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EXTRACTION, ISOLATION AND ANALYSIS OF VINCA ALKALOIDS: AN OVERVIEW

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ABSTRACT

Vinca alkaloids are obtained from the Madagascar periwinkle plant. They are naturally occurring or semi synthetic nitrogenous bases extracted from the pink periwinkle plant Catharanthus roseus G. Don. and have a hypoglycemic as well as cytotoxic effects. They have been used to treat diabetes, high blood pressure and have been used as disinfectants. The vinca alkaloids are also important for being cancer fighters. There are four major vinca alkaloids in clinical use: Vinblastine, vinorelbine, vincristine and vindesine. Vinca alkaloids are the second-most-used class of cancer drugs and will stay among the original cancer therapies. The present review work was mainly focused on compilation of data related to extraction of important alkaloids from Vinca (Catharanthus roseus) using different techniques and further their isolation, detection / identification, and analysis using modern analytical methods.

Keywords: *Catharanthus roseus, vinblastine, vinca alkaloids, vincristine, Isolation and extraction, Analysis.*

1. INTRODUCTION

Catharanthus roseus (L.) G. Don (*C. roseus*) is a medicinal plant of the *Apocynaceae* family, originally from Madagascar. In the present, it has been naturalized in all tropical regions of the world. *C. roseus* produces 120 alkaloids, 70 of which have pharmacological activity, for example, vindosine, hörhammericine, lochnericine, vindolicine, anhydrovinblastine, vincristine, tabersonine, catharanthine, vindoline, yohimbine, vinblastine, ajmalicine. Terpenoid indole alkaloids (TIA) are specially cultivated in an industrial scale to obtain anticancer alkaloids for the pharmaceutical industry.

The main alkaloids obtained from *C. roseus* are shown in Figure 1: (1) vindolicine; (2) anhydrovinblastine; (3) vincristine; (4) ajmalicine; (5) tabersonine; (6) catharanthine; (7) vindoline; (8) vinblastine; and (9) ajmalicine [1].

Catharanthus roseus (L.) G. Don is regarded as a rich source of pharmaceutically important terpenoid indole alkaloids. Vindoline and catharanthine are the major monomer alkaloids as well as biosynthetic precursors for the "dimeric" alkaloids, vinblastine and vincristine, two well-known anticancer drugs used in the treatment of acute leukemia and Hodgkin's disease [2]. Low "dimeric"

alkaloid contents in the plant have encouraged intense research for alternative production methods involving cell cultures [3,4], metabolic engineering [5, semi-synthesis [6,7] or even total chemical synthesis [8]. Total synthesis has proved difficult due to structural complexity of the molecules and complicated reaction steps involving stereochemical constraints. Various semi-synthetic procedures have been developed for these alkaloids on the basis of chemical [6,7] or enzymatic [9] coupling of commercially available catharanthine and vindoline. As a means of simpler and economically feasible semi-synthesis of vinblastine and vincristine, a photochemical one pot synthesis is used [10 -13].

Vinblastine and vincristine, the wonder drugs for cancer, are being isolated from the leaves of field grown Catharanthus roseus plant by techniques such as tissue culture, cell culture, shoot culture [14], semi synthesis [15] as well as total synthesis [16]. Although the drugs can be obtained by these methods, their supply is limited and cannot meet the present requirements. A number of endophytic fungi have been isolated by Kharwar et al. from the *Catharanthus roseus* plant found in India [17]. But there are so far no reports of vinblastine and vincristine from above endophytic fungi. Zahng et al. [18] and Tung et al. [19] reported that vincristine is produced by Fusarium oxysporum, an endophyte of Catharanthus roseus while Guo and Kunming [20] obtained vinblastine from Alternaria sp. isolated from the same plant found in China and showed the production based upon TLC and HPLC only.

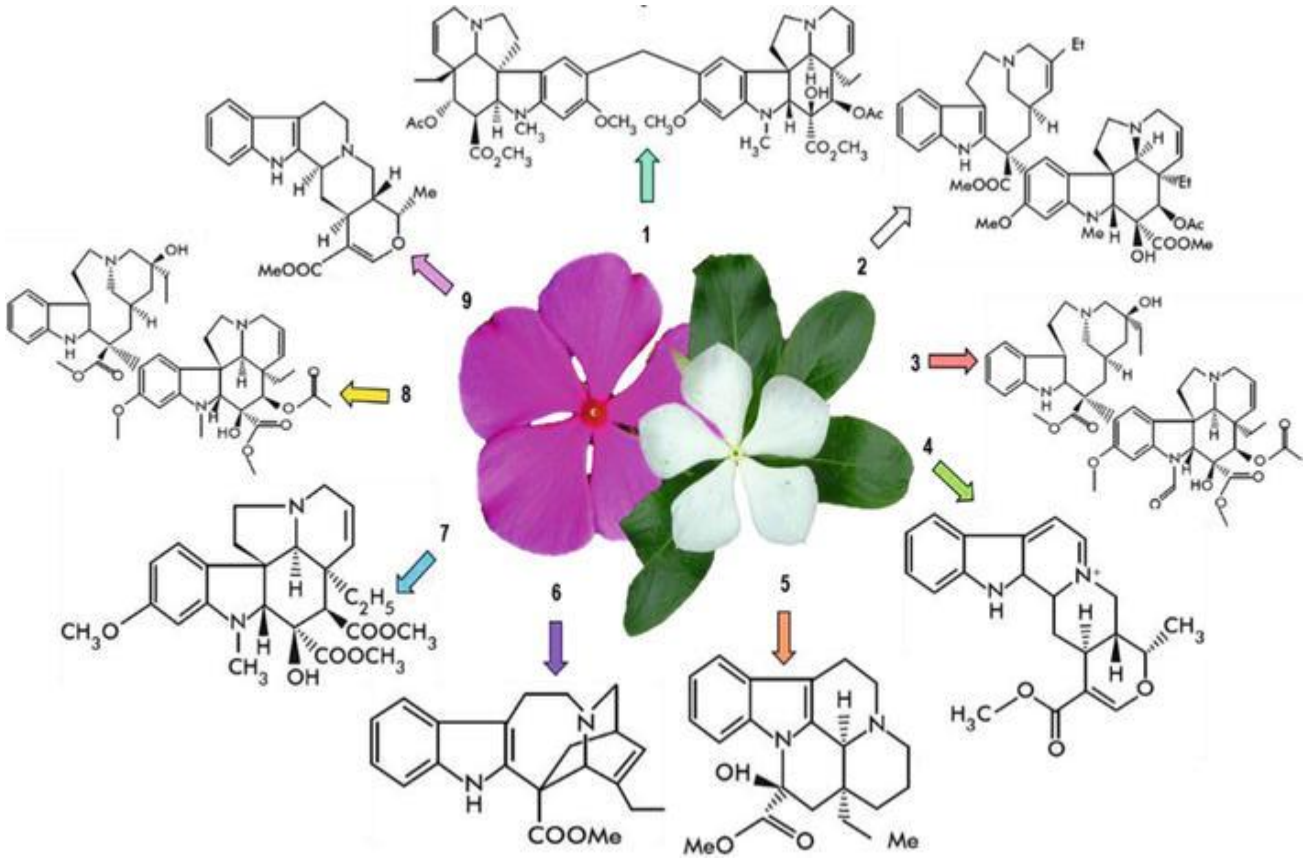

Fig. 1: Alkaloids produced by Catharanthus roseus (1) vindolicine ($C_{51}H_{64}N_4O_{12}$, 925.08 g/mol); (2) anhydrovinblastine ($C_{46}H_{56}N_4O_8$, 792.97 g/mol); (3) vincristine ($C_{46}H_{56}N_4O_{10}$, 824.95 g/mol); (4) ajmalicine ($C_{21}H_{24}N_2O_3$, 352.43 g/mol); (5) tabersonine ($C_{21}H_{24}N_2O_2$, 336.44 g/mol); (6) catharanthine ($C_{21}H_{24}N_2O_2$, 336.42); (7) vindoline ($C_{25}H_{32}N_2O_6$, 456.53 g/mol); (8) vinblastine ($C_{46}H_{58}N_4O_9$, 810.97 g/mol); and (9) ajmalicine ($C_{21}H_{24}N_2O_3$, 352.43 g/mol).

Catharanthus roseus produces vinblastine, utilized in treating Hodgkin's disease; testicular tumors, breast carcinoma, choriocarcinoma, Kaposi sarcoma and Letterer-Siwe disorder. Vincristine is used to treat acute lymphocytic leukemia, lymphosarcoma, lympho-granulomatosis and in solid infant tumors. The preparation process of 1 kg of vincristine has a cost of US$

3.5 million, while vinblastine has a cost of US$1 million. Therefore, 530 kg of dry leaves are necessary to produce 1 kg of vincristine and half a ton for getting 1 g of vinblastine. The high cost is due to the low concentrations in the aerial portion. Due to the high market value and its effectiveness in different medical treatments. The present review discusses about extraction, isolation, analysis, clinical pharmacology and applications of important alkaloids from Vinca.

2. EXTRACTION AND ISOLATION OF ALKALOIDS OF *CATHARANTHUS ROSEUS*

The extraction method of terpenoid indole alkaloids in *C. roseus* has been optimized by different authors. Most of the methods are time-consuming extractions with several steps and a high use of organic solvents. Despite the high aggregated value of the product, these multi-step processes generate a great amount of organic and acid residues, and as a consequence they rise production cost [21]. Some effective alkaloid extraction methods have been identified from pilose roots of *C. roseus*. For example, Tikhomiroff and Jolicoeur use methanol, lyophilize, dry the roots, and extract during 1 h in a sonication bath [22] use methanol, lyophilize, mash the roots, extract with 45 mL for 5 h in a sonication bath, and evaporate the mobile phase, use methanol and ethyl acetate, extract methanol during 20 min in a sonication bath at 50°C, evaporate methanol, resuspend with 20 mL 0.1 N of HCl, extract with 20 mL of ethyl acetate, adjust pH to 10, evaporate and resuspend in methanol extract with methanol and lyophilize [23]. Extraction can be made from dry material in water with sulfuric acid and four purification stages: fractioning by partition with benzene, two chromatographic columns and finally, crystallization in ethanol and sulfuric acid.

Various methods of extraction and isolation of different vinca alkaloids discussed below:

2.1 Svoboda's Method

Svoboda et al. devised an extraction scheme by which numerous individual Vinca alkaloids have been isolated. The plant material (ground whole plant, *Catharanthus roseus*) after de-fatting with Skelly B (essentially n-hexane), is extracted with 2% tartaric acid, before extraction with organic solvents under acidic and alkaline conditions, thus, initially separating those alkaloids whose tartrates are soluble in organic solvents and those which are not.

Some alkaloids are also removed from the plant material by the Skelly B together with fat. This extraction scheme and the subsequent chromatography and gradient pH extractions may serve to illustrate recent advances from the classical extraction technique. The principles are applicable to the separation of certain other groups of alkaloids.

In the following outline of the Svoboda extraction and separation schemes, only the details pertaining to those steps and fractions which yield vinblastine, vincristine, vinleurosine, and vinosidine are summarised. The details for those other fractions yielding a number of Vinca alkaloids other than these four are excluded.

The initial steps of extraction are summarised schematically in the flow sheet given in Fig. 2. The different fractions are then chromatographed on alumina, and eluted with various eluting solvents.

Some of these column fractions (i.e., eluted from the alumina column) are further subjected to gradient pH extractions to achieve separations of certain Vinca alkaloids.

In this scheme, vinblastine vinleurosine, vincristine, and vinrosidine are in fraction A, together with a number of other alkaloids.

Chromatography

A benzene solution of 10 gm of the extracted fraction A is chromatographed on 400 gm of alumina Grade F-20 (de-activated by treatment with 12.5 ml of 10% acetic acid). Eluting solvents used are benzene, benzene — chloroform, chloroform, and chloroform — methanol in 500 ml fractions. Vinleurosine (0.234 gm) comes down in fractions 34-42 (benzene – chloroform 1:1) while vinblastine (0.126 gm) is obtained as the sulphate from fractions 43- 45 (benzene – chloroform 1:1).

Post-vinblastine eluent fractions (3.6 kg of residue from evaporation of combined chloroform fractions) are re-chromatographed on de-activated alumina (120 kg) in a similar manner. The eluting solvents and fractions collected are — benzene (fraction 1), benzene-

chloroform 3:1 (fractions 2-15); benzene-chloroform 1:1 (fractions 16-29); benzene- chloroform 1:3 (fractions 3CM3); chloroform (fractions 44-57); and chloroform-methanol 19:1 (fractions 58-67).

In these re-chromatographed fractions, vinrosidine is isolated from fractions 33-45, and vincristine is isolated from fractions 33-42. The isolation of these alkaloids from these column fractions is achieved by subjecting each fraction to gradient pH extractions.

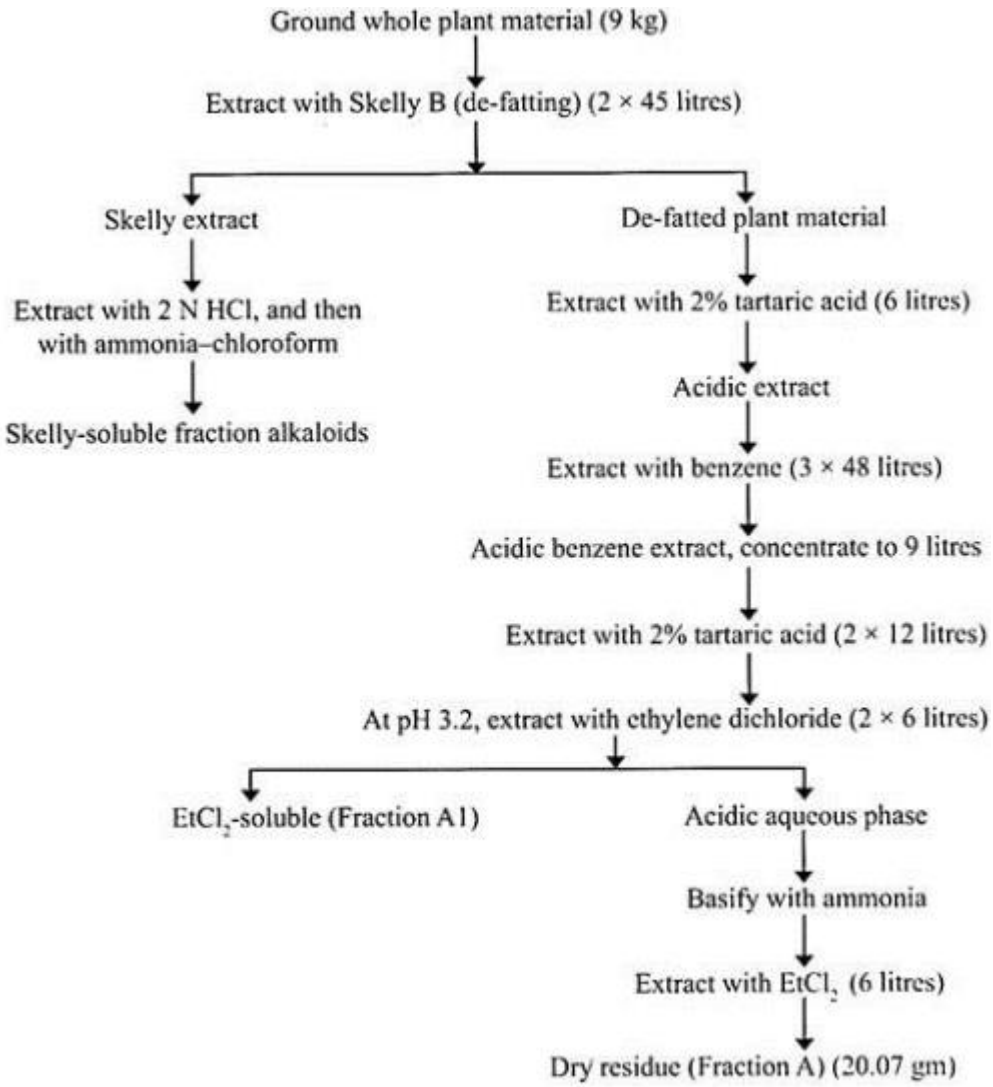

Fig 2: Flow chart for the Svobodas extraction

Gradient pH Extractions

The eluted crude fraction (10 gm) is dissolved in 500 ml of benzene, and any insoluble material is removed by filtration. The benzene solution is then extracted into 500 ml of 0.1 M citric acid by steam distillation under reduced pressure. This steam distillation procedure is used in order to avoid emulsion formation. After removal of all the benzene, any insoluble material is filtered off.

The acidic solution (pH – 2.75-2.85) is extracted with 500 ml of benzene. The aqueous phase is then adjusted with ammonia to pH 3.54, 3.9, 4.4, 4.9, 5.4, 5.9, 6.4 and 7.5 successively. At each pH level, the aqueous solution is extracted with 500 ml of benzene. The benzene extract in each case is dried over sodium sulphate and evaporated to dryness. Both vinrosidine and vincristine are extracted in this manner from the acidic solution, at pH levels 4.9-6.4. [24]

2.2 Isolation of Catharanthine, Vindoline and Vinblastine

Atta-ur-Rahma et al. (1983) described the following rapid procedure for isolating catharanthine, vindoline and vinblastine:

Procedure:

A) 20 kg of air-dried leaves of Catharnathus roseus are finely crushed with 40 litres of ethanol. The crushed material is filtered and washed thoroughly with 10 litres of ethanol. The ethanolic filtrates are combined and evaporated first in a flash evaporator and then in a rotary evaporator until it is concentrated to a gum. The gum is acidified with 5% HCl (5 litres) and washed with chloroform (3 × 1 litre). The ice-cold solution is then basified (1 litre, 33% NH, solution), and extracted with 2 × 3 litres of chloroform. Chloroform extracts are dried over anhydrous sodium sulphate and concentrated to a crude alkaloidal gum (120 gm).

The alkaloids (120 gm) are dissolved in 400 ml of chloroform and extracted with pH 3 phosphate buffer (one litre). The chloroform layer is dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum to afford 60 grams of the alkaloidal mixture. This is dissolved in 240 ml of chloroform. 480 ml of petroleum ether is added, which causes some alkaloids to precipitate out. Virtually no cathranthine, vindoline and vinblastine are present in the precipitated portion.

The precipitates are filtered off and the filtrate is again concentrated to a gum (36 gm). The gum is dissolved in 200 ml ethyl acetate and extracted with pH 2 phosphate buffer (one litre). The aqueous layer is separated and then extracted with chloroform (one litre) to afford a vindoline- and catharanthine-rich fraction (15 grams). The buffer layer is then basified with ammonia solution to pH 10 and extracted with chloroform (one litre) to afford the vinblastine-containing fraction (20 grams).

B) In order to obtain catharanthine and vindoline, a flash (dry) column chromatographic procedure is applied. This consists of packing silica gel (70-230 mesh) in a sintered column fitted with a ground glass bottom joint and tap. This column is fitted onto a Buchner flask. The substance is loaded on the column and the eluting solvent drawn down the column with the help of vacuum applied on the collecting Buchner flask.

C) 15 grams of vindoline- and catharanthine-rich fraction is loaded on the top of such a column, packed with 150 gms of silica gel (70 230 mesh). The column is eluted first with 28% ethyl acetate in petroleumether (2 litres), and then with 35% ethyl acetate in petroleum ether (5 litres) which affords pure catharanthine (0.6 gm). Elution with 45% ethyl acetate in petroleum ether (2 litres) removes the other alkaloids. Subsequent elution with 55% ethyl acetate in petroleum ether (5 litres), affords pure vindoline (2.4 grams).

D) The vinblastine containing fraction (20 gms) is loaded on a flash chromatography column packed with 200 grams of alumina (neutral activity 1). The column is eluted with 70% ethyl acetate in petroleum ether (7 litres). The eluates are concentrated to a gum (10 gms), and again loaded on the same type of column packed with 30 grams of TLC grade silica. Elution with 50% ethyl acetate in petroleum ether removes the faster moving substances. Subsequent elution with 0.5% ethanol in ethyl acetate (2 litres), affords a vinblastine-rich fraction (0.6 gram).

E) The vinblastine-containing fraction (0.6 gm) is loaded on a preparative HPLC (column diameter: 2.2 cm; column length: 50 cm; column packing – Lichroprep 15-25 pm silica; solvent pressure – 1000-1200 lb/sq inch; rate of flow – 13 ml/minute), and eluted successively with 50% ethyl acetate in petroleum ether (2 litres), 60% ethyl acetate in petroleum ether (4 litres), 65% ethyl acetate in petroleum ether (2 litres) and finally with 70% ethyl acetate in petroleum ether (2 litres). The last elution affords pure vinblastine (60 mg) as the slowest moving material. [25]

2.3 Extraction and Isolation of Vincristine and Vinblastine

The dried leaf material is taken and is extracted with a solution of hot ethanol–water–acetic acid in a ratio of 9:1:1. The solvent is removed and to the residue hot hydrochloric acid solution of 2% is added. The pH of the acidic extracts adjusted to 4, for the precipitation of the non-alkaloidal components, which can be separated by filtration. The pH of the aqueous acidic solution is now adjusted to 7 and then extracted with benzene. The benzene layer is evaporated to obtain vinblastine and other alkaloids.

Isolation of Vinblastine and Vincristine

The phenolic materials are removed by the washing the extract with dilute alkali. The washed extract is subjected to chromatography on alumina and elution is carried out in 18 fractions starting with benzene–methylene chloride (65:35) mixture to

pure methylene chloride. Vinblastine recovered in the ninth fraction. Further elution of the column results in separating the fractions of vincristine [26].

3. SEMISYNTHETIC PRODUCTION OF VINCA ALKALOIDS

3.1 Production of Vinblastin

Dry leaf powder of the Vinca is first extracted with 0.1M HCl for half an hour in ultrasonic bath, centrifuge the mixture for 10 minutes at 2000 rpm. Obtained sediment is re-extracted with more quantity of HCl. Mix both supranatant and filter it. Treat this filterate with petroleum ether to remove lipophilic compound and chlorophyll. Separate the acidic fraction and treat it with alkaline solution (pH 10.5) of 10 percent in embonic acid add slowly for the precipitate of alkaloid. Increase the pH upto 5.0. separate the precipitate with decantation and this precipitate can be used for the semi synthesis of vinblastin. Mix this precipitate with 0.1M HCl and 0.1 M Citric acid and cool the mixture from 0 to - 5°C using dichloro methane and ice bath. Slowly add 30 percent aqueous hydrogen peroxide, 10 percent aqueous sodium hypochlorite and 1 percent solution of sodium borohydride in methanol for three to five hours. Increase the pH of mixture upto 9.5, collect the organic layer step wise and dry it.

3.2 Production of Vincristine Sulphate

Treat dried homogenous ground material with dilute tartaric acid and extract with benzene. Concentrate the benzene extract and perform the steam distillation. The benzene will be separated as distillate and the residue will be left out. Dissolve the residue in methanol and treat it with dilute tartaric acid solution. Perform the distillation. The methanol will separate as distillate and treat the bottom product with dilute solution of ammonia. Extract it and then evaporate the extract to dryness. Dissolve the dry powder and separate the vincristine sulphate by chromatographic method by using alumina column and eluted with benzene, benzene + chloroform, chloroform and chloroform + methanol. Vincristine will be isolated and treat it with sulphuric acid, vincristin sulphate will be obtained. [27]

4. ANALYSIS OF VINCA ALKALOIDS

The purification processes were performed with preparative TLC and HPLC and the characterization was done by UV-Vis, ESI-MS and 1H NMR spectroscopy.

High performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) provides a highly selective and sensitive means for identification of the alkaloids [11,12]. Direct-injection ESI-MS has also been used for rapid identification of Catharanthus alkaloids [13]. Identity of the isolated compounds can also be confirmed through their melting points. Melting point of Vinblastine is 284–285°C and Vincristine is 273– 281°C.

Vinblastine and vincristine have been isolated in pure form to be detected through the use of several chromatographic techniques such as:

- Vacuum Liquid Chromatography with a silica gel column; aluminum oxide (1:1) mixed with Vacuum Liquid Chromatography (VLC); carbon column and purification by accelerated radial chromatography by centrifugation (chromatotron).
- Semi-quantitative methods have been established by the use of Thin Layer Chromatography (TLC) methods. TLC has a higher sensitivity for alkaloid detection; ajmalicine is detected at a 0.0007% in a volume of 10 µL. Vincristine is detected at 0.055% in a volume of 10 µL, while vinblastine and vindoline are not sensitive to this method since they are both in concentrations of 0.05% in a volume of 10 µL. The chromogenic reactive that is chromatographically used in alkaloid detection is the Cerium Ammonium Sulfate (CAS), which is known for reacting with analyte to produce visible colors in the TLC plate [28].
- Vincristin and vinblastin sulphate are estimated with the help of HPLC. The following solution are prepared for the estimation: Solution 1: 0.1 percent w/v of the substance being examine. Solution 2: Contain 0.2 percent w/v each of vinblastine sulphate RS

and vincristine sulphate RS solution. Solution 3, 4 and 5: 0.1 percent w/v, 0.002 percent w/v and 0.0001 w/v respectively of vinblastin sulphate (if vinblastin estimated) or vincristin sulphate (if vincristin sulphate estimated).

Column: Packed with stationary phase LC2. Flow rate: 1.0 ml/min.

Mobile phase: (For vinblastin) Mixture of 70 volume of methanol 30 volume of 1.5 percent w/v of diethylamine (pH adjusted 7.5 with phosphoric acid).

Detection wavelength: (For vinblastin sulphate) 297 nm.

Mobile phase: (For vincristin sulphate) Mixture of 50 volume of methanol 38 volume of 1.5 percent w/v of diethylamine (pH adjusted 7.5 with phosphoric acid) 12 volumes of acetonitrile. Detection wavelength: (For vincristin sulphate) 262 nm. [29]

- Thin Layer Chromatography of Vincristine: Vincristine dissolved in 25% water in methanol solution, spotted in Silica gel-G plate and developed using the solvent, acetonitrile: benzene (30:70). The dried plates are sprayed with 1% solution of ceric ammonium sulphate in 85% phosphoric acid. The Rf value of the appeared spot would be 0.39.

5. CLINICAL PHARMACOLOGY

Vinblastine is a drug used in the elective regime for the metastatic treatment of testicular cancer. The estimates of half-life after vinblastine administration to patients were 4 min, 1.6 h, and 25 h, which indicates a faster drug distribution in most tissues and a subsequent slower terminal elimination process. Distribution and initial cleaning phase for vincristine are kinetically comparable to the ones observed for vinblastine; half-lives for those phases have been reported at 4 min and 2.3 h in studies with vincristine. The terminal elimination phase for vincristine is reported to be three to four times longer than the one estimated for vinblastine, and the slow elimination of vincristine from the neuronal susceptible tissue suggest that it plays a role in neurotoxicity commonly seen in clinical adjustments with vincristine but not with vinblastine [30]. Hepatic metabolism and bile excretion play major roles in the elimination of both vinblastine and vincristine in humans [31]; small quantities of vincristine and vinblastine, in the order of 10% of the administered dose, are excreted with no alterations through urine. The renal clearance of vinblastine is reported as being less than 10% of the total elimination of the serum. It has been reported that vinblastine inhibits a polymorphic cytochrome P-450 in human hepatic microsomes, but the necessary concentrations were higher than those observed in clinical adjustments. It is recommended that vinblastine and vincristine doses must be reduced in patients with liver disease. Vincristine is conventionally administered intravenously, in adults, with a dose of 1.4 mg/m^2, the total dose must not exceed 2 mg in each administration. Sulkes and Collins have commented on the adjustments that can be provided for conventional doses of vincristine and other drugs. Of particular importance is the possibility that some patients can show a good clinical response and relatively low toxicity in dose regimes involving the cautious use of large quantities of vincristine. The initial dose of vinblastine for adults is 3.7 mg/m^2, with a range of the typical growing dose of 5.5–7.4 mg/m^2, administered weekly [32, 33].

6. APPLICATIONS OF VINCA ALKALOIDS

Vinca alkaloids are used in chemotherapy for cancer. They are a class of cell cycle–specific cytotoxic drugs that work by inhibiting the ability of cancer cells to divide: Acting upon tubulin, they prevent it from forming into microtubules, a necessary component for cellular division.[33] The vinca alkaloids thus prevent microtubule polymerization, as opposed to the mechanism of action of taxanes.

The vinblastin sulphate is highly used in the treatment of neoplasm, lymphocytic lymphoma, hodgkin's disease, testicular carcinoma. Vinblastin can show its potency in its individual form but generally it is dispensed in combination of other drugs to improve its therapeutic efficacy. It is dispensed through intravenous route by considering other factors like body surface, patient

age, WBC count etc. Vincristine sulphate is also available in ampoule and stored in refrigerator to improve its stability. It is also useful in lymphosarcoma, small cell lung cancer, neuroblastoma, hodgkin's disease, cervical and breast cancer. These alkaloids show antimitotic activity which inhibits cell growth. They disrupt the microtubules which causes the dissolution of cell mitotic spindle and the growth of cell arrest in metaphase.

Vinca alkaloids are now produced synthetically and used as drugs in cancer therapy and as immunosuppressive drugs. These compounds include vinblastine, vincristine, vindesine, and vinorelbine. Additional researched vinca alkaloids include vincaminol, vineridine, and vinburnine.

Minor vinca alkaloids include minovincine, methoxyminovincine, minovincinine, vincadifformine, desoxyvincaminol, and vincamajine. Vinpocetine is a semi-synthetic derivative of vincamine (sometimes described as "a synthetic ethyl ester of apovincamine") [34-38].

7. CONCLUSIONS

Catharanthus roseus is an important medicinal plant with several applications in pharmaceutical and industrial products. In the present, vinblastine and vincristine are two alkaloids for the treatment of childhood leukemia and Hodgkin lymphoma. Production rate of vinblastine and vincristine in *C. roseus* is very low, its extraction costly, and too inefficient to be industrialized. The semi synthesis also faces many obstacles because of the necessary presence of precursors and intermediaries. The great pharmacological importance of the terpenoid indole alkaloids vincristine and vinblastine, associated to its low content in plants (approximately 0.0005% of dry weight), in vitro tissue and cell cultures, will permit the stimulation of intense research regarding the biosynthesis pathways of terpenoid indole alkaloids yet unknown through in vitro culture studies under biotic or abiotic elicitation strategies with the objective of increasing the production of *C. roseus* alkaloids.

8. DISCLOSURE OF CONFLICT OF INTEREST

The author declares no conflict of interest.

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