Effect of Water Soluble Neem Metabolite (Soluneem) Compared to Chlorhexidine on Common Oral Bacteria: An *In-Vitro* Study

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Abstract

Aim: To assess and compare the antimicrobial efficacy of a unique water-soluble formulation of Neem metabolite, *Soluneem* (*Azadirachtin*) and Chlorhexidine (CHX) against common cariogenic bacteria like *Mutans streptococci* and *Lactobacilli.*

Methodology: Samples of un-stimulated expectorated saliva were collected from children with high caries index (DMFT of 3 to 5). *Mutans streptococci* and *Lactobacillus* were isolated from the saliva sample using *Mitis salivaris*, *Bacitracin* and *Rogossa agar* respectively. Inhibitory action of *Soluneem* and chlorhexidine on *Mutans streptococci* and *Lactobacilli* was observed by disc diffusion assay. The diameter of inhibition zone was recorded and subjected for statistical analysis.ANOVA and post-hoc Bonferroni test were used.

Results: The results of the disc diffusion assay showed that *Soluneem* (*Azadirachtin*) and chlorhexidine both had significant antimicrobial action on *Mutans streptococci.* Minimum inhibitory concentration of Soluneem was found to be 3%.The mean diameter of the inhibition zone was 25.69 mm at a concentration of 3% (300 micro grams) and above for Soluneem. However, no inhibition of *Lactobacilli* was observed. The mean diameter of inhibition zone was 27.39 mm and 20.29 mm for *Mutans streptococci* and *Lactobacilli* respectively for CHX. The results showed no statistically significant difference in the inhibition of *Mutans streptococci* between *Soluneem* and CHX.

Conclusion: This is the first study to evaluate the efficacy of *Soluneem* (3%) on the inhibition of common oral bacteria *Mutans streptococci. Soluneem* (*Azadirachtin*) showed effective inhibition of *Mutans streptococci* similar to CHX It also shows considerable potential to be incorporated into various oral formulations for maintenance of oral hygiene. However, furtherlong term studies are warranted to establish its antimicrobial action, safety and efficacy in the prevention of dental caries.

Key Words: Azadirachta (Soluneem), Chlorhexidine, Mutans streptococci, Lactobacillus, Dental Caries, Oral Health

Introduction

The oral cavity is an extremely complex entity with several major and minor compartments which together create a special microbial environment. It contains a large and diverse microflora, including Gram-positive and Gram-negative bacteria along with viruses and fungi. It is estimated that one millilitre of whole saliva may contain more than 200 million organisms representing more than 250 different bacterial species [1].

Dental diseases such as dental caries and periodontal diseases are both the result of bacterial infections. *Mutans streptococci* and *Lactobacilli* are mainly responsible for dental caries. Dental caries may lead to life threatening infections, and the costs for operative dental treatment are significant. Therefore, a need exists to identify individuals at risk for the disease and to target preventive measures for these individuals. The development of chemotherapeutic agents capable of inhibiting dental plaque formation has been of great interest to dental researchers and clinicians over the past decade.

These include various pharmacologically active, organic and inorganic substances including those from natural sources such as Neem, Miswak, Mango, etc. [2,3] which are used as chewing sticks in different parts of the world. Chlorhexidine is a widely used antimicrobial agent for the reduction and control of caries activity. In patients with high counts of *Mutans Streptococci* chlorhexidine (CHX) can be employed as an adjunct to other preventive measures. The various adverse effects of antibiotics and emerging antimicrobial drug resistance have posed a major global challenge. This justifies further research and development of natural antimicrobial agents targeting specific oral pathogens while, at the same time, being safe for the host. Recent studies have demonstrated promising antimicrobial activity of natural products against selected oral pathogens [4-6].

Neem (*Azadirachta indica*) belonging to Meliaceae family with '*azadirachtin*' as an active ingredient has been used in India and south Asia for thousands of years to clean teeth and fight oral infections. Studies suggest that '*azadirachtin*' is appropriate for treating gingivitis and oral infections because it inhibits bacterial growth [5].

Over centuries, neem has provided leaves, seed oil and barks with a range of healing properties. In India, this plant is referred to as "village pharmacy" because of its ability to cure many disorders. It is of particular interest to field of dentistry as it has long history of treating teeth and gum problem. Modern

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science validates that neem has antimicrobial properties. The use of neem products is considered eco-friendly, economical and an effective alternative to synthetic drugs.

Azadirachtin is a secondary metabolite present in neem tree seeds. Its chemical formula is C33H44O16. It acts mainly as 'Anti-feedant' and a growth disruptor. Currently available formulations containing *Azadirachtin* degrade very quickly in the presence of water or sunlight.

Earlier preparations from the neem seed kernel were available as emulsified concentrates and required many solvents. *Soluneem* is a unique, world's first water soluble antimicrobial agent consisting of various limonoids, primarily *azadirachtin-A*. It is derived from the neem seed kernel and produced by a novel patented technology by Vittal Mallya Scientific Research Foundation, Bangalore that enhances thermo stability and bioavailability and has a greater shelf life. It is highly stable even at 110 degrees centigrade (thermo stable) whereas, technically Neem Seed Kernel Extracts (NSKE) is thermo labile.

Soluneem is available as off-white, amorphous powder, which can be stored for more than 2 years without loss of bioactivity. It instantly dissolves in water to give a clear solution which is ready for use. *Soluneem* has been tested for acute oral and acute dermal toxicity by the Fredrick Institute for Plant Protection and Toxicology (FIPPAT), Chennai, which is an accredited institute following OECD guidelines for testing. The results indicate that *Soluneem* is extremely safe for humans, animals, birds, fishes and beneficial arthropods including honeybees [7]. *Soluneem* when used as a bio-insecticide, the concentration levels are very high and we are looking at a very low concentration for use in dentistry (caries prevention). Moreover, we are looking at its principal ingredient i.e., *Azadirachta indica* which is used in dentistry since ages.

This study seeks to evaluate the *in vitro* efficacy of water-soluble metabolite, *Soluneem* (*Azadirachtin*) against common cariogenic micro-organisms like *Mutans streptococci and Lactobacilli.*

Materials and Methods

Unstimulated pooled saliva was collected in sterile container from children aged 6-12 years with high caries index (dmft/DMFT > 3) reporting to the Department of Paediatric and Preventive Dentistry, DAPM RV Dental College and Hospital, Bangalore, India. Written informed consent was obtained from the parents or guardians. Ethical clearances were obtained from the institution's ethical committee and review boards.

The saliva was collected between 9.30-11.30 am for a period of 5 minutes. The children were instructed not to eat or drink and undergo any physical exercise or strenuous work prior to saliva collection. The children suffering from any internal soft tissue pathology, medically compromised patients and those on antibiotic therapy were excluded. With the help of a sterile syringe 1 ml of saliva was taken from the container and mixed with 4 ml transport media (Thioglycolate broth) and transported to the laboratory within an hour.

Microbial Analysis

The sample was then vortexed in a cyclomixer to get a uniform mix. The sample was then diluted to 1:5 concentration.10 µl of the diluted sample was streaked with the help of standard inoculation loop of 4 mm diameter on *Mitis Salivaris Bacitracin* (MSB) agar selective for *Mutans streptococci and Rogosa agar* selective for Lactobacilli [8].

The MSB agar plates were incubated under aerobic conditions for 48 hours at 37°Cand the *Rogosa* agar plates were incubated in anaerobic environment for 72 hours at 37°C. After incubating the plates, the colonies with morphologic characteristics of *Mutans streptococci* (0.5 mm raised convex undulated colonies of light blue colour with rough margins, granular frosted glass appearance) and Lactobacilli (regular or curved rods) were identified. Identification of *Mutans streptococci* was confirmed by biochemical tests like mannitol and sorbitol fermentation and catalase test. Gram staining was also performed. Catalase test and Gram staining confirmed *Lactobacilli* (Gram positive and catalase negative rods).

Disc Diffusion Assay

After isolation and identification of *Mutans streptococci and Lactobacilli*, pure cultures were prepared for *Mutans streptococci and Lactobacilli* using MSB and Rogosa agar respectively. A suspension of the pure culture was prepared in distilled water. Using an inoculation loop, a loop full of pure culture was inoculated in MSB and Rogosa broth and incubated overnight. A lawn culture of the organisms (*Mutans streptococci and Lactobacilli*) was done on *MSB* and *Rogosa agar* respectively from the overnight grown culture. The water-soluble sample of *Soluneem* to be tested was diluted with distilled water to obtain the desired concentrations, and then placed in different log concentrations (0.01 gm per 10 ml to 0.9 gm per 10 ml distilled water) at the centre of the previously made lawn culture plates by means of discs. Diameter of inhibition zone (mm) of *Mutans streptococci* by *Soluneem* at various concentrations has been shown in *Figure 1*. Similarly, *CHX* in different concentrations (0.01 to 0.09) was also incorporated on the plates, which acted as a positive control. Diameter of inhibition zone (mm) of *Mutans streptococci* by CHX at various concentrations has been shown in *Figure 2*. One plate each of *Mutans streptococci and Lactobacilli* were kept without adding any of the test

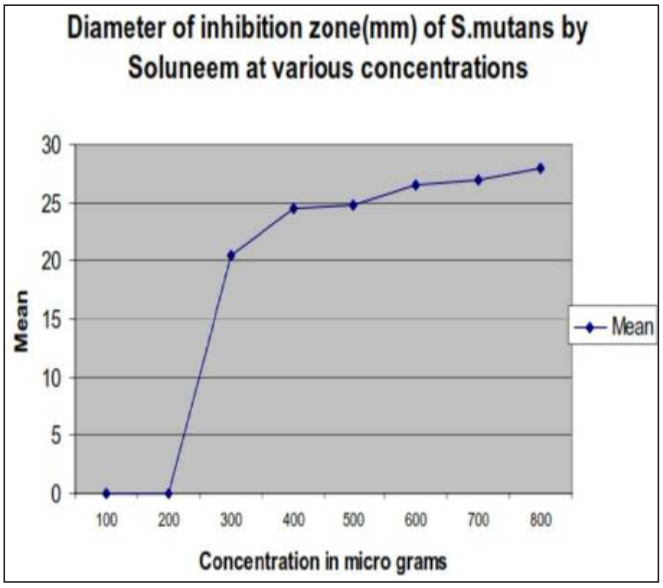

Figure 1. *Diameter of inhibition zone (mm) of Mutans streptococci by Soluneem at various concentrations.*

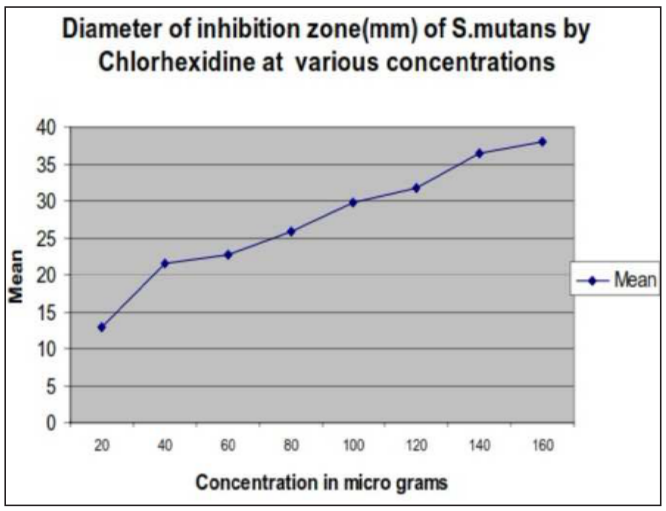

Figure 2. *Diameter of inhibition zone (mm) of Mutans streptococci by CHX at various concentrations.*

samples as control. Then, the MSB agar plates were incubated in aerobic conditions for 48 hours at 37°C in an incubator; the rogosa agar plates were incubated in anaerobic environment for 72 hours at 37°C. (Pour plate technique). After incubation of the plates the inhibitory zones were noted around the test samples (*Soluneem* and CHX) and the diameter of inhibition zone was measured and recorded to check the growth inhibition of *Mutans streptococci and Lactobacilli*. Three trials were performed similarly. Based on the area / diameter; inhibition zone is directly proportional to sensitivity.

Results

Inhibition of *Mutans streptococci* using *Soluneem* has been shown in *Table 1*. Lactobacilli were not inhibited by *Soluneem*. Inhibitions of *Mutans streptococci and Lactobacilli* using CHX have been shown in *Table 2*. Mean inhibition of *Mutans streptococci* by *Soluneem* is 25.69 mm and by CHX is 27.38 mm. The mean inhibition of lactobacillus by CHX was found to be 20.29 mm (*Table 3*).

Descriptive statistics and inter and intra group inhibitions using ANOVA are tabulated in *Table 4*. A significant intra and inter group mean inhibition values ($p<0.01$) was found between the three groups. In order to find out which pair of groups has a significant difference, we carried out multiple comparisons post-hoc- Bonferroni test. It was noted that no significant difference is observed between *Mutans streptococci- Soluneem*(group 2) and *Mutans streptococci* – chlorhexidine (group 1) ($p>0.05$) claiming *Soluneem* is as effective as CHX. However, significant difference between the inhibitions rate of lactobacilli and chlorhexidine (group 3) with that of *Mutans streptococci* – chlorhexidine (group 1) and *Mutans streptococci– Soluneem* (group 2) was observed as shown in *Table 4* (*Figure 3*).

Discussion

Anti-microbial control of dental plaque flora especially *Mutans streptococci and Lactobacilli* may be beneficial in the control or prevention of dental caries. A variety of chemotherapeutic agents have been tested which include antibiotics, metal ions, enzymes, plant extracts and phenolic compound [9,10]. The efficacy of these antimicrobial agents

Table 1. *Inhibition of Mutans streptococci using Soluneem*

Conc. of Soluneem (µ gm)	Zone of inhibition of *Mutansstreptococci*(mm)			
	Trial 1	Trial 2	Trial 3	Mean ± SD
100	0	0	0	0 ± 0
200	0	0	0	0 ± 0
300	20	20.5	21	20.5 ± 0.5
400	24	25	24.5	24.5 ± 0.5
500	25	25.5	24	24.83 ± 0.76
600	26	27	26.5	26.5 ± 0.5
700	26.5	27.5	27	27 ± 0.5
800	27.5	28.5	28	28 ± 0.5
900	28	29	28.5	28.5 ± 0.5

depends on many factors like vehicle used, concentration of active agents and duration of the treatment.

The present study is the first to evaluate the relative efficacy of *Soluneem* on *Mutans streptococci and Lactobacilli* and compare its efficacy with that of standard antimicrobial agent- CHX. The antimicrobial action of CHX on *Mutans streptococci and Lactobacilli* with the mean inhibition diameter of 27.38 mm and 20.9 mm respectively is in accordance with various other studies [11-18].

The experimental agent *Soluneem* showed antimicrobial activity only against *Mutans streptococci*, with the mean inhibitory diameter of 25.69 mm, which was supported by various studies on neem extract [3,19]. Further, comparison of the inhibitory actions of *Soluneem* and CHX on *Mutans streptococci* showed no statistically significant difference between the two antimicrobial agents, suggesting that *Soluneem* is equally potent and effective in inhibiting *Mutans streptococci* as chlorhexidine.

As per our knowledge only one *in vivo* study has claimed that neem (leaf extract) gel has an anti-bacterial effect on both *Mutans streptococci and lactobacilli*. However, the age group of the sample and whether the DMFT index had been used or not, has not been mentioned [5]. Evidence suggests that there is a statistically significant relation between *Mutans streptococci* and DMFT categories, but not *Lactobacilli* [20]. It has also been established that *Mutans streptococci* is responsible for the initiation of dental caries and lactobacillus in the progression of caries [21,22].Therefore, considering the above mentioned facts it may be assumed that *Soluneem* may be effective in prevention of dental caries as it has shown inhibitory action on *Mutans streptococci*. However, its ineffectiveness against *Lactobacilli* may limit its use in established or progressing carious lesions. A recent study has also shown that *Soluneem* is ineffective against periodontal pathogens like *Bacteroids fragilis*, *Prevotella corporis*, *Prevotella melaninogenica*, and *Pepto streptococus species* [23].

Within the limitations of our study, it can be commented that *Soluneem* has antimicrobial activity only on aerobic organisms and not on anaerobic organisms. However, further studies have to be undertaken on various other organisms (aerobic and anaerobic), to determine the effectiveness of *Soluneem* on different categories of micro- organisms.

The present study showed that antimicrobial action of *Soluneem* (*Azadirachtin*) against *Mutans streptococci* starts at a concentration of 3 % (300 micro grams). According to toxicology report, 2 gm per kg body weight is relatively safe for humans. Therefore, it can be assumed that *Soluneem* at the

Table 2. *Inhibition of Mutans streptococci and Lactobacilli using Chlorhexidine.*

Concentration of CHX (µ gm)	Zone of inhibition *Mutans streptococci* (mm)				Zone of inhibition *Lactobacilli* (mm)			
	Trial 1	Trial 2	Trial 3	Mean ± SD	Trial 1	Trial 2	Trial 3	Mean ± SD
20	13	12.5	13	12.83 ± 0.28	11	11.5	12	11.50 ± 0.50
40	22	21	21.5	21.50 ± 0.50	12.5	13	12	12.50 ± 0.50
60	23	22	23	22.66 ± 0.57	14	15	14.5	14.50 ± 0.50
80	27	25.5	25	25.83 ± 1.04	18	18.5	19	18.50 ± 0.50
100	30	29	30.5	29.83 ± 0.76	20	22	21	21.00 ± 1.00
120	32	31.5	32	31.83 ± 0.28	24	25	23.5	24.16 ± 0.76
140	36	36.5	37	36.50 ± 0.50	28	27	27.5	27.50 ± 0.50
160	37.5	38	38.5	38.00 ± 0.50	32	33	33	32.60 ± 0.57

Table 3*.Descriptive statistics for all three groups and inter and intra group inhibitions.*

Group (n=24)	Mean ± SD	95% Confidence Interval (CI) for Mean		Min	Max
		Lower Bound	Upper Bound		
Mutans streptococci & Chlorhexidine	27.38 ± 8.03	23.98	30.77	12.50	38.50
Mutans streptococci & Soluneem	25.69 ± 2.63	24.49	26.89	20.00	29.00
Lactobacillus & Chlorhexidine	20.29 ± 7.20	17.25	23.33	11.00	33.00

Table 4. *ANOVA and Bonferroni test.*

Group (n=24)	Mean ± SD	95% CI for Mean		F	P-Value	Sig diff between
		Lower Bound	Upper Bound			
1. *Mutans streptococci* & Chlorhexidine	27.38 ± 8.03	23.98	30.77	7.651	0.001*	1 vs 3
2. *Mutans streptococci* & Soluneem	25.69 ± 2.63	24.49	26.89			2 vs 3
3. *Lactobacillus* & Chlorhexidine	20.29 ± 7.20	17.25	23.33			

*Statistically significant at $p \leq 0.05$

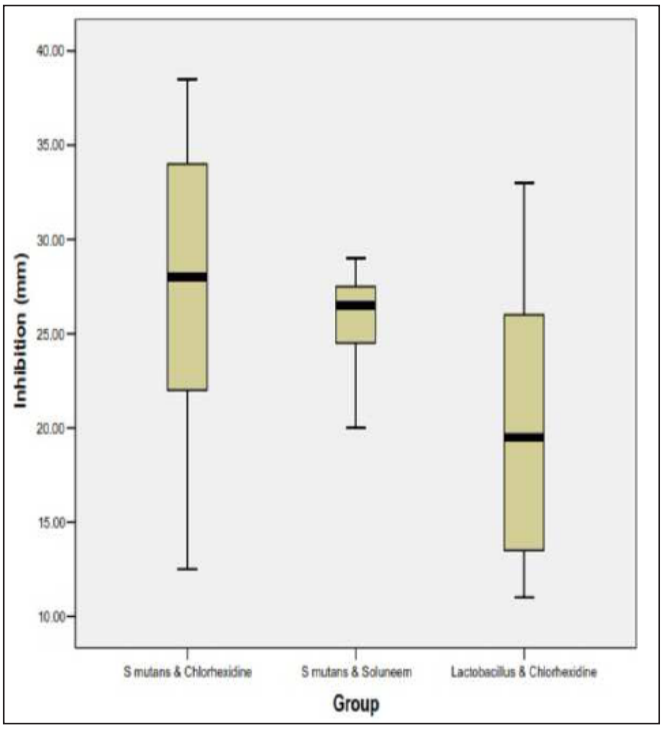

Figure 3*. Box and Whisker plot showing various inhibition zones of S mutans/ Chlorhexidine, S. mutans/ Soluneem, Lactobacillus/ Chlorhexidine.*

given concentration can be safely used in humans without any detrimental effect.

Brushing with neem (*Azadirachta indica*) twigs, chewing neem leaves and seeds after a meal has been the traditional dental care practice in most of the countries worldwide, especially in Asian countries. However, the use of harvested chewing sticks after prolonged storage period is not advisable for oral hygiene measures as it was noted that *Azadirachta indica* chewing twigs were more susceptible to post-harvest fungal overgrowth within 4 weeks of storage, which could lead to adverse reactions from the same [24].

In contrast, *Soluneem* has the advantage of being a unique water-soluble neem metabolite when compared to other neem extracts, which were formulated in various organic solvents such as alcohol, ether etc. which reduced their shelf life. *Soluneem* has a shelf life of more than 2 years. It is also cost effective as water is available readily as a universal solvent. Compared to other technical neem seed kernel extracts which are thermolabile, *Soluneem* is highly thermo stable even at 110 degrees. The above mentioned advantages of *Soluneem* make it a unique and effective antimicrobial agent. Although CHX has a good antimicrobial activity, its long term use is under scrutiny due to its various undesirable adverse effects. Genotoxicity and mutagenicity of CHX has been well documented [25]. Though rare but it has also shown severe reactions of contact dermatitis. Hence, it has been suggested to be used as a therapeutic agent and not for routine long term use. Emerging drug resistance is also a major concern. Taking the above afore mentioned facts into consideration, it is tempting to speculate that in future *Soluneem* could be used as a key ingredient of an antibacterial mouth rinse or incorporated into tooth paste as an alternative to other synthetic products for the prevention of dental caries. However, further long term studies to assess its safety, efficacy and its properties are warranted.

Conclusion

In conclusion, we report here for the first time the efficacy of *Soluneem* (3%) on the inhibition of *Mutans streptococci* which is relatively similar to that of standard antimicrobial agent Chlorhexidine.

CHX has been used as a therapeutic agent due to its antimicrobial activity. However, its long term use is under scrutiny due to its various undesirable adverse effects. *Soluneem*

could be a promising alternative to other antimicrobial agents for the prevention of dental caries. Modern science validates that neem products are eco- friendly, economical and effective alternative to synthetic drugs. It is also tempting to speculate that *Soluneem* can also be used in combination with other various naturally occurring antimicrobial agents which has a wide range of antimicrobial action.

Further long term *in vivo* multicentric studies incorporating *Soluneem* (*Azadirachtin*) in the form of mouth wash or gel may be conducted to establish its antimicrobial action, safety and efficacy in the prevention of dental caries.

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Conflict of Interest

I confirm that the manuscript is original and has not been published elsewhere, neither is it submitted to any other journal or meeting or postings for consideration. In addition, the authors report no conflict of interest, and the manuscript in its submitted form has been read and approved by all authors.

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References

1. Thylstrup A, Fejerskov O (Editors). Textbook of Clinical Cariology. The Oral Environment - An Introduction (2ndedn). Copenhagen Munksgaard -1994: pp.14.

2. Almas K. The antimicrobial effects of seven different types of Asian chewing sticks.*Odonto-StomatologieTropicale*.2001; **24**:17-20.

3. Prashant GM, Chandu GN, Murulikrishna KS, Shafiulla MD. The effect of mango and neem extract on four organisms causing dental caries: Mutans streptococci, Streptococcus saliva virus, *Streptococcus mitis*, and *Streptococcus sanguis*: An *in vitro* study.*Indian Journal of Dental Research*.2007; **18**:148-151.

4. Wolinsky LE, Mania LE, Mania S, Nachnani S, Ling S. The inhibiting effect of aqueous *Azadirachta indica* (Neem) extract upon bacterial properties influencing *in vitro*plaque formation. *Journal of Dental Research*.1996; **75**:816-822.

5. Pai MR, Acharya LD, Udupa N Evaluation of antiplaque activity of *Azadirachta indica* leaf extract gel-A 6-week clinical study.*Journal of Ethnopharmacology*.2004; **90**:99-103.

6. Seneviratne CJ, Wong RW, Hägg U, Chen Y, Herath TD, Samaranayake PL, Kao R. Prunusmume extract exhibits antimicrobial activity against pathogenic oral bacteria. *International Journal of Paediatric Dentistry*.2011; **21**:299-305.

7. http://www.vmsrf.org/html/solu-neem.html

8. Collins AA. A comparison of tomato yeast agar with rogosa's medium for lactobacillus counts. *Journal of Dental Research*. 1964; **43**:713.

9. Järvinen H, Tenovuo J, Huovinen P. *In vitro* susceptibility of Streptococcus mutans to chlorhexidine and six other antimicrobial agents. *Antimicrobial Agents and Chemotherapy*.1993; **37**:1158-1159.

10. Smullen J, Koutsou GA, Foster HA, Zumbé A, Storey DM. The antibacterial activity of plant extracts containing polyphenols against Streptococcus mutans. *Caries Research*.2007; **41**:342-349.

11. Maltz M, Zickert I, Krasse B. Effect of intensive treatment with chlorhexidine on number of Streptococcus mutans in saliva. *Scandinavian Journal of Dental Research*.1981; **89**:445-449.

12. Zickert I, Emilson CG, Krasse B. Effect of caries preventive measures in children highly infected with the bacterium Streptococcus mutans. *Archives of Oral Biology*. 1982; **27**:861-868.

13. Bowden GH. Mutans streptococci caries and chlorhexidine. *Journal of Canadian Dental Association*.1996; **62**:700-707.

14. Ernst CP, Prockl K, Willershausen B The effectiveness and side effects of 0.1% and 0.2% chlorhexidine mouth rinses: A clinical study. *Quintessence International*.1998; **29**:443-448.

15. Vanka A, Tandon S, Rao SR, Udupa N, Ramkumar P. The effect of indigenous Neem *Azadirachta indica* [correction of (*Adirachta indica*)] mouth washes on Streptococcus mutans and lactobacilli growth. *Indian Journal of Dental Research*.2001; **12**:133-144.

16. Kulkarni VV, Damle SG. Comparative evaluation of efficacy of sodium fluoride, chlorhexidine and triclosan mouth rinses in reducing the mutans streptococci count in saliva: an *in vivo* study. *Journal of Indian Society of Pedodontics and Preventive Dentistry*.2003; **21**:98-104.

17. Ersin NK, Uzel A, Aykut A, Candan U, Eronat C Inhibition of cultivable bacteria by chlorhexidine treatment of dentin lesions treated with the ART technique. *Caries Research*.2006; **40**:172-177.

18. Neeraja R, Anantharaj A, Praveen P, Karthik V, Vinitha M. The effect of povidone-iodine and chlorhexidine mouth rinses on plaque Streptococcus mutans count in 6- to 12-year-old school children: An *in vivo* study. *Journal of Indian Society of Pedodontics and Preventive Dentistry*.2008; **26**:14-18.

19. Polaquini SR, Svidzinski TI, Kemmelmeier C, Gasparetto A. Effect of aqueous extract from Neem (*Azadirachta indica A*. Juss) on hydrophobicity, biofilm formation and adhesion in composite resin by Candida albicans. *Archives of Oral Biology*.2006; **51**:482-490.

20. Hegde PP, Ashok Kumar BR, Ankola VA. Dental caries experience and salivary levels of Streptococcus mutans and Lactobacilli in 13-15 years old children of Belgaum city, Karnataka.*Journal of Indian Society of Pedodontics and Preventive Dentistry*.2005; **23**:23-26.

21. Krishnakumar R, Singh S, Subba Reddy VV. Comparison of levels of mutans streptococci and lactobacilli in children with nursing bottle caries, rampant caries, healthy children with 3-5 dmft/DMFT and healthy caries free children. *Journal of Indian Society of Pedodontics and Preventive Dentistry*.2002; **20**:1-5.

22. Byun R, Nadkarni MA, Chhour KL, Martin FE, Jacques NA, Hunter N. Quantitative analysis of diverse Lactobacillus species present in advanced dental caries. *Journal of Clinical Microbiology*.2004; **42**:3128-3136.

23. Sharma S, Vijayendra S , Bharwani A "An *In vitro* Microbiological Study Evaluating the Efficacy of Soluneem (A Water Soluble Neem Formulation from *Azadirachta Indica*) Against Periodontopathic Microorganisms." *Journal of Oral Health & Community Dentistry*.2012; **6**: 4-9.

24. Etebu E, Tasie AA, Daniel-Kalio LA. Post-harvest fungal quality of selected chewing sticks. *Oral Diseases*.2003; **9**:95-98.

25. Gahyva SM, Siqueira JF Jr. Direct genotoxicity and mutagenicity of endodontic substances and materials as evaluated by two prokaryotic test systems. *Journal of Applied Oral Science*. 2005; **13**:387-392.