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FINGERPRINT STUDY AND QUANTIFICATION OF BIOACTIVE GENISTEIN GLYCOSIDE IN *DERRIS SCANDENS* STEM AND POLYHERBAL FORMULATIONS BY TLC-IMAGE ANALYSIS

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ABSTRACT

The simple and efficient thin-layer chromatographic (TLC)–image analysis method was applied to the fingerprint study and quantitative determination of an active ingredient, genistein-7-O-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside, in the dried powder of *Derris scandens* stem and its polyherbal formulations. The fingerprint analysis was performed on silica gel 60 F_{254} TLC plates with the use of various solvent systems and detections; whereas, the quantitation of the active compound was achieved by using the previously reported TLC-image analysis method processed with two types of analysis software, UN-SCAN-IT and ImageJ. Evaluating the similarity between different samples could be clearly acquired from the fingerprints constructed from stored TLC images. The profile plots of all images generated by using the TLC-image analysis software was also useful for quantifying the amount of the active component in the complex polyherbal formulations. The resulting amount obtained from the TLC–image analysis method was found to be comparable to the ones obtained from the TLC-densitometric and HPLC methods, and the content of GTG found in different samples was significantly different and ranged from 0.01 to 2.2 %w/w.

Keywords: TLC-image analysis, *Derris scandens*, polyherbal formulation, fingerprint, quantification, genistein-7-O-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside

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Introduction

Derris scandens (Roxb.) Benth. (family Fabaceae), commonly known in Thai as *Thao-Wan-Priang,* has been in the List of Herbal Medicinal Products in the Thailand National List of Essential Medicines for musculoskeletal pain treatment.[1] The use of *D. scandens* as a traditional medicine is found in a bolus formulation combined with other herbal plants and as an herbal drug in a capsule formulation containing *D. scandens* dried powder or 50% hydroethanolic extract with or without other herbal components. Due to several reports on its efficacy and safety for the treatment of muscle ache and knee osteoarthritis[2-6] and the policy of the Thai Ministry of Public Health to promote the use of Thai herbs, *D. scandens* herbal formulation is widely prescribed for the treatment of musculoskeletal pain syndrome in a hospital. The current data on the HDC TTM Service website, the management information system (MIS), provided by the Department of Thai Traditional and Alternative medicine, show that the herbal drug usage value of *D. scandens* capsules is in the fifth rank.[7]

Due to increasing public interest, various herbal and polyherbal formulations containing *D. scandens* were produced and marketed by different manufacturers which could result in an inconsistency in the herbal product's quality. The study of product quality, in terms of fingerprint analysis and quantitation of the active component is, therefore, regarded as one of the important processes to gain useful information for standards and quality assurance of herbal drugs.[8,9]

The commercially available *D. scandens* formulation was mostly found in the polyherbal capsule dosage form claiming to contain *D. scandens* as a major active herbal ingredient. Genistein-7-*O*-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside (GTG) (Figure 1) has been reported to be a crucial phytoconstituent present in *D. scandens* and shown to possess anti-inflammatory activity.[10] In the Thai Herbal Pharmacopoeia, the assay of *D. scandens* dry extract and the capsule formulation requires the HPLC method to determine the GTG content, calculated as genistein, by using commercially available genistein as the reference standard. For the identification of raw material, the TLC method is employed.[11]

TLC has been regarded as one of the most useful techniques for analysis of medicinal plants. The fingerprint profile generated by TLC has been commonly used for ensuring identity and consistency of herbal drugs, especially in complex herbal material and polyherbal preparations.[12-14] The quantification of active constituent can also be achieved by using TLC. The TLC method for determination of GTG in *D. scandens* was developed and validated in our previous study.[15] Furthermore, with the help of the image analysis technique,[16-18] the TLC method provided more major advantages, as compared to HPLC, including simplicity, a low operating cost and the possibility to obtain digital data and images of

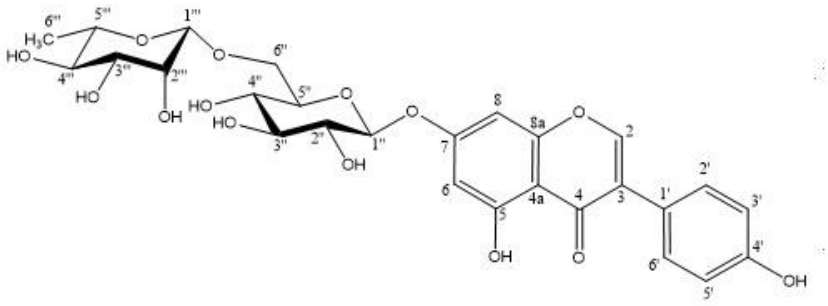

Figure 1 Genistein-7-O-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside (GTG)

numerous TLC fingerprints. Therefore, the present study focused on the fingerprint analysis and quantitative determination of the active compound, GTG, in *D. scandens* and in commercial polyherbal formulations claiming to contain *D. scandens* as the major component, by using the simple TLC-image analysis method. The GTG contents determined by the TLC-image analysis were also compared with those analyzed by the TLC-densitometric and HPLC methods to ensure the application of TLC-image analysis as an alternative and appropriate tool for the quality control of herbal drugs.

Materials and methods

Standards and samples

GTG (purity [HPLC] >98%) was isolated from *D. scandens* as described in a previous report.[15] Genistein (purity [HPLC] >98%) (Sigma-Aldrich; St. Louis, MO, USA) was purchased. All chemicals and solvents used were analytical reagent grade. A mixture of standard solution at 0.004–0.12 mg/mL of genistein and 0.008–0.25 mg/mL of GTG in 50% aqueous ethanol was prepared.

Ten samples were used in this study. DS1, *D. scandens* dry extract was prepared according to THP2018[11] by extraction of the dried powder of *D. scandens* stem with ethanol (50 per cent) and lyophilization. DS2, *D. scandens* dried powdered stem and DS3-DS10 polyherbal formulations, claiming to contain *D. scandens* as the major component, were purchased from a local herbal drugstore in Bangkok, Thailand. DS3-DS9 were in capsule formulation and DS10 was in bolus dosage form.

The preparation for sample analysis was modified from the method described previously.[19] The aliquot amount of the herbal powder taken from 20 capsules and 20 ground boluses was used for the sample preparation of DS3-DS10. The following samples, DS1 (0.2 g), DS2 (1 g), DS3-DS9 (the aliquot amount equivalent to 1 g of *D. scandens* calculated from the claimed label of each sample) and DS10 (4 g), were sonicated in 40 mL of 50% aqueous ethanol at room temperature for 30 min. Then, all sonicated samples were centrifuged at 3000 rpm for 5 min. The clear sample solution was subjected to the TLC and HPLC analysis. However, for HPLC, each sample solution was filtered through a 0.45 µm Nylon-66 filter (Chrom Tech Inc., USA) before injection.

Chromatographic conditions for fingerprint analysis

The analysis was performed on TLC silica gel 60 F_{254} aluminium plates (10 cm x 20 cm with 0.25 mm thickness, Merck, Germany). Five µL of each solution were manually applied as a 5 mm band onto a TLC plate using the TLC sampler Nanomat 4 (Camag, Switzerland). A distance between bands, from the side edge and from the bottom edge, were 15, 15 and 12 mm, respectively. The plate was immersed into a TLC chamber containing the mobile phase and developed to a distance of 9.8 cm from the bottom edge. Four different mobile phases, ethyl acetate-acetic acid-formic acid-water (10:1.1:1.1:2.4, v/v), ethyl acetate-methanol-water (8:1.3:1, v/v), *n*-hexane-ethyl acetate (6:4, v/v), and toluene-ethyl acetate (93:7, v/v) were used. After being completely developed, the plate was air-dried at room temperature and, then, visualized under UV light at 254 nm, 366 nm, and derivatized with Fast Blue B salt 0.5% (w/v) and anisaldehyde. For the derivatization of the TLC plate with the spray reagents, the plate sprayed with a solution of Fast Blue B until wet was placed over an ammonia vapor chamber (25% ammonia solution), whereas the wet sprayed plate with a solution of anisaldehyde was heated at 110ºC until spots developed under visible light. All images were captured by using a CAMAG TLC visualizer 2 and stored as JPEG files for further image processing.

Chromatographic conditions for quantification of GTG

The TLC condition for determination of GTG in DS1-DS10 was performed according to the previously reported method[15] by using a TLC silica gel 60 F_{254} aluminum plate (20 cm × 10 cm with 0.25 mm thickness) and a multiple development technique. Ethyl acetate–methanol–water (8:1.3:1, v/v) and *n*-hexane–ethyl acetate (6:4, v/v) was used as the first and the second mobile phase, respectively. A total of 12 bands, including 6 replicates of each sample solution and a mixture of standard solution at 6 concentrations, ranging from 0.004–0.12 mg/mL for genistein and from 0.008–0.25 mg/mL for GTG, were each applied at 5 µL and as 5-mm bands onto the TLC plate using the TLC sampler Nanomat 4. The distance between each band was 1.5 cm. The plate was developed to a distance of 5 cm from the origin in a TLC chamber previously saturated with the first mobile phase, ethyl acetate–methanol–water (8:1.3:1, v/v), and then it was removed from the tank and left to stand until dry. The air-dried TLC plate was developed in the second mobile phase, *n*-hexane–ethyl acetate (6:4, v/v), to a distance of 8.6 cm from the origin.

The developed plate was subjected to the TLC-densitometric method in the absorbance mode at 260 nm and TLC-image analysis by using the commercial software, UN-SCAN-IT (Silk scientific, USA) as previously described in detail.[15] In addition, a free and open platform, ImageJ (NIH, USA),[20] was also used for TLC-image analysis in this study as follows. The digital file of JPEG image at a resolution of 1000 pixels was opened by ImageJ. The rectangular selection tool was used to outline the desired track on the TLC plate. A lane box was drawn for each track and set with the same equal width and height for every lane at 65 × 410. A chromatogram was then generated using the "Plot Lanes" option and followed by the "Straight Line Selection Tool" to draw baselines under the peak of interest. The area under the peak in the form of total pixel numbers was measured for quantification by using the "Wand Tool."

The quantitative TLC image analysis method using ImageJ was also validated in this study according to the International Conference on Harmonization (ICH) guidelines[21] in terms of specificity, sensitivities (limit of detection [LOD] and limit of quantification [LOQ]), repeatability (intra-day precision) and the intermediate (inter-day) precision, accuracy and robustness as previously described.[15]

The content of GTG in each sample was determined and calculated from the polynomial regression equation obtained from a calibration plot between the peak area and the standard solution at the above-mentioned concentration range. The amount of GTG in DS1-DS10 was reported in terms of %w/w of GTG (calculated as GTG), A and B, when using GTG and genistein as a reference standard, respectively. The %w/w of GTG was calculated based on the weight of each sample used in the above sample preparation protocol.

The contents (%w/w) of GTG in DS1-DS10 were also analyzed by using the HPLC method as described previously.[15]

Statistical comparison

ANOVA: two-factor with replication was performed at a 95% confidence level to compare the amount (%w/w) of GTG in DS1-DS10 as determined by different methods, i.e., the TLC–image analysis method processed by UN-SCAN-IT and ImageJ, the TLC–densitometry, and the HPLC method, using GTG and genistein as reference standards. Statistical analysis was performed by using Excel 2016 (Microsoft, USA).

Results and Discussion

Owing to the complexity of herbal material, usually consisting of various classes of chemical constituents with low to high polarity, TLC fingerprints of *D. scandens* and polyherbal samples were obtained by using several solvent systems and multiple detections. With the help of an image

capturing device and the software developed for TLC image analysis, similarities and differences between samples could be simply made and compared from the visualized images of numerous fingerprints, while the chromatographic line profiles of the TLC images were also straightforwardly generated by the software.

For fingerprint analysis, when the solvent systems with greater strength, e.g., ethyl actetate-acetic acid-formic acid-water (10:1.1:1.1:2.4, v/v) (Figure 2-1) and ethyl acetate-methanol-water (8:1.3:1, v/v) (Figure 2-2), were employed, the band of GTG, at the R_F values of 0.50 and 0.52, respectively, was clearly seen on the TLC plates under 254 nm in all samples except the lowest intensity observed in DS10. However, genistein was moved to the solvent front due to having a much lower polarity when compared to GTG. The UV-absorbing property of GTG at 254 nm was due to the aglycone part, genistein, thus appearing as a dark-colored band and as a light-orange band with fast blue B.

When using less polar mobile phases, e.g., *n*-hexane-EtOAc (6:4, v/v) (Figure 2-3) and toluene-ethyl acetate (93:7, v/v) (Figure 2-4), GTG did not move from the origin while the band of genistein, at the R_F values of 0.38 and 0.05, respectively, was developed and observed clearly in the standard tracks (tracks 1 and 12); though, very little was detected in the samples.

By using various solvent systems and detections, information confirming similarity/ dissimilarity between samples could be obtained in more supportive details from side-by-side visual inspection of TLC fingerprinting images. Furthermore, the chemical pattern of each sample could be compared from the line profile plots of each chromatogram generated by using TLC image analysis software. For example, a line plot of each track was constructed from processing the TLC images taken under UV254 nm of Figures 2-2 and 2-3 by UN-SCAN-IT (Figure 3). The line profile showed that the chromatographic patterns of DS1-DS10 were quite similar and GTG was observed as a major peak in all samples except in DS10 (Figure 3 left). The profile plots of all images could be easily generated by using the TLC-image analysis software and was found to be useful for overall comparisons.

The visual information retrieved from the TLC fingerprints and the chromatographic profiles strongly supported that one of the herbal materials contained in the polyherbal formulations, DS3-DS10, was in fact *D. scandens*. Similar chromatographic profiles to the ones of *D. scandens* dry extract (DS1) and bulk drug (DS2), especially with respect to sequences, positions and colors of separated bands, were observed in the TLC fingerprints of all polyherbal samples. However, when comparing the intensity of the GTG band shown in the fingerprints of DS2-DS9, the intensities varied, thus, resulting in a range of GTG quantities present in each formulation.

Beside the use of the TLC-image for fingerprint analysis, its application was extended to quantify the amount of GTG in these formulations. The previously reported TLC-densitometric and image analysis methods by UN-SCAN-IT were used for the quantification.[15] The image analysis method using the free and open platform, ImageJ, was also attempted and validated in this study. The calibration curves showed good polynomial regression (R^2 > 0.99) between the peak area and the concentration. Limit of sensitivities (LOD and LOQ), precision, accuracy (recovery) and robustness of the TLC method by ImageJ are presented in Table 1 and were comparable to those obtained from the method using UN-SCAN-IT but were less sensitive and precise than the TLC–densitometric method.[15]

The TLC-densitometric and image analysis methods by UN-SCAN-IT and ImageJ were applied to determine the amount of GTG in DS1-DS10 by using GTG and genistein as reference standards. Unlike GTG, genistein was commercially available and easily obtained, and the results from the previous study also suggested that genistein was suitable as the reference standard for determination of the GTG

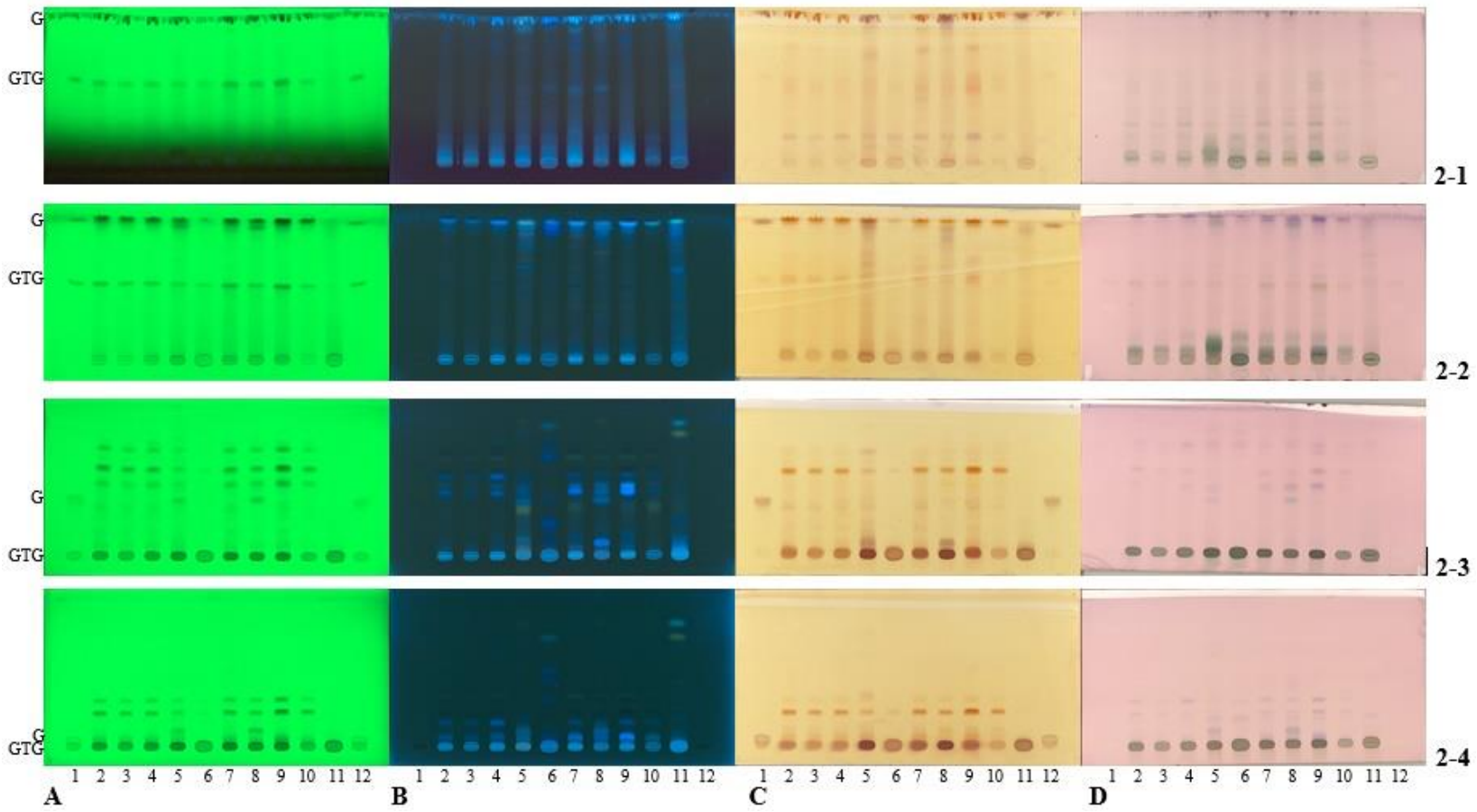

Figure 2 TLC fingerprints of a mixture of genistein and GTG (tracks 1 and 12) and DS1-10 (tracks 2-11), respectively, on silica gel 60 F_{254} aluminum plates using four different mobile phases. 2-1, ethyl actetate-acetic acid-formic acid-water (10:1.1:1.1:2.4, v/v); 2-2, ethyl acetate-methanol-water (8:1.3:1, v/v); 2-3, *n*-hexane-ethyl acetate (6:4, v/v); and 2-4, toluene-ethyl acetate (93:7, v/v). From left to right; Detection under UV 254 nm (A); under UV 366 nm (B); under white light after derivatization with fast blue B (C); and under white light after derivatization with anisaldehyde (D), respectively.

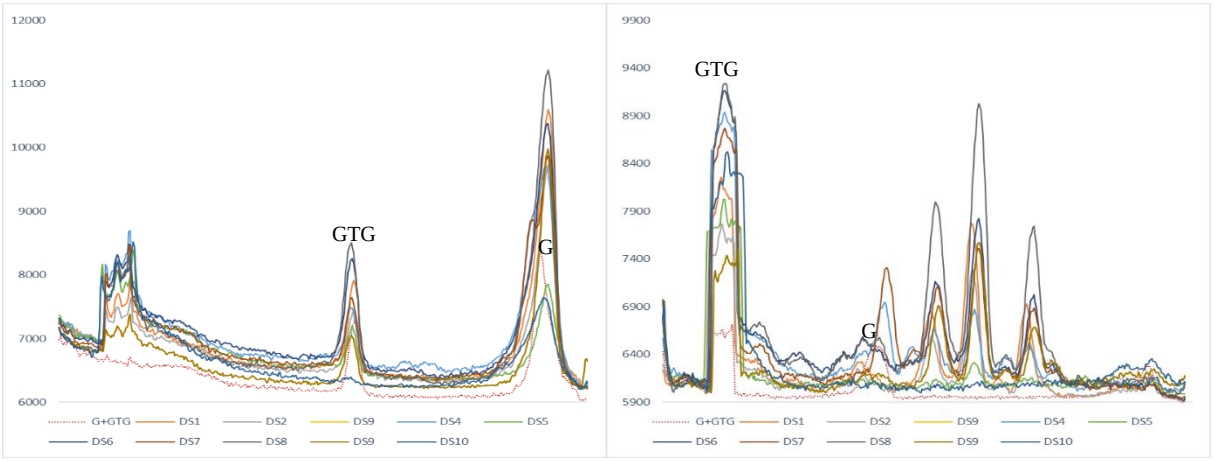

Figure 3 Chromatographic line plots of Figures 2-2 (GTG and genistein (G) at R_F values of 0.52 and 0.98, respectively) and 2-3 (GTG and genistein (G) at R_F values of 0.00 and 0.37, respectively) taken under 254 nm using UN-SCAN-IT, left and right, respectively.

Table 1 Method validation parameters of the TLC-image analysis by ImageJ

Parameters	**GTG**	**Genistein**
Concentration range (mg/mL)	0.015-0.25	0.007-0.12
R^2	0.9985 ± 0018	0.9982 ± 0004
LOD (mg/mL)	0.003	0.003
LOQ (mg/mL)	0.011	0.011
Precision (*%RSD*, *n* = 3)		
Intra-day	1.59-4.51	1.20-3.49
Inter-day	3.01-4.40	2.30-4.56
Recovery (%, *n* = 3)		
1	112.17	106.19
2	110.12	108.07
3	103.12	102.48
Average	108.47	105.58
Robustness (*%RSD*, *n* = 3)		
I	2.69	2.86
II	2.53	3.94

content in *D. scandens* by the TLC method.[15] However, due to a large difference in polarity between GTG and genistein, the TLC method using a multiple development technique was employed to clearly separate the two substances from the others in *D. scandens* on the same TLC plate. GTG and genistein were obtained at the R_F values of 0.26 and 0.62, respectively. Since genistein was the aglycone part of GTG, the ultraviolet (UV) absorption spectra of GTG and genistein were quite similar, and the spectra of the peaks corresponded to GTG in the samples were also similar to those of the standards, confirming the identities of GTG in these samples (Figure 4).

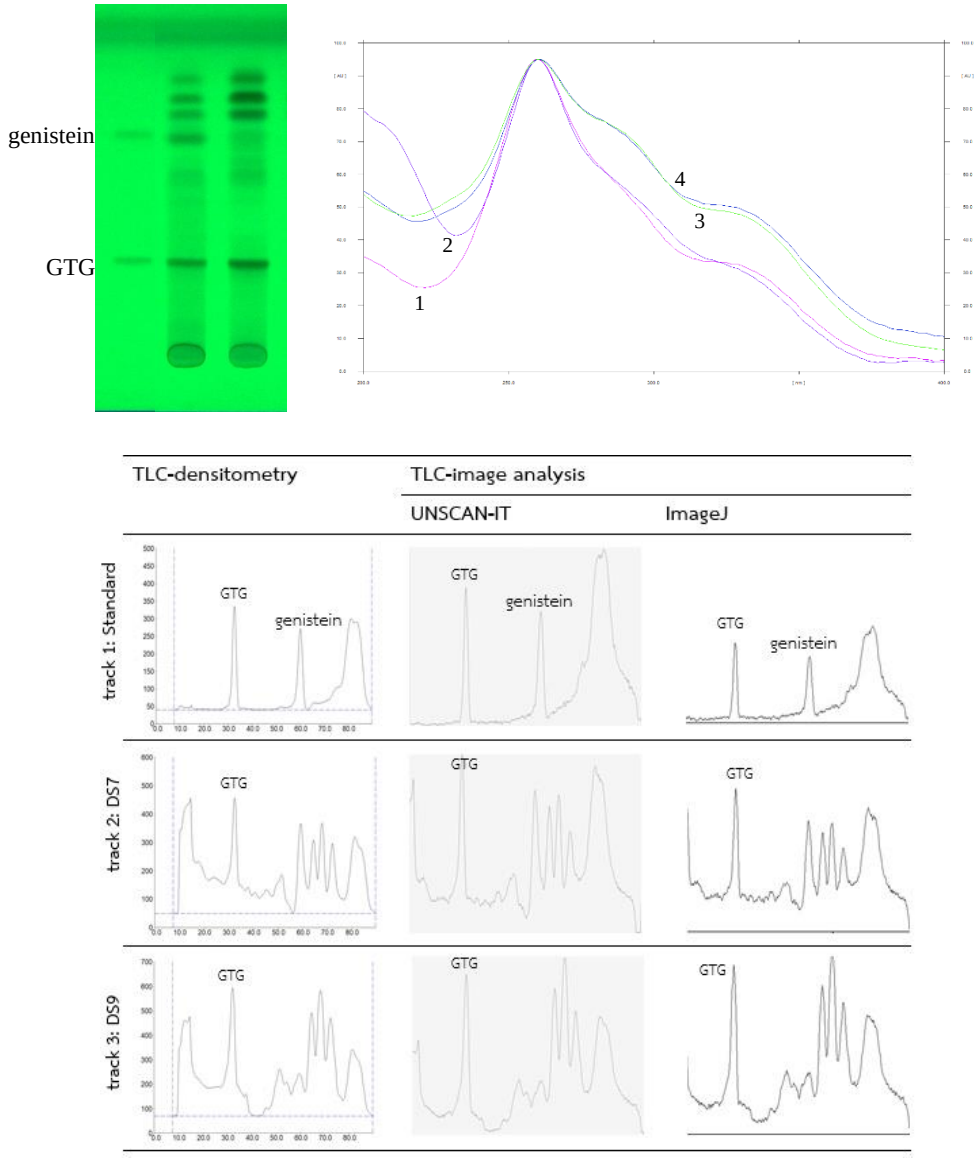

Figure 4 TLC and a chromatographic profile generated by TLC-densitometry and TLC-image analysis (UN-SCAN-IT and ImageJ) of a standard, DS7 and DS9 (tracks 1-3, respectively), using a TLC silica gel 60 F_{254} aluminum plate as the stationary phase, and ethyl acetate–methanol–water (8:1.3:1, v/v) and *n*-hexane–ethyl acetate (6:4, v/v) as the first and the second mobile phase taken under UV 254 nm; and, the overlay of the UV spectra of the peaks corresponded to GTG (1), genistein (2), and GTG in DS7 (3) and in DS9 (4), respectively.

Table 2 shows the percent of the content of GTG in DS1-DS10 as determined by the TLC–image analysis TLC–densitometry and HPLC. The %w/w of GTG (calculated as GTG), A and B, analyzed by different methods using GTG and genistein as the reference standard, in each sample, was not found to be significantly different ($p>0.05$). However, the content of GTG found in different samples was significantly different ($p<0.05$), ranging from 0.01 to 2.2 %w/w. DS1, *D. scandens* dry extract, had the highest content of GTG, approximately 5-6 times higher than DS2, *D. scandens* dried powder. GTG was one of the main constituents found in the *D. scandens* dry extract which was inconsistent with the previous report.[22]

Table 2 %w/w of GTG, A and B, using GTG and genistein as a reference standard, respectively (*n*=*6*). The calculation of %w/w was based on the weight of the amount of each sample used in the sample preparation protocol.

Samples	TLC-Image Analysis				TLC-densitometry		HPLC	
	UN-SCAN-IT		Image J					
	A	B	A	B	A	B	A	B
DS1	2.15±0.11	2.22±0.11	2.13±0.09	2.22±0.10	2.18±0.14	2.14±0.13	2.16±0.02	2.12±0.02
DS2	0.38±0.03	0.38±0.03	0.39±0.03	0.39±0.02	0.38±0.02	0.38±0.01	0.38±0.00	0.37±0.00
DS3	0.26±0.02	0.27±0.02	0.25±0.01	0.28±0.01	0.26±0.00	0.27±0.00	0.26±0.01	0.26±0.01
DS4	0.11±0.01	0.09±0.01	0.10±0.01	0.11±0.01	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00
DS5	0.21±0.01	0.20±0.01	0.21±0.02	0.22±0.01	0.21±0.02	0.22±0.03	0.21±0.01	0.20±0.01
DS6	0.29±0.01	0.32±0.01	0.29±0.01	0.28±0.01	0.31±0.00	0.29±0.00	0.31±0.02	0.30±0.02
DS7	0.08±0.01	0.08±0.01	0.08±0.01	0.08±0.01	0.09±0.01	0.07±0.01	0.08±0.00	0.08±0.00
DS8	0.25±0.01	0.27±0.01	0.27±0.02	0.27±0.02	0.25±0.01	0.24±0.01	0.25±0.00	0.25±0.00
DS9	0.18±0.01	0.17±0.01	0.18±0.02	0.18±0.01	0.18±0.00	0.18±0.00	0.17±0.00	0.17±0.00
DS10	0.02±0.00	0.01±0.00	0.01±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.01	0.02±0.00

Most of the sample formulations were discovered to contain the amount of GTG in a range of 0.2 to 0.4 %w/w. However, DS4 and DS7 had the amount of GTG at approximately 0.08-0.1 %w/w, while DS10 was found to have the lowest GTG content at about 0.02 %w/w. From the TLC fingerprint of DS10 (Figure 2, track 11), it was observed that the component of *D. scandens* seemed to be in a lesser proportion as compared to other herbals, resulting in the small amount of GTG detected in this formulation. In addition, the difference in the amount of GTG present in each product might also depend on the quality of plant raw materials used for the preparation of the herbal formulations.

Conclusion

An application of TLC-image analysis for the fingerprint study and quantification of bioactive genistein glycoside in *D. scandens* and its polyherbal formulations was described herein. The method was proven to be practical regarding to its simplicity, low-operating cost and uncomplicated set up. Digital images of TLC fingerprints and the profile plots obtained from the image analysis method provided useful qualitative and quantitative information and could be considered as a valuable tool for quality assessment of complex herbal material.

Acknowledgments

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