Study on the best extraction technology of total flavonoids from *Piper sarmentosum* Roxb. leaves and evaluation of antioxidant activity

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Abstract. This paper investigated the best extraction process of total flavonoids from *Piper sarmentosum* Roxb. leaves. The optimal extraction solvent was first determined to be ethanol, and then the effects of ethanol concentration, extraction temperature, extraction time and the ratio of liquid to solid on the extraction yield of total flavonoids were determined by single factor method. Based on the above experiments, the orthogonal experimental design of three factors and three levels was established. The best extraction conditions of total flavonoids from *Piper sarmentosum* Roxb. leaves were concluded: extraction time 1.5h, liquid-solid ratio 175:1, ethanol concentration 75%, extraction time 1.5h, and the effect of solid-liquid ratio was greater than that of ethanol concentration, and the effect of ethanol concentration was greater than that of time. Furthermore, the antioxidant properties of total flavonoids were also determined from *P. sarmentosum* leaves in the regions of Suixi, Lianjiang, and Cunjin based on the DPPH, FRAP and MCC methods.

1. Introduction

*Piper sarmentosum* Roxb., also known in Chinese as ba yanxiang, is an herb that belongs to the *Piperaceae* family and grows in Southeast Asia. The domestic planting areas in China are mainly distributed in Fujian, Guangdong, Hainan, Guangxi, Yunnan, Guizhou, and Tibet. It has been reported that *P. sarmentosum* has important medicinal value, and can be used for the treatment of cough, toothache, stomach pain, bloating, and loss of appetite [1]. *P. sarmentosum* is rich in micronutrients such as calcium, iron, zinc, manganese, and strontium. It has a unique aroma and is also edible [2]. In addition, *P. sarmentosum* has a unique shade-tolerant property which makes it useful as a landscape plant in places where sunshine is inadequate [3].

Studies have shown that *P. sarmentosum* leaves have antioxidant properties, and these antioxidant properties may be related to the flavonoids in the leaves[4]. Chen et al. explored *P. sarmentosum* extracts using three different polar solvents. It was found that the total antioxidant activity of the three extracts was related to the polarity of the extraction reagent. The higher the polarity, the higher the
antioxidant activity. In addition, they also found that *P. sarmentosum* extracts can affect the secretion of inflammatory factors in IPEC-J2 cells, thereby achieving anti-inflammatory effects[5].

Tuntiwachwuttikul *et al.* isolated 16 compounds from fresh *P. sarmentosum*, where aromatic alkenes (1), 1-allyl-2-methoxy-4,5-methylenedioxybenzene (4), β-sitosterol (5), a pyrrole amide (6), sarmentine (10), sarmentosine (13), and pellitorine (14) were isolated from the fruits and leaves of the plant. (+)-Sesamin (2), horsfieldin (3), two pyrrolidine amides (11) and (12), guineensine (15), and brachystamide B (16) were newly discovered chemical compounds. Sarmentamide A, B, and C (7-9) were new natural products. The study found that compounds 1 to 4 and 6 to 16 have antiplasmodial, anti-mycobacterial, and antifungal activities [6].

Research from Piyatida *et al.* found that *P. sarmentosum* has a sensitive allelopathic effect and can play a huge role in the development of bioherbicides[7]. Feng *et al.* studied the herbicidal activity of *P. sarmentosum* extracts by greenhouse seed germination and pot experiments. The results showed that *P. sarmentosum* extracts had strong herbicidal activity against 14 types of weeds, including *Echinochloa crusgalli*. Among them, the extract exhibited the highest herbicidal activity against *Echinochloa crusgalli*, *Amaranthus tricolor*, and *Chloris virgata*. This indicated that *P. sarmentosum* has broad application potential in the development of plant-based herbicides [8].

Niu *et al.* studied the extraction conditions of oleoresin from *P. sarmentosum* with supercritical CO₂ using a single-factor test and an orthogonal test. It was found that the optimal process conditions for supercritical CO₂ fluid extraction of oleoresin from *P. sarmentosum* were a pressure of 20 MPa, an extraction time of 1.5 h, and a temperature of 55 °C. Under these conditions, an extraction yield of 4.19% was achieved [9].

At present, there are many reports on the extraction process of total flavonoids from plants and their antioxidant properties. However, studies on the extraction of total flavonoids from *P. sarmentosum* leaves and their antioxidant properties are relatively few. This may be because of the low yield and small growing area of *P. sarmentosum*. Nevertheless, further exploration of *P. sarmentosum* leaves is needed owing to their good nutritional, ornamental, and medicinal value. Therefore, this paper reports the optimal extraction conditions of total flavonoids from *P. sarmentosum* leaves, as well as the antioxidant properties of total flavonoids in *P. sarmentosum* leaves from different regions.

2. Materials and methods

2.1. Materials

*P. sarmentosum* leaves were collected from Lianjiang region, Suixi region, and the Cunjin Park of Zhanjiang County of Guangdong Province. The leaves were washed, dried, and pulverized, then placed in a plastic bag for storage.

2.2. Methods

2.2.1. Extraction method of total flavonoids of *P. sarmentosum* leaves. A 0.30 g sample of the pulverized *P. sarmentosum* leaves powder was precisely weighed and placed in a 100 mL round-bottom flask, and an appropriate amount of extraction solvent was added. The extraction temperature, extraction time, liquid-solid ratio, and extraction solvent concentration were fixed. The solution was heated and stirred for a specified period of time, and then centrifuged to obtain the flavonoid extract of the *P. sarmentosum* leaves.

2.2.2. Selection of the maximum absorption wavelength of total flavonoids of *P. sarmentosum* leaves. The total flavonoids in the *P. sarmentosum* leaves are expressed in equivalent amounts of rutin. A colorimetric pretreatment experiment was performed according to literature reports using a 15 mL centrifuge tube with 1 mL of 0.2 mg/mL rutin standard solution [10-11]. The resulting mixture was then subjected to a full scan from 400 to 800 nm to determine the maximum absorption wavelength of
rutin, as shown in Figure 1. The maximum absorption wavelength of rutin was 510 nm. Therefore, 510 nm was selected as the optimum wavelength for flavonoid evaluation.

![Figure 1. Selection of Rutin absorption wavelengths](image1)

2.2.3. Measurement of total flavonoids content of P. sarmentosum leaves.

Plotting of the standard curves: The solutions used to generate the standard curve are shown in Table 1. The blank was set to zero, and the absorption value of each solution was measured at 510 nm.

| Table 1. Rutin standard samples |
|--------------------------------|
|                                |
| Blank                          |
| 0.2 mg/mL rutin standard solution (mL) | 0 | 0.25 | 0.50 | 1.00 | 2.00 | 4.00 |
| 5% NaNO₂ (mL)                  | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 10% Al(NO₃)₃ (mL)              | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 4% NaOH (mL)                   | 5 | 5 | 5 | 5 | 5 | 5 |
| 60% ethanol (mL)               | 5.00 | 4.75 | 4.50 | 4.00 | 3.00 | 1.00 |
| Final rutin concentration (mg/mL) | 0 | 0.005 | 0.010 | 0.020 | 0.040 | 0.080 |

![Figure 2. Rutin standard curve](image2)

The absorbances of the different concentrations of the rutin standard solution shown in Table 1 were measured at 510 nm. To plot the standard curves, the rutin concentration (mg/mL) was plotted as the horizontal axis, and the absorbance value was plotted as the vertical axis, as shown in Fig. 2. The linear regression equation for this rutin standard curve is: $Y = 10.3336X - 0.02054$, $R^2 = 0.9977$. Rutin showed a good linear relationship in the range of 0 to 0.08 mg/mL.

Measurement of the total flavonoids content in extracts: The test solution (1.00 mL) was precisely transferred to a 15 mL colorimetric tube and the absorbance measured as above. The average value of the absorbance was substituted into the linear regression equation of the rutin standard curve, and the total flavonoid content in the extract was calculated. The total flavonoids content is:
Total flavonoids yield (mg/g) = \( \frac{c \times V \times n}{w} \)

where: 
- \( c \): results calculated from the standard curve (mg/mL);
- \( V \): total volume of the extract (mL);
- \( n \): dilution factor;
- \( w \): sample weight (g).

2.2.4. Effects of different extraction solvents on the yield of total flavonoids of *P. sarmentosum* Leaves. 

To study the optimal extraction conditions of total flavonoids from *P. sarmentosum* leaves, we first investigated the total flavonoids content of *P. sarmentosum* leaves using three extraction solvents: ethanol, methanol, and water. After the optimal extraction reagent was determined, the extraction solvent with the highest total flavonoids yield was selected as the extraction solvent for subsequent experiments.

According to the method of 2.2.1, 0.30 g of *P. sarmentosum* leaves powder from Lianjiang was weighed, and 30 mL of 60% ethanol, 60% methanol, or distilled water was separately added. The mixtures were stirred at 60 °C for 2 h and centrifuged after the reactions were completed to obtain the extracts. The total flavonoids content in the extracts was determined as described in section 2.2.3.

Figure 3 demonstrates that the yields of total flavonoids extracted by ethanol, methanol, and distilled water were 25.59, 20.12, and 12.51 mg/g, respectively. Therefore, ethanol was selected as the extraction solvent for subsequent experiments.

![Figure 3. Total flavonoids yield of extracts from different solvents](image)

2.2.5. Extraction of total flavonoids of *P. sarmentosum* Leaves. A single-factor experimental design was adopted, and the *P. sarmentosum* leaves collected from the Lianjiang region were used as the materials and the total flavonoids extracted according to the method of 2.2.1.

Effect of ethanol concentration: Five test groups were prepared, and 0.30 g of reserved *P. sarmentosum* leaves powder was accurately weighed for each group. Then, 30 mL of different concentrations of ethanol was added and reacted at 60 °C for 1 h. Each group had three replicates, and the total flavonoids content was determined as described in section 2.2.3.

Effect of extraction time: Four test groups were prepared, and 0.30 g of *Piper sarmentosum* leaves powder was accurately weighed for each group. Then, 30 mL of 70% ethanol was added and reacted at 60 °C for different amounts of time. Each group had three replicates, and the total flavonoids content was determined as described in section 2.2.3.

Effect of liquid-solid ratio: Four test groups were prepared, and 0.30 g of *P. sarmentosum* leaves powder was weighed for each group. Then, 70% ethanol solvent of different volumes was added and reacted at 60 °C for 1 h. Each group had three replicates, and the total flavonoids content was determined as described in section 2.2.3.

Effect of extraction temperature: Four test groups were prepared, and 0.30 g of *P. sarmentosum* leaves powder was weighed for each group. Then, 30 mL of 70% ethanol with a liquid-solid ratio of
100:1 was added and the flavonoids extracted at different temperatures for 1 h. Each group had three replicates, and the total flavonoids content was determined as described in section 2.2.3.

2.2.6. Evaluation of Antioxidant Capacity of Total Flavonoids. Evaluation of DPPH free radical scavenging capacity: According to the method of Hou et al.[12], 0.5 mL of the test solution was added to 2.0 mL of 0.2 mmol/L DPPH in methanol. Then, the solution was mixed, and the absorbance was measured at 515 nm after 60 min of reaction in the dark. The control absorbance was determined by replacing the test solution with 60% ethanol and added to the DPPH in methanol by the same method. The formula for calculating the DPPH free radical scavenging rate is as follows:

\[
\text{Scavenging rate} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100\%
\]

where \(A_0\) is the absorbance of the control at the measurement wavelength; and \(A_1\) is the absorbance of the sample after the DPPH reaction.

Evaluation of Fe\(^{3+}\) reducing power (FRAP): According to the method of Ma et al.[13], a Trolox stock solution was diluted with methanol to prepare different concentrations of Trolox standard solution. In a 37 °C water bath, 20 μL of the standard solution was added to a 5 mL test tube, and then 1 mL of distilled water and 1.8 mL of the freshly prepared TPTZ mixture were added. After 10 min of incubation, the absorbance was measured at 593 nm. The Trolox standard curve is shown in Figure 4. The linear regression equation was obtained as: \(Y = 0.000313X + 0.01039\), \(R^2 = 0.9999\).

![Figure 4. Trolox standard curve](image)

Evaluation of the sample solution and the standard sample: Instead of the standard sample, 20 μL of the sample solution was transferred, and the measurement procedure was the same as above. The total antioxidant capacity of the sample is expressed in μmol/L.

Evaluation of Fe\(^{2+}\) chelating ability (MCC): According to the method of Amarowicz R et al.[14], 1 mL of the sample solution and the blank reagent were accurately transferred into test tubes. Then, 2.8 mL of distilled water, 50 μL of 2 mmol/L FeCl\(_2\) solution, and 150 μL of 2.5 mmol/L Ferrozine solution were added. At 25 °C, the reacting system was first shaken for 30 s, and after standing for 10 min, a colorimetric analysis was performed at 562 nm.

\[
\text{Chelating ability of the sample} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100\%
\]

where \(A_0\): UV absorption of the control; \(A_1\): UV absorption after sample reaction.

3. Results and discussion

3.1. Effects of different factors on the extraction of total flavonoids

3.1.1. Effect of extraction solvent concentration. As shown in Figure 5, the total flavonoids yield increased when the ethanol volume fraction increased from 60% to 70%. When the ethanol concentration increased further, the total flavonoids yield decreased. This may be related to the change in the polarity of the solution. Therefore, the optimal extraction concentration of ethanol is 70%.
3.1.2. **Effect of Extraction Time.** Figure 6 illustrates that the total flavonoids yield first increased and then decreased with the increase of extraction time, and the extraction yield was the highest at 2 h. This may be because the solid phase concentration and the driving force decreased as time increased, and more impurities were extracted. Therefore, the optimal time for extraction of total flavonoids from *P. sarmentosum* leaves is 2 h.

3.1.3. **Effect of liquid-solid ratio.** Figure 7 demonstrates that the total flavonoids yield first increased and then decreased with increasing liquid-solid ratio. When the liquid-solid ratio was 150:1, the total flavonoids content was the highest. Based on the comprehensive experimental results, the 150:1 of liquid-solid ratio was selected.
3.1.4. Effect of extraction temperature. Figure 8 shows that as the temperature increased, the extraction rate of total flavonoids also increased, and the total flavonoids yield was the highest at 70 °C. When the temperature was higher than 70 °C, the total flavonoids content began to gradually decrease. This may be because alcohol-soluble impurities dissolved faster when the temperature was too high and competed with the flavonoids for dissolution in the ethanol-water system. In addition, high temperature also damaged the structure of the flavonoids[15], causing the extraction yield to decrease. Therefore, the optimal temperature for extraction of total flavonoids from *P. sarmentosum* leaves is 70 °C.

![Figure 8. Effect of temperature on total flavonoids](image)

3.2. Orthogonal test results

Based on the single-factor tests, an L₉ orthogonal table (3³) was applied to further optimize the conditions. The specific conditions and levels are shown in Table 2.

| Levels (A) Liquid-solid ratio (B) Ethanol (%) (C) Time (h) | Total flavonoids content (mg/g) |
|---------------------------------------------------------|--------------------------------|
| 1 (A₁) 1 (B₁) 1 (C₁) | 25.04 |
| 2 (A₂) 2 (B₂) 2 (C₂) | 23.12 |
| 3 (A₃) 3 (B₃) 3 (C₃) | 21.61 |

Table 3 shows that the optimal conditions for extracting total flavonoids from *P. sarmentosum* leaves are A₃B₃C₁, and the order of importance of the factors is liquid-solid ratio > ethanol concentration > extraction time. Integrating other conditions, the optimal extraction conditions are a liquid-solid ratio of 175:1, an ethanol concentration of 75%, an extraction time of 1.5 h, and a temperature of 70 °C.

![Table 3. Results of orthogonal experimental design](image)
3.3. Study on the antioxidant activity of total flavonoids of P. Sarmentosum leaves from different regions

Figure 9. Total flavonoids from P. sarmentosum leaves from different regions

Figure 9 shows that under the optimal extraction conditions, the yields of total flavonoids in P. sarmentosum leaves from the regions of Cunjin, Suixi, and Lianjiang were 18.20, 31.02, and 32.87 mg/g, respectively. The total flavonoids content extracted from the P. sarmentosum leaves collected from Cunjin was the lowest, while the total flavonoids content in the P. sarmentosum leaves collected from Lianjiang was slightly higher than the total flavonoids content in the P. sarmentosum leaves of Suixi. This may be because the soil, water, and other conditions are different in different regions, which resulted in different total flavonoids content in the P. sarmentosum leaves. This indicates that the growing environment conditions also have certain effects on the total flavonoids content of the P. sarmentosum leaves.

The scavenging capacity of flavonoids from different regions on free radicals is shown in Figure 10(a). The scavenging ability of the total flavonoids from the P. sarmentosum leaves of the regions of Cunjin, Suixi, and Lianjiang on DPPH free radicals were 42.61%, 88.65%, and 82.78%, respectively. The scavenging capacity of the extract of P. sarmentosum leaves from Suixi was slightly higher than that of Lianjiang.

Figure 10(b) shows that the abilities of P. sarmentosum leaves extracts from the three regions of Cunjin, Suixi, and Lianjiang to reduce iron ions were 0.28, 0.79, and 0.85 μmol/mL, respectively. The reducing power of the total flavonoids of P. sarmentosum leaves from Lianjiang was the highest. However, it was comparable to the P. sarmentosum leaves from Suixi. In summary, the total flavonoids contents in the P. sarmentosum leaves of Suixi and Lianjiang were similar, and their total antioxidant capacities were almost the same. The total flavonoids content of P. sarmentosum leaves from Cunjin was the lowest, and its antioxidant capacity was also the lowest. The total flavonoids content was positively correlated with the total antioxidant capacity (FRAP).
Figure 10. Scavenging ability of DPPH (a) and reducing power of Fe³⁺ (b)

Figure 11. Chelating ability of Fe²⁺

Figure 11 shows that the ferrous ion chelating ability of *P. sarmentosum* leaves extracts from the regions of Cunjin, Suixi, and Lianjiang was 53.0%, 70.3%, and 65.4%, respectively. This indicated that the total flavonoids of *P. sarmentosum* leaves had some chelating ability to ferrous ions, but the chelating abilities of total flavonoids extracts of *P. sarmentosum* leaves from different regions were not the same.

4. Conclusion

The samples were collected from Lianjiang region, Suixi region, and the Cunjin Park of Zhanjiang County of Guangdong Province. This study primarily determined the optimal extraction conditions of total flavonoids from *P. sarmentosum* leaves and the antioxidant properties of the total flavonoids in *P. sarmentosum* leaves from the three different regions.

The optimal extraction conditions of total flavonoids from *P. sarmentosum* leaves, obtained by single-factor experiments and orthogonal experiments, are a liquid-solid ratio of 175:1, an ethanol concentration of 75%, an extraction time of 1.5 h, and a temperature of 70 °C. At these conditions, the measured values of total flavonoids content in the regions of Cunjin, Suixi, and Lianjiang are 18.20, 31.02, and 32.87 mg/g, respectively.

The antioxidant activities of the total flavonoids of *P. sarmentosum* leaves collected in the three regions of Suixi, Lianjiang, and Cunjin were evaluated by DPPH free radical scavenging, MCC, and FRAP methods. The results demonstrated that flavonoids in *P. sarmentosum* leaves of different regions have good antioxidant properties. The antioxidant properties of *P. sarmentosum* leaves of Suixi and Lianjiang are better than that of the *P. sarmentosum* leaves of Cunjin, and the total flavonoids content in the three regions positively correlates with FRAP.

This study illustrates that the total flavonoids content in *P. sarmentosum* leaves from different growth regions is different, which may be related to the local growing conditions. The results provide an theoretical basis for the efficient use of *P. sarmentosum* leaves.
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References
[1] Fu, L.G., Chen, T.Q., Lang, K.Y., et al. (2000) Higher Plants of China (Volume Vol. 3). Qingdao Publishing, Qingdao.
[2] Cheng, C.F., Miao, X.R., Li J.W. (2018) Development and utilization of Piper sar-mentosum Roxb. leaves series dishes. Xiandai Horticulture, 1: 37-38.
[3] Chen, H.M., Zhang, Y. (2018) Discussion on the Piper sar-mentosum Roxb. Multi-purpose shaded ground cover plants. Xiandai Horticulture, 10: 20-21.
[4] Lin, Z. (2018) Study on antioxidant activity of Piper sarmentosum Roxb. leaves extract. Journal of Food Safety and Quality, 9(12): 3135-3141.
[5] Chen, C.W. (2019) Study on antioxidant and anti-inflammatory effects of extracts from Piper sarmentosum Roxb. in vitro. China Animal Husbandry & Veterinary Medicine, 3: 677-683.
[6] Tuntiwachwuttikul, P., Phansa, P., On, Y., et al. (2006) Chemical Constituents of the roots of Piper Sarmentosum. Chemical and Pharmaceutical Bulletin., 54: 149-151.
[7] Pukclai, P., Kato-Noguchi, H. (2011) Allelopathic activity of Piper sarmentosum Roxb. Asian Journal of Plant Sciences, 10: 147-152.
[8] Feng, G. (2017) Herbicidal activity of extract of Piper sarmentosum. Chinese Journal of Tropical Crops, 10: 1811-1814.
[9] Niu, D.B. (2016) Study on the optimum extraction technology of oleoresin from Piper sarmentosum Roxb. with supercritical CO2 fluid. Science and Technology of Food Industry, 4: 34-37.
[10] Wu, T.N. (2014) Method for determining the flavonoids content. China Foreign Medical Treatment, 24: 197-198.
[11] Ma, T.T. (2007) Method for determining the total flavonoids content of traditional chinese medicine. Anhui Medical and Pharmaceutical Journal, 11(11): 1030-1032.
[12] Hou, X.L., Liu, J.Q., Gao, H., et al. Extraction and antioxidant activity of total phenol from black garlic. Modern Food Science & Technology, 11: 158-162.
[13] Ma, H., Ru, X., Wang, J., Zhao, L.M., Wang, S. (2019) Study on in vitro antioxidant activity of four types of tea aqueous extracts and tea polyphenols. Food Research and Development, 40(8): 65-70.
[14] Amarowicz, R., Troszyńska, A., Baryłko-Pikielna, N., et al. (2004) Polyphenolics extracts from legume seeds: correlations between total antioxidant activity, total phenolics content, tannins content and astringency. Journal of Food Lipids, 11(4): 278-286.
[15] Huang, C.C. (2012) Extraction, separation, and partial characterization of flavonoids from Camellia Oleifera L. and Camellia Seed Tea. Anhui Agricultural University, Hefei, 1-64.