



Research article

Effectiveness of combined bangle rhizome (*Zingiber montanum*) and black betel leaf (*Piper betle* var. *nigra*) water extract in combatting caries-causing oral pathogens using response surface methodology

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Abstract

Importance of the work: There is potential to use endemic herbal extracts to maintain dental and oral health.

Objectives: To determine the optimal conditions for the combination of bangle and black betel extracts to inhibit pathogenic bacteria associated with dental caries.

Materials and Methods: Water extracts of bangle rhizomes (BR) and black betel leaves (BL) were utilized to identify the most effective formula for suppressing three dental pathogenic bacteria using response surface methodology. The factors investigated were the BR and BL concentrations and the BR-to-BL ratio.

Results: A BR concentration of 10–20% significantly affected the antibacterial activity against *Enterococcus faecalis* and *Streptococcus mutans*, whereas a BL concentration of 10–20% had no significant effect on the growth zones of *E. faecalis* and *S. mutans*. The BR-BL combination (ratios 1:1–3:1) significantly influenced antibacterial activity in inhibiting the growth of both bacteria. *Porphyromonas gingivalis* was not affected by any treatment. The optimization of the combination of the three factors for the antibacterial activity against *E. faecalis* was achieved effectively under the conditions of BR 20.0%, BL 19.8% and a BR-to-BL ratio of 3, whereas for *S. mutans*, it was BR 20.0%, BL 10.0% and a BR-to-BL ratio of 3.

Main finding: BR alone was a promising natural ingredient for preventing oral infections against *E. faecalis* and *S. mutans*; however, its combination with BL is necessary to improve its antibacterial effectiveness against *E. faecalis*.

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Introduction

Dental caries is the most prevalent oral disease and a leading cause of tooth loss (Frencken et al., 2017; Ahirwar et al., 2019). According to Rai et al. (2024) the World Health Organization has reported that 60–90% of school children and nearly 100% of adults worldwide have dental caries. The incidence of dental caries in Indonesia (Decayed, Missing, Filled Teeth) averages 68.6%, broken down into the age classes of: children (3–9 yr), 67.9%; adolescents (10–24 yr), 57.5%; and adults (≥ 25 years), 73.3% (Ministry of Health of the Republic of Indonesia, 2018). Dental and oral health problems, particularly dental caries, affect nearly one-half of the global population, namely 3.58 billion people, with untreated, permanent teeth caries being the most common condition, impacting 2.5 billion individuals worldwide (Dye, 2017).

There is a long history of using plants for the treatment of various diseases in Indonesia, with plants serving as a valuable resource for the development of various modern and traditional medicines (Popoola et al., 2021; Arsy et al., 2022). Among these are medicinal plants containing essential oils, including those from the *Zingiberaceae* family, namely *Zingiberaceae cassumunar* (bangle) and from the *Piperaceae* family, represented by black betel (*Piper betle* var. *nigra*) (Raut et al., 2014; Batubara et al., 2021; Prasetya et al., 2021). Both these plants contain terpenes, acetone, phenols, aldehydes, alcohols, esters and acids (Nurhayati et al., 2022). Often, single-component extracts lack sufficient strength to provide antimicrobial effects, making synergy between multiple compounds a recommended approach to enhance antimicrobial efficacy. However, little is known about the interactions that yield the optimal effects. This knowledge could contribute to the design of new and more effective antimicrobial mixtures (Jack et al., 1995).

Optimal effects can be achieved through the combination of extracts from two different plants (Spinella, 2002) or by using plant extracts combined with antibiotics (Spinella, 2002; Taukoora et al., 2016). The interactions of the active ingredients in multicomponent drugs can exhibit either synergistic or antagonistic effects. Combinations of herbs or extracts that demonstrate synergistic effects on the active ingredients (Syahrir et al., 2016) continue to be explored (Han et al., 2021). Traditional Chinese medicine also uses formulas that contain many plants, including for treating COVID-19 (Mudja, 2021). Combining various extracts, other compounds or antibiotics to achieve therapeutic effects requires optimizing

the components, which is typically determined at the laboratory level, before they can be applied to higher life forms, such as humans. To date, there have been no reports on the antibacterial effects and optimal composition of the factorial variation of bangle plants combined with betel leaves on the growth of bacteria related to dental health, such as *Enterococcus faecalis*, *Porphyromonas gingivalis* and *Streptococcus mutans*. Other studies have reported the antibacterial effects of single extracts of bangle and black betel leaves (Pardosi et al., 2022; Tandirogang et al., 2022). The pursuit of optimal effects through a combination of extracts is a promising avenue for antibacterial, antioxidant, anti-inflammatory and antifungal applications, positioning it as a potential new therapy in dentistry.

Bangle extract is a promising source for developing pharmaceutical products and functional foods (Hari P Devkota et al., 2021) for traditional medicine aimed at preventing periodontitis, dental caries, dental pain, gingivitis and plaque (Singh et al., 2022). However, research on the combination of bangle-rhizome extracts remains limited. Most studies have focused on combinations of essential oils at low concentrations. For example, a combination of 1% bangle and 3% red ginger yielded the largest inhibition zone (15.47 mm) against *S. aureus*, which was larger than for its single form, whereas the combination of 1% bangle and 3% red ginger against *E. coli* (13.37 mm) did not produce a significant increase compared with its individual administration (Anggraini, 2015).

Black betel leaves (*Piper betle* L. var. *nigra*) is a tropical plant closely related to the common *P. betel* (Prasetya et al., 2021). The black betel variety contains antioxidants that can be developed into herbal health products that are beneficial for the body (Arsy et al., 2022), as they can inhibit the growth of bacteria, yeast and mold (Nazzaro et al., 2013) and are anti-cancer (Yusof et al., 2022). Black betel leaves have reported antimicrobial activity against oral cavity pathogenic bacteria, such as *S. mutans*, *Streptococcus sanguinis* and opportunistic pathogenic fungi such as *Candida albicans* (Prasetya et al., 2021).

Antimicrobial testing of 5% black betel leaf extract combined with variations in the carbol concentration inhibited the growth of *S. sanguinis*, producing an inhibition zone diameter of 6.26 mm and against *C. albicans*, with an inhibition zone diameter of 5.15 mm (Sari et al., 2020). Antimicrobial tests of a combination of 1% black betel with glycerin (4%, 8% or 12%) in mouthwashes at different ratios had growth-inhibition activity against mutant *Streptococcus* and *E. coli*, with 1:4, 1:8 and 1:12 producing 1.8 mm (weak), 2.5 mm (weak) and 1.7 mm (weak) inhibition zone diameters, respectively (Qhorina et al., 2021).

The antibacterial activity of single extracts of bangle rhizome has been reported against both Gram-positive and Gram-negative bacteria, including *E. coli*, *Klebsiella pneumoniae*, *Salmonella paratyphi*, *Salmonella typhi* and *Shigella flexneri* (Amatayakul et al., 1979; Devkota et al., 2021), methicillin-resistant *Staphylococcus aureus*, *S. aureus* MDR (Siddique et al., 2019) and (Amatayakul et al., 1979; Hari P Devkota et al., 2021) (Amatayakul et al., 1979; Hari P Devkota et al., 2021) (Siddique et al., 2019) (Siddique et al., 2019) (Siddique et al., 2019) *S. mutans* (Pardosi et al., 2022). Betel leaves have had antibacterial activity against *Propionibacterium acnes*, *Staphylococcus aureus*, *Candida albicans* and *Streptococcus mutans* (Pardosi et al., 2022). Generally, a high concentration is required to achieve strong inhibitory power in single extract tests; however, in many experiments, doubling the concentration does not result in a two-fold increase in the inhibitory zone (Pithayanukul et al., 2007; Sujono et al., 2019; Pardosi et al., 2022; Tandirogang et al., 2022). In some cases, a single medicinal plant with a low extract concentration may inhibit bacterial growth; however, increasing the concentration does not result in a significant increase. Additionally, the high concentration of each extract component can render its use inefficient and ineffective for mixed-drug preparations. Therefore, a combination of extracts from multiple sources is necessary to obtain the optimal conditions for antibacterial effects.

The purpose of the current study was to determine an effective combination of bangle rhizome water extract and black betel leaf water extract for inhibiting the growth of bacteria related to dental health, specifically *S. mutans*, *E. faecalis* and *P. gingivalis*.

Materials and Methods

Materials

Bangle rhizome (*Zingiber montanum*) was obtained from Prangat subdistrict, Kutai Timur regency (0°12'11.43" S;

117°18'46.91" E), while the heart-shaped, dark-green colored, black betel leaf (*Piper betle* var. *nigra*) was sourced from Mugirejo Village, Sungai Pinang subdistrict, Samarinda City (0°28'47.48" S; 117°12'23.22" E). The oral cavity infection bacteria *E. faecalis* ATCC 29212, *P. gingivalis* ATCC 33277 and *S. mutans* ATCC 25175 were obtained from the Regional Technical Implementation Unit of the East Kalimantan Provincial Health Laboratory. The 6 mm disc filter papers and other nutrients were sourced from Merck. Chlorhexidine and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich.

Research design and data analysis

An experimental optimization study was applied using response surface methodology (RSM) with a Box-Behnken design comprising three factors and three levels. Khan et al. (2022) used the same experimental design (Khan et al., 2022) (Khan et al., 2022) (Khan et al., 2022) to optimize the antibacterial and wound-healing activities of nitrofurazone- and lidocaine-loaded silica microspheres. The Design Expert Version 13 software (Stat-Ease, Inc.) was used for RSM data processing. For each experiment, analysis of variance was used to model linear equations to determine the effect of the three factors (bangle concentration, black betel leaf and the bangle-to-black betel leaf ratio). Significance was tested at $p < 0.05$.

The factors examined were the concentration of bangle rhizome water extract (BR), the concentration of black betel leaf water extract (BL) and the BR-to-BL ratio (BR:BL), as shown in Table 1. The observed response was the zone of bacterial growth inhibition.

Each inhibition zone value from 17 experiments (three repetitions each) was obtained from the test results; then, the data were analyzed using Design Expert Version 13 to obtain the actual and predicted inhibition zone responses. The actual inhibition zones of the three bacteria that had optimal conditions were analyzed to obtain predicted values using Design Expert Version 13 recommendations to produce solution values with 95% confidence intervals.

Table 1 Three factors and three levels for bangle combination experiment with betel

Factor	Unit	Symbol	Range and level		
			-1	0	1
A - Bangle rhizome water extract (BR)	%	X1	10	15	20
B - Black betel leaf water extract (BL)	%	X2	10	15	20
C - Ratio BR-toBL (BR:BL)*		X3	1	2	3

Note: *) BR and BL express in mL

Research procedure

Extraction of bangle rhizomes and black betel leaves

The extraction was performed based on boiling (Yim et al., 2013). First, the samples were cleaned under running water. Then, each sample was dried at room temperature without direct sunlight, before grinding into 100 g crumbs, divided into 10 bags containing 10 g each. Each sample (10 bags) was boiled in a closed container (pressure cooker) with 500 mL water for 30 min. The residue was boiled again with fresh water under the same conditions; this procedure was repeated three or four times. Subsequently, the sample solution was evaporated in an oven at 60°C, until a solid (dry) filtrate was obtained.

Each extract was diluted with 10% DMSO to obtain multiple concentrations (Based on weight per volume, w/v) of 10%, 15% and 20%. The ratios of the combination of bangle rhizome extract-to-black betel leaf (BR:BL) were determined to adjust the test volume to 0.5 mL with several ratios (1:1, 2:1 and 3:1).

Media preparation and sterilization

Nutrient agar (NA) medium was prepared with 13 g nutrient broth (NB), glucose 2 g and 20 g agar. This mixture was dissolved in 1,000 mL of distilled water and sterilized in an autoclave at 121°C for 15 min.

Bacterial cultures

Bacterial culturing was carried out by transferring the source liquid of the pure bacterial culture on NA by streaking an ose, already made in the pure bacterial culture. The NA was incubated overnight at 37°C. The growth phase of the bacterial log was prepared by inoculating pure culture colonies (taken from NA) in 10 mL of NB and incubating by shaking at 150 revolutions per minute at room temperature for 18 hr. Next, the log-phase culture suspension was diluted six times using a 0.90% sodium chloride

solution until it reached the McFarland turbidity standard of 0.5, equivalent to $1-2 \times 10^8$ colony forming units/mL.

Determination of inhibition zone

The antibacterial activity test of the BR and BL was conducted by measuring the bacterial growth inhibition zone using a paper disc on 2% NA medium against *E. faecalis*, *S. mutans* and *P. gingivalis*, based on the observation of the inhibition zone (Ncube et al., 2008; Taukoora et al., 2016). The antibacterial test procedure involved three steps: 1) the bacterial suspension with a standard of 0.5 McFarland was pipetted as much as 100 mL into an Erlenmeyer tube containing 110 mL of liquid 2% NA and stirred until homogeneous, after which 20 mL of NA was poured into a Petri dish and left at room temperature until it solidified; 2) the paper disc was soaked for 30 min in 0.5 mL of a combination solution of BL and BR, according to the treatment, using a 1.5 mL Eppendorf tube containing a concentration of bangle extract; and 3) the paper disc was placed on top of the NA in a Petri dish and incubated at 37°C for 24 h. Testing for each bacterium was repeated three times. Chlorhexidine and 10% DMSO were used as the positive and negative controls, respectively. Antibacterial resistance was assessed by measuring any clear or cloudy zones using a screw gauge micrometer (Taukoora et al., 2016).

Results

Effect of combination of bangle rhizome water extract and black betel leaf water extract on antibacterial activity

BR and its combination with BL had a significant effect on the antibacterial activity observed in the growth inhibition of *E. faecalis*. Similarly, for *S. mutans*, BR and its ratio to BL had a significant influence. BL alone was not significant regarding antibacterial activity against the two bacteria (Table 2).

Table 2 Types of models and coefficients of condition equation for combination of bangle rhizome extract and black betel leaf water extract for inhibition zones of *Enterococcus faecalis*, *Streptococcus mutans* and *P. gingivalis*

Source	<i>E. faecalis</i>	<i>S. mutans</i>	<i>P. gingivalis</i>
Model	Linear	Linear	Mean
Intercept	$p = 0.0355$ 21.2241	$p = 0.0024$ 22.0835	19.4388
Coefficient			
A - Bangle rhizome water extract (BR)	1.20375 $p = 0.0232$	1.9525 $p = 0.0009$	
B - Black betel leaf water extract (BL)	0.07875 $p = 0.8689$	-0.11125 $p = 0.8124$	
C - Ratio BR-to-BL (BR:BL)	1.04 $p = 0.0446$	1.20625 $p = 0.0209$	
Lack of fit	$p = 0.2246$	$p = 0.1378$	$p = 0.9987$

BR = bangle rhizome water extract; BL = black betel leaf water extract; inhibition zone measured in millimeters; A = BR concentration in 10% dimethyl sulfoxide (DMSO) as a percentage; B = BL concentration in 10% (DMSO) as a percentage; C = combination of A and B in 0.5 mL, where A and B are in milliliters.

The model of the combined BR and BL condition equations, as well as the ratios of both for the growth inhibition zone of the two cariogenic bacteria, *E. faecalis* and *S. mutans* were linear as shown below:

$$\text{Inhibition zone of } E. faecalis = 21.22 + 1.20 A + 0.0788 B + 1.04 C$$

$$\text{Inhibition zone of } S. mutans = 22.08 + 1.95 A - 0.1112 B + 1.21 C$$

Where:

A = BR concentration in 10% dimethyl sulfoxide (DMSO), as a percentage

B = BL concentration in 10% DMSO, as a percentage

C = combination of A and B in 3 mL, where A and B are in milliliters

In total 17 combinations of the BR and BL treatments and ratios produced actual growth inhibition zones for *E. faecalis*, *P. gingivalis* and *S. mutans* in the ranges 17.52–23.48 mm, 16.64–20.75 mm and 17.44–25.06 mm, respectively, while the predicted growth inhibition zones for *E. faecalis* and *S. mutans* were 18.98–23.47 mm and 18.92–25.24 mm (Table 3). Notably, for *P. gingivalis*, the predicted growth inhibition zone produced only one value, 19.44 mm, which was the average of the actual values obtained, as a valid equation

model for the growth inhibition zone of *P. gingivalis* has not yet been established.

Changes to the concentration of BR or to the BR-BL ratio significantly affected the growth inhibition zone of *E. faecalis*, whereas the shift in BL concentration had no significant effect. An extremely negative influence on the growth inhibition zone of *E. faecalis* was observed under the conditions of the minimal BR (10%), the minimal BL (10%) and the minimal BR-to-BL ratio (1:1), as shown in Fig. 1A, whereas an extremely positive influence was noted using the maximum conditions for each factor (Fig. 1B).

A similar trend was observed in *S. mutans*. The shift in the concentration of BR or the BR-to-BL ratio significantly affected the growth inhibition zone of *S. mutans*, whereas the shift in BL concentration had no significant effect. An extremely negative influence on the growth inhibition zone of *S. mutans* was observed under the conditions of the minimal BR (10%), the maximum BL (20%) and the minimum BR-to-BL ratio (1:1), as shown in Fig. 2A, whereas an extremely positive effect was noted for the maximum BR concentration (20%), the minimum BL concentration (10%) and the maximum BR-to-BL ratio (3:1), as shown in Fig. 2B.

Table 3 Effect of combination of bangle rhizome water extract (BR) and black betel leaf water extract (BL) on growth inhibition zone of *Enterococcus faecalis*, *Streptococcus mutans* and *Porphyromonas gingivalis*

Unit	BR (%)	BL (%)	BR:BL	Inhibition zone (mm)					
				<i>E. faecalis</i>		<i>S. mutans</i>		<i>P. gingivalis</i>	
				Actual	Prediction	Actual	Prediction	Actual	Prediction
1	15	15	2	21.06	21.22	21.17	22.08	19.43	19.44
2	20	15	1	20.60	21.39	21.71	22.83	19.01	19.44
3	15	15	2	21.27	21.22	23.36	22.08	16.64	19.44
4	15	10	1	19.86	20.11	20.53	20.99	19.03	19.44
5	15	10	3	20.43	22.19	21.18	23.40	19.40	19.44
6	15	20	1	21.32	20.26	21.98	20.77	19.88	19.44
7	10	10	2	22.58	19.94	21.54	20.24	19.68	19.44
8	20	15	3	22.94	23.47	25.06	25.24	20.41	19.44
9	20	10	2	22.54	22.35	24.97	24.15	18.87	19.44
10	10	20	2	18.41	20.10	17.44	20.02	19.36	19.44
11	10	15	3	20.77	21.06	22.22	21.34	18.72	19.44
12	15	15	2	22.71	21.22	22.36	22.08	20.19	19.44
13	15	15	2	22.22	21.22	22.90	22.08	20.75	19.44
14	15	20	3	23.48	22.34	24.12	23.18	19.15	19.44
15	15	15	2	20.27	21.22	22.38	22.08	21.60	19.44
16	20	20	2	22.83	22.51	23.79	23.92	20.10	19.44
17	10	15	1	17.52	18.98	18.71	18.92	18.24	19.44

Prediction data obtained using response surface methodology, run using the Design Expert Version 13 program. BR and BL had concentrations of 10%, 15% or 20%. Administration of the two extracts resulted in BR-to-BL volume ratios (BR:BL) of 1:1, 2:1 and 3:1; then, the extract was added to a volume of 0.5 mL.

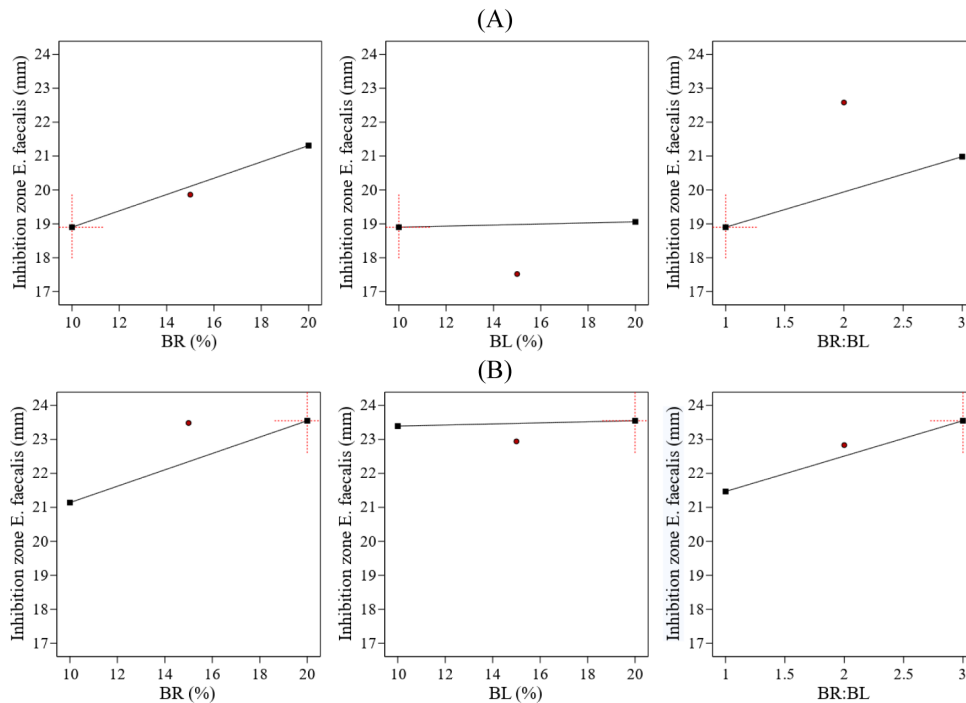


Fig. 1 Effect of combinations of bangle rhizome water extract (BR) and black betel leaf water extract (BL) and their volume ratio (BR:BL) on growth inhibition zone of *E. faecalis*: The condition of (A) minimum response at actual factors of BR=10%, BL=10% and volume ratio of BR:BL=1; (B) maximal response at actual factors of BR=20%, BL=20% and volume ratio of BR:BL=3, where dotted red cross indicates.

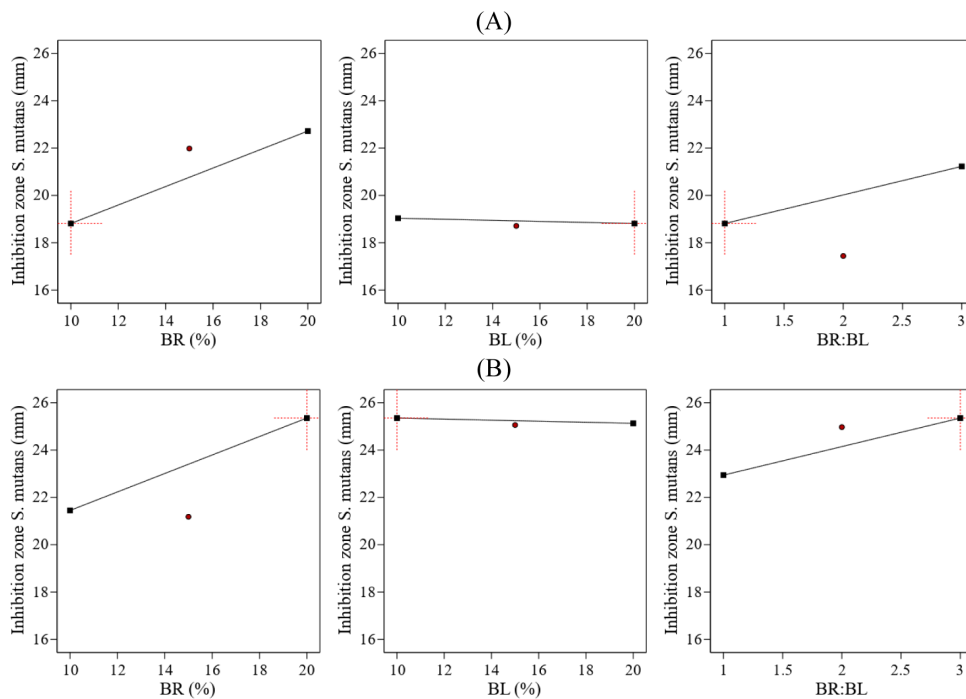


Fig. 2 Effect of combinations of bangle rhizome water extract (BR) and black betel leaf water extract (BL) and volume ratio (BR:BL) on growth inhibition zone of *S. mutans*: The condition of (A) minimum response at actual factors of BR=10%, BL=20% and volume ratio of BR:BL=1; (B) maximum response at actual factors of BR=10%, BL=10% and volume ratio of BR:BL=3, where dotted red cross indicates.

The optimization criteria for the combination of BR and BL and their ratio used in the research are presented in Table 4.

Condition optimization of bangle rhizome and black betel leaf water extract combination for antibacterial activity

Optimization of the combination of BR and BL water extract and their ratio for simultaneous antibacterial activity against two cariogenic bacteria (*S. faecalis* and *E. mutans*) resulted in 92 solutions with a desirability value of 0.898–1.000. The

contour profile of the condition optimization analysis for the combination of BR and BL water extracts is presented in Fig 3 and Fig 4.

Of the 92 available solutions, the 11th solution was selected for the optimization of antibacterial activity against *E. faecalis*; for example, BR 19.9957%, BL 19.8727% and the BR-to-BL ratio (BR:BL) was 2.99486, while the 85th solution, for example, BR 20%, BL 10.0224% and the BR-to-BL ratio (BR:BL) was 2.99999 for optimizing antibacterial activity against *S. mutans* (Table 5).

Table 4 Optimization criteria for antibacterial activity from combination of bangle rhizome water extract (BR) and black betel leaf water extract (BL) and ratios of both

Source	Goal	Limits	Weight	Importance (1–5)
BR	In range	10–20	1–1	3
BL	In range	10–20	1–1	3
BR:BL	In range	1–3	1–1	3
Inhibition zone <i>Enterococcus faecalis</i>	Maximize	17.52–23.48	1–1	5
Inhibition zone <i>Streptococcus mutans</i>	Maximize	17.44–25.06	1–1	5
Inhibition zone of <i>Porphyromonas gingivalis</i>	None	16.64–21.60	1–1	3

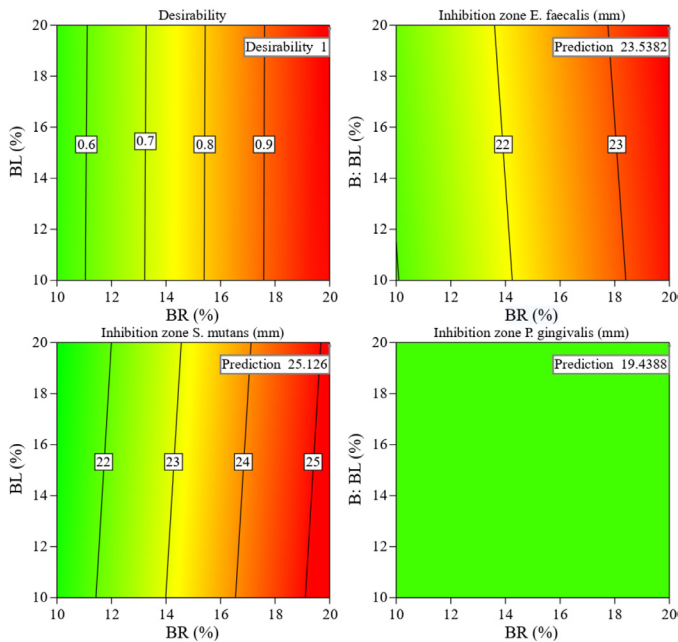


Fig. 3 Optimization of combined conditions of bangle rhizome water extract (BR), black betel leaf water extract (BL) and their ratio (BR:BL) in inhibiting growth of cariogenic bacteria of 11th solution, where data are actual, with BR:BL = 2.99486

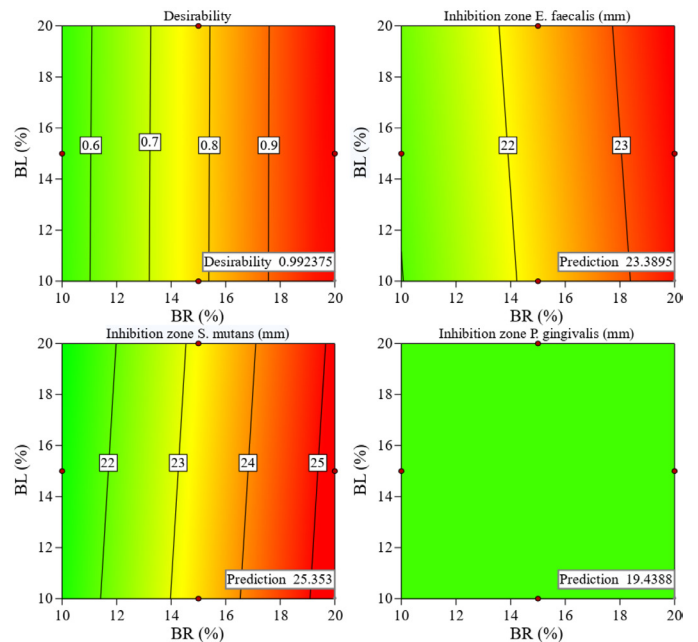


Fig. 4 Optimization of combined conditions of bangle rhizome water extract (BR), black betel leaf water extract (BL) and their ratio (BR:BL) in inhibiting the growth of cariogenic bacteria of 85th solution, where data are actual, with BR:BL = 2.99999

Table 5 Confirmation of optimum value of growth inhibition zone of cariogenic bacteria in optimization of combination of bangle rhizome water extract (BR) and black betel leaf water extract (BL) and ratio of both

Type of bacteria	Growth inhibition zone (mm)		
	Predicted mean	95% CI low for mean	95% CI high for mean
11th solution*			
<i>Enterococcus faecalis</i>	23.5382	21.6723	25.4041
<i>Streptococcus mutans</i>	25.1260	23.2939	26.9581
<i>Porphyromonas gingivalis</i>	19.4388	18.8765	20.0012
85th solution**			
<i>E. faecalis</i>	23.3895	21.5090	25.2699
<i>S. mutans</i>	25.3530	23.5066	27.1994
<i>P. gingivalis</i>	19.4388	18.8765	20.0012

CI = confidence interval.

* = data obtained from conditions of BR 19.9957%, BL 19.8727% and BR-to-BL ratio (BR:BL) 2.99486

** = data obtained with BR 20%, BL 10.0224% and BR-to-BL volume ratio (BR:BL) 2.99999

The 11th solution produced growth inhibition zone responses for *E. faecalis* and *S. mutans* of 23.54 and 25.13 mm, with ranges of 21.67–25.40 mm (95% confidence interval, CI) and 23.29–26.96 mm (95% CI), respectively. The 85th solution produced inhibition zones for *E. faecalis* and *S. mutans* of 23.39 and 25.35 mm, with ranges of 21.51–25.27 mm (95% CI) and 23.51–27.20 mm (95% CI), respectively (Table 5). For *P. gingivalis*, an optimal value was not obtained because of the absence of a suitable optimization model. The growth inhibition zone value was 19.44 mm, with a range of 18.88–20.00 mm (95% CI).

Discussion

Bangle (*Zingiber montanum*) and *Piper betle* var. *nigra* contain compounds that have potent antibacterial properties (Sujono et al., 2019), making them potential candidates for treating infections caused by pathogenic bacteria in the oral cavity (Yim et al., 2013). Secondary metabolites from the rhizomes of *Zingiber montanum* (Fam. *Zingiberaceae*) led to the isolation of mono-, sesqui- and diterpenes. The compounds were characterized as (E)-8(17),12-labdadiene-15,16-dial (1), zerumbol (2), zerumbone (3), buddledone A (4), furanodienone (5), germacrone (6), borneol (7) and camphor (8). Among these terpenes, compounds 1 and 2 had potential antibacterial activity against multidrug-resistant (MDR) and methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates (Siddique et al., 2019). Secondary metabolites from the extract of black betel were identified as piperine (14.22%) and diisooctyl phthalate (14.67%) as the major compounds, with piperolein B, piperanine, β -caryophyllene oxide and α -caryophylladienol as minor compounds, whereas

the aquadest extract identified hydroxychavicol (81.89%) and chavibetol (12.01%) as predominant. Piperolein B and piperine had strong binding affinities to the MRSA proteins 4DKI, 6H5O and 4CJN, comparable to that of ciprofloxacin (Yasir et al., 2024). Other research has highlighted the potential of bangles (*Zingiber montanum*) (Hari Prasad Devkota et al., 2021) and black betel (*Piper betle* var. *nigra*) (Taukoorah et al., 2016) to serve as an alternative to conventional antibiotics, which have become less effective owing to increased antibiotic resistance. By comparing the effectiveness of these natural medicines with other traditional treatments, the current study aimed to provide valuable insights into their potential use in combating dental infections caused by oral cavity pathogens.

Bangle ethanol and black betel extracts have demonstrated good activity against both Gram-positive and Gram-negative bacteria, consisting of *E. coli*, *K. pneumonia*, *S. paratyphi*, *S. typhi* and *S. flexneri* (Amatayakul et al., 1979; Hari P Devkota et al., 2021), as well as methicillin-resistant *S. aureus*, *S. aureus* MDR (Siddique et al., 2019) and *S. mutans* (Pardosi et al., 2022).

In contrast to the ethanol extract, the black betel leaf water extract did not have any antibacterial activity against *E. faecalis*, *S. mutans* and *P. gingivalis*. In the current study, each extract was obtained using the decoction method, reflecting its empirical use in the community. The stew results, combined under various conditions and subsequently tested for antibacterial activity, indicated potential antibacterial effects based on the inhibition zones against the three tested bacteria. The water extracts in the current study were designed for direct application as herbal mouthwashes. The current findings suggested that further innovations are needed to combine BR with herbal extracts other than BL, as the two do not demonstrate positive interactions and BL did not have a significant effect on the bacterial growth inhibition zone.

The black betel leaf ethanol extract had a wide range of antibacterial activities. The ethanol extract had antibacterial activity against *P. acnes*, *S. aureus*, *C. albicans*, *S. mutans* (Rahmatiyah et al., 2023) and *P. gingivalis* (Herryawan et al., 2023) although it did not affect *E. faecalis* (Putri et al., 2024). It remains a promising candidate for developing topical preparations or mouthwashes for periodontal therapy and for managing other oral cavity infections.

Another consideration is the increase in the concentration of the extract. Herryawan et al. (2023) reported that the antibacterial effectiveness of the extract increased with increasing concentrations. For example, they reported that 100% red betel extract produced the highest inhibition zone (25.16 mm), while their black betel extract had an inhibition zone of 19.65 mm.

Consideration should be given to the combination of two different herbs or their combination with chemicals, such as glycerin, in the search for herbal mouthwashes because herbal extract combinations often provide positive interaction effects on antibacterial agents. Anggraini (2015) reported that a combination of bangle essential oil and red ginger at a ratio of 1:3 had greater antibacterial activity against *S. aureus* than other ratios (1:1, 3:1, 1:0 and 0:1). Furthermore, Qhorina et al. (2021) reported that mouthwash composed of ethanol (70%) extract of black betel leaf, glycerin, Tween 80, sorbitol, asam benzoat, natrium benzoat, menthol and peppermint oil with glycerin effectively inhibited the growth of *S. mutans*, but not *Candida albicans*.

The optimal inhibitory power of the combination of the BR and BL water extracts against *E. faecalis* and *S. mutans* was achieved at a BR-to-BL ratio of 3:1, with concentrations of 20.0% BR and 19.8% BL for *E. faecalis* and 20.0% BR and 10.0% BL for *S. mutans*. In contrast, *P. gingivalis* did not have any optimal conditions. Using concentrations of 15% BR and 15% BL, with a BR-to-BL ration of 2:1, the maximum inhibition of *P. gingivalis* growth was 21.60 mm, which was higher than reported in other studies, where a concentration of black betel single extract inhibited *P. gingivalis* growth with an inhibition zone of 19.65 mm (Herryawan et al., 2023). Combining the two extracts aimed to achieve synergy based on intended use. Synergy achieved through herbal combinations is an important aspect of traditional medicine for the treatment of specific diseases (Chung et al., 2004; Yusof et al., 2022), particularly as an antibacterial agent (Pithayanukul et al., 2007). The boiling method used in the current study to produce the two extracts has potential for community applications, as opposed to the extraction method using ethanol solvents, reported elsewhere.

Conclusion

The concentration of BR (10–20%) significantly affected the antibacterial activity against *E. faecalis* and *S. mutans*, whereas BL (10–20%) had no significant effect on the growth zones of *E. faecalis* and *S. mutans*. The BR-BL combination (at volume ratios of 1:1–3:1) significantly influenced antibacterial activity in inhibiting the growth of both bacteria. The optimization of the combination of three factors (BR concentration, BL concentration and the volume ratio of both) for antibacterial activity against *E. faecalis* was achieved under the conditions of BR 19.9957%, BL 19.8727% and a BR-to-BL volume ratio of 2.99486, whereas for *S. mutans*, these conditions were BR 20%, BL 10.0224% and a BR-to-BL volume ratio of 2.99999. Under optimal conditions, the growth inhibition zones were 23.5382 mm for *E. faecalis* and 25.3530 mm for *S. mutans*. These results indicated that BR alone was a promising natural ingredient for preventing oral cavity infections; however, its combination with BL is necessary to enhance its antibacterial effectiveness against *E. faecalis* and *S. mutans*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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