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Extrication process of chlorogenic acid in Crofton weed and antibacterial mechanism of chlorogenic acid on *Escherichia coli*

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Abstract

Crofton weed is a perennial herb and a biological intruding species. The present study firstly used the orthogonal test to compare the differences in extraction of chlorogenic acid in leaves and stems of Crofton weed by using three kinds of solvents, namely water, ethanol and ethyl acetate. The best effect was found by using ethonolic extraction of chlorogenic acid in Crofton weed. Further, by choosing *Escherichia coli* as test object, *in-vitro* antibacterial test was conducted to study the antimicrobial activities of chlorogenic acid by testing a series of indexes before and after the interaction between chlorogenic acid and *Escherichia coli*, to clarify the antibacterial mechanism of chlorogenic acid on *Escherichia coli*. Finally, by comparing the antibacterial activities of isochlorogenic acid A on *Escherichia coli*, it was concluded that both chlorogenic acid and isochlorogenic acid A showed antibacterial activities against *Escherichia coli*, wherein chlorogenic acid had a better antibacterial effect on *Escherichia coli* than isochlorogenic acid A.

Key words

Antibacterial activity, Chlorogenic acid, Crofton weed, Extraction, Orthogonal test

Introduction

Originated in Mexico, Crofton weed was introduced into southern Yunnan in the middle of 20th century. It quickly spread in southern and southwestern China and affected the ecological balance and threatened the growth of crops, causing serious ecological pressure and economic loss. It is the most infectious and harmful exotic weed (Chao *et al.*, 2007). In recent years, strengthening the control on spread of Crofton weed, the research of comprehensive utilization of Crofton weed has been launched to get treasure out of the harm. As Crofton weed contains a chemical substance, chlorogenic acid (Fu *et al.*, 1999) that belongs to phenolic compounds. It is a kind of phenylpropanoids formed by the interaction between cinnamic acid and quinic acid via the shikimic acid pathway in the process of aerobic respiration of plants. Chlorogenic acids are mainly effective components in many medicines (such as Flos Lonicerae, Herba Artemisiae, Eucommia Bark) and in traditional Chinese medicines (such as Fu Gan Ning, Shuang Hua injection, acne oral liquid) which has significant pharmacological activities viz., antibacterial, antiviral, antioxidant, anti mutagenesis, antitumor and cholagogic, as well as in antihypertensive, increasing white blood cell, stimulating central nervous system. They are widely used as additives in foods, beverages, etc., and are in great demand (Huang *et al.*, 2012; Cao *et al.*, 1999).

Although chlorogenic acid exists widely in plants, but only few kinds of plants have higher content. It is mainly extracted from cocoa beans, honeysuckle flower and eucommia bark leaf which are all active pharmaceutical ingredients of traditional Chinese medicine. Earlier research have found that chlorogenic acid is relatively abundant in

Crofton weed stems and leaves, therefore, here we intend to use abundant resource and harmful weeds-Crofton weed as raw material. However, the antibacterial effect and antibacterial mechanism of chlorogenic acid monomer is not yet fully understood and there is no available systematical research report. Accordingly, in the present study, *Escherichia coli*, a common pathogenic bacteria, was selected as test object to test the antibacterial activity of chlorogenic acid, and thus clarifying the inhibition mechanism of chlorogenic acid on *Escherichia coli,* a theoretical basis for the development and utilization of chlorogenic acids has been provided (Da *et al.*, 2003).

Materials and Methods

Experiment materials : Crofton weed leaves were collected from XiChang, Sichuan Province in 2015. Standard sample of Chlorogenic acid (brought from Beijing XianTong Times Pharmaceutical Technology And Development co., Ltd.). High performance liquid chromatograph (HPLC) (Agilent 1100 Series). Rotary evaporation apparatus (Shanghai YaRong Biochemical Instruments co., ltd.). Temperature controller heater and reflux condensa-tion device (self-made). All reagents are analytic pure (Beijing ChongXi Technology Co. Ltd.) Visible ultraviolet detector, column temperature box, chromatography workstation.

Chromatography Conditions : Using 150 mm×4.60 mm LUNA SuC_{13} (2) column, choosing V (methanol): V (methanol) : V (methanol) = 19:81:1.5 as mobile phase, testing with wavelength of 325 nm, at velocity of 0.5 mL·min-1, and under 25.0 °C (Li Y.S., 2013).

Extraction of chlorogenic acid : Ten grams of Crofton weed leaves were weighed and then grinded. According to $L_9(3^4)$ successively performed the extraction 3 times, blending the filtrate and abandoning the residue, then decompressing and rotary distilling the filtrate, desolventizing and concentrating, using HPLC to determine the content of chlorogenic acid in the extract, calculating extraction ratio of chlorogenic acid and content of chlorogenic acid in extract. The experiment factor level of water extraction is shown in Table 1.

Table 2 shows, choosing water as solvent, the extraction technology is $A_2 8_2 C_1 D_3$, the extraction duration is 2 hours, 10 times of water, at temperature of 40 °C with the PH value of 7. The order of the factors which affect the extraction ratio of chlorogenic acid is B>A>D>C, the solid-liquid ratio>extraction time>pH>extraction temperature. In ethanol extraction method, the solid-liquid ratio has the largest influence on the extraction ratio of chlorogenic acid and on the content of chlorogenic acid in extract, while the temperature has the least influence.

Ethanol Extraction : The 10.00g of Crofton weed leaves were weighted and grinded. According to L_{16} (4^5) design method, we successively performed the extraction 3 times, blending the filtrate and abandoning the residue, then decompressing and rotary distilling the filtrate, desolventizing and concentrating, using HPLC to determine the content of chlorogenic acid in the extract, calculating extraction ratio of chlorogenic acid and content of chlorogenic acid in extract. The experiment factor level of ethanol extraction is shown in Table 3. Table 4 shows that after choosing ethanol as solvent, the extraction technology is $A_2B_3C_3D_4E_2$, here the ethanol concentration is 40% and the extraction duration is 4 hours, i.e. 10 times of ethanol, at temperature of 80 °C with the pH value of 3. The order of the factors which affect the extraction ratio of chlorogenic acid is B>E>C>D>A, the extraction time > pH > solid-liquid ratio > extraction temperature > ethanol concentration. Having an analysis from the angle of content of chlorogenic acid in extract (mass fraction), the order of the factors is B>C>E>D>A, the extraction time > solid-liquid ratio > pH > extraction temperature > ethanol concentration. In ethanol extraction method, the extraction time has the largest impact on the extraction ratio of chlorogenic acid and on the content of chlorogenic acid in extract, while the ethanol concentration has the least impact.

Ethyl Acetate Extraction : The 10.00g of Crofton weed leaves were weighted and grinded. According to $L_9(3^4)$ design method, we successively performed the extraction 3 times, blending the filtrate and abandoning the residue, then decompressing and rotary distilling the filtrate, desolventizing and concentrating, using HPLC to determine the content of chlorogenic acid in extract, calculating extraction ratio of chlorogenic acid and the content of chlorogenic acid in the extract.

Table 6 shows, if ethyl acetate is chosen as solvent, no matter having consideration from extraction ratio of

Table 1 : Experiment factor level of water extraction

Level	Extraction Duration h (A)	Ratio of Solid to Liquid(B)	Temperature (°C)	pH (D)
1	0.5	1:5	40	4
2	2	1:10	60	6
3	4	1:13	80	7

Table 2 : Orthogonal test table and results of water extraction

Number	A	B	C	D	Extracti-veyield ratio (%)	Content of chlorogenic acid in extract (%)	Extraction ratio of chlorogenic acid (%)
1	1	1	1	1	29.86	11.02	3.25
2	1	2	2	2	39.39	39.22	15.40
3	1	3	3	3	39.29	33.24	13.06
4	2	1	2	3	31.05	38.63	11.96
5	2	2	3	1	44.72	54.62	24.56
6	2	3	1	2	42.62	42.26	16.33
7	3	1	3	2	39.62	16.36	4.95
8	3	2	1	3	35.12	78.51	27.65
9	3	3	2	1	46.78	31.15	15.98
Extractiveyield ratio	K_1	108.54	107.70	107.20	121.32	-	-
	K_2	118.36	117.19	117.46	121.25	-	-
	K_3	121.79	123.86	123.84	105.23	-	-
	R	13.25	16.23	16.54	16.27	-	-
Content of chlorogenic acid	K_1	863.92	126.78	130.75	100.66	-	-
	K_2	134.11	118.56	152.63	92.23	-	-
	K_3	125.05	100.45	123.21	150.28	-	-
	R	50.19	30.28	30.25	58.62	-	-
Extraction ratio of chlorogenic acid	K_1	31.62	48.46	42.23	42.85	-	-
	K_2	56.22	43.26	45.23	37.62	-	-
	K_3	48.55	42.15	45.21	52.71	-	-
	R	21.95	47.23	5.48	15.09	-	-

Table 3 : Experiment factor level of ethanol extraction

Level	Ethanol Concen-tration % (A)	Extraction Duration h (B)	Ratio of Solid to Liquid (C)	Tempera ture (°C) (D)	pH(E)
1	25	0.5	1:4	40	2
2	40	2.5	1:6	50	3
3	70	4	1:10	70	5
4	95	7	1:12	80	7

chlorogenic acid or from content of chlorogenic acid in the extract (mass fraction), the optimum extraction technology is $A_2B_2C_1D_1$, namely the extraction temperature at 50 °C, ethyl acetate concentration is 80%, the extraction duration is 2 hours, and the PH value of 2. The order of the factors which affect the extraction ratio of chlorogenic acid is A>D>C>B, the extraction temperature > pH > extraction time > ethyl acetate concentration. In the usage of ethyl acetate extraction, the extraction temperature has maximum effect on extraction ratio of chlorogenic acid and content of chlorogenic acid in the extract, while the concentration of ethyl acetate has the least effect.

Chlorogenic Acid Purification : NKA-9 macroporous resin 9.63g packing column, 5 times the volume of distilled water, and ethanol solution (φ = 40%) which serve as eluent were adopted for purification, and the purification results are shown in Table 7.

Chlorogenic acid and isochlorogenic acid A were purchased from National Institute for the Control of Pharmaceutical and Biological Products; 1-N-phenyl naphthylamine (NPN) was prepared from Sigma Company; *Escherichia coli* was collected by Jiangxi Agricultural University microbiological laboratory; beef extract, peptone and other biological reagents were purchased from Beijing AOBOX Biotechnology Co., Ltd; alkaline phosphatase assay kit was collected Nanjing Jiancheng Bioengineering Institute. UV - 2551 type ultraviolet-visible spectrophotometer was bought from Japanese SHIMADZU Company; 970 the CRT fluorescence spectrophotometer was bought from Shanghai Precision and Scientific Instrument Corporation; DDS - 11 - a conductivity meter was obtained from Shenzhen KaDiYa Technology Co. Ltd.

Determination of antibacterial activity: chlorogenic acid was dissolved in isochlorogenic acid A standard sample in distilled water to make 0.1 g/ML solution as the sample of antibacterial teat. Potassium sorbate was mixed with sodium benzoate to make 0.0015 g/ML solution, which was used for the comparison of antibacterial activity. The size of antibacterial circle was measured after the culture, and results showed that the bigger the antibacterial circle was, the better was the antibacterial effect.

Table 4 : Orthogonal test table and results of ethanol extraction

Number	A	B	C	D	E	Extracti-veyield ratio (%)	Content of chlorogenic acid in extract (%)	Extraction ratio of chlorogenic acid (%)
1	1	1	1	1	1	32.65	34.65	11.32
2	1	2	2	2	2	48.26	35.65	15.62
3	1	3	3	3	3	42.26	30.60	15.30
4	1	4	4	4	4	36.25	29.10	16.26
5	2	1	2	3	4	43.710	33.89	12.65
6	2	2	1	4	3	48.08	59.86	13.66
7	2	3	4	1	2	45.43	27.32	13.98
8	2	4	3	2	1	41.82	26.35	14.11
9	3	1	3	4	2	53.22	33.02	11.65
10	3	2	4	3	1	53.11	25.64	13.25
11	3	3	1	2	4	33.56	26.66	11.03
12	4	4	2	1	3	37.08	26.35	9.65
13	4	1	4	2	3	14.19	25.16	3.02
14	4	2	3	1	4	13.62	65.32	5.62
15	4	3	2	4	1	52.31	23.02	33.21
16	4	4	1	3	2	27.13	25.32	6.28
Extractiveyield ratio	K_1	171.56	132.26	131.26	183.26	-	-	-
	K_2	175.26	149.65	135.45	160.36	-	-	-
	K_3	165.23	185.23	172.54	139.65	-	-	-
	K_4	106.26	156.32	481.56	135.46	-	-	-
	R	62.95	50.21	50.42	45.62	-	-	-
Content of chlorogenic acid	K_1	123.32	118.32	145.62	161.23	-	-	-
	K_2	156.32	129.45	121.25	146.85	-	-	-
	K_3	123.62	128.59	120.45	116.63	-	-	-
	K_4	139.65	108.23	156.32	118.26	-	-	-
	R	31.25	82.35	33.04	44.96	-	-	-
Extraction ratio of chlorogenic acid	K_1	54.26	39.65	52.32	76.25	-		
	K_2	65.56	50.24	46.23	62.58	-	-	-
	K_3	50.38	88.54	52.32	64.02	-	-	-
	K_4	48.32	41.97	74.29	41.23	-	-	-
	R	20.15	48.56	29.63	35.56	-	-	-

Determination of the conductivity of bacterium solution: A 0.9% NaCl solution was used to wash the *E.coli* at exponential stage, so as to obtain the bacterial suspension, wherein it should meet the condition of OD_{600nm}=0.35. 5ml prepared *E.coli* bacterial suspension was taken to react with 5ml chlorogenic acid material at concentration of 2 and 0.2 mg ml^{-1} respectively for a period of time. After that centrifugation (10000 r/min, 5 min) was performed, and then the conductivity of the supernatant liquor was determined. Regarding the control group where the chlorogenic acid solution was replaced with distilled water, it was tested 3 times (once for every 10 mins) , so as to finally determine the change in tendency of the metal ion exudation.

Measurement of obsorbancy of bacterium solution 260nm: the collected E. coli was washed with 0.9% NaCl for three times before being re-suspending (OD_{420nm} = 0.670). By mixing 2mg/ml antibacterial activity materiel solution with bacterial suspension 1:1 (v/v), then values of OD_{260nm} at different times were determined.

Determination of microbial solution APK activity: 0.5 ml of 108 cfu ml^{-1} *E. coli* bacterial suspension was mixed with equivalent volume of 2 mg ml^{-1}, the same volume of chlorogenic acid and isochlorogenic acid A, where sterile water was adopted as blank control. Through time spliced

Table 5 : Experiment factor level of ethyl acetate extraction

Level	Temperature °C (A)	Ethyl Acetate Concentra-tion % (B)	Extraction Time h (C)	pH (D)
1	30	50	2	2
2	50	80	4	5
3	80	90	5	7

Table 6 : Orthogonal test table and results of ethyl acetate extraction

Number	A	B	C	D	Extracti-veyield ratio (%)	Content of chlorogenic acid in extract (%)	Extraction ratio of chlorogenic acid (%)
1	1	1	1	1	10.32	60.32	6.13
2	1	2	2	2	11.25	37.56	4.36
3	1	3	3	3	8.36	11.26	0.95
4	2	1	2	3	12.35	8.23	0.96
5	2	2	3	1	11.65	20.32	2.14
6	2	3	1	2	9.65	24.65	2.95
7	3	1	3	2	12.42	5.45	0.65
8	3	2	1	3	9.65	7.65	0.75
9	3	3	2	1	11.30	8.69	0.95
Extractiveyield ratio	K_1	30.25	36.21	39.26	33.26	-	-
	K_2	34.21	22.32	35.16	33.77	-	-
	K_3	33.69	26.35	35.15	30.11	-	-
	R	4.05	5.13	5.74	3.36	-	-
Content of chlorogenic acid	K_1	109.65	78.62	92.26	89.56	-	-
	K_2	55.64	65.32	52.15	71.23	-	-
	K_3	21.26	48.62	34.26	26.51	-	-
	r	88.25	25.86	58.26	65.26	-	-
Extraction ratio of chlorogenic acid	K_1	11.26	7.48	9.06	9.63	-	-
	K_2	6.03	7.56	6.24	7.56	-	-
	K_3	2.36	5.26	4.04	2.54	-	-
	R	9.15	3.15	5.26	6.19	-	-

Table 7 : Chlorogenic acid purification results

φ = 40% ethanol NKA-9	Upper column chlorogenic acid mass/g	Collected chlorogenic acid mass /g	Purified dry extract mass/g	Chlorogenic acid purity/%	Chlorogenic acid collection ratio/%
Macroporous resin	0.0104	0.0055	0.0241	24.98	53.88

Table 8 : Antibacterial activity of chlorogenic acids and compound preservative on *E.coli*

	Antibacterial Circle			
Bacterial Type	Chlorogenic acid	Isochlorogenic acid A	Sodium benzoate	Potassium sorbate
E.coli	23.4±0.2	21.2±0.1	13.5±0.3	14.8±0.2

timing sampling, it can obtain the supernatant liquor of the sample interaction, and the AKP content was determined in accordance with requirements on the spectrophotometer kit.

NPN to *E. coli* cell wall permeability test: First the test bacteria was cultivated at OD_{630nm} = 0.5, and bacteria was obtained through centrifugation in 1000 x g at room temperature . Then half volume of HEPES buffer solution of pH = 5.3 was added in it. Pipette was used to take 100 ul of bacterial suspension into fluorescence black slot which contained 2mg ml^{-1} chlorogenic acid solution with 10mmol l^{-1} NPN buffer solution. 10mmol l^{-1} NPN buffer solution was regarded as a comparison group for making the total volume to 200 u L. Fluorescence photometer was used to determine the relative fluorescence (excitation wavelength=355nm, emission wavelength=405nm).

Results and Discussion

Chlorogenic acid and isochlorogenic acid A showed antibacterial activity on *E.coli*, and was compared with the commonly used chemical preservative sodium benzoate and potassium sorbate.

Table 8 shows that chlorogenic acid and isochlorogenic acid A performed well on antibacterial activity, while chlorogenic acid had better effect than isochlorogenic acid A. Chlorogenic acid and isochlorogenic acid A showed better antibacterial activity on *E. coli* as compared to chemical synthetic preservatives such as sorbic acid potassium and sodium benzoate (Xiong *et al.*, 2012).

The absorption value of chlorogenic acid material acted on *E.coli* bacteria solution 260nm is shown in Fig. 1. After interaction of chlorogenic acid with *E.coli*, OD of bacterial solution increased along with the time and reached maximum at 60 min, where chlorogenic acid showed maximum antibacterial activity.

Cell membrane acts as a contact with protective barrier in bacteria. When bacteria comes in bacteriostatic agent, the cell membrane is destroyed and the protective barrier of bacteria is broken and thus, its internal electrolyte leaks into the culture solution, leading to increased conductivity of culture solution. Therefore, the conductivity change of culture solution reflects the permeability change in bacterial cell membrane.

As shown in Fig. 2 when the concentration of chlorogenic acid was 2 mg ml^{-1}, the conductivity of bacterial solution after treatment for any times was higher than that of control group, which increased with duration. This indicates that chlorogenic acid can increase the cell membrane permeability and result in intracellular leakage of electrolytes. Chlorogenic acid has a stronger destructive effect on cell membrane between the two reagents.

Fig. 3 shows the change in activity of *E.coli* alkaline phosphatase before and after adding chlorogenic acid. Fig. 3 shows that after adding chlorogenic acid solution into *E.coli* bacterial solution, the AKP content of bacterial solution become significantly higher than that of the control group, and showed increasing tendency. During the early interaction phase, the rising speed was slow, while the rising speed was faster after 150 min and then followed a steady trend; while the AKP of control group did not change. This suggests that chlorogenic acid can destroy the completeness of bacterial cell wall in a shorter time and with a more significant destroying effect as compared with isochlorogenic acid A (Sheng *et al.*, 2008).

NPN absorption method was adopted to study the effect of chlorogenic acids on *E.coli* cell wall permeability . If the bacterial cell wall is damaged or dysfunctional, NPN will permeate into the cell wall to increase the fluorescence intensity of the system (Run *et al.*, 2006).

As shown in Fig. 4, after adding NPN into the *E. coli* bacterium suspension with chlorogenic acids, the fluorescence intensity increased with longer duration of action, and the fluorescence intensity reached its maximum value after 14 mins of interaction.

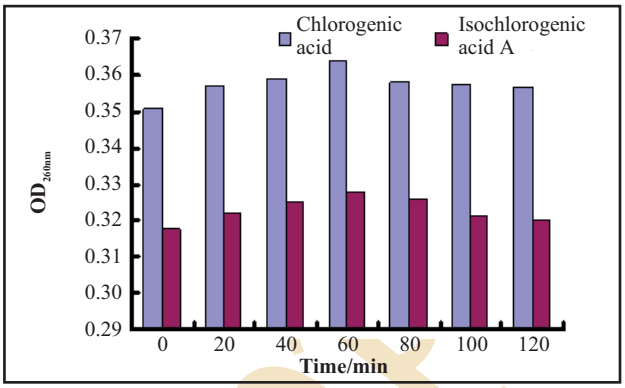

Fig. 1 : Change of absorbancy of nutrient solution

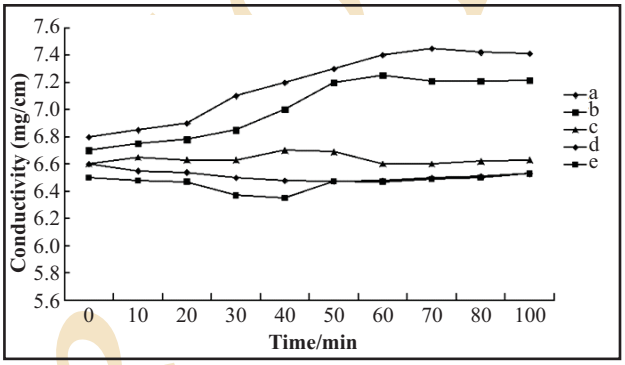

Fig. 2 : Change of conductivity of *E.coli* bacterial solution

Notes : (a) 2 mg ml^{-1} of chlorogenic acid; (b) 0.2 mg ml^{-1} chlorogenic acid; (c) control group; (d) 0.2mg ml^{-1} isochlorogenic acid A; e: 2mg ml^{-1} isochlorogenic acid A.

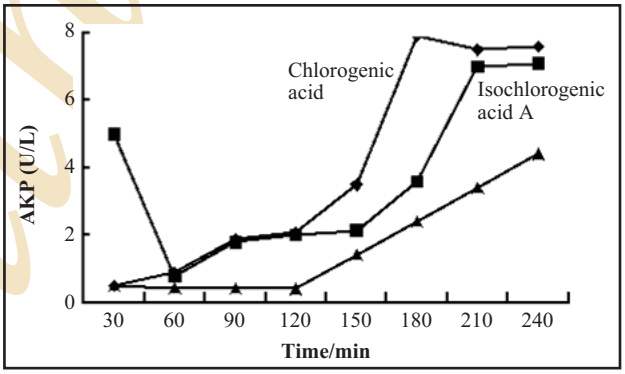

Fig. 3 : Change of AKP activity

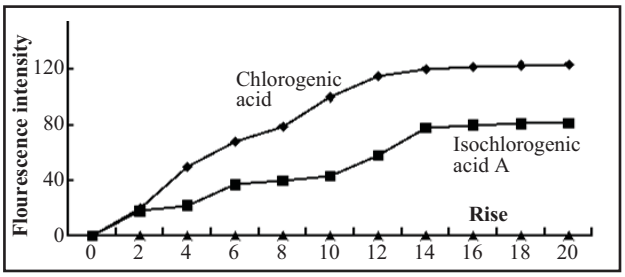

Fig. 4 : Change of NPN fluorescence intensity after the action of chlorogenic acid materials on *E.coli*

Through comprehensive comparison and considering extraction rate of chlorogenic acid, chlorogenic acid content in extract and cost issue, ethanol extraction has many more advantages as compared to water and ethyl acetate extract. Water extract has many impurities, which is difficult to purify, easy to breed bacteria, but hard to be preserved. In addition, water is not a preferred material for being solvent, because it has disadvantages such as high boiling point and desolventizing difficulty. On the other hand, ethyl acetate is more costly than ethanol, which is not suitable for industrial production. Overall ethanol extraction can overcome the drawbacks in water extraction, such as easy to mildew and also enjoy price advantage as compared to ethyl acetate, with higher solvent yield easiness in performing later operations such as filtration, solvent recollection and drying. The optimum extraction parameters are as follows: 40% concentration of ethanol, ethanol pH value is 3, solid to liquid mass ratio is 1:10, the extraction duration is 4 hours, and extraction temperature is 80 °C. The purity of chlorogenic acid after the purification by NKA-9 macroporous resin is 24.98%.

Both Chlorogenic acid and isochlorogenic acid A showed good antibacterial activity against *E.coli*. Results prove that chlorogenic acids can destroy the structure of *E.coli* cell wall and cell membrane within a short period of time, and thus increase the permeability of cell wall. On this basis, cell electrolytes, enzymes, DNA, RNA and NPN can more easily leak into the cell wall, thus affecting the stability of cell structure and causing death of cell. Compared with isochlorogenic acid A, Chlorogenic acid showed better antibacterial effect and stronger action intensity to cell wall and membrane, so as to achieve a better antibacterial effect.

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