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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2018.300.306

Research Article
Anticariogenic Activities of *Derris reticulata* Ethanolic Stem Extract Against *Streptococcus mutans*

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Abstract

Background and Objective: *Streptococcus mutans* is a dominant causative pathogen of dental caries, which is a major oral health problem affecting million people worldwide. *Derris reticulata* is a medicinal plant possessing antimicrobial activity against several Gram-positive pathogenic bacteria. None the less, its effects on growth and cariogenic properties of *S. mutans* has not been clearly established. This study aimed to investigate the antibacterial and anti cariogenic activities of the *D. reticulata* ethanolic stem extract. **Materials and Methods:** The TLC analysis was performed to authenticate the *D. reticulata* sample. Minimum inhibition concentration and minimum bactericidal concentration were determined by using broth dilution and drop plate methods, respectively. Sucrose dependent and sucrose independent-adherences, biofilm formation and glycolytic pH drop assays were performed to evaluate the anticariogenic activity. **Results:** The ethanolic stem extract of *D. reticulata* possessed the antibacterial activity against *S. mutans* with the MIC and MBC of 0.875±0.250 and 1.750±0.500 mg mL^{-1}, respectively. The extract at the lower concentrations of sub-MIC also had significant inhibitory actions against the cariogenic properties of *S. mutans*, including surface adherence, biofilm formation and glycolytic acid production. **Conclusion:** The *D. reticulata* stem extract had a substantial anticariogenic activities and thus potentially be developed as an oral health care product for dental caries prevention in the near future.

Key words: Dental caries, *Derris reticulata*, *Streptococcus mutans*, cariogenic property, antimicrobial activity

Citation: Pawitra Pulbutr, Sakulrat Rattanakiat, Natnicha Phetsaardeiam, Patcharapa Modtaku, Rattazart Denchai, Achida Jaruchotikamol and Wanwisa Khunawattanakul, 2018. Anticariogenic activities of *Derris reticulata* ethanolic stem extract against *Streptococcus mutans*. Pak. J. Biol. Sci., 21: 300-306.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dental caries or tooth decay is a major oral health problem caused by a chronic infection of acid-producing bacteria leading to a progressive destruction of the affected teeth[1]. According to the WHO[2] report, 60-90% of schoolchildren and almost 100% of adults have experienced dental caries worldwide. Although several micro-organisms have been found to involve in the etiology of dental caries, a dominant pathogenic species in many patients with dental caries is *Streptococcus mutans*, the facultative anaerobic gram-positive bacteria[3]. The crucial virulence factors of *S. mutans* include tooth surface adherence, biofilm or dental plaque formation and acid production (acidogenesis)[4]. Therefore, in addition to an inhibition against *S. mutans* growth, a suppression of their cariogenic properties is another promising strategy for the prevention and treatment of dental caries. Although certain chemicals have been used in oral health care products for a prevention of dental caries, some drawbacks of their uses lead to an ongoing search for more effective anticariogenic agents with less adverse effects. Plant based anti-caries agent is an interesting alternative, which potentially provides a relatively safe and more accessible approach for the management of dental caries.

Derris reticulata or Cha-em-nuea in Thai is a medicinal plant in family Leguminosae (sub family Papilionoideae), which distributes naturally in every region of Thailand. The roots and stems of *D. reticulata* have been used in Thai folk medicine for several purposes such as to sweeten the recipe and to treat productive cough and throat diseases[5]. The major phytochemical constituents reported in *D. reticulata* stem were prenylated flavanones named lupinifolin, 2‴,3‴-epoxy lupinifolin, dereticulatin and 1‴-hydroxy-2‴,3‴-epoxylupinifolin[6,7]. Lupinifolin isolated from *D. reticulata* stem had an antibacterial activity against various Gram-positive bacteria especially *Staphylococcus aureus* with the MIC and MBC of 8 and 16 μg mL^{-1}, respectively[8]. Nonetheless, the effects of the ethanolic stem extract of *D. reticulata* on growth and virulence properties of *S. mutans* have not been clearly established. This study thus aimed to investigate the antibacterial and anticariogenic activities of the *D. reticulata* ethanolic stem extract.

MATERIALS AND METHODS

Preparation of the *D. reticulata* ethanolic stem extract: The stems of *D. reticulata* were obtained from the local herb store in Bangkok, Thailand. The specimens were primarily authenticated macroscopically and microscopically by Assistant Professor Dr. Wanida Caichompoo, a botanist at the Faculty of Pharmacy, Mahasarakham University, Thailand. The authentication of the sample was confirmed by thin-layer chromatography (TLC) analysis as described in the following section. The stems were cleaned and dried at 60°C in the hot air oven for 24 h and subsequently ground. The 200 g of the ground sample were macerated with 95% (v/v) ethanol (800 mL) at room temperature for 7 days. The mixture was filtered through a filter paper (Whatman No. 1). The filtrate was evaporated by using rotary evaporator (Heidolph®, Germany). The extracts were kept at -20°C before using in the experiment. Yields (%) of the extract was 1.96%.

Thin layer chromatography (TLC) analysis: The authentication of *D. reticulata* ethanolic stem extract was performed by using TLC analysis as described by Chansuwan *et al*.[9]. The TLC silica gel 60 GF254 (10×10 cm plate) was used as stationary phase for TLC fingerprint of the *D. reticulata* ethanolic stem extract. Five microliters of the extract (5 mg mL^{-1}) were spotted on the TLC plate. The TLC plate was developed with a mobile phase of hexane: ethyl acetate (6:4). Subsequently, the TLC plate was observed under ultraviolet light (365 and 254 nm) and detected with 10% sulfuric acid in ethanol and then heated at 110°C for 10 min.

Determination of the MIC and MBC values: The minimum inhibitory concentration (MIC) was determined by using a broth dilution method. *Streptococcus mutans* (DMST 1877) were inoculated into BHI broth media in test tubes and grown to a stationary phase up to 1.5×10^8 CFU mL^{-1} (McFarland no. 0.5) at 37°C with 5% CO_2. The bacteria suspension was further diluted with BHI broth to 1.5×10^6 CFU mL^{-1} (1 mL) and inoculated into fresh BHI (1 mL) containing various concentrations of the tested extracts. The MIC was recorded as the lowest concentration totally inhibiting visible bacterial growth in the test tube after 24 h of incubation. Chlorhexidine was used as the positive control whereas 10% DMSO (vehicle) was used as the negative control.

A drop plate method was used for the MBC value determination. The mixture (100 μL) from the test tube containing no visible bacterial growth from the earlier MIC determination experiment was spread onto the BHI agar and incubated at 37°C, 5% CO_2 for 24 h. The MBC was indicated by the lowest concentration of the extract resulting in no colony growth onto the plate. Three independent experiments were performed to obtain the mean MIC and MBC of the extracts.

Biofilm formation assay: The biofilm formation assay was performed following the method of Hasan *et al.*[10] with slight modifications. The *S. mutans* (1.5×10^8 CFU mL^{-1}) were grown in 96-well microplate containing BHI (150 µL) with 5% (w/v) sucrose. The bacteria were incubated with the extract or vehicle in 5% CO_2 incubator at 37°C for 6, 12, 20 or 24 h. After the incubation at the specific period, the media were decanted from the microplate to remove the planktonic cells. The microplate wells were washed gently with sterile deionized water to remove the remaining unattached cells. The adhered biofilm was fixed by adding formalin (37%, diluted 1:10) with 2% sodium acetate. The fixed biofilm was stained with 0.1% crystal violet (200 µL) and left for 15 min. The microplate wells were washed twice with sterile deionized water and then the biofilm-bound dye was removed by adding 150 µL of 95% ethanol. The microplate was shaken for 10 min and the volume of 100 µL was taken out to measure its optical density at 600 nm. Inhibition of biofilm formation was calculated as percentage from the following equation:

$$\text{Inhibition of biofilm formation (\%)} = \frac{OD_{600}\ \text{vehicle-}OD_{600}\ \text{extract}}{OD_{600}\ \text{vehicle}}\times100$$

Sucrose-dependent and sucrose-independent adherence assays: The adherence assay of *S. mutans* on smooth glass surface was conducted according to the method of Prabu *et al.*[11] with some modifications. *Streptococcus mutans* (1.5×10^8 CFU mL^{-1}) were incubated with the extract or vehicle in BHI broth (10 mL) with or without 5% (w/v) sucrose for the sucrose-dependent and sucrose-independent adherence assays, respectively. The glass tubes containing the bacteria were placed at an angle of 30° for 24 h at 37°C in 5% CO_2 incubator. After the 24 h incubation, the media were decanted and collected to measure the optical density of the decanted cells at the wavelength of 600 nm. Subsequently, 0.5 M NaOH (2 mL) was added into the glass tube to remove the adhered cells followed by vortexing. The optical density of the adhered cells was then measured at the wavelength of 600 nm. The sum of the optical density of the decanted cells and the adhered cells was designated as the optical density of the total cells. Adherence (%) was calculated as follows:

$$\text{Adherence (\%)} = \frac{OD_{600}\ \text{of adhered cells}}{OD_{600}\ \text{of total cells}}\times100$$

Glycolytic pH drop assay: The glycolytic pH drop assay was carried out according to the method of Ban *et al.*[12]. *Streptococcus mutans* were grown in BHI broth at 37°C in 5% CO_2 incubator for 24 h. The bacteria were washed with a salt solution containing KCl (50 mM) and $MgCl_2$ (1 mM) and the bacterial cell suspension (7.5×10^8 CFU mL^{-1}) was then prepared in the same salt solution. The extracts (at sub-MICs) or vehicle was added into the bacterial cell suspension and pH of the suspension was adjusted to 7.2-7.4 by using KOH (0.2M). A solution of glucose (final concentration of 0.5% w/v) was then added into the bacterial cell suspension. The pH of the bacterial cell suspension was continuously recorded every 1 min over a period of 60 min. The initiate rate of pH drop, which indicates the best measure of the bacterial acidogenesis was calculated from the change of pH in the first 10 min (0-10 min).

Statistical analysis: The data were expressed as Mean±SD (MIC, MBC, initial rate of pH drop) or Mean±SEM (% adherence and inhibition (%) of biofilm formation). The statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. The data were considered as significant difference when p-value is less than 0.05.

RESULTS

TLC characteristics of the *D. reticulata* ethanolic stem extract: The TLC chromatogram of the ethanolic extract of *D. reticulata* stem was shown in Fig. 1. The marked spot of hRf at 49-56 was detected.

Antibacterial activity of the *D. reticulata* ethanolic stem extract against *S. mutans*: The ethanolic extract of *D. reticulata* stem inhibited the growth of *S. mutans* with the MIC of 0.875±0.250 mg mL^{-1}. The bactericidal activity of the extract was also observed with the MBC of 1.750±0.500 mg mL^{-1}. The MIC and MBC of chlorhexidine (positive control) were 0.625 and 1.25 µg mL^{-1}, respectively.

Effects of the *D. reticulata* ethanolic stem extract against biofilm formation of *S. mutans*: The ethanolic extract of *D. reticulata* stem at the concentrations of 10, 50, 100, 250, 500 and 750 µg mL^{-1} significantly inhibited *S. mutans* biofilm formation at every incubation period tested ($p<0.05$) (Fig. 2). The extract at the lowest concentration tested (1 µg mL^{-1}) also produced a significant inhibition against biofilm formation at the incubation period of 12, 20 and 24 h ($p<0.05$). The maximal inhibitory action against biofilm formation of 102.77±2.68% was detected when the highest concentration of the extract (750 µg mL^{-1}) were tested at 24 h incubation.

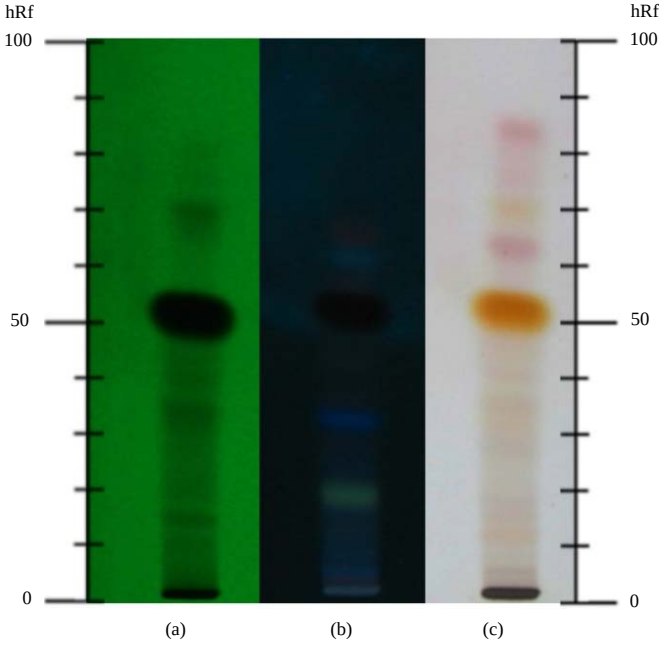

Fig. 1(a-c): Thin layer chromatographic chromatogram of the ethanolic extract of *D. reticulata* stem, (a) Detection under UV 254 nm, (b) Detection under UV 366 nm and (c) Detection with 10% sulfuric acid in ethanol and heated

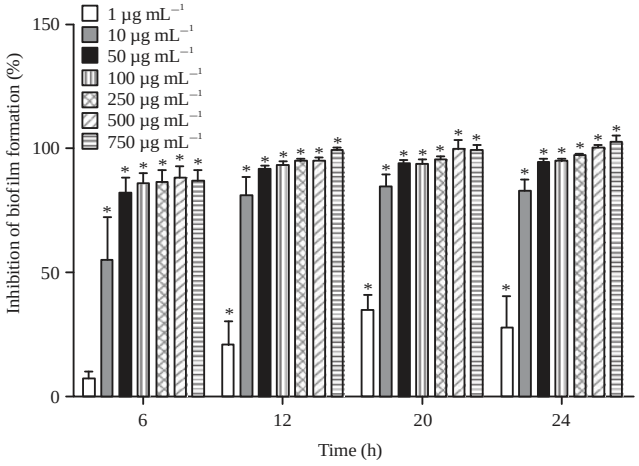

Fig. 2: Effects of the ethanolic extract of *D. reticulata* stem on biofilm formation of *S. mutans*.
*p<0.05 when compared with the negative control (One-way ANOVA followed by Bonferroni *post -hoc* test, n = 3)

Effects of the *D. reticulata* ethanolic stem extract against sucrose-dependent and sucrose-independent adherences of *S. mutans*: The *D. reticulata* stem ethanolic extract at the concentrations of 5, 10, 50, 250 and 750 µg mL^{-1} significantly

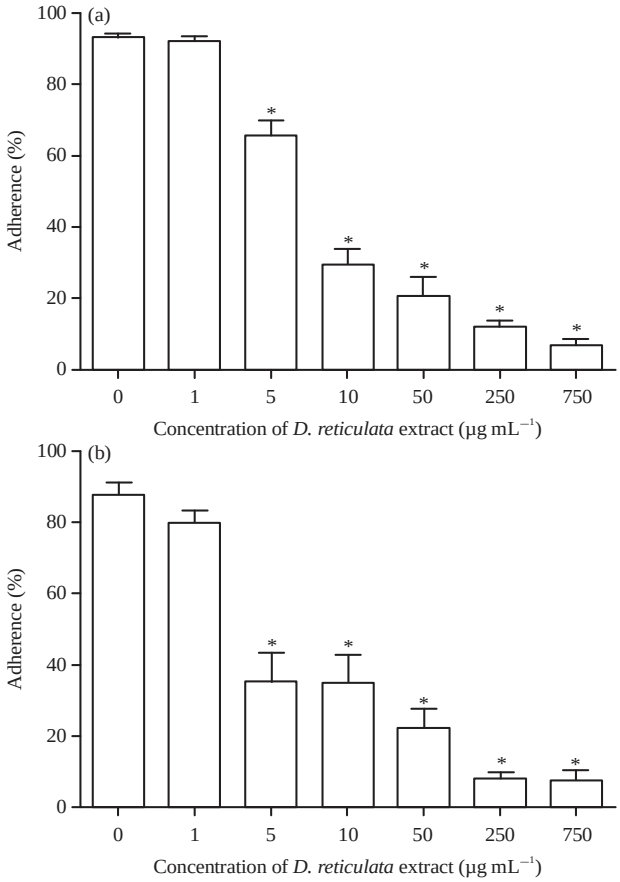

Fig. 3(a-b): Effects of the ethanolic extract of *D. reticulata* stem on (a) Sucrose-dependent and (b) Sucrose-independent surface adherences of *S. mutans*
*p<0.05 when compared with the negative control (One-way ANOVA followed by Bonferroni *post hoc* test, n = 4)

inhibited both sucrose-dependent and sucrose-independent adherences (p<0.05) (Fig. 3). The adherences of *S. mutans* in the presence and the absence of sucrose were drastically decreased to 7.19±1.27 and 7.69±2.46%, respectively when the extract at the concentration of 750 µg mL^{-1} was tested.

Effects of the *D. reticulata* ethanolic stem extract on *S. mutans* glycolytic pH drop: The ethanolic extract of *D. reticulata* stem at the concentrations of 100, 250, 500 and 750 µg mL^{-1} significantly reduced the initial rate of glycolytic pH drop (p<0.05) (Table 1). The onset pH of 7.27±0.03 was decreased to 4.37±0.10 after 60 min incubation in the control group, whereas the initial pH of 7.20±0.01 was decreased to 5.30±0.31 after 60 min

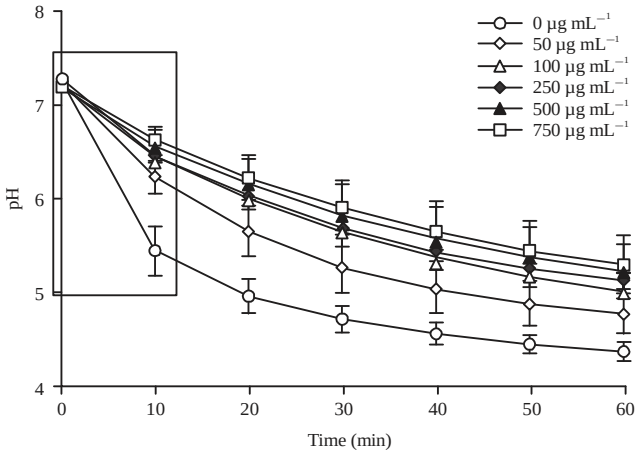

Fig. 4: Effects of *D. reticulata* stem ethanolic extract on glycolytic pH drop (the values enclosed in box corresponds to the initial rate of the pH drop)
Data represent Mean±SD (n = 3)

Table 1: Effects of the *D. reticulata* stem extract on initial rate of glycolytic pH drop

Concentration of the extract (µg mL^{-1})	Initiate rate of pH drop (pH unit/min) (Mean±SD)
0	0.182±0.024
50	0.130±0.049
100	0.082±0.011*
250	0.075±0.009*
500	0.064±0.018*
750	0.058±0.014*

*p<0.05 when compared with the negative control (One-way ANOVA followed by Bonferroni *post -hoc* test, n = 3)

incubation with the *D. reticulata* ethanolic stem extract at the concentration of 750 µg mL^{-1} (Fig. 4).

DISCUSSION

The TLC analysis can be used for the authentication of a crude drug derived from *D. reticulata* stem[13]. The authentication of *D. reticulata* commercialized as a crude drug in herb stores is essential in Thailand since the word "Cha-em" in Thai can be inferred to 4 different species of medicinal plants, including *D. reticulata* (Cha-em-nuea), *Myriopteron extensum* (Cha-em), *Glycyrrhiza glabra* (Cha-em-thet) and *Albizia myriophylla* (Cha-em-Thai). The TLC fingerprint of the *D. reticulata* ethanolic stem extract was similar to the previous report of Chansuwan *et al.*[9] in which the spot of hRf at 49-56 was detected. This indicated that the *D. reticulata* ethanolic stem extract used in this study was prepared from the genuine *D. reticulata* sample.

The ethanolic stem extract of *D. reticulata* had an antibacterial activity against *S. mutans* with the MIC and MBC of 0.875±0.250 and 1.750±0.500 mg mL^{-1}, respectively. The extract at the sub-MICs also exhibited the inhibitory actions against the crucial cariogenic properties of *S. mutans* including biofilm formation, sucrose dependent and sucrose-independent adherences and glycolytic acid production. Several phytochemicals, especially flavonoids, were reported to be present in *D. reticulata*[6,7]. Lupinifolin, a prenylated flavanone have been identified as a major flavonoid isolated from the stem of *D. reticulata* with the yield (%) of 0.55[6]. In addition to its presentation in the stem of *D. reticulata*, lupinifolin can be found in other medicinal plants including *Albizia myriophylla, Eriosema chinense, Erythrina fusca* and *Myriopteron extensum*[14-17]. Lupinifolin isolated from *A. myriophylla* stem showed a potent antibacterial activity against *S. mutans* with the MIC and MBC of 0.98 and 1.96 µg mL^{-1}, respectively[18]. This flavanone exhibited its bactericidal activity via disrupting the integrity of the bacterial cell membrane[19]. Thus, lupinifolin potentially also played a dominant role in the antibacterial activity of the *D. reticula* stem extract against *S. mutans* in this study. It was reported that Gram-positive bacteria were more sensitive to the antibacterial activity of lupinifolin than Gram-negative bacteria[8,14]. Lupinifolin derived from *D. reticulata* stem exhibited the antibacterial activity against *Staphylococcus aureus* with the MIC and MBC of 8 and 16 µg mL^{-1} whereas it had no antibacterial activity against some Gram-negative bacteria such as *Enterobacter aerogenes* and *Pseudomonas aeruginosa*[8].

The inhibitory actions of the *D. reticulata* stem extract against cariogenic properties of *S. mutans* was also evidently demonstrated in this study. The *D. reticulata* ethanolic stem extract at the concentrations of sub-MIC (lower than 625 µg mL^{-1}) significantly inhibited both sucrose-dependent and sucrose-independent adherences in a concentration-dependent manner. The anti-adherence action of the extract was in agreement with its inhibitory action against biofilm formation demonstrated in this study. A production of extracellular polysaccharides (EPSs) or glucan via the function of *S. mutans* glucosyltransferase (GTF) are essentially required for sucrose-dependent adherence and biofilm formation[20]. Several flavonoids, such as kaempferol, apigenin and myricetin were found to inhibit the GTF activity of *S. mutans*[21,22]. The inhibitory actions of the extract against both sucrose-dependent adherence and biofilm formation thus were possibly attributed to its inhibition against GTF activity. In the absence of sucrose, antigen I/II protein plays a pivotal role for sucrose-independent adherence of *S. mutans*[23]. It was reported that the inhibitory actions of some phytochemicals, including flavonoids and polyphenols,

against sucrose-independent adherence of *S. mutans* involved their suppressive actions on antigen I/II expression and/or activity[24]. Therefore, further experiments regarding the effects of lupinifolin, the primary flavonoid presented in *D. reticulata* stem, on the activities of GTF and antigen I/II of *S. mutans* should be performed hereafter.

The ethanolic extract of *D. reticulata* stems at the sub-MICs significantly inhibited the glycolytic pH drop caused by *S. mutans*. The ability to produce acid as a consequence of carbohydrate fermentation is a crucial cariogenic property of pathogenic *S. mutans*[25]. Certain flavonoids, specifically catechin and apigenin, exhibited their suppression against glycolytic pH drop of *S. mutans* via the inhibition against F_1F_0-ATPase, an essential pump for extracellular acid efflux[26,27]. Disruptions of F_1F_0-ATPase function or glucose metabolism thus potentially accounted for the inhibitory actions of the extract against glycolytic acid production of *S. mutans*.

CONCLUSION

The ethanolic stem extract of *D. reticulata* possessed the antibacterial activity against *S. mutans* with the MIC and MBC of 0.875 ± 0.250 and 1.750 ± 0.500 mg mL^{-1}, respectively. The extract at the lower concentrations of sub-MIC also had significant inhibitory actions against the cariogenic properties of *S. mutans*, including surface adherence, biofilm formation and glycolytic acid production. Thus, the *D. reticulata* ethanolic stem extract may conceivably be used as an effective plant-derived anticaries agent for dental caries prevention in the near future.

SIGNIFICANCE STATEMENT

This study revealed the antibacterial and anticariogenic activities of the *D. reticulata* ethanolic stem extract against *S. mutans*, the primary causative pathogen of dental caries. Both bacteriostatic and bactericidal actions of the extract were demonstrated. The anticariogenic activities against *S. mutans* were discovered even at the low concentrations of sub-MIC. Thus, a novel oral care product containing the active ingredient derived from *D. reticulata* may be arrived at.

ACKNOWLEDGMENT

We would like to express our gratitude to Assistant Professor Dr. Wanida Caichompoo for her kind help in the specimen authentication.

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