International Journal of Agricultural Technology 2024 Vol. 20(6):2245-2258
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Docosanol and docosanol-mediated synthesis of gold nanoparticles target biofilm formation in drug resistant pathogens

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Judan Cruz, K. G. (2024). Docosanol and docosanol-mediated synthesis of gold nanoparticles target biofilm formation in drug resistant pathogens. International Journal of Agricultural Technology 20(6):2245-2258.

Abstract An emerging approach in the design of novel antimicrobial drugs to control pathogenesis is through targeting biofilm formation in bacteria. Biofilm formation is a quorum sensing-linked virulence factor that plays a major role in the development of antibiotic resistance. The research findings highlighted the action of a bioactive compound Docosanol and Docosanol gold nanoparticles on the inhibition of biofilm formation against drug-resistant pathogens, *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae*. The successful synthesis of gold nanoparticles using Docosanol was confirmed through characterization such as change in solution color to pink, UV–visible spectra peak at 550 nm and DLS analysis that showed particle mean size at 48.3 nm $\pm$ 2.1 nm. Significantly lower biofilm formation was noted using Docosanol against all test bacterial pathogens. Docosanol-AuNPs showed higher biofilm formation inhibition in *S. aureus* and *P. aeruginosa* compared to Docosanol alone. This study emphasized the potential of Docosanol and Docosanol-AuNPs in developing strategies against biofilm formation, microbial pathogenesis and resistance.

Keywords: Antibacterial, Biofilm formation, Docosanol, Gold nanoparticles

Introduction

Despite the initial success of antibiotics, its unrestrained use led to the development of antimicrobial resistance that poses serious burden to world healthcare systems. The incidences of antibiotic resistance are increasing rapidly with long term impacts on health systems (Gómez-Núñez *et al*., 2020), and this is expected to aggravate in the coming years. Although drug resistance increase is evident, the progress of drug development for drug-resistant pathogens still needs to be accelerated. Hence, the need for new approaches to lower incidence of drug-resistant infections and resistance is now crucial. Serious bacterial infections from drug-resistant pathogens highlight the need for effective and innovative drugs.

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Biofilm production is a quorum sensing (QS)-mediated virulence factor and considered one of the key mechanisms of pathogenesis in biofilm-forming bacteria. Biofilm is a polysaccharide matrix that offers protection and stability to a community of bacteria (Høiby *et al*., 2015) and contributes to their resistance to antibiotics by minimizing diffusion of substances across its matrix (Sun *et al.,* 2013; Uruén *et al*., 2021). Biofilms, thus, have become the main focus in anti-QS drug development (Diaz *et al*., 2015) and considered a promising approach to address antimicrobial resistance. To efficiently target bacterial virulence, disruption of the QS system was developed as a practical approach to manage pathogens. QS allows bacteria to coordinate vital processes and to synchronize behaviors such as virulence in order to effect pathogenicity. This is made possible by the production of signaling molecules that enables them to communicate among the species (interspecies) and between species (intraspecies). Targeting this communication system allows for the control of virulence without affecting growth, thereby addressing antimicrobial resistance that evolve by imposing selective pressure through the overuse of antimicrobials.

Plant compounds are recognized inhibitors of QS systems and provide new prospects in developing drugs against biofilm formation. They have been known to possess active groups of metabolites that act against several mechanisms of pathogenesis in microbes. The resemblance in the structure of the phytochemicals to QS signals and their ability to block signal receptors account for their action as effective QS inhibitors (Kalia, 2013). Phytochemicals have been documented to block QS activities and are recognized as one of the most effective natural sources of QS inhibitors that suppress intra- and inter-species QS communication systems (Teplitski *et al*., 2000) and reduce microbial pathogenesis (Rasmussen and Givskov, 2006). These natural compounds also offer an advantage due to their chemical stability (Rasmussen and Givskov, 2006) and non-toxicity (Hentzer *et al*., 2003), and thus safe for human health. Docosanol is a plant-derived compound mainly known for its antiviral activity against herpes virus infections (Pope *et al*., 1998; McKeough and Spruance, 2001; Leung and Sacks, 2004; Hammer *et al*., 2018; Sadowski *et al*., 2021). It works by interfering the fusion between the plasma membrane and the virus envelope, avoiding viral cell entry and subsequent viral replication (Pope *et al*., 1998; Leung and Sacks, 2004; Sadowski *et al*., 2021). Aside from the reported inhibition of biofilm and virulence factors against Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Lakshmi *et al*., 2020) and *Klebsiella pneumoniae* (Umaru *et al*., 2019), the actions of docosanol on bacteria has not been fully explored.

Among the innovative strategies for therapeutic applications, nanotechnology-based drugs emerged as one of the promising approaches that

offers advantages in enhanced transport of drugs. Nanotechnology had paved for development of drugs to new heights as nanoparticles proved to be a potent tool for drug delivery. It enhances drug delivery through extremely small dimensions that enables it to immediately penetrate target microbes or organs. The green synthesis of nanoparticles draws appeal in biomedical applications as it presents an advantage over toxic chemical and physical processes that involves the use of hazardous substances (Salem and Fouda, 2021). The green synthesis of gold nanoparticles (AuNPs) is of particular interest since metal-based NPs offers better control of bacteria. Aside from its extremely small dimensions that improves cell surface association, AuNPs offer structural properties that compromises the bacterial architectural integrity (Gómez-Núñez *et al*., 2020). Several plants have been evaluated in the synthesis of metallic NPs with much success. These biologically synthesized nanoparticles have shown to have higher antimicrobial activity and have shown to improve biofilm inhibition actions of some plant compounds (Fernando *et al*., 2020; Fernando and Judan Cruz, 2020; Velasco *et al*., 2020; Judan Cruz *et al*, 2021; Santos *et al*, 2021). This paper highlighted the potential of biofilm inhibition strategies through the use of Docosanol and Docosanol-synthesized gold nanoparticles.

Materials and methods

Gold Nanoparticle (AuNP) synthesis using Docosanol

Docosanol was obtained from Sigma-Aldrich (Germany). Docosanol was prepared as stock solution at a concentration of 3.4 mg/ml by DMSO. The method for the biological synthesis of AuNPs was adapted from Fernando *et al*., (2020). Docosanol was mixed in 10^{-3}M concentration of Gold chloride III. This was incubated in a 250-ml Erlenmeyer flask under dark conditions at room temperature with constant stirring with a magnetic stirrer for 60 minutes. The change in color indicated synthesis of AuNPs. This was purified by centrifugation at 4000 rpm for 20 minutes. Pellets were dispersed in deionized water. The synthesized nanoparticles was evaluated visually for precipitate formation or aggregation prior to use.

UV-visible spectroscopy analysis

The synthesis of gold nanoparticles was confirmed by UV–Vis Spectroscopy analysis using BioSpectrometer (Eppendorf). The resulting absorption value was noted.

Dynamic Light Scattering (DLS) analysis

The particle size of the AuNPs produced in the synthesis was determined at a scattering angle of 173° using Nanoparticle Analyzer SZ-100 (Horiba Scientific).

Disk diffusion assay for antibacterial activity of Docosanol and Docosanol-AuNPs against Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and *Klebsiella pneumoniae*

The study evaluated the following bacterial strains: *Staphylococcus aureus* PNCM 1582, *Pseudomonas aeruginosa* PNCM 1335*, Escherichia coli* PNCM 1634*,* and *Klebsiella pneumoniae* PNCM 1754*.*

Each test bacteria were grown for 16-18 hours in Mueller Hinton Agar (MHA) at 37ºC and then transferred to sterile distilled water with the turbidity adjusted to McFarland 0.5 standard. MHA plates were inoculated with the standardized culture. A sterile cotton swab dipped into the bacterial suspension was streaked in three different directions over the entire surface of the agar to ensure even distribution. On empty, sterile petri plates, 20µl of each treatment was pipetted onto 6-mm sterile blank antibiotic discs and allowed to stand for a few minutes to eliminate excess liquids. Using sterile forceps, infused discs were then transferred carefully to each other into MHA previously inoculated with the given pathogens separately. Treatments were done in triplicates. Distilled water served as negative control. After 24 to 48 hours of incubation, the appearance of the zone of inhibition around each paper disc was noted.

Biofilm formation assay

Overnight cultures (180 µl) of each test bacteria were added with 20 µl of corresponding sub-MICs of Docosanol and Docosanol-AuNPs individually in microtiter plates and incubated at 30 °C for 40 h. After incubation, the wells were rinsed with sterile distilled water five (5) times to remove planktonic cells, air-dried for 45 min, and stained with 150 µl of 1% crystal violet solution. The plates were rinsed to remove excess stain for five (5) times. To destain the wells for quantification of biofilm production, 200 µl of 95% ethanol was added. Subsequently, 100 µl from each well was transferred to a new microtiter plate and the OD values were measured at 570 nm (MultiSkan FC, Thermo Scientific). Culture medium added with only bacteria served as control.

Statistical analysis

Data were shown in means ± standard deviations (SD). Differences in quantified biofilms were analyzed through independent sample Tukey's Honest Significance Difference Test with 0.05 level of significance.

Results

Characterization of Synthesized Gold Nanoparticles (AuNPs) using Docosanol

The color of the solution changed from yellow to pink after 60 minutes of incubation at room temperature indicating AuNP formation (Figure 1a). The UV–vis spectra also confirmed the formation of gold nanoparticles which showed the SPR peak at 550 nm (Figure 1b). The size of Docosanol AuNPs measured using the dynamic light scattering (DLS) is at approximately 48.3 nm ± 2.1 nm with monodisperse distribution (Figure 1c).

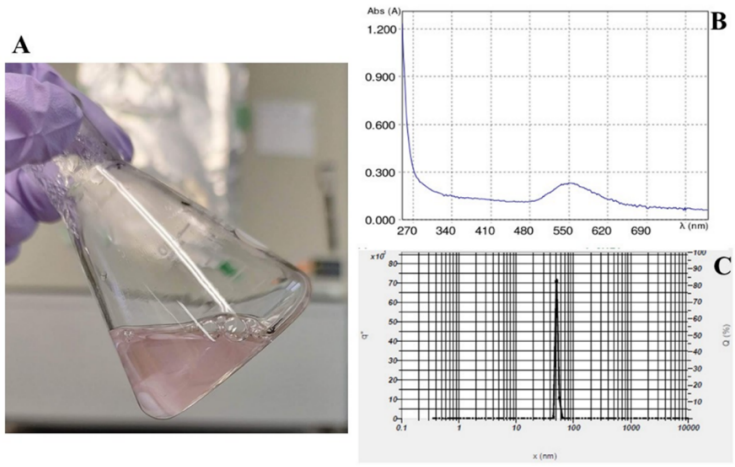

Figure 1. Synthesized gold nanoparticles using Docosanol (a) change in color to pink; (b) UV-Vis absorbance peak (c) DLS analysis

Antibacterial activity

No antibacterial activity was observed using Docosanol and Docosanol-AuNPS against the test bacteria. This was noted in the absence of the zone of inhibition for all the evaluations done in the disk diffusion assay. The absence of antibacterial activity is necessary for the accuracy of the subsequent biofilm formation assay to rule out the effects of an antibacterial-mediated decrease in biofilm production.

Docosanol and Docosanol-AuNPs inhibit biofilm formation in the test bacteria

The mean OD values in all test bacteria treated with Docosanol and Docosanol-AuNPs show a significant decrease in biofilm formation (Figure 2). Docosanol inhibited biofilm formation in *S. aureus* (OD 0.926), *P. aeruginosa* (OD 1.11), *E. coli* (OD 0.234) and *K. pneumoniae* (OD 0.103) compared to the negative control (media + bacterial suspension only) (*S. aureus* OD 1.949; *P. aeruginosa* OD 2.874; *E. coli* OD 0.77; and *K. pneumonia* OD 0.725). All AuNPs treatments significantly inhibited biofilm formation in all test bacteria compared to the negative control: *S. aureus* (OD 0.248); *P. aeruginosa* (OD 0.28); *E. coli* (0.136) and *K. pneumoniae* (0.097). Notably, Docosanol-AuNPs treatments in *S. aureus* (0.248) and *P. aeruginosa* (0.28) showed a significantly higher degree of biofilm inhibition compared to Docosanol alone (0.926; 1.11).

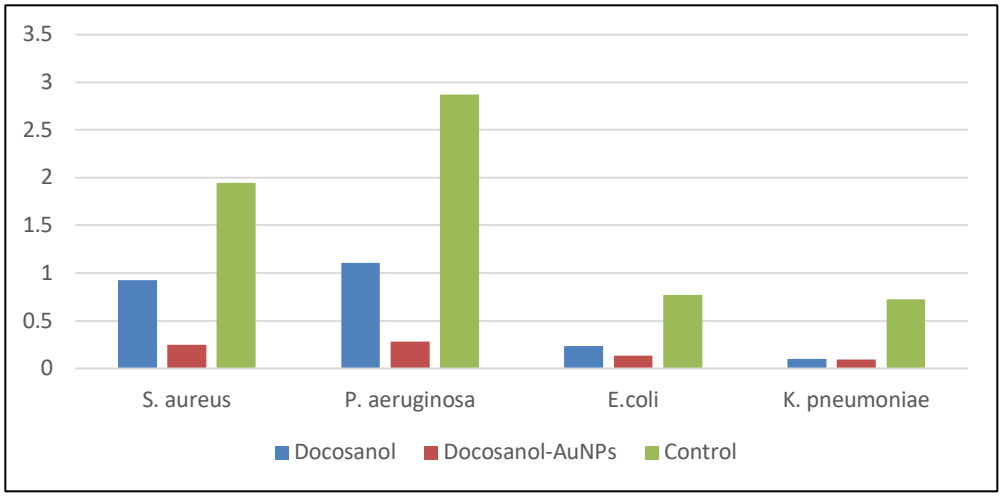

Figure 2. Mean OD values of Docosanol and Docosanol-AuNPs against *S. aureus, P. aeruginosa, E. coli* and *K. pneumoniae*

Discussion

Docosanol-AuNPs synthesis was confirmed through several indications. The change in color from yellowish to pink indicates the successful formation of AuNPs through the use of the bioactive compound as reducing agent (Mubarak *et al*., 2011; Mukherjee *et al*., 2016; Ovais *et al.,* 2016). The distinctive pinkish hue is due to the surface plasmon resonance (SPR) property of the AuNPs (Ngumbi *et al*., 2018; Inbakandan *et al.,* 2010). The SPR peaks detected with UV-vis is one of the principal approaches to confirm the synthesis of AuNPs and had been highly utilized in the determination of the metallic NP sizes (Ngumbi

et al., 2018). Due to the SPR, AuNPs develop a somewhat pink to reddish color as particles become smaller as it leads to the absorption of the blue light and reflection of red (Lee *et al*., 2018). Hence, the color change is proportional to the size in the NPs, where an increase in size will cause a shift in wavelength where red is absorbed showing a bluish coloration (Lee *et al*., 2018). The decrease in intensity and conjugation length from 595 to 550 nm also suggests decrease in particle size during synthesis (Emmanuel *et al*., 2017).

The DLS analysis also indicated the mean size at approximately 48.3 nm $\pm$ 2.1 nm, which is a confirmed nanosize. This extremely reduced size of AuNPs in this study emphasizes their biomedical potential as nanostructures have a high degree of uniformity and stability, and can effectively infiltrate the cell and cell processes improving the delivery of target molecules (Decuzzi *et al*., 2017). The nanosize also influence their entry and interactions within the biological systems, and host responses (Moreno-Vega *et al*., 2012). This suggests that the efficacy of compounds, or drugs, may be enhanced through encapsulation in a nano-sized carrier. The achievement of size at a nanoscale is critical in fully attaining the biomedical prospects of NP products, specifically to combat microbial pathogenesis.

All treatments exhibited higher inhibition in biofilm formation in all test bacteria. This observed biofilm inhibition may be attributed to the action of Docosanol and the extremely reduced dimensions of the nanoparticles. Docosanol is known for its pharmacological potential that includes antiviral activities (Pope *et al*., 1998; McKeough and Spruance, 2001), anti-inflammatory action (Spruance, 2002; Awan *et al*., 1998) and potential antidiabetic action (Jhong *et al.,* 2015). For antimicrobial activities, Docosanol was found effective against MRSA and *K. pneumonie* in previous studies (Lakshmi *et al*., 2020). This is the first paper that reports the action of Docosanol and Docosanol AuNPs against drug-resistant pathogenic bacteria biofilm formation inhibition and the first to report the successful synthesis of gold nanoparticles using Docosanol.

Natural products are chief sources of innovative antipathogenic agents. Drugs based on phytoactive compounds form a highly recognized approach in pharmacology (Cruz *et al*., 2007) to target QS systems in bacteria and therefore can be incorporated in schemes towards bacterial management. Hence, the search for antipathogenic therapies based on natural products should be maximized to overcome biofilm-associated infections, and Docosanol provides a new prospect. Several other plant metabolites have been reported to inhibit QS systems in bacteria. Major metabolites such as alkaloids, tannins and flavonoids are known anti-quorum sensing agents (Balangcod *et al*, 2012; Morah and Otuk, 2015) that specifically are known to inhibit biofilm formation by reducing the exopolysaccharides (EPS), proteins and DNA in the extracellular matrix (ECM)

(Tiwari *et al*., 2017). Potent phytoactive compounds against biofilm were identified, these include quercetin against *E. faecalis* (Qayyum *et al*., 2018) and *P. aeruginosa* (Ouyang *et al*., 2016); phloretin inhibits biofilm formation in *E. coli* (Lee *et al*., 2011) and downregulated biofilm-associated genes against *Listeria monocytogenes* (Wei *et al*., 2020) and *Salmonella* (Shuai-Cheng *et al*., 2016) and other QS-regulated genes in *Pectobacterium brasiliense* (Pun *et al*., 2021); hordenine effectively suppressed biofilm in *P. aeruginosa* (Zhou *et al*., 2018). Genistein, protocatechuic acid, p-hydroxybenzoic acid, and resveratrol demonstrated biofilm formation inhibition in *S. aureus* (Morán-Pereira *et al*., 2018). Hamamelitannin reduces biofilm activity in several microbes (Cobrado *et al*., 2012). Other plant metabolites with known QSI activities include curcumin (Nazzaro *et al*., 2013), furanones (Nazzaro *et al*., 2013), phenols and phenolic acids (Yu *et al*., 2013; Kumar *et al*., 2014; Ramanujam *et al*., 2014), terpenes and sesquiterpenes (Amaya *et al*., 2012; Paza *et al*., 2013) and rosmarinic acid (Nazzaro *et al*., 2013). A growing number of active components from plants are continuously tested for their anti-biofilm formation and other QS-related activities that prove their potential in biomedical applications.

Interestingly, biofilm formation was found significantly lower in treatments with biosynthesized gold nanoparticles using Docosanol against *S. aureus* and *P. aeruginosa*. This shows the potential of using synthesized nanoparticles in improving the efficiency of controlling biofilm formation in drug resistant pathogens. This may be attributed to an improved delivery system of the compounds through reduced particle size and exhibit larger surface area to volume ratio (Kamat *et al*., 2002; Geoprincy *et al*., 2013) that permits facilitated access to the highly complex cell membrane. The other Docosanol- AuNPs treatments have comparable effects with Docosanol alone i.e., *E. coli* and *K. pneumoniae*. In these cases, nanoparticle synthesis and its actions can still be optimized through the use of a higher concentration of gold chloride. Nevertheless, this study reported on the successful synthesis of Docosanol-AuNPs, and its actions can still be explored through the subsequent experiments, e.g. increase in concentration of the gold chloride in the synthesis of nanoparticles and other bioassays.

The decrease in biofilm production in the test bacteria displays the potential of Docosanol and the synthesized Docosanol-AuNPs to effectively permeate within the bacterial membranes. One of the key determinants of pathogen virulence and antimicrobial resistance is the formation of biofilms. This is a particularly important factor in antimicrobial drugs as the biofilm offers an efficient blocking layer for resisting antibiotics (Ahmed *et al*., 2016; Sriramulu, 2013; Algburi *et al*., 2017). It is important to note that these results were demonstrated in human pathogenic bacteria such as *S. aureus*, *P. aeruginosa, E.*

coli and *K. pneumoniae*. These bacteria are drug-resistant that cause acute and chronic medical device-associated infections in hospitals (Bryers, 2008; Chibber *et al*., 2013; Ahmed *et al*., 2016; Chambers and DeLeo, 2009) and pose serious concerns in global healthcare. *S. aureus* has long been recognized as one of the most important bacteria that cause disease in humans. It is the leading cause of soft tissue infections and can cause serious infections such as bloodstream infections, pneumonia, or bone and joint infections and if passes through the bloodstream or internal tissues, these bacteria may cause a variety of potentially serious infections (Taylor and Unakal, 2022). *P. aeruginosa* form resistant biofilms (Parkins *et al*., 2001) and cause a long list of life threatening chronic infections through biofilms that include cystic fibrosis pneumonia, otitis media, and bacterial prostatitis (Ma *et al*., 2012; Tolker-Nielsen, 2014; Chadha *et al*., 2022). It is also an ideal organism to explore biofilms and effects on biofilms ((Parkins *et al*., 2001; Chadha *et al*., 2022). Biofilms caused by *E. coli* are difficult to eradicate and contribute to urinary tract and intestinal infections (Sharma *et al.,* 2016) with an armory of antimicrobial resistance mechanisms that include a large number of antibiotic inactivating enzymes such as beta-lactamases (Katongole *et al.,* 2020). This is also the case for *K. pneumoniae* (Nirwati *et al.,* 2019). Aside from UTI and gastrointestinal tract infections, *K. pneumoniae* can be observed in immunocompromised individuals (Guerra *et al.,* 2022). It should be noted that infections caused by these bacteria are mostly due to biofilm formation, hence, ways to inhibit biofilms and curb antibiotic resistance becomes a critical concern.

Antibiotic use has risen in recent years as a result of a rising number of infections, necessitating measures to combat bacterial resistance (Sriramulu, 2003). Since present antimicrobial approaches and drugs cannot adequately address this issue, the discovery of potential agents remains necessary to control the evolution of microbial pathogenicity. This paper emphasizes the pharmacological potential of Docosanol and the green synthesis of AuNPs using Docosanol in the control of biofilms in bacterial human pathogens while avoiding the risk of developing antimicrobial resistance. Although this study conveyed the significant inhibition of biofilm formation in pathogenic bacteria, the exact processes and genetic pathways leading to inhibition were not explored and presents an avenue for detailed and deeper investigations.

Acknowledgements

The author is deeply grateful for the technical support of the Department of Biological Sciences, College of Science and the CLSU Nanotechnology Facility of the Central Luzon State University Science City of Muñoz, Nueva Ecija, Philippines.

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(Received: 9 May 2023, Revised: 10 September 2024, Accepted: 13 September 2024)