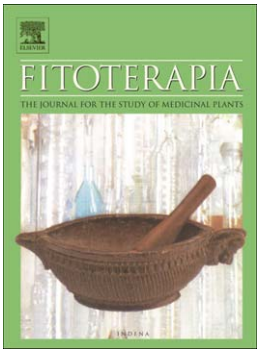


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# The antibacterial activity of syringopicroside, its metabolites and natural analogues from Syringae Folium

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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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## 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.


**Keywords:** Syringae Folium; UPLC-TOF-MS; Metabolites; Syringopicroside; iridoid glucoside; anti-MRSA.

## 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, anti-oxidant, 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 *Staphylococcus 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.

## 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 Mili-Q water purification system (Milipore, 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 hour 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, 4×40cm) 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). Compound **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 $^{1}H$ and $^{13}C$ NMR using a Bruker DRX600 instrument (Bruker, USA), operating at 600 MHz for $^{1}H$ 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**

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 5-11min, 30%-60% B at 11-14min and decreased quickly to 5% B at 15.1-18 min.

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 1750V 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 were 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. (5g, 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 was placed on silica gel C.C. (38 g, 2*25 cm) and subjected to gradient elution with P.e.-EtOAc (1:1~1:4, v/v). **MA5** (12 mg) was obtained after further purification using the Sephadex LH-20 and Prep.TLC methods. UE5 was placed on silica-gel C.C. (4 g, 0.9*12 cm) and subjected to gradient elution with EtOAc-$Me_2CO$ (5:1~1:1, v/v) and further purification by Sephadex LH-20 using MeOH yield **MA7** (4 mg). **MA6** (34 mg) was obtained as crystals from UE6 after Sephadex LH-20 elution.

### 2.6 *In vitro* Antibacterial assays

#### 2.6.1 Bacterial stains and culture conditions

The test organisms used in this study were as follows: *S. aureus* ATCC 6538 (MSSA) and clinical isolated MRSA were obtained from the Chinese People's Liberation Army 463 hospital. The stains were used to the test minimum inhibitory concentrations (MICs) and were cultured overnight in nutrient broth at 37 °C and stored at 4 °C.

#### 2.6.2 Broth micro dilution assay for determination of the MIC

The MIC values for the microorganisms were determined as sensitivity to compounds in microdilution assay [10]. Briefly, the 96-well plates were prepared by dispensing 120-μ1 broth and 30-μ1 inoculum into each well. A 150-μ1 extract initially prepared at the concentration of 4 or 5 mg/ml was added into the first well. Then, 150-μ1 from their serial dilutions was transferred into eight consecutive wells. The final volume in each well was 150μ1. Equal volumes of each bacterial strain culture, which contained approximately $10^6$ CFU/ml, were applied to sterile NB supplemented with multiple proportion dilutions of the samples at concentrations ranging from 0.03125 $mg \cdot ml^{-1}$ to 2 $mg \cdot ml^{-1}$ in 96-well plates. These serially diluted cultures were then incubated at 37 °C for 18 h. The MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms.

### 2.7 *In vivo* antibacterial experiment

The mice were randomly divided into nine groups with 8 mice in each group. The mice in the ESF groups received the ESF fraction, according to the formula recorded in the pharmacology experimental methodology edited by Chen [7]. The oral administration dose of the low-dose group

was calculated by coefficient commutation of somatotypes based on the clinical dose of SF [11] as 82.2 mg·kg$^{-1}$ (in dry extraction), whereas the middle-dose and the high-dose groups received twice and quadruple of the low dose, i.e., 164.4 mg·kg$^{-1}$ and 328.8 mg·kg$^{-1}$, respectively. The doses of the Sy groups were calculated by the content of Sy in ESF as 24.7, 49.32 and 98.64 mg·kg$^{-1}$. The YLX groups were received 82.2 mg·kg$^{-1}$ YLX capsule contents, while positive control group was given VH through intraperitoneal injection, which is also based on the clinical dose as 300 mg·kg$^{-1}$; and the blank group received normal saline. The administration lasted for seven days. On the 3rd and 4th days, all of the mice were intraperitoneally injectted with MRSA ($10^9$ CFU·ml$^{-1}$) at doses of 0.1 ml·10 g$^{-1}$ and 0.15 ml·10 g$^{-1}$, respectively. The survival rate of each group was then calculated for 7 days after infection.

## 3. Results

### 3.1 Phytochemical investigation of ESF by column chromatograph and UPLC-TOF-MS analysis

From the ESF, 6 iridoid glucosides, including Syringopicroside (**1**), Syringopicroside B (**2**), Syringalactone B (**3**), Ligustroside (**4**), Griffithoside B (**5**) and Formoside (**6**) were obtained. The structures (Fig.1a) were elucidated on the basis of $^1$H, $^{13}$C-NMR and MS data and by comparison with the references [16-19]. The NMR data of these compounds was provided in supplement data.

The UPLC-TOF-MS analysis showed the presence of the isolated compounds in the ESF. The compounds were identified in comparison of their retention time and MS spectra with external standards. Sy, the maximum amount of compound, together with other 5 IGs were identified as the major components of ESF (Fig.1b).

### 3.2 The isolation and elucidation of *in vivo* metabolites of Sy

A total of seven metabolites (**MA1**-**MA7**) were isolated from the urine of rats after oral administrated with Sy. To our best knowledge, all isolated compounds were identified as the *in vivo* metabolites of Sy for the first time. The structures of **MA1**-**MA7** were identified as syringopicrogenin B (**MA1**), sclerochitonoside B (**MA2**), syringopicrogenin A (**MA3**), tyrosol (**MA4**), *p*-hydroxybenzoic acid (**MA5**), benzoic acid (**MA6**), and 4-hydroxy-acetophenone (**MA7**)

by comparison with those existing NMR data in references [19,22-25] (Fig.2).

### 3.3 The *in vitro* antibacterial activity of ESF, Sy and its analogues (IGs) from ESF

The *in vitro* anti-MSSA and anti-MRSA activity of the ESF and YLX were evaluated and compared (Table 1). The dosage of each fraction was decided based on the previous anti-MSSA study [14]. Against the two test organisms, the MIC values of YLX were 2.45 and 4.90 $mg \cdot ml^{-1}$, respectively, which were twice or even quadruple higher than that of ESF (1.28 and 1.28 $mg \cdot ml^{-1}$). It means the antibacterial constituents of YLX have been effectively enriched by ESF, and thus we assume anti-MSSA activity of ESF could be sensitive to MRSA.

In order to determine the antibacterial materials basis of the ESF and figure out whether the IGs are the active ingredients, the isolated IGs were first evaluated using the MIC values (Fig.3). Generally speaking, the susceptibility of the tested samples to MRSA is lower than which to MSSA. Among 6 IGs, compound **1**, compound **2** and **3** showed excellent antibacterial activities, especially to the MRSA, while the compound **4**, **5** and **6** performed weaker activities. The results showed that, the existent of 3′-OH of *p*-hydroxyphenylethyl alcohol fragment can improve the antibacterial activity of the IGs. In addition, the seco-IGs are not as good as IGs in the case of the antimicrobial activity. However, all IGs showed a much weaker *in vitro* antibacterial activities, comparing with the positive control (VH).

### 3.4 The *in vitro* antibacterial activity of Sy metabolites of against MRSA and MSSA

In order to determine whether the *in vivo* metabolites of Sy are the real bioactive constituents and whether Sy plays the important role in anti-MRSA bacteria activity of ESF, the *in vitro* anti-MRSA and anti-MSSA activities of the isolated metabolites of Sy were tested and compared(Fig.4). Among the isolated metabolites, the **MA1** and **MA2** performed a lower MICs comparing with their original compound **1** (Sy), which indicated that the hydrolyzed aglycone possessed better antibacterial activities. Especially, the **MA5** and **MA6** performed very low MICs both in the MSSA and MRSA testing, both of which may be the final metabolites of Sy. But another three metabolites, **MA3**, **MA4** and **MA7,** did not have the antibacterial activity against MRSA, and meanwhile **MA3** and **MA4** possessed strong activity against MSSA.

### 3.5 The *in vivo* antibacterial activity of ESF and Sy.

The *in vivo* antibacterial activity of the ESF was characterized by survival rates in the experimental peritonitis model. Three ESF groups which received different dosage were used as therapeutic plan, while YLX and VH were tested as two positive control groups. The survival rates of the blank control group and the VH group were 42.8% and 100%, respectively, which demonstrates that the experimental models were successfully established. In the therapeutic plan, the oral administration dose of the low-dose group was calculated by coefficient commutation of somatotypes based on the clinical dose of SF [6] as 82.2 $mg \cdot kg^{-1}$ (in dry extraction), whereas the middle-dose and the high-dose groups received twice and quadruple of the low dose, i.e., 164.4 $mg \cdot kg^{-1}$ and 328.8 $mg \cdot kg^{-1}$, respectively, which performed the final survival rates in mice with values of 57.1%, 100% and 83.3%, respectively, while the YLX group value is 78.6%. The protective effect of the ESF-M (same dose as YLX) was stronger than that of YLX, and identical to the positive control of VH. Fig.5 describes evident time-dependent manner from the 3$^{rd}$ to the 7$^{th}$ days after the MRSA-infection. Meanwhile slightly dose-dependent manner can be also observed between the low- and medium-dose groups, while survival rates of the high-dosage groups creep down below that of the medium-dose groups.

Sy is typical IGs from SF and accounts for over 30% of ESF, reported in our previous paper [20]. To determine whether Sy is the anti-MRSA active component of ESF, the *in vivo* antibacterial activity of the Sy was characterized as a breakthrough point by the survival rates in the experimental peritonitis model. In the Fig.6, the survival rate of the model group (K) gradually reduced as time goes on, and finally fell straight to the 42.8%; while the survival rate of the positive control group (ESF-M) remained at 100%. In the therapeutic groups, orally administered Sy (27.4, 54.8, 109.6 mg/kg) performed different protective effects: Sy-H group kept zero death to the 4$^{th}$ day after MRSA-infected, while the Sy-M group kept the high survival rate of 92.8% from the 3$^{rd}$ day till the end of the experiment. The final survival rates in mice as 75%, 92.8% and 87.5%, respectively, showed greatly significant with the blank control, but weaker than the effective fraction (ESF-M). In agreement with the previous study, the main antibacterial active constituents in SF were widely reported as phenolic acids [21], which could partly contribute to the anti-MRSA activity. Fig.6 illustrated evident time-dependent manner from the 3$^{rd}$ to the 7$^{th}$ days after the MRSA-infection. Similar with the case of ESF, slightly dose-dependent manner can be observed

between the low- and medium-dose groups, while survival rates of the high-dosage groups creep down below that of the medium-dose groups.

## 4. Discussion

In order to validate the speculation that the anti-MSSA effective fraction ESF is also sensitive to MRSA, the *in vitro* and *in vivo* anti-MRSA activity of the ESF and YLX were evaluated and compared. The results show that the *in vitro* sensitivity of ESF to MRSA is as same as that to MSSA, but the susceptibility to MRSA of YLX is obviously weaker than to MSSA, which demonstrates that the active components of SF (or YLX) are effectually enriched.

According to our results, the medium dose of ESF performed a similar survival rates (100%) as the positive control group (VH). But VH possesses inevitable ototoxicity which greatly limits its usage. Therefore, the ESF, without apparent side effects, has huge potentials for medicine research and clinical applications in the treatment of the MRSA.

The main antibacterial active constituents in SF are widely reported as phenolic acids [21]. At the same time, a handful of reports show the antibacterial activity of the IGs, especially isolated from the genus *Syringa* [8, 26]. However, the antibacterial activity of IGs in SF has not been yet reported so far, although other bioactivities of IGs in SF are studied, such as antifungal, antioxidant and anti-inflammatory [27, 28]. Firstly, IGs displayed excellent antibacterial activity not only to MSSA, but also to MRSA in the *in vitro* experiments, but not much stronger than the ESF. In the other hand, Sy, as the major IG of ESF **(**Fig.1b**),** displayed the promising increase of survival rate, only slightly weaker activity than ESF. Based on these facts, we can firmly assume that IGs, especial for Sy, is the major active component both against MSSA and MRSA. And also, this is the first time to estimate the antibacterial activity of IGs from SF.

As we studied before, comparing with the positive control (VH), the MICs of isolated IGs are much higher, which means their *in vitro* antibacterial activities are much weaker than VH. But their *in vivo* effects are almost the same as VH. In addition, the preclinical study shows that the activity would be lost by changing the administration route from oral to injection, which proves that the absorbed ingredients in blood are different with the oral administration ones. Thus, we hypothesize that the metabolites are the real material basis of the multiple functions of different constituents.

Seven metabolites of Sy from the urine of rats are reported, and then the *in vitro* antibacterial activity of these isolated metabolites were evaluated and compared with those of the parent compound (Sy). The hydrolyzed product (**MA1**) and the derivative (**MA2**) performed lower MICs than that of Sy, which demonstrates that **MA1** and **MA2** have better antibacterial activities. Smaller molecules of aglycone support a better membrane permeability of the bacteria, which could be speculated to be the in vivo material basis of the bioactivity.

Our previous study [14] showed that the antimicrobial fraction of SF exhibits a more long-lasting activity than the clinical used antibiotic, which might be induced by a multiple components effects. LIU *et al*. found that Sy are rapid distribution, fast elimination and the retention time was very short [29]. Based on these facts, the metabolites are the main existing form instead of the mother compound. Considered the similarity and diversity of the IGs in structure (Fig.1a), the deglycosylation may widely exist but the reaction rates differed from each other under the action of different hydrolytic enzymes. It is supported that the actual compounds responsible for the antibacterial activity were produced in a more gradual way. The greater antibacterial activity of the metabolites of Sy could partly explain this phenomenon.

## Conclusion

In current study, the *in vivo* and *in vitro* anti-MRSA material basis of the effective fraction of SF was studied by testing the anti-MRSA activity. A total of 6 IGs isolated from ESF and 7 metabolites of a typical IG, Sy, were isolated from rat urine and characterized by NMR and MS. It is the first time that we conduct a comprehensive investigation of the *in vivo* metabolites of Sy. The anti-MRSA activity of its metabolites were surveyed and compared with each other. This study conducts preliminary research on the metabolic process of the main constituents of SF and also lays the foundation for the further research and development of Syringae Folium to justify its use in the traditional medicine.

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## Table 1

Minimum inhibitory concentration(MIC) of the ESF, YLX and VH in reference strains of MSSA and MRSA.

| Fraction/compound | MIC (mg/ml) | |
|---|---|---|
| | MSSA | MRSA |
| **ESF** | 1.28 | 1.28 |
| **YLX** | 2.45 | 4.90 |
| **VH** | <0.026 | <0.039 |

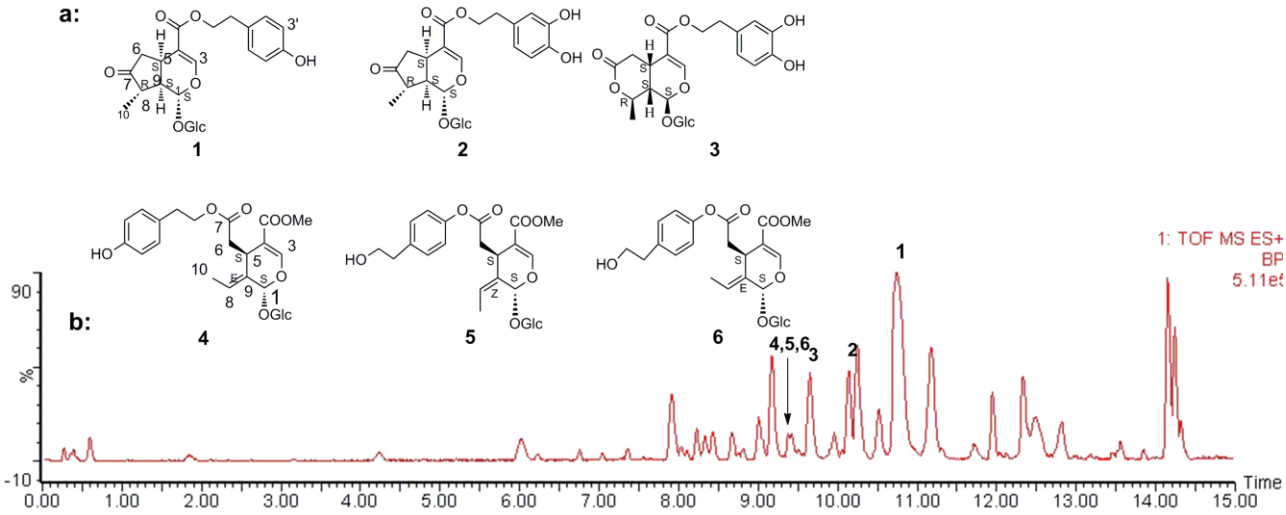


Fig.1a The chemical structures of compounds **1**-**6**: Syringopicroside A (**1**), Syringopicroside B (**2**), Syringalactone B(**3**), Ligustroside (**4**), Griffithoside B (**5**), Formoside (**6**). 1b: The UPLC-TOF-MS chromatogram of the ESF.

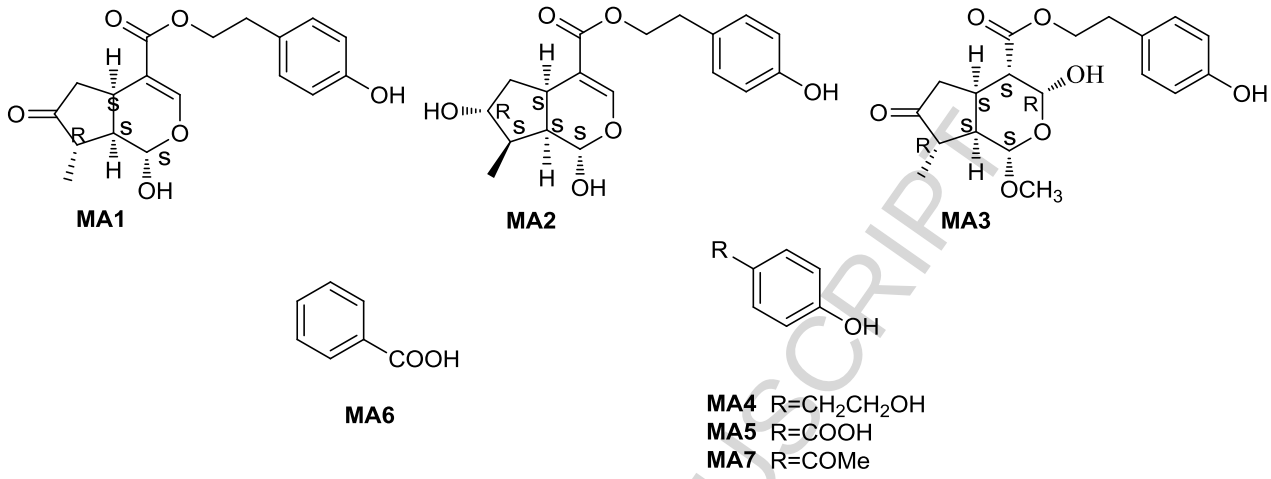


Fig.2 The chemical structure of the major *in vivo* metabolites of Sy: Syringopicrogenin B (**MA1**), Sclerochitonoside B (**MA2**), Syringopicrogenin A (**MA3**), Tyrosol (**MA4**), *p*-Hydroxybenzoic acid (**MA5**), Benzoic acid (**MA6**) and 4-Hydroxy-acetophenone (**MA7**).

VH group (300 mg·kg$^{-1}$, *n=7*) and blank control group (untreated, *n=7*).

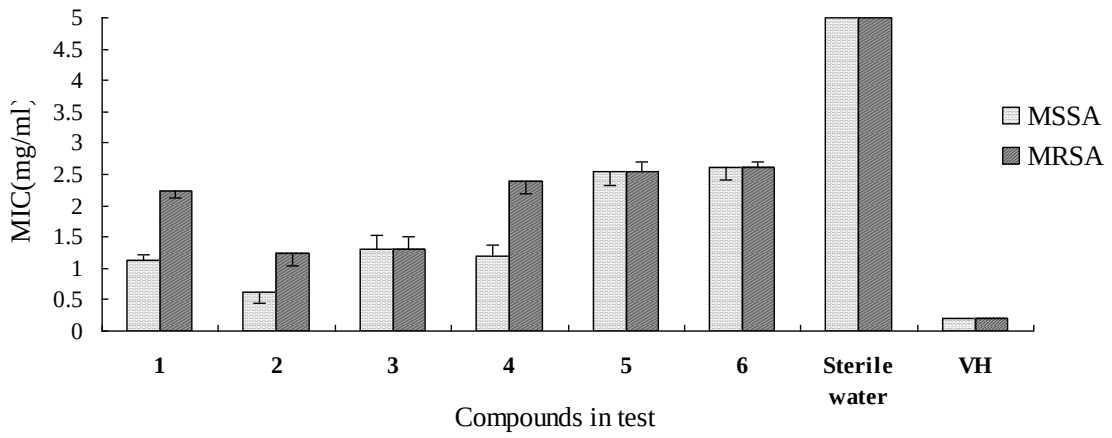


Fig.3 The *in vitro* antibacterial activities of the isolated iridoid glycosides from the ESF against MSSA and MRSA. Syringopicroside A (**1**), Syringopicroside B (**2**), Syringalactone B(**3**), Ligustroside (**4**), Griffithoside B (**5**), Formoside (**6**). Each MIC value expressed as the means ± SD (n=3).

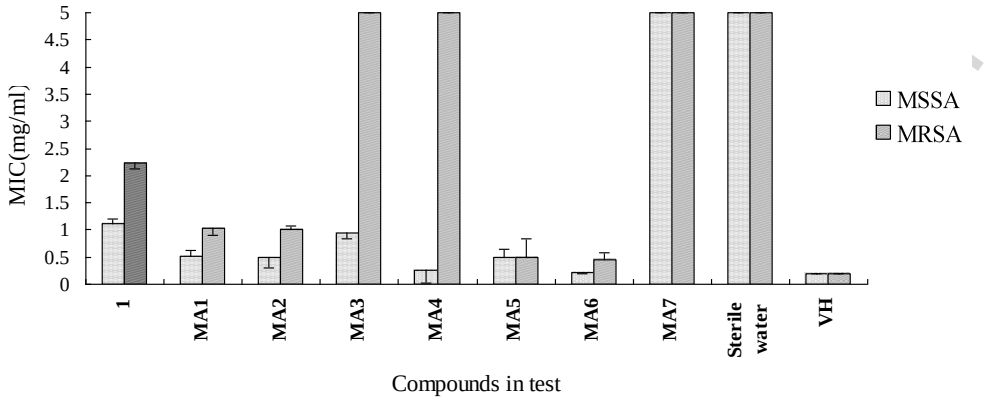


Fig.4 The *in vitro* antibacterial activities of the isolated *in vivo* metabolites isolated from Sy-fed rats' urine against MSSA and MRSA. Syringopicroside (**1**), Syringopicrogenin B (**MA1**), Sclerochitonoside B (**MA2**), Syringopicrogenin A (**MA3**), Tyrosol (**MA4**), *p*-Hydroxybenzoic acid (**MA5**), Benzoic acid (**MA6**) and 4-Hydroxy-acetophenone (**MA7**). Each MIC value expressed as the means ± SD (n=3).

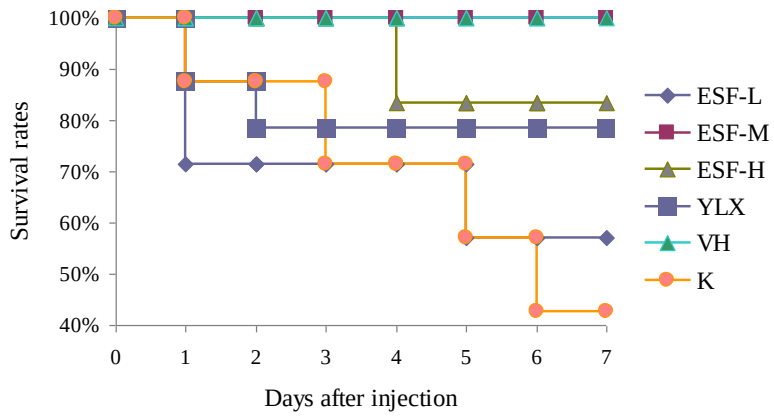


Fig.5 The survival rates of MRSA-infected mice. ESF-L group (82.2 mg·kg$^{-1}$, *n=7*); ESF-M group (164.4 mg·kg$^{-1}$, *n=6*); ESF-H group (328.8 mg·kg$^{-1}$, *n=6*); YLX group (82.2 mg·kg$^{-1}$, *n=7*);

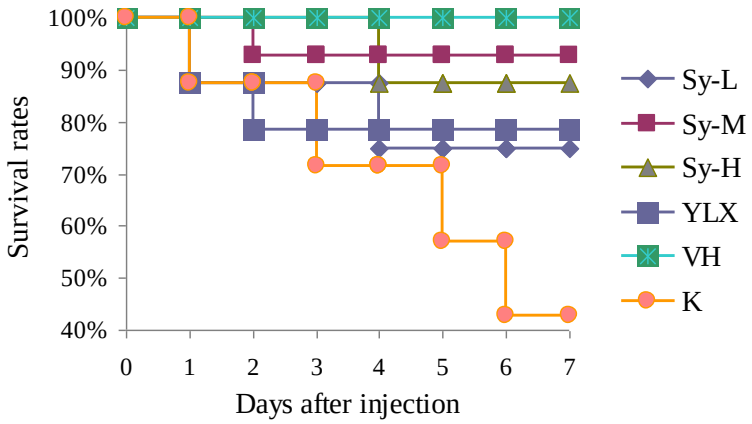


Fig.6 The survival rates of MRSA-infected mice. Sy-L group (27.4 mg·kg$^{-1}$, *n=8*); Sy-M group (54.8 mg·kg$^{-1}$, *n=7*); Sy-H group (109.6mg·kg$^{-1}$, *n=8*); YLX group (82.2 mg·kg$^{-1}$, *n=7*); VH group (300 mg·kg$^{-1}$, *n=7*) and blank control group (untreated, *n=7*).

Graphical abstract