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# *In vitro* effects of *Salvia officinalis* L. essential oil on *Candida albicans*

Tularat Sookto[1], Theerathavaj Srithavaj[1], Sroisiri Thaweboon[2], Boonyanit Thaweboon[2*], Binit Shrestha[1]

[1]*Maxillofacial Prosthetic Clinic, Department of Prosthodontics, Faculty of Dentistry, Mahidol University, Thailand*
[2]*Department of Oral Microbiology, Faculty of Dentistry, Mahidol University, Thailand*

PEER REVIEW

**Peer reviewer**

Prof. Futoshi Nakazawa, Department of Oral Microbiology, School of Dentistry, Health Sciences University of Hokkaido, Japan.
Tel: +81-133-23-2513
E-mail: nakazawa@hoku-iryo-u.ac.jp

**Comments**

This is an informative study that evaluates the anticandidal effect of *S. officinalis* L. on the clinical and standard strains of *C. albicans*. The study also investigates the effects of the extract on adhesion of *C. albicans* to polymethyl methacrylate resin. The results show that the extract has inhibitory, cidal and antiadherent properties against *C. albicans* and suggests its potential usage as an alternative to commercial denture cleansers.

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ABSTRACT

**Objective:** To determine the anticandidal activities of *Salvia officinalis* L. (*S. officinalis*) essential oil against *Candida albicans* (*C. albicans*) and the inhibitory effects on the adhesion of *C. albicans* to polymethyl methacrylate (PMMA) resin surface. **Methods:** Disc diffusion method was first used to test the anticandidal activities of the *S. officinalis* L. essential oil against the reference strain (ATCC 90028) and 2 clinical strains of *C. albicans*. Then the minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) were determined by modified membrane method. The adhesion of *C. albicans* to PMMA resin surface was assessed after immersion with *S. officinalis* L. essential oil at various concentrations of 1×MIC, 0.5×MIC and 0.25×MIC at room temperature for 30 min. One-way ANOVA was used to compare the *Candida* cell adhesion with the pretreatment agents and Tukey's test was used for multiple comparisons. **Results:** *S. officinalis* L. essential oil exhibited anticandidal activity against all strains of *C. albicans* with inhibition zone ranging from 40.5 mm to 19.5 mm. The MIC and MLC of the oil were determined as 2.780 g/L against all test strains. According to the effects on *C. albicans* adhesion to PMMA resin surface, it was found that immersion in the essential oil at concentrations of 1×MIC (2.780 g/L), 0.5×MIC (1.390 g/L) and 0.25×MIC (0.695 g/L) for 30 min significantly reduced the adhesion of all 3 test strains to PMMA resin surface in a dose dependent manner ($P<0.05$). **Conclusions:** *S. officinalis* L. essential oil exhibited anticandidal activities against *C. albicans* and had inhibitory effects on the adhesion of the cells to PMMA resin surface. With further testing and development, *S. officinalis* essential oil may be used as an antifungal denture cleanser to prevent candidal adhesion and thus reduce the risk of candida-associated denture stomatitis.

KEYWORDS

*Salvia officinalis* L., *Candida albicans*, Essential oil, PMMA resin, Adhesion

## 1. Introduction

Denture-associated stomatitis is a common clinical disorder seen among denture wearers with a prevalence of 15% to over 77%[1]. Poor systemic immunity and nutritional status, local factors night-time denture wear, defective denture and poor denture hygiene can act as predisposing factors to denture stomatitis. Inherent porosities within the dentures can act as reservoirs of various microorganisms. *Candida albicans* (*C. albicans*) is the most prevalent and pathogenic species associated with denture stomatitis. The abilities of *C. albicans* to adhere directly or via a layer of dental plaque to the polymethyl methacrylate (PMMA) resin and to colonize on the resin surface are important factors in the pathogenesis of denture stomatitis[1,2].

Treatment of denture stomatitis involves maintainance of denture hygiene, correction of denture faults and medical therapy[1]. Regular cleaning of denture to remove adherent denture plaque is important to maintain health of oral tissues. Denture-cleansing methods include scrupulous removal of adherent plaque by mechanical and chemical methods. Although mechanical cleansing by brushing with dentifrice is popular, it might not be sufficient to remove the adherent microorganisms[3]. Furthermore, prolonged mechanical cleansing can create surface scratches that can enhance microbial attachment and biofilm growth[1].

The use of chemical denture cleanser is suggested as an alternative method. Chlorhexidine gluconate and sodium hypochlorite have shown powerful antimicrobial effect against most oral microorganisms and can be used as

*Corresponding author: Boonyanit Thaweboon, Department of Oral Microbiology, Faculty of Dentistry, Mahidol University, 6th Yothi Street, Phayathai, Rajathewee, Bangkok 10400, Thailand.
E-mail: boonitdt@yahoo.com
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effective denture-cleansing agents. However, discoloration of the dentures and even damage to the prosthesis have been reported after routine immersion of dentures in these solutions[4,5].

The search of new therapeutic alternatives has revealed that herbal extracts have potential antimicrobial actions. sage, *Salvia officinalis* L. (*S. officinalis*), is a perennial plant native to the Mediterranean region. Extracts from Sage have been reported to have a wide range of medicinal properties including spamolytic, antimicrobial and astringent[6-8]. Gas chromatography and mass spectrometry analysis have revealed *S. officinalis* L. essential oil to be composed mostly of oxygen-containing monoterpenes with major constituents such as 1, 8-cineole, β-thujone, borneol, β-elemene and camphor[9].

The objectives of this study were to evaluate the potential inhibitory effects of *S. officinalis* L. against *C. albicans* and to investigate the inhibitory effects on the adhesion of *C. albicans* to PMMA resin surface after immersion with *S. officinalis* L. essential oil.

## 2. Materials and methods

### 2.1. Materials

The essential oil extracted from the leaves of *S. officinalis* L. by steam distillation was obtained from Hong Huat Company Limited, Thailand. The test microorganisms used for this study were reference strain (ATCC 90028) and 2 clinical strains of *C. albicans* obtained from the Department of Oral Microbiology, Faculty of Dentistry, Mahidol University, Thailand.

### 2.2. Disc diffusion test

The test microorganisms were prepared by overnight incubation in Sabouraud dextrose agar (SDA) (BD, New Jersey, USA) at 37 °C for 48 h. Fresh colonies were transferred to sterile distilled water and candidal suspension was prepared at concentration of McFarland No.1 ($10^7$ CFU/mL). Then the candidal suspension was spread evenly on SDA. The stock reagent was prepared by mixing *S. officinalis* L. essential oil with an excipient, Tween-80 (Sorbitan mono-9-octadecenoate polyoxy-1.2-ethanediyl) (Sigma-Aldrich Pte Ltd., Science Park Road, Singapore), at 95% (v/v) concentration (855.00 g/L). Dilutions of the base reagent were done with sterile distilled water to obtain essential oil concentrations of 85.50 g/L (1:10), 17.10 g/L (1:50) and 8.55 g/L (1:100), respectively. 0.2% chlorhexidine and 5% (v/v) Tween-80 were prepared as positive and negative controls respectively. A volume of 20 μL of the dilutions were inoculated on paper discs (Whatman® Grand AA, Disc size 6 mm, Whatman International Ltd, England), placed on to the inoculums plate and incubated for 48 h at 37 °C.

### 2.3. Measurement of minimum inhibition concentration (MIC) and minimum lethal concentration (MLC)

The modified membrane method was used to determine the MIC of *S. officinalis* L. extract against the test strains. The test consists of cultivating the microorganisms on membranes (Sartorius AG, Göttingen, Germany) of 0.45 μm porosity placed on agar media containing different concentrations of the essential oil.

The stock essential oil was mixed with Tween-80 to obtain various concentrations of *S. officinalis* L. ranging from 44.50 to 0.17 g/L, which were then mixed with SDA. A volume of 20 μL of diluted cell suspension ($10^7$ CFU/mL) of the test microorganisms was then gently dropped on the membrane to obtain approximately $1\times10^5$-$2\times10^5$ CFU/spot. The inoculated plates were incubated at 37 °C for 48 h and evaluated for candida growth.

The membranes from the MIC test were obtained and placed in test tubes with Sabouraud dextrose broth (SDB) (BD, New Jersey, USA) and incubated at 37 °C for 48 h. MLC was determined as the minimum concentration of agent at which there was no microbial growth, as interpreted by a clear broth solution.

### 2.4. Inhibitory effect of S. officinalis L. essential oil on the adhesion of C. albicans to denture PMMA resin surface

#### 2.4.1. Sample preparation

The heat-polymerized PMMA resin (Vertex RS, Dentimex, Netherlands) blocks were prepared using a stone mold. The tissue surface of the samples were made uniform by packing the PMMA acrylic resin on stone mold surfaces, which had been poured on an even plastic retainer (Tru-Tain Othod, Rochester, USA). Two hundred and tweenty five PMMA samples (15 mm×15 mm×1 mm) were cut out from the resin blocks with the diamond disc (Axis Dental, Kerr Corporation, USA). The test surfaces of the samples were not polished so as to give an accurate impression of the tissue surface of the dentures. The sample strips were placed in an ultrasonic bath (VWRB9500E-DTH, VWR International, USA) for 20 min and then immersed in distilled water for 1 week to leach out the excess monomer. Prior to use, they were disinfected by dipping in 70% alcohol for 30 min, washed with sterile distilled water. Then they were dried and checked for sterility.

#### 2.4.2. Preparation of C. albicans suspension

*C. albicans* ATCC 90028 and the clinical strains were cultured on Sabouraud dextrose broth at 37 °C for 48 h. Then the culture was centrifuged (Hereaus Instruments, Heidelberg, Germany) at 3 000 r/min for 10 min and the resultant cell pellet was washed twice with phosphate-buffered saline solution (pH 7.2). *C. albicans* was resuspended in buffered saline solution at concentration of $1\times10^7$-$2\times10^7$ cells/mL (McFarland No.1).

#### 2.4.3. Adherence assay procedures

The adhesion assay was based on the method of Samaranayake and MacFarlane with modifications[10]. The PMMA resin samples were treated with essential oils at concentrations of 1×MIC, 0.5×MIC and 0.25×MIC, and another 2 groups of samples were treated with 0.2% chlorhexidine and sterile distilled water respectively. All PMMA resin samples were soaked in 2 mL of solution for 30 min. Then the samples were washed with phosphate buffer solution and placed in sterile microwell plate (Nunclon, Kamstrup, Denmark).

Approximately 2 mL of the standardized *C. albicans* cell suspension was added to each microwell containing the resin samples. The yeast cells were left to adhere for 60 min at 37 °C in a shaking incubator (120 r/min). The non-adherent cells were removed from the resin samples by gently dipping 3 times with 2 mL of phosphate buffer solution.

The remaining adherent cells on the surface of the PMMA

resin samples were fixed and stained. The samples were then mounted on glass slides and adherent yeast cells were quantified under microscope (Olympus CX-31, Japan). Twenty fields at 400 magnification were randomly selected from each strip and counted for cell adherence.

#### 2.2.3.4. Interpretation of data

The mean numbers of yeast cell per 20 fields were expressed as cells/mm$^2$. Blastopores and budding daughter cells were counted as individdual cells; hyphae forms were neglected.

### 2.5. Statistical analysis

All tests were done in triplicate on 3 separate occasions. One-way ANOVA was used to compare the mean number of the candidal cell adhesion among 5 pretreatment agents. Pairwise comparisons of the mean differences in candidal adhesion between the reagents were performed by Tukey's test with the confidence interval at 95%.

## 3. Results

### 3.1. Disc diffusion test

The inhibition zones of *S. officinalis* L. essential oil at the concentration of 855.00 g/L against *C. albicans* ATCC 90028, clinical strain I and II were (31.50±9.97) mm, (24.00±1.26) mm and (24.33±4.71) mm, respectively. On the contrary, at the concentrations of 85.50 g/L, 17.10 g/L and 8.55 g/L no inhibition zones were observed. 0.2% chlorhexidine, which was used as a positive control, exhibited inhibition zones of (22.83±2.31) mm, (21.16±1.17) mm and (20.66±1.03) mm against ATCC 90028, clinical strains I and II respectively. 5% (v/v) Tween-80 did not show inhibition zone.

### 3.2. MIC and MLC

The result showed that *S. officinalis* L. essential oil had MIC and MLC values of 2.78 g/L against all test strains of *C. albicans*.

### 3.3. Inhibitory effect of S. officinalis L. essential oil on the adhesion of C. albicans to denture acrylic surface

Table 1 shows the mean number of adhering *C. albicans* after immersion with 1×MIC (2.78 g/L), 0.5×MIC (1.39 g/L) and 0.25×MIC (0.69 g/L) of *S. officinalis* L., 0.2% chlorhexidine and the control, distilled water.

Chlorhexidine at 0.2% showed 96%-98% reduction in the adherent candidal strains when compared with the control, distilled water. *S. officinalis* L. essential oil at concentrations of 1×MIC, 0.5×MIC and 0.25×MIC showed a reduction in the adherent test strains ranging from 89%-96%, 78%-85% and 58%-77% respectively (Table 2). *S. officinalis* L. essential oil at the concentration of 1×MIC, 0.5×MIC and 0.25×MIC had significantly reduced the number of all three strains of candida adhering to acrylic surface when compare to the control, distilled water ($P$<0.05). Comparison between the different concentrations of *S. officinalis* L. showed significant difference in the reduction of adhesion between each concentration. The highest inhibition of adhesion of all three strains was seen with 0.2% chlorhexidine, followed by *S. officinalis* L. at concentrations of 1×MIC, 0.5×MIC and 0.25×MIC.

**Table 1**

The mean number of *C. albicans* ATCC 90028 and two clinical strains adhered on acrylic surface after 30-min treatment of *S. officinalis* (cells/mm$^2$).

| *C. albicans* | 0.25×MIC | 0.5×MIC | 1×MIC | Chlorhexidine (0.2%) | Distilled water |
|---|---|---|---|---|---|
| ATCC 90028 | 117.70±1.60$^{ab}$ | 61.62±1.52$^{ab}$ | 29.54±1.62$^{ab}$ | 6.34±1.68$^{a}$ | 278.74±1.66 |
| Clinical strain I | 138.90±1.27$^{ab}$ | 38.99±1.49$^{ab}$ | 12.82±1.34$^{ab}$ | 7.07±1.73$^{a}$ | 366.10±1.61 |
| Clinical strain II | 79.74±1.19$^{ab}$ | 49.16±1.22$^{ab}$ | 14.70±1.17$^{ab}$ | 11.05±1.22$^{a}$ | 342.61±1.19 |

Values are expressed in Mean±SD, $n$=20. MIC=2.78 g/L.
$^{a}P$<0.05 compared with distilled water, $^{b}P$<0.05 compared with chlorhexidine.

**Table 2**

Mean percentage of reduction of *C. albicans* adhesion on PMMA resin surface treated by *S. officinalis* compared to control, distilled water (%).

| *C. albicans* | 0.25×MIC | 0.5×MIC | 1×MIC | Chlorhexidine (0.2%) |
|---|---|---|---|---|
| ATCC 90028 | 58 | 78 | 89 | 98 |
| Clinical strain I | 62 | 89 | 96 | 98 |
| Clinical strain II | 77 | 85 | 95 | 96 |

MIC=2.78 g/L

## 4. Discussion

The essential oil and leaves extracts of *S. officinalis* L. has been documented to have wide range of antimicrobial effects[6]. The antimicrobial actions are suggested due to its particular chemical constituents dominated by oxygenated hydrocarbons, monoterpene hydrocarbons and sesquinterpene hydrocarbons particularly 1-8, cineole, α-β-thujone and borneol[9]. The antimicrobial activity of *S. officinalis* L. essential oil extract was tested by microdilution method against 13 bacterial and 6 fungi strains, showing antifungal effects against clinical strains of *C. albicans*[7]. However in this study, the use of modified membrane method was preferred over the microdilution method because the solution of essential oil and Tween-80, when diluted in water, produced a turbid color, which made the detection of microbial growth difficult to assess. Membrane allowed ease of identification, removal and transportation to the broth for further MLC testing. The results of this study found MIC and MLC values of *S. officinalis* L. essential oil for all 3 strains of *C. albicans* at 2.78 g/L, which was lower than the results of a study by Hayouni *et al* that showed *S. officinalis* L. essential oil was effective against *C. albicans* at MIC of 9 g/L[9]. On the contrary, the results from this study is higher than that was reported by Jirovetz *et al* which showed the MIC value of *S. officinalis* L. essential oil against *C. albicans* ATCC 10231 at a concentration of 0.06 g/L[8]. The difference in antifungal activity of the essential oils may have been due to variations in testing methods and the chemical composition of the extracts that can be influenced

by geographic and seasonal factors as well as the extraction process[11-13].

Candidal adhesions on acrylic surfaces are well established[2]. Various quantification methods to evaluate candidal adhesion to biological and inert surfaces are available including indirect immunofluorescence, fluorescence-labeled cytometry, radioisotope analysis and photometric quantification. In this study, direct enumeration under light microscopy was used due to its cost effectiveness. Although this technique permits visualization of the yeast cells adherent to PMMA resin, it is subjective and time-consuming.

As compared to distilled water, there was statistical difference in the reduction of adhesion of all three strains of *C. albicans* to PMMA resin surface at all three concentrations of *S. officinalis* L. Although higher concentration of the essential oil led to greater anti-inhibitory effects, it was observed that inhibition of candidal adhesion occurred well below the MIC values. Immersion of the samples at even a low concentration of 0.25×MIC (0.695 g/L) for 30 min showed 58%-77% reduction in adhesion as compared to distilled water.

There have been many studies to explain the mechanism of adhesion of *C. albicans* to oral and denture acrylic surface[2,10]. The ability of candida to adhere and form biofilm is an important factor for its virulence. Initial adhesion of candida is mediated by cell surface hydrophobicity, van der Waals forces and receptor-ligand interactions. Although there are no studies specifically on the anti-adherent activity of *S. officinalis* L. on *C. albicans*, it has been purposed that the main steps involved in biofilm formation such as adherence and proliferation (germ-tube formation and hyphae formation) are affected after exposure of the microorganism to monoterpenes or monoterepene containing essential oils. These compounds interact with lipid components in the cellular structure, thereby increasing membrane permeability and electrolyte imbalance. This affects the membrane balance and interferes with formation of filamentous form of *C. albicans* essential for biofilm formation[14]. As *S. officinalis* L. is rich in monoterpenes, this could have been a possible mechanism responsible for its anti-adherent effects against *C. albicans*.

The type and processing technique can greatly influence the surface characteristics of PMMA resin, including surface roughness which is a major contributing factor in candidal retention; a rougher surface topography promotes more candidal adhesion compared to smooth surfaces[15]. In this study, heat-polymerized PMMA resin was used, as it is the most commonly utilized material for denture fabrication. It also has less porosities and candidal adhesion as compared to self-polymerized PMMA resin[16]. Although PMMA samples prepared under a glass slab has shown to have a smoother surface[15,16], a stone mold was used to represent the actual clinical situation during processing of dentures. The surface roughness of the test surfaces of the PMMA samples was controlled by processing them in a stone mold, which had been prepared on an even plastic retainer.

*S. officinalis* L. essential oil has also been traditionally consumed in form of herbal drinks in many European countries. However, the use of aromatic plants such as *S. officinalis* L. and *Artemisia absinthium* had been scrutinized in the past, due to reports of thujone-related convulsions and hallucinations. Although there are no restrictions for sage preparation to be used in foods, the European Medicines Agency has recommended an acceptable daily intake of 5.0 mg/person[17]. The literature regarding the maximum daily intake and systematic exposure assessment from medicines containing *S. officinalis* L. is also lacking[18]. The purpose of this study was to use *S. officinalis* L. essential oil as a denture-cleansing agent rather than for direct consumption. Even though thujone is present in the *S. officinalis* L. essential oil in relatively low amounts[7,8], it may be recommended to wash the dentures thoroughly after immersion in the essential oil to prevent thujone related side effects.

In conclusion, the results of this study showed that *S. officinalis* L. essential oil had inhibitory as well as fungicidal effects on all the test strains of *C. albicans* at 2.78 g/L. Immersion of the heat-polymerized PMMA test samples in *S. officinalis* L. essential oil showed a dose dependent reduction in the number of all three strains of candida adhering to resin surfaces as compared to distilled water. Although significant difference ($P<0.05$) in the mean number of adhering candida between all tested concentrations of *S. officinalis* L. and 0.2% chlorhexidine was noted, this study exhibited that there was a 89%-96% reduction in the adherence of all tested *C. albicans* strains with *S. officinalis* L. at 1×MIC (2.78 g/L) compared to distilled water, whereas 0.2% chlorhexidine showed an reduction of 96%-98%.

Despite the limitations, the results of this initial study have been encouraging. More studies on the antimicrobial action of higher concentrations of *S. officinalis* L. essential oil, its effect on the physical properties of PMMA resin and related toxicities are needed to validate its use as a potential denture-cleansing agent.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

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## Comments

### *Background*

Candida associated denture stomatitis is prevalent worldwide and seen in almost 60% denture wearers. Inherent porosities within the denture base resin can serve as a reservoir for Candida. Mechanical debridement alone may not suffice to remove the organisms completely and gives them the ability to proliferate under the nutrient-rich oral conditions and re-infect the inflamed oral tissues. Thus, the need for denture cleansers or disinfectants has arisen. Studies have shown that popularly used chemical disinfectants and procedures can lead to alterations in the properties of denture following long-term usage. The overt use of chemical has also led to the development of resistant species. This has established the need to seek for alternatives. The interest on the potential antimicrobial effects of herbs has been ever increasing due to their

antimicrobial action, relatively low toxicity, acceptance and ability to be manufactured locally.

### *Research frontiers*

Studies have been conducted to study the chemical composition of the *S. officinalis* L., and other species of sage. Phenolic and flavonoids compounds isolated from the extracts have been shown to have potential antimicrobial and antioxidant activities. Recent studies by Kontogianni *et al.* (2013) have also shown these extract to have cytotoxic and immunomodifying activities against cancer cells.

### *Related Reports*

The results from this study had variance with past literature. The minimal inhibitory concentration was lower than those reported by Hayouni *et al.* (2008) and, on the contrary, higher than those reported by Jirovetz *et al* (2006). These variations may be due to the differences in the testing methods employed, microbial strains, and the chemical compositions of the extracts.

### *Innovations and breakthroughs*

There have been limited studies on the anticandidal properties of *S. officinalis* L.. Data regarding its effects on the adhesion of *C. albicans* to polymethyl methacrylate resin is also lacking. The results of the study showed the extract has potential anticandidal activities and also suggested that immersion of dentures in the extract can have a profound reduction in the adhesion of *C. albicans* compared to distilled water. The research also compared the result with those of 0.2% chlorhexidine.

### *Applications*

The ability of the candida to adhere directly or via a layer of plaque to the polymethyl methacrylate resin adds to its virulence in the initiation and continuation of denture stomatitis. The anticandidal and antiadherent effects of *S. officinalis* L. can have a profound impact on reducing the load of *C. albicans* on denture base and may help to reduce the prevalence of denture stomatitis. However, more studies on the effects of extract on the properties of denture base resin need to be addressed.

### *Peer review*

This is an informative study that evaluates the anticandidal effect of *S. officinalis* L. on the clinical and standard strains of *C. albicans*. The study also investigates the effects of the extract on adhesion of *C. albicans* to polymethyl methacrylate resin. The results show that the extract has inhibitory and antiadherent properties against *C. albicans* and suggest its potential usage as an alternative to commercial denture cleansers.

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