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Formulation and antimicrobial evaluation of mouthwash containing propolis and roselle extract

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ABSTRACT: Poor oral hygiene can promote the transition of commensal microorganisms into opportunistic pathogens and contribute to the development of oral diseases. Antimicrobial mouthwashes may serve as preventive or adjunctive therapies in the early stages of oral infections. On the other hand, propolis and roselle (*Hibiscus sabdariffa*), both rich in phenolic compounds, possess well-documented antimicrobial activities and are promising candidates for natural mouthwash formulations. This study evaluated the antimicrobial activity of the combination of ethanolic extract of propolis (EEP) and aqueous extract of roselle (AER) against *Staphylococcus aureus*, *Streptococcus mutans*, and *Candida albicans*, and developed a combined-extract mouthwash formulation. The MIC values of EEP against *S. aureus*, *S. mutans*, and *C. albicans* were 1280, 640, and >20,480 µg/mL, respectively, whereas AER exhibited MIC values of 320, 1280, and 10,240 µg/mL. The MBC values of EEP were 1280 µg/mL for *S. aureus* and 2560 µg/mL for *S. mutans*, while AER showed MBC/MFC values of 1280, 10,240, and 10,240 µg/mL against *S. aureus*, *S. mutans*, and *C. albicans*, respectively. Checkerboard assays indicated additive interactions against *S. aureus* (FICI = 2) and *S. mutans* (FICI = 3). A mouthwash containing 2% EEP and 1% AER was successfully formulated, exhibiting a transparent light-brown appearance, menthol aroma, homogeneous characteristics, pH of 5.44 ± 0.03, and viscosity of 1.36 mPa·s. The undiluted formulation achieved complete microbial reduction within 2–10 min of contact. These findings suggest that the EEP–AER combination has potential as a natural antimicrobial mouthwash for oral health management.

KEYWORDS: Antimicrobial; mouthwash; oral disease; propolis; roselle.

▪ INTRODUCTION

The oral cavity is recognized as the primary entry point of food into the body and simultaneously serves as a habitat for a diverse range of microorganisms. Approximately 1,000 microbial species are estimated to reside in the human mouth, forming a complex ecosystem known as the oral microbiome [1]. The oral microbiome plays a crucial role in maintaining health, yet imbalances within this ecosystem can contribute to the onset of both local (e.g., dental caries, gingivitis, periodontitis) [2] and systemic diseases (e.g., heart diseases, diabetes, autoimmune diseases, pregnancy complications) [3]. Those conditions, often described as dysbiosis, enable normally commensal microorganisms to shift toward pathogenic behavior. One of the main causes of this imbalance is poor oral hygiene. Therefore, maintaining oral hygiene is essential to preserving the equilibrium of the oral microbial community.

Despite widespread public health efforts, individual knowledge and awareness of oral hygiene remain highly variable and, in many populations, relatively low. As a result, oral diseases are among the most prevalent global health problems and can substantially impair quality of life. According to the World Health Organization's 2022 Global Oral Health Status Report, nearly 3.5 billion people worldwide are affected by oral diseases [4], and the 2024 WHO Global Oral Health Meeting [5] have declared oral diseases a global public health priority.

To prevent or manage oral diseases associated with microbiome dysbiosis, several oral hygiene practices have been recommended, including the use of antiseptic mouthwash that exert bactericidal and fungicidal

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effect against oral pathogens. Antiseptic mouthwashes are commonly used as a daily adjunct to mechanical cleaning, such as toothbrushing and flossing [6], [7]. Conventional formulations often rely on chemical agents like phenol, chlorhexidine, and povidone-iodine. However, their long-term daily use is not recommended due to potential adverse effects, including brown discoloration of teeth, restorative materials, and the tongue dorsum, hypersensitivity reactions, and substantial disruptions of the salivary microbiome, which may paradoxically increase the risk of dental caries [8]. This highlights the need for developing safer antiseptic mouthwash formulations.

In line with the growing "back-to-nature" trend, natural product-based formulations are gaining attention as promising alternatives. Several plant- and animal-derived substances exhibit antimicrobial activity and may serve as effective active ingredients in mouthwash. Notably, propolis and roselle (*Hibiscus sabdariffa* L.) are rich in phenolic compounds with well-documented antimicrobial properties [9], [10], [11]. Previous dental research [11], [12] have also demonstrated their capacity to inhibit oral pathogens, highlighting their potential for incorporation into oral care products.

Considering these findings, propolis and roselle extracts merit further investigation as potential alternatives to conventional antiseptic mouthwashes. Their combination may provide a broader antimicrobial spectrum through complementary mechanisms of action while potentially reducing the concentration required for each individual extract. Furthermore, their widespread availability in Indonesia also offers advantages in accessibility and cost-effectiveness. However, to date, no studies have evaluated the antimicrobial activity of a combined propolis–roselle extract in a mouthwash formulation. Therefore, this study aims to assess the antimicrobial activity of ethanol extract of propolis (EEP) and aqueous extract of roselle (AER), both individually and in combination, against three common oral pathogens: *Streptococcus mutans*, *Staphylococcus aureus*, and *Candida albicans*. Additionally, this research focuses on formulating and characterizing a mouthwash containing these extracts and evaluating its antimicrobial efficacy.

▪ MATERIALS AND METHODS

Materials

Raw propolis was obtained from a local beekeeper in Garut, West Java, Indonesia. Aqueous roselle extract was purchased from PT Sari Alam (Sukabumi, West Java, Indonesia). Ethanol 70% and deionized water were obtained from OneMed (Surabaya, Indonesia). Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), Sabouraud dextrose agar (SDA), and Sabouraud dextrose broth (SDB) were purchased from HiMedia (Mumbai, India). Phenol, used as the standard reference antimicrobial compound, and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Normal saline (0.9% NaCl) was purchased from PT Widatra Bhakti (Pasuruan, East Java, Indonesia). Glycerin, methylparaben, propylparaben, sodium saccharin, and menthol were supplied by Brataco (Jakarta, Indonesia). A commercial propolis-containing product (Product "P"), used as a reference comparator, was purchased over-the-counter in Bandung, West Java, Indonesia.

Preparation of EEP

Raw propolis was weighed (10 g) and macerated in 70% ethanol at a 1:10 ratio (w/v) for 72 h at room temperature, with stirring every 12 h for 5 min. The mixture was filtered using Whatman filter paper. Then, the filtrate was concentrated using a vacuum rotary evaporator (Hei-Vap Advantage, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 55 ^{o}C, 55 rpm, and 100 mBar. The extraction process yielded 36.00% ± 0.01 (w/w) EEP.

Phenolic content determination of extracts

The total polyphenol content was determined according to SNI 8490:2018 [13]. Total polyphenol content was determined using the Folin–Ciocalteu method with gallic acid as the standard. A stock solution (250 µg/mL) was prepared by dissolving 62.5 mg of gallic acid in a 250 mL volumetric flask, followed by 10 min of sonication to ensure complete dissolution. From this stock, standard solutions ranging from 10-100 mg/kg were prepared. Each (1 mL) was mixed with 5 mL of 10% Folin-Ciocalteu reagent (Merck, Germany), allowed to stand for 3–8 min, and then treated with 4 mL of 7.5% sodium carbonate solution. The mixtures were incubated in the dark for 2 h and absorbance was measured at 740 nm using a UV–Vis spectrophotometer (Beckman Coulter DU 720, Beckman Coulter Inc., Brea, CA, USA). For the sample

analysis, 10 g of extract was dissolved in 25 mL of distilled water, sonicated for 10 min, diluted to 100 mL with distilled water, and homogenized. Each extract (1 mL) was treated with Folin–Ciocalteu reagent and sodium carbonate following the same procedure as the standards. After 2 h of incubation in the dark, absorbance was measured at 740 nm. The polyphenol content of the sample was then calculated from the calibration curve and expressed as gallic acid equivalents (GAE).

Bacterial strains and growth condition

Streptococcus mutans ATCC 25175, *Staphylococcus aureus* ATCC 6538, and *Candida albicans* ATCC 10231 were obtained from the Microbiology Laboratory, School of Pharmacy, Bandung Institute of Technology. Bacterial strains were cultured on MHA and incubated at 37 °C for 18-24 h, while fungal strains were grown on SDA under the same conditions.

Antimicrobial activity of extracts

Minimum inhibitory concentration (MIC) determination

MIC determination was performed according to CLSI guidelines [14]. Bacterial suspensions were prepared by transferring colonies from MHA into MHB, incubating at 37 °C for 18–24 h, and adjusting turbidity to 0.08–0.13 at 625 nm (equivalent to 0.5 McFarland, ~10^8 CFU/mL), followed by a 1:100 dilution to obtain ~10^6 CFU/mL. Fungal suspensions were prepared by suspending colonies from SDA in sterile saline, incubating at 37 °C for 18-24 h, diluting in SDB, and adjusting turbidity to 0.08–0.13 at 530 nm (0.5 McFarland, ~10^6 CFU/mL), then further diluted (1:100 and 1:20) to achieve ~5.0×10^2 to 2.5×10^3 CFU/mL. Antimicrobial activity was assessed using the broth microdilution method, where serial concentrations of the extracts (40-20,480 µg/mL in 0.1% DMSO) were added to 96-well microplates containing microbial suspensions and incubated at 37 °C for 24 h. Each concentration was tested in triplicate, and the MIC was defined as the lowest concentration that completely inhibited visible growth. Culture medium without inoculum, microbial suspension without treatment, and phenol were used as the negative control, positive control, and reference standard, respectively.

Minimum bactericidal or fungicidal concentration (MBC or MFC) determination

To determine MBC and MFC, 5 µL aliquots from the MIC well and higher concentrations were spot-inoculated onto MHA (bacteria) or SDA (fungi) plates and incubated at 37 °C for 18–24 h. The MBC and MFC were defined as the lowest concentration showing no visible microbial growth, with all tests performed in triplicate.

Checkerboard assay for combination testing

Combination effects were assessed using a checkerboard microdilution assay in 96-well plates (49 wells; rows A–H × columns 1–8) (**Figure 1.**) following the method described by [15]. Each well was filled with 100 µL of MHB. AER stock solution was prepared at 8× MIC, along with double-strength AER stock solution (2AER) at 16× MIC. Then, 100 µL of AER was added to row A, columns 1–7 (final 4× MIC), and 100 µL of the 2AER stock was added to row A, column 8 (final 8× MIC). Twofold serial dilutions of AER were performed down rows A→G by transferring 100 µL from the previous row and discarding the excess from row G. EEP stock solution was prepared at 8× MIC. A total of 100 µL was dispensed across columns 8→2 (final 4× MIC), followed by twofold serial dilutions across the row, with excess discarded from column 2. Finally, 100 µL of microbial suspension was added to all wells. Plates were incubated at 37 °C for 18-24 h and interactions were determined using the fractional inhibitory concentration index (FICI):

$$\text{FICI} = \frac{MIC\ of\ EEP\ in\ combination}{MIC\ of\ EEP\ alone} + \frac{MIC\ of\ AER\ in\ combination}{MIC\ of\ AER\ alone}$$

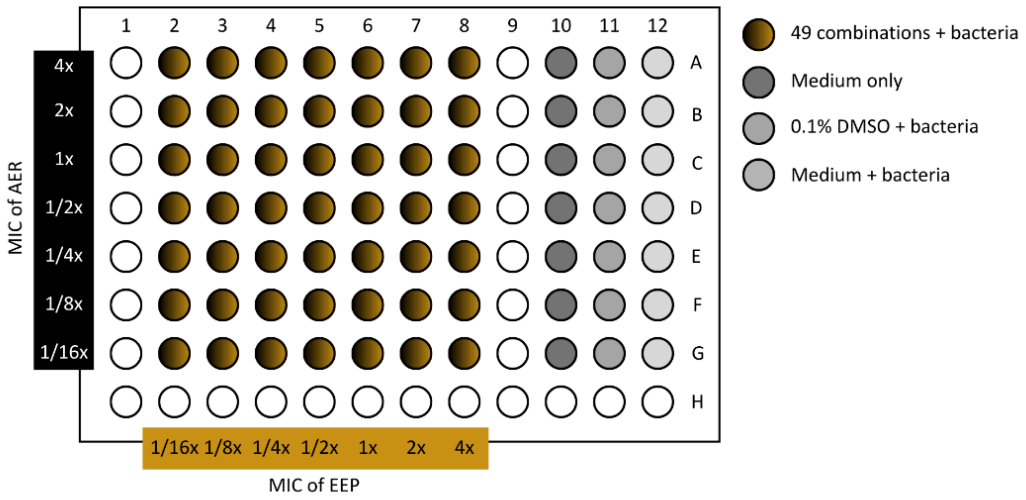

Figure 1. Checkerboard assay of EEP and AER.

Phenol coefficient determination of extracts

The phenol coefficient was determined according to the method described by [16] with modification. A 5% phenol solution was used as the reference standard and serially diluted to obtain concentrations ranging from 1:20 to 1:200. Samples of 5% EEP and 5% AER were prepared similarly and diluted in the same range. For each concentration, three tubes containing 3 mL of sterile MHB were prepared to assess antiseptic activity at 2-, 5-, and 10-min contact times. *S. aureus* suspension was added, vortexed, and after each specific contact time, transferred into 5 mL of fresh MHB. This transfer into a larger volume of fresh medium served to dilute any residual phenol or extract carried over from the contact-time tube, reducing its concentration below the inhibitory threshold and thereby arresting further antimicrobial action. Tests were run in duplicate and incubated at 37 °C for 48 h, and bacterial growth was assessed by medium turbidity. The phenol coefficient was expressed as the ratio of the highest dilution of the test extract to that of phenol showing no growth at 10 min but growth at 5 min.

Formulation of mouthwash

The mouthwash was formulated using a combination of EEP and AER at the lowest effective antimicrobial concentration with the desired contact time. Excipients were added to complete the mouthwash formulation (**Table 1.**). The formulation was evaluated through organoleptic testing (appearance, color, aroma, and taste), pH measurement using a pH meter (Mettler Toledo FiveEasy Plus F20, Mettler-Toledo GmbH, Greifensee, Switzerland), and viscosity determination using Ostwald viscometer. The homogeneity of the formulation was also checked by visually examining the mouthwash against a white background under adequate lighting, observing the top, middle, and bottom portions of the container for uniform color and clarity and the absence of particulate matter, sedimentation, or phase separation.

Table 1. Formulation composition of the EEP–AER mouthwash.

No.	Composition	Concentration (%)	Function
1	Ethanol	5	Cosolvent
2	Glycerin	10	Humectant, viscosity enhancer
3	Sodium saccharin	0.05	Sweetener
4	Menthol	0.05	Flavoring agent
5	Citric acid monohydrate	q.s	Buffering agent
6	Sodium citrate	q.s	Buffering agent
7	Methylparaben	0.015	Preservative
8	Propylparaben	0.02	Preservative
9	Purified water	ad 100	Solvent

No.	Composition	Concentration (%)	Function
10	EEP	As determined by the experimental result	Active ingredient
11	AER	As determined by the experimental result	Active ingredient

Confirmation of mouthwash antiseptic activity using the standard antiseptic test

The protocol for confirming the mouthwash's antiseptic activity followed the direct and rapid contact method adapted from the phenol coefficient test. The mouthwash was diluted with sterile distilled water to concentrations of 1:2, 1:4, 1:6, 1:8, and 1:10, homogenized by vortexing. For each concentration, three test tubes were prepared to evaluate antiseptic activity at 2-, 5-, and 10-min contact times. Each tube contained 5 mL of MHB, into which *Staphylococcus aureus* suspension was added and mixed. After the specified contact times, samples were transferred into sterile tubes containing 5 mL of MHB. Similarly, this transfer step diluted the carried-over mouthwash formulation, reducing its active concentration below the inhibitory threshold and functioning as a dilution-based neutralization step to prevent continued antimicrobial action during the subsequent incubation period. Tests were performed in duplicate, followed by incubation at 37 °C for 48 h. Antiseptic activity was determined by observing bacterial growth based on medium turbidity at each contact time. A base formulation without active ingredients was used as a negative control to confirm that the observed antimicrobial activity was attributable to EEP and AER rather than to the preservatives present in the formulation. Product "P" served as the standard reference.

Statistical analysis

All antibacterial determinations (MIC and MBC/MFC), considered semiquantitative due to the discrete nature of the dilution series, were performed in triplicate, with the reported value representing the mode (most frequently observed value) across the three replicate trials, whereas the phenol coefficient and antiseptic tests were performed in duplicate. Quantitative data are expressed as mean ± standard deviation (SD) of triplicate measurements.

▪ RESULTS

Total polyphenolic content of EEP and AER

The total polyphenolic content, expressed as GAE, was determined for both extracts. This parameter is important to characterize, as it may provide an indication of the potential biological activity of the extracts. EEP contained 133.90±1.73 mg GAE/g, while AER showed a higher content of 247.37±30.16 mg GAE/g.

Minimum inhibitory and bactericidal/fungicidal of EEP and AER

The antimicrobial activity of EEP and AER was evaluated against *S. aureus*, *S. mutans*, and *C. albicans* by determining the minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC or MFC) (**Table 2.**). EEP exhibited antibacterial properties against *S. aureus* and *S. mutans*, but showed no activity against *C. albicans*. AER inhibited all test microorganisms. In comparison, the standard phenol showed higher MIC, indicating that both EEP and AER have stronger antibacterial activity than the standard. Attempts to assess EEP's antifungal activity at higher concentrations (up to 100,000 µg/mL) were limited by poor solubility, preventing valid measurements. Given the study's focus on developing an effective antiseptic mouthwash combining EEP and AER and the low antifungal activity of EEP, further testing against *C. albicans* was not continued.

Table 2. MIC, MBC, and MFC of extracts against oral microbes.

Sample	Oral microbes (µg/mL)								
	Staphylococcus aureus			*Streptococcus mutans*			*Candida albicans*		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MFC	MFC/MIC
EEP	1.280	1.280	1	640	2.560	4	>20.480	-	-
AER	320	1.280	4	1.280	10.240	8	10.240	10.240	1
Phenol	5.210	10.240	2	5.120	20.480	4	1.280	1.280	1

Combination effects of EEP and AER

The combination of EEP and AER was evaluated using the checkerboard assay, a method that provides a reliable in vitro and in vivo estimation of antimicrobial interactions. A total of 49 concentration combinations were tested. The MIC of EEP-AER combination against *S. aureus* was observed at 1× MIC for both extracts, resulting in FICI of 2. For *S. mutans*, the MIC combination was observed at 2× MIC for EEP and 1× MIC for AER, resulting in FICI of 3.

Antiseptic properties of EEP and AER

The short-contact antiseptic activity of EEP and AER was evaluated using the standard phenol coefficient test, which compares the potency of an antiseptic to phenol. The phenol coefficient is calculated as the ratio of the highest dilution of the test substance that inhibits *S. aureus* at 10 minutes but not at 5 minutes, relative to standard phenol. A coefficient >1 indicates greater antiseptic activity than phenol, whereas <1 indicates lower activity [17]. EEP showed no antibacterial activity at contact times of 2, 5, and 10 minutes up to a 1:100 dilution. Thus, its phenol coefficient could not be determined.

In contrast, AER demonstrated activity at a maximum dilution of 1:120 (**Table 3.**), corresponding to a phenol coefficient of 1.2 compared with phenol (**Table 3.**). This indicates that AER demonstrates stronger antiseptic activity than standard phenol. The 1:120 dilution corresponds to 0.83% AER, which reflects its short-contact antimicrobial activity and provides a rational basis for formulation. Which produced comparable effects at a higher concentration.

Table 3. Growth of *Staphylococcus aureus* in the 5% AER and 5% standard phenol solution during the phenol coefficient test.

Dilution	Conc. (%)	Contact time (min; duplicate)					
		5% AER			5% standard phenol		
		2	5	10	2	5	10
1:20	5.00	-	-	-	-	-	-
1:30	3.33	+	-	-	-	-	-
1:40	2.50	+	-	-	-	-	-
1:50	2.00	+	+	-	-	-	-
1:60	1.67	+	+	-	-	-	-
1:70	1.43	+	+	-	+	-	-
1:80	1.25	+	+	-	+	-	-
1:90	1.11	+	+	-	+	+	-
1:100	1.00	+	+	-	+	+	-
1:110	0.91	+	+	-	+	+	+
1:120	0.83	+	+	-	+	+	+
1:130	0.77	+	+	+	+	+	+
1:140	0.71	+	+	+	+	+	+
1:150	0.67	+	+	+	+	+	+
1:160	0.63	+	+	+	+	+	+
1:170	0.59	+	+	+	+	+	+
1:180	0.56	+	+	+	+	+	+
1:190	0.53	+	+	+	+	+	+
1:200	0.50	+	+	+	+	+	+

Note: (–) indicates no bacterial growth; (+) indicates presence of bacterial growth.

Formulation and physicochemical assessment of EEP-AER-contained mouthwash

Since the short-contact antiseptic activity of EEP alone could not be determined and its interaction with AER was classified as additive or indifferent, the optimal EEP concentration for formulation was assessed by testing different EEP:AER ratios (1:1, 1.5:1, and 2:1). These combinations were further assessed against *S. aureus* using the short-contact antiseptic activity test (**Table 4.**).

Table 4. Short contact time antiseptic activity of three extract combination ratios againsts *Staphylococcus aureus* for the formulated mouthwash.

EEP: AER	Contact time (min; duplicate)		
	2	5	10
1:1	+	+	+
1.5:1	+	+	-
2:1	-	-	-

Note: (–) indicates no bacterial growth; (+) indicates presence of bacterial growth.

The formulation with EEP:AER ratio of 1:1 showed bacterial growth at all contact times, indicating insufficient effectiveness against bacteria in short-contact conditions and was therefore not selected. The 1.5:1 ratio inhibited bacterial growth at 10 minutes but not at 2 or 5 minutes, whereas the 2:1 ratio demonstrated complete inhibition at 2, 5, and 10 minutes. Both ratios exhibited antibacterial activity, though with different contact-time effectiveness. Considering its faster onset of action, a 2:1 ratio was selected for the mouthwash formulation, corresponding to a composition containing 2% EEP and 1% AER.

Formulation was carried out by incorporating excipients to support stability, as shown in **Table 1.** The mouthwash was then evaluated to ensure compliance with standard quality requirements. The parameters assessed included organoleptic properties, pH, viscosity, and homogeneity (**Table 4.**).

Table 5. Formulated mouthwash characteristics.

Parameter	Result
Organoleptic	
Color	Light brown, transparent
Taste	Slightly acidic with a cooling and astringent sensation
Aroma	Menthol
Texture	Liquid
pH	5.44±0.03
Viscosity (mPa.s)	1.36±0.00
Homogeneity	Homogeneous

Confirmation of antiseptic actibity of mouthwash

To exclude any potential antimicrobial contribution from excipients, an additional test was performed using the vehicle formulation without EEP and AER active ingredients. The results showed visible growth of *S. aureus* at 2, 5, and 10 minutes, confirming that the excipients alone had no antimicrobial contribution. Thus, the observed antiseptic activity could be attributed solely to the EEP–AER extracts. Then, confirmation of the antiseptic effect was further carried out using the standard antiseptic test. The mouthwash was diluted up to 1:10 to evaluate its ability to maintain activity at lower extract concentrations (**Table 6.**). All diluted samples showed bacterial growth, indicating that the undiluted formulation is the optimal mouthwash.

Table 6. Growth of *Staphylococcus aureus* in the determination of the antiseptic effectiveness of the vehicle, formulated mouthwash, and commercial product.

Sample	Contact time (min; duplicate)		
	2	5	10
Basis	+	+	+
UM	-	-	-
1:2 DM	+	+	+
1:4 DM	+	+	+
1:6 DM	+	+	+
1:8 DM	+	+	+
1:10 DM	+	+	+
UP	+	+	-

Sample	Contact time (min; duplicate)		
	2	5	10
1:2 DP	+	+	-
1:4 DP	+	+	+

Note: Basis = base formulation without active ingredients; (–) indicates no bacterial growth; (+) indicates presence of bacterial growth; UM = undiluted formulated mouthwash; DM = diluted formulated mouthwash; UP = undiluted product P; DP = diluted product P

A comparative evaluation was also conducted against a commercially available natural-based mouthwash containing propolis extract (Product P). No commercial roselle-based products were available in Indonesia, hence not included for comparison (**Table 6.**). Product P exhibited microbial growth at 2 and 5 minutes without dilution, though no growth was observed at 10 minutes. In contrast, the test mouthwash containing 2% EEP and 1% AER demonstrated relatively superior activity, showing antibacterial effects at earlier contact times without dilution. However, upon 1:2 dilution, Product P retained its activity profile, whereas the test mouthwash showed bacterial growth at all contact times.

▪ DISCUSSION

Phenolic compounds are well-documented to exert a wide range of biological activities, including antimicrobial effects, which are particularly relevant in dental applications [11], [12], [18], [19]. In this study, the total phenolic content of EEP was determined to be 133.90 ± 1.73 mg GAE/g. EEP exhibited inhibitory effects on bacterial growth, with MIC values of 1280 µg/mL for *S. aureus* and 640 µg/mL for *S. mutans* but showed no inhibitory effect against the fungus *C. albicans*. In contrast, Nubla et al. [20] reported antifungal activity of EEP derived from Lombok, Indonesia, against *Candida* sp. with total phenolic contents ranging from 222 to 278 mg GAE/g, which were higher than those observed in this study. Thus, the absence of antifungal effects in our study may be associated with the relatively lower phenolic content of the extract.

Meanwhile, the total phenolic content of AER was found to be 247.37±30.16 mg GAE/g, and the extract exhibited inhibitory effects against all tested bacteria in this study, with an MIC value of 320 µg/mL against *S. aureus*. Sitoresmi et al. [21] evaluated roselle extract from Yogyakarta, Indonesia, which contained 23.77±0.25 mg GAE/g and demonstrated antibacterial activity against *S. aureus*, with MIC of 5500 µg/mL. In comparison, our findings showed a higher phenolic content along with a substantially lower MIC. These results support a potential association between phenolic content and antimicrobial effect, indicating that higher phenolic content may contribute to stronger antimicrobial activity.

The determination of MIC and MBC or MFC values is essential for establishing effective antimicrobial doses [22]. Moreover, these values provide insights into the antibacterial properties of the tested samples. Silva et al. [23] proposed a classification system for the strength of natural extracts based on MIC values: <100 µg/mL = very strong, 100–500 µg/mL = strong, 501–999 µg/mL = moderate, and 1.000–2.000 µg/mL = weak. Accordingly, EEP demonstrated weak activity against *S. aureus* and moderate activity against *S. mutans*. In contrast, AER showed strong activity against *S. aureus*, but weak activity against *S. mutans* and *C. albicans*. The antimicrobial activity of EEP varies considerably across studies, which may be attributed to differences in geographical origin. For example, Chilean EEP exhibited weak activity against *S. aureus*, with MIC values ranging from 200 to 26,900 µg/mL [24]. In contrast, Taiwanese EEP demonstrated very strong activity, with MIC values ranging from 3.75 to 60 µg/mL [25], while Polish EEP showed strong activity with an MIC of 128 µg/m [26]. Interestingly, the activity of EEP against *S. mutans* observed in the present study is generally consistent with previous reports. A Polish study reported a mean MIC of 1.10 mg/mL against mutans streptococci isolated from saliva, suggesting a weak to moderate antibacterial capacity [27]. Unfortunately, studies on Indonesian propolis remain limited against both bacteria. Evidence regarding the antimicrobial activity of AER is more limited and predominantly based on agar disc diffusion assays rather than MIC or MBC determinations. Abbass et al. [11] reported that AER exhibited antibacterial activity against both *S. aureus* and *S. mutans*, with MIC values of 32 mg/mL. In contrast, another study found that AER produced no inhibition zone against *C. albicans*, indicating limited antifungal activity [28].

In addition, the MIC and MBC/MFC ratios can be used to determine the mode of antimicrobial action. An extract is considered bactericidal when the MBC/MIC ratio ≤ 4 and bacteriostatic when the ratio > 4 [29]. Based on these criteria, EEP exhibited bactericidal effects against *S. aureus* and *S. mutans*. AER, meanwhile, was bactericidal against *S. aureus* but bacteriostatic against *S. mutans*. Under specific laboratory conditions,

the traditional paradigm suggests that combinations of two bactericidal agents are generally synergistic, whereas combinations of bactericidal and bacteriostatic agents tend to exhibit antagonistic interactions [29]. However, the checkerboard assay in this study did not fully align with that hypothesis, as the combination of EEP and AER exhibited additive or indifferent effects against both *S. aureus* and *S. mutans* (FICI = 2 and 3, respectively). According to the FICI interpretation criteria, values between 0.5 and 4 indicate additive interactions, whereas values ≤0.5 and >4 indicate synergistic and antagonistic effects, respectively [30]. This discrepancy may be due to the diverse range of compounds present in each extract, leading to complex pharmacodynamic interactions. Propolis-derived flavonoids and phenolic acids primarily disrupt microbial membrane integrity [31], while roselle's anthocyanins and organic acids [11], such that their combined effect reflects the sum of these distinct actions rather than mutual potentiation. Nevertheless, the additive interaction between EEP and AER remains advantageous therapeutically, as it still provides a beneficial antimicrobial effect compared to individual use and may broaden activity against the diverse polymicrobial communities associated with oral dysbiosis owing to their relative potencies against some pathogens [32]. Although the combination demonstrated additive antibacterial activity, the clear mechanism underlying the interaction between EEP and AER remains to be clarified through future studies.

The phenol coefficient method is a long-established but still relevant standard for evaluating the antiseptic or disinfectant activity of phenolic compounds. In this study, the phenol coefficient of EEP could not be determined, and to date, no comparable studies have reported this parameter for propolis extracts. However, a time-kill assay on *S. aureus* demonstrated that ethanolic propolis extract reduced bacterial counts by 1 log CFU/mL at 4× MIC (approximately 2.5% v/v) after 2.5 h, with further reductions over time [33] . This suggests that the killing activity of propolis extract may be influenced not only by concentration but also by exposure time. In our study, the combination of EEP with AER reduced the required contact time for antiseptic activity in a concentration-dependent manner, further supporting the benefit of their combined use.

The formulated mouthwash was subsequently evaluated to ensure its suitability for oral application and consumer comfort [7]. In the oral cavity, saliva maintains pH near neutrality (6.81–7.45) [34]. The pH of the mouthwash formulation was 5.44±0.03, which is slightly acidic but still compliant with the European Standard for Oral Care Products (NEN-EN-ISO16408:2015), which requires a pH between 3.0 and 10.5 [35]. It also aligns with the Indonesian National Standard (SNI 12-3524-1995) for oral care products, which currently applies to the toothpaste category due to the absence of a specific standard for mouthwashes, specifying an acceptable pH range of 4.5–10. Viscosity is another critical factor influencing mouthwash comfort; the closer its viscosity is to water [36] (0.89 mPa.s at 25 °C), the easier it is to use. The formulated mouthwash exhibited a viscosity of 1.36 mPa.s, which may be attributed to the addition of glycerin. The formulation also included flavoring agents to maintain oral freshness [7].

Finally, the antiseptic efficacy of the mouthwash formulation was evaluated using standard antiseptic assays. The base formulation without active ingredients showed no inhibitory effect on bacterial growth, confirming that the observed antimicrobial activity in the mouthwash formulation is indeed attributable to EEP and AER rather than to the base itself, particularly in the presence of preservative. The formulation was further diluted up to 1:10 to evaluate whether antimicrobial activity was retained at lower extract concentrations. Compared with the commercial comparator (Product P), the test mouthwash containing 2% EEP and 1% AER showed superior antimicrobial activity at undiluted concentrations, demonstrating a faster onset of action. At 1:2 dilution, the comparator maintained similar efficacy, whereas the test formulation showed bacterial growth at all contact times. This difference may be attributable to differences in excipients and the exact propolis extract concentration in the comparator, which may not be disclosed by the manufacturer. Nonetheless, the undiluted formulation containing 2% EEP and 1% AER confirmed short-contact antimicrobial activity, supporting its potential for further development as a commercial natural mouthwash.

This study has several limitations. First, the antimicrobial efficacy of the EEP–AER mouthwash was demonstrated only in its undiluted form, as dilution markedly reduced or abolished activity, unlike the commercial comparator, which remained active even when diluted. This suggests that the recommended use of EEP and AER should account for potential dilution by water or saliva, ensuring the applied concentration remains within the effective range. Second, safety assessment (e.g., cytotoxicity on oral mucosal cells) and stability evaluation were not performed, leaving the formulation's biocompatibility and shelf-life stability unaddressed. Furthermore, the antimicrobial activity was evaluated only against single-species planktonic

cultures, which do not fully replicate the complexity of the oral environment, which contains polymicrobial or biofilm. Therefore, this study provides a useful preliminary basis for the further development of this formulation. Future studies should address formulation optimization for dilution tolerance, safety, stability, and antibiofilm activity to support clinical translation and commercialization.

▪ CONCLUSION

The mouthwash formulation containing 2% EEP and 1% AER demonstrates promising antimicrobial potential against some oral pathogens, particularly at short-contact times, likely due to its phenolic content. It also exhibits favorable physicochemical characteristics, including organoleptic quality, pH, viscosity, and homogeneity. This formulation may have potential applications in preventive dentistry to reduce the risk of oral diseases.

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