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BSc (Hons) in Applied Chemistry
Project Report

Pattern Analysis of Chromatograms of Euphorbiae Genus

by
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Pattern Analysis of Chromatograms of Euphorbiae Genus

by

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Abstract

A TLC method has been developed for the identification of two bio-markers, myricitrin and quercitrin, in *Euphorbia hirta* L., the raw material of Euphorbiae Hirtae Herba (飛揚草). These two bio-markers showed good separation with developing solvent composed of dichloromethane: ethyl acetate: methanol: formic acid in a ratio of 5:3:1.5:1, v/v, spraying with 10% sulphuric acid in ethanol, and detected in UV light at 366 nm. Three bio-markers, isoquercitrin, ellagic acid and afzelin, were also added for the fingerprint pattern analysis.

HPLC method has been developed for the analysis of the five selected bio-markers in *Euphorbia hirta* L. samples. The bio-markers were detected with good separation when using a Dikma Platisil ODS column at 40^{o}C, with a binary gradient mobile phase composed of 0.2% phosphoric acid and methanol, and detected at wavelength 360 nm. The HPLC method has been validated to test the reproducibility of fingerprints.

TLC analysis indicated some differences between the Euphorbiae genus studied (*Euphorbia hirta* L., *Euphorbia thymifolia* L., *Euphorbia humifusa* W., Euphorbia maculata L., *Euphorbia prostrata* A. and *Euphorbia hypericifolia* L.) in a relatively rapid way. Differentiation between the species of same genus and detection of bio-markers in the Euphorbiae genus samples were performed in a more precise and accurate way with the assist of HPLC analysis.

Comparison on patterns between Euphorbiae genus, same species and different species, have been briefly investigated. The difference between the fingerprint patterns of the same species samples from different collection regions was also investigated.

1. Introduction

1.1. Description and Background

Euphorbia hirta L. (飛揚草) is the raw material of the Chinese medicine Euphorbiae Hirtae Herba (大飛揚草). It is a very common plant growing in tropical and subtropical region over the world, mainly distributed in roadsides, fields and wastelands. In China, it is distributed mainly in the southern parts, such as Guangdong, Guangxi, Hunan, Jiangxi Province [1-5].

Euphorbia thymifolia L. (千根草) is the raw material of the Chinese medicine Euphorbiae Hirtae Herba (小飛揚草). Similar as *Euphorbia hirta* L., it is a plant growing in warm countries globally, and mainly distributed in roadsides, fields and grasslands. In China, the habitat of *Euphorbia thymifolia L.* is alike to that of *Euphorbia hirta* L [1-3].

Euphorbia maculata L. (斑地錦) is the raw material of the Chinese medicine Euphorbiae Humifusae Herba (地錦草). It is also a plant growing in subtropical region globally, and mainly distributed in roadsides, rocky hillside and grasslands. In China, it is distributed mainly in the south-eastern parts such as Fujian, Jiangxi, and Zhejiang [1,4,5].

Euphorbia hirta L. has the plant size larger than *Euphorbia thymifolia L.* The stems of former species are erect or ascending, while that of latter species are prostrating on ground. The size of leaf is relatively larger for *Euphorbia hirta* L. (For details, please refer to the table on next page)

Botanical Name: *Euphorbia hirta* Linn Plant Family: Euphorbiaceae Chinese Name: 飛揚草（正名）；大飛揚，大飛揚草，乳籽草，飛相草（俗名） Pharmacopoeia Name: Euphorbiae Hirtae Herba Part Used: Dried whole plants Other name: Garden spurge (English), Dudhi (Hindi) etc.	 A
Botanical Name: *Euphorbia thymifolia* Linn Plant Family: Euphorbiaceae Chinese Name: 千根草（正名）；小飛揚，小飛揚草，細葉地錦草（俗名） Pharmacopoeia Name: Euphorbiae Thymifoliae Herba [3] Part Used: Dried whole plants Other name: Thyme-leaved Spurge (English), Dugdhika (Hindi) etc.	 B
Botanical Name: *Euphorbia maculata* Linn Plant Family: Euphorbiaceae Chinese Name: 斑地錦（正名）；鋪地錦，奶汁草（俗名） Pharmacopoeia Name: Euphorbiae Humifuse Herba [3] Part Used: Dried whole plants Other name: Creeping Euphorbia (English)	 C

Figure 1. General information of *Euphorbia hirta* L., *Euphorbia thymifolia* L. and *Euphorbia maculata* Linn (Left Colum) and their Chinese Medicine (Righ Column)

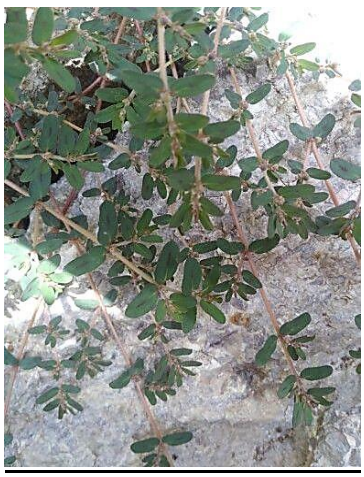
		
Euphorbia hirta L.	*Euphorbia thymifolia* L.	*Euphorbia maculata* L.

Figure 2. Photographs of *Euphorbia hirta* L., *Euphorbia thymifolia* L. and *Euphorbia maculata* L.

Table 1. **Comparison of morphological differences between *Euphorbia hirta* L., *Euphorbia thymifolia* L. and *Euphorbia maculata* L.** [1-5].

	Euphorbia hirta L.	*Euphorbia thymifolia* L.	*Euphorbia maculata* L.
Description	Annual plant, 15-50 cm high	Annual plant, 15 cm long	Annual plant, 15 cm long
Root	Fibrous, 3-5 mm thick	Slim, torturous branched	Fibrous, 2-3 mm thick
Stem	1-3 mm thick, cylinder. Stem base ascending upward and branches in the middle. Light red in branches, covered with yellow coarse hairs.	1-2 mm thick, cylinder. Stem mostly prostrating on ground and branches plentiful generally. Light red in color, slightly covered by soft hairs	1-2 mm thick, cylinder. Stem mostly prostrating on ground and branches plentiful generally. Light red in color, slightly covered by soft hairs
Leaf	1-4 cm long, 0.5-1.5 cm wide. Ovate to oblong ovate. Apex pointed. Middle part sometimes with purple spots. Coarse upper surface. Both surfaces covered with soft hairs.	4-8 mm long, 3-4 mm wide. Invert ovate. Apex round. No middle purple spots. Smooth upper surface. Both surfaces covered by sparse short soft hairs.	6-12 mm long, 2-4 mm wide. Ovate to oblong ovate. Apex round. Middle part with one large purple spots. Both surfaces covered by sparse short soft hairs.
Other characteristic	Containing white latex when cut.		

As a Chinese medicine with long history of curing diseases, Euphorbiae Hirtae Herba is commonly used to clear heat, relieve summer-heat and itching . It is also adopted to cure dysentery, diarrhea; eczema, tinea pedis etc. [3-5]. In India, the fresh milky latex of it is used to wounds and warts [7]. Euphorbiae Thymifoliae Herba exhibits similar medical values as Euphorbiae Hirtae Herba to cure dysentery, diarrhea, eczema, malaria etc. [3], in other countries, such as India and West Africa, it is used as an antiviral agent to treat bronchial asthma, astringent in diarrhea and dysentery. The essence oil of it can even be used as natural insect repellent [10]. Euphorbiae Humifusae Herba also used to clear heat, and exhibits its medical values to cure dysentery, diarrhea, similar as Euphorbiae Hirtae Herba and Euphorbiae Thymifoliae Herba [4-5].

1.2. Bioactive Components in Euphorbiae Genus

Euphorbia hirta L. and *Euphorbia thymifolia* L. are comprised of a wide array of chemical consitituents such as flavonoids, tannins, organic acids, triterpenoids and diterpenoids. These chemicals are bio-active and they are mainly found in the aerial parts of the plants [7-11].

Table 2. Comparison of chemical compositions between *Euphorbia hirta* L., *Euphorbia thymifolia* L., and *Euphorbia maculata* L.

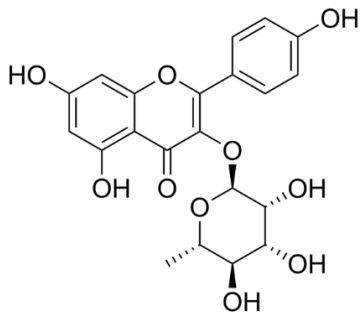
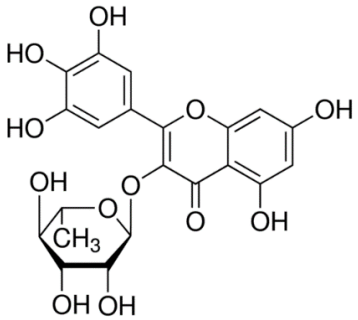
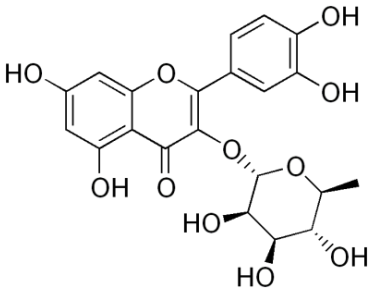
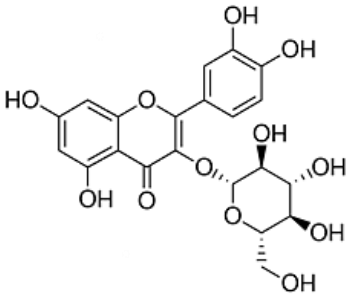
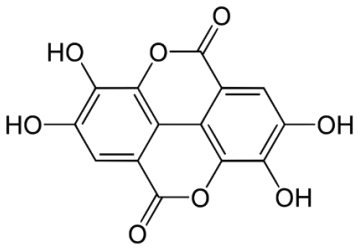
Component	*Euphorbia hirta* L.	*Euphorbia thymifolia* L.	*Euphorbia maculata* L.
flavonoids	Afzelin, quercitrin, myricitrin, isoquercitrin. kaempferol, quercetin, myricetin, rutin etc.	Quercetin, kaempferol, 4-trihydroxy flavone-7-glycoside etc.	Quercetin, kaempferol
tannins	Euphorbin (A-E), euphorbianin, leucocyanidol, camphol etc.	Casuariin, eugeniin, geraniin, etc.	Euphorbin (A,B), geraniin
Organic acids	Ellagic acid, gallic acid, protocatechuic acid	Palmitic acid , isomallotinic acid, salicylic acid etc	Ellagic acid, gallic acid, chebulagic acid
Titerpenoids	α-amyrin, β-amyrin, β-sitosterol, cycloartenol etc.	β-amyrin, β-sitosterol, euphorbol, taraxerol etc.	
Diterpenoids	Euphorbol hexacosanoate, ingenol triacetate etc.	12-deoxyphorbol-13,2O-diacetate	

Four flavonoid glycosides, namely afzelin, isoquercitrin, myricitrin and quercitrin, as well as one phenolic acid, which is ellagic acid, have been identified in *Euphorbia hirta* L. owing to the abundance and the commercial availability [7].

They are chosen as bio-markers for chromatographic analysis of Euphorbiae genus not only for their important bioactivities to the human body, but also simultaneous determination of these five bio-markers has not been explored so far. The previous literature main focus on qualitative and quantitative determinations of two prevalent bio-markers, which are myricitrin and quercitrin [12].

Therefore, a chromatographic method is required to develop to show the presence of the five selected bio-markers.

Table 3. Chemical structures of the five selected bio-markers

 Afzelin	 Myricitrin
 Quercitrin	 Isoquercitrin
 Ellagic Acid	

1.3. Selection of Bioactivities of Components as Markers for Identification

Afzelin, Quercitrin and Myricitrin are reported in *Euphorbia hirta* L. showed antimalarial activity. They both inhibited the proliferation of *P. falciparum* (with the gene CDC1, chloroquine sensitive), which is a unicellular protozoan parasite that causes an infectious disease known as malaria, having their IC50 values 1.1, 4.1, 5.4 mg/mL, respectively. Whiles these flavonoid glycosides have little influence on the growth of KB 3-1, the host cell, showing cytotoxic activity with the IC50 values 276.1, 88.2, 156.4 mg=/mL, respectively [13].

Quercitrin

Quercitrin indirectly showed anti-diarrheal activity. At dose dependent concentration, it againsted castor oil- and PGE2-induced diarrhoea in mice. It also delayed small intestinal transit in the rat without modifying the fluid transport across the colonic mucosa, when the diarrhea was accelerated with castor oil. It also proposed that glycoside of quercitrin, is removed in the intestine, in order that aglycone, quercetin, increased colonic fluid absorption under the presence of secretagogue compounds [14].

The anti-inflammatory activity of quercitrin, through the inhibition of the NF-κB pathway, was also explained by the release of quercetin from quercitrin [15]. Furthermore, quercitrin demonstrated its anti-proliferative and apoptotic effects on lung cancer cells by controlling the immune response [16].

Myricitrin

Myricitrin, myricetin -3-O-rhamnoside, manifested anti-inflammatory effect. In a streptozotocin-induced mouse model (streptozotocin is commonly used to induce experimental diabetes in animals), myricitrin suppressed inflammation, cardiac oxidative stress and apoptosis by activated Akt and inhibited ERK signaling, so as to against diabetic cardiomyopathy [17]. The bio-marker also demonstrated its anti-inflammatory effect for acne vulgaris by the inhibiting the release of inflammatory cytokines IL 8 and TNF α [18].

Moreover, it has been found that myricitrin exerts antipsychotic-like effects in animal models [19], showed inhibitory effects on oxidative stress-induced endothelial damage and early atheroschlerosis [20], and alleviates methylglyoxal-induced mitochondrial dysfunction [21].
Myricitrin also demonstrated neuroprotective action on mouse brains from the loss of dopamine-producing neurons caused by neurotoxicity [22].

Afzelin

Afzelin (kaempferol-3-*O*-rhamnoside) is a flavonoid glycoside that demonstrated its antibacterial activity agaist *P. aeruginosa,* a Gram negative bacteria that can be found commonly in soil and water. The minimum inhibitory concentration (MIC) of afzelin against the bacteria was found to be 31 μg/mL [23].

Moreover, it showed its anti-cancer and apoptotic induction activities. Afzelin inhibited cell proliferation in MCF-7 cells (a type of breast cancer cells), in a dose-dependent manner. It also promoted apoptosis via the activation of the caspase signaling cascade, (caspase-9, caspase-3 and PARP). Hence, it is expected to be a potential anti-cancer therapeutics [24].

Isoquercitrin

Isoquercitrin (quercetin-3-O-glucoside) is potential for treating allergies. In a murine model of asthma, leucocytes in the mice with daily receiving of isoquercitrin were analyzed, and discovered that the eosinophil counts were lower in the bronchoalveolar lavage fluid (BALF), blood and lung parenchyma. (Eosinophils are pathogen that causes allergic diseases). This suggests the anti-inflammatory activity of isoquercitrin [25].

The flavonoid glycoside also shows its potentialities on colon cancer treatment. It inhibited Wnt/β-catenin, thus suppressing the related signaling pathway that leads to an array of human cancers. It also showed specific effect anti-tumoral effects on colon cancer cells (SW480, DLD-1, and HCT116) *in vitro*. Together they suggest the anti-tumor activity of isoquercitrin [26].

Ellagic Acid

Ellagic acid is potential for treating acute lung injury (ALI). In a nonlethal acid aspiration model of ALI in mice, the ellagic acid treatment reduced the vascular permeability and BALF levels, and also accelerated the resolution for lung neutrophilia and reduced the COX-2-induced exacerbation of inflammation. Together, these findings reveal the anti-inflammatory activity of ellagic acid [27].

The phenolic acid also shows its potentialities on enhancement on colorectal cancer treatment. Together with urolithins, they inhibited the formation and growth of colonospheres inside the colon cancer stem cells *in vitro* and *ex vivo*. It also reduced aldehyde dehydrogenase activity (ALDH, a marker of chemoresistance). This specifically showed its controllability of colon cancer chemoresistance. Together they support the anti-tumor activity of isoquercitrin [28].

1.4. Objectives of this study

Nowadays, Chinese medicine is paying particular attention on authentication in order to prevent the misprocessing of the genuineness of the Chinese Materia Medica (CMM). The authentication method is no longer limited to morphological identification. Different analytical methods, such as microscopic identification and fingerprint analysis, have also been developed.

In this project, the pattern analysis of euphorbiae genius sample is considered as a small scale of the herbal authentication since a complete standard is required to establish for identification of euphorbiae species samples.

The project has two main objectives:

1) To develop a precise and accurate method for identification of selected bio-markers in *Euphorbia hirta* L.
2) To conduct inter and intra-species comparison the fingerprint patterns under the same genus.

2. Materials, Reagents and Apparatus

2.1. Sample Information

A total of 10 batches of *Euphorbia hirta L. sample* were kindly prepared by Dr. Cheung Hon Yeung. (Table 4A) While 4 batches of *Euphorbia thymifolia L.* Samples were collected in Hong Kong from 20170708-20170713 (Table 4B).

Besides, a batch of *Euphorbia maculata* L. was also used in this study. With examining another three Euphorbiae species (one batch per species), there are six in total (Table 4C).

The euphorbiae species samples have been authenticated by Dr. Zhao Zhong Zhen, the associate dean of School of Chinese medicine, Baptist University in Hong Kong.

Table 4A. Information of *Euphorbia hirta* L. samples from Mainland China

Batch No.	Production/ Collection Area
FYC-L-001	Shenzhen, Guangdong (廣東深圳)
FYC-L-002	Yulin, Guangxi (廣西玉林)
FYC-L-003	Yulin, Guangxi (廣西玉林)
FYC-L-004	Taizhou, Zhejiang (浙江台州)
FYC-L-005	Meizhou, Guangdong (廣東梅州)
FYC-L-006	Maoming, Guangdong (廣東茂名)
FYC-L-007	Shangrao, Jiangxi (江西上饒)
FYC-L-008	Yulin, Guangxi (廣西玉林)
FYC-L-009	Yongzhou, Hunan (湖南永州)
FYC-L-010	Guilin, Guangxi (廣西桂林)

Table 4B. Information of Euphorbia thymifolia L. samples from Hong Kong

Batch No.	Production/ Collection Area
FYC-S-002	Lam Tei
FYC-S-004	Tung Chung
FYC-S-005	Pak Nai
FYC-S-006	Ping Shan

Table 4C. Information of other Euphorbiae species used in this study.

Batch No.	Name:	Production/ Collection Area
FYC-S-001	*Euphorbia humifusa* W.	Yangsuo, Guangxi (廣西陽朔)
FYC-S-007	*Euphorbia maculata* L.	Lishui, Zhejiang (浙江麗水)
FYC-S-003	*Euphorbia prostrata* A.	Tuen Mun
FYC-L-000	*Euphorbia hypericifolia* L.	Yuen Long

2.2. TLC analysis

2.2.1. Reagents

The standard stock of myricitrin, quercitrin, isoquercitrin, ellagic acid and afzelin were in HPLC grade, with purity ≥ 98%, were purchased from Herbest (ShaanXi province, China).

Other reagents were in analytical grade. Dichloromethane (≥99.5%), ethyl acetate (≥99.5%), formic acid (≥98%), methanol (≥99.5%), and sulphuric acid (1.84 g/mL) were purchased from Fuyu Chemical Reagent. Ethanol (≥99.5%) was purchased from Baishi Chemical Reagent.

TLC reference standard solution of myricitrin, quercitrin, isoquercitrin, ellagic acid and afzelin were prepared as 0.25, 0.25, 0.508, 0.4 and 0.134 mg/mL respectively. Developing solvent system was composed by dichloromethane: ethyl acetate: methanol: formic acid, 5: 3: 1.5: 1 in volume ratio, while spraying reagent was composed by 10% sulphuric acid in ethanol.

2.2.2. Material and Apparatus

Analytical balance (Sartorius BSA2245-CW, readable to 0.01 mg) was used for weighting bio-marker stocks and samples. Centrifugal tubes (50 mL, Eppendorf), disposable plastic syringes (5 mL), nylon syringe filter (Diameter 13 mm, pore size 0.45μm), an ultrasonic bath (200 W, Kudos SK5200H), were used for extraction. Pipettes (5 and 10 mL, Eppendorf) were used for preparing sample extraction, developing solvent and spraying reagent.

Bands were developed on HPTLC plates (10 cm x 20 cm), pre-coated with silica gel 60 F_{254nm} (Merck), with the samples extracts spotted by a TLC sample applicator (Cammag Linomat 5). The plates were placed on a TLC twin trough developing chamber. An Oven (heating up to 105.0^{o}C), as well as UV light system (λ=366 nm, Cammag Reprostar 3) was used for TLC analysis.

2.3. HPLC analysis

2.3.1. Reagents

The standard stock of myricitrin, quercitrin, isoquercitrin, ellagic acid and afzelin were in HPLC grade, with purity ≥ 98%, were purchased from Herbest (ShaanXi province, China).

Methanol for mobile phase solvent (≥ 99.9%) was in HPLC grade, purchased from purchased from RCI Labscan (Bangkok, Thailand). Double distilled water (Milli-Q) was used throughout the study (Millipore, Bedford, MA, USA).

Other reagents were in analytical grade. Ethanol and phosphoric acid were purchased from Merck Emsure® (Darmstadt, Germany).

2.3.2. Material and Apparatus

An analytical balance readable to 0.01 mg was used for weighting bio-marker stocks, whiles an analytical analytical balance readable to 0.1 mg was used for weighting samples. A blender and a sieve (mesh size: 850 μm) were used for sample preparation.

Centrifuge tubes (50 mL), a centrifuge machine (Avanti J-E and Allegra X-15R), PTFE filter (0.45-μm), Syringes (5 mL) and an ultrasonic bath (100 W) and volumetric flask (10 mL) were used during sample extraction. Pipettes (20, 200, 1000 μL and 5 mL, Eppendorf) were used for preparing sample extraction, precision test, as well as addition of bio-markers in recovery test.

Measuring cylinders (1000 mL) were used for preparation of mobile phase in HPLC analysis. The column adopted for analysis was ODS HPLC column, DIKMA Platisil ODS, 5 μm, 4.6 x 250 mm, with 15% of carbon load. The HPLC analysis was carried under Agilent 1260 Infinity High performance liquid chromatographic (HPLC) system equipped with a diode array detector (DAD) detector, having a dual-channel pump, capable of gradient flow rate up to 1.0 mL/min and an injector with a 10-μL sample loop, it was required to be capable for detecting wavelength ranging from 190 to 400 nm.

3. Experimental Procedure

3.1. TLC analysis

3.1.1. Sample Preparation

A *Euphorbia hirta* L. sample (Batch no. FYC-L-005) was adopted for TLC testing. The sample has been air dried and foreign matters inside the sample have been removed. The sample has been powdered in a blender and then passed through a sieve (mesh size: 850 μm). The powered sample was adopted for analysis.

Approximately 0.5 g of the powered sample was added to the 50-mL centrifugal tube followed by adding 10 mL of 70% Ethanol. The mixture was then sonicated (100 W) for 30 min followed by a 10-min centrifuging process (4000 rpm, about 4ºC). Finally, the mixture was then filtered through a 0.45-μm PTFE filter.

3.1.2. Method Development

Firstly, the temperature and relative humidity were recorded. With aid of a TLC applicator, myricitrin standard solution (6 μL), quercitrin standard solution (3 μL)**,** as well as sample extract (5 μL), were spotted separately onto a TLC plate spaced about 1 cm from the bottom of the plate.

Next, the developing solvent was added into one of the troughs of the TLC developing chamber, while the TLC plate was placed vertically into another trough. The chamber was then covered with a lid and the TLC plate was equilibrated for about 15 min.

After that, the chamber was cautiously slanted so that the development solvent pass to the bottom of the TLC plate. When the plate was developed over a path of about 8.0 cm, it was removed from the chamber, with marking the solvent front marked and drying the plate in air.

Then, the plate was evenly sprayed with 10% sulphuric acid and heated in an oven at about 105°C for 30 seconds to allow the spots or bands to become visible. Finally, the TLC plate was examined under UV light at 366 nm.

The R_f value was calculated as follows:

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

3.1.3. Comparison of Different Species

TLC analysis was carried out on 10 batches of *Euphorbia hirta* L. samples (Table 4A) and 4 batches of *Euphorbia thymifolia* L. samples (Table 4B) respectively on an independent TLC plate.

Comparison of band patterns, and corresponding color of myricitrin and quercitrin, isoquercitrin, ellagic acid and afzelin was conducted and R_f values of the bio-markers were recorded.

Comparison of band patterns of different Euphorbiae genus (Table 4C) were conducted.

3.2. HPLC analysis

3.2.1. Sample Preparation

Another *Euphorbia hirta L*. sample (Batch no. FYC-L-009) was adopted as HPLC testing. The extraction process was same as above.

3.2.2. Method Development

HPLC analysis was performed by an Agilent 1260 Infinity HPLC system equipped with a 1260 diode array detector (DAD). A DIKMA technology, Platisil ODS column (5 µm, 4.6 x 250 mm) was adopted. The mobile phase was made up of 0.2% (v/v) Phosphoric Acid (A) and Methanol (B) using a linear gradient elution of 30-36% (B) in 0-55min, 36-45% (B) in 55-65 min, 20-35% (B) in 65-70 min. The flow rate was 1.0 mL/min and the injection volume was 10µL. UV spectra were recorded between 190 and 400 nm and the detection wavelength was set at 360 nm. The column temperature was set at 40^{o}C. Before analysis, the column was equilibrated with 30% (A) for at least 30 min (Longer time during first injection of a day). After each injection, the column was washed with 100% (B) for 5 min and then equilibrated with 30% (A) prior to next injection.

3.2.3. Comparison of Different Species

Similar to TLC analysis, HPLC analysis was carried out on 10 batches of *Euphorbia hirta* L. sample (Table 4A) and 4 batches of *Euphorbia thymifolia* L. samples (Table 4B) were undergone HPLC analysis on an independent day according to the established extraction and HPLC methods.

Comparison of fingerprint patterns of different Euphorbiae genus (Table 4C) was also conducted.

3.2.4. Method Validation

Several system suitability tests were carried out. Firstly, Resolution (R), No. of Theoretical Plates (n) and tailing factor (T) of the marker peaks among 10 batches of *Euphorbia hirta* L. were determined.

Secondly, intra-day (5 injections) and inter-day (3 consecutive days) reproducibility of peak area and retention time of the marker peaks were obtained by using a *Euphorbia hirta* L. sample (Batch no. FYC-L-007), the reproducibility was measured as percentage of relative standard deviation (%RSD) of peak area (PA) and retention time (RT)

Thirdly, precision test was performed by injecting the mixture of standard solution for 5 times in 1 day.

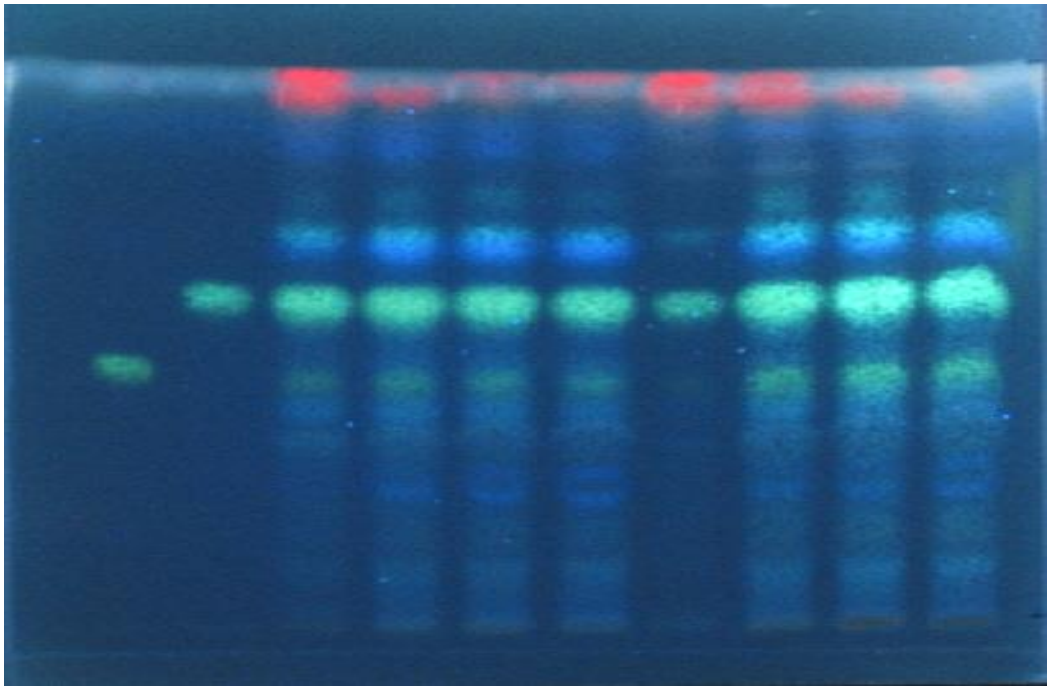
Moreover, the accuracy was determined by evaluation of recovery test by spiking myricitrin, quercitrin, isoquercitrin, ellagic acid and afzelin with the concentration of 10 μg/mL, 8.128 μg/mL, 8 μg/mL, 33 μg/mL, 2.68 μg/mL, respectively to the 10 mL sample extract.

4. Result and Discussion

4.1. TLC Method Development

4.1.1. Extraction condition

Firstly, the extraction solvent was compared. According to previous studies, methanol or ethanol were mostly used to extract the flavonoids. Moreover, the percentage by volume of alcohol affect the extraction efficiency. It is important to select a suitable percentage of alcohol. Methanol and ethanol, of 100%, 70%, 50%, 30% were used. The result found that the band intensity of myricitrin and quercitrin extracted by 70% ethanol was the most obvious (Lane 8).

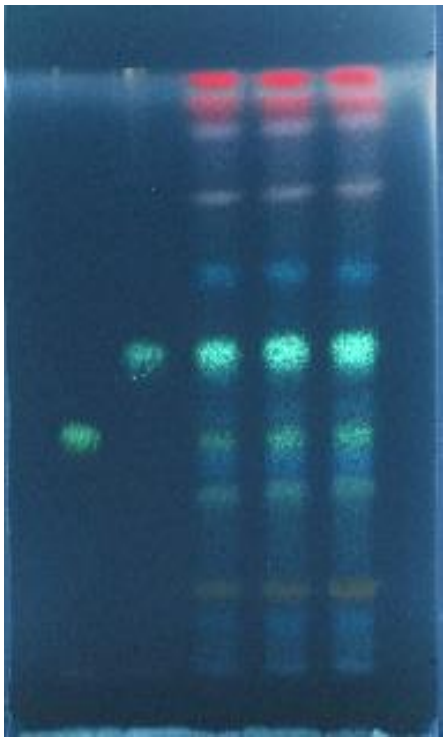


1 2 3 4 5 6 7 8 9 10

Lane	Solution	Extraction solvent
1	Myricitrin standard solution	--
2	Quercitrin standard solution	--
3	Extracts of Sample FYC-L-005	100% Methanol
4		70% Methanol
5		50% Methanol
6		30% Methanol
7		100% Ethanol
8		70% Ethanol
9		50% Ethanol
10		30% Ethanol

Figure 3A. TLC chromatogram of *Euphorbia hirta* L. extracts prepared by different composition of extraction solvents under UV light (366 nm) after spraying.

Next step was considering the extraction time. 15, 30 and 60 minutes (Lane 3, 4, 5) were compared. It was found that there was little difference in band intensity under 366 nm when 30 and 60 min extraction time were compared. The bands were apparently blurred when 15 min was adopted. Considering the efficiency, the extraction time of 30 minutes was adopted.



1 2 3 4 5

Lane	Solution	Sonication time
1	Myricitrin standard solution	--
2	Quercitrin standard solution	--
3	Sample FYC-L-005	15 min
4		30 min
5		60 min

Figure 3B. TLC chromatogram of *Euphorbia hirta* L. extracts prepared by different extraction time under UV light (366 nm) after spraying.

4.1.2. Developing Solvent Systems, Spray Reagent and Observation Condition

Different developing solvent systems were adopted.

Starting from Trial A, the R_f value of myricitrin was lower than 0.3. To increase R_f values of myricitrin and quercitrin, methanol was added and portion of formic acid was increased. The R_f value of quercitrin was increased to 0.90, but it was too high (Trial B). Therefore the portion of methanol and formic acid was reduced, the Trial C result obtained, however myricitrin was hardly seen and quercitrin was not well separated.

Considered to change the hexane into dichloromethane and it was found out that the bands were eluted with good separation and satisfactory R_f values (Trial D). The substitution of hexane with acetone got poor separation of bands (Trial E). When cyclohexane was adopted, myricitrin band was hardly observed (Trial F), which was similar to Trial C.

To conclude, the developing solvent system trial D (dichloromethane: ethyl acetate: methanol: formic acid, 5: 3: 1.5: 1, v/v was adopted owing to the better separation and satisfactory R_f values of myricitrin and quericitrin.

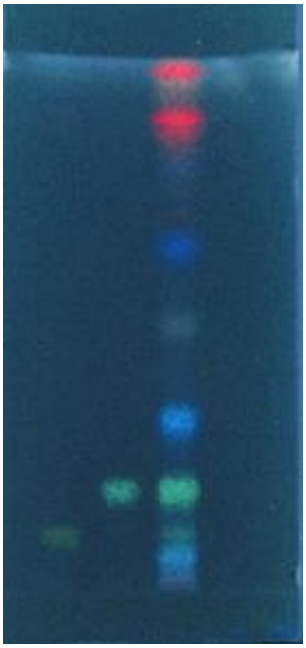
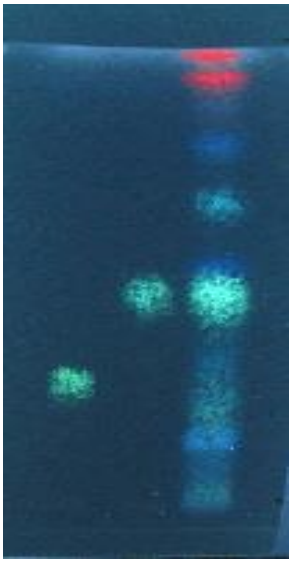
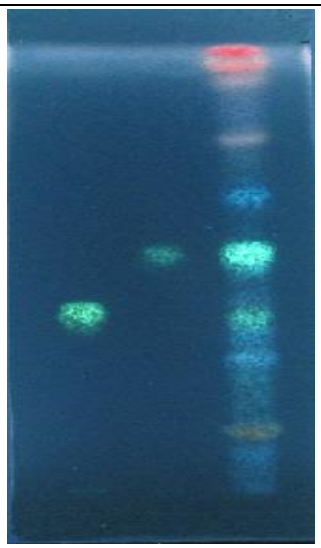
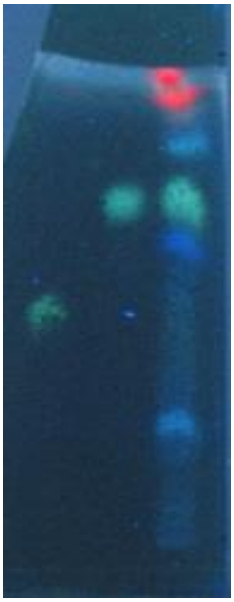
Trial A Hexane: ethyl acetate: formic acid (6:10:1)	Trial B Hexane: ethyl acetate: methanol: formic acid (3:5:1:1)	Trial C Hexane: ethyl acetate: methanol: formic acid (3:5:0.5:0.5)
 1 2 3	 1 2 3	 1 2 3
Trial D ✓ Dichloromethane: ethyl acetate: methanol: formic acid (5:3:1.5:1)	Trial E Acetone: ethyl acetate: methanol: formic acid (4:2:1:0.5)	Trial F Cyclohexane: ethyl acetate: methanol: formic acid (3:5:1:0.5)
 1 2 3 **Better resolution**	 1 2 3	 1 2 3

Figure 3C. TLC chromatograms of *Euphorbia hirta* L. extracts prepared by different composition of developing solvents under UV light (366 nm) after spraying. Note: Lane 1: Myricitrin, Lane 2: Quercitrin, Lane 3: FYC-L-005

The next step was to select the spraying agent. The common spraying agents were 1% aluminum chloride dissolved in ethanol, and 10% sulphuric acid dissolved in ethanol.

For plate A, 1% aluminum chloride – ethanol was adopted as spraying agent, bands were blurred in particular the myricitrin. Whiles for plate B, 10% sulphuric acid – ethanol was adopted as spraying agent, bands were clear and myricitrin band was easily identified. . This implied that 10% sulphuric acid – ethanol was the better spraying agent.

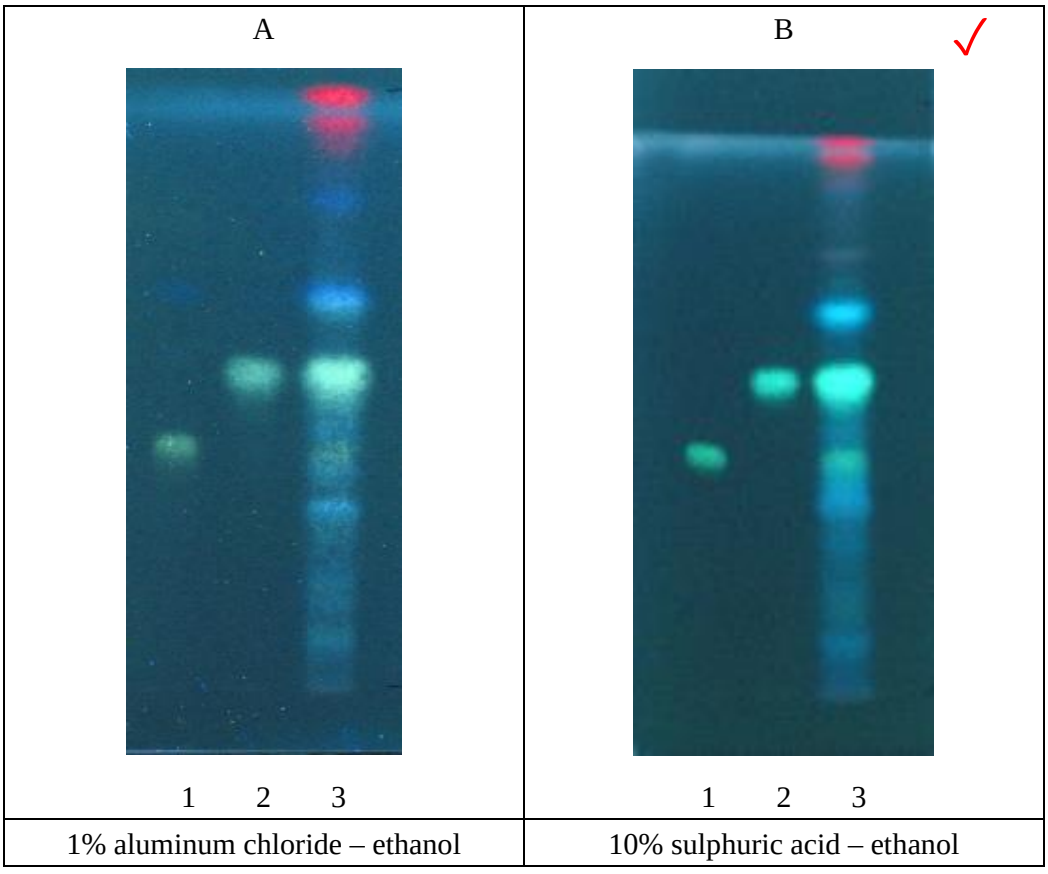


Figure 3D. Effect of spaying agents on bands after TLC development. Note: Lane 1: Myricitrin, Lane 2: Quercitrin, Lane 3: FYC-L-005

After adopted 10% sulphuric acid – ethanol as spraying agent, the observation condition was considered.

For plate A, bands were visible under visible light but very few bands were observed. For plate B, under 254 nm, the bands were difficult to observe. For plate C, under 366 nm, green and blue colored bands were easily observed. This implied that UV light with 366 nm gave the best observation condition.

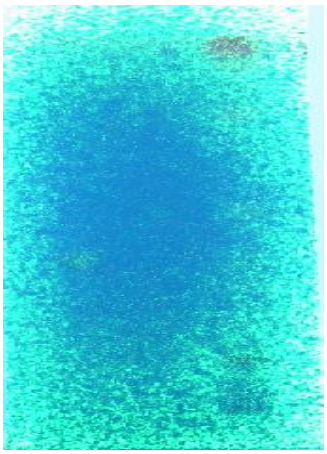
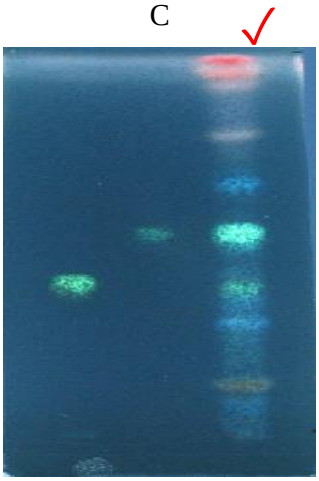
A	B	C
		
1 2 3	1 2 3	1 2 3
Under visible light	Under UV light 254 nm	Under UV light 366 nm

Figure 3E. Effect of detection wavelengths on bands after TLC development. Note: Lane 1: Myricitrin, Lane 2: Quercitrin, Lane 3: FYC-L-005

4.2. TLC and Euphorbiae genus

4.2.1. Addition of Isoquercitrin, Ellagic Acid and Afzelin in to TLC plate

Since the developed method was only suitable for myricitrin and quercitrin. Another three standards isoquercitrin, ellagic acid and afzelin were added into a same TLC plate.

From the result (Please refer to figure 5A), the R_f value of myricitrin (Lane 1) and isoquercitrin (Lane 3) were similar (0.42 and 0.41, respectively), but their bands differed in color (green and cyan, respectively).

Furthermore, the ellagic acid (Lane 4) was shown as a strip on the TLC pate under UV light. The R_f value of ellagic acid were undetectable.

Afzelin (Lane 5) was shown as a cyan band on the TLC pate under UV light, with R_f value 0.66.

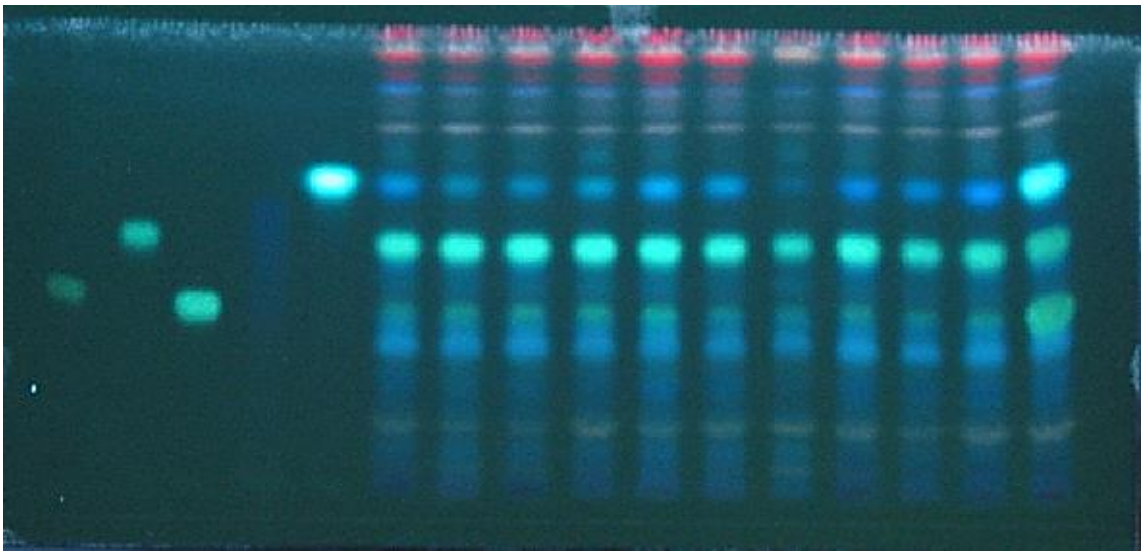
4.2.2. Species comparison: Intra species -- *Euphorbia hirta* L.

On the same plate again, a total of 10 batches of *Euphorbia hirta L.* sample (Lane 6-15), as well as the spiking of the last sample (FYC-L-010) (Lane 16) were undergone TLC analysis. It was observed that all the 10 batches containing the similar visible bands under UV light 366 nm.

Quercitrin bands were sharp and having the R_f value 0.54. The bands just below the quercitrin, R_f value 0.40, were myricitrin. Isoquercitrin bands were unable to identify out owing to the close approach between myricitrin bands and unknown bands at the R_f 0.36.

Only by comparing R_f values, the bands at R_f value 0.66 were identified as afzelin. Nevertheless, the color of afzelin standard and that of samples were mismatched. When compared the non-spiked (Lane 15) and the spiked (Lane 16), the bands at such R_f value differed in color (dim blue and cyan, respectively). Though concentration of the standard bio-marker may affect the color intensity, it might be due to the unknown compounds with similar R_f value as afzelin. Hence, afzelin was not identified out in the samples.

It was also found out that the bands of FYC-L-007 (Lane 12) was relatively dimmer when compared to the others using the similar condition. This indicated that the contents of the bio-markers were relatively lower.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

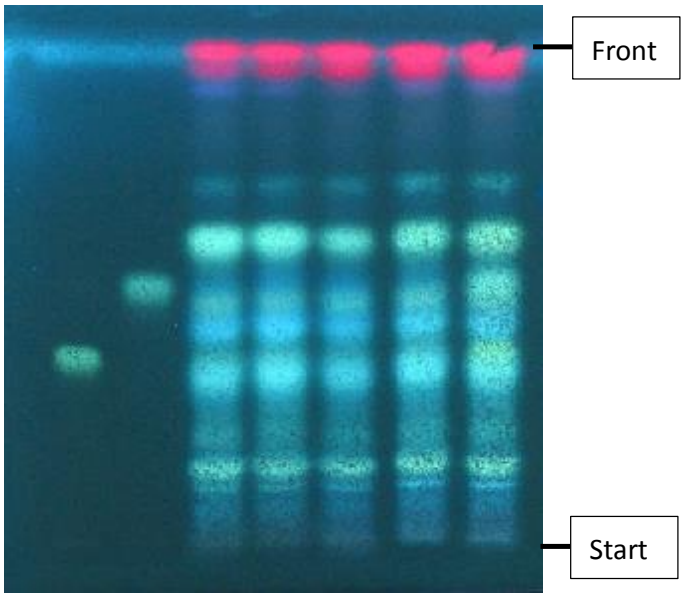
Lane	Solution	Batch no.	Result	R_{f1}	R_{f2}	R_{f3}	R_{f4}	R_{f5}
1	Myricitrin	Standard	Myricitrin positive	0.42	---	---	---	---
2	Quercitrin	Standard	Quercitrin positive	---	0.55	---	---	---
3	Isoquercitrin	Standard	Isoquercitrin positive	---	---	0.41	---	---
4	Ellagic Acid	Standard	EllagicAcid positive	---	---	---	NA	---
5	Afzelin	Standard	Afzelin positive	---	---	---	---	0.66
6	*Euphorbia hirta* L.	FYC-L-001	Quercitrin And Myricitrin Positive	0.40	0.54	---	---	0.66
7		FYC-L-002		0.40	0.54	---	---	---
8		FYC-L-003		0.40	0.54	---	---	---
9		FYC-L-004		0.40	0.54	---	---	---
10		FYC-L-005		0.40	0.54	---	---	---
11		FYC-L-006		0.40	0.54	---	---	---
12		FYC-L-007		0.40	0.54	---	---	---
13		FYC-L-008		0.40	0.54	---	---	---
14		FYC-L-009		0.40	0.54	---	---	---
15		FYC-L-010		0.40	0.54	---	---	---
16	Spiked *E. hirta* L. FYC-L-010 with all the standard	---	All positive	0.41	0.55	0.41	NA	0.67

Figure 4A. TLC results of ***Euphorbia hirta* L.** sample observed under UV light (366 nm) after spraying. Note : $R_{f\ 1\text{-}5}$: myricitrin (R_{f1}), quercitrin (R_{f2}), isoquercitrin (R_{f3}), ellagic acid (R_{f4}), afzelin (R_{f5}).

4.2.3. Species comparison: Intra species -- *Euphorbia thymifolia* L.

A total of 4 batches of *Euphorbia thymifolia* L. samples (Lane 3-6), as well as the spiking of the last sample (FYC-S-006) (Lane 7) were undergone TLC analysis. Quercitrin and myricitrin were spotted (Land 1, Lane 2), and their R_f values were 0.37 and 0.51 respectively. However, only the spiked sample (FYC-S-006) contained these two bands (Lane 7), this implied the absence of the quercitrin and myricitrin in all *Euphorbia thymifolia* L samples.

Nevertheless, all the *Euphorbia thymifolia L.* samples contained similar band pattern under UV light 366 nm. All possessed two thick bands between myricitrin and quercitrin (R_f about 0.42 and 0.48); three bands over quercitrin, with a thick one obviously with R_f about 0.59; two bands below myricitrin, with R_f about 0.35 and 0.16. These bands contributed to the characteristic pattern of *Euphorbia thymifolia* L.



Lane	Solution	Batch no.	Result	R_{f1}	R_{f2}
1	Myricitrin	Standard	Myricitrin positive	0.37	---
2	Quercitrin	Standard	Quercitrin positive	---	0.51
3	*Euthorbia thymifolia* L.	FYC-S-002	Negative	---	---
4		FYC-S-004	Negative	---	---
5		FYC-S-005	Negative	---	---
6		FYC-S-006	Negative	---	---
7	Spiked *E. thymifolia L.* FYC-S-006 with myricitrin and quercitrin	---	Both positive	0.37	0.51

Figure 4B. TLC results of *Euthorbia thymifolia* L. samples observed under UV light (366 nm) after spraying. Note: myricitrin (R_{f1}), quercitrin (R_{f2}).

4.2.4. Species comparison: Inter species -- Euphorbiae genus

A total of six different species under the Euphorbia genus (i.e. *Euphorbia hirta* L., and *Euphorbia thymifolia* L. (Lane 3 and 4), as well as *Euphorbia humifusa* W., *Euphorbia prostrata* A., *Euphorbia maculate L*. and *Euphorbia hypericifolia* L. (Lane 5-8, respectively) were undergone TLC analysis on the same plate.

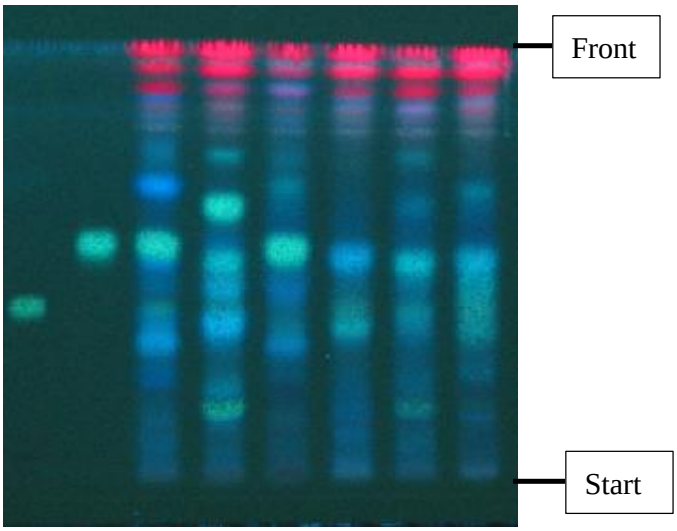
Overall, each of the Euphorbiae species has its unique chromatographic pattern under the same TLC condition.

The chromatographic pattern of *Euphorbia thymifolia* L. (Lane 4) were obviously different from that of *Euphorbia hirta* L. (Lane 3) and *Euphorbia humifusa* W. (Lane 5) Between R_f 0.3 and R_f 0.8, they contained the same band. *Euphorbia thymifolia* L. contained more bands than *Euphorbia hirta* L. (8 bands vs. 4 bands)

The bands of *Euphorbia thymifolia* L. (Lane 4) were similar to that of *Euphorbia maculata L.* (Lane 7). They both contained five common bands between R_f 0.3 and R_f 0.8.

Euphorbia prostrata A. (Lane 6) and *Euphorbia hypericifolia* L. (Lane 8) showed different chromatographic pattern to other Euphorbiae species. On the TLC plate, only *Euphorbia prostrata* A. demonstrated the absence of bands between R_f 0.55 and R_f 0.8. Whiles the stacking of bands between R_f 0.35 and R_f 0.5 was only appeared in *Euphorbia hypericifolia* L.

Nevertheless, by adding the myricitrin and quercitrin standard (Lane 1 and 2), it is found that only the *Euphorbia hirta* L. (Lane 3) and the *Euphorbia humifusa* W. (Lane 5) contained quercitrin, and only the *Euphorbia hirta* L. contained myricitrin when observed under UV light at 366 nm.



Lane	Solution	Batch no.	Result	R_{f1}	R_{f2}
1	Myricitrin	Standard	Myricitrin positive	0.41	---
2	Quercitrin	Standard	Quercitrin positive	---	0.55
3	*Euphorbia hirta* L.	FYC-L-010	Quercitrin and Myricitrin positive	0.40	0.55
4	*Euphorbia thymifolia* L.	FYC-S-006	Negative	---	---
5	*Euphorbia humifusa* W.	FYC-S-001	Quercitrin positive	---	0.55
6	*Euphorbia prostrata* A.	FYC-S-003	Negative	---	---
7	*Euphorbia maculate* L.	FYC-S-007	Negative	---	---
8	*Euphorbia hypericifolia* L.	FYC-L-000	Negative	---	---

Figure 4C. TLC results of Euphorbiae Species sample observed under UV light (366 nm) after spraying. Note: myricitrin (R_{f1}), quercitrin (R_{f2}).

4.2.5. Conclusion of TLC analysis

TLC could be a simple and rapid method to distinguish different Euphorbiae species form each other. This is because the band pattern of each species was unique Therefore, it is suitable for identification of a certain Euphorbiae species by comparing the band patterns .

Nevertheless, the separation of all five bio-markers was required to be improved by lengthening the solvent front in order to examine the presence of them accurately. With limited to myricitrin and quercitrin, only *Euphorbia hirta* L. countained both of them and *Euphorbia humifusa* W. contained quercitrin only. Therefore, it is concluded that the band patterns of TLC analysis indicated some differences between the Euphorbiae genus studied.

Table 5. Conclusion of the observation in the band patterns of Euphorbiae genus by TLC analysis.

Different species under Euphorbiae genus	Comment
Euphorbia hirta L. and *Euphorbia humifusa* W.	Show similarity in pattern
Euphorbia thymifolia L. and *Euphorbia maculata* L.	Show similarity in pattern
Euphorbia prostrata A.	Different from other species
Euphorbia hypericifolia L.	Different from other species

4.3. HPLC Method Development

4.3.1. Extraction Condition

Similar as TLC analysis, different concentrations of methanol and ethanol, respectively from 100%, 70%, 50%, 30% were carried out for extraction. The result found out that when using methanol as extraction solvent, myricitrin peaks always gave lower purity. Overall, 70% ethanol extract has higher purity compared with other compositions of methanol and ethanol, as well as relatively higher peak area. Hence, it was decided to choose 70% ethanol as extraction solvent.

Table 6A. Selection of sonication solvent in HPLC analysis

Extraction solvent	100% Ethanol		70% Ethanol		50% Ethanol		30% Ethanol	
Peak Number	Area	Purity	Area	Purity	Area	Purity	Area	Purity
Myricitrin	165.8	982.475	918.9	982.598	923.1	975.864	859.2	984.537
Isoquercitrin	28.0	NA	140.7	984.716	134.8	983.862	129.2	997.535
Ellagic Acid	69.3	NA	704.2	999.811	772.9	999.826	2485.9	999.846
Quercitrin	580.8	999.483	2627.5	999.690	2621.6	999.428	2192.4	999.745
Afzelin	33.6	NA	135.8	998.846	136.5	999.04	105.7	999.410

Extraction solvent	100% Methanol		70% Methanol		50% Methanol		30% Methanol	
Peak Number	Area	Purity	Area	Purity	Area	Purity	Area	Purity
Myricitrin	702.2	970.709	956.4	877.478	866.5	891.81	686.7	983.043
Isoquercitrin	95.3	997.530	137.5	979.585	119.5	969.656	107.6	997.969
Ellagic Acid	460.3	999.747	782.6	999.828	1088.8	999.854	1022.6	999.837
Quercitrin	2056.4	999.746	2693	999.373	2466.8	999.499	1475.5	999.699
Afzelin	116.3	999.389	139	999.193	124.9	999.387	72.3	998.949

Extraction time was compared using 15, 30, 45, and 60 minutes sonication. Similarly, peak purity and peak area were already good when 30 min was used. Myricitrin peak purity was worse when adopting 45 min extraction. Considering the efficiency, the extraction time of 30 minutes was adopted.

The extraction method, sonication or reflux, was also investigated under the 30 minutes. Though peak area of reflux was generally larger than that of sonication, peak purity of myricitrin and isoquercitrin were unacceptable. It was deduced that reflux method may allow the co-elution of other chemical compound more readily when compared with sonication.

Table 6B. Selection of sonication time in HPLC analysis

Sonication Time	15 min		30 min		45 min		60 min	
Peak Number	Area	Purity	Area	Purity	Area	Purity	Area	Purity
Myricitrin	937.6	981.642	945	999.057	985.3	987.165	963.6	986.931
Isoquercitrin	131.5	998.071	136.4	997.804	149	997.584	137	997.612
Ellagic Acid	635.9	999.805	674.7	999.804	781.7	999.845	801.6	999.855
Quercitrin	2701.7	999.600	2752.6	999.498	2852.6	999.588	2810.1	998.059
Afzelin	144.3	999.324	144.1	999.026	146.5	998.988	147.9	999.159

Table 6C. Selection of extraction method in HPLC analysis

30 min	Sonication		Reflux	
Peak Number	Area	Purity	Area	Purity
Myricitrin	945	999.057	1113.8	972.173
Isoquercitrin	136.4	997.804	166.5	977.872
Ellagic Acid	674.7	999.804	1216.3	999.902
Quercitrin	2752.6	999.498	3163.4	999.724
Afzelin	144.1	999.026	167.3	999.206

4.3.2. HPLC Conditions (Column, temperature, mobile phase, gradient elution and wavelength

4.3.2.1. Selection of Column

Two brands of C18 column were compared: (A) Agilent Zorbax SB-C18 column, 5 µm, 4.6 x 250 nm, with 10 % of carbon load, (B) Dikma Platisil ODS column, 5 µm, 4.6 x 250 nm, with 15 % of carbon load. When column A was adopted,several problems arised. Firstly, the peak resolution was poor. Isoquercitrin and ellagic acid could not be well separated. Secondly, the purity of myricitrin is utterly low. Moreover, peaks of ellagic acid and quercitrin showed obvious tailing.

When column B was adopted under the same condition, peaks were apparently well separated. All the peaks were having acceptable purity. Hence, column B was adopted to HPLC analysis.

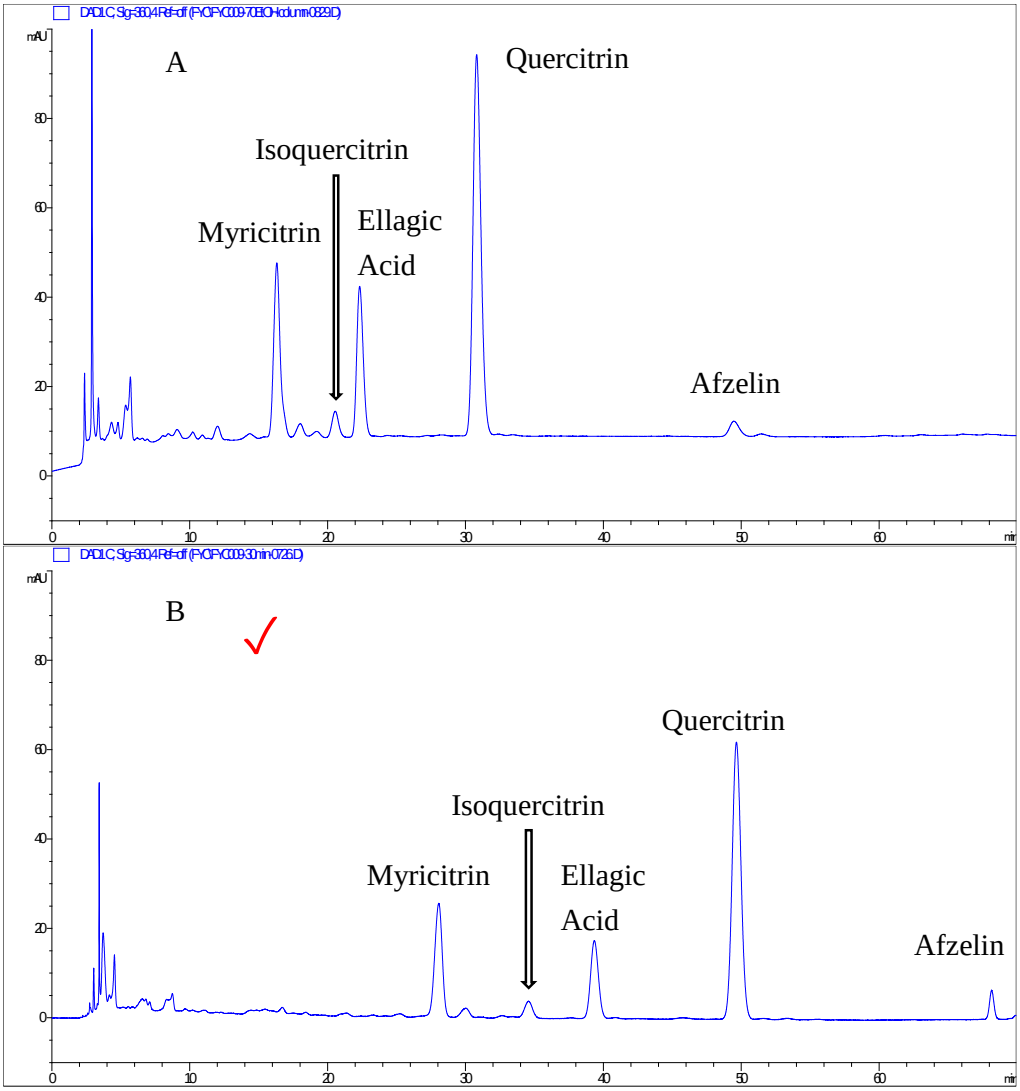


Figure 5A. HPLC Chromatograms of *Euphorbia hirta* L. obtained using different column. (A) Agilent Zorbax SB-C18 column, 5 µm, 4.6 x 250 nm, and (B) Dikma Platisil ODS column, 5 µm, 4.6 x 250 nm.

4.3.2.2. Selection of Column Temperature

Then, the column temperature, either 25°C and 40 °C, was compared. Obviously, the lower temperature, peaks eluted later and become broadened. Due to the low polarity, afzelin was unable to be eluted out before 70 min. When the temperature increased to 40°C, all the selected peak were eluted. Hence 40°C was selected as column temperature.

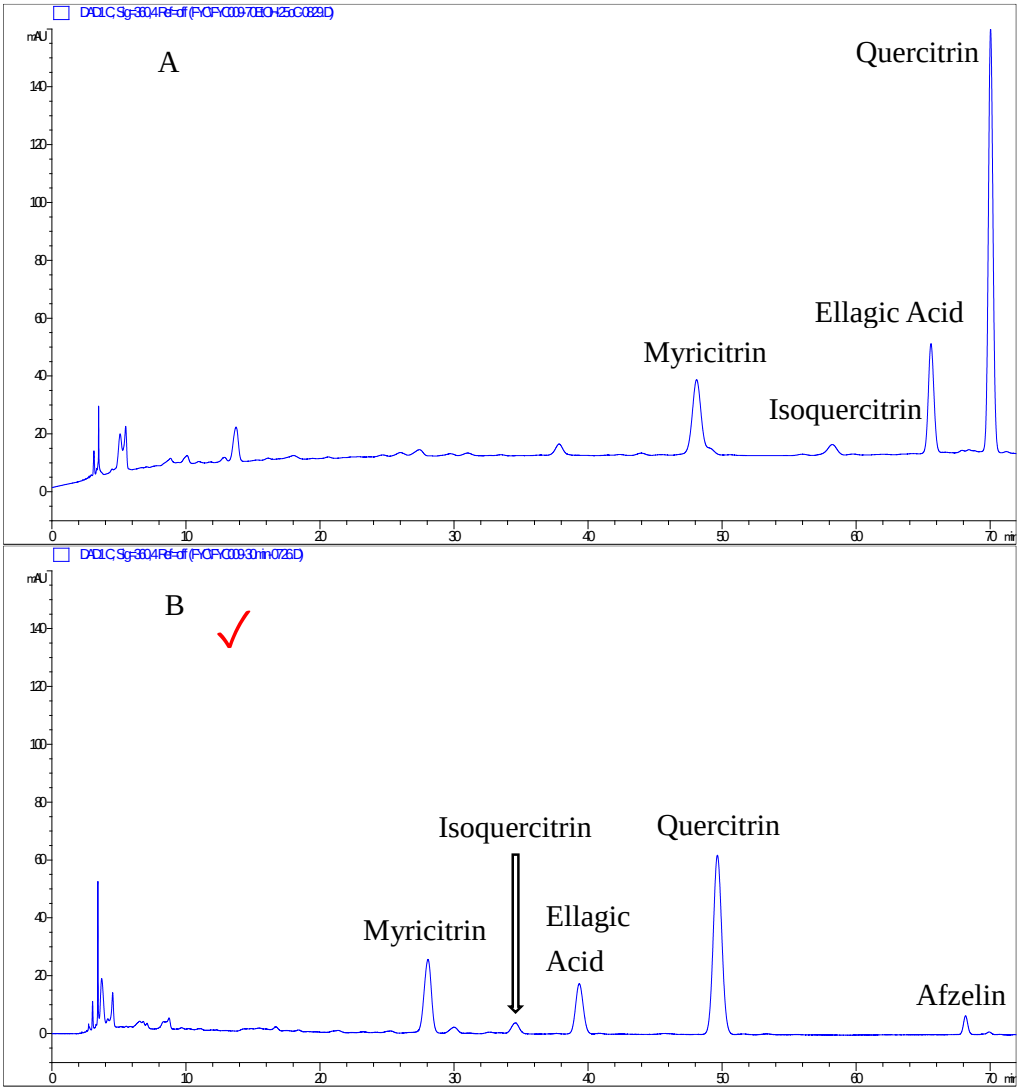


Figure 5B. HPLC Chromatograms of *Euphorbia hirta* L. obtained using different column temperature. (A) 25°C and (B) 40 °C.

4.3.2.3. Selection of Mobile Phase Solvent

After that, the three mobile phase of the HPLC was compared: (A) Methanol (HPLC grade) and water, (B) Methanol (HPLC grade) and 0.2% Phosphoric Acid, and (C) Acetonitrile and 0.2 % Phosphoric Acid.

Through the above comparison, the combination of (B) Methanol and 0.2% Phosphoric Acid was the best. For (A), ellagic acid peak was absent. For (C), acetonitrile could not separate myricitrin peak well and possibly stacked with the ellagic acid. The baseline was apparently shifted 5 mAU upward.

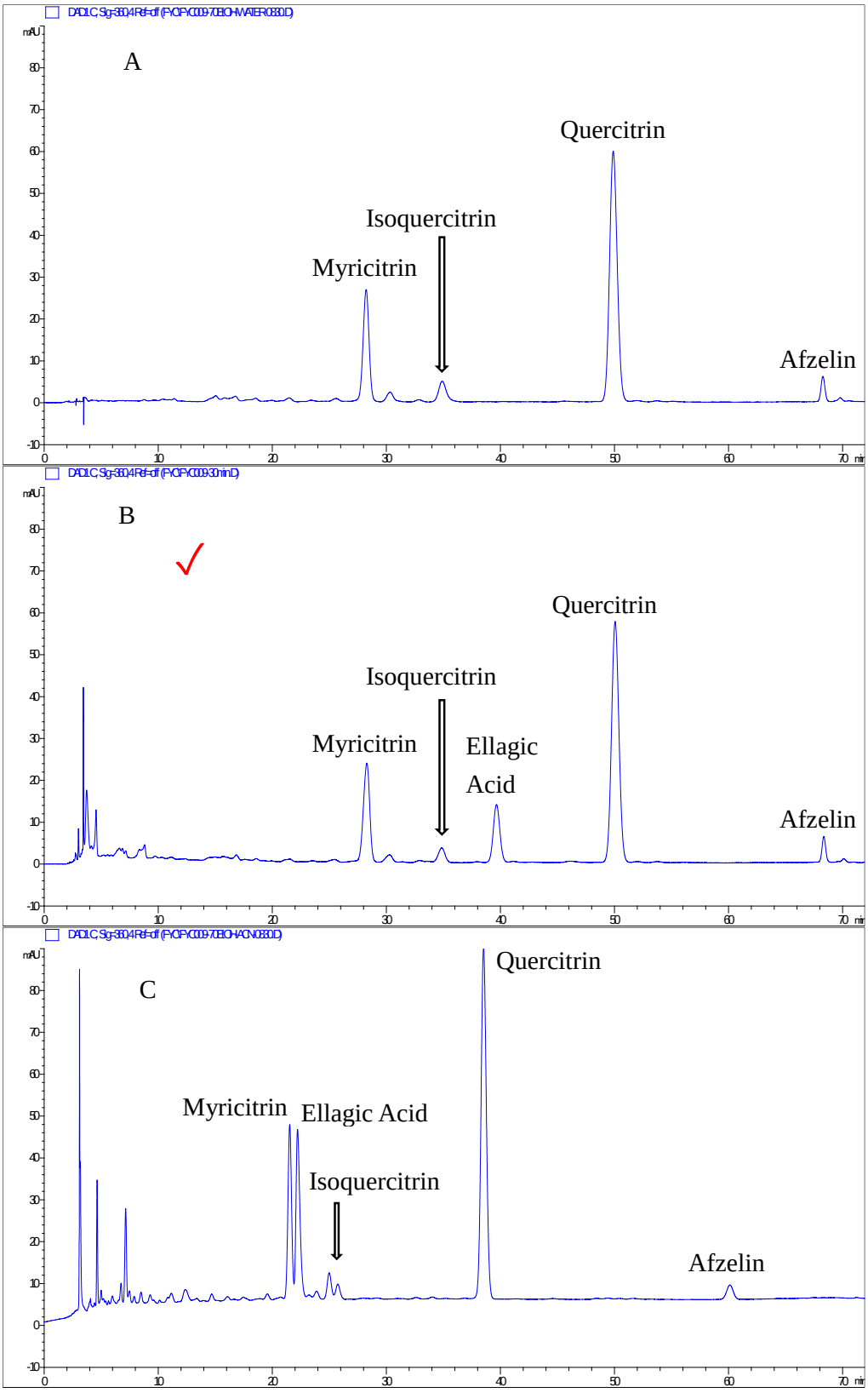


Figure 5C. HPLC Chromatograms of *Euphorbia hirta* L. obtained using different mobile phase solvent. (A) Methanol (HPLC grade) and water, (B) Methanol (HPLC grade) and 0.2% Phosphoric Acid, and (C) Acetonitrile and 0.2 % Phosphoric Acid as mobile phase. All three chromatograms shared the same elution profile.

4.3.2.4. Selection of Mobile Phase Gradient Profile

For mobile phase gradient profile, chromatograms of four trials were demonstrated and the elution profiles were listed below:

Table 7A. Gradient elution profile of the four trials

1			2		
Time (min)	% of A	% of B	Time (min)	% of A	% of B
0	65	35	0	68	32
60	55	45	50	62	38
			60	55	45
3			4		
Time (min)	% of A	% of B	Time (min)	% of A	% of B
0	68	32	0	70	30
50	65	35	55	64	36
60	54	46	65	55	45
70	54	46	70	55	45

Note: (A): 0.2% (v/v) Phosphoric Acid, (B) Methanol (HPLC grade)

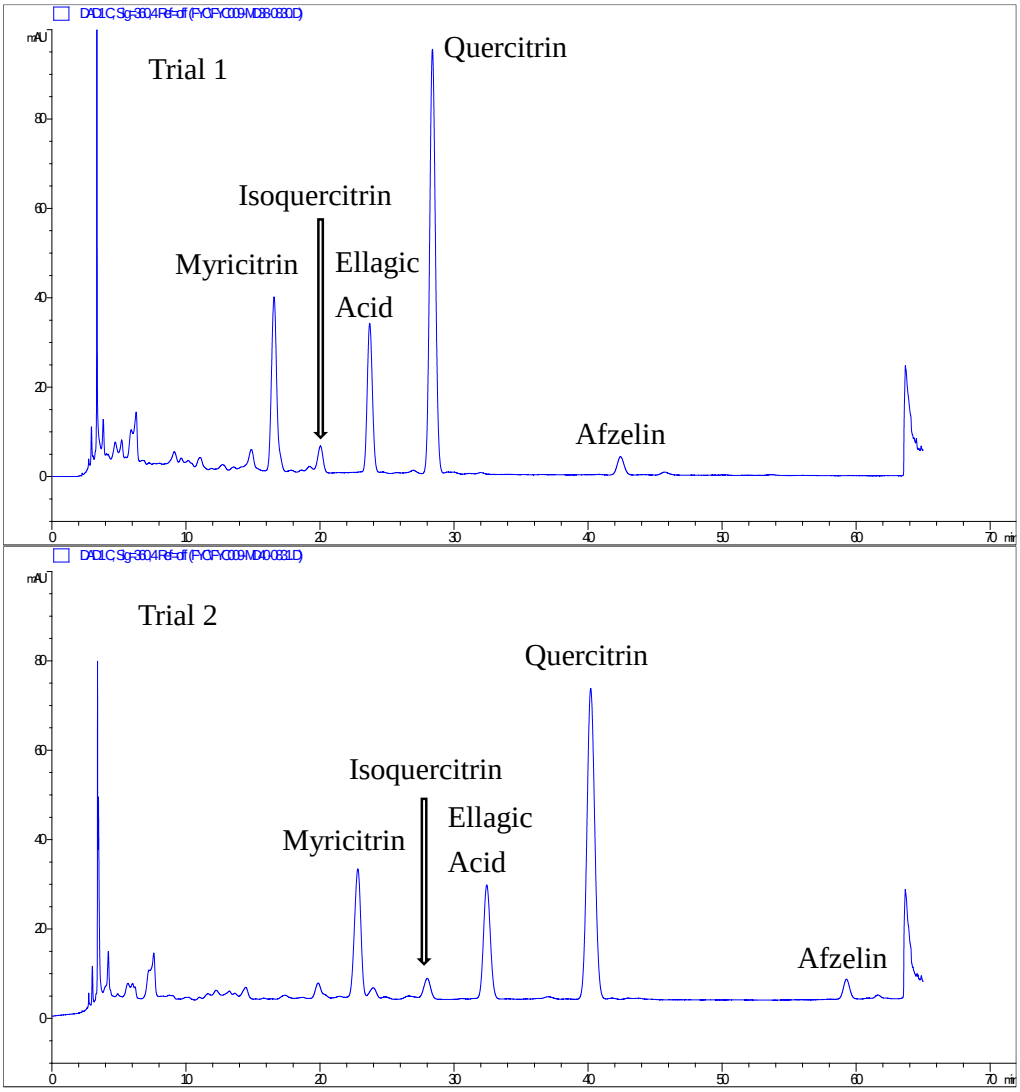
In trial 1, myricitrin peaks was tailing, with poor purity (866), indicated the possibility of overlapping with another chemical component. The peaks of myristrin and isoquercitrin were eluted too earily (Myricitrin at R_t about 16min, isoquercitrin at R_t about 20 min), so slower gradient was required.

In trial 2, peaks were eluted with better resolution (Myricitrin peak raised from 2.5 to 3.7). Tailing of myricitrin peak was solved without the co-elution with small peaks. Afzelin eluted a bit late yet still within the set time range. It was considered to allow the faster elution of afzelin and better separation of myricitrin and the small peak just behind myricitrin.

For trial 3, number of plates of myristrin were dropped (10615→9776) and the resolution was worse. Purity was not changed greatly compared to trial 2. The raise in % B from 45% to 46% did not greatly affect the retention time of afzelin. Moreover, myricitrin and the small peak were still unable to be separated out.

For trial 4, it was the best method among all trials since myricitrin and the small peak were successfully separated from each other (myricitrin peak resolution: 29.27) Number of plates were more than 10000 and peaks purities were larger than 990.

Therefore, the gradient profile of trial 4 was adopted owing to the better resolution of peaks, purity, as well as higher number of plates.



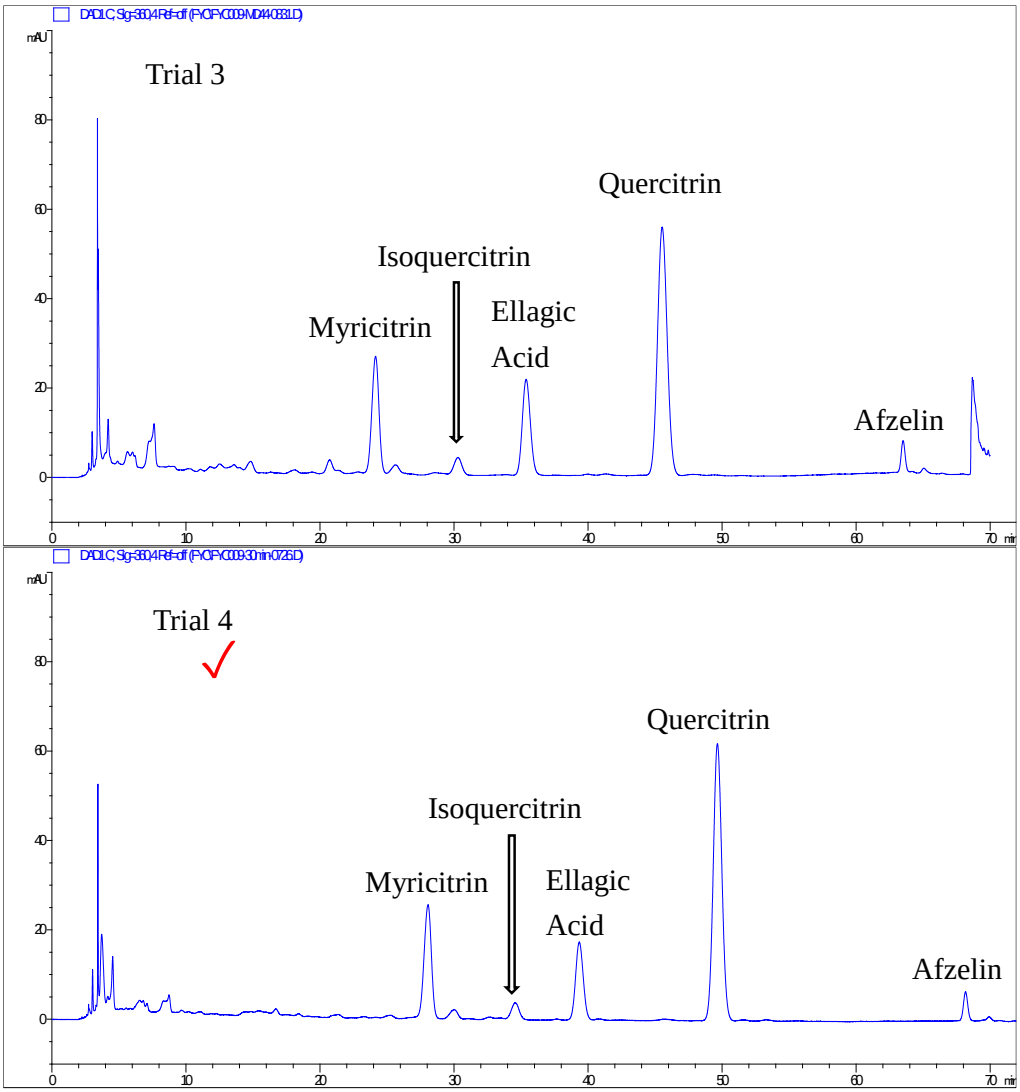


Figure 5D. HPLC Chromatograms of *Euphorbia hirta* L. using different gradient of mobile phase.

4.3.2.5. Selection of detection wavelength

Detection wavelength, 256, 270 and 360 nm, were compared. It was found out that at 256 nm wavelength, the peak intensity was higher than that of 270 and 360 nm. This is because by observation the wavelength graphs of all the markers, the maximum absorption was between 250 and 260 nm. Nevertheless, 256 nm were not selected due to uneven baseline. A flattened baseline is important since it determines the peak area of the eluted peak. In assay study, peak area is crucial for calculation the content of the content of the selected component. Errors could be prominent when integrating the peak area under the uneven baseline.

By observing the baseline, the selection of 270 nm was also eliminated. At 360 nm, the baseline was smooth, and hence select 360 nm as detection wavelength.

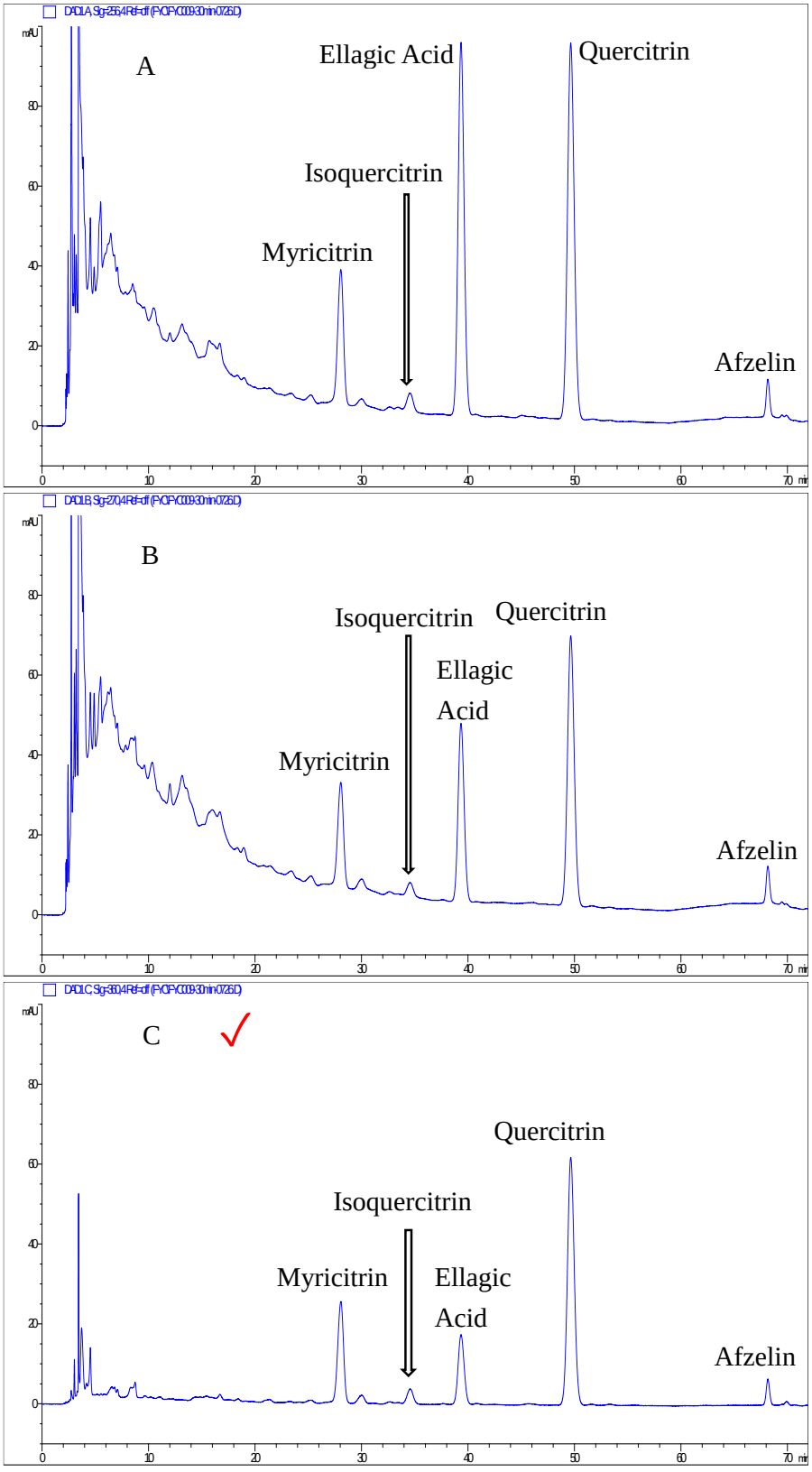
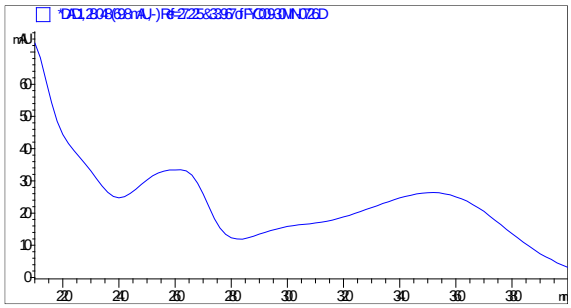


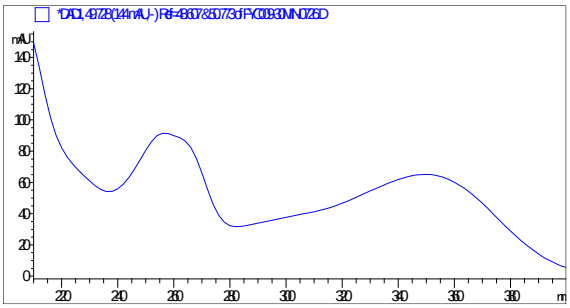
Figure 5E. HPLC Chromatograms of *Euphorbia hirta* L. obtained using different detection wavelength. (A) At wavelength 256 nm, (B) At wavelength 270 nm, (C) At wavelength 360 nm.

Intensity of all the markers detected from wavelength 190 to 400 nm

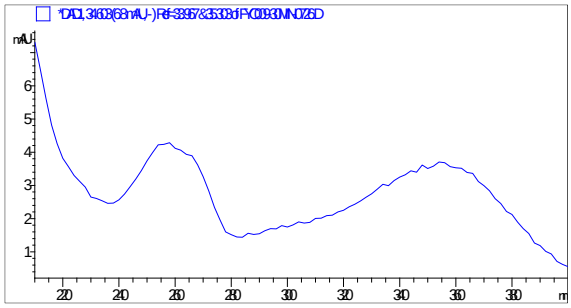
(A) Myricitrin



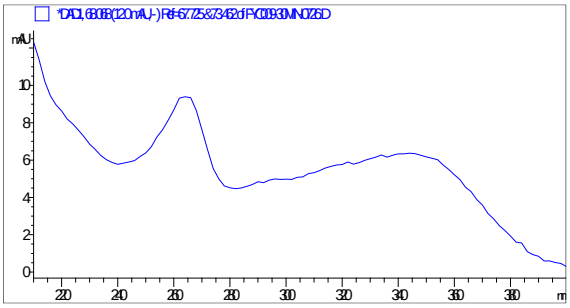
(D) Quercitrin



(B) Isoquercitrin



(E) Afzelin



(C) Ellagic Acid

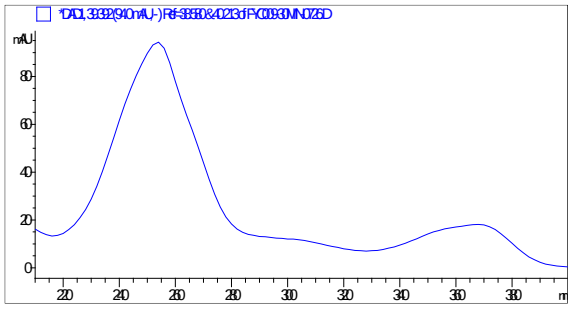


Figure 6A-E. Wavelength graphs of five bio-markers.

4.3.3. Summary of extraction condition and HPLC condition

Table 7B. Summary of extraction condition and HPLC condition of *Euphorbia hirta* L.

Extraction condition			
Extraction solvent	70% Ethanol		
Extraction time	30 min		
Extraction method	Sonication		
HPLC condition			
Instrument	Aglient 1260 Infinity system with 1260 DAD VL detector		
Column	Platisil ODS column, 5 µm, 4.6 x 250 nm, Dikma technology		
Injection volume	10 µL		
Detector	DAD (Diode array detector)		
Column temperature	40		
Flow rate	1.0 mL/min		
Mobile phase solvent	0.2% Phosphoric Acid (A) and Methanol (HPLC grade) (B)		
Gradient elution profile	Time (min)	% of (A)	% of (B)
	0	70	30
	55	64	36
	65	55	45
	70	55	45
Detection wavelength	360 nm		

4.4. HPLC and Euphorbiae genus

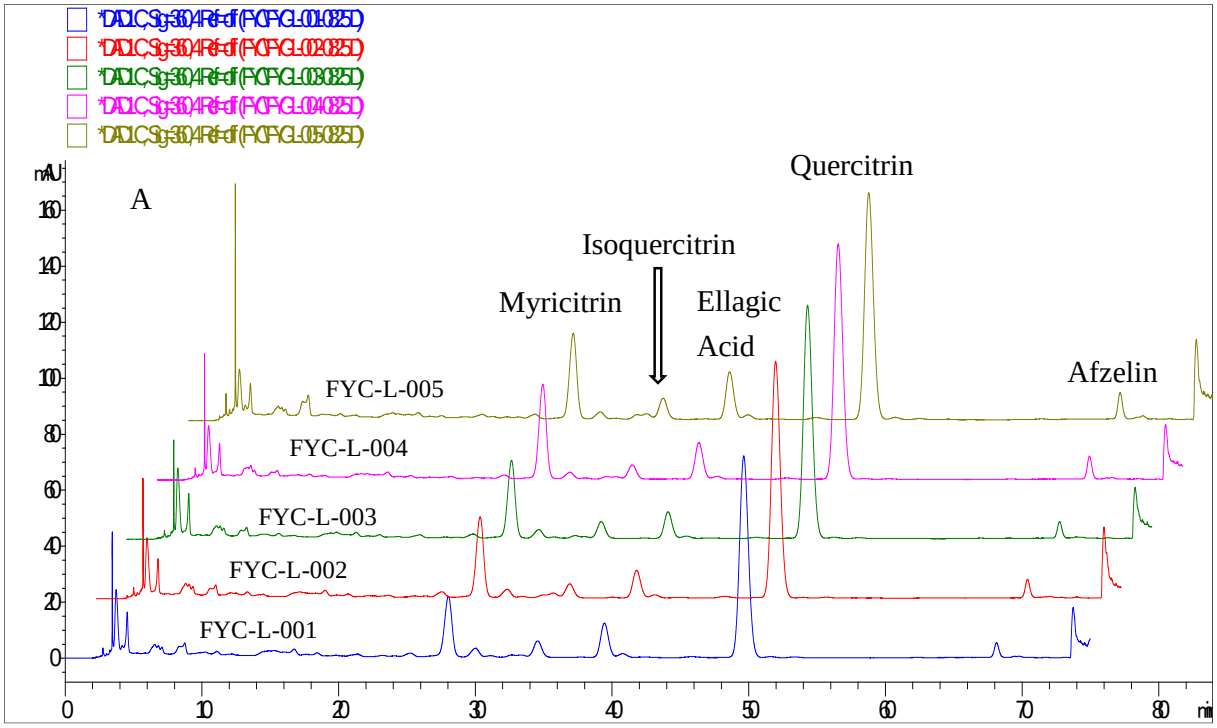
4.4.1. Species comparison: Intra species -- Euphorbia hirta L.

The 10 batches of *Euphorbia hirta* L. sample showed high similarities on the fingerprints.

Firstly, rather than the five marker peaks and a small peaks behind myricitrin, there were no other major components could be detected between 25 and 70 min..

Secondly, under the setup condition, the peak area of quercitrin was the largest among the other 4 markers, then followed by myricitrin and ellagic acid, and finally isoquercitrin and afzelin.

Thirdly, the RSD of retention time the respective five markers among the sample were less than 2%. This represented the high reproducibility of the system.



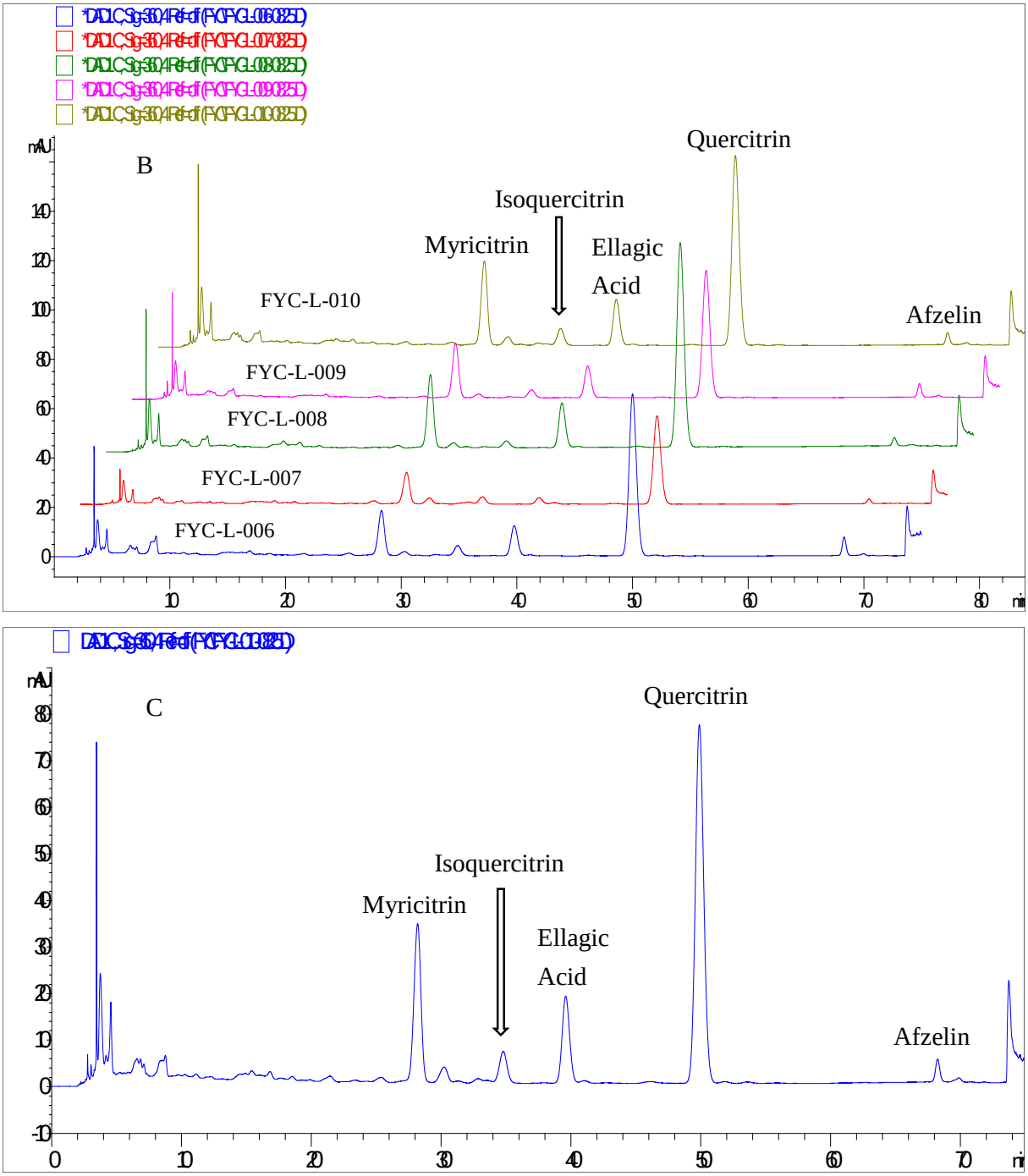


Figure 7A. Stacked HPLC Chromatograms of *Euphorbia hirta* L. (A) Batch 1-5 (B) Batch 6-10, as well as HPLC Chromatogram of (C) Reference batch FYC-L-010

4.4.2. Species comparison: Intra species -- Euphorbia thymifolia L.

The 4 batches of *Euphorbia thymifolia L.* sample showed high similarities on the fingerprints.

For the fingerprint pattern of each batch, the sharp peak was detected at 46 min. Also, peaks around 22 and 31 min were stacked and could not be well separated.

The high similarity on the fingerprint patterns between batches was due to the small collection area.

The climate difference between areas was small in Hong Kong.

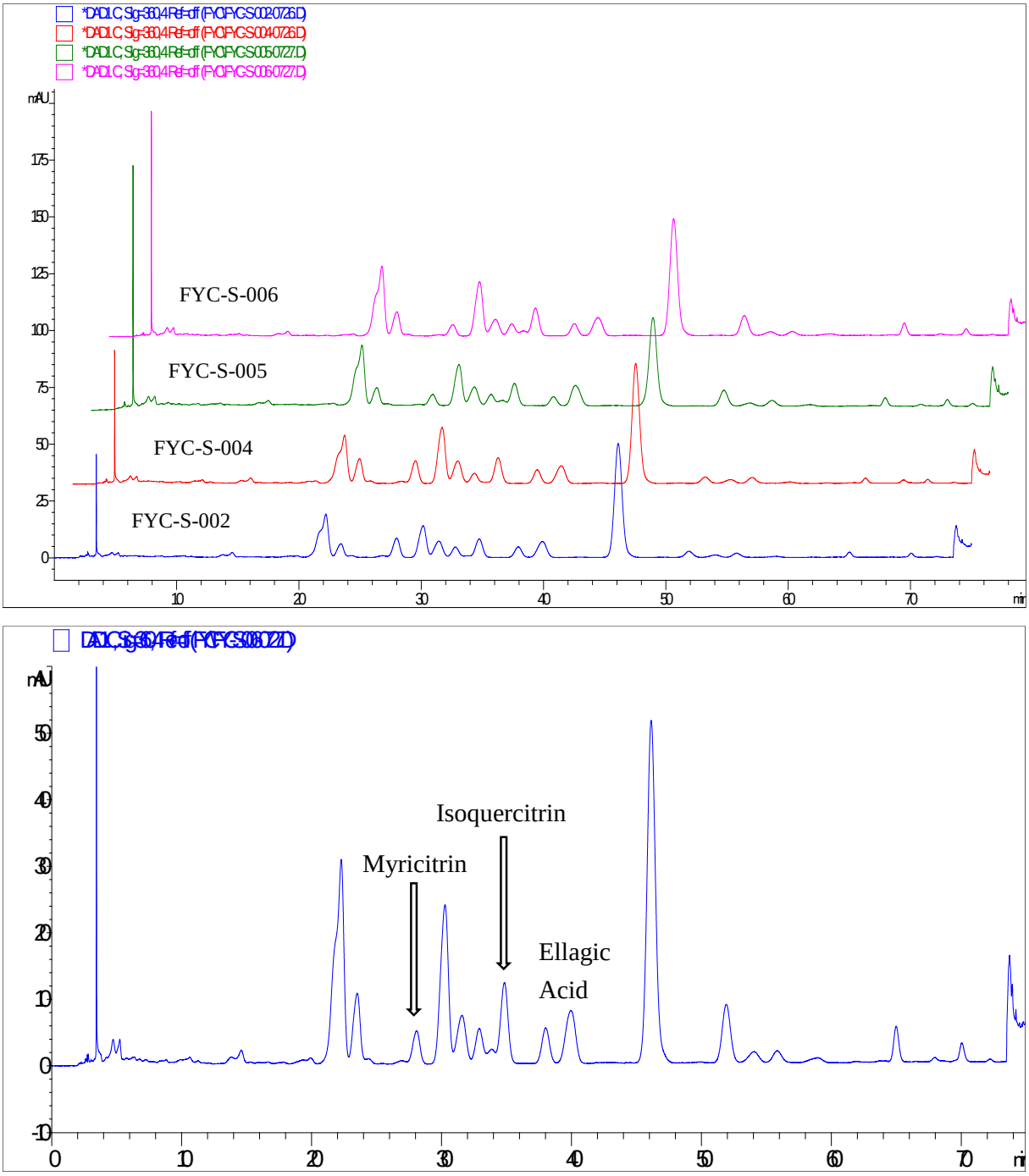


Figure 7B. Stacked HPLC Chromatograms of 4 batches of *Euphorbia thymifolia* L. (A) Batch 2,4,5,6, as well as a HPLC Chromatogram of (B) Reference batch FYC-S-006.

4.4.3. Species comparison: Inter species -- Euphorbiae genus

4.4.3.1. Euphorbia hirta L. and Euphorbia thymifolia L.

Though their Chinese medicinal names were similar, it was found that their fingerprint patterns were significantly different. Less peaks were detected in *Euphorbia hirta* L. compared with *Euphorbia thymifolia* L. Three markers were discovered in both species. They were myricitrin, isoquercitrin and ellagic acid.

Quercitrin and afzelin were only found in *Euphorbia hirta* L., whiles the other peaks in in *Euphorbia thymifolia* L. were unable to found in *Euphorbia hirta* L. This indicated that the presence of quercitrin and afzelin could be the evidence to distinguish *Euphorbia hirta* L. from *Euphorbia thymifolia* L.

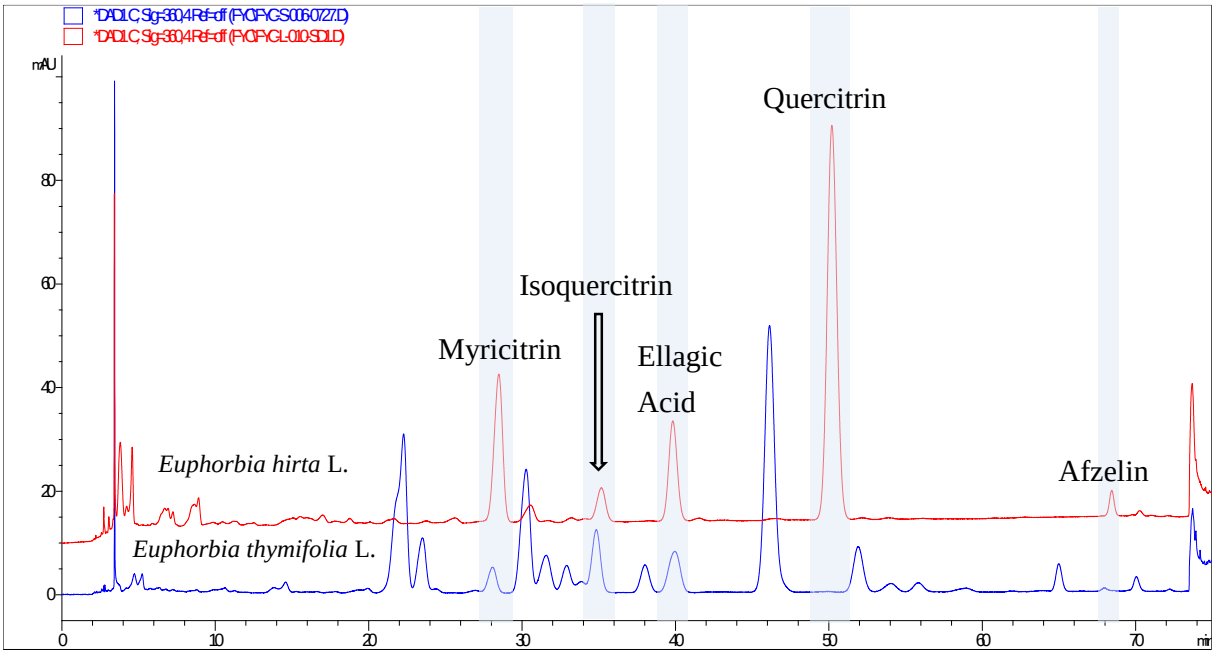


Figure 7C. Stacked HPLC Chromatogram of *Euphorbia hirta* L. and *Euphorbia thymifolia L.* extracts.

4.4.3.2. Different species under Euphorbiae genus

It was obvious that the fingerprint of each species was unique. By observing the fingerprint chromatograms of Euphorbiae genus samples, only the *Euphorbia hirta* L. contained myricitrin.

Euphorbia hirta L. and *Euphorbia humifusa* W. had a degree of similarities since they both contained quercitrin, and afzelin. While the other species were contained barely, or even undetectable content of these two compounds. Furthermore, myricitrin, isoquercitrin and ellagic acid were all detected in the two species.

The fingerprint pattern of *Euphorbia thymifolia* L. and *Euphorbia maculata* L. had a degree of similarities due to the obvious stacking peaks at about 22 min, the peak eluted at about 52 min (which is only observable in this two species), as well as the same elution pattern from 40 to 52 mins. It was deduced that they contained the similar chemical components.

The fingerprint pattern of *Euphorbia prostrata* A. was also clean. Also, it was found out that all the peaks could be detected in the other species (excluding *Euphorbia hirta* L.)

The fingerprint pattern of *Euphorbia hypericifolia* L. demonstrated its uniqueness due to the stacking peaks presented at about 25 min, a new retention time and the strong, continuous peak elution during 30 to 40 min. Also the peak eluted at about 43 min was only involved in this species.

It was also discovered that they both contained ellagic acid at about 40 min, as well as isoquercitrin at about 35 min. Ellagic acid is omnipresent in hebal plants, yet whether the prescence of isoquercitrin (or, even other compound eluted at the similar retention time) is the characteristic of the Euphorbiae genus is requied to be proven by analysing more samples.

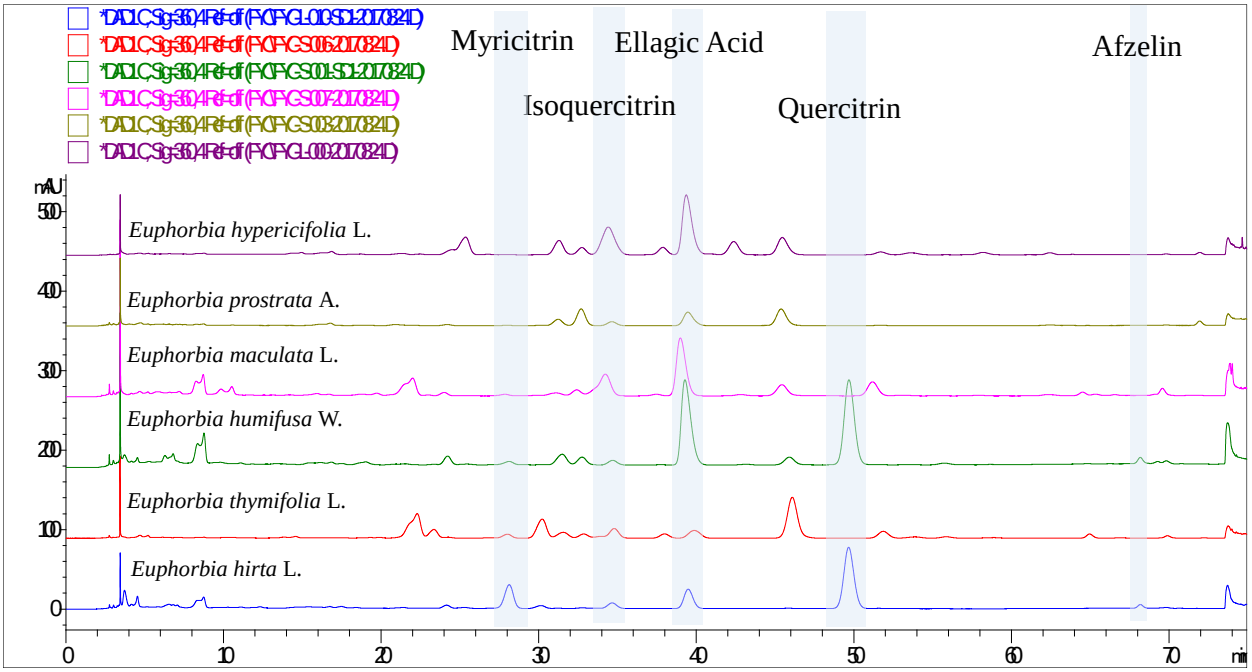


Figure 7D. Stacked HPLC Chromatograms of all Euphorbiae genus extracts collected in this study.

Table 8A. Checkbox table of the markers of the Euphorbiae genus by HPLC analysis

	Myricitrin	**Isoquercitrin**	**Ellagic Acid**	**Quercitrin**	**Afzelin**
Euphorbia hirta L.	✓	✓	✓	✓	✓
Euphorbia thymifolia L.	✓	✓	✓	×	×
Euphorbia humifusa W.	✓	✓	✓	✓	✓
Euphorbia maculata L.	✓	✓	✓	×	×
Euphorbia prostrata A.	×	✓	✓	×	×
Euphorbia hypericifolia L.	×	✓	✓	×	×

Notes: ✓ = Consists this chemical component, detectable by HPLC
× = Does not consist this chemical component

Table 8B. Conclusion of the observation in the fingerprint patterns of Euphorbiae genus by HPLC analysis, which was matched to TLC analysis

Different species under Euphorbiae genus			**Comment**
Euphorbia hirta L.	and	*Euphorbia humifusa* W.	Show similarity in pattern
Euphorbia thymifolia L.	and	*Euphorbia maculata* L.	Show similarity in pattern
Euphorbia prostrata A.			Different from other species
Euphorbia hypericifolia L.			Different from other species

4.5. HPLC Method Validation

4.5.1. Resolution (R), No. of Theoretical Plates (n) and tailing factor (T)

The no. of Theoretical Plates (n), Resolution (R) and Tailing factor (T) were obtained by injection of the 10 *Euphorbia hirta* L. batches. In each batches, those value were more than 10000, 1.5 and in between 0.9-1.1, respectively. Results were expressed as the mean of 10 batches.

Table 9A. No. of Theoretical Plates (n), Resolution (R) and Tailing factor (T) of the HPLC method using FYC-L-007

Markers	Retention time (n=10)	Half Width [min]	No. of Theoretical Plates (n)	Resolution (R)	Tailing factor (T)
Myricitrin (1)	28.1359	0.5886	12656.4	22.6325	0.9464
Isoquercitrin (2)	34.6962	0.6205	17628.6	5.5428	0.9763
Ellagic Acid (3)	39.5617	0.5797	22568.8	4.609	1.0712
Quercitrin (4)	49.776	0.7072	27449.3	9.0435	1.0796
Afzelin (5)	68.1824	0.3608	197858.7	20.2515	1.053
Criteria	--	--	≥ 10000	≥ 1.5	0.9-1.1

4.5.2. Intra-day and Inter-day Reproducibility

The relative standard deviation of retention time and peak area of Intra-day and Inter-day result, using the same extraction of *Euphorbia hirta* L. (batch no. FYC-L-007), were less than 2% and 5%, respectively. This indicated the highly reproducible of chromatograms under the established HPLC condition.

Table 9B. Intra-day and Inter-day reproducibility result using FYC-L-007

		Intra-day		Inter-day (3 consecutive days)	
Sample: FYC-L-007		Injection: = 5		Injection: 5 x 3 = 15	
	Markers	RT	PA	RT	PA
Mean	Myricitrin (1)	28.282	468.76	28.16553	468.653
	Isoquercitrin (2)	34.850	99.8	34.70647	102.14
	Ellagic Acid (3)	39.7302	98.96	39.63313	101.047
	Quercitrin (4)	50.025	1603.1	49.825	1611.57
	Afzelin (5)	68.329	39.48	68.21847	40.26
SD	Myricitrin (1)	0.131	3.577	0.134	0.110
	Isoquercitrin (2)	0.167	4.096	0.166	2.072
	Ellagic Acid (3)	0.190	1.942	0.150	1.837
	Quercitrin (4)	0.240	5.522	0.228	7.673
	Afzelin (5)	0.088	0.944	0.117	0.727
RSD	Myricitrin (1)	0.463	0.763	0.477	0.024
In 100%	Isoquercitrin (2)	0.478	4.105	0.478	2.028
	Ellagic Acid (3)	0.478	1.963	0.378	1.818
	Quercitrin (4)	0.480	0.344	0.458	0.476
	Afzelin (5)	0.129	2.392	0.172	1.807
Criteria		≤ 2%	≤ 5%	≤ 2%	≤ 5%

Note: RT = Retention Time, PA = Peak Area; SD = Standard Deviation, RSD = Relative Standard Deviation (%RSD = SD / Mean ×100%)

4.5.3. Precision of markers

The precision of markers was also investigated by injecting the known concentration standard solution. The result obtained as below. Again, the relative standard deviation of retention time and peak area of markers precision were less than 2% and 5%, respectively. This further demonstrated the reliability of system under the established HPLC condition.

Table 9C. Precision result of markers using standard solution

Sample: FYC-L-007		Injection = 6	
	Markers	RT	PA
Mean	Myricitrin (1)	28.104	462.383
	Isoquercitrin (2)	34.625	65.067
	Ellagic Acid (3)	39.573	69.717
	Quercitrin (4)	49.704	668.233
	Afzelin (5)	68.100	36.050
SD	Myricitrin (1)	0.335	6.621
	Isoquercitrin (2)	0.377	0.372
	Ellagic Acid (3)	0.360	0.331
	Quercitrin (4)	0.434	9.860
	Afzelin (5)	0.182	0.706
RSD	Myricitrin (1)	1.192	1.432
In 100%	Isoquercitrin (2)	1.088	0.572
	Ellagic Acid (3)	0.910	0.475
	Quercitrin (4)	0.873	1.475
	Afzelin (5)	0.268	1.959
Criteria		$\leq 2\%$	$\leq 5\%$

4.5.4. Recovery test

A preliminary recovery test was carried out using *Euphorbia hirta* L. (batch no. FYC-L-007) as sample.

The concentrations of sample and the spiked sample could be calculated from the standard as follow:

Table 9D. Recovery test result

Markers	Sample Conc. (μg/mL)	Spiked sample Conc. (μg/mL)	Standard Conc. (μg/mL)	%Recovery
	(injection = 3)	(injection = 3)	(injection = 6)	
Myricitrin (1)	26.5374	35.959	10	98.418
Isoquercitrin (2)	10.587	20.429	8.128	109.161
Ellagic Acid (3)	8.458	14.561	8	88.4753
Quercitrin (4)	86.576	124.169	33	103.842
Afzelin (5)	3.769	7.147	2.68	110.817

And hence, the % Recovery of each markers were calculated through the following equation

$$\% \text{ Recovery } = \frac{Concentration\ of\ spiked\ sample}{Concentration\ of\ sample + standards} \times 100\%$$

And finally obtained the value between 88 to 111%. The value was considered to be slightly deviated.

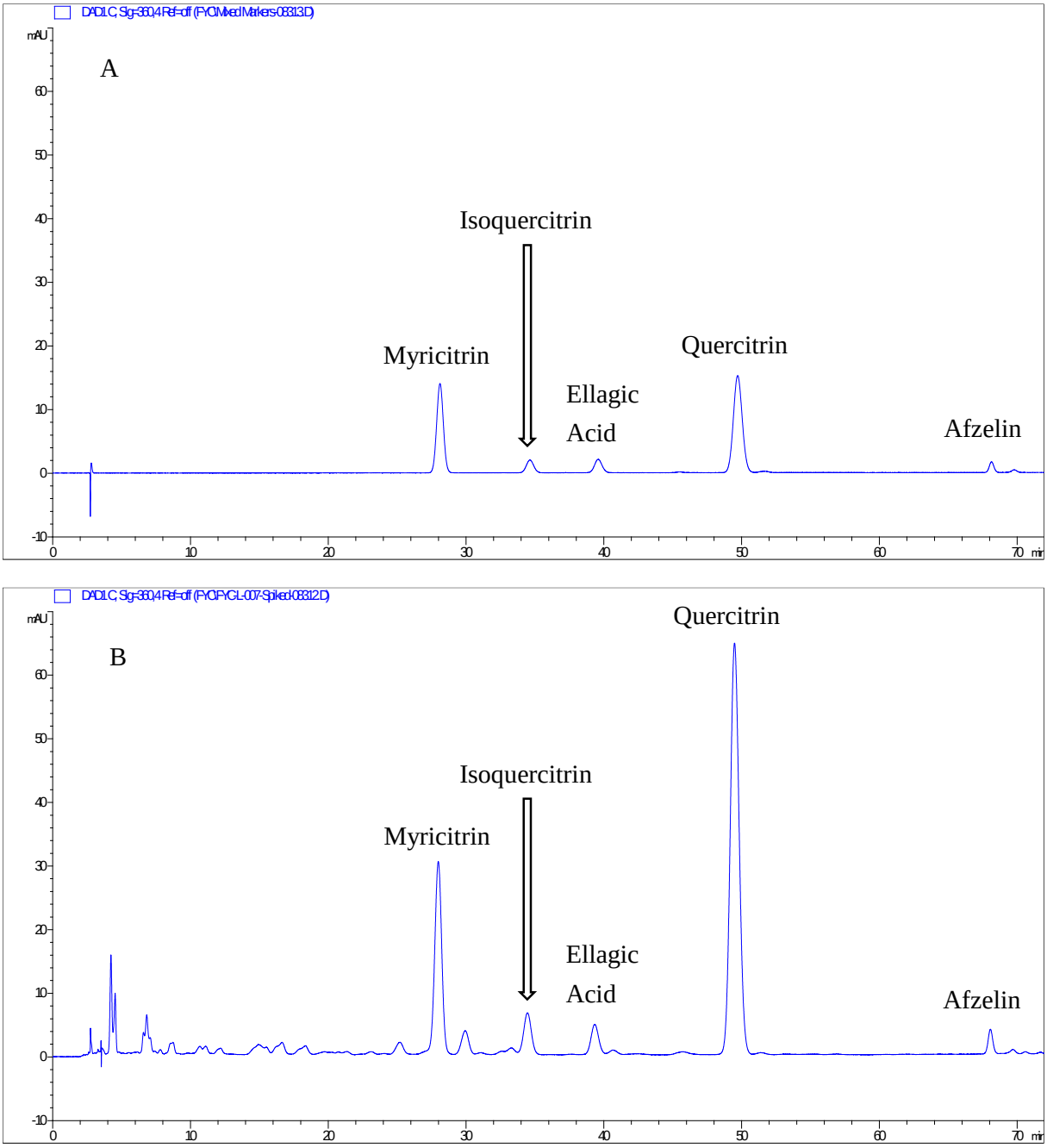


Figure 8B. HPLC chromatograms of the standard mixture of 5 markers (A) and spiked *Euphorbia hirta* L. Sample extract (batch no. FYC-L-007) (B).

4.6. Discussion of markers polarity and band separation (peaks elution)

The sequence of the elution of markers were myricitrin, isoquercitrin, ellagic acid, quercitrin and afzelin, which is the decrease in polarity according to their chemical structure. In TLC, the developing solvent was mainly made up of the dichloromethane, which is relatively less polar compared with formic acid and methanol. Hence the less polar the compound, the higher is the preference of compounds to retain in the developing solvent, and therefore the higher is the R_f value.

In HPLC, the column is packed with numerous long hydrocarbon chains with 18 C atoms, which is relatively non-polar compared with the 0.2% phosphoric acid and methanol in mobile phase. During injection, the polar components (e.g. ethanol in the sample) retain in the mobile phase and elute readily. As the polarity of mobile phase decreases (i.e. increasing the ratio of methanol), more non-polar compounds elute. The elution time is also affected by the carbon load of the column which represents the amount of C atoms in stationary phase. Therefore, the higher the carbon load of the column, the longer time is required for the whole elution process. Experimentally, peaks eluted faster in column A due to a lower carbon load. (10% and 15%, for column A and B, respectively)

4.7. Comparison between results of TLC and HPLC

In view of time, focusing on the separation, TLC analysis is much faster than HPLC analysis. Experimentally, the former one required less than 30 mins and 70 mins per injection for HPLC.

However, considering the resolution of the fingerprint patterns, it is much obvious that HPLC analysis is better than TLC analysis in the following reasons.

(1) Result Reproducibility

The result reproducibility of TLC is lower than HPLC due to several reasons.

For TLC, the separation process is carried out within a glass chamber (semi-closed system), the result (R_f value of bands) is easily affected by humidity and temperature. For example, the R_f value of quercitrin obtained through different plates during species comparison varied from 0.51 to 0.55 due to the weather change, yet how these environmental factors affect the R_f value of the bands is required for investigation.

The position of the TLC plate placing into the chamber also plays an important role. When the plate is not totally vertically placed, border effect, in particular for long plates is hardly evitable. The phenomenon is that the bands at the first line and last line (they are at the edges of the plate) elute faster and show slightly slanted, resulting a higher R_f value of bands.

Lane	Solution	R_{f1}	R_{f2}	Comment
Left	*E. hirta* L. sample	0.40	0.54	Border effect: higher R_f value of bands at the edge
Right	*E hirta* L. spiking	0.41	0.55	

Figure 9. Broder effect happened on the last lane, spiking bands of *Euphorbia hirta* L.

Whiles HPLC, the parameters such as flow rate and column temperature are well controlled by the closed-system, the result (elution of peaks) is not affected by out-coming factors.

(2) Detection limit

TLC cannot observe the biomarkers with very low content compared with the HPLC. For example, the small amount of myricitrin in *Euphorbia thymifolia* L. could be detected by HPLC, but not TLC. The presence of isoquercitrin and afzelin in *Euphorbia hirta* L., of those were limited, also could not be identified by TLC. Therefore, it explains the observation in species comparison, is mainly focused on HPLC analysis.

Furthermore, TLC is not suitable for accurate analysis of the chemical component quantitatively. It only limits to rapid qualitative analysis of the Chinese Materia Medica with high concentration of biomarkers. Other methods such as LC-MS and HPLC should be applied for quantitative studies.

Besides, there may be a chance that the species containing another compound that having the similar R_f value, (e.g. myricitrin and isoquercitrin) and same color after spraying.

4.8. Improvements

Once again, the species comparison is according to method development of *Euphorbia hirta* L. The peak resolution and purity of other euphorbia genus sample are not as high as *Euphorbia hirta* L.

As mentioned, the TLC method development was only based on two bio-markers, quercitrin and myricitrin in *Euphorbia hirta* L. A new method is required to establish in order to observe all the five bio-markers with good separation and suitable R_f range at the same time, for the purpose to examine the presence of these bio-marker preliminarily.

Furthermore, the recovery test in HPLC could be more accurate by adopting calibration curves to find out the concentration of standard bio-marker.

5. Conclusion

Method developments, both TLC and HPLC, for identification of *Euphorbia hirta* L., the raw material of Euphorbiae Hirtae Herba, have been established and modified. Those methods are then applied into Euphorbiae genus, and five biomarkers are detected successfully in both methods. In particular HPLC, the separation is satisfactory.

TLC analysis gives brief fingerprint patterns of the Euphorbiae genus samples. It supports the detailed observation in HPLC. Also, the HPLC method has been validated to testify the reproducibility of fingerprints.

Similarities and differences on patterns between Euphorbiae genus, same species and different species, have been briefly investigated, exhibiting different fingerprints in HPLC and TLC. The region of collection does not affect the fingerprint patterns

6. Suggestions for Further Studies

More experimental works, rather than using TLC and HPLC analysis, are suggested for confirming the presence of markers in Euphorbiae genus.

Just considering the fingerprint patterns, further studies can be carried out by providing a more detailed sample information. Apart from the region of collection, the growth environment (e.g. on roadside or under the bushes) may affect the constituent component in the species. The comparison of preservation methods, either air-dried or oven dried of the sample, may also spot out the difference in the components.

The bio-markers are important to reflect the medical values and the authenticity of a Chinese Materia Medica. Furthermore, the efficacies of Chinese Materia Medica are reflected by the content of certain markers, it is therefore important to carry out content determination by HPLC after the identification.

Moreover, more batches of Euphorbiae Species can be involved, in order to obtain a more precise quality on pattern study.

Last but not least, identification of more possible and specific bio-markers in Euphorbiae Species can be considered since the flavonoid and phenolic acid bio-markers are commonly existed in herbal plants. Different classes of bio-markers, for example, terpenoids and sterols are worth to be investigated.

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