

# Isoforsythiaside, an antioxidant and antibacterial phenylethanoid glycoside isolated from *Forsythia suspensa*

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## ABSTRACT

The isolation, structural elucidation, antioxidant effect and antibacterial activity of isoforsythiaside, a novel phenylethanoid glycoside isolated from *Forsythia suspensa*, were described. The antioxidant activity was estimated using the 1-diphenyl-2-picrylhydrazyl scavenging activity method and the in vitro antimicrobial activity was evaluated by the microtitre plate method. The results showed that this compound had strong activities. Owing to these properties, the study can be further extended to exploit for the possible application of isoforsythiaside as the alternative antioxidants and antibacterial agents from natural origin.

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## 1. Introduction

*Forsythia suspensa* (Thunb.) Vahl. is widely distributed in China, Korea, Japan and many European countries. The fruit of this plant is a well-known traditional Chinese medicine (TCM), known as “Lianqiao” in Chinese [1]. More than 40 Chinese medicinal preparations containing *Forsythia suspensa* (*F. suspensa*) are listed in Chinese Pharmacopoeia, such as Shuanghuanglian oral solution, Yinqiao Jiedu tablet and Qinlian tablet. The crude drug is widely used as an antipyretic, antidotal and anti-inflammatory agent for the treatment of ulcers and infections, such as acute nephritis and erysipelas [2,3]. It was also reported that *F. suspensa* was able to suppress vomiting, resist hepatic injury, inhibit elastase activity, and exhibit diuretic, analgesic, antioxidant, anti-endotoxin and antiviral effects [4–8].

A number of compounds including phenylethanoid glycosides, lignans, flavonoids, terpenes, and volatile oils had been isolated from the plant [4]. Among them, forsythiaside, forsythin and rutin proved to be largely responsible for the various biological activities of the herb. For example, forsythiaside showed strong antibacterial, antiviral, antioxidant, anti-inflammatory and cyclic adenosine monophosphate phosphodiesterase (cAMP) inhibitory effects [2,9–12]; forsythin exhibited antioxidant and weight losing abilities [13]; and rutin showed strong antioxidant effects [14].

In this paper, we report on the isolation of isoforsythiaside (Fig. 1), a new glycoside isomer of forsythiaside, from *F. suspensa*. The chemical structure was elucidated by applying IR, HRMS, 1D and 2D NMR techniques. The antioxidant and antibacterial activities of this novel phenylethanoid glycoside were also examined.

## 2. Material and methods

### 2.1. General

IR spectra were recorded on a UNICAM FTIR1020 spectrometer. NMR spectra were recorded on a Bruker DPX 500 NMR instrument (500 MHz for <sup>1</sup>H NMR and 125 MHz for <sup>13</sup>C NMR). Chemical shifts were given as  $\delta$  values with reference to tetramethylsilane (TMS) as an internal standard, and coupling constants were given in Hz. Optical rotations were measured at 589 nm (Na line) at 20 ± 2 °C with a Perkin-Elmer Model 343 digital polarimeter, using a 10 cm, 1 mL cell. High resolution mass spectra (HRMS) were recorded with a Bruker micrOTOF spectrometer in electrospray ionization (ESI) mode. Preparative HPLC (Amersham Biosciences AKTA Purifier with Frac-900) was performed on a Spherigel 100A C18 column (5  $\mu$ m, 1500 × 300 mm, Switzerland).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co. (St. Louis, MO). Vitamin C (VC) was purchased from Dengfeng Chemical Co. (Tianjin, China). Tetracycline (TC) was purchased from Amresco Inc. (USA). Forsythiaside was isolated from *F. suspensa* in our laboratory and its structure was char-

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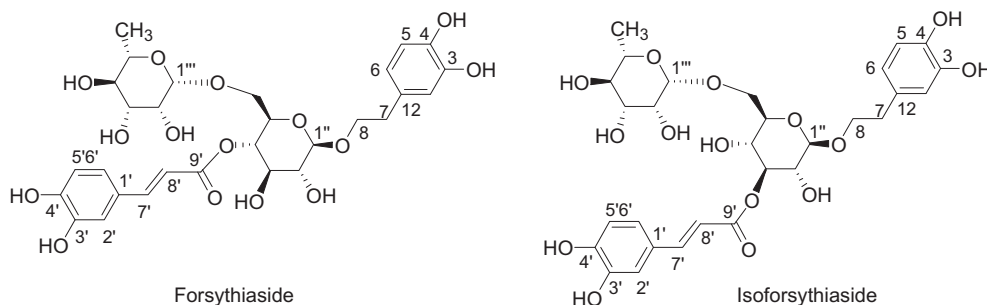


Fig. 1. Chemical structures of forsythiaside and isoforsythiaside.

acterized by IR,  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and MS as described by Liang [15].

All reagents used were of analytical grade, with the exception of methanol used for HPLC-MS analysis, which was chromatographic grade (Merck, Darmstadt, Germany). Water for HPLC-MS analysis was purified by a Milli-Q water purification system (Millipore Corporation, MA, USA).

## 2.2. Plant material

The dried fruits of *F. suspensa* used for isolation in this study were purchased from the local market (San menxia, China), in 2006. The plant materials were identified by Professor Yazhou Wang (Northwest University, Xi'an 710069, China). The voucher specimen (20090901) was deposited at Biomedicine Key Laboratory of Shaanxi Province, Northwest University, Xi'an, China.

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

## 2.3. Microorganisms

The bacterial strains: *Escherichia coli*-10B (*E. coli*), *Pseudomonas aeruginosa* (PAO), and *Staphylococcus aureus*-Rn 4220 (SA) were tested for purity by Gram reactions and by biochemical tests. The strains were kept at  $-70^\circ\text{C}$  in LB agar, activated by transferring into nutritive agar, and incubating at  $37 \pm 1.0^\circ\text{C}$  for 18 h.

## 2.4. Isolation of isoforsythiaside

The ground fruits of *F. suspensa* (250 g) were extracted four times with 75% aqueous EtOH (2 L) at  $60^\circ\text{C}$ , where after the extracts were combined and concentrated under vacuum. The concentrate (34 g) were chromatographed over a polyamide (100–120 mesh) column that was eluted with a EtOH– $\text{H}_2\text{O}$  gradient solvent system. Fractions containing compounds with similar *R<sub>f</sub>* values by TLC were evaporated and combined to give six fractions (F1–F6).

Fraction F3 (5 g) was subjected to over silica gel column, using ethyl acetate/methanol = (8:1; v/v) as the mobile phases. Again, fractions containing compounds displaying similar TLC *R<sub>f</sub>* values were evaporated and combined to give five fractions (F3.1–F3.5). Fraction F3.4 (240 mg) was thereafter explored by preparative HPLC on a C18 column (5  $\mu\text{m}$ ,  $1500 \times 300\text{ mm}$ , flow rate  $3.8\text{ mL min}^{-1}$ ), using methanol/water = (21:79; v/v) as the mobile phases, to afford isoforsythiaside (53 mg,  $t_R = 32\text{ min}$ ) and forsythiaside (602 mg,  $t_R = 55\text{ min}$ ).

Isoforsythiaside: light yellow powder,  $[\alpha]_D^{20} = -18.6$  ( $c = 0.1$ , MeOH). IR (KBr):  $\nu_{\text{max}}\text{ cm}^{-1}$ : 3410 (OH), 1690 ( $\text{C}=\text{O}$ ), 1604, 1510, 1449, 1377, 1283. HRESIMS (negative):  $m/z$  623.1961 (calcd. for  $\text{C}_{29}\text{H}_{35}\text{O}_{15}$ , 623.1976,  $[\text{M}-\text{H}]^-$ ).  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ , 500 MHz) and  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ , 125 MHz): see Table 1.

Table 1

$^1\text{H}$  NMR (500 MHz) and  $^{13}\text{C}$  NMR (125 MHz) data of isoforsythiaside in  $\text{CD}_3\text{OD}$  ( $\delta$  in ppm).

Position	Isoforsythiaside	
	$\delta_{\text{C}}$	$\delta_{\text{H}}^a$
1	131.96	–
2	116.88	6.68 d (2.0)
3	145.21	–
4	146.63	–
5	117.62	6.66 d (7.8)
6	121.80	6.55 dd (7.8, 2.0)
7	37.17	2.78 m
8	72.83	3.68 m
		3.97 m
1'	128.37	–
2'	115.96	7.04 d (1.6)
3'	147.36	–
4'	150.10	–
5'	117.04	6.76 d (7.8)
6'	123.39	6.94 dd (7.8, 1.6)
7'	147.48	7.58 d (16.0)
8'	115.67	6.31 d (16.0)
9'	169.57	–
1''	104.89	4.40 d (8.0)
2''	74.03	3.36 t (9.4)
3''	79.49	5.02 br.t (9.5)
4''	70.56	3.51 m
5''	77.23	3.51 m
6''	68.35	3.63 m
		3.97 br.d (10.5, 2.0)
1'''	102.75	4.74 br.s
2'''	72.89	3.84 d (1.5)
3'''	72.72	3.72 m
4'''	74.56	3.36 m
5'''	70.36	3.67 m
6'''	18.54	1.26 d (6.2)

<sup>a</sup> Signals were assigned by HMQC, HMBC, NOESY and 1H–1H COSY experiments. Proton coupling constants (*J*) in Hz are given in parentheses.

## 2.5. Determination of antioxidant activity with the DPPH radical scavenging method

DPPH $^\bullet$  scavenging activity was determined using a modified method of Brand–Williams et al. [16]. Different volumes of MeOH solution of isoforsythiaside, forsythiaside and VC (0.49, 0.36, 0.42  $\text{mg mL}^{-1}$ ) were taken into different test tubes. Added 3 mL methanol solution of DPPH $^\bullet$  (31.52  $\mu\text{g mL}^{-1}$ ) to each tube and shaken vigorously. The volume was adjusted to 5 mL by adding MeOH [17,18]. Methanol was used as a negative control for baseline correction, while VC was incorporated as the positive control [19,20]. The tubes were allowed to stand at  $27^\circ\text{C}$  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 [21–23].

Radical scavenging activity was calculated in percentage using the following formula:

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

The data was presented as the mean of the triplicates and the concentrations required for a 50% reduction ( $\text{EC}_{50}$ ) of  $\text{DPPH}^{\cdot}$  were obtained graphically. The  $\text{EC}_{50}$  value defined as the concentration of antioxidant necessary to decrease the initial  $\text{DPPH}^{\cdot}$  concentration by 50%. The lower the value, the stronger the antioxidant activity was.

## 2.6. Determination of the in vitro antimicrobial activities by the microtiter plate method

The antimicrobial activities against *E. coli*, *PAO* and *SA* of different concentrations of isoforsythiaside and forsythiaside were determined by the microtiter plate method described by the United States Pharmacopeia [24]. A twofold microdilution broth method [25–28] was used to determinate the minimum inhibitory concentrations (MIC) value for each test substance. Each well contained  $\sim 10^6$  CFU  $\text{mL}^{-1}$  of test bacteria and LB medium (100  $\mu\text{L}$ ). A 100  $\mu\text{L}$  of MeOH solution of isoforsythiaside, forsythiaside and TC (0.49, 0.46, 0.50  $\text{mg mL}^{-1}$ ) were added to wells of the first row. Dilutions were used to dispense 100  $\mu\text{L}$  into the other sterile 96 wells of a Elisa plate using a multichannel micropipette, resulting in eight concentrations to be tested for each compound. A negative control containing TC and a positive control containing only inoculated growth medium 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.

## 3. Results and discussion

### 3.1. Structural elucidation of isoforsythiaside

The molecular formula of isoforsythiaside was determined as  $\text{C}_{29}\text{H}_{36}\text{O}_{15}$  by HRESIMS (negative):  $m/z$  623.1961 (calcd. for  $\text{C}_{29}\text{H}_{35}\text{O}_{15}$ , 623.1976,  $[\text{M}-\text{H}]^-$ ). The IR spectrum (KBr) indicated the presence of hydroxyl (3350  $\text{cm}^{-1}$ ), conjugated carbonyl (1690, 1283  $\text{cm}^{-1}$ ), and double bond (1605, 1590 and 1510  $\text{cm}^{-1}$ ) functions.

The  $^{13}\text{C}$  and DEPT-135 NMR experiments indicated the presence of three  $\text{CH}_2$  groups. The  $^1\text{H}$  NMR spectrum of isoforsythiaside displayed aromatic signals at  $\delta_{\text{H}}$  7.04 (d,  $J = 1.6$  Hz, H-2'), 6.94 (dd,  $J = 7.8, 1.6$  Hz, H-6'), 6.76 (d,  $J = 7.8$  Hz, H-5'), 6.68 (d,  $J = 2.0$  Hz, H-2), 6.66 (d,  $J = 7.8$  Hz, H-5), and 6.55 (dd,  $J = 7.8, 2.0$  Hz, H-6), which demonstrated the presence of two 1,3,4-trisubstituted aromatic rings. Two doublets, 7.58 (d,  $J = 16.0$  Hz, H-7'), and 6.31 (dd,  $J = 16.0$  Hz, H-8'), showed the presence of a trans-double bond. The above information, together with the observation of  $^{13}\text{C}$  NMR spectrum signals at  $\delta_{\text{C}}$  128.37 (C-1'), 115.96 (C-2'), 147.36 (C-3'), 150.10 (C-4'), 117.04 (C-5'), 123.39 (C-6'), 147.48 (C-7'), 115.67 (C-8'), 169.57 (C-9'), indicated the presence of a caffeoyl unit in the molecule. Signals at  $\delta_{\text{C}}$  37.17 and  $\delta_{\text{H}}$  2.78 showed the presence of a benzyl methyl unit, together with the observation of  $^{13}\text{C}$  NMR spectrum signals at  $\delta_{\text{C}}$  131.96 (C-1), 116.88 (C-2), 145.21 (C-3), 146.63 (C-4), 117.62 (C-5), 121.80 (C-6), 37.17 (C-7), 72.83 (C-8), indicated the presence of a 3,4-dihydroxy-phenylethanol unit in the molecule.

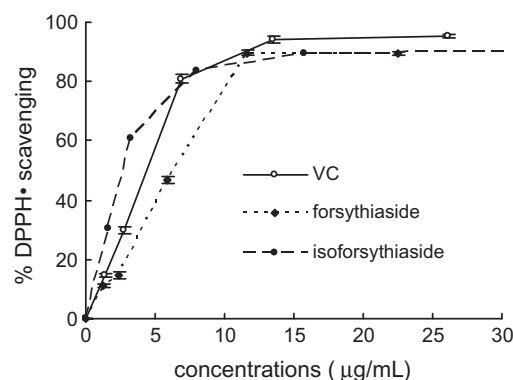


Fig. 2. Scavenging effects of samples on DPPH radicals.

Anomeric protons signals of the sugar protons H-1''' ( $\delta_{\text{H}}$  4.74), H-1'' [ $\delta_{\text{H}}$  4.40 (1H, d,  $J = 8.0$  Hz)] and a  $-\text{CH}_3$  signal ( $\delta_{\text{H}}$  1.26), corresponding to their  $^{13}\text{C}$  NMR spectrum signals at  $\delta_{\text{C}}$  102.75 (C-1'''), 104.89 (C-1''), 18.54 (C-6'''), indicated that the two sugar moieties were glucose and rhamnose. The respective positions of the substituents of glucose were determined using long-range heteronuclear correlations observed by HMBC. The correlations showed three-bond coupling from H-1'' to C-8 and H-3'' to C-9' which indicated that the oxymethylene C-8 and carboxylic C-9' were attached to the glucose C-1'' and C-3'', respectively. The HMBC correlation between the second anomeric (H-1''') proton and C-6'' proved that the linkage between rhamnose and glucose was 1'''  $\rightarrow$  6''. Moreover, the spectral data of isoforsythiaside (Table 1) were very similar to those of the spectral data of the known compound, forsythiaside. The structure was therefore assigned for isoforsythiaside.

### 3.2. Antioxidant activity

There have been numerous methods for the measurement of antioxidant activities 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. 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 [29]. In this research, free radical scavenging potentials of isoforsythiaside and forsythiaside at different concentrations were tested by the DPPH $^{\cdot}$  method (Fig. 2). The degree of discoloration indicates the scavenging potentials of the compound. The concentrations required for  $\text{EC}_{50}$  of DPPH $^{\cdot}$  were obtained and shown in Table 2.

The results showed that isoforsythiaside demonstrated a more notable antioxidant activity than forsythiaside and VC. The significance of phenolic compounds as dietary antioxidants has been

Table 2

Free radical scavenging activities ( $\text{EC}_{50}$ ) and antibacterial activities (MIC) of pure phenylethanoid glycosides isolated from *F. suspensa*.

Properties	Components			
	Isoforsythiaside	Forsythiaside	VC	TC
<b>Antioxidant property</b>				
$\text{EC}_{50}$ value ( $\mu\text{g/mL}$ )	2.74	6.30	4.38	n <sup>a</sup>
<b>Antibacterial properties</b>				
MIC value ( $\mu\text{g/mL}$ )				
<i>E. coli</i>	40.83	38.33	n	29.76
<i>PAO</i>	40.83	38.33	n	29.76
<i>SA</i>	81.66	76.67	n	119.05

<sup>a</sup> Not determined.

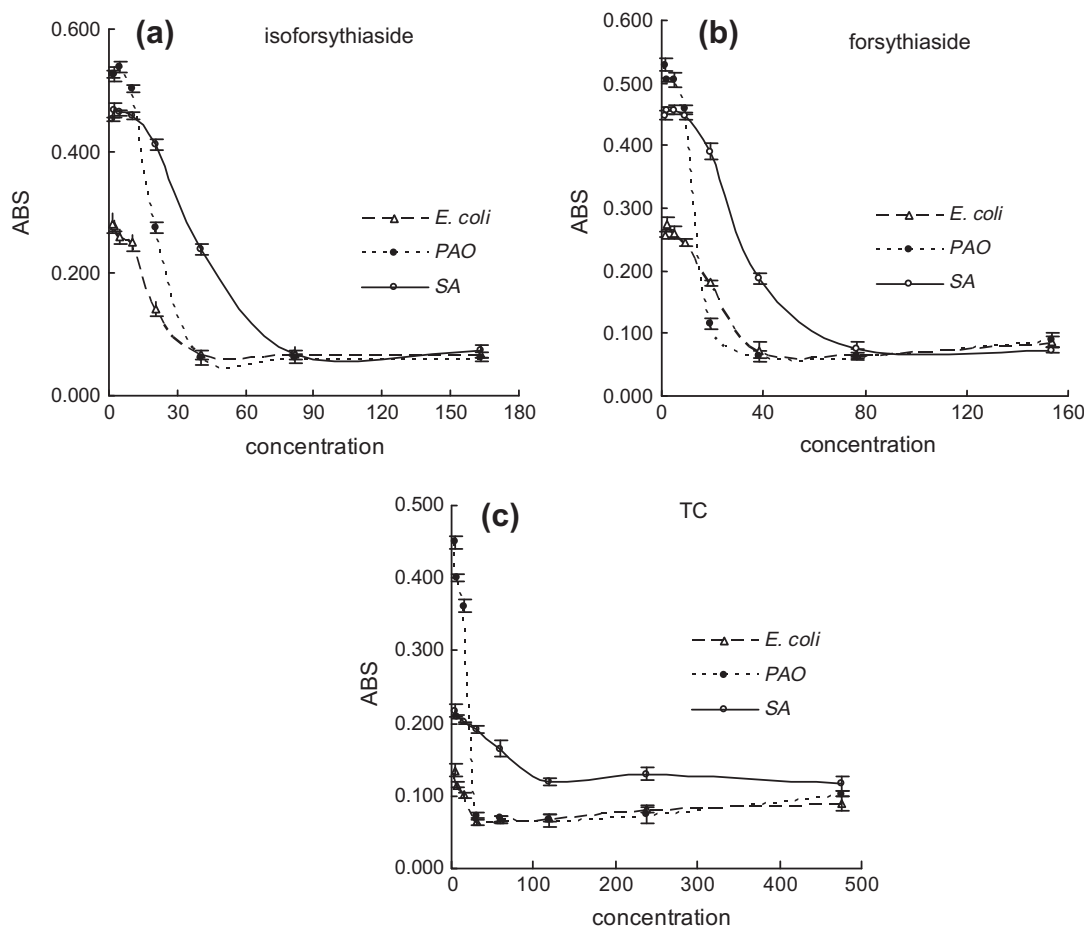


Fig. 3. Antibacterial activities of different concentrations ( $\mu\text{g mL}^{-1}$ ) of isoforsythiaside (a), forsythiaside (b) and TC (c).

highlighted in recent years [30–32]. In the study of Zhang and his coworkers [33], they 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 containing meta-substituting hydroxyl. The amount of phenol hydroxyl and amount of intramolecular H-bond 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 researches [33–35] 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.

### 3.3. In vitro antimicrobial activities

When studying the influence of the concentration of isoforsythiaside and forsythiaside on the antimicrobial activities against *E. coli*, *PAO* and *SA* we used twofold microdilution broth method. And the data were described graphically in Fig. 3. It was observed that as the concentration increased, there was an increase in the inhibition of the bacterial growth, however at different rates depending on the compound.

The MIC data of the two compounds and TC were presented in Table 2. The results showed both isoforsythiaside and forsythiaside had comparatively high antibacterial activities against the three common bacteria. Their MIC values against *SA* were even lower than TC.

## 4. Conclusions

Isoforsythiaside, a novel phenylethanoid glycoside, was successfully isolated and separated from *F. suspensa*. Our research demonstrated that preparative HPLC is a powerful technique to separate and isolate this constituent, and the pharmacology test showed that isoforsythiaside exhibited high antioxidant and antibacterial activities. In the light of these results, in vivo actions and clinical applications of this new compound are required further investigation.

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