ISSN 1330-9862
(FTB-1631)

scientific note

Preparing Apigenin from Leaves of *Adinandra nitida*

Benguo Liu, Zhengxiang Ning, Jianhua Gao and Keyong Xu*

College of Light Industry and Food Science, South China University of Technology, Guangzhou, Guangdong, CN-510640, PR China

Received: February 17, 2006
Accepted: July 6, 2006

Summary

Leaves of *Adinandra nitida* were used as raw material, and a new industrially significant method of preparing apigenin was established by hydrolyzing a water extract and recrystallizing it with ethanol in order to obtain a new source for the production of this flavone. A yield of about 2.5 % (dry mass) was obtained with the purity of 93.05 %, determined by high performance liquid chromatography (HPLC). Moreover, the main flavonoids in leaves of *Adinandra nitida* and the product after acid hydrolysis were identified as camellianin A and apigenin, respectively, by ultraviolet-visible spectrometry (UV/VIS) and electrospray ionization mass spectrometry (ESI-MS).

Key words: *Adinandra nitida*, flavonoid, apigenin, electrospray ionization mass spectrometry

Introduction

A number of studies have shown that natural antioxidants from plant sources can effectively inhibit oxidation in food and reduce the risk of age-dependent diseases (*1*,*2*). Flavonoids, widespread in fruits, vegetables, teas and medicinal plants, have received the greatest attention and have been studied extensively, since they are a kind of highly effective antioxidants and less toxic than synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *etc*. (*3*). Apigenin, 5,7,4′-trihydroxyflavone, has antioxidant, antitumor, and spasmolytic activity, and can reduce high blood pressure (*4*). To the best of our knowledge, flowers of *Matricaria chamomilla*, which have been used for producing apigenin, contain no more than 0.3 % of this flavone, by dry mass. For this reason, the extraction of apigenin from chamomile flowers is not an economically viable industrial process.

The leaves of *Adinandra nitida*, known as shiyacha in China, is a kind of traditional tea substitute in South China, and has been reported to have many curative effects, such as reducing blood pressure, as well as antibacterial, antioxidant, analgesic properties, *etc*. (*5–8*). Though some published reports have proved that the leaves of *Adinandra nitida* are abundant in apigenin glucosides, such as camellianin A and camellianin B (*9*), its main use has been as a cheap substitute for tea and not as a new source of preparing apigenin. In this paper, a new industrially significant method of preparing apigenin from the leaves of *Adinandra nitida* is introduced in order to make full use of this plant, and to make it a new source of apigenin.

Materials and Methods

Adinandra nitida plant material

The leaves of *Adinandra nitida* (2005 production, moisture content 9.3 %) were purchased from Dayaoshan MingXiangGe tea firm in Laibin, China.

Reagents

Apigenin (HPLC 98 %) was purchased from Shanxi Huike Botanical Development Co., Ltd. (Xian, China). Methanol (Tianji Shield Company, Tianji, China) was of HPLC grade and other chemicals were of analytical grade.

Identification of the main flavonoids in the leaves of Adinandra nitida

The main flavonoids in the leaves of *Adinandra nitida* were identified by ultraviolet-visible spectrometry

*Corresponding author; Phone: ++86 020 88 566 216; Fax: ++86 020 87 113 848; E-mail: zzgclbg@126.com

(UV/VIS) and electrospray ionization mass spectrometry (ESI-MS). About 72 g of leaves (moisture content 9.3 %) were extracted with 1000 mL of boiling water for 1 h and then filtered. The filtrate was left for 24 h at ambient temperature and then filtered through a porous filter. The precipitate retained on the filter was washed and purified to get the main flavonoid in the leaves by recrystallization. As a result, about 0.38 g of a yellow powder were obtained. An aliquot (10 mg) was dissolved in methanol and diluted to 20 mL for identification. UV/VIS analysis was performed on a TU-1810PC UV/VIS spectrophotometer (Purkinje, China) and analyzed according to a published method (*10*). The mass spectra were obtained with an LC-MS system (LCMS-2010A, Shimadzu, Japan). Methanol solution of the sample was injected directly into the mass spectrometer to obtain the negative full scan electrospray mass spectra, which could rapidly present the molecular masses of the ingredients of the sample. The ESI-MS parameters in this study were as follows: nebulizing gas flow, 1.5 L/min; curved desolvation line temperature (CDL temperature), 250 °C; heat block temperature, 200 °C; detector voltage, 1.5 kV; ion gauge vacuum (IG vacuum), $1.2 \cdot 10^{-3}$ Pa; the scan range was set from 100 to 1000 m/z. The ESI-MS data were recorded and processed by LCMSsolution software ver. 3.1.

Method for preparing apigenin from the leaves of Adinandra nitida

The leaves of *Adinandra nitida* (150 g, moisture content 9.3 %) were extracted twice with 1500 mL of boiling water for 1 h and then filtered. After cooling, sulphuric acid was added to the extract in the ratio of 1:50 (by volume). The mixture was heated for 20 min and then filtered to collect the yellow precipitate. The precipitate was washed on the filter until neutral pH was obtained and then dried. About 15.5 g of raw product (product A) were obtained from the yellow powder. By recrystallizing for three times from ethanol, about 3.4 g of pure product (product B) were obtained.

Identification of the main flavonoids in the product after hydrolysis

The main flavonoids in the product after hydrolysis were identified by ultraviolet-visible spectrometry and electrospray ionization mass spectrometry using the methods previously described. The yellow powder of the product B (10 mg) was dissolved in methanol and diluted to 20 mL for identification.

Determination of the purity of product A and product B

For HPLC analysis, 37.7 and 29.8 mg of product A and product B, respectively, were dissolved in methanol to produce solutions of 0.377 and 0.298 mg/mL.

Apigenin standard was dissolved in methanol to produce a stock solution of 0.567 mg/mL. For the HPLC calibration curve, a concentration range from 0.0567 to 0.567 mg/mL had been prepared.

The analytical method used was modified from a published method (*11*). The samples were separated on a reversed phase column, Symmetry® C18 column (3.9× 150 mm; 5 μm particle size), manufactured by Waters, USA. The mobile phase consisted of methanol and water in a volume ratio of 50:50 with a flow rate of 0.8 mL/min. The HPLC system consisted of a Waters 1525 Binary HPLC Pump and a Waters 2487 Dual Wavelength Absorbance Detector. The injection volume was 10 μL and the detector was set at 259 nm. Before HPLC analysis, all samples had to be passed through a 0.45-μm Millipore filter. The quantification of apigenin in the product was based on an external standard. The chromatographic data were recorded and processed by Breeze Systems software ver. 3.3.

Results and Discussion

Identification of the main flavonoids in the leaves of Adinandra nitida

The main flavonoids in the leaves of *Adinandra nitida* were identified by using UV/VIS and ESI-MS analyses. Through UV/VIS spectra, the places of free hydroxyl groups on benzo-γ-pyrane could be determined. Six UV/VIS spectra of the flavonoid in methanol and methanol containing five different shift additives (MeONa, $AlCl_3$, $AlCl_3$/HCl, NaOAc and NaOAc/H_3BO_3) were obtained. The UV/VIS spectra of the main flavonoid obtained in this study are as follows (λ_{max}/nm): (MeOH) 263, 329; (MeONa) 271, 322, 381; ($AlCl_3$) 263, 328; ($AlCl_3$/HCl) 264, 305, 332, 399; (NaOAc) 271, 350; (NaOAc/H_3BO_3) 263, 330. According to the method introduced by Markham (*10*), this main flavonoid in the leaves of *Adinandra nitida* was identified as 5,7,4′-trihydroxyflavone, whose 5-hydroxyl was replaced.

ESI-MS is the new development of mass spectrometry, which can accurately measure the molecular mass of small and big biological molecules. Due to the acidic nature of the flavonoids, deprotonation was found to be a much more effective ionization mode than protonation. Fig. 1 shows ESI-MS ($[M\text{-}H]^-$) of the main flavonoid in leaves of *Adinandra nitida* with the molecular mass of 620.4.

The UV/VIS spectra of the main flavonoid obtained in this study match the UV/VIS spectra of camellianin A (*9*). The relative molecular mass of camellianin A is 620.563, coinciding with the result of ESI-MS. Thus the main flavonoid in leaves of *Adinandra nitida* was identified as camellianin A (CAS 109232-77-1, Fig. 2).

In a previous work (*9*), some flavonoids such as camellianin A, camellian B, or apigenin were found in leaves of *Adinandra nitida*, camellianin A being identified as the main flavonoid, which is in agreement with the result of this study.

Identification of apigenin

The UV/VIS spectra of the main flavonoid obtained from product B are as follows (λ_{max}/nm): (MeOH) 268, 335; (MeONa) 276, 325, 393; ($AlCl_3$) 276, 302, 346, 383; ($AlCl_3$/HCl) 277, 301, 343, 382; (NaOAc) 275, 382; (NaOAc/H_3BO_3) 268, 341. According to the method introduced by Markham (*10*), this main flavonoid in the product after acid hydrolysis was identified as 5,7,4′-trihydroxyflavone.

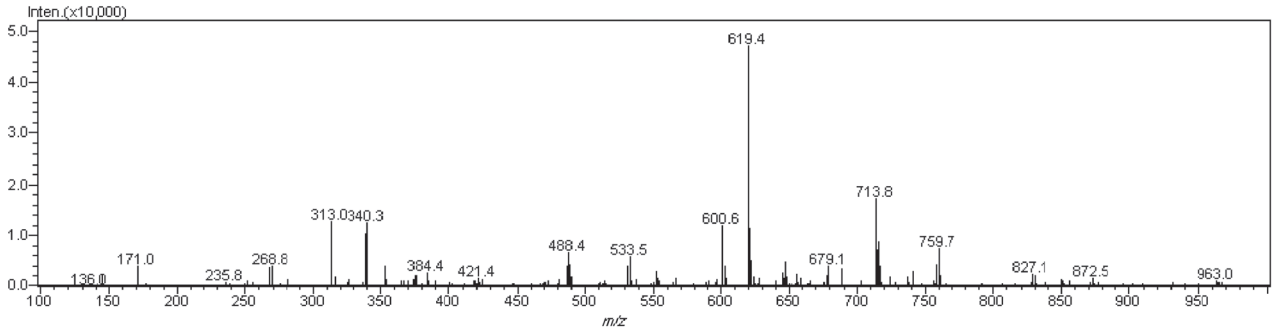

Fig. 1. ESI-MS ([M-H]$^-$) of the main flavonoid in leaves of *Adinandra nitida*

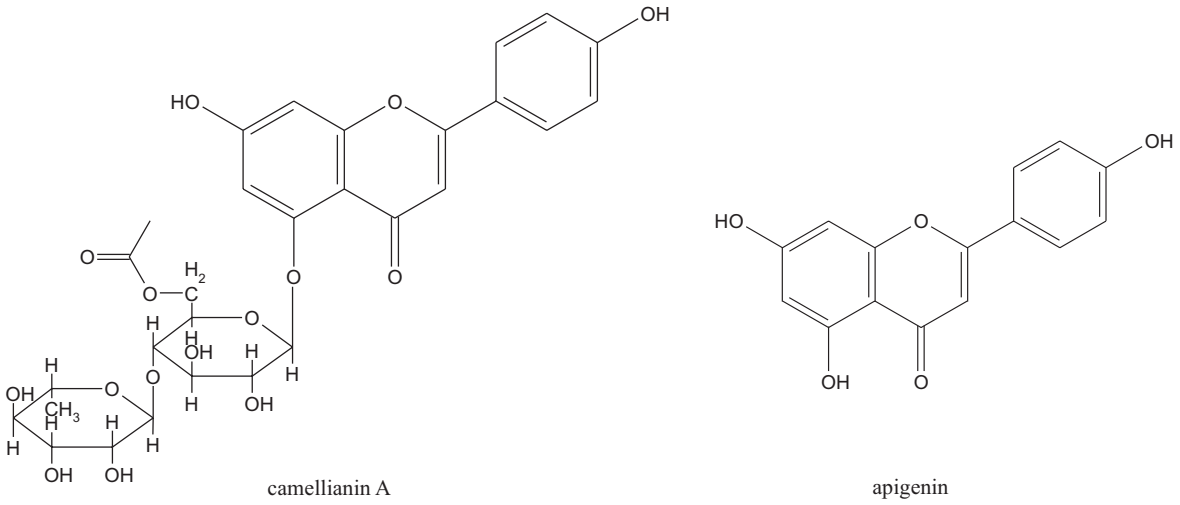

Fig. 2. Chemical structures of the studied flavonoids

The UV/VIS spectra of the main flavonoid obtained in this study match the standard UV/VIS spectra of apigenin (λ_{max}/nm): (MeOH) 267, 296 (sh), 336; (MeONa) 275, 324, 392; ($AlCl_3$) 276, 301, 348, 384; ($AlCl_3$/HCl) 276, 299, 340, 381; (NaOAc) 274, 301 (sh), 376; (NaOAc/H_3BO_3) 268, 302 (sh), 338 (*12*). The standard molecular mass of apigenin is 270.24, coinciding with the result of ESI-MS (Fig. 3). Thus, the main flavonoid in the product was identified as apigenin (CAS 520-36-5, Fig. 2).

Fig. 4 shows the ESI-MS ([M-H]$^-$) of the raw extract of leaves of *Adinandra nitida*. It can be seen that there are many kinds of compounds, including camellianin A. After acid hydrolysis of the extract, the yellow precipitate, which was collected as product A, was filtered, washed on the filter until neutral pH was obtained, and then dried. Fig. 5 shows the ESI-MS ([M-H]$^-$) of product A, of which apigenin is the main compound, which is in agreement with the results of HPLC determination of purity of product A and product B (Table 1).

To this day, more than 4000 kinds of flavonoids have been identified or synthesized, but few of them can be widely used in areas of food production or medicine. The reason for this is that although flavonoids exist ubiquitously in plants, few kinds of plants contain enough flavonoids to achieve large-scale production. In this study, the leaves of *Adinandra nitida* were used as a source of flavonoids. To the best of our knowledge, flowers of *Matricaria chamomilla*, which have been used for apigenin extraction, contain a percentage not more than 0.3 % of this flavone in the dry mass, and in mixture with other flavones, which have strictly analogous physical properties. Under such conditions, a cost-effective indu-

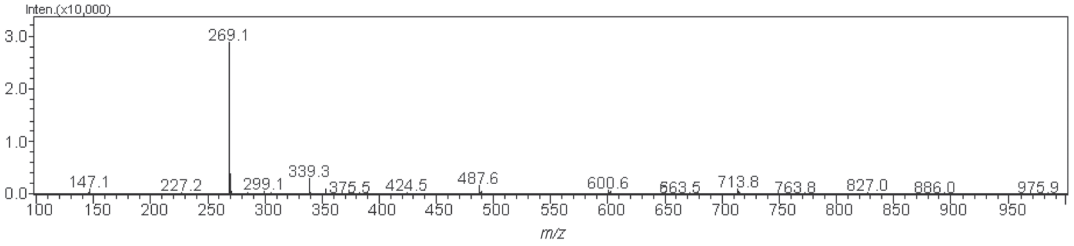

Fig. 3. ESI-MS ([M-H]$^-$) of product B

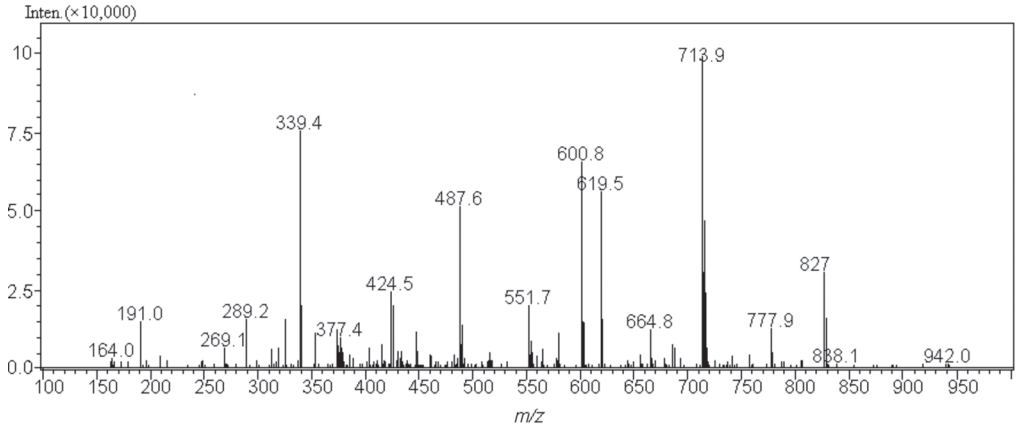

Fig. 4. ESI-MS ([M-H]$^-$) of the raw extract of leaves of *Adinandra nitida*

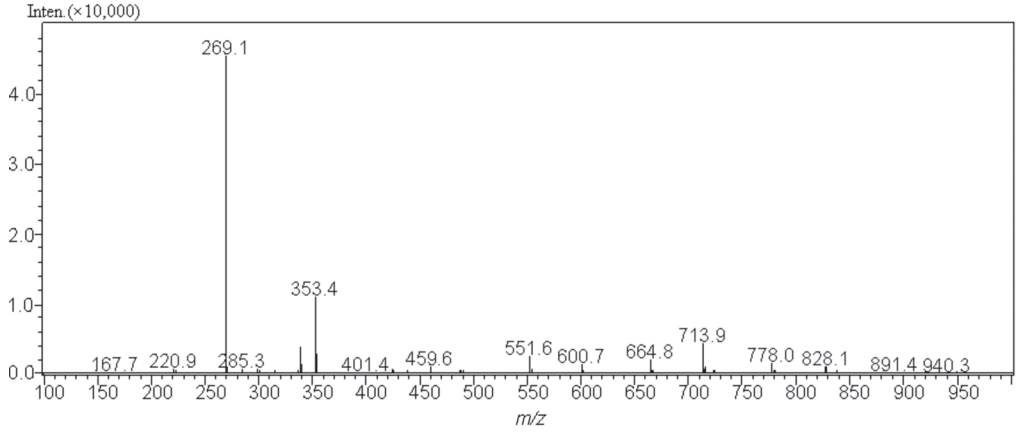

Fig. 5. ESI-MS ([M-H]$^-$) of product A

Table 1. The purities of apigenin in product A and product B

	w(product A)/%	*w*(product B)/%
Purity of apigenin*	63.93±4.41	93.05±1.35

*values are the mean±SD of *N*=3 samples

strial process for the extraction of apigenin from camomile flowers is not feasible.

The method for preparing apigenin proposed in this study is extremely simple and economical, because of the nature of the raw material, the solvents used, the number of extractive stages and the total processing time. Furthermore, the purity of the products was high (Table 1).

With the technology used in the present study, about 2.5 % of pure apigenin (product B) were obtained from leaves of *Adinandra nitida*. Such a yield is to be considered very interesting from the point of view of industrialization of the process, especially because of the very low commercial value of the unprocessed material.

It is well known that apigenin has antioxidant, anticarcinogenic and spasmolytic activities, and can reduce high blood pressure (4). That is why leaves of *Adinandra nitida* are a good source of apigenin, which can be added to food as a kind of functional ingredient, and have many beneficial effects on human health. It can also be used in medicine in standard forms of administration, such as capsules, tablets, and oral suspensions.

Conclusion

By using leaves of *Adinandra nitida* as raw material, which is abundant in camellianin A (a kind of apigenin glucoside), a new method of obtaining apigenin was established, making it a new source of apigenin. The process is extremely simple, economical and industrially significant.

References

1. S. Burda, W. Oleszek, Antioxidant and antiradical activities of flavonoids, *J. Agric. Food. Chem. 46* (2001) 2774–2779.
2. Y. Zou, Y. Lu, D. Wei, Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. *in vitro*, *J. Agric. Food. Chem. 52* (2004) 5032–5039.
3. S.S. Pekkarinen, I.M. Heinonen, A.I. Hopia, Flavonoids quercetin, myricetin, kaemferol and (+)-catechin as antioxidants in methyl linoleate, *J. Sci. Food Agric. 79* (1999) 499–506.
4. C. Engelmann, E. Blot, Y. Panis, S. Bauer, V. Trochon, H.J. Nagy, H. Lu, C. Soria, Apigenin – Strong cytostatic and anti-angiogenic action *in vitro* contrasted by lack of efficacy *in vivo*, *Phytomedicine*, *9* (2002) 489–495.

5. M. Chen, J. Yu, G. She, Y. Zhu, Analysis of nutritional components and medicinal components in wild Shiyacha, *Nat. Prod. Res. Dev. 8* (1996) 84–86 (in Chinese).
6. Y. Chen, G. She, H. Chen, Study on antitumor activities of substances extracted from *Adinandra nitida* Merr. *ex* H. L. Li, *Nat. Sci. 12* (1997) 43–45 (in Chinese).
7. Y. Chen, G. She, H. Chen, Antitumor activities of flavonoids extracted from *Adinandra nitida* and their effects on the the expression of *p53* gene in mice sarcoma-180, *Nat. Prod. Res. Dev. 19* (1998) 52–56 (in Chinese).
8. M. Chen, J. Yu, Studies on flavonoids extraction from *Adinandra nitida* Merr. *ex* H. L. Li and on their antioxidative and bacteriostatic bioactivities, *Nat. Sci. 12* (1997) 52–57 (in Chinese).
9. J. Jin, Y. Wen, G. Cheng, Studies on the flavonoids from *Adinandra nitida*, *Guihaia*, *5* (1985) 301–303 (in Chinese).
10. K.R. Markham: *Techniques of Flavonoid Identification*, Science Press, Beijing, PR China (1990) pp. 42–57 (in Chinese).
11. J. Liu, D. Wu, L. Wang, J. Liu, Quantitative determination of contents of flavone glycosides in *Chrysanthemum morifolium*, *Chin. Tradit. Herbal Drugs*, *32* (2001) 308–310 (in Chinese).
12. L. Huang, D. Yu: *Application of UV Spectrometry in Organic Chemistry*, Science Press, Beijing, PR China (1988) p. 540 (in Chinese).