Antibacterial and Antiviral Activities of Artemisia Annua Aqueous Extract in Vitro

Ali Tao, Zurong Song, Xuehua Feng, Anna Zhang, Huimin He, Yumei Chen

College of Pharmacy, Anhui Xinhua University, Hefei, Anhui Province, 230088, China
corresponding author’s e-mail: taoali84@163.com

Abstract. The aim of this study was to investigate the inhibition rate of Artemisia annua aqueous extract against Staphylococcus aureus, Escherichia coli, Candida albicans and influenza A virus in vitro. The inhibitory ring test and minimal inhibitory concentration (MIC) were used to evaluate the inhibition rate of Artemisia annua aqueous extract on the tested bacteria in vitro, and the inhibition rate of Artemisia annua aqueous extract on the cytopathy caused by influenza A virus (FM1) was detected by cytopathy method and cell culture technology. According to the experimental results, the Artemisia annua aqueous extract has antimicrobial and antiviral effects. Aqueous extract of Artemisia annua has certain inhibitory effect on Staphylococcus aureus, Escherichia coli and Candida albicans, especially on Staphylococcus aureus; and the aqueous extract of Artemisia annua has shown inhibitory effect on the cytopathy caused by influenza A virus FM1 at the concentration of 0.976 mg/ml.

1. Introduction
Artemisinin is an annual plant of the genus Artemisia, also known as grass artemisinin, inchen artemisinin, etc[1]. It has the efficacy of clearing heat and removing steaming and malaria [2]. Artemisinin and its derivatives are a kind of new anti-malaria drugs independently developed by Chinese scientific researchers, and the aqueous extract of Artemisia annua is one kind of artemisinin derivatives [3,4]. According to current studies, artemisinin and its derivatives have a wide range of pharmacological effects such as anti-tumor, regulating immunity, anti-schistosomiasis, anti-arrhythmia, and anti-malaria [5,6]. Bacteria and viruses are the main sources of infectious diseases, causing serious harm to animals and humans. According to the research at home and abroad, artemisinins have antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and fungi, and its antibacterial activity has specificity and concentration dependence [7]. Artemisinin and its derivatives have direct or synergistic antimicrobial effects on many common clinically resistant and pathogenic bacteria such as Mycobacterium tuberculosis, Staphylococcus aureus, Escherichia coli and Helicobacter pylori [8,9]. Moreover, artemisinins have an inhibitory effect on hepatitis C virus JFH-1 [10], and condensate tannins from Artemisia annua aqueous extracts contain significant anti-HSV-2 activity and potential anti-HBV resistance[11]. Therefore, strengthening the research on this kind of drugs may provide new ideas for the clinical application of artemisinins.
2. Experimental section

2.1. Materials

2.1.1. Raw Materials and Reagents
Artemisia annua (sun dried and cut off) was purchased from Bozhou medicinal materials market. It was identified as Artemisia annua by Professor Li Qizhao, Anhui Xinhua University. Ribavirin Injection, Shandong Xinhua Pharmaceutical Limited by Share Ltd; agar nutrient medium; PBS phosphate buffer solution, self made reagent, domestic trypsin; trypsin solution, American GIBCO product; ciprofloxacin, Jiangsu the Yellow River Pharmaceutical Enterprise Co, Ltd.

2.1.2. Cells, Viruses and Strains
HeLa cells (human cervical cancer cells), derived from Anhui Tumour Hospital; influenza A virus FM1 strain, Chinese Academy of Medical Sciences Beijing virus Institute; Staphylococcus aureus, Candida albicans, Escherichia coli, Chinese strain Preservation Center.

2.1.3. Main Instruments
Electronic analytical balance, Beijing Hai Fuda Technology Co, Ltd.; ultra clean workbench Wujiang hung Ding purification equipment Co, Ltd.; constant temperature CO₂ incubator, DuPont Co, cell culture bottle and 96 hole plate, American Falcon company; high pressure sterilizer, Shanghai Bo Xun industrial limited company medical equipment factory; inverted microscope, Olympus company.

2.1.4. Sample Preparation
For the preparation of the water extract of Artemisia Annua L., 50g coarse powder of dried Artemisia Annua L. was used, the crude powder was carefully weighed, the decoction was soaked in multi-fold distilled water for 50Min, boiled for 30min, the decoction was filtered by Gauze, the unfiltered decoction was continued to be boiled for 30min, the decoction was filtered, the filtrate, which was combined twice, was simmered and condensed to 100mL.

2.2. In Vitro Antibacterial Experiments

2.2.1. Preparation of Bacteria Solution
The strains were inoculated into the culture dish and cultured overnight at 37 °C. The culture dish with better colony growth was selected and inoculated in the prepared culture dish. After cultured at 37 °C for 18 hours, the typical colony growth medium was selected, adding 5 ml sterile physiological saline, gently scraping away the Moss, and the suspension was prepared. 100 L turbidimetric liquid was prepared by turbidimetric method in turbidimetric tube[12].

2.2.2. Bacteriostatic Ring Experiment

2.2.2.1. Preparation of Bacteriostatic Tablets
Take aseptic dry filter paper, add 20µL of Artemisia annua aqueous extract, aseptic distilled water and ciprofloxacin respectively, and aseptic distilled water is blank control. Ciprofloxacin is a positive control. Then the treated filter paper take placed in a sterile culture dish and dried in a temperature incubator at 37 °C. The diameter of the filter paper is 5 mm.

2.2.2.2. Inoculation of Test Bacteria
The prepared test bacterial suspension was Inoculate with sterile cotton swab on the culture medium. Dry at room temperature for 5 min[13]. Then the prepared paper pieces containing Artemisia annua aqueous extract, positive control group and blank control group were evenly pasted on the surface of the culture dish according to the routine operation. 16~18 h was incubated in incubator at 37 °C, and
the results were observed. The experiment was repeated 3 times. The diameter of the bacteriostatic ring was measured by vernier caliper D (including patches) and the results were recorded. The standard of determination is: D≤8 mm is insensitive, 8 mm< D≤ 13 mm is low sensitivity, 13 mm< D≤19 mm is moderately sensitive, D>19 mm is highly sensitive.

2.2.3. Determination of Minimum Inhibitory Concentration
The test was carried out in the test tube method for the determination of MIC. A number of small test tubes were prepared and washed with distilled water to ensure that the test tubes were washed clean. Each tube was sterilized at 121 °C for 25 min by adding 1 mL nutrient broth medium into a pipette gun. Make 7 test tubes containing the water extract of Artemisia annua by two times dilution method at 121 °C. The concentration was 500, 250, 125, 62.5, 31.25, 15.62, 7.81 mg/mL, and ciprofloxacin was prepared in the same way. The concentration of 500, 250, 125, 62.5, 31.25, 15.62, 7.81 g/mL was diluted to 1000 times the bacteria with a concentration of 1 * 108 CFU/mL. The final concentration of the bacterial solution was 105 CFU/mL, and 0.1 mL bacteria was added to each tube. When incubated at 37 °C for 24 h, the lowest concentration of Artemisia annua water extract was the minimum inhibitory concentration. The test was repeated 3 times, and the average value was obtained.

2.3. In Vitro Antiviral Experiments

2.3.1. HeLa Cell Culture
HeLa cells were washed with phosphoric acid buffer for 3 times, and 1-2mL trypsin solution was digested into single cells, diluted into 1×105 cell/mL cell suspension with fresh medium, inoculated in 96 hole cell culture plates, 0.2mL at each hole, and 24 hours in 5% CO2 culture at 37 °C.

2.3.2. Toxicity Test of Water Extract on Cells
On the 96 hole plate, add 0.8 × 105 / mL HeLa cell 0.1 mL per pore, add water extract maintenance solution containing Artemisia annua. The concentration of water extract of Artemisia annua was diluted to 8 different concentrations, 4 holes were repeated at each concentration, and 4 hole cell control and Leigh Bhave Lin positive control were also set up at the same time, incubated at 37 degrees centigrade, incubated in 5% CO2 incubator for 48 h, and the pathological changes were observed under inverted microscope daily. (cytopathic effects, CPE): (-): cell-free lesions; (+): cells below 25% produce lesions; (+ +): 25%~50% cells produce lesions; (+ + +): 50%~75% cells produce lesions; (+ + + +): 75%~100% cells produce lesions, with no apparent cytopathic (CPE) minimum dilution concentration as the maximum non-toxic concentration (TC0) of the drug.

2.3.3. Inhibition of Influenza Virus by Water Extract
On the 96 cell culture plate with HeLa cell, 100 TCID50 influenza virus FM1 solution 100 µl per hole were added, incubated in 37 °C, 5% CO2 incubator for 1h, and then discard the maintenance solution containing virus. The Artemisia annua water extract began to dilute at 4 concentration with the maintenance solution from the maximum non-toxic concentration, and added to the infected cells. 200 µl per hole were added, and 4 holes were longitudinally repeated. At the same time, Leigh Bhave Lin positive control group, cell control group and virus control group were set up. Each group was incubated in 37 C, 5% CO2 incubator, and the cell pathological changes were observed under inverted microscope, and the results were recorded.

3. Results and discussion

3.1. Study on The Antibacterial Activity of Artemisia Annua Aqueous Extract

3.1.1. Determination of Inhibition Zone Diameter of Water Extract from Artemisia Annua
The larger the diameter of the bacteriostatic zone is, the greater the bacteriostatic capacity of the drug.
is. As can be seen from table 1 and picture 1, the diameter of bacteriostatic circle of Artemisia water extract to Escherichia coli is 15mm, which is moderately sensitive; the diameter of bacteriostatic circle of Artemisia water extract to Candida albicans is 19mm, and the diameter of bacteriostatic circle to Staphylococcus aureus is 21mm, which are highly sensitive. Therefore, the water extract of artemisia annua had inhibitory effect on all three experimental bacteria, and the most significant inhibitory effect on staphylococcus aureus.

| Bacterial species     | Sterile distilled water | Water extract of artemisia annua | Ciprofloxacin |
|-----------------------|-------------------------|----------------------------------|---------------|
| E. coli               | 0                       | 15                               | 27            |
| Candida albicans      | 0                       | 19                               | 10            |
| Staphylococcus aureus | 0                       | 21                               | 30            |

3.1.2 Determination of The Minimum Inhibitory Concentration of Artemisia Annua Water Extract

The minimum inhibitory concentration of different concentrations of Artemisia annua aqueous extract on Staphylococcus aureus, Escherichia coli and Candida albicans is shown in table 2. According to table 2 and picture 2, the minimum inhibitory concentration of Artemisia annua water extract on E. coli is 31.52 mg/ml, and the minimum inhibitory concentration to Candida albicans and Staphylococcus aureus was 15.62 mg/ml. The inhibitory effect of Artemisia annua extract on E. coli was lower than that of Candida albicans and Staphylococcus aureus.

| Bacterial species     | Minimum inhibitory concentration (MIC, mg/ml or g/ml) |
|-----------------------|-------------------------------------------------------|
|                       | Sterile distilled water | Artemisia water extract | Ciprofloxacin |
| E. coli               | --                      | 31.25                   | 7.81          |
| Candida albicans      | --                      | 15.62                   | 31.25         |
| Staphylococcus aureus | --                      | 15.62                   | 7.81          |
3.2. Studies on The Antiviral Activity of Artemisia Annua Aqueous Extract

3.2.1. Toxicity of Artemisia Annua Water Extract on Cells

The normal cells in the negative control group were cells growing on the wall. The cells had clear contour. 24 hours later, the cells were round, aggregated, necrotic, shedding, and arranged irregularly when the cytotoxicity was induced by drug toxicity. The results of CPE test of Artemisia annua water extract showed that the maximal non-toxic concentration of Artemisia annua extract to cells was 3.90 mg/ml., according to the results of table 3.

| drug                  | Diluted multiples | Cell control |
|-----------------------|-------------------|--------------|
| water extract of artemisia annua | 21 22 23 24 25 26 27 28 | + + + + + + + + + + + + + + + + + + + + + + + |
| ribavirin             |                   |              |

3.2.2. Inhibition of Artemisia Annua Water Extract on Virus

With 3.90 mg/ml as the initial concentration and four times dilution, the cytopathic effect induced by Artemisia annua aqueous extract on influenza A virus FM1 was observed under microscope. The results showed that the effect of Artemisia annua aqueous extract on influenza virus was shown in table 4. Compared with the virus control group, the water extract of Artemisia annua L. had a strong inhibitory effect on the influenza A virus at 3.90 mg/ml. When the concentration was 1.95 mg/ml, the inhibitory effect was slightly weaker than that at 3.90 mg/ml. When the concentration was 0.976 mg/ml, the water extract of Artemisia annua still had certain cytopathic effect on influenza A virus. When the concentration was 0.488 mg/ml, the water extract of Artemisia annua had no inhibitory effect on influenza A virus when compared with the virus control group.

| drug                  | Drug concentration /mg.ml-1 | Cell lesions | Virus control | Cell control |
|-----------------------|-----------------------------|--------------|---------------|--------------|
| water extract of artemisia annua | 3.90                       | +            | +++           | -            |
|                       | 1.95                        | ++           | +++           | -            |
|                       | 0.976                       | +++          | +++           | -            |
|                       | 0.488                       | +++          | +++           | -            |
| ribavirin             | 3.90                        | -            | +++           | -            |
|                       | 1.95                        | -            | +++           | -            |
|                       | 0.976                       | +            | +++           | -            |
|                       | 0.488                       | +            | +++           | -            |
4. Conclusion
From the above experimental results, we can see that in the antimicrobial experiment, the water extract of Artemisia annua has a more significant antimicrobial effect on Staphylococcus aureus and a slightly weaker inhibitory effect on Escherichia coli and Candida albicans; in the antiviral experiment, the water extract of Artemisia annua has a concentration of 0.488 mg/ml. At the same time, it can inhibit the cytopathy of influenza A virus, and the inhibitory effect is dose-dependent. It indicates that the water extract of Artemisia annua may have good application prospects in the development of antimicrobial and antiviral agents, and it is worthy of further development.

Acknowledgements
This work was supported by Natural Science Research Foundation of the Department of Education of Anhui Province (No.KJ2019A0874), Scientific research team of Anui Xinhua university (kytd201908,2019xqjdxf03) and Demonstration project of grassroots teaching and research Office of Anui Xinhua university (2019jyssfx02).

References
[1] Zhao Zhaowu, Ni Fuyong, song Yaling, et al. Studies on chemical constituents of Artemisia annua [J]. Chinese Journal of traditional Chinese Medicine,2014,39(24):4816-4821(in Chinese).
[2] Wu Yekuan, Li Longyun, Zhong Guoyue. Research overview of Artemisia annua [J]. Research on Chinese herbal medicine in Chongqing, 2004 (2): 58(in Chinese).
[3] Zhang Huijun, Wang Shali. Effect of Artemisia annua water extract on urinary system and digestive system of rabbits [J]. Journal of Chongqing Medical University, 2009,34 (10): 1374-1377(in Chinese).
[4] Huang Yan, Zhang Huijun, Wang Shali, et al. Effect of Artemisia annua water extract on reproductive function and reproductive development of mice [J]. Reproduction and contraception, 2010,30 (08): 505-508(in Chinese).
[5] Wang Xu, He Lin. research progress on the pharmacodynamic effect of artemisinin compounds on diseases other than malaria [J]. Chinese Journal of new drugs, 2018,27 (19): 2258-2263(in Chinese).
[6] Wang Xiaohue, Ma Minghua, Zhang Jingting, et al. Research progress on pharmacological action of Artemisia annua [J]. Chinese Modern Applied Pharmacy, 2018,35 (05): 781-785(in Chinese).
[7] Appalasamy S,Lo Ky,Cheng SJ,et al. Antimicrobial activity of artemisinin and precursor derived from in vitro plantlets of artemisia annua L [J] ,Biomed Res Int. 2014,2014 (31):215872.
[8] GOSWAMI S,BHAKUNI R S,CHINNIAH A,et al.Anti-heli-cobacter pylori potential of artemisini, and its derivatives [J].Antimicrob Agents Chemother,2012,56(9):4594 — 4607
[9] Jiang Weimei, Qian Yan. Research progress on antibacterial activity of artemisinin and its derivatives [J]. Chinese pharmacy, 2019,30 (14): 2003-2007(in Chinese).
[10] Obeid S, Alen J, Nguyen VH,et al. Artemisinin analogues as potent inhibitors of in vitro hepatitis C virus replication [J] .Plos One,2013,8(12):e81-83
[11] Zhang Junfeng, Tan Jian, Pu Qiang, et al. Study on antiviral activity of Artemisia annua tannin [J]. Research and development of natural products, 2004 (04): 307-311(in Chinese).
[12] Saruli, Yang Bin, Yu Mei, et al. Study on antibacterial, antiviral and antitumor activities of total flavonoids of Allium mongolicum in vitro [J]. Feed industry, 2018,39 (08): 54-57(in Chinese).
[13] Huang Yixuan, Chen Huimin, Yao Liang, et al. In vitro antibacterial and antiviral effects of allicin extracted from scallion [J]. Modern food technology, 2018,34 (02): 69-74(in Chinese).