



OPEN Synthesis, characterization and molecular docking study of novel bile acid curcumin conjugates as potent antibacterial and antibiofilm agents

Neha V. Rathod & Satyendra Mishra

Two novel bile acid–curcumin conjugates were synthesized via esterification of curcumin with cholic acid and deoxycholic acid. FT-IR, ^1H & ^{13}C NMR and HPLC spectral data were employed to characterize the synthesized conjugates. Conjugate 3a exhibited significant antibacterial activity against *Pseudomonas putida* (ATCC 25922) with an IC_{50} 4.48 ± 0.2 $\mu\text{g/mL}$. Conjugate 3b showed efficacy against *Bacillus megaterium* (QMB 1551) with an IC_{50} of 7.02 ± 0.4 $\mu\text{g/mL}$. Both conjugates demonstrated higher antibacterial efficacy compared to curcumin, which had IC_{50} values of 46.09 ± 2.6 and 76.02 ± 5.2 $\mu\text{g/mL}$, respectively. Conjugate 3a showed strong antibiofilm activity it reduced biofilm biomass of *Bacillus megaterium* (QMB1551) and *Lactococcus lactis* (NZ900) by nearly 90% at a concentration of 80 $\mu\text{g/mL}$. It also inhibited approximately 50% of biofilm formation in *L. lactis* and *P. putida*, with MIC_{50} values of 24.8 ± 1.3 and 28.3 ± 0.4 $\mu\text{g/mL}$, respectively. In silico analysis showed Conjugate 3a exhibited a binding affinity of -7.11 kcal/mol against bacterial outer membrane protein (PDB ID: 4RHB), compared to curcumin's -2.99 kcal/mol. Moreover, the conjugate 3a exhibited a higher affinity -8.12 kcal/mol than curcumin -6.49 kcal/mol against the cell division protein (PDB ID: 6LL6). These results highlight the enhanced ability of conjugate 3a to interact with and inhibited essential bacterial targets, supporting its potential as a promising antibacterial agent.

Keywords Curcumin, Bile acid, Curcumin-bile acid conjugates, In silico study, Antibacterial activity

Antibiotic resistance has emerged as a major global health crisis in worldwide. The World Health Organization (WHO) has recognized antimicrobial resistance (AMR) as one of the top ten public health threats. The rapid rise of multidrug-resistant (MDR) bacterial strains has rendered many conventional antibiotics ineffective, creating an urgent need for alternative therapeutic strategies¹². Natural products, particularly plant-derived phytochemicals, have long played a vital role in treating infectious diseases. Preclinical and experimental studies have demonstrated their ability to disrupt bacterial physiology through diverse mechanisms^{3,4}.

Bile acids, synthesized from cholesterol, are amphipathic molecules essential for lipid metabolism and gut microbial regulation^{5–7}. Their ability to disrupt bacterial membranes grants them antibacterial efficacy against both Gram-positive and Gram-negative species⁸. Moreover, their function as permeability enhancers and solubilizing agents contributes to improved drug absorption and bioavailability⁹. Their low toxicity, biological tolerance, and structural versatility have led to their physiology and physiochemical properties¹⁰, such as bio-nanomaterials¹¹, biomedical devices and sensors¹², and pharmacological uses of bile acid derivatives¹³, such as antibacterial^{14–18}, antibiofilm¹⁹, antifungal^{20,21}, and antitumor activities^{22–24}. Turmeric (*Curcuma longa*), widely used in traditional medicine, contains curcumin as its primary bioactive compound²⁵. Curcumin exhibits broad-spectrum pharmacological effects²⁶, it has had a broad range of biological activities^{25,27}. Curcumin is a symmetrical molecule with two methoxylated phenolic rings connected by a β -diketone bridge, forming a planar structure suitable for designing pharmacological inhibitors^{28,29} including antibacterial³⁰, antioxidant³¹, antibiofilm activity^{32,33} and anti-inflammatory activities³⁴. Mechanistically, it acts by inhibiting quorum sensing, disrupting membranes, and modulating oxidative stress³⁵. However, curcumin's clinical potential is limited therapeutic due to its poor aqueous solubility or bioavailability³⁶, rapid metabolism³⁷. To overcome these

School of Biotechnology and Bioengineering, Institute of Advanced Research, Gandhinagar 382426, Gujarat, India.
✉ email: satyendra.mishra@iar.ac.in; satyendramishra1@gmail.com

limitations, curcumin has been chemically modified through golden spice and through esterification^{38,39} and conjugated with therapeutic agents, enhancing its cellular uptake and antibacterial efficacy^{40,41}. Some recent curcumin delivery systems or bile acid–based nanocarriers such as biosomes⁴², antimicrobial^{43–46}, anticancer⁴⁷, antioxidant⁴⁸, anti-inflammatory⁴⁹, antimalarial⁵⁰.

Curcumin inhibits bacterial cell division by targeting FtsZ, a GTPase crucial for Z-ring formation, disrupting GTP polymerization and protofilament assembly⁵¹. This conjugation provides an innovative approach to overcome these restrictions and enhance antibacterial properties. Curcumin's bioactive polyphenolic structure and bile acids' amphiphilic characteristics may be coupled to generate hybrid molecules with improved pharmacokinetic profiles and synergistic antibacterial and biofilm activities. The conjugates exhibit dual antibacterial action by enhancing membrane permeability and targeting key proteins. Docking studies confirmed strong interactions with FtsZ and DNA gyrase, suggesting inhibition of bacterial cytokinesis and DNA replication. These results highlight the potential of curcumin–bile acid conjugates as effective antibacterial and antibiofilm agents.

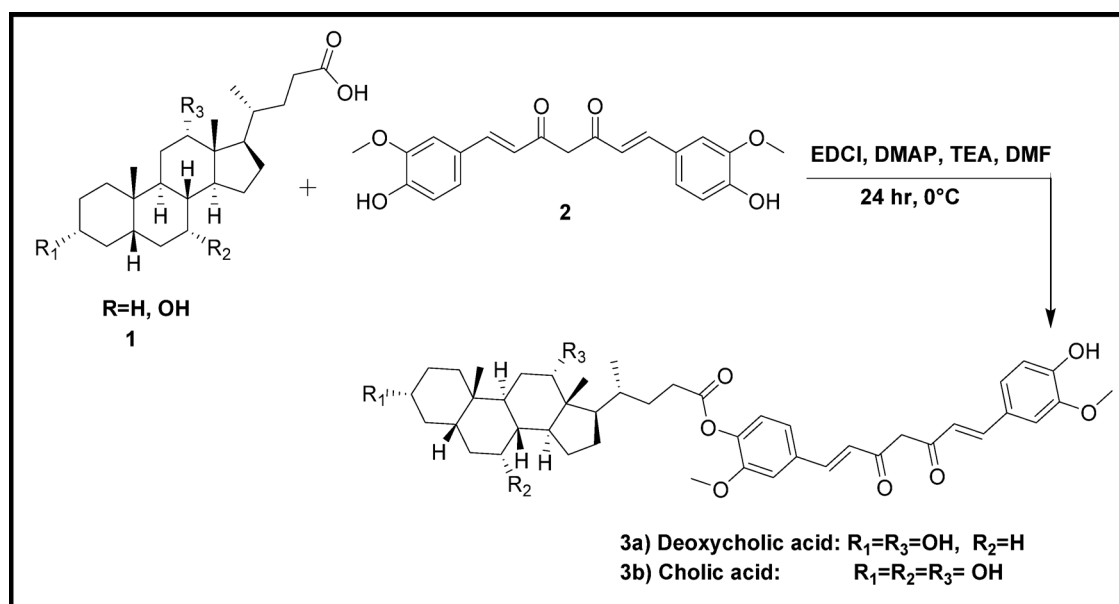
Results and discussion

Chemistry

To synthesize the compounds (3a–b), we utilized 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) as the coupling agent and triethylamine (TEA) as the base in a condensation reaction. Additionally, a catalytic amount of 4-dimethylaminopyridine (DMAP) was incorporated illustrated in Scheme 1, this carefully orchestrated method allowed us to successfully produce the target compounds (3a–b) with high yield and purity. **3a** DCA-Curcumin conjugate ¹H-NMR in DMSO-*d*₆ spectra shows a peak of curcumin aromatic, alkenyl, steroidal protons confirm the structure of synthesized conjugates (as shown in Supplementary Fig. 2). The ¹³CNMR peaks at 192.77 ppm, 192.33 ppm of curcumin diketone's carbonyl carbons, and 172.32 ppm of curcumin to bile acid ester carbonyl carbon association validate curcumin's conjugation with bile acid (Supplementary Fig. 3). Additionally, the FTIR spectrum shows C=O stretching in ester and ketone groups at 1700–1750 cm^{−1}, C–O stretching in ester and phenolic groups at 1200–1300 cm^{−1}, and C–O–C stretching in methoxy groups at 1000–1150 cm^{−1}. In aromatic and conjugated systems, additional peaks include 1600–1650 cm^{−1} for C=C stretching and 3300–3500 cm^{−1} for O–H stretching (Supplementary Fig. 1). These results validate the functional groups of curcumin–bile acid conjugates. Detailed spectrum characteristics, such as signals for aromatic and alkenyl protons, methoxy substituents, hydroxyl groups, and the carbonyl carbons of both the ester and diketone groups, provide solid evidence that the curcumin moiety has been coupled with the steroidal scaffold. FTIR stretching vibrations for C=O, C–O, and O–H functionalities, consistent chemical shifts in both ¹H and ¹³C NMR, and an exact mass-to-charge ratio from ESI-MS that matches the calculated molecular formula further extend further support to this synthesis.

In vitro antibacterial activity

Bile acid–curcumin conjugates (**3a–b**) were specifically designed to target and inhibit the growth of a variety of bacterial strains, including the Gram-positive *Lactococcus Lactis* (NZ900), *B. Megaterium* (QMB1551), and the Gram-negative *Pseudomonas putida* (ATCC25922) and *M. Serratia* (NCTC10211). Table 1 demonstrates the results of the investigation on these conjugates antibacterial efficacy. According to our finding outcomes, these conjugates considerably inhibited the development of the bacterial strains that were investigated. It is interesting to note that conjugates **3a** and **3b** had remarkable efficacy, as seen by their half-maximal inhibitory concentrations



Scheme 1. Bile acid –curcumin conjugate by condensation reaction.

Compounds	Gram positive		Gram negative	
	<i>L. Lactis</i> (NZ900)	<i>B. megaterium</i> (QMB 1551)	<i>P. putida</i> (ATCC25922)	<i>M. Serratia</i> (NCTC10211)
, m Conjugate 3a	9.48 ± 0.09	7.02 ± 0.4	4.48 ± 0.2	17.26 ± 1.2
Conjugate 3b	29.87 ± 2.76	34.94 ± 0.63	10.20 ± 0.1	11.09 ± 0.8
Curcumin	50.19 ± 2.61	76.02 ± 5.2	46.09 ± 2.6	64.23 ± 3.2
Ampicillin	14.94 ± 0.14	41.56 ± 4.6	37.23 ± 3.4	29.92 ± 0.29

Table 1.. Antibacterial potency (IC₅₀ µg/mL) of curcumin and synthesized bile acid (3a-b) Conjugates.

(IC₅₀ values). Figure.1 A and 1B show the results of an evaluation of the synthesised compounds' antibacterial activity against two strains of Gram-positive bacteria, *L. lactis* and *B. megaterium*. Among of all the compounds that were investigated, conjugate **3a** showed highest antibacterial activity. The IC₅₀ value of conjugate-**3a** against *B. megaterium* was 7.02 ± 0.40 µg/mL, which was much higher than that of ampicillin (IC₅₀ of 41.56 ± 4.6 µg/mL) and curcumin (IC₅₀ of 76.02 ± 5.2 µg/mL). This indicates an approximately 10.8-fold enhancement of activity over curcumin and a 5.9-fold improvement over ampicillin. Conjugate **3a** showed a similar pattern against *L. lactis*, with an IC₅₀ of 9.48 ± 0.09 µg/mL, indicating once higher efficacy in comparison to ampicillin (14.94 ± 0.14 µg/mL) and curcumin (50.19 ± 2.61 µg/mL). A 5.3-fold increase in activity over curcumin and a 1.57-fold increase in potency over ampicillin are indicated by these values. Furthermore, compared to conjugate **3a**, conjugate **3b** exhibited mild inhibitory effects with higher IC₅₀ values. The IC₅₀ for *L. lactis* was 29.87 ± 2.76 µg/mL, whereas the IC₅₀ for *B. megaterium* was 34.94 ± 0.63 µg/mL. Although conjugate **3b** was less effective than **3a**, it nevertheless was superior to curcumin, indicating that some degree of antibacterial activity was preserved after structural modification. Steric hindrance or an inadequate binding affinity with bacterial targets, however, could be the reason of **3b**'s lower effectiveness when compared to **3a**. With IC₅₀ values of 50.19 ± 2.61 µg/mL and 76.02 ± 5.2 µg/mL against *L. lactis* and *B. megaterium*, respectively, the parent compound, curcumin, showed the lowest effective antibacterial response of all the studied compounds. These findings support the limited bactericidal potency of natural curcumin on its own. The reference standard, ampicillin, showed moderate activity with IC₅₀ values of 14.94 µg/mL (*L. lactis*) and 41.56 µg/mL (*B. megaterium*). These results indicate that conjugate **3a** has the most promising antibacterial activity against the strain of gram-positive bacteria. In the case of *P. putida* and *S. marcescens*, the IC₅₀ values show that the synthesised compounds are effective at inhibiting Gram-negative strains. As depicted in Fig.1C and 1D.

Conjugate **3a** had IC₅₀ values of 17.26 ± 1.2 µg/mL against *S. marcescens* and 4.48 ± 0.20 µg/mL against *P. putida*, making it the most effective antibacterial agent among the compounds tested. Regarding *P. putida*, this activity corresponds to a potency improvement of around 10.29 times over curcumin (46.09 ± 2.6 µg/mL) and 8.3 times over ampicillin (37.23 ± 3.4 µg/mL). Additionally, **3a** demonstrated a 3.72-fold improvement over curcumin (64.23 ± 3.2 µg/mL) and a 1.73-fold enhancement over ampicillin (29.92 ± 0.29 µg/mL), demonstrating its better activity against *S. marcescens*. These findings strongly imply that conjugate **3a** has broad-spectrum antibacterial action and is effective against both Gram-positive and Gram-negative bacteria. Conjugate **3b** exhibited substantial inhibitory potential despite being less active than **3a**. The IC₅₀ values for *P. putida* and *S. marcescens* were 10.20 ± 0.1 µg/mL and 11.09 ± 0.8 µg/mL, respectively. Conjugate **3b** demonstrated almost 4.5 times the activity of curcumin and 3.65 times that of ampicillin against *P. putida*. Remarkably, conjugate **3b** superior to all other drugs, including **3a**, against *S. marcescens*, indicating that certain interactions involving outer membrane porins or efflux regulation might have contributed to its increased effectiveness against this strain. The impact of bacterial species-specific processes on drug activity is highlighted by this change in relative potency. Although curcumin is well-known for its wide range of biological effects, it was once again shown to be the least effective, with IC₅₀ values for *P. putida* and *S. marcescens* being 46.09 µg/mL and 64.23 µg/mL, respectively. The β-lactam antibiotic ampicillin, which is frequently used to treat Gram-negative infections, demonstrated moderate action, with IC₅₀ values of 29.92 ± 0.29 µg/mL (*S. marcescens*) and 37.23 ± 3.4 µg/mL (*P. putida*). These results demonstrate that conjugate-**3a** has strong, broad-spectrum activity that is substantially more potent than ampicillin and curcumin in both tested Gram-negative bacterial strains. These findings firmly suggest that both compounds need to be investigated further as possible candidates for next-generation antibacterial drugs. As shown in Table 4, the conjugates exhibit markedly enhanced antibacterial activity relative to native curcumin and its known analogues. The conjugate **3a** exhibited a MIC₅₀ of 4.48 ± 0.2 µg/mL against *P. putida* (ATCC 25922), while the conjugate **3b** showed a MIC₅₀ of 7.02 ± 0.4 µg/mL against *B. megaterium* (QMB 1551). These values are notably lower than those reported for native curcumin and many of its analogues, which typically range from 78 to 156 µg/mL against Gram-positive organisms such as *S. aureus* and *E. faecalis*⁵². In comparison, curcumin-based analogues reported in literature demonstrate variable activity, with MIC values against *S. aureus* ranging from 0.0625 to 0.9 mg/mL^{53,54}. The lower MIC₅₀ values observed for our conjugates indicate greater potency at significantly reduced concentrations, suggesting that structural modification of curcumin with bile acid moieties substantially improves its cell permeability, stability, and bacterial target affinity. These findings support the conjugates' potential as more effective antibacterial agents than unmodified curcumin, against both Gram-positive and Gram-negative bacteria.

Quantitative analysis of antibiofilm activity

In Fig.2A-D, the OD₆₀₀ values are depicted, which were utilized to determine the minimum inhibitory concentrations (MIC₅₀) for the antibiofilm properties of the synthesized bile acid–curcumin conjugates (3a and

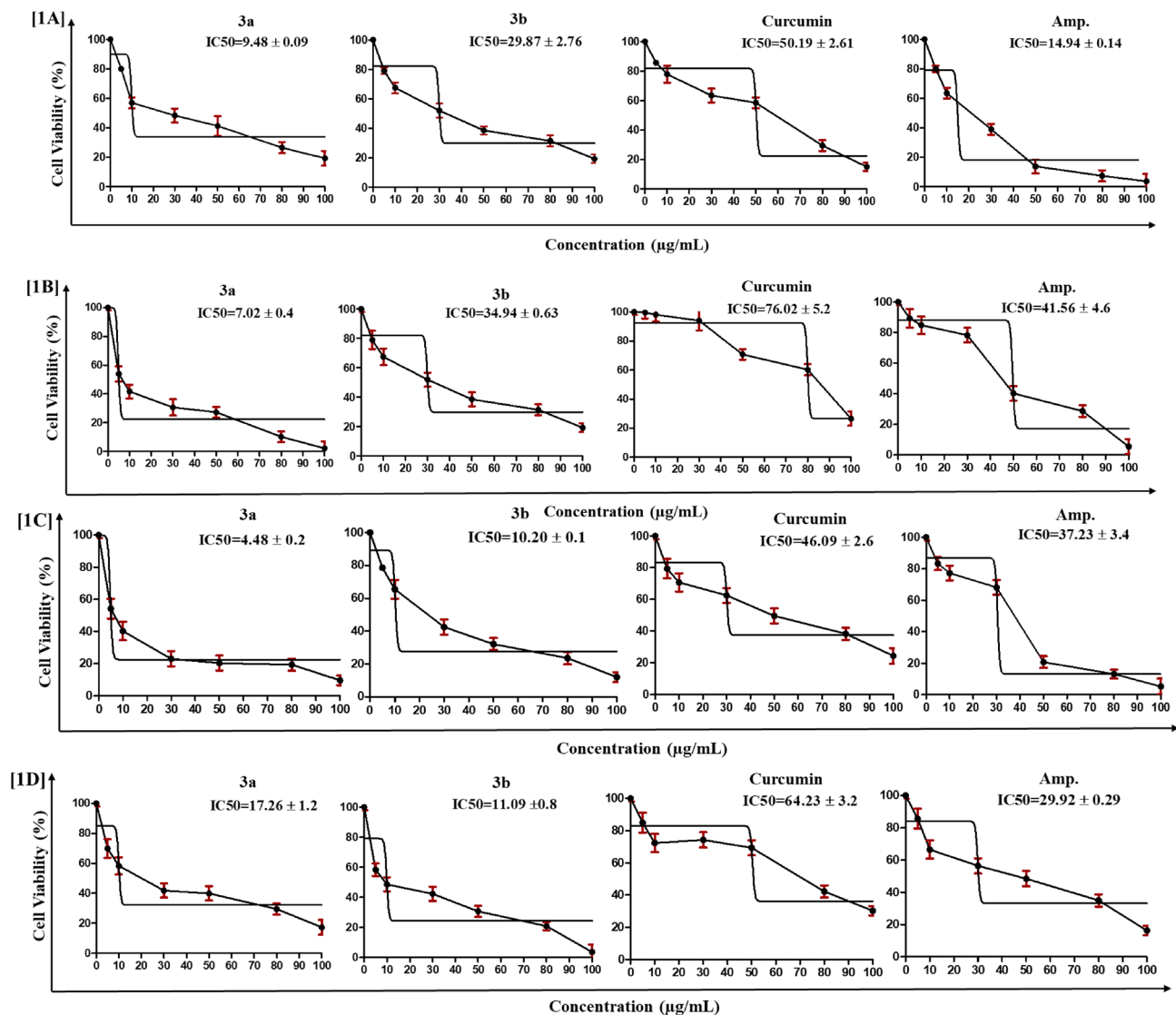


Fig. 1. Bacterial cells were treated with various concentration 10 to 100 μg/mL of curcumin, conjugate **3a** and conjugate **3b** with positive control standard drug ampicillin (Amp) analogues for 24 h at 37 °C was determined by microdilution assay (1 A-D), where, (A) *Lactococcus Lactis* (NZ900), (B) *Bacillus Megaterium* (QMB1551), (C) *Pseudomonas putida* (ATCC25922) and (D) *Marcescens Serratia* (NCTC10211). Statistical analysis performed using one way ANOVA test (n = 3); significance levels: p < 0.05.

3b), along with curcumin and ampicillin, after a 24-hour exposure to test compounds at concentrations between 5 and 100 μg/mL. We find that conjugate **3a** strongly reduced biofilm formation in both *L. lactis* (NZ900) and *B. megaterium* (QMB1551), achieved > 90% decrease in biofilm biomass at 80 μg/mL, as illustrated in Fig. 3A and 3B biofilm reduction graph in (%). In particular, conjugate **3a** had substantial antibiofilm activity at 25 μg/mL, providing over 50% inhibition in *L. lactis* with an estimated IC₅₀ of 24.8 ± 1.3 μg/mL. The IC₅₀ for conjugate **3a** in *B. megaterium* was slightly higher at 48.6 ± 3.1 μg/mL, indicating biofilm resilience peculiar to strain. Conjugate **3b** had modest action in both strains, achieving around 80% inhibition at 75–100 μg/mL. In comparison to conjugate **3a**, conjugate **3b** was less effective, as evidenced by its IC₅₀ values of 49.7 ± 2.4 μg/mL in *L. lactis* and 73.2 ± 5.2 μg/mL in *B. megaterium*. The parent molecule, curcumin, showed significantly lower antibiofilm capability; in both strains, IC₅₀ values were greater than 70 μg/mL, indicating low membrane permeability and restricted biofilm penetration. The common antibiotic ampicillin, on the other hand, was unable to successfully suppress the formation of biofilms within the studied concentration range and was unable to achieve 50% inhibition even at higher concentrations, suggesting that biofilm-embedded cells are inherently resistant. These findings suggest that, in comparison to curcumin and traditional antibiotics, bile acid–curcumin conjugates, especially conjugate **3a**, exhibit improved antibiofilm efficacy in a concentration-dependent manner with lower MIC₅₀ values.

Bile acid–curcumin conjugates appear to have an effect on Gram-positive bacterial biofilms by targeting on intracellular division and replication as well as extracellular biofilm architecture. These conjugates can overcome

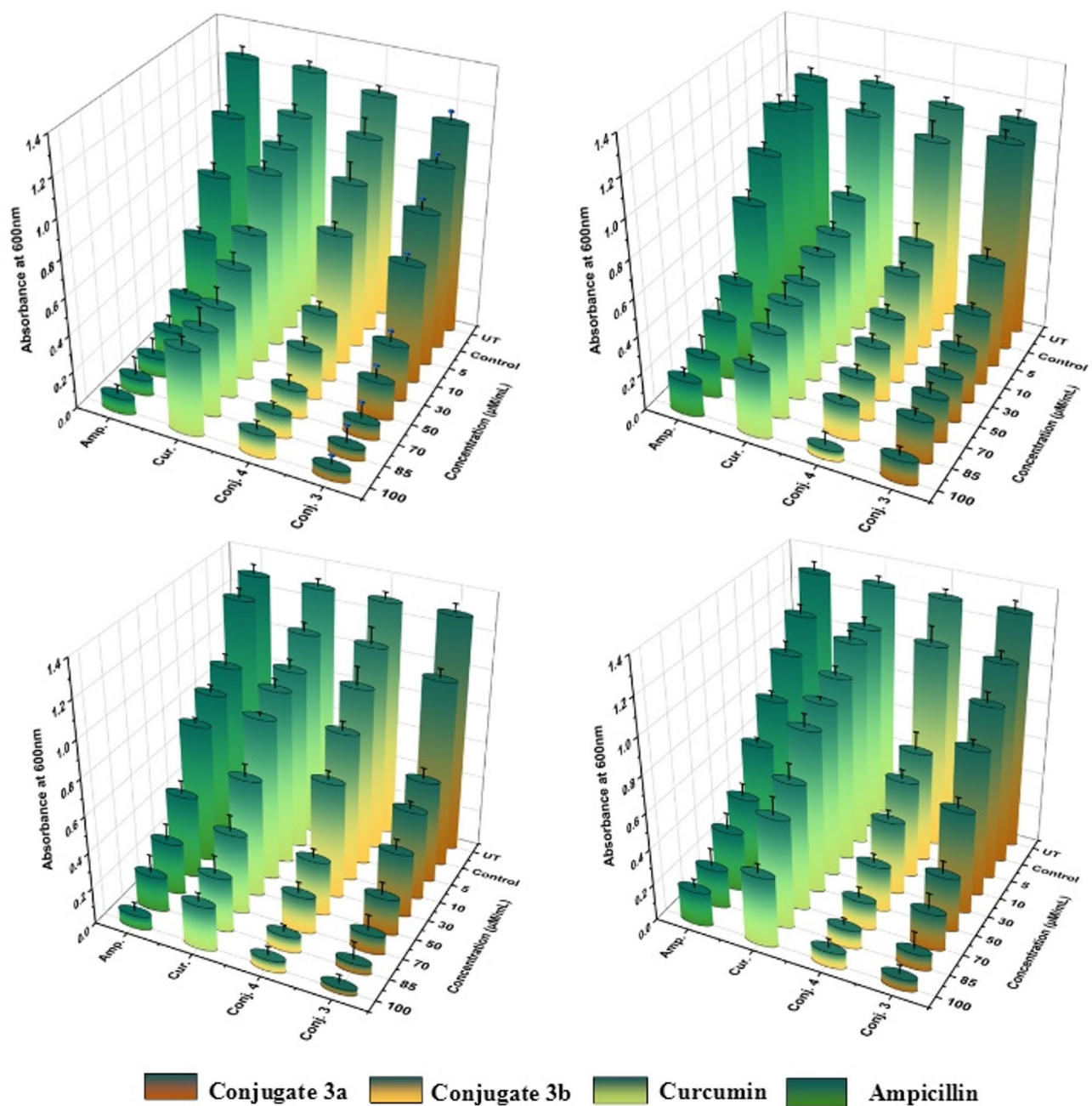


Fig. 2. Quantitative analysis of antibiofilm inhibition (%) of different concentrations of test compounds against (A) *Lactococcus Lactis* (NZ900), (B) *Bacillus Megaterium* (QMB1551), (C) *Pseudomonas putida* (ATCC25922) and (D) *M. Serratia* (NCTC10211). The experiment was repeated ($n = 3$) and are presented as the mean $\pm$ SEM. Statistical significance was determined by a one-way ANOVA with Turkey's multiple comparison ($n = 3$, *** $p < 0.05$; ** $p < 0.01$; * $p < 0.001$ the control group).

common biofilm-associated resistance mechanisms because of their intricate mechanism of action, which comprises enhanced membrane penetration, reduction of cell division (FtsZ), and interference with genetic machinery (DNA gyrase). Strain-dependent differences in IC_{50} values could be explained by the unique matrix composition and biofilm architecture of each bacterium, with *B. megaterium* most likely generating a denser exopolysaccharide matrix than *L. lactis*. Additionally, it was quantitatively assessed against *S. marcescens* (NCTC 10211) and *P. putida* (ATCC 25922), two strains of Gram-negative bacteria. The conjugate **3a** exhibited the strongest inhibition, lowering biofilm development by more than 90% at 85 $\mu\text{g/mL}$ in both strains, as illustrated in Fig. 3C and 3D. Despite the inherent resilience of Gram-negative bacterial membranes, the corresponding IC_{50} values for *P. putida* and *S. marcescens* have been found to be $28.3 \pm 0.4 \mu\text{g/mL}$ and $31.9 \pm 0.6 \mu\text{g/mL}$, respectively, suggesting high efficacy. conjugate **3b** demonstrated significant antibiofilm action, with IC_{50} values of $61.7 \pm 1.9 \mu\text{g/mL}$ for *P. putida* and $54.1 \pm 0.8 \mu\text{g/mL}$ for *S. marcescens* despite being less effective than conjugate

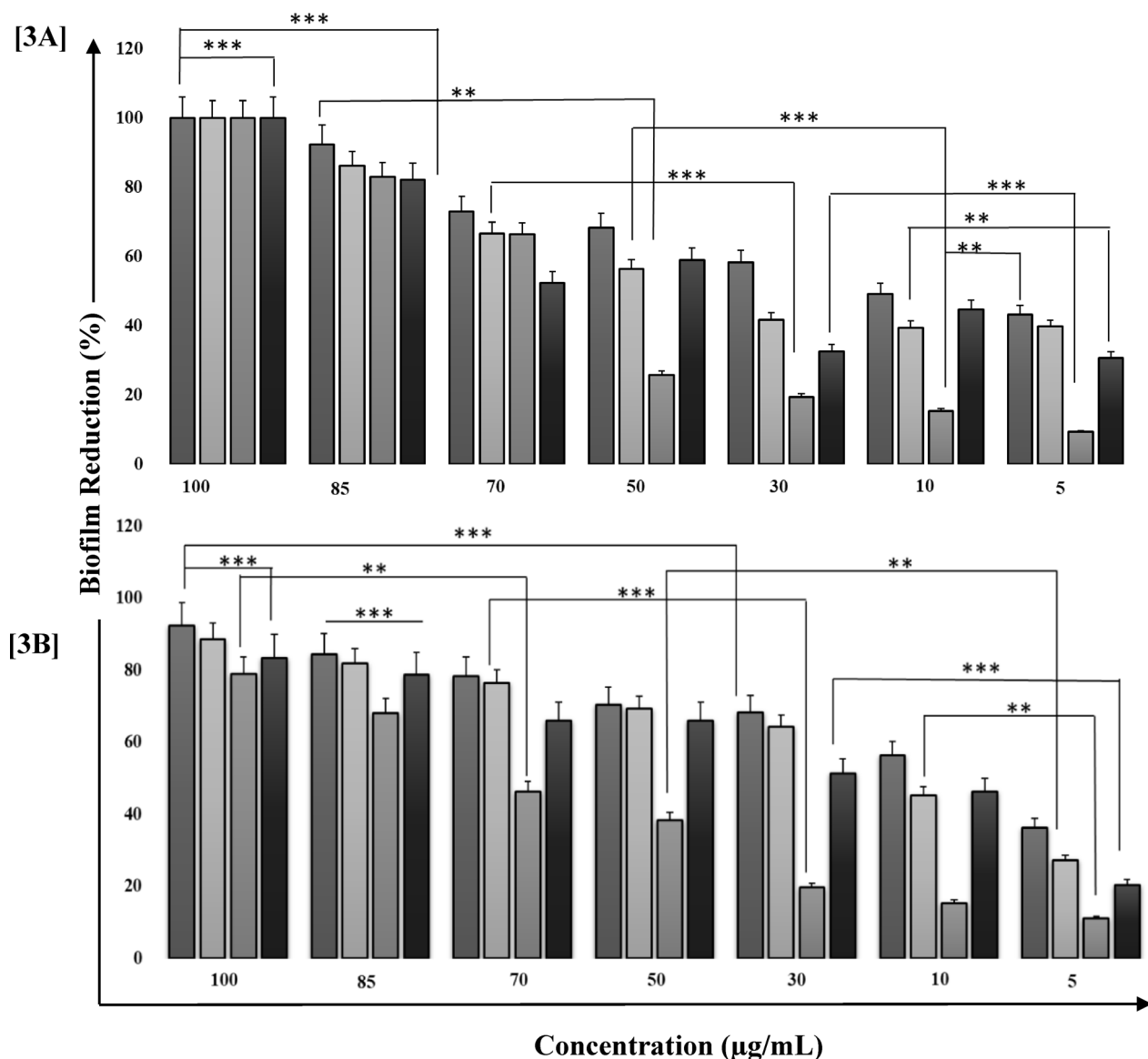


Fig. 3. Quantitative analysis of antibiofilm inhibition (%) of different concentrations of test compounds against (A) *Lactococcus Lactis* (NZ900), (B) *Bacillus Megaterium* (QMB1551), (C) *Pseudomonas putida* (ATCC25922) and (D) *Marcscens. Serratia* (NCTC10211). The experiment was repeated ($n=3$) and are presented as the mean $\pm$ SEM. Statistical significance was determined by a one-way ANOVA with Turkey's multiple comparison ($n=3$, *** $p<0.05$; ** $p<0.01$; * $p<0.001$ the control group).

3a. Curcumin's IC_{50} values for *P. putida* and *S. marcescens* were $84.5 \pm 3.6 \mu M$ and $88.2 \pm 1.4 \mu g/mL$, respectively. These results highlight curcumin's low bioavailability and limited capacity for disrupting biofilm structure when unconjugated. In all strains studied, ampicillin showed weaker antibiofilm activity than both conjugates, with IC_{50} values over $70 \mu g/mL$. Ampicillin exhibited inhibition for *P. putida* and *S. marcescens*, its IC_{50} values were $44.5 \pm 0.9 \mu g/mL$ and $58.2 \pm 2.4 \mu g/mL$, respectively and required significantly larger concentrations to produce similar effects to conjugates. These findings highlight the low bioavailability and restricted capacity of curcumin to alter the structure of biofilms when it is not conjugated. The findings show that bile acid-curcumin conjugates have improved antibiofilm activity. The most effective candidate is the conjugate **3a**, which consistently shows the lowest IC_{50} values across both Gram-positive and Gram-negative bacterial biofilms. By strategically conjugating with deoxycholic acid, curcumin's bioavailability, membrane affinity, and penetration into the extracellular polymeric matrix of biofilms have been substantially improved. The conjugate's broad-spectrum, multi-target mechanism of action is attributed to this structural alteration, which interferes with DNA replication, disrupts membrane integrity, and inhibits cell division. Significantly, conjugate **3a** important antibiofilm activity against resilient strains and model organisms shows potential as a next-generation therapeutic agent for the management of bacterial diseases associated with biofilms and resistant to antibiotics.

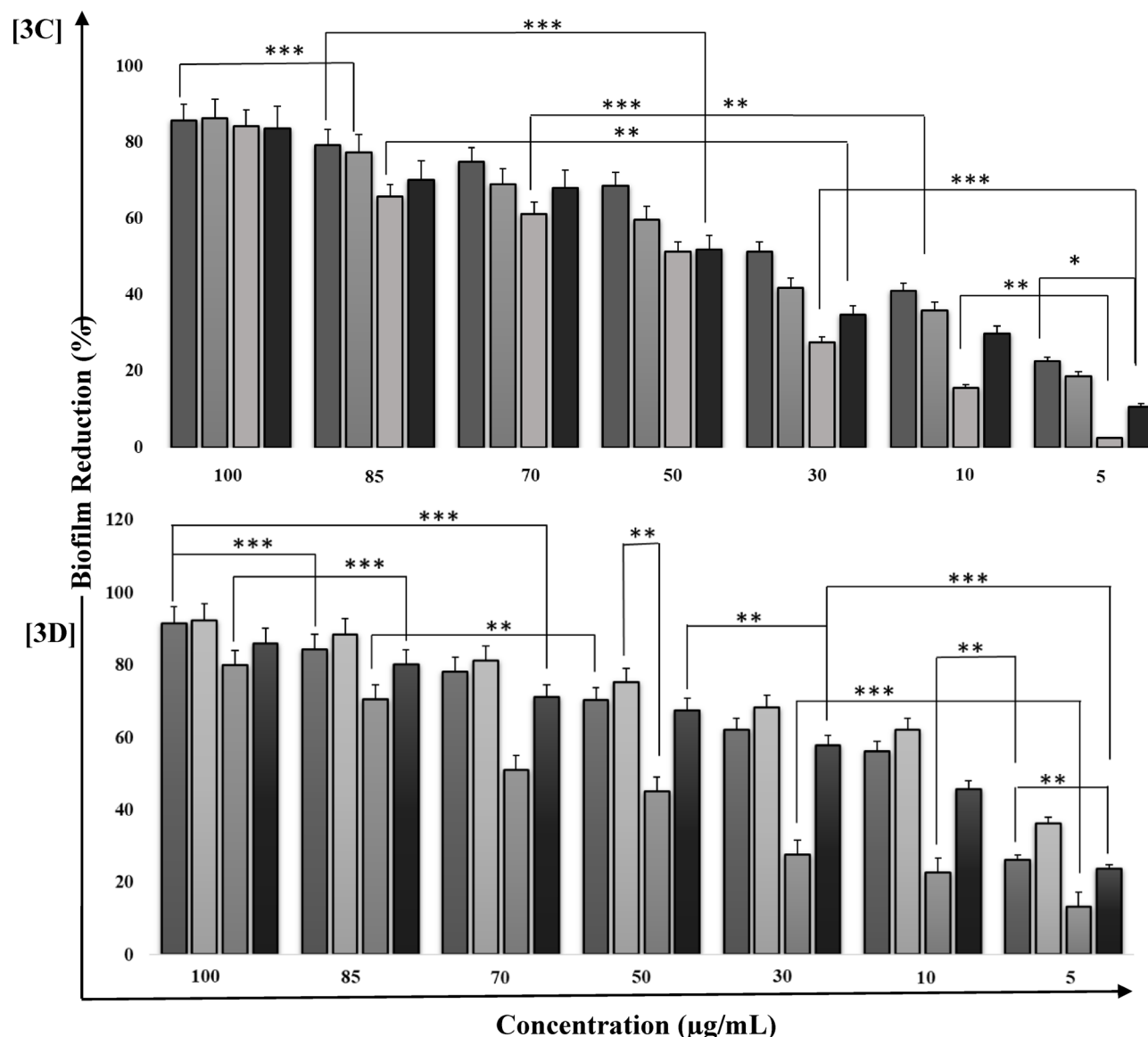


Fig. 3. (continued)

Molecular in silico analysis

The binding affinity (Docking Free energy) and amino acid interactions of the compounds with the selected bacterial drug targets are shown in (Table 2; Fig. 4). The lowest or comparable docked scores of -6.05 and -6.4 kcal/mol were against the DNA gyrase protein (curcumin dock score -6.42 kcal/mol), while the highest docked scores of -8.11 and -7.11 kcal/mol were demonstrated by **3a** against cell division (6LL6) and outer membrane (4RHB) protein, respectively. Figure 4. docked images 4a-c and 4a'-c' show how ligand **3a** interacts with important amino acids like arginine (Arg:31), phenylalanine (Phe:182), lysine (Lys:51), glycine (Gly:103), asparagine (Asn:45), aspartate (Asp:186), and alkyl and π -alkyl as well as conventional hydrogen bonding. Significant amino acids including proline (Pro:247), valine (Val:188), asparagine (Asp:187), serine (Ser:245), and methionine (Met:242) interact with ligand (3b) through π donor carbon hydrogen bonds that prevent cell division in protein (6LL6). Docking of ligand **3a** with outer cell membrane protein (4RHB) revealed binding interactions with significant and functionally relevant amino acids, including lysine (Lys:79) π -alkyl bonding, proline (Pro:46), valine (Val:80), glycine (Gly:41), threonine (Thr:103), and aspartate (Asp:42) through conventional hydrogen bonding. The docking studies of the **3b** ligand revealed that the binding interactions with important and functionally relevant amino acids, including Aspartate (Asp:25), Threonine (Thr:26), Serine (Ser:31), Leucine (Leu:23), Proline (PRO-202), Threonine (Thr:27), Threonine (Thr:32), Valine (Val:29), and Glycine (Gly:29), interact against (4RHB) through the van der Waals force and the isoleucine (Ile:164)

ΔG docking score [kcal/mol]			
Compounds	6LL6	4RHB	1KZN
Conjugate 3a	−8.12	−7.11	−6.89
Conjugate 3b	−6.73	−5.6	−6.4
Curcumin	−6.49	−2.99	−6.42
Clorobiocin	−6.18	−6.70	−6.62

Table 2.. Calculated docking scores for synthesized compounds (3a-b).

pi-sigma bond. Though the van der Waals force interacts with amino acids like arginine (Arg:192), lysine (Lys:212), phenylalanine (Phe:41), histidine (His:37), isoleucine (Ile:186), and threonine (Try:184), the docked interaction studies of **3b** with the DNA gyrase protein (1KZN) revealed additional pi-cation/anion interactions with histidine (His:38) and glutamic acid (Glu:183). detailed data on the number of hydrogen bonds formed between the ligand and the amino acid/nucleotide residues within the active site regions of the target protein (as shown in Supplementary Figs.9 and 10). In Fig.5 Clorobiocin, a well-known aminocoumarin antibiotic and DNA gyrase inhibitor, was included as a reference compound and docked against all three bacterial targets: (5a-5a') DNA gyrase (PDB: 1KZN), illustrates the binding of clorobiocin within the active site of the target protein, showing multiple stabilizing interactions. Key residues such as Glu50, Asp73, Thr165, and Arg76 form strong hydrogen bonds and electrostatic (π -anion) interactions, while Ala53, Ile78, and Val43 contribute hydrophobic contacts through alkyl and π -alkyl interactions. Amide- π stacking with Asn46 and Ala47 further stabilizes the ligand. These interactions indicate a well-anchored binding conformation, supporting clorobiocin's role as a potent reference inhibitor. Figure.5b-5b' the cell division protein FtsZ (PDB: 6LL6) with forming stable interactions within the active site of the target protein. Key hydrogen bonds are observed with residues Glu38, Gly35, and Gln56, while Arg33 engages in a strong π -anion interaction. Hydrophobic contacts, including alkyl and π -alkyl interactions, are contributed by Val30, Ile34, and Arg31, stabilizing the ligand within the pocket. Additionally, van der Waals interactions with surrounding residues support tight binding., and Fig. 5c and c' the outer membrane protein (PDB: 4RHB) showed Conventional hydrogen bonds are observed with Asn527, Asn592, Asp592, Tyr388, and Tyr528, suggesting strong polar interactions critical for ligand stabilization. Additionally, alkyl and π -alkyl interactions with residues such as Val379, Phe417, Ile595, and Ala390 contribute to hydrophobic stabilization within the binding pocket. These combined interactions support the stable and specific binding conformation of clorobiocin.

Mechanism action of targeted protein

The molecular docking study investigated the synthetic bile acid–curcumin conjugates binding affinities against three important bacterial targets: DNA gyrase subunit B (PDB ID: 1KZN), outer membrane porin protein (PDB ID: 4RHB), and FtsZ (PDB ID: 6LL6). The results of this study provided mechanistic insight into the antibacterial activity of the conjugates. These targets were carefully selected since they are essential to the survival and growth of bacteria. Both conjugates interact with bacterial outer membrane and cell division proteins, specifically targeting the GTPase enzyme responsible for GTP polymerization and Z-ring formation during cytokinesis. By binding to this enzyme, the conjugates inhibit septum formation, thereby blocking bacterial cell division and compromising membrane integrity. With a docking score of −8.12 kcal/mol, the conjugate **3a** showed the highest binding affinity towards FtsZ in comparison to both conjugate **3b** and curcumin (−6.49 kcal/mol). In order to initiate cell division by forming the Z-ring at the site of septum formation, the highly conserved bacterial protein FtsZ is required^{51,55,56}. Considering conjugate **3a** has a high affinity for the GTP-binding site of FtsZ, it may be effective in preventing its assembly and polymerisation, which would stop cytokinesis and prevent the growth of bacteria^{57,58}. The fact that FtsZ-mediated septum formation is a key process of binary fission in both Gram-positive and Gram-negative bacteria, this result is very pertinent. Conjugate **3a** has the ability to damage the integrity of the bacterial outer membrane because it not only targets FtsZ but also exhibits a strong interaction with the outer membrane porin protein (−7.11 kcal/mol). Transmembrane proteins called porins (PDB: 4RHB) control the flow and inflow of small molecules across Gram-negative bacteria's outer membrane. This transport function gets impeded by 3a binding to the β -barrel pore structure, which could result in increased membrane permeability, osmotic imbalance, and ultimately cell lysis.

The functional advantage provided by bile acid conjugation was highlighted by the much lower affinity that curcumin alone showed for the membrane protein (−2.99 kcal/mol). Though not as strongly as conjugate **3a**, conjugate **3b** also demonstrated a moderate interaction with the porin (−5.60 kcal/mol), indicating that the steric conformation and hydrophobicity of the conjugate **3a** moiety may improve membrane integration and disruption in comparison to the conjugate **3b**. Additionally, all conjugates showed moderate binding inside the ATPase domain of the GyrB subunit, including the second major protein DNA gyrase (PDB ID: 1KZN), a type II topoisomerase essential for decreasing torsional strain during DNA replication and transcription. Interestingly, conjugate **3b** (−6.40 kcal/mol) and curcumin (−6.42 kcal/mol) showed a slightly stronger affinity than conjugate **3a** (−6.89 kcal/mol). This suggests that even though conjugate **3a** is more effective at targeting membranes and divisions, conjugate **3b** and curcumin may still have an effective inhibitory interaction with enzymes involved in DNA processing. These interactions most likely disrupt transcription and replication by interacting with ATP hydrolysis and preventing bacterial DNA from supercoiling. This mechanism of action contributes to the conjugates overall antibacterial potency, even if it can be considered secondary in comparison to membrane rupture and cell division inhibition. The docking data indicate that the conjugates of bile acid and curcumin

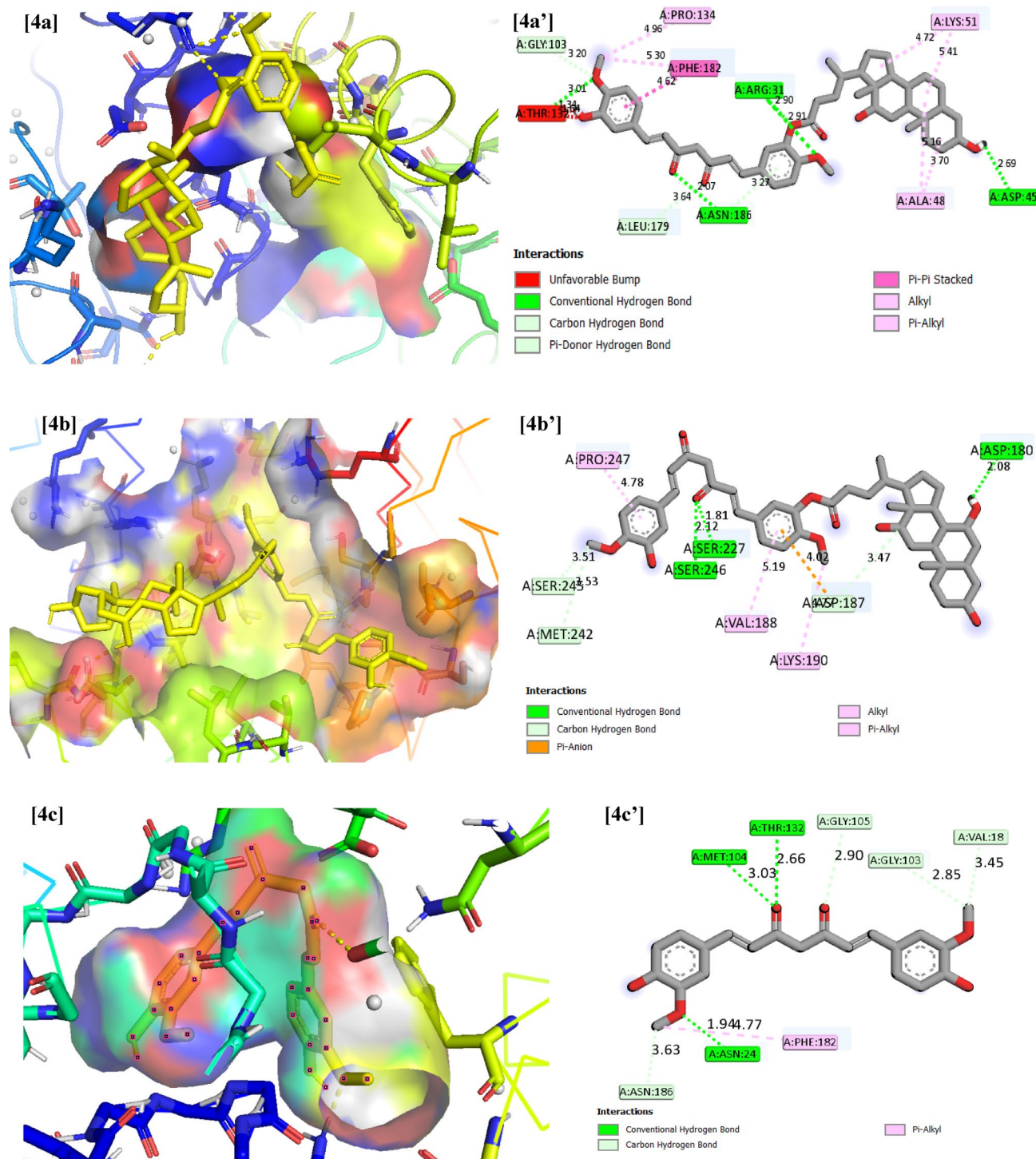


Fig. 4. Docking of the (4a) conjugate 3a, (4b) conjugate 3b and (4c) Curcumin with the PDB: 6LL6 protein. (a) Displays the binding pocket of 6LL6, showing the compound positioned and highlights the 3D interactions between the compound and the protein within the active site, demonstrating stable binding within the active site. (4a'–c') Provides a 2D schematic representation of the bonds and interactions.

work through a synergistic multi-target mechanism that includes DNA replication suppression, cytokinesis inhibition, and membrane destabilisation. When compared to curcumin alone, the conjugation of curcumin with bile acids, particularly conjugate 3a, appears to improve hydrophobic interactions, membrane permeability, and protein binding efficiency, leading to a significantly higher antibacterial activity. Targeting multiple critical bacterial processes simultaneously, this multi-site interference approach is very effective at preventing resistance development. These results validate the rational development of bile acid–curcumin hybrids as broad-spectrum, antibacterial, and antibiofilm agents.

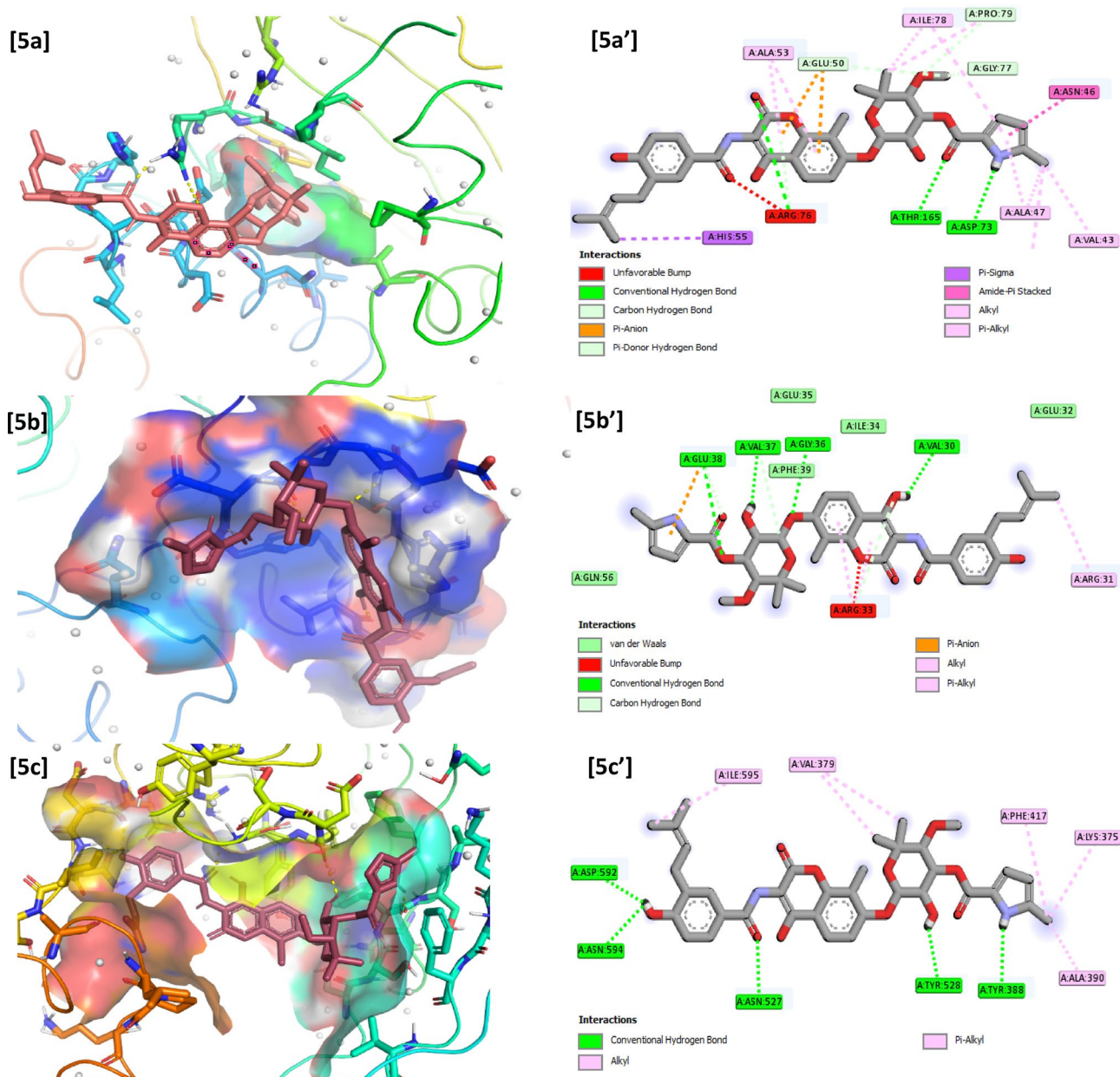


Fig. 5. Docking with Inhibitor Clorobiocin (**5a**) DNA gyrase (PDB: 1KZN), (**5b**) cell division (PDB: 6LL6) and (**5c**) cell membrane (PDB: 4RHB) protein. (**a**) Displays the binding pocket, showing the compound positioned and highlights the 3D interactions between the compound and the protein within the active site, demonstrating stable binding within the active site. (**5a'-c'**) Provides a 2D schematic representation of the bonds and interactions.

Ligand efficiency (LE) analysis

To evaluate the binding quality of the synthesized conjugates independent of molecular size, ligand efficiency (LE) was computed by normalizing the binding affinity to the number of heavy atoms (non-hydrogen atoms) in each ligand, and the ligand efficiency was calculated using formula 1 with the best binding pose⁵⁹.

$$LE = \frac{\Delta G \text{ (binding energy)}}{N \text{ (Heavy atoms)}} \quad (1)$$

Where:

LE = Ligand Efficiency (in kcal/mol per heavy atom).

Δ = Binding energy of the ligand (typically from docking, in kcal/mol; negative value).

N = Number of non-hydrogen atoms in the ligand.

Compound	Binding energy (kcal/mol)	Heavy atoms	Ligand efficiency (kcal/mol per heavy atom)		
			6LL6	4RHB	1KZN
Conjugate 3a	−8.12	54	−0.15	−0.132	−0.128
Conjugate 3b	−6.73	55	−0.122	−0.102	−0.116
Curcumin	−6.49	27	−0.24	−0.111	−0.238
Clorobiocin	−6.18	49	−0.126	−0.137	−0.135

Table 3.. Ligand efficiency (LE) of selected compounds against three bacterial target proteins: LE values represent binding energy normalized per heavy atom.

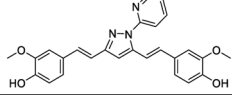
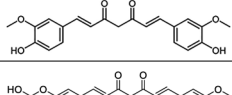
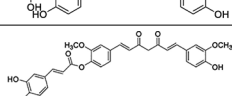
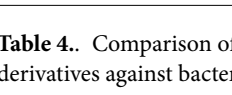
Compound	Target organism	MIC value	References
Conjugate 3a	<i>Pseudomonas putida</i> (ATCC 25922)	4.48 ± 0.2 (MIC ₅₀ : µg/mL)	This Work
Conjugate 3b	<i>Bacillus megaterium</i> (QMB 1551)	7.02 ± 0.4 (MIC ₅₀ : µg/mL)	
	<i>Staphylococcus aureus</i>	0.0625 (MIC: mg/mL)	53
	<i>Staphylococcus aureus</i>	0.25 (MIC: mg/mL)	
	<i>Staphylococcus aureus</i>	78 (MIC: µg/mL)	52
	<i>Enterococcus faecalis</i>	156 (MIC: µg/mL)	
	<i>Staphylococcus aureus</i>	0.9 (MIC: µg/mL)	54
	<i>Streptococcus viridans</i>	0.9 (MIC: µg/mL)	

Table 4.. Comparison of IC₅₀ values of the synthesized conjugates with literature-reported curcumin derivatives against bacterial strains.

LE provides a size-independent metric for assessing the efficiency of a molecule in engaging its biological target, thereby enabling more rational optimization of drug-like properties. The LE values were calculated for each compound docked to three bacterial protein targets: cell division protein (PDB: 6LL6), cell membrane-associated target (PDB: 4RHB), and DNA gyrase (PDB: 1KZN) in Table 3. The conjugate-3a exhibited consistently superior ligand efficiency across all three targets, with values of −0.150 kcal/mol/HA (6LL6), −0.132 kcal/mol/HA (4RHB), and −0.128 kcal/mol/HA (1KZN). This suggests that despite its higher molecular weight (742.95 g/mol), 3a maintains a favorable binding-to-size ratio, likely because of its optimized interaction profile with critical binding site residues. The conjugate 3b showed slightly lower LE values, ranging from −0.102 to −0.122 kcal/mol/HA, indicating moderate interaction efficiency that may be attributed to differences in linker flexibility or functional group orientation affecting the binding pose. Interestingly, curcumin, although significantly smaller, yielded the highest LE for PDB: 6LL6 (−0.240 kcal/mol/HA) and PDB: 1KZN (−0.238 kcal/mol/HA), suggesting highly efficient per-atom interactions for these targets. However, its LE dropped markedly to −0.111 kcal/mol/HA for 4RHB, indicating a target-dependent variability in the interaction quality. Clorobiocin, a known antibiotic reference, displayed relatively uniform LE values across all three targets (−0.126 to −0.137 kcal/mol/HA), serving as a robust baseline for evaluating the novel ligands.

Among all the tested ligands, conjugate 3a exhibited the highest ligand efficiency (LE = −0.150 kcal/mol per heavy atom) against the cell division protein (PDB ID: 6LL6). These results position the conjugate 3a as a compelling lead compound, demonstrating consistently favorable ligand efficiency (LE) values across multiple bacterial protein targets. The ability of 3a to maintain high binding efficiency while accommodating substantial molecular complexity underscores its structural and pharmacodynamic optimization potential. This convergence of target affinity and atom economy reinforces its potential for advancement as a broad-spectrum antibacterial agent with multi-target inhibitory capacity.

Conclusion

In this study, bile acids were employed as bioactive carriers to successfully synthesize the two-novel steroidal-curcumin conjugates of 3a and 3b via a condensation reaction. FT-IR, ¹H NMR, ¹³C NMR, HPLC, and mass spectrometry investigations were used to corroborate the structural elucidation. In comparison to native curcumin, both conjugates showed increased antibacterial and antibiofilm activity. Conjugate-3a showed strong inhibition of *P. putida* (ATCC 25922) at an IC₅₀ of 4.48 ± 0.2 µg/mL, while conjugate 3b showed activity against *B. megaterium* (QMB 1551) at an IC₅₀ of 7.02 ± 0.4 µg/mL. Curcumin alone, on the other hand, showed much less activity; IC₅₀ values of 46.09 ± 2.6 µg/mL and 76.02 ± 5.2 µg/mL against the strains, respectively, demonstrate that both conjugates are approximately tenfold more active. Molecular docking investigations demonstrated that conjugate 3a had a significantly higher binding affinity for important bacterial targets, which corroborated

the experimental observations. Particularly, conjugate **3a** had a docking score of -7.11 kcal/mol for the outer membrane protein (PDB ID: 4RHB), while curcumin had a score of -2.99 kcal/mol. Furthermore, conjugate **3a** generated a binding energy of -8.12 kcal/mol, which was higher than curcumin's -6.49 kcal/mol, indicating improved interaction with the bacterial cell division protein FtsZ (PDB ID: 6LL6). All of these findings point to the fact that bile acid conjugation substantially improves curcumin's bioactivity, target specificity, and membrane interaction. This leads to a feasible strategy for developing next-generation antimicrobial drugs that combat resistant bacterial infections more effectively.

Conjugate **3a** achieved $>90\%$ biomass reduction at $80\text{ }\mu\text{g/mL}$ and strongly reduced the production of biofilms in *Lactococcus lactis* (NZ900) and *Bacillus megaterium* (QMB1551). Subsequently demonstrated its significant efficiency by achieving $>50\%$ biofilm inhibition in *L. lactis* with an estimated IC_{50} of $24.8 \pm 1.3\text{ }\mu\text{g/mL}$ at a concentration of only $25\text{ }\mu\text{g/mL}$. The IC_{50} values for conjugate **3a** against Gram-negative organisms were $28.3 \pm 0.4\text{ }\mu\text{g/mL}$ for *P. putida* (ATCC 25922) and $31.9 \pm 0.6\text{ }\mu\text{g/mL}$ for *S. marcescens* (NCTC 10211) in biofilm formation, indicating that it was able to penetrate across the protective outer membrane barrier that is usually associated with biofilm resilience. Comparatively, conjugate **3b** likewise demonstrated significant antibiofilm effects, with IC_{50} values of $54.1 \pm 0.8\text{ }\mu\text{g/mL}$ for *S. marcescens* and $61.7 \pm 1.9\text{ }\mu\text{g/mL}$ for *P. putida*. However, its efficacy was consistently lower than that of conjugate **3a**. The structural and physicochemical benefits of deoxycholic acid are believed to be responsible for conjugate **3a** improved antibiofilm performance. These benefits probably include improved membrane penetration, dispersion of the biofilm matrix, and multi-target interaction, including disruption of FtsZ and DNA gyrase function. Remarkably, both conjugates displayed significant antibiofilm capabilities along with their strong antibacterial effects, effectively decreasing biofilm biomass in both Gram-positive and Gram-negative strains at doses lower than those required for curcumin or ampicillin. The antibacterial and antibiofilm efficacy observed, particularly for conjugate **3a** showed in IC_{50} Table 1. Bile acids are inherently amphiphilic, and their conjugation to curcumin significantly increases the overall lipophilicity of the resulting compounds. This enhanced lipophilicity likely facilitates more effective integration into and disruption of bacterial membranes, particularly in Gram-negative strains where the outer membrane acts as a barrier to many hydrophilic drugs. The greater membrane permeability and improved cellular uptake of the lipophilic conjugates may contribute to their higher activity. Among the two, conjugate **3a** derived from deoxycholic acid showed better performance than conjugate **3b**, may due to its relatively higher hydrophobicity compared to cholic acid. This suggests that subtle structural differences in bile acids can influence physicochemical behavior, membrane interaction, and ultimately biological activity. Their dual activity demonstrates their potential as a treatment for both planktonic and sessile bacterial species. These findings validate conjugate **3a** as a promising lead candidate with broad-spectrum antibiofilm potential, offering an effective strategy for targeting persistent and drug-resistant bacterial biofilms in both Gram-positive and Gram-negative infections.

Experimental section

Materials and methods

The study's reagents and chemicals were all purchased from SRL, Sigma-Aldrich, and Alfa Aesar, and they were all used exactly as supplied, without no further purification. curcumin ($\geq 98\%$ purity) and bile acids (cholic acid and deoxycholic acid, $\geq 98\%$ purity) were procured from Sigma-Aldrich (USA). Solvents were also purchased from commercial sources and used straight away unless otherwise noted. TLC was used on Merck silica gel 60 F254 plates to monitor the reactions' progress. Column chromatography employing silica gel with a particle size range of 230–400 mesh was then used to purify the compounds that were synthesized during the course of the study. A FTIR (ATR)-IR Spirit was used to perform Fourier Transform Infrared Spectroscopy (FTIR) measurements, and a Shimadzu UV-1900i Spectrophotometer was used to collect UV absorbance data (spectral analyses). All synthesized conjugates ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using Bruker 400 MHz NMR spectrometers, with tetramethyl silane (TMS) helping as the internal reference. Chemical shifts and coupling constants (J) are measured in parts per million (ppm, δ) and Hertz (Hz), respectively and Microtiter plate reader (96 well plate).

General procedure for synthesis of conjugates of bile acids and Curcumin (3a-b)

10 mL of anhydrous DMF containing 1.0 mmol of cholic acid/deoxycholic acid and 1.1 mmol of curcumin round bottom flask was cooled (ice bath) in an argon environment before DMAP 0.2 mmol, EDCI 1.2 mmol, and TEA 2.0 mmol were added. At room temperature, the reaction mixture was stirred constantly for 18 to 24 h. To monitor the reaction's progress, TLC was employed. The mixture was diluted with 50 mL of ethyl acetate after the reaction completed, and then it was washed out with 50 mL of citric acid and 50 mL of brine. After filtering and drying the ethyl acetate fraction over anhydrous Na_2SO_4 , the organic solvent was evaporated under vacuum. Ethyl acetate/hexane was used as the eluent in silica column chromatography to purify the residue, yielding 70–78%. Mass spectrometry, ^1H NMR, ^{13}C NMR spectroscopy, and HPLC were employed to characterise the purified products.

Spectral data of synthesized conjugate (3a-3b)

4-((1E,6E)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dien-1-yl)-2-methoxyphenyl
4-((3R,10 S,12 S,13R)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1 H-cyclopenta[a]phenanthren-17-yl)
pentanoate (conjugate 3a)

Purified by chromatography on silica gel, eluting ethyl acetate/hexane 8:2 (v/v), yellow solid, m.p. = $189\text{--}192\text{ }^\circ\text{C}$; 78% yield (280 mg), purity: 99%; ^1H -NMR (400 MHz, $\text{DMSO}-d_6$): δ 9.76 (1 H, s, OH), 7.61 (1 H, d, $J = 15.6\text{ Hz}$,

aromatic H), 7.59 (1 H, d, $J=8.2$ Hz, aromatic H), 7.58–7.35 (6 H, m, aromatic H), 7.14–7.13 (2 H, m, aromatic H), 6.84–6.82 (4 H, m, alkenyl H), 4.33 (1 H, s, 12-OH), 2.51 (1 H, s, 3-OH), 3.82, 3.81 (each 3 H, s, OCH₃), 1.99–1.16 (26 H, m, CH/CH₂ in steroidal ring), 1.38 (3 H, s, CH₃), 0.85 (3 H, s, 18-CH₃), 0.63 (3 H, d, $J=5.8$ Hz, 21-CH₃), 0.62 (3 H, s, 19-CH₃) (Supplementary Fig.2). ¹³C-NMR (125 MHz, DMSO-d₆): δ 192.77, 192.33 (C=O, diketone); 172.32 (ester C=O); 151.90 (aromatic C-OH); 148.23 (aromatic C-OCH₃); 142.71, 141.33 (conjugated alkene); 139.78, 133.97 (aromatic C); 126.59, 122.74, 122.53 (inner aromatic C); 118.93–112.40 (conjugated aromatic/alkene C); 70.92 (C-OH in steroid core); 59.94, 56.45, 55.68 (OCH₃); 44.27–38.90 (bridgehead C in steroid); 31.77 (aliphatic CH/CH₂); 21.78, 17.31, 12.92 (methyl C) (as shown in Supplementary Fig.3). FTIR (cm⁻¹): 1700–1750 (C=O in ester/ketone); 1200–1300 (C-O in ester/phenol); 1000–1150 (C-O-C in OCH₃); 3300–3500 (O-H); 1600–1650 (C=C in aromatic/conjugated systems) (Supplementary Fig.1). In a 15-minute run, a 10.85-minute retention time and 99.53% purity were obtained (Supplementary Fig.4).

4-((1E,6E)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dien-1-yl)-2-methoxyphenyl (R)-4-((3R,5 S,7R,8R,9 S,10 S,12 S,13R,14 S,17R)-3,7,12-trihydroxy-10,13-dimethylhexadecahydro-1 H-cyclopenta[a]phenanthren-17-yl)pentanoate (conjugate 3b)

Purified by chromatography on silica gel, eluting ethyl acetate/hexane 6:4 (v/v), yellow solid, Mp = 186–190 °C; 70% yield (251 mg), purity: 99%; ¹H-NMR (400 MHz, DMSO-d₆): δ 9.78 (1 H, s, OH), 7.60 (1 H, d, $J=15.6$ Hz, aromatic H), 7.57 (1 H, d, $J=8.2$ Hz, aromatic H), 7.57–7.31 (6 H, m, aromatic H), 7.29–7.10 (2 H, m, aromatic H), 6.82–6.80 (4 H, m, alkenyl H), 4.34 (1 H, s, 12-OH), 2.51 (1 H, s, 3-OH), 3.85, 3.82 (each 3 H, s, OCH₃), 1.71–1.47 (26 H, m, CH/CH₂ in steroidal ring), 0.89 (3 H, s, CH₃), 0.81 (3 H, s, 18-CH₃), 0.64 (3 H, d, $J=5.8$ Hz, 21-CH₃), 0.63 (3 H, s, 19-CH₃) (Supplementary Fig.6). ¹³C-NMR (125 MHz, DMSO-d₆): δ 194.37, 194.22 (C=O, diketone); 171.93 (ester C=O); 151.75 (aromatic C-OH); 148.47 (aromatic C-OCH₃); 142.02, 141.30 (conjugated alkene); 139.51, 134.21 (aromatic C); 126.68, 122.74, 124.98 (inner aromatic C); 116.17–101.18 (conjugated aromatic/alkene C); 71.49 (C-OH in steroid core); 60.24, 56.45, 56.15 (OCH₃); 47.94–36.60 (bridgehead C in steroid); 33.39 (aliphatic CH/CH₂); 23.55, 14.55, 12.92 (methyl C) (Supplementary Fig.7). FTIR (cm⁻¹): 1700–1750 (C=O in ester/ketone); 1200–1300 (C-O in ester/phenol); 1000–1150 (C-O-C in OCH₃); 3300–3500 (O-H); 1600–1650 (C=C in aromatic/conjugated systems) (Supplementary Fig.5). In a 15-minute run, a 9.75 min retention time and 99.94% purity were obtained (Supplementary Fig.8).

In vitro antibacterial assay

A broth microdilution assay was evaluated using 96-well plates to evaluate the antibacterial properties of the bile acid-curcumin conjugate. The half-maximal inhibitory concentrations (IC₅₀), expressed in μ g/mL, were determined through a two-fold serial microdilution method following standard protocols. Initially, the conjugate was dissolved in DMSO to create a stock solution with a concentration of 1000 μ g/mL, which was then mixed with Mueller-Hinton broth (MHB). The conjugates were then serially diluted twice to achieve 5–100 μ g/mL concentrations. The prepared microorganisms were diluted to the McFarland standard (0.5) optical density of 1.5×10^8 CFU/mL. A prepared bacterial culture of 970 μ L was then taken in each sterilized Eppendorf. The final volume was built up to 1 mL after 30 μ L of each dilution was added to the Eppendorf tube (Final concentration of DMSO as V/V: 3%). The inoculum was mixed evenly with the broth by shaking the Eppendorf, and it was then incubated for 24 h at 37 °C. A triplicate set of experiments was conducted. The IC₅₀ value was determined as the lowest concentration of conjugates that inhibited visible growth after incubation. *Pseudomonas putida* (ATCC25922), *Bacillus Megaterium* (QMB1551), *Lactococcus Lactis* (NZ900), and *Marcescens Serratia* (NCTC10211) are the four bacterial strains that were tested. These strains were obtained from the Institute of Advanced Research's (IAR) Microbiology Laboratory in Gandhinagar, and they provided a varied bacterial panel to evaluate the synthetic compounds' antibacterial activity.

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of treated cell}}{\text{Absorbance of untreated cell}} \times 100$$

The half-maximal inhibitory concentration (IC₅₀) was calculated by graphing the test compound's concentration in μ g/mL versus cell viability (%). Statistical validity was ensured by doing each measurement in triplicate.

Antibiofilm assay

A standard biofilm inhibition experiment was used with the same bacterial strain to systematically assess the antibiofilm activity of the synthesized bile acid-curcumin combination (3a–b) against both Gram-positive and Gram-negative bacterial strains. In our approach, biofilm production is quantitatively measured using a microplate reader, as described by Stepanovic et al.⁶⁰. The amount of biofilm that forms is measured with a microreader plate. Luria-Bertani (LB) Broth is used to develop the microbial culture, which is then adjusted to the McFarland standard (0.5). The comparable cell density of 1.5×10^8 organisms/mL CFU is obtained with optical density (OD) at 600 nm⁶¹. There were 180 μ L of bacterial culture in each well of the 96-well flat-bottom sterile polystyrene plates. Subsequently 20 μ L of each triclosan conjugate in DMSO at different doses (5, 10, 15, 30, 50, 85, and 100 μ g/mL) was added. As previously demonstrated to be suitable for these cultures, microplates were incubated for 24 h at 37 °C without agitation. The plate washed three times with distilled water after the planktonic cells were removed, and then each well was stained with 100 μ L of 1% aqueous crystal violet for 15 min at room temperature. Adhesive cells were stained along the plate walls for 20 min, and then the plates were rinsed with sterile distilled water. To dissolve the adherent cells, 200 μ L of 30% acetic acid was added to each well after drying. Then, using a microplate reader, the absorbance of each well was determined at 600 nm. The normalised optical density value (OD₆₀₀) of adherent cell biofilm development was measured, excluding planktonic cell contributions. Each experiment was carried out in triplicate.

Molecular docking study

Docking studies were conducted to assess the potential antibacterial activities of the two representative compounds with clorobiocin, a known DNA gyrase inhibitor, included as a reference compound for comparative analysis. These studies focused on their antibacterial properties, leading to the selection of three common proteins: DNA Gyrase (PDB ID: 1KZN), a transport protein (PDB ID: 4RHB), and the E. Coli cell division protein FtsZ (PDB ID: 6LL6). Based to the docking scores that were computed (detailed provided in Table 2), the majority of the compounds that were analyzed showed stronger efficacy and potential antibacterial activity. Both docked compounds occupied the same binding pocket as the parent co-crystallized ligands, confirming their specific interaction and potential efficacy.

Choose target for docking study

Three important bacterial proteins were carefully chosen for molecular docking experiments in order to assess the antibacterial potential of the synthesized compounds. These studies focused on the proteins' vital functions in bacterial physiology and their applicability as verified targets in antibacterial research. The first protein, E. coli cell division protein FtsZ (PDB ID: 6LL6), is a tubulin homolog that forms the Z-ring at the site of cell division, which is essential for bacterial cytokinesis⁶². It is a prime target for selective antibacterial drugs due to its structural differences from human proteins and importance in cell survival. DNA Gyrase (PDB ID: 1KZN), the second protein, is a type II topoisomerase that is essential for preserving DNA supercoiling and supporting transcription, replication, and chromosomal segregation. Its importance as a target for antibacterial drugs discovery is established by its crucial function in bacterial survival and its absence in human cells⁶³. The third protein, transport protein (PDB ID: 4RHB), plays a key role in ion transport, cellular homeostasis, and nutrition uptake all of which are essential for bacterial metabolism and survival⁶⁴. A thorough evaluation of the drugs' antibacterial effectiveness can be obtained by targeting these three proteins, which stand for essential biological functions like cell division, DNA replication, and nutrition uptake. The study's translational potential is improved by this systematic and comprehensive strategy, which addresses important pathways in bacterial resistance.

Protein preparation

In the present study, we chose a variety of bacterial proteins for this investigation, including those involved in cell division, DNA replication, transcription, and the outer membrane. All three proteins, which were extracted as PDB files, are crucial to the bacterial life cycle. Then, each of these biomolecules was imported into AutoDock 1.5.7, a molecular docking program. Using Biovia visualizer software v24.1.0.23298, the proteins were first further trimmed by removing the co-crystallized ligands. After removing water molecules and heteroatoms, hydrogen was added, charges (Kollman charges) were estimated, and the protein was converted to a pdbqt file. The co-crystallized ligands were then positioned in the grid box's middle. The grid box measurements were saved in config.txt format for AutoDock Vina docking.

Ligand preparation

The bioactive ligand molecules (**3a-b**) that were synthesized were transformed into three dimensions utilizing 3D chem software. The ligands from 3D SDF files were converted to the Protein Data Bank (PDB) format using Openable 2.4.1. The ligand preparation process involved the individual uploading of these ligand molecules into AutoDock Tools. The compounds were charged with Gasteiger accusations. Additionally, polar hydrogen atoms were combined, and rotational interactions were identified and altered.

Molecular docking analysis

All compounds undergo molecular docking study using AutoDock Vina 1.5.7 and the script standard approach. After combining polar hydrogens, the target proteins and chosen compounds were both saved in pdbqt format. To perform molecular docking, a grid box was used. It was essential to design grid boxes with specific measurements and a spacing of 0.3 Å. Protein–ligand complex docking experiments were conducted using the Lamarckian Genetic Algorithm (LGA)⁴⁰. All the binding affinities were determined. The docking positions of the complex structures and the interactions between the amino acids were examined using Biovia Discovery Studio v24.1.0.23298.

Statistical analysis

All experiments were performed in three independent biological replicates, each consisting of three technical replicates ($n = 3$ biological $\times$ 3 technical). Data are expressed as mean $\pm$ standard error of the mean (SEM) based on biological replicates. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A p -value of less than 0.05 was considered statistically significant. Significance levels were denoted as follows: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$, compared to the control group.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

Received: 27 May 2025; Accepted: 6 August 2025

Published online: 01 October 2025

References

- Bertagnolio, S. et al. WHO global research priorities for antimicrobial resistance in human health. *Lancet Microbe*. **5** (11). [https://doi.org/10.1016/S2666-5247\(24\)00134-4](https://doi.org/10.1016/S2666-5247(24)00134-4) (2024).
- Shallcross, L. J. & Davies, S. C. The world health assembly resolution on antimicrobial resistance. *J. Antimicrob. Chemother.* **69** (11), 2883–2885. <https://doi.org/10.1093/jac/dku346> (2014).
- Rahmani, A. H., Alsahli, M. A., Aly, S. M., Khan, M. A. & Aldebasi, Y. H. Role of Curcumin in disease prevention and treatment. *Adv. Biomed. Res.* **7**, 38. https://doi.org/10.4103/abr.abr_147_16 (2018).
- Abdallah, E. M., Alhatlani, B. Y., de Paula, R., Menezes & Martins, C. H. Back to nature: medicinal plants as promising sources for antibacterial drugs in the Post-Antibiotic era. *Plants* **12**, 17. <https://doi.org/10.3390/plants12173077> (2023).
- Fleishman, J. S. & Kumar, S. Bile acid metabolism and signaling in health and disease: molecular mechanisms and therapeutic targets. *Signal. Transduct. Target. Ther.* **9** (1), 97. <https://doi.org/10.1038/s41392-024-01811-6> (2024).
- Hofmann, A. F. Bile acids: trying to understand their chemistry and biology with the hope of helping patients. *Hepatology* **49** (5), 1403–1418. <https://doi.org/10.1002/hep.22789> (2009).
- Staley, C., Weingarden, A. R., Khoruts, A. & Sadowsky, M. J. Interaction of gut microbiota with bile acid metabolism and its influence on disease States. *Appl. Microbiol. Biotechnol.* **101** (1), 47–64. <https://doi.org/10.1007/s00253-016-8006-6> (2017).
- Begley, M., Gahan, C. G. M. & Hill, C. The interaction between bacteria and bile. *FEMS Microbiol. Rev.* **29** (4), 625–651. <https://doi.org/10.1016/j.femsre.2004.09.003> (2005).
- Pavlović, N. et al. Bile acids and their derivatives as potential modifiers of drug release and Pharmacokinetic profiles. *Front. Pharmacol.* **9**, 1283. <https://doi.org/10.3389/fphar.2018.01283> (2018).
- di Gregorio, M. C., Cautela, J. & Galantini, L. Physiology and physical chemistry of bile acids. *Int. J. Mol. Sci.* **22** (4). <https://doi.org/10.3390/ijms22041780> (2021).
- Pagano, K. et al. Bile acid binding protein: A versatile host of small hydrophobic ligands for applications. *Comput. Struct. Biotechnol. J.* **6** (7), e201303021. <https://doi.org/10.5936/csbj.201303021> (2013). The Fields Of Mri Contrast Agents And Bio-Nanomaterials.
- Baker, K., Sikkema, R. & Zhitomirsky, I. Application of bile acids for biomedical devices and sensors. *Med. Devices Sens.* **3**, 1–9. <https://doi.org/10.1002/mds3.10119> (2020).
- Mishra, R. & Mishra, S. Updates in bile acid-bioactive molecule conjugates and their applications. *Steroids* **159**, 108639. <https://doi.org/10.1016/j.steroids.2020.108639> (2020).
- Tsakamoto, S., Matsunaga, S., Fusetani, N. & van Soest, R. W. Acanthosterol sulfates A-J: ten new antifungal steroidal sulfates from a marine sponge *Acanthodendrilla* sp., *J. Nat. Prod.* **61** 11, 1374–1378, (1998). <https://doi.org/10.1021/np980178n>
- Mohamed, N. R., Elmegeed, G. A., Abd-ElMalek, H. A. & Younis, M. Synthesis of biologically active steroid derivatives by the utility of Lawesson's reagent., *Steroids* **70**, 3, 131–136, (2005). <https://doi.org/10.1016/j.steroids.2004.11.001>
- Brunel, J. M., Loncle, C., Vidal, N., Dherbomez, M. & Letourneux, Y. Synthesis and antifungal activity of oxygenated cholesterol derivatives., *Steroids*, **70** 13, 907–912, (2005). <https://doi.org/10.1016/j.steroids.2005.06.007>
- Zhang, Y. et al. Atropurosides A-G, new steroidal saponins from *Smilacina atropurpurea*. *Steroids* **71** (8), 712–719. <https://doi.org/10.1016/j.steroids.2006.04.005> (2006).
- Sugandhi, E. W., Slebodnick, C., Falkinham, J. O. 3rd & Gandour, R. D. Synthesis and antimicrobial evaluation of water-soluble, dendritic derivatives of epimeric Salpha-cholestan-3-amines and Salpha-cholestan-3-yl aminoethanoates., *Steroids*, **72**, 8, 615–626 (2007). <https://doi.org/10.1016/j.steroids.2007.04.001>
- Rathod, N. V. & Mishra, S. Synthesis and biological evaluation of bile Acid–Triclosan conjugates: A study on antibacterial, antibiofilm, and molecular Docking. *Bioconj. Chem.* **36** (2), 276–290. <https://doi.org/10.1021/acs.bioconjchem.4c00539> (2025).
- Salunke, D. B. et al. New steroidal dimers with antifungal and antiproliferative activity., *J. Med. Chem.*, **47**, 6, 1591–1594. (2004). <https://doi.org/10.1021/jm030376y>
- Visbal, G. et al. Synthesis, in vitro antifungal activity and mechanism of action of four sterol hydrazone analogues against the dimorphic fungus *paracoccidioides* *Brasiliensis*. *Steroids* **76**, 10–11. <https://doi.org/10.1016/j.steroids.2011.04.012> (2011).
- Brossard, D. et al. Jul., Synthesis of bile acid derivatives and in vitro cytotoxic activity with pro-apoptotic process on multiple myeloma (KMS-11), glioblastoma multiforme (GBM), and colonic carcinoma (HCT-116) human cell lines., *Eur. J. Med. Chem.*, **45**, 7, 2912–2918, (2010). <https://doi.org/10.1016/j.ejmech.2010.03.016>
- Mrózek, L. et al. Investigation of new acyloxy derivatives of cholic acid and their esters as drug absorption modifiers. *Steroids* **76**, 10–11. <https://doi.org/10.1016/j.steroids.2011.04.014> (2011).
- Rathod, N. V. & Mishra, S. Bile Acid-conjugate as a promising anticancer agent: recent progress. *Curr. Med. Chem.* **31** (26), 4160–4179. <https://doi.org/10.2174/0109298673274040231121113410> (2024).
- Chattopadhyay, I., Biswas, K., Bandyopadhyay, U. & Banerjee, R. Turmeric and Curcumin: Biological actions and medicinal applications, *Curr Sci*, **87**, (2003).
- Sharifi-Rad, J. et al. Turmeric and Its major compound curcumin on health: bioactive effects and safety profiles for food, pharmaceutical, biotechnological and medicinal applications, *Front. Pharmacol.*, **11** 1–23, (2020). <https://doi.org/10.3389/fphar.2020.01021>
- Dosoky, N. S. & Setzer, W. N. Chemical composition and biological activities of essential oils of curcuma species. *Nutrients* **10** (9). <https://doi.org/10.3390/nu10091196> (2018).
- Anand, P., Kunnumakkara, A. B., Newman, R. A. & Aggarwal, B. B. Bioavailability of curcumin: problems and promises. *Mol. Pharm.* **4** (6), 807–818. <https://doi.org/10.1021/mp700113r> (2007).
- Sharma, R. A., Gescher, A. J. & Steward, W. P. Curcumin: the story so far. *Eur. J. Cancer.* **41** (13), 1955–1968. <https://doi.org/10.1016/j.ejca.2005.05.009> (2005).
- Peng, Y. et al. Anti-Inflammatory effects of Curcumin in the inflammatory diseases: status, limitations and countermeasures. *Drug Des. Devel Ther.* **15**, 4503–4525. <https://doi.org/10.2147/DDDT.S327378> (2021).
- Sharifi-Rad, J. et al. Turmeric and its major compound Curcumin on health: bioactive effects and safety profiles for food, pharmaceutical, biotechnological and medicinal applications. *Front. Pharmacol.* **11**, 1021. <https://doi.org/10.3389/fphar.2020.01021> (2020).
- Jakubczyk, K., Drużga, A., Katarzyna, J. & Skonieczna-Żydecka, K. Antioxidant Potential of Curcumin-A Meta-Analysis of Randomized Clinical Trials., *Antioxidants (Basel, Switzerland)*, **9** 11 (2020). <https://doi.org/10.3390/antiox9111092>
- Adamczak, A., Ożarowski, M. & Karpiński, T. M. Curcumin, a natural antimicrobial agent with Strain-Specific activity. *Pharmaceuticals (Basel)*. **13** (7). <https://doi.org/10.3390/ph13070153> (2020).
- Pramana, A., Firminda, A., Arnata, I. W., Sartika, D. & Sari, E. O. Reduction of biofilm and pathogenic microorganisms using curcumin-mediated photodynamic inactivation to prolong food shelf-life. *Int. J. Food Microbiol.* **425**, 110866. <https://doi.org/10.1016/j.ijfoodmicro.2024.110866> (2024).
- Shome, S., Das Talukdar, A., Nath, R. & Tewari, S. Curcumin-ZnO nanocomposite mediated Inhibition of *Pseudomonas aeruginosa* biofilm and its mechanism of action. *J. Drug Deliv Sci. Technol.* **81**, 104301. <https://doi.org/10.1016/j.jddst.2023.104301> (2023).
- Bučević Popović, V., Karahmet Farhat, E., Banjari, I., Jeličić Kadić, A. & Puljak, L. Bioavailability of oral Curcumin in systematic reviews: A methodological study. *Pharmaceuticals* **17** (2). <https://doi.org/10.3390/ph17020164> (2024).
- Górnicka, J., Mika, M., Wróblewska, O., Siudem, P. & Paradowska, K. Methods to Improve the Solubility of Curcumin from Turmeric., *Life (Basel, Switzerland)*, **13**, 1 (2023). <https://doi.org/10.3390/life13010207>
- Prasad, S., Tyagi, A. K. & Aggarwal, B. B. Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. *Cancer Res. Treat.* **46** (1), 2–18. <https://doi.org/10.4143/crt.2014.46.1.2> (2014).

39. Agrawal, N. & Jaiswal, M. Bioavailability enhancement of Curcumin via esterification processes: A review. *Eur. J. Med. Chem. Rep.* **6**, 100081. <https://doi.org/10.1016/j.ejmcr.2022.100081> (2022).
40. Mishra, S., Narain, U., Mishra, R. & Misra, K. Design, development and synthesis of mixed bioconjugates of piperic acid–glycine, curcumin–glycine/alanine and curcumin–glycine–piperic acid and their antibacterial and antifungal properties. *Bioorg. Med. Chem.* **13** (5), 1477–1486. <https://doi.org/10.1016/j.bmc.2004.12.057> (2005).
41. Dubey, S. K., Sharma, A. K., Narain, U., Misra, K. & Pati, U. Design, synthesis and characterization of some bioactive conjugates of Curcumin with glycine, glutamic acid, valine and demethylenated piperic acid and study of their antimicrobial and antiproliferative properties. *Eur. J. Med. Chem.* **43** (9), 1837–1846. <https://doi.org/10.1016/j.ejmech.2007.11.027> (2008).
42. Hashem, F. M., Elkhateeb, D. & Ali, M. M. Abdel-Rashid, In-vivo and in-vitro assessment of curcumin loaded bile salt stabilized nanovesicles for oral delivery. *Daru* **33**(1), 9. <https://doi.org/10.1007/s40199-024-00544-9>. <https://doi.org/10.1016/j.freeradbiomed.2005.01.022> (2024).
43. Diaz-Ramirez, J. et al. Bacterial cellulose/thiolated Chitosan nanoparticles hybrid antimicrobial dressing for Curcumin delivery. *Int. J. Biol. Macromol.* **289**, 138836. <https://doi.org/10.1016/j.ijbiomac.2024.138836> (2025).
44. Amini, S. M. & Shahroodian, S. Antibacterial activity of silver and gold nanoparticles that have been synthesized by Curcumin. *Inorg. Nano-Metal Chem.* **55** (5), 520–526. <https://doi.org/10.1080/24701556.2024.2352352> (2025).
45. Vatani, E. et al. Antimicrobial effect of Curcumin nanoparticles and ferulago angulate Boiss extract against Methicillin-Resistant Staphylococcus aureus (MRSA) isolated from wound infections. *Bionanoscience* **14** (3), 2228–2236. <https://doi.org/10.1007/s12668-024-01563-1> (2024).
46. Pradhan, A. et al. Feb., Amphiphilic Poly(ethylene glycol)-Cholesterol Conjugate: stable micellar formulation for efficient loading and effective intracellular delivery of curcumin, *ACS Appl. Bio Mater.*, **8**, 2, 1418–1436, (2025). <https://doi.org/10.1021/acsabm.4c01657>
47. Sun, Y., Qin, R., Gao, Y., He, Q. & Sun, R. Influence of monoglyceride on curcumin-loaded nanostructured lipid carriers: stability, antioxidant activity, digestion behavior, and intestinal absorption. *Food Biosci.* **71**, 107150. <https://doi.org/10.1016/j.fbio.2025.107150> (2025).
48. Surya, M. et al. One pot hypnea Valentiae mediated synthesis of silver doped Curcumin nanoparticles and their evaluation of antibacterial, anti-inflammatory and anticancer activity. *J. Ind. Eng. Chem.* **149**, 374–385. <https://doi.org/10.1016/j.jiec.2025.01.051> (2025).
49. Nur, A. et al. Development of a curcumin-piperine nanoparticle system using dissolving microneedles for transdermal drug delivery in malaria treatment: in vitro evaluation. *Int. J. Pharm.* **671**, 125258. <https://doi.org/10.1016/j.ijpharm.2025.125258> (2025).
50. Sahebi, K. et al. In vitro and in vivo anti-parasitic activity of Curcumin nanoemulsion on leishmania major (MRHO/IR/75/ER). *BMC Complement. Med. Ther.* **24** (1), 238. <https://doi.org/10.1186/s12906-024-04522-1> (2024).
51. Kaur, S., Modi, N. H., Panda, D. & Roy, N. Probing the binding site of Curcumin in Escherichia coli and Bacillus subtilis FtsZ - A structural insight to unveil antibacterial activity of Curcumin. *Eur. J. Med. Chem.* **45** (9), 4209–4214. <https://doi.org/10.1016/j.ejmech.2010.06.015> (2010).
52. Polaquini, C. R. et al. Antibacterial activity of 3,3'-dihydroxycurcumin (DHC) is associated with membrane perturbation. *Bioorg. Chem.* **90**, 103031. <https://doi.org/10.1016/j.bioorg.2019.103031> (2019).
53. Hamed, O. A. et al. Synthesis and antibacterial activity of novel Curcumin derivatives containing heterocyclic moiety. *Iran. J. Pharm. Res.* **12** (1), 47–56 (2013).
54. Feng, L. et al. Synthesis and biological evaluation of curcuminoid derivatives. *Chem. Pharm. Bull.* **63**(11), 873–881 (2015).
55. Rai, D., Singh, J. K., Roy, N. & Panda, D. Curcumin inhibits FtsZ assembly: an attractive mechanism for its antibacterial activity. *Biochem. J.* **410** (1), 147–155. <https://doi.org/10.1042/BJ20070891> (2008).
56. Romberg, L., Levin, P. A. & Drive, O. B. Poised at the edge of stability. *Annu. Rev. Microbiol.* **57**, 125–154. <https://doi.org/10.1146/annurev.micro.57.012903.074300.Assembly> (2017).
57. Silber, N., Matos, C. L., De Opitz, C., Mayer & Sass, P. Cell division protein ftsz: from structure and mechanism to antibiotic target. *Future Microbiol.* **15** (9), 801–831. <https://doi.org/10.2217/fmb-2019-0348> (2020).
58. Yoshizawa, T. et al. Crystal structures of the cell-division protein FtsZ from Klebsiella pneumoniae and Escherichia coli. *Acta Crystallogr. Sect. F Struct. Biol. Commun.* **76**, 86–93. <https://doi.org/10.1107/S2053230X2000076X> (2020).
59. Hopkins, A. L., Keserü, G. M., Leeson, P. D., Rees, D. C. & Reynolds, C. H. The role of ligand efficiency metrics in drug discovery. *Nat. Rev. Drug Discov.* **13** (2), 105–121. <https://doi.org/10.1038/nrd4163> (2014).
60. Stepanović, S. et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. *APMIS* **115** (8), 891–899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x (2007).
61. Sanchez, L. M. et al. Biofilm formation and detachment in gram-negative pathogens is modulated by select bile acids. *PLoS One.* **11** (3). <https://doi.org/10.1371/journal.pone.0149603> (2016).
62. Löwe, J. & Amos, L. A. Crystal structure of the bacterial cell-division protein FtsZ. *Nature* **391**, 203–206. <https://doi.org/10.1038/34472> (1998).
63. Nitiss, J. L. DNA topoisomerase II and its growing repertoire of biological functions. *Nat. Rev. Cancer.* **9** (5), 327–337. <https://doi.org/10.1038/nrc2608> (2009).
64. Stautz, J. et al. Molecular mechanisms for bacterial potassium homeostasis. *J. Mol. Biol.* **433** (16), 166968. <https://doi.org/10.1016/j.jmb.2021.166968> (2021).

Acknowledgements

NVR gratefully acknowledges the research fellowship provided by the Knowledge Consortium of Gujarat (KCG) through the SHODH program (Ref No: 202101446), as well as the Institute of Advanced Research, Gandhinagar, which provided critical financial support.

Author contributions

Neha Rathod: Writing – Original draft, designed and performed the experiments, analyzed the data, Validation
Satyendra Mishra: Conceptualization, Review and Editing, Formal analysis, Supervision, Recourses, Project Administration.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-15297-x>.

Correspondence and requests for materials should be addressed to S.M.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2025