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The antibacterial activity of syringopicroside, its metabolites and natural analogues from Syringae Folium

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ABSTRACT

In the present study, the *in vitro* antibacterial activity of an effective fraction (ESF) from Syringae Folium (SF) on Methicillin-resistant *Staphylococcus aureus* (MRSA) was evaluated and then its *in vivo* activity was evaluated by using the MRSA-infected mouse peritonitis model. The ESF showed a significant *in vitro* and *in vivo* activity on decreasing the Minimum Inhibitory Concentrations (MICs) and increasing the survival rate of mouse from 42.8% to 100%. Six iridoid glucosides (IGs) of ESF were characterized by UPLC-TOF-MS method and also isolated by column chromatography. Most of them showed *in vitro* anti MRSA activity. Syringopicroside (Sy), the major compound of IGs, was found to increase the survival rate from 42.8% to 92.8% of the MRSA-infected mouse, which revealed Sy is also the main active components of ESF. In order to know why the effect of oral administration of SF is better than its injections in clinic and the metabolites of Sy, seven metabolites of Sy were isolated from rat urine and identified on the basis of NMR and MS spectra. Most of metabolites possessed stronger *in vitro* anti-MRSA activity than that of Sy, which furtherly proved the clinical result.

1. Introduction

The Syringae Folium (SF) has been traditionally used for heat clearing and detoxifying, and also, has been utilized to treat bacterial infectious diseases, such as acute enteritis [1], bacillary dysentery and upper respiratory tract infection in China [2]. Various preparations of SF extracts have been widely applied in current clinical applications. For example, eye drops are able to cure 93% patients suffering from herpes simplex keratitis [3], tablets display a higher recovery rate for children's acute infections than other antibiotics among 400 clinical cases [4] and SF demonstrates a broad-spectrum natural antibacterial activity in 50 pharyngitis cases [5]. Yan Li Xiao (YLX), with the meaning of "quick-acting anti-inflammation" in Chinese, is one of commercial medicines which consists of water extraction and powder of SF. One of its disadvantage lies in large amounts of tablets (9 to 12 per day) used for patients [6], due to their crude extracts. In order to reduce the pill burden and improve the efficacy, the new extraction technology is necessary under the bioassay-guiding fractionation.

Phytochemical studies demonstrated that iridoid glycosides (IGs) are the main active constituent of SF [7]. IGs are one of the most important natural compounds that are widely distributed in various plant families, which are extensively present in almost all *Syringa* species and have antimicrobial, antihypertensive, anti-inflammatory, antioxidant, and antitumor activities [8]. *Syringa* iridoids are generally substituted by various acid fragments and phenolic moieties such as 3, 4-dihydroxy-phenethyl, or caffeic acid, which contribute to their low polarity [8], and the structural similarity of which are very high.

Methicillin-resistant *Staphylococcus aureus* (MRSA), which is resulted from the selective pressure of antibiotics currently used, has increased relentlessly and is well recognized as a global nosocomial problem in recent years, especially in patients of intensive care units (ICU) admission and of those who are elderly and repeatedly hospitalized. It has now been the predominant and serious pathogenic bacterium, leading to high morbidity and mortality. [9–12]. Therefore, the development of anti-MRSA agents with novel mechanisms of action is highly necessary [13].

To solve above stated issues, an antibacterial effective fraction of SF (ESF) was successfully screened out by using the *in vivo* and *in vitro* Methicillin-sensitive *S. aureus* (MSSA) experiments in our previous work [14]. Our current interests lie in that whether the ESF can also show the anti-MRSA activity due to anti-drug-resistant bacterial activity of SF reported in clinics [15]. In order to investigate the anti-MRSA activities of the ESF and its resulting bioactivity constituents, the *in vitro* and *in vivo* antibacterial activity of the fraction, isolated compounds and metabolites of Syringopicroside (Sy, the typical IG of SF) on MRSA have been reported in our present paper.

Abbreviations: SF, Syringae Folium; ESF, the effective fraction of Syringea Folium; MRSA, Methicillin-resistant *Staphylococcus aureus*; MSSA, Methicillin-sensitive *Staphylococcus aureus*; VH, Vancomycin hydrochloride; Sy, Syringopicroside A; IG, Iridoid glucoside; MIC, Minimum Inhibitory Concentration; YLX, "quick-acting anti-inflammation" capsule (a commercial capsule made up of water extraction and powder of SF).

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2. Experimental

2.1. Chemicals and material

SF was identified by Jun Yin, the professor of Shenyang Pharmaceutical University and deposited in the laboratory of the Department of Pharmacognosy. YLX capsules and the Vancomycin hydrochloride (VH) were produced by Xiuzheng Pharmaceutical Group (Tonghua, China) and Eli Lilly (Seishin Laboratories, Japan), respectively. All solvents used for the preparation were of analytical grade (Tianjin Bodi Chemical Ind., Co., Ltd., Tianjin, China). Silica gel (100–200 mesh and 200–300 mesh; Qingdao Marine chemical Co., Ltd) was provided by Yuwang chemicals Industries, Ltd. Acetonitrile, methanol (HPLC-grade) and formic acid were purchased from Fisher Chemicals (USA) and ultra-pure water (18.2 M) was prepared with a Milli-Q water purification system (Millipore, France). The NMR spectra were recorded using 300, 400 and 600 MHz Bruker TOPSPIN 2.1 spectrometer. Chemical shifts are expressed in δ (ppm) with reference to the tetramethylsilane (TMS) peak. Column chromatography (C.C.) was carried out on Sephadex LH-20, silica gel 200–300 mesh.

2.2. Preparation of ESF

SF (1800 g) was prepared and soaked in 75% ethanol (m: v = 1: 10) for 30 min, followed by being refluxed for 2 times, 1 h for each time and then filtered. The filtrate was combined and evaporated in 80 °C to a density of 1.01–1.10 g/ml. The residue was precipitated with water 5 times amount of the residue for 30 min and then centrifuged for 30 min (3000 r/min). The supernatant was evaporated to a density of 1.01–1.10 g/ml (667 g/L for crude drug) and then the concentrated solution was prepared. After that, it was loaded on macroporous resin D101 (2000 g) with the speed of 1 BV/h and adsorbed for 1 h. The ratio of dry weight of the solution and macroporous resin was 1:10, the ratio of diameter and height of the resin column was 1:8. Solution was eluted by 6 BV of 25% ethanol and then 8 BV of 55% ethanol under vacuum of 0.07 MPa. Finally, the 55% ethanol eluate was collected and concentrated to extract on 80 °C water bath and then evaporate to dryness in vacuum to get the ESF (120.6 g).

2.3. Fractionation and isolation

The ESF (100 g) was further separated by C.C. over a silica gel (1500 g, 10 * 60 cm) eluted with a CH_2Cl_2–MeOH gradient system to give eight fractions: Fr.1 (2 L, 25:1, *v*/v), Fr.2 (4 L, 20:1, *v*/v), Fr.3 (4 L, 15:1, *v*/v), Fr.4 (10 L, 12:1, *v*/v), Fr.5 (6 L, 8:1, *v*/v), Fr.6 (4 L, 5:1, *v*/v), Fr.7 (4 L, 3:1, *v*/v) and Fr.8 (4 L, 2:1, *v*/v). Fr.4 (28.3 g) was subjected on silica-gel C.C. (450 g, 7.5 * 25 cm) and eluted with CH_2Cl_2–MeOH (15:1–8:1, *v*/v) gradient system to obtained **1** (20.3 g). Fr.3 (9.7 g) was further isolated by C.C. silica gel (200 g, 4 × 40 cm) and eluted with PE-EtOAc 5:1(1.20 L), 3:1 (1.20 L), 3:1 (1.20 L) and 1:2 (1.20 L) to yield **1** (2.1 g) together with Fr.3-1(1.2 g) and Fr.3-2 (0.7 g). Fr.3-2 was purified on Sephadex LH-20 C.C. with MeOH to afford **4** (25 mg). Compounds **5** (15 mg) and **6** (17 mg) were obtained by further purification using Prep.HPLC (MeOH–H_2O, 60:40). Fr.5 (10.7 g) was placed on silica-gel C.C. (200 g, 2.5 × 20 cm) and subjected to gradient elution with PE-EtOAc 1:1 (2.0 L) and 1:2 (2.0 L). Another 3.6 g compound **1** together with Fr.5–1 (3.2 g) was obtained. Fr.5–1 was subjected to Sephadex LH-20 C.C. and partly (200 mg) subjected to Prep.HPLC (MeOH–H_2O, 40:60) to obtained **2** (43 mg) and **3** (22 mg). The purity was higher than 98% as checked by HPLC-UV, and the structures were elucidated by ^1H and ^{13}C NMR using a Bruker DRX600 instrument (Bruker, USA), operating at 600 MHz for ^1H spectra and 150 MHz for ^{13}C spectra. The chemical structures were depicted in Fig. 1a.

2.4. UPLC-QTOF–MS qualitative analysis of the active fraction

2.4.1. UPLC conditions

Separations were performed on an ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) with an Acquity UPLC column (HSS C_{18} 100 mm × 2.1 mm, 1.8 μm) and the column temperature was maintained at 40 °C. The mobile phase consisted of H_2O (A) and MeOH (B). The flow rate of the mobile phase was 500 μL/min and the injection volume was 4 μL. Analytes were eluted from the column with a gradient. The gradient program was conducted as following: the initial composition of B was 5% and increased to 10% in 2 min, 10%–20% B at 2–5 min, 20%–30% B at 5–11 min, 30%–60% B at 11–14 min and decreased quickly to 5% B at 15.1–18 min.

2.4.2. Q-TOF/MS^E analysis

Analyses were performed using a Micromass-Q-TOF Premier mass spectrometer (Waters) coupled with an electrospray ionization (ESI) source in positive mode. The MS tune parameters were as follows: the cone and desolvation gas flow were 50 L/h and 700 L/h, respectively; the temperature of the source and desolvation were set at 130 °C and 350 °C, respectively. The capillary and the cone voltage were set at 3.0 kV and 40 eV, respectively; the micro-channel plates (MCPs) were operated at 1750 V and the Q-TOF mass spectrometer was operated in MS^E mode with a low collision energy set at 6 eV in the first function and a collision energy ramp from 20 to 40 eV in the second function. Centroid mode data were collected over the range of m/z 100–1000 in both functions, and the scan time was 0.2 s with an interscan delay of 0.02 s.

The standard stock solution of the separated compounds was prepared and diluted with methanol to appropriate concentrations. Dried powder (2 mg) of ESF was accurately weighed and diluted with methanol to 0.2 mg/ml. The resultant solution was filtered through a 0.22-μm filter for UPLC-TOF–MS analysis.

2.5. Isolation and purification of the metabolites of Sy from the urine samples

Twenty male SD rats weighing 200–220 g were used. All of the experimental animals were purchased from the Pharmacology Experimental Center of Shenyang Pharmaceutical University. All of the mice and rats were acclimatized for one week in a light- and temperature-controlled room with a 12-h dark/12-h light cycle. Rodent laboratory chow pellets and tap water were supplied *ad libitum*. The animal care and protocols used in this study were approved by the Institutional Animal Ethics Committee of Shenyang Pharmaceutical University. (Number: SLXK (BJ) 2013–0605). After 7 days of adaption, twenty SD rats were administrated a dose of 100 mg/kg per day of Syringopicroside A for 20 days. A total of 2.4 L urine were collected and stored at −20 °C.

The urine sample was firstly extracted with 3 L of EtOAc for four times and then extracted with 2 L of n-butyl alcohol for four times. The extractions were evaporated to dryness under 50 °C. The EtOAc extraction (2.1 g) was applied to Sephadex LH-20 C.C. for 7 times using a MeOH system and separated into three fractions: UE1 (163 mg), UE2 (302 mg) and UE3 (233 mg). The n-butyl alcohol extraction was applied onto the silica gel C.C using a CH_2Cl_2–MeOH (4:1 ~ 1:1, *v*/v) system and was separated into three fractions: UE4 (748 mg), UE5 (168 mg) and UE6 (522 mg). **MA1** (7 mg) and **MA2** (6 mg) was obtained from UE2 by repeated silica gel C.C (15 g, 2 * 30 cm), eluted using P.e.-EtOAc (3:1 ~ 1:2, *v*/v) and Prep-HPLC eluted using MeOH–H_2O (55:45, *v*/v) as mobile phase. While UE1 was placed on ODS C.C. (5 g, 2 * 15 cm) and subjected to gradient elution with Me_2CO–H_2O (0:100–30:70, *v*/v), then the target sub-fraction was placed on Sephadex LH-20 C.C. again and eluted with P.e.-CH2Cl2–MeOH (3:3:1, *v*/v/v) to obtained **MA4** (32 mg). **MA3** (10 mg) was obtained as crystals from the UE3 fraction after repeated silica gel C.C (12 g, 1 * 22 cm) eluted using P.e.-EtOAc (5:1 ~ 1:1, *v*/v) and Sephadex LH-20 C.C eluted using MeOH. UE4