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Assessment of antimicrobial efficacy of neem, propolis, and chlorhexidine against oral microbiota - A comparative in vitro study

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Abstract

Background and objective: Dental caries is the most common and transmissible disease of childhood in the world and is a multifactorial disease caused due to the occurrence and interaction between dental biofilm and oral microflora. CHX digluconate is the most commonly used chemotherapeutic antimicrobial agent It has broad-spectrum antimicrobial activity without any systemic side effects but it has other local side effects. The extracts of active ingredients from medicinal herbs have gained the attention of researchers and were found to have great results against the formation of biofilm and antimicrobial activity, as these herbal medicinal ingredients have similar efficacy as chemotherapeutic agents without any side effects. So the purpose of this study is to compare and evaluate the efficacy of natural agents such as neem leaves extracts and propolis solution against oral microbiota.

Aim of the study: To assess and compare the antimicrobial efficacy of neem, propolis, and chlorhexidine against 4 different oral micro-organisms; S. mutans, S. oralis, L. acidophilus, C. Albicans.

Methodology: We determined the antimicrobial activity of Neem leaves extracts, propolis solution, and a chlorhexidine (gold standard) against pure cultures of Streptococcus mutans MTCC No 497, Streptococcus

oralis MTCC No. 2696, Lactobacillus acidophilus MTCC No. 10307, and Candida albicans MTCC No. 183 which were obtained and grown in selective culture media. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of both materials were evaluated by serial dilution and disc diffusion method, respectively.

Results: Concerning S. mutans, propolis showed higher disc diffusion- 21(IQR 5.5) followed by CHX-12(IQR 4), whereas, Neem and propolis showed no diffusion for L. acidophilus and Strept. Oralis. CHX showed 30(IQR 5) and 13(IQR 5.5) diffusion for L. acidophilus and Strept. Oralis respectively. Similarly, the CHX group showed higher diffusion- 17(IQR 2.5) as compared to neem- 5(IQR 3.5) and propolis- 4(IQR 1.5) for C. albicans. Statistical significant difference was seen among the groups with respect to S. mutans (p=0.002), L. acidophilus (p=0.001), Strept. Oralis (p=0.001) and C. albicans (p=0.009). In the serial dilution method, with regard to S. mutans, neem showed higher dilution – 500 (IQR 125) followed by CHX-250 (IQR 125), whereas, Neem and propolis showed no diffusion for L. acidophilus and Strept. Oralis. CHX showed 125(IQR 125) and 500(IQR 250) diffusion for L. acidophilus and Strept. Oralis respectively. The Propolis group showed higher diffusion- 500(IQR 125) as compared to neem- 500 and CHX- 125(IQR 125) for C. albicans. Kruskal-wallis test was applied to compare the serial dilution among the groups. Statistical significant difference was seen among the groups with respect to S. mutans (p=0.007), L. acidophilus (p=0.001), Strept. Oralis (p=0.001) and C. albicans (p=0.003). Intergroup comparison was done using post-hoc Mann Whitney test.

Conclusion: Propolis had maximum efficacy against streptococcus mutans while chlorhexidine had the best efficacy against the rest of the organisms which concludes that chlorhexidine is the best agent that can be used as a mouth wash for eliminating the organisms responsible for biofilm formation while on the other hand Neem and propolis can be used as an adjunct, but not as efficient as chlorhexidine.

Keywords: Biofilm, Neem, Chlorhexidine, Minimum inhibitory concentration, Minimum Bactericidal concentration, Propolis.

Introduction

Plaque removal is of utmost importance for control of dental caries and other associated diseases of oral cavity Error! Reference source not found.. Oral biofilms are primary cause of gingivitis, periodontitis, caries, halitosis and systemic disease [2]. The microflora includes primary colonizers as well as secondary colonizers, of which streptococcus mutans and streptococcus oralis are one of the early colonizers responsible for plaque formation. S. mutans is considered as bacteria with high cariogenic potential because of its acidogenicity and aciduricity, ability to form extracellular glucans from sucrose and conversion of sucrose to lactic acid. Apart from S. mutans other bacteria such as lactobacilli and candida albicans are responsible for plaque formation and maturation [1]. Although tooth brushing is the most effective way to clean teeth and to control dental plaque, mouth washes are widely used to complement tooth brushing [2].

Chlorhexidine (CHX) is the most popular type of mouthwash frequently prescribed by dentists and is the golden standard antiplaque in the treatment of gingivitis and periodontitis. Chlorhexidine is used to kill bacteria that cause infections. It is found in many medicines that are applied directly to the affected area of the body. It is an antiseptic treatment. It is used to treat and prevent infections. In general this drug is used where infections

of the skin, mouth or throat are present or may arise. The treatment and prevention of infections of minor cuts, grazes, burns and scalds, athlete's foot, blisters, stings and insect bites, spots, chapped or rough skin and minor infections of the mouth or throat Its side effects include staining, dysgeusia, painful mucous membranes and burning sensation during mouth washing. Therefore, its regular and extended use should be avoided [2].

In recent years, extracts of active ingredients from medicinal herbs have gained attention of researchers all over the world in an attempt to find an alternative to chemotherapeutic agents [1].

Azadirachta indica commonly known as Neem has been extensively used in Ayurveda, Unani, and Homeopathic medicines and has become a wonder tree of modern medicine. Neem leaves have been reported to posses antihyperglycemic, immunomodulatory, anti inflammator, antimalarial, antioxidant, antiviral, antimutagenic, anticarcinogenic, antibacterial and antifungal properties [6]. The neem leaves' antimicrobial properties have long been recognized to be beneficial to the skin and hair. Due to its antiplaque, anti-carious, and antibacterial effects, it has been widely used in different parts of the world as an oral hygiene tool[4].

Propolis, a natural resinous substance collected by honey bees to fill their hives cracks and crevices, is a complex chemical composition. Propolis was first used as a medicine by the Egyptians and use of it was continued by the Greeks and Romans. The major constituents of propolis are flavones, flavanones, and flavanols. It is used in homeopathic and herbal practice as an antiseptic, anti-inflammatory, antimycotic, and bacteriostatic agent[7]. The antibacterial effect of propolis is bactericidal by inhibiting their mobility. Propolis kills the fungi and also the viruses while the growth of the latter is also inhibited [4].

Mouthwashes are used in dentistry for prevention and curative purpose. Presently available mouthwashes are all medicated and effective. However, the affordability when it comes to a country like India and their side-effects has raised questions. Essential oils and botanical extracts have the potential to benefit oral health[3].

So the rationale of the in vitro study is to evaluate the antimicrobial effect of Neem leaves, propolis and chlorhexidine against oral bacteria such as S. mutans, S. oralis, lactobacillus acidophilus and Candida albicans.

Materials & Methods

The study was conducted at department of Microbiology, Kempagowda in stitte of Medical Science, Bengaluru. Microbial Type Culture Collection (MTCC) Strains of S. mutans (MTCC No: 497), S. oralis (MTCC No:2696), L. acidophilus (MTCC No: 10307), C. albicans (MTCC No: 183) were used in this study. MTCC strains were procured from the Institute of Microbial Technology (IMTECH), Chandigarh. Commercially available CHX gluconate 0.2%, freshly prepared neem extracts, freshly prepared propolis were the antimicrobial agents used in this study.

Methodology

MTCC strains were procured from the Institute Of Microbial Technology (IMTECH), Chandigarh

Specimen preparation

Preparation of neem leaf extract: Mature fresh Azadirachta indica leaves were collected and leaves were washed in sterilized distilled water and weighed in a sterile disposable cup. 25gms of fresh neem leaves were added to 50ml of absolute ethanol. Mixture is macerated for 1-2 mins using a mortar and pestle.

Preparation of aqueos solution of propolis: A 1:60 aqueous solution of propolis is prepared by dissolving 1 capsule of propolis (1000mg) which is available commercially in to 60 ml of sterile warm normal saline.

The capsule was mixed thoroughly in a glass beaker to obtain the propolis solution.

Commercially available 0.2% chlorhexidine was used

Preparation of microbial inocula

A direct colony suspension of each bacterial isolate was prepared in brain–heart infusion broth and turbidity was adjusted to 0.5 McFarland Standard for all the bacteria.

Determination of minimum inhibitory concentration of antimicrobial agents by serial dilution method

To determine the antibacterial activities, serial dilutions of Neem, Propolis and chlorhexidine were prepared in brain–heart infusion broth. S. mutans, S. oralis, L. acidophilus, and C. albicans strains were suspended in brain–heart infusion broth. About 1000 µg/ml concentration of Neem, Propolis and CHX were diluted in twofold serial dilution manner. So after each dilution the concentration of the antimicrobial agents becomes half of the previous dilutions. Five microliter of each bacterial inocula were added to the test tube containing antimicrobial agents, respectively. The test tubes were shaken properly and incubated at 37°C for 24 h. Minimum inhibitory concentration (MIC) was determined by visual inspection and confirmed by spectrophotometry. The least dilution with absence of bacterial growth was considered as most effective. Procedure was repeated five times to minimize error.

Determination of minimum bactericidal concentration using agar disc diffusion method

Agar disc diffusion method is used to determine the antibacterial activity of Neem, Propolis and CHX. Fifty microliter of bacterial aliquots from inoculum were spread evenly on culture plates with sterile swab in order to achieve an even bacterial lawn culture. Sterile diffusion discs of diameter 6 mm, soaked in different concentrations of Neem, propolis and chlorhexidine were kept at an equal interval and incubated at 37°C for 24 h in an aerobic condition. Petri plates were observed for zone of inhibition, which were measured using zones scale in millimeters. The discs with largest zone of inhibition were considered as most effective. The tests were repeated five times to minimize errors.

Results

Data was subjected to normalcy test (Shapiro-wilk test). Data showed non-normal distribution. Hence non-parametric tests (Kruskal-Wallis with post-hoc Mann-Whitney) were applied.

Table 1 shows the comparison of the antimicrobial efficacy of neem, propolis and CHX with respect to S. Mutans, L. acidophilus, Strept. Oralis and C. albicans. With regard to S. mutans, propolis showed higher disc diffusion- 21(IQR 5.5) followed by CHX-12(IQR 4), whereas, Neem and propolis showed no diffusion for L. acidophilus and Strept. Oralis. CHX showed 30(IQR 5) and 13(IQR 5.5) diffusion for L. acidophilus and Strept. Oralis respectively. Similarly, CHX group showed higher diffusion- 17(IQR 2.5) as compared to neem-5(IQR 3.5) and propolis- 4(IQR 1.5) for C. albicans. Kruskal-Wallis test was applied to compare the disc diffusion among the groups. Statistical significant difference was seen among the groups with respect to S. mutans (p=0.002), L. acidophilus(p=0.001), Strept. Oralis (p=0.001) and C. albicans(p=0.009).

Inter group comparison was done using post-hoc Mann Whitney test. Statistical significant difference was seen between neem and propolis with respect to s. mutans whereas neem V/s CHX, Propolis V/s CHX showed statistical significant difference with respect to all the microorganisms (S. mutans, L. acidophilus, Strept. Oralis and C. albicans).

Table 3 shows the comparison of the antimicrobial efficacy of neem, propolis and CHX with respect to S. Mutans, L. acidophilus, Strept. Oralis and C. albicans in

serial dilution method. With regard to S. mutans, neem showed higher dilution – 500 (IQR 125) followed by CHX-250 (IQR 125), whereas, Neem and propolis showed no diffusion for L. acidophilus and Strept. Oralis. CHX showed 125(IQR 125) and 500(IQR 250) diffusion for L. acidophilus and Strept. Oralis respectively. Propolis group showed higher diffusion-500(IQR 125) as compared to neem- 500 and CHX-125(IQR 125) for C. albicans. Kruskal-Wallis test was applied to compare the serial dilution among the groups. Statistical significant difference was seen among the groups with respect to S. mutans (p=0.007), L. acidophilus (p=0.001), Strept. Oralis (p=0.001) and C. albicans (p=0.003).

Inter group comparison was done using post-hoc Mann Whitney test. Statistical significant difference was seen with neem v/s propolis, Propolis V/s CHX with respect to s. mutans whereas neem V/s CHX showed statistical significant difference with respect to all the microorganisms (S. mutans, L. acidophilus, Strept. Oralis and C. albicans).

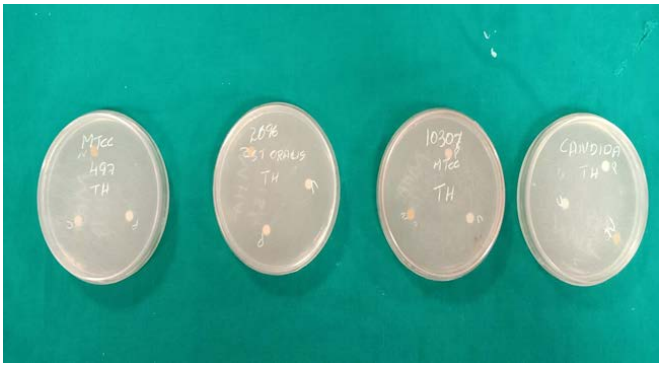


Figure 1: determination of minimum bactericidal concentration using agar disc diffusion method.

Figure 2: determination of minimum inhibitory concentration of antimicrobial agents by serial dilution method.

Figure 3: zone of inhibition for streptococcus oralis.

Figure 4: zone of inhibition for streptococcus mutans.

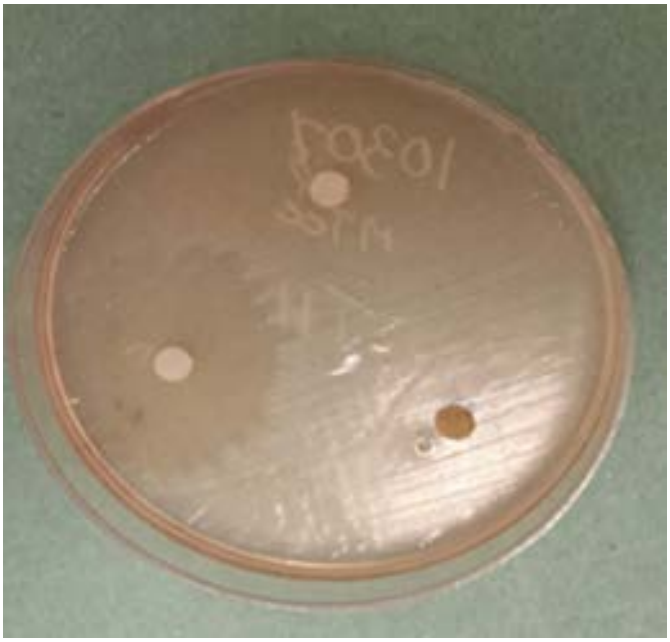


Figure 5: zone of inhibition for lactobacillus acidophilus.

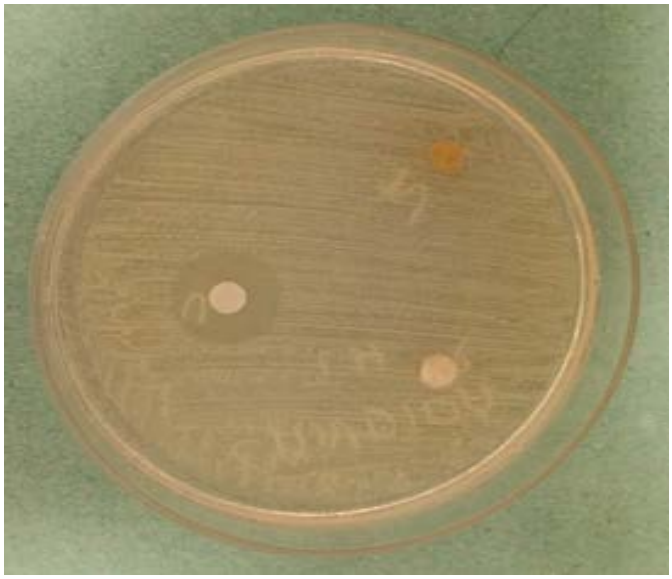


Figure 6: zone of inhibition for candida albicans.

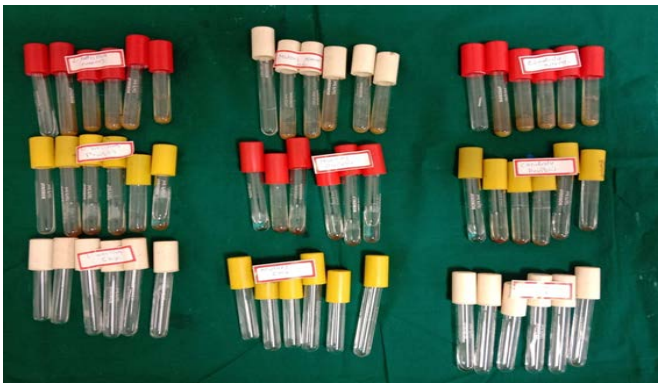


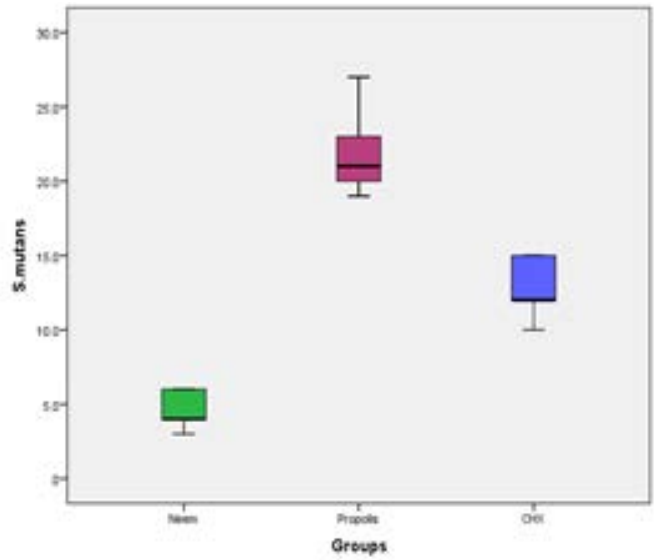
Figure 7: Final outcome after serial dilution method.

Table 1: Comparison of the disc diffusion method among the groups using Kruskal Wallis

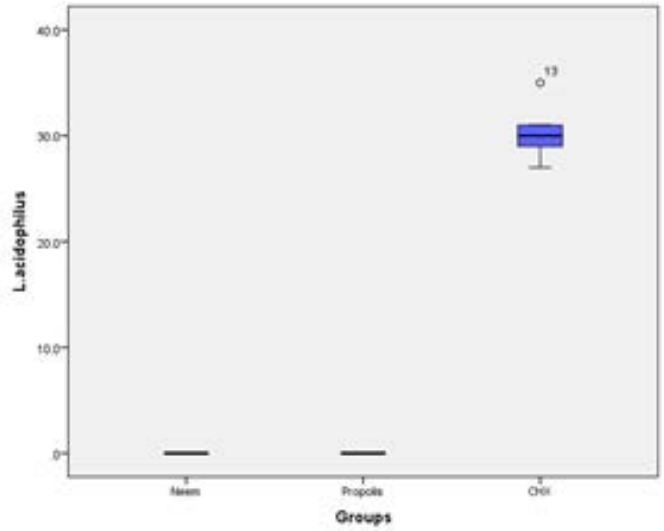
Micro-organisms	Groups	Minimum	Maximum	Median	IQR	P value
S. Mutans	Neem	3.0	6.0	4.0	2.5	0.002*
	Propolis	19.0	27.0	21.0	5.5	
	CHX	10.0	15.0	12.0	4.0	
L. acidophilus	Neem	0	0	0	0	0.001*
	Propolis	0	0	0	0	
	CHX	27	35	30	5	
Strept. Oralis	Neem	0.0	0.0	0.0	0.0	0.001*
	Propolis	0.0	0.0	0.0	0.0	
	CHX	9.0	16.0	13.0	5.5	
C. albicans	Neem	2.0	7.0	5.0	3.5	0.009*
	Propolis	4.0	6.0	4.0	1.5	
	CHX	14.0	18.0	17.0	2.5	

*Significant

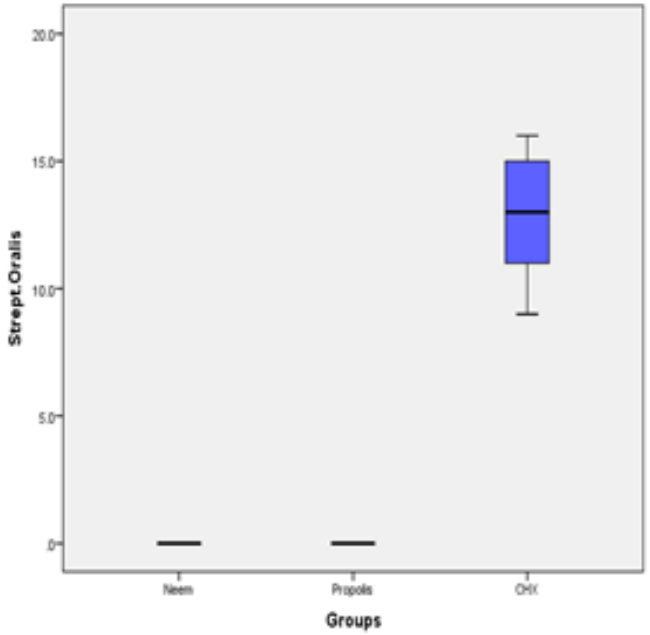
Graph 1: Comparison of the disc diffusion method among the groups using Kruskal Wallis.



Graph 2: Graphical representation of comparison of efficacy of Neem, propolis and chlorhexidine against S. Mutans in disc diffusion.



Graph 3: Graphical representation of comparison of efficacy of Neem, Propolis and Chlorhexidine against L. Acidophilus in disc diffusion.



Graph 4: Graphical representation of comparison of efficacy of Neem, Propolis and Chlorhexidine against S. Oralis in disc diffusion.

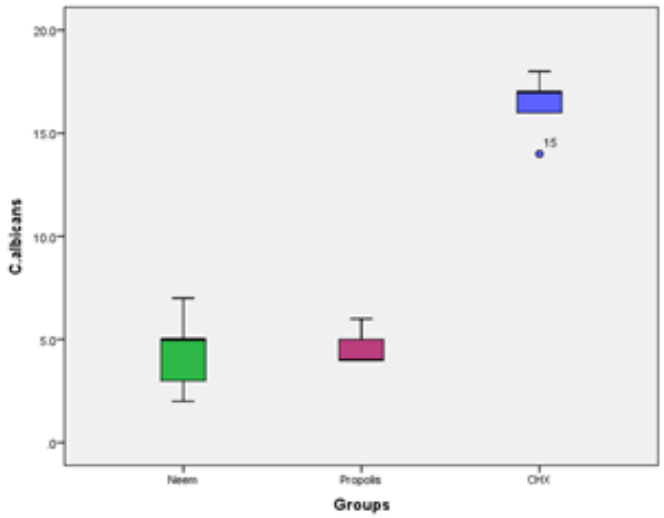


Table 2: inter-group comparision of disc diffusion using post-hoc Mann Whitney test.

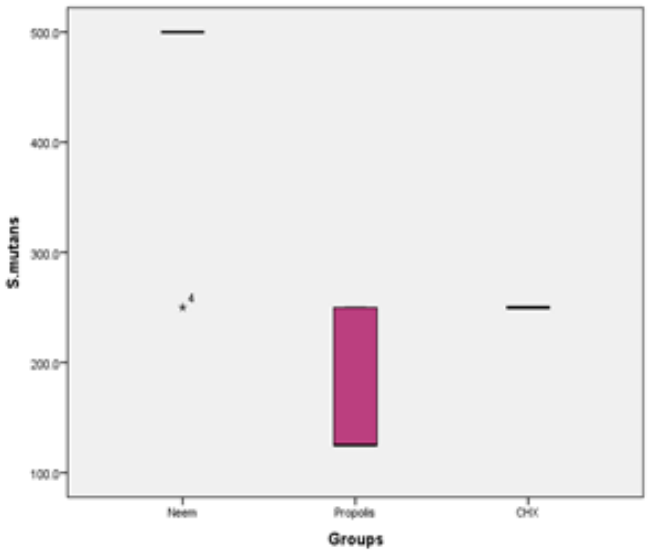
		S. mutans	L. acidophilus	Strept. Oralis	C. albicans
Neem V/s Propolis	U value	0.00	12.50	12.50	12.00
	p value	0.009*	1.000	1.000	0.915
Neem V/s CHX	U value	0.00	0.00	0.00	0.00
	p value	0.008*	0.005*	0.005*	0.009*
Propolis V/s CHX	U value	0.00	0.00	0.00	0.00
	p value	0.009*	0.005*	0.005*	0.008*

*p value set significant at 0.05/3=0.016

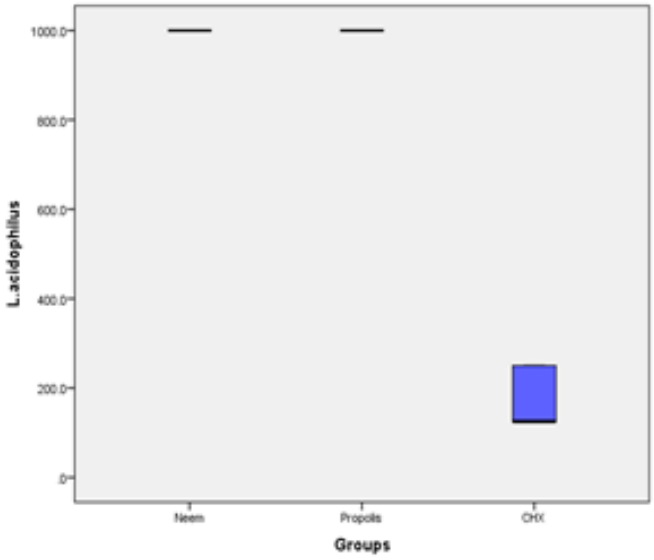
*Significant

Table 3: Comparison of the serial dilution method among the groups using Kruskal Wallis test.

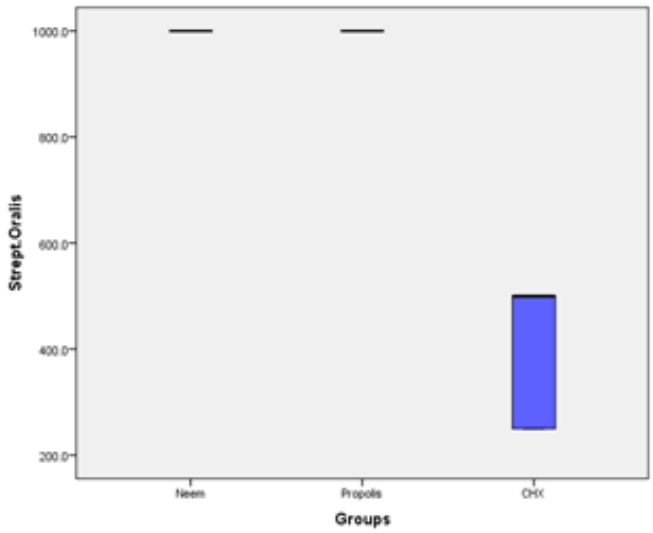
Micro-organisms	Groups	Minimum	Maximum	Median	IQR	p value
S. Mutans	Neem	250.0	500.0	500.0	125.0	0.007*
	Propolis	125.0	250.0	125.0	125.0	
	CHX	250.0	250.0	250.0	-	
L. acidophilus	Neem	1000	1000	1000	-	0.001*
	Propolis	1000	1000	1000	-	
	CHX	125.0	250.0	125.0	125.0	
Strept. Oralis	Neem	1000	1000	1000	-	0.001*
	Propolis	1000	1000	1000	-	
	CHX	250.0	500.0	500.0	250.0	
C. albicans	Neem	500	500	500	-	0.003*
	Propolis	250.0	500.0	500.0	125.0	
	CHX	125.0	250.0	125.0	125.0	



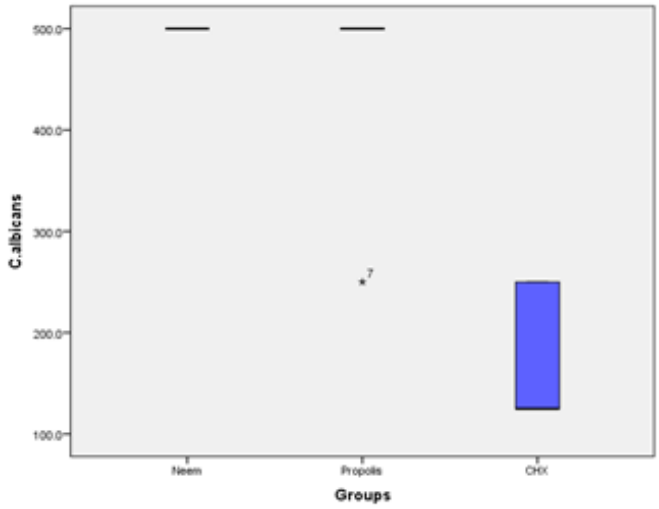
Graph 5: Graphical representation of comparison of efficacy of Neem, Propolis and Chlorhexidine against S. Mutans in serial dilution.



Graph 6: Graphical representation of comparison of efficacy of Neem, Propolis and Chlorhexidine against L. Acidophilus in serial dilution.



Graph 7: Graphical representation of comparison of efficacy of Neem, Propolis and Chlorhexidine against S. Oralis in serial dilution.



Graph 8: Graphical representation of comparison of efficacy of Neem, Propolis and Chlorhexidine against C. Albicans in serial dilution.

Discussion

Plaque is the principal causative factor in gingival and periodontal diseases the most rational methodology towards the prevention of periodontal diseases would be regular effective removal of plaque by personal oral hygiene[5.]

The most diverse collections of oral microorganisms are found in the biofilms on teeth (dental plaque).[36] The oral

microbiota represents an important part of the human microbiota, and includes, according to different references, several hundred to several thousand diverse species.[22] Major etiological agent of human dental caries, S. mutans lives primarily in biofilms on the tooth surfaces, the so-called dental plaque. Strains of S. mutans produce up to three glucosyltransferases, Gt fB, -C and -D, that utilize the glucose moiety of sucrose as the substrate to synthesize glucose polymers of glucans.[38]

Lactobacillus was the first known microorganism associated with dental caries development. It is gram's positive, rod shape facultative anaerobic, non-spore forming bacilli. They appear during the first years of a child's life and are present in high numbers in saliva, on the dorsum of the tongue, mucous membranes, the hard palate, in dental plaque and, in fewer numbers, on tooth surfaces.[39]

Candida albicans and distinct Candida species are present in the mouth of up to 75% of the populace without any symptom of disease. This fungus is an opportunistic and decisive human pathogen residing as a commensal in the genitourinary tract, the gastrointestinal tract, on the skin as well.[40]

Chlorhexidine is considered as the gold standard among mouth rinses due to its property of increased persistence in the oral cavity (substantivity) that prolongs the antimicrobial action of this mouth rinse. However, side effects like mucosal irritation, burning sensation and altered taste perception have been reported with short-term (1 week) usage of nonalcohol-based 0.2% chlorhexidine mouthrinse.[16] chlorhexidine gluconate, the uptake by bacteria and yeasts was shown to be extremely rapid, with a maximum effect occurring within 20 s. Damage to the outer cell layers takes place (but is insufficient to induce lysis or cell death.[41] The agent then crosses the cell wall or outer membrane, presumably by passive diffusion, and subsequently attacks the bacterial cytoplasmic or inner membrane or the yeast plasma membrane.[41] Clinicians frequently administer CHX mouth rinses in order to inhibit the development of plaque However, the cytotoxic characteristics and side effects of CHX are the basic disadvantages that limit the administration of this pharmaceutical so some manufacturers are in an attempt to produce natural oral care products from plant extracts in order to avoid the side effects of synthetic products.[19]

Neem leaf is rich in antioxidants and helps to boost the immune response in gum and tissues of the mouth. Neem offers a good remedy for curing mouth ulcers, tooth decay and acts as a pain reliever in toothache problems. The antimicrobial effects of Neem have been reported against S. mutans and S. faecalis. Aqueous extract of Neem stick and the Gallo tannin-enriched extract from Mel aphis chinensis inhibited insoluble glucan synthesis and results in bacterial aggregation. It reduces the ability of streptococci to colonize tooth surfaces.[31] The principle constituents of neem includes [Carbohydrate], Crude protein, Crude fiber, Fat, Ash, Moisture, Amino acids, Glutamic acid, Tyrosine, Aspartic acid, Alanine, Proline, Glutamine Minerals, Calcium, Iron, Phosphorus, Thiamine, Niacin, Vitamin C, Carotene.[41]

Propolis -a natural resinous material produced by honey bee- has recently been proposed as an alternative anti-plaque mouthwash. The chemical composition of propolis includes 50% resin, 30% wax, 10% aromatic and essential oils, 5% pollen and 5% other constituents. Propolis has shown strong antimicrobial and anti-inflammatory properties, making it a good candidate for treatment and prevention of oral diseases.[18] the antibacterial and antifungal activities of propolis are

mainly due to flavonones, flavones, phenolic acids esters and prenylated p-coumaric acids .[42]

Even though many studies are conducted individually of various natural oral care products on different bacterias, not many studies are reported on the comparison of natural oral products for its efficacy on multiple oral microorganisms. So, the present study was conducted to compare the antimicrobial efficacy of Neem, Propolis and Chlorhexidine against streptococcus mutans, lactobacillus acidophilus, streptococcus oralis and candida albicans.

The results of our present study showed that neem extracts had a highest zone of inhibition of 6mm and minimum of 3mm with an average of 4 mm in disc diffusion method and in serial dilution of the neem extracts it showed antibacterial efficacy at an average of 500 microgram/ml against Streptococcus Mutans.

The antibacterial property of neem could be possible due the constituents in neem extracts which inhibits bacterial growth. Phytol, which is a diterpenes, was found to be around 16.8%. Phytol can decrease the level of bacterial counts in vivo. Dodecanoic acid or lauric acid, a type of medium-chain fatty acids, was also obtained but in a small amount. This constituent reduces biofilm formation in vitro and restrains oral bacterial growth.[34]

In our study neem exhibited no zone of inhibition against lactobacillus acidophilus and bacterial turbidity was seen in highest concentration of the solution which is 1000 microgram/ml which indicated that neem extracts are ineffective against L. Acidophilus. So our study does not co relate with the study done by Tasa Narong T etal (2021) who stated that neem paste is effective against cariogenic bacteria such as Lactobacillus Acidophilus,[34] but supports the study done by Lakshmi et. Al who stated that neem leaves extracts are effective against Stretococcus Mutans, Mitis and Sanguis but not effective against L. Acidophilus.[31]

Not many literatures have been reported on the efficacacy of Neem leaves or extracts on its efficacy against S. Oralis. Mostly the studies were conducted against Mutans, Feacalis and Mitis and has shown positive results against it. In our present study Neem leaves extracts didn't show any zones of inhibition against Streptococcus Oralis in disc diffusion method and on serial dilution of neem extracts bacterial turbidity or growth was seen at 1000 microgram/ml which was highest concentration of solution used indicating that Neem extracts are not effective against Streptococcus oralis.

Ethanolic and aqueous extract of Neem leaf showed significant anti-candidial effect against C. albicans. A clinical study demonstrated the effects of the leaf aqueous extract from Azadirachta indica (Neem) on adhesion, cell surface hydrophobicity and biofilm formation, which may affect the colonization by Candida albicans.[31] The present study demonstrated an highest zone of inhibition of 7mm and a lowest of 2mm and an average of 5mm for neem leaves extraxts against candida albicans and in serial dilution it showed no bacterial turbidity at an average of 500 microgram/ml which indicated that Neem leaves has satisfactory antifungal efficacy against Candida Albicans. This corelates with the study done by Dikshitha Ray Barua et. Al (2021) who demonstrated that 15% w/w of neem leaf extract showed a maximum inhibition of 21 mm after 24 hours and minimum of 17 mm after seven days.[23] Quercetin and ß-sitosterol, were the first polyphenolic flavonoids purified from neem fresh leaves and were known to have antibacterial and antifungal properties. The same authors purified the active fractions of neem organic extracts using HPLC and found that their content

of major compounds such as 6-deacetylnimbin, azadiradione, nimbin, salannin and epoxy-azadiradione were with appreciable active when bio assayed on many pathogenic fungi.[43]

Propolis reduces human dental plaque accumulation and its insoluble external polysaccharide content. Its antimicrobial activity is attributed to the presence of flavonoids and terpenoids.[29.] in our present study 1: 60 aqueous solution of propolis was prepared by dissolving 1 tablet of propolis (1000mg) in 60 ml of sterile warm normal saline and on testing its antibacterial propert against S. Mutans its showed a maximum zone of inhibition of 27mm and a minimum of 19mm with an average of 21mm in disc diffusion method and on determination minimum bactericidal concentration using serial dilution method it showed an average of 125 microgram/ml indicating that Streptococcus Mutans is highly sensitive to propolis. This corelates with the study done by Mahabala et. Al (2016) stated that propolis is effective against gram positive organisms.[35.]

The mechanism of activity of propolis against microorganisms is very complex. Some components present in propolis extracts such as flavonoids (quercetin, galangin, and pinocembrin) and caffeic acid, benzoic acid, and cinnamic acid probably act on the microbial cytoplasmic membrane or cell wall site, causing functional and structural damages. The antibacterial activity could also be related to the synergistic effect of all components than an individual compound.[35.]

In our study propolis didn't exhibit any zone of inhibition against lactobacillus acidophilus in agar disc diffusion method and bacterial turbidity was seen at the highest concentration of propolis in serial dilution which clearly indicated that propolis doesn't have any antibacterial efficacy against lactobacillus acidophilus. Not many literatures were found on propolis having inhibitory effect on L. Acidophilus only Mahabala et. Al (2016) have stated that propolis have bacteriostatic effect but no bactericidal effect against L. Acidophilus.[35.]

our study didn't exhibit any zone of inhibition for propolis against streptococcus oralis in disc diffusion method and bacterial turbidity was seen at 1000 microgram/ml in serial dilution method which clearly indicated that Streptococcus oralis is not sensitive against propolis. No literatures were found supporting the antibacterial efficacy of propolis against S. oralis even though Izabela et. Al (2019) stated that propolis acts on both against Gram-positive and Gram-negative, as well as aerobic and anaerobic bacteria. The activity of propolis depends on chemical composition such as flavonoids and esters of phenolic acids.[44.]

Propolis exhibits antimicrobial, anti-inflammatory, healing, anesthetic and cariostatic properties. According to Takaisi-Kikuni and Schilcher,[25] it prevents fungal cell division and also breaks down fungal cell wall and cytoplasm similar to the action of some antibiotics.

In our present study propolis exhibited good antifungal efficacy in both serial dilution and disc diffusion method against candida albicans. In disc diffusion it showed a minimum zone of inhibition of 4mm and a maximum of 6mm with an average of 4 mm and in serial dilution no bacterial turbidity was seen at an average of 500 microgram/ml which indicates that candida albicans are sensitive to propolis. This corelates with the study done by Flavia k et. Al (2016) who stated that Brazilian propolis is highly efficient against candida albicans infections and has been attributed to the synergistic activity between its various potent biological ingredients, mainly phenolic and flavonoid compounds.[45.] The flavonoids constitute a very important class of polyphenols, widely present in

propolis and the greater part of propolis biological activity is attributed to polyphenols.[45.]

Chlorhexidine gluconate is, to date, the most thoroughly studied and the most effective anti-plaque and anti-gingivitis agent. The most commonly prescribed concentration is 0.2% and is considered as the gold standard of all mouth washes.[7.] so in this study Chlorhexidine was taken as the bench mark antimicrobial agent for the comparison of efficacy of Neem and Propolis.

In our present study chlorhexidine showed a mean zone of inhibition of 12mm with a maximum of 15mm and a minimum of 10mm against streptococcus mutans in disc diffusion method and in serial dilution method it showed no bacterial turbidity at an average of 250 microgram/ml. so after comparison of three antimicrobial agents, propolis showed maximum efficacy against Streptococcus mutans followed by Chlorhexidine and neem leaves extracts. This corelates with the study done by Akca et. Al (2016) who stated that propolis is highly efficient against Streptococcus group and its antibacterial effects against mutans could be complex, leading to the disintegration of the cytoplasm, cytoplasmic membrane and cell wall, partial bacteriolysis, and inhibition of protein synthesis.[19.]

On the study of chlorhexidine against Lactobacillus acidophilus it showed a minimum zone of inhibition of 27mm and a maximum of 35mm with an average of 30mm in disc diffusion method and in serial dilution of chlorhexidine it showed absence of bacterial turbidity at an average of 125 microgram/ml which indicated that lactobacillus acidophilus is highly sensitive to chlorhexidine. In our study neem leaves and propolis didn't exhibit any zone of inhibition in disc diffusion method and no antibacterial efficacy was seen in serial dilution method. So on comparing with Neem and propolis Chlorhexidine is the best antimicrobial agent against Lactobacillus Acidophilus.

In this study chlorhexidine exhibited a mean zone of inhibition of 13mm with a minimum of 9mm and a maximum of 16mm in disc diffusion method and in serial dilution it showed absence of bacterial growth at an average of 500 microgram/ml against streptococcus oralis which clearly indicated that streptococcus oralis is highly sensitive to chlorhexidine and on comparison with neem leaves and propolis, chlorhexidine exhibited the best antibacterial efficacy against streptococcus mutans as neem leaves and propolis was completely ineffective against it.

Our present study showed good antifungal property for chlorhexidine against candida albicans as it showed a mean zone of inhibition of 17 mm with a maximum of 18mm and a minimum of 14mm in disc diffusion method and absence of fungal growth at an average of 125 microgram/ml concentration of chlorhexidine which clearly indicated that candida albicans are sensitive to chlorhexidine. On comparison with neem and propolis chlorhexidine exhibited the maximum antifungal property against candida albicans followed by neem and thirdly propolis.

After inter comparison of all three antimicrobial agents the results showed that chlorhexidine is the most effective antimicrobial agent against S. mutans, S. oralis, L. Acidophilus & C. Abicans as it showed zones of inhibition against all organisms used in the study whereas neem and propolis showed sensitivity only against S. mutans & C. Albicans.

So, the results of this study co relates with the study done by Shradha et al (2017) who stated that Chlorhexidine is a positively-charged molecule that binds to the negatively-charged sites on the cell wall that destabilizes the cell wall and interferes with osmosis

which is responsible for its anti-bacterial property. The antifungal property is by impairing the integrity of the cell wall and the plasma membrane entering the cytoplasm resulting in leakage of cell contents and cell death.[27.]

So, after analyzing the results of the study we can conclude that chlorhexidine is the best antimicrobial agent in overall against oral microbiota. Neem and Propolis exhibited antimicrobial properties against S. Mutans and C. Albicans and among that propolis exhibited maximum efficacy against S. mutans in comparison with Neem and Chlorhexidine.

Conclusion

The present invitro study was conducted to asses and evaluate the antimicrobial efficacy of neem propolis and chlorhexidine against Streptococcus mutans, Streptococcus oralis, Lactobacillus Acidophilus and Candida Albicans.

Within the limitations of the study it was found that propolis had maximum efficacy against streptococcus Mutans, Neem extracts showed satisfactory efficacy against mutans and albicans while chlorhexidine had excellent efficacy against all the organisms.

So in our present study we can conclude that chlorhexidine is the best agent that can be used as a mouth wash for eliminating the organisms responsible for biofilm formation while on the other hand Neem and propolis can be used as an adjunct, but not as efficient as chlorhexidine.

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