Pharmacological applications and in vitro biotechnological production of anticancer alkaloids of Catharanthus roseus

REVIEW

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ABSTRACT

Catharanthus roseus (periwinkle) is a plant of the Apocynaceae family. This plant synthesizes two indole terpene alkaloids: vinblastine and vincristine, used against cancer. *C. roseus* is endemic to Madagascar and is known as vicaria in Cuba; in Mexico, it is found in Veracruz, Tabasco, Quintana Roo and Yucatan. *C. roseus* is of considerable interest for *in vivo* or *in vitro* studies, and from which over 130 alkaloids of the indole terpene group have been isolated. The total alkaloid content in roots is 2-3 %, 9 % in the fibers, the leaves contain 1 % alkaloids, the stem, fruit, seeds, and pericarp containing 0.48, 0.40, 0.18 and 1.14 %, respectively. About 500 kg of dried leaves are used to isolate 1 g of vinblastine and two tons of macerated leaves provide 1 g of the active principle required for the treatment of a child with leukemia for 6 weeks. Semi-synthesis and organic synthesis are costly and low yielding, so alternatives have been used to enhance biotechnological production by adding inducers to stimulate the production of metabolites in the biosynthetic pathway. Therefore, the objectives of this paper are: to mention the biological mechanism of action, biological activity, biosynthetic pathway of the vinblastine and vincristine alkaloids, report on the production of alkaloids from in vitro callus cultures and cells in suspension. Finally, the methods for the quantification of terpene indole alkaloids are highlighted.

Keywords: terpene indole alkaloids, mechanism of action, anti-leukemia drugs, vinblastine, vincristine, *in vitro culture*

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RESUMEN

Aplicaciones farmacológicas y producción biotecnológica in vitro de los alcaloides anticancerígenos de *Catharanthus roseus*. *Catharanthus roseus* es una planta que pertenece a la familia Apocynaceae. Esta planta sintetiza dos alcaloides indol-terpénicos cuyo uso es relevante para el tratamiento contra el cáncer: vinblastina y vincristina. C. roseus es endémica de Madagascar y se conoce como vicaria en Cuba; en México, se distribuye en Veracruz, Tabasco, Quintana Roo y Yucatán. Es de gran interés para estudios *in vivo* o *in vitro*, y de ella se han aislado más de 130 alcaloides del grupo indol-terpénico. Su contenido total de alcaloides en raíces es de 2-3 % (9 % en sus fibras), las hojas contienen 1 % de alcaloides, el tallo, fruto, semillas, pericarpio contiene 0.48; 0.40; 0.18 y 1.14 %, respectivamente. Se utilizan cerca de 500 kg de hojas secas para aislar 1 g de vinblastina y dos toneladas de hojas maceradas proporcionan 1 g del principio activo, cantidad requerida para el tratamiento de un niño con leucemia durante 6 semanas. La semisíntesis y la síntesis orgánica son costosas y de baja producción, por lo que se han utilizado alternativas biotecnológicas para incrementar la producción mediante la adición de inductores que estimulan la producción de los metabolitos en su ruta de biosíntesis. En este trabajo se abordan el mecanismo de acción y las actividades biológicas de esta planta, la ruta de biosíntesis de los alcaloides vinblastina y vincristina, y la producción de alcaloides a partir de cultivos in vitro de callos y células en suspensión. Finalmente, se destacan los métodos de cuantificación de los alcaloides indol-terpénicos.

Palabras clave: alcaloides indol-terpénicos, mecanismo de acción, fármacos antileucémicos, vinblastina, vincristina, cultivo *in vitro*

*I*ntroduction

Catharanthus roseus L. (G.) Don., is an important medicinal plant belonging to the Apocynaceae family; this plant is a dicotyledonous angiosperm and synthesizes two terpene indole alkaloids: vinblastine and vincristine that are used to fight cancer [1]. The plant species *C. roseus* is endemic to Madagascar and is commonly known in Cuba as vicaria; it is found in Mexico in the States of Veracruz, Tabasco, Quintana Roo and Yucatán [2] as well as in El Salvador [3]. This plant is also found in other tropical and subtropical regions such as: South Africa, Southern Asia, South America and Australia where it is used as an ornamental plant; it grows well in India, Israel, Sri-Lanka, Mozambique [4] and Egypt [1]. In Cuba, it is frequently grown by the population in yards and gardens as an ornamental and medicinal plant [5].

The *C. roseus* species is of great interest for *in vivo* or *in vitro* studies from which more than 130 alkaloids of the terpene indole group and 25 alkaloids of dimeric nature have been isolated [6-8]; several of them are used in human medicine. All parts of the plant contain more than 100 alkaloids in variable proportions. The total alkaloid content of roots reach 2-3 % or nearly 9 % in root fibers, while the leaves contain 1 % alkaloids, the stem, fruit, seeds, and pericarp contain 0.48, 0.40, 0.18 and 1.14 %, respectively [9].

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This plant produces anti-cancer dimeric alkaloids such as vinblastine and vincristine in concentrations of 0.0004 to 0.0003 % of the dry weight of leaves and stores anti-hypertensive alkaloids such as ajmalicine and serpentine in the roots. Certain chemical constituents are vinblastine, vindoline, catharantine, ajmalicine and serpentine [8]; the species are also rich in bisindole alkaloids (aproximately 40 compounds), many of which contain a molecule of vindoline or catharantine [8]. The market for its leaves is monopolized by the United States, and by a country in Eastern Europe: Hungary. The main demand for leaves is by the United States, and the United Kingdom is also interested in buying 10 tons of leaves each year and Germany shows great interest in the roots of this plant [3].

The biosynthetic route for indole alkaloids has been studied by De-Luca and Cutler [10]. Because of the presence of cytotoxic bis-indole alkaloids of therapeutic importance, the production of vinblastine, vincristine and vindesine has become one of the main fields of interest in modern cell biotechnology [11]. The plant produces the active dimeric alkaloids in low concentrations (0.0005 %), where nearly 500 kg of dry leaves of *C. roseus* are used to isolate 1 g of vinblastine [8] and 2 tons of macerated leaves produce 1g of the alkaloid as the active principle, which is the amount required for the treatment of a child with leukemia for 6 weeks [9]. Because of the large number of alkaloids it contains, the isolation of vinblastine and vincristine in the laboratory is very costly.

Although all parts of the plant produce alkaloids (leaves and stems) in different proportions [12], the maximum concentrations are found in the cortex of the roots, particularly when blooming [13]. A wide array of different alkaloid sub-classes have been identified: vincosan, corynanthean, vallesiachotaman, strychnan, aspidospermatan, plumeran, ibogan, eburnan and bisindole alkaloids [14]. Up to 40 different bisindole alkaloids have been found in *C. roseus*, many of which contain a moiety of plumerane (vindoline) and ibogane (catharantine). In relation to plant chemistry, *C. roseus* contains carbohydrates, flavonoids, saponins, phenol compounds, terpene indole alkaloids [15], antocyanines, glucosides [16], heart glycosides, steroids, mono-terpene glucosides [8]. It is also has no tannins. Two flavonols have also been isolated and identified [17] as well as glycosidic flavonols that have been identified in seeds, stems, leaves and flowers of *C. roseus* [18]. The extracts of the sprouts of *C. roseus* are used as a potential source of natural available antioxidants and with excellent pharmaceutical applications [19].

The biological mechanism of action of the vinblastine and vincristine consists of the binding of the tubulin during mitosis. These compounds inhibit the chromatin filaments drawn to their respective poles [20], leading to the inhibition of cellular mitosis during the metaphase, and thereby starting the programmed cellular death or apoptosis [21]. Vincristine inhibits polymerization of the microtubules, producing an arrest in phase G2/M and inducing apoptosis [22].

Because the semi-synthesis of vincristine and vinblastine, starting with the precursors and the organic synthesis, is highly expensive and the production is poor, and also the extraction of the metabolites are complicated by the low concentrations of the pharmaceuticals in *C. roseus*, alternative biotechnology strategies have been used to be able to increase the production of these secondary metabolites. They include the addition of biotic or abiotic inducers that stimulate the production of the metabolites in the biosynthesis pathway of the alkaloids. Therefore, the aims of this paper are: to mention the biological mechanism of action, biological activities and the biosynthetic pathways for the vinblastine and vincristine alkaloids, to inform on the production of alkaloids through *in vitro* cultures of calluses and cells in suspension, and finally on the quantification methods of the terpene indole alkaloids.

On the relevance of C. *roseus* alkaloids for anticancer therapy

Particularly, the use of *C. roseus* as source of anticancer drugs has become relevant due to the high morbidity and mortality rates of certain types of cancers, which are sensitive to treatment with compounds like vinblastine and vincristine. This is the case of acute myeloid leukemia [23] and Hodgkin's lymphoma.

To have an idea of the possible impact of these therapies, there have been reported 350 434 cases of leukemia in the world, with a standard incidence rate both in underdeveloped and developed countries of 5.0 per 100 000 inhabitants [24], accounting for approximately 210 000 cases in the last ones. The highest rates have been found in the United States, Canada, Europe and Australia; with the lowest in African countries [25]. Moreover, acute myeloid leukemia has a peak incidence in the group of children of less than five years of age, descending in the group of 5 to 9 years of age and as of that age it increases exponentially with age [26]. This makes the availability of such compounds more urgent for the scientific and medical community.

In relation to Hodgkin's Lymphoma, it has an incidence of 3 cases per 100 000 yearly, and comprises 10 % of the lymphomas in the United States, of which 85 % are found in males, with a bimodal incidence curve: 15 to 34 years of age and after 50 years of age. Estimates of the American Cancer Association in 2010 established that there were 8490 cases of which 4670 were men, with 1320 deaths [27]. In the European Union, the incidence is of 2.2 per 100 000/year, with mortality rates of 0.7 per 100 000/year [28]. Noteworthy, during the last decade, the survival of patients treated for Hodgkin's Lymphoma substantially improved, and the percentage of cure for this neoplasia is of 80 to 85 % [29].

Particularly in Mexico, until 2003 there were 935 cases reported, with a higher incidence in the group of males of 15 to 19 years of age and females had the same incidence in the groups of 15 to 19 and 20 to 24 years of age [24]. In fact, according to the INCan, the Mexican National Institute of Cancer, until 2004 it represented 0.8 % of the lymphomas, with 162 cases diagnosed, of which 88 were men and 74 were women [30, 31].

All these have fostered the search for new compounds and the development of efficient production processes for its obtainment, using *C. roseus* as

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biological source for alkaloids displaying therapeutic activity relevant for cancer treatment.

Mechanisms of biological action of the alkaloids of C. *roseus*

The cytotoxic alkaloids of *C. roseus* (vinblastine, vincristine and more recently, vinorelbine) are anti-mitotic, anti-cancer agents inducing tubuline to form spiral polymers at physiological protein concentrations [32] thus interfering in the formation of micro-tubules.

Vinblastine is an analogous chemical compound of vincristine (Figure). It binds to the tubulin and thus interferes in the assembly of the microtubules [33].

During mitosis metaphase, the pharmaceutical inhibits polymerization of the tubulin to microtubules, in contrast to the anti-cancer drug, taxol, which has the function of stabilizing the microtubules, reducing their dynamism by later impeding depolymerization and promoting the arrest of the mitosis, and therefore producing, cell death [34].

The vinblastine works specifically during the metaphase stage of the cell cycle, since the microtubules are components of the mitotic spindle, and the kinetochores that are necessary for the separation of the chromosomes during the anaphase in mitosis. Vincristine enters the cell through a transportation mechanism and it binds to the tubular proteins.

The prescribed dosages of vinblastine range from 3.7 to 18.5 mg/kg with an interval of at least seven days between each dose; having a half clearance life of 25 hours and 95 % of it is excreted in the feces and less than 1 % is eliminated in the urine without metabolization [23].

Vincristine is mainly metabolized in the liver. Its main metabolite is the diacetylvinblastine, with a greater biological activity [35]. It also interferes with the nucleic acid and protein synthesis by blocking the use of glutamic acid. Other effects in the DNA and RNA syntheses were recently described, also in the inhibition of the proteosome [36], anti-angiogenesis [37] and the decrease of the resistance of the cells to chemotherapy [38].

Furthermore, the vincristine sulfate is used to treat Wilm's tumor, neuroblastoma, breast cancer, rabdomyosarcoma and osteogenic sarcoma.

Uses of C. *roseus* in traditional medicine

Traditional medicine uses the alkaloids from *C. roseus* for different non-malignant diseases: in Africa the leaves are used for menorrhagia and rheumatism [39].

C. roseus has been used to fight diabetes for many years; it was proven by the induction of diabetes in rats that it had hypoglycemic activity [40], on having an anti-diabetic effect as the result of the increase of glucose and the promotion of insulin production [41, 42].

In India, wasp bites are treated with the sap from the leaves, and in Brazil it is used against diabetes, in the treatment of hemorrhages and wound healing. In Hawaii an extract of the boiled plant has been prescribed to stop bleeding. In Central and South America, they use it to relieve throat pain and laryngitis. In Cuba, Puerto Rico, Jamaica and other islands, the aqueous extract of the white flower is commonly used as an eye wash for infants and as fomentation for easing eye diseases [39, 43].

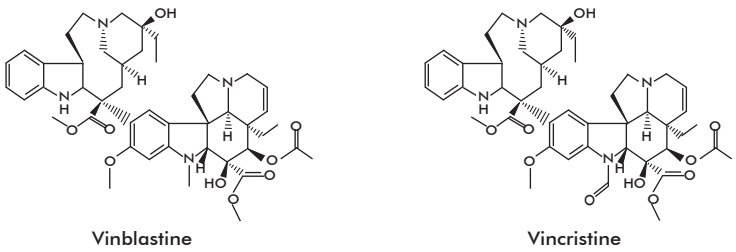

Figure. Structure representation of vinblastine and vincristine, cytotoxic alkaloids found in *Catharanthus roseus* and used in anticancer therapy.

In the Bahamas they use the infusion of flowers for asthma, and of the entire plant to fight tuberculosis. In Mauritius, the infusion of leaves are used to combat dyspepsia and indigestion. In Vietnam, it is used for malaria and diabetes; and the inhabitants of Bermuda and Curacao use the plant when they have high blood pressure [44].

It has been also used for treating fever, malaria, menstrual cycle regulation, as a euphoric drug [45], as tranquilizer, and for its ability to reduce arterial blood pressure [42].

The plant is also known for its anti-spasmodic properties because of the presence of alkaloids such as ajmalicine, serpentine and reserpine. The alkaloids: vincamine and vindoline are anti-ulcer compounds. In France, the hot water extract of the whole plant is used as an anti-galactogogue [46].

Finally, *C. roseus* shows anti-spasmodic properties because of its reserpine and serpentine contents. Furthermore, it shows high anti-plasmodial activity *in vitro* because of the presence of terpenoids, flavonoids and sesquiterpenes [47]. *C. roseus* should not be consumed orally without cooking because it may be hallucinogenic [47, 48].

Uses of C. *roseus* in allopathic medicine

The anti-tumor alkaloids vinblastine and vincristine are used in malignant diseases; they are used in chemotherapy for leukemia since they reduce the number of leukocytes in the blood (a high number of leukocytes indicate leukemia) and in the treatment of Hodgkin's disease [13] characterized by being a monoclonal B cell neoplasia, and by the presence of abnormal cells called Reed-Sternberg cells [49].

Vinblastine (vinblastine sulfate) is experimentally used for neoplasia treatment and for resistant pregnancy choriocarcinoma, a malignant neoplasia of the trophoblast, which is a highly aggressive and fetal lesion, since even when there is a timely diagnosis and it is appropriately treated with chemotherapy, it produces mortality in 10 to 15 % of the cases [50].

It is also effective in the treatment of advanced testicular tumors, breast cancer, Kaposi sarcoma, and the Letterer-Siwe disease [51]. Vincristine, formally known as leurocristine (vincristine sulfate) is used in leukemia treatment in children.

Vincristine is produced by the bonds of the terpene indole alkaloids: vindoline and catharantine in the *C. roseus* plant [52]. The use of the vinblastine and vincristine combined with chemotherapy has given 80 % remission in Hodgkin's disease, 99 % in acute lymphocytic leukemia, 80 % in Wilm's tumor in children,

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70 % in pregnancy corium cancer and the remission of 50 % in Burkitt's lymphoma [39, 43]. The indole alkaloid called amotin also has a strong anti-leukemia activity [1].

At the neurologic level, the ajmalicine and serpentine are drugs used in treating depression, anxiety, as well as being effective as anti-stress drugs [53, 54]. The supplements based on active ingredients of *C. roseus* such as vincamine, are used for the prevention and treatment of cerebro-vascular disorders and failures, vertigo, ischemic deficiencies and headaches, because they help oxygenate and increase brain glucose levels [55]; besides preventing abnormal clotting, they also increase the levels of serotonin, a brain neurotransmitter. It is important to mention that the deficiency of serotonin produce schizophrenia, phobia, migraine and bulimia.

On the other hand, vincamine is now known to increase the memory retention properties and it is effective in the treatment of vascular dementia [56]. Anhydrous vinblastine is used in the treatment of lung and cervix cancer [57]. The catharantine isolated from *C. roseus* is cytotoxic in P-388 and KB human cancer cell lines [58]. Furthermore, ajmalicine is used in the treatment of circulatory disorders and as an antihypertensive since it acts as an antagonist of the α1-adrenergic receptor, known as alpha blocker [59], with a preferential action on α2-adrenergic receptors [60].

Biological activities of the C. *roseus* alkaloids

The main secondary metabolites of *C. roseus* are terpene indole alkaloids with important applications in human medicine as mentioned above, and also presenting biological activities such as: anti-tumor, anti-diabetes, anti-helminthic, anti-hypertensive, anti-diarrhea, and anti-microbial actions, among others. The clinical evidence of the biological activities of *C. roseus* is presented below.

Anti-tumoral activity

Cancer is a genetic pathology characterized by the uncontrolled proliferation of a certain group of cells in the body. If the control regulating cell multiplication does not work well, the cells start growing and dividing unnecessarily, and when the young cells inherit the trend to proliferate without any control, the result is a clone that expands indefinitely forming a tumor.

These tumors may be benign, or they may become malignant if they are able to invade and disseminate throughout the body, in a process known as metastasis [61].

Different percentages of crude methanol extracts of Catharanthus have shown anti-cancer activity against many types of cells under *in vitro* conditions [62] and a greater activity is especially found against multidrug resistant tumors [63].

As early as in 1979, ethanol extract from leaves (70 %) was found as very active in CA-Ehrlich ascitis when administered intra-peritoneally in female mice [64]. The alkaloid fraction of the dry leaves is also active at surface level. Nineteen patients with genital, flat and common warts were treated; of these, all warts disappeared in six patients, almost completely in seven patients, 50 % of the warts were eliminated in five patients and one patient did not show any response to the chloroform extract [65].

The chloroform extract of leaves was active in Leuk-P3887 [66]. The total amount of alkaloids of the whole plant were administered to mice by the intraperitoneal route at a dose of 10 mg/kg and by the oral route at 75 mg/kg, both concentrations found to be active in Leuk-P15348.

Anti-helminthic activity

The infections produced by helminths are chronic diseases affecting human beings. *C. roseus* is used as an anti-helminthic agent in medicine.

Agarwal *et al*. evaluated the anti-helminthic property of *C. roseus* through the use of *Pherethima posthuma* as the experimental model with piperazine citrate as the standard reference. The ethanol extract, at a concentration of 250 mg/mL showed significant anti-helminthic activity at 46.3 min, while the standard drug showed activity at a concentration of 50 mg/mL at 40.7 min. This ethno-medical research considers *C. roseus* as an effective anti-helminthic drug [67].

Anti-diarrheal activity *in vivo*

The *in vivo* anti-diarrheal activity of the ethanol extract of leaves has been tested in Wistar rats, using castor oil as the experimental diarrhea inducing agent, besides the pre-treatment of the extract. Loperamide and the atropine sulfate were used as the standard drug. The anti-diarrhea effect of the ethanol extract of *C. roseus* showed the dose dependent inhibition of the diarrhea inducing castor oil at a dose of between 200 and 500 mg/kg [68].

Anti-oxidant activity

Kumar *et al*., 2012 studied the anti-oxidant activity of *C. roseus*. These researchers found that the temperature of the habitat had a specific effect on the antioxidant activity, which would make it possible to see that the super oxide dismutase and polyphenol oxidase enzymes had a greater antioxidant activity when the temperature was increased in contrast to the catalase [69].

The methanol extract of the leaf was used to direct the study. Rasool *et al*., in 2011 carried out several *in vitr*o tests of antioxidants to study the effect of the solvent on the extraction and total anti-radical potential of the different extracts of *C. roseus*. The trials showed that the extracts and the fractions are a good source of natural antioxidants. The 100 % methanol extract and the fraction of 100 % ethyl acetate of the shoots of *C. roseus* showed high antioxidant activity [70].

Antimicrobial activity

Muhammad *et al*. reported the anti-bacterial potential in crude extracts of different parts (leaves, stalks, roots and flowers) of *C. roseus* against clinically relevant bacterial strains [71]. The antimicrobial activity of extracts of leaves of *C. roseus* was tested against microorganisms such as *Pseudomonas aeruginosa* NCIM 2036, *Salmonella typhimurium* NCIM 2501 and *Staphylococcus aureus* NCIM 5021 and it was found that the crude extract may be used as a prophylactic agent in the treatment of many diseases [72].

Kumari and Gupta evaluated the potential of *C. roseus* against several pathogenic germs under *in vitro*

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conditions, at the dose of 50 mg/mL of the extract of *C. roseus* var. rosea which was effective against *Bacillus fusiformis*, while at the dose of 20 mg/mL of the extract it was selective against *Aspergillus fumigatus*, *Candida albicans*, *Escherichia coli*, *Bacillus fusiformis* in two varieties of *C. roseus*: Rosea and Alba [42].

The ethanol extract of flowers of *C. roseus* is reported to have healing properties in rats [73]. The extract promotes contraction of wounds, increases the content of hydroxyproline and the anti-bacterial activity against *P. aeruginosa* and *S. aureus*.

Furthermore, the ethanol extract of leaves, stems, flowers and root extracts of *C. roseus* have a wide scope of anti-bacterial activity against *E. coli*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Salmonella typhi* and *Aeromonas hydrophila* [71]. In a similar study, the extracts of different parts of the plant were reported to inhibit both the gram-positive and gram-negative bacteria [74].

Cytotoxic activity

The alkaloid fraction of dry leaves in a cell culture was active in CA-9KB, the median effective dose (ED50) at 0.0435 µg/mL [75]. The chloroform extract and filtrate of the *in vitro* culture of calluses, at the dose of 50 mg (dry weight of the plant) was active on the aqueous extract of the culture Leuk-L12. The methanol extracts of the leaves of *C. roseus* produce positive anti-proliferative activity against HeLa human cancer cells (a HeLa cell is a cell type classified in an immortal cellular line used in scientific research. Its growth is aggressive, and its resistance to apoptosis is mainly due to a combination of papilloma virus 18 that produces a protein that degrades p53 without mutating it, and several alterations in chromosomes 1, 3, 5 and 6), MCF-7 (it is an epithelial cancer cell line that has been widely used, and it is derived from breast adenocarcinoma) with values of the concentration showing 50 % of maximum inhibition of cell proliferation (GI50) of 3.5 ± 0.1 and 4.7 ± 0.6 µg/mL, respectively [76].

Anti-hyperglycemic effect

The daily oral administration of the extract of dichloromethane:methanol (1:1) has been evaluated by using the leaves of *C. roseus* (500 mg/kg of body weight) for 20 days and its effect was tested in blood glucose and normal liver enzymes in diabetic rats [77]. The extract showed a significant increase in the body weight and a decrease in glucose, urea and cholesterol levels of the treated animals. The activity of the liver enzymes such as hexokinase, was increased while that of glucose-6 phosphatase and fructose 1,6-biphosphatase decreased significantly.

Anti-diabetic effect

Several studies in animals have shown that the ethanol extracts of leaves and flowers of *C. roseus* decrease the levels of glucose in the blood [78, 79]. Furthermore, the aqueous extracts have the capacity to decrease the blood glucose in 20 % in trials with diabetic rats, while the reductions of the levels of glucose in the blood with dichloromethane and methanol extracts are of 49 and 58 %, respectively [80].

On the other hand, the vindolicine alkaloid demonstrated a potent inhibition activity in PTP-1B, which is due to the effect of vindolicine as a new inhibitor of PTP-1B, which can serve as a "sensitizer of insulin" in the management of type 2 diabetes. Paranitrophenyl phosphate (pNPP) was used as a substrate for the trial on phosphatase activity (the recombinant enzyme PTP-1B) and it was added at the start of the reaction [81].

The main structural skeleton of the terpene indole alkaloids together with its biological activity are shown in table 1.

General biosynthesis of the alkaloids of C. *roseus*

The secondary metabolic route of the indole alkaloids in *C. roseus* has been documented [82, 83] and it is very complex. The biosynthesis route of the indole alkaloids has been studied by De-Luca and Cutler [10]. The tryptophan (precursor of the indoles) turns into a tryptamine, and later it is condensed with secologanin (a precursor of the iridoids) to form strictosidine, the common precursor of all indole alkaloids, divided into three branches of several enzymatic reactions which leads to the production of ajmalicine, tabersonine and catharanthine, respectively. The transformation of vindoline, a downstream enzymatic intermediary product of tabersonine, into the downstream processing pathway of catharantine followed by several enzymatic reactions generates vinblastine, which is finally transformed in a single reaction to vincristine. Notably, the production of terpene indole alkaloids is strongly regulated by environmental conditions and the cell growth stage [84, 85].

Production of alkaloids in *in vitro* cultures of C. *roseus* calluses

The *in vitro* culture technique of tissues and cells is considered to be an effective tool for the production of secondary metabolites. The *in vitro* culture of plant cells, whether this is done in a solid medium with calluses or in a liquid medium with cell suspensions, is a potential source of substances of interest for the pharmaceutical industry [86].

The *in vitro* cultures of calluses are obtained by explants of leaves of different plant species [87] and by obtaining *in vitro* cultures of viable cells in suspension that are obtained through the inclusion of friable calluses (with disintegration capacity) in liquid culture media.

Current research in relation to obtaining *in vitro* cultures of calluses and cells from C. *roseus* is presented below. Fatemeh and Abdolreza [88], obtained the maximum induction of calluses as well as a high percentage of fresh and dry weights of calluses obtained from explants of roots with 1.5 m/L of naphthalene acetic acid (NAA) and 0.1 mg/L from kinetin and 2 g/L of glutamic acid under conditions of darkness. Haq *et al*. [89] implemented an efficient protocol of the micropropagation through explants, from nodal portions, shoots and callus-genesis by means of explants of leaves and knots [89]. There were multiple outgrowths obtained from several concentrations of benzyl amino purine (BAP) and NAA, but BAP (1 mg/L) showed the best responses (90 and 80 %)

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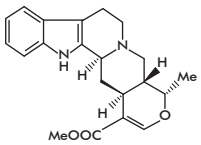
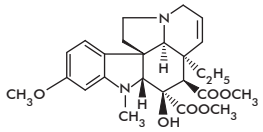
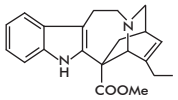
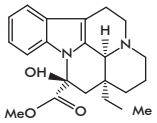
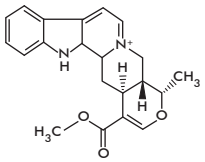
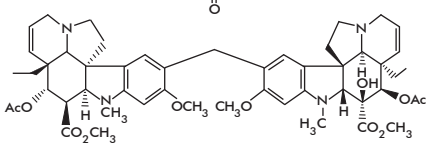
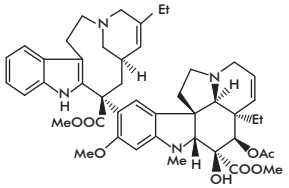
Table 1. Structural determinants of the main indole terpen alkaloids of *Catharanthus roseus*

Secondary metabolite/ molecular formula	Molecular mass (g/mol)	Chemical structure	Biological activity [source]
Ajmalicine $C_{21}H_{24}N_2O_3$	352.43		Antispasmodic properties, depresion treatment, anti-stress effects [53]
Vindoline $C_{25}H_{32}N_2O_6$	456.53138		Anti-ulcerative properties [53]
Catharanthine $C_{21}H_{24}N_2O_2 \cdot H_2O$	336.42746		Cytotoxic action on the HCT-116 colorectal carcinoma cell line [44]
Vincamine $C_{21}H_{26}N_2O_3$	354.44274		Prevention and treatment of cerebral disorders and insuficiencies [55]
Serpentine $C_{21}H_{22}N_2O_3$	349.40304		Anti-hypertensive, anti-spasmodic properties, anxiety treatment [54]
Vindolicine $C_{51}H_{64}N_4O_{12}$	925.07346		'Insulin sensitizer' in diabetes type 2 management [81]
Anhydrovinblastine $C_{46}H_{56}N_4O_8$	795.977325		Lung and cervico-uterine cancer treatment [81]

of both explants. The best response of calluses was observed in the Murashige-Skoog (MS) medium supplemented with 2, 4-D plus Kin (1 + 1 mg/L) in all explants (the result of obtaining 95 % leaves, 80 % nodes) and the plantelets obtained were transferred to different pots under greenhouse conditions for their adaptation.

Soleimani *et al*. [12] indicated that the callus cultures with applications of 2,4-D have a significant effect on the percentage of callus-genesis and on the amounts of vinblastine and vincristine alkaloids [12], but the kinetin in high concentrations produces a considerable decrease in the percentage of callus and alkaloid production [12]. The maximum concentration of vincristine (0.7088 µg/g of dry weight) was produced with 1.0 mg/L of the 2,4-dichlorophenoxi acetic acid (2,4-D plus 0.5 mg/L of kinetin and the best treatment for the maximum production of vinblastine (0.7088 µg/g of dry weight) was obtained with 1.0 mg/L of 2,4-D without kinetin. Verma *et al*. [90] obtained an early induction of calluses (99 %) on the tenth day through the use of explants of hypocotyl under conditions of darkness in the MS medium, where the best one was that containing: benzyl aminopurine (BAP 1.5 mg/L) plus NAA (1.0 mg/L), followed by the MS medium fortified with BAP (3.0 mg/L) plus NAA (4.0 mg/L). The MS medium with half the content of macro- and micro-nutrients supplemented with indole butyric acid (IBA; 2.5 mg/L) plus NAA (0.5 mg/L) showed the best response in rooting with high quality roots [90].

Verma *et al*. [91] analyzed combinations of auxins and cytokinins to find the best callus growth and increase the alkaloid content. The highest content of total alkaloids found in the production of the biomass of calluses was through the leaves having the concentrations of 0.50 mg/L 2,4-D and 1.0 mg/L BAP and 6 % sucrose. Negi [92] suggested a simple protocol to obtain a good production of calluses from the nodal explants of *C. roseus*: an MS culture medium supplemented with a combination of 1.0 mg/L kinetin and a combination of 1.0 mg/L BAP; 1 mg/L 2,4-D and 1 mg/L

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IAA [92]. Similarly, MS culture media supplemented with a combination of 2 mg/L kinetin and BAP; MS plus 2, 4-D, IAA and 0.5 mg/L showed green calluses and resin secretion. However, the MS culture media plus 2 mg/L of BAP and 1 mg/L of 2, 4-D produced better calluses of a green color with resin secretion. Furthermore, the leaves explants growing in the MS medium plus 1.0 mg/L of BAP and 1.0 mg/L of NAA showed a large number of roots. When combining in the MS medium, 0.1 mg/L of kinetin and 1.0 mg/L of 2,4-D; and MS plus 0.1 mg/L BAP and 1 mg/L IAA, a simple process for the induction of *C. roseus* calluses was observed.

On the other hand, Kalidass *et al*. [93], established *in vitro* cultures of calluses of *C. roseus* in MS media supplemented with 1.0 µM of 2,4-D and 1.0 µM of 6-furfurylaminopurin to grow callus cultures, and they obtained 598.04 mg of dry biomass to quantify the content of vincristine by HPLC through the use of methanol extracts. The concentration of 0.5 µM of benzyl adenine (BA) produced 0.57 mg/g dry weight of vincristine while the concentration of 1.0 µM NAA and 0.5 µM BA produced 20.38 mg/g of dry weight of vincristine. Ataei-Azimi *et al*. studied the production of indole alkaloids in *in vitro* cultures of calluses and roots. The alkaloids came from calluses and roots of petioles of *C. roseus* in the presence of 0.1, 5, 10 and 20 mg/L of kinetin and NAA [15]. The MS medium with 0.1 mg/L NAA plus 0.1 mg/L of kinetin showed the highest production of vindoline, catharantine, vincristine and the capacity of the organogenesis of roots, but the levels of those alkaloids and of ajmalicine were low in comparison with the petioles of the whole plant and the levels of serpentine were similar. Those results suggest that the capacity to synthesize vinblastine and vincristine, vindoline and catharantine are associated to morphologic differentiation, light and high temperature (35 ºC).

New roots, callus roots and calluses from the MS medium were obtained with 0.1 mg/L of NAA plus 0.1 mg/L of kinetin and were sub-cultured in a hormone free medium with the same hormonal concentrations for their growth and organogenesis. The best treatment for the formation of calluses was with 2 mg/L BAP plus 2 mg/L kinetin and 0.1 mg/L indole acetic acid (IAA) [12]. Ferderique and Leslie [94] reported that cytokinins have a greater accumulation effect than the indole alkaloids such as ajmalicine and serpentine in cultures of calluses of *C. roseus* taken from cotyledons.

Previous studies show that adding the precursors of the biosynthesis of alkaloids may be a production strategy for the secondary metabolites. The treatment of tryptophan on calluses of *C. roseus* can increase the content of catharantine up to 950.536 µg/g of dry weight [95]. Generally, the plants with poor callus growth produce more catharantine than those of greater growth. From such a study it was found that the optimum growth was observed with the treatment of 175 mg/L of the tryptophan precursor.

Finally, some alkaloids that have been recently discovered and isolated from *in vitro* cultures of calluses of *C. roseus*: akuammicine; cavincine; tubotaiwine; catharantine; tabersonine; perivine; 21-hydroxy cyclolochnerine; lochneridine; alstonine; serpentine; vallesiachotamine; isovallesiachotamine; ajmalicine; 19-epi,3-iso ajmalicine; 3-epi ajmalicine; O-diacetyl akuammiline; tetrahidroalstonine; sitsirikine; yohimbine; dihydro-sitsirikine,; 7-hydroxiindolenine ajmalicine; pseudo-indoxil ajmalicine; 10-hydroxydeacetyl akuammiline; mitraphilline; strichtosidine; vinblastine and two previously unidentified compounds: cavincidine and perosine [8].

Production of alkaloids in *in vitro* cell cultures of C. *roseus*

The *in vitro* suspension cell cultures of *C. roseus* are used to study the structural modifications of several synthetic and natural products [96] such as oxidation, hydroxylation, reduction, isomerization, esterification and glycosylation [97], as well as obtaining the maximum production of secondary metabolites of medical importance. *C. roseus* calluses and cells have been studied for their biotransformation capacity [98]. The increase of the accumulation of ajmalicine in 1040 ± 26.6 mg/L in *in vitro* in suspension cell cultures of *C. roseus* through the use of cyclodextrins and methyl jasmonate together with the exposure to UV light [99] was evidenced. Catharantine was obtained from *in vitro* cell cultures, but it has been very difficult to reach a stable accumulation with significant amounts of the vindoline metabolite [100].

On the other hand, the interaction between the cytokinins and ethylene in the accumulation of alkaloids in *in vitro* cultures has been investigated and a higher increase in the accumulation of ajmalicine in cells sub-cultivated in the free medium of 2,4-D was obtained [101].

The cells that were specialized and differentiated with the addition of precursors can produce more secondary metabolites than the *in vivo* cultures [102]. Although cells submitted to differentiation show slower growth than those that are not differentiated, some studies demonstrated that their morphology may have an influence; for example, it has been established that the cylindrical (*i.e.*, elongated) cells of *C. roseus* contain a higher concentration of alkaloids than the round cells [103]. The inclusion of the tryptophan precursor may increase the content of catharantine [104] and it may also increase the number of elongated or cylindrical cells.

As far as we know, the way in which the content of catharantine is increased because of the tryptophan treatment and the release of other secondary metabolites on the same culture or the culture of cellular aggregates of *C. roseus*, remains to be well characterized [104].

Therefore, *in vitro* suspended cell cultures of *C. roseus* are considered as an excellent biotechnological tool for the *in vitro* and *in viv*o analyses of the biosynthetic pathway for the formation of anti-tumor alkaloids. With the aid of this tool, the relevant enzymes in the biosynthesis pathway were identified, but other studies are required to get into further detail at relevant enzymatic steps, even for the production of the anti-tumor drug taxol [105, 106]. For a comprehensive list of compounds isolated from these systems, also including the previously unidentified catindine and cavincidine compounds see [8].

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Elicitation of anti-cancer alkaloids of C. *roseus* in *in vitro* cultures

Some new, recently discovered secondary metabolites of *C. roseus* have been obtained and their biological function established (Table 2).

Semi-synthesis and the organic synthesis of vincristine and vinblastine are highly costly and of low production yields, and their extraction from *C. roseus* is complicated, due to the low concentrations of these compounds within the plant. Therefore, alternative biotechnological strategies have been used to increase their levels as secondary metabolites, by adding biotic and abiotic inducers that stimulate the production of the metabolites in the alkaloids biosynthesis pathway (Table 3).

For example, the inclusion of pectinase increases 2.5 times the levels of tabersonine; the inclusion of chitin makes the levels of ajmalicine rise in 50 %, and the addition of jasmonic acid increases the levels of lochnericine and horthammericine, but not those of tabersonine [121].

Additionally, alkaloids have been detected in the pollen of *C. roseus*, mainly vincristine [122], which may provide a breakthrough for the pharmaceutical industry. Furthermore, phytochemical ingredients were identified, such as: total proteins, free amino acids, among others. The steroids and total pollen proteins were extracted by spectrophotometry, yielding 35.56 %. Free amino acids analysis detected 17 amino acids identified by the TLC method [122].

It has been demonstrated that the biosynthesis of the terpene indole alkaloids in *C. roseus* is tightly controlled at the cellular, tissue and organ levels [84], depending greatly on the phenology (the plant's own biological state of development) and on the surrounding environment (biotic, abiotic and epigenetic factors).

On the other hand, it is important to remark that in feedback studies, it was found that high concentrations of the intermediaries or precursors of metabolites are able to generate greater accumulation of alkaloids in the biosynthesis pathway. But some experiments reveal that the presence of high concentrations of intermediary metabolites does not stimulate biosynthesis. In fact, the high content of metabolites, such as tryptamine and geraniol in culture media *in vitro* leads to cellular toxicity. This is attributed to the lack of differentiation of tissues, the direct exposure to metabolites or the high permeability of the membrane, but the most important aspect that is not considered is the lack of storage capacity of the synthesized tissue [84].

The production of alkaloids by *in vitro* cell culture may be considered as a differentiation process which is ruled by the environmental conditions (culture medium, phyto-hormones, light, temperature, precursors). The accumulation of alkaloids is a complex phenomenon, which is not totally disconnected to cell growth, but it is difficult to connect it with the appearance or disappearance of some metabolites or substances in the medium [84].

The production of alkaloids and the accumulation of biomass *in vitro* are directly influenced by the pH units of the medium; values with a range of 5.5 to 6.5 do not have much effect on the production of alkaloids. The value of 5.5 has been optimum for the production of serpentine [123].

Table 2. New metabolites obtained from *in vitro* culture of *Catharanthus roseus* cells

Metabolite	Function	Type of culture	Reference
Phosphatidyl kinase	Phospholipid metabolic enzyme	Plasmatic membranes of *C. roseus* cell suspension cultures	[107]
Trichosetin	Antibiotic	Dual cultures of *Trichoderma harzianum* and *C. roseus* calluses	[108]
Phytic acid	Phosphorus storage, mRNA cellular export, chromatin remodeling	Cell suspension cultures	[109]

Table 3. Biotechnological approaches for the increased production of terpenoid indole alkaloids by *Catharanthus roseus* (Apocyanaceae)

Method	Metabolite	Levels	Reference
Cultures of biofilms (6 mm thick)	Alkaloids	0.18 %	[110]
Immobilized cells	Serpentine	300 μg/mL	[111]
Root cultures *in vitro*	Alkaloids	2-3 times increase	[112]
Suspension cell cultures by adding loganin and tryptamine	Alkaloids	350 mM	[113]
Suspension cell cultures supplemented with: betain, n-propyl gallate, tetramethyl ammonium bromide, linoleic acid, arachidonic acid, succinic acid, malic acid	Ajmalicine Serpentine Catharanthine	23-63.6 mg/L 16-32 mg/L 8.5 times increase 24 mg/L	[11]
Suspension cell cultures supplemented with 10 mM sodium nitroprusside	Total catharanthine	40.3 mg/L	[114]
C. roseus plants treated with gibberellic acidat 1 kg/m^3	Total alkaloids	3.44 %	[115]
C. roseus plants treated with mycorrhizal arbuscular fungi (MAF), alone or combined with P_2O_5 at 200 kg/ha	Ajmalicine	1.22 ± 0.66 mg/ g of plant 1.68 ± 0.44 mg/ g of plant	[9]
Suspension cell cultures irradiated with UV	Catharanthine Vindoline	0.12 ± 0.0054 mg/ g of plant (d.w.) 0.06 ± 0.0023 mg/ g of plant (d.w.)	[116]
In vitro hairy roots culture by metabolic engineering	Total alkaloids	9.51 mg/g of plant (d.w.)	[117]
In vitro culture of calluses	Vincristine	20.38 mg/g	[93]
In vitro hairy roots culture with the activation or repression of the CrWRKY1 transcription factor	Serpentine Ajmalicine Catharanthine Tabersonine	291.5 ± 73.2 μg/ g d.w. 15.4 ± 1.6 μg/ g d.w. 100.2 ± 15.1 μg/ g d.w. 19.3 ± 1.6 μg/ g d.w.	[118]
Co-culture in medium 1/2 MS supplemented with 100 μM acetosyringone	Vindoline	1.42-2.72 μg/ mg d.w.	[63]
In vitro hairy roots co-culture with *A. rhizogenes* A4 in B5 medium	Catharanthine	0.17 mg/g f.w.	[119]
Suspension cell cultures supplemented with regulation elicitors and inhibitors	Tabersonine Vindoline Vinblastine	9.02 mg/g f.w. 0.42 mg/g f.w. 0.81 mg/g f.w.	[120]

d.w.: dry weight; f.w.: fresh weight

It has been reported that the alkaloids produced in *in vitro* cultures of cells in suspension are stored in the vacuole and the storage capacity simultaneously changes when pH varies in the medium and the vacuole. The maximum and minimum units of pH

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were used by Asada and Shuler [124], to release the alkaloids within the cells in the culture medium. The optimum value (5.5-5.8) sometimes varies during the culture period and it influences the *in vitro* response on the production of terpene indole alkaloids [124].

Methods of qualitative and quantitative identification of the content of terpene indole alkaloids in C. *roseus*

In the following are present someof the most common methods which make the quantification of indole alkaloids secondary metabolism and their products in *C. roseus* feasible. Vinblastine and vincristine have been isolated in a pure form by 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 radial chromatography accelerated by centrifugation (Cromatotron). Semi-quantitatively procedures have been also implemented by using thin layer chromatography (TLC) methods [125]. TLC is highly sensitive to the detection of alkaloids; the ajmalicina is detected at 0.0007 % in a volume of 10 µL. The vincristine at 0.055 % in a volume of 10 µL while vinblastine and vindoline are not sensitive to this method because they are both found at concentrations of 0.05 % in a volume of 10 µL [126]. The chromogenic reagent that is chromatographically used in the detection of alkaloids is the cerium ammonium sulphate (CAS) that is known to react with the analyte to produce visible colors in the TLC plaque [15].

Other methods involve the analysis of the indole alkaloids by High Performance Liquid Chromatography (HPLC) [85]. The equipment for this method comprises a self-sampler able to analyze multiple samples. The separation of the indole alkaloids is based on the reverse phase chromatography using C18 columns as the steady phase. The mobile phases usually consist of a mixture of buffer solutions such as n-heptanesulfonic acid, diammonium phosphate, or ammonium acetate supplemented with triethylamine and an organic phase (methanol or acetonitrile). The detection is carried out using a fixed wave UV detector or a fluorescent detector.

Additionally, the interaction of the growth hormones on the regulation of the indole alkaloids has also been extensively studied [13], as well as their extraction using super-critical fluids, which has been one of the most efficient quantification techniques used. Moreover, an understanding of the regulation of the metabolic flows is obtained with the metabolic flow analysis (MFA) [127], which requires the determination of the rates of the biosynthetic reaction. Nevertheless, a large number of measurements are required.

On the other hand, *C. roseus* has been phytochemically analyzed, and it has been found that it contains: carbohydrates, flavonoids, saponins, phenolic compounds, indole alkaloids, terpenoides [15], antocyanins (rosindin, 3-O-glucosides and 3-O-(6-O-p-coumaroyl) hirsutidin, malvidin and petunidin glucosides [16]. It lacks tannins, cardiac glycosides, steroids (catasterone, brasinolide) and monoterpene glucosides (loganin, secologanin, sweroside, dioxyl and dihydro-loganin) [8]. Two trisaccharide flavonoles of kaempferol and quercetin were also isolated [17, 60]; 15 glycosidic flavonoles were identified in seeds, stems, leaves and flowers [18], with other alkaloids been identified: 4'-deoxy-vinblastine, leurosine, pleurosine, leurocristine, leurosidine, vincoline, vincarodine, roseadine, vindolicine, rosicine, 5'-oxo-leurosine, N'b-oxide leurosidine and pericyclivine [6].

Conclusions

C. roseus has been a research icon because of the large number of phytochemical compounds, secondary metabolites and the therapeutic effects they produce. The secondary metabolites of *C. roseus* are terpene indole alkaloids with pharmacologic activity and with several applications in human medicine. The plant has a wide variety of properties: anti-cancer, anti-diabetes, anti-helminthic, anti-hypertensive, anti-diarrheic, anti-microbial, among others. The indole dimeric alkaloids: vinblastine and vincristine have become valuable drugs in cancer chemotherapy because of their potent anti-tumor activity against several types of leukemia and solid tumors. Remarkle examples are vinblastine, which is formed by catharantine and vindoline and catharantine, which is a member of the iboga family of the indole alkaloids.

Because of the use of plants and *in vitro* cell cultures, the biosynthesis pathway has been determined, but not completely clarified. Furthermore, a considerable number of enzymes have been characterized and their respective cloned genes defined, with the production of the alkaloids been found as highly regulated at the transcriptional level.

In order to increase the availability of alkaloids for therapeutic use, the production of biomass from *in vitro* cultures of calluses from leaves has arisen as biotechnological tool to increase the accumulation of alkaloids in *C. roseus*. The combination of different basal media as carbon sources, phytohormones and inducers of the biotic and abiotic type may provide useful ways for the rational technical development and the increase in production yields of several of these bioactive molecules *in vitro*. The *in vitro* cultures of calluses or cells in suspension could be used at a large industrial scale to obtain bioactive compounds that are of great importance in human health, and are envisaged as models to circumvent the limitations of other production systems.

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