

## Original Research

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## Evaluation Of the Antimicrobial Efficacy of *Nymphaea nouchali* var. *caerulea* (Blue Water Lily) Extract Against *Streptococcus Mutans* (MTCC 10449): An In-Vitro Study

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### ABSTRACT:

**Introduction:** Dental plaque initially begins pellicle formation and with *Streptococcus mutans* colonization and biofilm maturation, driving gingivitis and periodontitis. Plant derived agents targeting plaque are promising; *Nymphaea nouchali* is phenolic and flavonoid rich with documented antimicrobial, antioxidant, and antibiofilm effects, but its activity against oral primary colonizers remains underexplored.

**Aim:** To evaluate the antimicrobial efficacy of *Nymphaea nouchali* var. *caerulea* flower extract against *S. mutans* using agar well diffusion technique, in comparison with 0.2% chlorhexidine.

**Materials and Method:** Dried *Nymphaea nouchali* var *caerulea* (blue water lily) petals are macerated with 70% ethanol, kept in orbital shaker at 30°C for 24 h and filtered and reduced on a magnetic stirrer to obtain crude extract. *S.mutans* inoculum (0.5 McFarland) is lawn cultured on blood agar. Wells received the extract, saline (negative control) and 0.2% chlorhexidine (positive control). Plates are incubated anaerobically at 37°C for 24 h. Zones of inhibition are measured and analyzed as mean  $\pm$  SD in triplicates.

**Results:** One-way ANOVA revealed highly significant differences in antimicrobial activity across the groups ( $p < 0.001$ ). The *Nymphaea nouchali* var. *caerulea* petal extract exhibited a robust zone of inhibition (ZOI) of  $4.200 \pm 0.237$  mm, which post hoc Bonferroni analysis confirmed was significantly superior to the saline negative control (0.000 mm,  $p < 0.001$ ). While the Chlorhexidine positive control yielded the largest overall ZOI ( $6.167 \pm 0.153$  mm), the extract demonstrated definitive and reproducible antimicrobial potential.

**Conclusion:** The hydroalcoholic extract of *Nymphaea nouchali* var. *caerulea* exhibits a statistically highly significant in vitro antimicrobial effect against *Streptococcus mutans*. These findings establish its definitive potential as a natural phytotherapeutic candidate in inhibitory plaque formation, justifying further quantitative validation and formulation development.

## 1. INTRODUCTION

Oral biofilms are structured microbial communities that play a central role in the pathogenesis of dental caries and periodontal disease.<sup>(1)</sup> Among the organisms involved in cariogenic biofilm development, *Streptococcus mutans* is widely recognized for its strong adherence, acidogenicity, aciduricity, and extracellular polysaccharide production that stabilize the matrix.<sup>(2)</sup> Although classically associated with supragingival plaque and dental caries, *S. mutans* has also been identified in saliva and subgingival plaque of patients with gingivitis and chronic periodontitis, supporting its relevance in broader oral microbial ecology.<sup>(3)</sup>

Natural products remain an important source of bioactive compounds with potential antimicrobial applications.<sup>(3,4)</sup> *Nymphaea nouchali* var. *caerulea* (blue water lily) is an aquatic medicinal plant rich in secondary metabolites such as flavonoids, phenolics, tannins, alkaloids, and related phytoconstituents, with reported antibacterial, antioxidant, anti-inflammatory, antinociceptive, and other pharmacological activities. Studies have reported antibacterial, antioxidant, anti-inflammatory, antinociceptive, and related activities associated with its flavonoids, phenolics, tannins, alkaloids, and other phytoconstituents.<sup>(4-9)</sup> Experimental work on flower and seed extracts of *Nymphaea nouchali* has demonstrated antibacterial activity against several Gram-positive and Gram-negative bacteria microorganisms, suggesting that the plant may serve as a source of therapeutically useful antimicrobial compounds.<sup>(4-9)</sup>

However, the available literature has focused predominantly on non-oral pathogens or general pharmacological properties, while targeted evaluation against major oral pathogens remains sparse.<sup>(5-8)</sup> The current study identifies a gap in evidence concerning the antimicrobial efficacy of *Nymphaea* flower or leaf extracts against *S. mutans*. Therefore, the present in-vitro study was planned to evaluate the antimicrobial effect of *Nymphaea nouchali* var. *caerulea* petal extract against *S. mutans* (MTCC 10449) and compare it with 0.2% chlorhexidine using agar well diffusion analysis.

## 2. MATERIALS AND METHODS

### 2.1 Study Material

Fresh flower petals of *Nymphaea nouchali* var. *caerulea* extract

### 2.2 Extraction Procedure

Fresh flower petals of *Nymphaea nouchali* var. *caerulea* were collected and shade-dried. The partially dried petals were further dried in a hot air oven at 40°C for 1.5 hours. The following approaches were used for drying medicinal flowers to preserve the phytochemicals.<sup>(1,2)</sup> The dried petals were then crushed into a fine powder using a sterile mortar and pestle under laminar air flow conditions. 5g of the powdered sample were mixed with 100 mL of 70% ethanol in sterile conical flask, similar to previously described ethanolic extraction protocols for *Nymphaea* species.<sup>(3,4)</sup> The mixture was placed on an orbital shaker at 30°C and 100–150 rpm for 24 hours to facilitate extraction.<sup>(3-5)</sup> After extraction, the solution was filtered using Whatman No. 1 filter paper, and approximately 50 mL of filtrate was obtained. The filtrate was stored in a sterile vial at 4 °C for short term preservation.

Subsequently, the 50 mL extract was concentrated by heating in a beaker on a magnetic stirrer at 40–44°C for 1.5 hours until the volume was reduced to 15mL. A low temperature approach was chosen to remove excess ethanol while minimizing degradation of heat-sensitive constituents.<sup>(1,2,7)</sup> The resulting crude plant extract was finally collected after cooling to room temperature (Fig:1) and stored in sterile Eppendorf tubes at 4 °C for further microbial analysis.<sup>(3-6)</sup>

### Microorganism and inoculum preparation

The test microorganism used in the study was *Streptococcus mutans* (MTCC 10449) (Fig:2), a standard reference strain commonly used in in-vitro antimicrobial testing.<sup>(8,9)</sup> The organism was inoculated on blood agar and incubated anaerobically at 37°C for 18–24 hours. A single well-isolated colony was then transferred into 5 mL brain heart infusion (BHI) broth and incubated at 37 °C until visible turbidity developed. The bacterial suspension was then adjusted to 0.5 McFarland standard (approx.  $1.5 \times 10^8$  CFU/mL).<sup>(10,11)</sup>

## Agar well diffusion assay

A sterile cotton swab was dipped into the standardized bacterial suspension (0.5 McFarland), excess fluid was removed by pressing the swab against the test tube, and the inoculum was streaked over the agar plate surface in three directions at 60° angles to obtain a uniform lawn culture. The inoculated plates were allowed to dry for 10 minutes at room temperature. <sup>(10)</sup>

Using a sterile cork borer of 8mm diameter, four equidistant wells were made in each agar plate, and the agar plugs were removed with sterile forceps. Two wells received 50 µL of crude *Nymphaea nouchali* var. *caerulea* petal extract filtrate (Fig.3), one well received 50 µL of 0.9% saline as negative control, and one well received 50 µL of 0.2% chlorhexidine gluconate as positive control (Fig.4). The plates were kept at room temperature for 30 minutes to permit diffusion of the test solutions into the agar. The plates were then incubated in an incubator at 37°C for 24 hours. <sup>(8,9)</sup> Triplicate plates were prepared. (Fig.5)

## Outcome assessment

After incubation, the plates were examined for zones of inhibition around each well. (Fig.6) The total diameter of each clear zone (including the well) was measured in millimetres along two perpendicular axes using a vernier calliper, and the mean value was recorded. The net zone of inhibition was calculated by subtracting the well diameter from the mean total diameter, following methods used in previous *Nymphaea* and lotus-extract antimicrobial studies. <sup>(3-6)</sup> Measurements from triplicate plates were used to calculate mean  $\pm$  standard deviation for each group. <sup>(3,6)</sup>

| EXPERIMENTAL GROUPS                                                        | PLATE 1 | PLATE 2 | PLATE 3 |
|----------------------------------------------------------------------------|---------|---------|---------|
| <i>Nymphaea nouchali</i> var. <i>caerulea</i> extract- Well 1 [Test Group] | 4.1 mm  | 4.5 mm  | 4.3 mm  |
| <i>Nymphaea nouchali</i> var. <i>caerulea</i> extract -Well 2 [Test Group] | 4 mm    | 3.9 mm  | 4.4 mm  |
| 0.2% Chlorhexidine<br>[Positive Control]                                   | 6 mm    | 6.2 mm  | 6.3 mm  |
| 0.9% Saline<br>[Negative Control]                                          | 0 mm    | 0 mm    | 0 mm    |

**Table 1:** Zone of inhibition produced by *Nymphaea nouchali* var. *caerulea* extract and control groups against *Streptococcus mutans*.

ZOI: Zone of inhibition. Values represent net inhibition zones (mm) calculated by subtracting the initial 8-mm well diameter from the total measured inhibition diameter. Measurements were performed in triplicate.

The *Nymphaea nouchali* var. *caerulea* extract produced consistent inhibition against *Streptococcus mutans* across all experimental replicates, with net inhibition zones ranging from 3.9 – 4.5 mm. 0. 9% chlorhexidine demonstrated higher antimicrobial activity, whereas saline showed no inhibition beyond the original well diameter (Table 1).

## 2.3 Analytical Methods

### • Zone of Inhibition (ZOI) Analysis

The antimicrobial activity of the *Nymphaea nouchali* var. *caerulea* (blue water lily) petal extract was evaluated by measuring the Zone of Inhibition (ZOI) across three independent experimental replicates (Plate 1, Plate 2, and Plate 3). The raw inhibition zones for the experimental extract (evaluated in duplicate as Well 1 and Well 2), the positive control (0.2% Chlorhexidine), and the negative control (0.9% Saline).

## 2.4 Statistical Analysis

All data were statistically analyzed using IBM SPSS Statistics software version 32.0.x. Continuous variables for the ZOI measurements were expressed as Mean  $\pm$  Standard Deviation (SD) along with their corresponding 95% Confidence Intervals (CI), minimum, and maximum values. To assess the statistical differences in the mean ZOI among the three distinct groups

(Group 1: *Nymphaea nouchali* var. *caerulea* petal extract, n=6; Group 2: Chlorhexidine positive control, n=3; and Group 3: Saline negative control, n=3), a one-way Analysis of Variance (ANOVA) was executed.

The homogeneity of variances and normality assumptions were satisfied prior to the parametric analysis. For post hoc intergroup pairwise comparisons, the Bonferroni adjustment test was employed to account for multiple comparisons and prevent Type I errors. The threshold for statistical significance was established *a priori* at  $\alpha = 0.05$  ( $p < 0.05$ ).

## • Descriptive and Inferential Findings

These descriptive statistics are systematically organized in Table 2.

| PARAMETER      | SUM OF SQUARES | df | MEAN SQUARE | F VALUE | p VALUE |
|----------------|----------------|----|-------------|---------|---------|
| Between groups | (SPSS value)   | 2  | —           | —       | <0.001  |
| Within groups  | (SPSS value)   | 9  | —           |         |         |
| Total          |                | 11 |             |         |         |

**Table 2:** Comparison of antimicrobial efficacy among groups using one-way ANOVA

One-way Analysis of Variance (ANOVA) was performed to compare mean net ZOI among the three groups. A  $p$  value  $< 0.05$  was considered statistically significant.

One-way ANOVA revealed a statistically significant difference in antimicrobial activity among the tested groups ( $p < 0.001$ ), indicating that the observed differences in inhibition zones were unlikely to be due to chance.

To evaluate if the observed discrepancies among the mean values were statistically significant, the one-way ANOVA framework was utilized. The test indicated highly significant variations in antimicrobial efficacy between the examined groups  $\{F(2, 9) = 837.310, p < 0.001, \text{Sum of Squares} = 60.783\}$ . The within-group variance (residual error) remained remarkably minimal (Mean Square = 0.036), indicating high experimental reproducibility.

Because the ANOVA confirmed significant main effects, post hoc intergroup pairwise matrix evaluations via the Bonferroni method were conducted to identify specific group differentials.

The pairwise analyses validated that the antimicrobial efficacy of the *Nymphaea nouchali* var. *caerulea* petal extract (Group 1) was significantly superior to the saline negative control (Group 3), showcasing a mean difference of 4.200 mm ( $p < 0.001$ , 95% CI: 3.805 to 4.595 mm). Conversely, standard Chlorhexidine (Group 2) maintained a significantly larger efficacy footprint than the plant extract, outperforming it by a mean difference of 1.967 mm ( $p < 0.001$ , 95% CI: -2.362 to -1.572 mm). Finally, the positive control generated a significantly wider ZOI than the negative control, with a mean difference of 6.167 mm ( $p < 0.001$ , 95% CI: 5.710 to 6.623 mm). These paired findings are consolidated below in Table 3.

| COMPARISON               | MEAN DIFFERENCE (mm) | p VALUE |
|--------------------------|----------------------|---------|
| Extract vs Chlorhexidine | -1.967               | <0.001  |
| Extract vs Saline        | +4.200               | <0.001  |
| Chlorhexidine vs Saline  | +6.167               | <0.001  |

**Table 3:** Bonferroni post-hoc comparison of net ZOI among groups

Bonferroni correction was applied for multiple pairwise comparisons. Negative values indicate comparatively lower inhibition in the first group.

Post-hoc analysis demonstrated that the antimicrobial activity of *Nymphaea nouchali* extract was significantly greater than saline ( $p < 0.001$ ), confirming its antibacterial effect against *S. mutans*. However, chlorhexidine exhibited significantly higher inhibition compared with the plant extract ( $p < 0.001$ ).

## 3. RESULTS AND DISCUSSION

### 3.1 Biological Activity

The highest antimicrobial activity was observed with 0.2% chlorhexidine ( $6.167 \pm 0.153$  mm). The *Nymphaea nouchali* extract demonstrated a measurable inhibitory effect ( $4.200 \pm 0.237$  mm), while the saline control showed complete absence of antimicrobial activity as detailed in Table 4.

| GROUP                                                 | N  | MEAN NET ZOI (mm) | SD    | 95% CONFIDENCE INTERVAL |
|-------------------------------------------------------|----|-------------------|-------|-------------------------|
| <i>Nymphaea nouchali</i> var. <i>caerulea</i> extract | 6  | 4.200             | 0.237 | 3.952 – 4.448           |
| 0.2% Chlorhexidine                                    | 3  | 6.167             | 0.153 | 5.787 – 6.546           |
| 0.9% Saline                                           | 3  | 0.000             | 0.000 | —                       |
| Total                                                 | 12 | 3.642             | 2.357 | —                       |

**Table 4:** Descriptive analysis of antimicrobial activity based on net zone of inhibition

Values are expressed as mean  $\pm$  standard deviation (SD). CI: Confidence interval; ZOI: Zone of inhibition.

Descriptive profiling of the groups revealed that the positive control (Chlorhexidine) yielded the highest mean zone of inhibition at  $6.167 \pm 0.153$  mm (95% CI: 5.787 to 6.546 mm). The *Nymphaea nouchali* var. *caerulea* petal extract demonstrated a prominent and robust antimicrobial effect, exhibiting a mean ZOI of  $4.200 \pm 0.237$  mm (95% CI: 3.952 to 4.448 mm). No zone of inhibition was observed for the saline negative control group ( $0.000 \pm 0.000$  mm). The total pooled mean across all experimental units (N=12) was calculated at  $3.642 \pm 2.357$  mm.

### 3.2 Discussion

This in-vitro study was designed to explore a specific gap in the literature: the limited evidence on the antimicrobial effect of *Nymphaea nouchali* against oral pathogens such as *Streptococcus mutans*.<sup>(3-6,11)</sup> Experimental studies on *N. nouchali* flowers, seeds, and leaves have demonstrated antibacterial activity against several Gram-positive and Gram-negative microorganisms, supporting the biological plausibility of testing its extract against oral bacteria.<sup>(3-6,11,12)</sup> In addition, Nymphaeaceae plants, including *N. nouchali* and *N. pubescens*, have been reported to contain abundant phenolics, flavonoids, tannins, and other secondary metabolites with antioxidant and potential antimicrobial properties.<sup>(2,6,7,13)</sup>

The choice of *S. mutans* (MTCC 10449) as the test organism is relevant because this species is a key cariogenic pathogen with high adherence, acidogenicity, and a central role in the formation and maturation of cariogenic biofilms.<sup>(1,14)</sup> Evidence also indicates that *S. mutans* can be recovered from saliva and subgingival plaque, particularly in individuals with gingival inflammation and periodontitis, highlighting its broader ecological significance in the oral cavity.<sup>(14,15)</sup> In this study, the *N. nouchali* extract exhibited a robust mean zone of inhibition (ZOI) of  $4.200 \pm 0.237$  mm. One-way ANOVA confirmed highly significant variations in antimicrobial efficacy across groups  $\{F(2, 9) = 837.310, p < 0.001\}$ . Post hoc Bonferroni pairwise analysis validated that the plant extract's activity was statistically superior to the saline negative control (0.000 mm,  $p < 0.001$ ), though significantly lower than the Chlorhexidine positive control ( $6.167 \pm 0.153$  mm,  $p < 0.001$ ). This measurable inhibition of *S. mutans* supports further investigation of this plant as a potential phytotherapeutic adjunct for plaque control and caries-related microbial modulation.

Any observed antimicrobial effect may plausibly be attributed to phytoconstituents such as flavonoids, phenolic acids, and tannins, which have been associated with membrane disruption, enzyme inhibition, and interference with microbial adhesion and biofilm formation in other plant-based studies.<sup>(2,6-7,13)</sup> However, it must be emphasized that agar well diffusion results depend not only on intrinsic antimicrobial potency but also on the diffusion characteristics of the extract through the agar matrix, which can underestimate or overestimate activity for certain compounds.<sup>(10,16-17)</sup> The use of a crude hydroalcoholic flower extract also precludes identification of specific active molecules and does not provide minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), or quantitative antibiofilm data.<sup>(10,16,18)</sup>

Several limitations therefore need to be acknowledged. First, as an in-vitro model, the study cannot reproduce the full complexity of the oral environment, including saliva, host immune factors, and multispecies biofilm interactions. <sup>(14,19)</sup> Only a single organism, *S. mutans*, is evaluated, so the findings will not reflect the polymicrobial nature and ecological dynamics of natural dental biofilms. <sup>(1,14,19)</sup> Second, the agar well diffusion method is influenced by solubility and diffusibility of components; poorly diffusible yet potentially potent constituents may show small or absent zones. <sup>(10,16)</sup> Third, the protocol does not include MIC, MBC, time-kill, antibiofilm, or cytotoxicity assays, which are essential steps before considering translational or clinical application of a plant-derived agent. <sup>(10,16,18,20)</sup>

## 4. CONCLUSION

The present in-vitro study was undertaken to evaluate the antimicrobial efficacy of *Nymphaea nouchali* var. *caerulea* petal extract against *Streptococcus mutans* using an agar well diffusion method, with 0.2% chlorhexidine serving as the positive control. By targeting a defined gap in existing literature on Nymphaeaceae and oral pathogens, this work is expected to provide preliminary insight into the potential of *Nymphaea nouchali* var. *caerulea* as a plant-derived antimicrobial candidate in dentistry.

If the extract demonstrates meaningful inhibition of *S. mutans* compared with saline and shows a biologically relevant effect relative to chlorhexidine, the findings will justify more detailed studies, including MIC and MBC estimation, antibiofilm assays, phytochemical standardization, and formulation development. In the longer term, such work may contribute to the development of complementary herbal agents that could support or partially substitute conventional chemotherapeutics in plaque control, subject to rigorous evaluation of efficacy, safety, and clinical outcomes.

In conclusion, the findings of this in vitro investigation demonstrate that the hydroalcoholic extract of *Nymphaea nouchali* var. *caerulea* exhibits a highly significant antimicrobial effect against the primary oral pathogen *Streptococcus mutans*. While the standard 0.2% chlorhexidine control maintained superior overall efficacy, the definitive zone of inhibition produced by the plant extract highlights its potential as a viable, natural phytotherapeutic candidate. These results successfully establish a baseline that justifies future research into its minimum inhibitory concentration, antibiofilm properties, and long-term biocompatibility for clinical dental applications.

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Dr. Shilpi Singh, Professor and Head, Department of Public Health Dentistry, IDST Modinagar, Uttar Pradesh, India.

## AUTHOR CONTRIBUTIONS

**Dr. Ruchi Pandey/ Dr. Shruti Ananya:** Conceptualization, Investigation.

**Dr. Nidhi Didwania/ Dr. Shakila Mahesh:** Methodology, Laboratory Analysis.

**Dr. Lipika Gopal:** Data Curation, Formal Analysis.

**Dr. Ruchi Pandey/Nipun Dhalla:** Supervision, Writing—Review & Editing.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ETHICS STATEMENT

The study is an in-vitro investigation submitted for IEC clearance, and participant-related confidentiality, consent, and risk provisions were marked as not applicable in the uploaded document.

## DATA AVAILABILITY STATEMENT

Data generated or analysed during the current study is available in the manuscripts. For any further query, the corresponding authors can be contacted.

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**Fig. 1 :** Crude extract of *Nymphaea nouchali* var. *caerulea*



**Fig.2:** *Streptococcus mutans* (MTCC 10449) colony



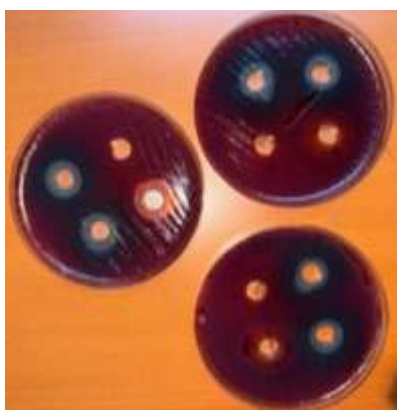
**Fig.3:** Well 1 and 2 receive the crude *Nymphaea nouchali* var. *caerulea* petal extract



**Fig.4:** Well 3 receives 0.2% Chlorhexidine Gluconate and Well 4 receives 0.9% Saline



**Fig.5:** Triplicate plates were prepared



**Fig.6:** Plates showing Zone of Inhibition