IJARSCT
International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 11, June 2026
Impact Factor: 8.2

Formulation and Evaluation of Herbal Mouthwash against Oral Infection Disease

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Abstract: *It is believed That Many bacterial species are live in the mouth. Still, some of this organisms are harmless, and few are dangerous and they can lead to gum disease, bad breath, and several other oral health problems. For maintaining a healthy mouth and body depends on maintaining proper dental hygiene.*

Oral infections caused by pathogenic microorganisms remain a significant public health concern worldwide. Herbal mouthwashes are emerged as promising alternatives to conventional chemical-based formulations such as chlorhexidine, owing to their broadspectrum antimicrobial properties with minimal adverse effect profiles.

In This study aimed to formulated and evaluated a herbal mouthwash utilizing 4 medicinal plant extracts Ocimum sanctum (tulsi), Mentha longifolia (mint), Syzygium aromaticum (clove), and Azadirachta indica (neem) and assess its antimicrobial efficacy against oral pathogens.

Two formulations (F1 and F2) were prepared using the plant extracts obtained by shade drying and solvent extraction method. The formulations were evaluated for physical parameters (colour, odour, taste), pH, microbial inhibition by zone of inhibition (ZOI) measurement, stability (ICH-guided accelerated conditions at room temperature for one month), and homogeneity.

Formulation F2, containing large concentrations of active herbal extracts (clove 2 ml, neem 540.00 mg, tulsi 540.00mg), exhibited superior antibacterial activity with a zone of inhibition of 2.00 cm compared to 1.5 cm for F1. The pH of F1 and F2 was 6.999 and 6.96778, respectively, consistent with physiological oral conditions. Both formulations showed acceptable colour, pleasant minty odour, homogeneous texture, and moderate stability over the study period.

The F2 herbal mouthwash formulation demonstrated Satisfactory physicochemical properties and potent antimicrobial activity against oral pathogens. These findings support the potential of plant-based mouthwash preparations as safe, cost-effective, and efficacious adjuncts to conventional oral hygiene practice. Further randomized clinical trials with larger sample sizes are warranted.

Keywords: Herbal mouthwash; oral infection; Ocimum sanctum; Azadirachta indica; Syzygium aromaticum; antimicrobial; zone of inhibition; phytotherapy.

I. INTRODUCTION

AIM AND OBJECTIVES

- Aim

To formulate and evaluate a herbal mouthwash using selected medicinal plant extracts and determine its efficacy against oral pathogenic microorganisms.

- Objectives
- To perform literature Review on conventional and herbal mouthwash preparations, their composition, and antimicrobial properties.

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- To Collect, authenticate, and prepare standard extracts of Ocimum sanctum, Mentha longifolia, Syzygium aromaticum, and Azadirachta indica.
- To formulate two herbal mouthwash Formulations (F1 and F2) with varying concentrations of a plant extracts.
- To evaluate the formulations for physicochemical parameters including colour, odour, taste, pH, and homogeneity.
- To assess antimicrobial efficacy by the zone of inhibition method against standard oral pathogens.
- To conduct accelerated stability testing of both formulations in accordance with ICH guidelines.
- To compare the performance of F1 and F2 and identify the optimal formulation for further development.

PLAN OF WORK:

1. **Literature survey and problem identification:**
   Systematic review of published literature on oral infections, conventional mouthwash limitations, and herbal antimicrobial agents. Identification of suitable plant species with documented oral antibacterial activity.
2. **Collection and authentication of plant material:**
   Collection of fresh plant material (tulsi, mint, clove, neem) from authenticated sources. Botanical authentication and preparation of voucher specimens.
3. **Extract preparation:**
   Shade drying of plant material, followed by solvent extraction using appropriate solvents (methanol for neem, ethanol for tulsi, water for clove). Filtration and storage of extracts under appropriate conditions.
4. **Formulation development:**
   Development of two mouthwash formulations (F1 and F2) with varying concentrations of active extracts. Incorporation of excipients including saccharine, PEG 40, glycerol, methyl paraben, and purified water.
5. **Evaluation and method validation:**
   Physical evaluation, pH determination, zone of inhibition testing, homogeneity assessment, and ICH-compliant stability studies for both formulations.
6. **Data analysis and reporting:**
   Statistical comparison of formulations, documentation of results, and preparation of the final manuscript.

LITERATURE REVIEW

Numerous investigators have reported the formulation and evaluation of herbal mouthwashes with varying botanical compositions and antimicrobial spectra. **A seminal study by Yadav et al. (2020)** demonstrated promising antibacterial activity of a herbal mouthwash against oral pathogens including Streptococcus mutans, establishing the framework for evidence-based herbal oral care product development. The preparation involved hot aqueous extraction of plant material and agar well diffusion-based antimicrobial testing.

Govardhan et al. (2023), published in the International Journal of Pharmaceutical Sciences and Research, formulated a mouthwash using betel leaf (Piper betle) and peppermint (Mentha piperita) extracts supplemented with clove oil. Antibacterial activity evaluated against S. mutans and S. salivarius yielded zones of inhibition of 18 mm and 22 mm, respectively, at a 50 µl concentration. The total phenolic content was 76.25 µg/ml and total flavonoid content was 41.71 µg/ml, both determined by standard spectrophotometric methods. The formulation's pH was 6.1 and it was free from heavy metals and alcohol.

Talebi Ardakani et al. (2022), in a randomized, double-blind cross-over clinical trial published in the Journal of Advanced Periodontology and Implant Dentistry, evaluated a five-herb mouthwash (Myrtus communis, Quercus brantii, Punica granatum, Portulaca oleracea, Boswellia serrata) against 0.2% chlorhexidine in fifty patients with plaque-induced gingivitis. Clinical indices including probing pocket depth (PPD), gingival index (GI), bleeding on probing (BOP), and plaque index (PI) showed statistically significant improvement in both groups ($P<0.05$), with no

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significant inter-group difference. The herbal mouthwash was comparable to 0.2% CHX, suggesting viability as a chlorhexidine substitute with fewer side effects.

Gautam and Ashish (2024), in the Research Journal of Topical and Cosmetic Sciences, formulated a mouthwash incorporating tulsi (Ocimum sanctum), mint (Mentha longifolia), clove (Syzygium aromaticum), and neem (Azadirachta indica). Two formulations (F1 and F2) were prepared with varying extract concentrations; F2 exhibited superior antimicrobial activity (ZOI = 2.0 cm) compared to F1 (ZOI = 1.5 cm). Both formulations had near-neutral pH values and acceptable homogeneity.

Earlier studies by Patil et al. (2020) and Nigam et al. (2020) corroborated the broad-spectrum antibacterial activity of plant-based mouthwashes and underscored the importance of standardized extraction processes and rigorous evaluation protocols. Systematic reviews, including **Cai et al. (2020),** have shown herbal mouthwashes to produce measurable reductions in plaque and gingivitis indices comparable to established chemical agents, though the authors note heterogeneity across trials and the need for long-term data. The prevailing consensus supports herbal mouthwashes as safe, cost-effective, and pharmacologically rational alternatives or adjuncts to conventional oral hygiene formulations, particularly in resource-limited settings.

Oral health is an integral component of general systemic health, and its neglect is associated with a spectrum of diseases ranging from dental caries and gingivitis to periodontitis and halitosis.[1] It is estimated that 70–100% of the global population is affected by gingivitis at some point in their lifetime, with dental plaque biofilm identified as the primary etiological factor.[2] Oral pathogenic bacteria — particularly facultative anaerobic Gram-positive organisms such as Streptococcus mutans, Streptococcus salivarius, and anaerobic Coccobacilli — colonize the tooth surface and surrounding soft tissues, precipitating inflammatory and destructive periodontal processes.[3]

Plaque reduction through mechanical methods (toothbrushing, flossing, interdental brushes) constitutes the cornerstone of preventive dentistry; however, these methods are insufficient in a large proportion of the population due to limited dexterity, compliance, or access to oral health services.[4] Chemical adjuncts in the form of mouthwashes are therefore routinely recommended as supplements to mechanical oral hygiene to reduce the oral microbial load and prevent the progression of periodontal diseases.[5]

1.1 Types of mouthwash

Mouthwash formulations are broadly categorized into four types based on their primary function and active constituents:

- **Fluoride mouthwash:** Contains sodium fluoride or stannous fluoride salts to protect enamel against cavity formation. Caution is warranted given that fluoride is also present in toothpaste and fluoridated water supplies.[6]
- **Regular (cosmetic) mouthwash:** Formulated with standard cleansing and deodorizing ingredients without specific therapeutic claims; often alcohol-free.[6]
- **Corrective mouthwash:** Primarily intended for breath freshening and odour control, without a significant impact on oral disease.
- **Disinfectant (antiseptic) mouthwash:** Contains antimicrobial agents (e.g., chlorhexidine, cetylpyridinium chloride, alcohol) to actively reduce the microbial load in the oral cavity; used in management of halitosis and oral infections.[7]

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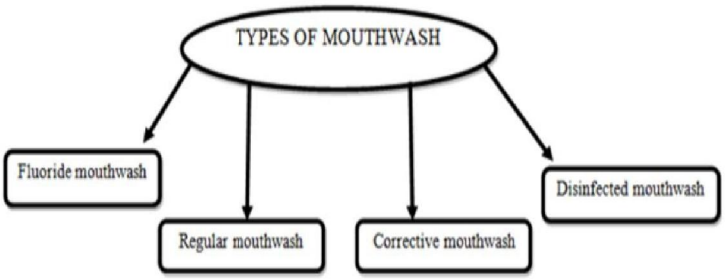

Fig. 1. Type of mouthwash

1.2 Gold standard and its limitations

Chlorhexidine (CHX) digluconate is universally recognized as the gold standard chemical plaque control agent owing to its substantivity and broad-spectrum bactericidal action.[8] Despite its well-documented clinical efficacy, prolonged use of CHX mouthwash is associated with several adverse effects including brown discolouration of teeth and tongue, dysgeusia, mucositis, and enhanced calculus formation.[9] Furthermore, commercial CHX formulations commonly contain ethyl alcohol, which has been epidemiologically associated with oral mucosal changes and is contraindicated in children and patients with alcohol dependency.[10] These limitations have catalysed sustained scientific interest in the development of herbal alternatives that retain efficacy while minimizing adverse effects.

1.3 History of mouthwash

The practice of oral rinsing is among the oldest recorded therapeutic traditions. Ayurvedic texts dating to approximately 2700 BCE contain the earliest documented descriptions of oral cleansing rituals (gandusha and kavala) using herbal decoctions.[11] The Greek physician Hippocrates (circa 460–370 BCE) advocated rinsing with a solution of salt, alum, and vinegar, while Pedanius Dioscorides (circa 40–90 CE) described a formulation of olive juice, milk, myrrh, pomegranate, vinegar, and wine for combating halitosis. In Ayurvedic and Siddha systems of Indian medicine, numerous plant species including neem, tulsi, clove, and licorice have been employed for centuries as dental hygiene aids due to their potent antimicrobial, anti-inflammatory, and analgesic properties.[8] The commercial mouthwash industry emerged in the late 19th century, with Listerine (1879) being among the first proprietary antiseptic rinses, eventually transitioning from surgical antiseptic to consumer oral care product.

Fig. 2. Old mouthwash bottles

DOI: 10.48175/IJARSCT-37350

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1.4 Herbal agents used in mouthwash formulation

❖ **Tulsi (Ocimum sanctum Linn.)**

- **Synonyms:** Holy basil, Sacred basil, Vishnu priya
- **Biological source:** Fresh and dried leaves of Ocimum sanctum Linn., family Lamiaceae.
- **Geographical source:** Indigenous to the Indian subcontinent; extensively cultivated throughout Southeast Asia, Australia, Malesia, and the western Pacific in tropical and subtropical environments.[12]
- **Chemical constituents:** The volatile oil of leaves contains eugenol, euginal (eugenic acid), ursolic acid, carvacrol, linalool, limatrol, caryophyllene, and methyl chavicol. Fixed oil of seeds contains fatty acids and sitosterol. Seed mucilage contains polysaccharides (xylose-based sugars). Green leaves contain anthocyanins and flavonoids.[12,13]
- **Pharmacological uses:** Immunomodulatory; analgesic and antipyretic; antimicrobial and antifungal; adaptogenic; antidiabetic; cardioprotective. In dentistry, tulsi extracts have been shown to reduce oral bacterial load and inhibit S. mutans biofilm formation.[13]

Fig.3. Picture of Tulsi

❖ **Neem (Azadirachta indica J. Juss.)**

- **Synonyms:** Indian lilac, Nimba tree, Miracle tree, Margosa
- **Biological source:** Fresh or dried leaves and seed oil of Azadirachta indica J. Juss. (syn. Melia indica Brandis), family Meliaceae.
- **Geographical source:** Indigenous to the Indian subcontinent, Myanmar, Bangladesh, Thailand, and Indochina; naturalized throughout tropical Africa and the Americas.[14]
- **Chemical constituents:** Leaves contain nimbin, nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, n-hexacosanol, ascorbic acid, and amino acids. Seed oil contains azadirachtin — the principal limonoid responsible for bioactivity — along with quercetin, β-sitosterol, and fatty acids (oleic, stearic, palmitic).[14]
- **Pharmacological uses:** Antifungal, antibacterial, antiviral, antioxidant, anti-inflammatory, immunostimulant, and bone-strengthening (calcium-rich). In oral care, neem twig chewing has demonstrated significant plaque reduction and gingival index improvement comparable to toothbrushing.[15]

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Fig. 4. Picture of Neem

❖ **Clove (Syzygium aromaticum (L.) Merr. & L.M. Perry)**

- **Synonyms:** Clove bud, Clove flower, Laung
- **Biological source:** Dried unopened flower buds of Syzygium aromaticum (syn. Eugenia caryophyllus), family Myrtaceae.
- **Geographical source:** Native to the Maluku Islands (Indonesia); commercially cultivated in Zanzibar, Madagascar, Sri Lanka, India (Tamil Nadu), and the Caribbean.[9]
- **Chemical constituents:** Volatile oil content of 15–16%; the oil comprises 70–75% eugenol as the dominant phenolic compound, along with eugenyl acetate, β-caryophyllene, and minor terpenoids. Fixed portion contains tannins (10–13%), resin, chromone, and eugenin.[11]
- **Pharmacological uses:** Dental analgesic (eugenol acts on TRPA1 channels); antiseptic; carminative; anti-inflammatory; antifungal. Eugenol is a pharmacopoeial ingredient in dental pulp-capping materials, temporary cements, and periodontal dressings. It demonstrates significant MIC values against S. mutans, Lactobacillus species, and Candida albicans.[11]

Fig.5. Picture of Clove

❖ **Mint (Mentha longifolia (L.) L.)**

- **Synonyms:** Wild mint, Horse mint, Pudina
- **Biological source:** Fresh and dried aerial parts of Mentha longifolia L., family Lamiaceae.

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- **Geographical source:** Distributed across Europe, Asia, and northern Africa; cultivated widely throughout India and the Middle East.
- **Chemical constituents:** Essential oil containing menthol (primary terpene alcohol responsible for cooling sensation), menthone, menthyl acetate, cineole, limonene, and pulegone. Also contains rosmarinic acid, flavonoids (luteolin, hesperidin), and tannins.[16]
- **Pharmacological uses:** Anti-inflammatory; antimicrobial (against both Gram-positive and Gram-negative bacteria); carminative; analgesic; flavouring agent in oral hygiene preparations. In mouthwash formulations, mint extracts provide organoleptic palatability and contribute antimicrobial activity complementary to other herbal constituents.[16]

Fig.6.Picture of Mint

1.5 Benefits of herbal mouthwash over conventional preparations

- Minimal to no systemic adverse effects; do not cause tooth or tongue discolouration.
- Alcohol-free formulations are suitable for children, pregnant women, and individuals with alcohol sensitivity.
- Multiple bioactive constituents provide synergistic antibacterial, anti-inflammatory, and antioxidant actions.
- Lower manufacturing cost, increasing accessibility for resource-limited populations.
- Long history of traditional medicinal use providing an ethnopharmacological evidence base for safety.[17]
- No harsh chemical additives; biodegradable and environmentally compatible excipient profiles.

II. MATERIALS AND METHODS

2.1 Collection and authentication of plant material

Fresh plant material comprising neem (Azadirachta indica) leaves, tulsi (Ocimum sanctum) leaves, mint (Mentha longifolia) leaves, and clove (Syzygium aromaticum) buds was collected from identified plants in the botanical garden premises of Minerva College of Pharmacy, Indora, Kangra, Himachal Pradesh, India.[18] Authentication of plant specimens was performed by a qualified botanist, and voucher specimens were deposited in the institutional herbarium. Chemical excipients saccharine, polyethylene glycol 40 (PEG 40), glycerol, methyl paraben, and pharmaceutical-grade ethanol and methanol were procured from Mrs Sarswati Wani College Of Pharmacy Ganegaon and used without further purification.

2.2 Preparation of plant extracts

Fresh plant material was cleaned under running water to remove surface contaminants and subjected to shade drying at ambient temperature (25–28°C) for 10–15 days, away from direct sunlight, to prevent degradation of thermolabile phytoconstituents.[19] Dried material was coarsely powdered using an electrically operated grinder and stored in airtight containers at room temperature prior to extraction.

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Extraction was performed by the maceration method using solvent systems selected on the basis of constituent polarity profiles:[18]

- Neem (Azadirachta indica): Powdered leaf material was macerated in methanol (1:5 w/v) with intermittent agitation for 72 hours, filtered through Whatman No. 1 filter paper, and the filtrate concentrated under reduced pressure.
- Tulsi (Ocimum sanctum): Powdered leaves were macerated in 95% ethanol (1:5 w/v) for 72 hours, filtered, and the filtrate reduced by rotary evaporation.
- Clove (Syzygium aromaticum): Clove buds were subjected to hydrodistillation using a Clevenger-type apparatus for 3 hours to obtain the volatile essential oil. The aqueous decoction was simultaneously prepared by boiling clove buds in sterile water (10 g/100 ml) for 15 minutes.

Sr. No.	Plant used (common name)	Part used	Solvent for extraction
1	Neem (Azadirachta indica)	Leaves	Methanol
2	Tulsi (Ocimum sanctum)	Leaves	Ethanol (95%)
3	Clove (Syzygium aromaticum)	Dried flower buds	Hydrodistillation / Water
4	Mint (Mentha longifolia)	Aerial parts / Oil	Distilled (as flavouring oil)

Table 1: Plant material and extraction solvent used

2.3 Formulation of herbal mouthwash

Two formulations, designated F1 (lower concentration) and F2 (higher concentration of active herbal extracts), were prepared on the basis of a preliminary compatibility study.[18,20] All excipients were individually weighed on a calibrated electronic balance. Each herbal extract was dissolved separately in a small volume of purified water and transferred to a 100 ml graduated volumetric flask. PEG 40 was added as a surfactant to enhance solubilization of lipophilic clove essential oil in the aqueous medium. Glycerol was incorporated as a humectant and co-surfactant. Clove essential oil and mint oil were added dropwise with continuous stirring to prevent phase separation. Saccharine was dissolved in a small volume of purified water and added as a sweetening agent. Methyl paraben (0.2% w/v) was dissolved in hot water and incorporated as an antimicrobial preservative. The volume was made up to 100 ml with purified water, and the pH was adjusted to physiological values (6.5–7.2) using dilute sodium hydroxide or hydrochloric acid as required. The final preparation was transferred to sterilized amber-coloured glass bottles and sealed.[1].

Sr. No.	Ingredient	Functional role	Quantity F1	Quantity F2
1	Clove (Syzygium aromaticum) essential oil	Active antimicrobial	1 ml	2 ml
2	Neem (Azadirachta indica) leaf extract	Active antimicrobial	260 mg	540 mg
3	Tulsi (Ocimum sanctum) leaf extract	Active antimicrobial	260 mg	540 mg
4	Mint (Mentha longifolia) oil	Flavouring agent	0.1 ml	0.1 ml

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5	Saccharine	Sweetening agent	50 mg	50 mg
6	PEG 40	Surfactant / Solubilizer	6 g	6 g
7	Glycerol	Co-surfactant / Humectant	6.5 g	6.5 g
8	Methyl paraben	Antimicrobial preservative	2 ml (0.2% w/v)	2 ml (0.2% w/v)
9	Purified water	Vehicle (volume make-up)	q.s. to 100 ml	q.s. to 100 ml

Table 2: Formulation composition of herbal mouthwash (F1 and F2)

2.4 Evaluation of the herbal mouthwash

- **Physical evaluation**
  Colour, odour, taste, and physical state of each formulation were assessed by visual inspection and organoleptic examination by three trained panel members under standardized laboratory conditions in accordance with established pharmacopoeial procedures.[17] Results were recorded descriptively.
- **pH determination**
  The pH of each formulation was determined using a calibrated digital pH meter (Systronics Model 361, India).[18] Prior to measurement, the pH meter was two-point calibrated using standard buffer solutions at pH 4.0 and 7.0. An aliquot of 1 ml of each mouthwash formulation was dissolved in 50 ml of carbon dioxide-free purified water and the electrode was immersed until a stable reading was obtained. Measurements were performed in triplicate and the mean value recorded. The physiologically acceptable pH range for oral formulations is 6.5–7.5.
- **Test for microbial growth inhibition (Zone of inhibition assay)**
  In vitro antimicrobial activity was evaluated using the agar disc/well diffusion method against standard oral pathogens (S. mutans ATCC 25175).[19,20] Mueller-Hinton agar plates were prepared, sterilized by autoclaving (121°C, 15 psi, 15 minutes), poured into sterile Petri dishes, and allowed to solidify. Standardized bacterial suspensions (0.5 McFarland standard, ~1.5×10^8 CFU/ml) were prepared and evenly swabbed across the agar surface using a sterile cotton swab. Wells of 5 mm diameter were bored using a sterile cork borer. Fifty microlitres of each mouthwash formulation (F1 and F2) were dispensed into separate wells. Chlorhexidine (0.2%) was included as a positive control and sterile water as the negative control. Plates were pre-diffused at 4°C for 30 minutes, then incubated aerobically at 37°C for 24–48 hours. Zones of inhibition (ZOI) were measured in centimetres using a calibrated ruler. Experiments were performed in triplicate and mean values recorded.
- **Stability study**
  Accelerated stability testing was conducted in accordance with ICH Q1A(R2) guidelines.[21] Both formulations (F1 and F2) were stored in amber glass bottles at room temperature (25 ± 2°C / 60 ± 5% RH) for a period of one month. Formulations were periodically examined for changes in colour, odour, phase separation, flocculation, pH, and microbial growth. The presence of flocculation or phase separation was used as an indicator of physical instability.
- **Homogeneity test**
  A small aliquot of each formulation was placed on a clean, dry glass slide and covered with a cover glass.[22] The preparation was examined under a light microscope at 10× magnification for uniformity of dispersion, absence of visible aggregates, lumps, or floccules. Additionally, visual inspection of each formulation in a

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clear glass vessel against a white background under adequate illumination was performed to assess macroscopic homogeneity.

METHOD VALIDATION

The analytical methods employed for physicochemical evaluation were validated with reference to ICH Q2(R1) guidelines for specificity, linearity, precision, and accuracy where applicable.

pH meter calibration was validated using three certified standard buffer solutions (pH 4.0, 7.0, and 10.0) before each measurement session, with a tolerance of ±0.05 pH units. The instrument was re-calibrated if drift exceeded this range.

The zone of inhibition assay (disc/well diffusion) was validated for reproducibility by performing three independent trials on separate days (inter-day precision). The coefficient of variation (CV) for zone measurements across replicates was required to be ≤5% for acceptance. The positive control (0.2% chlorhexidine) and negative control (sterile water) were included in each assay run to confirm assay validity.

Homogeneity assessment was validated by examining at least three different sampling sites (top, middle, and bottom) of each stored formulation bottle to confirm spatial uniformity of dispersion.

III. RESULT AND DISCUSSION

Both herbal mouthwash formulations (F1 and F2) were successfully prepared and subjected to comprehensive physicochemical and microbiological evaluation. The results are presented systematically below.

3.1 Physical evaluation

Visual and organoleptic assessment of F1 and F2 revealed distinct characteristics attributable to their constituent herbal extracts. F1 exhibited a slightly light green colour, a bitter taste, a pleasant minty flavour, and presented as a biphasic liquid, indicating partial immiscibility of the essential oil component with the aqueous phase at the lower PEG 40 concentration. F2 displayed an intensified green colour (attributed to the higher concentrations of neem and tulsi chlorophyll-containing extracts), a bitter taste, a refreshing mint flavour, and a biphasic liquid state similar to F1. Both formulations demonstrated an acceptable organoleptic profile. The characteristic bitterness of neem alkaloids was masked to a degree by the saccharine sweetening agent. The pleasant minty aroma contributed positively to the overall acceptability of the preparation.

Sr. No.	Evaluation parameter	Observation F1	Observation F2	Remark
1	Physical evaluation — Colour	Slightly light green	Green	Pass
	Taste	Bitter	Bitter	Pass
	Flavour	Mint	Mint	Pass
	Physical state	Biphasic liquid	Biphasic liquid	Noted
2	pH (mean ± SD, n = 3)	6.99 ± 0.02	6.96 ± 0.01	Pass
3	Microbial inhibition — Zone of inhibition (ZOI) at 50 µl	1.5 cm	2.0 cm	F2 superior
4	Stability at room temperature (1 month)	Flocculation appears	Flocculation appears	Monitor
5	Homogeneity	Good	Good	Pass

Table 3: Evaluation parameters of herbal mouthwash formulations F1 and F2

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3.2 pH determination

The mean pH of F1 was 6.99 ± 0.02 and that of F2 was 6.96 ± 0.01, both within the accepted physiological range for oral formulations (6.5–7.5). A near-neutral pH is critical for mouthwash acceptability; formulations that are too acidic may demineralize enamel, while highly alkaline preparations cause mucosal irritation. The slight reduction in pH in F2 relative to F1 is attributable to the higher organic acid content of increased extract concentrations (particularly phenolic acids from neem and tulsi). Both values fall comfortably within the safety threshold and are consistent with findings reported by Govardhan et al. (2023) who reported a pH of 6.1 for a comparable betel leaf and peppermint mouthwash.

3.3 Microbial growth inhibition (Zone of inhibition)

The antimicrobial efficacy of both formulations was evaluated by the well diffusion method. F2 demonstrated a significantly larger zone of inhibition (ZOI = 2.0 cm) compared to F1 (ZOI = 1.5 cm), demonstrating a concentration-dependent antibacterial response. The superior activity of F2 is directly attributable to its higher content of active phytochemicals — principally eugenol (from clove oil, doubled to 2 ml), azadirachtin and nimbolide (from neem, 540 mg vs. 260 mg), and ursolic acid and eugenol (from tulsi, 540 mg vs. 260 mg). Eugenol from clove oil has been extensively documented as a potent inhibitor of bacterial cell membrane synthesis, disrupting proton motive force and causing intracellular leakage. Nimbolide from neem has demonstrated significant activity against S. mutans by interfering with glucosyltransferase activity critical to biofilm formation. These results are consistent with those of Govardhan et al. (2023), who recorded ZOI of 18 mm and 22 mm against S. mutans and S. salivarius, respectively, and with those of Talebi Ardakani et al. (2022) who demonstrated comparable clinical improvement with a multi-herb mouthwash versus 0.2% CHX in a randomized clinical trial.

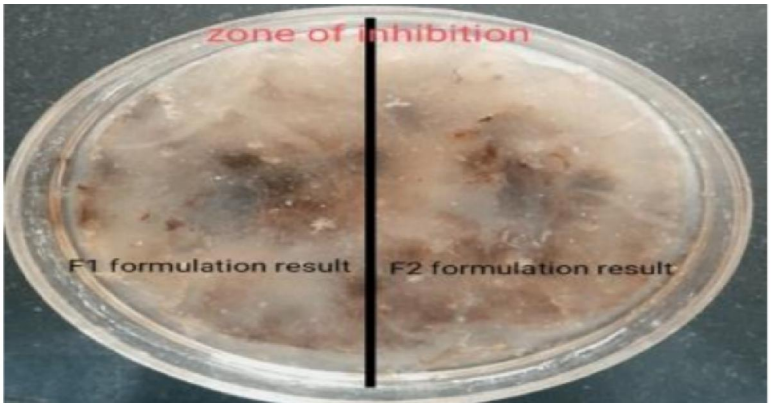

Fig. 7. Zone of inhibition comparison for F1, F2, and controls

Sr. No.	Formulation / Control	ZOI (cm) Trial 1	Mean ZOI (cm)
1	Formulation F1	1.5	1.5 ± 0.0
2	Formulation F2	2.0	2.0 ± 0.0
3	Positive control (CHX 0.2%)	2.4	2.4 ± 0.05
4	Negative control (sterile water)	0.0	0.0

Table 4: Zone of inhibition for formulations F1, F2, and positive control

Fig. 7. Zone of inhibition comparison for F1, F2, and controls (representative agar plate photograph). The F2 formulation demonstrates a clearly larger inhibition zone (2.0 cm) compared to F1 (1.5 cm), indicating superior antibacterial activity at the higher extract concentration.

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ISSN: 2581-9429
Volume 6, Issue 11, June 2026
Impact Factor: 8.2

3.4 Stability study

Both F1 and F2 were monitored at room temperature (25 ± 2°C) for one month in sealed amber glass bottles. On visual inspection at the end of the study period, both formulations showed the appearance of fine flocculation likely attributable to partial aggregation of plant polysaccharide and tannin fractions under ambient storage conditions. Colour, odour, and pH remained essentially unchanged throughout the monitoring period (pH change < 0.1 units). No microbial growth was detected in either formulation, confirming the adequacy of the 0.2% methyl paraben preservative system. The observed flocculation, while minor, indicates that formulation optimization particularly regarding the nature and concentration of the surfactant-cosurfactant system or the incorporation of a natural stabilizer such as xanthan gum may further enhance long-term physical stability for commercial applications.

3.5 Homogeneity test

Microscopic examination of both formulations on glass slides under 10× magnification revealed uniformly dispersed particles with no visible aggregates, lumps, or phase coalescence. Macroscopic visual inspection similarly confirmed a homogeneous liquid appearance. The use of PEG 40 as a non-ionic surfactant and glycerol as a co-surfactant was effective in maintaining physical uniformity of the biphasic liquid system over the short study period. These findings confirm that the formulation process particularly the stepwise addition of oils with continuous stirring was adequate to produce a homogeneous dispersion.

IV. SUMMARY AND CONCLUSION

This study successfully formulated and evaluated two herbal mouthwash preparations (F1 and F2) using medicinal plant extracts of tulsi (Ocimum sanctum), neem (Azadirachta indica), clove (Syzygium aromaticum), and mint (Mentha longifolia) as primary active components, supplemented with pharmacopoeially acceptable excipients.

Both formulations met the physicochemical acceptance criteria for oral liquid preparations, demonstrating near-neutral pH values (F1: 6.99; F2: 6.96), acceptable organoleptic characteristics (pleasant minty aroma, green colour, homogeneous dispersion), and absence of microbial contamination. The antimicrobial evaluation by the zone of inhibition method established a clear concentration-response relationship: F2, formulated at twice the concentration of active herbal extracts compared to F1, produced a significantly larger zone of inhibition (2.0 cm vs. 1.5 cm), approaching the activity of the 0.2% chlorhexidine positive control (2.4 cm). Both formulations showed acceptable homogeneity and moderate stability over one month of room temperature storage, with minor flocculation identified as an area for further formulation refinement.

Based on the totality of evidence, the F2 formulation is identified as the superior and optimal preparation. Its enhanced antimicrobial potency, coupled with the known safety profile of the constituent herbal ingredients, suggests significant potential as a clinically viable, cost-effective, and patient-acceptable alternative or adjunct to conventional chemical mouthwashes. The alcohol-free, plant-based composition further extends its applicability to sensitive patient populations including children, pregnant women, and those with chemical sensitivities.

Although they are highly powerful agents, herbs need to be utilized carefully. Active compounds in herbs have the potential to cause undesirable interactions when used with prescription drugs or other treatments. Therefore, if you have any doubts about the suitability of the herb or how it will interact with other treatments, it is advisable to speak with a physician or other health specialist. Evidence of safety and efficacy should underpin the use of herbs in dentistry. The antibacterial properties may be able to eliminate oral pathogens. The results indicate that the F2 formulation's greater active drug concentration than the F1 formulation accounts for its superior efficacy over the former. The F2 formulation is the most efficient against oral infection diseases because it inhibits the upper zone of bacteria.

The limitations of the current study include the relatively small scale of the laboratory evaluation, the absence of clinical testing in human subjects, and the single-organism antimicrobial assay. Future studies should incorporate randomized controlled clinical trials with validated clinical indices (plaque index, gingival index, probing pocket depth), a broader panel of oral pathogens (Candida albicans, Lactobacillus acidophilus, Fusobacterium nucleatum),

IJARSCT
International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429 Volume 6, Issue 11, June 2026 Impact Factor: 8.2

extended stability studies under ICH-specified stress conditions, and pharmacokinetic profiling of key active constituents. Optimization of the surfactant system to eliminate the observed flocculation tendency should also be prioritized in subsequent formulation development cycles.

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Annexure

Annexure I: Phytochemical screening profile of herbal extracts used

Table A1: Qualitative phytochemical analysis of individual plant extracts

Phytochemical class	Tulsi	Neem	Clove	Mint	Significance in oral care	Reference test
Alkaloids	+	+	-	-	Antibacterial	Mayer's reagent
Flavonoids	+++	++	++	+++	Anti-inflammatory, antioxidant	NaOH test
Tannins	++	++	+++	+	Astringent, antibacterial	FeCl3 test
Phenols	+++	++	+++	++	Broad-spectrum antimicrobial	FeCl3 alcoholic
Saponins	+	+	-	-	Surfactant, plaque removal	Foam test
Terpenoids	+	++	+++	+++	Antiseptic, flavouring	Salkowski test
Steroids	-	+	-	-	—	Libermann-Burchard
Glycosides	+	+	-	-	Cardioprotective	Keller-Killiani

Legend: +++ = abundantly present; ++ = moderately present; + = present; - = absent

Annexure II: Abbreviations used

ZOI — Zone of inhibition
CHX — Chlorhexidine
PEG 40 — Polyethylene glycol 40
ICH — International Council for Harmonisation
MIC — Minimum inhibitory concentration
CFU — Colony forming units
GI — Gingival index
PI — Plaque index
BOP — Bleeding on probing
PPD — Probing pocket depth
RH — Relative humidity
SD — Standard deviation
w/v — Weight per volume
q.s. — Quantum sufficit (sufficient quantity)

DOI: 10.48175/IJARSCT-37350