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Quercetin in a Lotus Leaves Extract May be Responsible for Antibacterial Activity

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In the course of a search for chemotherapeutic agents inhibiting suspected peridontitis bacteria, extracted and purified substances from lotus leaf were identified by antimicrobial activity tests with use of the broth micro-dilution methods on 96-microwell plate. The antimicrobial activity of extracts was tested against five microorganisms, namely: *Actinobacillus actinomycetemcomitans Y4, Actinomyces viscosus 19246, Porphyromonas gingivalis 33277, Fusobacterium nucleatum 25586,* and *Actinomyces naeslundii wvl 45*. The most active antimicrobial extract was subjected to spectroscopic analysis using UV, mass spectrometry, and by ^{1}H, ^{13}C-, nuclear magnetic resonance spectroscopy. Our data showed that the minimum inhibitory concentrations of the most active extract were 0.625, 1.25, 1.25, 0.625 and 2.5 mg/mL for *A. actinomycetemcomitans, A. viscosus, P. gingivalis, F. nucleatum,* and *A. naeslundii*, respectively. The component that had a greatest antimicrobial activity was determined to be quercetin. Thus, we conclude that quercetin extracted from lotus leaves may be a potential antibacterial agent for periodontitis.

Key words: Lotus leaf extracts, Periodontitis bacteria, Spectroscopic analysis

INTRODUCTION

Periodontal disease is usually seen as a chronic inflammatory disease. Several bacteria are thought to play a role in disease formation. These bacteria occur in periodontal lesions (aggressive and chronic periodontitis) and gingivitis. *Actinobacillus actinomycetemcomitans* is a gram negative bacterium that grows better in anaerobic conditions. *Actinomyces viscosus* has been regarded as a potential pathogen in oral infections because it adheres well to oral epithelial cells. *Actinomyces naeslundii* is a gram positive rod shaped bacterium which occupies the oral cavity and which has been implicated in periodontal disease. *Fusobacterium nucleatum* is a bacterium isolated from patients with periodontal disease and has great capacity for adhering to oral epithelial cells. *Porphyromonas gingivalis* is a gram negative oral anaerobe found in periodontal lesions.

The use of medicinal herb extracts for treatment of dental caries and periodontal disease is a well-known practice in traditional Chinese medicine. In the Chinese market, 55% are dentifrice-containing herbs. Investigators and manufacturers pay close attention to the study and development of herbs for oral health. Reports on the isolation and identification of natural, plaque-inhibiting substances from herbs have generated much interest in the development of various mouth rinses and dentifrices containing substances (Namba *et al*., 1982; Wolinsky *et al*., 1984; Wennstrom 1985; Wu-yuan *et al*., 1990; Kubo *et al*., 1992; Wu *et al*., 1993; Ooshima *et al*., 1994). Several toothpaste or mouth rinses made in China contain extracts from *Radix Zamthoxyli*, Chlorogenic Acid (extract from *Flos Lonicerae*), tea polyphenol (Namba *et al*., 1982; Kubo *et al*., 1992; Ooshima *et al*., 1994). However, hardly any in vitro microbiological research has been reported concerning the active ingredients of such products.

Traditionally, lotus leaf is used for treating mouth inflammation, tumescence and halitosis among civilians. We isolated and identified an antibacterial compound, a quercetin, from lotus leaves. The extract was evaluated for inhibition of growth of several oral reference microorganisms. The elucidation of the structure of the isolated compound was performed by spectroscopy (UV, EIMS, and NMR).

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The compound may be a potential antibacterial agent for periodontitis.

MATERIALS AND METHODS

Preparation of lotus leaf extracts

Dry lotus leaves were ground with a mill until they were reduced to 2 mm particles. They were macerated with deionized water (10 g/50 mL). The preparation was then decocted twice at 100°C × 10 min, remerged with water, and concentrated by lyophilization. The aqueous phase was exhaustively extracted twice in butanol and dried by decompression concentration. The remaining material after butanol extraction was dried and circumfluence extracted twice with 40% ethanol, once by filtration, and concentrated to dryness. Butanol extracts, 40% ethanol extracts and the remainder (which was insoluble in 40% ethanol) was dried and stored for further evaluation using antimicrobial activity tests.

Microbial species, culture conditions and agents

Five strains of bacteria susceptive involved in periodontal disease were used in the present study. These were (1) *Actinobacillus actinomycetemcomitans* Y4, (2) *Actinomyces viscosus* ATCC 19246, (3) *Porphyromonas gingivalis* ATCC 33277, (4) *Fusobacterium nucleatum* ATCC 25586, and (5) *Actino*myces *naeslundii* wvl 45. Green tea polyphenol (96% in purity, Zhong Ke Natural Plant Technology Company, Hangzhou, China), a well known antibacterial plant material, was used as a control, at 40 mg/mL in the original concentration.

Antimicrobial activity test

The antibacterial activity of each extract was assayed by using a broth micro-dilution method on 96-microwell plates based on recommendations of the National Committee for Clinical Laboratory Standards (NCCLS 1990). Stored bacteria that were previously stored at -80°C were reconstituted, identified and incubated anaerobically in 5 mL of pre-reduced Wilkins-Chalgren Anaerobe Broth (Oxford, England) in an incubator (Dual Gas Model, English) at 37°C for 24 h to a density equal to that of a no. 1 McFarland standard. Identification of bacteria was done by Gram staining, checking for aerotolerance, sensitivity to antibiotics, fermentation of carbohydrates, production of indoles and nitrates, and by a biochemical appraisal verification system (KLOBME - Key Laboratory of Oral Biomedical Engineering, Ministry of Education, China). The bacterial suspension was further diluted with anaerobic broth to give a final inoculum of 10^6 CFU/mL. For the evaluation of bacterial sensitivity, the study extracts and agents were dissolved in distilled water and sterilized by boiling for 5 min. Serial twofold dilutions of 80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 (mg/mL) were prepared with each anaerobe broth. Each microwell of a 96-microwell plate contained 50 μL of diluted agents and 50 μL of bacterial suspension. Final test volumes of 100 μL were dispensed into each well. In the final two ranks the microplate. The herbal agent as a negative control or only bacteria as a positive control. After 24 h of incubation in an anaerobic chamber at 37°C, bacterial growth was observed by stereoscopy and recorded. For each microwell, a 50 μL of treated suspension was removed and smeared on new anaerobe basal plates (Oxford, England). Bacterial colony counting was then carried out. For each extract we determined the minimum inhibitory concentration (MIC) for each individual bacterial strain. Assays were done in triplicate assays and the results are shown as means.

Purification of butanol extract and antibacterial sensitivity test

Butanol extracts were purified by chromatography on silica gel, under pressure (0.8 kg/cm^2) and a flow rate of 1 mL/min. Solvents for gradient elution were Cyclohexane: diethylether: Ethanol: Acetic acid mixtures proportion ranging from 3:1:0.5:0.02 to 1:1.5:0.5:0.02. The results were checked by thin-layer chromatography (TLC) (silica gel HSGF254 glass sheets). The whole extracts were divided into four fractions and each tested for antibacterial activity.

Characterization of the chemical structure of fraction-3

The chemical structure of fraction-3 was determined using spectroscopic analyses. This included UV, EIMS, ^{1}H-NMR and ^{13}C-NMR spectroscopy. For UV spectroscopy, a U-3000 (Hitachi, Japan) was used. EIMS was recorded on a Finnigan MAT 90 (Bremen, Germany) with electron ionization at 70 eV. Samples were dissolved in DMSO for proton NMR analysis. The NMR analyses were run on a Varian-Unity 300 spectrometer (^{1}H-NMR and ^{13}C-NMR at 300 MHZ and 75 MHZ).

RESULTS

All butanol extracts, 40% ethanol extracts and insoluble substances

All extracts showed antibacterial activity against periodontal disease causing bacteria (Table I). The extracts inhibited bacterial growth. Butanol extract exhibited greater antibacterial potency, with MICs ranging from between 1.25 to 5.00 mg/mL.

Substances purified from a butanol extract

Fraction-3 showed the greatest antibacterial activity against *A. actinomycetemcomitans, A. viscous, A. naeslundii,*

Table I. Results of sensitivity tests of lotus leaf extracts

Stains	Agents (mg/mL)			
	40% ethanol extract	butanol extract	40% ethanol insoluble Substances	green tea polyphenol (control)
Actinobacillus actinomycetemcomitans	10	5	20	2
Actinomyces viscosus	5	5	>20	2
Actinomyces naeslundii	1.25	1.25	1.25	0.5
Fusobacterium nucleatum	2.5	2.5	10	1
Porphyromonas gingivalis	5	5	20	2

Lotus leaf extracts inhibited bacterial growth. Butanol extracts exhibited the highest antibacterial activity against periodontal disease causing bacteria.

Table II. Sensitivity tests for purified butanol extracts of lotus leaves

Stains	Agents (mg/mL)			
	Fraction-1	Fraction-2	Fraction-3	Fraction-4
Actinobacillus actinomycetemcomitans	2.5	5	0.625	1.25
Actinomyces viscosus	1.25	5	1.25	1.25
Actinomyces naeslundii	1.25	5	1.25	1.25
Fusobacterium nucleatum	2.5	2.5	0.625	1.25
Porphyromonas gingivalis	2.5	10	1.25	2.5

Values shown are minimum inhibitory concentrations (MIC). Fraction-3, a butanol-extracted separated on silica gel showed the highest antibacterial activity.

F. nucleatum, and P. gingivalis (Table II). The MICs against these organisms were 0.625, 1.25, 1.25, 0.625, and 2.5 mg/mL, respectively.

Chemical structure characterization of fraction-3

Fraction-3 had a positive reaction on magnesium-hydrochloric acid interactions, a characteristic of flavonol compounds. The elucidation of its structure was done by spectroscopic (UV, EIMS, and NMR) methods. The UV spectrum showed λ_{max}=258 and 362 nm suggestive of the presence of a flavonol bond (Fig. 1). This was supported by the ^{13}C/NMR spectrum that revealed a characteristic peak value of δ60-102 ppm which can be assigned to flavonol (Fig. 2). Electron Impact Mass Spectrometry (EIMS) spectrua clearly indicated a characteristic peak value of M/e302, denoting the molecular ion of quercetin without any sugar components (Fig. 3). Mass Spectroscopy spectra indicated the presence of the molecular ion [$C_{15}H_{10}O_7$]. Furthermore, ^{1}H NMR spectroscopic analysis showed a characteristic peak (J=2.5 HZ) with value of (DMSO-D6) δ6.41 (H-8) ppm, implying the presence of a 5.7-dihydricflavone dihydrolavone in an A ring, which

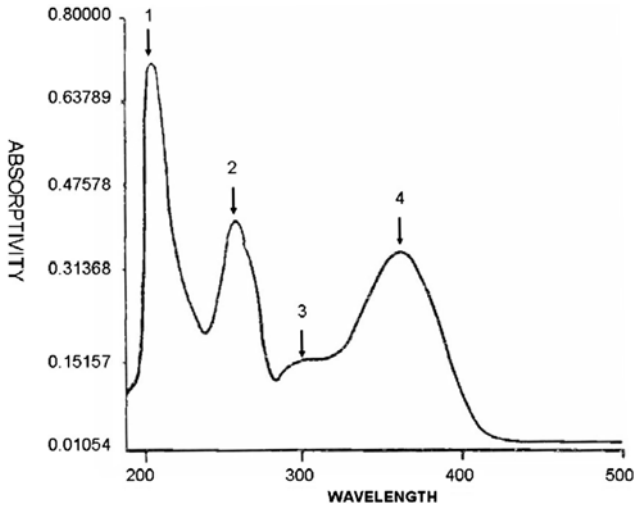

Fig. 1. Chromatogram of UV spectroscopic analysis of Fraction-3. Dried lotus leaves were hydrated and extracted using 40% butanol and 40% ethanol. The extracts and the residue were evaluated for inhibition of growth of several bacteria. The butanol-extracted material was separated into four fractions on silica gel. Fraction-3, the fraction containing the most potent inhibitor, was analysed by spectroscopic methods. The chromatograms show flavonol. Peak position and relative peak heights of annotated wavelengths are as follows: 1: Wavelength = 206 Result= 0.723953, 2: Wavelengths= 258 Result= 0.420609, 3: Wavelengths= 306 Result= 0.155807, 4: Wavelengths= 362 Result= 0.352341.

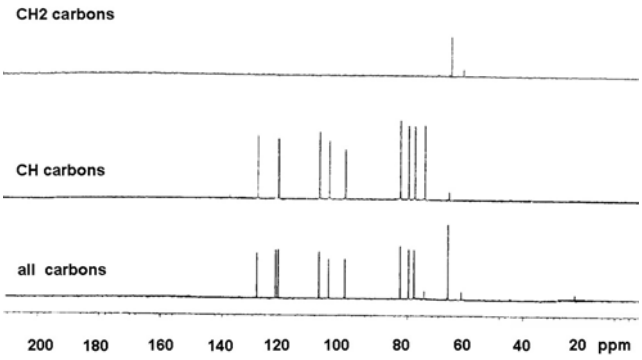

Fig. 2. ^{13}C-NMR spectrum of Fraction-3. The elucidation of the structure of Fraction-3 was done using NMR spectroscopy. The spectrum revealed correlation peaks at ä60-120ppm characteristic of a flavonol.

connects to no glycosides (Fig. 4). One peak at δ7.55 and peaks at δ7.68 in the H NMR data denoted C-2 and C-6, positions and suggested a 3, 4-dihydroxy substitution.

DISCUSSION

The Lotus plant Nelumbo nucifera Gaertn is a widely distributed plant in the Lakes Region of southern China. In Chinese traditional medicine (CTM) theory, it is claimed to have a number of properties, such as action on carbuncles,

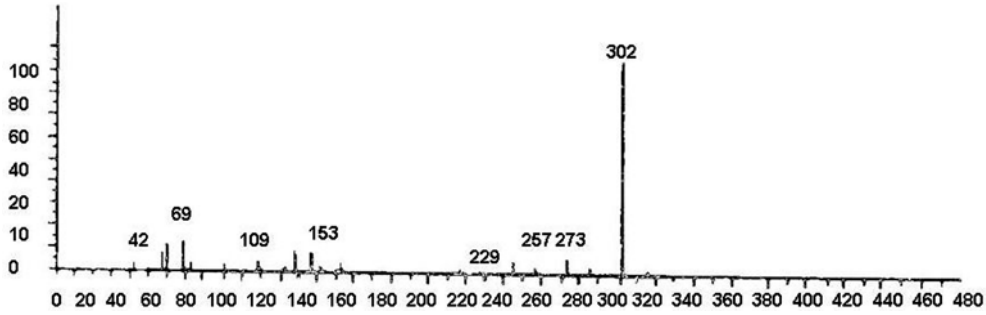

Fig. 3. Electron Impact Mass Spectrometry (EIMS) spectrum clearly indicating the characteristic peak value of M/e302, indicative of a flavonol.

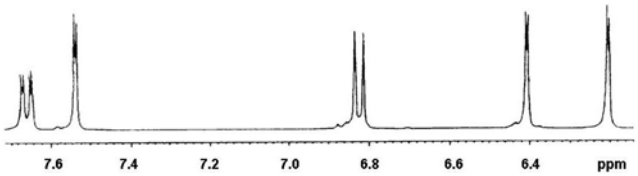

Fig. 4. Chromatograms from ^{1}H-NMR spectroscopic analysis of fraction-3. ^{1}H-NMR spectroscopic analysis showing the characteristic two peaks (J=2.5 HZ) value of (DMSO-D6) at δ6.02 (H-6) and δ6.40 (H-8) ppm which implying the presence of a 5.7-dihydroflavonol in the A ring, that connects to no glycosides. Two peaks at δ7.50 and four peaks at δ7.60 in the H-NMR data denoted C-2 and C-6, positions and suggested a 3, 4-dihydroxy substitution. Two complex peaks at δ5.30 were in a proton at C-1, characteristic of a quercetin.

sores, and eczema (Cui *et al*., 1996; Li *et al*., 1998). Traditionally, in Chinese folk medicine, lotus leaves are used to treat mouth inflammation, achieving detumescence, and for removing halitosis (Li *et al*., 2004). Before this study, to evaluate the possibility and efficiency of a Chinese herb as a new drug for preventing caries and periodontitis, investigators did comparative studies to determine whether Chinese herbs inhibited the growth of bacteria that were known as pathogens involved in caries and periodontitis. Results show that the Lotus leaf is better than other common herbs in that they have a greater effect and a lower costand thus deserves additional research (Xu *et al*., 2000). In the present study, the ability of some lotus leaf extracts to inhibit the growth of oral pathogenic bacteria was demonstrated. The most active antimicrobial extract, fraction-3, was characteristic of quercetin which chemical formula is $C_{15}H_{10}O_7$ and its molecular weight 302.24. Numerous flavonoids including quercetin or quercetin 3-O-glycosides have already been isolated from lotus leaves (Tian, 2007; Kashiwada, 2005; Wassel, 1996). Quercetin may have antioxidant, anti-inflammatory, immunemodulatory, anticancer and gastroprotective activities (Ng *et al*., 2003; Bathori *et al*., 2004; Kanadaswami *et al*., 2005). Rauha (Rauha *et al*., 2000) reported antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. The present study reports the antibacterial activity of a quercetin, isolated form the Lotus Leaf. Further studies are needed to evaluate the effectiveness of this compound as an antimicrobial agent.

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