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# Evaluation of Antibacterial and Antibiofilm Activity of *Cucurbita Maxima* Leaf Extract Against *Streptococcus Mutans* Isolated from Orthodontic Patients

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**ABSTRACT**

**Aim:** Streptococcus mutans in dental plaque has been implicated as one of the etiologic factor for development of white spot lesions in orthodontic subjects. The aim of this investigation was to evaluate the antimicrobial activity and anti-biofilm activity of *Cucurbita Maxima* leaf extract on *S.mutans* isolated from dental plaque of subjects undergoing orthodontic treatment.

**Methodology:** Dental plaque samples were collected from out-patients undergoing orthodontic treatment. From the collected plaque samples, Biochemical Characterization of *S. mutans* was performed. The Medicinal Plants were sourced, and the solvent was extracted. The stock solution was then prepared from the extracted solvent. Evaluation of Minimum Inhibitory Concentration (MIC) of the plant extract and Biofilm Inhibition Assay with Gas Chromatography-Mass Spectrum Analysis (GCMS) was carried out.

**Results:** The MIC was identified to be 1.25mg/ml. The biofilm inhibition assay showed that the extract did not inhibit the formation of the biofilm. The GCMS analysis identified twenty-five constituent compounds from the crude extract.

**Conclusion:** The *C. maxima* leaf extract showed antibacterial activity against *S. mutans,* and the minimum inhibitory concentration was identified at 1.25mg/ml. The extract did not inhibit the formation of biofilm.

**Keywords:** Antibacterial, Biofilm, Extract, Leaf, Plant.

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## INTRODUCTION

*Streptococcus mutans (S. mutans)* is commonly found in the human oral cavity and is one of the main species that contributes to the development of tooth decay, also known as dental cavities.[1] The bacterium *S. mutans* produces an acid which metabolizes sugar and can break down tooth enamel and can lead to the formation of cavities.[2,3] Maintaining good oral hygiene, including brushing and flossing regularly and limiting sugary foods and drinks, can help reduce the presence of *S. mutans* in the mouth and prevent tooth decay but is not routinely practiced by many orthodontic patients hence antimicrobial agents such as mouthwashes are used to control the effects of *S. mutans* and other harmful bacteria in the mouth. [4,5]

*Cucurbita maxima (C. maxima)*, also known as pumpkin or squash, is a species of flowering plant in the cucurbit family (*Cucurbitaceae*). It is native to South America and is widely cultivated for its edible fruit, which is used in a variety of dishes, including soups, pies, and breads. *C. maxima* is a large, vining plant that produces large, yellow or orange fruits that can weigh up to several hundred pounds.[6–8] In addition to its dietary use *C. maxima* has also been used medicinally to treat a variety of conditions, including wounds, burns, and infections. It is important to note that the medicinal uses of *C. maxima* have not been extensively researched, and more studies are needed to fully understand its potential health benefits.[9–13] The study objective was to evaluate the antimicrobial and anti-biofilm effects of *C. maxima* leaf extract on *S. mutans* isolated from dental plaque of subjects undergoing orthodontic treatment.

## MATERIALS AND METHODS

### Study design and Clinical Isolates

This study involved the participation of patients reporting to a dental teaching hospital in the capital city of Chennai, Tamil Nadu, India, during the year 2022. Dental plaque samples were collected from out-patients undergoing orthodontic treatment. The plaque samples were collected using a sterile No. 23 Shepherd's hook explorer. The collected samples were transferred to Brain Heart Infusion (BHI) broth and incubated at 37℃ for 24 hours. After 24 hours of incubation 1 µl of the sample was spread on the Mitis - Salivarius Agar plate. Then the plates were incubated at 37°C for 24 hours. The isolated colonies were stored at 4℃ for further analysis.

### Biochemical Characterization of *S. mutans*

A preliminary screening of the clinical isolates of *S. mutans* was identified on the standard microbiological tests. This included characteristic growth patterns on Mitis-Salivarius agar, Luria-Bertani agar and colony morphology, texture, Gram staining, cell size, oxidase, catalase, motility, temperatures, citrate, indole, methyl red, Voges proskaur's, nitrate reduction test, gelatin hydrolysis, Hydrogen Sulfide ($H_2S$) test, lactose, sucrose, lipid hydrolysis, luminescence and pH.

### Collection of Medicinal Plants

The leaves of *C. maxima* were collected from the Saveetha Institute of Medical and Technical Sciences orchard, Chennai, Tamil Nadu, India.

**Preparation of Solvent Extract**

The leaves were separated from the stems and kept for shade drying inside the dark room for 10 days. Once the leaves were dried, pulverized and 10 grams of coarse powder was dissolved in 100 ml of menthol (Nice chemicals, Cochin, Kerala, India). The flask was incubated at a normal room temperature for 48 hours. The extract was filtered by using Whatman® No.1 filter paper (Whatman® qualitative filter paper grade 1, Jigchem Universal, Mumbai, India) and filtered extract was kept for evaporation. The dried extract was stored in 4℃ for further analysis.

**Evaluation of Minimum Inhibitory Concentration (MIC)**

The broth microdilution (two-fold) method was used to determine the minimum inhibitory concentrations (MICs) of methanol and leaf extract of *C. maxima*. MICs of the methanol and *C. maxima* leaf extract against *S. mutans* used in the assay were assessed in different concentrations ranging from 10 to 0.01 mg/ml. The growth of the bacteria was visualized by adding 2,3,5-triphenyl tetrazolium chloride (TTC) salt (Belami Fine Chemicals Pvt Ltd., Mumbai, India) that acts as an indicator. The lowest concentration with no visible growth was recorded as MIC.

**Biofilm Inhibition Assay**

The effect of methanol *C. maxima* leaf extract on biofilm formation by *S. Mutans* was determined by the crystal violet staining assay.[14–18] A 20 µl overnight culture of S. mutans and C. maxima leaf extract were loaded in a dose-dependent manner (2, 1, and 0.5 mg/mL) into 180 µl of the BHI broth medium and incubated at 37℃ for 24 hours. After 24 hours incubation, the planktonic cells were removed by washing with sterile water and the surface-adherent biofilm was stained with a 0.1% crystal violet solution. After 5 minutes the unbound crystal violet was washed with sterile-distilled water. Finally, the adherent biofilm-bound crystal violet was eluted in 200 µl of ethanol (70%) and quantified by measuring the intensity of crystal violet at 492 nm and 630 nm using a UV−vis spectrophotometer.

**Gas Chromatography-Mass Spectrum Analysis (GCMS)**

The methanol extract of *C. maxima* was sent for GCMS to identify the various constituents present in the sample. The analysis was done based on the protocol described previously by Ganesh and Vittal.[14] The *C. maxima* leaf extract was evaluated by a Shimadzu GCMSQP-2010 plus detector gas chromatograph equipped with A RTx5MS (30.0 * 0.32mm ID., 0.5 m thickness) crossband diphenyl dimethyl polysiloxane-fused silica capillary column. Helium, ≥99.99%, was used as a carrier gas with a constant flow rate of 0.8 mL/min. The previously concentrated *C. maxima* leaf extract was dissolved in n-hexane, and 1 µl of the sample was injected (split ratio of 100:1) into GC− MS using an AOC5000 auto-injector. The GC−MS chromatogram obtained was matched by the NIST/WILEY 7 library database.

## RESULTS

### Minimum Inhibitory Concentrations of *C. maxima* leaf extract on *S. mutans*

Minimum inhibitory concentrations (MIC) of the methanol extracts of leaf extracts against *S. mutans* was determined by the broth microdilution method. The extract inhibited the growth of *S. mutans* at the lowest concentrations of 1.25mg/ml (Table 1). For further study, concentrations below the MIC of the methanol crude extracts were used for antibiofilm activity against the *S. mutans*.

**Table 1 - MIC Assay table**

| S. No | Concentration (mg/ml) | Test 1 | Test 2 |
|---|---|---|---|
| 1 | 10 | No Growth | No Growth |
| 2 | 5 | No Growth | No Growth |
| 3 | 2.5 | No Growth | No Growth |
| 4 | 1.25 | No Growth | No Growth |
| 5 | 0.625 | Growth | Growth |
| 6 | 0.3125 | Growth | Growth |
| 7 | 0.15625 | Growth | Growth |
| 8 | 0.078125 | Growth | Growth |
| 9 | 0.0390625 | Growth | Growth |
| 10 | 0.01953125 | Growth | Growth |
| Control | 0 | Growth | Growth |
| Sterility Control | 0 | No Growth | No Growth |

### Effect of *C. maxima* leaf extracts on Biofilm Formation in *S. mutans*

The *C. maxima* leaf extract did not inhibit *S. mutans* biofilm formation in a dose-dependent manner. When the effect of the *C. maxima* leaf extract on biofilm formation was analyzed using light microscopy, we observed bacterial aggregation (biofilm matrix) on the surface of the cover slip in the control (without treatment) group and absence of bacterial aggregation in the sterility control group. We also did not observe any reduction in the biofilm cluster in the *C. maxima* leaf extract treated *S. mutans* as compared to that of the control.

### Identification of Chemical Constituents by GCMS

The primary identification of chemical constituents in *C. maxima* leaf methanol extract was confirmed using GC-MS, which revealed 23 different chemical compounds when compared to published spectra. Methyl 3-Butenyl-1-d2 Ether, n-Hexadecanoic acid, and Ethane, fluoro- (CAS) Fluoroethane were identified as major components likely to inhibit microbial activity among the 25 constituents studied.

***Table 2 - Constituents of C. maxima leaf extract identified through GCMS***

| S. No | Retention Time | Area % | Compound Name |
|---|---|---|---|
| 1 | 1.139 | 9.77 | Ethane, fluoro- (CAS) Fluoroethane |
| 2 | 1.270 | 4.31 | PROPENE-1.1-D2 |
| 3 | 1.342 | 2.31 | Silane, dimethoxy methyl- |
| 4 | 9.358 | 0.32 | Dodecanoic acid (CAS) Lauric acid |
| 5 | 9.949 | 0.48 | 1,2-Benzenedicarboxylic acid, diethyl ester |
| 6 | 11.714 | 0.92 | Tetra decanoic acid |
| 7 | 12.333 | 0.41 | (-)-Loliolide |
| 8 | 12.543 | 14.9 | 2-Tridecen-1-ol, (E)- |
| 9 | 12.648 | 1.46 | 6-Dodecanone |
| 10 | 12.811 | 3.93 | 1-Hexadecyne |
| 11 | 13.018 | 5.99 | Phytol |
| 12 | 13.483 | 1.17 | Pentadecanoic acid, 14-methyl-, methyl ester |
| 13 | 13.729 | 0.26 | 1,7-Octadien-3-ol, 2,6-dimethyl- |
| 14 | 13.908 | 17.51 | n-Hexadecanoic acid |
| 15 | 14.067 | 0.51 | Phthalic acid, butyl trans-dec-3-enyl ester |
| 16 | 14.211 | 0.63 | 1-Tetradecanol (CAS) Alfol 14 |
| 17 | 15.204 | 0.83 | 1-Nonadecanol |
| 18 | 15.348 | 0.89 | 9,12,15-Octadecatrienoic acid, methyl ester |
| 19 | 15.463 | 20.52 | Methyl 3-Butenyl-1-d2 Ether |
| 20 | 15.730 | 4.66 | cis-7-Tetradecen-1-ol |
| 21 | 15.807 | 3.6 | 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- |
| 22 | 15.957 | 3.62 | Octadecanoic acid (CAS) Stearic acid |
| 23 | 18.025 | 0.2 | Eicosanoic acid, methyl ester (CAS) Arachidic |
| 24 | 18.132 | 0.55 | E-5-dodecenal |
| 25 | 19.165 | 0.23 | FARNESYL ACETONE B |

## DISCUSSION

In India, *C. maxima* is widely cultivated and used as food sources. Seed, flower and fruit extracts of *C. maxima* have been found to be high in bioactive components such as unsaturated fatty acids, sterols, tocopherols, squalene, and carotenoid.[19,20] Amin et al., found that *C. maxima* seed oils were high in tocopherol and polyunsaturated fatty acids.[21,22] These substances have anti-diabetic, anti-hypertensive, anti-oxidant, anti-microbial, and anti-tumor properties. In this investigation, the main objective centered around the invitro evaluation and assessment of the antibacterial and antibiofilm activity of *C. maxima* leaf extract on *S. mutans* that was obtained from dental plaque samples collected from orthodontic patients. This is the first report on the antibacterial and antibiofilm activity of *C. maxima* leaf extract on *S. mutans* activity as various studies have already established the antibacterial activity of *C. maxima* flowers, seeds, and pulp against *Staphylococcus aureus, Salmonella typhi, Escherichia coli, Enterococcus faecalis,* and *Bacillus cereus.*[13, 23–25] Okon et al. also demonstrated that *C. maxima* leaf extract had antibacterial activity against normal human pathogens *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli.*[26] In this study, they also observed that S. aureus was the most sensitive to *C. maxima* extract and the antibacterial activity is greater as the concentration is higher.[26] Antifungal activity of *C. maxima* flowers against *Candida albicans* and *Cochliobolus lunata* has also been established.[23]

Infections caused by pathogenic bacteria have been a challenge for mankind. Antibiotics and antibacterial compounds have been successfully utilized for tackling infections but the development of resistance to such compounds has developed a scare amongst researchers and clinicians. The infectious diseases caused by resistant microbes can be treated with antimicrobial-rich plant extracts. Folklore has traditionally used herbal medicine to treat a variety of infectious diseases. Although the majority of the cases were not scientifically evaluated, the chemical constituents of even simple medicinal preparations are beneficial.[27]

In our study, using the broth microdilution technique we identified that bacterial activity of *S. mutans* was inhibited at 77.5% dilution at the concentration of 1.25mg/ml. We were also able to identify that at sub-inhibitory concentrations the *C. maxima* leaf extract did not show any inhibitory activity on the formation of biofilm.

The GCMS evaluation was performed to identify the constituents of the *C. maxima* leaf extract. This assessment report had identified three compounds that showed an area percentage which was greater than 10%. The three compounds that were identified are Methyl 3-Butenyl-1-d2 Ether (20.52%), n-Hexadecanoic acid (17.51%) and 2-Tridecen-1-ol, (E)- (14.9%). Area percentage is the percentage of total peak area on the chromatogram made up by the analyte.[28] Thus area percentage is an indicative of the proportional concentration of a compound within an extract. The antibacterial activity of the *C. maxima* leaf extract could be attributed to these phenolic compounds. Bahri-Sahloul et al., reported that that the plant extracts that enriched in phenolic compounds have potential antibacterial activity.[29]

## CONCLUSION

This investigation has revealed that the leaf extract obtained for the *C. maxima* plant has antibacterial activity against *S. mutans* isolated from dental plaque in patients undergoing orthodontic treatment. The minimum

inhibitory concentration was found to be 1.25mg/ml. The biofilm inhibition assay showed that the extract did not inhibit the formation of oral biofilm. The GCMS analysis of the crude extract was able to identify twenty-five constituent compounds. Three compounds Methyl 3-Butenyl-1-d2 Ether, n-Hexadecanoic acid and 2-Tridecen-1-ol, (E)- had an area% of greater than 10%.

## CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

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