

Vincristine and Vinblastin Isolation by Fermentation

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ABSTRACT:

Endophytic fungi exists with there host in a symboitic relation where the Mimics the chemistry and also produce the same product as there host which Makes it very important for the production of useful compound like taxon, Camptothecin etc.Vincristine and vinblastin are anti cancer drug which are very expensive to Produce to make it affordable it is isolated from catharanthus roseus to make it available to all patient's in affordable price.
Keywords:- Endophytic fungi,symbiotic ,taxon,camptothecin,vincristine,vinblastin

I. INTRODUCTION:

Cancer ranks 2 in the world as most death causing disease according to WHO ,it is uncontrolled division of cells which causes abnormal growth of the cells in that particular region which causes tumor. But nature has cure for this disease which are plant derived secondary Metabolites' e.g. Vincristine, vinblastine , taxol etc. Due to increase in medicinal production the exploitation of plants also increased so the knowledge of endophytic fungi producing same metabolite's as plants is not less than a boon.

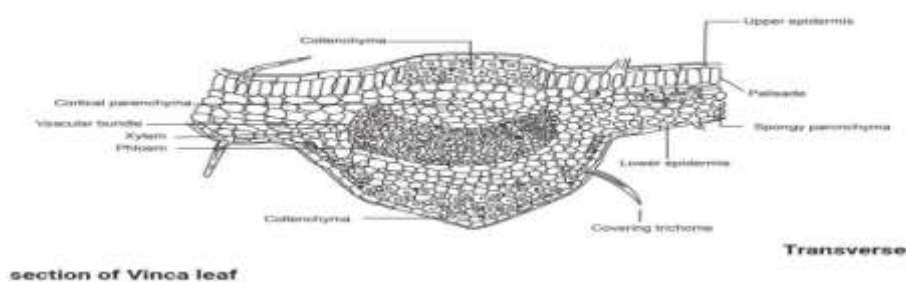
Endophytic fungi:-endophyte is an symbiotic microorganism, often a bacterium or fungus, that reside within various parts of plants for at least part of its life cycle without causing apparent disease. They produce a number of important secondary metabolites, including anticancer, anti-fungal, anti-diabetic 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.

Catharanthus roseus :-

Synonym:- *Vinca roseus*,Madgaskar
Biological source :- *Vinca* is the dried entire plant of
Catharanthus roseus linn belonging to family Apocynaceae
Geographical source :- It is mainly found India ,South Africa etc.



Catharanthus roseus



section of Vinca leaf

Transverse

Microscopy

Vinca has dorsiventral leaf structure. Epidermis is a single layer of rectangular cells covered with thick cuticle. It consists of unicellular covering trichome and cruciferous stomata. In the mesophyll region single layer of elongated and closely packed palisade parenchyma cells are present just below the upper epidermis. In the midrib region two to three layers of collenchyma is present, both below the upper epidermis and above the lower epidermis. Vascular bundle consisting of xylem and phloem is present in the middle of midrib region and rest of the intercellular space is covered by five to eight layers of spongy parenchyma. Calcium oxalate crystals are absent.

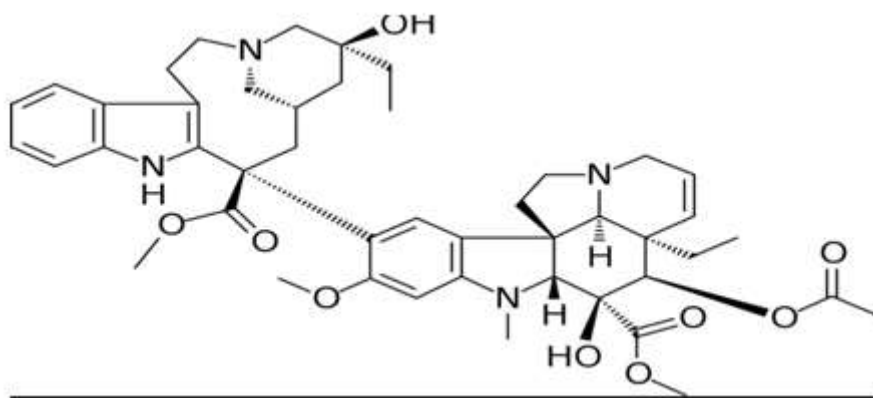
Chemical Constituents:-

Alkaloids are present in entire shrub but leaves and roots contain more alkaloids. About 90 alkaloids have been isolated from Vinca from which some like Ajmalicine, Serpentine and

Tetrahydroalstonine are known and are present in other species of Apocynaceae. The important alkaloids in Catharanthus are the dimer indole indoline alkaloids Vinblastine and Vincristine and they possess definite anticancer activity. Vindoline and Catharanthine are indole monomeric alkaloids. It also contains monoterpenes, sesquiterpene, indole and indoline glycoside.

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*.



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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.



Matetial and method of isolation :-

1. Sample collection :-
Fresh and healthy leaf of catharanthus roseus were collected in a sterile polythene bag
2. Surface sterilization :-
The fresh leaf root and stem were was under slow running yap water for few minutes then it was washed by tween 20 (2-3) drops then again it is washed with sterile water for 2 -3 times and then by 0.1% mercuric chloride solution for 60 seconds

followed by washing with sterile water again for several time

3. Culturing of endophytes:-
PDA (Potato Dextrose Agar) and NA (Nutrient Agar) media were prepared and autoclaved at 121°C for 15 psi for 15 minutes . Then aseptically 20 ml pda and na were added in the sterile petriplate and were allowed to solidify on room temperature and the leaves and stem were cut into pieces by sterile forcep and scarpel and the leaves was also macerated



The plates are then incubated at room temperature for 10 - 12 days . The bacterial and fungal growth were seen on the petri dish edges and sides .

Amongst all of them the best 6 were selected and again incubated for pure culture



Fig 7 – pure cultures of the selected endophytes.



Fig 6 – endophytes grown after 10 days.

Fermentation :-

The fermentation of endophytes are done in two different stages and it is carried out on the pure culture

Stage 1 :-

MGYP extract was prepared (Maltose , Glucose , Yeast, peptone) 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 pantothenate - 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.



Fig 8- culture flasks before fermentation.



Fig 9 – culture flasks after fermentation.

Extraction and purification of alkaloids :-

1. Dry leaf of vinca is extracted with 0.1ml HCl for half an hour in ultrasonic bath centrifuge the mixture for 10 min
2. Sediment is re - extracted with more quantity of hcl mix with supernatant and filter it

3. To remove lipophilic compound and chlorophyll treat this filter with petroleum ether
4. Separate the acidic fraction treat it with alkaline solution 10% of embonic acid add slowly for the precipitate to form

5. Increase the pH to 5 and separate the precipitate by decantation which is used in semisynthesis of vinblastine
6. Mix this precipitate with 0.1M HCL and 0.1 M citric acid and cool it from 0 to -5 ° C using dichloromethane and ice bath
7. Add 30% aq hydrogen peroxide , 10% aq sodium hypochlorite, 1 % solution of sodium

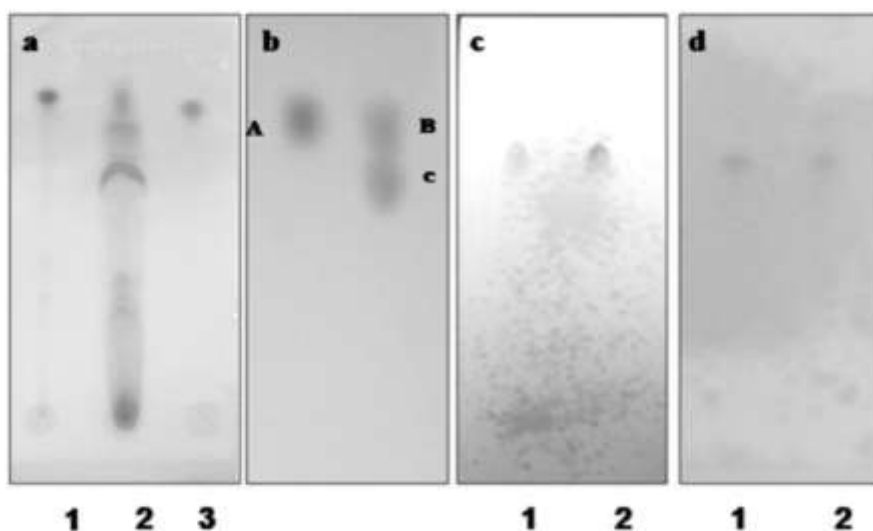
borohydride in methanol slowly for 3 to 5 hours

8. Increase the pH to 9.5
9. Collect the organic layer step wise and dry it

Purification :-

Purification of vinca alkaloids are done by 2 methods

1. TLC



(a) TLC of crude fungal vinblastine from culture filtrates along with standard Vinblastine

- 1: Vinblastine standard,
- 2: Crude sample,
- 3: Vincristine standard,

Detection: Ceric ammonium sulphate reagent.

(b) TLC of partially purified fungal vinblastine from culture filtrates along with standard vinblastine on silica gel using chloroform:methanol (8:2) solvent system.

- A: Standard vinblastine
- B: Partially purified vinblastine
- C: Partially purified vincristine,

Detection: Ceric ammonium sulphate reagent.

(c) TLC of fungal vinblastine purified from culture filtrates along with standard vinblastine on silica gel using chloroform:methanol (8:2) solvent system.

- 1: Purified fungal vinblastine
- 2: Standard vinblastine,

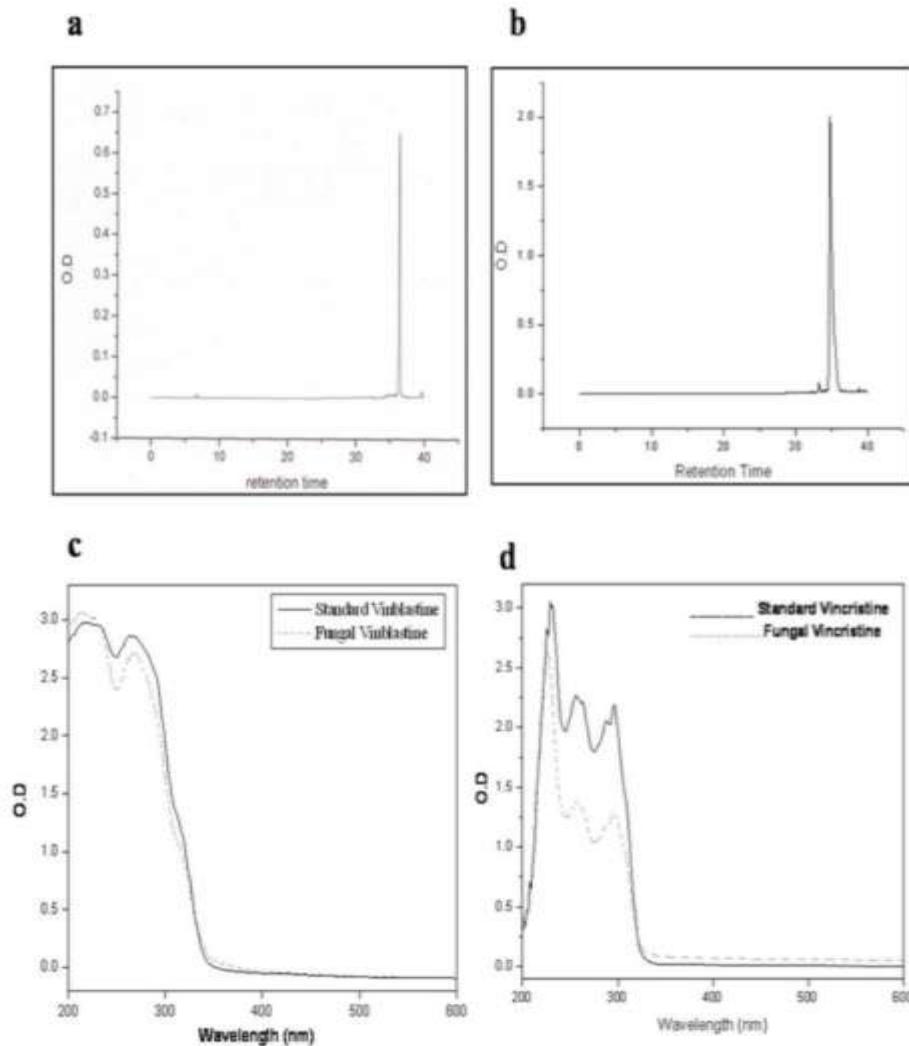
Detection: Ceric ammonium sulphate reagent.

(d) TLC of fungal vincristine purified from culture filtrates along with standard vincristine on silica gel using chloroform:methanol (8:2) solvent system.

- 1: Standard vincristine,
- 2: Purified fungal vincristine,

Detection: Ceric ammonium sulphate reagent.

2. HPLC and UV spectroscopy



- a) HPLC profile of pure fungal vinblastine with retention time of 36.6 min.
- (b) HPLC profile of pure fungal vincristine with retention time of 34.9 min.
- (c) UV absorption spectrum of standard vinblastine and fungal vinblastine.
- (d) UV absorption spectrum of standard vincristine and fungal vincristine

Test for presence of vinca alkaloid:-

1. Wager's test :-

When wayer reagent is added to vinca alkaloid then it shows cloudy white precipitate giving positive result



Fig 10 – cloudy white precipitate of col 4.

2. Hager's test :-

When hager reagent is added to vinca alkaloid yellow precipitate is observed giving positive result



Fig 11 – yellow precipitate of col 4.

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

Colony no.	Mayer's test	Picric acid test
Col 1	No	No
Col 2	Yes	No
Col 3	No	No
Col 4	Yes	Yes
Col 5	No	Yes
Col 6	Yes	Yes

II. CONCLUSION :-

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 quantitative and qualitative

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