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Original Article

DETERMINATION OF VINDOLINE AND RUTIN CONTENT IN FIVE DIFFERENT MORPHOTYPES OF *CATHARANTHUS ROSEUS* LEAVES USING HPLC

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ABSTRACT

Objective: To determine total phenolic and flavonoids contents and also quantify vindoline and rutin in different morphotypes of *Catharanthus roseus* using High-performance liquid chromatography (HPLC) method.

Methods: Total flavonoids content (TFC) was determined by Aluminium chloride colorimetric and total phenolic content (TPC) was estimated by Folin-Ciocalteu reagent assay. The chromatographic separation was done by using a C18 column at room temperature and eluted with a mobile phase consisting of a mixture of phosphate buffer (pH=5.8) and acetonitrile at a flow rate of 1.0 ml/minute and detection was carried out at 254 nm.

Results: TPC and TFC content was found highest in Cr00DP and lowest in Cr00WFSRE. Results also showed that the purple morphotypes Cr00DP gives more vindoline (0.3 mg/g) and rutin (18.57 mg/g) concentration compared to the pink morphotype Cr00PFRE contained 18.3 mg/g rutin and 0.2 mg/g vindoline. White morphotypes contained 0.383 mg/g rutin and 0.004 mg/g vindoline which was significantly less as compared to purple and pink morphotypes.

Conclusion: The plant has the significant number of alkaloids and flavonoids. The obtained outcomes from different morphotypes are thus significant for the purpose of vindoline and rutin isolation from *Catharanthus roseus* plant. These isolated bioactive phytoconstituents are a good candidate for a further pharmacological and clinical study.

Keywords: *Catharanthus roseus,* Total phenolic content, Total flavonoid content, High-performance liquid chromatography

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INTRODUCTION

Plants, since time perpetual, have been exploited for their therapeutic potential and play a critical job in the prosperity of man. Researchers are showing incredible interest in plant-derived substances because of their versatile applications [1]. Plants are one of the richest bio-resources of current medicines, nutraceutical, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [2]. Plants have a wide assortment of phytocompounds like alkaloids, phenols, flavonoids, glycosides, and tannins, etc. which are responsible for the various biological properties and amelioration of human beings. Recently plants with antimicrobial activities have become more interesting because of the emergence of multiple drug-resistant strains of microorganisms. Usually, due to indiscriminate and over-prescription of some antibiotics have generated a renewed interest in herbal medicines [3].

Catharanthus roseus, commonly known as periwinkle belongs to the family Apocynaceae. It grows naturally as a wildflower throughout the sub-tropical region like Southern Europe, Australia, Africa, America and Asia [4]. This plant is broadly investigated due to its essential indole alkaloids like vindoline and catharanthine. These alkaloids are mainly known as the initiator in the synthesis of other significant alkaloids like vincristine and vinblastine [5]. Vincristine and vinblastine have been used in chemotherapy to cure various types of cancers [6]. The different varieties of the plants showed variation in biological properties and morphological traits. This plant has also been used in traditional and herbal medicine for curing various maladies such as bleeding problems, cancer, diabetes, fever, heart disease, malaria and stomach problems [7]. The aerial parts of the *C. roseus* exhibit good antioxidant and antimicrobial properties [8]. Plant kingdom contains large number of phenolics compounds and has multiple uses in cosmetic, food and pharmaceutical industries.

The common traditional methods for extracting herbal medicine products include soxhlet extraction, hot extraction, maceration, ultrasonication, and reflux. Long preparation time and low yield are few common drawbacks in these extraction process. In the current study methanolic leaves extract of five morphotypes of *Catharanthus roseus* analyzed for simultaneous determination of rutin and vindoline by high-performance liquid chromatography.

To the best of our knowledge, there is no previous research on the vindoline and rutin quantification in *Catharanthus roseus* within different morphotypes. In the present study, we checked and compared the vindoline and rutin content in the methanolic: water (1:1) leaves extract from five morphotypes of *Catharanthus roseus* which have different flowers colour, petal arrangement and eye colour by using HPLC method.

MATERIALS AND METHODS

Reagents and chemicals

Chemicals acetone, ethanol, methanol, H_2SO_4, NaOH, HCl, $FeCl_3$, Wagner's reagent (Iodine in potassium iodide), glacial acetic acid, tannic acid, chloroform, quercetin and ninhydrin. The standard Rutin and Vindoline were purchased from Signa-Aldrich (purity>99%). Solvent Acetonitrile and Phosphate buffer (pH=5.8) used as mobile. Athena C18 250X 4.6 column was used for analysis.

Plant material

The leaves of *Catharanthus roseus* were collected from the conservatory of Punjabi University, Patiala (fig. 1). Fresh and young leaves of selected plants were used for phytochemical analysis. The voucher specimen of *C. roseus* was identified and deposited in Herbarium (PUN) at the Department of Botany, Punjabi University, Patiala, India and allotted accession numbers to these plants were 62713, 62733, 62721, 62717 and 62719 for Cr00PFRE, Cr00TDRF, Cr00DP, Cr00WFRE and Cr00WFSRE respectively.

Fig. 1: Morphological features of five *Catharanthus roseus* morphotypes

Preparation of plant extract

The fresh mature leaves of plant *Catharanthus roseus* were washed with running tap water and then with distilled water to remove unwanted foreign materials like soil and dust. After washing plant material was dried under shade at room temperature without any direct exposure to sunrays. It was then coarsely grounded by using the electrical grinder. The powdered plant material was stored in an airtight container for further use. For extraction, 5g leaves powder were used along with 50 ml of methanol and water (50: 50). Initially, the dried leaves powder was ultrasonicated for 60 min, macerated at room temperature for 24 h and then ultrasonicated again. After this, centrifugation was performed for five minutes at 12,000 rpm followed by filtration through a Whatsman filter paper.

Qualitative phytochemical analysis

For the presence of various phytochemicals like alkaloids, glycosides, phenols, flavonoids, saponins and steroids, tests were performed as per standard methods described in given (table 1).

Table 1: Phytochemical analysis of different extracts of *Catharanthus roseus*

Phytochemicals	Name of test	Methodology	Results	Reference (s)
Alkaloids	Wagner's Test	1-2 ml plant extract was treated with Wagner's reagent (Iodine in Potassium Iodide	The formation of brown/reddish precipitate	[9]
Amino acid and Protein	Ninhydrin test	2-3 drops of 1% ninhydrin were added into 2 ml of plant extracts then placed at 100 °C for 1-2 min in water bath.	Observed for the formation of purple colouration	[10]
Glycosides	Salkowski test	In 1 ml of plant extract, 2 ml of chloroform was added. After that 2 ml of concentrated H_2SO_4 was added.	Formation of reddish brown coloured steroidal ring appeared.	[11]
Saponins	Foam Test	1-2 ml plant extract was mixed with 5 ml of distilled water in a test tube and shaken vigorously.	The formation of stable foam.	[12]
Quinones		1-2 ml plant extracts were added with a few drops of concentrated HCl	Yellow precipitate (or coloration)	[13]
Phenols	Ferric Chloride Test	Plant extracts were added with 3-4 drops of ferric chloride solution.	Formation of dark green colour.	[14]
Tannin	Braemar's Test	10% alcoholic ferric chloride was added to 2-3 ml of extract.	Formation of dark blue or greenish grey coloration	[15]
Flavonoids	Alkaline Reagent Test	To the plant extract add 2-3 ml of dilute NaOH, followed by addition of 3-4 ml dilute HCl.	Formation of dark yellow colour, colourless on addition of dilute acid	[16]
Terpenoids:		1 ml of extract was dissolved in 2 ml of methanol and then evaporated to dryness. Then add 3 ml of concentrated H_2SO_4.	A reddish-brown colour.	[17]
Carbohydrates	Fehling's Test	Fehling A and B reagents were mixed with 2 ml of plant extract and boiled for few minutes in water bath.	Precipitates of brick red in colour.	

Quantitively phytochemical analysis

Total phenolic content (TPC)

Total phenolic content was estimated by the Folin-Ciocalteu method [18]. Phenols react with phosphomolybdic acid in Folin-Ciocalteu reagent in alkaline medium produce a blue-colored complex (molybdenum blue) which can be estimated spectrophotometrically at 620 nm. A stock solution of plant extracts was prepared to 1 mg/ml. 5 ml of Folin-Ciocalteu and 2 ml of Na_2CO_3 was added to the 1 ml of plant sample. The solution was vortexes and incubated in dark for 15 min. Blank consisted of 5 ml Folin-Ciocalteu, 1 ml solvent and 2 ml of Na_2CO_3 solution. Gallic acid was used as standard. The

total phenolic content was calculated from the calibration curve (y=0.0164x+0.0557, R^2=0.9964) and the result was expressed in terms of mg of Gallic acid equivalent (GAE)/gram dry weight of sample. All tests were performed in triplicates.

Total flavonoid content (TFC)

Total flavonoid content was estimated by Aluminium Chloride Colorimetric method with some modifications of the method of [18] to determine total flavonoid content. A stock solution of plant extracts was prepared to 1 mg/ml. 1 ml of plant sample with 3 ml of methanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1M potassium acetate and 5.6 ml of distilled water was added and the mixture was vortex and left at room temperature for 30 min. The absorbance was measured at 420 nm. Quercetin was used as standard. All tests were performed in triplicates. Flavonoid contents were determined from the calibration curve (y=0.0047x+0.0391, R^2=0.996) and results were expressed as mg of Quercetin equivalent (QE)/gram dry weight of the sample.

Preparation of standard and sample solutions for HPLC

Standard Rutin and Vindoline (10 mg) were dissolved in 25 ml of the mobile phase. Similarly, 10 mg of leaf extract solution of *Catharanthus roseus* were dissolved in 25 ml mobile phase.

Chromatographic conditions

The HPLC analysis was performed using a column Athena C18 250X 4 and used at a column temperature of 35 °C. The injection quantity was 0.02 ml. Millipore filter of 0.45 µm used for filtration of both standard and samples. Filtered sample (0.02 ml) was injected in column with a flow rate of 1 ml/min, eluted isocratically with phosphate and acetonitrile (55:45% v/v). The elution was observed by UV absorption at 254 nm. The column operating temperature was maintained at room temperature. Compounds identification was done by comparison retention values of standards with the samples.

Assay formula

Amount of rutin and vindoline was calculated as follows: (mg/g)

$$\frac{\text{Sample area}}{\text{Standard area}} \times \text{Standard concentration}$$

RESULTS

The results accessed from preliminary phytochemical analysis of the crude leaf extracts of different accession of *C. roseus* are presented in (table 2). Of the 11 analysed phytochemicals, 9 were present in methanolic extracts, viz. alkaloids, flavonoids, phenols, carbohydrates, saponins, quinones, tannins and cardiac glycosides. Only one Phyto-constituents viz. phlobatannins were absent in all extracts. The maximum number of phytochemicals were present in methanolic extract hence, methanolic extract used for HPLC analysis.

Table 2: Phytochemical analysis of different extracts and 5 different morphotypes of *Catharanthus roseus*

Phytochemical test	Aqueous	Methanol	Ethanol	Chloroform	Petroleum ether
Alkaloid	+	+	+	+	+
Amino-acid	+	+	-	-	-
Carbohydrate	-	+	-	-	-
Flavonoids	-	+	-	-	-
Phenol	+	+	+	-	-
Phlobatannins	-	-	-	-	-
Tannin	+	+	+	-	-
Saponins	+	-	-	-	-
Cardiac glycosides	+	+	+	+	+
Terpenoids	-	+	-	-	-
Quinones	+	+	-	-	-

The outcome of total flavonoids contents (TFC) of the five morphotypes was analysed only in methanolic extracts is given in (table 3). The TFC in the different morphotypes varied from 13.5 to 40.4 mg QE/g dried plant sample. Among the five morphotypes Cr00DP contained the highest (40.4 mg/g) amount of flavonoids content compounds followed Cr00TDRF (38.21 mg/g), Cr00PFRE (36.3 mg/g), Cr00WFRE (16.7 mg/g) and Cr00WFSRE (13.5 mg/g).

The total phenol contents (TPC) of the five morphotypes determined by Folin-Ciocalteu method were recorded as gallic acid equivalents. The phenolic content in the different morphotypes varied from 14.7 to 41.8 mg TAE/g dried plant sample. Among the five morphotypes Cr00DP contained the highest (41.8 mg/g) amount of flavonoids content compounds followed Cr00TDRF (34.0 mg/g), Cr00PFRE (32.9 mg/g), Cr00WFRE (16.94 mg/g) and Cr00WFSRE (14.7 mg/g) table 3.

Table 3: Total phenolic and flavonoid content of the 5 different morphotypes of *Catharanthus roseus* (L.) G. don

S. No.	Accession no.	TFC (mg queretcin equivalent per g of leaves)	TPC (mg Gallic acid equivalent per g of leaves)
1	Cr00PFRE	36.3±0.722	32.9±0.654
2	Cr00TDRF	38.21±0.34	34.0±0.585
3	Cr00DP	40.4±0.52	36.8±1.12
4	Cr00WFRE	13.5±0.430	14.7±1.09
5	Cr00WFSRE	16.7±0.723	16.94±0.672

Values are the average of triplicate experiments and values expressed as mean±SEM

Quantification of rutin and vindoline in *Catharanthus roseus*

The retention time (Rt) of standards vindoline and rutin were found to be 13.577 and 2.12 with 100% area at wavelength 254 nm (fig. 2-3 and table 4). The method was used for the analysis of rutin and vindoline in the leaves of five different morphotypes of *C. roseus*. While the Rt of rutin in different morphotypes of *C. roseus* extract was found to be 2.240, 2.247, 2.370, 2.230 and 2.213 (fig. 4 and table 5), which are matching with standards Rt values of rutin. While the Rt of vindoline in different morphotypes of *C. roseus* extract was found to be 13.637, 13.650, 13.643, 13.647 and 13.610 (fig. 4 and table 6), which are also matching with standards Rt values respectively. The highest concentration of rutin was found in Cr00DP (18.57 mg/g) followed by Cr00PFRE (18.3 mg/g), Cr00TDRF (8.68 mg/g), Cr00WFSRE (0.720 mg/g) and Cr00WFRE (0.383 mg/g). The highest concentration of vindoline was found in Cr00DP (0.30 mg/g), followed by Cr00PFRE (0.20 mg/g), Cr00WFSRE with (0.036 mg/g), Cr00TDRF (0.025 mg/g) and Cr00WFRE (0.004 mg/g). The mobile phase includes phosphate buffer and acetonitrile showed the good peaks shape and good resolution.

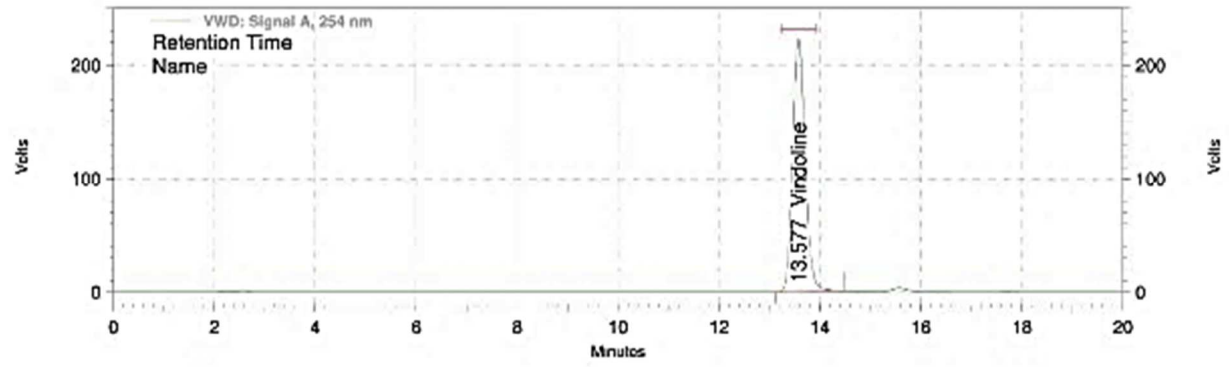

Fig. 2: HPLC chromatogram of standard vindoline

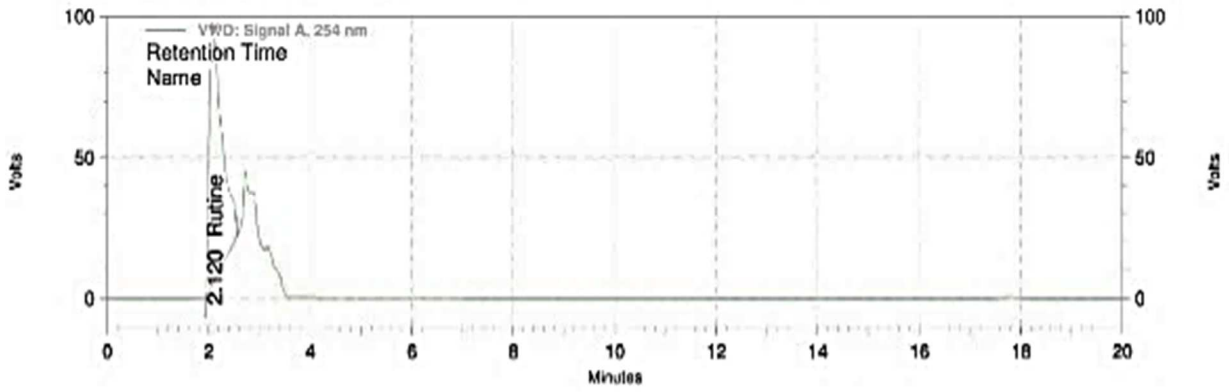

Fig. 3: HPLC chromatogram of standard rutin

Table 4: Retention time, area, % area and height of standards rutin and vindoline

Standards	Retention time (min)	Area	Area %	Height
Vindoline	13.577	64269995	100.00	3730336
Rutin	2.120	26328633	100.00	1437008

Table 5: Retention time, area, % area and height of rutin in an extract of 5 different morphotypes C*atharanthus roseus*

Standards	Morphotypes	Retention time (min)	Area	Area %	Height
Rutin	Cr00PFRE	2.230	134015163	96.41	6708335
	Cr00TDRF	2.247	404304021	98.05	22299690
	Cr00DP	2.370	1204672726	97.35	47631315
	Cr00WFRE	2.240	72394979	92.33	3738133
	Cr00WFSRE	2.213	45118540	88.73	2315896

Table 6: Retention time, area, % area and height of vindoline in an extract of 5 different morphotypes C*atharanthus roseus*

Standards	Morphotypes	Retention time (min)	Area	Area %	Height
Vindoline	Cr00PFRE	13.647	4991707	3.59	301946
	Cr00TDRF	13.650	8033624	1.95	530622
	Cr00DP	13.643	32813784	2.65	2203122
	Cr00WFRE	13.637	6015622	7.67	375297
	Cr00WFSRE	13.610	5732623	11.27	317263

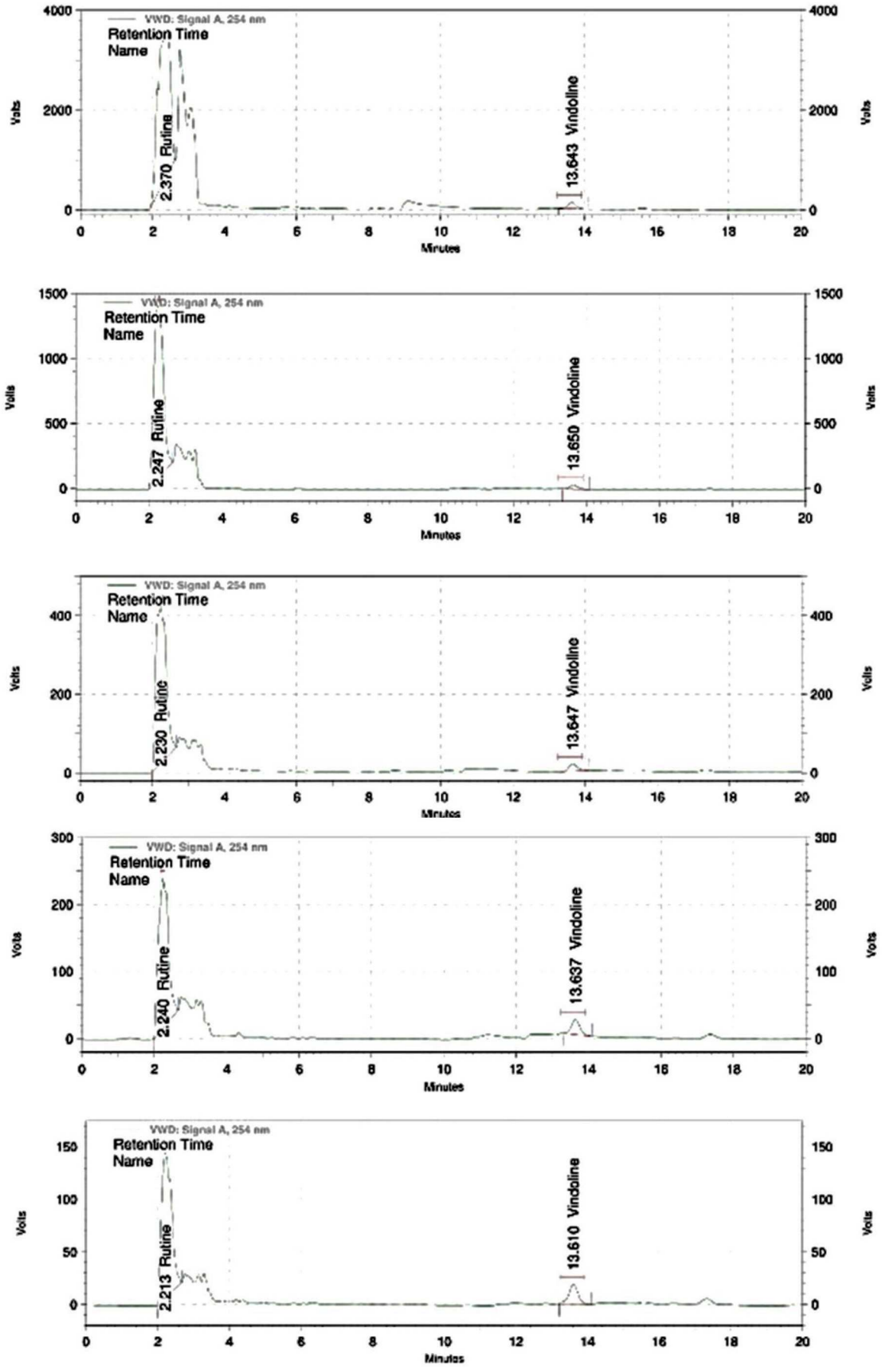

Fig. 4: HPLC chromatogram of five different morphotypes of *Catharanthus roseus*

DISCUSSION

In the current study, the qualitative phytochemical analysis revealed the presence of tannins, phenols, alkaloids, flavonoids, terpenoids, glycosides, carbohydrates and protein. This bioactive secondary metabolite of the methanol extract of *Catharanthus roseus* provides medicinal properties. The presence of alkaloids and flavonoids compounds responsible for pharmacological activities like antioxidant, anti-microbial, anti-neoplastic and antifertility. Similarly, [20] reported the preliminary phytochemical analyses of *Catharanthus roseus* were evaluated the presence of sugar, protein, amino acids, lipids, phenol and tannin in the ethanolic extract. [21] Reported the presence of seven different phytochemicals like alkaloids, tannins, phenols, saponins, sterols, terpenoids and cardiac glycosides from *Sarcostemma brevistigma* leaves. In this study, the methanol extract showed a great number of phytoconstituents of *Catharanthus roseus*. Similarly, [22] estimated vindoline content in a purple, red and white variety of *C. roseus*. Outcomes very much similar to our finding indicated maximum content was found in purple then followed by white and red variety. Concentration of rutin estimated by HPLC in methanolic leaf extract of *C. roseus* (21.59 % w/v) by [23]. According to [24] high amount of vindoline (39.2% w/v) was found in methanolic leaf extract of *C. roseus*.

The finding revealed that the methanolic extract of *C. roseus* dark purple morphotype Cr00DP is showed more phenolic and flavonoids as compared to the other pink and white morphotype. Outcomes also indicating that there is a huge variation in the quantity of phenol, flavonoids and alkaloids composition of these studied morphotypes. According to obtained finding, *C. roseus* leaves has a good amount of natural bioactive phytoconstituents viz. phenol, flavonoids and alkaloids. Therefore, it is recommended to use this plant as an alternative source for the production of new drugs.

CONCLUSION

The finding of the study indicated the existence of rutin and vindoline in methanolic leaves extract of Catharanthus roseus using HPLC. Purple morphotype has more rutin and vindoline concentration as compared to red, pink and white morphotypes. A simple extraction method has effectively yielded rutin and vindoline in comparison with other methods.

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AUTHORS CONTRIBUTIONS

Both the authors contribute equally in the research, result interpretation, wrote and finalized the manuscript.

CONFLICTS OF INTERESTS

Both the authors declare that there is no conflict of interest regarding the publication of this research.

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