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Development of Mouth Care Product Mixing with *Boesenbergia Pandurata* Extract for Inhibiting of *Streptococcus mutans*

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ABSTRACT

The dental caries subjected to *Streptococcus mutans* (*S. mutans*), is one of the oral cavity pathogenic bacteria. Thai traditional medical knowledge sculpture together with the modern scientific approach were applied to development of mouth care product mixing with Boesenbergia Pandurata Extract (BPE). The research objectives were to find the optimal condition of two Boesenbergia pandurata extraction processes and to evaluate the efficiency of two mouth care products such as herbal toothpaste and mouth wash product. The extraction optimal condition and efficiency of mouth care product were determined by inhibition of *Streptococus mutans* ATCC 25175. The inhibition of *Streptococus mutans* analyzed by determination of disc diffusion method and broth microdilution method and time kill analysis. The optimal solvent extraction process of *B. pandurata* was 95% of ethanol extraction solvent, 7 days of immerging time and maximum dilution as disinfectant at 1:7 of extract per water (v/v). *B. pandurata* extract by solvent extraction can be inhibited *S. mutans* from 10^7 to 10^2 CFU mL^{-1}. The optimal hydrodistillation process of *B. pandurata* was 90°C of extraction temperature. This condition gave both *B. pandurata* oil and distillate product. Both products can inhibit *S. mutans*. The mouthwash mixed with *B. pandurata* extract inhibited *S. mutans* better than powder toothpaste. Finally, the *B. pandurata* extract had a good potential of being used as a disinfectant. It can be application for development of tooth care product as toothpaste and mouthwash products.

Key words: *Streptococcus mutans*, *Boesenbergia pandurata*, dental caries, mouth care product, extraction techniques

INTRODUCTION

Boesenbergia Pandurata (*B. pandurata*) or fingerroot or Chinese ginger is one of traditional culinary and medicinal herbs in Southeast Asia. Boesenbergia Pandurata was in Zingiberaceae and treated on abdominal pain, sputum laxative, wounds and diarrhea colic disorder (Atun *et al*., 2013; Tewtrakul *et al*., 2009). The chemical compounds of *B. pandurata* consisted of many Flavonoids such as boesenbergin A, boesenbergin B, panduratin A, cardomin and cardomonin, especially. Their Flavonoids have scientifically proved as the antibacterial agents (Tuchinda *el al*., 2002).

Streptococcus mutans was potentially impacted as a plaque forming bacterium, effect of dental caries in animals and humans. The activities of *S. mutans* was sticking on tooth surfaces in the presence of sucrose, releasing of acids from fermentation of various dietary sugars and inducing of dental caries (Hamada *et al*., 1984; Oh *et al*., 2003). There are many elimination techniques of *S. mutans* especially natural antibiotic agents. Various natural antibiotic agents from medicinal plants have been selected for antibacterial activity by extraction techniques. Many researches have been studied on the antibacterial activity (Hwang *et al*., 2004), anti-allergic activity and anti-inflammatory effect (Tewtrakul *et al*., 2009) of *B. pandurata*.

Tooth care product classified two types including toothpaste and mouthwash. Thai folk toothpaste commonly produced in powder form. The common powder toothpaste ingredients contain many dried herbal plants mixing with binder compounds for example calcium carbonate, sodium bicarbonate and alumina, perlite (Joiner, 2007). The compositions of mouthwash were surfactant, drug extract, astringent antiseptic agent, flavor and color (Yigit *et al*., 2008). The antibacterial agent was added into all tooth care products for decreasing of biofilm and reducing of occurrence of dental carries (Verkaik*et al*., 2011; Dalirsani *et al*., 2011).

This research aimed to find the optimal condition of two *B. pandurata* extraction processes and to evaluate the inhibition efficiency of *S. mutans* in mouth care products mixing with *B. pandurata* extractions.

MATERIALS AND METHODS

Preparation of *B. pandurata* extract: Fresh rhizomes of *B. pandurata* in Fig. 1 were bought from the Talat Thai market, Bangkok, Thailand in January 2012. The

Fig. 1: Fresh rhizomes of *B. pandurata*

solvent extraction and hydrodistillation applied for preparation of *B. pandurata* extract. The cleaned rhizomes were sliced and separated into fresh and dry form.

The fresh rhizomes of *B. pandurata* were mixed with water at ratio 1:2 (*B. pandurata*: water) and distillated on varied temperature at 30, 60 and 90°C. The hydrodistillation samples were collected in three parts including *B. pandurata* oil (Top product), distillates (Bottom product) and residue (Crude). All hydrodistillation samples were test inhibition of *S. mutans* analyzed by disc diffusion method.

The fresh rhizomes of *B. pandurata* were dried by oven at 55°C for 24 h, finely grinded into powder. The *B. pandurata* dried powder was immerged in 40, 60, 80 and 90% of ethanol for 24 h, followed by filtered using filter paper (Whatman No. 4). The *B. pandurata* solutions were extracted at 40°C, pressures at 0.85 bar and 30 min by rotary evaporator (BüCHI Rotavapor R-200 including bath B-490). The extractions were kept in 4°C. After the finding of optimal usage ethanol, the immerging time of *B. pandurata* was found at 1,3,5 and 7 days. The optimal condition of solvent extraction of *B. pandurata* was determined by disc diffusion method and Broth Microdilution Method. The *B. pandurata* extraction by solvent extraction and hydrodistillation were mixed in powdered toothpaste and mouthwash products.

Bacterial strains and culture conditions: The *Streptococcus mutans* ATCC 25175 was used in this study as dental carries microorganism (Rockville, MD, USA). *S. mutans* were cultured in Triptic Soy Broth (TSB) 10 mL, at 37°C for 24 h. The *S. mutans* was purified by streak plate method on Mitis Salivarius Agar (MSA) and the isolated *S. mutans* colony was kept in glycerol 30% TSB% at -4°C. *S. mutans* was multiplied to suspension containing 1×10^7 colony forming units CFU mL^{-1} before using in determination of inhibition of *S. mutans*.

Determination of inhibition of *Streptococcus mutans* ATCC 25175 by Disc diffusion method and Broth Microdilution method (Barry, 1991): The solvent extraction samples were performed on Disc diffusion method and Broth Microdilution in duplicate. The hydrodistillation samples were only analyzed on Disc diffusion method in duplicate. Simple statistical analysis was used to report the results in term of Mean±SD, at n = 2.

Disc diffusion method: The *B. pandurata* extract with ethanol condition at 40, 60, 80 and 95% (v/v) with immerging time at 24 h were dilute with deionized water at 1:1, 1:3, 1:5 and 1:7. The filter papers were immerged on those extracts until saturation. The *S. mutans* solution was spread on MSA agar and the saturated filter papers were set on MSA agar. All of MSA agars were incubated for 24 h at 37°C. The MIC was determined by judging visually occurrence of clear zone of bacterial in the series of test.

Broth microdilution method: A bacterial suspension (0.1 mL) containing 1×10^7 colony forming units CFU mL^{-1} was added to TSB broth and incubated for 24 h at 37°C. The *B. pandurata* extract with ethanol condition were added into TSB broth. The MBC was the concentration in which *S. mutans* was unable to remain viable.

Preparation of Mouth care product: Two mouth care products were prepared into powder toothpaste and mouthwash. The major composition of powder toothpaste contained 18.86% Siam rough bush, 18.86% Liquorice, 18.86% Clove tree, 18.86% Cuttlebone, 18.86% Marl limestone,

Table 1: Main composition of powder toothpaste

	Recipe (% by weight)	
Compositions	A1	A2
Siam rough bush	18.86	18.86
Clove tree	18.86	18.86
Liquorice	18.86	18.86
Cuttlebone	18.86	18.86
Marl limestone	18.86	18.86
Comphanone	4.14	4.14
Menthol	0.06	0.06
B. pandurata extract by solvent extraction	1.50	-
B. pandurata extract by hydrodistillation	-	1.50
Total	100.00	100.00

Fig. 2: Powder toothpaste with *B. pandurata* extract

4.14% Cinnamomum camphor, 0.06% menthol and 1.50% *B. pandurata* extract. The *B. pandurata* extract were used one optimal condition of solvent extraction and one optimal condition of hydrodistillation. Table 1 showed the ratio of composition of powder toothpaste samples. The mixed powder toothpaste showed in Fig. 2. The preparation of mouthwash mixed many aqueous chemicals such as Surfactant (Polysorbate 20), Sorbitol, Eucalyptus oil, Tartrazine, Menthol, ethanol, salt, deionized water and *B. pandurata* extract. The ratio of mouthwash compositions were shown in Table 2. The mixed mouthwash showed in Fig. 3. Two mouth care product were analyzed the product quality by time kill analysis for determination of inhibition of *S. mutans*.

Time kill analysis: The *Streptococcus mutans* ATCC 25175 suspension containing 1×10^7 colony forming units CFU mL^{-1} was added to TSB broth and incubated for 24 h at 37°C. The powder toothpaste and mouthwash put into all suspension at ratio 1:1. Appropriate volumes of the suspensions were diluted with 0.9% of NaCl. Test solutions and growth controls were then placed into a shaker and incubated at 37°C. Samples were withdrawn at 5,15,30,50,75,105,140 and 180 minutes serially diluted (when necessary) and plated onto MSA agar using spread plate method. The plates were incubated at 37°C for 24-48 h and colony counts were determined. All kill curves were performed in duplicate.

Fig. 3: Mouthwash with *B. pandurata* extract

Table 2: Main composition of mouthwash

	Recipe (% by volume)	
Compositions	B1	B2
Surfactant (Polysorbate 20)	0.07	0.07
Sorbitol	10.00	10.00
Eucalyptus oil	0.07	0.07
Tartazine	0.07	0.07
Menthol	0.07	0.07
Ethanol	1.00	1.00
Salt	0.07	0.07
Deionized water	87.67	87.67
B. pandurata extract by solvent extraction	1.00	-
B. pandurata extract by hydrodistillation	-	1.00
Total	100.00	100.00

RESULTS AND DISCUSSION

Optimal condition of *Boesenbergia pandurata* extract by solvent extraction: These extracts by solvent extraction process were strong yellow and odor of *B. pandurata*. The inhibition of *S. mutans* ATCC 25175 was applied for decisions of optimal condition including percent of ethanol in solvent, immerging time and dilution ratio of Boesenbergia pandurata extract. The inhibition of *S. mutans* ATCC 25175 was analyzed by Disc Diffusion Method and Broth Microdilution Method. Table 3 was the results of inhibition of *Streptococcus mutans* ATCC 25175 with *B. pandurata* extract from solvent extraction at 1 day of immerging time. Table 4 showed inhibition of *Streptococcus mutans* ATCC 25175 with *B. pandurata* extract from 95% of ethanol extraction at several of immerging time.

The increasing of percentage of ethanol in solvent extraction inhibited on the growth of *Streptococcus mutans* ATCC 25175 in Disc diffusion method and Broth Microdilution Method. The results of inhabitation of *Streptococcus mutans* ATCC 25175 with *B. pandurata* extract at 40,60, 80 and 95% wasn't different. The optimal dilution ratio was interested in the high volume and high bacteria inhabitable. Table 4 indicated that the optimal immerging time was 7 days. The MIC at 7 days and 1:7 (extract: water) is the least value (1.20 cm). The amount of *Streptococcus mutans* was calculated by Broth Microdilution Method. The results of inhibition of *Streptococcus mutans*

Table 3: Inhibition of *Streptococcus mutans* ATCC 25175 with *B. pandurata* extract from solvent extraction at 1 day of immerging time by disc diffusion method

	Minimum inhibitory concentration (MIC) (cm.)			
	Dilution ratio (extraction: ionized water)			
Percentage of ethanol in extraction (%, v/v)	1:1	1:3	1:5	1:7
40	0.65	0.60	0.65	0.65
60	0.77	0.60	0.65	0.65
80	0.80	0.70	0.65	0.65
95	0.60	0.70	0.65	0.70

Table 4: Inhibition of *Streptococcus mutans* ATCC 25175 with *B. pandurata* extract from 95% of ethanol extraction at several of immerging time by disc diffusion method

	MIC diameter (cm.)			
	Dilution ratio (extraction: ionized water)			
Immerging time (day)	1:1	1:3	1:5	1:7
1	0.60	0.70	0.70	0.70
3	0.90	1.00	1.20	1.00
5	1.00	1.10	1.10	1.15
7	1.18	1.10	1.20	1.20

Table 5: Inhibition of *Streptococcus mutans* ATCC 25175 with *B. pandurata* extract from solvent extraction at 1 day of immerging time and dilution ratio at 1:7 by broth microdilution method

	Amount of *Streptococcus mutans* ATCC 25175 (Log CFU mL^{-1})		
Percentage of ethanol in extraction (%, v/v)	No. 1	No. 2	Average (Mean±SD;n = 2)
No extraction (control)	7.10	7.13	7.12±0.01
40	3.38	3.56	3.47±0.13
60	2.60	2.70	2.65±0.07
80	2.70	2.60	2.65±0.07
95	2.60	2.60	2.60±0.00

ATCC 25175 with *B. pandurata* extract from solvent extraction at 1 day of immerging time and dilution ratio at 1:7 by Broth Microdilution Method was presented in Table 5. The amount of *Streptococcus mutans* ATCC 25175 at control condition was 10^7 CFU mL^{-1} and the critical value of inhibition of *Streptococcus mutans* ATCC 25175 was 10^4 CFU mL^{-1}. The inhibition of *Streptococcus mutans* ATCC 25175 at 40% of ethanol extraction approximately determined on 10^4 CFU mL^{-1}. The increasing of percentage of ethanol in extraction from 60 to 95% decreased the amount of *Streptococcus mutans* ATCC 25175 up to 10^2 CFU mL^{-1}. The results of inhibition of *Streptococcus mutans* ATCC 25175 with *B. pandurata* extract from 95% of ethanol extraction with dilution ratio at 1:7 and several of immerging time by Broth Microdilution Method was showed on Table 6. The objective of this experiment was to know the optimal *B. pandurata* immerging time in ethanol. The amount of *Streptococcus mutans* ATCC 25175 was shocked in 95% of ethanol at various immerging time. The inhibition of *Streptococcus mutans* ATCC 25175 was started on the 1 day of immerging time and the amount of *Streptococcus mutans* ATCC 25175 reduce from 10^7 to

Table 6: Inhibition of *Streptococcus mutans* ATCC 25175 with *B. pandurata* extract from 95% of ethanol extraction with dilution ratio at 1:7 and several of immerging time by broth microdilution method

	Amount of *Streptococcus mutans* ATCC 25175 (Log CFU mL^{-1})		
Immerging time (day)	No.1	No.2	Average (Mean±SD;n = 2)
No extraction (control)	7.18	7.14	7.16±0.03
1	2.59	2.64	2.62±0.03
3	2.67	2.76	2.72±0.05
5	2.97	2.86	2.92±0.05
7	2.00	2.32	2.16±0.16

Table 7: Inhibition of *Streptococcus mutans* ATCC 25175 with *B. pandurata* hydrodistillation extract by disc diffusion method

		MIC diameter (cm.)			
B. pandurata hydrodistillation product		Dilution ratio (extraction: ionized water)			
Hydrodistillation temperature (°C)	Product	Crude	1:1	1:3	1:5
30	Oil	NA	NA	NA	NA
	Distillate	NA	NA	NA	NA
	Bottom	ND	ND	ND	ND
60	Oil	NA	NA	NA	NA
	Distillate	NA	NA	NA	NA
	Bottom	1.10	1.00	1.11	0.90
90	Oil	1.26	ND	ND	ND
	Distillate	0.90	0.80	0.70	0.7
	Bottom	ND	ND	ND	ND

NA: It cannot extract by hydrodistillation, ND: It cannot detect the amount of bacteria

10^2 CFU mL^{-1}. The amount of *Streptococcus mutans* ATCC 25175 at 7 day around 10^2 CFU mL^{-1} was highest efficiency of inhibition of *Streptococcus mutans* ATCC 25175. According to Table 5 and Table 6, the amount of *Streptococcus mutans* ATCC 25175 inhibited by the extract from solvent extraction at 95% of ethanol and dilution ratio at 1:7 (v/v). The increasing of immerging time can produced the high efficacy of inhibition in extraction. The best immerging day was 7 days. The extraction at that condition can reduce the amount of *Streptococcus mutans* ATCC 25175 from 10^7 to 10^2 CFU mL^{-1}. Finally, it concluded that the optimal condition of solvent extraction was 95% of ethanol, immerging time at 7 days and dilution ratio at 1:7 (v/v).

The previous study on the antimicrobial activities of ten herbal extracts (Dalirsani *et al*. 2011) was found that rosemary extract had inhibited on *Streptococcus mutans*. The inhibitory clear zone on Disc diffusion method of rosemary extraction was similar to *B. pandurata* extract by solvent extraction process.

Optimal condition of *Boesenbergia pandurata* extract by hydrodistillation: The hydrodistillation product generally received oil, distillate and bottom produce or residue. Three products were analyzed on inhabitation of *Streptococcus mutans* ATCC 25175 by disc diffusion method. All of laboratory testing results was explained on Table 7. The hydrodistillation temperature related to the occurrence of hydrodistillation products. The hydrodistillation temperature at 30 and 60°C cannot produce in oil and distillate of *B. pandurata* hydrodistillation products. The *B. pandurata* hydrodistillation bottom product of 30 and 90°C cannot inhibited the

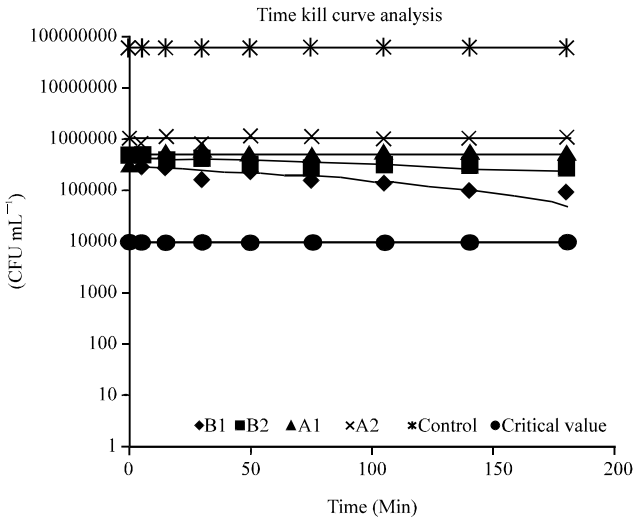

Fig. 4: Time kill curve analysis of tooth care product

Streptococcus mutans ATCC 25175 activity. It was different to 60°C. The MIC of *B. pandurata* bottom product at 60°C was 1.10 cm, but the MIC of *B. pandurata* distillate product at 90°C was around 0.9 to 0.7 cm. The hydrodistillation temperatures of *B. pandurata* at 90°C easily produced oil and distillate. It differed on 30 and 60°C that cannot receive those products. The bottom product of 30 and 90°C cannot inhibit the growth of *Streptococcus mutans* ATCC 25175 but it was different at 60°C. The oil and distillate only receive from the hydrodistillation temperatures at 90°C and they can inhibit the growth of *Streptococcus mutans* ATCC 25175. The results of this experiments concluded that the optimal extraction temperature for *B. pandurata* hydrodistillation was 902°C.

Time kill analysis: Time kill analysis related with time factor that impacted on the inhibition of *Streptococcus mutans* ATCC 25175 in developed tooth care products. This section concerned on the efficiency of inhibition of *Streptococcus mutans* ATCC 25175 in mouthwash and powder toothpaste. The results were shown in Fig. 4. The inhibition of powder toothpaste A1 and A2 product can inhibit of *Streptococcus mutans* ATCC 25175. The efficiency of inhibition of A1 product was less than A2. The amount of bacteria of A1 product decreased from 10^7 to 10^5 CFU mL^{-1} while A2 product reduced from 10^7 to 10^6 CFU mL^{-1}. However, both of the inhibition of mouthwash B1 and B2 product declined from 10^7 to 10^5 CFU mL^{-1}. The comparison between developed toothpaste and mouthwash product indicated that the inhibition of *Streptococcus mutans* ATCC 25175 of mouthwash product *Streptococcus mutans* ATCC 25175 was better than the powder toothpaste product. The product mixing with *B. pandurata* extract by solvent extraction process was more efficacy inhibition than hydrodistillation process because of hydrophobia in oil product.

CONCLUSION

From these findings, they were found to be feasible to apply *B. pandurata* extract as disinfectant in tooth care product. The following conclusions can be employed to explain the inhibition of *S. mutans* by *B. pandurata* extract in both powder toothpaste and mouthwash products:

- The optimal solvent extraction process of *B. pandurata* was 95% of ethanol extraction solvent, 7 days of immerging time and maximum dilution as disinfectant at 1:7 of extract per water (v/v). *B. pandurata* extract by solvent extraction can be inhibited *S. mutans* from 10^7 to 10^2 CFU mL^{-1}
- The optimal hydrodistillation process of *B. pandurata* was 90°C of extraction temperature. This condition gave both *B. pandurata* oil and distillate product. Both products can inhibit *S. mutans*
- The mouth wash mixed with *B. pandurata* extract inhibited *S. mutans* better than powder toothpaste

In summary, the *B. pandurata* extract had a good potential of being used as a disinfectant. It can be application for development of tooth care product as toothpaste and mouthwash products.

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