

# In Vitro Biotechnological Production and Pharmacological Studies of Antileukemic Alkaloids of *Catharanthus roseus*

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**Abstract** Different techniques of in vitro cultures of the medicinal plant *Catharanthus roseus* are available. In this regard, the plant is a source of important secondary metabolites that are compounds widely used in pharmacology. For instance, vinblastine and vincristine are alkaloids employed in the treatment of leukemia. This chapter discusses the techniques mostly used in the field of modern biotechnology, such as the in vitro culture of callus and suspension cells, as well as those related to organs, roots, and seedlings. Similarly, the chapter encompasses the types of explant cultures used, induction rates, and the culture environment, jointly with hormones and concentration employed. Also discussed is the level of production

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of each category of alkaloids according to the type of in vitro culture. Similarly, new metabolites obtained from suspension cell cultures of *Catharanthus roseus*, along with major pharmacological studies recently conducted, are contained in the chapter.

**Keywords** *Catharanthus roseus* • Callus culture • Root cultures • Cell suspension cultures • Antileukemic alkaloids

## 1 Introduction

Biotechnology of plant in vitro cultures represents a successful tool in the production of callus and cell cultures that have the capacity to produce secondary metabolites, compounds of great interest in the pharmaceutical and medical fields (Barrales-Cureño and Ramírez 2013). Alkaloids vincristine and vinblastine, elements produced from *Catharanthus roseus*, are a good example of this. Vinblastine and vincristine are potent mitotic inhibitors that are used in chemotherapy for leukemia. They are complex, chemically synthesized structures, similar to other drugs used in the fight against cancer, such as Taxol (Barrales-Cureño and Soto 2012; Barrales-Cureño et al. 2012, 2015, 2016). In that regard, biotechnological approaches represent the best way in obtaining these compounds.

Recently, the production of vinblastine and vincristine has been induced and research carried out as it has never been over the plant in vitro cultures by means of hormone combination of auxins and cytokinins (Villa-Ruano et al. 2011). Cell potency represents the basis of in vitro culture, a term defined as the potential capacity of a single plant cell to regenerate into a whole plant. Several in vitro techniques, such as micropropagation of adventitious meristems or organs, including tissues and cell cultures, provide a large amount of material of *Catharanthus roseus* that is used in the isolation of dimeric and indole mono-type alkaloids with multi-therapeutic properties. In this regard, research has demonstrated that *Catharanthus roseus* have the potential to regenerate through somatic organogenesis during the induction of friable calluses. Likewise, in vitro cultures of multiple shoots can be induced directly. The great pharmacological importance of terpene indole alkaloid, associated with low content in plants (approximately 0.0005% of dry weight), stimulates intensive research regarding metabolic routes of the alkaloids occurring in various studies over in vitro culture. These allow determining the concentrations that occur in in vitro callus and cell suspension cultures.

In this chapter, the importance of different types and conditions of in vitro cultures in the production of vinblastine and vincristine as antileukemic alkaloids is highlighted, as well as that of other related metabolites including the main medical applications of *Catharanthus roseus* species.

## 2 Overview of In Vitro Culture

Generally, the process of in vitro culture implies the inoculation of a medium gelled culture (using agar, gelrite, or phytagel®) and a piece of tissue or plant organ, known as explant, previously treated to remove any unwanted body present on the surface (disinfestation). The culture is incubated under controlled environmental conditions of light, temperature, and humidity, along with other physicochemical and nutritional conditions, in order to construct an amorphous cell mass called callus; or toward differentiation, in an organized, embryo and organ-producing tissue (Calva and Pérez 2005). Organ cultures redifferentiate to complete plants (micropropagation) which are then transferred to a greenhouse, a condition known as acclimatization phase. The temperature is usually controlled and set between 25 and 28 °C, while the pH is between 5.2 and the 6.5 and the light ranges from 0 to 12.000 lux (Calva and Pérez 2005).

Several studies have investigated the effects of pH on cell growth and metabolite production in suspension cells (Morgan et al. 2000). Some studies confirmed that changes of pH made on cultures increased the release of secondary metabolite. In addition, few studies were conducted in order to examine the effects that produce buffers over the growth of crops or the metabolic pathways during secondary metabolism. Similarly, several authors have studied the effect of buffers on in vitro root cultures of *Catharanthus roseus*, in order to quantify the content of serpentine, ajmalicine, abersonine, horhammericine, and lochericine (Morgan et al. 2000).

Light, as a parameter, plays a prominent role in the in vitro production of secondary metabolites of *Catharanthus roseus*. Other factors, such as temperature, also have a significant influence on the growth of suspension cell cultures and in the production of ajmalicine, as is indicated by certain authors (Ten Hoopen et al. 2002). The optimum temperature for both processes, the growth of biomass and the production of secondary metabolites was 27.5 °C. Young or developing plants with meristem tissues showing vigorous vegetative growth are the best source of explants. Even though, both juvenile and adult growth can be found in the same plant, the first one is characterized by being active and not having reproductive structures, while adult growth is usually slower and plants feature sexual structures for their reproduction. Disinfestation of tissue to be used as a source of explants is performed with disinfectant agents, such as sodium hypochlorite or calcium. Disinfectant agent penetration in rough or hairy surfaces of plant tissue can be increased with the addition of surfactants, such as Tween 20. Meanwhile, activated carbon or citric acid is used as antioxidants.

Phytohormones and their inhibitors are substances produced by plants. By controlling their response to environmental stimuli, such as light, temperature, and humidity, they help regulate and coordinate processes essential for the development of the plants. They consist of auxins, gibberellins, cytokinins, abscisic acid, ethylene, brassinosteroids, polyamines, salicylic acid, and jasmonic acid. In particular, auxins and gibberellins promote the elongation of cells while inhibiting differentiation. Meanwhile, cytokinins stimulate the division process by which new cells are produced, and can thus

avoid cellular aging. Ethylene stimulates fruit ripening mainly, while abscisic acid inhibits the action of auxin, gibberellins, and cytokinins, to serve as a natural defense system against physiological effects of stress. Cytokinins and auxins are compounds commonly used in plant cell cultures. In addition, the 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most widely used plant hormone in the induction and maintenance of callus tissue due to its property to suppress organogenesis rigorously. With regard to cytokinins, compound 2-Indolaminopurine (2iP) is the more active structure. Notwithstanding the foregoing, the 6-Benzylaminopurine (BAP) and kinetin (KIN), the latter is a synthetic cytokinin affected by light in the wavelength range 300–800 nm, represent the most widely used in plant cell culture compounds.

In vitro cultures provide insight into the production of secondary metabolites. In this respect, there are various sources in which different alkaloids of *Catharanthus roseus* have been isolated in vitro. Examples include the ajmalicine alkaloid (molecular formula:  $C_{21}H_{24}N_2O_3$ , molar mass:  $352.43 \text{ g mol}^{-1}$ , present antispasmodic properties, depression treatment, antistress effects), which has been extracted using analysis conducted from callus, cell suspensions, shoots and hairy roots; and the alstonine, from in vitro callus; as well as anthirine, in cell suspensions. To this, we can add the cathindine in suspensions; serpentine (molecular formula:  $C_{21}H_{22}N_2O_3$ , molar mass:  $349.40 \text{ g mol}^{-1}$ , with antihypertensive properties, antispasmodic properties, anxiety treatment), in callus, suspensions, shoots and hairy roots; the aquamidine, in callus, suspensions and shoots; and lochericine, in callus, suspensions and hairy roots. Similarly, the horhammericine, in suspensions and shoots; the tabersonine, in callus and suspensions; the vindoline (molecular formula:  $C_{25}H_{32}N_2O_6$ , molar mass:  $456.53 \text{ g mol}^{-1}$ , exhibiting anti-ulcerative properties), in suspensions cell and shoots; catharanthine (molecular formula:  $C_{21}H_{24}N_2O_2$ , molar mass:  $336.42 \text{ g mol}^{-1}$ , with cytotoxic action on the HCT-116 colorectal carcinoma cell line), in suspensions, shoots and roots; and 3,4 anhydrovinblastine (molecular formula:  $C_{46}H_{56}N_4O_8$ , molar mass:  $795.97 \text{ g mol}^{-1}$ , to combat lung and cervico-uterine cancer treatment), in shoots; are part of isolation sources. Similarly, the leurosine in shoots; the catharine, in shoots; vinblastine (molecular formula:  $C_{46}H_{58}N_4O_9$ , molar mass:  $810.97 \text{ g mol}^{-1}$ ), in callus, shoots and somatic embryos; and vincristine (molecular formula:  $C_{46}H_{56}N_4O_{10}$ , molar mass:  $824.96 \text{ g mol}^{-1}$ ), in somatic embryos and shoots are also considered sources (Barrales-Cureño 2015).

### 3 Applications of In Vitro Culture

The main applications of the technique of in vitro culture of cells, tissues, and organs are found in the plant micropropagation, obtaining pathogen-free plants, the preservation of germplasm, as well as plant breeding. In this regard, the biosynthesis of secondary metabolites and basic research in disciplines, such as genetics, physiology, and biochemistry, are also implications of the technique. As regards micropropagation, embryogenesis, and organogenesis, these methods can be used in obtaining somatic clones, as well as in the regeneration of complete plants with

uniform characteristics. From this, valuable cultivars free of microorganisms and difficult to obtain using traditional farming methods plants are established.

In vitro cultures can also be stored for long periods. This is accomplished by any of the methods of preservation used for microorganisms such as refrigeration, slow or reduced growth methods for preserving for months, and cryopreservation. The latter consists of crop storage in liquid nitrogen for cooling to  $-196^{\circ}\text{C}$ , which ensures storage for several years. The method eliminates problems related to physical space, excess labor, crop contamination, as well as effects resulting from genetic erosion (Osorio et al. 2011).

There are several advantages in cell and plant culture in fundamental research, micropropagation, and production of biological compounds, such as secondary metabolites, proteins, and genetically modified products activity. In this regard, studies are carried out in a greatly reduced time under controlled conditions, which compared favorably with those using traditional methods for a plant grown.

## **4 In Vitro Culture of Callus of *Catharanthus roseus***

A callus represents a set of friable dedifferentiated cells growing in a solid medium, and serve as starting material for the establishment and growth of suspension cells (Barrales-Cureño and Ramírez 2013). The calluses obtained may be subcultured for maintenance and propagation. Furthermore, differentiation can be induced to embryos and organs formation (organogenesis and embryogenesis, respectively). Similarly, these can be transferred to a liquid culture medium in order to obtain suspension cells and small aggregates. Table 1 presents the main in vitro cultures of callus from *Catharanthus roseus*.

### **4.1 In Vitro Culture of Cell Suspension from *Catharanthus roseus***

The in vitro cultivation of plant cells in liquid medium for suspension cell represents a potential topic of interest to the pharmaceutical industry showing all the advantages inherent in biotechnological processes (Barrales-Cureño et al. 2011). Cell culture (especially, cell suspensions) offer several advantages. These include that of a similar handling with respect to that performed with microorganisms; rapid cell multiplication (doubling time); and the ability to scale novel techniques, such as in the case of bioreactors and temporary immersion systems (Pérez-Alonso and Jiménez 2011). Notwithstanding the foregoing, not all compounds are produced in undifferentiated cells in terms of equal quantity and quality as those that are obtained from mother plants. Similarly, it should be considered the fact that many metabolites are synthesized from their integration into differentiation events. In this regard, several authors have pointed that cell lines are a vehicle for the production of metabolites in equal to or greater than

**Table 1** In vitro cultures of callus from *Catharanthus roseus*

Explant type	In vitro culture type	Percentage	Medium	References
Hypocotyl	Callus induction	99%	Murashige and Skoog Medium supplemented with BAP (1.0 mg L <sup>-1</sup> ) + NAA (1.0 mg L <sup>-1</sup> )	Singh et al. (2011)
Leaf	Best callus response	95%	Murashige and Skoog medium supplemented with 2,4-D (1 mg L <sup>-1</sup> ) + Kin (1 mg L <sup>-1</sup> )	Haq et al. (2013)
Node	Callus	80%	Murashige and Skoog medium supplemented with 2,4-D (1 mg L <sup>-1</sup> ) + Kin (1 mg L <sup>-1</sup> )	Haq et al. (2013)
Fruit	Callus	60%	Murashige and Skoog medium supplemented with 2,4-D (1 mg L <sup>-1</sup> ) + Kin (1 mg L <sup>-1</sup> )	Haq et al. (2013)
Leaf	Callus growth	Enhancement of alkaloid content	0.50 mg L <sup>-1</sup> of 2,4-D and 1.0 mg L <sup>-1</sup> of BA 47.92 ± 2.85	Verma and Singh (2012)
Hypocotyls of in vitro germinated seeds	Embryogenic callus	2,4 D (1.0 mg L <sup>-1</sup> ) and Murashige and Skoog medium	The advanced cotyledonary embryos showed prominent root and shoot axis, which germinated into plantlets.	Aslam et al. (2014)
Petiole segments of seedlings	Callus roots	10-fold catharanthine, 125-fold serpentine, 0.5-fold vindoline and 0.34-fold ajmalicine were produced by new roots	Medium Murashige and Skoog containing 0.1 mg L <sup>-1</sup> NAA and 0.1 mg L <sup>-1</sup> Kin	Ataei-Azimi et al. (2008)
Leaf explants	Callus was induced in MS medium with plant growth regulator (PGR) 2 mg L, 2,4-D and 0.2 mg L Kinetin	MS medium with tryptophan 50–250 mg L <sup>-1</sup>	Catharanthine content of <i>Catharanthus roseus</i> aggregate cells after 14 days of culture was increasing and has optimum content in treatment C (150 mg L <sup>-1</sup> ) that was equal to 50.96 µg diagonal g dw	Pandiangan et al. (2013)

Nodal section	Callus induction	Medium containing MS more Kinetin ( $1 \text{ mg L}^{-1}$ ) and NAA ( $2 \text{ mg L}^{-1}$ )	66% explants were responded in Murashige and Skoog medium supplemented with NAA and kinetin	Sandhya et al. (2016)
Leaf and stem segments from mature plants	Callus cultures	MS medium supplemented with 2,4-Dichlorophenoxy acetic acid (2,4-D) $1.0 \text{ } \mu\text{M}$ and 6-furfurylaminopurine (kinetin) $1.0 \text{ } \mu\text{M}$ was used to support the growth of callus cultures	The maximum amount of dry biomass ( $598.04 \text{ mg}$ ) was produced after 7 weeks of culture	Kalidass et al. (2010)
Stem and leaf explants in a modified MS liquid induction medium supplemented with $5.37 \text{ } \mu\text{M}$ $\alpha$ -naphthaleneacetic acid and $4.65 \text{ } \mu\text{M}$ kinetin	In the induction medium, most leaf explants developed into friable half-closed hollow callus clusters	The compact callus clusters could synthesize indole alkaloids 1.9 and 2.4-fold higher than the half-closed hollow callus clusters and dispersed cell cultures	The degree of compaction expressed by the ratio of fresh weight to dry weight of these suspension cultures was correlated to indole alkaloid production	Zhao et al. (2001)

the amount achieved under natural conditions. In addition, new substances have been detected that are not synthesized by plants in their natural habitat. From this, it can be said that the cultivation of cell lines reflexes a biotechnology of great importance for the development of new secondary metabolites (Pérez-Alonso and Jiménez 2011).

In vitro cell suspension cultures are maintained under the same physical and physicochemical conditions for callus induction. In the case of cell suspension cultures of *Catharanthus roseus*, all terpene indole alkaloids derived from intermediates as can be strictosidin, serpentine, catharanthine, ajmalicine, and tabersonine, as well as vincristine and vinblastine (Zhi-Gang et al. 2013). Strictosidine precursor is then hydrolyzed by strictosidine  $\beta$ -glucosidase producing cathenamine as the main product (El-Sayed et al. 2004). Once the cell culture has been established, it is possible to observe the presence of a continuous process of epigenetic or genetic changes, which causes the population to become heterogeneous. As a result, the selection of clones with high growth and production of metabolites of interest becomes a necessary aspect to observe. Meanwhile, cell lines are obtained by selecting several strategies, including macroscopic, enzyme and microscopic examination (cell viability, for example, using fluorescein diacetate) (Pérez-Alonso and Jiménez 2011). Table 2 presents the main in vitro cultures of cell suspension from *Catharanthus roseus*.

## 4.2 In Vitro Culture of Organ from *Catharanthus roseus*

Aspects associated with the accumulation of secondary metabolites imply the presence of certain types of cells and organelles, including the expression and regulation of catabolic and biosynthetic genes. Therefore, organ culture means an interesting alternative in the production of plant secondary metabolites. In this regard, the shoots and roots represent two types of bodies that are of major importance and that can be cultured on a large scale. In particular, organ culture produces substances of interest that have not been obtained from undifferentiated cultures. Notwithstanding the foregoing, shoot culture does not have the capacity to produce all compounds obtained in natural conditions in the leaves of plants. If the compound of interest is synthesized in roots, therefore shoot culture will tend to not appear. Moreover, it is important to consider that even if the compound is synthesized in the leaves, it is likely that the pattern and concentration result different from those obtained in intact plants. The main advantage indicates that organ culture is more stable in genetic terms if compared with the cultivation of suspension cell and callus (Pérez-Alonso and Jiménez 2011). Table 3 presents the main in vitro cultures of shoots from *Catharanthus roseus*.

Vindoline is a major alkaloid in vitro cultures of *Catharanthus roseus* outbreaks from which some authors reached 2 mg g<sup>-1</sup> dry weight after 27 days of culture (Hernández-Domínguez et al. 2004).



**Table 2** In vitro cultures of cell suspension from *Catharanthus roseus*

Explant type	In vitro culture type	Alkaloid type	Level production	References
Leaf explants	Suspension cell cultures	Indole alkaloids production	Murashige and Skoog medium containing 1 mg L <sup>-1</sup> Kinetine under light condition. The highest value of mass cell cultures and indole alkaloids production were achieved with modified MS medium containing 300 mg L <sup>-1</sup> of either L-glutamine for mass cell induction or L- tryptophan for enrichment of total indole alkaloids	Taha et al. (2009)
CRPP Cell suspension line	Cell suspensions	The cell lines were grown in MS or B5 medium supplemented with either 20 g L <sup>-1</sup> glucose or 30 g L <sup>-1</sup> sucrose in 250 mL Erlenmeyer flasks with 100 or 70 mL culture volume per flask	24 µmol g <sup>-1</sup> dry weight	Zuwairi et al. (2014)
Shake flask suspension cultures of <i>Catharanthus roseus</i> cells in two-stage process	The processes for production of indole alkaloids	Both culture processes produced ~20 g dw <sup>-1</sup> of biomass. Total and individual indole alkaloid production were ten times higher (740 mg L <sup>-1</sup> and 25–4000 µg g <sup>-1</sup> dw, respectively) for two-stage than for one-stage cultures	Zenk's alkaloid production medium (APM)	Tom et al. (1991)
Cell cultures in shake flasks and bioreactors	Ajmalicine production	The production of ajmalicine on production medium in a shake flask was not reproduced in a bioreactor	In turbine stirred bioreactor, at low oxygen concentration an intermediate from the tryptophan pathway, tryptamine, is accumulated. At high oxygen, ajmalicine is formed	Ten Hoopen et al. (1994)

**Table 3** In vitro cultures of shoots from *Catharanthus roseus*

Explant type	In vitro culture type	Response	Medium	Reference
Hypocotyl	Shoot proliferation	89.2% in light and 71.6% in light, respectively	Murashige and Skoog basal medium supplemented with BAP (1.5 mg L <sup>-1</sup> ) + NAA (1.0 mg L <sup>-1</sup> ) and BAP (3.0 mg L <sup>-1</sup> ) + NAA (4.0 mg L <sup>-1</sup> )	Singh et al. (2011)
Hypocotyl calli	Shoot regeneration	10–15 shoots regenerated per calli.	Murashige and Skoog medium supplemented with BAP (1.5 mg L <sup>-1</sup> ) + NAA (1.0 mg L <sup>-1</sup> )	Singh et al. (2011)
Shoot tip	Multiple shoots	Percentage: 90%	BAP (1 mg L <sup>-1</sup> )	Haq et al. (2013)
Nodal portion	Multiple shoots	Percentage: 80%	BAP (1 mg L <sup>-1</sup> )	Haq et al. (2013)
Nodal explants	Shoot bud	Percentage: 100%	Murashige and Skoog medium supplemented with BAP (1.0 mg L <sup>-1</sup> )	Pandey et al. (2014)
Nodal segments	Multiple Shoots	Number of shoots/explant: 7.30 ± 0.64, shoot length (cm): 5.97 ± 0.17 and shooting response (%): 99%	Murashige and Skoog medium supplemented with 0.5 mg L <sup>-1</sup> BAP ± 1 mg L <sup>-1</sup> NAA	Bagum and Mathur (2014)
Juvenile explants such as shoot tip and nodal sections	Multiple shoots	57% of shoots tips were responded on medium containing BAP and kinetin	BAP 2 mg L <sup>-1</sup> and kinetin 1 mg L <sup>-1</sup>	Sandhya et al. (2016)
Shoots about 1.5–2 cm	Axillary buds	Results showed that adding 2 mg L <sup>-1</sup> (BA) to the medium caused significantly increasing of parameters. The average of shoots number was recorded (4.75), leaves number (9.25), the fresh and dry weight were recorded (1103.75 and 112.00 mg), respectively	Murashige and Skoog medium in culture vessels supplemented with different concentrations of BA (0, 1, 2, 3, or 4) mg L <sup>-1</sup> and 0.2 mg L <sup>-1</sup> of NAA	Al-oubaidi and Mohammed-Amin (2014)

### **4.3 *In Vitro Culture of Roots from Catharanthus roseus***

The roots synthesize, accumulate, and secrete a variety of secondary metabolites, in addition to providing mechanical support and allow water and nutrients collection from the soil. In addition, it has been reported that biosynthetic activity of roots is also maintained in in vitro culture, a reason from which *Catharanthus roseus* root crops grow rapidly in a Murashige and Skoog medium. Several types of research determined that in vitro cultures have the ability to synthesize metabolites by producing roots. In that regard, cultures may serve as a biotechnology option for the production of alkaloids for future research. Table 4 presents the main in vitro cultures of roots from *Catharanthus roseus*.

Table 5 presents the main in vitro cultures of plantlets from *Catharanthus roseus* (Table 6).

## **5 Pharmacological Studies in *Catharanthus roseus***

Cancer is a term applied generically to a great number of different diseases. Due to its nature, it comprehends several malignant tumors found in different locations, such as leukemia, bone sarcoma, Hodgkin's disease, and non-Hodgkin's lymphoma (Barrales-Cureño 2015). In particular, six are the alterations identified in cancerous cells that determine their potential. In that respect, they: (1) show signs of very active growth; (2) evade apoptosis; (3) reflect loss of responsiveness to antigrowth signals; (4) release substances to the medium for tissue vascularization; (5) invade tissues and organs; and (6) experience unlimited replicative growth (Hanahan and Weinberg 2000).

### **5.1 *Antioxidant Enzyme Activity***

An experiment that involved different concentrations of sodium chloride was conducted over two varieties of *Catharanthus roseus* (the alba and rosea varieties). It was found that the enzyme activity of superoxide dismutase levels increased to 50 mM of sodium chloride, which contributes to a higher level of this enzyme with antioxidant value (Abdul 2009).

### **5.2 *Antiviral Activity***

Ozcelik et al. (2011) indicated the antiviral effect of *Catharanthus roseus* in herpes simplex virus (type I) with an effect of cytopathogenicity at  $0.8 \mu\text{g mL}^{-1}$ . The catharoseumine, a monoterpene indole alkaloid, which has a single peroxy, was identified as a potential inhibitor against falcipain-2 protozoan parasites that cause malaria,

**Table 4** Cultivos in vitro de raíces de *Catharanthus roseus*

Explant type	In vitro culture type	Response	Medium	Reference
Hypocotyl	Root	Best rooting response with quality roots	Half strength Murashige and Skoog medium supplemented with IBA ( $2.5 \text{ mg L}^{-1}$ ) + NAA ( $0.5 \text{ mg L}^{-1}$ )	Singh et al. (2011)
Nodal segments	Maximum rooting	Number of roots/explant: $3.60 \pm 0.51$ , root length (cm): $1.68 \pm 0.32$ and rooting response (%): 90%	Murashige and Skoog medium supplemented with $5.0 \text{ mg L}^{-1}$ IBA	Bagum and Mathur (2014)
The Plant Growth Regulators used were paclobutrazol (PBZ), gibberellic acid ( $\text{GA}_3$ ) and <i>Pseudomonas fluorescens</i> elicitors (PF Elicitors). The estimated alkaloids were ajmalicine, catharanthine, tabersonine, serpentine, and vindoline	Roots	The root vindoline contents increased with PBZ and PF Elicitors treatments but the decreased under $\text{GA}_3$ treatments when compared to control plants. In roots, the ajmalicine content increased significantly under all the treatments on all sampling days. The catharanthine contents increased with the age in control and growth regulator treatments, but the increase was not prominent and significant in PGR treatments when compared to controls. The serpentine contents of the plant increased with PGR treatments, but the increase was more prominent in PBZ treatments when compared to other treatments	The increase was in the order $\text{PBZ} > \text{PF Elicitors} > \text{GA}_3$	Jaleel et al. (2009)

showing an  $\text{IC}_{50}$  value of  $4.06 \text{ } \mu\text{M}$  (Wang et al. 2012). Meanwhile, vinblastine and vincristine showed antiparasitic effects against *Trypanosoma*, which causes trypanosomiasis in humans, inhibiting its mitosis and affecting cell shape in a dose-dependent manner (Grellier et al. 1999). Also, the use of  $15 \text{ } \mu\text{M}$  of vinblastine and  $50 \text{ } \mu\text{M}$  of vincristine inhibited cytokinesis and nuclear division. Consequently, the compounds affected the cell morphology, whereas the effect of  $3 \text{ } \mu\text{M}$  of vinblastine and  $10 \text{ } \mu\text{M}$  of vincristine inhibited the cytokinesis without affecting cell cycle progression.

**Table 5** In vitro cultures of plantlets from *Catharanthus roseus*

Explant type	In vitro culture type	Response	Level production	References
Nodal explants of stem through axillary shoot proliferation	Shootlets	MS medium supplemented with $0.5 \text{ mg L}^{-1}$ BAP $\pm 1 \text{ mg L}^{-1}$ NAA	99% of shooting response	Mehta et al. (2013)
Multiple shoot cultures	Production of ajmalicine in shake flasks	Effect of different concentrations of IAA and BA in the production of ajmalicine	Murashige and Skoog medium supplemented with IAA at a low concentration and BA at a low concentration, accumulated high levels of ajmalicine	Satdive et al. (2003)
(a) Hypocotyl sections	(a) In vitro callus grown seedlings	MS supplemented with naphthaleneacetic acid ( $\text{NAA } 2 \text{ mg L}^{-1}$ ), 6-benzyl-aminopurine (BAP, $5 \text{ mg L}^{-1}$ ), casein hydrolysate ( $\text{CH, } 1000 \text{ mg L}^{-1}$ ), and asparagine ( $100 \text{ mg L}^{-1}$ ) for callus induction	Vinblastine Yield:(a) $0.9 \mu\text{g g}^{-1}$	Datta and Srivastava (1997)
(b) Cotyledonary leaves			(b) $0.1 \mu\text{g g}^{-1}$	
(c) Hypocotyl sections	(b) In vitro callus grown seedlings		(c) $1.6 \mu\text{g g}^{-1}$	
(d) Immature fruits	(c) In vitro callus grown seedlings		(d) $0.2 \mu\text{g g}^{-1}$	
	(d) Mature plant			

**Table 6** New metabolites obtained from in vitro culture of *Catharanthus roseus* cells

Metabolite	Function	Type of culture	References
Phosphatidyl kinase	Phospholipid metabolite enzyme	Plasmatic membranes of <i>Catharanthus roseus</i> cell suspension cultures	Wissing et al. (1994)
Trichoselin	Antibiotic	Dual cultures of <i>Thrichoderma harzianum</i> and <i>Catharanthus roseus</i> callus	Marfori et al. (2002)
Phytic acid	Phosphorous storage, mRNA cellular export, chromatin remodeling	Cell suspension cultures	Mitsuhashi et al. (2005)
Cathachunine	The bisindole alkaloid cathachunine which lost C-18' and C-19' was isolated from <i>Catharanthus roseus</i> . It exerted a potent antitumor effect toward human leukemia cells through the induction of apoptosis via an intrinsic pathway	Dried whole plants	Xiao-Dong et al. (2016)

### 5.3 Hypoglycemic Activity

Several animal studies showed that ethanolic extracts of leaves and flowers decreased the levels of glucose in the blood (Ghosh and Gupta 1982; Chattopadhyay et al. 1991). In particular, the aqueous extract decreased the glucose levels in diabetic rats by 20%. This ratio is compared with the reduction of 49–58% in glucose (Singh et al. 2001), which was related to the dichloromethane and ethanolic extracts. Meanwhile, the hypoglycemic effects derived from an increased use of glucose in the liver (Chattopadhyay 1991).

Currently, research works have been conducted on new alkaloids in *Catharanthus roseus*. Some examples include that of the vindogentianine, a hypoglycemic metabolite extracted from the leaves of the plant. Works showed hypoglycemic activity in  $\beta$ -TC<sub>6</sub> and C2C12 cells by a higher glucose consumption, as well as significant in vitro inhibition. This suggests that the hypoglycemic activity of vindogentianine derives from the increased consumption of glucose, including the PTP-1B-type inhibiting effect, a potential therapeutic agent that fights diabetes mellitus type 2 (Huat et al. 2015).

### 5.4 Antidiarrheal Activity In Vivo

In vivo antidiarrheal activity, produced by ethanolic extracts of leaves and using castor oil as an inducing agent of experimental diarrhea, was tested in Wistar rats. For the same purpose, loperamide, and atropine were used as standard drugs. The antidiarrheal effect caused by ethanolic extract showed a dose-dependent inhibition of castor oil, which induced diarrhea at doses of 200 and 500 mg kg<sup>-1</sup> (Kyakulaga et al. 2011).

### 5.5 Antimicrobial Activity

The antimicrobial activity of the leaf extracts was tested against various microorganisms such as *Pseudomonas*, *Salmonella*, and *Staphylococcus*, these extracts emerge as prophylactic agents in the treatment of various diseases (Patil and Ghosh 2010). Ramya et al. (2008) evaluated the in vitro antibacterial activity by use of crude extracts of *Catharanthus roseus*. The results indicated that the leaf extract that was prepared exhibited better antibacterial activity if compared with that of the extracts from other parts of the plant. Based on the above, the aqueous extracts of leaves, stems, roots, and flowers showed low growth of microorganisms, for example, *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus* and *Bacillus subtilis* (Ramya et al. 2008).

Kumari and Gupta (2013) tested *Catharanthus roseus* leaf extracts (rosea variety), which showed excellent activity against *Aspergillus*. Meanwhile, extracts from alba variety stems showed maximum inhibitory activity against *Bacillus*, while

rosea variety flowers indicated superior activity against *Bacillus* in the methanolic extract. In particular, the Minimum Inhibitory Concentration of the extract against microorganisms tested was found at the range between 100 and 20 mg mL<sup>-1</sup>.

Acetonic, ethanolic, and chloroformic, which has been used for leaf extracts against pathogenic microorganisms were evaluated in a different study, which was to determine their antimicrobial potential. Thus, the ethanolic extract deployed maximum antibacterial activity when compared with acetone and chloroform extracts. Thus, it proved that *Staphylococcus* was the prominent, susceptible microorganism, being followed by *Escherichia coli*, *Pseudomonas* sp. and *Streptococcus* sp. (Shanmugaraju and Bhakyaraj 2016).

## 5.6 Antineoplastic Effect

*Catharanthus roseus* plants contain various dimeric indole alkaloids, which show antitumor activity significantly. In this regard, it was found that these alkaloids have apoptosis-inducing activity against in vitro and in vivo tumor cells. This is measured by the nuclear factor kappa enhancer of activated B cells, and from c-Jun N-terminal kinase routes, where damage on DNA and mitochondrial dysfunctions are present significantly.

The nuclear factor kappa B was discovered about 20 years ago. It was identified as a protein that has the ability to bind in order to improve the immunoglobulin light chain in B cells. The factor belongs to the family of transcription factors NF- $\kappa$ B, which is ubiquitous and participates in the inflammatory and immune response in the formation, development, progression, and apoptosis of tumors (Echeverri and Mockus 2008). Meanwhile, kinases c-Jun N-terminal bind and phosphor c-Jun protein in Ser-63 and -73 residues were also present in the transcriptional activation domain. These activated kinases, such as cytokines, react to stress stimulus, including UV irradiation and thermal and osmotic shocks. They participate both in the differentiation of T cells, and apoptotic processes (Echeverri and Mockus 2008).

Furthermore, different percentages of crude methanolic extracts have been identified. Metrics indicate the presence of significant anticancer activity against numerous cell types under in vitro conditions (Ueda et al. 2002), versus different types of multidrug-resistant tumors (Wang et al. 2004). Moreover, Ruskin and Aruna (2014), showed that the ethanolic extract of *Catharanthus roseus* has in vivo antitumor activity in the Ehrlich tumor model. In contrast, the in vitro study of ethanolic extract exhibited significant antitumor activity.

The aqueous extract obtained from the aerial parts caused a hypoglycemic activity in rabbits intragastrically at a dose of 10 g kg<sup>-1</sup>, contrary to that produced by an aqueous-ethanolic extract at a dose of 5 g kg<sup>-1</sup>. The hypoglycemic action was also observed in rats exposed to aqueous and ethanol extracts of the leaf of the plant. Vinblastine molecule is reported to be an effective component of certain cancer chemotherapy regimens, specifically when used with bleomycin, and methotrexate

in VBM chemotherapy for Stage IA or IIA Hodgkin lymphomas. The inclusion of vinblastine allows for lower doses of bleomycin and reduced overall toxicity with larger resting periods between chemotherapy cycles (Gobbi et al. 2003).

## 5.7 Toxicity

Acute toxicity studies were performed in mice. Research indicated that the median lethal dose of intravenous vinblastine was  $9.5 \text{ mg kg}^{-1}$ , and vincristine intraperitoneally was  $5.2 \text{ mg kg}^{-1}$ . In this regard, 20% of mice died when they were administered the total fraction of alkaloids, which was obtained from the root of the plant subcutaneously, and provided in doses of  $50 \text{ mg kg}^{-1}$ . The ethanolic extract of the leaves was orally administered in daily doses of  $75 \text{ mg kg}^{-1}$  for 24 days. The application provoked a marked reduction in the weight of the animal's testicles and prostate on the 25th day as the autopsy revealed. Similarly, the action indicated antispermatogenic activity in rats derived from the fraction of total alkaloids of plant intraperitoneally administered.

## References

- Abdul JC (2009) Soil salinity regimes alters antioxidant enzyme activities in two varieties of *Catharanthus roseus*. *Bot Res Int* 2:64–68
- Al-oubaidi HKM, Mohammed-Amin AS (2014) Effect of benzyl adenine on multiplication of *Catharanthus roseus* L. in vitro. *World J Pharm Sci* 3:2101–2107
- Aslam J, Mujib A, Prasad SM (2014) Somatic embryos in *Catharanthus roseus*: a scanning electron microscopic study. *Not Sci Biol* 6:167–172
- Ataei-Azimi A, Delnavaz HB, Ebrahimzadeh H et al (2008) High in vitro production of anticanceric indole alkaloids from periwinkle (*Catharanthus roseus*) tissue culture. *Afr J Biotechnol* 7:2834–2839
- Bagum T, Mathur M (2014) In vitro regeneration of *Catharanthus roseus* and *Bacopa monnieri* and their survey around Jaipur district. *Int J Pure App Biosci* 2:210–221
- Barrales-Cureño HJ (2015) Aplicaciones farmacológicas y producción biotecnológica in vitro de los alcaloides anticancerígenos de *Catharanthus roseus*. *Biotechnol Appl* 32:1101–1110
- Barrales-Cureño HJ, Ramírez Sepúlveda MF (2013) Una revisión sobre la producción de taxoides anticancerígenos en cultivos in vitro de callos y células de *Taxus* spp. *Rev Colomb Biotechnol* 15:67–177
- Barrales-Cureño HJ, Soto HRM (2012) Taxoides: metabolitos secundarios del árbol del tejo (*Taxus* spp.). *Rev Chapingo Ser Cie* 18:207–218
- Barrales-Cureño HJ, HRM S, VAC R et al (2011) Extracción y cuantificación de taxoides por HPLC en hojas in situ y en callos inducidos in vitro de *Taxus globosa* Schlecht. *Span J Rural Dev* 2:103–114
- Barrales-Cureño HJ, de la Rosa MCR, Villegas OS (2012) Hacia una genética celular del cáncer. *Revista Científica y Tecnológica de la Universidad Veracruzana La Ciencia y el Hombre* 25:1–4
- Barrales-Cureño HJ, Castillo HFJ, Barros GLC (2015) El Paclitaxel. *Bol Soc Quim Peru* 29:7–10
- Barrales-Cureño HJ, Farrera RA, Reyes RC et al (2016) Generalidades del fármaco Taxol: una revisión sistemática. *Rev Méd Universidad Veracruzana* 16:75–91



- Calva CG, Pérez VJ (2005) Cultivo de células y tejidos vegetales: fuente de alimentos para el futuro. *Rev Digit Universit* 6:2–16
- Chattopadhyay RR (1991) A comparative evaluation of some blood sugar lowering agents of plant origin. *J Ethnopharmacol* 67:367–372
- Chattopadhyay RR, Sarkar SK, Ganguli S (1991) Hypoglycemic and antihyperglycemic effect of leaves of *Vinca rosea* Linn. *Indian J Physiol Pharmacol* 35:145–151
- Datta A, Srivastava PS (1997) Variation in vinblastine production by *Catharanthus roseus* during in vivo and in vitro differentiation. *Phytochemistry* 46:135–137
- Echeverri RNP, Mockus SI (2008) Factor nuclear  $\kappa B$  (NF- $\kappa B$ ): señalosoma y su importancia en enfermedades inflamatorias y cáncer. *Rev Fac Med* 56:133–146
- El-Sayed M, Choi YH, Frédérich M et al (2004) Alkaloid accumulation in *Catharanthus roseus* cell suspension cultures fed with stemmadenine. *Biotechnol Lett* 26:793–798
- Ghosh RK, Gupta I (1982) Effect of *Vinca rosea* and *Ficus racemosus* on hyperglycemia in rats. *Indian J Anim Health* 19:145–148
- Gobbi MDPG, Broglia MDC, Merli F et al (2003) Vinblastine, bleomycin, and methotrexate chemotherapy plus irradiation for patients with early stage, favorable Hodgkin lymphoma. *Cancer* 98:2393–2401
- Grellier P, Sinou V, Garrea de Loubresse N et al (1999) Selective and reversible effects of vinca alkaloids on *Trypanosoma cruzi* epimastigote forms: blockage of cytokinesis without inhibition of the organelle duplication. *Cell Motil Cytoskeleton* 42:36–47
- Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100:57–70
- Haq R, Naz S, Aslam F et al (2013) Comparison of in vitro response of micropropagation and callusgenesis of medicinal plant, *Vinca rosea* L. *J Agric Res* 51:9–17
- Hernández-Domínguez E, Campos-Tamayo F, Vázquez-Flota F (2004) Vindoline synthesis in in vitro shoot cultures of *Catharanthus roseus*. *Biotechnol Lett* 26:671–674
- Huat TS, Yeng LC, Arya A et al (2015) Vindogentianine, a hypoglycemic alkaloid from *Catharanthus roseus* (L.) G. Don (Apocynaceae). *Fitoterapia* 102:182–188
- Jaleel CA, Gopi R, Gomathinayagam M et al (2009) Traditional and non-traditional plant growth regulators alters phytochemical constituents in *Catharanthus roseus*. *Process Biochem* 44:205–209
- Kalidass C, Ramasamy MV, Daniel A (2010) Effect of auxin and cytokinin on vincristine production by callus cultures of *Catharanthus roseus* L. (Apocynaceae). *Trop Subtrop Agroecosyst* 12:283–288
- Kumari K, Gupta S (2013) Phytopotential of *Catharanthus roseus* L. (G.) Don. var. Rosea and Alba against various pathogenic microbes in vitro. *Int J Res Pure Appl Microbiol* 3:77–82
- Kyakulaga A, Hassan AT, Brenda VP (2011) In vivo antidiarrheal activity of the ethanolic leaf extract of *Catharanthus roseus* Linn. (Apocynaceae) in Wistar rats. *Afr J Pharm Pharmacol* 5:1797–1800
- Marfori EC, Kajiyama S, Fukusaki E et al (2002) Trichosetin, a novel tetramic acid antibiotic produced in dual culture of *Trichoderma harzianum* and *Catharanthus roseus* Callus. *Z Naturforsch C* 57:465–470
- Mehta J, Upadhyay D, Paras P et al (2013) Multiple shoots regeneration of (anticancer plant) *Catharanthus roseus*—an importante medicinal plant. *Am J Pharmtech Res* 3:785–793
- Mitsuhashi N, Ohnishi M, Sekiguchi Y et al (2005) Phytic acid synthesis and vacuolar accumulation in suspension-cultured cells of *Catharanthus roseus* induced by high concentration of inorganic phosphate and cations. *Plant Physiol* 138:1607–1614
- Morgan JA, Barney CS, Penn AH et al (2000) Effects of buffered media upon growth and alkaloid production of *Catharanthus roseus* hairy roots. *Appl Microbiol Biotechnol* 53:262–265
- Osorio SA, Mascorro GJO, Rodríguez de la O et al (2011) Crioconservación de ápices de crisantemo (*Dendranthema grandiflorum* Kitam) por encapsulación-deshidratación y por vitrificación. *Rev Chapingo* 17:33–43
- Ozcelik B, Kartal M, Orhan I (2011) Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolics acids. *Pharm Biol* 49:396–402
- Pandey S, Bahadur AN, Kanungo VK et al (2014) *In-vitro* propagation of a medicinal plant *Catharanthus roseus* L. (G.) Don. *Indian J Life Sci* 4:125–128

- Pandiangan D, Tilaar W, Nainggolan N et al (2013) Relations between catharanthine content enhancement with the other associated secondary metabolites in *Catharanthus roseus* cell culture that treated tryptophan. *Int J Sci Res* 4:2208–2212
- Patil PJ, Ghosh JS (2010) Antimicrobial activity of *Catharanthus roseus*—a detailed study. *Br J Pharmacol Toxicol* 1:40–44
- Pérez-Alonso N, Jiménez E (2011) Producción de metabolitos secundarios de plantas mediante el cultivo in vitro. *Biotecnología Vegetal* 11:195–211
- Ramya S, Govindaraji V, Navaneetha K et al (2008) In vitro evaluation of antibacterial activity using crude extracts of *Catharanthus roseus* L. (G.) Don. *J Ethnobot Leaflets* 12:1067–1072
- Ruskin RS, Aruna SR (2014) *In-vitro* and *in-vivo* antitumor activity of *Catharanthus roseus*. *Int Res J Pharm App Sci* 4:1–4
- Sandhya M, Deepti L, Bhakti D et al (2016) Effect of growth regulator combination on in-vitro regeneration of *Catharanthus roseus*. *Int J Life Sci* 6:1–4
- Satdive RK, Fulzele DP, Eapen S (2003) Studies on production of ajmalicine in shake flasks by multiple shoot cultures of *Catharanthus roseus*. *Biotechnol Prog* 19:1071–1075
- Shanmugaraju V, Bhakyaaraj R (2016) Antimicrobial potential activity of leaf extracts of *Catharanthus roseus* against human pathogens under laboratory conditions. *Int J Curr Res Biol Med* 1:35–51
- Singh R, Kharb P, Rani K (2011) Rapid micropropagation and callus induction of *Catharanthus roseus* in vitro using different explants. *World J Agr Sci* 7:699–704
- Singh SN, Vats P, Suri S (2001) Effect of an antidiabetic extract of *Catharanthus roseus* on enzymic activities in streptozotocin induced diabetic rats. *J Ethnopharmacol* 76:269–277
- Taha HS, El Bahr MK, Seif-El-Nasr MM (2009) In vitro studies on Egyptian *Catharanthus roseus* (L.) G. Don. IV: manipulation of some amino acids as precursors for enhanced of indole alkaloids production in suspension cultures. *Aust J Basic Appl Sci* 3:3137–3144
- Ten Hoopen HJG, Van Gulik WM, Schlattmann JE, Moreno RHP, Vinke JL, Heijnen JJ, Verpoorte R (1994) Ajmalicine production by cell cultures of *Catharanthus roseus*: from shake flask to bioreactor. *Plant Cell Tiss Org Cult* 38:85–91
- Ten Hoopen HJG, Vinke JL, Moreno PRH, Verpoorte R, Heijnen JJ (2002) Influence of temperature on growth and ajmalicine production by *Catharanthus roseus* suspension cultures. *Enzyme and Microbial Technology* 30:56–65
- Tom R, Jardin B, Chavarie C et al (1991) Effect of culture process on alkaloid production by *Catharanthus roseus* cells: I suspension cultures. *J Biotechnol* 21:1–19
- Ueda JY, Tezuka Y, Banskota AH (2002) Antiproliferative activity of Vietnamese medicinal plants. *Biol Pharm Bull* 25:753–760
- Verma AK, Singh RR, Singh S (2012) Improved alkaloid content in callus culture of *Catharanthus roseus*. *Bot Serb* 36:123–130
- Villa-Ruano N, Pacheco-Hernández Y, Lara-Zaragoza EB et al (2011) Biotecnología de plantas medicinales: generando fármacos de un futuro tornado presente. *Temas de ciencia y tecnología* 15:13–20
- Wang L, He HP, Di YT (2012) Catharoseumine, a new monoterpene indole alkaloid possessing a peroxy bridge from *Catharanthus roseus*. *Tetrahedron Lett* 53:1576–1578
- Wang S, Zheng Z, Weng Y (2004) Angiogenesis and anti-angiogenesis activity of Chinese medicinal herbal extracts. *Life Sci* 74:2467–2478
- Wissing JB, Kornak B, Funke A (1994) Phosphatidate kinase. A novel enzyme in phospholipid metabolism (characterization of the enzyme from suspension-cultured *Catharanthus roseus* cells). *Plant Physiol* 105:903–909
- Xiao-Dong W, Chen-Yang L, Miao-Miao J et al (2016) Induction of apoptosis in human leukemia cells through an intrinsic pathway by cathachunine, a unique alkaloid isolated from *Catharanthus roseus*. *Phytomedicine* 23:641–653
- Zhao J, Wei-Hua Z, Hu Q et al (2001) Compact callus cluster suspension cultures of *Catharanthus roseus* with enhanced indole alkaloid biosynthesis. *In Vitro Cell Dev Biol* 37:68–72
- Zhi-Gang G, Liu Y, Mei-Zheng G et al (2013) Regulation of vinblastine biosynthesis in cell suspension cultures of *Catharanthus roseus*. *Plant Cell Tiss Org Cult* 112:43–54
- Zuwairi SM, Rianika MN, Pomahocová B et al (2014) Analysis of metabolites in the terpenoid pathway of *Catharanthus roseus* cell suspension. *Plant Cell Tiss Org Cult* 117:225–239

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Current Research and Future Prospects

Naeem, M.; Aftab, T.; Khan, M.M.A. (Eds.)

2017, XII, 412 p. 43 illus., 30 illus. in color., Hardcover

ISBN: 978-3-319-51619-6