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Full Length Research Paper

Efficacy of a mouthrinse based on leaves of the neem tree (*Azadirachta indica*) in the treatment of patients with chronic gingivitis: A double-blind, randomized, controlled trial

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The aim of this study was to compare the short-term efficacy and safety of a *Azadirachta indica* mouthrinse on gingival inflammation and microbial plaque, compared to 0.12% chlorhexidine. A double-masked, randomized, parallel armed study was carried out to assess the efficacy of an oral mouthrinse based on leaves of the neem tree reducing gingivitis. Study subjects were recruited from a slum in Brazil. Fifty-four subjects were enrolled and randomly assigned in two groups (26 neem group, 28 chlorhexidine control). Interventions consisted of a seven day therapy of the *A. indica*-based mouth rinse and chlorhexidine 0.12%, respectively. Plaque index, gingival index and gingival bleeding index were obtained at baseline, as well as after one and four weeks. Additionally, the count of cariogenic bacteria (*Streptococcus mutans*) in the saliva was assessed before and after treatment. All clinical index scores were reduced in both groups seven and 30 days after treatment. There was no statistically significant difference between groups in clinical and microbiological parameters. Adverse events were mild and of transient nature. This short-term study demonstrated that *A. indica*-based mouth rinse is highly efficacious and that it may be used as an alternative therapy in the treatment of periodontal disease.

Keywords: *Azadirachta indica*, gingivitis, *Streptococcus mutans*, neem tree, clinical trial.

INTRODUCTION

Destructive periodontal diseases are characterized by loss of the supporting tissues of teeth, and they are recognized as major public health problem worldwide (Botelho, 2008). Epidemiological studies have shown that periodontal diseases are among the most common afflictions of mankind (Petersen and Yamamoto, 2005). Even in the first world countries, like the United States, it is is estimated that 35% of the dentates aged 30 to 90 years have periodontitis (Albandar et al., 1999) and about 50% some degree of gingival inflammation (Albandar et al., 1999).

Teeth loss by periodontitis often compromises function and esthetics and may also be associated with discomfort (Botelho et al., 2007b). In addition, recent studies suggest that such chronic, low-grade, localized infections such as periodontitis may be associated with systemic health problems including cardiovascular disease (Hujoel et al., 2002), pre-term low birth weight (Offenbacher et al., 1996), diabetes mellitus (Grossi et al., 1996) and

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chronic obstructive pulmonary disease (Scannapieco et al., 2001). Hence, control and treatment of this chronic infection has gained more priority.

The most effective method of prevention and maintenance of periodontal diseases is mechanical oral hygiene combined with proper professional maintenance (Botelho et al., 2007a). However, in reality the degree of motivation and dexterity required for an optimal oral hygiene level may be beyond the ability of the majority of patients (Koch and Lindhe, 1967).

From this perspective, the utilization of antimicrobial mouthrinses has been considered a useful adjunct to oral hygiene. Several compounds have been evaluated for their effectiveness on supragingival plaque and gingivitis including bisbiguanides (such as chlorhexidine gluconate) (Lang et al., 1982), and essential oils (Botelho et al., 2007c). Some of these substances have been associated with side-effects that incapacitate their long-term use, new formulations are needed. The neem tree (*Azadirachta indica*) has the widest spectrum of use of all natural products. The first known use of neem by the Harrappa culture in ancient India dates back 4500 years (Vanka et al., 2001). Today, neem extracts are used to treat various skin diseases, as an antiseptic substance, against endo and ectoparasites or simply as a herbal mouthwash (Vanka et al., 2001). Neem extract has also an excellent effect as a non-toxic repellent, insecticide and pesticide (Mulla and Su, 1999).

A controlled study was conducted to assess the efficacy of an *A indica*-based mouthrinse regarding the antiplaque and anti-gingivitis properties. Additionally, the *Streptococcus mutans* count in the saliva was assessed before and after treatment.

MATERIALS AND METHODS

Plant material

The preparation of the *A. indica*-mouthrinse was carried out at the Department of Pharmacy of the Federal University of Ceará, Brazil. *A. indica* leaves were collected from the medicinal garden of the same institution. Taxonomic identification of plants was performed by botanists of the Prisco Bezerra Herbarium, Department of Biology, Federal University of Ceará (Fortaleza, Brazil), where voucher specimens are deposited (#F244).

The leaves were dried under controlled conditions, then, 20.0 g of dry powder was mixed with 100 ml of 70% (w/v) ethanol for a week in a round bottom flask with occasional shaking. The flask was kept in dark to avoid effect of light on the active ingredients. The extract was then filtered through a muslin cloth for coarse residue and finally through Whatman No. 1 filter paper, measured and kept in an airtight amber colored container. The final mouth wash final formulation included 25% of *A. indica* extract, 20.0% of Saccharin, peppermint oil (<0.1%) as flavor and amaranth red color, according to the manufacturer instructions.

Drugs

A commercially available 0.12% chlorhexidine gluconate mouthrinse (periogard®) was used as an antimicrobial oral agent. The mouthrinse of *A. indica* used in this study previously reported study (Pai et al., 2003). Chlorhexidine gluconate mouthrinse 0.12% and *A. indica 25*% mouthrinse were prepared at the Faculty of Pharmacy, Federal University of Ceará, according to the manufacturer instructions. The appearance, color, smell and taste of both drugs were made identical as far as possible.

Patients

Iinitially, a written informed consent was provided for individuals willing to participate in a protocol approved by the Institutional Review Board of the Federal University of Ceará, Brazil. This was a randomized, controlled, observer-blind, parallel-group 1-month clinical trial.

All examinations were conducted by a single, experienced dental examiner. Reliability was established for the gingival index (GI) with a κ statistic of 0.86, which indicates satisfactory calibration. Fifty – five patients, aged 18 - 65 years old, who met the following inclusion criteria, were included into the study: a minimum of 10 natural teeth; a mean plaque index (PI) of at least 1.05; a mean GI (Sillness and Löe, 1964) of at least 1.0. The teeth that were fully crowned or extensively restored and third molars were not included in the tooth count.

Patients unwilling to complete the treatment protocol, pregnant women, subjects with any disease that would impede the use of the substances used in the study (that is, mental diseases), as well as subjects that had used any type of antibacterial mouthrinse within four weeks of recruitment into the study were excluded from the study. All subjects signed an informed consent form after the nature of the study was fully explained to them. The protocol was reviewed and approved by the Institutional Review Board of the federal University of Ceará (# 551/04).

Qualifying subjects presented to the clinical site for baseline examinations having refrained from any oral hygiene procedures prior to their visit on that day. The baseline examinations consisted of the following: (i) A complete intraoral examination performed to document the mucosae condition; (ii) A saliva sampling collection before and after 7 days evaluation study; (iii) Gingival bleeding GBI (Ainamo and Bay, 1975) and supragingival plaque PI (Sillness & Löe, 1964) were scored on the buccal and lingual surfaces of all scorable teeth; (iv) Gingivitis of the buccal and lingual marginal gingiva and interdental papillae of all scorable teeth was scored using the in which the gingiva are scored on a four-point scale from 0 (absence of inflammation) to 3 (severe inflammation) (Sillness and Löe, 1964). These examinations were all repeated at 7 days and 1 month. On the examination days, subjects refrained from oral hygiene procedures and the use of their assigned was obtained from the fresh leaves, which is similar to a mouthrinse the morning of their examinations in order to eliminate possible bias resulting from each mouthrinse.

Study population and design

Study subjects were recruited from *Morro do Sandra's*, a typical slum community in Fortaleza, capital of the state of Ceará in northeast Brazil. According to key informants, the community is a high risk area for caries and periodontal diseases.

Patients with a minimal mean gingival index of 1.0 and at least 10 remaining teeth in the functional dentition were included. Patients unwilling to complete the treatment protocol, pregnant women, subjects with any disease that would impede the use of the substances used in the study (that is, mental diseases), as well as subjects that had used any type of antibacterial mouthrinse within four weeks of recruitment into the study were excluded from the study .In total, 55 patients (aged 17 to 65 years old) were enrolled. Eligible patients were then randomly assigned to test and control groups using blocked randomization from a computer-generated list. The experimental group received a seven day b.i.d. treatment course of *A. indica*-based mouthrinse (n = 26) while the positive control group received the same regimen with 0.12% chlorhexidine

mouthrinse (n = 28). The variation in the number of participants in each group was a result of the randomization process. These patients were also recruited for another clinical study about gingivitis published elsewhere. In addition to reduce inter-observer bias, all saliva samples collection was done by a single experienced dentist (M.A.B). After the baseline visit, subjects were instructed to rinse approximately 15 mL of mouthrinse for 30 seconds, twice per day (once after breakfast and once in the late afternoon) during a period of 7 days. All mouthrinses were supervised by a research assistant. Study subjects, the investigators and assistants involved in the trial were masked with respect to the treatment. During the seven days of the trial, the subjects continued to exercise their regular non-supervised, self-performed plaque control measures. The saliva samples collection was repeated at seven days after baseline; the study was carried out according to the guidelines of the Declaration of Helsinki for biomedical research involving human subjects.

Each one of the daily rinses was supervised on each weekday. The risings were not done at the time of tooth brushing but at separate times. Subjects were provided with a supply of coded mouthrinse and plastic dosage measuring cups for their twice-daily weekend/holiday supervised at home rinsing. In addition, one of the examiners maintained a diary to document these risings. Subjects were constantly supervised in the morning and afternoon rising. Absences at risings were measured as an indication of compliance. During the study, subjects followed their usual oral hygiene and dietary habits and were instructed to refrain from using commercial mouthrinses (other than their assigned study mouthrinses) and to advise the investigator if they initiated antibiotic or anti-inflammatory drug therapy.

Microbiology study

The microbiological evaluation for *S. mutans* count was carried out in the Department of Pathology, Laboratory of Microbiology, School of Medicine, Federal University of Ceará, Fortaleza. Stimulated saliva was utilized to record *S. mutans* counts. Initially, subjects were asked to motivate saliva flow trough simulated mastication for 1 minute. This procedure was carried out in order to clear the oral cavity of any residual saliva. Subsequently, subjects were then asked to chew a sterile cotton roll for 1 minute and finally to expectorate into a sterile *Eppendorf* tube.

Saliva samples were collected at baseline and immediately after 7 days treatment use of the mouthrinses (*A. indica or* Chlorhexidine). For the microbiology analysis, solid selective culture medium (*mitis-salivarius* with bacitracin agar enriched with sucrose 15%) was utilized for the cultivation of S. *mutans* (Kölher and Brathall, 1979)

The salivary samples were used on two different dilutions (1:100 and 1:1000). The samples were then incubated at 37°C for 48 h in a 5% CO_2 atmosphere. A semi-quantitative, four quadrant streaking method was adopted from previous studies on *S. mutans* estimation, using a Drigalsky spatula to streaked the diluted saliva samples onto *mitis-salivarius* agar. The bacterial growth was recorded in all the four quadrants. All the experiments were done in duplicate.

The duplicate plate counts derived from each dilution saliva sample were converted to Colony Forming Units CFU/mL and the resulting numbers were arithmetically averaged. The CFU samples scores were logarithmically (base 10) transformed, with all analyses performed on the transformed scores.

Statistical analysis

The results of this study were compared between treatment groups with respect to demographic background information (age, percentage of females, tobacco use, number of teeth present, level of education, family income, PI, GI, and GBI). For this analysis, proportions were compared using Fisher's exact test and the Mann-Whitney U-test for independent samples were used to test for significant differences.

The Stata Statistical Software was used for data analysis (College Station, TX; Stata Corporation). The data were first averaged within each patient, and then patient means were analyzed between treatment groups to determine baseline comparability of the two groups. Differences with a P value <0.05 were considered significant. Efficacy analyses were performed for each post-baseline visit upon an intent-to-treat (ITT) population. The Wilcoxon test was used to identify which means (7 and 30 days treatment) were significantly different from baseline. The bacteriological counts were $\log_{10}$ transformed prior to statistical analysis to homogenized variances and eliminate the super dispersion.

RESULTS

Demographics and baseline comparison

About thirty percent of the initial population did not meet the inclusion criteria or were excluded. The most common reason for exclusion was the lack of a minimal number of teeth required for the study. None of the eligible patients refused to participate in the study. The studied population comprised of fifty-four subjects that were randomized into the treatment groups, 26 to the *A. indica* group and 28 to the chlorhexidine group. During the study, five participants were non-compliant with the treatment protocol and were excluded from analysis (3 tests and 2 controls).

Baseline demographic and clinical characteristics of both groups are shown in Table 1. The overall mean age of the test group was 31.7 ± 12.8 years (16 - 65 years) as compared to 31 ± 9.6 years (17 - 54 years) of the control group (P = 1.0). All parameters including gender, smoking status, level of education, monthly family income, number of teeth present, plaque index, gingival index and gingival bleeding index were not significantly different between the groups at baseline (all P>0.1).

Treatment outcomes

The gingival index scores at baseline, as well as seven and thirty days after treatment are depicted in Figure 1(A). Within subjects effect data demonstrated a statistically significant decrease in mean gingival index scores for both groups at seven and thirty days as compared to baseline (both P<0.001). Similarly, the plaque index and gingival bleeding index scores were reduced significantly in both groups seven and thirty days after baseline (Figures 1[B] and [C]); all P<0.001). The gingival index was reduced from 1.52 before treatment to 0.45 30 days after treatment in the neem group as compared to a reduction from 1.63 to 0.42 in the control group (P<0.001). The values for the plaque index were 1.62 to 0.71 and 1.75 to 0.45 (P<0.001) respectively. The gingival bleeding index was reduced from 0.56 to 0.31 in the neem group (P<0.001) and from 0.62 to 0.22 in the

Table 1. Baseline demographic and clinical characteristics by treatment group.

	Test *A .indica* (n=26)	Controlchlorhexidine (n=28)	Significance
% Females	38.5%	39.3%	*P=1.0**
Age (years):			
Mean (SD)	31.7 (±12.8)	31 (±9.6)	*P=0.8†*
Range	16-65	17-54	
Current or previous smoker: n (%)	12 (59.3%)	13 (43.4%)	*P=0.7†*
Number of teeth present: Mean (SD)	18,2 (± 3.5)	20,4 (± 5.0)	*P=0.6†*
Level of Education Illiterate (%)	11.5%	14.5%	*P=0.2**
Monthly Family Income (USD)	1.644 USD	1.778 USD	*P=0.6**
Plaque index: Median (IR)	1.70 (1.47-1.92)	1.63 (1.44-1.75)	*P=0.3**
Gingival index: Median (IR)	1.57 (1.37-1.77)	1.75 (1.60-1.91)	*P=0.1**
Gingival bleeding index: Median (IR)	0.67 (0.52-0.75)	0.62 (0.54-0.75)	*P=0.5**
CFU/ml Median before - after	9550-1200	3800- 900	*P=0.7**

* Mann-Whitney for non paired data.
† t-test.
SD = standard deviation.
IR = interquartile range.

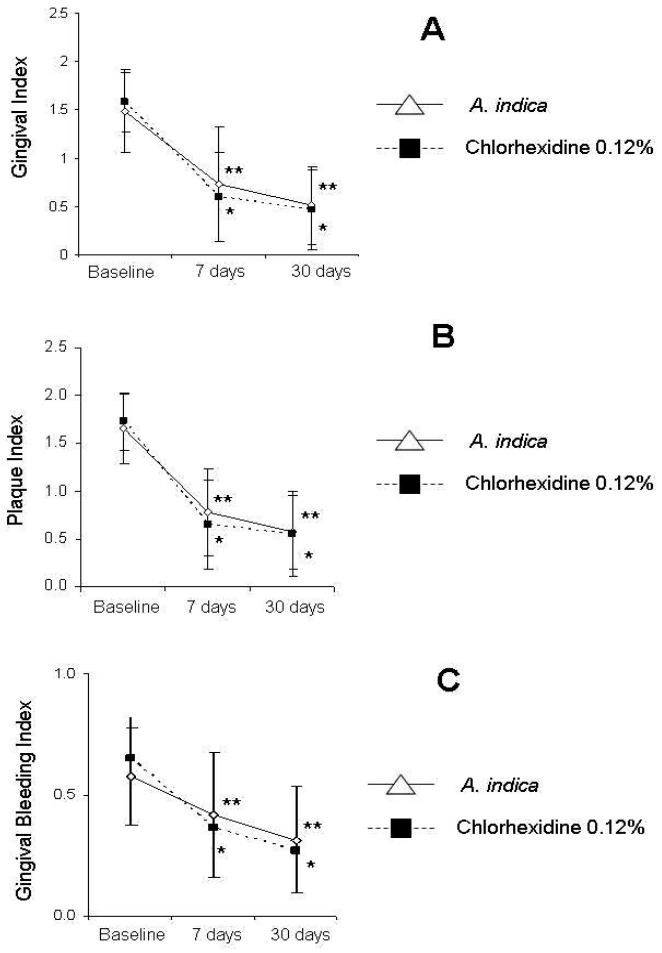

Figure 1. Effect of *A. indica*-based mouthrinse or 0.12% chlorhexidine mouthrinse on clinical parameters. A: Gingival index; B: Plaque index; C: Gingival bleeding index. * = *P*<0.05 compared to baseline, ** = *P*<0.05 compared to baseline. Graphic are displayed as mean and standard errors of the mean

control group (*P*<0.001). There was no statistically significant difference between the *A. indica*-based mouthrinse and 0.12% chlorhexidine for any of the clinical parameters throughout the study (*P* = 0.5).

Adverse events

The most common adverse event reported was mild burning sensation, totalizing 38% and 14% for the test and control groups, respectively (Table 2). Altered taste was accounted for in 15% of the test patients and 21% of the control group and was mild and transient in nature.

S. mutans counts

The *S. mutans* count in the *A. indica* group was reduced from a median of 9550 CFU/ml to 1200 CFU/ml (*P*<0.001). The results in the control group CFU median were ranging from 3800 to 900 (*P*<0.001). There were no statistically significant differences when the microbiologic results were compared between groups *P* = 0.7*

DISCUSSION

Our data show that a mouthrinse based on the neem tree is equally effective in reducing periodontal indices as chlorhexidine. The results demonstrated a significant reduction of gingival, plaque and bleeding indexes in both groups over a period of four weeks. Additionally, the count of cariogenic bacteria in the saliva was reduced drastically.

Chlorhexidine has been utilized for several decades and it is still considered one of the most effective anti-plaque agents in dentistry. However, long term use of chlorhexidine is limited by altering taste and staining of teeth

Table 2. Adverse events related to the use of *Azadirachta indica*-based and chlorhexidine mouthrinse.

Adverse Events	Test *indica* (n = 26)	Control 0.12% chlorhexidine (n = 28)
Burning sensation	10 (38%)	4 (14%)
Altered taste	4 (15%)	6 (21%)
Epithelium desquamation	6 (23%)	0 (0%)
Nausea	2 (7%)	0 (0%)
Altered tongue sensitivity	2 (7%)	0 (0%)
Altered saliva color	0 (0%)	0 (0%)
Dry mouth	0 (0%)	1 (3%)

(Fardal and Turnbull, 1986). Therefore, new formulations with similar or superior efficacy and possibly fewer long-term effects need to be investigated.

One of the candidates is the leaves of the Indian neem tree. This plant has been used in India and other parts of Asia for thousands of years for medical purposes. In traditional literature, preparations of the tree are claimed to be effective in a wide spectrum of inflammatory and infectious diseases. Recent investigations have shown that *A. indica* elaborates a vast array of biologically active compounds that are chemically diverse (Subapriya and Nagini, 2005). Therefore, *A. indica* can be regarded as a valuable plant source for the rationalization of its use in traditional medicine and for modern drug development as well (Van der Nat et al., 1991).

Current data have suggested that by-products of A. indica possess several biological properties, such as antiviral (Parida et al., 2002) and oral antimicrobial (Wolinsky et al., 1996). (Figure 2 A and B)

In dentistry, *A indica* has also demonstrated a good efficacy in the treatment of periodontal disorders (Patel and Venkatakrishna, 1988). In a small trial from India, it was suggested that a dental gel containing *A. indica* extract has significantly reduced plaque index and bacterial count as compared to positive controls (chlorhexidine 0.2%) (Pai et al., 2004). However a double blind study on a mouthrinse solution based on neem has never been published. The present study fills this gap and supports the earlier data on the efficacy of neem formulations delivering the natural bioactive components locally in the oral cavity.

A. indica demonstrated in an *in vitro* study antibacterial activity on different bacterial pathogens isolated from various clinical sources (Rao, 1986). According to other previous studies on natural agents, the microbial count of *S. mutans* in the saliva was found to be reduced significantly (Pai et al., 2004). The positive effect on dental health has been reported in epidemiological studies such efficacy of the herbal mouthrinses extract and the low dental caries among other natural bioactive products users (Botelho et al., 2007b).

Due to the bitter taste associated with this plant, several

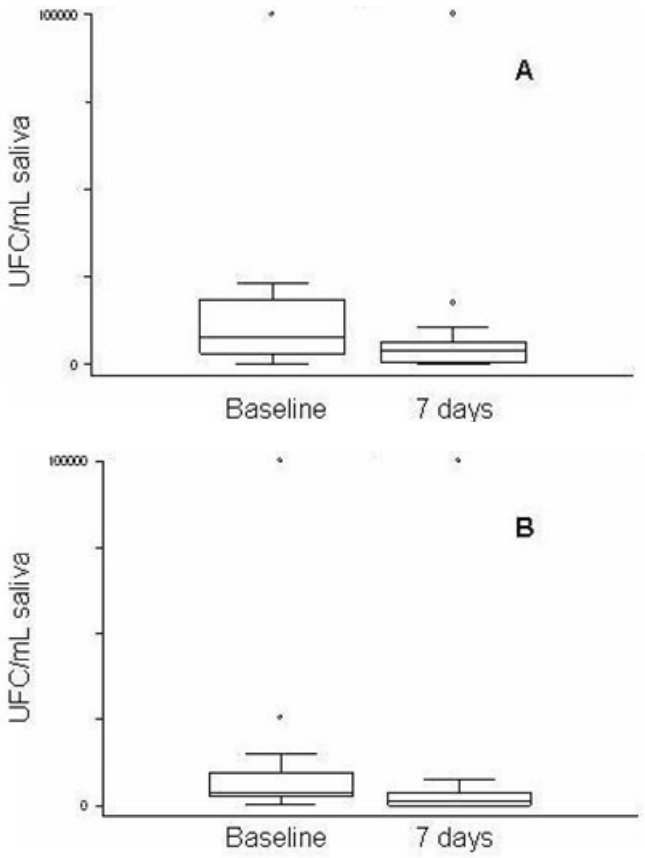

Figure 2. Effect of *A. Indica* based mouthrinse or 0.12% chlorhexidine mouthrinse on *S. mutans* salivary counts. A: *A. Indica* mouthrinse (UFC/mL saliva); B: Clhorhexidine mouthwash (UFC/mL saliva); $p<0.05$ compared to baseline.

different formulations have been developed. In this clinical trial, the *A. indica* extract gel was formulated along with the sweetener and flavor to increase the patient compliance and acceptability. It can be speculated from this study that regular administration of an extract hydroethanolic solution can reduce periodontal indexes combined with acceptable patient compliance.

The compound demonstrated to be relatively safe. Adverse events were common and more frequent in the neem group, but were mild in all cases and resolved without any therapy. However, long-term adverse events are not known and need to be addressed by appropriately designed studies. Due to a bitter taste of the mouthrinse, and for our study was made in a very low resources community, it can justify some patients did not adhered to the treatment.

The treatment doses were applied in after the breakfast and after lunch. In order to eliminate the bias decreased flow of the saliva during overnight that could be attributed to was also successful in reducing the plaque index and microbial count compared to the control group.

Within the limitation of this trial, it was demonstrated that -based mouthrinse is safe and effective in reducing

plaque index, gingival index and gingival bleeding index scores in a high-risk population. The relative reduction of the scores was statistically similar to the reduction in the 0.12% chlorhexidine group. Future long-term prospective studies with larger sample size are being planned to confirm these findings. The results may have an important impact in order to create an effective and inexpensive oral health intervention in low-socioeconomic communities.

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REFERENCES

Ainamo J, Bay I (1975).Problems and proposals for recording gingivitis and plaque. Int Dent J. 25: 229-35.

Albandar JM, Brunelle JA, Kingman A (1999). Destructive periodontal disease in adults 30 years of age and older in the United States, 1988-1994. J. Periodontol. 70: 13-29.

Botelho MA, Bezerra-Filho JG; Correa LL, Heukelbach J (2007). Effect of a novel essential oil mouthrinse without alcohol on gingivitis: a double-blinded randomized controlled trial. *J. Appl. Oral Sci*. 15: 175-180.

Chattopadhyay RR (1999). Possible mechanism of antihyperglycemic effect of Azadirachta indica leaf extract: part V. J Ethnopharmacol. 67: 373-376.

Fardal O, Turnbull RS (1986). A review of the literature on use of chlorhexidine in dentistry. *J of the Amer Dent Assoc*. 112: 863-869.

Farnsworth NR, Waller DP (1982). Current status of plant products reported to inhibit sperm. Res front in fertil reg: RFFR / PARFR. 2: 1-16.

Grossi SG, Skrepcinski FB, DeCaro T, Zambon JJ, Cummins D, Genco RJ (1996). Response to periodontal therapy in diabetics and smokers. J Periodontol. 67: 1094-1102.

Heukelbach J, Oliveira FA, Speare R (2006). A new shampoo based on neem (Azadirachta indica) is highly effective against head lice in vitro.Parasitol Res. 99:353-6.

Hujoel PP, Drangsholt M, Spiekerman C, DeRouen TA (2002). Pre-existing cardiovascular disease and periodontitis: a follow-up study. J Dent. *Res*. 81: 186-191.

Kant R & Bhatt RM (1994). Field evaluation of mosquito repellent action of neem oil. India.n J. Malariol. 31: 122-125.

Klock B, Serling J, Kinder S, Manwell MA, Tinanoff N (1985). Comparison of effect of SnF2 and NaF mouthrinses on caries incidence, salivary S. mutans and gingivitis in high caries prevalent adults. Scand J. Dent Res. 93: 213-217.

Koch G, Lindhe J (1967). The effect of supervised oral hygiene on the gingiva of children. J. Periodontal Res, 2: 64-69.

Kohler B, Bratthall D (1979). Practical method to facilitate estimation of *Streptococcus mutans* levels in saliva. J. Clin Microbiol. 9: 584-588.

Lang NP, Hotz P, Graf H, Geering AH, Saxer UP, Sturzenberger OP, Meckel AH (1982). Effects of supervised chlorhexidine mouthrinses in children. A longitudinal clinical trial. J. Periodontal Res. 17:101-111

Miller LS, Manwell MA, Newbold D, Reding ME, Rasheed A, Blodgett J, Kornman KS (1992). The relationship between reduction in periodontal inflammation and diabetes control: a report of 9 cases. J. Periodontol. 63: 843-848.

Mulla MS, Su T (1999). Activity and biological effects of neem products against arthropods of medical and veterinary importance. J. the Amer Mosquito Contr Assoc. 15: 133-152.

Njoku OU, Alumanah EO, Meremikwu CU (2001). Effect of Azadirachta indica extract on plasma lipid levels in human malaria. Boll. Chim. farm. 140: 367-370.

Offenbacher S, Katz V, Fertik G, Collins J, Boyd D, Maynor G, McKaig R, Beck J (1996). Periodontal infection as a possible risk factor for preterm low birth weight. J.Periodontol. 67: 1103-1113.

Pai MR, Acharya LD, Udupa N (2004). Evaluation of antiplaque activity of Azadirachta indica leaf extract gel-a 6-week clinical study. J Ethnopharmacol. 90: 99-103.

Parida MM, Upadhyay C, Pandya G, Jana AM (2002). Inhibitory potential of neem (Azadirachta indica Juss) leaves on dengue virus type-2 replication. J Ethnopharmacol. 79: 273-278.

Patel VK, Venkatakrishna-Bhatt H (1988). Folklore therapeutic indigenous plants in periodontal disorders in India (review, experimental and clinical approach). Int J clin pharmacol ther and toxicol. 26: 176-184.

Petersen PE (2004). Challenges to improvement of oral health in the 21st century--the approach of the WHO Global Oral Health Programme. Int Dent J. 54: 329-343.

Petersen PE, Yamamoto T (2005). Improving the oral health of older people: the approach of the WHO Global Oral Health Programme. Comm dent and oral epidemiol. 33: 81-92.

Rao DV, Singh I, Chopra P, Chhabra PC , Ramanujalu G (1986). In vitro antibacterial activity of Neem oil. The Indian .J of med. research, 84: 314-6.

Ravindran J, Eapen A, Kar I (2002). Evaluation of repellent action of neem oil against the filarial vector, Culex quinquefasciatus (Diptera:Culicidae). Indian J. malariol. 39: 13-7.

Scannapieco FA, Ho AW (2001). Potential associations between chronic respiratory disease and periodontal disease: analysis of National Health and Nutrition Examination Survey III. J. Periodontol. 72: 50-56.

Scannapieco FA, Wang B, Shiau HJ (2001). Oral bacteria and respiratory infection: effects on respiratory pathogen adhesion and epithelial cell proinflammatory cytokine production. Ann. Periodontol. 6: 78-86.

Sharma SK, Dua VK, Sharma VP (1995). Field studies on the mosquito repellent action of neem oil. *The South Asian J Trop Med Pub health.* 26: 180-182.

Silness J, Löe H (1964). Periodontal disease index. Ann Periodontol. 4: 655-669.

Subapriya R, Nagini S (2005). Medicinal properties of neem leaves: a review. Curr Med Chem and Anti-Cancer Agent. 5: 146-149.

Van der Nat JM, van der Sluis WG, de Silva KT (1991). Ethnopharmacognostical survey of Azadirachta indica A. Juss (Meliaceae). J Ethnopharmacol. 35: 1-24.

Vanka A, Tandon S, Rao SR, Udupa N, Ramkumar P (2001). The effect of indigenous Neem *Azadirachta indica* [correction of (*Adirachta indica*)] mouth wash on *streptococcus mutans* and lactobacilli growth. Ind. J. Dent. Res. 2: 133-144.

Wolinsky LE, Mania S, Nachnani S, Ling S (1996). The inhibiting effect of aqueous Azadirachta indica (Neem) extract upon bacterial properties influencing in vitro plaque formation. J. Dent. Res. 75: 816-822.

World Medical Association Declaration of Helsinki (2001). Ethical principles for medical research involving human subjects. Bull of the World Heal Org. 79: 373-374.