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In vitro antibacterial activities of *Scutellaria baicalensis Georgi* against cariogenic bacterial

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Abstract *Scutellaria baicalensis Georgi* has been used for thousands of years in traditional Chinese medicine practice for several purposes. It possesses several biological activities such as anti-oxidative, anti-inflammatory, antibacterial and antiviral activities. Although the antibacterial activity of *Scutellaria baicalensis Georgi* has already been demonstrated, little is known about its antibacterial activity against oral pathogens *in vitro*. Therefore, the aim of this study is to evaluate the antibacterial activity by six different kinds of *Scutellaria baicalensis Georgi* extracts *in vitro*. The three kinds of bacterial strains were used as follows: *Streptococcus mutans* ATCC 25175, *Streptococcus sobrinus* ATCC 33478 and *Streptococcus salivarius* ATCC 7073. The antibacterial activity was determined by the agar diffusion method, and the zones of growth inhibition were measured. The MIC's and MBC's were determined by the broth dilution and agar dilution methods. The bactericidal activity was determined by time-kill assay. In all the *Scutellaria baicalensis Georgi* solvent extracts, except for water and ethyl acetate, a significant inhibitory activity was observed. The acetone and 80% ethanol, and the ethanolic extracts showed higher activity than the methanol extracts and produced inhibition zones ranging from 7.11 ± 0.18 to 14.79 ± 1.02 mm in diameter at a concentration of 750μg/disk. The MIC value of the *Scutellaria baicalensis Georgi* extracts ranged from 125 to 1,000μg/m*l*. The MBC values for different strains and extracts ranged from 250 to 2,000μg/m*l*. Thus, *Scutellaria baicalensis Georgi* would be useful for the suppression of oral pathogens, and has the potential for use in the prevention of dental caries.

Key words
Antibacterial activity,
Scutellaria baicalensis Georgi,
Streptococci

Introduction

Dental caries is one of the most common oral diseases worldwide[1]. *S. mutans* has been described as an 'obligate biofilm' organism[2–3] and strongly implicated as the principal etiological agent in human dental caries[4–5]. The ability of *S. mutans* to initiate dental caries depends on several virulence factors, including: the initiation of biofilm formation by adherence and accumulation on the tooth surface that is promoted by its synthesis of insoluble, extracellular polysaccharides, the high efficiency in catabolizing carbohydrates and producing acids, and the ability to grow and continue to metabolize carbohydrates at low pH[6–9]. Therefore, one of the strategies to prevent caries is to inhibit the growth and adherence of mutans streptococci, dental plaque formation and the expression of virulence factors. Many attempts have been made to eliminate mutans streptococci from the oral flora. Various antibiotics have been very effective in preventing dental caries[10–11]. They reduce the number of oral pathogens and inhibit dental plaque formation[11–12]. However, the excessive use of these chemicals can result in derangements of the oral and intestinal flora and

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cause undesirable side effects[12–13]. To overcome this problem, substances which could offer an alternative to antibiotics have been sought. Currently, biological agents from natural sources are receiving scientific attention as modifiers of the formation and development processes of dental caries, as well as their antimicrobial and affecting biofilms formation properties[14–23].

Scutellaria baicalensis Georgi is widely distributed through out the world, mainly in Asia, known as Huangqin in Chinese and Ogon in Japanese. It is officially listed in the Pharmacopoeia of the People's Republic of China and the Japanese Pharmacopoeia JPXIII. It is one of the most widely used traditional Chinese herbal medicines[24]. Its roots are a nontoxic nature product with multiple pharmacological effects and a complex chemical composition. It exhibits a wide range of biological activities, including anti-oxidative, anti-inflammatory, antibacterial and antiviral activities[25–30]. The antibacterial activity of the *Scutellaria baicalensis Georgi* extract has been studied by several authors[25,29], however, few studies have investigated its activity towards oral pathogens.

The objective of the present study was to investigate the antibacterial effect of the *Scutellaria baicalensis Georgi* (huangqin) extract on cariogenic bacterial.

Materials and Methods

Plant material

The *Scutellaria baicalensis Georgi* root was purchased from the Beijing Chinese Herbal Medicine Company, Beijing, China. The Dried powdered root of *Scutellaria baicalensis Georgi* was used for the preparation of the herbal extracts in acetone, ethanol, methanol, ethyl acetate and water as the extraction solvents. One thousand milliliters of the extraction solvent was added to 100 g of the ground herbal plant. The mixture was magnetically stirred at room temperature for 24 hours followed by filtration using a 0.45μm cellulose filter (Tokyo Roshi Co., Tokyo, Japan) and the solvent was concentrated at or below than 40°C (below 80°C for the water extracts) using a rotary evaporator (EYELA, NE-1, Tokyo Rikakikai Co., Ltd.). The sticky condensate was then freeze-dried. The weight of the solid residue was recorded and taken as the yield of the crude extract. The extracts were stored at −20°C and were freshly dissolved in suitable solvents just prior to screening for their antibacterial activity.

Microorganism

Streptococcus mutans ATCC 25175, *Streptococcus sobrinus* ATCC 33478 and *Streptococcus salivarius* ATCC 7073 were used for testing the antibacterial effect of the *Scutellaria baicalensis Georgi* extracts.

Medium

The brain heart infusion (BHI, Difco Laboratories, Detroit, MI, USA) was used for the culture of the streptococci bacterial strains. The Mueller-Hinton (Difco, MA, USA) agar, MS[4] agar, and BHI agar were used for the antibacterial assays. The MSB[4] agar was used for the time-kill assays. One percent dimethyl sulfoxide (DMSO) was used for dissolving the extracts of *Scutellaria baicalensis Georgi*.

Antimicrobial assay

A disk has been utilized for the disk diffusion susceptibility tests performed by the NCCLS[31,32] standardized methods. Briefly, Petri dishes were prepared with a base layer of Mueller-Hinton agar (Difco, MA, USA) (10 ml) and a top layer of 0.2% BHI agar (3 ml) inoculated with 30μl of each bacterial suspension (10^5 CFU/ml). The concentration of the suspension was standardized by adjusting the optical density to 0.6 at 600 nm (HITACHI U-1000 spectrophotometer). Sterile filter discs (6 mm in diameter) were impregnated with 15μl of dilutions of known extract concentrations (750μg/disc), the 99.9% extraction solvents, 1% dimethyl sulfoxide (DMSO) and water served as the negative controls, while 0.2% chlorhexidine gluconate was the positive control[10]. Each plate contained five paper discs with the plant extract and the controls. The same procedure was used for all the streptococci strains. The plates were diffused at 4°C for 2 h to allow diffusion of the extracts, and their subsequently incubated in an atmosphere of 95% nitrogen and 5% carbon dioxide at 37°C for 18 h. The antimicrobial activity was evaluated by measuring the diameter of the zone of growth inhibition around the disks. The growth inhibition diameter was an average of four measurements, taken in four different directions. All tests were performed in triplicate.

Determination of minimum inhibitory concentration (MIC) of the extracts

The MIC of the extracts was determined by the broth dilution method[33,34]. Serial two-fold dilutions

of the extracts were prepared in BHI broth (Difco Laboratories, Detroit, MI, USA) with concentrations ranging from 7.81 to 4,000μg/m*l* (water extract from 15.62 to 10,000μg/m*l*). A final volume is 2 m*l* in each tube. The test streptococci bacterial strains were inoculated into the tubes containing BHI broth and grown to log phase for 7 h at 37°C (OD = 0.6, 600 nm). A 100μ*l* aliquot of the standardized inoculum (2×10^5 CFU/m*l*) was added to each tube. The tubes were aerobically incubated at 37°C for 18 h. Two control tubes were maintained for each test batch. These included the antibiotic control (tube containing extract and the growth medium without inoculum) and organism control (the tube containing the growth medium, physiological saline and the inoculum). The MIC values were interpreted as the highest dilution (lowest concentration) of the sample, which showed a clear fluid with no development of turbidity. All tests were performed in triplicate.

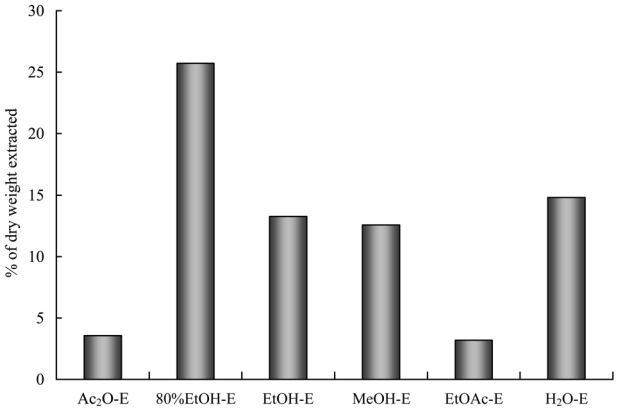

Fig. 1 Percentage of 100 g dried root of *Scutellaria baicalensis Georgi* extracted with 1,000 m*l* of acetone (Ac_2O), 80% ethanol (EtOH), Methanol (MeOH), ethyl acetate (EtOAc) and water (H_2O)

Determination of minimal bactericidal concentration (MBC) of the extracts

The agar dilution method was used to determine the minimal bactericidal concentration (MBC). These assays were performed by a modification of that described by a previously published method[35]. Briefly, 2 m*l* of the extracted solutions at the different concentrations (0.78 mg/m*l* to 40 mg/m*l*) was mixed with 18 m*l* of sterile MS agar in a conical flask. Solutions of the extract were mixed with agar which was autoclaved and allowed to cool to 48–50°C. The final concentrations of extract in the MS agar were 0.078 mg/m*l* to 4 mg/m*l*, respectively. The mixture was then magnetically stirred to obtain a homogenous mix. Two milliliters of 10% dimethyl sulfoxide (DMSO) and water without the added extract was used as the routine control. It was carefully mixed and poured into the agar plates immediately on a level surface. The plates should be used on the same day or kept them wrapped in plastic in a cold room. The surface of the agar plates must be completely dry before inoculation. Mark the plates for orientation. The test microorganisms were inoculated into the tubes containing the BHI broth and grown to log phase for 7 h at 37°C (OD = 0.6, 600 nm). A 100μ*l* aliquot of the standardized inoculum (2×10^5 CFU/m*l*) was added to each plate. Start by inoculating a plate without extract (growth control), and continue with the plate containing the lowest concentration of extract and then those with the highest concentrations. A second control plate was inoculated at the end. Leave the plates to dry at room temperature before incubating them at the desired atmosphere and temperature. The plates were subsequently incubated in an atmosphere of 95% nitrogen and 5% carbon dioxide at 37°C for 48 h. To establish the MBC's, a sample swab was taken from the area with no bacterial growth and streaked onto an extract-free MS agar plate. The MBC represented the concentration of extracts that killed at least 99.9% or more of the initial inoculum[34]. All assays were repeated three times. The MBC values were then determined.

The time-kill assay

The time-kill curve method was used to study the bactericidal effects of the extracts of *Scutellaria baicalensis Georgi*. Test tubes containing 4 m*l* volumes of BHI were supplemented with the extracts of *Scutellaria baicalensis Georgi* at concentrations equal to 1x and 2x multiples of the MIC. Aliquots of the overnight broth cultures of *S. mutans* ATCC 25175 were inoculated into the tubes to obtain a final inoculum of 2.0×10^6 CFU/m*l* (OD 600 = 0.62) and were incubated for 24 h. Samples of 100μ*l* were collected from each tube at T = 0, 1, 3, 6, 9, 12, 18 and 24 h post inoculation and plated onto MSB agar. The plates were subsequently incubated in an atmosphere of 95% nitrogen and 5% carbon dioxide at 37°C for 48 h, when the colony forming units (CFU's) were determined. Killing curves were constructed by plotting the $\log_{10}$CFU per milliliter versus time over 24 h. All of the assays were done

Table 1 Antibacterial assay of the extracts of *Scutellaria baicalensis Georgi*

Test compound	Inhibition Zone (mm, Mean ± SD)		
	S. mutans	*S. sobrinus*	*S. salivarius*
Ac_2O extract	14.79 ± 1.02	9.05 ± 2.04	9.62 ± 0.64
80% EtOH extract	13.70 ± 1.23	8.25 ± 0.92	8.83 ± 0.48
EtOH extract	11.06 ± 1.22	7.11 ± 0.18	8.27 ± 0.41
MeOH extract	8.39 ± 0.57	—	—
EtOAc extract	—	—	—
H_2O extract	—	—	—
1% DMSO	—	—	—
0.2% CHX	16.63 ± 2.45	12.58 ± 2.64	14.55 ± 0.77

Ac_2O, acetone; EtOH, ethanol; MeOH, methanol; EtOAc, ethyl acetate; H_2O, distilled water; DMSO, dimethyl sulfoxide; CHX, chlorhexidine; —: no growth inhibition was observed. Values represent mean ± SD of three separate experiments. Disks (6 mm) soaked with 15 μl of the 50 mg/m*l* extracts of *Scutellaria baicalensis Georgi* (750 μg/disk).

Table 2 The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of different solvent extracts of *Scutellaria baicalensis Georgi* against the streptococci strains

	Microorganism					
	Streptococcus mutans ATCC 25175		*Streptococcus sobrinus* ATCC 33478		*Streptococcus salivarius* ATCC 7073	
Solvent extracts	MIC[a]	MBC[a]	MIC	MBC	MIC	MBC
Ac_2O extract	125	250	250	500	500	1,000
80% EtOH extract	125	250	250	500	500	1,000
EtOH extract	500	1,000	500	1,000	500	1,000
MeOH extract	500	1,000	500	1,000	500	1,000
EtOAc extract	1,000	2,000	1,000	2,000	1,000	2,000
H_2O extract	>10,000	—	>10,000	—	>10,000	—

[a]: The MIC and MBC values are expressed in μg/m*l*. The concentration of the extracts ranged from 7.81 to 10,000 μg/m*l*. Ac_2O, acetone; EtOH, ethanol; MeOH, methanol; EtOAc, ethyl acetate; H_2O, distilled water; —: not tested.

in quadruplicate on at least three occasions using two growth controls (BHI and BHI with 1% v/v DMSO).

Results

Quantity and rate of extraction

The results are shown in Fig. 1. Eighty percent ethanol (80% EtOH) and water (H_2O) extracted the most material, i.e., 25.72% and 14.79% of the dry weight of the *Scutellaria baicalensis Georgi*. The next was with ethanol (EtOH) followed by methanol (MeOH), acetone (Ac_2O) and ethyl acetate (EtOAc), which extracted similar quantities.

The antibacterial activity of the *Scutellaria baicalensis Georgi* extracts

The antibacterial activity of six solvent extracts of the *Scutellaria baicalensis Georgi* was studied using the agar diffusion method and the results are shown in Table 1. All the *Scutellaria baicalensis Georgi* solvent extracts, except for water and ethyl acetate, showed a significant inhibitory activity. Inhibition was observed against the *S. mutans* ATCC 25175, *S. sobrinus* ATCC 33478 and *S. salivarius* ATCC 7073 bacterial strains. The acetone and 80% ethanol, and the ethanol extracts showed a higher activity than the methanol extracts and produced inhibition

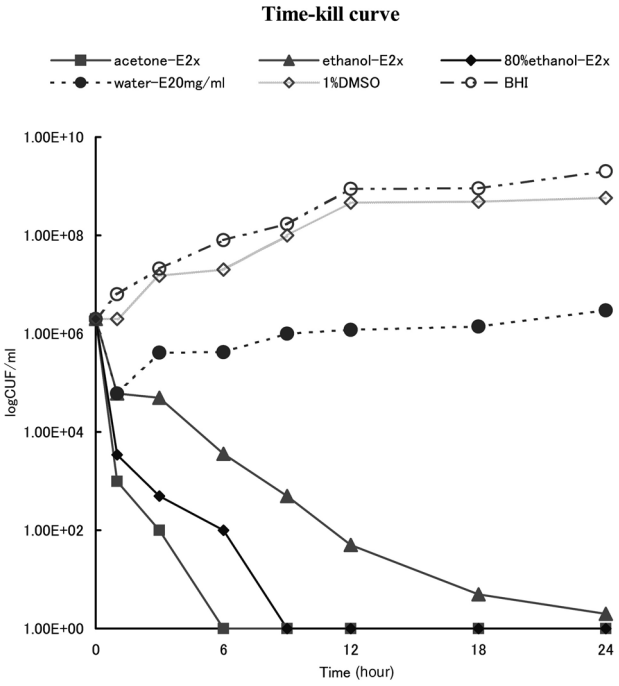

Fig. 2-1 Different extracts of *Scutellaria baicalensis Georgi* at concentrations equal to 2x MIC inhibits growth of *S. mutans* ATCC 25175

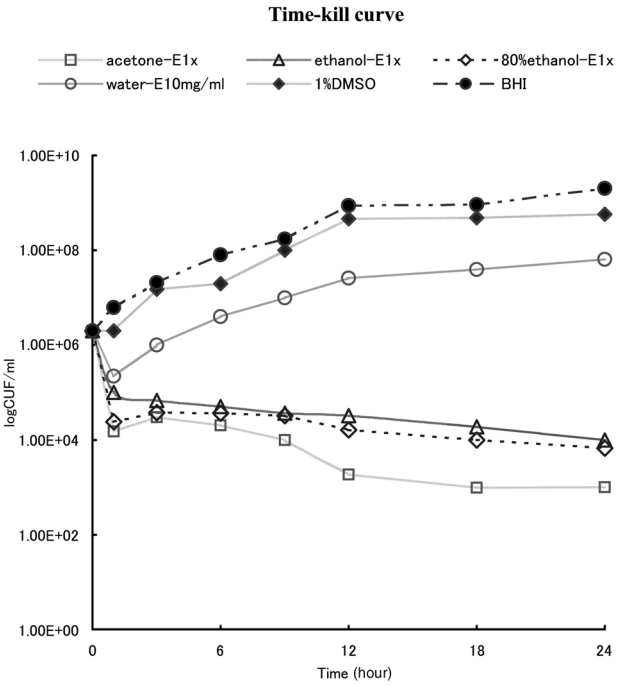

Fig. 2-2 Different extracts of *Scutellaria baicalensis Georgi* at concentrations equal to 1x MIC inhibits growth of *S. mutans* ATCC 25175

zones ranging from 7.11 ± 0.18 to 14.79 ± 1.02 mm in diameter at a concentration of 750μg/disk.

MIC's and MBC's

The MIC and MBC values of the different solvent extracts of *Scutellaria baicalensis Georgi* against the streptococci strains shown in Table 2 indicate that the extracts exhibited the following order of potency for the streptococci strain growth inhibition: Ac_2O extract > 80% EtOH extract > EtOH extract > MeOH extract > EtOAc extract > H_2O extract. *S. mutans* was more susceptible to the extracts of *Scutellaria baicalensis Georgi* than *S. sobrinus* and *S. salivarius*. As excepted, the bactericidal concentration of the extracts was higher (two times) than the MIC values.

The time-kill assays

The bactericidal activity of *Scutellaria baicalensis Georgi* was evaluated *in vitro* by the time-kill experiments. The results of the time-kill kinetic studies are summarized in Fig. 2-1. The $\geq 2 \log_{10}$CFU/m*l* decrease in the initial inoculum at 6 h was the definition of being bactericidal. The acetone, 80% ethanol and ethanol extracts of *Scutellaria baicalensis Georgi* at concentrations equal to two-fold the MIC (or MBC) rapidly reduced the viable counts of *S. mutans* within 1, 3, and 6 h incubations (reduction of 2 log in the number of CFU per milliliter) on *S. mutans*. Water extracts of *Scutellaria baicalensis Georgi* (10–20 mg/m*l*) reduced only within 1 h of incubation (reduction of 1 log in the number of CFU per milliliter). The acetone, 80% ethanol and ethanol extracts of *Scutellaria baicalensis Georgi* at concentrations equal to the MIC exerted bactericidal effects (a > 2 log decrease in the number of CFU per milliliter) on *S. mutans* after 24 h incubation (Fig. 2-2).

Discussion

It is well known that mutans streptococci are the major etiological agents in dental caries; both *S. mutans* and *S. sobrinus* produce acids, and extra cellular glucans and fructans from sucrose, which are critical factors in the expression of virulence by these microorganisms[4]. Hence, controlling *S. mutans* and/or inhibiting the growth, adherence, dental plaque formation, and the expression of the virulence factors of the mutans streptococci can accomplish the prevention of dental cavities. In nature there are a large number of different types of antimicrobial

compounds that play an important role in the natural defense of all kinds of living organisms. This study focused on *Scutellaria baicalensis Georgi*. It has been widely used for thousands of years in China and other countries. Its mechanism of antimicrobial activity, though not completely understood, seems to be complex and depend on its composition. *Scutellaria baicalensis Georgi* contains a variety of flavones, phenylethanoids, amino acids, sterols and essential oils. Its dried roots contain over 30 kinds of flavonoids, baicalin, baicalein, wogonin and oroxylin A that are the main biologically active compounds in *Scutellaria baicalensis Georgi*[36–38]. Plant extracts generally contain flavonoids in the glycosidic form. This may be the reason why the plant extracts did not produce as significant an inhibition as many of the pure compounds. In spite of this, the extracts of *Scutellaria baicalensis Georgi* have shown significant activity against *S. mutans* growth *in vitro* when compared to its control and the inhibitory zone for every group of bacterial strains tested. The size of the zones of the growth inhibition was variable, depending on the strain tested, although this does not necessarily mean that one bacterial strain is more susceptible than another.

In our study, acetone and 80% ethanol were proved to be good solvents in inhibitory extraction from *Scutellaria baicalensis Georgi*. The acetone and 80% ethanol extracts showed inhibition zones against *S. mutans* at lower doses (750μg/disc) while other extracts were only active at double the concentration (data not shown). Acetone was much easier to use and is the volatility, miscibility with polar and no-polar solvents and its relatively low toxicity to the test organisms[39].

In conclusion, the present results demonstrated that the extracts of *Scutellaria baicalensis Georgi* was effective for killing *S. mutans*. At the same time, the present data proved that the extracts of *Scutellaria baicalensis Georgi* contain bioactive compounds that possess an antibacterial activity *in vitro*.

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