Electronic Supplementary Information

Cooperative enhancement of antibacterial activity of sanguinarine drug through *p*-sulfonatocalix[6]arene-functionalized silver nanoparticles

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Experimental

Sanguinarine chloride hydrate (sanguinarine >98 % purity, MW= 367.8 Da) was obtained from Sigma-Aldrich/Advent ChemBio Pvt. Ltd. The concentration of SGR solution was determined spectrophotometrically using its molar absorption coefficient (ε= 30,700 M^{-1} cm^{-1} at 327 nm in acidic aqueous solution).[1] *p*-sulfonatocalix[6]arene (SCx6) was obtained from Alfa Aeser, India. Nanopure water (Millipore Gradient A10 System; conductivity of 0.06 µS cm^{-1}) was used to prepare the sample solutions. HCl as well as NaOH used for pH adjustment were obtained from Merck India. Solution pHs were measured by a pH meter model Eutech industrie, PC 2700. Before measurements, the pH meter was calibrated at two pHs, namely, at pH 4 and 7, by using standard pH buffer solutions from Merck (India). $AgNO_3$ and $NaBH_4$ were obtained from Chemco Fine Chemicals, India and Sisco Research Laboratory (SRL), India and were of pure grades. Luria Bertani broth, Luria agar, phosphate buffered saline were from HiMedia, Mumbai, India; 2,3,5 triphenyl tetrazolium chloride dye from Sigma chemicals.

***Synthetic procedure for silver nanoparticles (AgNPs) and the p-sulfonatocalix[6]arene-functionalized silver nanoparticles (SCxAgNPs)*:**

AgNPs: Aqueous solutions of silver nitrate ($AgNO_3$) (200 µl, 2 mM) and sodium borohydride ($NaBH_4$) (200 µl, 12 mM) was added drop wise simultaneously to water (1 ml) with stirring at room temperature. The resultant golden yellow solution was stirred for 10 minutes and diluted with water to a total volume of 2 ml.[2]

SCxAgNPs: An aqueous solution of $AgNO_3$ (5mL, 2mM) added in 5 mL aqueous solution of SCX6 (1 mM) and the volume was adjusted to 20 mL by adding water and the mixture was stirred for 30 minutes. Cooled aqueous solution of sodium borohydride (5mL, 12mM) was added drop wise to the

above mixture with stirring at room temperature. Stirring was continued for about 1hr and golden yellow color of SCx6-functionalized silver nanoparticle solution was appeared. The nanoparticle solution displayed characteristic surface plasmon band (SPB) with maximum at 394 nm (Fig. 1A).

FT-IR measurements: The SCxAgNPs kept in a freezer for 1hr for agglomeration and the agglomerated nanoparticles were centrifuged and dispersed in water repeatedly to remove the uncomplexed SCx6. Shift in the characteristic IR bands of SO_3^- at 1170 and 1044 cm^{-1} and OH groups at ~3427 cm^{-1} verified the presence of SCx6 moieties on the AgNP surface (Fig. 1B).

Optical absorption spectra were recorded with a Shimadzu UV-VIS-NIR Spectrophotometer (UV-3600, Tokyo, Japan). Approximate concentrations of these nanoparticles were estimated by using the molar extinction coefficient (ε) at 380 nm as ~2×10^4 $M^{-1}cm^{-1}$.[3] Steady-state fluorescence spectra were recorded using a FS5 spectrofluorometer (Edinburgh Instruments, UK). The time-resolved (TR) fluorescence measurements were carried out using a time-correlated-single-photon-counting (TCSPC) spectrometer (Horiba Jobin Yvon IBH, UK). In the present work, a 405 nm diode laser (~100 ps, 1 MHz repetition rate) was used for sample excitation and a PMT was used for fluorescence detection. Transmission electron microscopic (TEM) measurements, both bright field low magnification and lattice imaging, were performed using 200 keV electrons in JEOL 2010 UHR TEM microscope.

Antibacterial activity measurements. Antibacterial assay was carried out using 96-well Microtiter plates to determine the minimum inhibitory concentration (MIC) which is defined as the lowest concentration of drug or antibacterial agent at which there is no visible growth of bacteria. Standard bacterial cultures *Escherichia coli* (ATCC 31258) and *Staphylococcus aureus* (MTCC 96) and multidrug resistant *Salmonella oslo* isolated from marine fish were used as the test bacteria. This was confirmed using 2,3,5 triphenyl tetrazolium dye.

The cells were inoculated from glycerol stock on Luria agar plate and loopful transferred to Luria Bertani broth and 50 μl was inoculated in fresh 5.0 ml broth incubated at 35 ± 2˚C on shaker 220 rpm for 18 hrs. These were then diluted to obtain a bacterial culture of 1 x 10^7 cfu/ml equal to turbidity of 0.5 McFarland's standard and used as working solution. 100 μl Luria broth was added to all the wells. 100 μl of sample was added to the first well and serially diluted two-fold from (80-2.5 μM) up to 6^{th} well. 10 μl of either *E. coli* or *S. aureus* was added. 7^{th} and 8^{th} well served as negative and positive controls respectively. The plate was incubated for 18hrs at 35 ± 2˚C with shaker at 220rpm. 20 μl of tetrazolium dye was added to each well and further incubated for 20-minutes at 35 ± 2˚C. The change in colour of the dye indicates bacterial growth.

Antibiotic Sensitivity Testing was performed by agar diffusion method as described by National Committee for Clinical and Laboratory Standards Institute (2013).[4] In short, the single isolated

colony of the organism (*Escherichia coli*, *Staphylococcus aureus* or multidrug resistant *Salmonella oslo*) from the Luria agar plate was transferred to 5.0 ml of Luria broth and incubated at 35 ± 2°C for 18–20 h. 5 µl of the culture was then transferred to fresh medium (5.0 ml LB) and incubated at 35 ± 2°C for 18–20 h. The culture was diluted to obtain 10^7 cfu/ml using Phosphate buffered saline. 100 µl of this was plated on Luria agar plates using sterile spreader to obtain a matt growth. Wells were bored using sterile cork borer (8.0 mm) diameter. 20 µl of the samples were added to the wells) and incubated at 35 ± 2°C for 18–20 h. The zone of inhibition was measured using the scale provided by Himedia Laboratories. All the experiments were performed in duiplicates and were repeated 3 times with fresh samples.

MTT Assay for cell viability study: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay presents a quantitative colorimetric method for studying cytotoxic agents, where the amount of MTT reduced by cells to its blue formazan derivative is quantified spectroscopically at 570 nm and is indicative of the number of viable cells. In our experiments we have used normal Chinese Hamster Ovary (CHO) cell and lung cancer (A549) cell for cytotoxicity evaluation. Both cell types were cultured as monolayers in phenol red free DMEM medium supplemented with 10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C under 5% CO_2 and humidified air. For the assay, cells (~$5x10^3$) seeded per well in to 96 well plate, kept overnight for attachment and then treated with plain SGR, SGR with SCx6-coated and uncoated silver nanoparticles and SGR-loaded SCxAgNP in the presence of Ca^{2+} ion. Following this, cells were incubated at 37 °C for 48 hours, imaged using Olympus fluorescence microscope (Model - CKX41, Japan) attached to Progres® digital camera and processed for colorimetric detection as reported previously.[5] The control group represents cells grown in DMEM medium. The percentage (%) viability was calculated from the decrease in absorbance at 570 nm of treated groups as compared to that of control group.

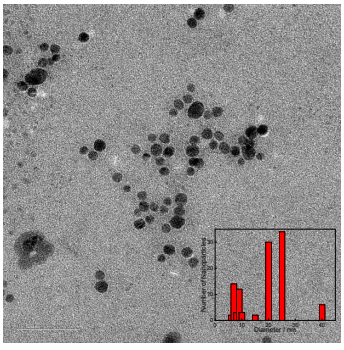


Fig S1. TEM images of uncoated AgNP and inset shows its particle size distribution.

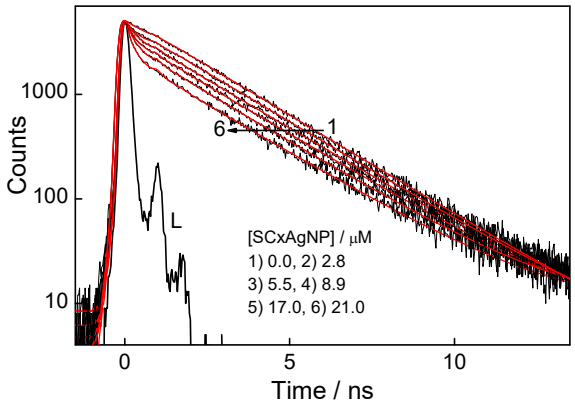


Fig. S2 Fluorescence decay traces of SG^+ at different concentrations of SCxAgNP. λ_{ex} = 405 nm, λ_{mon} = 605 nm, L represents lamp profile.

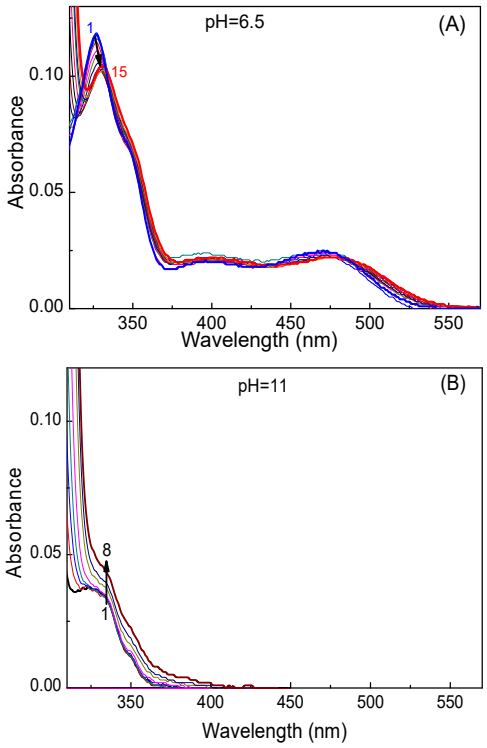


Fig.S3 (A) Absorption spectra of SG^+ at different concentrations of SCx6 at pH 6.5. [SCx6]/ μM: (1) 0.0, (2) 0.6, (3) 1.8, (4) 3.6, (5) 5.5, (6) 10.9, (7) 24.1, (8) 42.0, (9) 65.3, (10) 93.9, (11) 127.3, (12) 161.8, (13) 207.0, (14) 262.4 and (15) 327.3. (B) Absorption spectra of SGOH at different concentrations of SCx6 at pH 11.0. [SCx6]/ μM: (1) 0, (2) 25, (3) 50, (4) 100, (5) 200, (6) 500 and (7) 1000.

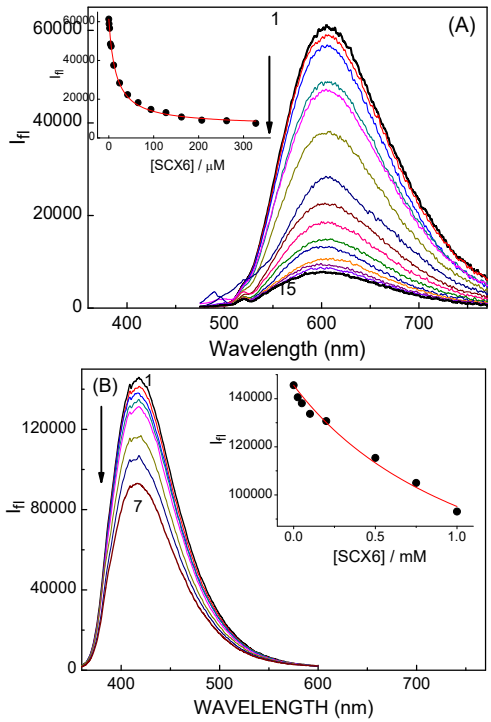


Fig.S4 (A) Fluorescence spectra of SG^+ at different concentrations of SCx6 at pH 6.5. [SCx6]/ μM: (1) 0.0, (2) 0.6, (3) 1.8, (4) 3.6, (5) 5.5, (6) 10.9, (7) 24.1, (8) 42.0, (9) 65.3, (10) 93.9, (11) 127.3, (12) 161.8, (13) 207.0, (14) 262.4 and (15) 327.3. (B) Fluorescence spectra of SGOH at different concentrations of SCX6 at pH 11.0. [SCx6]/ μM: (1) 0, (2) 25, (3) 50, (4) 100, (5) 200, (6) 500 and (7) 1000. Insets show the binding isotherms of the respective forms of SGR with SCx6 (λ_{mon} = 605 nm for SG^+ and 416 for SGOH).

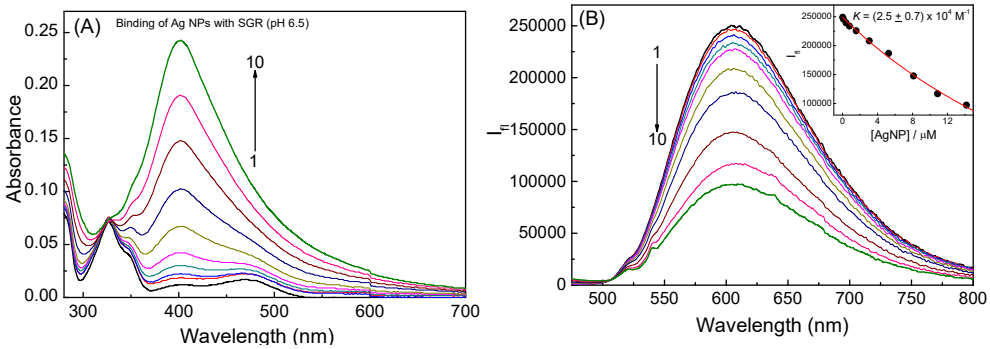


Fig.S5 Absorption (A) and fluorescence (B) spectra of SG^+ at different concentrations of uncoated-AgNP at pH 6.5. [AgNP]/μM:(1) 0.0, (2) 0.08, (3) 0.39, (4) 0.78, (5) 1.54, (6) 3.04, (7) 5.25, (8) 8.1, (9) 10.85 and (10) 14.14. Inset shows the binding isotherm of SG^+ with uncoated-AgNP (λ_{mon} = 605 nm).

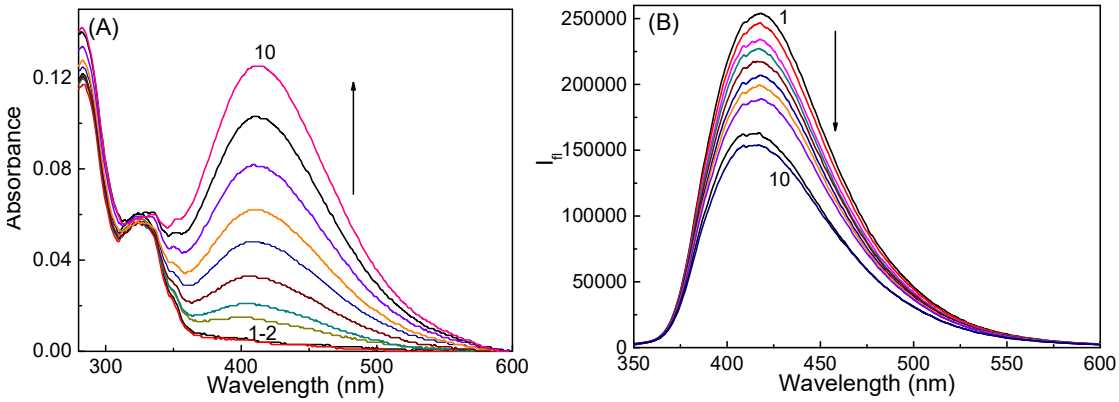


Fig. S6 Absorption (A) and fluorescence (B) spectra of SGOH at different concentrations of uncoated-AgNP. [AgNP] / μM: (1) 0, (2) 0.05, (3) 0.5, (4) 0.9, (5) 1.6, (6) 2.4, (7) 3.3, (8) 4.3, (9) 5.5 and (10) 6.9.

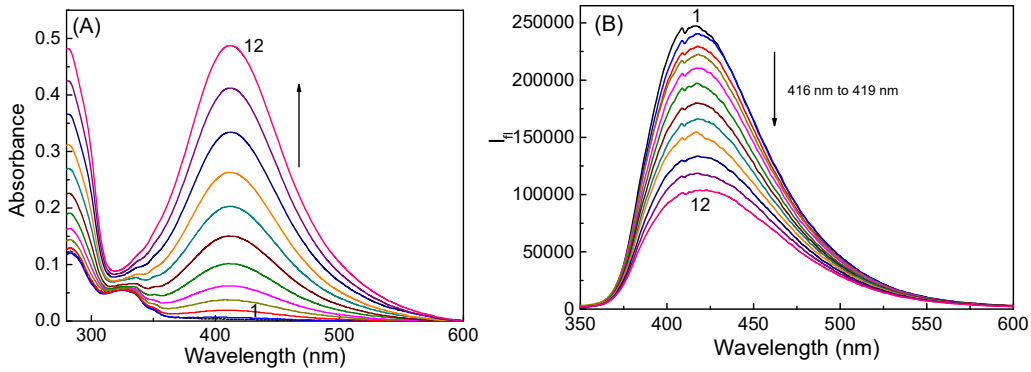
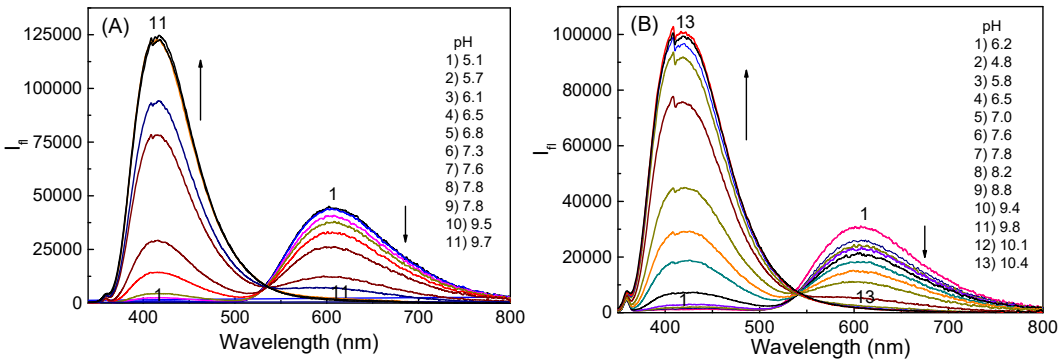


Fig. S7 Absorption (A) and fluorescence (B) spectra of SGOH at different concentrations of SCxAgNP. [SCxAgNP] / μM: (1) 0, (2) 0.1, (3) 0.5, (4) 1.3, (5) 2.5, (6) 4.4, (7) 6.9, (8) 9.3, (9) 12.2, (10) 15.7, (11) 19.6 and (12) 23.4.



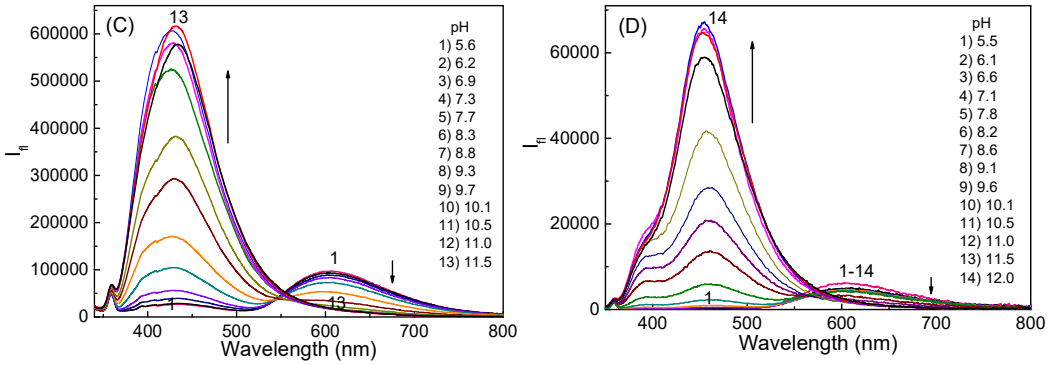


Fig. S8 Fluorescence spectra of SGR in the absence (A) and presence of 25 μM (B), 50 μM (C) and 100 μM (D) SCxAgNP at different pHs.

Table S1: Binding constant values of SG^+/SGOH with SCx6 and SCxAgNP.

System	***K*** **(M^{-1})**
SG^+-SCx6 (10 mM Phosphate buffer, pH ~6.5)	$(6.3\pm0.3) \times 10^4$
SGOH-SCx6 (10 mM bicarbonate buffer, pH ~11)	$(7.8\pm3.2) \times 10^2$
SG^+-SCxAgNP	$(3.4\pm0.2) \times 10^5$
SGOH-SCxAgNP	-----[#]

[#] *In case of SGOH-SCxAgNP system, we could not analyze/fit the binding isotherm properly due to the interference of strong absorption of SCxAgNP as SGOH also emits in the same spectral region (414 nm).*

Table S2. Changes in the pK_a value obtained from fluorescence studies of SGR at different solution conditions.

Systems	**pK_a**	**ΔpK_a**
SGR	7.7	
SGR-SCxAgNP (25 μM)	8.3	0.6
SGR-SCxAgNP (50 μM)	8.9	1.2
SGR-SCxAgNP (100 μM)	10.1	2.4
SGR-SCxAgNP (100 μM)-Ca^{2+} (25 mM)	9.4	1.7
SGR-SCxAgNP (100 μM)-Ca^{2+} (50 mM)	9.0	1.3
SGR-SCxAgNP (100 μM)-Ca^{2+} (100 mM)	8.7	1.0
SGR-uncoated AgNP (100 μM)	8.3	0.6
SCX6 (100 μM)	8.6	0.9

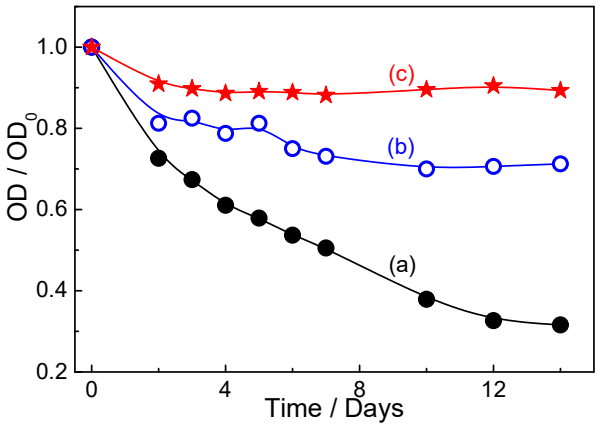


Fig. S9 The changes in absorbance at the respective peak positions with time of SGR in the absence (a) and presence of 25 μM (b) and 100 μM of SCxAgNP, at ambient conditions at pH 7.4.

Note-S1

Employing the tunability of the noncovalent interactions, we further establish that the fine tuning in the pK_a values of SCxAgNP:SGR system is indeed feasible by introducing Ca^{2+} metal ion through competitive binding and the pK_a shift is reversed to ~8.6 with the addition of 100mM Ca^{2+} (Fig. S10 and Table S2)

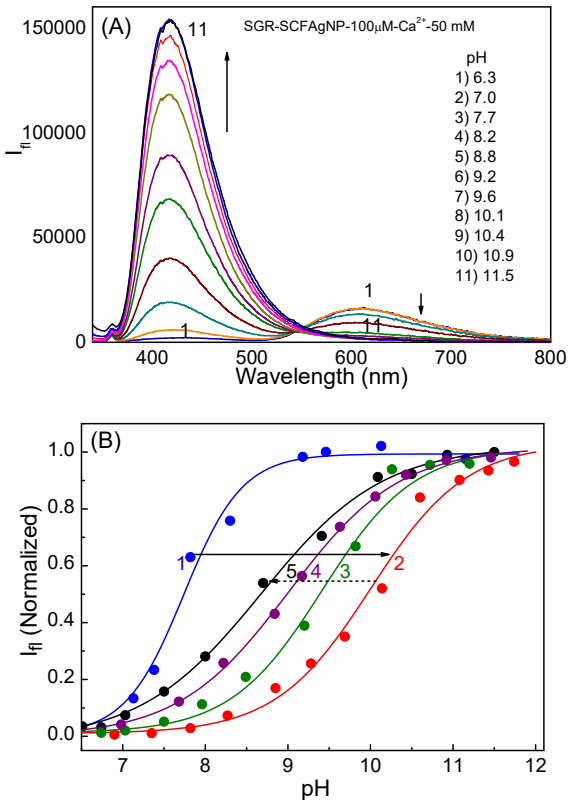


Fig. S10 (A) Fluorescence spectra of SGR in the presence of SCxAgNP (100 μM) and Ca^{2+} ion (50 mM) at different pHs. (B) pH titration curves from fluorescence studies of SGR in the absence (1) and presence of SCxAgNP (100μM) with [$CaCl_2$] / mM: (2) 0, (3) 25, (4) 50 and (5) 100.

Table S3. Minimal inhibitory concentration of sanguinarine (SGR) at different solution conditions towards two pathogenic bacteria at physiological pH 7.4. All the experiments are done in duplicate and repeated thrice. All values are expressed as average of 6 replicates.

Systems	**Minimum inhibitory concentration in μM (μg/ml)**	
	E. coli (Gram -ve)	*S. aureus* (Gram +ve)
SGR	20 (7.4)	80 (29.4)
SGR-SCxAgNP (50 μM)	20 (7.4)	80 (29.4)
SGR-SCxAgNP (100 μM)	10 (3.7)	40 (14.7)
SGR-SCxAgNP (100 μM)-Ca^{2+} (25 mM)	20 (7.4)	---
SGR-SCxAgNP (100 μM)-Ca^{2+} (50 mM)	---	80 (29.4)
SGR-uncoated AgNP (100 μM)	10 (3.7)	40 (14.7)
SCx6 (100 μM)	---	---
SCxAgNP	100 μM	25 μM
Uncoated-AgNP	100 μM	---

Table S4. Antibacterial activity (in terms of inhibition zone) of CPFL with and without additives against two pathogenic (Gram -ve) micro-organisms at pH 7.4.

Systems	Zone of inhibition / mm	
	E. coli	*S. oslo*
CPFL / H_2O (20 μM)	25±0.5	21±0.4
CPFL (20 μM) / SGR (80 μM) / SCxAgNP (100 μM)	28±0.6	23±0.5
CPFL (20 μM) / SCxAgNP (100 μM)	25±0.5	20±0.4
CPFL (20 μM) / SGR (80 μM)	26±0.5	19±0.4
CPFL / H_2O (5 μM)	15±0.3	15±0.3
CPFL (5 μM) / SGR (20 μM) / SCxAgNP (25 μM)	16±0.3	18±0.4
CPFL (5 μM) / SCxAgNP (25 μM)	15±0.3	16±0.3
CPFL (5 μM) / SGR (20 μM)	18±0.4	16±0.3
CPFL / H_2O (2.5 μM)	13±0.3	12±0.2
CPFL (2.5 μM) / SGR (10 μM) / SCxAgNP (12.5 μM)	13±0.3	17±0.3
CPFL (2.5 μM) / SCxAgNP (12.5 μM)	13±0.3	14±0.3
CPFL (2.5 μM) / SGR (10 μM)	15±0.3	14±0.3

Evaluation of loading efficiency. The loading efficiency of SGR on SCxAgNP has been evaluated by centrifuging the SCxAgNP:SGR complex for about 90 minutes. The absorption of complex without centrifugation and the supernatant after centrifugation has been recorded. From the decrease in absorbance at SGR peak position, the concentration of the SGR and hence the loading on to the SCxAgNP has been estimated.

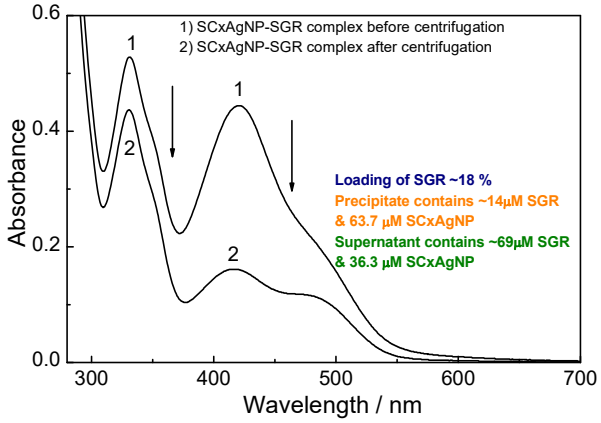


Fig. S11 The absorption spectra of SCxAgNP:SGR complex before centrifugation (spectrum 1) and the supernatant containing free SGR after centrifugation (spectrum 2).

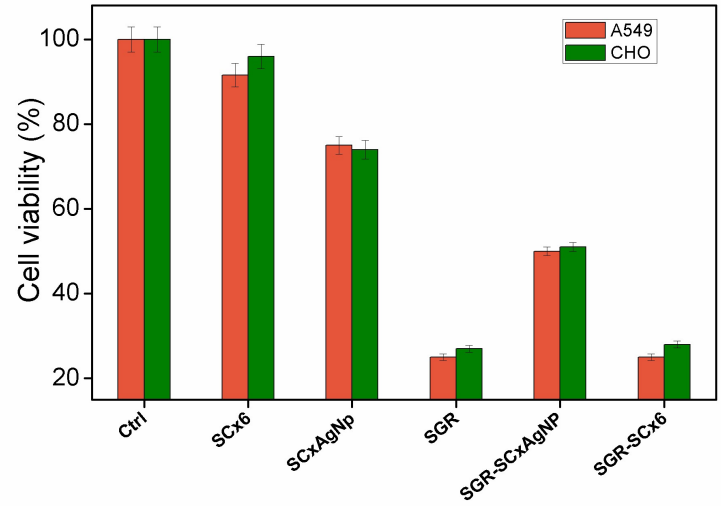


Fig. S12 The viability of A549 (orange bar) and CHO (green bar) cells treated with SCx6, SCxAgNP, SGR, SGR loaded SCxAgNP and SGR:SCx6 complex.

References

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