Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(3):634-639

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Antibacterial activity and mechanism of 36 Chinese herbs

Lin Ma[1,2]

[1]*College of Chemistry and Chemical Engineering, Baoji University of Arts and Sciences, Baoji, China*
[2]*Shaanxi Key Laboratory of Phytochemistry, Baoji, China*

ABSTRACT

The extracts from 36 kinds of Chinese Taibai herbs (CTHs) were obtained with the help of methanol and tested for antibacterial activity against four bacteria and two fungi inhibition through methods of inhibition zone, MIC and MBC. Then, the antibacterial mechanism of the extracts from CTHs was studied based on the growth curve and cell membrane permeability. The results showed that the best antibacterial activity against S. aureus and B. cinerea was the Notholirion hyacinthinum (Wils.)Stapf and the best activity against P. aeruginosa and E. coli was Cynanchum wilfordii (Maxim.) Hems L. The antibacterial mechanism of two CTHs were against four pathogen is as follows: drugs cause a decrease in the number of cell divisions in logarithmic phases and induce the voids of cell walls to become, thus the materials in the cell to leak out.

Key words: 36 Chinese Taibai herbs; CTHs extracts; antibacterial activity; antibacterial mechanism

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INTRODUCTION

Since the penicillin was found in the early 20th century, the antibiotics had been the first line of treatment against many Bacterial infections and prokaryotic system. The compounds that specifically target fundamental cellular processes of bacteria, with negative consequences for pathogen survival, and no plausible side effects in host are considered as potentially useful antibiotics (Dimauro and Davidzon, 2005; Fischel-Ghodsian, 2005; Wang et al., 2006). It has been observed that more than 40% of antibiotics interfere with bacterial protein biosynthesis machinery and more specifically the ribo some is one of the most important targets (McCoy et al., 2011). In the prokaryotic system, the translation is a complex process and involves several steps of initiation, elongation and termination which had been well established. However, antibiotics had various impacts on bacteria, probiotics and human cells. There will be bacteria mutation and an increase in bacterial resistance, if identical antibiotic is used repeatedly and redundantly. Using antibiotic excessively will not cure diseases faster and better, but inhibit the growth of probiotics and lead to intestinal flora imbalance and secondary infection [1, 2]. So it is very important to find the new drug that has antibacterial activity against bacteria without affecting probiotics.

Chinese herbal medicine (CHM) is an important part of Chinese medicine, and has been used widely in China for more than thousands of years. There are some active substances extracted from CHM, such as Gallic acid (Li, 1575), camphor (Hong, 1711), Artemisinin (Tu et al., 1967) and so on [3,4]. The CTHs used in this paper were collected from Taibai Mountain, the main peak of Qinling Mountains, which have more than 600 kinds of Chinese herbal medicine. There is also a tradition of using herbal medicine in folk. The methanol extracts were isolated independently from 36 kinds of CTHs, and they were evaluated for their antimicrobial activity and mechanism against four bacteria S. aureus, B. subtili, P. aeruginosa and E. coli and two fungi inhibition S. cerevisiae and B. cinerea.

EXPERIMENTAL SECTION

MICROORGANISMS AND MEDIA

The indicator strains (two gram-positive bacteria (G+), two gram-negative bacteria (G-) and two fungi) tested for antimicrobial activity were Staphylococcus aureus (ATCC 25923; G+; pathogen), Bacillus subtili (ATCC 6051; G+; probiotics), Pseudomonas aeruginosa (ATCC 27853; G-; pathogen), Escherichia coli (ATCC 25922; G-; pathogen), Saccharomyces cerevisiae (ATCC 204508; fungus; probiotics), and Botrytis cinerea (ATCC 90480; fungus; phytopathogen) respectively. The nutrient agar (NA) medium, the potato dextrose agar (PDA) plate, the potato dextrose agar (PD) plate and the nutrient agar (LB) medium were all sterilized at 121°C for 20 minutes [5].

VALUABLE COMPONENTS EXTRACTION

The 36 kinds of CTHs were collected from Taibai Mountain which is in the middle of Qinling Mountains, China in September, 2012 (see Table 1). Most of them were healthy rhizomes and stored after being dried in the shade. After the herbs were crushed, 500ml methanol and 100g powdery of the CTHs were put into the 1000ml round-bottomed flask and reflux condensation was achieved through spherical condensers. The complete setup was heated with thermostat water bath cauldron in order to reach constant temperature 55℃ lasted for 2 hours. Then the vacuum extraction filtering method was used for herb solution to get the filtrate. The filter cake and 500 ml methanol were put into the 1000ml round-bottomed flask for reflux condensation for 2 hours. This process was repeated three times [5]. Then merge the obtained filtrate which was under rotary evaporation in vacuum under the condition of 0.04Mpa and 55℃. At last, added 150mg medicine extract to 1000μl which was mixed 800μl and 200μl DMSO under ultrasonic wave to reach the concentration of 150mg/ml.

Table1.1: The names of 36 CHTs

Number	Name
1	*Daphne tangutica Maxim (zu si ma)*
2	*Abies sutchuenensis (pu song shi)*
3	*Cladonia alpestris(L.)Rebenh (tai bai hua)*
4	*Akebiaquinata (Thunb.) Decne (ba yue gua)*
5	*Cladonia gracilis(L.)Willd (tai bai lu jiao)*
6	*Gymnadenia conopsea R. B (shou zhang sen)*
7	*Rodgersi aaesculifolia Batal (suo gu qi)*
8	*Bergenia scopulosa T.P. Wang (pan long qi)*
9	*Tupistra chinensis Bak (zhu gen qi)*
10	*Cynanchum wilfordii (Maxim.)Hemsl (ge shan qiao)*
11	*Umbillicaria hypococcina (Jatta) Llano (hong shi er)*
12	*Pteroxygonum giraldii Dammer et Diels (qiao mai qi)*
13	*Pedicularis davidii Franch (tai bai yang sen)*
14	*Cladonia fallax Abbayes (jin shua ba)*
15	*Wikstroemia nutans Champ.(jin yao dai)*
16	*Centipeda minima (L.) A.Br. et Aschers (di hu jiao)*
17	*Pothos repens(Lour.) Merr (fei tian wu gong qi)*
18	*Biondiahenryi (Warb.exSchltr.et Diels)Tsiang et P.T.Li (kun xian sheng)*
19	*Dasiphorafruticosa (L.)Rydb (yaowang cha)*
20	*Rhodiola dumulosa (Franch.) Fu (feng wei qi)*
21	*Pyrola japonica Klenze ex Alef.(lu shou cha)*
22	*Pyropolyporus fomentarius (L.ex Fr.) Teng (hua jun zhi)*
23	*Rosa omeiensis Rolfe (ci shi liu)*
24	*Diphylleia sinensis Li (wo er qi)*
25	*Tupistra fimbriata Hand.-Mazz. (tie bian dan)*
26	*ThamnoliasubuliW .Cuib (tai bai cha)*
27	*Polygonatum cirrhifo-lium (Wall.) Royle (lao hu jiang)*
28	*Gentiana apiata N. E. Brown (zhu ling cao)*
29	*Phlegmariurus carinatus(Desv.)Ching (da shen jin cao)*
30	*Alectoria asiatica Du Rietz (tou fa qi)*
31	*Stellaria alsine Grimm (tian peng cao)*
32	*Solidago virga-aurea L.var.leiocarpa(Benth.)A.Gray (yi zhi huang hua)*
33	*Lepisorus eilophyllus (Diels) Ching (shi jiang dou)*
34	*Panax pseudo-ginseng Wall.var.japonicus(C.A.Mey.)Hoo&Tseng (ji zi qi)*
35	*Notholirion hyacinthinum (Wils.)Stapf (tai bai mi)*
36	*Roots of Schisandra chinensis (wu wei zi gen pi)*

ANTIMICROBIAL ACTIVITY

Antimicrobial activities of the CTHs extracts were studied through the microbial technology of measuring inhibition zone [6] of herbal solution. The inhibition zone diameter formed by the CTHs extracts at concentration of 150mg/ml against the tested microbial strains was used to determine their antimicrobial activities. The value of the inhibition zone diameter was the average of three measured replicates.

Select the first 6 kinds of CTHs extracts which had the bigger inhibition zone for each microbial strain to determine the Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) by double broth dilution method with Microdilution Checkerboard Techniques (MCT). The microorganism was seeded into 96 well plates and incubated in an inverted position for 20 hours at 35°C to observe whether it turned red after an increase of 5% MTT. The concentration of the last no-reddened well plate was MIC [7]. After MIC experiment, 200μl taken from each well with the concentration being no more than MIC bacterial suspension was inoculated in blood agar plates for 24h for observation. The lowest drug concentration that yielded no growth was documented as MBC [7]. All CTHs liquid were conducted three times. The MIC and MBC of 3 antimicrobial agents against strains were examined by the same method. The antibiotics were Penicillin (used for G+), Gentamicin (used for G-) and Natamycin (used for fungus) respectively through the evaluation of the effect on the inhibiting between antibiotics and the CTHs.

ANTIMICROBIAL MECHANISM

After the antimicrobial activity test, and on the basis of the inhibition zone size, MIC and MBC as well as the comparison of corresponding antibiotic effects, some traditional Chinese medicines were chosen for antibacterial mechanism experiments.

At first, the bacterial growth curve was drawn by classical method [6, 8] and the fungus growth curve was drawn by dry weight method [9]. If the microorganism was a bacterium, the ultraviolet spectrophotometer would be adopted to measure the absorbance whose wavelength is 610nm at each time-point, and then a curve of absorbance would be drawn accordingly. If the microorganism was a fungus, the filter cake which had gone through fungus filtration would be dried at 95°C and weighed at each time-point to draw a curve. Studies on cell membrane permeability of CTHs extract was mainly done through the conductivity [10] and reducing sugar content [8, 11]. Each bacterium was divided into two groups: a control group (without drug) and a dosing group. In each group, there were seven test tubes, which were marked 0min, 5min, 15min, 30min, 60min, 90min and 180min respectively. Each tube contained 18ml sterile water and 2ml bacteria. In the dosing group 1ml liquid at a concentration of 100mg/ml was added in. Both of the tubes were then cultivated at a constant temperature of 35°C. Later, corresponding samples were picked out to measure the conductivity at each time point. When measuring the reducing sugar content, each bacterium was divided into two groups: a control group (without drug) and a dosing group. In each group, there were seven test tubes, which were marked 0h, 1h, 2h, 3h, 4h, 5h, 6h. Each tube contained 18ml sterile water and 2ml bacteria. 1ml liquid at a concentration of 100mg/ml was added into the dosing group, the two groups cultivated at a constant temperature of 35°C. Later, corresponding samples were picked out to measure the reducing sugar content at each time point through Fehling's solution [12].

RESULTS AND DISCUSSION

ANTIMICROBIAL ACTIVITY

The experimental results of the inhibition zone in the antimicrobial activity test are shown in Figure 1.

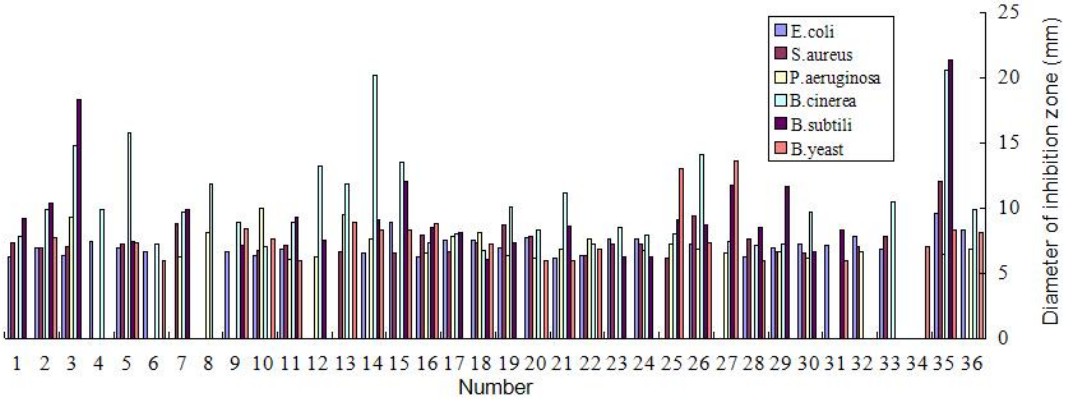

Fig.1: The inhibition zone width of 36 CTHs

There are four scenarios. At first, fourteen kinds of CTHs No.3, No.10, No.11, No.16, No.17, No.18, No.19, No.20, No.22, No.24, No.26, No.29, No.30 and No.35 can antagonize S. aureus, P. aeruginosa, E. coli and B. cinerea. Moreover, four kinds of CTHs No.4, No.8, No.32 and No.33 have unique antibacterial activities. For example, CTHs No.4 can suppress E. coli and B. cinerea, and CTHs No.8 can effectively antagonize P. aeruginosa and B. cinere. The CTHs No.32 not only shows strong antagonistic action to pathogenic bacteria S. aureus and P.

aeruginosa, but also has no inhibitory impact on B. subtili and S. cerevisiae. Then, the CTHs No.35 could effectively antagonize S. aureus and fungus B. cinerea but had no inhibitory impact on B. subtili and S. cerevisiae. At last, some CTHs extracts No.29, No.30 and No.34 had no inhibitory impact on bacteria and fungus, but they could increase S. cerevisiae. After the inhibition zone test, each bacterium selected six CTHs extracts which had the largest inhibition zone. The MIC and MBC of the six CTHs extracts were measured through double broth dilution method. Results are shown in the Figure 2 and Table 2. Through comparing the inhibition zone, MIC and MBC of six CTHs extracts which enjoyed good antibacterial effects of antibiotics and bacterium, No.35 and No.10 were chosen to carry out the inhibitory mechanism experiment. The results showed that for No.35, which had sound inhibitory effect on S. aureus and B. cinerea, the inhibition zones were 12mm and 20.6mm respectively, MICs were 0.15mg/ml and 0.08mg/ml respectively, and MBCs were 0.3mg/ml and 0.3mg/ml respectively. For No.10 which had sound inhibitory effect on E. coli and P. aeruginosa, the inhibition zones were 10mm and 9.6mm respectively, MICs were 0.59mg/ml and 0.29mg/ml respectively, and MBCs were 1.2mg/ml and 0.15mg/ml respectively. Moreover, No.10 could also increase the activity of probiotics B. subtili.

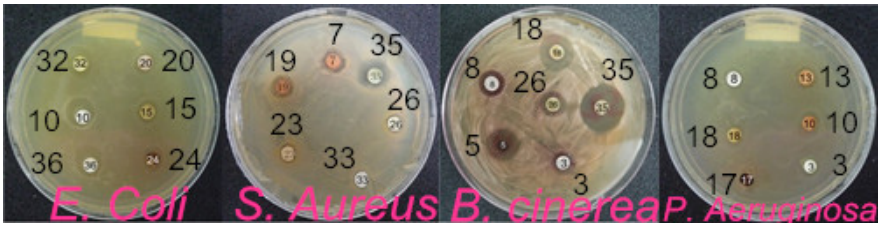

Fig. 2: Six CTHs with the maximum inhibition zone

Table.2: The results of inhibition zones width, MIC and MBC

Microorganisms	Number	Inhibition zone (mm)	MIC (mg/ml)	MBC (mg/ml)
S. aureus	7	8.44	2.34	4.69
	19	7.93	2.34	2.34
	23	8.7	1.17	2.34
	26	9.42	0.29	1.17
	33	7.8	4.69	4.69
	35	12	0.15	0.29
	Penicillin	8.5	0.59	1.17
P. aeruginosa	3	9.3	1.17	2.34
	8	8.14	4.69	4.69
	10	10	0.59	1.17
	13	9.5	1.17	1.17
	17	7.7	9.38	18.8
	18	7.57	9.38	9.38
	Gentamicin	9.7	1.17	1.17
E. coli	15	7.5	4.69	4.69
	10	9.6	0.29	0.29
	20	7.7	1.17	2.34
	24	7.66	1.17	1.17
	32	7.93	0.59	1.17
	36	8.3	0.59	0.59
	Gentamicin	9.4	0.29	0.59
B. cinerea	3	14.8	0.29	0.59
	5	15.8	0.15	0.15
	8	11.6	1.17	1.17
	18	10.8	0.59	0.59
	26	14.1	0.59	1.17
	35	20.6	0.08	0.29
	Natamycin	15.1	0.29	0.29

ANTIMICROBIAL MECHANISM

After the antimicrobial activity test, we got the No.35 (Notholirion hyacinthinum (Wils.) Stapf, tai bai mi) and No.10 (Cynanchum wilfordii (Maxim.) Hemsl, ge shan qiao) to study the antimicrobial mechanism against S. aureus, B. cinerea, P. aeruginosa and E. coli respectively.

Normal E. coli, S. aureus, B. cinerea and P. aeruginosa were used as the control group. And a comparison experiment was made between S. aureus, B. cinerea with No.35 added, and E. coli and P. aeruginosa with No.10

added. The growth curve in Figure 3 shows that the CTHs extracts inhibited the growth of bacteria and fungus, whose logarithmic growth stage did not reach the number in normal conditions. So, the No.35 and No.10 extracts could inhibit division of cells in logarithmic growth phase. Figure 3 shows the permeability changes of E. coli, S. aureus, B. cinerea and P. aeruginosa outer membrane. It could be found that under the influence of No.35 and No.10, the conductivity of E. coli, S. aureus, B. cinerea and P. aeruginosa liquid reached the maximum within an hour and the reducing sugar content could significantly increase after 4 hours. Thus, it can be concluded that after drugs were added, the micromolecule seeped quickly from cell of pathogens and leaded the conduction rate to increase, then as time passed by, reducing sugar content of macromolecules gradually seeped as well.

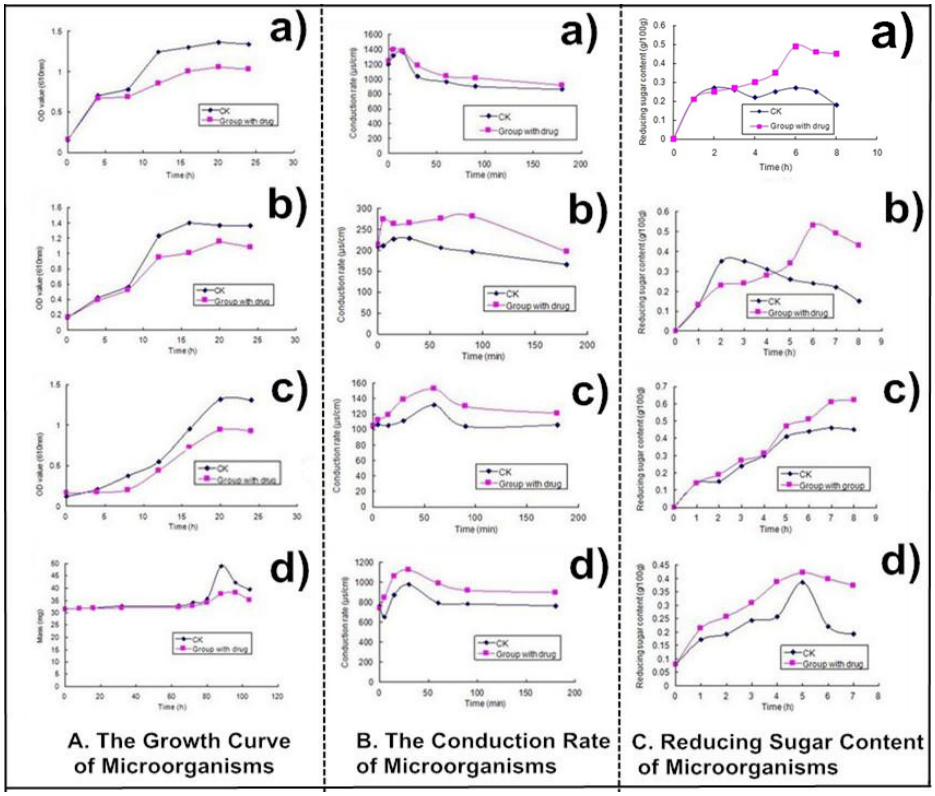

Fig. 3: A. The growth curve of microorganisms. a). *E. coli* , No.10. b). *S. aureus*, No.35. c). *B.cinerea*, No.35. d). *P. aeruginosa*, No.10. B. The conduction rate of microorganisms. a). *E. coli* , No.10. b). *S. aureus*, No.35. c). *B. cinerea*, No.35. d). *P. aeruginosa*, No.10. C. Reducing sugar content of microorganisms. a). *E. coli* , No.10. b). *S. aureus*, No.35. c). *B. cinerea*, No.35. d). *P. aeruginosa*, No.10

In summary, the inhibitory mechanism of No.35 and No.10 can be deduced as follows: both drugs destroy the cell membrane structure of the four pathogens (E. coli, S. aureus, B. cinerea and P. aeruginosa), which can increase the permeability of the cell membrane or the formation of membrane pores, leading to intracellular material leakage, the inhibition of cell growth and finally the death of cells.

Acknowledgments

The author is thankful to the project of Shaanxi Key Laboratory of Phytochemistry (No.13JS005); the key project of Baoji University of Art and Sciences (No. ZK14021).

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