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Original Paper

Antibacterial, Antioxidant, and Anti-inflammatory Evaluation of a Newly Formulated Polyherbal Mouthwash

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ABSTRACT
The expanding concerns regarding the safety of health care products used for oral disorders inspire the researcher to find alternatives that being safe and natural for oral care, this property was found it is way in searching the use of herbal products. The aim of the current research to formulate and evaluate herbal mouth was products using different herbs such as chamomile cinnamon, and clove. The prepared formulations were evaluated for physicochemical properties like organoleptic properties as taste and consistency, viscosity, and thermal stability. By agar diffusion procedure, the antibacterial efficacy of herbal mouth formulation was evaluated using an oral pathogen commonly encountered in pathogenesis of dental plaque. While radical scavenging technique was evaluated by using hydrogen peroxide to evaluate antioxidant activity of herbal mouth wash. The anti-inflammatory potential was assessed by the egg albumin denaturation method. The results elaborated that the best herbal mouth formulation has acceptable organoleptic that will satisfy consumer needs. The other properties were within acceptable ranges regarding pH, viscosity, and thermal stability. The antibacterial activity showed concentration-dependent inhibition for bacterial growth. Also, it showed better and significant antioxidant activity. The prepared herbal mouthwash formulation showed a potential regarding establishment of natural alternative for treatment oral health diseases.

KEYWORDS: Herbal mouthwash, Phytochemicals, Antioxidant, Antibacterial, Anti-inflammatory, Neem

1-INTRODUCTION

The essential characteristic of general health is the hygiene of oral cavity which affects the systemic well-being and the dental health. Gingivitis, dental caries and periodontitis are the common oral infectious disorders that are responsible for the development of the dental biofilms in the oral cavity. These biofilms are rich in pathogenic microorganisms such as *Streptococcus* and *Candida* that induce inflammatory process and tissue damage, necessitating the need for effective and preventive therapeutic actions [(1)].

Mouthwashes are aqueous antiseptic solutions intended to refresh the breath as cosmetics product, diminish microbial burden by antiseptic activity, and regulate plaque development[(2)]. Besides these activities it also has analgesic, anti-inflammatory, and antifungal effects, making them an exceptional choice for the treatment of different oral disorders. Some of the mouth wash products used for treatment of Xerostomia by providing long standing moisturizing activity and hence will enhance the overall oral comfort [(3)(4)].

The traditional mouthwashes preparations contain synthetic chemical actives such as hydrogen peroxide, chlorhexidine, triclosan, and benzydamine HCl. Despite of being effective in treatment of oral disorders, their continuing usage were linked with several adverse effectssuch as Chlorhexidine[(5)] may result in tooth staining, mouth irritation, dry mouth; and unpleasant taste in the mouth, whereas benzydamine HCl[(6)] may cause throat irritation, burning and stinging sensations within the throat, coughing and headache[(7)]. Likewise, triclosan was linked with endocrine disturbances such as thyroidal signaling and causing immune deficiency[(8)(9)].

The choice that satisfy these urgent needs are herbal mouthwashes formulations which utilizes phytochemicals derived from herbs that possess desirable biological properties. The advantages of these products being well-tolerated alternatives, low cost and biocompatible product with fewer side effects compared to the traditional products. They are non-staining and non-irritating products that are safer for individuals with sensitive mouths, besides they do not contain abrasive additives that can lead to dryness[(10, 11)].

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Numerous herbs of medicinal plant types have established antimicrobial properties and are designed to include into mouthwash products such as Cinnamon (*Cinnamomum verum*), clove (*Syzygium aromaticum*), and Chamomile (*Matricaria chamomilla*) [12].

This study concentrates on the development and evaluation of herbal mouthwash using extracts of clove, cinnamon, and chamomile. The study search for the different biological activities of these product in term antibacterial, antioxidant and anti-inflammatory activity and to assess its probability as an effective natural alternative for traditional mouthwash formulations.

2-MATERIALS AND METHODS

Study Protocol and Ethics

This was a comparative cross-sectional study, conducted from January to June 2024 at a teaching hospital in Salah al-Din Governorate, Iraq. The Research Ethics Committee at the University of Samarra granted approval (Approval No. EC-2023/115) in line with the Declaration of Helsinki. All participants were asked for written informed consent.

Participants and Groups

2.1 Collection of Plants

Dried leaves of *Azadirecta indica* (neem), *Mentha piperita* (peppermint), *Matricaria chamomilla* (chamomile), *Camellia sinensis* (Green Tea), *Origanum vulgare* (Oregano), dried flower buds of *Syzgium aromaticum* (clove), and dried barks of *Cinnamomum zeylanicum* (Cinnamom) were purchased from local market[13]. The plants utilized in this research was showed in Figure 1.

Figure 1: Botanicals utilized in the preparation of herbal mouthwash

2.2 Preparation of Plant Extract

The gathered leaves from various plants, cinnamon bark, and dried clove buds were meticulously washed with sterile water to eliminate dirt and impurities, thereafter dried, broken into small pieces using a mortar, and stored separately in airtight containers. The aqueous extract of each plant material was made by boiling 20 grams of chopped parts in 200 milliliters of water for approximately 10 to 15 minutes. The mixture is permitted to cool, subsequently filtered using a Whatman filter, and stored in the refrigerator [14, 15].

2.3 General procedure for the formulation of herbal mouthwash

The complete care herbal product as mouthwash was formerly organized to include three herbal extracts selected for their established antibacterial, anti-inflammatory, and antioxidant capabilities[16]. Various herbal mouthwashes were formulated (F1-F5) as presented in Table 1. Each composition comprises a blend of various botanical extracts. Initially, approximately 70 mL of D.W was heated to the temperature range of 40-50°C. Subsequently, the remaining water-soluble chemicals were incrementally introduced to heated water (glycerin, sodium benzoate, sodium saccharin, and Tween 80) while stirring until all substances were fully dissolved. Disperse the required volumes of herbal extracts into the solution with vigorous stirring using magnetic stirrer, followed by addition of flavors and then make up the final volume to 100 mL with distilled water. Ultimately, filter and package in a pristine amber bottle, ensuring the label is properly affixed. The chemicals listed in Table (2) were incorporated into each formulation in fixed proportions.

Table 1. Different herbal mouth wash components

Formula	Anti-bacterial Plant Extract(5 mL)	Anti-inflammatory Plant Extract(5 mL)	Anti-oxidant Plant Extract(5 mL)
F1	Neem	Chamomile	Green Tea
F2	Oregano	Chamomile	Green Tea
F3	Peppermint	Chamomile	Green Tea
F4	Cinammom	Chamomile	Green Tea
F5	Cinammom	Chamomile	Clove

Table 2. Substances which included in all formulation in constant volumes and quantities

Component	Quantity (for 100 mL)
Glycerin	2.0 mL
Polysorbate 80	0.5 mL
Sodium Benzoate	200 mg
Sodium Saccharin	50 mg
Menthol	20 mg
D. W	Up to100 mL

2.4 Evaluation of herbal Mouthwash

2.4.1 Determination of organoleptic properties

The organoleptic qualities of the formulated mouthwashes were assessed through visual inspection. Our senses are employed to evaluate the flavor and fragrance of the prepared mouthwash. Five healthy volunteers were selected, and after tasting and smelling the mouthwash, they were interrogated regarding their assessment using a hedonic scale for organoleptic evaluation as outlined in Table 3. Additionally, sensory and visual assessments were juxtaposed with the commercially available mouthwash formulation (Chlorhexidine) [17].

Table 3. Organoleptic evaluation scale

Category	Scale
Very sweet	5
Sweet	4
Neutral	3
Bitter	2
Extremely bitter	1

2.4.2 pH

A pH meter equipped with a glass electrode was exploited to measure the pH of the formulated herbal mouthwash products. To evaluate pH, the electrode of the pH meter was dipped in the mouthwash formulations and maintained in the solutions until the displayed reading stabilized. The procedure was quantified in triplicate [18].

2.4.3 Viscosity

Viscosity of mouthwash formulations was estimated utilizing a computerized rotational viscometer. A 50 mL sample of herbal mouthwash was poured in a beaker and allowed to equilibrate for 10 minutes prior to obtaining digital readings with rotor No. 4 at four distinct rotations (6, 12, 30, and 60 rpm) for each formulation. The viscometer reading was recorded at the appropriate speed. The procedure was quantified in triplicate[19].

2.4.4 *In vitro* antibacterial activity

In vitro antibacterial activity was conducted on isolated colonies of *Streptococcus mutans*. The Agar well diffusion method was employed to ascertain the zone of inhibition [20]. The variants of *S. mutans* were introduced onto nutritional agar. Plates were dried, and four wells were created using a 6 mm agar well cutter. 25 µL, 50 µL, 75 µL, and 100 µL of the produced mouthwash were dispensed into the corresponding wells. The agar plates were maintained undisturbed to facilitate the passive diffusion of herbal mouthwash into the agar culture media. The dishes were afterward raised at 37°C for twenty-four hours. The inhibition zones were measured in millimeters using suitable rulers. The procedure was quantified in triplicate.[21].

2.4.5 Evaluation of antioxidant activity

The hydrogen peroxide scavenging analysis was conducted in concordance with method established by Ruch *et al.* with slight modifications[22]. A 40 mM hydrogen peroxide solution was made by dissolving 4.42 mL of 30% H_2O_2 in phosphate buffer (pH 7.4), to form final volume (50 mL). Various volumes (10µL, 20µL, 30µL, 40µL, and 50µL) of the chosen herbal mouthwash formulation were diluted to 500 µL with phosphate buffer and combined with 500 µL of 40 mM H_2O_2. The mixtures were vortexed, raised for ten minutes at ambient temperature, and absorbance was measured at 230 nm relative to phosphate buffer as blank. A negative control including buffer and H_2O_2 (absent of sample) indicated 0% scavenging. Ascorbic acid at a concentration of 0.1% served as the standard or positive control[23]. The capacity to decompose hydrogen peroxide was determined utilizing the following equation below:

% scavenged (H_2O_2) = (Ao − A1)/Ao × 100

Ao: is the absorbance of the control

A1: the absorbance of the sample.

The assess was performed in triplicates and results were tabulated as mean ± standard deviation (SD).

2.4.6 EGG albumin denaturation assay

A 5ml solution was made, containing 2.8 ml of freshly prepared phosphate buffered saline of pH - 6.3 and 0.2 ml of egg albumin extracted from hen's egg. Specific concentrations were prepared separately for selected herbal mouthwash (10 µL, 20 µL, 30 µL, 40 µL, 50 µL). Aspirin was used as the positive control. Then the mixtures were heated in a water bath at 37ºC for 15 min, then the temperature raised to 70 ºC for 5 min followed by cool down to room temperature and absorption was measured at 660 nm[24].

2.4.7 Stability Studies (effects of the temperature)

An accelerated stability research was conducted over a duration of three months for the chosen herbal mouthwash formulation, subjecting the products to two different temperatures (3-5 °C and ambient temperature). The criteria assessed included various organoleptic characteristics and pH. The samples were held at different temperatures: 3-5 °C and 25 °C with a relative humidity (RH) of 60%. Ultimately, the samples subjected to expedited study were removed following the conclusion of the three-month duration and evaluated for various organoleptic features [25, 26].

2.5 Statistical analysis

The outcomes of this investigation were presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed to assess the differences among the samples at a significance level of $p<0.05$.

3. Results and Discussion

3.1 Determination of organoleptic properties

All five herbal mouthwash formulations and the commercially available chlorhexidine product underwent organoleptic assessment, with the results presented in Table 4. The formulations mostly varied in taste strength, olfactory characteristics, and clarity. All the produced formulations and the commercial product were aqueous, smooth, and exhibited low viscosity. The preparation of all these items was conducted sequentially to achieve optimal formulations with desirable attributes that meet the needs of patients with oral disorders . The formulation F3, which includes peppermint, had superior refreshing characteristics compared to other products due to the cooling effects of peppermint oil and menthol [(27)]. Notwithstanding the attractive qualities of this formulation, bitterness persists due to the presence of caffeine and catechins from green tea [(28)]. A negative organoleptic attribute of this formulation is astringency, which denotes a dryness of the mouth that produced via the the interaction between polyphenols and salivary or mucosal proteins, resulting in diminished oral lubrication[(29)]. This trend is also observed in F1, F2, and F4 due to the presence of green tea. The bitterness of F1 was rated at the highest level (extremely bitter) due to the herb Neem, which contains elevated concentrations of potent bitter compounds such as Azadirachtin, nimbin, meliacin, and nimbidin[(30)]. In relation to the palatability of the herbal products, F5 formulation showed enhanced organoleptic properties such as distinctive warm and spicy fragrance in comparison to the commercial chlorhexidine product and other herbal formulations. Sodium saccharin as artificial sweeteners possibly countered the bitterness of the herbal extracts, while clove and cinnamon enhanced the palatability scale. Depending on these organoleptic results, F5 was the best formulation that showed consumer acceptability and will choose it to complete the further microbiological and stability assessment.

Table 4: Organoleptic properties of the prepared herbal mouthwash

F	Taste	Odor	Clarity
F1	Extremely bitter	Herbal scent	Green to yellowish slightly turbid
F2	Pungent, and bitter	Strong pungent odor	Dark yellowish slightly turbid
F3	Fresh minty with less bitterness	Minty and cool	Slightly green and clear
F4	Bitter spicy taste	Warm spicy aroma	Yellowish to light brown slightly turbid
F5	Sweet	Strong hot spicy odor	Dark brown and clear
M*	Sweet	Minty	Greenish and clear

*Marketed chlorhexidine mouthwash

3.2 pH measurements

The pH readings of the herbal mouthwash are presented in Table 5. The pH range was 5.94-6.53, which is regarded as close as the neutral pH of the oral cavity [(31)]. The pH of the mouthwash should be neutral to slightly acidic, aligning with the typical range of the oral cavity and ensuring safety for oral tissues and teeth. To ensure that the product, upon consumption, does not induce any form of irritation to the oral mucosa. An unreasonably alkaline or acidic mouthwash may cause irritation to the gums, mucosa of oral cavity, teeth, dryness and a burning feeling in the mouth[(32)]. Notwithstanding the numerical discrepancies across the formulations, one-way ANOVA statistics indicated that the variance in pH was not statistically substantial ($p = 0.667$). It suggests that altering the herbal combination did not significantly affect the acidity or alkalinity of the final product within the examined formulation parameters. F5 exhibited the highest mean pH (6.53 ± 0.94), while the commercially available chlorhexidine product displayed the lowest mean pH (5.73 ± 0.16).

3.3 Viscosity measurements

The viscosity of F5 was measured using a rotational viscometer to ensure their flow is smooth, their spreading in the oral cavity is complete, and its capacity to disseminate and coat the oral cavity appropriately. The formulation should not too viscous which may be challenging to maneuver, whereas too fluid product may fail to distribute and cover the oral cavity effectively [(33)]. Table 5 showed the results of viscosity study at various rotational speeds which determined by a rotational viscometer. In all herbal mouth wash formulations, the viscosity decreased with increasing rotation speed, showed non-Newtonian shear-thinning characteristics (pseudoplastic behavior), which attributed to variations in their herbal constituents. The marketed product showed

result of decreased viscosities in comparison to F1 and F2, at the same time the viscosities of the marketed product was greater than F3, F4, and F5 for all speeds. The explanation for this reduction in viscosity with increasing speed of rotation related to disruption of intermolecular connections within the liquid system during shear. This rheological property as pseudoplastic behavior is advantageous for mouth wash formulations products as the product will maintain satisfactory structure during rest whereas it facilitating the easy flow during shaking, pouring, and rinsing (force of shear)[34]. Statistical investigation showed significant differences among the evaluated formulations at all rotational speeds ($p < 0.05$).

Table 5: Viscosity and pH measurements of different herbal mouthwash

F	Viscosity (mPa.s) *				pH*
	Speed 6 rpm	Speed 12 rpm	Speed 30 rpm	Speed 60 rpm	
F1	938±53	530±14	202±13	20±03	6.35±0.12
F2	921±34	503±42	242±09	81±08	5.94±0.23
F3	878±23	561±27	219±05	40±05	6.05±0.78
F4	865±71	485±11	212±16	43±05	6.42±1.05
F5	750±53	517±26	189±08	51±07	6.53±0.94
M	863±41	632±17	234±23	67±10	5.73±0.16

***(Mean ± SD, n=3)**

3.4 Antibacterial activity

The antibacterial activity of F5 against *S. mutans* was conducted using the agar well diffusion method, as seen in Figure 2, with the results presented in Figure 3. The antibacterial efficacy is a vital quality characteristic of a mouthwash designed for oral hygiene, as the inhibition of cariogenic bacteria may diminish plaque accumulation and the ensuing advancement of oral diseases[35]. An increase in the zone of inhibition was detected, based on concentration. The average inhibition zones for F5 at 25, 50, 75, and 100 µL were 13 ± 1.52, 19 ± 2.03, 23 ± 2.08, and 26 ± 3.15 mm, respectively. One-way ANOVA revealed a statistically significant impact of concentration on antibacterial activity ($p = 0.0014$), indicating that the observed increase in inhibition zone was improbable to result from random variation alone. The results demonstrate that the antibacterial effect greatly intensified with an increase in given volume, while the incremental benefit between 75 and 100 µL was minimal.

The current data demonstrate that the chosen herbal mouthwash formulation F5 exhibits significant inhibitory effectiveness against *S. mutans*. This observation holds pharmaceutical significance due to *S. mutans* leads to the formation of dental plaque and is a significant factor in the genesis of dental caries[36]. The antibacterial efficacy of herbal mouthwash may stem from growth inhibition, membrane disruption, and metabolic suppression, and the concentration-dependent response reported herein corroborates the biological significance of the chosen plant combination[37].

Figure 2: Agar diffusion method for evaluation of selected formulation of herbal mouth wash at four different concentrations 25, 50, 75, and 100 µL which showed inhibition zones of 12, 16, 22, and 28mm; respectively

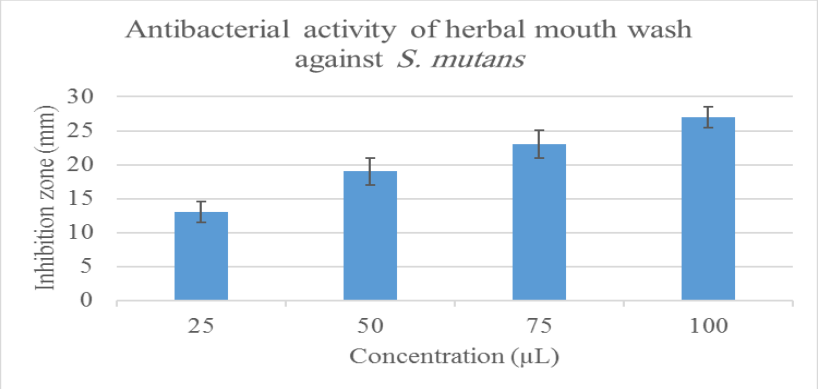

Figure 3: Antibacterial activity of the prepared herbal mouth wash F5

3.5 Antioxidant activity

The antioxidant efficacy of the chosen polyherbal mouthwash (F5) was assessed by the hydrogen peroxide (H_2O_2) scavenging assay. The principle relies on quantifying the reduction in H_2O_2 concentration. Hydrogen peroxide exhibits an absorbance peak at 230 nm. Upon the addition of an antioxidant, it decomposes H_2O_2, resulting in a reduction of absorbance. A lower absorbance indicates a greater scavenging activity[38]. The percentage of scavenging for various concentrations of herbal mouthwash F5 is illustrated in Figure 4. The percentage inhibition values at doses of 10, 20, 30, 40, and 50 µL were 21.3 ± 3.1%, 34.7 ± 2.8%, 48.9 ± 4.2%, 61.5 ± 3.9%, and 72.8 ± 2.4%, respectively, while the standard ascorbic acid had a markedly superior scavenging activity of 89.6 ± 5.5%. The antioxidant activity was increased as the concentration of herbal mouth wash product increased, demonstrating a concentration-dependent scavenging effect.

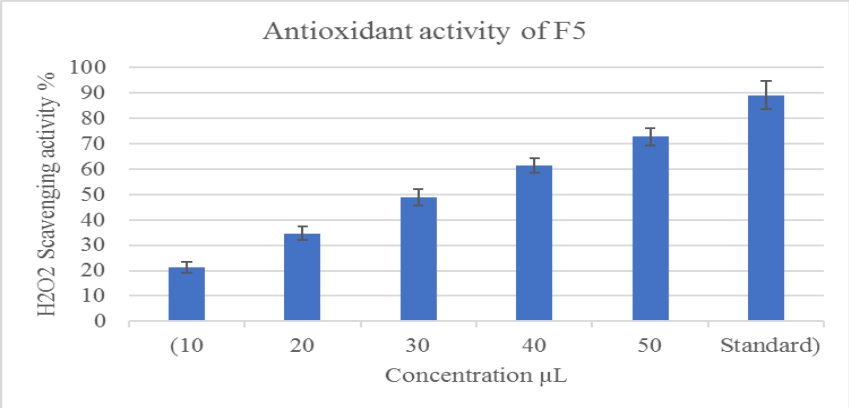

Figure 4: Scavenging activity of the prepared herbal mouth wash F5

The increasing the activity of antioxidant with increasing concentrations of the herbal product was statistically ($p < 0.0001$), supporting that notable antioxidant activity was related to the different herbal constituents of the formulation F5. One the other hand F5 mouthwash product exhibited a noticeably lower antioxidant activity in comparison to the standard ($p<0.05$). The dietary carbohydrate was converted to acids by *S. mutans* which is the main bacterium responsible for dental caries. These bacteria clinging to tooth enamel and produces hydrogen peroxide and lactic acid, which causes acidic environment that demineralizes tooth structure, resulting in decay [39]. Furthermore, hydrogen peroxide has moderate oxidizing activity that produces highly reactive hydroxyl radicals within cells, resulting in oxidative consequences that end with damage to DNA, lipids, and proteins. Therefore, the antioxidant activity of F5 by scavenging H_2O_2 designates its potential to alleviate cellular damage resulted from oxidative stress [40]. The detected antioxidant activity of the herbal mouthwash F5 was possibly related to the synergistic effects of different phytochemicals present in the herbs used in the preparation of these products such as essential oils, polyphenols, and flavonoids. In F5 formulation, green tea contains high amounts of catechins, which has robust free radical scavenging capabilities. While clove contains eugenol, which characterized by powerful antioxidant activity[41]. The synergistic antioxidant activity of Cinnamon was related to cinnamaldehyde and other phenolic substances. The overall antioxidant efficacy of the formulation F5 was collectively related to the collective interaction among these phytochemicals [42].

3.5 EGG albumin denaturation assay

The egg albumin denaturation assay was used to assess the anti-inflammatory effects of herbal mouthwash made with different herbal extracts of cinammom, clove and chamomile (F5) showed in Figure 5. The effectiveness of the experiment was determined by the percentage of inhibition at various concentrations (10–50 µL) of the herbal mouthwash and the standard (Aspirin). The herbal mouthwash F5 exhibited a significant inhibition of 43% at concentration of 10 µL, while Aspirin as standard demonstrated a slightly higher inhibition of 51% than F5. Increasing the concentration of the herbal mouthwash to 20 µL resulted in a higher inhibition of 55%, while the inhibition percent of standard was 63%. The anti-inflammatory activity of the herbal mouthwash remained consistent at 30 µL, with a slightly increased inhibition of 59%, while the standard showed a slight increase to 67%. At 40 µL and 50 µL concentrations the inhibition increased steadily as 66 and 73%, respectively. While for standard the percent was 72 and 82%, respectively. For all concentrations of herbal mouth wash formulation, the inhibition percent was significantly lower than the standard ($p<0.05$). Overall, the results indicate that the herbal mouthwash preparation F5 possesses concentration-dependent anti-inflammatory effects, with its effectiveness comparable to the standard Aspirin at various concentrations making it a potential natural alternative for managing inflammatory conditions in the oral cavity.

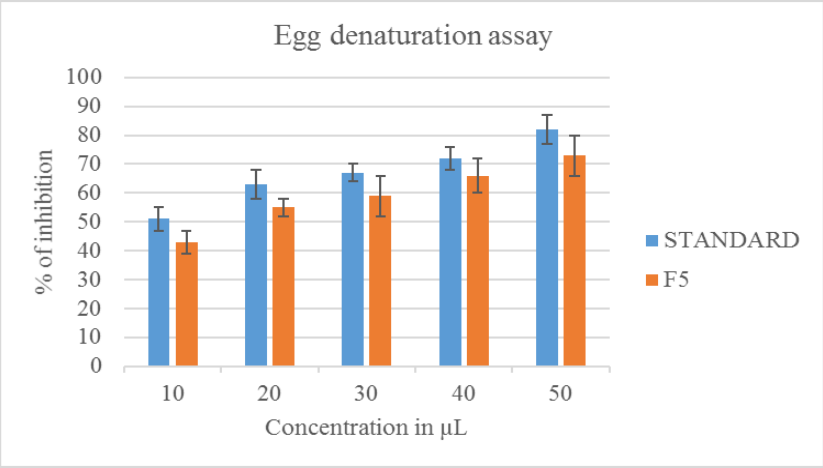

Figure 5: Anti-inflammatory activity of mouthwash formulation using Egg albumin denaturation assay

3.6 Stability studies

The accelerated stability studies of herbal mouth wash F5 was conducted by exposing the product to different temperatures in accordance with ICH recommendations[43]. Accelerated stability studies (short term) provide preliminary data regarding preserving the product of better attributes during the long period of storage. In the current study, F5 exhibited physical stability throughout three months of storage at both ambient and refrigerated temperatures, with no significant variations in their shade, aroma, clarity, or phase separation. The measured pH values after period of three months storage persisted within a satisfactory range, demonstrating the short-term stability of the selected herbal mouthwash formulation F5. The physical evaluation and stability studies of herbal mouth wash F5 after three months was depicted in Table 6.

Table 6: Stability studies of herbal mouth wash F5 after three months

Temperature	Evaluation Parameter	Observation
3-5 °C	Taste	Very sweet
	Odor	Strong aroma
	Clarity	Brown to slightly yellowish and clear
	pH	6.33±0.67
Room Temperature 25°C, RH=60%	Taste	Sweet
	Odor	Spicy aroma
	Clarity	Brown and clear
	pH	6.14±0.14

4. CONCLUSION

In the present work herbal mouthwash formulation containing chamomile, cinnamon, and clove extracts was effectively prepared and showed acceptable physicochemical properties. The selected mouth wash product showed significant antibacterial activity against oral pathogen as *S. mutans*. Besides, it has considerable antioxidant activity along with considerable anti-inflammatory effects. These activities are possibly related to the synergistic effects of different phytochemicals present in the herbs used in the preparation of these products. The results in this study specify that the prepared herbal mouthwash products may be a practicable natural alternative for traditional oral sanitation products. Nevertheless, further research, which includes *in vivo* studies and long-term stability evaluations, is recommended to authenticate its therapeutic practicality and safety.

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