# Antimicrobial Activities against Periodontopathogens of Essential Oil from Lemon Grass (*Cymbopogon citratus* (DC.) Stapf.)

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## ABSTRACT

***The purpose of this study was to investigate the antimicrobial activities of the essential oil from Cymbopogon citratus (DC.) Stapf. (lemon grass) against some periodontal pathogens, including Actinomyces naeslundii (WVU 45), Porphyromonas gingivalis (WP 50) and the clinical isolates from 3 gingivitis and 3 periodontitis patients, using the broth dilution and antibiotic-sensitivity tests. The results revealed that the minimum inhibitory concentration (MIC) values of the essential oil against Actinomyces naeslundii and Porphyromonas gingivalis were 0.44 and 0.22 mg/ml, respectively. Furthermore, the results showed that some bacterial strains from clinical isolates could resist both concentrations. However, the black-pigmenting bacteria (BPB) could not be detected in any group. These resistant bacteria could be differentiated into 10 different groups, depending upon their antibiotic-sensitivity patterns to the following four antibiotics, i.e., tetracycline hydrochloride, ceftazidime, ampicillin and erythromycin. Five of the 10 groups were susceptible to the tested oil at the original concentration whereas only one group was sensitive to 10% tetracycline hydrochloride. It can be concluded that essential oil of Cymbopogon citratus has activities against both reference strains and majority of clinical-isolate groups, especially the tetracycline hydrochloride-resistant strains. The present study suggests the benefit of the use of essential oil to treat any tetracycline hydrochloride-resistant bacteria in combination with other antibiotics.***

**Key words:** Lemon grass (*Cymbopogon citratus*), Antibacterial activity, Tetracycline hydrochloride-resistant bacteria.

## INTRODUCTION

Periodontal disease, including gingivitis and periodontitis, is one of the most common oral diseases. Gingivitis is an inflammatory disease of the gum without periodontal destruction (Page, 1986). The major etiologic bacteria of gingivitis are *Actinomyces, Streptococcus, Fusobacterium, Veillonella* and *Treponema* species (Moore et al., 1989). Periodontitis causes destruction of the periodontium and leads to tooth loss (Page and Schroeder, 1976). The predominant microorganisms associated with periodontitis are mainly anaerobic and facultative anaerobic bacteria such as *Porphyromonas gingivalis, Prevotella intermedia, Aggregatibacter actinomycetemcomitans* (former name *Actinobacillus actinomycetemcomitans*) and *Tannerella forsythia* (former name *Bacteroides forsythus*) (Dzink et al., 1988). The aim of periodontal treatment is to eliminate the microorganisms and their by-products mainly by means of mechanical instrumentation. Antiseptics such as chlorhexidine (Palcanis, 1994) and antibiotics are also used as adjunctive therapy, especially in recurrent cases. Chlorhexidine mouthrinse is well accepted for the prevention of plaque accumulation and gingivitis (Brownstein et al., 1990; Chaves et al., 1994). Tetracyclines, metronidazole, amoxycillin and clindamycin are recommended as additional treatment (Walker and Gordon, 1990; van Winkelhoff et al., 1992; Christersson and Zambon, 1993).

Medicinal plants have been considered as optional antimicrobial agents. It has been shown that an extract from the root of *Sanguinaria canadensis* can inhibit several microorganisms (Tin-Wa et al., 1970) and reduce gingivitis (Moran et al., 1988). The same effect was found with an extract from *Psidium guajava* (Triratana and Amonchat, 1991). Recently, the antimicrobial activities of several Thai medicinal plants against oral microorganisms were reported (Sookhee et al., 2003). Carbajal et al., (1989) reported the activity of the essential oil from *Cymbopogon citratus* (lemon grass) in reducing blood pressure in animal models after venous application and antiinflammatory activity after oral application. Furthermore, high doses of the essential oil from Cymbopogon citratus showed no adverse effects on either the intestinal or central nervous systems in animal studies (Carlini et al., 1986). The aim of the present study was to determine the antimicrobial activities of the essential oil from *Cymbopogon citratus* (DC.) Stapf. (lemon grass) against some periodontopathogens, including the reference strains and clinical isolates associated with gingivitis and periodontitis.

## MATERIALS AND METHODS

### Essential oil extraction and quality control of the oil

The fresh leaves of *C. citratus,* grown in the northern part of Thailand (Fig. 1), were subjected to 3-hour hydrodistillation to produce the essential oil. The oil was first quality-controlled by GC-MS analysis. n-Hexane extract of the essential oil was dissolved in n-hexane (1 mg/ml). GC-MS was carried out with a gas chromatograph (HP 5890 Series II Gas Chromatograph, Hewlett Packard, USA), coupled with a mass spectrometer (HP 5971A Mass Selective Detector,

**Figure 1.** Cymbopogon citratus (lemon grass).

Hewlett Packard, USA) and a mass data system (HP G1030A MS Chemstation, Hewlett Packard, USA). The conditions used were as follows: column, DB-1 fused silica capillary (0.22 mm x 30 m, J&W Scientific); column temperature, 100°C (kept for 3 min), 100-300°C (increased at a rate of 15°C/min) and 300°C (kept constant for 5 min); carrier gas, He 33.1 ml/min; ionizing mode, El (70 eV).

**Patient selection**

Gingivitis and chronic periodontitis patients were recruited from the patients of the Faculty of Dentistry, Chiang Mai University. Three gingivitis patients (females, aged 24-30 yrs) exhibited inflammation of the gum and bleeding upon probing, with neither periodontal pocket formation nor periodontal destruction; while three patients with chronic periodontitis (2 males and 1 female, aged 40-55 yrs) showed destruction of the periodontal structures with deep periodontal pockets (>5mm). The criteria for patient selection were: non-smoking, non-pregnant persons with no previous use of antibiotics or no periodontal treatment 3 months prior to sample collection. All volunteers signed a consent form. The study was approved by the Ethics Committee of the Faculty of Dentistry, Chiang Mai University.

**Sample collection**

In the gingivitis patient group, supragingival plaque from the midlingual area of a lower molar was scraped once with a sterile Gracey's curette no. 7/8 (Aesculap®, Germany) throughout the crown height. The sample was transferred into a tube containing 0.2 ml of reduced transport fluid (RTF). In the chronic periodontitis patient group, supragingival plaque was removed with a sterile cotton pellet. Subgingival plaque was obtained by inserting a fine sterile paper point (Densply Caulk®, Milford, DE) into the periodontal pocket until resistance was felt and left in the pocket about 20 seconds. Then the paper point was transferred into a tube containing 0.2 ml of RTF. All of the samples were immediately carried to the Dental Research Center, Faculty of Dentistry, Chiang Mai University within 10 minutes for further processing.

**Bacterial preparation**

The clinical samples were homogenized and 100 µl of the supragingival sample from the gingivitis group was incubated in 10 ml of Brain Heart Infusion Broth (BHI broth) at 37°C in an aerobic condition for 2 days. One hundred microlitres of the subgingival sample from the periodontitis group was incubated in 10 ml of BHI broth, supplemented with 10 µg/ml of hemin and 1 µg/ml of menadione at 37°C in an anaerobic condition for 5 days. The reference bacteria, *Actinomyces naeslundii* (WVU 45), was incubated in BHI broth and *Porphyromonas gingivalis* (WP 50), which is black-pigmenting Bacteroides (BPB), was incubated in BHI broth supplemented with menadione and hemin. After the incubation period, the concentrations of all bacterial samples were diluted to a McFarland turbidity standard of 0.5 ($1.5x10^8$ colony forming unit (CFU) per ml) in order to calibrate the original amount of bacteria for the broth dilution test. For the negative control, a fine sterile paper point was used and incubated in the same condition on blood agar plate.

**Minimum inhibitory concentration (MIC) study**

Two-fold serial dilution of the essential oil was performed by mixing the essential oil with an equal volume of BHI broth. All bacterial samples, both reference strains and clinical isolates, were tested for determination of MIC which can reduce 90% of bacteria ($MIC_{90}$).

First, the broth dilution test was performed with the reference bacterial strains as shown in Table 1. Zero point two percent chlorhexidine (CHX) and 10% tetracycline hydrochloride (100 mg/ml TCN-HCl) were used as the positive control for gingivitis and periodontitis pathogens, respectively. After incubation, 100 µl of the mixed samples were cultured in duplicate on blood agar plates supplemented with 5% defribrinated human blood, 5 µg/ml hemin and 0.5 µg/ml of menadione and incubated in appropriate conditions and time. The bacterial colonies were counted in CFU/ml in both plates and recorded as the mean CFU/ml. The $MIC_{90}$ of the broth dilution test was interpreted as 90% reduction of the mean CFU/ml for each reference strain. Second, the known $MIC_{90}$ values of the reference strains were selected for use in the tests with clinical isolates following the methods described above.

**Antibiotic sensitivity test**

For the resistant bacteria from the MIC test, the antibiotic-sensitivity test was performed to confirm the antibacterial activity of the essential oil. The resistant bacteria were further tested with 4 standard antibiotics; tetracycline hydrochloride, ceftazidime, ampicillin and erythromycin by disk diffusion testing and classified according to their resistance patterns. The classified antibiotic-resistant bacteria were further tested with 10% TCN-HCl and the essential oil at the original concentration by the disk diffusion method (Kirby-Bauer test) (Yilmaz et al., 2007).

**Table 1.** The proportion of agents for the broth dilution test

| Substances | Amount of substances (ml) | | | |
|---|---|---|---|---|
| | Test sample | Negative Control | Positive Control 1 | Positive Control 2 |
| Diluted essential oil | 0.5 | - | - | - |
| BHI broth[a] | 0.5 | 1.0 | 0.5 | 0.5 |
| 0.2% CHX[b] | - | - | 0.5 | - |
| 10% TCN-HCl[c] | - | - | - | 0.5 |
| Bacterial suspension[d] | 0.5 | 0.5 | 0.5 | 0.5 |

[a]BHI broth = Brain Heart Infusion broth
[b]CHX = chlorhexidine
[c]TCN-HCl = Tetracycline hydrochloride
[d]Bacterial suspension = 1.5 x $10^8$ cells/ml

## RESULTS

The average yield of *C. citratus* oil obtained was 0.4%. The physical appearance of the oil was of a pale-yellow clear liquid. The oil obtained was composed mainly of citral a (62.9%) and citral b (33.9%) at the retention time of 5.21 and 4.76 min, respectively, as the identifiable major constituents in the GC-MS analysis.

In the bacterial reference strains group, it was found that the $MIC_{90}$ of the essential oil (citral a mixed with citral b) that could inhibit growth was 0.44 mg/ml for *A. naeslundii* (WVU 45) and 0.22 mg/ml for *P. gingivalis* (WP 50). For the bacteria obtained from gingivitis and periodontitis patients, it was found that the concentration of 0.44 and 0.22 mg/ml could not inhibit all of the anaerobic bacteria. Some white bacterial colonies could resist the essential oil as well as the positive control. However, the black- pigmenting anaerobic bacteria could not be seen in the blood agar plates as shown in Table 2.

The resistant bacteria, with white colony characteristics, were further tested with 4 standard antibiotics and classified according to the antibiotic-resistant pattern into 10 groups as shown in Table 3. The zone-diameter interpretive standard breakpoint, which is measured in mm for the organisms, is shown in Table 4. Then the 10 bacterial groups were tested again with 10% TCN-HCl and with the essential oil from *C. citratus* (894 mg/ml). The results demonstrated that the essential oil could inhibit half of the resistant groups as presented in Table 5 while 10% TCN-HCl could inhibit only one of 10 groups.

**Table 2.** The antimicrobial activities of the essential oil and positive control.

| Clinical isolation (G[a]/P[b]) | Essential oil[c] | | Positive control[d] | |
|---|---|---|---|---|
| | BPB | Other bacteria | BPB | Other bacteria |
| G1 | - | + | - | + |
| G2 | - | - | - | + |
| G3 | - | + | - | + |
| P1 | - | + | - | + |
| P2 | - | + | - | + |
| P3 | - | + | - | + |

[a]G =Gingivitis
[b]P =Periodontitis
[c]at $MIC_{90}$ 0.44 or 0.22 mg/ml
[d]0.2% Chlorhexidine or 10% TCN-HCl
+ = Presence - = Absence

**Table 3.** Antibiotic-resistant patterns.

| Group | Antibiotic-resistant pattern | | | |
|---|---|---|---|---|
| | TCN-HCl | Ceftazidime | Ampicillin | Erythromycin |
| 1 | S[a] | S | S | S |
| 2 | R[b] | RR | R | RR |
| 3 | R | R | R | RR |
| 4 | I[c] | RR | R | RR |
| 5 | R | RR | RR | RR |
| 6 | RR[d] | S | R | RR |
| 7 | R | R | R | I |
| 8 | R | S | R | S |
| 9 | I | R | S | R |
| 10 | R | S | R | RR |

[a]S = Susceptible
[b]R = Resistant but with some clear zone
[c]I = Intermediate
[d]RR = Absolute resistance

**Table 4.** The zone-diameter interpretive standard breakpoint for otherorganisms.

| Antibiotic | Disk concentration (μg/ml) | Zone diameter (mm) | | |
|---|---|---|---|---|
| | | Resistant (≤) | Intermediate | Susceptible (≥) |
| TCN-HCl | 30 | 14 | 15-18 | 19 |
| Ceftazidime | 30 | 14 | 15-17 | 18 |
| Ampicillin | 10 | 13 | 14-16 | 17 |
| Erythromycin | 15 | 13 | 14-22 | 23 |

(Adapted in part from NCCLS Document M100-S6: Sixth International Supplement, Performance Standards for Antimicrobial Susceptibility Testing).

**Table 5.** Inhibition zone of 10% Tetracycline hydrochloride and the essential oil.

| Group | Average zone of inhibition (mm) | |
|---|---|---|
| | 10% TCN-HCl (100 mg/ml) | Lemon grass extract (894 mg/ml) |
| 1. S,S,S,S | 32.5[a] | 25[a] |
| 2. R,RR,R,RR | 8 | 50[a] |
| 3. R,R,R,RR | 9 | 35[a] |
| 4. I,RR,R,RR | 0 | 16 |
| 5. R,RR,RR,RR | 7 | 14 |
| 6. RR,S,R,RR | 10 | 0 |
| 7. R,R,R,I | 10 | 8 |
| 8. R,S,R,S | 10 | 0 |
| 9. I,R,S,R | 13 | 21[a] |
| 10. R,S,R,RR | 9 | 50[a] |

[a]Sensitive (clear zone ≥ 19 mm).

## DISCUSSION AND CONCLUSION

The results showed that the essential oil from *C. citratus*, when used in different concentrations, could inhibit the oral bacteria, especially the reference strains. This finding was similar to several studies on the antimicrobial activities of the essential oil from *C. citratus* (Kokate and Verma, 1971; Gyane, 1976; Chiori et al., 1977; Agarwal et al., 1980). Furthermore, Sookkhee et al., (2003) reported that the essential oil from *C. citratus* showed the most anticandidal activity among 18 tested essential oils from Thai medicinal plants.

For the positive control of *A. naeslundii* (WVU 45), 0.2% chlorhexidine is well accepted because of its properties against Gram-positive and -negative bacteria and candida. Normally, chlorhexidine has a bacteriostatic activity, but in high concentrations (18-32 μg/ml), it can be bactericidal. The positive charge of chlorhexidine interacts with the negative charge of the bacterial cell wall and causes damage or death of the bacterial cell by precipitating the cytoplasm (Lang

and Brecx, 1986; Kornman, 1990). Stanley et al., (1989) studied the MIC of chlorhexidine in 52 bacterial strains and found that the concentrations of 8-62 µg/ml and 8 µg/ml could inhibit *Actinomyces* spp. and *P. gingivalis*, respectively, but for the clinical isolates, the required concentration of chlorhexidine was much higher (250 µg/ml). The major side effects of chlorhexidine, especially in mouthrinse form, are tooth or soft tissue staining and unpleasant taste.

The positive control for subgingival pathogens in this study was 10% TCN-HCl. It is a broad-spectrum antibiotic which can inhibit bacterial protein systhesis. Kleinfelder et al., (1999) studied the antibiotic susceptibility of oral bacteria and found that TCN-HCl in concentrations of 0.016-2 µg/ml could inhibit *P. gingivalis*. Gordon et al. (1980) also reported that 48 hours after single or multiple doses, TCN-HCl concentration in crevicular fluid was about 4-8 µg/ml. In addition to the side effects and TCN-HCl allergy, it was found that this antibiotic could not inhibit some bacterial species such as *Streptococcus* spp., *Actinomyces* spp., and *Eikenella corrodens* (Seymour et al., 1992). Walker (1996) reported that 20-30% of microorganisms from clinical isolates from adult periodontitis patients could be resistant to TCN-HCl. Because of the side effects and the bacterial resistance to the chemical agents, medicinal plants are suggested as a treatment modality to control microorganisms. Onawunmi et al., (1984) identified the constituents of the essential oil from *C. citratus*. They included myrcene, α-citral and β-citral. Both α-citral and β-citral have antibacterial activity while myrcene has no antibacterial effect.

In our study, the clinical isolates were obtained from 3 gingivitis and 3 periodontitis volunteers. It was found that some bacterial strains could be resistant to both essential oil concentrations (0.44 and 0.22 mg/ml) and to the positive controls. It was noted that the bacterial samples from the patients were obtained in the form of dental plaque or biofilm. Biofilm is composed of microcolonies of bacterial cells (15-20% by volume) and is often composed of several different bacterial species (Marsh and Bradshaw, 1995). Some functions of biofilm depend upon the ability to transfer informations among bacteria in microcolonies within the biofilm such as antibiotic-resistant ability (Wilson, 1996). Slots and van Winkelhoff (1993) suggested using the higher concentrations of the antimicrobial agents that are hundreds of times greater than the MIC. Olsen et al. (2002) also suggested that because of the different susceptibilities of bacteria in dental plaque, a combination of antibiotics should be selected. The resistant bacteria in our study demonstrated white and round colonies in different sizes on the agar surface and showed resistance to TCN-HCl. Similarly, Olsvik et al., (1995) found that 23% of the subgingival bacteria from periodontitis patients were resistant to 10 µg/ml TCN-HCl. Recently, Ready et al., (2002) demonstrated that the composition and the resistance profile of the microoraganisms were changed from 6 to 45% after exposure to TCN-HCl. They concluded that TCN-HCl could change the composition of the biofilm and elevate the proportion of the TCN-HCl-resistant strains. Although TCN-HCl proved to be effective against the reference bacteria *in vitro*, but in the clinical isolates, as a biofilm, all of the bacteria could not be inhibited.

However, in the present study, black-pigmenting anaerobic bacteria (including *P. gingivalis*) were not found in any test.

The resistant bacteria in this study were further investigaterd by antibiotic-sensitivity testing with standard disks of TCN-HCl, ceftazidime, ampicillin and erythromycin. The resistant bacteria were divided into 10 groups according to their resistance patterns. All resistant groups were tested again with the essential oil and 10% TCN-HCl. The results showed that 5 of 10 groups were susceptible to the essential oil at the concentration of 894 mg/ml while 10% (100 mg/ml) TCN-HCl could inhibit only one group (group S,S,S,S see Table 4). Several studies reported that other antibiotics such as penicillin (Listgarten et al., 1993), metronidazole (Listgarten et al., 1993; Kleinfelder et al., 1999), amoxicillin plus clavulanate (Kleinfelder et al., 1999), cephalosporins and chloramphenicol (Pacini et al., 1997) have the antimicrobial activities against the TCN-HCl-resistant strains. In the present study, it was found that the essential oils from medicinal plants offer a new choice for combination therapy against periodontal pathogens, especially the essential oil from lemon grass (*Cymbopogon citratus*).

From the study, it was concluded that the essential oil from *Cymbopogon citratus* (DC.) Stapf. (lemon grass) showed antimicrobial activity against periodontal pathogens, especially the reference strains *A. naeslundii* and *P. gingivalis*. From the clinical isolates, there were some TCN-HCl-resistant strains. Five of 10 groups of the resistant strains were inhibited by the essential oil at the original concentration. The results showed the superior properties of the essential oil and suggested the use of the essential oil with other antibiotics against resistant bacteria.

## ACKNOWLEDGEMENTS

The authors are grateful to Professor Kevin O' Carrol, Faculty of Dentistry, Chiang Mai University, for excellent guidance and improvement of this investigation as well as language correction. Furthermore, the authors wish to thank the Dental Research Center, Faculty of Dentistry, Chiang Mai University, as well as the sixth-year dental students Surichai Saisamut, Tongchat Suwantantula, Ratakhet Trisutacheep and Akin In-dee for providing laboratory support. The financial support of the Internal Research Grant, Faculty of Dentistry, Chiang Mai University is gratefully acknowledged.

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