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ORIGINAL ARTICLE

Effect of Limonene, Camphor and Menthol on Cariogenic Oral Pathogens

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ABSTRACT
Streptococci, Lactobacillus, and fungi take a significant role in caries development. It confirms the importance of controlling these microorganisms to prevent dental caries. Chemical methods are used to prevent plaque accumulation in addition to physical methods, which include the use of chemical mouthwashes like chlorhexidine. Chemicals, while beneficial, have many side effects. This study aimed to find the herbal compounds having fewer side effects. Three monoterpenes, menthol, limonene, and camphor in plants that have been shown to have an antibacterial effect have been prepared and by sensitization drug in 96-well plates exposed to Streptococcus mutans, Streptococcus salivarius, Streptococcus sobrinus, Lactobacillus casei, and several Candida spp.. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) were identified. The menthol had the best inhibitory effect on yeast growth (MIC = 0.5-1 µg/ml), and the limonene composition had the lowest fungicidal concentration against Candida spp. (MFC = 1.9 µg/ml). Menthol at a concentration of 128 µg/mL had no bactericidal effect while it killed Lactobacillus. Strep.salivarius were killed at a concentration of 128 µg/ml of limonene. At the same concentration of camphor, Strep.mutans were killed. Monoterpenes exhibited great antimicrobial activity, which can use in mouthwashes formulation.
Keywords: Monoterpenes, Streptococcus mutans, Candida albicans, Anti-Bacterial Compounds, Limonene, Menthol

INTRODUCTION
Every individual's mouth has a large number of microorganisms, which can be harmful to teeth and oral soft tissue. Oral microbial flora contains more than 700 types of bacteria [1-3]. Dental decays are one of the most widespread infections of the oral cavity which is proceeding apace. A pale yellowish smooth layer, which is strongly bonded to the tooth surface is called dental plaque [4]. It includes several different kinds of organisms, including bacteria and fungi[5]. Amidst all other species, Streptococcus mutans is the main species in the dental plaque and decays formation. the lactobacillus species also presented a prominent task in the process of decay [6]. For decades, Candida species have also been found in the human oral cavity, which is known to be the cause of decay development [7].

The principal method of avoiding the infectious disease of the mouth is controlling the accumulation of plaque on the teeth. Mechanical methods to prevent plaque-based infectious disease and preserve good oral hygiene include regular brushing and flossing, which are introduced as the gold standard of plaque removal. Chemical methods like mouthwashes also used to retain good oral hygiene because they have anti-plaque properties. Contrary to the mouthwashes benefits, some side effects exist such as teeth discoloration, taste alteration, and xerostomia by chlorhexidine [8].

These days there is a lot of discussion on using medicinal herbs instead of chemical compounds. In this study, essential oils have been suggested as antimicrobial agents against cariogenic microorganisms with the purpose of decreasing harmful complications of chemical mouthwashes.

Terpenes are polymers of five carbon hydrocarbon isoprene. Terpenes found extensively in the environment and nature such as limonene which could be found in huge amounts in nature. Monoterpenes in plants have dissuasive function against herbivores. Scientists found that Terpenes have antimicrobial activity and also a significant role in the prevention and treatment of various diseases such as cancer [9].

In the present study, we discuss the antimicrobial activity of three monoterpenes; camphor, limonene, and menthol, against streptococcus, lactobacillus, and also candidiasis.

METHODS AND MATERIALS
This original in-vitro research was performed at Azad Dental University of Shiraz, Iran.

Three compounds of monoterpene include Camphor, Limonene, and Menthol were purchased from Sigma Aldrich (an American chemical, life science, and biotechnology company owned by Merck KGaA.) which were used to determine their antimicrobial activity. Four strains of bacteria [Strep.mutans (ATCC 35668), Strep.salivarius (ATCC 9222), Strep.sobrinus (ATCC 27607), Lactobacillus casei (ATCC 39392)] were purchased from the Iranian research organization for science and technology (IROST) and four Candida species [C.albicans (ATCC 10261), C.glabrata (ATCC 90030), C.parapsilosis (ATCC 4344), C. tropicalis (ATCC 750)] were obtained from Department of Mycology, Shiraz university of medical science, Shiraz, Iran. Different culture media such as Sabouraud dextrose agar, RPMI, and BHI were used to culture yeast and bacteria species.

Due to the sensitivity of the experiment and risk of culture contamination by unwanted microorganisms, all the processes of the experiment took place under a laminar flow cabinet, near the fire flame and in an uncrowded place.

Bacterial strains cultured on BHI medium except for the Lactobacillus, which cultured on MRS medium. Then incubated at 37°C for 24hours. After that, cultured bacteria obtained and diluted by adding normal saline until the turbidity became equated to 0.5 McFarland(10^6 colony forming-unit (CFU)/mL of bacteria). Streptococcus species were diluted by using Tryptic Soy Broth while other bacteria strains were diluted by Muller Hinton Broth.

Fungal strains cultured on Sabouraud dextrose agar medium with Chloramphenicol and incubated at 35°C for 24hours and then dilute to 0.5 McFarland. Some fresh yeast added to 5 ml of distilled water to make a suspension. With a spectrophotometer set at 530nm, the turbidity of suspension adjusted to 0.5 McFarland. To screening antifungal activity of monoterpenes, the MIC of each substance for fungal strains was determined by a microdilution method using Mueller Hinton broth on 96-well culture plates. The wells were inoculated with 200µl of liquid growth media in the first column and 100µl of liquid growth media in 2-12 columns. Serial dilution of monoterpenes was prepared from columns 2 to 11. In the end, 100µl of microbial suspension was added to each well on column 2-12. The highest concentrations of monoterpenes were in wells on the 2nd column while wells on the 11th column had the lowest concentrations of monoterpenes. Wells on the first column consider as a blank and on the 12th column consider as the positive control group. The plates with fungi were incubated at 30°C for 24-48hours. The concentration of the first clear well (represents growth inhibition of microorganisms) consider as MIC.

The same method was also used for bacteria. The plates with bacteria also were incubated at 37°C for 24 hours.

To determine the minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC) 10µl of contents were obtained from the well before the well of MIC to 2nd well and mixed them. Then cultured on a medium and incubated at 32°C for 24hours. Wells without microorganisms or maximum with three colonies consider as MFC or MBC.

RESULT

Antibacterial and antifungal activity of camphor, menthol, and limonene against Streptococcus species, Lactobacillus, and Candida species were measured by an in-vitro experiment.

MIC and MFC of camphor, menthol, and limonene against Candida strains are given in

Table **1**.

Table 1: Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of Menthol, Limonene, and Camphor against Candida species (µg/mL).

organisms	ATCC†	menthol			limonene			camphor			Fluc-onazole
		MIC50	MIC90	MFC	MIC50	MIC90	MFC	MIC50	MIC90	MFC	MIC50
Candida albicans	10261	0.25	0.50	2.00	8.00	16.0	16.0	0.50	1.00	4.00	2.00
Candida glabrata	90030	0.12	0.50	2.00	0.50	1.00	2.00	0.06	0.50	4.00	2.00
Candida tropicalis	750	0.12	0.50	1.00	0.50	1.00	4.00	0.06	0.25	2.00	1.00
Candida parapsilosis	4344	0.50	1.00	4.00	0.03	0.06	0.12	1.00	2.00	4.00	0.25

† ATCC: American Type Culture Collection

In this study, all of these three compounds of monoterpene (Camphor, Limonene, and Menthol) inhibited the growth and also killed all Candida species. The geometric mean of MIC50, MIC90, and MFC values for Menthol against Candida species were respectively 0.20 µg/ml, 0.59 µg/ml, and 2 µg/ml. The geometric mean of MIC50, MIC90, and MFC values for Limonene against Candida species were respectively 0.49 µg/ml, 0.98 µg/ml, and 1.97 µg/ml. The geometric mean of MIC50, MIC90, and MFC values for Camphor against Candida species were respectively 0.20 µg/ml, 0.70 µg/ml, and 3.36 µg/ml.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Menthol, Limonene, and Camphor against Lactobacillus and Streptococcus species (µg/mL).

organisms	ATCC‡	Menthol			Limonene			Camphor		
		MIC50	MIC90	MBC	MIC50	MIC90	MBC	MIC50	MIC90	MBC
Streptococcus salivarius	9222	32	64	R	16	32	128	64	128	R†
Streptococcus mutans	35668	32	64	R	32	64	R	16	32	128
Streptococcus sobrinus	27607	64	128	R	16	64	R	32	64	R
Lactobacillus casei	39392	8	32	128	64	128	R	16	64	R

†R: resistant ‡ATCC: American Type Culture Collection

Moreover, Menthol showed the best growth inhibitory effect against Candida species. Limonene had the lowest MFC among other monoterpenes.

Also, MIC and MBC of these three compounds of monoterpene against lactobacillus and streptococcus strains are measured and presented in Table 2.

The geometric mean of MIC50 for menthol against Lactobacillus and Streptococcus species was 26.9 μg/mL. Menthol at the geometric mean of 64 μg/mL (concentration range between 32 to 128 μg/mL) had no bactericidal activity against Streptococcus species while it killed Lactobacillus at a concentration of 128 μg/mL. The geometric means of MIC50 for Limonene and Camphor against Lactobacillus and Streptococcus species were the same and equaled to 26.9 μg/mL Strep.salivarius were killed at a concentration of 128 μg/ml of Limonene. At the same concentration of Camphor, Strep.mutans were killed.

Monoterpenes were more effective on fungi rather than on bacteria.

DISCUSSION

All of the investigated fungi in this study were sensitive to 3 compounds of Monoterpene, Camphor (MIC= 0.25 – 2 μg/ml), Limonene (MIC= 0.06 – 16 μg/ml) and menthol (MIC= 0.5 – 1 μg/ml) and they were able to eliminate all the fungi under study. Also, the anti-fungal effect of the mentioned compounds was observed in this study, the result was in line with the study of Diogo Mirona et al. in 2014. They found that monoterpenes have the greatest effect on Dermatophyte fungi (MIC= 100.4 μg/ml). They also found that the Trichophyton rubrum is the most sensitive type of fungus to the monoterpenes (MIC= 22.9 μg/ml) [10].

Camphor with MFC= 36.3 μg/ml was able to eliminate all of the investigated Candida. Also, among the studied bacteria, Camphor at the concentration of 128 μg/ml led to the elimination of Strep.mutans which is the main cause for decay. As such, we found that camphor had antifungal and antibacterial properties similar to those obtained in the study by Schafer E et al. in 1999. They presented research on antibacterial substances for root canal cleaning and they added 15% camphor to the 10% chloroxylenol which used to wash the canal. And they found that adding camphor killed most of the bacteria and fungi in the canal, especially C.albicans [11]. While in the year 2007 in Turkey, Tabanca et al. examined a compound containing 14% camphor on C.albicans and obtained MIC = 125 μg/ml, but we tested pure camphor on this species and obtained MIC = 1 μg/ml and we found that this compound was able to destroy the fungi even at 4 μg/ml. So we stated that camphor has an acceptable antifungal effect [12]. In our study limonene with MIC = 0.98 μg/ml, completely inhibited the growth of candida species, and killed all the four candida species with MFC = 1.97 μg/ml and inhibited bacterial growth with MIC =64 μg/ml. A study conducted by Slobodan Milosavljevic et al. in 2007, on limonene in aerial stems of the Cecilia anemone, found that for the effect of limonene on 5 fungal samples there was a need for MIC = 10 - 42 μg/ml. However, in our study, this value was stated as MIC = 0.06 -16 μg/ml. Thus, it can be concluded that limonene also has good antifungal properties [13]. In our research, menthol was more potent than the above-mentioned compounds and inhibited the growth of the fungi at a lower concentration (MIC= 0.59 μg/ml), whereas for Camphor the amount of inhibition of the fungi was 0.7 μg/ml and for Limonene it was 0.98 μg/ml Similar results were obtained by Kazemi et al., in 2012, by examining the monoterpene compounds in LAVANDULA. As such, they also found that menthol had the highest antimicrobial and antifungal activity [14].

In a study by Demenice Trombetta et al., in 2005, by examining the effect of these compounds on Staphylococcus aureus and Escherichia coli, it was found that menthol had the highest effect on these species [15]. As shown in our results, the effect of menthol composition was stronger for Lactobacillus bacteria, and this compound destroyed lactobacilli at a concentration of 128 μg/ml. But in general, regarding the effect on the bacteria, in our study there was not much difference in the mean concentrations of the three compounds and the effect was almost the same.

Chemicals are used in mouthwashes today. In 2000, Akihiro Yoshihara et al. found that fluoride in mouthwashes reduced Strep.mutans but had no effect on lactobacilli. But in our study, we found that monoterpenes were able to destroy Lactobacillus species in addition to Strep.mutans by menthol. So the advantage of using herbal mouthwashes over fluoride mouthwashes has been identified, but further research is still needed to fully prove this [16].

In 2000, D.H fine et al., investigated the effect of daily washing of Antiseptic Listerine of 2 times a day on the Strep.mutans and they found that this mouthwash reduced Strep.mutans by 75% with the concentration of 20 ml. But in our study, the effect of monoterpene compounds was significantly better than that of mouthwash. Menthol with a concentration of 128 μg/ml destroyed the examined Strep.mutans. And generally, monoterpenes with a concentration of 64 ug/ml reduced the Strep.mutans by 90%. This comparison shows that the effect of these compounds on Strep.mutans is much stronger than Listerine mouthwash [17]. In 2001, Yanla A et al. studied chlorhexidine mouthwash, which is the most common type of mouthwash and observed that chlorhexidine destroys lactobacillus, while we found that the camphor also affected similarly to chlorhexidine. So, Camphor can then replace chlorhexidine [18], because, in a study by Salehi et al., 2006, it was found that chlorhexidine causes discoloration in 86% of people using this type of mouthwash, and in 13% of people it changes the taste. Even in Persica's mouthwashes, these percentages were 13% and 14%, respectively, so the need to find materials to replace these high-risk substances was identified [19]. Also, the effect of chlorhexidine on Strep.mutans was investigated by Jaronin et al. in 1993. Chlorhexidine is indeed active with MIC <0.001 μg/ml and this value for monoterpenes is MIC = 32-64 μg/ml, and we see a lot of differences, but one has to look at which are the most profitable given the price and the side effects, and also what kind (chemical or herbal) is the people's preference [20]. In 2017, Rahul j hedge et al. claimed that the compounds in tea may be able to inhibit oral bacterial growth. Through comparison, they observed that the effect of green tea mouthwash was not different from that of CHX+NaF mouthwash, and this suggested that the use of herbal mouthwashes can be a promising preventive treatment to prevent caries [21]. The results of this study are in line with the study of Faezeh Ranjbar in 2015. They presented reports in laboratory animals and humans that consuming green tea (without adding sugar) reduces tooth decay. Repeated consumption of green tea can significantly reduce the formation of caries, even in the presence of sugar in the diet. GTP (Green Tea Poly Phenols) found in green tea inhibits the growth of oral bacteria such as Strep.salivarius and Strep.Mutans [22].

Contrary to all the research we have mentioned so far, in 2011, Mostafa Sadeghi et al. investigated the antimicrobial effect of Persica and Matrica herbal mouthwashes on common oral bacteria and concluded that herbal mouthwashes were less potent than chlorhexidine mouthwashes to inhibit the growth of oral bacteria. But further research is needed to fully prove this [23].

Our goal and also the purpose of many previous articles have been to find some compounds or essential oils that can be added as a supplement to today's mouthwashes to improve recovery and help with the mechanical control of plaque with fewer side effects than chemicals. In 2007, Mr. Eccles and his colleagues introduced menthol as a compound that could be added to toothpaste and mouthwash formulations to improve their properties [24]. In the present study, it was shown that menthol, limonene, and camphor with different concentrations can be added to the formulation of antimicrobial and antifungal products. However, in 1993, Nicole Diary et al., have put forward a new issue that if we use two or more compounds of monoterpene as a combination, they have much better properties than the one in which we use as one compound. Their study was conducted on Strep.mutans and Strep.sanguinis species [25].

It was found that using monoterpenes in mouthwashes can have the same effect as the chemical mouthwashes and thus can be a good substitute for them. According to this study, if we use these three compounds of monoterpene in new mouthwashes, we can achieve the antimicrobial properties of chemical mouthwashes with much fewer side effects.

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Conflict of Interest: The authors deny any conflict of interest.

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