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Research paper

Lupinifolin from *Albizia myriophylla* wood: A study on its antibacterial mechanisms against cariogenic *Streptococcus mutans*

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ABSTRACT

Objective: To determine the anti-Streptococcus mutans mechanisms of action of lupinifolin from *Albizia myriophylla* Benth. (Fabaceae) wood and provide scientific evidence to support the traditional use of the plant against dental caries.

Methods: The minimum inhibitory concentration (MIC) was evaluated using the broth micro-dilution method. The effects of lupinifolin on bactericidal activity, bacterial cell walls, and membranes were investigated by time-kill, lysis, and leakage assays, respectively. Electron microscopy was utilized to observe any cell morphological changes caused by the compound. Localization of lupinifolin in S. *mutans* was detected using the thin layer chromatography technique.

Results: The MIC range of lupinifolin against S. *mutans* (n = 6) was 2–4 μg/ml. This compound displayed bactericidal effects on S. *mutans* ATCC 25175 by 90–99.9% killing at 4MIC-16MIC after 8–24 hours. Lupinifolin-treated cells demonstrated no lysis. However, significant cytoplasmic leakage through the bacterial membrane was observed after treatment with lupinifolin at 4MIC-16MIC. As revealed by ultrastructural analysis, lupinifolin produced some changes in bacterial cell walls and membranes. Moreover, the compound was observed in the cytoplasmic fraction of the lupinifolin-treated cells. These results suggest that lupinifolin can enter the cell of bacteria but does not accumulate in the cell envelope and subsequently disrupts the integrity of the cytoplasmic membrane, leading to cell death.

Conclusion: The scientific evidence from this study offers valuable insights into the potential role of lupinifolin in pharmaceutical and antibiotic applications and supports the therapeutic effects of A. *myriophylla*, which has traditionally been used as an alternative treatment for dental caries.

1. Introduction

Medicinal plants are of great interest for the discovery of antimicrobial compounds. Recently, it has been accepted that there are many plant secondary metabolites responsible for the therapeutic effects of plant based drugs. The variation of several factors such as genetic and environmental conditions may affect chemical composition of the herbal raw materials and in turn herbal products' quality (Charoonratana et al., 2014). In general, chemical information on the major active ingredients of the herbal medicines would serve as a useful parameter for their quality assessment (Joycharat et al., 2016; Zhao et al., 2012). In addition, knowledge about the mechanisms of action of active substances could be helpful to ensure the safety and efficacy of herbal plants used in traditional systems of medicines (Clegg & Mac Gabhann, 2015). Many plant compounds have been studied and some demonstrate strong antimicrobial activity and have good potential to be developed into effective drugs (Chorachoo et al., 2016; Joycharat et al., 2013; Joycharat et al., 2016; Limsuwan et al., 2009; Limsuwan, Hesseling-Meinders, Voravuthikunchai, Van Dijl, & Kayser, 2011). However, the mechanisms or modes of action of the main active compounds of many medicinal plants are unclear, which poses a challenge to the pharmaceutical and agrochemical industries.

Abbreviations: ATCC, American Type Culture Collection; cfu/ml, colony-forming unit per milliliter

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Albizia myriophylla Benth. (Fabaceae) is a large liana widely found in Southeast Asia (Yoshikawa et al., 2002). In Thailand, this medicinal plant is commonly known by its local name, 'Cha-Em-Thai'. In Thai folk medicine, various parts of this plant species including its leaf, flower, root and wood have been recommended for treating many human diseases (Medicinal Registration Division, 1998; Saralamp, Chuakul, Temsiririrkkul, Clayton, & Paonil, 1996). The root of this plant has been used against fever, to alleviate thirst, and as a laxative and mucolytic. The leaf has been applied to wounds to stop bleeding and to treat earache. The flower has been used as a digestive agent. Interestingly, the wood of *A. myriophylla* has been documented as *materia medica* used alone or in combination with other medicinal plants, in various Thai herbal formulas, as folk remedies for various ailments occurring in the oral cavity and throat such as toothache, sore throat, and aphthous ulcer (Medicinal Registration Division, 1998). Moreover, the Health Information System Development Office (HISO) of Thailand has noted that *A. myriophylla* is one of the most widely used medicinal plants in oral care products in Thailand due to its antimicrobial, anti-inflammatory, astringent, and wound healing activities (Health Information System Development Office: HISO, 2017). In addition, the study of 35 Thai herbal formulas used by southern Thai traditional healers for treating dental caries (Joycharat et al., 2012) found that *A. myriophylla* and its active compound, lupinifolin, demonstrated very good antibacterial activity against *Streptococcus mutans*, the most common bacterial pathogen responsible for the induction of tooth decay (Joycharat et al., 2012; Joycharat et al., 2013; Joycharat et al., 2016). Lupinifolin was found to demonstrate potent activity closely comparable to that of chlorhexidine, a gold standard antiseptic used as an antiplaque, and showed strong activity against clinical isolates of *S. mutans* at minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) ranging from 0.25–2 and 0.5–8 μg/ml, respectively (Joycharat et al., 2013). In addition, lupinifolin showed no acute and subacute toxicity in mice or rats at administration doses of 5 and 30 mg/kg/day, respectively (Chivapat et al., 2009). However, the anti-*S.mutans* mechanism of action of this compound has never been investigated. Therefore, the present work was carried out in order to determine its antibacterial mechanism against cariogenic *S. mutans*. The results from this study constitute scientific evidence for the efficacy of the traditional use of *A. myriophylla* against dental caries which may foster the acceptance of traditional medicine and natural products as an alternative system of health care.

2. Materials and methods

2.1. Lupinifolin

The wood of *A. myriophylla* was collected from the southern region of Thailand in July 2013. Botanical identification was performed by Assistant Professor Dr. Oratai Neamsuvan, an ethnobotanist at the Faculty of Traditional Thai Medicine, Prince of Songkla University, where the voucher specimen (NJ0911) was deposited. The purified lupinifolin from *A. myriophylla* wood was obtained from our previous work (Joycharat et al., 2016). The compound was kept at −20 °C and dissolved in dimethyl sulfoxide (DMSO, Fischer Chemical, UK) before use.

2.2. Bacterial strain and culture condition

Five clinical isolates of *S. mutans* from carious lesions of patients' teeth were obtained from the collection of the Excellent Research Laboratory on Natural Products, the Research Center of Excellence, and the Department of Microbiology, Faculty of Science, Prince of Songkla University, Thailand. *Streptococcus mutans* ATCC 25175 was used as a reference strain. The bacterial cultures were stored in a brain heart infusion broth (BHI, Difco, USA) containing 20% glycerol at −80 °C until use. All isolates were cultured on BHI agar incubated with 5% CO_2 at 37 °C for 24 h.

2.3. Antibacterial activity

A modified broth micro-dilution method conforming to Clinical and Laboratory Standards Institute: CLSI (2009) was used to determine the MIC and MBC of lupinifolin against *S. mutans*. Lupinifolin was dissolved in 10% DMSO and serial two-fold dilutions were made. A suspension of *S. mutans* in BHI broth was prepared from the overnight broth culture and adjusted to 0.5 McFarland standard (approximately 1.5×10^8 cfu/ml) using Wickerham card. The bacterial suspension (180 μl) was mixed with the diluted test compound (20 μl) in a 96-well flat bottom microtiter plate (Costa, NY, USA). The final bacterial cell concentration was approximately 5×10^5 cfu/ml. The final concentration of the test compound ranged from 0.5–1024 μg/ml. 1% DMSO (final concentration) and chlorhexidine (Sigma-Aldrich, USA) were used as negative and positive controls, respectively. The microtiter plates were incubated with 5% CO_2 at 37 °C for 24 h. The MIC was recorded as the lowest concentration that produced a complete suppression of visible growth. The test was performed in triplicate independent experiments.

2.4. Time-kill assay

A time-kill assay was employed to investigate the bactericidal activity of lupinifolin. The bacterial cultures (approximately 5×10^5 cfu/ml) in BHI broth supplemented with the compound at concentrations equivalent to MIC, 2MIC, 4MIC, 8MIC and 16MIC were incubated with 5% CO_2 at 37 °C. Surviving bacteria were observed at 0, 3 and 5 min and 1, 5, 8, 12, 16, 20 and 24 h by culturing on BHI agar plates incubated with 5% CO_2 at 37 °C. A control with 1% DMSO was prepared with the same conditions. All assays were carried out in duplicate.

2.5. Bacteriolytic activity

A modified version of the method described by Carson, Mee, and Riley (2002) was used to determine the bacteriolytic activity of lupinifolin. Briefly, the suspensions of *S. mutans* ATCC 25175 in Phosphate-buffered saline (PBS; Sigma-Aldrich, USA) supplemented with 0.001% Tween 80 solution (Sigma-Aldrich, USA) (PBS-T) were mixed with the compound at concentrations equivalent to 16MIC and the optical density at 620 nm (OD_{620}) was measured at 0, 1, 2, 3, 4, 5, 6, 8, 12, 16, 20, and 24 h. Bacterial cell lysis was indicated by a decrease in OD_{620}. Corresponding dilutions of the compound were used as blanks. A negative control with 1% DMSO (final concentration) was applied with the same conditions. 10% Triton X-100 (Sigma-Aldrich, USA) was used as a positive control. The results were expressed as a ratio of the OD at each time versus the OD at 0 h (in%). All assays were carried out in triplicate.

2.6. Effect on membrane leakage

A modified version of the method described by Carson et al. (2002) and Oonmetta-aree, Suzuki, Gasaluck, and Eumkeb (2006) was used to determine the effect of lupinifolin on bacterial cytoplasmic membranes by inducing loss of cytoplasmic materials. A suspension of *S. mutans* ATCC 25175 was prepared from the culture on BHI agar. The suspension was washed twice using a washing-centrifugation procedure (5000 revolutions per minute (rpm) for 5 min) with PBS then was resuspended in PBS (10 ml) and adjusted to 0.5 McFarland standard (approximately 1.5×10^8 cfu/ml) using Wickerham card. Lupinifolin was then added to the prepared bacterial suspension at final concentrations equivalent to 4MIC, 8MIC, and 16MIC. Then, samples (0.1 ml) were collected at 0, 1, 2, 4, 8, 12, 16, 20, and 24 h, and diluted with PBS (1:100). The diluted samples were filtered through a 0.2 μm pore-size filter. The optical density at 260 nm (OD_{260}) of the filtrates was determined. Filtrates of each concentration of lupinifolin (without bacteria) were