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Original Article

ANTIBACTERIAL EFFECTIVENESS OF DRAGON SCALE LEAVES (*DRYMOGLOSSUM PILOSELLOIDES* L.) MOUTHWASH AGAINST *STREPTOCOCCUS MUTANS* AND *ENTEROCOCCUS FAECALIS* (PRELIMINARY STUDY)

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ABSTRACT

Objective: Dental caries are mainly caused by *Streptococcus mutans*, whereas *Enterococcus faeca*lis is detected in root canals. Using mouthwash can reduce oral bacteria. The purpose of this study was to determine the stability of mouthwash containing *Drymoglossum piloselloides* L. extract at concentrations of 10%, 20%, and 30%, assess its acute toxicity, and evaluate its antibacterial effectiveness against *Streptococcus mutans* and *Enterococcus faecalis*.

Methods: This research uses laboratory experimental research with a post-test-only controlled group design. Twenty-five samples were used, divided into five at 10%, 20%, and 30%, positive and negative control. The stability of the mouthwash was evaluated through a cycling test consisting of six cycles, while the acute toxicity was assessed using the Brine Shrimp Lethality Test (BSLT). The antibacterial test was used to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) by using a liquid dilution method analyzed with a UV-Vis spectrophotometer, and the antibacterial inhibition zone was evaluated using the Kirby-Bauer disk diffusion method. Data analysis utilized One-way ANOVA followed by post-hoc Games-Howell (normal data distributed) or Kruskal-Wallis tests followed by Mann-Whitney tests (not normally data distributed).

Results: Based on organoleptic, pH, and viscosity tests, *Drymoglossum piloselloides* L. extract mouthwash was stable and non-toxic. MIC testing using the liquid dilution method showed that the 30% concentration had a significantly different minimum inhibitory effect than the negative control. The 30% *Drymoglossum piloselloides L.* leaf extract concentration showed significant suppression against *Streptococcus mutans* (10.483±0.076 mm) and *Enterococcus faecalis* (10.367±0.058 mm) in disk diffusion technique findings. None of the mouthwash concentrations group reached the ≥99% bacterial reduction threshold required for MBC determination.

Conclusion: It concluded that *Drymoglossum piloselloides* L. extract at 30% concentration has an antibacterial effect.

Keywords: *Drymoglossum piloselloides* L, antibacterial, *S. mutans*, *E. faecalis*, mouthwash

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INTRODUCTION

Oral and dental health problems are diseases experienced by half of the world's population [1]. In 2017, the Global Burden of Disease Study found that 3.56 billion people worldwide suffer from oral diseases, in which dental caries are the most discovered [2, 3]. The 2018 National Basic Health Survey (RISKESDAS) results reveal that 45.3% of Indonesians have dental caries [4]. *Streptococcus mutans* (*S. mutans*) is prominent bacteria in dental caries formation. *S. mutans* produces glucosyltransferase enzyme (GTF) that changes sucrose into soluble and insoluble extracellular polysaccharides such as glucan and fructan, which are constituents of plaque and dental caries formation [5]. The development of dental caries appears as microorganism entrance to dental nerves. *Enterococcus faecalis* (*E. faecalis*) bacteria usually exist in root canals and stay persistently inside even though treatment has been administered [6, 7]. *E. faecalis* persistence can cause root canal infection [7].

Caries are inseparable from the presence of bacteria, which leads to using antibacterial agents as a primary approach. One of the widely used antibacterial products is mouthwash. Using mouthwash is considered adequate because it thoroughly cleanses the mouth, thereby reducing the pathogens that cause oral health problems. The use of chemical-based mouthwash ingredients can eliminate microorganisms. The ability to eliminate microorganisms is influenced by the solution's concentration, where higher concentrations enhance effectiveness and increase toxicity [8]. Toxicity is a critical factor in mouthwash selection; therefore, alternatives with effective ingredients and relatively low toxicity are needed. Plant-based extracts are believed to be effective as alternative mouthwash ingredients due to their biocompatibility and relatively low toxicity [9].

Indonesia is the second-largest country in terms of natural resources worldwide. Indonesia owns over 30,000 of the 40,000 types of plants worldwide, and 940 are helpful as medication [10]. Traditional medication offers various advantages that increase its popularity, including the relatively low side effects [11]. One of the plants used as traditional medication is dragon scale leaves (*Drymoglossum piloselloides* L.) [12].

Drymoglossum piloselloides L. is an epiphytic plant that grows on another plant as its physical support and is commonly found growing in Universitas Sumatera Utara, Medan, Indonesia. This plant might appear superfluous, but eventually, it will have beneficial efficacy and can be utilized as medication either in fresh or dried form [12]. According to Lumbantoruan and Sari, phytochemical analysis results of *Drymoglossum piloselloides* L. leaves contain saponin, flavonoid, and tannin that overcome various diseases, namely sore throat, mouth ulcers, tuberculosis, mumps, as well as become wound medicine and anti-cancer [15]. Sari *et al.* examined *Drymoglossum piloselloides* L. and revealed that mouthwash with *Drymoglossum piloselloides* L. extract performs potent inhibition concerning *S. mutans* and *S. viridans* bacteria at a concentration of 10% and 15% [14]. Referring to the study conducted by Sari *et al.* (2023), the present research utilized the lowest concentration reported in their findings (10%) and extended the upper limit to 30%, exceeding the previously tested 15%, due to the well-

documented resistance of *Enterococcus faecalis* to elimination. This study aims to determine the stability of mouthwash containing *Drymoglossum piloselloides* L. extract at concentrations of 10%, 20%, and 30%, assess its acute toxicity, and evaluate its antibacterial efficacy against *S. mutans* and *E. faecalis.*

MATERIALS AND METHODS

This study used a laboratory experimental study with a post-test-only group design. *Drymoglossum piloselloides* L. leaves were identified at the Plant Systematics Laboratory of the Herbarium MEDANESE, Universitas Sumatera Utara (no.2581/MEDA/2024). The extraction and acute toxicity tests of *Drymoglossum piloselloides* L. were conducted at the Cendekia Research Laboratory in Medan. The phytochemical qualitative screening was conducted at the Laboratory for Pharmacy Biology, Faculty of Pharmacy, Universitas Sumatera Utara. The stability test of *Drymoglossum piloselloides.* leaves extract mouthwash was conducted at the Laboratory for Microbiology, Faculty of Pharmacy, Universitas Sumatera Utara.

The sample of this study consisted of *S. mutans* ATCC®25175 and *E. faecalis* ATCC®29212 bacteria. According to the Federer formula (n-1)(5-1)≥15, n=4,75 (5 samples each group) with α 0.05. The sample amount was 25, divided into five samples representing 5 study groups. The study groups included *Drymoglossum piloselloides* L. extract with concentrations at 10%, 20%, and 30%, positive control (*Chlorhexidine* 0.2% mouthwash), and negative control (*Dimethyl Sulfoxide*/DMSO). The Health Research Ethics Committee of Universitas Sumatera Utara has authorized the research consent form (No. 953/KEPK/USU/2024).

Formulation of *Drymoglossum piloselloides L.* leaves extract

The sampling method of this study was purposive sampling without comparing the same components from other areas. The sample was *Drymoglossum piloselloides* L., taken from the environment of Universitas Sumatera Utara, Medan, Indonesia. The inclusion criteria for this study were the green and undamaged *Drymoglossum piloselloides* L. leaves. Meanwhile, the exclusion criteria for this study were the dried, broken, and damaged *Drymoglossum piloselloides* L. leaves. The result of plant identification at Plant Systematics Laboratory (Herbarium Medanense) USU, according to the letter no. 2581/MEDA/2024 stated that the specimens for the testing were dragon scale leaves (*Drymoglossum piloselloides* (L) C Presl).

Drymoglossum piloselloides L. was washed using hygienic water, drained, and then measured its wet weight. The leaves were air-dried in a drying chamber at±40 °C until they got dried, and the dry sortations were measured as the dry weight. The dried samples were ground using a blender, and the powder weight was measured and stored in a plastic container.

The extraction process was undertaken through maceration using 96% ethanol solvent. 500 g of Drymoglossum piloselloides L. Compared to methanol and water, 96% ethanol was more advantageous for plant extraction due to its ability to yield a higher diversity of chemical constituents, its selectivity, non-toxicity, and efficient absorption. This made it suitable for extracting nonpolar, semipolar, and polar compounds. Simplicia powder leaves with an acceptable degree were placed into a vessel, then poured with 75 parts of 96% ethanol, closed, and left for 5 days. The formulation must be protected from light and mixed occasionally daily. After 5 days, the formulation was filtered, and the wastes were drained. The wastes were washed using sufficient solvent, stirred, and filtered to obtain 100 parts. The macerates were collected into a closed vessel, let to cool and protected from light for 2 days, then poured, and the solvent was evaporated with the rotary evaporator (Heidolph VV-300, Heidolph Instruments GmbH and Co. KG, Germany) at the temperature of 40-50 °C, then concentrated on a water bath until a thick extract was acquired [16].

Preparation of test solution concentrations and mouthwash formulation

Thick extract of *Drymoglossum piloselloides* L. leaves was then diluted with Dimethyl Sulfoxide (DMSO) at concentrations of 10%, 20%, and 30%, following the dilution formula of N1 × V1 = N2 × V2 (N1: 100% Concentration (%); V1: Volume of solution with needed 100% of concentration, (ml); N2: Targeted concentration (%); V2: Targeted volume of concentration (ml).

The formulation of *Drymoglossum piloselloides* L. mouthwash with 10% concentration consisted of 10% *Drymoglossum piloselloides* L. extract, zinc chloride (0.5%), menthol (0.05%), PEG 40 Hco (1 %), Glycerin (10%), cinnamon oil (0.1%) and followed by distilled water addition until 100%. Formulation of *Drymoglossum piloselloides* L. was started by weighing the whole ingredients and then diluting zinc chloride into half of the water (solution 1). The next step was mixing PEG-40, menthol, glycerin, and cinnamon oil (solution 2). Solution 1 was then added to solution two while stirring slowly. After that, *Drymoglossum piloselloides* L. leaves extract was added and stirred. The remaining water was poured until 450 ml, filtered, and put into a formulation bottle [17]. The formulation of 20% and 30% concentrations worked in the same technique as the formulation of 10% concentration mouthwash; the only difference was the *Drymoglossum piloselloides* L. extract concentration amount, while the negative control was the primary material without the addition of active ingredients of *Drymoglossum piloselloides* L. extract.

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Characteristics of mouthwash of dragon scale leaves (*Drymoglossum piloselloides* L.) and stabilization tests

Organoleptic testing was done using senses to observe the form, homogeneity, scent, taste, and color of the provided mouthwash [18]. pH measurement was undertaken for the mouthwash formulation by firstly calibrating into buffer solution with a pH level of 7 and 4, and then separating 30 ml of sample, putting the sample into a beaker followed by placing a pH meter (ATC, PT Digital Akurasi, Indonesia) into the sample until the constant pH value has been acquired [19]. For the viscosity test, 50 ml of the formulation was put into a beaker glass. Set the rotor No. 2 at a speed of 60 rpm. Then, the sample was dipped to the rotor limit mark. The viscometer (NDJ 8s, Shanghai Martests Instrument Co., Ltd, China) was turned on for 10 seconds, and after that, the viscosity value appeared on the viscometer screen [19].

Physical stability (organoleptic), pH, and viscosity testing of mouthwash adopted the six cycles of cycling test method by storing the formulation at the temperature of 4°±2 °C for 24 h and then storing it at the temperature of 40°±2 °C for 24 more hours. The duration of each different temperature was considered as one cycle and conducted for 12 d. The physical state of the mouthwash was determined based on the organoleptic, pH, and viscosity comparison with the previous formulation.

Acute toxicity test with brine shrimp lethality test (BSLT) method

The dark and light vessels were used to hatch the shrimp eggs inside the vessels. The dark zone was used to place the eggs and aerator, while the light zone had a lamp to radiate during the hatching process and separate the cysts. Approximately ±50-100 mg of shrimp eggs that would be hatched were placed within the vessels, and the inside vessels were divided into two zones, dark and light, in which the lights would be turned on for 48 h. Subsequently, ten larvae were pipetted into 2500 µl of seawater. Two drops of DMSO

were added to dissolve the sample. The extract to be tested was made in 20, 200, 400, 1000, and 2000 ppm concentrations.

Furthermore, the sample solution to be tested was pipetted as much as 2500 µl each and adjusted to 5000 µl so that concentrations of 10, 100, 200, 500, and 1000 ppm were obtained. Each concentration had conducted three repetitions. Control groups were carried out without adding samples, and only two drops of DMSO were added as a negative control. The solution was left alone for 24 h and then measured using probit analysis to determine LC50 [21].

Antibacterial activity test

S. mutans and E. facelis bacteria stored in Nutrient Agar (NA) media were taken using an ose sterilized needle and planted in the slant agar media by scratching. Later, the bacteria were incubated inside the incubator (Memmert, Memmert GmbH+Co. KG, Germany) at 37 °C for 24 h. The bacteria rejuvenated on the slant agar media, and the colonies were obtained using a sterile ose needle. Colonies of *S. mutans* and *E. faecalis* were collected from the culture medium using a sterile inoculating needle and suspended in a test tube containing 10 ml of nutrient broth (NB). The suspension was incubated until it matched the turbidity of the McFarland standard solution no. 0.5 (To prepare the McFarland standard, 9.95 ml of 1% H_2SO_4 was mixed with 0.05 ml of 1% $BaCl_2$ solution. The turbidity of the bacterial suspension was visually compared to the standard. If the suspension exceeded the reference turbidity, it was diluted using 0.9% NaCl solution to achieve the desired concentration), indicating a bacterial concentration of 10^8 CFU/ml. To dilute the bacterial suspension, 0.1 ml of the inoculum was added to a test tube containing 9.9 ml of nutrient broth then vortexed until homogeneous, resulting in a bacterial concentration of 10^6 CFU/ml [19].

Determination of minimum inhibitory concentration (MIC) by *Disc Diffusion* method and liquid dilution using UV-Vis spectrophotometry

Kirby-Bauer disc diffusion was a method that was applied to conduct diffusion. Filter paper discs containing *Drymoglossum piloselloides* L. extract at concentrations of 10%, 20%, and 30%, as well as a positive control (0.2% chlorhexidine) and a negative control (DMSO), each with a volume of 25 µl, were placed on Mueller-Hinton Agar (MHA) media inoculated with *S. mutans* and *E. faecalis.* The discs were incubated at 37 °C for 24 h. Following the incubation period, observations were conducted to assess the efficacy of each solution, and the Minimum Inhibitory Concentration (MIC) was determined by measuring the diameter of the inhibition zones around the discs using a vernier caliper [6].

10 ml of sterilized nutrient broth (NB) media was poured into each sterilized test tube with 2 ml of previously labeled media with concentration notes. The previously used cotton swab was dipped to swab the clear zone results obtained through disc diffusion testing. Before incubation, 25 test tubes were measured for absorbance at a wavelength of 580 nm using Uv-Vis spectrophotometry (Thermo Fisher Scientific, PT. Indomultimeter Test Instruments, Indonesia). Following, the tubes were incubated in an incubator for 24 h at a temperature of 37 °C. After 24 h of incubation, the absorbance value was measured again using Uv-Vis spectrophotometry [22].

Table 1: Phytochemical test for *Drymoglossum piloselloides* L.

Secondary metabolite	Reagent	Result
Alkaloid	Dragendorff	-
	Bouchardat	-
	Meyer	+
Flavonoid	Mg powder+Amyl Alkohol+HCl2	+
Glycoside	Molisch+H2SO4	+
Saponin	Hot water/shaken	+
Phenol/Tannin	FeCl3	+
Triperten/Steroid	Liebermann-Bourchat	-

Determination of minimum bactericidal concentration (MBC)

Minimum Bactericidal Concentration (MBC) values were decided by calculating the number of colonies. The composed clear zone from each concentration amid MIC testing was managed to streak employing a sterilized cotton swab, and at that point, plunged for 10 min in each reaction tube containing Mueller and drop it into sterile Petri dishes; add Plate Count Agar (PCA) at that point, homogenize, and incubate for 24 h at the temperature of 37 °C. The colonies shaped within the Petri dishes at that point were calculated utilizing the colony counter machine (Scan®300 Interlab, France). A concentration is deemed to have MBC when it can reduce the initial bacteria count by 98-99% [18, 19].

Data analysis

The data collected from all tests were analyzed using the Statistical Product and Service Solutions (SPSS) software for Windows. The Shapiro-Wilk test (n<50) was used to evaluate the normality of the data. Mouthwash stability analysis was performed using the Wilcoxon test, paired T-test, One-Way ANOVA, and Kruskal-Wallis tests.

The MIC analysis, conducted with a UV-Vis spectrophotometer, employed the Kruskal-Wallis test and One-Way ANOVA, followed by Mann-Whitney and Games-Howell post hoc tests. The inhibitory activity, measured using the disc diffusion method, was analyzed with One-Way ANOVA and Bonferroni post hoc testing. The MBC analysis used the Kruskal-Wallis test and One-Way ANOVA for statistical evaluation.

RESULTS

Phytochemical screening for *Drymoglossum piloselloides* l

The phytochemical test for *Drymoglossum piloselloides* L. was conducted at the Laboratory for Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara. The qualitative testing results are displayed in table 1.

Characteristic testing results of *Drymoglossum piloselloides* L. mouthwash formulation

The organoleptic testing of Drymoglossum piloselloides L. leaves mouthwash formulation using a 6-cycle cycling test resulted in color, odor, taste, and appearance in table 2. The stability evaluation of *Drymoglossum piloselloides* L. leaves mouthwash formulation at each concentration revealed no significant differences in pH and viscosity before and after the six cycles (p<0,05).

After the six testing cycles, the concentration of *Drymoglossum piloselloides* L. leaves mouthwash formulation decreased compared to the initial observation. The absence of significant difference arose after the six cycles of the cycling test, indicating that *Drymoglossum piloselloides* L. leaves mouthwash is stable. Variety testing outcomes of significant differences in pH and viscosity of (p<0,001 among the 10%, 20%, and 30% concentrations of *Drymoglossum piloselloides* L. leaves mouthwash are presented in table 2.

BSLT acute toxicity test results

Acute toxicity test of *Drymoglossum piloselloides* L. leaves, the 30% concentration mouthwash using the *Artemia salina* Leach shrimp death ratio method showed that *Drymoglossum piloselloides* L. leaves extract is non-toxic (table 3). The shrimp larva's LC50 (Lethal Concentration 50) value was 1065.86 µg/ml, which was more than 1000 µg/ml, categorizing the extract as non-toxic. Given that the 30% mouthwash concentration exhibited no toxicity, it can be inferred that the lower concentrations of 10% and 20% are likewise non-toxic.

Table 2: Organoleptic and stability characteristics testing results of *Drymoglossum piloselloides* L. leaves mouthwash using 6-cycle of cycling

Observation	Concen tration	Before cycle (Average)	After 6th cycle (Average)	p-value	Average of cycle value	p-value
ORGANOLEPTIC						
Odor	10%	Distinct odor	Distinct odor	Stable/	-	
Color		Light brown	Light brown	unchanged		
Shape		Liquid	Liquid			
Homogeneity		Homogenous	Homogenous			
Odor	20%	Distinct odor	Distinct odor	Stable/	-	
Color		Brown	Brown	unchanged		
Shape		Liquid	Liquid			
Homogeneity		Homogenous	Homogenous			
Odor	30%	Distinct odor	Distinct odor	Stable/	-	
Color		Dark brown	Dark brown	unchanged		
Shape		Liquid	Liquid			
Homogeneity		Homogenous	Homogenous			
Odor	Blanco	Distinct odor	Distinct odor	Stable/	-	
Color		White	White	unchanged		
Shape		Liquid	Liquid			
Homogeneity		Homogenous	Homogenous			
Characteristics of *Drymoglossum piloselloides* L. leaves mouthwash.						
pH	10%	6.407+0.005	6.317+0.005	0.109[a]	6.358+0.032	<0.001*[c]
	20%	6.389+0.005	6.293+0.005	0.102[a]	6.338+0.031	
	30%	6.350+0.000	6.263+0.011	0.102[a]	6.312+0.031	
	Blanco	6.480+0.000	6.340+0.010	0.109[a]	6.405+0.049	
Viscosity	10%	0.597+0.001 cP	0.595+0.001cP	0.157[a]	0.596+0.001cP	<0.001*[d]
	20%	0.599+0.001 cP	0.597+0.001 cP	0.157[a]	0.598+0.001cP	
	30%	0.603+0.001 cP	0.600+0.001 cP	0.102[a]	0.601+0.001 cP	
	Blanco	0.593+0.001 cP	0.592+0.000 cP	0.225[b]	0.592+0.001 cP	

Note: *significant; a=Wilcoxon test; b=Paired T-test; c=One-way ANOVA test; d=Kruskal-Wallis test

Table 3: BSLT acute toxicity test results

Concentration (ppm)	Death ratio (%)	Probit	LC50 value
10	0	0	
100	12,5	3.845	
200	12,5	3.845	1065.86 µg/ml
500	15	3.96	
1000	40	4.75	

ANOVA post hoc with Games Howell results among pH of *Drymoglossum piloselloides* L. leaves mouthwash groups with 10% vs. 20% = 0.247, 20% vs. 30% = 0.068, and the remaining comparison with other groups are significantly different. Mann-Whitney post hoc among mouthwash viscosity groups are 10% vs. 20% at the value of p = 0.009; 10% vs. 30% mouthwash = 0.04, 10% mouthwash vs. blanco = 0.003; 20% vs. 30% mouthwash = 0.004, 20% mouthwash vs. blanco = 0.003; 30% mouthwash vs. blanco = 0.003.

Antibacterial activity results of *Drymoglossum piloselloides* L. leaves against *E. faecalis* and *S. mutans* bacteria

Minimum Inhibitory Concentration (MIC) test using UV-Vis spectrophotometry showed that three of *Drymoglossum piloselloides* L. leaves extract concentrations exhibited significant differences in inhibition of *E. faecalis* and *S. mutans* bacteria (table 4). UV-Vis spectrophotometric analysis revealed a reduction in absorbance following incubation, suggesting that bacterial growth was suppressed and that the bacteria could not proliferate effectively after exposure to the inhibitory agent. One-way ANOVA post hoc of MIC towards *E. faecalis* using Games-Howell resul ted in 10% vs. 30% concentration= p 0.000; 10% vs. Positive control= p 0.039; 20% vs. Positive control=p 0.043; 30% vs. Negative control= p 0.023; Positive vs. Negative control= p 0.027, while the remains of the groups have no significant differences. Based on the preceding post hoc results, it is obtained that a 30% concentration of *Drymoglossum piloselloides* L. leaves extract against *E. faecalis* performed different inhibition compared with negative control.

Table 4: MIC testing results using spectrophotometer UV-Vis on *Drymoglossum piloselloides* L. leaves extract against *E. faecalis* and *S. mutans*

Sample	*E faecalis*					*S. mutans*				
	ΔAbsorbance ±SD before incubation	p	ΔAbsorbance ±SD after incubation	ΔAbsorbance ±SD difference	p	ΔAbsorbance± SD before incubation	p	ΔAbsorbance± SD after incubation	ΔAbsorbance± SD difference	p
10%	0.068+0.004	0.5	1.283+0.018	1.214+0.016	<0.00	0.064+0.003	0.10	1.217+0.045	1.152+0.045	
20%	0.070+0.005	95[a]	1.141+0.050	1.071+0.054	1[b]	0.072+0.004	8[a]	1.131+0.039	1.059+0.037	<0.00
30%	0.069+0.002		0.964+0.015	0.895+0.017		0.065+0.001		0.800+0.105	0.734+0.105	1[b]
Positive control (Chlorhexidine 0,2%)	0.066+0.002		0.538+0.149	0.472+0.150		0.063+0.002		0.435+0.092	0.372+0.091	
Negative control (DMSO)	0.069+0.001		1.267+0.054	1.198+0.055		0.065+0.001		1.279+0.067	1.124+0.066	

Table 5: Inhibition activity test using disc diffusion method on *Drymoglossum piloselloides* L leaves against *E. faecalis* and *S. mutans* bacteria

Extract concentrations	*E. faecalis*		*S. mutans*	
	Inhibition zone (Average±SD) mm	*p*	Inhibition zone (Average±SD) mm	*p*
10%	9.233±0.058	<0.001*	9.083±0.529	<0.001*
20%	10.008±0.063		9.842±0.052	
30%	10.367±0.058		10.483±0.076	
Positive control (Chlorhexidine 0,2%)	15.30±0.1		14.40±0.2	
Negative control (DMSO)	0.00±0.00		0.00±0.00	

Table 6: MBC test for *Drymoglossum piloselloides* L leaves extract against *E. faecalis* and *S. mutans* bacteria

Extract concentrations	*E. faecalis (A)*			*S. mutans (B)*		
	Bacteria colony amount (CFU/ml)	% Reduction	*p*	Bacteria colony amount (CFU/ml)	% Reduction	*p*
10%	1055.67+17	40.10%	0.009*[a]	818.33+64.06	61%	<0.001*[b]
20%	588+38.30	66.64%		401.67+45.36	80.86%	
30%	243+32.08	86.21%		184.67+11.37	91.20%	
K+	109+9.85	93.82%		119+26.91	94.33%	
K-	1762.33+209.34	0%		2098.33+451.78	0%	

Note: a. Kruskal-Wallis test; b. One-way ANOVA test, *p≤0,05=significant based on the One-Way ANOVA test, Note: * significant; a. Kruskal-Wallis test; b. One-way ANOVA test

One-way ANOVA post hoc of MIC against *S. mutans* using Games-Howell produced the value of 10% vs. 30% concentration=p 0.04; 10% vs. Positive control=p 0.004; 20% vs. Positive control= p 0.008; 30 % vs. Negative control=p 0.02; Positive vs. Negative control=p 0.002; while the remains of the groups have no significant differences. A similar result was obtained on *S. mutans* in that 30% concentration of *Drymoglossum piloselloides* L. leaves extract against *S. mutans* performed different inhibitions compared with negative control.

The results of the antibacterial activity test of *Drymoglossum piloselloides* L. leaf extract against *E. faecalis* and *S. mutans* using the disc diffusion method showed significant differences among *Drymoglossum piloselloides* L. leaf extract with concentrations of 10%, 20%, and 30% toward the inhibition against both the bacteria (table 5). One-way ANOVA post hoc results using Bonferroni obtained the comparison of the whole extract concentrations for testing, positive control, and negative control toward the inhibition zone of *E. faecalis* bacteria, which has a significant difference (p<0,001). Bonferroni's post hoc results on the testing group toward the inhibition zone of *S. mutans* obtained only 20% and 30% concentrations that are not significantly different (p=0.120), while the other groups' comparisons were significantly different.

This research could not discover the Minimum Bactericidal Concentration (MBC) test results because all concentrations of *Drymoglossum piloselloides* L. leaves could not reduce 99%-100% of the *E. faecalis* and *S. mutans* bacterial colonies. The most bacterial reduction was found in the 30% concentration extract, with 91.20% in S. mutans and 86.21% in *E. faecalis* (table 6). Post hoc testing of MBC on *E. faecalis* and *S. mutans* bacteria using various concentrations of *Drymoglossum piloselloides* L. leaves extract showed that the entire concentrations compromise significant difference values (p≤0.05), the higher the concentration value of *Drymoglossum piloselloides* L. leaves extract, the higher possibility of bacteria reduction.

Post hoc results using Mann Whitney on MBC against *E. faecalis* obtained significant differences in all test groups. Post hoc resultsusing Games-Howell among MBC groups against *S. mutans* obtained the values of 10% concentration vs. Negative control = p 0.112; 20% vs. Negative control = p 0.067; 30% vs. Positive control = p 0.126; and among other groups were significantly different.

DISCUSSION

Herbal plants have been popularly used as an alternative ingredient for producing mouthwash. *Drymoglossum piloselloides* L. leaves can be exploited as a mouthwash because of active substances such as flavonoids, glycosides, saponins, phenols, and tannins found in this study's results of phytochemical tests (table 1). These results align with other studies that revealed the active substances of flavonoids, saponins, phenols/tannins, and triterpenes/steroids contained in *Drymoglossum piloselloides* L. leaves [23, 24]. These compounds integrate to equip antibacterial effects. Arif *et al.* also conducted phytochemical tests on dragon scale leaf extract (Pyrrosia piloselloides), resulting in phenols, flavonoids, tannins, saponins, and sterol compounds [25]. Research by Kurniasari *et al.* displayed that the results of quantitative phytochemical tests of *Drymoglossum piloselloides* L. leaf extract contained tannin compounds at 4.01%, which was the highest active substance, followed by flavonoids at 3.18%, phenols at 2.96%, saponins at 2.56%, terpenoids at 1.03%, and sterols at 0.88% as the lowest active substance [26].

The extract of *Drymoglossum piloselloides* L. leaves contains chemical compounds such as flavonoids, glycosides, saponins, phenols, and tannins, which are believed to exhibit antibacterial activity against *S. mutans* and *E. faecalis*. Flavonoid compounds act as antibacterial agents by forming complexes with extracellular proteins, causing structural damage and altering the cell membrane's mechanisms. They also inhibit bacterial nucleic acid synthesis and reduce bacterial motility [27]. The antibacterial effects of flavonoids and glycosides are linked to their ability to disrupt the permeability barrier of bacterial cell membranes [28]. On the other hand, Saponins lower the surface tension of bacterial cell walls, leading to lysis or rupture. This disruption allows antibacterial agents to penetrate the cell, interfere with metabolism, and ultimately cause bacterial death. Saponins absorbed on the cell surface increase the membrane permeability, resulting in the leakage of essential substances and cell death. Phenolic compounds act as antibacterial agents by poisoning protoplasm and damaging bacterial cells. Phenolic compounds exhibit antibacterial properties by damaging the protoplasm and interacting with bacterial cell membranes, leading to lysis, protein denaturation, and the inhibition of cytoplasmic protein, nucleic acid, and ATP-ase synthesis. Tannins act by coagulating bacterial protoplasm, precipitating and binding proteins, which disrupt cell wall formation, impair cell permeability, and ultimately cause bacterial cell death [27, 29].

The stability evaluation of *Drymoglossum piloselloides* L. leaf extract mouthwash was conducted using a 6-cycle cycling test. An organoleptic test revealed no color, odor, shape, and homogeneity change after the six cycles. The *Drymoglossum piloselloides* L. leaf mouthwash formulation has a distinctive odor. In terms of color, the mouthwash extract shows a light brown color that remains stable. This is linear with Wijewardane and Weerasakera's research, which reported the results of the *Drymoglossum piloselloides* L. leaf mouthwash having a similar color corresponding to an extract and a characteristic odor like coconut oil [30].

The pH stability test yielded noteworthy findings and was conducted using a pH meter. Over six cycles, the pH of the *Drymoglossum piloselloides* L. leaf extract mouthwash remained relatively stable, showing only minor decreases without significant variations. The average pH values for 10%, 20%, and 30% concentrations were 6.358±0.032, 6.338±0.031, and 6.312±0.031, respectively (table 2). According to Baliga (2013), cited by Seethalaksmi (2016), Saliva typically has a pH range of 6.2 to 7.6 under normal conditions, with an average pH of approximately 6.7. The oral cavity generally maintains a near-neutral pH between 6.7 and 7.3 [31]. In line with these findings, the pH meter test in this study showed that the pH of the *Drymoglossum piloselloides* L. leaf extract mouthwash ranged from 6.3 to 6.4 across all tested concentrations (10%, 20%, and 30%), indicating that the extract solution remains within a safe pH range for use in the oral cavity.

Viscosity testing indicated that *Drymoglossum piloselloides* L. leaf extract mouthwash was safe and comfortable. According to Rowe (2009), as referenced by Andini (2020), the ideal viscosity for a mouthwash is comparable to that of water, which has a viscosity of approximately 0.89 cP. The mouthwash formulation is closer to the viscosity value of 0.89 cP and is safer and more comfortable to use [32]. Based on this statement, the viscosity test results in this study showed that the average viscosities of *Drymoglossum piloselloides* L. leaf extract at concentrations of 10%, 20%, and 30% were 0.596±0.001 cP, 0.598±0.001 cP, and 0.601±0.001 cP, respectively. These findings indicate that as the concentration of *Drymoglossum piloselloides* L. leaf extract increases, its viscosity approaches that of water. The 30% concentration demonstrated the most optimal viscosity with an average value of 0.601±0.001 cP (table 2). This finding highlights the importance of maintaining optimal viscosity for mouthwash formulations to ensure safety and usability so the formulation is neither too condensed nor too watery [33]. The stability of mouthwash viscosity is essential to guarantee that the final product remains safe and functional.

Using natural compounds potentially leads to a toxic state depending on the body dose threshold. Advanced testing is required to comprehend the safety guidelines of certain extracts by assessing their acute toxicity, determining the LC50 value, diverse toxic symptoms, toxic spectrum, and death effects [34]. The toxicity evaluation of this study revealed that *Drymoglossum piloselloides* L. leaf extract contains antibacterial secondary metabolites. Henceforth tested on animal testing, *Artemia salina* Leach shrimp larvae were used in the Brine Shrimp Lethality Test (BSLT) method. This method is employed to identify the toxic effects and determine the bioactivity of a compound derived from natural ingredients. *Artemia salina* Leach shrimp larvae were chosen for toxicity and bioactive compounds studies from plant extracts [35]. In this study, the acute toxicity test on *Drymoglossum piloselloides* L. leaf extract using the BSLT method with the death ratio of *Artemia salina* Leach shrimp larvae obtained the result of Lethal Concentration 50 (LC50) value of shrimp larvae, which was 1065.86 µg/ml (table 3). LC50 value above 1000 µg/ml is categorized as non-toxic. The high LC50 value in this extract implies that the extract has a low risk to the test organism. Consequently, the *Drymoglossum piloselloides* L. leaf extract is reported as a non-toxic and safe mouthwash ingredient. Although the BSLT showed non-toxic results for this mouthwash, follow-up cytotoxicity assays in mammalian cell lines are recommended.

S. mutans and *E. faecalis* are the predominant g-positive bacteria in the oral cavity, contributing to the development of dental caries and periodontal infections [36]. This study assessed the antibacterial activity of *Drymoglossum piloselloides* L. leaf extract against *S. mutans* and *E. faecalis* using MIC and MBC assays. The MIC test was carried out using a UV-Vis Spectrophotometer and the disc diffusion method, while the MBC test was performed using the streaking method, with samples collected from the inhibition zone observed during the MIC test.

UV-Vis spectrophotometry is more accurate than visual observation in interpreting the ability to detect MIC. Several benefits of this method include reproducibility, supporting optimal growth conditions for slow-growing bacteria, and even exposing bacterial cells to antibacterial molecules in the medium. Akinduti (2019) explained that visual assessment is regarded as less accurate, which can cause the inaccurate determination of MIC, but this can be anticipated by using UV-Vis spectrophotometry. This method is highly sensitive; it requires less time and fewer samples and reagents. This test method is based on Beer's Law principle, which states that the amount of light absorbed in a medium is symmetrical to the bacteria that absorb the light [37].

The MIC is determined using UV-Vis spectrophotometry, which compares the average absorbance value before and after incubation. If the absorbance value before incubation exceeds the absorbance value after incubation, bacterial growth is likely inhibited. However, bacterial growth can still occur if the absorbance value before incubation is lower than the absorbance value after incubation [38].

The results of the Uv-Vis spectrophotometer test in this study showed that the *Drymoglossum piloselloides* L. leaf mouthwash formulation had begun to inhibit *S. mutans* and *E. faecalis*, with the 30% extract showing the slightest absorbance difference (Δ0.734±0.105 for S. mutans and Δ0.895±0.017 for *E. faecalis*) (table 4). *Drymoglossum piloselloides* L. leaf extract at a concentration of 10%-30% has begun to inhibit the bacterial growth. However, because of the smaller absorbance value before incubation at the three concentrations compared with the absorbance value after incubation, bacterial growth still occurs in both *S. mutans* and *E. faecalis*.

MIC tests were also carried out by employing the disk diffusion method. The disk diffusion method is most commonly applied to examine a plant extract's inhibitory level based on the diameter of the inhibition zone created on a disc paper [39]. Based on the antibacterial activity criteria by David and Stout (1971), cited by Octiara (2023), if the diameter of the inhibition zone of an extract against bacteria is<5 mm, then the extract has been categorized as a weak antibacterial category. If the diameter of the inhibition zone is 5-10 mm, then the extract has a moderate antibacterial category; if the diameter of the inhibition zone is 10-20 mm, then the extract has a strong antibacterial category; and if the diameter of the inhibition zone is>20 mm, it can be referred that the extract has a powerful antibacterial category [40].

Disk diffusion results in this study revealed the inhibitory activity of *Drymoglossum piloselloides* L. leaf extract against *S. mutans* and *E. faecalis*. The inhibition zone of *Drymoglossum piloselloides* L. leaf extract with 30% concentration showed the inhibition zones were 10.483±0.076 mm for *S. mutans* and 10.367±0.058 mm for *E. faecalis*, indicating strong antibacterial potential (table 5). Although the diameter of the inhibition zone of *Drymoglossum piloselloides* L. leaf extract with 30% concentration was not as high as the positive control (0.2% chlorhexidine), which constructed the inhibition zone diameter of 14.40±0.2 mm in *S. mutans* and 15.30±0.1 mm in *E. faecalis*. However, this extract has a potent potential bacterial inhibitory agent. Among the tested concentrations, the 30% *Drymoglossum piloselloides* L. leaf extract demonstrated the most pronounced antibacterial activity against *Streptococcus mutans* and *Enterococcus faecalis*, as evidenced by the largest inhibition zones. This effect is likely attributable to the higher levels of bioactive compounds—such as saponins, phenols/tannins, and flavonoids—present at increased extract concentrations.

The MBC test in this study revealed that *Drymoglossum piloselloides* L. leaf extract at concentrations of 10%, 20%, and 30% reduced the number of bacterial colonies of *S. mutans* and *E. faecalis*. The concentration increase of the leaf extract elevated the reduction value for both types of bacteria. The higher the extract concentration, the higher the average bacterial reduction value. In this study, a concentration of 30% extract resulted in the most significant reduction, with a value of 86.21% for *E. faecalis* and 91.20% for *S. mutans* (table 6). However, the extract could not achieve 99-100% bacterial eradication for both bacteria. These results showed that *Drymoglossum piloselloides* L. leaf extract can inhibit the growth of *S. mutans* and *E. faecalis* but cannot kill the bacteria completely. Subsequent research may explore the use of higher extract concentrations to achieve the ≥99% bacterial reduction required for MBC determination. However, it is essential to reassess the potential toxicity of these concentrations in human applications.

The efficacy of antibacterial activity against *S. mutans* and *E. faecalis* is affected by several factors, such as bacterial characteristics (including susceptibility and resistance, tolerance, persistence, biofilm formation and inoculum size, antibacterial concentration, and host factors [41]. In *E. faecalis* bacteria, the virulence factors that affect the pathogenicity of the bacteria are not fully apprehended. However, these bacteria are capable of growing in extreme conditions and are resistant to different antibacterial agents. [42] Several active antibiotics against cell walls, such as oxacillin and cephalosporins, have no activity against *E. faecalis* bacteria. *E. faecalis* bacteria have also resisted several typically used active antibiotics against cell walls, including vancomycin [43-45]. Based on these findings, the large or small reduction value of *E. faecalis* bacteria compared to *S. mutans* in this study is presumably due to *E. faecalis* bacteria's high resistance level.

CONCLUSION

Drymoglossum piloselloides L. leaves extract mouthwash with concentrations of 10%, 20%, and 30% exhibited non-toxic and well-stabilized ingredients. *Drymoglossum piloselloides* L. leaves extract with a 30% concentration demonstrated the highest antibacterial activity against *S. mutans* and *E. faecalis*, but it did not completely reduce the bacteria eradication. A limitation of this study is that it is a preliminary investigation. Further research is planned, including a second phase involving animal testing and a third phase involving *in vivo* studies in humans.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally

CONFLICT OF INTERESTS

Declared none

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