IN VITRO ANTIMICROBIAL ACTIVITY OF *OCIMUM AMERICANUM* L. ESSENTIAL OIL AGAINST ORAL MICROORGANISMS

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Abstract. The aim of the present study was to evaluate the efficacy of the essential oil of *Ocimum americanum* L. on *in vitro* activity against *Streptococcus mutans, Lactobacillus casei* and *Candida albicans*. An agar disk diffusion method was employed for screening antimicrobial activity. Minimum inhibitory concentration (MIC) and minimum cidal concentration (MCC) values of the oil against planktonic cells were determined using the Millipore membrane method. The antimicrobial potential of the essential oil was also investigated with a biofilm model. The results indicate that essential oil has antimicrobial activity against all tested microorganisms. The MIC values of the oil against the three organisms was 0.04% v/v whereas the MCC values for *S. mutans, L. casei* and *C. albicans* were 0.08%, 0.3% and 0.08% v/v, respectively. *S. mutans* and *C. albicans* were more sensitive to the essential oil than *L. casei*. With the biofilm assay, a 5-minute exposure to 3% v/v essential oil eliminated 3 $\log_{10}$ of the tested microorganisms. At a lower concentration (0.3% v/v), a 2 $\log_{10}$ reduction in *S. mutans* and *C. albicans* was observed while the lactobacilli were more resistant. This finding indicates the possibility of using the essential oil of *O. americanum* L. in oral health care products for reducing these pathogenic microorganisms in the oral cavity.

INTRODUCTION

Dental caries and candidiasis are common oral health problems of people in many countries. These conditions are caused by bacteria and yeast residing in the oral cavity. Dental caries is the destruction of the hard tissue of teeth. It is initiated by direct demineralization of the enamel of teeth due to acids produced by bacteria. *Streptococcus mutans* and *Lactobacillus* spp are known by their acidogenic and acidophilic properties, more than those of other oral bacteria. They have been shown to have cariogenic potential in both humans and animals (Harper and Loesche, 1984; Loesche, 1986). *S. mutans* appears to be important in the initiation of dental caries since its activities lead to colonization of the tooth surface, dental plaque or oral biofilm formation and demineralization of tooth enamel. Lactobacilli, which are mainly found in carious dentin and cementum, have been suspected to be secondary invaders that contribute to the progression of lesions. Oral candidiasis is a common opportunistic infection of the oral cavity caused by *Candida* spp. The incidence varies depending on age and certain predisposing factors. Four types of oral condidiasis are

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common, namely pseudomembranous, erythematous, hyperplastic and angular cheilitis. In the debilitated, compromised host, oral candidal infection may spread to the gastrointestinal tract, trachea, lungs, liver and central nervous system, which may result in septicemia, meningitis, hepatosplenic disease and endocarditis (Samaranayake, 2000). The most frequent *Candida* spp isolate of the oral cavity is *C. albicans* (Samaranayake, 2000). There is a large body of evidence indicating *Candida* can adhere and colonize oral surfaces including the mucosa and acrylic denture which represents the first step in the infectious process. In addition, yeast cells have the ability to co-aggregate with oral bacteria (Cannon and Chaffin, 1999).

Natural products have been used as folk medicine for many years. Among them, hoary basil has attracted increased interest due to its antimicrobial activity against a wide range of pathogenic microorganisms. *Ocimum americanum* L., hoary basil or mosquito plant is an annual herbaceous plant native to Asia and Africa. It is a robust, aromatic plant reaching 0.7 m high with an erect stem and very green, ovate leaves, grayish-green beneath, and white, greenish or purplish pink-white flowers. The whole plant has a strong aromatic scent and three distinct chemotypes: flora-lemony, camphoreceous or spicy. Essential oil is obtained by steam distillation of the leaves and flowering tops. The oil is colorless to pale yellow and easily evaporates. From a pharmacological perspective, essential oil obtained from *O. americanum* L. has shown antibacterial activity against *Staphylococcus aureus*, *Streptomyces pyogenes*, *Escherichia coli* and *Salmonella typhosa*. Antitubercular activity against *Mycobacterium* spp was found at a concentration of 100 ppm. The oil has antifungal activity against a great number of fungi including some human pathogens (Ntezurubanza *et al*, 1986).

Even though the antimicrobial activity of essential oil against microorganisms has been investigated by many researchers for medical purposes, little information is available regarding its efficacy against oral pathogens. The aim of the present study was to evaluate the efficacy of the essential oil of *O. americanum* L. on the *in vitro* activity against the oral microorganisms *S. mutans*, *L. casei* and *C. albicans* as planktonic cells and in a biofilm model.

MATERIALS AND METHODS

Microorganisms

The tested microorganisms were obtained from the culture collections of the Department of Microbiology, Faculty of Dentistry, Mahidol University, Thailand. The organisms were: *S. mutans* KPSK2, *L. casei* ATCC 6363 and *C. albicans* ATCC 13803. They were maintained on Brain Heart Infusion (BHI) agar (BBL, USA). Overnight cultures were prepared by inoculating approximately 2 ml Mueller Hinton broth (Difco, USA) with 2-3 colonies of each organism taken from BHI agar. Broth was incubated overnight at 37ºC. Inocula were prepared by diluting overnight cultures in saline to approximately 10^8 cfu/ml for bacteria and 10^7 cfu/ml for yeast. These suspensions were further used for antimicrobial determination.

Essential oil

The extraction of the essential oil was carried out at the laboratory of the Thailand Institute of Scientific and Technological Research by steam distillation of *O. americanum* leaves.

Disk diffusion method

The microbial growth inhibitory potential of the oil was determined using the disk diffusion method as described by Washington (1981). Freshly prepared bacterial and

yeast suspensions were spread on Mueller Hinton agar. Essential oil (15 µl) was dropped onto sterile paper disks (6 mm diameter) and the same volume of 0.2% chlorhexidine gluconate and saline solution served as positive and negative controls, respectively. The disk was dispensed onto the Mueller Hinton agar and incubated at 37ºC for 24-48 hours. All tests were performed in triplicate and zones of inhibition were measured after incubation.

Millipore membrane method

The minimum inhibitory concentration (MIC) and minimum cidal concentration (MCC) values of essential oil were determined using the Millipore method as described by Tantaoui-Elaraki *et al* (1992). In brief, serial dilutions of essential oil, ranging from 2% v/v to 0.04% v/v, were prepared in Mueller Hinton agar incorporated with 0.5% v/v Tween-20 to enhance oil solubility. Cellulose acetate membrane filters (0.45 µm porosity) (Sartorius, Germany) were placed on the surface of the agar plate and 5 µl of each microbial suspension was dropped onto each filter. Mueller-Hinton agar with 0.5% v/v Tween-20 without essential oil (15 µl) was used as a positive growth control. The plates were incubated at 37ºC for 24-48 hours. The MICs were determined as the lowest concentration of essential oil inhibiting the visible growth of each organism on the membranes.

For the determination of MCCs, cellulose acetate filters without any microbial growth were transferred into BHI broth and incubated at 37ºC for 24-48 hours. The lowest concentration of essential oil that showed no visible growth of the organisms in the tube was interpreted as the MCC.

Biofilm model

The antimicrobial activity of essential oil was also evaluated on biofilm against *S. mutans* KPSK2, *L. casei* ATCC 6363 and *C. albicans* ATCC 13803 on the bottom of a microtiter plate. Biofilm assays were done using a protocol modified from Loo *et al* (2000) and Guggenheim *et al* (2004). Briefly, all microorganisms were cultivated in fluid thioglycolate medium (Difco, USA) supplemented with 0.3% glucose for 7 hours. Each microbial suspension (10^6 cfu/ml) was combined and placed at a cold temperature for 6 hours. Approximately 500 ml of unstimulated saliva was collected from three volunteers 2 hours after eating, drinking or having a hygiene procedure. The pooled saliva was centrifuged at 20,000*g* for 30 minutes at 4ºC. The supernatant was filtered through a 0.45 µm-pore membrane (Sartorius, Germany). Absence of any viable microorganisms in the saliva was checked by culturing on blood agar. Salivary components were absorbed onto polystyrene, flat-bottom 96-well microtiter plates for 4 hours at room temperature in order to form an acquired pellicle. After removal of excess saliva, the coated microtiter plates were inoculated with a mixture of saliva, thioglycollate medium supplemented with 3% glucose and a pool of microorganisms. The final density of inoculum in each well of the microtiter plate was 10^5 cfu/ml. The microtiter plates were then incubated at 37ºC for 24 hours under anaerobic conditions.

Saline solution and 0.2% chlorhexidine gluconate solution were used as negative and positive controls, respectively. The biofilms were exposed to controls and essential oil (0.3% and 3% v/v) for 5 minutes and incubated for 24 hours. After removal of the media and unattached cells, the biofilms were washed twice, scrapped and sonicated in PBS. Serial dilutions of sonicated cells were cultivated in Mitis-Salivarius Bacitracin agar (Difco, USA), Rogosa SL agar (Difco, USA) and Sabouraud dextrose agar (Difco, USA) to determine the number of *S. mutans*, *L. casei* and *C. albicans*, respectively.

Table 1
Antimicrobial activity of the essential oil of *O. americanum* against oral microorganisms in planktonic state.

	Zones of inhibition (mm)	MIC (% v/v)	MCC (% v/v)
1. Essential oil			
S. mutans	28	0.04	0.08
L. casei	19	0.04	0.30
C. albicans	>30	0.04	0.08
2. 0.2% chlorhexidine			
S. mutans	21	0.062	0.062
L. casei	20	0.125	0.125
C. albicans	16	0.062	0.062

MIC, minimum inhibitory concentration
MCC, minimum cidal concentration

Table 2
Antimicrobial activity of the essential oil of *O. americanum* against oral microorganisms in a biofilm model.

Microorganisms	0.3% v/v Essential oil	3% v/v Essential oil	0.2% Chlorhexidine gluconate	Normal saline solution
S. mutans	7.2×10^4	2.9×10^3	1.7×10^3	8.5×10^6
L. casei	5.1×10^5	6.3×10^3	2.5×10^3	6.0×10^6
C. albicans	4.5×10^4	3.4×10^3	2.1×10^3	2.8×10^6

Data presented as cfu/ml of microorganisms

RESULTS

Using the disk diffusion method as a screening test, the essential oil of *O. americanum* showed antimicrobial activity against all of the tested microorganisms with a zone of growth inhibition > 30-19 mm (Table 1). Greater inhibition zones were observed when *S. mutans* and *C. albicans* were exposed to the oil compared with *L. casei*.

The MICs of essential oil obtained by the method described by Tantaoui-Elaraki *et al* (1992) are also shown in Table 1. All the tested organisms were inhibited at ≥ 0.04% v/v of the oil, whereas the MCC values for *S. mutans, L. casei* and *C. albicans* were 0.08%, 0.3% and 0.08% v/v, respectively. *S. mutans* and *C. albicans* were more sensitive to essential oil than *L. casei*.

We also employed the highest MCC value of the tested organisms (0.3% v/v) and 3% v/v which was 10 x the MCC concentration when investigating the antimicrobial effect of essential oil against biofilm formed by the species in this study. Table 2 shows the antimicrobial effect of essential oil against multi-species biofilm. After 5 minutes of exposure, the essential oil at MCC value (0.3% v/v) showed a 2 $\log_{10}$ reduction in *S. mutans* and *C. albicans* compared to

standard growth of biofilm with the negative control. *L. casei* was found to be more resistant. At a higher concentration, a 3 $\log_{10}$ reduction in *S. mutans*, *L. casei* and *C. albicans* was observed which is similar to the results obtained from chlorhexidine solution.

DISCUSSION

Plant essential oils are valuable natural components used as raw materials in many products, perfumes, cosmetics, spices, treatments, aromatherapy, phytotherapy, spices and in the field of nutrition (Buchbauer, 2000). The oils are complex mixtures comprised of many compounds. Each constituent contributes to the beneficial or adverse effects of these oils.

O. americanum has long been used as a medicinal and aromatic plant in many countries. Previous studies reveal the major compound found in the essential oil of this plant is eugenol, reaching nearly 30% of the compound. Methyl chavicol is the second most common compound and accounted for 17% of the compound. Trepinol has been identified as the third main constituent in the oil comprising 15% of the compound (Shadia *et al*, 2007).

In recent years, due to the widespread and often indiscriminate use of antimicrobial drugs, many microorganisms have acquired resistance to specific antibiotic treatment (Evan's, 1999). This has created major clinical problems in the treatment of infectious disease (Davis, 1994). Antibiotics are sometimes associated with adverse effects including hypersensitivity reactions, reduction of beneficial gut and mucosal microorganisms, immunosuppression and allergic reactions (Idose *et al*, 1968). Research of medicinal plants has increased and their antimicrobial activity has been evaluated in many studies (Koo *et al*, 2000; Smullen *et al*, 2007; More *et al*, 2008).

A variety of methods have been used to determine antimicrobial activity of essential oils depending on the type of essential oil and target organism. Frequently used methods include agar disk diffusion (Rafii and Shahverdi, 2007; Fontenelle *et al*, 2008; More *et al*, 2008) agar well diffusion (Singh *et al*, 2006; Fontenelle *et al*, 2008) and incorporation of the essential oil in agar media prior to inoculation (Rosato *et al*, 2008). In the present study, essential oil from *O. americanum* was first screened for its antimicrobial activity using the agar disk diffusion method and then the MIC and MCC were examined using the method reported by Tantaoui-Elaraki *et al* (1992). In this method, the organisms were cultured on Millipore membranes of 0.45 μm porosity placed on agar media containing different concentrations of the oil.

Agar disk diffusion is the method most frequently used to screen plant extracts for antimicrobial activity. However, the usefulness of this method is limited to the generation of preliminary, qualitative data only, as the hydrophobic nature of most essential oils prevents the uniform diffusion of these substances through agar medium (Janssen *et al* 1987; Hili *et al* 1997). Moreover, essential oils are poorly soluble in water. This causes many problems in studying their biological and pharmacological properties. To overcome these problems, many researchers have recommended the use of various solvents in the dilution of essential oils such as acetone, alcohol, ethylene glycol, ethanol, methanol and DMSO, or using an emulsifier detergent or Tween 20 as was chosen to use in the present study (Lahlou, 2004). In addition, their activities show some challenges, for example, essential oils present a complex chemical composition, some of their constituents are volatile and have to be used in low doses.

According to Coller *et al* (1989), any antimicrobial agent is considered effective if the size of inhibition zone produced, measured after deduction of well or disk diameter, is 2 mm or more. In the present study, the result obtained from the disk diffusion method demonstrated the essential oil of *O. americanum* possessed strong antimicrobial activity since the zones of inhibition produced by the oil against *S. mutans*, *L. casei* and *C. albicans* were 28, 19 and >30 mm, respectively (disk diameter was 6 mm). Even though the essential oil seems to be more effective than chlorhexidine solution, it should be noted that the concentration of chlorhexidine used was 0.2% whereas the oil had not been diluted. The antifungal activity of the oil against *C. albicans* was similar to that reported by Tajo and Thoppil (1999) and Hammer *et al* (1999). However, the MIC value found in the current study (0.04% v/v) was much lower than the previous report (0.5% v/v). The difference may be from the experimental conditions (method, temperature, photoperiod, etc), materials, the method of extraction of the essential oil, which influence the chemical composition, the dissolution and dispersion of the oil and the tested organisms. Furthermore, the composition of plant oils is known to vary according to local climatic and environmental conditions (Janssen *et al*, 1987; Sivropoulou *et al*, 1995).

Although the antimicrobial activity of essential oil of *O. americanum* has already been reported for various pathogenic microorganisms, no data has been obtained regarding the activity against oral bacteria, especially *S. mutans* and *L. casei*. The results of this study demonstrate, in free form or planktonic state, the oil has antibacterial activity against *S. mutans* and *L. casei*. *S. mutans* is more susceptible to the oil than *L. casei*, the MCC value for *S. mutans* was 0.08% v/v but the *L. casei* was 0.3% v/v. The components of the essential oil are known to act on the cell membrane of both gram-positive and gram-negative bacteria. Essential oil was found to be composed of mainly eugenol, methyl chavicol and trepinol (Shadia *et al*, 2007). Previous studies revealed the mechanisms of action of eugenol and trepinol on disrupting bacterial outer membranes and mitochondria as well as disturbing cell structures (Oyedemi *et al*, 2009). In addition, the inhibition of energy generation by inhibition of glucose uptake and glucose utilization has been observed (Gill and Holley, 2004). For chavicol, the proposed antimicrobial action also involved the alteration of the bacterial cell membrane structures which result in the disruption of membrane permeability (Sharma *et al*, 2009).

In the oral cavity, most colonizing and infecting microorganisms are found not as single-living cells but rather as complex structured microbial communities. They are often encapsulated within a matrix of exopolymeric material and attached to biotic or abiotic surfaces (Kolenbrander, 2000). These communities are referred to as "biofilms". Of clinical relevance is the fact that biofilm is more resistant to immune defense mechanisms and less susceptible to antimicrobial agents, which can display enhanced pathogenicity (Quave *et al*, 2008). Many microbiological studies tested oral antimicrobial agents against growing cells in a planktonic state. The use of an *in vitro* biofilm model provides an alternative approach for examining the antimicrobial effects of agents against oral biofilm which is a key to the existence and survival of these microorganisms with inter- and intra-species interactions in the oral cavity. We employed an *in vitro* biofilm formed by three oral pathogens (*S. mutans*, *L. casei* and *C. albicans*) more suitable for testing the agent aims to treat biofilm-related diseases in the oral cavity.

Our results show, despite the inhibitory and effects on the tested microorganisms in planktonic state, the essential oil from *O. americanum* leaves exhibited a less pronounced effect upon these microorganisms in biofilm form. Exposure to the essential oil at 0.3% v/v, the highest MCC values in the planktonic state, for 5 minutes eliminated only 2 $\log_{10}$ of *S. mutans* and *C. albicans* (Table 2). However, at a higher concentration (3% v/v), the effect was similar to that of chlorhexidine gluconate (a 3 $\log_{10}$ reduction in all tested microorganisms). This indicates that microorganisms in the biofilm are strongly protected and less susceptible to antimicrobial agents than in planktonic cells. The cells in biofilm exhibit properties distinct from those of planktonic cells, including resistance to antimicrobial agents, physiological properties and interaction with host tissues (Wilson, 1996). Experiments comparing biofilms of oral bacteria strains with broth cultures using chlorhexidine gluconate have demonstrated that much higher concentrations of chlorhexidine are required to significantly inhibit existing biofilm cells (Pratten *et al*, 1998; Shani *et al*, 2000).

Taken together, our results indicate the essential oil of *O. americanum* acts as a potent antimicrobial agent that can disrupt existing biofilm. However, this study was conducted on planktonic and artificial biofilm *in vitro*. A clinical study may be required to confirm the validity of the results obtained. Additionally, further investigations of the toxic or irritant properties are imperative when considering their use in humans.

In summary, the results of the present study suggest the essential oil of *O. americanum* at a concentration of 3% v/v may be a beneficial component of oral health care products, such as mouthrinse and toothpaste, that provide prevention against cariogenic bacteria and candidal infection. The use of this oil would depend on cost considerations, odor and flavor.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Siripen Jarikasem and Dr Bhusita Vannissorn of the Thailand Institute of Scientific and Technological Research for providing the essential oil for this study.

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