Vol 5 | Issue 1 | 2015 | 33-39.

e-ISSN 2248 – 9142
print-ISSN 2248 – 9134

International Journal of
Current Pharmaceutical & Clinical Research

www.ijcpcr.com

EFFICACY OF CITRUS ESSENTIAL OIL ON THE GROWTH OF ORAL CANDIDIASIS IN ELDERLY DIABETIC POPULATION - RANDOMIZED PARALLEL GROUP CLINICAL TRAIL

Raghavendra Shanbhog*[1], Nandlal B [2], Kulkarni P[3], Sumana K[4]

[1]Reader, [2]Principal and professor, Department of Pedodontics, JSS Dental College & Hospital, JSS University, Mysore, Karnataka, India.
Professor, Department of Pharmaceutics, JSS College of Pharmacy, JSS University, Mysore, Karnataka, India.
Professor, Department of Microbiology, JSS Medical College JSS University, Mysore, Karnataka, India.

ABSTRACT

This randomized control 2 month's intervention clinical trial was done with objective to evaluate the effect of 0.2% chlorhexidine gluconate, and 2% citrus essential oils (EO) (*C. limonum* or lemon) mouthwashes on the growth of oral candidiasis in elderly long standing diabetic population. This pilot double-blind parallel design study was done with 20 subjects in each group. The selected subjects were randomly allocated into 2 groups, group A- using 0.2% chlorhexidine mouthwash, group B- 2% mouthwash prepared from *C. limonum* EO. At the beginning and end (after 60 days) of the study non-stimulated saliva samples were obtained and C. albicans colonies from the saliva samples were counted and calculated as the number of colony-forming units (CFUs). Both baseline and postoperative data were compared using appropriate statistical analysis. The results of the present study indicate that there was significant decrease in the number of colony-forming units of C. albicans in both the groups (chlorhexidine & lemon) compared to baseline count. However chlorhexidine showed better results as compared to lemon group. It was concluded that 2% lemon mouthwash can act as an alternative herbal drug formulation which is natural biodegradable and safe that is effective against C albicans.

Key words: C. albicans, Elderly diabetic, *C. limonum*, Essential oil, Mouthwash.

INTRODUCTION

Improved health care has resulted in increasing elderly population in India and is expected to touch 137 million by 2021. Today, India is home to one out of every ten senior citizens of the world [1]. Diabetes mellitus is a common and growing global health problem which causes several complications and is very common in elderly population. Immunodeficiency and increased susceptibility to opportunistic infections are seen in long term diabetic patients. Colonization of Candida is more prevalent in people with diabetes mellitus because of poor glycemic control [2-5]. This predisposition also contributes to xerostomia, which may be due to increased glucose levels in oral fluids or immune dys-regulation [6]. Although, various topical and systemic antifungal drugs are available for the treatment of oral candidiasis [7,8] the disease is still a major problem among those subjects. Development of drug resistance, high recurrence rate after stopping the medication is frequently noted. Thus, control of the symptoms rather than cure may be the goal in the treatment of oral candidiasis in long term diabetic elderly subjects. Chlorhexidine gluconate at 0.2% concentration has long been recommended to be used as a mouthwash for this purpose. But bitter taste and tooth staining properties of CHX may cause poor compliance [9,10].

Corresponding Author: - **Raghavendra shanbhog** Email: - drraghu.kiddoc@gmail.com

As an alternative, medicinal plants are explored to produce valuable herbal products, which are frequently used as natural alternatives to treat several diseases. An *in vitro* study by Oliveira S et al. (2014), evaluating the effects of *C. limonum* (lemon) and *C. aurantium* (bergamot or bitter orange) essential oils (EOs) compared to 0.2% chlorhexidine and 1% sodium hypochlorite (NaOCl) solutions on multi-species biofilms formed on acrylic resin by *Candida albicans*, *Enterococcus faecalis* and *Escherichia coli* reference strains reported that *C. aurantium* and *C. limonum* EOs were most effective solutions and have promoted a 100% inhibition of *C. albicans*, in multi-species bio-films [11]. However *in vivo*, the altered nature of bio-film and salivary flow may alter these results. Also EO when formulated as mouthwashes its efficacy & compliance may change and needs to be evaluated. So this pilot study was designed with an objective to

- Formulate 2 % EOs of. *limonum* (lemon)
- Compare its efficacy, compliance, and the possible adverse effects with 0.2% chlorhexidine mouth wash in elderly long standing diabetic population.

MATERIALS AND METHODS

Study design & Ethical clearance

The study design was of randomized control 2 month's intervention clinical trial. The study plan was approved by the Ethical Committee of JSS Dental College (Ref. No: DCH/Ethical/2014-115) and registered in the hospital register for clinical trials. All information about the patients and their identity were anonymous. Subjects were given both verbal and written information about the nature of the study and written consent obtained. They were allowed to leave the study at any time during the procedures.

Study setting & Sample size determination

Old age homes surrounding Mysore city were targeted for high, expected levels of cooperation and low population mobility. The sample size of 20 was selected in each group based upon 95% confidence interval and 90% power making total sample of 40 from 2 groups.

Preparation of mouth wash

Chlorhexidine gluconate mouthwash

Commercially available mouthwash (Proprietary name: Clohex, concentration 0.2%) was procured from the market and to mask the brand name the mouthwash was dispensed in a coded opaque amber color glass bottle by the study coordinator.

C. limonum Mouthwash

Mouthwashes *of C. limonum* were prepared through essential oil of fruit peels. Essential oil from fruits was collected with the help of experts from the JSS Pharmacy College Mysore. Fresh lemon were purchased, identified & authenticated by an experienced botanist and washed thoroughly with distilled water to remove any external macro contamination. The fresh peels were separated from pulp carefully taking care not to waste peel content. Then peels placed in a round-bottom distillation flask to which distilled water was added. The EOs was obtained by hydro-distillation for 3 hrs with the Clevenger apparatus. The oils separated; dried over anhydrous sodium sulfate, and stored in an amber color glass bottle at 4°C.

2 parts of essential oil was mixed with 98 parts of sterile water to make 2% essential oil mouth wash. No other additives like sweeteners or flavours were added. For every 10 days mouthwash was prepared freshly and was dispensed in a coded opaque amber color glass bottle by the study coordinator similar to chlorhexidine.

The study was divided into three phases

Phase I Selection of study subjects and consent

The subjects for the study were selected from 3 different old age homes of Mysore taluka. After identifying and personal meeting with primary care giver of all three old age homes a letter was sent explaining the aims of the study and asking them for their consent for concerned senior citizens to participate in the study. Also the subjects who participated in the study were also informed regarding the aim of the study and consent was obtained. The subjects were included based on voluntary participation. A total of 40 elderly diabetic subjects who fulfilled the eligibility criteria were selected.

Inclusion criteria

- Subjects of either sex over 65 years of age known diabetic from past 5 years and above.
- Subjects in good general health.
- Subjects capable of caring for his/her daily oral hygiene, themselves independently
- Subjects with positive candida count in the mouth after pre base line saliva sample microbiological evaluation.

Exclusion criteria

- Smokers
- Subjects with hypersensitivity or allergy to the study medications
- Subjects with already established candidal lesion in the mouth
- Individuals with oral mucosal disease and on antibiotic, antifungal drug treatments or chlorhexidine like antiseptic mouth washes in the month prior to inclusion in the study.

Selected subjects were then enrolled and assigned to a computer generated table by the examiner who assigned the coded mouth rinses according to treatment groups after baseline examination into:

- **Group 1**- 20 subjects-receive 0.2% chlorhexidine mouthwash.
- **Group 2**- 20 subjects-receive 2% mouth wash prepared from *C. limonum* (lemon) EO.

Phase II Medical record and clinical examination

The medical records; history regarding daily used drugs and dental details of all subjects were recorded using a pre-printed format. Compliance with the inclusion criteria was confirmed once again. A Chair side clinical dental examination was carried out by the trained dentist using an otological light source, oral cavity mirror and probe. Oral health status, oral symptoms findings at the beginning of the trial was recorded. Then all the subjects received instructions for correct oral hygiene protocols. After 7 days a non-stimulated saliva sample was obtained from all the 40 subjects for determination of the base line number of colony-forming units (CFUs) of C. albicans in the mouth, using the oral rinse technique [12].

Phase III Intervention

Then respective group subjects received the bottles of treatment rinse, and the instructions for correct use of the same. Subjects were advised to use the 10 ml of mouth wash for 1 minute twice daily for 60 days. After the use of mouth wash subjects were advised not eat or drink for at least 30 minutes. For every 10 days the investigator visited the old age homes to assess compliance, and the possible adverse effect from the subject's that was recorded by the subjects themselves in a pre printed format given to them at the beginning of the study. After 60 days of use of treatment rinse, once again non-stimulated saliva sample was collected using oral rinse technique for determination of the number of colony-forming units of C. albicans post intervention. The compliance reported regular use of the study preparations as confirmed by two means one the primary care giver of the old age home kept a diary of the preparations consumed. Secondly as a measure of compliance with the prescribed treatment, the patients were instructed to return the bottles at the end of the treatment phase. Oral health status and oral symptoms findings were recorded once again and compared with base line data.

The efficacy of mouthwash was evaluated through microbiological evaluation. The compliance of mouthwash was evaluated through palatability, odor and acceptability by subjects. The adverse effect was evaluated through symptoms like irritation, burning sensation, dryness of mouth, tissue staining etc. All of the above sign and symptoms were recorded as present or absent.

Saliva Sample collection and microbiological evaluation

Sampling for oral yeasts was undertaken 2 times (at the beginning of the study, and after 60 days between 8 and 11 a.m. The participants were told to refrain from eating, drinking (except water) for 1 hr prior to the investigation. The samples were collected using the oral rinse technique with 10 ml of saline solution during 60 s. The non-stimulated saliva samples were obtained according to the oral rinse technique and were seeded using sterile loops in agar Sabouraud chloramphenicol–gentamycin culture plates under aerobic conditions at pH 6.0 and 37^0C for 48 h. The resulting colonies were counted, and the strains were identified under the light microscope according to morphological criteria. The yeast colonies from the saliva samples were counted and calculated as the number of colony-forming units (CFUs) per millilitre.

Statistical analysis

The SPSS_ version 17.0 statistical package (SPSS_Inc., Chicago, IL, USA) was used for the general descriptive analysis of the sample and for evaluation of each of the variables. The Kruskal–Wallis non-parametric test was used for the comparison of continuous variables at each treatment time (baseline and final evaluation), while the Wilcoxon test for paired samples was used to analyse the evolution within each randomized group. In turn, the chi-squared test was applied for comparing the distributions of the categorical variables and treatment groups. Statistical significance was considered for $p < 0.05$.

RESULTS

A total of 40 subjects completed the study (20 in the chlorhexidine group and 20 in the lemon group). The mean age of participated subjects was 68.5 years (range 65–72), with no significant differences between the two groups.

Oral habit & hygiene

Oral habits were recorded only at baseline. Of the participated subjects 100 % both in the test and control groups brushed their teeth daily twice as it was a compulsory rules of old age home. No noticeable variation observed in the oral hygiene and health of the subjects at the end of the study compared to base line.

Candida albicans colony forming unit count

Table 1 shows the mean colony-forming units of Candida albicans according to the randomized group. A statistically significant decrease in the number of colony-forming units of C. albicans was observed in both the groups (p-0.001) after the use of respective mouthwash by subjects daily twice for 60 days. When the mean differences of both the groups compared with Kruskal–Wallis test (Table 2), (Graph 1) again a statistically significant difference was noted (p-0.05) indicating the candida count reduction seen in both groups even is though statistically significant, as compared to chlorhexidine lemon group showed poor efficacy.

Compliance of Mouthwash

After using respective mouth wash for 60 days by the subjects of both the groups no problem in compliance was noted. The subjects reported that the mouthwash that they have used had good palatability, odor were acceptable without any change acceptability. All of them felt Mouth being felt fresh after the use of both the mouth wash.

Adverse effect of mouth wash

20% of the subjects receiving chlorhexidine mouthwash complained of occasional burning sensation in oral mucosa and dryness of the mouth. (Graph 2). However

above mention symptoms were absent in fruit essential oil mouth wash. Both the groups had no tissue staining after the use of respective mouth wash for 60 days.

Table 1. Distribution of the number of colony-forming units of candida albicans at base line and post intervention period

.	0.2%Chlorhexidine Mouth wash		2% (*C. limonum*) *Mouth wash*	
	Mean	**SD**	**Mean**	**SD**
Base line CFU count *(n=20)*	663.8	472.6	620.3	596.8
End of the study CFU count *(n=20)*	120.1	124.5	242.2	293.2
Appreciated Reduction in CFU count *(n=20)*	543.7	365.7	378.1	355.7
p-Value	0.001*		0.001*	

p-value corresponding to Wilcoxon paired samples test. SD: standard deviation.
*P < 0.05 was considered as statistically significant.

Table 2 . Comparison of mean difference between Chlorhexidine & Lemon group

.	**Mean CFU count**	**Chi-Square**	**df**	**p-Value**
0.2%Chlorhexidine Mouth wash (n=20)	543.7	5.979	2	0.050*
2% Lemon (*C. limonum*) *Mouth wash* (n=20)	378.1			

p-Value corresponding to Kruskal–Wallis test df- degree of freedom

Figure 1. Study Protocol and the Intervention

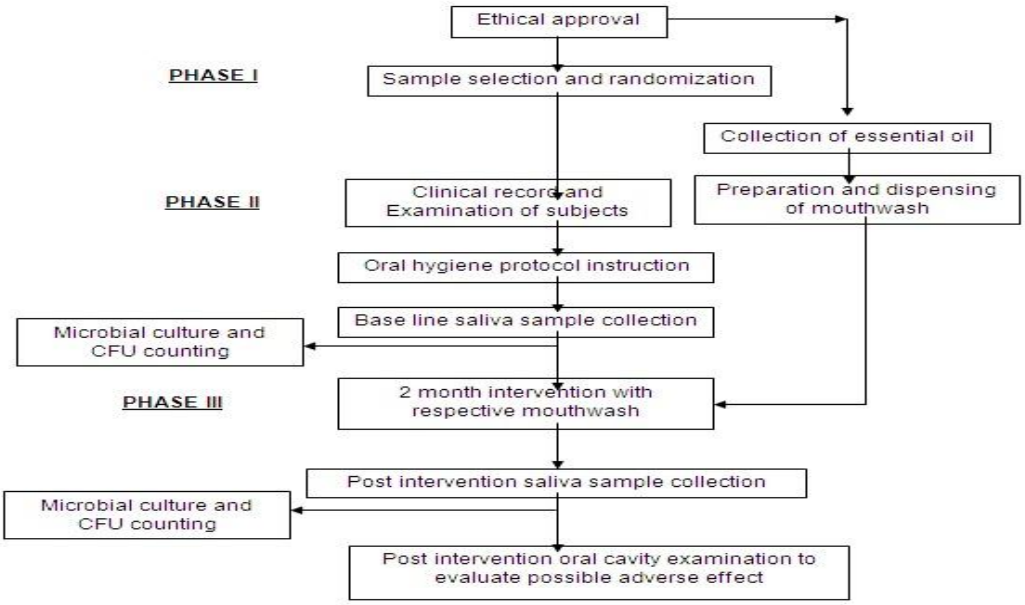
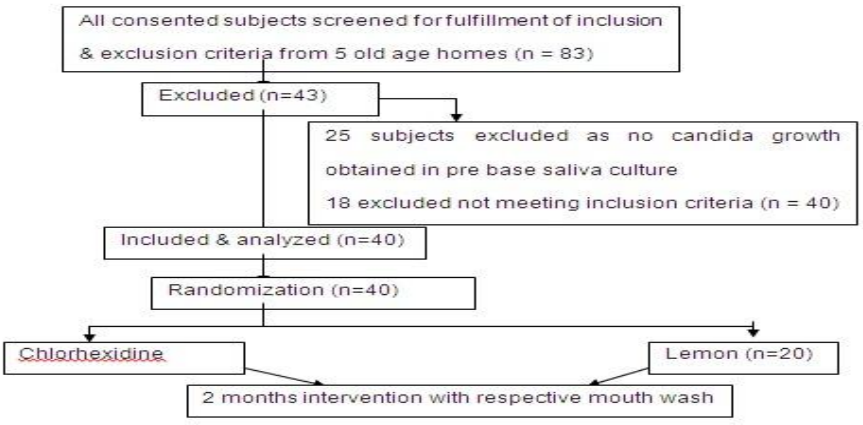
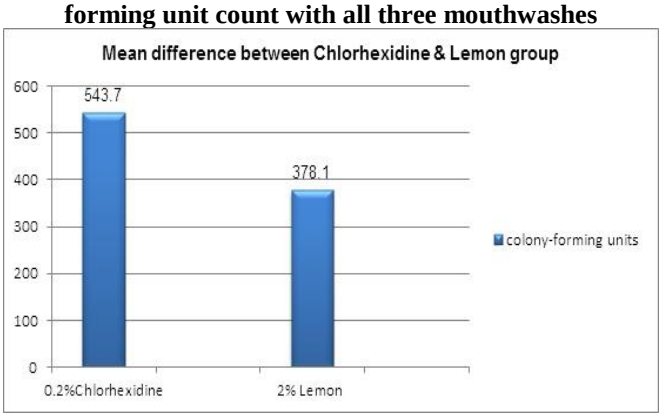


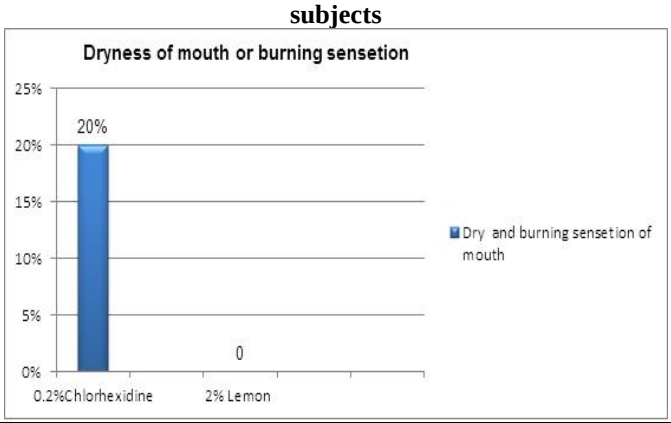
Figure 2. Sample selection process



Graph 1. Mean difference of candida albicans colony forming unit count with all three mouthwashes



Graph 2. Adverse effects of mouth wash reported by the subjects



DISCUSSION

This study was undertaken to investigate the efficacy of mouth wash prepared of lemon essential oils on oral candida count of elderly long standing diabetic population because the size of the elderly diabetic population is fast growing in India and there is an emerging need to pay greater attention to aging-related oral health issues. In India, as a result of advances in medical science and improved social conditions during the past few decades there has been a progressive rise in the number and proportion of elderly, aged 60 years and above and 75% reside in rural areas[13,14]. Government of India adopted 'National Policy on Older Persons' in January, 1999. The policy defines 'senior citizen' or 'elderly' as a person who is of age 60 years or above. The prevalence of diabetes mellitus increases with age. In India, 20% of the elderly population has DM [15,16]. In addition; over 25% of older persons have impaired glucose tolerance (IGT) [17].

Candida species are frequent members of the commensal oral microflora of humans; they are opportunistic pathogens that under conditions of host debilitation can cause a spectrum of oral infection like elderly diabetic. Oral candidiasis has been frequently recognized in diabetic patients, which can be due to their increased glucose in their oral fluids and their immune dysfunction. Environmental alteration of diabetic oral cavity favours Candida colonization and causes a change from a harmless commensally existence to a pathogenic state that known as predisposing factors for Candida infection [2,18,19].

Even though various topical and systemic antifungal drugs are available for the treatment of oral candidiasis development of drug resistance, high recurrence rate after stopping the medication is frequently noted [7,8]. Given the increasing incidence of resistant species in oral infection and the development of resistance against some of the traditionally used antifungals and preventive agents, there is a constant need for research into new and effective agents to treat oral candidosis. So we decided to focus on candidal species from elderly diabetic subjects. Some authors have suggested that mouth rinses containing chlorhexidine, trichlosan, probiotics and those incorporating essential oils may be an appropriate alternative to conventional antifungals in the management of oral candidiasis [13].

In this sense, 0.2% chlorhexidine gluconate a cationic chlorophenyl bisbiguanide that exhibits a broad spectrum of antimicrobial activity through binding to negatively charged *Candida* surfaces, and inducing a loss of structural integrity, decreases adherence and disrupts the cell wall has been successfully & most frequently used as a mouth rinse in the treatment. So it is said that chlorhexidine is a safe material, with low toxicity potential when used correctly and till today considered as gold standards [20-24]. So we compared EO mouth wash with chlorhexidine. However, chlorhexidine has been reported to have a number of local side-effects on its long-term use like brown discoloration of teeth, some restorative materials and dorsum of tongue; taste perturbation; oral mucosal ulcerations and paresthesia; unilateral/bilateral parotid swelling, and enhanced supra-gingival calculus formation [25].

As an alternative, medicinal plants have been explored to produce valuable herbal products, which are frequently used as natural alternatives to treat several diseases. Essential oil mouthwashes containing a range of natural plant extracts, including thymol, eucalyptol, bioflavanoids and tea tree (*Melaleuca alternifola*) oil derivatives, have also been shown to have anticandidal activity *in vitro* [26-32]. As an addition to this a *in vitro* study was reported evaluating the effects of *C. limonum* (lemon) and *C. aurantium* (bergamot) essential oils (EOs) compared to 0.2% chlorhexidine (CHX) on multi-species biofilms reported that *C. aurantium* and *C. limonum* EOs were most effective solutions and have promoted a 100% inhibition of *C. albicans, in* multi-species biofilms[11]. However *in vivo* due to the dilution factor of essential oils and altered nature of bio-film, salivary flow, with expectation of altered results the present study was designed.

Study was double blinded as primary investigator was unaware about the allocation details of the study subjects & Study subjects were unaware of which group they belonged to. The person who conducted the microbial evaluation was unaware about which group the study participants belong to.

Studies reported that successful treatment of candidosis can be hampered where there is an established biofilm. Candida biofilms exhibit significantly higher tolerance to both antimicrobial mouthwashes and also traditional antifungal agents [33]. Subjects with already established candidal lesion in the mouth were excluded from the study. For this *in vivo* study, the sampling method used for candida in the oral cavity was oral rinse technique. This method is extensively used, and even though it does not directly target specific mucosal lesions, it provides a count of the candidal carriage. A study compared oral rinse technique with imprint culture for detection of oral microorganisms and found oral rinse technique to be better for yeast recognition. This technique was verified to be the most sensitive and ideal technique to find and determine overall candidal carriage [34].

Study in 1987 reported that 0.2% chlorhexidine gluconate mouth rinse used daily significantly reduced plaque, but there was no significant effect on the number of Candida organisms [35]. In contrast, the results of our study show a statistically significant decrease in the number of colony forming units of C. albicans between the start and end of treatment, in both patient groups. Our finding coincides with studies reported by Pia Lo´ pez-Jornet 2012[36]. Looking at the amount of reduction observed in candidal colony forming count even though chlorhexidine shown the best efficacy lemon EO mouth wash also showed promising results. By the use of increased concentration of oil and increasing the frequency and duration of use more efficacy can be expected.

Studies showed the most common adverse effect of chlorhexidine to be staining of the oral surfaces, especially the teeth [36-39]. In our study, no subject experienced mucosal desquamation or irritation, or alterations in taste sensation although it must be taken into account that the study duration was rather limited (60 days). Staining is dose dependent and can vary markedly between individuals [40]. Looking at these results the concentration of essential oil for the preparation of the mouthwash can be safely increased also the EO mouth washes can be used for longer duration. Also as mouth wash preparations are cleared from the oral cavity with the faster rate due to flow of saliva, developing an alternative mode of drug delivery system with EO can be thought of.

Limitation

- Limited sample size and duration of use
- Based on type of diabetes reduction in colony count was not examined
- Microbial evaluation done with conventional culture method

CONCLUSION

Within the limitations of the study

- Use of both the study products i.e. 0.2 %chlorhexidine 2% lemon essential oil mouthwashes for 60 days showed significant reduction in candida colony forming unit compared to baseline count.
- 0.2 %chlorhexidine was more effective in reducing candida count compared to 2% lemon essential oil mouthwash.
- 2% lemon essential oil mouthwash can act as An alternative formulation which is natural, biodegradable and safe that is effective against C albicans

REFERENCES

1. Anonymous 1. http,//www.ida.org.in/newYouroralhealth/Elderly.aspx
2. Kumar BV, Padshetty NS, Bai KY, Rao MS. Prevalence of Candida in the oral cavity of diabetic subjects. *J Assoc Physicians India*, 53, 2005, 599–602.
3. Southerland JH, Taylor GW, Offenbacher S. Diabetes and Periodontal Infection, Making the Connection. *Clinical Diabetes*, 23, 2005, 171–8.
4. Geerlings SE, Hoepelman AI. Immune dysfunction in patients with diabetes mellitus (DM) FEMS. *Immunol Med Microbiol*, 26(3-4), 1999, 259–65.
5. Soell M, Hassan M, Miliauskaite A, Haikel Y, Selimovic D. The oral cavity of elderly patients in diabetes. *Diabetes Metab*, 33(1), 2007, S10–S18.
6. Barbeau J, Seguin J, Goulet JP, de Koninck L, Avon SL, Lalonde B, et al. Reassessing the presence of Candida albicans in denture-related stomatitis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 95(1), 2003, 51–9.
7. Epstein JB, Vickars L, Spinelli J, Reece D. Efficacy of chlorhexidine and nystatin rinses in prevention of oral complications in leukemia and bone marrow transplantation. *Oral Surg Oral Med Oral Pathol*, 72, 1992, 291–5.
8. Greenspan D, Greenspan JS. Management of the oral lesions of HIV infection. *J Am Dent Assoc*, 122, 1991, 26–32.
9. Langslet A, Olsen I, Lie SO, Lokken P. Chlorhexidine treatment of oral candidiasis in seriously disease children. *Acta Paediatr Scand*, 63, 1974, 806–11.
10. Nittayananta W, DeRouen TA, Arirachakaran P, et al. Arandomized clinical trial of chlorhexidine in the maintenance of oral candidiasis-free period in HIV infection. *Oral Dis*, 14, 2008, 665–70.

11. Oliveira S, Zambrana J, Di Iorio F, Pereira C, Jorge A. The antimicrobial effects of *Citrus limonum* and *Citrus aurantium* essential oils on multi-species biofilms Braz. *Oral res*, 28(1), 2014.
12. Navazesh M, Kumar SK. University of Southern California School of Dentistry Measuring salivary flow, challenges and opportunities. *J Am Dent Assoc*, 139, 2008, 35S–40S.
13. Jeyalakshmi S, Chakrabarti S, Gupta N. Situation Analysis of the Elderly in India. Central Statistics Office Ministry of Statistics and Programme Implementation Government of India. 2011. [Last accessed, 13-02-2014]. Available from, http,//mospi.nic.in/mospi_new/upload/elderly_in_india.pdf.
14. Population projection for India & states - 1996-2016. Census of India 1991 (Report). New Delhi, Registrar General, India 1996.
15. Meneilly GS, Tessier D. Diabetes in the elderly. *Diabet Med*, 12, 1995, 949-960.
16. Laasko M, Pyörälä K. Age of onset and type of diabetes. *Diabetes Care*, 8, 1985, 114-117.
17. Harris MI, Hadden WC, Knowler WC, et al. Prevalence of diabetes and impaired glucose tolerance and plasma glucose levels in the US population aged 20 to 74 years. *Diabetes*, 36, 1987, 523-534.
18. Sahin I, Oksuz S, Sencan I, Gulcan A, Karabay O, Gulcan E, et al. Prevalance and risk factors for yeast colonization in adult diabetic patients. *Ethiop Med J*, 43(2), 2005, 103-9.
19. Samaranayake LP, MacFarlane TW. Host factors and oral candidosis. *Oral candidosis*, 1990, 66-103.
20. MacNeill S, Rindler E, Walker A, Brown AR, Cobb CM. Effects of tetracycline hydrochloride and chlorhexidine gluconate on Candida albicans, an *in vitro* study. *J Clin Periodontol*, 24, 1997, 753–760.
21. Pusateri CR, Monaco EA, Edgerton M. Sensitivity of *Candida albicans* biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. *Arch Oral Biol*, 54, 2009, 588–594.
22. Barkvoll P, Attramadal A. Effect of nystatin and chlorhexidine digluconate on *Candida albicans*. *Oral Surg Oral Med Oral Pathol*, 67, 1989, 279–281.
23. Budtz-Jorgensen E. Etiology, pathogenesis, therapy, and prophylaxis of oral yeast infections. *Acta Odontol Scand*, 48, 1990, 61–69.
24. Ellepola ANB, Samaranayake LP. Oral candidal infections and antimycotics. *Crit Rev Oral Biol Med*, 11, 2000, 172–198.
25. Flotra L, Gjermo P, Rolla G, Waerhaug J. Side effects of chlorhexidine mouthwashes. *Scand J Dent Res*, 79, 1971, 119-25.
26. Filoche SK, Soma K, Sissons CH. Antimicrobial effects of essential oils in combination with chlorhexidine digluconate. *Oral Microbiol Immunol*, 20, 2005, 221–225.
27. Pan PH, Finnegan MB, Sturdivant L, Barnett ML. Comparative antimicrobial activity of an essential oil and an amine fluoride ⁄ stannous fluoride mouthrinse *in vitro*. *J Clin Periodontol*, 26, 1999, 474–476.
28. Fine DH. Mouthrinses as adjuncts for plaque and gingivitis management. A status report for the American Journal of Dentistry. *Am J Dent*, 1, 1988, 259–263.
29. Kubert D, Rubin M, Barnett ML, Vincent JW. Antiseptic mouthrinse-induced microbial cell surface alterations. *Am J Dent*, 6, 1993, 277–279.
30. Netuschil L, Weiger R, Preisler R, Brecx M. Plaque bacteria counts and vitality during chlorhexidine, meridol and listerine mouthrinses. *Eur J Oral Sci*, 103, 1995, 355–361.
31. Fine DH, Markowitz K, Furgang D, Goldsmith D, Charles CH, Lisante TA and Lynch MC. Effect of an essential oil-containing antimicrobial mouthrinse on specific plaque bacteria *in vivo*. *J Clin Periodontol*, 34, 2007, 652–657.
32. Pizzo G, La Cara M, Licata ME, Pizzo I, D_Angelo M. The effects of an essential oil and an amine fluoride ⁄ stannous fluoride mouthrinse on supragingival plaque regrowth. *J Periodontol*, 79, 2008, 1177–1183.
33. David Williams, Michael Lewis Pathogenesis and treatment of oral candidosis. *Journal of Oral Microbiology*, 3, 2011, 5771 - DOI, 10.3402/jom.v3i0.5771
34. Campisi G, Pizzo G, Milici ME, Mancuso S, Margiotta V. Candidal carriage in the oral cavity of human immunodeficiency virus-infected subjects. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 93, 2002, 281-86.
35. Addy M, Hunter L. The effects of a 0.2% chlorhexidine gluconate mouthrinse on plaque, tooth staining and Candida in aphthous ulcer patients. A double-blind placebo-controlled cross-over study. *J Clin Periodontol*, 14, 1987, 267–273.
36. Pia Lo´ pez-Jornet et al. Short-term side effects of 0.2% alcohol-free chlorhexidine mouth rinse in geriatric patients, a randomized, double-blind, placebo-controlled study. *Gerodontology*, 2012, 29, 292–298
37. Gu¨ rgan CA, Zaim E, Bakirsoy I, Soykan E. Short term side effects of 0.2% alcohol-free chlorhexidine mouth rinse used as an adjunct to non-surgical periodontal treatment, a double-blind clinical study. *J Periodontol*, 77, 2006, 370–384.
38. Addy M, Praytino SW. Light microscopic and color television image analysis of the development of staining on chlorhexidine treated surface. *J Periodontol*, 51, 1980, 39–43.
39. Addy M, Prayitno S, Taylo L, Cadogan S. An *in vitro* study of the role of dietary factors in the etiology of tooth staining associated with the use of chlorhexidine. *J Periodontal Research*, 14, 1979, 403–410
40. Moran J, Addy M, Courtney M, Smith S, Newcombe R. A clinical study to assess the ability of a powered toothbrush to remove chlorhexidinetea dental stain. *J Clin Periodontol*, 31, 2004, 95–98.