

Chemical constituents and biological activities of *Stephania yunnanensis* H. S. Lo

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Abstract

To study the chemical constituents of *Stephania yunnanensis* H. S. Lo and their in vitro antibiotic activities. Silica gel, alumina and Sephadex LH-20 chromatographic methods are used to isolate alkaloidal constituents. Structures of the compounds are identified based on physicochemical properties and spectral analysis. Kirby-Bauer test and porous plate method are used to study the inhibitory activities of isolated chemical constituents on methicillin-resistant *S. aureus* (MRSA). Five chemical constituents are isolated from the tuber extract of *Stephania yunnanensis* H. S. Lo, namely tetrahydropalmatine (1), palmatine (2), sinoacutine (3), dicentrine (4) and jatrorrhizine (5). Inhibition zone diameters of tetrahydropalmatine and palmatine against MRSA are larger at a concentration of 50 mg/mL, which are 13 mm and 16 mm, respectively; tetrahydropalmatine and palmatine both have inhibitory effects on MRSA, with MIC of 0.312 µg/ml and 0.156 µg/ml. In the porous plate method, IC₅₀ of tetrahydropalmatine and palmatine are 0.436 µg/ml and 0.201 µg/ml. Alkaloidal constituents in *Stephania yunnanensis* H. S. Lo are mainly berberine type, morphinan type and protoberberine type alkaloids. Besides, tetrahydropalmatine and palmatine have rather significant anti-MRSA activities.

Keywords: *Stephania yunnanensis* H. S. Lo, Chemical constituent, Alkaloid, MRSA, Biological activity

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Introduction

Stephania yunnanensis H. S. Lo is a famous traditional Chinese herbal medicine in Yunnan, China, which is often called Shanwugui, Jinbuhuan, Didan and Baididan in folk medicine. The herb is bitter, acrid, cold and slightly toxic, which has heat-clearing, detoxifying, sedative, analgesic and qi-regulating functions. In the folk medicine, it is used to treat snake bites, rheumatic pain, stomach ache, etc. Studies have shown that *Stephania yunnanensis* H. S. Lo contains a variety of chemical constituents, which are mainly alkaloids, and some polysaccharides [1-3]. Modern medical research has revealed that *Stephania* has insecticidal, antibiotic, and antiviral activities [4-7].

Snake bites are prone to cause infection; in folk medicine, *Stephania* is used to treat snake bites, suggesting that *Stephania* may contain some natural products with good antibiotic activities. To further discover natural products with antibiotic activities, this paper extracts and isolates monomer compounds in *Stephania yunnanensis* H. S. Lo, and conducts in vitro antibiotic activity tests on these monomer chemicals.

Materials

Melting point was measured with SGW X-4 micro melting point apparatus (Wuguang, Shanghai), and was uncorrected; MS was measured with Waters Synapt G2 mass spectrometer; and NMR was measured with Bruker-400 SYS-600 NMR spectrometer using TMS as the internal standard. Miniature vortex mixer was manufactured by Huxi Analytical Instrument Factory, Shanghai; ZHJH-CH09B clean bench was manufactured by Zhicheng Analytical Instrument Manufacturing Co., Ltd., Shanghai; and ZD-85A dual-function gas bath thermostatic oscillator was manufactured by Jieruier Electrical Equipment Co., Ltd., Jintan.

Column chromatography and TLC silica gel plates were manufactured by Qingdao Haiyang Chemical Plant; column chromatography alumina was manufactured by Kaimei Chemical Reagent Factory; and Sephadex LH-20 was manufactured by Amersham Pharmacia, Sweden. Reagents like ethanol, acetone, chloroform and cyclohexane used were industrially or chemically pure solvents; and TLC reagents used were 10% sulfuric acid in ethanol and modified bismuth potassium iodide solution. Nutrient broth medium was provided by Aobox

Biotechnology LLC, Beijing; and agar was provided by Solarbio Technology Co., Ltd., Beijing.

Stephania yunnanensis H. S. Lo medicinal herb was purchased from a medicinal material wholesale market in Gejiu, Yunnan, which was identified by Professor Zhang Dongfang of the China Medical University as the *Stephania yunnanensis* H. S. Lo in the genus *Stephania* of the family Menispermaceae. The specimens were preserved in the College of Pharmacy of Dalian Medical University. *Methicillin-resistant S. aureus* (MRSA) was isolated at the First Affiliated Hospital of Dalian Medical University, whose coincidence rate was 99% upon identification by automated microbial analyzer VITEK-AMS.

Extraction and Isolation

Stephania yunnanensis H. S. Lo was sliced, dried and crushed, then 5 Kg of the powder was taken and extracted for 2 h with 95% ethanol three times. The extracts were combined, and solvent was recovered under reduced pressure to give 700 g of extract. After suspending in water, the extract was extracted sequentially with petroleum ether, ethyl acetate and n-butanol to give respective fractions. 190 g of ethyl acetate fraction was isolated repeatedly by silica gel and alumina column chromatographies, and purified by Sephadex LH-20 column chromatography to obtain 5 compounds: tetrahydropalmatine (120 mg), palmatine (360 mg), sinoacutine (80 mg), dicentrine (56 mg) and jatrorrhizine (89 mg).

Structure Elucidation

(1) Tetrahydropalmatine: Colorless tabular crystals (methanol), Mp. 149.6~142.1°C, ESI-MS (m/z) 356 [M+H]⁺, C₂₁H₂₅NO₄. ¹H-NMR (400 MHz, CD₃OD) δ: 6.77 (1H, s, H-1), 6.65 (1H, s, H-4), 2.79 (2H, t, J=6.0 Hz, H-5), 3.13 (2H, t, J=6.0 Hz, H-6), 4.31 (1H, d, J=15.8 Hz, H-8α), 3.54 (1H, d, J=15.8 Hz, H-8α), 6.89 (1H, d, J=8.4 Hz, H-11), 6.81 (1H, d, J=8.4 Hz, H-12), 2.90 (2H, m, H-13), 3.23 (1H, d, J=5.4 Hz, H-14), 3.83 (3H, s, 9-OCH₃), 3.87 (3H, s, 10-OCH₃), 3.89 (3H, s, 2-OCH₃), 3.85 (3H, s, 3-OCH₃); ¹³C-NMR (100 MHz, CD₃OD) δ: 109.1 (C-1), 125.9 (C-1a), 147.9 (C-2), 147.1 (C-3), 110.6 (C-4), 129.0 (C-4a), 29.2 (C-5), 51.7 (C-6), 54.1 (C-8), 128.2 (C-8a), 151.0 (C-9), 145.5 (C-10), 110.8 (C-11), 121.6 (C-12), 126.9 (C-12a), 36.5 (C-13), 60.2 (C-14), 59.9 (9-OCH₃), 56.1 (3-OCH₃), 56.1 (10-OCH₃), 56.1 (2-OCH₃). ¹H-NMR and ¹³C-NMR data were basically consistent with those reported in the literature [8], so the compound was identified as Tetrahydropalmatine.

(2) Palmatine: Pale yellow needle crystals (methanol), Mp. 205.2-207.9°C, ESI-MS (m/z) 352 [M+H]⁺, C₂₀H₁₉NO₄. ¹H-NMR (400 MHz, DMSO) δ: 7.75 (1H, s,

H-1), 7.12 (1H, s, H-4), 3.22 (2H, t, J=6.5 Hz, H-5), 4.99 (2H, t, J=6.5 Hz, H-6), 9.85 (1H, s, H-8), 8.21 (1H, d, J=9.1 Hz, H-11), 8.11 (1H, d, J=9.1 Hz, H-12), 9.12 (1H, s, H-13), 4.15 (3H, s, 9-OCH₃), 4.09 (3H, s, 10-OCH₃), 3.95 (3H, s, 2-OCH₃), 3.81 (3H, s, 3-OCH₃); ¹³C-NMR (100 MHz, DMSO) δ: 109.9 (C-1), 149.2 (C-2), 149.8 (C-3), 112.1 (C-4), 132.8 (C-4a), 26.4 (C-5), 55.5 (C-6), 145.9 (C-8), 119.1 (C-8a), 152.0 (C-9), 143.5 (C-10), 123.6 (C-11), 126.9 (C-12), 129.1 (C-12a), 119.9 (C-13), 137.5 (C-14), 121.3 (C-14a), 61.5 (9-OCH₃), 57.2 (3-OCH₃), 56.1 (10-OCH₃), 55.6 (2-OCH₃). ¹H-NMR and ¹³C-NMR data were basically consistent with those reported in the literature [9], so the compound was identified as Palmatine.

(3) Sinoacutine: Colorless blocky crystals (methanol), Mp. 197.9-199.3°C, ESI-MS (m/z) 328 [M+H]⁺, C₁₉H₂₁O₄N. ¹H-NMR (400 MHz, CDCl₃) δ: 6.74 (1H, d, J=8.3 Hz, H-1), 6.66 (1H, d, J=8.3 Hz, H-2), 6.32 (1H, s, H-5), 7.61 (1H, s, H-8), 3.61 (1H, d, J=5.3 Hz, H-9), 2.93 (1H, dd, J=17.6 Hz, 5.3 Hz, H-10α), 3.32 (1H, d, J=17.6 Hz, H-10β), 1.78 (1H, td, J=12.6 Hz, 4.5 Hz, H-15α), 2.41 (1H, d, J=12.6 Hz, H-15β), 2.52 (1H, dd, J=12.4 Hz, 3.0 Hz, H-16α), 2.62 (1H, dd, J=12.4 Hz, 3.0 Hz, H-16β), 3.76 (3H, s, 3-OCH₃), 3.89 (3H, s, 6-OCH₃), 6.41 (1H, br.s, 4-OH), 2.46 (3H, s, N-CH₃); ¹³C-NMR (100 MHz, CDCl₃) δ: 121.2 (C-1), 109.0 (C-2), 144.9 (C-3), 144.1 (C-4), 118.6 (C-5), 161.5 (C-6), 182.1 (C-7), 123.4 (C-8), 61.2 (C-9), 37.9 (C-10), 130.8 (C-11), 124.1 (C-12), 44.2 (C-13), 152.1 (C-14), 32.7 (C-15), 47.2 (C-16), 41.8 (N-CH₃), 56.4 (3-OCH₃), 54.9 (6-OCH₃). ¹H-NMR and ¹³C-NMR data were basically consistent with those reported in the literature [10], so it was identified as Sinoacutine.

(4) Dicentrine: Pale yellow blocky crystals (methanol), Mp. 168.1-170.3°C, ESI-MS (m/z) 340 [M+H]⁺. ¹H-NMR (400 MHz, CDCl₃) δ: 6.55 (1H, s, H-3), 2.67 (2H, m, H₂-4), 3.23 (4H, m, H₂-5, H₂-7), 3.98 (1H, m, H-6α), 5.95, 6.10 (each 1H, d, J=1.9 Hz, -OCH₂O-), 6.82 (1H, s, H-8), 7.76 (1H, s, H-11), 2.53 (3H, s, N-CH₃), 3.90 (6H, s, 9-OCH₃, 10-OCH₃); ¹³C-NMR (100 MHz, CDCl₃) δ: 142.8 (C-1), 117.7 (C-1a), 127.2 (C-1b), 148.1 (C-2), 105.9 (C-3), 123.9 (C-3a), 29.1 (C-4), 53.7 (C-5), 63.4 (C-6a), 35.9 (C-7), 128.1 (C-7a), 111.4 (C-8), 147.9 (C-9), 148.8 (C-10), 110.6 (C-11), 126.9 (C-11a), 101.7 (-OCH₂O-), 44.1 (N-CH₃), 56.2 (9-OCH₃), 56.9 (10-OCH₃). The above data were consistent with those reported in the literature [11], so the compound was identified as Dicentrine.

(5) Jatrorrhizine: Red prismatic crystals (methanol), Mp. 205.3-207.9°C, ESI-MS (m/z) 338 [M]⁺. ¹H-NMR (400 MHz, DMSO) δ: 7.72 (1H, s, H-1), 6.96 (1H, s, H-4), 3.13 (2H, t, J=6.5 Hz, H-5), 4.98 (2H, t, J=6.5 Hz, H-6), 9.82 (1H, s, H-8), 8.21 (1H, d, J=9.2 Hz, H-11), 8.10 (1H, d, J=9.2 Hz, H-12), 9.01 (1H, s, H-13), 4.13 (3H, s, 9-OCH₃), 4.07

(3H, s, 10-OCH₃), 3.94 (3H, s, 2-OCH₃); ¹³C-NMR (100 MHz, DMSO) δ: 109.7 (C-1), 147.9 (C-2), 149.5 (C-3), 114.4 (C-4), 134.3 (C-4a), 26.7 (C-5), 56.3 (C-6), 145.8 (C-8), 117.9 (C-8a), 151.1 (C-9), 143.9 (C-10), 123.1 (C-11), 126.0 (C-12), 129.6 (C-12a), 118.2 (C-13), 137.3 (C-

14), 122.1 (C-14a), 61.9 (9-OCH₃), 57.2 (2-OCH₃), 56.7 (10-OCH₃). The above data were consistent with those reported in the literature [12], so the compound was identified as Jatrorrhizine.

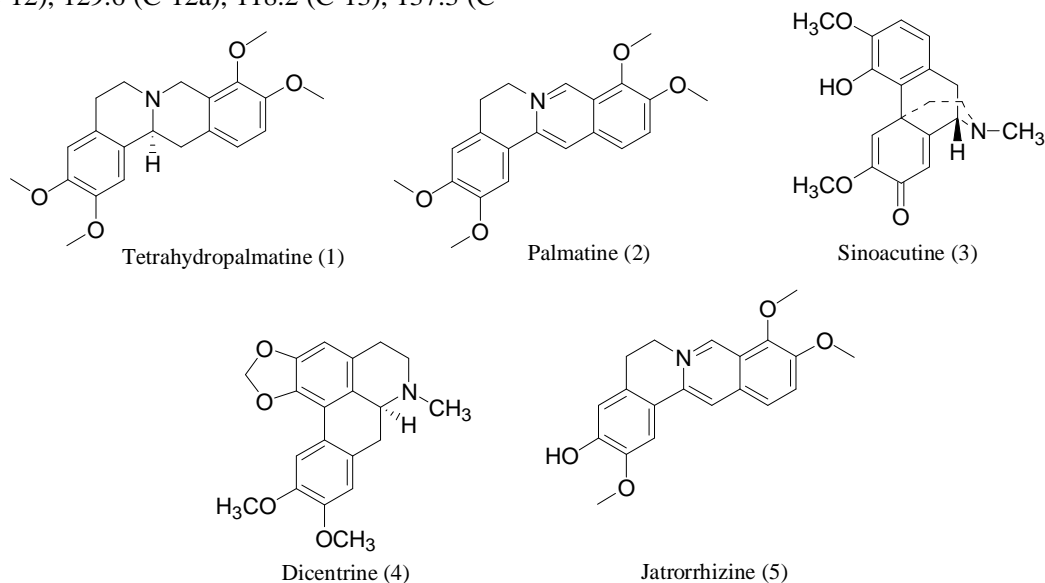


Figure 1. The structure of compounds from *Stephania yunnanensis* H. S. Lo

Determination of Antibiotic Activities

Preparation of samples

Tetrahydropalmatine and palmatine were dissolved separately in DMSO to prepare 50 mg/mL stock solutions for later use.

Activation of test strains

MRSA strains were seeded on slant medium, and cultured in a 37°C incubator for 24 h for later use.

Preparation of bacterial culture plate

The prepared agar medium was transferred into conical flask, autoclaved at 121°C for 15 min, and cooled to 50~60°C, then poured into plate on a clean bench, and cooled to give solid medium for late use.

Preparation of bacterial suspension

After activation, the strains were seeded on broth medium, then prepared into a bacterial suspension of a certain concentration, and stored in a 4°C refrigerator (not longer than 12 h).

Preparation of drug-containing paper discs

The samples were diluted, and sterilized for later use. Each paper disc (6 mm in diameter) was added with 5 µL of solutions, whereas blank control group was added with 5 µL of sterile distilled water. Negative control group was added with 5 µL of analytical grade DMSO.

K-B method

On a clean bench, sterilized agar media were poured into Petri dishes. After marking the dishes, 0.1 mL of prepared bacterial suspension was pipetted with a sterile pipette into the Petri dishes, and spread uniformly with a spreader. The above paper discs were placed equidistantly on the bacterium-containing plates to prepare bacterial plates. After setting aside at room temperature for 3~5 min, drug-containing paper discs were added, and the plates were incubated at a 37°C incubator for 24 h, then colony growths were observed, and inhibition zone diameters were determined accurately [7]. Each antibiotic test was repeated twice in parallel for each diluent.

Determination of minimum inhibitory concentration (MIC)

After samples were diluted proportionally with suitable amounts of solvent (DMSO), each paper disc was added with 5 µL of drug solutions, blank control group was added with 5 µL of sterile distilled water, and negative control group was added with 5 µL of analytical grade DMSO. The concentration at which there was no detectable inhibition zone diameter was the MIC. All operations were carried out under sterile conditions.

Determination of median inhibitory concentration (IC₅₀) [8-16]

Clean and sterile 96-well culture plates were added separately with 90 µL of MRSA that was cultured in broth

medium for 18 h, and then added with 10 μ L of sample stock solutions with different concentration gradients; the final concentration of solvent (DMSO) was 1/10 the concentration of stock solutions. Meanwhile, negative, blank and solvent controls were set up, each with three replicates. After the 96-well plates were cultured at 37°C for 20 h, absorbance (A) was measured at 595 nm using a microplate reader. Supernatant A of each sample under the same conditions was taken as the blank control, after subtracting the blank control, A of bacterial amount was obtained, corresponding supernatant was prepared under 10000/6 min conditions, and drawn 100 μ L correspondingly. Inhibition rate (%) was calculated according to the following formula:

$$\text{Inhibition rate} = [(A_{\text{solvent control}} - A_{\text{control supernatant}}) - (A_{\text{drug}} - A_{\text{negative supernatant}})] / (A_{\text{positive control}} - A_{\text{negative supernatant}}) \times 100\%$$

Table 1. Inhibition zones of monomer compounds against MRSA

Sample	Tetrahydropalmatine	Palmatine	Blank	DMSO	Positive control (berberine)
Concentration (mg/ml)	50	50	-	-	50
Inhibition zone (mm)	13	16	0	0	15

Determination of MIC

Each sample was tested as per the method under "Determination of minimum inhibitory concentration (MIC)" for antibiotic activity against MRS; the data are shown in Table 2.

Table 2. MIC of Tetrahydropalmatine and Palmatine against MRSA

Sample	0.312
Tetrahydropalmatine	0.156
Palmatine	0.156
Positive control (berberine)	0.312

Determination of IC₅₀

Each sample was tested for IC₅₀ in accordance with the "Determination of median inhibitory concentration (IC₅₀); the results are shown in Table 3.

Table 3. IC₅₀ of Tetrahydropalmatine and Palmatine against MRSA

Sample	IC ₅₀ (μ g/ml)
Tetrahydropalmatine	0.436
Palmatine	0.201
Positive control (berberine)	0.223

Stephania may achieve treatment of snake bites in the folk medicine by two ways: 1. it can detoxify snake venom, and avoid the venom from damaging other organs; 2 it

$$\text{solution} - A_{\text{drug supernatant}}] / (A_{\text{positive control}} - A_{\text{negative supernatant}}) \times 100\%$$

Curves were plotted with sample concentration versus inhibition rate, linear regression equation was solved, and concentration causing 50% inhibition, i.e. IC₅₀ value, was calculated.

Results

Determination of inhibition zone

Tests were carried out as per the procedure under "K-B method", respectively. Inhibition zone data of each sample are shown in Table 1.

can heal wounds, and kill bacteria around the wounds. The aim of this study is to find active constituents in *Stephania yunnanensis* H. S. Lo by studying its chemical constituents and antibiotic activities, so as to lay the foundation for development and utilization of *Stephania yunnanensis* H. S. Lo resources.

Five compounds isolated from *Stephania yunnanensis* H. S. Lo are all alkaloids, of which tetrahydropalmatine and palmatine are berberine alkaloids, sinoacutine and dicentrine are morphinane alkaloids, and jatrorrhizine protoberberine alkaloid. This suggests that alkaloids are the main chemical constituents of *Stephania yunnanensis* H. S. Lo. Alkaloids have a high biological activity, and berberine alkaloids have a good antibiotic activity [17-18]. To study in depth the chemical constituents in *Stephania yunnanensis* H. S. Lo, we choose tetrahydropalmatine and palmatine as the study objects. Main reasons are as follows: 1. tetrahydropalmatine and palmatine are alkaloids, which have good biological activities. 2 Tetrahydropalmatine and palmatine have similar structures, so differences in antibiotic activities can be explored from the structural perspective.

Experimental results show that the inhibition zone diameters of tetrahydropalmatine and palmatine against MRSA are larger at a concentration of 50 mg/mL, which are 13 mm and 16 mm, respectively. Tetrahydropalmatine and palmatine both have inhibitory effects on MRSA, with MIC of 0.312 μ g/ml and 0.156 μ g/ml. In the porous plate method, IC₅₀ of tetrahydropalmatine and palmatine

are 0.436 µg/ml and 0.201 µg/ml. All anti-MRSA activity indicators of palmatine are higher compared with the positive control berberine, whereas tetrahydropalmatine has anti-MRSA activity indicators all slightly lower than berberine. Main reasons may be: 1. compared with tetrahydropalmatine, palmatine has conjugated structure, and uniform electron cloud distribution, its solubility is better than tetrahydropalmatine, which can easily penetrate bacterial membrane and fully act with bacteria. 2. Palmatine is smaller in terms of spatial volume, which is substantially in the same plane, while tetrahydropalmatine occupies larger space structurally. Spatial difference may result in deviation in targets, leading to difference in antibiotic activities.

There is still space for in-depth study of this topic. To be able to further explore its antibiotic mechanism, the following experiments can be conducted: 1. TEM observation of microscopic damage of bacteria by drug. 2. Flow cytometric detection of changes in bacterial metabolic cycle. 3. Establishment of wound infection model to clarify the healing effect of drug on infected wounds, so as to lay a solid foundation for development and utilization of *Stephania yunnanensis* H. S. Lo.

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