SUPPLEMENTARY MATERIAL

Epigallocatechin gallate has antibacterial and antibiofilm activity in methicillin resistant and susceptible *Staphylococcus aureus* of different lineages in non-cytotoxic concentrations

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ABSTRACT

Staphylococcus aureus is an opportunistic agent that can cause a variety of infections, both hospital and community-acquired. Epigallocatechin gallate (EGCG), a flavonoid present in the leaves of *Camellia sinensis*, has different biological activities, including antimicrobial potential. Here we evaluate the **antibacterial** and antibiofilm potential of EGCG in nine clinical strains of *S. aureus* with different genetic profile and antimicrobial susceptibilities. The minimum inhibitory concentrations (MIC) of EGCG ranged from 7.81 to 62.5 μg/mL, and bactericidal activity was observed at 4 times the MIC. Sub-inhibitory concentrations were able to inhibit biofilm production. Concentrations ≤ 62.5 μg/mL of EGCG were non-cytotoxic for murine macrophages. EGCG significantly reduced the mortality of infected *Galleria mellonella* larvae with the *S. aureus*, having shown relevant antibiofilm properties and efficacy in inhibiting the growth of different clinical isolates of *S. aureus*, thus being a promising substance for the treatment of infections caused by this agent.

KEYWORDS: Antibacterial; Antibiofilm; Epigallocatechin gallate; *Staphylococcus aureus*.

EXPERIMENTAL SECTION

Bacterial strains and EGCG. Nine *S. aureus* isolates with distinct phenotypic and genotypic characteristics were used in this study (Table S2) (Schuenck et al. 2012). The strains were kept in the laboratory at -20 °C in cryotubes with 1 mL TSB (Tryptone Soy Broth, HiMedia Laboratories, India) supplemented with 20% glycerol. The epigallocatechin gallate (EGCG) (> 95% purity by HPLC) was purchased from Sigma-Aldrich (Missouri, USA).

Minimum Inhibitory Concentrations. The minimum inhibitory concentrations (MICs) were determined according to the broth microdilution methods suggested by CLSI (CLSI 2015). The EGCG was serially diluted with cation-adjusted muller-hinton broth (Oxoid, England) from a concentration of 1000 μg/mL to 3.90 μg/mL. The *S. aureus* strains were inoculated on every well at a 5×10^5 CFU/mL, and bacterial growth was visually determined after 24 hours of incubation at 35 °C. The MIC was defined as the lowest EGCG concentration that inhibited bacterial growth.

Time-kill assays. Time-kill assays were performed using the microplate method (Zhou et al. 2013), at concentrations of 1 time and 4 times the MIC of EGCG for each isolate, and 10^5 CFU/mL was used as the starting inoculum. The cultures were incubated at 35°C for 24 h. At time intervals of 0, 2, 4, 6, 12 and 24 h post inoculation, 10 μL samples were collected, serially diluted, and plated onto nutrient agar (HiMedia Laboratories, India). The colonies were counted after the plates were incubated at 35°C for 24 h.

Inhibition of biofilm formation by EGCG. The production of biofilm by *S. aureus* was analysed using 96-well polystyrene microtiter plates, according to the method proposed by Stepanović et al. (2007). After 24 hours of incubation at 35°C, with different EGCG concentrations (0.5 times the MIC for 0.97 μg/mL), the control wells contained medium in the absence of EGCG. After incubation, staining with 0.1% crystal violet and the optical density (OD) of each well was measured at 570 nm. *Escherichia coli* DH5α and *Staphylococcus epidermidis* (ATCC 35984) were used as negative and positive controls, respectively. Based on the OD obtained, the isolates were classified into four categories: weak, moderate, strong or non-producer of biofilm. The cutoff was defined based on the OD of the negative control, plus three times the value of the standard deviation (ODc). The isolates were classified as follows: $OD \leq ODc$ = non-biofilm producer; $ODc<OD \leq 2ODc$ = weak biofilm producer; $2ODc <OD <4ODc$ = moderate biofilm producer; and $OD \geq 4ODc$ = strong biofilm producer.To calculate the

percentage of biofilm inhibition, the OD value obtained was subtracted from the OD of the negative control, which is a non-biofilm producer. The percentage of inhibition was calculated using the equation: (OD of the treated control /OD of the non-treated control) x 100.

Cytotoxicity assay. Cytotoxicity tests were performed with the ATCC J774A.1 (ATCC® TIB-67 ™) of the mouse macrophage cell line, using protocol ISO 10993-5 (2009). The cells were grown in culture flasks with RPMI 1640 medium (Sigma-Aldrich, USA), supplemented with fetal bovine serum, L-glutamine and $NaCO_3$. For the assay, the cells were placed at a concentration of 5 x 10^4 cells /mL per well on a 96-well plate, and the plate was then incubated for 24 hours, so that they could adhere to it. Then, different concentrations of EGCG (0.97 to 1000 μg/mL) previously diluted in a RPMI medium were added. After incubation for 48 hours, cell viability was verified using the alamarBlue® dye (Invitrogen™, USA).

Toxicity in *Galleria mellonella.* *In vivo* toxicity tests were performed with *G. mellonella* as infection model, kept at room temperature with an artificial diet. The larvae were inoculated with different doses of EGCG (10, 20, 40, 60, 80, 100, 200 and 400 mg/kg), previously diluted in a phosphate-buffered saline solution (PBS 1X), through the abdomen's ventral surface. Ten larvae were used for each experimental condition, and ten control larvae were inoculated with PBS only. The larvae were kept at 35 °C for 72 h and mortality was observed every 24 h.

Treatment Assays. The *G. mellonella* larvae were infected with 10^5 CFU/larva from isolates 1155 or 1168. These isolates were selected based on the results obtained in the time-kill assay, which showed distinct patterns of bacterial growth when incubated under minimal inhibitory concentrations. Between the isolates analysed, the 1155 sample obtained higher growth inhibition, and 1168 obtained lower growth inhibition when submitted to EGCG concentrations at 1 x MIC. The test was carried out following the methodology of Ramarao et al. (2012) and Betts et al. (2017) with adaptations. Thirty minutes after the onset of the infection, a second injection containing either EGCG (200 mg/kg) or PBS was administered. The larvae were incubated at 35 °C for estimation of the survival rate (alive/dead) after 0, 24, 48 and 72 h. The tests were performed in three biological replicates, using fifteen larvae for each experimental condition.

Statistics. The data analyses were performed on GraphPad Prism 7, using One-Way ANOVA, for the antibiofilm assays. The *G. mellonella* larvae's survival curves were

plotted using the Kaplan-Meier method, and the differences in survival were calculated using the log-rank test. Significance was defined as $P < 0.05$.

REFERENCES

Betts JW, Hornsey M, Wareham DW, La Ragione RM. 2017. *In vitro* and *in vivo* activity of theaflavin–epicatechin combinations versus multidrug-resistant *Acinetobacter baumannii*. Infect Dis Ther. 6:435–442.

CLSI. 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approv Stand Ed CLSI Doc M07-A10.:1–87.

ISO 10993-5. 2009. Biological evaluation of medical devices - Part 5: Tests for cytotoxicity: *in vitro* methods. Int Stand. 3 Ed:42.

Ramarao N, Nielsen-Leroux C, Lereclus D. 2012. The insect *Galleria mellonella* em as a powerful infection model to investigate bacterial pathogenesis. J Vis Exp.:1–7.

Schuenck RP, Cavalcante FS, Emery E, Giambiagi-de Marval M, Netto dos Santos KR. 2012. *Staphylococcus aureus* isolates belonging to different multilocus sequence types present specific virulence gene profiles. FEMS Immunol Med Microbiol. 65:501–504.

Stepanović S, Vuković D, Hola V, Bonaventura G DI, Djukić S, Ćirković I, Ruzicka F. 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 115:891–899.

Zhou Y, Hou Z, Fang C, Xue X, Da F, Wang Y, Bai H, Luo X. 2013. Comparison of microplate and macrodilution methods in time–kill study of new antimicrobial drugs. Folia Microbiol (Praha). 58:9–16.

Table S1: Minimum inhibitory concentration (MIC) of epigallocatechin gallate (EGCG) in *Staphylococcus aureus* strains and biofilm production

Strain[a]	MIC of EGCG (µg/mL)	Biofilm production
1125 (MRSA)	31.25	Weak
1128 (MSSA)	7.81	Moderate
1154 (MSSA)	31.25	Moderate
1155 (MRSA)	62.5	Weak
1158 (MRSA)	15.62	Moderate
1168 (MSSA)	7.81	Moderate
39A (MRSA)	31.25	Moderate
92A (MRSA)	31.25	Strong
102A (MRSA)	7.81	Strong

[a]MSSA = methicillin-sensitive *Staphylococcus aureus*
MRSA= methicillin-resistant *Staphylococcus aureus*

Table S2: General characteristics of the *Staphylococcus aureus* strains used in this study.

Strain number	Source	Virulence genes[a]	Pulsotype (PFGE)[b]	ST[c](MLST)	Susceptibility to oxacillin/SCC*mec*	MIC (µg/mL)[d]	
						OXA	VAN
1125	joint prosthesis	*ica, cna, fnbA, ebpS*	A1	1	MRSA/ SCC*mec*IV	64	1
1128	joint prosthesis	*ica, cna, fnbA, ebpS*	A2	1	MSSA	<0,5	2
1154	osteomyelitis	*ica, fnbA, ebpS*	B	5	MSSA	<0,5	2
1155	osteomyelitis	*pvl, ica, cna, fnbA,bbp, ebpS*	C	30	MRSA/ SCC*mec*IV	8	1
1158	blood	*ica, fnbA, ebpS*	D	5	MRSA/ SCC*mec*IV	32	1
1168	surgical wound infection	*pvl, ica, cna*	E	1462	MSSA	<0,5	1
39A	blood	*fnbA, ebpS*	F	5	MRSA/ SCC*mec*II	>256	1
92A	tracheal secretion	*fnbA, ebps*	G	5	MRSA/ SCC*mec*IV	128	1
102A	blood	*fnbA, fnbB, pvl*	H	8	MRSA/ SCC*mec*IV	96	1

[a]Virulence genes: *cna* (collagen-binding protein), *bbp* (bone-sialoprotein-binding protein), *ebpS* (elastin binding protein), *fnbB* (fibronectin-binding protein B), *fnbA* (fibronectin-binding protein A), *pvl* (panton-valentine leucocidin) and *ica* (production of the intercellular polysaccharide adhesin related to biofilm formation); [b] Pulsed-field gel electrophoresis (PFGE) after genomic DNA macrorestriction with *Sma*I enzyme); [c]Sequence type (ST) obtained using multi-locus sequence typing (MLST) method; [d]minimum inhibitory concentration, OXA (oxacillin), VAN (vancomycin).

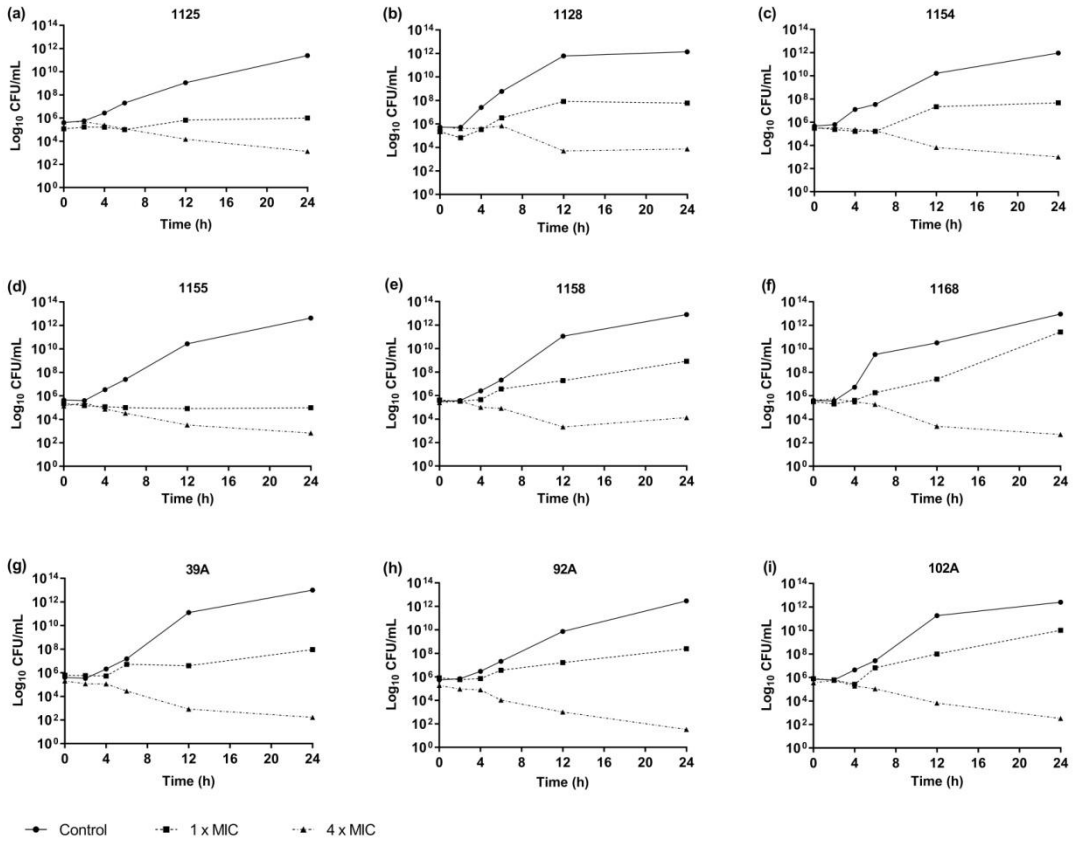

Figure S1: Time-kill curves of epigallocatechin gallate against *Staphylococcus aureus* isolates (0, 2, 4, 6, 12 and 24 hours) submitted to 1 time and 4 times the minimum inhibitory concentration.

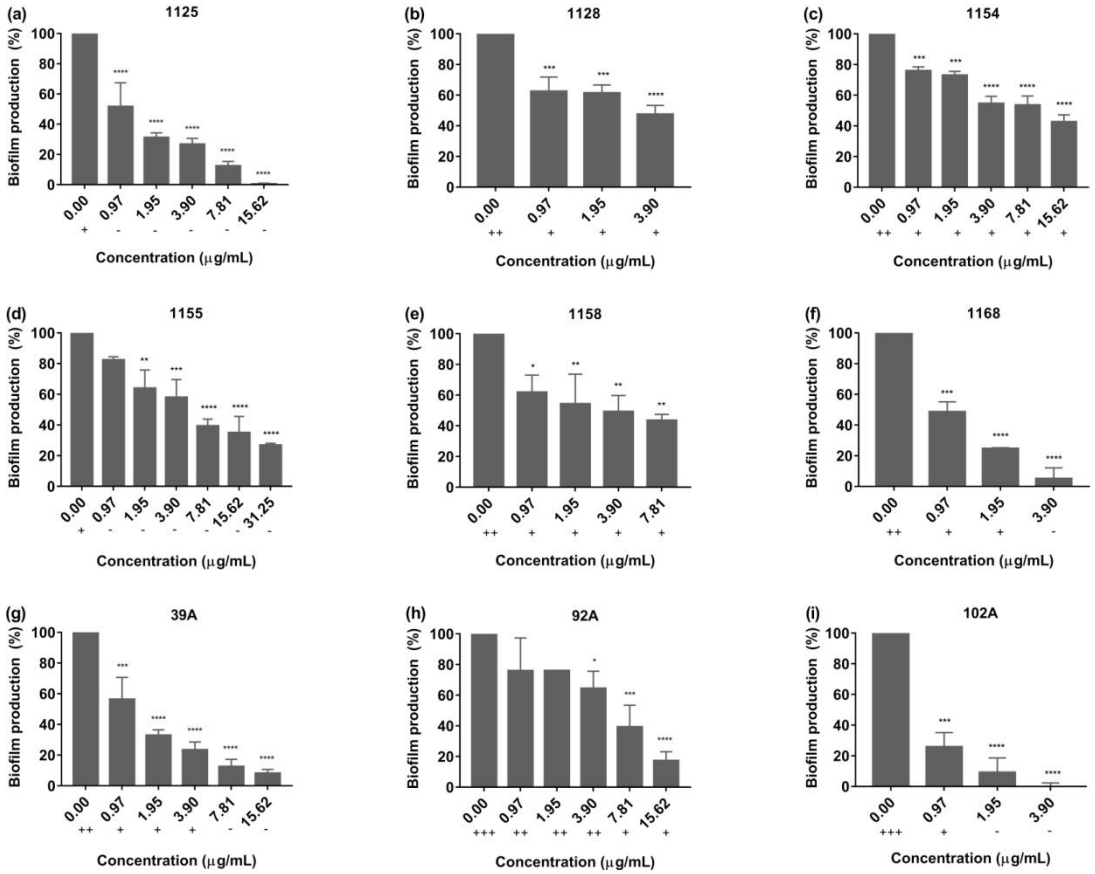

Figure S2: Inhibitory effect of epigallocatechin gallate (EGCG) on the production of biofilm by different strains of *Staphylococcus aureus*. +++ strong biofilm producer, ++ moderate biofilm producer, + weak biofilm producer, - non biofilm producer. * values of P between 0.05 and 0.01; ** values of P between 0.01 e 0.001; *** values of P between 0.001 e 0.0001; **** values of P <0.0001.

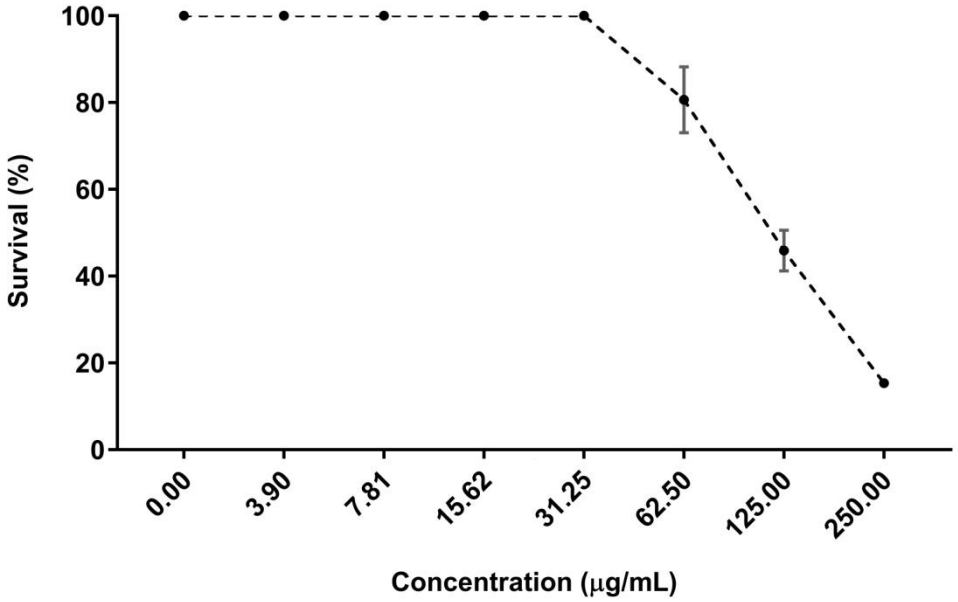

Figure S3: Evaluation of the cytotoxic effects of epigallocatechin gallate on macrophages of the J774A.1 cell line. The cells were used at a $5x10^4$ cells/mL concentration, distributed on a 96-well plate and incubated for 48 hours at different EGCG concentrations (from 1000 to 0.97 μg/mL). The figure shows the cytotoxicity of the concentrations from 250 to 3.90 μg/mL.

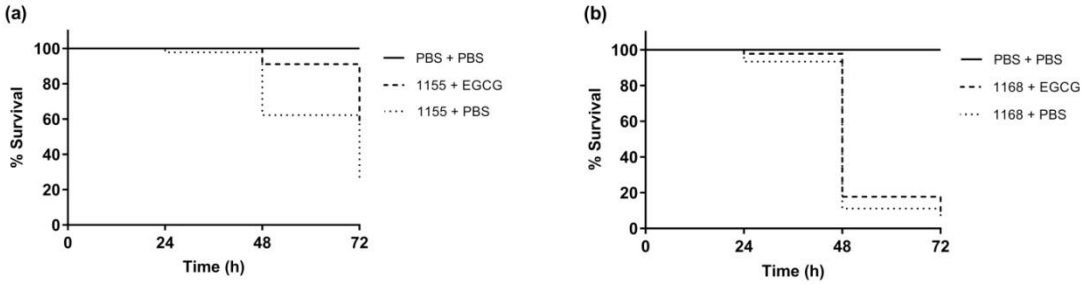

Figure S4: Survival of *Galleria mellonella* infected with 10^5 CFU/larva of isolates 1155 and 1168 after treatment with: PBS 1X; epigallocatechin gallate (200 mg/kg) and uninfected but treated twice with PBS 1X.