ORIGINAL RESEARCH ARTICLE

ANTIMICROBIAL MOUTH RINSES - ARE THEY POTENT AGAINST CANDIDA ALBICANS?

Authors:
Mithun Pai BH[1],
Mahesh Kumar M. [2],
Amit V. Mahuli[3],
Prashanth GM[4],
Chandu GN[5].

[1]Assistant Professor, Dept. of Public Health Dentistry, Manipal College of Dental Sciences, Mangalore 575 001, Karnataka, India.

[2]Assistant Professor, Dept. of Public Health Dentistry, CSI College of Dental Sciences and Research, East Veli Street, Madurai 625 001, Tamil Nadu, India.

[3]Senior Lecturer, Dept. of Public Health Dentistry, Dr. DY Patil Dental College and Hospital, Pimpri, Pune, India.

[4]Reader, Department of Preventive and Community Dentistry, College of Dental Sciences, Davangere. 577 004, Karnataka, India.

[5]Professor, Department of Preventive and Community Dentistry, College of Dental Sciences, Davangere. 577 004, Karnataka, India.

Address for correspondence:
Dr. Mithun Pai BH, Assistant Professor, Dept. of Public Health Dentistry, Manipal College of Dental Sciences, Mangalore, Karnataka 575 001.
E mail dr6446@yahoo.co.in

ABSTRACT

Background: There has been an increase in *Candida* infections over the period of several decades. This is probably because of the increasing number of elderly and seriously ill patients, immunosuppressive therapies, as well as the increased use of antibiotics and more invasive therapeutic medical procedures. The study was conducted with an objective of determining the antifungal efficacy of five mouth rinses on *Candida albicans* in vitro.

Methods: The study is an experimental in-vitro study conducted to evaluate antifungal efficacy of five mouthrinses against *Candida albicans.* A three pronged objective included screening the mouth washes for antifungal properties, to check the minimum inhibitory concentration (MIC) and to evaluate the fungicidal efficacy of the mouth rinses on *Candida albicans,* using disc diffusion, broth macro dilution and modified Bernstein method was done respectively. The mean and standard deviation of the diameter of inhibition zone was calculated. The data analyzed using one way Analysis of Variance (ANOVA) followed by Tukey post hoc test for pair wise comparison.

Results: The study showed that all the mouthrinses showed zones of inhibition against *Candida albicans.* Zones of inhibition for Chlorhexidine were highest and were significantly greater. All the mouth washes used in the present study showed kill times that were lesser than thirty seconds.

Conclusion: The findings of the study demonstrate that mouthrinses have sufficient antifungal properties that might represent appropriate alternative to conventional antifungal drugs in the management of *Candida albicans*.

Key words: antimicrobial Mouth rinse, *Candida albicans*, antifungal activity.

J Odontol Res 2013;1(1):4-10

INTRODUCTION

Oral Candidiasis is a relatively common opportunistic mycotic infection in man. It is usually caused by *Candida albicans,* which is predominant over other yeasts in the oral cavity. Although oral carriage of this opportunistic pathogen varies greatly, it is often present in the oral microflora of healthy individuals. A characteristic feature of candidiasis is its development when the host provides the environmental conditions and nutrients essential for attachment, growth and reproduction. [1]

In comparison to bacteria pathogenic fungi have received little attention as biocide targets. Biocides, better known as antiseptics, disinfectants, or preservatives, are commonly added to mouthwashes, toothpastes, hand soaps, and related consumer products.

C. albicans is commonly found at low levels among the normal oral flora, but its overgrowth in immuno compromised individuals or following broad-spectrum antibiotic therapy leads to oropharyngeal Candidiasis.[2] As oral microbiota not only consist bacteria but also contain other micro organisms which cause mixed infection in the oral cavity a broader spectrum of activity of oral care products are needed, the establishment and maintenance of oral microbiota is related not only to interbacterial co aggregations but also to interactions of these bacteria with yeasts such as *C. albicans*. Fungi are frequently isolated in several oral sites, including the tongue, buccal mucosa, palate, dental biofilm, sub gingival microbiota, carious lesions and prosthetic appliances. Studies have suggested a possible relation between *C. albicans* and periodontal disease, dentin and/or root caries. *C. albicans* has similar capacity of colonizing hydroxyapatite as that of *Streptococcus mutans*, however using different mechanisms. Enamel and dentin demineralization produced by fungal organic acids, as well as the presence of cells with *C. albicans* hyphae invading dentinal tubules, prove this ability of fungi to invade and destroy organic and inorganic dental tissues. This microorganism adheres to hydroxyapatite, especially through electrostatic interactions and at smaller numbers. *Candida albicans* has also the ability to dissolve hydroxyapatite at a larger rate when compared to *S. mutans* .[3]

Despite the availability of a number of effective antimycotic for the treatment of oral candidiasis, failure of therapy is not uncommon owing to the unique environment of the oral cavity where the flushing effect of saliva and the cleansing action of the oral musculature tend to reduce the drug concentration to sub-therapeutic levels. Since mouthwashes have already proved their efficacy against wide range of bacteria their anti fungal efficacy have yet to be substantiated. The study was conducted with a three pronged objectives of screening the antifungal efficacy of five mouth washes on *Candida albicans.* To check The Minimum Inhibitory Concentration (MIC) of five mouthrinses on *Candida albicans and* check the fungicidal efficacy of five mouth washes on *Candida albicans in an in vitro medium.*

MATERIALS AND METHODS

The materials and armamentarium used in the study constituted of the following:

The Test mouth rinses that were used in this study are Cetylpyridinium chloride mouth wash Crest Pro Health, Procter And Gamble (Cetylpyridinium chloride 0.06 %). Hexidine ICPA Health Product (Chlorhexidine Gluconate 0.2%). Listerine mouthwash Johnson and Johnson, (Thymol 0.6%, eucalyptol 0.09 %, Menthol 0.4%, Ethanol 26%). Betadine,Win Medicare Pvt Limited, (Povidone iodine, Absolute Alcohol 38%). Mougel ayurvedic mouth wash Immis Pharmaceuticals, (Embilica Officinalis, Terminalia chebula, Terminalia Belerica, Acacia Catechu, Borax) were used to determine antifungal activity on Candida albicans.

Filter discs uniformly loaded with 30µl of mouthwash were placed with sterile tweezers onto the prepared plates. Two filter discs were placed in each Sabouraud agar plates. Based on expected difference from pilot study, 5% level of significance (α), and 80% power of study (1-β), for each mouth rinse and control (sterile deionised distilled water). The zone of microbial inhibition was measured after the time intervals of 18 hours, 24 hours, and 48 hours on the underside of the Petri dishes using vernier

callipers. The zone of inhibition on the growth of test strains were defined by the area where visible growth had been inhibited if that is obvious; if it was not then, measurement was made to the point of 80% inhibition of growth. First the whole of the diameter of the zone of inhibition was measured and later the diameter of filter disc was deducted from it. This revealed the actual zone of inhibition around the filter disc.

All the measurements of zone of inhibition were carried out by a single examiner. Calibration of examiner was done prior to and during the study by re-examining 5% of the samples, to minimize intra examiner variability. Intra examiner agreement was determined using kappa statistics (k). Intra examiner agreement score ($\kappa = 0.94$) was almost perfect, according to Landis and Koch, thus meeting the scientific requirement for validity and reliability.[4]

Determination of minimum inhibitory concentration (MIC):

The first dilution (the highest concentration) was made by adding equal volume of stock solution to the broth in the first tube. The further dilutions were made by adding 1.5 ml of previous dilution to the next tube containing 1.5 ml broth where the v/v ratio was 1: 2, 1:4, 1:8, 1:16, 1:32, to 1:64 respectively. The final volume of broth and mouth rinse in each tube should be 1.5 ml, so the excessive amount of mouth rinse in the last test tube was discarded.[5]

Mouth rinses were thus diluted in series of exponential dilutions from undiluted to1:64 dilution using broth dilution technique. Equal volumes (1.5 ml) of fungal suspensions and mouth rinses are mixed and kept at 37^0C for 18hours. Optical density at 560nm was determined using spectro photometer. Duplicates were examined and averages of two test tubes were taken. The optical density was determined relative to fungal growth. Controls consisted equal volume of fungal suspension and distilled water. Blanks consisted of Sabouraud broth and mouth rinse results were expressed as percentage.

$$= \frac{(\text{OD CONTROL} - \text{OD BLANK}) - (\text{OD SAMPLE} - \text{OD BLANK})}{(\text{OD CONTROL} - \text{OD BLANK})}$$

Test for fungicidal activity:

The fungicidal activity was determined by modified Bernstein method, wherein, 0.5 ml of mouth rinse and fungal suspension are measured with a micropipette and kept in plastic vials at room temperature, the mixture was centrifuged after a time periods of 30 seconds, five minutes and 15 minutes in a Remi R-83 centrifuge at 1000 RPM for three minutes. Mixture was inoculated on Sabouraud s agar medium to form a lawn culture, and incubated at 37 °C for 48 hours. Average of two test tubes were taken as mean. Controls consisted of equal volumes of fungal solutions in Phosphate Buffered Saline and number of colony forming units were determined from each sample and control, and was expressed in terms of percentage.

Statistical Analysis

For each mouth rinse and control, the mean and standard deviation of the diameter of inhibition zone was calculated. The data were analyzed using one way Analysis of Variance (ANOVA) followed by Tukey's post hoc test for pair wise comparison**.** p-values <0.05 were considered statistically significant.

RESULT

The results of this study as per the differing methodologies were as follows**,**

Results of disc diffusion method:

Graph 1, demonstrates the zones of inhibition of mouth rinses at time intervals of 18 hours, 24 hours and 48 hours on the agar plates. The disc diffusion method demonstrated that all the mouthrinses used in the present study showed zone of inhibition against *Candida albicans*. The zones of inhibition was largest for Chlorhexidine mouthwash and Mougel ayurvedic mouth wash demonstrated the least zone of inhibition at 18 hours, 24hours and 48 hours respectively**.** Difference in zones of inhibition between Chlorhexidine mouthwash, Povidone iodine mouthwash, Listerine mouthwash and Mougel ayurvedic mouth wash at the time intervals of 18 hours, 24hours and 48 hours was statistically significant, but the zones of inhibition between Chlorhexidine mouthwash and Cetylpyridinium

chloride mouthwash at the end of 18 hours, 24hours and 48 hours was statistically not significant.

MIC, Optical Densities and Percentage Inhibition of the mouthwashes using Broth Macro Dilution technique is illustrated in table 1, Chlorhexidine mouthwash and Cetylpyridinium chloride mouth wash even at the dilutions of 1:64 (15.1 µg/ ml) did not demonstrate any change in their optical densities, followed by Listerine mouthwash which demonstrated changes in optical density at dilutions of 1:32(31.2 µg/ ml), succeeded by Povidone iodine mouthwash wherein changes were observed at the dilutions of 1:8 (125 µg/ ml) and Mougel ayurvedic mouth wash showed changes in optical density at dilutions of 1:2(500 µg/ ml). The table I also demonstrates the percent inhibition of mouthrinses on *Candida albicans* wherein Chlorhexidine mouthwash and Cetylpyridinium chloride mouthwash demonstrated 100 percent inhibition, and Mougel mouthwash demonstrated an inhibition of 100 percent at its original concentration only.

The number of viable colonies were recovered after the exposure of *Candida albicans* to mouthwashes to demonstrate Fungicidal Activity or the kill times at 30 seconds, 5 minutes and 15 minutes. All the mouth washes used in the present study showed kill times that were lesser than thirty seconds as there were no visible colony growth even after incubation of organisms in mouthwash for 15 minutes.

Graph 1: Comparative effect of mouthwashes against Candida albicans at 18 Hours, 24 hours and 48 hours.

Table 1: Minimum Inhibitory Concentration (MIC) and Percentage Inhibition of the five mouthrinses using Broth Macro Dilution technique.

DISCUSSION

The antimicrobial properties of individual mouthrinses were measured by testing the zone of inhibition on the microbial strains incubated on Sabourauds dextrose agar. In regards to the testing of zones of inhibition, many methods have been devised and employed with concerns for cost and time. One of the most popular methods used today, as explained by Cormican M et al, is to measure zones of inhibition by a disc diffusion method. This method is quick, easy, and inexpensive.

In the present study the zones of inhibition of Chlorhexidine was highest and was significantly greater at 18 hours, 24hours and 48 hours than Cetylpyridinium chloride mouth wash, Povidone iodine mouthwash, Listerine mouthwash Mougel ayurvedic mouth wash demonstrated least zone of inhibition at the end of 18 hours, 24hours and 48

Graph 1` :Zone of inhibition of mouthrinses at 18hours, 24 hours and 48 hours

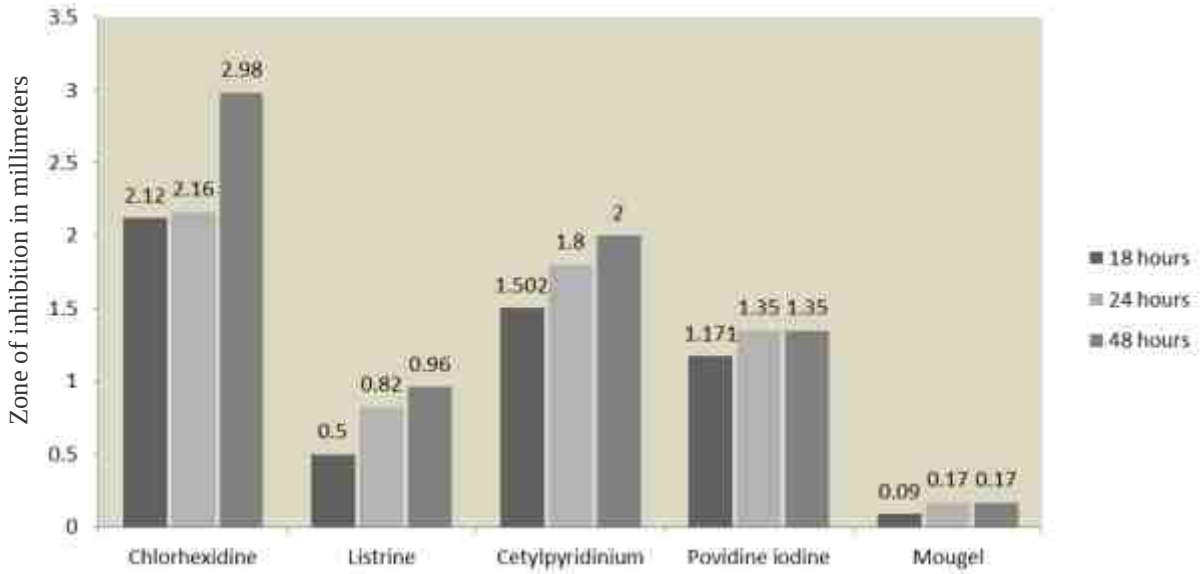

Table 1: Minimum Inhibitory Concentration (MIC) and Percentage Inhibition of the mouthwashes using Broth Macro Dilution.

	1:1	1:2	1:4	1:8	1:16	1:32	1:64
CHLORHEXIDINE % INHIBITION	100	100	100	100	100	100	100
CETYL PYRIDINIUM % INHIBITION	100	100	100	100	100	100	100
POVIDONE IODINE % INHIBITION	100	100	100	90.9	86.9	73.6	26.3
LISTERINE % INHIBITION	100	100	100	90.9	86.9	94.7	94.4
MOUGEL % INHIBITION	100	97.6	92.5	80	50	14.2	0

hours respectively against Candida albicans. In a study conducted by Nakamoto K et al in 1995 [7] chlorhexidine mouthwash did not show any zones of inhibition on cultures of Candida albicans, which is in stark contrast to the present study. The results of the study conducted by Nakamoto K et al in 1995 is similar to with the present study, where in Cetylpyridinium chloride mouth wash demonstrated zones of inhibition of less than 8mm with and without sodium benzoate at the end of 18 hours. The difference in the result may be attributed to the concentration of mouth rinses used as the methodology of both the studies are similar.

In the present study the Mougel showed the least zones of inhibition of less than 1mm even after 48 hours of incubation and is in contrast with the studies of Saeed S et al in 2007 [8], Patel M et al in 2007 [9] and Saini ML et al in 2008 [10] wherein zones of inhibition for Candida albicans were much higher than in the present study. The authors in the above mentioned studies used individual ingredients of the plants directly and used 100 percent concentrations where as in the present study the ingredients were a mixture of Embilica officinalis, Terminalia chebula, Terminalia Belerica, Acacia catechu, Borax and was commercially available as mouthwash and in the present study agar diffusion was carried out with filter paper discs according to CLSI guidelines but in the studies conducted by Saeed S et al in 2007, Patel M et al in 2007 agar diffusion by well method was performed.

In the present study the minimal inhibitory concentration of Cetylpyridinium chloride mouth wash was less than 15.1 (µg/ ml) which was similar to the studies conducted by Nakomato et al [7], Gulliana et al [11], Gulliana et al [12], Edlind MP et al [2] wherein the procedure or methods to achieve minimal inhibitory concentration varied between all the other authors except Edlind MP et al[2]. Where in the agar plates were used for determining the minimal inhibitory concentration where as in the present study differences in optical densities were considered for determining the minimal inhibitory concentration of each of the mouthwashes.

The minimal inhibitory concentration for Chlorhexidine in this study was similar to that of Cetylpyridinium chloride mouth wash where in no changes in optical density was observed at 15.1 (µg/ ml) concentration which is similar to the findings of the studies conducted by Miller et al [13], Gulliana et al [12] but in stark contrast to study conducted by

Gulliana et al [11]. where in the minimal inhibitory concentration for Chlorhexidine mouth wash was higher than the present study, this difference can be attributed to the difference in the methodology of the two studies where in Gulliana et al[11]. have used agar plates to demonstrate the minimal inhibitory concentration of the mouthwashes, whereas the differences in optical densities were considered for the present study and the incubation time for the present study was 18 hours as compared to 48 hours for the study conducted by Gulliana et al[12] the ideal incubation duration is of 16-18 hours, but prolonged incubations as in the study of Gulliana et al [11], should be avoided as antimicrobial deteriorate over time and may result in false interpretation.

The fungicidal efficacy of the mouth washes on *Candida albicans* modified Bernstein method was used where in the kill time or the fungicidal efficacy of the mouth washes were evaluated. In the studies conducted by Yamanaka et al in 1994 on listerine mouthwash and Nakamoto et al in 1995 [7] on Cetylpyridinium chloride and Waltimo et al on povidone iodine kill time or the fungicidal efficacy of the mouth washes, was similar to that of the present study, where as in the study conducted by Gulliana et al 1999[11], cetyl pyridinium chloride showed a longer kill time than the present study, this diffrence in the kill times may be attributed to their assesments of microorganisims on the cultures where in even the presence of discrete colonies were considered to asess the kill times and their kill times ranged for every 5 seconds which is contrary to the present study where the colonies were assesed for only 30 seconds, 5 minutes and 15 minutes and no intermediate readings were assessed.

CONCLUSION

From the results of the present study, it can be concluded that all the five commercially available mouthwashes demonstrated antifungal activity. Cetylpyridinium chloride mouthwash and Chlorhexidine mouthwash demonstrated significantly higher antifungal efficacy than other mouthwashes used in this study. Upon demonstration of Minimal Inhibitory Concentration two mouthwashes namely Cetylpyridinium chloride mouthwash and Chlorhexidine mouthwash demonstrated strong antifungal activity at lower concentrations. The fungicidal activities or the kill times of all mouthrinse was similar and showed that the antifungal efficacy was not time dependent. The findings of the present study suggest that mouthrinses containing antimicrobial agents might represent appropriate alternative to conventional antifungal drugs in the management of diseases caused by *Candida Albicans*. Although results of the present study show best overall antifungal activity of Cetylpyridinium chloride mouthwash and Chlorhexidine mouthwash against *Candida albicans* tested in present study, in vivo studies are needed to prove or refute its efficacy in real environmental circumstances.

REFRENCES

1. Waltimo D, Orstavik EK, Siren, Haapasalo MPP. In vitro susceptibility of Candida albicans to four disinfectants and their combination. International Endodontic Journa 1999;32:421-9.
2. Edlind MP, Smith WL, and Edlind TD. Effects of Cetylpyridinium Chloride Resistance and Treatment on Fluconazole Activity versus Candida albicans Antimicrobial Agents and Chemotherapy.2005;49 (2):843-5.
3. Vasconcelos LSD, Sampaio FD, Sampaio MCC, Pereira MSP, Higino JS, Peixoto MHP. Minimum Inhibitory Concentration of Adherence of Punica granatum Linn (pomegranate) Gel Against S. mutans, S. mitis and C. albicans. Braz Dent J 2003; 17(3):223-7.
4. http://en.wikipedia.org/wiki/ Cohen's_kappa(assessed on October 8th, 2011)
5. Flisfisch S, Meyer J, Meurman JH, Waltimo T. Effects of fluorides on Candida albicans. Oral Diseases. 2008;14(4):296-301.
6. National Committee for Clinical Laboratory

Standards. Reference method for broth dilution antifungal susceptibility testing for yeasts. Proposed standard. Document M-27-P. National Committee for Clinical Laboratory Standards, Villanova.1992.

7. Nakamoto K, Tamamoto M , Hamada T. In vitro effectiveness of mouthrinses against Candida Albicans .Int J Prosthodont 1995; 8(5):486-9.

8. Saeed S, Tariq P Antimicrobial activities of emblica officinalis and coriandrum sativum against gram positive bacteria and Candida albicans. Pak. J. Bot.2007; 39(3): 913-7.

9. Patel M, Shackleton J, Coogan MM , Galpin J, Annual Report March 2006 – March 2007 Division of Oral Microbiology The main research interest of this division is HIV and oral Candidiasis. The research projects in 2006-2007.

10. Saini ML, Saini R, Roy S, Kumar A. Comparative pharmacognostical and antimicrobial studies of acacia species (Mimosaceae). Journal of Medicinal Plants Research2008;2(12):378-86.

11. Gullian G, Pizzo G, Milici ME, Musottoc GC, Gaingreco R. Invitro antifungal properties of mouthrinses containing antimicrobial agents. J Periodontol 1997; 68:729-33.

12. Gullian G, Pizzo G, Milici ME,Gaingreco R . Invitro activities of antimicrobials against Candida species. Oral surg oral Med Oral Pathol Oral Radiol Endol 1999;87:44-9.

13. Meiller T F, Kelley J I, Jabra-Rizk MA, De Paola LG , Baqui AAMA , Falker WA. In vitro studies of the efficacy of antimicrobials against fungi. Oral Surg Oral Med Oral Path Oral Radiol Endo2001;91:663-70.