Chinese Medicine Based on *Radix Puerariae*

Yang Dajian

A thesis submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

in the Department of Applied Biology and Chemical Technology

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Declaration

I hereby declare that this thesis has been written based on my own research project which was conducted by myself since my registration for the degree of Doctor of Philosophy in May 2003 and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

Yang Dajian

April, 2006

Abstract

A systematic survey on the commonly used Chinese medicinal herb, *Radix Puerariae* has been carried out for the preparation of my doctorial research project. The project include the authentication and identification of the raw materials, the analysis of its active ingredients and the development of fingerprinting methodology and the establishment of the appropriate quality control technology for *Radix Pueraria*. According to the study of structure-activity relationship (SAR) of puerarin and its analogues and derivatives, related chemicals with higher oral bioavailability and better activity than puerarin have been determined. After a comprehensive research on various formulations containing puerarin, including PEG dispersion formulation (dripping pills) and phospholipid complex (capsules), a unique technology to increase the bioavailability of puerarin was developed. The study on the clinical applications of various compound formulations containing *Radix Pueraria* as the major ingredient for the regulation of blood lipid level has resulted in a formulation with the best efficacy. The successful competion of the current project will help any further demand for the advancement of research and development of Chinese medicine containing Radix Puerariae.

Publications and patents from the project

Publications

1. Sibao Chen, **Dajian Yang,** Albert S. C. Chan, et al. HPLC fingerprint analyses of *Radix Puerari*e. *Chinese Traditional and Herbal Drugs*, 2003, 34(7): 661-663.
2. Haoliang Song, **Dajian Yang**, Albert S.C. Chan, et al. Protective effect of puerarin oral formulation on myocardial ischemia in rats and on cardiomyocytes of neonatal rats during hypoxia. *Chinese Traditional and Herbal Drugs*, 2003, 34(12): 1104-1107.
3. Sibao Chen, **Dajian Yang**, Albert S.C. Chan, et al. Seasonal variation in isoflavonoid compositions of *Pueraria lobata* (Wild.) Ohwi, assessed by HPLC analysis with DAD detector and by UV-Spectrophotometry analysis. Accepted by *Phytochemical Analysis*.
4. Sibao Chen, **Dajian Yang**, Albert S.C. Chan, et al. High-performance thin-layer chromatographic fingerprints of isoflavonoids for distinguishing *Radix Puerariae Lobate* from *Radix Puerariae Thomsoniae*. Accepted by *Journal of Chromatography A.*.
5. Ying Li, **Dajian Yang**, Albert.S.C. Chan, et al. Pharmacokinetic, tissue distribution and excretion of puerarin and puerarin-phospholipid complex in rats. Accepted by Drug *Development and Industrial Pharmacy*

Patents filed

1. **Dajian Yang**, Albert S. C. Chan, Shilin Chen. Herbal Formulations for Modulating Blood Lipids (US and PCT patent)._US patent Application No. 10/925,674

   Filing date: 25 August 2004

2. **Dajian Yang**, Albert S. C. Chan, Shilin Chen Herbal Formulations for Modulating Blood Lipids (Chinese patent Application No. 200410094763.3

   Filing date: 18 November 2004

   Priority date: 25 Auggust 2004

3. **Dajian Yang**, Yueming Li, Albert S. C. Chan. Puerarin Derivatives and Their Medical Applications

   US patent Application No. 10/969,571

   Filing date: 20 October 2004

4. **Dajian Yang**, Yueming Li, Albert S. C. Chan. Puerarin Derivatives and Their Medical Applications

   Chinese patent Application No. 200410096209.9

   Filing date: 25 November 2004

5. **Dajian Yang**, Albert S. C. Chan, Shilin Chen. Process of puerarin phospholipids complex

   Chinese patent Application No. 200610008872.8

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Contents

Abbreviation

4Ac	Tetra-acetylpuerarin
5Ac	Penta-acetylpuerarin
6Ac	Hexa-acetylpuerarin
CM	Chinese medicine
FT	Fourier transform
HPLC	High performance liquid chromatograph
HRMS	High resolution mass spectroscopy
IR	Infrared
MS	Mass spectroscopy
NIR	Near Infrared (Spectroscopy)
NMR	Nuclear magnetic resonance
ORTEP	Oak Ridge thermal ellipsoid plot
PEG	Polyethylene glycol
PEG-P	Polyethylene glycol-puerarin
PPC	Puerarin phospholipid compound
Pur(Pue)	Puerarin
RSD	Relative standard deviation
TCM	Traditional Chinese medicine
TLC	Thin layer chromatograph
UV	Ultraviolet

Chapter 1 Introduction

1.1 The Significance of Chinese Medicine containing Radix Puerariae

1.1.1 Background

Radix Puerariae, a commonly used herb in traditional Chinese medicine (TCM), is derived from the dried root of *Pueraria lobata* (Willd.) Ohwi and *P. thomsonii* Benth. (Family Fabaceae). According to TCM classics, it has cool-property commonly used to treat exterior syndromes, relieve fever, promote the production of body fluid, facilitate eruption and arrest diarrhea. The Ministry of Health of P. R. China has included this material in the list of "herbs used as both food and drug".[1]

The earliest record of Radix Puerariae (**Figure 1.**) was found in *Shen Nong Ben Cao Jing* (*Shengnong's Chinese Materia Medica*, 神農本草經), in which Radix Puerariae was categorized as medium grade and described as 'sweet and acrid in taste, being able to relive all kinds of rheumatism and elevate *Yin-Qi* while acting as an antipyretic, anti-emetic and antidote (葛根味甘平。主消渴身大熱，嘔吐諸痹，起陰氣，解諸毒)'.[2]

Figure 1. *Radix Puerariae*

The description of the medical applications of Radix Puerariae also appeared in the Han dynasty (漢朝) and Tang dynasty (唐朝) when the root of *P. edulis* was refered as a medicinal herb. In the Song dynasty, the roots of *P. thomsonii* and *P. phaseoloides* were also used as medicinal hebs. However, it was found that, *P. lobata*

seemed to be the most popular species in the neoteric period.[3]

There existed many publications in which the property of Radix Puerariae in different dynasties was described. For example, in the Liang dynasty, Tao Hongjing remarked: *Currently, people mainly took the braised root of kudzu; it was most popular in Nan Kang and Lu Ling area, and it had more succulence and less veins, and a sweet taste. But it was not suitable for medical use*. It was proved that Radix Puerariae was both used as a medicine and a food, and that it was produced in great quantity in the Jiang Xi area during that period of time. When stating that it was 'succulent, less fibrous and sweet' (多汁，少筋，味甘) Tao Hongjing was probably referring to the *Pueraria thomsonii* Benth species. In the old days they were considered to be less effective as far as its medical function was concerned.[3]

Tu Jing Ben Cao (圖經本草), which was included in the re-composition of *Zheng He Jing Shi Zheng Lei Ben Cao* (重修政和經史證類本草), described for the first time the morphological characteristic of this plant: *A young, hairy perennial vine with a woody twinning stem of around 8 to 10 metersn length, purple in colour with green, trifoliate leaves; it blossomes without fruit in July while the configuration of the flower was somewhat like that of pea; its root is arm-like in shape and dark purple in colour* (Fig 2). This description corresponds to the characteristics of *Pueraria lobata* (Willd.) and *Pueraria thomsonii* Benth. Li Shizhen (李時珍) noted: The plant, which is often collected in July and August, can grew in

Figure 2. Leaves of *Pueraria lobata*

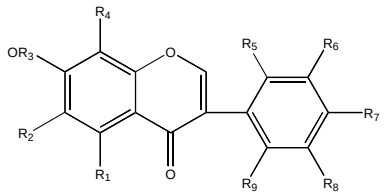
the wild or be cultivated, its root is purple outside and white inside; it produces large wisteria-like purple flowers on long racemes and has papery pods covered with a tawny down flat green beans like the stone of salted plum; it has a stinking taste when eaten raw; it is thus incorrect for Su Song to say that this plant is infructuous.[4] In addition to the description of its root, flower and fruit, Li Shizhen also recorded for the first time the morphological characteristic of the seed, and pointed out the mistakes Su Song made. Li Shizhen also discussed the wild type and the cultured type of Radix Puerariae. There were two pictures of Radix Puerariae in *Zhi Wu Ming Shi Tu Kao*, which coincided with the modern recordation in the Picture Book of Chinese Advance Plant and Flora of China.[3]

Radix Puerariae contains abundant nutritional and functional components such as starch, protein, isoflavone and other organic compounds. The isoflavone components, such as puerarin and daidzin, are considered to be the principal active chemicals in Radix Puerariae, which were reported to promote blood flow in the cardiovascular and cerebrovascular systems, reduce blood sugar level, improve human memory, inhibit cancer cells and induce the differentiation of cancer cells.[5]

1.1.2 Chemical constituents in Radix Puerariae

The major chemical constituents in Radix Puerariae are isoflavones, including daidzein, daidzin and puerarin;[6-26] Puerariae glycosides[27-31], being the ramification of dihydrochalcones, triterpenoids[32-35] of oleanene type, such as kudzusapogenol, sophoradiol, cantoniensistriol, soyasapogenol A/B; as well as alkaloids[36-38].

The content of flavonoids in Radix Puerariae could be as high as 12%, in which isoflavones is the major constituent. Up to now, fifty-two flavones have been isolated from the genus *Pueraria* (Fig. 3, Table 1):



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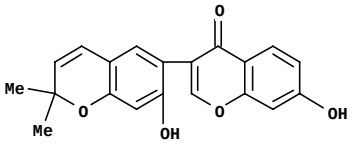

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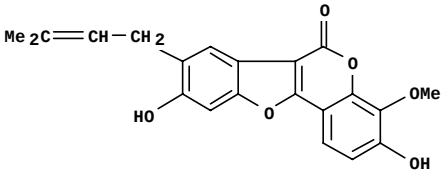

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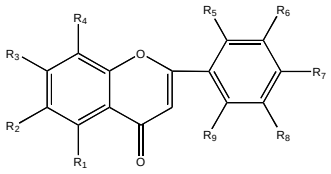

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Structures of Flavonoids from Radix Puerariae

No	**name**	**Structure**	**plant source**	**Reference**
1	Daidzin	R_1=R_2=R_4=R_5=R_6=R_8=R_9=H, R_3=Glu, R_7=OH	*P. lobata*	6
2	Formononetin	R_1=R_2=R_4=R_5=R_6=R_8=R_9=H, R_3=OH, R_7=OMe	*P. lobata*	6, 7
3	Puerarin	R_1=R_2=R_5=R_6=R_8=R_9=H, R_3=OH, R_4=Glu, R_7=OH	*P. lobata*	8
4	Mirificin	R_1=R_2=R_5=R_6=R_8=R_9=H, R_3=OH, R_4=Glu-Glu, R_7=OH	*P. tuberose*	9
5	3'-Methoxy puerarin	R_1=R_2=R_5=R_6=R_8=R_9=H, R_3=OH, R_4=Glu, R_6=Ome, R_7=OH	*P. lobata*	10
6	Daidzein	R_1=R_2=R_4=R_5=R_6=R_8=R_9=H, R_3=OH, R_7=OH	*P. lobata*	11
7	Daidzin	R_1=R_2=R_4=R_5=R_6=R8=R_9=H, R_3=Glu, R_7=OH	*P. lobata*	6
8	Genistein	R_2=R_4=R_5=R_6=R_8=R_9=H, R_1=R_3=R_7=OH	*P. peduncularis*	12
9	Tectorigenin	R_4=R_5=R_6=R_8=R_9=H, R_1=R_3=R_7=OH, R_2=OMe	*P. lobata*	6
10	Tectoridin	R_4=R_5=R_6=R_8=R_9=H, R_1=OH, R_2=OMe, R_3=Glu, R_7=OH	*P. lobata*	13

11	Glycitin	R_1= R_4=R_5=R_6=R_8=R_9=H, R_2=Ome, R_3=Glu, R_7=OH	*P. lobata*	6
12	Glycitein	R_1=R_4=R_5=R_6=R_8=R_9=H, R_2=Ome, R_3=Glu, R_7=OH	*P. peduncularis*	6
13	4H-1-Benzopyran-4-one, -(β-D-glucopyranosyl oxy)-5-hydroxy-3-(4-hydroxyphenyl)-(9CI)	R_2=R_4=R_5=R_6=R_8=R_9=H, R_1=OH, R_3=Glu, R_7=OH	*P. peduncularis*	12
14	Formononetin	R_1=R_2=R_4=R_5=R_6=R_8=R_9=H, R_3=OH, R_7=OMe	*P. lobata*	10
15	4H-1-Benzopyran-4-one, 5-hydroxy-3-(4-hydroxyphenyl)-6-methoxy-7-[(6-O-β-D-xylopyranosyl-β-D-glucopyranosyl)oxy]-(9CI)	R_4=R_5=R_6=R_8=R_9=H, R_1=OH, R_2=Ome, R_3=Glu-Glu, R_7=OH	*P. thunbergiana*	7
16	3-(4-Hydroxyphenyl)-6-methoxy-7-[(6-O-β-D-xylopyranosyl-β-D-glucopyranosyl)oxy]-(9CI)	R_1=R_4=R_5=R_6=R_8=R_9=H, R_2=Ome, R_3=Glu-Glu, R_7=OH	*P. thunbergiana*	7
17	Kwakhurin	R_1=R_2=R_4=R_6=H, R_3= R_5= R_7=OH, R_8=Ome, R_9= CH_2-CH=CMe_2	*P.mirifica*	14
18	6"-O-Malonyldaidzin	R_1=R_2=R_4=R_5=R_6=R_8=R_9=H, R_3=β-D-glu, R_7=OH	*P. lobata*	15
19	8-β-D-Glucopyranosyl-3-[4-(β-D-glucopyranosyl oxy)-3-hydroxyphenyl]-7-hydroxy- (9CI)	R_1=R_2=R_5=R_6=R_9=H, R_3=R_8=OH, R_4=R_7=β-D-Glu	*P.lobata*	15
20	3'-Methoxydaidzin	R_1=R_2=R_4=R_5=R_6=R_9=H, R_3=β-D-glu, R_8=OMe , R_7=OH	*P.lobata*	15
21	7-9CI	R_2=R_4=R_5=R_6=R_8=R_9=H, R_1=R_7=OH, R_3=[6-O-(carboxyacetyl)-β-D-glu]oxy	*P.lobata*	16
22	Propanedioic acid	R_1=R_2=R_5=R_6= R_8=R_9=H, R_3=R_7=OH, R_4=[6-O-(carboxyacetyl)-β-D-glu]oxy	*P. lobata*	16
23	Propanedioic acid	R_2=R_5=R_6=R_8=R_9=H, R_4=[6-O-(carboxyacetyl)-β-D-glu	*P. lobata*	16
24	Acacetin	R_2=R_4=R_5=R_6=R_8=R_9=H, R_1=R_3= OH, R_7=OMe	*P. lobata*	16

25	Ononin	R_1=R_2= R_4=R_5=R_6= R_8=R_9=H, R_7=Ome, R_3= [6-O-(carboxyacetyl)-β-D-glu]oxy	*P. lobata*	16
26	Biochanin A 7-O-glucoside 6"-O-malonate	R_2= R_4=R_5=R_6= R_8=R_9=H, R_1=OH, R_7=Ome, R_3= [6-O-(carboxyacetyl)-β-D-glu]oxy	*P. lobata*	16
27	Lupiwighteone	R_2=R_5=R_6=R_8=R_9=H, R_4=3-methyl-2-butenyl, R_1=R_3= R_7= OH	*P. lobata*	17
28	8-Prenyldaidzein	R_1=R_2=R_5=R_6=R_8=R_9=H, R_4=3-methyl-2-butenyl, R_3= R_7=OH	*P.lobata*	17
29	Isoflavone aglycon kwakhurin hydrate	R_1=R_2=R_4=R_6=H, R_3= R_5=R_7=OH, R_8=Ome, R_9=3-hydroxy-3-methylbutyl	*P. mirifica*	18
30	O-malonylglucoside	R_1=R_2=R_5=R_6=R_8=R_9=H, R_3= R_7=OH, R_4=(6-O-acetyl-β-D-glu)oxy	*P. mirifica*	18
31	7-(6-O-malonyl-β-D-glucopyranosyloxy) -3-(4-hydroxyphenyl)-4 H-1-benzopyran-4-one	R_1=R_2=R_4=R_5=R_6=R_8=R_9=H, R_3=β-D-glu, R_7=OH	*P. lobata*	19
32	Kakkalide	R_4=R_5=R_6= R_8=R_9=H, R_1=OH, R_2=R_7=OMe, R_3=(6-O-β-D-xyl-β-D-glu)oxy	*P. tuberose*	20
33	Puerarin I	R_1=R_2= R_5=R_6= R_8=R_9=H, R_4=R_3=R_7=OMe	*P. tuberose*	21
34	Puerarin f	R_1=R_2=R_5=R_6=R_8=R_9=H, R_4=CHO, R_3=R_7= OMe	*P. tuberose*	21
35	Biochanin A	R_2=R_4=R_5=R_6=R_8=R_9=H, R_1=R_3=OH, R_7= OMe	*P. lobata*	22
36	3'-Methoxydaidzein	R_1=R_2=R_4=R_5=R_6=R_9=H, R_3=R_7=OH, R_8= OMe	*P. lobata*	22
37	3'-Hydroxypuerarin	R_1=R_2=R_5=R_8=R_9=H, R_3=R_6=R_7=OH, R_4=β-D-glu	*P. lobata*	22
38	Sayanedin	R_1=R_2=R_4=R_5=R_6=R_9=H, R_3=R_8=OMe, R_7=OH	*P. lobata*	22
39	Cladrin	R_1=R_2=R_4=R_5=R_6=R_9=H, R_3=OH, R_7=R_8= OMe	*P. lobata*	22
40		R_1=R_2=R_5=R_8=R_9=H, R_3=R_7=OH, R_4=glu-glu, R_6=OMe	*P. lobata*	22
41	Glycerol 1-tetracosanoate	R_1=R_4=R_5=R_8=R_9=H, R_2=R_3=OMe, R_6=R_7=-O-CH_2-O-	*P. lobata*	23
42	Puerarone (I)		*P. tuberose*	24
43	Puerarostan (II)		*P. tuberose*	24
44	Apigenin	R_2=R_4=R_5=R_6=R_8=R_9=H, R_3=β-D-glu,	*P. lobata*	16

	7-O-malonylglucoside	R_1=R_7=OH		
45	Acacetin 7-O-malonyl glucoside	R_2=R_4=R_5=R_6=R_8=R_9=H, R_3= O-malonylglucoside, R_1=OH, R_7=OMe	*P. lobata*	16
46		R_2=R_4=R_5=R_6=R_7=R_8=R_9=H, R_3= β-D-glu, R_1= OH	*P. lobata*	16
47	Chrysin	R_2=R_4=R_5=R_6=R_7=R_8=R_9=H, R_1=R_3= OH	*P. lobata*	16
48	Rutin	R_2=R_4=R_5=R_8=R_9=H, R_1=R_3=R_6=R_7= OH, R_{10}=[6-O-(6-deoxy-α-L-man)-β-D-glu]oxy	*P. lobata*	25
49	Robinin	R_2=R_4=R_5=R_6=R_8=R_9=H, R_1=R_7= OH, R_3=β-D-glu, R_{10}=[6-O-(6-deoxy-α-L-man)-β-D-glu]oxy	*P. lobata*	25
50	Nicotiflorin	R_2=R_4=R_5=R_6=R_8=R_9=H, R_1=R_3=R_7=OH, R_{10}=[6-O-(6-deoxy-α–L-man)-β-D-glu]oxy	*P. lobata*	25
51	Apigenin	R_2=R_4=R_5=R_6=R_8=R_9=H, R_1=R_3=R_7=OH	*P. thunbergiana*	26

1.1.3 Analysis of Chemicals in Radix Puerariae

Radix Puerariae has been proved to be clinically effective in alleviating hypertension related symptoms such as neck stiffness, dizziness, coronary heart disease, sudden deafness, migraine and the blocking of retina vena and artery.[39]

Many analytical methods such as colorimetry, UV spectrophotometer, TLC-UV spectrophotometer, TLC- double wavelength scaning, HPLC and HPCE have been developed to determine the active constituents of Radix Puerariae. In a TLC analysis, Radix Puerariae was grounded and extracted with methanol, and the extract was applied and developed on a TLC plate using chloroform-methanol-water (7:4:0.25) as the mobile phase. The spots were scraped from the TLC plate and extracted with methanol. The fluorescent spot was measured under Ex value 355 nm and Em value

460 nm respectively. The linear range was 0.01-0.08 μg/ml (r=0.9998); the observed recovery rate was better than 98.0 % with RSD < 5 %. With the help of the TLC-Fluorescence spectrophotometer method, the average content of puerarin and the relative standard deviation were found to be 0.844 % and 2.06 %, respectively. By comparing with other analytical methods, this method had a much lower limit of detection and high sensitivity that confirmed the usefulness of this method for the microanalysis of puerarin.[40]

1.1.3 Natural Resourse

Eighteen species of *Pueraria* genus have been found around the world, most of which are distributed in the temperate or sub-torrid zone. They prefer to grow among bushes, in the sunny side of slopes at 100-2000 meters above sea level. China is the center of this genus. Almost nine species including two variaties are distributed in the mainland. These species are mainly distributed in Yunnan Province and its adjacent provinces.[41] Eight of them have been collected for medical use (Table 2).While the analytical results of the chemical constituents in Radix Puerariae are shown in Tables 3 to 9)

Table 2 Distribution of *Pueraria* spp. in China[41]

Species	Distribution
P. labata (Willd.) Ohwi	All over of China. In Anhui province, it is located mainly in the south of this province and Da Bie mountain
P. thomsonii Benth.	Guizhou, Guangxi, Guangdong and Yunnan provinces, currently most of them are cultivated, and there is also introductive cultivation in Jing county and Jinde of Anhui province
P. edulis Pump.	Yunnan, Guizhou, Sichuan and Guangxi provinces
P. phaseoloides (Roxb.) Benth.	Zhejiang, Fujian, Taiwan and Guangdong provinces
P. omeiensis Wang et Tang	Sichuan, Yunnan and Guizhou provinces
P. montana (Lour.) Merr.	Fujian, Guangxi, Guangdong and Taiwan provinces
P. peduncularis Grah.	Yunnan, Guizhou, Xizang and Guangxi provinces
P. alopecuroides Craib	Yunnan and Guizhou provinces

Table 3 Contents of total isoflavones in different species (colorimetry)[42,43]

Species	Contents of total isoflavones（%）
P. lobata	7.60
P. thomsonii	1.22
P. omeiensis	1.70
P. montana	0.80
P. phaseoloides	1.58
P. peduncularis	0.50

Table 4 Contents of total isoflavones in different parts of *Pueraria lobata* (colorimetry)[44]

Sample	Phloem	Xylem	Root
Total isoflavones（%）	5.60	3.96	4.81

Table 5 Contents of puerarin in Radix Puerariae from different regions (%) (HPLC method)[45]

P. lobata Jinan	2.81	Hubei	2.22	Anhui	2.90
Yidu	4.61	Guizhou	3.44	Shandong	3.00
Dezhou	2.02	Changsha	2.81	Shanxi	2.90
Youyang	6.57	Ankang	4.28	Hubei	1.50
Guzhang	3.97	Zhenping	7.78	*P. thomsonii*: Guangxi	0.61

Table 6 Contents of puerarin and daidzin in root and stem of Radix Puerariae (%) (HPLC method)[46]

Species		*P.omeiensis*	*P. phaseoloides*	*P. lobata*	*P. montana*	*P. thomsonti*	*P.edulis*
Puerarin	Root	2.30	0.74	3.52	0.51	5.03	0.94
	Stem	1.49	0.50	0.65	0.08	1.03	0.43
Daidzin	Root	0.49	0.18	0.63	0.57	1.04	0.22
	Stem	0.30	0.11	0.54	0.11	0.33	0.14

Table 7 Contents of puerarin, daidzin and daidzein in root and stem of Radix Puerariae (%) (Double wavelength scanning method) [47]

	Daidzein	Daidzin	Puerarin
Root	0.195	3.933	2.481
stem	0.059	0.714	4.315

Table 8 Contents of polysaccharides in Radix Puerariae (Phenol-sulfuric acid colorimetry) [48]

P. lobata	3.57-18.72 (Jinzhai, Anhui province)
P. thomsonii	15.38-38.73 (Fengxin, Jiangxi province)

Table 9 Contents of puerarin in Yufengningxin tablet (mg/tablet) (HPLC method)[49]

Beijing Tongrentang Co., Ltd	19.49	Medicinal material	
Hebei Kuangcheng Pharmaceutical Factory	21.21	*Pueraria lobata* (Willd.) Ohwi	2.36-4.55%
Tianjin Traditional Chinese Pharmaceutical Factory	18.50	*P. thomsonii* Benth	0.18-0.41%

Roasting is commonly used in the traditional processing of Radix Puerariae. Orthogonal test was introduced to study the content of flavones in the bran-baked Radix Puerariae, which was found to be influenced by temperature, time and quantity of bran.[50] And the optimum conditions were considered to be : 0.4g/g unit of bran, 160 °C, 40 minutes.

According to the results of modern research, *Pueraria lobata* can be widely planted in many places except Tibet, Xinjiang Autonomous Area and Qinghai Province.,

1.1.4 Application of Puerarin as Therapeutics

Study showed that Radix Puerariae could significantly relieve angina, improve the

electrocardiogram of myocardial ischemic and reduce the oxygen demand of myocardium.[51]

Another effective aglycone ingredient of Radix Puerariae was reported to reduce the dynamic index to an extent of 14%, lower the coronary vascular resistance, increase the circulation of coronary artery, which would benefit the oxygen supply balance of ischemic myocardium.[52]

As one of the active ingredients in Radix Puerariae, puerarin has been developed as a drug for the treatment of cardiovascular and cerebrovascular diseases and clinically used in mainland of China in the form of injection.

While peurarin won't reduce the branch coronary artery blood circulation of the ischemic region, it does have some effect to slow down the heart rate, enhance the myocardial contraction and lower the main artery pressure (MAP). Puerarin can reduce the tension-time index (TTI) and the rising speed of the left ventricle pressure (LV dP/dt). Furthermore, puerarin can lower the resistance of the exterior side coronary artery in the ischemic myocardium.[53]

The most significant effect of puerarin on the heart is its ability to improve myocardial ischemic. Intravenous infusion of puerarin preparation in acute myocardial ischemic dog could reduce the heart rate and arterial pressure without affecting the collateral coronary blood. The puerarin infusion also significantly lowered the tension time index and the rate of rise in the left ventricular pressure. Restoration of the systemic blood pressure to the same level that existed prior to the infusion results in a substantial increase in the collateral blood flow to the ischemic

myocardium. In addition, puerarin could dramatically reduce the secretion of lactic and CPK released from the myocardial when there is myocardial ischemic or infusion. Moreover, it could diminish the myocardial oxygen demand and water content when infused.[54- 56]

Puerarin could improve the 6-K-PGFla and high density lipid (HDL) level while reducing the TXB2/6-K-PGF1α ratio, resulting in the effects of anti-angina and anti-hypertension with the dilatation of the peripheral vascular reduction of the myocardial oxygen demand and the improvement of ischemic ECG.[54,57]

By comparison to similar preparations, namely Shengmai Injection (生脈注射液), Compound Danshen Injection (複方丹參注射液) and Fructose Sodium Diphosphate (果糖磷酸氫二鈉注射液), [58]etc., the puerarin injection is more effective on coronary heart diseases. [59] It could remove hydroxyl radical (·OH) more efficiently than Danshen Injection and Tetramethylpyrazine Injection.[60] For the treatment of acute ischemic cerebrovascular diseases, it is not as efficacious as defibrinogenase, but equivalent to Mailuoning (脈絡寧).[61]

1.2 Development of Radix Puerariae as New Drugs

1.2.1 Background

The medical application of Radix Puerariae was originally documented in *Shennong's Chinese Materia Medica*. Traditionally, Radix Puerariae was used as a component in many compound formulas, such as Gegen Tang, Gegen Qinlian Tang, etc. There existed no ancient records on the use of Radix Puerariae for cardiovascular

and cerebrovascular diseases, but it was used in some prescriptions for apoplexy.[62]

As described in the *Pharmacopoeia of the People's Republic of China*, Radix Puerariae has a sweet and pungent taste, and bears a cool nature based on the TCM theory. It mainly governs the spleen and stomach channels. It can expel evil factors from muscles, allay fever, promote production of body fluid, ameliorate skin rashes, stop diarrhea, and invigorate the vital function. It can be clinically used for exopathic fever, headache and neck stiffness, thirsty mouth, measles without adequate eruption, dysentery, alvine flux etc.[62]

In the early 1970's, researchers from the Chinese Academy of Medical Sciences began to use Radix Puerariae decoctum for the treatment of neck stiffness and hypertension. Trials indicated that its curative effect was rather encouraging. The chemical, pharmacological and clinical studies further indicated that the iso-flavonoids presented in Radix Puerariae could effectively cure cardiovascular and cerebrovascular diseases. Various preparations, including Yufen Ningxin Tablets and puerarin injection were developed. These formulations have been clinically applied for the treatment of coronary heart diseases, sudden deafness, hemicranias and retina artery or vein blockage.[39]

1.2.2 Preparations and products

By developing the HPLC method for determining the content of puerarin, different methods had been investigated and compared as far as the extraction of total flavones was concerned. An optimized process of extraction had been established[63] and AB-8 macroporous resin was found to be the best material for the purification of the

flavones of Radix Puerariae.[64]

The following are some preparations made from Radix Puerariae:

(1) Injection of Radix Puerariae (Pu Le Lin Injection)

2ml per ampoule, containing 100-200 mg puerarin

(2) Tablet of Radix Puerariae (tablet of *Radix Puerariae* extract, or tablet of flavones from Radix Puerariae, Yu Feng Ning Xin tablet)

10 mg, 50 mg or 100 mg total flavones

(3) Essential tablet of Radix Puerariae (Kang Xin tablet)

45 mg puerarin.

(4) Dadzein tablet from Radix Puerariae

25 mg dadzein each tablet.

(5) Solid dispersion capsules of dadzein from Radix Puerariae

30 mg of dadzein per capsule.

(6) Capsule of dadzein powder from Radix Puerariae

30mg of dadzein per capsule

1.3 Problems and solutions

1.3.1 Problems

Although substantial success in the clinical applications and market sales of Radix Puerariae products have been achieved during past decades, there are still a number of issues/problems associated with products made from Radix Puerariae to be addressed. (1) There exists no satisfactory method to assess and control the quality of Radix Puerariae raw material and crude drug. Development of an apropriate method

for the quality control of Radix Puerariae would be highly desirable. As one would expect that the chemical components or active ingredients contained in one species could be quite different from the other. For example, the puerarin content in *Pueraria lobata* (Willd.) Ohwi could be as high as ten time of that in *P. thomsonii* Benth., while both species are the official source of Radix Puerariae. Thus it is so important to identify the correct species of high quality that the quality and consistency of the preparations of Radix Puerariae can be properly appreciated. Over 15 chemical constituents of Radix Pueraria had been identified and it is possible and necessary to develop quality control method using multi-componet analysis and fingerprinting instead of using only puerarin and daidzein, currently available method of quality control for Radix Puerariae.

(2) Technologies to enhance the efficacy of Radix Puerariae preparations and the bioavailability of the active ingredients should be further developed. Radix Puerariae could have a wide range of medical applications, however, the only available product made from single plant material, Radix Puerariae is indicated for cardiovascular disease only. Therefore, it is desirable to expand the usage of such a useful herb by, for example, studying the effect-enhancing technologies, to develop new applications of Radix Puerariae preparations, and to improve the efficacy of existing products. Even the quality control of a single herb formulation of Radix Puerariae, such as Yufeng Ningxi Tablets, has left much to be demanded due to the complex nature of the chemicals in the extract/tablet. Futhermore, the efficacy of the oral dosage form is hampered, as research indicated, by the poor bioavailability of puerarin, the major

active chemical in Radix Puerariae preparations. While the injectable preparation of Radix Puerariae, such as puerarin injection, could have better efficacy, the adverse drug reaction of the product was reported to be the second highest among the injectable preparations made from Chinese medicinal herbs. Therefore, developing oral dosage forms of puerarin would become medically desirable and commercially viable.

1.3.2 Proposed Research Directions

(1) Developing better analytical method for quality control of Radix Puerariae

Systematic and comprehensive in-depth study on the following issues,

1. Measurement of the contents of total flavones, content of puerarin,

2. Identification method for active ingredients,

3. HPLC and TLC dual wavelength scanning fingerprint measurement for the Radix Puerariae crude materials,

(2) Studies on process and quality control methodology for standard extract

The quality of the extracts of Radix Puerariae on the market differs by their origin and extraction process. For some extracts, their fingerprints were found to be quite different from each other and markedly from that of the Radix Puerariae crude materials. It is necessary to carry out a systematic study on the optimum processing method to produce the standard extracts and the development of the qualitative and quantitative analytical method for the extracts. The co-relationship of the fingerprints and content of the chemical or active markers of the standardised extracts and that of Radix Puerariae should be established accordingly.

(3) Efficacy-enhancing and bioavailability improvement study

This is achieved through three different approaches, i.e. bioavailability and efficacy improvement by enhancing bioaffinity through chemical structure modification, bioavailability improvement by enhancing solubility with modern formulation process and efficacy improvement by synergistic reactions through skilled combination designs.

According to the correlationship analysis on the structure of puerarin and its analogues and the knowledge of drug absorption, distribution and excretion after oral administration, it can be speculated that the solubility property of puerarin, i.e., poor solubility in both water and non-polar organic solvents, may be related to the hydroxyl hydrophilic groups on the glucose moiety of its structure and that the low solubility of puerarin be the principal factor attributing to its poor bioavailability through oral administration. For this reason, it is of significance to develop the formulation technology to enhance its solubility and the chemical technology to modify the hydrophilic hydroxyl groups on the glucose moiety in the puerarin structure, so that the bioavailability of puerarin can be improved. New puerarin oral formula with high bioavailability may be developed with modern formulation technology such as the PEG dispersion and phospholipid compound formulation. The latter is also an important method for developing new puerarin preparations.

Effect improvement by specifically designed combinations is a measurement to strengthen the functions of Radix Puerariae. Radix Puerariae can adjust blood lipid and blood sugar levels, resist oxidation and myocardial ischemia, and improve blood circulation. It is a kind of TCM ingredient that is able to adjust the body function in many aspects but at a moderate degree. To develop any kind of new preparations, its clinical application scope must be defined. For the development of new preparations

made from Radix Puerariae, one or two kinds of functions must be selected for detailed studies. Medical combination can enhance its functions and generate better clinical efficacy for newly developed preparations. According to the TCM medical combination theory, the extracts from some TCM herbs may improve the function of Radix Puerariae to adjust the blood lipid level.

1.4 The Aim and Objective of the Project

This project aims to develop an adequate quality control technology for Radix Puerariae materials and find a scientific method to properly ensure the quality consistency of Radix Puerariae materials by a systematic study on the Radix Puerariae resource and their products. This project also aims to explore the empirical rules for effect-enhancing medical combination of Radix Puerariae, formulation technology of high-bioavailability puerarin oral formula, structural modification of puerarin for developing high-bioavailability derivatives of puerarin. The ultimate objective of this prject is to lay a foundation for innovative Chinese medicine based on Radix Puerariae.

In this thesis, the study on the control of the quality consistency of Radix Puerariae is described, along with the formulation and chemical modification technologies for improving the efficacy and bioavailability of puerarin.

Chapter 2 Quality Analysis and Control Methods of Radix Puerariae

2.1 Introduction

Though substantial successes on clinical application and market sales have been achieved, manifold problems on Radix Puerariae products are still present leaving a vast space for further research and development.

So far, there is a lack of an efficient way to assess and control the quality of the Radix Puerariae crude materials, and the development of an authenticated method for the quality control would be highly desirable. Becauce of a vast variety of the Radix Puerariae species, the chemical components or active ingredients contained in one species differ tremendously from the others. According to the previous research results, over 15 chemicals were identified from Radix Puerariae. With the current available method of quality control, which relied on mainly the measurement of a few chemical markers, such as puerarin, daidzein, it will not be considered sufficient to ensure the quality consistency of the Radix Puerariae crude materials. It is therefore of importance to develop a multi-component identification technique and a fingerprint identification technique for the Radix Puerariae crude materials and their preparations.

In China, there are a total of 9 *pueraria* species and 2 varieties distributed mainly in Yunnan Province and its adjacent areas. The main species growing at north of Yangtze River is *Pueraria lobata*. The major preparations of Radix Puerariae in the TCM market are mainly made from *Pueraria lobata* and *Pueraria thomsonii*. All

other species grow in the Southwest of China and a few species are locally used as TCM ingredients. According to chemical analysis, the contents of puerarin and flavones in *Pueraria lobata* are substantially higher than that in all other species. All those species are not suitable for TCM crude materials except *Pueraria lobata* and *Pueraria thomsonii*.

Currently, the quantity of produce of *Pueraria lobata* is rather large with superior quality in Huoshan County of Anhui Province (including Dabieshan Mountain area, such as Jinzhai County) and Ankang area of Shanxi Province of China. Some local enterprises can produce *Radix Puerariae* in a mass production scale.

2.2 Determination of Flavone/Isoflavone Content

2.2.1 Method: UV Spectrophotometry

Samples of *Pueraria lobata* and *Pueraria thomsonii* from different origins were subjected to this study. And puerarin (made by China National Institute for the Control of Pharmaceutical and Biological Products, Batch #: 752-200108, used for content determination) was used as Standard Reference Chemical. Analytically pure solvents were used in all the experiments, and the samples were analyzed with an UV/VIS Spectrometer (Model Lamba 35, Perkin Elmer).

2.2.2 Preparation of sample solution and reference material solution

a. Reference material solution

Puerarin sample was accurately weighed (0.0318 g), and was dissolved in ethanol. A 100 ml stock solution was prepared in a 100-ml volumetric flask.

b. Sample solution

Accurately weighing five samples of *Radix Puerariae* (100 mg for each group); and each sample was put into a flask. Methanol (50 ml) was added and the mixture was refluxed for one hour. The mixture was then filtered to remove the solid, and the residue, after the removal of methanol, was re-dissolved in 30% ethanol. The solution, after filtration to remove any insoluble substance, was diluted to a constant volume of 50 ml in a volumetric flask. A solution was prepared for flavone/isoflavone content measurement by diluting 0.07 ml of the sample to 10 ml (in a volumetric flask).

2.2.3 Methodology

All the samples were subjected to UV analysis at the absorption wavelength of 250 nm. The reference solution was first tested to get the relationship between sample concentration and UV absorbance. Thus, 150 μL, 200 μL, 250 μL, 300 μL, and 400 μL of the reference solution were accurately taken and diluted to 10 ml with 30% ethanol (in volumetric flasks). After the absorbance at 250 nm was measured, the data was calculated and a curve of absorbance versus concentration was prepared.

The results suggested that puerarin showed a proper linear relationship between the UV absorbance and its concentration within the concentration range of 4.8-12.7 μg/ml with a regression equation Y = 7.245594 X + 0.1. (r = 0.9991, n = 5, **Table 2-1**).

Table 2-1 Results of linear relationship of puerarin measured with UV spectrophotometer

	1	2	3	4	5
Puerarin Concentration(mg/ml)	0.0048	0.0064	0.0080	0.0095	0.0127
A	0.3574	0.4647	0.5890	0.6909	0.9082
r			0.999080		

Stability experiment was also carried out. Accurately measured reference solution (0.2 ml) was dissolved in 30% ethanol to 10 ml in a volumetric flask, and the UV spectra were taken every 30 minutes. The results indicated that the sample was stable within 3 hours with RSD of 0.19% (n=5, **Table 2-2**).

Table 2-2 Results of stability experiment

	1	2	3	4	5
Time	14:50	15:20	15:50	16:20	16:55
A	0.4718	0.4701	0.4698	0.4697	0.4698
RSD			0.19%		

Reproducibility experiments were carried out to validate the method. Accurately weighing five groups of the same sample of *Radix Pueraria*; 5 sample solutions were prepared the same as the preparation of the sample solution; and UV absorption was measured as per the specifications. The RSD was 2.5% (n=5, **Table 2-3**).

Table 2-3 Results of reproducibility

	1	2	3	4	5
Radix Pueraria (mg)	511	500	509	508	495
Flavone (mg)	80.71	77.14	80	82.86	75.71
Content of Flavone (%)	15.8	15.4	15.7	16.3	15.3
RSD			2.5%		

Precision experiment was carried out by consecutively taking 5 measurements of the absorbance value of the above #1 reproducibility sample solution with an UV spectrophotometer, the result is RSD=0.05% (n=5). The experiments indicated that this method can precisely measure the flavone/isoflavone contents of *Radix Puerariae*.

Table 2-4 Results of precision

	1	2	3	4	5
A	0.6566	0.6571	0.6572	0.6573	0.6575
RSD			0.05%		

Sample-addition recovery test was then carried out. Accurately measure six groups of content-known samples; certain amount of reference was added, and the recovery sample solution was prepared according to the method mentioned above. The results of recovery were calculated by taking the added amount from the measured results (**Table 2-5**).

Table 2-5 Results of sample-addition recovery test

	1	2	3	4	5	6
Sample (mg)	78.19	78.97	110.37	110.84	142.24	142.40
Addition (mg)	48.00	48.00	64.00	64.00	80.00	80.00
Measurement(mg)	128.75	130.00	173.33	180.00	227.50	225.00
Recovery	103.3%	103.8%	99.1%	104.7%	103.7%	101.8%
Average Recovery			102.7%			
RSD %			1.96			

2.2.4 Determination of Content of Flavone in *Radix Puerariae*

After establishing the relationship between UV absorbance and puerarin concentration, Radix Puerariae samples from different regions were subjected to the flavone/isoflavone content measurement. The UV absorbance of the sample was first measured with an UV spectrophotometer. The flavone contents of these samples were then calculated from the regression equation Y = 7.245594 X + 0.1. The results were shown in **Table 2-6** and **Figure 2-1**.

Table 2-6 Flavone Contents in *Pueraria lobata* & *Pueraria thomsonii* produced in different regions

Sample	No.	Origin	Flavone Content (%)	Average
Pueraria lobata	1	Shanxi (n=5)	10.4	7.57
	2	Jiangxi (n=5)	3.30	
	3	Nanjing (n=5)	9.00	
	4	Shenyang (n=5)	9.40	
	5	Huoshan, Anhui-1 (n=3)	7.00	7.24
	6	Huoshan, Anhui -2 (n=3)	8.10	
	7	Huoshan, Anhui -3 (n=3)	5.40	
	8	Huoshan, Anhui -4 (n=3)	7.40	
	9	Huoshan, Anhui -5 (n=3)	9.60	
	10	Huoshan, Anhui -6 (n=3)	6.10	
	11	Huoshan, Anhui -7 (n=3)	6.20	
	12	Huoshan, Anhui -8 (n=3)	6.20	
	13	Huoshan, Anhui -9 (n=3)	7.40	
	14	Huoshan, Anhui -10 (n=3)	8.70	
	15	Huoshan, Anhui -11 (n=3)	6.00	

	16	Huoshan, Anhui -12 (n=3)	6.30	
	17	Huoshan, Anhui -13 (n=3)	9.30	
Pueraria thomsonii	18	Huoshan, Anhui (n=5)	0.80	0.93
	19	Shenyang (n=5)	1.20	
	20	Nanjing (n=5)	0.80	
	21	Kunming (n=5)	0.80	
	22	Chengdu (n=5)	0.70	
	23	Jiangxi (n=5)	1.60	
	24	Kunming (n=5)	0.60	

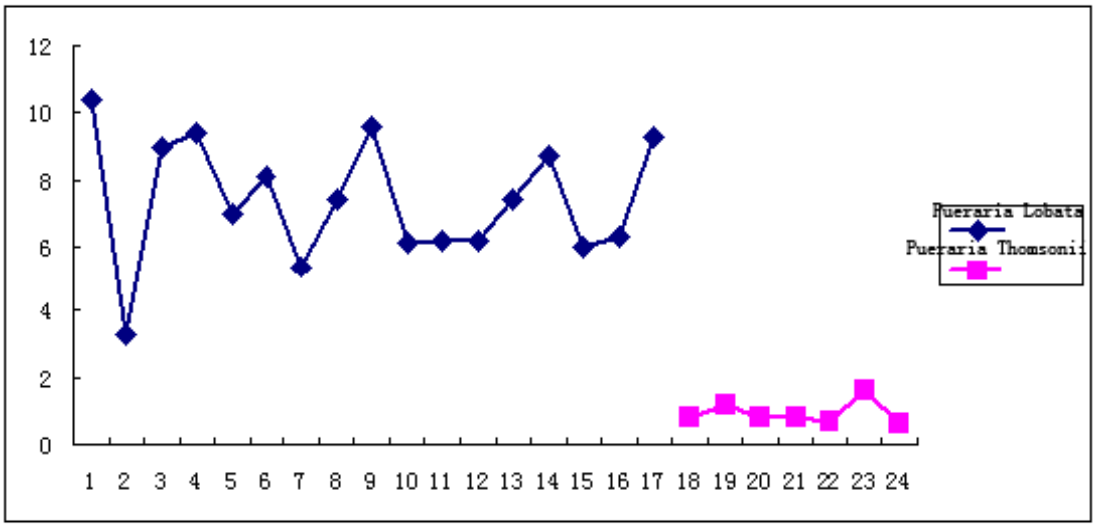


Figure 2-1 Flavone content in Radix Puerariae

The results indicated that the flavone content in *Pueraria lobata* and *Pueraria thomsonii* varied significantly from region to region. The average content of flavone in *Pueraria lobata* was 7.42%, and that in *Pueraria thomsonii* was only 0.93%. Flavone is the principal active ingredient of puerariae, and the content of flavone is positively correlated to the material quality. Therefore, *Pueraria lobata* is better than *Pueraria thomsonii* for medical purpose on the basis of the flavone content.

The flavone content in *Pueraria lobata* (or *Pueraria thomsonii*) from different regions differs dramatically. The flavone content in *Pueraria lobata* from Shanxi

was up to 10.4%, while only 3.3% was detected that from Jiangxi. The difference of the flavone content in samples produced in Huoshan of Anhui is small, and the average content is about 7.24%. This indicates that different environmental conditions in different areas have significant impacts on the quality of the materials, and *Pueraria lobata* produced in Huoshan of Anhui is more suitable for medical purpose regarding the consistency of the flavone contents.

2.3 Assay of Main Active ingredients in *Radix Puerariae*

2.3.1 Sample, Reference, Reagent and Instrument:

Pueraria lobata and *Pueraria thomsonii* were taken from the main origins across China. Several compounds such as C_1: 3'-hydroxypueraria, C_2: puerarin, C_3: puerarin-7-*O*-apioside, C_4: daidzin, C_5: genistin, C_6: formononetin-7-*O*-glucoside, C_7: puerarin-7-*O*-glucoside, C_8: daidzein (See **Figure 2-2** for structures of these compounds) were used as references.

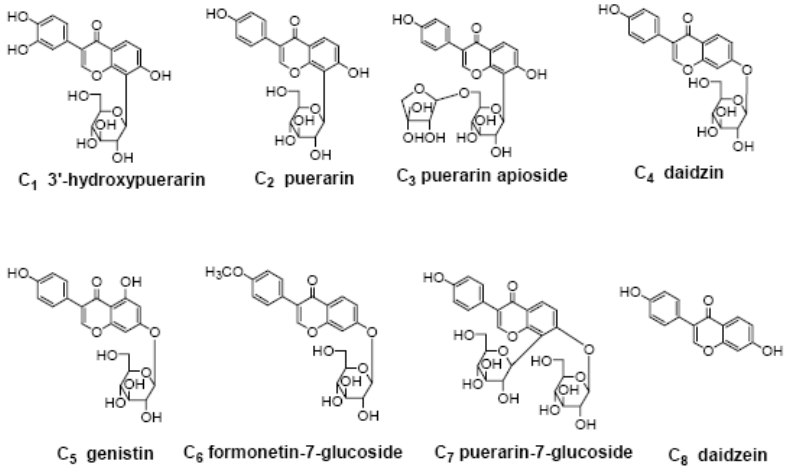


Figure 2-2 Chemical structures of the active ingredients in *Radix Pueraria*

The experiments were carried out on an Agilent HPLC instrument with a DAD diode array detector, and the data were processed with a HP1100 chromatography station.

Chromatographical grade methanol solvent was purchased from Fischer, and ethanol was analytically pure. Water was doubly distilled to exclude any possible metal ions, and the liquid phase was filtered through a 0.45 μm filter membrane before use.

2.3.2 Preparation of Sample Solution and Reference Solution

Reference solution was prepared first. At first, several reference compounds were weighed: C_1 (2.1 mg), C_2 (2.0 mg), C_3 (2.4 mg), C_4 (2.0 mg), C_5 (0.5 mg), C_6 (0.4 mg), C_7 (0.2 mg), C_8 (2.4 mg); and these accurately weighed compounds were dissolved in 30% ethanol at a constant volume of 25 ml in volumetric flasks. The mixture of the reference solution was prepared and filtered with a 0.45 μm filter membrane before use.

Sample solutions were also prepared. Accurately transfered 100 mg of Radix Puerariae powder (50 meshes) into a round-bottom flask; accurately added 100 ml of 30% ethanol solution and measured the weight. The mixture was refluxed for 30 minutes; and the weight of the mixture was maintained by adding 30% ethanol. The mixture was thoroughly stirred and filtered. The mixture was further filtered with a 0.45 μm filter membrane before subjecting to the HPLC analysis.

2.3.3 Methodology

The HPLC experiments were carried out using Zobax XDB-C18 (4.6 mm × 250 mm, 5 μm) as stationary phase and water/methanol as mobile phase with gradient elution at a flow rate of 0.5 ml/min. The sample was detected at 250 nm using an UV detector with an injection volume of 20 μL, and the HPLC pattern was shown in **Figure 2-3**.

Gradient elution conditions:

0~10 min: B (25%~30%)

20~50 min: B (30%~80%)

50~60 min: B (80%~100%)

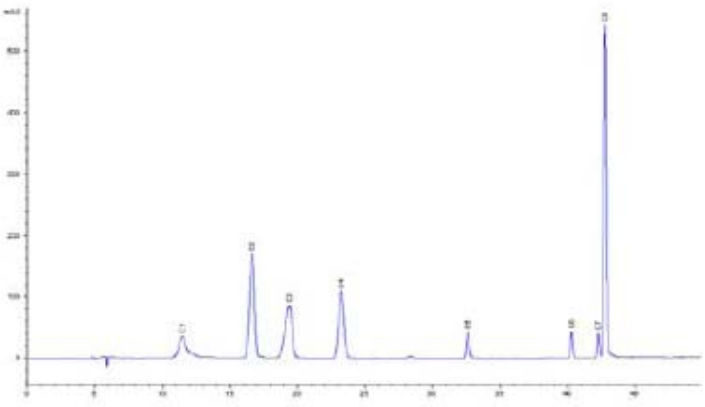


Figure 2-3 HPLC Chromatogram and Specifications of References

Linear relationship between the flavonoids/isoflavonoids concentration and the integrated HPLC peak areas were observed for all the reference samples (**Table 2-7**). The mixtures of reference solution to the following volume: 0.8, 1.6, 2.4, 3.2, 4.0, 4.8 ml were first measured, and each sample was diluted with 30% ethanol to 5 ml.

Table 2-7 Linear relationships between the HPLC integration peak areas and the reference sample concentrations

Reference	Linear Equation	Co-efficient factors	Linear Range (μg)
C_1	Y=73.73555X ─ 135.00845	r = 0.99687	0.26~1.62
C_2	Y=165.04816X ─ 59.58439	r = 0.99947	0.25~1.54
C_3	Y=100.04325X ─ 72.79888	r = 0.99917	0.20~1.85
C_4	Y=120.96193X ─ 80.66717	r = 0.99949	0.25~1.54
C_5	Y=128.15012X ─ 30.85446	r = 0.99910	0.06~0.39
C_6	Y=93.62508X ─ 9.43401	r = 0.99793	0.05~0.31
C_7	Y=164.89503X ─ 6.54282	r = 0.99881	0.02~0.16
C_8	Y=226.84228X ─ 81.48411	r = 0.99909	0.20~1.85

After establishing the linear relationship between the reference sample concentration and the HPLC integrated peak areas, samples of *Puerarie lobata* and *P. thomsonii* were also subjected to the HPLC analysis, and the HPLC patterns were shown in **Figure 2-4** and **Figure 2-5**. The HPLC analysis clearly indicated that all the eight reference compounds could be detected from the *Radix Puerariae*, but the contents of the active ingredients were different.

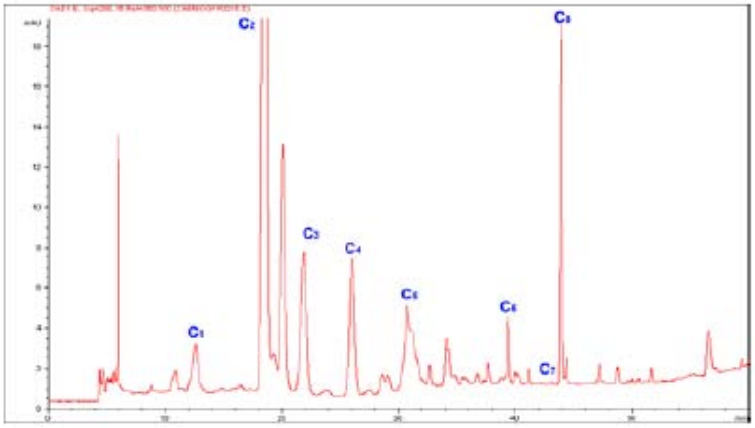


Figure 2-4 HPLC pattern of *Pueraria lobata*

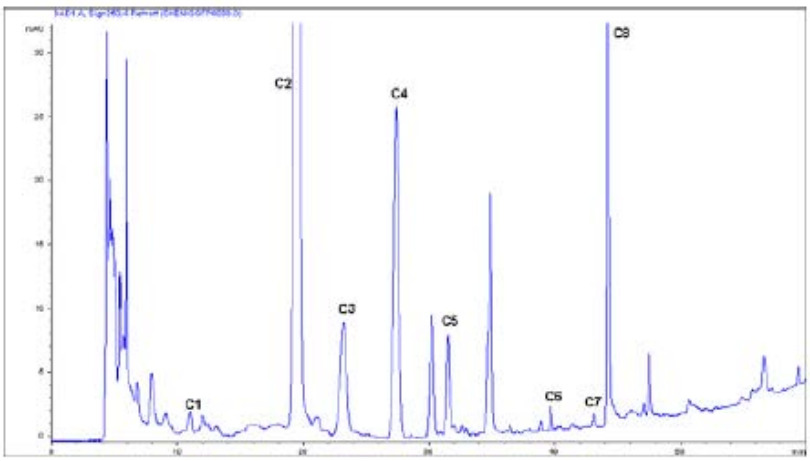


Figure 2-5 HPLC spectra of *Pueraria thomsonii*

Precision test was carried out by consecutively injecting the reference solution for six times, and subsequently measuring the peak area of each reference solution. As shown in **Table 2-8**, good consistency could be observed for all eight reference compounds.

Table 2-8 Results of peak area measured in reference precision test

	1	2	3	4	5	6	Ave	RSD%
C_1	17.9499	17.5824	17.6434	17.6860	17.9595	17.50379	17.721	1.08
C_2	26.2817	26.7818	26.5724	26.7558	26.6066	26.63726	26.606	0.67
C_3	31.3518	31.9926	31.6953	31.9496	31.7247	31.77502	31.7428	0.80
C_4	22.7114	23.2114	23.0699	23.7327	23.6688	24.02001	23.4024	2.08
C_5	5.0071	5.0086	5.0777	5.3780	5.4067	5.56252	5.2401	4.56
C_6	5.1083	5.2367	5.1898	5.2520	5.2171	5.17889	5.1971	0.99
C_7	2.4568	2.5172	2.5848	2.5048	2.2528	2.51550	2.4720	4.65
C_8	29.6717	30.8745	30.0342	30.7685	30.6862	30.51219	30.4245	1.55
Total	140.5387	143.2052	141.8673	144.0275	143.5224	143.7052	142.779	0.99

Stability test was carried out by analyzing the reference solution at 0, 2^{nd}, 4^{th}, 6^{th}, 8^{th},

and 12th hours. The calculated RSD for each peak area was less than 5%, indicating that the reference sample was stable enough within 12 hours.

Reproducibility test was carried out by measuring 6 groups of the Anhui-1 sample. The sample solution was prepared as the method for the preparation of reference solution, and the RSDs were calculated after the HPLC analysis of the sample solution. As shown in **Table 2-9**, the RSD for each peak area was less than 5%, indicating that the experiments could be well re-produced.

Table 2-9 Results of peak area measured in reference reproducibility test

	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
1	0.326	3.073	1.000	0.732	0.095	0.035	0.037	0.130
2	0.355	3.045	0.955	0.671	0.098	0.033	0.039	0.142
3	0.358	3.010	0.956	0.662	0.093	0.030	0.038	0.135
4	0.358	3.055	0.982	0.668	0.091	0.038	0.040	0.157
5	0.342	2.989	0.933	0.656	0.089	0.035	0.039	0.167
6	0.336	2.951	0.964	0.657	0.100	0.032	0.041	0.124
Ave	0.346	3.021	0.965	0.674	0.093	0.034	0.039	0.142
RSD(%)	3.840	1.520	2.420	4.280	4.480	1.020	4.050	3.130

Recovery test also gave good results. Five groups of samples with the amount of contents known were accurately taken and dissolved in the reference solution as per the preparation of the sample solution. The sample-added recovery sample solution was prepared by adding a specified amount of the reference compounds to the prepared solution, and the recovery data were calculated based on the HPLC results.

As shown in **Table 2-10**, the recovery for all the reference compounds were over 95%.

Table 2-10 Results of recovery measurement (n=5)

	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
Average Recovery(%)	95.7	98.5	97.5	99.5	96.1	96.3	95.1	95.7
RSD(%)	2.8	1.4	0.9	0.9	3.0	1.7	4.0	3.5

2.3.4 Determination of active ingredients in *Radix Puerariae*

After establishing the linear relationship between the concentrations and HPLC peak areas for all the major components, the active ingredients of Radix Puerariae from different regions were determined based on the HPLC analysis. The solution of Radix Puerariae samples was prepared using crude Radix Puerariae from different regions as per the method for sample solution preparation. The contents of active ingredients could be calculated based on the equations in **Table 2-7**. The results are shown in **Table 2-11**.

Table 2-11 Contents of active ingredients in Radix Puerariae from different regions (n=5)

		C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8
Pueraria lobata	Yun'nan	0.373	2.723	0.875	0.582	0.164	0.043	0.012	0.638
	Shanxi	0.342	4.500	0.493	0.574	0.045	0.032	0.000	0.117
	Nanjing	0.775	5.577	0.622	0.540	0.120	0.038	0.040	0.155
	Shenyang	0.563	7.493	0.662	1.063	0.203	0.044	0.036	0.115
	Jiangxi	0	1.624	0	0.397	0.273	0	0	0.208
	Houshan1	0.346	3.021	0.965	0.674	0.093	0.034	0.039	0.142

	Houshan 2	0.472	5.262	0.937	0.362	0.114	0.020	0	0.082
	Houshan3	0.352	3.694	0.462	0.386	0.105	0.031	0.000	0.097
	Houshan 4	0.357	4.620	0.438	0.407	0.080	0.090	0.007	0.241
	Houshan 5	0.666	6.253	0.800	0.619	0.031	0.033	0.010	0.086
	Houshan 6	0.369	3.276	0.649	0.272	0.026	0.028	0.006	0.117
	Houshan 7	0.391	4.115	0.412	0.407	0.085	0.029	0.035	0.125
	Houshan 8	0.309	4.300	0.340	0.448	0.098	0.070	0	0.123
	Houshan 9	0.380	4.680	0.602	0.532	0.152	0.027	0.034	0.084
	Houshan 10	0.467	6.218	0.317	0.862	0.189	0.042	0	0.113
	Houshan 11	0.243	3.909	0.288	0.427	0.126	0.028	0	0.181
	Houshan 12	0.325	4.049	0.664	0.332	0.118	0.019	0	0.093
	Houshan 13	0.463	3.250	1.141	0.643	0.023	0.042	0.012	0.019
Pueraria thomsonii	Guangzhou	0	0.560	0.026	0.090	0.004	0.004	0.004	0.089
	Shenyang	0	0.973	0.051	0.136	0.004	0.005	0.004	0.086
	Nangjing	0	0.050	0.027	0.072	0.004	0.004	0.004	0.058
	Yun’nan	0	0.700	0.022	0.030	0.003	0.003	0.021	0.082
	Chengdu	0.026	0.483	0.024	0.043	0.024	0.003	0.004	0.041

With the use of gradient elution, the baseline separation of flavone in Radix Puerariae can be achieved, and the content of most flavones can be determined simultaneously. The precision and reproducibility of this method is desirable.

Results indicated that the contents of flavonoids and puerarin in *Pueraria lobata* and *Pueraria thomsonii* differed dramatically. The content of puerarin in Radix Puerariae from main planting base normally fell in the range of 2.7%~7.5%. All these contents were much higher than that as specified in Pharmacopoeia of the P.R. China. The total flavonoid content of Radix Puerariae from Shenyang (Located in northern

China), 7.49%, was the highest, and it was in accordance with descriptions in previous literatures. The average total flavonoid content of Radix Puerariae from Huoshan Anhui was 4.36%, and had only small variations among different samples (**Figure 2-6**), indicating that Radix Puerariae produced in this region was more preferable

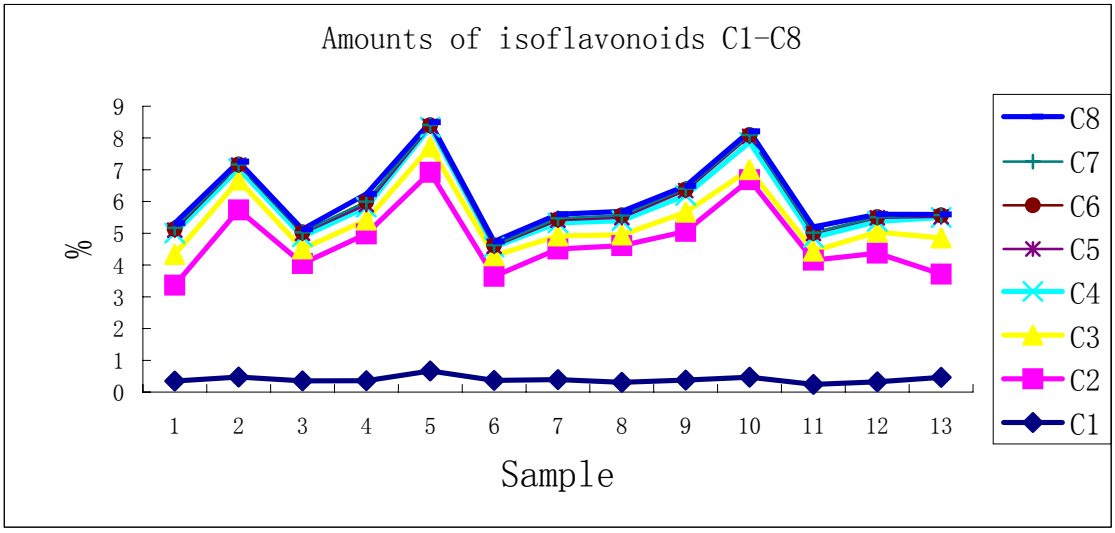


Figure 2-6 Variations of flavonoids in Radix Puerariae from Anhui province

2.4 Study on Radix Puerariae Fingerprint

The principal active ingredient in Radix Puerariae is isoflavone. The study results indicated that the content of the active ingredients contained in Radix Puerariae materials differed substantially with the origin, climate and ecological environment where the herb was harvested. The quality could not be sufficiently controlled if only a limited number of active ingredients in the herb were measured in the qualitative and quantitative analyses. At present, identification by fingerprint has been globally

recognized as the most effective means for the quality control of traditional Chinese medicinal materials and Chinese patent medicines. Radix Puerariae collected from Huoshan of Anhui was inspected, and its fingerprint was established.

Agilent HPLC instrument equipped with a DAD diode array detector was used, and a HP1100 chromatogram station was used for data processing. The HPLC solvents were purchased from Fischer (HPLC grade), and the others were chemically pure. The water used was of super clean water quality, and the herb was *Pueraria lobata* originated from Huoshan, Anhui, China.

The HPLC experiments were carried out using Zorbax XDB-C_{18} (4.6 mm × 250 mm, 5 μm) as stationary phase, and water/methanol were used as mobile phase with gradient elution.

0 min:	B (25%)
10 min:	B (30%)
20 min:	B (30%)
50 min:	B (80%)
60 min:	B (100%)

Flow rate: 0.5 ml/min; wave length: 250 nm, injection volume: 20 μL

Puerarin was used as the reference. A reference solution of 20 μg/ml was prepared by diluting the precisely weighed sample to a certain volume. The sample to be analyzed was prepared from 100 mg of Radix Puerariae powder (50 meshes). The sample was transferred to a round bottom flask; 100 ml of 30% ethanol solution was accurately injected and the total weight was measured. The mixture was refluxed for

30 min and the weight was maintained through the addition of 30% ethanol. The mixture was stirred and filtered, and the obtained solution was further filtered with a 0.45 μm filter membrane.

HPLC analysis of each sample was carried out by injecting 20 μL of the sample solution, and the results were calculated based on the integral area of each peak. Blank test was first carried out by analyzing 20 μl of 30% ethanol, and the comparison experiments were carried out by analyzing 20 μL of the reference solution. Precision test of the samples was realized by consecutively injecting six times of the same sample solution. The total number of common peaks detected was 15, and the peak area RSD for all common peaks was less than 5% (**Tables 2-12** to **2-16**). These results suggested that the precision was well acceptable.

Table 2-12 Retention time of common peaks of *Pueraria lobata*

Peak No.	Retention Time					
	1	2	3	4	5	6
1	10.16	9.64	10.02	9.87	10.19	10.12
2	11.96	11.32	11.76	11.57	11.99	11.88
3	17.46	16.62	17.23	16.88	17.54	17.33
4	18.09	17.38	17.91	17.53	18.24	18.01
5	18.97	18	18.81	18.4	19.09	18.89
6	20.4	19.5	20.21	19.73	20.54	20.27
7	27.73	26.28	27.55	26.88	27.74	27.32
8	29.59	28.82	29.57	29.12	29.72	29.41
9	31.87	31.79	32.03	31.45	32.05	31.78
10	38.93	38.91	38.79	38.68	38.88	38.73
11	39.47	39.42	39.29	39.11	39.37	39.21
12	40.71	40.7	40.57	40.44	40.66	40.52
13	43.97	43.93	43.77	43.63	43.86	43.71
14	46.81	46.8	46.59	46.44	46.65	46.47
15	51.32	51.29	51.15	51.04	51.21	51.06

Table 2-13 Relative retention time of common peaks, precision test

Peak	Relative Retention Time							
	1	2	3	4	5	6	Average	RSD
1	0.58	0.58	0.58	0.58	0.58	0.58	0.582	0.16%
2	0.68	0.68	0.68	0.69	0.68	0.69	0.684	0.16%
3	1	1	1	1	1	1	1	0.00%
4	1.04	1.05	1.04	1.04	1.04	1.04	1.04	0.28%
5	1.09	1.08	1.09	1.09	1.09	1.09	1.088	0.29%
6	1.17	1.17	1.17	1.17	1.17	1.17	1.171	0.19%
7	1.59	1.58	1.6	1.59	1.58	1.58	1.586	0.75%

8	1.69	1.73	1.72	1.72	1.69	1.7	1.71	1.55%
9	1.82	1.91	1.86	1.86	1.83	1.83	1.853	3.01%
10	2.23	2.34	2.25	2.29	2.22	2.24	2.261	4.26%
11	2.26	2.37	2.28	2.32	2.24	2.26	2.289	4.29%
12	2.33	2.45	2.35	2.4	2.32	2.34	2.364	4.47%
13	2.52	2.64	2.54	2.58	2.5	2.52	2.551	4.83%
14	2.68	2.82	2.7	2.75	2.66	2.68	2.715	5.28%
15	2.94	3.09	2.97	3.02	2.92	2.95	2.98	5.71%

Table 2-14 Peak area of common peak, precision test

Peak No.	Peak Area					
	1	2	3	4	5	6
1	24.66	31.46	24.03	23.46	25.61	23.93
2	107.38	150.91	136.49	147.5	130.85	133.1
3	4533.56	4523.66	4462.05	4532.5	4434.78	4376.98
4	145.48	140.68	142.09	143.5	164.3	125.84
5	245.94	184.16	186.55	205.24	189.79	173.27
6	853.33	840.6	836.01	866.35	818.67	850.41
7	788.13	0	743.54	752.11	736.91	738.79
8	108.72	38.95	62.29	60.22	77.01	120.73
9	328.24	168.94	165.33	170.07	227.47	169.45
10	69.36	29.94	105.73	83.1	69.38	129.47
11	27.56	15.44	37.26	43.23	23.81	24.12
12	34.16	18.15	32.67	83.06	17.96	18.11
13	27.41	847.51	163.83	26.61	33.32	31.02
14	13.74	24.75	35.59	21.41	11.24	9.79
15	17.42	17.61	18.1	18.88	11.52	12.22

Table 2-15 Peak area ratios of common peaks, precision test

Peak No.	Peak Area Ratio						Average	RSD%
	1	2	3	4	5	6		
1	0.01	0.01	0.01	0.01	0.01	0.01	0.006	0.06%
2	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.31%
3	1	1	1	1	1	1	1	0.00%
4	0.03	0.03	0.03	0.03	0.04	0.03	0.032	0.25%
5	0.05	0.04	0.04	0.05	0.04	0.04	0.044	0.49%
6	0.19	0.19	0.19	0.19	0.18	0.19	0.189	0.33%
7	0.17	0	0.17	0.17	0.17	0.17	0.14	6.28%
8	0.02	0.01	0.01	0.01	0.02	0.03	0.017	0.65%
9	0.07	0.04	0.04	0.04	0.05	0.04	0.046	1.29%
10	0.02	0.01	0.02	0.02	0.02	0.03	0.018	0.72%
11	0.01	0	0.01	0.01	0.01	0.01	0.006	0.20%
12	0.01	0	0.01	0.02	0	0	0.008	0.50%
13	0.01	0.19	0.04	0.01	0.01	0.01	0.042	6.60%
14	0	0.01	0.01	0	0	0	0.004	0.20%
15	0	0	0	0	0	0	0.004	0.06%

Table 2-16 Similarity calculation in precision test

	Calculation Results					
	1	2	3	4	5	6
Similarity	0.99	0.99	0.99	0.99	0.99	0.99

Reproducibility experiments were carried out by analyzing six samples of the same solution (Huoshan-Anhui-1). The solution was prepared the same as that as described in **Section 2.3**, and the HPLC analysis of these solutions showed that the relative peak area RSD of common peaks was less than 5%, indicating the reproducibility of the experiments.

Stability experiment was carried out by analyzing the sample solution (Huoshan, Anhui-1) after standing the samples for 0, 2, 4, 6, 8, and 12 hours. The relative peak area RSD of common peak was less than 5%, indicating that the sample solution was stable within 24 hours.

Fingerprint and technical parameters were then established using an analysis software of fingerprint similarity developed by Zhejiang University. The analysis on the relevant parameters from the HPLC analysis of 10 batches of sample solution was carried out, and the number of common peaks was 15 (**Tables 2-17** to **2-20**).

Table 2-17 Retention time of common peaks during hplc test, 10 batches of *Pueraria lobata* from Huoshan, Anhui

Peak No.	Retention Time									
	1	2	3	4	5	6	7	8	9	10
1	10.64	10.68	10.73	10.73	10.73	10.2	10.74	10.91	10.77	10.73
2	12.58	12.65	12.67	11.68	12.67	12.02	12.73	12.87	12.66	12.67
3	18.50	18.77	17.04	17.07	17.09	17.63	18.75	19.11	18.69	18.68
4	19.29	19.65	18.56	18.59	18.6	19.21	19.57	19.94	19.49	19.51
5	20.08	20.39	20.23	20.23	20.23	20.23	20.31	20.7	20.26	20.23
6	21.86	22.14	19.88	19.92	19.92	20.76	22.23	22.76	22.17	22.11
7	25.98	25.74	26.37	26.37	26.37	24.74	26.52	27.14	26.48	26.37
8	30.7	30	29.62	29.07	29.07	30.03	30.97	31.21	30.88	30.85
9	32.64	31.72	31.37	31.33	31.45	32.38	32.58	32.82	32.62	32.5
10	34.05	33.57	33.09	33.04	33.15	33.59	34.28	34.51	34.24	34.22
11	37.64	37.06	36.48	36.43	36.95	37.42	37.74	37.85	37.69	37.68
12	39.35	38.76	38.69	38.62	38.69	39.15	39.47	39.58	39.43	39.43
13	39.9	39.23	39.18	39.11	39.18	39.68	40.03	40.14	39.98	39.98
14	41.12	40.55	40.49	40.42	40.49	40.93	41.22	41.35	41.2	41.2
15	43.88	43.09	43.05	42.99	43.07	43.67	43.98	44.14	43.95	43.94

Table 2-18 Relative retention time of common peaks during HPLC test, 10 batches of *Pueraria lobata* from Huoshan, Anhui

Peak No.	1	2	3	4	5	6	7	8	9	10	Average
1	0.58	0.57	0.63	0.63	0.63	0.58	0.57	0.57	0.58	0.57	0.59
2	0.68	0.67	0.74	0.68	0.74	0.68	0.68	0.67	0.68	0.68	0.69
3	1	1	1	1	1	1	1	1	1	1	1
4	1.04	1.05	1.09	1.09	1.09	1.09	1.04	1.04	1.04	1.04	1.062
5	1.09	1.09	1.19	1.19	1.18	1.15	1.08	1.08	1.08	1.08	1.12
6	1.18	1.18	1.17	1.17	1.17	1.18	1.19	1.19	1.19	1.18	1.179
7	1.4	1.37	1.55	1.55	1.54	1.4	1.41	1.42	1.42	1.41	1.45
8	1.66	1.6	1.74	1.7	1.7	1.7	1.65	1.63	1.65	1.65	1.669
9	1.76	1.69	1.84	1.84	1.84	1.84	1.74	1.72	1.75	1.74	1.775
10	1.84	1.79	1.94	1.94	1.94	1.9	1.83	1.81	1.83	1.83	1.865
11	2.03	1.97	2.14	2.13	2.16	2.12	2.01	1.98	2.02	2.02	2.06
12	2.13	2.07	2.27	2.26	2.26	2.22	2.11	2.07	2.11	2.11	2.161
13	2.16	2.09	2.3	2.29	2.29	2.25	2.13	2.1	2.14	2.14	2.19
14	2.22	2.16	2.38	2.37	2.37	2.32	2.2	2.16	2.2	2.21	2.259
15	2.37	2.3	2.53	2.52	2.52	2.48	2.35	2.31	2.35	2.35	2.407

Table 2-19 Peak area in HPLC , 10 batches of *Pueraria lobata* from Huoshan, Anhui

Common Peak No.	Peak Area									
	1	2	3	4	5	6	7	8	9	10
1	21.94	51.23	21.8	29.08	24.76	13.56	41	59.8	22.35	67.57
2	165.78	443.14	182.07	188.33	170.87	144.07	232.32	292.61	110.03	252.99
3	4535.7	6163.8	3846.6	4030.5	3661.9	4272.6	4641.8	6383.4	3960.9	4951.5
4	153.88	300.33	118.58	155.79	155.53	518.66	199.58	164.91	109.94	327.1
5	700.91	407.87	618.95	651.78	747.34	537.41	244.87	715.05	436.72	433.1
6	695.73	1318	618.95	651.78	747.34	537.15	987.59	509.53	453.57	1505.6
7	481.85	766.47	451.23	459.55	453.32	537.41	646.48	1139.8	521.47	392.22
8	518.94	824.63	381.19	642.06	168.78	317.03	394.55	153.23	59.1	228.97
9	49.01	28.61	12.35	14.02	26.91	31.61	26.34	86.39	32.83	30.21
10	88.79	4.57	120.45	125.14	6.26	106.27	178.91	237.34	148.3	125.67
11	45.03	42.16	9.84	11.66	13.8	27.15	55.55	134.49	47.73	78.46
12	68.89	51.53	61.1	62.33	48.97	60.6	18.3	36.69	20.39	11.72
13	17.26	26	20.35	23.11	23.87	29.02	25.9	32.39	19.99	23.12
14	22.35	46.26	15.6	16.28	13.49	13.44	19.73	54.26	14.1	22.95
15	464.48	116.75	193.16	198.19	160.94	197.54	133.43	188.66	302.06	127.61

Total Area of Peak	8030.5	10591	6672.3	7259.6	6424.1	7343.5	7846.4	10189	6259.5	8578.8
Total of Peak Area	8524.6	11093	6964.8	7420.6	6639.2	7546.5	8341.9	11003	6825.3	9475.2
Proportion of Peak	94.20%	95.48%	95.80%	97.83%	96.76%	97.31%	94.06%	92.60%	91.71%	90.54%

Note: The average proportion of peak area in all peak area is 94.63%, and RSD is 2.59%.

Table 2-20 Relative peak area in HPLC measurement, 10 batches of *Pueraria lobata* from Huoshan Anhui

Peak No.	1	2	3	4	5	6	7	8	9	10	Average	RSD%
1	0	0.01	0.01	0.01	0.01	0	0.01	0.01	0.01	0.01	0.01	0.42%
2	0.04	0.07	0.05	0.05	0.05	0.03	0.05	0.05	0.03	0.05	0.05	2.47%
3	1	1	1	1	1	1	1	1	1	1	1	0.00%
4	0.03	0.05	0.03	0.04	0.04	0.12	0.04	0.03	0.03	0.07	0.048	2.69%
5	0.15	0.07	0.16	0.16	0.2	0.13	0.05	0.11	0.11	0.09	0.12	4.60%
6	0.15	0.21	0.16	0.16	0.2	0.13	0.21	0.08	0.11	0.3	0.173	3.64%
7	0.11	0.12	0.12	0.11	0.12	0.13	0.14	0.18	0.13	0.08	0.12	2.05%
8	0.11	0.13	0.1	0.16	0.05	0.07	0.08	0.02	0.01	0.05	0.08	4.52%
9	0.01	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.007	0.31%
10	0.02	0	0.03	0.03	0	0.02	0.04	0.04	0.04	0.03	0.025	1.31%
11	0.01	0.01	0	0	0	0.01	0.01	0.02	0.01	0.02	0.009	0.57%
12	0.02	0.01	0.02	0.02	0.01	0.01	0	0.01	0.01	0	0.01	0.51%
13	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0	0.005	0.09%
14	0	0.01	0	0	0	0	0	0.01	0	0	0.005	0.17%
15	0.1	0.02	0.05	0.05	0.04	0.05	0.03	0.03	0.08	0.03	0.047	2.41%

The HPLC fingerprints of Radix Puerariae were also shown in **Figures 2-7** and **2-8**. As shown in **Figures 2-7**, all the 15 active ingredients could be detected, and **Figure 2-8** showed that 10 batches of Radix Puerariea from the market contained most of the active ingredients, and the composition of active ingredients varied from batch to batch.

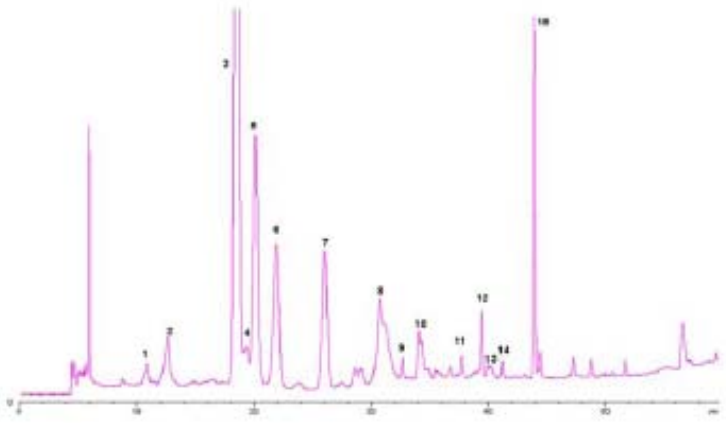


Figure 2-7 Typical HPLC Fingerprint Illustrations

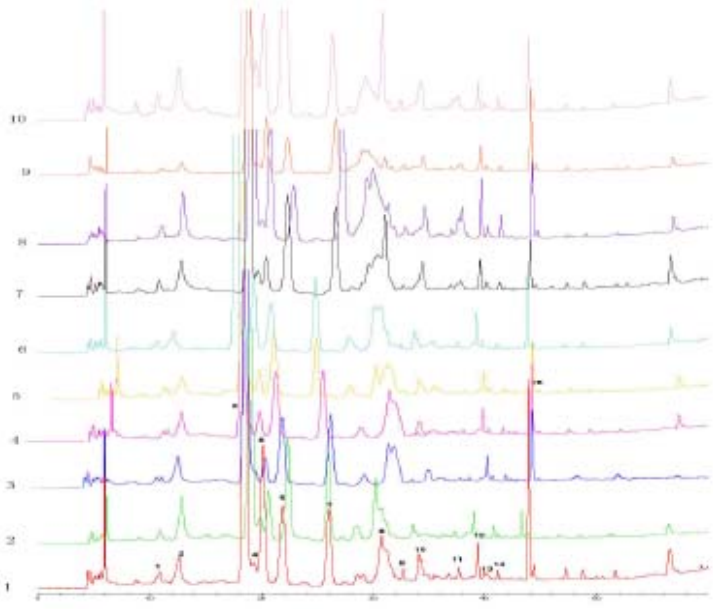


Figure 2-8 Overlapping illustration of fingerprint of 10 batches of medicinal materials on market

Methanol-water gradient elution was used throughout the experiments. Most flavone/isoflavone compounds in Radix Puerariae could be properly separated, and complete HPLC chromatogram could be obtained. The precision and reproducibility of these samples were rather good. All these proved that this method could be adopted as the fingerprint identification method for the quality control of Radix Puerariae.

The results showed the relative retention time, peak area and relative peak area of common peaks. Peak No. 3 was puerarin. This was the principal component of *Radix Puerariae*, its peak area accounted for 55% of the total peak area, and this compound was relatively stable. Therefore, this peak could be served as a reference peak to evaluate the raw materials. According to the data in Tables **2-19** and **2-20**, the RSD of the relative peak areas of the common peaks for 10 batches of samples was less than 5%. The average proportion of common peak area in total peak area was 94.6%, and RSD was 2.59%. These results complied with the relevant specifications of fingerprint.

The samples used in this test were taken from Huoshan, Anhui. The chromatograms showed good similarity. The average common peak area was over 94%. It may be concluded that this method can be used to control the quality of Radix Puerariae.

Experimental results of *Pueraria lobata* and *Pueraria thomsonii* indicated that the compositions of these two species were substantially different. It was therefore reasonable to assume that only crude medical materials from the same species can be used for the production of TCM preparations. The fingerprints of *Pueraria lobata*

from different origins were quite similar and they showed the same common peaks. But the peak areas and their relative values varied from batch to batch.

2.5 Summary

Recording the UV-visible spectral analysis of a sample at 250 nm could be used to determine the composition of the active ingredients of Radix Puerariae, and good reproducibility was achieved. HPLC analysis of an alcohol extract of Radix Puerariae could be used to determine the composition of the active ingredients of the raw material, and this method could also provide fingerprint information. HPLC fingerprint experiments indicated that *Pueraria lobata* was more suitable for medical application than *Pueraria thomsonii*, and fingerprint patterns of different batches of *Pueraria lobata* produced in Huoshan, Anhui showed a good similarity, and the average common peak area was over 94%. This possibly indicated that *Pueraria lobata* produced in Huoshan could be of the highest quality.

Chapter 3 Bioavailability and Pharmacological Activity of Puerarin in Different Dispersion Formulas

3.1 Introduction

As the major active ingredient in Radix Puerariae, puerarin can be used as an ideal drug for preventing coronary heart disease, cardiac infarction, cardiac arrhythmia, hypertensive, atheoscleorosis and cerebrovascular disease. Most importantly, this compound, which is abundant from natural source, bears various pharmacological effects. However, the poor solubility in both organic solvent and water causes the poor bioavailability of the compound. Improving the bioavailability of puerarin has become a key to solving the problem. By the perspective of preparation research, the dispersion status of medicine in a preparation is the one of the main factors influencing the dissolution and absorption of the oral solid preparation. It is therefore significant to optimize the preparation technique, and to study the dispersion status of medicine in preparation.

In this project, two formulation technologies, i.e., PEG dispersion and phospholipid complex, were used to study puerarin oral preparation, and comparison of the pharmacological activity of products with the above two technologies were also made. The results indicated that the pharmacological activities of both preparations resulting from the use of these two techniques were better than the puerarin, and puerarin phospholipid complex was better than PEG dispersed substance of puerarin.

3.2 PEG dispersion of Puerarin

Polyethylene glycol (PEG) is a water soluble substance and could be used for

enhancing the solubility of traditional Chinese medicine. The dosage form of PEG dispersion substance is normally in the form of dripping pill.[59]

3.2.1 Preparation of Puerarin Dripping Pill

The component of the ground substance was a mixture of PEG 4000 and propylene glycol with a proportion of 1:4.4:0.6 for puerarin: PEG 4000:propylene glycol. The coolant was methylsilicone oil 201-100.

An appropriate amount of puerarin was weighed and 3 times of alcohol was used to dissolve the material. The mixture was then added to molten ground substance at 80 °C according to the proportion in prescription. The solvent was removed, and the preparation was dropped under the following conditions: material temperature was 90 °C, dropping temperature 100 °C, inner and outer diameter of dropper 1.6 mm and 3.1 mm, respectively, dropping distance 10 cm, dropping speed 45 drops/min, and the temperature for the condensing agent was 0 °C.

3.2.2 *In vitro* Dissolution Test

Study revealed that the dissolution rate of the formulated solid dispersion was greater than the original one. However, further experiments were still needed to verify the extent of the increase of the rate. *In vitro* dissolution test was a significant aspect for evaluating the preparation and was carried out to evaluate the dissolution rate of the new preparation.

3.2.2.1 Instruments and Reagents

The experiments were carried out on a ZRS-8G intelligent dissolution instrument (made by Radio Plant of Tianjin University) and Agilent 1100 Series HPLC System. The test medicine included reference puerarin (from National Institute for the

Control of Pharmaceutical and Biological Products), pepsin, puerarin dropping pill, normal puerarin tablet, puerarin dispersible tablet, total flavone dispersible tablet, total flavone dropping pill (made specially for the test), and Yufeng Ningxin tablet (made by Beijing Tong Ren Tang).

3.2.2.2 Methodology of *in vitro* Dissolution

Due to the poor water solubility of puerarin and the small size of the dropping pill, the Small Cup Method was used for test according to the Pharmacopoeia of the People's Republic of China.

Formulation of artificial gastric fluid was prepared by mixing 16.4 ml of dilute hydrochloric acid with about 800 ml of water and 10 g of pepsin. The mixture was shaken evenly and water was added to 1000 ml.

About 35 mg of the sample was added to each cup, and 100 ml of degassed dissolution medium was added at a temperature of 37.0±0.5 °C with rotational speed of 100 RPM. Taking 2 ml of sample at the 15^{th} minute and filtered the solution immediately. The clean liquid obtained through a high speed centrifugation was then subjected to HPLC analysis using acetonitrile:water (15:85) as the mobile phase.

A standard curve which could correlate the puerarin concentration with the integrated peak area of the HPLC plot was first established. Accurately weighed puerarain (41.2 mg) was first dissolved in the artificial gastric fluid and the total volume was 100 ml (using a volumetric flask). The reference solution of 0.1 ml, 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, and 2.5 ml was diluted to 10 ml with the artificial gastric fluid (in volumetric flasks). The standard curve was finally obtained by HPLC analysis of 20 μl of each solution, and correlating the HPLC peak area with the concentration of puerarin. The

results were summarized in **Table 3-1**.

Table 3-1 Plotting of standard curve for dissolution

Injecting concentration C (g/μl)	0.00412	0.0206	0.0412	0.0618	0.0824	0.103
Peak area A	516.5	2424.4	4862.6	7389.7	9907.1	11750.2

An equation of the standard curve: A=116286.198C+62.714, r=0.9993 was finally obtained after processing the data of the injecting concentration (μg/μl) versus the integral peak area (A) of the HPLC plot.

After establishing the relationship between the HPLC analysis results and the concentration of puerarin, dissolution experiments were then carried out. Dissolved 6 grains of the sample in 100 ml of degassed artificial gastric fluid at a temperature of 37.0±0.5°C with a rotation speed of 100 RPM. Two ml of sample was taken at different times (adding dissolution medium with the same volume at the same temperature) and the solution was filtered immediately. The clean liquid was centrifuged at high speed and the clear solution was subjected to HPLC analysis to measure the puerarin content and the relative percentage of accumulative dissolution. Acetonitrile: water (15:85) was used as the mobile phase, and normal puerarin tablet, dispersible puerarin tablet and Yufeng Ningxin tablet were all subjected to this experiment under the same conditions. The results were summarized in **Table 3-2**.

Table 3-2 Result of *in vitro* dissolution test

Injecting Time	Accumulative Dissolution Percentage of Puerarin (%) (n=3)			
	Puerarin dropping pill	Puerarin normal tablet	Puerarin dispersible tablet	Yufeng Ningxin tablet
1min	15.0	None	1.12	None
3min	41.0	None	5.8	None
6min	67.3	0.06	16.3	None
10min	83.0	0.10	27.5	None
15min	92.4	0.82	40.9	None
20min	93.1	1.35	52.4	0.3
30min	93.5	3.57	52.6	-
45min	Not Tested	3.59	Not Tested	6.48
60min	Not Tested	3.60	Not Tested	16.7
75min	Not Tested	Not Tested	Not Tested	27.6
90min	Not Tested	Not Tested	Not Tested	35.7
120min	Not Tested	Not Tested	Not Tested	56.7
150min	Not Tested	Not Tested	Not Tested	78.0
180min	Not Tested	Not Tested	Not Tested	84.4

The greatest accumulative dissolution volume was calculated by combining the HPLC analysis results with the index of the period of peak area. The results were summarized in **Table 3-3** and **Figure 3-1**.

Table 3-3 Result analysis of *in vitro* dissolution test

Item	Puerarin dropping pill	Puerarin normal tablet	Puerarin dispersible tablet	Yufeng Ningxin Tablet
Period for Complete Dissolution (min)	15	30	20	180
Maximum Accumulative Dissolution Volume (%)	93.5	3.6	52.6	84.4

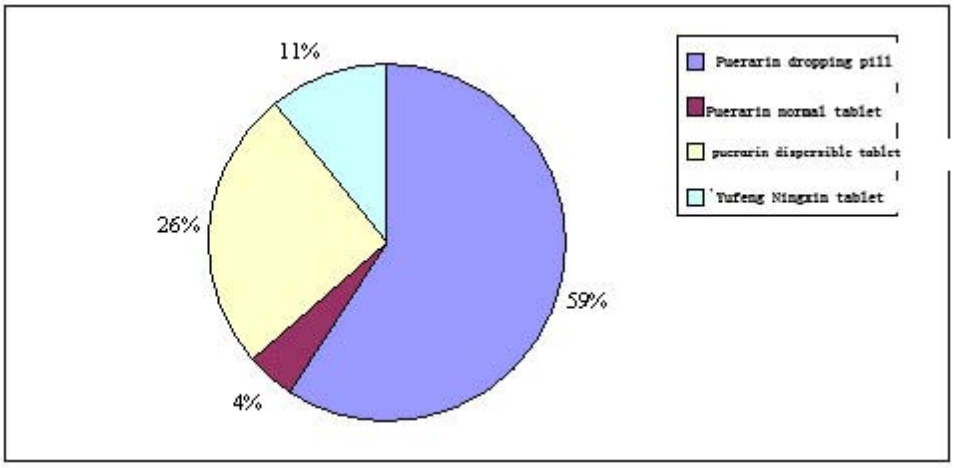


Figure 3-1 Comparison of analysis on *in vitro* dissolution curve

Experiment results indicated that the *in vitro* dissolution of the puerarin dropping pill was 310 times quicker than that for Yufeng Ningxin tablet, 69 times for the normal puerarin tablet, and 1.8 times for the dispersible tablet at 20^{th} minute time point. The experiments also assumed that the period of complete dissolution took 15 minutes, the dissolution speed of the dropping pill would be 165 minutes faster than that of Yufeng Ningxin tablet, 15 minutes faster than that of the normal tablet and 5 minutes faster than that of the normal puerarin dispersible tablet.

The peak area of the dropping pill during the testing period did not show too much difference to that of the puerarin dispersible tablet, but the accumulative dissolution volume of the dropping pill was 2 times of that for the dispersible tablet. The accumulative dissolution volume of the normal tablet was extremely small, as 3.85% of that of the dropping pill, which was consistent with the poor dissolvability of puerarin in water. The above results indicated that the dropping pill significantly improved the dispersion effect of puerarin.

The time required for the complete dissolution of Yufeng Ningxin tablet was

12 times of that of the dropping pill, 9 times of that of the dispersible tablet. The accumulative dissolution volume of Yufeng Ningxin tablet was smaller than that of the dropping pill but much greater than that of the dispersible tablet. It was normally slow for the normal tablet made from puerarin extract to dissolve. However, the accumulative dissolution volume calculated for puerarin was better than that in the pure chemical, probably due to the interaction between various components in the preparation. All the experiment results indicated that the puerarin dropping pill was faster to dissolute and had a large accumulative dissolution volume.

3.3 Puerarin Phospholipid Complex

3.3.1 Introduction

Many TCM active ingredients have been reported/recorded to possess various pharmacological activities, but when admitted orally most of them had poor absorption, unwanted gastrointestinal tract actions or some other side effects due to their physicochemical property such as big polarity and poor water solubility. These drawbacks significantly limited the clinical applications of the products.

It was reported that, phospholipid complex of a drug would significantly increase the lipophilicity, absorption and permeability as well as the bioavailablity of drugs.[65-67] Gatti et al., who studied the effect of phospholipids on the bioavailability of silymarin, had found that the absorption of the original drug was improved due to the high fat-solublility of the complex and its bioavailability was 280% of that of the original drug.[68] The experiments on the absorption of goldbeater's large intestine surface of dolichol phospholipids indicated that the absorption was also very

good.[69] Therefore, phospholipid complex with natural active ingredients has shown promising effect on the enhancement of lipophilicity as well as bioavailability.

The phospholipid complex of natural active ingredients was made by compounding the natural active ingredient with phospholipids. By testing the anti-inflammatory effect of such phospholipid complex as enoxolone and silymarin, and the capability of preventing acute hepatic injury, it was proved that the pharmacological activity of the phospholipid complex was better than that of the original drug and the effect lasted longer.[70]

3.3.2 Preparation of Puerarin Phospholipid Complex

Puerarin and soy lecithin with the mass proposition of 1:1.2 were added to an adequate volume of absolute ethyl alcohol. The concentration of puerarin was 100 mg/ml, temperature was 30 °C, and the agitation time was 0.5 h. The solvent was then removed *in vacuo* at 45 °C. The puerarin phospholipid complex (80 meshes) was finally obtained after drying the produced to shallow yellow solid at 50 °C *in vacuo*.

3.4 Study on the Absorption of Puerarin, Puerarin Phospholipids Complex (PPC) and PEG-Puerarin Dropping Pill (PEG-P) *in vivo* in Dogs

The experiments were carried out using the puerarin phospholipid complex and PEG-puerarin as testing samples and puerarin as reference.

3.4.1 Analyzing Method

Chromatographic conditions: Zorbax SB-C_{18} column (5 μm, 250 mm × 4.6 mm) was used as stationary phase, and acetonitrile: water (15:85) was used as the mobile

phase at a flow rate of 0.7 ml/min. An UV detector was used and the measured wave length was 250 nm. The injection quantity was 20 μl with hydroxy benzaldehyde as the internal standard.

Pre-treatment of the sample: 10 μl (1.0 mg/ml) internal standard dissolved in methanol was added in a 1.5 ml centrifuge tube with a stopper and the sample tube was purged with N_2. The dog blood sample was stood still for 1 hour in advance, and then centrifuged for 10 min at 3000 RPM. The supernant serum was used for test. To a 1.5 ml centrifuge tube was added the internal standard, and 500 μl serum for test. The solution was well mixed, and 500 μl methanol was added to deposit the proteins. After thorough mixing with an Eddy oscillator, the tube was centrifuged for 10 min at 10000 RPM, 20 μl of the sample taken from the supernate was filtered through 0.2 μm PTF filter membrane.

Preparation of the reference solution and the internal standard solution used to determine the *in vivo* absorption of the puerarin and puerarin phospholipid complex:

Accurately transfer certain volume of reference puerarin, and the reference solution was obtained by diluting the transferred puerarin with methanol to a concentration of 500 μg/ml. Measured quantity of the methanol solution of puerarin (0.2 and 1.0 ml, respectively) was then transferred to a 10 ml volumetric flask and methanol was added to prepare the reference solution. The concentrations of the obtained solutions were 10.0 and 50.0 μg/ml, respectively.

Measured quantity of the internal standard methanol solution was also transferred to a 10 ml volumetric flask and diluted with methanol to obtain a concentration of 1.0

mg/ml of the internal standard methanol solution

Accurately transfer a volume of reference puerarin solution to a 10 ml volumetric flask and prepare the puerarin methanol solution to reach the concentration of 500 μg/ml. Accurately transfer 0.1, 0.2, 0.4, 1.0, 2.0 and 4.0 ml of the solution into 10 ml volumetric flasks, and methanol was added to volume. The standard solutions were then prepared to the concentrations of 5.0, 10.0, 20.0, 50.0, 100.0 and 200.0 μg/ml, respectively.

The methanol solution of the internal standard was also prepared in the same manner at a concentration of 1.0 mg/ml.

Method for determining the *in vivo* absorption of puerarin and puerarin phospholipid complex: 10 μl of the internal standard methanol solution (1.0 mg/ml) and certain amount of the reference solution was added to a 1.5 ml centrifuge tube and the tube was purged with N_2. The blank serum was then added and was treated and the regression equation wae obtained through the regression analysis by correlating the concentration with the ratio of the peak areas of reference Ab and the peak area of the internal standard sample. Results summarized in **Table 3-4** indicated a perfect linear relationship between the concentration of puerarin and the peak area Ab/As in the range of 0.1-20.0 μg/ml.

Table 3-4 Calibration curve of puerarin in beagle dog serum (n=3)

C (μg/ml)	0.1	0.2	1.0	10.0	20.0
Ab/As	0.0457	0.0760	0.1596	1.1676	2.4816
C= (Ab/As-0.0294)/0.1209 R=0.9992					

Determination of the *in vivo* absorption of puerarin injection was then carried out. To a 1.5 ml centrifuge tube was added 10 μl of the internal standard methanol solution (1.0 mg/ml), the reference solution was also added at a designed amount, and the solution was purged with N_2. Blank serum was added and the sample was treated as previously described. Analyzing the sample concentration versus the ratio of the peak area of reference (Ab) with the peak area of the internal standard sample gave the corresponding regression equation. The results described in **Table 3-5** indicated that a good linear relationship between the sample concentration and the peak area Ab/As could be observed in the range of 20.0-400.0 μg/ml.

Table 3-5 Calibration curve of puerarin in beagle dog serum (n=3)

C (μg/ml)	20.0	50.0	100.0	200.0	400.0
Ab/As	2.4816	6.4877	12.639	27.733	59.0773
C=(Ab/As+1.3689)/0.1497 R=0.9994					

Precision and recovery rate were measured by accurately transferring certain volume of the reference solutions of high, moderate and low concentrations into the blank serum, and successively calculating the RSD within one day. The determination was also carried out within 5 days successive and the RSDs were calculated within days.

The results were summarized in **Table 3-6**.

Table 3-6 Precision of puerarin determination in beagle dog serum (n=5)

Added (μg/ml)	Within-day		Between-day	
	Mean±SD	RSD (%)	Mean±SD	RSD(%)
0.200	0.201±0.0045	2.2	0.203±0.0072	3.5
1.000	0.844±0.0075	0.9	0.833±0.017	2.0
10.000	10.722±0.148	1.4	10.477±0.223	2.1

Similarly, by adding certain amount of the reference solution of high, moderate and low concentrations into 0.5 ml blank serum, and the recovery rate could be determined and calculated. The results were summarized in **Table 3-7**.

Table 3-7 Recovery of puerarin from beagle dogs

Added (μg/ml)	Found (μg/ml)	Recovery (%)	Mean±SD (%)	RSD (%)
0.200	0.201	100.41	100.57±2.17	2.1
0.200	0.197	98.49		
0.200	0.206	102.81		
1.000	0.939	93.86	96.23±2.7	2.0
1.000	1.031	101.3		
1.000	0.935	93.49		
10.000	10.635	106.35	107.22±1.48	1.3
10.000	10.894	108.94		
10.000	10.639	106.39		

All the results indicated that this method was easy to carry out and highly exclusive. The test on recovery rate and precision proved that both the accuracy and precision of this analytical method met the analysis requirement of biological samples, and it

could be used to determine the content of puerarin in the sample serum in the body of dog.

3.4.2 Determination of Puerarin Content in the Sample Serum of Beagle

The testing beagles (six) were first starved for 12 hours. After the beagles took the puerarin with the dosage of 52.5 mg/kg (puerarin or the equivalent to puerarin, the beagles were allowed to take the puerarin phospholipid complex and puerarin PEG pills, after 7 days for an interval of the intersecting test), 5 ml venous blood from the forelimbs were taken at 0, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, 600 and 720 minutes, respectively. The blood samples were stood still for about 60 minutes and were centrifuged 10 minutes at the speed of 3000 RPM. The serum was taken and was stored in a refrigerator at -20 °C for determination.

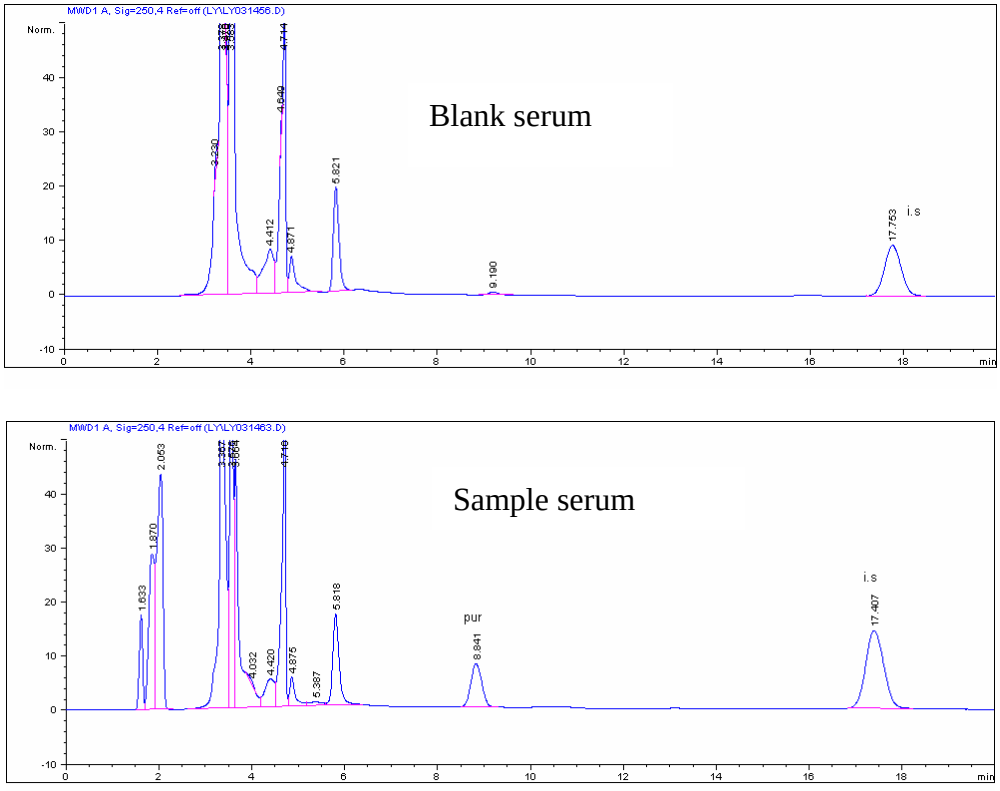


Figure 3-2 HPLC chromatograms of puerarin determined samples in serum

The blood samples were first treated, and the blood concentration of puerarin was determined using the HPLC internal standard method. The pharmacokinetic parameters were calculated with the pharmacokinetics program 3P97. The *in vivo* absorption information of each dosage forms was compared and the results were listed in **Table 3-8**.

Table 3-8 Puerarin in serum after oral administration of puerarin

No	Time (min)					
	0	10	20	30	45	60
1	0	0.328	0.456	1.199	2.055	2.557
2	0	0.033	0.69	0.533	1.547	2.275
3	0	0.46	0.468	1.203	2.24	2.265
Mean (g/ml)	0	0.274	0.538	0.978	1.947	2.366
±SD		±0.257	±0.132	±0.386	±0.386	±0.166

No	Time (min)						
	90	120	180	240	360	480	600
1	4.052	4.381	2.94	3.414	0.556	0.144	0.039
2	2.287	1.859	1.089	0.723	0.49	0.245	0.038
3	3.354	2.649	1.533	0.586	0.184	-	-
Mean	3.231	2.963	1.854	1.574	0.41	0.130	0.026
±SD	±0.889	±1.290	±0.966	±1.594	±0.198	±0.123	±0.022

- Non-determinable as the sample was below the detecting limit of 0.1 μg/ml

Table 3-9 Puerarin in serum after oral administration of puerarin soy-phospholipid complex

No	Time (min)						
	0	10	20	30	45	60	90
1	0	-	0.080	0.746	1.730	2.758	3.838
2	0	-	0.215	0.205	1.360	1.728	2.451
3	0	0.060	0.221	0.893	2.089	3.202	2.230
Mean	0	0.02	0.172	0.615	1.726	2.563	2.839
±SD		±0.035	±0.080	±0.362	±0.365	±0.756	±0.872

No	Time (min)						
	120	180	240	300	360	480	600
1	4.984	3.176	1.490	1.526	0.676	0.197	-
2	2.689	2.507	1.539	0.754	0.431	0.239	-
3	3.491	3.259	2.529	1.574	0.630	0.501	0.133
Mean	3.721	2.981	1.853	1.285	0.579	0.312	0.044
±SD	±1.165	±0.412	±0.586	±0.460	±0.131	±0.165	±0.077

Table 3-10 Puerarin in serum after oral administration of PEG-P

No	Time (min)						
	0	10	20	30	45	60	90
1	0.000	0.811	1.638	2.227	2.089	1.620	1.592
2	0.000	0.273	2.214	0.776	0.057	1.697	2.414
3	0.000	0.172	1.038	0.855	1.016	2.032	3.365
Mean	0.000	0.419	1.63	1.286	1.054	1.783	2.457
±SD		±0.343	±0.588	±0.816	±1.017	±0.219	±0.887

No	Time (min)						
	120	180	240	300	360	480	600
1	0.840	0.614	0.403	0.366	0.259	0.222	-
2	1.043	0.846	0.323	0.328	0.302	0.270	0.116
3	3.466	2.540	1.500	1.465	1.133	0.715	0.117
Mean	1.783	1.333	0.742	0.720	0.565	0.402	0.077
±SD	±1.461	±1.051	±0.658	±0.646	±0.493	±0.272	±0.067

Table 3-11 Puerarin in serum after intravenous administration of puerarin

No	Time (min)						
	0	3	5	10	15	20	30
1	0	234.89	227.60	208.13	205.33	204.74	140.54
2	0	168.17	-	158.07	136.14	155.26	-
3	0	-	343.10	194.71	329.88	195.51	168.58
Mean	0	201.53	285.35	186.97	223.78	185.17	154.56
±SD		±47.18	±81.67	±26.92	±98.18	±26.31	±19.83

No	Time (min)							
	45	60	75	90	120	150	180	240
1	101.24	83.80	64.78	69.85	60.23	37.70	17.069	8.036
2	125.29	-	56.83	53.55	46.70	36.08	25.04	12.908
3	156.25	122.10	78.67	83.32	45.42	26.53	12.644	6.142
Mean	127.60	102.95	66.76	68.91	50.78	33.44	18.25	9.03
±SD	±27.58	±27.08	±11.05	±14.91	±8.21	±6.03	±6.28	±3.49

- Not measured.

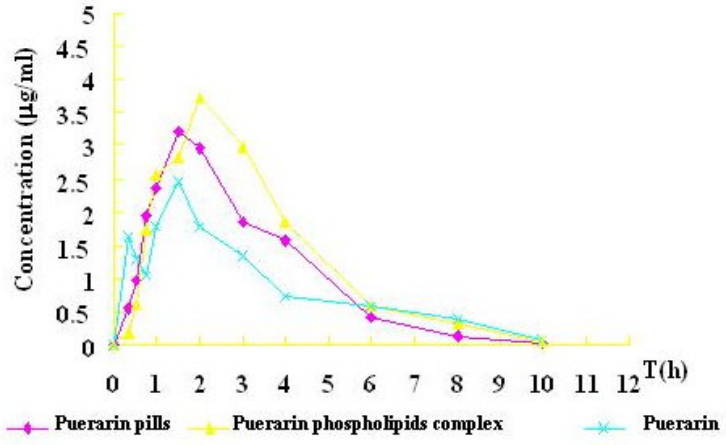


Figure 3-3 Serum level of Puerarin after oral intake 52.5 mg/kg of puerarin, PPC and PEG-P (all equivalent to 52.5 mg/kg of puerarin)

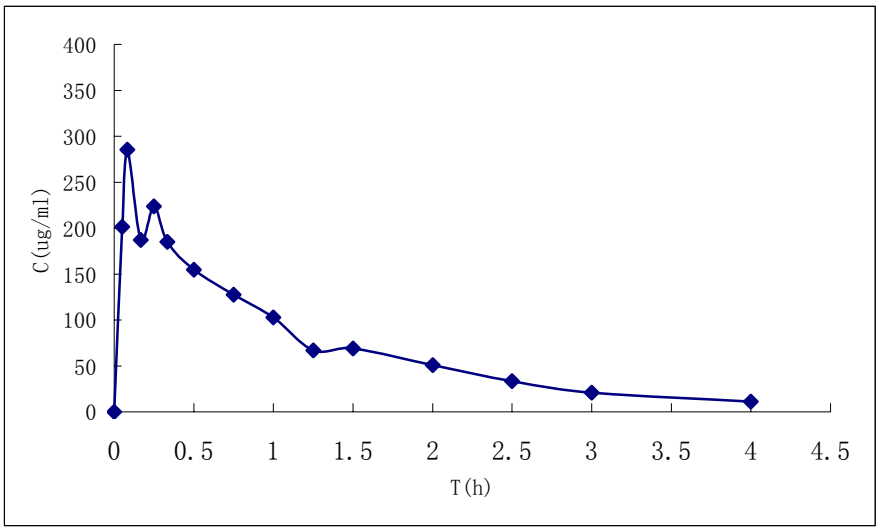


Figure 3-4 Serum level of puerarin after intravenous intake 52.5 mg/kg of puerarin

results analysis

The pharmacokinetic parameters were calculated with the pharmacokinetics programme 3P97 and the pharmacokinetic parameters of *in vivo* absorption of each puerarin dosage were compared. The puerarin injection in the body of beagle was in a single cell mode and the absorption was of the first level. Puerarin, and its

phospholipid complex and PEG-P in beagle dog were in a two-chamber mode and the absorption was of first level (**Table 3-12**).

Table 3-12 Comparison of pharmacokinetic parameters between puerarin and other dosage forms of puerarin (n=3)

	Puerarin	PEG-P	PPC
A (μg/ml)	15.29±7.88	4.03±0.76	11.34±9.36
$\alpha(h^{-1})$	1.69±1.35	1.38±0.98	0.52±0.28
B (μg/ml)	30.56±1.72	9.90±15.62	9.82±15.16
$\beta(h^{-1})$	0.68±0.18	0.32±0.18	0.42±0.17
Ka (h^{-1})	1.02±0.39	1.54±0.95	0.68±0.16
V/F(c)(mg/(μg/ml)	138.02±5.20	363.07±277.97	156.30±103.41
$T_{1/2\alpha}$(h)	0.49±0.38	0.71±0.48	1.56±0.64
$T_{1/2\beta}$(h)	1.06±0.27	2.65±1.33	1.82±0.62
$T_{1/2Ka}$(h)	0.74±0.24	0.58±0.32	1.04±0.22
K_{21} (h^{-1})	0.74±0.1	6.75±11.07	0.50±0.28
K_{10} (h^{-1})	0.61±0.32	0.34±0.24	0.44±0.17
K_{12} (h^{-1})	24.48±3.97	0.22±0.24	0.03±0.01
AUC ((μg/ml)*h)	7.94±0.21	9.11±4.46	13.67±2.72
CL(s) (mg/h/(μg/ml))	65.11±25.47	79.03±30.22	58.52±21.12
T (peak)(h)	1.62±0.30	1.31±0.71	1.91±0.51
C (max)(μg/ml)	3.00±1.13	2.21±0.82	2.38±1.27

Table 3-13 Pharmacokinetic parameters of puerarin injection

	Puerarin injection
C_0 (μg/ml)	201.73±42.84
Ke (h^{-1})	0.74±0.08
V(c)(mg/(μg/ml))	3.23±0.78
$T_{1/2(Ke)}$(h)	0.94±0.10
CL(s) (mg/h/(μg/ml))	2.35±0.39
AUC ((μg/ml)*h)	272.54±45.77

The results indicated that the absolute bioavailability of the oral intake of puerarin was 7.94/272.54=2.91%, whereas it increased to 13.67/7.94=172.16% and 9.11/7.94=114.74% in the case of PPC and PEG-P, respectively. PPC was better than PEG-P regarding the *in vivo* bioavailability enhancement.

3.5 Efficacy of Different Dispersion Formula of Puerarin on the Oral Intake

3.5.1 Effect of PEG-P and PPC on the arrhythmia of mice caused by chloroform

3.5.1.1 Material

PEG-P was added to a certain volume of distilled water and the mixture was thoroughly grounded to ensure the dissolution of the drug. The concentrations of the tested samples were 2.4g/10 ml, 1.2g/10 ml, and 0.6g/10 ml. The solution was dosed by oral at a dose of 20ml/kg. The PCC solution was prepared similarly, the concentrations of the drug solution were 0.88g/10 ml, 0.44g/10 ml and 0.22g/10 ml, and the dose was also 20 ml/kg.

The Yufeng Ningxin tablet was used as positive reference, which was produced by Tong Ren Tang Traditional Chinese Medicine Refinery, Beijing Tong Ren Tang Co., Ltd. The weight of each tablet was 0.28 g (Batch number: 0120035). The tablet was

first grounded and was then added to distilled water, and a solution with a concentration of 15 tablets/100 ml was finally prepared.

Kunming species Mice, at the SPF level, half of them male and the other half female, weighted between 18~22g were used in the experiments. The mice and feeds were provided by the Laboratory Anmimal Research Center, Guangzhou University of Chinese Traditional Medicine. Certificate Number: 2002A005. The mice were fed for a week after purchase to ensure that the animals got accustomed to the environment.

The test was performed at the SPF-level in a scientific research animal laboratory of Modern Research Institute of Chinese Tradition Medicine, The Hong Kong Polytechnic University. The temperature was 23±1 °C and the relative humidity was 73%. It was quiet in surroundings, and light and there was an alternation of light and shade to go with the day and night.

3.5.1.2 Method and Results

A total of 80 mice were randomly divided into eight groups according to their weight and sex, 10 mice in each group with an equal number of male and female. The animals were subjected to the following experiments: Group I: Blank control group: distilled water, 0.2 ml/10g; Group II: positive drug control group: Yufeng Ningxin tablet, 0.15 piece/1 ml, 0.2 ml/10 g; Groups III-V: testing drug group: PEG-P, three dosage groups of 4.8 g/kg, 2.4 g/kg, 1.2 g/kg, and 0.2 ml/10g weight; Groups VI-VIII: test drug group: PPC, three dosage groups of 1.76 g/kg, 0.88 and 0.44 g/kg, and 20 ml/kg weight. The tested samples were poured into the stomach of the animals for 4 days. The mice were anaesthetized with 4 ml of chloroform half an hour later after

pouring the last batch of the samples into the stomach. After the mice stopped breathing, their thoraxes were immediately opened and the ventricular fibrillation positive rate was examined with X^2. The results were listed in **Table 3-14**.

Table 3-14 Pre-test results of puerarin dropping tablet and phospholipids complex in resisting arrhythmia of mice caused by chloroform

Group	Number	Mice with Ventricular Fibrillation	Mice without Ventricular Fibrillation
Blank control	10	10	0
Yufeng Ningxin tablets	10	5*	5*
PEG-P high dosage	10	3**	7
PEG-P moderate dosage	10	5*	5*
PEG-P low dosage	10	6	4*
PPC high dosage	10	3**	7
PPC moderate dosage	10	5*	5*
PPC low dosage	10	5*	5*

Note: compared with blank control group. * $P < 0.05$ ** $P < 0.01$

The results in **Table 3-14** indicated that PEG-P of 4.8 g/kg and 2.4 g/kg could exhibit significant effect on mouse arrhythmia caused by chloroform and the ventricular fibrillation rate of mice could also be reduced.

PPC of 1.76 g/kg, 0.88 g/kg and 0.44 g/kg also showed significant effect on mice arrhythmia caused by chloroform and the ventricular fibrillation rate of mice could be reduced. The results indicated that, at a similar dose, PPC had better effect than the puerarin drop pills.

3.5.2 Effect of PEG-P and PPC on Myocardial Ischemia in Rats Caused by Pituitrin.

3.5.2.1 Material

Puerarin solution was prepared with sterilized distilled water, the tested concentrations were 0.8 g/10 ml and 0.4 g/10 ml, and the dose was 10 ml/kg.

PEG-P solution was prepared with sterilized distilled water and the concentrations were 4.8g/ 10 ml and 2.4g/10 ml (corresponding to 0.8 g/10 ml and 0.4 g/10 ml of puerarin), and the dose was 10 ml/kg.

PPC was supplied by the Institute of Chinese Traditional Medicine. Batch number: 20021009. The sample solution was prepared with concentrations of 0.88 g/10 ml and 0.44 g/10 ml (corresponding to 0.4 g/ml and 0.2 g/ml of puerarin), and the dose was 10 ml/kg.

Puerarin injection: 100 mg/2 ml/piece, produced by Yiqiao (Hunan) Pharmaceutical Co., Ltd. (Batch Number: 20020109) , was used as positive reference.

Yufeng Ningxin tablet: produced by Tong Ren Tang Traditional Chinese Medicine Refinery, Beijing Tong Ren Tang Co., Ltd. (0.28g each piece) (Batch Number 0120035). During the test, the tablet was grounded to powder and certain volume of distilled water was added to make the concentration up to 0.3 piece /ml.

3.5.2.2 Animal

Rats at the SPF level, half of them male and the other half female, weighing 200±20g were used. The rats was provided by the Guangzhou University of Traditional Chinese Medicine. Animal Quality Certificate Number: 2002A011. The rats were fed for one week after purchase to make them got accustomed to environment.

The experiment was carried out in a SPF-level scientific research animal laboratory of The Modern Research Institute of Chinese Tradition Medicine, Hong Kong Polytechnic University. The temperature was 23±1 °C and the relative humidity 73%. It was quiet in surrounding, and there was an alternation of light and shade to go with the day and night.

3.5.2.3 Method and Results

A total of 90 SD rats of the SPF level, weighing 200±20g, with an equal number of male and female, were chosen for the experiments. The animals were randomly divided into nine groups according to their weight and sex. The nine groups were subjected to the following experiments: Group I: control group: distilled water, 10 ml/kg; Group II and III: positive control group: the tail vein of the mice was injected with the puerarin injection of at the dose of 0.67 ml/kg; Yufeng Ningxin tablet, 0.3 piece/ml, at the dose of 10 ml/kg weight; Groups IV and V: puerarin solution group: 0.8 g/kg, 0.4 g/kg and 10 ml/kg; Groups VI and VIII: PEG-P group: 4.8 g/kg, 2.4 g/kg and 10 ml/kg; Groups VIII and IX: PPC solution group: 0.88 g/kg, 0.44 g/kg and 10 ml/kg. The rats were fed with drugs for 5 consecutive days. After one hour of drug administration at the sixth day, 3% pentobarbital sodium was injected into the abdominal cavity of the rats with a dose of 40 mg/kg, and two lead electrodes were connected to record the normal electrocardiogram. After the electrocardiogram got stable, pituitrin was injected into the tail vein (dose 1 μg/kg) and the electrocardiogram was immediately recorded for 30 minutes. The change of T wave before and after drug administration was calculated and the changing value of time during P-R and Q-T before and after drug administration was also calculated. Statistic analysis on the changing value of each group was carried out and the results were listed in the following **Tables 3-15** to **3-17**:

Table 3-15 Effect of peg-p and ppc on resisting myocardial ischemia of rat caused by pituitrin (change of T wave, UV)

Time	Group							
	Pituitrin	Puerarin Inj.	YFNX Tab.	Puerarin	PEG-P (high dose)	PEG-P (low dose)	PPC (high dose)	PPC (low dose)
5s	15.29±6.79	18.06±10.09	20.94±15.88	11.06±9.64	12.55±12.49	15.39±11.12	11.67±10.33	19.23±9.58
10s	85.68±20.12	26.88±20.62**	22.58±14.49**	20.55±10.13**	21.64±13.78**	22.38±14.77**	19.38±14.64**	25.44±15.45**
15s	120.09±39.98	9.82±7.59**	30.90±17.62**	26.75±20.41**	19.62±12.69**	23.08±10.31**	22.54±17.01**	20.51±9.39**
30s	56.41±35.56	19.08±14.44**	25.11±22.30*	25.29±17.67*	17.70±8.52**	14.34±9.43**	19.45±10.62**	17.79±12.65**
1min	69.32±40.44	6.99±3.88**	20.05±14.86**	27.09±18.19**	23.13±19.50**	26.69±20.31**	20.18±16.05**	26.43±20.08**
2min	78.11±39.28	19.01±8.62**	22.79±8.60**	20.34±13.45**	13.91±6.62**	19.34±8.68**	16.64±13.89**	20.55±15.04**
5min	75.52±29.33	9.69±6.58**	19.31±16.96**	19.72±13.50**	16.05±9.64**	19.25±10.91**	15.67±13.21**	17.52±13.32**
10min	12.68±7.09	16.62±8.31	10.10±10.01	22.82±11.41	11.85±9.72	15.34±10.62	11.85±9.72	14.27±11.09

Compared with control *P < 0.01 ** P < 0.001

The results indicated that the change of T wave of PEG-P and PPC group at 10s, 15s, 30s, 1min, 2min and 5min differed remarkably from that of control.

Table 3-16 Effect of PEG-P and PPC on resisting myocardial ischemia of rat caused by pituitrin (changed during P-R, s)

Time	Group							
	Pituitrin	Puerarin Inj.	YFNX Tab.	Puerarin	PEG-P (high dose)	PEG-P (low dose)	PPC (high dose)	PPC (low dose)
5s	0.0018±0.0008	0.0013±0.0007	0.0013±0.0006	0.0012±0.0005	0.0013±0.0007	0.0012±0.0006	0.0012±0.0005	0.0011±0.0008
10s	0.0033±0.0018	0.0015±0.0010*	0.0016±0.0012*	0.0018±0.0009*	0.0017±0.0011*	0.0019±0.0015*	0.0018±0.0010*	0.0019±0.0011*
15s	0.0032±0.0019	0.0012±0.0008*	0.0014±0.0015*	0.0015±0.0011*	0.0013±0.0013*	0.0019±0.0009*	0.0014±0.0011*	0.0015±0.0010*
30s	0.0185±0.0054	0.0016±0.0011**	0.0016±0.0009**	0.0013±0.0013**	0.0020±0.0011**	0.0016±0.0012**	0.0022±0.0015**	0.0015±0.0011**
1min	0.0165±0.0033	0.0013±0.0011**	0.0015±0.0009**	0.0020±0.0012**	0.0018±0.0010**	0.0014±0.0011**	0.0017±0.0009**	0.0016±0.0010**
2min	0.0169±0.0046	0.0012±0.0009**	0.0013±0.0011**	0.0013±0.0012**	0.0015±0.0011**	0.0013±0.0011**	0.0017±0.0011**	0.0015±0.0009**
5min	0.0103±0.0023	0.0015±0.0012**	0.0017±0.0011**	0.0012±0.0012**	0.0015±0.0011**	0.0012±0.0011**	0.0015±0.0011**	0.0017±0.0013**
10min	0.0102±0.0069	0.0013±0.0011**	0.0012±0.0011**	0.0013±0.0012**	0.0015±0.0014**	0.0013±0.0012**	0.0018±0.0014**	0.0017±0.0012**

Compared with control group * $P < 0.05$ ** $P < 0.01$

The changing value during P-R of PEG-P ang PPC at 10s, 15s, 30s, 1min, 2min, 5min and 10min differed remarkably from that of control group.

Table 3-17 Effect of PEG-P and PPC on resisting myocardial ischemia of rat caused by pituitrin (changed during Q-T, s)

Time	Group							
	Pituitrin	Puerarin Inj.	YFNX Tab.	Puerarin	PEG-P (high dose)	PEG-P (low dose)	PPC (high dose)	PPC (low dose)
5s	0.0043±0.0011	0.0016±0.0011**	0.0017±0.0004**	0.0015±0.0011**	0.0012±0.0009**	0.0013±0.0009**	0.0015±0.0005**	0.0011±0.0009**
10s	0.0011±0.0009	0.0015±0.0011	0.0013±0.0012	0.0018±0.0012	0.0015±0.0014	0.0012±0.0011	0.0012±0.0009	0.0017±0.0014
15s	0.0052±0.0019	0.0017±0.0013**	0.0020±0.0015**	0.0020±0.0015**	0.0014±0.0011**	0.0012±0.0013**	0.0013±0.0013**	0.0012±0.0010**
30s	0.0015±0.0004	0.0016±0.0011	0.0017±0.0006	0.0017±0.0013	0.0022±0.0013	0.0020±0.0014	0.0020±0.0014	0.0014±0.0012
1min	0.0023±0.0011	0.0014±0.0009	0.0018±0.0011	0.0022±0.0011	0.0020±0.0015	0.0018±0.0015	0.0019±0.0010	0.0016±0.0013
2min	0.0023±0.0006	0.0010±0.0009*	0.0013±0.0009*	0.0016±0.0012	0.0017±0.0012	0.0016±0.0009	0.0014±0.0011*	0.0013±0.0009*
5min	0.0050±0.0021	0.0015±0.0012**	0.0021±0.0015**	0.0013±0.0010**	0.0015±0.0010**	0.0015±0.0014**	0.0012±0.0011**	0.0017±0.0014**
10min	0.0020±0.0009	0.0012±0.0011	0.0018±0.0010	0.0016±0.0012	0.0015±0.0009	0.0013±0.00011	0.0017±0.0014	0.0012±0.0009

Compared with control group *P < 0.01 ** P < 0.001

The above table shows that the changing value during Q-T of both PEG-P and PPC groups at 15s, 15s, 5 and 2 min differed remarkably from that of control group.

Results indicated that the PEG-P of 4.8g/kg and 2.4g/kg and PPC of 0.88 g/kg and 0.44 g/kg could significantly resisted myocardial ischemia of rat caused by pituitrin.

3.6 Summary

In summary, both puerarin dropping pill and puerarin phospholipid complex showed improved solubility and bioavailability of the drugs. The change of T wave of the PEG-P and PPC groups at 10s, 15s, 30s, 1 min, 2 min and 5 min differed remarkably from that of the control. The change value during Q-T of both the PEG-P and PPC groups at 15s, 15s, 5 and 2 min differed remarkably from that of the control group. The PEG-P of 4.8g/kg and 2.4g/kg and PPC of 0.88 g/kg and 0.44 g/kg could significantly resist myocardial ischemia in rats caused by pituitrin.

Chapter 4 Radix Puerariae Compound Formula for Regulating Blood Lipid

4.1 Introduction

Compound formulas are the main feature of TCM prescriptions. All compound formulas in TCM should strictly comply with the traditional Chinese medicine theory. Presently significant efforts have been made to develop Chinese medicine with modern technology with some successful examples, such as puerarin injection. It is much more complex to develop a formula made of a compound prescription with modern technology.

Radix Puerariae has shown many important bioactivities. It can be applied to treat a wide range of disorders, but presently the simple recipe of Radix Puerariae is only limited to the treatment of cardiovascular diseases. It is of significance to study the effect-enhancing technologies by medical combination, to develop new applications for Radix Puerariae preparations, and to improve the efficacy with new prescriptions. This chapter describe the best compound prescription of Radix Puerariae for regulating blood lipid by screening known prescriptions.

4.2 Compound Radix Puerariae Prearation for Regulating Blood Lipid in Mice with Hyperlipemia

4.2.1 Materials

Drug and Reagents: Radix Puerariae extraction, Cassia seed extraction, Fructus Alpiniae Oxyphyllae extraction, safflower oil, evening primrose capsule (made by Guangzhou Xing Qun Pharmaceutical Company Limited, Batch Number: 0208001),

cholesterin reagent (made by Biosino Biotechnology & Science Inc., Batch Number: 180051), triglyceride (made by Biosino Biotechnology & Science Inc., Batch Number: 220241), low density lipoprotein cholesterol reagent (made by Biosino Biotechnology & Science Inc. Batch Number: 190031).

Instrument: 5415D desk centrifuge (made by Eppendorf), XD811 biochemical analyzer (made by Shanghai Xunda medical instrument company).

Animal: 95 KM mice of SPF level, 18-22 g, half male and half female, provided by Guangzhou TCM University Animal Center. Certificate number: Certificate of Laboratory Animal Monitoring Institute of Scientific Committee of Guangdong Province (YJZ) No. 2002A005 and the SPF level animal laboratory (YJZ No. 2002C019).

4.2.2 Method

Grouping: Randomly divide the mice into 9 groups: normal control group, safflower oil group, model group, Radix Puerariae extraction group, cassia seed extraction group, Fructus Alpiniae Oxyphyllae extraction group, Radix Puerariae extraction plus cassia extraction group (4:1), Radix Puerariae extraction plus Fructus Alpiniae Oxyphyllae extraction (4:1) and evening primrose group.

Eggnog model: formulate 75% eggnog with fresh natural egg and normal saline. Ip 0.5 ml per mouse starved for 16 hours.

Method: Drug was first administered to each group for 3 consecutive days, and ip 0.5 ml per mouse was given within 1 hour after drug delivery in the 3rd day. Drug was administered one more time in 3 hours after model-forming, and blood sample was

taken from the mice's eye socket 20 hours after model-forming. The blood sample was centrifuged at 3000 RPM for 10 minutes, and the serum was subjected to TC, TG and HDL-C analysis.

Information analysis: information was expressed by x±s and t inspection.

4.2.3 Result

4.2.3.1 Effect of *Radix Puerariae* compound on TC of mice with hyperlipemia

The TC level of the normal control group, safflower oil group and all the drug-delivery group was obviously lower than that of the model group, and the difference was significant ($P<0.01$). No obvious difference between the normal control group and all the drug-deliveried groups (**Table 4-1**).

4.2.3.2 Effect of *Radix Puerariae* compound on TG of mice with hyperlipemia

Compared with the model group, the TG level of the mice of the normal control group, safflower oil group and all drug-delivery groups showed no obvious variations. There was a dramatic difference between the *Radix Puerariae* extraction group and the normal control group ($P<0.01$). There was an obvious difference between the normal control group and the group of extraction of Radix Puerariae plus Fructus Alpiniae Oxyphyllae extraction ($P<0.05$) (**Table 4-1**).

4.2.3.3 Effect of *Radix Puerariae* compound on HDL-C of mice with hyperlipemia

Compared with mice in the model group, mice in the normal control group, safflower oil group and the entire drug–delivery group, showed a higher HDL-C level. The HDL-L levels of mice in the safflower oil group, Fructus Alpiniae Oxyphyllae group, the *Radix Puerariae* extraction +cassia seed extraction group, Radix Puerariae extraction +Fructus Alpiniae Oxyphyllae extraction group showed the most obvious

differences.(P<0.01), and the HDL-L levels of mice in the Radix Puerariae extraction group, evening primrose oil group exhibited obvious differences.(P<0.05). Compared with the normal control group, safflower oil group, Radix Puerariae plus cassia extraction group, the HDL-L levels had dramatic differences (P<0.01). The HDL-L levels in the Radix Puerariae extraction group, Fructus Alpiniae Oxyphyllae extraction and Radix Puerariae extraction group+Fructus Alpiniae Oxyphyllae extraction group had obvious differences (P<0.05).

Table 4-1 Effect on TC, TG and HDL-C in mice with hyperlipemia

group	n	dose (g/kg)	TC	TG	HDL-C
Normal control	10		4.45±2.48**	1.40±0.34	1.52±0.29
Safflower Oil	10		3.65±1.37**	1.27±0.18	2.00±0.30**▲▲
Model	10		7.45±1.66	1.17±0.37	1.32±0.37
Extraction of *Radix Puerariae*	10	0.576	4.78±1.73**	1.06±0.22▲▲	2.08±0.95*▲
Cassia seed extraction	10	0.18	3.80±1.31	1.12±0.24	1.52±0.24
Fructus Alpiniae Oxyphyllae extraction	10	0.252	4.75±0.95**	1.43±0.39	1.77±0.26**▲▲
Radix Puerariae extraction +cassia seed extraction	11	0.4608+0.036	4.94±1.42**	1.16±0.37	1.92±0.35**▲▲
Extraction of *Radix Puerariae*+ Alpiniae Oxyphyllae	11	0.4608+0.0504	4.64±1.63**	1.06±0.49▲	1.77±0.31**▲
Evening primrose oil	11	0.667	4.47±0.87**	1.24±0.51	1.83±0.66*

* P<0.05 compared with model group; ** P<0.01 compared with model group;

▲ P<0.05 compared with normal control group; ▲▲P<0.01

4.3 Compound Radix Puerariae Preparation for regulating blood lipid in rats

with hyperlipemia

4.3.1 Material

Drugs and reagents: Radix Puerariae extraction, cassia seed extraction, Fructus Alpiniae Oxyphyllae extraction, safflower oil, evening primrose capsule (made by Guangzhou Xingqun Pharmaceutical Company Limited, Batch Number: 0208001), cholesterol (made by Beijing Ding Guo Biotechnology Development Center), Deoxycholic acid Sodium salt (made by China National Medicine Group Shanghai Chemical Reagent Company, import subpackage, F20021218), Propylthiouracil (Guangzhou Shi Qiao Pharmaceutical Co.,Ltd. Batch Number: 020601), Cholesterin reagent, (made by Biosino Biotechnology & Science Inc., Batch Number: 180051), Triglyceride reagent (made by Biosino Biotechnology & Science Inc. Batch Number: 220241), Low lipidlipoprotein cholesterol reagent (made by Biosino Biotechnology & Science Inc. Batch Number: 190031), low density lipoprotein (made by Beijing Zhongsheng High-tech Bioengineering Company, Batch Number: 020604).

Main equipment: 5415D Desk Centrifuge (made by Eppendorf), XD811 Biochemical Analyzer (made by Shanghai Xun Da medical instrument company).

Animal: 90 SD rats of SPF level, with 180-220g, half male and half female, provided by Center of Laboratory Animal Science the First Military Medical University) Certificate Number: Certificate of Laboratory Animal Monitoring Institute of Scientific Committee of Guangdong Province(YJZ No. 2001A051).

4.3.2 Method

Grouping: Randomly divide the rats into 9 groups: normal control group, safflower

oil group, model group, Radix Puerariae extraction group, cassia seed group, Fructus Alpiniae Oxyphyllae extraction, Radix Puerariae extraction+cassia seed extraction (4:1), Radix Puerariae extraction+Fructus Alpiniae Oxyphyllae extraction (4:1) and evening primrose oil group.

Hyperlipemia model: Formulate fat emulsion with 10% cholesterol, 20% lard, 2% cholic acid Na, 1% propylthiouracil and water. Administer the material into rats' stomach at 0.4 ml/200 g for 7 days.

Method: conducting the drug administration to the rats during their model-forming period (model was not formed in the normal control and safflower group). The blood sample was taken at the 8th day from veniplex after the rats' eye socket.

4.3.3 Results

4.3.3.1 Effect of compound Radix Puerariae preparation on serum TC of hyperlipemia rats

For most of the tested groups, such as the normal control group, safflower oil group and all drug delivery groups, the TC levels in serum of rats were obviously lower than those of the model group. Compared with the model group, the normal group, the safflower oil group, *Radix Puerariae* extraction group, and Radix Puerariae extraction+cassia seed extraction group, the Radix Puerariae extraction+Fructus Alpiniae Oxyphyllae extraction group, the TC levels showed the most obvious differences ($P<0.01$); the TC levels in the cassia seed extraction group, Fructus Alpiniae Oxyphyllae extraction group and evening primrose oil group had obvious differences ($P<0.05$). Compared with the normal control group, Radix Puerariae extraction group, the Radix Puerariae extraction+cassia seed extraction, the TC

levels showed the most obvious differences (P<0.01).

For the TG level in the serum of the rats, the normal control group, safflower group and all drug delivery groups were lower than that of the model group. Only in the Fructus Alpiniae Oxyphyllae extraction group, the TG level showed a significant difference from the model group.

4.3.3.2 Effect of compound Radix Puerariae preparation on serum HDL-C and LDL-C of rats with hyperlipemia

As for the HDL-C level in the rats' serum, the cassia seed extraction group, Fructus Alpiniae Oxyphyllae extraction group, Radix Puerariae extraction +Fructus Alpiniae Oxyphyllae extraction group and evening primrose oil group were higher than that of the model group. Compared with the model group and the Fructus Alpiniae Oxyphyllae extraction group, Radix Puerariae extraction+Fructus Alpiniae Oxyphyllae extraction group, the TG levels showed the most obvious difference (P<0.01); for the Evening oil group showed an obvious difference (P<0.05). Compared with the normal control group, the Fructus Alpiniae Oxyphyllae extraction group and Radix Puerariae extraction+Fructus Alpiniae Oxyphyllae extraction group, the TG levels showed the most obvious difference (P<0.01); for the Evening oil group showed an obvious difference (P<0.05).

As for the LDL-C levels in the rats' serum, the normal control group, safflower oil group and all the drug delivery groups, were lower than that of the model group. Compared with the model group, the normal control group, safflower group, Radix Puerariae extraction group, Fructus Alpiniae Oxyphyllae extraction group, Radix Puerariae extraction+cassial seed extraction group and Radix Puerariae

extraction+Fructus Alpiniae Oxyphyllae extraction, the LDL-C levels showed the most obvious differences (P<0.01). Compared with the model group, the cassia seed extraction group, the LDL-C levels had an obvious difference (P<0.05). Compared with the normal control group, the Radix Puerariae extraction+cassia seed extraction group, the LDL-C levels showed a more obvious difference (P<0.01) (**Table 6-2**).

Table 4-2 Effects of different samples on the TC, TG, HDL-C and LDL-C levels in rat serum with hyperlipemia

Group	n	Dose (g/kg)	TC	TG	HDL-C	LDL-C
Normal control	10		1.57±0.32**	0.74±0.26	0.91±0.17	1.85±0.33**
Safflower oil	10		1.49±0.22**	1.15±0.87	0.93±0.22	1.84±0.29**
Model group	10		2.26±0.57	1.33±1.41	1.04±0.20	2.72±0.38
Extraction of *Radix Puerariae*	10	0.576	1.07±0.22**▲▲	0.57±0.18	0.94±0.17	1.56±0.38**
Cassia seed extraction	10	0.18	1.73±0.29*	0.66±0.18	1.06±0.44	2.31±0.57*
Fructus Alpiniae Oxyphyllae extraction	10	0.252	1.79±0.26*	0.52±0.08*	1.51±0.43**▲▲	2.18±0.19**
Extraction of *Radix Puerariae*+cassial seed extraction	11	0.4608 +0.036	1.19±0.16**▲▲	0.65±0.21	0.86±0.23	1.51±0.19**▲▲
Extraction of *Radix Puerariae*+Fructus Alpiniae Oxyphyllae extraction	11	0.4608 +0.0504	1.49±0.21**	0.97±0.93	1.22±0.22**▲▲	1.98±0.43**
Evening primrose oil	11	0.667	1.90±0.22*	0.79±0.48	1.21±0.35*▲	2.61±0.43

* P<0.05 compared with model group; ** P<0.01 compared with model group

▲ P<0.05 compared with normal control group; ▲▲P<0.01 compared with normal control group

4.4 Summary

Both Radix Puerariae and compound Radix Puerariae preparartion can reduce blood lipid level, and the compound Radix Puerariae preparation showed better result than that of Radix Puerariae extract. The results indicated that the compound of Radix Puerariae extract with Fructus Alpiniae Oxyphyllae extract was more effective to reducing TC, TG, LDL-C and increasing HDL-C. This compound formula could be comprehensively used to regulate the blood lipid level.

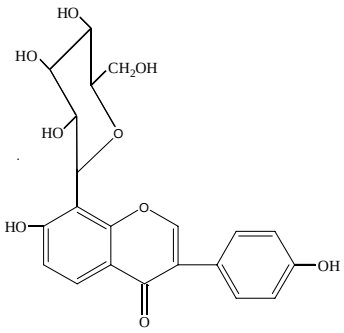
Chapter 5 Preparation of Puerarin Derivatives and Their Physico-chemical Properties

5.1 Introduction

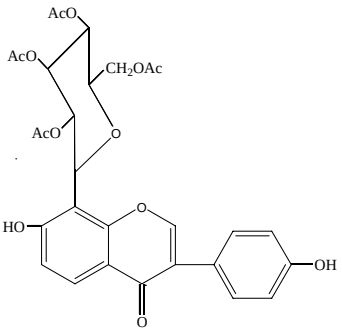
Puerarin is a C-glycoside of daidzein with glucose. The special structural feature of puerarin has shown significant impact on its bioavailability. Its chemical structure is stable, but its lipophilicity and water solubility are very poor, which is not idea in terms of *in vitro* dissolution and *in vivo* absorption, especially when administered orally. Studies had indicated that very little puerarin could be absorbed by oral intake,[71] but the bioavailability of daidzein administered orally was found to be much higher than that of puerarin.[72] These results possibly indicated that the glucosyl moiety in puerarin would be responsible for the poor *in vivo* absorption of puerarin. Due to the low bioavailability of puerarin administered orally, the current dosage form of puerarin used clinically is mainly injectable preparation. We considered it is possible to improve the bioavailability and the pharmaceutical effect of puerarin by modifying the glucose moiety of puerarin to mask the hydrophilic property of the functional groups.

The functional groups of puerarin are 7, 4'-dihydroxy, of which the 7-hydroxy has a poorer activity than 4'-hydroxy due to the steric hindrance of 8-glucosyl moiety. In order to improve the bioavailability of puerarin and enhance its pharmacological activity, we have modified the phenolic hydroxyl groups of puerarin and alcoholic hydroxyl groups of glucose through acetylation of these hydroxyl groups. Three compounds had been synthesized, and their structures were confirmed with

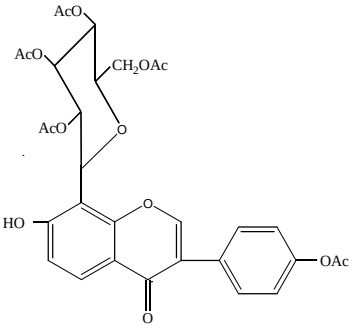
ultraviolet spectrometry, infrared spectrometry, mass spectrometry, nuclear magnetic resonance and single crystal X-ray diffraction experiments. **Figure 5-1** showed the structures of puerarin and its derivatives.



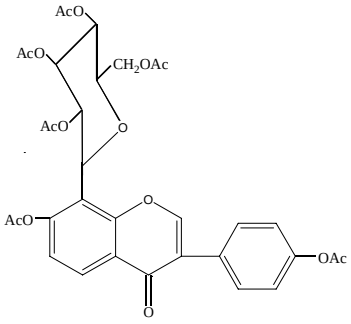
Puerarin (Pur)



Tetraacetylpuerarin (4Ac)



Pentaacetylpuerarin (5Ac)



Hexaacetylpuerarin (6Ac)

Figure 5-1 Structures of puerarin and its derivatives, 4Ac, 5Ac and 6Ac

5.2 Preparation of Puerarin Derivatives

Puerarin (50 g, 0.12 mol) (purchased from Beijing Union Pharmaceutical Factory) was dissolved in 300 ml pyridine, and 100 g of acetic anhydride was slowly added in 30 minutes at room temperature. After being stirred for 24 hours, the crude product was slowly precipitated by transfered the reaction mixture to a large amount of ice water. The precipitate was collected through filtration, and 80 g of 6Ac was obtained with white solid, and the yield was around 99%.

6Ac (8 g, 0.012 mol), dissolved in dichloromethane (50 ml), was added to aqueous

sodium carbonate (5%, 50 ml). After the mixture was stirred at room temperature for one hour, the organic layer was separated, and dried over sodium sulphate. A white foam (7 g) was obtained by concentrated it.

Chromatographic separation of the reaction mixture was carried out by silica gel H (produced by Qingdao Marine Chemical Factory, 10~40 μm). The eluent was a mixture of dichloromethane-ethyl acetate (9:1, 5:1, 3:2). Combining the same ingredients was to give 1.5 g of 4Ac, 2. 0g of 5Ac, and 2.5 g unreacted 6Ac.

5.3 Structure Identification of New Puerarin Derivatives

5.3.1 Materials and Instruments

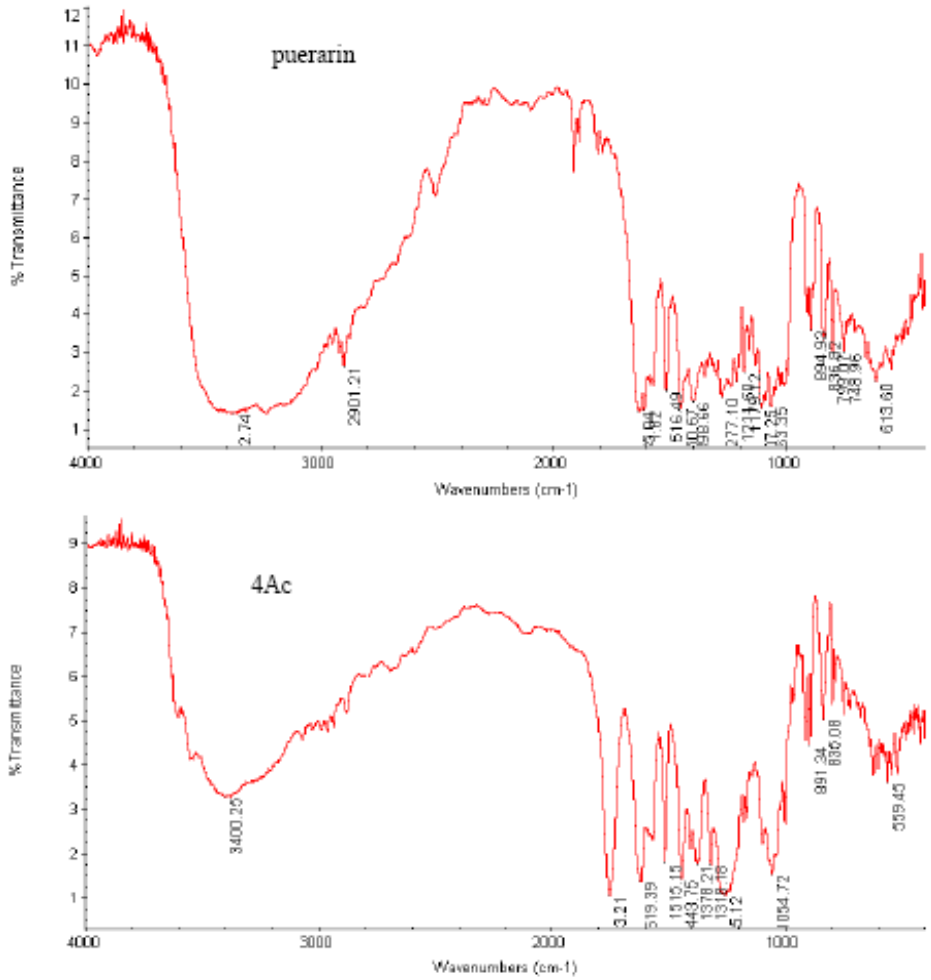
Lambda 35 ultraviolet spectrophotometer (Perkin-Elmer U.S.); AVATAR360 FT-IR infrared spectrometer; nuclear magnetic resonance instrument (Bruker 400 MHz); TLC scanner (CAMAG); MODEL 341 polarimeter (Perkinelmer, Germany); YRT-3 melting point apparatus (Tianjin University Precision Instrument Factory); powershot G_2 Canon Digital Ixus and SW22 thermostat water shaker (Germany).

Puerarin derivatives were prepared as indicated above, and puerarin was purchase from Beijing Union Pharmaceutical Factory (batch number: 030208); all the compounds are purified before subjecting to structural analysis. The physical constants of these compounds were measured and the results were listed in **Table 5-1**.

Table 5-1 Physical properties of puerarin and its derivatives, 4Ac, 5Ac and 6Ac

	Pur	4Ac	5Ac	6Ac
Appearance	White crystal powder	Colorless needles	Colorless flake	White crystal powder
MP(°C)	188-9	144-5	105-6	118-9
$[\alpha]_D^{20}$	+22.4	+37.7	+ 49.3	-45.5

The IR spectra of these compounds were recorded on an AVATAR360 FT-IR infrared spectrometer. The samples were first passed through a 120-mesh sieve, and the KBr pellets of these samples were prepared for recording the IR spectra. **Figure 5-2** represents the results.



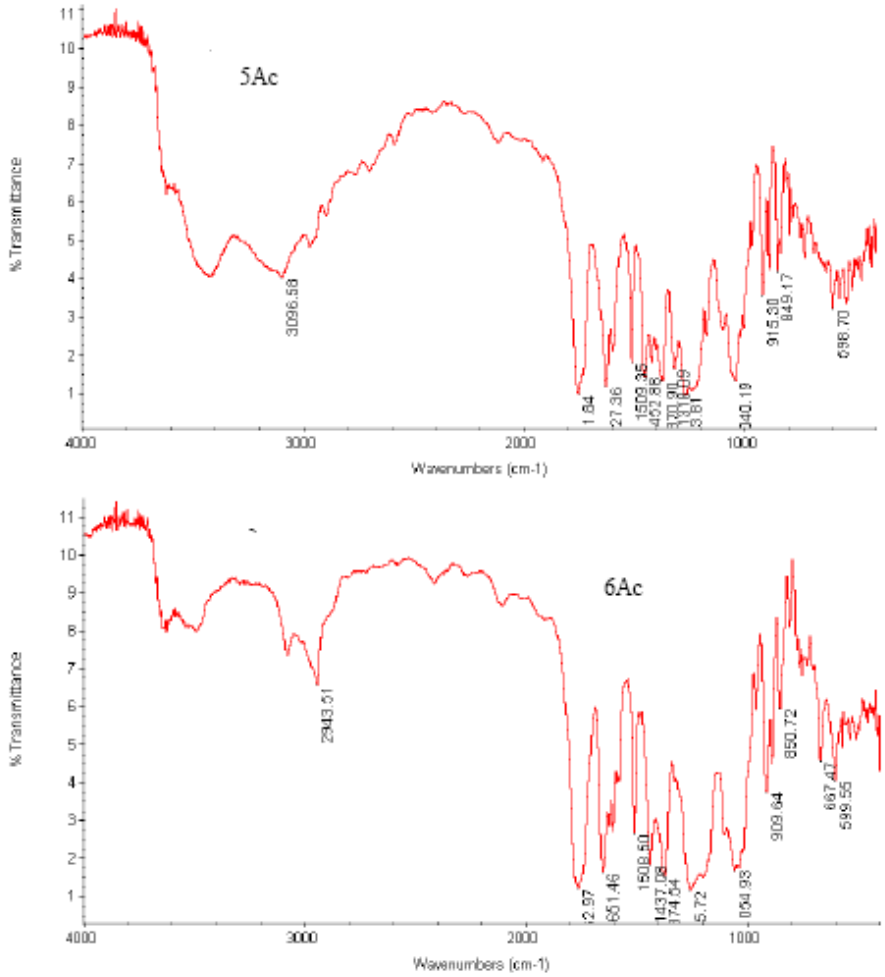
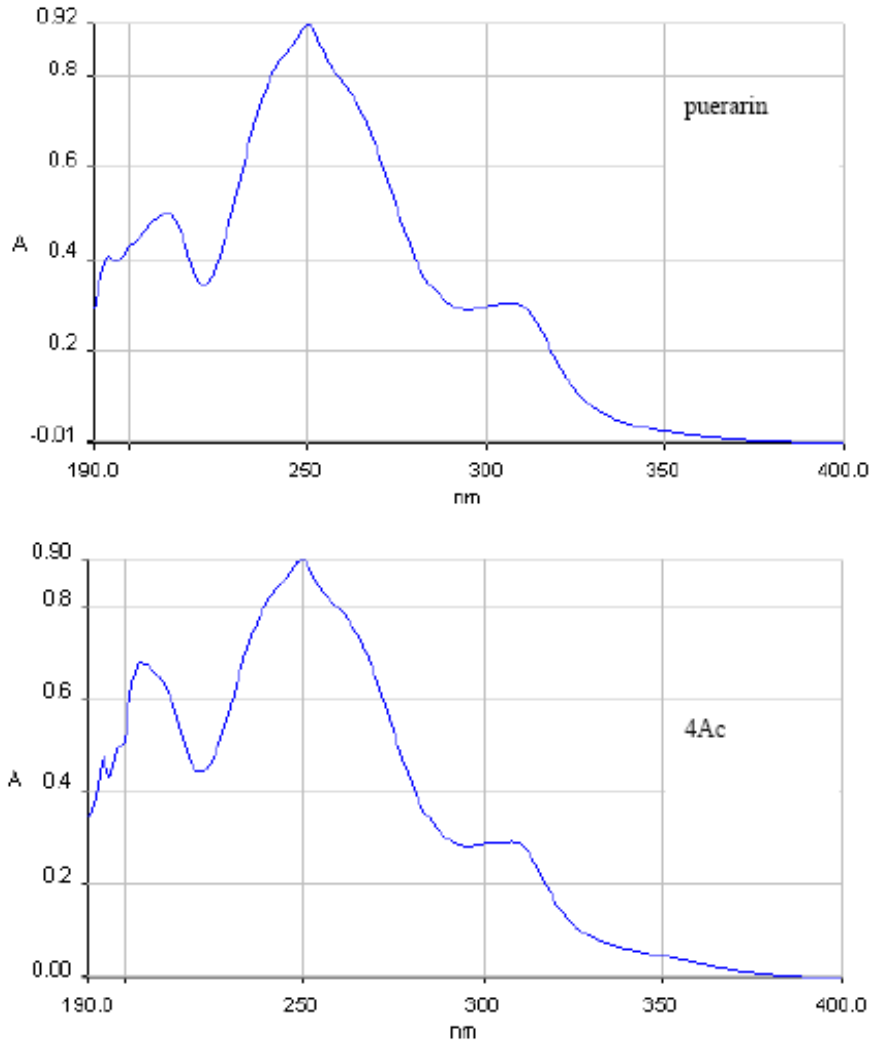


Figure 5-2 Infrared spectra of puerarin and its derivatives, 4Ac, 5Ac and 6Ac

Compared with puerarin, infrared spectra indicated that the stretching vibration peak (ν_{OH}) of 4Ac and 5Ac shifted to the direction of a high wave number with gradually weakened intensity, and the stretching vibration peak of 6Ac completely vanished in the end, all these indicated the reduction of the number of OH groups. All the three derivatives showed a high peak near the position of 1750 cm^{-1} which corresponded to the $\nu_{C=O}$ stretching vibration peak in the carboxyl group. The electron-withdrawing effect shifted the high wave number direction.

After dissolving puerarin and its derivatives with methanol, the UV visible spectra of these samples were also recorded with a scanning range of 190~400 nm. **Figure 5-3** presented the UV spectra of these samples.



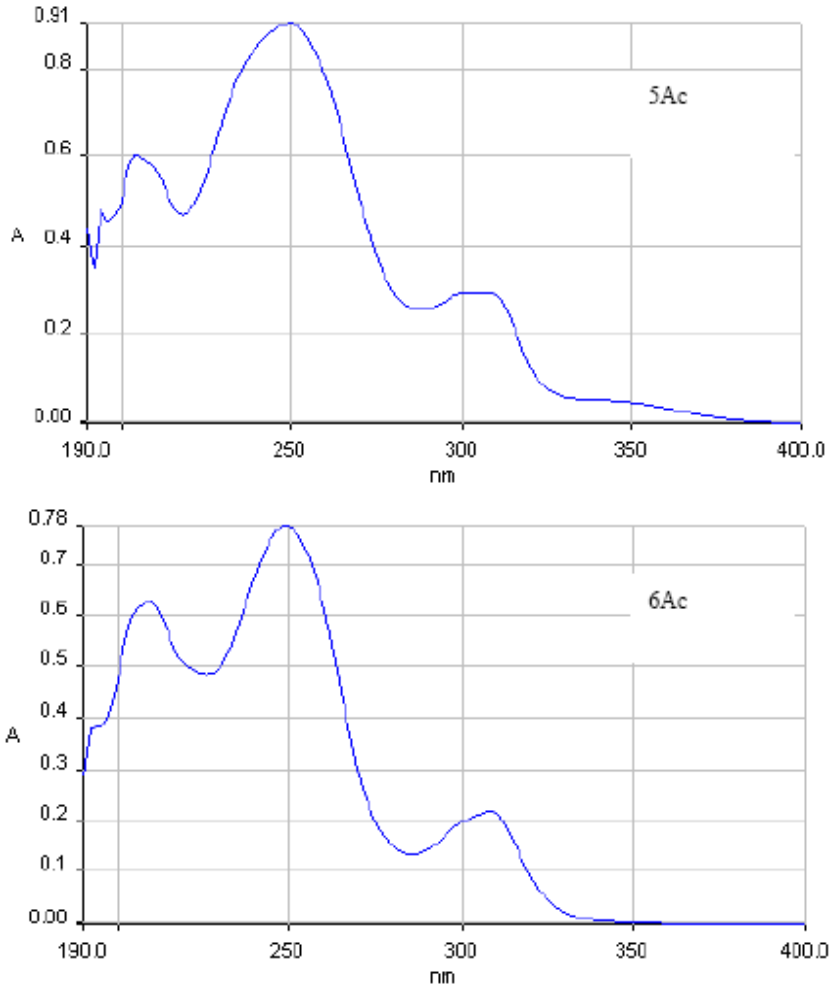


Figure 5-3 UV Spectra of puerarin and its derivatives, 4Ac, 5Ac and 6Ac

The UV spectra of these samples showed that puerarin and its derivatives all exhibited the largest absorption at around 250 nm and a shoulder peak at 308 nm, indicating that these compounds bore the same isoflavone skeleton.

5.3.2 Data of NMR, MS (see appendices for their spectra)

5.3.2.1 Data of ^{1}H NMR ($CDCl_3$, δ, ppm) spectra of 4Ac

8.21 (s, 1H), 8.19 (d, 1H, *J* = 9 Hz), 7.96 (s, 1H), 7.39 (d, 2H, *J* = 8.5 Hz), 7.01 (d, 1H, *J* = 9 Hz), 6.88 (d, 2H, *J* = 8.5 Hz), 5.76 (s, br, 1H), 5.43 (m, 3H), 5.34 (m, 1H),

4.36 (dd, 1H, J_1 = 3.5 Hz, J_2 = 12 Hz), 4.21 (dd, 1H, J_1 = 2.5 Hz, J_2= 13 Hz), 3.99 (m, 1H), 2.14 (s, 3H), 2.09 (s, 3H), 2.03 (s, 3H), 1.69 (s, 3H).

Data of ^{13}C NMR ($CDCl_3$, δ, ppm) spectra of 4Ac

176.6, 170.9, 170.6, 169.8, 161.4, 156.8, 152.1, 130.5, 128.8, 125.1, 122.9, 117.4, 115.9, 108.2, 76.6, 73.9, 70.2, 68.1, 61.8, 20.8, 20.3

Data of Mass Spectra of 4Ac

MS: 585.1808 (100%), 413.3010, 393.3213, 360.3675, 264.8656.

HRMS

Found for $C_{29}H_{28}O_{13}$ + H, 585.1616, calculated: 585.1608.

Found for $C_{29}H_{28}O_{13}$ + Na, 607.1408, calculated: 607.1428.

Color needles was crystallized by acetone/hexane and was subjected to X-ray diffraction analysis, see appendix for the ORTEP drawing of the crystal.

5.3.2.2 Data of ^{1}H NMR ($CDCl_3$, δ, ppm) spectra of 5Ac

8.20 (d, 1H, J = 9.5 Hz), 8.0 (s, 1H), 7.57 (d, 2H, J = 8.5 Hz), 7.17 (d, 2H, 8.5 Hz), 7.01 (d, 1H, 8.5 Hz), 5.40 (m, 3H), 5.35 (m, 1H), 4.37 (dd, 1H, J_1 = 3.5 Hz, J_2 = 12.5 Hz), 4.21 (dd, 1H, J_1 = 2 Hz, J_2 = 12.5 Hz), 3.99 (m, 1H), 2.32 (s, 3H), 2.14 (s, 3H), 2.09 (s, 3H), 2.02 (s, 3H), 1.67 (s, 3H).

Data of ^{13}C NMR ($CDCl_3$, δ, ppm) spectra of 5Ac

175.8, 171.0, 170.6, 169.8, 169.1, 161.5, 152.5, 150.9, 130.3, 129.3, 128.9, 117.7, 108.6, 74.0, 70.3, 68.3, 62.0, 21.4, 20.9, 20.8, 20.4

Data of Mass Spectra of 5Ac

MS: 627.1688 (100%), 556.2772, 425.1861, 397.1928, 360.3266.

HRMS:

Found for $C_{31}H_{30}O_{14}$ + H: 627.1688, calculated: 627.1714.

Found for $C_{31}H_{30}O_{14}$ + Na: 649.1506, calculated: 649.1533.

Colorless flake was obtained by ethanol and subjected to X-ray diffraction analysis, see appendix for the ORTEP drawing.

5.3.2.3 Data of ^{1}H NMR ($CDCl_3$, δ, ppm) of 6Ac

8.34 (d, 1H, *J* = 9 Hz), 8.09 (s, 1H, br), 7.60 (d, 2H, *J* = 8 Hz), 7.23 (m, 4H), 5.87-7.72 (1H, br), 5.43-5.28 (m, 2H), 3.88 (m, 1H), 2.45 (s, 3H), 2.33 (s, 3H), 2.08 (s, 6H), 2.03 (s, 3H), 1.70 (s, br, 3H).

Data of Mass Spectra of 6Ac

MS: 669.1808 (100%), 556.2771, 481.1439, 413.2685, 360.3273, 297.6115.

HRMS:

Found for $C_{33}H_{32}O_{15}$ + H: 669.1801, calculated: 669.1819.

Found $C_{33}H_{32}O_{15}$ + Na: 691.1622, calculated: 691.1639.

5.4 Study on the Stability of Puerarin and Its Derivatives

5.4.1 Selection of Evaluation Index and Establishment of Evaluation Method

Puerarin and its derivatives were placed under the conditions of high temperature (60 °C), high humidity (90% R.H.) and high light (4500±500 LX) for ten days, and the purity changes were monitored. Sample appearance, identification, content, and weight gain through moisture absorption were chosen as evaluation indices.

5.4.2 Thin-Layer Chromatography Analysis of the Samples

The samples of puerarin, 4Ac, 5Ac, and 6Ac (5 mg) were accurately weighed and

dissolveed with methanol to adjust the concentrations to 1 mg/ml, and the solutions were used as samples for furthere experiments.

Developing solvent: Chloroform: methanol: water (7: 1: 0.2); ethyl acetate: acetone: water: toluene (6.5:4.5:1.2:0.7); toluene: methanol: glacial acetic acid (7:1:1).

The sample solutions were spotted on a Silica gel GF_{254} plate, and developed with the three developing solvents, then the sample spots were visualized under the UV light (254 nm). The results were shown in **Figures 5-4** to 5-**6** and **Tables 5-2** to 5-**4**.

Figure 5-4 Stability of samples towards high temperature

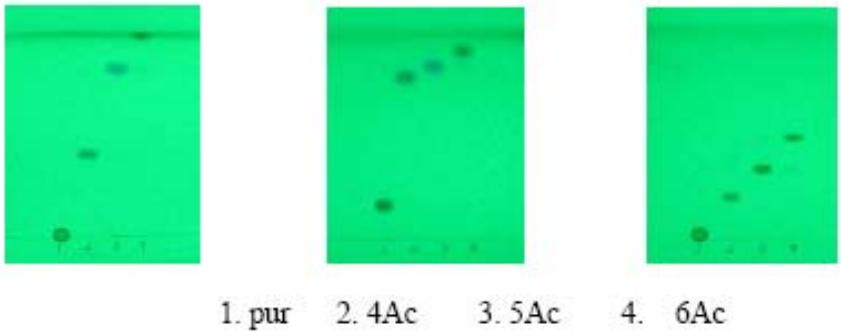


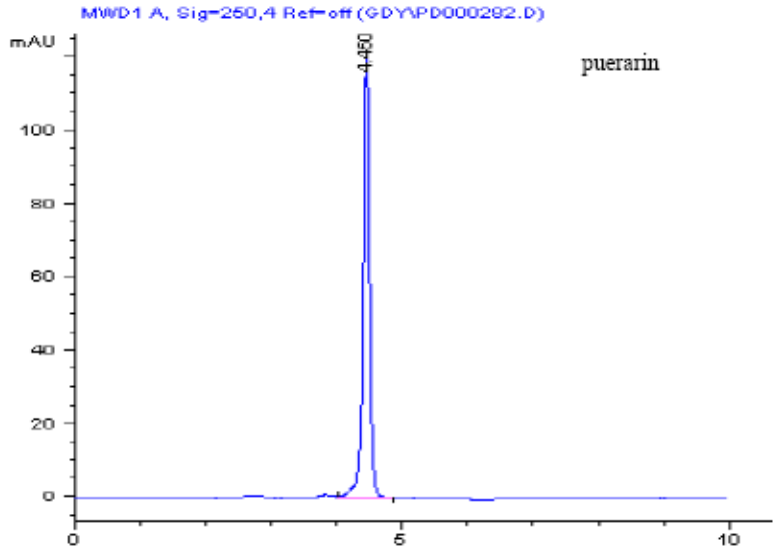
Figure 5-5 Stability of samples against high humidity

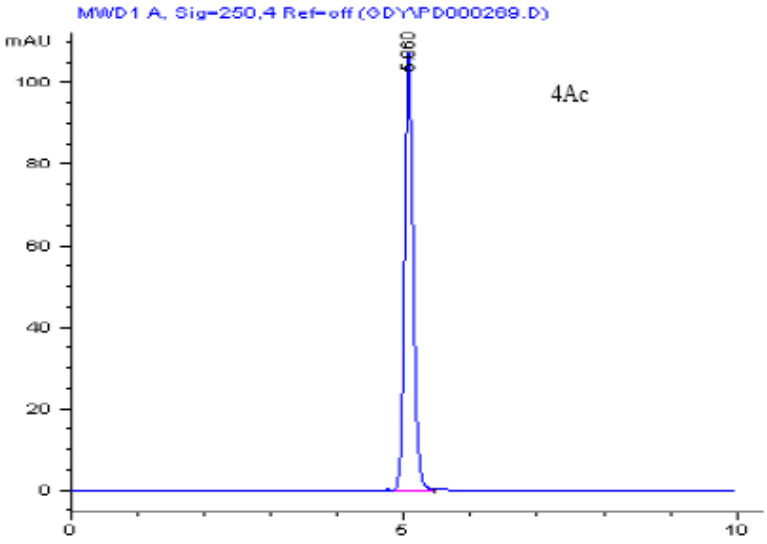
1. pur 2. 4Ac 3. 5Ac 4. 6Ac

Figure 5-6 Stability of samples against highlight

5.4.3 HPLC Determination of Content

HPLC analysis of the samples was carried out on an Agillent chromatographic column (4.6 × 250 mm, 5.0 μm) with a HP 1100 chromatography workstation. The mobile phase of puerarin was methanol: water (60:40) at a flow rate of 0.5 ml/min. The mobile phase of the puerarin derivatives was methanol: water (60:40) at a flow rate of 0.7 ml/min. **Figure 5-7** presented the HPLC analysis results of puerarin and its derivatives, and **Tables 5-2** to **5-4** summarized the details.

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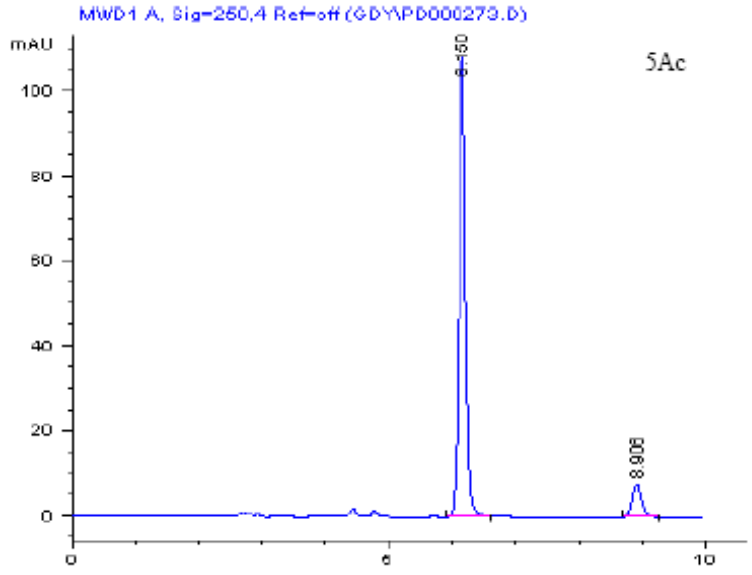
40
20
0
5.060
4Ac
0
5
10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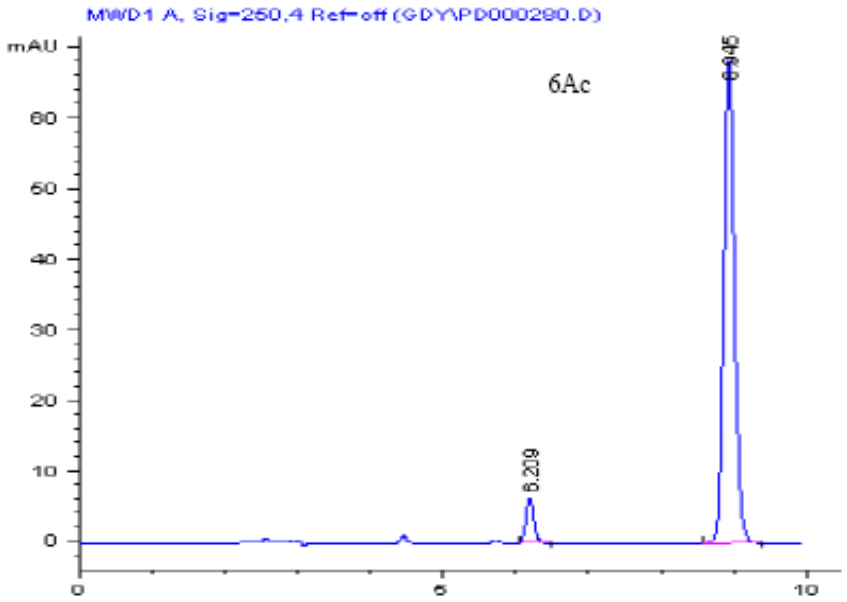


Figure 5-7 HPLC Spectra of puerarin, 4Ac, 5Ac and 6Ac for the test of stability

Table 5-2 Stability of puerarin and its derivatives at high temperature (60 °C)

	Sample	Pur	4Ac	5Ac	6Ac
5th Day	Appearance	white powder	Colorless needles	Colorless flake	white powder
	TLC	One spot	One spot	One spot	One spot
	Content	98.6%	98.3%	93.6%	91.7%
10th Day	Appearance	white powder	Colorless needles	Colorless flake	white powder
	one spot	One spot	one spot	One spot	one spot
	Content	98.5%	98.1%	93.3%	91.6%

The test results under high temperature showed that puerarin and 4Ac were more stable than 5Ac and 6Ac.

Except for a spot at the expected position of 6Ac, the TLC analysis results of 6Ac revealed that there was another spot at the position of 5Ac, which might be caused by the partial deacetylation of 6Ac. High performance liquid chromatogram also showed

that puerarin and 4Ac were more stable than 5Ac and 6Ac, because only a single component was detected for puerarin and 4Ac, but two components for 5Ac and 6Ac.

Table 5-3 Stability of puerarin and its derivatives under high humidity (RH 92.48%)

	Sample	Pur	4Ac	5Ac	6Ac
5^{th} Day	Appearance	white powder	Colorless needles	Colorless flake	white powder
	Increased Weight	1.2%	1.3%	1.2%	1.4%
	TLC	One spot	One spot	One spot	Two spot
	Content	98.6%	98.2%	94.2%	92.3%
10^{th} Day	Appearance	white powder	Colorless needles	Colorless flake	white powder
	Increased Weight	1.3%	1.3%	1.4%	1.2%
	one spot	One spot	one spot	One spot	Two spot
	Content	98.4%	98.6%	93.5%	91.8%

The high humidity stability experiments had indicated that puerarin and 4Ac were more stable than both 5Ac and 6Ac under the high humidity.

Except for the major spot of 6Ac, the TLC analysis of 6Ac had indicated that a small amount of 5Ac was also detected, which must been caused by the partial deacetylation of 6Ac during storage. HPLC analysis also indicated that puerarin and 4Ac were more stable than 5Ac and 6Ac, since only one component was detected from puerarin and 4Ac, and two components were detected from 5Ac and 6Ac due to partial deacetylation. The results of weight gain by humidity absorption indicated that humidity absorption of all derivatives were not apparent and negligible.

Table 5-4 Test results under highlight (4000±500LX)

	Sample	Pur	4Ac	5Ac	6Ac
5th Day	Appearance	white powder	Colorless needles	Colorless flake	white powder
	TLC	One spot	One spot	One spot	Two spot
	Content	98.3%	98.1%	93.6%	92.1%
10th Day	Appearance	white powder	Colorless needles	Colorless flake	white powder
	one spot	One spot	one spot	One spot	Two spot
	Content	98.8%	98.2%	94.2%	91.8%

The highlight stability tests had also revealed that puerarin and 4Ac were more stable than 5Ac and 6Ac.

5.4.4 Stability of Puerarin and its Derivatives in Water with Different pH Values

The tested materials were dissolved in aqueous solution with different pH values and were stored for 10 days under natural light. The samples were then subjected to TLC analysis. **Table 5-5** and **Figure 5-8** were summarized these results.

Table 5-5 Effect of different pH value on the appearance of the compounds

pH Value	1	2	3	4	5	6	7	9	11	13
Pur	-	-	-	-	-	-	-	+	+	++
4Ac	-	-	-	-	-	-	-	+	+	++
5Ac	-	-	-	-	-	-	-	+	+	++
6Ac	-	-	-	-	-	-	-	+	+	++

- White suspension, + Faint yellow solution, ++ Yellow solution

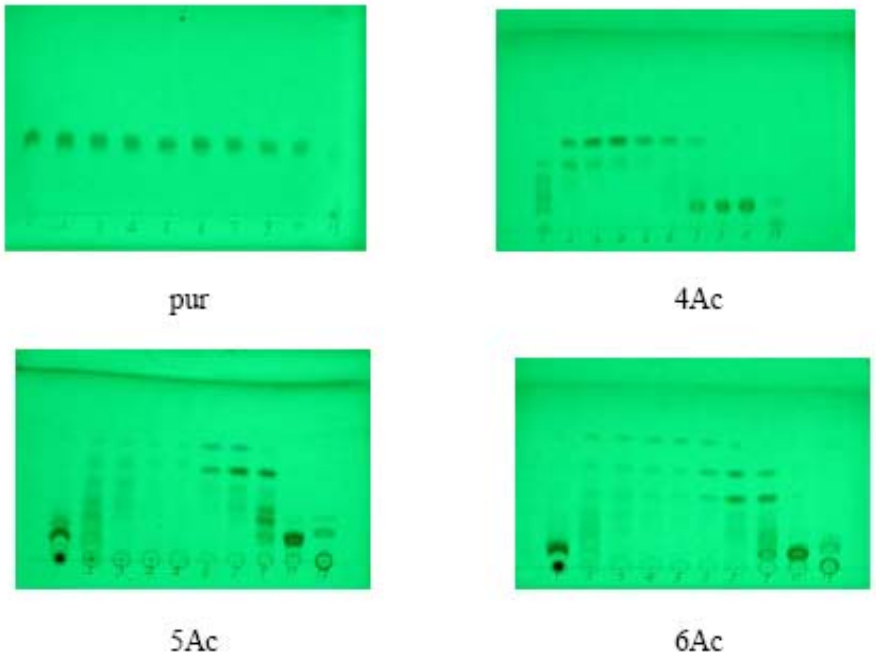


Figure 5-8 TLC results of puerarin and its derivatives under different pH values

From the TLC plates, puerarin appeared stable against acid and alkali and showed a single spot in the pH range from 1 to 11. But at pH above 13, no spot was shown at the puerarin position due to the salification (formation of phenolic salts). Compound 4Ac was slightly less stable against acid and alkali, and showed a single spot at pH from 5 to 6. 4Ac showed different degrees of hydrolysis and salification at other pH values, 5Ac and 6Ac were unstable against both acid and alkali.

5.4.5 Determination of Stability of Puerarin and its Derivatives in Air

The samples were stored in an open vessel for 30 days, and the samples were subjected to TLC analysis. The results were summarized in **Table 5-9**.

Table 5-9 Stability puerarin and its derivatives against Air

Sample	pur	4Ac	5Ac	6Ac
Appearance	white powder	Colorless needles	Colorless flake	white powder
TLC	One spot	one spot	one spot	two spot
Content	98.5%	98.2%	93.8%	91.6%

The results indicated that puerarin and 4Ac were stable against oxygen in the air, whereas both 5Ac and 6Ac were less stable than 4Ac. The results of the TLC analysis revealed that near the spot of 6Ac, a small amount of 5Ac could be detected. HPLC analysis also revealed that puerarin and 4Ac were more stable than 5Ac and 6Ac, while the former two compounds showed single peak, two peaks were detected from 5Ac and 6Ac.

5.4.6 Summary of the stability test results

Stability experiments had indicated that puerarin and its derivative 4Ac were stable under the tested conditions such as high temperature, high humidity and highlight as well as at wide range of pH values and against oxygen in the air, whereas 5Ac and 6Ac were less stable under these conditions.

5.5 Determination of Solubility of Puerarin and 4Ac

5.5.1 Solubility of Puerarin and 4Ac in water

Puerarin and 4Ac (about 150 mg), accurately weighed, were put in two 10 ml standard full plug test tubes, containing 10 ml distilled water, respectively, and were

shaken on the 25 °C thermostat shaker at 100 RPM. The sample was centrifuged for 15 min (3000 RPM) after being shaken for 48 hours, and the supernate was filtered with a microporous filtering film. The solution was diluted and its UV absorbance at 250 nm was measured. The solubility could then be calculated accordingly. The experiments were carried out for 5 times and the results were summarized in **Table 5-10**.

Table 5-10 Solubility of puerarin and 4Ac in Water

Times	1	2	3	4	5	Average
pur(mg/ml)	4.20	4.25	4.20	4.25	4.30	4.24
4Ac(mg/ml)	0.060	0.058	0.059	0.061	0.058	0.0592

The solubility experiments indicated that the water solubility of 4Ac was significantly lower than that of puerarin.

5.5.2 Effect of pH on the Solubility of Puerarin and 4Ac

Puerarin and 4Ac are isoflavone C-glycoside and bear a glucopyranose moiety at the C_8-position of the aromatic ring. The glycoside exhibited strong hydrophility, and the glucosyl moiety was the foundation of the hydrophility of puerarin. There were also phenolic hydroxyl groups at C_7 and C_4, and these functional groups should also contribute to the solubility of the compounds. Aqueous NaOH (0.1 M) and diluted hydrochloric acid (0.1 M) were used to adjust the pH value of the solution to study the relationship between the solubility of puerarin/4Ac and the pH values of the solvent. The average results were summarized in **Table 5-11**.

Table 5-11 Effect of pH value on the solubility of puerarin and 4Ac

pH Value	1	3	5	7	9	11
pur(mg/ml)	4.10	4.25	4.25	5.80	6.96	7.60
4Ac(mg/ml)	0.620	0.590	0.060	0.077	0.085	1.340

These results indicated that puerarin solubility did not have significant change at the pH range from 1 to 5, and the solubility of 4Ac gradually reduced when the pH value was increased from 1 to 5. The solubility of both compounds could be increased with the pH values above 7.

5.5.3 Solubility of Puerarin and 4Ac in Different Various Medias

Puerarin and 4Ac were dissolved in distilled water, dilute hydrochloric acid (pH 1.2) and phosphate buffer with pH 5.0, 6.0, 6.8, and 4.6. After being shaken for 48 hours in the 37 °C water bath in close condition, the samples were stood still for 12 hours in the same 37 °C water bath. The mixtures were then centrifuged for 10 minutes (3000 RPM), and the supernates were diluted to a certain volume. The concentration of the compounds was then measured and the solubility of these compounds in different release media was calculated (See **Table 5-12** for details).

Table 5-12 Solubility of puerarin and 4Ac in different release media

Media	pH 7.0	pH 1.2	pH 5.0	pH 6.0	pH 6.8	pH 7.4
pur(mg/ml)	4.30	4.20	4.40	4.50	5.55	6.15
4Ac(mg/ml)	0.060	0.600	0.060	0.060	0.075	0.083

Results in **Table 5-12** indicated that the solubility of puerarin and 4Ac showed no apparent change in the distilled water at pH from 1.2 to 6.8. At pH above 6.8, the solubility of both compounds increased with increasing the pH values.

5.5.4 Oil/Water Partition Ratio of Puerarin and 4Ac

A Certain volume of saturated solution of puerarin and 4Ac was added into a mixture of a certain amount of *n*-Octanol and hydrochloric acid (pH 1.2) or phosphate buffer (with pH of 5.0, 6.0, 6.8 and 7.4). The mixtures were first well mixed, and kept on a 37 °C thermostat shaker at 100 RPM for 48 hours. The mixtures were then kept still to allow the separation of organic and aqueous phases. The water phase of each sample was centrifuged for 10 minutes at a speed of 3000 RPM and subjected to the concentration measurements (**Table 5-13**).

Table 5-13 Oil/water partition ratio of puerarin and 4ac

Medium	pH 7.0	pH 1.2	pH 5.0	pH 6.0	pH 6.8	pH 7.4
pur	0.3842	0.3904	0.3846	0.387	0.4197	0.5570
4Ac	18.70	19.20	19.00	19.00	36.50	36.43

The results in **Table 5-13** revealed that the apparent oil/water partition ration of 4Ac was much higher than that of puerarin, which has stronger lipophilicity than that of puerarin.

5.6 Summary

Acetylation of puerarin in pyridine at the presence of an excess amount of acetic anhydride produced puerarin peracetate 6Ac, and partial deacetylation of 6Ac produced the corresponding puerarin penta- and tetracetate. These compounds were characterized through infrared spectra, ultraviolet spectra, and nuclear magnetic resonance. TLC/HPLC analysis of the compounds indicated that all the compounds were essentially pure, and could be used for most of the studies. For compound 4Ac, the alcoholic hydroxyl groups in the glucose moiety were acetylated. In 5Ac, both

the alcoholic hydroxyl groups in the glucose moiety and the 4’-OH were acetylated. In 6Ac, all of the alcoholic hydroxyl groups in the glucose moiety and at 7- and 4’-positions were acetylated.

Puerarin and its derivatives were all white powders in appearance under high temperature, high humidity, and highlight. TLC analysis indicated that puerarin and its derivatives 4Ac and 5Ac showed one spot in three developing system with different polarities, whereas 6Ac showed two spots under similar conditions. Content determined by HPLC analysis showed that puerarin and 4Ac had only one peak, whereas 5Ac and 6Ac had two peaks and the smaller peaks represent the partial deacetylation prducts, respectively, of the corresponding parent compounds. All these experiments indicated that puerarin and 4Ac were more stable than other derivatives, and 4Ac showed good stability, which made it possible for the compound to be used clinically.

Physicochemical property study showed that 4Ac had lower water solubility than that of puerarin, and its oil/water partition ratio was higher than that of puerarin, all above revealed that the liposolubility of 4Ac was stronger than that of puerarin. This could be the main reason for the high *in vivo* absorption of 4Ac than that of puerarin.

Chapter 6 Bioavailability and Bioactivity of Puerarin and its Derivatives

6.1 Study on Bioavailability of Puerarin and its Derivatives

6.1.1 Method and result

Puerarin was purchased from Beijing Union Pharmaceutical Factory (Batch No.:030208) and the purity was over 98% (based on HPLC analysis). Puerarin derivatives 4Ac, 5Ac and 6Ac were prepared as described in Chapter 5 with 99.8% purity. Solvents acetonitrile and methanol were HPLC grade, and water was double distilled and de-ionized.

HPLC was performed on Agillent 1100 with an Agillent XDB-C_{18} chromatographic column (250 mm×4.6 mm D, 5 μm) using DAD Diode Array Detector, and the data were processed with the HP 1100 chromatogram station. The samples were first passed through a pre-column Agillent XDB-C_{18} (12.5 mm×4.6 mm D, 5 μm), and gradient elution was adopted during the analysis: 0 min: acetonitrile:water (10:90) 0.7 ml/min; 15 min: acetonitrile:water (60:40) 0.7 ml/min; 20 min: acetonitrile:water (70:30) 0.7 ml/min; 30 min: acetonitrile:water (100:0) 0.7 ml/min. Column temperature was at room temperature and detector wavelength was 250 nm.

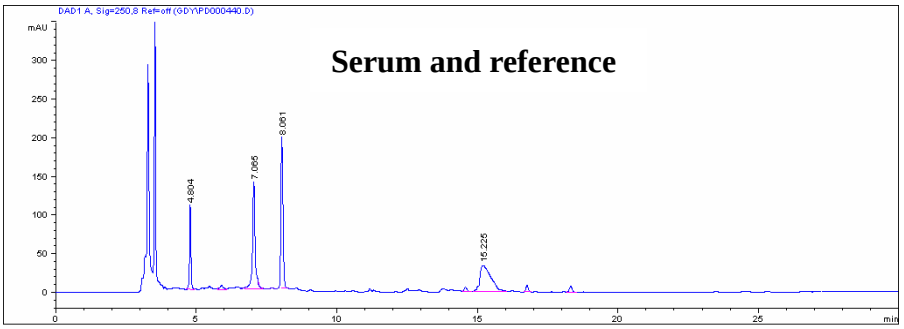
Animal: SD rat, weight: 180±20g, half male and half female, were provided by animal centre of Guangzhou University of Traditional Chinese Medicine.

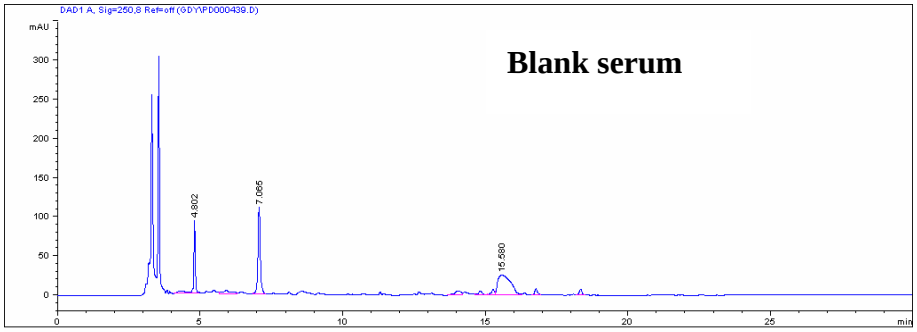
Sampling: 260 SD rats were randomly divided into 20 groups with 13 rats in each group. Stop feeding for 1 day before the experiment. Puerarin, 4Ac, 5Ac, 6Ac were poured into the stomach of the rats at the dose of 400 mg/kg, 560 mg/kg, 600 mg/kg

and 640 mg/kg, respectively. The blood sample (3.0 ml) was taken from femoral vein after 10 minutes, 20 minutes, 30 minutes, 45 minutes, 60 minutes, 90 minutes, 120 minutes, 4 hours, 6 hours, 7 hours, 8 hours and 12 hours. The samples were kept in clean centrifuge tubes at room temperature for 0.5 hour, and were centrifuged for 15 minutes at 3000 RPM. The supernatant serum was then collected for use.

Sample preparation: 2.0 ml methanol was added to 0.5 ml serum. The mixture was oscillated in swirl for 1 minute and was centrifuged for 15 minutes at 3000 RPM. Supernatant serum was purged with nitrogen, and 0.2 ml of methanol was added into the residue. The mixture was centrifuged for 10 minutes at 10000 RPM, the supernatant serum was collected and subjected to HPLC analysis.

HPLC analysis on the samples of puerarin reference, blank serum and serum with drug indicated that the impurities in serum did not affect the measurement of puerarin (retention time 8.5 minutes, **Figure 6-1**).





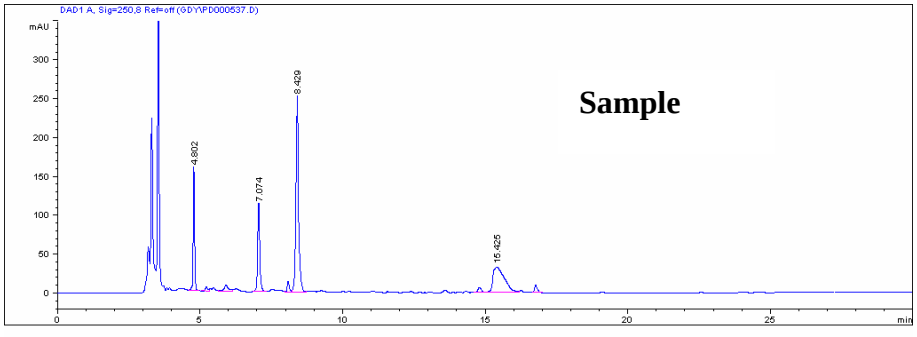


Figure 6-1 HPLC plots puerarin for the bioavailability test

Standard curve plotting: puerarin reference, accurately weighed, was dissolved in methanol to give the stock solution, and the stock solution was diluted to reach the concentration of 0.1 mg/ml (in a volumetric flask). This stock solution was then diluted with methanol to obtain a series of standard solutions with concentrations of 0.025, 0.0125, 0.00625, 0.003125, 0.00156 mg/ml puerarin, respectively. Mixing 0.5 ml puerarin solution with 0.5 ml blank serum and a reference solution was obtained after proper treatment. The above solution was then subjected to HPLC analysis: 20 μl of the above solutions were injected in the HPLC, and the absolute peak areas of these samples were measured. Plotting standard curve of puerarin reference with puerarin concentrations as X axis and HPLC peak areas as Y axis gave an equation which could be used for puerarin concentration determination: Y=33426X+32.108 (R^2=0.9996). A linear relationship was good in the concentration range of 1.95 μg/ml to 31.25 μg/ml, and the minimum detection limit was 195ng/ml (**Figure 6-2**).

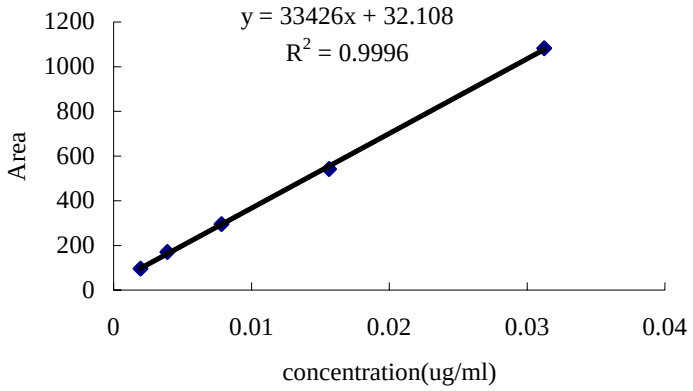


Figure 6-2 Calibration curve of puerarin in serum

Recovery test: 6 fractions of 0.5 ml serum were mixed with a certain amount of puerarin solution and the solutions were mixed in swirl. To this solution was added 2.0 ml methanol, and the mixture was oscillated in swirl for 1 minute and centrifuged for 15 minutes at 3000 RPM. The supernatant liquid was purged with nitrogen and the residue was dissolved with 0.2 ml methanol. The mixture was then centrifuged for 10 minutes at 10000 RPM, and the supernatant liquid was subjected to HPLC analysis. The peak area was used for recovery rate calculation. As shown in **Table 6-1**, the average recovery rate of high, medium and low concentration is 92.03%.

Table 6-1 Test of recovery (n=6)

S/N	Added amount	Measured amount (area)	Recovery rate (%)	Mean (%)	RSD (%)
1	710.6	636.6	89.59	92.03	1.40
2	695.1	645.7	92.89		
3	346.0	318.8	92.14		
4	355.6	328.1	92.27		
5	185.2	172.7	93.25		
6	184.6	169.9	92.04		

The results also indicated that the recovery rate was within the required range and the method was reasonable and reliable.

Precision Test: to 0.5 ml serum samples containing puerarin, 4Ac, 5Ac and 6Ac, respectively was added 2.0 ml methanol. The mixture was oscillated in swirl for 1 minute and was centrifuged for 15 minutes at 3000 RPM. The supernatant liquid was purged with nitrogen, and the residue was re-dissolved with 0.2 ml methanol. The mixture was then centrifuged for 10 minutes at the speed of 10000 RPM and the supernatant liquid was then subjected to HPLC analysis. A single-point external standard method was used for calculating the RSD (%). **Tables 4-2** and **4-3** were summarized the results.

Table 6-2 Within-day precision (n=5)

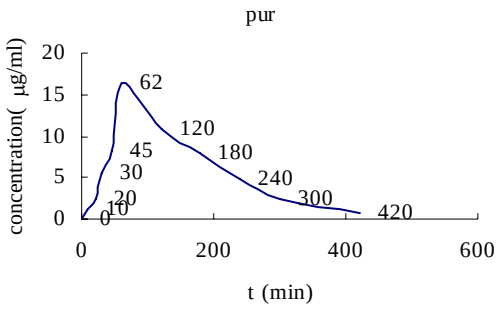
S/N	1	2	3	4	5	Average	RSD
Peak areas	318.8	328.1	348.1	348.4	361.7	341.0	5.06%

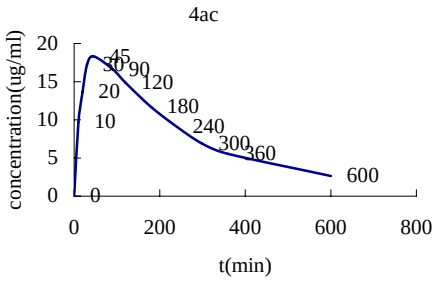
Table 6-3 Day to day precision (n=5)

S/N	1	2	3	4	5	Average	RSD
Peak areas	645.7	636.6	610.5	542.8	562.1	599.54	7.57%

These experiments indicated that both the within-day precision and the day to day precision were less than 10%, and the precision of the method was acceptable.

Data analysis: The single-point external standard method was used to calculate the blood drug level. The blood drug data was processed with a 3P97 pharmacokinetic statistics program, and the result indicated that process of puerarin, 5Ac and 6Ac in body was in accordance with the two-compartment model, and 4Ac was in accordance with the one-compartment model. See **Table 6-4** and **6-5** for the main pharmacokinetic parameters.





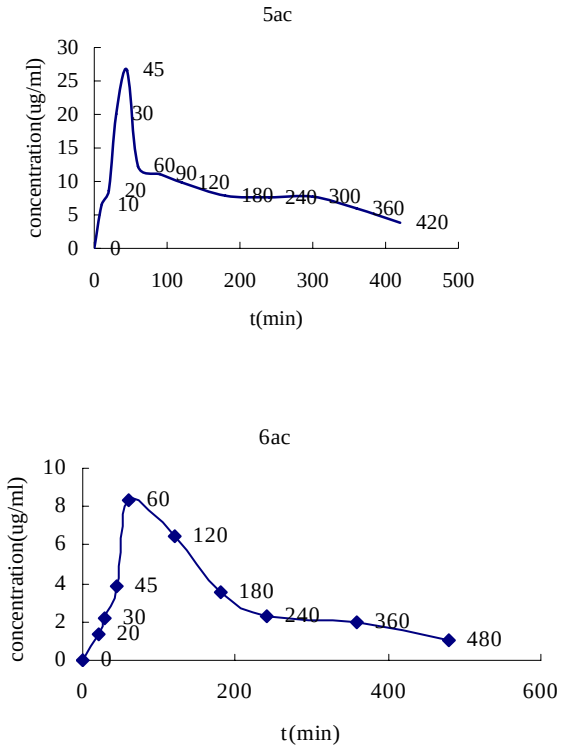


Figure 6-3 Drug-time cures

Table 6-4 Pharmacokinetics parameters of puerarin derivatives

Parameter / Sample	$t_{1/2(a)}$ min	$t_{1/2(\beta)}$ min	CL g/kg/min/ug/ml	AUC(0~∞) (g/ml) min	T(peak) min	C(max) g/ml
pur	53.42±11.32	60.92±0.43	0.20±0.05	2729.94±491.99	84.91±7.69	9.22±2.37
4Ac	38.95±12.25	32.32±0.48	0.10±0.01	6104.81±275.29	51.48±13.03	17.61±2.06
5Ac	22.12±18.72	425.03±253.68	0.10±0.04	4566.95 ±762.64	42.23±18.07	15.04±1.81
6Ac	111.97±108.66	2664.97±379.94	0.17±0.12	3149.69±467.26	81.39±10.02	4.93±0.07

Table 6-5 Comparison of main pharmacokinetics parameters

(Sample)	Pur	4Ac	5Ac	6Ac	4Ac/Pur	5Ac/Pur	6Ac/Pur
AUC (g/ml) min	2730	6150	4567	3150	2.24	1.67	1.15
T (peak) min	85	51	42	81	0.6	0.49	0.95
C (max) g/ml	9.22	17.61	15.04	4.93	1.91	1.63	0.53

According to the data in the Tables, *in vivo* plasma concentration and the area below time curve in 4Ac were far higher than that of other compounds, and there was an obvious difference between the 4Ac group and the puerarin group ($t<0.01$).

6.1.2 Summary

Puerarin had the advantages of low toxicity and fast metabolism. The bioavailability of puerarin was low when administered orally by dogs, with the absolute bioavailability at about 3%. Most of the drug was discharged through dejecta in its original form. The cause for the low bioavailability of puerarin may be that the glucose moiety attached to isoflavone parent nucleus caused the low affinity of the drug with cell, and very little could be absorbed.. Given that most of the drug effects come from the interaction of the drug molecule with the biological receptors, the solubility of the drug molecule and the size, form and spatial structure of the molecule would be very important. We assumed that the water-solubility and lipo-solubility could be improved through the structural modification, and the spatial structure could also be changed through structural modification. The current experimental results indicated that through the partial acetylation, the

water-solubility of the puerarin derivatives was reduced, with the lipo-solubility significantly increased, and the bioavailability of the derivatives also improved.

The pre-test experiments indicated that the stability of 4Ac, 5Ac, 6Ac was gradually reduced. In mixed reference, gradient elution was used. Their retention time is 8.5 minutes, 16.4 minutes, 19.2 minutes and 21.4 minutes respectively. In general, 4Ac, 5Ac, 6Ac existed in the form of puerarin in rats. The reason might be that the puerarin derivatives were hydrolyzed and the acetyl groups were removed by metabolism of the rats.

6.2 Study on the bioactivity of puerarin derivatives

6.2.1 Effect of puerarin and its derivatives on myocardial ischemia caused by pituitrin

Puerarin formulate solution: 0.8 g/10 ml was prepared by mixing puerarin with a solution of PEG 400 and sterilized distilled water (1:1 w/w) with dosage of 10 ml/kg for stomach pouring.

Puerarin derivatives (4Ac): specially formulated solution of 1.12g /10 ml (corresponding to puerarin 0.8 g/10 ml was prepared with PEG 400 and sterilized distilled water at a proportion of 1:1, dosage 10 ml/kg for stomach pouring.

Puerarin derivatives (5Ac): specially formulated solution of 1.20 g/10 ml (corresponds to puerarin 0.8g/10mL) was prepared with PEG 400 and sterilized distilled water at proportion of 1:1, dosage of 10 ml/kg for stomach pouring.

Puerarin derivatives (6Ac): specially formulated solution of 1.28 g/10 ml (corresponding to puerarin 0.8 g/10 ml) was prepared with PEG 400 and sterilized

distilled water at proportion of 1:1, dosage 10 ml/kg for stomach pouring.

SD rats at SPF level with weight of 200±20g were used. The rats, half female and half male, were provided by Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine. Certificate No of animal quality: No. 2002A005, (YJZZ) Certificate issued by Institute Experimental Animal Monitoring in Committee of Science and Technology, Guangdong Province. The animals should be bred under normal condition for one week after purchase in order to help them adapt to the environment.

60 SD rats at SPF level were divided into the following 6 groups according to their weight and sex: Group I: normal control group: distilled water, dosage 10 ml/kg; Group II: positive control group: propranolol (2 mg/ml), dosage: 10 ml/kg; Grop III: puerarin solution group, puerarin of 0.8 g/10mL dosage of 10 ml/kg; Group IV: puerarin derivative (4Ac) solution group: 4Ac concentration was 1.12 g/10 ml and the dosage was 10 ml/kg; Group V: puerarin derivatives (5Ac) solution group: 5Ac of 1.20 g/10 ml and dosage of 10 ml/kg; Group VI: puerarin derivative (6Ac) solution group: 6Ac of 1.28 g/10 ml and dosage of 10 ml/kg.

The rats were first fed with drugs/controls for five consecutive days. On the sixth day, after feeding drugs for 1 hour, 3% amobarbital (dosage 40 mg/kg) was injected into the abdominal cavity for anaesthesia. The rats were connected with two lead electrodes and the normal electrocardiograms were recorded. When the electrocardiogram was getting stable, pituitrin (dosage 1 μg/kg) was injected into the tail vein and the electrocardiogram was recorded immediately for 30 minutes. The T

wave variations before and after drug delivery, were calculated, and the data were processed. The results were presented in **Table 6-6**.

Table 6-6 T-wave variation of puerarin and its derivatives resisting rat myocardial ischemia by pituitrin($\bar{x}$±sd, n=10)

Group	Dosage /(g.kg^{-1})	T wave range T(μV)					
		5s	15s	30s	2min	5min	10min
Control group	—	15.69±7.17	105.23±33.09	66.25±31.98	75.15±27.29	70.62±28.30	19.26±10.11
Positive group	2 Tab/kg	16.11±5.30	18.51±9.26**	22.10±11.23**	30.41±11.58**	26.62±10.99**	22.09±11.31
Puerarin group	0.8	18.06±10.09	25.69±11.21**	31.22±14.59**	39.20±16.11**	29.10±16.23**	25.01±10.40
4AC	1.12	15.09±7.62	23.23±10.69**	35.40±10.93*	36.51±16.02**	25.60±14.23**	25.41±10.21
5AC	1.20	19.09±6.20	29.42±9.39**	37.61±13.43*	40.11±16.04**	24.50±13.63**	24.39±14.28
6AC	1.28	16.33±9.18	29.09±16.50**	39.12±15.21*	41.05±15.60**	29.14±10.60**	22.11±9.81

Compared with control group ** $P< 0.01$ * $P< 0.05$

Data in **Table 6-6** showed that there were obvious differences among 15s, 30s, 2min and 5min with the control group after pituitrin was injected into the tail vein of the rats.

6.2.2 Study of puerarin and its derivatives against arrhythmia induced by chloroform

Yufeng NingXin Tablet (as positive reference drug, made by Beijing Tong Ren Tang Co., Ltd. Batch No.: 0120035) was grounded to powder, and an appropriate amount of distilled water was used to prepare a solution with a concentration of 0.15 mg puerarin per ml.

KM mice at SPF level, 18~22g, half female and half male, were provided by Guangzhou TCM University Animal Centre with certificate No. 2002A005, (YJZZ) issued by Institute of Experimental Animal Monitoring in Committee of Science and Technology, Guangdong Province. The mice were fed in laboratory for a week after purchase under normal condition to allow them to adapt to the environment.

60 Mice were divided into the following 6 groups according to their weight and sex, each group had 10 mice with half male and half female: Group I: control group: distilled water, dosage 20 ml/kg; Group II: positive-drug control group: Yufeng Ningxin tablet, 0.28 g/tablet, 0.15 tablet/ml, dosage 20 ml/kg. Groups III to VI: testing drug groups for puerarin, 4Ac, 5Ac, 6Ac, dosage 20 ml/kg. The drug was poured into the mice stomach in each group for 4 consecutive days. On the 5th day after stomach pouring for 1 hour, the mice were anaesthetized with 4 ml chloroform. The thoraxes were opened, and the positive ratio of ventricular fibrillation was

checked when the mouse stopped breathing. The results were summarized in **Table 6-7**.

Table 6-7 Test result of puerarin and its derivatives against mouse arrhythmia

Group	Quantity (mouse)	Ventricular fibrillation	without ventricular fibrillation
Control	10	7	3
Yufengningxin	10	4	6*
Puerarin	10	5	5*
4Ac	10	3	7**
5Ac	10	4	6*
6Ac	10	5	5*

Note: Compared with control group, we get *P < 0.05 **P < 0.01

Current study indicated that puerarin 0.8 g/kg, puerarin derivative 4Ac 1.12 g/kg (corresponding to puerarin 0.8 g/kg), puerarin derivative 5Ac 1.20 g/kg (corresponding to puerarin 0.8 g/kg) and puerarin derivative 6Ac 1.28 g/kg (corresponding to puerarin 0.8 g/kg) could all resist rat myocardial ischemia resulted from pituitrin injection.

Furthermore, puerarin and its derivatives could obviously resist mouse arrhythmia induced by chloroform, and decrease its ventricular fibrillation ratio.

6.3 Acute Toxicity of Puerarin and its Derivatives

In order to be registered as a grug, a drug candidate must have shown its safety, effectiveness and stability. The current comprehensive evaluation of the stability, effectiveness and bioavailability of puerarin derivatives indicated that 4Ac was more

promissing for further development. The compound had a better lipophilicity, and better stability than that of other puerarin derivatives, and the bioavailability of 4Ac was significantly improved as comared to puerarin. This compound was then subjected to an acute toxicity experiment.

The most concentrated 4Ac solution was poured into the mouse's stomach at the highest possible dosage, and its toxic reaction was monitored. The experiment results would be directly used to provide a theoretical basis for further drug development, as well as to act as a reference for clinical drug administration.

The compand 4Ac (specially made) was dissolved in PEG-400: distilled water (1:1) to prepare the drug solution containing 60% (g/v) of 4C.

Animal: 20 mice at first level, 19.81±0.80g), half female and half male, were provided by Guangzhou TCM University Animal Centre with certificate of No. 2002A005, (YJZZ) issued by Institute of Experimental Animal Monitoring in Committee of Science and Technology, Guangdong Province. The mice were fed in laboratory for a week after purchase in order to help them adapt to the environment (temperature: 23~25 °C, relative humidity of 60~70%).

Water, instead of food, was provided for the mice 12 hours prior to the experiment. During the experiment period, the drug solution was poured into the stomach of the mice, based on the weight of the mouse. Test drug solution was to give to mice by 0.4 ml/10g. 20 Mice were fed with the drug solution once a day. The toxic reaction such as their behaviour was observed for 7 consecutive days. After being weighed at the 8th day, the mice were anatomized and their main viscera were observed to find

any visible pathological changes.

The 20 mice were fed with the drug solution once a day, and no obvious variation and abnormality were observed. Their fur was staining, and the natural openings, such as eyes, noses, ears and genitalias, were clean without any abnormal secretion. Bowels, breath frequency, and amplitude were normal. During the 7-consecutive day observation, no mouse was found dead. The average weight was 26.11±1.31 g in the 8th day, this was remarkably different ($P<0.01$) from the average weight before the drug delivery. The mice were also anatomized, and no visible pathological change was found in the main visceras. The weight changes before and after the drug delivery, were listed in **Table 6-8**.

Table 6-8 The weight changes of mice in the acute toxicity test of 4Ac

Animal	Quantity	Weight before drug (g)	Weight in the 8th day after drug(g)	Average increase(g)
Mouse	20	19.81±0.80	26.11±1.31	6.3

6.4. Summary

As a polar molecule, puerarin showed poor bioavailability when administered orally. Structural modification could partially mask its hydrophilic property, resulting in the derivatives being more lipophilic. Study showed that 4Ac, the tetracetate derivative of puerarin, was more suitable for further clinical study. The compound showed good stability, higher bioavailability and low toxicity.

Chapter 7 Discussions

Radix Puerariae is a very famous Chinese medicine which has been used as an ingredient in many compound recipes traditionally. However it has not been considered as a good candidate for the development of a modern oral drug containing only *Radix Puerariae* extract. The reasons for this include lacking of proper quality control method, the low bioavailability of its active compounds and the weak biological action for any well defined therapeutic end point. The current project is designed to try to find some solutions to the problems mentioned above so that more effective new drugs could be developed from this safe herb.

7.1 The quality consistency of *Radix Puerariae*

Quality consistency of raw materials is crucial to the new drug development from traditional Chinese medicinal ingredients. The chemical profile of the raw material of *Radix Puerariae* from different regions or different species varies substantially. The content of the total flavones in the root of *Pueraria lobata* from Shanxi was found as high as 10.4%, whereas that from Jiangxi as low as 3.3%. The variation of the content of flavones in the root of *P. lobata* from Huoshan, Anhui Province seemed relatively small with an average figure at 7.24%. The result provided us with a guidance that Huoshan may be a better choice for the cultivation of *P. lobata* wth high consistency of the chemical profile. The average content of flavones in the root of *P. lobata* from several provinces was 7.42%. It was also found that the root of *P. thomsonii*, which is also listed in the Chinese Pharmacopoeia as one of the two

sources of *Radix Puerariae*, contained only 0.93% total flavone, much lower than that in the root of *P. lobata*. Similar results had been reported that the content of total flavones in the root of *P. lobata* was higher than that in the root of *P. thomsonii*.[46,47] In addition to the assay of total flavone, determination of as much as possible the chemical markers is just as important in light of new drug development from botanical origin. In this project, eight chemical cnstituents, puerarin, daidzin, daidzein, 3´-hydroxypuerarin, puerarin-7-O-apioside, genistin, formononetin-7-O-glucoside and puerarin-7-O-glucoside, which may well be the main active compounds in *Radix Puerariae*, had been identified and used as a set of chemical markers to compare the chemical profile of the *Radix Puerariae* produced from either *P. lobata* or *P. thomsonii* collected in the Mainland of China. Although Zhang and Zhong[49,55] reported the determination of the content of active compounds in *Radix Puerariae* by HPLC, only two compounds were used, i.e. puerarin and daidzein, as the markers without information on the validation of the methodology. The content of the two major chemicals, puerarin and daidzein, in the root of *P. lobata* was found higher than that in the root of *P. thomsonii.* The content of puerarin in the root of *P. lobata* from Shenyang is about 7.49%, whereas that from Jiangxi is only 1.62%. The results are in line with the content of the total flavones mentioned above. These findings demonstrated again the huge difference in the quality of Radix Puerariae from different species and regions. It was also found that the variation of the content of the eight compounds in the root of *P. lobata* from Huoshan, Anhui was relatively small, with puerarin content at 4.36%.

Based on the chemical profile only of the two species, it can be concluded now that *P. lobata* should be considered as a better source for the production of *Radix Puerariae* to be used in new drug development. And the best site for the production of *Radix Puerariae* should be around the area of Huoshan, Anhui Province. With the application of Good Agriculture Practice (GAP) in the cultivation of *P. lobata* in Huoshan, the production of better quality raw material of *Radix Purariae* with certain degree of consistency can be achieved.

HPLC was found to be the most appropriate analytical method for the determination of active compounds and fingerprint pattern of *Radix Puerariae* and suitable for examining quality and consistency. There are fifteen peaks identifiable in the HPLC pattern of *Radix Puerariae* with a gradient elution of water and methanol as mobile phase. The fingerprint pattern of ten batches of samples showed a good similarity. For the first time, the fingerprinting patterns of *Radix Puerariae* was established using HPLC with the methodology validated, which is expected to be applicable, with necessary modifications, to the quality control of extracts and finished products made from *Radix Puerariae* The data obtained and the methodology established in this study could be used as a part of the protocol of the specification of *Radix Puerariae*.

7.2 The efficacy of *Radix Puerariae* and its active compounds

Good efficacy is essential to a drug. Usually the increase of efficacy depends on the increase of bioavailability for pharmaceutics, whereas that probably depends on the coefficient for a compound formula.

Traditionally, *Radix Puerariae* was described as “sweet and acrid in taste”, being able to relieve all kinds of rheumatism and has been reported to be clinically effective in Cardiovascular diseases[73,74] and to be pharmacologically effective in regulating blood lipids, relieving muscle spasm, increasing the total bone mineral content (BMC), bone mineral density (BMD), bone bio-mechanics strength and the weight of ovary.[75] The multi-pharmacological activities suggested that *Radix Puerariae* could have potential medical applications such as hyperlipemia, osteoporosis, menopause symptoms, etc.

For the first time we investigated the potential application of *Radix Puerariae* for the indication of hyperlipemia by examining the effect of the prescriptions on the regulation of blood lipids in mice and rats. The results indicated that the effect of the extract of the single raw material, such as *Radix Puerariae*, Cassia seeds and Fructus Alpiniae Oxyphyllae (Yi Zhi), on the regulation of of the level TC, TG and HDL-C was less than that of the compound formula. Although the effect of *Radix Puerariae* on the level of TC, and HDL-C was significantly different from the control group, but that of TG was not, which meant that the effect of the extarct of *Radix Puerariae* on the regulation of blood lipids was not as potent as to have desired clinical significance. Further study was carried out on the effect of the mixture of the extracts of *Radix Puerariae* and Fructus Alpiniae Oxyphyllae (Fructus Alpiniae Oxyphyllae is a TCM herb which has never been reported having the effect of regulating blood lipids). The results showed that the regulation effect on the level of TC, TG and HDL-C with the test sample was significantly stronger than that of the control group,

suggesting possible synergistic actions between fructus Alpiniae Oxyphyllae and *Radix Puerariae.* It becomes logical to believe that the combination of the two ingredients may provide a new drug candidate for further development.

In the traditional application, Fructus Alpiniae Oxyphyllae was recorded or reported neither to have impact on the regulation of blood lipids traditionally nor to enhance the effect of *Radix Puerariae* on the regulation of blood lipids. The answer to the current discovery of synergy between the two ingredients could be speculated on the basis of the traditional Chinese medicine theory. According to TCM theory, hyperlipemia may be caused by the blockage of phlegm and blood, and manifested as the symptoms of weakness with strong pathogenic factor.[76] Restoring the spleen and kidney and resolving the blockage of phlegm and blood become the principle consideration for the treatment of the condition. Fructus Alpiniae Oxyphyllae has the properties of acrid in taste and enters the fibril of the spleen and kidney and restores the function, removes the block of phlegm and blood. This is a good example of the combination of traditional theory and modern science in terms of new botanical drug development.

Puerarin, one of the most important active compounds in *Radix Puerariae*, has been proved to be clinically effective, if injected, in the treatment of cardiovascular disease in China. For the first time we investigated the effect of puerarin administrated orally in different dispersion forms on arrhythmia of mice caused by chloroform and myocardial ischemia of rat caused by pituitrin.The results showed that both polyethylene glycol (PEG-P) dispersion and phospholipids complex (PPC)

had better effect than Yufenningxin tablet, in which the active compound is thought to be puerarin, while PPC showed stronger action than PEG-P. These experiments and results have added a new dimension to the endeavor to improve the efficacy of puerarin in oral dosage forms.

In the consideration of structure-activity relationship (SAR), the 7, 4'-dihydroxyl in the molecule of both puerarin and daidzein, the aglycone of puerarin, was regarded as essential to the activity because the distance between the two hydroxyls is very similar to that in the molecule of estradiol, about 12Å. Isoflavones, including puerarin and daidzein, might have the same binding pattern to the estrogenic receptor (ER) and similar action in vivo.[77]

7.3 The bioavailability of puerarin

Bioavailability of a substance is often influenced by its solubility in water and lipophilic fluid. PEG has the property of increasing the solubility of insoluble drugs in water and has been used widely, while phospholipid, a lipophilic substance that could increase the permeability of the polar drugs, can be utilized to disperse the drugs for better bioavailability. Puerarin is a relatively polar substance with inadequate ability to penetrate the lipo-bilayer and due to the polarity of the molecule, has poor water solubility. It seems that it is useful to the enhancement of bioavailability of puerarin to increase the solubility both in water and in lipo solvent. We tried to add hydrophilic substances to PPC for better water solubility, but the result was negative. This could be suggestive that increasing the permeability is more important than increasing the water solubility for the better bioavailability of

puerarin.

We investigated, for the first time, the effect of puerarin and its derivatives on arrhythmia in mice caused by chloroform and myocardial ischemia in rats caused by pituitrin.The results showed that the derivatives of puerarin, 4Ac, 5Ac and 6Ac, which are more lipophilic than puerarin, had better effect than puerarin. The results suggested that increasing the lipophility is very important to the increase of the effect of puerarin. At the same time it was also found that the effect of 4Ac with medium lipophility was better than that of either 5Ac or 6Ac with higher lipophility. This finding, a proper lipophility was crucial to the improvement of the effect of puerarin derivatives, could provide a guidance for the future development of better drug candidates containing puerarin and its derivatives. It is also understood that the proper lipophilty would result in the better absorption, improved bioavailability and thus enhanced effect. This mechanism could also apply to the understanding of the better effect of PEGP and PPC.

7.4 Conclusion

The current project has addressed three key issues in relation to the drug development from *Radix Puerariae* with satisfactory results and I believe the approach to the subject could be generally adapted to the research and development of new botanical drugs from promising TCM ingredients

1. The development of an appropriate analytical method for the quality identification and control of the raw material of *Radix Puerariae*. This is more of an importance when a botanical drug raw material could be produced from

different species/varieties and from various production sites such as *Radix Puerariae*. It has been determined that *P. lobata* should be used as the better species for new drug development and the best cultivation site in Huoshan area, Anhui Province. The establishment and validation of the HPLC method for the determination of the eight chemical markers and the fingerprinting patterns of *Radix Puerariae* not only for the first time make it possible to control the quality of the raw material but also to use the similar method in the QC of extracts and finished dosage forms.

2. The experimental exploration for the improvement of the efficacy of the orally administrated products of *Radix Puerariae* by the application of technology in pharmaceutics science. The combination of modern science and traditional knowledge has resulted in a new potential combination formula to enhance the effect of *Radix Puerariae.*
3. The study on the bioavailability of puerarin and synthesized derivatives has resulted in the determination of the appropriate lipophility of the candidate compounds. This could have furnished a new opportunity to develop drugs from pure chemicals isolated from *Radix Puerariae*.

Chapter 8 Conclusions

Radix Puerariae, a very famous TCM herb, was widely used in clinic in China and forms the basis of several newly developed drugs such as puerarin injection. Generally, there were three main problems concerning the raw material of Radix Puerariae and its related products. The first problem was the variation of the quality of Radix Puerariae with the different species and origins. As a result, a comprehensive method for quality control needed to be developed. The second problem was the low bioavailability of puerarin when administrated orally, to which there existed no effective solutions. The last one was the low effectivenees of the drugs containing Radix Puerariae for a specific therapeutic endpoint.

In this study, technologies have been developed and the solutions to solving the above-mentioned problems to a extent, have been found.

A survey on the resource of Radix Puerariae was made nationwide. Samples of raw materials, specimen of *Puraria lobata* and *Pueraria thomsonii* were made. Based on the results from the survey and sample analysis, the origin of the superior quality of the Radix Puerariae raw materials was identified. According to the study, the active compounds of Radix Puerariae from Huoshan of Anhui Province were relatively high, the content variations between different batches were small, and the quality was rather consistent.

A comprehensive in-depth study have been carried out to measure the content of total flavones and puerarin, develop the identification method for 8 active compounds in Radix Puerariae, establish HPLC fingerprint patterns for the raw materials. The

results of the current project are likely to offer an advanced and appropriate method for the quality control of both the raw material and products containing Radix Puerariae.

Three derivatives of puerarin were prepared aiming at improving the bioaffinity and bioavailability of puerarin. Studies indicate that the bioavailability and efficacy of the derivatives are much better than that of puerarin.

Also carried out was the comparison study on the bioavailability and pharmacology of puerarin dispersion for its anti-myocardial ischemia effect using such technologies as phospholipid recombination and PEG dispersion. The result indicated that phospholipid complex of puerarin had a much better bioavailability and anti-myocardial ischemia effect than puerarin. The bioavailability and anti-myocardial ischemia effect of puerarin PEG dispersions (Dripping pills) were also better than that of puerarin, but not as good as phospholipid puerarin. This may have introduced a new dimention in the search for ways of improving bioavailability of such drugs as puerarin.

In order to enhance the efficacy of Radix Puerariae for a specified indication, the screening of compound prescriptions based on puerarin and other blood-lipid regulating TCM ingredients was conducted. The result showed that the extracts of *Fructus Alpinae Oxyphyllae* could effectively improve the activity of Radix Puerariae in regulating blood lipid level.

In summary, a comprehensive method for the quality control of Radix Puerariae was established, as well as two technologies for increasing the bioavailability of puerarin

and one technology for enhancing the efficacy of Radix Puerariae for regulating blood lipid.

It is hoped that the results of the current project have paved a step stone for the future research and development of drugs containing Radix Puerariae, including, for example, the mechanism of action of puerarin, the screening of compound prescription based on Radix Puerariae for other specified indication and the detection of other active compounds in Radix Puerariae.

Appendices

Spectra of puerarin and its derivatives

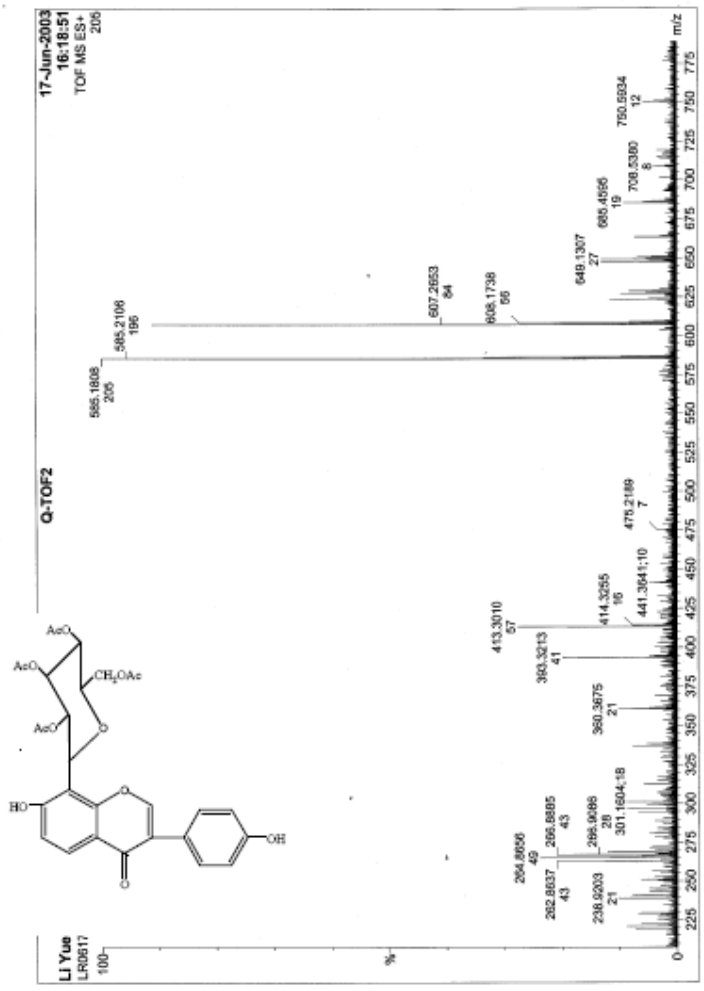
Spectra for 4Ac

Spectra for 5Ac

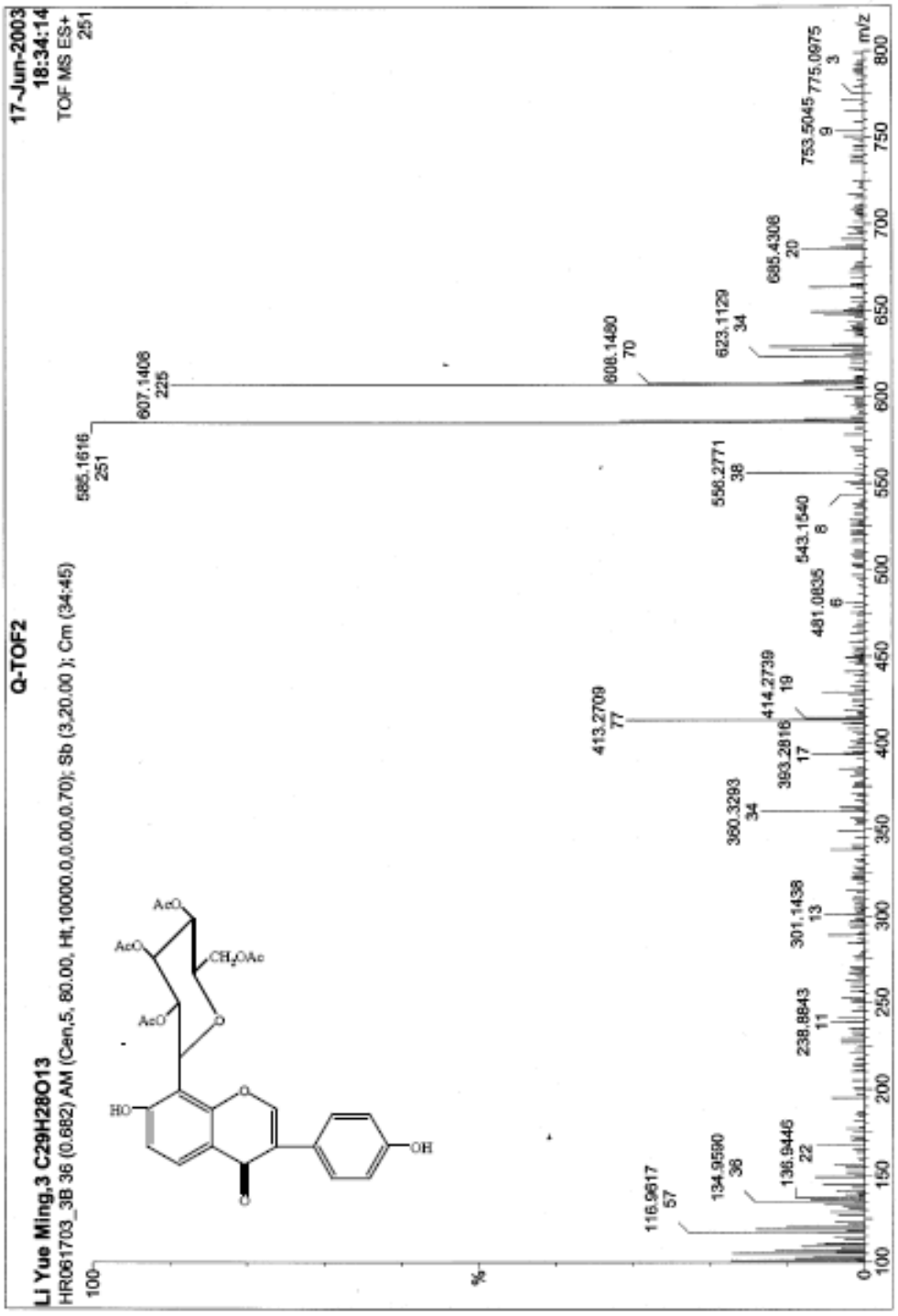
Spectra for 6Ac

Spectra for Puerarin

Spectra of puerarin and its derivatives



Mass Spectrum of 4Ac



Mass spectra of 4Ac

Elemental Composition Report Page 1

Single Mass Analysis (displaying only valid results) - displaying only valid results
Tolerance = 2.0 mDa / DBE: min = -0.5, max = 120.0

Monoisotopic Mass, Odd and Even Electron Ions
41 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Minimum:				-0.5	
Maximum:		2.0	1000.0	120.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
585.1616	585.1608	0.8	1.4	15.5	C29 H29 O13

Elemental Composition Report Page 1

Single Mass Analysis (displaying only valid results)
Tolerance = 2.0 mDa / DBE: min = -0.5, max = 120.0

Monoisotopic Mass, Odd and Even Electron Ions
34 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Minimum:				-0.5	
Maximum:		2.0	1000.0	120.0	
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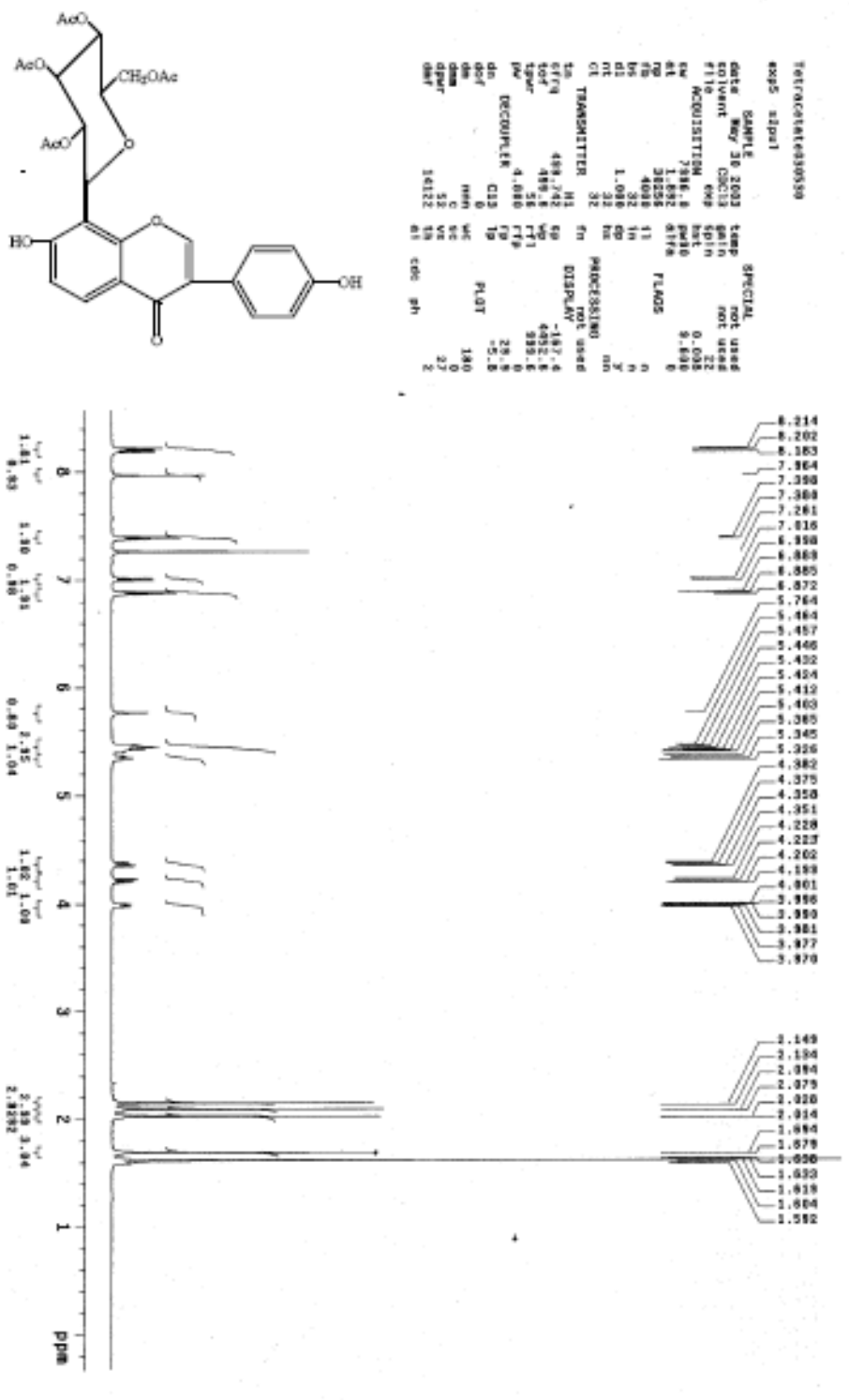
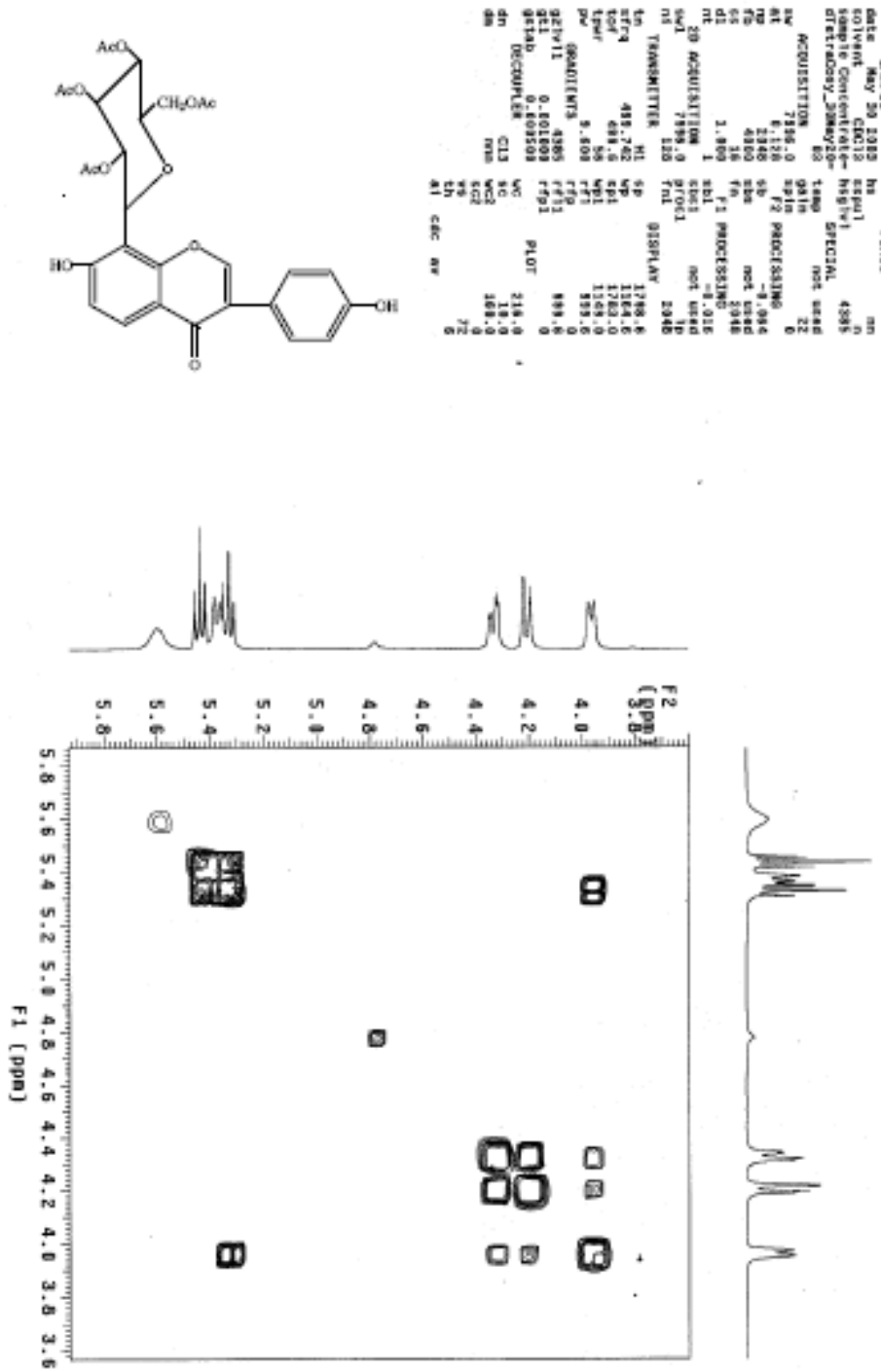
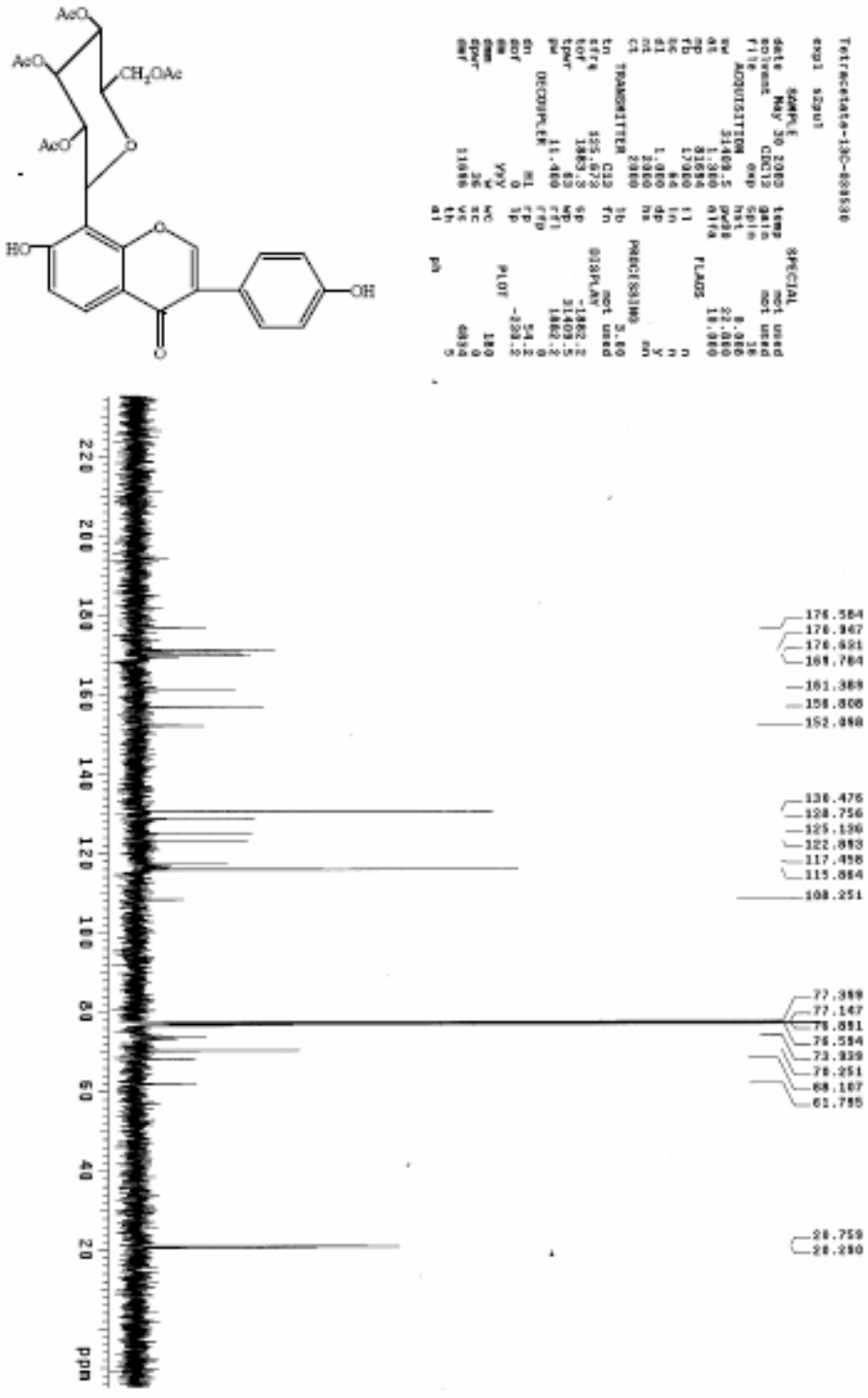
607.1408	607.1428	-2.0	-3.2	15.5	C29 H28 O13 Na



^{1}HNMR Spectra of 4Ac

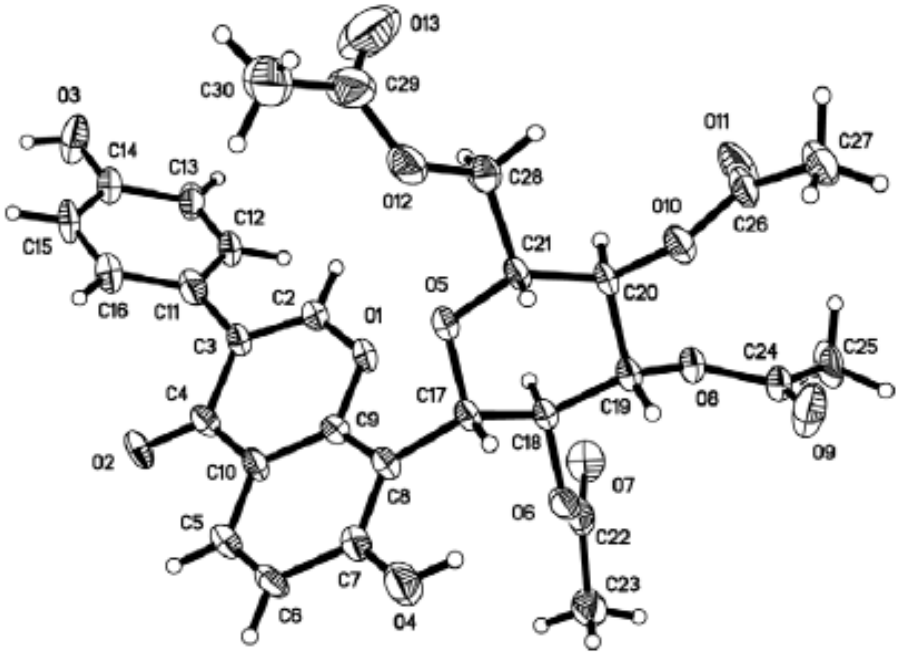


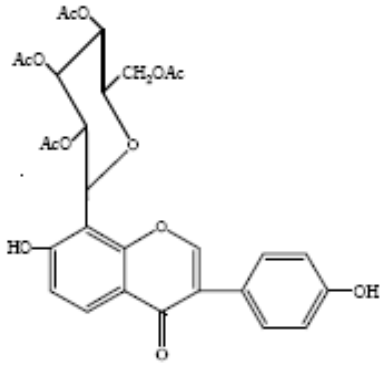
H-H Cosy Spectra of 4Ac



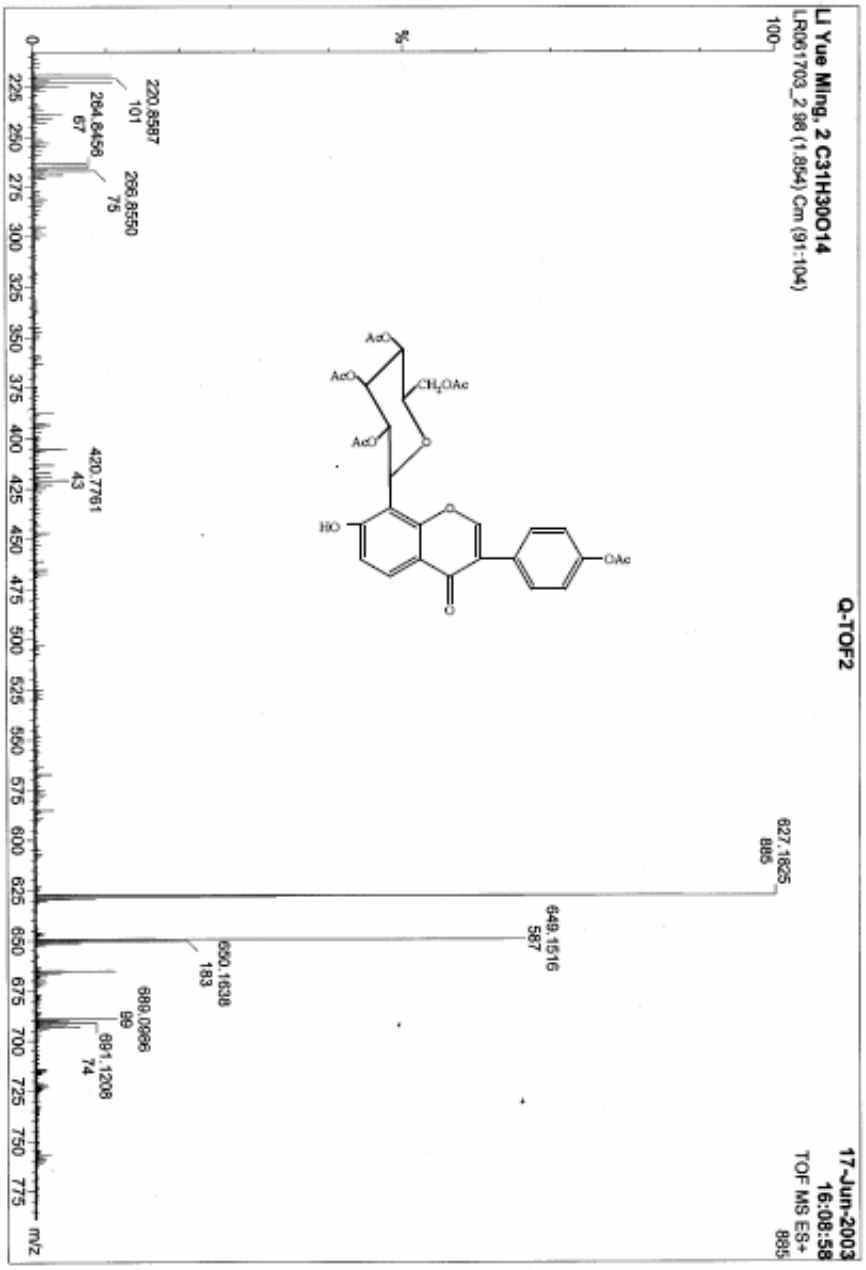
^{13}C-NMR Spectrum of 4Ac

ORTEP drawing for puerarin tetracetate (4Ac)

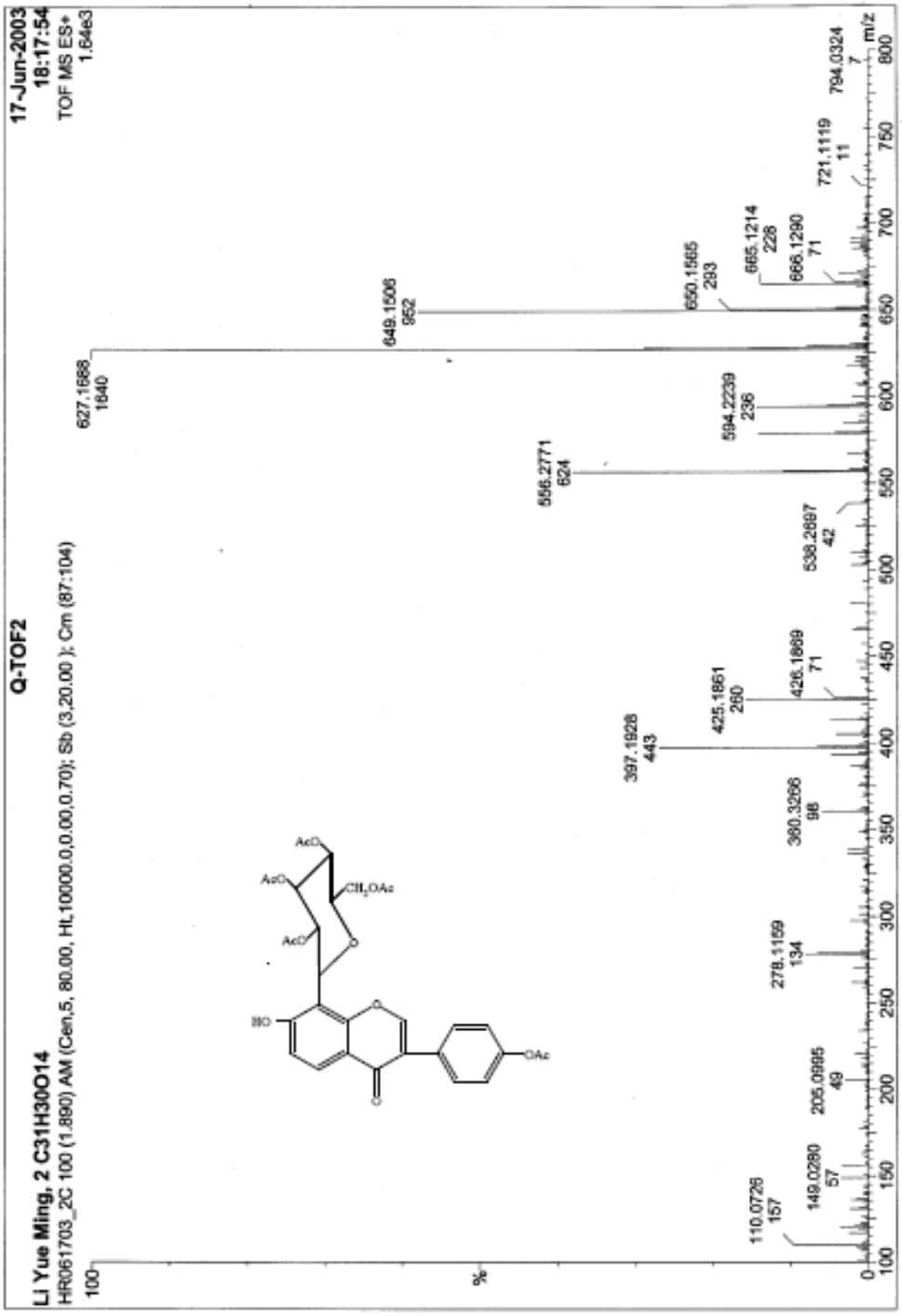




Spectra for 5Ac



Mass Spectrum of 5Ac



Mass Spectrum of 5Ac

Elemental Composition Report

Single Mass Analysis (displaying only valid results)
Tolerance = 10.0 mDa / DBE: min = -0.5, max = 120.0

Monoisotopic Mass, Odd and Even Electron Ions
20 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

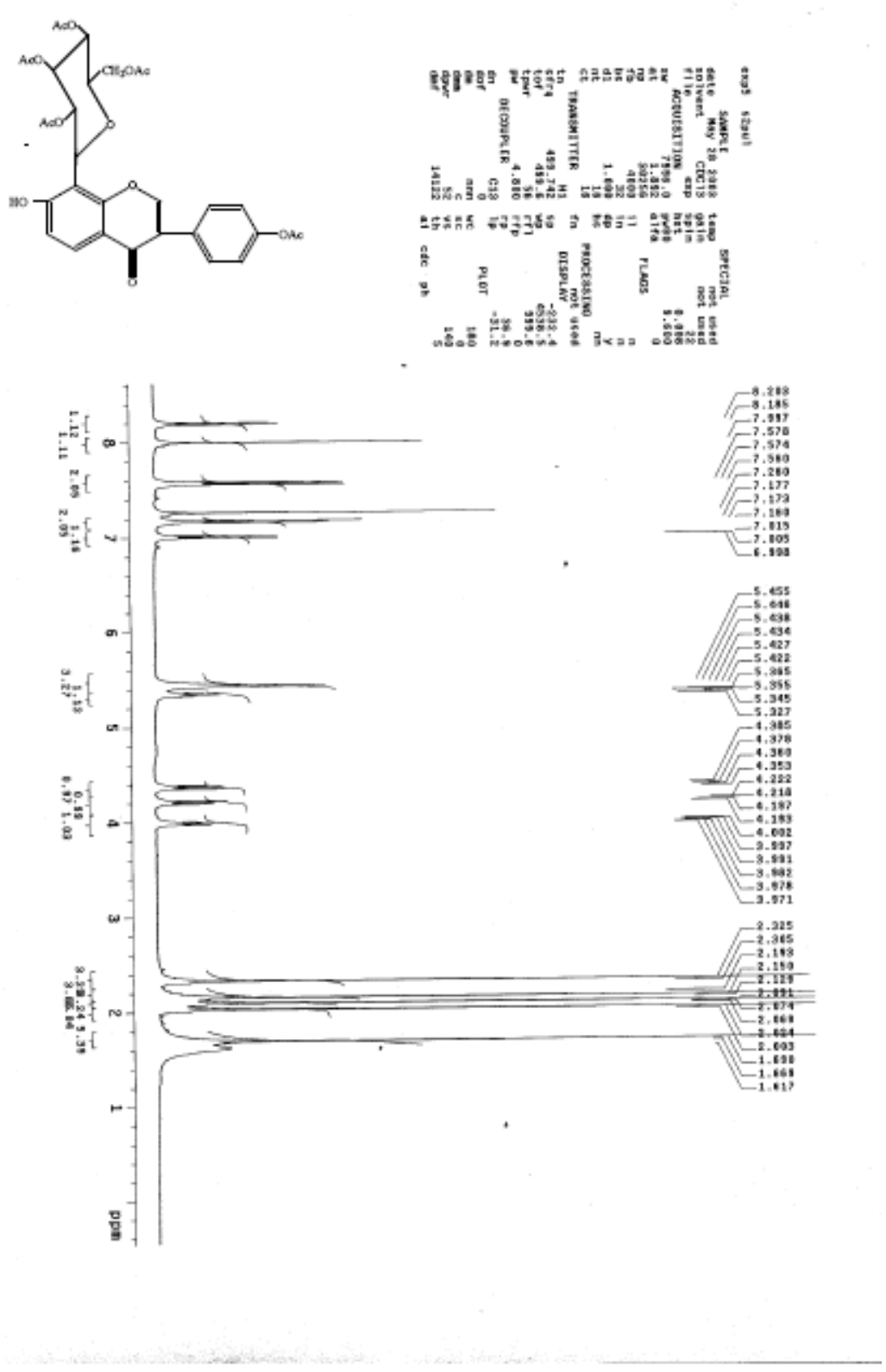
Minimum:				-0.5	
Maximum:		10.0	1000.0	120.0	
Mass	**Calc. Mass**	**mDa**	**PPM**	**DBE**	**Formula**
627.1688	627.1714	-2.6	-4.2	16.5	C31 H31 O14

Elemental Composition Report

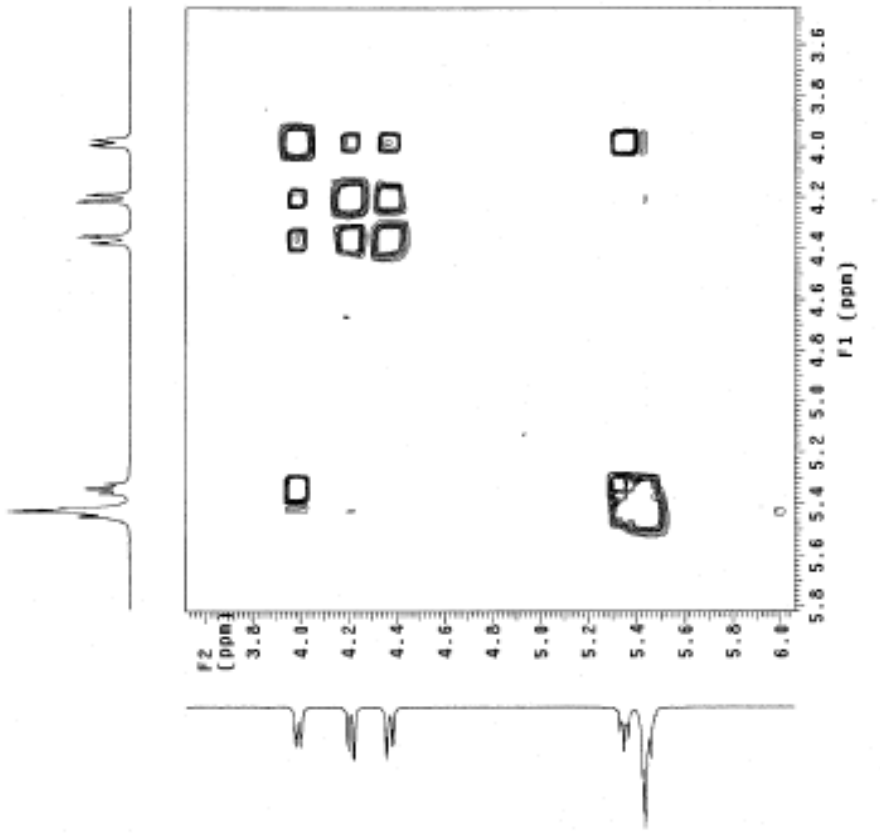
Single Mass Analysis (displaying only valid results) - displaying only valid results
Tolerance = 5.0 mDa / DBE: min = -0.5, max = 120.0

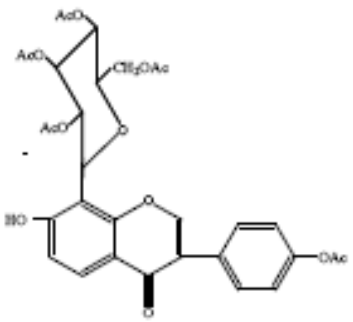
Monoisotopic Mass, Odd and Even Electron Ions
36 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Minimum:				-0.5	
Maximum:		5.0	1000.0	120.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
649.1506	649.1533	-2.8	-4.2	16.5	C31 H30 O14 Na

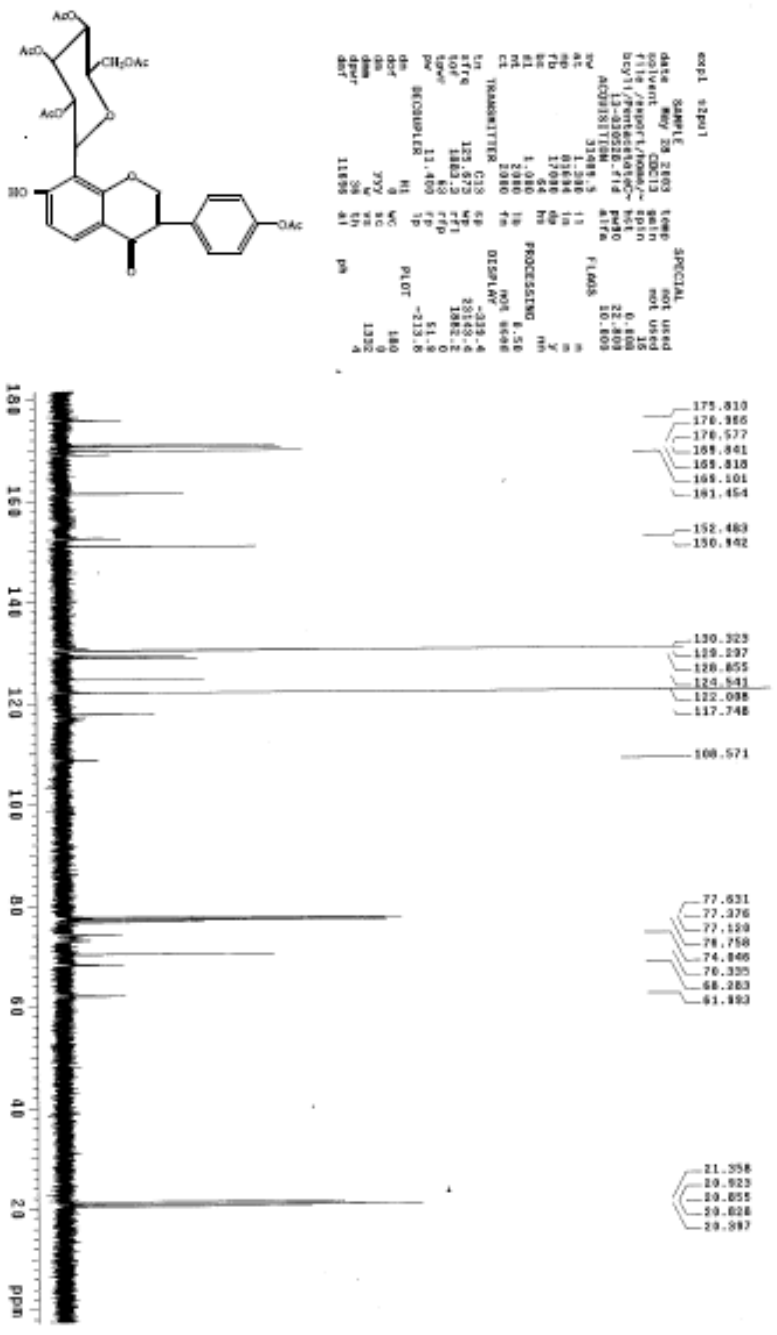


^{1}HNMR Spectrum of 5Ac



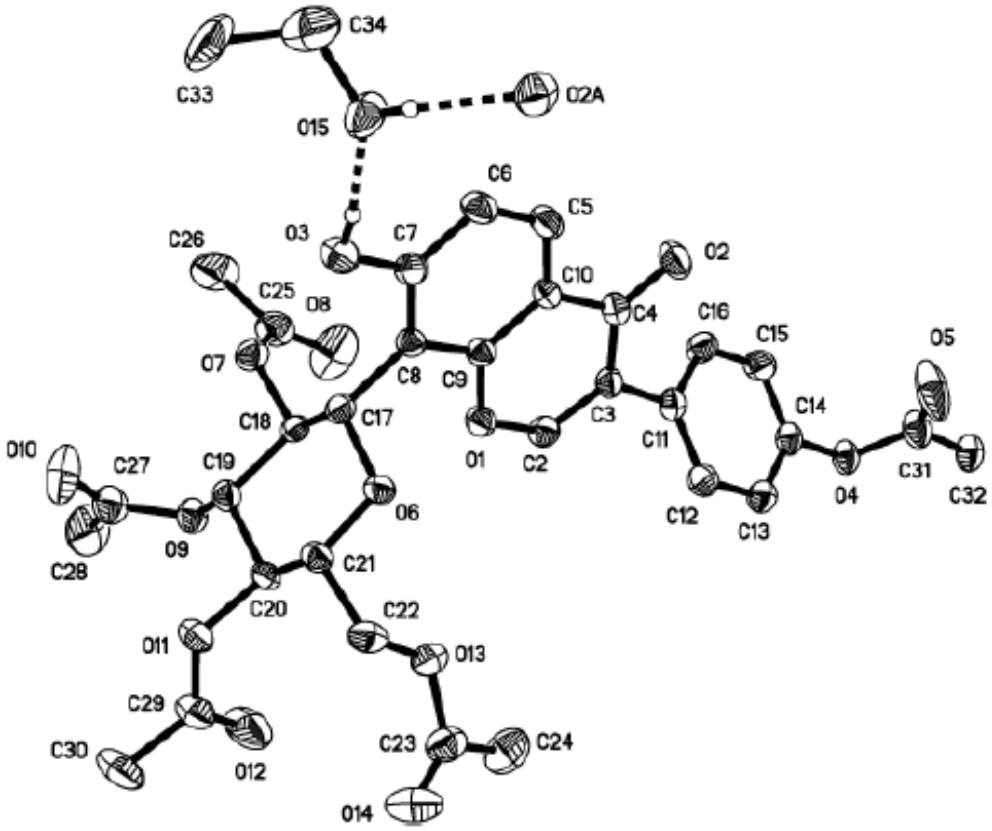


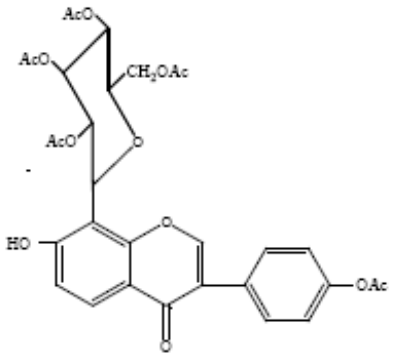
H-H COSY of 5Ac



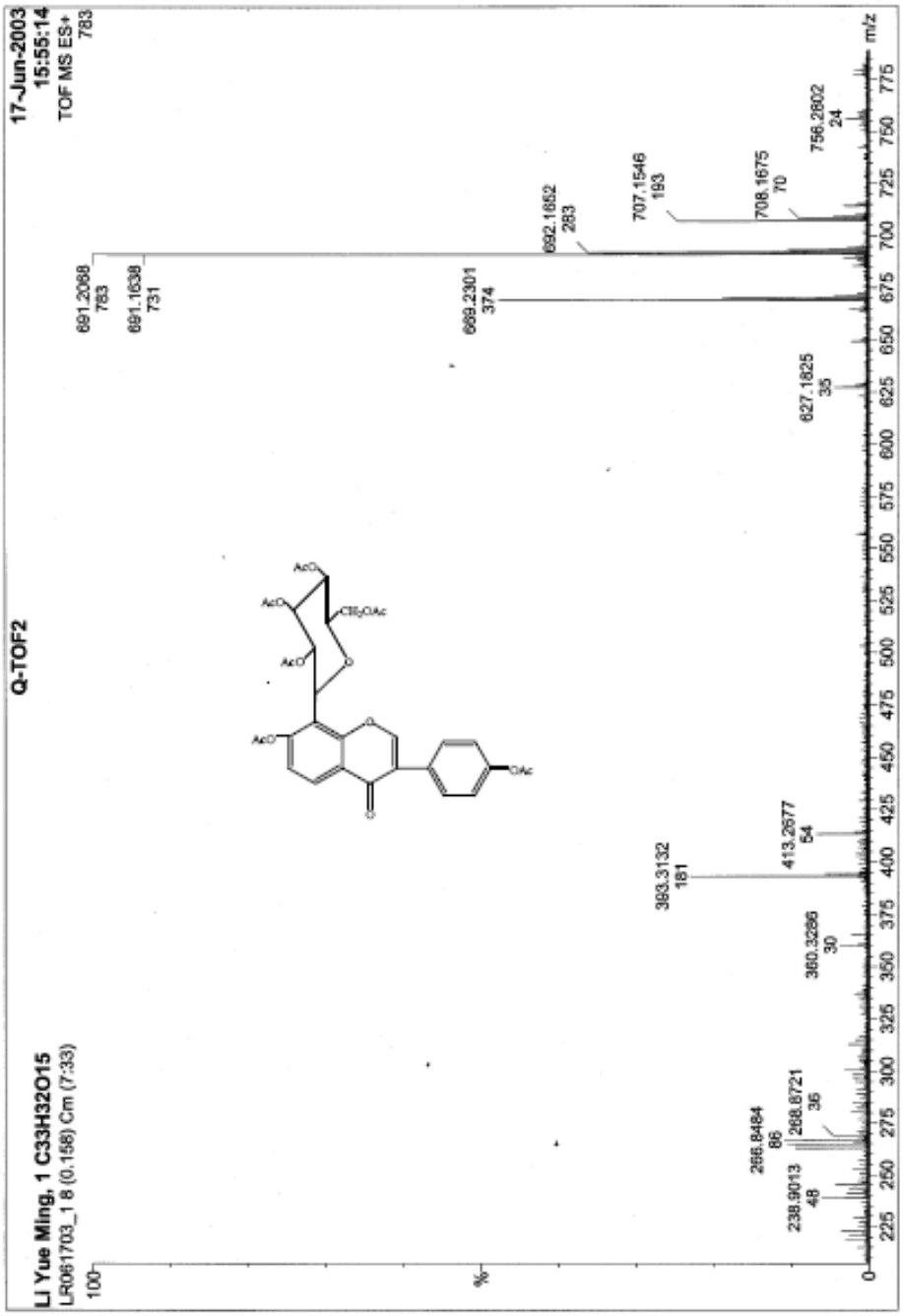
13C-NMR Spectrum

ORTEP drawing for puerarin pentacetate (crystallized from ethanol)

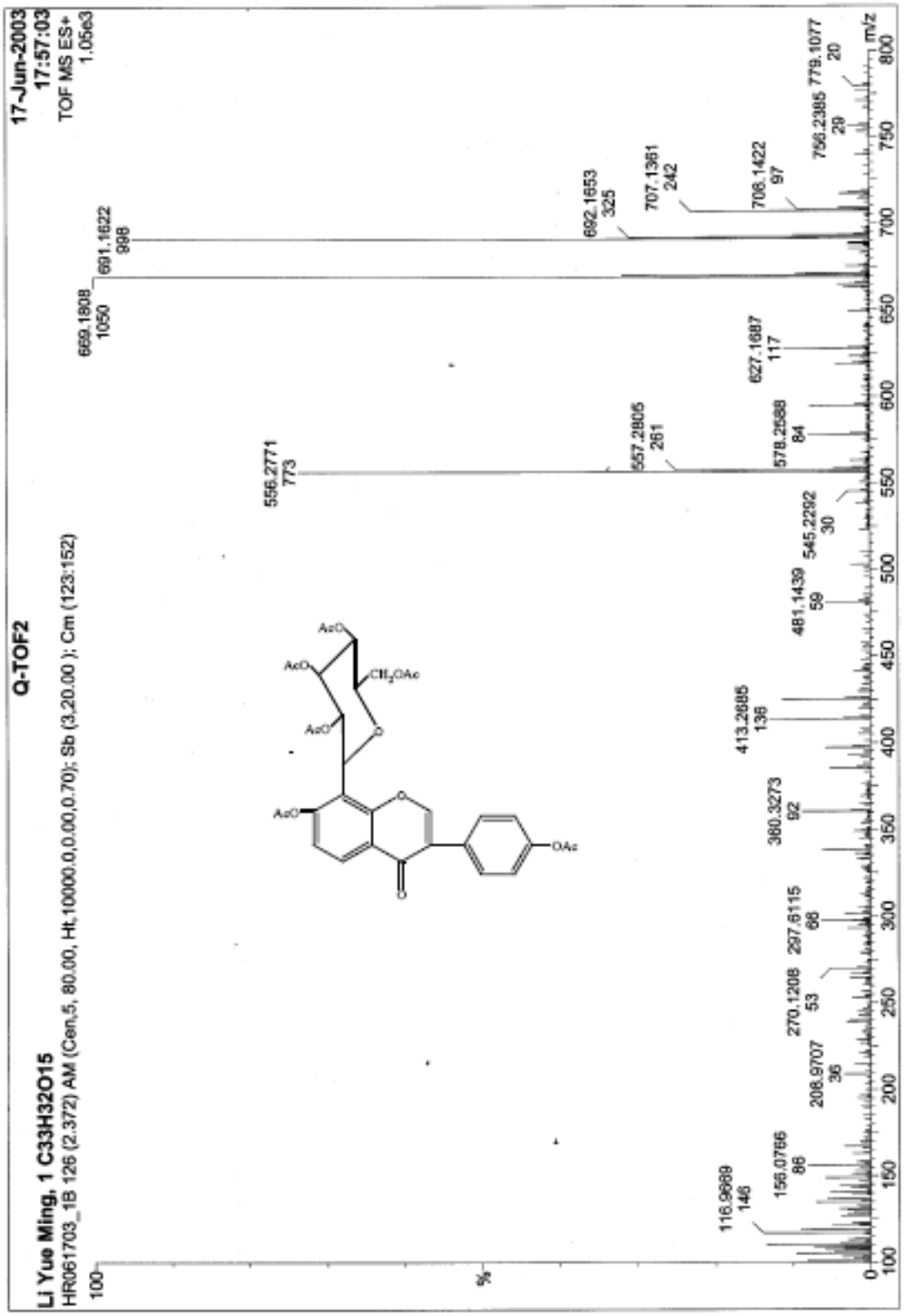




Spectra for 6Ac



Mass Spectrum of 6Ac



Mass Spectrum of 6Ac

Elemental Composition Report

Single Mass Analysis (displaying only valid results)
Tolerance = 10.0 mDa / DBE: min = -0.5, max = 120.0

Monoisotopic Mass, Odd and Even Electron Ions
14 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

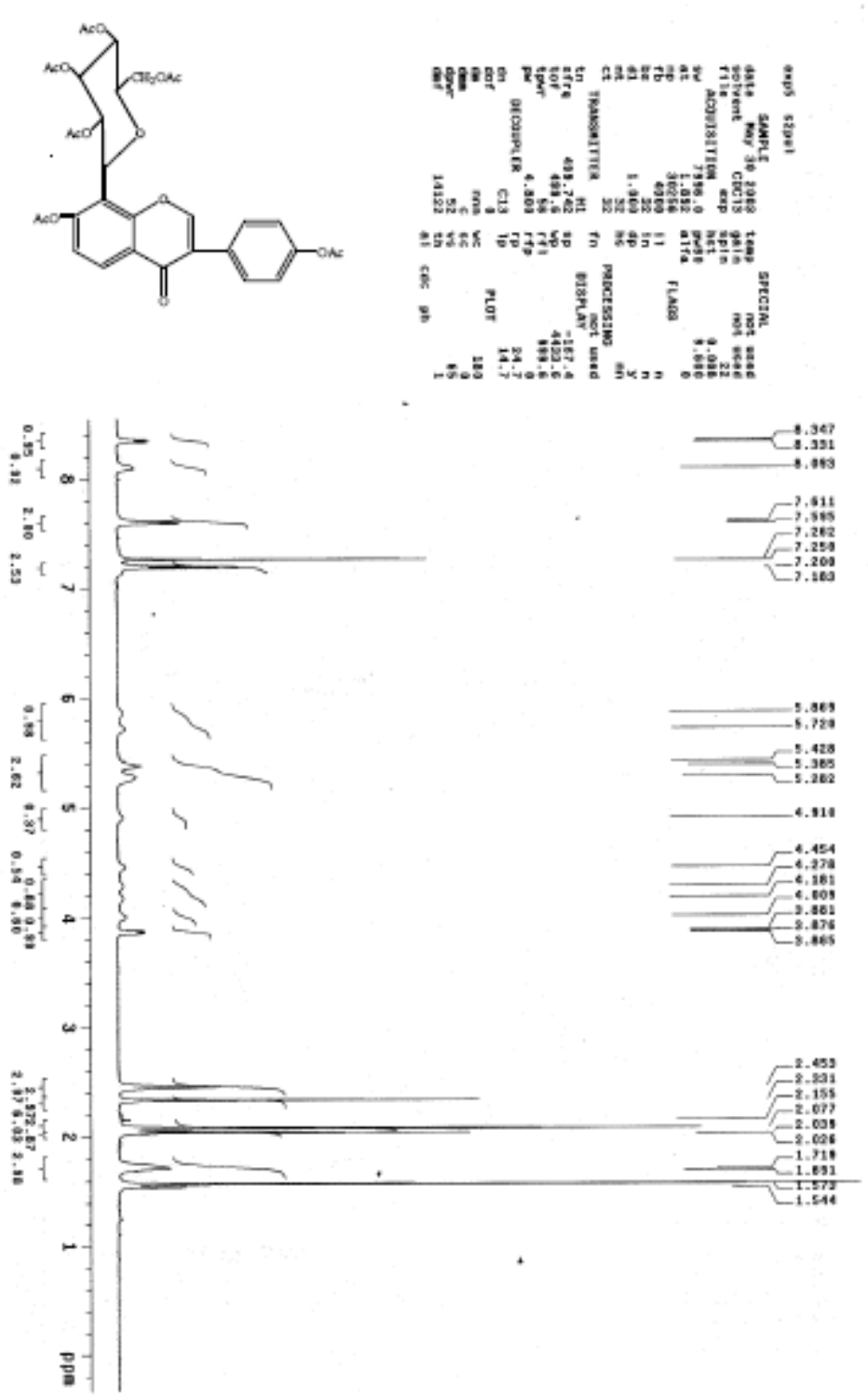
Minimum:				-0.5	
Maximum:		10.0	1000.0	120.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
669.1808	669.1819	-1.2	-1.7	17.5	C33 H33 O15

Elemental Composition Report

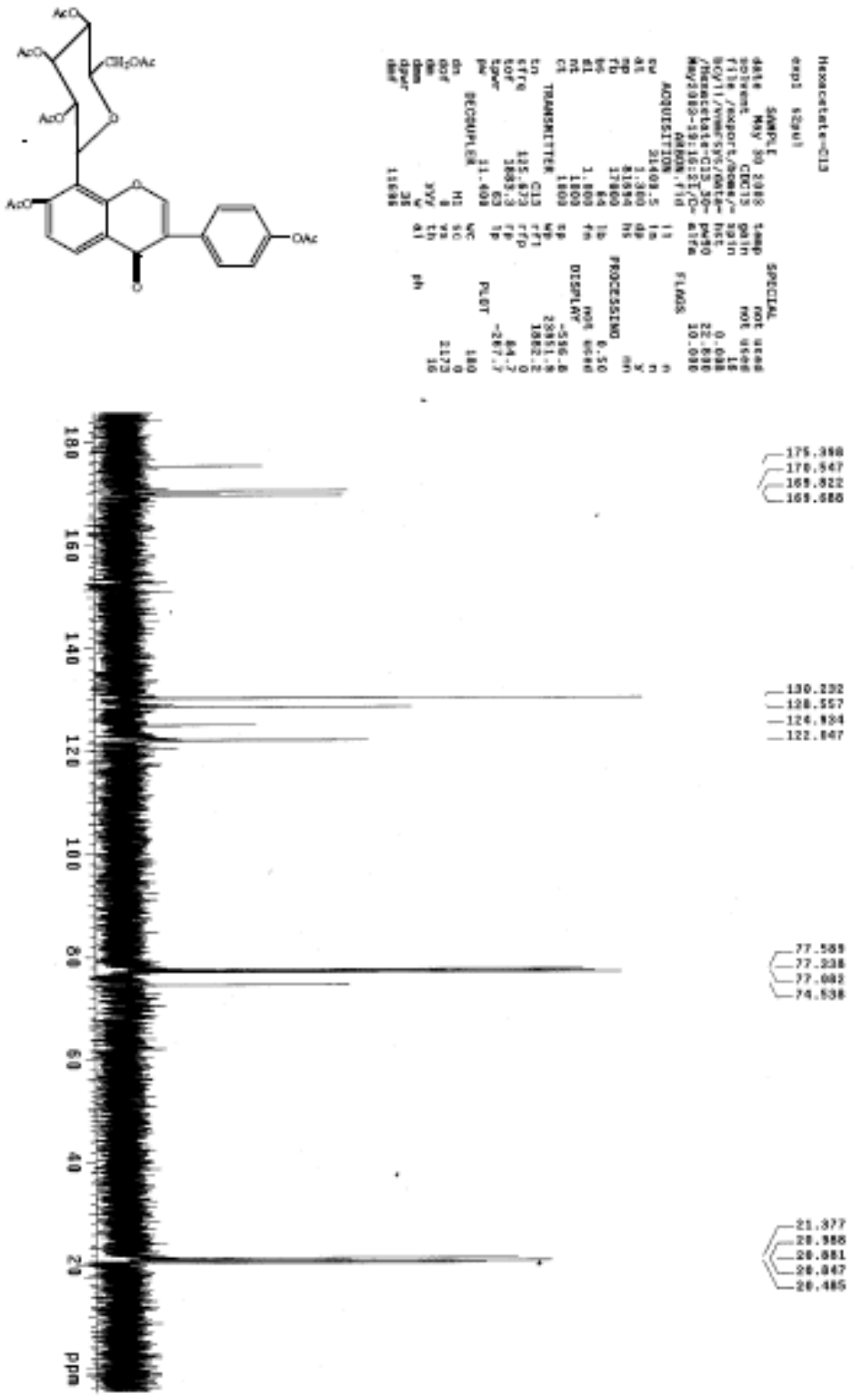
Single Mass Analysis (displaying only valid results)
Tolerance = 10.0 mDa / DBE: min = -0.5, max = 120.0

Monoisotopic Mass, Odd and Even Electron Ions
28 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Minimum:				-0.5	
Maximum:		10.0	1000.0	120.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
691.1622	691.1639	-1.7	-2.5	17.5	C33 H32 O15 Na

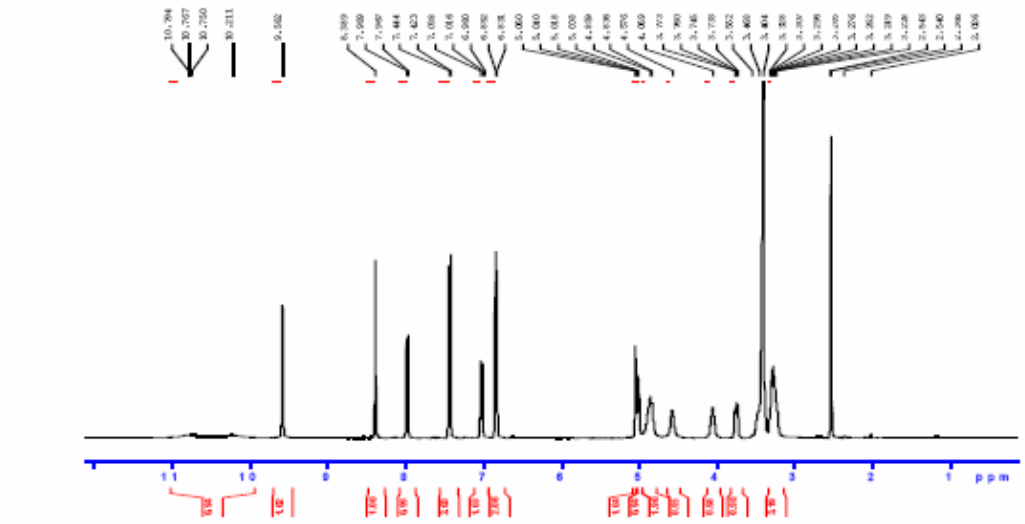


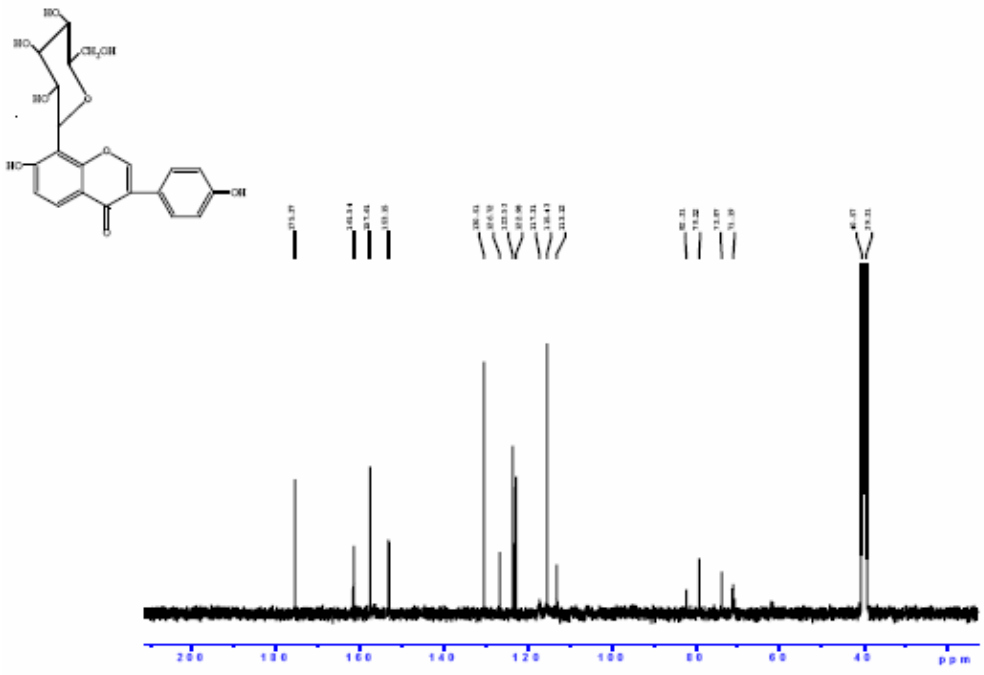
^{1}H-NMR Spectrum of 6Ac



^{13}C-NMR Spectrum

^{1}H-NMR spectra of Puerarin





^{13}C-NMR spectra for puerarin

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