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EXTRACTION OF ANTHRAQUINONE DERIVATIVES FROM *RHUBARB* RHIZOMES AND THEIR ANTIBACTERIAL TESTS

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Photometry was employed to study the optimum extraction conditions of anthraquinone derivatives from rhizomes of *Rheum officinale* Baill in this study. The influences of extraction solvents (chloroform, benzene, ethanol, methanol, and glycerol), acid, and extraction time on the extraction yield were discussed. The results indicate that, to the Rhubarb rhizomes powder with the average particle size 0.18 mm, the conditions of the extraction solvent composed by chloroform, glycerol, and sulfuric acid (20%) in the ratio of 4:1:1 ($v:v$), the weight of dried Rhubarb to the solvent volume in the ratio of 1:12 ($w:v$), extraction time of 110 min, the anthraquinone derivatives extraction could achieve the best yield. And the antibacterial tests showed the raw extraction products had the MIC (minimal inhibitory concentration) of 20 μg/mL and 30 μg/mL to *Staphylococcus aureus* and *Escherichia coli*, respectively.

Keywords: Anthraquinone derivatives; photometry; optimum extraction conditions; *Rheum officinale* Baill; antibacterial.

1. Introduction

Rhubarb is one of the important and well-known Chinese herbal medicine resources; and it has been used as medicine for thousands of years. It has the effect of strengthening the stomach, purging heat, removing bacterial dysentery, loosening the bowels, promoting blood circulation, etc. Additionally, rhubarb has the action of antitumor and antimutagenicity.[1−5] Besides the pharmacological value, rhubarb also can be made into a nourishing food. But the most attractive research is the antibacterial activity of the extraction products. It is generally believed that the anthraquinone derivatives, including emodin, aloe-emodin, rhein, physcion, chrysophanol, aloe-emodin and their glucosides, are identified as the important active components.[6−11] Other reports demonstrated that these anthraquinone derivatives could not only inhibit the α-glucosidase in diabetes disease but also inhibit some protein kinases.[12,13] Therefore, many compound Chinese herbal medicines contain rhubarb due to its multipharmic effects.[13,14]

Anthraquinone derivatives are often obtained from natural sources and as rhubarb has splendid pharmic effects, much interest has been focused on the extracting and separating of the effective components, especially in the aspect of optimum extracting conditions. However, rhubarb has complex constituents and what's more, different methods and extraction solvents have great influence on the extraction yield. Some methods were developed to extract and separate the gross

anthraquinone derivatives, such as supercritical fluid extraction, ethanol extraction, aether extraction, sulfuric-acid/chloroform extraction, and so on.[15−17] Generally, to study the optimum extracting conditions of the herbals, a large number of experiments are required to carry out, such as multi-aspect and multi-level orthogonal experiments,[17] and these time-cost and reagent-consumed experiments increase the cost greatly. The methods commonly used for the separation of anthraquinone compounds in rhubarb are high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), thin-layer chromatography (TLC), and their improved or related methods,[18−22] which are apparatus-cost or low precision or complex to operate. So, a method with precision, simplicity, time-saving, and low cost, is deadly required to determine the optimum extracting conditions in practice. Therefore, more information should be obtained from the less trials. Normally, in the experiments of studying the optimum conditions, to determine the gross yield is necessary. And photometry value is in linear relation with the molar concentration, and to measure this value in a certain range can calculate the gross extractings.[23] In this paper, quantitative analysis of the gross anthraquinone derivatives had been determined by using photometry in real-time during the process of extracting, and the results indicated the successful determination of the optimum extracting conditions. This work has provided an alternative method for the related herbal extracting and also can be applied to quantifying other active components that have absorbance. This method not only greatly reduces the number of trials but also can be time-saving, easy to operate, cost-saving, and direct-viewed, so it will be a powerful tool in medicine researches.

2. Methods and Materials

2.1. *Materials and reagents*

The rhizomes of rhubarb were purchased from the Hubei Chinese Herbal Medicine Resource Ltd. (Wuhan, Hubei, China) and identified as *Rheum officinale* Baill; the pure anthraquinone derivatives were purchased from the Examination Institute of Medicine and Biological Produce of Wuhan (Wuhan, Hubei, China). Chloroform, benzene, methanol, ethanol, glycerol, vitriol (20%), were ordered from China National Pharmaceutical Group (Shanghai, China). All the reagents were of analytical grade.

2.2. *Experiment methods*

2.2.1. *Extraction*

The air-dried, powdered roots of rhubarb (average particle size 0.18 mm, 25 g) were put into the 500 mL round-bottom flask, and then the extracting solvents (chloroform, benzene, ethanol, etc.) were added to the flask and slowly heated to reflux. Extracting solvents of 0.1 mL were taken out every 5 min when reflux began and then diluted to 4 mL to measure the absorbance value in the range of 350−550 nm.

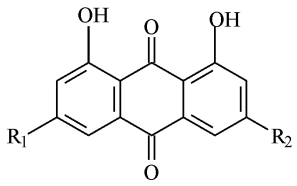
The absorbance was determined by UV−vis spectrophotometer (UV-2550, Shimadzu, Japan).

2.2.2. *Antimicrobial assays*

The bacterium, *Staphylococcus aureus* (ATCC6538) and *Escherichia coli* (ATCC8099), respectively, was cultured on peptone nutrient agar medium for the activation, and then the activated bacterium were inoculated into the peptone liquid nutrient medium to culture for 24 h, and the final bacterium suspend solution concentration was 10^6-10^7 CFU/mL. The rhubarb extractings which were dissolved in distilled water were diluted by 10-fold serial dilution technique at the total volume of 1 mL each tube; then 2 mL of liquid peptone nutrient medium and 1 mL of bacterium suspend solution were added to each of the tubes, which were shaken to uniformity and then cultured under 38°C for 24 h in the shaking bed. Then 0.1 mL of the mixture was taken out from the tube, diluted to 1 mL with physiological brine, and then the dilution was added to the plates of peptone nutrient agar medium under 38°C for 24 h, and then the lawns were counted on the plates.

3. Results and Discussion

Structures of the main anthraquinone derivatives in rhubarb are shown in Fig. 1. R_1 and R_2 are the different substitutions. These derivatives have the same core skeleton, and they have the same property in some aspects, such as the strong absorbance at about 430 nm. Therefore, the gross anthraquinone derivatives in extracting solvents can be determined by the absorbance value at this wavelength, which is the base of this study to determine the accumulated gross yields in different extracting conditions.



	R_1	R_2
Rhein	H	COOH
Chrysophanol	H	CH_3
Aloe_emodi n	H	CH_2OH
Emodin	OH	CH_3
Phscion	OCH_3	CH_3

Fig. 1. Structures of the important anthraquinone derivatives in Rhubarb.

3.1. *The influence of different extracting solvents*

Generally, the best extraction yield is determined by the extraction solvents and their ratios, which are prepared according to the active components exist states in herbals. The commonly used extracting solvents are benzene, chloroform and methanol, ethanol, glycerol, and their mixtures.[16] The absorbance curves of the standard anthraquinone derivative in different solvents in the same concentration almost overlapped (Fig. 2), indicating that the solvents themselves will not influence the absorbance value at about 430 nm, and so the value is only determined by the total mass of anthraquinone derivatives in solvent. These results show that the absorbance obtained in the different solvents aforementioned is comparable.

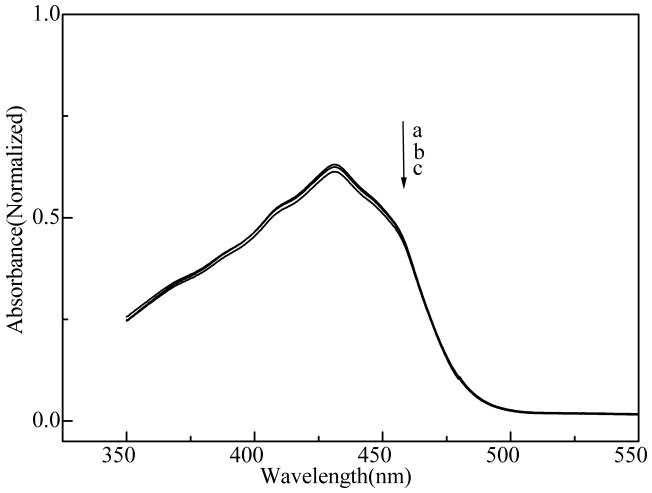


Fig. 2. The absorbance curves of standard samples in different solvents (chloroform (a), benzene (b), ethanol (c)) in the same concentration. The standard samples contain Rhein and Emodin in arbitrary ratio and the total concentration is 10 mg/mL.

3.2. *Solvents influence*

The solvent in polar difference decides its extracting ability of effective components in rhubarb. Under the same conditions, the absorbance of chloroform (a) extraction is the strongest, the benzene (b) is weaker, and the ethanol (c) is the weakest (as shown in Fig. 3). These results show that, to extract the anthraquinone derivatives in rhubarb, the chloroform extraction effect is the best, the benzene follows next, and the ethanol the last. It also can be seen from Fig. 3 that the benzene and the chloroform extraction absorbance curve has one weak absorbance peak in 350−400 nm range, which indicates the extracting of impure ingredients in the process of extracting anthraquinone derivatives from rhubarb. On comparison, the ethanol extraction absorbance curve is quite smooth in this range, and this indicates that ethanol is better than chloroform and benzene to the impurity inhibitory. Therefore, if the extracting solvent contains chloroform as well as ethanol, it may achieve both good extraction quantity and effective impurity inhibition.

3.3. *The effects of impurity inhibition*

It is normally inevitable to get other structure-similar components in the process of extracting;

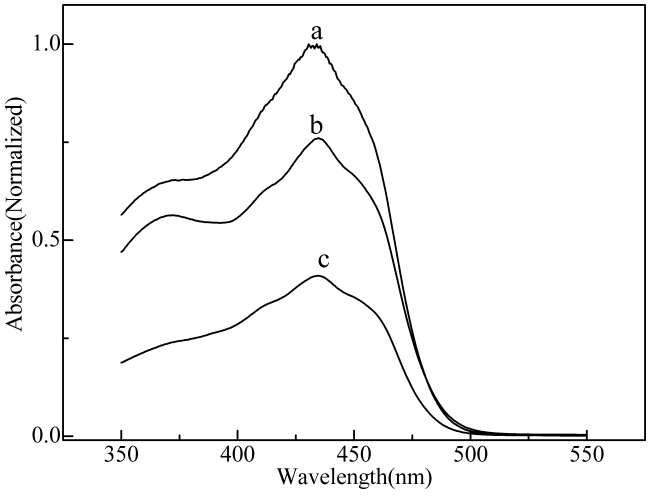
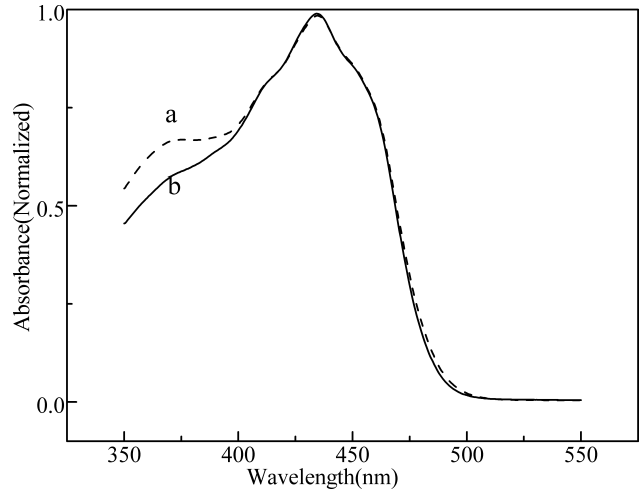
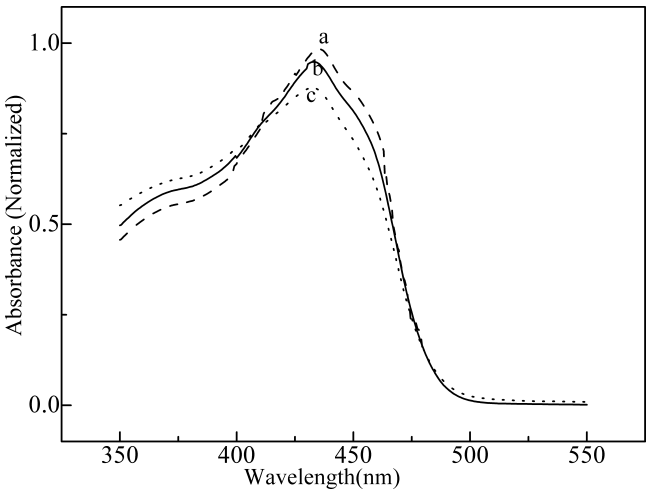


Fig. 3. The influence of different extraction solvents on the yield. The absorbance curve of the extraction: Chloroform (a), benzene (b), ethanol (c). The data were the highest concentration of each solvent and normalized. The volume is 200 mL; dry Rhubarb 25 g. The following figures are the same.

therefore, to add another solvent that has the impurity inhibitory effect is considered when choosing the extracting solvent. And it may achieve the best extraction yield with less impurities. From Fig. 4(A), it can be seen that the absorbance curve of extraction solvent containing glycerol decreases remarkably in the 350−400 nm range, but the 430 nm absorbance basically keeps the same. This indicates that when glycerol was added to the extracting solvent the yield could be maintained while the impurities had been inhibited. Glycerol, methanol, and ethanol were studied according to the reports,[15−17] and the final results indicate that in the same conditions, glycerol is better than ethanol, and the methanol is better than ethanol in the ratio of 1:4 to chloroform (see Fig. 4(B)).



(A)



(B)

Fig. 4. The impurity inhibitory effect of alcohol. (A) The absorbance curve of only chloroform extraction (a) and chloroform/glycerol extraction (b); (B) The absorbance curve of chloroform/glycerol (a), chloroform/methanol (b), chloroform/ethanol (c) extraction. The volume of glycerol, methanol, ethanol is the same — 50 mL, chloroform is 200 mL (1:4, $v:v$).

3.4. *The effects of acid to the extraction yield*

The anthraquinone derivatives in rhubarb exist mainly in free or combination, such as glucoside derivatives. The free anthraquinone derivatives can be directly extracted, but the combinatives must be hydrolyzed before the extraction, like the glucoside derivatives, which only can be decomposed under the sulfuric acid hydrolysis. There are reports about the sulfuric acid (25%, v) that it can greatly improve the extraction yield during the solvent extraction process.[15−17] In the results show in Fig. 5, there is a very strong absorbance in the extraction solvent that had sulfuric acid added to it (chloroform/ sulfuric acid 4:1), but it was weaker in the solvent that did not add. It indicates that the extraction yield of anthraquinone derivatives increased greatly after acidolysis. Therefore, the extraction solvent needs to add one quota of the acid which is in the ratio of 1:4 to the main solvent chloroform, to increase extraction yield.

3.5. *The optimum time of extraction*

The extraction speed of anthraquinone derivatives in rhubarb is quite slow under the normal extraction

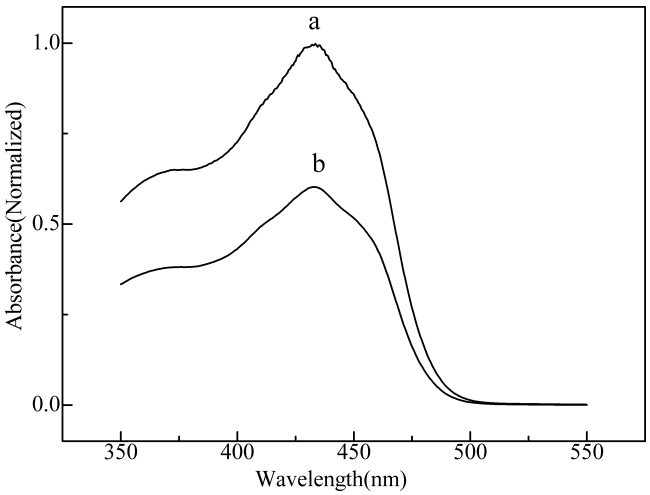


Fig. 5. The absorbance curve of the extraction solvent that added sulfuric acid (a) and did not add sulfuric acid (b). The volume of sulfuric acid (20%) is 50 mL (1:4, $v:v$).

temperature; therefore, it is quite necessary to heat up for certain time to reflux the extraction solvents. The anthraquinone derivatives are not merely derived by the simple process of solute dissolution, but also by swollen as well as the sulfuric acid hydrolysis of the combinatives. Furthermore, in the rhubarb plant tissue, it also includes the diffusion of solvents during the first stage and adsorption of anthraquinone derivatives in the final stage.[15] Therefore, the extraction time is also an essential factor that affects the extraction yield. By using the optimum combination extraction solvents — chloroform 200 mL, sulfuric acid 50 mL, and glycerol 50 mL (4:1:1), the absorbance at 430 nm was monitored in different extraction times — the absorbance−time curve increases in the first 110 min since the beginning of reflux (Fig. 6), and this indicates the increasing total mass of anthraquinone derivatives in this period of time. But, after the time of 115 min, the curve decreased, which means the reduction of the gross anthraquinone derivatives, and this possibly is caused by the destruction of sulfuric acid to the anthraquinone derivatives or the adsorption speed is quicker than the extraction speed. Therefore, the optimum extraction time is about 110 min.

3.6. *Antibacterial experiments*

The antibacterial experiments were carried out by a standard procedure.[24] Briefly, the rhubarb raw extraction products and phenol (commonly used as antibacterial disinfector) were diluted by 10-fold serial dilution technique in tubes and each of the dilution was studied under the same conditions for the MIC tests. The final test results can be seen in Fig. 7. The number of the lawns on solid nutrient agar medium that had the addition of rhubarb extraction solvent (left) was obviously less than that which did not have the addition (right). These results indicate very strong antibacterial activity of rhubarb raw extraction products. Serial trials had been done to determine the minimal inhibitory concentration (MIC, μg/mL), and the results indicate that the MIC for rhubarb chloroform/glycerol extraction is 20 μg/mL and 30 μg/mL to *Staphylococcus aureus* and *Escherichia coli*, respectively, while the MIC for phenol is 12 μg/mL and 55 μg/mL. These results show that the rhubarb chloroform/glycerol extraction has a very good antibacterial performance.

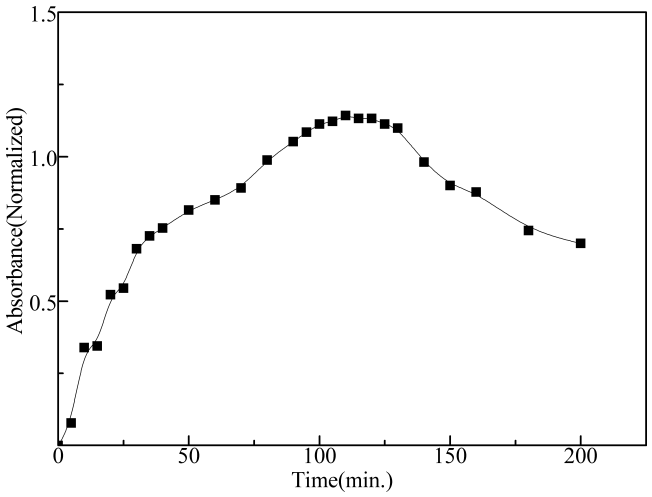


Fig. 6. The plot of absorbance peak area with the extraction time.

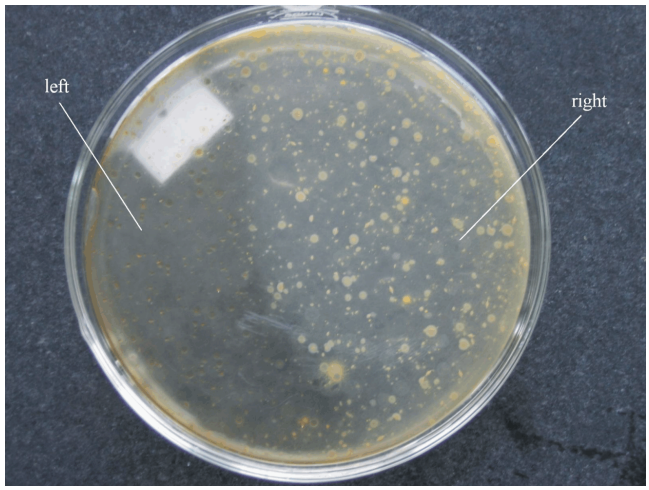


Fig. 7. The antibacterial activity of chloroform/glycerol extraction solvent. The left of the plate was added chloroform/glycerol extraction solvent and the anthraquinone derivatives concentration was about 50 μg/mL; the right was the blank that did not add any antimicrobial reagent. The cultural medium was solid peptone nutrient agar medium and the bacteria was *Staphylococcus aureus*.

4. Conclusions

In conclusion, the optimum extraction conditions of anthraquinone derivatives in *Rheum officinale* can be quickly determined by the absorbance. The influence of composition ingredients as well as the proportion of extraction solvent, the different reagents to the impurity inhibitory, the extraction time, and so on, can be directly monitored in the absorbance curve. Comparing to the traditional orthogonal experiments, this method saves the reagents and shortens the time greatly, and it also can examine the

concentration of anthraquinone derivatives in the extraction system in real time, and thus it is very suitable for monitoring in industrial process. This method also provides an alternative method for the extraction and separation of natural products. The antibacterial experiments confirmed that the chloroform/glycerol extraction obtained by this method has very strong antibacterial performance to *Staphylococcus aureus* and *Escherichia coli*, thus further confirming the validity of this method to extract the active components.

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