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Development and Validation of Thin Layer Chromatography-Densitometric Method for Quantification of Kaempferol and Chlorogenic Acid in Methanolic Extract of *Dragea Volubilis*

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Abstract

The present study aims to prepare a methanolic extract of *Dragea volubilis* and to quantitatively estimate kaempferol and chlorogenic acid using newly developed and validated method of high-performance thin layer chromatography (HPTLC) using TLC densitometry technique. Dried leaves obtained from *D. volubilis* were defatted with petroleum ether and extracted in a Soxhlet extractor with methanol. The solvent was evaporated using a Rota evaporator. Quantitative analysis of the extract was done using TLC and HPTLC. A mixture of toluene: ethyl acetate: glacial acetic acid (5.5:4:0.5) for kaempferol and ethyl acetate: acetic acid: water (7:1.5:1.5) for chlorogenic acid was used as a mobile phase. The chromatography was performed on a TLC plate precoated with silica gel GF_{254} and the developed TLC plates were visualized and quantified at 367 nm and 327 nm for kaempferol and chlorogenic acid, respectively. The percentage yield was found to be 0.0397 ± 0.0001 and 0.0755 ± 0.0000 for kaempferol and 0.0755 ± 0.0000 for chlorogenic acid. R_f values 0.95 and 0.61 were observed for kaempferol and chlorogenic acid, respectively. The method was validated for instrumental precision, repeatability, coefficient of determination (r^2), linearity range (ng), LOD (ng), LOQ (ng), and intra-day and inter-day precision for both kaempferol and chlorogenic acid, and all the parameters were found to be within the acceptable range as per the ICH guidelines. The newly developed HPTLC method was found to be prompt, cost-effective, precisely accurate, and reproducible for the qualitative as well as quantitative analysis of kaempferol and chlorogenic acid in methanolic extract of *D. volubilis*.

Keywords: *Dragea volubilis,* Kaempferol and chlorogenic acid, High-performance thin layer chromatography, Validation

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Introduction

Modern advancements in techniques of isolation of active constituents from plants with medicinal value have led to fruitful results in many diseases of the central nervous system such as Alzheimer's disease and Parkinson's disease. These are still a major challenge for drug development. Phytochemical studies play an important role in drug development.[1] *Dragea volubilis* (Linn.) Benth. (Asclepiadaceae) has been used in the traditional system for healing various diseases such as physical weakness, skin ailments, hemorrhoids, anti-inflammatory, antipyretic, analgesic, and antibacterial potential. *Dregea volubilis* has also shown promising results as larvicidal,[2] anti-leukemic,[3] antitumor, antioxidant[4] and anti-diabetic,[5] antihyperlipidemic,[6] and hepatoprotective agents.[7] Most of the pharmacological activities of *D. volubilis* are due to its strong antioxidant properties.[8] Studies performed earlier claimed the presence of alkaloids, terpenoids, steroids, coumarins, tannins, flavonoids, proteins, carbohydrates, glycosides, phytosterol, anthocyanidins, amino acids, phenolic compounds lipids, and certain unidentified compounds.[9-11] Comprehensive chemical exploration of *D. volubilis* revealed the presence of phenolic acids such as chlorogenic acid, flavanol glucosides such as kaempferol and triterpenoids. Kaempferol has shown the protective effect of the transgenic.[12] It exhibited reduction in neurotoxic motor and cognitive impairements in *Drosophila melanogaster* model of Alzeimer's disease.[13] Kaempferol has shown anti-cholinesterase activity in Ellman's spectrophotometric method. Kaempferol and chlorogenic acids have potential anti-oxidant and anti-acetylcholinesterase activity.[13] Kaempferol has protective effects against amyloid beta-induced neurotoxicity in mice toxicity.[14] Chlorogenic acid has shown neuroprotective effects of scopolamine-induced amnesia through anti-acetylcholinesterase and anti-oxidative activities in mice (Kwon *et al.*, 2010). Some studies revealed that chlorogenic acid inhibited AChE and BChE activities in *in-vitro* assays.[15]

The present study was aimed to evolve and substantiate the HPTLC method for estimation of kaempferol and chlorogenic acid in methanolic extract of *D. volubilis* using the TLC densitometry technique. Phytochemical and pharmacological screening studies had an important role in drug development. In ayurvedic formulations generally, there is the presence of whole drug rather than its active or isolated principles advancement of chromatographic techniques and spectral analysis has changed the entire perspective of herbal drugs in drug discovery. *D. volubilis* (L.f.) Benth is a fairly large woody plant having a smaller

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size than a tree with several main stems arising from the ground. It is well distributed in Southern parts of India and is also found in other Asian countries. It is found in almost all native countries such as Srilanka, Myanmar, Indonesia, Thailand, and China.[16] There is a variety of active constituents obtained from *D. volubilis* such as glycosides, volubilioside A, B, C, phenolic acids such as chlorogenic, hydroxycinnamic acids, flavonol glucosides, rutin, and kaempferol 3-rutin.[8] Many studies have shown a wide variety of pharmacological activities with active constituents obtained from *D. volubilis*.[17] This plant has proved its usefulness in the traditional system of medicines to cure many ailments such as inflammation, pain, and wound healing and it is treated as a wonder herb.[18] The anti-leukemic activity was reported from an active fraction obtained from leaves of *Wattakaka volubilis* which was comparable to standard drugs.[3] *Wattakaka volubilis (D. volubilis)* has shown potential to improve dyslipidemia and diabetes in rats.[19] Polypregnane glycosides isolated from *D. volubilis* exhibited neuroprotective activities in cerebral ischemic rats.[20] Exhaustive chemical exploration of *D. volubilis* disclosed the presence of phenolic acids such as chlorogenic acid, ursolic acid, flavanol glucosides such as kaempferol, and triterpenoids.[21] *D. volubilis* is found to be a strong antioxidant and its pharmacological activities can be ascribed to its strong antioxidant potential. Moreover, phytochemical exploration reported the presence of terpenoids, steroids, glycosides alkaloids, tannins, flavonoids, phytosterol, anthocyanidins, etc. However, still a little is known about the pharmacological activities of isolated compounds. Chlorogenic acid has shown neuroprotective activity against Aβ using an MTT assay.[22] Kaempferol and chlorogenic acid obtained from plant sources exhibited many pharmacological activities [Figure 1].

Hence, there is a need to develop and standardize a chromatographic technique for quantification of kaempferol and chlorogenic acid obtained from *D. volubilis*. There are no such methods reported for quantification of kaempferol and chlorogenic acidic *D. volubilis* so far. Hence, the present research aims to develop and validation of a high-performance thin-layer chromatographic (HPTLC) method for the estimation of kaempferol and chlorogenic acidic methanolic extract of *D. volubilis*.

Materials and Methods

Materials

Fresh leaves of *D. volubilis (Asclepiadaceae)* were collected from Nilgiri hills, Tamil Nadu, and authenticated from Punjabi University, Patiala vide specimen no: 115. The standard marker compounds used for the study, that is, kaempferol and chlorogenic acid were purchased from Sigma Aldrich. All chemicals utilized for the study were of analytical grade and were received from different sources.

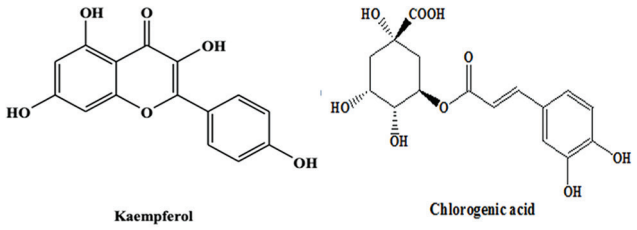

Figure 1: Chemical structure of: (a) Kaempferol and (b) chlorogenic acid

Extraction Procedure

Leaves were dried in shade and crushed to powder with the aid of a grinder. The fatty and sticky material was removed with petroleum ether (60–80°C) and then extracted with hot methanol in a Soxhlet apparatus.[23] The clear solution in the side tube of the apparatus was the endpoint of extraction with methanol. Finally, methanolic extract was concentrated with a Rota vacuum evaporator.[24]

TLC

Precoated TLC silica gel GF_{254} (stationary phase) plates (10 cm × 20 cm; E. Merck) were used for the analysis. The plates were eluted in a mixture of toluene: ethyl acetate: glacial acetic acid (5.5:4:0.5) for kaempferol and ethyl acetate: acetic acid: water (7:1.5:1.5) for chlorogenic acid. The extract was applied on the TLC plate using a capillary tube and elution was carried out with a solvent system. Then, (20%) sulfuric acid (H_2SO_4 were sprayed within methanol and heated in an oven at 105°C on the TLC plates. Accordingly, R_f values were calculated by measuring the distance traveled by a solute (sample) and solvent front (Anuradha *et al.*, 2018).

HPTLC

Optimized solvent system = toluene: ethyl acetate: glacial acetic acid (5.5:4:0.5) for kaempferol and ethyl acetate: acetic acid: water (7:1.5:1.5) for chlorogenic acid. HPTLC technique for qualitative and quantitative analysis was developed and substantiated for quick analysis of methanolic extract obtained from *D. volubilis* leaves. Methanol was used as a solvent for the sample application. Chromatographic separation was achieved on precoated TLC plates with silica gel GF_{254} as the stationary phase using the above-mentioned solvent system as mobile phase, that is, toluene: ethyl acetate: glacial acetic acid (5.5:4:0.5) for kaempferol and ethyl acetate: acetic acid: water (7:1.5:1.5) for chlorogenic acid. Continuous radiation of UV-spectrum between 190 and 600 nm was emitted from a deuterium lamp. All determinations were carefully done at room temperature with detection wavelengths at 367 and 327 nm. Peak areas were plotted against the corresponding values of concentration to get the linear calibration regression.[25]

Spraying Agent

Development of plates was performed using a Camag glass tank and developed dried plates were immersed in 0.5 % anisaldehyde-sulfuric acid reagent. Plates were heated at 110°C for 2 min till the spots were distinguishably visible and were then visualized in TLC scanner at 367 nm and 327 nm, respectively, for kaempferol and chlorogenic acid.

Assay

Standard and test solutions were observed on a pre-coated TLC plate. The percentage quantity of kaempferol and chlorogenic acid present in *D. volubilis* methanolic extract was estimated by comparing the areas computed for the test and standard solution.

Detection of Linearity

Evaluation of linearity of different concentrations of the standard solution of kaempferol and chlorogenic acid was done. The linear calibration curves of the standard were observed to be linear over

a range from 150 to 900 ng/spot on TLC plates in the form of sharp bands. The plates were developed in a mixture of a solvent system containing toluene: ethyl acetate: glacial acetic acid (5.5:4:0.5) for kaempferol and ethyl acetate: acetic acid: water (7:1.5:1.5) for chlorogenic acid up to a distance of 8 cm, the plates were analyzed at room temperature. After development, the plates were dried after development and the components were visualized. The response for detection of kaempferol and chlorogenic acid was measured for each band at the wavelength of 367 and 327 nm, using Camag TLC Scanner equipped with win CAT software. The peak areas were observed for all concentrations of kaempferol and chlorogenic acid. A graph depicting peak areas of kaempferol and chlorogenic acid for linearity was plotted against the applied concentration of kaempferol and chlorogenic acid in ng.[26]

Method Validation

The newly developed method was checked for accuracy, perfection, and meticulousness. Repeated scanning of the same spot of kaempferol and chlorogenic acid 7 times each was done to confirm the reproducibility of the method. Solutions of standard kaempferol and chlorogenic acid were analyzed at a concentration of 300 ng and 450 ng/spot 5 times on the same day and on the different days to check inter-day and intra-day precision. Results confirmed the accuracy of the method. To analyze the accuracy of the method, the recovery studies were executed by the standard addition method.

The percentage recovery and average percentage were calculated. A recovery trial was done for the added quantities of standards and was studied at three variant levels similar to that of the reported assay. Additions were repeated 3 times on three different days. Recovery of the added amount of standard was calculated for the same. Evaluation of the limit of detection and limit of quantization was also calculated by the proposed method.[27]

Table 1: Summary of validation parameters

S. No.	Parameter	Kaempferol	Chlorogenic acid
1.	Instrumental precision (% CV, n=7)	1.09	1.12
2.	Repeatability (% CV, n=5)	1.56	1.89
3.	Coefficient of determination (r^2)	0.996	0.996
4.	Linearity range (ng)	200–700	200–700
5.	LOD (ng)	31	26
6.	LOQ (ng)	105	80
7.	Intra-day precision (% CV, n=9)	1.33	1.35
8.	Inter-day precision (% CV, n=9)	1.78	1.60
9	Specificity	Specific	Specific

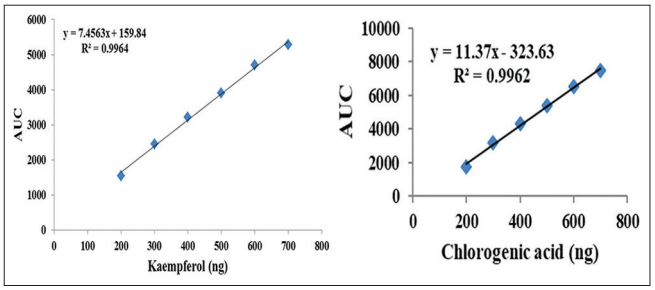

Figure 2: Calibration curve for kaempferol and chlorogenic acid

Results and Discussion

The percentage yield was found to be 0.0397 ± 0.0001 for kaempferol and 0.0755 ± 0.0000 for chlorogenic acid.

TLC

The R_f value was calculated as 0.95 and 0.61 for kaempferol and chlorogenic acid, respectively, which was in accordance with the reported value of kaempferol and chlorogenic acid marker confirming the content of kaempferol and chlorogenic acid in extract.

HPTLC

Silica gel GF_{254} HPTLC plates were used as stationary phase in this method and mobile phase toluene: ethyl acetate: glacial acetic acid (5.5:4:0.5) for kaempferol and ethyl acetate: acetic acid: water (7:1.5:1.5) for chlorogenic acid, respectively, (Larsen *et al.*, 2004). In *D. volubilis* leaves extract was quantified at an Rf value of 0.95 and 0.61, respectively. The outcomes of method validation parameters are shown in Table 1.

Calibration Curve

The calibration curve showed linearity in the range of 200–700 ng for both kaempferol and chlorogenic acid and the correlation coefficient was found to be 0.996 for both [Figure 2]. The identity of the band of kaempferol in the sample extract was observed by overlaying the tracks showing peak values of the sample with that of marker [Figure 3a and b]. The limit of detection was 31

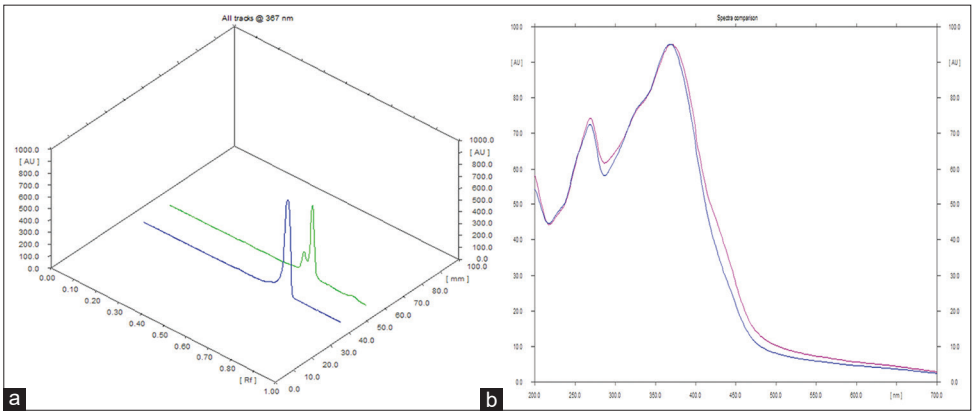

Figure 3: (a) Spectra overlay of kaempferol and (b) peak overlay of kaempferol

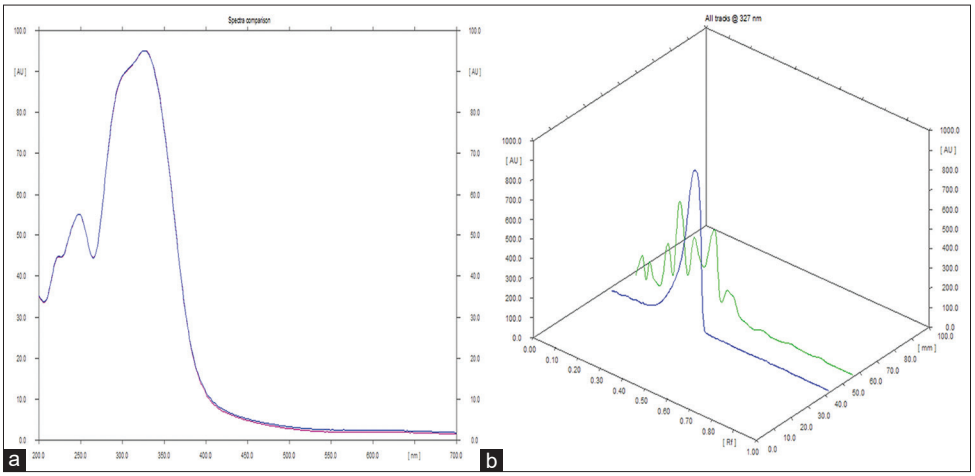

Figure 4: (a) Spectra overlay of chlorogenic acid and (b) peak overlay of chlorogenic acid

and 26 ng/spot and the limit of quantification was found to be 105 and 80 ng, respectively, for kaempferol and chlorogenic acid. The method was substantiated in terms of accuracy, perfection, and meticulousness expressed as % CV (coefficient of variation) which were found to be <1.09 %. and 1.12%. The recovery values obtained were 95.85–99.28% with an average percentage recovery of 99.46% showing the accuracy of the method [Figure 4].

Table 1 summarizes the observed values for validation parameters.

Conclusion

The developed HPTLC method was found to be swift, remunerative, precise, and can be utilized for the quantitative as well as qualitative estimation of kaempferol and chlorogenic acid.

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