P_28

In Vitro Antibacterial Activity of Selected Herbal Extracts on *Streptococcus mutans*

Jitrada Wannachot [1,2], Sakulrat Rattanakiat[1*]

Abstract

Introduction: Dental caries is an infectious disease associated with numerous microorganisms, mainly *Streptococcus mutans*. Since consumers mostly have a concern of trustworthy and least harmful products for health care, health industries, including dental care business, have contributed huge effort on production of natural substances. The aim of this study was to investigate the inhibitory effect against *S. mutans in vitro* of the 95% ethanol extracts from five herbs, *Psidium guajava* L., *Momordica cochinch inensis* Spreng.,*Glycyrrhiza glabra* L., *Syzygium aromaticum* L. and *Piper retrofractum* Vahl. **Methods:** Antimicrobial activity was evaluated using disc diffusion and broth dilution method. The combination inhibitory effects of extracts against *S. mutans* were also determined using checkerboard assay. **Results:** Some extracts showed activity against *S. mutans*. The extract of *S. aromaticum showed* the largest mean diameter of inhibition zone (16.7±0.5 mm) while the extract of *G. glabra* was the most effective with the lowest minimum inhibitory concentration (MIC) of 0.195 mg/mL and minimum bactericidal concentration (MBC) of 3.125 mg/mL. A combined extract of *S. aromaticum* and *P. guajava* showed a synergistic effect in inhibiting the growth of *S. mutans*. **Conclusion:** The results showed that these herbal extracts provide the potential candidate for developing an oral antimicrobial agent to control or prevent dental diseases associated with oral pathogenic bacteria like *S. mutans*.

Keywords: Dental caries, Dental plaque, *Streptococcus mutans*, Synergistic effect, Herbal extract

[1] Pharmaceutical Chemistry and Natural Products Research Unit, Faculty of Pharmacy, Mahasarakham University, Kantarawichai, Maha Sarakham, Thailand.

[2] Kutchum Hospital, Kutchum, Yasothon, Thailand.

* **Corresponding author**: Pharmaceutical Chemistry and Natural Products Research Unit, Faculty of Pharmacy, Mahasarakham University, Kantarawichai, Maha Sarakham, Thailand. 44150. Tel. +66 43754360. E-mail: nuut_16@hotmail.com

1. Introduction

Dental caries is the most common oral infectious disease in the world that results in localized dissolution and destruction of the calcified tissues of the teeth (Loesche, 1986). *Streptococcus mutans* is commonly known as the causative bacteria in the formation of dental plaque and dental caries. *S. mutans* is able to synthesize water-insoluble glucans that mediate adhesion to and colonization of teeth. Moreover, *S. mutans* ferments carbohydrates such as sucrose, fructose, and glucose into acid, particularly lactic acid which causes teeth damage by dissolving tooth structures (Jordan and Leblanc, 2002). Therefore, the use of antimicrobial agents to control these cariogenic bacteria is the one of the strategies for caries prevention. Previous study reported that natural products might be good alternative for caries prevention (Badria and Zidan, 2004). Although, many Thai herbs have been reported to have antimicrobial activity but lack of report about activity against *S. mutans.* In this study, five plants were selected and evaluated for activity against *S. mutans,* which four of them including *Psidium guajava* L., *Glycyrrhiza glabra* L., *Syzygium aromaticum* L. and *Piper retrofractum* Vahl. had been used as Thai folk medicine for dental care formula at Kudchum Hospital. In addition, *Momordica cochinchinensis* Spreng. was also selected due to potential to have antimicrobial activity (Nantachit and Tuchinda, 2009), In Thailand, Clove oil from *S. aromaticum*, which belongs to family Myrtaceae has been traditionally used for dental health because of its antibacterial, anti-inflammatory, and analgesic effects (Aneja and Joshi, 2010). Guava leaf (*P. guajava*), which belongs to family Myrtaceae has been used in folklore practices to maintain the oral hygiene (Razak and Rahim, 2003). Gac fruit (*M. cochinchinensis*) belongs to family Cucurbitaceae and is indigenous to Southeast Asia. Its seed membrane and oil are rich sources of carotenoids (lycopene and beta-carotene) (Kubola and Siriamornpun, 2011; Vuong *et al*., 2006). *P. retrofractum* belongs to the Piperaceae family. The fruits of *P. retrofractum* have used for their anti-flatulent, expectorant, antitussive and counter-irritant properties in traditional medicine (Tewtrakul *et al*., 2000). Licorice (*G. glabra*) is a perennial herb which possesses sweet taste. The most common medical use for treating upper respiratory ailments including coughs, sore throat and bronchitis (Anderson and Smith, 1961). The objective of this study was to investigate the inhibitory effect of the crude extracts from some herbs on *S. mutans in vitro*.

2. Materials and Methods

2.1 Bacterial strain and growth condition

S. mutans DMST 18777 used in this study was obtained from Department of Medical Sciences Thailand Culture Collection. The culture was grown in brain heart infusion (BHI) broth at 37 °C in the presence of 5% CO_2.

2.2 Plant materials and preparation of herbal extracts

Fresh leaves of guava (*Psidium guajava* L.), and fruits of gac (*Momordica cochinchinensis* Spreng.) were collected from Yasothon Province, Thailand. Dry rhizomes of *Glycyrrhiza glabra* L. were purchased from a herb shop in Bangkok,

Thailand. Dry buds of *Syzygium aromaticum* L. and dry fruits of *Piper retrofractum* Vahl. were purchased from a herb shop in Ubonratchathani, Thailand. Leaves of guava and fruits of gac were collected during June 2013. The plants were identified by researchers and the herbaria were made and deposited at Faculty of Pharmacy, Mahasarakham University for future reference. The aril, skin plus yellow pulp (mesocarp) and seeds were separated from the ripe fruit of *M. cochinchinensis,* due to this fruit has not reported antibacterial activity against *S. mutans,* although there were some reports about biological activities of each part. Then they were separately extracted. The plant material were dried using hot air oven at 55ºC for 72 hours. All dried herbs were grounded to coarse powders. To obtain crude extracts, pulverized herbs were macerated in 95% ethanol (1:10 w/v) for 7 days. The macerates were then filtered and dried using a rotary evaporator. The obtained crude extracts were stored at -20°C until further use.

2.3 Screening for antimicrobial activity [Disc diffusion technique]

Antimicrobial activity of extracts was determined by agar disc diffusion method. Broth culture of bacterial strain was adjusted to the density of McFarland No 0.5. Then, the suspension was spread on the BHI plates. The 6 mm diameter filter discs loaded with 15 mg of extract were placed on the plates which were then incubated at 37°C overnight. Zones of inhibition of growth were measured in millimeters. Standard disc of 0.2% chlorhexidine was used as a positive control, while disc of 95%ethanol and distilled water were used as negative controls. All the tests were done in triplicate and the mean values of the diameter of inhibition zone with ± standard deviation were determined. Extracts presented with obvious clear zone were selected to determine MIC and MBC.

2.4 Determination of minimum inhibitory concentration (MIC)

The MIC was performed according to the standard reference method (Clinical and Laboratory Standards Institute, 2012). The extract dilutions were made in several two-fold decreasing concentrations with BHI broth containing 5% dimethyl sulfoxide (DMSO) from 12.5 mg/mL to 0.097 mg/mL. One milliliter of standard inoculum of the bacterial strain matching the no.0.5 Mc Farland was seeded into 1-ml dilution. All test tubes were incubated at 37 °C in the presence of 5% CO_2 for 24 hours and observed for turbidity indicating bacterial growth. MIC was determined as the highest dilution (that is, the lowest concentration) of the extract that showed no visible growth. Two control tubes, positive and negative control, were also performed. Tube with no bacteria cells represented the positive control while the one without the extracts represented the negative control for the test.

2.5 Determination of Minimum Bactericidal Concentration (MBC)

The MBC were determined by the drop plate method from the selecting tubes that showed no growth during MIC determination. Ten microliters of culture was subcultured onto BHI agar and incubated for further at 37° C in the presence of 5% CO_2 for 24 hours. The least concentration, at which no growth was observed, was noted as the MBC.

2.6 Synergy test

Synergistic effect was primarily screened using double disc diffusion method. Place discs separated by a distance equal to the sum of the zone radii for each disc tested separately. After incubation observe the interface of the zones of inhibition. Theoretically, antagonism shows a truncated zone, indifference shows no change, and synergism shows an enhanced zone. The couple of extract that showed synergistic effect was further evaluated using Checkerboard macro-dilution assay. Equal concentrations of each of extract were combined in test tubes at concentra tions of 4×MIC, 2xMIC, MIC, 0.5xMIC, 0.25xMIC, 0.125×MIC, 0.0625xMIC and 0.0312xMIC. The MIC was determined after incubation overnight at 37°C in the presence of 5% CO_2. A fraction inhibitory concentration index (FICI) was used to interpret the synergistic effect. The FIC index (FICI) was calculated using the following formula:

$$\text{FIC index}=\text{FICA}+\text{FICB}=\frac{[A]}{\text{MICA}}+\frac{[B]}{\text{MICB}},$$

where [A] is the concentration of drug A and MICA and FICA are the MIC and the FIC of drug A for the organism, respectively, whereas [B], MICB, and FICB are similarly defined for drug B. The FIC index obtained was interpreted as follows: <0.5 denotes synergy; 0.5–1 denotes an additive effect; 1–2 denotes indifference; and >2 denotes antagonism (Moody, 2004).

3. Results

Table 1 showed antibacterial activity of extracts which was screened by disc diffusion technique. The largest inhibition zone 16.7±0.5 mm in diameter was observed in *S. aromaticum* extract. The extract of *P. retrofractum* produced a small inhibition zone. Nevertheless, inhibition zone of extracts of aril, skin and mesocrap of *M. cochinchinensis* were not observed. Therefore, the extracts of *P. guajava* leaf, seed of *M. cochinchinensis*, *G. glabra* and *S. aromaticums* were selected to further determine of MIC and MBC using broth dilution method. The MIC and MBC values of herbal extracts were given in table 2 which ranged from 0.195 to >12.5 mg/ml for MIC and 3.12 to >12.5 mg/ml for MBC. The ethanolic extract of *G.glabra* showed the lowest MIC and MBC values.

Table 1 Antibacterial activity of herbal extracts against *Streptococcus mutans*.

Herbal extracts or chemical agent	Zone of Inhibition (mean ±SD; mm)
P. guajava (leaves)	12.0±1.0
S. aromaticum (flower buds)	16.7±0.5
G. glabra (rhizomes)	16.3±0.5
P. retrofractum (fruits)	6.7±0.5
M. cochinchinensis (fruits)	
- seeds	11.0±0.0
- aril	No zone
- skin and mesocarp	No zone
Chlorhexidine (0.06 μg/disc)	26.3±0.5

Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of herbal extracts

Herbal extracts or chemical agent	MIC (mg/mL)	MBC (mg/mL)
P. guajava	*1.56*	*>12.5*
S. aromaticum	1.56	*>12.5*
G. glabra	0.195	3.125
M. cochinchinensis (seeds)	6.25	*>12.5*
Chlorhexidine	< 0.00156	< 0.00156

Screening synergy test using double disc diffusion methods showed enhanced zone between zone of *P. guajava* and *S. aromaticum (figure 1).* The other couple of extracts showed no enhanced zones of inhibition. To confirm synergistic inhibitory effect against *S. mutans* of *P. guajava* and *S. aromaticum* extracts, checkerboard macrodilution assay was further performed. The results of the checkerboard assay are summarized in Tables 3. A combined extract of *S. aromaticum* and *P. guajava* showed a synergistic effect with the lowest FIC index of 0.25.

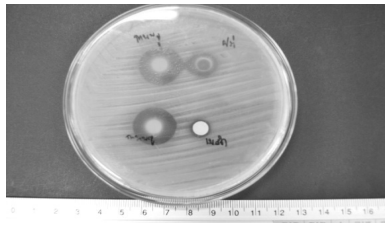

Figure 1 Inhibition zones around the disc with *S. aromaticum extract (left) and P. guajava* extract (right) against *S. mutans*.

Table 3 Fractional inhibitory concentration (FIC) and FIC index (FICI) of combination between *S. aromaticum and P. guajava* extracts.

***S. aromaticum* extract**		***P. guajava* extract**		**FICI (FICA+FICB)**	**Interpretation**
Conc. (mg/mL)	**FICA**	**Conc. (mg/mL)**	**FICB**		
0.195	0.125	0.195	0.125	0.25	Synergistic effect

Conc: concentration; FICA: FIC of *S. aromaticum* extract; FICB: FIC of *P. guajava* extract; ADI: additive effect; IND: indifferent effect; MIC: minimum inhibitory concentration. The FICI was interpreted as follows: synergistic effect ($0 < FICI \leq 0.5$), additive effect ($0.5 < FICI \leq 1$), and indifferent effect ($1 < FICI \leq 2$).

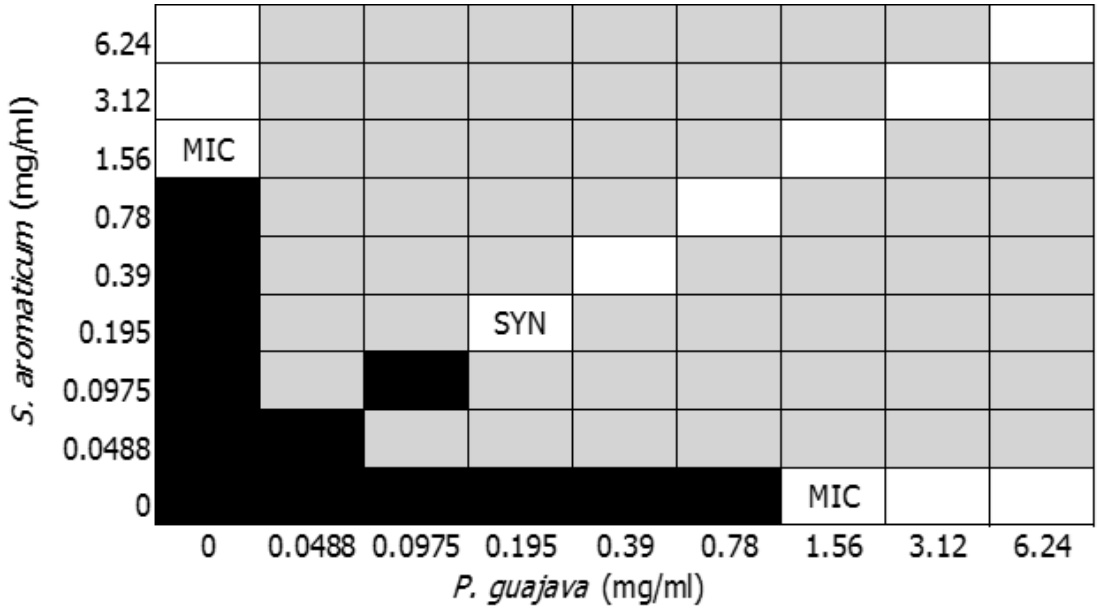

Figure 2 Combination interaction of *S. aromaticum* extract (A) and *P. guajava* extract (B) against *S. mutans* by checkerboard macrodilution method. As black zones indicate bacterial growth, grey zones indicate not determined and without color zones are free of bacteria. SYN: synergistic effect; MIC: minimum inhibitory concentration.

4. Discussion and Conclusion

It is clear that oral microorganism causes dental caries. Therefore, antimicrobial agents are needed to keep these pathogens at an optimal level which consistent with oral health. Many plants have been reported to inhibit growth of oral bacteria (Limsong *et al*., 2004). In this study, antimicrobial activity and the effect of combining selected herbal extracts were investigated. As the results, extract of liquorice *(G. glabra)* showed the highest activity with the lowest MIC and MBC values of 0.195 and 3.12 mg/ml, respectively. The extracts from clove (*S. aromaticum*) and guava (*P. guajava*) were the seconds with the equal values of MIC. *G. glabra* antimicrobial activity was in accordance with the previous report (Geetha and Anitha, 2012). The similar results with *G. glabra* and *S. aromaticum* extract on *S. mutans* showed moderate effective at concentration of 20 mg/mL (10-15 mm inhibition zone). Their extracts demonstrated MIC values less than 12.5 mg/mL, and the MBC values ranged from 25-50 mg/mL (Chaiya *et al*., 2013). Previous study found that *G. glabra* (licorice) can inhibit the growth of *S. mutan*s and *S. sanguis* (Geetha and Anitha, 2012). The main active ingredient of *G. glabra* is glycyrrhizin (Kumar and Dora, 2012). Glycyrrhizin in *G. glabra* showed dose-dependent inhibitory effect on glucosyltrans ferase activity of *S. mutans* (Sela *et al*., 1987). Clove oil from *S. aromaticum* has been identified as a potential antibacterial agent, as it contains the known main constituent and active compound, eugenol (Chaiya *et al*., 2013). A previous study reported that *S. aromaticum* can inhibit several types of oral pathogens such as *S. mutans, Porphyromonas gingivalis, Prevotella intermedia* and *Actinomyces viscosus* (Cai, 1996). Aqueous extracts of guava have shown *in vitro* antibacterial effect on the growth of plaque bacteria (Fathilah, 2009). They also exhibit antibacterial activity against *S. mitis*, *S. sanguinis* and *Actinobacillus* sp., which are well known as plaque bacteria. In addition, *P. guajava* was shown anti-adherence effect on the adhesion of early plaque settlers (Fathilah, 2011; Fathilah *et al*., 2009; Razak *et al*., 2006; Razak and Rahim, 2003). The MIC against *S. mutans* of aqueous extracts was 5 mg/ml which was equal to MBC, indicating bactericidal effect (Saraya *et al*., 2008).The active flavonoid compound, guaijaverin, demonstrated high potential antiplaque agent by inhibiting the growth of *S. mutans* and showed bacteriostatic activity (Prabu *et al*., 2006). Moreover, eugenol as well as guaijaverin can inhibit *S. mutans* caused carries by adherence suppression, reduction of water-insoluble glucan synthesis by glucosyltransferases (Prabu *et al*., 2006; Shuxu *et al*., 2013).

P. guajava and *S. aromaticum* extracts showed synergistic interaction with other extracts or antimicrobial drugs (Hemaiswarya and Doble, 2009). However, very few studies have been done to see synergistic effect of herbs on *S. mutans*. The previous study reported synergism between quercitrin and deoxynojirimycin was observed on *S. mutans*, with a FICI of 0.313. Their MIC values were found to be 64 mg/mL and 16 mg/ml, respectively (Hasan *et al*., 2014). We found that *P. guajava* extract in combination with *S. aromaticum (1:1 ratio)* recorded significant synergistic effect

with the lowest FIC index of 0.25 when combined 0.195 mg/ml of each extract. These data suggest that the combined extracts could be a useful option for the effective treatment of bacterial infection. The synergistic combination of two or more compounds are essential for treatments because of the following reasons: first, to prevent or decrease the emergence of resistant strains, second, to decrease dose-related toxicity, as a result dosage, and third, to attain a broad spectrum of activity (Eliopoulos, 1996). The mechanism of synergistic enhancing the antibacterial properties of each other was not evaluated. However, the membrane damaging effect of eugenol in *S. aromaticum extract* might be a mechanism of this synergistic effect (Hemaiswarya and Doble, 2009). Although MIC of both extracts were the same, the difference in the mechanism of actions of these compounds on *S. mutans* at the cellular level is likely to be.

In previous study, methanolic leave extract of *M. cochinchinensis* inhibited 4 pathogenic fungi tested, *T. rubrum, T. mentagrophytes, M. gypseum* and *E. floccosum* (Sutabhaha and Khantawa, 2011). However, *M. cochinchinensis* fruit has not reported antibacterial activity against *S. mutans*. *P. retrofractum* carry a variety of piperidine alkaloids, such as piperine, pipernonaline and dehydropipernonaline (Kim *et al*., 2011). Ethanolic extract of *P. retrofractum* fruits inhibit bacteria pathogens such as *Staphylococcus albus, Salmonella typhi, P. aeruginosa, E. coli, Bacillus megaterium* and *Aspergillus niger* (Khan and Siddiqui, 2007). Nevertheless, *P. retrofractum* extracts did not produced inhibition zone in present study. However, extract of *M. cochinchinensis* seed showed some antibacterial activity with MIC value of 6.25 mg/mL.

Since, *P. guajava* and *S. aromaticum* showed good activity with synergistic effect. In addition, *G. glabra* exhibited the lowest MIC and MBC. The results suggested that those three herbals have the potential to be developed into agents that can be used as preventative or treatment therapies for oral diseases. Further clinical studies of the safety and efficacy of these agents will be important to establish whether they offer therapeutic benefits, either alone or in combination with conventional therapies that can help to reduce the overall burden of oral diseases worldwide. Simultaneously, further works including the isolation of the bioactive phytochemical compounds, investigation of the mechanisms of actions and *in vivo* studies to validate the therapeutic potentials of these combinations may provide valuable data to obtain new therapeutic agents or strategies.

Acknowledgements

This project was funded by Faculty of Pharmacy, Mahasarakham University and Agricultural Research Development (Public Organization).

Refernces

Anderson DM, Smith WG. The antitussive activity of glycyrrhetinic acid and its derivatives. *J Pharm Pharmacol* 1961; 13: 396-404.

Aneja KR, Joshi R. Antimicrobial activity of *Syzygium aromaticum* and its bud oil against

dental caries causing microorganisms. *Ethnobot Leaflets* 2010; 14: 960-975.

Badria FA, Zidan OA. Natural products for dental caries prevention. *J Med Food* 2004; 7(3): 381-384.

Cai L. Compounds from *Syzygium aromaticum* possessing growth inhibitory activity against oral pathogens. *J Nat Prod* 1996; 59(10): 987-990.

Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility test. Approved standard M02-A11. 11th ed. CLSI, Wayne, PA. 2012, 32: 1.

Chaiya A, Saraya S, Chuakul W, *et al*. Screening for dental caries: preventive activities of medicinal plants against *Streptococcus mutans*. *Mu J pharm* 2013; 40(1): 9-17.

Eliopoulos GM, Moellering Jr. RC, editors. Antimicrobial combinations. In: Antibiotics in laboratory medicine. 4th ed. Baltimore (MD): William & Wilkins; 1996. 330-338.

Fathilah AR. *Piper betle* L. and *Psidium guajava* L. in oral health maintenance. *J Med Plants Res* 2011; 5(2): 156-163.

Fathilah AR, Rahim ZH, Othman Y. Bacteriostatic effect of *Piper betle* and *Psidium guajava* extracts on dental plaque bacteria. *Pak J Biol Sci* 2009; 12(6): 518-521.

Fathilah AR, Yusoff M, Rahim HA. The effect of *Psidium guajava* and *Piper betle* extracts on the morphology of dental plaque bacteria. *Pak J Med Sci* 2009; 25(6): 928-933.

Geetha RV, Anitha R. In vitro evaluation of antibacterial activity of ethanolic root extract of *Glycyrrhiza glabra* on oral microbes. *Int J Drug Dev & Res* 2012; 4(4): 161-165.

Hasan S, Singh K, Danisuddin M, *et al*. Inhibition of major virulence pathways of *Streptococcus mutans* by quercitrin and deoxynojirimycin: a synergistic approach of infection control. *PLoS ONE* 2014; 9(3): e91736.

Hemaiswarya S, Doble M. Synergistic interaction of eugenol with antibiotics against gram-negative bacteria. *Phytomedicine* 2009; 16: 997–1005.

Jordan C, Leblanc DJ. Influences of orthodontic appliances on oral populations of mutans streptococci. *Oral Microbiol Immun* 2002; 17(2): 65-71.

Khan M, Siddiqui M. Antimicrobial activity of piper fruits. *Nat Prod Radi* 2007; 6(2): 111-113.

Kim KJ, Lee MS, Jo K, *et al*. Piperidine alkaloids from *Piper retrofractum* Vahl. protect against high-fat diet-induced obesity by regulating lipid metabolism and activating AMP-activated protein kinase. *Biochem Biophys Res Commun* 2011; 411(1): 219-225.

Kubola J, Siriamornpun S. Phytochemicals and antioxidant activity of different fruit fractions (peel, pulp, aril and seed) of Thai *M. cochinchinensis* (*Momordica cochinchinensis* Spreng). *Food Chem* 2011; 127: 1138–1145.

Kumar A, Dora J. Review on *Glycyrrhiza glabra* (G. glabra). *J Pharm SciInnovat* 2012; 1: 1-4.

Limsong J, Benjavongkulchai E, Kuvatanasuchati J. Inhibitory effect of some herbal extracts on adherence of *Streptococcus mutans*. *J Ethnopharmacol* 2004; 92 (2-3): 281-289.

Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* 1986; 50(4): 353-380.

Moody J. Synergism testing: broth microdilution checkerboard and broth macrodilution methods. In: Isenber HD, editor. *Clinical microbiology procedures handbook*. 2nd edition. Volume 2. Washington DC: American Society for Microbiology Press; 2004.1-23.

Nantachit K, Tuchinda P." Antimicrobial activity of hexane and dichloromethane extracts from *Momordica cochinchinensis* (Lour.) Spreng leaves". *Thai Pharm Health Sci J* 2009; 4(1): 15-20.

Prabu GR, Gnanamani A, Sadulla S. Guaijaverin – a plant flavonoid as potential antiplaque agent against *Streptococcus mutans*. *J Appl Microbiol* 2006; 101: 487–495.

Razak FA, Othman RY, Rahim ZH. The effect of *Piper betle* and *Psidium guajava* extracts on the cell-surface hydrophobicity of selected early settlers of dental plaque. *J Oral Sci* 2006; 48(2): 71-75.

Razak FA, Rahim ZHA. The anti-adherence effect of *Piper betle* and *Psidium guajava* extracts on the adhesion of early settlers in dental plaque to saliva-coated glass surfaces. *J oral sci* 2003; 45(4): 201-206.

Saraya S, Kanta J, Sarisuta N, *et al*. Development of guava extract chewable tablets for anticariogenic activity against *Streptococcus mutans*. *Mu J Pharm* 2008; 35(1-4): 18-23.

Sela MN, Steinberg D, Segal R. Inhibition of the activity of glucosyltransferase from *Streptococcus mutans* by glycyrrhizin. *Oral Microbiol Immunol* 1987; 2: 125–128.

Shuxu J, Li Y, Xue C, *et al*. The effect of eugenol on the cariogenic properties of *Streptococcus mutans* and dental caries development in rats. *Exp Ther Med* 2013; 5: 1667-1670.

Sutabhaha B, Khantawa B. Antibacterial and antifungal activities Fak Khaao leaf extract. *Faculty of Associated Medical Sciences* 2011; 44(1): 31-37.

Tewtrakul S, Hase K, Kadota S, *et al*. Fruit oil composition of *Piper chaba* Hunt. P. longum L. *J Essent* 2000; 2: 603-608.

Vuong LT, Franke AA, Custer LJ, *et al*. *Momordica cochinchinensis* Spreng (*M.cochinchinensis*) fruit carotenoids reevaluated. *J Food Compos Anal* 2006; 19: 664–668.