Bio-hybridization of nanobactericides with cellulose films for effective treatment against members of ESKAPE drug resistant pathogens

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1. Introduction:

Bio-hybridization is a process of modulation of bioactive molecules with structural components (Gao and Maruyama, 2014). The outcome of the hybridization results in enhancement or up gradation of desired activity (Ma et al., 2016). The exact definition of bio-hybridization is yet to be completely elucidated but however, there has been significant progress to obtain multifold applications based on the principle of bio-hybridization (Syed et al., 2016). Some of the bio-hybrid molecules are used in developing implants, regenerative medicines, bioreactors, biosensors, development of functionalized films/composites (Khan et al., 2015; Volova et al., 2018). The process of bio-hybridization not only enhances the applicative properties, it also influence on the physicochemical properties of the hybrid complex (Witte et al., 2012). The ideal bio-hybridized prodcut in the biomedical application should be biocompatibility, sustainable and controlled release of drugs, longevity, biodegradable and profound desired activity (Owen et al., 2016; Shidlovskiy et al., 2017). In recent years, owing to the rapid expansion of drug resistant pathogens, there has been serious concern in developing novel antimicrobial agents which can control and combat drug resistance (Founou et al., 2016). Most of the available standard antibiotics are ineffective against wide range of microbial pathogens (Gupta and Birdi, 2017). Based on which, the mortality and morbidity rates are at alarming pace (Kim et al., 2015). The major contributors of drug resistance are grouped as ESKAPE members which includes *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species (Santajit and Indrawattana, 2016). These members are reported to be one of the prime source of nosocomial infection in hospitals which are not only infecting patients, they are also having adverse effects on working staff and visitors which are reported to be acquiring infection directly or indirectly (Syed et al., 2017). The burden posed by these pathogens on health care system is reported to be large scale which is global economic crisis (Singer et al., 2016; WHO,2017a). In order to combat these pathogens, WHO has categorized antimicrobial resistance as one of the top priority research (WHO,2017b). Based on this, scientific groups are engaged in developing novel strategies to control the expansion of drug resistance (Rather et al., 2017). One such strategy include implementation of technologically advanced scientific domain "Nanotechnology" (Syed et al., 2017). The recent studies have highlighted the bactericidal potential of wide range of nanomaterials especially metallic nanomaterials (Kavitha et al., 2013). These nanobactericides are at nanoscale which are more upgraded than its bulk counterpart (Syed et al., 2016). Extensive research on nanomaterials have demonstrated the activity of nano-silver against myriad pathogenic bacteria with multiple mode of actions (Zhang et al., 2016). Scientific literatures provide the evidence of nano-silver acting on the

metabolism of pathogens which in turn paralysis its functioning (Kota et al., 2017). Based on these facts, the present study was designed to develop bio-hybridization of silver nanobactericides onto bacterial cellulose film and their activity against clinically isolated multi-drug resistant pathogens which are tested to be having resistant to more than ten antibiotics. These pathogens were isolated from the individual suffering from myriad microbial infections which was cultured and identified to affiliate its identity to be members of ESKAPE pathogens. In order to omit the adverse effects of conventional mode of synthesizing nanobactericides, phyto-mediated process. The phyto-mediated process of producing silver nanobactericides is one of the facile and benign route wherein the metal salt is reduced by phyto-constituents which are also reported to aid in bearing stabilization and applicative properties (Baker et al., 2013). The synthesized nanobactericides were hybridized onto bacterial cellulose films. The bacterial cellulose is a network of cellulose fibrils with less than 100 nm which has resulted in unique properties thus forming one of the promising material (Volova et al., 2018). Scientific reports highlight the importance of BC due to its versatile properties, ease in large production via fermentation, inexpensive, generation in different shapes, absorptivity, elasticity, biocompatible, inert, hypoallergenity and mechanical properties (Volova et al., 2018). In recent years, use of bacterial cellulose has rapidly expanded which has marked its application in biomedical sector to develop implants, catheters, scaffolds to tissue engineering, bio-composite etc (Moniri et al., 2017). The biocompatibility of bacterial cellulose films is regarded as one of the best suit for biomedical applications to designed novel biomaterials such as artificial skin substitutes for treatment of wounds, ulcers and burns (Volova et al., 2018). In such situations, prevention of microbial infections is one of the top priority and bacterial cellulose lack antimicrobial potential (Hu and Hsieh, 2015; Heli et al.,2016). Hence hybridization of bacterial cellulose films become prime important (Faria-Tischer et al., 2016; Tsai et al., 2017). Therefore the was carried out in present study by selecting *Chamerion angustifolium* as source of synthesizing silver nanobactericides. The selection of plant species was carried out based on the scientific and traditional knowledge *Chamerion angustifolium* is reported to be one of the therapeutic plant and consumed as tea in various parts of Asia (Martin and Husband, 2013; Pinno et al., 2014).

2. Materials and Methods

2.1.Plant processing

The plant materials (Stem and leaves) were collected from the abundant growing area of Krasnoyarsk region, Siberia, Russia. Plant materials were washed thoroughly under running tap water to remove the soil debris. The plant materials were chopped into small segments and 20 g of finely cut materials was added to one-liter beaker containing 500 ml of sterile distilled water (Syed et al., 2017a). The mixture was boiled for 30 minutes to obtained aqueous extract which was stored at 4^{o}C until further use.

2.2.Synthesis of silver nanobactericides

The aqueous extract was subjected to synthesis of nanobactericides wherein, for the synthesis of silver nanobactericides, 1 mM silver nitrate was incubated with aqueous extract at ratio 7:3. The conversion of Ag+ to Ag^{o} was initially confirmed with a change in the color of the reaction mixture and further confirmation was achieved with the UV-Visible spectrophotometer.

2.3. Characterization of nanobactericides

The synthesized nanobactericides were subjected to characterization using various hyphenated techniques. The morphological structure and local elemental composition were determined via a high-resolution transmission electron microscope (HRTEM) JEOL JEM-2100 operating at an acceleration voltage of 200 kV. The HRTEM with an energy-dispersive spectrometer Oxford Inca x-sight. Selected area electron diffraction (SAED) was used to determine the crystal structure of nanoparticles (Mikhlin et al., 2014). The possible role of the aqueous extract as reducing agent was studied using FTIR spectroscopy. The crystalline nature was studied using X-ray diffractometer instrument operating at a voltage of 30 kV.

2.4.Production of bacterial cellulose films

The production of bacterial cellulose film (BCF) was carried out according to the protocol described by Shidlovskiy et al., 2017. In brief, BCF was synthesized by *Komagataeibacter xylinus* B-12068 culture strain which was previously isolated from fermented tea (kombucha). The actively growing isolate was cultured in Hestrin-Schramm (HS) liquid medium and for 7 days at a temperature of 30°C under static conditions. Later after the incubation period, BCF was separated from bacterial cells and media component with the treatment of 1.0 M NaOH at 70 ^{o}C which was followed by repeated washing with deionized water. Then, BCF was placed in 0.5 % solution of hydrochloric acid for 24 hours to neutralize which was later rinsed with double distilled water. Then the films were stored until further use.

2.5. Bio-hybridization of nanobactericides onto BCF and their biophysical characterization

The synthesized nanobactericides were centrifuged and subjected to washing repeated for three times with sterile distilled water. The pellet was dissolved and pipette on the bacterial cellulose membranes until the complete membrane is immersed which was incubated at 37 ^{o}C for one hour in the water bath. The obtained film was dried and subjected to

biophysical characterization. The BCF were excised into small blocks (5×5 mm) which were subjected to morphological characteristics using scanning electron microscopy of ultrahigh resolution S-5500 (Hitachi, Япония, 2009). The sample was processed by placing onto the sample stage and sputter-coated with gold, using an Emitech K575X sputter coater (10 mA, 2×40 s). The sample was examined and the morphological characteristics were measured and recorded with image analysis program (Image Processing and Data Analysis) in Java. Mechanical properties of the BCF were investigated using an electromechanical tensile testing machine Instron 5565 (U.K.). Samples 75 mm long, 12 mm wide were prepared for studying physical and mechanical properties of the films.

2.6.Multi-drug resistant pathogens

The selected strains are reported to be multi-drug resistant strains bearing resistant mechanism to nearly 10 different antibiotics. The test pathogens are *Acinetobacter baumannii* strain 210, *Acinetobacter baumannii* strain 211, *Pseudomonas aeruginosa* strain 55, *Pseudomonas aeruginosa* strain 40, *Klebsiella pneumoniae* strain 104, *Methicillin-resistant Staphylococcus aureus, Escherichia coli* strain 55. All the test pathogens were handled with prime care and preserved according to standard guidelines and maintained at culture collection center of Krasnoyarsk Medical University.

2.7.Preparation of test bacterial suspension

The test inoculum suspensions were prepared according to the protocol described by Teh et al., 2017 with slight modification. In brief, the actively growing test bacterial strains were inoculated into 10 ml sterile Mueller Hinton broth (MHB) and incubated overnight at 37 ^{o}C. The overnight test bacterial suspensions were adjusted to 0.5 McFarland Standard with sterile Mueller Hinton broth under aseptic conditions. The preparation of inoculum was carried as per the Clinical and Laboratory Standards Institute (CLSI) guidelines

2.8. Antimicrobial activity of silver nanobactericides

The synthesized nanobactericides were centrifuged at 15,000 rpm for 20 minutes. The obtained pellet was washed thrice with double distilled water and 5 mg/ml concentration was evaluated for antimicrobial activity via well diffusion assay and micro-broth dilution assay. In brief pre-warmed MHA (Mueller-Hinton agar) plates were seeded with test bacterial suspension ($1.5x10^6$ CFU/ml) and swabbed uniformly, later by using sterile cork borer agar was punched to obtained wells and 100 µl nanobactericides were added into each well and incubated at 37°C for 24 hours. After incubation, the zone of inhibition was measured and interpreted with different antibiotics.

2.9.Minimal inhibitory concentration

The minimal inhibitory concentration (MIC) was carried out according to the protocol described by Syed et al., 2017b. In brief, the plates were prepared under aseptic conditions and volume of 100 µL of test material (nanobactericides 1 mg/ml). The test material was pipette it out in the first row followed by addition of 50 µL of nutrient broth to all other wells. Further, serial dilutions were performed using a multichannel pipette and 10 µL of resazurin as growth indicator was seeded to each well. The final volume of the broth was adjusted with the addition of 30 µL isosensitised broth to each well ensuring the final volume of the nutrient broth. Finally, 10 µL of bacterial suspension ($1.5x10^6$ CFU/ml) was added to each well. The plate was incubated at 37° C for 18 to 24 hours. The color change was then assessed visually from purple to pink or colorless. The lowest concentration at which color change occurred was taken as the MIC value (Sarker et al., 2007).

2.10. Antibacterial activity of silver nanobactericidal cellulose films

The BCF embedded with nanobactericides were subjected to antibacterial activity according to the protocol described by Volova et al., 2018 with slight modification. In brief, small blocks of BCF with nanobactericides were excised under aseptic condition. The pre-warmed MHA (Mueller-Hinton agar) plates were seeded with ($1.5x10^6$ CFU/ml) which was swabbed uniformly. The prepared blocks of BCF were placed onto the swabbed media and incubated at 37° C for 24 hours. The activity was measured as the zone of inhibition across the block in millimeters.

3. Results and Discussion

The results of bio-hybridization of nanobactericides with bacterial cellulose are promising enough to attribute against multi-drug resistant pathogens. The synthesis of nanobactericides was achieved by treating 1mM silver nitrate with aqueous plant extract *Chamerion angustifolium*. The scientific literature on the selected plant insights its traditional usages which is popularly consumed as chai (Tea) it has. It is reported to possess therapeutic activity against benign prostatic hyperplasia and prostate cancers (Rogers, 2014). To best of our knowledge, there have been scanty reports on *Chamerion angustifolium*, based on which the present plant became the subject of present investigation. The aqueous extract of the plant was filtered and treated with 1 mM silver nitrate and synthesis of silver nanobactericides was monitored until the color change was observed. The initial confirmation of synthesis was achieved by UV-Visible spectrophotometry with maximum absorption at 408 nm. The UV-visible spectroscopy is one the reliable tool to confirm the formation of silver nanobactericides (Mahmudin et al., 2016). The

maximum absorbance in the range of 100 to 800 nm is due to the surface plasmon resonance which defines the collective excitation of electrons (Azar and Mohebbi, 2013). In the present investigation, the influence of different variables on synthesis was observed. The synthesis was rapid and maximum at pH 9 and temperature 90^{o}C which was studied individual (Figure 1). The synthesis was rapid and completed within 30 minutes of incubation time which was recorded as change in the color of the reaction mixture to brown. The influence of different variables influencing the synthesis of nanomaterials is well demonstrated and the results obtained in the present investigation were in accordance with these scientific reports (Qian et al., 2013). The reduction of metal salts to produce nanobactericides is achieved due to the presence of phyto-components which reduced the silver ions to form nano-sized silver. The diverse classes of phyto-constitutents present in aqueous extract also influences on the stability of synthesized silver nanobactericides (Baker et al., 2013). The bio-molecular interaction between the phyto-components and silver nanobactericides was predicted with FTIR analysis which displayed vibrational stretches in the IR range which corresponds to functional groups (Table 1). The scientific studies demonstrate the interaction of phyto-constitutes facilitates the bio-hybridization process. The obtained results coincide with earlier findings (Sumi et al., 2016). The crystalline nature of synthesized nanobactericides was predicted with XRD analysis which confirmed the face centric cubic of silver by displaying intensive peaks at 2θ angle which denoted (111), (200) and (311) planes. The obtained results justify the findings of plant-mediated crystalline nano-silver (Jeevan et al., 2012). The morphological characteristics of silver nanobactericides were defined with TEM micrographs which displayed polydispersity of silver nanobactericides with size ranging from 2 to 40 nm as shown in the figure….These morphological characteristics were well defined and are in accordance with previous scientific reports (Awwad et al., 2013). In the present investigation, as an applicative point of view, the synthesized silver nanobactericides were tested against multi-drug resistant pathogens (ES>>>) which are resistant to more than ten antibiotics namely ampicillin, cefoperazone, cefepime, chloramphenicol, imipenem, meropenem, gentamicin, tetracycline, tobramycin and vancomycin. The bactericidal activity was measured as the zone of inhibition via well diffusion assay as shown in the figure…..Among the test pathogens, *Methicillin-resistant Staphylococcus aureus* was most sensitive with 21 mm zone of inhibition and least activity was observed against *Acinetobacter baumannii* strain 211 with 10 mm zone of inhibition. The activity was also determined by minimal inhibitor concentration with rezasurin as the growth indicator. The optical density of the test organisms seeded with nanobactericides was measured at 600 nm, interestingly, the MIC results (Table 2) were in accordance well diffusion assay. In the present investigation, bio-hybridization of silver nanobactericides with bacterial cellulose films was carried by external pipetting and embedding of silver nanobactericides onto the previously synthesized bacterial cellulose films. The films were dried and biophysically characterized to determine the physical and mechanical properties. Interestingly, biohybridization of silver nanobactericides with cellulose films changed the mechanical properties of bacterial cellulose which resulted in the increase in the Young's modulus and tensile strength in comparison with control bacterial cellulose films (Table 3). The obtained results were in accordance with earlier findings which states the influence of nanomaterials on the physicochemical properties of bacterial cellulose (Moniri et al., 2017). The process of bio-hybridization resulted in nesting of silver nanobactericides onto bacterial cellulose forming a layer which might result in the change of mechanical properties. The bio-hybridization results in immobilization of nanobactericides thus offering advantage wherein nanobactericides are freely available onto the surface of bacterial cellulose thus increasing the rigidity and prevents from oxidation by providing chemical stability and it can offer better bactericidal properties (Liyaskina et al., 2017). The advantage of bio-hybridization in conferring the desired activity is well demonstrated in various studies. The SEM analysis of depicted the size of the silver nanobactericides ranged between 30 to 100 nm (Fig.6). The bio-hybridized bacterial films were excised into the small block which was subjected to bactericidal activity against multi-drug resistant pathogens. The activity was measured as the zone of inhibition across the bio-hybridized films (Fig.7). The obtained results were in accordance with well diffusion assay of silver nanobactericides which indicated that bio-hybridization process had no impact bactericidal activity. The exact mechanism of biohybridization is yet to be completely elucidated, but studies report that there might be electrostatic interaction between the silver nanobactericides and bacterial cellulose matrix. In the present investigation, the addition of silver nanobactericides onto the surface makes them to form a layer which can easily react and express significant activity. The obtained results are in accordance with studies conducted by Yang et al., 2012, where nano-silver was hybridized onto bacterial cellulose films which showed significant activity against *E.coli* and *S.aureus* . As similar to present investigation Kirby Bauer method was followed to evaluate the bactericidal activity which can be best suited for developing functional antimicrobial agents. The use of bacterial cellulose as ideal tool to develop bio-hybrid nano composite bearing antimicrobial properties is gaining tremendous importance in recent years (Liyaskina et al., 2017). The mode of external immobilization of silver nanobactericides onto bacterial cellulose can offer better results for instance desired size tunable nanomaterials can be synthesized and loaded onto the cellulose membrane. As antimicrobial activity of nanomaterials is influenced by size of the particles. It is reported that as the size decreases, the activity increases. Similar observation was carried out by study conducted

by Shao et al., 2016, wherein copper nanoparticles were loaded onto regenerated bacterial cellulose membranes and tested against range of bacterial pathogens viz *S. aureus* ATCC 6538, *B. subtilis* ATCC 9372, *C. albicans* CMCC(F) 98001, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. The study concluded that antibacterial activity was influenced by the presence of copper nanoparticles which can be of great potential for developing dressing materials. Hence in the present investigation, the bactericidal activity of bio-hybridized nano-bactericidal films are promising enough which can contributes towards the growing scientific knowledge to develop new leads of developing functional antimicrobial agents especially against ESKAPE. According to the scientific survey, increasing resistance among the ESKAPE group of pathogens is reported to cause severe threats to all forms of lives irrespective to their habitats which can lead to huge economical crisis. The situation becomes worst in developing countries which lacks sophisticated facilities and management system. Hence in the present investigation silver nanobactericides were synthesized and tested against members of ESKAPE pathogens. The tested pathogens were isolated from different pathogens suffering from severe microbial infection and tested for drug resistance according to standard protocols. The efficacy of nano-silver as antibacterial agents is well documented but scanty reports are available on its potential against pathogens which are bearing resistant to different classes of antibiotics. Hence the present study was carried out wherein the silver nanobactericides displayed activity against both Gram +ve and Gram -ve test pathogens. Thus, the results can offer great potential in developing novel and functional antimicrobial agents.

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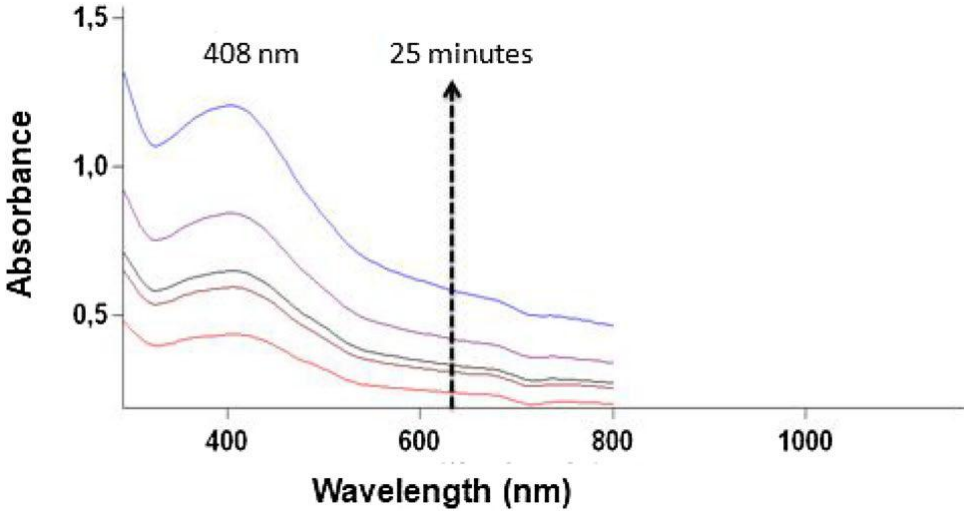


Fig. 1 UV–visible spectrum of silver nanobactericides

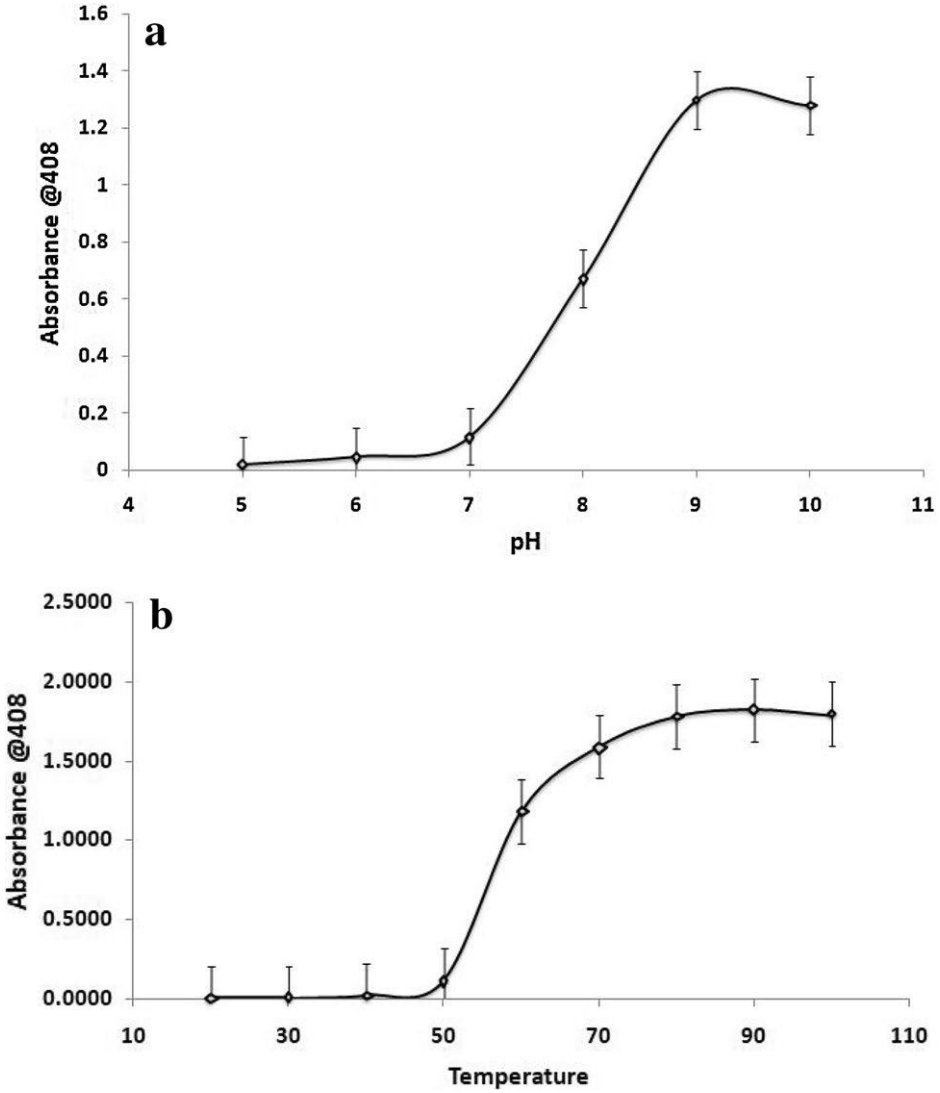


Fig. 2 **a** Influence of pH on synthesis of silver nanobactericides. **b** Influence of temperature on synthesis of silver nanobactericides

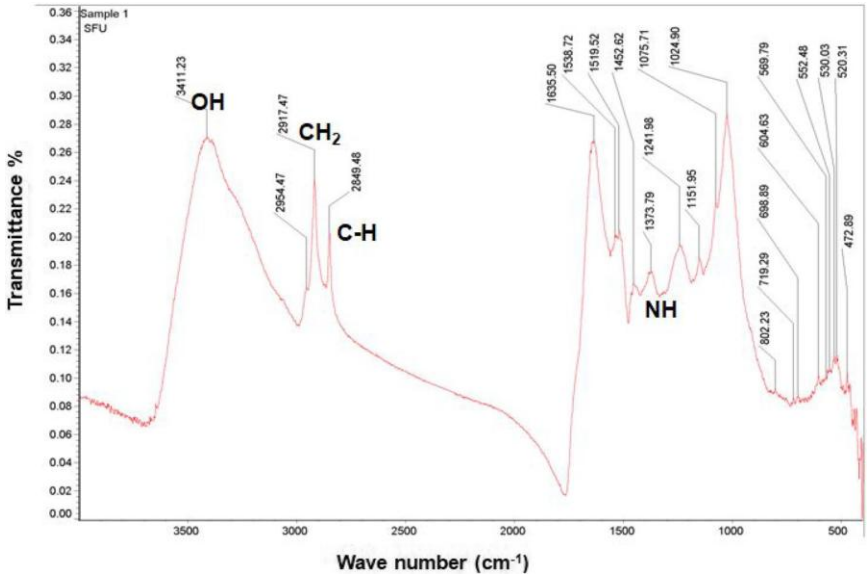
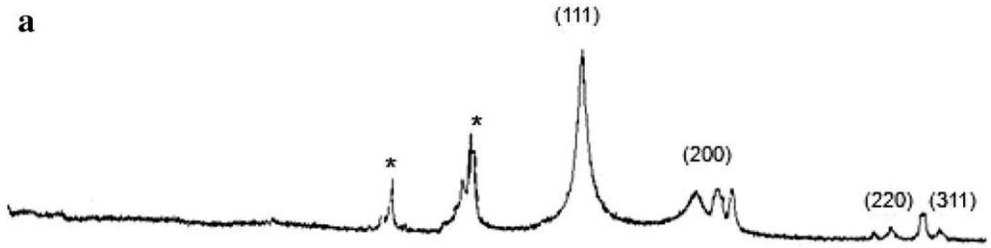


Fig. 3 FTIR analysis of silver nanobactericides



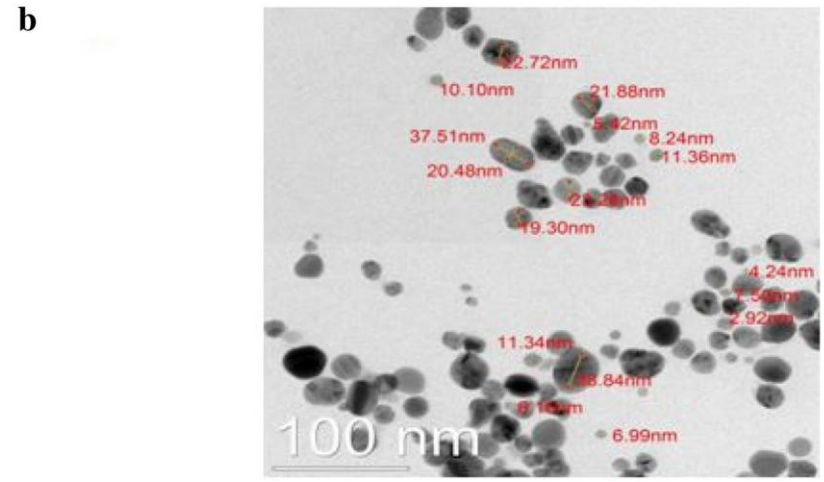


Fig. 4 **a** XRD analysis. **b** TEM analysis of silver nanobactericides

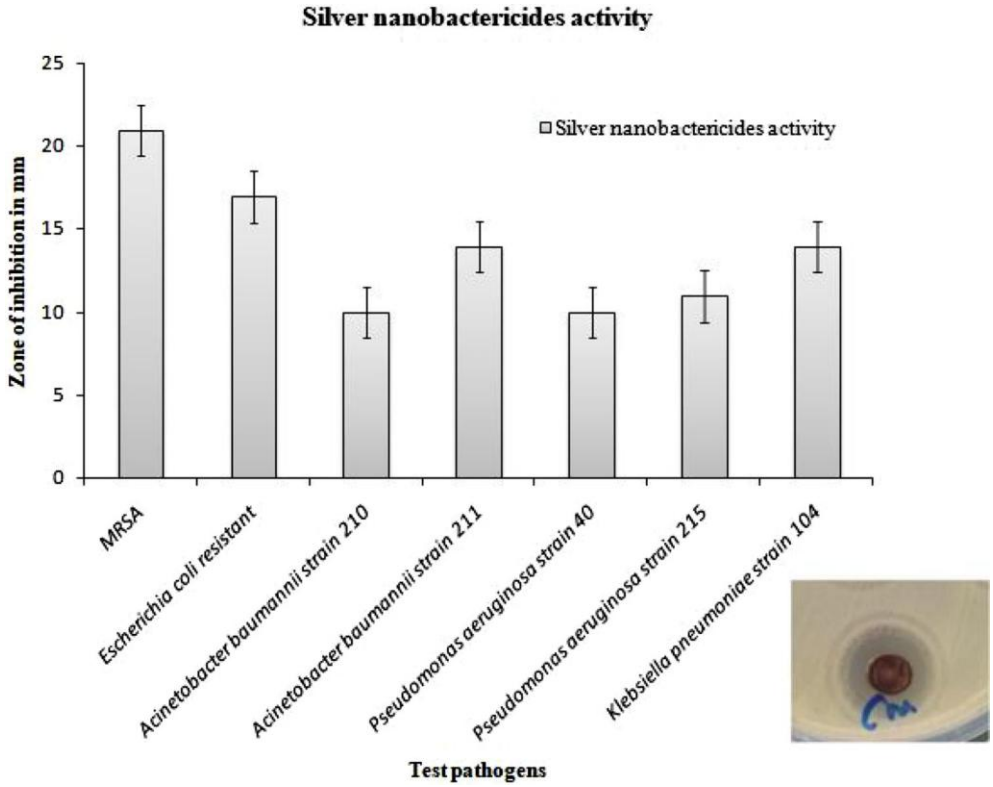


Fig. 5 Bactericidal activity of silver nanobactericides against multi-drug-resistant pathogens

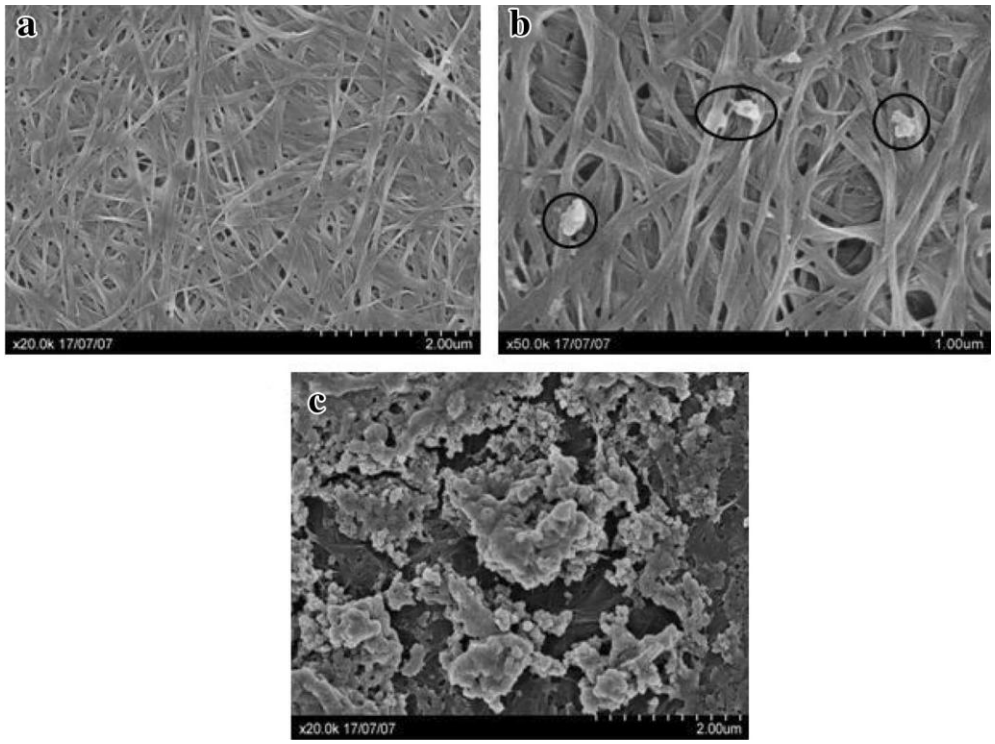


Fig. 6 **a** Control bacterial cellulose film. **b** Bio-hybridization of silver nanobactericides onto cellulose films. **c** Bio-hybridization and agglomeration of silver nanobactericides

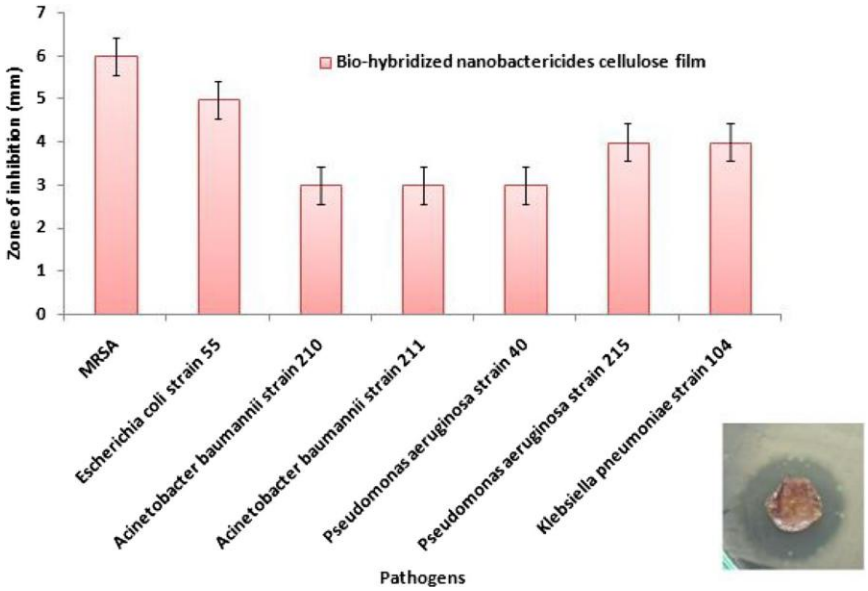


Fig. 7 Bactericidal activity of bio-hybridized nanobactericidal film against multi-drug-resistant pathogens