Journal of Oral Biology and Craniofacial Research 14 (2024) 522–529

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr

Clinical and microbial evaluation of mouthwash containing *Achyranthes aspera* and *Trachyspermum ammi*: A randomized controlled non-inferiority trial

Ram Surath Kumar [a], Anil V. Ankola [a,*], Roopali M. Sankeshwari [a], Vinuta Hampiholi [b], Sagar Jalihal [a], Atrey J. Pai Khot [a,c], Varkey Nadakkavukaran Santhosh [a], Kavitha Ragu [a]

[a] Department of Public Health Dentistry, KLE Vishwanath Katti Institute of Dental Sciences, KLE Academy of Higher Education and Research, Belagavi, 590010, India
[b] Department of Periodontics, KLE Vishwanath Katti Institute of Dental Sciences, KLE Academy of Higher Education and Research, Belagavi, 590010, India
[c] Department of Public Health Dentistry, Goa Dental College and Hospital, Bambolim, Goa, India

ARTICLE INFO

Keywords:
Antibacterial
Chlorhexidine
Dental plaque
Gingivitis
Herbal
Periodontal pathogen
Real-time polymerase chain reaction
RT-PCR

ABSTRACT

Objectives: *Achyranthes aspera* (Apamarga) and *Trachyspermum ammi* (Ajwain) have been used in many clinical conditions, and it displays valuable properties as an alternative to Chlorhexidine (CHX) in the management of gingivitis. Therefore, this study aims to assess the effect of *Achyranthes aspera* and *Trachyspermum ammi* (AA + TA) based herbal mouthwash, 0.2 % CHX, and placebo mouthwash on gingival health, plaque control and antibacterial activity against specific periodontal pathogens (*Porphyromonas gingivalis* and *Tannerella forsythia*) using quantitative real-time PCR (RT-PCR).

Methods: This was a randomized controlled non-inferiority trial involving 108 children with plaque-induced gingivitis who were randomly assigned to three groups of 36 children each: Group A, AA + TA mouthwash; Group B, CHX mouthwash; and Group C, placebo mouthwash. Gingival index and plaque index were recorded at baseline, 7th and 21st day. RT-PCR was employed to determine the bacterial counts of each plaque sample at baseline and after 21 days.

Results: All three groups exhibited a gradual and significant reduction in both gingival and plaque scores from baseline to days 7 and 21. However, the placebo group did not demonstrate a significant difference in scores between days 7 and 21. Furthermore, a significant reduction in bacterial counts of *P. gingivalis* and *T. forsythia* was observed in the groups receiving CHX and AA + TA mouthwash after 21 days of intervention compared to the placebo group.

Conclusion: AA + TA mouthwash demonstrated non-inferiority in anti-gingivitis and anti-plaque properties compared to CHX, suggesting its potential suitability as an alternative to CHX when used in conjunction with mechanical plaque control measures.

1. Introduction

Gingivitis, a commonly occurring oral disease marked by bleeding and inflammation of the gingiva, is primarily caused by microorganisms and inadequate oral hygiene. The formation of dental plaque on the teeth and gingiva is the key contributor to gingivitis. If not addressed, this condition can advance to periodontitis, potentially leading to premature tooth loss.[1]

Porphyromonas gingivalis and *Tannerella forsythia*, members of red complex pathogens are strongly associated with the onset and progression of periodontal diseases. Moreover, a significant positive correlation exists between the red complex pathogens and indicators such as bleeding on probing and periodontal pocket depth.[2]

Mechanical plaque control techniques are the mainstay in maintaining oral hygiene which requires time, skill, and motivation for optimal effectiveness.[3] As a result, antimicrobial agents are commonly utilized as adjuncts to mechanical plaque control techniques. Chlorhexidine (CHX) has been the gold standard since the 1940s because of its

* Corresponding author.
E-mail addresses: ramsurathkumar1996@gmail.com (R.S. Kumar), dranilankola@kledental-bgm.edu.in (A.V. Ankola), drroopali@kledental-bgm.edu.in (R.M. Sankeshwari), drvinutahampiholi@kledental-bgm.edu.in (V. Hampiholi), drsagarjalihal@kledental-bgm.edu.in (S. Jalihal), atreypaikhot01@gmail.com (A.J. Pai Khot), nsvarkey29@gmail.com (V.N. Santhosh), kavi7komala@gmail.com (K. Ragu).

https://doi.org/10.1016/j.jobcr.2024.06.006
Received 30 May 2024; Received in revised form 26 June 2024; Accepted 27 June 2024

antibacterial efficacy and substantivity. Despite its various advantages, CHX being a chemical, bears various adverse effects when used for a longer period.[4]

Traditional medicine has gained popularity, due to its affordability, therapeutic value, and reputation for having fewer side effects compared to synthetic drugs.[5] The reason for choosing *Achyranthes aspera* (Apamarga roots) and *Trachyspermum ammi* (Ajwain seeds) is their traditional long-standing history as effective antimicrobial and anti-inflammatory agents.[6–8] In ancient times, fresh roots of *A. aspera* were used as a toothbrush in routine oral hygiene practices.[8,9] Currently, there are no studies in the literature that have explored the clinical and microbiological effectiveness of the newly introduced mouthwash containing *A. aspera* and *T. ammi* (AA + TA) for the prevention and management of gingivitis. Therefore, the present study aims to assess the effect of herbal mouthwash containing *A. aspera* and *T. ammi* (AA + TA), 0.2 % CHX, and placebo mouthwash on gingival health, plaque control and antibacterial activity against specific periodontal pathogens (*P. gingivalis* and *T. forsythia*) using quantitative real-time PCR (RT-PCR). The hypothesis of the present study states that the *A. aspera and T. ammi* (AA + TA) based herbal mouthwash is non-inferior to 0.2 % CHX mouthwash in children with plaque-induced gingivitis.

2. Materials and methods

2.1. Study design and ethical approval

This study was structured as a randomized, triple-arm, parallel-group, placebo-controlled, non-inferiority clinical trial conducted at a government school in the Belagavi District, India, spanning from October to November 2022. Ethical approval was obtained from the Institutional Research and Ethics Committee (IRB number: EC/NEW/2021/2435) with the reference number 1447, dated 28.08.2021. The research adhered to the ethical principles governing human experimentation, aligning with the Helsinki Declaration of 1975, as revised in 2000. The study was registered on the Clinical Trials Registry-India platform as CTRI/2022/03/041423. CONSORT statement to report trials of herbal interventions was adhered.[10]

2.2. Study participants

The study involved children aged 12–15 years who had moderate gingivitis, as assessed by the gingival index.[11] Children and their caregivers who gave their assent and provided written informed consent were recruited into the study. Children with other systemic conditions; allergic reactions in the past; under medications for the last three months or the use of antibacterial mouthwash within the last four weeks, and those with physical or cognitive disabilities were excluded.

2.3. Sample size calculation

The GPower software (G*Power Version 3.1.9.4) was used to calculate the sample size for the study. The sample size was estimated based on a previous study by Singhal et al., 2018,[12] which reported a mean reduction in gingival score of 0.45 ± 0.08. To detect an effect size of 0.75 with a non-inferiority margin of 0.40, a minimum of 36 children per group was required, resulting in a total sample of 108 children, factoring in an anticipated 10 % dropout rate. The calculations were based on 80 % power and a 5 % alpha error.

2.4. Mouthwash preparation

AA + TA and placebo mouthwashes were prepared. The formulation of the mouthwash was based on antibacterial efficacy, cytotoxicity, and palatability. The undiluted prepared mouthwash was filtered and 120 mL was transferred to each of the 36 sterile amber-coloured containers. Similarly, the placebo containers contained all of the components of herbal mouthwash except for AA and TA extracts. The taste and smell of the AA + TA mouthwash and placebo mouthwash were evaluated and the containers were labelled A, B, or C prior to the distribution. Solution A represented AA + TA mouthwash, Solution B - 0.2 % CHX mouthwash, and Solution C was a placebo mouthwash. CHX gluconate mouthwash (0.2 % Hexidine) was procured from ICPA Health Products Ltd., Mumbai, India.

2.5. Randomization and blinding

In total, 108 children (36 in each group) were recruited after an oral examination by a single investigator based on the eligibility criteria. The chief coordinator was not involved in the selection of participants, examinations or evaluation of the outcome variables but was responsible for assigning the mouthwash following the order established in a random sequence generated through Microsoft Excel. Children were assigned to A, B, or C, corresponding to one of the study groups. The assignment sequence was sealed in opaque envelopes marked with the child's number using SNOSE (Sequentially Numbered Opaque Sealed Envelope) technique. The investigator and participants were masked to the content of mouthwashes.

2.6. Execution of the study

The investigator underwent training prior to the study under the chief coordinator in the Department of Public Health Dentistry to record indices [Gingival index (GI) and plaque index (PI)] on 20 children, and the intra-examiner agreement was 0.92. At the baseline, the following clinical parameters were recorded: GI[11] and PI[13] following which oral bacteriological plaque sampling was collected from each child from specific tooth surfaces (buccal groove of the mandibular first molar) under aseptic conditions using a sterile spoon excavator. The specimen was placed in 1 mL of Tris EDTA buffer (TE buffer). Before collecting plaque samples, a preprocedural mouthrinse with drinking water was performed to remove all food debris. Children were advised to abstain from eating, drinking, or practicing oral hygiene for a minimum of 1 h before the baseline sample collection.

All participants received oral prophylaxis followed by 30 s of rinsing with 2.5 mL of their assigned mouthwashes within the school premises. Verbal and written instructions, along with a tabulated record were distributed for using the mouthwash under the supervision of a caregiver at home. They were encouraged to rinse for 30 s twice daily (after breakfast and before bedtime 45 min after brushing) with 2.5 mL of mouthwash (diluted in a 1:1 ratio (v/v) with drinking water). Standard oral hygiene instructions were given to all three groups along with instructions to refrain from using any other form of oral hygiene aids during the course of the study.

The clinical examinations and scorings were performed at baseline, 7th day, and 21st day under the same working conditions by the investigator, who was masked to group allocation. Plaque specimens were also collected at baseline and after 21 days. All specimens were duly labelled, stored at 4 °C, transferred immediately to the research laboratory, and processed within an hour of collection. Fig. 1 illustrates a CONSORT flow diagram.

A written questionnaire was administered to all three groups to assess palatability, acceptability, and any negative effects linked to mouthwash usage. During follow-up visits, the children were instructed to bring the used containers with them to ensure compliance.

2.7. RT-PCR for bacterial load determination

DNA was extracted from plaque specimens using the Modified Proteinase K method.[14] As previously described by Lau et al., 2004,[15] RT-PCR was carried out using specific primers targeting the 16S ribosomal ribonucleic acid region of *P. gingivalis* and *T. forsythia*. PCR reactions were carried out in a 25 μL reaction mixture containing 2 μL of

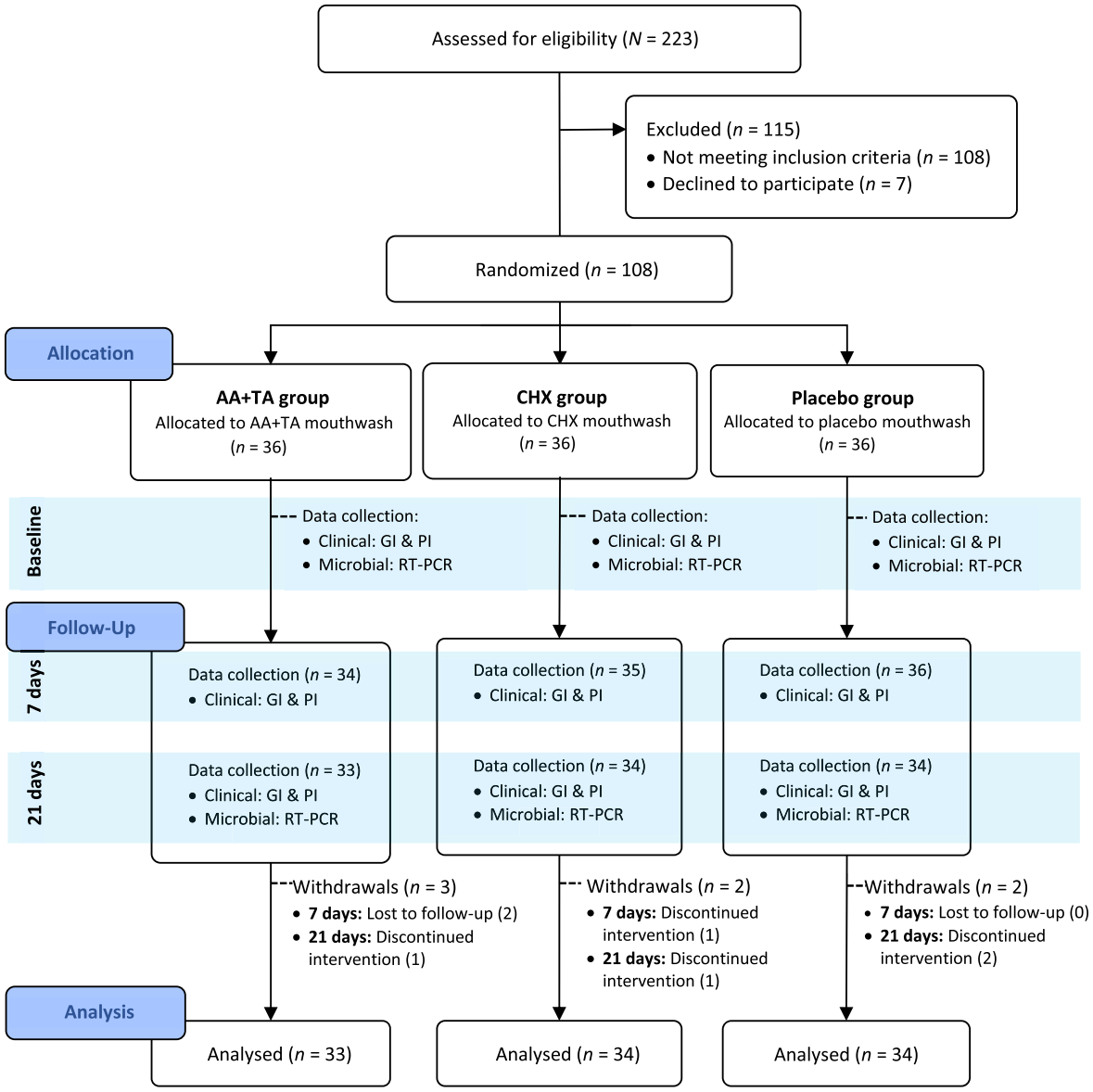

Fig. 1. CONSORT diagram showing the methodology adopted for conducting clinical trial AA + TA: Herbal mouthwash containing *Achyranthes aspera* and *Trachyspermum ammi*; CHX: 0.2 % Chlorhexidine gluconate mouthwash; GI: Gingival index; PI: Plaque index; RT-PCR: Real-Time Polymerase Chain Reaction.

template DNA, 2 μL of each of the specific primers (Bioserve™, Hyderabad, India), and 12.5 μL of PCR master mix [TB Green Premix Ex Taq (Tli RNaseH Plus) (Takara Bio inc., Kusatsu, Japan)]. The master mix contains TaKaRa Ex Taq HS, dNTP Mixture, Tli RNase H, Mg2+, and TB Green. The PCR tube strips were placed in a Realplex mastercycler (Eppendorf, Germany). RT-PCR conditions included initial denaturation followed by 40 Cycles of thermal cycling conditions: denaturation, annealing, and extension (See Table 1).

Melting curve analysis was conducted at 60–95 °C range to ensure the specificity of the amplification reaction. Quantification was accomplished through serial dilutions of DNA samples from standard strains of *P. gingivalis* ATCC 33277 and *T. forsythia* ATCC 43037 (LGC

Table 1
Information on the primers utilized in PCR amplification, including the target band size and the corresponding annealing temperatures.

Target Genes and Primer sequences	Thermal cycling conditions				Amplification length
		40 Cycles			
	Initial denaturation	Denaturation	Annealing	Extension	
Porphyromonas gingivalis F: 5'-AGG CAG CTT GCC ATA CTG CG-3' R: 5'-ACT GTT AGC AAC TAC CGA TGT-3'	95 °C for 30s	95 °C for 20s	60 °C for 30s	72 °C for 30s	404 bp
Tannerella forsythia F: 5'-GCG TAT GTA ACC TGC CCG CA-3' R: 5'-TGC TTC AGT GTC AGT TAT ACC T-3'	95 °C for 30s	95 °C for 20s	61 °C for 30s	72 °C for 30s	641 bp

F, forward primer; R, reverse primer; s, seconds; bp, basepairs.

PromoChem, Bangalore, India). Using serially diluted DNA samples (10^9-10^5 CFU/mL) of the standard strains, the cycle thresholds (Ct values) were obtained and used to generate standard curves (Ct values against quantity). The Ct values of unknown plaque specimens were plotted on the standard curve to obtain absolute quantification of *P. gingivalis* and *T. forsythia*.

2.8. Study outcome

The primary endpoint was the difference in mean gingival score after rinsing with mouthwash containing AA + TA, CHX, or a placebo. The secondary endpoints were the difference in mean plaque score and bacterial load after mouthrinsing as determined using RT-PCR. The non-inferiority margins for mean gingival (d = 0.40) and plaque (d = 0.50) scores at 21 days were set prior to the start of the study, based on existing literature[16,17] and clinical judgement.

2.9. Statistical analysis

The SPSS® software was used for the statistical analysis (IBM Corp. Released 2012, V 21.0. Armonk, NY, USA). The normality of the data was assessed using the Shapiro–Wilk test, revealing that the clinical parameters followed a normal distribution, whereas the microbiological parameters had a skewed distribution. Furthermore, logarithmic transformation was carried out for microbiological parameters. One-way ANOVA/Kruskal-Wallis test was carried out for the comparison between the groups. The changes in each group over time were analyzed using Repeated measures ANOVA/Wilcoxon Sign Rank test. The statistical significance level was set at $p \leq 0.05$ and the tests were one-tailed.[18,19]

3. Results

3.1. Population characteristics

In total, 223 children were screened, of which 108 were eligible and randomized. At 21 days post-intervention, seven (6.5 %) children were lost to follow-up resulting in 101 (93.52 %) children being evaluated (See Fig. 1). The participants across the three groups did not exhibit statistically significant differences in the distribution of demographic variables (age, $p = 0.030$; gender, $p = 0.485$, socioeconomic status, $p = 0.079$); (See Supplementary Table 1).

3.2. Measured clinical outcomes

Table 2 presents mean scores of GI and PI at different time intervals. At the baseline assessment, there were no statistically significant variations in the scores of GI ($p = 0.285$)and PI (p=.458) among the groups. All three groups exhibited a gradual and statistically significant reduction in both GI and PI scores from baseline to follow-up visits (7 days and 21 days); $p < 0.001$. However, the placebo group did not demonstrate statistically significant difference in scores between days 7 and 21 [GI ($p = 0.500$)and PI (p=.091)]. A significant reduction in mean GI and PI scores was observed for CHX and AA + TA groups at all time intervals when compared to the placebo group, $p < 0.001$. Between CHX and AA + TA, CHX group demonstrated significant reductions in both GI and PI scores compared to AA + TA group in both the follow-up visits (See Fig. 2).

3.3. RT-PCR for bacterial load determination

DNA copies per reaction values were used to reflect the bacterial load. The median bacterial counts and the change in bacterial counts are described in Table 3. At baseline, there were no statistically significant differences between the bacterial counts [*P.gingivalis* (p=.187) and *T. forsythia* (p=.210)] obtained using RT-PCR and were comparable.

Table 2
Comparison of mean gingival and plaque scores at baseline with the gingival score on the 7th day and 21st day, from the start of mouthwash administration in various mouthwash groups.

Group (n)		Baseline	7 days	21 days	p-Value[b]
Gingival score					
AA + TA (n = 33)	Mean ± SD	1.53 ± 0.09$^{a\alpha}$	0.87 ± 0.12$^{a\beta}$	0.78 ± 0.12$^{a\gamma}$	<0.001*
	95 % CI	1.50–1.56	0.83–0.91	0.73–0.82	
CHX (n = 34)	Mean ± SD	1.51 ± 0.13$^{a\alpha}$	0.65 ± 0.18$^{b\beta}$	0.47 ± 0.13$^{b\gamma}$	<0.001*
	95 % CI	1.47–1.56	0.59–0.71	0.43–0.52	
Placebo (n = 34)	Mean ± SD	1.51 ± 0.10$^{a\alpha}$	0.96 ± 0.12$^{c\beta}$	0.99 ± 0.13$^{c\beta}$	<0.001*
	95 % CI	1.46–1.54	0.92–1.00	0.94–1.03	
p-Value[a]		0.285	<0.001*	<0.001*	
Plaque score					
AA + TA (n = 33)	Mean ± SD	1.71 ± 0.14$^{a\alpha}$	0.90 ± 0.13$^{a\beta}$	0.79 ± 0.11$^{a\gamma}$	<0.001*
	95 % CI	1.66–1.76	0.85–0.94	0.75–0.83	
CHX (n = 34)	Mean ± SD	1.70 ± 0.08$^{a\alpha}$	0.73 ± 0.11$^{b\beta}$	0.48 ± 0.12$^{b\gamma}$	<0.001*
	95 % CI	1.67–1.73	0.69–0.77	0.43–0.52	
Placebo (n = 34)	Mean ± SD	1.69 ± 0.15$^{a\alpha}$	1.09 ± 0.20$^{c\beta}$	1.11 ± 0.19$^{c\beta}$	<0.001*
	95 % CI	1.64–1.75	1.02–1.16	1.04–1.18	
p-Value[a]		0.458	<0.001*	<0.001*	

AA+TA: polyherbal mouthwash containing *Achyranthes aspera* and *Trachyspermum ammi*; CHX: 0.2% Chlorhexidine gluconate mouthwash. Different lowercase signify significant differences among the mouthwashes within the column. The statistical analysis employed: [a]One-way ANOVA test followed by Tukey's *post hoc* test. Different Greek symbols signify significant differences within the group at various time intervals (in the row). The statistical test employed: [b]Repeated measures ANOVA test followed by Bonferroni *post hoc* test. The significance threshold was set at *$p \leq 0.001$ (1-tailed), indicating a highly statistically significant result.

Statistically significant change in *P. gingivalis* count was observed in the CHX group (4.27×10^6) and AA + TA group (1.65×10^6) when compared to the placebo group (0.02×10^6), $p < 0.001$. Similarly, CHX and AA + TA groups showed a significant change in *T. forsythia* count (CHX group: 453.0×10^6; AA + TA group: 346.0×10^6) when compared to the placebo group (-58.3×10^6), $p < 0.001$. Between CHX and AA + TA, CHX group demonstrated significant reductions in bacterial counts of both the organism compared to AA + TA group. Log_{10} bacterial counts of *P. gingivalis* and *T. forsythia* are depicted in Fig. 3.

4. Discussion

The overall findings affirmed that AA + TA mouthwash demonstrated non-inferiority in effectiveness compared to 0.2 % CHX in reducing gingival and plaque scores. Additionally, it was effective in controlling the growth of *P. gingivalis* and *T. forsythia*. These findings suggest that such mouthwash can serve as an effective anti-plaque agent, reducing dental plaque and gingival inflammation. The observed reduction can be ascribed to the antibacterial and anti-inflammatory properties inherent in *A. aspera* and *T. ammi*.[8,9] Notably, the placebo group also exhibited reduction in GI and PI scores, a phenomenon likely influenced by oral prophylaxis received subsequent to the baseline assessment.[20]

In the current study, CHX serves as a positive control as it is the gold standard for antiplaque agents, It is known to be effective against a spectrum of oral microorganisms, including periodontal pathogens. However, studies reveal that *P. gingivalis* can acquire resistance to CHX.[21] The existence of multidrug resistance in dental plaque bacteria further complicates this resistance.[22] Moreover, CHX carries the disadvantage of being a chemical that can cause dysgeusia, staining of teeth and tongue, xerostomia, and precipitation of calcium and phosphate ions from the tooth surface when used for a longer period.[4] To overcome

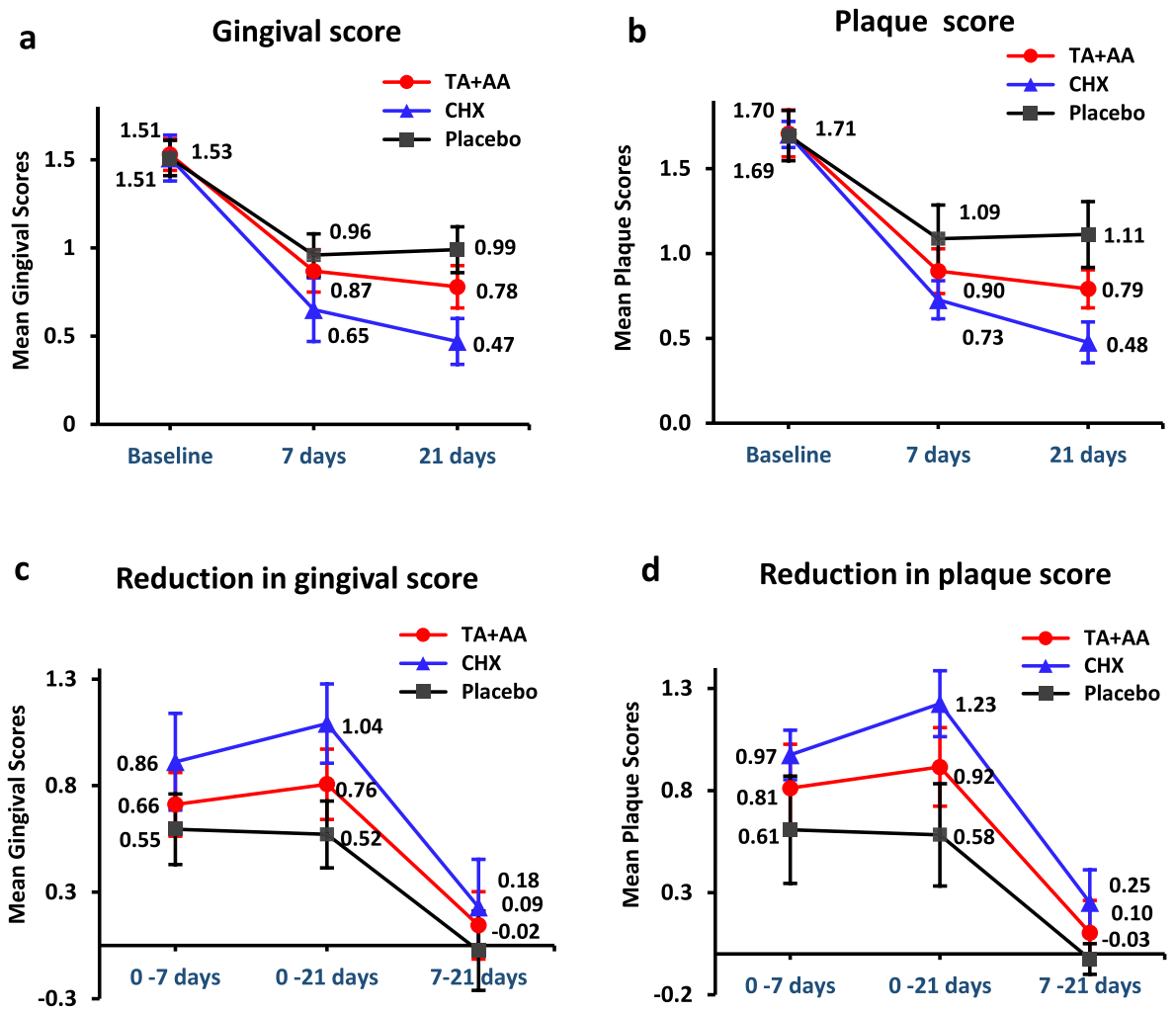

Fig. 2. Line charts depicting the mean gingival and plaque scores **(a, b)** and mean reduction in gingival and plaque scores **(c, d)** pre- and post-administration of tested mouthwashes for 7 days and 21 days.
AA + TA: polyherbal mouthwash containing *Achyranthes aspera* and *Trachyspermum ammi*; CHX: 0.2 % Chlorhexidine gluconate mouthwash.

such adverse effects, extensive research is being conducted in the field of alternative medicine.

In this study, we have employed RT-PCR as a powerful tool for the identification of specific gene targets associated with *P. gingivalis* and *T. forsythia*. Notably, recent advancements in research methodologies witnessed a transition from colony-forming unit assessments to molecular approaches like RT-PCR. Unlike conventional PCR approaches, which primarily provide qualitative information, RT-PCR detects and helps in identifying specific gene targets and facilitates the precise quantification of target DNA sequences in samples. This technique boasts high sensitivity and specificity. Moreover, it offers enhanced safety measures by minimizing the risk of cross-contamination.[23]

The specific mechanism of action (MOA) responsible for the antibacterial activity against periodontal pathogens remains unclear for *A. aspera* and *T. ammi*. Generally, the antimicrobial MOA of flavonoids falls into categories such as inhibiting cytoplasmic membrane function, synthesis of nucleic acid, and energy metabolism.[24] According to Pandey et al. the antibacterial activity in *A. aspera* is attributed to its flavonoid content.[25] The quantitative analysis of *A. aspera* revealed a diverse range of total phenolic content, ranging from 0.39 to 5.26 mg/g.[26] Similarly, Modareskia et al. highlighted that the major constituents in *T. ammi* primarily consist of phenolic compounds (thymol: 59.9–96.4 %, *p*-cymene: 0.6–21.2 %, γ-terpinene: 0.2–17.8 %, and carvacrol: 0.4–2.8 %), exhibiting strong antibacterial activity against various pathogens.[27]

Several studies reported that *A. aspera*[28–30] and *T. ammi*[31,32] exhibited antibacterial and antifungal activity against various oral microorganisms.[33] Soorgani et al. and Boyapati et al. observed a significant reduction in the subgingival microflora count of *P. gingivalis* when *A. aspera* was used as a non-surgical local drug delivery system for the management of chronic periodontitis, with no reported adverse effects.[34,35] Yavagal and Rajeshwar reported the antibacterial activity of commercially available *T. ammi* oil against *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, and *Fusobacterium nucleatum*.[36] Bansal et al. reported that an *A. aspera* based mouthwash was comparable to CHX mouthwash in reducing *Streptococcus mutans* count after a 7-day period.[30] Similarly, Saffarpour et al. observed that *T. ammi* oil based herbal mouthwash demonstrated anti-gingivitis activity comparable to CHX after 14 days.[37]

The use of natural medicinal remedies is gaining prominence as substitutes for chemical interventions in the management of various diseases, including periodontal diseases, because of comparable potential with fewer associated adverse effects. Currently, herbal based mouthwashes are being actively utilized to address issues such as gingival inflammation and bleeding.[38–40] Through the standardization and assessment of active plant-derived compounds, herbal agents have the potential to contribute to the development of a new healthcare paradigm, offering effective treatments for oral diseases in the future. The limitation of the current study is that only the short-term effects of

Table 3
Comparison of total bacterial counts of *Porphyromonas gingivalis* and *Tannerella forsythia* between the groups at baseline and 21 days.

Group		Total bacterial count						
		Baseline		21 days		*p*-Value[a]	Change	
		Counts (x10^6)	Log_{10}	Counts (x10^6)	Log_{10}		Counts (x10^6)	Log_{10}
Porphyromonas gingivalis								
AA + TA	Median (IQR)	5.58 (4.48–8.73)$^{\alpha}$	6.75 (6.65–6.94) $^{\alpha}$	3.83 (3.00–5.58)$^{\alpha}$	6.58 (6.48–6.75) $^{\alpha}$	<0.001*	1.65 (0.70–3.03)$^{\alpha}$	0.16 (0.06–0.32) $^{\alpha}$
	Min	1.47	6.17	1.45	6.16		−1.72	−0.32
	Max	114.0	8.06	14.10	7.15		103.0	1.02
	n (%)	33 (100 %)		31 (93.9 %)				
CHX	Median (IQR)	5.69 (4.64–8.40)$^{\alpha}$	6.75 (6.66–6.92) $^{\alpha}$	1.51 (1.00–3.03)$^{\beta}$	6.18 (6.00–6.48) $^{\beta}$	<0.001*	4.27 (3.32–5.31)$^{\beta}$	0.60 (0.40–0.77) $^{\beta}$
	Min	3.94	6.60	0.75	5.87		0.72	0.08
	Max	20.20	7.31	4.48	6.65		17.60	0.95
	n (%)	34 (100 %)		28 (82.4 %)				
Placebo	Median (IQR)	6.22 (4.70–10.1)$^{\alpha}$	6.79 (6.67–7.00) $^{\alpha}$	5.93 (4.66–10.6)$^{\gamma}$	6.78 (6.67–7.02) $^{\gamma}$	0.375	0.02 (−0.11-0.13)$^{\gamma}$	0.00 (−0.01-0.01) $^{\gamma}$
	Min	4.28	6.63	4.28	6.63		−3.69	−0.07
	Max	23.90	7.38	27.60	7.44		5.16	0.15
	n (%)	34 (100 %)		30 (88.2 %)				
p-Value[b]		0.187	0.172	<0.001*	<0.001*		<0.001*	<0.001*
Tannerella forsythia								
AA + TA	Median (IQR)	422.0 (275.0–931.5)$^{\alpha}$	8.62 (8.44–8.97) $^{\alpha}$	76.6 (29.3–152.5)$^{\alpha}$	7.88 (7.47–8.19) $^{\alpha}$	<0.001*	346.0 (199.5–665.0)$^{\alpha}$	0.91 (0.48–1.11) $^{\alpha}$
	Min	9.91	7.00	2.44	6.39		2.21	0.04
	Max	14600.0	10.16	663.0	8.82		14500.0	2.16
	n (%)	33 (100 %)		29 (87.9 %)				
CHX	Median (IQR)	429.0 (151.3–1132.5)$^{\alpha}$	8.63 (8.18–9.06) $^{\alpha}$	10.1 (3.2–40.3)$^{\beta}$	7.00 (6.51–7.61) $^{\beta}$	<0.001*	453.0 (135.0–1330) $^{\alpha}$	1.71 (1.13–1.88) $^{\beta}$
	Min	17.90	7.25	0.32	5.51		15.10	0.07
	Max	9580.0	9.98	8240.0	9.92		2990.0	2.99
	n (%)	34 (100 %)		27 (79.4 %)				
Placebo	Median (IQR)	189.0 (89.6–913.3)$^{\alpha}$	8.28 (7.95–8.95) $^{\alpha}$	370.0 (157.5–783.5)$^{\gamma}$	8.57 (8.20–8.90) $^{\gamma}$	0.328	−58.3 (−355.5-346) $^{\beta}$	−0.11 (−0.52-0.41) $^{\gamma}$
	Min	20.40	7.31	24.40	7.39		−3160.0	−1.19
	Max	3470.0	9.54	3710.0	9.57		3210.0	1.55
	n (%)	34 (100 %)		33 (97.1 %)				
p-Value[b]		0.210	0.214	<0.001*	<0.001*		<0.001*	<0.001*

AA+TA: Polyherbal mouthwash containing *Achyranthes aspera* and *Trachyspermum ammi*; CHX: 0.2% Chlorhexidine gluconate mouthwash; IQR: Interquartile range (Q1-Q3). *n* denotes the number of positive samples. Different Greek symbols signify significant differences among the mouthwashes within the column. The statistical analysis employed for Counts (x10^6): [a]Wilcoxon Sign Rank test; [b]Kruskal-Wallis test followed by Dunn's *post-hoc* test. The statistical analysis employed for Log transformation: [b]One-way ANOVA test followed by Tukey's *post hoc* test. The significance threshold was set at *$p \leq 0.001$ (1-tailed), indicating a highly statistically significant result.

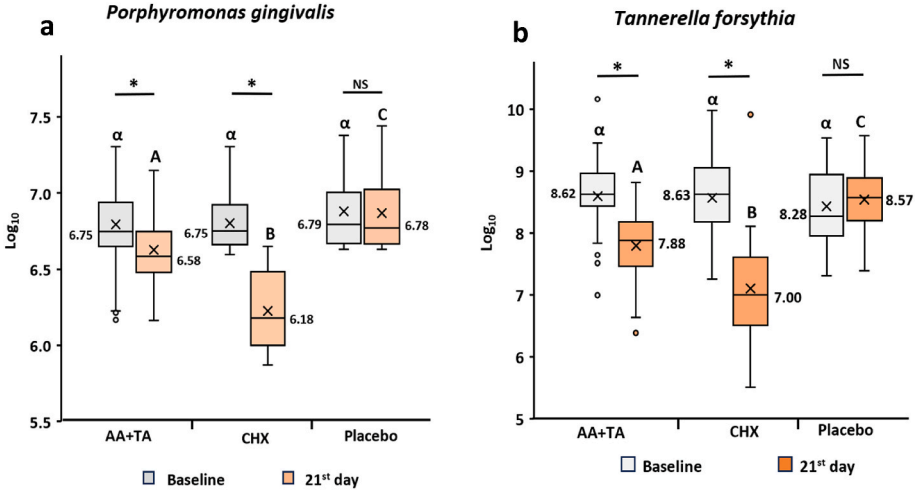

Fig. 3. Boxplots demonstrating the statistical difference analysis for the tested mouthwashes pre- and post-administration. Log_{10} bacterial counts of *Porphyromonas gingivalis* **(a)** and *Tannerella forsythia* **(b)**. Median values are indicated by the line within the boxplot. Different Greek symbols and Different uppercase indicate a difference between the mouthwashes. The statistical test employed: Dunn's *post-hoc* method following Kruskal-Wallis test; Wilcoxon Sign Rank test. *Statistically significant, $p \leq 0.05$; NS, not significant.

herbal mouthwash on gingival health, plaque control, and oral microbiota were assessed. Further long-term prospective investigations are needed to corroborate the findings of the current clinical trial.

5. Conclusion

The mouthwash containing *T. ammi* and *A. aspera* demonstrated non-inferiority compared to 0.2 % CHX in the management of plaque induced gingivitis, establishing itself as a comparable antiplaque agent. Thus, AA + TA mouthwash demonstrates promising anti-gingivitis and anti-plaque properties, suggesting its potential suitability as an alternative to CHX when used in conjunction with mechanical plaque control measures.

Sources of funding

This research was supported by the Indian Council of Medical Research (ICMR), India [grant number- 3/2/June-2021/PG-Thesis-HRD (49D)] under the Principal Investigator Ram Surath Kumar K. The funder had no role in study design, data collection and analysis, preparation of the manuscript or decision to publish.

IRB approval

The study was approved by the Institutional Research and Ethics Committee (IRB number: EC/NEW/2021/2435) with the reference number: 1447, dated: 28.08.2021. This study adhered to the ethical standards of human experimentation as well as the Helsinki Declaration of 1975, as revised in 2000. The study was registered on the Clinical Trials Registry-India platform as CTRI/2022/03/041423.

Authorship contribution statement

1. Ram Surath Kumar: Conceptualization, Investigation, Project administration, Visualization, Writing - original draft.
2. Anil V Ankola: Resources, Supervision, Validation, Data curation, Formal analysis, Methodology, Writing -review & editing.
3. Roopali M Sankeshwari: Resources, Supervision, Validation, Writing -review & editing.
4. Vinuta Hampiholi: Conceptualization, Investigation, Project administration, Visualization, Writing - review & editing.
5. Sagar Jalihal: Resources, Supervision, Methodology, Validation, Writing - review & editing.
6. Atrey J. Pai Khot: Software, Data curation, Writing – original draft & Data curation.
7. Varkey Nadakkavukaran Santhosh: Software, Data curation, Writing – original draft.
8. Kavitha Ragu: Software, Data curation, Writing – original draft.

Acknowledgment

The authors extend their heartfelt appreciation to Lokesh Kumar for his meticulous grammar check of the manuscript, which significantly contributed to the enhancement and refinement of its overall quality.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jobcr.2024.06.006.

References

1. Larsen T, Fiehn NE. Dental biofilm infections – an update. *APMIS*. 2017;125(4):376–384.
2. Socransky Ss, Haffajee Ad, Cugini Ma, Smith C, Kent JrRL. Microbial complexes in subgingival plaque. *J Clin Periodontol*. 1998;25(2):134–144.
3. Drisko CH. Nonsurgical periodontal therapy. *Periodontol*. 2000 2001;25(1):77–88.
4. Jones CG. Chlorhexidine: is it still the gold standard? *Periodontol*. 2000 1997;15(1):55–62.
5. Khan MS, Ahmad I. *Herbal medicine: current trends and future prospects. InNew Look to Phytomedicine*. Academic Press; 2019:3–13.
6. Sharma RN, Bala J, Singh A, Prabhjot K. Antibacterial potential of Achyranthusaspera Linn procured from Himachal Pradesh, Punjab and Haryana, India. *Res J Chem Sci*. 2011;8:80–82.
7. Dhale DA, Bhoi S. Pharmacognostic Characterization and phytochemical screening of Achyranthes aspera Linn. *Curr Agric Res J*. 2013;1(1):51.
8. Bairwa R, Sodha RS, Rajawat BS. Trachyspermum ammi. *Pharm Rev*. 2012;6(11):56.
9. Ha HA, Al-Humaid LA, Aldawsari M, Bharathi D, Lee J. Evaluation of phytochemical, antibacterial, thrombolytic, anti-inflammatory, and cytotoxicity profile of Achyranthes aspera aerial part extracts. *Environ Res*. 2024;243, 117802.
10. Gagnier JJ, Boon H, Rochon P, Moher D, Barnes J, Bombardier C. Reporting randomized, controlled trials of herbal interventions: an elaborated CONSORT statement. *Ann Intern Med*. 2006;144(5):364–367.
11. Löe H. The gingival index, the plaque index and the retention index systems. *J Periodontol*. 1967;38(6):610–616.
12. Singhal R, Siddibhavi M, Sankeshwari R, Patil P, Jalihal S, Ankola A. Effectiveness of three mouthwashes – manuka honey, Raw honey, and Chlorhexidine on plaque and gingival scores of 12–15-year-old school children: a randomized controlled field trial. *J Indian Soc Periodontol*. 2018;22:34–39.
13. Silness J, Löe H. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condtion. *Acta Odontol Scand*. 1964;22(1):121–135.
14. Ahmad NN, Cu-Unjieng AB, Donoso LA. Modification of standard proteinase K/phenol method for DNA isolation to improve yield and purity from frozen blood. *J Med Genet*. 1995;32(2):129–130.
15. Lau L, Sanz M, Herrera D, Morillo JM, Martín C, Silva A. Quantitative real-time polymerase chain reaction versus culture: a comparison between two methods for the detection and quantification of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythensis in subgingival plaque samples. *J Clin Periodontol*. 2004;31(12):1061–1069.
16. Singh O, Reddy VK, Pradhan D, Sharma L. Comparative evaluation of green tea and chlorhexidine mouthwashes on gingivitis: a randomized controlled trial. *J Indian Assoc Public Health Dent*. 2019;17(4):269.
17. Sreenivasan PK, Prasad KVV. Effects of a chlorhexidine mouthwash on clinical parameters of gingivitis, dental plaque and oral polymorphonuclear leukocytes [PMN]. *Contemp Clin Trials Commun*. 2020;19, 100473.
18. Cuzick J, Sasieni P. Interpreting the results of noninferiority trials—a review. *Br J Cancer*. 2022;127(10):1755–1759.
19. Gupta SK. Non-inferiority clinical trials: practical issues and current regulatory perspective. *Indian J Pharmacol*. 2011;43(4):371.
20. Pradeep Ar, Suke DK, Martande SS, Singh SP, Nagpal K, Naik SB. Triphala, a new herbal mouthwash for the treatment of gingivitis: a randomized controlled clinical trial. *J Periodontol*. 2016;87(11):1352–1359.
21. Grenier D, Bertrand J, Mayrand D. Porphyromonas gingivalis outer membrane vesicles promote bacterial resistance to chlorhexidine. *Oral Microbiol Immunol*. 1995;10(5):319–320.
22. Saleem HGM, Seers CA, Sabri AN, Reynolds EC. Dental plaque bacteria with reduced susceptibility to chlorhexidine are multidrug resistant. *BMC Microbiol*. 2016;16(1):214.
23. Valones MAA, Guimarães RL, Brandão LAC, et al. Principles and applications of polymerase chain reaction in medical diagnostic fields: a review. *Braz J Microbiol*. 2009;40:1–11.
24. Hendra R, Ahmad S, Sukari A, Shukor MY, Oskoueian E. Flavonoid analyses and antimicrobial activity of various parts of Phaleria macrocarpa (Scheff.) Boerl fruit. *Int J Mol Sci*. 2011;12(6):3422–3431.
25. Pandey G, Rao C, Gupta SS, Verma K, Singh M. *Antioxidant and Antibacterial Activities of Leaf Extract of Achyranthes aspera Linn*. Prickly Chaff Flower); 2014.
26. Upadhya V, Pai SR, Hegde HV. Effect of method and time of extraction on total phenolic content in comparison with antioxidant activities in different parts of Achyranthes aspera. *J King Saud Univ Sci*. 2015;27(3):204–208.
27. Modareskia M, Fattahi M, Mirjalili MH. Thymol screening, phenolic contents, antioxidant and antibacterial activities of Iranian populations of Trachyspermum ammi (L.) Sprague (Apiaceae). *Sci Rep*. 2022;12(1), 15645.
28. Ambulkar S, Tale V, Khilari S. Evaluation of the antibacterial potential of traditional medicinal plants against bacteria isolated from dental caries. *J Pure Appl Microbiol*. 2021;15(3):1204–1211.
29. Lakshmi T, Roy A, Merlin ARS. Antibacterial activity of achyranthes aspera extract against oral pathogens–an in vitro study. *Plant Cell Biotechnol Mol Biol*. 2020;21(25):37–40.
30. Bansal A, Marwah N, Nigam AG, Goenka P, Goel D. Effect of Achyranthes aspera, 0.2% aqueous chlorhexidine gluconate and punica granatum oral rinse on the levels of salivary Streptococcus mutans in 8 to 12 Years old children. *J Contemp Dent Pract*. 2015;16(11):903–909.
31. Dadpe MV, Dhore SV, Dahake PT, Kale YJ, Kendre SB, Siddiqui AG. Evaluation of antimicrobial efficacy of Trachyspermum ammi (Ajwain) oil and chlorhexidine against oral bacteria: an in vitro study. *J Indian Soc Pedod Prev Dent*. 2018;36(4):357.
32. Khan R, Zakir M, Khanam Z, Shakil S, Khan AU. Novel compound from Trachyspermum ammi (Ajowan caraway) seeds with antibiofilm and antiadherence activities against Streptococcus mutans: a potential chemotherapeutic agent against dental caries. *J Appl Microbiol*. 2010;109(6):2151–2159.
33. Kumar R.S., Ankola A.V., Nagamoti M.B., et al. Evaluation of antibacterial and cytotoxic potency of polyherbal gel formulation containing Achyranthes Aspera and Trachyspermum Ammi as intracanal medicament: an in vitro study. J Dent. 2024. [Ahead of print] https://doi.org/10.30476/dentjods.2024.100380.2218.

34. Soorgani NR, Khashu H, Agrawal R, Ninan RK, Peter S, Mukka N. Clinical effects of subgingivally delivered Achyranthes aspera gel in chronic periodontitis: a clinical and microbiological study. *J Pharm BioAllied Sci*. 2023;15(suppl 1), S693.
35. Boyapati R, Gojja P, Chintalapani S, Nagubandi K, Ramisetti A, Salavadhi SS. Efficacy of local drug delivery of Achyranthes aspera gel in the management of chronic periodontitis: a clinical study. *J Indian Soc Periodontol*. 2017;21(1):46.
36. Yavagal PC, Rajeshwar SS. Antibacterial efficacy of Trachyspermum ammi Oil against Porphyromonas gingivalis, Fusobacterium nucleatum, and Aggregatibacter actinomycetemcomitans: an in vitro study. *J Indian Assoc Public Health Dent*. 2022;20 (4):398.
37. Saffarpour A, Amini N, Keshtkar A, Mokhber Dezfuli N, Manayi A. Comparative effects of mouthwash containing trachypermum ammi oil with chlorhexidine on dental plaque induced gingivitis. *Res J Pharmacogn*. 2020;7(4):23–30.
38. Al-Maweri SA, Alhajj MN, Deshisha EA, et al. Curcumin mouthwashes versus chlorhexidine in controlling plaque and gingivitis: a systematic review and meta-analysis. *Int J Dent Hyg*. 2022;20(1):53–61.
39. Cai H, Chen J, Panagodage Perera NK, Liang X. Effects of herbal mouthwashes on plaque and inflammation control for patients with gingivitis: a systematic review and meta-analysis of randomised controlled trials. *Evid Based Complement Alternat Med*. 2020;2020, e2829854.
40. Haas AN, Wagner TP, Muniz FWMG, Fiorini T, Cavagni J, Celeste RK. Essential oils-containing mouthwashes for gingivitis and plaque: meta-analyses and meta-regression. *J Dent*. 2016;55:7–15.