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Original Article

SCREENING OF SELECTED PLANT ESSENTIAL OILS FOR THEIR ANTIFUNGAL ACTIVITY AGAINST CANDIDA SPECIES ISOLATED FROM DENTURE STOMATITIS PATIENTS

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Abstract

Background: Many efforts have been made to discover new antimicrobial compounds from various sources such as micro-organisms, animals and plants. Use of herbal medicines has a long history in Asian population in treating different ailments. Many plants have been found to have properties like antibacterial, antifungal and antioxidant activities.

In a complete denture wearer, due to either local or systemic factors, denture stomatitis has been found to a have high prevalence. In such patients, higher incidence of Candida infection and increased levels of Candida spp. also have been identified. Existing antifungal agents like Nystatin & Fluconazole have been found to have toxic effects on long term application and chances of development of drug resistance is high. Medicinal plants have been used to inhibit growth and development of fungal infection from Candida isolated from sites other than oral cavity.

Objectives: To evaluate the antifungal activity of some of the medicinal essential oils against oral isolates of Candida obtained from denture wearing patients identified through a survey.

Materials and Methods: The oral isolates of Candida species were collected after conducting a survey of complete denture wearers, wearing the denture for at least more than a year. The speciation of the organism was done after identifying it from the specimens. It was confirmed with the help of germ tube test, other biochemical tests and chlamydospore formation tests. A lab isolate also was included in the study. The essential oils of different herbs were extracted using hydro distillation methods. The antifungal activity of these oils was tested and compared with antifungal activity of Nystatin and fluconazole using modified Kirby-Baeur method. The inhibition zones of different oils were measured and compared among themselves against all the clinical isolates and the lab isolate.

Results: About 55 complete denture wearing patients have been surveyed and among them 27 have been found to be positive for Candida-associated denture stomatitis. Among the Candida species, Candida albicans was more prevalent followed by C. tropicalis and C. glabrata.

Among the test essential oils, Origanum vulgare (stored & fresh) and clove oil gave positive results against all isolates. The fresh samples gave better results than the stored and all three oils showed more antifungal activity than Nystatin & fluconazole.

Interpretation: The candida-associated denture stomatitis is prevalent in patients who are wearing denture prosthesis for more than a year.

Candida albicans is the most prevalent causative species followed by C. tropicalis and C. glabrata.

Origanum vulgare and clove oil are potent antifungal agents against oral species of Candida. They can be either used separately or their synergistic activity could be explored against denture stomatitis.

Key words: Denture stomatitis, herbal essential oils, clove oil, origanum vulgare, Candida spp., nystatin, fluconazole

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Introduction

High prevalence of denture stomatitis has been observed in patients who are wearing complete denture prosthesis. About 60 to 70 % of prevalence has been reported by various authors in the past [1-6]. In geriatric patients, when there is immune suppression due to any reason, there is a conversion of the normal oral commensal Candida into an infection causing pathogen [7-15]. The pathogenic fungi Candida grow in large numbers forming biofilms on the surface of the prosthesis and on the oral mucosa. These biofilms have been found to be highly resistant to existing antifungal agents like Nystatin and Fluconazole. Moreover, these drugs when administered on a long term basis have been known to cause toxicity in human beings.

Efforts are being made to discover new antifungal agents from sources like micro organisms, animals and plants, which are either less toxic or not toxic at all. Use of plant extracts has a long history in Indian scenario for treatment of different ailments. Plants have been found to have rich source of antibacterial, antioxidant and antifungal activities. Several studies using plant essential oils have been carried out against Candida isolated from areas other than oral cavity [16-20]. No studies are reported in the literature regarding their effect against oral species of Candida in a denture wearer. Hence this study was undertaken with the aim of evaluating the effect of eight different plant essential oils on oral isolates of Candida retrieved from a denture wearing patients. Conventional antifungal agents like Nystatin and Fluconazole were also added as controls. A lab isolate was taken for comparative purposes and Agar Well diffusion method was implemented to study the susceptibility tests.

Materials and Methodology:

Ethical clearance for the present study was obtained from the ethical committee of NITTE University, Mangalore, Karnataka, India. The clinical isolates of Candida required for the study were collected through a survey conducted in the department of Prosthodontics. Fifty five patients wearing complete denture prosthesis for more than a year were selected based on the selection criteria.

Selection criteria:

Inclusion Criteria:

1. Patients wearing a denture >1 year
2. Patients not on any antifungal agent
3. Patients willing to participate in the study

Exclusion criteria:

1. Patients unwilling to participate in the study
2. Patients wearing a denture for <1 year
3. Patients already on antifungal therapy

A structured questionnaire was prepared to obtain the necessary information from the patients. Once selected, they were explained about the study and a written consent was obtained. About 27 patients were found to be positive for denture stomatitis observed through clinical signs and symptoms.

Collection of Candida samples: The isolates were collected from saliva samples of the selected patients. The patient was made to sit comfortably on the dental chair. The maxillary denture was removed and using a sterile cotton bud sample was collected from the palatal aspect of the tissue surface of the denture. (Fig.01). It was immediately placed in a test tube containing Phosphate Buffer Solution and was transported to the laboratory where it was cultured on the Saboraud's Dextrose Agar (SDA) to separate the Candida.

Isolation of different species:

Once the Candida was identified, it was once again incubated in SDA medium at 37°C for 48 hours. Candida was confirmed using Germ Tube Test, Biochemical assays and CHROMagar tests (Fig. 02). Based on color of the colonies in CHROMagar, species in the samples were identified as C. albicans (pale green, 13/27), C. tropicalis (09/27), and C. glabrata (05/27). Once isolated, they were separated and subcultured for further use.

A lab isolate ATCC90028 of C. albicans was added in the study for comparative purposes.

Collection of plant material:

The dry leaves of Origanum vulgare were purchased from JKH Impex Pvt. Ltd. Mumbai. Leaves of Pogostemon

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patchouli, Oscimum sanctum and Neem were collected locally and the leaves were identified taxonomically at the University of Agricultual Sciences, Bengaluru. Commercially available Origanum vulgare, clove and caprylate were purchased from departmental store.

Preparation of herbal essential oils:

The fresh leaves of Pogostemon patchouli, Oscimum sanctum and Neem were dried at room temperature and were powdered. Essential oils from all plants were extracted using hydrodistillation procedure and the oils were stored at -4 ° C till use. Nystatin and fluconazole were added as controls.

Antifungal susceptibility testing (Well diffusion method):

A modified Kirby-Beur method was used. Here, the

Table 1 : Antifungal activity of the essential oils of the collected plants

Plant name	Botanical name	Zone of inhibition (mm/20µl)			
		Candida albicans	Candida glabrata	Candida tropicalis	Candida albicans (Lab isolate ATCC90028)
Origanum (stored extract)	Origanum vulgare	30±5	35±2	30±2	30±4
Origanum (fresh extract)	Origanum vulgare	30±3	36±2	32±3	30±2
Origanum (commercial)	Origanum vulgare	----	----	----	----
Patche Kadiru (Kannada)	Pogostemon Patchouli	----	----	----	----
Tulasi	Oscimum Sanctum	----	----	----	----
Clove	Syzygium aromaticum	33±4	30±2	30±2	33±1
Neem	Azadirachta indica L	----	----	----	----
Coconut	Cocosnicifera Sanctum	----	----	----	----

Table II : Descriptive analysis of data

Comparison of three essential oils in each group

		N	Mean	std. Deviation	df	Mean Square	F	Sig.
candida albicans	origanum vulgare (o p) stored at 4	10	29.9	3.281	2	9.733	1.547	0.23
	origanum vulgare fresh extract	10	30.1	2.025				
	clove oil	10	31.7	2.003				
	Total	30	30.57	2.555				
candida glabrata	origanum vulgare (o p) stored at 4	10	35	1.491	2	103.333	35.769	<0.00
	origanum vulgare fresh extract	10	36	2.055				
	clove oil	10	30	1.491				
	Total	30	33.67	3.133				
candida tropicalis	origanum vulgare (o p) stored at 4	10	30	1.491	2	14.033	4.802	0.01
	origanum vulgare fresh extract	10	32	2.16				
	clove oil	10	29.9	1.37				
	Total	30	30.63	1.921				
candida albicans lab isolate (control) (ATCC90028)	origanum vulgare (o p) stored at 4	10	29.9	2.644	2	29.233	8.919	0.00
	origanum vulgare fresh extract	10	30.2	1.476				
	clove oil	10	33	0.816				
	Total	30	31.03	20251				

There is significant difference in the candida glabrata, tropicalis and lab isolate

Keywords: Denture stomatitis, herbal essential oils, clove oil, origanum vulgare, Candida spp., nystatin, fluconazole - Vinaya Bhat

Candida species were suspended in SDA broth and it was made equivalent to 0.5 McFarland units. Six millimeter wells were created on the SDA medium and 20 microlitre each of the antifungal agents was delivered into each well. It was incubated again for further 48 hours at 37 ° C with agitation. After 48 hours, zone of inhibition for each essential oil and for each species was measured using a flexible scale (Fig.03). The results were tabulated (Table I) and were subjected to statistical analysis.

Results:

Among eight essential oils (Origanum vulgare in three forms, i.e., fresh extract, stored extract & commercial, Pogostemon patchouli, Oscimum sanctum, Neem, clove and caprylate) three showed positive effects against Candida species. Origanum vulgare in two forms, i.e. Fresh extract, and stored extract and Clove oil showed significant results ($p<0.001$) on all species of Candida. All showed more antifungal effect than Nystatin (22mm zone of inhibition) and fluconazole (19mm zone of inhibition). Among fresh and preserved forms of Origanum vulgare, fresh samples showed better results (30±3, 36±2, 32±3, 30±2) against all species. There was no significant difference ($P<0.231$) in action of three oils on Candida albicans where as there was significant difference in Candida glabrata, C. tropicalis and lab isolate ($P<0.001$, 0.016, 0.001). This indicates that all three oils have similar activity against Candida albicans and the action is different for other three species. C. glabrata and C. tropicalis are more susceptible to Origanum vulgare rather than clove oil, where as lab isolate is more susceptible to clove oil. Hence any of the three oils can be used effectively against oral Candida albicans and Origanum vulgare is also effective on other oral isolates. Clove oil is more effective on lab isolate of Candida albicans ATCC90028. Origanum vulgare also showed significant difference as compared to Fluconazole & Nystatin ($P<0.001$) (Table II) & (Table III).

Discussion :

Denture stomatitis is a very common type of infection of the oral mucosa found in 60 to 65% of the denture wearing population [21, 22]. Various reasons have been cited by the authors for this prevalence. Denture hygiene, trauma and allergy to the denture base materials are the commonest causes. In addition, when the immune status of the patient is lowered due to systemic diseases or due to immunosuppressive therapy, the otherwise normal oral commensal Candida turns pathogenic and causes denture stomatitis. Histologic sections of the oral mucosa have been shown to exhibit Candida blastospores, hyphae and pseudohyphae.

In diabetes mellitus, the patients have been found to be more susceptible to oral candidiasis than patients without diabetes [23-24]. If the patient is a denture wearer, it has been found to favor the growth of candida to cause candidiasis [24-25]. In a study by Sanita et al [6], they said that Candida species were more prevalent in patients with denture stomatitis in both, diabetic and non diabetic conditions. Diabetes mellitus has been a risk factor for the development of DS. In a study by Emami et al[5], they found that the patient who is wearing a removable denture whether partial or complete is at a risk of developing DS. Presence of dental prostheses has also been found to be associated with HIV/AIDS [15]. They are of the opinion that CD4 count plays an important role in the development of oral candidiasis.

Candida albicans has been found to be the most prevalent species in Candida-related denture stomatitis. Under normal circumstances, it is a common oral commensal not causing any pathosis. However, during favourable conditions it transforms itself into a virulent pathogen using various mechanisms.

Even though Candida albicans is the frequently associated species in denture stomatitis, C. glabrata is the emerging species next to it [26-29]. However, Sanita et al[6], in their study concluded that C. albicans and C. tropicalis were more prevalent in a denture wearer with denture stomatitis. In severe candidiasis, C. tropicalis was more prevalent. Mixed infections caused by C. albicans and C. glabrata have been demonstrated in vitro oral epithelium [30]. Reconstructed Human Oral epithelium was used to demonstrate the ability of C. albicans to enhance the invasiveness of C. glabrata in causing the infection.

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To arrive at a conclusion regarding the dominance of the species in the local population the authors conducted a survey to include denture wearers from where the samples were collected for species characterization of Candida. In this survey, we found that after C. albicans, it was C. tropicalis and then C. glabrata which were the next most prevalent species of Candida.

Existing antifungal agents like Nystatin and Fluconazole have been found to be toxic on long term use [31-35]. Also, development of resistance by the organisms has been recorded. ® Hence, there is a need to develop a new antifungal agent which may be less or not toxic at all. Plants have a long positive history for being used as natural remedies for various ailments [36-38, 18]. Several herbal agents have been tried against Candida of various regions in the human body [18-20]. However, no studies have been reported regarding the investigation of antifungal effects of any herbs on the Candida strains obtained from the oral candidiasis of a denture wearing patient. This study was undertaken to screen some of the herbal products that are commonly available against Candida spp. of oral candidiasis.

A modified Kirby-Beur method was implemented to study the antifungal susceptibility potential of the eight herbs. Zone of inhibition for each was measured and tabulated. Three oils (two types of Origanum vulgare and clove oil) showed strong positive antifungal effects on all strains (Clinical and lab) of Candida, where as, the other oils did not show any measurable activity at all. Zone of inhibition for stored Origanum vulgare was 30mm±5 for Candida albicans, 30mm±2 for Candida tropicalis, 35mm±2 for Candida glabrata and 30mm±4 for Candida lab isolate. For fresh sample of Origanum vulgare was, 30mm±3 for C. albicans, 32mm±3 for C. tropicalis, 36mm±2 for C. glabrata and 30mm±2 for the lab isolate. Clove oil exhibited 33mm±4mm for C. albicans, 30mm±2 mm for C. tropicalis, 30mm±2 mm for c. glabrata and 33mm±1 mm for lab isolate. Nystatin has been found to show an antifungal effect of up to 22mm [18] and fluconazole upto 19mm [39]. In this study, the antifungal effects of Origanum vulgare and clove oil were found to be substantially high as compared to Nystatin and Fluconazole, indicating that they can have better results in eradicating candidiasis. And being of plant origin, they may be less toxic. Further studies on the toxic effects of these oils on animals are indicated.

Origanum vulgare is a plant belonging to the family Lamiaceae which contains the phenolic compound Carvacrol as a primary compound [40]. In a few earlier studies, the essential oil of Origanum vulgare has been studied against Candida spp. isolated from sites such as, vaginal mucous membrane of dogs, cutaneous tegument of a dog, cutaneous tegument of a capuchin monkey [20], culture collection Candida albicans ATCC10231[38], ATCC 14053[18], female mice [19] and from human stool specimen [18]. In all the above studies Origanum vulgare has been found to be possessing anti-candidal activity. In the present study, the Candida spp. was obtained from the oral cavity and this plant was found to be very effective against different species of Candida and so also a lab species of Candida albicans. Both, the stored and the fresh extracts showed good antifungal effect as compared to fluconazole and nystatin, indicating that proper storage does not decrease the potency of the oil.

Syzygium aromaticum (clove) had been used against lab isolate of Candida albicans ATCC 10231 by Hammer et al.[38]. In their study of antimicrobial activity of essential oils and plant extracts carried on several organisms they found that clove had an antifungal effect against Candida albicans. In our study also, the essential oil of clove showed a strong antifungal activity against several species of Candida obtained from the oral cavity thus confirming its possession of positive antifungal effects.

Conclusion :

This study has scientifically proved the antifungal efficacy of three essential oils obtained from fresh & stored samples of Origanum vulgare and Clove oil. Further, it was found that the effects are much more than the existing antifungals like Nystatin & Fluconazole. The usage of Origanum vulgare or clove oil or the combination of the two essential oils has the potential of being used in treating

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oral candidiasis. The present in-vitro study has confirmed the potentiality of the two essential oils to be used as antifungal agents against Candida spp. However, further studies to determine their MIC& MFC and also the phase II studies on animals have to be carried out. Also, their pharmacological effects like antibacterial, anti carcinogenic and anti-inflammatory activities can be investigated.

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