ASSESSMENT OF ANTIMICROBIAL ACTIVITY AND CYTOTOXICITY ACTIVITY OF AZADIRACHTA INDICA AND STEVIA REBAUDIANA BASED MOUTHWASH PREPARATION

*Running title***:** Antimicrobial activity and cytotoxic effect of stevia and neem

ABSTRACT

INTRODUCTION: Mouth rinses are generally utilized as subordinates to oral cleanliness and in the conveyance of dynamic specialists to the teeth and gums. These flushes can impact plaque development and adjust the course of gingival irritation. Today, Neem is utilized to treat different skin illnesses, as a disinfectant substance and as a natural mouthwash. Stevia can inhibit the growth of microorganisms that are responsible for dental caries.

AIM: The main objective of this study is to demonstrate the new herbal combination that could be a better alternative to chlorhexidine.

MATERIALS AND METHODS: Plant extract was prepared and an antimicrobial and cytotoxic effect was done by considering various parameters. The antimicrobial activity of nanoparticles prepared using plant extract was investigated and the results of the test were described as the standard deviation and analyzed. For the cytotoxic activity, an ELISA plate was used, wherein the mortality rate of the shrimps was estimated when the plant extract mediated of nanoparticles of different concentrations was added.

RESULTS: Results were statistically analyzed and correlation is done in SPSS. Antimicrobial activity increases in a dose-dependent manner as the concentration increases. The cytotoxic activity showed the number of live nauplii decreased in the second day when compared to the first suggesting extract has potent cytotoxic activity.

CONCLUSION: This study was done to give pharmacological evidence against antimicrobial and cytotoxic effects thereby helping in a wide array of medical and dental applications and used as a bio toxic alternative.

Keywords: Neem, *Stevia*, Mouthwash, nanoparticles, cytotoxicity, Innovative method.

HIGHLIGHTS:

- An Indigenous Herbal mouthwash was found to enhance plaque and gingival scores to some extent in this study.
- Herbal mouthwash did not cause tooth discolouration or a bad taste, and it proved helpful at reducing plaque formation.
- The study needs to be evaluated further for isolating the possible compounds to test the effectiveness of antimicrobial activity in the oral cavity of the human body to prevent various diseases.

INTRODUCTION:

Mouthwashes are commonly recommended by dentists for the prevention and treatment of a variety of oral diseases. Mouthrinses are generally utilized as subordinates to oral cleanliness and in the conveyance of dynamic specialists to the teeth and gums. These flushes can impact plaque development and adjust the course of gingival irritation(1–4). Some ingredients that act as digestive aids are also found in mouthwash. Today, we use an industrial mouthwash containing several chemical compounds that are toxic to our oral cavities such as thymol, methyl salicylate, and hydrogen peroxide(5–9). The majority of industrial mouth rinses use alcohol to destroy bacteria, and anyone who has used an alcohol-based mouthwash knows how irritating it could be. Although alcohol can be beneficial in the short term, it eventually causes our bodies to develop resistance to the antibiotics present in these mouthwashes(10–13). Some medications, chemotherapy, and lifestyle decisions could cause dry mouth (xerostomia). The use of an alcohol-based mouth rinse regularly would cause a reduction in

saliva production(14–16). The main objective of this study is to demonstrate the new herbal combination that could be a better alternative to chlorhexidine.

The previously known utilization of Neem by the Harappan culture in old India goes back 4500 years. The historical backdrop of the Neem tree is inseparably connected to the historical backdrop of the Indian lifestyle. Today, neem removes are utilized to treat different skin illnesses, as a disinfectant substance and as a natural mouthwash. Dental caries are a human multifactorial infection that has influenced many populations all across the globe extensively. In recent years, *Azadirachta Indica*, commonly known as neem, has gained worldwide prominence due to its wide range of medicinal properties(17–20). About 150 compounds have been isolated from different components of neem to display various properties like immunomodulatory, anti-inflammatory, antihyperglycemic, anti-fungal, antibacterial and cytotoxicity(21–25). On the inhibitory activity of Neem and *Stevia* extract on *Streptococcus mutans*, multiple cross-sectional and longitudinal studies have been performed to date. The study also determines the minimum inhibitory concentration of herbal mouthwashes prepared from neem against *Streptococcus mutans*. Previous studies have shown that herbal mouthwashes prevent bacterial infections and dental caries. But, the procedure for salivary analysis of pH and bacteria count had no clarity because there was a lag in the sampling method. Previous studies concluded that *Stevia rebaudiana* reduced the acidic pH and improved the buffering capacity in high-risk individuals(26–29).

Stevia rebaudiana is a plant that has medicinal value and was used as a cure for a great range of ailments and sweeteners in ancient times. *Stevia* has both sensory and functional properties superior to other plant extracts(30–34). *Stevia* can inhibit the growth of microorganisms that are responsible for dental caries. The herbal mouthwash with antimicrobial properties could prevent the growth of bacteria-like organisms and prevent centers infections.(35–39) Therefore this study was designed to assess the clinical efficacy of the herbal mouthwash. Natural mouthwashes are gentle for even sensitive oral health. This natural herbal mouthwash has antimicrobial properties and has no harsh additives. So, here an In vitro experiment has been carried out so far to test the antibacterial efficacy and cytotoxicity of *Azadirachta indica* and *Stevia rebaudiana(40–42).*

MATERIALS AND METHODS:

Preparation of plant extract:

Fresh powdered extract of 2.5 grams Neem and *Stevia* was added to 100 ml of distilled water to the beaker and boiled for 10-20 minutes in the heating mantle. The boiled extract was filtered using filter paper. (Figure 1)

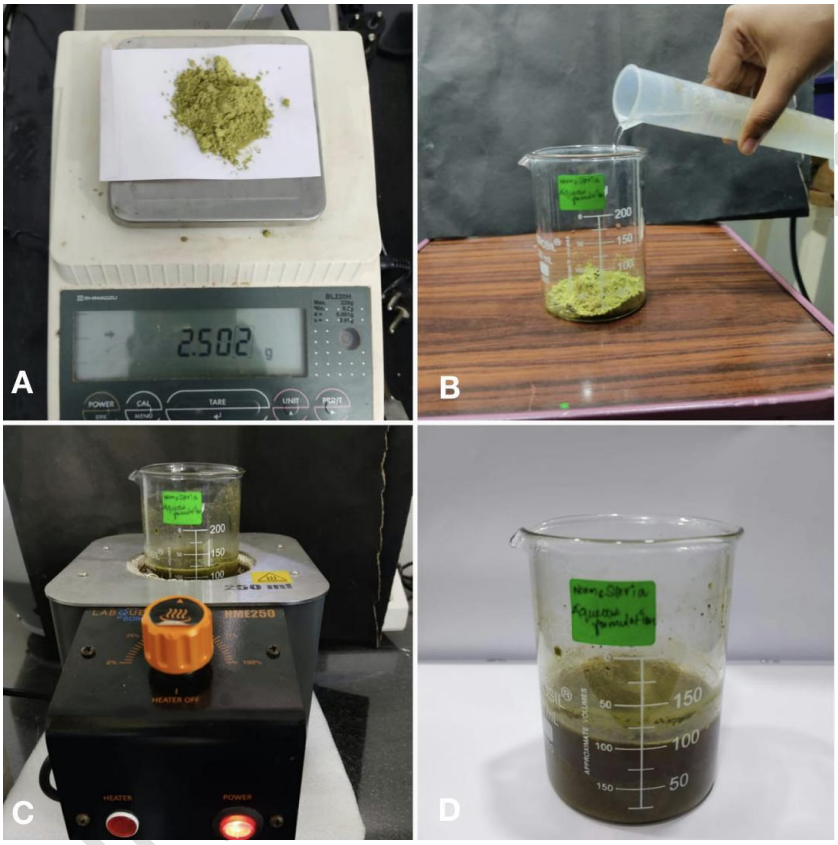

FIGURE 1:A) Represents 2.5gms of Neem that has been measured in a weighing machine. B) addition of distilled water into the extract. C) aqueous formulation of Neem and *Stevia* boiling at 60-70 degrees celsius. D)Neem and *Stevia* extract formulation.

Freshly powdered extract of 2.5 grams Neem and *Stevia* was added to 100 ml of distilled water to the beaker and boiled for 10-20 minutes in the heating mantle. The boiled extract was filtered using filter paper.

CYTOTOXIC ACTIVITY:

Saltwater preparation :

2g of iodine-free salt was weighed and dissolved in 200ml of distilled water. 6 well ELISA plates were taken and 10-12 ml of saline water was filled. To that 10 nauplii were slowly added to each well (20µL,40 µL,60 µL,80 µL,100 µL). Then the nanoparticles were added according to the concentration level. The plates were incubated for 24 hours (Figure2). After 24 hours, the ELISA plates were observed and noted for the number of live nauplii present and calculated by using the following formula,

A number of dead nauplii/Number of dead nauplii+number of live nauplii×100.

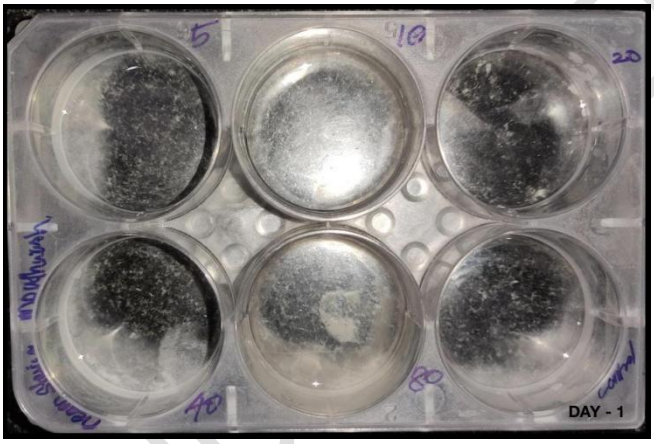

FIGURE 2: Image showing analysis of cytotoxic activity using Neem and *Stevia* mediated nanoparticles of day 1 activity with 10 nauplii in each well.

Validation was done by nano experts. Correlation analysis was done using SPSS software. Only an antimicrobial and cytotoxic effect was done, In further studies, the anti-diabetic and anti-inflammatory activity can be done.

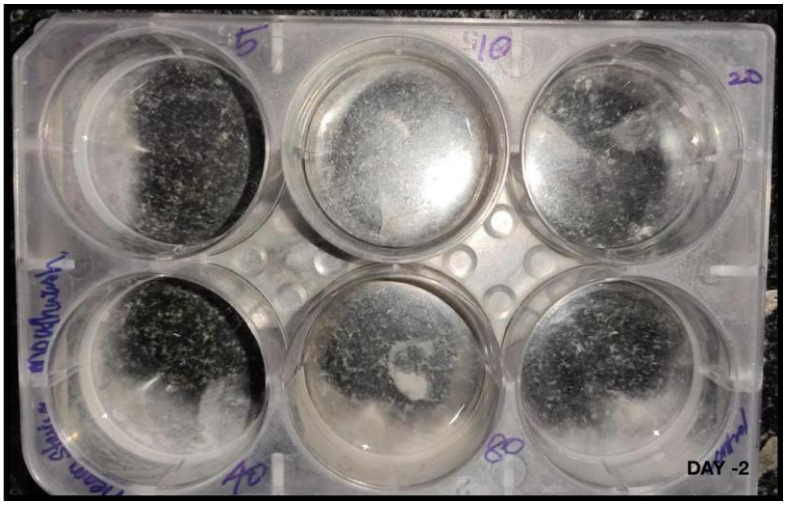

FIGURE 3: Image showing the analysis of cytotoxic activity using Neem and *Stevia* mediated nanoparticles of day 2 activity. In 5µl concentration, there were 9 nauplii present with 8 nauplii in 10 µl concentration, 7 nauplii were found alive in both 20µl and 40 µl concentration, none of the nauplii were alive in 80µl concentration.

ANTIMICROBIAL ACTIVITY:

Antibacterial action against *Staphylococcus aureus*, Bacillus, and *E.coli* strains of the respective nanoparticles. To evaluate the zone of inhibition, MHA agar was used for this operation. Micro pipetting can be done with caution to prevent biasing and previously collected reference values that are used for comparison. Multiple culture plate study has to be done. Muller Hinton agar was prepared and sterilized at 120lbs for 45 minutes. The media poured into the sterilized plates and allowed solidification to remain stable.

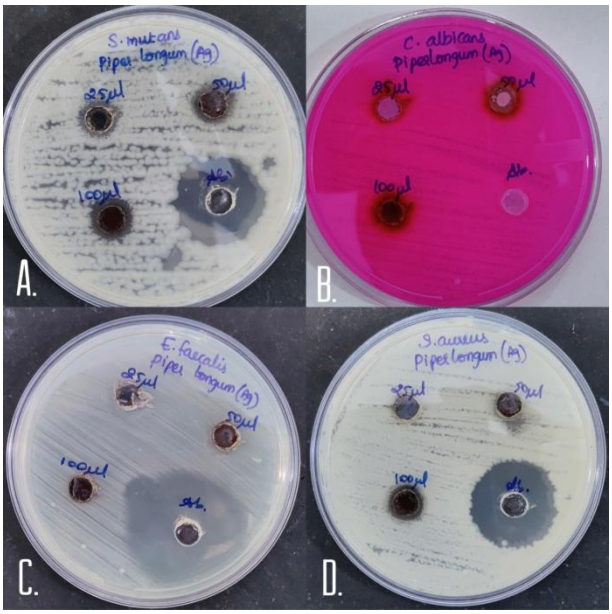

Figure 4: Antimicrobial activity observed in agar plates containing different microorganisms. A) Zone of inhibition of *S.mutans*. B) Zone of inhibition of *C.albicans* C) Zone of inhibition of *E.faecalis* D) Zone of inhibition of *S.aureus*.

RESULTS:

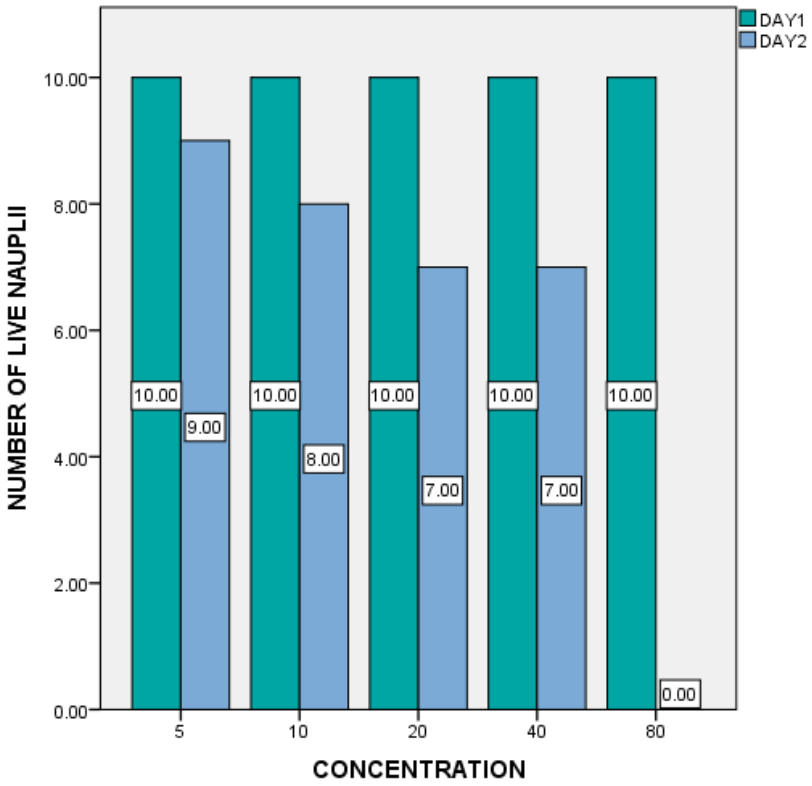

FIGURE 5: Graph represents the cytotoxic effect of the plant extract. The X-axis represents various concentration levels and the Y-axis represents the presence of a number of live nauplii. At 5µl - 9 nauplii were alive, 10µl -8 nauplii were alive, 20µl, 40µl-7 nauplii were alive and 80µl- 0 nauplii.

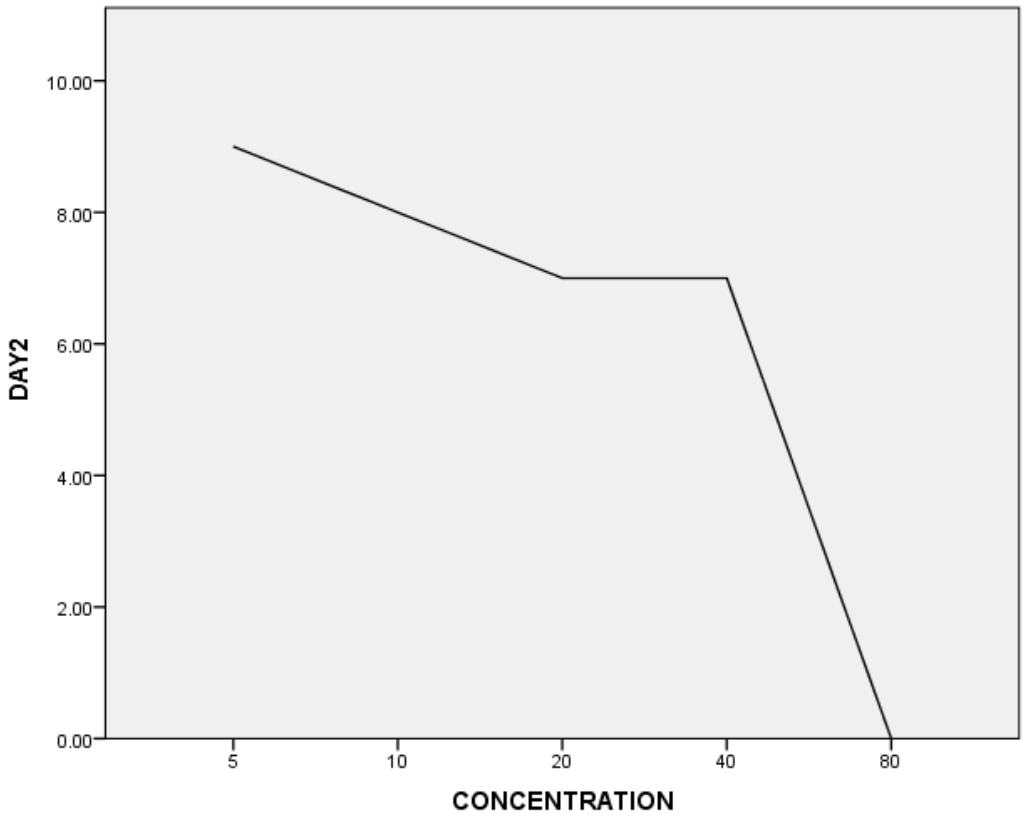

FIGURE 6: The given line diagram represents the day 2 activity of the cytotoxic effect of plant extract. The X-axis represents the different concentration levels and the Y-axis represents the decrease in a number of nauplii present.

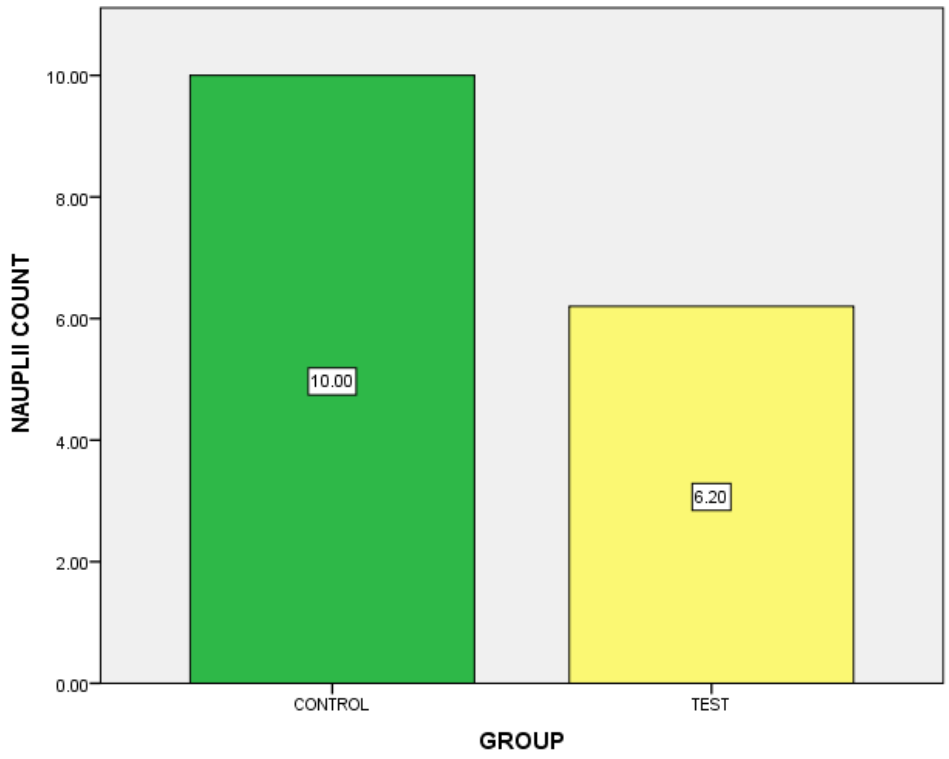

FIGURE 7: The given bar graph represents the total mean value of the cytotoxic effect of plant extract. The X-axis represents the control and test group and the Y-axis represents the number of nauplii present.

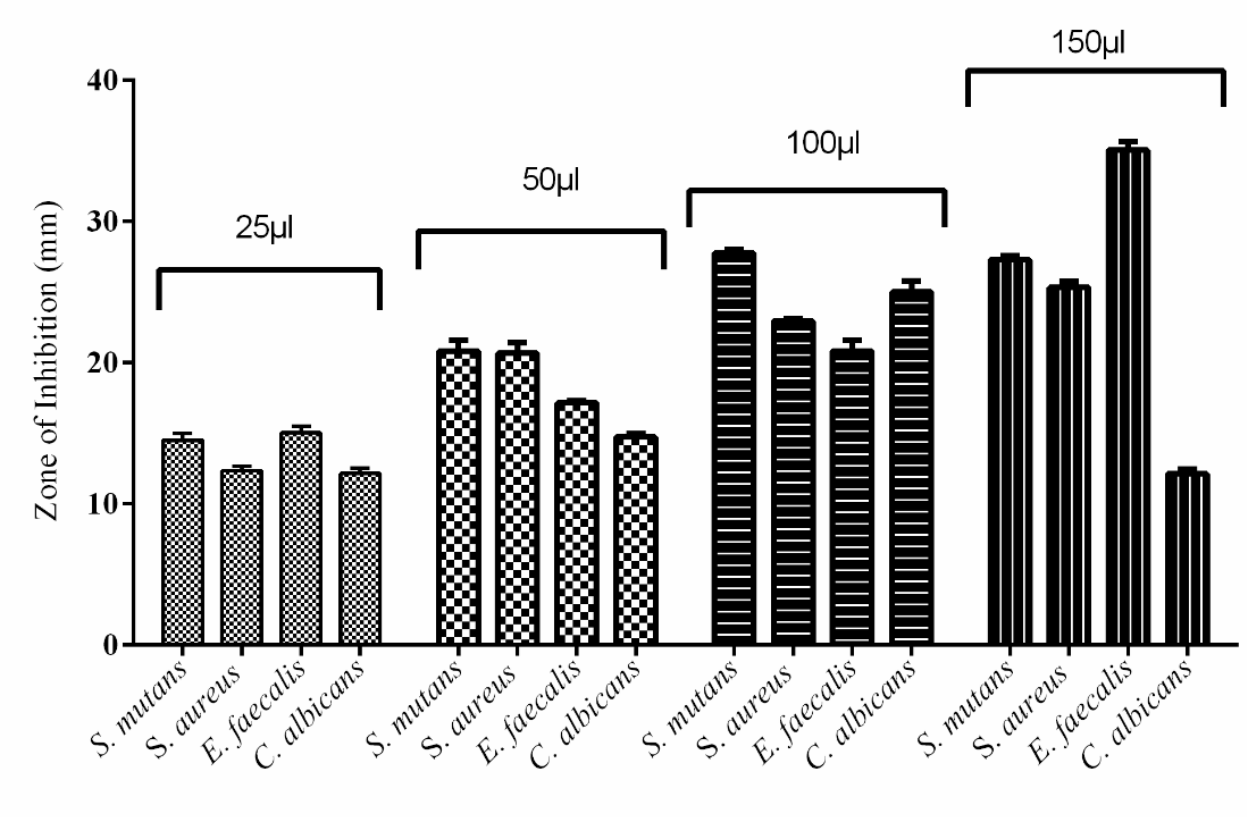

FIGURE 8: Graph showing the antimicrobial activity of *Neem and Stevia* mediated nanoparticles. The percentage of a zone of inhibition in Millimeter(mm) and different concentration levels of the plant extract are represented. The X-axis shows concentration, the Y-axis shows the percentage of a zone of inhibition. At 150µl *Enterococcus foecalis* showed an increased zone of inhibition.

DISCUSSION:

To find the cytotoxic activity in the herbal mouthwash, 6 various parameters have been taken in the study. These parameters include (5µl, 10µl, 20µl, 80µl, control). In each concentration, 10 nauplii (live nauplii) have been dropped. On day 1, all the 10 nauplii were alive in all the concentration levels (Figure5). On day 2, the significant increase in the concentration, decreased the nauplii count in the extract (Figure 6). This clearly showed the influence of the cytotoxic effect in the solution. The count of nauplii remains as 10 on the first day of study in all five concentration levels. In 5µl concentration, the nauplii count decreased from 10 to 9 in number. In 10µl concentration, the nauplii count reduced to 8 in number. In 20µl concentration, the nauplii count was 7 in number. In 40µl concentration, the nauplii count remained as 7 and finally, in 80µl concentration, the nauplii count was recorded as zero. This massive variation in the nauplii count in various concentration levels proved the presence of cytotoxicity (Figure 7).

For the Antimicrobial activity, four different species have been taken. *Enterococcus faecalis, Staphylococcus aureus, Streptococcus mutans, and Candida albicans* were the four different species that were taken as a sample in this experiment. All these four species were allowed to concentrate in four different concentrated solutions (25µl, 50µl, 100µl, 150µl). The zone of inhibition increased as the concentration level increased. But, there was a variation found in *Candida albicans* found in 150µl concentration level. From 25µl to 100µl concentration, there was a significant increase in zone of inhibition but, there was a sudden fall in the inhibition zone for Candida albicans in 150µl concentration. In the 25µl concentration, four different species exhibited four different zones of inhibition. *Enterococcus faecalis* showed a value of 15mm, *Staphylococcus aureus* exhibited 13mm, *Streptococcus mutans* value was recorded as 15mm and *Candida albicans* showed till 13mm of the zone of inhibition. So, here there was consistency in zones of inhibition found in the study.

At 50µl concentration level, *Enterococcus faecalis* showed 17 mm, *Candida albicans* showed 15mm of the zone of inhibition as in 25µl concentration level. Staphylococcus aureus and *Streptococcus mutans* showed similar values of 20mm of the zone of inhibition at 50µl concentration level. At 100µl concentration level, the zone of inhibition gradually increased for all 4 species. For *Enterococcus faecalis*, the zone of inhibition was 20mm. For

Staphylococcus aureus, the zone of inhibition was 23mm and for *Streptococcus mutans*, the zone of inhibition was 27mm and for *Candida albicans*, the zone of inhibition was recorded as 25mm. At 150µl concentration level, the zone of inhibition for *Enterococcus faecalis* was 35mm, For Staphylococcus aureus, 25mm was the zone of inhibition value and for *Streptococcus mutans*, the zone of inhibition was recorded as 27mm. So, for all three bacterial species, there was a sign in the zone of inhibition levels but *Candida albicans* showed the antagonistic effect in the Antimicrobial activity. In the 150µl concentration, the *Candida albicans* species showed a 12mm zone of inhibition. This clearly showed that the herbal mouthwash prepared in the laboratory would inhibit the growth of bacteria in the oral cavity but not the fungal infections. So, the prepared mouthwash showed a minimum resistance to fungal infections. As per the CAMBRA guidelines (Caries management by risk assessment), Antimicrobial mouthwash is an important caries preventive therapy. Herbal mouthwashes have control of dental caries in high-risk individuals. In this study, there was a statistically significant difference in the mean parameters. So from the following references, the previous studies showed very less evidence in vitro effect of neem and stevia extract (Figure 8). The current findings are the first randomized trial and meta-analysis to examine the therapeutic benefits of herbal mouthwashes as an alternative to maintain oral hygiene. Some results revealed a lot of variabilities, which may be attributed to variations in baseline indices across experiments(27,43,44). There are limitations such as teeth staining, increased alcoholic content, taste fluctuations, xerostomia, and stability problems in commercially available liquid mouthwashes containing synthetic active ingredients. The minimum sample size was also a major limitation in the study. Further clinical trials have to be done to test the antimicrobial and cytotoxic activity. Our team has extensive knowledge and research experience that has translate into high quality publications (45).(46–59) ,(60–64)

CONCLUSION:

Within the limitations of the study, Neem and *Stevia* extract helped us to detect the antimicrobial activity and cytotoxic effect on the various species in different concentration levels. The study needs to be evaluated further for isolating the possible compounds to test the

effectiveness of antimicrobial activity in the oral cavity of the human body to prevent various diseases.

NOTE:

The study highlights the efficacy of "herbal" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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