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Biofilm reduction, *in-vitro* cytotoxicity and computational drug-likeness of selected phytochemicals to combat multidrug-resistant bacteria

Itumeleng T. Baloyi [a], Idowu J. Adeosun [a], Francesca Bonvicini [b], Sekelwa Cosa [a,*]

[a] *Division of Microbiology, Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Private Bag X20, Hatfield Pretoria 0028, South Africa*

[b] *Department of Pharmacy and Biotechnology, Alma Mater Studiorum, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy*

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ABSTRACT

Multidrug-resistant (MDR) bacteria in biofilms are frequently linked with persistent infections across healthcare settings, due to their virulence factors. Since the post-antibiotic era coerces the quest for novel therapeutics, the use of medicinal plants and their phytochemicals emerges as prospective alternatives for the failing antibiotics. Preliminary screening of untargeted drugs for their drug-likeness and biosafety properties is a necessary step in the advancement of the drug discovery process. Thus, the study aimed to assess the noteworthy phytochemicals with antibacterial potential to reduce the biofilm formation of selected MDR bacteria, evaluate their safe use and drug-likeness properties thereby providing advanced knowledge to contribute to the search for safe, antipathogenic drugs. Three phytochemicals of 1,2,3-benzenetriol, guanosine and phytol revealed significant minimum inhibitory concentrations (MIC) between 0.250 – 0.040 mg/mL against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pyogenes*. Guanosine and phytol, both revealed noteworthy MIC values of 0.016 and 0.031 mg/mL for *S. pyogenes* and *S. aureus*, respectively. Five MDR bacterial pathogens treated with 1,2,3-benzenetriol, guanosine and phytol at a concentration of 0.250 mg/mL reduced anti-adhesion and biofilm development up to 78.88% and 31.82%, respectively. *In situ* visualisation by scanning electron microscope (SEM) displayed guanosine to significantly disrupt the biofilm structures of *S. aureus, S. pyogenes* and *P. aeruginosa*. Atomic force microscope (AFM) detected differences between the topographies of *S. aureus, S. pyogenes* and *P. aeruginosa* biofilms treated with guanosine and phytol. Guanosine and phytol showed approximately 100% of cell viability in a dose-dependent manner (0.25 – 0.001 mg/mL) while causing no cell damage on African monkey kidney Vero (epithelial) cells and showed a cytostatic rather than a cytotoxic effect. Drug-likeness *in-silico* screening revealed that the compounds obeyed Lipinski's rules and have bioavailable scores of 0.55F. Guanosine and phytol showed antivirulent, biosafety and drug-likeness properties with significant pharmacokinetic predictions. This study highlights the significance of phytotherapeutics for the development of novel antipathogenic agents.

* Corresponding author.
E-mail address: sekelwa.cosa@up.ac.za (S. Cosa).

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Introduction

Bacterial diseases are primarily associated with the ability of bacteria to inhabit and spread across their hosts through diverse motility forms, activating various virulence factors, and developing a well-structured biofilm; thus leading to cell and tissue damage, moreover evasion of the host's immune system [1]. Biofilms are a diverse three-dimensional consortium of microbes that attaches to surfaces and have a sheath layer of exopolymeric substance (EPS) [2]. It is estimated that nearly eighty percent (80%) of the globe's microbial biomass is in the form of biofilms, whereas in nature, the planktonic cells are deemed as the main mode of existence for pathogens [3]. Moreover, biofilms reveal phenotypes that are unique from their incoherent free-floating cells and have a greater ability to settle on surfaces and withstand exogenous stress [3]. Microbial adhesion, following the assembly and build-up of an extracellular matrix comprised of several polymeric substances such as extracellular DNA, proteins, humic substances and polysaccharides make up the stages in the formation of biofilms [4]. Development of biofilms at early phases can be prevented by hindering forces such as Brownian movements, Van der Waals force of attraction, sedimentation and electrostatic attraction which are responsible for the support of bacterial attachment to various surfaces [5]. Several bacterial pathogens like *Streptococcus pyogenes, Staphylococcus aureus* and *Pseudomonas aeruginosa* use quorum sensing (QS) tactics to activate resistance genes, form biofilm and their associated virulence factors [6]. Therefore, there is an increasing need to re-search for agents or compounds with novel antivirulence/antipathogenic properties, particularly by blocking bacterial QS systems thereby regulating virulence factors including biofilm formation [7]. Antibiofilm agents developed from bacteria, fungi, marine organisms and plants have shown effectiveness in inhibiting the formation of EPS, cell attachment and reducing virulence factors by interrupting the QS system [8]. Notable techniques such as atomic force microscope (AFM) form an integral part which provides nanoscale surface characterisation of the biofilms and gives a clear indication of how they attach to natural surroundings [9]. Phytochemicals play a significant role in inhibiting bacterial adhesions and suppressing genes related to biofilm formation [10], with the capability to stop the accessibility to nutrients essential for adhesion and bacterial growth [11]. Antibiofilm agents can hinder bacterial virulence with no effect on the cell's viability [12], more so, these molecules require to be assessed for any adverse effects such as cytotoxicity is vital before their use and using them for commercialisation [5]. Natural products are the main source for finding and developing drugs that could tackle infections, thus the pursuit of novel bioactive compounds and investigation of their pharmacological effects have advanced a propitious approach for acquiring innovative drugs [13]. Furthermore, the drug innovation and advancement process require precise therapeutic target selection through high throughput screening (HTS) of hit-compounds for their absorption, distribution, metabolism and excretion (ADME) properties [14]. Phytochemical drug-likeness properties are one fundamental aspect of the preliminary screening stages [15], hence, the drug likeness validation of the compounds in this study.

To pose solutions the quest for the identification of lead target compounds that demonstrates the inhibition of the target infection, shows no undesirable side effects and significant drug-likeness properties, this study evaluated the compound's antibacterial and antibiofilm activities against MDR bacteria. Thereafter, validated the antibiofilm activity by employing *in situ* visualisation techniques (scanning electron microscope and atomic force microscope) and assessed the safe use of these compounds through *in vitro* cytotoxicity assays and drug-likeness properties.

Material and methods

Microbial strains, cell culture and maintenance

The microbial strains: *Pseudomonas aeruginosa* ATCC 9721, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 33495, *Streptococcus pyogenes* ATCC 19615 and *Escherichia coli* ATCC 10536 were procured from Sigma-Aldrich (Johannesburg, South Africa). The University of Pretoria, Faculty of Natural and Agricultural Sciences Ethics Committee provided approval to utilise these microorganisms (reference number: NAS158/2021). These pathogens were grown as described by the Clinical and Laboratory Standards Institute [16] for batch-batch reproducibility. Mueller Hinton (MH) medium was used to prepare the active bacterial cultures of *S. aureus, S. pyogenes, P. aeruginosa, E. coli* and *K. pneumoniae* and incubated at 37 ℃. Glycerol stock cultures of each organism were maintained and placed in a -80 ℃ freezer. Prior to each assay, an overnight-grown bacterium was prepared on a respective agar plate and incubated at 37 ℃. A single or two colonies were transferred to sterile distilled water to obtain an absorbance (OD_{600nm}) of 0.1. A 0.5 Mc Farland standard equivalent was achieved by adjustment of cell suspension.

African green monkey kidneys cells (Vero ATCC CCL-81) were selected as a model system to evaluate the toxicity of the compounds as they are epithelial and non-malignant cells, with well-defined culturing characteristics, and they are internationally recommended as a standard to study cytotoxicity of molecules and biomaterials according to ISO10993-5 (International Organization for Standardisation). The Vero cells were purchased from the American Type Culture Collection (ATCC) and were cultivated in Eagle's minimal essential medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Before the experiments, the exhausted medium was removed and cells were rinsed with phosphate-buffered saline (PBS; pH 7.2). Subsequently, trypsin ethylenediamine tetraacetic acid (EDTA) solution was added to the flask, to detach the cells from the surfaces. The light microscope was used for the observation of cells (Televal 31, Zeiss, Germany) at a 10x magnification to ensure that the cell layer is dispersed.

Phytochemical compounds

The following compounds of 1,2,3-benzenetriol, guanosine and phytol were previously identified by Baloyi et al. [17] from

bioactive plant extracts of *Melianthus comosus* and *Vachellia karroo* and selected based on their chemical structure as well as their *in-silico* findings [17,18]. These chemical compounds of 1,2,3-benzenetriol (Lot no: BCCG0904), guanosine (Lot no: BCCB9660), phytol (Lot no: 0001452396) and quercetin (reference compound) (Lot no: LRAB7760) were purchased from Sigma-Aldrich (Johannesburg, South Africa).

Minimum inhibitory concentration (MIC) of selected chemical compounds against multidrug-resistant (MDR) bacteria

Evaluation of minimum inhibitory concentrations (MICs) of the above-mentioned compounds using micro broth dilution assay was done, following the method described by Cosa et al. [19], with some modifications. Concisely, in all the wells 100 μL of Mueller Hinton broth (MHB) was transferred then in Row A of the micro-titer plate, an aliquot of 100 μL of each compound (in triplicate) was transferred into wells together with 1% dimethyl sulfoxide (negative control) and quercetin (reference compound). A standardised bacterium (control) and a sterile MH broth (blank) were also transferred to the wells with an aliquot of 200 μL, respectively. Two-fold serial dilutions were performed, with a 0.250 – 0.001 mg/mL concentration range and then a 100 μL of the standardised bacterium was added to every well. After 24 h incubation at 37 ℃, 40 μL of P-iodonitrotetrazolium (INT, 0.2 mg/mL) was added and incubated for a further 30 min to 1 h until the colour of the solution converted to pink. Visual assessment for bacterial growth inhibition which indicates clear wells with no colour change was recorded. The lowest concentration to inhibit bacterial growth was noted as the MIC value for the compound.

Inhibitory effects of phytochemical compounds on cell attachment and biofilm development

The antibiofilm assay was assessed for both cell attachment and biofilm growth inhibition using selected compounds, this method was followed according to Baloyi et al. [20] with minor adjustments. Succinctly, compounds of guanosine, phytol and quercetin which revealed noteworthy MIC values ($\leq$ 0.064 mg/mL) were tested against *S. aureus, S. pyogenes, E. coli, K. pneumoniae* and *P. aeruginosa*, for both cell attachment and biofilm development inhibition.

For the cell anti-adhesion inhibition assay, 100 μL of standardised bacterial suspension ($OD_{600nm} = 0.1$), 100 μL of MH broth and 100 μL of extract were transferred to the wells. The reference compound (quercetin) and negative control (1% DMSO) were also added to the wells. A 200 μL of sterile MH broth (blank) was transferred to the wells and incubated at 37 ℃ for 24 h. For biofilm development bioassays, 100 μL of standardised bacterial suspension and 100 μL of MH broth was added to the wells and incubated at 37 ℃ for 8 h. After incubation, 100 μL of extracts and controls were transferred into respective wells and incubated further for 24 h. Biofilm biomass was evaluated using the adjusted crystal violet (CV) assay. The 96-well plates containing formed biofilm were washed with sterile distilled water to remove planktonic cells and media. The plates were then oven-dried at 60 ℃ for 45 min. After drying, 1% CV solution (Sigma-Aldrich, Johannesburg, South Africa) was employed to stain the residual biofilm for 15 min in the dark. The wells were then rinsed with sterile distilled water to detach some of the unabsorbed stains. A semiquantitative evaluation of biofilm formation was performed by the addition of 125 μL of 95% ethanol to destain the wells. In a new plate, a total of 100 μL destaining solution was added and the absorbance ($OD_{585\ nm}$) was determined using a multimode microplate reader (SpectraMax® paradigm).

Biofilm inhibition was calculated using Equation 1. The following criterion for interpretation of results was used; whereby values between 0 and 100% were interpreted as an inhibitory activity, then further, breaking down as follows: values between 0 – 39% (weak activity), 40 – 69% (moderate activity), 70 – 100% (good activity) whereas negative values indicate the growth improvement than inhibition of biofilm.

$$\text{Biofilm inhibition } (\%) = (\text{Control} - \text{Test})/\ (\text{Control}) \times 100 \quad (1)$$

Where OD is the optical density read at an absorbance of 585 nm

In situ visualisation of biofilm using a scanning electron microscope (SEM)

A scanning electron microscope (SEM) was used to investigate the structural modifications of biofilms after treatment with compounds following the method described by Wijesundara & Rupasinghe [21], with minor adjustments. Biofilms were grown on coverslips, put in 12-well polystyrene plates and incubated at 37 ℃ for 8 h. After 8h incubation, the preformed biofilm was treated with compounds and the positive control, (quercetin) then incubated further for 24 h at 37 ℃. Following incubation, plates were rinsed three times with phosphate-buffered saline (PBS; pH 7.2) for 10 min. The biofilm grown on coverslips was fixed using 2.5% glutaraldehyde for 1-2 h at room temperature. Thereafter, coverslips were dehydrated by using graded series of ethanol (30, 50, 75, 90 and 100%) for 10 min each rinsing at room temperature. Thereafter, coverslips containing biofilms were allowed to dry overnight, then samples were mounted onto aluminium studs and coated with carbon and visualised using a crossbeam 540 scanning electron microscope (Carla Zeiss, Germany). The SEM images were recorded at 20.000x and 50.000x magnification.

Topographical characterisation of bacterial biofilms using atomic force microscopy (AFM)

The effect of the best three compounds (guanosine, phytol and quercetin) were monitored on *P. aeruginosa, S. aureus* and *S. pyogenes* using atomic force microscopy (AFM) as described by Santana et al. [22], with minor modifications. The overnight bacterial strains were grown in LB media, centrifuged (2000 × *g*, room temperature, 15 min) and rinsed thrice using phosphate buffer solution (5 mM,

pH 6.5). Tubes comprising the same buffer were resuspended with approximately 10^8 colony-forming units (CFU/mL). The compounds were diluted using their respective MIC values and 100 μL of compounds were transferred to 3 mL of the cell suspensions. The samples were incubated for 4 hours at 37 ℃. Untreated cell suspensions were used as controls. After incubation, 1 mL from each of the treated samples was collected and centrifuged (6000 × *g*) for 15 min at room temperature. A smear of cells was prepared on a glass slide. The slides were air-dried and visualised using the atomic force microscope. Samples were viewed in a contact imaging mode using a Veeco atomic force microscope (Dimension icon with Scan Asyst) and silicon tip on nitride lever (cantilever 0.55 – 0.75 μm). A nominal constant of 32 Nm-1 and resonance frequency of ≈300 kHz was used with a scan rate of 0.100 Hz and scan size of 5.00 μm. Nanoscope analysis Scan Asyst software (Nanoscope version 8.15) was used for the imaging analysis.

Evaluation of cytotoxic effects of selected compounds on mammalian cells

Water-soluble tetrazolium salt (WST-8) was used to evaluate the metabolic activity of cells as an indicator of cell viability using the cell counting kit-8 (CCK-8; Dojindo Molecular 150 Technologies, Italy). The cytotoxicity activity was determined using the lactate dehydrogenase enzyme (LDH Assay kit-WST, Dojindo Molecular Technologies, Italy). LDH is a firm cytoplasmic enzyme released into the cell culture medium because of the loss of membrane integrity [23].

Bioactive compounds were evaluated for their effects on epithelial African green monkey kidney cells (Vero ATCC CCL-81) following the method as described by Bonvicini et al. [24], with slight modifications. Succinctly, 24 h before experiments, trypan blue was used for cell counting using the Bürker chamber to determine the number of cells to be seeded on the 96-well microplates. An aliquot of 100 μL cell suspension was seeded into 96-well microplates at 10^4 cells/well and incubated in 5% CO_2 at 37 ℃ for 24 h. After incubation, the exhausted medium was replaced with the renewal of a complete medium supplemented with varying concentrations of compounds (0.25 mg/mL – 0.0019 mg/mL) and doxorubicin (0.025 – 0.00019 mg/mL), a cytotoxic drug control. All experiments included wells with untreated Vero cells (positive control), background medium of the extracts and a range of 0.1 – 0.0008% DMSO. These varying concentrations of DMSO were relative to the redissolved plant extracts and were added to verify that the DMSO does not have any influence on the Vero cells. After 48 h incubation, cellular morphology evaluation of the micro-wells containing cells with treatment was visualised at a 10X magnification and captured using the Hamamatsu corporation image software (Olympus i73 mic). Subsequently, 10 μL of lysis buffer was transferred to several wells of Vero cells representing the positive control in the LDH and incubated for 10 min at 37 ℃. All supernatants were removed from the 96-well plate and stored for LDH measurements. Hundred (100) μL of fresh medium containing 10 μL of CCK-8 solution were added to the cell monolayer and incubated for 2 h. The optical density (OD) values at 450 nm were measured after 2 h of incubation and data were expressed as percentage values of cell viability relative to the untreated controls using equation 2.

$$\text{Cell viability} = \text{OD (450 nm) untreated cells}/\text{OD (450nm) treated cells} \times 100 \quad (2)$$

In parallel, the collected cell-free supernatants were assayed for LDH released through damaged plasma membranes by adding a volume of the reconstituted working solution. After 30 min of incubation at room temperature in the dark, the stop solution was added, and absorbance was measured at 490 nm. Data were expressed as percentage values relative to both the 100% lysis controls, included in the test, and untreated controls using equation 3. Plates were read at 490 nm using a Multiskan Ascent Microplate Reader (Thermo Fischer, Italy).

$$\text{Cytotoxicity } (\%) = (A - C)/(B - C) \times 100 \quad (3)$$

Where: A indicates treated cells (test samples), B: high control (lysed Vero cells) and C: low control (untreated Vero cells).

Cell viability and cytotoxicity were also expressed as CC_{50} for the compounds reducing Vero metabolism by 50% compared to the untreated controls or determining an LDH activity of 50% relative to the lysed cell control. The CC_{50} values were obtained by interpolation on the dose-response curves generated by plotting the percentage values, relative to the positive control (set to 100% of growth), as a function of the tested concentrations (GraphPad Prism version 5.0 for Windows, San Diego, CA, USA).

In-silico screening of drug-likeness properties of selected phytochemical compounds

The evaluation of the pharmacokinetic and physicochemical properties of guanosine, phytol and quercetin (reference compound) were performed using the SwissADME web server (http://www.swissadme.ch/index.php) [25]. The compound's smiles were obtained from the PubChem database and inserted in the web server for analysis to generate the predicted parameters. Determination of water solubility, medicinal chemistry and lipophilicity of the compounds was generated. The bioavailability scores and drug-likeness properties (Lipinski's, Ghose's, Veber's, Egan's and Muegge's rules) of the compounds were calculated.

Statistical analysis

All the experiments were done in triplicates with at least two independent experiments. The results are presented as mean ± standard deviation. The values of compounds and positive controls were adjusted for comparisons using the pairwise comparisons of the one-way ANOVA - Turkey's method using the GraphPad Prism Software, version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). P values < 0.05 were considered significant. The following criteria for the antibacterial activity of compounds were used, MIC < 0.01 mg/mL (noteworthy activity), 0.01 mg/mL $<$ MIC $\leq$ 0.10 mg/mL (moderate activity), and MIC > 0.10 mg/mL (insignificant) [26].

Results

Antibacterial activity of compounds

Phytochemicals of 1,2,3-benzenetriol, guanosine and phytol were previously identified by Baloyi et al. [17] from bioactive plant extracts. The three phytochemical compounds were evaluated for their antibacterial activity on selected MDR pathogens. The MICs for the three tested compounds of 1,2,3-benzenetriol, guanosine and phytol revealed MIC values ranging from 0.250 – 0.004 mg/mL against the five selected MDR bacteria (Table 1).

The Gram-positive bacteria (GPB) of *S. pyogenes* and *S. aureus* were the most susceptible to the treatment. For *S. pyogenes*, a noteworthy MIC value of 0.016 mg/mL was observed for both guanosine and phytol while 1,2,3-benzenetriol showed a MIC value of 0.031 mg/mL. All three compounds of 1,2,3-benzenetriol, guanosine and phytol, revealed a MIC value of 0.031 mg/mL against *S. aureus*. The reference compound of quercetin also exhibited a significant MIC value of 0.004 mg/mL for both *S. pyogenes* and *S. aureus*. The Gram-negative bacteria (GNB) (*E. coli, K. pneumoniae* and *P. aeruginosa*) were less susceptible to the treatment. All compounds, as well as quercetin, showed MIC values of 0.250 mg/mL for both *E. coli* and *K. pneumoniae*. A moderate MIC value of 0.031 mg/mL and 0.063 mg/mL was shown by guanosine and phytol, respectively while 1,2,3-benzenetriol showed MIC of 0.125 mg/mL against *P. aeruginosa*. Quercetin showed a lower MIC value of 0.008 mg/mL against *P. aeruginosa*. The best compounds that showed significant MIC values were guanosine, followed by phytol and 1,2,3-benzenetriol against all five tested bacteria.

Cell anti-adhesion inhibition of compounds

Cell attachment (anti-adhesion) inhibition activity was assessed for the four compounds at sub-MIC concentrations against the five tested bacteria (Fig. 1). The tested compounds were 1,2,3-benzenetriol, guanosine phytol and quercetin. Based on the results, all tested compounds showed good anti-adhesion inhibition against *S. pyogenes* (Fig. 1). Guanosine and phytol exhibited the best cell attachment inhibition with 76.89% and 76.84%, respectively while 1,2,3-benzenetriol showed a 51.52% inhibition against *S. pyogenes*. The reference compound, quercetin also exhibited good inhibitory activity of 74.65% against *S. pyogenes*. The three tested compounds showed good to moderate inhibitory activity with 76.48, 72.61 and 50.10% for guanosine, phytol and 1,2,3-benzenetriol, respectively against *S. aureus*. Quercetin exhibited moderate inhibitory activity of 61.65%. Compounds of guanosine and phytol performed even better ($p < 0.05$) than the reference compound against *S. aureus*.

The GNB (*E. coli, K. pneumoniae* and *P. aeruginosa*) presented weak and strong activity towards compounds ranging between 6.25 – 73.88% (Fig. 1). The compounds of guanosine and phytol exhibited weak activity of 40.34% and 31.26%, respectively against *E. coli.* Guanosine and phytol exhibited an anti-adhesion inhibition of 38.58 and 41.57%, respectively while quercetin showed a 29.92% inhibition, with a significant difference ($p < 0.05$) against *K. pneumoniae*. A compound of 1,2,3-benzenetriol showed weak inhibition of $\leq$ 15% for both *E. coli* and *K. pneumoniae*. Strong inhibition of 73.88% and 70.20% was shown by guanosine and phytol, respectively while 1,2,3-benzenetriol showed weak inhibition of 49.93% against *P. aeruginosa.* Both compounds of guanosine and phytol showed better activity than quercetin (55.14%) with a significant difference ($p < 0.05$).

Biofilm development inhibition of compounds

The three tested compounds exhibited weak inhibitory activities against the five tested MDR pathogens (Fig. 2). All tested compounds exhibited a $\pm$ 30% weak inhibitory effect on both GPB of *S. pyogenes* and *S. aureus*. Phytol presented a preformed biofilm inhibition of 39.71 and 35.33% for *S. pyogenes* and *S. aureus*, respectively. Followed by guanosine with 36.35% and 34.57% while 1,2,3-benzenetriol showed performed inhibition of 31.61% and 31.18% for *S. pyogenes* and *S. aureus*, respectively. Quercetin exhibited a percentage inhibition of 43.38% and 42.30% against *S. pyogenes* and *S. aureus*, respectively.

The GNB also showed resistance to the treatment of compounds, with lower percentage inhibition (Fig. 2). For *E. coli*, the weak activity of 5.47 – 21.73% was presented by the three tested compounds, whilst between 5.35 – 23.68% inhibitory effects were exhibited by the compounds against *K. pneumoniae*. Similarly, the tested compounds showed an inhibitory effect between 18.83 – 34.85% against *P. aeruginosa*. For preformed biofilm assessment, the tested bacteria exhibited some resistance towards the compounds.

Table 1
Minimum inhibitory concentrations (mg/mL) of compounds against MDR bacteria.

Antibacterial activities MIC (mg/mL) Compounds	*E. coli* ATCC 10536	*K. pneumoniae* ATCC 33495	*P. aeruginosa* ATCC 9721	*S. aureus* ATCC 25923	*S. pyogenes* ATCC 19615
1,2,3-Benzenetriol	0.250	0.250	0.125	0.031	0.031
Guanosine	0.250	0.250	0.031	0.031	0.016
Phytol	0.250	0.250	0.063	0.031	0.016
Quercetin	0.250	0.250	0.008	0.004	0.004

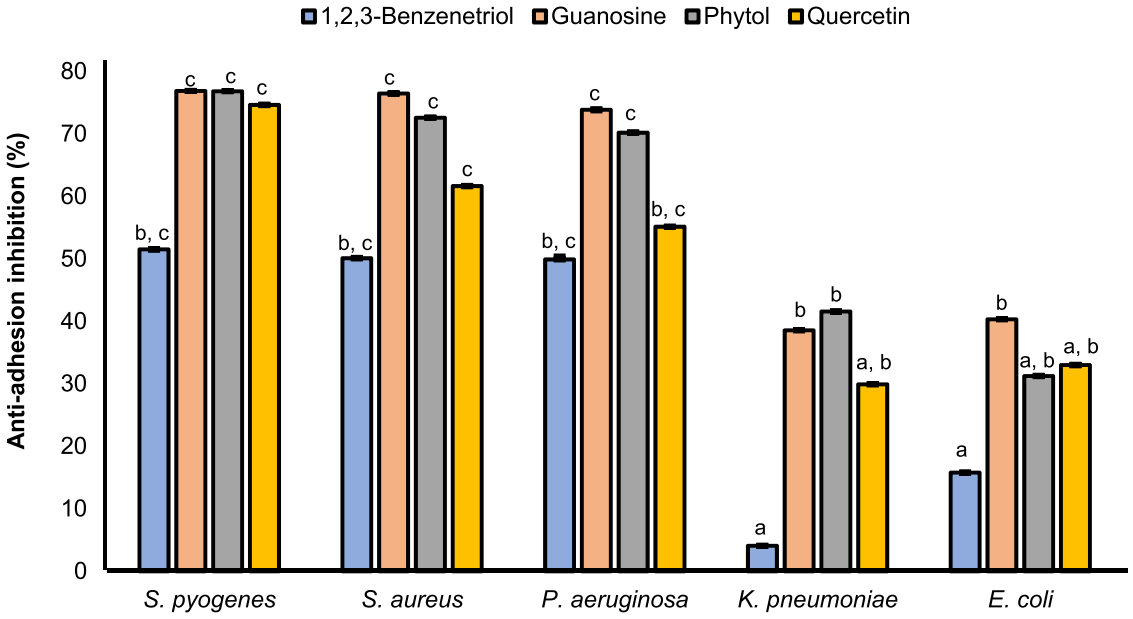

Fig. 1. Anti-adhesion inhibition of compounds against MDR bacteria. Means are values of triplicate independent experiments ± SD. The contrast of percentage inhibition at 0.250 mg/mL for compounds, against GPB (*S. pyogenes* and *S. aureus*) and GNB (*P. aeruginosa, E. coli* and *K. pneumoniae*). Comparison for each pathogen across the different individual treatments at the same concentration, presented with different letters. Various letters (a–c) show significant differences at $p < 0.05$.

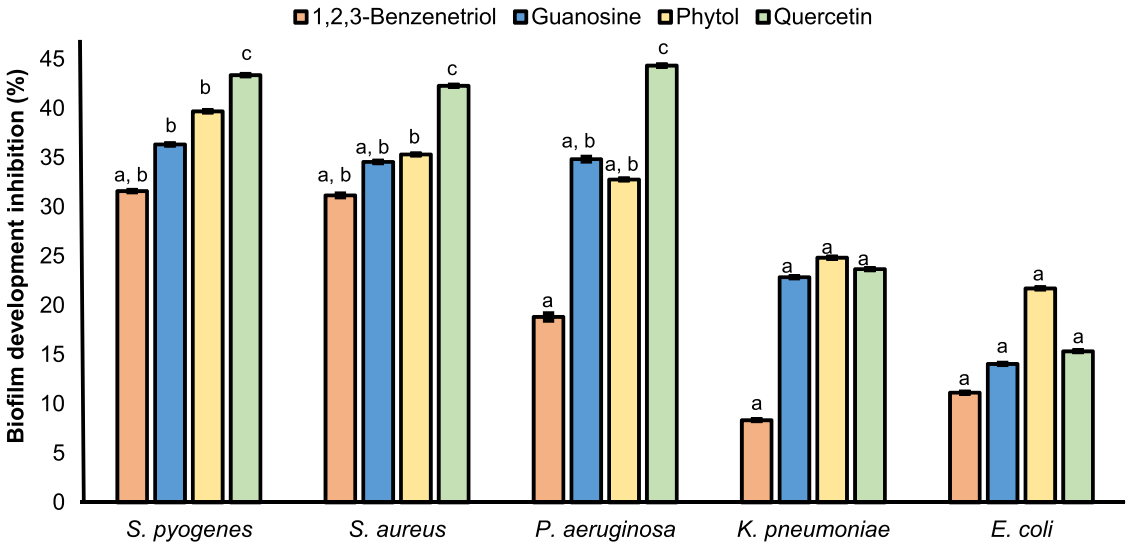

Fig. 2. Biofilm development inhibition of compounds against MDR bacteria. Means are values of triplicate independent experiments ± SD. The contrast of percentage inhibition at 0.250 mg/mL for compounds, against GPB (*S. pyogenes* and *S. aureus*) and GNB (*P. aeruginosa, E. coli* and *K. pneumoniae*). Comparison for each pathogen across the different individual treatments at the same concentration, presented with different letters. Various letters (a–c) show significant differences at $p < 0.05$.

Scanning electron microscopy analysis

Scanning electron microscopy (SEM) analysis was carried out and further investigated for the active phytochemicals of guanosine and phytol, this was based on their inhibitory effect on biofilm development assay. SEM analysis assisted with validation of the results obtained for biofilm development and capturing micrographs of biofilm layers formed by *P. aeruginosa, S. aureus* and *S. pyogenes*, without treatment (untreated control) as presented in Fig. 3A–C, respectively. The untreated controls were then exposed to treatment using the positive control of quercetin, which showed a disruption and reduction of the biofilm layers of the targeted bacteria

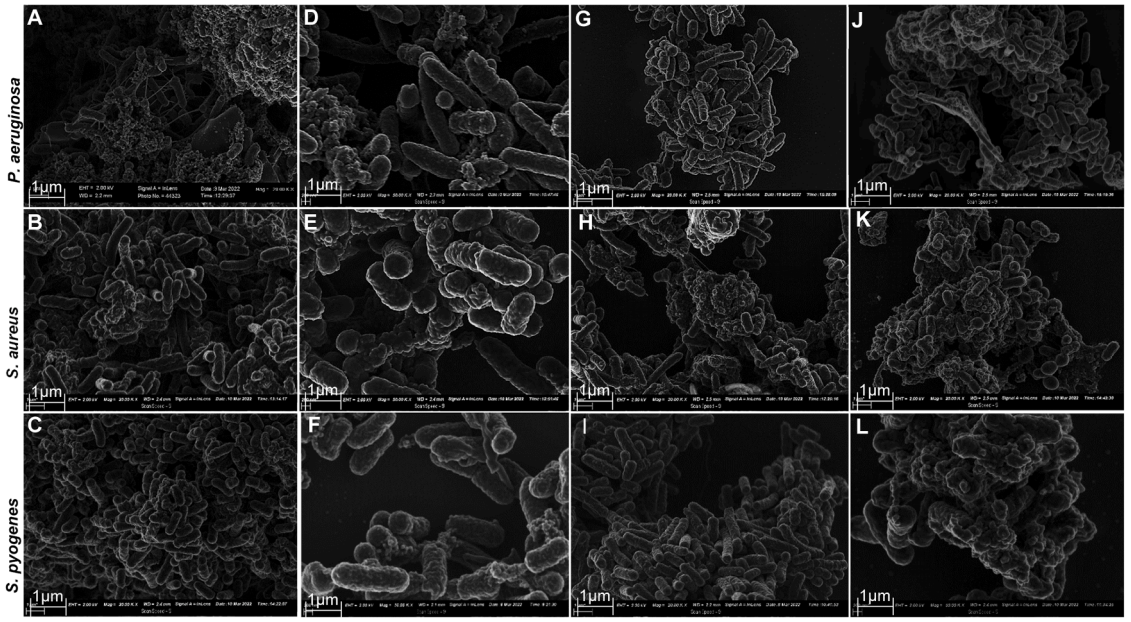

Fig. 3. Micrograph images by scanning electron microscope following exposure to compounds (0.25 mg/mL) after 8 h biofilm development to validate their inhibitory effects on biofilms. Three bacteria (untreated control) were tested as shown in A: *P. aeruginosa*; B: *S. aureus*; and C: *S. pyogenes*. The following compounds of guanosine (D-F), phytol (G-I) and quercetin (J-L) were tested. SEM micrographs were recorded at 20.000x and 50.000x magnification. Scale bars: 1.00 µm.

(Fig. 3D–F). Biofilm layers were treated with compounds of guanosine (Fig. 3G–I) and phytol (Fig. 3J–L). The disruption of *S. aureus* and *S. pyogenes* biofilms were more observed from guanosine in Fig. 3H and I, respectively and phytol in Fig. 3K and L, respectively. Based on these overall micrographs, this corroborates with the weak inhibition obtained from the *in vitro* biofilm development assay for compounds against the tested bacteria.

Atomic force microscopy analysis

The two and three-dimensional views of the surface topographies of studied *P. aeruginosa, S. aureus* and *S. pyogenes* biofilms are shown in Fig. 4. Conspicuous differences were detected between the topographies of untreated and treated biofilms using the atomic

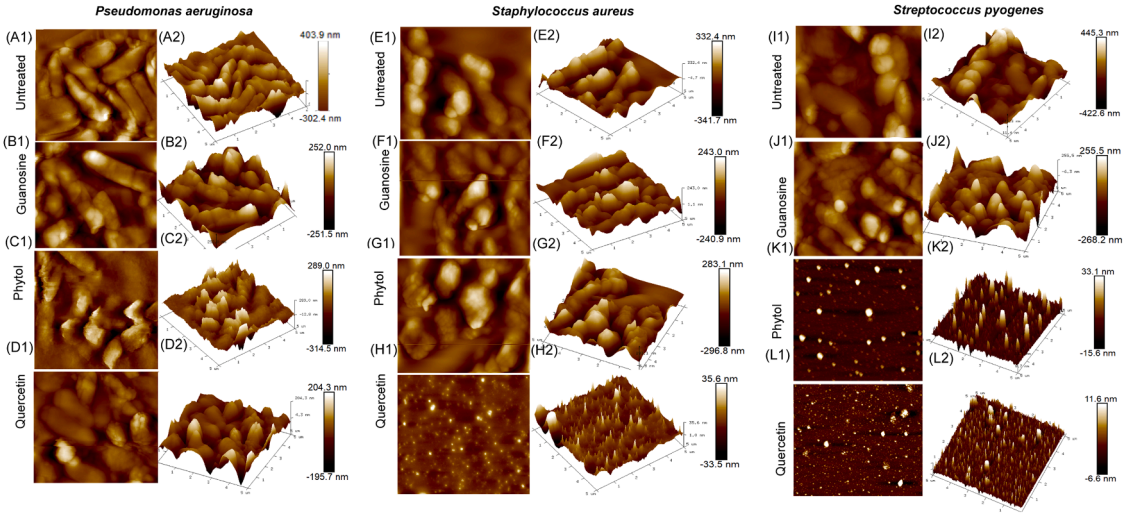

Fig. 4. AFM images showing two-dimensional (2D) and three-dimensional (3D) surface topography of untreated and treated biofilms of *P. aeruginosa, S. aureus* and *S. pyogenes* at a scan size of 5.00 µm (5000 nm). 2D images of untreated and treated biofilms are shown in A1–L1 while the 3D images are shown in A2–L2.

force microscope (AFM). The AFM analysis revealed the surface roughness of biofilms formed by *P. aeruginosa, S. aureus* and *S. pyogenes*, mainly composed of unevenly distributed lumps (Fig. 4(A1, E1 and I1)). The biofilms showed a maximum height of 403.9nm for untreated *P. aeruginosa*, 332.4nm for untreated *S. aureus* and 445.3nm for untreated *S. pyogenes* as shown in the 2D images (Fig. 2(A1, E1 and I1)). An average roughness (Ra) of 28.7nm (*P. aeruginosa*), 80.6nm (*S. aureus*) and 96.2nm (*S. pyogenes*) is shown in the 3D images (Fig. 4(A2, E2 and I2)) were obtained using the nanoscope analysis (v 8.15) software.

Biofilms treated with guanosine and phytol at their respective MIC values revealed visible differences in height and surface roughness in comparison with the untreated biofilms. For biofilms treated with guanosine, maximum lump heights of 252.0nm, 243.0nm and 255.5nm were recorded for *P. aeruginosa, S. aureus* and *S. pyogenes,* respectively (Fig. 4(B1, F1 and J1)). However, biofilms treated with phytol had a maximum height of 289.0 nm and 283.1 nm and 33.1nm for *P. aeruginosa, S. aureus* and *S. pyogenes* respectively. (Fig. 4(C1, G1, and K1)). The average roughness (Ra) for phytol-treated biofilms was 54.7nm for *P. aeruginosa,* 62.7nm for *S. aureus* and 2.68nm for *S. pyogenes.* Fig. 4(C2, G2 and K2) revealed a reduction in surface roughness when compared with the untreated biofilms.

Notably, biofilms treated with the positive control (quercetin) revealed the highest reduction in the surface roughness and height, with a maximum lump height at 204.3nm, 35.6nm and 11.6nm for *P. aeruginosa, S. aureus* and *S. pyogenes* respectively (Fig. 4(D2, H2 and L2)). Their average roughness was also remarkably reduced to 36.5nm, 4.29nm and 1.25nm.

Cell viability and cytotoxicity activity of compounds

The Vero cell line was used as a model system to evaluate the effects of the three compounds, guanosine, phytol and quercetin, on mammalian cells; indeed, these epithelial non-malignant cells are well-defined experimental settings internationally recommended as a standard to study cytotoxicity. Cells were treated for 48 h with different concentrations of compounds, in the range of 0.25 – 0.019 mg/mL. Based on the result of the CCK-8 assay (Fig. 5a), both guanosine and phytol did not interfere with cell viability even at the highest tested concentration (0.25 mg/mL). On the contrary, treatment with quercetin affected Vero metabolism in a dose-dependent manner reducing cell viability by 10.23%, at 0.25 mg/mL. For this compound, the CC_{50} was 0.05 mg/mL as obtained by interpolation

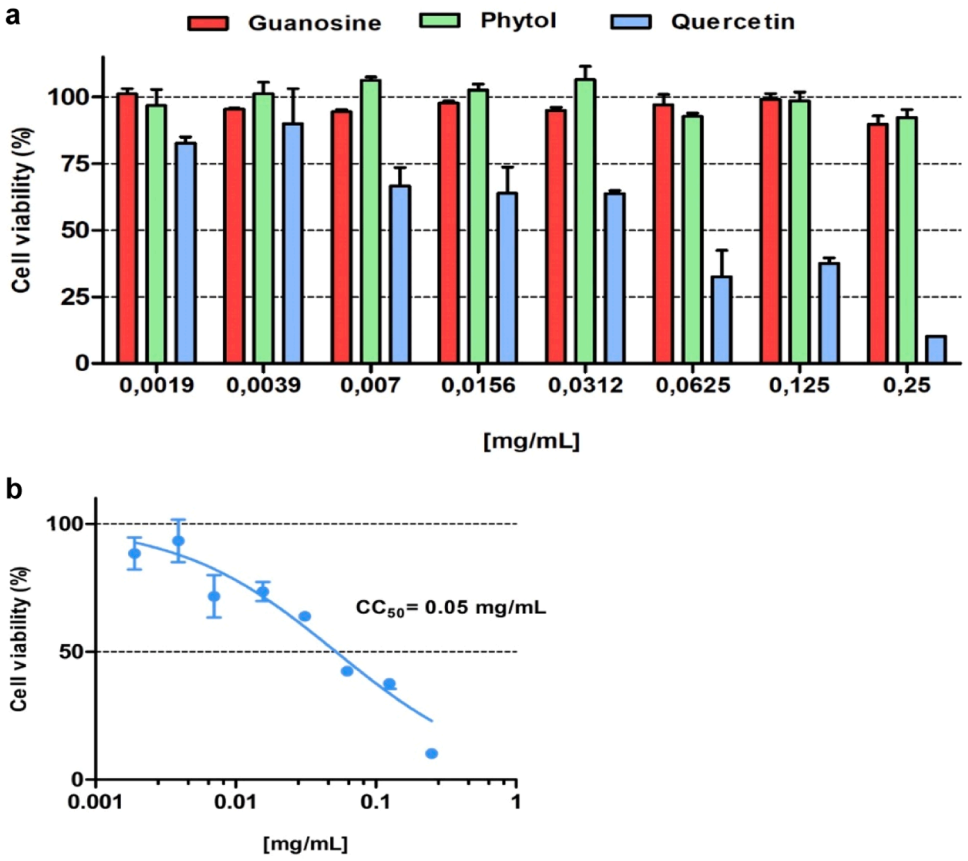

Fig. 5. (a) Cell viability (%) relative to the untreated Vero cells (ATCC CCL81) at different concentrations (mg/mL) of compounds. **(b)** Dose-response curve of quercetin obtained on Vero cells. Symbols represent mean values with standard deviations, and lines define the curves obtained from nonlinear regression analysis. Percentage values are relative to the untreated cells. Means are values of triplicate independent experiments ± SD.

on the dose-response curve generated by plotting the percentage values of Vero viability as a function of the tested concentrations (Fig. 5b).

The cytotoxicity activity (LDH) of compounds is presented in Table 2. As the amount of released enzyme from cells is one of the major methods to assess cell death and only a negligible presence of lactate dehydrogenase enzyme was measured in our experimental conditions, results confirm the safety of guanosine and phytol in the tested range of concentrations and suggest that quercetin exerted a cytostatic effect on Vero metabolism rather than a cytotoxic effect.

The reliability of the model system used in the study as well as of the experimental procedures was assessed by testing the doxorubicin, a well-known cytotoxic antineoplastic agent. The clinical drug control interfered with Vero cells and results are presented in Fig. 6. The effect of the drug at 0.025 mg/mL was cytotoxic as the LDH was 33.20 ± 3.39% and the safety threshold is usually set at 30%.

Drug-likeness predictions of the studied compounds

The *in-silico* ADME predictions of the three respective compounds of guanosine, phytol and quercetin (reference compound) were determined as presented in Table 3. Based on the analysis, both phytol and guanosine had low gastrointestinal (GI) absorption whereas quercetin has high GI absorption, guanosine and quercetin are P-glycoprotein substrates except for phytol and all three compounds cannot permeate the blood-brain barrier (BBB) with Log KP values of -2.29, -7.05 and -9.37 cm/s for phytol, quercetin and guanosine, respectively (Table 3). Guanosine was predicted as a non-inhibitor of all cytochrome enzymes whereas phytol was an inhibitor for only CYP2C19 and quercetin was an inhibitor for CYP1A2, CYP2D6 and CYP3A4. Of the three compounds, guanosine appears to be the most soluble in water and phytol to be moderately soluble as shown by Log S (Esol and SILICOS-IT) in Table 3.

All three compounds had heavy metals of ≥ 20, phytol had 13 rotational bonds and for both H-bond acceptors and donors was 1 with molar refractivity of 98.94 and TPSA of 20.23 Å2. Conversely, guanosine had 2 rotational bonds, 7 and 5 H-bond acceptors and donors, respectively with molar refractivity of 65.50 and topological polar surface area (TPSA) of 159.51 Å2. Additionally, quercetin obeyed all the rules, while guanosine and phytol disobeyed the Veber and Egan rules, all compounds had a bioavailability score of 0.55 F. Guanosine had zero alerts for both pan assay interference compounds (PAINS) and brenk with synthetic accessibility of 3.85, in contrast, phytol had zero alert PAINS but 1 alert brenk and synthetic accessibility of 4.30 then quercetin had 1 alert of catechol A for both PAINS and brenk.

Discussion

Broad research exploring innovative strategies to prevent pathogens from initiating biofilms continues. This has highlighted the efficacy of medicinal plants and their phytochemicals in reducing biofilm formation on critical pathogens like *S. aureus, E. coli, S. pyogenes, P. aeruginosa* and *K. pneumoniae* [27]. Finding alternative treatments for MDR bacteria is imperative where hindering the bacterial quorum sensing (QS) systems is an evolving approach to eradicating pathogenicity than bactericidal effect [28]. For this reason, pharmaceutical industries and the scientific community are searching for plant-derived compounds like saponins, terpenoids, alkaloids, tannins, polyphenols and flavonoids, which are known to combat pathogens and improve the host's defense system [29]. This study evaluated the minimum inhibitory concentrations of the prospective antivirulence compounds and then assessed their antibiofilm activities on MDR bacteria.

Based on the results, the preliminary screening of phytochemical compounds against selected MDR bacteria gave a clear indication of the compound's antibacterial activities. Guanosine and phytol showed significant antibacterial activities with MIC values ranging between 0.016 – 0.063 mg/mL against the tested bacteria (Table 1). Noteworthy antibacterial activities for both guanosine and phytol revealed minimum inhibitory concentrations of 0.016 and 0.031 mg/mL against *S. pyogenes* and *S. aureus*, respectively. These two compounds of phytol and guanosine are previously identified by Baloyi et al. [17] from the methanolic extract of *Melianthus comosus*. Nocedo-Mena et al. [30] reported the antibacterial activity of phytol against the clinical strain of methicillin-resistant *Staphylococcus aureus* (MRSA) (14-2095) with MIC value of 0.2 mg/mL. In this study, phytol exhibited a MIC value of 0.031 mg/mL against *Staphylococcus aureus* (ATCC 25923). The low MIC values suggest that a low concentration of the compounds is required for inhibiting the growth of the organism, hence drugs/compounds with lower MIC scores are more effective antimicrobial agents [31]. According to Gibbons [32] in Mamabolo et al. [33], a MIC value of 0.064 mg/mL or lower is considered significant for individual phytochemicals.

Table 2
LDH activity on Vero cells (ATCC CCL81) at different concentrations of the compounds.

Concentrations (mg/mL)	Compounds		
	Guanosine	Phytol	Quercetin
0.002	6.31±3.00	2.08±1.17	-10,95±1,92
0.004	1.91±0.72	-1.43±0.26	-6,71±3,35
0.007	-0.89±0.51	-1.61±0.45	-8,18±3,19
0.016	-3.39±1.75	-4.85±0.16	-7,98±3,33
0.031	-0.51±0.36	-1.63±0.35	-11,35±1,47
0.062	-0.37±0.07	-1.69±0.18	-6,54±1,84
0.125	-0.20±0.09	-0.75±0.27	-3,38±2,59
0.25	-2.59±0.34	-4.13±0.99	-4,02±2,00

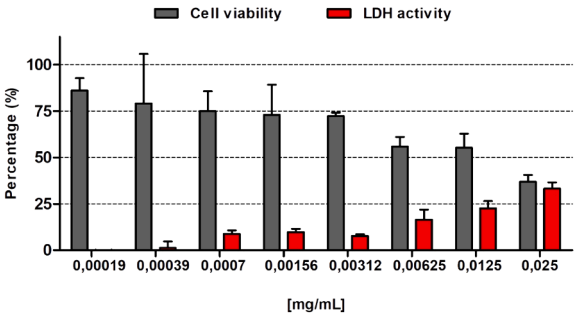

Fig. 6. Cell viability and LDH activity measured on Vero cells (ATCC CCL81) at different concentrations (mg/mL) of doxorubicin.

For this reason, both phytol and guanosine showed potent activities and qualify as potential antibacterial agents against the studied test pathogens.

Quantification of biofilm upon treatment with test compounds in anti-adhesion (Fig. 1) and preformed biofilm (Fig. 2) steps, portrayed varying results. Notably, guanosine and phytol had good anti-adhesion activity against *S. aureus, S. pyogenes* and *P. aeruginosa*, which performed better than quercetin. The GNB of *E. coli* and *K. pneumoniae* showed weak to no inhibitory activity of compounds due to the three-component membrane, potential extrusion via efflux pumps (EPs), porins alterations and other mechanisms being resistive to the diffusion of the compounds [34]. A 1,2,3-benzenetriol phytochemical had insignificant inhibitory activities against the tested bacteria. Overall, the two phytochemical compounds (guanosine and phytol) significantly disrupted cell anti-adhesion and slightly reduced the preformed biofilm biomass of the tested bacteria. The findings of this study signify that guanosine and phytol could play a role in preventing the cell-cell communication system and associated factors. However, when biofilms are in their natural surroundings they include multiple species, whereby the inter-species may be the contributing factor to the growth and formation of this consortium of microorganisms [35].

Further microscopic analysis using SEM and AFM was applied to visualise the morphological changes and surface morphologies of bacterial aggregation. The SEM analysis validated the biofilm inhibitory effect of guanosine and phytol and indicated a reduction in the biofilm aggregation of the *S. aureus, S. pyogenes* and *P. aeruginosa* (Fig. 3). Surface morphology permits a key insight into bacteria compound interactions and allows better understanding into the developments of bacterial surface settlement, however, such studies are limited [9]. Similar to this study, Adeosun et al. [36] indicated phytol significantly decreases the height and the roughness of *K. pneumoniae* using AFM.

The cytotoxicity of numerous natural products is likely to have adverse side effects on test subjects [37]. Consequently, it is imperative to establish whether plant extracts and secondary metabolites showing promising drug activities are effective in the tolerable toxicity and selectivity index range [38]. Famuyide et al. [39] pointed out that *in vitro* cytotoxicity on cell lines cannot be compared to animal toxicity as several factors like gut interactions and bioavailability play a role. The study constitutes an *in vitro* proof-of-concept on the safety of the selected bioactive extracts and compounds. According to the results of the CCK-8 assay which determined the dose concentration required for Vero cell's viability and revealed that the tested compounds exhibited no cytotoxic effects on the Vero cells.

The LDH activity indicated that the tested compounds did not cause cell death even in a dose-dependent manner as presented in Table 3. Whilst at higher concentrations, doxorubicin exerted a cytotoxic effect, thus indicating the suitability of the performed assays. A $\geq$ 25% LDH activity is considered acceptable and safe to use [40]. These prospective compounds require to be completely investigated on other cell lines related to the traditional use in humans and performed *in vivo* assays, to verify their pharmacognostic and pharmaceutical significance [41].

The computational tool applied in this study is the most commonly used, to sieve compounds with unsuitable properties, particularly poor absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles [13]. The virtual screening of the drug-likeness characteristics of the studied compounds provided significant predictions required to select the suitable compounds which have favourable ADME properties. The drug-likeness screening of compounds revealed that guanosine and quercetin obeyed Lipsinki's rule, with phytol having one violation. Lipinski suggested the well-known drug-likeness filter the "Rule of Five", which states that for a drug to be orally absorbed it should adhere to these provided rules of the molecular weight (MW) $\leq$ 500, octanol/water partition coefficient (logP) $\leq$ 5, number of hydrogen bond donors $\leq$ 5 and number of hydrogen bond acceptors $\leq$ 10 [13], except for a drug having one violation. Deficient pharmacokinetic properties are deemed as one of the major reasons compounds fail during the drug development process [14]. Findings from this study gave an insight into the structural features of the chemical compounds which are imperative from the pharmacokinetic prospect for additional hit-to-lead development [14].

Conclusion

The findings of this study confirmed guanosine and phytol as promising antibiofilm compounds contributing to the discovery and documentation of antibiofilm agents that can assist impede bacterial diseases. This study also highlighted the *in vitro* proof-of-concept of compounds that had a cytostatic rather than a cytotoxic effect on Vero cells within a specific dose. Guanosine and phytol showed no

Table 3
Drug-likeness properties of the studied compounds.

Compounds	Pharmacokinetics GI absorption	BBB permeation	P-gp substrate	CYP1A2 Inhibitor	CYP2C19 Inhibitor	CYP2C9 Inhibitor	CYP2D6 Inhibitor	CYP3A4 Inhibitor	Log Kp (Skin permeation) (cm/s)
Phytol	Low	No	Yes	No	No	Yes	No	No	-2.29
Guanosine	Low	No	No	No	No	No	No	No	-9.37
Quercetin	High	No	No	Yes	No	No	Yes	Yes	-7.05
	Water solubility								
	Log S (Esol)	**Solubility (mg/mL)**	**class**	**Log S (Ali)**	**Solubility (mg/mL)**	**Class**	**Log S (Silicos-it)**	**Solubility (mg/mL)**	**class**
Phytol	-5.98	3.10e-04	MS	-8.47	9.94e-07	PS	-5.51	9.06e-04	MS
Guanosine	-0.61	7.01e+01	VS	-0.94	3.25e+0.1	VS	0.51	9.10e+0.2	S
Quercetin	-3.53	7.51e-02	S	-3.60	6.41e-02	S	-3.24	1.73e-01	S
	Physicochemical properties								
	No. heavy atoms	**No. rotatable bonds**	**No. H-bond acceptors**	**No. H-bond donors**	**Molar Refractivity**	**TPSA (Å^2)**			
Phytol	21	13	1	1	98.94	20.23			
Guanosine	20	2	7	5	65.50	159.51			
Quercetin	22	1	7	5	78.03	131.36			
	Drug-likeness								
	Lipinski	**Ghose**	**Veber**	**Egan**	**Muegge**	**Bioavailability score**			
Phytol	Yes;1 violation: MLOGP>4.15	No; 1 violation: WLOGP>5.6	No; 1 violation: Rotors>10	No; 1 violation: WLOGP>5.88	No; 2 violations: XLOGP3>5, Heteroatoms<2	0.55			
Guanosine	Yes: 0 violation	No; 1 violation: WLOGP>-0.4	No; 1 violation: TPSA>140	No; 1 violation: TPSA>131.6	No; 1 violation: TPSA>150	0.55			
Quercetin	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55			
	Medicinal Chemistry								
	PAINS	**Brenk**	**Lead-likeness**	**Synthetic accessibility**					
Phytol	0 alert	1 alert: isolated alkene	No; 2 violations: Rotors>7, XLOGP3>3.5	4.30					
Guanosine	0 alert	0 alert	Yes	3.85					
Quercetin	1 alert: catechol A	1 alert: catechol A	Yes	3.23					

VS: very soluble: MS: Moderately soluble; PS: poorly soluble; S: soluble

toxic effects on both bacterial and mammalian cells. These compounds indicated that they can be used safely on epithelial with no malignant cells without causing cell damage. Furthermore, the drug-likeness prediction scores of these two compounds demonstrated they could be potentially used in the early stages of drug discovery as antipathogenic compounds. Understanding the molecular mechanisms of the compound's biological activity is crucial, particularly if these compounds are to be further explored for other purposes. Thus, providing a distinct overview of the molecular mechanism is important.

CRediT authorship contribution statement

Itumeleng T. Baloyi: Conceptualization, Investigation, Data curation, Validation, Writing – original draft. **Idowu J. Adeosun:** Investigation, Validation, Writing – review & editing. **Francesca Bonvicini:** Methodology, Investigation, Software, Writing – review & editing. **Sekelwa Cosa:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare no financial conflict of interest or personal relationships that could have appeared to influence the work reported in this paper.

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