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Chapter

Alkaloids of Pharmacological Importance in *Catharanthus roseus*

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Abstract

Catharanthus roseus is a plant of the *Apocynaceae* family. It produces over 120 alkaloids, 70 of which are pharmacologically active. *C. 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, this chapter deals with the pharmacological application of the *C. roseus* alkaloids.

Keywords: antileukemic, indole-monoterpene alkaloids, Letterer-Siwe disorder, vinblastine, vincristine

1. Introduction

Catharanthus roseus (L.) G. Don 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 [1]. The market of its leaves is monopolized by the United States and countries of Eastern Europe, like Hungary. In addition, attempts to obtain these alkaloids by *in vitro* tissue culture (cells in suspension) have not been very promising, since

there are many yet unknown enzymes involved in their biosynthesis. *C. roseus* alkaloids isolated from leaf, root, and flower can be analyzed through chemical, chromatographic, and spectroscopic analytical methods. It has been estimated that active alkaloid content in leaves is very low—2 tons of leaves are needed to isolate and purify 1 g of vincristine, the amount needed for the treatment of an infant during 6 weeks. Vinblastine and vincristine alkaloids are potent chemotherapeutics with anticancer activity [2–5], and they also have tumor inhibition properties for the treatment of leukemia [6], lymphosarcoma (cancer in the lymphogenous system), lymphogranulomatosis (cancer in cervical lymphatic ganglia) and other malign tumors. Vinblastine is used in the treatment of Hodgkin's disease (it has a ganglion onset and it extends initially through the lymphatic system and later through the blood) the diagnosis must be made when typical Reed-Sternberg cells are found [7–9]. Letterer-Siwe disease (the average age for this disease is 2 years; it is a generally acute and disseminated dermatosis, which is characterized by lesions simulating seborrhoeic dermatitis distributed in hairy skin, neck, and trunk. The presence of purple papules, pustules, vesicles, and petechiae, and also systemic signs that include fever, anemia, lymphadenopathies, osteolytic lesions, and hepatosplenomegaly, has been described) [10]. It is effective in the treatment of advance testicular tumors, breast carcinoma, choriocarcinoma (malign neoplasia originated from the gestational trophoblast, of great aggressivity when not treated at the right time [11]. Kaposi's sarcoma (mesenchymatous tumor with the involvement of blood and lymphatic vessels, originated by the human herpesvirus 8, also known as Kaposi's sarcoma-associated herpesvirus [12], while drugs with hypotensor effect are prepared with ajmalicine and reserpine [13, 14]. These alkaloids are produced and accumulated exclusively in *C. roseus* plants, and only in trace amounts, around 0.0005% of the dry weight, which makes their extraction hard and costly. According to Loyola-Vargas and colleagues, the process for extracting 1 kg of vinblastine costs 1 million dollars, while 3.5 million are needed to produce the same amount of vincristine [15]. The high cost of these substances is due to them being found in very low concentrations in the aerial part of the plant (around 0.0005% of dry weight); which is why half a ton of dry leaves of *C. roseus* are needed for the obtention of 1 g of vinblastine [16] while to produce 1 kg of vincristine 530 kg are used [17]. Besides, their extraction is very complicated since it is carried out in the presence of 200 molecules with similar chemical and physical properties. The low production of vinblastine and vincristine, the high value in the market, and their effectiveness in different medical treatments have fostered research to determine their biosynthesis and to develop alternate production methods [18]. The production of vinblastine and vincristine has recently been induced and studied in *in vitro* cultures of plant tissues through hormone combinations of auxins and cytokinins [1]. Plant tissue *in vitro* culture biotechnology is a successful tool for the productive generation of calli and cells that produce secondary metabolites of pharmaceutical and medical importance, such as the alkaloids vincristine and vinblastine from *Catharanthus roseus* [18]. The objectives of this chapter emphasize and point out the main pharmacological applications of the species *Catharanthus roseus*. Its phenotype, biological action mechanism, biosynthesis of terpenoid indole alkaloids, and alkaloid extraction, analysis and production in *in vitro* cultures of *C. roseus* are described.

2. Phenotypic characteristics of *Catharanthus roseus*

C. roseus is an annual herb, woody in its base and ramified, it measures 80 cm in height. It has well developed roots and it flowers all year long, which is why it is

used as an ornamental plant. Its leaves are opposite, oblong, with round apex, simple, whole, dark green color, shiny in the upper side, and of short petioles. Its branches can be erect or decumbent, and its relatively big flowers are axillary, solitary, of short peduncle and with five petals. Its fruit is a dehiscent follicle that contains numerous seeds (more than 20) of color black [19, 20]. There are several forms differentiated by flower coloration, probably due to genotypic variations, prevailing those of white color, white with red centre, red centre, dark rose (almost purple), white with disperse centre to violet rose or with dark centre bordered with red. This species of wild plant can tolerate many types of biotic and abiotic stress.

3. Mechanism of biological action

Vincristine and vinblastine are potent mitotic inhibitors used in leukemia chemotherapy; they are structures hard to synthesize chemically, like other cancer-fighting drugs such as taxol [21] thus biotechnological approaches represent the best route for its obtention. Vincristine binds to the tubulin β -subunit, the precursor protein of microtubules responsible of mitosis and other essential cellular functions like substrate transport, cellular mobility, and structural integrity, and it inhibits microtubule formation—this disruption causes cellular death and mitosis arrest [22].

4. Biosynthesis of terpenoid indole alkaloids of *Catharanthus roseus*

It has been shown that the biosynthesis of terpenoid indole alkaloids (TIA) in *C. roseus* is subject to strict control at the level of cells, tissues, and organs—in addition, it depends to a great extent on the own developmental stages of the plant and the surrounding environment. Several studies have dealt with the regulation of some of the genes coding for the enzymes involved in the synthesis of TIA and recently some of the molecular mechanisms controlling gene expression in cell suspension cultures of *C. roseus* have been elucidated [23]. The first step in TIA biosynthesis is the formation of tryptamine [24] from the L-tryptophane amino acid in a reaction catalyzed by the TDC enzyme. This cytosolic enzyme binds the primary metabolism with the secondary metabolism and its activity is considered as a limiting step, although not the only one, in the control of TIA biosynthesis [25]. Another limiting step in the biosynthesis is the tryptamine that binds to the secologanin monoterpene, the final product of the biosynthetic route of iridoids, in a reaction catalyzed by the STR1 enzyme. Tryptamine condensation with iridoid glucoside secologanin under the catalysis of the strictosidine synthase (STR) results in the formation of strictosidine, the central intermediary in the biosynthesis of all types of indole alkaloids [26]. Subsequently, strictosidine is metabolized through different enzymatic steps, including those catalyzed by D4H and DAT enzymes that lead to the formation of vindoline and catharanthine, the monoterpene alkaloids precursors to vinblastine and vincristine [27]. 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.

4.1 Vindoline formation

Strictosidine β -D-glucosidase (SGD) is the enzyme that performs an important role in guiding monoterpene indole alkaloids biosynthesis in a specific direction.

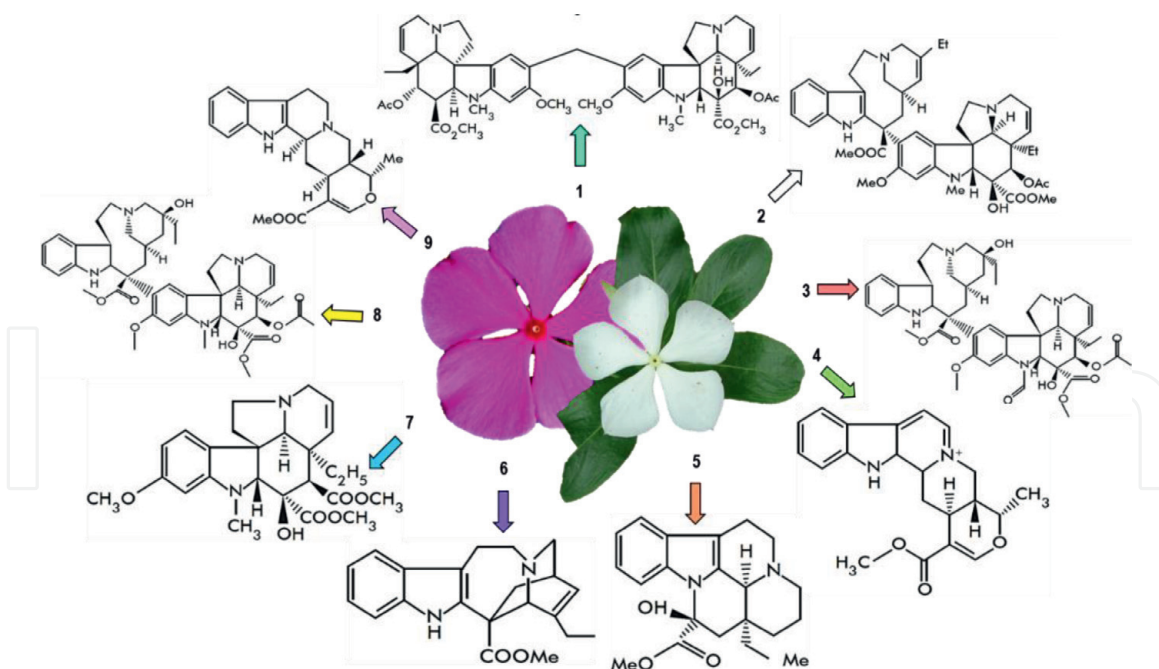


Figure 1.

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).

The elimination of the rest of the glucose of strictosidine by SGD leads to an unstable, highly reactive aglucone, that is believed to convert into 4,21 dehydrogeissoschizine. It is believed that the latter is converted into cathenamine by the cathenamine synthase. Cathenamine is then converted into tabersonine through several steps, transforming into vindoline by a six-step sequence [28].

4.2 Regulation of *tdc*, *str-1*, *d4h*, and *dat* genes in *Catharanthus roseus*

Gene and enzyme regulation participating in TIA biosynthesis in *C. roseus* depends on the biological system employed, i.e., it differs from cell cultures to plants and within these it depends on the tissue and developmental stages analyzed. In addition, it has been found that the molecular mechanisms of regulation respond differentially to the presence of elicitors or to conditions of light and hormone stress, among others [29].

4.3 *tdc* and *str1* genes

In *C. roseus* plants, high levels of mRNA for *tdc* and *str1* have been observed in roots and leaves, the latter induced by UV light. The *tdc* and *str1* transcripts and their respective proteins show high cellular and tissue specificity, for example, they have been detected exclusively in the superior and inferior epidermis of leaves, in stem epidermis, and flower buds [30].

In cell cultures of *C. roseus*, *tdc* and *str1* are highly regulated at the transcriptional level. The expression of these two genes is inhibited by the presence of auxins, although they are induced by elicitation with fungi, yeast extraction, methyl jasmonate (MeJa), salicylic acid, and chitosan. These results suggest that the expression of *str1* and *tdc* in cell cultures of *C. roseus* is regulated in a coordinated way by similar molecular mechanisms. The presence of some elements responding to elicitation in the promoter of the *tdc* gene has been determined. The architectural analysis of this promoter using *tdc*-*gusA* fusions in transgenic tobacco showed that

the region between the positions –538 and –112 is determinant to control expression levels in different plant organs [31]. In addition, three functional regions of this promoter were identified starting from the position –160. A region between positions –160 and –99 increased transcription, and two regions, one between –99 and –87, and the other one between –87 and –37, responded differently to elicitation. To determine the regulatory mechanisms of *str1*, progressive deletions in the 5' region of *str1* promoter were joined to the reporter gene 3-glucuronidase (*gusA*) and their studied activity in transgenic tobacco. The analysis of the promoter of the *str1* gene of *C. roseus* showed that the activator sequences are located between the positions –339 and –145. In other experiments, [32] showed that the biosynthetic route of jasmonate (octadecanoic acid pathway) was an integral part of the signal transduction pathway leading to the expression of *tdc* and *str1* genes in cells in suspension of *C. roseus*.

The expression of the promoter of the gene *str1* in transgenic cellular cultures of *C. roseus*, transformed with a construction containing the fusion *str1/gusA*, showed that a fraction of *str1* promoter located in the position –369 is sufficient to induce the expression of the *gusA* gene in response to MeJa. In a subsequent report, [33] identified an element of 42 base pairs (pb) within the 396 pb fragment—the Jasmonate and Elicitor Responsive Element (JERE). The JERE region showed a GCC sequence in the *str1* promoter that was necessary and sufficient for gene expression in the treatment with elicitors and jasmonate. Using the hybridization system of double hybrids and the JERE region as “bait”, two cDNA coding for ORCA proteins (Octadecanoic derivative Responsive Catharanthus AP2-domain) were isolated. The AP2 domain is found exclusively in plant transcription factors and is involved in the regulation of several stress responses. In cell suspension cultures of *C. roseus*, *str1* expression due to the jasmonate effect is mediated by the ORCA2 protein. Also, expression of the ORCA2 transcript was induced by elicitors, including yeast extract and MeJa. Recently, a new transcription factor (ORCA3) was discovered in cell suspension cultures of *C. roseus*. ORCA3 coordinately regulates multiple genes, including *dxs*, *tdc*, *str1*, *sgd*, *cpr*, and *d4h*, which code for pathway routes both of primary and secondary metabolism related to TIA formation [23].

4.4 *d4h* and *dat* genes

In *C. roseus* plantlings, *d4h* is induced by the presence of light and their transcription levels are increased after treatment with MeJa [34]. *In situ* hybridization and immunolocalization studies have showed that *d4h* and its protein are located in specialized cells (laticifers and idioblasts) shown in young leaves, stems and flower buds of *C. roseus* [30]. Both the transcripts of *dat* gene and its protein are co-located with *d4h* and D4H in laticifers and idioblasts [30]. In addition, studies with intact plants and with plantlings have shown that the induction of the mRNA of *dat*, and DAT activity and accumulation occur preferentially in leaves and cotyledons of etiolated plants treated with light, but they are not present in roots nor in cell suspension cultures, which explains the impossibility of producing vinblastine and vincristine in cell systems.

5. Pharmacological application of *Catharanthus roseus* alkaloids

5.1 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 [35]. Hepatic metabolism and bile excretion play major roles in the elimination of both vinblastine and vincristine in humans [36]; 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 [37]. 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 [38]. 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 [39]. 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 $5.5\text{--}7.4 \text{ mg/m}^2$, administered weekly [37, 38].

5.2 Antidiabetic activity

Considering the traditional use against diabetes, *C. roseus* was included in a research program in Canada, with the objective of finding insulin substitutes. Nevertheless, although no extract derived from the plant showed sensitivity in that regard, an occasional observation in blood indicated that some leaf-derived extracts accumulated alkaloids, sensibly decreasing the number of white globules, granulocytes in particular. This finding motivated scientists to carry out *in vitro* studies with leukemia cells, which lead to the isolation of vinblastine and vincristine in the 60s, among the more than 70 alkaloids of indolic nature that this plant produces. Later, Svodoba in Lilly successfully performed assays in rats with P-1534 leukemia, finding that the tumor was sensitive to these extracts. *Catharanthus roseus* (L.) G. Don is a plant traditionally used by populations in India, South Africa, China, and Malaysia to treat diabetes. Most of the reports on the antidiabetic activity of this plant have been made using crude extracts [39–42].

Soon et al. [43] found that the dichloromethane leaf extract of *Catharanthus roseus* (L.) G. Don showed antidiabetic activity, with an increase in glucose uptake in pancreas (β -TC6) and myoblastic cells (C_2C_{12}). Four alkaloids—vindoline I, vindolidine II, vindolicine III and vindolinine IV—were isolated and identified from the dichloromethane leaf extract of this plant. The dichloromethane extract and the compounds I–III were not cytotoxic in the pancreatic β -TC6 cells under the highest dose ($25.0 \text{ }\mu\text{g/mL}$). The four alkaloids induced a relatively high glucose uptake in pancreatic β -TC6 cells or myoblast C_2C_{12} , being III the one that showed the highest activity [43, 46, 66].

5.3 Antileukemic activity

Vincristine is employed to treat lymphocytic acute leukemia (the most frequent malign hemopathy in childhood), of which several chromosomic alterations with prognostic importance are known. Among them there are the translocation (4;11) and the translocation (9;22), which are indicators of a bad prognosis, while hyperdiploidy is associated with a good prognosis [44] and it attacks lymphomas including solid tumors in children.

5.4 Antioxidant enzymatic activity

An experiment with different concentrations of sodium chloride in two varieties of *Catharanthus* (var. *alba* and *rosea*) was carried out. It was found that the enzymatic activity of the superoxide dismutase increased at levels of 50 mM of sodium chloride, which helps to raise the levels of this enzyme with antioxidant value [45].

5.5 Antiviral activity

Ozcelik et al. [46] showed the antiviral effect of *Catharanthus* in the simplex herpes virus (type I) with a cytopathogenicity effect at 0.8 µg/mL. Catharoseumine, a monoterpene indole alkaloid, has a unique peroxy bridge, which was identified as a potential inhibitor against falcipain-2 protozoa parasites (causes of malaria), showing an IC₅₀ value of 4.06 µM. Vinblastine and vincristine showed an antiparasitic effect against *Trypanosoma*, that causes trypanosomiasis in humans, inhibiting its mitosis and affecting its cellular shape in a dose-dependent manner. The use of 15 µM of vinblastine and 50 µM of vincristine inhibited cellular division and cytokinesis, and affected cellular morphology, while the effect of 3 µM of vinblastine and 10 µM of vincristine inhibited cytokinesis without affecting cell cycle progression [46].

5.6 Hypoglycemic activity

It was shown in several animal studies, that ethanolic leaf and flowers extracts decreased the levels of glucose in blood. Hypoglycemic effects are a result of increasing the use of glucose in liver [47]. The aqueous extract decreased glucose in blood in approximately 20% of diabetic rats, compared to methane and dichloromethane extracts in which glucose in blood decreased 49–58% [48]. In the present there are new alkaloids that have been studied in the *Catharanthus* plant, for example, vindogentianine, an hypoglycemic metabolite extracted from leaves, showed hypoglycemic activity in β-TC₆ and C₂C₁₂ cells by induction of a higher glucose consumption and a significant *in vitro* inhibition, suggesting that the hypoglycemic activity of vindogentianine is due to the increase in glucose consumption and PTP-1B-type inhibition, which can be a potential therapeutical agent against typ. 2 diabetes [49].

5.7 Antidiarrhoeic *in vivo* activity

The antidiarrhoeic *in vivo* activity of ethanolic leaf extracts was tested in Wistar rats with beaver oil as an experimental diarrhea inductor agent. Loperamide and atropine were used as standard drugs. The antidiarrhoeic effect of the ethanolic extract showed a dose-dependent inhibition of the beaver oil, inducing diarrhea at doses of 200 and 500 mg/kg [50].

5.8 Antimicrobial activity

The antimicrobial activity of leaf extracts was tested against microorganisms such as *Pseudomonas*, *Salmonella*, *Staphylococcus* and thus, these extracts show promissory effects as prophylactic agents in the treatment of many diseases. Ramya et al. [53] evaluated the *in vitro* antibacterial activity through the use of crude extracts of *Catharanthus* [51].

The results indicated that leaf extracts showed a higher antibacterial activity than the extracts prepared from other parts of the *Catharanthus* plant. Thus, the aqueous extracts of leaves, stems, roots and flowers showed low microorganism growth [52] tested leaf extracts of *Catharanthus* var. *rosea*, which showed an excellent activity against *Aspergillus*. Stem extracts of var. *alba* showed a maximum inhibitory activity against *Bacillus* while the flowers of *Catharanthus* var. *rosea* showed a higher activity against *Bacillus* in the methanolic extract. The MIC (Minimal Inhibitory Concentration) against the tested microorganisms was in the range of 100–20 mg/mL. In a different study, foliar acetonetic, ethanolic, and chloroformic extracts were tested against pathogenic microorganisms to determine its antimicrobial potential. The ethanolic extract showed the maximum antibacterial activity when compared to the acetonetic and chloroformic extracts, in such a way that *Staphylococcus* was the most susceptible bacteria, followed by *Escherichia*, *Pseudomonas* and *Streptococcus* [53].

5.9 Antineoplastic effect

Catharanthus plants contain a series of dimeric indole alkaloids with significant antitumor activities. It has been found that these alkaloids have an *in vitro* and *in vivo* apoptosis-inductive activity against tumor cells, mediated by the nuclear factor kappa potentiator of B activated cells, and by the c-Jun N-terminal kinase pathways, in which DNA damage and mitochondrial dysfunction play important roles. The nuclear factor kappa B was discovered approximately 20 years ago, as a protein that binds to the enhancer of the κ light chain of immunoglobulins in B cells. It belongs to the family of NF- κ B transcription factors, which is ubiquitous and participates in the immune and inflammatory response, during tumor development, formation, progression, and apoptosis [54]. The c-Jun N-terminal kinases, originally identified as kinases that bind to and phosphorylate c-Jun protein in the Ser63 and Ser73 residues in their transcriptional activation domain are mitogen-activated kinases responding to stress stimuli, such as cytokines, UV radiation, thermal shock, and osmotic shock, and are involved both in T lymphocyte differentiation processes, and in apoptosis processes. Different percentages of the crude methanolic extracts have been found to show significant anticancer activity against several cell types under *in vitro* conditions and with a high activity against multidrug-resistant tumor types. On the other hand, Ruskin and Aruna showed that the ethanolic extract of *Catharanthus* has *in vivo* antitumor activity in the Ehrlich carcinoma tumor model, while the *in vitro* study of the ethanolic extract showed significant antitumor activity [55].

6. Extraction and analysis 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 [56].

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 [57] 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 [58]. 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. 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 [59].

7. Generalities of the *in vitro* culture

The general process of the *in vitro* culture consists in inoculation on a gelified culture media (generally with agar Gelrite or Phytigel[®]) with a fragment of plant tissue or an organ, called explant, previously treated to eliminate all the organisms found in its surface (disinfestation). The culture is incubated under controlled environmental conditions of light, temperature, and humidity, that together with the physiochemical and nutritional conditions lead to the development of the explant towards the formation of an amorphous cell mass called callus, or towards the differentiation in an organized tissue that will produce organs or embryos. Organ cultures can be re-differentiated into complete plants (micropropagation) that can be later transferred to a greenhouse, a phase known as acclimation. Culture temperature generally is controlled between 25 and 28°C, the pH between 5.2 and 6.5, and the light from 0 to 12,000 lux. Several studies have researched the effects of pH on cell growth and metabolite production in cell suspension cultures [60].

In addition, several studies modify the pH of the culture media to increase the release of secondary metabolites, and few studies have been carried out to examine the effects of buffers in plant culture growth or in secondary metabolism biosynthesis pathways. Several authors have studied the effect of buffers in *in vitro* cultures of *Catharanthus* roots, to quantify the content of serpentine, ajmalicine, tabersonine, lochnericine, and horhammericine. The main alkaloids serpentine, lochnericine, and horhammericine from *C. roseus* are shown in **Figure 2**.

Light is a very important factor in the *in vitro* production of secondary metabolites of *Catharanthus*. In that regard, other authors showed that temperature has an important influence on cell suspension culture growth and ajmalicine production. The optimal temperature for both biomass growth and secondary metabolite

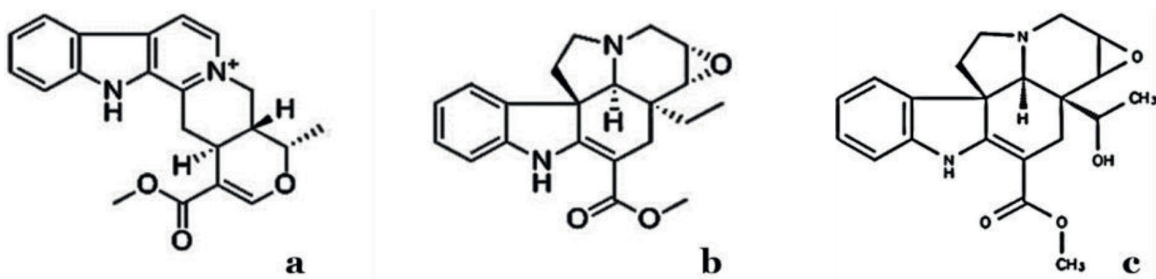


Figure 2.

Alkaloids produced by *C. roseus* (1) serpentine ($C_{21}H_{21}N_2O_3$, 349.4 g/mol); (2) lochnericine ($C_{21}H_{24}N_2O_3$, 352.43 g/mol) and (3) horhammericine ($C_{21}H_{24}N_2O_4$, 368.42 g/mol).

production is 27.5°C. Young or developing plants with meristematic tissues and vigorous vegetal growth are the best source of explants. Although both juvenile and adult growth can be found in the same plant, the former is characterized by its activity and by the absence of reproductive structures, while the adult growth is slower and presents sexual structures for plant reproduction. The disinfection of the tissue to be used as a source of explants is made with disinfectant agents like sodium or calcium hypochlorite. The penetration of the disinfectant agent in rugged or hairy surfaces of the plant tissue can increase with the addition of tensioactive agents such as Tween 20. Activated carbon or citric acid are used as antioxidants [61]. In addition, the *in vitro* cultures allow us to know the production of secondary metabolites. In this respect, the sources from which the different *Catharanthus* alkaloids have been isolated *in vitro* are diverse; for example, the alkaloid ajmalicine has been extracted through the analysis from calli, cell suspensions, sprouts, and pilose roots; alstonine from *in vitro* calli; antirrhine in cell suspensions; cathindine in suspensions; serpentine in calli, suspensions, sprouts, and pilose roots; acoumicine in calli, suspensions, and sprouts; lochnericine in calli, suspensions, and pilose roots; horhammericine in suspensions and sprouts; tabersonine in calli and suspensions; vindoline in suspensions and sprouts; catharine in suspensions, sprouts, and roots; 3,4 anhydrovinblastine in sprouts; catharine in sprouts; vinblastine in calli, sprouts, and somatic embryos; and vincristine in sprouts and embryos, among others [61].

7.1 *In vitro* culture of alkaloid production of *Catharanthus roseus*

It stands out that among the major advantages of plant cell and tissue cultures in basic research, of micropropagation and production of compounds with biologic activity such as secondary metabolites, proteins, and transgenic products, they allow studies in a shorter time and under more controlled conditions than the ones used in traditional methods. A callus is defined as a group of dedifferentiated friable cells growing in a solid medium and it is the initial material for the establishment and growth of suspension cells. The obtained calli can be subcultured for its maintenance and propagation or induced into differentiation to form organs (organogenesis), embryos (embryogenesis) or to be transferred into a liquid culture medium to obtain cells and small aggregates in suspension. The *in vitro* culture of plant cells in a liquid medium for cell suspensions is a potential source of substance of interest for the pharmaceutical industry, showing all the advantages inherent to biotechnological processes. The advantages offered by cell culture, specifically cell suspension culture, is that it allows a similar handling to the one performed with microorganisms, a fast cell multiplication (duplication time), and it is possible to carry out a scaling into new techniques such as bioreactors and temporal immersion systems. However, not all compounds are produced in undifferentiated cells in

the same quantity and quality than the ones obtained from mother cells. This is due to many metabolites being synthesized integrally to differentiation events. Several authors have pointed out the identification of cell lines that can produce metabolites in the same amount, or higher, than in natural conditions. New substances have also been detected, which are not synthesized by the plants in their natural environment, which is why it is asserted that cell lines culture constitutes a highly relevant biotechnology for the obtention of new secondary metabolites. *In vitro* cell suspension cultures are kept under the same physical and physicochemical conditions used for calli induction.

There are different *in vitro* culture techniques of the medicinal *C. roseus* plant, which provides a range of important secondary metabolites of pharmacological application, such as the antileukemic alkaloids vinblastine and vincristine, useful in leukemia treatment [62]. Specifically, in cell suspension cultures of *Catharanthus*, all the terpenoid indole alkaloids derivate from an intermediary like strictosidine, including serpentine, catharanthine, ajmalicine, tabersonine, vincristine, and vinblastine [63]. Once the cell culture has been established, a continuous process of epigenetic or genetic changes is observed, which causes the population to be heterogeneous. This creates the necessity of selecting clones with a high growth rate and with a high production of metabolites of interest. Cell lines are obtained through the selection of several strategies, including microscopic (cell viability, for example, with fluorescein diacetate), macroscopic, and enzymatic tests. The aspects associated to secondary metabolites accumulation are the presence of certain cell types, organelles, biosynthetic, or catabolic gene expression and regulation. Thus, organ culture represents an interesting alternative to the production of plant secondary metabolites. Two types of organs are considered to be of greater importance: sprouts and roots, which can be cultivated at a large scale. Organ culture can produce substances of interest that have been obtained through undifferentiated cultures. However, sprout cultures cannot produce all the compounds that are obtained in plant leaves under natural conditions. If the compound of interest is synthesized in roots, then it will not appear in sprouts cultures. On the other hand, it is necessary to take into account that, even though the compound is synthesized in leaves, it is possible that its patterns and concentration are different to the ones obtained from intact plants. As a major advantage, it is pointed out that organ cultures is more genetically stable compared with cell suspension and calli cultures [64].

Several *in vitro* culture techniques such as adventitious meristems or organ propagation, cell and tissue cultures, provide a large amount of *Catharanthus* material for the isolation of mono and dimeric indole type alkaloids with multi-therapeutic properties. Several studies have shown that *Catharanthus* is regenerated generally through somatic organogenesis by the induction of friable calli. In addition, *in vitro* cultures of multiple sprouts can be directly induced. Vindoline is an important alkaloid in *in vitro* cultures of *Catharanthus* sprouts. Some authors obtained 2 mg g^{-1} of dry weight after 27 days of culture [65]. Roots synthesize, accumulate, and secrete a large variety of secondary metabolites, in addition to providing mechanical support and allowing water and soil nutrients uptake. It is known that biosynthetic activity in roots is also maintained in *in vitro* cultures. *In vitro* cultures of *Catharanthus* with fast growth have been established in Murashige & Skoog (MS) medium. Several studies mention that *in vitro* cultures are able to synthesize metabolites through root production. These cultures could be a biotechnological alternative of alkaloid production for future research.

Mekky et al. [66] cultured leaves of *C. roseus* in Murashige & Skoog medium supplemented with 1.5 mg/L of BAP and 1.5 mg/L of 2,4-D. The callus obtained was subcultured in 15 different combinations of growth hormones during 28 days.

Alkaloids were extracted from the calli and different treatments were analyzed with HPLC in regard to the capacity of vincristine and vinblastine production compared to the wild plant. Biomass was maximized with combinations of the growth hormone 2,4-D/NAA and IAA/NAA but alkaloid biosynthesis was reduced to the minimum. Vincristine production was potentiated in almost all growth hormone combinations with Kin/IAA, and they produced the highest concentration. However, vinblastine was potentiated in the combinations of growth hormones Kin/IAA, IAA/Gb, BAP/Gb and NAA/Gb only; with Kin/IAA being the one that showed the highest concentration of vinblastine. The main motivating result was the biosynthesis of dimeric anticancer alkaloid essence, vincristine was barely detected in the wild plant and vinblastine, that showed an increase of 3.39-fold compared with the wild plant. In addition, there is a growing demand for these two alkaloids [66].

8. Conclusions

C. 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 semisynthesis 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.

Acknowledgements

This work was supported by the “Programa para el Desarrollo Profesional Docente” PRODEP-Mexico of the project 2017-2018 of Hebert Jair Barrales-Cureño PhD of the Universidad Intercultural del Estado de Puebla.

Conflict of interest

All contributing authors declare no conflict of interest.

List of abbreviations

BAP	6-Benzylaminopurine
CAS	Cerium Ammonium Sulfate
Gb	Gibberellin
HPLC	High-Performance Liquid Chromatography
IAA	Indole-3-acetic acid
KIN	Kinetin
NAA	1-Naphthaleneacetic acid
TIA	Terpenoid indole alkaloids

TLC Thin Layer Chromatography
VLC Vacuum Liquid Chromatography
2,4-D 2,4-Dichlorophenoxyacetic acid

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