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EVALUATION OF ANTI - INFLAMMATORY AND ANTI –BIOFILM ACTIVITY OF SILVER NANOPARTICLE USING *WATTAKAKA VOLUBILIS* LINN. F.

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ABSTRACT

Silver nanoparticles (AgNPs) are an important class of nanomaterial for a wide range of biomedical applications. Synthesis of AgNPs through biological route is preferred due to its eco-friendly and economic aspects. Green synthesis provides advancement over chemical and physical method.Persistent infections caused by bacterial biofilms have been associated with a number of medical conditions including periodontal disease, endocarditis, osteomyelitis and cystic fibrosis. Preventing biofilm formation would be a more logical option than treating it. The main strategy to prevent biofilm formation is to clean and disinfect regularly before bacteria attach firmly to surfaces. Anti-inflammatory activity was calculated by the percentage of HRBC membrane stabilization or protection and the biofilm formation was determined by the congo red agar method and tissue culture plate method. The present study explored the *Wattakaka volubilis* which are efficient producers of AgNPs and could act as safe and cost-effective with potential biological applications.

KEYWORDS: ***Silver nanoparticles, Wattakaka volubilis , Biofilm, AgNPS, TCP, CRA, HRBC***

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INTRODUCTION

Nanoparticles are gaining interest in the field of nanodrug delivery systems without harming the cells of the body organs. Hence, there is a need to develop green chemistry approaches in the synthesis for the nanomaterials. In this aspect, synthetic methods based on naturally occurring biomaterials are the alternative eco-friendly method[1].The synthesis of noble metal nanoparticles and their description attracts an increasing interest in the field of nanotechnology because of their potential applications in various fields such as biotechnology, chemistry, physics and medicine[2]. Among several nanoproducts, a most prominent nanoproduct is nanosilver. Silver nanoparticles (AgNPs) have been used for antimicrobial, antioxidant, anti-diabetic, antibiolfilm and anti-hemolytic effects. AgNPs are an important for a wide range of industrial and biomedical applications[3]. AgNPs are known to have antioxidant and antimicrobial properties[4]. AgNPs have become increasingly popular as an antibiotic agent in textiles and wound dressings, medical devices, and appliances, such as refrigerators and washing machines[5]. However, physical methods give a low yield and chemical methods are toxic to the environment, and also it is difficult to prepare AgNPs with well-defined size, whereas biological methods involve synthesis of AgNPs by means of enzymatic reduction, with better control over the shape and size of the NPs[6,7]**.** New innovations in vaccines and therapeutics that also improve patient compliance can significantly improve infectious disease treatment. Patient compliance can be substantially improved if therapies were more potent, cheaper, and required less rigorous or less frequent dosing regimens. Nanoparticles used as novel immunotherapeutic platforms are attractive for several reasons. First, these systems can encapsulate a high density of bioactive compounds that can stimulate immunity against infection. Second, these systems can be fabricated from materials that can release encapsulated compounds in a sustained fashion over several days to months. Finally, because of the flexibility over their synthesis and formulation, these systems can be extensively modified to enhance their bioactivity or transport to specific cells and organs within the body. In nature, bacteria often exist as biofilms. PGA serves as an adhesin that stabilizes biofilms of *E. coli* and other bacteria[8]. Bacteria and fungi occur as individual, free-floating (planktonic) cells or clustered together in aggregates of cells (biofilms). A microbial biofilm is 'a structured consortium of microbial cells surrounded by a self-produced polymer matrix[9]. Some biofilms adhere to natural or artificial surfaces in the host (including devices), while others may consist of aggregates associated with but not directly adherent to the surface[10-11]. During the following decades it became obvious, that biofilm infections are widespread in medicine and odontology, and their importance is now generally accepted[12]. In this work, nanoparticles were synthesized from *Wattakaka volubilis.* Antioxidant, anti-inflammatory and antibiofilm activities the synthesized nanoparticles were studied. This potentially can lead to novel therapeutic, imaging, and biomedical applications.

MATERIALS AND METHODS

Collection of plant material

Fresh leaves of *Wattakaka volubilis* Linn. F. where collected from Kalvarayan Hills, Villupuram district, Tamilnadu, India, in June 2013. The plant was identified by Dr. G. Surendiran (Taxonomist) University of Madras, Tamilnadu, India. The voucher specimen (WV 01) has been deposited in herbarium, PG and Research Centre in Biotechnology, M.G.R College, Hosur, TamilNadu, India, for future references.

Organisms used

Organisms used for the present study were *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Citrobacter*, *Citrobacter koseri*, *Escherichia harmanni*, *Proteus mirrabules*, *Klebsiella rihnoscleroma*, *Klebsiella oxytoca*, *Klebsiella pneumonia.*

Synthesis of silver nanoparticles

Silver nitrate ($AgNO_3$ Anlytical Grade) was procured from Sigma (USA). All aqueous solution where prepared using triple distilled deionized water. In typical reaction procedure, five ml of *Wattakaka volubilis* Linn. f. Leaf extract was added in 95ml of aqueous solution of 1mM silver nitrate for reduction into silver ion (Ag^+). This aqueous solution was placed in 250 ml of Erlenmeyer flask and heated on water bath for one hour at 100 °C. Reduction of silver nitrate to silver ions was confirmed by the colour change from colourless to brown. The formation of silver nanoparticles was also confirmed by spectrophotometer. UV-Vis spectra with strong SPR band at 400 - 450 nm will indicate the formation of silver nanoparticles. Colour in density increases with the increase of silver nitrate concentration at a fixed volume fraction (f=0.2) of plant.

Anti-inflammatory activity

The collected blood was mixed with equal volume of sterilized Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.05% citric acid and 0.42 % sodium chloride in water) The blood was centrifuged at 3000 rpm and packed cells were washed with isosaline (0.85%, pH 7.2) and a 10 % v/v suspension was made with isosaline. The assay mixture contains the drug (at various concentration as mentioned in table1), 1 ml phosphate buffer (0.15 M, pH 7.4), 2 ml of hyposaline (0.36%) and 0.5 ml of HRBC (Human Red Blood Cells) suspension. Diclofenac was used as the reference drug. Instead of hyposaline2 ml of distilled water was used in the control. All the assay mixtures were incubated at 37°C for 30 min and centrifuged. The haemoglobin content in the supernatant solution was estimated using spectrophotometer at 560 nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100 %. The percentage of HRBC (Human Red Blood Cells) membrane stabilization or

protection was calculated by using the formula, Anti-inflammatory activity = 100- (Test OD/Control ODx100)

Determination of Biofilm formation

Congo Red Agar method (CRA)

This method utilizes aspecially prepared solid medium – Brain Heart Infusion broth (BHI) supplemented with 5% sucrose and 0.08% Congo red for screening the formation of biofilm by clinical pathogens. The medium composes of BHI (37 g/L), Sucrose (50 g/L), Agar No.1 (10 g/L) and Congo red stain (0.8 g/L). Congo red will be prepared in the form of concentrated aqueous solution and will be autoclaved at 121˚C for 15 min, separately from other medium constituents. Following autoclave, the concentrated solution will be added to agar which was previously cooled to 55˚C. Plates will be inoculated and incubated aerobically for 24 – 48 hours at 37˚C.

Tissue Culture Plate method (TCP) – in vitro biofilm formation assay

Individual wells of sterile, polystyrene, 96-well-flat bottom tissue culture plates will be filled with 180µL of BHI broth and inoculated with 10µL of overnight culture. To the mixture 10µL of silver nanoparticles were added from the stock so that final concentration was made between 10 nM and 100 nM. The tissue culture plates will be incubated for 24 hours at 37˚C. After incubation, content of each well will be gently removed. The wells will be washed four times with 0.2mL of Phosphate Buffer Saline (PBS, pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate will be fixed with sodium acetate (2%) and stained with crystal violet (0.1%, w/v). Excess stain will be rinsed off by thorough washing with triple distilled deionized water and plates will be kept for drying. After drying, 95% ethanol will be added to the wells and the optical density (OD) of stained adherent bacteria will be determined with a micro plate reader (Model 680, Bio-Rad) at 595 nm (OD_{595} nm). These OD values will be considered as an index of bacteria adhering to surface and forming biofilms. Experiments will be performed in triplicates and the data will be then averaged and the standard error will be calculated.

RESULTS AND DISCUSSION

Anti-inflammatory activity

The human red blood corpus cell (HRBC) membrane is similar to lysosomal membrane compounds, the prevention of hypo tonicity induced HRBC membrane lysis is taken as a measure of anti-inflammatory activity of drug. This study revealed that the 1mM concentration of silver nanoparticles of leaves powder of *Wattakaka volubilis* Linn.f. produces 39.7%, 45.5% , and 51.8% and protection of the HRBC membrane from lysis due to hypo saline at dose level of 500 µg/ml when compared to 100% lysis induced in control. The nanoparticles of silver nitrate and sodium borohydride can effectively stabilize HRBC membrane only up to 24.3% and 31.3% respectively from the same dose level of hypo saline. The standard drug produces 83.33% protection at a concentration of 500 µg/ml (Table 1).

Table 1

Anti-inflammatory activity of 1mM concentration of samples

Concentration of 1mM AgNPs (µg/ml)	Test value(OD_{560})				Control value (OD_{560})				Percent Stabilization			
	1mM	D.F	S.N	S.B	1mM	D.F	S.N	S.B	1mM	D.F	S.N	S.B
62.5	0.131	0.048	0.314	0.419	0.201	0.072	0.364	0.461	34.9	33.34	13.8	9.2
125	0.120	0.038	0.309	0.402	0.216	0.073	0.373	0.466	44.5	47.95	17.2	13.8
250	0.116	0.026	0.300	0.364	0.236	0.080	0.386	0.474	50.9	67.5	22.3	23.3
500	0.111	0.014	0.296	0.332	0.242	0.084	0.391	0.483	54.2	83.33	24.3	31.3

Table 2

Anti-biofilm effect in tissue of 1mM concentration of silver nanoparticles

Organisms Used	10nM	20nM	30nM	40nM	50nM	60nM	70nM	80nM	90nM	100nM
Citrobacter	16.8	23.2	24.0	26.0	30.4	24.4	32.8	34.6	42.4	46.0
Citrobacter koseri	23.2	19.2	10.0	5.6	6.0	8.8	37.0	12.4	13.3	48.8
Escherichia harmanni	19.2	16.0	7.0	10.4	10.8	15.2	36.4	36.0	47.0	56.8
Proteus mirrabules	42.0	44.8	16.0	24.0	27.2	27.6	38.8	48.8	60.0	62.8
Klebsiella rihnoscleroma	23.2	31.2	27.6	2.0	26.0	49.2	38.8	38.8	47.0	53.2
Klebsiella oxytoca	22.4	26.8	26.0	27.6	31.6	31.2	33.4	34.0	33.2	35.4
Klebsiella pneumonia	42.0	8.0	19.6	38.8	22.4	30.0	41.4	42.4	36.0	47.2

Antibiofilm activity

The anti-biofilm activity was analysed using various nanomolar concentrations (10nM-100nM) of silver nanoparticles. Silver nanoparticles treatment has inhibited the exopolsacharides, indicated the absence of dry crystalline black colonies. The presence of nanoparticles at a certain level inhibited bacterial growth by more than 90% (Table 2, Figure1)

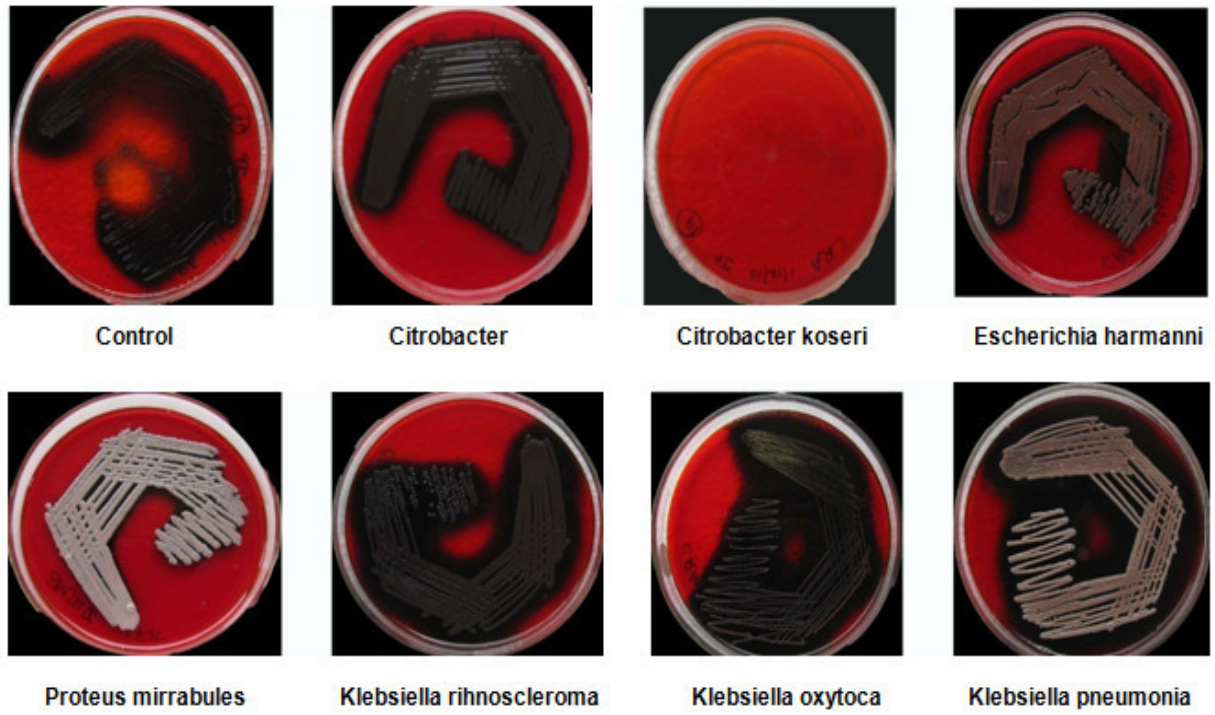

Figure 1
Biofilm forming pathogenic microorganisms

DISCUSSION

Nanotechnology is the development of a reliable and eco-friendly process for synthesis of metallic nanoparticles. We have demonstrated that use of a natural, low cost biological reducing agent, *Wattakaka volubilis* leaf extracts (aqueous) can produce metal nanostructures, through efficient green nano chemistry methodology, avoiding the presence of hazardous and toxic solvents and waste. It is well known that silver nanoparticles exhibit yellowish brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles[13]. As the *Wattakaka volubilis* leaf extract was mixed in to aqueous solution of the silver nitrate, it started to change the color from watery to brown due to reduction of silver ion; which indicated the formation of silver nanoparticles. It is generally recognized that UV–Vis spectroscopy could be used to examine size and shape-controlled nanoparticles in aqueous suspensions[14]. The UV-Vis spectra recorded from the reaction medium after heating. It was observed from the that the hot water extract shows significant anti-inflammatory activity at the concentration of 500 µg / ml. Silver nanoparticles leaf extract of *Wattakaka volubilis linn.f.* produces 63.7% and 54.2% protection of the HRBC (Human Red Blood Cells) membrane from lysis due to hypo saline at dose level of 500 µg/ml when compared to 100% lysis induced in control. The anti-inflammatory activity of the nanoparticles were concentration dependent, with the increasing concentration the activity is also increased. The silver nanoparticles of leaf of *Wattakaka volubilis linn.f.* has shown significant anti-inflammatory activity in comparison to the acetone extract of the same plant. The Tissue culture plate assay described by Chraistensen *et al.,* is most widely used and was considered as standard test for detection of biofilm formation. The increase in concentration of sliver nanoparticle of *Wattakaka volubilis linn .f* has shown reduction in biofilim formation. This is evident from where the synthesized silver nanoparticles treatment almost completely inhibited the formation of biofilm.

CONCLUSION

The silver nanoparticles of leave powder of *Wattakaka volubilis linn.f.* at 1mM concerntation has shown significant ant-inflammatory and ant-biofilim activity which was compared with standard drug. 1mM concentration of silver nanoparticles of leaves powder of *Wattakaka volubilis* Linn.f. produces 39.7%, 45.5% , and 51.8% and

protection of the HRBC membrane from lysis due to hypo saline at dose level of 500 µg/ml when compared to 100% lysis induced in control. The nanoparticles of silver nitrate and sodium borohydride can effectively stabilize HRBC membrane only up to 24.3% and 31.3% respectively from the same dose level of hypo saline. The standard drug produces 83.33% protection at a concentration of 500 µg/ml., Silver nanoparticles treatment has inhibited the exopolsacharides, indicated the absence of dry crystalline black colonies. The presence of nanoparticles at a certain level inhibited bacterial growth by more than 90%.

CONFLICT OF INTEREST

Conflict of interest declared none.

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