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Original Article

High-Performance Thin-Layer Chromatographic Fingerprints of Flavonoids and Phenol Carboxylic Acids for Standardization of Iranian Species of the Genus *Crataegus* L.

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Abstract

Eight samples of flowering tops from six species of the genus *Crataegus* L., commonly called Hawthorn, from different geographic locations of Iran were standardized according to German Pharmacopoeia monograph on *Crataegi folium cum flore* (hawthorn leaf with flower) by high-performance thin-layer chromatographic (HPTLC) fingerprinting combining with digital scanning profiling of their major polyphenolics as flavonoids and phenol carboxylic acids (PCAs). All of the species, except one, contained rutin and hyperoside as flavonoids, and chlorogenic and caffeic acid as PCAs in their flowering tops samples. HPTLC fingerprinting, rather than conventional TLC in the DAB 1997 hawthorn monograph, gave faster and more efficient qualitative and quantitative analysis of hawthorn plant material and their extracts that was appropriate for standardization of *Crataegus* spp. The corresponding digital scanning profiling provided an easy way for quantitative comparisons among the species. The HPTLC fingerprint is also suitable for rapid and simple authentication and comparison of subtle differences among samples of identical plant resource, but from different geographic locations.

Keywords: Caffeic acid; *Crataegus*; Digital scanning profile; Hawthorn; HPTLC fingerprint.
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1. Introduction

Several species of the genus *Crataegus* L. (Hawthorn) have been reported to possess a wide range of pharmacological actions on the cardiovascular system [1]. Preparations of hawthorn including leaf, flower, and berry have been used traditionally in minor forms of coronary heart disease [2], heart failure [3] and cardiac arrhythmia [4], but the only approved monograph by German Commission E is hawthorn leaf with flower with the unique indication of decreasing cardiac output, classified by the New York Heart Association (NYHA) as stage II [5]. Also, the approved monograph of hawthorn by the German

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Pharmacopoeia (DAB 1997) is hawthorn leaf with flower (*Crataegi folium cum flore*) which consists of dried approximately 7 cm long, flowering twig tops of *C. monogyna* Jacq. or C. laevigata (Poir.) DC. (syn.: *C. oxyacantha* L.) and, more seldom, of other *Crataegus* species, such as *C. pentagyna* Waldst. et Kit. ex Willd., *C. nigra* Waldst. et Kit. and *C. azarolus* L. [6]. Hawthorn is known for its polyphenolics among which the major active constituents are: Flavonoids (1-2%) including hyperoside and rutin as flavonol-*O*-glycosides (Figure 1A); vitexin (Figure 1B), vitexin-2″-*O*-rhamnoside and acetylvitexin-2″-*O*-rhamnoside as flavone-*C*-glycosides; proanthocyanidins (1-3%) including oligomeric procyanidins (n=2 to n=8 catechins and/or epicatechins), especially dimeric procyanidin B-2 (Figure 1C); and phenol carboxylic acids (PCAs) including caffeic acid and chlorogenic acid (Figure 1D and 1E) [7]. Hawthorn species have different flavonoid compositions, and there are also qualitative and quantitative differences in the flavonoid compositions in the flowers, leaves and fruits of the same plants [8]. According to the DAB 1997 monograph, the flowering tops of the official medicinal species should contain at least 0.7% flavonoids, calculated as hyperoside, determined by the aluminum chloride spectrophotometric method, and major polyphenolics of rutin and hyperoside as flavonoids and chlorogenic acid and caffeic acid as PCAs characterized by a thin-layer chromatography (TLC) method [6].

In the present study, Iranian *Crataegus* spp. (Table 1) were studied analytically to evaluate the presence of two requirements mentioned by DAB 1997 in order to standardize them as official medicinal species. All of the species studied, except *C. pseudoheterophylla*, contained flavonoid content equal to or greater than 0.7 percent in flowering tops (Table 2). Also, all of the leaf samples, except leaves of *C. pseudoheterophylla*, and only the flowers of *C. curvicepala* and *C. pseudoheterophylla* contained flavonoid contents greater than 0.7% (Table 2). High-performance thin-layer chromatographic (HPTLC) fingerprinting combining digital scanning profiling was developed to identify the major polyphenolics as flavonoids and PCAs in flowering tops, leaves and flowers samples, rather than conventional TLC as a standard pharmacopoeia method.

Although the principles of TLC and HPTLC methods are identical, because of the use of kinetically optimized fine-particle layers in HPTLC, separation is faster and more efficient and the results are more reliable and reproducible. In combination with digital scanning profiling, HPTLC also provides accurate and precise R_F values and quantitative analysis of sample constituents by *in situ* scanning densitometry aided by the formation of easily detected derivatives by post-chromatographic chemical reactions as required, as well as a record of the separation in the form of a chromatogram with fractions represented as peaks with defined parameters including absorbance (intensity), R_F, height and area [9]. Furthermore, the feature of a pictorial fluorescence image of HPTLC coupled with a digital scanning profile is more and more attractive to herbal analysts for constructing a herbal chromatographic

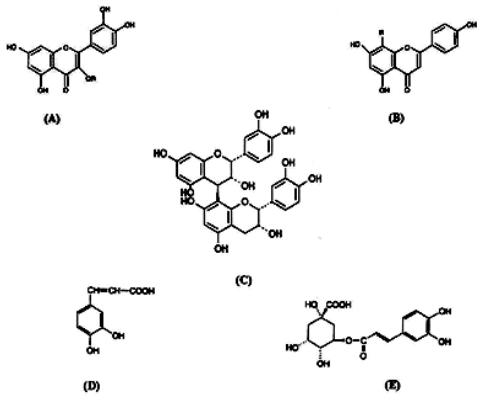

Figure 1. (A): Flavonol-*O*-glycoside: hyperoside (R=β-D-galactosyl) and rutin (R=β-D-rutinosyl); (B): Flavone-*C*-glycoside: Vitexin (R=β-D-glucosyl); (C): Procyanidin B-2; (D): Caffeic acid and (E): Chlorogenic acid (3-*O*-caffeoyl quinic acid).

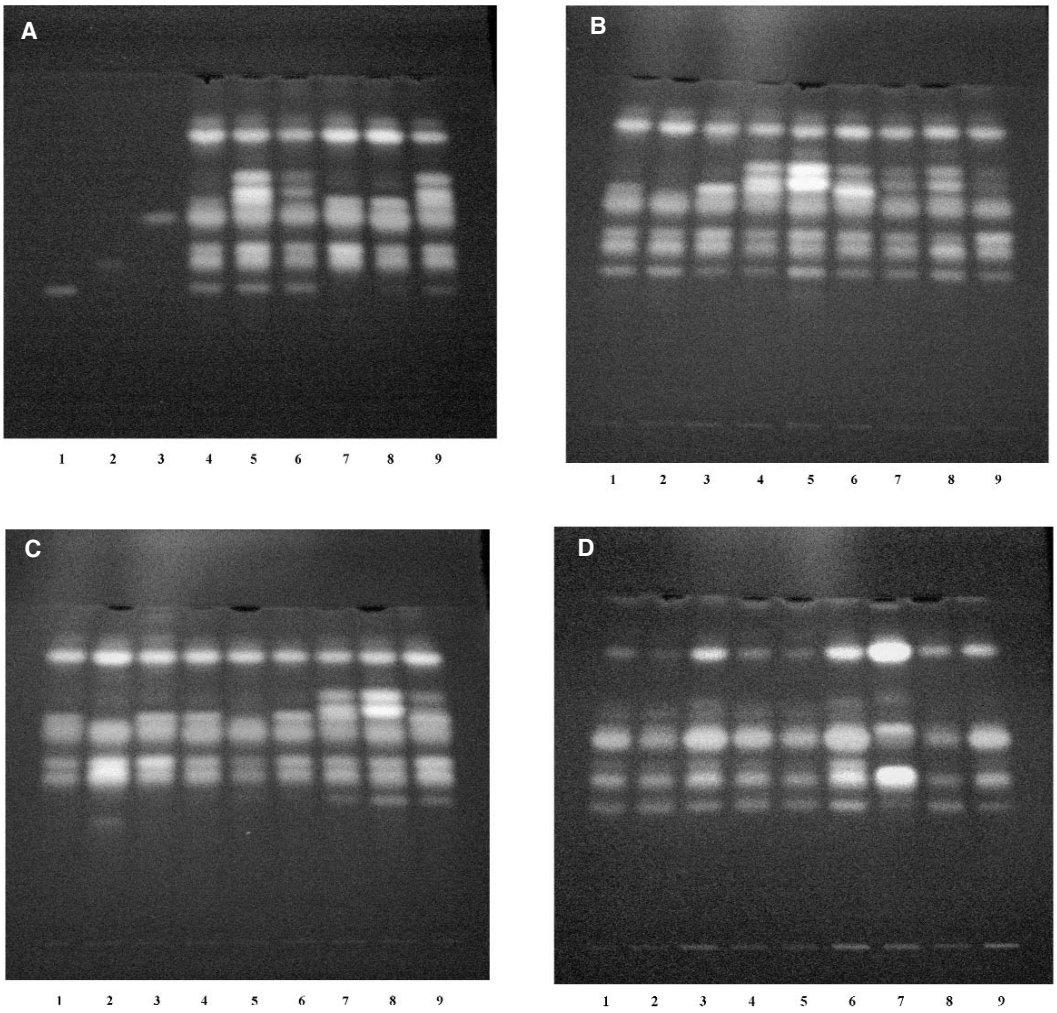

Figure 2. HPTLC fluorescence images under the excitation wavelength 366 nm of flowering tops (FT), leaves (L) and flowers (F) samples of *Crataegus* spp. **(A)**: tracks 1-3 represents of reference compounds: rutin (1), chlorogenic acid (2), hyperoside (3); tracks 4-11 represents of FT samples of *Crataegus* spp.: *C. atrosanguinea* (4), *C. pentagyna* (f) (5), *C. meyer*i (f) (6), *C. pentagyna* (g) (7), *C. curvicepala* (8), *C. meyeri* (g) (9). **(B)**: 1: FT, 2: L and 3: F of *C. atrosanguinea*; 4: FT, 5: L and 6: F of *C. pentagyna* (f); 7: FT, 8: L and 9: F of *C. meyeri* (f). **(C)**: 1: FT, 2: L and 3: F of *C. pentagyna* (g); 4: FT, 5: L and 6: F of *C. curvicepala*; 7: FT, 8: L and 9: F of *C. meyeri* (g). **(D)**: 1: FT, 2: L and 3: F of *C. pseudoheterophylla*; 4: FT, 5: L and 6: F of *C. pseudoheterophylla* (two identical species from one location but from diffrent source); 7: FT, 8: L and 9: F of *C. microphylla*.

fingerprint by means of HPTLC [10]. The main objective of this study was to evaluate and optimize the HPTLC fingerprint method in standardization of *Crataegus* spp. These HPTLC fluorescence images coupled with the scanning profiles provided adequate information and parameters for comprehensive identification, assessment and comparison of major active constituent fingerprints in the samples studied to serve as a basis for their use in medicinal preparations for cardiovascular diseases.

2. Materials and methods

2.1. Materials

Eight flowering tops samples from six species of the genus *Crataegus* were collected from the north, north-west and west of Iran on May 2006 (At first, two flowering tops samples were collected from Sanandaj,

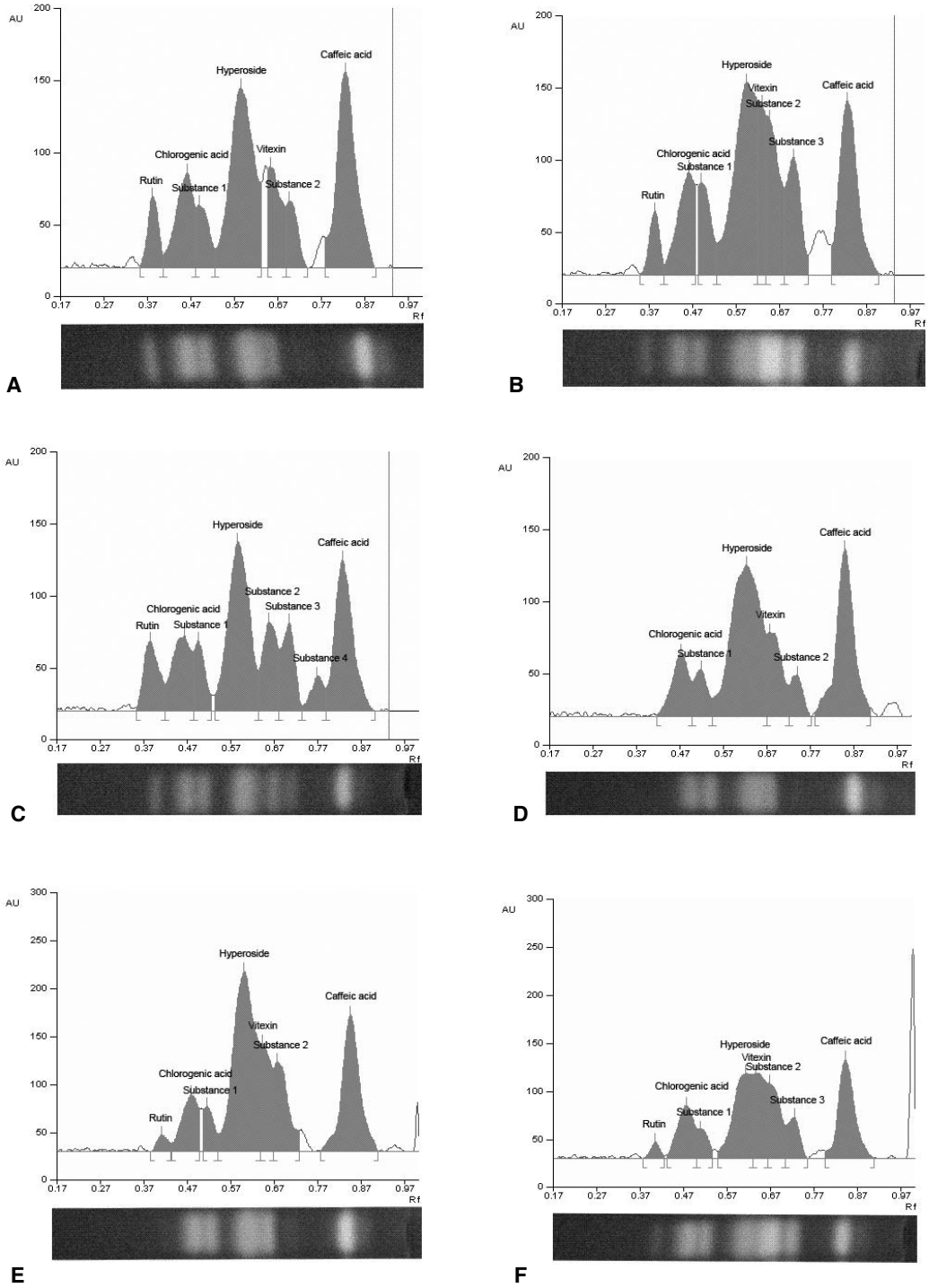

Figure 3. Typical HPTLC images and corresponding digital scanning profiles of flowering tops samples of *Crataegus* spp. **(A)**: *C. atrosanguinea*; **(B)**: *C. pentagyna* (f); **(C)**: *C. meyeri* (f); **(D)**: *C. pentagyna* (g); **(E)**: *C. curvicepala*; **(F)**: *C. meyeri* (g); **(G)**: *C. pseudoheterophylla* and **(H)**: *C. microphylla*.

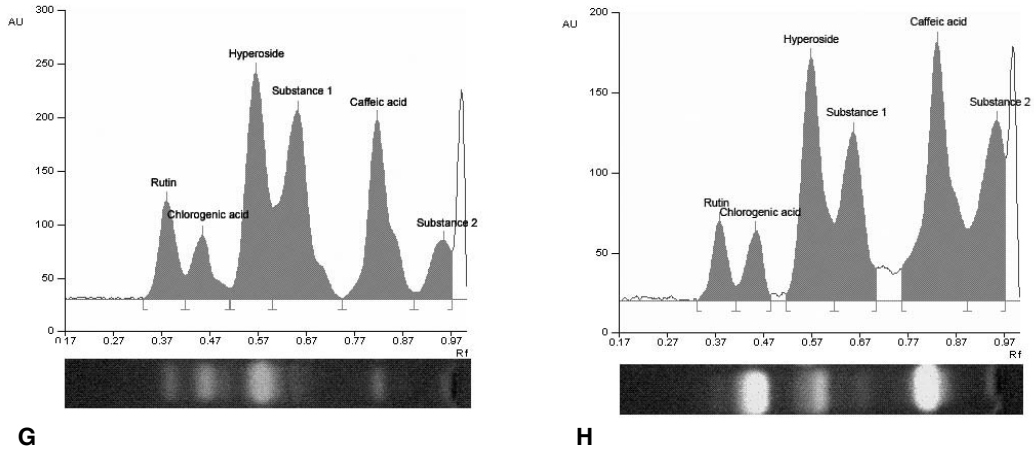

Figure 3. Continued

Kordestan, Iran and then authenticated as *C. pseudoheterophylla* and the HPTLC chromatograms of them (Figure 1D) were shown to be identical. Therefore, one of the samples was studied) (Table 1). In each sample, flowering tops and separated leaves and flowers were dried at the room temperature at a relative humidity of 40%. All samples were authenticated by Dr. Gh. Amin (Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran, where voucher specimens are deposited) according to their morphological characteristics. Rutin, hyperoside and chlorogenic acid were used as reference substances, diphenylboryloxyethylamine (Natural Product, NP) and polyethylene glycol-4000 (PEG-4000) as spray reagents. Solvents of analytical grade were purchased from Merck (Merck, Darmstadt, Germany).

2.2. Preparation of reference solution

Reference solutions were: rutin, hyperoside (2.5 mg of each separately) and chlorogenic acid (1 mg) were dissolved in methanol (10 ml).

2.3. Preparation of sample solution

Powdered samples (1 g of flowering tops, leaves, or flowers, separately) were extracted with methanol (10 ml) for 5 min. on a water bath at about 60 °C and then filtered. A total of the 5 ml of the methanolic extract was concentrated to about 2 ml under vacuum; water (1 ml) and ethyl acetate (10 ml) were added and shaken several times. The ethyl acetate phase was separated and reduced in volume (to 1 ml) under vacuum.

2.4. Chromatography

Aliquots (2 µl) of the reference and sample

Table 1. A summary of tested samples.

Sample	Collected location	Herbarium no.
C. atrosanguinea Pojark.	Fandogh-Loo, Ardebil *, Iran	6664-TEH
C. pentagyna Willd.	Fandogh-Loo, Ardebil, Iran	6659-TEH
C. meyeri Pojark.	Fandogh-Loo, Ardebil, Iran	6660-TEH
C. pentagyna Willd.	Gardane-Heyran, Ardebil, Iran	6663-TEH
C. curvicepala Lindman.	Gardane-Heyran, Ardebil, Iran	6661-TEH
C. meyeri Pojark.	Gardane-Heyran, Ardebil, Iran	6662-TEH
C. pseudoheterophylla Pojark.	Sanandaj, Kordestan **, Iran	6658-TEH
C. microphylla Koch	Hashtpar, Gilan ***, Iran	6657-TEH

Note: * North-West of Iran; ** West of Iran; *** North of Iran

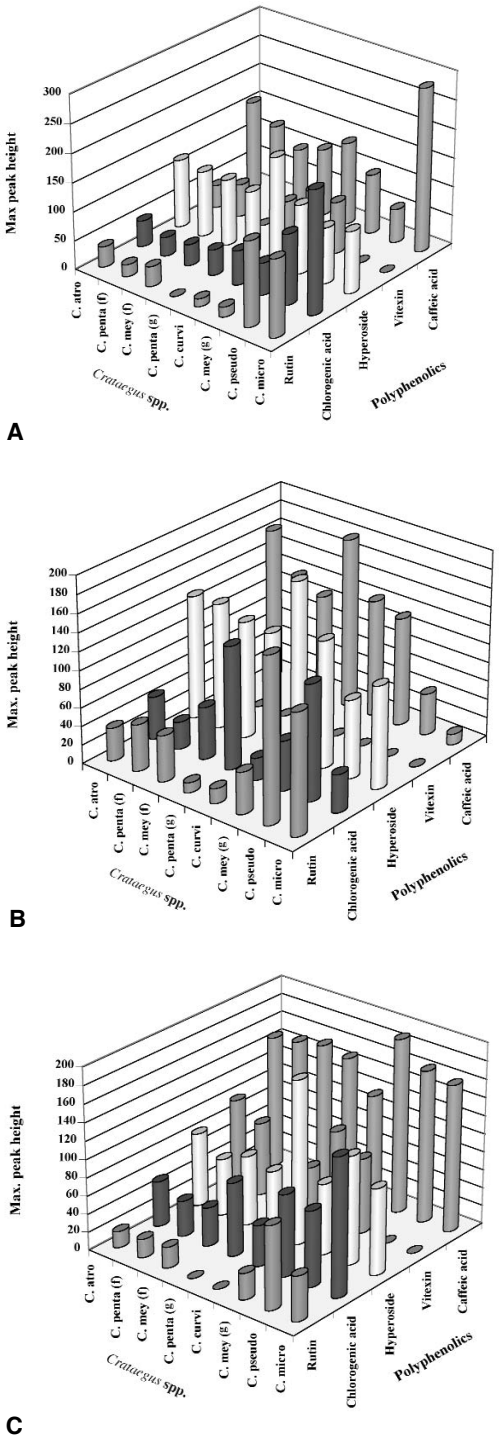

Figure 4. The quantifiable comparison of 3D graphs of HPTLC fingerprints of flowering tops **(A)**, leaves **(B)**, and flowers **(C)** samples of *Crataegus* spp.

solutions were applied separately bandwise by Camag Linomat IV (Camag, Muttenz, Switzerland) with parameters of bandlength: 6 mm, track distance: 3 mm, distance from left edge: 11 mm and low edge: 8 mm, and delivery speed: 15 sec/µl to silica gel 60 F_{254}-precoated HPTLC plates, 10×10 cm (Merck, Darmstadt, Germany). After saturation for 20 min. with the mobile phase vapor, the plates were developed horizontally in Camag horizontal developing chamber (10×10 cm) for 80 mm at the room temperature, i.e. 18-22 °C with ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:26, v/v) as the mobile phase. After heating the plate at 100 °C for 5 min., derivatization of the chromatogram was performed by Camag glass reagent spray by spraying still-hot plate with 1% methanolic NP, followed by 5% methanolic PEG-4000. The plate was observed after 30 min. under UV-366 nm light in Camag UV cabinet and the HPTLC fluorescence image documented. The corresponding digital scanning profiling was carried out with a Camag TLC scanner 3 fitted with winCATS V1.2.3 software at a single-wavelength UV-366 nm. Documentation of chromatograms was carried out with a Sony digital camera, 5 Mpixel (Japan).

3. Results and discussion

3.1. Interpretation of HPTLC fingerprint chromatograms

The HPTLC images shown in Figure 2 indicate that all reference substances and sample constituents were clearly separated without any tailing and diffuseness. The R_F values and fluorescence colors of the reference substances according to Wagner's Atlas of thin-layer chromatography are: Orange fluorescence band with R_F 0.3-0.4 as rutin, blue fluorescence band with R_F 0.4-0.5 as chlorogenic acid, orange fluorescence band with R_F 0.5-0.6 as hyperoside, yellow-green fluorescence band with R_F 0.6-0.7 as vitexin and blue fluorescence band with RF 0.8-0.9 as caffeic acid [11]. By comparison of sample

constituents with reference substances on the same plate, constituents of sample extracts were identified. As seen in Figure 3, all of the flowering tops samples contained rutin, hyperoside, chlorogenic acid and caffeic acid, except of *C. pentagyna* (g) in which no rutin was detected on the chromatogram and digital scanning profile (Figures 2C and 3D). Also, all of the leaf and flower samples, except for flowers of *C. pentagyna* (g) and *C. curvicepala*, contained four major polyphenolics (Figure 2). Rutin in flowering tops and leaf samples of *C. curvicepala* were hardly detected on the chromatogram (Figure 2C), related to the low concentration in the sample extract, but the corresponding peak was detected on digital scanning profiles (Figure 3E). The fluorescence band corresponding to hyperoside in the flower sample of *C. pseudoheterophylla* showed much great intensity than the corresponding band in flowering top and leaf samples (Figure 2D) related to much higher concentration as shown by quantitative data in Table 2. However, in other samples, the hyperoside percentages in flower samples were much less than in flowering tops and leaf samples, except for *C. curvicepala* (Table 2). The highest intensity corresponding to the hyperoside band was detected in samples of *C. curvicepala* (Figure 2C) supported by quantitative data in Table 2 (about 1.2%). The highest intensity for chlorogenic acid and caffeic acid bands was detected in the flowering top sample of *C. microphylla* corresponding to their high concentrations in the sample extract (Figure 2D). Vitexin was only detected in flowers and because of that in flowering tops samples (Figures 2A, B and C). The yellow fluorescence bands at greater R_F than the hyperoside band were related to other flavonoids and blue fluorescence bands at greater R_F than the chlorogenic acid and caffeic acid bands were related to other phenol carboxylic acids (Figure 2).

3.2. Interpretation of digital scanning profiles of HPTLC fingerprint chromatograms

Figure 3 shows HPTLC fluorescence images coupled with digital scanning profiles of flowering top samples of the species studied. Every fraction in each track is represented as a specific peak with defined values of absorbance, R_F, height and area. By digital scanning of the chromatogram, the trace quantity of fractions was readily determined as peaks, whereas could not be visually detected on the plate (rutin on Figures 2C and 3E). Since the digital scanning profile was intuitively converted from the HPTLC image, all the peak intensities were in accord with fluorescence bands and their brightness. Thus, it can be easily evaluated by comparison of the polyphenolic content of samples by quantitative comparison of the peak intensities (maximum peak heights) (Figure 4). The hyperoside peak-to-peak ratios expressed by the fingerprint patterns and their integrated peak area values obtained from digital scanning profiling of sample chromatograms (Figure 5) underscored the precision of

Table 2. Flavonoid content calculated as hyperoside (g/100g dry matter).

Sample	Flowering tops (g %)	Leaves (g %)	Flowers (g %)
C. atrosanguinea	0.77	0.87	0.53
C. pentagyna (f)	0.74	0.88	0.41
C. meyeri (f)	0.75	0.82	0.50
C. pentagyna (g)	0.72	0.81	0.46
C. curvicepala	1.21	1.23	1.18
C. meyeri (g)	0.78	0.89	0.51
C. pseudoheterophylla	0.63	0.55	0.78
C. microphylla	0.70	0.72	0.62

Note: (f): Fandogh-Loo; (g): Gardane-Heyran

quantitative data obtained by the spectrophotometric method (Table 2).

4. Conclusions

Although TLC is a conventional method used generally in pharmacopoeias as one of the standardization methods, the HPTLC method is more practical. HPTLC is feasible for development of chromatographic fingerprints to determine major active constituents of medicinal plants. The separation and resolution are much better, and the results are much more reliable and reproducible than TLC. Combined with digital scanning profiling, it has the main advantage of *in situ* quantitative measurement by scanning densitometry. Furthermore, the colorful pictorial HPTLC image provides extra, intuitive visible color and/or fluorescence parameters for parallel assessment on the same plate. In the present study, the proposed HPTLC fingerprint method combined with digital scanning profiling was used for standardization. In conclusion, the results obtained from flavonoid content determination (Table 2), qualitative evaluation of HPTLC fingerprint images (Figures 2 and 3) and quantitative comparison of hyperoside peak areas (Figure 5) of flowering tops samples of species studied showed that according to the DAB 1997 monograph on hawthorn leaf with flower, *C. atrosanguinea*, *C. pentagyna* (f), *C. meyeri* (f, g), *C. curvicepala* and *C. microphylla* contained two Pharmacopoeial requirements for standardization and can be introduced as new official medicinal species for the preparation of effective herbal cardiovascular medicines. *C. curvicepala* is of special note with the highest hyperoside content (about 1.2%) which means that it could serve as a suitable alternative to the conventional official species, i.e. *C. monogyna* and *C. laevigata*. Also, all of the leaf samples, except for *C. pseudoheterophylla*, and the flowers of *C. pseudoheterophylla* met the two Pharmacopoeial requirements for standardization and can be used as medicinal parts.

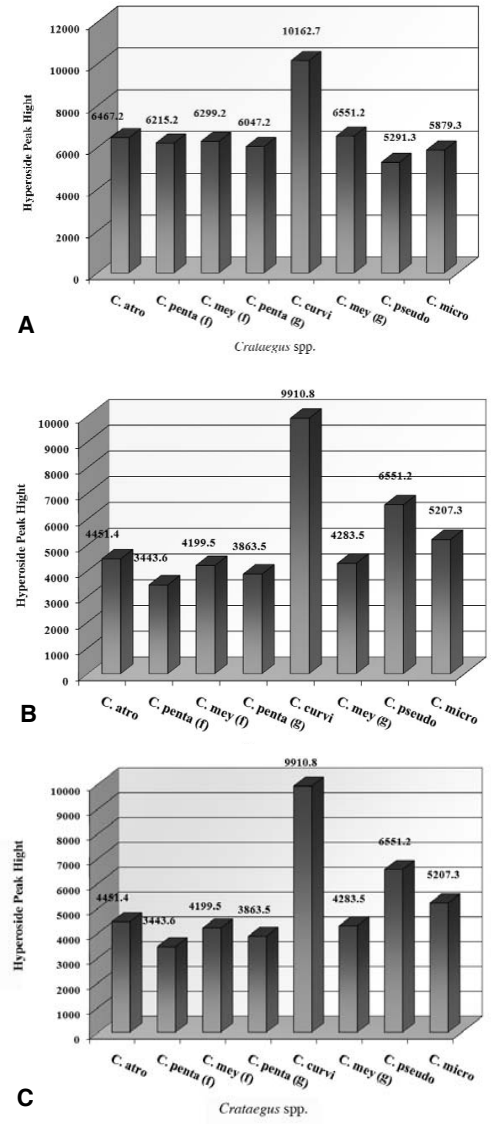

Figure 5. Comparison of hyperoside integrated max peak area values of flowering tops (A), leaves (B), and flowers (C) samples between *Crataegus* spp. HPTLC fingerprints.

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