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Anti Biofilm Activity and Time-Kill Study of Silver Nanoparticles of *Strychnos nux vomica* Root Ethyl Acetate extractagainst Clinically Resistant *Staphylococcus aureus* Mutants

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Abstract

Introduction and Aim: The development of biofilms is a crucial component of adherent pathogens and is regarded as one of the indirect mechanisms by which bacteria are resistant to different types of current antibiotics. The time kill studies are also important to evaluate the drug efficiency towards particular bacteria. The current investigation is carried out to determine the antibiofilm and time dependent death initiation ability of silver nano particles (AgNo's) prepared using S. *nux-vomica* root ethyl acetate extract.

Materials and Methods: Crystal violet assay was used to determine the antibiofilm assay using 1 X MIC, 2 X MIC, and 4 X MIC concentrations of prepared AgNo's. Ampicillin is used as reference drug. time kill study is also carried out using 1 X MIC, 2 X MIC, and 4 X MIC concentrations of prepared AgNo's.

Results: Antibiofilm activity results of AgNP's prepared using ethyl acetate extract of S. *nux-vomica* root revealed to exhibit concentration dependent biofilm inhibition of *S. aureus* mutant strains. As per the results, we noticed that the tested AgNP's are more significant MMSA with inhibition percentage 44.7%, 85.1%, and 83.4% recorded at 1 X MIC, 2 X MIC, and 4 X MIC respectively. Based on the result, we noticed that AgNo's was significantly killed MMSACFU at 1 × MIC after 5h of treatment time of interval with 31.9%. However, the death rate percentage of MMSA was steadily raised to 56.5% at 8h treatment time and dropped to 44.8% after 9h of treatment.

Conclusion: we conclude that, S. *nux-vomica* root ethyl acetate extract AgNo's were very significant against MMSA and MRSA and slightly effective against VRSA.

Key words: S. *nux-vomica*, anti-biofilm, time kill study

1.0 INTRODUCTION

The development of biofilms is a crucial component of adherent pathogens and is regarded as one of the indirect mechanisms by which bacteria are resistant to different types of current antibiotics (1-2). Proteins, carbohydrates, and other organic molecules create a network called biofilm that aids in the pathogen's binding and establishment of a firm attachment to surfaces (3). Medical microbiologists were unaware of the

biofilm lifestyle of microorganisms until Nils Hiby discovered a connection between the aetiology of a chronic infection and bacterial aggregation in cystic fibrosis patients in the early 1970s (4). Since that time, biofilms have been identified as being involved in a variety of clinical illnesses (5-6), and mounting data suggests that biofilms have a role in pathogenesis, particularly in chronic infections.
Time-kill Kinetics tests aid in the comprehension of the interactions between various microbial strains and antimicrobial substances. The test demonstrates an antimicrobial agent's concentration- or time-dependent test action on different microorganism strains. It categorises antimicrobial drugs as either bactericidal/fungicidal or bacteriostatic/fungistatic (7). Drug resistance in bacteria can occur in a variety of ways, including biofilm development, active drug efflux, drug inactivation brought on by enzyme release, and drug target site modification (8). Antibiotics' method of action is unaffected by bacteria that produce biofilms because the creation of biofilms inhibits the penetrating capacities of the bacterium (9). The ability of nanoparticles (NPs) to quickly diffuse or pass through cell membranes makes them crucial for medication administration. Additionally, the effectiveness of drug release at the intracellular location is increased by the use of biodegradable polymers in the manufacturing of NPs. The green chemistry technique, uses minimal energy, avoids the use of harmful chemicals, produce NP using environmentally favourable methods. Gold, silver, copper, zinc, and copper are frequently utilised to create NPs from plants. Staphylococcus *aureus* (*S. aureus*) is a common component of human microflora that spreads from person to person by direct touch; however colonised items or surfaces are also significant factors in *S. aureus* transmission. According to the CDC NNIS System (2001) (10), 30–40% of persons worldwide are nosal carriers of this bacterium. Antibiotics frequently work on *S. aureus* developed resistance to the most significant family of antibiotics, isoxazoylpenicillins, including flucloxacillin, methicillin, and oxyacillin, throughout the years (11). The current scenario demands for the discovery or development of drugs with immense mechanisms of action against *Staphylococcal* infections in light of the clinical failure of antibiotics. The novel medications can be created artificially (chemical synthesis) or naturally (microbial synthesis and plant derived). Drug development also involves structurally altering current antibiotics to address resistance mechanisms effectively. With reference to drug discovery against *Staphylococcal* infections the present study was designed to ascertain the antibiofilm and bactericidal effects of silver nano particles prepared using *Strychnosnux-vomica* (S. *nux-vomica*) root extract.

2.0 MATERIAL AND METHODS

2.1 Collection of plant material

The S. *nux-vomica* root material was bought from the village Siddapuram, Warangal rural, Telangana. The root material was washed with tap water to remove the adhered soil. The material was dried in a shadow for approximately 45 days. The dried root material was made fine powder.

2.2 Extraction

We have used Ethyl acetate solvent to extract 250g of S. *nux vomica* root powder using Soxhlet continuous hot extraction method. The solvent evaporation was carried out using rota evaporator to collect the crude extract.

2.3Synthesis of Silver nanoparticles

The synthesis of silver nanoparticles (AgNP's) of S. *nux-vomica* root ethyl acetate extract been already reported by us (14). For understanding, here we have included a brief method of synthesis. The silver nano particles of S. *nux-vomica* are synthesised in accordance to the method described by Song and Kim, (2009) (12). Briefly, to 40 mL of 1 mM $AgNO_3$, 10 mL of 10% (w/v) ethyl acetate extract dissolved in DMSO was added and maintained at room temperature till the development of golden colour. This solution is incubated overnight in a dark chamber closed tightly. The supernatant is discarded and the sediment is further processed for centrifugation at 10,000 RPM for about 15-20 min. The supernatant formed is discarded. The sediment particles were dissolved with double distilled water and once again the subjected to centrifugation at 10,000 RPM for 5min. The supernatant is discarded and the sediment was dissolved with ethanol and subjected for further centrifugation at 10, 000 RPM for about 5 min. The supernatant is discarded and the silver nano particles (AgNP's) were oven-dried overnight at 50-55^{0}C (Song and Kim, 2009).

2.4 Antibiofilm assay

2.4.1Bacterial Strains

The Antibiofilm and Bactericidal efficacy of S*nux-vomica* root AgNP's was investigated against Methicillin-susceptible *S. aureus* (MSSA), Methicillin- resistant *S. aureus* (MRSA) and Vancomycin Resistant *S. aureus* (VRSA) The bacterial strains were maintained on LB medium. The selected bacterial cultures were grown and the turbidity was adjusted with sterile broth to obtain a half of MC Farland standard (1x108 - 5x108 cfu/ml). This is used as inoculum for the assay.

2.4.2 Growing a biofilm

The test was conducted using the previously reported methodology (13). In a nutshell, the plate was turned and shaken to spill the cells. The microtiter plates should be gently washed 3–4 times with sterile distilled water, allowed to air dry, and then baked at 60°C for 35–45 minutes to complete drying. This step minimises the background staining of the well and eliminates unattached cells and medium components that can be stained in the next step. Transfer 125mL of a 0.1% crystal violet solution to each well, and then let it sit there for 10 to 15 minutes. To get rid of all extra cells and colour, thoroughly rinse the plate four or five times with sterile distilled water. Biofilm may be seen at this point as purple rings on the well's walls. The plate is left to dry overnight before being evaluated quantitatively. Ampicillin is used as a positive control at concentration of 10µg/ml. S *nux-vomica* root AgNPs at 1 X MIC, 2 X MIC, 4 X MIC (The Minimum Inhibitory Concentration of AgNPs against *S. aureus*; previously reported (14) are used to assess the antibiofilm property (Table S1). The negative controls are DMSO and sterile distilled water. The biomass of the biofilm was calculated using the crystal violet (CV) staining method (13).

2.4.3 Crystal violet staining assay

2.4.3.1 Staining the Biofilm

The test was conducted using the previously reported methodology (15). In a nutshell, the plate was turned and shaken to spill the cells. The microtiter plates should be gently washed 3–4 times with sterile distilled water, allowed to air dry, and then baked at 60°C for 35–45 minutes to complete drying. This step minimises the background staining of the well and eliminates unattached cells and medium components that can be stained in the next step. Transfer 125mL of a 0.1% crystal violet solution to each well, and then let it sit there for 10 to 15 minutes. To get rid of all extra cells and colour, thoroughly rinse the plate four or five times with sterile distilled water. Biofilm may be seen at this point as purple rings on the well's walls. The plate is left to dry overnight before being evaluated quantitatively.

2.4.3.2 Quantitative assessment of biofilm

By adding 125mL of 33% acetic acid to each well, a quantitative evaluation of biofilm development was carried out. The micro titre plate is incubated for 10 to 15 minutes at room temperature. In order to measure the absorbance at 590 nm, a 125mL aliquot of the solubilized solution was transferred to a brand-new, sterile microplate. The samples' average absorbance was calculated, and the following equation (13) was used to calculate the percentage of biofilm inhibition.
Percentage (%) of inhibition = OD Negative control-OD Experimental/OD of Negative control x 100

2.5 Time-Kill Kinetics Assay

Using the previously mentioned technique (16), the time-kill kinetics of ethyl acetate extract of root AgNP's was assessed. Aliquots of the 1 MIC, 2 MIC, and 4 MIC root extracts were made. To tubes containing nutrient broth, 1.0 106 CFU/ml of an inoculum was added, and the tubes were then incubated at 37 °C for 24 hours. The organisms underwent a control test without the extracts or standard antibiotics. Aliquots of 1.0 ml of the medium were taken at time intervals of 0, 1, 2, 3, 4, 5, 6, 12, and 24 h and inoculated aseptically into freshly prepared 20 ml nutrient agar plates and incubated at 37oC for 24 h. The CFU of the organisms was determined and the experiments were performed in triplicate. A graph was plotted between log CFU/ml versus time.

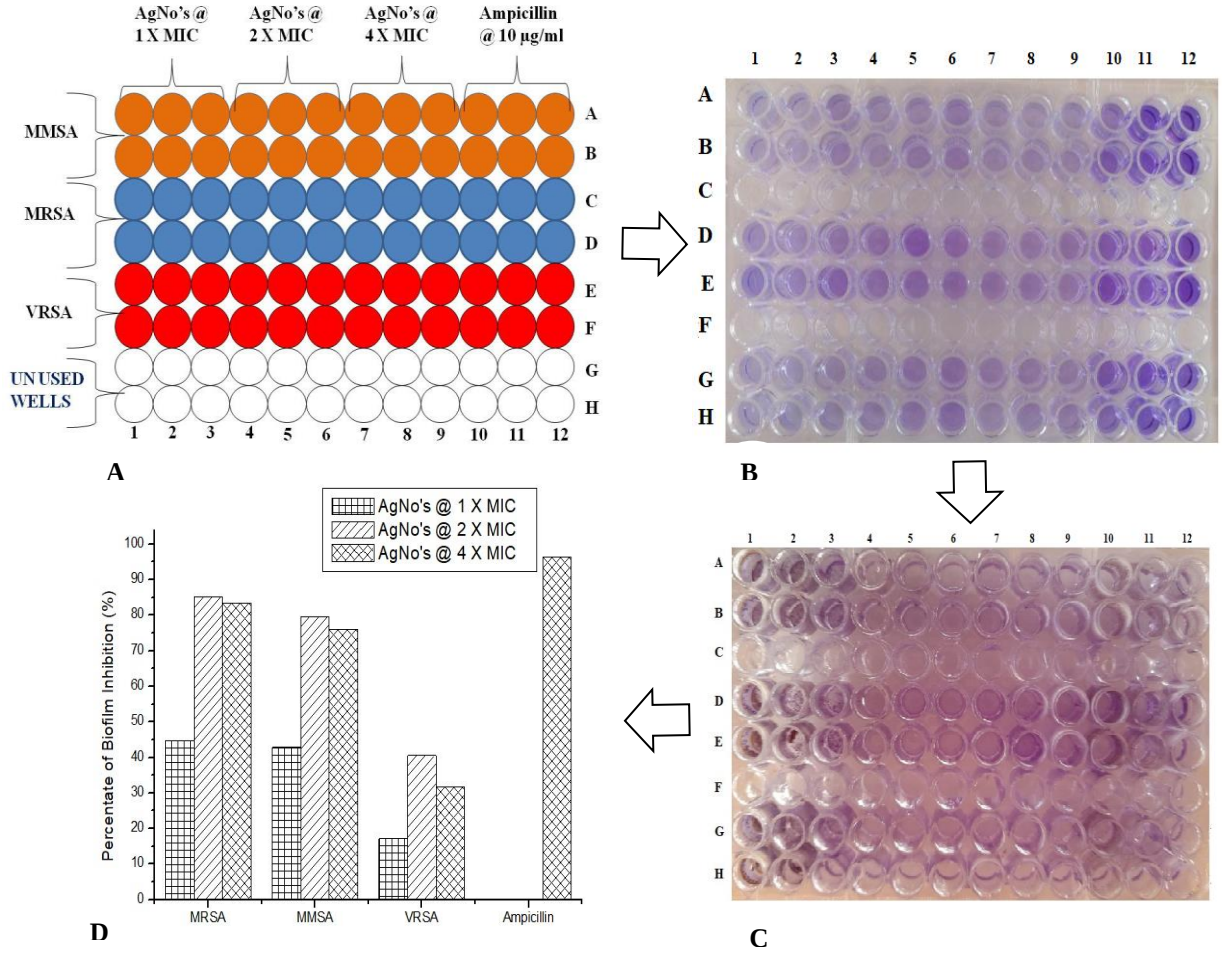

Fig 1 Schematic representation of Antibiofilm activity by AgNo's of S. *nux-vomica* root ethyl acetate extract against *S. aureus* mutant strains. A-Inoculation of selected *S. aureus*in to the 96 well plate, B-Crystal violet assay plate, C-quantification of biofilm, D-percentage of biofilm inhibition by AgNo's

3.0 RESULT

3.1 Antibiofilm assay

Antibiofilm activity results of AgNP's prepared using ethyl acetate extract of S. *nux-vomica* root revealed to exhibit concentration dependent biofilm inhibition of *S. aureus* mutant strains. As per the results, we noticed that the tested AgNP's are more significant MMSA with inhibition percentage 44.7%, 85.1%, and 83.4% recorded at 1 X MIC, 2 X MIC, and 4 X MIC respectively. Following, the effect of biofilm inhibition by AgNo's was also found significant against MRSA with biofilim inhibition percentage 42.8%, 79.6%, and 75.9% recorded at 1 X MIC, 2 X MIC, and 4 X MIC respectively. The biofilm inhibition capacity of AgNo's against VRSA was restricted to minimum. VRSA with inhibition percentage 17.2%, 40.5%, and 31.7% recorded at 1 X MIC, 2 X MIC, and 4 X MIC respectively (Fig 1).

3.2 Time Kill Studies

The time kill study result of selected mutant strains of Staphylococcus *aureus* is shown in Fig 2 and Fig 3. The study at different concentrations of MIC (1 X MIC, 2 X MIC, and 4 X MIC) of the AgNo's of S. *nux-vomica* root ethyl acetate extract was tested against selected mutant strains of *S. aureus*. Based on the result, we noticed that AgNo's was significantly killed MMSACFU at 1 × MIC after 5h of treatment time of interval

with 31.9%. However, the death rate percentage of MMSA was steadily raised to 56.5% at 8h treatment time and dropped to 44.8% after 9h of treatment. Following to MMSA, AgNo's prepared from S. *nux-vomica* root ethyl acetate extract was also more significant against MRSA. Approximately, diminish of MRSACFU at 1 × MIC was recorded after 5h of treatment time of interval with 29.9%. However, the death rate percentage of MRSA was constantly raised to 50.7% at 8h treatment time and dropped to 37.3% after 9h of treatment. The death rate percentage of VRSA comparing to MSSA and MRSA was found least. Death of VRSACFU at 1 × MIC was recorded after 5h of treatment time of interval is 18.2%. However, the death rate percentage of VRSA was slightly raised to 26.1% after 8h treatment time and dropped to 20.4% after 9h of treatment. On the other hand, the death rate of *S. aureus* mutant strains at 2 X MIC and 4 X MIC was found high. Among the strains tested, MMSA was found most susceptible to AgNo's prepared from S. *nux-vomica* root ethyl acetate extract. Diminish of MMSACFU at 2 × MIC and 4 X MIC after 5h of treatment time of interval revealed with 76.0% and 72.5% respectively. However, the death rate percentage of MMSA increased to 85.7% and 75.6% respectively after 8h treatment time and dropped to 71.9% and 68.1% after 9h of treatment. Following to MMSA, the death percentage rate was found high with MRSA. Diminish of MRSACFU at 2 × MIC and 4 X MIC after 5h of treatment time of interval revealed with 68.8% and 65.2% respectively. However, the death rate percentage of MRSA increased to 75.5% and 69.6% respectively after 8h treatment time and dropped to 70.1% and 60.3% after 9h of treatment. Next to MRSA, the death percent rate was found high with VRSA. Diminish of VRSACFU at 2 × MIC and 4 X MIC after 5h of treatment time of interval revealed with 49.1% and 47.3% respectively. However, the death rate percentage of MRSA increased to 55.1% and 50.4% respectively after 8h treatment time and dropped to 48.2% and 46.1% after 9h of treatment.

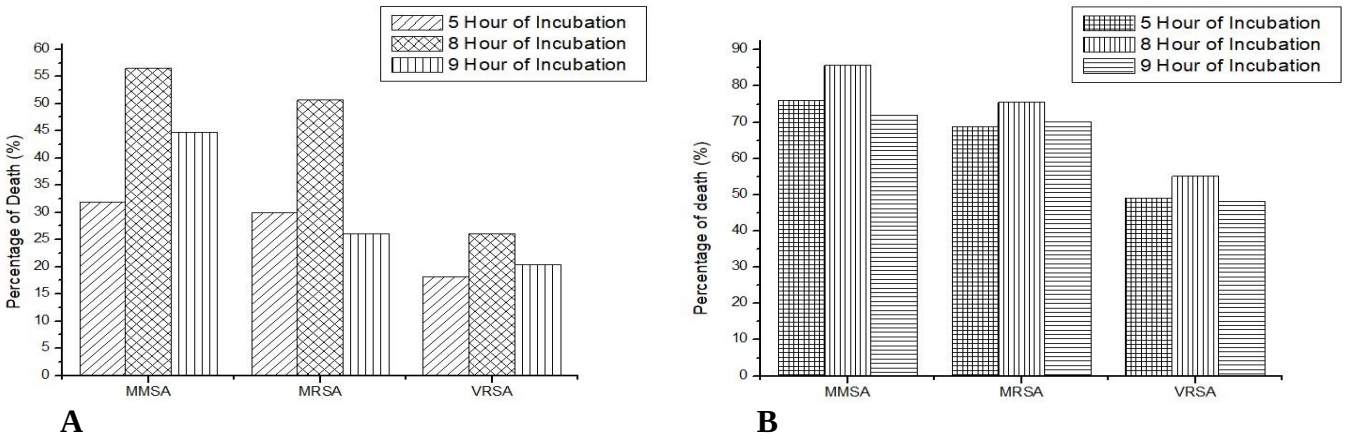

Fig 2Time kill studies; A- Efffect of S. *nux-vomica* root ethyl acetate extract AgNo's against *S. aureus* mutant strains at 1 X MIC, B- Effect of S. *nux-vomica* root ethyl acetate extract AgNo's against *S. aureus*mutant strains at 2 X MIC.

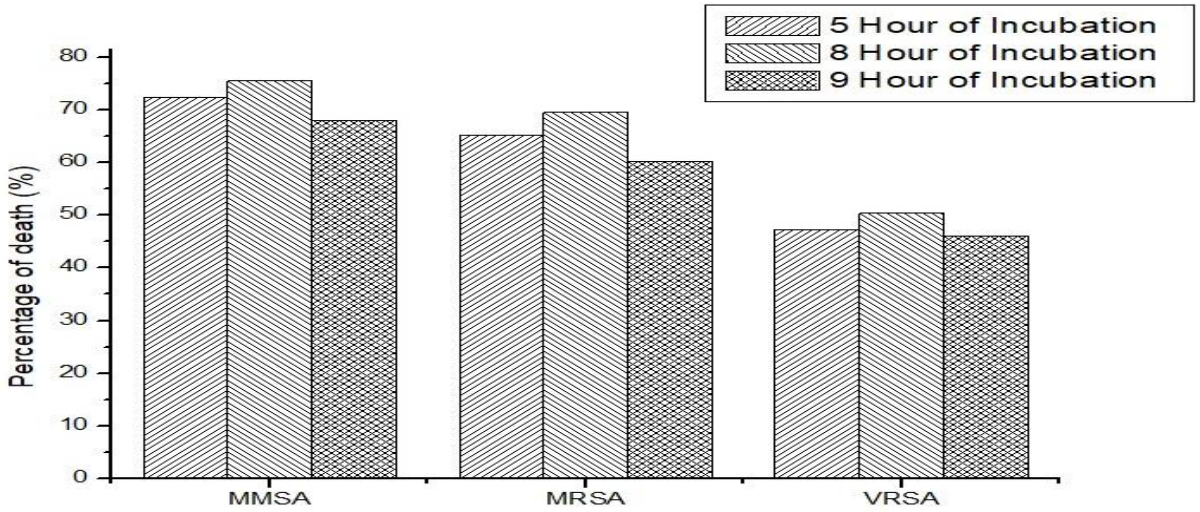

Fig 3 Time kill studies; Effect of S. *nux-vomica* root ethyl acetate extract AgNo's against *S. aureus*mutant strains at 4 X MIC

4.0 Discussion

The selection of AgNP's of S. *nux- vomica* root ethyl acetate is based significant antibacterial activity previously reported against *S. aureus* species (14). Numerous types of infections can be brought on by staphylococci. (17) S *aureus* lead to the development of several localised abscesses in different sites places and superficial skin diseases (boils, styes) in humans. (18) Moreover, this bacterium is the root cause of furunculosis and deep-seated infections like osteomyelitis and endocarditis. (19) *S. aureus*, together with S. *epidermidis*, cause nosocomial infections. (20) Other pathogenic effects of S *aureus* releases enterotoxins into food and lead to the subsequent food poison. (21) Moreover, it also releases super antigens into the blood and cause toxic shock syndrome. (22) saprophiticus, particularly in girls, causes urinary tract infections. (23). Development of drugs towards the treatment of *S. aureus* infections, have become difficult due to the development of Multi Drug Resistant (MDR) *S. aureus*. Apart from MDR, hazardous effects of contemporary drugs for the treatment of bacterial infections also have been major concern. Thus, there is an urgent need for the discovery or development of drugs which are safe and affordable. Secondary metabolites of plants act as a significant alternative medicine for the treatment of several human diseases. Nano formulation of plant extracts (Green Synthesis) is a very good approach for significant delivery of drug to the target site.
In the current study, we have examined the biofilm inhibition and time kill studies of S. *nux-vomica* root ethyl acetate extract silver nano particles (AgNo's) against *S. aureus* mutant strains. In accordance to our studies, we revealed that AgNo's are highly significant inhibited the biofilm formation ability of S. *auerus* mutant strains. MMSA and MRSA are found to be highly failed to develop biofilm in the presence of AgNo's. However, VRSA was found slightly resistant towards tested AgNo's. Biofilm is one of the important strategies of any bacterium to develop the resistance against any antibiotic treatment. Thus the determination of bioflm inhibition capability of any drug or plant extracts or nano particles chemical or prepare via plant extracts hold great importance. On the other hand, the determination of time dependent kill of any bacterium by any drug also takes greater part in the development of a particular drug. In the current investigation, we observed that, AgNo's significantly initiated the process of death from 5^{th} h of incubation. The exponential death of S. *aureus* was found at 8^{th} h of incubation. However, the death rate of these mutant strains was constant from 8^{th} to 9^{th} h and declined after 9^{th} of incubation.

Conclusion

Based on the results, we conclude that, S. *nux-vomica* root ethyl acetate extract AgNo's were very significant against MMSA and MRSA and slightly effective against VRSA.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Schilder, AG., Chonmaitree, T., Cripps, AW., Rosenfeld, RM., Casselbrant, ML., Haggard, MP., Venekamp, RP. Otitis media. *Nat. Rev. Dis. Primers.* 2016; doi: 10.1038/nrdp.2016.63.
2. Monasta, L., Ronfani, L., Marchetti, F., Montico, M., Brumatti, LV., Bavcar, A., Grasso, D., Barbiero, C., Tamburlini, G. Burden of Disease Caused by Otitis Media: Systematic Review and Global Estimates. *PLoS ONE.* 2012; 7:e36226. doi: 10.1371/journal.pone.0036226.
3. Rovers, MM., Schilder, AG., Zielhuis, AG., Rosenfeld, RM. Otitis media. *Lancet.* 2004; 363 (9407): 465–73. doi: 10.1016/S0140-6736(04)15495-0.
4. Høiby N. A short history of microbial biofilms and biofilm infections. *APMIS.* 2017; 125 (4): 272–275. doi: 10.1111/apm.12686.

5. Costerton, JW., Stewart, PS., Greenberg, EP. Bacterial biofilms: A common cause of persistent infections. *Science.* 1999; 284 (5418): 1318–22. doi: 10.1126/science.284.5418.1318.
6. Stoodley, P., Hall-Stoodley, L. Evolving concepts in biofilm infections. *Cell. Microbiol.* 2009; 11 (7):1034–43. doi: 10.1111/j.1462-5822.2009.01323.x.
7. Auinger, P., Lanphear, BP., Kalkwarf, HJ., Mansour, ME. Trends in otitis media among children in the United States. *Pediatrics.* 2003; 112 (3pt 1): 514–20. doi: 10.1542/peds.112.3.514.
8. Donlan, RM and Costerton, JW. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clin. Microbiol. Rev.* 2002; 15 (2): 167–193. doi: 10.1128/CMR.15.2.167-193.2002.
9. DeAntonio, R., Yarzabal, JP., Cruz, JP., Schmidt, JE., Kleijnen, J. Epidemiology of otitis media in children from developing countries: A systematic review. *Int. J. Pediatr. Otorhinolaryngol.* 2016; 85: 65–74. doi: 10.1016/j.ijporl.2016.03.032.
10. CDC NNIS System: National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992–April 2001,issued August 2001. *Am. J. Infect. Control.* 29:400–421.

11. Diekema, DJ. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. *Clin. Infect. Dis.*2001;32: S114–S132.

12. Song Y and B.S. Kim. (2009): Rapid biological synthesis of silver nanoparticles using plant leaf extracts. Bioproc. Biosyst. Eng., 32 (1): 79–84.
13. Sandasi, M., Leonard, C., Viljoen, A. The effect of five common essential oil components on Listeria Monocytogenes biofilms. Food Microbiol. 2008; 19 (11): 1070-1075
14. Praveeenkumar, G., Kireety Sharma, A., Murali Krishna, T. Antibiotic effects of silver nanoparticles synthesized from *Stychonsnux vomica* ethyl acetate root column fraction against clinical resistant *Staphylococcus* strains, *int. J. Adv. Res.* 2022; 10(2): 1096-1099.
15. Djordjevic, D., Wiedmann, M., Mclandsborough, L. Microtiter plate assay for assessment of Listeria Monocytogenes biofilm formation. Appl Environ Microbiol. 2002; 68 (6): 2950-2958.
16. Tsuji, BT., Yang, JC., Forrest, A., Kelchlin, PA., Smith, PF. *In vitro* pharmacodynamics of novel rifamycin ABI-0043 against *Staphylococcus aureus*. Antimicrob Agents Chemother. 2008; 62 (1): 156-160.
17. Bhakdi, S., Tranum-Jensen, J., Alpha-toxin of *Staphylococcus aureus*. Microbiol Rev. 1991; 55 (4): 733.
18. Easmon, CSF and Adlam C. Staphylococci and staphylococcal infections. Vols 1 and 2. Academic Press, London, 1983.
19. Foster, TJ. Potential for vaccination against infections caused by *Staphylococcus aureus*. Vaccine. 1991; 9: (4) 221.
20. Foster, TJ and McDevitt, D. Molecular basis of adherence of staphylococci to biomaterials. p. 31, In Bisno AL, Waldvogel FA (eds): Infections Associated with Indwelling Medical Devices, 2nd Edition. American Society for Microbiology, Washington, D.C., 1994.
21. Lyon, BR and Skurray, R. Antimicrobial resistance in *Staphylococcus aureus*: genetic basis. Microbiol Reviews. 1987; 51 (1): 88.
22. Prevost, G., Couppie, P., Prevost, P. Gayet, S., Petiau, P., Cribier, B., Monteil, H., Piemont, Y. Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. J Med Microbiol. 1995; 42(4):237-45.
23. Rupp, ME and Archer, GL. Coagulase-negative staphylococci: pathogens associated with medical progress. Clin Infect Dis. 1994; 19 (2): 231.