Extraction and determination of total flavonoids in jujube by alcohol extraction

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Abstract. Jujube is a ripe fruit of Rhamnaceae. Its main active component is flavonoids, so the extraction and determination of total flavonoids in jujube will help to develop and utilize the medicinal value of jujube. In this study, the total flavonoids were extracted from jujube by alcohol extraction method. Through single factor investigation and orthogonal test, it was found that the total flavonoids content in jujube was the highest under the condition of 70°C, material ratio of 1:40, and extraction of 30 min by 70% ethanol. The content of total flavonoids in the extract of jujube was 1.57% at the wavelength of 510 nm by UV and rutin as the standard. The method was evaluated by methodological study, and it was determined that this method could be used as the detection of total flavonoids in jujube extraction.

1. Introduction
In the Classical Chinese medicine and ancient agriculture have recorded, jujube has characteristics of health-preservation, health-care, anti-cancer, promoting blood circulation, promoting digestion, improving body immunity and so on, which was widely used traditional chinese herbal medicines, and was multi-version of the Chinese Pharmacopoeia contained[1-2]. Modern research found that jujube contains a variety of chemical components, such as carbohydrates, esters, organic acids, alkaloids, saponins and flavonoids, the most important active ingredient is flavonoids in these components[3-4]. Flavonoids have good therapeutic effect on coronary heart disease and spasm, and also have characteristics of anti-bacterial, anti-inflammatory and anti-tumor[5-8]. The method of extraction and content determination is an important part of the quality standard of jujube and its preparation. So, in this study, we will study the extraction method and content determination method of jujube, and provide scientific basis for the further development and utilization of the medicinal value of jujube.

2. Instruments and materials

2.1. Materials
Jujube was purchased from Shanghai Di Bai Chemical Technology Co. Ltd. Rutin(≥98%) was purchased from Dr.Ehrenstorfer company. C₂H₅OH and CH₃OH were purchased from Xilong Chemical Co. Ltd. Petroleum ether. Al(NO₃)₃ was purchased from Chongqing Chuandong Chemical Co. Ltd. NaNO₂, NaOH, CH₃CH₂CH₂CH₂OH and CH₃COCH₃ were purchased from Tianjin Fine Chemical Development Center. HCL was purchased from Shanghai Modern Pharmaceutical Co. Ltd.
Magnesium and CH₃CH₂OOCCH₃ were purchased from Tianjin Tianli Chemical Reagent Co. Ltd.

2.2. Instruments
High speed universal grinder was purchased from Guangzhou Hu Ruiming Instrument Co. Ltd. Electric heating oven was purchased from Shanghai right a Instrument Co. Ltd. Electric constant temperature water bath was purchased from Suzhou win An Yang Instrument Co. Ltd. UV-Vis spectrophotometer was purchased from Shimadzu Corporation. ultrasonic cleaner was purchased from Shanghai Ba Jiu Industrial Co. Ltd. Circulating water type multipurpose vacuum pump was purchased from Zhengzhou the Great Wall branch industry and Trade Co. Ltd. Vacuum rotary evaporator was purchased from Taikang Xi'an biological science and Technology Group. Vacuum freeze drier was purchased from Ningbo Shuang Jia Instrument Co. Ltd. Precision electronic balance was purchased from Shanghai Precision Science Instrument Co. Ltd.

3. Experimental method

3.1. Establishment of content determination method

3.1.1. Configuring the standard solution. 20.1 mg rutin was took to 100 mL volumetric bottle, 95% ethanol was added to the scale and then rutin standard solution was obtained, the concentration is about 0.20 mg/mL. Shaken well and spared.

3.1.2. Selection of maximum absorption wavelength. Appropriate rutin standard solution was took in 10 mL volumetric flask, then added 0.4 mL of 5% NaNO₂ solution, mixed evenly, placed after 6 min calmly. Then added 0.4 mL of 10% Al(NO₃)₃ solution, mixed evenly, placed after 6 min calmly. Then added 5 mL of 5% NaOH solution, constant volume with 95% ethanol finally. Placed after 15 min calmly, the reagent solution as blank and subjected to spectral scanning at a wavelength of 400-700 nm. According to the scanning results, the maximum absorption wavelength selected as the measurement of this experiment wavelength.

3.1.3. Drawing standard curve. The rutin standard solution of 0.2 mg/mL were placed in 10 mL volumetric flask respectively (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mL), and then added 4 mL of 30% ethanol. Then, 0.3 mL of 5% NaNO₂ solution was added and placed for 6min. Added 0.3 mL of 10% Al(NO₃)₃ solution and placed for 6 min calmly, add 4 mL of 4% NaOH solution, distilled water diluted to 10 mL respectively, shaken evenly, and then placed for 15 min calmly. The blank control group is not added reference substance, at the maximum absorption wavelength, the absorbance value of the blank group was measured, and the vertical coordinate was taken as the absorbance and the abscissa was the concentration of the rutin reference substance solution, then drawing standard curve.

3.1.4. Precision test. Appropriate ethanol solution was added to rutin standard solution, the absorbance was determined by UV at 510 nm and repeated 5 times. Calculating the relative standard deviation RSD value.

3.1.5. Stability test. After the determination of the sample solution, the blank reagent as a control, the absorbance value of sample was measured per 10 min and determined to 60 min continuously.

3.1.6. Repetitive test. The extraction of total flavonoids were repeated 6 times, and the prepared samples were collected in turn. The absorbance was measured at 510 nm, repeated 6 times, and the relative standard deviation RSD value was calculated.

3.1.7. Sample detection. 1 mL test solution was placed in 10 mL volumetric flask, added 4 mL of 30% ethanol, then 0.3 mL of 5% NaNO₂ solution added to the obtained liquid and placed for 6 min calmly. 0.3 mL of 10% Al(NO₃)₃ solution was added and placed for 6 min, added 4 mL of 4% NaOH solution
and diluted to 10 mL with distilled water and shaken for 15 min. Distilled water used as a blank control, the absorbance value of the sample was measured at the maximum absorption wavelength, then the concentration of the total flavonoids in the extract were calculated by the regression equation of standard curve, and the content of total flavonoids in jujube were calculated by formula. Total flavonoid content (mg/g) = C×V/M (C is the concentration of flavonoids in extract, V is the total volume of extract, M is the amount of total flavonoid extract of jujube).

3.2 Extraction method and optimization scheme

3.2.1. Extraction of total flavones in jujube. A certain amount of jujube directly crushed with a grinder, the petroleum ether was added to the flask at a ratio of 10: 1 and the fat-soluble pigment from the slag was removed. Then install the condensate reflow device, in 70°C water bath reflowing. After reflowed for 2 h, the petroleum ether was filtered off and the dregs were placed in a fume hood and the residual petroleum ether was evaporated. The obtained drug and 70% ethanol were added to a flat-bottomed flask in an amount of 40: 1, fitted with a reflux condenser and heated under reflux at 70°C. After 30 min, the residue was filtered and the filtrate was collected. The residue was re-placed in a flat-bottomed flask and reflowed for 3 times. The filtrates were combined, concentrated under reduced pressure, lyophilized in vacuo, the total flavonoids of jujube were obtained.

3.2.2. Qualitative identification method. (1) Hydrochloric acid-magnesium powder reaction: 2 ml test solution was took to the test tube, respectively, added a little magnesium powder and 1-2 ml concentrated hydrochloric acid for color reaction, the test color change observed, it can check whether there are total flavonoids. If the color of the solution becomes red, it indicates that the extract contains flavonoids or flavonols and other ingredients. (2) Aluminum trichloride reaction: 2 ml test solution was took to the test tube, then added a drop of aluminum trichloride for color reaction, the test color change observed, it can check whether there are total flavonoids. If the solution appears yellow precipitate, indicating that there are three or five hydroxyl-containing flavonoids. (3) Lead acetate reaction: 2 ml test solution was took to the test tube, then added a drop of lead acetate solution to the color reaction in the test tube, the change of the color observed, and it can check whether there are total flavonoids. If the solution appears yellow precipitate, explain the flavonoids containing hydroxyl groups.

3.2.3. Single factor test. Single factor study is a widely used method of study, in the selected several factors only to change one of the factors, the rest of the factors fixed to the single factor experiment. Single factor experiments examined the effect of different levels of various factors on total flavonoids extracted from ethanol in jujube. Four single factors were ethanol concentration, water bath temperature, extraction time and material ratio. Ethanol concentration was 30%, 50%, 70% and 90% respectively. The bath temperature was 50°C, 60°C, 70°C and 80°C respectively. The extraction time was 10 min, 30 min, 60 min and 90 min respectively. Material ratio were 1: 20, 1: 30, 1: 40, 1: 50. As shown in Table 1.

| Level | A Ethanol concentration(%) | B Bath temperature(°C) | C Material ratio (g:mL) | D Time (min) |
|-------|--------------------------|-----------------------|------------------------|-------------|
| 1     | 30                       | 50                    | 1:20                   | 10          |
| 2     | 50                       | 60                    | 1:30                   | 30          |
| 3     | 70                       | 70                    | 1:40                   | 60          |
| 4     | 90                       | 80                    | 1:50                   | 90          |

(1) Effect of ethanol concentration on flavonoid contents: 50 mL ethanol solution with
concentration of 30%, 50%, 70%, 90% respectively, extracted the total flavonoids of 2 g jujube powder in 80 °C water bath reflowed for 30 min, filtrated and the volume was 100 mL. The absorbance value was determined by UV, and the flavonoid contents were calculated.

2) Effect of water bath temperature on flavonoid contents: The treated jujube powder 2 g was placed in a flat-bottomed flask, added 50 mL of 50% ethanol solution at 50 °C, 60 °C, 70 °C, 80 °C water bath heated for 30 min respectively, filtrated and the volume was 100 mL. The absorbance value was measured, and the flavonoid contents was calculated.

3) Effect of different material ratio on flavonoid contents: The treated jujube powder 2g was took to a flat-bottomed flask, 50% ethanol to the material ratio 1: 20, 1: 30, 1: 40, 1: 50 mixed. And heated to reflow in water bath at 70 °C for 30 min. After filtrated, the volume was 100 mL. The absorbance was measured and the flavonoid contents was calculated.

4) Effect of reflow time on flavonoid contents: The treated jujube powder 2 g took to the flat-bottomed flask, added 50mL of 50% ethanol solution in 70 °C water bath heated for 10 min, 30 min, 60 min, 90 min respectively. Filtrated and the volume was 100 mL. The absorbance value was measured, and the flavonoid contents was calculated.

3.2.4. Orthogonal test. On the basis of single factor test, the effect of water bath temperature, material ratio and reflow time on flavonoid contents was obvious. So this orthogonal test will select the water bath temperature, material ratio and reflow time as a variable for orthogonal test, and repeat the operation 3 times, the specific experimental design as shown in Table 2.

| Test number | A | B | C |
|-------------|---|---|---|
| 1           | 1 | 2 | 3 |
| 2           | 1 | 2 | 2 |
| 3           | 1 | 3 | 3 |
| 4           | 2 | 1 | 2 |
| 5           | 2 | 2 | 3 |
| 6           | 2 | 3 | 1 |
| 7           | 3 | 1 | 3 |
| 8           | 3 | 2 | 2 |
| 9           | 3 | 3 | 1 |

3.2.5. Orthogonal test data processing. The data were analyzed by SPSS 21.0, and the optimum conditions were obtained by extracting the total flavonoids from jujube.

4. Results of extraction method and optimization scheme

4.1. Establishment of a method for the determination of content

4.1.1. Determination of maximum absorption wavelength. The rutin reference substance solution in the ultraviolet spectrophotometer for full-wavelength scanning and found that the maximum absorbance at 510 nm, consistent with the literature, so choose the absorption wavelength of 510 nm as a quantitative detection wavelength. See Table 3 for details.

| Wavelength(nm) | 470 | 480 | 490 | 500 | 510 | 520 | 530 | 540 |
|----------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Absorbance(A)  | 0.220 | 0.237 | 0.254 | 0.266 | 0.270 | 0.265 | 0.253 | 0.232 |
4.1.2. Standard curve. The absorbance value was measured at 510 nm. The standard curve was plotted using the standard solution absorbance as the ordinate Y and the mass concentration of rutin in the standard solution as the abscissa X, as shown in Figure 1. The regressive equation of the curve is: \( y = 0.0112x + 0.0049 \), \( R^2 = 0.9995 \), indicating that the method has good linearity and can be used as the method for the determination of flavonoids in jujube.

![Figure 1. Rutin's standard curve.](image)

4.1.3. Precision experiment. It can be seen from Table 4 that the content of total flavonoids in the sample is relatively average and the relative standard deviation is small, which indicates that the method has good precision and accuracy.

| Sample number | Absorbance | Average absorbance | RSD (%) |
|---------------|------------|--------------------|---------|
| 1             | 0.318      |                    |         |
| 2             | 0.312      |                    |         |
| 3             | 0.312      | 0.313              | 1.24%   |
| 4             | 0.308      |                    |         |
| 5             | 0.316      |                    |         |

4.1.4. Stability experiment. It can be seen from Table 5 that the results of the absorbance values measured at different times are not very different. The colored material produced by the color reaction is stable, and the change of the absorbance value does not occur within 60 min, indicating that the spectrophotometric method Total flavonoid content is stable and reliable, suitable for quantitative analysis.

| Time (min) | Absorbance | Average absorbance | RSD (%) |
|------------|------------|--------------------|---------|
| 10         | 0.318      |                    |         |
| 20         | 0.314      |                    |         |
| 30         | 0.308      | 0.310              | 1.82%   |
| 40         | 0.314      |                    |         |
| 50         | 0.304      |                    |         |
| 60         | 0.304      |                    |         |

4.1.5. Repeated experimental. The results of repeated trials are shown in Tab 6. The average contents(%) was calculated by Table 6 is 1.565%, RSD%=0.56%, which proves the repeatability of this experiment is better.
### Table 6. Repetitive test results.

| Serial number | 1   | 2    | 3    | 4    | 5    | 6    |
|---------------|-----|------|------|------|------|------|
| Absorbance (A)| 0.240 | 0.300 | 0.310 | 0.246 | 0.326 | 0.304 |
| Contents(%)   | 1.35% | 1.63% | 1.67% | 1.34% | 1.75% | 1.65% |

4.2 Extraction methods and optimization of the results of the program

4.2.1. Qualitative identification of the results. (1) Hydrochloric acid-magnesium powder reaction: The extract was dissolved in ethanol, an appropriate amount of magnesium powder was added and a few drops of concentrated hydrochloric acid were added dropwise. After 1 to 2 minutes, the color change of the solution was observed: orange red turned purple, indicating that there was total flavonoids in the extract, It is feasible to extract the total flavonoids from jujube. (2) Aluminum trichloride reaction: The extract was dissolved in ethanol, and a drop of aluminum trichloride was added to the color reaction to observe the change in the color of the test. The yellow precipitate appeared in the solution, indicating that there was total flavonoids in the extract, and ethanol was reflowed to extract the total flavonoids of jujube. (3) Lead acetate reaction: The extract was dissolved in ethanol, and then a drop of lead acetate solution was added dropwise to observe the color change. The yellow precipitate appeared in the solution, indicating that the total flavonoids in the extract and the ethanol extract were effective.

4.2.2. Results of single factor experiments. (1) Effect of ethanol concentration on flavonoid contents: It can be seen from Figure 2 that flavonoid contents gradually increased with the increase of ethanol concentration, and the contents reached the highest value of 1.25% at 70% concentration. At this time, the concentration increased again and the contents decreased, When the concentration reached 90%, the contents was the lowest, only 1.06%. In summary, the ethanol concentration of 70% when the contents was the highest, reaching 1.25%.

![Figure 2. Effect of ethanol concentration on flavonoid contents.](image)

(2) Effect of water bath temperature on flavonoid contents: You can see from Figure 3, with the increase of temperature, and flavonoid contents also increased, but when the temperature exceeds 70°C, the contents of flavonoid is no longer increasing but decreased, because the temperature may be too high in flavonoids oxidation. To sum up, the contents was the highest at 70 °C and reached 1.39%.
Figure 3. Effect of water bath temperature on flavonoid contents.

(3) Effect of different material ratio on flavonoid contents: As can be seen from Figure 4, the flavonoid contents increases with the increase of the material ratio, but when the material ratio increased to 1:40, the flavonoid contents began to slow down. To sum up, the flavonoid contents reached the highest at the material ratio of 1:40, reaching 1.42%.

Figure 4. Effect of material ratio on flavonoid contents.

(4) Effect of reflow time on the flavonoid contents: It can be seen from Figure 5, the reflow time before 30min, the flavonoid contents is on the rise. The contents was highest at 30 min, reached 1.51%. The flavonoid contents was decreased gradually in the period of 30-90 min and the lowest content was 1.46% at 90 min. In summary, when the reflow time was 30min, the flavonoid contents was the highest, reaching 1.51%.

Figure 5. Effect of reflow time on flavonoid contents.

4.2.3. Orthogonal test results. Arrange the test according to the design table of orthogonal test. See Table 7 for the arrangement of specific factors.
Table 7. Orthogonal test results and range analysis.

| Test number | A  | B  | C  | Absorbance | Flavonoid contents(%) |
|-------------|----|----|----|------------|-----------------------|
| 1           | 1  | 1  | 1  | 0.336      | 1.48                  |
| 2           | 1  | 2  | 2  | 0.352      | 1.54                  |
| 3           | 1  | 3  | 3  | 0.340      | 1.50                  |
| 4           | 2  | 1  | 2  | 0.342      | 1.51                  |
| 5           | 2  | 2  | 3  | 0.352      | 1.54                  |
| 6           | 2  | 3  | 1  | 0.336      | 1.47                  |
| 7           | 3  | 1  | 3  | 0.336      | 1.48                  |
| 8           | 3  | 2  | 2  | 0.364      | 1.57                  |
| 9           | 3  | 3  | 1  | 0.328      | 1.44                  |

| K1          | 4.520 | 4.470 | 4.390 |
| K2          | 4.520 | 4.650 | 4.620 |
| K3          | 4.490 | 4.410 | 4.520 |
| R           | 0.030 | 0.240 | 0.230 |

Table 8. Analysis of variance of orthogonal results.

| Source of variance | Sum of squares of deviations | Degrees of freedom | Mean square | F value |
|--------------------|------------------------------|-------------------|-------------|---------|
| A                  | 0.072                        | 2                 | 0.007       | 8.615   |
| B                  | 0.089                        | 2                 | 0.029       | 17.307  |
| C                  | 0.101                        | 2                 | 0.002       | 5.395   |
| Error              | 0.006                        | 2                 | 0.016       |         |

In the analysis of Table 7, extreme R can be inferred that the results of this experiment did not analyze the significant, it may be that each single factor level of the experiment has little effect on the experiment. The results showed that the order of the three factors on the content of total flavonoids was: B> C> A, that is, the temperature of the water bath> the ratio of material to material> reflow time. According to the orthogonal results of Table 8, it can be deduced that the optimum conditions for the extraction of total flavonoids are: A3B2C2, reflow time is 30 min, temperature is 70 °C, material ratio is 1:40, can achieve the best extraction effect.

5. Discussion
Jujube is the mature fruit of plum plant, which has extensive planting area and abundant resources in China. Jujube has a variety of pharmacological activities, such as blood supplement, anti-cancer, invigorating the spleen and strengthening the body, and many other functions. It belongs to the traditional Chinese medicinal materials which are widely used[9-12]. The study shows that many Chinese herbal medicines contain flavonoids including jujube. At present, the commonly used methods of extracting flavonoids are organic solvent extraction method, ultrasonic extraction method, microwave method, critical fluid extraction and so on. Jujube contains a variety of chemical components, flavonoids are a major component, flavonoids chemical components have characteristics of protecting liver, relieving spasm, anti-bacterial and anti-inflammatory[13-15]. In this experiment, the total flavonoids were extracted from jujube by reflow extraction method. The method was simple and easy to realize. Through single factor investigation and orthogonal test, it was found that the total flavonoids content in jujube was the highest under the condition of 70 °C, material ratio of 1:40, and extraction of 30 min by 70% ethanol. The content of total flavonoids in the extract of jujube was 1.57% at the wavelength of 510 nm by spectrophotometry and rutin as the standard. The method was evaluated by the methodological study and the method was used to detect the total flavonoids in jujube extract. Jujube as a traditional Chinese medicine or food are very common in our daily life, I believe its medicinal value will have a good development prospects.
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