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(RESEARCH ARTICLE)

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Isolation, purification and characterization of vindoline from *Catharanthus roseus*

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Abstract

Catharanthus roseus is a popular medicinal plant available all over the world. Across the globe, different parts of the plant are used to treat diabetes, hypertension, cancer, and menstrual abnormalities. In the current study, aim was to isolate, purify and characterize vindoline from the aerial parts (stem, leaves and flowers) of *C.roseus*. Vindoline was extracted by hot ethanolic extraction procedure and then purified using preparative thin layer chromatography (pTLC). The functional group analysis was performed to authenticate the presence of specific functional groups by using fourier transform- infrared spectroscopy (FT-IR). Liquid chromatography-mass spectroscopy (LC-MS) and high performance liquid chromatography (HPLC) were performed to conform the purity of the isolated compound and the scientific data presented.

Keywords: *Catharanthus roseus* ; Vindoline; Hot ethanolic extraction; Preparative TLC; HPLC; LCMS; FT-IR

1. Introduction

Catharanthus roseus belongs to the apocynaceae family, which is also known as periwinkle or Madagascar periwinkle. The plant is considered to be a good source of pharmaceutically useful terpenoid indole alkaloids [1] and may be dated back to 2600 B.C.E. in Mesopotamia where it was grown as herbal medicines[2]. *C.roseus* is now widely distributed and cultivated in China, India, Indonesia, Australia, North and South America. Monomeric indole alkaloids named by vindoline and catharanthine are the most abundant constituents in the extract of this plant [3]. Biosynthesis of vindoline is catalysed by the enzyme acetyl-coenzyme A: deacetylvindoline 4-0-acetyltransferase (DAT) [4]. Dimers of vindoline & catharanthine and vinblastine & vincristine are used to treat certain malignancies [4]. Vindoline is also used to make vinorelbine and vinflunine semi-synthetically which are used in treating breast cancer and urothelial carcinoma of the bladder [5]. Vinorelbine is an anticancer drug that is used to treat non-small cell lung cancer and breast cancer [3]. Vindoline has also been demonstrated in certain trials to be effective in the treatment of cancer, diabetes mellitus, hyperlipidaemia, cardiovascular illnesses, and renal disease. The antibacterial, antifungal, antiviral and antioxidant properties of this herb have also been well documented [6-9]. The study aiming to isolate vindoline from aerial parts of the plant by hot ethanolic extraction method followed by preparative TLC for purification. Obtained vindoline from crude alkaloid mixture was subjected to HPLC to check the purity and the molecular weight of vindoline was determined by LC-MS and FT-IR spectroscopy was performed to authenticate the presence of specific functional groups.

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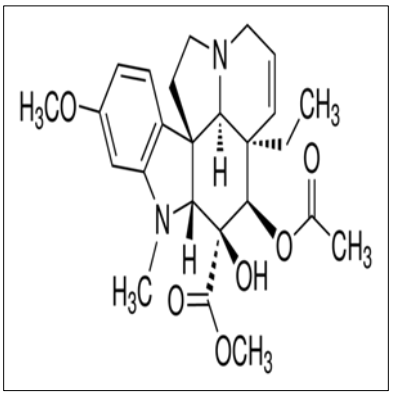


Figure 1 Structure of vindoline [10]

2. Material and methods

2.1. Chemicals and laboratory equipment

Solvents such as ethanol, ethyl acetate, hexane and dichloromethane, petroleum ether, acetone, acrylonitrile (HPLC grade) were used along with bismuth (III) nitrate. Standard vindoline (purity 98%) was purchased from Krishgen Bio-systems, Mumbai. 20*20 cm pre-coated aluminium TLC plates Silica gel 60 F254 were used. Hei-VAP Core rotary evaporator was used to evaporate the solvent under controlled conditions.

2.2. Sample collection`

Plant specimens were collected from Mysore, Karnataka, India located between 12° 18’ 26’’ north latitude and 76° 38’ 59’’ east longitude. Aerial parts (stem, leaves and flowers) of the plant were washed thoroughly and dried in a through-flow laboratory dryer set at 35 °C.

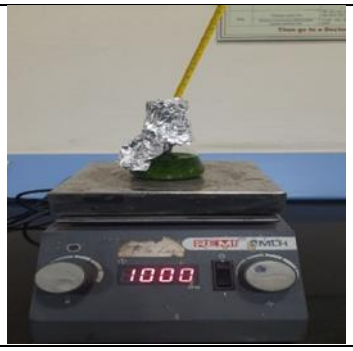



(A)	(B)	(C)	(D)

Figure 2 (A)Plant specimen, (B) stems of *C.roseus*, (C) leaves of *C.roseus*, (D) drying of samples

2.3. Alkaloid extraction

Alkaloid from the aerial parts of *C.roseus* was extracted according to the methodology described by Misra *et al* 2014. with necessary modifications [10]. To extract alkaloids, dried and powdered plant material of 25 g was mixed with ethanol (96%) through overnight maceration at 55°C and filtered by using Whatman no.1 filter paper and steps were repeated till powdered plant became colourless. A rotary evaporator was used to evaporate the ethanolic extract to dryness. The obtained green extract containing chlorophyll and alkaloids were re-dissolved in 25 mL ethanol, diluted with double distilled water and acidified with 3% HCl at pH-1. The chlorophyll from the mixture was extracted using 75 mL of hexane, the steps were repeated 3 times and hexane fraction was discarded safely. The obtained aqueous layer was cooled to 10 °C, pH increased to 10 using 25% aqueous ammonia solution, and alkaloids were extracted using 25 mL of dichloromethane for 3 times. The dichloromethane extract was washed with 100 mL of brine, the water content was removed using anhydrous sodium sulphate, and dichloromethane was evaporated and dried using a rotary evaporator under controlled conditions. The obtained semisolid plant extract was weighed and stored in the refrigerator at 4 °C [1, 11].



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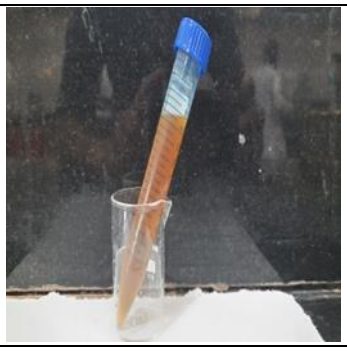


C

Figure 3 (A) Overnight maceration of plant sample, (B) concentration of the product, (3) crude alkaline product obtained

2.4. Phytochemical analysis

The ethanolic plant extract was screened for the following phytochemicals:

Alkaloids, carbohydrates, flavonoids, saponins, and terpenoids.

2.4.1. Test for alkaloids (Dragendoff's test):

0.2 mL ethanolic extract + 0.2 mL HCl 2–3 drops of Dragendoff's reagent were added to the mixture. The presence of alkaloids was indicated by the formation of an orange precipitate [12, 13].

2.4.2. Test for carbohydrates (Molisch's test)

0.2 mL of ethanolic extract was mixed with a 2-3 drops of Molisch's reagent and 0.2 mL of sulphuric acid was added along the sides of the test tube and appearance of a purple colour ring indicating a positive test was observed [13, 14].

2.4.3. Test for flavonoids

In a test tube, 0.2 mL of the ethanolic extract was combined with 10% sodium hydroxide solution and HCl was added to this mixture. The presence of flavonoids was indicated by the formation of a yellow solution that becomes colourless subsequently [13].

2.4.4. Test for saponins

In a test tube, 0.2 mL of ethanolic extract was mixed with 0.6 mL of water. The concoction was rapidly agitated and the presence of saponins was confirmed by the formation of persistent froth [13].

2.4.5. Test for terpenoids (Salkowski test)

A 0.2 mL ethanolic extract was combined with 0.2 mL chloroform solution and concentrated H_2SO_4 was carefully added to produce two layers. The presence of terpenoids was indicated by the formation of a reddish-brown colour at the junction [15].

2.5. Isolation of vindoline by thin-layer chromatography (TLC)

TLC analysis was performed on the obtained alkaloid-rich plant extract and a standard vindoline. The analysis was according to the procedure described by Mai Ngoc Tam *et al.* 2006 [16] with necessary modifications in mobile phase [petroleum ether, ethyl acetate, acetone, and ethanol in the ratio of 70:20:10:1 v/v] (ethyl ether was replaced by ethyl acetate). The chromatographic chamber was saturated with the mobile phase and 5 µL of standard vindoline and 40 µL of crude alkaloid extracts were spotted on the TLC plate just 1 cm above the edge and allowed to air dry [17]. TLC plate was placed inside the chamber and the plate was allowed to develop. Once the solvent front reached three-quarters of the plate, the plate was removed from the chamber and solvents were allowed to evaporate. Dragendorff's reagent was sprayed on the plate to develop spots [12]. R_f value of standard vindoline and the corresponding band to standard vindoline was calculated by using the formula:

R_f = distance travelled by the solute in cm / distance travelled by the solvent in cm [12]

R_f value of (A) = 2.3/5.4 = 0.42

R_f value of (B) = 2.2/5.4 = 0.40

2.6. Preparative thin-layer chromatography

Based on the obtained R_f value, vindoline was isolated by preparative TLC. Silica gel coated TLC plates were sliced used. Using a short pipette, thin line of approximately 10mg of crude alkaloid sample dissolved in ethanol was deposited on the plate and sample was allowed to air dry[18]. The chromatographic chamber was saturated with vapours of mobile phase [petroleum ether, ethyl acetate, acetone, and ethanol (70:20:10:1 v/v)]. The TLC plate was placed in the chamber and closed using the lid. Once the solvent had reached three-quarters of the plate, the plate was removed from the chamber and the solvents were allowed to evaporate. The band was scraped off based on the R_f value of the vindoline (0.44) using a scraper and placed in a test tube and the silica gel was rinsed in ethanol and then filtered. To obtain the pure form of vindoline, the filtrate was concentrated using a cold concentrator. The concentrate of the vindoline was then subjected to high-pressure liquid chromatography (HPLC) to check the purity of the vindoline.

2.7. Characterisation of purified vindoline

2.7.1. FT-IR analysis

IR spectrum were recorded using Perkin Elmer FT-IR Two ULTRA spectrophotometer in the 400-4000 cm-1 range at a flow rate of 16 (cm- 1/min) with Dichloride methane as solvent.

2.7.2. LC-MS

The *C.roseus* alkaloids were analysed by Acquity HPLC-MS/MS (Waters) system, equipped with Xevo triple quadrupole mass spectrometer with an ESI source. Mobile phase was used as described in the HPLC procedure. The flow rate was adjusted to 1 mL/min and the column temperature was maintained at 30℃. The heated capillary and voltage were maintained at 350ºC and 2.6 kV and nitrogen was used for desolvation with a flow of 650 L/h. The collision energy was adjusted at 3V, while the cone voltage was adjusted at 20V.

2.7.3. HPLC Analysis

HPLC analysis was performed Waters systems - model Acquity-LC with PDA detector and the column used was michron (503) 20*50 mm 400 Å. Solution A containing 0.1% formic acid in water and solution B containing acetonitrile were used as mobile phase solutions. The Mobile phase was filtered through nylon membrane and degassed under vacuum before using as eluent. 5 µL of purified vindoline was injected into HPLC. Flow rate mobile phase was adjusted to 1 mL/min and gradient elution was performed as in Table 1.

Table 1 Gradient elution of solvent A and B

Time	% A	% B
0.0	90	10
5.0	90	10
20	05	95
30	05	95
32	90	10
40	90	10

3. Results

3.1. Alkaloid extraction

Alkaloids of *C.roseus* were extracted by the hot ethanolic extraction method until the plant specimen got colourless. The product was then concentrated using a rotary evaporator, yielding 60mg/gram (6.0%) crude alkaloid indicating that

alkaloids can be isolated more effectively using hot ethanolic extraction. The yield obtained was much better than the previous reported ultra-sonication method of extraction [3].

3.2. Phytochemical analysis

Phytochemical analysis of plant extract of *C.roseus* has revealed the presence of alkaloids, flavonoids, and terpenoids (Table 2) showing a promising result.

Table 2 Phytochemical screening of *C. roseous* extract for bioactive compounds

Type of tests	Result
Alkaloids	+
Carbohydrates	-
Flavonoids	+
saponins	-
Terpenoid	+

Note: '+' positive; '-' negative.

3.3. TLC analysis

TLC analysis was performed for crude alkaloid mixture and a standard vindoline. The R_f value of the obtained band of standard vindoline (A) and the corresponding band of the crude alkaloid (B) was calculated. R_f value of standard and purified vindoline was found to be 0.42 and 0.40 respectively (figure 4).

Figure 4 TLC analysis. Lane A -standard vindoline; lane B -ethanolic extract

Preparative TLC was carried out to isolate the pure form of vindoline based on the R_f value (figure 5) of crude vindoline obtained by TLC analysis. The total yield of purified vindoline was found to be 12.8mg/gram (1.28%) of dried powder plant sample.

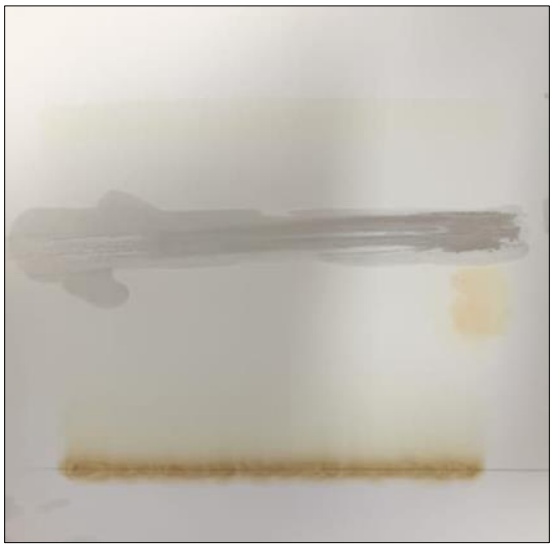


Figure 5 Scrap off of Corresponding vindoline band

3.4. FT-IR analysis

The functional group analysis was performed to authenticate the presence of specific functional groups correlating with the literature of vindoline. The FT-IR data shows the major peaks at 1204.88 and 1736.81 cm^{-1} which represents the presence of C-N and C=O stretching respectively (Table 3). C-N stretching confirms the compound as alkaloid with the ester group. Isolated vindoline confirms the functional group as per the standard used comparisons with the structure of vindoline (Figure 1), the FITR spectral results of the isolated vindoline confirms the functional group as per the standards used.

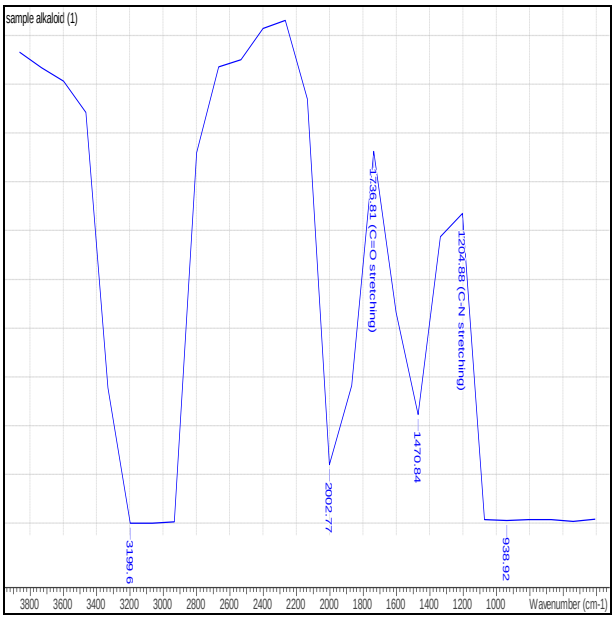


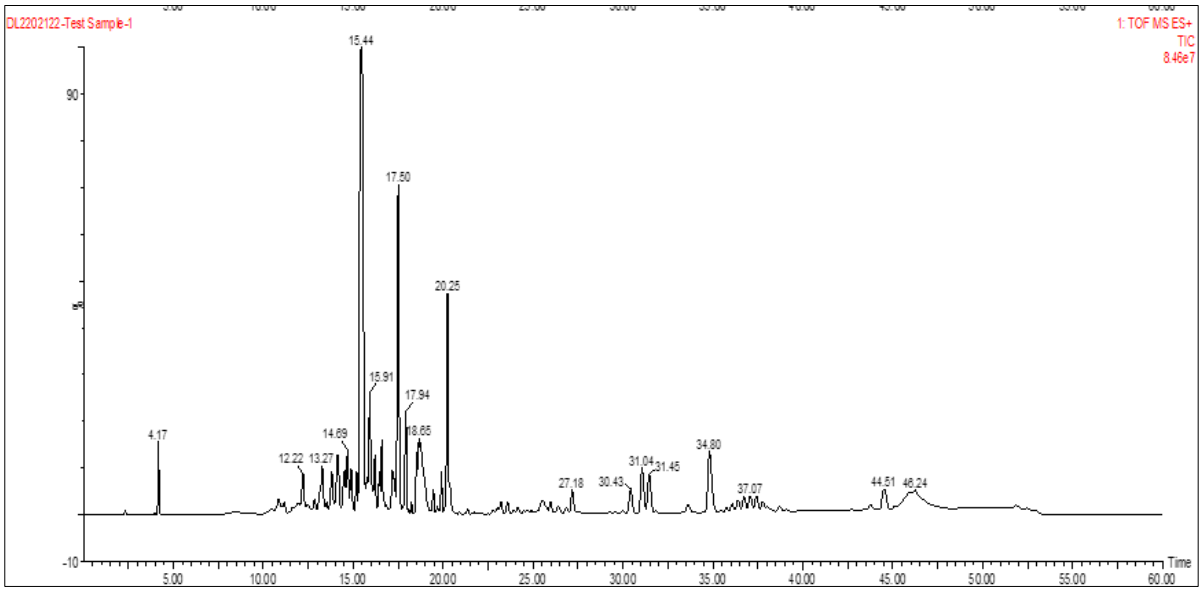
Figure 6 FT-IR spectrum of purified vindoline

Table 3 Functional group prediction for the peaks of FT-IR spectrum

No.	Fragments	cm^{-1}	%T	Formula	Vir/Trans
1	[1,3,5,8-9,14,16,25,27]	1204.88	31.787	C8H3N	C-N stretching
2	[21]	1736.81	38.173	O	C=O stretching

3.5. LC-MS analysis

The elution in the liquid chromatography part of the LCMS data shows the major peak at 15.41 min which has corresponded to the 457.29 m/z ratio on mass spec analysis of the major peak (Supplementary figure 1). Whereas the LCMS analysis of the isolate also has a major elution peak at 15.43min with 457.29 m/z ratio. This confirms the isolated compound as vindoline. The isolate has top peaks in MS at 188.13, 397.26, 457.29, 458.29 and 439.27. Even the MS spectrum of the isolate also correlates with that of the previously reported data where the major peaks at 188.13, 397.26, 457.29 and 439.27 of the mass spectrum matches [19].



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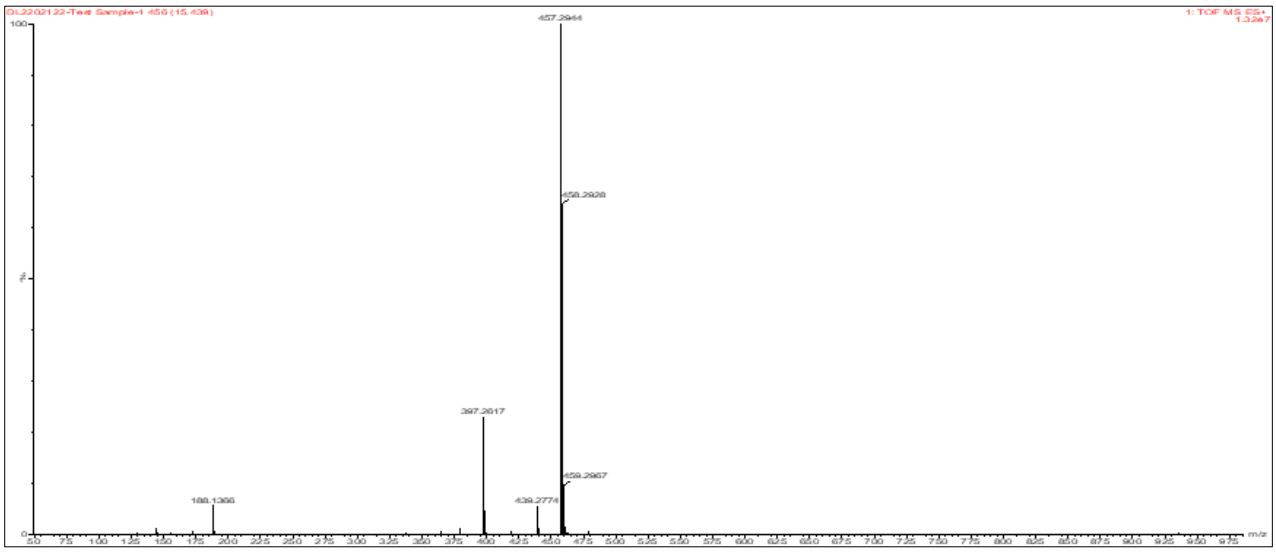

B

Figure 7 (A) LC chromatogram of purified vindoline; (B) M/S chromatogram of purified vindoline

3.6. HPLC analysis

HLPC analysis was carried out using a PDA detector to evaluate purified vindoline's peak and retention time. The optimisation of the mobile phase is achieved by the combination of 0.1% formic acid in water and acetonitrile. Purified vindoline has a spike at a retention time of 15.48 min. Result was compared against the retention time of standard (Supplementary figure 2).

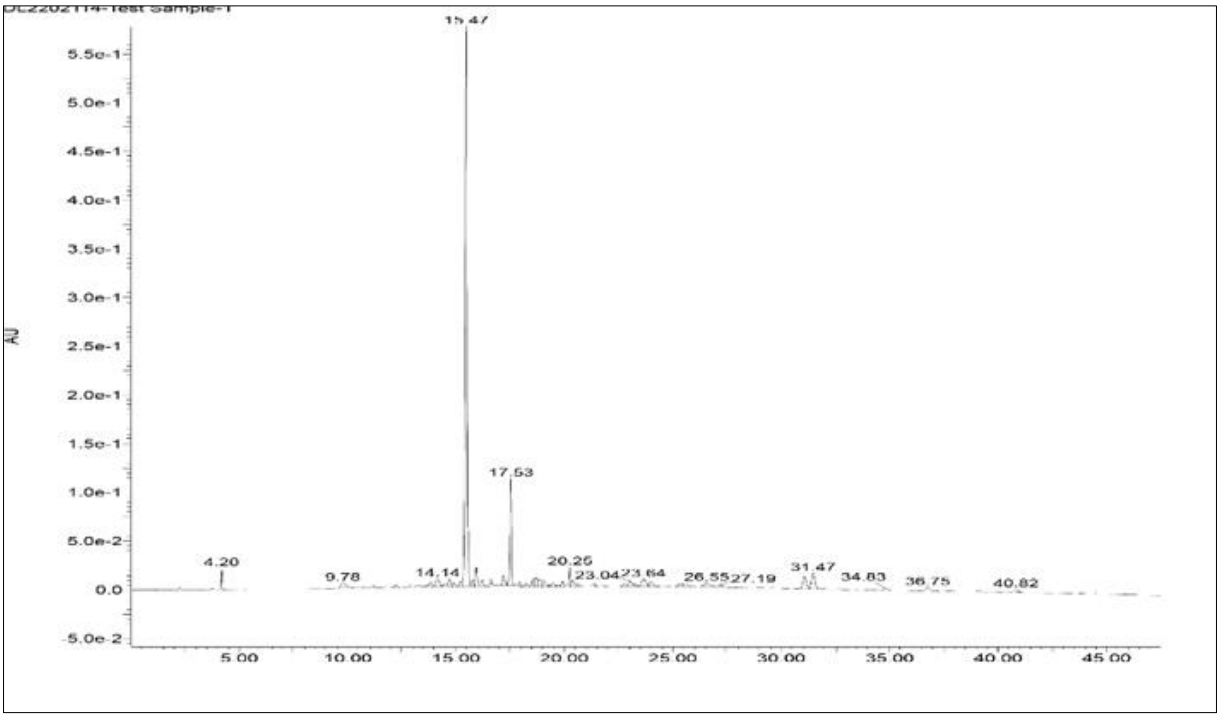

Figure 8 HPLC chromatogram of purified vindoline

4. Discussion

Vindoline is a monomeric indole alkaloid abundantly present in *C.roseus*. In the current study, vindoline has been extracted using hot ethanolic extraction procedure, notably, overnight maceration at 55°C till the sample turns colourless has increased the yield of the compound compared with method described earlier [1, 3, 11]. The main advantage of pTLC incorporated in the current study over other separation methods described *elsewhere is that it* provides a more effective and robust system than column chromatography with regard to separation efficiency and peak distribution of mixtures composed of low-retarded analytics[20]. Also, replacing ethyl ether with ethyl acetate has shown separation of vindoline from other compounds more efficient due to its moderate polarity. HPLC data showed that the compound isolated was with minimal contamination. LC-MS peaks of isolate correlated with the standard which conforms that the isolated compound was vindoline. The procedures established in this work may thus be used to extract vindoline quickly and effectively and the approach might potentially be used to produce energy-efficient and ecologically friendly alkaloids extraction systems.

Abbreviations

- TLC: Thin Layer Chromatography.
- R_f: Retention factor.
- HPLC: High Pressure Liquid Chromatography.
- FT-IR spectroscopy: Fourier Transform-Infrared.
- LC-MS: Liquid Chromatography-Mass Spectroscopy.
- PDA detector: Photodiode-Array detector.
- DTA: Deacetylvindoline 4-0-acetyltransferase.

5. Conclusion

In the present study we have standardized an effective method for extraction and isolation of biologically potent vindoline alkaloid from *C.roseus* with maximum purity. This established procedure would be very beneficial in pharma for isolation of the potent precursor alkaloid with high purity from natural sources.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

Authors have declared that there is no conflict of interest.

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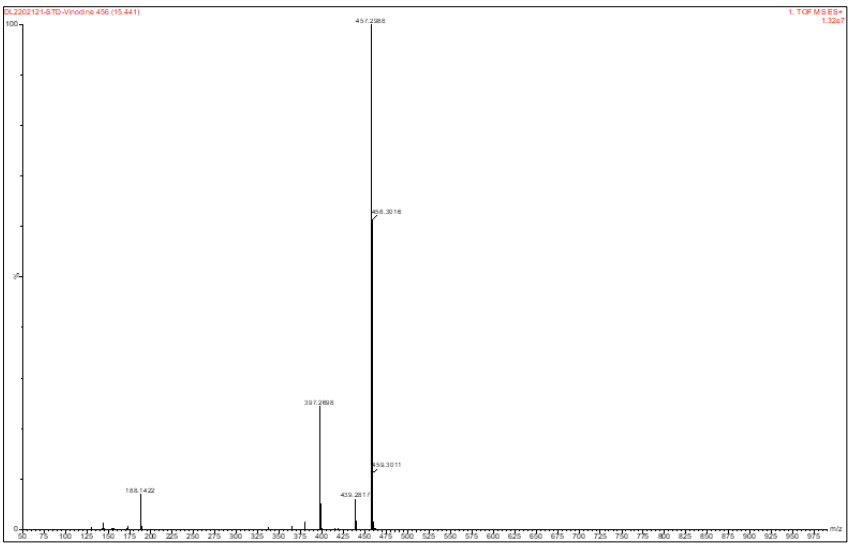
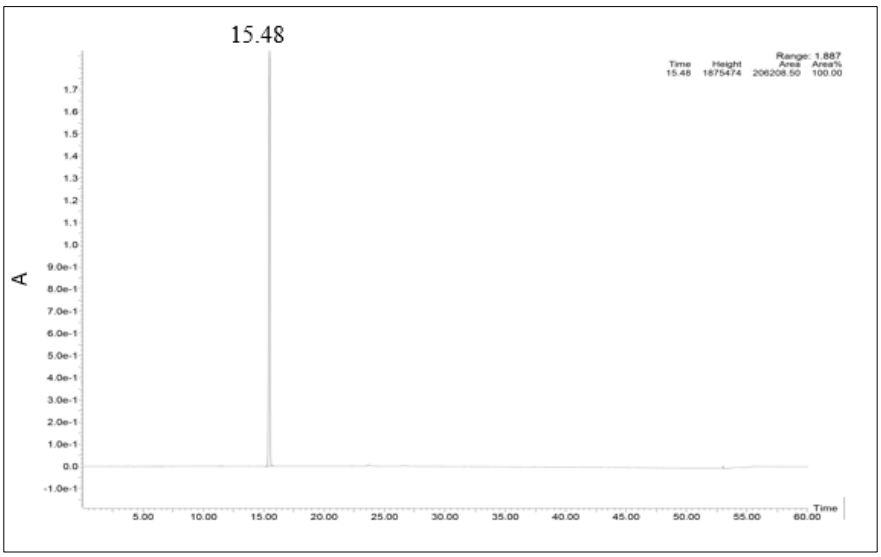
Appendix

Supplementary data for 'Isolation purification and characterisation of vindoline from *Catharanthus roseus* '



Supplementary figure 1 LC-MS chromatogram of standard vindoline

DL2202113-STD-Vinodine 3: Diode Array 250



Supplementary figure 2 HPLC Chromatogram of standard vindoline