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ORIGINAL ARTICLE

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

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Abstract

The rapid expansion of drug-resistant pathogens has created huge global impact and development of novel antimicrobial leads is one of the top priority studies in the current scenario. The present study aims to develop bio-hybridized nanocellulose films which comprise of phytogenic silver nanobactericides. The nanobactericides were synthesized by treating 1 mM silver nitrate with aqueous extract of *Chamerion angustifolium* which reduced the metal salt to produce polydispersed nanobactericides which were tested against the members of ESKAPE drug-resistant communities. The synthesized silver nanobactericides were subjected to characterization with UV–visible spectra which displayed maximum absorbance at 408 nm. The bio-molecular interaction of phyto-constituents to mediate synthesis and stabilization of nanobactericides was studied with Fourier-transform infrared spectroscopy (FTIR) which depicted functional groups associated with nanobactericides. The crystalline nature was studied with X-ray diffraction (XRD) which showed Bragg's intensities at 2θ angle which denoted (111), (200), (220), and (311) planes. The morphological characteristics of silver nanobactericides were defined with transmission electron Microscopy (TEM) image which displayed polydispersity of silver nanobactericides with size ranging from 2 to 40 nm. The synthesized nanobactericides showed a significant activity against MRSA strain with 21 mm zone of inhibition. The minimal inhibitory concentration of silver nanobactericides to inhibit the growth of test pathogens was also determined which ranged between 0.625 and 1.25 µg/ml. The silver nanobactericides were bio-hybridized onto nanocellulose films produced by *Komagataeibacter xylinus* B-12068 culture strain. The films were dried to determine the mechanical properties which showed increased in Young's modulus and tensile strength in comparison with control bacterial cellulose films. Overall, the results obtained in the present investigation are promising enough to report bactericidal activity of bio-hybridized nanobactericidal films against ESKAPE. These communities are reported to cause severe threats to all forms of lives irrespective to their habitats which can lead to huge economical crisis.

Keywords ESKAPE · Bio-hybridization · Silver nanobactericides · Phytogenic · Bactericidal activity

Introduction

Bio-hybridization is a process of modulation of bioactive molecules with structural components (Gao and Maruyama 2012). The outcome of the hybridization results in enhancement or upgradation of desired activity (Ma et al. 2016). The exact definition of bio-hybridization is yet to be completely elucidated, but, however, there has been a significant progress to obtain multifold applications based on the principle of bio-hybridization (Syed et al. 2016). The concept of bio-hybridization is implemented in developing implants, regenerative medicines, bioreactors, biosensors,

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and development of functionalized films/composites (Vergara et al. 2012; Khan et al. 2015; Volova et al. 2018). The bio-hybridization not only enhances the applicative properties, it also influences on the physicochemical properties of the hybrid complex (Witte et al. 2012). The ideal bio-hybridized product in the biomedical application should be biocompatibility, sustainable and controlled release of drugs, longevity, and biodegradable and attenuate profound desired activity (Owens 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-resistant infections (Founou et al. 2016). Most of the available standard antibiotics are ineffective against wide range of drug-resistant microbial pathogens (Gupta and Birdi 2017). The impact of drug resistance has increased the mortality and morbidity rates which are expected to shoot up every year (Kim et al. 2015). The major contributors of drug resistance are grouped as ESKAPE members which include *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 sources of nosocomial infection in hospitals which are not only infecting patients; they are also posing adverse effects on working staff and visitors whose are susceptible to infection directly or indirectly and can act as carrier (Syed et al. 2017). The burden posed by these pathogens on healthcare system is reported to be large scale which is affecting global economic crisis by increasing the healthcare costs (Singer et al. 2016; WHO 2017a). To combat these pathogens, WHO has categorized antimicrobial drug resistance as one of the top priority research (WHO 2017b). Based on these facts, scientific groups are engaged in developing novel strategies to control the expansion of drug resistance (Rather et al. 2017). One such strategy includes 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; Du et al. 2018). Therefore, in the present investigation, *Chamerion angustifolium* was selected as subject of interest to synthesize silver nanobactericides. The selection of plant species was carried out based on the scientific and traditional knowledge *C. angustifolium* is reported to be one of the therapeutic plants and consumed as tea in various parts of Asia (Martin and Husband 2013; Pinno et al. 2014). These nanobactericides are particles at nanoscale which are more upgraded than its bulk counterpart (Syed et al. 2016). Extensive research on nanomaterials has demonstrated the activity of nanosilver against myriad pathogenic bacteria with multiple modes of actions (Zhang et al. 2016). Scientific literatures provide the evidence of nanosilver acting on the metabolism of pathogens which in turn paralysis its metabolic functioning (Kota et al. 2017). Based on these considerations, the present study was designed to develop bio-hybridization of silver nanobactericides onto bacterial cellulose film (BCF) and test their activity against clinically isolated multi-drug-resistant pathogens which are reported to bear resistance to different available standard antibiotics. These pathogens were isolated and identified from the individual suffering from myriad microbial infections according to the standard protocol described by Clinical and Laboratory Standards Institute (CLSI) guidelines to reveal their affiliation to the members of ESKAPE pathogens. To omit the adverse effects of the conventional mode of synthesizing nanobactericides, phyto-mediated synthesis process was implemented in the present investigation. The phyto-mediated route to synthesize nanobactericides is one of the facile and benign processes, 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 bio-hybridized onto bacterial cellulose films produced by *Komagataeibacter xylinus* B-12068 culture strain. The bacterial cellulose (BC) is a network of cellulose fibrils with less than 100 nm which possess unique properties for multiple applications (Volova et al. 2018). Scientific reports highlight the importance of BC owing 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). Lately, the use of bacterial cellulose has rapidly expanded in biomedical sector to develop implants, catheters, scaffolds to tissue engineering, functional nanopolymers, nanocomposite, etc. (Mai et al. 2017; Moniri et al. 2017). The biocompatibility of bacterial cellulose films is regarded as one of the best suited for biomedical applications to designed novel biomaterials such as artificial skin substitutes for treatment of wounds, ulcers, and burns (Volova et al. 2018). In severe situations of microbial infections, controlling the growth of pathogens becomes top priority and bacterial cellulose lacks antimicrobial potential (Hu and Hsieh 2015; Heli et al. 2016). Hence, hybridization of bacterial cellulose films with biomolecules to attenuate antimicrobial effects becomes prime important (Faria-Tischer et al. 2016; Tsai et al. 2017).

Materials and methods

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 1-l beaker containing 500 ml of sterile distilled water (Syed et al. 2017a). The mixture was boiled for 30 min to obtained aqueous extract which was stored at 4 °C until further use.

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.

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 (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.

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 °C which was followed by repeated washing with deionized water. Then, BCF was placed in 0.5% solution of hydrochloric acid for 24 h to neutralize which was later rinsed with double distilled water. Then, the films were stored until further use.

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 to achieve concentration 5 mg/ml and 30 ml of nanobactericides solution was pipette on the bacterial cellulose membranes until the complete membrane is immersed and incubated at 37 °C for 1 h in the water bath. Later, the films were washed to three times with sterile distilled water to remove unbound nanoparticles. The obtained film was dried and subjected to biophysical characterization. The BCF were excised into small blocks (5 × 5 mm) to study morphological characteristics using scanning electron microscopy of ultrahigh resolution S-5500 Hitachi 2009 model. 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 (UK). Samples 75 mm long, 12 mm wide were prepared for studying physical and mechanical properties of the films.

Multi-drug-resistant pathogens

The selected strains are reported to be multi-drug-resistant strains bearing resistant mechanism to nearly ten different antibiotics. The test pathogens are *Acinetobacter baumannii* strain 210, *A. baumannii* strain 211, *Pseudomonas aeruginosa* strain 215, *P. aeruginosa* strain 40, *Klebsiella pneumoniae* strain 104, *Methicillin-resistant Staphylococcus aureus*, and *Escherichia coli* strain 55. All the test pathogens were handled with prime care and preserved according to the standard guidelines and maintained at culture collection center of Krasnoyarsk Medical University.

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 °C. The overnight test bacterial suspensions were adjusted to 0.5 McFarland Standard with sterile Mueller–Hinton broth under aseptic conditions.

Antimicrobial activity of silver nanobactericides

The synthesized nanobactericides were centrifuged at 15,000 rpm for 20 min. 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 (1.5×10^6 CFU/ml) and swabbed uniformly, later 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 h. After incubation, the zone of inhibition was measured and interpreted with different antibiotics.

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 pipetting it out in the first row followed by addition of 50 µl of nutrient broth to all other wells. Furthermore, 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 (1.5×10^6 CFU/ml) was added to each well. The plate was incubated at 37° C for 18–24 h. 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).

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 (1.5×10^6 CFU/ml) which was swabbed uniformly. The prepared blocks of BCF were placed onto the swabbed media and incubated at 37 °C for 24 h. The activity was measured as the zone of inhibition across the block in millimeters.

Results and discussion

The results obtained in the present investigation highlight the bio-hybridization of nanobactericides with bacterial cellulose to attenuate antibacterial activity against selected drug-resistant pathogens. The results are promising enough and add scientific inputs towards the growing knowledge on development of drug-resistant pathogens. The synthesis of nanobactericides was achieved by treating 1 mM 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 is also reported to possess therapeutic activity against benign prostatic hyperplasia and prostate cancers (Rogers 2014). To the best of our knowledge, there have been scanty reports available on *Chamerion angustifolium* which prompted us to select it as a subject of interest in the present investigation. The aqueous extract obtained from *Chamerion angustifolium* was filtered and treated with 1 mM silver nitrate to synthesize nanobactericides. Initially, synthesis of silver nanobactericides was monitored with change in the color of reaction mixture to dark brown color. The synthesis was further confirmed by UV–visible spectrophotometry which displayed maximum absorbance at 408 nm (Fig. 1). The UV–visible spectroscopy is one the most reliable tools to confirm the formation of silver nanobactericides as per the scientific literature (Mahmudin et al. 2016). The maximum absorbance in the range of 100–800 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 studied to achieve rapid and maximum synthesis. In the present study, it was noted that synthesis was rapid and maximum at pH 9 and temperature 90 °C (Fig. 2a, b). The synthesis was completed within 25 min of incubation time which was recorded visually with increase in the intensity of reaction mixture in comparison to other range of pH and temperature. The influence of different variables on the synthesis of nanomaterials is well demonstrated and the results obtained in the present investigation are in accordance with earlier scientific reports (Qian et al. 2013). Unlike the conventional methods, the plant-mediated synthesis of nanomaterials can be attributed to the diverse classes of phyto-compounds which reduce the metal salt to produce nanobactericides (Baker et al. 2013). The bio-molecular interaction between the phyto-components and silver nanobactericides was studied with FTIR analysis which displayed vibrational stretches (Fig. 3) in the IR range corresponding to the functional groups associated with nanobactericides (Table 1). The scientific studies demonstrate the interaction of phyto-constitutes facilitates the bio-hybridization process. The obtained results coincide with earlier findings (Sumi

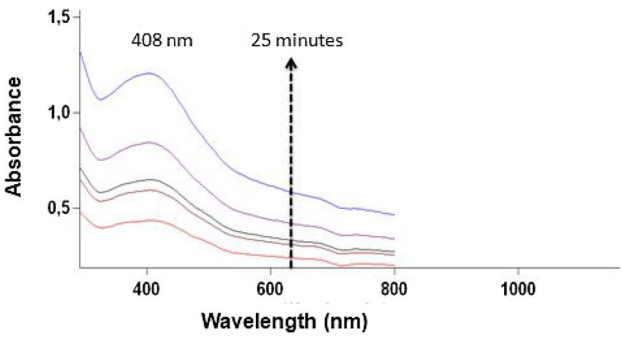

Fig. 1 UV–visible spectrum of silver nanobactericides

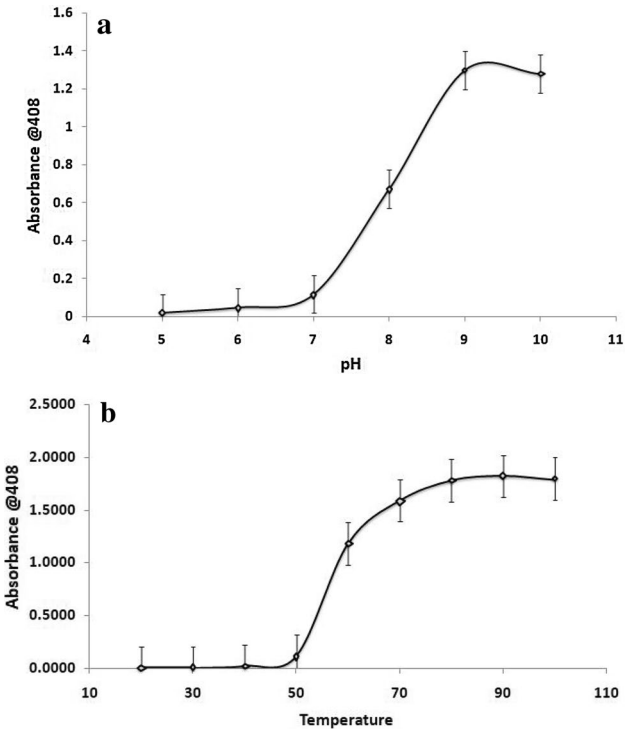

Fig. 2 **a** Influence of pH on synthesis of silver nanobactericides. **b** Influence of temperature on synthesis of silver nanobactericides

and Devadiga 2016; Yuanyuan et al. 2017). 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), (220), and (311) planes (Fig. 4a). The obtained results justify the findings of plant-mediated crystalline nanosilver (Jeevan et al. 2012). The morphological characteristics of silver nanobactericides were defined with TEM images which displayed polydispersity of silver nanobactericides with size ranging from 2 to 40 nm, as shown in Fig. 4b. These morphological characteristics were well defined and are in accordance with the 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 belonging to members of ESKAPE pathogens 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 Fig. 5. 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 resazurin 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 bio-hybrid cellulose films were characterized which revealed the mechanical properties of bacterial cellulose with increase in the Young's modulus and tensile strength in comparison

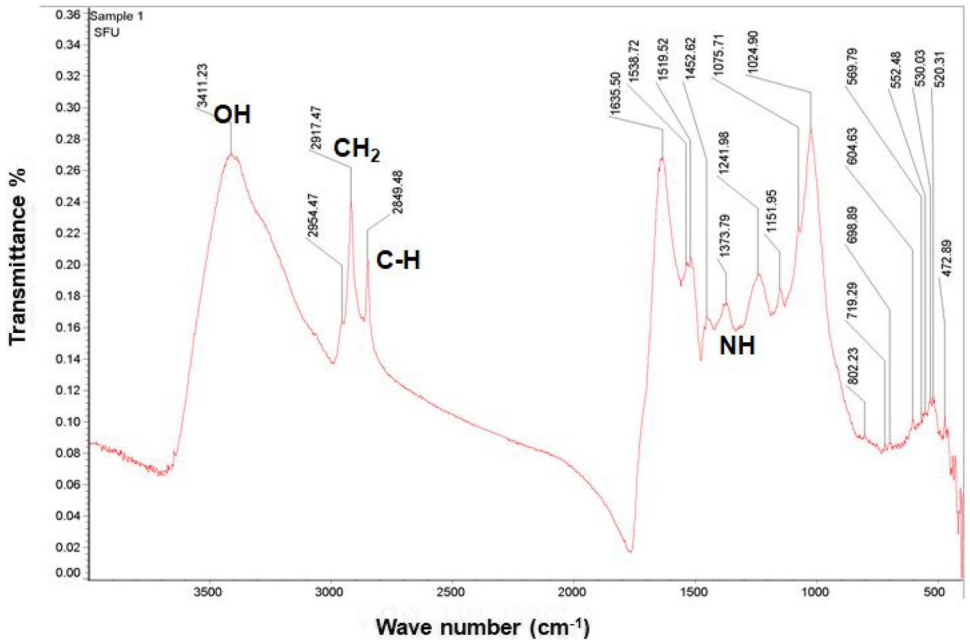

Fig. 3 FTIR analysis of silver nanobactericides

Table 1 FTIR peak position of all functional groups

FTIR	Functional Group	References
3411	OH	Barua et al. (2008)
2917	CH_2	Viana et al. (2012)
2849	C-H	Kaniappan and Latha (2011)
1635	H–O–H	Chandrasekaran et al. (2013)
1373	NH	Kanagathara et al. (2011)
1241	C=O	Todica et al. (2015)

with control bacterial cellulose films (Table 3). The obtained results were in accordance with earlier findings which state 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. In the present investigation, the phytocomponents might also associate with bio-hybridization process which plays an important role in applicative properties. This can be justified with the recent study conducted by Yuanyuan et al. 2017 which reported the nanocomplexes of epigallocatechin with 3-Mercapto-1-Hexanol and β-Lactoglobulin which improved the antitumor activity. The SEM analysis of depicted the size of the silver nanobactericides ranged between 30 and 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 bio-hybridization 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 nanosilver was hybridized onto bacterial cellulose films which showed a significant activity against *E. coli* and *S. aureus*. As similar to the present investigation, Kirby Bauer method was followed to evaluate the bactericidal activity which can be best suited for developing functional

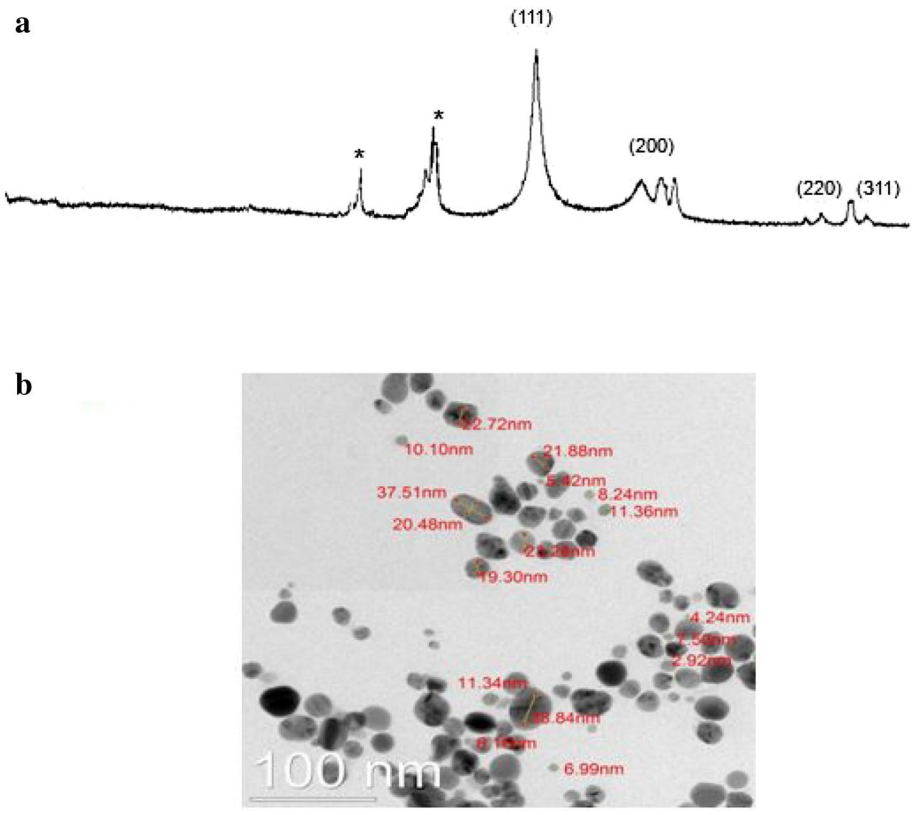

Fig. 4 **a** XRD analysis. **b** TEM analysis of silver nanobactericides

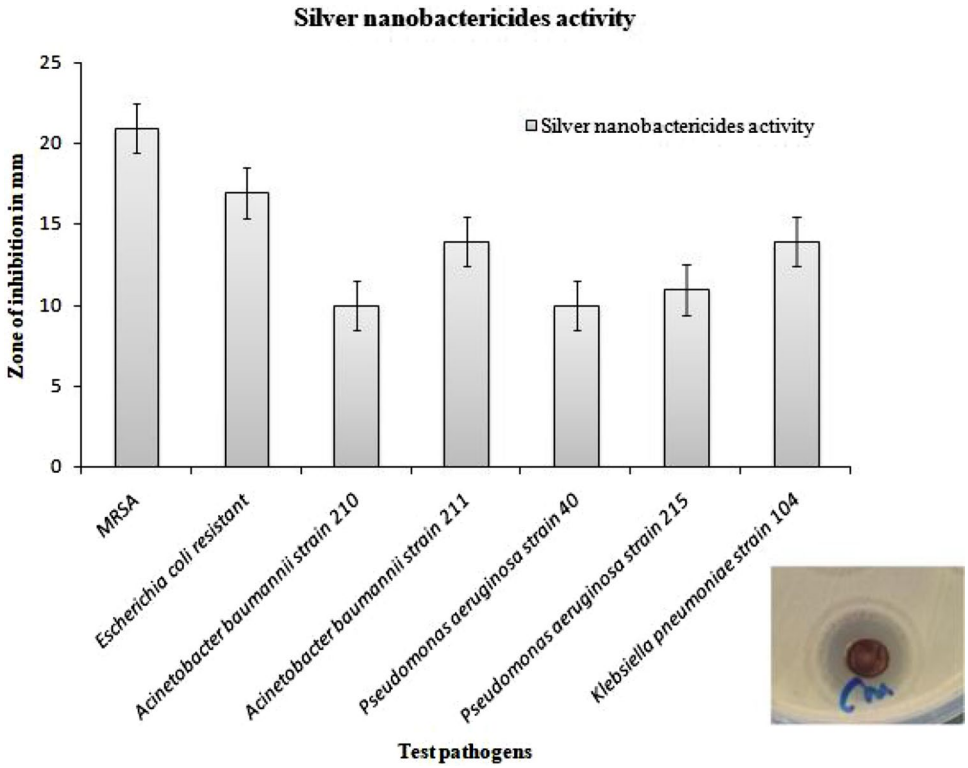

Fig. 5 Bactericidal activity of silver nanobactericides against multi-drug-resistant pathogens

Table 2 Minimal inhibitory concentration of nanobactericides against multi-drug-resistant pathogens

List of pathogens	Silver nanobactericides (µg/ml)
Acinetobacter baumannii strain 210	1.25
Acinetobacter baumannii strain 211	1.25
Escherichia coli strain 55	0.625
Klebsiella pneumoniae strain 104	0.625
Methicillin-resistant Staphylococcus aureus	0.625
Pseudomonas aeruginosa strain 40	1.25
Pseudomonas aeruginosa strain 215	1.25

antimicrobial agents. The use of bacterial cellulose as ideal tool to develop bio-hybrid nanocomposite 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. Furthermore, activity was assessed after 30 days of developing bio-hybrid films which showed that the films were stable and retained the bactericidal activity against the test pathogens (Suppl-1). The antimicrobial activity of nanomaterials is also reported to be 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 nanobactericidal films are promising enough which can contributes towards the growing scientific knowledge to develop new leads of

Table 3 Mechanical properties of bio-hybridized nanocellulose films

Sample	Thickness (µm)	The Uoung's modulus (MPa)	Tensile strength (MPa)	Elongation at break (%)
Bio-hybridized nanobactericidal cellulose films	20	5653.43 ± 152.57	105.17 ± 26.26	3.49 ± 0.36
Control bacterial cellulose	20	5605.34 ± 60.15	42.87 ± 4.88	1.06 ± 0.25

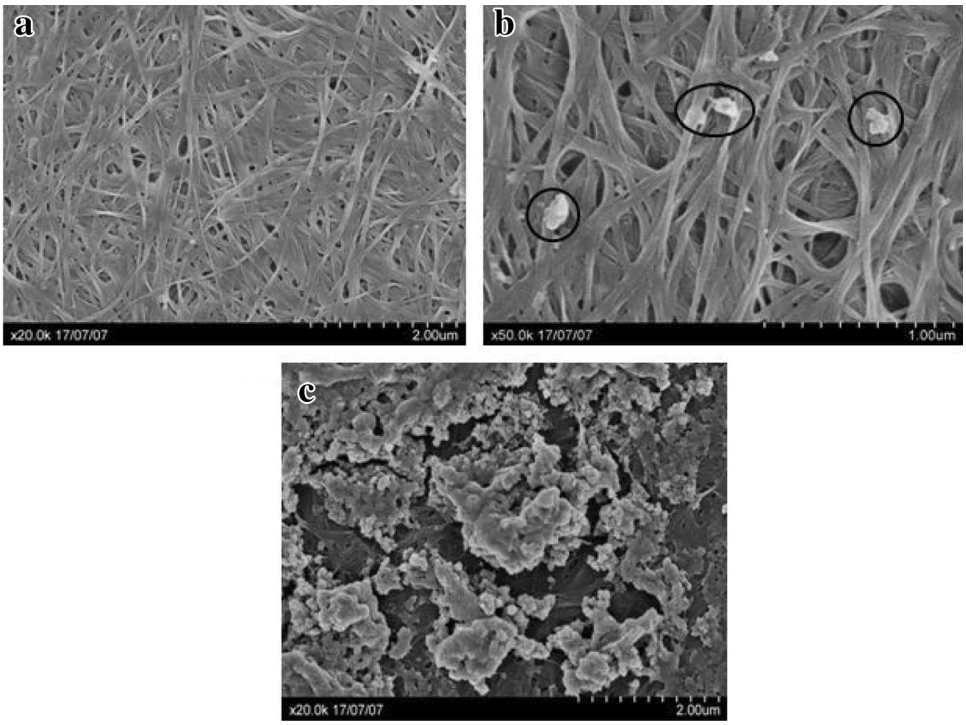

Fig. 6 **a** Control bacterial cellulose film. **b** Bio-hybridization of silver nanobactericides onto cellulose films. **c** Bio-hybridization and agglomeration of silver nanobactericides

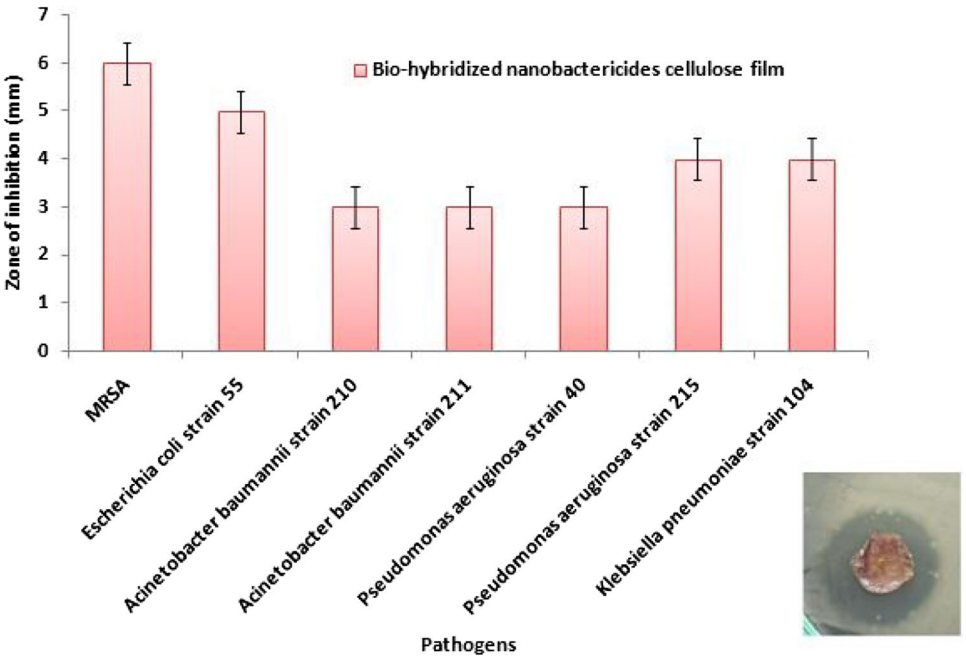

Fig. 7 Bactericidal activity of bio-hybridized nanobactericidal film against multi-drug-resistant pathogens

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 nanosilver 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.

Conclusion

The results of present study highlights the phytogenic-based synthesis of silver nanobactericides from *Chamerion angustifolium* which displayed a significant activity against ESKAPE pathogens The activity was further assessed by the development of bio-hybridized cellulose films. Thus, the study attributes towards the growing scientific evidence of nanomaterials as an alternative tool to combat drug-resistant pathogens. In the study, the initial attempt has been made to develop novel dressing materials in near future with profound activity.

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Compliance with ethical standards

Conflict of interest All authors state that they do not have any conflict of interest.

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