

Assessment of Antifungal Properties of Crude Neem Extract Versus Neem Mouthwash – An In Vitro Study

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Abstract : Dental caries is the most common oral disease in mankind caused primarily by *Mutans streptococci* and *lactobacilli* and the prevalence of *Candida albicans* in the biofilms of early childhood caries is greater than in non-caries children suggesting the possibility of synergism. Great importance is given to herbal extracts for prevention and control of oral diseases. The various side effects organic herbal extracts have created a need for simple plant extraction practices which can be done in a normal household kitchen. The present study was aimed at comparing in vitro the antifungal properties of crude neem extract made by a simple household procedure with a pharmaceutically prepared neem mouthwash. The growth and biofilm formation of *Candida albicans* in the presence of the test materials were evaluated by direct contact inhibition test and tissue culture plate assay. The home-made crude extract of neem was found to have significant antifungal properties comparable to that of the mouthwash.

Keywords: Neem, Crude extract, Mouthwash, *Candida albicans*

I. Introduction

Dental caries is the most common oral disease of mankind. *Mutans streptococci* and *lactobacilli* are the primary etiological agents, their acidogenic and aciduric properties being the factors responsible for their cariogenicity. Oral candidiasis is the most common oral fungal infection in humans, primarily caused by *Candida albicans*. Although *Candida* are normally present in the oral cavity of healthy individuals, they are opportunistic pathogens that cause infections with high frequency in immune deficient individuals[1]. Studies show that *Candida albicans* in the biofilm in Early Childhood Caries are more prevalent than in caries free children. Their colonization in the oral cavity of children might be related to pacifier usage and feeding habits in infants and young children[2].

Advances in the field of preventive dentistry have led to the advent of various methods and formulations that will help reduce or inhibit the counts of these microorganisms in the oral cavity. Unfortunately, the economic burden associated with dental diseases can be extremely high among the poor and under privileged population[3]. Thus the deployment of natural substances in dentistry is gaining momentum. Neem (*Azadirachta indica*) has been used in Ayurveda, Unani and Homeopathic medicine in a wide range of applications. Various dental care products have been extracted from the neem leaves, seeds and bark. The phytochemical constituents present in neem which are responsible for its biological activities include nimbidin, nimbin, nimbolinde, Azadirachtin, gallic acid, epicatechin, catechin and margolone[4]. Studies have shown that the extracts of neem leaves have antibacterial effects and antifungal effects. Organic extracts of herbal products such as alcoholic and acetonic extracts as mouth rinses are prepared in laboratory set ups. Flavoring agents, buffers and vehicles are added according to pharmaceutical guidelines of mouthwash preparations, which require accurate scales, measurements and pharmaceutical expertise. But, when these herbal mouth rinses, become available commercially, their prices are sometimes even higher than the conventional chemical mouth rinses.

Thus, in order that the underprivileged population in rural areas are benefited, there is an increasing need for simple plant extraction methods that can be done in a normal household kitchen.

II. Aim And Objective

The present *in vitro* study was aimed at assessing and comparing the antifungal properties of home-made crude neem extract with a pharmaceutically prepared neem mouthwash.

III. Materials And Methods

Neem leaves that were collected from the garden were dried under controlled parameters.

3.1 Preparation of crude aqueous neem extract

The crude aqueous extract was prepared by macerating 20g of dry powder of neem leaves with 100ml of distilled water with occasional shaking. This is then filtered to remove the coarse residue and to obtain the filtrate. The filtrate was then condensed by evaporation to 20 ml. (Figure 1)

3.2 Preparation of neem mouthwash

Neem extract was first prepared by macerating 20g of dry powder with 100ml of sterile distilled water in a round bottomed flask with occasional shaking. The extract was then filtered through a muslin cloth and finally through Whatman No.1 filter paper and stored in a airtight amber coloured container. The final mouthwash formulation included neem extract and compound sodium chloride mouthwash. (Figure 2)

Evaluation of Antifungal Activity

Agar Well Diffusion Test[5] was initially used to assess the antifungal activity of the neem crude extract and pharmaceutically prepared neem mouthwash..A standard strain of *Candida albicans* maintained by the Department of Microbiology, K. S. Hegde Medical Academy was used. As no inhibition zones were observed around any of the wells containing the test material, the direct contact test was done. A pure growth of the organism in the absence of the test material was used as control. Six trials were done for statistical evaluation.

To assess the Minimum Inhibitory Concentration and Minimum Fungicidal Concentration, the organisms were incubated with 8 serial dilutions of the neem crude extract and the neem mouthwash at 37° C for 18 hours. (Figure 3)

2.3 Effect on adherence of C. Albicans

For adherence or biofilm detection, the Tissue Culture Plate assay[6] (also called semi quantitative microtitre plate test) described by Christensen et al (1985) was used for detection of biofilm formation (Figure 4).The optical density was read at 540nm in ELISA microplate reader and the results were interpreted according to Table 1.

IV. Results

One way ANOVA Kruskal Wallis H test was used to find significant differences for the group comparisons and post-hoc Mann Whitney U test was used to find significant differences for the pair-wise comparisons.

The microbial counts were recorded as colony forming units per ml and the resulting numbers were arithmetically averaged. The CFU scores were logarithmically (base 10) transformed, and all analyses were performed with the transformed scores. The *Candida albicans* colony counts in the presence of Neem crude extract and Neem mouthwash are shown in Table 2 and Figure 5. Both 50% concentration and 100% concentration of both the crude extract and the mouthwash were found to be inhibitory, while only the 100% of both the test materials were found to be fungicidal.

The mean absorbance value of the stained adherent fungal biofilms, as read by ELISA reader, are shown in Table 3. Neither the neem extract nor the neem mouthwash was found to inhibit biofilm formation by *Candida albicans* (All optical density values are > 0.240).

V. Discussion

Dental caries is a multifactorial infectious disease, which arises from the interplay between the oral microflora, teeth, dietary factors and oral hygiene measures. Recent evidence indicates that there is a high prevalence of *S. mutans* in dental biofilms where *Candida albicans* also resides, suggesting that the cariogenic process may be mediated by an interaction between these diverse species[7,8]. Several *in vitro* studies[8,9] have shown that *C. albicans* enhances the adherence of *S. mutans*, and some *in vivo* studies also support the active role of *Candida* species in dental caries[9-11].

The pharmaceutically prepared mouthwash that was used in this study contained an aqueous extract of neem, but the final formulation was prepared with addition of sodium bicarbonate, compound sodium chloride and peppermint oil as vehicle and flavouring agent. Whereas, the crude extract of neem was made by simple methods that could be done in a household kitchen. The aim of our study was to assess and compare the antifungal activities of home-made crude extract of neem with that of neem mouthwash *in vitro*.

Initially, when the agar diffusion test was used for assessment of antimicrobial sensitivity, both the crude extract and the mouthwash did not show any activity. The absence of diffusion areas around the tested

materials could mean that the active components were not diffusible in the agar, probably due to their larger particle size. Similar findings were seen in the study done by Nayak Aarati et al[12] with aqueous and alcoholic extracts of neem. With this observation, we decided to use the direct contact test to assess the antimicrobial activity.

The direct contact test (DCT), which was used by Weiss et al, 1996[13], and Cobankara et al, 2004[14], is a valuable *in vitro* assay to study antimicrobial properties of solid materials. It allows direct contact between the microorganisms and the tested material and thereby measurement of the effect of the tested material on microbial growth. It also allows a distinction to be made between their static and cidal effects[3]. In our study, both the test materials showed significant inhibitory effects on growth of *C. albicans*. Our results are similar to those of the studies by Nayak Arati et al[12], Autade et al[15], Bohora et al[16] and Tyagi SP et al[17], all of which have shown that organic extracts of neem have good antifungal properties. The study by Nayak Arati et al concluded that the alcoholic extract of neem had better antifungal activity when compared to the aqueous extract of neem although the difference was not statistically significant.

The tissue culture plate method described by Christensen et al[6] was used to assess the effects of the test materials on biofilm formation by *C. albicans*. The optical density of the adherent fungal biofilms on the walls of the microtitre plates was read. Neither of the test materials showed any inhibition on the biofilms of *C. albicans*. This could be because of the reduced concentration of the active components in the test materials, as they were aqueous extracts of. The type of extraction had an influence on the activity of plant extracts. Our results are contrary to those of Polaquini S et al[18] who found that the aqueous neem extract used in their study did not inhibit the growth of *C. albicans*, but had an anti-adhesive effect on the surface of composite resin when studied *in vitro*. This could be because of the difference in the concentration of the active component of neem present in the final formulation used in our study[19], and also because we have used a different standard strain of *C. albicans*.

Thus, our *in vitro* study conducted with the aqueous neem mouthwash and the crude aqueous neem extract showed that the crude extract, which was made by a simple extraction technique that can be replicated in a household kitchen, had significant antifungal properties, which are comparable to those of the mouthwash.

Further *in vivo* studies need to be conducted to assess the activities of the crude extracts in the oral cavity. Factors such as stability, viscosity and any side effects if present, need to be assessed. The bitterness of crude plant extracts, especially those of neem can be masked by the addition of natural flavouring agents like honey, and made more acceptable for children.

3. Figures And Tables



Figure 1 :Crude aqueous neem extract



Figure 2 :Pharmaceutically prepared neem mouthwash

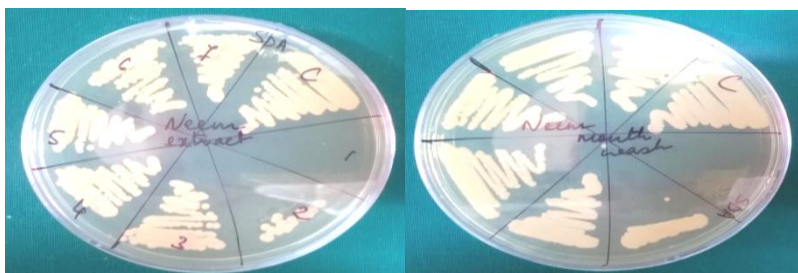


Figure 3: Different concentrations subcultures to assess MIC and MBC

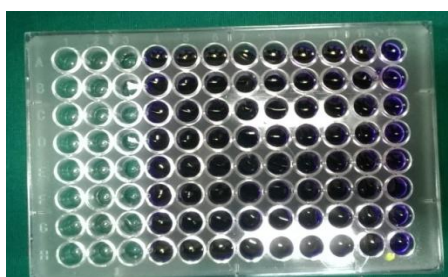


Figure 4 : Stained adherent bacteria in the presence of the test materials

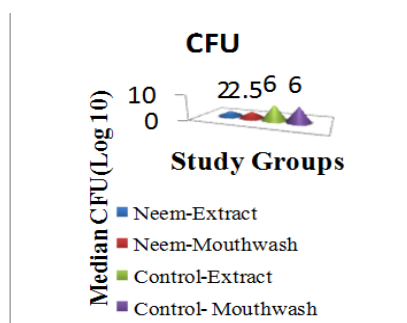


Figure 5 :Candida Albicans Colony Counts In The Presence Of Neem Crude Extract And Neem Mouthwash

Table 1 : Fungal biofilm formation according to optical density values. (Blank value – 0.120)

Mean of OD value at 540nm	Adherence	Biofilm Formation
< 0.120	Non	Non
0.120 – 0.240	Moderately	Moderate
> 0.240	Strong	High

Table 2 : Comparison of antifungal property of crude extracts with their corresponding mouthwashes
*P < 0.05 statistically significant

Test Material	Mean(SD)	Kruskal Wallis Test
Extract	2.33 (0.51)a,c	Chi-Square – 17.00 p-value- 0.00*
Mouthwash	2.50(0.54)b	
Control	6(0)	

^a Extract vs Control - P < 0.02

^b Mouthwash vs Control - P < 0.02

^c No statistically significant difference between Extract and Mouthwash

Table 3 : Effect of neem extract and neem mouthwash on *Candida albicans* adherence

Test Material	Mean OD (of 8 trials)	Adherence	Inference
Extract	0.7376	Strong	No effect on biofilm
Mouthwash	0.9963	Strong	No effect on biofilm

VI. Conclusion

Aqueous extracts of neem which can be made by simple household methods could prove to be a boon for the under-privileged sections of our society in terms of their use as a mouth rinse.

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