**Supplementary Information for**

# Avoiding Ventilator-Associated Pneumonia: Curcumin-Functionalized Endotracheal Tube and Photodynamic Action

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## Supplementary Materials and Methods

**Reagents.** Cesium carbonate and solvents were acquired from Sigma-Aldrich (Lisbon, Portugal) and FluoroChem (Derbyshire, UK) and used without further purifications. NaCl, KCl, $NaH_2PO_4$ and $KH_2PO_4$ were acquired from Synth (Diadema, São Paulo, Brazil). The photosensitizer ((1*E*,6*E*)-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) herein designated by curcumin (95%) was supplied by PDT Pharma®. The culture media BHI (brain heart infusion) was purchased from Kasvi®.

**Endotracheal tube.** PVC-based endotracheal tube (ETT) (Solidor®) was supplied by Orthomed (São Carlos, São Paulo, Brazil).

**Apparatus.** UV-Vis diffuse reflectance spectra were recorded on a Cary 5000 UV-Vis-NIR spectrophotometer equipped with a sphere of integration between 200-2500 nm. Fluorescence spectra were performed on an Agilent Technologies Cary Elipse Fluorometer Spectrometer. FT-IR spectra were recorded between 400-4000 $cm^{-1}$ on a FTIR Nicolet 5700 (ThermoElectron Corporation) spectrophotometer equipped with a Smart Orbit accessory. SEM images were acquired from a ZEISS LEO 440 (Cambridge, England) using OXFORD (model 7060) as detector, operating with a 15kV electron beam, current of 2.82 A and I probe of 200 pA. An Instrontensile testing equipment was used for the stress-strain analysis.

## Experimental Procedures

**Functionalization of PVC-based Endotracheal Tube (ETT) with Curcumin.** The commercial ETT (~11 g) was introduced into a hand-made reactor (*SI Appendix*, Fig. S1) and three vacuum/$N_2$ gas cycles were performed. Separately, curcumin (396 mg; 1.07 mmol) and $Cs_2CO_3$ (1.99 g; 6.01 mmol)

was dissolved in DMSO (80 mL) (solution A). Then, solution A was introduced into the hand-made reactor *via* cannula and the reaction mixture was kept for 4 hours at 30 ºC, under inert atmosphere. After this time, the reactor was cooled to room temperature and the ETT-curc was removed from the reactor and washed four times with DMSO (20 mL) and two times with ethanol (20 mL). The ETT-curc was dried under vacuum, at room temperature, and stored under inert atmosphere protected from the light. The indirect quantification of the amount of curcumin bonded to the ETT was determined by UV-Vis of the residual washing solvent, using standard curcumin solutions and the value of attached curcumin was found to be about 0.5% (w/w).

**Characterization of the Curcumin-functionalized Endotracheal Tube (ETT-curc)**

**UV-Vis.** UV-Vis spectra were recorded on a Cary 5000 UV-Vis-NIR spectrophotometer equipped with a sphere of integration between 200-2500 nm. The conversion to absorption was performed assuming the Kubelka-Munk function. The UV-vis absorption spectrum of ETT-curc shows a band at 430 nm, in similarity with the non-immobilized curcumin.

**Fluorescence.** Fluorescence spectra were performed on an Agilent Technologies Cary Elipse Fluorometer Spectrometer. In order to confirm the presence of curcumin onto ETT the previously prepared ETT-curc was cut in small pieces followed by emission fluorescent analysis.

**FT-IR.** Attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectra were measured over the range 400-4000 $cm^{-1}$ on the FTIR Nicolet 5700 (ThermoElectron Corporation) spectrometer equipped with a Smart Orbit accessory.

**SEM.** Microscopy images were obtained from the ZEISS LEO 440 (Cambridge, England) with an OXFORD detector (model 7060) with a 15kV electron beam, at the Central of Instrumental Chemical Analysis of the Institute of Chemistry of São Carlos (CAQI/IQSC/ USP) current of 2.82 A and a probe of 200 pA. The samples were covered with 6nm of gold in a Coating System metallizer BAL-TEC MED 020 (BAL-TEC, Liechtenstein) kept in a desiccator until the moment of analysis. The metallization conditions were pressure in the chamber = 2.00 x $10^{-2}$ mbar; current = 60 mA; with deposition rate of 0.60 nm/s.

**Stress-strain analysis**. An In strontensile testing machine (*SI Appendix*, Fig. S3) was used in displacement control mode to apply the desired mechanical load to the specimen. The ETT and ETT-curc were tested in a tensile test where they were subjected to a force that tended to move them to rupture. The tubes were secured by two claws positioned at the axial ends of the tube and a force in the same direction was applied. The force velocity that is applied is controlled by the operator and the equipment generates a table and a graph with the data over time. The test occurs until the material ruptures itself.

**Microorganisms**

**Pre-inoculum.** The microorganisms *Staphylococcus aureus* (ATCC 25925), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC14502) were subjected to a pre-inoculum development. The pre-inoculum development was carried out in a 50 mL Falcon tube containing 10% of the frozen bacteria (in 20% glycerol) and 90% of the culture medium. The pre-inoculum tube was kept at 37 °C for 15 h, in a shaker incubator at 140 rpm. After this period, each Falcon tube was subjected to three successive centrifugations (1500 rpm) along 15 min. After each centrifugation, the supernatant containing the culture medium was discarded and the same of volume of phosphate buffered saline PBS (ideal pH for the bacteria used) was added to each tube.

***In vitro* antimicrobial activity tests.** Sterile ETT were cut into pieces of one centimeter long each in a biological safety cabinet (ESCO Airstream® Class II). Then, one piece of the ETT and ETT-Curc was inserted into each well of the 24 well plates and BHI growth medium (900 µL) and bacterial inoculum (100 µL) were added.The solution was homogenized 6 times with a 1000 µL pipette in each well of the plate. Finally, the biofilms formed were characterized by counting bacterial colonies.

**Bacterial biofilm formation.** The same protocol made for the pre-inoculum and the biofilm formation was repeated in this experiment. The groups tested were: 1) only bacteria on the ETT; 2) bacteria with PS-functionalized on ETT. We have tested the groups in 7 times. The bacterial biofilms developed in the tubes were washed twice with a phosphate-buffered saline solution (PBS), and all the PDT group were submitted to PDT with the Biotable® device. The tubes of group time 1 were diluted and plated in Petri dishes with BHI agar medium. The rest of the tubes have returned to a new BHI infusion medium. In time 2, the tubes were rewashed and were submitted to the Biotable device. The group of time 2 was diluted and plated; the rest of the tubes have returned to a new BHI infusion medium. This procedure was repeated until the time 7. The methodology was described in the (SI Appendix, Fig. S6). Table S2 (SI Appendix) described the different light doses of each respective time group were received.

**Statistical Analysis.** The data were demonstrated as means and SD (standard deviation). Two-group comparisons were performed by student's t test and using One-way ANOVA followed by Tukey test. Two-tailed p values $< 0.05$ were considered statistically significant. The analyses were performed using the software Origin® academic, license granted by USP.

**Light.** The illumination system was composed with two types of irradiation and both emitting with a uniform and continuous irradiation at 450 nm. Each of them was performed using a light source developed by the Laboratory of Technological Support - LAT/USP (São Carlos Institute of Physics - University of São Paulo). The first system was composed by 24 LEDs for a multi-well irradiation, with a fluency of 35 mW/cm$^2$. The second system was composed by just one LED emitting at 70 mW/cm$^2$ for a direct illumination inside the tube.

**Optical fiber system.** The second illumination system was composed with a blue laser, 450 nm, and optical fibber developed by the Laboratory of Technological Support - LAT/USP (São Carlos Institute

of Physics - University of São Paulo). The fluence of the irradiation was tested inside and outside the tube.

**Photodynamic Therapy for ETT-curc.** In the present study, four experimental groups were divided and received a different type of treatment: **1)** biofilms formed on the surface of the ETT; **2)** biofilms formed on the surface of ETT-curc; **3)** biofilms formed on the surface of irradiated ETT under light dose of 50 J/cm$^2$; **4)** biofilms formed on the surface of ETT-curc tubes irradiated under light dose of 50 J/cm$^2$. Before each treatment describe above, the tubes were washed twice with PBS (2 mL) to remove all the non-adherent cells and treated. After each treatment, each tube was added in a Falcon with 10 mL of PBS for the bacterial biofilm be removed from the surface and under mechanical agitation. Just after the procedures, the samples were analyzed by the plating method on solid agar for counting bacterial colonies for antimicrobial evaluation of the groups.

**Counting Colony Forming Units (CFU).** The count of the bacterial colonies was performed after 24 hours of incubation, at 37 °C. Each experimental group was made in triplicate and colonies between 3 and 30 were counted, and the mean of each group was calculated in CFU/mL.

**Supplementary Figures and Tables**

**Table S1.** Stability study of ETT in different solvents and temperatures.

| Entry | Solvents | Time (h) | Temperature (°C) | Macroscopic Physical Observations |
|---|---|---|---|---|
| 1 | Dimethoxyethane | 3 | 30 | Dilated tube diameter |
| 2 | Dichloromethane | 3 | 30 | Degraded tube |
| 3 | Ethyl Acetate | 3 | 30 | Dilated tube diameter |
| 4 | Tetrahydrofuran | 3 | 30 | Degraded tube |
| 5 | Dimethylsulfoxide | 3 | 30 | No physical deformation on ETT |
| 6 | Dimethylsulfoxide | 4 | 30 | No physical deformation on ETT |
| 7 | Dimethylsulfoxide | 5 | 40 | Partially Degraded tube |
| 8 | Dimethylsulfoxide | 4 | 40 | Partially Degraded tube |
| 9 | Dimethylsulfoxide | 4 | 50 | Degraded tube |

**Table S2.** The dose light described for each irradiated group.

| **ETT with light (J/cm²)** / **ETT-curc with light (J/cm²)** | 50 | 100 | 150 | 200 | 250 | 300 | 350 |
|---|---|---|---|---|---|---|---|
| **Time Groups** | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

**Figure S1.** Hand-made reactor for functionalization of ETT with curcumin.

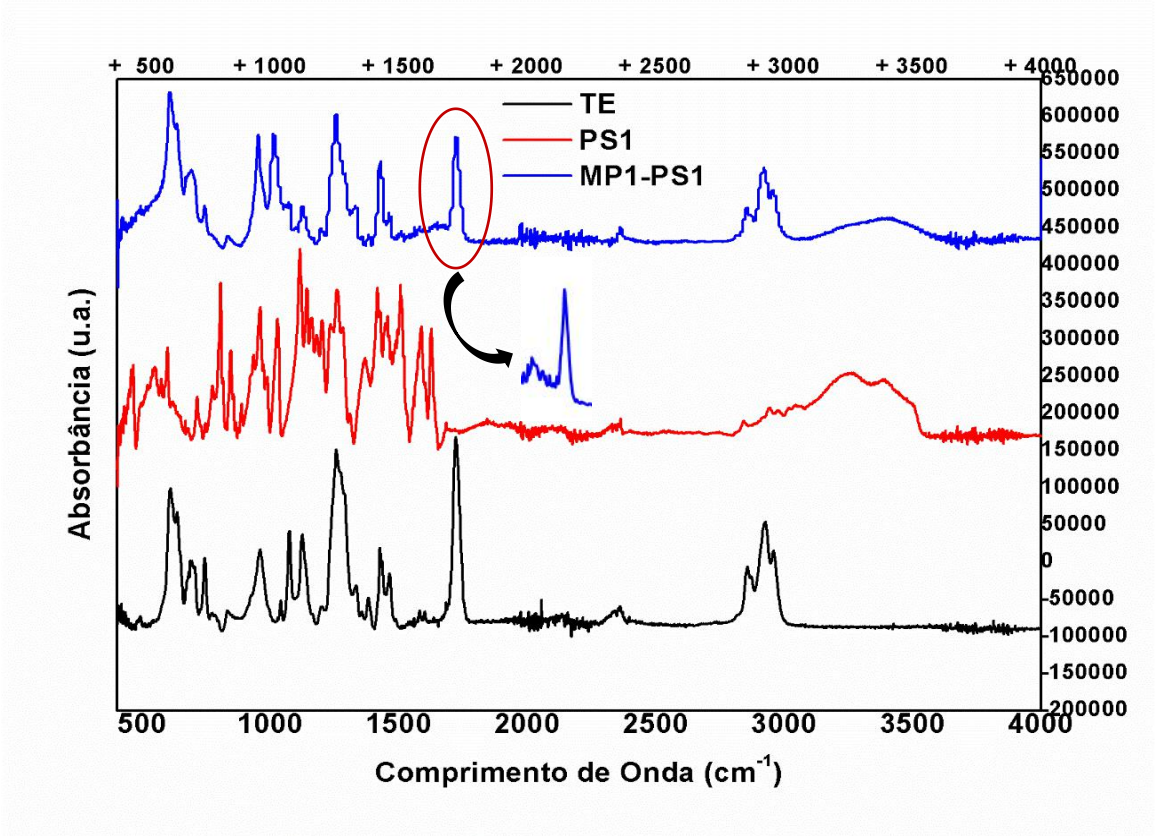


**Figure S2.** FT-IR spectra of: ETT (black), curcumin (red) and ETT-curc (blue).


**Figure S3.** Stress-strain analysis for ETT and ETT-curc.

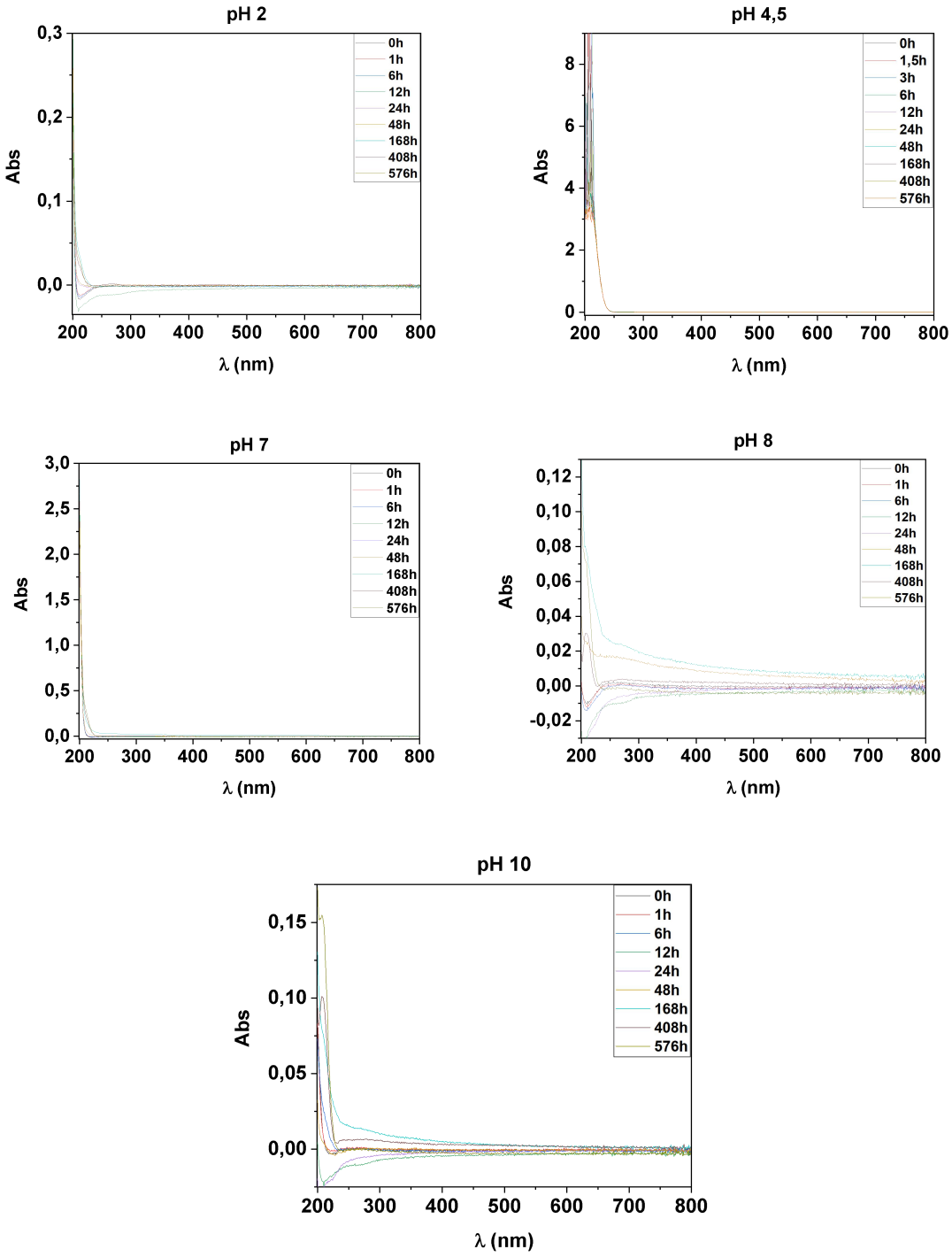


**Figure S4.** Stability evaluation of ETT-curc at different pHs (2.0, 4.5, 7.0, 8.0 and 10.0).

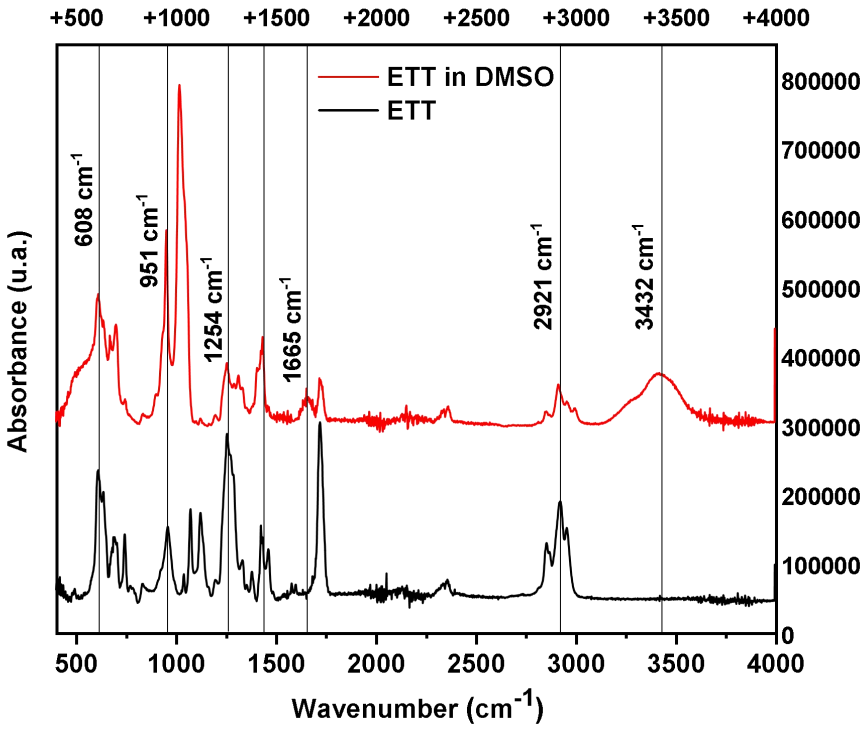


**Figure S5.** FT-IR spectra of: ETT (black); ETT after reaction with DMSO (red). The peak at 3432 $cm^{-1}$ is typical of water.

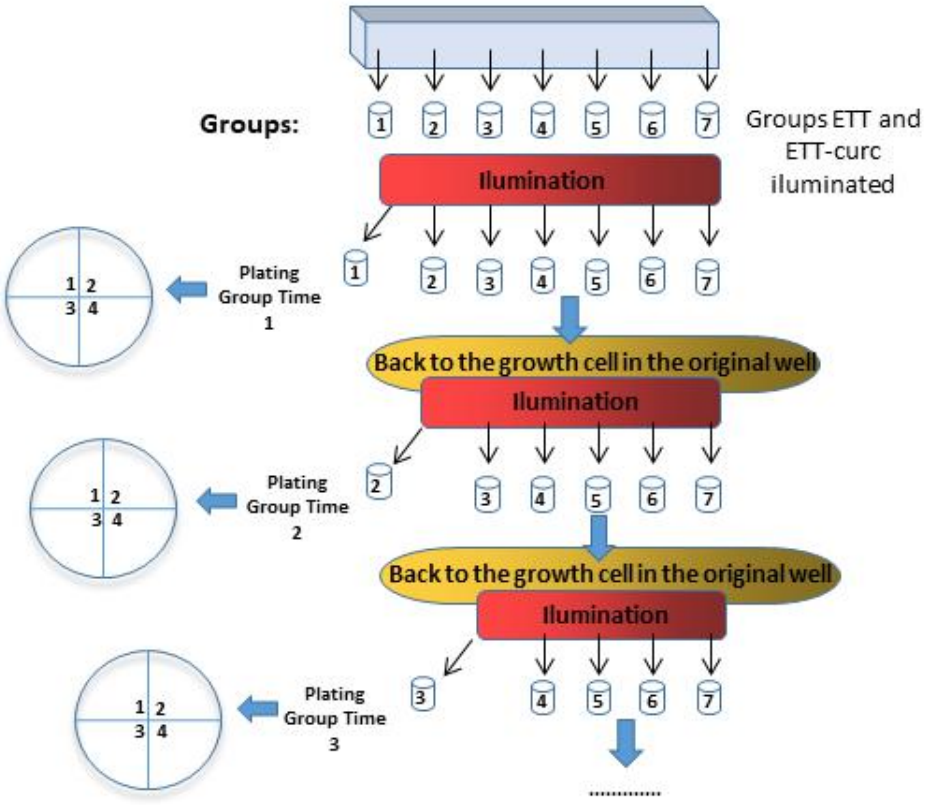


**Figure S6.** The experimental methods of the *S. aureus* kinetic growth in ETT and ETT-curc tube over time.

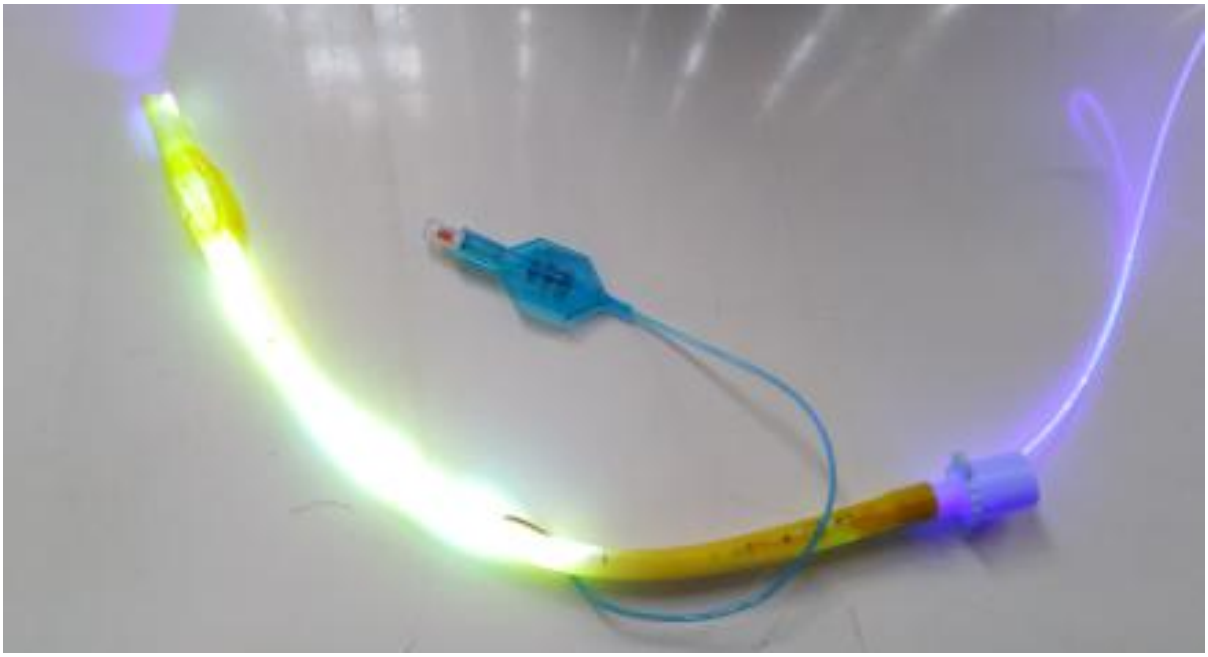

**Figure S7.** ETT-curc illuminated through a cylindrical diffuser fiber, placed inside the tube.

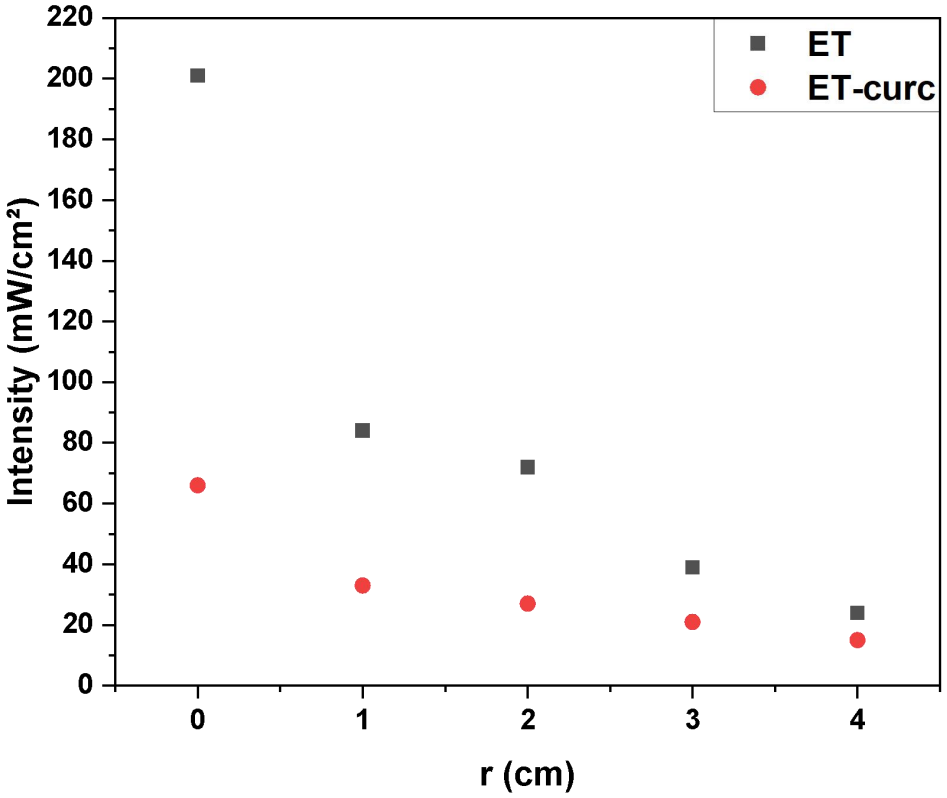


**Figure S8.** Light intensity measured outside of ETT (black squares) and ETT-curc (red circles) in different distances illuminated by an optical fiber inserted inside the tube.

In order to measure the light intensity that passed through the endotracheal tube (ETT and ETT-curc) and reached their outer surface, we used a fiber inserted inside the ETT and ETT-curc measuring the light intensity in different distances (Fig. S8). This experiment demonstrated that the ETT-curc absorbs nearly 66% of the light delivered by the fiber. The absorption of light can be explained by the presence of curcumin on the ET's internal and external surface. The light intensity on the internal surface of ETT-curc is higher than 12 J/cm² per minute, while on the external surface is nearly 4 J/cm² per minute. Therefore, these results indicate that the antimicrobial threshold dose (50 J/cm$^2$) is reached in 4.5 minutes for the internal surface, while 13.5 minutes was needed for the external surface. Overall, these results can guarantee to reach the threshold (50 J/cm$^2$) for microbial reduction and elimination in a few minutes. So, the experiment proves to be efficient in eliminating bacterial biofilm inside and outside the ETT-curc.