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Antioxidant and antibacterial activity of two compounds (forsythiaside and forsythin) isolated from *Forsythia suspensa*

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Abstract

Forsythia suspensa (Thunb.) Vahl. has been widely used in traditional medicines in Asia to treat gonorrhoea, erysipelas, inflammation, pyrexia, ulcer and other diseases. Recently the investigation has been focused on the antioxidant and antibacterial activity of this plant. However, limited scientifically proven information is available. We isolated two compounds (forsythiaside and forsythin) from this plant. The aims of this investigation, therefore, were to assay antioxidant activity and antibacterial properties of the two main and distinctive compounds isolated and to exploit antioxidants and antibacterial agents from natural compounds. The antioxidant activity was estimated using the 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity method and the in-vitro antimicrobial activity was evaluated by microtitre plate method. Forsythiaside was found to possess strong antioxidant and antibacterial activity but forsythin was much weaker. Owing to these properties, the study can be further extended to exploit the possible application of forsythiaside as an alternative antioxidant and antibacterial agent of natural origin.

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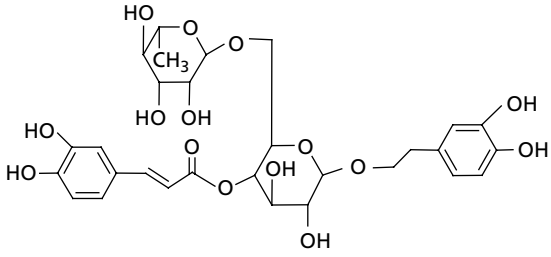
Introduction

Many research works (Conklin 2000) have mentioned the disadvantage of synthetic antioxidants and their possible injurious properties for human health in addition to their possible toxicity, and general consumer rejection has led to their decreasing use. Likewise, antibiotic resistance is another tough problem for all medical experts. Consequently, the development of alternative antioxidants and antibacterial agents of natural origin has attracted considerable attention during the past decade (Koop 2002). A systematic search for useful bioactivity from medicinal plants is now considered to be a rational approach in nutraceuticals and drug research. According to a conservative estimation, 300 000–400 000 plant species grow on Earth; however, only a small percentage have had their phytochemistry and biological function investigated (Kitani et al 2001).

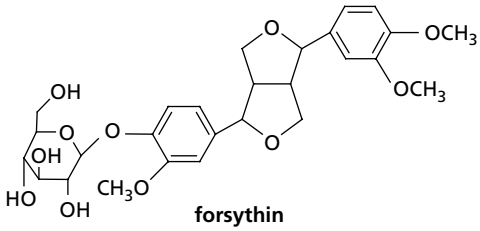
Forsythia suspensa (Thunb.) Vahl. is widely distributed in China, Korea, Japan and many European nations. Its fruits have long been used in Chinese and Japanese folk medicine to treat gonorrhoea, erysipelas, inflammation, pyrexia and ulcer (Nishibe et al 1992; Ozaki et al 1997). Its leaves are used as tea in the Orient for health supplements. This plant is also cultivated for its beautiful yellow flowers. *F. suspensa* is one of the few herbal medicines that has been confirmed to have anti-inflammatory, antibacterial and antiviral activity by Western medical experts (Li & Zhang 2006).

Though recent study has been focused on the antioxidant and antibacterial activity of *F. suspensa* (Ming et al 1998; Li & Cheng 2005), limited scientifically proven information is available on its bioactivity and pharmacological functions. There are different opinions about the main bioactive components of this plant (Ming et al 1998; Zhang et al 2003; Zhao et al 2005).

In this study, we isolated two distinctive compounds (forsythiaside and forsythin, Figure 1) from *F. suspensa*. Then we estimated the antioxidant activity using the DPPH• scavenging activity method, and the antimicrobial activity against *Escherichia coli, Pseudomonas and Staphylococcus aureus* was evaluated by microtitre plate method.



forsythiaside



forsythin

Figure 1 Structures of isolated compounds.

The aims of this investigation were: firstly, to assay antioxidant activity and antibacterial properties of the two isolated compounds; secondly, to determine the contents of the two compounds in different parts of *F. suspensa* MeOH extracts and compare the free radical scavenging potentials of different parts; and, thirdly, to identify and determine major bioactive components in this plant contributing to its antioxidant and antibacterial activity. The ultimate goal of our study was to develop safe, effective, inexpensive and alternative antioxidants and antibacterial agents of natural origin (Amakura et al 2000; Häkkinen et al 2000; Kalt 2005).

Materials and Methods

Reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co. (St Louis, MO). Vitamin C was purchased from Dengfeng Chemical Co. (Tianjin, China). Tetracycline was purchased from Amresco Inc. (USA). All the other solvents used were of analytical grade and obtained from Xi'an Chemical Co. (Xi'an, China).

Plant material

Dried fruits of *F. suspensa* used for isolation in this study were purchased from the local market, in 2006. The roots, barks, branches, leaves, flowers, fruits, seeds and fruit shells of *F. suspensa* were collected in Northwest University, Xi'an, China, in 2006. The plant materials were identified by Professor Yazhou Wang, Northwest University, Xi'an 710069, China. Voucher specimens have been deposited in the Biology and Medicine Key Laboratory of Shaanxi province, China.

The plant materials were powdered in a grinder to get 20 mesh size powders.

Microorganisms

The bacterial strains *Escherichia coli*-10B, *Pseudomonas aeruginosa* and *Staphylococcus aureus*-Rn4220 were tested for purity by Gram staining and by biochemical tests. The strains were kept at −70°C in LB agar, activated by transferring into nutritive agar, and incubating at 37 ± 1.0°C for 18 h.

Isolation of forsythiaside and forsythin

The fruits of *F. suspensa* (250 g) were extracted with 75% EtOH (2 L), 60°C, four times, and the solutions were combined and concentrated under vacuum. The concentrated solutions were chromatographed over polyamide (100–120 mesh) column eluted with EtOH–H_2O gradient solvent system. Fractions with similar R_f values by TLC were evaporated and combined to give 6 fractions (F1–F6). Fraction F2 (10 g) was subjected to column chromatography over silica gel 60 eluted with EtOAc–MeOH in turn to obtain forsythin (132 mg).

Fraction F3 (40 g) was also subjected to silica gel column using the same method as above; fractions with similar R_f values by TLC were evaporated and combined to give 5 fractions (F3.1 ~ F3.5). Fraction F3.4 (240 mg) was thereafter explored by preparative HPLC (Amersham Biosciences, Switzerland) using a C18, 5 μm column and eluted with methanol–water (21:79, v/v) for 100 min, followed by a washing and a reconditioning of the column. After several successive injections, samples corresponding to the same chromatographic peaks were controlled by analytical HPLC, concentrated under reduced pressure and lyophilized to obtain forsythiaside (177 mg).

The structures of the two isolated compounds were characterized by UV, IR, ^{1}H NMR, ^{13}C NMR and MS in comparison with the data in references (Liang 2003). Their purities were determined to be more than 98% by reversed-phase HPLC.

HPLC analysis

The two isolated compounds (forsythiaside and forsythin) were used as reference compounds for the HPLC analysis. They were accurately weighed, dissolved in methanol–water (60:40, v/v) solution and diluted to appropriate concentration. Stock solution of the mixture of the two references, containing forsythiaside (0.99 mg mL^{-1}) and forsythin (0.44 mg mL^{-1}) was stored in the refrigerator at 4°C. The solution was brought to room temperature and filtered through a 0.45-μm membrane filter before HPLC analysis.

Each powder (1 mg) of different parts of *F. suspensa* was ultrasound-extracted with methanol (30 mL) for 30 min, then added to 20 mL double-distilled water. Last, the samples were ready for HPLC analysis after being filtered by means of a Millipore filter (0.45 μm).

The liquid chromatographic system consisted of Beckman-125 model (Beckman Corp, USA), fitted with a Diamonsil-C18 (4.6 mm × 250 mm, 5 μm) column (Dikma Technologies, China) and a 32Karat system controller. The injection system used a system gold 508 auto sampler. Detection was performed

by a UV-visible spectrophotometer PDA-168 set at a sensitivity of 0.02 AUFS and a wavelength of 270 nm. Column temperature was held at 27°C. Elution was carried out at a flow rate of 1 mL min^{-1} with mobile phases consisting of (A) water and (B) methanol. The gradient elution was as follows: 0–14 min, 34.6% B, 14–15 min, 34.6–43.5% B, 15–23 min, 43.5% B, 23–24 min, 43.5–45% B, 24–34 min, 45% B, 34–36 min, 45–80% B, and 36–40 min, 80% B.

The two standard calibration curves showed a high degrees of linearity ($r^2 > 0.99$). Sample compounds were identified on the basis of the retention times of standard materials and were quantified by comparing their peak areas with those of standard curves.

Determination of antioxidant activity with the DPPH radical scavenging method

DPPH• scavenging activity was determined using a modified method of Brand-Williams et al (1995). Different volumes of MeOH solution of forsythiaside, forsythin, vitamin C (0.36, 5.66, 0.42 mg mL^{-1}) and MeOH extracts of different parts of *F. suspensa* were placed in different test tubes. Methanol solution of DPPH• (3 mL, 31.52 μg mL^{-1}) was added to each tube and shaken vigorously. The volume was adjusted to 5 mL by adding MeOH (Zafrilla 2001; García-Alonso et al 2003). The experiment was tested with MeOH as control and vitamin C as antioxidant reference (Zschunke 2000; Kim & Lee 2004), and MeOH was used for the baseline correction. The tubes were allowed to stand at 27°C in the dark for 60 min. Changes in the absorbance of the samples (ABS) were measured with a UV-Vis spectrophotometer (Hitachi Ltd, Tokyo, Japan) set at 516 nm. Each experiment was performed in triplicate. The radical stock solution was prepared fresh daily (Chang et al 2001; Wang et al 2002; Friaa & Brault 2006).

Radical scavenging activity (percentage) was calculated using the following formula:

$$\text{DPPH radical scavenging activity } (\%) = [(\text{ABScontrol} - \text{ABSsample})/\text{ABScontrol}] \times 100 \quad (1)$$

The data were presented as the mean of triplicates and the EC50 value of DPPH• was obtained graphically (concentration of antioxidant necessary to decrease the initial DPPH• concentration by 50%). The lower the value, the stronger the antioxidant activity.

Determination of the in-vitro antimicrobial activity of forsythiaside and forsythin by the microtitre plate method

The antimicrobial activity against *E. coli, P. aeruginosa* and *S. aureus* of different concentrations of forsythiaside and forsythin were determined by the microtitre plate method described by the USP (USP 2006). A two-fold microdilution broth method (Friedman et al 2002; Burt 2004; Ooi et al 2006; Shan et al 2007) was used to determinate the minimum inhibitory concentration (MIC) value. Each well contained $\sim 10^6$ CFU mL^{-1} of test bacteria and LB medium (100 μL). MeOH solution of forsythiaside, forsythin and tetracycline (100 μL; 0.46, 8.10, 0.50 mg mL^{-1}, respectively) were added to wells of the first row. Dilutions were used to dispense 100 μL into the other sterile 96 wells of a standard tray using a multi-channel micropipette, resulting in 8 concentrations to be tested for each compound. A control containing tetracycline and a positive control containing inoculated growth medium without test samples were prepared. Each experiment was performed in triplicate. The MIC value is a measure to define the antibacterial activity of a compound and is defined as the lowest concentration of drug that inhibits visible growth. The amount of growth in the wells containing test samples was compared with the amount of growth in the control wells when determining the growth end points. When a single skipped well occurred, the highest MIC was read.

The plates were agitated in a shaker (THZ-82, Guohua Co., China) at 200 rev min^{-1}, 37 ± 1.0°C, for 24 h. The absorbance at 595 nm was taken using a microplate reader (Wallac Victor3 1420 multilabel counter, Turku, Finland).

Statistical analysis

One-way analysis of variance was performed on the data using SPSS (release 11.0). Student's *t*-LSD (least significant difference) ($P = 0.05$) was calculated to compare the means of the different samples.

Results and Discussion

Content of forsythiaside and forsythin in different parts of *F. suspensa*

The content of forsythiaside and forsythin was determined using HPLC. Typical HPLC chromatograms are shown in Figure 2. The contents of the two chemical constituents in different parts are presented in Table 1. There was a much

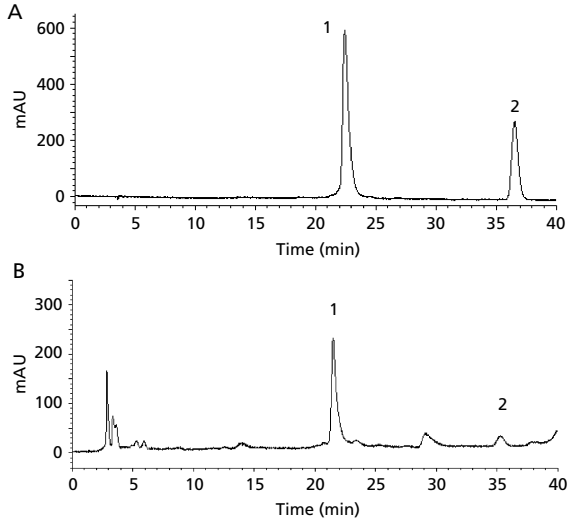


Figure 2 HPLC chromatograms of reference substances (A) and samples (*F. suspensa* fruits) (B). 1, forsythiaside; 2, forsythin.

Table 1 Results of content determination and free radical (DPPH•) scavenging activity (EC50) of different parts of *F. suspensa*

Part	Content (%)[a]		EC50 (μg mL^{-1})
	Forsythiaside	Forsythin	
Root	0.573 ± 0.005A	0.676 ± 0.001A	89.75
Bark	7.421 ± 0.096B	0.721 ± 0.012B	60.78
Branch	0.000 ± 0.000C	0.000 ± 0.000C	B[b]
Leaf	3.170 ± 0.032D	2.159 ± 0.020D	71.52
Flower	3.161 ± 0.035D	0.000 ± 0.000C	66.25
Fruit	3.783 ± 0.001E	0.365 ± 0.004E	54.91
Seed	4.717 ± 0.003F	0.467 ± 0.006F	43.05
Fruit shell	0.257 ± 0.002G	0.043 ± 0.001G	364.75

[a]Mean of triplicates. Mean values (± standard deviations) with different letters in the same columns are significantly different ($P < 0.05$) from one another. [b]Beyond the range

greater amount of forsythiaside in the barks, leaves, flowers, fruits and seeds than in the roots, branches and fruit shells. The content of forsythin from leaves was much higher than from the other parts. The content of forsythiaside and forsythin from branches was so small as to be undetectable.

In all, there were many differences in the content of forsythiaside and forsythin in the different parts.

Antioxidant activity

There are numerous methods for the measurement of antioxidant activity of biological materials. Most well-known chemical assays among them are mainly based on the ability to scavenge various kinds of free radicals. Free radicals cause autoxidation of unsaturated lipids in food. On the other hand, antioxidants are believed to intercept the free radical chain of oxidation, thereby forming a stable end product, which does not initiate or propagate further oxidation of the lipid (Sherwin 1978). In this research, the free radical scavenging potential of the isolated compounds at different concentrations were tested by the DPPH• method (Figure 3). The degree of discoloration indicates the scavenging potentials of the compound. The EC50 for DPPH• was obtained and is shown in Table 2.

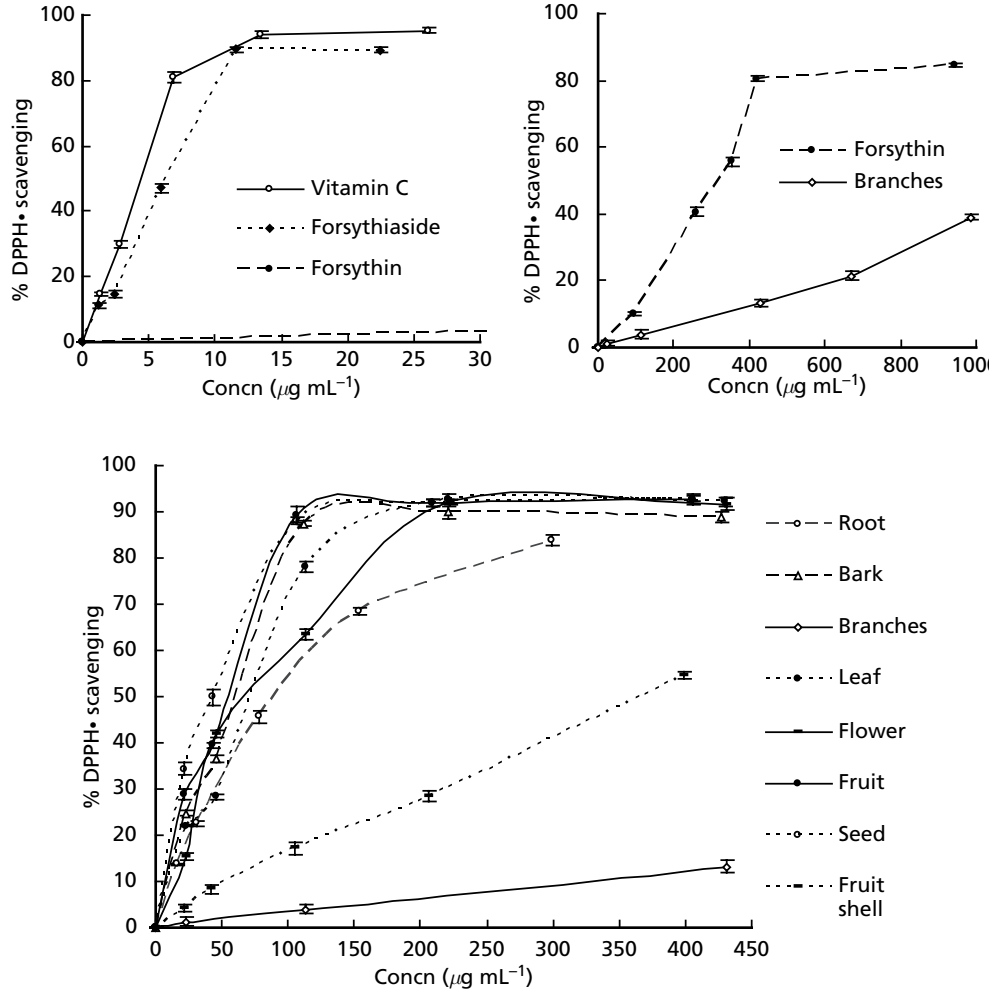


Figure 3 Scavenging effect of samples on DPPH radicals.

Table 2 Free radical (DPPH•) scavenging activity (EC50) and antibacterial activity (MIC) of different components of *F. suspensa*

Property	Component		Vitamin C	Tetracycline
	Forsythiaside	Forsythin		
Antioxidant activity				
EC50 value (μg mL^{-1})	6.30	317.69	4.38	n
Antibacterial activity				
MIC value (μg mL^{-1})				
E. coli	38.33	na	n	29.76
P. aeruginosa	38.33	na	n	29.76
S. aureus	76.67	na	n	119.05

n, not determined; na, no activity.

The results showed that forsythiaside demonstrated a notable antioxidant activity. The scavenging efficacy of forsythin was much weaker, in contrast with previous data (Zhao et al 2005). The significance of phenolic compounds as dietary antioxidants has been highlighted in recent years (Cao et al 1996; Prior et al 1998; Velioglu et al 1998). In a study by Zhang et al (2003), the authors suggested that the better uniformity of the spin densities distribution of half-quinone free radical was the essential cause that accounted for the better antioxidation of forsythiaside. Accordingly, the antioxidation of polyphenols containing ortho-substituting hydroxyl is better than that of those containing meta-substituting hydroxyl. The amount of phenol hydroxyl and amount of intramolecular H-bonding in a molecule plays a major role in the antioxidation of the molecule, which contributes to the important factor of the strong antioxidation of polyphenols. Many researchers (Zhang et al 2003; Li et al 2006; Zhang & Wang 2007) have reported the strong antioxidation of rutin, quercetin and chlorogenic acid. These compounds are common natural phenolic antioxidants and they all have ortho-substituting hydroxyl structures.

In continuation of our work, we sequentially evaluated the antioxidant activity of different parts of *F. suspensa* extracts, and the results are shown in Figure 3. The results of EC50 were summarized in Table 1. The antioxidant activity followed the order: fruits > seeds > flowers > barks = leaves > roots > fruits shells > branches. There are significant differences in antioxidant activity among the different part extracts. This indirectly indicated that the antioxidant activity of the different parts mainly depended on the content of forsythiaside in them.

In-vitro antimicrobial activity of forsythiaside and forsythin

When studying the influence of the concentration of forsythiaside and forsythin on the antimicrobial activity against *E. coli, P. aeruginosa* and *S. aureus*, we used a two-fold microdilution broth method. The data are represented graphically in Figure 4. It was observed that as the concentration increased, there was an increase in the inhibition of the bacterial growth, albeit at different rates depending on the compound.

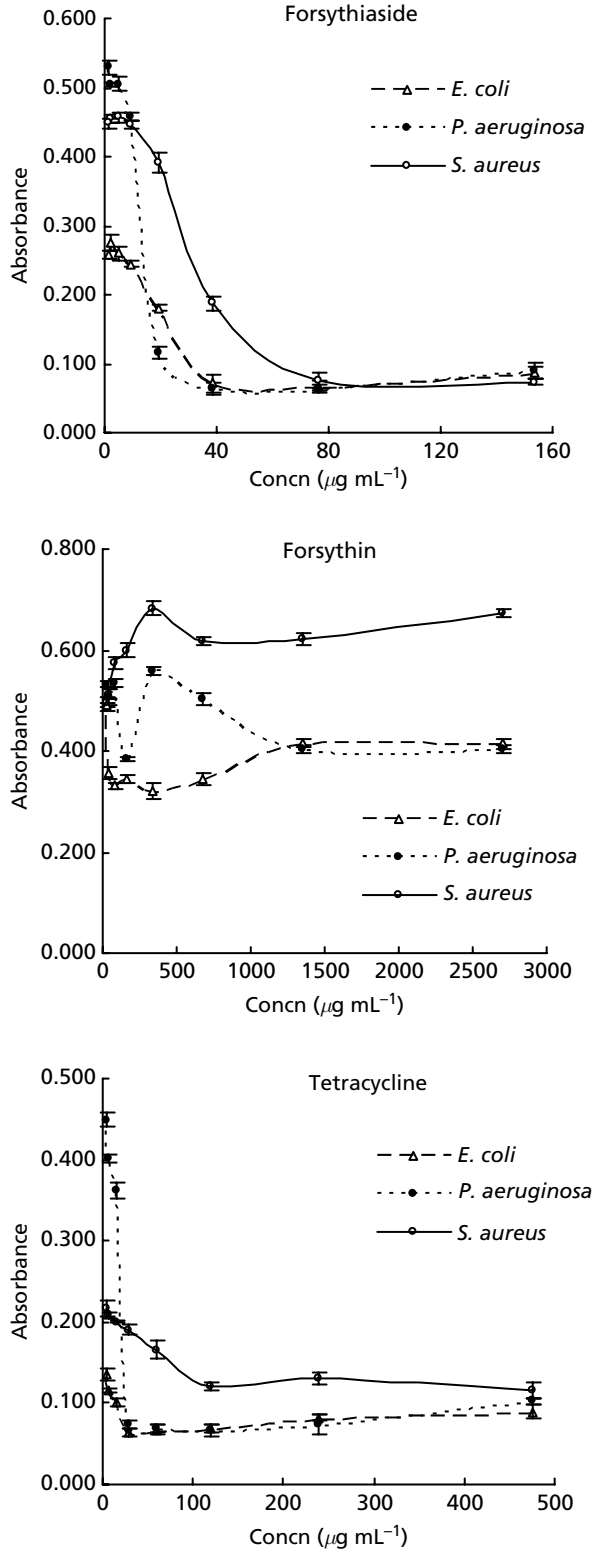


Figure 4 Antibacterial activity of different concentrations of forsythiaside, forsythin and tetracycline.

The MIC data of the two compounds and tetracycline are presented in Table 2. The results showed forsythiaside had comparatively high antibacterial activity against the three common bacteria. Its MIC value against *S. aureus* was even lower than that of tetracycline. Forsythin, did not show any activity against the three selected bacterial strains. This was the first study that compared the antibacterial activity of forsythiaside with the activity of forsythin.

Conclusion

The antioxidant activity data obtained for the two compounds suggested that forsythiaside acts as an antioxidant. It contributed more than 50% of the antioxidant activity of *F. suspensa*. Additionally, forsythiaside presented strong antibacterial activity against the three common selected bacteria, and was even more effective against *S. aureus* than tetracycline. However, the activity of forsythin was not remarkable.

F. suspensa is one of the plant medicines that has been accepted not only by oriental medical experts but also Western experts. We present evidence of how forsythiaside could have a beneficial role as an antioxidant or antibacterial agent. Further studies are needed on the isolation and characterization of other bioactive compounds in *F. suspensa*.

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