Brief Report

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Anti-virulence activities of biflavonoids from *Mesua ferrea* L. flower

Xiaochun Zhang[1], Rongrong Gao[1], Yan Liu[1], Yuhe Cong[1], Dongdong Zhang[2], Yu Zhang[2], Xuefei Yang[2,*], Chunhua Lu[1,*], Yuemao shen[1]

[1] *Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, Jinan, Shandong, China;*

[2] *Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, China.*

Summary **Based on the anti-virulence activity on *Salmonella*, the ethyl acetate extract (EAE) of *Mesua ferrea* flower was investigated for its chemical constituents. Ten purified compounds were identified and assayed for their inhibitory activity against Type III secretion system (T3SS) by polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots experiments. We found the biflavonoids, rhusflavanone and mesuaferrone B, exhibited inhibitory effects on the secretion of *Salmonella* pathogenicity island 1 (SPI-1) effector proteins (SipA, B, C and D) without effecting the bacterial growth. In addition, 5, 6, 6'-trihydroxy-[1,1'-biphenyl]-3,3'-dicarboxylic acid (6) is a new natural product from *M. ferrea* flower.**

Keywords: *Mesua ferrea* (Roxb.) L., anti-virulence activity, biflavonoids, T3SS

1. Introduction

In the ongoing battle between people and pathogens, natural resources play an important role for saving people's life, therefore, natural medicinal plants used by ancient people are still valuable treasure to seek drugs (*1*). Bactericides, such as penicillin, can inhibit bacterial growth with high selection pressure and led to rapid increase in antibiotic resistance. Therefore, we need to change the idea to discover new antibiotics. An alternative idea on drug development is to inhibit bacterial virulence (including adhesion, secretion systems, or quorum sensing) (*2,3*), because virulence blockers inhibit pathogens by disarming the bacteria and preventing normal infection.

As we all know, *Salmonella enterica* is an important pathogen of humans and live-stocks. The gastroenteritis, dysentery and diarrhea caused by *Salmonella* are serious problems for public health, especially in tropical regions (*4*). The pathogenicity of *S. enterica* mainly depends on two type three secretion systems (T3SSs), encoded by *Salmonella* pathogenicity island 1 (*SPI-1*) and *SPI-2* (*5*). During infection, pathogens use the T3SS to inject effector proteins into target host cells, disrupting host defense mechanisms and allowing invasion. Therefore, to reduce virulence or inhibit specialized secretion systems are an obvious aim for us to develop drugs (*6,7*), and T3ss is a well-studied and attractive anti-virulence target (*6*).

The whole plant of *M. ferrea* is wildly used as medicinal plant in tropical Asia and India. The dried flowers are used for fever, astringent, anti-inflammation and also used in dysentery and anti-typhoid (*8,9*). In recent years, many bioactivities of *M. ferrea* were reported including antibacterial (*8,10-13*), antiarthritic (*14*), antioxidant and immunomodulatory (*15*), antispasmodic (*16*), estrogenic and progestational activity (*17*) and tyrosinase and elastase inhibitory activity (*18*). Our screening results on 93 Myanmar medicinal plants suggested that the flower extract of *M. ferrea* exhibited potent antibacterial and anti-T3SS activity (*12*). Therefore, this study was aiming

§These authors contributed equally to this work.
*Address correspondence to:
Dr. Xuefei Yang, Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China.
E-mail:xuefei@mail.kib.ac.cn

Dr. Chunhua Lu, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China.
E-mail: ahua0966@sdu.edu.cn

to explore the anti-virulence active components from *M. ferrea*. The present study investigated the chemical isolation of compounds (**1-10**) from the flowers of *M. ferrea* and identified rhusflavanone (**4**) and mesuaferrone B (**5**) as the chemical inhibitors of T3SS of *Salmonella*.

2. Material and Methods

2.1. *General experimental procedures*

The NMR spectra were obtained on a Bruker Avance DRX-400 NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C in CD_3OD using tetramethylsilane (TMS) as an internal standard. HRESIMS were carried out on an LTQ-Orbitrap XL. Column chromatography (CC) was carried out on medium preparative liquid chromatography (MPLC) (RP-18, 40~63 μm; Merck KGaA, Germany), Sephadex LH-20 (25-100 μm; GE Healthcare, Sweden) and normal silica gel (200-300 mesh; Qingdao Haiyang Chemical Co. Ltd., China).

2.2. *Bacterial strains and growth condition*

The strain used in this study is *Salmonella enterica* serovar Typhimurium UK-1 χ8956 (*S. Typhimurium*). The bacteria was grown on LB agar media (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4). *S. Typhimurium* was grown in LB broth or on LB agar plates supplemented with 0.2% L-arabinose at 37°C or 25°C with shaking at 220 rpm (*19*).

2.3. *Plant material*

The *M. ferrea* flowers were collected from the Hopong Township, Taunggyi Region, Shan State (20o47'27.47"N; 97o10'29.61"E 1115m), Myanmar, in May 2017. It was authenticated by Yu Zhang and the voucher specimen (MB201705TGY016) was deposited in Kunming Institute of Botany, Chinese Academy of Sciences.

2.4. *Extraction and isolation*

The *M. ferrea* flowers (1.0 kg) were dried and powdered and then extracted three times with 95% ethanol at room temperature. The ethanol extracts were combined and concentrated using a rotary evaporator, under reduced pressure at 40°C to yield a residue. Extractions were dispersed in water and extracted with petroleum ether (PE), ethyl acetate (EA) and n-butanol (Bu) respectively to result in three different crude extracts.

The active screening suggested that EA extract (EAE) and BuE exhibited anti-T3SS activity. In order to identify the anti-virulence components from *M. ferrea*, EAE was subjected to chromatography by MPLC (140 g RP-18) eluted with 30%, 50%, 70% and 100% MeOH (2 L each) to obtain 14 fractions and marked as Fr.1-14.

Fr.3 (467 mg) was further purified by MPLC (40 g RP-18 silica gel) and eluted with 10%, 15%, 20%, 25%, and 30% MeOH, 200 mL each to yield Fr.3A-3G. Fr.3C (22.5 mg) was subjected to CC over silica gel (PE:EA, 5:1, 4:1, 3:1, 2:1 and 1:1) to afford **10** (4.7 mg) and 7 (7.0 mg). Fr.3G (55.6 mg) was subjected to CC over silica gel (PE:EA, 5:1, 4:1, 3:1, 2:1 and 1:1) to afford **8** (4.4 mg). Fr.4 (399 mg) was further purified by MPLC (40g RP-18 silica gel; 10%, 20%, 25%, and 35%MeOH, 200 mL each, respectively) and further normal silica gel CC (PE:EA, 3:1, 2:1 and 1:1) to obtain **6** (7.3 mg). Fr.7 (420 mg) was subjected to MPLC over RP-18 silica gel (40 g) eluted with 40%, 45%, 50% MeOH to obtain Fr.7A-7H. Fr.7D (57.0 mg) was subjected to CC over silica gel (PE:EA, 2:1, 1:1, 1:2 and 1:3) to afford **2** (17.0 mg). Fr.7G (60.3 mg) and further purified by CC over silica gel (PE:EA, 2:1, 1:1 and EA) to afford **1** (16.7 mg). Fr.7H (50.4 mg) was purified by CC over silica gel (PE:EA, 5:1, 4:1, 3:1 and EA) to afford **3** (16.6 mg). Fr.9 (607.8 mg) was subjected to CC over Sephadex LH-20 eluted with MeOH to produce Fr.9A-E. Fr.9C (23.0 mg) was further purified by CC over silica gel (PE:EA, 2:1, 1:1, EA, MeoH) to obtain **4** (12 mg). Fr.9D (35.3 mg) was subjected to CC over silica gel (PE:EA, 10:1 , 10:2 , 2:1 , 1:1 and EA) to afford **5** (6 mg). Fr.9E (20.1 mg) was purified by CC over silica gel (PE:EA, 2:1 and 1:1) to obtain **9** (2.1 mg, from 1:1 eluent).

According to the TLC detection, BuE showed similar behavior with EAE. HPLC detection for fractions EAE and BuE also suggested that compounds **4** and **5** are the main constituents in BuE (Figure S24, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=45*), which can explain the anti-virulence activity of BuE.

2.5. *Detection of secreted SPI-1-associated effector proteins*

The extracts PEE, EAE and BuE together with the purified 10 compounds (**1-10**) were screened for their inhibitory effects on the secretion of the SPI-1 effector proteins of *S. Typhimurium*. Cytosporone B was used as the positive control (*12,20*). The 25°C overnight cultures of *S. enterica* were diluted 10 times with LB medium containing 0.2% L-arabinose and cultivated for 4 h at 37°C/220 rpm in the absence or presence of extracts (100 μg/mL) or compounds (100 μM). The supernatant of 1 mL culture was used to obtain secreted proteins. The proteins were precipitated with a final concentration of 10%TCA by repeated at 4°C and centrifuged at 12,000 g for 15 min and washed with 250 μL ice-chilled acetone. The precipitates were dried for 15 min. The pellets were dissolved with loading buffer to an optical density (OD_{600}). The protein samples were

heated to 95°C for 5-10 min to denature the proteins and then analyzed by 10% SDS-PAGE. Protein were visualized either by Coomassie blue or by Western blots.

2.6. *Western blots*

This procedure was the same as our previous described (*12,20*). *S. Typhimurium* was cultured and treated as described above. The proteins were separated by 10% SDS-PAGE and blotted onto PVDF membranes. The blotted membrane was washed with 5% w/v BSA (bovine serum albumin) in TBST (Tris-buffered saline mixed with Tween 20) at room temperature for 1 h. Then, membranes were incubated in 5% BSA containing anti-SipC or anti-FliC monoclonal antibody overnight at 4°C and then washed with TBST (5 min, three times). The membrane was incubated for 1 h in TBST containing the secondary antibody at room temperature with shaking. Then, membranes were washed three times with TBST again. Finally, the membrane was incubated in ECLA reaction buffer (0.1 M Tris-HCl, pH 8.5, 25 mM luminol, 4 mM p-coumaric acid) and ECLB reaction buffer (0.06% v/v H2O2 in 0.1 M Tris-HCl, pH 8.5) for 2 min, and proteins were detected by ECL method (Molecular Imager ChemiDoc XRSt; Bio-Rad, Hercules, CA). Relative intensity of protein levels of SipC and FliC were analyzed using Image Lab Software.

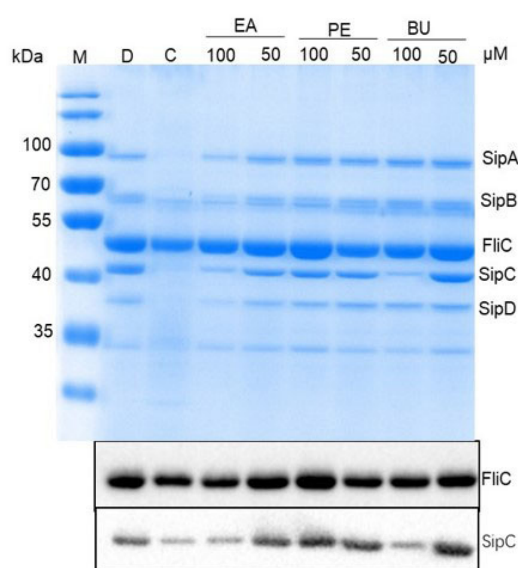

Figure 1. Extracts inhibited secretion of SPI-1 effector proteins. SipA/B/C/D, SPI-1 effector proteins; FliC, flagellar filament protein; M, marker; D, DMSO control; C, CsnB.

3. Results and Discussion

3.1. *Structure elucidation of the isolated compounds*

The anti-T3SS activity screening and western blot results suggested that the EAE and BuE with 100 μg/mL from *M. ferrea* flower showed evident anti-T3SS effects on the secretion of effector proteins SipA, B, C and D (Figure 1). From EAE, three flavonoids (**1-3**), two biflavonoids (**4** and **5**) and five phenolic compounds (**6-10**) (Figure 2) were obtained and their structures were identified by their ^{1}H ,^{13}C NMR (see supporting information), HSQC, HMBC and ESIMS spectroscopic analyses and further comparing with those reported data. They were identified as kaempferol-3-O-rhamnoside (**1**) (*21*), quercitrin (**2**) (*22*), quercetin (**3**) (*23,24*), rhusflavanone (**4**) (*25*), mesuaferrone B (**5**) (*18,26*), 5,6,6'-trihydroxy[1.1'-biphenyl]-3,3'-

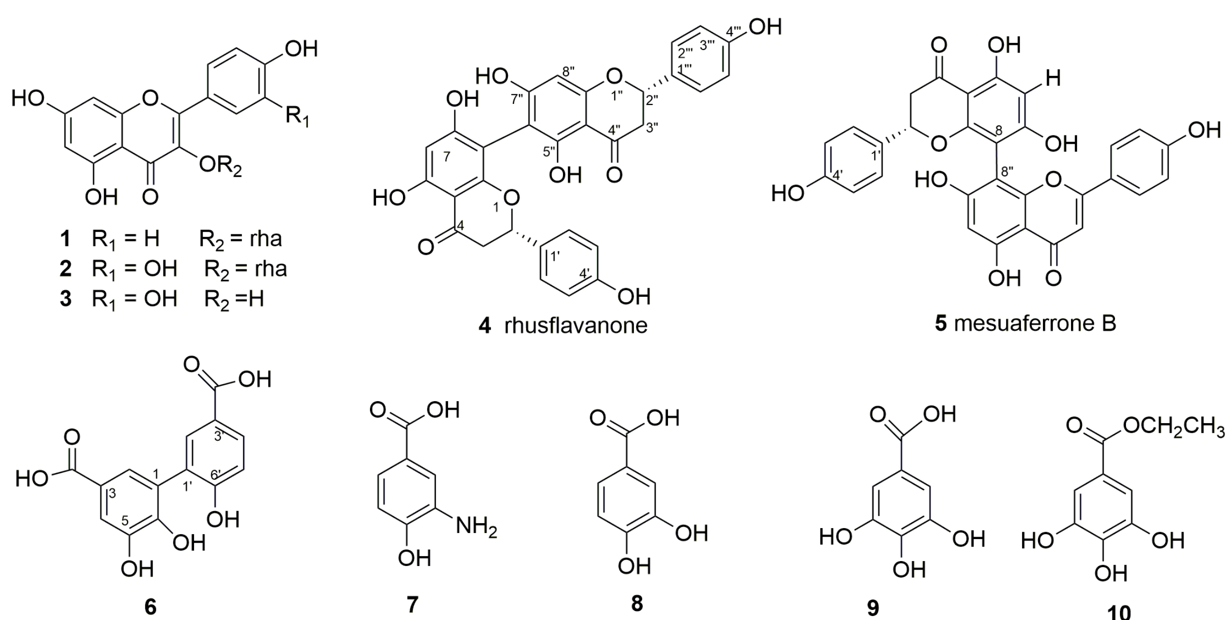

Figure 2. The chemical structures of compounds 1-10.

dicarboxylic acid (**6**), 3-amino-4-hydroxybenzoic acid (**7**) (*27,28*), procatechuic acid (**8**) (*29,30*), gallic acid (**9**) (*31*) and procatechuic acid ethyl ester (**10**) (*32,33*).

It is emphasized here that **6** was obtained as a colorless powder and it was identified as a new natural product. The molecular formula was determined to be $C_{14}H_{10}O_7$ according to the HRESIMS data of *m/z* 291.1571 $[M+H]^+$. ^{1}H NMR data (400 MHz in CD_3OD): δ_H 7.51 (d, *J* = 1.9 Hz, H-2), 7.17 (d, J = 1.8 Hz, H-4), 7.84 (d, *J* = 1.9 Hz, H-2'), 7.49 (dd, *J* = 8.2, 1.8 Hz, H-4'), 6.87 (d, *J* = 8.2 Hz, H- 5'); ^{13}C NMR data (100 MHz in CD_3OD): δ_C: 133.6s (C-1), 112.4d (C-2), 112.1s (C-3), 111.6d (C-4), 146.5s (C-5), 142.1s (C-6), 170.9s (C-7), 133.2s (C-1'), 118.8d (C-2'), 123.4s (C-3') 124.7d (C-4'), 115.6d (C-5'), 153.4s (C-6'), 170.9s (C-8).

3.2. *Rhusflavanone and Mesuaferrone B inhibited the secretion of SPI-1 effector proteins*

The inhibitory effects on secretion of pathogenicity island-1 (SPI-1)-associated effector proteins on *Salmonella in vitro* were investigated for compounds **1-10**. We found that rhusflavanone (**4**) and mesuaferrone B (**5**) exhibited inhibitory effects on the secretion of the T3SS effectors SipA, B, C and D without effecting on FliC (Figure 3). Mesuaferrone B can inhibit the secretion of effector proteins in a dose dependent manner (Figure 4a) and no effect on the growth of *Salmonella* (Figure 4b). Therefore, mesuaferrone B from *M. ferrea* may be an inhibitor of T3SS of *Salmonella*.

In conclusion, rhusflavanone and mesuaferrone B are the anti-virulence components from *M. ferrea* with inhibitory activity on the secretion of effector proteins on *Salmonella*. Although, some researches have reported the diverse activity of biflavonoids (*18,25,34,35*), no anti-virulence activity has not been reported. Our results provide promising baseline information for the potential use of *M. ferrea* in the treatment of bacterial infections through anti-virulence pathway.

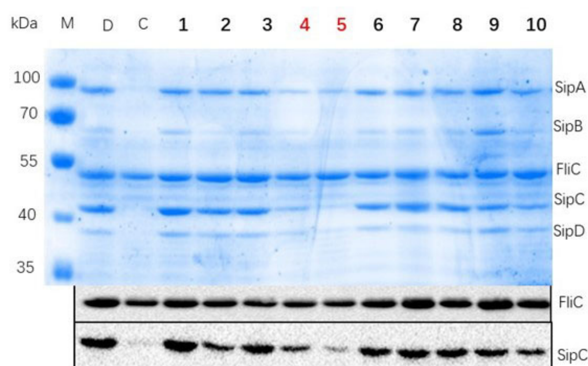

Figure 3. Compounds inhibited secretion of SPI-1 effector proteins. SipA/B/C/D, SPI-1 effector proteins; FliC, flagellar filament protein; M, marker; D, DMSO control; C, CsnB.

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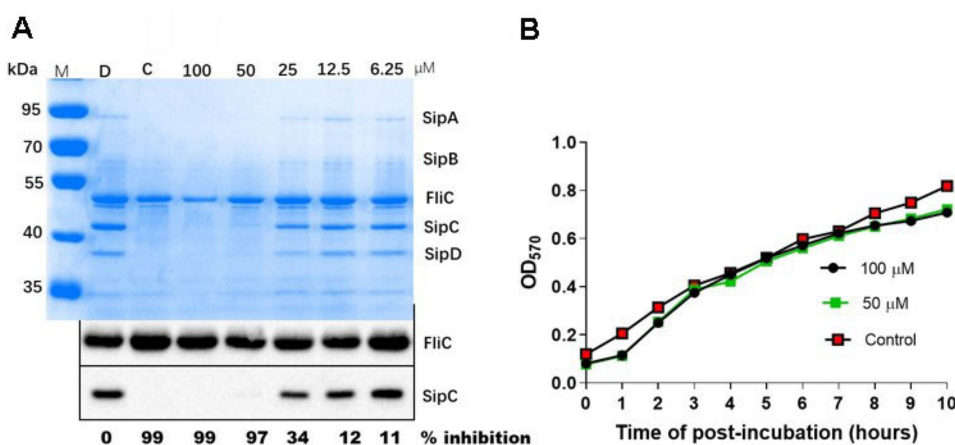

Figure 4. Mesuaferrone B (5) inhibited the secretion of SPI-1 effectors in a dose-dependent manner and did not affect growth of S. Typhimurium. A) Mesuaferrone B inhibited secretion of SPI-1 effector proteins at a range of concentrations from 6.25 to 100 μM detected by SDS-PAGE followed by Coomassie blue staining or Western blotting. M, marker; C, DMSO control. Inhibition percentage was calculated by comparing the SipC blot with that of the DMSO control. **B)** Mesuaferrone B did not inhibit the bacterial growth at a range of concentrations at 50 and 100 μM.

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