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SEPARATION, PURIFICATION AND CHARACTERIZATION OF VINCRISTINE AND VINBLASTINE FROM *FUSARIUM OXYSPORUM*, AN ENDOPHYTIC FUNGUS PRESENT IN *CATHARANTHUS ROSEUS* LEAVES

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ABSTRACT

Endophytic fungi reside in their host plants in a symbiotic manner, imitating their chemistry and produce several natural products some of which are camptothecin, taxol, podophyllotoxin, etc. Many of these compounds possess pharmaceutical applications. Vincristine and Vinblastine are two such organic compounds, which possess efficient anti-cancer activity. These compounds are isolated from the endophytic fungi, extracted from the *Catharanthus roseus* species in higher amounts which are sufficient to meet the bulk requirements of the pharmaceutical industry. Using the TLC technique, the isolated compounds were purified and the structural moieties were characterized using MS/MS and ESI-MS. It was noticed from one liter of the filtered culture, vincristine and vinblastine were obtained as 66.5 mg and 75.5 mg respectively. Further, the cultural, morphological features were analyzed along with internal transcribed spacer (ITS) sequential analysis, and the fungal strain was found as *Fusarium oxysporum*.

Keywords: *Catharanthus roseus*, Endophytic fungi, Vincristine, Vinblastine, Anti-cancer activity.

1. INTRODUCTION

The health of the maximum strength of the world's population is in the hands of the medicinal plants, as they are the major source of curable drugs. Due to medicinal importance and other multiple uses, the demand for the medicinally important plant is constantly rising. However, the supply of these value-aided plants has become erratic and inadequate. Population explosion increased anthropogenic activities and destructive harvesting combined with habitat loss in the form of deforestation have aggravated the problems of availability of the plants. Overharvesting has become out of control. If the pattern of over usage goes the same way, medicinal plant's wealth will surely be in danger. According to a World Bank report, many of the medicinal flora are being utilized and over-harvested, so they will become extinct and endangered. Therefore, the need for the conservation of medicinal plants is crucial.

Some of the most important plant genera, which yield frequently prescribed medicines, are *Dioscorea deltoidea*, *Papaver somniferum*, *Atropa belladonna*, *Rauwolfia serpentina*, *Cinchona officinalis*, etc. *Dioscorea* species are being frequently used as medicine by the Chinese. The active ingredients present in these tubers are-diosgenin, which is an important medicinal steroid-like prednisone, dexamethasone. Capsaicin obtained from *Capsicum* species is also used as a digestive stimulator and for controlling the rheumatic disorder. Berberine is (anti-bacterial drug) extracted from Berberis. Its productivity was increased in cell cultures by optimizing the nutrients and level of phytohormones in the growth medium [1]. Taxol, a complex diterpene alkaloid (an anticancer drug) was isolated from *Taxusbrevifolia*. There are some major plant drugs of which no synthetic substitute is currently available.

Catharanthus roseus (L.) G. Don, with trade name Vinca, belongs to the family of *Apocyanaceae*. It is widely cultivated in some states of India. It is also known to produce a variety of alkaloids (>200). The alkaloids isolated from *C. roseus* can exhibit hypotensive, sedative, tranquilizing, and anti-cancer activities. This plant species has found some traditional medical uses like depressants of the central nervous system, relieving muscle pain, and wasp stings. It is an anti-cancer and anti-diabetic agent as well. The species has been a highly

studied medicinal plant, as it could produce several monoterpenoid indole-based alkaloids (>100). Among these, two commercial alkaloids were known to exhibit potent anti-cancer activity. The plant species was also a rich source of enzymatic and non-enzymatic anti-oxidants [2, 3].

The two commercial alkaloids that can be extracted from *Catharanthus roseus* in large amounts are namely, *vincristine* [4] and *vinblastine* [5] which belongs to the vinca group of alkaloids. Catharanthine [6] and vindoline [7] are two other alkaloids, which can be extracted from the plant species. However, the major limitation in their extraction is the lack of sensitivity and accuracy in the adopted analytical procedures due to their complex chemical structures.

These drugs can be injected in different routes and can interfere with the collapse of cancer-causing cells. In some research findings, it was noticed that few chemical constituents extracted from *C. roseus* species have caused the prevention in the growth of new blood vessels which supported the development of tumors [8]. Vincristine was mainly observed to be formed by *Fusarium oxysporum*, an endophytic host [9]. Its companion, vinblastine was found to be isolated from another host [10].

These alkaloids can also treat various types of leukemia and lymphoma [11]. They can also cure both malignant and non-malignant disorders and in the platelet and their associated disorders. The absolute values of their extracts were considered to be minimum in many of the methodologies [12].

Methods like non-aqueous capillary electrophoresis [13], HPLC [14], HPLC coupled with EIMS [15] were used in some of the research procedures and have shown less resolution due to the lower levels of reproducibility. To overcome these conditions, much focus was kept on the isolation, purification, and extracting the anti-neoplastic alkaloids using various analytical separation techniques. However, these methods were observed to be time-consuming and formed the extracts in fewer concentrations.

Based on the above observations, the present research work was designed to extract the endophytic fungus from the selected plant species, *Catharanthus roseus*. The major target in this extraction process was to isolate the most effective anti-cancer agents, namely vincristine and vinblastine at appreciable amounts. Their structural moieties were characterized using MS/MS and ESI-MS after purification by the TLC method. To obtain better resolution and reproducibility, a chromolith column has been used for the extraction of the alkaloids. The reproducibility was found to be better for greater than 300 samples with the same column. The HPLC run was conducted using a photodiode array (PDA) detector.

2. MATERIAL AND METHODS

The main plant species under study, *Catharanthus roseus* was gathered from selected areas of Rajasthan (India) and the endophytic fungi were isolated from its leaves. The fungi, along with *Fusarium oxysporum* were brought into PDA medium (potato dextrose agar) at an optimum neutral pH (7.0) and room temperature (27°C). The stock cultures were stored at around 15°C after the sub-culturing at monthly intervals. Solvents like chloroform ($CHCl_3$), methanol (MeOH), ethyl acetate ($CH_3COOC_2H_5$, EA), acetonitrile (CH_3CN, AN) were used in the TLC analysis. Ceric ammonium sulfate (CAS) was used as a spraying agent in this analysis to identify the colored spots of the samples under study and their standards.

2.1. Characterization of the compounds

The mass of the compounds was found out using ESI-MS (Applied Biosystems). Tandem mass spectra (MS/MS) was recorded on a XevoQT mass spectro-meter. Finally, TLC was performed using solvent system $CHCl_3$: MeOH (8:2) by taking the minute amount of extract dispersed in EA.

2.2. Identification of the endophytic fungus

Identification of fungus was done as done by Ahmad et al [16] using PDA medium, Microscopy, and using given PCR primers of ITS (Internal transcribed spacer region) of tested fungus genomic DNA namely, the reverse primer, ITS4 TCCTCCGCTTATTGATATGC, and the forward primer, ITS1- TCCGTAGGTGAACCTGCGG. The obtained PCR products were sequenced using Sanger's sequencing and were carried out with the help of Chromous Biotech Pvt. Ltd. (Bangalore, India). The obtained sequences were then analyzed for their homogeneity.

2.3. Isolation of pure vinblastine and vincristine from the fungus and their characterization

This was done as done previously by Ahmad et al [16]. In this, inoculum from 7 days old culture was introduced into yeast culture, glucose (1.0 g), malt extract (0.3 g), peptone (0.5 g), yeast extract (0.3 g), and 100 ml (MGYP medium). The contents were kept under rotary shaking apparatus for 5 days with 240 rpm

at room temperature. Then, seed culture (10 ml) was transferred into 100 ml of vinca medium-1 and was incubated for 20 days as shake culture at around 25°C. The composition of vinca medium-1 is 3% of glucose, 1% of succinic acid, 100 mg of sodium benzoate, 1 % of peptone, 3.6 mg of magnesium sulfate, 1 mg of biotin, 1 mg of thiamine, 1 mg of pyridoxal, 1 mg of calcium pantothenate, 1 mL of pH 6.8 phosphate buffer, 0.1 % of L-Tryptophan, 0.05 % of geranium oil. Lyophilization of culture filter was done after filtered with a muslin cloth. The solvent, Ethyl Acetate was used to extract Lyophilized (culture) filtrate and the organic layer was separated. This process was done thrice. Using sodium sulfate (anhydrous), the solvent was dried and under rotavapor (at around 40°C), it was concentrated under vacuum to obtain the crude extract. Then TLC analysis was conducted.

Using CAS, the colored spots of alkaloids as brilliant violet color and/or purple color were observed on the TLC plates. For compounds purification, the silica gel was rinsed with $CHCl_3$ and then eluted with the solvent mixture of $CHCl_3$: MeOH (both 100%, 9:1, 8:2, 7:3, 1:1, and 3:7). The Rf values of the samples similar to that of the standard targeted alkaloids were collected and loaded on a silica plate and were developed in the same solvent system (8:2). The presumed bands of the fungal strains of the alkaloids were removed and eluted out using MeOH. The purity of the compounds isolated was verified by using TLC with the solvent systems (a) $CHCl_3$: MeOH (8:2 and 9:1) (b) EA: AN (8:2).

2.4. ESI-MS and MS-MS analysis

It was done as previously stated by Ahmad et al [16]. The samples under study were dissolved in a solvent mixture of MeOH, AcOH, and water (50:50:0.1). The samples were then injected into the MS (flow rate: 5 µl per minute and 3800 V voltage, TOF mode). The spectra were obtained in the range of m/z of 100 to 1400. The molecule's fragmentation under study was acquired using MS-MS interfaced technique. The spectrum was observed from m/z of 100 to 900. The molecular ion peaks of the standard alkaloids were obtained.

2.5. Callus production

The callus cultures (13 weeks old) were used as the source of explants. Fresh leaves of shoots (non-flowering) were used for the extraction of the culture. After 4 weeks of initiation, the first subculture was performed and for the next 3 weeks, the same medium was maintained. The process was done at the Laboratory of Structural Analysis and Development of Plant, School of Life Sciences and Technology ITB, Culture media. Zenk medium was used and it was mixed with 50% sucrose and 2.5 % and gelrite respectively, for the culture, and medium under treatment. To induce them, growth-regulating substances like 1 µM NAA and 10 µM Kinetin [16], were used. All the glass equipment and the culture media were sterilized in the autoclave (121°C, 15 psi) for 15 min respectively.

2.6. Drought stress treatment

To initiate the process, polyethylene glycol (PEG, molecular weight= 4000 u) was sterilized using a polytetrafluoroethylene (PTFE) membrane filter (0.45 µM) and then transferred into an Erlenmeyer flask (100 mL) occupied with 25 mL of the sterile medium. Various concentrations of PEG like 0, 6, 9, and 12 % were used for the treatment process for 0, 24, 48, and 72 h respectively. The compact callus culture was dismembered to aggregated form and then weighed. The treatment process was initiated by placing 1g of this aggregate into 25 mL of the treatment media present in an Erlenmeyer flask (100 mL). Then these aggregated cultures were turned into subcultures in the media which were constituted with solid Zenk and NAA (1 µM) and Kinetin (10 Mm). On the 8^{th} and 11^{th} consecutive days, the callus was isolated.

2.7. Secondary metabolites extraction

The isolated callus culture obtained in the previous step was dried in a freezer for 32 h. The so obtained dry sample was collected and from this, a known quantity of the solid (100 mg) was powdered in a mortar pestle and further extracted with absolute methanol of required volume. The crude extract was kept under sonication for 1 h and then subjected to centrifugation for 5-10 min (at 10,000 rpm) near room temperature. Then the filtrate was divided from the pellet and then collected separately. The finally obtained metabolite extracts were stored at around -20°C.

2.8. Analysis of protein (TCA SDS PAGE method)

To evaluate the quality profiles of the putative enzymes [DAT (50 kDa), D4H (45.5 kDa), and PRXI (37.43 kDa)] included in the ending steps of biosynthesis of the targeted alkaloids, the protein analysis studies were conducted.

2.9. Protein extraction

One gram of the callus treated in the Drought stress treatment stage was collected. This sample was precipitated using 10% CCl_3COOH (Trichloroacetic Acid) and 2-Mercaptoethanol (2 %, C_2H_6OS) for 16 h at around -20°C. The obtained samples were centrifuged (at 5000 rpm) for 30 min at around 4°C. The supernatant liquid was separated slowly and the residue was treated with 10 mL of ice-cold acetone and then again centrifuged for a further 10 min under similar conditions. This stage was repeated in three successive cycles and then the supernatant liquid obtained was discarded and a lysis buffer was added (250 mL) to the residue to maintain a constant pH [17, 18].

2.10. Protein concentration measurement.

The protein obtained in the previous step was added (0.1 mL) to the Bradford reagent (1.0 mL) and further incubated near room temperature for 2-5 min. The OD of the sample was recorded using a UV-Vis spectrophotometer at an absorption wavelength (λ_{max}) of 595 nm. The concentration of protein in the samples was determined by interpolating the absorbance of the sample with the standard absorption peaks of bovine serum albumin [19].

2.11. SDS-PAGE electrophoresis

In this stage, to the protein samples (each 0.45 mg), a loading buffer was added (1:1, V/V). It was then heated to 100°C for 10 min and then cooled to 4°C. SDS-PAGE gel (10 %) was then placed into the chamber of electrophoresis and then it was filled with the corresponding buffer. The protein samples (12 µ) were slowly immersed into the wells of the gel. The studies were conducted at 100 V for 90 min.

2.12. Protein staining and analysis.

The previously obtained gel was introduced into 0.05 % of Coomassie Brilliant Blue R-250 for 4 h. It was submerged in the destaining solution for the next 12 h and shook (at 50 rpm) so that the excess dye can be removed.

3. RESULTS AND DISCUSSION

3.1. Identification of endophytic fungus and isolating purified vinblastine and vincristine

On conducting morphological, molecular, and cultural studies, the presence of AA-CRL-6 (fungal strain) was detected using a PDA medium which can produce the alkaloids under study. There formed white-colored colonies. Many of the morphological characteristics of the strain were in close agreement with the prevailing features in *Fusarium oxysporum*. Upon amplification of the DNA by the primers, ITS-4 and ITS-1, 491 base pairs fragments were identified. The complete analysis revealed the presence of *Fusarium oxysporum* (99.9 %). These studies concluded that the isolated AA-CRL-6 belongs to *Fusarium* genus and the *oxysporum* species.

3.2. Vincristine and Vinblastine from cultured filtrates

The cultured filtrates were extracted using EA after the solvent removal and a brown-colored residue was obtained, known as crude extract [20]. This extract was subjected to TLC treatment (Figure 1a) and five brilliant purple-colored spots were noticed with clear discrimination [21].

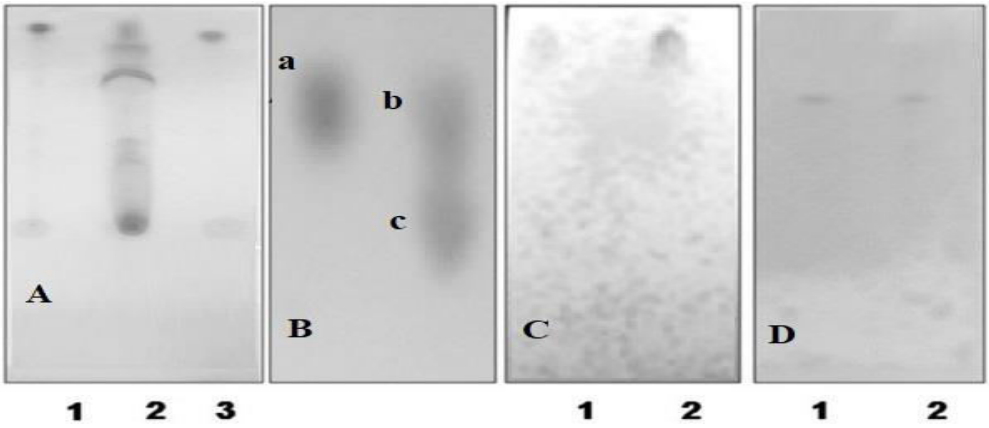


Fig. 1: Analysis by TLC

From the TLC analysis, the Rf values were calculated as 0.76 and 0.75 for vinblastine and vincristine respectively and the same was observed to be very close to their standard Rf values. Further, the spots of the alkaloids in the whole crude extract have displayed similar color as with their standard compounds with

CAS reagent. Figure 1b represents the positions of the partially purified forms of the alkaloids which were very much similar to their standard compounds. These partial forms were operated with preparative TLC and the pure compounds were obtained. In this preparative TLC analysis, vinblastine was identified with a bright purple-colored single spot, and vincristine was marked by the appearance of a dark violet spot at their respective Rf values as noticed above (Fig. 1c). These spots were identified by spraying the CAS reagent (Figure 1d). The purity of the Vincristine and Vinblastine was found to be having similar chromatographic features as that of their standard compounds on the TLC.

(a) Crude fungal vinblastine along with its standard. (1) Standard compound, (2) Crude sample, (3) Standard of vincristine.

(b) Vinblastine along with its standard. (1) A standard compound, (2) partially purified form, (3) partially purified vincristine.

(c) Fungal vinblastine purified along with its standard. (1) Purified fungal compound, (2) Standard form.

(d) Fungal vincristine purified along with its standard. (1) A standard compound, (2) Purified fungal form.

***TLC conditions**: (i) 8:2 ratio of $CHCl_3$: MeOH solvent system on silica gel.*

(ii) Detection by CAS reagent.

(iii) All samples were collected from culture filtrates.

3.3. ESI-MS and MS-MS analysis

At m/z 811, the molecular ion peak was noticed in the ESI-MS spectra (Fig. 2a). Along this [M+H] molecular ion peak, other production peaks for the purified fungal vinblastine were obtained at m/z 350, 524, 545, 730, 750, and 794 respectively in the MS-MS spectra (Fig. 2b).

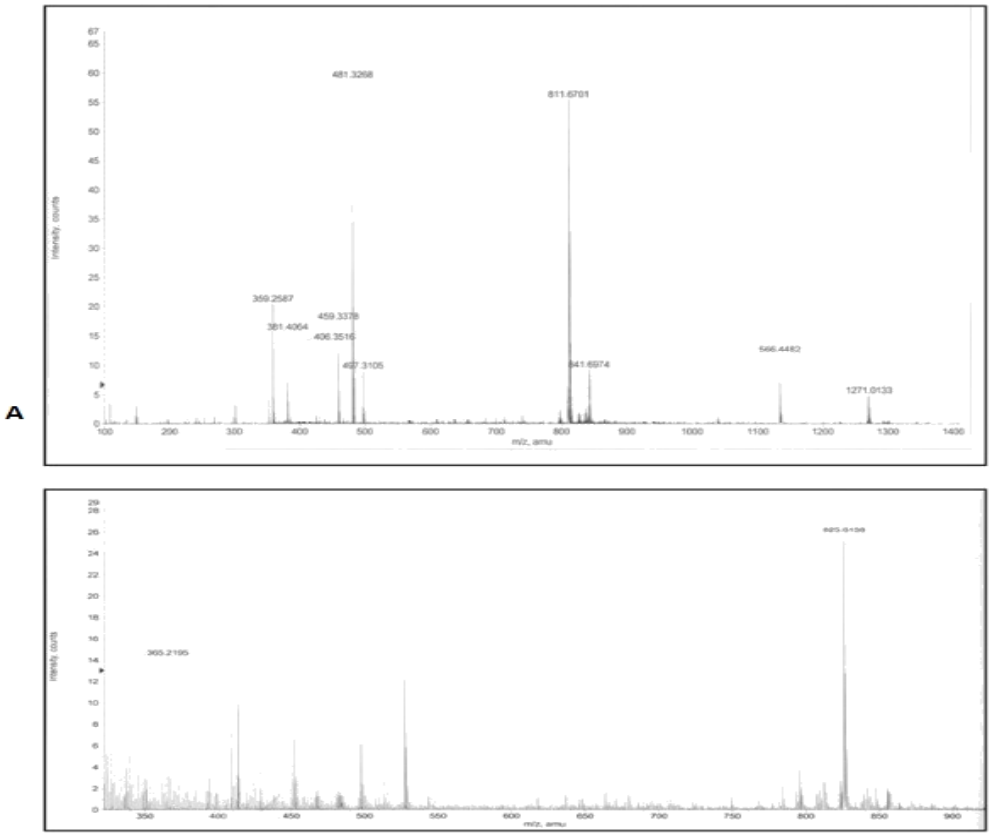


Fig. 2: ESI-MS spectrum of (a) fungal vinblastine; (b) fungal vincristine

In a similar manner for the purified fungal vincristine the molecular ion peak was observed at m/z 824 in the ESI-MS spectra (Fig. 3a) along with the fragment ions at M/Z 765, 809, and 826 were observed in the MS-MS spectra as shown in Fig.3b [22, 23].

3.4. Characteristics of the callus culture

The callus culture was utilized, to produce the secondary metabolites through the idioblast and laticifer discrimination process, Khafagi, *et al* in 2007 [24] conducted their research and observed that the culture

of cell suspension of *Peganum harmala* L. However, the phenomenon cannot be generalized.

Rischer *et al.*, in 2006 and Zhou *et al.*, in 2010 research groups have conducted their research on the cell cultures of *C. roseus* and identified that the suspension of the cell culture cannot produce the targeted amount of the alkaloids. The gene encoding involved in the last stages of the development of the alkaloids was not denoted in the cell cultures of *C. roseus*. Rather, it was denoted only in the idioblast and laticifer [25, 26].

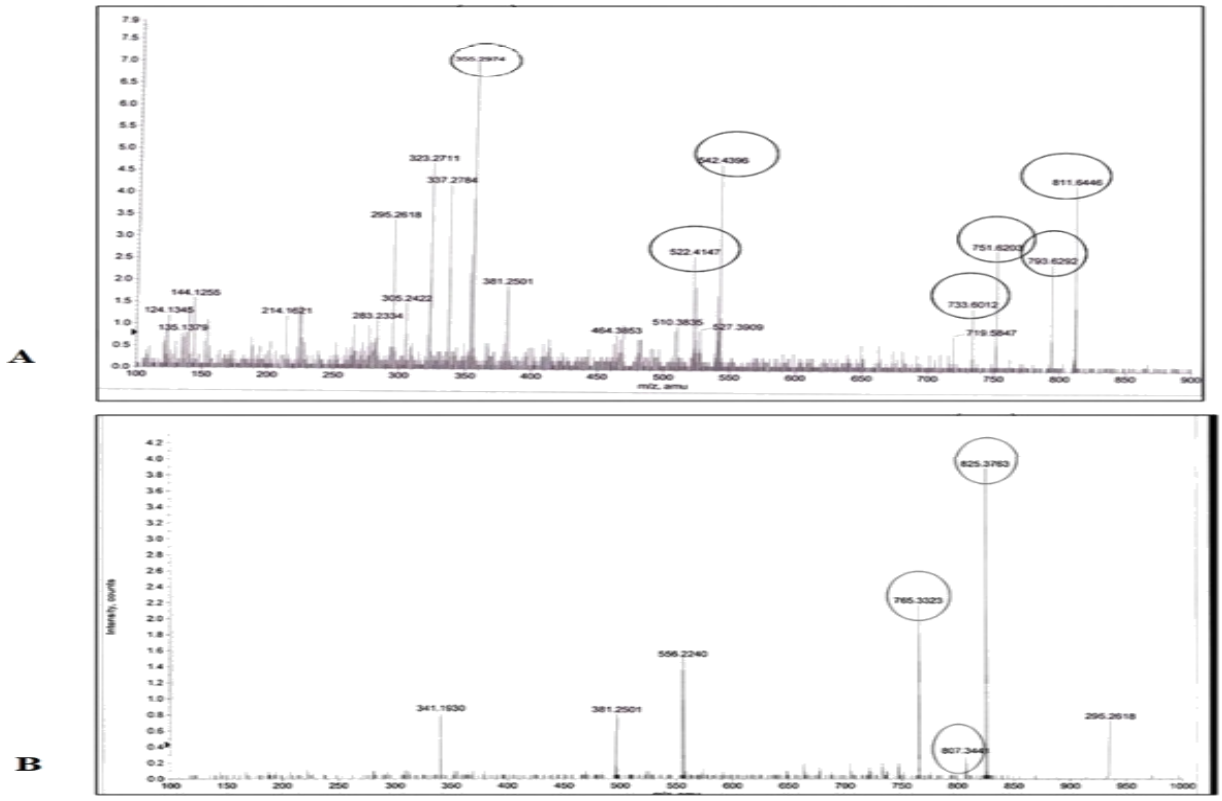


Fig. 3: MS-MS spectrum of (a) vinblastine; (b) vincristine

3.5. Effect of the concentration of polyethylene glycol and duration of therapy on vinblastine and vincristine production

The retention times of vinblastine (in AN: Na_2HPO_4 eluent) and vincristine were obtained at ± 27 minutes and ±17 minutes, respectively in the HPLC analysis as shown in Fig. 4.

Table 1: Concentration of vinblastine in the sample on the 8th and 11th Day

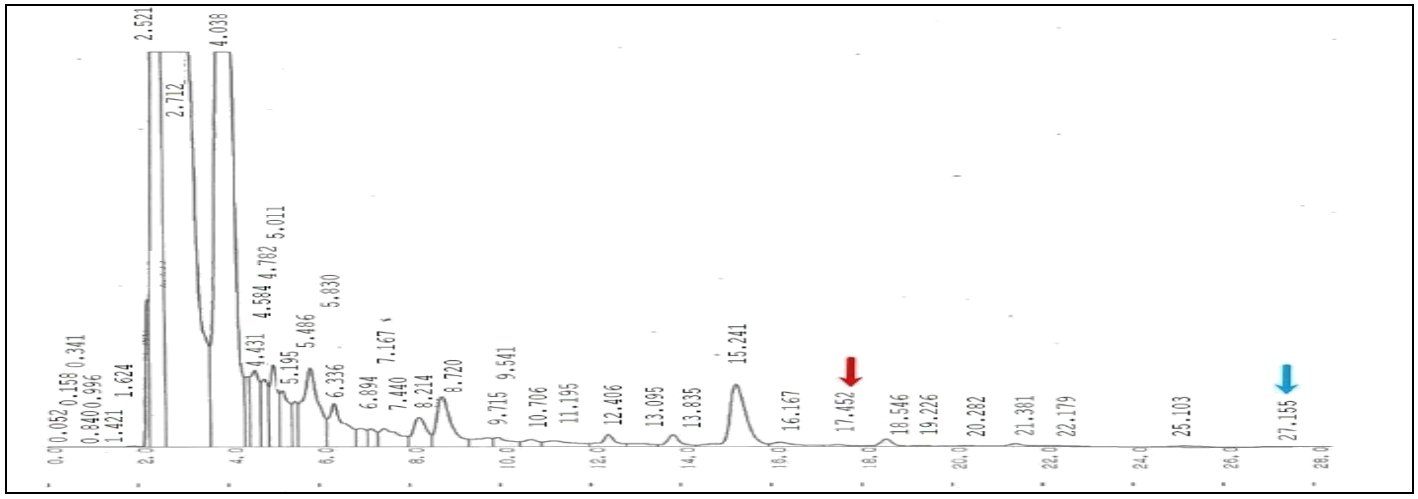
Concentration of PEG (4000 u)	Duration of treatment (hours)	Concentration of vinblastine (µg/g dry weight)	
		Day 8	Day 11
0%	24	0.0188^{ab}	ND
	48	ND	0.0666^{ab}
	72	**0.5625^{f}**	0.0325^{ab}
6%	24	ND	0.0076^{ab}
	48	0.2210^{c}	0.0050^{a}
	72	0.0957^{ab}	0.0293^{ab}
9%	24	0.0191^{ab}	0.0129^{ab}
	48	0.0129^{ab}	0.0099^{ab}
	72	0.1280^{bc}	0.0889^{ab}
12%	24	ND	0.0193^{ab}
	48	0.0645^{ab}	ND
	72	0.3550^{d}	0.0276^{ab}

On comparing the results of the sample extracts with their standards of the targeted alkaloids, it was observed clearly that all the cultures treated with 0, 6, 9 and 12 % of polyethylene glycol were present. After the

treatment with PEG, the concentration of vinblastine was found to be less on the 11th day than on the 8th day, whereas the concentration of vincristine was more on the 11th day than on the 8th day (Table 1). The fall in the concentration of vinblastine was due to its transfor-mation into vincristine [27, 28], leurosine, or its analogs.

Table 2: Concentration of vincristine within the sample on the 8th and 11th Days

Concentration of PEG (4000 u)	Treatment duration (hours)	Concentration of vincristine (µg/g dry weight)	
		Day 8	Day 11
0%	24	0.0083^{a}	0.0189^{ab}
	48	0.0139^{a}	0.0472^{ab}
	72	0.0792^{abc}	0.0949^{bcd}
6%	24	0.0189^{a}	0.0356^{ab}
	48	0.0289^{ab}	0.0221^{ab}
	72	0.0359^{ab}	0.1378^{cd}
9%	24	0.0279^{ab}	0.0158^{a}
	48	0.0511^{ab}	0.0169^{a}
	72	0.0589^{ab}	0.0249^{ab}
12%	24	0.0261^{ab}	0.0459^{ab}
	48	0.0439^{ab}	0.0179^{a}
	72	0.0199^{ab}	0.1598^{d}



Vincristine (red arrow) and vinblastine (blue arrow)

Fig. 4: HPLC chromatogram obtained after 72 h of treatment of the aggregate culture with 0 % polyethylene glycol on the 8th day

3.6. Protein profile analysis

The analysis revealed that all therapies had the same protein band pattern. It indicates that in the aggregate culture, the protein profile was not altered on treatment with polyethylene glycol. The research findings of Raina *et al.*, in 2012 proved that the wounding stress could encourage the activity of MAP Kinase CrMPK3 along with the drought stress treatment [29]. The activity of protein kinase can insert the regulation of ORCA3 to make it possible in the transcript of TDC, DAT, and D4H. This phenomenon explains the similarity in the treated control cultures (with 0 % polyethylene glycol) and other cultures (with 6, 9, and 12 % polyethylene glycol). Further, the results exhibited the expected presence of DAT protein (±50 kDa) [30] and D4H (±45 kDa). The analysis of SDS-PAGE also showed the presence of PRX1 (±37.43 kDa) [31] and a 36 kDa band was thought to be as phosphatase, that meets with the laticifer (Figure 5). The leaf sample proteins analysis revealed the existence of DAT protein (±50 kDa)[32] and D4H protein (±45 kDa), but the band signified the presence of phosphatase and PRX1 that was not observed in the sample (Fig. 5).

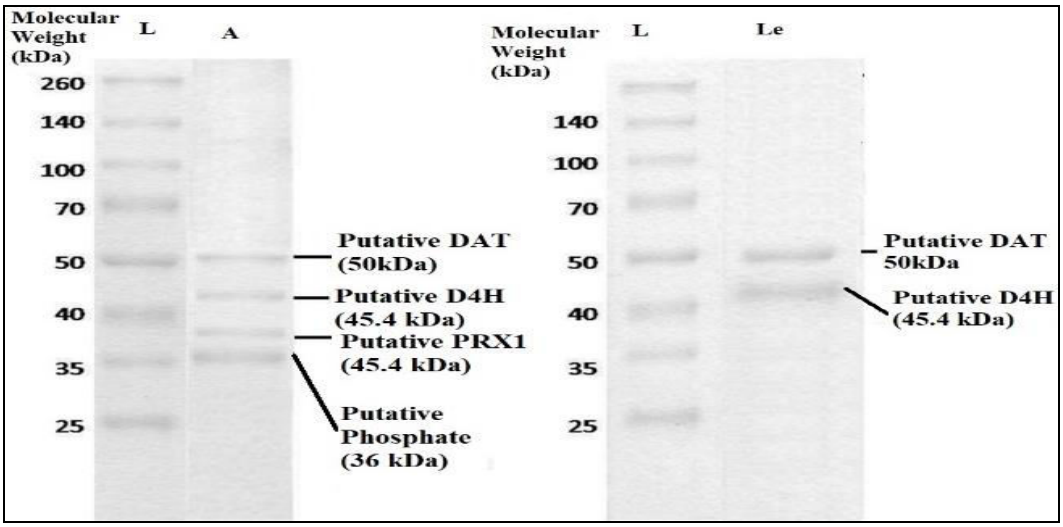


Fig. 5: Left: aggregate culture protein profile; Right: a fresh leaf of *C. roseus* (*L = Ladder Broad Range, A = Aggregate Sample, Le = Leaf Sample)*

4. CONCLUSION

The research work suggests that the fungal strain, AA-CRL-6, can reproduce vincristine and vinblastine compounds. It was developed in the PDA medium that created slow budding snow-white colonies. It was observed that the majority of the morphological features of the strain were very close to the *Fusarium oxysporum* features. Its identity was also revealed in the sequential analysis as 99.9 %. Therefore, it can be concluded that AA-CRL-6 belongs to the *Fusarium* genus and the *oxysporum* species.

In the TLC analysis, the Rf values of all the spots when compared with their standard Rf values were found to be in precision. The purity of their fungal strains showed nearer chromatographic features than that of their standards in the selected solvent systems. Hence, the extraction method adopted was effective in the isolation of the vinblastine and vincristine.

5. ACKNOWLEDGEMENT

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Conflict of interest

No conflicts of interest associated with this publication.

6. REFERENCES

1. Sato F, Asada K, Yamada Y. *Plant and Cell Physiology*, 1979; **20(1):**193-200.
2. Jaleel CD, Sankar B, Murali PV, Gomathinayagam M, Lakshmanan GMA, Panneerselvam R. *Colloid Surf. Biointerfaces,* 2006; **62**:105-111.
3. Jaleel CA, Panneerselvam R. *Chinese Journal of Pharmacology and Toxicology,* 2007; **1(6):**487- 494.
4. Krishnan R. Advances in Horticulture, Medicinal, and Aromatic Plants. Malhotra Publishing House; 1995.
5. Manganey P, Andriamialisoa RZ, Langlois Y, Langlois N, Potier P. *J. Am. Chem. Soc.,* 1979; **101**:2243-2245.
6. Singh DV, Maithy A, Verma RK, Gupta MM, Kumar S. *J. Liq. Chromatogr. Relat. Technol.,* 2000; **23:**601-607.
7. Uniyal GC, Bala S, Mathur AK, Kulkarni RN. *Phytochem. Anal.,* 2001; **12**:206-210.
8. Zhang LB, Gou LH, Zeng SV. *Chinese Trad Herb Drugs,* 2000; **11**:805-807.
9. Tung CY, Yang DB, Gou M. *J Chuxiong Normal Univ*., 2002; **6**:39-41.
10. Guo B, Kunming LH. *J Yunnan Univ*, 1998; **20**:214-215.
11. Farnsworth NR. *Lloydia;* 1961; **24(3):**105-138.
12. El-Sayed A, Cordell GA. *J Nat Prod*, 1981; **44**:289-293.
13. Barthe L, Ribet JP, Pelissou M, Degude MJ, Fahy J, Duflos A. *Journal of Chromatography A,* 2002; **968(1-2):**241-250.
14. Gupta MM, Singh DV, Tripathi AK, Pandey R, Verma RK, Singh S, et al. *J Chromatogr Sci.*, 2005; **43**:450-453.

15. Chua IH, Bodnarb JA, Bowmanc RN, White EL. *J. Liq. Chrom. &Rel. Tech.*, 1997; **20**:1159-1174.
16. Kumar A, Patil D, Rajamohan PR, Ahmad A. *PLoS ONE,* 2013; **8(9):**e71805.
17. Bhardwaj J, Yadav SK. *Am. J. Agric. Biol. Sci.*, 2013; **8**(4):293-310.
18. Cilia M, Yang X, McLaughlin M, Thannhauser TW, Gray S. *J. Biomol. Tech.,* 2009; **20(4):**201-215.
19. Bradford MM. *Anal. Biochem.*, 1976; **72(1-2):**248-254.
20. Guttman A, Khandurina J, Budworth P, Xu W, Hou Y. *Europe*, 2004; **17**:39-41.
21. Volkov SK. *Pharm Chem J.,* 1996; **30**:391-399.
22. Favretto D, Piovan A, Filippini R, Caniato R. *Rapid Commun Mass Spectrom*, 2001; **15**:364-369.
23. Zhou H, Tai Y, Sun C, Pan Y. *Phytochem Anal.,* 2005; **16**:328-333.
24. Khafagi IK. *Am. J. Plant Physiol.,* 2007; **2(1):**17-26.
25. Rischer H, Oresic M, Seppanen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, et al. *PNAS,* 2006; **103(14):**5614-5619.
26. Zhou ML, Hou HL, Zhu XM, Shao JR., Wu YM, Tang YX. *Academic J.,* 2010; **4(25):**663-673.
27. Tikhomiroff C, Joliceur M. *J. Chromatogr.,* 2002; **955(1):**87-93.
28. O'Connor SE, Maresh JJ. *National Product Report*, 2006; **23**:532-547.
29. Raina SK, Wankhede DP, Jaggi M, Singh P, Jalmi SK, Raghuram B, Sheikh AH, Sinha AK. *BMC Plant Biol.,* 2012; **12(134):**1-13.
30. St-Pierre B, Vazques FA, Luca VD. *Plant Cell,* 1999; **11(5):**887-900.
31. Kumar S, Dutta A, Sinha AK, Sen J. *The FEBS Journal,* 2007; **274**:1290-1303.
32. Britto AJ, Kumar PBJR, Gracelin DHS. *J. Chem. Biol. Physic. Sci.*, 2012; **2(2):**792-796.