ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4

Journal of Coastal Life Medicine

Evaluation of Antimicrobial Efficacy of Andrographis Paniculate (Nilavembu Decoction) against Various Microbial Biofilm Formed on the Tooth Substrate -An in Vitro Study

Received: 26 October 2022, **Revised:** 29 November 2022, **Accepted:** 31 December 2022

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Keywords

Antimicrobial efficacy, Nilavembu decoction, biofilm model, Minimum Inhibitory Concentration, Biofilm, Zone of Inhibition

Abstract

Aim: To evaluate the antimicrobial efficacy of Andrographis paniculata (nilavembu decoction) against various microbial biofilm formed on tooth substrate and to compare it with gold standard chlorhexidine mouthwash

Settings: Laboratory setting

Design: An in vitro experimental study

Methods and Material: An experimental invitro study was conducted among six test pathogens that include 3-gram positive bacteria, 2-gram negative bacteria and fungi. The anti-microbialproperty was assessed by the Zone of Inhibition, Minimum Inhibitory Concentration and the number of colonies formed on the tooth substrate on 3rd day and 5th day was assessed as Colony Forming Units per milliliter (CFU/ml) and comparison between the nilavembu decoction, chlorhexidine and saline was done.

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

Statistical analysis used: All the microbiological procedures were repeated thrice and the mean and standard deviation was calculated. Kruskall Wallis ANOVA was used for comparison of number of colonies forming units of various microorganisms between three groups followed by Mann Whitney U test as post hoc test for pair wise comparison

Results: The results showed that nilavembu decoction has showed larger zone of inhibition(16.1mm) compared to chlorhexidine (14.3mm) and it was statistically significant (P<0.05) Nilavembu decoction also showed its effect at lesser concentration against Candida albicans (50mg/ml) and Enterococcus faecalis (25mg/ml) and reduced number of microbial colonies was found on 3rd day microbial biofilm compared to 5th day biofilm which shows that nilavembu decoction have better effect on immature biofilm.

Conclusions: Nilavembu decoction showed better antimicrobial effect against *Candida albicans*, *Enterococcus faecalis* and *Streptococcus mutans* compared to other test pathogens.

1. Introduction

The field of medicine is a unique field with numerous advancements that commence every day. But even with such tremendous growth, certain infection such as dental caries poses a challenge to the general public. This bestows an immense burden to health care services around the world of which developing countries are the most affected. [1] Microorganisms appear in each and every corner of human life and it affects every aspects of human life.[2] The oral cavity possesses a number of features such as saliva, pH which make it a distinct habitat for a menagerie of microorganisms.[3]The emergence and dissemination of antibiotic Resistance as well as the evolution of new strains of microorganisms have become a significant public health threat as there are fewer antimicrobial agents available for the infections caused by microorganisms.[4]

Biofilms, or surface –attached communities of cells represent a bacteria's common lifestyle. These biofilms are associated with a wide range of persistent infections and reported to show increased resistance to many antimicrobial agents. [5]

According to the World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs. Andrographis paniculata belongs to the family Acanthaceae. Andrographis paniculata is commonly known as Kalmegh, "King of bitters" in English, locally in Tamil Nadu, India it is called as Nilavembu.Traditionally, the plant was used as an infusion, decoction, or powder separately or incombination with other medicinal plants. The decoction is prepared by boiling the powder with water.[6]

Nilavembu decoction is recommended for all types of fevers and infections. Numerous studies have shown that nilavembu is effective against several viruses and bacteria, but studies against the microorganisms responsible for oral diseases are scarce.

Therefore, assessing the antimicrobial potential of a promising herbal extract

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

(which is popular, economical and easily available) on a biofilm model is essential to prove its clinical utility. Thus, an invitro study was conducted with an aim to evaluate the antimicrobial efficacy of Andrographis paniculata (nilavembu decoction)

against various microbial biofilm formed on tooth substrate and to compare it with gold standard chlorhexidine mouthwash.

2. Methodology

An experimental study invitro study was conducted to assess the antimicrobial efficacy of Andrographis paniculata (nilavembu decoction). Bacterial and fungal isolates were used for the study. They includedEnterococcus faecalis, Streptococcus mutans, and Streptococcus mitis, for gram positive bacteria and Porphyromonasgingivalis, Campylobacter rectus for gram negative bacteria. Fungal isolates used included Candida albicans. Standard microbial strains (MTCC) were used. All the microbial strains were suspended in the nutrient broth at 37^0C for 48 hours. Brain Heart Infusion Agar (BHI) and Sabouraud Dextrose Agar (SDA) were used for testing the antibacterial and antifungal activity respectively.

Preparation of Nilavembu decoction

The Nilavembukudineer powder was procured from Government Siddha Medical College. The preparation of decoction was done according to the instructions given by Health and Family Welfare department. It was prepared by Boiling 10 grams of nilavembukudineer powder in 100ml of water until it gets reduced to half. The decoction was separated using sieve. The concentration of nilavembu decoction decided was 10%.

Microbiological procedures:

Agar –well diffusion test

Antimicrobial activity was carried out by Agar Well Diffusion Method. Sterile Brain Heart Infusion Agar (BHI) for test bacteria and Sabouraud Dextrose Agar (SDA) for Candida albicans were prepared and cultures of microorganisms were spreaded on the solid plate. A total of three wells were made in each of the six nutrient agar plate using sterile cork borer (6mm in diameter). 10% Nilavembu decoction is considered as a test solution and 0.2% Chlorhexidine is used as a positive control and saline as a negative control. All the three test agents were poured in each well of six agar plates. The agar plates were incubated at 37^0C for 24 hours.

Measurement and Interpretation of Zones of Inhibition:

After incubation period, plates were removed and zones of inhibition were measured using scale ruler in millimeter (mm). Clear zones of inhibition indicated the susceptibility while absence of such zones showed resistance or no inhibitory effect of the test solutions on the microorganisms. It was tested three times and the mean values were recorded.

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

Minimum Inhibitory Concentration

The concentration of drug required to produce the effect is defined as the Minimum Inhibitory Concentration.

For determining Minimum Inhibitory Concentration, the respective bacterial and Candida albicans strains from the stock were revived by plating on Brain Heart Infusion agar (BHI). After 18 hours of incubation for bacteria and 48 hours of incubation for Candida at 37^0C, isolated colonies were selected and the identities of the organisms were confirmed by gram staining. Isolated colonies were transferred to sterile Brain Heart Infusion broth and Sabouraud dextrose broth for the bacterial and Candida strain and incubated at respective temperatures. An aqueous solution of 10% concentration was prepared from the Nilavembu powder as the stock solution.100 µl of the broth was added to each well. In the first well containing 100µl of broth, 100µl of test solutions were added. After mixing well 100µl was transferred to the second well and this was continued till the last (8^{th}) well. From the last well 100µl final solution was discarded. Dilutions of the extract prepared were 100mg, 50mg, 25mg, 12.5mg, 6.25mg, 3.125mg, 1.625mg, 0.725mg. 10µl of diluted extract sample were added to the plate. Following the 18 to 24 hours of incubated exposure, the plates containing the challenge microorganisms, were examined to determine the highest dilution of product (and conversely, the lowest concentration of product) that completely inhibits growth of microorganisms, as determined by the naked eye. The dilution value (and/or product concentration value) was recorded as the Minimum Inhibitory Concentration value.

Assessing the biofilm formation:

Selection of the teeth:

54 single rooted human mandibular premolars extracted due to orthodontic reason with fully formed apices and free of caries, calculus was taken. The teeth were cleaned of superficial debris and tissue tags and stored in normal saline to prevent dehydration before use and then biomechanically prepared, Teeth samples were randomly divided into three groups

3 tooth specimens were used for each microorganism making it a total of 18 specimens for each group samples

Group A-Nilavembu decoction

Group B-Chlorhexidine mouthwash

Group C-Saline

The samples were autoclaved at 121^0cfor a period of 20 minutes.

The sterilized tooth was stored in 5ml falcon tubes.

3ml of culture medium (Brain Heart Infusion broth for bacteria and Sabouraud Dextrose broth for Candida albicans) were added to the vials containing tooth samples and inoculated with 2.5 microlitre of microbial suspension and incubated at 37^0C for 3 days.

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

The culture medium was replaced every 24 hours to prevent nutrition depletion.

After 3 days, 3ml of test solutions was added in each tube.

3ml of Nilavembu decoction was added to each tube in group A

3ml of Chlorhexidine was added to each tube in group B

3ml of saline was added to each tube in group C

For quantitative analysis, after 10 minutes of adding the test solutions, the contents of the tubes were evacuated and 2ml of sterile saline was added and after severely vortexing (about one minute), 10 microlitre of solutions was swabbed in consecutive plates. After 18 hours of inhibition for all the bacteria and 48 hours of incubation for Candida albicans at 37^0C, the number of colonies was counted by dividing the plates into four parts and the colonies were counted manually for one part and it was multiplied by four to derive Colony Forming Units per milliliter (CFU/ml). The same procedure was done for 5 days using the same teeth after sterilization. The counting was repeated thrice and the mean and standard deviation was calculated.

STATISTICAL PROCEDURES:

Statistical analyses were performed using Statistical Package for Social Sciences software (SPSS version 19, IBM, USA).

1. The number of colonies forming units of various microorganisms in each group was expressed as mean and standard deviation.

2. Kruskall Wallis ANOVA was used for comparison of number of colonies forming units of various microorganisms between three groups followed by Mann Whitney U test as post hoc test for pair wise comparison.

P value of < 0.05 was considered to be statistically significant.

3. Results

In the present study antimicrobial efficacy of nilavembu decoction against various microbial biofilm formed on the tooth substrate was assessed and compared with the positive and negative control.

Comparison of antimicrobial activity of nilavembu decoction with positive and negative control against the test pathogens

Compared to chlorhexidine and saline, nilavembu decoction showed larger zone of inhibition against*Streptococcus mutans, Enterococococus faecalis* and *Candida albicans*with maximum zone ofinhibition showed against*Candida albicans*whereaschlorhexidine showed larger zone of inhibition against *Campylobacter rectus* and *Porphyromonasgingivalis*compared to nilavembu decoction and saline.(Table 1) (Figure 1)

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

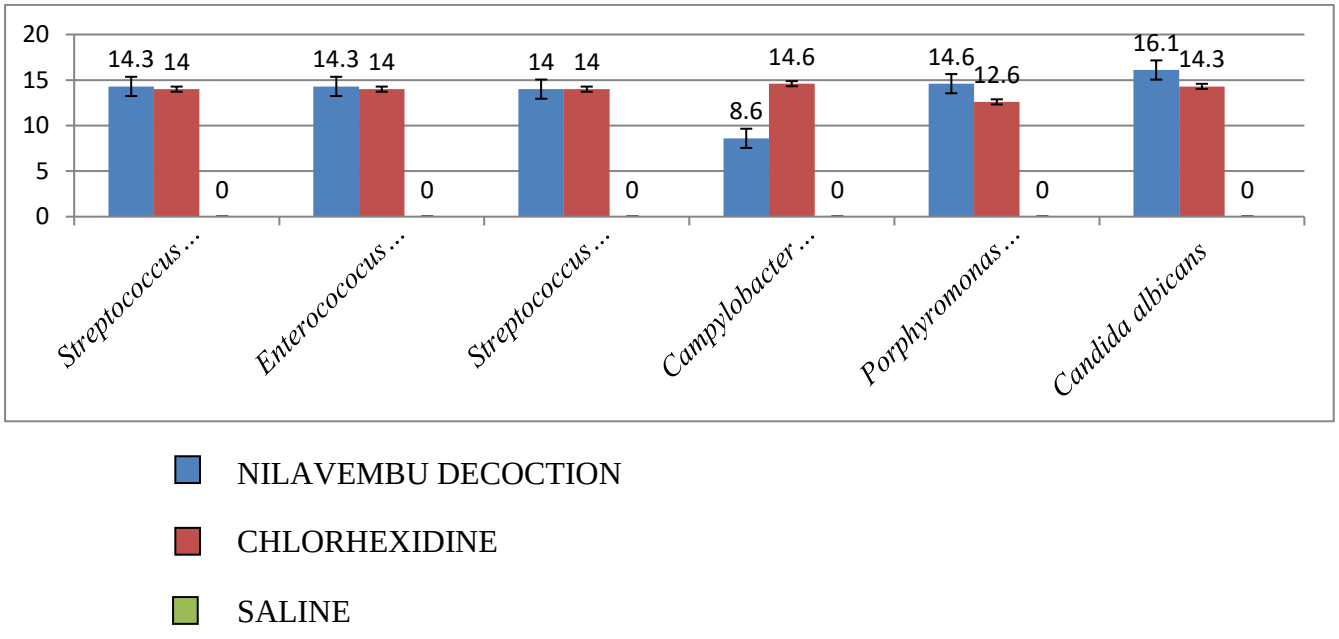
TABLE 1: Mean, Standard Deviation and Statistical Analysis of Antimicrobial Efficacy against test pathogens by different groups

Test pathogens	Mean Zone of Inhibition in(mm) with SD			P-Value*
	Nilavembu decoction	Chlorhexidine	Saline	
Streptococcus mutans	14.3±0.58	14±1	0	.05
Enterococococus faecalis	14.3±0.58	14±1	0	.05
Streptococcus mitis	14±1	14±1	0	.06
Campylobacter rectus	8.6±.58	14.6±1.15	0	.02
Porphyromonasgingivalis	14.6±1.15	12.6± .58	0	.02
Candida albicans	16.1 ± 1.5	14.3±0.58	0	.02

P<0.05 is considered as statistically significant

SD-Standard Deviation, *Kruskal Wallis ANOVA test

Figure: 1 -Zone of Inhibition in (mm) exhibited bydifferent groups against test pathogens

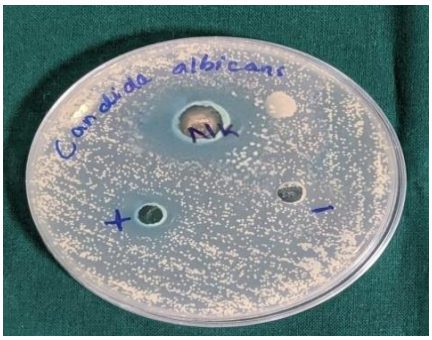


ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

Chlorhexidine showed statistically significant difference against *Campylobacter rectus* and *Porphyromonasgingivalis* where Chlorhexidine showed larger zone of inhibition than Nilavembu decoction.

Nilavembu decoction showed larger zone of inhibition against *Candida albicans* and it was statistically significant (Photograph 1

Photograph 1: Nilavembu decoction showing maximum zone of inhibition against Candida albicans compared to Chlorhexidine and saline



Comparison between Nilavembu decoction and saline showed statistically significant difference against all the test pathogens where Nilavembu decoction showed larger zone of inhibition compared to saline.

Minimum Inhibitory Concentration (MIC) The MIC value for the nilavembu decoction against *Streptococcus mutants, Streptococcus mitis, Porphyromonasgingivalis*and *Campylobacter rectus* was determined as 100mg/ml and showed resistance with other concentrations and against *Enterococococcus faecalis* was 100mg/ml and 50mg/ml and 25mg/ml and showed resistance with other concentrations

The MIC value for the nilavembu decoction against *Candida albicans* was 100mg/ml and 50mg/ml and showed resistance with other concentrations

The results showed that nilavembu decoction is effective against *Candida albicans* and*Enterococococcus faecalis* even at lesser concentration compared to other test pathogens.

Biofilm formation on the tooth substrate:

Microbial count was maximum in the saline group and minimum in Nilavembu decoction and Chlorhexidine groups. Quantitative analysis of 3-days and 5-days biofilm formed on the tooth substrate showed significant difference between all the three groups with respect to the test pathogens where the mean number of *Streptococcus mutans, Enterococcus faecalis*, and *Candida albicans* colonies is lesser in nilavembu

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

decoction group and the mean number of *Streptococcus mitis, Campylobacter rectus* and *Porphyromonasgingivalis* colonies is lesser in chlorhexidine group The differences noted were statistically significant. Saline showed highest number of colonies with regard to all the test pathogens. (TABLE 2, 3

Table: 2: Quantitative analysis of 3-days microbial biofilm formed on the tooth substrate for different groups

Test pathogens	**Number of Microorganisms in CFU/ml (Mean±SD)**			**P-Value**
	Nilavembu decoction	**Chlorhexidine**	**Saline**	
Streptococcus mutans	61.7 $x10^3$±2.8 $x10^3$	70 $x10^3$±2 $x10^3$	200 x 10^3	0.023
Enterococcus faecalis	61.7$x10^3$±2.8 $x10^3$	68.3 $x10^3$±7.6 $x10^3$	200 x 10^3	0.041
Streptococcus mitis	76.7$x10^3$±6.1$x10^3$	75 $x10^3$±5 $x10^3$	200 x 10^3	0.05
Campylobacter rectus	96.7$x10^3$±11.5$x10^3$	73.3$x10^3$±2.5$x10^3$	200 x 10^3	0.023
Porphyromonasgingivalis	153.3$x10^3$±25.1$x10^3$	83.3$x10^3$±2.8$x10^3$	200 x 10^3	0.023
Candida albicans	59$x10^3$±3.6$x10^3$	63.3$x10^3$±2.8$x10^3$	200 x 10^3	0.038

P<0.05 -Statistically significant, SD-Standard Deviation, CFU-Colony Forming Units

*Kruskal Wallis ANOVA test

Table 3: Quantitative analysis of 5-days microbial biofilm formed on the tooth substrate for different groups

Test pathogens	**Number of Microorganisms in CFU/ml (Mean±SD)**			**P-Value***
	Nilavembu decoction	**Chlorhexidine**	**Saline**	
Streptococcus mutans	86.7$x10^3$±5.7 $x10^3$	92.3$x10^3$±3.05 $x10^3$	313.3$x10^3$±11.5$x10^3$	0.04

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

Enterococococus faecalis	$71.7x10^3±10.4\ x10^3$	$91\ x10^3±6.5\ x10^3$	$313.3x10^3±11.5x10^3$	0.02
Streptococcus mitis	$87.6x10^3±3.7x10^3$	$85.3\ x10^3±5.5\ x10^3$	$313.3x10^3±11.5x10^3$	0.05
Campylobacter rectus	$133.3x10^3±15.2x10^3$	$94.3x10^3±1.1x10^3$	$313.3x10^3±11.5x10^3$	0.026
Porphyromonasgingivalis	$183.3x10^3±15.2x10^3$	$86x10^3±5.5x10^3$	$200\ x\ 10^3$	0.027
Candida albicans	$65x10^3±5x10^3$	$78x10^3±2x10^3$	$313.3x10^3±11.5x10^3$	0.027

P<0.05 -Statistically significant, SD-Standard Deviation, CFU-Colony Forming Units

*Kruskal Wallis ANOVA test

Post hoc analysis comparison between Nilavembu decoction and Chlorhexidine of 3-days and 5-days biofilm formed on the tooth substrate showed lesser number of *Streptococcus mutans, Enterococococus faecalis* and *Candida albicans* colonies in nilavembu decoction group compared to chlorhexidine group. Chlorhexidine has better effect against *Campylobacter rectus* and *Porphyromonasgingivalis* compared to nilavembu decoction against these pathogens. The differences noted were statistically significant. No significant difference was noted against *Streptococcus mitis* between these groups on 3 days biofilm. (Table4,5). When 3 days and 5 days were compared number of microbial colonies was lesser in 3 days biofilm compared to 5 days biofilm in all the groups

Table 4: Comparison of Nilavembu Decoction with Chlorhexidine (Post Hoc Analysis) (At the end of 3 days)

Test pathogens	**Number of Microorganisms in CFU/ml (Mean±SD)**		**P-Value***
	Nilavembu decoction	**Chlorhexidine**	
Streptococcus mutans	$61.7\ x10^3±2.8\ x10^3$	$70\ x10^3±2\ x10^3$	0.04

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

Enterococcus faecalis	$61.7x10^3±2.8\ x10^3$	$68.3\ x10^3±7.6\ x10^3$	0.2
Streptococcus mitis	$76.7x10^3±6.1x10^3$	$75\ x10^3±5\ x10^3$	0.6
Campylobacter rectus	$96.7x10^3±11.5x10^3$	$73.3x10^3±2.5x10^3$	0.04
Porphyromonasgingivalis	$153.3x10^3±25.1x10^3$	$83.3x10^3±2.8x10^3$	0.04
Candida albicans	$59x10^3±3.6x10^3$	$63.3x10^3±2.8x10^3$	0.17

Table 5: Comparison of Nilavembu Decoction with Chlorhexidine (Post Hoc Analysis) (At the end of 5 days)

Test pathogens	**Number of Microorganisms in CFU/ml (Mean±SD)**		**P-Value***
Streptococcus mutans	$86.7x10^3±5.7\ x10^3$	$92.3x10^3±3.05\ x10^3$	0.2
Enterococcus faecalis	$71.7x10^3±10.4\ x10^3$	$91\ x10^3±6.5\ x10^3$	0.05
Streptococcus mitis	$87.6x10^3±3.7x10^3$	$85.3\ x10^3±5.5\ x10^3$	0.37
Campylobacter rectus	$133.3x10^3±15.2x10^3$	$94.3x10^3±1.1x10^3$	0.046
Porphyromonasgingivalis	$183.3x10^3±15.2x10^3$	$86x10^3±5.5x10^3$	0.05
Candida albicans	$65x10^3±5x10^3$	$78x10^3±2x10^3$	0.05

P<0.05 -Statistically significant**, ***Mann Whitney U test

4. Discussion

In recent years, there has been a tremendous increase in the studies related to the use of natural plant extract as an antimicrobial agent against microorganisms responsible for oral diseases. These studies have showed that herbal drugs are safer than synthetic chemical drugs. This may be because of the presence of more secondary metabolites which include alkaloids, glycosides,

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

flavonoids, steroids, tannins and saponins in herbal drugs.[7]

The present invitro study was carried out to evaluate the antimicrobial efficacy of nilavembu decoction against *Streptococcus mutans, Streptococcus mitis, Enterococcus faecalis, Campylobacter rectus, Porphyromonasgingivalis*and *Candida albicans* and to compare it with gold standard chlorhexidine.

Nilavembu decoction was chosen because of its well-knownantipyretic, analgesic and anti-inflammatory properties and it is also easily available.[8] The present study was done with nilavembu decoction as such it is administered by the government.

The microorganisms selected in this study are those implicated in common dental diseases like gingivitis, dental caries, periodontal diseases, endodontic re-infections and also opportunistic infections, hence they were taken as the test pathogens.

In the present study nilavembu decoction showed better zone of inhibition against certain test pathogens such as *Streptococcus m*utans, *Enterococcus faecalis* and *Candida albicans* compared to chlorhexidine. This may confirm the effective protection of nilavembu decoction against the test pathogens and it is synchrony to the study conducted by Ramanathan et al (2019)[9] where nilavembukudineer was effective against certain gram positive and gram negative bacteria but biofilm model was not used thereby not stimulating the clinical condition.[9]

It is more prudent to check the efficacy of antimicrobial agents against the microorganisms in their biofilm mode and it is established that the biofilm forming capacity and its structural organization are influenced by the chemical nature of the substrate. Biofilm experiments conducted on polycarbonate or glass substrate will not provide a true indication of the bacteria-substrate interaction. Hence, biofilm was formed on a tooth substrate in this study to. All the groups were tested in direct contact with the biofilm formed on tooth substrate at different durations (3 days and 5 days)[5]

Quantitatative assay with 3 days biofilm showed less microbialgrowth when treated with nilvembu decoction against *Streptococcus mutans, Enterococcus faecalis* and *Candida albicans* compared to chlorhexidine whereas 5 days biofilm showed more microbial growth when treated with nilvembu decoction and it is similar to the study conducted by J Prabhakar et al (2014)[3] on triphala against *Streptococcus mutans* where triphala showed 100% efficacy against 3 days biofilm but not on 7 days biofilm.This might be because of the age of biofilm which plays a vital role in the resistance of micro-organisms to antimicrobials.

In this study, we have allowed the growth of biofilm only for a period of 3 days and 5 days. Mature biofilms may develop their

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

own localized environments and better protect them against changes in the environment. Shen et al(2011) [10] demonstrated that if young, nonmatured biofilms are used to assess the antibacterial efficacy of test agents, the results give a far too optimistic picture of their effect. It is, therefore, important to use mature biofilms when evaluating the antimicrobial efficacy.[10]

In addition to this, the time period of the test solutions kept in contact with the biofilm may be a significant factor to be considered. In the present study, only one-time interval of 10 min for all the test agents was allowed. If the plant extracts are allowed to act for a longer time on the biofilm microorganisms, they could perform better.[10]

In the present study nilavembu decoction has exhibited better antibacterial activity on the Gram-positive bacteria than Gram-negative bacteria. This might be because of the difference in morphological constituents between Gram-positive and Gram-negative bacteria where the outer layer of gram negative bacteria is made up of lipopolysaccharide components whereas the outer layer of Gram-positive bacteria is made up of peptidoglycan layer which makes the cell wall more permeable to antimicrobial substances. Gram-negative bacteria are also less susceptible to antimicrobial chemical substances than Gram-positive bacteria and it was similar to the study conducted by Bipul Biswas et al (2013) [11] in which it was reported that gram-negative bacteria are usually more resistant to the plant-origin antimicrobials and even show no effect, compared to Gram-positive bacteria.[11]

The present study showed that nilavembu decoction can be used as adjunct to mechanical plaque control measures against the test gram positive bacteria and *Candida albicans.*

However, a direct comparison of the study results was not possible because review of available literature showed very less work was done previously in this area and therefore, this study holds ground for future research.

5. Conclusion

Based on the invitro study conducted in a laboratory setting the following conclusions can be drawn.

Under the limitation of study, it was concluded that

Compared to other test pathogens, nilavembu decoction showed better Zone of inhibition against *Candida albicans* this shows nilavembu decoction is a better antifungal agent than anti-bacterial agent.

The low MIC values for organisms such as *Enterococcus faecalis* and *Candida albicans* is an indication of the efficacy of the nilavembu decoction against these pathogens even at lower concentrations.

The colony counting was assessed after 10 minutes of adding the test and control agents

ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

at 3rd day showed better effect than the 5th day which shows that nilavembu decoction have better effect on immature biofilm.

Compared to chlorhexidine nilavembu decoction showed reduction in number of microbial colonies against the test gram positive bacteria and *Candida albicans* in biofilm model on extracted tooth surface. This shows nilavembu decoction has a good antimicrobial action against these pathogens.

The high antimicrobial activity of nilavembu decoction against gram positive bacteria compels us to think of its probable applications in the field of dentistry as an adjunct to the regular mechanical plaque control measures and as a propitious endodontic irrigant.

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ISSN: 2309-5288 (Print)
ISSN: 2309-6152 (Online)
CODEN: JCLMC4
Journal of Coastal Life Medicine

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Acknowledgment

We would like to thank Dr. Anandhi and Mr. Arun from Central Research Laboratory, for the help in microbiological procedures

Funding

The study was funded by **ICMR** under Short Term Studentship (STS) Project