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Evaluation of Plant Essential Oils as Natural Alternatives for Alcohol-based Mouthwashes: Spotlight—Lemongrass and Citronella Java

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Abstract

Objective The purpose of our study was to evaluate plant-derived essential oils (EOs) as natural alternatives to commercial alcohol-based mouthwashes in the prevention of dental caries since several recent studies have linked high incidence of oral cancer among users with a history of prolonged use of alcohol-based mouthwashes.

Materials and Methods Lemongrass, Citronella Java, Gingergrass, and Caraway seed EOs were tested against commonly occurring multidrug-resistant (MDR) oral bacteria namely *Micrococcus luteus*, *Enterococcus faecalis*, *Streptococcus oralis*, and *Streptococcus salivarius*. Agar well diffusion method was used to determine the antibacterial effectiveness of these EOs. Samples of Citronella Java and Lemongrass EO were also analyzed by gas chromatography (GC).

Results Lemongrass and Citronella Java exhibited the highest antibacterial activity against all four bacterial strains. Inhibition zones of Lemongrass were 12, 21.3, 28.3, and 32 mm in diameter against *E. faecalis*, *M. luteus*, *S. oralis*, and *S. salivarius*, respectively. In comparison, inhibition zones of Citronella Java were 11.5, 17, 20.7, and 20.2 mm in diameter against *E. faecalis*, *M. luteus*, *S. oralis*, and *S. salivarius*, respectively. A significant finding in our study was that antibacterial activity of Lemongrass was much higher than that of tetracycline, a broad-spectrum antibiotic, against *S. oralis* and *S. salivarius*, while the inhibitory effects of Citronella Java against these two oral streptococci were comparable to tetracycline. The major components of Citronella Java identified by GC were citronellal, citronellol, and geraniol, whereas Lemongrass was primarily composed of cis and trans forms of citral.

Conclusion Our results suggest that Lemongrass and Citronella Java could be promising natural alternatives to alcohol-based mouthwashes against MDR oral bacteria in the prevention of dental caries.

Keywords
- essential oils
- oral cancer
- alcohol-based mouthwashes
- oral microbiome
- gas chromatography
- Gram staining
- agar well diffusion

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Introduction

Despite recent implementation of public health strategies, dental caries remains the most costly and prevalent noncommunicable oral disease worldwide.[1] Dental caries (also known as tooth decay or dental cavities) is a multifactorial disease with several biological, environmental, and sociobehavioral risk factors.[2] Dental caries develops when tooth-adherent cariogenic bacterial aggregates produce extracellular polymeric substances known as biofilms.[3] Through frequent consumption of sugars and use of food preservatives, such as benzoates, sulfites, and nitrites, bacterial metabolism produces lactic acid, which adversely demineralizes and decays tooth structure over time.

Prevalence of Dental Caries

Dental caries is a major global public health crisis. According to the National Institutes of Health and Centers for Disease Control and Prevention, the prevalence of untreated and treated dental caries among youth aged 2 to 5 years is 21.4%, 6 to 11 years is 50.5%, and 12 to 19 years is 53.8%. In adults, this prevalence rises to a staggering 90% among adults aged 20 to 64 years and 96% among adults aged 65+ years. Data from both youth and adult age groups were evaluated in the most recent National Health and Nutrition Examination Survey (2011–2016).

The Oral Microbiome

When Anton van Leeuwenhoek, the father of microbiology, observed dental plaque under his homemade microscope in 1674, he referred to the tiny moving structures as "animalcules." Today, these "animalcules" are known as microbes and include bacteria, archaea, fungi, viruses, and protozoa, and many are thought to underpin oral and systemic diseases.[4] Harboring over 700 species of bacteria, the oral cavity is the second largest and diverse microbiota after the gut in humans.[5] The hard surfaces of the teeth and soft tissue of the oral mucosa make it an ideal environment for the colonization of common microbes that promote dental caries such as *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus salivarius*, and *Enterococcus faecalis*, among many others. This ecological community of symbiotic, commensal, and pathogenic bacteria make up the oral **microbiome**, a term coined by Nobel prize laureate, Joshua Lederberg.[5]

Composition of the Oral Microbiome

The diverse oral microbiome is one of the most well-studied niches in the oral cavity and comprises a total of 392 taxa and 1,500 genomes.[6] Within it, approximately 700 species of prokaryotes have been identified, of which 54% are officially named, 14% are unnamed (but cultivated), and 32% are uncultivated.[7] The principal bacterial genera found in healthy oral cavities include both gram-positive and gram-negative cocci and rods.[8] Nonbacterial members include protozoa, fungi, and viruses. As mentioned earlier, the diversity of the human oral microbiome is individually specific due to factors such as usage of antibiotics, dietary lifestyle, smoking, and diseases.[9]

Biological Evolution of the Oral Microbiome

Today, oral diseases impact over half of the world's population (3.58 billion people), and the etiology of oral diseases has become increasingly important to prevent their onset. Microbes first came into existence approximately 1.5 billion years ago and have been performing metabolic functions in animals for at least 500 million years.[10] Bacteria that reside in the oral mucosa form highly regulated, structurally, and functionally organized communities known as biofilms which attach to hard tooth surfaces and break down enamel. Bacteria in the oral microbiome communicate with each other through a process called quorum sensing which is conducive for host colonization, biofilm formation, defense against competing bacteria, and environmental changes. Quorum sensing activities in biofilms also stimulate the virulence and pathogenic potential of bacteria. However, not all bacteria in the oral microbiome are virulent and pathogenic.[11]

The oral microbiome plays key roles in human biology, health, and disease, but little is known about the global diversity, variation, or evolution of this microbial community. However, research studies have shown through the reconstruction of oral metagenomes from up to 100,000 years ago that the microbial profiles of Neanderthals and modern humans are highly similar, specifically in the salivary amylase-binding capability with oral bacterial streptococci.[12] These findings indicate that both Neanderthals and modern humans share functional adaptations in nutrient metabolism and suggest microbial coadaptation with host diet. These findings further suggest that due to the preservation of oral metagenomes of later African hominids, the discovery of natural oral antiseptics is vital in an era of increasing population toward the prevention of dental caries.

Herbal Mouthwashes

In the last decade, there has been renewed interest to replace artificial preservatives with natural and nontoxic compounds, such as essential oils (EOs), to aid in food preservation and prevent the onset of systemic disease. Recent research has also shown a possible correlation between prolonged alcohol use in oral antiseptics and oral cancer.[13] While the possibility of alcohol-containing mouthwashes contributing to the development of oral cancer is not a new proposition, some studies have shown the possible link between the daily use of alcohol-containing mouthwashes and development of head and neck cancer.[13–15]

Herbal medicines, which are generally less concentrated than EOs, are derived from botanical sources and contain a mixture of active ingredients, such as catechins, tannins, and sterols.[16] Historically, they have been applied in dentistry to inhibit microorganisms, reduce inflammation, soothe irritation, and relieve pain.[17–19] It has been recently reported that

several herbal mouthwashes have achieved encouraging results in the control of plaque and gingivitis.[20] Further, the efficacy of these herbal mouthwashes on the management of various oral pathologies is comparable to commonly used antibacterial synthetic chemicals and alcohol-based mouthwashes.[21,22]

In a recent study conducted by Tidke et al (2022), aloe vera, a potent antimicrobial agent, was shown to be effective in removing plaque and ameliorated symptoms of dental caries, including tooth hypersensitivity, toxicity, and tooth staining.[22] Notably, aloe vera was found to be equally effective as chlorhexidine (CHX) mouthwash, a nonherbal antiseptic medication that is considered the gold standard in the prevention of dental plaque and treatment of gingivitis and periodontitis.[2,23] A similar study by Jeddy et al (2018) also showed the effectiveness of reducing bacterial load by herbal mouthwashes containing the active ingredient red ginseng, a potent anti-inflammatory agent and antioxidant, in preventing dental caries.[24]

Although a 2008 study reported neem, a tropical plant native to India, to be less effective than CHX, it was still shown to be effective against the onset of dental caries.[18] However, it should be noted that CHX is not meant to be applied on a long-term basis due to side effects including brownish discoloration of teeth, oral mucosal lesions, parotid swelling, and colorization of teeth and fillings.[25–28] Consequently, adverse side effects, including mild irritant contact dermatitis and life-threatening anaphylaxis, have also been reported with prolonged CHX usage.[29] In efforts to find natural alternatives against dental caries, several additional herbal extracts with significant antimicrobial and anti-inflammatory properties, such as *Carica papaya* leaf extract, chamomile, Echinacea, sage, and *Camellia sinensis*, have all demonstrated to be effective alternatives to CHX in the prevention of dental plaque and treatment of gingivitis and periodontitis.[19] Accordingly, these results suggest that herbal mouthwashes provide a safer alternative and are equally effective as nonherbal mouthwashes for reducing dental plaque and various oral pathologies in the short term. Additional qualitative research is necessary to investigate the therapeutic effects of herbal mouthwashes against dental caries in the long term.

Like herbal medicines, EOs contain antimicrobial properties and are also known as volatile oils. EOs are aromatic oily liquids derived from plant materials such as flowers, buds, seeds, leaves, twigs, bark, herbs, fruits, and roots.[22] An estimated 3,000 EOs are known today, and several are used medicinally. Tea tree oil, lavender oil, thyme oil, peppermint oil, and clove (eugenol) oil have been used by people to treat ailments and tooth decay. Specifically, clove oil has been used topically in dental practice to treat pulpits and dental hypersensitivity for thousands of years.[22] To our knowledge, caraway seed, lemongrass, gingergrass, and Citronella Java EOs have not been investigated on common oral bacteria, including *E. faecalis*, *Lactobacillus acidophilus*, *S. salivarius*, and *Streptococcus oralis*. We wanted to study the chemical composition and antimicrobial activity of these EOs on four common oral bacteria and evaluate their effectiveness as natural plant alternatives to alcohol-based mouthwashes.

Materials and Methods

Materials

EOs were purchased from Bulk Apothecary (Aurora, OH). Bacterial strains were obtained from Carolina Biological Supply Company (Burlington, NC) and American Type Culture Collection (Manassas, VA). Chemicals and reagents were from Fisher Scientific (Pittsburgh, PA) and Carolina Biological Supply Company (Burlington, NC).

Essential Oils

Four EOs were evaluated in this study namely, Lemongrass (*Cymbopogon flexuosus*), Citronella Java (*Cymbopogon winterianus*), Gingergrass (*Cymbopogon martini*), and Caraway seed (*Carum carvi*). Purity of these extracts was ascertained during steam distillation. They were stored in a cool, dry place in dark glass bottles to minimize their oxidation.

Growth and Maintenance of Bacterial Cultures

Four bacterial strains were chosen for this study namely, *Micrococcus luteus*, *E. faecalis*, *S. oralis*, and *S. salivarius*. *Micrococcus luteus* was grown on tryptic soy agar (TSA, Difco, Detroit, MI). *Enterococcus faecalis* was grown on brain heart infusion agar (Difco, Detroit, MI). Both *S. salivarius* and *S. oralis* were grown on TSA with 5% sheep blood (Carolina Biological Supply Company, Burlington, NC) in 5% CO_2. All strains were propagated at 37°C overnight (~16–18 h). Bacterial cultures were maintained on sterile agar plates at 4°C and fresh plates were restreaked every 2 to 3 weeks.

Gram Staining

All bacterial strains were streaked for single colonies under aseptic conditions. Plates were then incubated for 24 hours at 37°C. Standard Gram staining procedure was followed with well-isolated single colonies to corroborate colony morphology.[30]

Agar Well Diffusion Assay

Agar well diffusion assay was adapted from Kirby–Bauer disc diffusion method.[30] A 100 µL of each bacterial strain (OD_{600} (Optical Density at 600 nm wavelength) = 0.25 – roughly 10^7 cells per plate) was spread evenly onto sterile agar plates. The plates were then allowed to dry for approximately 10 minutes. One such agar plate with each bacterial strain without any EO served as a negative control for that set. For experimental trials, a sterile Pasteur pipette was used to cut 10-mm wells at the center of each agar plate. In total, 20 µL of the EO was added to the well. Discs containing 30 µg of tetracycline were tested in parallel as a positive control on all four bacterial strains. Trials with tetracycline were done at least twice to ensure that results were consistent. Plates were left undisturbed and incubated for approximately 20 hours at 37°C. Diameters of the zone of inhibition was measured for each case and recorded. Experimental trials were performed on all the bacterial strains with all four EOs at least three times, and values were averaged.

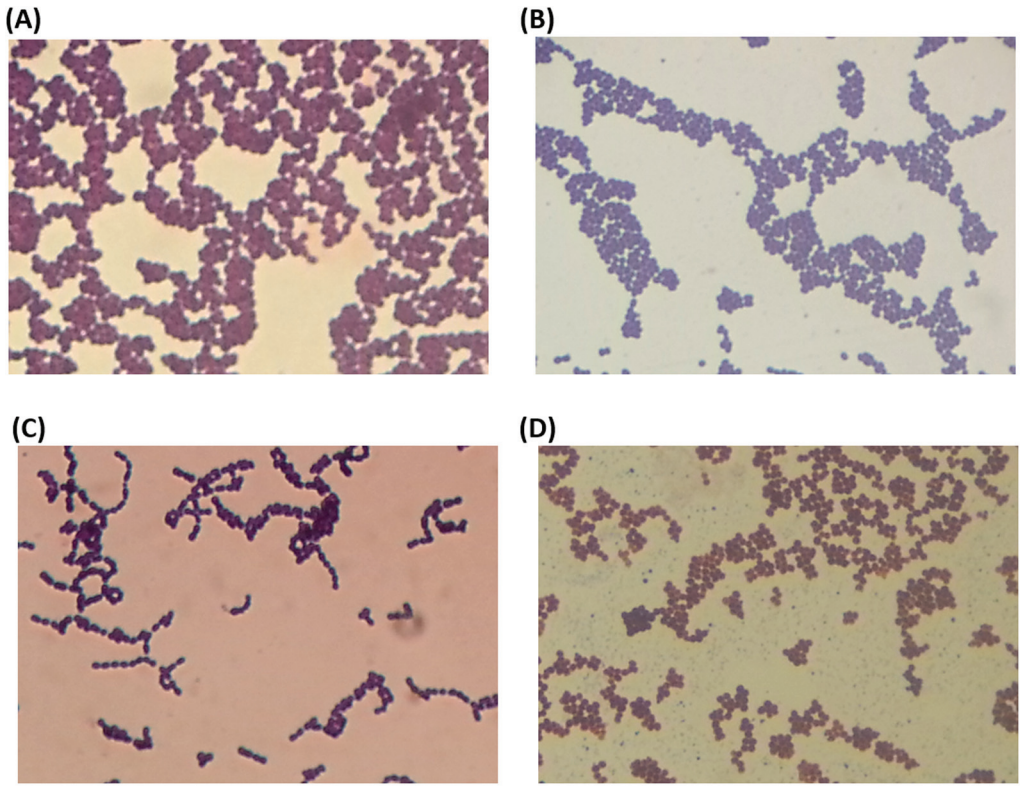

Fig. 1 Gram stains of oral bacteria used in this study. **(A)** *Enterococcus faecalis*. **(B)** *Streptococcus salivarius*. **(C)** *Streptococcus oralis*. **(D)** *Micrococcus luteus*.

Gas Chromatography Analysis

Citronella Java, *C. winterianus* (Aura Cacia, Norway, IA) and Lemongrass EO, *C. flexuosus* (Nature's Oil, Statesboro, OH) steam distilled extracts were diluted 1:200 in diethyl ether. Samples for gas chromatography (GC)-flame ionization detection (FID) were analyzed on a Shimadzu GC-2010 Plus (Columbia, MD) equipped with an AOC-20i Autosampler. Shimadzu LabSolutions Lite software was used for analysis. The column flow rate was 1 mL/min (helium carrier gas, and the split ratio was 1:39). A Shimadzu SHRXI-5MS column (15 m, 0.25 mm inner diameter (ID), 0.25 µm film thickness) was used for all separations. The program was started at 50°C for 2 minutes. The temperature was increased at 20°C/min to 200°C and held for 1 minute. The total run time was 10.5 minutes. Injector and detector temperatures were held constant at 250 and 280°C, respectively. Authentic samples of citronellal, citronellol, citral (mixture of cis and trans isomers), and geraniol were all purchased from Acros Organics with ≥95% purity.

Cymbopogon winterianus and *C. flexuosus* samples were also analyzed using a Shimadzu GCMS–QP2010SE equipped with an AOC-20i Autosampler. Shimadzu LabSolutions GCMSsolution Version 4.20 software was used for analysis. The column flow rate was 1 mL/min (helium carrier gas, and the split ratio was 1:10). A Shimadzu SH-Rxi-5silMS column (30 m, 0.25 mm ID, 0.25 µm film thickness) was used for all separations. The program was started at 50°C for 2 minutes. The temperature was increased at 20°C/min to 250°C and held for 5 minutes. The total run time was 17 minutes. Ion source and interface temperatures were held constant at 230 and 250°C, respectively.

Results

Gram Staining

Gram staining protocol was followed with well-isolated single colonies as per standard procedure.[30] All the bacterial strains used in this study stained purple and were spherical/ovoid in shape. Hence, they were identified as gram-positive cocci. *Enterococcus faecalis* and *S. salivarius* colonies appear as clusters. *Streptococcus oralis* colonies appear as short and medium chains. *Micrococcus luteus* colonies appear as tetrads and short chains (►**Fig. 1**). These findings are consistent with previously published morphology results for these bacterial strains.[30]

Agar Well Diffusion Assay

Agar well diffusion assay was performed with four EOs, namely Lemongrass, Gingergrass, Caraway seed, and Citronella Java, on all the bacterial strains described earlier. Among all the EOs that were tested, Lemongrass and Citronella Java exhibited the highest antibacterial activity against all four bacterial strains as evident from the diameters of their zones of inhibition (►**Figs. 2** and **3**). Inhibition zones made by Lemongrass were 12, 21.3, 28.3, and 32 mm in diameter against *E. faecalis*, *M. luteus*, *S. oralis*, and *S. salivarius*, respectively. Inhibition zones made by Citronella Java were 11.5, 17, 20.7, and 20.2 mm in diameter against *E. faecalis*, *M. luteus*, *S. oralis*, and *S. salivarius*, respectively. Both oils were most effective against *S. oralis* and *S. salivarius* (►**Tables 1–4**). Of notable mention is that antibacterial

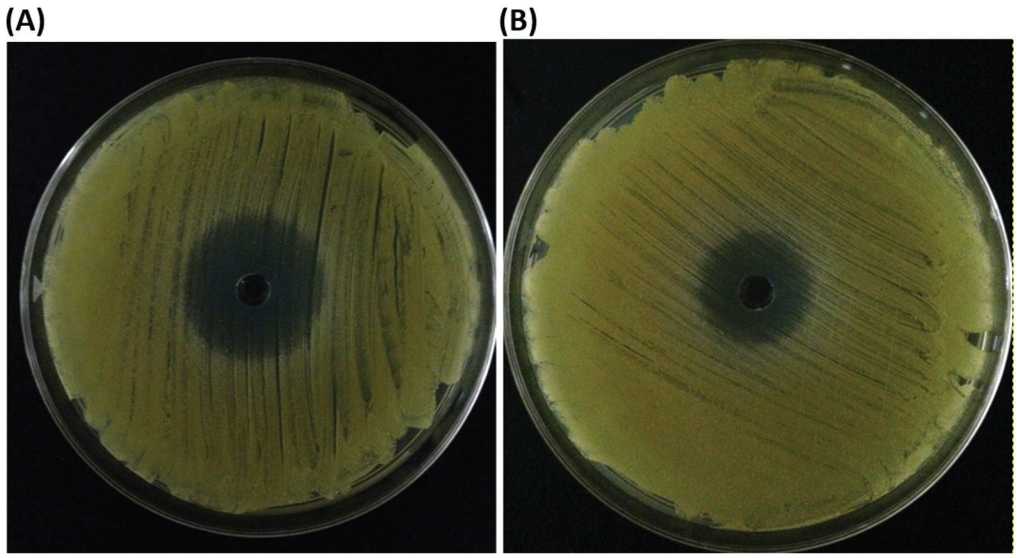

Fig. 2 Agar well diffusion assay with *Micrococcus luteus.* (**A**) Lemongrass. (**B**) Citronella Java.

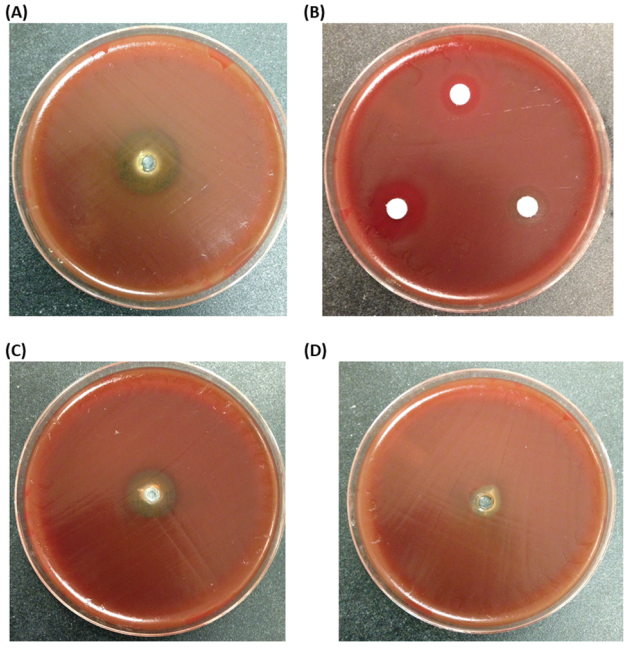

Fig. 3 Agar well diffusion assay with *Streptococcus oralis*. (**A**) Lemongrass. (**B**) Penicillin, neomycin, and tetracycline (clockwise from top). (**C**) Citronella Java. (**D**) Gingergrass.

activity of Lemongrass was much higher than that of the broad-spectrum antibiotic, tetracycline, on both these strains, while Citronella Java's inhibitory effects were comparable to that of tetracycline (►**Tables 3** and **4**). Tetracycline was used a positive control standard to make comparisons in this study.

Gas Chromatography

Samples of Citronella Java, from *C. winterianus*, and Lemongrass EO, from *C. flexuosus*, were analyzed by GC (►**Figs. 4**–**6**). The major components of *C. winterianus* were citronellal, citronellol, and geraniol, whereas *C. flexuosus* is primarily composed of cis and trans forms of citral (►**Table 5**). These compounds were initially identified using the GCMS library match software and subsequently verified by purchasing purified compounds (authentic samples) and comparing their GC retention times. The compounds identified within these oil extracts agree with other literature reports for these same species.[31,32] Additionally, a 1:1 mixture of the *C. winterianus* and *C. flexuosus* shows the individual compounds have different retention times and the compositions of the extracts from the different species are unique (►**Fig. 6**).

Table 1 Diameters of zones of inhibition of compounds tested against *Micrococcus luteus*

	Trial I	Trial II	Trial III	Average
Lemongrass	22	22	20	21.3
Gingergrass	11	10	11	10.7
Caraway seed	11	11	10	10.7
Citronella Java	18	16	17	17
Tetracycline	21	21	22	21.3

Diameter of zone of inhibition (mm).

Table 2 Diameters of zones of inhibition of compounds tested against *Enterococcus faecalis*

	Trial I	Trial II	Trial III	Average
Lemongrass	12	11	13	12
Gingergrass	11	9	11.5	10.5
Caraway seed	9.5	7	9	8.5
Citronella Java	11.5	12	11	11.5
Tetracycline	21	19.5	NA	20.3

Abbreviation: NA, not available. Diameter of zone of inhibition (mm).

Table 3 Diameters of zones of inhibition of compounds tested against *Streptococcus oralis*

	Trial I	Trial II	Trial III	Average
Lemongrass	28	28	29	28.3
Gingergrass	12	13	14	13
Caraway seed	11	12.5	10.5	11.3
Citronella Java	22	20	20	20.7
Tetracycline	18	17	NA	17.5

Diameter of zone of inhibition (mm).

Table 4 Diameters of zones of inhibition of compounds tested against *Streptococcus salivarius*

	Trial I	Trial II	Trial III	Average
Lemongrass	32	31.5	32.5	32
Gingergrass	12	14	12	12.7
Caraway seed	11	11	13	11.7
Citronella Java	17.5	21	22	20.2
Tetracycline	22	22	NA	22

Diameter of zone of inhibition (mm).

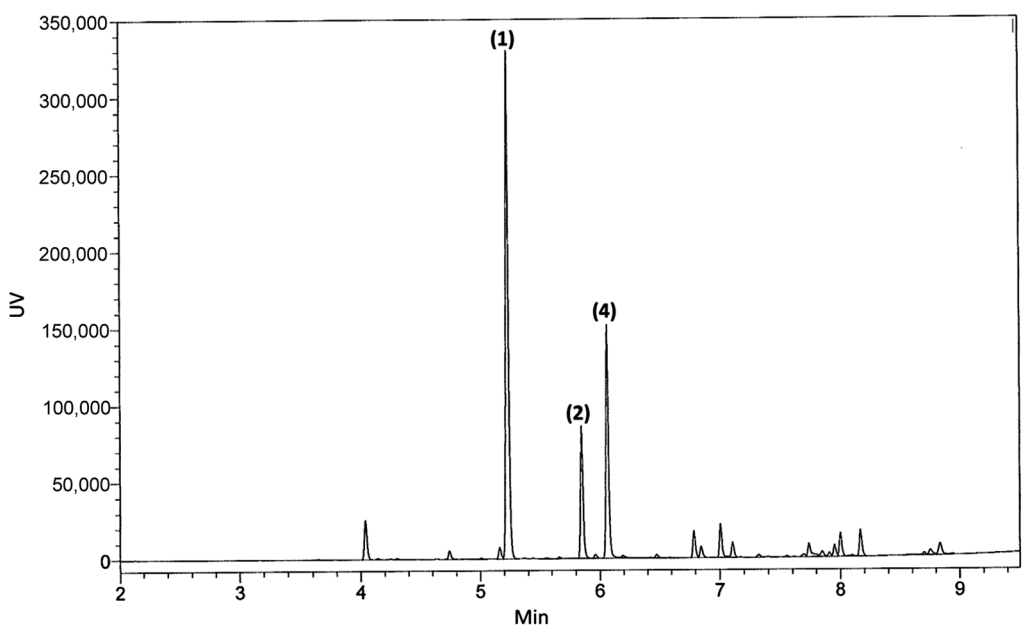

Fig. 4 GC chromatograph of the steam extracted *Cymbopogon winterianus* sample. Major components present are citronellal **(1)**, citronellol **(2)**, and geraniol **(4)**. GC, gas chromatography; UV, ultraviolet.

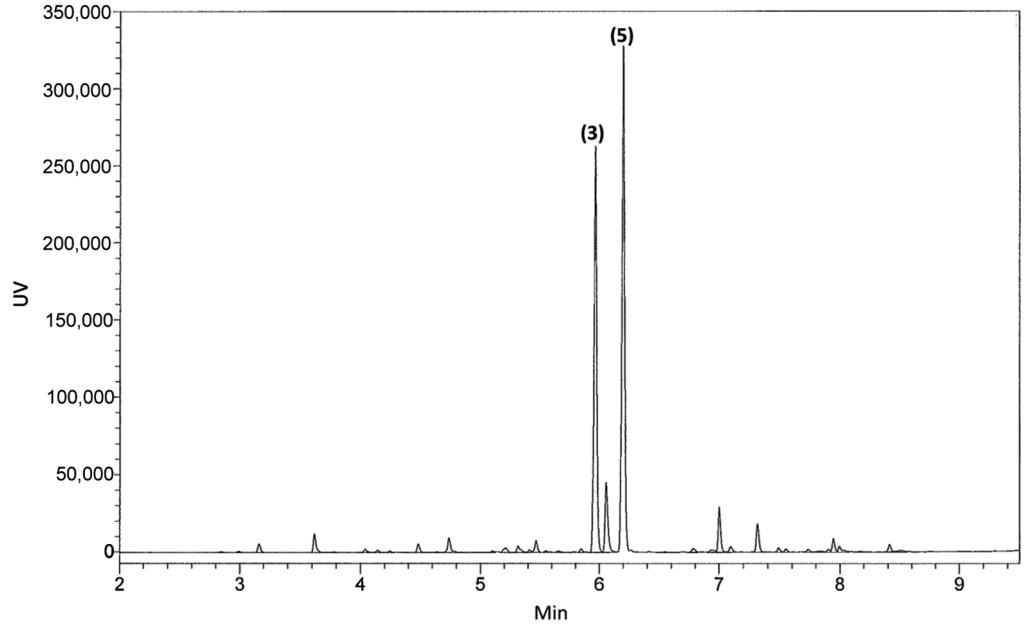

Fig. 5 GC chromatograph of the steam extracted *Cymbopogon flexuosus* sample. Major components present are the two isomers (neral and geranial) of citral **(3)** and **(5)** in nearly equimolar concentrations. GC, gas chromatography; UV, ultraviolet.

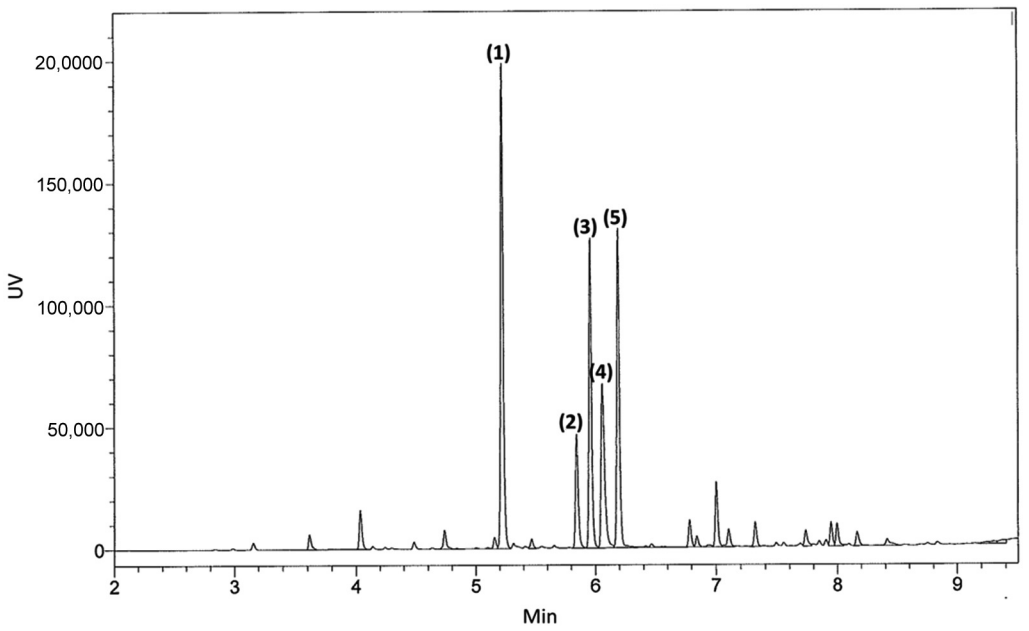

Fig. 6 GC chromatograph of a 1:1 mixture of the steam extracted *Cymbopogon winterianus* and *Cymbopogon flexuosus* samples. Chromatograph shows the major components from the extraction are unique to each species. Citronellal **(1)**, citronellol **(2)**, and geraniol **(4)** are present in *C. winterianus*, whereas the two isomers of citral **(3)** and **(5)** are present in *C. flexuosus*. GC, gas chromatography; UV, ultraviolet.

Discussion

The aim of this study was to evaluate plant-derived EOs as natural alternatives to commercial alcohol-based mouthwashes. Several recent studies have linked high incidence of oral cancer among users with a history of prolonged use of alcohol-based mouthwashes.[15,32–35] We wanted to identify compounds that occur naturally in plants which would have similar effectiveness as chemical mouthwashes. We evaluated the antibacterial effects of four EOs namely Lemongrass, Gingergrass, Caraway seed, and Citronella Java on bacterial strains that are commonly found in the oral cavity. The oral bacteria chosen for this study were *E. faecalis*, *M. luteus*, *S. oralis*, and *S. salivarius*. All of these are multidrug resistant (MDR), making them ideal candidates for our study.[36,37]

Table 5 Gas chromatography retention times of individual *Cymbopogon winterianus* and *Cymbopogon flexuosus* steam extractions

	GC retention times (minutes)			
Compound	***Cymbopogon winterianus* alone**	***Cymbopogon flexuosus* alone**	***Cymbopogon winterianus: Cymbopogon flexuosus* (1:1)**	**Authentic samples**
Citronellal	5.230 (44%)	–	5.221	–
Citronellol	5.848 (11%)	–	5.844	5.865
Citral (isomer 1)	–	5.965 (37%)	5.959	5.970
Geraniol	6.063 (20%)	–	6.058	6.085
Citral (isomer 2)	–	6.199 (38%)	6.189	6.202

Abbreviation: GC, gas chromatography.
Mixture of *Cymbopogon winterianus* and *Cymbopogon flexuosus* samples, and purchased purified compounds (authentic samples). Values in parenthesis indicate the relative abundance of the compound within the extraction mixture.

Among the four EOs that were tested, Lemongrass and Citronella Java exhibited the highest antibacterial effect on all bacterial strains. Both had similar activity profiles to that of the positive control, Tetracycline on *M. luteus*. Although inhibitory, they were unable to prevent growth of *E. faecalis* to the same extent as Tetracycline. However, both were highly effective against *S. oralis* and *S. salivarius* (►**Tables 1**–**4**).

Multidrug resistance to antibiotics exhibited by oral bacteria is a serious global threat.[38] Among innovative approaches that are currently being tested against *E. faecalis*, disruption of quorum sensing and dalbavancin (a vancomycin analogue) look encouraging.[39] Our preliminary study demonstrates that EOs from Lemongrass and Citronella Java disrupt growth of *E. faecalis* to a reasonable extent (►**Table 2**).

Oral streptococci are currently classified into six phylogenetic groups. *Streptococcus oralis* and *S. salivarius* used in our study belong to the Mitis and Salivarius groups, respectively.[40] While both these strains are typical nonpathogenic oral and intestinal commensals, several occurrences of invasive infections such as meningitis, endocarditis, and bacteremia have been reported. Recent approaches to fight oral streptococci include novel antibiotics isolated from cultures of soil microbe extracts, antibiotic analogues across various classes, and bacteriophage–antibiotic-augmented treatment. While studies demonstrate that they are effective in targeting structural integrity and various metabolic processes of some drug-resistant oral streptococcal genera, no one approach is universal.[37,38] Of noteworthy mention is the fact that Lemongrass was a lot more efficient than the broad-spectrum antibiotic Tetracycline at preventing the growth of both streptococcal strains that were tested by us (►**Tables 3** and **4**). This would suggest that Lemongrass can potentially be used in oral mouth rinses synergistically with other successful novel antibiotics and antibiotic analogues against MDR oral streptococci.

Among several antibiotics that were tested by European Committee on Antimicrobial Susceptibility Testing (EUCAST), *M. luteus* was found to be susceptible to Tetracycline.[41] Both Lemongrass and Citronella Java inhibited *M. luteus* to the same extent as Tetracycline (►**Table 2**). This is a very encouraging finding since it further supports that both these EOs could potentially replace alcohol in oral mouth rinses.

Conclusion

To our knowledge, EOs tested by us have not been studied elsewhere. Our preliminary study on these EOs holds much promise since bacterial resistance to EOs has not been reported so far. Our results indicate that Lemongrass and Citronella Java could potentially be used in alcohol-free mouth rinses. A potential limitation of our study includes possible mild allergic reactions in users to these EOs. We also do not know how Lemongrass and Citronella Java interact with each other as well as with other components usually found in mouth rinses. We hope that our candidate EOs exhibit a synergistic or additive antibacterial effect. Clinical trials with volunteers would address these potential concerns. Assays that evaluate the antibacterial properties of the major components of both these oils namely citronellol, citronellal, geraniol, and citral (►**Figs. 4**–**6**) on these oral bacteria are needed. Our candidate oils will also need to be tested together on the same panel of bacteria. Qualitative studies that evaluate their mechanism of action on these bacteria would provide further insight. These findings could also provide valuable information for novel drug design against MDR oral bacteria.

Conflict of Interest
None declared.

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