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Vol. 9(20), pp. 620-628, 25 May, 2015
DOI: 10.5897/JMPR2011.1783
Article Number: B8DAEF153489
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Refining the methods of extraction and purification of apigenin from *Viola yedoensis* makino

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Received 24 December, 2011; Accepted 18 May, 2015

In this study, the effects of supercritical CO_2 extraction and macroporous adsorbent resin on separation and purification of apigenin were compared. A better separation and purification macroporous adsorbent resin was selected according to static adsorption and desorption results. Silica gel column chromatography and LSA-10 macroporous adsorbent resin performed better in the separation and purification procedure. The purity of apigenin could reach 92.8% after separation and purification. The optimal supercritical CO_2 extraction conditions of apigenin from *Viola yedoensis* makino are solid to liquid ratio: 5:3, extraction pressure: 20 MPa, extraction temperature: 55°C, extraction time: 2 h, separation vessel temperature: 55°C, pressure: 9 Mpa, extraction vessel temperature: 40°C and pressure：5 MPa. Supercritical CO_2 extraction had a great effect on the separation and purification of apigenin from *V. yedoensis* makino; the purity could reach 97.6%. Assaying apigenin by high performance liquid chromatography (HPLC) showed that the sample volume and peak area of the response have a good linear relationship, r = 0.9996, between 1.58 to 15.80. The results indicated that the procedure of separation and purification of apigenin from *V. yedoensis* by supercritical CO_2 extraction, which is better than that of macroporous adsorbent resin, is suitable for the separation and purification of high purity apigenin.

Key words: Extraction, purification, macroporous adsorbent resin, supercritical CO_2 extraction.

INTRODUCTION

Viola yedoensis makino, also known as *Porella Viola*, *Corydalis dishes*, *Lepidium Tiger*, *Purple Corydalis*, *Orychophragmus*, and *Brachybotrys*, belongs to the violaceae family, which consists of rosette-like perennial herbs with 7 to 14 cm high, non-ground stems Institute of Chinese academy of sciences (ICAS, 1987, Ren, 1986; The Chinese herbal medicine assembly, 1978; Du, 1996). The underground stem is very short, while the main root is coarse and the whole-plant is covered with short white feathers. *V. yedoensis* makino contains 29.27 g of protein, 2.38 g of soluble sugar, 33.95 mg of amino acids and a variety of vitamins per 100 g of dry matter. Therefore, when the whole plant has been dried so it can be used as a

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medicine, it has been characterized in Chinese as being "cold, bitter pungent and nontoxic".

The plant has been used for clearing away heat and toxic materials as well as for the soothing and cooling of swellings. It has also been used for curing carbuncle sores, furuncle, erysipelas, phlegmon, poisonous snakebites, icterus and urinary tract infections. It has been praised as a drug for treating abscesses resulting from pyogenic infection and furunculosis. “Materia Medica justice" recognizes that the plant provided a useful generic drug against carbuncle swelling and boil poison (Drug administration department of the People's Republic of China, 2000; Zhuang, 2002; Wen et al., 2006).

It was reported in the current paper that macroporous adsorbent resin or a silica gel extraction column was used in the separation and purification of the apigenin (Zhu et al., 2005; Dawa et al., 2008; Zhu et al., 2005; Li et al., 2007; Zhang et al., 2004).. The majority of apigenin in the violaceae viola exist as various kinds of glycosides. In order to achieve the purpose of separating the impurities and thereby purifying the apigenin, so that it retains as much of its physiological activity as possible, it is necessary to find the most economical and efficient way of separating and purifying the apigenin from the violaceae viola family. In this study, the methods of continuous separation, followed by purification with macroporous resin (Zhang et al., 2002), silica gel extraction column and the supercritical CO_2 fluid extraction after solvent extraction crystal were studied.

MATERIALS AND METHODS

Reagents

V. yedoensis makino was collected from the Wuling Mountains, parched at the temperature of 60°C, crushed through a 40 μm mesh sieve and dried to a constant weight. Extra material was kept under seal as backup. Methanol, acetic acid, ethyl acetate, petroleum ether, acetone, chloroform, ethanol, 5% HCl, 2% NaOH, phosphoric acid (all reagents were Domestic AR), acetonitrile (HPLC) and different concentrations of methanol all have various volume ratios. Silica gel (Qingdao drier factory Marine Chemical Group, used for column chromatography), D-101 macroporous resin (Pesticide Factory Tianjin), LSA-10, LSA-21 (lan xiao Technology Co., Ltd.xi an, China), the remaining macroporous adsorbent resin was provided by Forest Key Laboratory of Chemical Engineering, Hunan Province, China. Apigenin standard reference samples were obtained from JF natural products research and development of Tianjin Co, Ltd, purity 98%. Apigenin purified samples (after extraction, separation, purification from the *V. yedoensis* Makino) were prepared as described in the study.

Apparatus

HA121-50-02 Supercritical CO_2 extraction instrument (Jiangsu，China), UV-Lamba25 Perkin-Elmer (U.S.), LC-20A high-performance liquid chromatography (HPLC) Shimadzu (Japan), LD5-2A centrifuge (Beijing Medical), JY98-Ⅲ DN Cell Ultrasonic grinder, AEB-220 electronic balance Shimadzu (Japan), AEL-200 one hundred thousandth electronic balance Shimadzu (Japan), RE540-AW Rotary Evaporator Shimadzu (Japan) and DP-32 Vacuum Drying Oven Yamato. (Japan) were used in the study

Experimental methods

Determination of the apigenin standard absorption wavelength and measurement of the standard curve using UV Absorbance

5.55 mg of an apigenin standard was measured and dissolved in 70% heated methanol. The solution was measured in a 100 ml volumetric flask. Two drops of acetic acid solution was added (hereafter, the same amount will be added), dubbed in 55.5 μg / ml solution for backup. Apigenin standard solution was added into the volumetric flask as shown in Table 2. First, 5 ml solution from No. 4 was scanned using the UV spectrophotometer at 800 to 200 nm. The maximum UV absorption peak obtained from the scan was 345 nm. Therefore, a 345 nm wavelength was chosen for detection

Research to determine better high performance liquid chromatography (HPLC) chromatographic conditions and measurment of an apigenin standard curve

The chromatographic column used was the Kromasil C18 column (250X4.6 mm i.d, 5μm); the column temperature was room temperature; mobile phase was acetic acid: methanol: acetonitrile: phosphoric acid: water = 10:200:100:10:200 v: v; flow rate was 0.60 mL / min; detection wavelength was 350 nm. 18.9 ml of a 55.5 μg / ml Apigenin standard solution was added into a 50ml volumetric flask dubbed into 21 μg / ml solution. Apigenin standard solution was detected by HPLC and the regression equation was calculated as described in Table 3.

Research to determine better techniques for supercritical CO_2 extraction of apigenin from Viola Philippica

Selection of the better entrainer: Five kinds of reagents: industrial alcohol, 80% ethanol, anhydrous ethanol, 70% methanol and methanol were used as entrainer. The usable entrainers were selected when the extraction pressure was 25 MPa, extraction temperature was 40°C, separation vessel pressure was 7 MPa, temperature of separation vessel was 40°C, separation vessel pressure was 4 MPa, temperature of separation vessel was 35°C, CO_2 flow rate was 20 L / h under the extraction time of 2 h, and the material which was not added as entrainer was used as controls.

Selection of the better supercritical CO_2 extraction parameter of apigenin: The entrainers were chosen as shown in Table 1. The three level orthogonal experiment was designed of $L_9(3^4)$. By examining the impacts between the supercritical CO_2 extraction technical parameters and the yields of apigenin, the best supercritical CO_2 extraction parameter of apigenin was determined.

Supercritical CO_2 extraction of apigenin in *V. yedoensis* makino: According to item 2.3.2, the parameters were selected for the supercritical CO_2 extraction process of apigenin from *V. yedoensis* makino. 500 g of *V. yedoensis* makino was added in 2 L of the barrel, loaded and extracted in the extraction vessel according to the selected conditions of supercritical CO_2. The extract was collected from the discharge port of the extraction vessel, extracts were dissolved in 70% heated methanol of constant volume in a 100 ml volumetric flask. Then 20μl of solution was tested by HPLC according to 2.2 of the high-performance liquid chromatography conditions.

Table 1. Orthogonal factor levels.

Factor	1	2	3
Amount of entrainer (solid to liquid ration)	5:01	5:02	5:03
Pressure of the extraction vessel (MPa)	20	28	35
Extraction vessel temperatre (°C)	35	45	55
Extraction time (h)	1	2	3

Studies on the separation and purification technique of apigenin in V. yedoensis makino with macroporous adsorption resin and silica gel column chromatography

Preparation of extract: 500.0 g of the *V. yedoensis* makino powder was weighed and added to 5000 ml of a 70% methanol solution, and then it was processed by ultrasonic for 10 min under 50°C and centrifuged for 10 min (3000 r / min) after being extracted for 30 min. The sediment was extracted once again, before the supernatant fluid was combined.

The solvent extraction of extract solution: The extract of *V. yedoensis* was concentrated to about 100 ml, and 150 ml of the petroleum ether was added. The mixture was then shaken and quieted by extraction. The lower solution was taken and extracted four times; it was added by 150 ml of the acetic ether and shaken and quieted in extraction. The upper solution had been taken and extracted four times. Extracted solution was concentrated and stood-by for experimentation.

Silica-gel: The extract of *V. yedoensis* was completely dissolved in 50% of the heated alcohol. 100.0 g of the treated silica gel column chromatography was added to the dissolved solution which was then slowly heated to 60°C by water bath. The methanol was volatilized and dried by distillation, prior to it being loaded onto the extraction column by dry method. Chromatography column was first extracted by 100 ml of the mixed liquid of chloroform-methanol (25:10, v:v). Specifically, the extract solution completely immersed the silica gel chromatography as was the criterion. It was extracted by 200 to 300 ml mixed liquid of chloroform-methanol (V:V = 25:25) (Eloesser et al.,1975). The latter extract was collected and then concentrated to dryness. Part of the solvent was recovered and heated until completely dissolved by 70% of the methanol, and readied for stand-by.

Selection of macroporous adsorption resin: The pre-processing of various macroporous adsorption resins were prepared according to the literature of Lu (2005). 1.0 g of treated resins (equivalent dry weight, the same as below) and 15 ml of the apigenin standard solution were added to a 50 ml conical flask. The resin was sealed and static absorbed for 24 h, before being filtered. The concentration of apigenin in the filtrate was detected and the adsorption rates of various resins were calculated. Desorption experiments were carried out by adding 30 ml of the 70% of methanol solution to the adsorbed apigenin resins. The concentration of apigenin in the desorpted solution was measured, and the rate of desorption was calculated. The proper resins were selected according to the resin adsorption and desorption rates.

Selection of sample concentration: 1, 4, 6, 8, 10, or 12 mls of the apigenin standard solution were placed in 50 ml conical flasks. The volume in each flask was adjusted to a constant volume of 15 ml, before an equivalent volume of filter resin was added, and static adsorpted in 24 h. Filter resin adsorption rates for different concentrations of apigenin were calculated.

Selection of eluant concentration: 30 mls of 0, 10, 30, 50, 70, or 90% methanol solution were respectively added to 2.0 g of six copies of the adsorbed apigenin standard solution filter resins (wet weight) and static desorpted in 24 h. The concentration of Apigenin of desorpted solution was measured and the optimum eluent concentration was determined.

Selection of eluent flow rate: 10.00 g of four copies of the filter resin (wet weight) were columned according to wet-load. 10 ml of apigenin standard solution was respectively added to the resin volume that is 10 ml and then eluted with 1, 2, 3 or 4 BV / h of the flow rate after the 4^{th} h of adsorption. Eluent was collected at 5 ml per tube and diluted to 10 ml. The apigenin content of elution was measured and the optimum flow rate determined.

Column chromatography LSA-10 macroporous resin: LSA-1macroporous resin was columned according to wet-load, adsorption and desorption were subject to the chosen conditions and eluent was collected and concentrated to dryness. Acetone was added and refluxed in 2 h under 56°C to make sure that the resin was fully dissolved. Resin was filtrated while hot but the filtrate was placed in the refrigerator overnight. The yellow crystals were precipitated and leached. Once the purified apigenin was obtained, it was dried to constant weight by vacuum drying (50cm Hg, 47°C).

RESULTS

UV standard curve of apigenin

The absorbance values of the apigenin standard solution at 345 nm are reflected in Table 2. In the regression equation of the standard curve (shown in Figure 1), concentration was taken (μg / ml) for abscissa and absorbance value (A345) for the vertical, axis is Y = 0.0685X + 0.0035, r = 0.9999. The solution concentration and absorbance have a good linear relationship especially in the concentration range of 3.33 to 19.98 μg / ml as determined by UV spectrophotometry of the apigenin standard solution.

HPLC testing pattern and the regression equation of the apigenin standard solution

The retention time and peak area of each sample volume are depicted in Table 3. The regression equation according to the concentration and peak area of apigenin is y = 15.845x-225.76, r = 0.9996. The concentration of standard solution and its size of the peak area have a linear relationship as determined by HPLC detection. This linear relationship was good in the concentration range of

Table 2. A_{345} values of apigenin standard solution.

Test number	1	2	3	4	5	6	7
Standard sample of apigenim (ml)	0	3	6	9	12	15	18
Concentration (μg/ml)	0	3.33	6.66	9.99	13.32	16.65	19.98
Absorbance values	0	0.232	0.46	0.695	0.917	1.133	1.379

Table 3. HPLC determination of the standard curve of apigenin.

Number	1	2	3	4	5
The injection volume (μl)	5	10	15	20	25
Apigenin concentration (10^{-3} μg/ml)	105	210	315	420	525
Peak area	1271	3000	4701	6403	8227

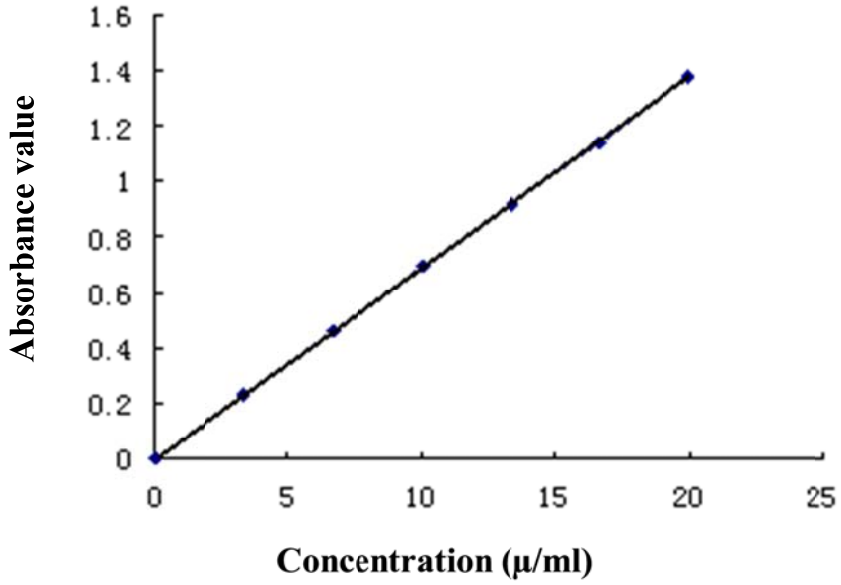

Figure 1. UV standard curve of the apigenin standard solution.

105 X 10^{-3} to 525 X 10^{-3} μg / ml. The HPLC testing pattern of the apigenin standard solution is shown in Figure 2.

Selection results of entrainer of apigenin in V. yedoensis Makino as obtained by supercritical CO_2 extraction

A better solution for the supercritical CO_2 extraction of apigenin from *V. yedoensis* makino involves using a co-solvent. Optimal conditions of extraction were selected by orthogonal experimentation as follows: solid-liquid ratio was 5:3, extraction pressure: 20 MPa, extraction temperature 50°C, extraction time 2h, separation vessel temperature：55°C, pressure: 9 Mpa, separation vessel temperature：40°C and pressure: 5 Mpa.

Determination of apigenin from V. yedoensis makino obtained by supercritical CO_2 extraction

Apigenin in *V. yedoensis* makino obtained by supercritical CO_2 extraction was detected in step 2.1 using an ultraviolet spectrophotometer. The average yield of apigenin in *V. yedoensis* makino was 0.0268% according to the regression equation. Thus, using ultraviolet spectrophotometry for the determination of the yield of apigenin in *V. yedoensis* makino obtained by supercritical CO_2 extraction, the study found that it was significantly higher than that of the conventional method extraction yields. It was also higher than the microwave-assisted extraction and ultrasonic-assisted extraction yield, as well as being significantly higher than the celery root apigenin content 75mg/Kg. This result indicated that supercritical CO_2 extraction is suitable for extraction, separation and

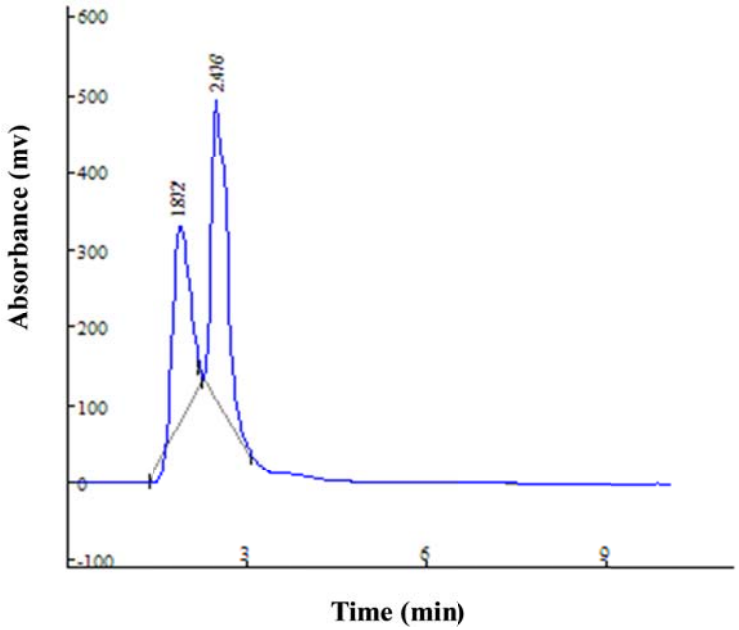

Figure 2. HPLC chromatogram of the apigenin standard solution.

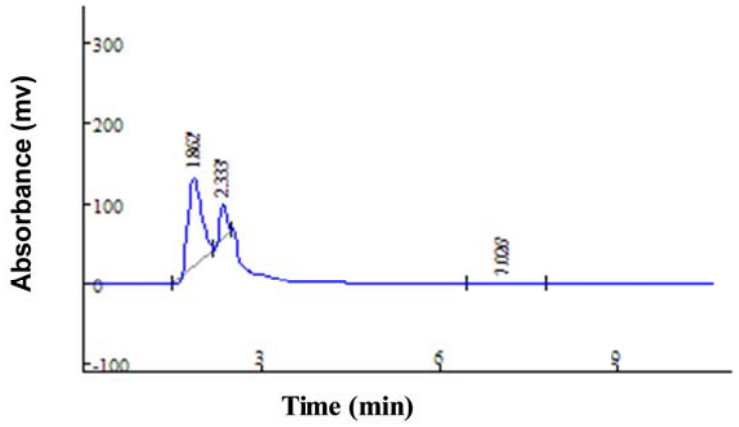

Figure 3. HPLC chromatogram of apigenin from *V .yedoensis* makino obtained by supercritical CO_2 extraction.

purification of apigenin in *V. yedoensis* makino.

Determination of apigenin from V. yedoensis makino obtained by supercritical CO_2 extraction using HPLC

Apigenin in *V. yedoensis* makino obtained by supercritical CO_2 extraction was detected by HPLC as described earlier. The HPLC chromatogram is shown in Figure 3. By comparing Figure 2 with Figure3, the study found that the retention time of the first two peaks of the supercritical CO_2 extract and the apigenin standard reference substance was consistent. However, they showed an obvious difference in peak area; the supercritical CO_2 extract had a peak at 7.026 min. Because very few contents of apigenin in *V. yedoensis* makino were from free apigenin, many of them were in the form of C-Glycosyl compounds, such as: apigenin 6,8-2-C-α-L-THP-glucoside, apigenin-6-C-α-L-THP-glycosyl-8-C-β-D-glucopyranoside, apigenin-6-C-β-D-THP-glycosyl-8-C-α-L-glucopyranoside, apigenin-6-C-β-D-THP-glycosyl-8-C-β-L-glucopyranoside, apigenin-6-8-2-C-β-D-glucopyranoside, apigenin-6-C-α-L-THP-glycosyl-8-C-β-D-xylopyranoside and apigenin-6-C-β-D-xylopyranosyl-8-C-α-L-THP-glucoside (Li et al., 2008).

The HPLC chromatogram of apigenin from *V. yedoensis* makino obtained by supercritical CO_2 extraction (Figure 3)

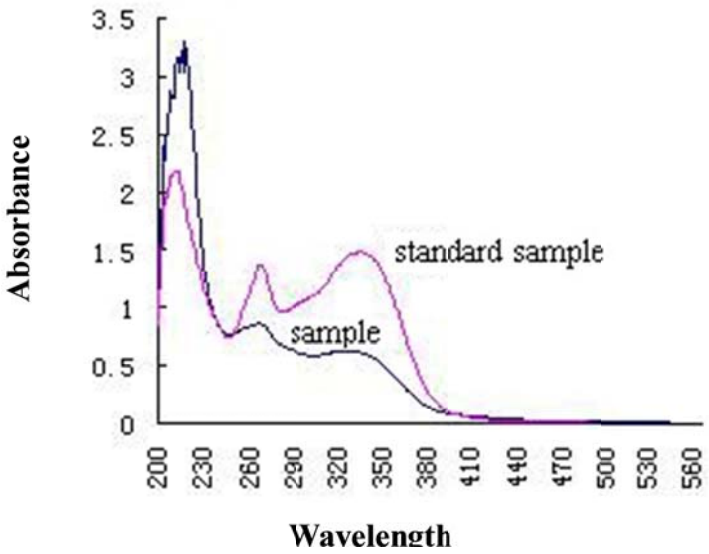

Figure 4. Ultraviolet scan atlas of apigenin standard sample and *V. yedoensis* makino apigenin purified sample.

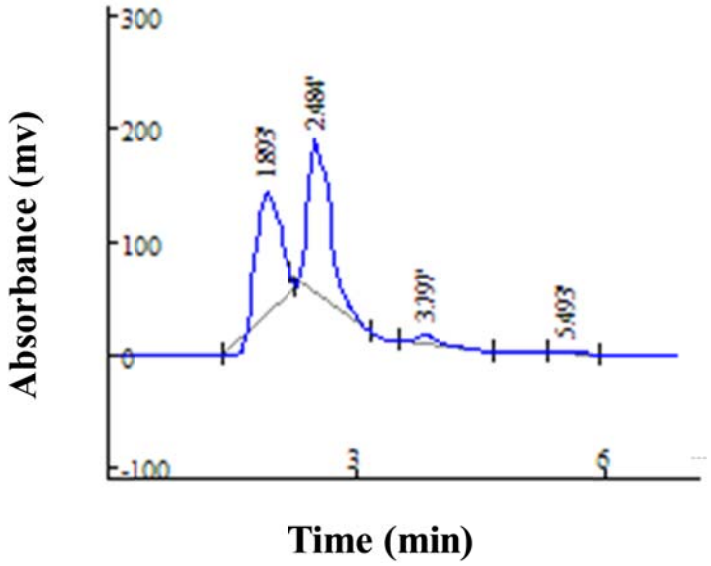

Figure 5. Representative HPLC trace of Viola philippica apigenin purified samples.

was compared with the HPLC chromatogram of the purified and isolated sample of apigenin from *V. yedoensis* makino obtained using Silica Gel Column Chromatography and LSA-10 macroporous adsorption resin (Figure 4) and the HPLC chromatogram of the apigenin standard solution and purified and isolated sample solution mixture (Figure 5). The study found that the peak number of the supercritical CO_2 extraction samples was less than the Silica Gel Column Chromatography and LSA-10 macroporous adsorption resin purified and isolated apigenin sample. In addition, the two peak areas changed. The study showed that the extraction and distillation of apigenin in *V. yedoensis* makino by supercritical CO_2 extraction was very effective.

Results of different macroporous adsorption resin for apigenin static exchange adsorption

It can be seen from the results depicted in Table 4 that

Table 4. Different macroporous resin adsorption determination results.

Resin	Size (mm)	Aperture (nm)	BET (m^2/g)	Polarity	Absorbance A^0	ODA
NKA-9	0.3-1.25	15.5-16.5	250-290	Non	0.832	0.378
NKA-2	0.3-1.25	14.5-15.5	160-200	Strong	0.828	0.419
AB-8	0.3-1.25	13.0-14.0	480-520	Weak	0.833	0.365
X-5	0.3-1.25	10.5-12.5	540-580	Non	0.83	0.339
D101	0.3-1.25	10.5-11.5	400	Weak	0.829	0.325
D4020	0.35-0.55	10.0-10.5	600-700	Non	0.832	0.412
D392	0.3-1.0	10.0-10.5	540-580	Alkaline	0.831	0.441
LSA-10	0.3-1.25	14.8-16.2	Highly active BET	Polar	0.833	0.268
LSA-21	0.3-0.9	12.6-14.8	Highly active BET	Polar	0.829	0.315

macroporous adsorption resin has the best capacity of exchange for apigenin. Therefore, this study used novel LSA-10 macroporous adsorption resin to adsorb, separate and purify the extracted apigenin from *V. yedoensis* makino. Desorption results with different methanol concentrations are presented in Table 5. From Table 5, we learned that a solution of 70% methanol has the strongest adsorption ability for LSA-10 macroporous polymeric adsorbent adsorption of apigenin**,** so then 70% methanol was used in the elution for macroporous polymeric adsorbent LSA-10 after adsorption in this study.

Ultraviolet scan of apigenin standard solution and purification of sample solution test results

Ultraviolet spectrophotometry of the apigenin standard solution and Silica gel column chromatography, LSA-10 macroporous polymeric adsorbent of *V. yedoensis* makino in separation and purification of samples of apigenin was shown in Figure 4 as map scanning. From map scans of the apigenin standard and purified samples in the ultraviolet spectrophotometer，we can see ultraviolet absorption peaks in the *V. yedoensis* makino purification of apigenin that are
consistent with peaks found with the apigenin standard. This experiment can be analyzed from using the separation and purification sample products and the apigenin standard sample products as the components of each are basically the same.

HPLC detection Silica gel column chromatography and macroporous polymeric adsorbent LSA-10 to V. yedoensis makino in separation and purification of apigenin sample maps

The yield of *V. yedoensis* makino in the extraction solution of apigenin respectively could reach 61.3 and 85.8% after silica gel column chromatography extraction and macroporous polymeric adsorbent LSA-10 adsorption and elution (the column was determined on the ratio of the content of apigenin). After twice the column chromatography and drying the material, the purity based on HPLC detection reached 92.8% (Figure 5).

HPLC detection silica gel column and LSA-10 separation and purification of apigenin samples and standard of mixture (V：V=1：1) atlas

Using high performance liquid chromatography instrument detection and knowing from scanning atlas (Figure 2) that apigenin in the reference standard had a retention time (tR) of 1.872 min and 2.476 min, the purity of apigenin could be calculated at 98%. Apigenin purified from *V. yedoensis* makino had a retention time (tR) of 1.893 min and 2.484 min (Figure 4). This is consistent with the standard product. By mixing the purification samples and the comparison solution according to the 1:1volume ratio，20 μL of the sample was tested by scanning atlas and this is depicted in Figure 6. From Figure 6, we can see that two peaks of the mixture material completely overlap, the peak of retention time (tR) and celery grain standard of peaks of retention time (tR) are consistent. With this experiment it can be determined that the sample obtained from the separation and purification of the *V. yedoensis* Makino is apigenin, but purification of the *V. yedoensis* Makino apigenin sample contained four other substances. The purity of the *V. yedoensis* makino apigenin sample was calculated to reach 95.80%.

DISCUSSION

In the previous studies, the methods used for apigenin extraction were mainly by maceration or reflux extraction with methanol, ethanol and other organic solvents (Peng, 2009; Shen et al., 2010; Jiang et al., 2013), while the ultrasonic- and microwave-assisted extractions were also applied by some investigators (Liu et al., 2010).

Table 5. Desorption result of different methanol concentrations to apigenin.

Number	Methanol concentrations (%)	OD
1	0	0.062
2	10	0.173

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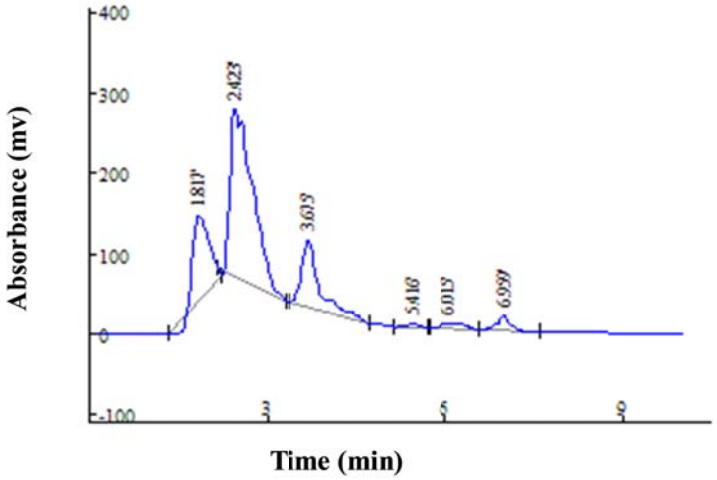

Figure 6. Representative HPLC trace of apigenin standard sample and purified sample.

Compared with the solvent reflux and cold maceration extractions, both the ultrasonic- and microwave-assisted extractions reduced impurities in the extracts and increased apigenin contents (Yang, 2007; Wang et al., 2003). However, they still have some disadvantages, for example, it is necessary to be extracted with a solvent, which contains more impurities, meanwhile the solvent recovery is very difficult. The present study adopted, for the first time, the supercritical CO_2 extraction of apigenin from *V. yedoensis* makino with integration of separation and removal of the solvent by one process to address the problems of apigenin extraction mentioned above; it simplified the procedure and improved production efficiency and did not cause environmental pollutions. However, the limited volume of extract and the high running costs by supercritical CO_2 extraction method made it for further improvement on large scale application.

Additionally, the separation and purification of apigenin were often through solvent extraction, macroporous resin, polyamide column chromatography and silica gel columns procedures (Peng, 2009; Li et al., 2007; Zhu et al., 2005). Although the conventional extraction, separation and purification methods were simple and mature, the significant difficulties were incomplete extraction of materials and the loss of apigenin in the separation and purification process (Liu, 2012; Liu et al., 2010).

Conclusion

In this study, the UV and HPLC analysis showed that the purity of apigenin by supercritical CO_2 extraction reached 97.6%; and the purity of conventional ultrasonic-assisted extraction reached 95.80%, and the yield of apigenin, 0.27± 0.01‰, by supercritical CO_2 extraction was significantly higher than the conventional ultrasonic-assisted extraction yield of apigenin, 0.22±0.01‰. Further analysis of the results of this study indicated that supercritical CO_2 extraction simplified the procedures, shortens the extraction time and improved the product yield and purity, suggesting that supercritical CO_2 extraction is entirely suitable for the extraction and purification of apigenin from *V. yedoensis* makino. Meanwhile the results of this study were consistent with other conclusions by Meng Fan-shu (2011). In conclusion, the supercritical CO_2 extraction method for apigenin separation and purification is superior to the conventional method such as ultrasonic-assisted extraction under the same experimental conditions, but limited product volume of extract is worthy of further

study for improvement.

Conflict of Interest

The authors have not declared any conflict of interest.

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