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# An Alternative Approach to Combat *C. albicans* and *S. aureus* Infections in Chronic Wounds.

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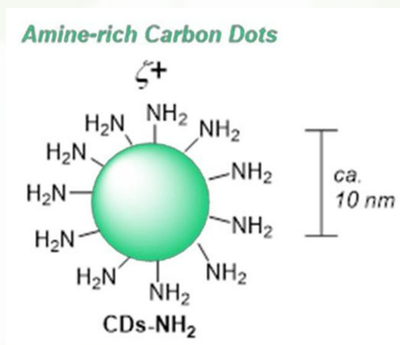
## *Introduction*

*Candida albicans* and *Staphylococcus aureus* are frequently found in diabetic foot ulcers, with prevalence rates of 47% and 95%, respectively. These species often cause systemic infections through interactions like cell-cell adhesion and cross-feeding, with *S. aureus* attaching to *C. albicans* in biofilms. Biofilms significantly enhance microbial survival, showing resistance to most antimicrobials and increasing strain resistance up to 1500-fold. Accordingly, developing new antibiofilm agents is of urgent importance [2], especially those that inhibit biofilm growth without killing the microorganisms. Carbon dots are a recently discovered class of carbon nanoparticles with positively charged surfaces that show versatility due to their varied functional groups. Used in fields like catalysis, biomedicine, and microbiology, CDs have demonstrated antibacterial properties. This study evaluates a CD variant, CD-$NH_2$, for its antifungal and antimicrobial potential, focusing on inhibiting biofilm growth rather than killing the microorganisms.

## *Materials and methods*

Carbon dots (CDs) are quasi-spherical carbon nanoparticles synthesized using top-down and bottom-up methods. The top-down method exfoliates larger carbon sources, while bottom-up methods retain functional groups like -COOH, -OH, or -NH2 on the CDs' surface [6]. This study used *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 strains. *In Vitro* antimicrobial activity against *S. aureus* was performed following CLSI guidelines [3], testing compound concentrations ranging from 500 µg/mL to 0.976 µg/mL. After incubation, Minimal Inhibitory Concentration (MIC) was established. *In vitro* antimicrobial activity against *C. albicans* and *S. aureus* biofilm formation was carried out as previously described [1,2,6]. After incubation, the biomass production was evaluated using the Crystal Violet (CV) assay. The optical density was measured at 590 nm. *In vivo* activity of CDs against *S. aureus and C.albicans* associated with chronic wound infections using *Galleria mellonella* were carried out as reported below [4,5]. Larvae were burt and treated with CD-$NH_2$, followed by the application of *C. albicans* and *S. aureus* cultures. Survival was monitored over 120 hours, with controls including larvae with and without wounds. Larvae death was indicated by color change and lack of movement. Antibiofilm activity was analyzed using the one-sample t-test and the Wilcoxon test. Significance values are indicated as follows: $P < 0.0001$ very highly significant (****), $0.0001 \le P < 0.001$ highly significant (***), $0.001 \le P < 0.01$ moderately significant (**), $0.01 < P < 0.05$ weakly significant (*). *G. mellonella* survival was displayed via Kaplan-Meier curves with a curves comparison test; p-value: $P < 0.0001$ very highly significant (****). Statistical data analysis was performed using GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA). Each experiment was performed at least three times, in triplicate, on separate dates.

## *Results*

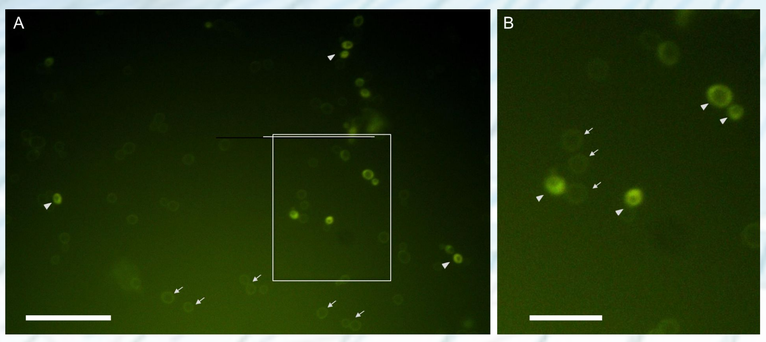



**1. CD-$NH_2$ inhibits *C. albicans* and *S. aureus* planktonic cells growth with GMMIC values of 397 µg/mL and 210 µg/mL respectively.**

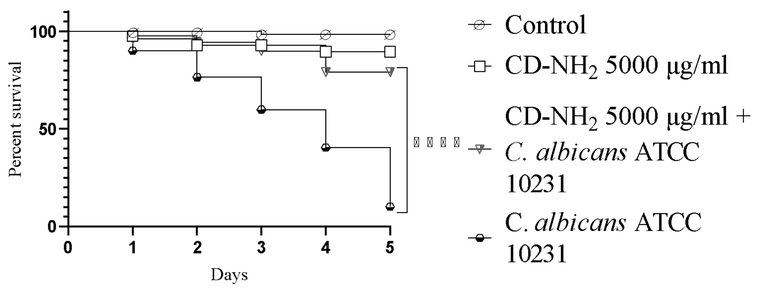
| | GMMIC (µg/mL) |
|---|---|
| | CD-$NH_2$ |
| *C. albicans* | 397 |
| *S. aureus* | 210 |

*Candida albicans* ATCC 10231 and *Staphylococcus aureus* ATCC 6538
GMMIC= geometric mean

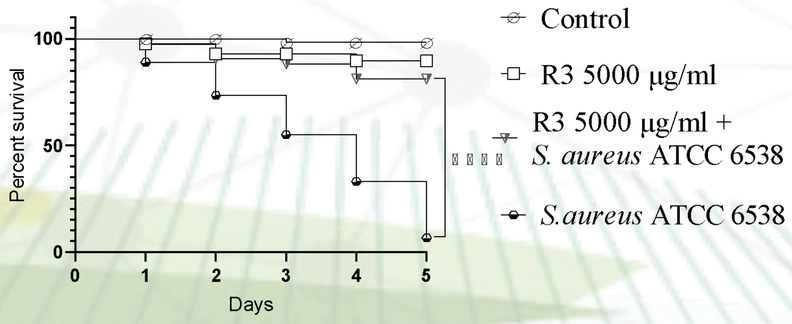
**2. Fluorescence microscopy images for internalization studies of CD-$NH_2$ in *C. albicans* planktonic cells. The white rectangle in (A) represents the area of the section in (B). All cells show a slight fluorescent signal in the cell walls (arrows) and some of them also show a more intense cytoplasmic signal (arrowheads). Bars 40 µm (A) and 5 µm (B).**



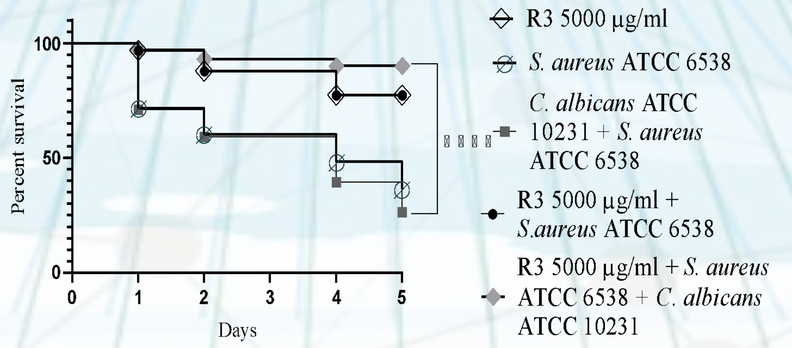

**3. In *vivo* experiments, *G. mellonella* larvae were infected with *C. albicans*. The burnt larvae were then treated with CD-$NH_2$, resulting in a significant reduction in larval mortality compared to the burnt larvae without CD-$NH_2$.**



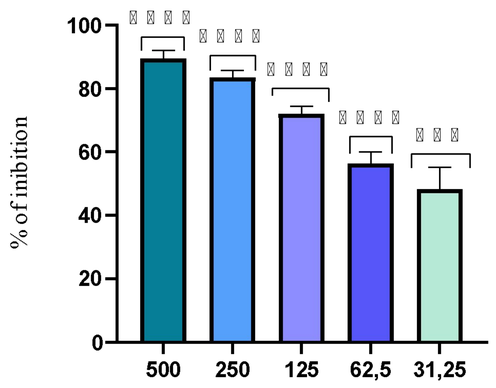

**4. In *vivo* experiments, *G. mellonella* larvae were infected with *S. aureus*. The burnt larvae were then treated with CD-$NH_2$, resulting in a significant reduction in larval mortality compared to the burnt larvae without CD-$NH_2$**




**5. In *vivo* experiments, *G. mellonella* larvae were infected with *C. albicans* and *S. aureus*. The burnt larvae were then treated with CD-$NH_2$, resulting in a significant reduction in larval mortality compared to the burnt larvae without CD-$NH_2$.**

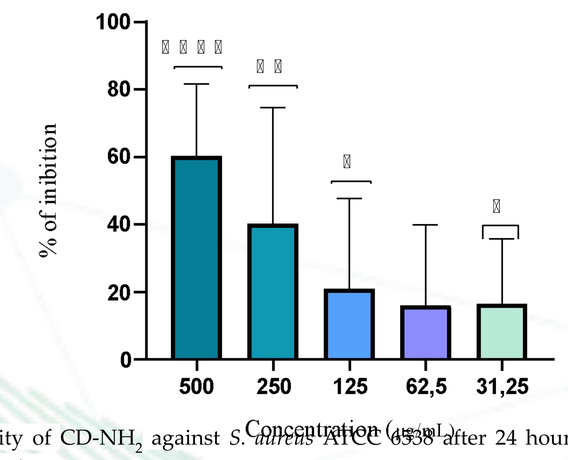



**6. CD-$NH_2$ at the concentration of 250 µg/mL, inhibits *C. albicans* biofilm formation more than 80%.**




Activity of CD-$NH_2$ against *C. albicans* ATCC 10231 after 48 hours of incubation.

**7. CD-$NH_2$ at the concentration of 500 µg/mL, inhibits *S. aureus* biofilm formation more than 50%.**




Activity of CD-$NH_2$ against *S. aureus* ATCC 6538 after 24 hours of incubation.

**8. CD-$NH_2$ at the concentration of 250 µg/mL, inhibits *S. aureus* and *C. albicans* mixed biofilm formation more than 80%.**

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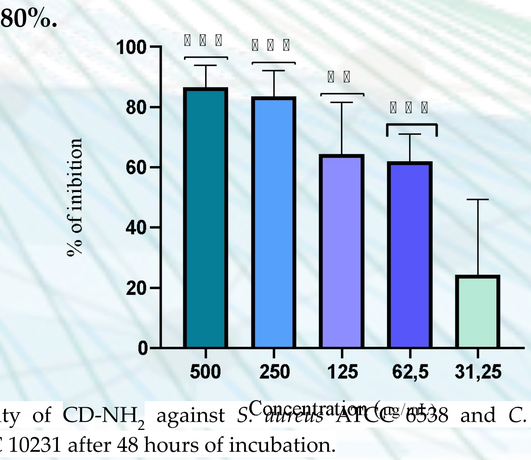


Activity of CD-$NH_2$ against *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 after 48 hours of incubation.

## *Conclusions*

In conclusion, this study has characterized CD-$NH_2$ and evaluated its potential as an effective antifungal and antibacterial nanomaterial against *C. albicans* and *S. aureus*. In fact, CD-$NH_2$ inhibits the mixed biofilm that is typical of chronic wounds, both *in vivo* and *in vitro*. The promising antimicrobial activity of CD-$NH_2$. is likely due to the presence of amine groups. Nonetheless, further investigations are warranted to fully explore the potential application of CD-$NH_2$. as an antimicrobial agent against *C. albicans* and *S. aureus* infections in chronic wounds.

## *References*

1. Heong, J.Z.A. et al. Priority effects dictate community structure and alter virulence of fungal-bacterial biofilms. *ISME J* 15, 2012–2027 (2021). 2.Xiying, W. et al. *Staphylococcus aureus* biofilm: Formulation, regulatory, and emerging natural products-derived therapeutics. Volume 7, 100175, ISSN 2590-2075 (2024) 3. CLSI. Methods for Diluition Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 11° ed. CLSI standard M07. Wayne, PA: Clinical and Laboratory Standards Institute; (2018). 4. Sheehan, G. et al. *C. albicans increases the pathogenicity of S. aureus during polymicrobial infection of G.mellonella larvae.* Microbiology (Reading) (2020). 5. Maslova, E. et al. Using the *Galleria mellonella* burn wound and infection model to identify and characterize potential wound probiotics. Microbiology (Reading) (2023). 6. Sturabotti, E. et al.Targeting the Antifungal Activity of Carbon Dots against *Candida albicans* Biofilm Formation by Tailoring Their Surface Functional Groups. Chemistry (2024). 7. CLSI. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition. CLSI document M27-A3. Wayne, PA: Clinical and Laboratory Standards Institute; (2008)

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