Journal of Food and Drug Analysis

Volume 20 | Issue 3 Article 18

2012

# Investigation of two species of Huang-qi (Aatragalus membranaceus and Hedysarum polybotrys) by HPLC, ITS, microscopic morphology and antioxidant activities

*Journal of Food and Drug Analysis, Vol. 20, No. 3, 2012, Pages 603-610* doi:10.6227/jfda.2012200306

# Investigation of Two Species of Huang-qi (*Aatragalus membranaceus* and *Hedysarum polybotrys)* by HPLC, ITS, Microscopic Morphology and Antioxidant Activities

**I-JUNG LEE[1], PIN-CHIEH HUANG[2], LI-JIE ZHANG[1], CHIA-CHING LIAW[3], MING-REN LIN[3], CHIA-JUNG YU[1], CHUNG-I HUANG[2]* AND YAO-HAUR KUO[1,4]***

[1.] *National Research Institute of Chinese Medicine, Taipei, Taiwan, R.O.C.*

[2.] *Department of Food Science, National Ilan University, Ilan, Taiwan, R.O.C.*

[3.] *R & D Department, Star Biotechnologies Inc., Taipei, Taiwan, R.O.C.*

[4.] *Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan, R.O.C.*

(Received: October 18, 2011; Accepted: March 12, 2012)

## ABSTRACT

*Hedysarum polybotrys* is commonly substituted for *Astragalus membranaceus* in Taiwan for many years. This study described several different ways, including microscopic morphology, ITS (internal transcribed spacer) sequencing and HPLC fingerprint methods to identify these two species. Furthermore, the antioxidant activities of the two plants were evaluated and *H. polybotrys* had higher antioxidant activity than *A. membranaceus*. All the methods gave consistent conclusions that test samples KRA2, 3 and 8 belonged to *A. membranaceus,* while KRA1, 4, 5, 6 and 7 belonged to *H. polybotrys*. The results revealed that all the above methods may successfully discriminate between *A. membranaceus* and *H. polybotrys*.

Key words: ITS, microscopic, HPLC fingerprint, antioxidant activity, *Astragalus membranaceus*, *Hedysarum polybotrys*, Astragali Radix

## INTRODUCTION

Astragali Radix (Huang-qi) is a well-known traditional Chinese medicine. The species *Astragalus membranaceus* is indigenous to China, Korea and Mongolia, and is used as adjunctive therapy in the treatment of colds and influenza, chronic diarrhea, edema, abnormal uterine bleeding and diabetes mellitus, as well as a cardiotonic agent[(1)]. Recent papers have reported that *A. membranaceus* possesses hepatoprotective, immunostimulating, cardiotonic and anti-aging activities[(2-4)]. In Taiwan, *Hedysarum polybotrys* is also known as Huang-qi and commonly substituted for *A. membranaceus*, which is thought to have similar pharmacological function. Reported methods for identifying Huang-qi included the observation of microscopic and morphological traits. However, these species are not easily distinguishable by smell or appearance. Recently, the nucleotide sequences of the internal transcribed spacer (ITS) has become an important gene locus for molecular systematic investigation. Specific PCR primers are positioned on the conserved rDNA genes to amplify the entire ITS spacer region. The ITS region of rDNA, defined as the unit containing the ITS1 spacer, 5.8S rDNA gene and ITS2 spacer, has proven to be a useful gene locus for screening different species of Chinese medicine[(5-8)]. The major ingredients of the root of *A. membranaceus* include saponins, flavonoids, polysaccharides, amino acids and trace elements[(9)], while *H. polybotrys* contains isoflavonoids as its main constituent[(10)]. In this study, we report the use of several different methods including microscopic morphology, ITS, as well as HPLC fingerprinting with ELSD and UV detection to discriminate between the two kinds of Huang-qi, *A. membranaceus* and *H. polybotrys*. Their antioxidant activities were also evaluated for by the DPPH method.

* Author for correspondence. Tel: +886-2-28201999 ext. 7051; Fax: +886-2-28236150; E-mail: kuoyh@nricm.edu.tw.
Tel: +886-3-9357400 ext. 7767; E-mail: cyhuang@niu.edu.tw

## MATERIALS AND METHODS

I. *Samples*

All samples (KRA1-KRA8, Table 1) of *A. membranaceus* and *H. polybotrys* were purchased from Dihua Street Market, Taipei in August 2009.

II. *Microscopic Identification*

All of the specimens were sliced manually, fixed in 50% glycerin water solution and observed under a microscope (Carl Zeiss, Inc., Germany).

III. *ITS Sequencing*

(I) *DNA extraction*

Samples were first cut into thin slices with a sharp knife, and then ground into powder with liquid nitrogen. Genomic DNA extraction procedure was followed and modified as reported by Lu *et al*.[11]. One hundred milligrams of sample powder was digested in 1 mL of lysis buffer (100 mM of Tris-HCl, pH 8.0, 100 mM of EDTA, 1% N-lauroylsarcosine sodium salt and 1 mg/mL of proteinase K) and incubated at 56 °C in a water bath for 1 h. The sample solution was extracted with 1 mL of phenol/chloroform/isoamyl alcohol (25 : 24 : 1; v/v/v) mixture solution and centrifuged at 13,000 × g for 5 min. The aqueous layer was mixed with 0.14 mL of 5 M of NaCl and 0.1 mL of 10% hexadecyltrimethylammonium bromide (CTAB) in 0.7 M of NaCl, and further incubated at 65 °C in a water bath for 15 min. The solution was extracted with 1 mL of chloroform/isoamyl alcohol (24 : 1; v/v) solution and centrifuged at 13,000 × g for 5 min. The aqueous solution was precipitated by adding 70 μL of 3 M of sodium acetate and 0.5 mL of isopropanol. After centrifugation at 13,000 × g for 15 min, the pellet was dissolved in 0.1 mL of sterile distilled water.

(II) *Polymerase Chain Reaction (PCR) Amplification*

Each PCR reaction (50 μL) consisted of 10 μL of 5X Master Mix buffer (synthesized by Genemark Technology Co. Ltd., Taiwan). Primer P1F (5'- CCT TAT CAT TTA GAG GAA GGA G -3') and P1R (5'- TCC TCC GCT TAT TGA TAT GC -3') were designed to amplify the ITS region. The positions of ITS regions were relative to the ITS1 spacer, 5.8S rDNA gene and ITS2 spacer. Genomic DNA (100 ng) was used as a template for each PCR reaction carried out in a thermocycler (Bio-Rad Co. Ltd., USA). The PCR program consisted of a denaturation step at 94 °C for 2 min, 40 cycles of 94 °C for 0.5 min, 58 °C for 0.5 min, 72 °C for 0.5 min, and a final extension step at 72 °C for 5 min. The PCR products were electrophoresed in 1.5% agarose with ethidium bromide staining and visualized under UV light.

(III) *DNA Sequencing and Sequence Alignments*

The PCR products of specimens were sequenced by Mission Biotech Co., Taiwan. The DNA sequences were aligned by BLAST search in the web site of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/blast/ blast.cgi).

IV. *HPLC Analysis*

The dried roots powder of all samples (100 g) were refluxed and extracted two times with 95% ethanol (450 mL) for 1 h. After evaporation of ethanol *in vacuo* at 45 °C, the residue (semi-solid, 10 g) was suspended in 10% aqueous ethanol and then loaded on the Diaion HP-20 column (3 × 5 cm, Mitsubishi Chemical Corporation, Japan), which was pre-wet with de-ionized water, and successively washed with 100% $H_2O$, 40% ethanol, 70% ethanol, and 95% ethanol to yield 4 fractions. The 70% ethanol fraction was analyzed by using HPLC.

The reference compounds, astragaloside I[12], II[13], III[12] and IV[12], isoastragaloside II[14], ononin[15], (−)-methylnissolin-3-*O*-glucoside[16], isomucromulatol-7-*O*-glucoside[17] and calycosin[18], were isolated from the 70% ethanol fraction of *A. membranaceus*, while formononetin[19] and medicarpin[10] were isolated from *H. polybotrys*, which were confirmed by comparing their $^{13}C$ NMR data with the reported data. Acetonitrile and methanol (LC grade) were purchased from Merck. Milli-Q ultra-pure water (Millipore, Q-gard 1/Quantum EX) was used throughout the study.

The HPLC profile was performed on a Shimadzu 10A series system equipped with two pumps (Shimadzu, LC-6AD, Japan), an evaporative light scattering detector (ELSD, Varian, 380-LC, England), a SPD-10A UV detector,

**Table 1.** The raw material samples of Astragali Radix and Hedysari Radix and their antioxidant activities

| Sample | Chinese name | Latin name | | Price NTD/600 g | DPPH (Removal effect, % , 200 μg/mL) |
|---|---|---|---|---|---|
| | | Plant | Crude drug | | |
| KRA1 | You-qi (油耆) | *H. polybotrys* | Hedysari Radix | 250 | 50.71 ± 0.36 |
| KRA2 | Huang-qi (黃耆) | *A. membranaceus* | Astragali Radix | 200 | 30.77 ± 3.06 |
| KRA3 | Bei-qi (北耆) | *A. membranaceus* | Astragali Radix | 400 | 38.51 ± 4.66 |
| KRA4 | Jin-qi (晉耆) | *H. polybotrys* | Hedysari Radix | 200 | 65.64 ± 1.02 |
| KRA5 | You-qi (油耆) | *H. polybotrys* | Hedysari Radix | 300 | 72.14 ± 1.46 |
| KRA6 | Jin-qi (晉耆) | *H. polybotrys* | Hedysari Radix | 150 | 82.77 ± 0.44 |
| KRA7 | Jin-qi (晉耆) | *H. polybotrys* | Hedysari Radix | 250 | 57.19 ± 1.60 |
| KRA8 | Bei-qi (北耆) | *A. membranaceus* | Astragali Radix | 250 | 31.71 ± 0.01 |

a four-channel vacuum degasser (Biotech, model 2003, Sweden), a 7715i manual injector (Shimadzu) with 200-μL sample loop, a SCL-10AVP system controller and SISC software (Scentific Information Service Corporation, Taiwan) for data analysis. The column used was Cosmosil 5C18-AR-II (5 μm, 250 × 4.6 mm i.d., Japan) with a flow rate of 0.8 mL/min. The mobile phase system used acetonitrile (A) and $H_2O$ (B), using a gradient program of 25-32% (A) in 0-20 min, 32-50% (A) in 20-50 min, and 50-80% (A) in 50-60 min. The detection wavelength was 210 nm. The ELSD conditions were as follows: the flow rate of nebulizer gas ($N_2$) was maintained at 1.8 L/min, the nebulizer temperature was set at 30 °C, and the drift tube temperature was set at 40 °C.

The 70% ethanol fraction was dried under vacuum, then accurately weighed about (100 mg) and dissolved in methanol, in a 10-mL volumetric flask. All sample solutions were filtered through a 0.45-μm filter (Millipore) before use. The injective volume was 20 μL.

V. *Scavenging Activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical*

The radical scavenging activities of partially purified 70% ethanol fractions of the eight samples on the DPPH free radical were measured using the method of Rangkadilok *et al*.[20] with minor modifications. An aliquot of each sample (120 μL, 100-10 μg/mL) or (±)-*α*-tocopherol (40-10 μg/mL) was mixed with 30 μL of 0.75 mM of DPPH methanol solution in a 96-well microplate. The mixture was shaken vigorously with an orbital shaker in the dark at room temperature for 30 min and the absorbance was measured at 517 nm with an ELISA reader. Methanol was used as the negative control by replacing the sample in the reaction solution. The DPPH radical scavenging activities of the test samples were compared to the negative control and (±)-*α*-tocopherol as the positive control.

## RESULTS

I. *Microscopic Identification*

There are some typical microscopic features for *A. membranaceus* and *H. polybotrys*.

(I) *A. membranaceus*

Figure 1 shows the appearance and microscopic features of KRA3 (*A. membranaceus*). Figure 1A shows the appearance of the prepared drug in pieces. The plant was observed to be long and flexible. The main root was columnar round in shape and the length and diameter were about 8-10 cm and 1.8-2 cm, respectively. The root was covered by a tough and wrinkled, yellowish to brown-colored skin, which tended to break out into many woolly fibers that could become very noticeable during close observation. The body of the root possessed a woody interior, which was yellowish to white in color. Its longitudinal streaks appeared to be deeper on the surface and dented in shape. In addition, the main root had a dark brown circular ring in its transverse direction. Its transverse section was light brown in color. Figures 1B-1E are pictures of microscopic features. Figure 1B shows the outline of the transverse section. Figure 1C shows the transverse section. The outermost region was composed of 7-11 layers of elongated, collapsed cork cell. The cork cortex was composed of 4-7 layers of tangentially elongated cell. Phloem fibers were grouped from a few to numerous in amounts. The fiber bundled away 260-320 μm from cork cambium and were arranged in a tighter and tidy manner, in a continuously circular band. The phloem radiation zone was composed of 3-5 layers of radially elongated cells, which were arranged in a radiation manner. Xylem vessels were composed of single or a combination of 2-3 reticulated, pitted and scalariform vessels with diameter of 10-60 μm. Fiber bundles were not usually found near the cork cambium but were mostly distributed near the inner portion. Parenchyma cells contained starch grains, which included simple or combined starch grains. Figure 1D shows the surface view. Figure 1E shows the microscopic details of *A. membranaceus*: E1, reticulated vessels; E2, xylem fiber and E3, starch grains. From its morphological microscopic features, KRA3 was identified as *A. membranaceus*[21-22].

(II) *H. polybotrys*

Figure 2 shows the appearance and microscopic features of KRA5 (*H. polybotrys*). Figure 2A shows the appearance of prepared drug in pieces. It was long and narrow in shape and the length and the diameter were about 6-12 cm and 1-1.2 cm, respectively. The roots were straight pieces of wrinkled, fibrous wood, which were long and cylindrical (round) and grew horizontally underground. Its body was grayish red-brown in color on the outside and yellowish to white color on the inside. A few markers of the lateral root were found. The cork split often and dropped easily, exposing the light yellow cortex fibers beneath.

Figure 2B-2E are pictures of microscopic features. Figure 2B shows the outline of the transverse section. Figure 2C shows the transverse section. The cork cambium was composed of 6-7 layers of tangentially-elongated collapsed cells, which consisted of light brown matter. The cortex occupied about one half of the radius in breadth. The outermost section of the phloem consisted of fissures. Phloem fibers were scattered around in bundles with calcium oxalate square crystals found around the fiber bundles. These crystals were 15-20 μm in length and 5-10 μm in width, and they formed crystal fibers. This feature was not found in *A. membranaceus*. Phloem fibers were not well lignified. Phloem was a broad radiation zone with 3-5 layers of cell. The cork cambium was circular in shape. The xylem consisted mainly of reticulated vessels with a diameter of 8-60 μm. Xylem fibers were located around the vessels, which were surrounded by parenchyma cells containing calcium oxalate square crystals. Xylem fibers were mildly lignified. The starch grains were round or oval in

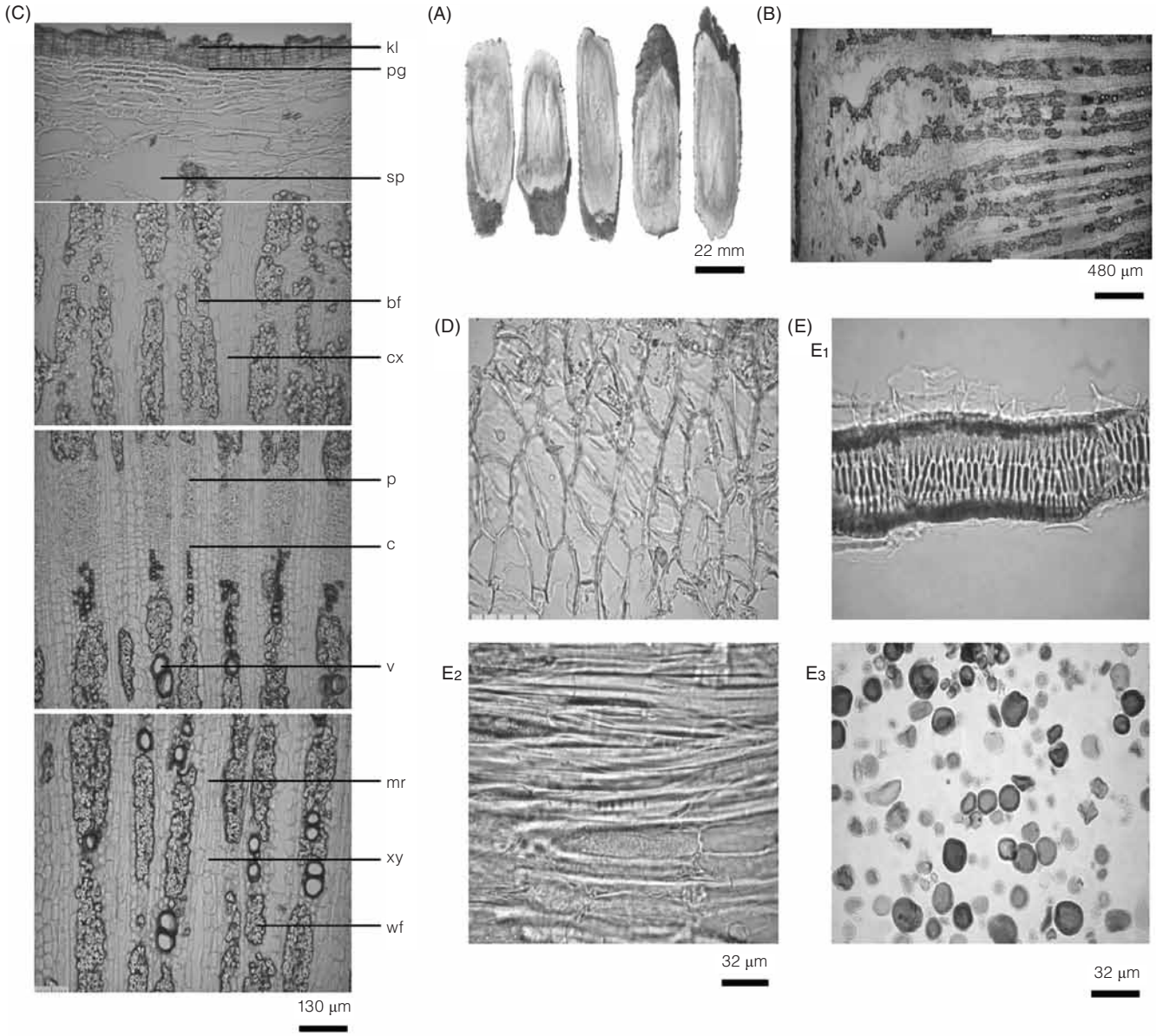


**Figure 1.** Microscopic features of Astragali Radix.
(A) Appearance, (B) Outline of transverse section, (C) Transverse section, (D) Surface view, (E) Details of transverse section, E1: Reticulated vessel, E2: Xylem fibers, E3: Starch grains.
kl: cork layer, pg: phellogen, sp: clefts, bf: bast fiber, cx: cortex, p: parenchyma, c: cambium, v: vessel, mr: medullary ray, xy: xylem, wf: wood fiber.

shape and had a diameter of around 5-20 μm. The compound starch grain was composed of 2-3 simple starch grains. Figure 2D shows the surface view section. Figure 2E shows the microscopic details; E1, reticulated vessels; E2, calcium oxalate square crystal and xylem fiber and E3, starch grains. From these features, KRA5 was identified as *H. polybotrys*[23,24].

The most significant difference in the exterior appearance of Astragali Radix and Hedysari Radix was: the bark of Astragali Radix was light yellowish brown to dark brown, while that of Hedysari Radix was reddish brown. The diameter of Astragali Radix was also longer than that of Hedysari Radix. Their differences in microscopic features were as follows: Parenchymatous cells containing prisms of calcium oxalate were found in Hedysari Radix, but could not be found in Astragali Radix. In addition, the size of starch particles in Astragali Radix was bigger than those in Hedysari Radix. The diameter of starch particles in Astragali Radix and Hedysari Radix were 12-30 μm and 10-12 μm, respectively. The vessels and fibers were denser in arrangement in Astragali Radix, but randomly loose in arrangement in Hedysari Radix. From the eight microscopic features above, we could identify the two species concerned. KAR2, 3 and 8 were identified as *A. membranaceus*, while KAR1, 4, 5, 6 and 7 were suggested as *H. polybotrys*. This shows that the microscopic method is

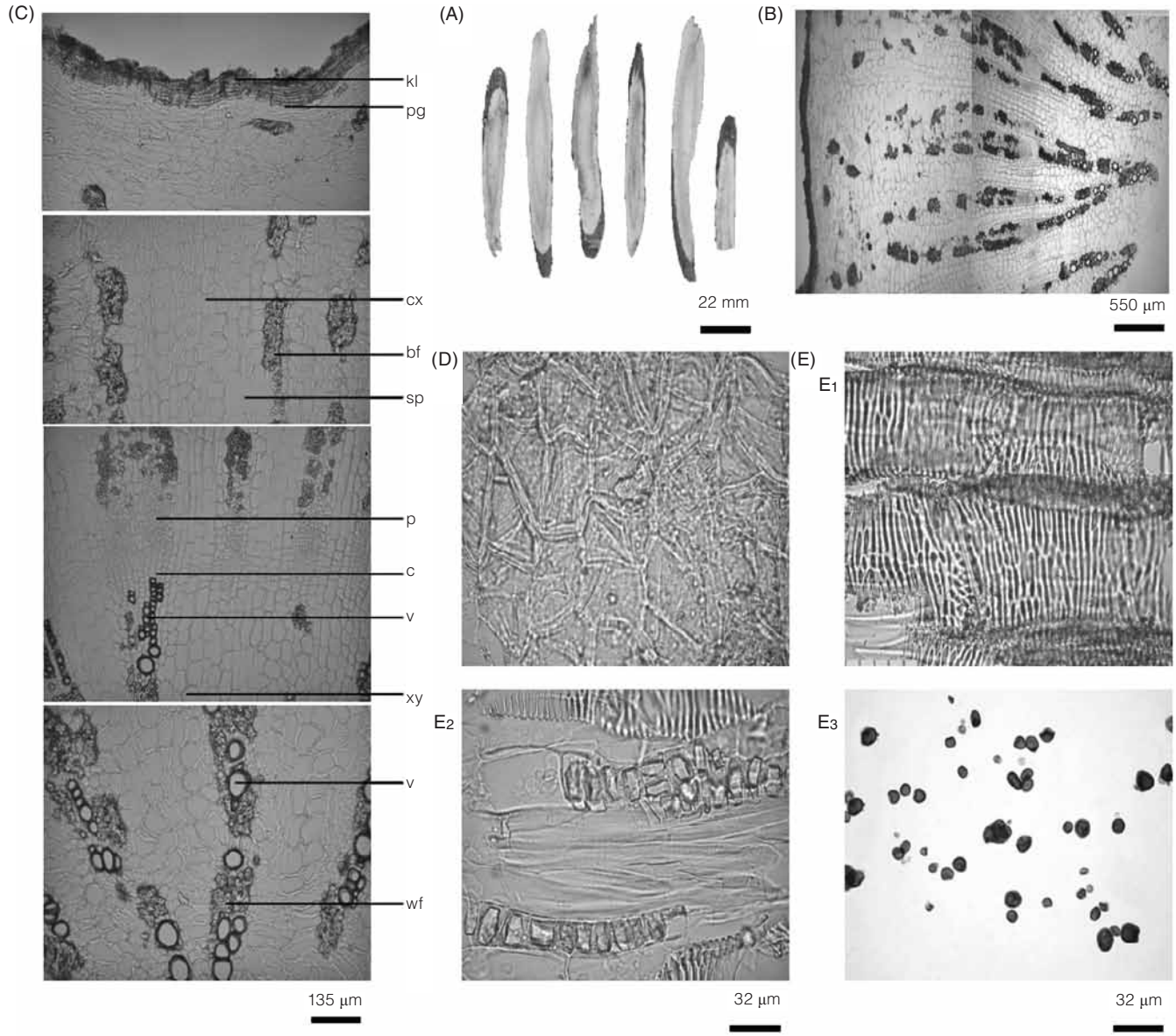


**Figure 2.** Microscopic features of Hedysari Radix.
(A) Appearance, (B) Outline of transverse section, (C) Transverse section, (D) Surface view, (E) Details of transverse section, E1: Reticulated vessel, E2: Crystal of calcium oxalate and fiber of xylem, E3: Starch grains.
kl: cork layer, pg: phellogen, cx: cortex, bf: bast fiber, sp: clefts, p: parenchyma, c: cambium, v: vessel, xy: xylem, wf: wood fiber.

an alternative method to distinguish between the two species mentioned.

II. *ITS Sequencing and Sequence Alignments*

Primers P1F and P1R were used to amplify the ITS region of the 8 specimens. Based on the PCR fragments of the internal transcribed spacer sequence, we tried to identify the differences between these two cultivars. Figure 3 shows the alignment of the ITS sequences of the 8 specimens. The results showed that KRA 2, 3 and 8 were similar to the GU289664.1 sequence, while KRA 1, 4, 5, 6 and 7 were similar to GQ434367.1. The average percentage difference between the ITS of the two kinds of Huang-qi mentioned was only 1% (The gray shaded areas represent the regions of different sequences in the two species). We could then distinguish the two kinds of Huang-qi from some insertion or deletion sites in the sequences, such as the sequence TACGCTCCCCATAATATGGCTT in GU289664.1 for *A. membranaceus*, compared with CGCCTCTGCCAGATTTGGCCTC in GQ434367.1 for *H. polybotrys*. Therefore, ITS sequencing is also an effective method for authentication.

III. *HPLC Fingerprint Analysis*

We studied the HPLC profile of the 70% ethanol

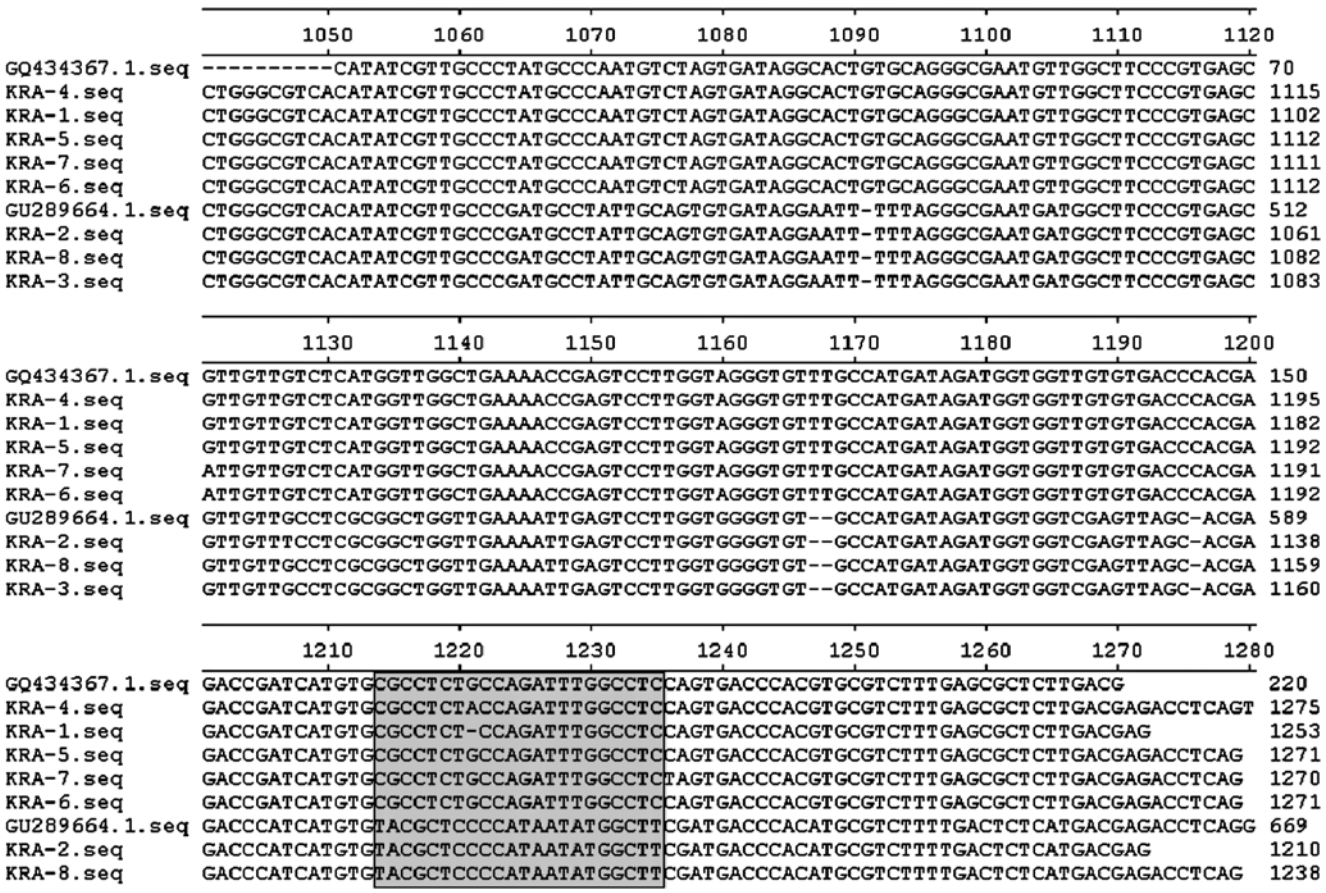


**Figure 3.** Sequence alignment of ITS fragments of the 8 specimens. Gray shading represents different sequences were aligned to identify the Astragali Radix and Hedysari Radix.

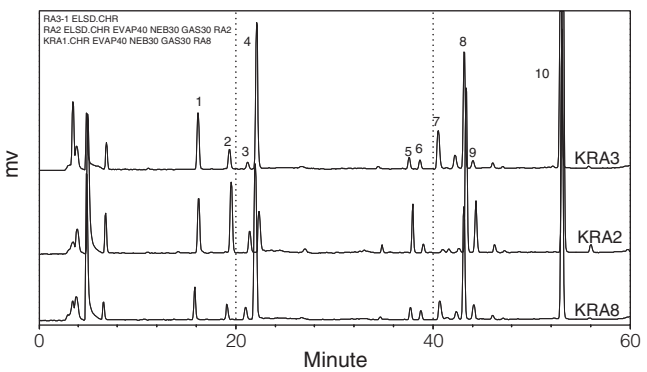


**Figure 4.** HPLC chromatograms of KRA2, KRA3 and KRA8 (ELSD detector). 1: ononin, 2: methylnissolin-3-*O*-glucoside, 3: isomucronulatol 7-*O*-glucoside, 4: calycosin, 5: astragaloside IV, 6: astragaloside III, 7: formononetin, 8: astragaloside II, 9: isoastragaloside II, 10: astragaloside I.

fraction of the eight samples (KRA1-8), and identified 11 main ingredients (peaks 1-11: ononin, methylnissolin-3-*O*-glucoside, isomucronulatol 7-*O*-glucoside, calycosin, astragaloside IV, astragaloside III, formononetin, astragaloside II, isoastragaloside II, astragaloside I and medicarpin, respectively) by comparing their retention times with the reference compounds. The structures identification of the reference compounds were described in the experimental part. As shown in Figure 4, samples KRA2, 3 and 8 had similar secondary metabolite patterns but different contents depending on the source. In Figure 5, KRA1, 4, 5, 6 and 7 showed similar secondary metabolite patterns. Figures 4 and 5 indicated that KRA2, 3 and 8 belonged to the same species, *A. membranaceus*, whereas KRA1, 4, 5, 6 and 7 belonged to *H. polybotrys*. The obvious difference between the two species was that the peaks of astragalosides were found in *A. membranaceus*, but not in *H. polybotrys*.

### IV. *Scavenging Activity of 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Bioassay*

Samples KRA1-8 were evaluated for their antioxidant activities by using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method. As shown in Table 1, the radical scavenging abilities of KRA2, 3 and 8 were smaller than 40%, whereas those of the other samples, KRA1, 4, 5, 6 and 7 were greater than 50%, at 200 μg/mL. The results concluded that the antioxidant activity of the 70% ethanol fraction of *H. polybotrys* was better than that of *A. membranaceus*.

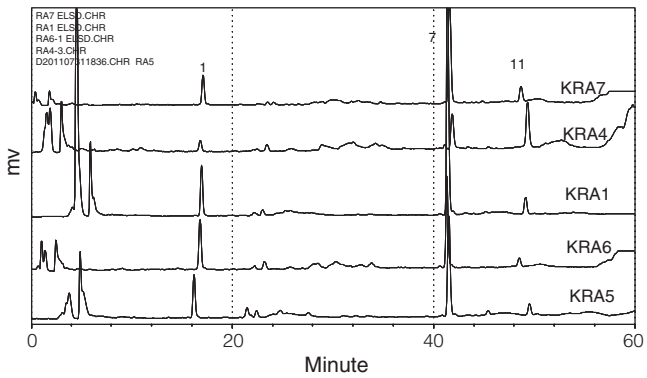


**Figure 5.** HPLC chromatograms of KRA1 and KRA4-7 (ELSD detector). 1: ononin, 7: formononetin, 11: medicarpin.

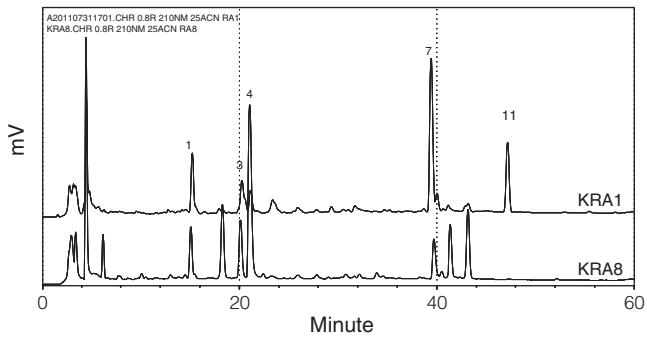


**Figure 6.** HPLC chromatograms of KRA1 and KRA8 (detected at UV 210 nm). 1: ononin, 3: isomucronulatol 7-*O*-glucoside, 4: calycosin, 7: formononetin, 11: medicarpin.

## DISCUSSION

The morphological microscopic method is often employed to assist in the identification of plant species due to its conservation of time and money. The two kinds of Huang-qi used in this study could be distinguished conveniently by their unique microscopic features.

Sequencing of the ITS region is widely applied in the authentication of Chinese medicine from its adulterants[25,26]. The two ITS regions between the 18S and 26S rDNA are useful markers for identification. They are hyper-variable among different plant species and can be amplified by conserved primers in the flanking regions[27]. In this study, the eight samples were determined based on the patterns of ribosomal DNA ITS regions which could be a successful tool to authenticate *A. membranaceus* and *H. polybotrys*.

From the HPLC profile analysis of the two species, the main different components of *A. membranaceus* and *H. polybotrys* are shown in Figures 4 and 5. The 70% ethanol fraction of *H. polybotrys* mainly comprised of isoflavonoids, while that of *A. membranaceus* mainly comprised of astragalosides. In Figure 6, five major peaks 1, 3, 4, 7 and 11 were identified as ononin, isomucronulatol 7-*O*-glucoside, calycosin, formononetin and medicarpin, respectively, for *H. polybotrys* (KRA1). These results, together with the antioxidant activities of isoflavonoids[28,29], accounted for the higher antioxidant activity of *H. polybotrys* compared to that of *A. membranaceus*. However, *A. membranaceus* (Huang-qi) can not be replaced by *H. polybotrys* (Jin-qi), as *A. membranaceus* possesses other pharmacological components such as astragalisides[1-4] which are not found in *H. polybotrys*.

All the above mentioned methods had consistent conclusions that the test samples, KRA2, 3 and 8 belonged to *A. membranaceus*, while KRA1, 4, 5, 6 and 7 belonged to *H. polybotrys*. In the Taiwanese market, Huang-qi commercial materials from the original plant, *H. polybotrys*, are known as Jin-qi (KRA4, 6 and 7) and You-qi (KRA1 and 5), while the original plant of Astragali Radix (Huang-qi) in the Pharmacopeia of the People's Republic of China[30] is known as Bei-qi in Taiwan and is from *A. membranaceus* (KRA2, 3 and 8). Our study reveals that the substitution of Astragali Radix by Hedysari Radix is very common, and the main original plant of Astragali Radix is *A. membranaceus* (Fisch.) Bge. The other Astragali Radix originated from *A. membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao, and is very rarely found in the Taiwanese market.

## ACKNOWLEDGMENTS

This study was supported by a research grant from the National Research Institute of Chinese Medicine (NRICM), Taiwan, Republic of China.

## REFERENCES

1. Chang, H. and But, P. 1987. Pharmacology and Applications of Chinese Materia Medica. World Scientific, Singapore, p. 1041.
2. Cui, R., He, J. C., Wang, B., Zhang, F., Chen, G. Y., Yin, S. and Shen, H. 2003. Suppressive effect of Astragalus membranaceus Bunge on chemical hepatocarcinogenesis in rats. Cancer Chemoth. Pharmacol. 51: 75-80.
3. Sinclair, S. 1998. Chinese herbs: a clinical review of Astragalus, Ligusticum, and Schizandrae. Altern. Med. Rev. 3: 338-344.
4. Zee-Cheng, R. K. 1992. Shi-quan-da-bu-tang (ten significant tonic decoction), SQT. A potent Chinese biological response modifier in cancer immunotherapy, potentiation and detoxification of anticancer drugs. Methods Find. Exp. Clin. Pharmacol. 14: 725-736.
5. Cheng, K. T., Su, B., Chen, C. T. and Lin, C. C. 2000. RAPD analysis of Astragalus medicines marketed in Taiwan. Am. J. Chin. Med. 28: 273-278.
6. Na, H. J., Um, J. Y., Kim, S. C., Koh, K. H., Hwang, W. J., Lee, K. M., Kim, C. H. and Kim, H. M. 2004. Molecular discrimination of medicinal Astragali Radix by RAPD analysis. Immunopharmacol. Immunotoxicol. 26: 265-272.
7. Lu, K. T., Lee, H. C., Liu, F. S., Lo, C. F. and Lin, J. H.

2009. Discriminating Astragali Radix from Hedysarum Radix in Chinese medicine preparations using nested PCR and DNA sequencing methods. J. Food Drug Anal. 17: 380-385.

8. Zhang, Y. B., Shaw, P. C., Sze, C. W., Wang, Z. T. and Tong, Y. 2007. Molecular authentication of Chinese herbal materials. J. Food Drug Anal. 15: 1-9.
9. Shao, B. M., Xu, W., Dai, H., Yu, P., Li, Z. and Gao, X. M. 2004. A study on the immune receptors for polysaccharides from the roots of Astragalus membranaceus, a Chinese medicinal herb. Biochem. Biophys. Res. Commun. 320: 1103-1111.
10. Miyase, T., Fukushima, S. and Akiyama, Y. 1984. Studies on the Constituents of Hedysarum polybotrys Hand.-Mazz. Chem. Pharm. Bull. 32: 3267-3270.
11. Lu, K. T., Lo, C. F., Chang, H. C. and Lin, J. H. 2005. Identification of Saposhinkoviae Radix in concentrated Chinese medicine preparations by nested PCR and DNA sequencing methods. J. Food Drug Anal. 13: 219-224.
12. Hirotani, M., Zhou,Y., Lui, H. and Furuya, T. 1994. Astragalosides from hairy root cultures of Astragalus membranaceus. Phytochemistry 36: 665-670.
13. Hirotani, M., Zhou, Y., Rui, H. and Furuya, T. 1994. Cycloartane triterpene glycosides from the hairy root cultures of Astragalus membranaceus. Phytochemistry 37: 1403-1407.
14. Kitagawa, I., Wang, H. K., Saito, M., Tagaki, A. and Yoshikawa, M. 1983. Saponin and sapogenol. XXXV. Chemical constituents of Astragali Radix, the root of Astragalus membranaceus Bunge. (2). Astragalosides I, II, and IV, Acetylastragaloside I, and Isoastragalosides I, and II. Chem. Pharm. Bull. 31: 698-708.
15. Lee, H. J., Lee, O. K., Kwon, Y. H., Choi, D. H., Kang, H. Y., Lee, H. Y., Paik, K. H. and Lee, H. J. 2006. Isoflavone glycosides from the bark of Amorpha fruticosa. Chem. Nat. Compd. 42: 415-418.
16. Zhang, L. J., Liu, H. K., Hsiao, P. C., Kuo, L. M. Y., Lee, I. J., Wu, T. S., Chiou, W. F. and Kuo, Y. H. 2011. New Isoflavonoid Glycosides and Related Constituents from Astragali Radix (Astragalus membranaceus) and Their Inhibitory Activity on Nitric Oxide Production. J. Agr. Food Chem. 59: 1131-1137.
17. Subarnas, A., Oshima, Y. and Hikino, H. 1991. Isoflavans and a pterocarpan from Astragalus mongholicus. Phytochemistry 30: 2777-2780.
18. Kamnaing, P., Fanso Free, S. N. Y., Nkengfack, A. E., Folefoc, G. and Fomum, Z. T. 1999. An isoflavan-quinone and a flavonol from Millettia laurentii. Phytochemistry 51: 829-832.
19. Herath, H. M. T. B., Dassanayake, R. S., Priyadarshani, A. M. A., De Silva, S., Wannigama, G. P. and Jamie, J. 1998. Isoflavonoids and a pterocarpan from Gliricidia sepium. Phytochemistry. 47: 117-119.
20. Rangkadilok, N., Sitthimonchai, S., Worasuttayangkurn, L., Mahidol, C., Ruchirawat, M. and Satayavivad, J. 2007. Evaluation of free radical scavenging and anti-tyrosinase activities of standardized longan fruit extract. Food Chem. Toxicol. 45: 328-336.
21. Zhao, Z. Z. 2005. An Illustrated Microscopic Identification of Chinese Materia Medica. International society for Chinese medicine, Hong Kong, pp. 180-182.
22. Pharmacopoeia Commission. 2009. Illustrated handbook on microscopic identification of Chinese crude drugs for Chinese pharmacopoeia. People's Medical Publishing H ouse, Beijing, pp. 372-373.
23. Pharmacopoeia Commission. 2009. Illustrated handbook on microscopic identification of Chinese crude drugs for Chinese pharmacopoeia. People's Medical Publishing H ouse, Beijing, pp. 211-212.
24. Zhao, Z. Z. 2005. An Illustrated Microscopic Identification of Chinese Materia Medica. International society for Chinese medicine, Hong Kong, pp. 225-227.
25. Chiou, S. J., Yen, J. H., Fang, C. L., Chen, H. L. and Lin, T. Y. 2007. Authentication of medicinal herbs using PCR-amplified ITS2 with specific primers. Planta Med. 73: 1421-1426.
26. Alvarez, I. and Wendel, J. F. 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29: 417-434.
27. Lin, T. C., Hsieh, C. C., Agrawal, D. C., Kuo, C. L., Chueh, F. S. and Tsay, H. S. 2007. ITS sequence based phylogenetic relationship of Dangshen Radix. J. Food Drug Anal. 15: 428-432.
28. Sato, T., Kawamoto, A., Tamura, A., Tatsumi, Y. and Fujii, T. 1992. Mechanism of Antioxidant Action of Pueraria Glycoside (PG)-1 (as Isoflavonoid) and Mangiferin (a Xanthonoid). Chem. Pharm. Bull. 40: 721-724.
29. Han, R. M., Tian, Y. X., Liu, Y., Chen, C. H., Ai, X. C., Zhang, J. P. and Skibsted, L. H. 2009. Comparison of flavonoids and isoflavonoids as antioxidants. J. Agr. Food Chem. 57: 3780-3785.
30. Pharmacopoeia Commission. 2010. The Pharmacopoeia of the People's Republic of China (PRC). Chinese Medical Science and Technology Press, Beijing, vol. 1, pp. 283-284.