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Antimicrobial Activity and Cytotoxicity of Mouthwash Prepared from *Azadirachta indica and Stevia rebaudiana* Extract– An *In vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. Author BM designed the study, performed the methods and wrote the first draft of the manuscript and author RP performed the statistical analysis and wrote the protocol. Author SRK managed the analyses of the study and managed the literature searches and author PS managed the final drafting and editing of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: Mouth wash 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. *Azadirachta indica* (Neem) was utilized to treat different skin illnesses, as a disinfectant substance and as a natural mouthwash. *Stevia rebaudiana* can inhibit the growth of microorganisms that are responsible for dental caries.
Aim: This study aimed to assess the antimicrobial activity and cytotoxicity from *Neem and Stevia* based Mouthwash.
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

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nauplii was estimated with the plant extract mediated nanoparticles at different concentrations. Data's were statistically analyzed by Spearman correlation through SPSS version 23.
Results: Anti-microbial activity showed positive correlation with increase in concentration (r=1). The cytotoxic activity showed negative correlation with the number of live nauplii decreased in the second day when compared to the first day suggesting that the extract has potent cytotoxic activity (r=-1).
Conclusion: 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.

Keywords: Azadirachta indica; Stevia rebaudiana; mouthwash; cytotoxicity; animicrobial activity; green synthesis.

1. INTRODUCTION

Mouthwashes are commonly recommended by dentists for the prevention and treatment of a variety of oral diseases. 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 [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 a 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. *Neem* helps in treating different skin illness, 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 prevents infection [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 [40-42]. 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*.

2. MATERIALS AND METHODS

2.1 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. (Fig. 1)

Freshly powdered extract of 2.5 grams *Neem* and *Stevia* was added with a ratio of 1:1 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.

2.2 Cytotoxic Activity

2.2.1 Saltwater Preparation

2g of iodine-free salt was weighed and dissolved in 200ml of distilled water. Six well ELISA plates were taken and 10-12 ml of saline water was filled. To that ten 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 (Fig. 2). 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.

Validation was done by nano experts. Spearman correlation analysis was done using SPSS software version 23.

2.3 Antimicrobial Activity

Antibacterial action against *Staphylococcus aureus*, *Streptococcus aureus, C. albicans and E. foecalis* strains of the respective nanoparticles was evaluated with the zone of inhibition, Muller Hilton Agar (MHA) 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. MHA was prepared and sterilized at 120lbs for 45 minutes. The media poured into the sterilized plates and allowed solidification to remain stable.

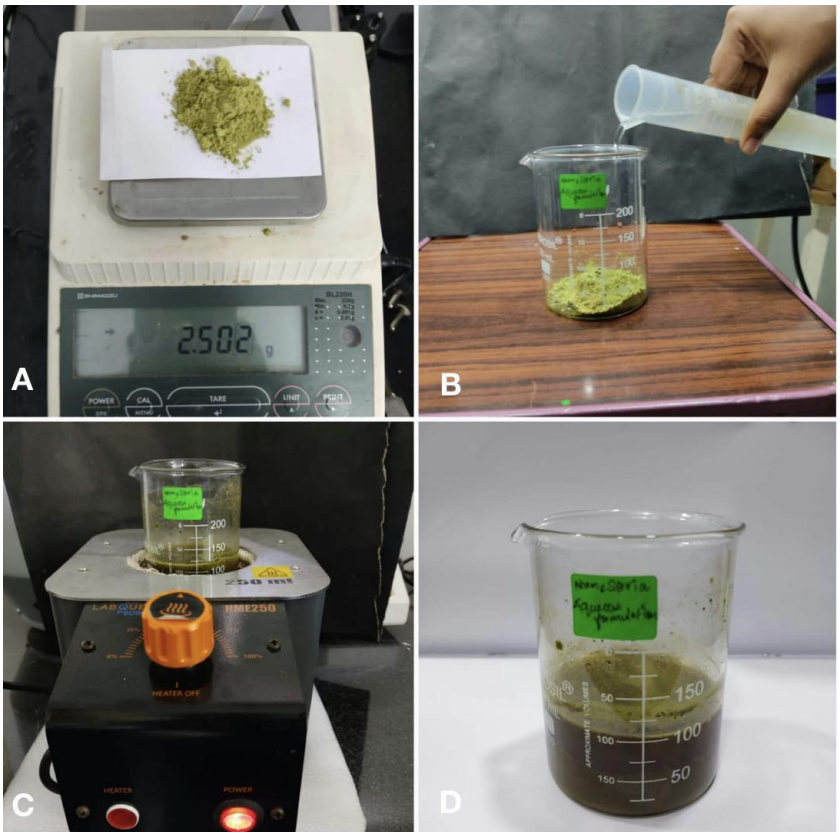

Fig. 1. A) Represents 2.5gms of Neem that has been measured in a weighing machine B) addition of distilled water into the extract, C) aqueous formulation of *Neem* and *Stevia* boiling at 60-70 degrees Celsius, D) *Neem* and *Stevia* extract formulation

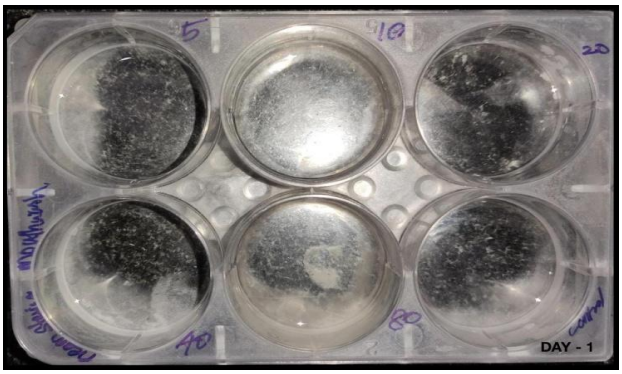

Fig. 2. Image showing analysis of cytotoxic activity using Neem and *Stevia* mediated nanoparticles of day 1 activity with 10 nauplii in each well

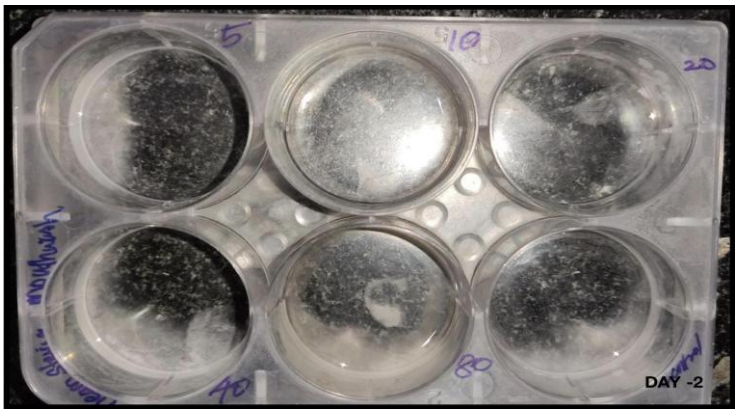

Fig. 3. Image showing the analysis of cytotoxic activity using *Neem* and *Stevia* mediated nanoparticles of day 2 activity. At 5 µl concentration, there were nine live nauplii present, eight nauplii at 10 µl concentration, seven nauplii were found alive in both 20 µl and 40 µl concentration, none of the nauplii were alive in 80µl concentration

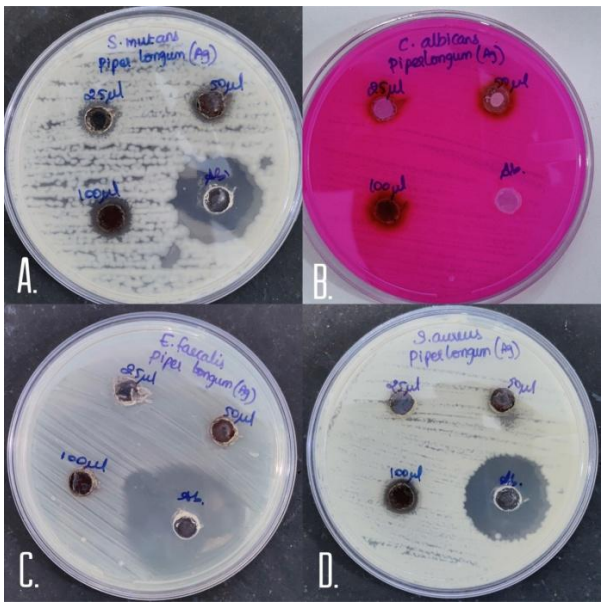

Fig. 4. Antimicrobial activity observed in agar plates containing different microorganisms, A) Zone of inhibition of *S.mutans*, B) Zone of inhibition of *C.albicans* C) Zone of inhibition of *E.faecalis* D) Zone of inhibition of *S.aureus*

3. RESULTS

Cytotoxic activity in herbal mouthwash, four different concentrations have been taken in the study (5µl, 10µl, 20µl, 80µl, control). At each concentration, 10 nauplii (live nauplii) have been dropped. *Artemia* nauplii is a stage of copepod life cycle of larvae is inexpensive, easy available, and can be used for short term study. 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 alive on the first day of study in all five concentration levels. At 5µl concentration, the live nauplii count decreased from ten to nine in number. At 10µl concentration, the live nauplii count reduced to eight in number. At 20µl concentration, the live nauplii count was seven in number. In 40µl concentration, the live nauplii count remained as seven and finally, at 80µl concentration, none of the nauplii were alive. This massive variation in the nauplii count in various concentration levels proved the presence of cytotoxicity (Fig. 7). Spearman correlation analysis reveals a negative correlation with the rise in concentration and decrease in the number of live nauplii (r=-1) which reveal the effective cytotoxic activity of *Azadirachta indica and Stevia rebaudiana* extract based mouth wash.

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 (25 µl, 50 µl, 100 µl, 150 µl). The zone of inhibition increased as the concentration level increased. At 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. *C.albicans* showed a maximum zone of inhibition at 100µl concentration. So, here there was consistency in zones of inhibition found in the study.

At 50 µl concentration level, *Enterococcus faecalis* showed 17 mm, *Candida albicans* showed 15 mm of the zone of inhibition as in 25 µl concentration level. Staphylococcus aureus and *Streptococcus mutans* showed similar values of 20mm of the zone of inhibition at 50 µl concentration level. At 100 µl 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 27 mm and for *Candida albicans*, the zone of inhibition was recorded as 25 mm.

At 150 µl concentration level, the zone of inhibition for *Enterococcus faecalis* was 35mm, For *Staphylococcus aureus*, 25 mm was the zone of inhibition value and for *Streptococcus mutans*, the zone of inhibition was recorded as 27 mm. 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. At 150µl concentration, the *Candida albicans* species showed a 12mm zone of inhibition (Fig. 8). 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.

4. DISCUSSION

The present study showed an effective antimicrobial and cytotoxic activity of *Azadirachta indica and Stevia* based green synthetic mouth wash. The antimicrobial activity was more effective in *E.foecalis* with a maximum zone of inhibition of 35mm at 150µl concentration. Similar study done by Kishore et al (2021) using silver nanoparticles synthesized from *Piper longum* extract and its antimicrobial activity were analyzed at various concentration (25 µl, 50 µl, 100 µl) against pathogens were the zone of inhibition was maximum for *C. albicans* [43]. Barma et al. analyzed the antibacterial activity of Silica nanoparticles against *E.foecalis, S.mutans and S.aureus* were mouthwash that has been incorporated showed effective antibacterial activity against gram positive pathogens [44]. Cytotoxic activity of *Azadirachta indica and Stevia* based green synthetic mouth wash showed an effective activity with decrease in number of nauplii with rise in concentration. Similar study, on cytotoxic activity of mouthwash from Lycopene-Chitosan nanocomposite revealed increased mortality rate in brine shrimp lethality assay and showed results directly propotional to the concentration of the Nanocomposite [45].

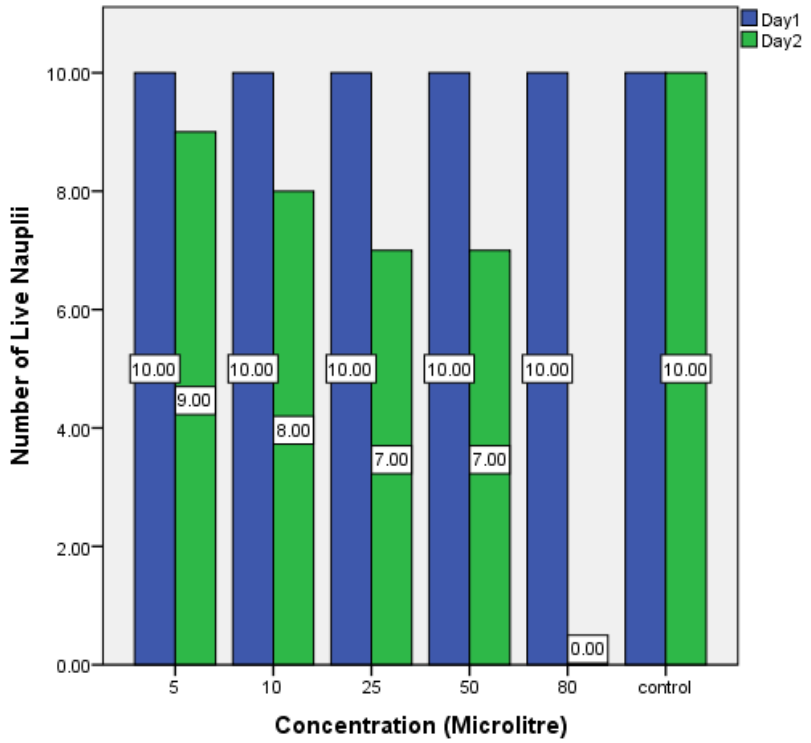

Fig. 5. Graph represents negative correlation (r=-1) of cytotoxic effect of the plant extract. The X-axis represents various concentration levels and the Y-axis represents the presence of a number of live nauplii. At 5 µl – nine nauplii were alive, 10 µl -eight nauplii were alive, 20 µl, 40 µl-seven nauplii were alive and 80 µl- none of the nauplii were alive

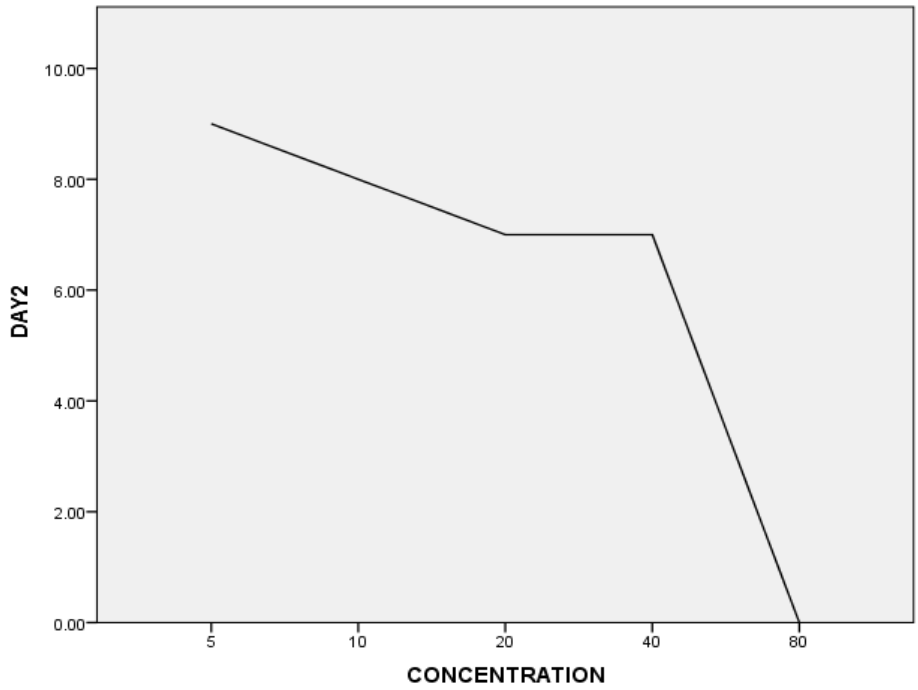

Fig. 6. The given line diagram represents the day 2 activity of the cytotoxic effect of plant extract. The X-axis represents the different concentration levels (Microlitre) and the Y-axis represents the decrease in a number of nauplii present

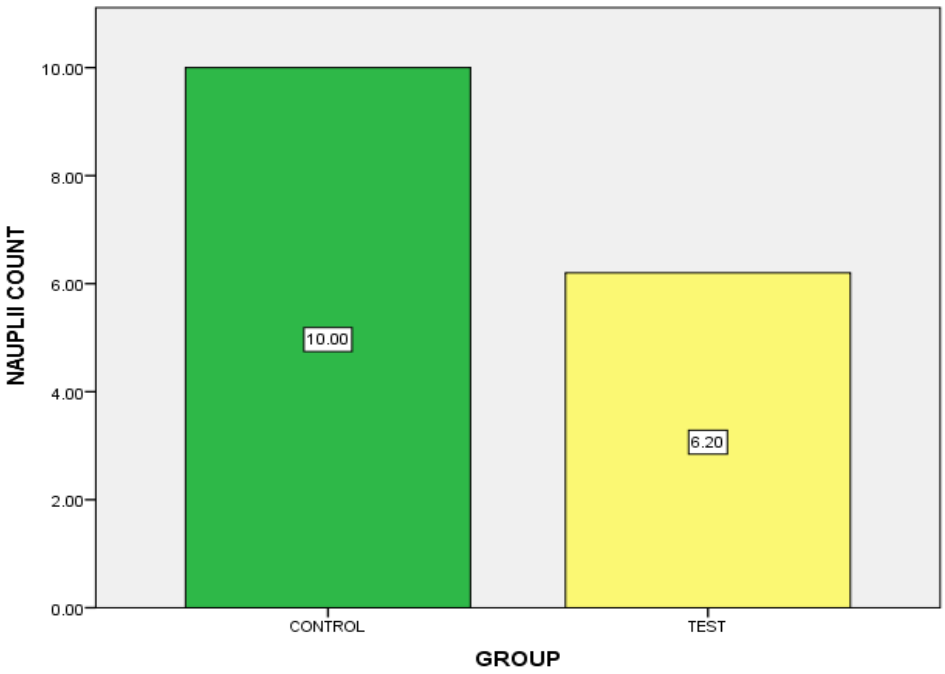

Fig. 7. The given bar graph represents the total mean value of the cytotoxic effect of plant extract. The X-axis represents the control and test group and the Y-axis represents the number of nauplii present

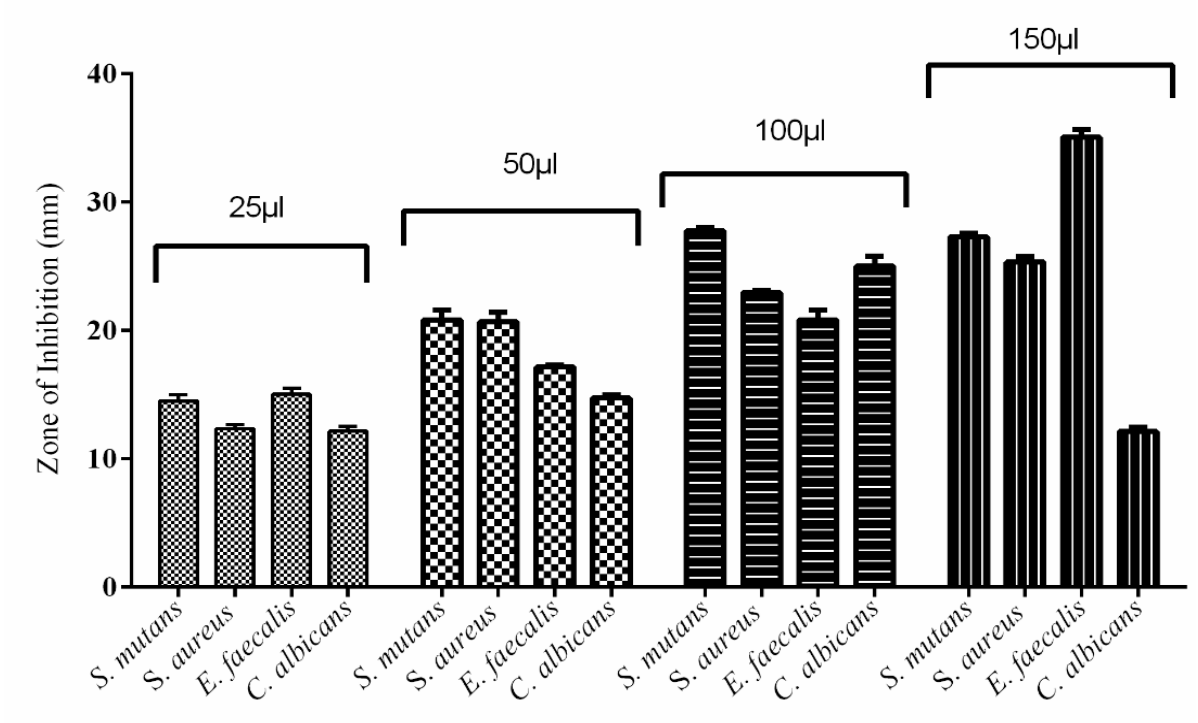

Fig. 8. Graph represents the antimicrobial activity of *Neem and Stevia* mediated nanoparticles. The percentage of a zone of inhibition in Millimeter(mm) and different concentration levels of the plant extract are represented. The X-axis shows concentration (microlitre), the Y-axis shows the percentage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

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 [27,46,47]. There are few drawbacks such as teeth staining, increased alcoholic content, taste fluctuations, xerostomia, and stability problems in commercially available liquid mouthwashes containing synthetic active ingredients. Minimum sample size was the major limitation in the study. Further clinical trials have to be done to test the antimicrobial and cytotoxic activity. Further invitro study, has to be carried out on anti-diabetic and anti-inflammatory activity of *Neem and Stevia* extract. Our team has extensive knowledge and research experience that has translate into high quality publications [48-65].

5. CONCLUSION

Neem and *Stevia* extract helped us to detect the antimicrobial activity and cytotoxic effect of various species at different concentration levels. This results explore us to the development of new antimicrobial biomedicine. Further clinical trials on animal model is essential for ensuring its safer clinical application.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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