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RESEARCH ARTICLE

Extraction, Isolation and Structure Elucidation of a Bioactive Constituent from Chloroform Extract of *Moringa concanensis* Leaves

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Abstract

Moringa concanensis Nimmo. (Moringaceae) is a wild plant found in India. Traditionally, tribes used this plant as an antioxidant, anticancer, anticonvulsant, antimicrobial, antifertility, anti-inflammatory, antipyretic, and analgesic. In the present study, the leaves of *Moringa concanensis* were subjected to successive solvent extraction with five different solvents of increasing polarity, i.e., petroleum ether, chloroform, ethyl acetate, methanol, and water, followed by a phytochemical screening of each extract. The phytochemical study indicated the presence of terpenoids, steroids, cardiac glycosides, flavonoids, alkaloids, carbohydrates, proteins, tannins, and phenolics. The chloroform extract showed the presence of cardiac glycosides (cardenolides); therefore, a RP-HPLC method was developed to identify the number of constituents in the extract. The extract was recrystallized to isolate the single component and to gain the desired purity for further spectral analysis. Different spectral data from UV-Visible, Infrared, Mass, and Nuclear Magnetic Resonance spectroscopy were analyzed and the isolated chemical constituent was identified.

Keywords: Cardiac Glycosides, Infrared Spectroscopy, Mass Spectroscopy, RP-HPLC, Structure Elucidation

1. Introduction

Plants are the basis of sophisticated medicinal systems that have been in existence for thousands of years, and they continue to provide humans with newer remedies. Conventionally, the isolation of bioactive chemical components is preceded by the determination of such compounds in plant extracts through several bioassays. The current advanced scientific techniques make the isolation of chemical constituents with medicinal importance simpler and easier. *Moringa concanensis* is a Moringa genus species with broader traditional medicinal uses, but due to less phytochemical exploration of species, it is unlikely to be incorporated in pharmaceutical formulations. According to the World Health Organization, herbal medicine is used by 80% of the world's population[1-3]. This study focuses on the exploration of the *Moringa concanensis* plant, including the identification of secondary metabolites that are bioactive. This plant has an impressive medicinal use like: antioxidant, anticancer, anticonvulsant, antimicrobial, antifertility, anti-inflammatory, antipyretic and analgesic along with a good nutritional value[4-14]. The present investigation provides information regarding the phytochemical evaluation of chloroform extract and the detailed structural elucidation of bioactive components present in the extract. The techniques used for the structure elucidation are HPLC (High Performance Liquid

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Chromatography)[17], UV, Infrared Spectroscopy, Mass Spectroscopy, ^{1}H, ^{13}C NMR spectroscopy[15].

2. Experimental

2.1 Collection, Authentication and Extraction

The *Moringa concanensis* leaves were collected from Navsari district, south Gujarat region and authenticated by taxonomist from Aspee College of Horticulture and Forestry, Navsari Agricultural University, Gujarat, India. After the authentication; the material was dried and open-air dried under continuous observation. The dried plant material was grounded and sieved through 40# mesh size for further extraction process. The successive solvent extraction was carried out with five solvents of increasing polarity i.e., petroleum ether, chloroform, ethyl acetate, methanol and water. The extracts obtained were further dried, and the yield was calculated. Further, the phytochemical screening of extracts was performed using conventional methods[16].

2.2 HPLC method for identification of components from extract:

High-performance liquid chromatography (HPLC) was used to separate and identify the components present in the extract. Many trials were conducted to establish the method for identifying the component using various mobile phase compositions and at different wavelengths. One gram of chloroform extract was dissolved in 100 ml methanol and this solution was injected into the HPLC system. System and condition details for the HPLC analysis are as follows:

- HPLC: Agilent Technology
- Model: 1220 Infinity LC
- Column: Neclosil-C_{18} (125mm×4.6mm, 5µm particle size)
- Wavelength: 220 nm
- Detector: PDA (Photodiode Array) Detector
- Flow rate: 0.5 ml/min
- Mobile Phase: Acetonitrile: Water: 1% Orthophosphoric Acid (50:50:0.1, V:V:V)

2.3 Recrystallization

The HPLC chromatogram shows the presence of one major component with an approximate purity of 80-83 %. Therefore, recrystallization of the extract was done to increase the purity of the major component. About 1.5 gm of extract was dissolved in 5 ml of methanol and the water was added dropwise up to 1 ml; when the crystal was fallen out in solution, further it was filtered and dried at 40°C. The recrystallised product was dissolved in methanol to obtain a 1 mg/ml concentration. This solution was again injected into the HPLC system.

2.4 Structure Elucidation

The recrystallised product was sent for identification of spectral characteristics using different analytical techniques like UV, IR, NMR and Mass. The assembled data was then interpreted, and the component was identified.

3. Results and Discussion

Table 1 shows the percentage yield of the extracts after successive solvent extraction. A phytochemical screening of different extracts was performed using conventional methods and the results are shown in Table 2. After phytochemical screening, it was confirmed that chloroform extract showed the presence of cardiac glycosides and further confirmatory tests confirmed the presence of cardenolide-type cardiac glycosides.

The high-performance liquid chromatography method was developed for the effective separation of components from chloroform extract. Different solvent

Table 1. Extraction details of *M. concanensis* leaves

Sr. No.	Solvent	Colour	Consistency	% Yield w/w
1	Petroleum ether	Greyish green	Sticky	16 %
2	Chloroform	Dark green	Sticky	3 %
3	Ethyl acetate	Dark brown	Sticky	3 %
4	Methanol	Yellowish brown	Sticky	8 %
5	Water	Brown	Sticky	10 %

Table 2. Phytochemical screening of extracts

Sr. No.	Phyto-constituents	Types of Extract				
		Petroleum Ether	**Chloroform**	**Ethyl Acetate**	**Methanol**	**Water**
1	Carbohydrates	-	-	-	+	+
2	Proteins	-	-	-	+	+
3	Terpenoids	-	-	+	-	-
4	Steroids	-	+	-	-	-
5	Cardiac Glycosides (Kedde's test, Legal's test)	-	+ + +	-	-	-
6	Anthraquinone Glycosides	-	-	-	-	-
7	Flavanoids	-	-	-	+	+
8	Tannins and phenols	-	-	-	+	+
9	Alkaloids	-	-	-	+	+

+ : Positive, - : Negative

systems and wavelengths were tried. The chromatogram of chloroform extract is shown in (Figure 1). It shows the major component of approximate purity of 83% at 12.267 min retention time and (Figure 2) is chromatogram of methanol as blank solvent run. Recrystallisation of chloroform extract using methanol and water showed a 15.33% yield, as shown in Table 3.

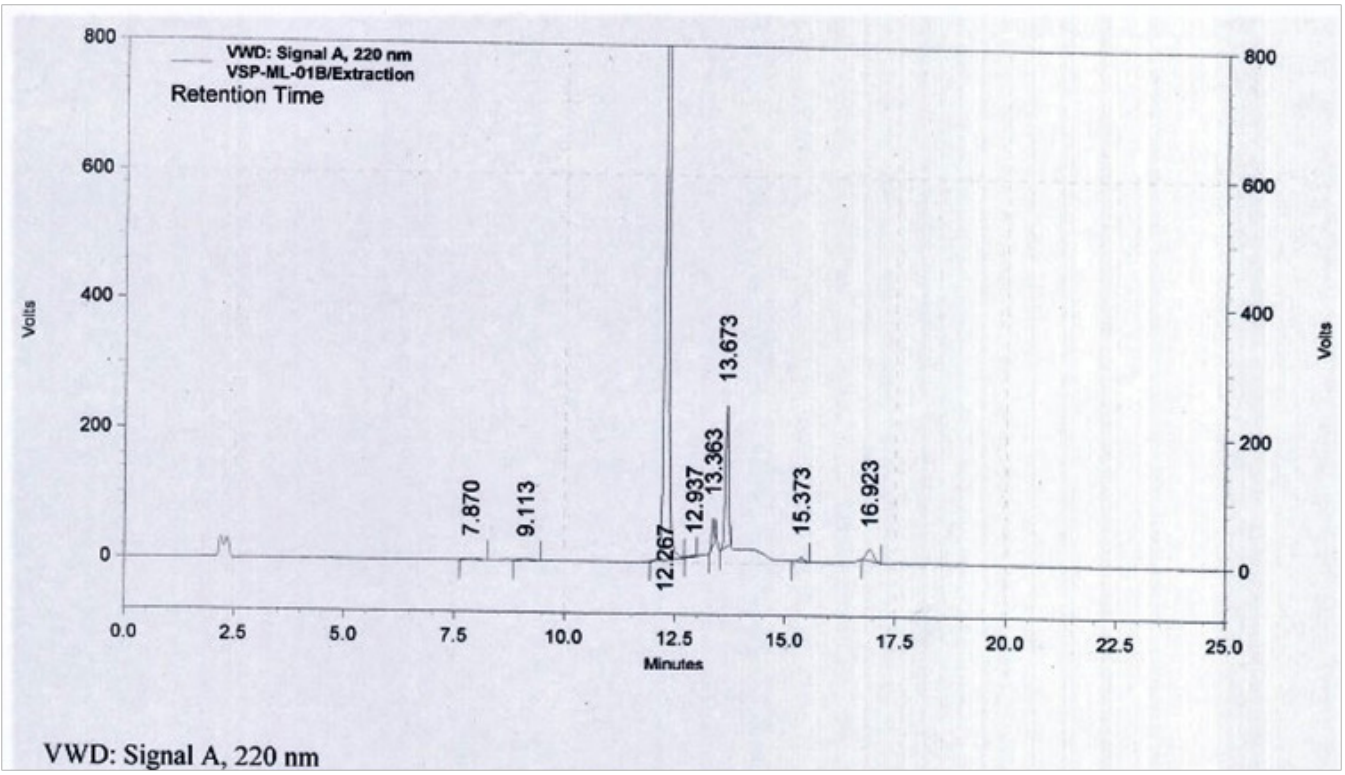


Figure 1. HPLC chromatogram of chloroform extract of *M. concanensis* leaves at 220nm.

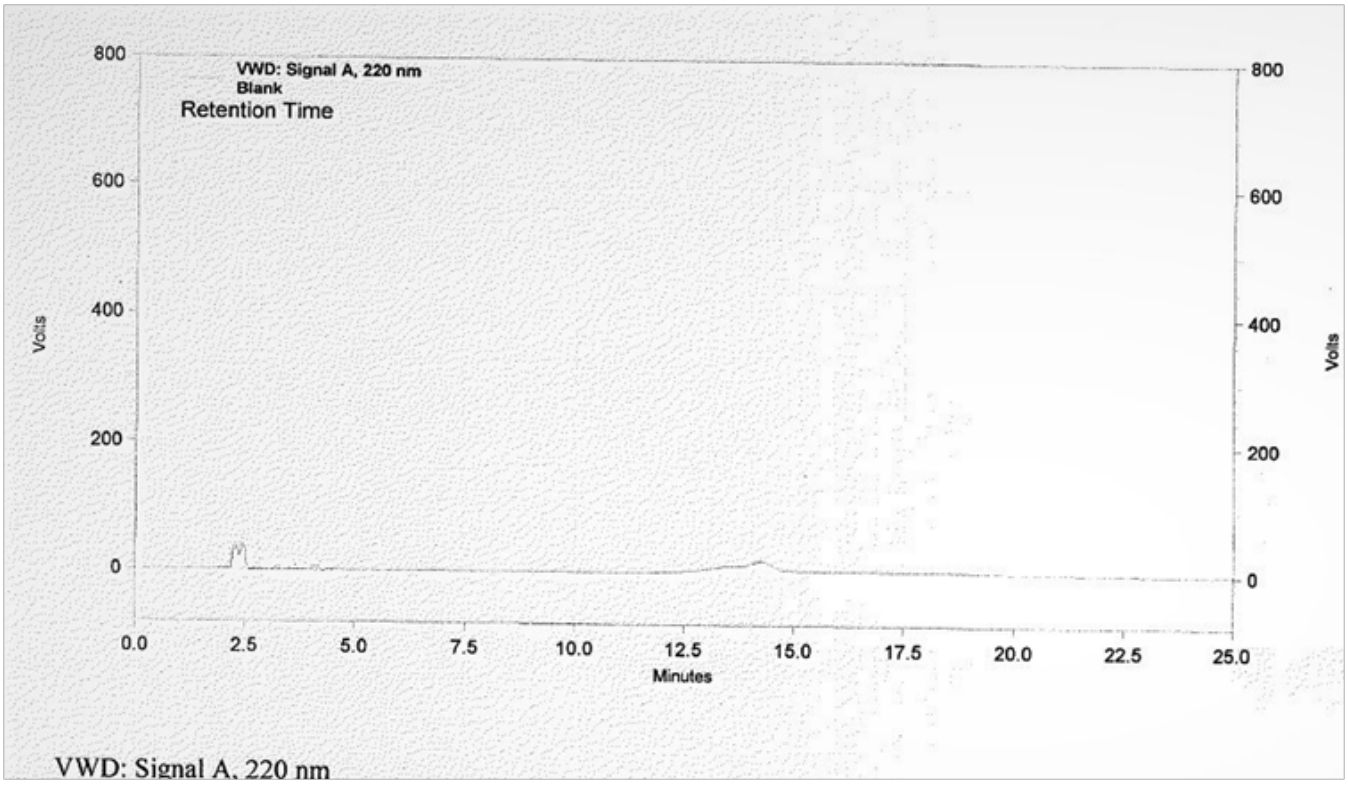


Figure 2. HPLC chromatogram of blank solvent Methanol at 220 nm.

Table 3. % yield of recrystallised chloroform extract

Amount of solvent (ml)	Amount of extract taken	Amount of recrystallised product	% Yield
Methanol + Water (5 + 1)	1.5 gm	0.23 gm	15.333 %

The result of the melting point of a recrystallized product is mentioned in Table 4. The melting point of the recrystallized product was 240 – 245 °C which was nearer to the reported melting point of digoxin, i.e. 230 – 265 °C.

Table 4. Melting point of recrystallized product

Compound	Melting Point
Recrystallized Product	240-245 °C (Measured)
Digoxin Standard	230-265 °C (Reported)[18]

Further, the HPLC chromatogram of recrystallised product showed an increased purity of the major component from 83% to 95% at same retention time, which is shown in Figure 3.

The recrystallised product of approximately 95% purity was further subjected to structure elucidation using different spectroscopical data like UV, IR, Mass and NMR.

The UV spectra were observed to determine the wavelength of the recrystallized product, and it was found to be 220 nm as shown in Figure 4. The result of Infrared Spectroscopy (IR) of the recrystallised product is given in Figure 5. The infrared spectra of the recrystallised product shows ~3400 frequency for -OH stretching, 2935.7 for -C-H stretching, 1719.19 for -C=O stretching and 1322 for -C-O stretching. The result of Mass Spectroscopy (MS) of the recrystallised product is given in Figure 6. The mass spectra reveal the mass of the compound, i.e., 781.80 which is nearer to the mass of digoxin, i.e., 780.93.

The proton NMR of the recrystallised product gives the number and types of protons present in the component. The different chemical shift values obtained with multiplets for 64 number of hydrogens which exactly matches with the number and types of hydrogen present in digoxin. Figures 7 and 8 show the results of NMR spectra. The carbon NMR of the recrystallised product gives the number and types of carbon present in the component. The different chemical shift values

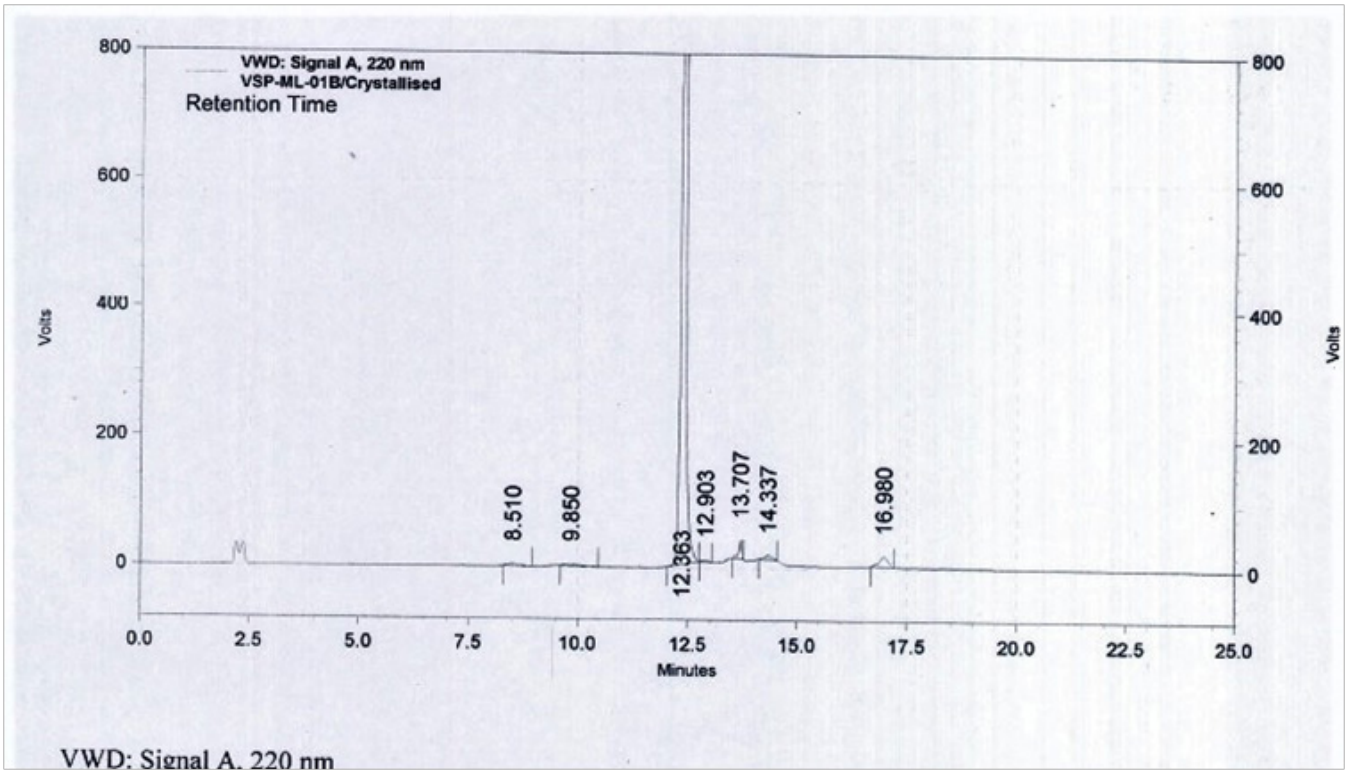


Figure 3. HPLC chromatogram of recrystallised product at 220 nm.

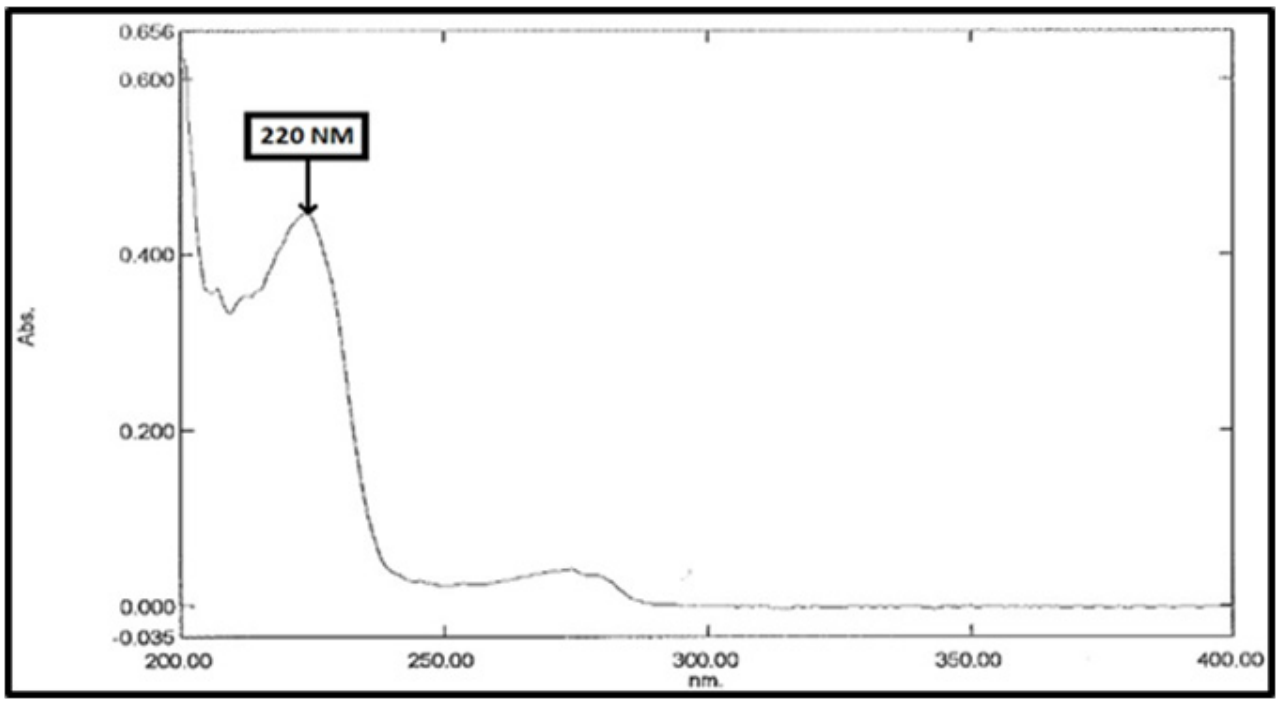


Figure 4. UV spectra of recrystallised product.

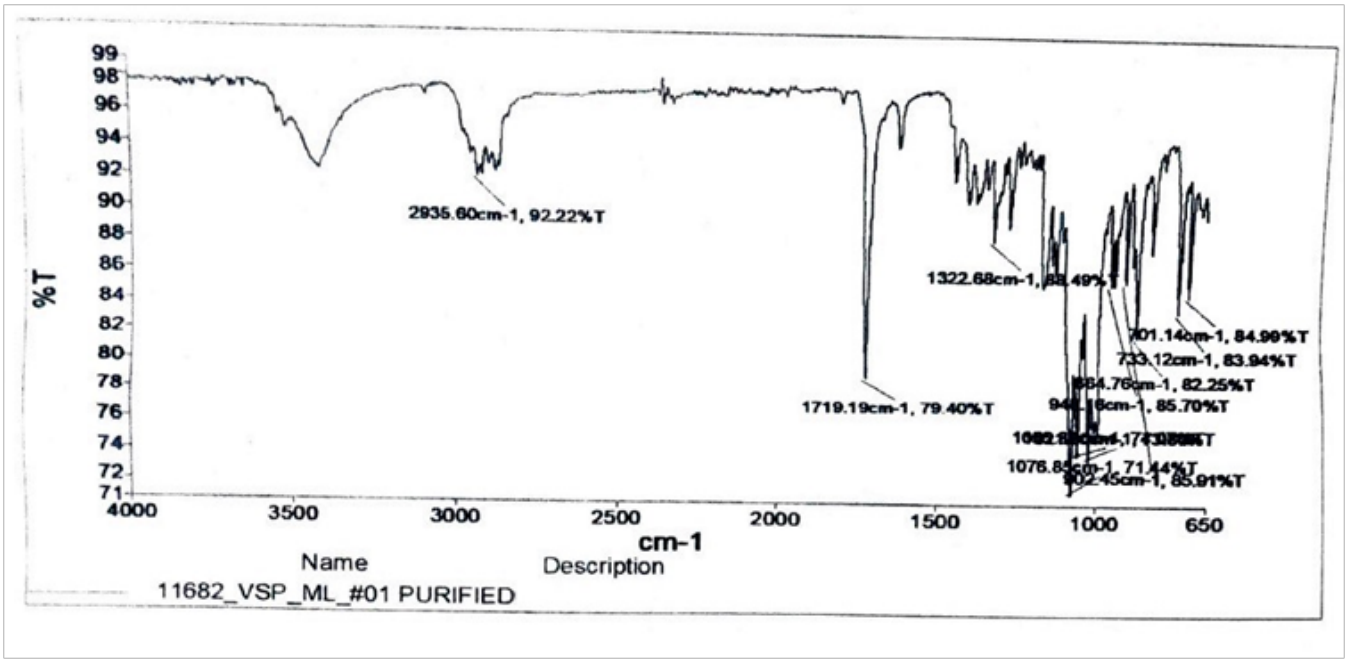


Figure 5. Infrared (IR) spectra of recrystallised product.

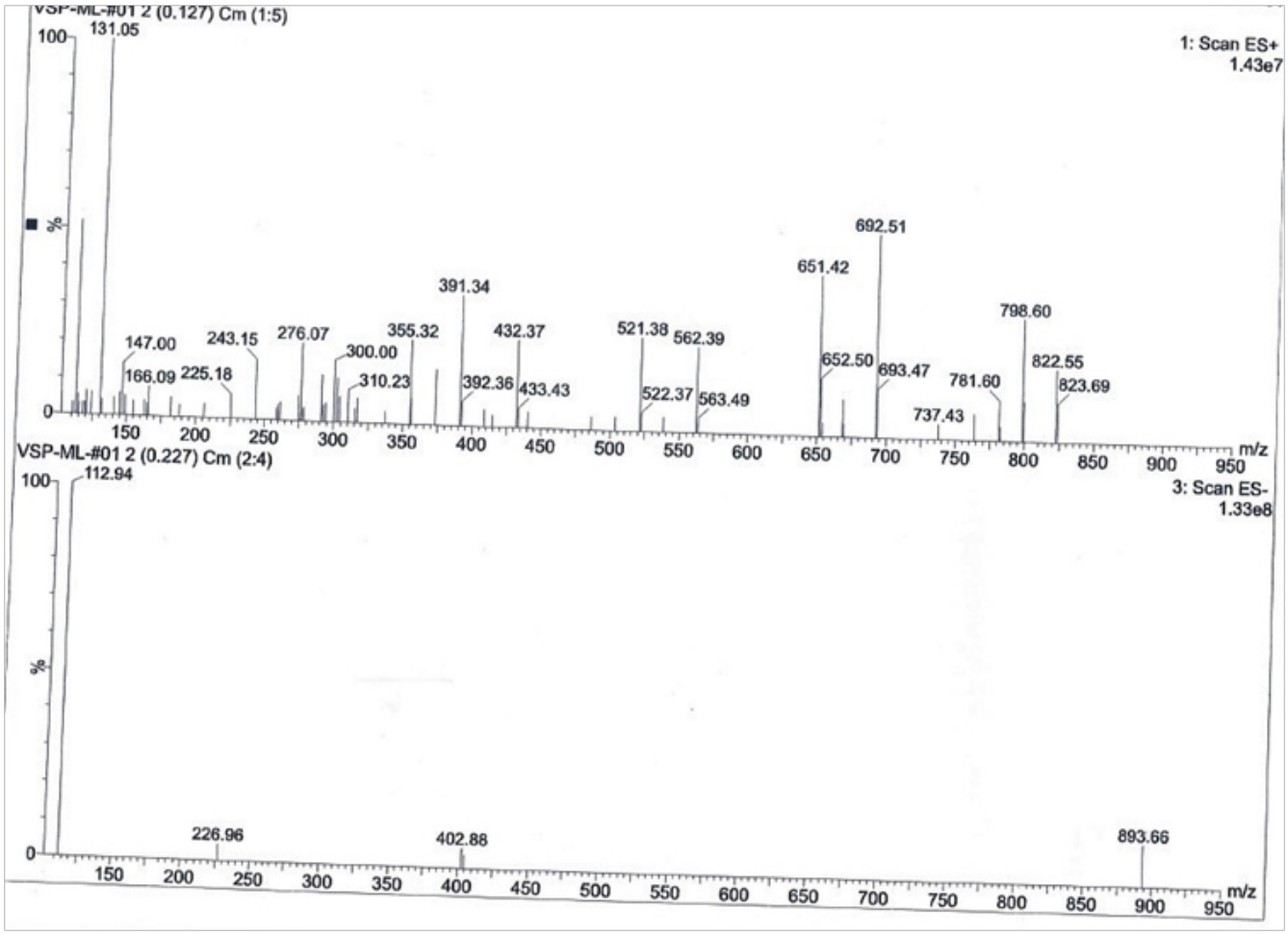


Figure 6. Mass spectra of recrystallised product.

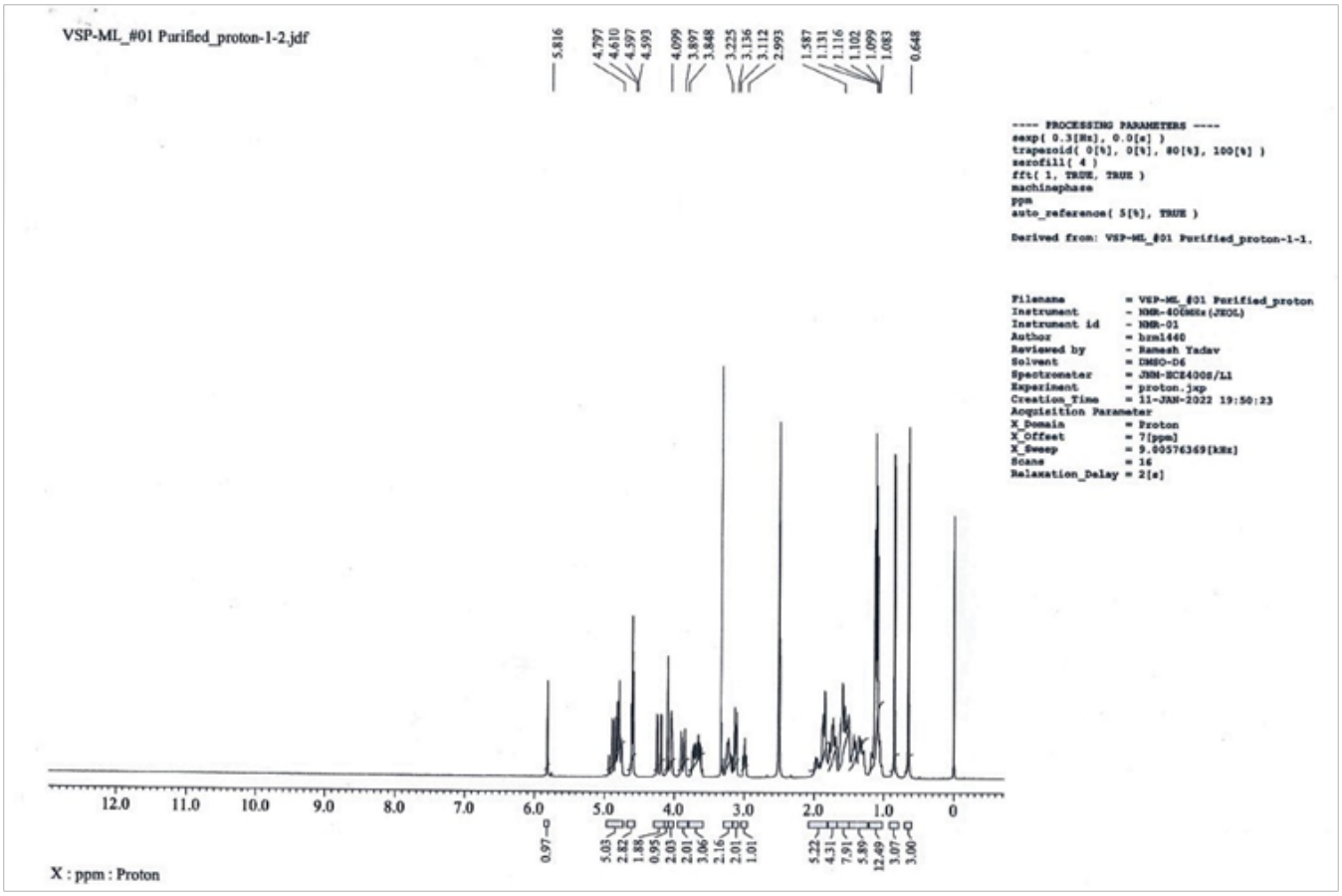


Figure 7. H^1 NMR spectra of recrystallised product.

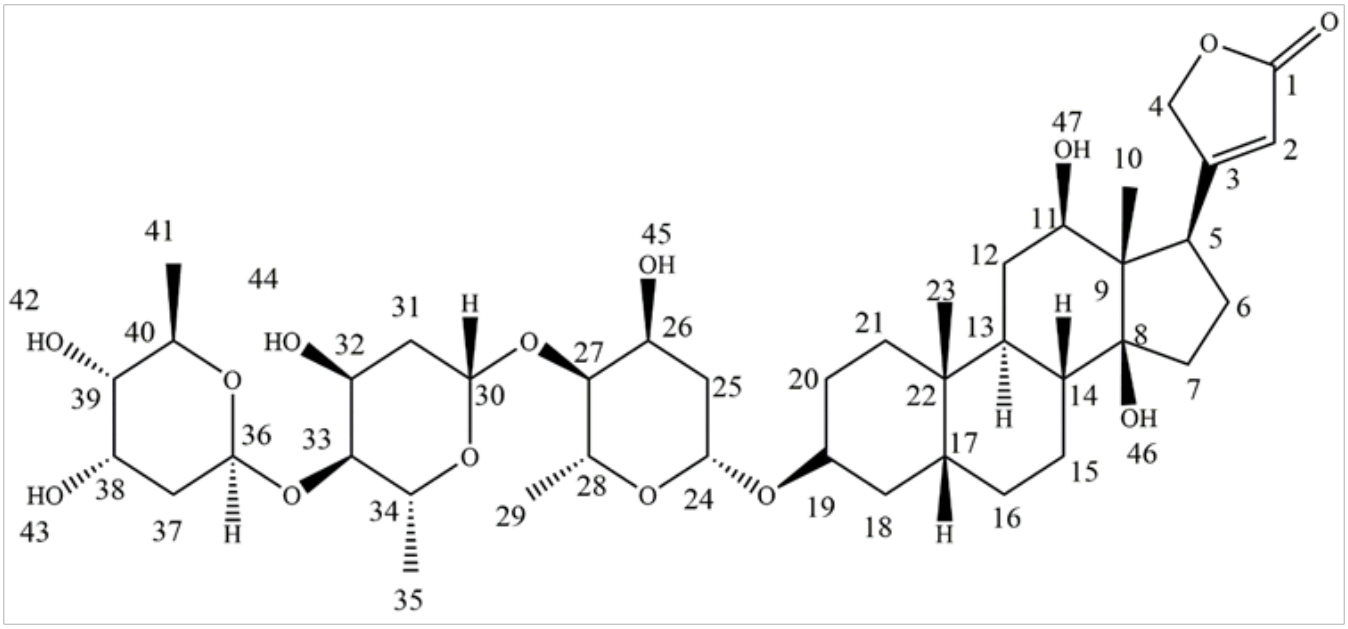


Figure 8. Structure of digoxin with number of hydrogens.

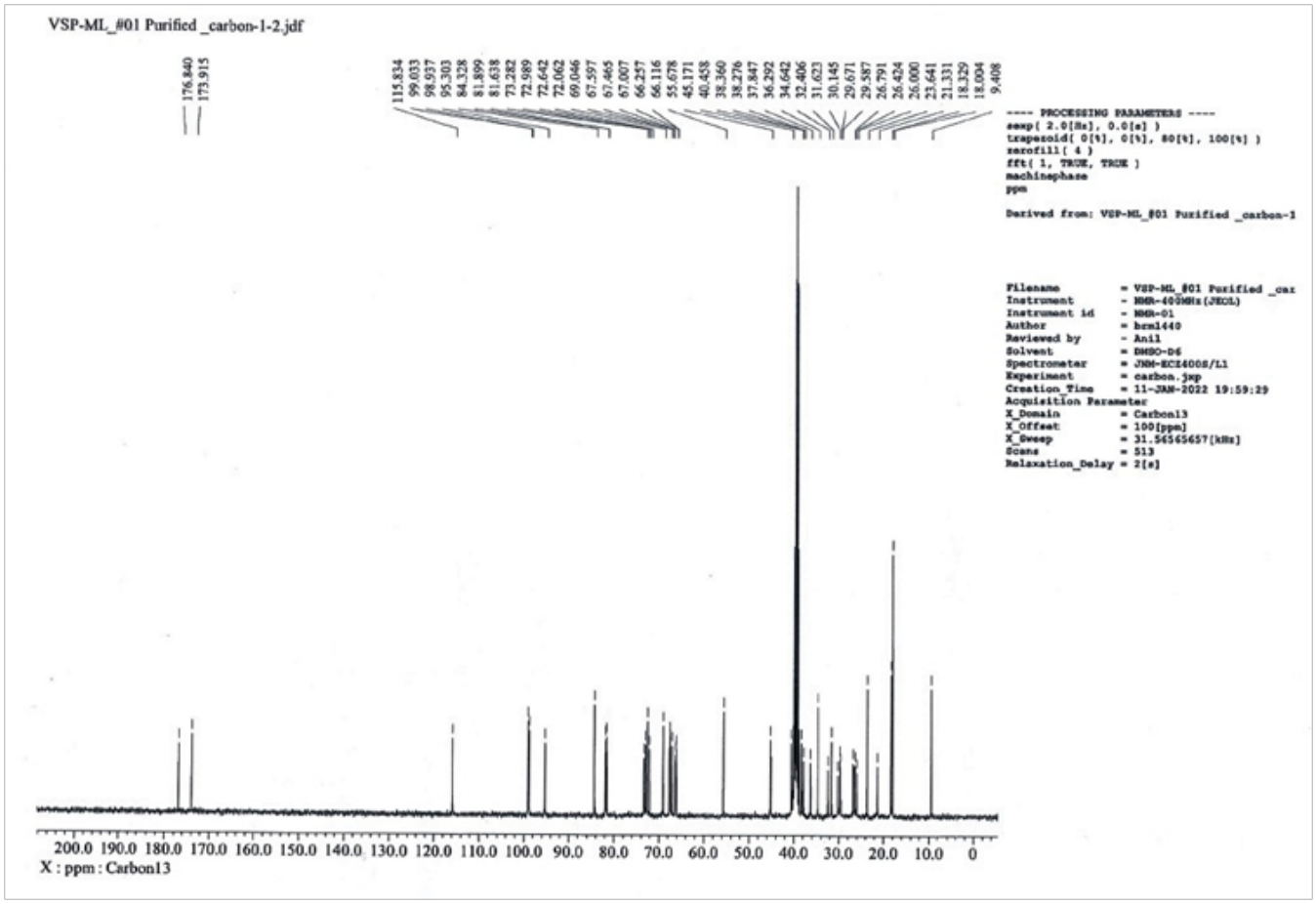


Figure 9. C^{13} NMR spectra of recrystallised product.

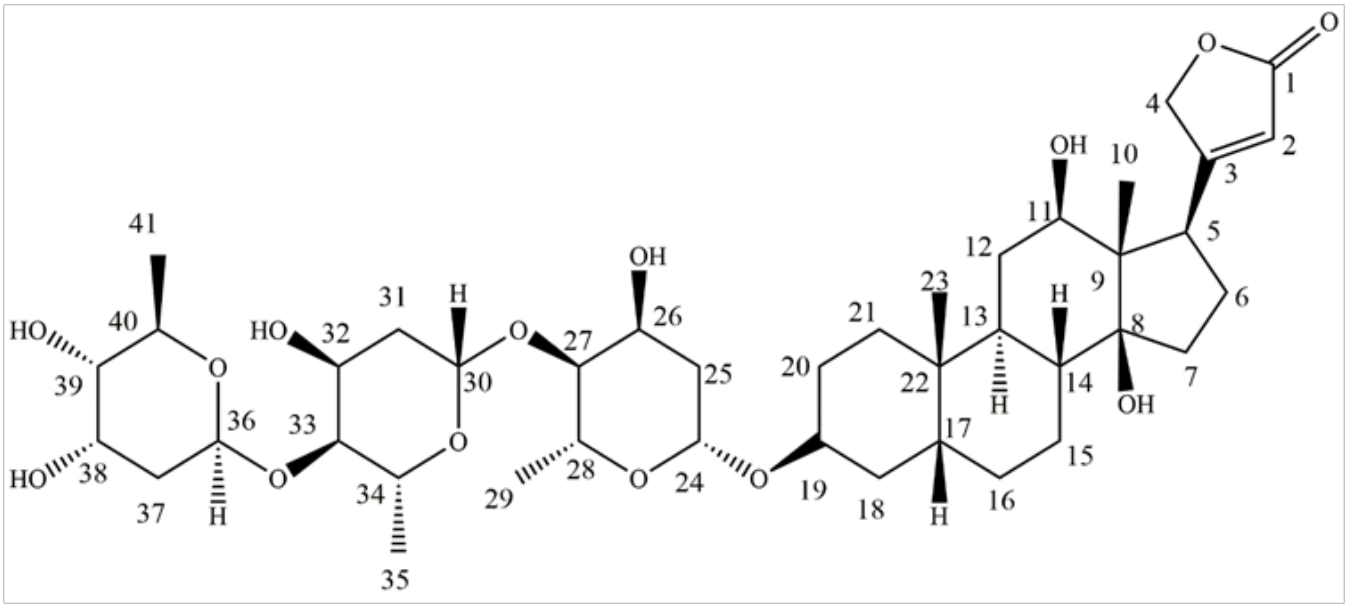
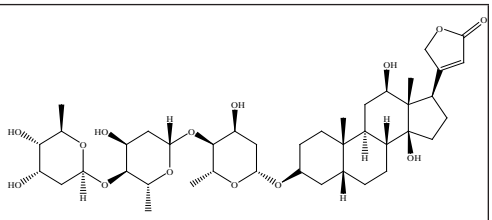


Figure 10. Structure of digoxin with number of carbons.

obtained for 41 number of carbons which exactly matches with the number and types of carbon present in digoxin (Figures 9, 10). After spectroscopical and chromatographic study, it was concluded that digoxin is present in the chloroform extract of *M. concanensis* leaves (Table 5).

Table 5. Summary of digoxin

Structure	
Chemical Name	4-((3R,5R,8R,9S,10S,12R,13S,14S,17R)-3-(((2R,4S,5S,6R)-5-(((2S,4S,5S,6R)-5-(((2S,4S,5S,6R)-4,5-dihydroxy-6-methyltetrahydro-2H-pyran-2-yl) oxy)-4-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-4-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-12,14-dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl) furan-2(5H)-one
Molecular Formula	$C_{41}H_{64}O_{14}$
Molecular Weight	780.95
Exact mass	780.43

4. Discussion

The sequential extraction of *M. concanensis* leaf powder was performed using five different solvents of increasing polarity. The yield of extracts in terms of %w/w was calculated on the basis of the mass of crude extract obtained for each solvent. Further, the phytochemical screening of individual extract was performed which indicated the presence of carbohydrates, proteins, terpenoids, steroids, cardiac glycosides, flavonoids, tannins, phenols, and alkaloids. The study further extended on chloroform extract as it indicated the presence of cardiac glycosides (Cardenolide type) which is unique from all the other species of the same genus and shows the uniqueness of plants in terms of phytochemical content. After the RP-HPLC method development, the chromatogram showed a single component with approximately 82.79 % purity. For the different spectroscopical determinations, the purity of the component should be above 90-95 %, so the extract was recrystallized using methanol and water. The recrystallized product showed approximately 95.52 % purity in the HPLC chromatogram. The melting point of the recrystallized product were observed between 240-245 °C which is nearer to digoxin's reported melting point. Further spectroscopical measurements i.e. UV, IR, NMR (^{1}H and ^{13}C), and the mass of the recrystallized product were done to identify the structure of the compound. In UV-Visible spectroscopy, it showed the wavelength maxima of 220 nm similar to digoxin. The mass of the recrystallized product using mass spectrometry is 781.60. In IR spectrometry different frequencies for –O-H stretching, -C-H stretching, -C=O stretching, and –C-O stretching. In NMR spectroscopy, the ^{1}H and ^{13}C NMR spectra show the number of hydrogens i.e., 64 and the number of carbons i.e., 41 respectively, which exactly matches the number of hydrogens and carbons present in the digoxin.

5. Conclusion

From the present study, it is concluded that the recrystallized fraction of chloroform extract of *Moringa concanensis* leaf contains digoxin, which is confirmed on the basis of spectral analysis (UV, IR, HNMR, 13C NMR, and Mass spectsocopy).

6. Funding

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7. References

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