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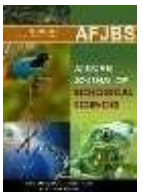

Title Page

Title: Efficacy of a Self–Prepared Azadirachta indica Mouthwash on *Porphyromonasgingivalis* Colonies: Invitro Microbiological Study.

Type of Article -Original Research

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Abstract

The current study aims to create and assess herbal mouthwash and assess its efficacy in combating the oral cavity's microbial load. The plant materials were gathered, and the water- soluble components were removed. The prepared mouthwash's physicochemical characteristics and antibacterial efficacy were further assessed. The mouthwash that is currently available has good antibacterial qualities. The stability study's findings support the efficacy of the preparation. Currently, available mouthwash is a liquid formulation that often includes antiseptic and antibacterial ingredients. In addition to being used to lessen microbial growth in the oral cavity, these solutions may also be administered for other purposes, such as their analgesic, and anti-inflammatory qualities.

Keywords-Indigenous mouthwash, Neem Mouthwash, Oral Microbiota, *Porphyromonasgingivalis*

Introduction

One condition that affects the tissues that support teeth is Periodontal Disease. Gingivitis, a minor form of periodontal disease, is typically the result of poor dental care. Gum swelling, redness, and bleeding are symptoms of gingivitis. Gingivitis is mostly caused by plaque, which frequently accumulates on the gums and tooth surfaces. Since the discovery of antibiotics, reducing plaque has been associated with preventative dentistry. It has also been realized that bacteria may be the cause of significant dental disorders, such as periodontal disease and caries. Bacteria in tooth plaque are one of the primary causes of periodontal inflammation, so careful plaque control is crucial.[1] Since oral bacteria are the primary cause of dental plaque, the best way to reduce plaque is to restrict their growth. Oral hygiene is commonly maintained with the use of mechanical plaque reduction techniques. Mechanical plaque control approaches are time-consuming, and performance requires a high level of motivation and skill sets. [2]Patients are advised to incorporate antimicrobial mouthwashes into their mechanical oral hygiene regimes because extra assistance is needed to control bacterial plaque. Therefore, it has been acknowledged that the most practical way to limit the growth of dental plaque is to remove nonspecific plaque or inhibition.[3] Many products have been on the market for years, including mouthwash, lozenges, gels, and pastes for application. In dental care, mouthwashes are used to both prevent and treat plaque development. All of the mouthwashes on the market nowadays are efficient and medicated.[4]

Neem (Azadirachta indica) has been used for a long time to cure swellings, infections, and skin blemishes. It has long been known that the antibacterial qualities of neem leaves are good for skin and hair. It is a frequently used oral hygiene tool in various regions of the world because of its antiplaque, antibacterial, and antithetic benefits. Neem functions as an anti-inflammatory by inhibiting prostaglandin E and 5 HT.[5] The antibacterial activity is explained by "azadirachtin," which is known to break down bacterial cell walls. Therefore, a shift in osmotic pressure inhibits the growth of bacteria, leading to cell death due to the breakdown of the cell wall. The development of pathogenic anaerobic microbes in the oral cavity is recognized as a significant health danger.[6,7] The purpose of this experiment is to assess how effectively Self-prepared Azadirachta indica mouthwash reduces *Porphyromonas gingivalis* microbiological colonies. We are concentrating on indigenous herbal mouthwash because the commercially available mouthwash contains chemicals that elicit an untoward reaction of discoloration of the oral tissues as well as a burning sensation.

Aims and Objectives of The Study

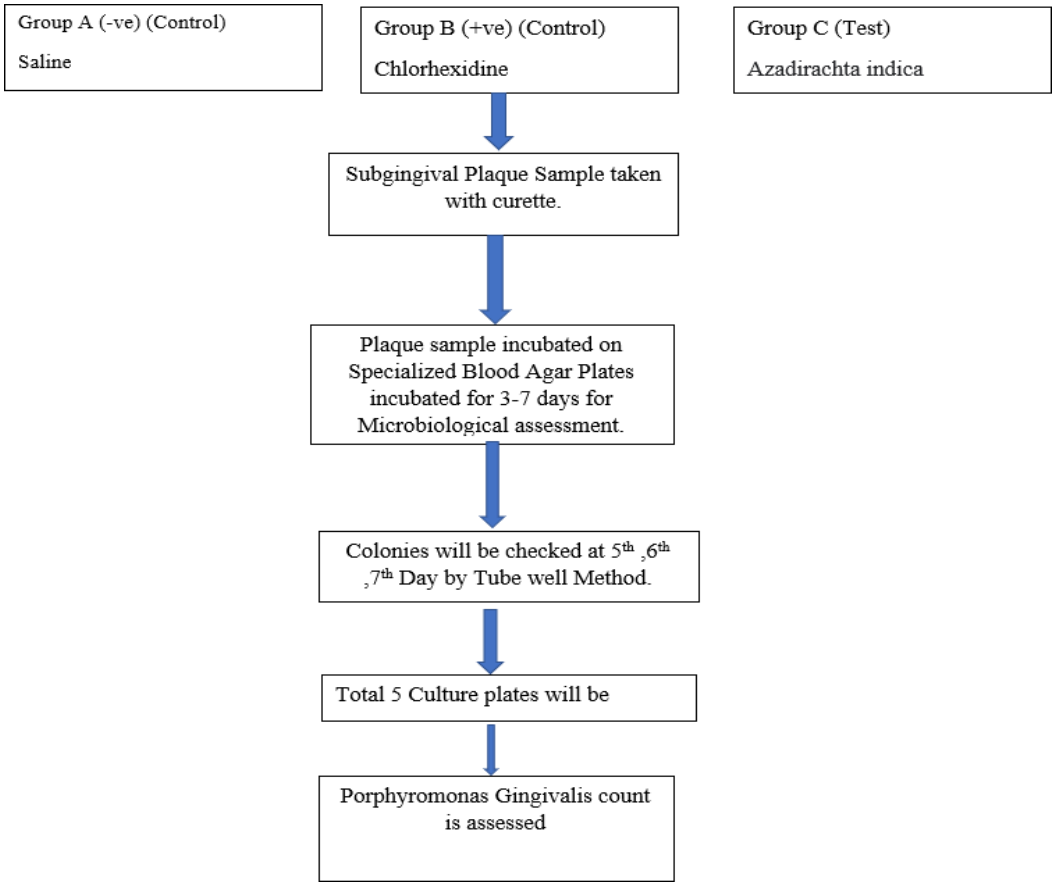
Aim of the Study-To check the efficacy of a self-prepared Azadirachta indica mouthwash on Porphyromonas gingivalis colonies.

Objectives of the Study-

1. To determine the MBC of Self-Prepared Azadirachta indica mouthwash.

2. To evaluate the efficacy of Self–Prepared Azadirachta indica mouthwash on *Porphyromonas gingivalis: Invitro* Microbiological Study.

Materials & Method of The Study (Table 1)



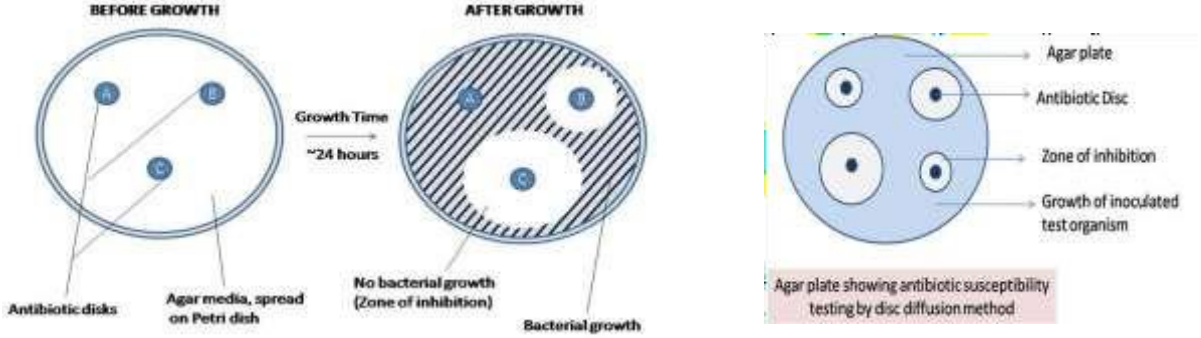


Table 1- **Materials and Method**

Ingredients of the Mouthwash

Neem powder-150 g

Mint oil-

Aspartame powder

Stevia

Ethanol-250 ml

Distilled Water-250 ml

Preparation for the Mouthwash [8] **(Figure 1-10)**

Step 1: Preparation of Neem Powder. Neem powder-The fresh neem leaves were sundried for 2 days until all the moisture from leaves get dried and crispy. Then the dried leaves were put into a blender and was grinded into a fine powder.

Step 2: Extraction To 150 g of Neem Powder, 50% of distilled water and 50% of ethanol was added i.e. 250 ml water and 250ml ethanol.

Step 3: Filtration The mixture is then kept for 2 days for effective maceration and complete extraction. - The macerated content was filtered through Muslin Cloth (Double Layered) - The pre-filtered macerate was again processed through vacuum filter for the removal of particulate matter. The obtained products were labelled as – A. Neem extract.

Step 4: Evaporation of Excessive Solvent- To remove the excessive amount of solvent the extracts were subjected to evaporation on a water bath for 60 degrees Celsius. The obtained product was the concentrated extracts for the formulation of mouth rinses.

Figure 1-Neem Powder (150 gms)

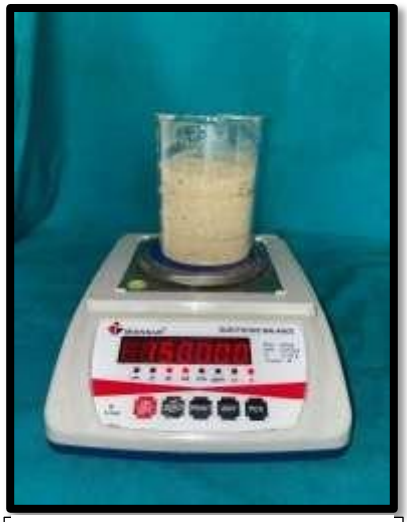


Figure 2-Neem Powder (150 gms) weighed on an electric weighing machine

Figure 3-250 ml of Ethanol

Figure 4-250 ml of Distilled Water

Figure 5-Preparation of the Neem mouthwash Extract

Figure 6-Filtration Apparatus

Figure 7-Filtration of the Mouthwash

Figure 8-Solvent Obtained Post Filtration

Figure 9-Rectangular Water Bath

Figure 10-Stirring the solution on the Water Bath

Evaluation of the Indigenous Mouthwash

Colour and Odour- Physical characteristics including colour and smell were assessed visually.[9]

Taste: The taste based on sweetness and sourness.[9]

pH: A digital pH meter was used to measure the mouthwash's pH after it was made using herbs. A standard buffer solution was used to calibrate the pH meter. Approximately 1 milliliter of mouthwash was weighed, diluted in 50 milliliters of purified water, and its pH was measured. [9]

Test for microbial growth in formulated mouthwash- Using the streak plate method, the mouthwash formulation was inoculated into the agar medium plates, and control was set up. After being put in the incubator, the plates are incubated for 24 hours at 37°C. Plates were removed from the incubation period and examined for microbial growth by contrasting them with the control.[10]

Stability Studies- Without appropriate stability assessments of the produced product, the formulation and preparation of any product are inadequate. Accelerated stability tests, in which the product is heated to high temperatures in accordance with ICH rules, are a generic technique for forecasting the stability of any product. For a duration of three months, an expedited short-term stability study was conducted on the developed formulation. The samples were kept in storage at 3–50 degrees Celsius, 250 degrees Celsius with a relative humidity of 60%, and 400 degrees Celsius with a relative humidity of 75%. Ultimately, the samples that were maintained for the expedited study were taken out and examined every month. [10]

In-Vitro Antibacterial Activity-

The MBC test makes it possible to ascertain the lowest agent concentration required to produce a bactericidal effect. It is important to note, nonetheless, that this procedure involves the antibiotic being in contact with the test organism for around eighteen hours. Since all other factors support the biocidal effect, the test establishes the lowest dose required to kill the test organism. For screening purposes, the MBC test is a useful and reasonably priced method for classifying a large number of antimicrobial agents according to potency.

Since the MBC test parameters are simple to regulate in the lab, it is easy to compare different antimicrobial drugs tested under the same circumstances and their individual effects on different microbes.

1. An overnight-grown pure culture of a designated microbe, diluted to a concentration of 1 x 10^5 to 1 x 10^6 cfu/ml in growth-supporting broth (usually Mueller Hinton Broth).
2. The antimicrobial test material is diluted to a stock level at roughly 100 times the predicted minimum inhibitory concentration (MIC), if that is known.
3. Test tubes or 96-well microtiter plates are used to create further 1:1 dilution. The designated microorganism is injected into equal amounts into each dilution of the test product(s).
4. For each test microorganism, a positive and negative control tube or well is included to show sufficient microbial growth during the incubation time and media sterility, respectively.
5. A baseline concentration of the microorganism is established by plating an aliquot of the positive control.

6. After that, the tubes or microtiter plates are incubated for the proper amount of time and at the proper temperature.
7. The bacterium is growing when there is turbidity, and the minimum inhibitory concentration (MIC) is the concentration at which no visible growth occurs.
8. The dilution that represents the MIC and at least two of the more concentrated test product dilutions are plated, and the viable CFU/ml is counted to estimate the MBC. When compared to the MIC dilution, the MBC is the lowest concentration that exhibits a predefined reduction (such as 99.9%) in CFU/ml. [10]

Viscosity: To check the nature of the mouthwash due to internal friction. The values of the assessed mouthwash were recorded. [10]

Irritational Test- The patient's untreated skin areas were used as the control group, and the mouthwash under test was applied topically to the patient in a single dose. At predetermined intervals, the level of irritation or corrosion is assessed and rated. At any point during the test, animals exhibiting persistent signs of extreme discomfort and/or anguish should be humanely put down, and the test chemical should be evaluated appropriately. [10]

TLC- Thin Layer Chromatography- An affinity-based technique called thin layer chromatography (TLC) is used to separate different chemicals in a mixture. TLC is a very adaptable separation technique that is frequently applied to sample analysis, both quantitative and qualitative. Almost any kind of substance, including pesticides, steroids, alkaloids, lipids, nucleotides, glycosides, carbohydrates, and fatty acids, can be analysed using TLC.

In TLC, an inert plate surface, commonly made of glass, plastic, or aluminium, is coated with a thin layer of an adsorbent substance, usually silica gel or aluminium oxide. One end of the TLC plate is dotted with the sample, which is then vertically inserted into a closed chamber containing an organic solvent (mobile phase). Due to their differing affinities for the stationary and mobile phases, sample constituents migrate over a range of distances when the mobile phase is propelled up the plate by capillary forces. The plate is taken out of the developing

chamber and dried when the solvent has reached the top of it. Each component's retention factor (Rf) is evaluated when the separated components show up as spots on the plate.

Results

The Physical Properties of three different formulations of the mouthwash were assessed such as **Colour, Taste and Odor** of the mouthwash. (Table 2-Physical Properties of the Mouthwash).

Formulation	Colour	Odour	Taste
Formulation 1	Olive Green	Fragrant-Minty	Astringent after Taste
Formulation 2	Olive Green	Fragrant-Minty	Astringent after Taste
Formulation 3	Olive Green	Fragrant-Minty	Astringent after Taste

Table 2- **Physical Properties of the Mouthwash**

The **pH of the Herbal mouthwash** over a course of seven weeks after storage is assessed. F1 had a pH range of 4.12 to 5, F2 was between 4.15-5.50 and F3 ranged between 4.56-6.2. (Table 3-pH of the Mouthwash)

pH of the Formulation	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
F1	4.12	4.2	4.4	4.5	4.7	4.9	5
F2	4.15	5.3	5.1	4.85	5.1	5.3	5.5
F3	4.56	5.75	5.42	5.42	5.64	5.75	6

Table 3- **pH of the Mouthwash**

The results of the **Stability Tests** are listed as follows (Table 4- Stability Tests of the Mouthwash)

TEMPERATURE	**EVALUATION PARAMETERS**	**OBSERVATION(Months)**			
3-5 ℃		0	1	2	3
	Visual Appearance	Olive Green	Olive Green	Olive Green	Olive Green
	Phase Separation	Nil	Nil	Nil	Nil
	Homogeneity	Good	Good	Good	Good
Room Temperature (25 ℃ RH=60%)	Visual Appearance	Olive Green	Olive Green	Olive Green	Olive Green
	Phase Separation	Nil	Nil	Nil	Nil
	Homogeneity	Good	Good	Good	Good
40 ℃ ±2 ℃ RH =75%	Visual Appearance	Olive Green	Olive Green	Olive Green	Olive Green
	Phase Separation	Nil	Nil	Nil	Nil
	Homogeneity	Good	Good	Good	Good

Table 4- **Stability Tests of the Mouthwash**

The results of the **Microbial Growth** are as follows. (Table 5-Microbial Growth of the Mouthwash)

Formulation	**Incubator**	**Temperature**	**Time**	**Microbes**
F1	Biological incubator	37°C	72 hr	No microbial growth
F2	Biological incubator	37°C	72 hr	No microbial growth
F3	Biological incubator	37°C	72 hr	No microbial growth

Table 5- **Microbial Growth of the Mouthwash**

The result of the **Irritation Test** is that the prepared herbal mouthwash was found to be non-irritating. No Redness, erosion, or damage to the surface was observed.

Invitro Antibacterial Activity- Minimum Bactericidal count was done for this herbal mouthwash due to its highly viscous nature. (Figure 11 -12).

MBC for mouthwash: 1:1S

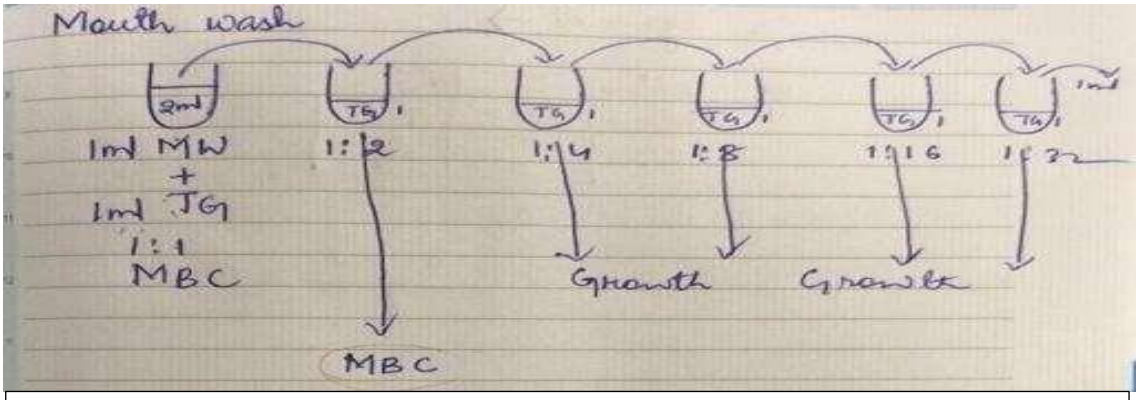


Figure 11- **The Process of MBC**

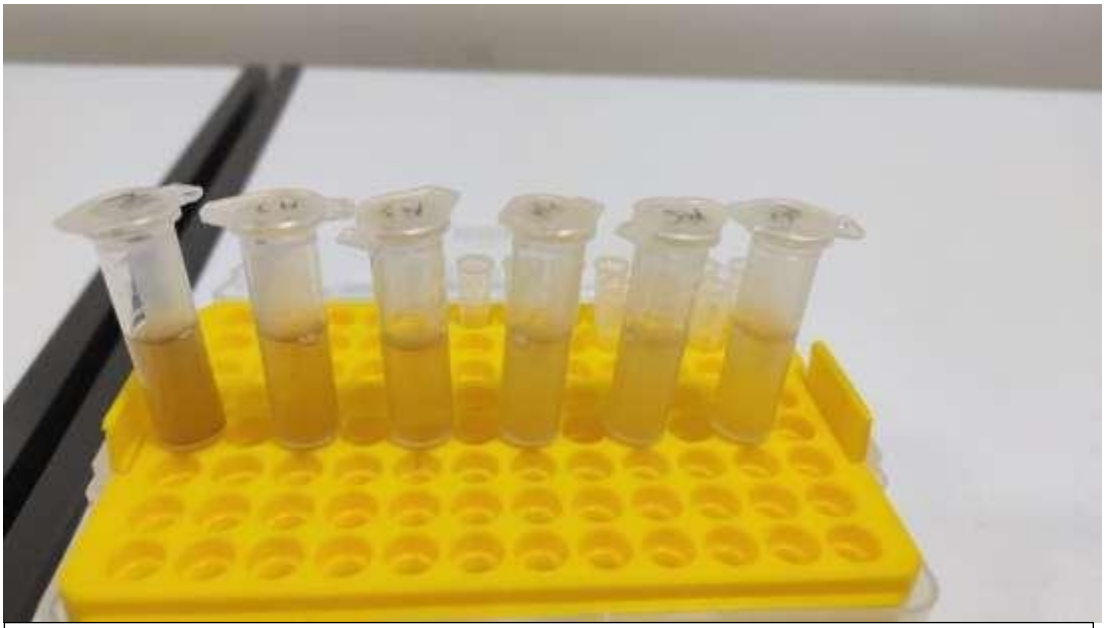


Figure 12- **Minimal Bactericidal Count**

TLC- Thin Layer Chromatography of the Herbal Mouthwash

Chemical test	**Reagent test**	**Neem Mouthwash**
Test for Carbohydrates	Molisch's Test	+ ve
	Fehling Test	+ ve
	Benedict's Test	+ ve
	Iodine Test	+ ve
	Tannic acid Test	+ ve
Test for Steroids	Salkowaski Test	+ ve
	Libbermann's Test	+ ve
Test For Protein	Biuret Test	+ ve
	Millon's Test	+ ve
Test For Amino acid	Ninhydrin Test	+ ve
Test For Glycoside	Brontrager Test	+ ve
	Keller-killani Test	+ ve
	Legal's Test	+ ve
Test For flavonoids	Shinoda Test	+ ve
	Sulphuric acid Test	+ ve

Table 6- **TLC- Thin Layer Chromatography of the Herbal Mouthwash**

Discussion

Periodontal disease has been taunted as a common disease affecting the bone and other supporting structures. Dental Plaque acts as a major synergistic agent to amplify the effect of pathogenic bacteria leading to bone and attachment loss. Many agents have been used to reduce plaque formation on hard and soft tissues. The most commonly used plaque reduction agents are primarily divided into Mechanical and Chemical Agents.[4,5] The mechanical agents include toothbrushes, flossing, etc. The most commonly used chemical agents are mouthwashes. There are several types of mouthwash which are available in the market. The commercially available mouthwashes although provide the required plaque control due to the inclusion of various chemicals lead to discoloration of the oral tissues and well as ulcer formations when used for a prolonged period. To combat the issues caused due to commercially available mouthwashes, indigenously produced mouthwashes provide respite in terms of the harmful implications of the aforementioned varieties.[11] This particular study aimed to prepare an herbal indigenous mouthwash that is suitable for use by patients. The use of neem in preparation of this particular mouthwash has been advantageous due to its several therapeutic properties. Neem or *Azadirachta indica* has several benefits such as free radical scavenger due to rich source of antioxidants, acting as an anti-inflammatory agent via regulation of proinflammatory enzyme activities including cyclooxygenase (COX), and lipoxygenase (LOX) enzyme. Neem has a strong anti-bacterial activity; it works by inhibiting the growth of various bacterial organisms. Through this study, we aimed to prepare an Indigenous Herbal mouthwash that supersedes the various disadvantages of the commercially available mouthwash. Our foremost aim was to determine the MBC of the mouthwash. Secondarily the effect of the self-prepared mouthwash on the *Porphyromonas gingivalis* colonies. In concurrence with this study, we also aimed to be able to prepare this mouthwash for commercial and to achieve this the mouthwash underwent various microbiological and pharmacological tests. The various tests included determining the physical properties of the mouthwash such as Colour, Odor and Taste, pH, Test for microbial growth in formulated mouthwash, Stability Studies, Viscosity, Irritational Test, TLC- Thin Layer Chromatography.

The results of the various tests suggested that the Self-Prepared mouthwash was Olive Green in Colour, Fragrant-Minty in Odour, and the Taste was Astringent after Taste. pH of the Herbal mouthwash was 6. By the Stability Test the formulation was found to be was found to stable

for a period of 12 weeks. There was no sign of salting out, immiscibility, precipitation, phase separation, or change in colour and pH. Additionally, no foul odour was observed during the time of the stability test at both at the Room Temperature of 25 ℃ RH=60%. There was no microbial growth which was seen at 37°C in the time duration of 72 hours for the three formulations. The Viscosity of the Herbal Mouthwash is 1.03Cp. The result of the Irritation Test is that the prepared herbal mouthwash was found to be non-irritating. No Redness, erosion, or damage to the surface was observed. Minimum Bactericidal count was done for this herbal mouthwash due to its highly viscous nature.MBC for mouthwash: 1:1S. The various tests done under the TLC- Thin Layer Chromatography of the Herbal Mouthwash were positive.

Saleem et al. (2018) provided an extensive report on the unique protective characteristics of A. indica as well as the most recent evidence supporting the use of neem as a cure for certain human disorders and the known mechanisms of action of neem components. The effective uses of neem in the food sector and the custom of using neem products for tooth hygiene. The capacity of pathogenic bacterial species to form biofilms has sparked interest in elucidating how these communities contribute to enhanced tolerance to antibacterial agents, in addition to typical antibiotic resistance.[11] Despite the widespread recognition of the significance of biofilm-associated illnesses in human disease, few innovative methods for successfully eradicating biofilms have been developed so far. Nonetheless, promising findings indicate that neem is consistently more successful than many other herbal extracts in inhibiting bacterial growth and specifically targeting biofilm-grown cells, making it a worthwhile candidate for drug research (Noor, 2011). [12]According to several studies (Jalaluddin et al., 2017; Hosny et al., 2021), neem extracts may be just as effective against plaque, gingivitis, and pain in vivo as chlorhexidine or hypochlorite, which are common ingredients in mouthwashes. They may also be just as effective against the biofilm-forming bacterium *Porphyromonas gingivalis*.[13]
Leali H et al assessed the antioxidant properties of neem leaf extract both on its own and in conjunction with bacteria and polycationic peptides that may be present at the site of inflammation, as well as the antibacterial effects of the extract on the periodontopathic bacteria *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Red blood cells or the polycationic peptides chlorhexidine and lysozyme were assessed using a chemiluminescence assay, and the Minimal Inhibitory Concentration (MIC) of neem leaf extract against each bacterial strain was assessed using a broth microdilution test. The extract from neem leaves shown strong antibacterial activity against *Porphyromonas gingivalis*, but it did not influence *Fusobacterium*

nucleatum growth or the coaggregation of the two bacteria. Still, it had a high level of antioxidant activity.[14]

These findings demonstrated the herbal mouthwash's strong antibacterial activity and its ability to prevent the formation of bacteria in the oral cavity. Oral microbial burden and oral illnesses are known to be associated. Herbal mouthwashes have a pleasant smell and can temporarily cover up unpleasant odours. On the other hand, herbal mouthwashes containing antimicrobials may be useful for the long-term management of unpleasant odours. When a patient uses this mouthwash, they tilt their head backward, allowing the mouthwash to remain in the back of their mouth while they exhale, causing the liquid to bubble. Herbal gargles are harmless because they have no negative effects due to their systemic availability in trace amounts.[15]

Conclusion

Self-prepared herbal mouthwash is quite effective in helping individuals get rid of various dental problems and foul breath. Furthermore, we can find solace in the knowledge that this mouthwash has no harmful substances. The findings of the physicochemical evaluation verify that the current herbal formulation's colour and Odor are appropriate, with a pleasing scent and improved aftereffects.[16,17]

Herbal mouthwash has shown superior antibacterial activity in several studies when compared to commercial mouthwash. When compared to commercial mouthwash, the effectiveness of herbal mouthwash in decreasing plaque and gingivitis was comparable, suggesting it could be a viable substitute.[18] While the currently being tested herbal mouthwash has no side effects, the chemical mouthwash has been reported to have numerous side effects, including tooth discoloration, altered taste perception, mucosal irritation, parotid swelling, and increased supra-gingival calculus formation. These side effects limit the mouthwash's acceptability and long-term use. The current herbal mouthwash is highly effective in curing gingival inflammation, reducing bacterial load, bad breath, and many dental problems. [19]

Future Perspective

This particular herbal indigenous mouthwash has been tested for various properties such as Thin Layer Chromatogenic Properties, Stability of the drug formulation, Increased biocompatibility, and Increased safety as well as the Shelf life of the product. We have thereby

tested the efficacy of the mouthwash In-Vitro by tests such as Minimum Bactericidal count to check its effective antibacterial properties when compared with chemical mouthwashes available in the market. We further aim to patent this product, so that it's available in the market as an indigenous herbal product cancelling all the negative side effects of its chemical counterpart.

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