ANTIMICROBIAL EFFECT OF CANNABIS ON STAPHYLOCOCCUS AUREUS ATCC25923, STREPTOCOCCUS MUTANS UA159, STREPTOCOCCUS PYOGENES CLINICAL STAIN

Kutana NAMARACH[1*] and Boonyanit THAWEEBOON[1]
1 Dentistry (International Program), Maxillofacial Surgery, Walailak University, Thailand; non_god@hotmail.com (Corresponding Author)

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ABSTRACT
Background: Resistance to multiple antimicrobial drugs is a growing challenge, highlighting the need for novel compounds. Cannabis, historically used for medical purposes, is being studied for antimicrobial potential. While many studies have noted its effects, further investigation is required to evaluate Cannabidiol (CBD) specifically on oral bacteria.
Aim: To evaluate CBD's ability to inhibit Staphylococcus aureus, Streptococcus mutans, and Streptococcus pyogenes.
Methodology: CBD (Merck, 1 mg/mL in methanol) was diluted to 5, 10, 15, and 20 μg/mL. Test strains were obtained from Chulalongkorn University and Prof. Jintakorn Kuvatanasuchati. Antimicrobial activity was assessed via disk diffusion using chlorhexidine gluconate (CHX) as control. MIC and MBC were determined by broth dilution.
Results: CBD showed dose-dependent inhibition, with zones from 8.6 to 16.6 mm. S. pyogenes was most susceptible, with similar inhibition to CHX at 20 μg/mL ($P = 0.96$). S. aureus and S. mutans showed smaller zones than CHX ($P < 0.05$). MICs were 5, 2.5, and 2.5 μg/mL; MBC was 5 μg/mL for all.
Conclusion: CBD exhibits antimicrobial activity against S. aureus, S. mutans, and S. pyogenes, particularly effective against S. pyogenes.
Keywords: Cannabis, Cannabidiol, Antimicrobial, THC, CBD

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INTRODUCTION

Cannabis (Cannabis sativa), or marijuana, produces both mental and physical effects (Hindocha et al., 2017). Historically valued for textiles and traditional medicine, it is now used in pharmacology and cosmetics. Ancient records describe cannabis oil for anesthesia in India before the 10th century B.C. In ancient India, it treated inflammation, wounds, sexually transmitted diseases, leukoderma, scabies, and smallpox (Dilara & Subhan, 2000).

There are three main types: C. sativa (marijuana, hashish, hash oil), C. indica (high-THC hashish for euphoria, medical, and research uses) (Chayasirisobhon, 2019). Cannabinoids are endogenous, synthetic, or plant-derived (phytocannabinoids), with cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC) being most important (Thant & Nussbaum, 2020). THC is psychoactive; CBD is non-psychoactive and shows anti-inflammatory, antimicrobial, antioxidant, anxiolytic, antidepressant, antipsychotic, and anticonvulsant effects (Barak et al., 2022).

Staphylococcus aureus is a Gram-positive bacterium in the upper respiratory tract and skin, causing infections such as abscesses, sinusitis, and food poisoning; MRSA strains resist treatment and lack a vaccine (Jaradat et al., 2020). *Streptococcus mutans*, a Gram-positive oral anaerobe, causes tooth decay; *S. sobrinus* has similar pathogenicity (Lemos et al., 2018). *Streptococcus pyogenes* (group A) is pathogenic but not a normal skin commensal (Newberger & Gupta, 2023).

Chlorhexidine (CHX) is an antiseptic used as mouthwash, gels, sprays, creams, and ointments (Brookes et al., 2021). CHX mouthwash (pH 5-7) binds oral tissues, with ~30% retained in saliva up to 5 hours and on mucosa up to 12 hours; it is poorly absorbed if swallowed and generally safe, with some side effects (Brookes et al., 2020).

The broth dilution method determines minimum inhibitory concentration (MIC) using serial two-fold dilutions in macro- or microdilution formats, with MIC as the lowest concentration without visible growth, and minimum bactericidal concentration (MBC) as the lowest killing 99.9% of bacteria (M26-A). Microdilution is more reproducible and efficient. The agar disk-diffusion method (CLSI standard) uses antimicrobial discs on inoculated agar to measure inhibition zones, classifying bacteria as susceptible, intermediate, or resistant. It does not distinguish bactericidal from bacteriostatic activity or directly quantify MIC but estimates it for guiding antibiotic choice.

Objectives

1) To measure the inhibition zone of tested bacteria on an agar plate by using Vernier calliper according to the agar disc diffusion method and compare to control (chlorhexidine 0.12%)
2) To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of CBD using the standard broth dilution method.

LITERATURE REVIEWS

The efficiency of CBD on micro-organisms

Van et al. (1976) reported MICs of 1-5 µg/mL for staphylococci and streptococci in broth, with reduced activity in media and no effect on Gram-negatives. Martinenghi et al. (2020) found MICs of 1 µg/mL for *S. aureus* and MRSA, and 2 µg/mL for *S. epidermidis*, without synergy with common antibiotics. Iseppi et al. (2019) observed no bactericidal effect on *Staphylococcus* but enhanced essential oil activity. Karas et al. (2020) and Klahn et al. (2020) noted high activity against Gram-positives, especially MRSA. Appendino et al. (2008) reported MICs of 2 µg/mL for multiple *S. aureus* strains, outperforming some antibiotics, supported by Farha et al. (2020). Wassmann et al. (2022) recorded MICs of 4 µg/mL for MRSA, L. monocytogenes, MRSE, and 8 µg/mL for E. faecalis, but >128 µg/mL for Gram-negatives.

Blaskovich et al. (2021) found MICs of 1-4 µg/mL for *S. aureus* and 1 µg/mL for *S. pyogenes*, with greater MSSA biofilm activity than daptomycin and vancomycin, plus synergy with

polymyxin B and colistin against *A. baumannii* and *E. coli*. Abichabki et al. (2022) confirmed synergy with polymyxin B against drug-resistant bacilli, with MICs of 4 µg/mL (CAMHB) and 64 µg/mL (MH-F) for *S. aureus*, and 32 µg/mL for *S. pyogenes.* MIC values vary across studies, with limited data for oral bacteria, warranting further study on *S. mutans, S. aureus,* and *S. pyogenes.*

The efficiency of CBD on oral bacteria

The oral cavity contains microorganisms linked to dental caries and periodontal diseases (Peng et al., 2022). Gram-positives like *S. mutans, S. sanguis,* and *S. mitis* are common in caries, while severe periodontitis often involves Gram-negatives like *A. actinomycetemcomitans* and *F. nucleatum* (Costalonga & Herzberg, 2014). Severe early childhood caries correlates with *Streptococcus, Porphyromonas,* and *Actinomyces* (Topcuoglu & Kulekci, 2015).

Vasudevan et al. (2020) showed 1% CBD polishing paste reduced oral bacterial counts more than standard paste ($P<0.001$), though anaerobe data were limited. CBD/CBG mouthwashes matched 0.2% chlorhexidine and outperformed most commercial rinses. Stahl et al. (2020) found cannabinoids had greater antibiofilm activity than toothpaste; Feldman et al. (2018) reported reduced MRSA biofilm formation and altered surface properties. Barak et al. (2022) found CBD MIC and MBIC values of 5 µg/mL against S. mutans, with dose-dependent biofilm inhibition and no regrowth at >20 µg/mL for 48 hours, suggesting potential for controlling oral pathogens.

MATERIALS & METHODS

Equipment

Autoclave, Incubator, Laminar air flow, Paper disc (6 mm, Whatman), Test tube (16 × 100 mm, Duran), Cell-culture dish

Materials

1) Cannabinol (CBD)

CBD (1 mg/mL in methanol) from Supelco Cerilliant (#C-045), Merck, Darmstadt, Germany, was used within the dosage range typical of commercial CBD supplements.

2) Chlorhexidine Mouthwash

Chlorhexidine Gluconate 0.12% Oral Rinse was purchased as a positive control.

3) Tryptic Soy Agar

General-purpose culture medium from Merck, Germany.

Bacteria

Microorganisms obtained from the Faculty of Dentistry, Chulalongkorn University, included *Streptococcus mutans* UA159, *Staphylococcus aureus* ATCC2592, and *Streptococcus pyogenes* (clinical strain). All were grown on nutrient agar and incubated at 37°C for 24 h prior to testing.

Preparation of Materials

CBD stock solution (1000 µg/mL in methanol) was diluted in 0.9% NaCl to obtain 5, 10, 15, and 20 µg/mL concentrations for disk diffusion testing.

Methods

1) Disc Diffusion Test

Sterile filter paper discs (6 mm) were impregnated with CBD solutions and placed on TSA plates inoculated with bacterial strains. Chlorhexidine 0.12% served as positive control. Plates were incubated at 37°C for 24 h and inhibition zones measured with a Vernier caliper. The experiment was performed in triplicate.

Dilution Calculation: $C_1V_1 = C_2V_2$

Where C_1 = 1000 µg/mL (stock), V_1 = volume of stock, C_2 = desired concentration, V_2 = 10 mL final volume.

Table 1 Preparation of Cannabidiol (CBD) Solutions at 5, 10, 15, and 20 µg/mL Final Concentrations for 10 mL Final Volume

Final Concentration (µg/mL)	Volume of CBD Stock Solution (µL)	Volume of 0.9% NaCl (mL)	Final Volume (mL)
5	50	9.95	10
10	100	9.90	10
15	150	9.85	10
20	200	9.80	10

2) Standard Broth Microdilution Method

Following CLSI M07-A8 guidelines, bacterial suspensions (10^8 CFU/mL; 0.5 McFarland) were diluted to 10^5 CFU/mL. CBD (20 µg) was serially diluted two-fold to 0.625 µg/mL in broth. Microtiter plates containing inoculum and CBD dilutions were incubated at 37°C for 24 h. MIC was the lowest concentration without visible growth; MBC was determined by sub-culturing wells without growth.

Statistical Analysis

Disc diffusion results were analyzed using one-way ANOVA with mean ± SD. Tukey's post hoc test was applied to compare inhibition zones ($p < 0.05$).

RESULTS

Antibacterial activity of CBD (Disc Diffusion Test)

CBD (5-20 µg/mL) and chlorhexidine 0.12% (CHX) were tested against *S. aureus, S. mutans*, and *S. pyogenes*. All treatments produced inhibition zones (Table 3). CHX consistently showed the largest zones. ANOVA and Tukey HSD confirmed significant differences ($p < 0.05$) except for CBD 20 µg/mL vs CHX in *S. pyogenes* ($P = 0.96$). *S. aureus*: CBD zones ranged 8.6-12.6 mm; CHX = 18.6 mm. CBD significantly less effective than CHX, with no differences among CBD doses. *S. mutans*: CBD zones 10.6-15.0 mm; CHX = 25.2 mm. All CBD doses significantly less than CHX; no intra-CBD differences. *S. pyogenes*: CBD zones 9.3-16.6 mm; 20 µg/mL comparable to CHX (17.3 mm). Lower doses significantly less than CHX. Overall, CBD showed dose-dependent effects, with *S. pyogenes* most susceptible at higher concentrations.

Table 2 Antimicrobial activity using the disk diffusion method (Mean ± SD in mm)

Treatment	*S. aureus (mm)*	*S. mutans (mm)*	*S. pyogenes (mm)*
Chlorhexidine 0.12%	18.6 ± 3.0	25.2 ± 2.1	17.3 ± 0.7
CBD 5 µg/mL	8.6 ± 0.6	10.6 ± 0.5	9.3 ± 0.6
CBD 10 µg/mL	10.6 ± 0.6	11.3 ± 0.6	11.6 ± 0.6
CBD 15 µg/mL	12.0 ± 1.7	13.0 ± 0.6	13.0 ± 0.6
CBD 20 µg/mL	12.6 ± 1.0	15.0 ± 0.8	16.6 ± 0.5

MIC and MBC

MIC and MBC values are shown in Table 4. *S. aureus* MIC = 5 µg/mL; *S. mutans* and *S. pyogenes* MIC = 2.5 µg/mL; MBC = 5 µg/mL for all.

Table 3 MIC and MBC of CBD against Bacterial Stains (µg/mL)

Bacterial Strain 10^5 UCF	MIC (µg/mL)	MBC (µg/mL)
Staphylococcus aureus	5.0	5.0
Streptococcus mutans	2.5	5.0
Streptococcus pyogenes	2.5	5.0

DISCUSSION & CONCLUSION

Discussion

1) *S. aureus*

Current MIC (5 µg/mL) aligns with Van et al. (1976) and Blaskovich et al. (2021), slightly higher than Media differences (Abichabki et al., 2022) influence MIC values. Disc diffusion zones here (8.6-12.6 mm) are smaller than CHX and comparable to reports by Blaskovich et al. (2021). Qi et al. (2022) demonstrated enhanced activity when CBD was incorporated into alginate copper hydrogel.

2) *S. mutans & S. pyogenes*

MICs were 2.5 µg/mL for both. Barak et al. (2022) reported a similar MIC for *S. mutans* and notable antibiofilm effects at ≥7.5 µg/mL. *S. pyogenes* susceptibility varied in literature: Blaskovich et al. (2021) MIC = 1 µg/mL; Abichabki et al. (2022) = 32 µg/mL (broth type dependent). In this study, CBD 20 µg/mL was as effective as CHX against *S. pyogenes*.

3) Gram-positive vs Gram-negative

Some studies (Gildea et al., 2022) suggest higher CBD activity against Gram-negative bacteria at very low doses, but most reports (Wassmann et al., 2022) show greater efficacy on Gram-positive strains.

4) Methanol effect

Toxicity occurs above ~20% v/v (Dyrda et al., 2019). Here, methanol was <20% v/v. Wadhwani et al. (2008) found inhibitory effects ≥4% for methanol/DMSO, with ethanol more potent.

5) CBD toxicity

CBD is well tolerated in animals (up to 120 mg/kg in mice/rats; 62 mg/kg in dogs) and humans (up to 1500 mg/day). WHO considers CBD safe with minimal side effects (Schofs et al., 2021).

6) Diffusion assay optimization

Brady & Katz (1990) showed agar nutrient content, agar thickness, sample volume, and incubation temperature influence inhibition zone size—factors relevant for optimizing CBD assays.

Conclusion

CBD showed significant antimicrobial effects but was less effective than chlorhexidine 0.12% against *S. aureus and S. mutans* ($P < 0.05$). At 20 µg/mL, CBD's effect on *S. pyogenes* matched chlorhexidine ($P = 0.96$), suggesting its potential as an alternative agent for this pathogen. Overall, CBD demonstrated activity against *S. aureus, S. mutans*, and *S. pyogenes*, warranting further studies on its broader effects on oral microflora.

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