

Detection of vinblastine and related bioactive compounds from culture extracts of endophytic fungi of *Catharanthus roseus*

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Abstract

This study investigates the synthesis of vinblastine by endophytic fungi *Alternaria alternata* (CRL 17), *Curvularia lunata* (CRL 28), *Aspergillus terreus* (CRL 52), and *Aspergillus clavatonanicus* (CRL 22), isolated from the leaf of *Catharanthus roseus*. The initial screening for secretion of plant alkaloids (vinca alkaloids) by fungi was performed by utilizing biochemical tests and thin-layer chromatography (TLC) which resulted total of 10 potential endophytes. Out of these ten, only four fungal extracts showed positive results for presence of vinblastine at same retention time (10 min.) compared to reference compound on HPLC analysis. The detected concentration of vinblastine was maximum (17 µg/ml) in *Aspergillus clavatonanicus* (CRL 22). The presence of vinblastine was further validated through ultra-high-performance liquid chromatography coupled with high-resolution accurate mass spectrometry (HRMS) analysis. The identification of anhydrovinblastine was validated through mass spectrometry, where molecular ions were observed at m/z 793.4185. The intermediate compounds which are involved in biosynthetic pathway of vinblastine were also identified in extract of *Alternaria alternata* (CRL 17). The study demonstrates that not only vinblastine but several other compounds of host origin have also been identified supporting the presence of biosynthetic pathway in endophytic fungus.

Highlights

Vinblastine, a plant origin anticancer drug is detected in extract of an endophytic fungus *Alternaria alternata* (CRL 17). The fungus was isolated from the host plant *Catharanthus roseus*. The intermediate precursors involved in biosynthesis of vinblastine are also detected in the fungal extract. This study demonstrates the future scope of vinblastine production by a fungal system.

Introduction

Plants have been revered for their medicinal properties since ancient, providing remedies for a wide array of ailments. However, a noteworthy shift has occurred in recent years, where the focus has turned towards microorganisms found in association with plants, rather than the plants themselves, as a source of valuable therapeutic compounds. This shift highlights a growing recognition of the significant therapeutic potential offered by microorganisms (Subbulakshmi et al. 2012). Endophytes are microorganisms that remain within the plant tissues without causing any discernible damage or infections to the host plant (Strobel 2002). They promote host plant growth and defence by forming secondary metabolites. They generate a variety of valuable bioactive compounds with wide range of applications in both the research and applied fields (Chandra 2012). Endophytic fungal species devote the majority of their life cycle living symbiotically inside the flourishing tissues of the host plant (Tan and Zhou, 2001). Endophytes, which are commonly found in plants, possess the remarkable ability to produce bioactive compounds that can enhance the plants' defence mechanisms against pathogens, as demonstrated by Guo et al. in 2008. These bioactive compounds have shown promise in drug discoveries. The utilization of these compounds holds great potential in meeting the growing demand for novel drugs. Moreover, the production of drugs sourced from endophytic fungi is cost-effective compared to traditional plant-based methods, as it involves a shorter production period, thereby reducing manufacturing expenses.

Catharanthus roseus L.G. Don. (Madagascar periwinkle) is a well-known medicinal plant native to Madagascar but also thrives in Indian subcontinents, Malaysia, where it is referred to as Kemunting Cina. Remarkably, its

flower has been adopted as the emblem for the National Cancer Council Malaysia (MAKNA), underscoring its significance in cancer research and treatment, as highlighted by Ayob and Simarani (2016). This plant is renowned for its abundant production of vital compounds, particularly vinca alkaloids such as vinblastine and vincristine. Additionally, *Catharanthus roseus* synthesizes vindoline and catharanthine, which serve as the principal monomer alkaloids and serve as biosynthetic precursors for vinblastine and vincristine, as discussed by Noble in 1990 (Duffin 2002). The introduction of vinblastine in 1960 marked a significant advancement in cancer treatment. Vinblastine plays a crucial role in various chemotherapy protocols and is extensively utilized in the treatment of a diverse array of malignancies. These include Hodgkin's lymphoma, non-Hodgkin's lymphoma, melanoma, testicular cancer, breast cancer, brain cancer, germ cell malignancies, and non-small cell lung cancer. This broad spectrum of applications underscores the versatility and effectiveness of vinblastine as a chemotherapeutic agent, contributing significantly to the management of cancer across different types and stages (Williams and Stephen 1987, Moudi et al. 2013). *C. roseus* exclusively produces vinblastine and vincristine, which rank as the second most commonly used anticancer drugs globally, after Taxol. However, the plant is known for yielding only minimal amounts of these crucial alkaloids, typically ranging from 0.0002–0.0005% (Sottomayor et al. 2004). Thus, this research was carried out to find novel endophytic fungi from the host plant *C. roseus* that could also produce vinca alkaloids specially vinblastine. Despite the importance of these alkaloids, research on their production by endophytic fungi remains limited.

Materials and methods

Sampling

Healthy, asymptomatic twigs of *C. roseus* were randomly brought together in sterile polythene bags from the agricultural research farm of Banaras Hindu University (BHU) Varanasi, India (elevation 25.5°N, 82.9°E) during August to September 2022. All of the samples were collected, brought to the lab in an icebox, and refrigerated at 4°C. The climate is moist and humid, with temperatures between 40°C and 25°C and up to 982 mm of precipitation annually. Additionally, it fosters the lush development of native herbs.

Isolation, purification and maintenance of the endophytic fungi

Plant sample was thoroughly washed with running tap water and then double-distilled water. All samples were first surface sterilized using standard procedure in order to remove epiphytic microbes (Fisher et al. 1992). The specimens underwent a series of sterilization steps: first, they were submerged in 70% ethanol for duration of 2 to 3 minutes. Following this, they were sterilized using aqueous sodium hypochlorite solution containing 4% available chlorine for another 2 to 3 minutes, after which they were rinsed in 75% ethanol for 30 seconds. Subsequently, the tissues underwent three rinses in sterile double distilled water and were then left to dry on a sterile surface. Only the leaf part of the plant was sampled. The leaf tissues were meticulously sectioned into small pieces (0.5×0.5 cm squares) using a sterile cutter. With the help of sterile scalpel and forceps, four to six segments of surface-sterilized leaf tissues were placed onto each PDA (Potato Dextrose Agar) plate amended with streptomycin sulphate (250 mg/l), and then sealed with Parafilm to prevent air contamination. Petri plates were then incubated in a BOD incubator (Super-tech) at 26 ± 2°C for a period of 5–6 days. Actively growing hyphal tips were transferred to fresh PDA Petri plates. To maintain stock cultures, subculturing was performed at monthly intervals, and cultures were preserved at 4°C. Furthermore, all isolated and identified fungi were preserved in cryovials layered with 15% glycerol and also in a lyophilized condition at -20°C in a deep freezer.

(Vestfrost). Surface texture, pigmentation, and spore morphology were served as key characteristics for identifying endophytic fungi.

Preparation of fungal crude extract

A two-stage fermentation process was conducted as previously described (Kumar et al. 2013). In the first stage, potato dextrose broth (PDB) was prepared. Pure cultures of endophytes were then inoculated into 100 ml of PDB medium. These flasks were placed on a rotary shaker and incubated at a temperature of 28°C while being agitated at 240 rpm for duration of 4 to 5 days. This initial incubation period served to establish the seed culture for subsequent stages of fermentation.

In the second stage aimed at vinca alkaloids production, 10 ml of the previously prepared seed cultures were transferred into 500 mL flasks containing 100 mL of Vinca Alkaloids-1 medium. The composition of the vinca medium (per 1000 ml) included: Glucose (30 g), Sodium benzoate (100 mg), Peptone (1 g), Magnesium sulfate (3.6 mg), Biotin (1 mg), Thiamine (1 mg), Pyridoxal (1 mg), Calcium pantothenate (1 mg), Phosphate buffer (1 ml, pH 6.8), L-Tryptophan (0.01 g), and Geranium oil (0.005%).

These flasks were then placed in a rotary shaker and incubated at 28°C with agitation at 240 rpm for a period of 20 days, constituting the second stage of fermentation. Following the 20-day incubation period, the culture was harvested, and the mycelia were separated from the culture broth by passing through four layers of muslin cloth. The culture filtrates obtained were then subjected to lyophilization, followed by extraction with equal volumes of ethyl acetate. The resulting extracts were combined and dried at room temperature to obtain crude extract.

Primary screening for vinca alkaloids producing endophytic fungi

The initial detection of presence of alkaloids in fungal extract was carried out by standard protocol (Shriner et al.1998).

Mayer's Test- Mayer's reagent is an alkaloid precipitating reagent used for the detection of alkaloids in natural products. Mayer's reagent is freshly prepared by dissolving a mixture of mercuric chloride (1.36 g) and of potassium iodide (5.00 g) in water (100.0 ml). 1 ml of fungal extract (5 mg/ml) was taken in a test tube. Few drops of Mayer's reagent were added along the sides of the test tube. The cloudy white precipitate observed in the Mayer's test is associated with the presence of alkaloids.

Hager's test - Hager's test is also used for detection of alkaloids. Hager's reagent is prepared by mixing 1gm of picric acid in 100 ml of distilled water. In a test tube, 1 ml of fungal extract (5 mg/ml) was taken and few drops of Hager's reagent were placed along the sidewalls of the test tube. A yellow precipitate observed confirms the presence of alkaloids.

Detection of fungal vinblastine by Thin Layer Chromatography

To detect fungal vinblastine, the methanolic crude extract (1 mg/ml) was analyzed using thin-layer chromatography (TLC) on silica gel plates with a thickness of 0.5 mm, provided by Merck. The chromatography was performed using a solvent system composed of chloroform:methanol:ethyl acetate in the ratio of 8:1:1. During the TLC analysis, vinblastine standard solutions (Sigma Aldrich, USA) were also included for comparison.

Detection of fungal vinblastine by High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography employing a C-18 symmetry column (Enable) coupled with an SPD-M20A prominence diode array detector (Shimadzu Corporation) was utilized for the separation, quantification, and analysis of vinblastine from partially purified fungal extract. The SPD-M20A detector offers exceptional sensitivity and stability, making it an advanced choice among today's PDA detectors. Gradient elution was conducted using a mixture of 5%-95% acetonitrile in water with 0.01% Trifluoroacetic acid (Sigma-Aldrich), at a flow rate of 0.5 ml/min. Detection of compounds was performed at a wavelength of 254 nm. A stock solution of standard vinblastine (Sigma-Aldrich) was prepared by dissolving 1 mg of the compound in 1 ml of acetonitrile, resulting in a concentration of 1 mg/ml. Subsequently, 15 microliters of these standards were injected into the HPLC column, and their chromatograms were recorded for reference. For analysis of the culture filtrate obtained after 28 days, alkaloids were extracted using ethyl acetate. The endophytic fungal crude extract was dissolved in HPLC-grade acetonitrile. Retention time (RT) values and the area under the peak obtained for the samples were then noted for further analysis and comparison. The fungal crude extracts were then purified using semi preparative high-performance liquid chromatography (Waters 2998) for vinblastine. The purified vinblastine was collected using Agilent Fraction Collector (G1364C) for further analysis.

Detection of fungal vinblastine and related bioactive compounds by HRMS

HRMS analysis of endophytic fungal extracts facilitates the detection and characterization of plant-derived compounds with potential pharmacological activity. This analytical technique involves precisely measuring the mass-to-charge ratio of ions present in the sample, providing high-resolution data on the molecular composition of the extract. The UHPLC chromatographic run was carried out using Dionex UltiMate 3000 RS UHPLC System with an analytical Hypersil GOLD™ C18 Selectivity HPLC Column, Particle size 1.9 µm, Diameter 2.1 mm, Length 100 mm. The oven temperature was set at 40°C, and the injected sample volume was 10 µL. The mobile phases were: (A) 100% Water + 0.1% Formic Acid and (B) 80% Acetonitrile + 0.1% Formic Acid and (C) 100% Methanol + 0.1% Formic Acid. Flow rate was 0.3 mL min⁻¹. The UHPLC system was connected to the single stage Orbitrap mass spectrometer through a heated electrospray interface (HESI) operating in positive ionization (3500 v) and negative ionization (2500 v). All the analyses were performed by the default parameters of "Compound discoverer 3.3.2.31" using online databases. The compounds were identified using mzCloud (ddMS2) and ChemSpider (formulaoexact mass). Similarity search was also performed for all compounds with ddMS2 data using mzCloud.

Molecular Identification of the vinblastine producing endophytic isolates

DNA extraction

For molecular identification of vinca alkaloids producing endophytic fungi, DNA extraction was performed. Initially, the fungal isolates were characterized based on their morphological features, alongside sequencing of the internal transcribed spacer (ITS) regions of each isolates. To facilitate DNA extraction, fungal cultures were cultivated in potato dextrose broth (PDB) at a temperature of 25 ± 2°C for duration of 5 days. Subsequently, the

mycelia were harvested by filtration through cheesecloth. Following this, the mycelia were dried by blotting and then pulverized in a pre-cooled mortar and pestle using liquid nitrogen. Total genomic DNA was extracted from the pulverized mycelial mass using the CTAB method. The concentration of the extracted DNA was determined using a nanodrop spectrophotometer.

PCR Amplification and Sequence Analysis of ITS rDNA

The fungal isolates underwent identification through a combination of morphological characterization and sequencing of their internal transcribed spacer (ITS) regions. Genomic polymerase chain reaction (PCR) was conducted using the universal ITS1 forward primer (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 reverse primer (5'-TCCTCCGCTTATTGATATGC-3'). The PCR reaction mixture consisted of genomic DNA (100 ng), ITS1 and ITS4 primers (100 µM), dNTPs (150 µM), and Taq DNA polymerase (2.5 U) in a total volume of 50 µl. PCR amplification was carried out using a PCR thermocycler, with the following cycling conditions: initial denaturation at 95°C for 5 min; 34 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min 30 sec; followed by a final extension at 72°C for 5 min.

The amplified PCR products were then subjected to separation by 0.8% agarose gel electrophoresis. The amplified PCR products were sent for sequencing at Biokart lab, Bangalore, India. Subsequently, the sequence data obtained were aligned and compared with sequences deposited in the GenBank database using the NCBI BLASTN program for further analysis and identification. The sequence data of fungal ITS region were submitted to NCBI GenBank with an accession number.

Results

An isolation effort yielded 56 endophytic fungal species inhabiting the leaf tissues of *C. roseus* (*data not shown*). Two biochemical tests, Mayer's test and Hager's test, were conducted to detect vinblastine in the fungal crude extract. The Hager's test yielded a yellow precipitate, while the Mayer's test resulted in a cloudy white precipitate (Supp. Figure 1. A-B). These positive results confirm the presence of vinca alkaloids in fungal extracts. The selected fungal crude extract which showed positive either Mayer's test or Hager's test underwent TLC analysis using a silica gel 60 plate and a chloroform:methanol:ethyl acetate (8:1:1) solvent system. Upon spraying with ceric ammonium sulphate reagent, the extract exhibited vibrant purple-colored spots. The R_f values of these spots were compared with the R_f value of standard vinblastine (0.79). Among the 10 fungal extracts compared with the standard vinblastine and plant extract, seven showed identical R_f values to vinblastine (Table 1).

Table 1
Detection of presence of alkaloids in fungal extract compared with plant extract and standard vinblastine

Isolates no.	Biochemical test		TLC
	Mayer's test	Hager'stest	Retention factor (Rf)
CRL 5		+	0.77
CRL 12			0.62
CRL 14		+	0.56
CRL 17	+	+	0.79
CRL 22	+		0.77
CRL28		+	0.80
CRL52	+	+	0.80
CRL 53	+	+	0.78
CRL 54	+		0.67
CRL 55	+	+	0.72
Plant extract	+	+	0.80
Standard vinblastine	+	+	0.79

HPLC Analysis of partially purified fungal extract showed the presence of vinblastine in four fungal extract as compared with standard vinblastine. The retention time of fungal vinblastine was identical to that of the standard vinblastine (10 min) (Fig. 1). The maximum concentration of vinblastine was observed in *Aspergillus clavatonanicus* (CRL 22) followed by *Aspergillus terreus* (CRL 52) (Table 2).

Table 2
HPLC Chromatogram analysis of fungal extract for vinblastine

Fungal isolates	Retention time	Area	Height	Conc.(µg/ml)
<i>Alternaria alternata</i> (CRL 17)	9.650	7803582	382795	7.497
<i>Aspergillus clavatonanicus</i> (CRL 22)	9.639	30378242	900739	17.107
<i>Curvularia lunata</i> (CRL 28)	9.409	6092542	290180	5.201
<i>Aspergillus terreus</i> (CRL 52)	9.636	6781538	513867	8.953
Standard vinblastine	10.039	10561036	2652516	65.263

The fungal extracts were analyzed for the presence of vinblastine by HRMS analysis. Vinblastine is a relatively large molecule with an elemental composition of $C_{46}H_{58}N_4O_9$ giving a molecular mass of 810.4204. In this analysis, anhydrovinblastine, the precursor compound of vinblastine, was exclusively detected in the extract derived from *Alternaria alternata* (CRL 17) (Table 3). The presence of anhydrovinblastine was confirmed through the identification of molecular ions at m/z 793.4185, corresponding to the $(M + H)^+$ ion (Fig. 2). This

confirmation validated its molecular weight as 793.4112 m/z. This finding suggests that *Alternaria alternata* (CRL 17) harbours the necessary biochemical pathways for the synthesis of anhydrovinblastine, which serves as a precursor in the biosynthesis of vinblastine. This analysis also reveals the detection of several intermediate compounds known to participate in the biosynthesis pathway of vinblastine and vincristine alkaloids, including, DL-mevalonic acid, DL-phenylalanine, and chorismic acid (Table 4). These intermediates undergo a series of enzymatic reactions, leading to the formation of various compounds, ultimately culminating in the production of vinca alkaloids such as vinblastine and vincristine. During HRMS analysis, a number of peaks corresponding to specific plant origin compounds are identified based on their mass spectra. The HRMS data offers valuable insights into the pharmacologically significant compounds found in the crude extract of *Alternaria alternata* (CRL 17). Among these compounds are aspirin, atropine, aromadendrin, aterperyleneol, taxifolin, fraxetin, and others, which have been documented in the literature for their analgesic, antipyretic, anticancerous, anti-inflammatory, and apoptotic activities (Supp. Table 1).

Table 3. Detection of vinblastine precursor from extract of *Alternaria alternata* (CRL 17) by HRMS analysis

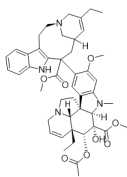
Compound	Chemical structure	Nature of compound	Formula	Mol. Wt.	m/z	RT (min)	Area (max.)
Anhydrovinblastine		Terpene indole alkaloid	C ₄₆ H ₅₆ N ₄ O ₈	793.4112	793.41852	19.079	2005548

Table 4. Intermediate compounds involved in biosynthetic pathway of vinblastine from *Alternaria alternata* (CRL 17)

Compound	Formula	Mol. Wt.	m/z	RT (min)	Area (max.)
DL-Mevalonic acid	C ₆ H ₁₂ O ₄	148.0734	147.0661	2.994	1.72E+08
5-Hydroxyindole-3-acetic acid	C ₁₀ H ₉ NO ₃	191.0581	192.0654	5.109	49482348
DL-Phenylalanine	C ₉ H ₁₁ NO ₂	165.0788	164.0715	5.12	7178253
5-Methoxy-3-indoleaceate	C ₁₁ H ₁₁ NO ₃	205.0734	204.0662	5.182	19559491
Chorismic acid	C ₁₀ H ₁₀ O ₆	226.0473	225.0399	5.68	1.47E+08
DL-Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	204.0897	205.097	6.465	44035158

Identification of fungal isolates

The four fungal isolates CRL 17, CRL 22, CRL 28, and CRL 52, showing presence of vinblastine in their extracts were identified through morphological and molecular approaches. The sequence analysis of ITS region of genomic DNA of these fungal isolates, revealed a high degree of similarity with the available data on Genbank (Supp. Table 2). The most positive isolate CRL17 where vinblastine was detected by both HPLC and HRMS analysis was identified as *Alternaria alternata*.

Discussion

In this study, four endophytic fungal isolates were identified producing vinblastine by HPLC while a derivative of vinblastine was detected only in the extract of *Alternaria alternata* CRL17 by HRMS. The vinblastine detected in isolates other than *Alternaria alternata* CRL17 might not be stable that's why it was not detected in HRMS analysis. Guo et al. (1998) documented the initial discovery of a vinblastine-synthesizing endophytic fungus, *Alternaria* sp., isolated from the phloem of *Catharanthus roseus*. Additionally, Zhang et al. (2000) and Tung et al. (2002) successfully identified a fungus, *Fusarium oxysporum* Syn. *Nothofusarium*, inhabit *C. roseus*, capable of synthesizing vincristine and its presence was only confirmed via HPLC analysis. Mayer's and Hager's tests are commonly used for initial detection of fungal alkaloids. An unidentified fungus was reported to produce vinblastine and vincristine by initial screening and subsequently by HPLC showing retention times (Rt) of 11.58 and 21.72 min, respectively (Ashoka et al. 2017). In contrast, study by (Kumar et al. 2013), *Fusarium oxysporum*, a member of ascomycota, was identified from *C. roseus*, exhibiting vinca alkaloids production in medium. Following TLC examination of the crude extract in an 8:2 chloroform:methanol solvent system, the Rf values of the spots were found to match with conventional Rf values of vinblastine and vincristine. Furthermore, after being sprayed with ceric ammonium sulphate reagent, the spots of vinblastine and vincristine in the crude extract showed the same colour as the standard. The presence of compounds was verified through HPLC analysis, revealing a single, symmetrical peak with a retention time of 36.6 min for vinblastine and 34.9 min for vincristine on a C18 symmetry column. Their study revealed that one litre of *Fusarium oxysporum* culture filtrate yielded approximately 76 µg of vinblastine and 67 µg of vincristine in the medium.

Chaetomium globosum, a member of ascomycota, an endophytic fungus identified from the stem of *C. roseus* in Iran. The HPTLC chromatogram revealed a calculated Rf value for fungal vinblastine (0.75), which closely matched that of the standard vinblastine (0.78). The quantification of fungal vinblastine was conducted via HPLC utilizing authentic vinblastine as a reference. The chromatogram of fungal vinblastine displayed a peak at a retention time of 12.678 min, mirroring the retention time of standard vinblastine (Rt 12.62 min.). The precise mass of vinblastine (C₄₆H₅₈N₄O₉) in this investigation was 810.975 while fungal vinblastine displayed m/z 811 that was nearly identical. The molecular ions at m/z 811 in ESI-MS were identified as (M⁺H)⁺, confirming that its molecular weight was 810 m/z (Zafari et al. 2019). In our study HPLC analysis of fungal extract and standard vinblastine showed RT at 10 min (Fig. 1). Similarly, endophytic fungi *Eutypella* sp., and *Talaromyces radicus*, member of ascomycota were found to produce vinblastine and vincristine in culture medium. TLC analysis revealed light brown dots, when Dragendorff's spray reagent was applied. These spots co-migrated with vincristine (Rf 0.68) and vinblastine (Rf 0.75) standards. HPLC analysis confirmed the presence of vincristine (RT 11.43 min) and vinblastine (RT 12.85 min) in the fungal extracts, as evidenced by their retention times matching those of the standards. UV absorption analysis revealed peaks at 252 nm for both compounds. Additionally, the LC-ESI-MS chromatogram of the crude extract displayed molecular ions m/z 825.46 and 811.51 at the appropriate retention times, confirming the presence of vincristine and vinblastine (Palem et al. 2015).

Curvularia verruculosa, classified as a member of ascomycota, was isolated as an endophyte from *C. roseus* leaf tissue. This strain was also demonstrated for production of vinblastine when cultured in vinca medium. The presence of vinblastine was confirmed in the *Curvularia* extract by observing a purple spot that co-migrated with authentic vinblastine on TLC plate. Furthermore, examination via HPLC revealed a high yield of vinblastine, measured at 182 µg/L (Parthasarathy et al. 2020).

Following the initial screening, further analysis was conducted using HPLC to assess the ethyl acetate extracts obtained from endophytic isolates CRL 17, CRL 22, CRL 28, and CRL 52. Interestingly, these extracts exhibited peaks in their chromatograms at retention times that closely matched that of the standard Vinblastine (RT 10 min.). This alignment in retention times strongly suggests the presence of compounds within these extracts that share similar chemical properties and elution behaviours with vinblastine. The nearly identical retention times observed in the chromatograms of the fungal extracts and the standard vinblastine indicate a high degree of similarity in their chemical compositions and structural characteristics. This finding suggests that the compounds present in the fungal extracts may indeed be related to or analogous to vinblastine, further emphasizing the potential of these endophytic fungi as sources of bioactive compounds with pharmaceutical relevance. In our analysis, the Ethyl acetate fungal extract was scrutinized for the presence of vinblastine using HRMS analysis. Interestingly, in this study, anhydrovinblastine, the derivative of vinblastine was identified solely in the extract of *Alternaria alternata* (CRL 17). This identification was confirmed by observing molecular ions at m/z 793.4185, which were attributed to $(M + H)^+$, thus confirming its molecular weight to be 793.4112 m/z (Fig. 2, Table 3). This finding highlights the unique capability of *Alternaria alternata* (CRL 17) isolated from the leaf segment of *Catharanthus* to produce anhydrovinblastine, a crucial intermediate in the biosynthesis of vinblastine, offering valuable insights into the biosynthetic pathways of vinca alkaloids within endophytic fungi associated with *C. roseus*. The HRMS analysis of *Alternaria alternata* (CRL 17) has provided valuable insights into the presence of various intermediates in the biosynthesis pathway of vinca alkaloids, along with the identification of other pharmacologically significant compounds, highlights the potential therapeutic applications of this crude extract. These intermediate precursors include DL-mevalonic acid, an essential precursor in the mevalonate pathway, vital for the synthesis of terpenoids and steroids, DL-phenylalanine, an aromatic amino acid serving as a precursor for numerous secondary metabolites and chorismic acid a significant intermediate in the shikimate pathway, which leads to the biosynthesis of aromatic amino acids and subsequent alkaloid formation (Table 4). During HRMS analysis, several peaks were detected corresponding to different compounds of plant origin. These compounds have been identified and characterized based on their mass-to-charge ratios (m/z) (Supp. Table.1). The identified compounds in the crude extract includes, Aspirin known for its analgesic and antipyretic properties (Thorat et al. 2013). It is a commonly used as non-steroidal anti-inflammatory drug (NSAID). Atropine is an alkaloid with anticholinergic properties, used medically to treat bradycardia (slow heart rate) and as an antidote for organophosphate poisoning (Smulyan et al. 2018). Aromadendrin is a flavonoid known for its anti-inflammatory and antioxidant activities (Kwak et al. 2009). Aterperyleneol, a lesser-known compound derived from *Alternaria alternata*, potentially exhibits anticancer activity (Junmin et al. 2022). Taxifolin, also known as dihydroquercetin is a flavonoid with significant antioxidant and anti-inflammatory properties (Li et al. 2017). By delineating the sequence of reactions and intermediates involved in vinca alkaloid biosynthesis, researchers can explore strategies to enhance the production of these valuable compounds. This knowledge not only contributes to our understanding of natural product biosynthesis but also holds promise for the development of biotechnological approaches for the sustainable production of vinca alkaloids. The identification of endophytic isolates CRL 17, 22, 28, and 52 as *Alternaria alternata*,

Aspergillus clavatonanicus, *Curvularia lunata*, and *Aspergillus terreus*, respectively, based on the internal transcribed spacer region (ITS1 and ITS4), adds significant depth to our understanding of the diversity of endophytic fungi associated with *C. roseus*. Furthermore, the identification of these fungal species opens up new avenues for exploring their potential in producing vinca alkaloids. Each fungal species may possess distinct metabolic pathways and biosynthetic capabilities, potentially leading to the production of unique secondary metabolites, including vinca alkaloids.

Conclusion

By understanding the diversity of endophytic fungi associated with *C. roseus*, we can better harness their biotechnological potential for the production of valuable bioactive compounds with pharmaceutical applications. These sources hold promise as substitutes for traditional plant-based methods in producing vinca alkaloids at potentially higher concentrations. However, further investigation is necessary to refine the cultivation conditions and enhance the yield of vinca alkaloids from the identified endophytic fungal strains. To escalate the concentration of these drugs from micrograms to grams per litre, it's essential to enhance the culture conditions by elucidating the biosynthetic pathway of the fungus. Additionally, employing genetic engineering techniques can facilitate abundant production of these crucial drugs. This eco-friendly, non-toxic, and cost-effective green synthesis approach stands in contrast to previous chemical synthesis methods, which often yielded inadequate quantities of the drugs. Our future work is to focus on the purification and crystallization of vinblastine/ anhydrovinblastine chemical and molecular characterization.

Declarations

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contribution Statement

UK-Conceptualization, experimental setup, data analysis, writing of original draft, editing

SKG- Conceptualization, data analysis, editing and review, funding acquisition and resource management, supervision

Author Contribution

UK-Conceptualization, experimental setup, data analysis, writing of original draft, editingSKG- Conceptualization, data analysis, editing and review, funding acquisition and resource management, supervision

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Data Availability

Data available on request

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Figures

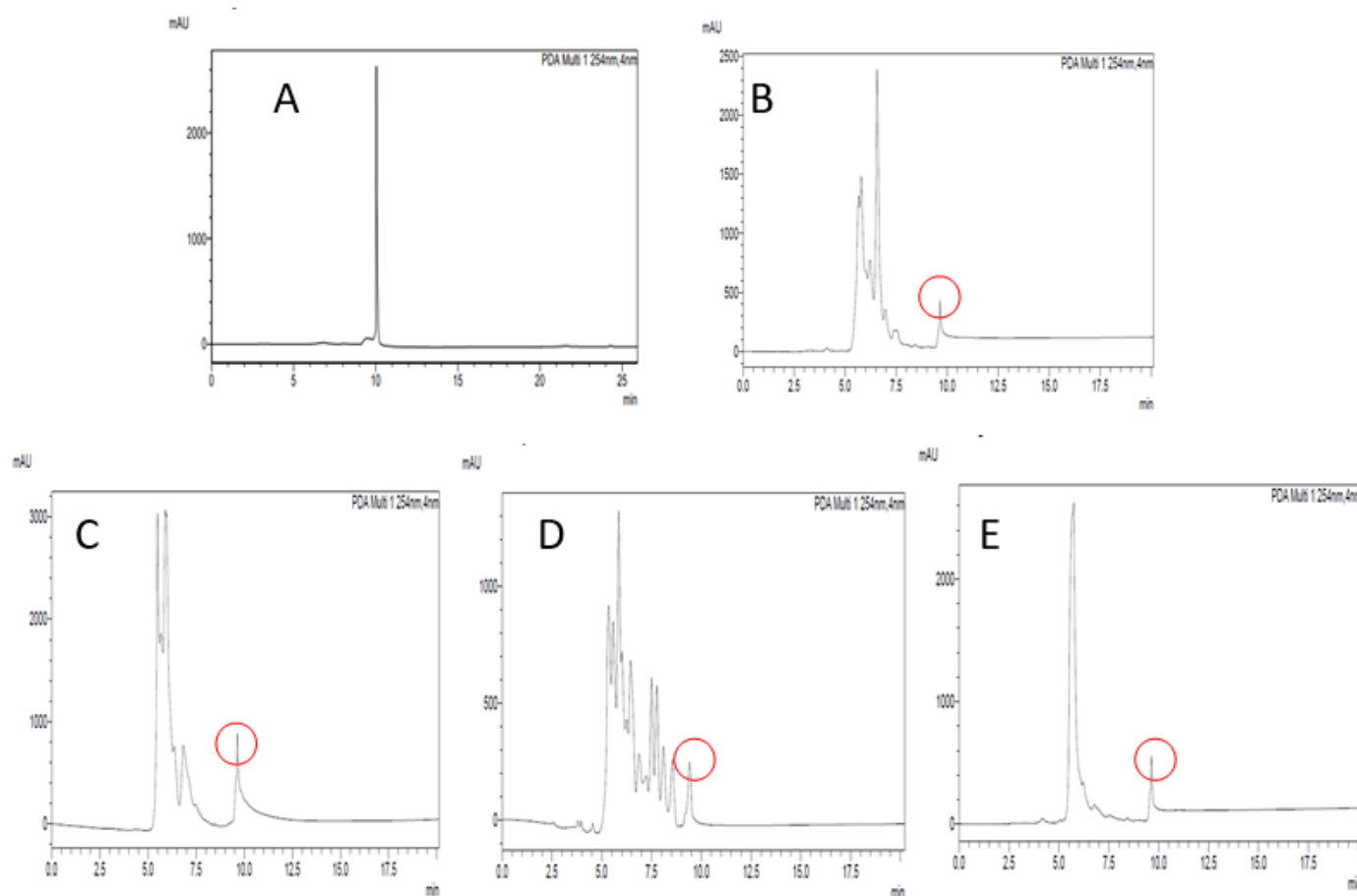


Figure 1

HPLC chromatograms

A. Chromatogram of Standard vinblastine revealing a retention time (Rt) of 10.0 minutes.

B-E. HPLC chromatograms of endophytic fungal extracts of (CRL 17), (CRL 22), (CRL 52) and (CRL 28) respectively

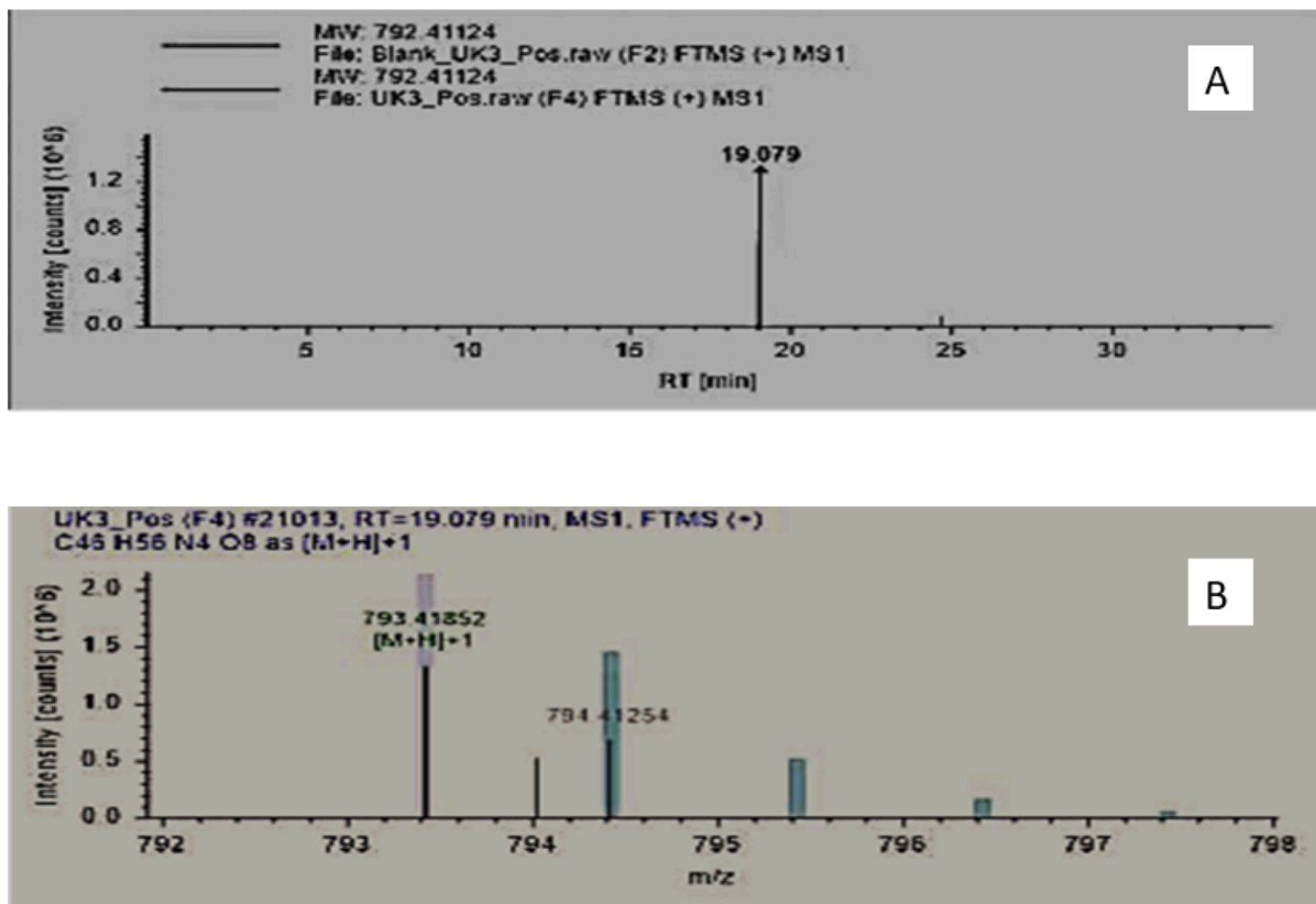


Figure 2

HRMS chromatograms of extract of *Alternaria alternata* (CRL 17)

A. Detection of anhydrovinblastine in fungal extract

B. Molecular mass determination by m/z value of anhydrovinblastine

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