The Taguchi orthogonal matrix, L_{27}, was used for designing the experimental trials considering the mentioned five variables at three different levels. The experimental

results obtained were subjected to analysis. Data processing is done by estimating the S/N ratio, which is the ratio of the target value to the deviation from its mean. In the Taguchi design, the target value (mean) represents the signal, and the standard deviation for the response variable represents Noise. For calculating the S/N ratio, the larger-the-better quality characteristic is selected, and it is calculated by using the following equation (1).

$$\frac{S}{N}_{Larger\ the\ better} = -10log\left\{\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_i^2}\right\} \tag{1}$$

Where n is the number of experiments, y_i is the response variable for the ith experiment

2.3 Green synthesis of silver nanoparticles and optimization experiments

The synthesized silver nanoparticles were centrifuged at 15000 rpm for 5-10min, and the obtained nanoparticles pellet was washed with ethanol solution twice and dried in a microwave oven to obtain a powdered form of nanoparticles.

2.4 Characterization of synthesized silver nanoparticles

The reduction of pure Ag+ particles was observed by estimating the UV-Vis range of the response. UV-Vis absorption spectrum of the samples is determined at a wavelength of 450nm, which was obtained as absorption maxima (300-500nm). The morphology and size of the obtained AgNPs were analyzed by using a Scanning electron microscope (SEM) and Transmission electron microscope (TEM) (SEM: Hitachi S3700N and TEM: Tecnai-12 FEI at Osmania University College of technology, Hyderabad).

2.5 Antimicrobial susceptibility testing

Agar well diffusion method was used to check the antibacterial activity of the obtained AgNPs. The most common wound forming dermal pathogens like S_1 (*Staphylococcus aureus*-Multi drug-resistant, hospital strain), S_2 (*Staphylococcus aureus*-pimple source), S_3 (*Staphylococcus aureus*-wound sample), S_4 (*Pseudomonas sp.*-hospital sample), and S_5 (*Pseudomonas sp.*-wound sample) were tested for their response to the green synthesized AgNPs. Standard drug (ampicillin 125μg/mL) and deionized water were used as a positive and negative control, respectively. The zones of inhibition (cm) were obtained after post-incubation at 37^{0}C for 24hrs.The experiment was carried in triplicates.

3 Results and Discussion

The AgNPs were synthesized using different plant extracts as listed in Table 1 as reducing and stabilizing agents, the aqueous silver nitrate solution turned to brown color upon incubation, which is the first visual indication for the formation of nanoparticles (Figure 1). Out of thirty different plant extracts, fifteen plant extracts showed significant results depending upon the rate of reduction of silver ions (Figure 2). Among the effective fifteen samples aqueous *Aloe vera* extract exhibited the maximum reduction of silver ions indicated by the highest absorbance value as 0.68. This may be due to the presence of biologically active constituents includes amino acids, enzymes, vitamins like A, C, E, quinones, lignin, sugars, salicylic acid, saponins, sterols[32] which also prevents aggregation of nanoparticles[33] and especially hydroquinone's in *Aloe vera* plant extract acts as the reducing agent[34].

Characterization studies of metallic nanoparticles are essential to comprehend their properties and applications[35,36]. AgNPs formation is primarily confirmed by the UV-Visible spectroscopy technique[37] where absorption values in the wavelength ranges of 300-500nm are used[38]. Silver nanoparticles at the nano range display an interesting optical phenomenon called surface Plasmon resonance (SPR) because of the total swaying of the directing metal surface electrons in resonance with the non-particulate radiation. This property was generally dependent upon the molecule type, size, shape, and local chemical nature. The absorption spectra of the AgNPs synthesized with *Aloe vera* extract were observed at 450nm, as indicated in Figure 3. The Scanning electron microscopy (SEM) analysis for size determination indicated that AgNPs with *Aloe vera* extract were spherical with a size range variation of 43.3-63.0 nm in diameter (Figure 4), and larger AgNPs could be due to an increase in the concentration of silver nitrate solution. The shape, size, and morphology of the green synthesized AgNPs were elucidated with the help of Transmission electron microscopy (TEM), further confirming the formation of silver nanoparticles. TEM images (Figure 5) of the sample also showed nanoparticles exhibiting a typical spherical morphology with a size range of 50nm.There
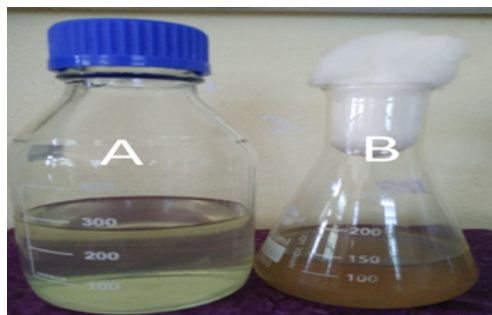
Fig 1. (A) Mixture of plant extract & silver nitrate, (B) Formation of silver nanoparticles
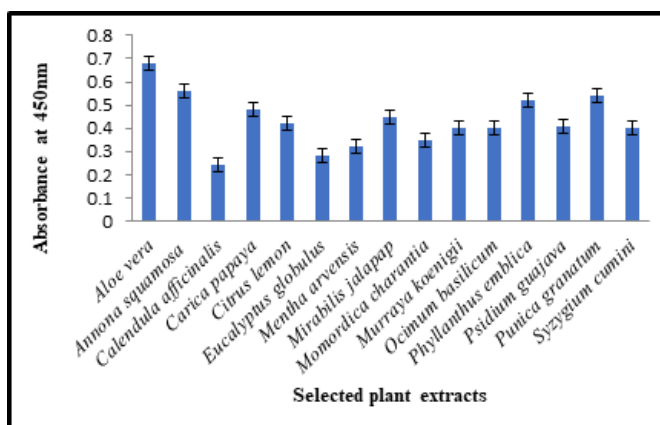

Fig 2. Absorbance of green synthesized AgNPs from various plant extracts

are similar such reports by Patcharaporn Tippayawat et al., who worked on *Aloe vera* extract and indicated the formation of spherical AgNPs with a size of 192.02-70.7nm[39].
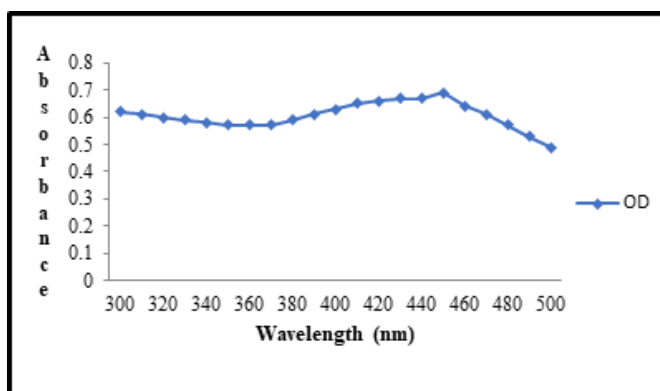

Fig 3. UV-Visible spectroscopy of AgNPs synthesized from *Aloe vera* plant extract
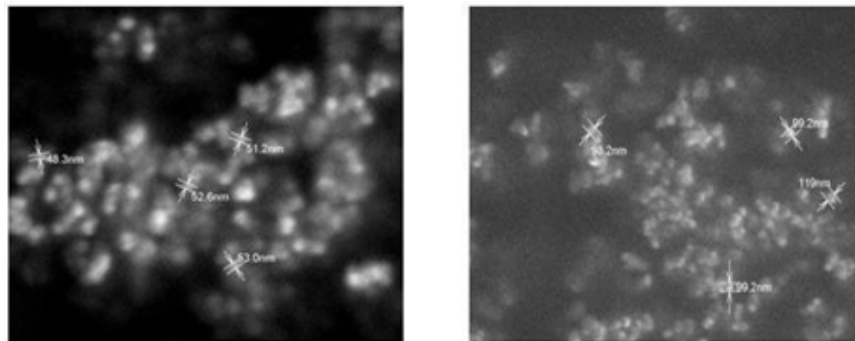
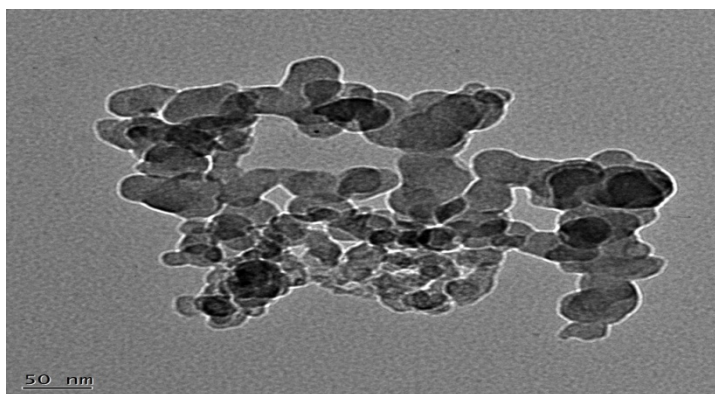**Fig 4.** SEM image of green synthesized silver nanoparticles

Fig 5. TEM image of green synthesized silver nanoparticles

3.1 Taguchi Experimental Design:

L_{27} orthogonal array design was used to study five variables at different levels (Table 3). The experimental results were statistically analyzed, and the main effect of each variable is indicated in the graphical plot (Figure 6).

Table 3. Taguchi L_{27} orthogonal array design experimental set up for green synthesis of sliver nano

Level	Factors					Average of set-A&B (OD values at 450nm)
	A	B	C	D	E	
L_1	2	25	5	10	RT	0.12
L_2	2	25	5	10	35	0.14
L_3	2	25	5	10	50	0.08
L_4	2	50	7	30	RT	0.38
L_5	2	50	7	30	35	0.46
L_6	2	50	7	30	50	0.31
L_7	2	75	9	60	RT	0.09
L_8	2	75	9	60	35	0.23
L_9	2	75	9	60	50	0.14
L_{10}	5	25	5	10	RT	0.18
L_{11}	5	25	5	10	35	0.26
L_{12}	5	25	5	10	50	0.14
L_{13}	5	50	7	30	RT	0.42
L_{14}	5	50	7	30	35	0.56
L_{15}	5	50	7	30	50	0.38
L_{16}	5	75	9	60	RT	0.1
L_{17}	5	75	9	60	35	0.23

Continued on next page

			Table 3 continued		
L$_{18}$	5	75	9	60	50	0.11
L$_{19}$	10	25	5	10	RT	0.21
L$_{20}$	10	25	5	10	35	0.32
L$_{21}$	10	25	5	10	50	0.21
L$_{22}$	10	50	7	30	RT	0.76
L$_{23}$	10	50	7	30	35	0.9
L$_{24}$	10	50	7	30	50	0.58
L$_{25}$	10	75	9	60	RT	0.12
L$_{26}$	10	75	9	60	35	0.24
L$_{27}$	10	75	9	60	50	0.16

RT-Room temperatures, A- Concentration of AgNO3, B- Concentration of plant extract, C- pH, D- Incubation time period, E- Incubation temperature, OD-Absorbance
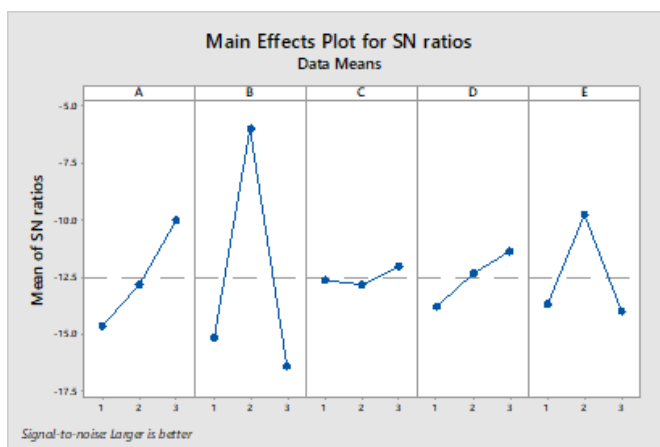

Fig 6. Taguchi design - Main effects Plot of Mean of S/N Ratio for the synthesis of AgNPs

3.2 Taguchi Experimental Design:

The quality characteristics "larger is better" from equation (1) were applied to the experimental data. From Figure 6, it was observed that the results based on the Signal to Noise Ratio (S/N), larger levels of factors A, B, C, D, and E give a comparatively greater production of AgNPs. Hence the optimal parameters for AgNPs production are observed as the incubation temperature of 35°C, the amount of AgNO$_3$ as 10mM, the pH as seven, and the concentration of plant extract as 50mL with an incubation period of 30mins as indicated in Table 4.

Table 4. Optimum values of factors and their levels

	Factor	Optimum Value
A	Concentration of AgNO$_3$ (mM/ml)	10 mM/ml
B	Concentration of plant extract(ml)	50 ml
C	pH	7
D	Incubation time period(min)	30 min
E	Incubation temperature (^{0} C)	35^0C

Metal nanoparticle synthesis and their characteristics were influenced by different parameters like concentration of silver nitrate, amount of plant extract, incubation period, and temperature. Their optimization studies, if done by a traditional uni-dimensional approach where one variable at a time is evaluated, keeping remaining variables constant would be both times consuming and laborious. Another major disadvantage is that it does not show what would happen if the other variables are

also changing simultaneously. Very few reports are available on the development of a protocol to obtain smaller size AgNPs using green synthesis by the Taguchi method [40].

Therefore, our study focused on robust design, the central theme of the Taguchi method, which emphasis on to make the process insensitive to those sources of variations. Therefore, Taguchi's statistical approach would be both time saving and cost-effective, and it gives scope for the study of different parameters and different levels in one experiment. The use of linear graphs and triangular tables suggested by Taguchi makes the assignment of parameters simple.

3.3 Antimicrobial activity:

The green synthesized AgNPs from aqueous *Aloe vera* plant extract exhibited considerably significant antibacterial activity against common wound forming dermal pathogens like S_1 (*Staphylococcus aureus*-Multi drug-resistant, hospital strain), S_2 (*Staphylococcus aureus*-pimple source), S_3 (*Staphylococcus aureus*-wound sample), S_4 (*Pseudomonas sp*.-hospital sample) and S_5 (*Pseudomonas sp.*-wound sample) (Figure 7). The results indicate that AgNPs have higher antibacterial activity against multidrug-resistant bacteria *S. aureus* S_1 (isolate 1) over the other pathogens (Figure 8). The obtained zones of inhibition when compared with the standard drug (ampicillin), it was found that only *Aloe vera* leaf extract was not very much effective, but the synthesized AgNPs were resulted as strong as the standard drug. Based on these observations, the minimum inhibitory concentration (MIC) of AgNPs is ≤ 0.0535 mg/L which may be due to uniform and predominant nano size silver metal ions that are synthesized using controlled statistical design of experiments.
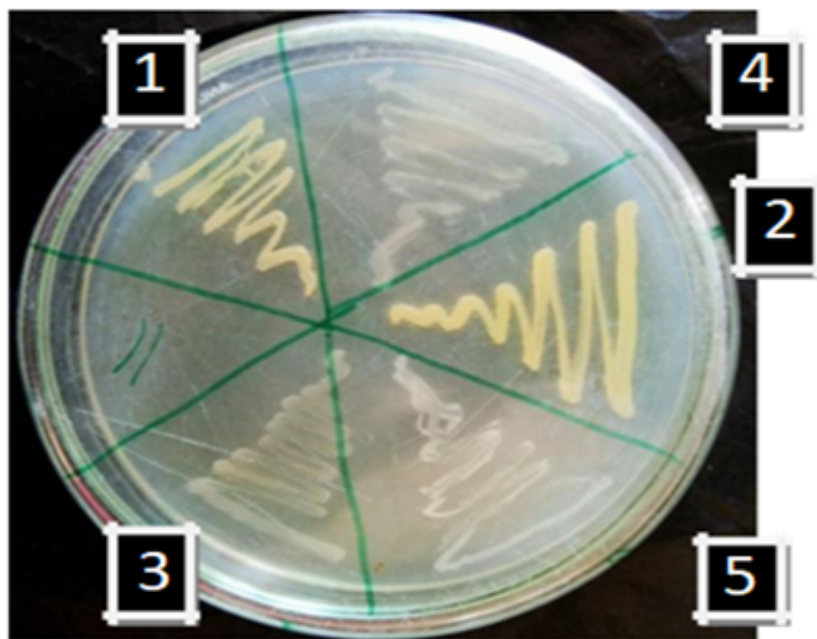

Fig 7. Common wound forming pathogens (isolate 1-S1 *Staphylococcus aureus*-Multi drug-resistant, hospital strain, isolate 2-S2 *Staphylococcus aureus*-pimplesource, isolate 3-S3 *Staphylococcus aureus*-wound sample, isolate 4-S4 *Pseudomonas sp*.-hospital sample, isolate 5-S5 *Pseudomonas sp*.-wound sample

Therefore, the antibacterial activity of green synthesized AgNPs using *Aloe vera* medicinal plant demonstrated antagonistic activity against both Gram-positive and Gram-negative wound forming bacteria. These results were agreed with the previous work carried out by Hungund et al., 2015; Balashanmugam et al., 2015, and Chitra et al., 2014. [41–43]

Hence the present study exhibits that a low concentration of AgNPs is alluring because of its expansive range of antibacterial action [44,45] and due to its bactericidal activity against multi drug resistant organisms like Methicillin-resistant *S. aureus* (MRSA), just as multidrug-resistant *Pseudomonas sp*. [46]. This might be because the positive charge on the Ag+ particle is critical for its antimicrobial property through the electrostatic fascination between the contrarily charged cell layer of the microorganisms and the decidedly charged nanoparticles. Accordingly, green synthesized AgNPs utilizing *Aloe vera* plant extract can likewise be utilized for other clinical applications.
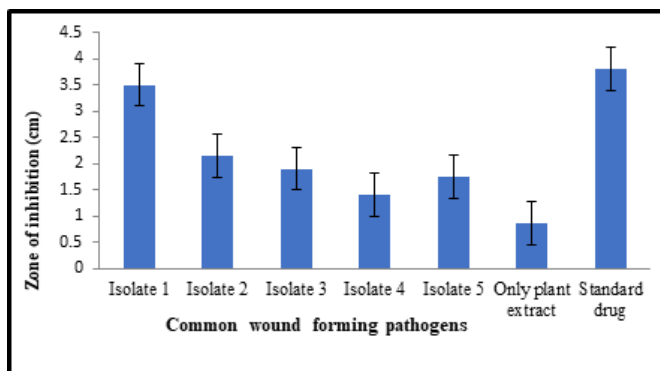
Fig 8. Antibacterial activities of green synthesized AgNPs against common wound forming pathogens

4 Conclusion

In the present study, AgNPs were synthesized by using green and eco-friendly technology, with qualitative data using statistical experimental design support. Our research concentrated on robust design, a vital concept of the Taguchi approach, which emphasizes the need to make the process insensitive to those sources of variation. As a result, Taguchi's L_{27} orthogonal array experimental design is applied to identify the effectiveness of every factor such as $AgNO_3$, pH, temperature, incubation time, and plant extract in the synthesis to obtain optimal values for the synthesis. AgNPs were observed as small, spherical in size 43.3-63.0 nm in diameter without any agglomeration. The AgNPs, which are produced by aqueous *Aloe vera* extract, demonstrated better results for broad-spectrum anti-bacterial activity against different bacteria strains selected for testing (Minimum inhibitory concentration (MIC) of AgNPs was found to be ≤ 0.0535mg/L). In the future, these non-toxic green synthesized AgNPs may have useful applications in the field of anti-bacterial formulations against various drug-resistant pathogens.

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