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Full Length Research Paper

Applying silica gel column chromatography purify resveratrol from extracts of *Morus alba L.* Leaf

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The resveratrol contained in mulberry leaves has manifold health care functions. In the experiment, resveratrol was extracted from mulberry leaf using a reflux extraction method of the organic solvent, then resveratrol was purified by silica gel column chromatography and determined by high performance liquid chromatography after mulberry leaf was extracted, filtered, and vacuum concentrated. The optimum extraction conditions were as follows: material quality concentration: 1:13, extraction temperature: 60°C, extraction time: 105 min, and concentration of ethanol: 80%. At these conditions, the yield of resveratrol was 0.0225%. Through the purity tests, mix chloroform and methanol in the ratio of 10:1 as the eluting solvent, the purified resveratrol by the silica gel column chromatography can be advanced from 0.8 to 99.3%.

Key words: Resveratrol, mulberry leaf, extraction technology, silica gel column chromatography, high performance liquid chromatography (HPLC).

INTRODUCTION

Mulberry (*Morus alba* L.) belonging to moraceae family, is a perennial deciduous plant. The Chinese Ministry of Health declared that mulberry leaves are edible and medicinal. They have high nutritional and medical values, and they contain active ingredients, such as polysaccharides, alkaloids, peptides, flavonoids, polyphenols, and so on. Various parts of the mulberry are used as medicine in the countries of China, Japan, and Korea to treat diabetes, paralytic stroke, and beriberi (Kim et al., 2003). However, the total area available for mulberry cultivation is decreasing, and mulberry trees are susceptible to frost damage (Lee et al., 2011). According to Shennong's Herbal, one of the world's earliest pharmacopeia, mulberry leaves, characterized by a sweet and bitter taste and are cold resistant, prevent obesity, soothe the liver, and improve eyesight (Zhao et al., 2008; Nair et al., 2004). They are also used in traditional medicine as a treatment for diabetes. Resveratrol (trans-3,4′,5-trihyrdoxystilbene) is a natural compound that is present in various plant species and is relatively abundant in the root of *Polygonum cuspidatum*. It has many biological and pharmaceutical properties (Gu et al., 2000; Clément et al., 1998; Bhat et al., 2001), and the most biologically active isomer is trans-resveratrol. Primary research on this substance began with the observation that a low incidence of cardiovascular diseases may co-exist with a high-fat diet and moderate consumption of red wine (it is known as the French paradox) (De and Villegas, 2007). When resveratrol was first extracted from the root of *Veratrum grandiflorum* in the 1940s, it started to gain attention because of its good biological action (Ferrero et al., 1998). Resveratrol can inhibit cancer, lower descendant blood fat, inhibit platelet

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aggregation, regulate the metabolism of lipoprotein, and protect the liver and other organs from lack of blood. It is an anti-oxidant that serves as a defense against HIV, bacteriostasis, and so on (Meng et al., 2002; Wang et al., 2003; Chen et al., 2005; Xu et al., 2008). In recent research, the percentage of apoptotic cells induced by resveratrol alone was higher in the group of patients with better prognostic markers than in those with worse prognostic markers (Monika et al., 2010). At present, natural resveratrol is extracted from Rhizoma *Polygoni cuspidati* (Polygonaceae) and grape skin (Vitaceae), which are limited raw materials. At present, mulberry is cultivated as forage in most districts of China, which means it is only utilized to raise silkworm, whereas many of its parts are not used. Therefore, examination and purification of resveratrol from mulberry is significant (Xiang et al., 2003; He et al., 2004; Zhang et al., 2004; Li et al., 2005). Breakthrough from research on mulberry plants will not only address the problem of producing resveratrol from raw materials but also have considerable social and economic benefits.

MATERIALS AND METHODS

Chemicals and methods

Rotary evaporator as an extracting device, high performance liquid chromatograph (HPLC) (Agilent 1100), water bath, mulberry leaves, ethanol (analytical grade), ethyl acetate (analytical grade), acetone (analytical grade), methanol (analytical grade), methanol (HPLC grade), and standard resveratrol (Sigma 99%) were used in the experiment. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

HPLC method for the determination of resveratrol

The resveratrol content was determined by high-performance liquid chromatography according to Zhao's method and was improved (Zhao et al., 2010). The chromatographic separation was achieved with an ODS chromatographic column (4.6×250 mm, 5 mm, Hypersil) operating at 35℃ and wavelength is 298 nm. The mobile phase used was methanol /deionized water, 6.4: 3.6 (v/v) at a flow rate of 1 ml/min, and the injection volume was 20 µl.

To make a standard curve, 0.02 g of standard resveratrol sample was weighed and placed in a 50 ml brown volumetric flask, methanol solution was added to scale, followed by regular shaking, then got the storing liquid was 400 mg/L; diluted the stock liquid to 200, 100, 300, and 400 mg/L standard solutions separately. A 20 ul sample was taken to test the peak area. A standard curve is drawn where the ordinate represents the peak area, and the abscissa represents the mass concentration of resveratrol. To determine resveratrol in the samples, 20 ul of the extracted solution after centrifugation was injected through a HPLC separations module. Resveratrol identification was made by comparing the relative retention times of sample peaks with standards. The chromatography of the extracted samples is shown in Figure 3. The solvent concentration of ethanol is 80%, the solid to liquid ratio is 1:15, the temperature is 60℃, and the extraction time is 90 min. For the test of precision and stability of the HPLC method, five replicate standard solutions of resveratrol were continuously injected in a day. The same standard sample was injected five times at different times of 0, 2, 4, 6, and 8 h, then RSD was calculated according to the peak area. For the test of repeatability, 10 g of the mulberry powder sample was placed in a round-bottom flask to which 100 ml of 80% ethanol was added in a 55℃ water bath for a 90 min reflux extraction.

The filtered solution was extracted and placed in a vacuum rotary evaporator to concentrate the crude extract, which includes resveratrol and other impurities. After the crude extract was dissolved in a 10 ml methanol solution, ultrasonically treated for 10 min, and stored in a refrigerator for 2 days, the extracted solution was collected after a 10 min centrifugation. For the test of sample recovery, standard samples in the same batch of sample solutions with known concentrations were added. Resveratrol was determined using HPLC.

Effects of different extract conditions on the extraction rate of resveratrol

Effects of different extraction solvents on the extraction rate: Ten grams of mulberry leaf powder were weighed and added up to four round-bottom flasks to which 150 ml ethanol, ethyl acetate, methanol, acetone extraction and ethanol, and methanol were added, with an acetone concentration of 80%, respectively. The solution was extracted once with a water bath temperature of 60℃ and an extraction time of 90 min. The extracted solution was collected after the crude extract, filtered and centrifuged. The extracted solution was stored in a refrigerator after dissolving it in a 10 ml methanol solution. HPLC was then used to determine the content of resveratrol.

Effects of different ethanol concentrations on the extraction rate: ten grams of mulberry leaf powder were weighed and added into, up to five round-bottom flasks. The solution was extracted once at an extraction time of 90 min and an extraction temperature of 60℃. The ethanol concentrations are 50, 60, 70, 80, and 90%. After the extract has been vacuum filtered, it was dissolved with 10 ml methanol and then centrifuged. Effects of different material qualities on the extraction rate: Ten grams of mulberry leaf powder were weighed and added into, up to five round-bottom flasks. The solution was extracted once at an extraction time of 90 min and an extraction temperature of 60℃. The ethanol concentration is 80%. The different ethanol volumes are 90, 110, 130, 150, and 170 ml. After the extract had been vacuum filtered, it was dissolved with 10 ml methanol and then centrifuged.

Effects of different temperatures on the extraction rate: Ten grams of mulberry leaf powder were weighed and added into up to five round-bottom flasks to which 150 ml of ethanol extract was added. The ethanol concentration was 80%, the extraction time was 90 min, and the extraction temperatures were 40, 50, 60, 70, and 80℃. After the extract had been vacuum filtered, it was dissolved with 10 ml methanol and then centrifuged. Effects of different extraction times on the extraction rate: Ten grams of mulberry leaf powder was weighed and added into up to five round-bottom flasks at extraction times of 30, 45, 60, 75, and 90 min. The extraction temperature was 60℃, and the alcohol volume was 150 ml. After the extract had been vacuum filtered, it was dissolved with 10 ml methanol and then centrifuged.

Preparation of the adsorbent and the column

Weight of 40 g of Silica Gel G and heated at 105℃ for 30 min, then it was hydrophobized by adding 100 ml chloroform, continuously heated for 20 min at 180℃ in a vacuum drying oven. Pack prepared Silica Gel G into the column (inside diameter/length=15/20) by wet packing method (Nakanishi et al., 1998).

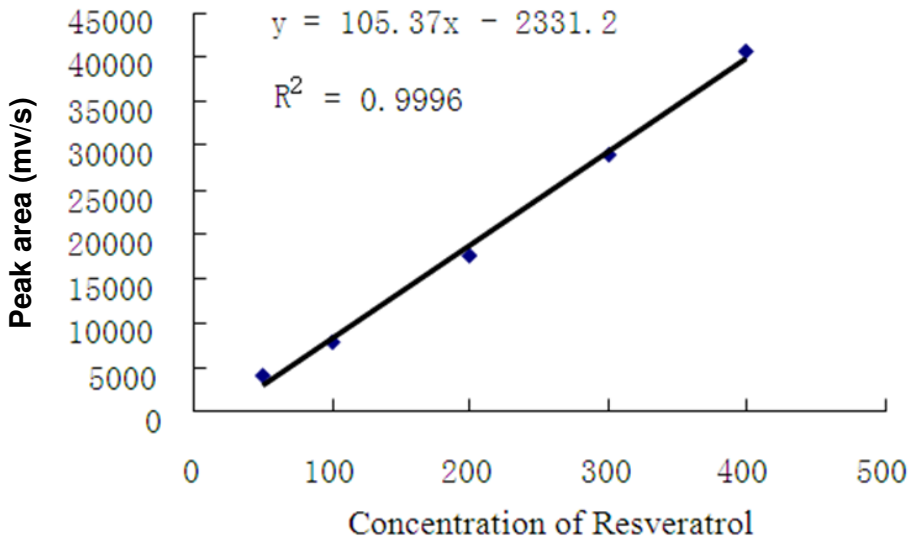

Figure 1. Standard curve graph.

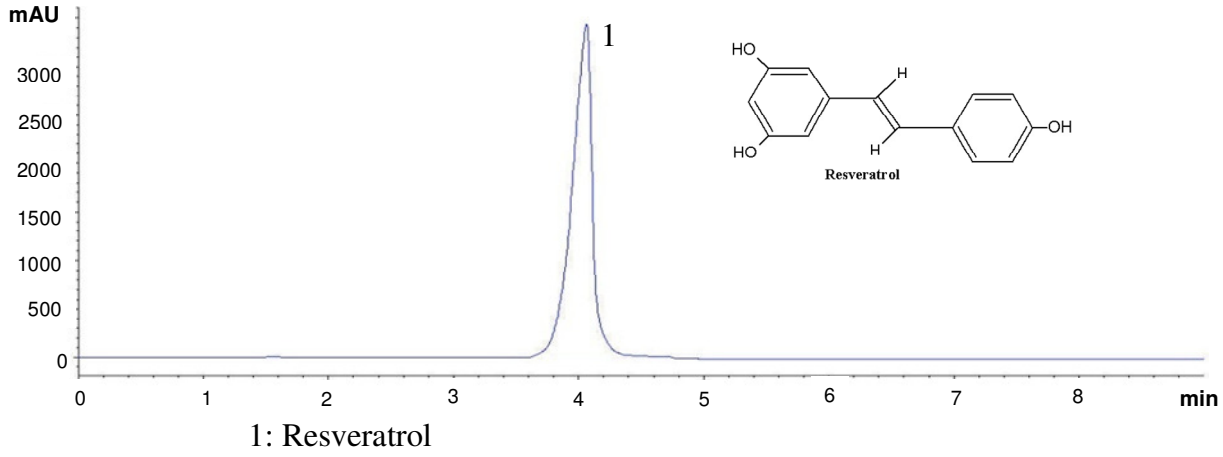

Figure 2. HPLC chromatogram of the resveratrol standard sample.

Treatment of the column

After loading 40 ml extracts on the column, the column was washed with 600 ml eluent (chloroform: methanol=10:1) at a rate of 2 ml/min, gathered the eluate 10 ml/bottle. After vaporizing the eluate, the remainder was dissolved in 10 ml 50% methanol, then the samples were injected into HPLC. In the end, the 80 samples gained all together were marked from 1 to 60.

RESULTS AND DISCUSSION

Determining the results of resveratrol with HPLC

As shown in Figure 1, the peak area and the concentration have a good linear relation. Figure 2 is the HPLC chromatogram of a resveratrol standard sample. The retention time of resveratrol was 4.059 min. Determination of resveratrol in the samples: Figure 3 is the HPLC chromatogram of mulberry extract. It has a very high absorption peak at 4.030 min, and its retention time is similar to that of the resveratrol standard sample.

The peak area is 21200.6. Using external standard method, the resveratrol content is determined to be 224.324 mg/l, and the percentage of resveratrol in dried mulberry leaf is 0.0224%. Experimental result on precision, stability, repeatability and sample recovery: Table 1 shows a good result of the precision and stability test in the resveratrol experiment, with an RSD of 0.51%. The results in Table 2 indicate that no significant change in the peak area occurred in 8 h, and the RSD is 0.57%. According to repeatability test, the results are shown in Table 3 and the RSD of the HPLC method is 0.49%. According to sample recovery test, the average recovery of sample is 98.02% and RSD is 0.57% (Table 3).

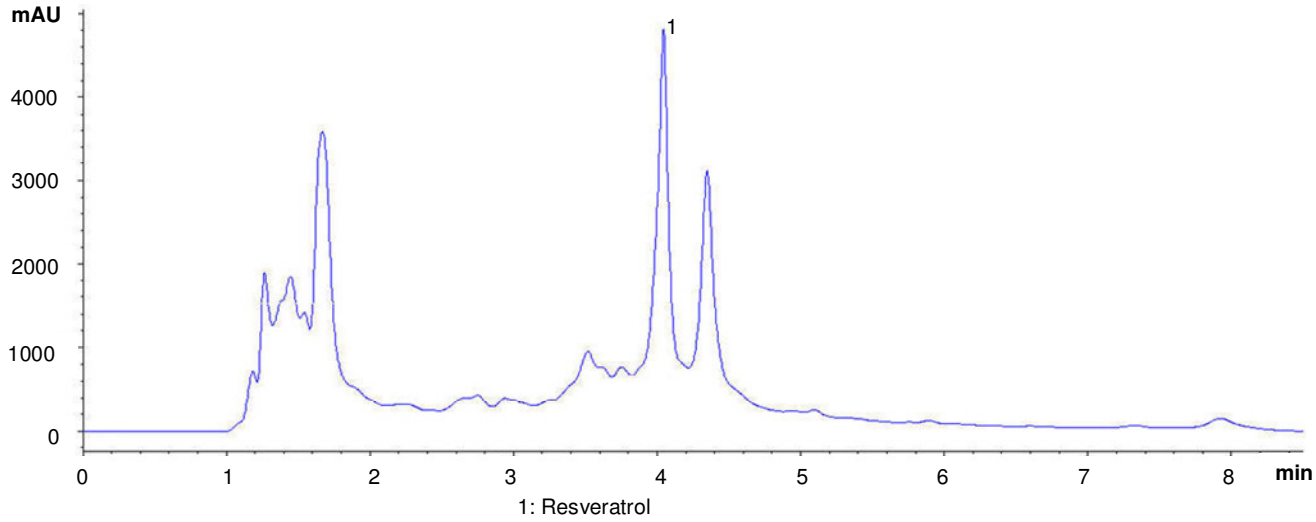

Figure 3. HPLC chromatogram of mulberry extract.

Table 1. Experimental result on precision in HPLC method for the determination of resveratrol.

Test number	1	2	3	4	5	RSD (%)
Area (mv.s)	38963.91	38852.50	38664.89	38576.46	38402.18	0.51

Table 2. The experimental result of stability in HPLC method for the determination of resveratrol.

Time (h)	0	2	4	6	8	RSD %
Peak area (mv.s)	38748.49	38961.04	38698.30	38529.05	38703.65	0.57

Table 3. Experimental result of sample recovery in HPLC method for the determination of resveratrol.

Sample number	1	2	3	4	5
Added mount (ml)	0.4	0.45	0.5	0.55	0.6
Peak area (mv.s)	33950	35416	36706	38767	40375
Percent recovery	98.54	97.98	97.09	98.22	98.29

Average recovery 98.02%; RSD 0.57%

Table 4. Effects of different agents on resveratrol extraction.

Different solvents	80% Ethanol	80% Carbinol	80% Acetone	Ethyl acetate
Peak area (mv.s)	21200.6	23625.8	25500.4	2567.2
Content (%)	0.0224	0.0247	0.0265	0.0048

Effects of different extraction conditions on the extraction rate of resveratrol

The results on the effects of different organic extraction solvents on the extraction of resveratrol are shown in Table 4. Various solvents have different effects on the extraction of resveratrol. Considering that the toxicity and volatility of ethanol are hygeian, we chose the safe,

Table 5. Effects of different concentrations of alcohol on resveratrol extraction.

Organic solvent	50% Ethanol	60% Ethanol	70% Ethanol	80% Ethanol	90% Ethanol
Peak area (mv.s)	12145.2	15643.5	18540.4	21200.6	21476.5
Content (%)	0.0139	0.0172	0.0199	0.0224	0.0227

Table 6. Effects of different material fluid ratios on resveratrol extraction.

RTW	1:9	1:11	1:13	1:15	1:17
Peak area (mv.s)	19796.4	20762.6	21200.6	21293.1	21356.3
Content (%)	0.0211	0.0220	0.0224	0.0225	0.0226

Table 7. Effects of different temperatures on resveratrol extraction.

Temperature	40℃	50℃	60℃	70℃	80℃
Peak area (mv.s)	7985.4	18144.7	19165.4	21200.6	21497.5
Content (%)	0.0194	0.0196	0.0205	0.0224	0.0227

Table 8. Effects of different extraction times on resveratrol extraction.

Time	45 min	60 min	75 min	90 min	105 min
Peak area (mv.s)	14325.9	17036.6	19565.4	21200.6	21297.5
Content (%)	0.0159	0.0185	0.0209	0.0224	0.0225

Table 9. Factors and levels of the orthogonal test.

Factor level	Ethanol concentration (%) (A)	Solid to liquid ratio (B)	Temperature (℃)	Extraction time (min) (D)
1	70	1:13	50	75
2	80	1:15	60	90
3	90	1:17	70	105

innocuous, easy-recovery ethanol as extraction solvent rather than acetone and methanol. The results on the effects of different concentrations of ethanol on the extraction of resveratrol are shown in Table 5. The extraction rate was increased with an increase in the concentration of ethanol. When the volume fraction of ethanol exceeded 80%, there was no obvious increase in the extraction rate. Therefore, we chose 70, 80, and 90% ethanol concentrations for the three-level orthogonal test.

The results on the effects of different liquid ratios on the extraction of resveratrol are shown in Table 6. The extraction rate of resveratrol increased with an increase in the ratio of solid to liquid. However, the effect of liquid ratios was not noticeable. Therefore, we chose 1:13, 1:15, and 1:17 for the three-level orthogonal test. The results on the effects of different temperatures on the extraction of resveratrol are shown in Table 7. The extraction rate of resveratrol increased along with an increase in temperature. However, the effect was not noticeable when the temperature was higher than 70℃. Therefore, we chose 50, 60, and 70℃ for the three-level orthogonal test. The results on the effects of different extraction times on the extraction of resveratrol are shown in Table 8. The extraction rate of resveratrol increased along with an increase in extraction time. However, when the extraction time was greater than 90 min, the increase was not noticeable. Therefore, we chose 75, 90, and 105 min for the three-level orthogonal test.

Effects of the orthogonal experiment

To ensure the accuracy and usefulness of the experiment, an orthogonal of four factors and three levels was developed. Integrated single-factor test results showed that four factors greatly affect resveratrol extracting yield: ethyl alcohol concentration, solid

Table 10. Orthogonal design and result of the extraction of resveratrol.

Test number	Concentration of ethanol (%)	Solid to liquid ratio	Temperature (℃)	Extraction time (min)	Peak area (mv.s)
	A	B	C	D	
1	1	1	1	1	13057.4
2	1	2	2	2	17086.7
3	1	3	3	3	17986.3
4	2	1	2	3	21223.5
5	2	2	3	1	18343.1
6	2	3	1	2	19032.9
7	3	1	3	2	21879.5
8	3	2	1	3	20542.6
9	3	3	2	1	18946.2
R_1	16043.5	18720.0	17544.3	16782.2	
R_2	19533.1	18657.5	19085.4	19333.0	
R_3	20456.1	18655.1	19402.9	19917.3	
R	4412.6	64.9	1858.6	3135.1	

to liquid ratio, extraction temperature, and extraction time. Then four factors three-level orthogonal test was performed using the four factors according to $L_9(3^4)$ table (Table 9), the levels of the table are as follows: the ethanol concentrations (A) are 70, 80, and 90%; the material ratios (B) are 1:13, 1:15, and 1:17; the temperatures (℃) are 50, 60, and 70℃; and the extraction times (D) are 75, 90, and 105 min. In the corresponding experimental conditions, 10 g of mulberry leaf powder was weighed for extraction, and then concentrated with a vacuum rotary evaporator after filtering and collecting the extracted solution. Finally, the concentrated solution was dissolved with 10 ml methanol and stored in a refrigerator before using HPLC to determine resveratrol content. The result of orthogonal test are shown in Table 10. From Table 10, in the reflux extraction of mulberry leaf, the most significant factor for the content of resveratrol is ethanol concentration, followed by extraction time and temperature, whereas the ratio of solid to liquid is not significant. The optimum extraction conditions can be set to $A_3B_1C_3D_2$. However, taking into account the effect of 80 and 90% ethanol, we chose to use $A_2B_1C_2D_3$ in which the ethanol concentration is 80%, the solid to liquid ratio is 1:13, the extraction temperature is 60℃, and the extraction time is 105 min. Under these conditions, the extraction of resveratrol extract was 0.0225% from dried leaf.

Purification of resveratrol from the extracts

Chromatography on silica is a conventional preparative separation method for resveratrol and polydatin. From Figure 3 we can see resveratrol has been a very good separation, and give a higher sensitivity compare Fulvio (1993). Despite applying silica gel column chromatography, purified polyphenols are the best choice, but so far are not seen in the separation of Mulberry extracts. Figure 3 gives us a good example; we obtained high-purity resveratrol by applying silica gel column chromatography. Through analysis of 60 samples with HPLC, it was shown that there was no resveratrol from sample 1 to 33, the resveratrol was increased continuously after sample 33.

No resveratrol existed in the last 15 samples, it indicated that all resveratrol were washed out. Representative HPLC chromatograms of the resveratrol in the samples are shown in Figure 3 and 4. The content of resveratrol was calculated from the corresponding calibration. It showed resveratrol was 41.5 mg in 200 g leaf of *Morus alba L.* and its improved from 0.8 to 99.3%. Compared with the published papers, Gu have reported adsorption chromatography on Superose 12, a cross-linked 12% agarose gel, was successfully used for the purification of the polyphenol resveratrol and its glycoside (polydatin) in one step directly from a crude root extract of the herb *P. cuspidatum* (Gu et al., 2006). In Delaunay's research, high purity resveratrol

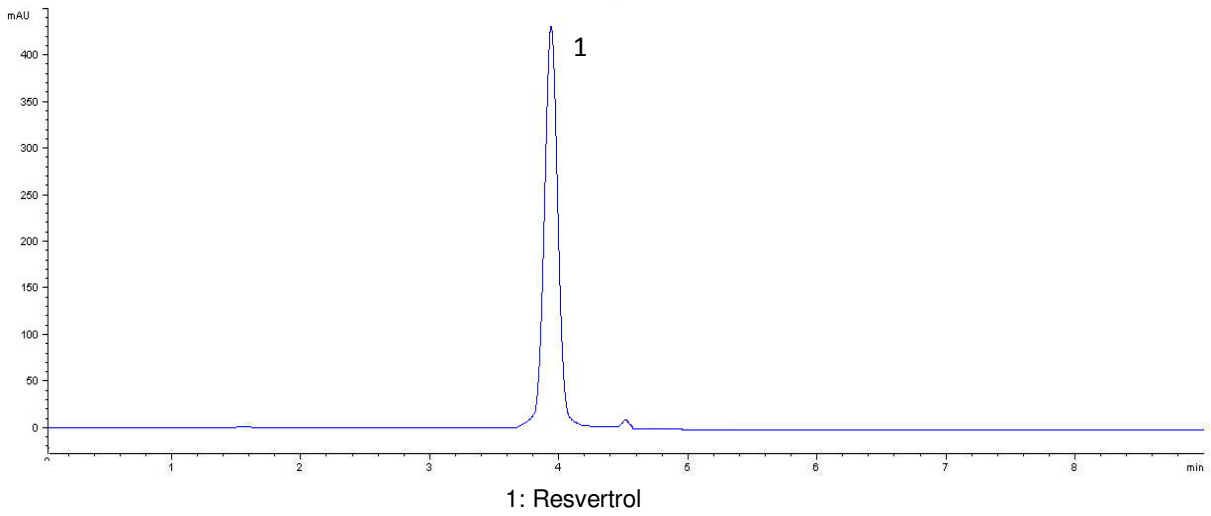

Figure 4. Representative HPLC chromatograms of the sample 36.

was obtained using centrifugal partition chromatography (CPC) in tertiary two-phase solvent systems and high-speed counter–current chromatography (HSCCC) was used for the separation and purification of resveratrol and polydatin (Delaunay et al., 2002). High performance thin layer chromatography (HPTLC) has been applied for rapid quantitative analysis of emodin, resveratrol and polydatin in crude extracts of the rhizome of *P. cuspidatum* (Zhao et al., 2005). The major disadvantage of CPC, HSCCC and HPTLC, in comparison to traditional column liquid chromatography, is the difficulty of scaling-up.

Conclusion

In this study, the simple and reliable method of HPLC was used for the determination of resveratrol content in mulberry leaves extract. Four factors affecting the active ingredients during refluxing extraction process were determined using the integrated single-factor test. These four factors were ethanol concentration, solid to liquid ratio, extraction temperature, and extraction time.

Finally, the optimized extraction conditions of the orthogonal design were used to determine the following extraction conditions: ethanol concentration of 80%, solid to liquid ratio of 1:13, extraction temperature of 60°C, and extraction time of 105 min. After mulberry leaf was extracted, filtered, and vacuum concentrated, the resveratrol was purified by silica gel column chromatography, the purified resveratrol by the silica gel column chromatography can be advanced from 0.8 to 99.3%.

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