Int. J. Chem. Sci.: 10(1), 2012, 306-316
ISSN 0972-768X
www.sadgurupublications.com

SEPARATION AND CHARACTERIZATION OF ANTHRAQUINONE DERIVATIVES FROM *CASSIA FISTULA* USING CHROMATOGRAPHIC AND SPECTRAL TECHNIQUES

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ABSTRACT

Present study involves characteristic evaluation of medicinally important ingredients such as anthraquinone derivatives from *Cassia Family*. *Cassia* family is well-known source of anthraquinone glycosides and its derivatives in the various parts of plants. A simple high performance liquid chromatography (HPLC) method was developed and validated for the determination of three anthraquinone derivatives (rhein, emodin and chrysophanic acid) in the extracts from *Cassia fistula.* The extracts were analyzed on C-18 column isocratic mobile phase in HPLC equipped with UV detector at 254 nm. The identification of each analyte was performed by use of standards. The limits of detection obtained for the analyte were in the range of 2.5-15 µg/mL. All three components present in the extracts of *cassia fistula* were also characterized by the ^{1}H-NMR and mass spectroscopic analysis.

Key words: HPLC, NMR, Mass spectroscopy, *Cassia fistula*, Anthraquinone derivative.

INTRODUCTION

Plants up-regulate and down-regulate their biochemical paths in response to the local mix of herbivores, pollinators and microorganisms[1]. The chemical profile of a single plant may vary over time as it reacts to changing conditions. The secondary metabolites and pigments can have therapeutic actions in humans, which can be refined to produce drugs. Plants synthesize a bewildering variety of phyto-chemicals but most are derivatives of a few biochemical motifs such as alkaloids, phenols and their derivatives, terpenoids, glycosides and others. These secondary metabolites possess some therapeutic properties therefore, some plants are also classified as Herbs.

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Herbs, and substances derived from natural sources (spices)[2] would have been one of the only treatments for diseases in all prehistoric cultures. An increasing number of modern drugs have been isolated and purified from plant extracts. Well-known examples of plant-derived medicinal agents include the anti-malarial drug quinine, isolated from the bark of *cinchona officinalis*, the analgesics codeine and morphine from p*apaver somniferum*, the cardiac glycoside digitoxin from *digitalis purpurea*, the antihypertensive reserpine from *rauwolfia serpentine*[3], and the anticancer drugs vinblastine-2 and vincristine-3, isolated from the Madagascar periwinkle, *catharanthus roseus*[4-7]. The plant-derived anticancer drugs, vinblastine-2 and vincristine-3 are still produced by their direct isolation from the plant material[8] and *catharanthus, laxative* anthraquinone glycoside from the plants of *cassia family.*

Plants from the *cassia family*; are identified as a potential source for herbal medicine. These plants contain anthraquinone glycosides and their derivatives and these derivatives are potentially known for their laxative property and skin and respiratory diseases[9,10].

In the present study, more emphasis has been given on the separation and characterization of anthraquinone derivatives. Validate the identity of the active ingredients through their isolation by using conventional column chromatography and HPLC techniques. These separated components were characterized by using NMR and Mass spectroscopic techniques.

EXPERIMENTAL

Chemicals & materials

Standard anthraquinone derivatives were purchased from Sigma-Aldrich Chemicals, having purity about 99%. These standards were used without further purification. All other chemicals and reagents of HPLC grade were purchased from E. Merck and SRL India Limited. The plants were collected from local region with authentication from botanist. To confirm the plant's identity the microscopic and morphological study were also carried out.

Extraction and hydrolysis

Conventional soxhlet extraction process was selected to isolate the mixture of anthraquinone glycosides. The extraction procedure was carried by using standard protocols with some modifications to confirm complete extraction of active ingredients from various parts of plants. The isolated products contain glycoside linkages with anthraquinone derivatives. To break this glycosidic linkages hydrolysis was carried out in the presence of

2M HCl for two hours and anthraquinone components were collected after recovery of solvent from rota-evaporator.

Column chromatography

Conventional column chromatographic technique was performed with some modifications. A column of 1707 mm height and 15 mm inner diameter packed with neutral silica gel of mesh size 60-120 was used for the separation. The silica gel slurry was prepared in chloroform and allowed to settle for the saturation over night. Care was taken to avoid air pockets or voids. The solubility of the isolated anthraquinone derivatives was taken into consideration for the selection of mobile phase system. The mobile phase in the ratio 9.5 : 0.5 of chloroform: methanol was optimized. The mobile phase was passed through the column and eluted each fraction was collected in individual vial. Each collected fractions were analyzed by TLC method to ascertain isolation.

HPLC Analysis

HPLC analyses were performed on isocratic system with UV detector for the detection of anthraquinone derivatives. The instrument specifications and analysis conditions were optimized. Isocratic system from Shimadzu (Asia-Pacific) P. Ltd. Model No. LC-10 AdVP with UV detector was used. The sample volume was kept 20-µL with 20-µL Reodyne injector system. The analytical column (C-18) with 250 mm x 4.6 mm i.d., 5 µm was used. The mobile phase was prepared from HPLC grade methanol and 0.5% acetic acid in the ratio of 85 : 15 to elute the anthraquinone derivatives. The flow rate was kept at 0.6 mL /min. and detection was carried out at 254 nm.

Preparation of standards

Stock solutions of standard anthraquinone derivatives were prepared in HPLC grade methanol having 500 mg/L concentration. These solutions were used for further analysis.

Preparation of Sample solution

The extracted component of each anthraquinone derivative was dissolved in HPLC grade methanol having approximate concentration of 500 mg/L in the stock solution. Prior to use, the mobile phase was filtered through 0.45 µm filter paper with filtration assembly followed by sonication for 10 minutes for the complete removal of air bubble/dissolved oxygen. Samples analyses were carried out by using same conditions and results were compared with the authentic standards.

NMR Spectroscopy

NMR spectra of few selected samples were recorded on a Bruker AMX 400 instrument. The samples were prepared in $CDCl_3$ solvent. All the results were compared with TMS signals.

Mass Spectroscopy

Mass spectra were recorded on Micro mass 70-70E mass spectrometer/FABMS spectrometer attached with Gas chromatograph having direct injection probe.

RESULTS AND DISCUSSION

The Anthraquinone derivatives separated from the *Cassia family* were purified by conventional column chromatography, which were further characterized by NMR and Mass spectroscopy. HPLC with C-18 column and equipped with UV detector is used for the separation and identification of compounds present in the sample extract. The conditions were already discussed in the experimental part.

Reproducibility of standards by HPLC- UV techniques

The validation of the analytical method is a key requirement prior to the estimation of actual samples for an analyst. Reproducibility and linearity curves for different anthraquinone derivatives are prepared on the basis of results obtained from HPLC analysis. It is advisable to the analyst that HPLC instrument should be standardized before proceeding for unknown sample analysis and therefore, we started with analysis of rhein, emodine and chrysophanic acid. These standards are injected individually and in combination to find out their retention time in the applied conditions with standard deviation. The average of eight injections for rhein is found 10.57 min. with standard deviation 0.190. Similarly, the average retention time for emodine is found 16.13 min. with standard deviation 0.123 and the average retention time for chrysophanic acid is found 21.16 min. with standard deviation 0.143.

The conventional column chromatography technique is used to separate primary components of anthraquinone derivatives *viz.* rhein, emodine and chrysophanic acid form extracted crude material. The each separated fractions of 10 mL is collected and each fraction is given numbered accordingly. Once complete elution of each separated fractions is achieved, the column is washed with appropriate solvent for further utilization.

All three major components of anthraquinone derivatives are found in the seeds and flowers of plant *cassia fistula* and therefore, we have taken seeds as well as flowers to collect the desired quantity of all three major components. The Fig. 1 shows the

chromatogram of fraction collected from seeds of *cassia fistula*. The figure clearly shows the presence of desired components in the fraction collected through column chromatography. The injected samples in the HPLC with C-18 column are detected at 254 nm in UV detector. The retention time of three components are matching with the retention time of standards. The retention time for rhein is found 10.75 min. with standard deviation 0.19 while retention time of emodine fraction is found 16.31 min. with standard deviation 0.13 and retention time of chrysophanic acid fraction is found 21.36 min. with standard deviation 0.15. Linearity curves for standards and sample are carried out for method validation. The standard addition-spike method is used to confirm the linearity of all three components. Table 1 represents the HPLC data of linearity for three standards.

Table 1: HPLC data for linearity of standards

S. No.	Retn. time (in min.)	Area	Area %	Compound name
1	10.75	3130017	5.58	Rhein
2	16.31	336560	0.6	Emodin
3	21.36	18286426	32.6	Chrysophanic acid

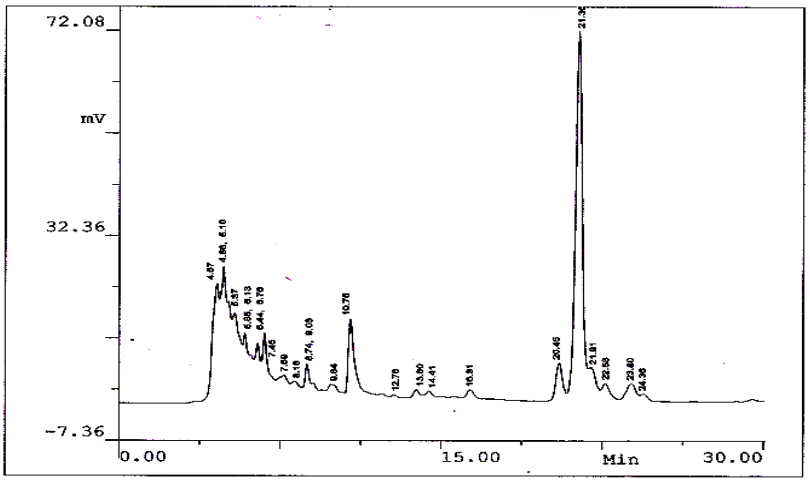

Fig. 1: HPLC chromatogram of extract from *cassia fistula* seeds

Under the specified chromatographic conditions, the peak areas are regressed against the concentrations of the three-anthraquinone derivatives to give the correlation coefficients of calibration curve data. The correlation coefficient R^2 is computed for all derivatives and the value are found around 0.99, which are in good correlation with each other. The above

results clearly suggest that method validation is possible in the HPLC system for all three components present in the fraction collected from column chromatography.

A representative figure (Fig. 2) is given below, which clearly indicates the linearity curve for chrysophanic acid in the range of 2.5 to 15 µg/mL concentration.

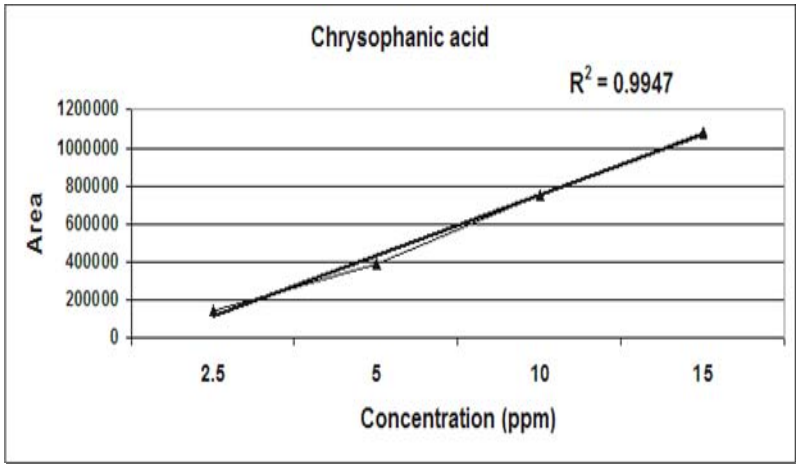

Fig. 2: Calibration curve of chrysophanic acid in the range of 2.5-15 µg/mL

Results of NMR spectroscopy

NMR spectroscopy is powerful tool to identify the carbon-proton skeleton of particular compound and surrounding environment. The proton NMR of extracted material is taken in $CDCl_3$ solvent with respect to TMS as a reference. The δ (ppm) value of NMR of all three major components present in the plant extract is shown in the respective tables and their explanations are also incorporated in the text.

The probable structure of rhein is given below and its proton NMR signals of eight different protons are shown in the Table 2.

H_d is significantly deshielded by the anisotropy of both the hydroxy and carboxylic acid groups and therefore, it appears in downfield. H_e is ortho to a carboxyl group while H_a and H_d are ortho to both hydroxyl group respectively. Both protons are deshielded in nature, but the carboxylic acid group shifts a proton further in downfield. Both H_a and H_b are doublets having fine structure consistent with their positions on the aromatic ring. H_b is relatively shielded and it appears in upfield as a widely spaced doublet. This proton does not experience any anisotropy effect because of its distance from the attached groups. H_f is a

singlet in downfield for the proton of carboxylic group. H_g and H_h both is singlet with fine structure consistent of hydroxyl group with similar environment.

Table 2: ^{1}H NMR signals for separated component (1) of anthraquinone derivative with reference to TMS in $CDCl_3$

Signal No.	Signal position δ (ppm)	Relative No. of proton	Multiplicity	Interpretation
1(a)	7.411-7.474	1	Doublet	Ar-H
2(b)	7.267	1	Doublet	Ar-H
3(c)	7.556-7.774	1	Multiplet	Ar-H
4(e)	7.986	1	Singlet	Ar-H
5(d)	8.477	1	Singlet	Ar-H
6(f)	11.997	1	Singlet	Ar-COOH
7(g)	5.35	1	Singlet	Ar-OH
8(h)	5.42	1	Singlet	Ar-OH

The probable structure of emodine is given below and its proton NMR signals of eight different protons are shown in the Table 3.

Table 3: ^{1}H NMR signals for separated component (2) of anthraquinone derivative with reference to TMS in $CDCl_3$

Signal No.	Signal Position δ (ppm)	Relative No. of proton	Multiplicity	Interpretation
1(a)	2.179	3	Singlet	CH_3
2(b)	6.679	1	Singlet	Ar-H
3(c)	7.402	1	Singlet	Ar-H
4(d)	7.875	1	Singlet	Ar-H
5(e)	7.094	1	Singlet	Ar-H
6(h)	5.423	1	Singlet	Ar-OH
7(f, g)	5.452	2	Singlet	Ar-OH

H_d is significantly deshielded by the anisotropy of both the hydroxyl groups and therefore, it appears in downfield as singlet. Electron resonance occurs more rapid and intensive in hydroxyl group in comparison to methyl group therefore; H_d gives singlet with downfield shift because H_b feels less anisotropy effect from methyl group present onto the adjacent carbon atom.

H_c gives singlet under the influence of hydroxyl group with 7.402 δ (ppm) value and H_e has methyl group onto its adjacent carbon atom therefore, it gives singlet at 7.094 δ (ppm) value in a upfield in comparison to H_c. Both protons are deshielded, but the hydroxyl group shifts a proton further downfield than a proton next to a methyl group. H_f, H_g and H_h protons are singlet with fine structure consistent. H_a have three relevant protons with 2.179 δ (ppm) value, which suggests presence of methyl group in skeleton.

The probable structure of chrysophanic acid is given below and its proton NMR signals of eight different protons are shown in the Table 4.

Table 4: ^{1}H NMR signals for separated component (3) of anthraquinone derivative with reference to TMS in $CDCl_3$

Signal No.	Signal position δ (ppm)	Relative No. of proton	Multiplicity	Interpretation
1(a)	2.441	3	Singlet	CH_3
2(e)	7.049	1	Doublet	Ar-H
3(b)	7.266	1	Singlet	Ar-H
4(f)	7.765-7.780	1	Triplet	Ar-H
5(h)	7.172	1	Singlet	Ar-H
6(g)	7.757	1	Doublet	Ar-H
7(c)	5.457	1	Singlet	Ar-OH
8(d)	5.428	1	Singlet	Ar-OH

Looking to probable structure of chrysophanic acid protons at position H_e and H_b are found at ortho position to both hydroxyl groups. Both the protons are deshielded and possesses similar environment but presence of methyl group shifts a proton further in downfield. Protons of hydroxyl group, H_d and H_c are having fine structure consistent with their positions on the aromatic ring. Due to steric hindrance with three protons of methyl

group, H_f and H_g are relatively deshielded and it appears in downfield as a widely spaced triplet and doublet respectively. These protons experience anisotropy effect because of its distance with the attached hydroxyl, methyl and carbonyl groups. The presence of methyl and a carbonyl group on the adjacent carbon atom of H_h, a singlet in downfield is observed. H_g and H_h both is singlet with fine structure consistent with similar environment. H_a have three relevant protons with 2.441 δ (ppm) value, which suggests presence of methyl group in skeleton.

Results of mass spectroscopy

The mass spectra of the extracted compound are presented here. The presence of each analyte in the extract is confirmed by its respective $[M-H]^-$ m/z ratio.

The fragmentation pattern of rhein is characterized by decarboxylation that is molecular ions of the analyte resulting in $[M-COOH]^-$ with m/z 239 a.m.u. The ions at m/z 253 and 267 are also observed in the mass spectrum of rhein, which are assumed to be a fragment derived from the molecular ion resulting in $[M-CH_2OH]^-$ and an adduct formation between the ion at m/z 239 and methanol ($[M-COOH + MeOH]^-$), respectively.

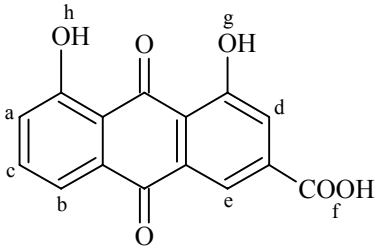

Fig. 3: Possible structure of 4, 5 dihydroxy 9-10 dioxoanthracene-2-carboxylic acid (Rhein)

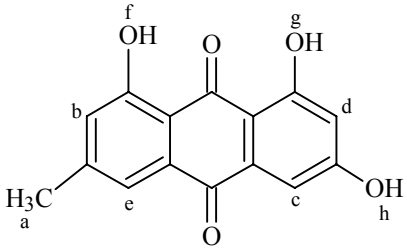

Fig. 4: Possible structure of 1, 3, 8-trihydroxy-6-methyl-anthraquinone (Emodine)

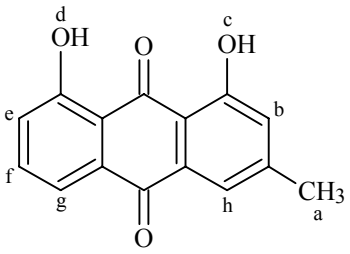

Fig. 5: Possible structure of 1, 8-dihydroxy-3-methyl-anthraquinone (Chrysophanic acid)

The fragmentation pattern of emodine is characterized by its mass spectrum. Molecular ion peak is recorded at 270 m/z ratio. In addition to the ions at [M-H]− of sample, the ion at m/z 269 is registered in the mass spectrum of emodin due to fragmentation of molecular ion four metastable species at 242, 241, 213 and 185 a.m.u. The loss of methyl group is identified by the fragment at 255 a.m.u peak.

The fragmentation pattern of chrysophanic acid is characterized by its mass spectrum. Molecular ion peak is recorded at m/z 254. The fragmentation pattern of chrysophanic acid is characterized by base peak at 254 a.m.u and molecular ion peak at 255 a.m.u. A loss of methyl group from parent ion (p-15) is confirmed by 239 a.m.u peak. A loss of OH group is also confirmed (p-17) by 237 a.m.u peak and one of mass 169 a.m.u to the fragment $(p\text{-}C_3O_3H)^+$.

CONCLUSION

Conventional column chromatography and simple HPLC technique are used to separate therapeutic agents like rhein, emodine and chrysophanic acid from *cassia fistula*. Characterization of separated components is successfully achieved with the help of NMR and Mass Spectroscopic techniques.

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Revised : 30.09.2011 *Accepted : 01.10.2011*