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Effects of sub-minimum inhibitory concentrations of lemon essential oil on the acid tolerance and biofilm formation of *Streptococcus mutans*

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ABSTRACT

Objectives: Lemon essential oil (LEO) is a kind of secondary metabolite from lemon peels and has been found to inhibit cariogenic bacteria for decades. However, its effects on main cariogenic virulence factors are rarely reported. The present study aimed to investigate the effects of sub-minimum inhibitory concentrations (sub-MICs) of LEO on the acid tolerance and biofilm formation of *Streptococcus mutans* (*S. mutans*) and preliminarily reveal the possible underlying mechanisms.

Designs: Effects of LEO on the acid tolerance and biofilm formation of *S. mutans* were investigated by the broth dilution method and crystal violet staining method respectively. The expression of *luxS*, *srtA* and *spaP* gene was also determined to explore the underlying mechanism. In addition, Tea polyphenols (TP), a major natural inhibitor of cariogenic virulence factors, and limonene (LIM), the major component of LEO, were selected as comparisons to evaluate the effects of LEO.

Results: Sub-MICs of LEO, LIM and TP exhibited a dose-dependent inhibition of growth of *S. mutans* at pH ranging from 4.0 to 7.0. The formation of *S. mutans* biofilm was remarkably inhibited and the inhibitory rates of LEO, LIM and TP were 97.87%, 94.88% and 96.01% respectively at 1/2 MIC. Similarly, a down-regulation was observed in the expression of *luxS*, *srtA* and *spaP* gene at sub-MIC levels.

Conclusions: Effects of LEO were similar or slightly stronger than LIM and TP, suggesting that LEO might represent a novel, natural anticarious agent that inhibited the specific genes associated with bacterial acid tolerance and biofilm formation without necessarily affecting the growth of oral bacteria.

1. Introduction

Dental caries is the most common infectious diseases affecting mankind. *S. mutans* is currently recognized as the main cariogenic bacteria and the cariogenic virulence factors of it mainly include acid production, acid tolerance, adherence and biofilm formation (Brighenti et al., 2008; Gibbons, 1984; Li, Lau et al., 2002; Saini, Saini, & Sharma, 2011). Quorum sensing is a bacterial mechanism for regulating gene expression in response to changes in population density(Merritt, Qi, Goodman, Anderson, & Shi, 2003). The regulatory genes of quorum sensing system mainly include *ComD*, *ComE* and *luxS* (Leung, Dufour, & Lévesque, 2015; Merritt et al., 2003). The *luxS* quorum-sensing system is present in approximately one half of all sequenced bacterial genomes. Many bacterial phenotypes have been attributed to cell signaling via the *luxS*-based autoinducer (AI)-2 system (Sha, Foltz, Erova, & Agar, 2008). The study showed that the biofilm formation and acid tolerant ability of *luxS*-deficient *S. mutans* were abnormal (Parveen & Cornell, 2011), indicating that the two physiological characteristics were regulated by *luxS*. The *comD* and *comE* mutants were defective in sensing and responding to the CSP and formed biofilms with reduced biomass (Li, Tang et al., 2002). However, the study of their effects on the acid resistance of *S. mutans* is rarely reported. Adherence of *S. mutans* to dental surfaces is the first step in the formation of biofilms by this organism and is mediated by sucrose-independent and sucrose-dependent mechanisms (Cvitkovitch, Li, & Ellen, 2003; Koga, Asakawa, Okahashi, & Hamada, 1986). The sucrose-independent adherence mainly involves the interaction between the surface protein and the host factors in the acquired membrane (Mitchell, 2003). The major cell surface protein P1 encoded by *spaP* has been shown to promote the adherence of *S. mutans* to hydroxyapatite in vitro and is implicated in *S. mutans* colonization of teeth in vivo (Lee et al., 1989; Song & Mcgavin, 2004). Recently it was shown that sortase encoded by *srtA* was responsible for sorting and

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anchoring P1 to the cell wall of *S. mutans* (Lee & Boran, 2003). These findings suggested that *spaP* and *srtA* gene could regulate the initial adherence and played a significant role in the formation of *S. mutans* biofilm.

Antimicrobial agents are considered as effective inhibitors of the major virulence factors in caries. However, the commonly used anticarious agents including chlorhexidine have resulted in a variety of problems such as tooth stain, changes in taste or the emergence of resistant strains because of the long-time or excessive use (Eriksen, Nordbø, Kantanen, & Ellingsen, 1985; Lang, Catalanotto, Knöpfli, & Antczak, 1988). It emphasizes the significance to develop a novel alternative or adjunctive anticarious chemotherapy. In recent years, with the continuous deepening of the research of Chinese herbal medicine, plant extracts, especially lemon extracts and tea polyphenols which are secondary metabolites abundant in plant-derived foods, have become a hot research based on the favorable biological traits. LEO, a mixture of terpenes and oxygenated derivatives (Ferhat, Meklati, & Chemat, 2007), has exhibited remarkably antibacterial and antioxidant properties and been popular in the fields of daily healthcare, food preservation and medicine health (Bertuzzi, Tirillini, Angelini, & Venanzoni, 2013; Miyake & Hiramitsu, 2011). TP is a kind of polyphenolic compounds extracted from green tea or oolong tea (Cho, Oh, & Oh, 2010; Sasaki et al., 2004). Studies have showed that TP can inhibit the adherence and biofilm formation of cariogenic bacteria (Cho et al., 2010; Xu, Zhou, & Wu, 2012).

Our previous studies have confirmed that LEO can inhibit *S. mutans* the acid production and the activity of enzymes related to it (Zhang, Yu, Wang, Liu, & Guo, 2010), and the sucrose-dependent adherence as well as the related genes (*gtfB* and *gtfC*) expression (Ying et al., 2013). However, little is still known about the effects of LEO on other main cariogenic virulence factors. In this study, we will determine the effects of sub-MICs of LEO on the acid tolerance and biofilm formation of *S. mutans*, preliminarily reveal the underlying mechanisms of LEO in the prevention of caries, and provide a theoretical basis for the development and application of natural anticarious agents.

2. Materials and methods

2.1. Plant extracts

LEO was obtained from the peels of lemon, which was grown in Sichuan province, China and in the intermediate maturation stage characterized by greenish-yellow coloration (Combariza, Tirado, Stashenko, & Shibamoto, 1994).The light yellow sample was collected and stored in a brown bottle at −4 °C. LIM (97%) was purchased from Alfa Aesar (UK). TP, provided by Solarbio (Beijing, RPC), was a green tea extract with a molecular weight of 238 and a molecular formula of $C_{11}H_9N_3O_2$.

2.2. GC–MS analysis of LEO

The essential oil was analyzed by gas chromatography (Varian 450, USA) equipped with a flame ionization detector. The chromatographic separations were performed on a DB-5 (30 m × 0.25 mm i.d. with 0.25 m) capillary column. Injector temperature was 250 °C and sample injection was performed in split mode. The oven temperature was maintained at 50 °C for 5 min after injection and then programmed at 4 °C/min to 220 °C, and raised to 290 °C which was held for 15 min. High-purity (over purity 99.99%) helium was used as carrier gas at the rate of 1 mL/min. The injection volume was 1 uL and performed in the split mode with a ratio of 20:1. The sample was mixed into the hexane prior to injection.

Mass spectra was carried out on a GC–MS system (Varian 320, USA). The capillary column used was a VF-1701MS (30 m × 0.25 mm i.d., 0.25 μm film thickness).The mass conditions were set as follows: ionization mode with EI, ionization energy of 70 eV, ion source and transfer line temperature at 250 °C, scan range between 30.0 u and 500.0 u. Compounds were retrieved and identified in the NIST2008 spectrum map library.

Table 1
Nucleotide sequences of primers used in this study.

Gene	Primer sequence	Size (bp)
16S rRNA	5′-CCTACGGGAGGCAGCAG-3′ 5′-ATTACCGCGGCTGCTGG-3′	196 bp
luxS	5′-ACTTGCTTTGATGACTGTGGC-3′ 5′-TCAGCGTATTGACGGGATG-3′	115 bp
srtA	5′-ATGGTGCTGGAACGATGAAA-3′ 5′-CATGCCTTCTTTTGCACGTT-3′	134 bp
spaP	5′-TTAGGCAGGGTCAAGG TGGT-3′ 5′-CTGTTATTAGGACCAGACATGCG-3′	136 bp

Table 2
Chemical compositions of LEO.

Retention time/min	Compounds	Molecular formula	Molecular weight	Relative content (%)
5.473	Thujene	$C_{10}H_{16}$	136	0.222
5.905	α-Pinene	$C_{10}H_{16}$	136	1.372
6.520	Camphene	$C_{10}H_{16}$	136	0.059
7.365	β-Pinene	$C_{10}H_{16}$	136	17.075
7.838	Myrene	$C_{10}H_{16}$	136	0.917
8.303	α-Phellandrene	$C_{10}H_{16}$	136	0.228
8.971	Limonene	$C_{10}H_{16}$	136	48.482
9.268	β-Phellandrene	$C_{10}H_{16}$	136	0.305
9.622	Cineole	$C_{10}H_{18}O$	154	0.324
9.812	3-Carene	$C_{10}H_{16}$	136	0.071
9.990	4-Carene	$C_{10}H_{16}$	136	8.455
10.201	1-Octen-3-ol	$C_8H_{16}O$	128	0.055
10.822	γ-Terpinene	$C_{10}H_{16}$	136	0.348
12.916	Terpinolene	$C_{10}H_{16}$	136	0.121
13.642	1-Linalool	$C_{10}H_{18}O$	154	0.396
14.532	β-Terpineol	$C_{10}H_{18}O$	154	0.306
15.407	Terpinen-4-ol	$C_{10}H_{18}O$	154	0.452
15.832	*cis*-Carveol	$C_{10}H_{16}O$	152	1.231
15.956	*trans*-Carveol	$C_{10}H_{16}O$	152	0.422
16.068	Geraniol	$C_{10}H_{18}O$	154	0.543
16.499	1-Nonanol	$C_9H_{20}O$	144	0.154
17.144	Neral	$C_{10}H_{16}O$	152	0.053
17.232	4-Ethylguaiacol	$C_9H_{12}O_2$	152	0.968
18.455	P-menth-1-en-8-ol	$C_{10}H_{18}O$	154	0.842
18.560	Anethole	$C_{10}H_{12}O$	148	0.182
19.005	Camphene	$C_{10}H_{16}$	136	0.037
19.011	β-Elemene	$C_{15}H_{24}$	204	0.042
19.430	Methyleugenol	$C_{11}H_{14}O_2$	178	0.546
19.810	Eugenol	$C_{10}H_{12}O_2$	164	0.002
20.510	Decanoic acid	$C_{10}H_{20}O_2$	172	0.546
21.648	Geranyl acetate	$C_{12}H_{12}O_2$	196	0.661
22.126	*trans*-Caryophyllene	$C_{15}H_{24}$	204	0.059
22.198	Caryophyllene	$C_{15}H_{24}$	204	0.140
22.303	Germacrene-D	$C_{15}H_{24}$	204	0.843
24.369	δ-Cadinene	$C_{15}H_{24}$	204	0.097
24.696	Elemicin	$C_{12}H_{16}O_3$	208	0.004
24.807	α-Cedrol	$C_{15}H_{26}O$	222	0.314
41.906	Methyl hexadecanoate	$C_{17}H_{34}O_2$	270	0.070
51.906	delta-Gluconolactone	$C_{19}H_{22}O_3$	298	6.851
57.606	Hyaluronic acid	$C_{16}H_{30}O_2$	254	0.159

2.3. Preparation of microbial strains suspension

The microorganism used in this study was *S. mutans* (UA159) provided by the Department of Stomatology, Wuhan University, China. Bacterial strains were grown in trypticase peptone yeast (TPY) medium under anaerobic conditions at 37 °C. Bacterial suspension was prepared with an optical density (OD) of 1.0 at 540 nm (approximately 10^8 cfu/

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