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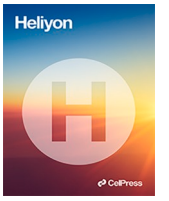

Antibacterial and antibiofilm effects of *Pseudomonas aeruginosa* derived outer membrane vesicles against *Streptococcus mutans*

Sangiliyandi Gurunathan [a,*], Pratheep Thangaraj [a], Joydeep Das [b], Jin-Hoi Kim [c,**]

[a] *Department of Biotechnology, Rathinam College of Arts and Science, Rathinam Techzone Campus, Eachanari, Coimbatore, 641 021, Tamil Nadu, India*
[b] *Department of Chemistry, Mizoram University, Aizawl, 796 004, Mizoram, India*
[c] *Department of Stem Cell and Regenerative Biotechnology, Konkuk University, Seoul, 05029, South Korea*

A R T I C L E I N F O

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A B S T R A C T

Antimicrobial resistance (AMR) is a serious and most urgent global threat to human health. AMR is one of today's biggest difficulties in the health system and has the potential to harm people at any stage of life, making it a severe public health issue. There must be fewer antimicrobial medicines available to treat diseases given the rise in antibiotic-resistant organisms. If no new drugs are created or discovered, it is predicted that there won't be any effective antibiotics accessible by 2050. In most cases, Streptococcus increased antibiotic resistance by forming biofilms, which account for around 80 % of all microbial infections in humans. This highlights the need to look for new strategies to manage diseases that are resistant to antibiotics. Therefore, development alternative, biocompatible and high efficacy new strategies are essential to overcome drug resistance. Recently, bacterial derived extracellular vesicles have been applied to tackle infection and reduce the emergence of drug resistance. Therefore, the objective of the current study was designed to assess the antibacterial and antibiofilm potential of outer membrane vesicles (OMVs) derived from *Pseudomonas aeruginosa* against*Streptococcus mutans.* According to the findings of this investigation, the pure *P. aeruginosa* outer membrane vesicles (PAOMVs) display a size of 100 nm. *S. mutans* treated with PAOMVs showed significant antibacterial and antibiofilm activity. The mechanistic studies revealed that PAOMVs induce cell death through excessive generation of reactive oxygen species and imbalance of redox leads to lipid peroxidation, decreased level of antioxidant markers including glutathione, superoxide dismutase and catalase. Further this study confirmed that PAOMVs significantly impairs metabolic activity through inhibiting lactate dehydrogenase activity (LDH), adenosine triphosphate (ATP) production, leakage of proteins and sugars. Interestingly, combination of sub-lethal concentrations of PAOMVs and antibiotics enhances cell death and biofilm formation of *S. mutans*. Altogether, this work, may serve as an important basis for further evaluation of PAOMVs as novel therapeutic agents against bacterial infections.

* Corresponding author.
** Corresponding author.
E-mail addresses: gsangiliyandi@yahoo.com, sangiliyandi.re@rathinam.in (S. Gurunathan), jhkim541@konkuk.ac.kr (J.-H. Kim).

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1. Introduction

Antimicrobial resistance (AMR) is a serious global threat to human health and highest burden in low resource settings [1,2]. Dental caries remains a relatively widespread pathology in communities all over the world despite several efforts. The dysregulation of the resident microbiota within the tooth biofilm is one of the many contributing factors that make caries a complex illness with a significant microbiological component [3]. The predominance of important acid-producing bacteria, like *Streptococcus mutans* (*S. mutans*), over non-acidogenic species of oral streptococci is a major factor in this dysregulation [4]. Increased dental biofilm acidity can therefore result in a progressive demineralization of the underlying tooth surface, which can cause cavitation and the disease to advance [5]. *S. mutans* antagonistic species and its predominance in the biofilm is mostly linked to healthy conditions [6–9]. Infections caused by multidrug-resistant bacteria have become a global burden and crisis. The ability of multidrug-resistant bacteria to form biofilms, which are 10- to 1000-fold more resistant to antimicrobials than planktonic organisms, is a well-known trait of these bacteria [10]. According to Mihai et al. [11], biofilm-related infections frequently originate from microbial colonization of soft tissues or medical implants and might present as chronic or persistent disease. Over 80 % of microbial infections are a result of biofilms, and the increase of multidrug resistance among species that generate biofilms has made treatment more difficult [10]. The management of public health is a crucial concern in the current world due to the rising prevalence of microbial resistance. No new medications have increased effectiveness against multidrug-resistant bacteria despite the fact that several have been created in the last few decades [12]. Given the existence of the Gram-negative outer membrane (OM) and efflux pumps, Gram-negative bacteria are naturally more difficult to eradicate with antibiotics than Gram-positive bacteria [13–15]. The need for innovative antibacterial medicines and therapeutic approaches is highlighted by the rising incidence of antibiotic resistance among Gram-negative organisms. To treat Gram positive and Gram negative infections, it is crucial to create alternative and more potent therapeutic approaches. In the past, nanoparticles used as antimicrobial and antibiofilm agent and also combination of nanoparticles and therapeutic drugs have been used to treat microbes and a variety of cancer treatment [16,17]. According to Imani et al. [18], a new hydroxyapatite/CuO/TiO2 nanocomposite was created and demonstrated antibacterial activity against the oral pathogen *S. mutans*. *S. mutans* was the target of an alginate-manganese oxide bionanocomposite with the highest level of antibacterial and antibiofilm activity [19].

Encapsulating existing antibiotics into nanoparticulate drug delivery systems is another effective method to overcome bacterial resistance [20,21]. Nanoparticles have shown potential drug carrier and also to improve drug transport to the site of infection [22,23]. Outer membrane vesicles (OMVs) revealed natural antibacterial capabilities [24], and it was demonstrated that loading the drugs polymyxin B and ampicillin into liposomes (LPs) dramatically increased the antibiotic activity against *P. aeruginosa* and *S. aureus*. LPs are spherical vesicles of a bilayer of phospholipids and artificial vesicles. Phospholipids, both natural and synthetic, can be used to create liposomes. LPs are usedas nanocarriers for various potentially active hydrophobic and hydrophilic molecules due to their unique properties. OMVs, on the other hand, are nanosized proteoliposomes made of bacterial proteins, lipids, nucleic acids, and other substances that are extracted from the outer membrane of Gram-negative bacteria used as drug delivery vehicle.

Recently, several studies reported that antimicrobial activity of OMVs mediated by small molecules, surfactants, and enzymes [25–30]. We postulated that OMVs produced from *P. aeruginosa* would be helpful in treating diseases caused by bacteria because of their antibacterial properties and common occurrence in bacterial biofilms. For instance, OMVs produced from *Burkholderia thailandensis* prevent the growth of fungi and bacteria that are both drug-sensitive and drug-resistant [31]. OMVs also have antibacterial and antibiofilm properties, these can help bacteria survive by removing poisonous substances, neutralizing environmental irritants, and removing misfolded periplasmic proteins, as well as by establishing a colonization niche, forming biofilms, delivering drugs, and modulatinghost retaliation and defence [25,32]. Gram-negative bacteria continuously release OMVs with sizes ranging from 20 to 450 nm by pinching off pieces of their outer membrane. According to recent studies, these OMVs are engaged in a variety of processes, including as cell-to-cell communication, biofilm formation, stress tolerance, horizontal gene transfer, and virulence [32,33]. Under various growth conditions, these vesicles continuously release biogenesis, and OMVs contain a variety of bioactive components, such as membrane lipids, lipopolysaccharides (LPS), OM proteins, peptidoglycan (PGN), degradative enzymes, nucleic acids, virulence factors, and more [32,33]. The OMV secretory pathway is a unique, bona-fide secretion system differs from other secretion systems. Extracellular proteases prevent the breakdown of proteins released through OMVs, allowing them to persist longer and travel farther. In addition, the secretion of OMVs enables the bacteria to export a mixture of molecules to a specific site at a considerably higher concentration than through conventional export methods [34]. According to Kadurugamuwa and Beveridge and Kesty [35,36], OMVs have the ability to precisely fuse to target cells (bacterial or mammalian) and then undergo endocytosis to convey their contents to the host cell. OMVs are powerful inducers of host immune responses because they include a number of highly conserved microbial signatures, also known as microbe or pathogen-associated molecular patterns (MAMPs or PAMPS), including LPS, PGN, flagellin, and other important surface proteins [37]. According to Filloux et al. and Lazdunski et al. [38,39], *P. aeruginosa* is well known for its capacity to generate and secrete a wide range of enzymes linked to the pathogenicity of this opportunistic disease. When *P. aeruginosa* is growing normally, it can release membrane vesicles (MVs) into the medium. When exposed to gentamicin, the release of MVs multiplied and also included DNA, lipopolysaccharide (LPS), and a number of hydrolytic enzymes, such as proteases and lipases. There are currently few studies on the isolation, characterization, and antibiotic activity of extracellular vesicles from bacteria. In particular, it is unknown how outer membrane vesicles from *P. aeruginosa* affect cell viability, growth and physiology of *S. mutans*. Therefore, the objective of the this study was designed to determine how *P. aeruginosa* generated outer membrane vesicles (PAOMVs) affect *S. mutans* cell viability, biofilm formation, and physiological activity.

2. Results and discussion

2.1. Isolation, purification and characterization of PAOMVs

First, we investigated the production and release of OMVs by using *P. aeruginosa*using culture supernatantwhen the bacterial culture reached an $OD_{600} = 0.5$, which corresponded to log phase. (The log phase of the culture was selected based on growth curve of *P. aeruginosa*). The *P. aeruginosa*were cultured in BHI broth at 37 °C, and PAOMVs were isolated. To guarantee precise accuracy of the analyses, two independent methods of purifications of the PAOMVs were performed using ultracentrifugation and ExoQuick (System BioSciences). To produce mass amount of bacterial vesicles, large volume of culture medium that has been first centrifuged and filtered through different sizes filtered to remove debris and biomolecules <100 kDa [40]. Ultracentrifugation was performed to remove protein aggregates, membrane fragments, and other debris. The purified vesicles were visualized by electron microscopy and revealed that *P. aeruginosa* produced PAOMVs of a typical shape, i.e., spherical cup-shaped structures (Fig. 1A). We observed that the presence of spherical vesicle-like structures in the putative EV pellet by TEM and TEM images of purified PAOMVs shown the size is around 100 nm (Fig. 1A), which is consistent with results obtained from DLS. SEM images confirmed that secretion of vesicles by *P. aeruginosa* (Fig. 1B). TEM images of purified OMVs shown the size is 100 nm, which is consistent with results obtained from Dynamic Light Scattering (DLS)and Nanoparticle Tracking Analyser (NTA) (Fig. 1C and D). DLS was used to determine the approximate size distribution of the vesicles. DLS analysis showed that most PAOMVs of *P. aeruginosa* showed an average diameter of 120 ± 20.0 nm. DLS revealed that various sizes of particles range from 10 to 200 nm. The results obtained from DLS showed that the OMVs presented a monodisperse profile with modal diameter of 120 ± 20.0 nm and concentrated in the range of about 100 nm. The hydrodynamic radius determined by this method depends on the ion type, surface structure, solvent viscosity, particle concentration, and diffusion coefficient. As a result, the size determined by this method may be higher than the result of electron microscopy. According to NTA results, *P. aeruginosa*-derived OMVs tended to concentrate in the 88–114 nm range. This size distribution matches that of vesicles made by other bacteria, as described by Kuehn and Kesty [32]. According to Shi et al. [41], spherical extracellular vesicles with spherical vesicle-like structures made of lipid bilayers and concentrated in the range of around 200 nm were seen in the supernatant of *Lacticaseibacillus paracasei P*C-H1.

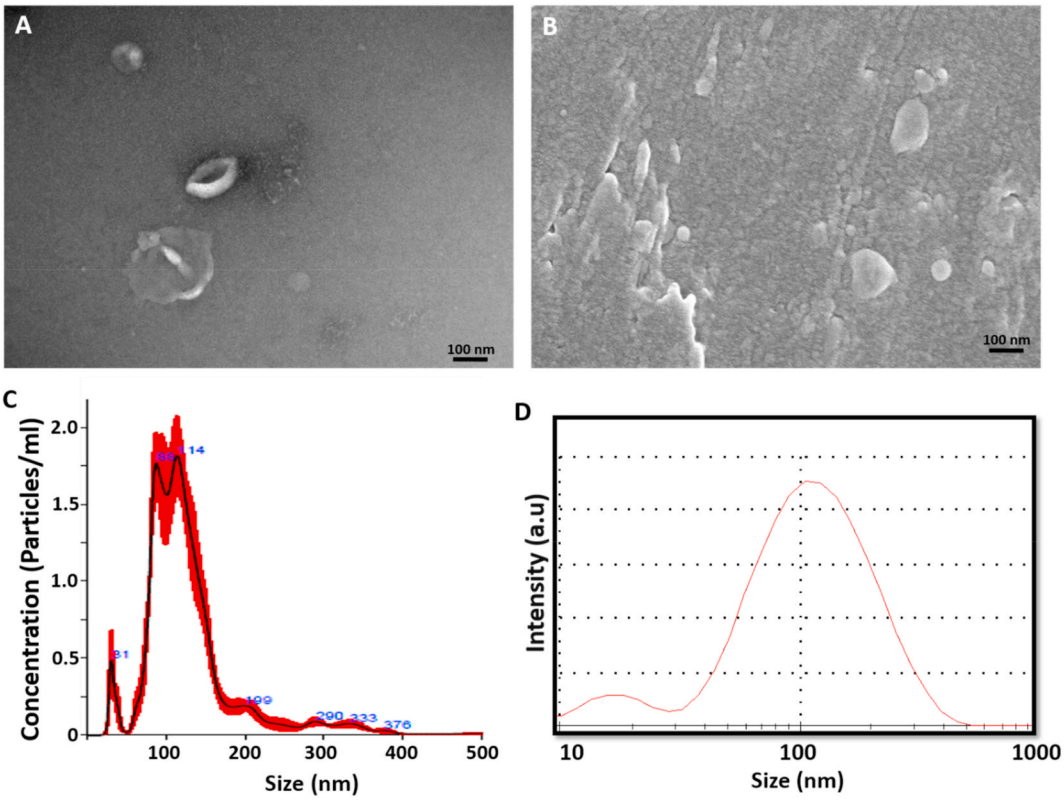

Fig. 1. Isolation and characterization of PAOMVs
*P. aeruginosa*was grownin BHI media and cells were centrifuged, and the supernatant was used for purification of vesicles. The purified BENVs were characterized various analytical techniques. (A) Transmission electron micrographs of PAOMVs showing cup-shaped and spherical in shape (B) Scanning electron microscopy of PAOMVs cells showing some spherical blebs surrounding the bacteria (C) Size and concentration of PAOMVs quantified using a NanoSight NS300. (D) Size distribution of PAOMVs was determined by DLS.

The size and morphology of PAOMVs vesicles were similar to nanovesicles from finger root [42]. The results from this experiment suggested that Gram-positive cells utilized vesicle secretion to deliver concentrated amounts of bioactive substances and other compounds into the extracellular space [43–47]. All purified PAOMVs were quantified based on protein yield. Protein concentrations of PAOMVs were 0.1 ± 0.05 mg/mL, which is comparable with outer membrane vesicles derived from *Klebsiella pneumonia* [48]. Our findings line with previous study suggest that TEM images of the OMVs obtained from *P. syringae* Lz4W, the range between 60 and 100 nm. The hydrodynamic radius of majority of these spherical vesicles was observed within the range of 60–80 nm. Several studies reported that isolation and purification of OMVs from variety of Gram negative bacteria, particularly, antibacterial and antibiofilm activity of OMVs from *P. aeruginosa* has not been reported yet against *S. mutans*. Therefore, we investigate the effects of PAOMVs on antibacterial and antibiofilm on *S. mutans*. *S. mutans* is a significant contributor to dental caries. *S. mutans*native habitat of oral cavity, specifically the dental plaque, a multispecies biofilm that develops on the tooth's hard surfaces [5].

2.2. Determination of MIC and sub-lethal concentration of PAOMVs and antibiotics

To determine the potential of additive or synergistic antibacterial effect of PAOMVs, various types of antibiotics were used including ampicillin, chloramphenicol, erythromycin, gentamicin, tetracycline and vancomycin. The MIC and sub-lethal concentration of PAOMVs and selected antibiotics were determined against *S. mutans* and presented in Tables 1 and 2 The MIC and sub-lethal concentration of PAOMVs against *S. mutans* found to be 10 μg/mL and 2.5 μg/mL respectively. The MIC and sub-lethal concentration of antibiotics against *S. mutans* is differing due to the potential and mechanism of each antibiotic.*S. mutans*were more susceptible to ampicillin than others tested antibiotics.

2.3. Dose- and time-dependent antibacterial effects of PAOMVs against S. mutans

As previously mentioned, *P. aeruginosa* derived membrane vesicles are known to exert antimicrobial properties through releases membrane vesicles (MVs) filled with periplasmic components, virulence factors, and hydrolytic enzyme factors, which are capable of hydrolyzing several glycyl peptides [35]. In the pathophysiology of dental caries, *S. mutans* are important tooth surface colonisers, and their dominance is essential for the development of the illness [3]. In order to evaluate the impact of isolated PAOMVs on bacterial growth of *S. mutans, S. mutans* were cultivated in the presence of increasing PAOMV concentrations. *S. mutans* was used to investigate the dose-dependent bactericidal activity of PAOMVs. Fig. 2A depicts the PAOMVs' inhibitory action on *S. mutans* at doses ranging from 1.56 to 50 μg/mL. When compared to the control, PAOMVs treatment in *S. mutans* clearly reduced cell viability. Complete growth inhibition of *S. mutans* was seen at 50 μg/mL of PAOMVs. On the other hand, the *S. mutans* treatment at MIC of 10 μg/mL decreased cell viability in a time-dependent manner (Fig. 2B). According to Guzman et al. [49], nanoparticles generally prevent bacterial cell growth by interfering with the synthesis of the cell's membrane, prohibiting the formation of proteins involved in bacterial cell division, interfering with the synthesis of nuclear material, and delaying a metabolic pathway ultimately leading to bacterial death. Furthermore, nanosized materials hyperosmotic stress and disturb cell permeability leading to cell death and also involved neutralization of cellular enzymes, which is mediated through formation of pits on bacterial cell wall may cause pits, which is eventually increases permeability and cause cell death [50,51]. The loss of bacterial cell respiration may be the cause of the decreased viability of bacterial cells. The use of several types of nanoparticles to suppress bacterial cell respiration for four clinical bacterial isolates has been reported in agreement with the current work [52–56]. However, the clear mechanism of bacterial derived nanovesicles on inhibition of cell viability is remaining elusive. According to Schwechheimer et al. [57], Gram-negative bacteria have an envelope comprised of an OM

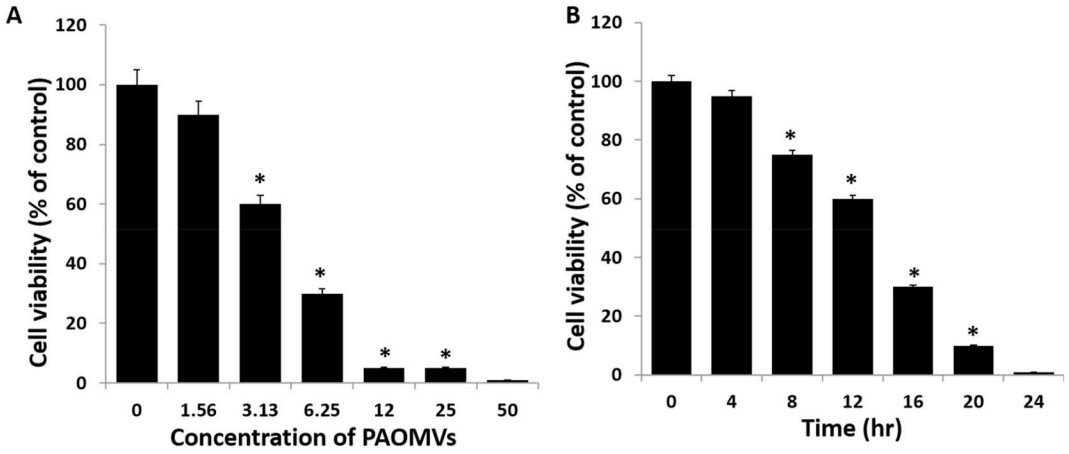

Fig. 2. Effect of PAOMVs on cell viability
(A) *S. mutans* cells were incubated with various concentrations of PAOMVs. Bacterial survival was determined at 24 h (B) Time dependent effect of PAOMVs on *S. mutans*. Results are expressed as the means ± SD of three separate experiments, with three replicates per experiment. Statistically significant differences between treatment and control groups were determined using student's t-test.

that has proteins bound as -barrels and lipopolysaccharides (LPS) bonded covalently by the lipidic moiety. OMV, which are composed of phospholipids, LPS, and outer membrane proteins (OMP), are nanostructures with sizes ranging from 20 to 250 nm that are secreted from the bacteria's OM [58]. During genesis of vesicles, various bioactive substances are localized in the lumen of the vesicles which are playing important role in antibiotic resistance and virulence dissemination [57–59]. OMV can bind to other bacteria and release enzymes that have been shown to have antimicrobial effects [60]. Studies suggest that vesicles from *P. aeruginosa* PAO1 with autolysins, such as peptidoglycan hydrolases, have antibacterial activity against various species of Gram-negative bacteria, including *E. coli* DH5 and *P. aeruginosa* PAO1 [27,35], and Gram-positive bacteria, including *Brachybacterium conglomeratum* CCM2134. Peptidoglycan hydrolases from OMV have been shown to have antibacterial activity against some chemotypes of peptidoglycan from target bacteria, including the A1Y chemotype, making it more vulnerable to cell lysis. Hemolysin, for instance, is found in the outer membrane vesicles (OMV) of *E. coli* and *P. aeruginosa* [35,61]. *P. aeruginosa* secretedantimicrobial compounds including quinolines, 4-hydroxy-2-heptylquinoline (HHQ) and 4-hydroxy-2- nonylquinoline (HNQ) from OMVexhibited antimicrobial effects against *Staphylococcus epidermidis*. Alkaline phosphatase from *E. coli* was shown to have antimicrobial activity against *P. aeruginosa* [62].

2.4. PAOMVs inhibit S. mutans biofilms in a dose-dependent manner

The majority of human infections are caused by pathogens that form biofilms, which are surface-associated microbial communities encased in a complex and highly viscous extracellular polymeric substance (EPS) made of polysaccharides, proteins, lipids, and other microbial-derived products [10]. Biofilm-related illnesses can appear as chronic or recurrent disorders and are frequently caused by microbial colonization of soft tissues or medical implants [11]. Furthermore, because dental caries is a biofilm-mediated condition, it's critical to evaluate both PAOMVs' potential antibiofilm capabilities as well as their capacity to limit bacterial development. Therefore, the impact of PAOMVs on *S. mutans* biofilm development following a 24-h incubation period was also evaluated. Our findings suggest that increasing concentration of PAOMVs inhibited biofilm formation, while reaching MIC concentration, the effect was significantly inhibits biofilm formation.*S. mutans* biofilm were quickly suppressed by PAOMVs treatment; 3.13 μg/mL caused 30 % inhibition and 6.25 μg/mL caused 60 % inhibition. After increasing concentration from 12 μg/mL to 50.0 μg/mL, there is no significant difference in inhibitory effects, it suggested that MIC concentration of 10 μg/mL enough to inhibit 90–100 % of inhibition. Against *S. mutans* biofilms, PAOMVs antibacterial activity was more effective. In a dose-dependent manner, PAOMVs showed significant bactericidal action against *S. mutans* biofilm cells (Fig. 3A). Dual-species biofilms produced by *S. mutans* and *S. sanguinis* were reported to be reduced in their growth and cariogenic pathogenicity by D-cysteine D-Cys [6]. When combined with nicin, three D- or L-enantiomers of Cys, Asp, and Glu at a concentration of 40 mM prevented the growth of *S. mutans* biofilms while also inhibiting antibacterial activity [63]. Silver nanoparticles potentially inhibit antibacterial activity and antibiofilm against Gram negative and Gram positive bacteria [16,17,64]. Gram-negative bacteria use OMV to release toxins, and additional virulence factors cause cytotoxicity. OMV transport chemicals involved in signaling, the formation of biofilms, or gene transfer that help the bacterial survival. Given their antimicrobial activity biofilms activity of PAOMVs, we hypothesized that *P. aeruginosa* derived PAOMVs could potentially be useful to treat biofilms formed by *S. mutans*. To further evaluate the PAOMVs-mediated loss of biomass, remarkably, increasing concentrations of PAOMVs led to a reduction in total biomass in *S. mutans* biofilms. The inhibition of biofilm formation was time-dependent manner. The inhibition of

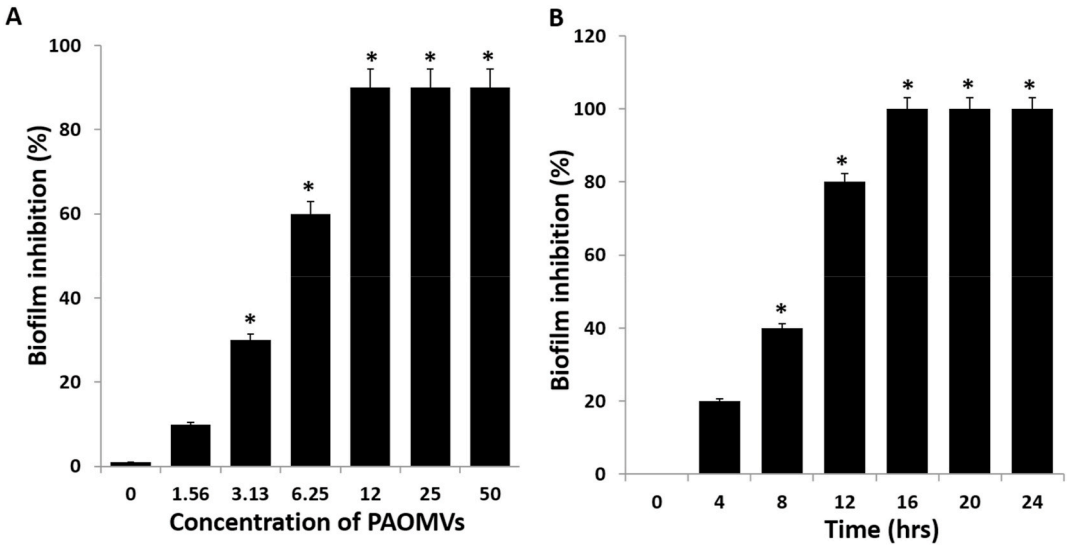

Fig. 3. Effect of PAOMVs on biofilm inhibition
The *anti*-biofilm activity of PAOMVs was assessed by incubating *S. mutans* with different concentrations of PAOMVs for 12 h in a 96-well plate. The results are expressed as the means ± SD of three separate experiments each of which contained three replicates. Treated groups showed statistically significant differences from the control group by the Student's *t*-test ($p < 0.05$).

S. mutans biofilm was readily observed within 4 h of OMV treatment, after 6 h biofilm inhibition showed 2 fold increase of biofilm inhibition reduction. PAOMVs antibiofilm activity was more effective against *S. mutans* biofilms. Strikingly, early hours of incubation with PAOMVs killed all *S. mutans* biofilm cultures (Fig. 3B). Previous studies demonstrated that OMVs derived from *Burkholderia thailandensis* inhibit the growth of drug-sensitive and drug-resistant bacteria and fungi due to the presence of number of antimicrobial compounds, including peptidoglycan hydrolases, 4-hydroxy3-methyl-2-(2-non-enyl)-quinoline (HMNQ), and longchain rhamnolipid are present in or tightly associate with *B. thailandensis* OMVs [31]. Kalishwaralal et al. [65] reported that AgNPsinhibited the biofilm formation by *P. aeruginosa* and *Staphylococcus epidermis*. Sriram et al. [66], observed that *Bacillus cereus* produced lipopeptide biosurfactant inhibits both antibacterial and antibiofilm against both Gram negative and Gram positive bacteria. AgNPs potentially inhibited cell viability and biofilm formation [17]. The dysfunction of the water channels within the biofilm, which is comparable to the mechanism of AgNPs, may be the cause of PAOMVs' inhibitory action on the biofilm [67]. Furthermore, PAOMVs could diffuse through the EPS layer and impart antimicrobial action like nanoparticles, in agreement with our findings, the toxicity of nanoparticles rises as the conduction band energy approaches that of biomolecules and the hydration enthalpy becomes less negative [68].

2.5. PAOMVs induce ROS generation in S. mutans

The molecular composition of the OMV, the type of bacteria, and environmental inducers all affect its actions. One OMV may perform multiple functions because of its intricate makeup [69]. Oxidative stress is a causative factor for cell death in bacteria [16]. We speculate about the possibility that OMV from *P. aeruginosa* can cause cell death by producing ROS. Oxidative stress is known to cause harm to intracellular systems such the respiratory system, proteins, DNA, and cell membranes [70]. Superoxide anions ($O_2\bullet$), a type of reactive oxygen species produced by NPs, have been identified as one of the main causes of major bacterial mortality. To examine the role of PAOMVs on reactive oxygen species generation, *S. mutans* treated with 10 μg/mL of PAOMVs for 12 h. The results depicted that PAOMVs induces 3 fold high levels of ROS compared to untreated cells. When used as a debriding agent, hydrogen peroxide (H_2O_2) is known to destroy bacterial structure by producing reactive oxygen species (ROS) and to create oxidative stress in microorganisms. It also has a powerful antibacterial impact. Furthermore, H_2O_2-induced oxidative damage causes apoptosislike death occurs in *E. coli* [71]. H_2O_2 exposure can cause *E. coli* to go into the SOS response, which speeds up the oxidation of bacterial DNA. The SOS response in *E. coli* can be triggered by H_2O_2 exposure, accelerating the oxidation of bacterial DNA [72]. Based on available evidences, we selected H_2O_2 as a positive control in all experiments. When *S. mutans* treated with H_2O_2 induced 4 folds level of ROS compared to control. Interestingly, when *S. mutans* pretreated with NAC, which is known as ROS inhibitor and antioxidant substance, the generation of ROS induced by PAOMVs or H_2O_2 significantly reduced to the level of control (Fig. 4A). It was found that NAC suppress PAOMVs-induced ROS generation in *P. aeruginosa*. High concentrations of H_2O_2 greatly inhibited bacterial growth, even led to bacterial mortality, and also induced *P. aeruginosa* to produce PAOMVs [73]. Similarly, nanosized AgNPs induces ROS generation up to several folds in various types of microorganism including Gram negative and Gram positive bacteria [16,17]. Antimicrobial strategies mainly depend on ROS mediated cell death. The creation of ROS is influenced by a number of biological factors, including pH and temperature, and cells

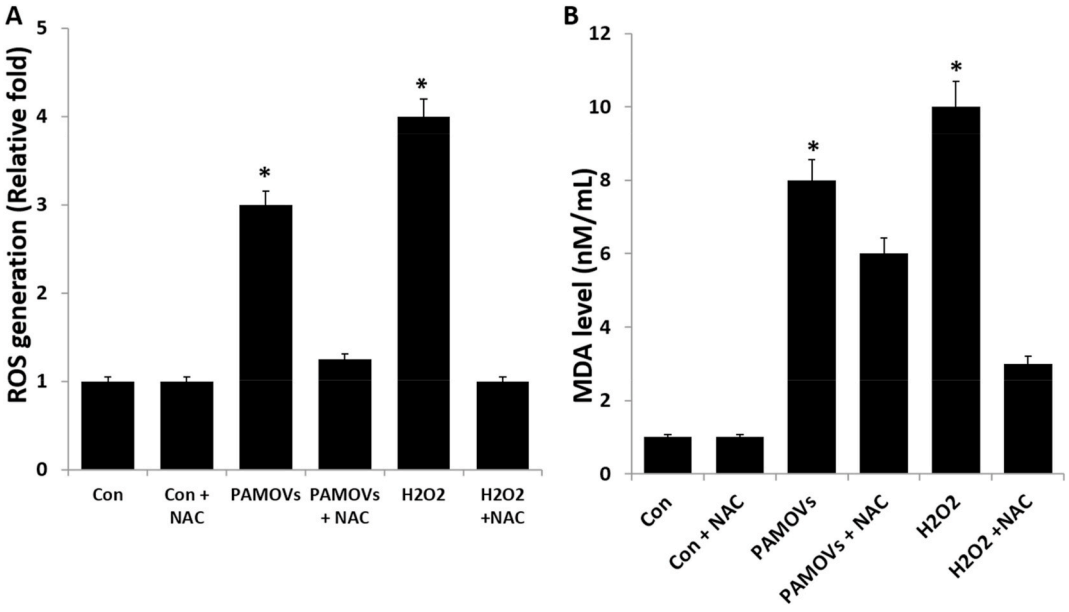

Fig. 4. Effect of PAOMVs on ROS generation and MDA
(A) *S. mutans* cells were treated with respective MIC of PAOMVs for 12 h. ROS generation was measured using DCFDA (B) MDA levels were measured using TBARS assay. Results are expressed as the means ± SD of three separate experiments, with three replicates per experiment. Statistically significant differences between treatment and control groups were determined using student's t-test ($p < 0.05$).

exposed to stress can produce significantly more ROS [74]. Due to their great abundance and quick rates of ROS response in bacteria, proteins are important biological targets for oxidative damage within cells [10,75–77]. OMVs can influence many target locations of the microbial cells, including the cell membrane, enzymes/proteins, lipids, DNA, and plasmids, despite the fact that the mechanism of their interaction with bacterial cells is not fully understood.

We assessed the amount of malondialdehyde (MDA) in treated and untreated cells to confirm the association between ROS and MDA content in PAOMVs-treated *S. mutans*. A popular indicator for oxidative stress is the lipid peroxidation of polyunsaturated lipids. Singlet oxygen and peroxy radicals can be produced during lipid peroxidation [78,79]. Malondialdehyde, a byproduct of lipid peroxidation that is easily detectable in bacterial culture, is a key biomarker for oxidative stress caused by lipid peroxidation [80]. Additionally, the alteration of cellular proteins and other components has been linked to the unsaturated aldehydes produced by these processes [81]. As a result, we assess the MDA levels in *S. mutans* treated with PAOMVs. MDA levels were substantially greater in *S. mutans* cultures than in the controls, as seen in Fig. 4B. Additionally, MDA levels were eight times higher in PAOMVs and 10 times higher in H_2O_2-treated cells, respectively. These findings imply that ROS generation via PAOMVs was what caused the suppression of bacterial growth. When *P. putida* was exposed to environmentally relevant amounts of AgNPs, the organisms antioxidant defence mechanism was repressed and lipid peroxidation increased [79,82]. Nanoparticles such as silver induced production of MDA in *Staphylococcus aureus* and *Pseudomonas aeruginosa* [83]and *Prevotella melaninogenica* and *Arcanobacterum pyogenes* [16]. Zhang et al. [70] reported that heat and cold stress induces generation of MDA in *E. coli*. Collectively, all these findings suggested that external inducers such as nanovesicles from bacteria, nanoparticles, antibiotics, heat and cold stress causes higher level of MDA which leads to cell death.

2.6. Effect of PAOMVs on antioxidants markers

Oxidative stress regulates a status of homeostasis of cells including maintenance of balanced level of prooxidant–antioxidant balance, if the cells disturbed by external stress resulted into DNA hydroxylation, protein denaturation, lipid peroxidation, and loss of cell viability and proliferation. Generally, most living organisms possess enzymatic defenses and non-enzymatic antioxidant defenses

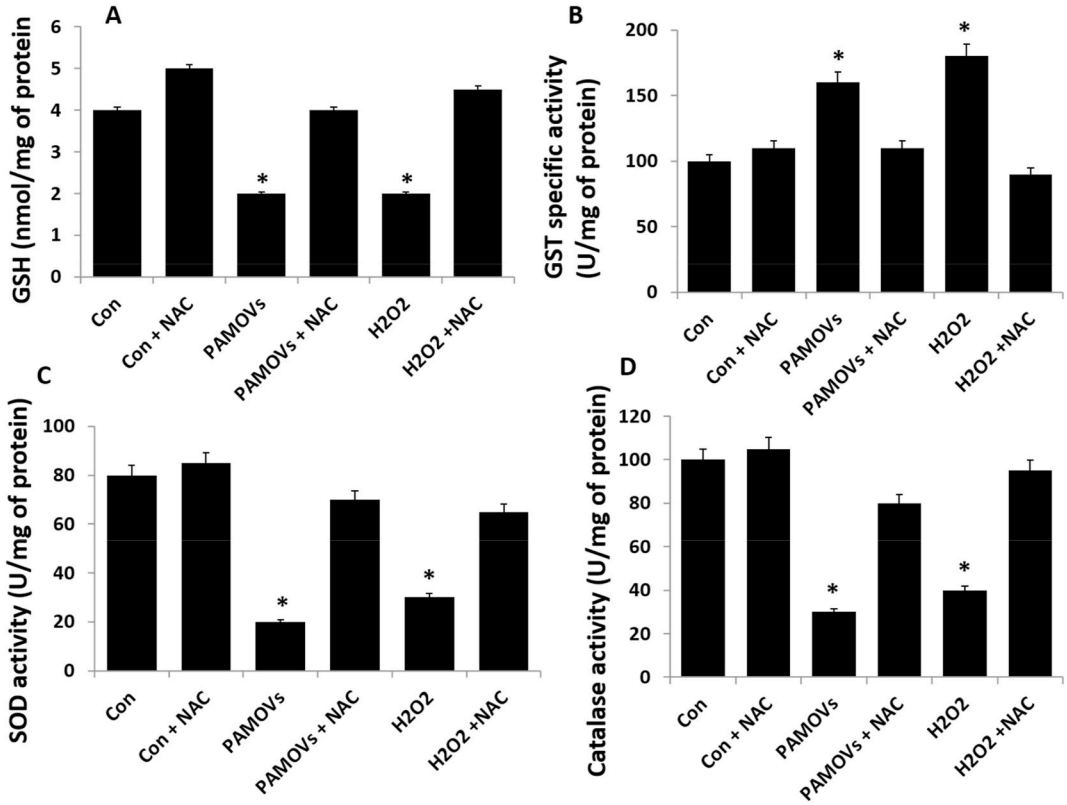

Fig. 5. Effect of PAOMVs on GSH and GST activity
(A) *S. mutans* cells were treated with respective MIC of PAOMVs for 12 h. GSH levels were measured enzymatically in the clear supernatant based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by the GSH reductase system (B) GST activity (C) SOD activity (D) catalase activity was determined as described in the Materials and Methods Section. Results are expressed as the means ± SD of three separate experiments, with three replicates per experiment. Statistically significant differences between treatment and control groups were determined using student's t-test ($p < 0.05$).

and repair systems to protect them against oxidative stress. In light of these findings, we hypothesized that oxidative stress caused by PAOMVs would have an impact on cellular antioxidant metabolites such glutathione (GSH). We measured GSH levels in cells treated with PAOMVs. Cells exposed to PAOMVs showed decreased 2 folds levels of GSH compared to untreated cells; similarly, H_2O_2 treated cells caused decreased levels of GSH levels (Fig. 5A). This was also in line with the findings of this and other studies, which showed that cells exposed to PAOMVs experience increased levels of oxidative stress, which causes GSH levels to drop. Of note, the cells treated with NAC increased the level of GST activity both in PAOMVs and H_2O_2 treated groupcompared to control. In line with our findings, Wongkaewkhiaw et al. [42] reported that nanovesicles from finger root significantly reduced total glutathione level compared to untreated cancer cells. These findings imply that fingerroot nanovesicles exhibited specific cytotoxicity against cancer cells through an increase in ROS production and a disruption of the redox balance that resulted in apoptotic cell death; as a result, PAOMVs have a substantial potential to be developed as specific antibacterial agents. AgNPs treated *P. aeruginosa* and *S. aureus* showed reduced amount of glutathione, which is associated with disturbing the redox balance and leads to cell death [79]. Similarly, external stress such as heat and cold causes reduced level of glutathione in *E. coli* [70]. Similar to PAOMVs, various types of nanoparticles such as graphene oxide [53], silver [16], palladium [84], platinum [85] down regulated the total glutathione level in variety of bacteria and cancer cells. Together, these findings show that PAOMVs produce an imbalance between oxidants and antioxidants in cells, which leads to the loss of cell viability through ROS production.

According to Mitra et al. [86] and Schröder et al. [87], GSTs are detoxification enzymes with the capacity to inactivate hazardous substances. A wide range of substrates' electrophilic centres are attacked by glutathione's sulphur atom through a nucleophilic reaction that is catalysed by GSTs. Further, we examined the activity of GST, an enzyme connected to GSH and predominantly involved in detoxification activities, to confirm the outcomes of the GSH assay [88]. Next, we examined the level of Glutathione *S*-transferases (GST) specific activity in PAOMVs treated *S. mutans*. Total specific GST activity was measured and represented as Units. Results from this study revealed a significant difference between the PAOMVs treated *S. mutans* and untreated cells. PAOMVs treated *S. mutans* showed higher GST activity (160 U/mg of proteins) (Fig. 5B), similarly, as a positive controlH_2O_2 treated cells also exhibited increased GST activity (180 U/mg of proteins). The increased level of GST activity is to overcome toxicity induced by PAOMVs. By greatly increasing the mRNA expression of the Nrf 2, HO-1, CAT, and SOD genes in HaCaT cells, small extracellular vesicles generated from Aloe vera displayed antioxidant capabilities against H_2O_2 [89]. GST play significant roles in mediating the tolerance of a *P. aeruginosa*

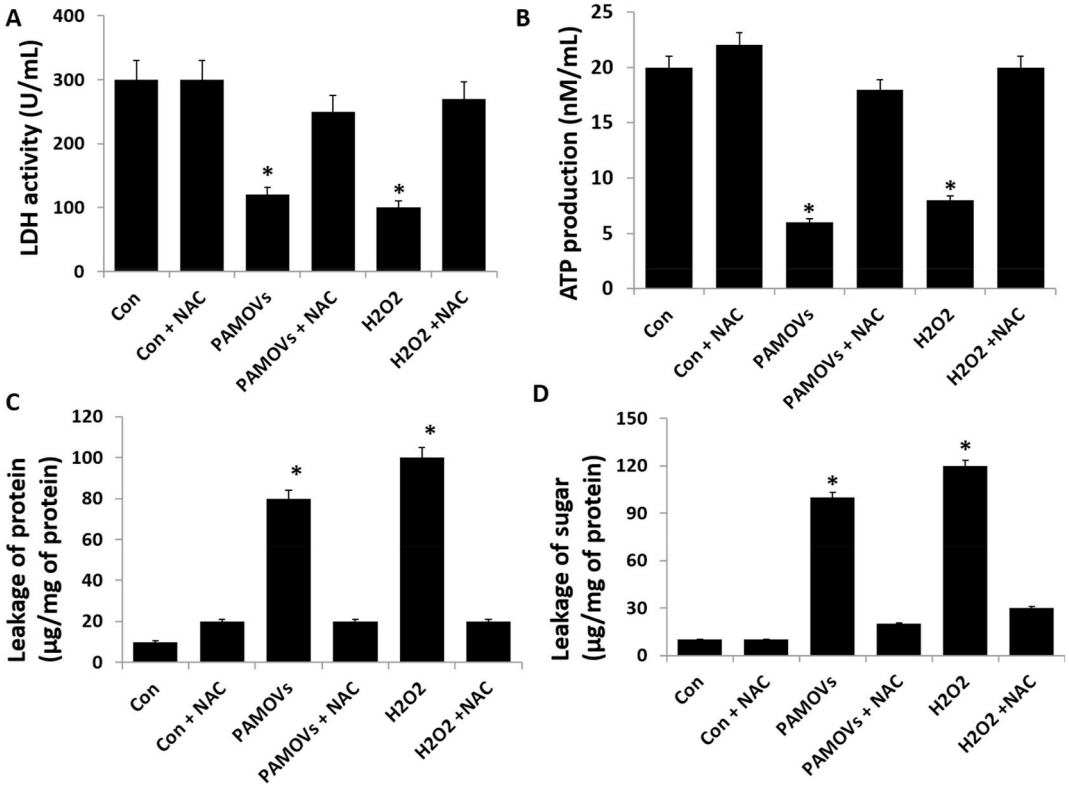

Fig. 6. Effects of PAOMVs on metabolic activity

(A) *S. mutans* cells were incubated with respective MIC of PAOMVs for 12 h. LDH activity was determined by measuring the reduction of NAD + to NADH and H+ during the oxidation of lactate to pyruvate (B) ATP levels were determined by measuring luminescence levels and comparing against an ATP standard curve (C) protein levels were measured using the Bradford assay (D) sugar concentrations Results are expressed as the means ± SD of three separate experiments, with three replicates per experiment. Statistically significant differences between treatment and control groups were determined using student's t-test ($p < 0.05$).

strain to various herbicides [90]. AgNPs treated *Folsomia candida* showed decreased levels of GSH and increased GST activity [91]. Similar to this, *P. putida* treated to AgNPs produced more ROS, had less glutathione, and had the antioxidant enzymes superoxide dismutase, catalase, and glutathione reductase inactivated [92]. Overall, the results indicate that *S. mutans* exposure to PAOMVs results in increased ROS production, decreased GSH levels, increased GST enzyme activity, and loss of cell viability, indicating the importance of oxidative stress as a significant mechanism of PAOMVs-induced toxicity in *S. mutans*. Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) are important modulators of nanoparticles induced oxidative stress and both enzymes are involved in maintaining the level of ROS and are used as bioindicators of increased ROS production [70,93]. To find out the role of SOD and CAT in PAOMVs induced redox imbalance, we examined the activity of SOD and CAT in the presence and absence of PAOMVs in *S. mutans*. The data derived from these experiments suggest that PAOMVs and H_2O_2-treated bacteria exhibit significantly lower SOD levels 20 U/mg of proteins and 30 U/mg of proteins respectively compared to the control (80 U/mg of proteins) (Fig. 5C). Other hand, PAOMVs and H_2O_2-treated cells showed decreased CAT activity up to four folds (20 U/mg of proteins) and three folds (30 U/mg of proteins) respectively compared to control (100 U/mg of proteins) (Fig. 5D). Surprisingly, *S. mutans* pre-treated with NAC recovered the loss of SOD and CAT activity. Thus, these observations suggest that decrease in antioxidant levels in PAOMVs treated *S. mutans* is due the disruption of the electron transport assemblies in the plasma membrane [79]. The antioxidant activity of ROS-metabolizing enzymes such NADPH-dependent flavoenzyme, catalase, glutathione peroxidase, and superoxide dismutase can be modified by nanoparticles-mediated ROS production [94]. For instance, *P. putida* cells exposed to environmentally relevant concentrations of AgNPs showed increased lipid peroxidation, suppression of the antioxidant defence system, including glutathione depletion, and inactivation of the enzymes SOD, CAT, and glutathione reductase [82]. The above findings are consistent with the existing studies showed that CAT activity decreases after interacting with TiO_2NPs [95], magnetic nanoparticles [96] or gold [70] and silver [83]. The data obtained from these studies indicated that down regulation of SOD and CAT activity were due to regulation of enzymatic activity by PAOMVs, which increased the susceptibilities of *S. mutans* to oxygen radicals or bioactive substances present in the PAOMVs can generate oxidative stress. Taken together, PAOMVs can act as oxidative stress inducer agents can cause cell death in *S. mutans*. Although several studies reported that biogenesis of extracellular vesicles from Gram negative and positive bacteria, the mechanisms of antibacterial activity of PAOMVs is still remain elusive and the detailed mechanisms of toxicity mediated through redox imbalance remain to be elucidated.

2.7. Effects of PAOMVs on metabolic activity

According to Abdullah-Al-Mahin et al. [97], the activity of lactate dehydrogenase (LDH), a valid marker for evaluating cell health under oxidative and thermal stress conditions, reflects the integrity of the cell membrane. To determine the effects of PAOMVs on LDH activity, *S. mutans* cells treated with PAOMVs in the presence or absence of NAC. As shown in Fig. 6A, there was significant difference in LDH activity PAOMVs-treated groups exhibited 120 U/mLcompared to control group (300 U/mL), indicating that the concentrations of PAOMVs used in this study did cause cell damage. Together, these findings revealed that PAOMVs at appropriate concentrations increase the cell death. Interestingly, the cells pre-treated with NAC have significant effects on LDH activity. Yuan et al. [83] reported that AgNPs potentially influence LDH activity in *P. aeruginosa* and *S. aureus*. These results suggest that *S. mutans*are susceptible to cell death. The possible mechanism of reduced LDH activity is due to disruption of respiratory chain dehydrogenases, which is agreement with previous study, demonstrated that AgNPsinhibited LDH activity in *E. coli* [98], *S. aureus* and *E. coli* [99]. Silver nanoparticles (AgNPs), zinc oxide (ZnO), cupric oxide (CuO), titanium dioxide (TiO2), and aluminium oxide (Al_2O_3) nanoparticles inhibited cellular metabolism by reduction of dehydrogenase activity in *Azotobacter chroococcum*, *Bacillus thuringiensis*, *P. mosselii*, and *Sinorhizobium meliloti* [67].

Lactate dehydrogenase activity is required to maintain glycolysis and ATP production. We hypothesize that the decreased LDH activity could leads to reduced level of ATP production. Thus, we measured the level of ATP levels in PAOMVs treated *S. mutans*. In addition to serving as a key signaling molecule, ATP is an essential chemical needed for a variety of biological processes, such as survival, growth, and replication [100]. *S. mutans* treated with PAOMVs had much lower amounts of ATP (6 nM/mL) than the control samples (Fig. 6B) and H_2O_2-treated cells, on the other hand, drastically alter *S. mutans* metabolic activity via modifying ATP synthesis (8 nM/mL), which in turn influences bacterial growth and reproduction. As we expected that, the loss of ATP synthesis is due to oxidative stress induced redox imbalance. Interestingly, cells treated with NAC ameliorate production of ATP. Consistent with our findings, several studies reported that AgNPs potentially inhibited ATP synthesis in *P. aeruginosa* and *S. aureus* [83]. The possible mechanism of PAOMVs directly affects membranes, specifically FOF1-ATPase activity and H + -coupled transport likely AgNPs [101]. Overall, the results of the current and earlier research point to FOF1-ATPase as a possible target for PAOMVs, which are engaged in cell metabolic functions, including bacterial growth and survival. FOF1-ATPase is found in bacterial membranes and is involved in ATP hydrolysis.

In order to investigate the effect of PAOMVs on the leakage of cytoplasmic proteins, *S. mutans* were treated with MIC of PAOMVs cells content of protein released into the supernatant was quantified by the Bradford method. The results showed that cells treated with PAOMVS causes significantleakage of protein compared to the control cells. Similarly, *S. mutans* treated with H_2O_2- showed high level of protein leakage compared to control (Fig. 6C). The data revealed that after 12 h of incubation in the presence of PAOMVs, the amount of protein leakage released into the supernatant was remarkable compared to untreated group. The cause of leakage of protein could be loss of membrane integrity and membrane disruption. Azam et al. [102] reported that both Gram negative and positive bacteria treated with *Penicillium oxalicum* mediated synthesis of cadmium oxide nanoparticles induce oxidative stress and proteins and sugar leakage. Similarly, AgNPs induce protein leakage in variety Gram negative and positive bacteria [17,83,103].

Further to confirm the effect of PAOMVs on membrane permeability, *S. mutans* cells were treated with MIC of PAOMVs for 12 h and

then leakage of sugars were measured which is anoften used stress marker to observe the membrane damage triggered by stressor molecules like nanoparticles and nanovesicles. The PAOMVs treated cells showed significant leakage of sugars compared to untreated cells (100 μg/mg of protein). Similarly, H_2O_2 treated cells showed noticeable increase level of sugars (120 μg/mg of protein) compared to control (Fig. 6D). The enhanced release of sugars from the cell interior suggests an increase in cell membrane permeability by PAOMVs.*Penicillium oxalicum* mediated synthesis of cadmium oxide nanoparticles increases leakage of sugars in *Staphylococcus aureus*, *Shigella dysenteriae*, and *P. aeruginosa*. AgNPs induces leakage of Gram negative and Gram positive bacteria by loss of membrane integrity [17,54,83]. Overall, our findings, which concurred with earlier research, showed that AgNPs break bacterial membranes, causing macromolecules to seep inside of cells [16]. Based on existing studies and this present study, we proposes possible mechanism of PAOMVs induced antibacterial and antibiofilm activity, which is similar to nanoparticles mediated toxicity in bacteria according to the size and interactions with target cells. As a result, PAOMVs can cause morphological destruction that may be related to the buildup of such toxic substances from PAOMVs in the bacterial membrane, which may have altered the membrane potential and ultimately resulted in cell death. In the case of *S. mutans*, a possible mechanism of death may be the disorganisation of the cell wall, which is associated with the cell debris as a result of cellular destruction. *S. mutans* causes the bacterial cell wall to be physically, mechanically, and morphologically damaged as a result of this destruction. As a result of the release of intracellular material and subsequent cell wall deformation brought on by PAOMVs, the cell wall may become more rough and amorphous mass may be present [67]. Another possible mechanism of PAOMVs induced cell death is due to the interaction of PAOMVs with target bacteria could cause loss of membrane permeability, destruction of cellular respiration, inhibition of the production of bioactive molecules like ATP, production of intracellular oxidative stress that damages cellular components and membranes, destruction of the ability to secrete exopolysaccharides, and elimination of the ability of bacteria to adhere to surfaces. In *S. mutans*, these processes could work separately or together.

2.8. PAOMVs increased sensitivity of S. mutans to antibiotics

Among various antibiotics tested,*S. mutans* showed more sensitivity towards ampicillin and vancomycin, hence we selected these two antibiotics for further experiments. To determine the combination effect of antibiotics and PAOMVs on cell viability of *S. mutans*, the cells were treated with sublethal concentration of PAOMVs and antibiotics for 12 h. *In vitro* killing studies were performed to explore the possibility of using PAOMVs and antibiotics as combination agent. *S. mutans* were treated with sublethal concentrations of PAOMVs or ampicillin or vancomycin or combination of PAOMVs and ampicillin. The addition of sublethal concentrations of PAOMVs to these antibiotics treatments resulted in significantly enhanced antimicrobial activity ($p < 0.05$) (Fig. 7A). Interestingly, PAOMVs showed an enhanced antibacterial effect with tested antibiotics against *S. mutans* compared to control or antibiotics alone. The most significant effects were observed with ampicillin and with vancomycin toward *S. mutans*.

Bacterial cells were cultured to form biofilms and then treated with PAOMVs alone or in combination with antibiotics to determine whether sublethal quantities of PAOMVs have synergistic effects. The results showed that the biofilm activity was greatly reduced by 20 % by PAOMVs (2.5 μg/mL) alone. The biofilm activity of *S. mutans* was reduced by 80 % when PAOMVs and ampicillin were

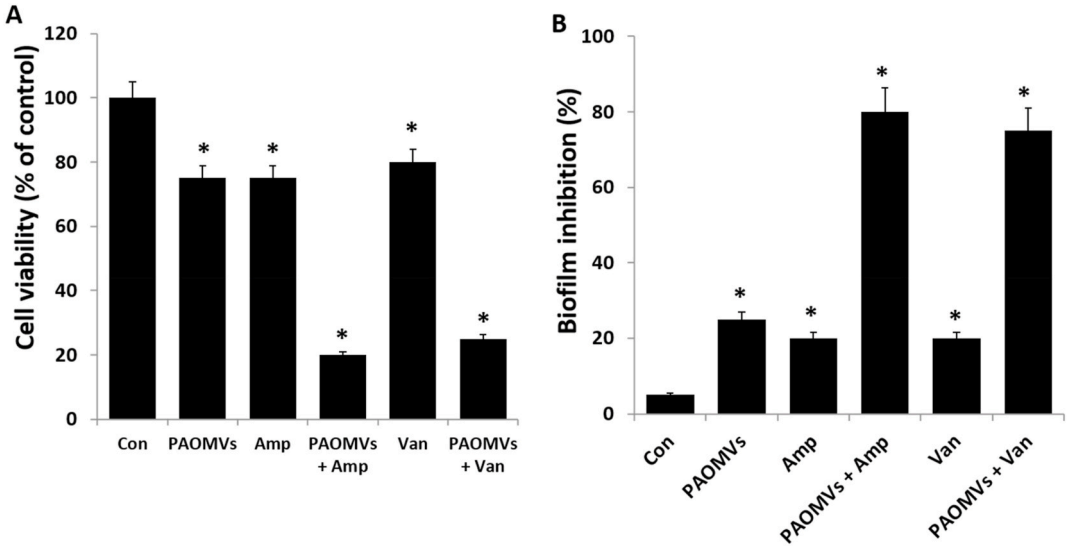

Fig. 7. PAOMVs enhances antibacterial and antibiofilm activity of antibiotic
(A) *S. mutans* cells were incubated with sublethal concentrations of PAOMVs or with sublethal concentrations of ampicillin or vancomycin or combinations of PAOMVs and ampicillin or combinations of PAOMVs and vancomycin for 12 h (A) Bacterial survival was determined at 12 h by the CFU assay (B) biofilm activity was determined. The results are expressed as the means ± SD of three separate experiments, each of which contained three replicates. Treated groups showed statistically significant differences from the control group by the Student's *t*-test ($p < 0.05$).

combined, and by 75 % when PAOMVs and vancomycin were combined. Overall, these results demonstrate that PAOMV and antibiotic treatments together improved levels of cell death and biofilm activity inhibition (Fig. 7B). Therefore, combining PAOMVs with various antibiotics at lower doses may result in an efficient *anti*-biofilm and antibacterial therapy. Both Gram-negative and Gram-positive bacteria responded favourably to ampicillin's antimicrobial activities. Combining these treatments had a similar inhibitory effect on biofilm activity. According to a previous study [83], *P. aeruginosa, Shigella flexneri, S. aureus, and Streptococcus pneumonia* all exhibit enhanced antibacterial and antibiofilm activity when drugs are combined with silver nanoparticles. According to Dhital et al. [104], direct transfer of the β-lactamase enzyme from OMVs to susceptible bacteria raised the MIC for AMX in susceptible *N. gonorrhoeae*. Our research is the first to demonstrate the significance of naturally occurring PAOMVs from *P. aeruginosa* in the fight against *S. mutans*. Kadurugamuwa and Beveridge [27] discovered that the OMVs were significantly more potent when the cells were cultured in the presence of a sub-inhibitory concentration of gentamicin. Furthermore, the gentamicin-resistant strain of *P. aeruginosa*, *P. aeruginosa* 8803, was successfully eradicated by the antibiotic-loaded OMVs. Murein hydrolase, found in *P. aeruginosa*-derived OMVs, may break apart the *S*-layer, the planar paracrystalline structures on both Gram-negative and Gram-positive bacteria [105]. This enzyme can also degrade peptidoglycan. These results revealed that the peptidoglycan-degrading enzymes present in the OMVs were the cause of the lytic behavior of the OMVs [28]. Due to the presence of cystobactamids within the OMVs, two additional myxobacterial strains, SBSr073 and Cbv34, as well as OMVs produced by CBv34 and Cbfe23, were also shown to inhibit the growth of *Staphylococcus aureus* cells intracellularly. According to Meers et al. (2018), OMVs produced from *Lysobacter enzymogenes* have chitinase activity and can stop the growth of the fungus *Saccharomyces cerevisiae* and *Fusarium subglutinans*.

3. Conclusion

The increasing prevalence of microbial resistance is an important issue to manage and reduce public health problems in the modern world and estimated to be a leading cause of mortality by 2050. New therapies with distinctive modes of action against numerous drug-resistant and biofilm-forming species are urgently needed to treat life-threatening infections as a result of rising antimicrobial resistance. Although a number of novel antibiotics were created in response to the appearance of new resistance strains, none have improved efficacy against bacteria that are multidrug resistant. To treat Gram negative and Gram positive infections, it is crucial to create alternative, biocompatible, and more successful therapeutic approaches. Hence, we prepared OMVs from *P. aerudginosa*. Bacterial extracellular vesicles are capable of acting as a vehicle for the administration of antibiotics due to their innate antibacterial activity. They may be protected from degradation by degrading enzymes as a result of their antimicrobial cargo chemicals' natural packing inside OMVs, which could reduce the chance of the creation of new resistances. Recently, outer membrane vesicles of Gram negative bacteria showed significant antibacterial effects; however, the underlying mechanism is still not understood. Thus, the aim of this investigation was to characterize the mechanism of antibacterial properties of *P. aeruginosa* derived OMVs as well as to determine antibiofilm properties against *S. mutans.*PAOMVs derived from PAOMVs have shown promising qualities in terms of size distribution and storage stability. The use of bacteria as EV sources is appropriate because they can be grown at high yields on an industrial scale, which helps the current method be translated into the clinic. We isolated OMVs from *P. aeruginosa*and evaluated antibacterial and antibiofilm activity of OMVs against dental plaque causative agent *S. mutans*. The study found that *P. aeruginosa* outer membrane vesicles (PAOMVs)dose-dependently inhibits cell viability and biofilm formationthroughincreased level ofgeneration of reactive oxygen species and decreased level of antioxidants. Furthermore, PAOMVs significantly affects metabolic activity and leads to cell death. This study presented evidence of antibacterial and *anti*-biofilm effects of PAOMVs and their enhanced ability against *S. mutans*. Furthermore, our study suggests that combination of PAOMVs and sub-lethal concentration of antibiotics showed significant effects either PAOMVs or antibiotics. These extracellular vesicles contained cargo molecules unique to the *P. aeruginosa* origin and with known antibacterial properties, which displayed a pronounced effect on *S. mutans* and could be associated with nanomechanical alterations consistent with membrane damage. Hence, our findings concluded that PAOMVs could be used as an enhancer with sub-lethal concentration of antibiotic for the treatment of infectious diseases. The mechanism of antibacterial and antibiofilm activity of PAOMVs is remainingelusive; therefore more studies are warranted to pinpoint the mechanism of antibacterial activity. Pharmaceutical industry need to pay more attention develop new antibiotics and vaccines using the multifaceted OMVs derived from *P. aeruginosa* against pathogenic Gram negative and positive bacteria. Bacterial extracellular vesicles play significant role on next-generation vaccines as nanoparticles against AMR, highlighting their engineering for vaccines development. These findings could pave the way for the creation of an entirely new class of antibiotics that are efficient against difficult-to-kill bacteria.

4. Materials and methods

Luria–Bertani (LB), nutrient broth and Brain Heart Infusion broth and agar was purchased from USB Corporation (Santa Clara, CA, USA). All other chemicals and toxicology assay kits were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. BacTiter-Glo™ Microbial Cell Viability Assay Reagent was purchased from Promega (Madison, WI, USA).

4.1. Bacterial strains and growth conditions

P. aeruginosa (ATCC 27853) and *S. mutans* (ATCC10449) strains obtained from the American Type Culture Collection. Stock cultures were maintained on Nutrient Agar (NA), Sigma-Aldrich, USA) slants, and sub-cultured onto freshly prepared NA plates and incubated at 37 °C for 24 h. A previously published procedure was followed to prepare the media and cultivate the bacteria [83]. Briefly, BHI media was used to develop all the cultures first under aerobic conditions at 37 °C. By streaking a bacterial colony on BHI

agar plates and sub-culturing once every two weeks, cultures were kept alive. The isolated, pure colonies were kept at 37 °C. Then *P. aeruginosa* cells were grown in BHI medium for overnight in BHI broth at 37 °C and centrifugation was performed at 6000 rpm for 10 min was used to harvest the cells, and then resuspended in sterile BHI media.

4.2. Isolation and characterization of OMVs from Pseudomonas aeruginosa

A few minor adjustments were made to the previously published approach for the isolation of outer membrane vesicles [106]. In a nutshell, a *P. aeruginosa* overnight culture was diluted 50 times in 500 mL of BHI and incubated at 37 °C for 16–18 h with 150 rpm of agitation. Centrifugation was used to remove the culture (8000 g for 15 min at 4 °C). A vacuum pump with 0.22-m-pore size filtering was used to remove the supernatant after it had been collected (Merck, Millipore, Billerica, MA, USA). Using 100 kDa centrifugal concentrators (Amicon Ultra, Merck Millipore, Cork, Ireland), the filtrates were concentrated at 5000 g for 30 min At 4 °C. Following that, the concentrated supernatants were pelleted (100,000×*g*, 4 °C) overnight in an ultracentrifuge (Beckman Coulter Optima L-90 K, USA). PAOMVs-containing pellets were re-dissolved in 500 μL of sterile PBS buffer (pH 7.4), aliquoted, and kept at 4 °C for further use. Using a Pierce ™ BCA Protein Assay Kit from Thermo Scientific, Waltham, Massachusetts, USA, the total protein content of PAOMVs was calculated. Using a Nanodrop 2000 spectrophotometer (Thermo-Scientific, US) at a wavelength of 280 nm, the quantity of protein and the concentration of isolated OMVs were determined. Transmission electron microscopy (TEM) and scanning electron microscopy were used to examine OMVs' structure and surface morphology. The dynamic light scattering (DLS) method was used to assess the size distribution. A NanoSight NS300 equipment (Malvern, UK) was used to detect the particle size and concentration [107].

4.3. Determination of MIC and sub-lethal concentration of PAOMVs and antibiotics

PAOMVs susceptibility tests were performed in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2003) using 96-well microtiter plates and a standard two-fold broth microdilution of the antibacterial agents in BHI. The disc diffusion experiments were conducted as previously described [17]. *S. mutans* cells were treated with various concentrations of PAOMVs ranging from 0 to 100 μg/mL and then we calculated the MICs of the PAOMVs. Phosphate-buffered saline (PBS) was used to generate PAOMVs solutions, and their antibacterial potency was evaluated. 1 mL of the bacterial suspension and the appropriate PAOMVs concentration were combined in BHI media to produce a final bacterial concentration of 10^5–10^6 colony forming units (CFUs)/mL, which was then incubated for 24 h. Immediately following treatment, 100 μL of the reaction mixture was diluted to 1 mL, and 100 μL of the entire mixture was utilized for plating. Utilizing the colony counting technique, cell viability loss was assessed. Stock solutions of antibiotics were made in microbial culture medium for each microbial species and sterilized by filtration to determine the antibiotics' minimum inhibitory concentrations (MICs; Sigma Chemical Co., St. Louis, MO, USA) [17]. The PAOMVs and antibiotic minimum inhibitory concentrations (MICs) were established as the lowest concentrations that prevented the bacteria's outward signs of growth **(Table 1).** PAOMVs or antibiotic dosages that reduced the number of sensitive cells by less than 20 % after 24 h of incubation were deemed to as "sub-lethal" **(Table 2).** A variety of sub-lethal concentrations of PAOMVs or antibiotics alone or in combination with one another were used in the viability testing.

4.4. Antimicrobial activity of PAOMVs

Overnight cultures of *S. mutans*were centrifuged at 6000 rpm for 5 min to assess the impact of PAOMVs on the growth of the isolates. The pellet was then resuspended in saline buffer. Finally, the sample's OD600 was changed to 0.1. Cells of *S. mutans* (5×10^5 cells in triplicate in 96-well round-bottom plates) were treated with various concentrations of PAOMVs. At the specified time points or dose responses, bacteria were collected, and the CFU count was performed. The controls were media only and media with PAOMVs. Values were expressed as the average of three separate trials, and each sample was plated in triplicate.

4.5. Determination of biofilm activity using the tissue culture plate method (TCP)

This assay was performed to determine the ability of PAOMVs to inhibit biofilm activity of *S. mutans*. This assay is based on colorimetric measurements of the crystal violet incorporated by sessile cells. The percentage inhibition of biofilm activity was calculated using the following equation: [1 - (A_{595} of cells treated with PAOMVs/A_{595} of non-treated control cells)] × 100 [17,65,66]. Experiments were performed in triplicate. The data are expressed as means ± SD.

4.6. In vitro cytotoxicity assays

With the necessary changes, *in vitro* cytotoxicity experiments were carried out as previously described [84]. *S. mutans* cells were

Table 1
Determination of MIC value of antibiotics (μg/ml) and PAOMVs (μg/ml).

Bacterial Species	Amp	Chl	Ery	Tet	Van	PAOMVs
Streptococcus mutans	0.5	2.0	1.0	1.0	1.0	1.0

Table 2
Determination of sub-lethal concentration of antibiotics (μg/ml) and PAOMVs (μg/ml).

Bacterial Species	Amp	Chl	Ery	Tet	Van	PAOMVs
Streptococcus mutans	0.2	0.5	0.4	0.4	0.3	2.5

first cultured in fresh medium for 24 h before centrifugation and suspension in deionized water after growing overnight at 37 °C in BHI broth. A cell suspension containing 10^6 cells per milliliter was treated with different PAOMV doses for 24 h at 37 °C. At the designated time points after incubation, bacteria were harvested, and 100-μL aliquots were obtained from each sample to count the CFUs.

4.7. Bacterial cell lysate preparation

Bacterial cells were cultivated and centrifuged at 5000 rpm for 10 min at 4 °C to generate bacterial cell lysates. The resulting pellet was then washed with PBS and resuspended in bacterial lysis buffer (Thermo Fisher Scientific). Lysozyme was then added, and the mixture was allowed to sit at 4 °C for 4 h before being subjected to 5 min of probe sonication (the samples were sonicated three times for 30 s each, with a 2 min break in between each sonication). Centrifugation at 10,000 rpm was employed to remove cell debris. The supernatant was then collected and used for enzyme tests. Antioxidant and oxidative indicators were assessed as previously mentioned [79].

4.8. Estimation of GSH levels

S. mutans cells were cultured at the necessary temperature with or without PAOMVs for 12 h to determine GSH levels enzymatically. Cells were centrifuged at 10,000 rpm for 5 min to pellet them, then they were PBS-washed and lysed. The lysate was created as previously mentioned. Based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by the GSH reductase system as previously described [88], GSH levels in the clear supernatant were calculated.

4.9. Determination of GST total activity

The GST activity was calculated as previously explained with the necessary changes [88]. 900 mL of pH 6.5 potassium phosphate buffer, 25 mL of 40 mM 1-chloro-2,4-dinitrobenzene, 50 mL of 1 mM GSH, and 25 L of enzyme extract were combined to perform a spectrophotometric test for GST at 37 °C. For 5 min, the increase in absorbance at 340 nm was measured to track the reaction mixture. The GST activity was measured in mol/min/mg of protein.

4.10. Determination of superoxide dismutase and catalase activity

A previously described procedure was modified appropriately to evaluate catalase activity [88]. The total protein content and enzyme activity in the bacterial cell lysate, as determined by the Bradford technique, were used to compute the specific enzyme activity [64].

4.11. Assay for the leakage of proteins and reducing sugars

The amount of protein leakage from bacterial cells was calculated as previously stated, and the method for calculating the amount of sugar leakage was also previously described [54,79].

4.12. Measurement of LDH activity

A previously established approach [53,108] was used to measure the reduction of NAD + to NADH and H+ during the oxidation of lactate to pyruvate in order to calculate the LDH activity.

4.13. Measurement of ATP levels

The ATP levels in the bacterial culture supernatant were measured using the techniques outlined in the previous sections [109, 110].

4.14. Statistical analysis

At least three duplicates of each experiment were performed on each subject. Results are shown as means and standard deviations. The Student's t-test was used to compare all experimental data. Statistically significant was defined as $p < 0.05$. One-way ANOVA was employed for groups with more than two participants. *P 0.05, **P 0.01 and ***P 0.001, vs. control.

Data availability

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

Not applicable.

CRediT authorship contribution statement

Sangiliyandi Gurunathan: Writing – review & editing, Writing – original draft, Conceptualization. **Pratheep Thangaraj:** Software, Formal analysis. **Joydeep Das:** Methodology. **Jin-Hoi Kim:** Validation, Supervision, Resources, Investigation, Funding acquisition.

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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