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HPLC ANALYSIS OF FLAVONOIDS IN *ACHYRANTHES ASPERA* AND *CISSUS QUADRANGULARIS*

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ABSTRACT

Reversed phase high performance liquid chromatography (RP-HPLC) with UV detector was done for the analysis of four naturally occurring flavonoids of *Achyranthes aspera* and *Cissus quadrangularis* namely lutioline, myricetin, quercetin, kaempferol. The separation of filtered methanolic extracts of selected plants as well as a mixture of authentic standard flavonoid samples of lutioline, myricetin, quercetin, kaempferol was done within 35 min. using an RP $\mathbf{C_{18}}$ column, at ambient temperature, running conditions included: injection volume 20µl; mobile phase: methanol: water (1:1) (0-10 and (7:3) (10-20 min), flow rate 1 ml/min; and detection at 339nm. Identifications of specific flavonoids are made by comparing their retention times with those of the standards. Three flavonoids in *Achyranthes aspera* and two flavonoids in *Cissus quadrangularis* were detected by HPLC.

KEYWORDS: *Achyranthes aspera*, *Cissus quadrangularis*, Flavonoids, Retention Time, HPLC

INTRODUCTION

Plants have been an integral part of traditional medicine across the continents since time immemorial. *Achyranthes aspera* and *Cissus quadrangularis* are important medicinal plants. These plants have been used in traditional Indian medicine for thousands of years to treat various disorders but very little is known about their chemistry. Preliminary phytochemical investigation of the extracts of *A. aspera* seeds and *C. quadrangularis* stem shown the presence of flavonoids. Flavonoids are a ubiquitous group of polyphenolic substances play significant role in plants and human health, thus it is important to better understanding of their potentials as therapeutic agents. It has been found that flavonoids possess diverse biological activities like antioxidant activity, free radical scavenging activity, antiulcer, antiinflammatory, antitumor, antiviral, cytotoxic, antidiabetic, and antihyperlipidemic activity. Therefore, the analysis of flavonoids would help in determining various biological activities of medicinal plants.

HPLC is gaining increasing interest for the analysis of plant extracts. It has added a new dimension to the investigation of flavonoids in herbal plants. Although TLC is a powerful and simple technique used for this purpose, there are situations in which it can produce doubtful results. The separations by HPLC are far more rapid and accurate than classical methods and provide high resolution and sensitivity. It has the advantage of generating a chemical fingerprint, which can be used in defining the identity and quality of a given sample. Therefore, the aim of the present study was to do a simple, routine and accurate HPLC analysis for the determination of four major naturally occurring flavonoids in *Achyranthes aspera* and *Cissus quadrangularis*.

MATERIAL AND METHOD

Plant material and extract preparation *Cissus quadrangularis* (stem) was collected from various parks of Bikaner where it is cultivated as ornamental plant whereas seed samples of *Achyranthes aspera* was purchased from the shop of herbal medicine and were identified by a well known taxonomist of Bikaner. The fresh samples were dried separately and used for further analysis. The dried samples were separately soxhlet extracted by Subramanian and Nagarajan (1969) method, in 80% ethanol (100 ml/g.d.w.) on a water bath for twenty four hours.

Extract Fractionation

Each of the extract was concentrated and re-extracted in petroleum ether (40-60°C, fraction first), ethyl ether (fraction second) and ethyl acetate (fraction third) in succession. Each step was repeated three times to ensure complete extraction in each case. Fraction first was rejected due to its richness in fatty substances, whereas fraction second was analyzed for free flavonoids and fraction third for bound flavonoids in each of the samples. Fraction third of each of the test samples was hydrolyzed by refluxing with 7% sulphuric acid (10 ml/gm residue) for two hours. The mixture was filtered and the filtrate was extracted with ethyl acetate in separating funnel. The ethyl acetate layer (upper layer) was washed with distilled water to neutrality, dried *in- vacuo* and analyzed for bound flavonoids.

Fractionation of Crude Extract by Thin Layer Chromatography (TLC) For Identification

Thin glass plates (20 X 20 cm) coated (wet thickness, 0.2-0.3 mm) with silica gel ‘G’ (30 gm /60 ml) were dried at room temperature. The dried plates were activated at 100 °C for 30 minutes in an oven and cooled at room temperature. Ethyl ether and ethyl acetate fractions from each of the test samples were separately applied 1 cm above the edge of the plates along with the standard reference compounds (apigenin, kaempferol, luteolin, quercetin, myricetin, scopoletin and negretin).

These glass plates were developed in an air tight chromatography chamber containing about 200 ml of solvent mixture of n-butanol, acetic acid and water (4:1:5,upper layer). Some other solvent mixtures such as ethyl acetate saturated with water, acetic acid, (6:4); acetic acid, concentrated hydrochloric acid and water (10:3:30) were also tried, but solvent mixture of n-butanol, acetic acid and water (4 : 1 : 5, upper layer) gave best results in all the cases examined. The developed plates were air dried and visualized under UV light (254 nm). The Rf values were calculated as an average of the five replicates.

Sample Preparation for HPLC Analysis

8.0 g of the powdered plant samples were extracted in a Soxhlet with HPLC grade methanol (150 mL) for 5 h. The extraction procedure was executed in triplicate. Each extract was then filtered and the volume was completed to 200 mL with methanol. 30 µl of these extracts were passed through .45µm filter and that filtrate was used for HPLC analysis.

HPLC Analysis

The HPLC system (Shimadzu lab chromo 2010 HT HPLC, UV detector) was used. The software package used for analyzing results was Shimadzu lab chromo HPLC control and auto-sampling. Chromatographic analysis was carried out using a c-18 column at ambient temperature. Running conditions included: injection volume 30µl; mobile phase: methanol: water (1:1) (0-10 and (7:3) (10-20 min), flow rate 1 ml/min; and detection at 339nm. The separation of filtered methanolic extract as well as a mixture of authentic standard samples of lutioline, myricetin, quercetin, kaempferol was done.

RESULTS AND DISCUSSIONS

The fractionation of crude ethanolic extract by Thin Layer chromatography (TLC) in ***A. aspera***. two fluorescent spots were shown in ethyl ether fraction (second) and one spot in ethyl acetate fraction (third) in all the instances, coinciding with those of the standard samples of quercetin (yellow, Rf 0.82); luteolin (yellow, Rf 0.78) and kaempferol (deep yellow, Rf 0.93) while ***C. quadrangularis*** showed only one fluorescent spot in both the fractions (second and third) in all the instances, coinciding with those of the standard samples of quercetin (yellow, Rf 0.82) and kaempferol (deep yellow, Rf 0.93). On spraying the developed plates with 5% ethanolic ferric chloride solution two spots were shown in ethyl ether fraction (second) and one spot in ethyl acetate fraction (third) in ***A. aspera***. Fraction second spots were coinciding with those of reference quercetin (brown) and luteolin (green); and that of fraction third with kaempferol (brownish) in ***A. aspera***. While ***C. quadrangularis*** showed only one spot in both the fractions (second and third). Fraction second spot was coinciding with that of reference quercetin (brown) and that of fraction third with kaempferol (brownish). After this, HPLC method was developed for the qualitative and quantitative analysis of those compounds in the selected plants.

The method developed for HPLC provided a quick analysis of the methanolic plant extract. The conditions used led to a good separation of the peaks in standard solution which could be identified in the chromatogram (Figure 1), Lutioline (Rt= 7.024), Myricetin (Rt= 9.821), Quercetin (Rt= 14.129), Kaempferol (Rt= 28.210). Plant samples were identified by comparison with the chromatogram of the four reference compounds obtained under the same conditions and the respective UV spectra, obtained on line. The separation of flavonoids showed 3 fine and distinguished peaks {(Figure 2), Lutioline (Rt= 8.147), Quercetin (Rt= 15.989), Kaempferol (Rt= 27.960)} in chromatogram of ***Achyranthes aspera*** and 2 peaks {(Figure 3) Quercetin (Rt= 14.989), Kaempferol (Rt= 29.216)} in ***Cissus quadrangularis***. The data were processed by the Shimadzu lab chromo HPLC control and auto-sampling software (LC chrome software). The peak area of standards and samples was calculated to determinate concentration. The results of the quantitative analysis are the average of three samples and the data are summarized in Table 2 and Table 3.

CONCLUSIONS

The results above showed, therefore, that *Achyranthes aspera* and *Cissus quadrangularis* are rich in the presence of the important biologically active flavonoids Lutioline, Quercetin, Kaempferol. The described HPLC procedure could be useful for the qualitative and quantitative analysis of flavonoids in plant materials. Flavonoid containing plants have many therapeutic properties like antimicrobial activity, antioxidant activity, free radicals scavenging activity. Therefore determination of flavonoids is very important related to the quality of medicinal plants.

Conflict of interest: None

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Acquired by	: Admin
Sample Name	: flavonoid standards
Sample ID	: flavonoid standards
Tray #	: 2
Vail #	: 1
Injection Volume	: 30 µL
Conc.	:
Data File Name	: flavonoid standards data new.lcd
Method File Name	: flavonoid standards Meth.lcm
Report File Name	: Default.lcr
Data Acquired	: 25/7/2016 11:15 PM
Data Processed	: 25/7/2016 11:28 PM

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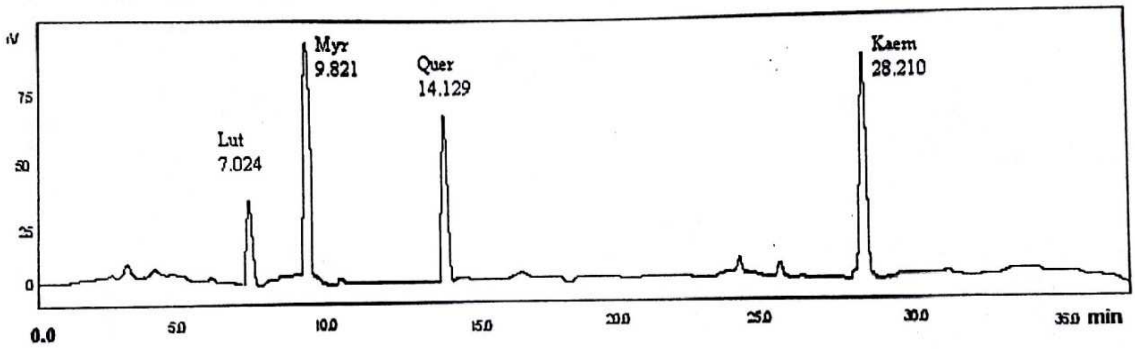

Peak	Name	Retention Time
1.	Lutioline (Lut)	7.024
2.	Myricetin (Myr)	9.821
3.	Quercetin (Quer)	14.129
4.	Kaempferol (Kaem)	28.210

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Acquired by	: Admin
Sample Name	: Achyranthes flavonoid
Sample ID	: Achyranthes flavonoid
Tray #	: 2
Vail #	: 1
Injection Volume	: 30 µL
Conc.	:
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Method File Name	: Achyranthes flavonoid Meth.lcm
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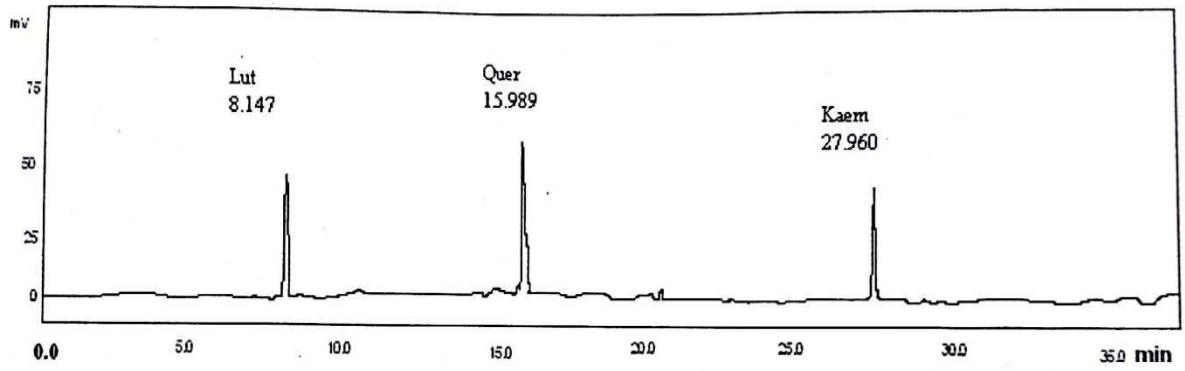

Peak	Name	Retention Time	Area(mm^2)	Conc.(µg g^{-1})
1.	LUT	8.147	9.01	6.96 ±0.22
2.	QUER	15.989	10.83	10.10 ±0.14
3.	KAEM	27.960	8.85	5.59 ± 0.82

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Acquired by	: Admin
Sample Name	: Cissus flavonoid
Sample ID	: Cissus flavonoid
Tray #	: 2
Vail #	: 1
Injection Volume	: 30 µL
Conc.	:
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Method File Name	: Cissus flavonoid Meth.lcm
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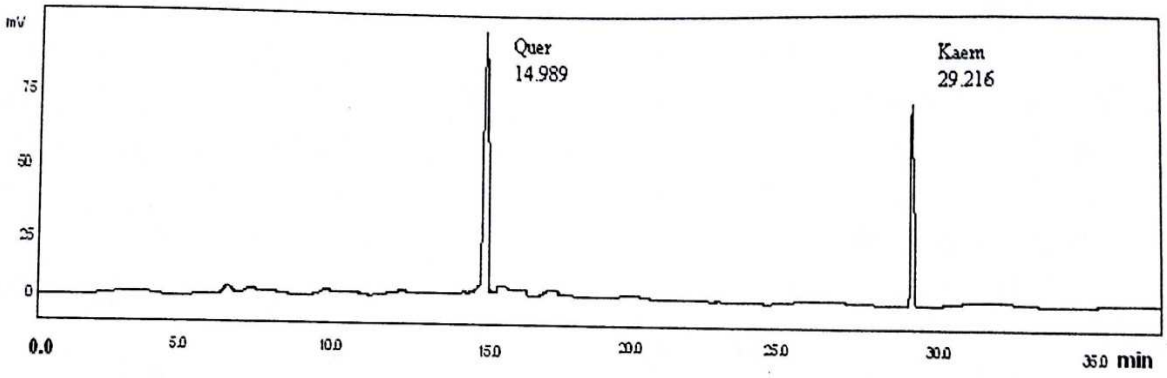

Peak	Name	Retention Time	Area(mm^2)	Conc.(µg g^{-1})
1.	QUER	14.989	11.01	10.99±0.06
2.	KAEM	29.216	8.88	7.25±0.19

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