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Identification of Flavonoids in the Fruit of *Cassia fistula* by High Performance Liquid Chromatography-Electrospray Mass Spectrometry

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Cassia fistula is traditionally known for its medicinal properties. It contains many phenolic compounds that may have potential as antioxidant. High performance liquid chromatography coupled with electrospray mass spectrometry (LC-ESI/MS) was used for the identification of flavonoids in the fruit of *Cassia fistula*. Mobile phase used in the chromatographic separation was 2 mM ammonium acetate buffer (solvent-A, pH 2.5 with acetic acid) and acetonitrile (solvent-B) with gradient programming. The peaks were identified by the comparison of retention time, UV-vis spectroscopic and mass spectrometric data with authentic standards and/or literature data. The identified flavonoids included two anthocyanins (cyanidine-3-O-galactoside, petunidin-3-O-glucoside), two flavan-3-ols (catechin and epicatechin) and one flavonol glycoside (kaempferol-3-O-glucoside).

Key Words: *Cassia fistula*, HPLC, LC-MS, ESI/MS, Anthocyanins, Flavan-3-ols.

INTRODUCTION

Cassia fistula Linn is used extensively in various parts of the world against a wide range of ailment[1]. It is believed that the major active dietary constituents attributed to these protective effects are flavonoids[2,3]. The health related properties of flavonoids are due to their antioxidant activity.

During the study on the antioxidant and other protective activity of some medicinal plants, *Cassia fistula* was found to have higher antioxidant activity. The objective of this research is to identify the flavonoids constituents in the fruits of *Cassia fistula* using high performance liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS).

EXPERIMENTAL

All flavonoid standards were of HPLC grade. Cyanidin-3-O-galactoside and petunidin-3-O-glucoside were obtained from polyphenol (Sandas, Norway). Kaempferol-3-O-glucoside was purchased from Indofine Chemical Co. Inc. (Hillsborough, NJ). Catechin and epicatechin were purchased from Sigma Chemical Co. (St Louis, MO). Distilled and deionized water was further purified by a Milli-Q water system (Millipore Ltd., Watford, UK) and used for all chromatographic analysis and sample standard preparations. All other solvents were of HPLC grade and were purchased from Qualigens fine chemicals.

The fruits of *Cassia fistula* Linn (Caesalpinaceae) were collected from Vidisha, India during the month of October and identified by Dr. S.K. Jain, Department of Botany, S.S.L. Jain P.G. College, Vidisha.

Extraction and isolation: The fruits were dried in shade for a week. Then powdered and extracted with 90 % aqueous alcohol by Soxhlet extraction (24 h) to yield extract. The extract was filtered with filter paper under reduced pressure. The residue was resuspended in 100 mL of the same solvent and extracted for 5 h. This extraction step was repeated twice. The combined alcoholic extract was concentrated with a rotary evaporator at 40 ºC under reduced pressure to remove ethanol. The resulting solution was mixed with 200 mL of water. After filtration, the aqueous solution was applied to a 23 cm × 2.7 cm i.d. Diaion HP-20 absorption resin column (Supelco, Bellefone, PA), which was preconditioned by washing with ethanol and then equilibrated with water. Non phenolic impurities including sugars, amino acids, proteins and minerals were washed out with water (500 mL). Phenolic compounds were eluted from the resin with ethanol (200 mL) and the eluent was dried with a rotary evaporator under reduced pressure. The phenolic residue was redissolved in water (40 mL) and freeze-dried to give a phenolic extract powder.

HPLC analysis was performed using a Waters 2690 separation module system (Waters Associates, Milford, MA) equipped with an autosampler and a waters model 996

photodiode array detector. A phenomenex Luna C_{18} (2) analytical column (250 mm × 4.6 mm i.d., particle size, 5 µm) with a C_{18} guard column (Phenomenex, Torrance, CA) was used for separation. The binary mobile phase consisted of 2 mM ammonium acetate buffer (solvent A, pH-250 with acetic acid) and acetonitrile (solvents B) and gradient program was as follows 0 % B to 15 % B in 45 min, 15 % B to 30 % B in 15 min, 30 % B to 0 % B in 5 min. The flow rate was 1.0 mL/min for a total run time of 65 min. The injection volume was 10 µL for all samples. All standards except for anthocyanins were dissolved in methanol. The anthocyanins were dissolved in 1 % HCl in methanol. The detection were set at 280, 360 and 520 nm for simultaneous monitoring of the different group of phenolic compounds.

LC-ESI/MS analysis: LC-ESI/MS analysis were performed with the same HPLC system as described above interfaced to a Waters micromass ZMD model mass spectrometer equipped with an ESI source, operated in both negative and positive ion modes.

Determination of flavonoids: The *Cassia fistula* fruit powder was soaked in 80 % methanol containing 0.1 % HCl and kept in an airtight capped bottle at room temperature for 2 h. The suspension was then incubated at 45 ºC in a water bath with continuous shaking for an additional 0.5 h after cooling to room temp the suspension was filtered by a syringe filter and 10 µL of the filtered extract was injected into HPLC analysis. Compounds were tentatively identified by congruent retention times and UV-vis spectra with those of standards. Confirmation of identity was achieved by comparing the retention time and UV spectra of both standards and samples determined by LC-MS. All samples were prepared and analyzed in duplicate.

RESULTS AND DISCUSSION

Once the LC-ESI/MS condition had been established for the compound studied, the fruit extract was analyzed by the method in the full scan mode. Fig. 1 shows the HPLC-UV-vis chromatograms of purified phenolic extract prepared from fruits of *Cassia fistula*. Identification of individual compounds was performed by comparison of LC retention time, photodiode array UV-vis spectroscopic and ESI-MS spectrometric data (Table-1) with those of authentic standard or with published data. A total of 5 flavonoids were identified in the fruit extract including two anthocyanin, two flavan-3-ols and one flavonol glycosides.

Flavan-3-ols: The HPLC-UV chromatogram acquired at 280 nm two major peaks (compound **1**, **4**) with typical flavon-3-ol spectra were observed. The maximum absorbance of these compounds was at about 276 nm characteristic of flavon-3-ols[4,5]. The compound **1** and **4** were observed in mass chromatogram selected at m/z 289 in negative mode $(M - H)^-$, suggesting the presence of catechin and epicatechin, respectively. The compound showed the same retention time, UV spectra and ESI mass spectra pattern as their standards.

Anthocyanins: In the HPLC-vis chromatogram acquired at 520 nm (Fig. 1) two peaks (compound **2** and **3**) were detected. The UV-vis spectra of these compounds showed strong absorption at 520 nm, which is characteristic of anthocyanin[6,7]. In mass chromatogram selected at m/z 449 and 479, two peaks (compounds **2** and **3**) were observed. The mass spectra of which showed their protonated aglycon ions $(A + H)^+$ to be m/z 287, 317, respectively corresponding to cyanidin and petunidin[5]. The protonated aglycon ion were all formed by loss of a sugar moiety with 162 units from their $(M + H)^+$, indicating that

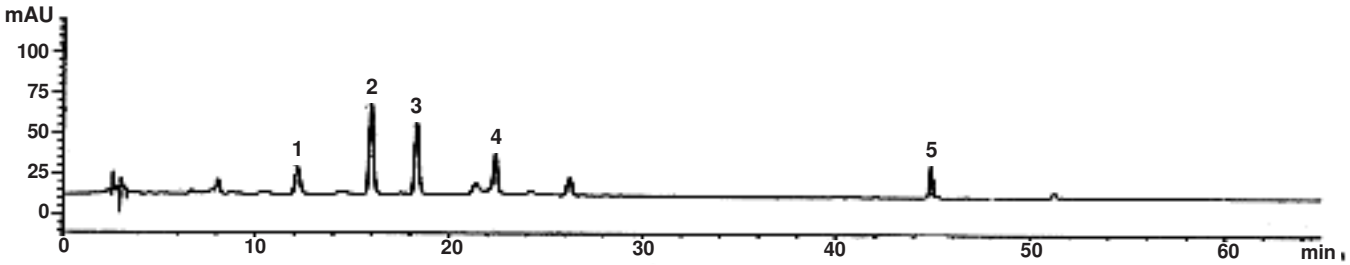


Fig. 1. HPLC chromatogram of purified phenolic extract of fruits of *cassia fistula* (1) catechin; (2) cyanidin-3-O-galactoside; (3) petunidin-3-O-glycoside; (4) epicatechin; (5) kaempferol-3-O-glucoside

TABLE-1
IDENTIFICATION OF ANTHOCYANINS, FLAVAN-3-OLS AND FLAVONOL GLYCOSIDES IN THE FRUIT EXTRACT OF *Cassia fistula* BASED ON HPLC RETENTION TIME (t_R), UV-VIS SPECTROSCOPIC CHARACTERISTICS (λ_{max}) and ESI/MS SPECTROMECTRIC

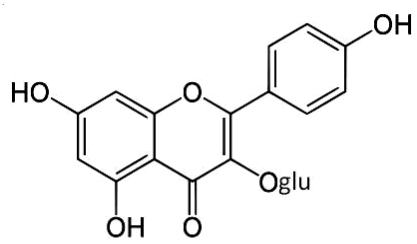
Flavan-3-ols					
Peak	t_R (min)	Identified compounds	λ_{max} (nm)	Molecular ion $(M-H)^-$	Compare with standard
1	12.12	Catechin	276	289	Yes
4	22.35	Epicatechin	276	289	Yes

Anthocyanins						
Peak	t_R (min)	Identified compounds	λ_{max} (nm)	Molecular ion $(M + H)^+$	Aglycon ion $(A + H)^+$	Compare with standard
2	15.92	Cyanidin 3-O-galactoside	281	449	287	Yes
3	18.21	Petunidin 3-O-glucosides	279	479	317	Yes

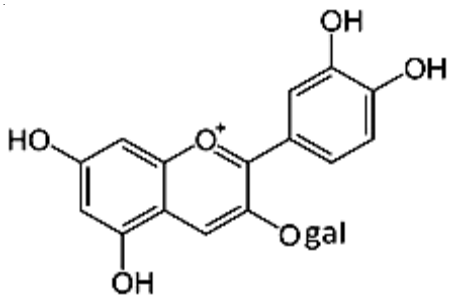
Flavonol glycosides						
Peak	t_R (min)	Identified compounds	λ_{max} (nm)	Molecular ion $(M-H)^-$	Aglycon ion $(A-H)^-$	Compare with standard
5	44.81	Kaempferol-3-O-glucoside	265	447	285	Yes

they are anthocyanidine monoglucoside. This suggest the presence of cyanidin-3-O-galactosides and petunidin-3-O-glucosides in addition to comparison of their retention time with those of authentic standards.

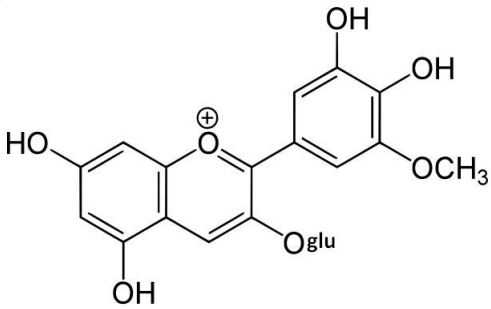
Flavonol glycoside: The HPLC-UV chromatogram acquired at 360 nm, one major peak (compound **5**) with typical flavonol UV spectra was observed. The maximum absorption of this compound were at about 265 and 360 nm, characteristic of flavonol compounds[8,9]. Compound **5** was observed in mass chromatogram selected at m/z 447. The compound showed losses of 162 form $(M - H)^-$ to $(A - H)^-$ of m/z 285, suggesting the presence of kaempferol-3-O-glucoside. The compound showed the same retention time, UV spectra and ESI mass spectra patterns as their standard.



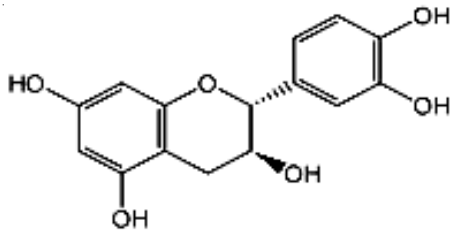
Kaempferol-3-O-glucoside (**1**)



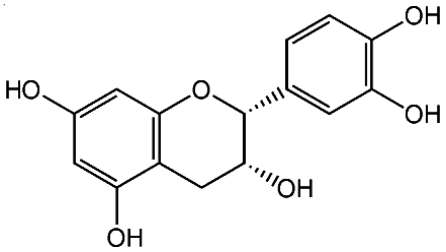
Cyanidin-3-O-galactoside (**2**)



Petunidin-3-O-glucoside (**3**)



Catechin (**4**)



Epicatechin (**5**)

Chemical structure of flavonoids identified in fruit of *Cassia fistula*

Conclusion

Dietary intake of flavonoid antioxidant is shown to be related to various beneficial effects including risk cardiovascular diseases and certain forms of cancer. With regards of flavonoids in *Cassia fistula*, most studies focus on leaves and bark of *Cassia fistula*[10,11]. Limited information is available on other parts. This is the study on the composition of flavonoids present in the fruit of *Cassia fistula*. The high content of flavonoids present in the fruits may contribute to high antioxidant activity observed for fruit of *Cassia fistula*.

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