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Original Article

Comparative Evaluation of Antimicrobial and Antibiofilm Efficacy of *Withania somnifera* (Indian ginseng) Mouthwash with *Chlorhexidine gluconate* Mouthwash against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia*: An *in vitro* Study

Kirti Kamat[1], Renuka Metgud[1,*], Archana Patil[2], Suneel Dodamani[3], Priya P Shetti[4], Sayali Manjarekar[3]

[1]Department of Periodontics, KLE VK Institute of Dental Sciences, KAHER, Belagavi, Karnataka, INDIA.
[2]Department of Pharmaceutics, KLE College of Pharmacy, KAHER, Belagavi, Karnataka, INDIA.
[3]Department of Microbiology, Dr. Prabhakar Kore Basic Science Research Centre, KAHER, Belagavi, Karnataka, INDIA.
[4]Department of Pharmacognosy and Phytochemistry, Dr. Prabhakar Kore Basic Science Research Centre, KAHER, Belagavi, Karnataka, INDIA.

ABSTRACT

Background: Periodontitis is a chronic inflammatory disease characterized by destruction of the supporting tissues of the teeth due to complex interactions between periodontal pathogens and the host immune response. Although chlorhexidine gluconate is considered the gold standard antiplaque agent, its long-term use is associated with several adverse effects that may compromise patient compliance. *Withania somnifera* (Ashwagandha), an important medicinal herb in Ayurveda, possesses anti-inflammatory, antioxidant, antimicrobial, and immunomodulatory properties. **Objectives:** To evaluate and compare the antimicrobial and antibiofilm efficacy of *Withania somnifera* mouthwash and 0.12% chlorhexidine gluconate mouthwash against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia*. **Materials and Methods:** An *in vitro* study was conducted against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans*, and *Prevotella intermedia*. Methanolic extract of *Withania somnifera* was prepared using the cold maceration technique and subsequently formulated into a mouthwash. The antimicrobial and antibiofilm efficacy of the formulated *Withania somnifera* mouthwash was compared with that of 0.12% chlorhexidine gluconate mouthwash against the tested periodontal pathogens. **Results:** 0.12% chlorhexidine gluconate mouthwash demonstrated superior antimicrobial and antibiofilm activity against *P. gingivalis, A. actinomycetemcomitans*, and *P. intermedia* compared to *Withania somnifera* mouthwash. However, the formulated *Withania somnifera* mouthwash exhibited significant antimicrobial and antibiofilm activity against all tested periodontal pathogens. **Conclusion:** Although chlorhexidine gluconate mouthwash showed significant antimicrobial and antibiofilm efficacy, its prolonged use is associated with several undesirable side effects. The formulated *Withania somnifera* mouthwash demonstrated promising antimicrobial and antibiofilm properties and may serve as a potential herbal alternative to 0.12% chlorhexidine gluconate mouthwash in periodontal therapy. Further studies incorporating biofilm models, cytotoxicity assessments and well-designed randomized controlled clinical trials are necessary to establish its clinical efficacy, safety and long-term benefits.

Keywords: Chlorhexidine, Dental plaque, Herbal mouthwash, Periodontal disease, *Withania somnifera*.

Correspondence:
Dr. Renuka Metgud
Department of Periodontics, KLE V K Institute of Dental Sciences, KAHER, Nehru Nagar, Belagavi-590010, Karnataka, INDIA.
Email: renukametgud@gmail.com

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INTRODUCTION

Periodontitis is an inflammatory disease of multifactorial origin marked by breakdown of supporting structures of the teeth caused by complex interplay between specific microbes or groups of specific microbes and host response (Shah *et al.*, 2015). This could lead to tooth loss, negatively affecting esthetics and impairing the overall quality of life (Papapanou *et al.*, 2018). The prevalence of periodontal disease ranges from 24.4% in adults between the age of 30 to 34 years to 70.1% in adults aged 65 years and older (Eke *et al.*, 2012).

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A significant change from a symbiotic community of microbes, which is mainly composed of facultative bacteria like *Actinomyces spp.* and *Streptococci spp.*, to a dysbiotic structure of microbial community, which is made up of anaerobic genera from the phyla *Proteobacteria, Spirochetes* and *Bacteroides,* is linked to the transition from periodontal health to disease (Hajishengallis *et al*., 2015).

The likelihood of an individual developing periodontal diseases depends upon the host's immunological and inflammatory responses to the periodontal pathogens present in dental plaque, which are influenced by a variety of risk factors, like stress, cigarette smoking, lack of oral hygiene, genetic predisposition and systemic conditions such as diabetes mellitus (Rajendiran *et al*., 2021). Gingivitis can progress to periodontitis when dental plaque accumulates, which may result in irreversible damage to the alveolar bone and supporting connective tissue (Rajendiran *et al*., 2021; Haffajee *et al*., 2008). Also, plaque levels and gingivitis severity have been shown to have a direct correlation (Chaudhari *et al*., 2012).

More recently the etiology of periodontal disease has undergone a major shift with emphasis on specific anaerobic microrganisms as the initiating factor. Several bacteria, that include *P. gingivalis, T. forsythia, A. actinomycetemcomitans, T. denticola, V. recta, P. intermedia, F. nucleatum*, and *spirochetes spp.* have been associated with severe forms of periodontal disease (Elburki *et al*., 2018).

The most appropriate strategy for preventing periodontal diseases is consistent and thorough removal of plaque by personalised oral hygiene measures (Chaudhari *et al*., 2012). Plaque management may be achieved through mechanical methods and chemical methods. Mechanical plaque control methods such as brushing and use of dental floss are considered the most advised and effective methods to maintain optimal oral hygiene and periodontal health (Rajendiran *et al*., 2021). Earlier, mechanical therapy alone was considered as the most effective therapeutic option, however, due to the tissue invasiveness of periodontal pathogens, antimicrobial therapy alongwith mechanical therapy has gained attention in treating periodontal diseases (Shah *et al.*, 2015).

Although mechanical plaque control plays an essential role in preventing gingivitis and periodontitis, there are certain drawbacks associated with it. Firstly, the interproximal areas of posterior teeth are difficult to reach for brushing and therefore necessitate the use of additional dental tools such as dental floss. Secondly, mechanical plaque control targets only the hard surfaces of oral cavity and does not consider the soft tissue surfaces of oral cavity which act as an important niche for microbial accumulation and colonization on the tooth surfaces (Chaudhari *et al*., 2012; Teles *et al*., 2009).

To overcome these drawbacks, numerous chemical agents like quaternary ammonium compounds, bisbiguanides, fluorides, essential oils, sanguinarine and triclosan have been approved. These agents are available either in the form of toothpaste/ dentifrice or mouthwash (Chaudhari *et al*., 2012). Numerous studies have evaluated the ability of mouthwashes to affect plaque formation and to modify the course of gingival inflammation (Kukreja *et al*., 2012).

CHX a bisbiguanide and "gold standard" anti-plaque agent was developed in the UK (1940s). Both bacteriostatic and bactericidal effects are demonstrated, along with broad spectrum activity against viruses, yeasts, and gram positive and gram negative bacteria. Commercially, it is available in a variety of forms, like dental varnishes (1%, 10%,40%), gels (0.12 to 1%), sprays (0.12 to 0.2%) and oral rinses (0.02 to 0.3%). Among these, CHX mouthwashes demonstrated significantly better plaque inhibition than CHX based dentrifices and gels (Rajendiran *et al*., 2021; Deus *et al*., 2022; Richards *et al*., 2015; Supranoto *et al*., 2015). Prolonged use of chlorhexidine gluconate mouthwash is associated with several side effects, such as transient taste alteration (dysgeusia), brownish deposits on teeth, tongue, prosthetic crowns and cytotoxicity against human cells *in vitro*, resulting in cell necrosis and death, thereby affecting patient compliance (Rajendiran *et al*., 2021).

In the current world of increasing interaction between oral health and herbalism, the naturally occurring compounds found in herbal agents help in restoring health in the most beneficial way while minimizing adverse effects. Herbal medicine is an integrated system of therapeutic and preventive approaches, which uses plants and their extracts to treat a variety of diseases. To address the limitations associated with the use of CHX mouthwashes, several herbal alternatives such as tulsi patra, clove oil, triphala, green tea, neem, pudina and aloe vera have been developed, which have shown to be effective in management of a variety of oral conditions, encompassing gingival bleeding, halitosis, mouth ulcers and dental caries (Dabholkar *et al*., 2016).

Withania somnifera, a plant belonging to Solanaceae family, is also referred to as winter cherry, Indian ginseng, or Ashwagandha (Pandit *et al*., 2013). In Ayurveda, it is considered to be an important medicinal herb for more than 3000 years. India is inhabited by just two species of *Withania*, namely *Withania somnifera* and *Withania coagulans*. It is found in many parts of India such as Rajasthan, Himachal Pradesh, Punjab, Mediterranean parts of North Africa and Nepal. The principal active compounds found in the plant include several withanolide type compounds (Khanchandani *et al*., 2019). The root shares many common characteristics with Ginseng and has not demonstrated any toxicity when taken for prolonged period of time. Numerous pharmacological studies have reported the biological properties of this plant, such as antiinflammatory, anticancer, antistress, antioxidative, antibacterial, immunomodulatory and cardiovascular effects (Pandit *et al*., 2013; Alam *et al*., 2012). According to reports, the high content of polyphenols and antioxidant activity in the roots,

fruits and leaves of *Withania somnifera* may provide numerous benefits to human health. In a study conducted on methanolic extract of the *Withania somnifera* plant, strong antimicrobial activity was demonstrated against a variety of pathogenic microbes, including *E. coli, S. aureus, P. aeruginosa, S. mutans*, and *C. albicans* (Alam *et al.*, 2012).

Hence the present *in vitro* study aims to determine and compare antimicrobial and antibiofilm efficacy of *Withania somnifera* mouthwash and CHX gluconate mouthwash against *P. gingivalis, A. actinomycetemcomitans* and *P. intermedia*.

MATERIALS AND METHODS

Materials

For Extract Preparation

Withania somnifera root powder, 90% Methanol, Weighing Scale, Beaker, Conical Flask, Whatman No. 1 filter paper.

For MIC and MBC

Methanolic extract of *Withania somnifera*, ATCC strains of *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia*, Weighing Scale, Eppendorf Tubes, Micropipettes (10 µL, 100 µL, 1000 µL), Brain heart infusion culture broth, Anaerobic Jar, Incubator, Blood agar plates, Platinum Inoculum loops, Electric loop sterilizer For antimicrobial activity of *Withania somnifera* mouthwash against Porphyromonas gingivalis, *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* 90 mm diameter petri plates, Sterile cork borer, *Withania somnifera* mouthwash, commercially available 0.12% Chlorhexidine gluconate mouthwash and distilled water.

For *Withania somnifera* mouthwash preparation

Methanolic extract of *Withania somnifera*, Distilled water, Glass measuring cylinder, Weighing scale, Sterile container, Beaker, Whatman No. 1 filter paper, D-Mannitol, Tween 80 and Clove oil.

For antibiofilm activity of *Withania somnifera* mouthwash against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia*

ATCC strains of *A. actinomycetemcomitans, P. gingivalis, P. intermedia, Withania somnifera* mouthwash, 0.1% crystal violet, 0.12% CHX gluconate mouthwash, Phosphate buffer saline (PBS), 96 well microtiter plate, Micropipettes (10 µL, 100 µL, 1000 µL), Mueller Hinton broth, 95% Ethanol and Microplate reader.

The Research and Ethical Committee of KAHER's KLE V K Institute of Dental Sciences, Belagavi approved all experimental procedures. The laboratory procedures were carried out at Dr. Prabhakar Kore Basic Science Research Center (BSRC), KAHER, Belagavi. *Withania somnifera* roots were procured and authenticated at Shri B. M. Kankanwadi Ayurveda Mahavidyalaya, KAHER, Belagavi. The roots were then dried and ground into a fine powder using mortar and pestle. Methanolic extract of *Withania somnifera* was prepared at CRF (Ayush Approved Drug Testing Laboratory) and was subjected to phytochemical screening at Shri B. M. Kankanwadi Ayurveda Mahavidyalaya, KAHER, Belagavi. *Withania somnifera* mouthwash was formulated at KLE College of Pharmacy, KAHER, Belagavi. Commercially available 0.12% CHX gluconate mouthwash was used. The experiment was conducted in three groups. Blood agar plates were used for the experiment. Three agar wells of 8mm diameter were prepared using sterile cork borer where distilled water was added to one well which was considered as blank, 0.12% CHX gluconate mouthwash and *Withania somnifera* mouthwash were added to the other two wells.

Group 1: Blank (distilled water added), *Withania somnifera* mouthwash, 0.12% CHX gluconate mouthwash against *A. actinomycetemcomitans*.

Group 2: Blank (distilled water added), *Withania somnifera* mouthwash, 0.12% CHX gluconate mouthwash against *P. gingivalis*.

Group 3: Blank (distilled water added), *Withania somnifera* mouthwash, 0.12% CHX gluconate mouthwash against *P. intermedia*.

Methodology

Extract preparation

The dried roots of *Withania somnifera* were collected and authentication was done from Shri B M Kankanwadi Ayurveda Mahavidyalaya, KAHER, Belagavi, following which the roots were preserved in an airtight container and powdered. Also, *Withania somnifera* root was subjected to phytochemical screening and was reported to contain flavonoids, hexose sugar and steroids**.** The methanolic extract of the *Withania somnifera* root was formulated using cold maceration technique. About 100 g of *Withania somnifera* powder was added to a solution containing 1000 mL of methanol. The mixture was left to soak and kept in the "New Brunswick scientific Excella E24 Incubator Shaker Series" for 48 hr at room temperature for homogenous mixing. Then, the mixture was filtered with Whatman No.1 filter paper and subjected to evaporation using a hot water bath to obtain methanolic extract of *Withania somnifera*. The stock solution of extract was prepared by dissolving 100 mg of crude extract in 10 mL methanol at pH 7.0 prepared with concentration of 10 mg/mL and kept at 4 °C in the dark to prevent oxidation before being used (Figure 1a) (Alam *et al.*, 2012).

Inoculum preparation

The inoculum preparation was carried out using ATCC strains of *P. gingivalis, A. actinomycetemcomitans, P. intermedia* and sterile BHI broth. The colonies were picked up using a sterile loop and then placed in a tube with 5 mL of BHI broth and subsequently

incubated at 37 °C for 8 to 14 hr until the turbidity reached that of 0.5 McFarland standard. The turbidity of actively growing bacterial culture was calibrated by utilizing broth to match the turbidity of 0.5 McFarland guidelines (Wiegand *et al.*, 2008; CLSI 2024).

Broth dilution method [Resazurin] for determining Minimum Inhibitory Concentration

For broth preparation, 5.5 g of BHI powder was added to 150 mL water and stirred well. Subsequently, the broth was autoclaved at 121 °C and 15psi pressure. The broth was then cooled at room temperature under laminar air flow in an aseptic condition. Broth dilution was carried out in a sterilized 96-well plate which was carried out in triplicates. In the beginning, ten wells were chosen, and 100 µL of broth was poured into each of the ten wells in triplicates. *Withania somnifera* extract (100 µL) was added to the first well, and the extract was serially diluted to the needed concentration until the ninth well. Separate wells were employed for positive as well as negative controls, and same process was performed for the well plates of remaining two rows. A bacteriological incubator was then utilized for incubating 96-well plates for 48 hr. Following the incorporation of 30 µL of Resazurin reagent into each well that contained 100 µL of extract, the plates were incubated for an additional 4 hr in order to detect any possible color changes. Any shift in colour from blue or violet to light pink, pink, or magenta was documented as MIC. Outcomes were recorded through the use of high-quality photographs (Wiegand *et al.*, 2008).

Minimum Bactericidal Concentration (MBC)

MBC was checked using the MIC values of *Withania somnifera* extract with the help of blood agar plates. The agar plates for *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* was prepared by adding 6.24 g of BHI Agar powder to 120 mL of distilled water and autoclaved at 121 °C and 15 psi pressure. It was then kept at room temperature for 10-15 min followed by which 6 mL of blood, 60 µL of Vitamin K and 0.6 mL of Horse serum was added. It was then poured and allowed to solidify. With the help of inoculating loop, a loopful of each MIC sample was streaked on the agar plates. The plates were sealed with the parafilm and were incubated in the bacteriological incubator for 24 to 48 hr. At the end, minimum concentration at which the bacteria did not show any growth was considered as MBC value (Figure 1b) (Wiegand *et al.*, 2008).

Mouthwash preparation

Withania somnifera mouthwash was prepared using cold maceration technique at KLE College of Pharmacy, KAHER, Belagavi, by considering the MBC results obtained from the above procedures.

10 g of methanolic extract of *Withania somnifera* was weighed using an electronic weighing machine and transferred into a clean and dry beaker. Approximately 70 mL of distilled water was added, and the solution was continuously agitated on a magnetic stirrer until a uniform dispersion was obtained. After adding D-mannitol (5% w/v) to the above solution, stirring was continued

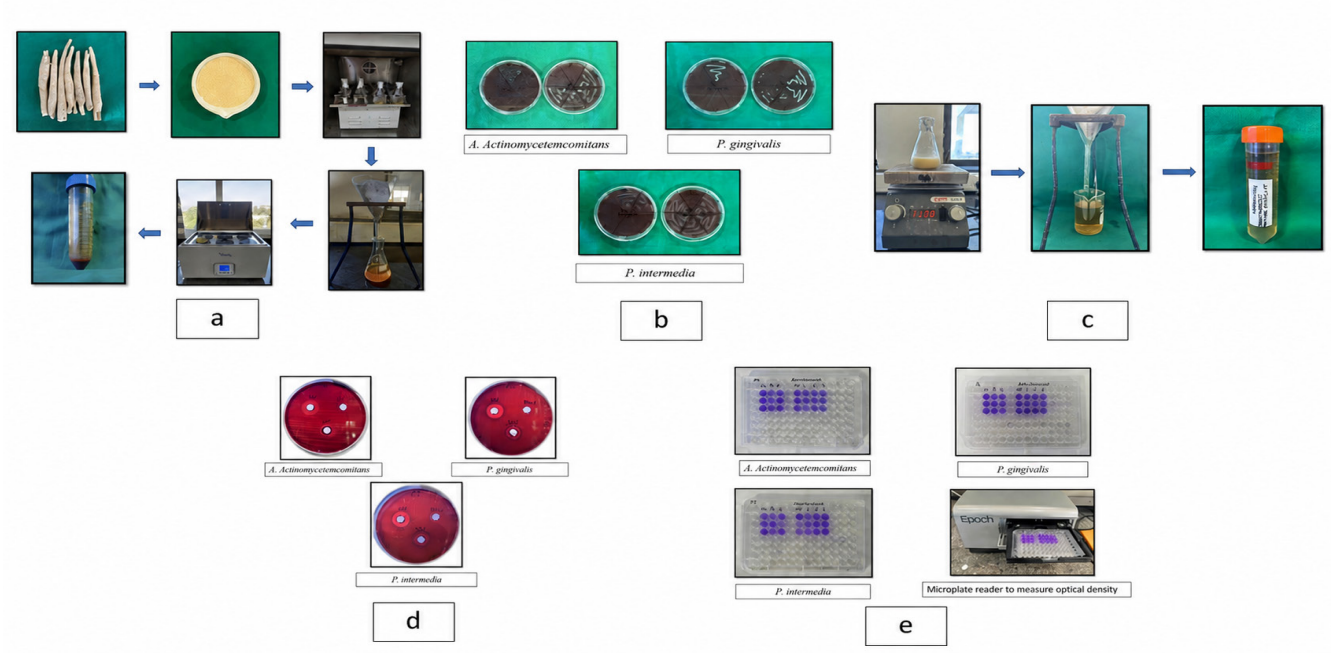


Figure 1: A: Steps involved in preparation of methanolic extract of *Withania somnifera*. B: MBC of methanolic extract of *Withania somnifera* against *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia*. C: Steps involved in formulation of *Withania somnifera* mouthwash. D: Agar well assay to assess antimicrobial efficacy of *Withania somnifera* and Chlorhexidine gluconate mouthwash against *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia*. e: Antibiofilm activity of *Withania somnifera* mouthwash assessed against *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia*.

Ingredients	Concentration	Uses
Withania somnifera extract	10 g	Active ingredient
D-Mannitol	5% v/v	Sweetening agent
Tween 80	1% w/v	Foaming and stabilizing agent
Clove oil	0.1% w/v	Flavouring agent
Distilled water	100 mL q.s.	Vehicle

until complete dissolution of the sweetening agent was achieved. Tween 80 (1%v/v) was then slowly added alongwith continuous stirring to ensure proper emulsification and stabilization of the solution. Clove oil (0.1% v/v) was gradually added to the solution, and the final volume of the mouthwash was made up to 100 mL using distilled water (Figure 1c) (Kukreja *et al.*, 2012).

Agar well diffusion assay to assess the antimicrobial activity of *Withania somnifera* mouthwash with Chlorhexidine gluconate mouthwash against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia.*

Agar well diffusion assay was carried out bacteriological agar plates in triplicates. The agar plates for *A. actinomycetemcomitans, P. gingivalis, P. intermedia* were made by dissolving 2.85g of MHA powder, 75 mL of distilled water. The plates were then sterilized in an autoclave set at 121 °C and 15psi of pressure following which 4 mL of blood, 60 µL of vitamin K, and 0.6 mL of horse serum were aseptically added to the medium after allowing it to cool at room temperature for 10 to 15 min. It was then transferred into sterile petri dishes and left to solidify. Bacterial suspensions of *A. actinomycetemcomitans P. gingivalis, P. intermedia* were taken [0.5 McFarland's] and spread all over the prepared agar plates using a sterile cotton swab by lawn culture method. Subsequently, uniform aseptic wells of 8mm diameter were created using a sterile cork borer. Each well was loaded with 100 µL of distilled water (blank), 100 µL of *Withania somnifera* mouthwash (test) and 100 µL of 0.12% chlorhexidine gluconate mouthwash (control). In a bacteriological incubator, the plates were kept for incubation at 37 °C for 24 to 72 hr, and observed for diffusion. Bacterial growth patterns were evaluated following incubation, and zones of inhibition surrounding each well were measured and results were obtained using 0.12% CHX gluconate mouthwash as the standard (Figure 1d) (Balouiri *et al.*, 2016, Perez *et al.*, 1990).

Table 1: Minimum Bactericidal Concentration (MBC).

Organism	MBC
Porphyromonas gingivalis	30
Aggregatibacter actinomycetemcomitans	30
Prevotella intermedia	50

All values are expressed in mg/mL against tested organisms.

Antibiofilm activity of *Withania Somnifera* mouthwash against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia*.

The antibiofilm activity of the prepared mouthwash was evaluated using crystal violet method on a 96 well plate in triplicates. Levofloxacin (0.00125 mg/mL), a positive control (PC), was added to one well, and 0.12% Chlorhexidine gluconate mouthwash was added to the other well. As a Negative Control (NC), 100 µL of Mueller Hinton broth was added. To these above-mentioned wells, 100 µL of bacterial culture were added. For the blank, 200 µL of Mueller Hinton broth without any bacterial culture was added. For the prepared mouthwash, three different concentrations, i.e 1%, 1.5% and 2% based on the MBC values, were taken. After that, the microtitre plate was carefully wrapped with a parafilm and incubated at 37°C for 48hr without any disturbance. The liquid was agitated out of the wells after incubation, and the plate was flipped. The liquid in each well was carefully removed, and the wells were gently rinsed with Phosphate-Buffered Saline (PBS) to wash away any loosely attached cells. The plate was then left to air dry. Next, 200µL of 0.1% crystal violet solution was added to every well and allowed to incubate at room temperature for 15 min to ensure proper staining. After incubation, excess stain was washed off by rinsing the wells three times with distilled water. To destain the wells, 150 µL of ethanol was added to each well. Finally, 100 µL of destaining solution was transferred to a fresh plate, and the absorbance was measured at 570nm on a microplate-based reader. Each assay was performed in triplicates following which mean optical densities

Table 2: Agar well diffusion assay to assess the antimicrobial activity of *Withania somnifera* mouthwash and 0.12% Chlorhexidine gluconate mouthwash against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia.*

Organism	Distilled water	0.12% CHX Mean±SD	*WS* mouthwash Mean±SD	*p* value
Porphyromonas gingivalis	0	18±1.0	14.67±0.58	0.023*
Aggregatibacter actinomycetemcomitans	0	19.0±1.73	14.67±0.58	0.023*
Prevotella intermedia	0	18.0±0.0	14.33±1.15	0.023*

All values are expressed in mm against tested organisms.

Table 3: Optical densities of negative control, positive control (Levofloxacin), Blank (Distilled water), 0.12% Chlorhexidine gluconate mouthwash and *Withania somnifera* mouthwash (1%, 1.5% and 2%) against *Porphyromonas gingivalis (PG), Aggregatibacter actinomycetemcomitans* (AA) and *Prevotella intermedia* (PI).

Organism	NC	PC	Blank	CHX	WS 1%	WS 1.5%	WS 2%
PG	2.08	0.95	0.89	1.01	1.28	1.08	1.03
AA	1.17	0.39	0.30	0.44	0.52	0.51	0.53
PI	2.15	0.49	0.29	0.40	0.69	0.73	0.90

Table 4: Percentage inhibition scores of five groups [Positive control (PC), 0.12% Chlorhexidine gluconate mouthwash (CHX) and three concentrations of *Withania somnifera* mouthwash 1%, 1.50% and 2%] against *Pg, Aa* and *Pi*.

Organism	PC	CHX	WS 1%	WS 1.5%	WS 2%	*p* value
PG	54.17±3.31	51.42±2.49	37.81±8.90	48.04±1.92	50.72±2.05	0.025*
AA	66.20±5.33	61.48±8.89	55.26±13.58	54.07±16.24	53.46±6.05	0.415
PI	76.65±7.69	81.61±2.85	67.75±1.81	66.08±2.10	57.62±7.44	0.037*

were calculated for NC, Blank, PC, 0.12% CHX mouthwash and three concentrations (1%, 1.5% and 2.5%) of the formulated *Withania somnifera* mouthwash (Figure 1e) (Table 3) and mean percentage inhibition scores were calculated for PC, 0.12% CHX mouthwash and three concentrations (1%, 1.5% and 2.5%) of the formulated *Withania somnifera* mouthwash (Table 4).

$$\text{Percentage inhibition} = \frac{OD_{\text{negative control}} - OD_{\text{sample x 100}}}{OD_{\text{negative control}}}$$

The percentage inhibition of *Withania somnifera* mouthwash was then compared with PC (levofloxacin) and 0.12% CHX mouthwash against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia.* (O'Toole *et al.*, 2011, Stepanović *et al.*, 2007).

Statistical Analysis

Comparison of three groups (Distilled water, 0.12% CHX gluconate mouthwash and *Withania somnifera* mouthwash) against *P. g* using Kruskal Wallis ANOVA. Comparison of three groups (Distilled water, 0.12% CHX gluconate mouthwash, gluconate mouthwash and *Withania somnifera* mouthwash) against A using Kruskal Wallis ANOVA. Comparison of three groups (Distilled water, 0.12% Chlorhexidine gluconate mouthwash and *Withania somnifera* mouthwash) against *P.i* using Kruskal Wallis ANOVA. Comparison of three organisms (*P.g, A.a and P.i*) against Blank (distilled water) using Kruskal Wallis ANOVA. Comparison of three organisms (*P.g, A.a and P.i*) against 0.12% Chlorhexidine gluconate mouthwash using Kruskal Wallis ANOVA. Comparison of three organisms (*P.g, A.a and P.i*) against *Withania somnifera* mouthwash using Kruskal Wallis ANOVA. Comparison of antibiofilm activity (percentage inhibition) of five groups: PC, 0.12% CHX mouthwash and three concentrations of *Withania somnifera* mouthwash (1%, 1.50% and 2%) in *P. g*. Comparison of antibiofilm activity (percentage inhibition) of five groups: PC, 0.12% CHX mouthwash and three concentrations of *Withania somnifera* mouthwash (1%, 1.50% and 2%) in *A. a*. Comparison of antibiofilm activity (percentage inhibition) of five groups: PC, 0.12% CHX mouthwash and three concentrations of *Withania somnifera* mouthwash (1%, 1.50% and 2%) in *P. i*. A probability value below 0.05 was deemed statistically significant.

RESULTS

MIC and MBC for the methanolic extract of *Withania somnifera* was determined. Agar well diffusion assay (zone of inhibition) was utilized for determining antimicrobial efficacy of 100 µl of 0.12% CHX gluconate mouthwash (standard), 100 µL of *Withania somnifera* mouthwash (test) and 100 µL of blank (distilled water) against standard ATCC strains, namely *P. gingivalis, A. actinomycetemcomitans*, and *P. intermedia*. The mean optical densities of NC, PC, Blank, 0.12% CHX gluconate mouthwash and 1%, 1.5% and 2% concentrations of *Withania somnifera* mouthwash were determined. Based on the mean optical density values, mean percentage inhibition scores were calculated and compared among five groups, i.e., PC, 0.12% CHX gluconate mouthwash and three concentrations of *Withania somnifera* mouthwash (1%, 1.50% and 2%) based on MBC values.

Minimum Inhibitory Concentration (MIC)

The MIC of methanolic extract of *Withania somnifera* against *P. gingivalis, P. intermedia, A. actinomycetemcomitans* could not be determined, as the extract interfered with the activity of resazurin dye, and colour change could not be appreciated to determine MIC values.

Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal concentration of methanolic extract of *Withania somnifera* for *P. gingivalis* was 30 mg/mL, for *A. actinomycetemcomitans* was 30 mg/mL and for *P. intermedia* was 50 mg/mL (Table 1).

Agar well diffusion assay to assess the antimicrobial activity of *Withania somnifera* mouthwash and 0.12% Chlorhexidine

gluconate mouthwash against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia*.

There was no growth noted w.r.t Blank (distilled water) against all the three organisms. The zone of inhibition of 0.12% CHX gluconate mouthwash was 18 mm for *P. gingivalis*, 19 mm for A. actinomycetemcomitans, and 18 mm for *P. intermedia*, whereas that of *Withania somnifera* mouthwash was 14.6 mm for *P. gingivalis*, 14.6 mm for *A. actinomycetemcomitans* and 14.3 mm for *P. intermedia* (Table 2).

Optical densities of negative control, positive control (Levofloxacin), Blank (Distilled water), 0.12% Chlorhexidine gluconate mouthwash and *Withania somnifera* mouthwash (1%, 1.5% and 2%) against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* for P. g, 2% *Withania somnifera* mouthwash demonstrated lowest mean optical density of 1.03 indicating maximum reduction in biofilm formation compared to 1.5% and 1% concentrations. For A. a, 1.5% *Withania somnifera* mouthwash demonstrated lowest mean optical density of 0.51 with maximum reduction in biofilm formation indicating that increasing concentration beyond 1.5% did not improve antibiofilm activity against A.a. For *P. i*, 1% *Withania somnifera* mouthwash demonstrated lowest mean optical density of 0.69 with maximum reduction in biofilm formation indicating that higher concentrations do not necessarily improve antibiofilm activity against *P. i* (Table 3).

Percentage inhibition scores (antibiofilm activity) of five groups [Positive control (PC), 0.12% Chlorhexidine Gluconate Mouthwash (CHX) and three concentrations of *Withania somnifera* mouthwash 1%, 1.50% and 2%] against Pg, Aa and Pi Based on the obtained mean optical densities (Table 3), the mean percentage inhibition scores were calculated for the five groups (Table 4). For P. g, 2% *Withania somnifera* mouthwash demonstrated highest percentage inhibition, while 1% *Withania somnifera* mouthwash demonstrated lowest percentage inhibition. For A. a, the percentage inhibition scores were comparable for all three concentrations of *Withania somnifera* mouthwash. For *P. i*, 1% *Withania somnifera* mouthwash demonstrated highest percentage inhibition. However, percentage inhibition scores were highest for PC and 0.12% CHX gluconate mouthwash against all the three organisms.

DISCUSSION

The present study aimed to assess and compare the antimicrobial efficacy of *Withania somnifera* mouthwash and chlorhexidine gluconate mouthwash against *P. gingivalis, A. actinomycetemcomitans* and *P. intermedia*.

Dental plaque consists of a microbial community adhering to tooth surface and encased in a matrix of polymers produced by host and bacteria. There are different stages of dental plaque formation such as acquired pellicle formation followed by co-adhesion where the secondary colonizers adhere to primary colonisers and sometimes detachment, which ultimately result in plaque formation (Marsh *et al*., 2006). Transition from periodontal health to disease is associated with a significant shift in microflora from gram-positive to gram-negative bacteria, which ultimately results in inflammatory response and periodontal destruction (Theilade *et al*., 1996).

Several bacteria, such as *P. gingivalis, T. forsythia, T. denticola, A. actinomycetemcomitans, F. nucleatum, P. intermedia, V. recta and Spirochetes spp.,* have been associated with severe forms of periodontal disease (Elburki *et al*., 2018). The most appropriate strategy for preventing periodontal diseases is consistent and thorough removal of plaque by personalised oral hygiene measures (Chaudari *et al*., 2012). Mechanical plaque control methods such as brushing and use of dental floss are considered the most advised and effective methods to maintain optimal oral hygiene and periodontal health (Rajendiran *et al*., 2021). Although mechanical plaque control plays an essential role in preventing gingivitis and periodontitis, there are certain drawbacks associated with it. To overcome these drawbacks, chemical plaque control agents like essential oils, fluorides, quaternary ammonium compounds, bisbiguanides, sanguinarine and triclosan have been approved, that are available either in the form of toothpaste/dentifrice or mouthwash (Chaudhari *et al*., 2012; Teles *et al*., 2009).

CHX, a bisbiguanide and "gold standard" anti-plaque agent, was developed in the UK (1940s). It exhibits both bacteriostatic and bactericidal effects, along with broad spectrum activity against yeasts, viruses and gram positive and gram negative bacteria. Its commercially accessible forms include dental varnishes (1%, 10%, 40%), sprays (0.12 to 0.2%), gels (0.12 to 1%) and oral rinses (0.02 to 0.3%). Among these, CHX mouthwashes demonstrated significantly better plaque inhibition than CHX based dentrifices and gels (Rajendiran *et al*., 2021; Deus *et al*., 2022; Richards *et al*., 2015; Supranoto *et al*., 2015).

In a study, 0.12% CHX mouthwash demonstrated significant antimicrobial activity with mean zones of inhibition of 18 mm, 20 mm & 22 mm against *P. i, A. a* and *P. g*, respectively, when compared to CPC and a herbal mouthwash (Sandhu *et al*., 2025). In a different research, significant reduction in PD and CAL was reported when CHX mouthwash was employed as an adjunct to SRP in comparison to SRP alone (Da Costa *et al*., 2017).

However, prolonged use of CHX mouthwash is associated with numerous side effects such as transient taste alteration, dry mouth, brownish deposits on teeth and tongue, parotid gland swelling and development of cross resistance to antibiotics (Deus *et al*., 2022).

To address the limitations associated with the use of CHX mouthwashes, several herbal alternatives such as clove oil, triphala, green tea, tulsi patra, neem, pudina and aloe vera have

been developed, which have shown to be effective in management of numerous oral conditions, encompassing gingival bleeding, halitosis, mouth ulcers and dental caries (Dabholkar *et al.*, 2016).

Withania somnifera, commonly referred to as Ashwagandha, Indian Ginseng or winter cherry, is a plant belonging to Solanaceae family. In Ayurveda, it has been regarded as an important medicinal herb for more than 3000 years (Pandit *et al.*, 2013). In a study it was found that methanolic extract of the *Withania somnifera* plant exhibited strong antimicrobial activity against a variety of pathogenic microbes, specifically *E. coli, P. aeruginosa, S. aureus, S. mutans, and C. albicans* (Alam *et al.*, 2012).

In the current study, the antimicrobial efficacy of *Withania somnifera* mouthwash was assessed and compared to 0.12% CHX gluconate mouthwash.

The MIC of methanolic extract of *Withania somnifera* could not be determined as the extract interfered with the activity of resazurin dye, and colour change could not be appreciated to determine MIC values.

The MBC of methanolic extract of *Withania somnifera* was 30 mg/mL for *P. gingivalis,* 30 mg/mL for *A. actinomycetemcomitans* and 50 mg/mL for *P. intermedia,* as shown in (Table 1).

The antimicrobial activity of *Withania somnifera* mouthwash was determined with the use of agar well diffusion assay. The zone of inhibition of 0.12% CHX gluconate mouthwash was 18 mm for *P. gingivalis,* 19 mm for *A. actinomycetemcomitans,* and 18 mm for *P. intermedia,* whereas that of *Withania somnifera* mouthwash was 14.6 mm for *P. gingivalis,* 14.6 mm for *A. actinomycetemcomitans* and 14.3 mm for *P. intermedia* (Table 2). These results suggest that 0.12% CHX gluconate mouthwash demonstrated a wider zone of inhibition compared to *Withania somnifera* mouthwash.

Mean and standard deviation of zone of inhibition for *P. g* with 0.12% CHX gluconate mouthwash was 18.00±1.00, and *Withania somnifera* mouthwash was 14.67±0.58. For *A. a, the* mean and standard deviation with 0.12% CHX gluconate mouthwash was 19.00±1.73 and *Withania somnifera* mouthwash was 14.67±0.58. For *P. i,* the mean and standard deviation with 0.12% CHX gluconate mouthwash was 19.00±1.73 and with *Withania somnifera* mouthwash was 14.67±0.58. There was no zone of inhibition noted with blank (distilled water).

The intergroup comparison of Blank (Distilled water), 0.12% CHX gluconate mouthwash was done using Kruskal Wallis ANOVA which showed statistically significant difference ($p<0.05$) for *P. g, A. a* and for *P. i* (Figures 2a-2c). These findings suggest statistically significant antimicrobial activity of *Withania somnifera* mouthwash when compared to 0.12% Chlorhexidine gluconate mouthwash. This significant antimicrobial activity may be attributed to the presence of flavonoids and steroids in *Withania somnifera*.

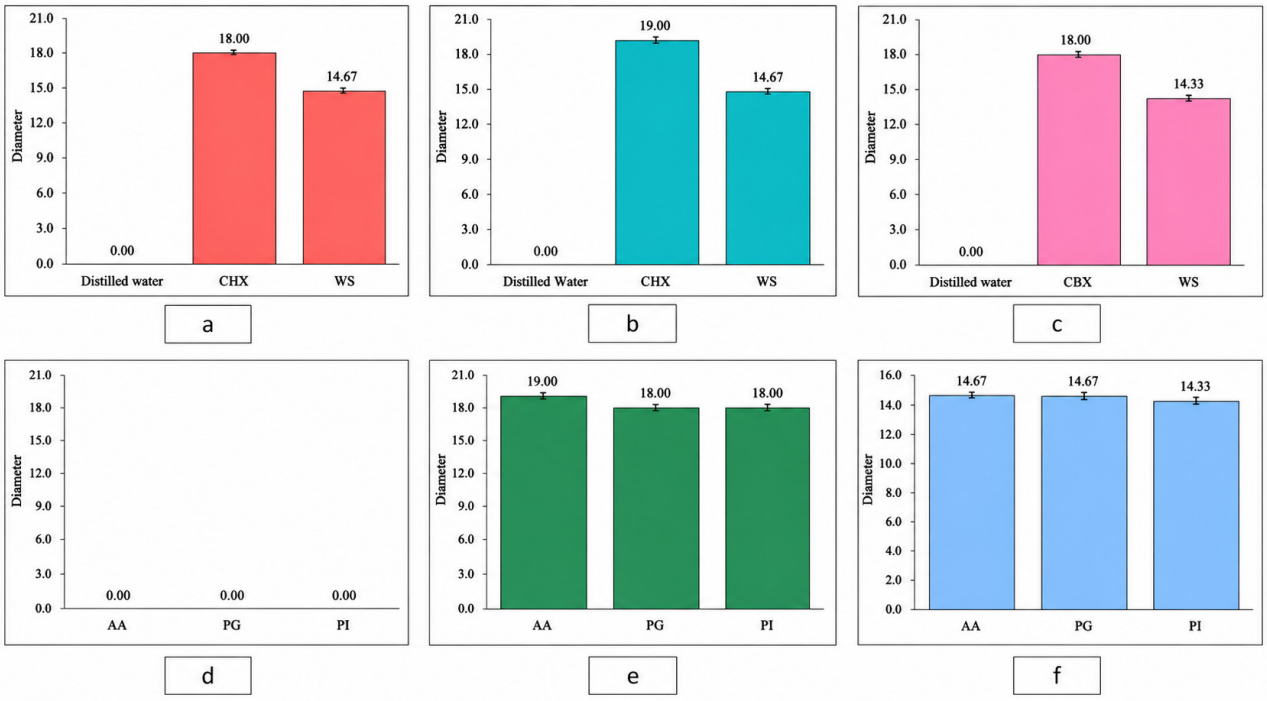


Figure 2: A: Comparison of three groups (Distilled water, 0.12% Chlorhexidine gluconate mouthwash and *Withania somnifera* mouthwash) against *P. gingivalis*. B: Comparison of three groups (Distilled water, 0.12% Chlorhexidine gluconate mouthwash and *Withania somnifera* mouthwash) against *A. actinomycetemcomitans*. C: Comparison of three groups (Distilled water, 0.12% Chlorhexidine gluconate mouthwash and *Withania somnifera* mouthwash) against *P. intermedia.* D: Comparison of three organisms (*A. actinomycetemcomitans, P. gingivalis* and *P. intermedia)* against Blank (Distilled water). e: Comparison of three organisms (*A. actinomycetemcomitans, P. gingivalis* and *P. intermedia)* against 0.12% Chlorhexidine gluconate mouthwash. f: Comparison of three organisms (*A. actinomycetemcomitans, P. gingivalis* and *P. intermedia)* against *Withania somnifera* mouthwash.

The intragroup comparison of three organisms (*P.g, A.a and P.i*) was done using Kruskal Wallis ANOVA, which showed no statistically significant difference with 0.12% Chlorhexidine gluconate mouthwash and *Withania somnifera* mouthwash (Figures 2d-2f).

The present study's results are in accordance with another study which evaluated the antimicrobial activity of methanolic extract of *Withania somnifera* leaf. According to the findings, methanolic extract of *Withania somnifera* leaf demonstrated a zone of inhibition of 20.6 mm against *MRSA* and 19.4 mm against *Enterococcus spp.* indicating its therapeutic use against wide range of microorganisms (Bisht *et al.*, 2014).

In another study, the antimicrobial activity of ethanolic and methanolic extracts of *Withania somnifera* leaf was assessed using agar well diffusion assay. Findings suggest that methanolic extract demonstrated significantly better antimicrobial activity than ethanolic extract with highest zone of inhibition (25.13 mm) against *S. typhimurium* and least zone of inhibition (21.33 mm) against *V. cholera* when compared to standard antibiotics (kanamycin and neomycin) (Moharana *et al.*, 2021).

The findings of the current study demonstrated a statistically significant difference in antimicrobial activity between 0.12% CHX gluconate mouthwash and *Withania somnifera* mouthwash ($p<0.05$). However, 0.12% CHX gluconate mouthwash demonstrated a wider zone of inhibition when compared to *Withania somnifera* mouthwash against *P.g, A.a and P.i* indicating superior antimicrobial activity of 0.12% CHX gluconate mouthwash when compared to *Withania somnifera* mouthwash.

Biofilm formation by periodontal pathogens is an important virulence factor that contributes to the chronic nature and treatment resistance of periodontal diseases. Due to presence of an EPS matrix, decreased metabolic activity and modified gene expression patterns, mature biofilms demonstrate increased resistance to antimicrobial agents (Costerton *et al.*, 1999).

In the present *in vitro* study, the formulated *Withania somnifera* mouthwash demonstrated a concentration dependent inhibition of biofilm formation against *P.g, A.a and P.i* which was assessed using crystal violet method. The antibiofilm activity of *Withania somnifera* mouthwash was evaluated by comparing the percentage inhibition scores of three concentrations of *Withania somnifera* mouthwash based on MBC values (1%, 1.5% and 2%) with PC and 0.12% CHX mouthwash against *P. g, A. a and P. i*. The results suggest that *Withania somnifera* interferes with bacterial adhesion and early stages of biofilm maturation (Donlan *et al.*, 2002). Phytochemical constituents like withanolides, flavonoids, alkaloids, and tannins, which are known to damage bacterial cell

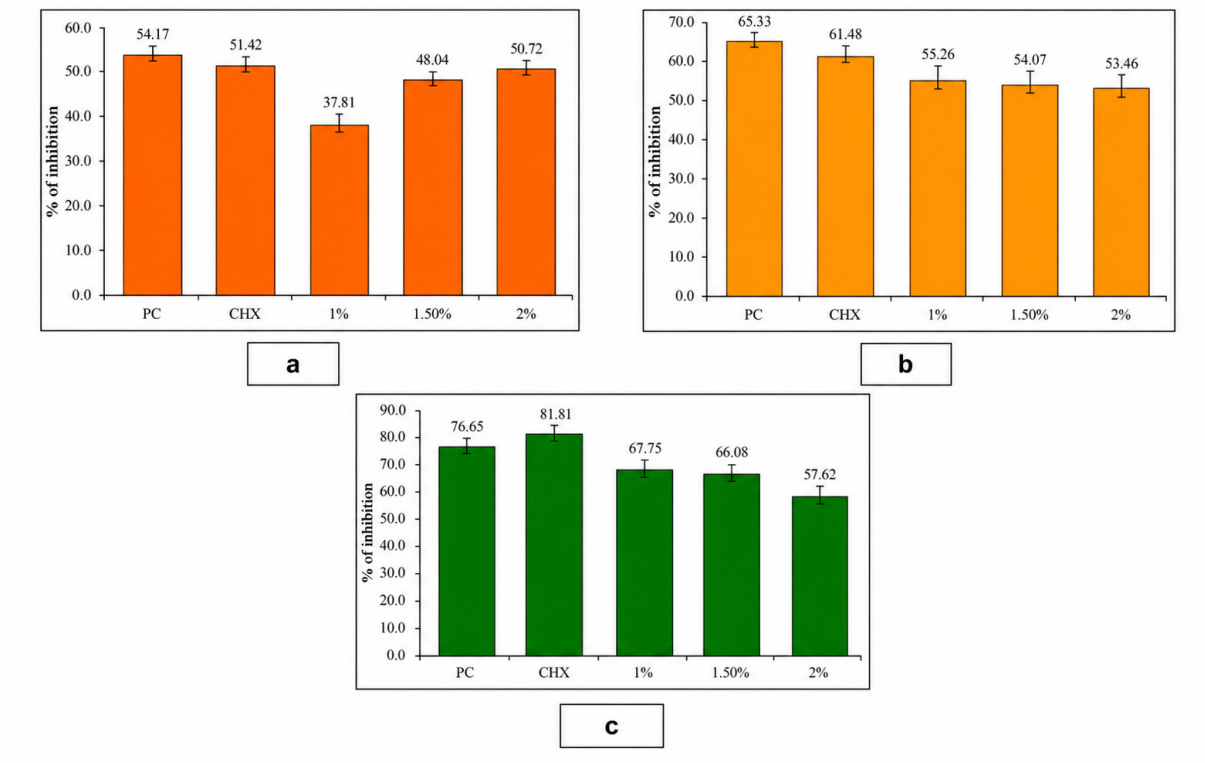


Figure 3: A: Comparison of antibiofilm activity (percentage inhibition) of five groups: Positive control (PC), 0.12% Chlorhexidine gluconate mouthwash (CHX) and three concentrations of *Withania somnifera* mouthwash (1%, 1.50% and 2%) against *P. gingivalis*. B: Comparison of antibiofilm activity (percentage inhibition) of five groups: Positive control (PC), 0.12% Chlorhexidine gluconate mouthwash (CHX) and three concentrations of *Withania somnifera* mouthwash (1%, 1.50% and 2%) against *A. actinomycetemcomitans*. C: Comparison of antibiofilm activity (percentage inhibition) of five groups: Positive control (PC), 0.12% Chlorhexidine gluconate mouthwash (CHX) and three concentrations of *Withania somnifera* mouthwash (1%, 1.50% and 2%) against *P. intermedia*.

membrane, impair quorum sensing and reduce generation of EPS may be responsible for the antibiofilm activity against tested periodontal pathogens (Mirjalili *et al*., 2009; Mishra *et al*., 2000).

Mean and standard deviation for PC were 54.17±3.31 and for CHX were 51.42±2.49 whereas that for 1% *Withania somnifera* mouthwash was 37.81±8.90, for 1.5% *Withania somnifera* mouthwash was 48.04±1.92 and for 2% *Withania somnifera* mouthwash was 50.72±2.05 for *P. g.* The intergroup comparison of percentage inhibition of five groups (PC, CHX, 1%, 1.5% and 2% *Withania somnifera* mouthwash) "showed statistically significant difference" for *P. g* (p<0.05) (Table 4) (Figure 3a).

Mean and standard deviation for PC was 66.20±5.33 and for CHX was 61.48±8.89, whereas that for 1% *Withania somnifera* mouthwash was 55.26±13.58, for 1.5% *Withania somnifera* mouthwash was 54.07±16.24 and for 2% *Withania somnifera* mouthwash was 53.46±6.05 for *A. a*. The intergroup comparison of percentage inhibition of five groups (PC, CHX, 1%, 1.5% and 2% *Withania somnifera* mouthwash) "showed no statistically significant difference" for *A. a* (Figure 3b). The observed difference in susceptibility may be related to the pronounced invasive characteristics, facultative anaerobic nature and intracellular persistence of *A. actinomycetemcomitans*, resulting in decreased sensitivity towards plant derived phytochemical compounds (Slots *et al*., 1999).

Mean and standard deviation for PC was 76.65±7.69 and for CHX was 81.61±2.85 whereas mean and standard deviation for 1% *Withania somnifera* mouthwash was 67.75±1.81, for 1.5% *Withania somnifera* mouthwash was 66.08±2.10 and for 2% *Withania somnifera* mouthwash was 57.62±7.44 for *P. i.* The intergroup comparison of percentage inhibition of five groups (PC, CHX, 1%, 1.5% and 2% *Withania somnifera* mouthwash) "showed statistically significant difference" for *P. i* (Table 4) (Figure 3c).

The present study's results are in accordance with those of a study that evaluated the antimicrobial and antibiofilm activity of *Withania somnifera* root extract against the multidrug-resistant pathogens. The average biofilm inhibition for *Withania somnifera* root extract was determined utilizing crystal violet method. The results indicate that aqueous root extract of *Withania somnifera* demonstrated an average biofilm inhibition of 55 to 68% compared to controls and also inhibited the production of EPS and reduced the density of the matrix which might be attributed to phytochemicals in plant (Singh *et al*., 2024).

Another study assessed antimicrobial and antibiofilm properties of methanolic leaf extract of *Withania somnifera* against *P. aeruginosa* and *E. coli.* The findings imply that methanolic extract of *Withania somnifera* leaf reported a significant biofilm reduction of 50-60% against *E. coli* and 45-55% against *P. aeruginosa* when compared to control which may be due to inhibition of bacterial adhesion and early biofilm maturation by the phytochemical constituents present in the plant leaf extract (Ahmed *et al*., 2018).

Although 0.12% chlorhexidine gluconate mouthwash demonstrated superior antimicrobial and antibiofilm activity, its prolonged use is associated with numerous side effects (Deus *et al*., 2022). Thus, results of the present study suggest that formulated *Withania somnifera* mouthwash demonstrated significant antimicrobial and antibiofilm activity against tested periodontal pathogens and could be used as an alternative to 0.12% chlorhexidine gluconate mouthwash in periodontal therapy.

CONCLUSION

The present study aimed to assess and compare the antimicrobial and antibiofilm efficacy of *Withania somnifera* mouthwash and CHX gluconate mouthwash against *P. gingivalis, A. actinomycetemcomitans*, and *P. intermedia.* The formulation of *Withania somnifera* mouthwash used in the present study was done at KLE College of Pharmacy, KAHER Belagavi, based on the MBC results obtained. All the laboratory procedures were performed at Dr. Prabhakar Kore Basic Science Research Center (BSRC), KAHER Belagavi

The MBC of methanolic extract of *Withania somnifera* for *P. gingivalis* was 30 mg/mL, for *A. actinomycetemcomitans* was 30 mg/mL and for *P. intermedia* was 50 mg/mL. Phytochemical analysis of methanolic extract of *Withania somnifera* demonstrated presence of flavonoids and steroids, which may have resulted in significant antimicrobial and antibiofilm activity. The zone of inhibition of 0.12% CHX gluconate mouthwash was 18 mm against *P. gingivalis,* 19mm against *A. actinomycetemcomitans,* and 18 mm against *P. intermedia*, whereas that of *Withania somnifera* mouthwash was 14.6 mm against *P. gingivalis,* 14.6 mm against *A. actinomycetemcomitans* and 14.3 mm against *P. intermedia*. Percentage inhibition of Positive control (Levofloxacin) was 54.17%, 0.12% CHX gluconate mouthwash was 51.42% whereas that of *Withania somnifera* mouthwash 1% was 37.81%, 1.5% was 48.04% and 2% was 50.72% against *P. gingivalis*. Percentage inhibition of Positive control (Levofloxacin) was 66.20%, 0.12% CHX gluconate mouthwash was 61.48% whereas that of *Withania somnifera* mouthwash 1% was 55.26%, 1.5% was 54.07% and 2% was 53.46% against *A. actinomycetemcomitans.* Percentage inhibition of Positive control (Levofloxacin) was 76.65%, 0.12% CHX gluconate mouthwash was 81.61% whereas that of *Withania somnifera* mouthwash 1% was 67.75%, 2% was 66.08% and 2% was 57.62% against *P. intermedia*.

The results of the present study indicate that although 0.12% chlorhexidine gluconate mouthwash demonstrated superior antimicrobial activity than *Withania somnifera* mouthwash against *P. gingivalis, A. actinomycetemcomitans* and *P. intermedia*, its prolonged use is associated with several side effects. Conversely, the formulated *Withania somnifera* mouthwash exhibited

significant antimicrobial and antibiofilm activity against tested periodontal pathogens, thus it could be used as an alternative to 0.12% CHX gluconate mouthwash in periodontal therapy.

Also, among the three concentrations of the formulated *Withania somnifera* mouthwash, 2% *Withania somnifera* mouthwash demonstrated a significant percentage inhibition of 50.72% against *P. gingivalis,* 1% *Withania somnifera* mouthwash demonstrated a significant percentage inhibition of 55.22% against *A. actinomycetemcomitans,* and 1% *Withania somnifera* mouthwash showed a significant percentage inhibition of 67.75% against *P. intermedia,* approaching the efficacy of 0.12% CHX gluconate mouthwash.

Within the limitations of the present *in vitro* study, further research incorporating biofilm models, cytotoxicity assessments and well-designed randomized controlled clinical trials are required to assess the clinical efficacy, safety and long-term benefits of the formulated *Withania somnifera* mouthwash.

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ABBREVIATIONS

ATCC: American Type Culture Collection; **A. a:** *Aggregatibacter actinomycetemcomitans*; **BHI:** Brain Heart Infusion; **CHX:** Chlorhexidine gluconate; **CAL:** Clinical Attachment Level; **CPC:** Cetylpyridinium chloride; **EPS:** Extracellular polymeric substance; **GI:** Gingival Index; **mm:** millimeter; **mg:** milligram; **mL:** milliliter; **MIC:** Minimum Inhibitory Concentration; **MBC:** Minimum Bactericidal Concentration; **MHA:** Mueller Hinton Agar; **MRSA:** Methicillin-Resistant *Staphylococcus aureus*; **NC:** Negative control; **OD:** Optical Density; **P. g:** *Porphyromonas gingivalis*; **P. i:** *Prevotella intermedia*; **PC:** Positive control; **PD:** Probing Depth; **PI:** Plaque Index; **PBS:** Phosphate Buffered Saline; **SRP:** Scaling and Root Planing; **WS:** *Withania somnifera*; **ZOI:** Zone of Inhibition.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

Kirti A. Kamat: Study designing, conceptualization, manuscript drafting, data collection, analysis and interpretation; **Ms. Sayali Manjarekar:** Data collection; **Renuka Metgud and Suneel Dodamani:** Study design, conceptualization, manuscript review and supervision. **Archana Patil and Priya Shetti:** Manuscript drafting, Manuscript review and supervision. All authors reviewed the results and approved the final version of the manuscript.

SUMMARY

The formulated *Withania somnifera* mouthwash demonstrated significant antimicrobial and antibiofilm activity against *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*, thus it could be used as an alternative to 0.12% CHX gluconate mouthwash in periodontal therapy.

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