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Original article

Chitosan loaded plant essential oils efficiently eradicate the multi-drug resistant bacterial infection and lung cancer cells

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ABSTRACT

In present research, of chitosan loaded plant essential oils (CsEOs) were performed against *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E.coli*), *K. pneumoniae* and *Staphylococci aureus* (*S. aureus*), also the anti-cancer properties were evaluated against A549 lung cancer cells. Consequently, the CsEOs against *P. aeruginosa, E.coli, K. pneumoniae* and *Staphylococci aureus* were shown 24 mm, 22 mm, and 26 mm and 22 mm zones of inhibition respectively. In addition, 500 µg/mL of CsEOs exhibited 92, 89, 88 and 94% of inhibition against *P. aeruginosa*, *E.coli*, *K. pneumoniae* and *S. aureus* were observed respectively using micro broth dilution broth experiment. Further, cytotoxicity ability of CsEOs against A549 human lung cancer cells were monitored by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, and result of 500 µg/mL concentration was exhibited 58% inhibition against A549 lung cancer cells. Furthermore, the microscopic evidence of 500 µg/mL concentrations of CsEOs treated A549 lung cancer cells was showed irregular morphology in phase contrast microscope. Altogether, both the invitro and morphological experiments of anti-bacterial and anti-cancer study results were conveyed that the CsEOs have excellent anti-bacterial and anti-cancer properties. Finally, the entire study was proposed, the chosen CsEOs was excellent bionanomaterial used to deliver the drugs and bioactive compounds in target sites for inhibit the infectious pathogens and cancer cells.

1. Introduction

Chitin and chitosan are polysaccharides composed of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN). Chitin is one of the most abundant biopolymer which is highly stable in nature (Mourya and Inamdar, 2008; Kanimozhi et al., 2018). Chitosan is a derivative formed by deacetylation of chitin. Initially in chitosan, membrane studies were done because of their properties (Candy and Sharma, 1990). Essential oils (EOs) from plants have been used in folk medicines. EOs primarily consist of terpenoids like monoterpenes, diterpenes, sesquiterpenes and other molecules aldehydes, acids, lactones, sulphur-containing compounds and homologues of phenylpropanoids (Nalini et al., 2019;

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Kanimozhi et al., 2019). Essential oils can inhibit or reduce the growth of bacteria, moulds and yeast. In addition, it also neutralises the toxic bacterial metabolites (Bakkali et al., 2008; Nazzaro et al., 2013). Essential oils components are convertible by isomerization, oxidation, and dehydrogenation reaction induced chemically or by enzymatically (Kanimozhi et al., 2018). Stability of the essential oils is determined by the presence of several chemicals and edaphic factors. In lemon oils, the amount of geranial, terpinolene will rise in p-cymene is observed (Turek and Stintzing, 2013; Kanimozhi et al., 2019). *Satureja montana* essential oils exhibited antimicrobial activity against all gram positive and gram negative microbes and fifty-eight clinical oral *Candida* sp. (Chouhan, Sharma and Guleria, 2017; Kayalvizhi et al., 2022). According to the available data and most of the studies represents that the gram positive bacteria shows easily destroyed bacteria in essential oils treatment (Seow et al., 2014; Wińska et al., 2019). Combination of chitosan and essential oils may provide exponential properties for antimicrobial activity with specificity. Recent report of Zhang et al., 2020 reported with excellent anti-bacterial activity of plant essential oils against some gram negative bacteria (Zhang et al., 2020). The two step nanoencapsulaiton process for preparation of essential oils loaded chitosan nanoparticles are oil-in water emulsification and ionic gelation method (Narmatha Christy et al., 2020; Bus et al., 2020). The synergetic effects of chitosan and essential oils have activity against *S. aureus, S. epidermidis, E. coli and P. aeruginosa.* Chitosan coating also worked as a reinforcement and improving flexibility of the electrospun membranes containing essential oils (Milanesi et al., 2021). *M. piperita* essential oil-chitosan nanoparticles showed a down regulation effect on eight genes involved in biofilm and virulence factors causing bacterial adherence (Ashrafi et al., 2019). A *Morinda citrifolia* essential oil chitosan (MCEO-CHs) nanoparticle exhibits excellent cytotoxicity activity against A549 lung cancer without damaging the red blood cells. Chitosan inclusion increases the cytotoxicity along with the biocompatibility (Rajivgandhi et al., 2020).

Above said advantages, this research was highly motivated with CsEOs to treat multi drug resistant bacteria and cancer cells through invitro analysis. The shematic representation of chitosan loaded palnt essential oils interaction between the MDRs bacteria and cancer cells (Fig. 1).

2. Materials and methods

2.1. Antimicrobial properties against various pathogens

Previously, the chitosan nanoparticle synthesis, essential oils extraction from the medicinal plant of Solanum nigrum, and their spectroscopic characterizations were effectively published in our previous reports (Khaled et al., 2021). Based on the previous reports, the CsEOs has more biofilm eradication property against biofilm producing *P. mirabilis*. For this advantage, CsEOs sample was obtained to complete inhibition of bacterial growth. The procedure of De La Cruz Sanchez et al. (2019) was also helped to this experiment, and followed by the protocol of agar well diffusion method using with spread the 48 h old cultures of *P. aeruginosa*, *E.-coli*, *P. aeruginosa* and *S. aureus* solidified nutrient agar media containing plate. Then, cut the 4 wells for add different concentration of CsEOs. For the antimicrobial resistant behavior of selected pathogens were confirmed by third generation antibiotic disc ceftazidime. All the plates were monitored under ordinary room temperature using 12–24 h time interval. After this time duration, the zones formed wells on the agar surface of the plates were noted clearly and noted.

2.2. Micro broth dilution method

The antimicrobial abilities of the CsEOs used to cross check, and confirmed their inhibition role against tested pathogens was monitored under liquid medium treatment process by 96-well plate method (Feng et al., 2020). In this method, 100 µl, 10 µl and 100–500 µl of muller hinton broth, 24 h pathogens and CsEOs were taken one by one in 96-well plate. The plate was gently shake and should not maintain the leakage of the solutions. Then, the sample was put into 37 ℃ maintaining incubator and monitored from 6 h to 24 h. After 6–24 h time interval, the turbidity levels were checked every time and noted. Finally, how much content of turbidity was identified in treated plates wells and also untreated plates wells. Based on the UV-spectroscopy analysis of treated result was compared with control result in percentage mode at 640 nm and calculated the percentage using bellowed formula,

$$Percentage\ of\ inhibition = Test\ O.D\ treated\ -- Control\ O.D \times 100 \quad (1)$$

2.3. Cytotoxicity effect against lung cancer cells using essential oils

The cytotoxicity nature of CsEOs and their ability against A549 lung cancer cells were detected by dimethylthiazol-diphenyltetra zolium bromide assay followed by effective methodology of Govindan et al. (2020). Initially, the amount of $\sim 2 \times 10^4$ cells of A549 was taken in previously filled DMEM medium of 96-well plate and monitored in room temperature with one day under the CO2 influence at 5% and 95% humidity. Consecutively, add different concentration 50–500 µg/mL of CsEOs into all the wells except control well. In room atmospheric temperature of 37 ℃ was set in CO2 incubator and stored in same incubator 24 h for detachment process. After detachment treatment process, the 25 µl of MTT solution was added gradually in all the wells and then diluted by PBS. Next, covered by aluminum foil and maintained

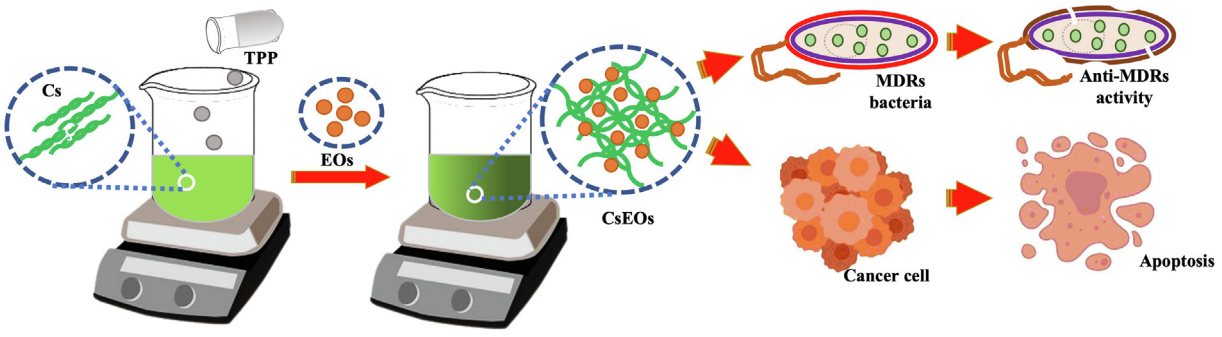

Fig. 1. Structural arrangement of chitosan nanoparticles loaded plant essential oils and their biomedical applications.

under room atmosphere for 4–5 h. Then, the plates were taken and cooled for find the crystal formazan present in the treated well or not. The untreated control and treated wells were clearly monitored in naked eye and confirmed the result based on the turbidity variation. After, the clear color intensity variations of the plates were confirmed by spectrometer analysis using 540 nm O.D values. Based on the interpretation, the IC50 value was identified and noted,

$$\%\ of\ IC_{50} = Control\ O.D - \ treated\ O.D \times 100,$$

The untreated control well was considered as 100% due to the well grown culture, and interpret the result with control result. Last, IC_{50} value of inhibition effect in the *Morinda citrifolia* extract was found based on the equation result.

3. Result

3.1. Bacterial inhibition by well diffusion method

After one day, CsEOs was performed against various pathogens, the zones were clearly formed around some of the bacteria and varied zones were also formed in some of the bacteria. In result, the 24 mm, 22 mm and 26 mm zones around the wells of chitosan nanoparticle loaded nanoparticles against *P. aeruginosa* (Fig. 2a), *E. coli* (Fig. 2b) and *K. pneumoniae* (Fig. 2c) were observed in respective plates at 500 µg/mL concentration. In addition, 18 mm zone of inhibition against *S. aureus* of the extract was also found after 24 h (Fig. 2d). Also, the inhibition zone based on the different concentration result was also exhibited in Fig. 2d. Therefore, the biological properties of the CsEOs were evidently suggested, bacteria were entirely killed. It also favored for MDRs bacteria and closely related to similarly reported researchers (Soltanzadeh et al. (2021); Soltanzadeh et al. (2021). As it has the inhibition ability against both GPB and GNB, the CsEOs is the important molecule in drug discovery studies. The evident of Hassan et al. (2021) was more similar to this result. Also, the CsEOs is the important research work against MDRs pathogens and it can be used as a future drug discovery candidate molecule. Based on the recent reports, CsEOshave excellent anti-microbial activity and anticancer activities. Because, the plant extracts, secondary metabolites, hormones and various other contents were involved to inhibit the infectious pathogens (Nidal et al., 2020; Anita et al., 2022). Also, chitosan

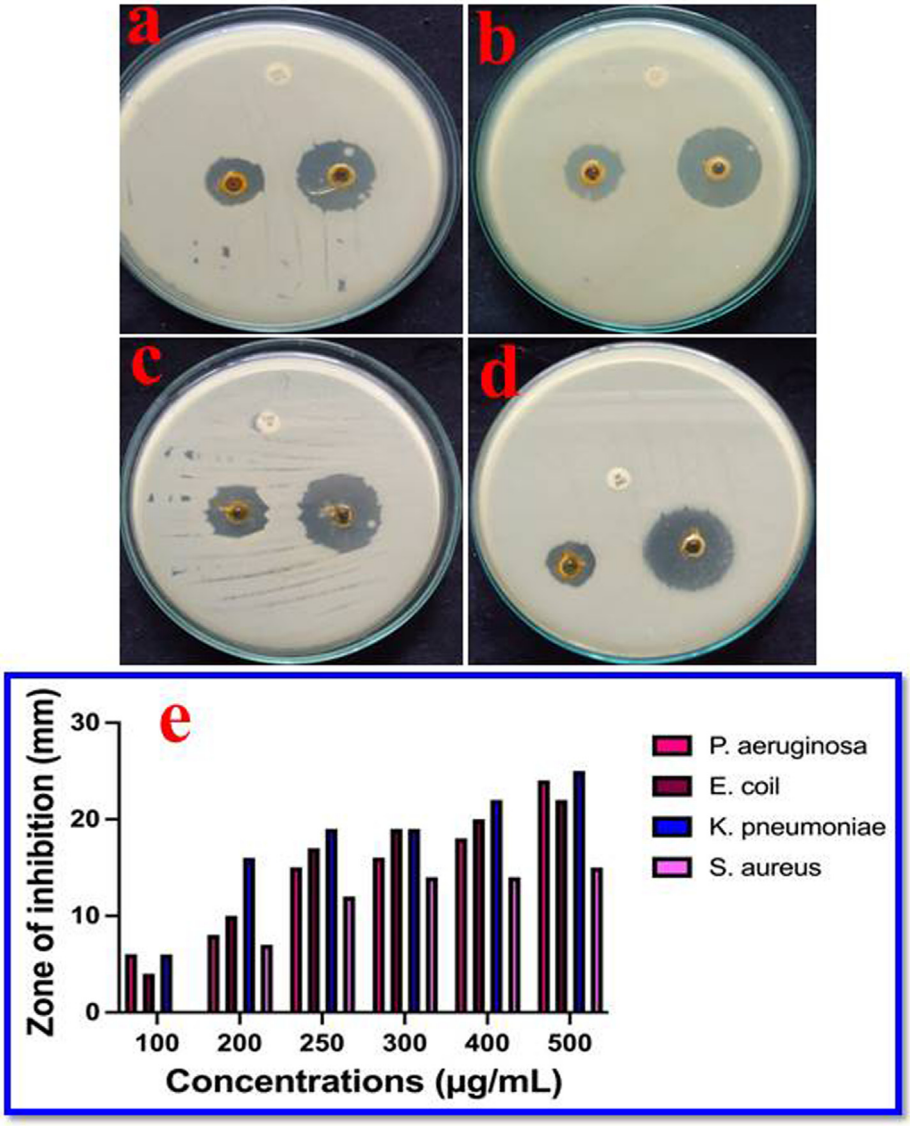

Fig. 2. Anti-microbial activity of chitosan nanoparticles loaded plant essential oils against various bacteria at increasing concentration. The zone of inhibition results against *P. aeruginosa* (a), *E. coli* (b), *K. pneumoniae* (c), *S. aureus* (d) and differentiation of increasing concentration of chitosan nanoparticles loaded plant essential oils against tested bacteria was available (e).

acted as an effective polymer used to improve the efficiency of EOs against pathogenic bacteria and lung cancer cells (Xiqiang et al., 2023; Anush et al., 2023). Also, chitosan as an excellent drug candidate for various biomedical applications, they are bactericidal (Rajivgandhi et al., 2021), anti-biofilm (Yang et al., 2020; Khaled et al., 2021) and anti-cancer activities (Rajivagndhi et al., 2020)). Chitosan itself has excellent anti-bacterial and anti-cancer properties against various diseases. Therefore, CsEOsas an excellent alternative drug to eradicate bacterial infections and lung cancer diseases (Ramachandran et al., 2020; Lin et al., 2019).

3.2. Micro broth dilution method

The consecutive biological properties identification of CsEOs and their effective nature was confirmed by microbroth dilution method. Based on the O.D value of this study proved, EOs as an effective antimicrobial agent for pathogenic bacteria. It was effectively confirmed by various time intervals. The time interval of 6 h exhibited 50% death cells in 500 μg/mL CsEOs. In addition, the 300 μg/mL of EOs effectively inhibit the pathogens with percentages of 65, 71, 60 and 58%. The initial inhibition was started at 100 μg/mL concentration only. Interestingly, 12 h and 24 h of the time interval is more relevant to this study and inhibit the bacterial growth stage by stage. At 12 h incubation, the complete inhibition of growth was observed in 500 μg/mL concentration. The range of inhibition percentages were 66, 65, 60 and 58 at 250 μg/mL concentration. As same as, at the time interval of 24 h, the inhibition was started at 25 μg/ml concentration and half inhibition percentage was started at 200 μg/mL concentration. Surprisingly, the highest inhibition rate of 92, 89, 88 and 94% of death cells were observed at 500 μg/mL. Current result of CsEOs treatment was influenced bacterial growth, and the growth went undergone the decline phase. Based on the microbroth dilution result, highest number of cell death with decreased dose of CsEOs was shown at 250 μg/mL (Fig. 3). Hence, the minimum inhibition concentration was fixed at 250 μg/mL concentration, and this concentration can be used efficiently in future study. The result was highly correlated with previous report of CsEOs study result. Recent researchers of Barani et al., 2014; Rajivagndhu et al. (2021); Junfeng et al. (2016) also agreed this statement and confirmed CsEOs, and the EOs rich plant of *Solanum nigrum* is the important phyto plant to active against various infectious diseases. More number of essential oils were present in the selected plant, and these all available in previously published report (Khaled et al., 2021), notably cubenol, a-pinene, a-terpineol, P-aucubin, a-terpinolene, a-pinene, lscopoletin, B-farnesene, a-terpenyl, B-asarone, sabinene, a-terpineol, bpinene are present in the plant.

3.3. Inactivation of A549 lung cancer cells

Based on the observation of MTT assay, the highest concentration of 500 μg/mL was excellent dose against A549 cells. The most confluent layer was formed in treated wells, especially at 250 μg/mL concentration, the IC_{50} concentration was nearly 56% (Fig. 4). The clear cytocompatibility and decreased growth of A549 lung cancer cells were observed in increasing concentration of CsEOs. In addition, the proliferation effect was very high compared with any other plant EOs (Suthagar Pillai, et al., 2012). Recent evidence result produced by Hesami et al., (2022), and it correlated with present result. EOs of α-norenone, L-scopoletin, nordamnacanthal and morindadiol may influence the high level of apoptosis through ROS production and mitochondrial damages. Previously, Campos et al. (2017); Jaya Kumar et al. (2012) also evidenced that the bioactive compounds of *Morinda citriflia* was influenced the necrosis process and lead to increase the programmed cells death. Zielińska et al. (2018) also agreed the current documented evidence of CsEOs as an excellent alternative drug molecule for treat cancer cells. After treatment and incubation with 24 h, the more

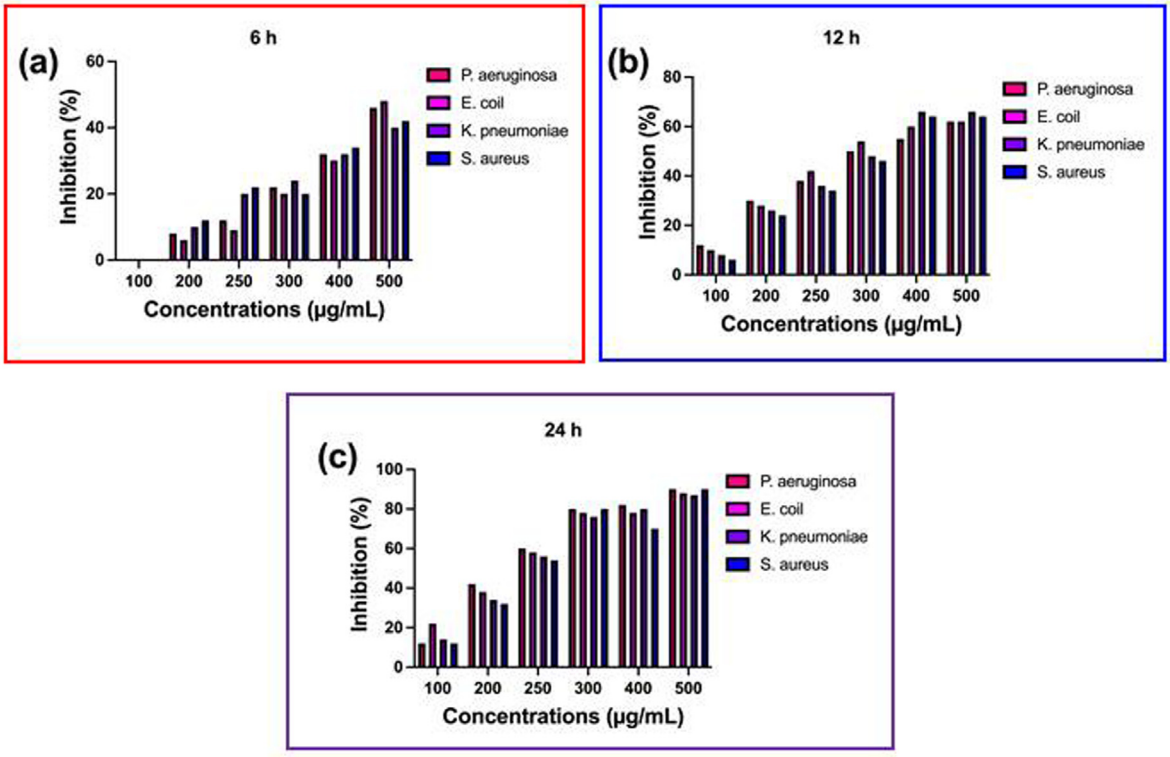

Fig. 3. Minimum inhibition concentration of chitosan nanoparticles loaded plant essential oils against selected multi drug resistant pathogens at different concentration.

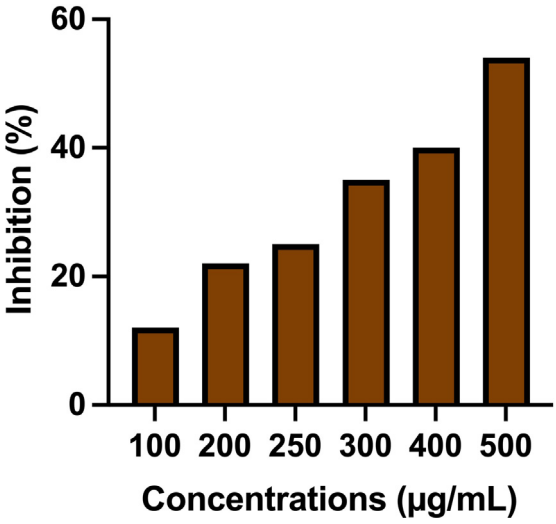

Fig. 4. Discovery of anti-cancer property of chitosan nanoparticles loaded plant essential oils against A549 lung cancer cells using cytotoxicity assay.

formazan production was observed when addition of MTT solution, it may happened through reduction of cellular enzymes. This process was also conveyed information about *Solanum nigrum* essential oils has the ability of anti-cancer role by formation of a blue color in treated wells. Previously reported evidences of Salehi et al. (2020); Alper Ozturk et al. (2020) were more supported to this research and CsEOs is the important material to use future drug discovery process of anti-cancer study.

3.4. Morphological variation by phase contrast microscope

The Fig. 5 of morphological evidences was exhibited with high condensed morphology of A549 lung cancer cells. In addition, obtained evidence of CsEOs may chance to produce bundle of apoptosis process within the cells. More cells were shown irregular shape compared with original morphology of A549 lung cancer cells. Also, the rough, attached uniform cells, high vegetative formed cells were shown in control (Fig. 5a), whereas the complete morphology alteration, smooth, inability to produce and excess level production of ROS formed structures were clearly shown (Fig. 5b). The morphological differentiation result of Fig. 5a and Fig. 5b were clearly indicates, received nanomaterial of CsEOs acted as an excellent anti-cancer module, and it can be used in future anti-cancer drugs after some toxicity evaluation. Previously, Amiri et al. (2020); Kavaz et al. (2019) also reported similar result

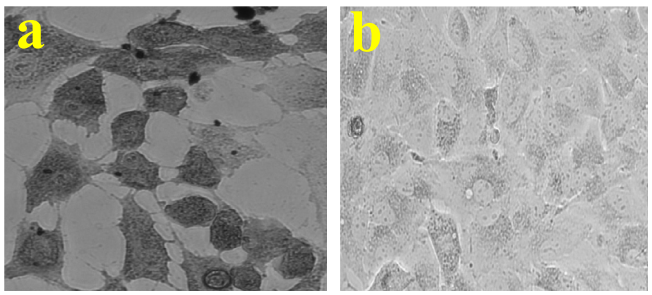

Fig. 5. Cytotoxicity effect of chitosan nanoparticles loaded plant essential oils against A549 lung cancer cells was morphologically confirmed by phase contrast microscope.

of the plant essential oils against cancer cells, and shown increased anti-cancer activities.

4. Conclusion

All the invitro experiments were purely accepted the obtained CsEOs has excellent biomedical properties. In addition, the biological properties of CsEOs were shown more inhibition against bacterial growth, and it confirmed by minimum inhibition concentration. Based on the evidences, it clearly evidence, the CsEOs was played an important roles in inactivation of bacterial virulence, and increased the apoptosis in cancer cells. Further, the previous reports of bioactive metabolites producing medicinal plant *Solanum nigrum* was also more supported to anti-cancer ability against A549 human lung cancer cells. Finally, the result was clearly conveyed that the obtained CsEOs as excellent biomaterial for lung cancer cells, and excellent capacity to inhibit the A549 lung cancer cells.

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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