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Optimized Separation and Quantification of Pharmacologically active markers Quercetin, Kaempferol, ß-sitosterol and Lupeol from *Cuscuta reflexa* Roxb.

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ABSTRACT

Cuscuta reflexa Roxb. (Cuscutaceae, Amarvel) is a golden yellow parasite commonly found throughout India. *Cuscuta reflexa* (CR) has been widely used for curing many diseases. CR has been reported as antibacterial, immunomodulatory, hepatoprotectant etc. In the present work a precise, accurate and reproducible densitometric HPTLC method is developed and validated for simultaneous quantification of four pharmacologically active markers Quercetin, Kaempferol, ß-sitosterol and Lupeol from the whole plant of CR using a common mobile phase. A binary detection mode was used for quantification of these markers. The amount of Quercetin, Kaempferol, ß-sitosterol and Lupeol was found to be 0.0296 ± 0.0015mg g^{-1}, 0.2901 ± 0.0011 mg g^{-1}, 0.3039 ± 0.0034 mg g^{-1} and 0.0553 ± 0.0107 mg g^{-1} respectively. There are no methods reported for simultaneous separation and quantification of these four pharmacologically active markers from any plant matrix using HPTLC. The current research work is an attempt for simultaneous quantification of Quercetin, Kaempferol, ß-sitosterol and Lupeol from CR in a single mobile phase.

Keywords: HPTLC, Kaempferol, Lupeol, Quercetin, ß-sitosterol

1. INTRODUCTION:

Various herbs and herbal extracts contain different biologically active phytoconstituents which exhibit therapeutic effects [1]. Among the various class of phytoconstituents, Flavonoids and Triterpenoids are widely distributed in plants. Flavonoids which occur both in the Free state and as glycosides are the largest group of naturally occurring phenols. They are formed from three acetate units and phenylpropane units [2]. Flavonoids (e.g. Quercetin and Kaempferol, **(Figure-1)** constitute phenolic compounds which are structurally derived from the parent substance, flavones. Triterpenoids (e.g. ß-sitosterol and Lupeol, (Figure-1) are compounds with a carbon skeleton based on six isoprene units which are derived biosynthetically from the acyclic C_{30} hydrocarbon, squalene. The above four biologically active phytoconstituents are reported to have several pharmacological activities. Quercetin is reported for its antiallergic [3], antitumor [4], immunomodulatory [5] activities. Similarly antidiabetic [6] and anticancer [7] activities of Kaempferol is also been reported. ß-sitosterol has anticancer [8], anticarcinogenic [9] activities while Lupeol is reported to show antimalarial [10] and hepatoprotectant [11] activities. These two major group of phytoconstituents largely differ in polarity due to their structural difference. Hence, quite often it is difficult to separate and retain them on normal silica gel stationary phase in a single mobile phase [12].

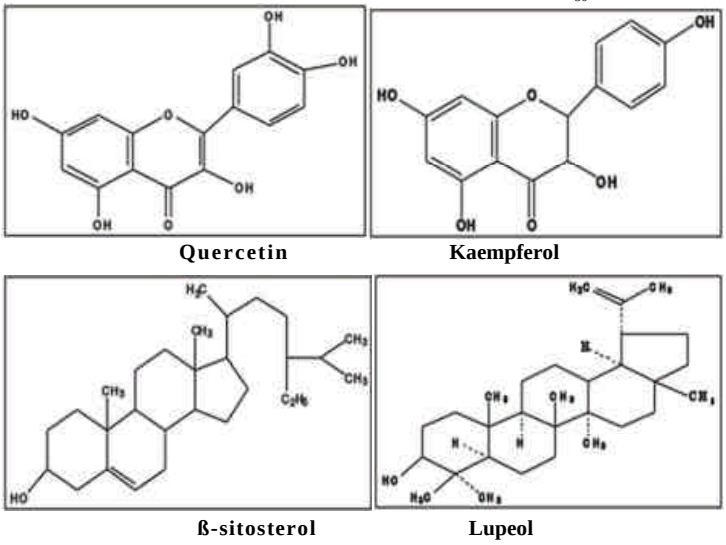

Figure: 1 Structures of Quercetin, Kaempferol, ß-sitosterol and Lupeol [27]

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Cuscuta reflexa Roxb. (Cuscutaceae) is an unusual parasitic vine, growing in a prolific manner over host plants with inter-twined stems and commonly found throughout India. *Cuscuta reflexa* (CR) is a voracious and destructive vine which usually overgrows on host and kills it [13]. Cuscutin, Kaempferol, Quercetin, Lupeol, ß-sitosterol, a-amyrin, ß-amyrin, Stigmasterol are the pharmacologically active markers reported from CR [14]. Inspite of its parasitic activity the plant is also reported to have many therapeutic activities such as antibacterial [15], immunomodulatory, antihelminthic, antioxidant [16], against hair loss, hepatoprotective [17], analgesic [18] etc.

Morphological, pharmacognostical and anatomical characteristics of CR and development of TLC fingerprint for identification of the plant on the basis of colors of spots and their R_f values is reported [19, 20]. But in both the above reported methods, no specific and detailed study has been elucidated on phytoconstituents present in CR.

There is HPTLC method reported for simultaneous quantification of Quercetin, ß-sitosterol and Lupeol from *Soymida febrifuga* [21]. Quercetin and Kaempferol was also simultaneously quantitated from two medicinal plants using HPTLC [22, 23]. An RP-HPTLC method (using RP-18 F_{254} TLC plates with dual run) for simultaneous determination of major flavonoids (including Apigenin and Quercetin) from herbal extracts is been reported [24].

But, there are no methods reported for simultaneous separation and quantification of these four pharmacologically active markers from any plant matrix using HPTLC. Therefore in the present work an attempt has been made for simultaneous quantification of Quercetin, Kaempferol, ß-sitosterol and Lupeol from CR in a single mobile phase. In the present work an HPTLC method was optimized to separate and quantitate Quercetin, Kaempferol, ß-sitosterol and Lupeol from CR in a single mobile phase. A binary detection mode was used for densitometric scanning of these pharmacologically active markers.

2. MATERIALS AND METHODS:

2.1. Plant Material:

Cuscuta reflexa (whole plant) growing on *Vitex negundo* Linn was collected from Lonavala in the month of February. The plant was authenticated and the specimen is preserved in Herbal Research Lab, Ramnarain Ruia College. The plant material was shade dried for five days and was kept thereafter in hot air oven maintained at 35±2^0 C for fifteen days. The plant material was then powdered, sieved through 80 mesh and was stored in airtight plastic bottle at room temperature for further analysis.

2.2. Chemicals:
All the chemicals used were of analytical grade and were procured from Qualigens Fine Chemicals, Mumbai. Standards Quercetin (? 98% purity), Kaempferol (96% purity), ß-sitosterol (98% purity) and Lupeol (97% purity) was procured from Sigma Aldrich, Germany. Derivatizing reagent i.e. Anisaldehyde sufuric acid was prepared as per the procedure described in [25].

2.3. HPTLC conditions:
Chromatographic separation was achieved on HPTLC plates precoated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with aluminium sheet support. Samples were spotted using CAMAG Linomat IV Automatic Sample Spotter (Camag Muttenz, Switzerland) equipped with syringe (Hamilton, 100 µL). Plates were developed in a glass twin trough chamber (CAMAG, 20 X 10 cm) presaturated with mobile phase for fifteen minutes. Scanning device used was CAMAG TLC Scanner 2 equipped with CATS 3 software. The experimental condition was maintained at 22 ± 2^0 C.

2.4. HPTLC fingerprinting profile

2.4.1. Extraction of phytoconstituents from CR
Extraction of phytocostituents from CR was optimized to achieve good fingerprinting and also to resolve the marker compounds efficiently. To the accurately weighed powdered drug (2 g), 5 mL of ethanol was added, vortexed for 5 minutes for thorough mixing and the mixture was kept standing overnight. Next day it was sonicated for 10 minutes, filtered through Whatmann filter paper No. 41 and the filterate (10 µL) was then used for HPTLC analysis.

2.4.2. Preparation of Standard solutions
Stock solutions of Quercetin, Kaempferol, ß-sitosterol and Lupeol (1000 µg mL^{-1}) were prepared by dissolving 10 mg each of accurately weighed standards in ethanol and making up the volume to 10 mL in standard volumetric flask respectively. For calibration curve aliquots of 10-70, 25-250, 12.5-250 and 10-100 µg mL^{-1} were prepared from this stock solution for Quercetin, Kaempferol, ß-sitosterol and Lupeol respectively.

Further three quality control samples (LQC, MQC, HQC) each of Quercetin (15, 30, 60 µg mL^{-1}), Kaempferol (35, 85, 200 µg mL^{-1}), ß-sitosterol (15, 55, 200 µg mL^{-1}) and Lupeol (15, 35, 80 µg mL^{-1}) were prepared for precision, accuracy and ruggedness studies.

2.4.3. Solvent system
A single solvent system consisting of Cyclohexane: Ethyl acetate: Formic acid (6: 4.5: 0.2 v/v/v) has been used in this method to resolve and to quantitate all the four marker compounds viz Quercetin, Kaempferol, ß-sitosterol and Lupeol from plant matrix of CR.

2.4.4. Simultaneous quantification of Quercetin, Kaempferol, ß-sitosterol and Lupeol
Though a TLC densitometric method was reported for quantification of kaempferol and quercetin from other plants **[24, 26]**, in the present research work an attempt has been made for simultaneous quantification of Quercetin, Kaempferol, ß-sitosterol and Lupeol from CR.

Sample solution (10 µL) was applied in triplicate to a precoated silica gel 60 F_{254} HPTLC plate (E.Merck) with the CAMAG Linomat IV automatic sample spotter. The plate was developed and scanned at 254 nm for Quercetin and Kaempferol and then after derivatizing with 1% anisaldehyde sulphuric acid reagent at 550 nm for ß-sitosterol and Lupeol .

3. METHOD VALIDATION
ICH guidelines were followed for the validation of the developed analytical method (CPMP/ICH/281/95 and CPMP/ICH/381/95).

3.1. Instrumental Precision
Instrumental precision was checked by repeated scanning (n = 7) of Quercetin (15 µg mL^{-1}), Kaempferol (35 µg mL^{-1}), ß-sitosterol (15 µg mL^{-1}) and Lupeol (15 µg mL^{-1}) and further expressed as relative standard deviation (%RSD).

3.2. Repeatability
The repeatability of the method was affirmed by analysing 15 µg mL^{-1} of Quercetin, 35 µg mL^{-1} of Kaempferol, 15 µg mL^{-1} of ß-sitosterol and 15 µg mL^{-1} of Lupeol individually on a HPTLC plate (n = 5) and expressed as % RSD.

3.3. Inter-Day and Intra-Day Precision
Variability of the method was studied by analysing quality control samples of Quercetin, Kaempferol, ß-sitosterol and Lupeol on the same day (intra-day precision) and on different days (interday precision) and the results were expressed as % RSD.

3.4. Limit of Detection and Limit of Quantification
For the evaluation of limit of detection (LOD) and limit of quantification (LOQ) different concentrations of the standard solutions of Quercetin, Kaempferol, ß-sitosterol and Lupeol were applied along with ethanol as blank and determined on the basis of signal-to-noise (S/N) ratio. LOD was determined at an S/N of 3: 1 and LOQ at an S/N of 10: 1.

3.5. Recovery
The accuracy of the method was assessed by performing recovery study at three different levels (25, 50 and 100%, spiking of Quercetin, Kaempferol, ß-sitosterol and Lupeol in plant matrix). The percent recovery and the average percent recovery for each were calculated.

3.6. Specificity
Specificity was ascertained by analyzing standard compounds and samples. The bands for Quercetin, Kaempferol, ß-sitosterol and Lupeol from sample solutions were confirmed by comparing the R_f and spectra of the bands to those of the standards. The peak purity of all the compounds **(Figure-2 and3)** was analysed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

3.7. Ruggedness
Ruggedness of the method was assessed by deliberately incorporating the small variations in the optimized chromatographic condition. Effect of change in analyst, change in mobile phase composition [Cyclohexane: Ethyl acetate: Formic acid (5.9: 4.6: 0.2 v/v/v) and Cyclohexane: Ethyl acetate: Formic acid (6.1: 4.4: 0.2 v/v/v)] and change in spotting volume (9 µL and 11 µL), on the response and R_f of quality control samples was observed.

4. RESULTS AND DISCUSSION
Of the various solvent systems tried, mixture containing Cyclohexane: Ethyl acetate: Formic acid (6: 4.5: 0.2 v/v/v) gave the best resolution of Quercetin (R_f = 0.19), Kaempferol (R_f=0.26), ß-sitosterol (R_f = 0.56) and Lupeol (R_f = 0.66) from the other components of the ethanolic extract of CR and enabled their simultaneous quantification. The identity of bands of Quercetin and Kaempferol in plant matrix was confirmed by overlay in UV absorption spectra with those of the standards Quercetin and Kaempferol while identity of bands of ß-sitosterol and Lupeol in plant matrix was confirmed by overlay in visible spectra with those of the standards ß-sitosterol and Lupeol using CAMAG TLC scanner 2. The purity of bands of Quercetin, Kaempferol, ß-sitosterol and Lupeol in the plant extract was confirmed by overlaying the absorption spectra at the start, middle and end position of the bands **(Figures 2 and 3)**.

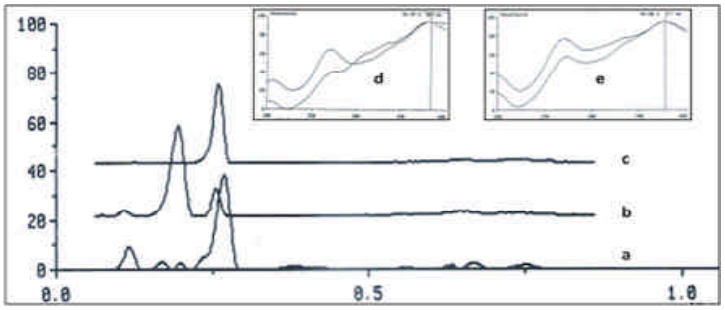

Figure: 2 HPTLC densitometric chromatogram scanned at 254 nm of (a) CR (b) Quercetin (c) Kaempferol and absorption spectra for identity and purity of marker compounds in the sample matrix with respective standards (d) Quercetin (e)

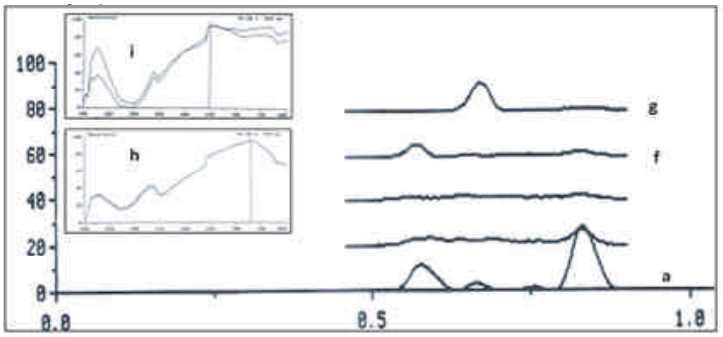

Figure: 3 HPTLC densitometric chromatogram scanned at 550 nm of (a) CR (f) ß-sitosterol (g) Lupeol and absorption spectra for identity and purity of marker compounds in the sample matrix with respective standards (h) ß-sitosterol (i) Lupeol

The four pharmacologically active phytoconstituents Quercetin, Kaempferol, ß-sitosterol and Lupeol were quantified from *Cusuta reflexa* by TLC densitometric method. The developed method was validated in terms of precision, repeatability and accuracy (**Table 1**). The linearity range for Quercetin, Kaempferol, ß-sitosterol and Lupeol was found to be 10-70, 25-250, 12.5-250 and 10-100 µg mL^{-1} respectively, with correlation coefficients (r^2 values) 0.9991, 0.9996, 0.9991, 0.9998 (**Table 2**). The TLC densitometric method was found to be precise with % RSD for intra-day precision in the range of 0.95-1.15, 0.91-1.21, 0.94-1.01, 1.03-1.20 and for inter-day precision in the range of 0.98-1.28, 0.95-1.29, 0.97-1.34, 1.15-1.32 for Quality control samples of Quercetin, Kaempferol, ß-sitosterol and Lupeol respectively (**Table 3**). This indicates that the method is precise. The LOD values for Quercetin, Kaempferol, ß-sitosterol and Lupeol were found to be 2, 5, 2, 2 µg mL^{-1} respectively while LOQ values were 5, 10, 5, 5 µg mL^{-1} respectively (**Table 1**). The average recoveries at three different levels of Quercetin, Kaempferol, ß-sitosterol and Lupeol were found to be 99.93%, 98.49%, 97.60% and 98.86% respectively (**Table 4**). Quercetin, Kaempferol, ß-sitosterol and Lupeol were simultaneously quantified from the plant matrix and the amounts were found to be 0.0296 ± 0.0015, 0.2901 ± 0.0011, 0.3039 ± 0.0034 and 0.0553 ± 0.0107 respectively (**Table 5**). Ruggedness of the method in terms of change in analyst and change in mobile phase composition showed variations within acceptable limits. Change in spotting volume at 9 and 11 µL did not affect the R_f of examined phytoconstituents but change in response was observed which was within acceptable limits. The present research work aims at separation, quantification and validation of four pharmacologically active phytoconstituents from plant matrix.

Table 1 : Method Validation parameters for examined phytoconstituents

Sr. No.	Parameter	Quercetin	Kaempferol	ß-sitosterol	Lupeol
1	Instrumental precision (% RSD, n = 7)	0.71	0.61	0.86	1.05
2	Repeatability (% RSD, n = 5)	0.93	0.75	1.03	0.98
3	Accuracy (average % recovery)	96.99	100.28	97.58	98.46
4	LOD (µg mL^{-1})	2	5	2	2
5	LOQ (µg mL^{-1})	5	10	5	5
6	Specificity	Specific	Specific	Specific	Specific
7	Ruggedness	Rugged	Rugged	Rugged	Rugged

Table 2 : Calibration parameters of examined phytoconstituents

Phytoconstituents	Linear Working Range (µg mL^{-1})	Regression equation	Correlation Coefficient (r^2)
Quercetin	10-70	y = 15.079x - 118.43	0.9991
Kaempferol	25-250	y = 5.5001x + 254.13	0.9996
ß-sitosterol	12.5-250	y = 6.5439x - 12.474	0.9991
Lupeol	10-100	y = 7.0534x + 0.9509	0.9998

Table 3 : Precision Studies for examined phytoconstituents

Phytoconstituents	Concentration (µg mL^{-1})	Intra-day (% RSD)[a]	Inter-day (% RSD)[a]
Quercetin	15	1.11	1.28
	30	0.95	0.98
	60	1.15	1.21
Kaempferol	35	1.07	1.13
	85	0.91	0.95
	200	1.21	1.29
ß-sitosterol	15	0.94	0.97
	55	1.01	1.34
	200	0.98	1.12
Lupeol	15	1.20	1.32
	35	1.03	1.15
	80	1.12	1.27

[a] ***Mean (n=3) ± S. D.***

Table 4 : Recovery studies for examined phytoconstituents

Phyto-constituent	Amount of phytoconstituent present (µg mL^{-1})	Amount of phytoconstituent Added (µg mL^{-1})	Amount of phytoconstituent Found (µg mL^{-1})	Recovery (%)	Accuracy (average % recovery)[a]
Quercetin	11.83	2.96	14.71	99.46	99.93
	11.83	5.92	17.32	99.82	
	11.83	11.83	23.78	100.5	
Kaempferol	116.03	29.01	140.95	97.18	98.49
	116.03	58.02	169.32	97.98	
	116.03	116.03	333.14	100.32	
ß-sitosterol	121.56	30.39	146.98	96.73	97.60
	121.56	60.78	177.52	97.30	
	121.56	121.56	240.13	98.77	
Lupeol	22.12	5.53	26.78	96.85	98.86
	22.12	11.06	34.65	104.43	
	22.12	22.12	42.16	95.29	

[a] ***Mean (n=3) ± S. D.***

Table 5 : Assay of examined phytoconstituents

Sample	Content of Quercetin (mg g^{-1})[a]	Content of Kaempferol (mg g^{-1})[a]	Content of ß-sitosterol (mg g^{-1})[a]	Content of Lupeol (mg g^{-1})[a]
***Cuscuta reflexa* Roxb.**	0.0296 ± 0.0015	0.2901 ± 0.0011	0.3039 ± 0.0034	0.0553 ± 0.0107

[a] ***Mean (n=3) ± S. D.***

The developed method uses a single mobile phase to separate and quantitate the four markers which is simple and time consuming. The proposed method can be used as a quality control tool for analysis of Quercetin, Kaempferol, ß-sitosterol and Lupeol in marketed herbal drugs, extracts, polyherbal formulations and in house formulations.

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