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Original article

Effective removal of biofilm formation in *Acinetobacter baumannii* using chitosan nanoparticles loaded plant essential oils

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ABSTRACT

The current study focused on anti-biofilm property of chitosan loaded essential oils, which were obtained and evaluated against biofilm forming *Acinetobacter baumannii*. The results of agar well punctured method demonstrated its efficiency at 150 µg/mL concentration. The complete biofilm arrangement was demolished at 150 µg/mL concentration as suggested by quantitative *invitro* inhibition assay measurement. The biofilm metabolic cells lost their virulence, which was confirmed by color changes of XTT assay. Furthermore, the biofilm metabolic product inactivation was proved by congo red agar assay with the evidence of complete exopolysaccharide arrest. The interior and exterior portions of the chitosan loaded essential oils were completely damaged, as suggested by confocal laser scanning electron microscope and scanning electron microscope observations. Hence, all the *invitro* inhibition assays, and morphological examination confirmed that the biofilm forming *Acinetobacter baumannii* lost its virulence factors and its bacterial growth was eradicated efficiently.

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1. Introduction

Worldwide, the most threatening bacteria is Gram negative, opportunistic nature of the *Acinetobacter baumannii* (Eze et al., 2021). It is a top threatening bacteria in healthcare delivery system and comes under highly critical list by World Health Organization

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(Al-Shamiri et al., 2021). In mostly caused nosocomial and urinary tract regions. In this virulence factors are the main factors for high occurrence of drug tolerance and produced the biofilm. *Acinetobacter baumannii* is resistant to almost all the existing classes of antibiotics, particularly beta lactum, tetracyclines, trimethoprim-sulfamethoxazole, fluoroquinolones (Elbehiry et al., 2021). The accumulation of biofilm forming *Acinetobacter baumannii* is 1000 times more resistant than normal pathogens and it completely developed the exopolysaccharide mediated slime layer (Gautam et al., 2021). This kind of consortium often helped to bacteria to prevent the other environmental stress, antibiotics and drugs. It can be spread easily in some related environments particularly, hospital atmosphere, catheter, foley catheter and cerebrospinal fluid (Mea et al., 2021).

Among the various constituents of plant materials, essential oils are heightened for biomedical application because of the more biological properties (Prakash et al., 2015; Khajenoori et al., 2009). The

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plant *Morinda citrifolia* is a tropical plant which is available mostly in South India, Australia and many parts of Southeast Asia (Purkayastha et al., 2012). It also simply called as “noni”, an important medicinal plant that proved as excellent phytochemical derivatives and various biomedical properties including antimicrobial, anti-viral, larvicidal and insecticide (Zhang et al., 2008). In industry, the products of *Morinda citrifolia* are an inevitable source to use in various processes. Till-date, the complete chemical properties, bioactive compounds, hormones and other biomedical properties are not driven clearly (Alves Veloso et al., 2020; Zhang et al., 2018). Till-date, the researchers are reported that the own metabolism of *Morinda citrifolia* was used for synthesis of essential oils, which is a complex molecules, arranged a clear organic structure. Previously, more researchers are reported that the *M. citrifolia* involved in the various biomedical properties, in particular essential oils shown excessive nature (Osorio et al., 2021; Silva et al., 2017). Previously, the chemical composition of *M. citrifolia* was purified as juice format in industry for eradicate the control of pathogenic microorganisms (Piaru et al., 2012; Jahurul et al., 2021).

Few studies of the essential oils with increased activity against phytopathogens, and more reports with biomedical properties including anti-cancer (Rajivgandhi et al., 2020), anti-oxidant (Suktham et al., 2021), anti-microbial (Sharmeen et al., 2020) and anti-viral properties. For these advantages, the current research was focused on South Indian medicinal plant of *Morinda citrifolia* essential oils for evaluated against biofilm producing bacteria. To improve the essential oils efficiency, the essential oils were loaded on natural polymer of chitosan, and then evaluated the biomedical activity against biofilm forming *Acinetobacter baumannii* to detect the essential oils efficiency.

Chitosan is a natural polymer which approved by Food and Drug Administration for use in industry, biopharmaceutical and biomedical process (Granata et al., 2021; Kanimozhi et al., 2018a,b). It highly covered by high units of glucosamine and connected with β (1–4) glycosidic bond. It is available more content in crustacean shells and somewhat in fungal cell wall (Pereira dos Santos et al., 2019; Zhang et al., 2020). In addition, the polymer nanoparticles have received a lot of attention for their use in biological applications. This is due to their exceptional electronic, thermal and optical properties along with their biocompatibility (Kanimozhi et al., 2018a,b; Nalini et al., 2019; Siddhardha et al., 2020). The chitosan structure was arranged with enormous amount of polysaccharides (Jamil et al., 2016). Compared with other carrier molecules, chitosan is acted as a excellent reservoir to deliver the drugs and antibiotics. It has highly non-toxic nature, increased adhesive property, flexible biocompatibility, biodegradability and various biological properties such as anti-oxidant, anti-cancer, antimicrobial, larvicidal (Bilal et al., 2020; Zhang et al., 2020; Keykhosravi et al., 2020). Recent years, all the fields including soil and agricultural, food and food additives, industrial process, biopharmaceutical and biomedical properties (Mondéjar-López et al., 2022; Kalagatur et al., 2018). In addition, the incorporation of plant extract, secondary metabolites, essential oils, drugs and other existing antibiotics are delivered in target points and also increasing efficiency level (Ebrahimzadeh et al., 2021; Rajivgandhi et al., 2021; Barzegar et al., 2021). Previously, more researchers are reported with chitosan loaded essential oils have improved biomedical properties and chitosan as an excellent carrier molecule (Kavaz et al., 2019; Kanimozhi et al., 2019; Kayalvizhi et al., 2022). The targeting factors are also very clear and effective against any pathogens using chitosan loaded essential oils (Ashrafi et al., 2019). Therefore, the current study was highly focused on chitosan nanoparticles loaded essential oils which was obtained from School of Life Sciences, Sun Yat-Sen

University, China for evaluated against biofilm producing *A. baumannii*.

2. Materials and methods

2.1. Collection of nanomaterial and pathogens

Initially, the chitosan loaded essential oils nanomaterial was obtained from Department of Ecology and Evolution, Sun Yat-Sen University, Gunagzhou, China for removal of biofilm producing gram negative bacteria. In addition, all the chemicals of this work were purchased from Suresh Scientific @ Co, Tiruchirappalli, Tamil Nadu, India who is the local supplier for Hi-Media, Mumbai, India. All the media and solutions, reagents were purchased from Ponmani @ Co, Tiruchirappalli, Tamil Nadu, India who is the local supplier for Merck, Mumbai, India.

2.2. Agar well puncture method for eradication of biofilm

The initial confirmation effect of essential oils loaded chitosan nanoparticle was evaluated against biofilm forming bacteria using agar well punctured method (Ma et al., 2020). The 48 h mate formed culture was swabbed on already prepared nutrient agar plates after solidification of 30 min. Then, the chitosan loaded essential oils were added various concentration 25–150 $\mu\text{g}/\text{mL}$ into the wells and gently shake for equally filled in all places. Next, all the plates were maintained in 37 °C setup incubator 1 day. After one day, the result was compared based on the positive and internal controls result and noted in mm of diameter.

2.3. Quantitative detection of biofilm eradication by 24-well polystyrene method

In adherent cells or attached cells of the biofilm forming *Acinetobacter baumannii* culture was grown in 24-well polystyrene plate with the presence of tryptic soy broth. Consecutively, 25–200 $\mu\text{g}/\text{mL}$ concentrations of chitosan loaded plant essential oils with diluted safely or gently by shaking method. This experiment was completely followed by Maruthupandy et al. (2020). Briefly, 24-well polystyrene plate was used to attached the biofilm cells on the wall was very effective for biofilm producing *Acinetobacter baumannii*. Based on the above statement, the *Acinetobacter baumannii* was used in this study to detect the eradication of biofilm formation or not using microtitre plate reader. After one day, the plates were shown highly turbidity with no any culture deposition or high deposition with no turbidity was seen on naked eye. In this experiment, the turbidity based detection of biofilm degradation was indicated that the chitosan loaded essential oils treated the sample. So, the turbidity range, inhibition concentration range and mate differentiation were quantitatively analyzed with the help of 4% crystal violet. Here, stained by 4% crystal violet solution was used for absorption of crushed biofilm places and discarded the crystal violet after 10 min. After treatment, first washed by double distilled water and second washed by PBS. Finally, 2 mL of ethanol solution was added into the wells for degradation of crystal violet solution after consecutive universal wash. Finally read the solution with microtitre plate for attached cells density and then calculated based on the control well and treated wells.

2.4. Metabolic activity inactivation of chitosan loaded essential oils

In live and dead conditions of biofilm forming *Acinetobacter baumannii* was monitored after treatment with chitosan loaded essential oils followed by the previously evidenced protocol of Govindan et al. (2020). After treatment in 24-well plate, the sample

was transferred to 15 mL falcon tube and centrifuged by 2500 rpm for 15 min and then separated supernatant and pellet in falcon tube. After the solution of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5- carboxanilide (XTT) was added each two ml in control and test samples. In lag phase, log (exponential phase), the biofilm producing bacteria was produced the different virulence factors vigorously. In the treatment of chitosan nanoparticles loaded essential oils, all the virulence factors were produced irregular role means of inactivation. Then, after 1 h time duration, the menadione acetone solution was added into the XTT plus chitosan nanoparticles treated biofilm pathogen containing mixture sample. Also added in untreated control samples also. Both the treated and untreated samples tubes were maintained 1 h and allowed to check the color production formed or not. The color changes were also confirmed by transferring color varied tubes by naked eye. Next, the color changes of the treated and absence of color in the untreated tubes were calculated using UV-spectrometer at 550 nm O.D of wavelength. Last, the O.D values were noted and calculated based on the formula for control and treated results.

2.5. Validation of XTT assay using agar plates assay

All the concentration of the XTT assay was evidently confirmed by agar streaking method using muller hinton agar and congo red agar assays. In both the plates, the control, half and complete inhibition concentrations were streaked and stored at 37 °C for 12 h. After, the color variations were indicated the nanomaterial efficiency (Jamal et al., 2021).

2.6. Internal biofilm factors modification

Increased efficiency of chitosan loaded essential oils against inside of the bacterial parts was evidently proved by fluorescence microscopic analysis using fluorescence dyes of AO. In this view, the bacterial culture was inoculated into the 24-well plate holding cysteine lactose electrolyte deficient broth and soak glass cover slip for adhere the biofilm on glass cover slip. Taken out the cover slip after 24 h, then initially washed by distilled water and followed by PBS. Back side of the cover slip was clearly washed until the adherent or attached cells removal and use front side of the cover slip. After successful washing, the slide was taken out and dried 10 min and followed by addition of AO stain to bind into the crushed parts of the intracellular bacterial body. Then, viewed by confocal laser scanning electron microscope at 40x magnification lens for clear image of the cells. The entire protocol was modified from previous protocol of Reichhardt et al. (2019).

2.7. External structure modification

The external structure and cell wall of the biofilm forming *Acinetobacter baumannii* by the influence of chitosan loaded plant essential oils. Initially, the mate formed *Acinetobacter baumannii* culture was grown in test tube with minimum inhibition concentration of chitosan loaded nanoparticles and allowed to grown together at 37 °C for 1 day. Then, taken out from tube and centrifuged at 5000 rpm 10 min for obtained pellet. Then, the pellet was carefully taken after PBS washing and made smear on cover slip. Then, 4% formaldehyde solution was gradually added on the smear and allowed to fix the sample with 4 h time duration. Then washed the fixation using prepared PBS and vacuum filtered by 0.1 mm polycarbonate membrane filter. Continuously done the dehydration process of filtered sample by 10–100% of ethanol (Namasivayam and Allen Roy, 2013). After successive time interval, the sample was dried properly and coated using gold palladium metal and directly viewed by scanning electron microscopy. The

complete process of chitosan loaded essential oils against biofilm producing bacteria (Fig. 1).

3. Result

3.1. Effect of chitosan loaded essential oils

In this study, the chitosan loaded essential oils were obtained from Department of Microbiology, Sun Yat-Sen University, China. The synthesis and characterization was already reported in our previously published article (Rajivgandhi et al., 2020). All the spectroscopic result of FT-IR, XRD and morphological evidences of SEM and TEM results were confirmed the preparation of essential oils successfully loaded into the chitosan molecules. In addition, the synthesized nanomaterial was performed against A549 lung cancer cells. In result, it was inhibited the A549 lung cancer cells very effectively and this evidences were proved by various invitro experiments, notably cell cycle arrest using flow cytometer analysis.

3.2. Agar well punctured method

Different zones of the chitosan nanoparticles loaded essential oils wells were conveyed that the biofilm bacteria was more sensitive to nanoparticles. Also, the result was suggested that the obtained chitosan nanoparticles loaded essential oils was not only effective against cancer cells, it also very efficient against biofilm producing bacteria (Rahman et al., 2017). This result was also interpreted with previously report, the selected chitosan nanoparticles loaded essential oils was concentration dependent inhibitor.

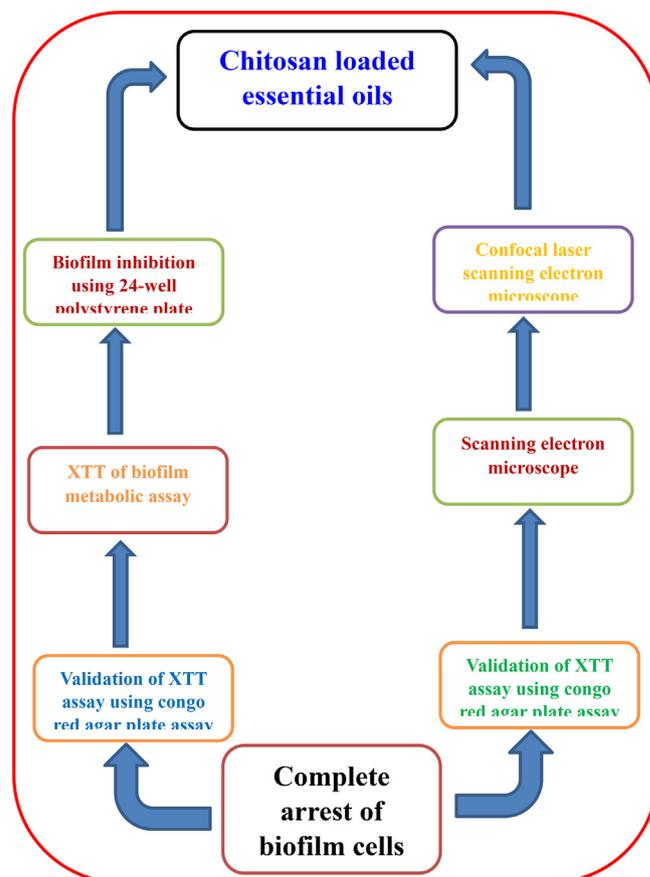


Fig. 1. Complete process of chitosan loaded essential oils against biofilm producing bacteria.

In this agar well punctured method, the 20 mm zones against 75 $\mu\text{g}/\text{mL}$ concentration (Fig. 2a), 26 mm zones against 100 $\mu\text{g}/\text{mL}$ concentration (Fig. 2b) and 30 mm zones against 150 $\mu\text{g}/\text{mL}$ concentration (Fig. 2c) were screened. Previously more researchers were reported that the *Morinda citrifolia* essential oils were very effective against biofilm bacteria due to the presence of rich bioactive compounds (Marisa Halim et al., 2017; Kovendan et al., 2014; Abou Assi et al., 2017; Sharmeen Jugreet and Fawzi Mahomoodally, 2021). The similar statement was reported by Pongnaravane et al. (2006), and chitosan loaded plant essential oils have improved bioactivity than other plant derivatives (Rajivgandhi et al., 2021). The inactivation of biofilm production due to the alter the virulence factors by plant essential oils loaded chitosan material as strong nanomaterials in biomedical activities.

3.3. Quantitative detection of biofilm eradication by 24-well polystyrene method

The primary biofilm eradication with increasing concentration process was evidently proved by quantitative confirmation using O.D value of microtitre plate results. When seen on the treated culture of the wells were not shown any mate formation compared to control. All the wells were shown clear turbidity and non-attached cells were shown clearly because of the chitosan nanoparticle loaded essential oils influence. In increasing concentration, the turbidity level was high and bacterial growth was low which is confirmed by microtitre plate reading result. As same as, the concentration of 150 $\mu\text{g}/\text{mL}$ was indicated 96% of inhibition against *Acinetobacter baumannii* culture wells. In 75 $\mu\text{g}/\text{mL}$ concentration, the inhibition percentage range was 54% and 34% of inhibition in 50 $\mu\text{g}/\text{mL}$ concentration. Among the calculated values, the lowest concentration of 150 $\mu\text{g}/\text{mL}$ with high turbidity based inhibition was fixed to further study (Fig. 3a, b). All the result was evidently supported the agar well punctured method and also similar to the statement of Rahman et al. (2017) with increasing concentration of the nanomaterial inhibited the biofilm growth highly. Usually, the chitosan loaded essential oils were inhibited the bacterial growth

completely due to the invasion drug efficiency (Ma et al., 2020a, b). In this result was suggested that the essential oils efficiency was heightened by chitosan and it deactivate the bacterial virulence factors (Bala Subramaniyan et al., 2021). Bacterial virulence was altered, bacteria lost their internal communication system and production of internal regulating factors failure (Luna et al., 2022). Therefore, the result of chitosan loaded essential oils were clearly similar to previously reported material in the process of anti-bacterial activity (Rahman et al., 2017). In addition, the obtained chitosan nanoparticles loaded essential oils have increased efficiency against cancer cells and bacterial cells very effectively.

3.4. Live/dead activity of biofilm producing *Acinetobacter baumannii*

Result of minimum biofilm inhibition concentration experiment was used to help the metabolic activity of biofilm forming *Acinetobacter baumannii*. In this experiment, the same 25–150 $\mu\text{g}/\text{mL}$ concentration was used to analyze the live condition of bacteria and their biofilm formation. Evidently, after addition of menadione acetone solution, the color was changed after incubation and confirmed the deactivation ability against virulence factors. As same as, the result was more similar to minimum biofilm inhibition concentration experiment and shown effectively inhibited the bacteria in increased concentration. In 75 $\mu\text{g}/\text{mL}$ concentration, almost all the bacteria was enter to decline phase due to the continuous arrest of internal formazan production. Also, the inhibition percentage was 60%, it was very high compared with previous reports of biofilm inhibition by plant essential oils alone (Zhang et al., 2018). In 150 $\mu\text{g}/\text{mL}$. In addition, the dose-dependent inhibition of chitosan loaded essential oils were suggested that the inhibitions were depends on the nature of the chitosan. The inhibition percentage was 99%, and absence of bacterial activity was observed (Fig. 4). Chitosan may influence the essential oils efficiency and delivered the essential oils in proper manner. This statement was agreed by Reichhardt and Parsek (2019), and chitosan is an excellent biopolymer which used as a carrier to various molecules particularly for essential oils. Similar evidences has been observed by Bala Subramaniyan et al. (2021) and reported that the chitosan nanoparticle loaded essential oils have high efficiency in decrease the pathogenicity in bacteria. Therefore, the XTT assay result was suggested that the obtained chitosan nanoparticle mediated essential oils have virulence factors mediated biofilm eradication an it can be confirmed by streaking on congo red agar assay.

3.5. Validation of metabolic activity assay

In congo red agar plates, the chitosan nanoparticle loaded essential oils treated XTT result was streaked for detection of virulence factors inactivated or not. As same as, the congo red agar plate result was agreed to XTT result due to the color changes. In 150 $\mu\text{g}/\text{mL}$ concentration, the bacterial growth was completely arrested (Fig. 5b). When seen on the untreated control tube, the clear growth was obtained (Fig. 5c). Both the results were further rechecked in microtitre plate with effective biofilm inhibition (Fig. 5a). Further, the biofilm formation was arrested or not by XTT using chitosan nanoparticle loaded essential oils were restreaked on the congo red agar plates and confirmed that the obtained nanomaterial were very effective against biofilm formation of *Acinetobacter baumannii*. Based on the color differences, control wells with XTT solution was shown bright black color appearance whereas no nay color formation of 150 $\mu\text{g}/\text{mL}$ and slightly pink color formation in 75 $\mu\text{g}/\text{mL}$ were observed. Based on the color variations, the black color formation of control indicates, the exopolysachharide, amino acid, proteins were continuously produced to *Acinetobacter baumannii* and able to produce

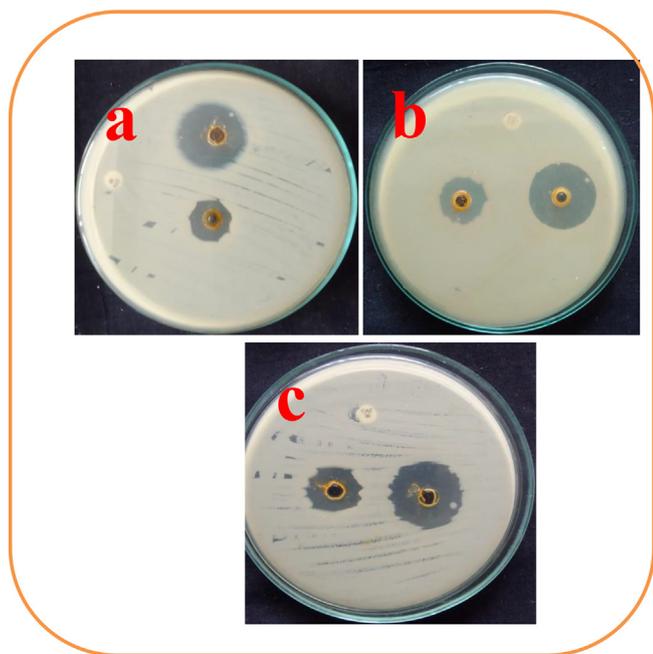


Fig. 2. Agar well punctured method for detection of chitosan loaded essential oils against biofilm forming *Acinetobacter Baumannii* at concentrations of 75 $\mu\text{g}/\text{mL}$ (a), 100 $\mu\text{g}/\text{mL}$ (b) and 150 $\mu\text{g}/\text{mL}$ (c).

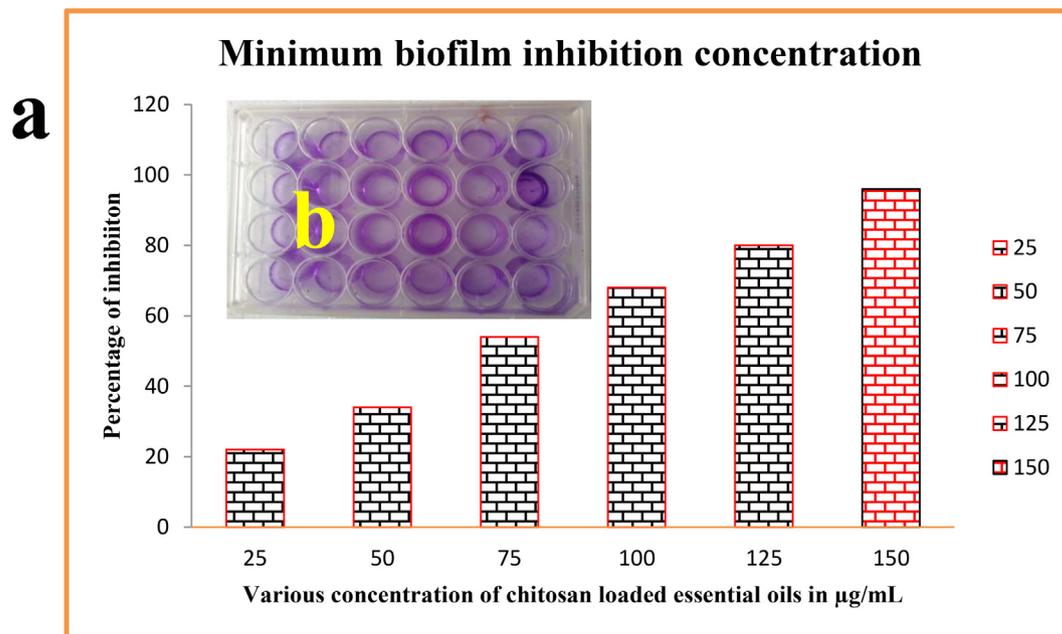


Fig. 3. Quantitative measurement of biofilm inhibition concentration against *Acinetobacter Baumannii* using chitosan loaded essential oils.

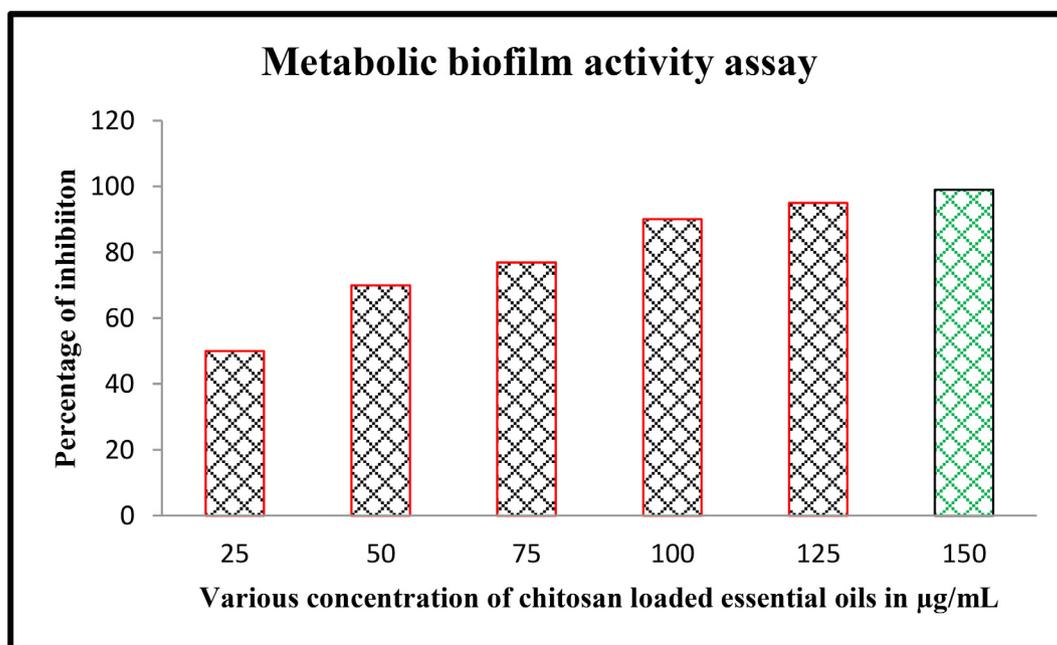


Fig. 4. Confirmation of metabolic activity inhibition measurement against *Acinetobacter Baumannii* using various concentration of chitosan loaded essential oils.

strong biofilm. Contrary result of no any color formation in the 150 µg/mL concentration plate was clearly indicated that the bacteria *Acinetobacter baumannii* lost their pathogenicity and also antigenicity. The plate was shown with no any color and no any bacterial growth was observed, this evident was conveyed the important information that the *Acinetobacter bamannii* was completely lost their structure. Surprisingly, the 75 µg/mL concentration treated *Acinetobacter baumannii* was shown pink color in congo red agar plate. This evident was proved that the bacteria lost their pathogenicity and remain hold their antigenicity. Therefore, all the three results were clearly suggested that the chitosan nanoparticle loaded essential oils not only contributed in biofilm

inhibition in *Acinetobacter baumannii*, it also destroy the bacteria also.

3.6. Internal biofilm factors modification

The internal structure of the biofilm arrangement was demolished by chitosan loaded essential oils were clearly seen in the picture of Fig. 6b. In result, the original rod shape structure of tightly closed chain formation was observed for control with developed internal materials (Fig. 6a). After treatment, the internal virulence factors, protein, amino acids, exopolysachharides, other responsible genes were stopped due to the complete essential oils delivery of chitosan was observed (Fig. 6b). After addition of AO dye, the

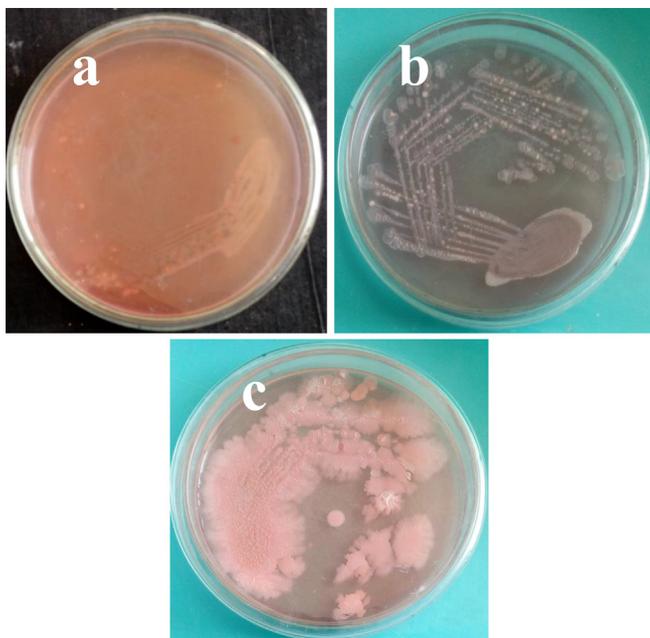


Fig. 5. Validation of biofilm metabolic activity using muller hinton agar plate for growth observation (a, b) and biofilm growth of control (c), pathogenicity of biofilm inhibition (d) and complete biofilm bacterial inhibition (e) by congo red agar plate assay.

damaged membrane was easily shown in fluorescence microscope with the help of glittering the damaged rod shaped colonies. After treatment with nanomaterial, the internal membrane layers were shown with confluent, and loosely arranged colonies were observed. Whereas, the dye was not shown with high intensity

level in untreated cells (Fig. 6a). All the arrangement of the biofilm was demolished and undergone to decline phase due to the depletion of virulence factors effect was conveyed that the chitosan loaded essential oils were very effective against biofilm forming *Acinetobacter baumannii*. Also, the 3D image of treated bacterial cells was clearly shown in Fig. 6c.

3.7. External morphological modification

Fig. 7b of chitosan loaded essential oils treated *Acinetobacter baumannii* was shown irregular shape, smooth surface with collapsed nucleolus. Contrary, the strong formation of punch of colonies with rod shaped colonies was observed in control image (Fig. 7a). Both the results were interpreted each other, and suggested that the original structure, size and shape of the bacterial morphology was affected clearly due to the effect of chitosan loaded essential oils and confirmed by SEM result of Fig. 7b. The intensity was low and exopolysaccharide production was stopped and hank like structure was absence. Also, the increased membrane polarization and fluid discharge was started on the periplasmic space and cell sheath. In bacteria, the polarization was very high and permeability was very easy in the treated cells. Entirely contrast result of control cells image was suggested that the present result was very useful and confirmation for external damages (Muthuchamy et al., 2020).

4. Conclusion

Based on the observed result, the chitosan was more efficient carrier to deliver the essential oils against biofilm producing bacteria. The essential oils efficiency was more compared with without loading chitosan and proved by quantitative biofilm inhibition and metabolic activity assay. Both the assays were more supported to agar well punctured result and proved that the chitosan loaded

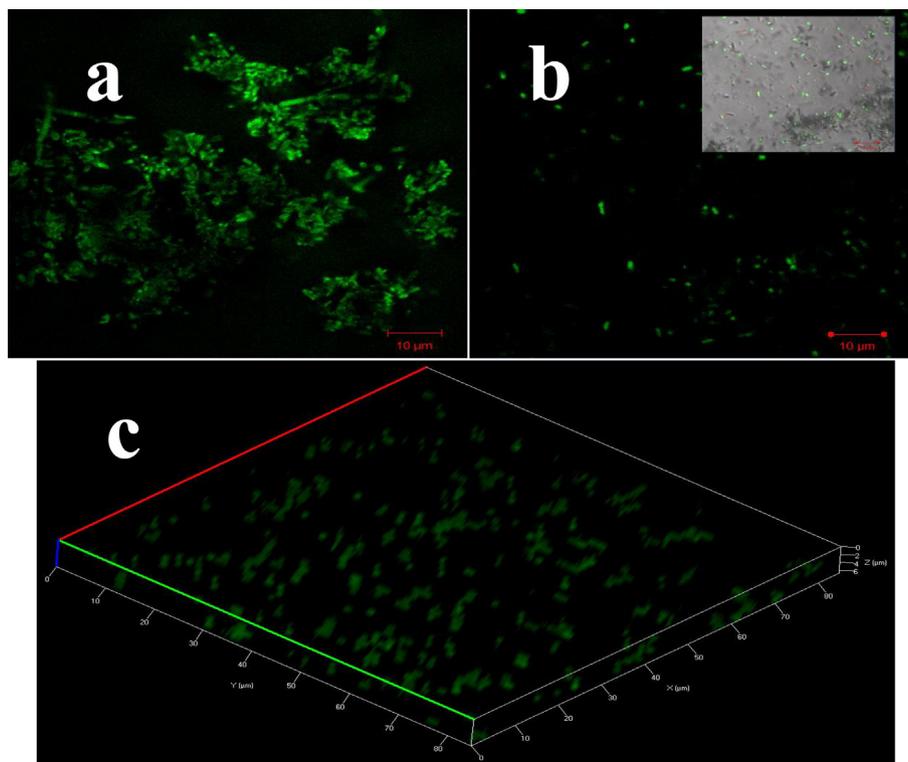


Fig. 6. Internal damages of biofilm forming cell and their parts of the *Acinetobacter baumannii* by confocal laser scanning electron microscope. The untreated control sample shown clear shape (a), treated sample shown damaged shape and arrangement (b) and 3D image of damaged cells (c).

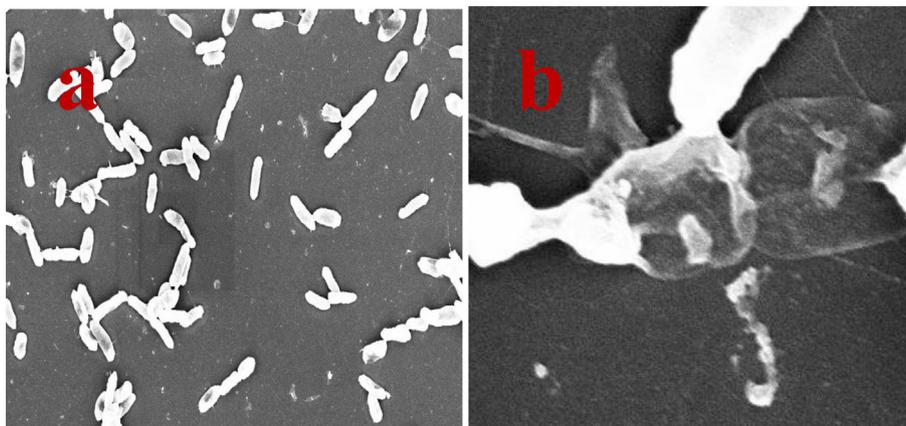


Fig. 7. External cell wall and surrounding layers cleavage by scanning electron microscope and result of clear size and rod shape morphology (a) and unclear rod shape and surface layers damaged (b) cells of *Acinetobacter baumannii*.

essential oils were inhibited the biofilm at concentrated dependent manner. The black, pink and no color formation of control, half treatment and complete treatment of the congo red agar plates were evidently proved that the *Acinetobacter baumannii* lost their virulence factors and more sensitive to tested nanomaterial. Both the intracellular and extracellular surface structures were also supported to the chitosan loaded nanomaterial not only inhibits the cancer cells, it also highly effective against biofilm forming *Acinetobacter baumannii*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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