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Lekha Sree Venkatesan

Saveetha Dental College and Hospital, SIMATS, Saveetha University

Sudharsan Ganapathi

Saveetha Medical College and Hospital, SIMATS, Saveetha University

Palanivel Sathishkumar

salemsathishkumar@gmail.com

Saveetha Dental College and Hospital, SIMATS, Saveetha University

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Effective eradication of multidrug-resistant *Acinetobacter baumannii* biofilm using hesperetin-silver nanoformulation

Lekha Sree Venkatesan^{a,#}, Sudharsan Ganapathi^{b,#}, Palanivel Sathishkumar^{a,*}

^aGreen Materials Laboratory, Department of Prosthodontics, Saveetha Dental College and Hospital, SIMATS, Saveetha University, Chennai, Tamil Nadu 600 077, India

^bSaveetha Medical College and Hospital, SIMATS, Saveetha University, Chennai, Tamil Nadu 600 077, India

***Corresponding author.** Email: salemsathishkumar@gmail.com (P Sathishkumar)

[#]These authors equally contributed to this work.

Abstract

The World Health Organization categorized *Acinetobacter baumannii* (carbapenem-resistant) is one of the priority pathogens and also advised to develop potential therapeutics to avoid the spreading of multidrug resistance. This study aims to assess the antibacterial and antibiofilm efficacy of hesperetin-mediated silver nanoparticles (Hes-AgNPs) against multidrug-resistant *A. baumannii* (MDRAB). In this study, 54 clinical samples were screened from hospitalized patients to isolate the MDRAB and further identified using the VITEK®2 Compact System. The antibacterial potential of different phytochemicals was explored against the isolated MDRAB strains. The antibacterial potential of phytochemicals was determined as follows: curcumin (12.9 mm) > morin (13.4 mm) > quercetin (13.7 mm) > naringin (15.7 mm) > hesperetin (16.9 mm). The promising phytochemical hesperetin was employed to synthesize Hes-AgNPs and further evaluated their antibiofilm potential against the MDRAB strains using MTT assay, CFU determination, and CLSM analysis. The MIC values of Hes-AgNPs were found as 75-105 µg/mL against MDRAB strains. A complete eradication of preformed MDRAB biofilm was achieved at 1.5xMIC (112-157 µg/mL) of Hes-AgNPs. Hes-AgNPs demonstrated great hemocompatibility. The present study suggests that Hes-AgNPs might be an effective drug to treat MDRAB biofilm and infections.

Keywords: Antibiofilm; *Acinetobacter baumannii*; Multidrug resistance; Hesperetin; Silver nanoparticles; Hemocompatible drug

Introduction

Acinetobacter baumannii, a Gram-negative coccobacillus, is an emerging multidrug-resistant bacteria [1]. It is considered to be a leading cause of both community and hospital-acquired infections, which pose clinical challenges and result in high mortality [2,3]. In general, hospital-acquired *A. baumannii* infections are higher persistence in nature due to biofilm formation, quorum signaling, and virulence factors [4,5]. In recent days, the multidrug-resistant *A. baumannii* (MDRAB) infection cases in hospitals have been increased alarmingly worldwide [3,6,7]. Drug-resistant *A. baumannii* increases morbidity and death rates in vulnerable groups since there are limited treatment options [8].

In recent update, World Health Organization (WHO) categorized *A. baumannii* (carbapenem-resistant) as a critical priority bacterium in the revised Bacterial Priority Pathogens List (BPPL) [9]. Also, BPPL recommended to develop a novel and advanced therapeutic approach to avoid the spreading of multidrug resistance [9]. Therefore, it is essential to explore the prevalence of *A. baumannii* infections and its multidrug resistance profile to achieve successful clinical treatment. The virulence of *A. baumannii* has increased not only due to multidrug resistance but also the potential to form biofilms [10]. Evidently, Mendes et al. [11] noted that biofilm-forming genes are potentially involved in the co-regulation of multidrug resistance. Thus, it is critical to find an alternative drug with significant therapeutic advantages for treating *A. baumannii* infections and its biofilm [12]. So far, exploring to find potential therapeutics that can effectively cure *A. baumannii* infection and biofilm is a hot topic of research.

Currently, several approaches are being investigated to control *A. baumannii* pathogenicity and biofilm formation [5,11]. In particular, phytochemicals such as carvacrol, curcumin, cinnamaldehyde, quercetin, catechin, geraniol, and eugenol demonstrate strong therapeutic potential to treatment drug-resistant *A. baumannii* and their biofilms [12,13]. Recent studies highlight that hesperetin exhibit excellent antimicrobial and antibiofilm potential [14,15]. Hesperetin, a trihydroxyflavanone mainly found in citrus fruits like grapefruits, lemons, and sweet oranges. demonstrates broad-spectrum antimicrobial activity [15,16]. It has been attracted as an alternative to conventional antimicrobial agents to overcome various infectious diseases [14]. However, in-depth investigation into the antibacterial and antibiofilm effectiveness of hesperetin against MDRAB has not yet been explored extensively. Phytochemicals have certain limitations during the real-world application in medical field due to limited bioavailability and poor solubility. To overcome such limitations, several nanoformulation techniques have been extensively explored [17]. Among that, phytochemicals coated metal nanoparticles exhibits great therapeutic potential to treat various microbial diseases and cancer [18]. Among metal nanoparticles, phytochemicals coated silver nanoparticle (AgNPs) demonstrated great therapeutic potential to treat various infectious diseases [19,20].

The present study aimed to successfully eradicate *A. baumannii* biofilm using hesperetin-mediated AgNPs (Hes-AgNPs). Herein, the prevalence of *A. baumannii* infections among hospitalized patients and their drug resistance profile was investigated. Besides, antibacterial potential of various phytochemicals was explored against the isolated MDRAW strains. Furthermore, the promising phytochemical hesperetin was used to synthesis Hes-AgNPs and assessed their antibiofilm efficacy against the isolated MDRAW strains. Finally, the hemocompatibility of Hes-AgNPs was determined on human red blood cells.

Materials and methods

Chemicals

Phytochemicals such as morin, naringin, curcumin, quercetin, hesperetin were procured from TCI (Tokyo Japan). Mueller–Hinton agar (MHA), Mueller-Hinton broth (MHB), nutrient agar, and nutrient broth, standard antibiotics discs, thiazolyl blue tetrazolium blue (MTT), silver nitrate, and crystal violet were purchased from HiMedia (Mumbai, India). LIVE/DEAD BacLight Bacterial Viability Kit was obtained from Thermo Fisher Scientific Company (Oregon, USA).

Ethical approval

The present study was approved by the Institutional Ethics Committee (Approval No.: 874/09/2026/UG/SRB/SMCH) of Saveetha Institute of Medical and Technical Sciences, SIMATS, Tamil Nadu, India.

Screening, isolation, identification, and antibacterial activity assessment

In this study, 54 clinical samples from skin/wound infections, respiratory infections and UTIs were screened to isolate the drug-resistant *A. baumannii*. The isolates were identified using the VITEK®2 Compact System at SIMATS, Tamil Nadu, India. The drug resistance profile of the isolated *A. baumannii* strains were screened with standard antibiotics discs on MHA plates using the disc-diffusion techniques. The antibacterial activity of phytochemicals such as morin, naringin, curcumin, quercetin, and hesperetin was tested against the isolated *A. baumannii* strains by zone of inhibition (ZOI) measurement on MHA plate through the agar-well diffusion technique and also determined by the minimum inhibitory concentration (MIC) using the broth micro-dilution technique. To examine ZOI, 250 µg of morin, naringin, curcumin, quercetin, and hesperetin were loaded into 9 mm wells of 0.5 McFarland *A. baumannii* as an inoculum seeded MHA plates. The cultured plates were then incubated overnight at 37 °C in a bacteriological incubator. The ZOI of phytochemicals was measured against each isolated strain, and the results were expressed as mm in diameter. The CLSI reference was applied to

determine the MIC of the phytochemicals via broth micro-dilution method [21]. Aseptically, 170 μ L of MHB were added to each well of the 96-well microtiter plate. Then, hesperetin (500 μ g/mL) and Hes-AgNPs (200 μ g/mL) suspension was added to the first well and serially diluted in MHB. Finally, 10 μ L of overnight grown bacterial culture (.5 McFarland) was inoculated into each well and incubated at 37 °C. Finally, the microplate was read at 600 nm using a BioTek Synergy microplate reader and determined the MIC values.

Synthesis and characterization of Hes-AgNPs

The AgNPs were synthesized using a 1:1 ratio (200 mM:200 mM) of hesperetin and silver nitrate in 100 mL of deionized water at pH 8.0 and 40 °C. The formation of AgNPs was confirmed after 10 min by the reaction solution turned dark brown. Further, the surface plasmon resonance (SPR) of Hes-AgNPs was observed by a UV-visible spectrophotometer within the range of 350–500 nm. The physicochemical property of the Hes-AgNPs was studied using high-resolution transmission electron microscopy (HREM, JEM-2100HR, Japan) equipped with energy-dispersive X-ray spectroscopy (EDX), attenuated total reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR; Bruker Alpha II, Germany), and X-ray diffractometer (XRD; Bruker D8 Advance, Germany).

Assessment of biofilm formation ability

The biofilm formation capability of the isolated *A. baumannii* strains was measured by the crystal violet staining method. Briefly, *A. baumannii* (5×10^5 CFU/mL as on inoculum) isolates biofilm was grown in nutrient broth at 37 °C for 72 h. After that, the culture supernatant was removed from the well and washed with phosphate-buffered saline (PBS, 50 mM). A 100 μ L solution of crystal violet (1% w/v) was added to the well to stain the attached bacterial cells in biofilm. After 5 minutes, the unbound stain was gently pipetted out of the well. To dissolve the cells adsorbed stain, 100 μ L of acetic acid (33% w/v) was added to the well and vortexed. Finally, the absorbance of the control and test samples was recorded at 590 nm, and the biofilm producers were classified as none, weak, moderate, or strong.

Antibiofilm studies

The antibiofilm potential of hesperetin and Hes-AgNPs was assessed against the isolated *A. baumannii* by inhibiting bacterial cells in preformed biofilm. In brief, the biofilm of the isolated strains was developed in a 96-well plate at 37 °C with nutrient broth for a week in a bacteriological incubator. The culture medium (nutrient broth) was periodically changed every 24 h interval. The preformed *A. baumannii* strains biofilm was then treated with different MIC of hesperetin and Hes-AgNPs, and further incubated for 24 h. After treatment, the culture supernatant was removed, and preformed *A. baumannii* was gently rinsed with 100 μ L of PBS (50 mM). The

treated biofilm was then subjected to colony forming unit (CFU) determination, MTT assay and CLSM analysis [22]. The CFU of bacteria in the control and treated preformed biofilm was determined according to the method described previously [23]. The MTT assay was used to determine the viable cells in the preformed biofilms. For MTT assay, 100 μ L of 0.05% MTT solution was dropped on the preformed *A. baumannii* biofilm (control and treated) and further incubated for 3 h under dark conditions. After incubation, formazan crystals were homogenized with 100 μ L DMSO and absorbance was recorded at 570 nm wavelength. The cell viability in the preformed biofilm was calculated as follows (eq. 1):

$$\text{Cell viability (\%)} = \frac{\text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Control}}} \times 100 \quad (1)$$

The CLSM was used to distinguish between viable and dead cells in the preformed *A. baumannii* biofilm after treatment. To facilitate CLSM analysis, treated and control preformed *A. baumannii* biofilms were stained with the LIVE/DEAD BacLight Bacterial Viability Kit for 30 min in the dark at room temperature. The CLSM (Leica Microsystem CMS GmbH-DMI8, Germany) was performed to visualize both viable and dead cells in the stained control and hesperetin-treated biofilms at 10x magnification.

Hemocompatibility assessment

In vitro hemolysis experiment was carried with human RBCs to confirm the hemocompatibility nature of hesperetin and Hes-AgNPs. In brief, the RBCs were separated from freshly drawn blood samples and diluted with PBS (50 mM). Then, it was treated with various concentrations of hesperetin (up to 1000 μ g/mL), Hes-AgNPs (up to 750 μ g/mL), and chemically synthesized AgNPs (Che-AgNPs) (up to 750 μ g/mL) at 37 °C for 60 mins. In parallel, 1% Triton X-100-treated RBCs was maintained as a positive control. The absorbance of control, hesperetin, Hes-AgNPs, Che-AgNPs treated samples was measured at 540 nm using a BioTek Synergy microplate reader. The percentage of hemolysis was calculated as follows (eq. 2):

$$\text{Hemolysis (\%)} = \frac{A_T - A_C}{A_{PC} - A_B} \times 100 \quad (2)$$

where, A_T , A_C , A_{PC} , and A_B are represents the absorbance of test, control, positive control, and blank, respectively.

Statistical analysis

The experiments were carried out in triplicate and results were expressed as the mean with standard deviation (SD). The data was plotted in OriginPro 2018 tool. The statistical analysis was assessed using ANOVA followed by the Tukey post-test using the GraphPad Prism 7.0. The significance was defined as follows: $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), and $P \leq 0.0001$ (****) as significant, highly significant, and extremely

significant.

Results and discussion

Prevalence of drug-resistant *A. baumannii*

Out of 54 clinical samples, bacterial growth was observed in 39 samples (72%). These 39 isolates obtained from the skin/wound infections, respiratory infections, and UTIs were identified as *Acinetobacter baumannii* (three isolates), *Escherichia coli* (five isolates), *Klebsiella pneumoniae* (four isolates), *Pseudomonas aeruginosa* (ten isolates), *Enterococcus faecalis* (three isolates), *Proteus mirabilis* (two isolates), *Serratia rubidaea* (one isolate) and *Staphylococcus aureus* (eleven isolates). In this investigation, *A. baumannii* was chosen to further explore drug susceptibility and biofilm formation in order to find a potential natural drug for the treatment since WHO categorized *A. baumannii* as a critical priority bacterium in the revised BPPL [9]. All of the *A. baumannii* strains were isolated from male patients with LRTIs between the age group of 55-74. The drug susceptibility of the three *A. baumannii* strains (isolate IA018, isolate IA036, and isolate IA057) was evaluated on MHA plates via the disc-diffusion technique. It is important to note that all three isolates were resistant to the tested antibiotics, except colistin (Figs. 1 and 2A).

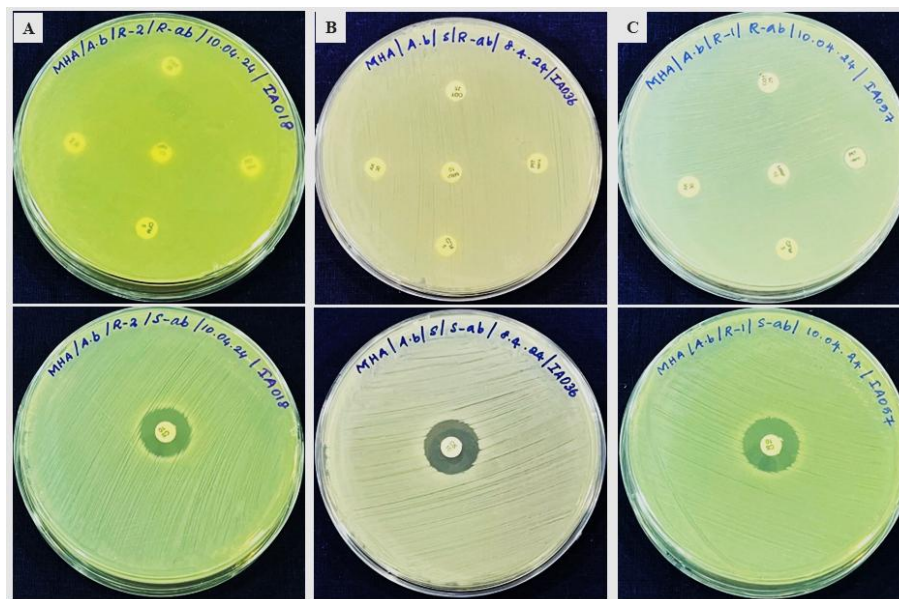


Figure 1. Drug-resistant profile screening of *A. baumannii* isolates (A) IA018, (B) IA036, and (C) IA057. Amikacin (AK-30mcg), Cefotaxime (CFM-5mcg), Co-trimoxazole (COT-25mcg), Piperacillin (PIT-100/10), Meropenem (MRP-10mcg), and Colistin (CS-10mcg).

This finding demonstrates that isolates *A. baumannii* IA018, *A. baumannii* IA036, and *A. baumannii* IA057 are multidrug-resistant strains. Several investigations have been reported the prevalence of MDRAB as well as

the genes responsible for the drug-resistance. For example, Bian et al. [24] isolated carbapenem-resistant *A. baumannii* (CRAB) from patients suffering from hospital-acquired infection in China, which can be associated with *bla*_{OXA-23} gene. Jain et al. [25] found carbapenemase encoded genes (*bla*_{OXA-23-like} and *bla*_{NDM-1}) in 96% of *A. baumannii* strains isolated from patients with LRTIs, and all of these isolates were colistin sensitive. Furthermore, the study noted that CRAB is a major cause of LRTIs. Jalal et al. [26] identified numerous antimicrobial genes with various resistance mechanisms (β -lactamases and antibiotic efflux pumps) in MDRAB strains isolated from a cancer hospital in Egypt. Leungtongkam et al. [27] found 84.9% of CRAB and 7.9% of MDRAB strains among the 339 *A. baumannii* isolates obtained from Thai hospitals. Yousefi Nojookambari et al. [28] reported 80% of multidrug resistant and 35.7% of extensively drug-resistant strains among the isolated *A. baumannii* stains from the Iranian children's hospital. Overall, the obtained drug-resistant results underline the need for effective alternative drugs to treat MDRAB infections and biofilms before spreading in the environmental compartments.

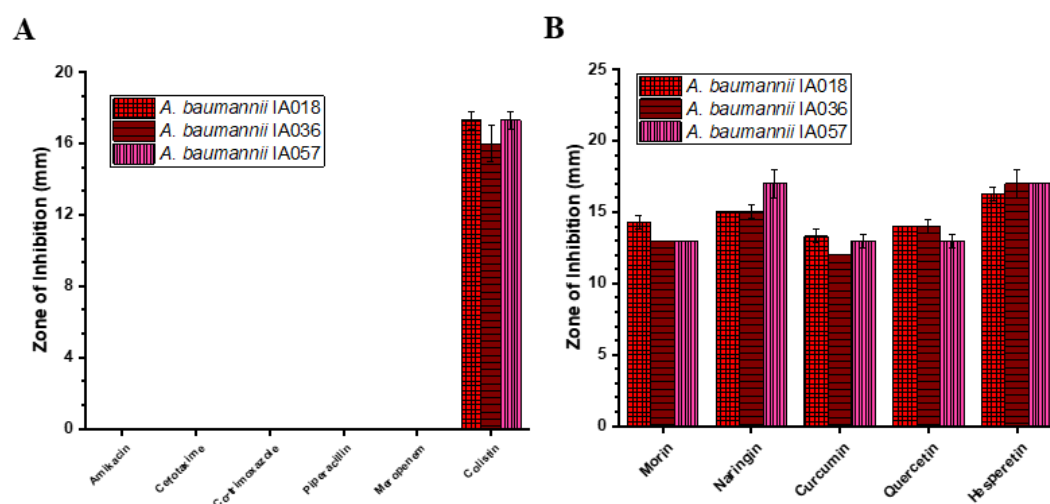


Figure 2. Antibacterial activity comparison. (A) Drug-resistant profile and (B) antibacterial activity of different phytochemicals. The control ant test groups demonstrated a significant difference ($P = \leq 0.001$).

Screening of potential antibacterial phytochemicals

The antibacterial effect of phytochemicals was evaluated against the isolated *A. baumannii* strains on MHA plates. The ZOI was noted for *A. baumannii* IA018, IA036, and IA057 strains as 14.3 mm, 13 mm, and 13 mm for morin; 15 mm, 15 mm, and 17 mm for naringin; 13.3 mm, 12 mm, and 13.3 mm for curcumin; and 14 mm, 14 mm, and 13 mm for quercetin; 16.3 mm, 17 mm, and 17.3 mm for hesperetin at a concentration of 250 μ g level, respectively (Figs. 2B and 3). Based on ZOI results, the average antibacterial potential of phytochemicals was determined as follows: curcumin (12.9 mm) > morin (13.4 mm) > quercetin (13.7 mm) > naringin (15.7 mm) > hesperetin (16.9 mm). Among the tested phytochemicals, hesperetin exhibits considerably higher antibacterial activity against the

isolated MDRAW strains. Therefore, hesperetin was chosen to explore further antibiofilm effectiveness evaluation.

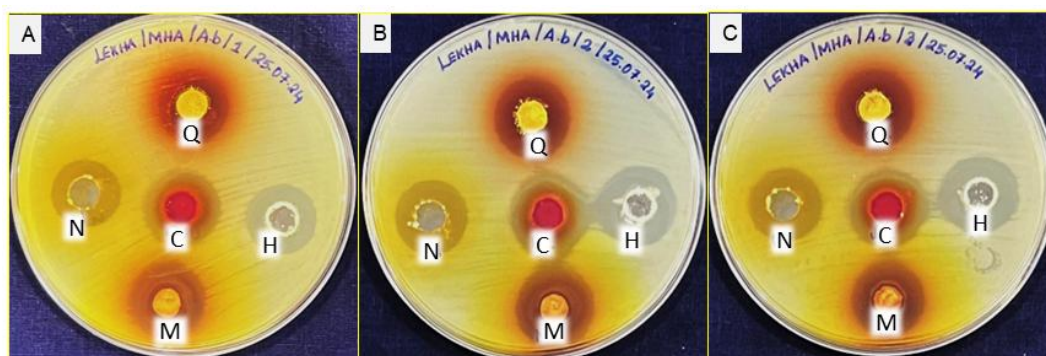


Figure 3. Antibacterial potential of phytochemicals against *A. baumannii* isolates (A) IA018, (B) IA036, and (C) IA057. C – curcumin, M – morin, Q – quercetin, N – naringin, and H – hesperetin.

Hesperetin (Fig. 4A insert) is a bioflavonoid, which is mainly found in citrus fruits [29]. It has numerous beneficial effects like anti-inflammatory, antimicrobial, antidiabetics, anticancer, anti-Parkinson effects, protects cardiovascular function, and excellent wound healing efficiency [14,15,29,30]. Previously, few studies explored with different phytochemicals to treat *A. baumannii* strains. For example, Álvarez-Martínez et al. [31] and Barbieri et al. [32] highlighted that phytochemicals are showed excellent antibacterial potential because they hinder protein synthesis, suppression of virulence factors, and damage the cell walls and membrane structure. Shamsudin et al. [33] noted that the functional groups and their positions of chemical structures determine the antibacterial potential of phytochemicals against the microorganisms. *Hibiscus sabdariffa* calyces can be employed as an alternative for synthetic antibiotics that are ineffective against MDRAW [34]. Antibacterial potential of some specific production against *A. baumannii* (including, drug resistance) have been explored in previous investigations. For example, Ušjak et al. [35] found that the bioactive substances chalcone (trifluoromethyl-substituted derivative; p-CF₃) potentially inhibited the twitching, surface motility, and biofilm formation of *A. baumannii*. Later, the same team noted that dihydrochalcones inhibited dual-species biofilm formation of *A. baumannii* and *Candida* species through the downregulation of ompA activity [36]. Miyasaki et al. [37] reported that norwogonin present in *Scutellaria baicalensis* potentially inhibits the clinically relevant MDRAW. Kızılyıldırım et al. [38] found that *Laurus nobilis* extract (rutin and ferulic acid) exhibited excellent antibacterial potential against CRAB. Also, molecular docking investigation demonstrated that the binding efficiency of rutin with OXA-51 was significantly higher compared to standard drug doripenem. Mahdally et al. [39] reported that justicidin B (arylnaphthalene lignan) present in *Origanum majorana* L. demonstrated potential antibacterial activity against *A. baumannii* through targeting fatty acid synthesis. Phatthalung et al. [40] highlighted that the combination of *Holarrhena antidysenterica* extract (HAE) with novobiocin might be an alternative for MDRAW treatment. It indicates that

phytochemicals present in the *H. antidyenterica* could play an effective role in the drug-resistant bacteria inhibition through synergetic interaction with conventional antibiotics. Gohari et al. [41] demonstrated that the dimethoxyphenyl structure, which contains an oxadiazole core as well as flavonoids (eriocitrin and narirutin), might be potential therapeutics to treat *A. baumannii*. Recent study noted that nanomedicine might be an effective approach to combat multidrug-resistant *A. baumannii* [42]. However, none of the investigation explored on the treatment of MDRAB using hesperetin their silver nanoformulation. Hesperetin has low water solubility and bioavailability due to its hydrophobic nature, and is not absorbed by the digestive tract [43]. To increase efficiency, hesperetin was nanoformulated with silver ions to form Hes-AgNPs, and their antibacterial and antibiofilm activity was assessed in this study.

Hes-AgNPs characterization

The reaction color of hesperetin added silver nitrate solution was turned from yellow to dark brown within 10 mins at pH 9.0 and 40 °C (**Fig. 4A** insert). The surface plasmon resonance (SPR) of the synthesized colloidal silver was confirmed at 431 nm (**Fig. 4A**), which indicates that hesperetin reduced the Ag ions to AgNPs. The HR-TEM image shows that the synthesized Hes-AgNPs was appeared as spherical in shape with the average size of 12 nm in diameter (**Figs. 4B** and **4C**). The EDX micrograph demonstrates that the intense peak for Ag at 3 keV and other peaks corresponds to carbon (C), oxygen (O), and aluminium (Al) elements. Also, the peaks appeared for elements O and C proves that hesperetin coated on the formed AgNPs. The remaining peak appeared for element Al might be due to the aluminium foil used for AgNPs attachment (**Fig. 4D**). As shown in XRD pattern, Hes-AgNPs have a distinct diffraction pattern with peaks at 2θ values of 38.2°, 44.4°, 64.6°, 77.5°, and 81.6°. These peaks are corresponding to the (111), (200), (220), (311), and (222) planes of the metal Ag face-centered cubic (FCC) structure (**Fig. 4E**). The absence of peaks that resemble other crystalline phases indicates the purity of the nanoparticles. Furthermore, the intensity of diffraction peaks indicated a high degree of crystallinity in AgNPs.

The average crystallite size of AgNPs was calculated using the Scherrer equation as follows [44] (eq. 3):

$$D = \frac{K\lambda}{\beta \cos \theta} \quad (3)$$

where, D , λ , k , β , and θ represents the average crystallite size, X-ray wavelength, Scherrer constant, line broadening in radians, and Bragg angle, respectively. The average crystallite size of Hes-AgNPs was determined to be 8 nm, which is lesser than the particle size. In the spectrum of Hes-AgNPs, stretching vibration appeared at

1630 cm^{-1} , 1384 cm^{-1} , and 1091 cm^{-1} , 823 cm^{-1} are corresponds to the C=O and –OH functional groups of hesperetin attached on the AgNPs after oxidation (**Fig. 4f**). Apart from this, the stretching appeared between 500-700 cm^{-1} are corresponds to the silver-oxygen (Ag-O). Furthermore, the results show that characteristic hesperetin peaks can be detected in the spectrum of Hes-AuNPs with slight shifting and decreased peak intensity. It demonstrates that C=O and –OH groups hesperetin plays an important role in the reduction of Ag ions to Ag atoms as well as structural stabilization through particle surface capping.

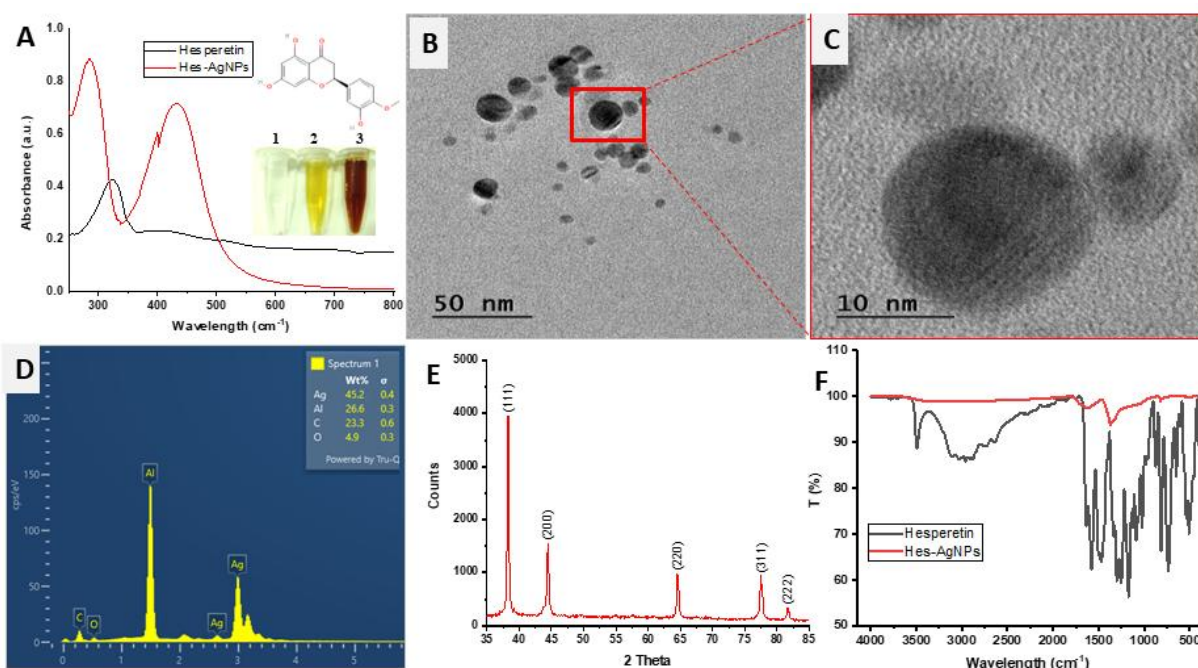


Figure 4. Physicochemical properties of Hes-AgNPs. (A) UV-vis spectrum, (B) and (C) HR-TEM images, (D) EDX micrograph, (E) FT-IR spectrum and (F) XRD pattern. Insert of figure A shows the photo of silver nitrate (1), hesperetin (2), and AgNPs (3).

Antibacterial potential of hesperetin and Hes-AgNPs

The ZOI of hesperetin (500 μg) and Hes-AgNPs (200 μg) was formed as 16 mm, 17 mm, and 17 mm and 22 mm, 21 mm, and 21 mm for *A. baumannii* strains IA018, IA036, and IA057, respectively (**Figs. 5 and 6A**). It proves that Hes-AgNPs exhibits significantly higher antibacterial potential against all isolated MDRAW strains. The MIC values of hesperetin were found as 335 $\mu\text{g/mL}$, 200 $\mu\text{g/mL}$, and 265 $\mu\text{g/mL}$; and Hes-AgNPs was denoted as 105 $\mu\text{g/mL}$, 75 $\mu\text{g/mL}$, and 85 $\mu\text{g/mL}$ for *A. baumannii* strains IA018, IA036, and IA057, respectively (**Figs. 6B and 6C**). This result proves that hesperetin synergically interacts with AgNPs for the successful inhibition of MDRAW. Different mechanisms of action against bacteria have been proposed for AgNPs by various research group. For

instance, after adhering to bacterial cells, AgNPs alter the structure of the cell membrane and block transport channels, resulting in cell death; (ii) AgNPs interact with phosphorus (P) or sulfur (S) groups of intracellular components (proteins and DNA), and altering their morphology and functional characters; (iii) AgNPs react with thiol groups of enzyme molecules, which disrupting the respiratory chain of the inner membrane by producing free radicals and reactive oxygen species (ROS) as well as activating the apoptotic pathway [20,45]. *A. baumannii* biofilm and their associated pathogenicity are particularly challenging to treat [46]. Therefore, antibiofilm efficacy of hesperetin and Hes-AgNPs was further explored against preformed biofilm of the isolated MDRAB strains.

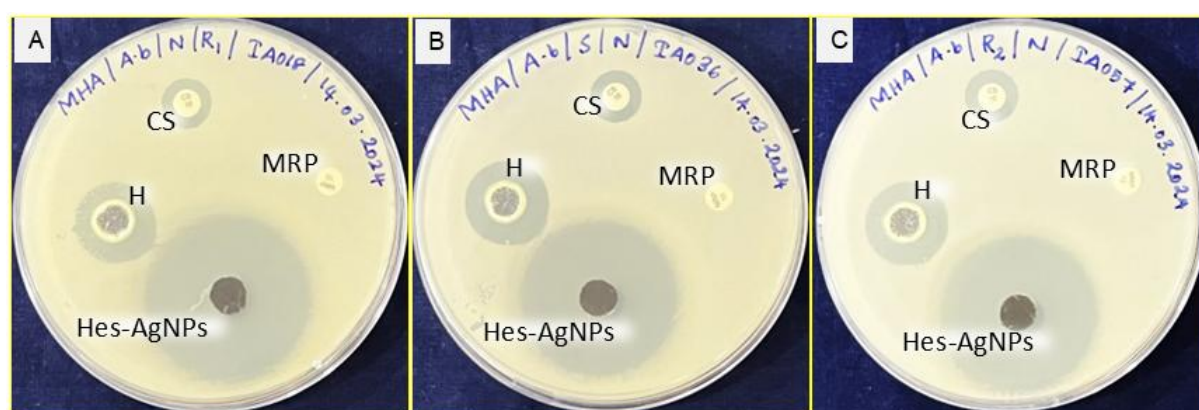


Figure 5. Antibacterial potential assessment (ZOI) of Hes-AgNPs against *A. baumannii*. H – hesperetin; Hes-AgNPs: hesperetin-silver nanoparticles; MRP: Meropenem, and CS: Colistin.

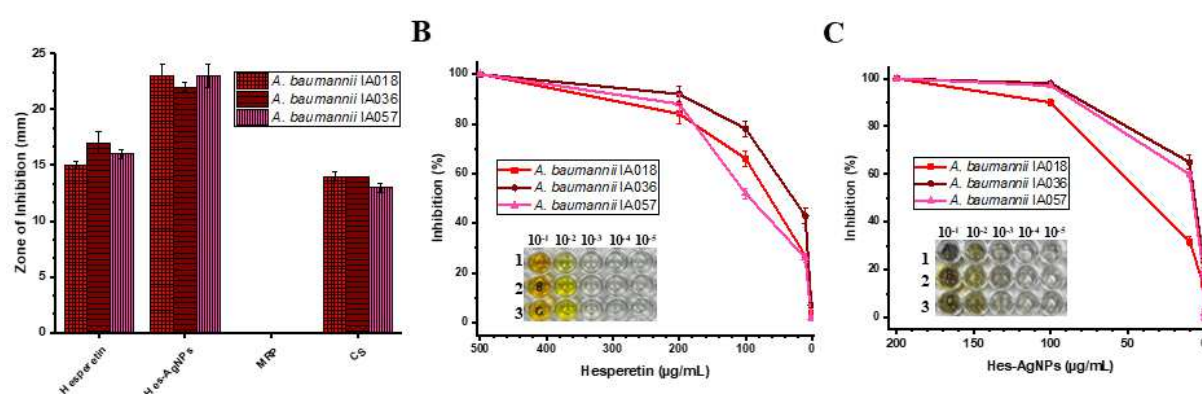


Figure 6. Antibacterial assessment: (A) ZOI and (B) MIC of hesperetin and (C) MIC of Hes-AgNPs activity against *A. baumannii*. MRP – Meropenem, and CS – Colistin. Insert of figures B and C shows the MIC photos of hesperetin and Hes-AgNPs treated *A. baumannii* isolates (1) IA018, (2) IA036, and (3) IA057, respectively. The control ant test groups demonstrated a significant difference ($P = \leq 0.001$).

Antibiofilm potential of hesperetin and Hes-AgNPs

The biofilm formation potential of the isolated MDRAB strains (IA018, IA036, and IA057) was investigated using

CV staining method. The results indicate that the absorbance was found as 0.348, 1.674, 1.711, 1.643 for NC, IA018, IA036, and IA057, respectively (**Fig. 7A**). It evidently indicates that all isolated *A. baumannii* strains showed strong biofilm formation ability. The antibiofilm efficacy of hesperetin and Hes-AgNPs was evaluated against the preformed biofilm of MDRAB at different MICs of each isolated strain. The MTT result demonstrates that around 49–57% and 21–32% cells are viable after treatment with 1xMIC of hesperetin (335 µg/mL, 200 µg/mL, and 265 µg/mL) and 1xMIC of Hes-AgNPs (105 µg/mL, 75 µg/mL, and 85 µg/mL), for *A. baumannii* isolates IA018, IA036, and IA057. It is important to note that hesperetin completely inhibits the bacterial cells at 2xMIC (670 µg/mL, 400 µg/mL, and 530 µg/mL); whereas, Hes-AgNPs showed complete eradication at 1.5xMIC onwards (157.5 µg/mL, 112.5 µg/mL, and 127.5 µg/mL), as illustrated in **Figs. 7B** and **7C**. Besides, CLSM was used to verify and distinguish between live and dead cells in the preformed *A. baumannii* biofilms both before and after treatment.

Figure 8 shows the CLSM image of control (A), 2xMIC hesperetin-treated (B), and 1.5xMIC Hes-AgNPs-treated (C) biofilms of *A. baumannii* strains IA018 (a), IA036 (b), and IA057 (c). The results also revealed that 98-100% of bacterial cells were inhibited in the preformed biofilms of *A. baumannii* isolates IA018, IA036, and IA057 after 24 h treatments with hesperetin (**Fig. 8B**) and Hes-AgNPs (**Fig. 8C**). As a direct confirmation, CFU results indicate that bacterial colonies were not found on the 2xMIC hesperetin (400-670 µg/mL) and 1.5xMIC Hes-AgNPs (112.5-157.5 µg/mL) treated *A. baumannii* isolates (**Fig. 9AB**). These results clearly indicate that 2xMIC of hesperetin (670 µg/mL, 400 µg/mL, and 530 µg/mL) and 1.5xMIC of Hes-AgNPs (157.5 µg/mL, 112.5 µg/mL, and 127.5 µg/mL) is a more effective dose for the eradication of bacterial cells in the preformed biofilm (7 days) of MDRAB strains. Natural substances found in plant materials, such as terpenes, flavonoids, alkaloids, and phenolic compounds, exhibit potent antibiofilm activity, according to the previous reports [47]. These phytochemicals interfere with the quorum sensing network by preventing the establishment of the polymer matrix, inhibiting cell adhesion and attachment, and reducing virulence factors. In the case of metallic nanoparticles, the antibiofilm mechanisms almost similar to the antibacterial activity mechanisms. Few studies have been reported the genes involved in the *A. baumannii* biofilm formation. For example, Altınok et al. [10] study confirmed that *csgA*, *csuE* and *ompA* genes associated with the *A. baumannii* biofilm formation. Lysitsas et al. [48] detected the biofilm associated genes such as *bap*, *bla_{PER}*, *ompA*, and *csuE* in *A. baumannii* (41) strains. Interestingly, the present study highlights that multidrug-resistance *A. baumannii* biofilm were completely eradicated at 2xMIC levels.

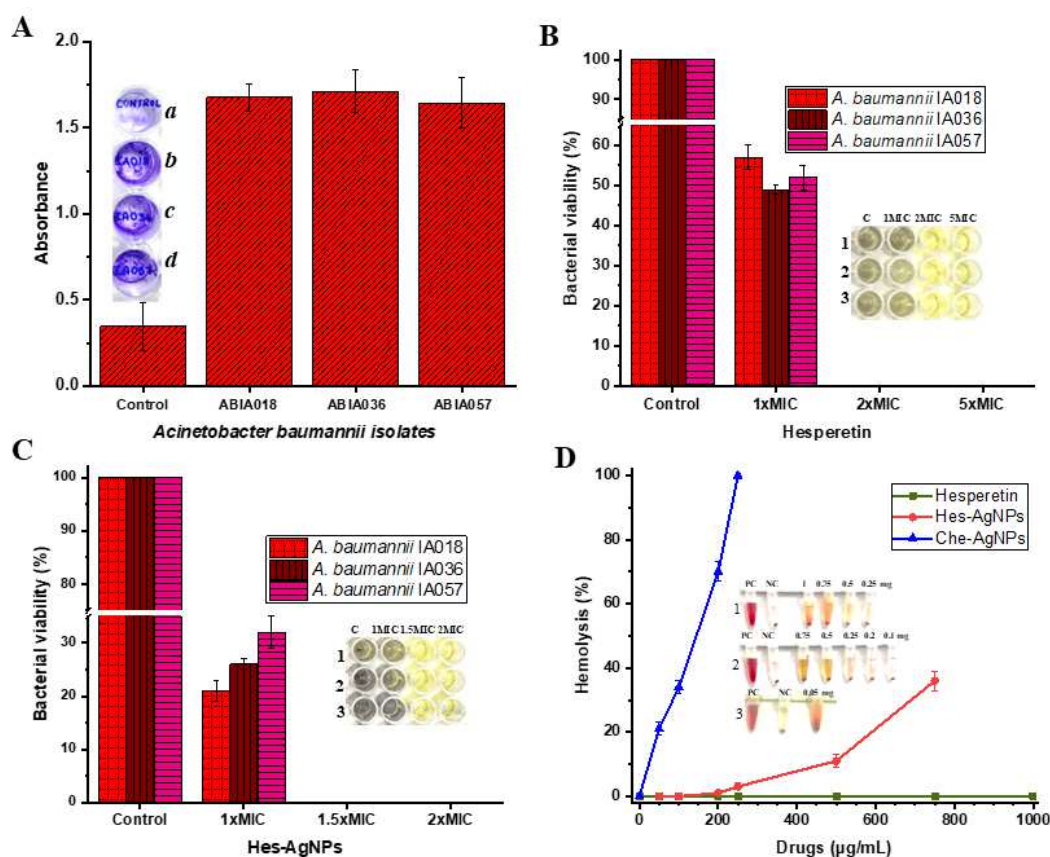


Figure 7. Antibiofilm potential assessment. (A) Biofilm formation potential of the isolates, and (B) hesperetin and (C) Hes-AgNPs against *A. baumannii* strains and (D) hemocompatibility analysis on human RBCs. Insert of figure A shows the photo of crystal violet staining of (c) control, and *A. baumannii* isolates (b) IA018, (c) IA036, and (d) IA057. Insert of figures B and C shows the MTT assay photos of hesperetin and Hes-AgNPs treated *A. baumannii* isolates (1) IA018, (2) IA036, and (3) IA057, respectively. Insert of figures D shows the photos of (1) hesperetin, (2) Hes-AgNPs, and (3) chemically synthesized AgNPs treated RBCs. PC – Positive control. NC – Negative control. The control and test groups demonstrated a significant difference ($P \leq 0.001$).

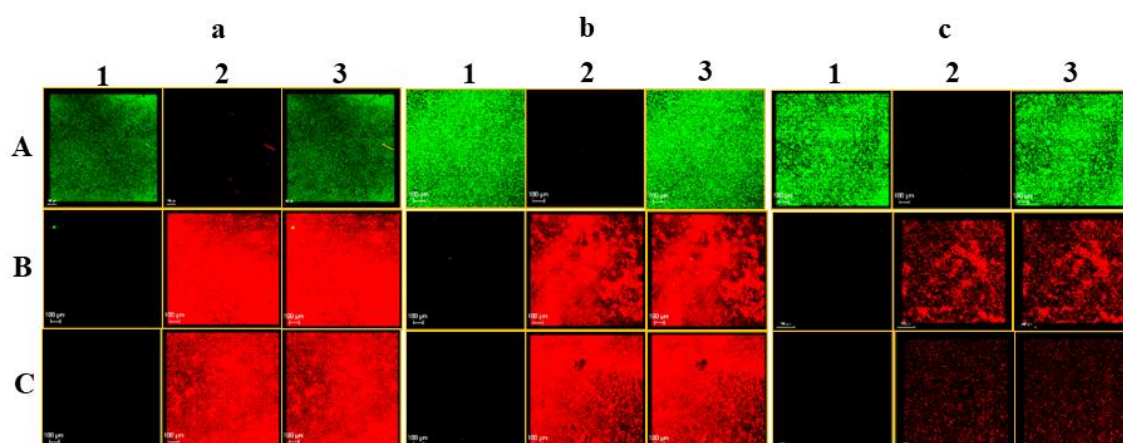


Figure 8. CLSM analysis. (A) Control (preformed *A. baumannii* biofilm after 7 days), (B) hesperetin (2xMIC) treated biofilms after 24 h, and (C) Hes-AgNPs (1.5xMIC) treated preformed biofilm. *A. baumannii* isolates (a) IA018, (b) IA036, and (c) IA057. (1) Viable cells (green color), (2) Dead cells (red color), and (3) Both viable and dead combined cells (red and green color).

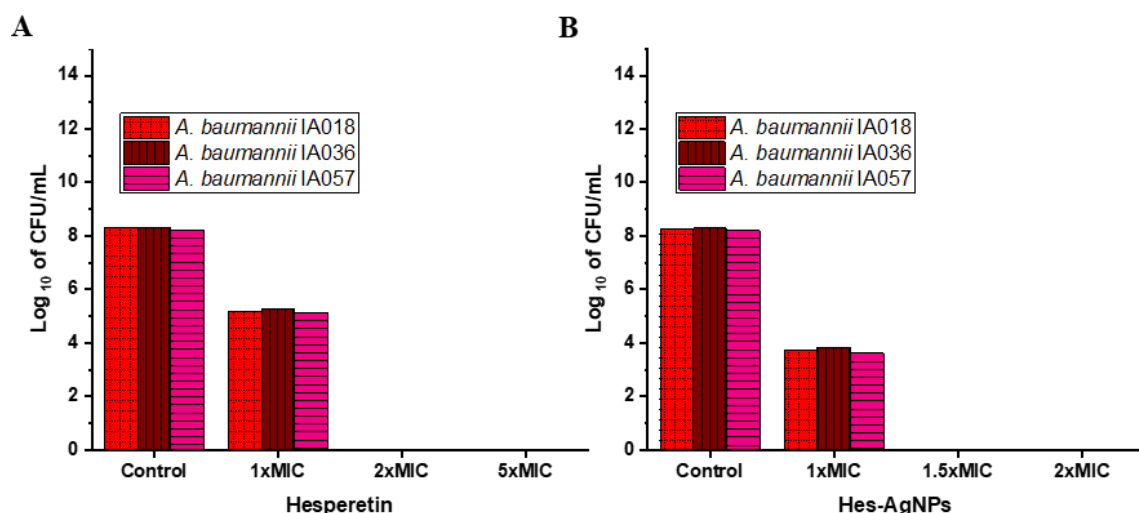


Figure 9. Colony forming units of (A) hesperetin and (B) Hes-AgNPs treated *A. baumannii* strains biofilm. The control and test groups demonstrated a significant difference ($P = \leq 0.001$).

Hemocompatibility of Hes-AgNPs

In general, drugs can interact directly with red blood cells (RBCs), thus hemolysis testing is an important in the biocompatible assessment process for the successful development of a novel drug [49]. Herein, the hemocompatibility of hesperetin and hesperetin, Hes-AgNPs, Che-AgNPs was assessed on RBCs. The results indicate that hesperetin and Hes-AgNPs was not significantly disturbed the RBCs at doses up to 1000 μ M and 250 μ g/mL levels, respectively. At the same time 11% of hemolysis was noted for Hes-AgNPs at a concentration of 500 μ g/mL. The antibiofilm potential of hesperetin and Hes-AgNPs for bacterial cells in preformed biofilm of MDRAW was found between the range of 670-400 μ g/mL and 157.5-112.5 μ g/mL, respectively (**Fig. 7D**). According to Ul-Islam et al. [50], the hemocompatibility threshold of drug/biomaterial should be less than 5% hemolysis. Based on this fact, it is remarkable to note that hesperetin and Hes-AgNPs are very safe and hemocompatible for the treatment of MDRAW infections and their biofilm in humans. Besides, Che-AgNPs showed hemolysis (21%) even at a concentration of 50 μ g/mL, which is similar to the previous report [17]. It suggesting that the free radicals formed by the metal nanoparticles may harm RBCs. Therefore, this study proves that hesperetin coating on the AgNPs hinders the metal toxicity and protects the RBCs.

Conclusions

The phytochemicals such as morin, naringin, curcumin, quercetin, and hesperetin exhibits considerable antibacterial potential against the multidrug-resistant *A. baumannii* strains. Interestingly, hesperetin and Hes-AgNPs are demonstrated excellent antibiofilm potential against the isolated *A. baumannii* strains. Furthermore,

hesperetin and Hes-AgNPs are highly hemocompatible. Therefore, the present study recommends that hesperetin and Hes-AgNPs might be successful therapeutics to eradicate multidrug-resistant *A. baumannii* strains causing infections and their biofilms in human.

Authors contribution statement

Lekha Sree Venkatesan: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft. **Sudharsan Ganapathi:** Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft. **Palanivel Sathishkumar:** Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Data Availability

Datasets generated in this study are available from the corresponding author on request.

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Declarations

Conflict of interest: The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Informed Consent: On behalf of all authors, the corresponding author states that informed consent was obtained from all participants involved in the study.

Ethical Approval: This study was approved by the Institutional Ethics Committee (Approval No.: 874/06/2026/UG/SRB/SMCH) of SIMATS.

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