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High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) for Qualitative and Quantitative Analysis of Chlorogenic Acid in *Calendula Officinalis Linn*

Darekar D. P.[1], Sheikh. M. V.[2], Hate. M.S.[3]

Ramnarain Ruia Autonomous College, Matunga, Mumbai 400 019, India
ddipika1992[at]gmail.com

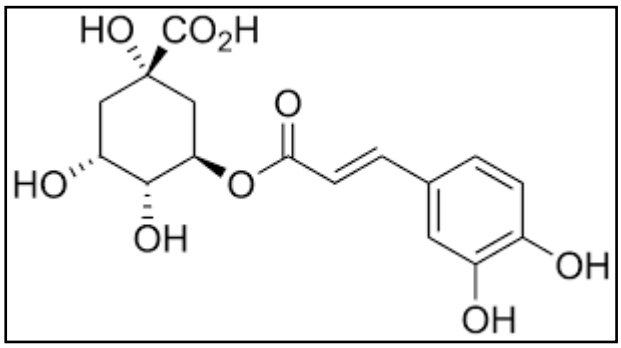
Abstract: ***A simple Chromatographic technique such as HPTLC and HPLC provide valuable additional information to establish the identity of the plant material.The primary goal of this study was to develop a simple and reliableHigh Performance Liquid Chromatography and High Performance Thin Layer Chromatography method to quantitate polyphenol such as chlorogenic acid present in Calendula Officinalis Linn. using analytical techniques. A combination of this two analytical methods may be useful in quality control analysis as it would give a simple, precise and rapid method.***

Keywords: Calendula officinalis, chlorogenic acid, phytoconstituents, polyphenols

1. Introduction

The term medicinal plant refers to a variety of plants that have medicinal properties. These plants are a rich source of compounds that can be used to develop drug synthesis. [1]Plant phytoconstituents as a source of medicinal actives have been reported in literature, since ancient times. [2] The most important of these bioactive constituents of plants are alkaloids, tannins, flavanoids and phenolic compounds.[3,4] *Calendula Officinalis Linn.* family asteraceae is a known medicinal plant and is used in folk medicine from the ancient times. It has been found to have a lots of medicinal properties , particularly for its antimicrobial and antioxidant activities. Therefore, investigation of the chemical Compounds within medicinal plants has become desirable.[5,6] (Poly) phenols are plant secondary metabolites that constitute one of the most common and widespread groups of substances in plants. The term 'polyphenol' could thus be used to define natural products featuring at least two phenyl rings bearing one or more hydroxyl substituents, including their functional derivatives (e.g., esters and glycosides). Phenolic compounds are essential for the growth and reproduction of plants, and are produced as a response for defending injured plants against pathogens. Some soluble phenolics are widely distributed, for example, chlorogenic acid, but the distribution of many other structures is restricted to specific genera or families, making them convenient biomarkers for taxonomic studies.Several analytical techniques have been established for the identification and quantification of natural products in herbal preparations.[7,8,9]HPTLC and HPLC has been used widely for analysis of herbal Medicinal extracts due to its simplicity of operation, speed, versatility and reproducibility, and relatively low cost. [10,11]

Structure of chlorogenic acid



2. Experimental Material

Collection of plants

The fresh flowers of Calendula officinalis were collected from Lonavala, Pune, in the month of February 2016 . The plant was botanically authenticated. A Voucher specimen of the plant has been deposited at the herbarium of the Botanical Survey of India, Pune. No. BSI/ WRC/ IDEN.CER./ 2017/600.

Chemicals and standards

Solvents used in HPTLC and HPLC mobile phase are analytical grade ethyl acetate (purity 99.6 %) , HPLC grade water, HPLC grade methanol (purity 99.9%) were procured from E.Merck, Mumbai, India. Analytical grade glacial acetic acid and formic acid was obtained from Qualigens Fine Chemicals, Mumbai, India. Reference standards Chlorogenic acid (purity >95%) were purchased from Sigma-Aldrich Chemie (Aldrich Division; Steinheim, Germany).

Equipments

CAMAG Linomat IV sample applicator, CAMAG Twin trough glass chamber, CAMAG TLC Plate Heater, CAMAG

TLC Scanner III equipped with winCats 3.0 version software, Shimadzu UFLC Prominence System, DGU-20 A3 PROMINANCE DEGASSER, LC-20AD A AND B BINARY Pump, SIL-20AC HT AUTOSAMPLER, CTO-20AC COLUMN Oven, SPD-M20 A Detector.

Plant extracts

*Calendula officinalis Linn.*flowers powder were extracted using soxhlet extraction. Five grams of plant powder was weighed and packed in a whatman paper thimble. It was then extracted with 100 ml methanol for 12 hours using soxhlet extractor.Extracts were filtered through a syringe filter of pore size 0.45 µm (Merck, India) and the filtrate were used as the sample solution for further analysis.

Standard solution

A stock solution of chlorogenic acid (1000 µg/ mL) was prepared in methanol. 10.0 mg of standard chlorogenic acid was accurately weighed and transferred to a 10.0 mL standard volumetric flask. The contents of the flask were initially dissolved in 5.0 mL of methanol, followed by sonication and then diluted up to the mark with methanol.

Parameters	*Chromatographic Condition for HPTLC*	*Parameter*	*Chromatographic Condition for HPLC*
Stationary phase	Silica gel 60 F_{254} pre-coated on aluminum sheet	Instrument	Shimadzu UFLC Prominence System
Mobile phase	Ethyl acetate: glacial acetic acid: formic acid: water10:1.1:1.1:2.3 (v/v/v/v)	Pump	LC-20AD A AND B 80 binary pump
Prewashing of plate	Methanol and activated at 120°C for 15 min	Injector	CTO-20AC auto sampler
Chamber saturation	15 min.	Injection Volume	10µl
Band	8 mm	Column oven	CTO-20AC column oven
Space	7 mm	Column	C18 column phenomenex, Luna C18(2), 5 µm, 4.6 × 250m
Speed	0.5 µL/sec	Mobile Phase	Methanol : Water : phosphoric acid (70:30: 0.1)
Drying of plate	At 120°C for 5 min	Flow Rate	0.7 ml/min
Lamp	Tungsten	Detector	20AC PDA detector
Wavelength	254 nm	Detection Wavelength	254nm

For HPTLC :Aluminum backed HPTLC plate precoated with silica gel 60F_{254} on aluminum sheets (E. Merck) were used as a stationary phase for the research work. The samples and standard solutions were applied using CamagLinomatIV sample applicator at the distance of 10 mm from the bottom edge of the HPTLC plate with the speed of 5µL/sec. The development of plate was carried out in a CAMAG glass twin trough chamber (10 X 10 cm). After development, the plates were dried in air first and then by keeping on the CAMAG TLC plate heater at 90°C for 5 min to allow complete evaporation of mobile phase components. The plates were then scanned at 254 nm with a CAMAG TLC Scanner with winCATS3 software, using the deuterium lamp.

For HPLC

High Performance Liquid Chromatography was performed using Shimadzu UFLC Prominence System instrument comprising of LC-20AD A AND B 80 binary pump, CTO-20AC ,LC-20AD A AND B , CTO-20AC auto sampler, CTO-20AC column oven and CTO-20AC PDA detector. An C18 column phenomenex, Luna C18(2), 5 µm, 4.6 × 250m was used as stationary phase employed. Separations were done in the isocratic mode, flow rate of 0.7ml/min; with an injection volume of 10 µl; UV detection was at 254 nm.

3. Optimized Chromatographic Conditions

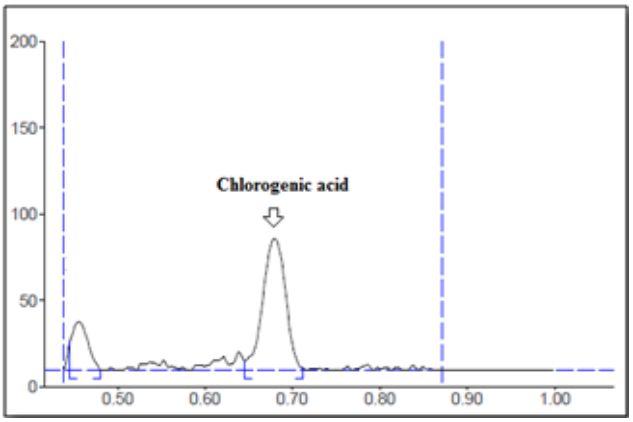


Figure 1: A typical HPTLC chromatogram of reference standard Chlorogenic acid At wavelength =254nm (R_f =0.70)

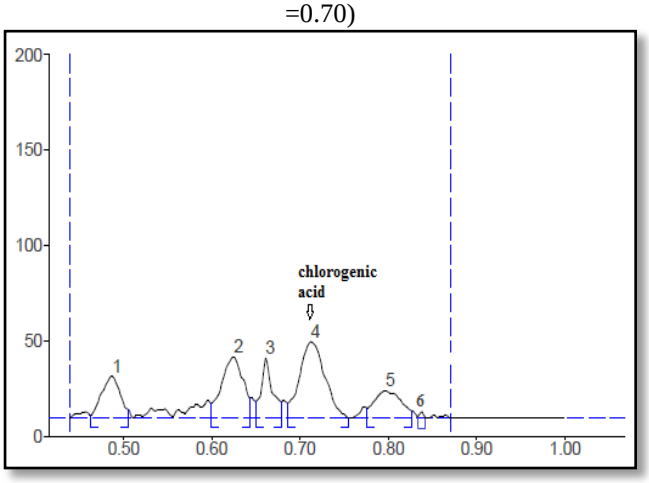


Figure 2: A typical HPTLC chromatogram of methanolic extract of flower of *Calendula Officinalis Linn.* At wavelength =254 nm(R_f =0.71)

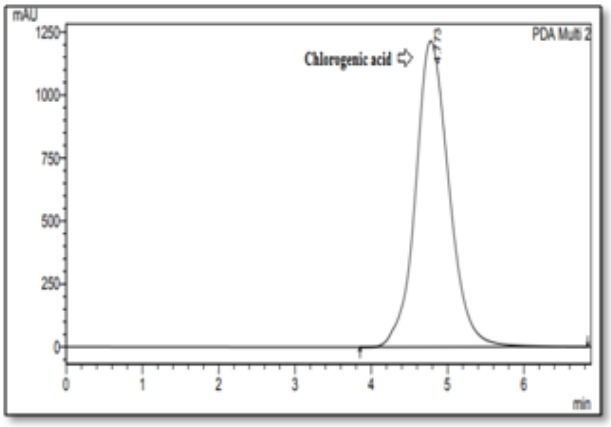


Figure 3: A typical HPLC chromatogram of standard solution of Chlorogenic acid (RT = 4.7mins)

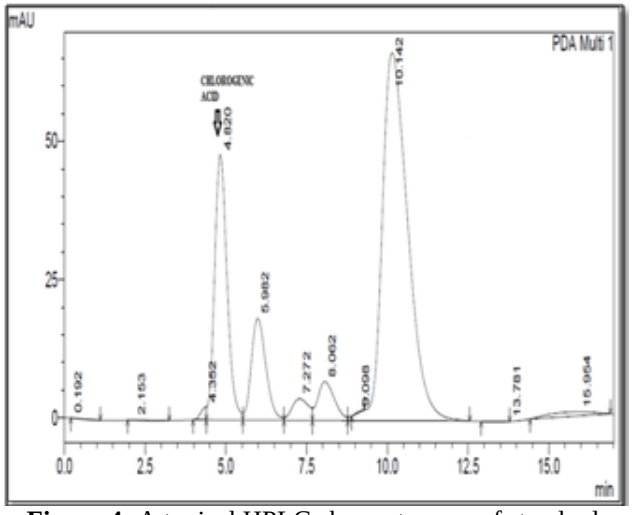


Figure 4: A typical HPLC chromatogram of standard solution of quantitation of Chlorogenic acid present in methanolic extract of *Calendula Officinalis Linn.* Flowers(RT = 4.8mins)
*RT = Retention Time

4. Results and Discussion

In the present research work high performance thin layer chromatographic (HPTLC) method is validated for the quantification of chlorogenic acid from flower powder of *Calendula Officinalis Linn*. Different polarities of solvents were tried from polar ,mid-polar and non-polar. Ethyl acetate: glacial acetic acid: formic acid: water10:1.1:1.1:2.3 (v/v/v/v) was adequate to resolve Chlorogenic acid (R_f =0.71) from the other constituents present in the methanolic extract of solvent strength of ethyl acetate, glacial acetic acid and formic acid in the volume ratio of flower powder of C.Officinalis Linn. The recovery chlorogenic acid was found to be 99.63% indicating good accuracy of the method. The peak area of chlorogenic acid in the flower powder solution increased after addition of standard chlorogenic acid to the flower powder solution without interference from other peaks.

The method used in the present research work was also found to be sensitive to measure the concentration of chlorogenic acid as low as 0.2µg per band.

A reverse phase high performance liquid chromatographic (HPLC) method is validated for the quantification of chlorogenic acid from flower powder of *Calendula Officinalis Linn*. In the present research work , reverse mode of separation is used for qualitative and quantitative identification of chlorogenic acid from flower powder of C. Officinalis Linn. Different polarities of solvents were tried from polar ,mid-polar and non-polar to resolve chlorogenic acid (Retention time 4.7 mins) .The recovery chlorogenic acid was found to be 99.99 % indicating good accuracy of the method. The peak area of chlorogenic acid in the flower powder solution increased after addition of standard chlorogenic acid to the flower powder solution without interference from other peaks. The method used in the present research work was also found to be sensitive to measure the concentration of chlorogenic acid as low as 0.1 µg/mL.

5. Conclusion

The developed method in this research work is precise, accurate and reproducible. HPTLC offers significant advantages in that it uses less solvent, and has the ability to simultaneously run multiple samples. Thus saving time and cost and on the other Hand, HPLC methods are generally considered more robust, and are capable of Higher degrees of precision on replication and quantitation. The method was found to be suitable for qualitative and quantitative analysis of polyphenols such as Chlorogenic acid from flower powder of C.Officinalis Linn, using high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC). The proposed method is simple, rapid, precise and accurate and can be further use for routine quality control analysis of flowers powder of *Calendula Officinalis Linn.*

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