Original Article

Characterization of Herbal Antifungal Agent, *Origanum vulgare* against Oral *Candida* spp. Isolated from Patients with *Candida*-Associated Denture Stomatitis: An *In vitro* Study

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Abstract

Background: Candida Associated Denture Stomatitis is the prevalent fungal pathosis in denture wearers, especially in immunocompromized patients. Existing antifungal agents are ineffective since the Candida species become resistant and also, they become toxic. Origanum vulgare is a herbal plant with high anti-fungal activity against Candida of blood and urine origin. However, it has never been explored against Candida from oral cavity. **Materials & Methodology:** Dry leaves of the plant were purchased and authenticated. Oil extraction was done using Hydro-distillation method. Clinical isolates of Candida from denture wearers was speciated using CHROMagar. Well Diffusion test was used to confirm the antifungal activity. Hydro-distillation & Maceration methods of extraction were compared. MIC/MFC was determined using CSLI guidelines. Infra-Red Spectroscopy was used to identify the active functional group. **Results:** O.vulgare showed 30±3mm of zone of inhibition as against 19mm for fluconazole. The suitable extraction method was Hydro-distillation. MIC & MFC were found to be 0.024% and 0.097% respectively which was much lesser than for fluconazole (0.25%). The active functional group had chemically similar structure as Carvacrol, usually found in antifungal herbs. **Conclusion:** within the limitations of the study, it was concluded that (a)O.vulgare is anticandidal for clinical isolates of oral Candida, (b) Hydro-distillation is an effective method as compared to Maceration (c) MIC & MFC are much lower than that of fluconazole (d) the major functional group was structurally similar to Carvacrol.

Keywords: *Antifungal herb, Candida-associated denture stomatitis, candidiasis, fluconazole, Origanum vulgare*

Introduction

Fungal pathosis caused by different *Candida* species results in localized and/or systemic lesions. In the oral infections, among various strains, *Candida albicans* is often found in abundance in the lesions.[1-3] Factors such as imbalanced diet, prolonged usage of broad-spectrum antibiotics, immunosuppressant medications and wearing of an ill-fitting, and poorly maintained oral prosthesis, can promote the conversion of these oral commensals into potential pathogens. Overgrowth of these pathogens on the oral mucous membranes can lead to Candida-associated denture stomatitis (CADS).[3,4]

Most antifungal agents currently available for the treatment of CADS have several drawbacks. Some antifungal agents have been found to be toxic on long-term application required to resolve the condition.[5,6] Furthermore, the fungi develop resistance to these drugs due to their inherent adaptive qualities such as formation of biofilms, expression of different genes, development of efflux pumps, or persistor cells.[7-12]

Herbal plants have a long history of being used as culinary flavoring agents and as medicines for different ailments in human beings.[13,14] Several plants have been found to possess antifungal properties against *Candida*-related pathosis in the urine, blood, and stools.[15,16]

Origanum vulgare is an herbal plant that has been used as food flavoring agent in many countries [Figure 1]. It has strong antiseptic and antimicrobial activity due to the presence of carvacrol and thymol, both phenolic compounds which directly inhibit germination and hyphal formation in *Candida*. Its antifungal actions have been found to be similar to nystatin and amphotericin B. A study in mice has shown

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to cure systemic candidiasis on a daily oral administration of 1.0 µl of *O. vulgare* essential oil for 30 days.[17] In human beings, the antifungal activities of *O. vulgare* have been studied against the *Candida* isolated from sources of various systemic conditions.[4,17,18] However, no study is available in the literature that has explored its possible anti-candidal activity against oral isolates of *Candida* that cause CADS.

Hence, *O. vulgare* was chosen to characterize its antifungal effects on oral isolates of *Candida* from a denture wearer with CADS. The organisms were collected from the patients affected with CADS. The essential oil was extracted from the leaves of the plant, and the antifungal activity was confirmed using well diffusion method against *Candida* species. The efficacy of two extraction methods, hydrodistillation and maceration, was compared. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the herb were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The active functional group in the experimental herb was identified as a compound similar to carvacrol through infrared (IR) spectroscopy method.

Materials and Methods

Collection and speciation of oral isolates of *Candida*

Ethical clearance for the study was obtained from Central Ethical Committee of the University. The procedure was explained to the patient in his/her mother tongue and a signed consent was obtained. Swabs from the tissue surface of the maxillary denture were taken from the patients who showed clinical symptoms of CADS.

Speciation of the *Candida* from the clinical samples was done, and they were further subcultured on Sauboraud Dextrose Agar (SDA) twice to confirm pure isolation of the yeast. The different species of *Candida* were first identified by Gram staining and later on confirmed by germ tube test, corn meal agar for chlamydospore formation and Chromagar, and other biochemical tests. Chrome *Candida* differentiation agar was used to differentiate *Candida* species. Chromagar for differentiation of *Candida* (Himedia, Mumbai, India) was prepared following manufacturer's instructions. *C. albicans* was differentiated as the colony exhibited light green color. Along with colonies of *C. albicans*, steel-/blue-colored colonies of *Candida tropicalis* and light pink-colored colonies of *Candida glabrata* were observed and identified [Figure 2].

Fungal inoculum was prepared from overnight culture (24 h) on SDA. Colonies were directly suspended in saline to obtain turbidity comparable to that of 0.5 McFarland Standards (approximately 1.5×10^6 CFU/ml).

Essential oil extraction

Dried leaves and bracts of *O. vulgare* were procured and essential oil [Figure 3] was extracted using hydrodistillation method. The dry leaves were used in a Clevenger apparatus for 4 h to extract the oils with $CHCl_3$, and then, it was dried over anhydrous sodium sulfate. The oil yield was stored in sealed amber-colored vial at 4°C until use.

Determining the antifungal activity of Origanum vulgare

Antifungal activity of *O. vulgare* was determined by agar diffusion test. Modified Kirby–Baeur method was used. SDA plates were prepared according to the CLSI

Figure 1: ***Origanum vulgare***

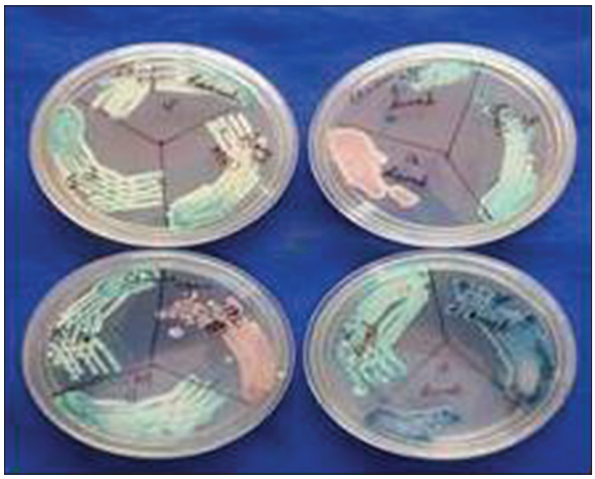

Figure 2: ***Candida*** **speciation**

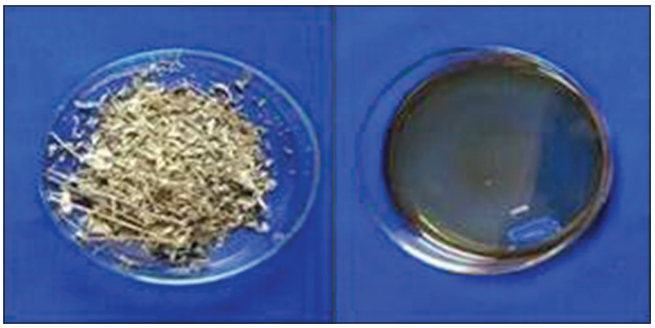

Figure 3: Dry leaves and essential oil of ***Origanum vulgare***

guidelines.[19] These plates were then inoculated within 15 min of the preparation of the suspension so that the density does not change. The medium was inoculated by even streaking with the help of a sterile cotton wool swab.

Six-millimeter wells were punched out on agar. These wells were then inoculated with 20 µl of the test material. The plates were incubated for 48 h at 37°C aerobically. After the incubation period, plates were removed and zones of inhibition were recorded.

The diameter of zone of inhibition was measured to the nearest millimeter with a thin transparent millimeter scale. Nystatin (HiMedia, Mumbai), a proven antifungal agent, was taken as the control for the test material.

Comparison of efficacy of extracts from maceration technique with hydrodistillation technique for Origanum vulgare [Table 1]

Maceration method

The different extracts of *O. vulgare* were obtained from maceration method using petroleum ether, chloroform, ethyl acetate, n-butanol, and methanol [Figure 4].

Preparation of ethanolic extract

The shade-dried powdered leaves (1 kg) of *O. vulgare* were soaked in ethanol (95%) for 4 days. After 4 days, the ethanolic layer was decanted off. The process was repeated for four times. The solvent from the total extract was distilled off, and the concentrate was evaporated on a water bath to a syrupy consistency and then evaporated to dryness.

Fractionation of ethanolic extract

The extract (70 g) was suspended in distilled water (1500 ml) and then extracted successively with petroleum ether (60°C–80°C, 8 × 500 ml), chloroform (8 × 500 ml), ethyl acetate (8 × 500 ml), n-butanol (8 × 500 ml), and methanol (8 × 500 ml). All the fractions were then washed with distilled water (30 ml), dried over anhydrous sodium sulfate, and freed of solvent by distillation. The ethanolic extract was thus fractionated into petroleum ether soluble extract (13 g), chloroform soluble extract (10 g), ethyl acetate soluble extract (7 g), n-butanol soluble extract (6 g), and methanol soluble extract (23 g).

The above extracts and the essential oil obtained through hydrodistillation method were tested and compared for their antifungal efficacy using well diffusion method against all the *Candida* species used in this study.

Minimum inhibitory concentration/minimum fungicidal concentration of Origanum vulgare [Table 2 and Figure 5]

Determining MIC and MFC of *O. vulgare* was done according to the CLSI guidelines.[19]

A pure culture of *Candida* spp. was grown in Sabouraud Dextrose Broth. The optical density of planktonic suspension of each culture was adjusted to 1.5×10^8 CFU/ml (McFarland 0.5 standard). A known concentration of the essential oil was serially diluted to two folds in sterile test tubes.

After the experimental agent was diluted, a volume of the standardized inoculum equal to the volume of the diluted antimicrobial agent was added to each dilution tube,

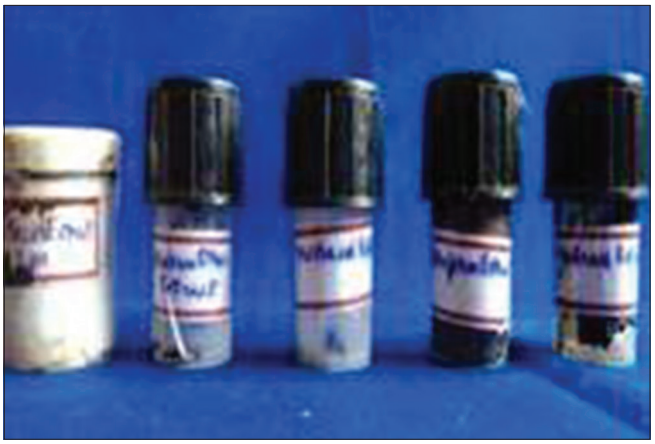

Figure 4: Maceration extracts of *Origanum vulgare*

Table 1: Comparison of efficacy of extracts from maceration technique with hydrodistillation technique for *Origanum vulgare*

Organism	Petrol ether	N-butanol	Ethanol	Chloroform	Methanol	Hydrodistillation
C. albicans	-	-	10 mm	-	-	30±3 mm
	-	-	9 mm	-	-	
	-	-	10 mm	-	-	
C. tropicalis	7 mm	-	12 mm	10 mm	-	32±3 mm
	8 mm	-	12 mm	9 mm	-	
	8 mm	-	14 mm	10 mm	-	
C. glabrata	-	-	-	-	-	36±2 mm
	-	-	-	-	-	
	-	-	-	-		

Results were tabulated and statistically analyzed using ANOVA. ANOVA: Analysis of variance. *C. albicans*: *Candida albicans*; *C. glabrata*: *Candida glabrata*; *C. tropicalis*: *Candida tropicalis*

Table 2: Minimum inhibition concentrations/minimum fungicidal concentration of *Origanum vulgare*

Organism	Concentrations of the extract in percentage											
	50	25	12.5	6.25	3.12	1.56	0.781	0.39	0.195	0.097	0.048	0.024
Minimum inhibition concentrations as expressed in triplicates												
C. albicans	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
C. glabrata	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
C. tropicalis	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Minimum fungicidal concentration as expressed in triplicates												
C. albicans	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	10^{3}*	10^{4}*
C. glabrata	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	10^{2}*	10^{3}*
C. tropicalis	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	10^{3}*	10^{3}*

*Growth expressed in CFU/ml as when compared to the 10^{6}CFU/ml of the positive control. NG: No growth;
C. albicans: *Candida albicans*; *C. glabrata*: *Candida glabrata*; *C. tropicalis: Candida tropicalis*

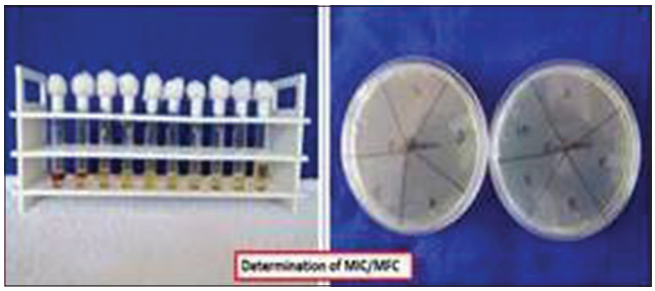

Figure 5: Minimum inhibitory concentration/minimum fungicidal concentration of *Origanum vulgare*

bringing the microbial concentration to approximately 500,000 cells/ml. Two control tubes were also included, one tube containing only the candidal culture which served as the positive control and other tube containing undiluted oil and the fungal culture which served as the negative control.

The inoculated, serially diluted extract was incubated aerobically for 37°C for 18 h.

After incubation, the series of dilution tubes were observed for microbial growth, indicated by turbidity and/or a pellet of microorganisms in the bottom of the vessel. The last tube in the dilution series that did not demonstrate growth corresponds with the MIC of the antimicrobial agent. The MIC endpoint is the lowest concentration of the oil at which there is no visible growth in the tubes.

The test tubes demonstrating no visible turbidity were subcultured to solid agar plates and MFC was determined by comparing the growth with the positive control. The MFC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial fungal population where no visible growth of the fungi was observed on the SDA plates. The tests were repeated in triplicates.

Infrared spectroscopy to determine the active functional group in Origanum vulgare

IR spectroscopy method was used to identify the active functional group using Bruker Fourier transform infrared alpha model, Germany [Figure 6]. The transmitted light frequencies were recorded to obtain IR spectrum [Figure 7], and it was later compared with existing database for the presence of active groups.

Results

Antifungal activity of *Origanum vulgare*

Agar well diffusion method was used to confirm the antifungal activity of the experimental herb. Authenticity of the test material was confirmed, as zone of inhibition was found to be 30 mm as compared to 22 mm of presently known antifungal agent nystatin**.**

Results were tabulated and statistically analyzed using analysis of variance.

None of the extracts from maceration technique showed zone of inhibition equivalent to zone of inhibition obtained through essential oil from hydrodistillation method (>30 mm). However, ethanol extract was the nearest and was efficient against only two species of *Candida*, that is, *C. albicans*, (10 mm) and *C. tropicalis* (14 mm). Ether (8 mm) and chloroform (10 mm) were effective only against *C. tropicalis*. n-butanol and methanol extracts were not effective against any *Candida* spp.

Infrared spectroscopy results

The peaks show OH, CH, and C = C groups suggesting that carvacrol and/or related compounds can be present in the test material *O. vulgare* [Figure 7].

Discussion

In this study, a novel medicinal plant *O. vulgare* was investigated for its antifungal activity against oral isolates of *Candida*, a unique research which has never been carried out in prosthodontics so far.

O. vulgare has been extensively used as antimicrobial agent against *Candida* infections in animals and human beings. The antifungal effects of *O. vulgare* have been studied against *Candida* spp. isolated from systemic infections. However, on review of existing literature, no study was available where it has been used against isolates of *Candida* from the oral cavity of a denture wearer.

Figure 6: Bruker Fourier transform infrared alpha model

CADS is a form of oral candidiasis and a chronic inflammatory reaction of the oral mucosa in a denture-wearing patient. It is usually characterized by erythema and edema of the palatal area localized to denture bearing tissues causing discomfort in wearing prosthesis. Localized trauma from a poorly maintained, ill-fitting denture and/or allergy to the material used for the prosthesis are the most common reasons for the causation of CADS.[2] In an immunosuppressed status such as diabetes mellitus,[20] long-term antibiotics therapy, chemotherapy for cancer, transplant recipient,[21,22] and AIDS/HIV,[3,23] the normal oral commensal fungus *Candida* turns virulent causing infection which if not treated can prove fatal. The present-day antifungal agent like fluconazole has been found to be causing toxicity on long-term application, and it also results in the development of resistance in the causative agent.[5-12]

Hence, this research was undertaken to explore the characteristics of *O. vulgare* for its possible antifungal activity against oral isolates of *Candida spp.* among patients with CADS.

The clinical isolates of *Candida* used in our study were collected from patients affected with CADS and they were subjected to speciation procedure using Chromagar to identify the species responsible. It was observed that *C. albicans* was the highest followed by *C. tropicalis* and *C. glabrata*.

The herb *O. vulgare* is rich in its antifungal activity which has been tried against clinical isolates from systemic diseases but never in oral isolates of *Candida* from denture wearers. Hence, it is justified to explore its possible antifungal activities against the microorganisms causing the disease in the denture wearing section of the population. It is a novel study of its kind in the literature. In this study, the antifungal effects of *O. vulgare* were assessed and efficacy of different extraction procedures was compared. The MIC and MFC of the herb were determined

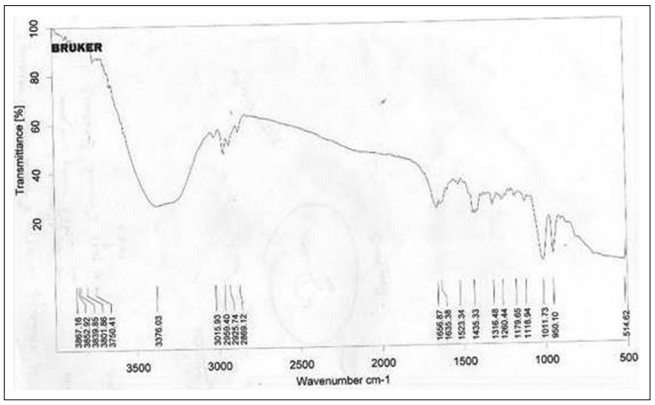

Figure 7: Infrared spectrum for *Origanum vulgare*

and the active functional component present in it also was identified.

Antifungal activity of *Origanum vulgare*

The antifungal activity of the experimental herb *O. vulgare* was confirmed by comparing it with the known antifungal agent like nystatin. Antifungal susceptibility testing was carried out using agar well diffusion method. Antifungal activity showed zone of inhibition as 30 mm for *O. vulgare* as compared to 22 mm for nystatin. This observation proved that the experimental herb *O. vulgare* is antifungal in nature and also it is superior in its activity when compared to nystatin. This result is in accordance with a similar study carried out by Stiles *et al*.[4] where they observed a zone of inhibition for nystatin as 22–25 mm and that for *O. vulgare* was 40–45 mm. However, their study was on *Candida* isolates that were obtained from human stools. Cleff *et al*.[24] studied the effect of *O. vulgare* against reference strains of *Candida* and found that all were susceptible to the essential oil of *O. vulgare*. Rosato *et al*.[25] and Souza *et al*.[26] evaluated and observed that the essential oil *O. vulgare* appeared to be effective, inhibiting all the *Candida* species in their study.

Efficacy of various extraction methods for *Origanum vulgare*

We compared essential oil of *O. vulgare* obtained through hydrodistillation and the various extracts obtained through maceration method. It was observed that the antifungal activity of the essential oil obtained by hydrodistillation method was higher against the *Candida* species included in this study (zone of inhibition 30 ± 6 mm) [Table 1]. The extracts obtained through maceration technique did not show significant activity as compared to the essential oil. Among extracts, isolate of *C. albicans* showed susceptibility to only chloroform extract (10 mm). *C. tropicalis* showed mild susceptibility to petrol ether (7–8 mm), ethanol (12–14 mm), and chloroform (9–10 mm). *C. glabrata* did not show susceptibility to any of the extracts but was susceptible to the essential oil (36 ± 2 mm) [Table 1].

Hence, in the present study, the hydrodistillation method was proved to be better than maceration method for this

novel herb. The hydrodistillation method for *O. vulgare* has been found to affect the quantity and quality of the essential oil obtained.[27-30] It has been observed by Kawase *et al*.[31] that when compared to other techniques, the quantity of the essential oil and its phytochemicals may contain higher concentration of active components. This could be the reason for the difference where essential oil of *O. vulgare* from hydrodistillation process showed better efficacy than extracts from maceration among *Candida* species.

However, in contrast, in a study by Malabadi and Kumar,[32] the effect of extracts of four herbal plants on *C. albicans* were assessed. The extracts included acetone, hexane, dichloromethane, and methanol. They observed that methanol extracts exhibited maximum antifungal effect against these *Candida* species. The conflicting result observed by them as compared to our study could be due to the fact that the plants used were different from *O. vulgare.* Herbal plants have different range of activity depending on their composition of phytochemicals and pathogens exhibit varied response to the different products.

Minimum inhibitory concentration/minimum fungicidal concentration of *Origanum vulgare*

After the antifungal action the essential oil was established, the research was continued to determine the MIC/MFC using serial dilution method. Serial dilution method for determination of MIC and MFC of the herbal oils have been implemented by Chitwood[33] and Johnson (2008)[34] and it was found to be satisfactory to use in laboratories. Furthermore, according to the NCCLS standards, it is an efficient method to evaluate MIC/MFC of the antifungal agents.[35] In the present study, the MIC of *O. vulgare* was found to be 0.024% and MFC was observed as 0.097% [Table 2] when it was tested against *Candida*. In a study by Manohar *et al*.,[17] the authors showed that *O. vulgare* can be fungicidal on *C. albicans* from mice with a MIC of 0.125% and MFC of 0.25%. In their study, they also observed that a daily dose of 1.0 µl of *O. vulgare* oil for 30 days can cure systemic candidiasis in mice.

We compared the MIC/MFC of *O. vulgare* with existing value for fluconazole (0.25%)[36] and it was observed that the MIC/MFC of *O. vulgare* (0.024% and 0.097%) was much lower. The MIC of *O. vulgare* (0.024%) which was determined in our study is in par with a study carried out by Hammer *et al*.,[37] wherein it was found to be 0.03% against *C. albicans*. In a study by Xu *et al*.,[38] MIC of fluconazole was found to be 125 times more in azole-resistant *C. albicans* than in susceptible *C. albicans.* The present experimental herb *O. vulgare* showed much lesser MIC/MFC as compared to fluconazole indicating that the herb can be effective even in a lower dose of MIC/MFC. In a comparative study by Lambert *et al*.,[39] they also observed that *O. vulgare* had a total inhibitory effect on the pathogens involved. However, their study was against bacteria and not the fungi affecting human beings.

Comparison of the MIC/MFC results obtained in the present study with earlier studies available may not be reliable, as the composition of the essential oil used in the present study could be different from those used in the previous studies. It is known that the composition of an essential oil can vary according to the local climatic and environmental situations and the time of harvesting the herb.[40,41] Furthermore, there are no comparable studies on effect of *O. vulgare* on human oral isolates from denture stomatitis patients, as the present study is the first of its kind in the literature.

Active functional group in *Origanum vulgare*

Herbal antifungal agents have been shown to contain active phytochemicals that are responsible for their activity against fungal pathogens. We analyzed our experimental herb *O. vulgare* using IR spectroscopy to identify the active functional group present in it. It was observed that *O. vulgare* contained a compound which had a similar chemical structure like carvacrol ($C_6H_3CH_3$ (OH) (C_3H_7)). The peaks in the spectrum showed OH, CH, and C = C groups indicating carvacrol[42,43] and/or related constituent is present in the test material. The hydroxyl group (OH) has been found to be responsible for the antifungal actions by carvacrol.[44,45] In this aspect, the present study is in consistency with earlier researchers where carvacrol has been found to be the primary active component giving antifungal property to *O. vulgare*[40,41,43] and that it inhibited *Candida*.[4,46]

Conclusion

In this study, effect of the antifungal agent *O. vulgare* was carried out on three oral isolates of *Candida* from denture wearers affected with CADS. Characterization of *O. vulgare* was carried out with respect to its antifungal activity, essential oil extraction methods, MIC/MFC, and the active functional ingredients present in it. Within the limitations of the study, it was concluded that:

1. *O. vulgare* is anti-candidal in nature against oral isolates of *Candida* in CADS
2. Antifungal effect of essential oil obtained through hydrodistillation is more than the effects of extracts obtained through maceration
3. MIC and MFC of *O. vulgare* for oral *Candida* are 0.024% and 0.097%, respectively, and were much lower than that of fluconazole
4. The major functional unit in *O. vulgare* is similar to carvacrol, which is a proven antifungal compound.

In this preliminary investigation, *O. vulgare* was found to be possessing high anti-candidal properties against the tested oral isolates of *Candida* spp. However, the authors suggest further in-depth research into this novel herb before it can be considered for therapeutic purposes in the oral cavity.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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