Original Article

Antimicrobial Properties of *Ocimum* Species: An *In Vitro* Study

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Abstract

Objective: This study aimed to determine the antimicrobial activity of ethanol-extracts obtained from *Ocimum gratissimum L.* (clove or African basil, Lamiaceae) and *O. santum L.* (holy basil) against some microorganisms present in oral cavity related to either medical or dental disease. **Materials and Methods:** Antimicrobial properties of both ethanol-extracts of *Ocimum* species against *Streptococcus mutans* KPSK2, *S. pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 16794, and *Candida albicans* ATCC 10231 were primarily determined by agar disk diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC or MFC) of these herbal extracts were further determined by broth micro-dilution method. **Results:** Ethanol-extracts of *O. sanctum L.* and *O. gratissimum L.* inhibited the growth of all tested microorganisms in various degrees ranging from the strongest antimicrobial activity of *O. sanctum* against *S. pyogenes* [MIC at 0.19% (w/v); MBC at 0.78% (w/v)] to the least inhibitory activity of *O. gratissimum* against *C. albicans* [MIC at 12.5% (w/v); undetectable MFC]. The ethanol-extract of *O. sanctum* showed stronger antimicrobial property against the tested bacteria and fungus than *O. gratissimum*. The ethanol-extracts of both *Ocimum* species showed stronger antibacterial than antifungal activity. However, the ethanol-extract of *O. gratissimum* even at a high concentration of 50% (w/v) was unable to eliminate the tested fungus. **Conclusion:** Ethanol-extracts of *Ocimum* species contain effective antibacterial and antifungal properties that may be beneficial for further development of antimicrobial agents in medical and dental fields.

Keywords: *Antimicrobial activity, ethanol-extract, Ocimum gratissimum L., Ocimum sanctum L.*

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Introduction

All civilizations and religions from the ancient period to the modern day have used various medicinal plants and herbs in the health care of humans and animals. Moreover, these nature-derived products are generally well-accepted by the general population, who observed them as crucial aspects of health care. These products also tend to have a high success rate in treating diseases, as they are sources of diverse molecules that show radical scavenger and antimicrobial properties.[1] Many medicinal plants and herbs have been extensively researched for their potential, mechanism, and effectiveness to cure several diseases. Among the commonly grown herbs in South East Asia, *Ocimum gratissimum L.* (Lamiaceae, clove or African basil) and *O. sanctum L.* (holy basil) are known for their numerous pharmacological properties including anti-oxidant, anti-inflammatory, analgesic, antidiabetic, antihypertensive, antimicrobial, anticancer, hepatoprotective, and wound healing properties.[2] The family "Lamiaceae" grows all around the world, consisting of nearly 236 genera and 7200

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species. The genus *Ocimum* belongs to Lamiaceae family that comprises of more than 150 species.

From the African to Asian continent, roots, leaves, barks, seeds, and other parts of medicinal herbs have been consumed either alone or mixed with drinks such as tea to cure fever, wounds, constipation, digestive problems, hemorrhoid, and many others diseases.[2,3] Since the emergence of antimicrobial resistance (AMR) strains of bacteria have made many infectious diseases difficult to manage[4] including opportunistic infections such as candidiasis that can affect the immunocompromised individuals. Many natural products with antimicrobial properties have been explored as alternative treatment.[5,6] Consecutively, several modern methodologies have also been developed to effectively extract and purify various compounds from plants which can further enhance the antimicrobial properties. However, it is also important to determine whether the extraction process retains the bioactive properties of the herbal compounds once used or consumed without any harmful effects. Therefore, this study aimed to analyze the antimicrobial property of the ethanol-extracts obtained from the leaves of *O. sanctum* and *O. gratissimum* [Figures 1A and B and 2A and B] against some pathogenic microorganisms of oral and medical importance.

Materials and Methods

O. gratissimum and *O. sanctum* leaves were obtained from an organic farm in Chonburi district, Thailand. The tested microorganisms including *Streptococcus mutans* KPSK2, *S. pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 16794, and *Candida albicans* ATCC 10231 were provided by the Department of Oral Microbiology, Faculty of Dentistry, Mahidol University. Brain heart infusion (BHI) agar and broth (BD BBL), Sabouraud dextrose agar (SDA) and broth (SDB) (Difco), trypticase soy agar with 5% sheep blood (SBA) (BD BBL), and Mueller-Hinton agar (MHA) (BD BBL) were purchased from Becton Dickinson and Company, France.

Herb collection and ethanol-extract preparation

Sun-dried leaves of *O. gratissimum* and *O. sanctum* were minced and soaked in 95% ethanol (300 g minced leaves/1 L ethanol) for 7 days at room temperature.[7] Spray drying technique was used to obtain a powder form of ethanol-extracts of *O. gratissimum* and *O. sanctum*. The stock solutions of herbal extracts were individually prepared by mixing 50 g of *O. gratissimum* or *O. sanctum* powder in 100 mL of sterile distilled water or 50% ethanol, respectively, to obtain a concentration of 50% (w/v).

Antimicrobial activity determination by agar disk diffusion method

Streptococcus mutans KPSK2 and *S. aureus* ATCC 16794 were freshly subcultured on BHI agar, whereas *S. pyogenes* ATCC 19615 was subcultured on SBA. They were then incubated at 37°C with 5% CO_2 for 24–48 h. *Candida albicans* ATCC 10231 was subcultured on SDA and incubated at 37°C for 24 h. A few colonies of each microorganism were inoculated in BHI broth and SDB for bacteria and fungus, respectively, then incubated as described for 24 h. These bacterial and fungal suspensions were re-adjusted to obtain 1–5 x 10^8 colony-forming unit (CFU)/mL by measuring their turbidity at λ=600 nm to obtain an optical density of 0.11 and 0.14, respectively, using cell densitometer. The inoculum was spread individually and evenly on MHA.

Each herbal extract solution [50% (w/v)] was prepared in two-fold serial dilutions to obtain various concentrations ranging from 12.5% to 50% (w/v). Each herbal solution (20 µL) was applied on a 6 mm-diameter paper disk and allowed to air dry prior to being placed on MHA. Sterile distilled water or 50% ethanol and 0.2% chlorhexidine were used as negative and positive controls, respectively. The disks containing

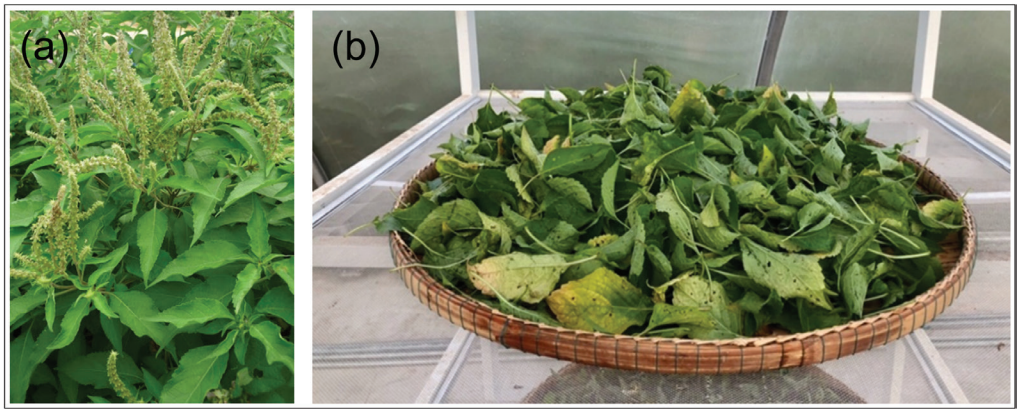

Figure 1: (A) *Ocimum gratissimum L.* plant. (B) Harvested sun-dried leaves of *O. gratissimum*

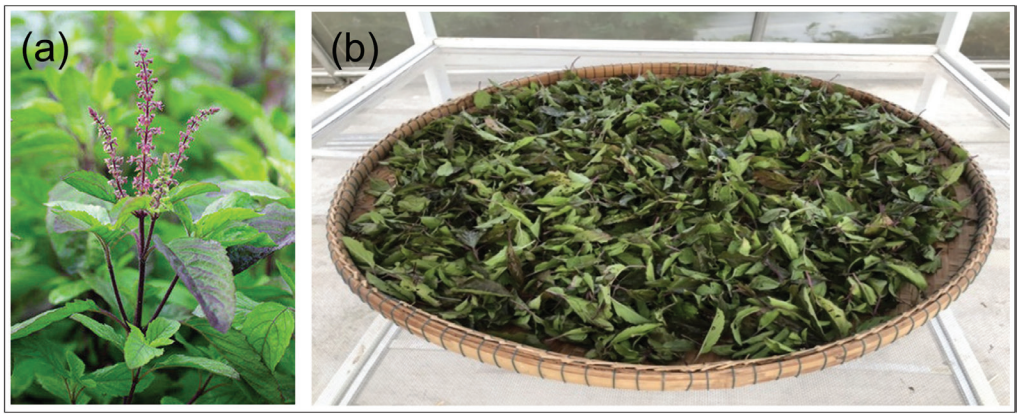

Figure 2: (A) *Ocimum sanctum L.* plant. (B) Harvested sun-dried leaves of *O. sanctum*

herbal extract were individually transferred and placed on the MHA primarily inoculated with certain microbial suspension. The plates were then incubated at appropriate environment for 24 h, after which the inhibition zones were observed and the diameters were measured in millimeter.

Minimum inhibitory concentration determination by broth micro-dilution method

First, two-fold serial dilution of the herbal extracts was prepared with sterile distilled water or 50% ethanol to obtain various concentrations of the herbal extract ranging from 0.096% to 50% (w/v). Then, 100 µL of herbal solution was added individually into each well of 96-well plate that was previously filled with 100 µL of BHI broth (2x). The final concentrations of herbal solution ranged from 0.048% to 25%. Then, a 100 µL of bacterial or fungal suspension was added to each well (10^4 CFU/mL) and kept in an incubator at appropriate environment for 24 h. The lowest concentration of herbal extract inhibiting the visible growth of a microorganism was recorded as MIC. A 0.2% chlorhexidine solution was used as the positive control whereas sterile distilled water or 50% ethanol was used as the negative control.

Minimum bactericidal or fungicidal concentration (MBC or MFC) determination

A 20 µL of culture broth from the wells at MIC and higher concentrations were transferred, inoculated on appropriate culture agar plates, and then incubated at appropriate environment for 24 h. The lowest concentration of herbal extract required to kill the tested bacteria or fungus by 99.9% (noted as a complete absence of bacterial or fungal colony) was recorded as the MBC or MFC.[8]

Statistical analysis

Each experiment was conducted in triplicate and repeated for five times at different periods of time. Inhibition zones of herbal extracts against the tested microorganisms were expressed in median of diameter (mm) with interquartile range. On the contrary, the MIC values of the herbal extracts were depicted in mode values.

Results

Antimicrobial activity determination by agar disk diffusion method

The preliminary antimicrobial activity of ethanol extracts from *O. sanctum* and *O. gratissimum* leaves were screened using agar disk diffusion method. Four concentrations including 50%, 25%, 12.5%, and 6.25% (w/v) of each herbal extract were tested for their inhibitory activity against *S. mutans* KPSK2, *S. pygogenes* ATCC 19615, *S. aureus* ATCC 16794, and *C. albicans* ATCC 10231. The ethanol extracts of both *Ocimum* species only inhibited the growth of the tested bacteria but did not have any inhibitory effect on the growth of fungus. The antimicrobial activity of *O. sanctum* and *O. gratissimum* extracts against the tested microorganisms was summarized in Tables 1 and 2.

Minimum inhibitory concentration determination by broth micro-dilution method

Following the screening test, the ethanol-extracts of *O. sanctum* and *O. gratissimum* leaves were further examined for their MIC and MBC/MFC values by broth micro-dilution method. The ethanol-extract of *O. sanctum* inhibited the growth of the tested bacteria and fungus with various degrees. *O. sanctum* extract strongly inhibited the growth of *S. pyogenes* with MIC of 0.19% (w/v) but expressed relatively less inhibitory activity against *C. albicans* with MIC of 3.12% (w/v). Similar to *O. sanctum*, ethanol-extract of *O. gratissimum* inhibited the growth of both bacteria and fungus tested in this study. However, the inhibitory activity of *O. gratissimum* extract against the tested oral

Table 1: Antimicrobial activity of ethanol-extract of *Ocimum sanctum* leaves against the tested microorganisms determined by agar disk diffusion method. Inhibition zone (diameter in mm) was expressed in median values and interquartile range

Concentrations of herbal extracts (% [w/v])	Inhibition zone (median of diameter [mm] ± interquartile range)			
	Streptococcus mutans	*Streptococcus pyogenes*	*Staphylococcus aureus*	*Candida albicans*
50.0	7 (0)	9 (0)	7 (0)	–
25.0	–	8 (0)	–	–
12.5	–	8 (1)	–	–
6.25	–	7 (8)	–	–
0.2% Chlorhexidine	15 (0)	13 (2)	14 (2)	14 (0)
50% Ethanol	–	–	–	–

– = absence of inhibition zone

Table 2: Antimicrobial activity of ethanol-extract of *Ocimum gratissimum* leaves against the tested microorganisms determined by agar disk diffusion method. Inhibition zone (diameter in mm) was expressed in median values and interquartile range

Concentrations of herbal extracts (% [w/v])	Inhibition zone (median of diameter [mm]± interquartile range)			
	Streptococcus mutans	*Streptococcus pyogenes*	*Staphylococcus aureus*	*Candida albicans*
50.0	8 (1.5)	0 (7.5)	7 (7)	–
25.0	0 (6.5)	–	–	–
12.5	–	–	–	–
6.25	–	–	–	–
0.2% Chlorhexidine	16 (1)	13 (1)	15 (1)	15 (0)

– = absence of inhibition zone

Table 3: Minimum inhibitory concentrate (MIC) and minimum bactericidal concentrate (MBC)/minimum fungicidal concentrate (MFC) values of ethanol-extract from *Ocimum sanctum* leaves against the tested microorganisms determined by broth micro-dilution method.

Microorganisms	*Ocimum sanctum* extract		50% Ethanol dilution	
	MIC [% (w/v)]	MBC [% (w/v)]	MIC [% (w/v)]	MBC [% (w/v)]
Streptococcus mutans	0.78	3.12	3.12	12.5
Streptococcus pyogenes	0.19	0.78	6.25	6.25
Staphylococcus aureus	0.78	1.56	0.78	0.78
Candida albicans	3.12	12.5	6.25	12.5

MIC and MBC/MFC values were expressed in mode values. (50% ethanol was used to dissolved *O. sanctum* extract powder)

Table 4: Minimum inhibitory concentrate (MIC) and minimum bactericidal concentrate (MBC)/minimum fungicidal concentrate (MFC) values of ethanol-extract from *Ocimum gratissimum* leaves against the tested microorganisms determined by broth micro-dilution method

Microorganisms	*O. gratissimum* extract	
	MIC [% (w/v)]	MBC [% (w/v)]
Streptococcus mutans	1.56	12.5
Streptococcus pyogenes	1.56	6.25
Staphylococcus aureus	1.56	1.56
Candida albicans	12.5	>12.5

MIC and MBC/MFC values were expressed in mode values

microorganisms was weaker than those of *O. sanctum* extract. The MIC and MBC/MFC values of *O. sanctum* and *O. gratissimum* extracts were summarized in Tables 3 and 4.

Discussion

Ocimum sanctum (holy basil or ka-phrao in Thai) and *O. gratissimum* (clove basil/African basil or yira/horapha chang in Thai), popular culinary herbs in Thai cuisine, were selected for this study. Ethanol-extracts of both herbs were tested for antimicrobial activity against *S. pyogenes* ATCC 14615, *S. aureus* ATCC 16799, *S. mutans* KPSK2, and *C. albicans* ATCC 10231. Ethanol-extract of *O. sanctum* leaves at 50% (w/v) concentration inhibited the growth of all three bacteria with various degrees of effectiveness. By agar disk diffusion method, *S. pyogenes* was sensitive most to ethanol-extract of *O. sanctum*. This result was also in agreement with the MIC value [0.19% (w/v)] determined by broth micro-dilution method. In addition, the growth of *S. aureus* and *S. mutans* were moderately inhibited by this extract using agar disk diffusion and broth micro-dilution methods [MIC values of 0.78%

(w/v)]. The antimicrobial activity of ethanol-extract of *O. sanctum* against the tested bacteria determined by agar disk diffusion method was similar to a previous study[9] but differed from several previous reports.[10-13]

The ethanol-extract of *O. sanctum* at 50% (w/v) concentration did not illustrate any inhibitory effect against *C. albicans* as primarily determined by agar disk diffusion method. The anti-*Candida* activity of the ethanol-extract of *O. sanctum* prepared in this study [MIC value of 3.12% (w/v)] was much weaker than previously reported by Sivareddy *et al.*[14] [MIC value of 0.2% (w/v)]. This may be due to the amount of raw material (dried leaves of herb) used for extraction process. The amount of *O. sanctum* dried leaves used for extraction in the current investigation was four times lower, compared with Sivareddy *et al.* (6 g in 20 mL of solvent vs. 25 g in 20 mL of solvent, respectively). Other notable differences were the extraction temperature (room temperature vs. cold extraction) and storage conditions (stored in auto-desiccator cabinet vs. at -20°C).

Extraction method is a vital key factor involved in the release of active components from the herbs. The ethanol-extract of *O. sanctum* prepared with Soxhlet extraction method showed the MIC and MBC against *S. mutans* at 25 µg/mL [0.0025% (w/v)],[15] which was much stronger than the values found in this study [MIC at 0.78% (w/v); MBC at 3.12% (w/v)]. Solvents used for extraction can also significantly affect the antimicrobial property of the extract. In 2016, Tantry *et al.*[16] found that the methanolic extract of *O. sanctum* was effective against *S. aureus* at the MIC value of 18.68 ± 0.95 mg/mL [1.868% (w/v)], which was slightly less effective than the ethanolic extract [MIC at 0.78% (w/v)] in this study. This seems to show that the active constituents of herb obtained may differ in quantity, quality, and categories dependent on the solvents used in the extraction process.

The ethanol-extract of *O. gratissimum* leaves at the concentration of 50% (w/v) expressed inhibitory effect only on the tested bacteria as determined by agar disk diffusion method. This result was further supported by the MIC values [1.56% (w/v)] of this herbal extract against all three tested bacteria. The ethanol-extract of *O. gratissimum* showed no inhibition zone against *C. albicans* by the agar disk diffusion method; however, this herbal extract expressed the inhibitory effect on the fungus with MIC value of 12.5% (w/v). Moreover, both antibacterial and antifungal properties of *O. gratissimum* ethanol-extract in this study were similar to those reported by Biqiku *et al.*[17] with the MIC values at >1024 µg/mL [>0.1024% (w/v)]. Furthermore, the antimicrobial property of *O. gratissimum* extract against *S. aureus* [MIC of 1.56% (w/v)] found in this study was slightly stronger than the study by Amengialue *et al.*[18] [MIC of 3.0% (w/v)]. However, the antimicrobial property against *S. aureus* of *O. gratissimum* ethanol-extract prepared by Soxhlet extractor[19] was much stronger [MIC of 0.2 (w/v); MBC of 0.4% (w/v)] than the results of this study. In addition, some reports have shown that herbal extracts with high quantity and quality can be obtained through the ethanol-maceration with either microwave or ultrasonic method.[20,21]

The form of the extract is also an important factor affecting the biological properties of herbs dependent on the diversity of active constituent released. Generally, essential oils of *O. sanctum* and *O. gratissimum* have shown greater antimicrobial activities than other forms including ethanol-, methanol- or n-hexane-extracts. Our previous study showed that the ethanol-extracts of *Ocimum* species contain functional groups indicative of aliphatic primary amines, aromatic compounds, alkanes, and alkyl halides on Fourier infra-red spectroscopy analysis.[22] Phenolic compounds including phenolic acids, rosmarinic acid, caffeic acid, and anigenin commonly present in both essential oil and ethanol-extract of *O. sanctum*, are active constituents effective against several pathogenic bacteria.[23-25] Phenolic compounds can damage permeability of bacterial membrane through mechanisms such as substrate complexing, membrane disruption, enzyme inactivation, and metal chelation resulting in ion leakage that can cause loss of membrane proton motive force leading to bacterial death.[26,27] Furthermore, hydrophobic property allows the oil to incorporate into the membrane and mitochondria of microorganisms increasing their permeability that leads to leakage of some cell contents and causes cell death.[26]

In this study, the ethanol-extracts of the leaves of *O. sanctum* and *O. gratissimum* extract were prepared and tested for antibacterial and antifungal effects against some important oral pathogens, and the results showed the former to have relatively stronger anti-bacterial effects. However, the study used powder based herbal extract which requires a proper solvent to attain good dispersion and maximize its bioactive properties. As with other studies of herbal extracts, there may be discrepancy in antimicrobial efficacy of the extracts based on variations in plantation factors such as geographic location and climate,[28] duration of harvesting,[29] active ingredients,[22] extraction processes,[23] strains of the tested microorganisms, and testing protocols. This study has shown promising

results for these herbal extracts but further investigations including antimicrobial property against microbial biofilm and cytotoxicity are required before they can be used safely and effectively as alternative antimicrobial agents.

Conclusion

O. sanctum and *O. gratissimum* ethanol-extracts effectively inhibited the growth of *S. pyogenes*, *S. aureus*, *S. mutans*, and *C. albicans*, respectively. The ethanol-extracts of both *Ocimum* species seemed to be more effective as antibacterial than antifungal agents. In addition, *O. sanctum* ethanol-extract expressed stronger antibacterial and antifungal properties than those of *O. gratissimum* extract.

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Conflicts of interest

The authors do not have any conflicts of interest to declare.

Authors' contribution

SC and TS were involved in study conception, MC was responsible for data collection and data acquisition, SC and TS were involved in data analysis and data interpretation, MC, BS, and SC were involved manuscript writing and revision. All the authors have read and approved the final version of the manuscript for publication.

Ethical policy and institutional review board statement

Not applicable.

Patient declaration of consent

Not applicable.

Data availability statement

The data set used in this study is available on a reasonable request from the corresponding author.

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