การพัฒนาน้ำยาบ้วนปากจากสารสกัดตำรับยาแก้รำมะนาด
Development of Mouthwash from The Yakaerammanad Formula Extract

นิพนธ์ต้นฉบับ

Original Article

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ภาควิชาการแพทย์แผนไทย คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยราชภัฏบ้านสมเด็จเจ้าพระยา เขต ธนบุรี กรุงเทพมหานคร 10600

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วารสารไทยเภสัชศาสตร์และวิทยาการสุขภาพ 2566;18(4):405-415.

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บทคัดย่อ

วัตถุประสงค์: เพื่อพัฒนาน้ำยาบ้วนปากจากสารสกัดตำรับยาแก้รำมะนาด **วิธีการศึกษา:** เตรียมสารสกัดน้ำด้วยการต้ม และสารสกัดเอทานอลด้วยการแช่สกัด นำสารสกัดของตำรับยาแก้รำมะนาดมาทดสอบปริมาณสารฟลาโวนอยด์รวมและปริมาณสารฟีนอลิกรวมด้วยวิธี Aluminium chloride และวิธี Folin-Ciocalteu ตามลำดับ ทดสอบประสิทธิภาพในการยับยั้งเชื้อ *Streptococcus mutans* และ *Candida albicans* ด้วยวิธี Disc diffusion method และหาค่าความเข้มข้นต่ำสุดที่มีฤทธิ์ยับยั้งเชื้อ (Minimal inhibitory concentration; MIC) และค่าความเข้มข้นต่ำสุดที่มีฤทธิ์ฆ่าเชื้อ (Minimal bactericidal concentration; MBC / minimum fungicidal concentration; MFC) ด้วยวิธี Broth microdilution method โดยใช้ยา ampicillin และ amphotericin B เป็นสารมาตรฐาน นำสารสกัดที่มีประสิทธิภาพมาพัฒนาเป็นน้ำยาบ้วนปาก 3 สูตร และทดสอบความคงตัวทางกายภาพและทางเคมีในสภาวะเร่ง 7 รอบ ที่อุณหภูมิ 5 และ 45 องศาเซลเซียส **ผลการศึกษา:** สารสกัดน้ำมีปริมาณสารฟลาโวนอยด์รวมและสารฟีนอลิกรวมสูงสุดเท่ากับ 0.029 ± 0.018 g QE/ 100 g ของสารสกัด และ 11.440 ± 0.488 g GE/ 100 g ของสารสกัด ตามลำดับ สารสกัดเอทานอลของตำรับยาแก้รำมะนาดแสดงฤทธิ์ยับยั้งเชื้อ *Streptococcus mutans* และ *Candida albicans* สูงสุดโดยแสดงค่า MIC เท่ากับ 1.953 และ 0.031 mg/mL ตามลำดับ ขณะที่ค่า MBC/MFC เท่ากับ 1.953 และ 15.625 mg/mL ตามลำดับ ผลการทดสอบความคงตัวพบว่า น้ำยาบ้วนปากสูตรที่ 2 มีสีเขียวอมเหลือง ไม่แยกชั้น ค่าความเป็นกรด-ด่างไม่เปลี่ยนแปลงอย่างมีนัยสำคัญ และน้ำยาบ้วนปากสูตร 2 มีปริมาณสารฟลาโวนอยด์และสารฟีนอลิก **สรุป:** น้ำยาบ้วนปากจากสารสกัดตำรับยาแก้รำมะนาดสูตรที่ 2 มีความคงตัวทางกายภาพและทางเคมีที่เหมาะสมจะนำไปพัฒนาเป็นผลิตภัณฑ์ดูแลสุขภาพช่องปากในอนาคตต่อไป

คำสำคัญ: ตำรับยาแก้รำมะนาด, น้ำยาบ้วนปาก, ปริมาณสารฟลาโวนอยด์รวม, ปริมาณสารฟีนอลิกรวม, ฤทธิ์ต้านจุลชีพ

Abstract

Objective: To develop mouthwash from the Yakaerammanad formula extract. **Methods:** The aqueous extract was extracted by decoction, and the ethanolic extract by maceration. The aqueous and ethanolic extracts of the Yakaerammanad formula were determined for their total flavonoid and total phenolic contents using the aluminum chloride method and Folin-Ciocalteu assay, respectively. Antimicrobial activity against *Streptococcus mutans* and *Candida albicans* was determined by disc diffusion method, and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) were determined by broth microdilution method, using ampicillin and amphotericin B as positive controls. Effective Yakaerammanad formula extracts were developed into 3 formulas of mouthwash and tested for physical and chemical stability under heating-cooling conditions for 7 cycles at temperatures of 5 and 45 °C. **Results**: Aqueous extracts of the Yakaerammanad formula had the highest total flavonoid and total phenolic contents, which were 0.029 ± 0.018 g QE/100 g of extract and 11.440 ± 0.488 g GE/100 g of extract, respectively. The ethanolic extract of Yakaerammanad formula had highest antimicrobial activity against *S. mutans* and *C. albicans*, which showed MIC values of 1.953 and 0.031 mg/mL, respectively, while MBC and MFC values were 1.953 and 15.625 mg/mL, respectively. The results of the stability test showed that mouthwash formula 2 from the Yakaerammanad formula extract is greenish-yellow and does not precipitate. The pH values were not significantly different. The mouthwash formula 2 had total flavonoid and total phenolic contents. **Conclusion:** The mouthwash formula 2 from the Yakaerammanad formula extract had the appropriate physical and chemical stability to be developed into oral health care products in the future.

Key words: Yakaerammanad formula, mouthwash, Total flavonoids content, total phenolic content, antimicrobial activity

Editorial note

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Introduction

The oral cavity is a reservoir of many microorganisms in the mouth. The mouth provides a wet environment and with food leftovers it becomes a hotspot for bacteria and fungi to grow and thrive.[1] *Streptococcus mutans* is included in the leading microorganisms which, together with other anaerobes and oral streptococci, are believed to play major roles in the establishment of the early biofilm community of the dental plaque, providing a matrix within which a plethora of other acidogenic and aciduric microorganisms later grow.[2] *Candida* species are the most common cause of fungal infections. *Candida albicans* remains the most common pathogen in oropharyngeal. The clinical manifestation of oral *Candida albicans* infection is oral thrush, often seen in infants due to inadequate immunity.[3] For those who wear dentures, dentures

may cause ulcers to occur first. The candida fungi that are present in the mouth grow to cause a disease called denture stomatitis (chronic atrophic candidiasis), which is inflammation of the submucosal tissue resulting in chronic inflammation a0t the junction of the gum and denture.[4] Thus, protecting the teeth and mouth generally from microbes and maintaining oral hygiene are essential for a healthy mouth. Oral hygiene products were used, including toothpaste, mouthwash, and floss. Using mouthwash is also highly recommended to kill bacteria in spots where the toothbrush cannot reach.[5]

A mouthwash is often used for its refreshing and antiseptic properties and its efficacy in controlling dental plaque.[6] However, regular commercialized mouthwash tends to include alcoholic substances and other chemicals that dry up the oral cavity and eventually promote bacterial growth. Some of these chemicals may also cause unwanted allergic reactions and even increase the risk of oral cancer.[7] There have been tremendous efforts towards replacing the commonly used mouthwash with safer products made of natural ingredients that can provide similar or even better oral protection but with minimal adverse effects.[8]

Periodontitis is an infectious inflammatory disease caused by the bacteria of the dental plaque, resulting in the progressive destruction of the tissues that support the teeth, i.e. the gingival, the periodontal ligament, cementum, and the alveolar bone.[9] Periodontitis, especially its mild and moderate forms, is highly prevalent in adult-aged populations all over the world, with prevalence rates around 50%, while its severe form has a global prevalence of around 10%. Genetics and environmental and behavioral factors are involved in the development of the disease, the exposure of susceptible individuals to its initiation, and the speed of its progression.[10] Signs and symptoms of periodontitis include plaque accumulation, calculus formation, gingival redness and swelling, gingival bleeding and suppuration, which may occur either spontaneously or when subjected to probing, halitosis (bad breath), and loss of alveolar bone. Others include deepening of the gingival crevice resulting in the formation of a pathological periodontal pocket, root exposure due to gingival recession, and increased tooth mobility. Severe forms of the disease may lead to tooth migration, compromised esthetics, impaired masticatory function, and ultimately tooth loss.[11]

In Thai traditional medicine, the Yakaerammanad formula was recorded in a textbook of traditional Thai medicine in the stone inscription of Wat Phra Chetuphon Wimonmangkalaram (Wat Pho). The Yakaerammanad formula is used to treat periodontitis and swollen gums in their early stages which consist of rock salt, *Brassica juncea* (L.) Czern. et Coss., *Aucklandia lappa* DC, *Acorus calamus* L, *Cyperus alternifolius* L, *Terminalia chebula* Retz., *Azadirachta indica* A. Juss. var. *siamensis* Valeton, *Piper retrofractum* Vahl, *Nigella sativa* L., *Terminalia bellirica* (Gaertn.) Roxb, *Terminalia arjuna* and *Zingiber officinale* Roscoe. All herbs were used in equal weight (1 part of each type), ground, and molded into sticks. For the use of Yakaerammanad formula, it was dissolved with alcohol and applied 3 – 4 times per day, taking 300 – 600 mg each time.[12] According to the pharmacological research report of herbs in the Yakaerammanad formula, it was found that *Brassica juncea* (L.) Czern. et Coss. has anticancer, antioxidant, anti-inflammatory, antimicrobial, and hypoglycemic effects.[13] *Aucklandia lappa* DC. possesses anti-inflammatory, analgesic, anticancer and gastroprotective effects.[14] *Acorus calamus* L. expresses antibacterial, antifungal, and antioxidant activities.[15] *Cyperus alternifolius* L. has antiulcer activity that reduced ulcer number, total ulcer score, and TNF-α content in the stomach of rats.[16] *Terminalia chebula* Retz. has antibacterial activities against *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Pseudomonas aeruginosa, Salmonella,* and *Escherichia coli*.[17] *Azadirachta indica* A. Juss. var. *siamensis* Valeton has antibacterial activities against *Streptococcus* spp., *S. aureus*, *S. hyicus*, *S. intermedius*, *P. aeruginosa*, *A. hydrophila*, *E. coli*, and *Klebsiella* spp. In addition, it has antifungal activities against *C. neoformans* and *C. albicans*.[18] *Piper retrofractum* Vahl. has hypolipidemic effects, anti-inflammatory and analgesic effects, anti-ulcer effect, and antimicrobial activity.[19] *Nigella sativa* L. has antiviral, anti-inflammatory, anti-cancer, and anti-oxidation activities. It has been reported that methanol and ethyl acetate extracts of *Nigella sativa* L. have antimicrobial activity against *Escherichia coli* and *Enterococcus faecalis,* respectively.[20] *Terminalia bellirica* (Gaertn.) Roxb has antibacterial activity against *Pseudomonas aureus*, *Streptococcus pyrogens*, and *Staphylococcus saprophyticus*.[21] *Terminalia arjuna* has antioxidant activity. The antibacterial potential of both the test materials was observed against the bacterial isolates from Crohn's disease (CD) and ulcerative colitis (UC) patients. *Terminalia arjuna* exhibited cytotoxicity in human colorectal adenocarcinoma cells (Caco-2).[22] *Zingiber officinale* Roscoe

inhibits the production of free radicals and oxidative stress, and along with these properties, it can reduce pro-inflammatory molecules like prostaglandins by inhibiting COX-1 and COX-2.[23]

Although there are numerous potential biological activities of herbs in the Yakaerammanad formula, information is still lacking regarding the treatment efficiency of the Yakaerammanad formula, especially for the development of mouthwash to protect against oral disease. Therefore, we aimed to investigate the antimicrobial activity of the Yakaerammanad formula extracts. We determined the total phenolic and total flavonoid contents of the Yakaerammanad formula extracts, and used effective extracts to develop mouthwash.

Methods

Preparation of herb samples

Herb samples in the Yakaerammanad formula consisted of *Brassica juncea* (L.) Czern. et Coss., *Aucklandia lappa* DC, *Acorus calamus* L, *Cyperus alternifolius* L, *Terminalia chebula* Retz., *Azadirachta indica* A. Juss. var. *siamensis* Valeton, *Piper retrofractum* Vahl, *Nigella sativa* L., *Terminalia bellirica* (Gaertn.) Roxb, *Terminalia arjuna* and *Zingiber officinale* Roscoe. Eleven herb samples were obtained from V.P. Pharmacy Co., Ltd. All herb samples were cleaned and dried in hot air oven at 40˚C for 48 to 72 hours. Then, all dried samples were ground using an electric grinder. Fifty grams of powder from each herb sample were mixed for further use in the preparation of the extract.

Extraction of samples

For conventional extraction, 200 g of the powder of the Yakaerammanad formula was boiled in 4 L of distilled water (plant: solvent ratio 1:20 w/v) for 45 min on hot plate. The aqueous extract was filtered and dried using the freeze-dried method to obtain a crude extract. Aqueous extract was stored at -20 ˚C until use.[24] For maceration techniques, 200 g of the powder of the Yakaerammanad formula was macerated in 2 L of 95% ethanol (plant: solvent ratio 1:10 w/v) at room temperature for 3 days. The ethanolic extract solution was filtered and evaporated using the rotary evaporator to yield crude extract. Ethanolic extract was stored at -20˚C for further study.[25]

Determination of total phenolic content using Folin-Ciocalteu method

The **concentration** of phenolic in the Yakaerammanad formula extracts was determined using Folin-Ciocalteu method.[26] For sample solution, 1 mg/mL of each extract solution was prepared in ethanol. Twenty-five microliters of extract were mixed with 25 µL of 2N Folin-Ciocalteu reagent and left for 8 minutes before the addition of 75 µL of distilled water and 100 µL of 20% sodium carbonate. The mixtures were kept in the dark for 90 minutes and the absorbance of the solution was measured at the wavelength of 765 nm using microplate reader. Gallic acid solutions (25-200 µg/mL prepared in distilled water) were used to prepare standard curve of gallic acid. Total phenolic contents of extracts were expressed in terms of gallic acid equivalent [gallic acid (g)/extract (100 g)]. Each sample of extracts and standard compounds was determined in triplicate.

Determination of total flavonoid content using aluminium chloride method

The concentration of flavonoid in the Yakaerammanad formula extracts were determined using aluminium chloride method.[27] Ten milligram per milliliter of each extract solution was prepared with ethanol. The reaction mixture contained 100 µL of sample solution and 100 µL of 2% aluminium chloride solution prepared in methanol. After incubation at room temperature for 30 minutes, the absorbance of the solution was measured at the wavelength of 415 nm using microplate reader. Quercetin solutions (10-60 µg/mL prepared in methanol) were used to prepare standard curve of quercetin. Total flavonoid contents of extracts were expressed in terms of quercetin equivalent [quercetin (g)/extract (100 g)]. Each sample of extracts and standard compounds was determined in triplicate.

Determination of antimicrobial activity

The tested microorganisms were *Streptococcus mutans* ATCC 25175 and *Candida albicans* ATCC 10231, both provided by Center of Analysis for Product Quality (CAPQ), Faculty of Pharmacy, Mahidol University. For *S. mutans* was grown and maintained on Tryptic Soy Agar medium (TSA) with 24 hours of incubation at 34 °C, while *C. albicans* was maintained on Sabouraud dextrose agar medium (SDA) with 48 hours of incubation at 27 °C.[28]

For antimicrobial screening using disc diffusion method, TSA and SDA base plates were seeded with the bacterial and fungal inoculum, respectively with inoculum size 1 × 10^8 CFU/mL for *S. mutans* and 1 × 10^7 CFU/mL for *C. albicans*. The extracts were dissolved in 20% DMSO to obtain 500 mg/mL solutions. Sterile filters paper discs (Whatman no. 1, 6 mm in diameter) were impregnated with 30 µL of each of the extract per disc and left to dry in vacuum so as to remove residual solvent. The Yakaerammanad formula extract discs were then placed on the seeded agar plates, while ampicillin (1 µg/mL) and amphotericin B (0.32 µg/mL) were used as standards for *S. mutans* and *C. albicans*, respectively. The plates were kept at 4°C for 1 h for diffusion of extract, thereafter were incubated at 34°C for *S. mutans* (24 h) and 27°C for *C. albicans* (48 h). Antibacterial and antifungal activities were detected by measuring the zone of inhibition (including the disc diameter) appeared after the incubation period. Each sample of extracts and standard compounds was determined in triplicate.[29]

To determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) / minimum fungicidal concentration (MFC), broth microdilution method was followed for determination of MIC values. The Yakaerammanad formula extracts were dissolved in 20% DMSO to make a stock solution (250 mg/mL). The stock solution of ethanolic extract was two-fold serially diluted at the ranges of 0.061 - 1.953 and 0.004 - 15.625 mg/mL for treating *C. albicans and S. mutans*, respectively. The stock solution of aqueous extract was two-fold serially diluted at the ranges of 0.488-62.50 mg/ml for treating *C. albicans and S. mutans*. Ampicillin and amphotericin B were two-fold serially diluted at the ranges of 0.003-0.781 and 0.003-0.098 µg/mL, respectively. The adjusted cultured bacteria and fungi (for bacteria 1 × 10^8 CFU/mL and for fungi 1 × 10^7 CFU/mL) were then added to each concentration of extracts. Bacterial and fungal suspensions were used as negative control, while broth ampicillin and amphotericin B were used as positive control. The suspensions were incubated at 34 °C for 24 h for bacteria and 27 °C for 48 h for fungi. After the incubation period, the MIC values were visually determined. The lowest concentration of each extract displaying no visible growth was recorded as the minimum inhibitory concentration. The concentration that inhibited bacterial/fungi growth completely (the first clear well) was taken as the MIC value. MIC values were determined in triplicate to confirm activity. The MBC/MFC was determined by subculturing 0.01 mL from each well. The lowest concentration of extract showing no visible growth on subculturing was taken as MBC or MFC. MBC and MFC was determined in triplicate.[30]

Preparation of mouthwash formulations

The extracts showing the highest total phenolic and total flavonoid content and the highest antimicrobial activity were selected to develop mouthwash products. Three different formulations of mouthwash were formulated with ingredients, such as the Yakaerammanad formula extract, propylene glycol, glycerin, sodium benzoate, 70% sorbitol, peppermint spirit, 5% sodium chloride, 70% ethanol and distilled water. For the formulation, the Yakaerammanad formula extract was dissolved in propylene glycol. 0.1 gram of sodium benzoate was dissolved in distilled water. After the ingredients were mixed following the formulation in Table 1, unlike other ingredients, 5% sodium chloride and 70% ethanol were added to the formulas 2 and 3, respectively. Finally, distilled water was added to make up the 100 mL total volume of the mouthwash formulations.

Table 1 Three different formulations of mouthwash from the Yakaerammanad formula extract.

Ingredients	Formulations		
	F1	F2	F3
Yakaerammanad formula extract (g.)	1.56	1.56	1.56
Propylene glycol (mL.)	10	15	18
Glycerin (mL.)	10	5	2.5
Sodium benzoate (g.)	0.1	0.1	0.1
70% Sorbitol (mL.)	10	10	10
Peppermint spirit (mL.)	0.8	0.8	0.8
5% Sodium Chloride (mL.)	-	10	-
70% Ethanol (mL.)	-	-	10
Distilled water qs to mL	100 mL	100 mL	100 mL

Stability test of mouthwash formulations

Different mouthwash formulations were subject to a stability test. Stability test aimed to ensure that the mouthwash formulations were usable and maintained the same characteristics in the long term. Physical stability test of the formulated mouthwash included recording sedimentation using a centrifuge machine (Hettich, Germany) at 3,000 rpm for 5 minutes, color was performed using a Chroma Meter CR-400 colorimeter (Konica Minotta, Japan) which was carried out in triplicate and expressed as L*, a*, b* (mean ± SD). The analyzed color parameters were L* (lightness: from 0 (black) to 100 (white), a* from – (green) to + (red) and b* from – (blue) to + (yellow). In addition, pH stability was also monitored using

a pH meter (Mettler Toledo, Switzerland). The pH readings were carried out in triplicate (mean ± SD). The determination of total phenolic and flavonoid content was expressed in terms of gallic acid equivalent and quercetin equivalent, respectively (mean ± SD). Stability test of three different mouthwash formulations were checked after heating and cooling cycles. All mouthwash was stored at 5 ˚C for 24 hours and were directly removed and placed at 45 ˚C for another 24 hours. This procedure was repeated for 7 cycles. The results were recorded and compared over the course of seven cycles.[31-33]

Statistical analysis

The data represented mean of three replicates ± standard deviation (SD). The significance of difference was used to compare mean (P-value < 0.05). The total phenolic content and flavonoid contents of the Yakaerammanad formula extracts were analyzed by independent samples t-test. Stability tests before and after the heating-cooling cycle of the total phenolic content and flavonoid contents of the same mouthwash were analyzed by paired-sample T tests, while each mouthwash was analyzed by one-way ANOVA. Stability tests before and after the heating-cooling cycle of the pH values and color of the same mouthwash were analyzed by repeated-measures ANOVA.

Results

Determination of total phenolic content using Folin-Ciocalteu method

Total phenolic contents of the Yakaerammanad formula extracts were determined by Folin-Ciocalteu method. It was found that aqueous extract from the Yakaerammanad formula significantly contained the higher total phenolic content than ethanolic extract from the Yakaerammanad formula with the total phenolic content 11.440 ± 0.488 and 3.939 ± 0.461 g gallic acid equivalent/ 100 g extract, respectively (Table 2).

Table 2 Total phenolic and total flavonoid contents of the Yakaerammanad formula extracts.

Samples	Total phenolic content (g GE/ 100 g of extract)	Total flavonoid content (g QE/ 100 g of extract)
Ethanolic extract	3.939 ± 0.461[b]	0.217 ± 0.050[a]
Aqueous extract	11.440 ± 0.488[a]	0.290 ± 0.018[a]

[a,b] Different letters in the same column indicate values are significantly different (P-value < 0.05).

Determination of total flavonoid content using aluminium chloride method

Total flavonoid contents of the Yakaerammanad formula extracts were determined by aluminium chloride method as showed in Table 2. The result showed that aqueous extract from the Yakaerammanad formula contained the higher total flavonoid content than ethanolic extract from the Yakaerammanad formula with the total flavonoid content 0.290 ± 0.018 and 0.217 ± 0.050 g quercetin equivalent/ 100 g extract, respectively.

Determination of antimicrobial activity

The antimicrobial activity was carried out using the disc diffusion assay. The clear zone formed around the disc proved that the ethanolic extract from the Yakaerammanad formula had antimicrobial activity against *s. mutans* and *c. albicans* with the inhibition zone 19.0 and 11.0 mm, respectively, while the aqueous extract from the Yakaerammanad formula did not exhibit microbial inhibition (Table 3). However, ampicillin had antimicrobial activity against *s. mutans* (inhibition zone 31.0 mm) and amphotericin B had antimicrobial activity against *c. albicans* (inhibition zone 15.5 mm).

Table 3 The antimicrobial activity of the extracts from the Yakaerammanad formula by disc diffusion method.

Samples	Diameter of inhibition zone (mm)	
	Streptococcus mutans	*Candida albicans*
Ethanolic extract	19.0	11.0
Aqueous extract	0	0
Ampicillin	31.0	-
Amphotericin B	-	15.5

Note: The concentrations of the extracts were 15 mg/mL, while ampicillin and amphotericin B were 0.32 and 1µg/mL, respectively.

The results of MIC and MBC/MFC of the extracts from the Yakaerammanad formula are displayed in Table 4. The ethanolic extract from the Yakaerammanad formula was bactericidal against *S. mutans* with a MIC of 1.953 mg/mL and MBC of 1.953 mg/mL, while the ethanolic extract from the Yakaerammanad formula was fungicidal against *C. albicans* with a MIC of 0.031 mg/mL and MFC of 15.625 mg/mL. The aqueous extract from the Yakaerammanad formula demonstrated less antimicrobial activity. For positive control antimicrobial agents, the ampicillin was bactericidal against *S. mutans* with a MIC of 0.012 µg/mL and MBC of 0.781 µg/mL, while the amphotericin B was fungicidal against *C. albicans* with a MIC of 0.006 mg/mL and MFC of 0.098 µg/mL. However, the

antimicrobial agents had higher antimicrobial activity than both extracts.

Table 4 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) / minimum fungicidal concentration (MFC) values of the extracts from the Yakaerammanad formula (mg/mL) and positive control antimicrobial agents (µg/mL).

Samples	*Streptococcus mutans*		*Candida albicans*	
	MIC	MBC	MIC	MFC
Ethanolic extract	1.953	1.953	0.031	15.625
Aqueous extract	> 62.500	> 62.500	> 62.500	> 62.500
Ampicillin	0.012	0.781	-	-
Amphotericin B	-	-	0.006	0.098

Stability test of mouthwash formulations

The ethanolic extracts had the highest antimicrobial activity. Therefore, it was chosen to develop a mouthwash product. 1.56 g of the ethanol extract was used to prepare 100 mL of mouthwash, which was calculated from the minimum fungicidal concentration (MFC) values of the ethanol extract (15.625 mg/mL). The mouthwash stability test was carried out under heating and cooling cycles, namely storage at a temperature of 5 ºC and 45 ºC for 48 hours each for 7 cycles. According to the results of the sedimentation test, it was found that formula 2 mouthwash did not precipitate, while formulas 1 and 3 mouthwash formed sediment. The results of the pH analysis on each mouthwash formula showed that the pH value of the formula ranged from 4.403 to 4.893 (Table 5). The pH values of the mouthwash formulas before and after the forced condition was formula 1 (4.530; 4.560), formula 2 (4.403; 4.370), and formula 3 (4.830; 4.703). The pH value of formula 3 mouthwash was the highest. However, the pH value of the formula 2 mouthwash was not significantly different after the condition was forced. This indicates that the formula 2 mouthwash has good pH stability.

Table 5 The pH values of three mouthwash formulations from the Yakaerammanad formula extract.

Cycles	Formula 1	Formula 2	Formula 3
0	4.530 ± 0.010^{a}	4.403 ± 0.035^{a}	4.830 ± 0.020^{a}
1	4.640 ± 0.035^{b}	4.500 ± 0.010^{a}	4.893 ± 0.015^{b}
2	4.613 ± 0.031^{b}	4.460 ± 0.010^{a}	4.827 ± 0.015^{a}
3	4.617 ± 0.006^{b}	4.483 ± 0.015^{a}	4.873 ± 0.023^{b}
4	4.593 ± 0.015^{b}	4.430 ± 0.010^{a}	4.813 ± 0.012^{a}
5	4.613 ± 0.006^{b}	4.460 ± 0.010^{a}	4.810 ± 0.026^{a}
6	4.560 ± 0.000^{b}	4.370 ± 0.010^{a}	4.703 ± 0.032^{c}
7	4.390 ± 0.000^{ab}	4.365 ± 0.015^{a}	4.490 ± 0.056^{d}

$^{a-d}$ Different letters in the same column indicate values are significantly different (P-value < 0.05).

The results of color parameters showed that the lightness (L*) values of the mouthwash formulations before and after the forced condition was formula 1 (23.400; 24.927), formula 2 (35.597; 34.080) and formula 3 (29.800; 30.110). The a* value of the mouthwash formulations before and after the forced condition was formula 1 (-0.027; -0.067), formula 2 (-2.293; -2.410) and formula 3 (-1.717; -1.767). The b* value of the mouthwash formulations before and after the forced condition was formula 1 (4.630; 4.830), formula 2 (9.090; 9.223) and formula 3 (3.443; 3.647). The results revealed that three mouthwash formulations have the same colors: green (-a*) and yellow (+b*). However, the lightness (L*) value of the formula 2 mouthwash was the highest, which showed that the formula 2 was clearly better than the formulas 1 and 3 (Table 6).

The total flavonoid and total phenolic contents of three mouthwash formulations from the Yakaerammanad formula extract were shown in Table 7. The total flavonoid content of the mouthwash formulas before (cycle 0) the forced condition was formula 1 (0.273 ± 0.009 g QE/ 100 g of extract), formula 2 (0.326 ± 0.010 g QE/ 100 g of extract), and formula 3 (0.384 ± 0.008 g QE/ 100 g of extract), which were statistically significantly different. The total flavonoid content of mouthwash formulas after (cycle 7) the forced condition found that formulas 1, 2, and 3 had flavonoid contents of 0.154 ± 0.018, 0.188 ± 0.008, and 0.218 ± 0.018 g QE/ 100 g of extract, respectively, and the mouthwash formula 3 was significantly different from the mouthwash formula 1. However, the total flavonoid content of each mouthwash formula before (cycle 0) and after (cycle 7) the forced condition was statistically significantly different. The results showed that mouthwash formula 3 had the highest total flavonoid content before and after the forced condition, with statistical significance. The total phenolic content of mouthwash formulas before (cycle 0) the forced condition found that formulas 1, 2, and 3 had phenolic contents of 5.699 ± 0.099, 4.695 ± 0.271, and 4.900 ± 0.324 g GE/ 100 g of extract, respectively, and mouthwash formula 1 had the highest total phenolic content with statistical significance. After (cycle 7) the forced condition, it was found that formulas 1, 2, and 3 had phenolic contents of 3.986 ± 0.450, 3.606 ± 0.770 and 3.907 ± 0.471 g GE/ 100 g of extract, respectively. The result showed that it had no significance. However, the total flavonoid content of mouthwash formulas 1 and 2 before

Table 6 The color parameters of three mouthwash formulations from the Yakaerammanad formula extract.

Cycles	Formula 1			Formula 2			Formula 3		
	L*	a*	b*	L*	a*	b*	L*	a*	b*
0	23.400 ± 0.142^{a}	-0.027 ± 0.006^{a}	4.630 ± 0.036^{a}	35.597 ± 0.095^{a}	-2.293 ± 0.040^{a}	9.090 ± 0.010^{a}	29.800 ± 0.050^{a}	-1.717 ± 0.015^{a}	3.443 ± 0.040^{a}
1	23.627 ± 0.025^{a}	-0.030 ± 0.000^{a}	4.710 ± 0.020^{ab}	35.7067 ± 0.021^{a}	-2.420 ± 0.026^{b}	9.113 ± 0.015^{a}	29.813 ± 0.015^{a}	-1.700 ± 0.010^{ab}	3.423 ± 0.031^{a}
2	23.700 ± 0.010^{a}	-0.040 ± 0.010^{ab}	4.733 ± 0.031^{ac}	35.797 ± 0.015^{a}	-2.477 ± 0.012^{c}	9.160 ± 0.010^{b}	29.900 ± 0.010^{a}	-1.690 ± 0.010^{ac}	3.490 ± 0.010^{a}
3	23.953 ± 0.047^{b}	-0.040 ± 0.010^{ac}	4.820 ± 0.020^{bc}	36.127 ± 0.117^{b}	-2.570 ± 0.010^{d}	9.060 ± 0.036^{a}	29.997 ± 0.006^{b}	-1.740 ± 0.010^{a}	3.490 ± 0.010^{a}
4	24.167 ± 0.153^{bc}	-0.043 ± 0.006^{ade}	4.950 ± 0.020^{d}	36.177 ± 0.025^{b}	-2.660 ± 0.026^{e}	9.123 ± 0.025^{ab}	29.993 ± 0.021^{bc}	-1.667 ± 0.015^{ad}	3.527 ± 0.025^{a}
5	24.513 ± 0.071^{c}	-0.060 ± 0.010^{bce}	4.727 ± 0.038^{ae}	35.517 ± 0.152^{a}	-2.283 ± 0.021^{a}	9.050 ± 0.040^{a}	30.177 ± 0.021^{d}	-1.640 ± 0.030^{bcd}	3.527 ± 0.025^{ab}
6	24.800 ± 0.010^{d}	-0.063 ± 0.006^{bc}	4.787 ± 0.015^{ce}	33.970 ± 0.061^{c}	-2.327 ± 0.025^{a}	9.170 ± 0.020^{b}	30.080 ± 0.026^{c}	-1.733 ± 0.006^{a}	3.603 ± 0.006^{bc}
7	24.927 ± 0.025^{e}	-0.067 ± 0.006^{bcd}	4.830 ± 0.030^{cd}	34.080 ± 0.026^{d}	-2.410 ± 0.036^{abc}	9.223 ± 0.032^{b}	30.110 ± 0.036^{c}	-1.767 ± 0.021^{a}	3.647 ± 0.042^{c}

$^{a-e}$ Different letters in the same column indicate values are significantly different (P-value < 0.05).

Table 7 The total flavonoid and total phenolic contents of three mouthwash formulations from the Yakaerammanad formula extract.

Mouthwash formulas	Flavonoid content (g QE/ 100 g of extract)		Phenolic content (g GE/ 100 g of extract)	
	Cycle 0	Cycle 7	Cycle 0	Cycle 7
Formula 1	0.273 ± 0.009$^{a, C}$	0.154 ± 0.018$^{b, B}$	5.699 ± 0.099$^{a, A}$	3.986 ± 0.450$^{b, A}$
Formula 2	0.326 ± 0.010$^{a, B}$	0.188 ± 0.008$^{b, AB}$	4.695 ± 0.271$^{a, B}$	3.606 ± 0.770$^{b, A}$
Formula 3	0.384 ± 0.008$^{a, A}$	0.218 ± 0.018$^{b, A}$	4.900 ± 0.324$^{a, B}$	3.907 ± 0.471$^{a, A}$

$^{a-b}$ Different letters in the same row indicate values are significantly different (P-value < 0.05).

$^{A-C}$ Different letters in the same column indicate values are significantly different (P-value < 0.05).

(cycle 0) and after (cycle 7) the forced condition was statistically significantly different. The results showed that formula 1 mouthwash had the highest total phenolic content. The flavonoid and phenolic contents of all 3 mouthwash formulas decreased after 7 cycles of heating and cooling. However, three mouthwash formulations from the Yakaerammanad formula extract contained higher phenolic contents than flavonoid contents.

Discussions and Conclusion

There has been a definite need in dentistry to develop an herbal mouthwash that has better biological activity and fewer side effects.[34] Medicinal plants have been identified and reported for their bioactivity in oral care studies.[35] Natural antimicrobial products may be an important agent in the treatment of caries, periodontal disease, and oral candidiasis.[36] This study showed that the ethanolic extract from the Yakaerammanad formula had antimicrobial activity against *S. mutans* and *C. albicans*. In addition, the ethanolic extract from the Yakaerammanad formula contains the phenolic and flavonoid contents. Flavonoids are structurally diverse secondary metabolites in plants that are reported to inhibit fungal growth by disrupting plasma membranes, inducing mitochondrial malfunction, and reducing cell wall construction, cell division, RNA (ribonucleic acid), and protein synthesis, as well as the efflux-mediated pumping system.[37] Phenols are a group of secondary metabolites distributed in plants that are used as antimicrobial agents due to their potential to damage membrane structural integrity in a nonspecific way and to inhibit certain electron transport enzymes.[38] It has been reported that *Terminalia chebula* and *Terminalia bellirica* are rich in gallic acid; moreover, ethyl gallate was found in *Terminalia bellirica*.[39] These compounds have antibacterial activity.[40] Gallic acid is a phenolic compound that inhibits the growth and biofilm formation of *S. mutans*. The minimum antimicrobial concentration of gallic acid against *S. mutans* was determined at 8 mg/mL.[41] The main compounds with proven antifungal activities were phenolic compounds such as gallic acid, thymol, and flavonoids (especially catechin).[42,43] However, the majority of the natural extracts exhibited antifungal activities against *C. albicans*, such as *Terminalia arjuna* and *Zingiber officinale* extracts.[44] However, the results of this study showed that while aqueous extract had higher levels of flavonoids and phenolic compounds, it had lower levels of antimicrobial activities, whereas ethanolic extract had lower levels of flavonoids and phenolic compounds but higher levels of antimicrobial activities. There is a research reporting that the Folin-Ciocalteau reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid, which after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum.[45] Folin-Ciocalteu reagent in nonspecific to phenolic compounds as it can be reduced by many nonphenolic compounds such as sulfur dioxide, organic acids including ascorbic acid, sugars (fructose and sucrose), and some amino acids present in the test sample.[46] The extract from decoction contains a large amount of water-soluble impurities. The decoction process might enhance the dissolution of some bioactive compounds compared with the maceration process.[47] This is consistent with the experimental results, which showed that the aqueous extract had a statistically significant higher phenolic content than the

ethanolic extract, while the flavonoid content of both extracts was not different. There are reports of phytochemical compounds found in herbs in the Yakaerammanad formula. *Brassica juncea* and *Acorus calamus* have alkaloid and triterpenoid compounds as phytochemical constituents, while *Terminalia chebula* and *Azadirachta indica* contain alkaloids.[48-51] The triterpenoid group was an antibacterial compound, which worked by reacting with transmembrane proteins (porin) on the outer membrane of the bacterial cell wall and forming strong polymeric bonds that caused damage to the porin.[52] Alkaloids worked as antibacterial by disrupting the constituent components of peptidoglycan in bacterial cells, therefore the bacterial cell wall layer was not formed intact and caused bacterial cell death.[53] For *Piper retrofractum* is rich in piperine, which it has been previously reported to give significant antibacterial activity against some bacterial strains.[54] The antibacterial potency of essential oil of *Nigella sativa* showed the action of essential oil of *Nigella sativa* rich in carvacrol, whose antimicrobial efficacy is explained by the actual position of the hydroxyl group on the phenolic structure of these molecules. and which modify permeability and cause leakage of intracellular components through their specific binding to the amine and hydroxylamine groups of bacterial membrane-bound proteins. In addition, it has been reported that O-cymene component of essential oil of *Nigella sativa* presents antifungal potency on *C. albicans* and other pathogenic fungal strains.[55] Moreover, the ethanolic extract of the Yakaerammanad formula showed less antimicrobial activity than the positive control. There are reports that herbs in the Yakaerammanad formula have anti-inflammatory effects. *Brassica juncea* leaf acts as an effective anti-inflammatory agent against acute and chronic inflammatory processes by suppressing the mRNA expression of a panel of inflammatory mediators, including TNF-α, IL-6, and IL-1β, in mice.[56] *Acorus calamus* L. leaves inhibited the production of pro-inflammatory cytokines such as interleukin-8 and activation of NF-kappaB.[57] *Terminalia chebula* fruit revealed the anti-inflammatory properties by regulating nitrite and TNF-α production; iNOS, COX-2 levels, and translocation of NF-KB protein.[58] *Azadirachta indica* inhibited pro-inflammatory cell signaling and apoptotic cell death mechanisms.[59] *Terminalia bellirica* treatment significantly diminished the elevated levels of inflammatory markers and downregulated the mRNA level expression of TNF-α, IL-6 and COX-2 genes.[60] *Zingiber officinale* can reduce pro-inflammatory molecules like prostaglandins by inhibiting COX-1 and COX-2.[23] However, *Piper retrofractum*, *Nigella sativa* and *Terminalia arjuna* have anti-inflammatory activity.[19,20,61] According to the data, herbs in the Yakaerammanad formula had antimicrobial and anti-inflammatory effects, which supports the use of Thai traditional medicine wisdom in the treatment of periodontitis. In this study, the ethanolic extract from the Yakaerammanad formula was selected to develop three formulations of mouthwash. Mouthwash selection criteria consisted of a clear, non-separating, sediment-free solution, pH stability, and content of flavonoid and phenolic compounds. Stability test results of three mouthwash formulas showed that mouthwash formula 2 was greenish-yellow, did not precipitate, and had a stable pH which was in accordance with the Thai Industrial Standards for oral rinse (TIS 2342–2550). The solution must be clear, not separated, free of sediment, and have a pH in the range of 3.0 to 10.5.[62] In addition, mouthwash formula 2 contains flavonoid and phenolic compounds. Furthermore, mouthwash Formula 2 contained sodium chloride, which has properties to reduce bacteria in the mouth, and did not contain alcohol, which causes a burning sensation in the mouth and increases the risk of oral cancer.[63,64] For mouthwash formulas 1 and 3, it was found that they were not stable physically or chemically. Therefore, mouthwash formula 2 may be suitable for product development. However, the pH of mouthwash formula 2 was below 5.5, which is considered a critical pH value for dissolution of enamel, and thus must be modified in other formulations. Various studies have found that phytochemicals inhibit bacterial multiplication. Thus, plant products can be used as a good alternative in dentistry for several uses, like treating oral infections, dental caries, and gingival and periodontal diseases.[65] Further research needs to be done using positive controls to compare the antibacterial effectiveness of herbal mouthwash and commercial mouthwash.

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