International Journal of Scientific Research in Chemistry
Available online at : www.ijsrch.com
OPEN ACCESS
ISSN: 2456-8457
doi : https://doi.org/10.32628/IJSRCH

Determination of Pharmacologically Active Anthraquinone Derivatives from Cassia roxburghii Using Chromatographic and Spectroscopic Techniques

Dr. Jalpa P. Harsora[1] Dr. Chirag R. Fultariya*[2]

[1]Chemistry Department, H. & H. B. Kotak Institute of Science, Dr. Yagnik Road, Rajkot, Gujarat, India
jalpaharsora@gmail.com [1]

*[2] Chemistry Department, H. & H. B. Kotak Institute of Science, Dr. Yagnik Road, Rajkot, Gujarat, India
chirag.fultariya@gmail.com [2]

ARTICLEINFO

Article History :
Accepted: 02 Sep 2024
Published: 04 Oct 2024

Publication Issue
Volume 9, Issue 5
September-October-2024

Page Number
32-39

ABSTRACT

This study focuses on the characterization of pharmacologically significant anthraquinone derivatives from Cassia roxburghii, a well-established source of anthraquinone glycosides and related bioactive compounds. A simple, reliable, and validated high-performance liquid chromatography (HPLC) method was developed for the quantification of three key anthraquinone derivatives—rhein, emodin, and chrysophanic acid. The analysis was performed using a C18 column with an isocratic mobile phase, and detection was carried out at 254 nm using a UV detector. The identification of individual analytes was confirmed by comparing retention times with reference standards. The limits of detection (LOD) for the analytes ranged from 2.5 to 15 µg/mL. Additionally, structural characterization was performed using ^{1}H-NMR and mass spectrometry, further confirming the presence of these bioactive compounds in Cassia roxburghii extracts.

Keywords: Cassia roxburghii, anthraquinone derivatives, rhein, emodin, chrysophanic acid, HPLC analysis, C18 column, UV detection, ^{1}H-NMR, mass spectrometry, bioactive compounds, phytochemical analysis, chromatographic techniques, pharmacological significance

I. INTRODUCTION

Plants continuously regulate their biochemical pathways in response to environmental factors such as herbivores, pollinators, and microorganisms (1). As a result, their chemical profiles may vary over time, adapting to changing conditions. Among these biochemical compounds, secondary metabolites and pigments play a crucial role in therapeutic applications and can be refined to produce pharmaceuticals. Plants synthesize an extensive range of phytochemicals, most of which belong to fundamental biochemical classes such as alkaloids, phenols, terpenoids, and glycosides. These secondary

Copyright © 2024 The Author(s): This is an open-access article distributed under the terms of the Creative Commons Attribution **4.0 International License (CC BY-NC 4.0)**

metabolites often exhibit therapeutic properties, leading to the classification of certain plants as medicinal herbs.

Throughout history, medicinal plants and natural substances have been primary sources of treatment for various ailments (2). Many modern pharmaceutical agents have been isolated and purified from plant extracts. Notable examples include quinine, an anti-malarial drug derived from *Cinchona officinalis* bark; codeine and morphine, analgesics extracted from *Papaver somniferum*; digitoxin, a cardiac glycoside from *Digitalis purpurea*; reserpine, an antihypertensive alkaloid from *Rauwolfia serpentina* (3); and the anticancer drugs vinblastine and vincristine, obtained from the Madagascar periwinkle (*Catharanthus roseus*) (4-7). Some plant-derived pharmaceuticals, such as vinblastine and vincristine, are still produced through direct isolation from plant material (8). Additionally, plants from the *Cassia* genus are recognized as a significant source of anthraquinone glycosides, which are widely known for their laxative effects and potential therapeutic applications in skin and respiratory diseases (9-12).

Among *Cassia* species, *Cassia roxburghii* is a promising medicinal plant due to its bioactive anthraquinone derivatives. These compounds exhibit various pharmacological activities, including antioxidant, antimicrobial, anti-inflammatory, and anticancer properties. In this study, we focus on the isolation and characterization of anthraquinone derivatives from *Cassia roxburghii*. The active compounds were isolated using conventional column chromatography and high-performance liquid chromatography (HPLC), followed by structural characterization using nuclear magnetic resonance (NMR) and mass spectrometry (MS) to confirm their identity.

Materials and Methods

Chemicals and Reagents:

Standard anthraquinone derivatives were purchased from Sigma-Aldrich Chemicals, with a purity of approximately 99%. These standards were used without further purification. All other chemicals and reagents of HPLC grade were obtained from E. Merck and SRL India Limited. The plant material was collected from the local region and authenticated by a botanist. To confirm the plant's identity, microscopic and morphological studies were also conducted.

II. Experimental

Extraction and Hydrolysis

The conventional Soxhlet extraction process was employed to isolate a mixture of anthraquinone glycosides. The extraction was carried out using standard protocols with slight modifications to ensure the complete extraction of active ingredients from various plant parts. The isolated products contained glycosidic linkages with anthraquinone derivatives. To cleave these glycosidic bonds, hydrolysis was performed using 2M HCl for two hours. After hydrolysis, the anthraquinone components were collected following solvent recovery using a rotary evaporator.

Column Chromatography

A conventional column chromatographic technique was employed with slight modifications. A column measuring 1707 mm in height and 15 mm in inner diameter was packed with neutral silica gel (mesh size 60-120) for the separation process. The silica gel slurry was prepared in chloroform and allowed to settle overnight to ensure complete saturation. Special care was taken to avoid air pockets or voids in the packed column. The selection of the mobile phase system was based on the solubility of the isolated anthraquinone derivatives. A mobile phase ratio of 9.5:0.5 (chloroform: methanol) was optimized for efficient separation. The mobile phase was passed through the column, and each eluted fraction was collected separately in individual vials. The collected fractions were analyzed by thin-layer chromatography (TLC) to confirm successful isolation.

HPLC Analysis

High-performance liquid chromatography (HPLC) was performed using an isocratic system equipped with a UV detector for the detection of anthraquinone derivatives. The optimized instrument specifications and analysis conditions were as follows:

- **Instrument:** Shimadzu (Asia-Pacific) P Ltd., Model No. LC-10 AdVP
- **Injection System:** Reodyne injector (20-µL)
- **Analytical Column:** C-18 (250 mm × 4.6 mm, 5 µm)
- **Mobile Phase:** 85:15 (HPLC-grade methanol : 0.5% acetic acid)
- **Flow Rate:** 0.6 mL/min
- **Detection Wavelength:** 254 nm

Preparation of Standard Solutions

Stock solutions of standard anthraquinone derivatives (rhein, emodin, and chrysophanic acid) were prepared in HPLC-grade methanol at a concentration of 500 mg/L. These solutions were used for further analysis.

Preparation of Sample Solutions

The extracted anthraquinone derivatives were dissolved in HPLC-grade methanol to achieve an approximate concentration of 500 mg/L in stock solutions. Before analysis, the mobile phase was filtered through a 0.45 µm filter paper using a filtration assembly, followed by sonication for 10 minutes to eliminate dissolved gases or air bubbles. All sample analyses were performed under identical conditions and compared with authentic reference standards.

NMR Spectroscopy

Nuclear Magnetic Resonance (NMR) spectra for selected anthraquinone derivatives were recorded on a Bruker AMX 400 instrument. Samples were prepared in $CDCl_3$ solvent, and all results were compared using tetramethylsilane (TMS) as an internal standard.

Mass Spectroscopy

Mass spectra were recorded using a Micromass 70-70E spectrometer with a Fast Atom Bombardment Mass Spectrometry (FABMS) detector. The system was attached to a gas chromatograph equipped with a direct injection probe.

The anthraquinone derivatives isolated from Cassia roxburghii were successfully separated using conventional column chromatography and HPLC techniques. The HPLC method employed a C-18 column and a UV detector to identify and quantify the anthraquinone compounds present in the sample extract. The detailed experimental conditions have been discussed in the previous sections.

Reproducibility of Standards Using HPLC-UV Techniques

Before analyzing unknown samples, method validation was performed to ensure the reproducibility and reliability of the analytical technique. Standardization of the HPLC system was conducted using rhein, emodin, and chrysophanic acid. The standards were injected individually and in combination to determine their retention times under the applied chromatographic conditions.

Retention Times and Standard Deviations:

- **Rhein:** 10.57 min (SD = 0.197)
- **Emodin:** 16.13 min (SD = 0.123)
- **Chrysophanic Acid:** 21.16 min (SD = 0.143)

Table-1 Retention time of three standards in the presence of each other with standard deviation

Sr. No.	Sample Name	RT-Rhein (in min.)	RT-Emodine (in min.)	RT-Chrysophanic acid (in min.)
1	Mix STD-1	10.86	16.31	21.37
2	Mix STD -2	10.85	16.28	21.34
3	Mix STD -3	10.50	15.98	21.00
4	Mix STD -4	10.50	15.99	21.03
5	Mix STD -5	10.52	16.13	21.17
6	Mix STD -6	10.57	16.17	21.22
7	Mix STD -7	10.28	16.04	21.02
8	Mix STD -8	10.46	16.13	21.13
Average		*10.57*	*16.13*	*21.16*
S. deviation		*0.0743*	*0.047*	*0.054*

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.

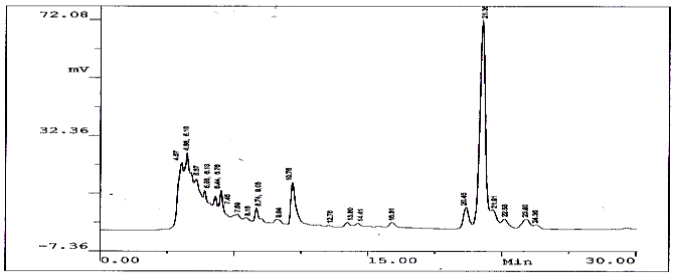


Figure-1 HPLC chromatogram of extract from seeds of *cassia fistula*

All three major components of anthraquinone derivatives are found in the root, seeds and flowers of plant Cassia roxburghii. The fig-1 shows the chromatogram of fraction collected from seeds of cassia plants. 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 method is used to confirm the linearity of all three components.

Table- 2 HPLC data for eluted fractions with their retention time and area percentage

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

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.

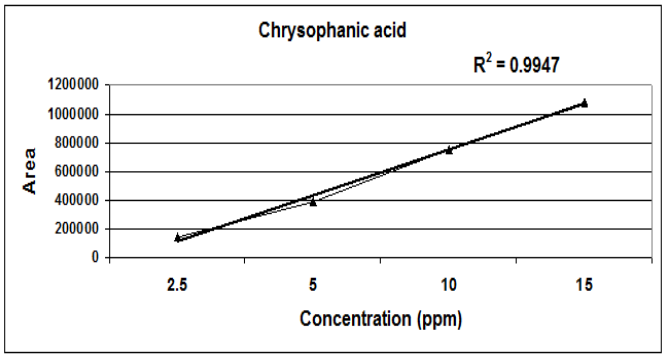


Figure-2 Calibration curve of chrysophanic acid in the range

2.5 to 15 of µg/mL

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. Calibration curves are constructed for each analyte by using a series of standard mixture solutions. Least-squares linear regression is used to determine the calibration parameters for each of standards. A summary of the calibration studies for the three analytes is presented in Table-3. The linearity of all calibration curves is determined by calculating the correlation coefficients, which varied from 0.9901 to 0.9947, which suggests that all three major components are in good correlation with each other.

Table-3 Correlation coefficient of three standards with their minimum detection limits

Compounds	Correlation coefficient	Range (µg/mL)
Rhein	0.9901	2.5 -15
Emodin	0.9914	2.5 -15
Chrysophanic Acid	0.9947	2.5 -15

The HPLC chromatogram of fractions collected from column chromatography under the specified conditions is given in the fig-3. The fig-3 clearly demonstrates the presence of three major components viz. rhein, emodine and chrysophanic acid along with some minor fractions of other anthraquinone derivatives like aleo-emodine.

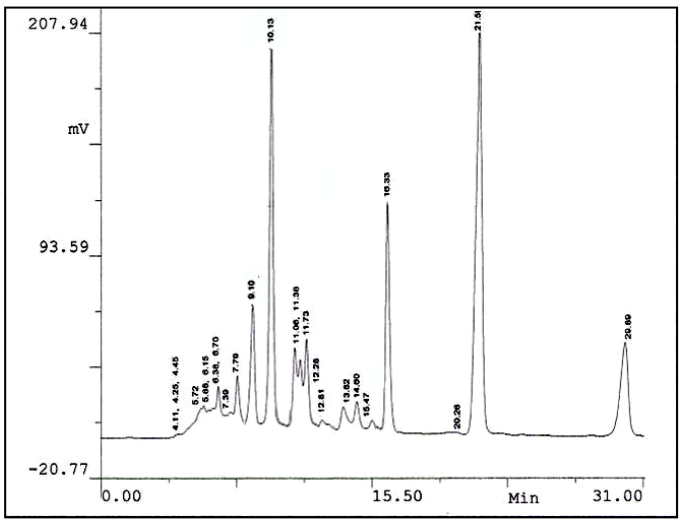


Figure-3 HPLC Chromatograph of eluted fractions from column chromatography, which indicates the presence of three major components.

A recovery study is performed to validate the accuracy of the developed method. Hence, extracted fractions are spiked with standard stock solutions of the analytes in triplicate at two different concentrations by using standard addition method. The spiked samples are extracted and purified with column chromatography. The spiked samples are analyzed using the same experimental and instrumental conditions as previously described for the analysis of the un-spiked samples. The recovery is determined by comparing the amount of analyte added to the samples and the amount of analyte detected during HPLC analysis and a summary of each is given in the table-4.

Table-4 Recovery data of standard addition by HPLC-UV detector techniques

Compounds	Concentrations µg/mL	Added amount	Theoretical (µg/mL)	Obtained (µg/mL)	Recovery (%)
Rhein	1.20	10 µg	1.59	1.56	98.1
		20 µg	1.98	1.92	96.9
Emodin	2.68	10 µg	3.06	2.98	97.5
		20 µg	3.45	3.37	97.6
Chrysophanic Acid	8.71	10 µg	9.07	9.1	99.3
		20 µg	9.43	9.32	98.8

Table-4 clearly shows that recovery of each component is varied from 96.9% to 99.3% under the specified conditions in which the experiments are carried out.

The method is validated by determining the intra- and inter-day precision. The relative standard deviation (R.S.D.) values for the retention times for the intra-day precision is 0.25%-0.703% (n=8). The R.S.D. values obtained for the inter-day precision study for the retention times is 0.29%-0.72% (n =8), which clearly suggests the method validation.

III.RESULTS AND DISCUSSION

Results of NMR spectroscopy

The proton NMR of extracted material is taken in CDCl3 solvent with respect to TMS as a reference. The δppm 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 structures of three anthraquinone derivatives are shown below and the 1H NMR signals are summarized in table-5.

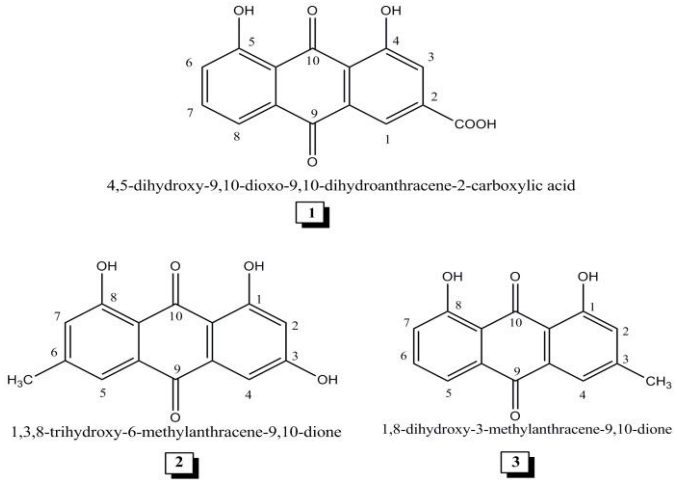


Figure-4 Probable structures of three anthraquinone derivatives

1		2		3	
Signal No.	Signal Position δppm	Signal No.	Signal Position δppm	Signal No.	Signal Position δppm
1(a) Ar-H	7.411-7.474	1(a) CH3	2.179	1(a) CH3	2.441
2(b) Ar-H	7.267	2(b) Ar-H	6.679	2(e) Ar-H	7.049
3(c) Ar-H	7.556-7.774	3(c) Ar-H	7.402	3(b) Ar-H	7.266
4(e) Ar-H	7.986	4(d) Ar-H	7.875	4(f) Ar-H	7.765 - 7.780
5(d) Ar-H	8.477	5(e) Ar-H	7.094	5(h) Ar-H	7.172
6(f) Ar-COOH	11.997	6(h) Ar-OH	5.423	6(g) Ar-H	7.757
7(g) Ar-OH	5.35	7(f, g) Ar-OH	5.452	7(c) Ar-OH	5.457
8(h) Ar-OH	5.42	-	-	8(d) Ar-OH	5.428

Table-5 1H NMR data of three anthraquinone derivatives

The first anthraquinone derivative, which was isolated using various chromatographic technique gives a molecular ion peak at m/z 284 (HR-MS) corresponding to the molecular formula C15H8O6 (calculated for Rhein [1], C15H8O6, M.W. 284.03). The UV-VIS spectrum showed absorption maxima at 220nm, 256nm and 345nm, which suggest the presence of extended π-bond system, aromatic rings and phenolic –OH (for $n \rightarrow \pi^*$ transition) (13). A bathochromic shift (407 to 531 nm) is observed on addition of NaOH suggests the presence of -OH. This is further confirmed by the IR spectra, which showed band of -OH group stretching at around 3400 cm-1, a chelated carbonyl at 1638 cm-1, carbonyl of an carboxylic group at 1703 cm-1and the absorption for free carbonyl at 1676 cm-1 groups are observed. In addition, the 1H NMR spectrum showed the characteristic up field signal for the hydroxyl group at 5.35 δppm (1H. S) and 5.42 δppm (1H. S). The 1H NMR spectrum also revealed the presence of caroboxylic acid proton at 11.997 δppm (1H, S) and five aromatic protons ranging between 7.267 δppm to 8.477 δppm elucidate the structure of anthraquinone ring and its substitution (13).

The second anthraquinone derivative, isolated using various chromatographic technique gives a molecular ion peak at m/z 270 (MS) corresponding to the molecular formula C15H10O5 (calculated for Emodine, C15H10O5, M.W. 270.05). The UV-VIS spectrum of second anthraquinone derivative shows absorption maxima around 220nm, 256nm and 345nm, which suggest the presence of extended π-bond system, aromatic rings and phenolic –OH (for n→π* transition) (13). A bathochromic shift (407 to 531 nm) is observed on addition of NaOH suggests the presence of -OH. This is further confirmed by the IR spectrum, which showed band of -OH group stretching at around 3400 cm-1, a chelated carbonyl at 1638 cm-1 and the absorption for free carbonyl at 1676 cm-1 is observed. In addition, the 1H NMR spectrum showed the characteristic up field signal for the hydroxyl group at 5.423 δppm (1H. S) and 5.452 δppm (2H. S). The 1H NMR spectrum also revealed the presence of an methylic proton at 2.179 δppm (3H,S) and five aromatic protons ranging between 6.679 δppm to 7.875 δppm elucidate the structure of anthraquinone ring and its substitution(13).

The third anthraquinone derivative is isolated by using chromatographic techniques shows a molecular ion peak at m/z 254 (MS) corresponding to the molecular formula C15H10O4 (calculated for Physicon, C15H10O4, M.W. 254.06). The UV-VIS spectrum shows absorption maxima around 256nm and 345nm, which suggest the presence of aromatic rings and phenolic –OH (for n→π* transition) (13). A bathochromic shift (407 to 531 nm) is observed on addition of NaOH suggests the presence of -OH. This is further confirmed by the IR spectrum, which showed band -OH group stretching at around 3400 cm-1, a chelated carbonyl at 1638 cm-1 and the absorption for free carbonyl at 1676 cm-1 is observed. In addition, the 1H NMR spectrum showed the characteristic up field signal for the hydroxyl group at 5.423 δppm (1H. S) and 5.452 δppm (2H. S). The 1H NMR spectrum also revealed the presence of an methylic proton at 2.179 δppm (3H,S) and five aromatic protons ranging between 6.679 δppm to 7.875 δppm elucidate the structure of anthraquinone ring and its substitution(13).

Results of Mass Spectroscopy

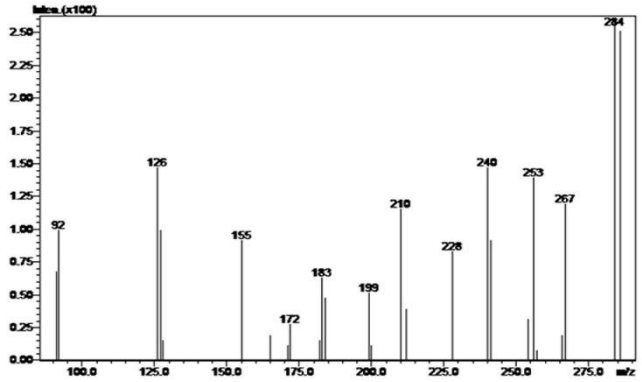


Figure-4 Mass spectrum of rhein

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-CH2OH]– and an adduct formation between the ion at m/z 239 and methanol [M-COOH + MeOH] – respectively.

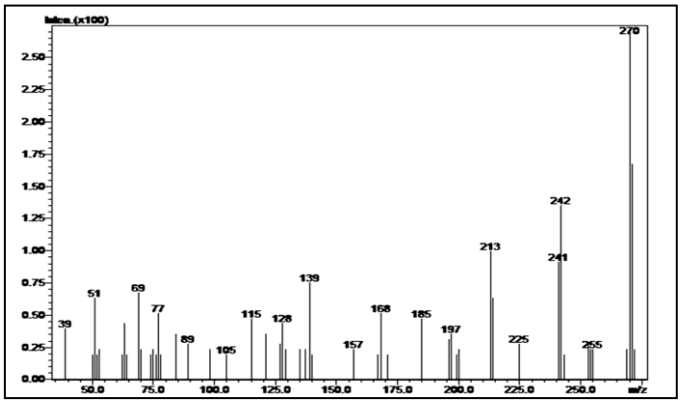


Figure-4 Mass spectrum of emodine

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 meta stable 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

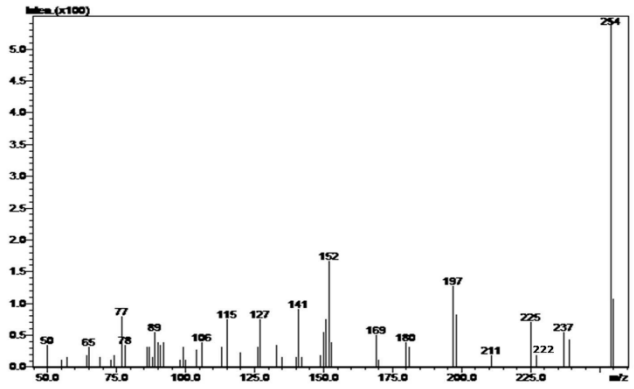


Figure-5 Mass spectrum of chrysophanic acid

The fragmentation pattern of physicon 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-C3O3H) +.

IV. Conclusion

In this study, conventional column chromatography and a simple HPLC technique were successfully employed to separate the therapeutic anthraquinone derivatives—rhein, emodin, and chrysophanic acid—from Cassia roxburghii. The isolated components were further characterized and confirmed using NMR and mass spectroscopic techniques, ensuring their structural identification and purity. This approach provides a reliable method for the extraction and analysis of medicinally important anthraquinones from plant sources.

V. Acknowledgement

The authors are thankful to Govt. of Gujarat and Principal of the H & H. B. Kotak Institute for providing constant support.

VI. References

[1]. Lincoln, T.; Eduardo, Z., Plant Physiology, Fourth Edition, 2006, Sinauer publication.

[2]. http://www.healthguidance.org/entry/6303/1/Prehistoric-Medicine.html

[3]. Farnsworth, N. R.; Akerele, O.; Bingel, A. S.; Soejarto, D. D. and Guo, Z. Bull WHO 1985, 63, 965-981.

[4]. Neuss, N.; Gorman, M.; Svoboda, Maciak G. H. G. and Beer C. T. V. J. Am. Chem. Soc., 1959 81:, 4754-4755.

[5]. Johnson, I. S.; Wright, H. F.; Svoboda, G. H. and Vlantis, J. Cancer Res. 1960, 20, 1016-1022.

[6]. Neuss, N.; Gorman, M. and Cone, N. J. Lloydia, 1964, 27(4), 389-392.

[7]. Neuss, N.; Gorman, M.; Hargrove, W. Cone, N. J.; Biemann, K.; Buechi, G. and Manning, R. E. Vinca Alkaloids XXI. J. Am. Chem. Soc. 1964, 86(7), 1440-1442.

[8]. Cragg, G. M.; Newman, D. J. and Weiss, R. B. Coral Reefs, Oncology 1997, 24 (2), 156-163.

[9]. Satoskar, R.S., Pharmacology and Pharmacotherapeutics 19th Edition 2005, Popular Prakashan Bombay pp994-995.

[10]. http://en.wikipedia.org/wiki/Skin

[11]. www.leeds.ac.uk/.../scabies/scabies.html

[12]. Fernand, V. E. Dinh, D. T., Washington, S. J., Fakayode, S. O., Losso, J. N., Van Ravenswaay, R. O. and Warner, I. M. Talanta 2008 74(4) 896-902.

[13]. Nok Hadiani Ismail., Abdul M. Ali., Nokio Aimi.T., Mariko Kitajima., Hiromitsu Takayama and Nordin H. Lajis., 1997. ANTHRAQUINONES FROM MORIlVDA ELLIPTICA., Phytochemistry 45(8) 1723-1725.