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Simultaneous Quantification of Pharmacologically Active Markers Quercetin, Kaempferol, Bergenin and Gallic Acid from *Cuscuta Campestris* Yuncker Using HPTLC

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Abstract

Background: *Cuscuta campestris* Yuncker. (Cuscutaceae), is a rootless obligate stem holoparasite that subsists on various plant species worldwide, including economically important crops and reduces their yields substantially causing damage in agriculture. Cuscuta campestris has been reported for its analgesic, anthelminthic, antipyretic, anti-inflammatory activity. Cuscuta campestris is reported to contain cuscutin, quercetin, kaempferol, kaempferol-3-O-glycoside, hyperoside, β-sitosterol, pinoresinol, arbutin, astragalin.

Aims and objective: This project work aims at capitalising the therapeutic potential of the plant by quantitating the various phytoconstituents present in the plant. Materials and methods: Separation and detection of four markers was achieved using Toluene: ethyl acetate: methanol: formic acid (6: 6: 2: 1, v/v/v/v). The method was validated as per the norms of the International Conference on Harmonisation (ICH) guidelines.

Results: The amount of quercetin, kaempferol, bergenin and gallic acid was found to be 0.5520 ± 0.0090 mg g^{-1}, 0.4980 ± 0.0126 mg g^{-1}, 3.5630 ± 0.0633 mg g^{-1}, 1.3498 ± 0.0234 mg g^{-1} respectively.

Conclusion: The method was found sensitive, accurate and reproducible. Therefore it can be recommended for marker-based standardization and quality assurance of Cuscuta campestris. The present research work is an attempt for simultaneous quantification of quercetin, kaempferol, bergenin and gallic acid from Cuscuta campestris in a single mobile phase. The validated HPTLC method can be further applied to evaluate the quality of any plant reported to possess these phytochemicals.

Keywords: Bergenin; *Cuscuta campestris*; Field dodder; Gallic acid; HPTLC; Kaempferol; Quercetin

Introduction

Cuscuta campestris Yuncker. (Cuscutaceae) is one of the most widespread parasitic weeds [1]. It is a stem holoparasitic plant without roots and leaves, but it can grow absorptive organ haustoria that provide physical and physiological bridge between itself and its host [2]. It can infect diverse host species [3,4], self-parasitize and hyper-parasitize [5], and it causes vast damage in agriculture [6]. These plants have evolved special adaptations to ensure their success, germination occurs late in the season when potential hosts are already established; seedlings selectively forage in plant communities and they may survive relatively long periods during the autotrophic stage. Invasion occurs via extremely elaborate mechanisms designed to match the biological processes of their host and bypass defense mechanisms. *Cuscuta campestris* is a weed of at least 25 crops in 55 countries [7].

Dodders contain different biologically active phytoconstituents which exhibit therapeutic effects [8]. There are different species of Cuscuta which have been reported for various therapeutic activities. All species of Cuscuta are likely to possess allied medicinal properties. Inspite of its parasitic activity, Cuscuta campestris possesses analgesic [9], antipyretic, hypothermic, anti-inflammatory, anthelmintic [10], antibacterial, antimalarial, antifungal, anti-leishmanial, insecticidal, phytotoxic [11] and CNS depressant activity [12] on different host. Chemical constituents reported in Cuscuta campestris are cuscutin, quercetin, kaempferol, kaempferol-3-O-glycoside, hyperoside, β-sitosterol, pinoresinol, arbutin, astragalin. C. campestris cause severe damages to carrots, alfalfa, sugarbeet, onions, legumes and other crops [7]. Cuscuta reflexa possess various therapeutic activities like antispasmodic, antihypertensive, muscle relaxant, cardiotonic, antiviral, anticonvulsant, hair growth [13], antisteroidogenic, anti-inflammatory, anti-epileptic, antitumor activity and is also known to have hypoglycemic [14], psychopharmacological, antifertility, dose dependent hypotensive and bradycardiac effects. The chemical constituent it contains are flavonoids, glycosides, alkaloids, cuscutalin, β-sitosterol, stigmasterol, kaempferol, dulcitol, myricetin, quercetin, quercetin-3-O-glucoside and bergenin [15] while Cuscuta chinensis possess anti-cancer [16], immunostimulatory and antioxidant activities [17] and the phytoconstituents reported are flavonoids, especially rutin, quercetin, isorhamnetin, kaempferol, lupeol, β-sitosterol, stigmasterol [18]. These biologically active phytoconstituents are also reported to have several pharmacological activities.

Quercetin is reported for its anti-allergic, anticancer [16], antitumor [19], immunomodulatory [20] activities. Similarly antidiabetic [21] and anticancer [17] activity of Kaempferol have also been reported. Bergenin is reported to possess biological activities such as antiulcer,

antihepatotoxic, anti-HIV, antiarrhythmic, neuroprotective, antifungal, anti-inflammatory, immunomodulatory and burn wound healing effects [22]. Gallic acid is known to have antioxidant, antitumoral, anti-inflammatory, anti-diabetic activity [23].

In recent years there has been a growing interest in the chemical composition and biological activities of C. campestris.There is HPTLC method reported for simultaneous quantification of quercetin, kaempferol, β-sitosterol and lupeol from Cuscuta reflexa [24]. Simultaneous quantification of bergenin, (+)-catechin, gallicin, gallic acid and β-Sitosterol using HPTLC from Bergenia ciliata [25,26] and simultaneous estimation of gallic acid, curcumin & quercetin by HPTLC is also reported [27]. Quercetin and kaempferol was also simultaneously quantitated from two medicinal plants using HPTLC [15,28]. A RP-HPTLC method (using RP-18 F254 TLC plates with dual run) for simultaneous determination of major flavonoids (including apigenin and quercetin) from herbal extracts has been reported [29].

But, there is no method reported for simultaneous separation and quantification of these four pharmacologically active markers quercetin, kaempferol, bergenin and gallic acid from any plant matrix using HPTLC. Therefore, in the present work an attempt has been made for simultaneous quantification of quercetin, kaempferol, bergenin and gallic acid from C. campestris in a single mobile phase.

Materials and Methods

Plant material

Cuscuta campestris (Whole plant) growing on host broad bean {Vicia faba L. (Fabaceae)} was collected from Indapur (Raigad). The plant was authenticated from Agharkar Research Institute, Pune. The plant material was shade dried for five days and was thereafter maintained at 35 ± 2°C for five days. The plant material was then powdered, sieved through 85 mesh and was then stored in airtight plastic bottle at room temperature for further analysis.

Chemicals

All the chemicals used were of analytical grade. Standards quercetin (≥ 98% purity), kaempferol (≥ 96% purity), gallic acid (≥ 98% purity) were procured from Sigma Aldrich, Germany and bergenin (≥ 97% purity) was procured from Chengdu Biopurify Phytochemicals Ltd, China. Figure 1 represents the Structures of Quercetin, Kaempferol, Bergenin, Gallic acid.

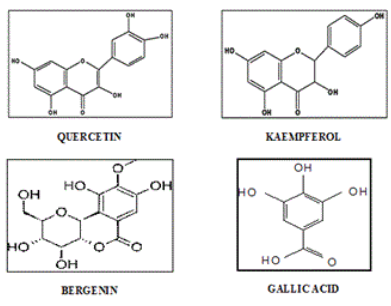

Figure 1: Structures of Quercetin, Kaempferol, Bergenin, Gallic acid.

Optimizing quantification conditions

Chromatographic separation was achieved on TLC plates (20 X 20 cm) precoated with silica gel 60 F254 (E. Merck) of 0.2 mm thickness with aluminium sheet support. Samples were spotted using CAMAG Linomat 5 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 twenty minutes. Scanning device used was CAMAG TLC Scanner 4supported by winCATS software version 1.4.7 equipped with CAMAG Linomat 5 sample spotter and CAMAG Reprostar 3 system for photo-documentation. The experimental condition was maintained at 22 ± 2°C.

Chromatographic evaluation of phytochemical markers

Extraction of phytoconstituents from Cuscuta campestris: Extraction of phytoconstituents from Cuscuta campestris was optimized to achieve good fingerprint and also to resolve the marker compounds efficiently. To the accurately weighed powdered drug (1 g), 10 mL of ethanol was added, vortexed for 5 minutes for thorough mixing and the mixture was kept standing overnight. Next day it was filtered through Whatman filter paper No. 1 and the filtrate (10 µL) was then used for HPTLC analysis.

Preparation of Standard solutions: Stock solutions of quercetin, kaempferol, bergenin and gallic acid (1000 µg mL^{-1}) were prepared by dissolving 10 mg each of accurately weighed standards in methanol and making up the volume to 10 mL in standard volumetric flask respectively. For calibration curve aliquots of 20-100, 20-100, 15-250 and 30-250 µg mL^{-1} were prepared from this stock solution for quercetin, kaempferol, bergenin and gallic acid respectively.

Further three quality control samples (LQC, MQC, HQC) each of quercetin (26, 45, 85 µg mL^{-1}), kaempferol (26, 45, 85 µg mL^{-1}), bergenin (20, 70, 210 µg mL^{-1}) and gallic acid (35, 85, 210 µg mL^{-1}) were prepared for precision, accuracy and ruggedness studies.

Solvent system: A single solvent system consisting of Toluene: Ethyl acetate: Methanol: Formic acid (6: 6: 2: 1, v/v/v/v) has been used in this method to resolve and to quantitate all the four marker compounds viz Quercetin, Kaempferol, Bergenin and Gallic acid from plant extract of Cuscuta campestris.

Method Validation

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

Instrumental precision

Instrumental precision was checked by repeated scanning (n=7) of the same spot of Quercetin (26 µg mL^{-1}), Kaempferol (26 µg mL^{-1}), Bergenin (20 µg mL^{-1}) and Gallic acid (35 µg mL^{-1}) and further expressed as relative standard deviation (%RSD).

Repeatability

The repeatability of the method was affirmed by analysing 26 µg mL^{-1} of Quercetin, 26 µg mL^{-1} of Kaempferol, 20 µg mL^{-1} of Bergenin and 35 µg mL^{-1} of Gallic acid individually on a TLC plate (n=5) and expressed as %RSD.

Inter-Day and Intra-Day precision

Precision of the method was evaluated by analysing Quality control samples of Quercetin, Kaempferol, Bergenin and Gallic acid on the same day (intra-day precision) and on different days (interday precision). Each level of precision was investigated by five sequential replicates of injections of Quercetin (26, 45, 85 µg mL^{-1}), Kaempferol (26, 45, 85 µg mL^{-1}), Bergenin (20, 70, 210 µg mL^{-1}), and Gallic acid (35, 85, 210 µg mL^{-1}) respectively and the results were expressed as % RSD.

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, bergenin and gallic acid were applied along with methanol as blank and were determined on the basis of signal-to-noise (S/N) ratio. LOD was determined at S/N of 3: 1 and LOQ at an S/N of 10: 1.

Recovery

The accuracy of the method was assessed by performing recovery study at three different levels (LQC, MQC and HQC-spiking of quercetin, kaempferol, bergenin and gallic acid in plant matrix). The percent recovery and the average percent recovery for each were calculated.

Specificity

Specificity was ascertained by analyzing standard compounds and samples. The bands for quercetin, kaempferol, bergenin and gallic acid from sample solutions were confirmed by comparing the Rf and spectra of the bands to those of the respective standards.

Ruggedness

Ruggedness of the method was assessed by incorporating the small variations in the optimized chromatographic condition. Effect of change in analyst, change in mobile phase composition [Toluene: ethyl acetate: methanol: formic acid (5.9: 5.9: 2: 1, v/v/v/v) and Toluene: ethyl acetate: methanol: formic acid (6.1: 6.1: 2: 1, v/v/v/v)] and change in spotting volume (9 µL and 11 µL) on the response and Rf of quality control samples was observed.

Sample solution (10 µL) was applied five times to a precoated silica gel 60 F254 TLC plate (E.Merck) with the CAMAG Linomat5 sample spotter. The plate was developed and scanned at 254 nm for quercetin, kaempferol, bergenin and gallic acid.

Results and Discussion

Of the various solvent systems tried, mixture containing Toluene: ethyl acetate: methanol: formic acid (6: 6: 2: 1, v/v/v/v) gave the best resolution of quercetin (Rf=0.60), kaempferol (R=0.63), bergenin (Rf=0.30) and gallic acid (Rf=0.46) from the other components of the ethanolic extract of Cuscuta campestris and enabled their simultaneous quantification. The identity of bands of quercetin, kaempferol, bergenin and gallic acid in plant matrix was confirmed by overlay in UV absorption spectra with those of the standards quercetin, kaempferol, bergenin and gallic acid using CAMAG TLC scanner 4 (Figure 2).

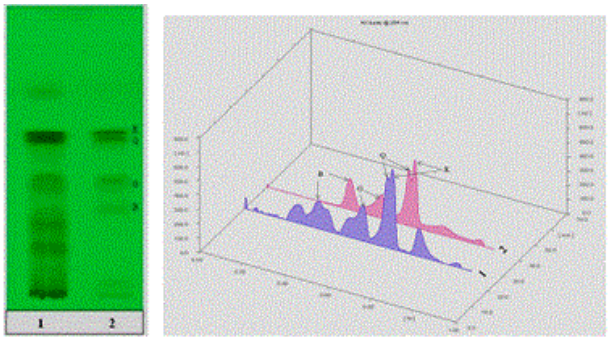

Figure 2: (A) HPTLC plate photo (B) overlay of the chromatograms. Track details (For figure A & B) 1) Extract of C. campestris 2) Bergenin (B) (100 µg/mL), Gallic acid (G) (100 µg/mL), Quercetin (Q) (100 µg/mL), Kaempferol (K) (100 µg/mL).

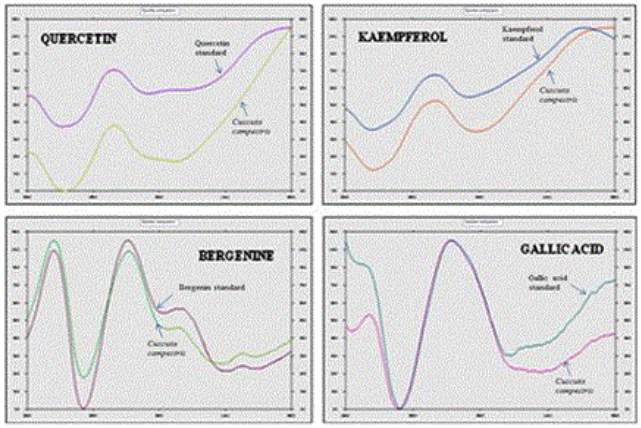

Figure 2C: Spectra of C. campestris with quercetin, kaempferol, bergenin and gallic acid at 254 nm.

The four pharmacologically active phytoconstituents quercetin, kaempferol, bergenin and gallic acid were quantified from Cusuta campestris by TLC densitometric method. The developed method was validated in terms of precision, repeatability and accuracy (Table 1).

Sl. No.	Parameter	Quercetin	Kaempferol	Bergenin	Gallic acid
1	Instrumental precision (% RSD, n = 7)	1.71	1.58	1.11	1.83
2	Repeatability (% RSD, n = 5)	1.49	1.52	1.07	1.30

3	Accuracy (average % recovery)	96.42	96.61	99.45	97.13
4	LOD (µg mL-1)	10	5	5	25
5	LOQ (µg mL-1)	20	10	10	30
6	Rf value	0.60	0.63	0.30	0.46
7	Specificity	Specific	Specific	Specific	Specific
8	Ruggedness	Rugged	Rugged	Rugged	Rugged

Table 1: Method Validation parameters for examined phytoconstituents.

The linearity range for quercetin, kaempferol, bergenin and gallic acid was found to be 20-100, 20-100, 15-250 and 30-250 µg mL^{-1} respectively, with coefficient of determination (r^2 values) 0.999, 0.999, 0.999, 0.999 (Table 2).

Phytoconstituents	Linear Working Range (µg mL^{-1})	Regression equation	Coefficient of determination (r^2)
Quercetin	20-100	y = 152.55x-332.57	0.999
Kaempferol	20-100	y = 73.937x+3483.5	0.999
Bergenin	15-250	y = 35.637x+1083.1	0.999
Gallic acid	30-250	y =79.04x+679.56	0.999

Table 2: Calibration parameters of examined phytoconstituents.

Phytoconstituents	Concentration (µg mL^{-1})	Intra-day (% RSD)a	Intra-day (% Nominal)a	Inter-day (% RSD)a	Inter-day (% Nominal)a
Quercetin	26	1.51	91.46	1.6	91.92
	45	0.33	88.53	1.04	87.97
	85	1.69	101.05	0.85	92.53
Kaempferol	26	1.74	97.15	1.57	99.94
	45	0.94	103.41	0.87	102.1
	85	1.61	98.68	1.61	99.31
Bergenin	20	0.48	105.71	1.07	106.64
	70	1.71	98.3	1.91	96.58
	210	1.52	102.71	1.69	104.3
Gallic acid	35	1.02	91.58	1.59	90.29
	85	1.07	89.25	1.61	90.04
	210	0.16	99.97	1.33	103.73
a Mean (n=5) ± S.D					

Table 3: Precision and accuracy studies for examined phytoconstituents.

The TLC densitometric method was found to be precise with % RSD for intra-day precision in the range of 0.33-1.69, 0.94-1.74, 0.48-1.71, 0.16-1.07 and for inter-day precision in the range of 0.85-1.60, 0.87-1.61, 1.07-1.91, 1.33-1.61 for quality control samples of quercetin, kaempferol, bergenin and gallic acid respectively (Table 3).

The % nominal for intra-day precision was in the range of 88.53-101.05, 97.15-103.41, 98.30-105.71, 89.25-99.97 % and for inter-day precision in the range of 87.97-92.53, 99.31-102.10, 106.32-109.67, 90.04-103.73%, for quality control samples of quercetin, kaempferol, bergenin and gallic acid respectively (Table 3). This indicates that the

method is precise. The LOD values for quercetin, kaempferol, bergenin and gallic acid were found to be 10, 5, 5, 25 µg mL^{-1} respectively while LOQ values were 20, 10, 10, 30 µg mL^{-1} respectively (Table 1). The average recoveries at three different levels of quercetin, kaempferol, bergenin and gallic acid were found to be 96.42, 96.61, 99.45 and 97.13% respectively (Table 4).

Concentrations			Accuracy (%)	Average recovery a(%)
Compounds	**Spiked (µg mL^{-1})**	**Founda(µg mL^{-1})**		
Quercetin	26	26.03 ± 0.064	100.12	96.42 ± 3.218
	45	42.4 ± 1.234	94.22	
	85	80.7 ± 0.954	94.94	
Kaempferol	26	23.63 ± 0.885	90.87	96.61 ± 7.653
	45	42.15 ± 0.524	93.66	
	85	89.5 ± 1.025	105.3	
Bergenin	20	19.65 ± 0.052	98.25	99.45 ± 1.224
	70	69.59 ± 0.547	99.41	
	210	211.45 ± 0.321	100.69	
Gallic acid	35	33.91 ± 0.810	96.88	97.13 ± 1.515
	85	81.40 ± 1.254	95.76	
	210	207.40 ± 0.552	98.76	
a Mean (n=5) ± S. D				

Table 4: Recovery studies for examined phytoconstituents.

Quercetin, kaempferol, bergenin and gallic acid were simultaneously quantified from the plant matrix and the amounts were found to be 0.5520 ± 0.0090 mg g^{-1}, 0.4980 ± 0.0126 mg g^{-1}, 3.5630 ± 0.0633 mg g^{-1}, 1.3498 ± 0.0234 mg g^{-1} respectively. 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 (9 and 11 µL) did not affect the Rf 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.

Conclusion

The developed method uses a single mobile phase to separate and quantitate the four markers which is simple and less time consuming. The proposed method can be used as a quality control tool for analysis of quercetin, kaempferol, bergenin and gallic acid in marketed herbal drugs, extracts, polyherbal formulations and in house formulations.

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