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Original Article

Microbiology Section

Evaluation of Huperzine A Concentration-dependent Antimicrobial and Antibiofilm Activity against *Streptococcus mutans* and *Candida glabrata*: An In-vitro Study

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ABSTRACT

Introduction: Antimicrobial Resistance (AMR) is a growing global public health crisis, with biofilm-forming microorganisms demonstrating significantly enhanced resistance to conventional antimicrobial agents. Biofilm-associated infections are notoriously difficult to eradicate and contribute to persistent, recurrent, and chronic infectious diseases. Repurposing drugs with established safety profiles represents a viable and cost-effective strategy for developing novel antimicrobial therapies. Huperzine A, a natural alkaloid derived from *Huperzia serrata*, is primarily known for its Acetylcholinesterase (AChE) inhibitory activity but has been increasingly investigated for repurposed antimicrobial applications.

Aim: To evaluate the concentration-dependent antimicrobial and antibiofilm activity of Huperzine A against selected bacterial and fungal pathogens, with particular focus on *Streptococcus mutans* and *Candida glabrata*.

Materials and Methods: This in-vitro experimental study was conducted at the Department of Pharmacology, Sri Ramachandra Medical College and Research Institute, Chennai, Tamil Nadu, India, from June 2025 to November 2025. Six standard microbial strains were included as the study sample: *Escherichia coli*, *Streptococcus mutans*, *Candida glabrata*, *Candida albicans*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Antimicrobial activity was assessed using the agar well diffusion method to determine the Zone Of Inhibition (ZOI) and the broth microdilution method to determine the Minimum Inhibitory Concentration (MIC). Biofilm inhibition was evaluated using Crystal Violet (CV) staining for biomass quantification and the MTT assay to measure metabolic activity of biofilm cells. Structural and viability changes in biofilms were further examined using Confocal Laser Scanning Microscopy (CLSM) and Colony-Forming Unit (CFU) enumeration. Statistical analysis was performed using One-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons test; a p-value of <0.05 was considered statistically significant.

Results: Huperzine A demonstrated measurable antimicrobial activity against all tested microorganisms. The highest inhibition was observed for *Candida glabrata* (17±1.2 mm) and *Streptococcus mutans* (13±0.8 mm) at 100 µg/mL. Corresponding MIC values were 145 µg/mL and 190 µg/mL, respectively. Biofilm assays revealed strong concentration-dependent inhibition, with up to 97% reduction in biofilm biomass for *S. mutans* and 96% for *C. glabrata* at 5× MIC. MTT assays demonstrated a marked decrease in metabolic activity, reducing cell viability to less than 10% compared to untreated controls.

Conclusion: Huperzine A demonstrated notable antimicrobial and antibiofilm activity against *Streptococcus mutans* and *Candida glabrata*. The compound effectively disrupts biofilm structure and reduces microbial viability in a concentration-dependent manner.

Keywords: Anti-infective agents, Biofilms, Drug repositioning, Microbial sensitivity tests, Plant extracts

INTRODUCTION

The AMR represents one of the most pressing global public health challenges of the 21st century. The World Health Organisation (WHO) has identified AMR as a critical threat to human health, food security, and sustainable development worldwide [1]. Biofilm formation is a major mechanism through which microorganisms resist antimicrobial agents. This is a major reason for resistance. Biofilms make cells more resistant to antibiotics than planktonic cells, which makes illnesses that happen in biofilms very hard to cure [2,3]. These infections contribute to chronic and recurrent disease states, including oral infections, systemic fungaemia and healthcare-associated infections. Recent projections estimate that AMR may be responsible for up to 10 million deaths annually and over 130 million hospitalisations worldwide, underscoring its immense global health burden. Resistance develops through multiple mechanisms, including target modification, enzymatic drug inactivation, reduced permeability, active efflux, and biofilm formation, all of which contribute to multidrug-resistant phenotypes [3].

Huperzine A is a natural sesquiterpene alkaloid obtained from the club moss *Huperzia serrata*, renowned for its strong, reversible suppression of AChE and its recognised therapeutic use in the therapy of Alzheimer's disease [4]. Its small molecular weight and lipophilic structure enable efficient penetration of the blood-brain barrier, which also raises its relevance for infections involving the central nervous system [5]. The investigation of Huperzine A for antibacterial action is based on growing information indicating that AChE and cholinergic signalling pathways are involved in microbial physiology. Researchers have found cholinergic receptors and acetylcholine-like compounds in bacterial membranes. Inhibiting AChE might damage microbial membranes, quorum sensing, stress responses, and enzyme function, all of which are important for biofilm development and microbial survival [6,7]. Microorganisms exhibit remarkable genetic adaptability through mutation, horizontal gene transfer, and epigenetic regulation, enabling rapid evolution of resistance under antimicrobial pressure. Such adaptability is driven by continuous selective pressure, allowing pathogens to rapidly acquire and disseminate resistance traits within microbial communities [3].

Several structurally different AChE inhibitors have exhibited antibacterial action that is independent of their enzyme-inhibiting capabilities, therefore supporting this idea. Benzofuran-triazole hybrids and benzodiazine derivatives have significant antibacterial activity against both gram-positive and gram-negative organisms, indicating that the antimicrobial efficacy may represent a class effect associated with drugs possessing AChE inhibitory properties [8]. Although these compounds exhibit structural differences from Huperzine A, they jointly support the idea that AChE inhibitors deserve comprehensive exploration for antimicrobial repurposing.

In clinical settings, the burden of AMR is further compounded by factors such as inappropriate antibiotic use, environmental exposure to antimicrobial agents, and inadequate infection control practices, all of which facilitate the emergence and spread of resistant pathogens [3].

Although there is more and more proof that AChE inhibitors may kill bacteria, Huperzine A has not been thoroughly tested in the lab for its ability to kill bacteria and biofilms that are clinically important. *Streptococcus mutans* and *Candida glabrata* were chosen for in-depth biofilm research due to their exhibiting the greatest sensitivity to Huperzine A in initial screening experiments, including six microbial strains. These organisms are of particular clinical relevance, as both are known to form resilient biofilms and are associated with persistent infections that are difficult to eradicate using conventional therapies [3].

The primary objective of this study was to evaluate the concentration-dependent antimicrobial and antibiofilm activity of Huperzine A against *Streptococcus mutans* and *Candida glabrata*, assessed through ZOI, MIC, biofilm biomass quantification, metabolic activity, viable cell enumeration, and confocal microscopy. The secondary objectives were to determine the broad-spectrum antimicrobial activity of Huperzine A, against six standard microbial strains and to assess the structural and viability changes in established biofilms using complementary assay methods.

MATERIALS AND METHODS

This in-vitro experimental study was performed at the Department of Pharmacology, Sri Ramachandra Medical College and Research Institute (Deemed to be University), Porur, Chennai, Tamil Nadu, India, spanning six months from June 2025 to November 2025. The research assessed the antibacterial and antibiofilm efficacy of Huperzine A against six common microbial strains employing recognised microbiological techniques.

Preparation of Huperzine A Solution: Two different stock solutions of Huperzine A in dimethyl sulfoxide (DMSO): one with 1 mg/mL for ZOI tests and one with 2 mg/mL for MIC tests. Working dilutions were prepared fresh from the respective stocks to achieve the required test concentrations. The compound was obtained from Life Care Phytolabs, Chennai, India, and stored at 4°C. A DMSO-only solvent control was included in all assays to distinguish any solvent effects from the activity of Huperzine A. The final DMSO concentration in test wells did not exceed 0.5% v/v, a concentration previously shown to be non inhibitory to microbial growth.

Microorganisms Used: The following standard reference microbial strains were used: *Escherichia coli* (ATCC 25922), *Streptococcus mutans* (ATCC 25175), *Candida glabrata* (ATCC 90030), *Candida albicans* (ATCC 10231), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 25923). All strains were subcultured and maintained on nutrient agar slants. Bacterial strains were incubated at 37°C for 24 hours, while *Candida* species were incubated at 35°C for 48 hours prior to use, in accordance with standard microbiological practice.

Antimicrobial Activity

Zone of Inhibition (ZOI): The agar well diffusion method was employed to assess the antimicrobial activity of Huperzine A against all six microbial strains, as it is well suited for evaluating novel compounds and natural products where standard disc diffusion may not reflect compound behaviour adequately [9]. Mueller-Hinton Agar (MHA; HiMedia, India) was prepared in distilled water (pH 7.0) and sterilised by autoclaving at 121°C for 15 minutes, then poured into sterile Petri plates and allowed to solidify. A standardised microbial suspension (10^6 CFU/mL) was spread uniformly over the MHA surface using a sterile cotton swab. Three wells (8 mm diameter) were punched into each plate: two wells were filled with 50 µL and 100 µL of Huperzine A solution (from 1 mg/mL stock), and one well was filled with the respective positive control antibiotic. Plates were left at room temperature for four hours to allow diffusion of the compound into the agar, then incubated at 37°C for 24 hours. Positive controls used in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI M02-A12, 2015) were: ciprofloxacin (5 µg; HiMedia) for gram-negative bacteria, ampicillin (10 µg; HiMedia) for gram-positive bacteria, and fluconazole (25 µg; HiMedia) for *Candida* species. DMSO-loaded wells served as negative controls. Following a 24-h incubation period at 37°C (35°C for *Candida* spp.), the diameter of inhibition zones was measured in millimetres.

Minimum Inhibitory Concentration (MIC): The MIC was determined using the broth microdilution technique in accordance with CLSI guidelines (CLSI M07-A10, 2015). Sterile 96-well microplates were used throughout. Each well received 180 µL Mueller-Hinton Broth (MHB; HiMedia, India) and 20 µL of Huperzine A stock solution (2 mg/mL) was added to the first well. Serial two-fold dilutions were then performed by transferring 20 µL from each well to the next using a multichannel pipette; 20 µL was discarded from the last well to maintain a uniform final volume of 180 µL, yielding a concentration range from 0.02 µg/mL to 200 µg/mL. Microbial suspensions were adjusted to a 0.5 McFarland standard (approximately 1×10^5 CFU/mL) and further diluted to achieve a final inoculum of 5×10^5 CFU/mL; 20 µL of this suspension was added to each test well. Positive control wells contained broth with inoculum but no compound; negative control wells contained broth only (no inoculum, no compound) as a sterility check. Plates were covered with a sterile lid and incubated for 24 hours at 37°C (35°C for *Candida* spp.). After incubation, wells were examined visually for turbidity. The MIC was defined as the lowest concentration of Huperzine A at which no visible turbidity (i.e., no visible microbial growth) was observed. All assays were performed in triplicate.

Biofilm Inhibition Assay

Biofilm formation: Biofilms of *S. mutans* and *C. glabrata* were formed in 96-well microtiter plates following protocols. For *S. mutans*, Brain Heart Infusion (BHI) broth supplemented with 1% sucrose was used to promote extracellular polysaccharide matrix development, as recommended by standard *S. mutans* biofilm protocols [10]. For *C. glabrata*, RPMI-1640 medium was used for biofilm induction, as is standard for *Candida* biofilm models [11]. Each strain was adjusted to 10^6 CFU/mL, and 10 µL of inoculum was added to 290 µL of the respective medium in each well. Plates were incubated at 37°C for both organisms, which is the established temperature for *S. mutans* and falls within the accepted range for *C. glabrata* biofilm formation. A 7-day incubation period was chosen to allow development of a mature, robust biofilm suitable for studying the effects of antibiofilm agents on established biofilm architecture, as opposed to inhibition of initial attachment.

Biofilm washing and treatment: After incubation, free-floating cells were discarded, and each well was gently rinsed twice with 200 µL of Phosphate-Buffered Saline (PBS). Preformed biofilms were treated with Huperzine A at MIC, 3× MIC, and 5× MIC. A positive antibiofilm control (chlorhexidine at 0.2% for *S. mutans* and fluconazole at 64 µg/mL for *C. glabrata*) and an untreated control (PBS only) were included in all biofilm assays.

Crystal Violet (CV) assay: Biofilms had been fixed with 99% ethanol for 10 minutes, stained with 0.1% CV for 20 minutes, and then rinsed. Retained dye was dissolved in ethanol, and absorbance was recorded at 590nm, utilising a microplate reader [12].

MTT assay: MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to estimate biofilm metabolic activity. Viable cells use mitochondrial enzymes to convert MTT into purple formazan crystals. Cell viability was quantified by measuring absorbance at 570 nm [13].

Confocal Laser Scanning Microscopy (CLSM): Biofilm structure and viability were assessed using a Leica Microsystems DMI8 confocal microscope. Acridine orange and ethidium bromide were used as fluorescent stains. Live cells fluoresced green, while dead or membrane-damaged cells appeared red. Imaging was performed at 10× magnification for overview assessment of biofilm distribution, and at 40× magnification for detailed evaluation of biofilm architecture and cell viability. Higher magnifications (40×–63×) provide more meaningful assessment of biofilm structure; the 10× images presented here are intended to provide spatial context across the full well area.

STATISTICAL ANALYSIS

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism Version 11.0. One-way ANOVA followed by Tukey's multiple comparisons test was used. A p-value <0.05 was considered statistically significant.

RESULTS

Huperzine A exhibited measurable antimicrobial activity against both gram-positive and gram-negative bacteria as well as fungi. The maximum ZOI was observed for *Candida glabrata* (17±1.2 mm) and *Streptococcus mutans* (13±0.8 mm) at 100 µg/mL concentration. Moderate inhibition zones were observed for *Staphylococcus aureus* (9±0.6 mm at 50 µg/mL; 12±0.7 mm at 100 µg/mL; MIC 210 µg/mL), *Candida albicans (*12±0.8 mm at 50 µg/mL, 15±0.9 mm at 100 µg/mL); *E. coli* (8±0.5 mm at 50 µg/mL; 11±0.9 mm at 100 µg/mL; MIC 225 µg/mL), and *Pseudomonas aeruginosa* (7±0.4 mm at 50 µg/mL; 10±0.6 mm at 100 µg/mL; MIC 240 µg/mL). Corresponding MIC values were 145 µg/mL for *C. glabrata* and 190 µg/mL for *S. mutans* [Table/Fig-1].

These results indicate that Huperzine A displays broad-spectrum antimicrobial potential, with higher efficacy against fungal strains compared to bacteria. The relatively higher MIC values observed for gram-negative organisms, particularly *Pseudomonas aeruginosa* (240 µg/mL), are consistent with the known outer membrane permeability barrier in gram-negative bacteria, which restricts entry of lipophilic compounds.

Biofilm Inhibition {Crystal Violet (CV) and MTT Assays}

Biofilm quantification using the CV assay revealed statistically significant, concentration-dependent inhibition across all tested concentrations. One-way ANOVA demonstrated highly significant differences among concentration groups for both organisms: F (7, 16)=9797, p-value <0.0001, R^2=0.9998 for *S. mutans*, and F (7, 16)=16885, p-value <0.0001, R^2=0.9999 for *C. glabrata* [Table/Fig-2]. [Table/Fig-3] visually demonstrates the progressive reduction in biofilm biomass (OD_{590}) with increasing concentrations of Huperzine A in both *S. mutans* and *C. glabrata*. The decline was concentration-dependent across the entire 0.5×-5× MIC range, with all pairwise comparisons remaining statistically significant (Tukey's post-hoc, p-value <0.0001).

The MTT assay confirmed a statistically significant, concentration-dependent reduction in biofilm metabolic activity for both organisms [Table/Fig-4]. One-way ANOVA revealed: F (7, 16)=6859, p-value <0.0001, R^2=0.9997 for *S. mutans*, and F (7, 16)=6770, p-value <0.0001, R^2=0.9997 for *C. glabrata*. Tukey's post-hoc test confirmed significant differences between all consecutive concentration pairs (all p-value <0.0001). At 5× MIC, metabolic activity was reduced to 8.0% in *S. mutans* and 8.8% in *C. glabrata* relative to untreated controls.

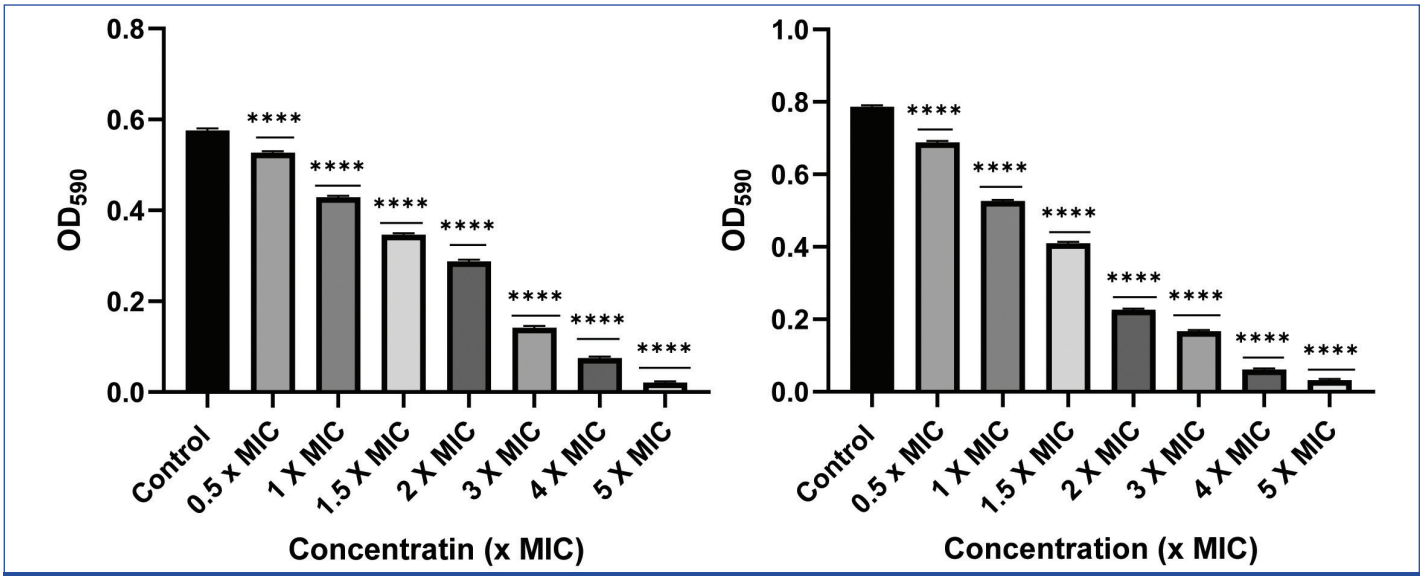
Tukey's post-hoc test confirmed that every concentration group was significantly different from every other (all p-value <0.0001), indicating a stepwise, concentration-dependent reduction in biofilm biomass. At 5× MIC, biofilm biomass was reduced by 97% for *S. mutans* and 96% for *C. glabrata* relative to untreated controls. All pairwise comparisons demonstrated statistically significant differences [Table/Fig-5].

Microorganism	Type	Positive Control Antibiotic (ZOI) (mm)	Zone of Inhibition (ZOI) (mm±SD) at 50 µg/mL	Zone of Inhibition (ZOI) (mm±SD) at 100 µg/mL	MIC (µg/mL)
Streptococcus mutans	Gram-positive bacterium	Ampicillin (10 µg/mL)=15 mm	10±0.7	13±0.8	190
Staphylococcus aureus	Gram-positive bacterium	Ampicillin 10 µg/mL=28 mm	09±0.6	12±0.7	210
Escherichia coli	Gram-negative bacterium	Ciprofloxacin (5 µg/mL)=21 mm	08±0.5	11±0.9	225
Pseudomonas aeruginosa	Gram-negative bacterium	Ciprofloxacin (5 µg/mL)=23 mm	07±0.4	10±0.6	240
Candida albicans	Yeast	Fluconazole (25 µg/mL)=22 mm	12±0.8	15±0.9	165
Candida glabrata	Yeast	Fluconazole (25 µg/mL)=24 mm	14±1.0	17±1.2	145

[Table/Fig-1]: Antimicrobial activity of Huperzine A against selected bacterial and fungal strains.
Values represent mean ± standard deviation of three independent experiments.
ZOI: Zone of inhibition; MIC: Minimum inhibitory concentration.
Based on these results, further assays were conducted to determine Huperzine A's effects on biofilm formation.

Source of variation	SS	DF	MS	F (DFn, DFd)	p-value	R^2
Streptococcus mutans						
Treatment (between groups)	0.9001	7	0.1286	F (7, 16)=9797	<0.0001	0.9998
Residual (within groups)	0.0002100	16	1.313×10^{-5}	—	—	
Total	0.9003	23	—	—	—	
Candida glabrata						
Treatment (between groups)	1.714	7	0.2448	F (7, 16)=16885	<0.0001	0.9999
Residual (within groups)	0.0002320	16	1.450×10^{-5}	—	—	
Total	1.714	23	—	—	—	

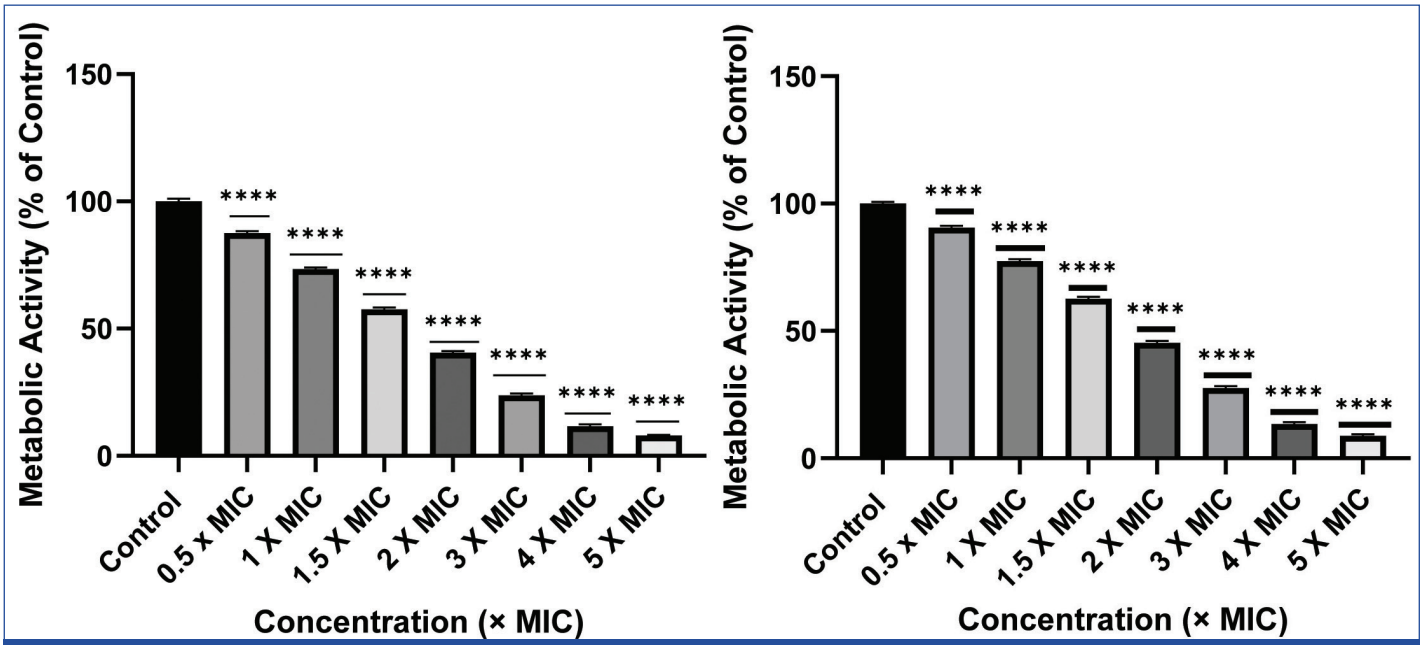
[Table/Fig-2]: One-way ANOVA summary- Crystal Violet (CV) assay (OD_{590}).
Abbreviations: *SS: Sum of Squares; DF: Degrees of Freedom; MS: Mean Square. n=3 replicates per group; 8 concentration groups (Control, 0.5×, 1×, 1.5×, 2×, 3×, 4×, 5× MIC). All pairwise comparisons by Tukey's post-hoc test: p <0.0001.*



[Table/Fig-3a, b]: Concentration-dependent inhibition of biofilm biomass by Huperzine A as measured by the CV assay (OD_{590}): a) *Streptococcus mutans;* and b) *Candida glabrata*.
Data represent mean ± SD of three independent experiments. ****p < 0.0001 (Tukey's post-hoc test). All pairwise comparisons were statistically significant.

Source of variation	SS	DF	MS	F (DFn, DFd)	p-value	R^2
Streptococcus mutans						
Treatment (between groups)	25568	7	3653	F (7, 16)=6859	<0.0001	0.9997
Residual (within groups)	8.520	16	0.5325	–	–	
Total	25577	23	–	–	–	
Candida glabrata						
Treatment (between groups)	25611	7	3659	F (7, 16)=6770	<0.0001	0.9997
Residual (within groups)	8.647	16	0.5404	–	–	
Total	25620	23	–	–	–	

[Table/Fig-4]: One-way ANOVA summary- MTT assay (% metabolic activity relative to control).
SS: Sum of Squares; DF: Degrees of Freedom; MS: Mean Square. n=3 replicates per group; 8 concentration groups (Control, 0.5×, 1×, 1.5×, 2×, 3×, 4×, 5× MIC). All pairwise comparisons by Tukey's post-hoc test: p <0.0001.



[Table/Fig-5a, b]: Concentration-dependent reduction in biofilm metabolic activity by Huperzine A as measured by the MTT assay (% of untreated control): a) *Streptococcus mutans;* and b) *Candida glabrata*.
Data represent mean ± SD of three independent experiments. ****p <0.0001 (Tukey's post-hoc test).

The concentration-dependent pattern was further confirmed by viable cell enumeration and CLSM imaging.

Viability Assessment (Colony Forming Unit Counting and Confocal Laser Scanning Microscopy (CLSM) Imaging)

CFU enumeration confirmed statistically significant, concentration-dependent reductions in viable cell counts for both organisms. It should be noted that the Control and 0.5× MIC groups were excluded from ANOVA as counts were Too Numerous To Count (TNTC); analysis was therefore performed across the 1× to 5× MIC range [Table/Fig-6].

One-way ANOVA revealed: F (5, 12)=6798, p-value <0.0001, R^2=0.9996 for *S. mutans*, and F (5, 12)=2650, p-value <0.0001, R^2=0.9991 for *C. glabrata* [Table/Fig-7]. For *S. mutans*, Tukey's

Huperzine A Concentration (× MIC)	Control	0.5×	1×	1.5×	2×	3×	4×	5×
CFU Count *(S. mutans)*	TNTC	TNTC	180	121	15	4	0	0
CFU Count *(C. glabrata)*	TNTC	TNTC	113	77	46	22	8	0

[Table/Fig-6]: Effect of Huperzine A on viable cell counts of *S. mutans* and *C. glabrata*.
Abbreviations: *CFU: Colony forming units; MIC: Minimum inhibitory concentration; TNTC: Too numerous to count. Control=untreated biofilm (no Huperzine A)*

Source of variation	SS	DF	MS	F (DFn, DFd)	p-value	R^2
Streptococcus mutans						
Treatment (between groups)	90646	5	18129	F (5, 12)=6798	<0.0001	0.9996
Residual (within groups)	32.00	12	2.667	—	—	
Total	90678	17	—	—	—	
Candida glabrata						
Treatment (between groups)	28708	5	5742	F (5, 12)=2650	<0.0001	0.9991
Residual (within groups)	26.00	12	2.167	—	—	
Total	28734	17	—	—	—	

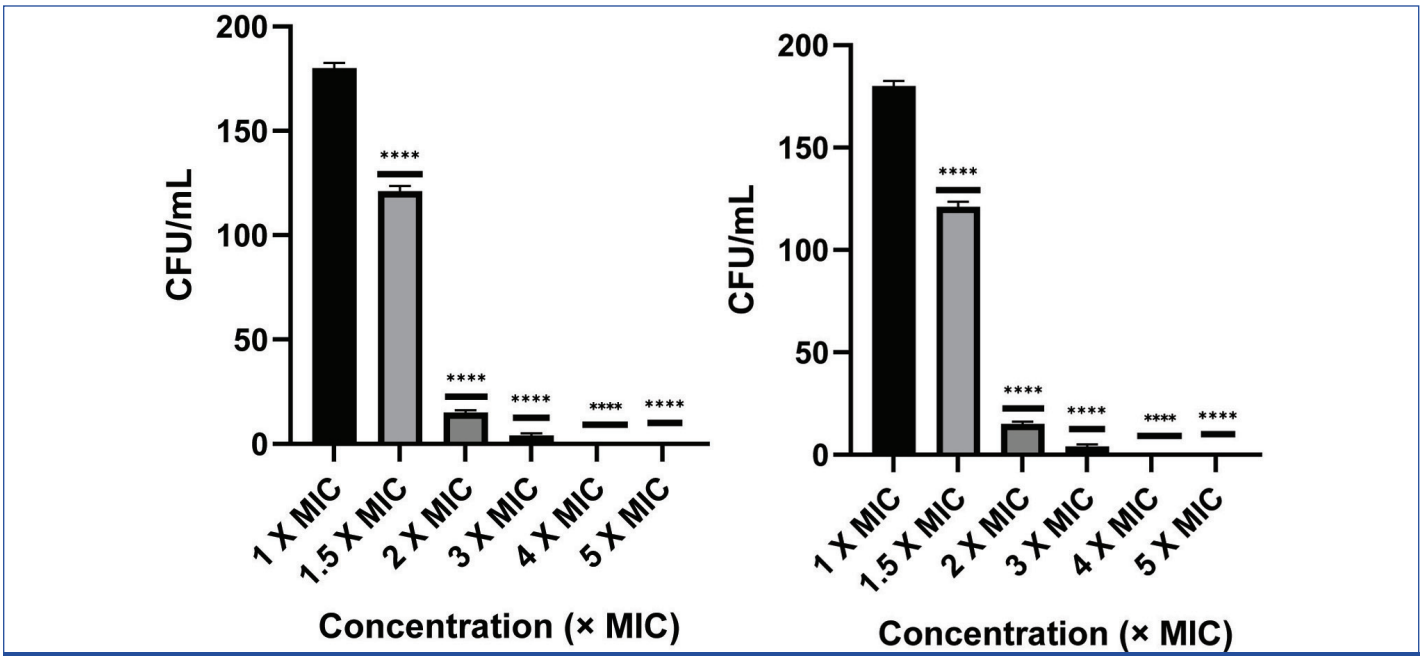
[Table/Fig-7]: One-way ANOVA summary- CFU enumeration.
Control and 0.5× MIC excluded from ANOVA (TNTC: Too Numerous To Count). Analysis across 1×–5× MIC (6 groups). SS: Sum of Squares; DF: Degrees of freedom; MS: Mean square. n=3 replicates per group.

post-hoc showed significant differences between all groups up to 3× MIC (all p-value <0.0001); comparisons between 3×, 4×, and 5× MIC were not significant (p-value=0.0906 and p-value > 0.9999 respectively), consistent with complete eradication achieved at 3× MIC. For *C. glabrata*, all comparisons were significant (p-value ≤ 0.0003), reflecting progressive reduction in viable counts through to complete eradication at 5× MIC [Table/Fig-8].

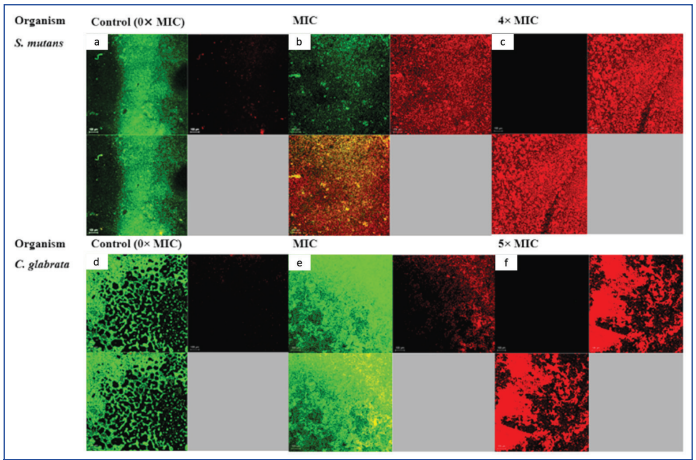
CLSM confirmed these observations, showing reduced biofilm thickness and extensive red fluorescence indicating cell death at higher concentrations [Table/Fig-9].

DISCUSSION

The present study demonstrated that Huperzine A possesses in-vitro antimicrobial and antibiofilm activity against *Streptococcus mutans* and *Candida glabrata* in a concentration-dependent manner. MIC values of 190 µg/mL for *S. mutans* and 145 µg/mL for *C. glabrata* indicate moderate potency. Biofilm biomass (CV assay) was reduced by up to 97% in *S. mutans* and 96% in *C. glabrata* at 5× MIC, while MTT metabolic activity fell below 10% of untreated controls. CFU enumeration confirmed near-complete eradication at 3× MIC for *S. mutans* and at 5× MIC for *C. glabrata*. CLSM imaging corroborated these findings, showing progressive loss of green (live) fluorescence and increasing red (dead) fluorescence at higher concentrations. Notably, at 2× MIC, *S. mutans* showed a sharper CFU reduction (15 CFU/mL) compared with *C. glabrata* (46 CFU/mL), whereas CV and MTT inhibition curves were broadly similar between the two organisms. This discrepancy may reflect the greater susceptibility of *S. mutans* to cell killing at lower concentrations, while *C. glabrata* retains metabolically inactive but structurally intact biofilm biomass that continues to stain with CV. Furthermore, *C. albicans* also demonstrated sensitivity (ZOI 15±0.9 mm; MIC 165 µg/mL). These findings are consistent with the hypothesis that AChE inhibitors may exert antimicrobial effects through disruption of cholinergic signalling pathways in microbial cells, interference with quorum sensing, or direct membrane disruption. Cholinergic receptors and acetylcholine-like compounds have been identified in bacterial membranes, and AChE inhibition may impair membrane integrity, stress responses, and enzyme activity critical for biofilm formation and microbial survival [6,7]. Prior studies on structurally related AChE-inhibiting compounds support this mechanism. Saeed S et al., reported that benzofuran-triazole hybrids with AChE inhibitory properties demonstrated significant antibacterial activity against both gram-positive and gram-negative organisms (MIC range 1.25-16 µg/mL), and Raza A et al., similarly showed that indole-derived



[Table/Fig-8a, b]: Concentration-dependent reduction in viable cell counts (CFU/mL) following Huperzine A treatment of preformed biofilms: a) *Streptococcus mutans*: complete eradication achieved at 3× MIC; b) *Candida glabrata*: complete eradication at 5× MIC. Data represent mean ± SD of three independent experiments. Control and 0.5× MIC groups excluded from ANOVA (TNTC: Too Numerous To Count). ****p<0.0001 (Tukey's post-hoc test).



[Table/Fig-9]: Confocal Laser Scanning Microscopy (CLSM) images showing the concentration-dependent antibiofilm effect of Huperzine A against *Streptococcus mutans* and *Candida glabrata.* Overview images were acquired at 10× magnification: (a-c) *S. mutans* biofilms at Control (0× MIC), MIC, and 4× MIC concentrations; (d-f) *C. glabrata* biofilms at Control (0× MIC), MIC, and 5× MIC concentrations. Green fluorescence=live cells; red fluorescence=dead or membrane-compromised cells.

benzodiazine compounds with AChE inhibitory activity (IC_{50} 6.11 µM) exhibited notable antibacterial activity against *Staphylococcus aureus* (ZOI 17–21 mm) [7,8]. Collectively, these findings establish a direct link between AChE inhibitory scaffolds and antimicrobial activity, a pattern that is further supported by the present study, where Huperzine A, a structurally distinct but pharmacologically related AChE inhibitor demonstrated concentration-dependent antimicrobial and antibiofilm activity against *S. mutans* and *C. glabrata*.

Both organisms selected for detailed biofilm assays are of established clinical relevance. Serotype k of *S. mutans* has been linked to cerebrovascular injury via collagen-binding proteins that damage vascular endothelium, and viridans streptococci have been associated with bloodstream infections and embolic CNS events [14]. *Candida glabrata* is an emerging fungal pathogen increasingly implicated in invasive candidiasis and CNS infections in both immunocompetent and immunocompromised individuals [15]. Huperzine A demonstrated ability to penetrate the blood-brain barrier raises the theoretical possibility that adequate CNS concentrations could be achieved in-vivo [16]. However, it must be emphasised that this remains speculative: the present study was purely in-vitro, pharmacokinetic data at antimicrobial concentrations are unavailable, and no toxicity assessment was conducted. Any conclusions regarding CNS therapeutic utility must await in-vivo validation.

The exact mechanism by which Huperzine A exerts its antimicrobial effect warrants further investigation. Future studies should incorporate mechanistic assays such as membrane permeabilisation and quorum sensing inhibition assays, as well as cytotoxicity evaluation on mammalian cell lines to determine the selectivity index. Combination studies with conventional antimicrobials may help establish whether Huperzine A demonstrates synergistic activity.

Limitation(s)

The present study had several limitations. Although One-way ANOVA with Tukey's post-hoc testing was performed and confirmed statistically significant concentration-dependent effects across all assays, the study remains exploratory and in-vitro in nature. Cytotoxicity testing on mammalian cell lines was not performed, leaving the selectivity index and therapeutic window of Huperzine A undefined. The selection of *S. mutans* and *C. glabrata* for biofilm assays was based on post-hoc sensitivity findings, introducing potential selection bias. Although DMSO-only controls were included, a contribution of the solvent to the observed activity at higher concentrations cannot be entirely excluded. The use of 10× magnification in CLSM is insufficient for detailed biofilm architectural assessment, and the entirely in-vitro design means that pharmacokinetic behaviour and in-vivo efficacy remain to be established. These limitations underscore the exploratory nature of this work and should be addressed in future studies.

CONCLUSION(S)

This study demonstrates, for the first time, that Huperzine A exhibits in-vitro antimicrobial and antibiofilm activity against *Streptococcus mutans* and *Candida glabrata* in a concentration-dependent manner, with up to 97% biofilm biomass reduction and near-complete elimination of metabolic activity at 5× MIC. These findings support the hypothesis that AChE inhibitors can be repurposed as antimicrobial agents, and Huperzine A, with its established safety profile and blood-brain barrier permeability represents an interesting candidate for further development. The compound showed broad-spectrum activity across six microbial strains, with the highest sensitivity in fungal strains. The ability to disrupt mature, established biofilms is particularly relevant given the clinical challenge posed by biofilm-associated infections. Taken together, these in-vitro results provide a foundation for future investigations into the pharmacological, toxicological, and in-vivo antimicrobial properties of Huperzine A. Confirmatory studies with rigorous statistical analysis, mammalian cytotoxicity testing, and in-vivo models are required before clinical relevance can be established.

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