Dual role of Pinostrobin-a flavonoid nutraceutical as an efflux pump inhibitor and antibiofilm agent to mitigate food borne pathogens

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Supplementary Information

Experimental methods

1. **Antimicrobial studies:** MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) were discerned by broth microdilution method. Briefly, pinostrobin solution was serialy diluted in a range starting from 0.25µg/ml to 64µg/ml and inoculated with test organisms in Mueller-Hinton broth and incubated at 37°C for 18-24h. The lowest concentration of pinostrobin, which prevented growth (visible turbidity), was assumed to be MIC.
2. **Synergy testing: Checkerboard Analysis:** The interactions between pinostrobin and various antibiotics were discerned using by checkerboard analysis on a 96-well plate by the standard method reported earlier. One of the components either pinostrobin was diluted along the y-axis and the other antimicrobial agent was diluted along x-axis. The FIC was calculated according to the equation: FIC index = $FIC_A + FIC_B$ = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/ MIC of drug B alone). An FIC index ≤ 0.5, indicated synergy, an FIC index between 0.5-2.0 indicated additive effect and an FIC ≥ 2.0 indicated antagonism.

3. **MIC reversal:** To recognise the drug potentiation activity of pinostrobin along with ciprofloxacin we used a set of strains viz *Enterococcus faecalis*, MRSA (ATCC 43300), SA1199 (wild-type *S.aureus*), SA1199B (*S.aureus* overexpressing NorA), K1758 (*S.aureus* NorA knockout strain), *Pseudomonas aeruginosa* (lab mutant) and *E.coli* (clinical isolate). Briefly, pinostrobin was used at 0.5X MIC along with ciprofloxacin in increasing

concentrations in cation adjusted Mueller-Hinton broth and incubated at 37°C for 18-24 hrs. The fold-change in MIC of ciprofloxacin along with pinostrobin was noted.

4. **Efflux pump inhibition assays:** EtBr cartwheel assay was used to study the efflux pump inhibition of pinistrobin in *S.aureus* and mutant strains. Here, nutrient agar was supplemented with pinostrobin at sub-inhibitory concentrations along with EtBr- an efflux substrate; followed by swabbing the cultures in a cartwheel pattern (Fig S1). After 24 hrs of incubation these plates were viewed under UV-transilluminater to observe the fluorescence of Etbr retained inside the cells.

   Real-time efflux assay was used to monitor the efflux of EtBr from test organisms using a spectrofluorimeter. Overnight cultured cells of SA1199, SA1199B and K1758 were re-inoculated into nutrient broth and allowed to reach log phase (0.2-0.3 OD). Then the cells were harvested and suspended in PBS (phosphate buffer saline pH 7.0) These cells were taken in aliquots and subjected to 1µg/ml of EtBr and 0.4% glucose along with pinostrobin at 0.5X MIC for 1 hr. Following this the cells were harvested again and washed twice with PBS to remove any traces of EtBr and re-suspended in PBS. The rate of Etbr efflux was quantified for 50min at Ex 530nm and Em 585nm using a spectrofluorometer. Reserpine and verapamil were used as positive controls for these experiments.

5. **Etbr accumulation assays:** SA1199 cells were grown in nutrient broth till they reached an OD of 0.2-0.3. Then they were harvested at 6000rpm for 15min and suspended in PBS. Aliquots of these cells were distributed into replica tubes and subjected to increasing concentrations of Pinostrobin (10µg/ml- 25µg/ml) along with 1µg/ml of EtBr and 0.4% glucose. Rate of EtBr accumulation inside the cells was quantified using Jasco FP-8200 spectrofluorimeter (Jasco, Tokyo, Japan) with Ex 530nm and Em 580nm immediately for over 30min at 5min intervals. Reserpine and Verapamil were used as positive controls at the same concentrations as mentioned earlier.

6. **Etbr efflux assays:** SA1199 cells were grown in nutrient broth till they reached an OD of 0.2-0.3. Then they were harvested at 6000rpm for 15min and suspended in PBS. These cells were distributed into replica tubes. Pinostrobin was added in increasing concentrations (10µg/ml- 25µg/ml) along with EtBr at 1µg/ml and 0.4% glucose. Then the tubes were incubated for 1 hr for maximum uptake of EtBr. Post incubation cells were again harvested and washed twice with PBS to remove any traces of EtBr and resuspended in EtBr free PBS. The rate of EtBr efflux was quantified using Jasco FP-8200 spectrofluorimeter (Jasco, Tokyo, Japan) with Ex 530nm and Em 580nm immediately for over 30min at 5min intervals.

Reserpine and Verapamil were used as positive controls at the same concentrations as mentioned earlier.

7. **Biofilm inhibition assays:** To comprehend the anti-biofilm nature of pinistrobin we used crystal violet staining assay wherein Tryptic soy broth was used to grow biofilms in the presence of increasing concentrations of pinostrobin in 96-well plates. After 18-24 hrs of incubation the planktonic cells were discarded and the plates were washed 2-3 times with PBS (phosphate buffer saline) and dried thoroughly. Then 0.1% crystal violet was used to stain the biofilms inside these wells followed by 15min of incubation. After incubation crystal violet washed off and again the plates were dried. The crystal violet up-taken by cells were extracted using 30% acetic acid and quantified at 595nm.

8. **Confocal imaging:** Static biofilms of *S.aureus*, *E.Faecalis*, *P. aeruginosa* and *E.coli* were grown on glass slides with Tryptic Soy Broth as the source of nutrients. In the same medium pinostrobin was added at 2µg/ml. After 24 hrs of incubation the slides were washed with sterile PBS to remove unbound cells and stained with a mixture of acridine orange (0.2 mg ml/1) and propidium iodide (0.33 mg ml/1) and imaged using Olympus FV 1000 confocal microscope with 10X, 40X and 60X objective. Excitation was done using Multi Argon laser for acridine orange (Ex 488nm Em 526nm) and Helium Neon laser for propidium iodide (Ex 594nm Em 600nm). Acridine Orange stains live cells and emits green fluorescence, hence live cells appear green. Propidium iodide stains dead cells and emits red fluorescence, hence dead cells appear red.

9. **Membrane Permeability studies:**

a. **NPN assay**: Change in outer membrane permeability of *P.aeruginosa* and *E.coli* in the presence of pinostrbin was measured using a fluorescent substrate viz. N-Phenyl-1-naphthylamine (NPN). In this assay log-phase (0.2- 0.3 OD) cells pelleted and re-suspended in 5mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES) buffer were subjected to NPN alone and then increasing concentrations of pinistrobin (1 µg/ml- 8 µg/ml). Any change in membrane permeability can be directly quantified by increasing fluorescence of NPN at Ex 350nm and Em 420nm.

b. **PI-CTAB assay:** Similarly, log-phase cells of *S.aureus* and *E.faecalis* were treated with pinostrobin in PBS for 1-2 hrs along with the substrate for uptake i.e propidium iodide (PI). Disruption of membrane allows the uptake of PI and hence binding to DNA which can be quantified (Ex 500nm Em 600nm) using a Jasco FP-8200 spectrofluorimeter (Jasco, Tokyo, Japan). The change in permeability is expressed as ratio of PI-treated to PI-untreated cells. \Here, Cetyltrimethylammonium bromide (CTAB) was used as a positive control.

Table S1: Pinostrobin exhibits antimicrobial effect against diverse bacteria and displays synergistic interactions with certain antimicrobial agents

Pathogen	**Antimicrobial Agents (MIC)(µg/ml)**											
		PIN‡	AMX#	AMP	MET	CIP	GEN	CHL	AZM	ERY	TET	PMB
Staphylococcus aureus	**I***	64	26	45	5	8	4	22	16	5	15	32
	R		**N**	**N**	**A**	**S**(16,2)	**A**	**N**	**N**	**A**	**N**	**N**
Bacillus subtilis	**I**	12	12	20	16	6	4	12	16	12	12	16
	R		**N**	**N**	**A**	**S**(2,3)	**A**	**N**	**A**	**A**	**A**	**A**
Enterococcus faecalis	**I**	12	18	18	24	6	20	6	14	18	18	12
	R		**N**	**N**	**A**	**S**(4,1)	**N**	**S**(4,1)	**N**	**A**	**S** (3,4.5)	**A**
Escherichia coli	**I**	12	12	16	12	6	6	6	12	12	12	10
	R		**A**	**N**	**N**	**S**(2,2)	**S**(2,2)	**S**(3,1.5)	**A**	**N**	**A**	**A**
Vibrio cholerae	**I**	12	15	8	24	4	3	4	20	40	30	8
	R		**A**	**A**	**N**	**S**(3,1)	**S**(3,0.75)	**A**	**N**	**N**	**T**	**S**(3,2)
Klebsiella pneumonia	**I**	24	40	24	20	12	6	6	8	12	46	2
	R		**A**	**A**	**A**	**S**(6,3)	**A**	**S**(3,2)	**S**(4,2)	**A**	**T**	**S**(6,0.5)
Proteus vulgaris	**I**	18	25	40	30	16	12	12	12	40	46	18
	R		**N**	**N**	**N**	**A**	**S**(4.5,3)	**A**	**A**	**A**	**N**	**A**
Proteus mirabilis	**I**	32	32	36	30	14	10	18	16	46	42	20
	R		**A**	**N**	**N**	**A**	**A**	**N**	**N**	**N**	**T**	**T**
Shigella dysenteriae	**I**	12	16	20	18	4	4	22	10	20	20	10
	R		**A**	**N**	**A**	**A**	**A**	**N**	**A**	**A**	**A**	**A**
Pseudomonas aeruginosa	**I**	32	30	18	12	6	4	6	12	20	16	6
	R		**A**	**T**	**N**	**S**(6,2)	**S**(6,1)	**S**(6,2)	**A**	**N**	**A**	**S**(6,2)

*I-Individual MIC, R – Results. **S**-Synergistic, **A**- Additive, **N** – No Interaction, **T**- Antagonistic. **FICI**- <0.5 (Synergy), 0.5 - 1.0 (Additive), 1.0 - 2.0 (No interaction), >2.0 (Antagonistic)

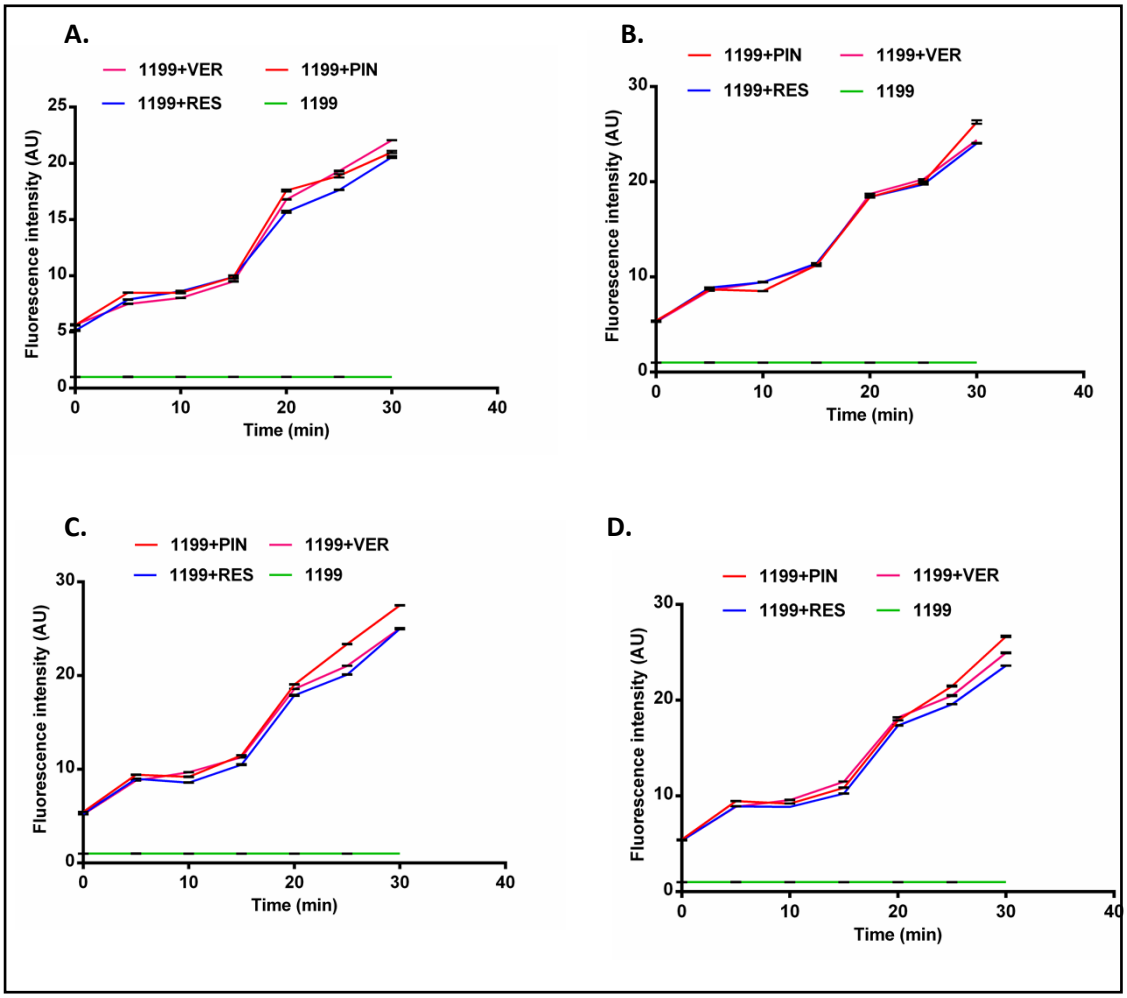
PIN- Pinostrobin,**Commercial Antibiotics** –AMX- Amoxicillin, AMP- Ampicillin,MET- Methicillin,CIP- Ciprofloxacin, GEN- Gentamycin, CHL- Chloramphenicol, AZM- Azithromycin, ERY- Erythromycin, TET- Tetracycline, PMB-Polymyxin-B.

S. Table 2: Pinostrobin reverses MIC of gallic acid and ferulic acid in diverse bacteria

	MIC (mg/ml) Ferulic acid	**MIC (mg/ml) Ferulic acid +PIN***	**Modulation Factor of PIN***	**MIC (mg/ml) Gallic acid**	**MIC (mg/ml) Gallic acid +PIN***	**Modulation Factor of PIN***
Enterococcus faecalis	>2.5	2	>1.25	2.5	2	1.25
Staphylococcus aureus (MRSA) ATCC 43300	>2.5	1.5	>1.67	>2.5	1.5	>1.67
S.aureus-1199 B	>2.5	1.5	1.67	2.5	2	1.25
S. aureus 1199	>2.5	1.5	1.67	>2.5	2	>1.25
S.aureus K1758	>2.5	1.5	1.67	>2.5	1.5	>1.6
**Pseudomonas aeruginosa*	>2.5	2	1.25	2	1	2
Escherichia coli	>2.5	1.5	1.67	>2.5	2	>1.67

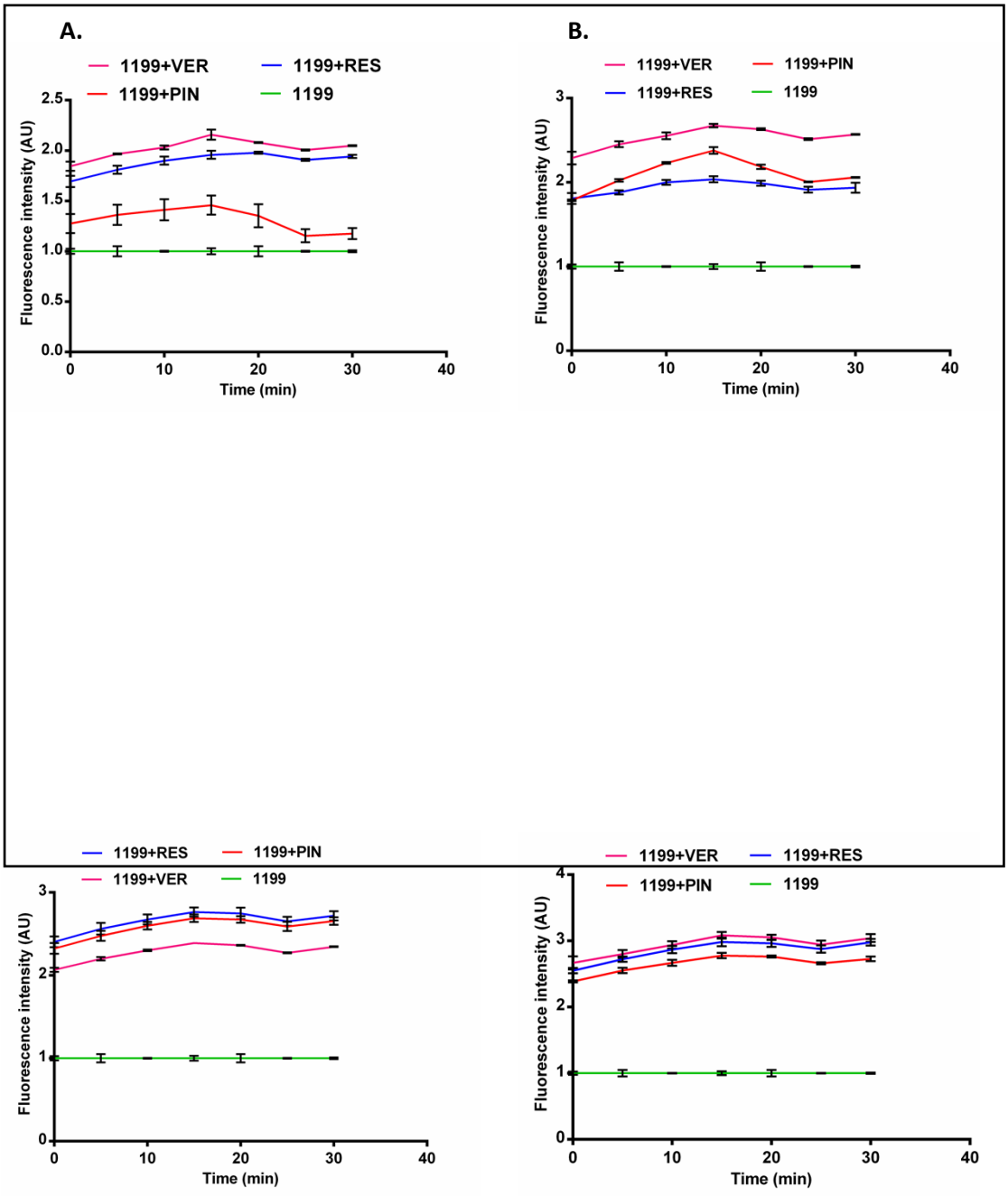
*PIN- Pinostrobin

FigS1: Dose-dependent accumulation of EtBr in SA1199 using Pinostrobin.



Figures represent the dose-dependent uptake of EtBr in wild type strain of *S.aureus* 1199 in the presence of Pinostrobin (PIN), Reserpine (RES) and Verapamil (VER). The test compounds have been used at the following concentrations: A. 10µg/ml; B. 15 µg/ml; C. 20µg/ml; D. 25µg/ml. The experiments were carried out over 30min of time and the residual fluorescence quantified by spectrofluorimetry using Jasco FP-8200 spectrofluorimeter (Jasco, Tokyo, Japan) with an Ex 530nm and Em 580nm. Fluorescence values of each sample was corrected for the background fluorescence of EtBr in buffer and normalized to EtBr fluorescence of untreated bacterial cells.

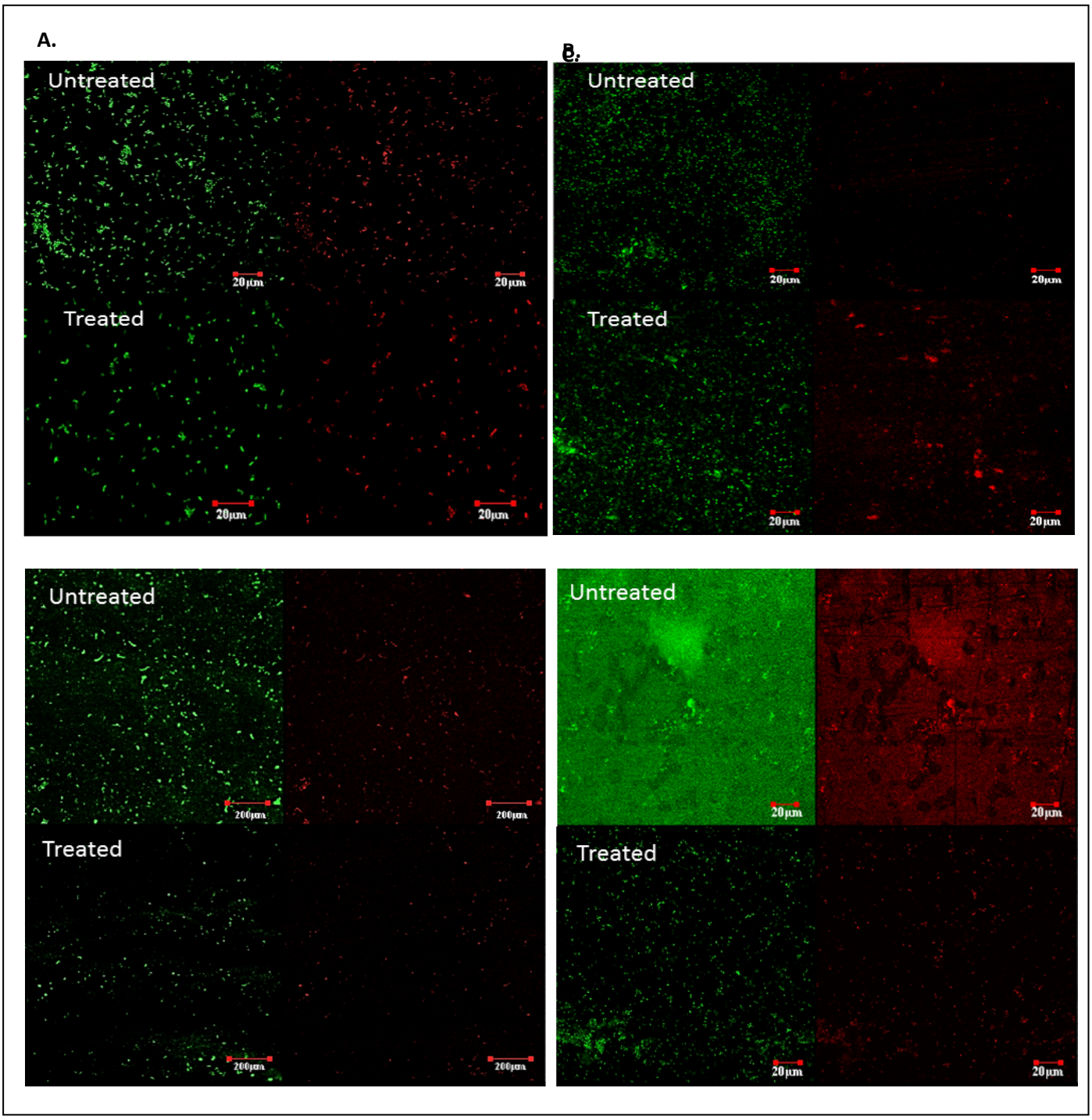
FigS2: Dose-dependent EtBr efflux in SA1199 using Pinostrobin.



Figures represent the dose-dependent efflux of EtBr in wild type strain of *S.aureus* (1199) in the presence of Pinostrobin (PIN), Reserpine (RES) and Verapamil (VER). The test compounds have been used at the following concentrations: A. 10µg/ml; B. 15 µg/ml; C. 20µg/ml; D. 25µg/ml. The experiments were carried out over 30min of time and the residual fluorescence quantified by spectrofluorimetry using Jasco FP-8200 spectrofluorimeter (Jasco, Tokyo, Japan) with an Ex 530nm

and Em 580nm. Fluorescence values of each sample was corrected for the background fluorescence of EtBr in buffer and normalized to EtBr fluorescence of untreated bacterial cells.

Fig S3: Pinostrobin treatment prevents colonization or increases the proportion of dead cells in biofilms formed by gram positive and gram negative bacteria.



Confocal image of 24 h biofilms formed in the presence and absence of pinostrobin (2µg/ml) stained with acridine orange (Ex 488nm; Em 526nm) and propidium iodide (Ex 594nm; Em 600nm)and imaged for live cells (green) and dead cells (red). In samples (A-D) top panel represents untreated control and bottom panel represents pinostrobin treated samples A): *E.coli* biofilms B): *P.aeruginosa* biofilms C) *E.faecalis* biofilms and D) *S.aureus* biofilms. Scale bar =20µm for *E. coli*, *P. aeruginosa* and *S. aureus*; Scale bar for *E.faecalis* biofilms =200µm.