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Rapid Method for Detecting Bactericidal Effects of Mangrove Derived Silver Nanoparticles Using Resazurin Microdilution Assay

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Abstract: ***Nanoparticles plays a vital role in the field of antimicrobial agents against pathogenic microorganisms. Screening of nanoparticles for antimicrobial activities is a time consuming and cumbersome process. Recently, a simple technique of using the dye resazurin has been used as an indicator of bacterial growth for testing antimicrobial activity on microtitre plate. However, this technique does not quantify the microbial load. Therefore, the present work was attempted to find a new antibacterial method employing the dye resazurin assay and haemocytometric counting of microbes for testing silver nanoparticles synthesised from Xylocarpus mekongensis .The bacterial strains E. coli, S. aureus and P. aeruginosa (multi-drug resistant strain) were used to evaluate the screening of mangrove extracts. Minimum inhibition concentration (MIC) was also calculated for the silver nanoparticles using ciproflaxcin as reference antibiotic. The antibacterial activity Xylocarpus mekongensis was carried out against all the three bacteria by the same method and the values were compared with reference antibiotic. The present study has suggested a rapid, dependable, easy and inexpensive method, suitable for testing the antibacterial activity of silver nanoparticles which are promising to develop as new antibacterials.***
Keywords: Mangroves, Silver, Nanoparticles, Resazurin, Antibacterials

I. INTRODUCTION

Antimicrobial formulations based on nanoparticles are efficient and effective in bactericidal activity (Franci *et al*., 2015). A considerable work is available on bactericidal activity of silver nanoparticles of either a simple or composite nature .(Kathiresan *et al*., 2010). Nanophasic and nanostructured materials have attracted a great deal of attention because of their potential for achieving specific processes and selectivity, especially in biological and pharmaceutical applications (Zhang *et al.*, 2016). Microorganisms are unlikely to develop resistance against silver, as they do against antibiotics, because the metal aims a wide range of targets in the organisms. (Medda *et al*., 2015, Hebeish *et al.*, 2014, Bekele *et al*., 2016) Thus, silver ions have been used as an antibacterial component in dental resin composites, and in coatings of medical devices (Bosetti *et al*., 2002). The silver nanoparticles go through a size-based interaction with human immunodeficiency virus type 1(Elechiguerra *et al*., 2005). Besides size, shape of the nanoparticles also determines the antibacterial activity of silver nanoparticles against *Escherichia coli* (Pal *et al*., 2007). We have proved in our laboratory that the tissue culture derived callus of the coastal halophyte *Sesuvium portulacastrum* is efficient in the production of silver nanoparticles with strong antimicrobial activity against 10 human pathogens (Asmathunisha *et al*., 2010).
Recently, reports suggest that resazurin based assay has been widely used to study the antibacterial effect of nanoparticles. (Anburaj *et al*., 2020, Travnickova *et al*., 2019, Sunita ojha *et al*., 2017, Loo *et al*., 2018).Silver nanoparticles are well known to exhibit strong microbial activity against various types of microorganisms.(Ahmed *et al*., 2016, Dhand *et al*., 2016, Selvam *et al*., 2017,Kalidasan *et al*., 2021)..Multidrug resistance pathogens are the main source for more infectious diseases in human being to cause increased number of mortality rate in humans (WHO, 2000, Tanwar *et al*., 2014). The present study employed the resazurin dye reduction as an indicator of bacterial growth to study the silver nanoparticles for their bactericidal activity.

II. MATERIALS AND METHODS

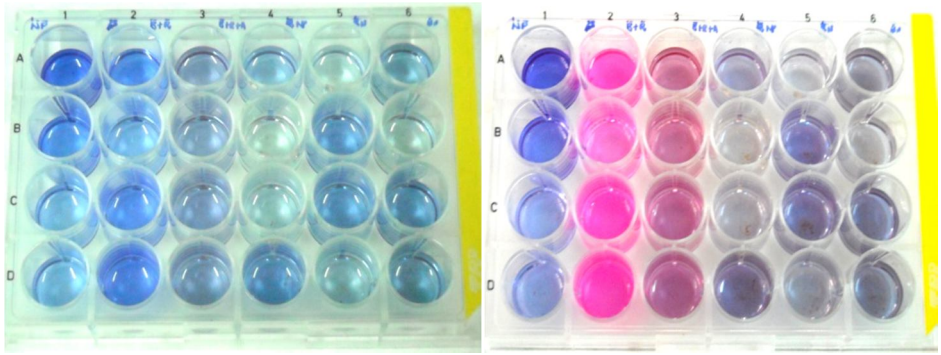
The bactericidal activity of silver nanoparticles produced by *Xylocarpus mekongensis* was estimated by a microtitre plate based method by using resazurin assay (Sarkar *et al*., 2007). Three clinical pathogens namely *E. coli* (AU314), *Pseudomonas aeroginosa* (AU101) and *Staphylococcus aureus* (AU210) obtained from Raja Muthiya Medical College, Annamalai Nagar were used as test bacterial organisms. Three sets of sterile 24 well plate was labelled. 100 µL of test compound in 10% (v/v) sterile water (usually a stock concentration of 1 mg/mL for purified compounds, and 10 mg/mL for crude extracts) was pipetted into the first row of the plate. 50 µL of nutrient broth or normal saline was added to all other wells.

The used tips were discarded and each well had 50 µL of the test material in serially descending concentrations. 10 µL of resazurin indicator solution was added to all wells. Nutrient broth with the strength of 30 µL of 3.3 was added to each well to ensure that the final volume was single strength. Finally, 10 µL of bacterial suspension was added to each well to achieve a concentration of 5×10^5 cfu/mL. Plates were wrapped with cling film to make sure that bacteria did not become dehydrated. Each plate had a set of controls: a column with a broad-spectrum antibiotic as positive control (usually ciprofloxacin in serial dilution), a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 µL of nutrient broth instead. The plates were prepared in triplicate, and incubated at 37°C for 24 h. The colour change was then assessed visually. The colour retaining property of the sample recorded as positive. The lowest concentration of silver nanoparticles at which colour was not changed was taken as the Minimum Inhibitory Concentration (MIC) value. The average of three values was calculated and that was the MIC for the test material. The maximum inhibition of silver nanoparticles concentration was measured at which the colour was totally changed.

III.RESULTS

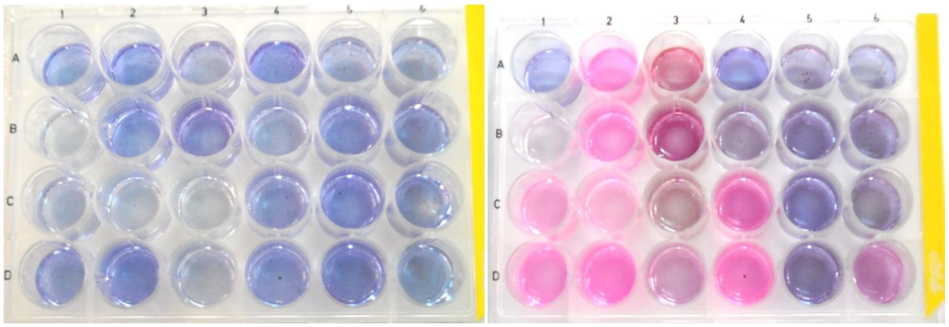
Bactericidal activity of silver nanoparticles was tested against *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* by using resazurin incorporation method. The data are shown in table 1 and Figure 1,2,3 and 4. The colour intensity revealed the viability of bacterial cells and the reduction in colour intensity indicated the killing of bacterial cells. In the nanoparticles treated wells, the colour intensity got reduced due to antibacterial activity of the nanoparticles. This effect was dependent upon the concentration of the silver nanoparticles used - 0.1, 0.2, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 70, 80 µl - which are equivalent to concentration of silver nanoparticles of 6.3, 12.6, 31.5, 63, 126, 252, 378,504, 630,1260, 1890, 2520, 3150, 3780, 4410, 5040 µg. Increasing concentration of nanoparticles resulted in decreasing of colour intensity of resazurin and thus increasing the antibacterial activity. The minimum inhibitory concentration was the concentration at which the reduction of colour intensity started and it was found at 15, 12.6, and 10 µg of silver nanoparticles for *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively.

Escherichia coli



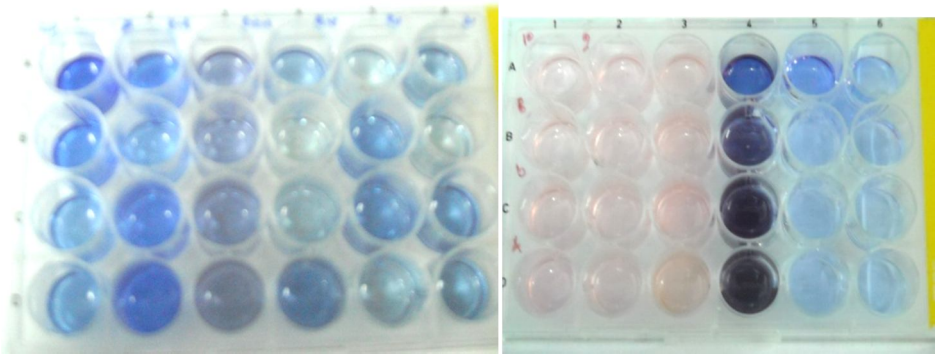
1.(a). Beginning of reaction (b). After 24h of reaction

Pseudomonas aeruginosa



2 (a). Beginning of reaction (b). After 24h of reaction

Staphylococcus aureus



3(a). Beginning of reaction (b). After 24h of reaction

Figure 1,2,3: Bactericidal effects of silver nanoparticles in terms of colour intensity of Resazurin against clinical pathogens - E. coli, P. aeroginosa and S. aureus

1) Column 1 wells with silver nanoparticles + resazurin dye
2) Column 2 wells with bacterial strain + resazurin dye (without silver nanoparticles)
3) Column 3 wells with commercial antibiotic (Cyprofloxacin) + resazurin dye
4) Column 4,5,6 wells with different concentrations of silver nanoparticles + bacterial strain + resazurin dye

Table 1: Effect of different concentration of nanoparticles on bactericidal activity in terms of optical density at 650 nm indicating bacterial viability of three clinical pathogens

Volume of nanoparticle suspension (µl)	Concentration of nanoparticles (µg)	OD value at 650nm (indicating the bacterial viability) *E.coli*	*S.aureus*	*P.aeruginosa*
0 (negative control)	0	0.524	0.579	0.589
0.1	6.3	0.524	0.579	0.589
0.2	12.6	0.499	0.510	0.512
0.5	31.5	0.498	0.508	0.510
1	63	0.498	0.507	0.509
2	126	0.497	0.507	0.508
4	252	0.494	0.506	0.508
6	378	0.495	0.505	0.507
8	504	0.496	0.503	0.507
10	630	0.495	0.501	0.501
20	1260	0.491	0.495	0.499
30	1890	0.486	0.479	0.492
40	2520	0.482	0.468	0.487
50	3150	0.476	0.452	0.474
60	3780	0.471	0.445	0.469
70	4410	0.443	0.439	0.458
80	5040	0.412	0.421	0.447
Commercial antibiotic (Ciprofloxacin) Positive control (0.6µg/ml)		0.462	0.485	0.483

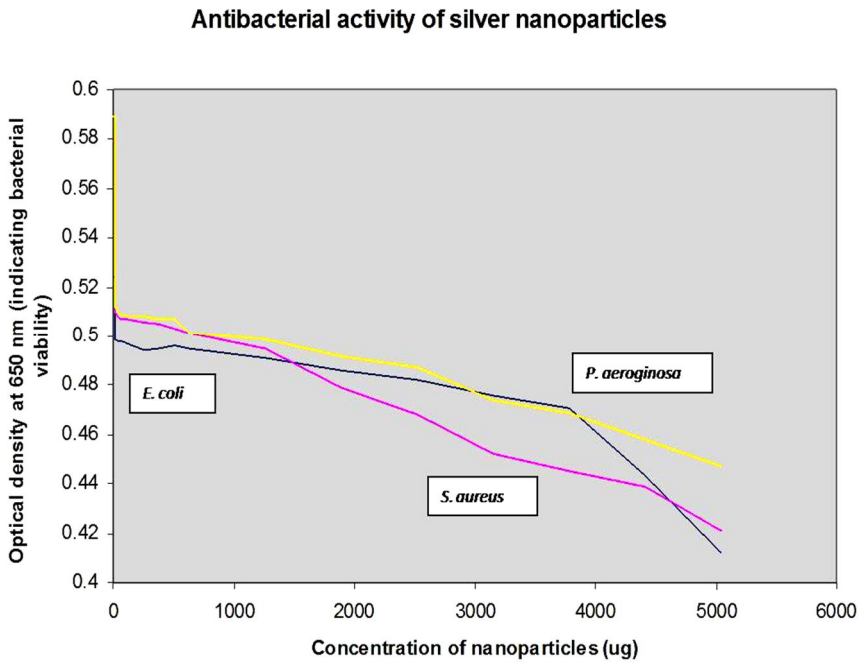


Fig. 4. Bactericidal effects of silver nanoparticles tested against three clinical pathogens (*Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*)

IV. DISCUSSION

Silver is documented as one of the foremost global antimicrobial substances. Silver ion and silver-based compounds are extremely toxic to microorganisms, showing strong biocidal effect against microbial species particularly multi-drug resistant human pathogenic bacteria (losasso *et al*., 2014). However, silver ions or salts have only limited usefulness as antimicrobial agents for many reasons: meddling effects of salts and discontinuous unleash of silver ions inadequate concentration from the metal. In contrast, these kinds of inadequacy can be overcome using silver nanoparticles as these are extremely high reactive species due to large surface area. The silver nanoparticles produced by using microbes and/or plant extracts are known to exhibit potent antimicrobial activity (Saxena *et al*., 2010). A similar observation was made here with the silver nanoparticles produced by plants of coastal origin and these nanoparticles also exhibited antimicrobial activity against the three clinical strains of bacteria (Tables 1). The antimicrobial activity of nanoparticles varied with test microbes. This differential activity of silver nanoparticles can be attributed to their differential sizes: the antimicrobial activity increases with decreasing size of the silver nanoparticles. The minimum inhibitory concentration of silver nanoparticle was found to be at 15, 12.6, and 10 µg of silver nanoparticles for *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively. Thus the Gram +ve bacteria like *S. aureus* and *P. aeruginosa* were found more susceptible to silver nanoparticles than the Gram –ve bacteria like *E. coli*.

V. CONCLUSION

Resazurin technique is simple and viable assay to determining the bacterial load in food quality control. A few reports are available on the use of this technique to test bactericidal effect of biosynthesized nanoparticles. In, this present study results are obtained in a short period of time (24 hrs) and with very good sensitivity. It is inexpensive and easy method to perform. This technique will not only reduce the experimental cost but also the time, compared to conventional agar plate methods.

VI. ACKNOWLEDGMENT

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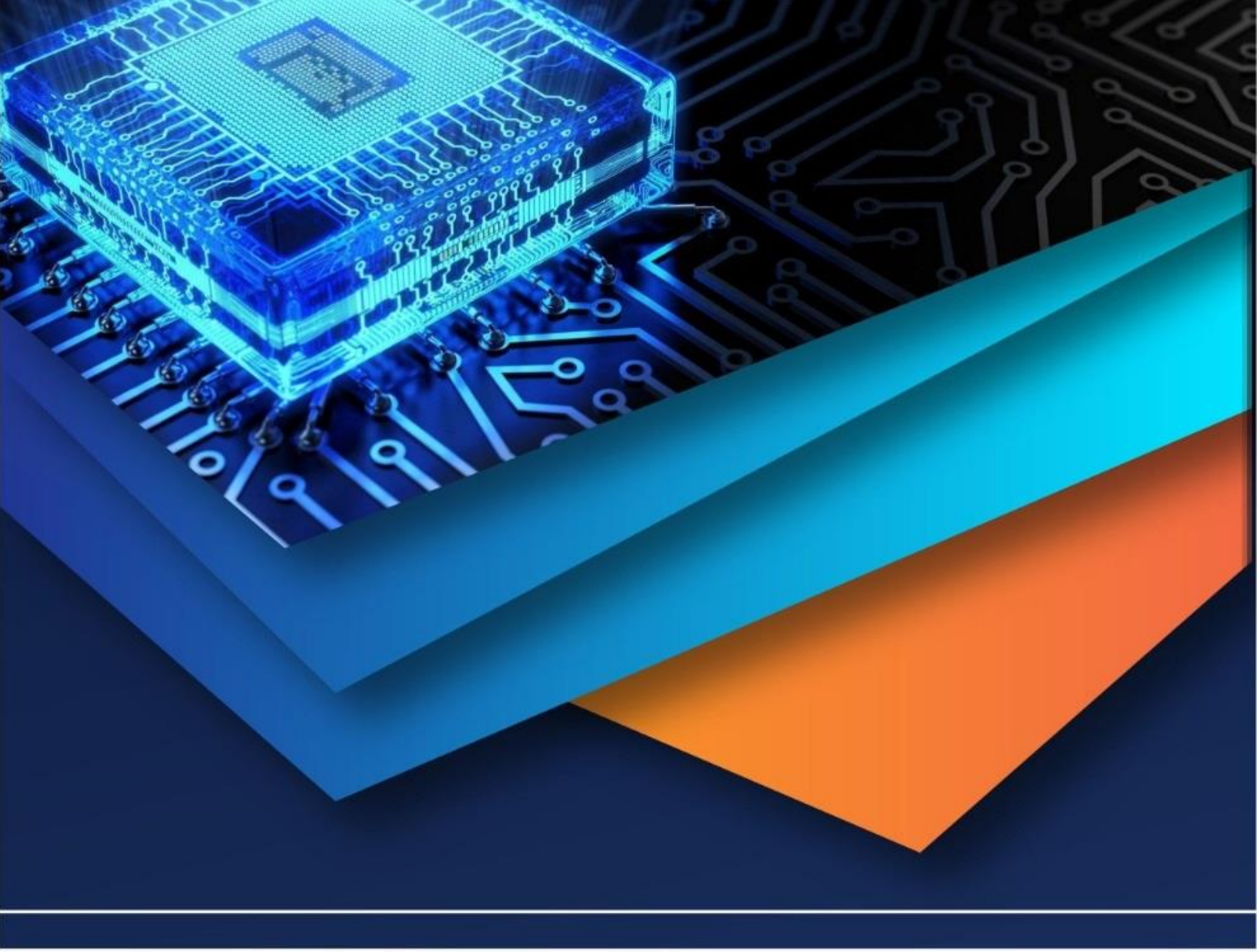

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