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Abstract

Background: *Sophora flavescens* Ait. is a traditional Chinese medicine with a long history in China. It is mainly used in the treatment of heat dysentery and similar ailments in the clinical. The objective of this paper was to isolate, purify and identify alkaloids from *Sophora flavescens* Ait. and to explore their inhibitory effects on C6 glioma cells.

Materials and Methods: Column chromatography, extraction and NMR spectroscopy were used to structurally identify the isolated compounds. MTT assay and flow cytometry were used to detect the inhibitory effect of matrine on C6 cells.

Results: Three compounds were isolated from *Sophora flavescens* Ait., namely matrine, oxymatrine and lupeol. Different concentrations of matrine solution all had inhibitory effects on growth of C6 cell lines, which showed apparent dose-effect relationship. Compared with the control group, proportion of G0/G1 phase cells increased in each matrine concentration group to a maximum of 79.8%; proportion of S phase cells reduced, and proportion of G2/M phase cells declined slightly to a minimum of 6.3%, suggesting that after the action of matrine proliferation of C6 cells was significantly inhibited and the cells were arrested in the G1 phase.

Conclusion: We concluded that *Sophora flavescens* Ait. has an inhibitory effect on C6 cell proliferation.

Keywords: *Sophora flavescens* Ait.; oxymatrine; lupeol; C6 glioma cells

Introduction

Chinese medicine *Sophora flavescens* Ait. is a plant in the genus *Sophora* of the family Leguminosae, which is a traditional Chinese medicine with a long history in China (Yuan et al., 2003), *Sophora flavescens* Ait. is recorded in the "Pharmacopoeia of the People's Republic of China" (2010 edition). It has heat-clearing, damp-drying (Zhao & Zhang, 1991), insecticidal and diuretic effects. Clinically, it is mainly used in the treatment of heat dysentery, hemafecia, jaundice, anuresis, leucorrhoea with reddish discharge, vulval swelling, pruritus vulvae, eczema, as well as external treatment of trichomonas vaginitis (Zhang et al., 2000; Zhu et al., 2000; Kang et al., 2003; Wang & Wang, 2000).

Matrine is one of main alkaloid constituents in *Sophora flavescens* Ait., which can also be isolated from the root of *Sophora subprostrata*, aerial parts of *Sophora tonkinensis*, whole plant of *Sophora alopecuroides*, and bark of *Sophora chrysophylla* and *Sophora tetraptera*. In this paper, three compounds from ethanol extract of *Sophora flavescens* Ait. were isolated by methods such as extraction, polyamide column chromatography, silica gel column chromatography and recrystallisation. Meanwhile, the effect of matrine on proliferation of human glioma cell line C6 was investigated.

Materials and Methods

Drugs and reagents

Sophora flavescens Ait. was purchased from medicine wholesale market, which was identified as *Sophora flavescens* Ait. in the genus *Sophora* of the family Leguminosae by Dr. Han Wang from Henan College of Traditional Chinese Meditation. Reagents included DMSO, MTT (Sigma, USA), fetal bovine serum (Gibco), and RPMI 1640 medium (Invitrogen).

Instruments

The main instruments used in the study include the following: CO-150 CO₂ incubator (NBS, USA), EPICS-XL flow cytometer (Beckman

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Coulter, USA), SW-CJ-SF clean bench (Suzhou Purification Equipment Factory, Sujing Group); model 680 microplate reader (Bio-Rad, USA).

Results

Extraction and isolation of alkaloids from *Sophora flavescens* Ait.

3 kg of *Sophora flavescens* Ait. was weighed out, ground, and extracted three times with 95% ethanol. Each extraction lasted 1.5 h. The extracts were combined, and then recovered under reduced pressure until no ethanol was smelt. PH value of aqueous ammonia was adjusted to 10.0. Extraction was performed five times with chloroform. Then, the extracts were combined and solvent was recovered to give crude product of total alkaloids. The total alkaloids were column chromatographed on silica gel and gradient eluted with chloroform-methanol-aqueous to give compounds 1-3.

Structural identification of alkaloids from *Sophora flavescens* Ait.

Compound 1: white columnar crystals (petroleum ether), easily soluble in methanol, chloroform, acetone, and slightly soluble in petroleum ether. m.p. 81-83°C. Data were attributed as follows: ¹H-NMR(300 MHz,,DMSO-d₆) δ: 2.07 (1H, m, 2Ha), 2.83 (1H, m, 2He), 1.96 (1H, m, 3Ha), 1.46 (1H, m, 3He), 1.46-1.38 (m, 4Ha), 1.93 (m, 4He), 1.78-1.55 (m, 6H), 1.78-1.55 (m, 6H), 1.78-1.55 (m, 7H), 1.47-1.34 (m, 8H), 1.84 (m, 9Ha), 1.43-1.36 (m, 9He), 2.13 (m, 10Ha), 2.84 (m, 10He), 3.85 (dt, J=9.7, 6.0Hz), 1.54 (m, 12Ha), 1.95 (m, 12He), 1.76-1.55 (m, 13H), 2.23 (m, 14Ha), 2.44 (m, 14He), 3.08 (t, J=12.4Hz, 17Ha), 4.45 (dd, J=12.7, 4.2Hz, 17He). ¹³C-NMR (75 MHz, DMSO-d₆) δ: 57.5 (C-2), 21.4 (C-3), 27.5 (C-4), 35.5 (C-5), 63.7 (C-6), 41.3 (C-7), 26.6 (C-8), 20.8 (C-9), 57.4 (C-10), 53.5 (C-11), 27.9 (C-12), 19.2 (C-13), 32.6 (C-14), 169.6 (C-15), 43.4 (C-17). Based on the above spectroscopic data and by contrasting the data with literature (Song et al., 2008), compound 1 was identified as matrine. The structure is shown in Figure 1.

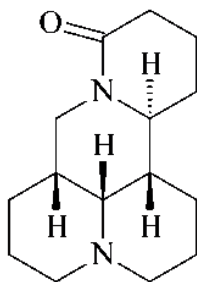


Figure 1: Matrine

Compound 2: white prismatic crystals (chloroform-ether), easily soluble in chloroform, methanol, slightly soluble in acetone, and insoluble in petroleum ether. m.p 207-208°C. Data were attributed as follows: ¹H-NMR (300 MHz, DMSO-d₆) δ: 3.12 (m, 2H), 2.73 (m, 3Ha), 1.87-1.54 (m), 1.86-1.54 (m, 4H), 1.86-1.54 (m, 5H), 3.12 (m, 6H), 1.86-1.53 (m, 7H), 1.86-1.53 (m, 8H), 2.08 (m, 8He), 2.73 (m, 9H), 3.13 (m, 10H) 5.14 (dt, J=10.2, 5.3Hz, 11H), 1.26 (m, 12Ha), 2.25 (m, 12He), 1.86-1.54 (m, 13H), 2.26 (m, 14Ha), 2.49 (m, 14He), 4.22 (t, J=12.6Hz, 17Ha), 4.43 (dd, J=12.6, 5.4Hz, 17He). ¹³C-NMR (75 MHz, DMSO-d₆) δ: 69.5 (C-2), 17.4 (C-3), 26.5 (C-4), 34.5 (C-5), 67.4 (C-6), 42.3 (C-7), 24.5 (C-8), 17.7 (C-9), 69.5 (C-10), 53.3 (C-11), 28.4 (C-12), 18.4 (C-13), 32.6 (C-14), 170.5 (C-15), 41.3 (C-17). Based on the above spectroscopic data and by contrasting the data with literature (Li et al., 2004), compound 2 was identified as oxymatrine. The structure is shown in Figure 2.

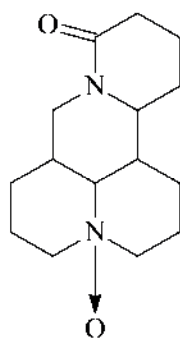


Figure 2: Oxymatrine

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Compound 3: white needle crystals (CHCl₃), m.p.: 173.4-174.5°C, easily insoluble in chloroform, diethyl ether, petroleum ether; hardly soluble in methanol, acetone. Data were attributed as follows: ¹H-NMR (300 MHz, DMSO-d₆) δ: 3.21 (dd, 1.3, 7.3Hz, 3H), 2.35 (m, 19H), 4.68 (s, 20Ha), 4.56 (s, 20Hb); ¹³C-NMR (75 MHz, DMSO-d₆) δ: 38.8 (C-1), 27.6 (C-2), 79.2 (C-3), 38.7 (C-4), 55.5 (C-5), 18.4 (C-6), 34.3 (C-7), 40.7 (C-8), 50.6 (C-9), 37.2 (C-10), 21.2 (C-11), 25.3 (C-12), 38.1 (C-13), 42.7 (C-14), 28.1 (C-15), 35.7 (C-16), 43.1 (C-17), 48.4 (C-18), 48.2 (C-19), 151.2 (C-20), 29.9 (C-21), 40.1 (C-22), 29.9 (C-23), 15.6 (C-24), 16.1 (C-25), 16.0 (C-26), 14.6 (C-27), 18.1 (C-28), 109.4 (C-29), 19.5 (C-30). Based on the above data, and by contrasting with the literature (Cheng, 1999), compound 3 was identified as lupeol.

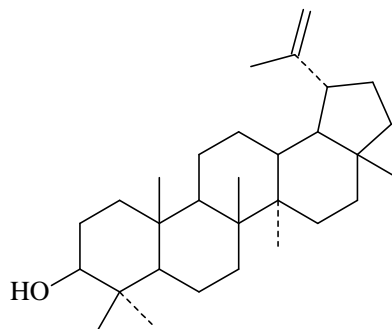


Figure 3: Lupeol

Cell cultivation

C6 glioma cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, and placed in the incubator set at 37°C, 5% CO₂ and subcultured routinely. The logarithmic growth phase cells were used in the experiment.

Determination of inhibitory effect of matrine on C6 cell growth by MTT assay

Logarithmic growth phase cells were digested, collected, made into a cell suspension with a concentration of 5×10^4 cells/ml, and seeded in 96-well plates at 200 μ L per well. 24 h later, matrine solutions that were prepared in RPMI 1640 medium were added. Their final concentrations were 0.2 mg/L, 0.4 mg/L, 0.6 mg/L, 0.8 mg/L and 1.0 mg/L, respectively. 6 replicate wells were set up for each concentration; cultivation was continued for additional 24, 48 and 72 h, respectively. After supernatant was discarded, each well was added with 20 μ L of MTT, mixed uniformly, and the plates were incubated at 37°C in 5% CO₂ incubator for another 4 h. Then, the culture medium was carefully aspirated, and 150 μ L of DMSO was added to each well. The plates were shaken for 10 min, and absorbance value (A value) of each well was measured at 570 nm using microplate reader. The rate for cell proliferation inhibition rate was calculated with the following formula:

$$\text{Cell proliferation inhibition rate (\%)} = (1 - \text{D value of the experimental group} / \text{D value of the control group}) \times 100\%.$$

The experimental results are shown in Fig. 4. As can be seen, the culture media containing different concentrations of matrine could all inhibit the growth of C6 cell lines, where apparent dose-effect relationship was seen. Such results indicate that matrine can effectively inhibit the proliferation of C6 cell lines in vitro.

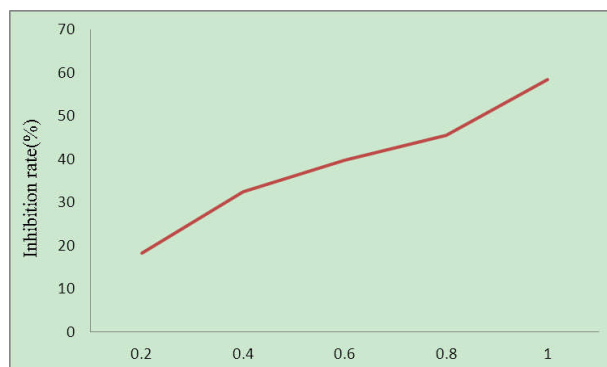


Figure 4: Inhibitory effect of matrine on C6 cell growth (mg/L)

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Determination of apoptosis rate by flow cytometry

C6 cells in the matrine treatment groups and control group which were cultured for 72 h were collected. Cell concentration was adjusted to 5×10^6 , and the cells were washed 3 times with PBS, and fixed with 70% ice-cold ethanol at -20°C overnight. They were then washed 3 times again with PBS, and stained at 4°C under dark conditions by addition of PI dye for 30 min. Cell concentration was adjusted appropriately, and the cells were passed through 300-mesh nylon sieve for six times. After filtration, cells were loaded on flow cytometry, and changes in the cell cycle after drug treatment were analysed.

As can be seen from the results in Fig. 5, compared with the control group, proportion of G0/G1 phase cells increased in each matrine concentration group to a maximum of 79.8%. S phase reduced and G2/M phase declined slightly to a minimum of 6.3%, suggesting that after the action of matrine, C6 cell proliferation was significantly inhibited and the cells were arrested in the G1 phase.

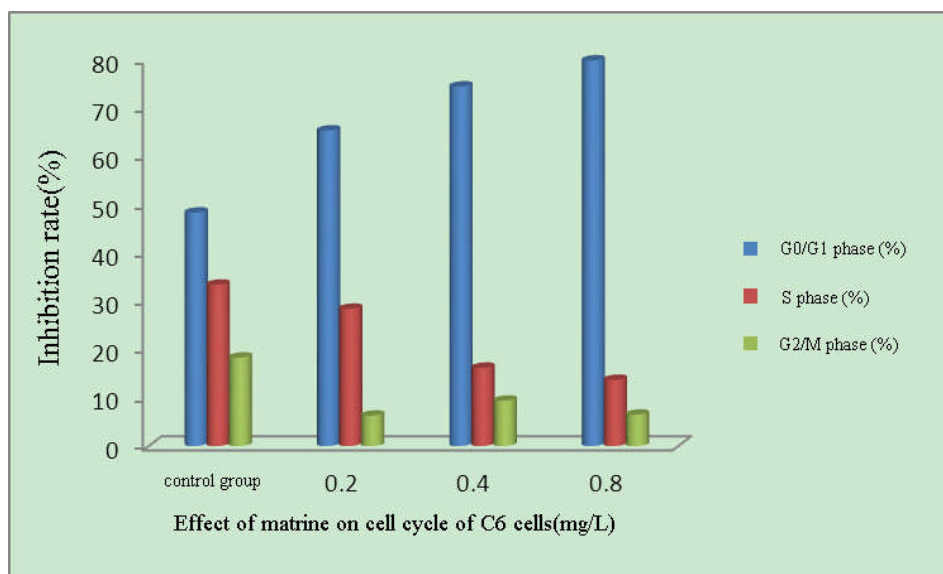


Figure 5: Effect of different concentrations of matrine on cell cycle of C6 cells

Discussion

In this experiment, alkaloids were extracted and isolated from commonly used Chinese medicine *Sophora flavescens* Ait. Three compounds were isolated from it. The isolated compounds were structurally identified by column chromatography, extraction and NMR spectroscopy. The three compounds were matrine, oxymatrine and lupeol.

To investigate the anti-glioma effect of *Sophora flavescens* Ait., this paper used different concentrations of self-prepared matrine to act on C6 cells. MTT assay results showed inhibition of C6 cell growth by different concentrations of matrine. Compared with the control group, each matrine experimental group all had a significant inhibitory effect on proliferation of C6 cells, which showed apparent dose-effect relationship as well.

Apoptosis is the programmed cell death regulated by related genes due to internal and external environmental changes. It can eliminate aging cells and potential abnormal growth cells in the body to achieve body's normal metabolism and avoid inflammation, which is an important guarantee for the maintenance of homeostasis in the body. Apoptosis regulation disorder can lead to a variety of human diseases, and thus is also an important cause of tumorigenesis (Thorburn, 2004; Green & Kroemer, 2004; Liu & Jiang, 2006).

It can be seen from the flow cytometric analysis of cell cycle that the proportion of S phase and G2/M phase C6 cells decreased after 72 h action of matrine to the minimum of 13.7% and 6.3%, respectively. The proportion of G0/G1 phase cells increased apparently, reaching 79.8%, suggesting that after the action of matrine, proliferation of C6 cells was significantly inhibited and that the cells were arrested in the G1 phase.

Acknowledgements

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