



# Investigation of Phloroglucinol Succinic Acid Dendrimer as Antimicrobial Agent Against *Staphylococcus Aureus*, *Escherichia Coli* and *Candida Albicans*

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## Abstract

In this study, antimicrobial investigations for the efficiently synthesized biocompatible Phloroglucinol Succinic acid (PGSA) dendrimer with anionic surfaces were performed using broth dilution method against a Gram-positive bacterium (*Staphylococcus aureus*), a Gram-negative bacterium (*Escherichia coli*) and a fungal human pathogen (*Candida albicans*) to determine the minimal inhibitory concentration (MIC) value. Additionally, fluorescence and UV absorbance spectroscopy techniques were used to monitor the release of intracellular materials from the pathogens owing to anionic dendrimers. The exact binding sites of this dendrimer on these pathogens by molecular modelling studies motivated us to report this nanocarrier as a new antimicrobial agent.

**Keywords:** Biocompatible polymers; Dendrimer; Anti-microbial; Tryptophan; Molecular modelling

## Introduction

In general, globally there is an intention on selecting new antibacterials with encouraging resistance mechanism against bacterial pathogens in terms of increased duration of action [1, 2]. Nanomedicine is one of the anticipating research, which offers to design novel nano materials with multifunctional properties for therapeutic application including the treatment of microbial infections and in many areas of medicine [3]. The macromolecules with hyperbranched structure and

monodisperse property in nanodimension modality are commonly addressed as dendrimers [4]. Compared to various small molecules and polymers, the high density surface groups in dendritic structure attracted to use this macromolecule as bactericides [5]. Among the two groups of bacteria (i.e., Gram-positive and Gram-negative) the bacteriostatic or bactericidal effect of inhibition of metabolic process or permanent damage to vital cell structures depends on the surface charge and concentration of the bactericides. For instance, many dendrimers, which are cationic in nature drive an

attractive force to penetrate the bacterial cell walls and then interact with plasma membrane bearing negative charges [6, 7]. Previous workers have investigated the interaction of dendrimers with both supported lipid bilayers and membranes in living cells. These results have shown that unacetylated dendrimers produce membrane holes while acetylated dendrimers do not, and that the size of the dendrimer also influences its hole-forming tendency [8]. Broth dilution method has been used as the standard method to evaluate the efficiency of dendrimer biocides as bactericides [9, 10].

Intensive research has been carried out to reveal the fact about the problems raised for dendrimers to act as a successful nano carrier and to have antimicrobial activity, which mainly depends on the structural factors (i.e., distribution of surface charge) [3]. There are some reviews proposing the application of newly designed dendrimers to act as new antimicrobial targets such as quaternary ammonium functionalized poly(propylene imine) dendrimers [11], lysine dendrimers [12] and glycodendrimers functionalized with carbohydrates [13]. Even though there is more talk about the advantages of cationic dendrimer for anti-microbial studies in literatures, some have made surface modifications to reduce its cytotoxicity which might cause membrane pores due to nonspecific binding to charged lipid bilayer of cellular membranes [7, 14]. Barbara et al modified the surface of the PPI dendrimer with maltotriose residues and reported that its genotoxicity has been highly reduced [15]. Yi-Hong K et al. [16] for the first time in their work, have undoubtedly reported about the phloroglucinol compound with *in vitro* studies in such a way that, it has a wide range of biological activities such as anti-cancer, anti-microbial, etc., particularly it can inhibit vascular endothelial growth factor (VEGF) in cancer cells and suppresses tumor angiogenesis. This instant supports our information to highlight that our anionic surface charged dendrimer as a nano carrier, which has phloroglucinol as a core molecule and anionic functional group moieties in the surfaces of both half and full generations responded as an antimicrobial agent against Gram-positive, Gram-negative bacteria and against a diploid fungus.

In the previous work, PGSA biocompatible dendrimer [17] with anionic surface charged (carboxylic acid and phenolic hydroxyl) groups has been synthesized and characterized, even the photodynamic efficacy of the photosensitizer,

Protoporphyrin IX incorporated inside it was evaluated in DLA cell lines (in vitro). In an ongoing effort to expand the biomedical applications of this PGSA dendrimer broth dilution method, spectroscopic and molecular modelling studies have been used to clearly highlight the finding of anti-microbial property in addition to its service as drug delivery system for therapeutic molecules.

## Materials and Methods

Phloroglucinol and succinic acid were purchased from Sigma Aldrich, India. We developed a water soluble dendrimer of generations up to 2.5 with negative charges. The method used to synthesis dendrimer with anionic surface charge and techniques used to characterize the end product of every half and full generations such as Fourier Transform-Infrared spectroscopy (FT-IR), to confirm the presence of functional groups and Zeta potential for surface charges measurement in the terminals was already discussed by Murugesan et al. [17]. This helps to understand the biocompatibility of the nano carrier which made to extend the present study to look for antimicrobial property in addition to its behaviour as a nano drug delivery system.

## Bacterial strains and growth conditions

The human pathogenic Gram-positive bacterium, *Staphylococcus aureus* (ATCC 12600), Gram-negative bacterium, *Escherichia coli* (ATCC 11775) and fungal pathogen *Candida albicans* (obtained from the Infectious Disease Laboratory, Chennai, India) were used. The human bacterial pathogens were inoculated into 5 mL of sterile Muller–Hinton broth, and incubated at 37 °C for 18 h whereas, the fungal pathogen, *C. albicans* was inoculated in Sabouraud's dextrose broth and incubated at 37 °C for 18 h.

## Antimicrobial activity

### Minimal inhibitory concentration (MIC) test

The MIC was determined by the method of Gabrielson et al. [18]. A sterile 96-well plate was used for the experiment. A volume of 100 µL of test material in 10% (v/v) DMSO or sterile water was pipetted into the first row of the plate. To all other wells 50 µL of Muller–Hinton broth or normal saline was added. Serial dilutions were performed using a multichannel pipette and the concentration of compound for serial

dilution was 10 mg in the first row and tips were discarded after use such that each well had 50  $\mu$ L of the test material in serially descending concentrations. Aliquot of 10  $\mu$ L of bacterial inoculum ( $5 \times 10^6$  cfu/mL) was then added to each well to achieve a concentration of  $5 \times 10^5$  cfu/mL. Each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. Each plate had a positive control (Streptomycin for *S. aureus*, *E. coli*; Fluconazole for *C. albicans* in serial dilution) in the first column. The plates were prepared in triplicate, and placed in an incubator at 37 °C for 18–24 h. Finally, to each well 10  $\mu$ L of 2, 3-bis (2-methoxy-4- nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (MTT) indicator solution was added. After incubation, the colour change was assessed visually. The MTT tetrazolium salt assay measures the cells ability to convert the tetrazolium salt into the formazan product. Any colour changes from yellow to purple were recorded as positive. The lowest concentration which inhibits the visible growth of microorganism was taken as the MIC value [19, 20].

### Steady state fluorescence measurement for tryptophan release

Tryptophan release study was carried out for recording the Steady state fluorescence spectra of tryptophan to examine the antimicrobial activity of different generations (G0.5, G1.0, G1.5, G2.0 and G2.5) of the anionic surface charged biocompatible dendrimer against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria using spectrofluorometer (FluoroMax-2, ISA Jobin Yvon-Spex, Edison, NJ). Antifungal activity of biocompatible dendrimer was also investigated against *C. albicans*. Samples were excited at 280 nm and emission was collected in the range of 300 nm to 540 nm.

### Cytoplasmic constituents release assay

The release of 260 nm absorbing material [1] from the pathogens *E. coli*, *S. aureus* and *C. albicans* was monitored through the detection of absorbance at 260 nm using UV-Vis spectrophotometer. Each bacterial suspension was taken in a separate container. To these suspensions (2.5 mL), the MIC values obtained from the MIC experiment for dendrimer of generation 0.5-2.5 were added. After the addition of dendrimer biocides, 2 mL of the suspension was removed to monitor the release of cytoplasmic constituents from the cell in a particular interval of time.

### Modelling Studies

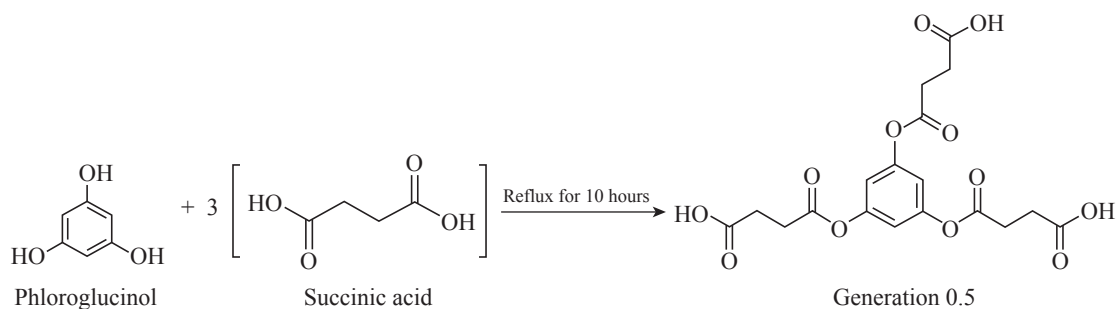
The X-ray crystal structures of *S. aureus*, *E. coli* and *C. albicans* were taken from the protein data bank (PDB) [21] and their structures were further subjected for docking study using GLIDE Schrodinger suite USA 2009. The protein structures were energy minimized using protein preparation wizard panel to add hydrogens, the charge state of protein residue is important for result generation by glide. Optimized potential of liquid simulations (OPLS 2005) force field is used for minimization process. Compound (Dendrimer) is the ligand taken for docking study for these three pathogens and the ligand structures were modelled using ACD/Chem basic. The structure of dendrimer compound was energy minimized using two algorithms, steepest descent and conjugate gradient. The compound binding sites were predicted using Q-site finder and further docked into their preferred binding sites using the induced fit docking (IFD) panel, here both the ligand and protein are flexible to dock and hence many confirmations were generated for individual ligand based on the docking score and glide energy.

### Results and Discussion

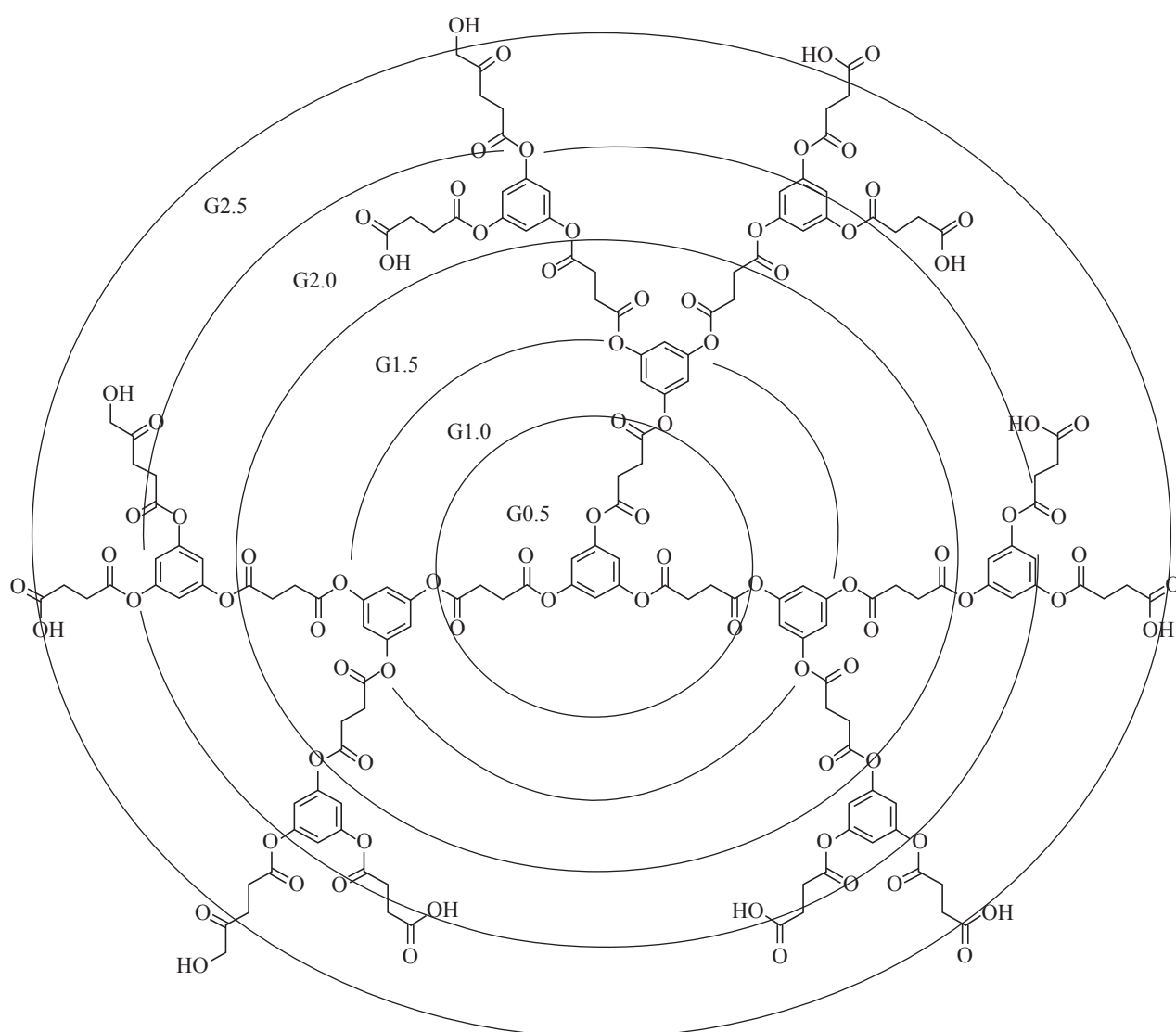
The dendrimer, which we formulated with two organic compounds was easily soluble in water and negatively charged, due to the esterification process of carboxylic acid with hydroxyl functional groups of phloroglucinol half generations (G0.5, G1.5, G2.5) were developed and vice versa was followed for the development of full generations (G1.0, G2.0). The structure of dendrimer of generation 0.5 with anionic surface is shown in Fig. 1, which is an initially obtained product by the condensation of phloroglucinol and succinic acid followed by the structure of G2.5 (Fig. 2).

### Minimal inhibitory concentration test (MIC Test)

The MIC test is relatively a straight forward and an easy way to test the antimicrobial activity for a formulation. The nano carrier which we developed will have many (carboxylic acid and phenolic –OH) functional groups on its surface of every half and full generations (G0.5-G2.5) with a negative charge. The antimicrobial activity for this nano carrier was tested and the minimum concentration at which it is able



**Fig. 1** Structure of Generation 0.5.



**Fig. 2** Structure of anionic PGSA Dendrimer of Generation 2.5.

to inhibit the visible growth of microorganism was determined from the MIC test. The MIC values of this nano carrier for G0.5-G2.5 were listed in the Table 1.

We noticed an increase in the MIC values on further development of generations when analyzed with Gram-positive bacterium (*S. aureus*), Gram-negative

bacterium (*E. coli*) and fungus (*C. albicans*), which describes that as the dendrimer structure developed generation by generation, the presence of functional groups will be more on its surface which increases the molecular weight and hence there is a need of higher concentration to inhibit the growth of microorganisms

**Table 1** The Minimum inhibitory concentration (MIC) of anionic PGSA dendrimer (G0.5-G2.5) on *S. aureus*, *E. coli* and *C. albicans*

Dendrimer Generation	Minimum inhibitory concentration (MIC) (mg/ml)		
	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
G0.5	0.312	0.312	0.312
G1.0	0.625	0.625	0.625
G1.5	0.312	0.625	0.312
G2.0	0.625	1.250	0.625
G2.5	0.625	1.250	0.625
Streptomycin	0.004	0.019	NA
Fluconazole	NA	NA	-

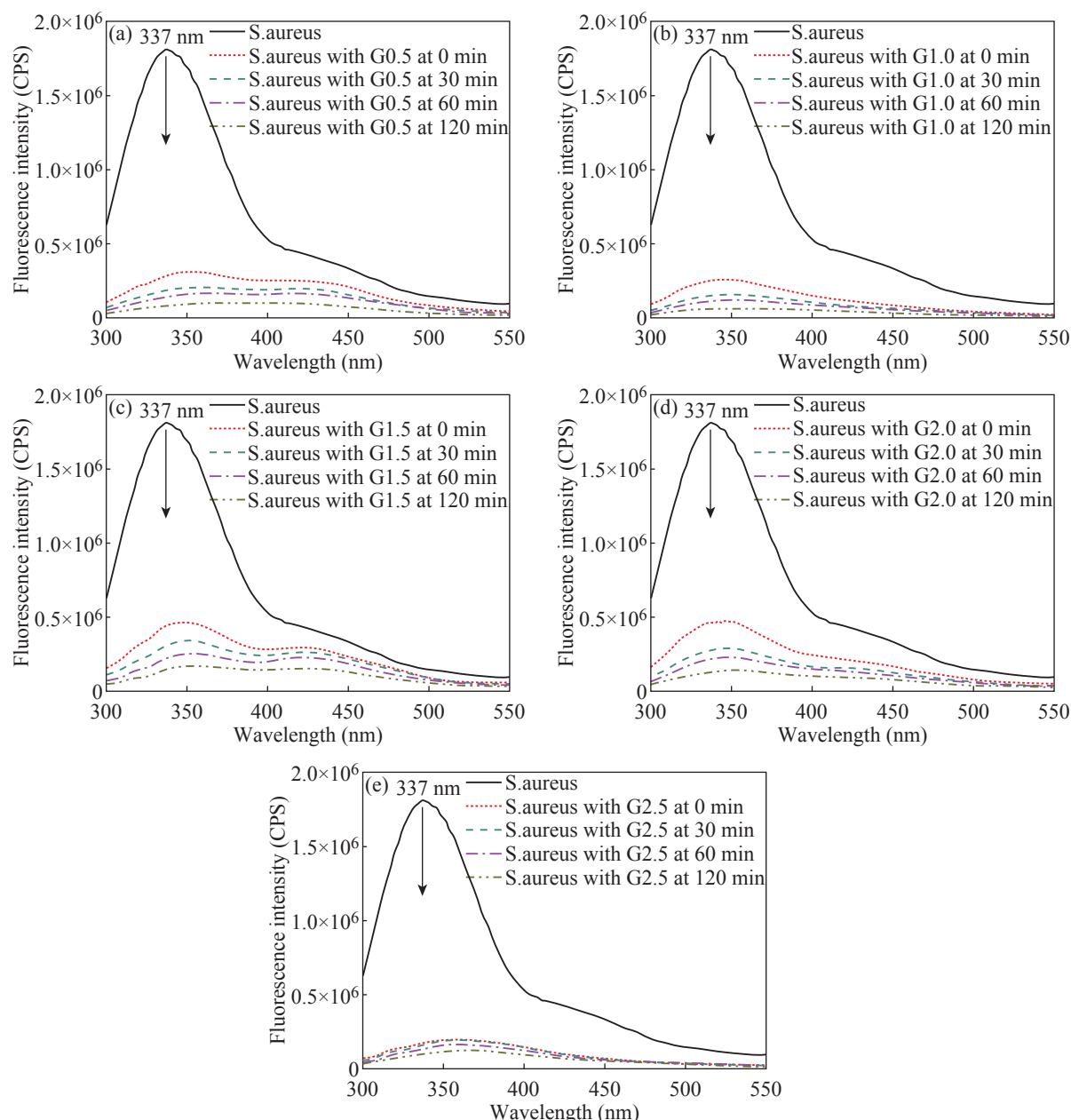
which is shown in Table 1. Moreover, it might be reasonable for us to state that our anionic PGSA dendrimer (G0.5-G2.5) surface and the microorganism cell membrane both tends to be anionic in nature; so in order to break/overcome the repulsive force between them and displace the divalent cations to get inside the cell membrane there is a need for increase in concentration for PGSA dendrimer when compared to that of the control (streptomycin) achieves with 0.004 mg/ml (*S. aureus*) and 0.019 mg/ml (*E. coli*).

The MIC obtained for Gram-positive bacterium (*S. aureus*) by G2.5 dendrimer is 0.625 mg/ml which is appreciable when compared to the inhibition value 1.25 mg/ml obtained for Gram-negative bacteria (*E. coli*) which offers to convey this nano carrier to have bactericidal effect but the control (streptomycin) achieves in 0.004 mg/ml (*S. aureus*). In the case of fungal, we noticed that even the positive control fluconazole did not cause any inhibition to *C. albicans* but the PGSA dendrimer influenced the fungicidal effects with the inhibition value 0.312 mg/ml (G0.5, G1.5) and 0.625 mg/ml (G1.0, G2.0, G2.5). Fluconazole should be considered as a drug control for *C. albicans* which showed resistance against it for the treated concentration and have acted as a multi drug resistance; so no particular value as MIC was obtained. But *C. albicans* was not able to show resistance to the PGSA dendrimer and hence MIC values were obtained. Since we have reasoned out the need of higher concentration for the PGSA dendrimer (as generations developed) to inhibit the microorganisms growth than the positive control needs, this study claims us to report that this anionic dendrimer tends to have both bactericidal and fungicidal effect appreciably.

### Tryptophan degradation study

Steady-state fluorescence emission spectra of tryptophan (maximum emission at 337 nm) from the pathogens *S. aureus*, *E. coli*, and *C. albicans* for the MIC values of PGSA dendrimers at particular interval of time are shown in Figs. 3, 4 and 5, respectively by means of reduction in fluorescence intensity. The effect of decrease in the fluorescence intensity of tryptophan (357 nm) with the blue shift (20 nm) for *S. aureus*, *E. coli* and *C. albicans* depicts that the generation G0.5-G2.5 showed an appreciable interaction with the bacterial biological membranes, which may further have the ability to denature the membrane proteins and displace the divalent ions such as calcium and magnesium which always have a significant role in preserving the stability of the outer membrane of bacteria [22]. Thus a small change in the membrane permeability permits the anionic PGSA dendrimer to penetrate the phospholipid bilayer and makes the tryptophan to degrade from the bacteria to produce bactericidal effect. From Figs. 3 and 4, it is possible to explore that the release of degraded tryptophan from *S. aureus* was more than 90% when compared to that of from *E. coli* by means of reduction in the fluorescence intensity for an immediate reaction (at 0 time period) of anionic PGSA dendrimer of G0.5 with *S. aureus* by losing the membrane permeability. This made us to state, our nano carrier with anionic surface gets more attracted to Gram (+) bacterium than Gram (-) bacterium and shows a good reduction in the tryptophan emission intensity for G0.5 itself and this was further continued as time increases. Similarly, the decrease in the fluorescence emission intensity of tryptophan



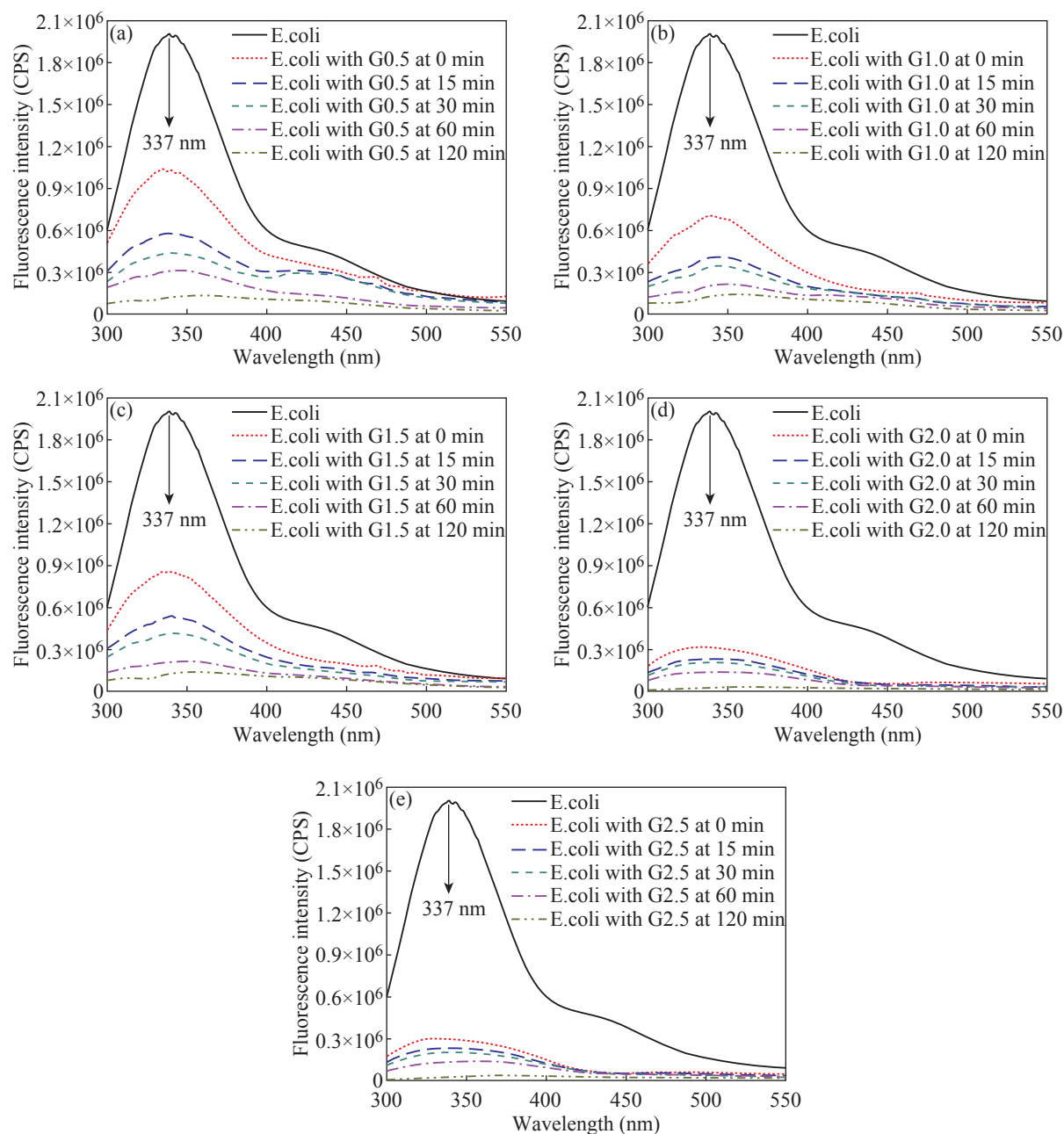


**Fig. 3** Steady-state fluorescence emission spectra of tryptophan from *S. aureus* after addition of PGSA dendrimer (a) G0.5, (b) G1.0, (c) G1.5, (d) G2.0, (e) G2.5.

as a sign of degradation from *C. albicans* (Fig. 5) was also able to monitor for the initial product of G0.5 dendrimer for an immediate reaction (at 0 time period); and for the remaining generations also (G1.0 to G2.5) as mentioned before; the same was continued as time increased. The results obtained from this technique confirms our study to highlight that, the entire half (G0.5, G1.5, G2.5) and full (G1.0, G2.0) generation of dendrimer exhibited significant bactericidal and fungicidal activity against Gram (+) bacterium (*S. aureus*), Gram (-) bacterium (*E. coli*) and dimorphic fungus (*C. albicans*).

## Cytoplasmic constituents release assay

The release of DNA or RNA (which strongly absorbs UV-Light at 260 nm), is a good indicator for measuring the bacterial membrane integrity using UV-Vis. spectrophotometer [1]. Using this method, the optical density (O.D) was recorded for the interaction of anionic PGSA dendrimer biocide with the *S. aureus*, *E. coli*, and *C. albicans* at 260 nm, to experimentally find out whether this dendrimer may influence the release of intercellular materials. The UV-Vis study of 260 nm release after the addition of dendrimers of

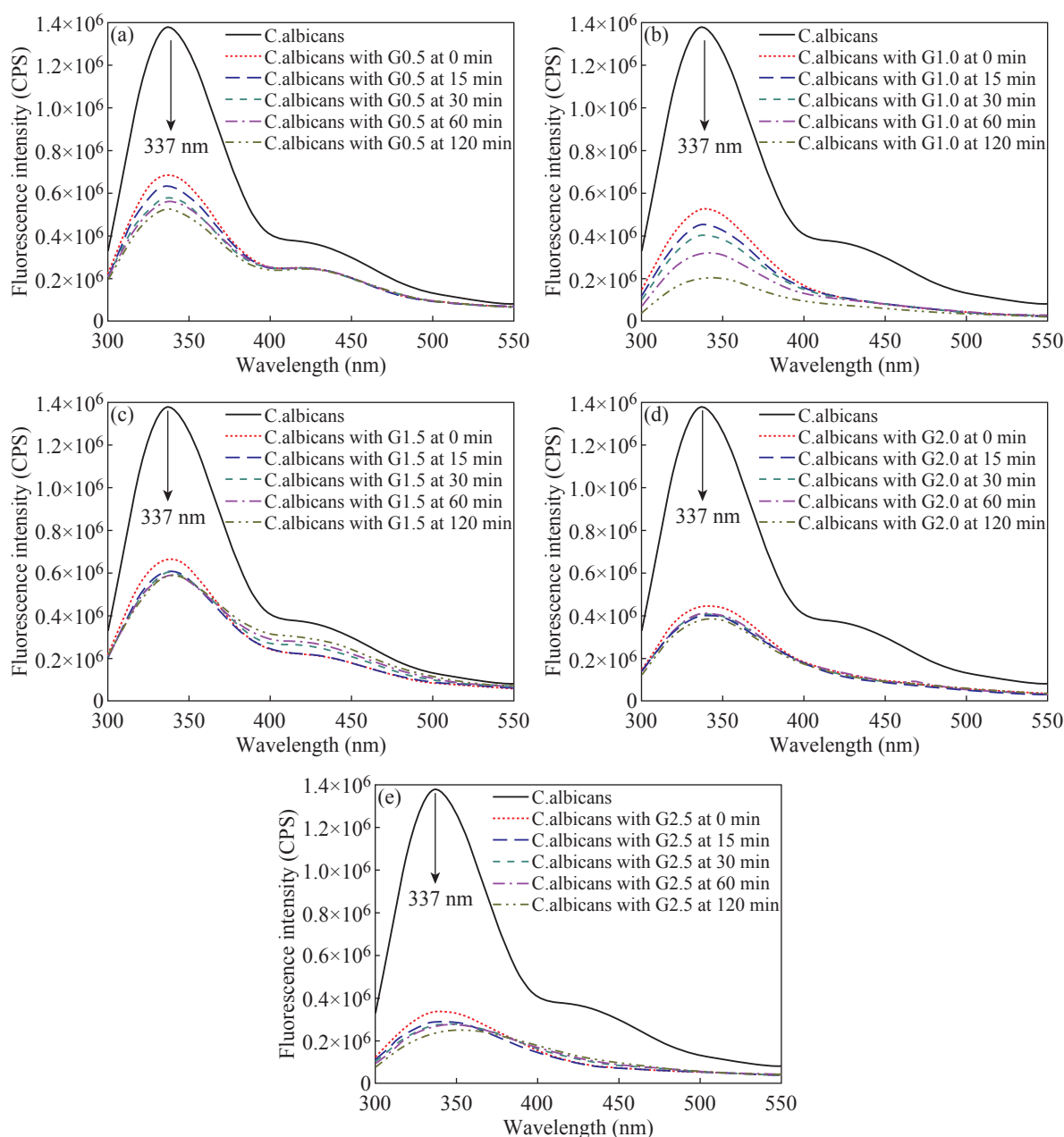


**Fig. 4** Steady-state fluorescence emission spectra of tryptophan from *E. coli* after addition of PGSA dendrimer (a) G0.5, (b) G1.0, (c) G1.5, (d) G2.0, (e) G2.5.

generations 0.5-2.5 to *E. coli* suspension is shown in Fig. 6(b).

Upon the addition of dendrimer biocide, O.D of the *E. coli* suspension at 260 nm quickly increased to a value of above 2 in 10 minutes and in the next 10 minutes the O.D gets saturated (i.e. which means, the interaction of dendrimer biocide with cell membrane finally has lead to cell death). Similarly, for *S. aureus* (Fig. 6(a)) the increase in the O.D was observed for 1 h (at intervals of 0, 30, 60 minutes) in which the all the generations of dendrimer influenced the release

of intercellular material with the representation of increase in O.D between 0.5 and 2.0 at once when added (i.e. at 0 time period) and not greatly increased beyond this for the remaining time intervals also. Thus when tested with the MIC values of dendrimer (G0.5-G2.5) on *S. aureus* for the cytoplasmic constituent release assay, the increase in O.D values also explicitly conveyed the cell death. Similarly for *C. albicans* the increase in the O.D was monitored up to 2 h (Fig. 6(c)). Even though we did not observe a linear increase in O.D as the generations increased, but beyond the period of 30 minute the O.D gets saturated



**Fig. 5** Steady-state fluorescence emission spectra of tryptophan from *C. albicans* after addition of PGSA dendrimer (a) G0.5, (b) G1.0, (c) G1.5, (d) G2.0, (e) G2.5.

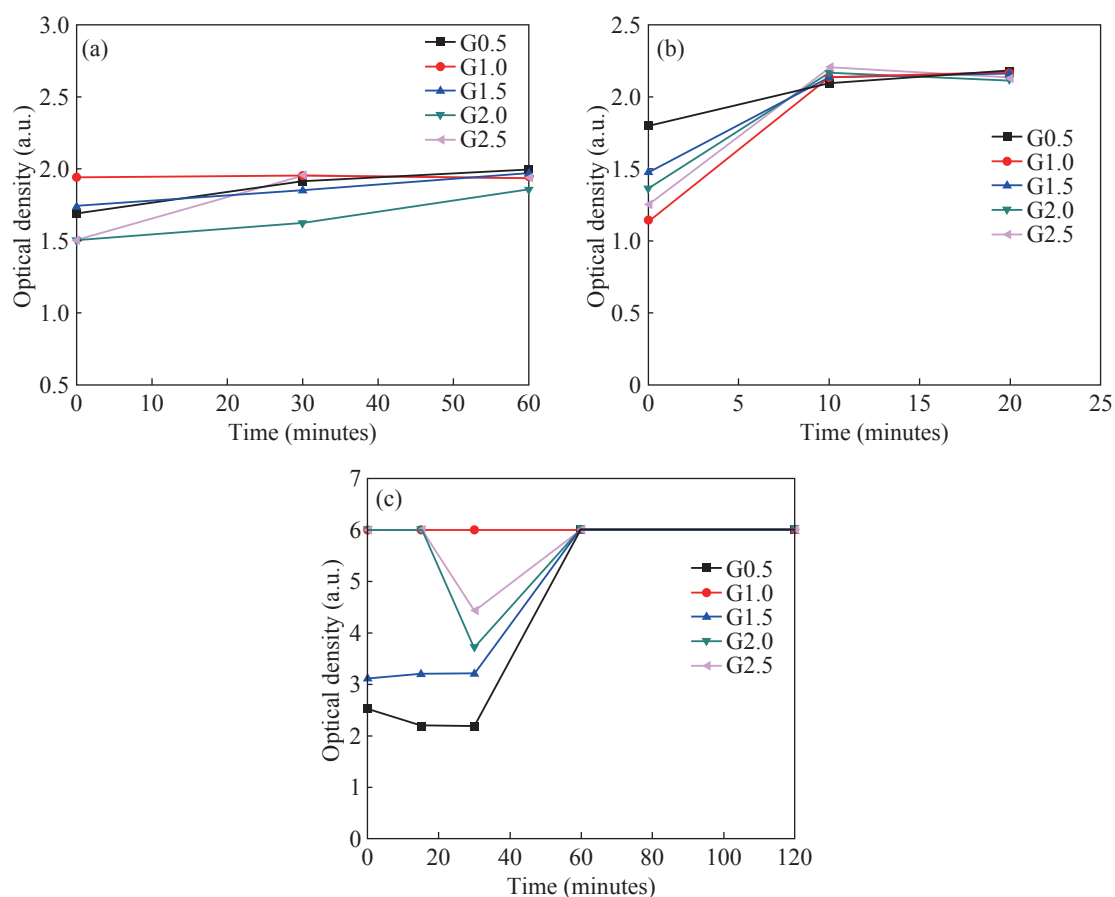
and it was noticed till 120 minutes. Since we have analysed this study in three different pathogens, each have taken different time periods to influence the cell death by the PGSA dendrimer which indicates that each pathogens requires a time period for releasing the intercellular materials fully and leading to cell death and hence reach a saturation in the O.D value. We also like to record the fact that, our anionic PGSA dendrimer also has the potential to disturb and displace the divalent cations present in the biological membrane as reported by Chen et al. [1]. Similarly, our anionic PGSA dendrimer also has the potential to disturb and

displace the divalent cations present in the biological membrane and the study proves the dendrimer ensures antimicrobial activity.

## Molecular docking studies

Biological and experimental observation were followed up with molecular docking studies, from which generation 0.5 (G0.5) was considered to have good antimicrobial activity when compared to other generations (G1.0, G1.5, G2.0, G2.5). So, PGSA dendrimer of G0.5 has been docked with the human

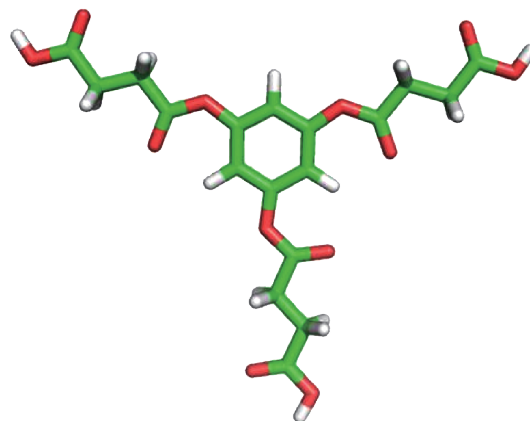




**Fig. 6** Release of 260 nm absorbing material from (a) *S. aureus*, (b) *E. coli*, (c) *C. albicans* after addition of PGSA dendrimer (G0.5-G2.5).

pathogens namely *S. aureus*, *E. coli*, and *C. albicans* in order to determine the preferred binding site on these three pathogens' structures. Since the above biological and experimental results could not lead to conclusion of the exact binding sites of dendrimer on these bacterial pathogens' structures, as well as the types of main binding forces. For this purpose, we selected/performed computer simulation experiments with molecular docking method to explore the interaction mechanism of PGSA dendrimer (G0.5) with these pathogens to support our experimental results.

Molecular models were built to discuss the binding modes for the interactions of PGSA dendrimer (G0.5) with three human pathogens by docking, using Schrodinger program. Here, the three dimensional structure of PGSA dendrimer (G0.5) was first modelled (Fig. 7) by Schrodinger suite 9.0 USA 2009 and by using two algorithms, steepest descent and conjugate gradient through optimized potential of liquid simulations (OPLS 2005) force field the energy minimization was achieved. The X-ray crystal structures of *S. aureus* (2ZCS), *E. coli* (4JX7) and *C.*



**Fig. 7** Energy minimized three dimensional structure of PGSA dendrimer (G0.5).

*albicans* (3QNE) were taken from the protein data bank (PDB ID) and their protein structures were energy minimized using protein preparation wizard panel to add hydrogen.

The charge state of protein residues is important for generating the result for Glide protein preparation facility which consists of two steps, refinement and

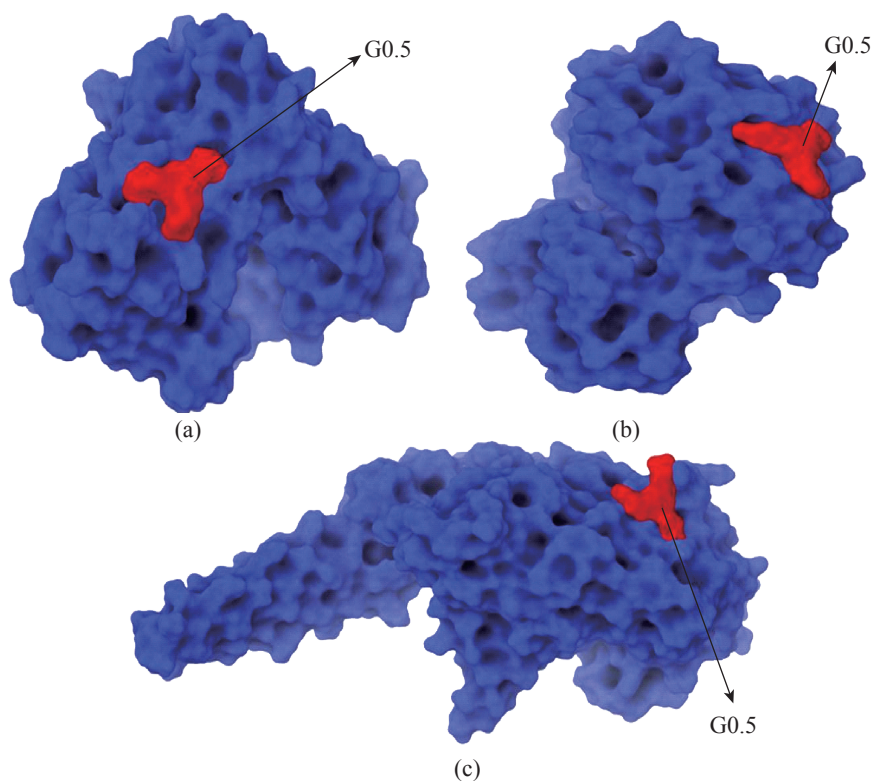
the energy minimization to reorient the side chain hydroxyl groups and alleviates potential steric clashes. In order to determine the binding sites of human bacterial pathogen structure, Glide (Grid-based Ligand Docking with Energetics) algorithm is used for the search of favourable interactions between one or more typically small molecules and a typically larger receptor molecule, usually a protein. The shape and properties of the receptor are represented by several different sets of fields that provide progressively more accurate scoring (binding) of the compound in order to determine the position and size of the active site which will be represented by receptor grids and set up Glide constraints. It was found that the compound, PGSA dendrimer had apparent interaction with all the three pathogens and was docked into their binding sites using the induced fit docking (IFD) panel. Here, both the compound and protein are flexible to dock, and hence many confirmations were generated for an individual structure and their compound. Based on the docking score, glide energy, hydrogen bond and hydrophobic interaction, the best confirmation was sorted and the corresponding results were analyzed. The overall structure of these three pathogens *S. aureus*, *E. coli* and *C. albicans* structures with PGSA

dendrimer of generation 0.5 was individually docked on their binding sites is shown in Fig. 8.

Glide energy, docking score and hydrogen bond interaction of the compound with key residues are given in Table 2. Docking result showed that all the three different structures and the compound have good binding affinity but when comparable with these three structures, *S. aureus* has good binding score and energy. Docking scores of these three different structures are comparable and hydrogen bond interactions with hydrophobic residues are shown in Fig. 9. The docking was in good agreement with the biological and experimental results and revealed the importance of interaction pathway between the compound, anionic PGSA dendrimer (G0.5) and pathogens' structures.

## Conclusions

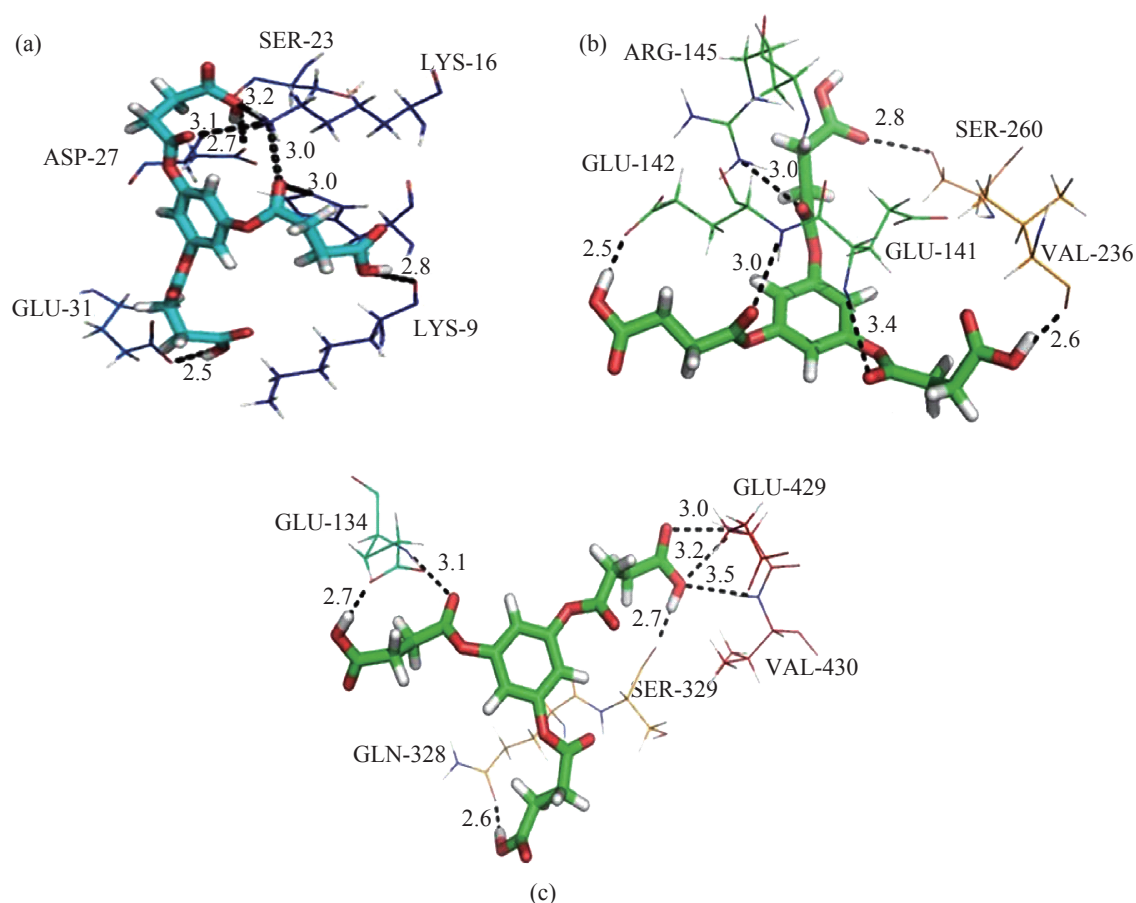
In summary, we depict that a polymeric nano carrier called PGSA dendrimer with negative charged surface was efficiently synthesized, characterized and that possessed significant antibacterial activity against *S. aureus* and *E. coli* and antifungal activity



**Fig. 8** Surface binding interaction of PGSA dendrimer of G 0.5 (red) with (a) *S. aureus* (2ZCS), (b) *E. coli* (4JX7), (c) *C. albicans* (3QNE).

**Table 2** Glide energy, docking score and hydrogen bond interaction of the PGSA dendrimer (G 0.5) with key residues of (a) *S. aureus*, (b) *E. coli*, (c) *C. albicans*

Dendrimer (G0.5) with pathogens	Hydrogen bond (D-H...A)	Distance (nm)	Docking score (Kcal/M)	Glide (Kcal/M)
(a) <i>S. aureus</i>	Lys9(O-H...O)	0.28	-6.23	-61.59
	His12(N-H...O)	0.29		
	Lys16(N-H...O)	0.29		
	Lys16(N-H...O)	0.30		
	Ser23(O-H...O)	0.29		
	Glu31(O-H...O)	0.25		
(b) <i>E. coli</i>	Glu141(N-H...O)	0.34	-6.10	-60.09
	(O-H...O)Glu142	0.25		
	Arg145(N-H...O)	0.30		
	(O-H...O)Val236	0.26		
	Ser260(O-H...O)	0.28		
(c) <i>C. albicans</i>	Glu134(N-H...O)	0.31	-6.02	-52.88
	(O-H...O)Gln328	0.26		
	(O-H...O)Ser329	0.27		
	Glu429(N-H...O)	0.30		

**Fig. 9** PyMol view of hydrogen bond interaction between PGSA dendrimer (G0.5) and amino acid residues of (a) *S. aureus*, (b) *E. coli*, (c) *C. albicans*.

against *C. albicans*. Moreover, the minimum inhibitory concentration value (0.312  $\mu\text{g/mL}$ ) obtained for G0.5 was identical against Gram(+), Gram(-) bacteria and

fungus, which pronounces to highlight for the first time from this study that the value obtained was appreciable and it is worthy to mention this nano carrier has both

the bactericidal and fungicidal effect for the initially developed product of G0.5 itself. This action was maintained even when the generation was further developed to G2.5, because the dendrimer biocide which we have formulated explicitly expressed negative charge density in every half and full generations. Additionally, tryptophan degradation study and 260 nm release study also reveals the same spectroscopically, by the release of intracellular components and leading to eventual cell death. We have also ascertained the exact binding location of the dendrimer of G0.5 on these pathogens with molecular docking studies. The binding score for the PGSA dendrimer (G0.5) with *S. aureus* gave better binding energy (-61.59 Kcal/M) compared to other two pathogens (*E. coli*, *C. albicans*). This study also gave supportive information for the biological and spectroscopic studies carried out for the antimicrobial activities with *S. aureus*, *E. coli* and *C. albicans*. This motivated us to report our biocompatible PGSA dendrimeric nano carrier as an attractive new antimicrobial agent against human pathogens, in addition to drug delivery application and hemocompatibility behaviour [26]. Till now there is search of new antibacterial agent globally alternative to the existing antibacterials for various clinical applications. As reported earlier, the infections caused by methicillin-resistant *S. aureus* (MRSA) are traceable and even caused death than HIV/AIDS in United States [23-25], it is hoped that the studies performed in this work may provide useful guidelines to look up better for the future.

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