Original Article

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Screening for Dental Caries: Preventive Activities of Medicinal Plants against *Streptococcus mutans*

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Abstract

Nine medicinal plants – *Glycyrrhiza glabra* L., *Terminalia chebula* Retz var. *chebula, Terminalia bellirica* (Gaertn.) Roxb., *Phyllanthus emblica* L., *Ocimum tenuiflorum* L., *Ocimum africanum* Lour., *Mentha arvensis* L. var. *piperascens* (Malinv. ex Holmes) Malinv. ex L.H. Bailey, *Mentha pulegium* L., and *Syzygium aromaticum* (L.) Merr. & L.M. Perry – were selected. To determine their effectiveness in preventing dental caries, ethanol extracts of these plants were screened for activity against *Streptococcus mutans* (DMST18777) using agar disk diffusion method. Three extracts –*T. bellirica*, *G. glabra* and *S. aromaticum*– showed moderate effectiveness at a concentration of 20 mg/ml (10–15 mm inhibition zone). *G. glabra* was the most effective, exhibiting minimum inhibitory concentration (MIC) of less than 12.5 mg/ml and minimum bactericidal concentration (MBC) of 25 mg/ml. A glass tube or smooth surface method was used to examine adherence inhibitory activity. Four extracts –*T. bellirica*, *G. glabra*, *S. aromaticum* and *P. emblica*– demonstrated strong effectiveness, distinguished by a clear glass surface after incubation. Phytochemical components (i.e. tannins and flavonoids) were found in most of the extracts. However, the active components against *S. mutans* should be further studied.

Keyword: Dental caries, *Streptococcus mutans*, *Terminalia bellirica*, Medicinal plants

INTRODUCTION

Dental caries are a major problem worldwide. *Streptococcus mutans* is a primary cause, having the ability to adhere to tooth surfaces while producing acid and surviving in acid conditions[1]. Left untreated, dental caries will gradually lead to tooth loss, with ensuing chewing difficulties and ultimately, a variety of health problems[2]. Dental caries prevention is preferable to treatment, because treatment might come too late to avoid the loss of the tooth. Conventional preventive methods such as the use of alcohol or antibiotics, e.g. chlorhexidine, erythromycin, ampicillin and penicillin, have proven effective in preventing dental caries[3]. However, excessive use of these chemicals has been reported to change the oral and intestinal flora, and can cause other problems such as vomiting, tooth staining or oral cancer[4,5]. Importantly, antibacterial agents can also promote the development of resistant bacterial strains[5]. For these reasons, alternative methods such as the use of medicinal plants are of increasing interest. Various compounds in plants that are produced for self-protection could support each other in inhibiting bacterial growth, while also reducing the chances of the development of resistant bacterial strains. However, few studies on these plants have been conducted. Several plants have demonstrated antibacterial activity, but their effects have not yet been proven against cariogenic bacteria. Some studies have focused on inhibition of the growth of bacteria that cause dental caries, but other important activities such as inhibition of tooth surface adherence or co-aggregation with other oral bacteria were not investigated.

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In Thailand, a variety of medicinal plants have been used to prevent or treat oral health problems. In this study, nine plants were selected and evaluated for their anti-cariogenic activity in terms of growth inhibition and antiadherence: *Glycyrrhiza glabra* L., *Terminalia chebula* Retz. var. *chebula*, *Terminalia bellirica* (Gaertn.) Roxb., *Phyllanthus emblica* L., *Ocimum tenuiflorum* L., *Ocimum africanum* Lour., *Mentha arvensis* L. var. *piperascens* (Malinv. ex Holmes) Malinv. ex L.H. Bailey, *Mentha pulegium* L., and *Syzygium aromaticum* (L.) Merr. & L.M. Perry.

MATERIALS AND METHODS

Materials

Flower buds of *Syzygium aromaticum*, leaves of *Ocimum africanum* and *Ocimum tenuiflorum*, rhizomes of *Glycyrrhiza glabra*, and fruits of *Terminalia chebula*, *Terminalia bellirica* and *Phyllanthus emblica* were obtained from a local market in Bangkok, Thailand. Leaves of *Mentha arvensis* var. *piperascens* and *Mentha pulegium* were obtained from SiriRuckhachati Nature Park in Mahidol University, Nakhon Pathom, Thailand. Each sample was macerated with 95% ethanol for 7 days and concentrated using a rotary evaporator. *Streptococcus mutans* DMST18777 (ATCC 25175T) was obtained from the Culture Collection for Medical Microorganisms, Department of Medical Sciences, National Institute of Health, Ministry of Public Health, Thailand. All plant materials were identified by Prof. Dr. Wongsatit Chuakul.

Antibacterial test: agar disk diffusion method

Antibacterial activity of the extracts was determined by the agar disk diffusion method. *S. mutans* bacteria were grown on tryptic soy agar (TSA) anaerobically at 37 °C for 24 h. Several colonies of overnight cultured bacteria were transferred into tryptic soy broth (TSB) and the density was adjusted to McFarland standard 0.5, or approximately equivalent to 10^8 CFU/ml. The density-adjusted bacteria were swabbed on Mueller-Hinton agar (MHA). The extracts were dissolved by 20% dimethyl sulfoxide (DMSO) to obtain 20 mg/ml solutions. To test each extract, sterile filter paper disks (6 mm in diameter) were placed on four MHA plates. Samples of each extract (5, 10 and 20 µl) were applied to three of the disks, and 20% DMSO was applied to the other disk as a negative control. A disk containing 15 µg erythromycin served as a positive control. The plates were then incubated anaerobically for 24 h. Antibacterial activity was evaluated by measuring the diameter of the inhibition zones. Each concentration was done in triplicate.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

The minimum inhibitory concentration was determined using broth dilution method in 96-well plates. The bacteria were first grown on tryptic soy broth (TSB) anaerobically at 37 °C for 24 h. The inocula of overnight cultured bacteria were adjusted in TSB to a density equivalent to 0.5 McFarland standard (10^8 CFU/ml). The extracts were dissolved in 20% DMSO to obtain 200 mg/ml, and were then diluted by serial twofold dilution to obtain concentrations ranging from 200 to 0.39 mg/ml. The adjusted cultured bacteria were then added to each concentration of extracts. Optical density (OD) was determined in a microplate reader at 600 nm before incubation (T_0). The 96-well plates were incubated at 37 °C for 24 h with constant shaking. After 24 h, the plates were again determined (T_{24}). The growth inhibition for the test wells at each dilution was determined using the formula:

$$\% \text{ inhibition} = \frac{1\text{-(ODtest well at}T_{24} \text{ - ODtest well at}T_0)}{(\text{ODcontrol well at}T_{24} \text{ - ODcontrol well at}T_0)} \text{ X } 100$$

The minimum concentration that showed 100% growth inhibition was indicated as MIC. The inocula from each concentration was subcultured to TSA and incubated anaerobically at 37 °C for 24 h. The highest concentration that showed no bacterial colonies was taken as MBC.

Determination of antiadherence activity

Antiadherence was determined by glass tube surface adherence assay as in Limsong (2004)[6], adapted from Murchison (1981)[7]. Each tube contained 0.3 ml of TSB, which was composed of 2% sucrose, 0.1 ml of extract, and 0.1 ml of the adjusted culture of *S. mutans*. All tubes were inclined at 30° and incubated anaerobically at 37 °C for 18 h. Bacterial adhesion was scored on a scale of 0 to +4 as described inthe Murchison method[7].

Phytochemical screening

The phytochemical groups of all extracts were determined for tannins and flavonoids. Phenolic compounds were tested by color precipitation with ferric chloride. Tannins were tested with gelatin and gelatin-salt reagent. Flavonoids were determined using hydrochloric acid and magnesium ribbon.

Thin layer chromatography (TLC) fingerprint

The extracts were concentrated to 1 mg/ml. The 5 µl of tested solutions were streaked 4 mm in length on the precoated silica gel aluminium plate $60F_{254}$. The extracts of *T. chebula* var. *chebula*, *T. bellirica*, *P. emblica*, and gallic acid (reference standard) were developed in a solvent system which was toluene: ethyl acetate: formic acid: methanol (30:30:8:2). The developed plates were examined under short (254 nm) wavelength ultraviolet light. The extracts of *S. aromaticum*, *M. arvensis* var. *piperascens*, *M. pulegium*, *O. africanum*, *O. tenuiflorum*, and eugenol (reference standard) were developed in a solvent system which was toluene: ethyl acetate (93:7). The plates were detected with vanillin-sulphuric acid spray reagent and observed under visible light. The extract of *G. glabra* was developed in a solvent system which was chloroform: methanol: water (64:50:10). The plate was detected with anisaldehyde-sulphuric reagent spray reagent and observed under visible light.

RESULTS

Antibacterial test: agar disk diffusion method

Three extracts – *T. bellirica*, *G. glabra*, and *S. aromaticum* – inhibited the growth of S. mutans; the inhibition zones varied from 8–15 mm. The largest inhibition zone at 15 mm was obtained with G. glabra at a volume of 20 µl; whereas erythromycin, which was used as a positive control, gave a 40 mm inhibition zone. The negative control (20% DMSO) produced no inhibition zone (Table 1).

Determination of antiadherence activity

The adherence inhibition of all extracts was determined; it was found that four extracts exhibited this activity. Data are shown in (Table 2.)

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

Extracts which exhibited antibacterial activity were further determined for MIC and MBC values. Three extracts demonstrated MIC values less than 12.5 mg/ml, and their MBC values ranged from 25–50 mg/ml (Table 3).

Table 1. Mean bacterial growth inhibition zones (mm) in agar disk diffusion method after treatment with 20 mg/ml of plant extracts

Plant species	Volume of extract (µl)		
	5	10	20
1.*Ocimum tenuiflorum* L.	-	-	-
2.*Ocimum africanum* Lour.	-	-	-
3.*Mentha arvensis* L. var. *piperascens* (Malinv. ex Holmes) Malinv. ex L.H. Bailey	-	-	-
4.*Mentha pulegium* L.	-	-	-
5.*Terminalia chebula* Retz. var. *chebula*	-	-	-
6.*Terminalia bellirica* (Gaertn.) Roxb.	-	8	10
7.*Phyllanthus emblica* L.	-	-	-
8.*Glycyrrhiza glabra* L.	10	12	15
9.*Syzygium aromaticum* (L.) Merr. & L.M. Perry	10	12	14
10. 20% DMSO (negative control)	0	0	0
11. Erythromycin (15 µg; positive control)		40	

Table 2. Antiadherence activity of crude ethanol extracts against *S. mutans*

Plant species	Volume of extract (µl)		
	5	**10**	**20**
1. *Ocimum tenuiflorum* L.	+2	+2	+2
2.*Ocimum africanum* Lour.	+4	+4	+4
3.*Mentha arvensis* L. var. *piperascens* (Malinv. ex Holmes) Malinv. ex L.H. Bailey	+4	+4	+4
4.*Mentha pulegium* L.	+3	+3	+3
5.*Terminalia chebula* Retz. var. *chebula*	+1	+1	+1
6.*Terminalia bellirica* (Gaertn.) Roxb.	0	0	0
7.*Phyllanthus emblica* L.	0	0	0
8.*Glycyrrhiza glabra* L.	0	0	0
9.*Syzygium aromaticum* (L.) Merr. & L.M. Perry	0	0	0

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of active plant extracts

Plant species	MIC (mg/ml)	MBC (mg/ml)
T. bellirica	< 12.5	25
G. glabra	< 12.5	25
S. aromaticum	< 12.5	50

Phytochemical screening

Preliminary phytochemical screening for tannins and flavonoids was performed. The results demonstrated that tannins were found in *T. chebula*, *T. bellirica*, *P. emblica*, *G. glabra* and *S. aromaticum*, while flavonoids were found in *G. glabra* (Table 4).

Table 4. Phytochemical components (tannins and flavonoids) of crude ethanol extracts

Plant species	Tannins	Flavonoids
1. *Ocimum tenuiflorum* L.	-	-
2.*Ocimum africanum* Lour.	-	-
3.*Mentha arvensis* L. var. *piperascens* (Malinv. ex Holmes) Malinv. ex L.H. Bailey	-	-
4.*Mentha pulegium* L.	-	-
5.*Terminalia chebula* Retz. var. *chebula*	+	-
6.*Terminalia bellirica* (Gaertn.) Roxb.	+	-
7.*Phyllanthus emblica* L.	+	-
8.*Glycyrrhiza glabra* L.	+	+
9.*Syzygium aromaticum* (L.) Merr. & L.M. Perry	+	-

Thin layer chromatography (TLC) fingerprint

TLC chromatograms of the extracts were developed in different mobile phases. The extract of *S. aromaticum* showed 10 bands under visible light and also eugenol comparing with standard at R_f value of 0.6 as shown in Figure 1 (A). The extract of *G. glabra* showed 5 bands under visible light including glycyrrhizin at R_f value of 0.3 which showed black-grey zone followed by Wagner H.[8] as shown in Figure 1 (B). The extracts of *T. chebula*, *T. bellirica*, *P. emblica*, and gallic acid were developed and observed under short (254 nm) wavelength ultraviolet light. Only the extracts of *T. chebula* and *T. bellirica* showed gallic acid at R_f value of 0.5 as shown in Figure 1 (C). The chromatograms of the extracts of *M. arvensis* var. *piperascens*, *M. pulegium*, *O. africanum*, and *O. tenuiflorum* were shown in Figure 1 (D).

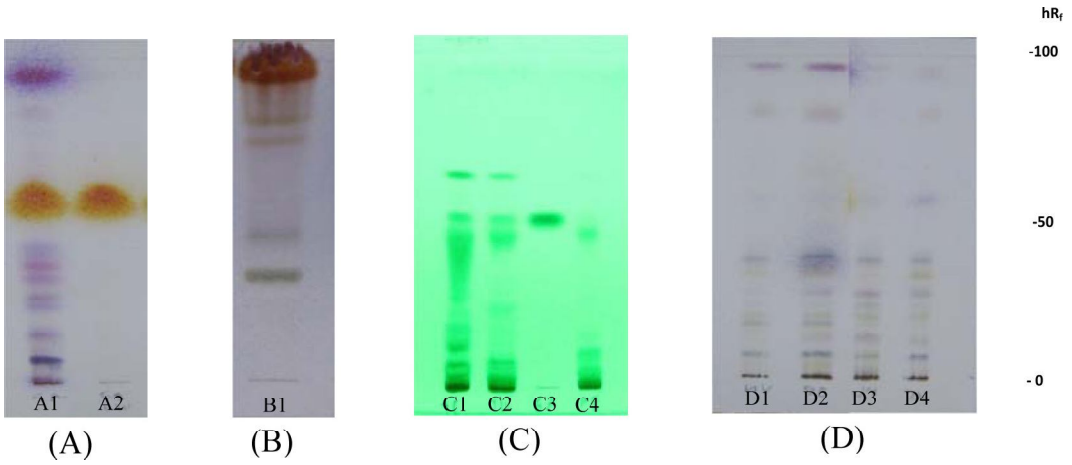

Figure 1. The TLC chromatograms of each extracts; stationary phase: silica gel GF254
(A); mobile phase: toluene: ethyl acetate = 93:7; spray reagent: vanillin-sulphuric acid; observed under visible light, A1 = *Syzygium aromaticum* extract, A2 = standard eugenol
(B); mobile phase: chloroform: methanol: water = 64:50:10; spray reagent: anisaldehyde-sulphuric acid; observed under visible light, B1 = *Glycyrrhiza glabra* extract
(C); mobile phase: toluene: ethyl acetate: formic acid: methanol = 30:30:8:2; observed under short wavelength (254 nm) ultraviolet light, C1 = *Terminalia chebula* extract, C2 = *Terminalia bellirica* extract, C3 = standard gallic acid, C4 = *Phyllanthus emblica* extract
(D); mobile phase: chloroform: methanol: water = 64:50:10; spray reagent: anisaldehyde-sulphuric acid; observed under visible light, D1 = *Mentha pulegium* extract, D2 = *Mentha arvensis* var. *piperascens* extract, D3 = *Ocimum africanum* extract, D4 = *Ocimum tenuiflorum* extract

DISCUSSION

Crude ethanol extracts of *Terminalia bellirica*, *Glycyrrhiza glabra* and *Syzygium aromaticum* moderately inhibited the growth of *S. mutans*, showing inhibition zones from 8–15 mm and MIC less than 12.5 mg/ml (Table 1). These results are in accordance with several previous studies. The ethanol extracts of *S. aromaticum* exhibited MBC of 25 mg/ml; but a previous study reported that clove oil (*S. aromaticum*) showed even greater effectiveness, with MIC of 3.125 mg/ml[9]. Clove oil has been identified as a potential antibacterial agent, as it contains the known active compound, eugenol[10,11,12,13]. The ethanol extract exhibited less effectiveness due to a lower concentration of clove oil or eugenol. Hwang (2004)[14] reported that methanol extract of *G. glabra* at 20 mg/ml inhibited bacterial growth of *S. mutans*, with a 20 mm inhibition zone; but no MIC value was given. In the present study the ethanol extract of *G. glabra* showed MIC of 12.5 mg/ml. *T. bellirica* in combination with *T. chebula* and *P. emblica*, known as Triphala in Thai traditional medicine exhibited MIC against *S. mutans* of 50 μg/ml[15], which was more effective than the ethanol extract of *T. bellirica* in this study (MIC of 12.5 mg/ml). But the present results indicated that extracts of *T. chebula* and *P. emblica* showed no antimicrobial activity against *S. mutans*, even though they contain tannins which commonly exhibit this activity. In contrast, *Terminalia chebula* and *Phyllanthus emblica* have been reported to exhibit antimicrobial activity against dental caries pathogens[16,17,18,19]. Because of these apparently conflicting results, the combination Triphala extract

should be studied further to conclusively determine its antimicrobial activity.

S. mutans has the ability to adhere to a smooth surface, providing suitable conditions for itself and for other bacteria to aggregate at the tooth surface. The present results showed that four plants (*T. bellirica*, *P. emblica*, *G. glabra* and *S. aromaticum*) strongly inhibited the adherence ability of *S. mutans*; tests on a smooth glass surface indicated a score of 0 on a scale of 0 to +4 (Figure 1). This result was similar to previous studies of *T. bellirica*, *P. emblica* and *S. aromaticum*[20,21]. No studies have been performed on the adherence inhibition of *S. mutans* by *G. glabra*. The mechanism of adherence inhibition should be studied further.

From phytochemical screening, all active extracts were found to contain tannins, while *G. glabra* also contained flavonoids. Several previous studies have also shown that *T. bellirica*, *P. emblica*, *G. glabra* and *S. aromaticum* contain tannins[22,23,24,25] which have been reported to possess antibacterial activity against both Gram-positive and Gram-negative bacteria[26,27]. Aqueous extract of green tea contains tannins, which conform to a previous study on growth inhibition of cariogenic bacteria, including *S. mutans*[23].

CONCLUSION

This study demonstrated that *Terminalia bellirica*, *Glycyrrhiza glabra* and *Syzygium aromaticum* inhibited the growth of *Streptococcus mutans* as well as the adherence ability of the bacteria. These plants should be studied for their active compounds and further developed into products for prevention of dental caries.

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