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Original Article

ANTIMICROBIAL ACTIVITY OF STANDARDIZED PIPER BETEL EXTRACT AND ITS MOUTHWASH PREPARATION

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ABSTRACT

Objective: Piper betel plant (Piper betel Linn.) is a native plant of South East Asia. It is empiritically long known for medication of dental caries and bad breath. The aim of this study wasto evaluate astandardized ethanol extract frompiper betelfor its antimicrobial activity toward *Streptococcus mutans* and several other microbes.

Methods: 150 g fresh leaves were steamly destilated resulting 15 g betel pipel oil and aqueous phase. The mixture, namely actifold 30 and 60, was characterized on its hydroxychavicolusing high performace liquid chromatography followed by formulation of mouthwash. Antimicrobial activities of Actifold 30 and 60 was then screened on*Pseudomonas aeruginosa, Eschericia coli, Staphylococcus aureus, Bacilus cereus, Candida albicans, Aspergilus niger, Staphylococcus cerevisae and Streptococcus mutan*.The piper betel oil was further analyzed using gas chromatography.

Results: The extract had a wide range of inhibitionswith low MIC against *P.aeruginosa, E.coli, S.aureus, B.cereus, C.albicans, A.niger, and S.cereviceae.*High purity active compound in Actifold, hydroxychavicoloil, gave significantly lower MIC value compared toother active ingredients in a commercial brand mouthwash. Mouthwash formulated with Actifold containing the same ethanol amount as the commercial brand mouthwash gave half the MBC value. Mouthwash formulated with hydroxychavicol without ethanol addition gave the same MBC value as the commercial brand mouthwash.The standardized piper betel extract Actifold 30x shows good potential in mouthwash formulation using a concentration 2.5 times its MBC value. It is also proven effective in low alcohol mouthwash formula.

Conclusion: Actifold 30x showed best inhibition towards the mold *A.niger*and showed fair inhibition towards yeast and bacteria tested.

Keywords: Betelpiper, Mouthwash, Hydroxichavicol, Antimicrobial activity.

INTRODUCTION

Piper betel plant (*PiperbetelLinn*.) is a native plant of South East Asia. In these countries, the plant has long been associated withmedication for dental caries and bad breath[1]. Also commonly used as masticatory, piper betel leaves are chewed with betel nut and lime for a mild stimulant effect.

Leaves of piper betel plant contain several active compounds such as eugenol and its isomers[2], chavibetol, hydroxychavicol [3],pentatriacontanol, piperol, piperbetol[4], carotenes, and ascorbic acid [5]. The compound hydroxychavicol has been examinedas an antimicrobial ingredient, and it shows promising for several applications. The possibility of using hydroxychavicol was evaluated from piper betel as an oral care agent and found that its antimicrobial profiles are well suited for an active ingredient for oral care products [6].

This research focuses on the efficacy of piper betel extract standardized in its hydroxychavicol content forantimicrobial activities toward *Streptococcus mutans*. The bacteria *Streptococcus mutans* is capable of synthesizing insoluble glucan that can very aggressively forms plaque and colonize the tooth surface[7]. Expansion of water-insoluble glucanformed by the reaction between sucrose and glucocyl transferase produced by the bacteria, and acidic condition caused by that reaction will ultimately result indetrimental tooth decay; this makes *Streptococcus mutans* the most strongly associated bacteria with dental carries[8].

Dental plaque formation can be reduced by good oral hygiene practices. Several practices include daily brushing, flossing, and mouthwash. Application of mouth rinse after tooth brushing can control the number of oral bacteria in the mouth by penetrating plaque biofilm [9]. In this experiment, a mouthwash preparation contains active ingredients namely Actifold 30x consist ofhydroxychavicol 0.3%, whereas actifold 60x consists hydroxychavicol 0.6%. The aim of this study was to evaluate antimicrobial activity of a standardized ethanol extract frompiper betelfor against several microbes.

MATERIALS AND METHODS

Materials

Menthol powder 99.0% and thymol powder 99.5% were obtained from Sigma-Aldrich(Saint Louis, MO). Liquid methyl salicylate 99.5% andliquid eucalyptol 99.0% were also purchased from Sigma-Aldrich(Saint Louis, MO). Eugenol oil 98.0% from a proprietary source was used as eugenol standard for GC and HPLC identifications. Hydroxychavicol standard 98.0% for HPLC identification was obtained from Biobiopha Co. Ltd. The commercial brand mouthwash was purchased froma local supermarket.Piper betelextracts were obtained from PT Haldin Pacific Semesta.

Isolation and analysis of hydroxychavicol oil

150 gfresh piper betel leaves was purchased from a local market. Extraction and isolation of high purity hydroxychavicol was done to 15 g of the leaves according to the method of Sharma *et al.* (2009).Piper betel oil wasobtainedby steam distillation of fresh leaves.Further, actifold 30x and actifold 60x were prepared. Quantification of hydroxychavicol in the oil and the ethanol extract was carried outusing reverse-phase HPLC at 30°C, Atlantis® dC18 (5-µm pore size, 150- by 4.6-mm internal diameter) column and UV detection at 280nm. Sample was eluted with 1% acetic acid in water:acetonitrile (60:40) at a flow rate of 1 mL/min for 30 minutes.

Preparation of mouthwash formula

Three formulations of mouthwashes were freshly prepared: mouthwash A and B both contain 1% Actifold 30x, while mouthwash C contains 4 active ingredients at the same amounts as in the commercial brand mouthwash formula. Mouthwash A and B differ only in the amount of ethanol used in the formula. The formulas were as followed (Table 1)

Table 1: Composition of formulas tested on its microbial activity

Ingredients	Composition (in %) of mouthwash formula		
	A	B	C
Thymol	-	-	0.064
Eucalyptol	-	-	0.092
Methyl salicylate	-	-	0.06
Menthol	-	-	0.042
Actifold 30x	1	1	-
Sodium benzoate	0.1	0.1	0.1
Benzoic acid	0.1	0.1	0.1
Sorbitol	4	4	4
Surfactant	0.1	0.1	0.1
Ethanol	20.6	-	21.6
Water	74.1	95.7	73.942

Microbial strains and inoculums preparation

Bacterial strains stock cultures were kept at 4°C on nutrient agar medium. The microorganisms used in this study were clinical isolates of *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus cereus, Candida albicans, Aspergillus niger, Saccharomyces cereviceae,* and *Streptococcus mutans*. Active cultures were prepared by inoculating fresh nutrient broth medium with a loopfull of cells from the stock cultures at 37°C for overnight.

Determination of MIC and MBC

Actifold 30x,actifold 60x, thymol, eucalyptol, menthol, methyl salicylate, and hydroxychavicolwere tested to determine Minimal Inhibitory Concentration (MIC) value toward*Streptococcus mutans*. Minimal Bactericidal Concentration (MBC) against *S. mutans*was also determined for all mouthwash formulas. Fresh grown bacteria (10^6cells/mL) at 100 µL volume in nutrient broth was inoculated in tubes with nutrient broth supplemented with different concentrations (10-500 µL) from the stock extract (1 mg/mL), and incubated for 24 h at 37°C. Turbidity appeared denoted presence of microorganism in the test tube after the period of incubation whereas the absence of turbidity indicates complete inhibition of microbial growth. The test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC. For MBC determination, broth (100µL) from the wells showing no visible growth was spread on a Trypticase soy agar plate. The minimum concentration that showed≥99.9% reduction of the originalpopulation was considered the MBC.All experiments were carried out in triplicate. Data points were represented by the mean of the measured values. Statistical analysis was carried out using MS-Excel software. Further, antimicrobial activity of standardized piper betel extract Actifold 30x and Actifold 60x against several microbes were performed using the same method.

RESULTS AND DISCUSSION

Analysis of hydroxychavicolshowed a linear response and provided a good calibration curve (y = 21188x – 5672.1,r =0.999) in a concentration range of 50 to 500 µg/mL, (Figure 1). Calculation of hydroxychavicol concentrations in Actifold 30x and 60x gave consistent levels of 0.3 dan 0.6%, respectively.

HPLC analysis and calculation confirmed that the ethanol extract Actifold 30x contains established hydroxychavicol concentrations of 0.3%. Aqueous extract of piper betel obtained by distillation and vacuum drying was confirmed mainly contained hydroxychavicol among other compounds. Their dried crude extract contained 39.31% hydroxychavicol but it does not contain eugenol [10].

The presence of eugenol in Actifold (tR: 19.2 minutes) extracts indicates that Actifold30x and 60x may also include other chemical compounds that are not present in an aqueous extract. The absence of hydroxychavicol in piper betel oil also shows that Actifold extracts contain compounds that are not present in the oil.

Actifold 30x and 60x both showed best antimicrobial activity result against the mold *Aspergillusniger*, with MIC values of 8 and 80µg/mL, respectively. Antimicrobial activities of Actifold 30x and 60x were in the range of 800-8,000 µg/mL against all other six microbes. Actifold showed similar inhibitions toward gram positive (*S.aureus* and *B.cereus*) and gram negative (*P.aeruginosa* and *E.coli*) bacteria. For most of the activities, Actifold 60x gavean average of lower MIC values of one log cycleagainst bacteria as compared to Actifold 30x.However, there was no difference in MIC values of Actifold 30x and Actifold 60x toward the yeast *C.albicans*. Actifold 60x even gave one log cycle higher MIC values against *S.cereviceae* and *A.niger* compared to Actifold 30x (Table 2).

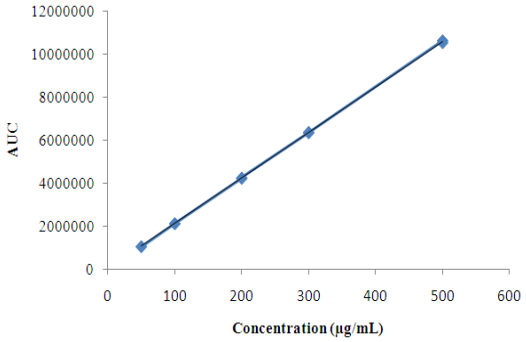

Fig. 1: Calibration curve of hydroxychavicol standard

Higher MIC values were obtained against*C.albicans* compared to *A.niger* and *S.cereviceae* with Actifold, which was different than obtained with betel oil [11]. Antimicrobial activity of hydroxychavicol present in Actifold may have contributed to the action toward *P.aeruginosa*. MIC values can be affected by many factors, among them are the type and strain of microorganisms tested. Actifold 60x with twice the hydroxychavicol concentration as Actifold 30x showed one log cycle better reduction against the gram positive and gram negative bacteria used in this study, but gave less or same inhibitions compared to Actifold 30x toward mold and yeasts. Actifold 30x and 60x both gave MIC number of 8000 µg/mL against *C.albicans*, this indicates that the extracts are not very effective for inhibition of the yeast, although the high value may be due to the strain of yeast applied. MIC values of 25 strains of *C.albicans* against pure hydroxychavicol were measured and reported values in the ranges of 125 – 500 µg/mL [12]. Purified hydroxychavicol in that study gave MIC values of 125-250 µg/mL against 7 different strains of *A.niger*, higher than the values obtained with Actifold in this study. Other phenolic compounds present in Actifold could contribute to its antimicrobial activity, so that Actifold is more effective in inhibition of this mold even though it has smaller concentration of hydroxychavicol. The discrepancy in these results may also be due to the differences of piper betel composition in India and Indonesia. Regions and maturity levels of piper betel leaves have been shown to affect the composition of piper betel oil of Srilanka [13]. There is also the possibility that in the case of yeast and molds in this study, the ethanol solvent of Actifold is a better inhibitory agent for the microbes than the active compounds of piper betel. Thus, increasing active compounds and decreasing ethanol concentration by intensifying Actifold 30x to Actifold 60x actually reduced the ability of the extract to inhibit the mold and yeasts. The commercialbrand mouthwash contains a total of 0.26% of four active plant extracts: eucalyptol, menthol, thymol, and methyl salicylate. Eucalyptol, menthol, and methyl salicylate exhibited the same MIC value against *S.mutans*, at 1 mg/mL. Thymol had significantly lower MIC value against *S.mutans*compared to the other 3 active ingredients in commercial brand mouthwash (Table 3). The hydroxychavicol oil has very low MIC value towards *S.mutans*, which indicates the inhibition efficacy of this compound against the bacteria. Actifold 30x has three times the MIC value compared to thethree active ingredients in the commercial brand mouthwash. Sharma et al. (2009) reported an MIC value of 250 µg/mL of their purified hydroxychavicol against *S.mutans*, this is ten times higher than the MIC value we obtained from hydroxychavicol in this study. Actifold 30x contains more than 300 times less hydroxychavicol than the almost pure compound (0.3% versus 98.0%). However, the MIC value of Actifold30x was only 120 times less than the high purity hydroxychavicol.

Table 2: Antimicrobial activity of standardized piper betel extract Actifold 60x and Actifold 30x against several microbes.

Microbes	Code	Control (-)[b]	Control (+)[c]	Microbial growth on dilution of sample[d]										MIC
				10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10	
P.aeruginosa	D	-	+	+	+	+	+	+	+	-	-	-	-	800
	E	-	+	+	+	+	+	+	+	+	-	-	-	8000
E.coli	D	-	+	+	+	+	+	+	+	-	-	-	-	800
	E	-	+	+	+	+	+	+	+	+	-	-	-	8000
S. aureus	D	-	+	+	+	+	+	+	+	-	-	-	-	800
	E	-	+	+	+	+	+	+	+	+	-	-	-	8000
B.cereus	D	-	+	+	+	+	+	+	+	-	-	-	-	800
	E	-	+	+	+	+	+	+	+	+	-	-	-	800
C.albicans	D	-	+	+	+	+	+	+	+	+	-	-	-	8000
	E	-	+	+	+	+	+	+	+	+	-	-	-	8000
A.niger	D	-	+	+	+	+	+	+	-	-	-	-	-	80
	E	-	+	+	+	+	+	-	-	-	-	-	-	8
S.cereviceae	D	-	+	+	+	+	+	+	+	+	-	-	-	8000
	E	-	+	+	+	+	+	+	+	-	-	-	-	800

D=actifold 60x, E=actifold 30x, [b] media MHB, [c] media MHB + inocula, [d] + = microbial growth; - = no microbial growth

Table 3: Antimicrobial activities of active ingredients in mouthwash preparation against *Streptococcus mutans*

Active ingredients	Amount (%)	MIC (µg/mL)
Eucalyptol	99	1000
Menthol	99	1000
Thymol	99.5	250
Methyl salicylate	99.5	1000
Hydroxychavicol	98.0	25
Actifold 30x	0.3	3000

The hydroxychavicol oil used in this study with 98.5% purity gave very low MIC value, 25 µg/mL (Table 3). This is a much lower value than reported by Sharma et al. (2009), their hydroxychavicol had MIC values of 250-500 µg/mL against 25 strains of *S.mutans*. Al-Bayati (2009) investigated the antimicrobial activity of menthol oil and found MIC value of 15.6 µg/mL against *S.mutans* [14]. The menthol powder used in this study was of 99% purity, but it gave about 100x higher MIC value of that menthol oil. Bacterial growth can be prevented to a great extent in oil due to zero water activity. Thymol has the highest antimicrobial activity toward *S.mutans* compared to the other 3 active ingredients of the commercial brand mouthwash, but it still gave higher MIC value than pure hydroxychavicol. This may be due to the oil form of hydroxychavicol, bacteria cannot survive in fat or oil since it needs water for its growth and reproduction. The MBC value of Actifold 30x was approximately 1.3 times higher than its MIC value toward *S.mutans*. The MBC to MIC ratio of less than 4 is regarded as an indication that the antimicrobial has good bactericidal activity [15]. In the case of Actifold30x, it has the effective bactericidal action for the gram positive *S.mutans*. Bacterial killing potency can be time dependent or dose dependent, with an antimicrobial agent having effect either from increasing exposure time or increasing concentration.

Rapid elimination of a bacterial pathogen should also reduce the chance of the emergence from resistance bacteria [16]. Commercial brand mouthwash reformulation used the same composition of its four active ingredients (menthol, eucalyptol, methyl salicylate, thymol) as it is written on thelabel. The MIC value of the commercial brand mouthwash and Formula C were similar. Total concentration of active ingredients in the commercial brand mouthwash was 0.26%, in comparison to 0.30% of hydroxychavicol in the mouthwashes using Actifold 30x (formula A and B). All four ingredients used in the reformulation were all high purity pharmaceutical grade components and the same alcohol content (21.6%) in the commercial brand mouthwash. The pH values of thecommercial brand mouthwash and Formula Cwere different. The commercial brand mouthwash used in this study had a pH of 3.6 – 3.8, whereas Actifold formulations with and without alcohol addition had pH values in the range of 4.1 – 4.6. Formulation of mouthwash with 1% Actifold 30x resulted in lower MBC value as compared to the commercial brand mouthwash with the same alcohol concentration (Table 4). Mouthwash containing about 1% alcohol (formula B) gave the same MBC value as the commercial brand mouthwash, but twice the MBC value of mouthwash containing 21.6% alcohol.

Table 4: Antimicrobial activity of mouthwash preparation against *Streptococcus mutans*

Mouthwash formula	MBC (µg/mL)
Commercial brand	4000
A	2000
B	4000
C	4000
Extract actifold30x	4000

The MBC value of mouthwash formula B with 1% Actifold was the same as the 100% Actifold. Dried ethanol extract of piper betel leaves was tested against four bacteria and showed strong antimicrobial activities [17] but dilution of the dried extract resulted in smaller inhibition zones for all four bacteria.

There are other ingredients that are includedin the mouthwash formula that has antimicrobial effect;for instance benzoic acidas preservative. Common food preservatives such as benzoates and sorbates had been shown to have antibacterial and plaque inhibiting properties [18].

The effect of pH on the effectiveness of essential oil as antimicrobial agent and oil concentration below MIC value can be used to retard microbial growth with reduced pH as low as 5.5 [17].In mouthwash formulation, inclusion of benzoic acid can accommodate the pH

decrease.The commercial brand mouthwash used in this study hadsignificantly pH of 3.6 – 3.8 whereas the Formula Cmouthwash had a higher pH of 4.9, possibly due to different composition of the ingredients. Ethanol is another ingredient in mouthwash that has its own antibacterial effect.This particular property of the solvent has prompt its use in mouthwash formula[19].Alcohol-free mouthwash has been tested against plaque accumulation with ethanol containing mouthwash showed better plaque inhibition [20]. However, high ethanol concentration in mouthwash itself has been discouraged due to its detrimental effects [20,21]. Actifold mouthwash formulas used 1% of Actifold 30x contains ethanol. The amount of active ingredient in a 1% Actifold 30x was 0.3%of hydroxychavicol, slightly higher than the combination amount of the four active ingredients of the commercial brand mouthwash (0.26%). The MBC value of mouthwash formula Athat is half of the MBC value of the commercial brand mouthwash containing the sameethanol concentration. It is possibly caused by the presence of other active compounds in Actifold 30x that may also have antimicrobial properties, such as eugenol. These compound was proved to be able provide additional antimicrobial activity against *S.mutans*. Actifold mouthwash formulated with higher ethanol content (21.6%) gave smaller MBC compared to mouthwash with only 1% ethanol content (Table 4), which indicates its higher effectiveness.

CONCLUSION

Actifold 30x and 60x can inhibit the growth of tested microorganisms: Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus cereus, Candida albicans, Aspergillus niger, Saccharomyces cereviceae, and Streptococcus mutans. Actifold 30X showed the best inhibition towards the mold A.nigerand showed fair inhibition towards yeast and bacteria tested. The standardized piper betelextract Actifold 30xshows good potential in mouthwash formulation using a concentration 2.5 times of its MBC value. There may great possibilities for use of Actifold extracts inother personal care product formulationsas a natural antimicrobial agent.

CONFLICT OF INTERESTS

Declared None

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