Endophytes are the microbes which reside inside the plant, imitate the same mechanism of product formation as that of the host plant. In this work the endophytes from the plant Catharanthus roseus analysed for the production of vinca alkaloids as that of host which are potential anticancer agents. Endophytes were screened and six frequently growing endophytes were selected. These were subjected to two stage fermentation for production of alkaloids. Out of six endophytes, two endophytic fungi showed the presence of alkaloids by mayer’s and picric acid test. These culture filtrates were further analysed by the method HPLC (High Performance Liquid Chromatography). The two major anti-cancerous alkaloids vincristine and vinblastine were qualitatively determined by HPLC. The Rt values determined to be 11.58 and 21.72 for vinblastine and vincristine respectively.

**KEYWORDS:** Endophytes, Catharanthus roseus, HPLC, vincristine, vinblastine.

1. INTRODUCTION

Cancer is generally defined as uncontrolled division of cells leading to abnormal growth or when they invade the adjoining parts of body (metastasis). Cancer is generally based on where the tumor is located or where it first started growing in the body. The induction of carcinogenesis is dependent on external and internal factors. The external factors responsible for carcinogenesis are tobacco, obesity, exposure to hazardous chemicals, infectious microorganism while the internal factors include mutations, hormonal and immune abnormalities.\(^3\)

Nature is the main source of all therapeutic compounds, as a huge chemical diversity is found in many species of plants, animals and microorganisms.\(^2\) Currently, more than 3000 plants worldwide have been reported to have anticancer properties. Secondary metabolites are of particular interest to scientists because of their unique medicinal properties. Plant-derived secondary metabolites have played an important role in the development of several clinically useful anti-cancer agents. The widely established ones include vinblastine, vincristine, camptothecin, podophyllotoxin and taxol\(^4\) derived from various plant sources. Plant based natural drugs are produced under specific environmental conditions, stress or nutrition availability. It is estimated that one kilogram paclitaxel is produced after extraction from 10,000 kg stem bark. Extensive use of medicinal plants for extraction of various pharmacologically active components like alkaloids has led to the extinction of certain number of species making them critically endangered.

Bioactive compounds are useful to provide support and relief in all aspects of the diseased conditions in humans. Threat to the environment sustainability, loss of biodiversity, land, water and air pollution are severe problems faced by the humans. By considering the limitations related to the productivity of plants as sources of these metabolites, microorganisms can serve as readily renewable, easily cultivable and inexhaustible source of these metabolites having pharmaceutical activities.\(^5\)\(^8\)

1.1 Endophytes - An endophyte is an symbiotic microorganism, often a bacterium or fungus, that reside within various parts of plants for at least part of its life cycle without causing apparent disease. They produce a number of important secondary metabolites, including anticancer, anti-fungal, anti-diabetic and immunosuppressant compounds. Some of these compounds are those produced by their respective host plants as well. The reason why some endophytes produce certain phytochemicals originally characteristic of the host might be related to a genetic recombination of the endophytes with the host.\(^10\) Even a biosurfactant producing fungi *Pseudozyma antarctica* resides as endophyte but originally reported to be isolated form antactica regions. The glycolipid biosurfactant...
Mannosylerythritol lipid produced by it has anticancerous properties.[22]

1.2 Catharanthus roseus - Catharanthus roseus (C. roseus) has the potential wherein their secondary metabolites could be used to treat various diseases. Alkaloids are the most pharmacologically active chemical constituents of Catharanthus roseus. Their mechanism of action involves binding to tubulin with disruption of mitotic spindle formation eventually leading to inhibition of mitosis, metaphase arrest and cell death.[6] The alkaloids like actineo plastidemeric, vinblastin, vincristine, vindesine, vindoline tabersonine are mainly present in aerial parts whereas ajmalicine, vincine, vincamine, raubasin, reserpine, catharanthine are present in roots and basal stem. Rosindin is an anthocyanin pigment found in the flower of C. roseus.

In this article we mainly focused on vincristine and vinblastine extraction and detection. These two are the world’s second largest best anticancer drugs.

1.3 Vinblastine - Vinblastine was first isolated by Robert Noble and Charles Thomas Beer at the University of Western Ontario from the Catharanthus roseus. The molecular structure is as shown in fig 3. Recently, vinblastine is used for the treatment of large variety of neoplasm and also suggested for the treatment of acute leukemia, Hodgkin's disease and breast and testicular cancer. Scientists reported that vinblastine is produced from Alternaria sp. isolated from Catharanthus roseus.

1.4 Vincristine - Vincristine, also called as leurocristine, is a chemotherapeutic drug used to treat number of cancer complications. These include acute lymphocytic leukemia, acute myeloid leukemia, Hodgkin's disease, neuroblastoma and small cell lung cancer among others wherein the drug is administered intravenously. Vincristine works partly by binding to the tubulin protein, stopping the cell from separating its chromosomes during the metaphase leading to the cell apoptosis. Because vincristine's mechanism of action targets all rapidly dividing cell types, it not only inhibits cancerous cells but can also affect the intestinal epithelium and bone marrow.
2. MATERIALS AND METHODS

2.1 Sample collection, surface sterilization, culturing and isolation of endophytes

Fresh healthy mature leaves of *Catharanthus roseus* was collected in sterile polythene bag from a garden in Jagajeevanram Nagar, Bangalore. Freshly collected leaves, stem and roots were washed under slow running tap water for few minutes followed by washing in Tween 20 (2-3 drops). Then washed thoroughly with sterile water for 2-3 times. Now these were rinsed with 0.1% of mercuric chloride for about 60 seconds and washed several times with sterile water. PDA (Potato Dextrose Agar, Himedia) and NA (Nutrient Agar, Himedia) media were prepared and autoclaved at 121°C at 15 psi for 15 min. Aseptically, 20 ml of sterilized PDA and NA were poured into their respective pre-sterilized glass petriplates and allowed to solidify at room temperature. Using a sterile scalpel and forceps, the leaves and stem were cut into small pieces, leaves were also macerated. These pieces were placed into petri dishes containing PDA and NA as shown in fig.5. On incubation of the plates for about 10-15 days at room temperature, fungal and bacterial growth on the media and on the edges of leaves, stems and macerated leaves was observed. Amongst many endophytes, the desirable six endophytic colonies were selected and their respective pure cultures were developed.

The pure cultures were then incubated at room temperature. Subculturing of stock cultures was done and preserved at 4°C. PDA medium is preferred for fungal and NA is preferred for bacterial growth.

2.2 Culturing and characterisation of vinca alkaloids from endophytes

2.2.1 Fermentation

A two stage fermentation procedure was carried out as reported.[1][7] The pure cultures of endophytes were subjected to fermentation.

Stage 1

MGYP (Malt extract, Glucose, Yeast extract, Peptone [Himedia]) media was prepared. Endophytes were inoculated with 7 days old culture into 100 ml of MGYP media in 500ml erlenmeyer flask and incubated at 28°C
on a rotary shaker (150 rpm) for 4–5 days, which were used as seed culture.

**Stage 2**
10 ml aliquots from Stage 1 flasks were inoculated into production media called vinca medium. Vinca media (1000ml) consists of Glucose – 30g, Sodium benzoate - 100 mg, Peptone - 1g, Magnesium sulphate - 3.6 mg, Biotin: 1 mg, Thiamine - 1 mg, Pyridoxal - 1 mg, Calcium pentothenate - 1 mg, Phosphate buffer - 1 ml (pH 6.8), L-Tryptophan - 0.01g, Geranium oil - 0.005g. These were incubated at 28°C. This is carried out for 20 days.

2.3 Extraction and purification of alkaloids
Our desired alkaloids are extracellular and would be present in the culture filtrate. Culture filtrates and mycelia were separated with the help of muslin cloth and then lyophilized. Lyophilized culture filtrate was extracted using ethyl acetate as a solvent system. The organic layer was separated from the aqueous layer using separating funnel. The lyophilized and extracted samples are subjected to rotary evaporation at 40°C with 5 bar pressure until the solvent is evaporated and the extract is concentrated.

2.4 Test for presence of vinca alkaloids
The extract consisting mainly vincristine and vinblastine alkaloids were detected by following established methods.

2.4.1 Mayer’s Test
Mayer's reagent is an alkaloidal 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). 3ml of extract was taken in a test tube. Few drops of Mayer’s reagent was added along sides of test tube.

2.4.2 Picric acid Test
Picric acid test is carried out by using Hager’s reagent. Hager’s reagent is prepared by adding 1g of picric acid in 100 ml distilled water. 3ml of extract was taken in a test tube. Few drops of Hager’s reagent was added along sides of test tube.

2.5 High Performance Liquid Chromatography (HPLC)
HPLC is used to separate, quantify and analyze the components from the mixture of compounds. C-18 symmetry column (Enable) coupled to SPD-M20A prominence diode array detector (Shimadzu Corporation) is used. The SPD-M20A high performance liquid chromatography PDA detector provides with the most advanced level of sensitivity and stability found in today's PDA detectors. The gradient elution was carried out by using 5%-95% acetonitrile in water with 0.01% Trifluoroacetic acid(Sigma-Aldrich) with flow rate 0.5ml/min. The wavelength recorder set at a 200nm to 300nm used to detect the compound. Stock solution was prepared by dissolving 1mg of standard vinblastine (Sigma-Aldrich) and vincristine (Sigma-Aldrich) in 1ml of acetonitrile (Himedia)(conc.1mg/ml). Different dilutions were made from stock solution as mentioned (mg/ml- 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8). 30microlitre of these standards were injected to HPLC column and their chromatograms were noted. From the culture filtrate obtained after 15 days, alkaloids were extracted by using ethyl acetate. The purified endophytic vinblastine and vincristine were dissolved with HPLC grade acetonitrile. The Rt values and the area under the peak obtained for the samples were noted on the calibration curve made by the different dilutions of the standard.[21]

3. RESULTS AND DISCUSSION
3.1 Sample collection, surface sterilization, culturing and isolation of endophytes
Growth of endophytes on the edges of the leaves were observed. Much growth was seen on the macerated leaves.

Fig 6 – endophytes grown after 10 days.
Amongst many endophytes, the desirable and oftenly grown six endophytic colonies were selected and their respective pure cultures were prepared with control and maintained at 26°C.

### 3.2 Fermentation

MGYP media was prepared and the selected endophytes were inoculated (fig.8). After stage one fermentation for 5 days, this seed culture was inoculated into stage two production media called vinca media\(^1\). This production was carried out for 20 days and thick culture broth observed as shown in fig 9. And our desired secondary metabolites were produced.
3.3 Test for presence of vinca alkaloids
Two biochemical tests, specific for detection of Vinca alkaloids, viz. Wager’s test and Hager’s test was performed. Yellow precipitate was observed in Hager’s test and cloudy white precipitate giving positive results, confirming the presence of vinca alkaloids.

Fig 10 – cloudy white precipitate of col 4.

Fig 11 – yellow precipitate of col 4.

Table 1 – table showing the results for presence of alkaloids.

<table>
<thead>
<tr>
<th>Colony no.</th>
<th>Mayer’s test</th>
<th>Picric acid test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col 1</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Col 2</td>
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<td>No</td>
</tr>
<tr>
<td>Col 3</td>
<td>No</td>
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<td>Yes</td>
</tr>
<tr>
<td>Col 6</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Fig.12. Chromatogram for Vinblastine standard.

The HPLC analysis of Vinblastine standard was carried out and retention time (Rt) was found to be 11.09 min (fig 12).
The HPLC analysis of Vinka alkaloids (vinblastine) extracted from the endophyte -3 was carried out and retention time (Rt) was found to be 11.58 min (fig 13).

Chromatogram for Vincristine standard:

The HPLC analysis of Vincristine standard was carried out and retention time (Rt) was found to be 20.8 min (fig 14).

Chromatogram for vincristine from endophyte.
The HPLC analysis of Vinca alkaloids (vincristine) extracted from the endophyte -6 was carried out and retention time (Rt) was found to be 21.72 min (fig 15).

4. CONCLUSIONS

Plant-derived secondary metabolites have played an important role in the development of several clinically useful anti-cancer agents like vinblastine, vincristine, camptothecin, podophyllotoxin and taxol. Plant endophytes, as a novel and abundant microorganism resource, owning the special ability to produce the same or similar compounds originated from their host plants, as well as other bioactive compounds. Compared with plant cell culture, the culture medium for the endophytes is simple, inexpensive with the abundant supply and the production cost is relatively low. The vinca alkaloids were extracted from the leaves of Catharanthus roseus and their presence was proved. The endophytes were isolated from the leaves which were incubated on PDA and NA media. The isolated endophytes resembles the desired morphological features through microscopic study. The pure cultures of these endophytes were prepared and two stage fermentation was carried out. C. roseus endophytes displayed most extreme potential of vinblastine and vincristine production by both the qualitative and quantitative analysis.

5. FUTURE SCOPE

As the experiment was carried out, vinca alkaloids were detected. These vinca alkaloids can be purified and tested for its anti-cancer property on tumor cell lines. The strains can be sent for genome sequencing for the identity of the genome. The fermentation process can be modified for better production and can be used for drug production process. The use of endophytes as an alternate source of drug can help mankind by reducing the side effect and making it available at an affordable price. With plant the main disadvantage is its longer time of growth, reproduction and less availability.

By the use of endophytes these drawbacks can be eliminated and better anti-cancer products will be introduced to the common people.

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7. REFERENCES


