ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

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RP-HPLC-DAD determination and quantification of Quercetin and Luteolin in plant extracts of Merremia aegyptia and Merremia dissecta

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Article History:

Received on: 24 Mar 2020
Revised on: 11 Apr 2020
Accepted on: 21 May 2020

Keywords:

HPLC,
Flavonoids,
Luteolin,
Quercetin,
DAD,
ng and μl

ABSTRACT

Medicinal plants produce various useful metabolites such as alkaloids, flavonoids, tannins etc those are widely used for the preparation of various pharmaceutical products, or as food additives. A simple and precise reverse phase high performance liquid chromatographic (RP-HPLC) separation method has been developed for determination and quantification of the flavonoids, quercetin and luteolin simultaneously, from methanolic extracts of *Merremia aegyptia* and *Merremia dissecta* after optimization of extracting solvent and chromatographic conditions through HPLC coupled to a Diode Array Detector(DAD). HPLC analysis estimated contents of the quercetin to be 20 ng/μl in *M. aegyptia* stem and 13.2 ng/ μl in *M. dissecta* callus whereas luteolin was found to be 0.4 mg/ml in *M. dissecta* callus. From the previous published literature, it appears that this is the first report of quantification of these flavonoid compounds as they have not been reported earlier in both the species under study.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v11i3.2752

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INTRODUCTION

High Performance Liquid Chromatography is an advanced form of liquid chromatography in which small particle columns are used through which the mobile phase is flown at high pressure for better isolation, separation, identification and quantification of component of interest from a mixture. It is a highly automated technique that generates reports on its own using autosamplers and data systems thus, extending detection limits to nanogram, picogram and even below levels. It was once gravity-driven process but now automation of this technique has lead to divergent methods of chromatography.

The methods of determination and quantification of plant biochemicals that are used on regular basis include Ultra Violet-Visible spectrophotometry, HPLC, High Performance Thin Layer Chromatography and Mass Spectrometry. High performance liquid chromatography coupled with diode array detector (HPLC-DAD) has been reported to quantify isolated compounds present in the polar soluble fraction of plant samples. Many compounds from various plants have been isolated by advanced HPLC methods from *Glycyrrhiza glabra* (Khalaf *et al.*, 2010) and *Cayratia trifolia* (Gour *et al.*, 2013), *Tridax procumbens* (Sanghavi *et al.*, 2014), *Citrus macroptera* (Nongalleima *et al.*, 2017) and *Matricaria chamomilla* (Dong *et al.*, 2017).

The plant species undertaken for the present study have large number of useful chemical constituents like various alkaloids, tannins, flavonoids, saponins such as stigmasterol, β-sitosterol, quercetin, γ sitosterol (clinosterol), β and α-amyrin, campesterol, ergosterol, oleamide, dibutyl phthalate, mandelic acid, guaiol, bergamotol and hexadecanoic acid, Lauric acid, 1,2benzenedicarboxylic, Cyclopentasiloxane, Phloroglucinol were some important metabolites identified from these species (Joshi *et al.*, 2018).

EXPERIMENTAL

Flavonoids are also collectively known as 'Vitamin-P'. They are polyphenolic compounds and are subdivided according to their substituents into flavanols, anthocyanins, flavones, flavonones and chalcones. Quercetin [2- (3,4-dihydroxyphenyl) -3,5,7-trihydroxy-4*H*-1-benzopyran-4-one] possess antioxidant, antidiabetic ,anticancer, anti-inflammatory and antiviral activity. Some of its other biological activities include strong inhibition of histamine which is released from mast cells and preventing oxidation of low-density proteins hampering atherosclerosis plaque formation. Quercetin has showed *in vitro* antiproliferative activity also against ovary and stomach cancer cell lines (Larson *et al.*, 2009; Bashir *et al.*, 2011).

Luteolin, 3',4',5,7-tetrahydroxyflavone, is a polyphenol present in various vegetables and medicinal herbs (López-Lázaro, 2009). It is used for treating hypertension, inflammatory disorders, and cancer cells. Its anticancer property includes inducing apoptosis, inhibiting cell proliferation, metastasis, angiogenesis and also have antiplatelet and vasodilatatory activities. Luteolin is also used as antioxidant inhibiting Reactive Oxygen Species(ROS) induced damage of lipids, DNA, and protein (Brown and Rice-Evans, 1998) and have showed *in vitro* activities like anti-inflammatory activity (Kumazawa *et al.*, 2006), a phosphodiesterase inhibitor (Yu *et al.*, 2004) and an interleukin 6 inhibitor (Xagorari *et al.*, 2001) . *In vitro* and *in vivo* experiments also suggest luteolin may inhibit the development of various types of cancer such as skin cancer (Han *et al.*, 2002).

All of these activities suggest that quercetin and luteolin are compounds with potential clinical application. Determination of contents of quercetin and luteolin in *Merremia aegyptia* and *Merremia dissecta* have not been reported so far. This work was therefore designed to develop an RP-HPLC-DAD system to quantify quercetin and luteolin from methanolic extractive solutions of *Merremia aegyptia* and *Merremia* dissecta Figure 1.

Chemicals and reagents

Methanol (Chromatographic grade, Merck), phosphoric acid (Analytical grade, Merck), acetonitrile (Chromatographic grade,Merck) and acetic acid (Analytical grade, Merck) were used for the mobile phase preparation. Quercetin (Sigma, St Louis), Luteolin (Sigma, St.Louis) were used as external standards. HPLC grade MilliQ water was obtained by Millipore water purification system.

Plant Material

The whole plants of *Merremia aegyptia* and *Merremia dissecta* were collected from Jaipur. The specimens were identified by Herbarium, Department of Botany,University of Rajasthan, Jaipur **(**RUBL211617 and RUBL211618**)**. The plants collected were washed with water and dried in the shady area for several days.

Standard stock solution and Sample preparation

Standard stock solutions of luteolin, quercetin were prepared by dissolving them in methanol, at concentration of 1.0 mg/mL. All standard solutions were filtered through 0.45 μm syringe filter. The purity was checked through HPLC analysis monitoring individual compounds absorption maximum.

All the plant samples viz., Leaf, Stem, Seed and Callus each from *M. aegyptia* and *M. dissecta* were weighed (5gm) and powdered in a mechanical grinder to fine powder and soxhlet extracted in 80 percent methanol for 24 hrs at 40°C. The methanol phases were filtered the next day and evaporated in a vacuum to obtain extracts. The dried extracts were dissolved in methanol and diluted. All sample solutions were also filtered through 0.45 μm syringe filter (Chen and Xiao, 2005).

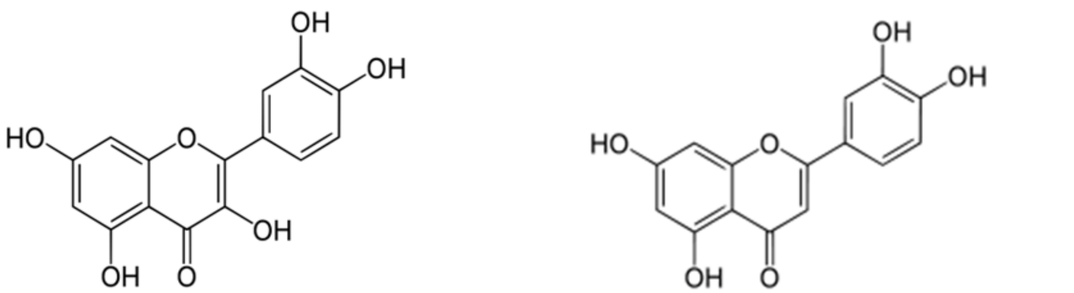
Instrumentation

5μl of each samples were injected with a HP Agilent 1200 infinity series quartenery pump autosampler with degasser and diode array detector(DAD), data analysis was performed with Agilent Chemstation software.

Determination and quantitative analysis

Determination and quantitative analysis of Quercetin and Luteolin in extracts were done through RP-HPLC following the protocol given by Chen and Xiao (2005)

Flavonoids were analyzed on a RP-C_{18} column((ZORBAX SB-C_{18},4.6*250mm, 5μM).; using a mobile phase, consisting of methanol-acetonitrile-acetic acid-phosphoric acid-H_2O (200:100:10:10:200, V/V); under the following conditions: detecting wavelength, 350 nm; flow rate, 1.0 ml/min; the sensitivity, 0.05 absorbance units full scale (AUFS) and the volume of injecting sample, 5.0 μl. The HPLC system was operated at ambient temperature (28$\pm$1°C).



Molecular formula :	$C_{15}H_{10}O_7$	Molecular formula:	$C_{15}H_{10}O6$
Molar Mass :	302.236g/mol	Molar mass :	286.25 g mol−1
Melting point-	309°C - 311°C	Melting point :	329.50 °C

Figure 1: Structure of Quercetin and Luteolin respectively

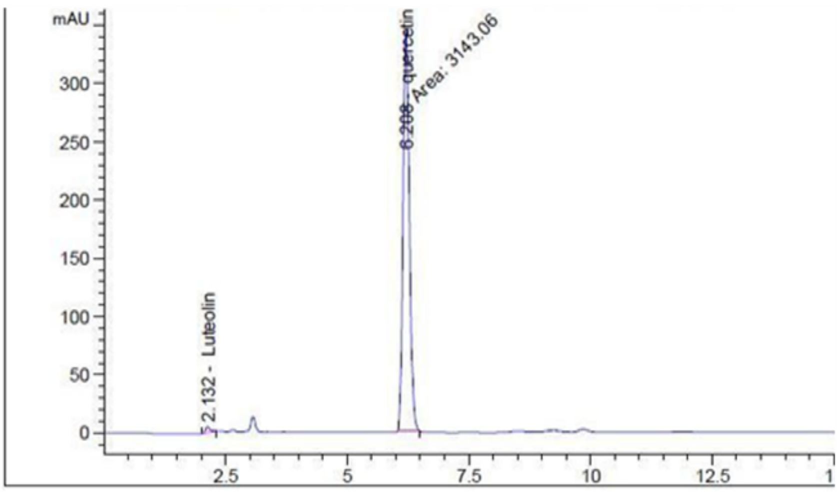


Figure 2: HPLC peaks and retention time of standard Quercetin and Luteolin

The method showed linearity for quercetin and luteolin in the range 2.1-20.6, 2.0-24.1 g/ml respectively, and the R.S.D. of the slope of these lines were 0.3%, 1.2% respectively. Precision and accuracy were determined for methanol 80% extractive solution. The recoveries were 95.92-98.10%, 92.18-95.13% for quercetin and luteolin respectively.

The mobile phase was chosen carefully to achieve maximum separation. It consisted of a gradient system of solution A- 0.025% Ortho-phosphoric acid and acetic acid in Milli Q water, solution B - Acetonitrile, solution C - Methanol, and at the flow rate of 1.0ml/min with starting pressure 114.0 bar and maximum pressure 126.0 bar, stop time 25 min with post time 5 min.

Analytes in each sample were identified by comparing the retention time and UV-Vis spectra at 260nm, reference 360nm with those of authentic compounds. Peaks were scanned between 190-400 nm for identification purpose and peak purity was checked to exclude any contribution from interfering peaks.

RESULTS AND DISCUSSION

HPLC is a versatile technique widely used for analysis of pharmaceuticals biomolecules, polymers and many organic and inorganic compounds. Chlorogenic acid and hippuric acid have been isolated and quantified from *Merremia emarginata* (Angappan *et al.*, 2018) and comparative analysis of *Merremia tridentata* and *Paederia foetida* have been also conducted to analyse the similarity between the active component found in both the plants used for treating vatarogas (Rajashekhara *et al.*, 2011) using HPLC methods.

The retention time and peak area observed for

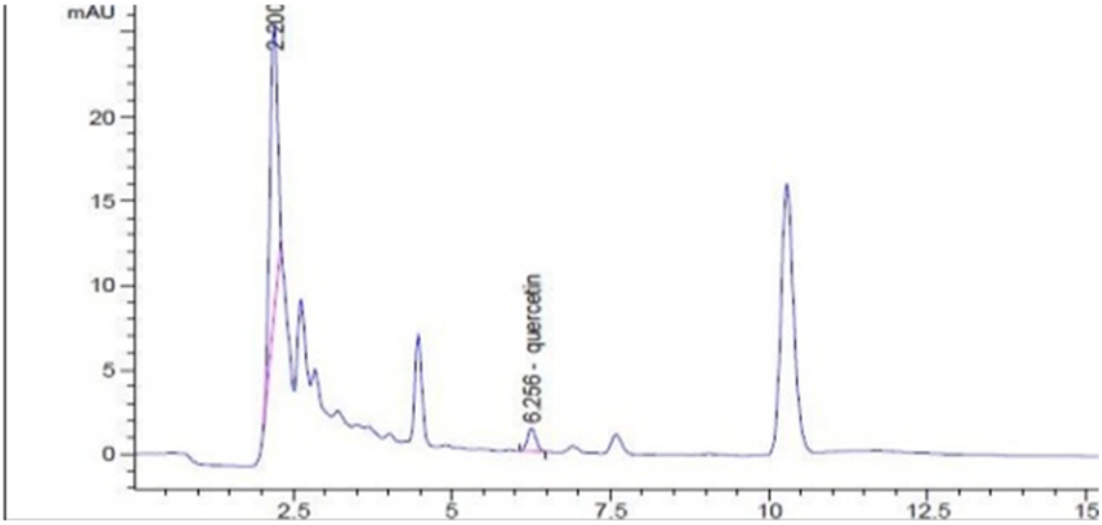


Figure 3: HPLC peaks and retention time of quercetin in *M.aegyptias* stem

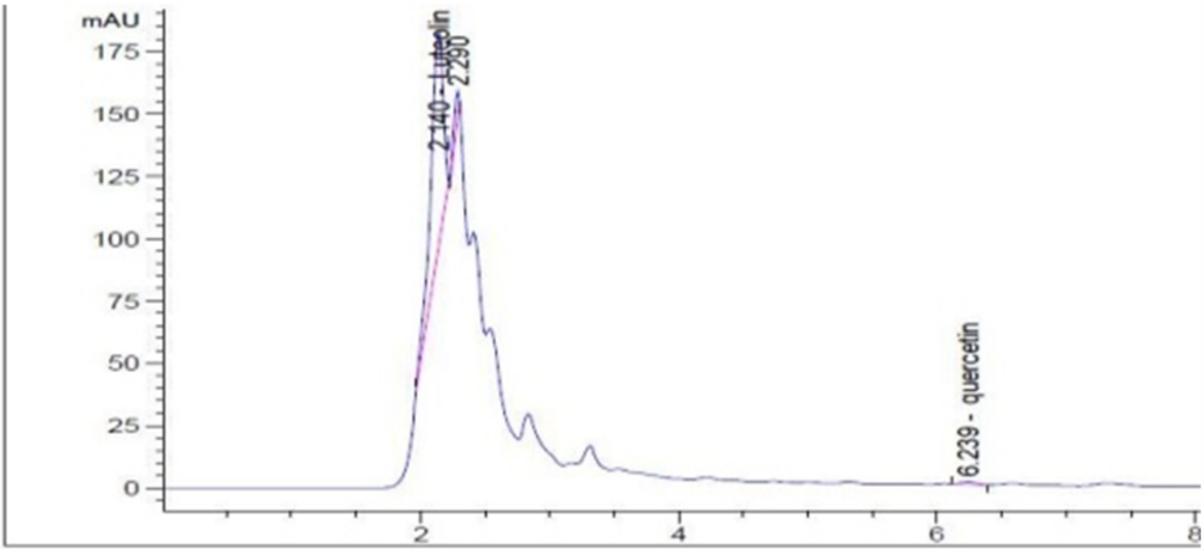


Figure 4: HPLC peaks and retention time of quercetin and luteolin in *M. dissecta* callus

standard quercetin was 6.208, 3143.062 (Figure 2) whereas for the stem extract of *M. aegyptia* (RT =6.256, 12.689 mAU) and for callus extract of *Merremia dissecta* (RT= 6.239, 8.415 mAU) (Figures 3 and 4).

The amount of quercetin in plant samples were calculated comparing standard curve with known amount of quercetin. The amount of quercetin was found maximum in *M. aegyptia* stem extract(20 ng/μl)(Figure 3)and (13.2 ng/ul) in callus extract of *M. dissecta*(Figure 4).

The retention time and peak area observed for standard luteolin was (RT=2.130), 44.418 mAU (Figure 2) and in *M. dissecta* callus extract showed (RT=2.140), 554.604 mAU.

The amount of luteolin in plant samples was analysed by comparing standard curve with known amount of luteolin. The amount of luteolin was found maximum in the *M. dissecta* callus extract (400.3 ng/μl or .4 mg/ml) (Figure 4).

CONCLUSIONS

The RP-HPLC method mentioned in this paper can be used for simultaneous determination of luteolin and quercetin in the extracts of *M. aegyptia and Merremia dissecta* plant samples.This method showed good sensitivity, precision, resolution and reproducibility. Better resolution among the two compounds with the analysis time (25 min) was reported and hence can play a reference role in the determination of polyphenolic compounds from other medicinal plants or pharmaceutical preparations as well.

ACKNOWLEDGEMENT

The corresponding author is highly thankful to UGC for the UGC-JRF grant and Centre for converging technologies, University of Rajasthan for the HPLC facility where the work was conducted.

Conflict of Interest

The authors declare that they have no conflict of interests.

Funding Support

None.

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