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A comparative estimation of quercetin content from *Cuscuta reflexa* Roxb.using validated HPTLC and HPLC techniques

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ABSTRACT

HPTLC and HPLC are two most widely accepted methods for determination of natural products. Present research work envisages microwave assisted extraction of quercetin from hydroethanolic extract of *Cuscuta reflexa* Roxb. (Cuscutaceae), an unusual parasitic vine. Further, chromatographic characterization of hydroethanolic extract of *C. reflexa* was carried out in terms of quercetin content using two validated methods (HPTLC and RP-HPLC). Confirmation of the presence of quercetin in the samples was carried out using Mass Spectrometry. HPTLC separation of quercetin was achieved on an aluminum-backed layer of silica gel 60 F_{254} using Toluene: ethyl acetate and formic acid as mobile phase while RP-HPLC was performed on Cosmosil C_{18}-column (150 mm x 4.6 mm, 5 µm) using mobile phase comprising of 0.025 M NaH_2PO_4 buffer – ACN (pH - 2.6) at a flow rate of 1.2 mL/min. ICH guidelines were followed for validation of both the the chromatographic methods. Samples of *C. reflexa* collected from different regions of India and growing on different hosts were also screened for their quercetin content. The developed chromatographic methods described here were found to be simple, rapid, accurate and sensitive.

INTRODUCTION

Chromatographic techniques such as HPTLC (Srinivas and Aparna, 2012; Shailajan *et al.*, 2013; Mamatha, 2011) and HPLC (Dhalwal *et al.*, 2010; Mehta *et al.*, 2001; Nicoletti, 2012) are reported to be useful for documentation and quantification of phytochemical markers form various medicinally important plants to identify morphological and geographical variations. However sample preparation techniques prior to subjecting the marker component in the instrument are time consuming. Conventional extraction techniques such as boiling, reflux etc. have been used for extraction but often results in the loss of phytochemical constituents due to oxidation or ionization, also prolonged extraction period makes the conventional extraction technique more cumbersome (Wang *et al.*, 2010). Microwave assisted extraction (MAE) has evolved as an efficient extraction techniques in recent decades (Huang and Zhang, 2004). It is the simplest and the most economical technique for extraction of many plant derived compounds. There have been previous reports on extraction of phytochemicals such as flavonoids (Wang *et al.*, 2010), Curcumin (Mandal *et al.*, 2008), Lupeol (Shailajan *et al.*, 2011a), saponins (Kerem *et al.*, 2005) etc. by MAE. But no literature is available for extraction of phytochemicals from *Cuscuta reflexa* by MAE. *C. reflexa* is an unusual parasitic vine, growing in a prolific manner over host plants with inter-twined stems and commonly found throughout India (Kapoor, 2005). Cuscutin, kaempferol, quercetin, lupeol, ß-sitosterol, α-amyrin, ß-amyrin, stigmasterol are the pharmacologically active markers reported from *C. reflexa* (Shailajan *et al.*, 2011). There are several reports indicating a wide range of pharmacological activities of *C. reflexa* such as antispasmodic, antihypertensive, antiviral, anticonvulsant, hypoglycemic, antifertility, anticancer, antiepileptic etc. (Sharma *et al.*, 2012).

Chromatographic methods such as RP-HPLC for estimation coumarins (Uddin *et al.*, 2007), TLC and HPLC methods for estimation of Kaempferol (Mitra *et al.*, 2011) have been reported from *C. reflexa* but there are no reports available on estimation of quercetin from *C. reflexa* simultaneously by HPTLC and HPLC. Quercetin (Figure 1) a bioactive flavonoid is reported to be present in its glycosidic form in most of the plants (Huang and Zhang, 2004). Hence in the present research work acid hydrolysis of the sample was carried out under optimized microwave

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conditions in order to obtain quercetin in the aglycone form. The purpose of the present research work was to develop extraction technique based on microwave assisted acid hydrolysis (MAAH). Two chromatographic methods (HPTLC and RP-HPLC) have been developed and validated for estimation of quercetin from different samples of *C. reflexa*. Mass spectrometric (MS) method was used for confirmation quercetin in plant samples.

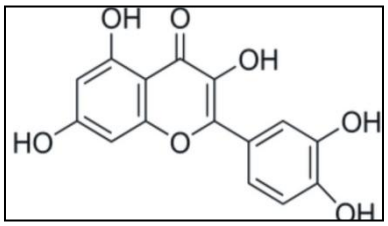

Fig. 1: Structure of quercetin.

MATERIAL AND METHODS

Samples and reagents

Quercetin (98% purity) was procured from Sigma Aldrich (Steinheim, Germany). Analytical grade ethyl acetate, formic acid, hydrochloric acid, phosphoric acid and toluene while HPLC grade acetonitrile and water were procured from Merck Specialities Pvt. Ltd. (Darmstadt, Germany). Sodium dihydrogen orthophosphate was purchased from Merck Specialities Pvt. Ltd.

Plant material

C. reflexa (whole plant) growing on different hosts namely *Clerodendrum inerme*, *Cordia domestica*, *Alstonia scholaris*, *Vitex negundo* and *Coccinia grandis* were collected from Aligarh (Uttar Pradesh), Baroda (Gujrat), Delhi, Roha (Maharashtra) and Mumbai (Maharashtra) respectively. The plant was authenticated from Agharkar Research Institute, Pune (Auth11-36). The plant materials were shade dried for three days and were kept thereafter in hot air oven preset at 35±2℃ for a week. The plant materials were then powdered sieved through 80 mesh (BSS) and were stored in airtight plastic bottles at room temperature for further analysis.

Apparatus

Conventional hydrolysis

A round bottom flask fitted with a cool water condenser was used for acid hydrolysis of samples by reflux method.

MAAH

Open vessel modified microwave system Model: GMC25E09- MRGX, Godrej equipped with a magnetron of 2450 MHz with nominal maximum power of 640 watts operated at 3 power levels and the time controller was used for MAAH.

Sample preparation procedure

Five hundred milligram of plant powder was accurately weighed in a round bottom flask containing 2N HCl (in 80% ethanol). The flask was placed in an oil bath and fitted with a cold water condenser and heated for 2 h at 100 ^{o}C. Similarly for MAAH, the flask was placed in the microwave oven, fitted with a water condenser and irradiated. The MAAH was optimized for solvent, solvent volume, sample soaking time, irradiation power and time of irradiation.

Sample cleanup for HPTLC and HPLC determination

After hydrolysis by conventional and microwave technique the samples were cooled at room temperature and filtered trough whatmann paper no 1, the marc was washed with a minimum amount of ethyl acetate (≈ 5 to 6 mL). The combined filtrates were transferred to a separating funnel, and distilled water (twice the quantity of filtrate) was added. Further the mixture was extracted with ethyl acetate (20 mLx 4). The ethyl acetate fractions were pooled and dehydrated over Na_2SO_4. It was then evaporated to dryness on a rotary evaporator at 40 ℃. The residue was reconstituted in ethanol. This solution was further used for chromatographic analysis.

Optimized Instrumental and Chromatographic Conditions

High Performance Thin Layer Chromatography (HPTLC)

Chromatographic analysis was performed on TLC plates pre-coated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with aluminum sheet support. Samples (10.0 µL) were spotted using CAMAG Linomat 5 (Switzerland) equipped with syringe (Hamilton, 100.0 µL). Plates were developed in a glass twin trough chamber (CAMAG) pre-saturated for 15 min with the mobile phase consisting of toluene-acetone-formic acid (5:2:0.5 v/v/v) and scanned at 378 nm using CAMAG TLC Scanner 4 conjugated with winCATS software Ver. 1.4.7. The temperature was maintained at 20 ±2℃. The plates were photo-documented using CAMAG reprostar 3 at 254 nm.

High Performance Liquid Chromatography (HPLC)

Chromatographic analysis was performed at 22±2℃using Jasco's HPLC system comprising of two PU-1580 pumps (HG-1580-31), rheodyne injector (20 µL loop) and photo diode array detector (MD-1510). Chromatograms were recorded by means of Jasco-Borwin chromatography software version 1.50. Separation was achieved on Cosmosil C_{18}-column (150 mm x 4.6 mm, 5.0 µm) using mobile phase as 0.025 M NaH_2PO_4:ACN (72:28 v/v) delivered at a flow rate of 1.2 mL/min. After a 10 min of equilibration period, the samples were injected into HPLC system. Peaks were recorded at 378 nm.

Mass spectrometric analysis (MS)

Mass spectrometric analysis was performed on an Applied Biosystems Hybrid Q-Trap API 2000 mass spectrometer (AB-MDS Sciex, Toronto, Canada) equipped with an electrospray ionization source (ESI). Data acquisition and analysis were performed using Analyst software 1.3 (AB Sciex, USA). The ESI source was operated in the negative ionization mode. Channel electron multiplier (CEM) was used as a detector. The compound-

dependent parameters were as follows: declustering potential and entrance potential were set at -50 V and -10 V. The common parameters set were as follows: curtain gas, ion spray voltage and nebulizer gas were 20 psi, -4500 V and 50 psi respectively.

Preparation of standard stock solutions

A stock solution of quercetin (1000 µg/mL) was prepared in ethanol. Seven calibrant samples (6 – 40 µg/mL for HPTLC and 0.5-100 µg/mL for HPLC) and three quality control samples namely low-LQC, mid-MQC and high-HQC (8.0, 16.0, 32.0 µg/mL respectively for HPTLC while 0.65, 7.0, 80.0 µg/mL respectively for HPLC) of quercetin were prepared in ethanol using the stock solution.

Method validation

The HPTLC and HPLC methods for determination of quercetin from *C. reflexa* samples were validated as per ICH guidelines (Shailajan *et al*., 2012b). The validation parameters addressed were linearity, sensitivity, precision, accuracy, ruggedness, recovery and stability.

Statistical analysis

Microsoft Excel-2007 was used to determine mean, standard deviation (SD), coefficient of variation (CV) and mean difference during the analysis.

RESULTS AND DISCUSSION

Plants and plant products are subject to wide variation in their phytochemical profile due to their variety, climatic conditions, maturity, post-harvest processing, storage, stability etc. Therefore, it is extremely important to standardize these drugs based on their marker compounds (Madhavan, 2011). Extraction is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. It forms the first basic step in medicinal plant research. Traditional techniques such as plant tissue homogenization, soxhlet extraction, reflux, maceration, digestion, percolation etc. are most widely used for extraction of phytoconstituents from plants but these techniques requires longer extraction time thus running a severe risk of their thermal degradation.

In the present research work conventional and microwave assisted acid hydrolysis methods for extraction of quercetin from *C. reflexa* samples were developed, optimized and compared. The enhancement of product recovery by microwave may be attributed to its heating effect, due to the dipole rotation of the solvent in the microwave field which causes the solvent temperature to rise, followed by an increase in the solubility of the compound of interest.

Optimized extraction conditions

To evaluate the merits of developed microwave assisted extraction method, the quercetin yield obtained was compared with those obtained by reflux method (conventional extraction). Following the extraction, the samples were subjected to HPTLC analysis. The quercetin yield obtained by conventional extraction technique was found to be 0.6248 ± 0.0035 mg/g and by MAAH 1.235 ± 0.081 mg/g from the sample collected from Mumbai. Since the quercetin content by MAAH was found to be increased, the MAAH technique was further optimized for the following parameters.

Effect of soaking time

The plant samples were subjected to different soaking times (1-5 min) before microwave irradiation. It was observed that the yield of quercetin increased when soaked upto 3 min however, further increase in soaking time did not show any promising effect on the extraction performance (Mandal *et al*., 2008).

Effect of solvent volume

Generally in conventional extraction technique, a higher volume of solvent may increase the recovery, but in MAE, a higher solvent volume may give lower recoveries (Wang *et al*., 2010). In the present work, it was observed that yield of quercetin increased with an increase in the solvent volume of 6 mL, after which the yield decreased abruptly. This may be due to the larger volume of ethanol (80%) which caused excessive swelling of the material and absorbing the effective constituent (Xiao *et al*., 2008).

Effect of microwave power and irradiation time

The plant samples were irradiated at 160, 320 and 640 Watts for different time intervals (1 - 8 min). It was observed that the microwave irradiation power influenced the yield of quercetin to a higher extent. Decrease in the extraction time was observed with the increase in power. Irradiation of *C. reflexa* sample at 640 W for 3 min gave maximum yield of quercetin. This may be attributed to microwave irradiation energy which enhances the penetration of the solvent into the matrix and delivers efficiently to materials through molecular interaction with the electromagnetic field and offer a rapid transfer of energy to the solvent and matrix, allowing the optimum dissolution of components to be extracted (Duan *et al*., 2001; Hu, 2003).

Thus, maximum extraction of quercetin was achieved by soaking the plant material for 3 min prior to MAE using 6 mL of solvent and further irradiating it in microwaves for 3 min at 640 W power.

Chromatographic characterization and MS

Simultaneous estimation of four markers namely, quercetin, kaempferol, ß-sitosterol and lupeol using HPTLC was reported earlier by our group. However, in the method reported, quercetin attained R_f of 0.19 on TLC plate, which was much closer to the spotting position as well as with resolution issues. In the present work, the optimized mobile phase resulted in an improved R_f of 0.35 along with an efficient resolution of quercetin (Fig. 2 and 3). Similary in the case of HPLC a well separated peak

of quercetin (R_t - 7.1 min) was obtained with no interference of other peaks showing the selectivity of the chromatographic method (Fig. 4).

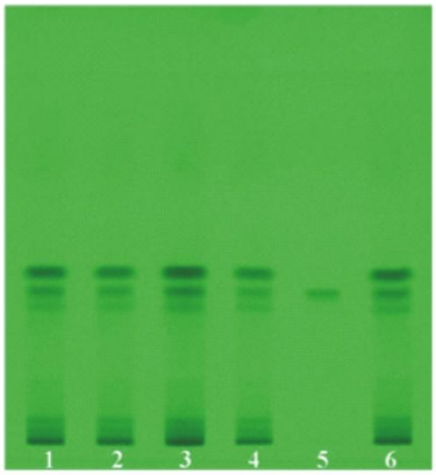

Fig. 2: HPTLC plate photo showing presence of quercetin from *C. reflexa* samples collected from different geographical regions (254 nm).

Both the methods were validated as per ICH guidelines and were found to be simple, rapid, specific, precise, sensitive and rugged during the validation experiment which on comparison showed that the HPLC method was more sensitive than HPTLC in estimating quercetin from plant matrix (Table 1). Both the methods were applied to evaluate the impact of regional variation on the content of quercetin in *C. reflexa* whole plant collected from different provinces of India (Table 2). It was found that the sample collected from Mumbai showed maximum content of quercetin, while the content of quercetin in other samples was as per this order Delhi > Baroda > Aligarh > Roha (Table 2).

Confirmation of quercetin in *C. reflexa* samples using MS

Mass spectrum for blank (without any injection), standard (quercetin) and quercetin from *C. reflexa*, were obtained. It was observed that the signal intensities of quercetin were prominent in the negative electrospray ionization mode. Thus, the quadrupole full-scan mass spectra were recorded in the negative ionization mode. The mass spectra revealed intense protonated molecular ion peaks, for quercetin at m/z 301.6 (Fig. 5). Similarly mass spectra for *C. reflexa* gave intense molecular ion peak at m/z 301.6 confirming the presence of quercetin in *C. reflexa*. Chromatographic methods developed in the current research work can be useful as routine quality control tools for the samples of *C. reflexa* and its formulations. These methods can also be applied to various plant matrices and polyherbal formulations containing quercetin.

CONCLUSION

MAAH was developed for sample preparation of *C. reflexa* prior to HPTLC and HPLC analysis. Compared to reflux heating, the time and energy were saved drastically in MAAH. Moreover the quercetin content was found to be much higher in MAAH. The developed HPTLC and RP-HPLC methods can be readily transferred to the industries for estimation of quercetin from any matrices containing *C. reflexa*. The concept can be applicable to all natural products and if explored properly, can prove to be an efficient tool for sample preparation and large-scale industrial application.

Table. 1: Method validation parameters for estimation of quercetin from *C. reflexa* using HPTLC and HPLC technique.

Parameters	**Results**	
	HPTLC	**HPLC**
LOD (µg/mL)	2.0	0.1
LOQ (µg/mL)	6.0	0.5
Linear range (µg/mL)	6.0 – 40.0	0.5 – 100.0
Regression equation	y = 279.6 x - 792.5	y = 37316 x – 6978
Mean coefficient of determination (r^2)	0.996	0.998
System suitability (% CV, n = 5)		
R_f / R_t	1.58	1.37
Area	0.98	1.16
Precision (% CV, n = 3)		
Within-Batch	1.25 – 1.36	0.18 – 1.48
Between-Batch	0.60 – 1.03	1.46 – 1.85
Recovery (%, n = 7)		
LQC	88.13	89.82
MQC	88.07	89.74
HQC	87.60	88.05
Stability		
Long-term stability		
Standard Stock Solution stability (For 15 days)	Stable at (4 ± 1^0C)	Stable at (4 ± 1^0C)
Short-term stability		
Bench top stability (For 6.00 h)	Stable at (25 ± 2^0C)	Stable at (25 ± 2^0C)
Short term stability (For 12.00 h)	Stable at (4 ± 1^0C)	Stable at (4 ± 1^0C)
Robustness	Robust	Robust

Table. 2: Content of quercetin in the samples of *C. reflexa* collected from different geographical regions using validated HPTLC and HPLC methods.

Samples	Concentration of quercetin in mg/g(Mean ± SD, n=7)	
	HPTLC	HPLC
Mumbai	1.235 ± 0.081	1.668 ± 0.035
Delhi	1.041 ± 0.049	1.089 ± 0.070
Baroda	1.014 ± 0.090	1.052 ± 0.016
Aligarh	0.864 ± 0.043	0.897 ± 0.022
Roha	0.816 ± 0.046	0.869 ± 0.022

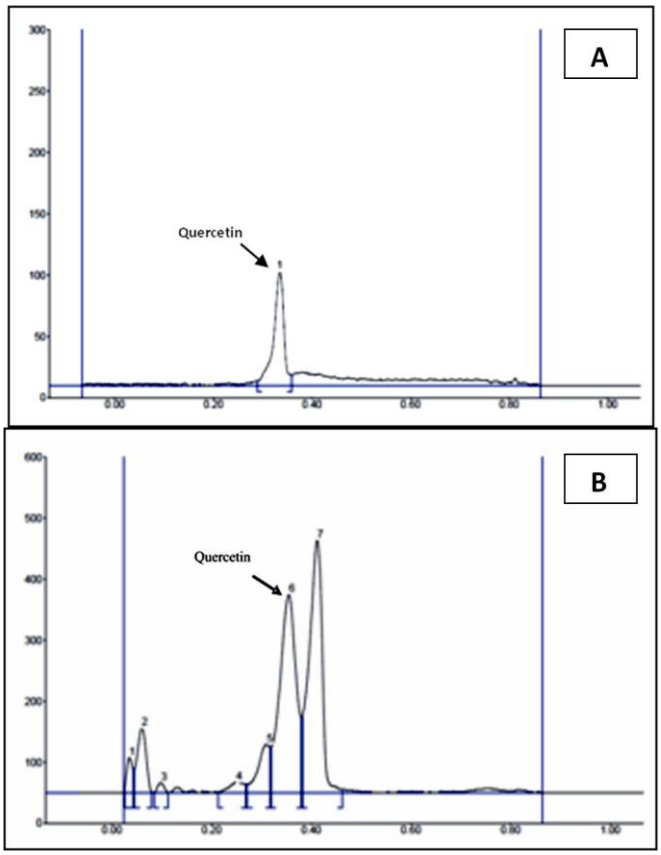

Fig 3: Representative HPTLC chromatograms of Quercetin (A) and *C. reflexa* (B) collected from Mumbai.

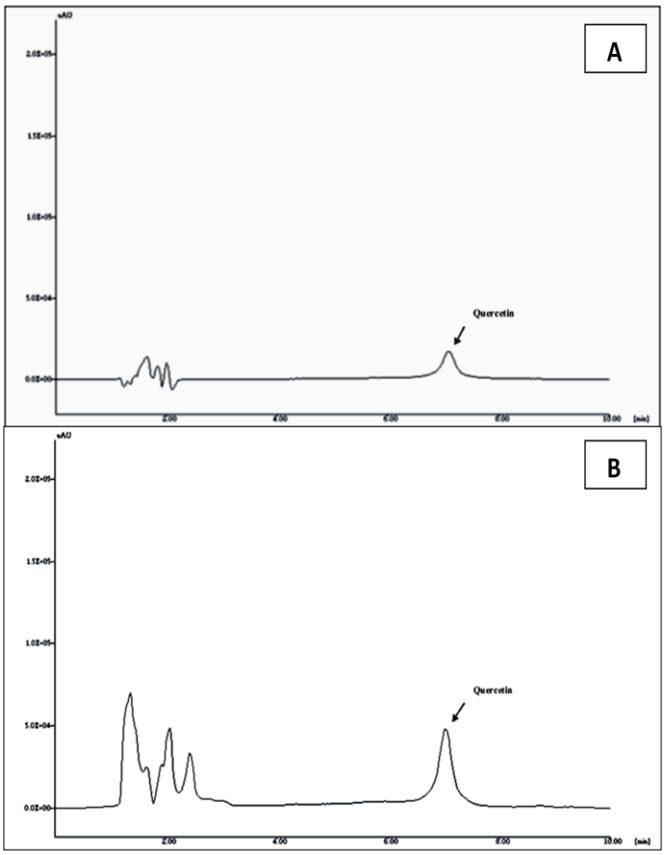

Fig. 4: Representative HPLC chromatograms of Quercetin (A) and *C. reflexa* (B) collected from Mumbai.

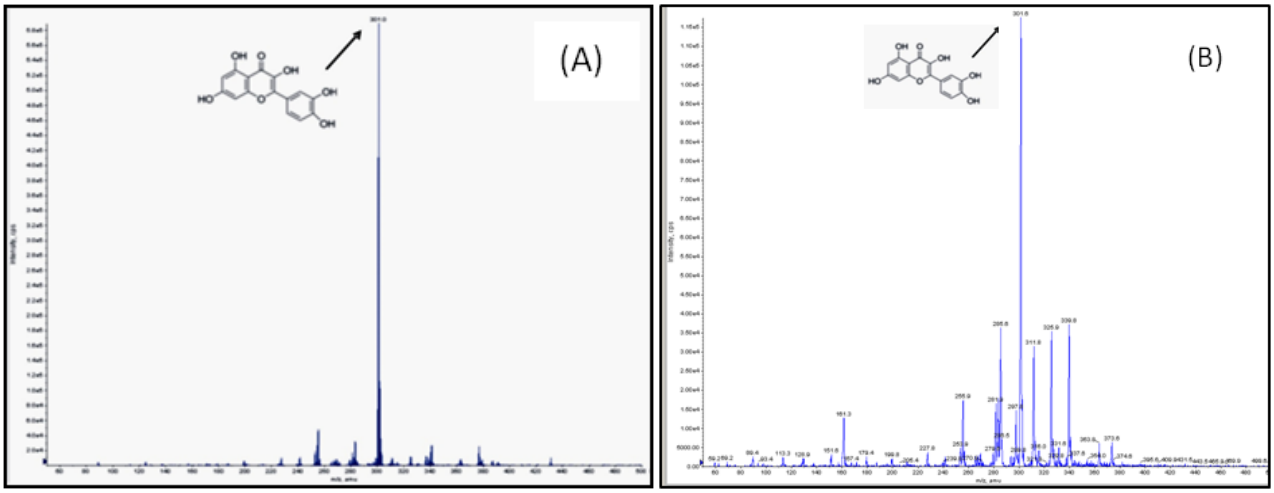

Fig. 5: Representative full scan Q1 MS mass spectrum of standard quercetin (A) and quercetin from *C. reflexa* (B) collected from Mumbai.

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